Effects of ambient temperature on mechanisms of pathogen transmission in house finches (*Haemorhous mexicanus*)

Sara Teemer Richards

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Dana M. Hawley, Chair
Jeffrey R. Walters
Joseph R. Hoyt
Leah R. Johnson

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Effects of ambient temperature on mechanisms of pathogen transmission in house finches (*Haemorhous mexicanus*)

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Academic Abstract

Ambient temperature is an important abiotic factor shaping the process of pathogen transmission because of its effects on hosts, pathogens, and interactions between them. However, most experimental studies demonstrating the effects of temperature on transmission remain correlative and often exclude endothermic taxa, which modify behavior and energy allocation strategies in colder environments in ways that could increase pathogen spread. Additionally, because many endotherms serve as important reservoirs for zoonotic diseases and are facing conservation threats due to disease, understanding how temperature influences transmission in these systems has downstream relevance to human and wildlife health. In this dissertation, I use three laboratory experiments to determine how temperature affects several mechanisms of transmission in a naturally occurring songbird-pathogen system. House finches (*Haemorhous mexicanus*) are small songbirds that rely on bird feeders to meet thermoregulatory demands during winter. However, interactions with other birds at the feeder and contact with contaminated feeder surfaces are important sources of transmission of the bacterial pathogen *Mycoplasma gallisepticum* (MG). These interactions likely contribute to the fall and winter outbreaks of mycoplasmal conjunctivitis, a disease characterized by severe conjunctival swelling and changes in behavior in house finches. In my first experiment, I simulated infection in house finches to determine how temperature (warm versus cold) affected contact-relevant sickness behaviors, and in turn, the potential for transmission. I found that ambient temperature had a complex effect on some but not all contact-relevant sickness behaviors in this system, which could have key implications for downstream pathogen spread. Next, I investigated how ambient temperatures influenced another mechanism of transmission, the viability and pathogenicity of MG harbored on bird feeder surfaces. I found that MG remained viable and pathogenic to birds significantly longer when incubated on feeder surfaces at colder versus warmer temperatures. In my final chapter, I determined how temperature influenced the pairwise-transmission of MG from an experimentally-inoculated “donor” bird to its susceptible “receiver” bird cagemate. Here I

examined how temperature influenced host infectiousness and estimated exposure dose, as well as the behaviors of both sick and healthy birds. I found that donor birds in colder temperatures were slower to recover from infection, and thus remained infectious for longer, compared to donor birds in warmer temperatures. I also found that receiver birds had more contacts with bird feeders and higher estimated doses of MG in colder temperatures. Despite evidence suggesting that MG transmission could be more successful in colder versus warmer temperatures, overall transmission success did not differ by temperature treatment. My work highlights the complex and non-uniform effects of temperature on aspects of the MG transmission process and suggests ways that temperature could have major implications for seasonal disease dynamics in this system. More broadly, my dissertation provides a framework for testing how different abiotic factors could influence the spread of other directly-transmitted diseases, which will be needed now more than ever in the face of global climate change.

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General Audience Abstract

Temperature can alter disease spread by changing how organisms interact with each other and their environment. Most scientific studies on this topic have focused on diseases in plants and cold-blooded animals, even though temperature can influence disease spread in warm-blooded animals as well. Warm-blooded animals must use large amounts of energy to stay warm in colder temperatures and will often change their behavior or how they spend their energy to save on energetic costs. In some cases, the way that warm-blooded animals respond to colder temperatures can also increase the risk of disease spread. Understanding how warm-blooded animals spread disease is important because many warm-blooded animals carry human diseases, and because climate change brings both conservation and disease threats. In this dissertation, I test how temperature influences factors that cause disease spread in a wild songbird. House finches (*Haemorhous mexicanus*) are social backyard birds that eat from bird feeders, particularly in winter months when ample food is needed to keep their bodies warm. However, busy bird feeders can cause sick and healthy birds to interact more frequently, and bird feeders themselves often carry the bacterium *Mycoplasma gallisepticum* (MG), which causes contagious pink-eye like symptoms in birds. Like many animals, house finches that are sick with MG save energy during infection by spending less time being active. Colder temperatures can be problematic for sick birds because they must spend energy to stay warm but save enough energy for fighting infection. In my first experiment, I examined this conflict between temperature and infection in birds, and in turn, how this conflict could shape disease spread. I found that temperature affected some but not all sickness-related behaviors in house finches, which could mean more disease spread at some temperatures, and less at others. My next experiment studied the bacterium itself, and how well it can survive outside of birds in winter versus summer temperatures. I found that not only was MG better at surviving on a bird feeder in colder temperatures, but it also caused worse disease symptoms in birds over time. In my last experiment, I infected one bird with MG and determined if disease was more likely to spread to

its healthy cagemate in warmer or colder temperatures. This was important for studying the effects of temperature on two other factors related to disease spread: the ability of sick hosts to remain contagious to others and the approximate number of pathogens eventually picked up by healthy individuals. I found that in colder temperatures, sick hosts had a harder time recovering, remaining contagious for longer. I also found that healthy bird partners were more likely to spend time at bird feeders in colder temperatures, where they encountered more pathogens on feeder surfaces. Despite these findings, overall MG spread was not higher in colder temperatures. This study provided some of the first evidence showing the complicated relationship between temperature and MG spread in house finches and suggests how temperature could play a role in the seasonal outbreaks of MG seen in nature. My study also provides a blueprint for studying how other environmental factors, such as humidity and rainfall, could shape the spread of other infectious diseases, which will be more important now than ever in a rapidly changing climate.

Dedication

This work is dedicated to Myla and Antonio Teemer, my parents and my first teachers. Thank you for always encouraging my endless passion for learning about the natural world and for giving me the little purple microscope that started it all.

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Attributions

Chapter 2: Effects of cold temperature and pseudo-infection on sickness behaviour and transmission potential in house finches

There is one additional author

Dana M. Hawley, Department of Biological Sciences, Virginia Tech, Blacksburg, VA, USA

Chapter 3: Colder temperatures augment viability of an indirectly transmitted songbird pathogen on bird feeders

There are four additional authors

Edan R. Tulman, Department of Pathobiology and Veterinary Science, Storrs, CT, USA

Alicia G. Arneson, Department of Biological Sciences, Virginia Tech, Blacksburg, VA, USA

Steven J. Geary, Department of Pathobiology and Veterinary Science, Storrs, CT, USA

Dana M. Hawley, Department of Biological Sciences, Virginia Tech, Blacksburg, VA, USA

Chapter 4: Colder temperatures augment some but not all mechanisms of pathogen transmission in house finches

There is one additional author

Dana M. Hawley, Department of Biological Sciences, Virginia Tech, Blacksburg, VA, USA

Chapter 1: Introduction

Background and Significance

Seasonal infectious diseases are shaped by cyclical interactions that occur between hosts, pathogens, and the abiotic environment. Among these environmental factors, ambient temperature has the potential to significantly influence disease dynamics because of its effects on numerous components of the transmission process (e.g., Schmid-Hempel, 2011; Thomas & Blanford, 2003; Mitchell et al., 2005; Figure 1). Most evidence demonstrating the effects of temperature on pathogen transmission stems from ectotherms (Dallas & Drake, 2016), plants (Jones & Barbetti, 2012), and/or vector-borne disease systems (Mordecai et al., 2013). In contrast, few studies examine the effects of ambient temperature on pathogen transmission in endothermic vertebrates despite the similar potential for temperature to impact disease-relevant traits in these systems, including behavior and energy allocation strategies (Dowell, 2001). Because endothermic vertebrate groups are often reservoirs for zoonotic pathogens (Dearing & Dizney, 2010) and face conservation and disease threats simultaneously (Devil Tumor Facial Disease, Hamilton et al., 2020; white-nose syndrome, Langwig et al., 2015), understanding how ambient temperature alters disease-relevant processes in endotherms is critical.

Across taxonomic systems, there is substantial evidence that ambient temperatures can impact pathogen transmission, typically by altering mechanistic aspects of host (infectiousness, recovery, behavior), vector (competence, survival, biting rate), or pathogen (environmental survival) biology (Altizer et al., 2013). However, empirical findings are often based on correlations between temperature and pathogen prevalence or other population-level disease metrics (Antolin, 2008; Dowell, 2001; Pascual & Dobson, 2005), making it difficult to tease apart specific interactions between temperature and transmission mechanisms in a given system. Further, studies of temperature and pathogen transmission rarely consider how temperature affects numerous potential transmission mechanisms simultaneously (Sipari et al., 2022), which is likely the case in nature. In addition, the effects of temperature on mechanisms of pathogen transmission are often non-uniform (Mordecai et al., 2013; Schmid-Hempel, 2011; Teemer & Hawley, 2024) and remain difficult to study outside of a controlled environment (Altizer et al., 2006). Overall, a mechanistic approach examining how ambient temperature influences the process of pathogen transmission can provide insights into the underlying factors shaping

seasonal disease dynamics, which are becoming increasingly difficult to predict in the face of a rapidly changing climate (Harvell et al., 2009; Lafferty, 2009; Lindgren et al., 2012). Here, I briefly review three transmission mechanisms relevant to the non-vector borne, endothermic host-pathogen system studied in this dissertation: host infectiousness, pathogen environmental survival, and host behavior.

Host Infectiousness

Colder temperatures are especially metabolically demanding for endotherms. At colder temperatures below a given population's "thermoneutral zone", individuals must utilize energy expensive methods, such as shivering, to maintain body temperature. However, meeting the metabolic demands of cold temperatures can directly compete with the maintenance of other energetically expensive processes within hosts, such as host immunity (e.g., Harvell et al., 2009; Martin et al., 2010, Figure 1A). For instance, the acute phase response is an energetically expensive component of the immune system that consists of a suite of behavioral and physiological changes that occur at the onset of infection (Adelman & Martin, 2009; Owen-Ashley & Wingfield, 2007). Because responding to infection and thermoregulation in winter are both energetically costly and necessary for survival, hosts often utilize energy allocation strategies to manage the energy trade-offs (Svensson et al., 1998). In turn, this could result in variation in susceptibility to pathogens and recovery as ambient temperatures change with season (Schmid-Hempel, 2011). This was seen in deer mice housed in low ambient temperatures, which had reduced immune function relative to mice at mild ambient temperatures (Demas & Nelson, 1998), and in hamsters challenged with SARS-Cov2 at cold temperatures, which had higher viral loads and took longer to recover than hamsters at warm temperatures (Chan et al. 2022). Ambient temperature may also affect other aspects of the immune process such as fever response (Owen-Ashley & Wingfield, 2007) and pathology (Hawley et al., 2012), which could in turn directly affect the amount of pathogens that are shed and available for susceptible hosts to encounter in the environment (Chan et al., 2022; Kang et al., 2024). Although temperature appears to play an important role in shaping host infectiousness, whether temperature-induced changes in susceptibility and recovery scale up to influence transmission dynamics remains understudied.

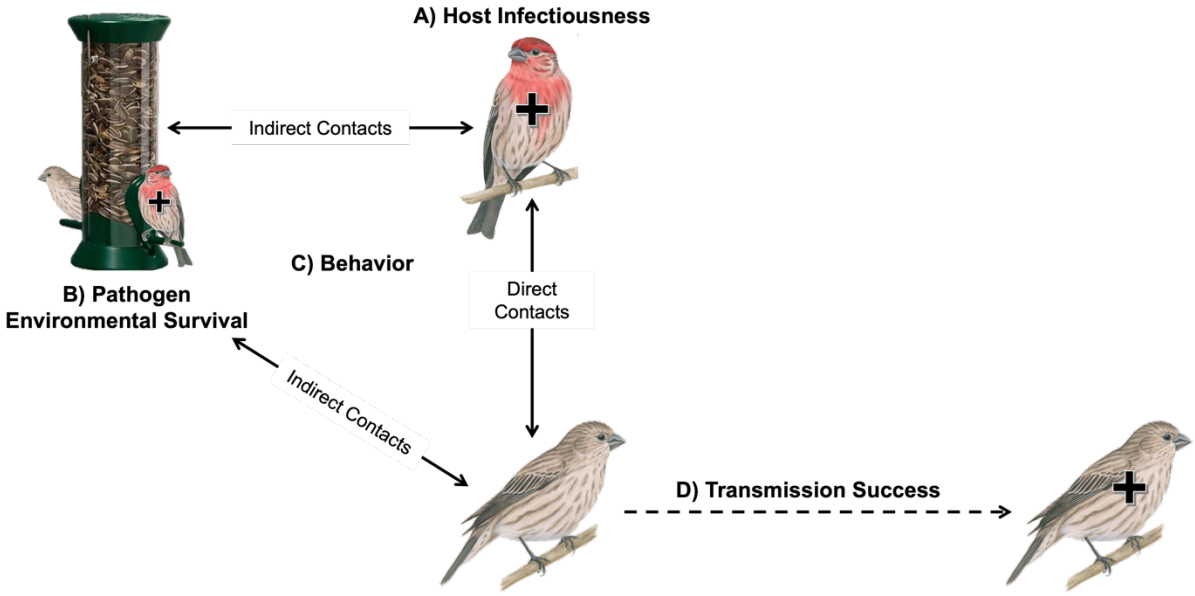

Figure 1. Potential transmission mechanisms influenced by ambient temperature in a wild songbird system, modified from McCallum et al. (2017). A) Temperature could affect host infectiousness and time to recovery. B) Temperature can augment pathogen survival outside of the host. C) The behavior of both sick and healthy individuals can also be shaped by temperature, which could affect contact rates between hosts. D) Temperature may affect numerous mechanisms simultaneously, which could scale up to have important implications for successful transmission. Infected birds are indicated by the plus symbol.

Pathogen Environmental Survival

In many disease systems, pathogens must survive at least some period of time outside of one host in order to reach the next (Breban, 2013). However, the survival of pathogens in the environment can vary with abiotic conditions, such as seen in the influenza virus, which has decreased survival in high sunlight (Sagripanti & Lytle, 2007) and humidity (Hemmes et al., 1960). Conversely, some environmental conditions can increase survival of pathogens in the environment, such as *Escherichia coli* (Williams et al., 2005) and the SARS-CoV2 virus (Riddell et al., 2020), which have greater viability in colder temperatures. For wildlife, exposure to pathogens can occur through interacting with contaminated substrates during feeding, including grass, soil, and water. For example, Fine et al. (2011) found that persistence of the bacterium

Mycobacterium bovis was significantly longer in colder temperatures relative to warmer temperatures, suggesting that the cooler seasons of the year may facilitate transmission between hosts. Pathogen transmission can also occur through supplemental feeding systems such as bird feeders, where pathogens are not typically directly ingested while feeding, but through contacts with pathogens on the surfaces of environmental fomites (e.g., Becker & Hall, 2014; Dhondt et al., 2007; Murray et al., 2016, Figure 1B). Thus, temperature could play an important role in shaping seasonal fomite transmission if pathogen persistence on fomites is higher in colder seasons but lower during the warmer seasons of the year. However, most studies testing these effects only show correlative links between increased pathogen survival in the environment and increased transmission (Pascual et al., 2002; Pascual & Dobson, 2005). Experimental studies are therefore needed to fully understand the role of temperature on fomite transmission dynamics (Teemer et al., 2024).

Host Behavior

Temperature has been shown to have a wide range of effects on individual behavior outside of the context of infectious disease. For example, at colder temperatures, ectotherms such as hermit crabs exhibit changes in anti-predatory behaviors (Briffa et al., 2013), and lizards increase aggressive behaviors such as posturing, vocalization, and attacks because physical movement, and thus the ability to escape, is limited at cooler temperatures (Crowley & Pietruszka, 1983). Endotherms also exhibit behavioral responses to colder temperatures, such as congregating under shelters, huddling (Beal, 1978), and modifying flocking behavior (Altizer, Hochachka, et al., 2004). For small birds, who have the additional challenge of increased rates of heat loss due to a small body-to-surface area ratio (Dawson et al., 1985), increasing food intake (Adelman et al., 2013) or time spent on bird feeders (Bonter et al., 2013; Grubb, 1975) are especially important for meeting the metabolic demands of cold temperatures. However, it is likely that the benefits of behavioral modification are also accompanied by costs, since certain behaviors, such as space use, sociality, and foraging, by healthy hosts can increase exposure and contact rates with infected hosts and pathogens in the environment (Figure 1C).

For birds with active infections, sickness behaviors could decrease the nature of contacts with other birds at the feeder or pathogens on surfaces. Sickness behaviors, including lethargy and anorexia, may not only help individuals survive infection (Hart, 1990) but also reduce

contact rates if birds spend less time at feeders (Ruden & Adelman, 2021). However, if birds prioritize feeding to survive harsh winter conditions over the expression of sickness behaviors, contact rates at the feeder could potentially increase. Further, prior work has shown that the expression of sickness behaviors is highly context-dependent (Owen-Ashley & Wingfield, 2006) and often is altered in ways that minimize costs and maximize benefits under different environments (Aubert et al., 1997; Lopes et al., 2023; Love et al., 2023). Thus, the role of ambient temperature on the expression of sickness behaviors, and the potential for contact between infected and susceptible individuals, is complicated but nonetheless important to include in mechanistic studies (Teemer & Hawley, 2024).

The broader relationship between behavior and pathogen transmission has been well-established (Dobson, 1988; Moore, 2002) and has been useful in providing predictive information for disease dynamics. However, this topic has largely been studied separately from the effects of the abiotic environment on transmission mechanisms, likely because behavior can be challenging to interpret, and because direct and indirect contacts between individuals are often infrequent and difficult to measure (Hoyt et al., 2018). There is a need for mechanistic studies examining the underlying abiotic factors shaping transmission to incorporate the behavior of both sick and healthy hosts, in conjunction with other mechanisms, for behavior to continue being a relevant tool for predicting pathogen spread (Bro-Jørgensen et al., 2019; Sipari et al., 2022, Figure 1D).

Study System

In this dissertation, I examine the effects of temperature on several mechanistic components of transmission (infectiousness, pathogen survival, and behavior) in a wild songbird species that naturally experiences fall and winter epidemics of a bacterial pathogen transmitted both directly and indirectly while foraging. The use of captive, controlled, experiments on a wild-caught bird species allows me to test the effects of temperature on specific mechanisms simultaneously, which is often difficult to do in non-captive settings (Altizer et al., 2006). Not only will this information illustrate the nature and importance of effects of ambient temperature on the process of transmission in this system, but it may also provide predictive power for understanding similarly transmitted diseases in a rapidly changing environment.

House finches (*Haemorhous mexicanus*) are common passerine birds found throughout most of North America (Pyle, 2022). In many parts of their range, they are found in association with humans and are almost exclusively granivorous, often relying on bird feeders for food (Dhondt et al., 2005). It is likely that typical feeder use and social behavior in house finches contributed to the emergence and rapid spread of the pathogen, *Mycoplasma gallisepticum* (MG), throughout eastern populations in 1994 (Adelman et al., 2015; Fischer, 1997; Hotchkiss et al., 2005). MG, which originally emerged from poultry populations, is a bacterium in the class Mollicutes that lacks a cell wall (Ley et al., 1996). MG is generally spread through direct contact with infected individuals or fomites, such as bird feeders (Dhondt et al., 2007). In house finches, MG infection typically results in severe swelling of ocular tissues that correlate with pathogen loads (Hawley et al., 2013) and the amount of MG deposited on to feeder surfaces (Adelman et al., 2013). In addition to severe pathology potentially affecting the ability of birds to acquire food (Bouwman & Hawley, 2010) and avoid predators (Adelman et al., 2017), MG infections (or pseudo-infections via lipopolysaccharide injections) have been associated with behaviors such as lethargy (Kollias et al., 2004), immobility (Teemer & Hawley, 2024), and increased time spent on bird feeders (Hawley et al., 2007).

MG now occurs in house finch populations as seasonal epidemics in late summer, fall, and winter, but little is known about the environmental factors that contribute to the seasonal epidemics that occur in this system (Altizer, Davis, et al., 2004). In colder temperatures, house finches have an increased metabolic rate (Hawley et al., 2012), which leads them to consume more food (Adelman et al., 2013) and spend more time at feeders in the wild (Bonter et al., 2013). Additionally, fall and winter are associated with an increase in numbers of susceptible juveniles in the population and increased flocking behavior (Altizer, Davis, et al., 2004), which can also facilitate epidemics. Whether the ability of MG to survive on bird feeder surfaces at colder versus warmer temperatures contributes to the seasonality of epidemics had not been tested prior to this dissertation. However, poultry strains of MG (which represent a distinct genetic clade of pathogen; Hochachka et al., 2013) appear to be sensitive to ambient temperatures outside the host (Chandiramani et al., 1966; Nagatomo et al., 2001), which could be critical for predicting fomite transmission given the importance of bird feeders in the house finch-MG system (Adelman et al., 2015; Teemer et al., 2024). Overall, temperature-related shifts in diverse transmission mechanisms could augment contacts with contaminated surfaces and

facilitate interactions between healthy and infected birds at feeders in ways that promote pathogen spread. However, the importance of these individual temperature effects and how they scale up to influence transmission dynamics is unknown and remains a critical gap in our understanding of the factors that shape seasonal epidemics more broadly.

Overall, house finches are a particularly tractable system for understanding the effects of temperature on the dynamic interactions between hosts and pathogens. Not only can house finches be easily captured and maintained in captivity, but MG can be cultured and maintained in the laboratory (Dhondt et al., 2007; Ley et al., 1996; Teemer et al., 2024). Additionally, non-infectious contact tracing methods can be used to quantify potential effects of ambient temperature on contact rates in both infected and susceptible birds, which are challenging to measure otherwise (Clay et al., 2009; Hawley et al., 2023; Hoyt et al., 2018). Because house finch-MG dynamics occur naturally in the wild, information learned in captivity can be effectively used to determine mechanisms of transmission, and further, patterns of transmission in nature.

Current Study

This dissertation contains three separate animal experiments that examine the effects of temperature on several mechanisms of pathogen transmission. The first two data chapters isolate temperature effects on host behavior (Chapter 2) and pathogen environmental survival (Chapter 3), respectively. Lastly, I examine how the effects of temperature on additional mechanisms, such as host infectiousness, simultaneously scale up to alter pairwise transmission success (Chapter 4). The chapter objectives include:

1. Determine how ambient temperature and pseudo-infection affect host behavior and the potential for pathogen transmission in house finches.
2. Investigate how ambient temperature alters the survival of MG in the environment and the potential role of fomites in driving seasonal epidemics.
3. Examine how the effects of ambient temperature on several mechanisms of MG transmission (infectiousness, recovery, behavior, and exposure dose) together influence pairwise transmission success.

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Chapter 2: Effects of cold temperature and pseudo-infection on sickness behaviour and transmission potential in house finches

Sara R. Teemer and Dana M. Hawley

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Abstract

Sickness behaviours are hallmarks of many infections and can influence transmission by altering behaviours of infected animals. Because sickness behaviour expression can depend on a host's environmental context, such as ambient temperature, it is critical to examine whether temperature alters sickness behaviour expression and resulting potential for pathogen transmission. House finches (*Haemorhous mexicanus*) are known to increase bird feeder use in colder (subthermoneutral) temperatures to meet thermoregulatory demands, but less is known about the behaviour of sick house finches under subthermoneutral conditions. Understanding whether cold temperatures alter sickness behaviours and conspecific contacts is key for predicting how abiotic factors such as temperature impact transmission potential between sick and healthy birds in ways relevant to the spread of pathogens such as *Mycoplasma gallisepticum* in house finches. We used a 2×2 design to examine effects of cold temperature and pseudo-infection on house finch sickness behaviours by manipulating both ambient temperature (thermoneutral or subthermoneutral) and infection status (control or pseudo-infected via lipopolysaccharide injection) of one bird per pair ("index bird"). We quantified several sickness behaviours and found that temperature and pseudo-infection had an interactive effect on the degree of index bird immobility, and temperature alone influenced the time index birds spent at the food dish. Neither temperature nor pseudo-infection status influenced index bird activity. To determine how temperature and pseudo-infection affect the potential for pathogen transmission to untreated cagemates, we applied a fluorescent and transferrable powder around the conjunctiva of the index bird in each pair and quantified the amount of powder transferred to its cagemate. We found that powder transfer, our proxy for pathogen transmission potential, was significantly higher in pairs housed at warmer temperatures, regardless of the index bird's pseudo-infection status. Ambient temperatures appear to have complex effects on sickness behaviours in house finches, with key potential implications for pathogen spread.

Introduction

The dynamics of infectious diseases that are transmitted directly between hosts are strongly influenced by the behaviours of both healthy and infected hosts. Diverse behaviours of susceptible individuals, such as space use, sociality, and feeding behaviour, have been shown to contribute to exposure and contact rates with both contagious individuals and with pathogens in the environment (e.g. Boyer et al., 2010; Keiser et al., 2016; Roznik & Alford, 2015; Zylberberg et al., 2012). The behaviours of sick individuals, including the lethargy, anorexia, and reduced activity often associated with sickness behaviours, play an equally crucial role in shaping the nature of contacts between individuals (Stockmaier et al., 2021). Further, the behaviours of both sick and healthy individuals can be strongly influenced by abiotic factors such as temperature, which may alter contact rates relevant for pathogen spread. Despite growing documented links between sickness behaviour and contact rates between individuals (Lopes et al., 2021; Stockmaier et al., 2018), the way that environmental factors influence these interactions has received markedly less attention (but see Hamilton et al., 2020).

One challenge to studying links between environmental context, sickness behaviours, and contact rates, is that the behaviours that underlie the likelihood of a host contracting or spreading pathogens are often difficult to directly observe, and thus can be difficult to quantify (called "cryptic connections", Hoyt et al., 2018). However, non-infectious contact tracing methods, such as the extent of fluorescent powder transferred from an "infected" to a "susceptible" individual, can be used to understand the importance of these behaviours to transmission potential (Clay et al., 2009; Hawley et al., 2023). A second challenge in studying effects of environmental context on sickness behaviours and contact rates is the ability to isolate how the behaviour of sick individuals *per se*, versus other characteristics of infected hosts such as the degree of pathology or pathogen load harboured (Hawley et al., 2023; Ruden & Adelman, 2021), influence a sick individual's transmission potential. The use of simulated or "pseudo" infection via injection of individuals with lipopolysaccharide (LPS) provides a controlled way to study sickness behaviours *per se*, and to isolate both their responses to environmental context and their influence on host transmission potential. LPS is a membrane component of several Gram negative bacteria and numerous studies have used it to induce temporary sickness behaviours in otherwise healthy hosts (e.g. Burness et al., 2010; Lopes et al., 2016; Owen-Ashley & Wingfield, 2006; Stockmaier et al., 2018).

Past studies using LPS injections to quantify sickness behaviours have documented that expression of these behaviours is often context dependent, varying with factors such as reproductive status and seasonality (Aubert et al., 1997; Owen-Ashley & Wingfield, 2006; Stockmaier et al., 2018). Expression of sickness behaviours requires the reallocation of an organism's limited resources towards fighting pathogens, which can be costly (Adelman & Martin, 2009). Thus, suppression of these behaviours may be more strategic under certain biotic and abiotic conditions when survival, social, or reproductive processes should be prioritised ("motivational state" Dantzer, 2001). For instance, Owen-Ashley and Wingfield (2006) found that LPS-challenged male song sparrows (*Melospiza melodia*) in breeding condition were relatively insensitive to the effects of LPS compared to males in non-breeding condition, providing evidence that the expression or suppression of sickness behaviours can be seasonal. Winter can be an especially challenging period for endothermic animals because of energetically demanding conditions that require individuals to maximise the benefits of sickness behaviours (pathogen reduction) while minimising the costs (e.g., reduced food intake and social interactions). For example, shortened day length, an important indicator for upcoming winter conditions, has been associated with attenuated immune responses in LPS-challenged Siberian hamsters (*Phodopus sungorus*), including diminished fever, anorexia, and ingestion of dietary iron (Bilbo et al., 2002).

Temperature is especially important in shaping behaviour of endothermic animals, but the direct effects of ambient temperature on host sickness behaviours have rarely been examined despite the importance of this abiotic factor in driving host behaviours relevant to pathogen spread. The thermoneutral zone is defined as the range of ambient temperatures in which an animal can maintain core body temperature without changes in metabolic rate. In colder temperatures below the thermoneutral zone for a given species, metabolic costs are expected to be highest for small endotherms because they lose heat more rapidly than large animals due to their higher surface to body ration, and therefore must more often employ energy-expensive strategies such as shivering to maintain their high internal body temperature (Dawson et al., 1985). As a result, for small endothermic animals, the expression of sickness behaviours such as anorexia and lethargy may be particularly costly to maintain under cold ambient temperatures. For instance, lactating female mice did not express sickness behaviours in cold ambient

temperatures in favour of other behaviours that promoted survival of their pups, including pup-rearing and nest building activities (Aubert et al., 1997).

Understanding the expression or suppression of sickness behaviours in response to ambient temperature is especially important because of the potential downstream effects of such behaviours on contact rates relevant for pathogen spread. For example, Lopes et al. (2016) showed that immune-challenged wild mice had reduced social connectivity after injection with LPS, and models determined that these behavioural changes limited transmission to a small group relative to larger outbreaks predicted by models that did not account for such behavioural changes. Similarly, compared to healthy Tasmanian devils (*Sarcophilus harrisii*), individuals with Devil Tumor Facial Disease and high tumor burdens typically show reduced interactions with conspecifics throughout the year (Hamilton et al., 2020). However, Hamilton et al. (2020) found that during late mating season, diseased devils had network metrics similar to those of healthy devils. This suggests that the seasonal expression (or suppression) of sickness behaviours may be important in driving contacts and subsequent transmission in that system. Further, in both endotherms and ectotherms, temperature- and infection-related changes in feeding behaviour are likely to interact in ways critical for disease spread if temperature alters contacts between susceptible individuals and viable pathogens in the environment. For instance, warmer temperatures result in increased foraging rate in daphnia (*Daphnia dentifera*) and therefore increase exposure to the pathogen *Metschnikowia bicuspidata* while feeding. However, this increase in feeding-based exposure is outweighed by effects of warm temperatures on other components of transmission, such that warmer temperatures do not increase overall outbreak risk in this system (Shocket et al., 2019).

Supplemental feeding systems, such as bird feeders, are unique in that pathogen exposure can occur via host contact with environmental fomites (i.e., feeder surfaces) while foraging, or via direct contacts with other individuals at supplemental food sites (Murray et al., 2016). Studies have demonstrated that relative to birds at warmer temperatures, birds at cooler temperatures often exhibit increases in food intake or time spent foraging (Adelman et al., 2013; Bonter et al., 2013; Grubb, 1975), changes in flocking behaviour (Altizer, Hochachka, et al., 2004; Grubb, 1987) and changes in other social behaviours, such as huddling (Beal, 1978). Because temperature has been shown to affect a wide variety of behaviours, it is likely that many of these behaviours are also relevant to pathogen spread. For example, in a supplemental feeding

context, infected birds expressing sickness behaviours such as lethargy and anorexia may spend less time at feeders and show lower overall activity relative to healthy birds, thus reducing spread of the pathogen (akin to model results from mice; Lopes et al., 2016). On the other hand, subthermoneutral temperatures may suppress sickness behaviours and thus augment the time that infected songbirds, which face the combined metabolic demands of infection and thermoregulation (Hawley et al., 2012), spend feeding relative to infected birds at thermoneutral temperatures. Consequently, temperature-induced changes in food intake, context-dependent expression of sickness behaviours, and interactions between the two, can be important to predicting transmission risk and seasonal epidemics, but little is known about these complex dynamics (Sipari et al., 2022).

We test these relationships in a songbird species that is affected by a common bacterial pathogen for which LPS injection can serve as a potentially informative model for sickness behaviours. House finches in North America are hosts for the directly-transmitted bacterial pathogen *Mycoplasma gallisepticum* (MG), which causes the disease mycoplasmal conjunctivitis (Dhondt et al., 2005). This species is highly gregarious and often forms large flocks for foraging, which likely contributes to the spread of MG (Altizer, Hochachka, et al., 2004). In these flocks, pathogen spread occurs through direct contacts between conspecifics and through short-term indirect contacts at feeders, when healthy birds feed at contaminated feeders following foraging visits from infectious individuals (Dhondt et al., 2007; Thompson, 1960). Natural infections with MG and pseudo-infections with LPS have both been associated with behavioural changes in house finches, including suppressed activity levels, decreased anti-predator behaviour, decreased food consumption, and reduced flock sizes when severe pathology is present (Adelman et al., 2013, 2017; Hawley et al., 2007; Moyers et al., 2015). Thus, LPS injection of house finches can be used to stimulate sickness behaviours broadly similar to those that occur during MG spread, although caution must be used in extrapolating LPS-mediated sickness behaviours to those induced by MG, which are longer and more severe in nature (Love et al., 2016). Importantly, because other factors such as host conjunctivitis severity are also known to influence MG transmission potential in house finches (Hawley et al., 2023), the use of LPS rather than MG to stimulate sickness behaviours allows isolation of the role of sickness behaviours *per se* in altering transmission potential in this system.

MG occurs in house finch populations as seasonal epidemics in fall and winter, such that transmission occurs across a wide range of ambient temperatures. Several factors contribute to the seasonality of epidemics in this system, including the influx of susceptible birds during late summer into the population (but see Adelman et al., 2013; Hosseini et al., 2004). Colder temperatures likely also increase MG exposure in winter: for example, in subthermoneutral conditions, healthy house finches have an increased metabolic rate (Hawley et al., 2012), which leads them to consume more food (Adelman et al., 2013) and spend more time at feeders in the wild (Bonter et al., 2013). These cold temperature-related changes in behaviour may augment indirect contact with feeders and facilitate direct contact rates when birds congregate at these common food sources (Adelman et al., 2015; Thompson, 1960), but the importance of these contacts on the potential for disease transmission remains unknown. Thus, it is critical to examine how ambient temperature specifically affects the behaviours of sick hosts in order to understand how temperature affects downstream transmission potential.

Here, we use LPS treatment as a non-pathogenic model to isolate the effects of ambient temperature on sickness behaviour expression in house finches, hypothesising that energy constraints at cold ambient temperatures will influence the extent of expression of sickness behaviours. We also use non-infectious contact tracing methods to quantify the potential effects of these sickness behaviours on host transmission potential, measured here as the degree of inert powder spread from the conjunctival region of the "index bird" to its cagemate to represent the mode of spread for MG, a common feeder pathogen in this species. We propose that interactions between temperature and pseudo-infection will affect sickness behaviours in house finches, and in turn, have implications for transmission potential.

Methods

Experimental Design

We used a 2 × 2 factorial design, manipulating both ambient temperature (thermoneutral or subthermoneutral housing) and the pseudo-infection status of the "index" bird (control or LPS-injected) in replicate male-female pairs (Table 1; $N = 37$ total pairs). We divided sampling into two temporal sessions to allow sufficient personnel and adherence to social distancing protocols in place November 2020, when the experiment was conducted. In some cases ($N = 13$),

birds initially assigned to cagemate in the first session served as the index bird in the following session with a new partner, but all 37 pairs contained a unique LPS-injected or control index-bird and untreated cagemate pairing, and all index birds were used only once. We also alternated the sex of the index bird such that males and females served as index birds in each temperature. Although we had a female-biased sex ratio overall due to constraints in the ability to sex juveniles at capture, we ensured that sex ratios within pseudo-infection treatment were equivalent across temperatures (Table 1), such that any detected effects of pseudo-infection could be attributed to treatment rather than confounds of sex.

We selected temperatures either within (thermoneutral treatment: 22 °C [night] - 28 °C [day]) or below (subthermoneutral treatment: 13 °C [night] - 19 °C [day]) the thermoneutral zone for house finches. These temperatures were selected because they were shown in previous studies to alter finch responses, including the amount of food consumption, metabolic rate, MG-induced disease severity, and circulating levels of IL-6 in plasma (Adelman et al., 2013; Hawley et al., 2012). Additionally, the selected subthermoneutral temperatures fall within the minimum range of temperatures for southwestern Virginia where the house finches were captured from the wild for this study (e.g., -5 to 15 °C in November 2020, National Centers for Environmental Information, 2023). The 6 °C difference between daytime (600h - 1800h) and nighttime (1800h - 600h) temperatures for each group allowed us to emulate daily temperature fluctuations.

Our first objective was to determine the additive or interactive effects of temperature and pseudo-infection on sickness behaviours (time at the food dish, activity, immobility) of the index bird. We used a non-pathogenic infection model by injecting index birds in the pseudo-infection treatment with lipopolysaccharide (LPS) to stimulate sickness behaviours similar to those that occur as a result of many bacterial infections (e.g., Owen-Ashley & Wingfield, 2006), including MG infection (Hawley et al., 2007; Kollias et al., 2004; Moyers et al., 2015). Our second objective examined how both temperature and pseudo-infection status affect pairwise transmission potential most relevant for MG spread. We mimicked transmission of MG by using an inert and readily transferable powder to trace contacts between the conjunctival regions of an index bird and its cagemate (see below). This method has been used as a less invasive way to understand behaviours that are otherwise difficult to observe readily, including movement in wild birds (Steketee & Robinson, 1995) and contacts between bats (Hoyt et al., 2018).

Capture and Housing

We captured hatch-year (defined as hatched within the calendar year; Pyle, 2022) house finches between July and August 2020 using wire-mesh feeder traps in Blacksburg, Virginia. The age of each bird was assessed based on plumage patterns following Pyle (2022). Following capture from the wild but prior to the start of the study, birds were housed in indoor animal rooms at 22 - 24 °C in groups of 1 - 2. At least twelve days prior to pseudo-infection, birds were transported to walk-in environmental chambers. Within each chamber, birds were housed in male-female pairs in wire cages (76 cm × 46 cm × 46 cm) under a 12D:12L light cycle, with access to two wooden perches, water, and food *ad libitum* (Roudybush Maintenance Diet). The male-female pairing was chosen to mimic the mixed-sex flocks that house finches typically participate in during fall and winter months (Altizer, Davis, et al., 2004), a time of year when we would not yet expect sex effects related to reproduction to influence interactions between birds.

Temperature Treatment

To facilitate temperature acclimation, all birds were housed at 28 °C for the first two days in the environmental chambers, and thereafter ambient temperatures were incrementally lowered on days -10, -8, and -6 prior to day 0 (day of pseudo-infection treatment). For birds in the thermoneutral treatment group, the ambient nighttime temperature was decreased by 2 °C daily. For birds in the subthermoneutral treatment group, the ambient daytime temperature was decreased by 2 - 4 °C daily and nighttime temperatures were decreased by 5 °C daily until experimental temperatures were reached (by day -6 prior to pseudo-infection treatment). Birds were then housed at assigned experimental temperatures for the remainder of the experimental timeline (thermoneutral: 22 °C [night] - 28 °C [day]; subthermoneutral: 13 °C [night] - 19 °C [day]).

Pseudo-infection

On day 0, approximately half of the index birds in each temperature treatment (Table 1) were subcutaneously injected in the pectoral muscle with approximately 40 μL of LPS, depending on each birds' body weight, for a final dose of 2 μg LPS/g body weight (combined 1:1 with Freund's incomplete adjuvant to prolong sickness responses beyond 24 hours; Adelman et

al., 2010; Owen-Ashley & Wingfield, 2006). Due to improper LPS and adjuvant mixing prior to injections of four index birds in the subthermoneutral treatment, four birds (and their cagemates) that were initially part of the study were not included in any subsequent analyses.

On LPS injection days, control index birds were captured and handled similarly, and received powder around their conjunctiva (see below) just as LPS-injected birds did. However, we did not inject control birds because injection with saline and adjuvant can itself alter behavioural responses (Adelman et al., 2010; Hegemann et al., 2013; Kelly et al., 2023; Vaziri et al., 2019), which would not allow our control birds to be representative of normal, healthy finch behaviour and transmission potential.

For the first 3 - 6 hours after LPS injection, pseudo-infected birds typically express peak sickness behaviours and do not show much variation between individuals (Moyers et al., 2015; Owen-Ashley & Wingfield, 2006). Thus, we chose to record and quantify behaviour 24 hours after injection to capture variation in sickness behaviour at the different temperatures (see *Behavioural Assessment* below). Additionally, because we hypothesised that time at the food dish might be influenced by both temperature and pseudo-injection, it was important to start recording when lights turned on in the environmental chambers (07:00 hours) to capture a peak foraging window for the day.

Transmission Potential Assessment

To assess how temperature and pseudo-infection influence transmission potential in this system, we quantified powder spread between the conjunctival region of the index bird and the conjunctival region of its cagemate, which are the most relevant areas for transmission of this conjunctival pathogen (Hawley et al., 2023). Note that we did not attempt to tease apart direct conjunctiva-conjunctiva contact versus indirect conjunctiva-surface-conjunctiva contact, as either type of contact can result in transmission in this system (Dhondt et al., 2007). Thus, any powder spread quantified is a summary metric of both direct and indirect contacts between the conjunctiva of index birds and their cagemates.

We used a small paint brush to apply inert, formaldehyde-free fluorescent powder (DayGlo Eco Pigment) within 4 mm around the conjunctiva of the index bird (Figure 1). Approximately 25 hours later, we measured the amount of powder transferred (hereafter termed ‘transmission potential’) between the index bird and cagemate using a handheld UV light. Each

bird was scored on a 0 - 3 scale based on the amount of powder present and the degree of fluorescence around the conjunctiva (0 = no powder or fluorescence, 1 = minimal powder or fluorescence, 2 = moderate powder or fluorescence, 3 = heavy powder and bright fluorescence, with 0.5 increments assigned if an observed value fell between two scores). The score for each eye was then summed for a maximum score of six for a given bird. Although powder scoring could not be done blind to ambient temperature, scores were always made blind to the treatment of the index bird. The 25-hour time period for powder assessment was chosen because 1) we expected variation in sickness behaviour in response to temperature to have already influenced the amount of powder transferred between birds and 2) this time period minimised the amount of back-transfer of powder from cagemate to index bird that might have occurred if left for a longer period of time.

Behavioural Assessment

To examine the effect of temperature and pseudo-infection behaviour, we recorded one hour of behaviour for index birds and cagemates beginning 24 hours after initial powder application and LPS or control treatment of the index bird. We analysed 30 minutes of this recording, starting 15 minutes after cameras were turned on and all people had left the room. We focused on behaviours that could facilitate contacts between individuals, especially those that could be affected by sickness, using a modified ethogram developed by Moyers et al. (2015) (Table 2). We then used time-budget behavioural analysis software (BORIS, Friard & Gamba, 2016) to analyse each video. All focal behaviours were measured as proportion of time the focal bird exhibited a given behaviour out of the total time that the bird was visible for that particular behaviour. For the two behaviours (immobility, activity) that could not be observed when a bird was out of view (Table 2), we subtracted any time spent out of view of the camera from the total time of the recording (maximum of 6.6 minutes out of view for any focal bird). All behavioural videos were analysed blind to index bird treatment, and behavioural data were collected similarly for both index birds and cagemates in each pair.

Analyses

All statistical analyses were conducted using RStudio Version 4.3.1 (R Core Team, 2023) and were visualised using the “ggplot2” (Wickham, 2016) and “patchwork” (Pedersen, 2022)

packages. To examine the overall significance of fixed effects in final models, we used the "car" package to conduct a Type II ANOVA Sum of Squares Test or Type III ANOVA Sum of Squares Test when interactions were present (Fox & Weisberg, 2019). We also used the "stats" package to compare additive and interactive models using likelihood ratio tests or AIC when appropriate to determine the best supported final model (R Core Team, 2023). The sex of each bird was initially included in all models but did not have a significant effect on any of the behaviours, so it was not included as a parameter in any of the final models.

For our first objective (effects of temperature and pseudo-infection on index bird behaviour), we used a generalised linear model ("MASS" package, Venables & Ripley, 2002) to examine the additive or interactive effect of temperature (thermoneutral or subthermoneutral) and index bird pseudo-infection status (pseudo-infected or control) on each behaviour (proportion of time spent at the food dish, active, or immobile). We used a quasibinomial distribution because we quantified the occurrence of all behaviours as a proportion of total time. Although cagemate behaviour was not the main focus for this study, the behaviours of cagemates can also be influenced by temperature and the infection status of social partners (Zylberberg et al., 2012); thus, such behaviours could contribute to differences in powder spread from index birds. To account for this, we used identical statistical methods to analyse the two most relevant behaviours (time spent at the food dish and activity) for cagemates that could have influenced contact rates with the index birds they were paired with. Because some cagemates in our sample *(N* = 13) served as index birds in a separate experimental session, analyses of index bird and cagemate behaviour were not entirely independent in our study. Thus, we focus on index bird behaviours here and provide separate analyses for cagemates. GLM parameter estimates, Likelihood-Ratio Test results, and figures for cagemate behaviour can be found in Supplementary Materials.

For our second objective, we used a beta regression model with "logit" link function ("betareg" package, Cribari-Neto & Zeileis, 2010) to examine how both temperature and pseudo-infection status affect index bird transmission potential (amount of powder spread to cagemates). Here, we limited the analysis to cagemate powder scores to assess effects of index bird treatment on transmission potential. To meet assumptions for beta regression models, we scaled powder scores into proportions such that the highest score possible (score of 6) indicated 100% transfer of powder (e.g., 5 = 83% transfer, 5.5 = 92% transfer, 4 = 67% transfer, etc.).

Ethical Note

This study was conducted in accordance with ABS guidelines for the Use of Animals in Research. All capture and housing of birds was done with permission from the United States Fish and Wildlife Service (MB158404-1) and Virginia Department of Game and Inland Fisheries (66646). All research and personnel operated under approved Institutional Animal Care and Use Committee protocols. The dose of LPS combined with Freund's incomplete adjuvant chosen for this study was used in previous studies in this system (Moyers et al., 2015) and similar passerine models (Adelman et al., 2010; Owen-Ashley & Wingfield, 2006). While the addition of Freund's incomplete adjuvant was necessary for observing sickness behaviours beyond 24 hours, these effects were temporary and generally subside within 48 hours (Owen-Ashley & Wingfield, 2006; Palacios et al., 2011). Birds were handled as minimally and as efficiently as possible throughout the experiment to minimise stress, especially during LPS administration. Immediately after injection, birds were returned to cages with their non-injected cagemates. Because house finches are highly social and prefer to associate with flockmates rather than alone when infected with MG (Langager et al., 2023), the pair-wise housing inherent to our experimental design may have also reduced individual stress in response to LPS injection. Lastly, reusing 13 birds, first as untreated cagemates and then as index birds in either the control or LPS treatment, allowed us to minimise the total number of wild-caught birds used for this study.

Results

Immobility

The percent time an index bird spent immobile (i.e., showed complete lack of movement; Table 2) depended on an interaction between its temperature treatment and pseudo-infection status (Type III ANOVA Sum of Squares Test; temperature*pseudo-infection: $\chi^2 = 4.2$, $N = 37$, $P = 0.041$), as well as pseudo-infection status as a main effect (Type III ANOVA Sum of Squares Test; pseudo-infection: $\chi^2 = 5.9$, $N = 37$, $P = 0.015$). Likelihood-ratio tests indicated more support for the interactive model compared to the additive model ($\chi^2 = 1.1$, $df = 1$, $P = 0.04$).

Parameter estimates from the GLM (Figure 2; intercept: subthermoneutral, control: $\beta = -2.3 \pm 0.59$), indicate that pseudo-infected birds at subthermoneutral temperatures spent more time immobile than control birds in the same temperature treatment (pseudo-infection, $\beta = 1.6 \pm$

0.70, $T = 2.3$, $N = 37$; temperature, $\beta = 1.2 \pm 0.70$, $T = 1.8$, $N = 37$). The GLM showed moderate support for interactive effects of temperature and pseudo-infection status on percent time immobile (pseudo-infection*temperature, $\beta = -1.7 \pm 0.88$, $T = -2.0$, $N = 37$), suggesting that the effect of pseudo-infection on immobility is in the opposite direction from that in thermoneutral temperatures.

Time Spent at the Food Dish

Analysis of fixed effects showed moderate support for an effect of temperature on the time an index bird spent at the food dish (Type II ANOVA Sum of Squares Test; temperature, $\chi^2 = 4.0$, $N = 37$, $P = 0.046$). In contrast, there was no statistical support for an effect of pseudo-infection treatment on time spent at the food dish (pseudo-infection, $\chi^2 = 0.057$, $N = 37$, $P = 0.81$). Likelihood-ratio tests indicated more support for the additive model compared to the interactive model ($\chi^2 = 0.011$, $df = 1$, $P = 0.80$).

GLM parameter estimates (Figure 3; intercept: subthermoneutral, control: $\beta = -0.78 \pm 0.27$) showed that control index birds in thermoneutral temperatures spent less time at the food dish relative to control birds in subthermoneutral conditions (temperature, $\beta = -0.66 \pm 0.33$, $T = -2.0$, $N = 37$). Pseudo-infected birds spent about the same amount of time at the food dish compared to control birds, regardless of temperature (pseudo-infection, $\beta = -0.080 \pm 0.33$, $T = -0.24$, $N = 37$).

Activity

Analysis of fixed effects showed no support for either additive or interactive effects of temperature and pseudo-infection on index bird activity (Type II ANOVA Sum of Squares Test; temperature, $\chi^2 = 0.37$, $N = 37$, $P = 0.54$; pseudo-infection, $\chi^2 = 0.76$, $N = 37$, $P = 0.38$). Likelihood-ratio tests indicated more support for the additive model compared to the interactive model ($\chi^2 = 0.39$, $df = 1$, $P = 0.17$). Parameter estimates from the GLM (Figure 4; intercept: subthermoneutral, control: $\beta = -0.26 \pm 0.26$); showed no differences in activity between birds, regardless of temperature ($\beta = 0.18 \pm 0.31$, $T = 0.61$, $N = 37$) or pseudo-infection treatment ($\beta = -0.26 \pm 0.30$, $T = -0.87$, $N = 37$).

Cagemate Behaviour

Temperature, but not the pseudo-infection status of the co-housed index bird, significantly affected time spent at the food dish by untreated cagemates (Figure S1; Type II ANOVA Sum of Squares Test; temperature, $\chi^2 = 8.6$, $N = 37$, $P = 0.0034$; index bird pseudo-infection status, $\chi^2 = 0.36$, $N = 37$, $P = 0.55$), with cagemates spending more time at the food dish when housed at subthermoneutral temperatures (see Supplemental Materials for GLM parameter estimates). In contrast, cagemate activity was not significantly affected by temperature or the pseudo-infection status of the co-housed index bird (Figure S2; Type II ANOVA Sum of Squares Test; temperature, $\chi^2 = 0.61$, $N = 37$, $P = 0.43$; index bird pseudo-infection status, $\chi^2 = 1.7$, $N = 37$, $P = 0.19$).

Transmission Potential

For our second objective, analysis of fixed effects showed statistically significant support for the main effect of temperature but not index bird pseudo-infection status (Type II ANOVA Sum of Squares Test; temperature, $\chi^2 = 5.2$, $N = 37$, $P = 0.022$; pseudo-infection, $\chi^2 = 0.78$, $N = 37$, $P = 0.38$). Our beta regression model (Figure 5; intercept: subthermoneutral, control: $\beta = -0.47 \pm 0.20$, $Z = -2.4$, $N = 37$) showed that powder scores of cagemates, representing transmission potential of the index bird, were higher at thermoneutral temperatures (temperature, $\beta = 0.51 \pm 0.23$, $Z = 2.3$, $N = 37$). However, pseudo-infection of the index bird did not meaningfully affect transmission potential to cagemates (pseudo-infection, $\beta = -0.20 \pm 0.22$, $Z = -0.88$, $N = 37$). Although we also tested for interactive effects of temperature and pseudo-infection status on transmission potential, there was less support for interactive effects in our models (AIC; temperature + pseudo-infection: $df = 4$, AIC = -22.1, temperature*pseudo-infection: $df = 5$, AIC = -21.6).

Discussion

The expression of sickness behaviours, such as decreased activity, decreased foraging, and increased resting, have generally been viewed as a costly but adaptive host mechanism for fighting infection (Dantzer, 2001; Hart, 1988). However, through these behaviours, sick individuals may also alter contact rates with their healthy counterparts in ways relevant for pathogen transmission (Stockmaier et al., 2021). Thus, understanding how environmental factors

such as temperature alter the expression of sickness behaviours and transmission potential is critical for understanding the dynamics of disease spread in a host population.

We found that ambient temperature significantly influenced some, but not all, aspects of sickness behaviour expression in house finches. Increased levels of immobility is a frequent characteristic of the sickness behaviours expressed in response to both infections and pseudo-infection (e.g., Ghai et al., 2015; Lopes et al., 2012). Interestingly, we found that sickness-induced immobility was detected only in subthermoneutral conditions for house finches, where pseudo-infected birds spent more time immobile compared to control birds. In contrast, control birds spent slightly more time immobile than pseudo-infected birds at thermoneutral conditions, indicating that an interaction between temperature and pseudo-infection status plays an important role in the expression of this sickness behaviour. The detected effects of pseudo-infection on immobility at cold temperatures but not warm temperatures may result from the additive metabolic costs of pseudo-infection and thermoregulation at subthermoneutral conditions (Hawley et al., 2012; Nord et al., 2013), which could favour immobility as an energy saving measure (Hart, 1990). However, the only prior study examining how sickness behaviours vary with temperature in birds found some support for the opposite pattern: zebra finches (*Taeniopygia guttata*) single-housed in similar ambient temperatures to our study (15 °C and 34 °C), showed average, but not statistically significant, increases in resting behaviour following LPS injection at the warmer ambient temperature (34 °C), but no detectable increase in resting following LPS injection at 15 °C (Burness et al., 2010). Another possibility is that the pairwise housing in our study facilitated the suppression of sickness behaviours in house finches, but only for those housed at thermoneutral temperatures. Prior work has shown that social housing in birds can lead to masking of sickness behaviours, relative to individually-housed birds (Lopes et al., 2012). Whether social housing interacts with energetic constraints to influence sickness behaviour expression has not been examined but is an important area for future study. Overall, the effects of temperature on particular sickness behaviours such as immobility may be non-uniform across species and social context, underscoring the importance of studying these responses in diverse taxa and social settings (Adelman & Martin, 2009; Aubert et al., 1997).

We also examined other potential components of sickness behaviour in house finches and found that temperature and pseudo-infection had no effect on activity, but temperature had a moderate effect on the time index birds spent at food dish, regardless of the bird's pseudo-

infection status. Because we measured behavioural responses approximately 24 hours post-LPS injection in this study, it is possible that pseudo-infection influenced other behaviours such as activity levels, but we were not able to detect it at the time window examined. Previous work in this system has shown that some house finch behaviours are sensitive to the effects of LPS. For example, in prior studies that did not vary temperature, pseudo-infected birds housed at standard vivarium room temperatures had significantly lower activity levels, but did not show reduced foraging behaviour, compared to controls (Moyers et al., 2015). The moderate increase in time spent at the food dish at colder temperatures, regardless of a bird's pseudo-infection status, is consistent with results from Adelman et al. (2013), which found that house finches in subthermoneutral temperatures had increased food intake relative to birds at thermoneutral temperatures. Notably, we found a similar relationship for untreated cagemates (Supplemental Materials), whereby cagemates housed at subthermoneutral temperatures spent more time at the food dish than those housed at thermoneutral temperatures. In contrast, we did not detect any effects of pseudo-infection status on the time that index birds spent at the food dish. This differs from Adelman et al. (2013), where MG-infected birds had significantly lower food intake and time spent on the feeder (detected via continuous PIT tag measurement through RFID antennae on the feeder port) compared to control birds. It is possible that the responsive and often subtle nature of feeding behaviours are difficult to capture through video recording at single time points, and thus time spent on a food dish over a 30-minute window may not be a suitable proxy for studying anorexia in this species.

While the exploration of factors that modulate sickness behaviours is a growing field (Lopes et al., 2021), few studies explore the consequences of context-dependent sickness behaviour on proxies of disease transmission. Such consequences are critical to quantify for our broader understanding of the seasonality of infectious disease transmission (Altizer et al., 2006) and the trajectory of pathogen transmission in light of global change (Sipari et al., 2022). Several studies have demonstrated that LPS injection reduces behavioural contacts between conspecifics, relative to control individuals not expressing sickness behaviours (Lopes et al., 2016; Stockmaier et al., 2018). However, the way that environmental context influences such reductions in contacts has not yet been addressed. We found that temperature treatment alone had a significant effect on the amount of powder spread between the conjunctival region of index birds and their cagemates, such that more powder was spread to cagemates at thermoneutral temperatures. This suggests

that the effect of ambient temperature on sickness behaviours in house finches, particularly the increased immobility detected at subthermoneutral temperatures at 24 hours post-LPS injection, may result in potential differences in index bird transmission potential. While changes in cagemate behaviour across temperatures may have also contributed to detected differences in powder spread, the significant reduction in time spent at the food dish by cagemates at warmer temperatures (where powder spread was higher) would be expected to hinder rather than facilitate powder spread from the index bird. Thus, temperature-induced behavioural changes in cagemates do not appear to explain, and may have even constrained, the higher degree of powder spread from index birds at warmer ambient temperatures.

Importantly, although our behavioural observations were limited to one time point approximately 24 hours post-injection and powder application, our metric of powder spread captures any contacts (direct or indirect) that occurred in the 25 hours following powder application, when sickness behaviours likely peaked. Interestingly, although pseudo-infection appeared to play a role in index bird immobility, it did not significantly influence transmission potential, suggesting that the observed differences in powder spread across temperatures are not solely explained by immobility in the pseudo-infected host. Because prior work has found that healthy male house finches avoid LPS-injected conspecifics (Zylberberg et al., 2012), avoidance behaviours of the cagemates in response to treatment of the index bird may also have influenced resulting powder spread in our study. However, we did not detect effects of index bird pseudo-infection status on the behaviours of their co-housed cagemates in this study (Supplemental Materials).

More broadly, our results indicate that changes in temperature may have significant downstream effects on proxies for pathogen transmission (Sipari et al., 2022) via behavioural mechanisms, which is especially concerning in the face of rising global temperatures (Lindgren et al., 2012). Based on powder spread alone, which only measures one potential behavioural mechanism of direct transmission (McCallum et al., 2017), our results suggest that relevant contacts for pathogen spread could increase, on average, at warmer ambient temperatures in house finches. However, it is important to note that temperature may also affect several other key components of pathogen transmission, including other infected host behaviours, disease severity (Hawley et al., 2012), pathogen survival in the environment (Dhondt et al., 2007), and the behaviour of healthy conspecifics (Adelman et al., 2015; Bonter et al., 2013). It is also important

to note that our study occurred over a relatively short window of time during which behaviour could have affected powder spread, whereas in nature, pathogen-associated sickness behaviours such as those caused by MG can persist for several weeks (Love et al., 2016), offering multiple opportunities for sick and susceptible individuals to interact over the course of infection. Thus, further studies should consider the potential effects of temperature on the many components of pathogen transmission to fully understand the role of the external environment.

The lack of detectable effects of pseudo-infection on transmission potential (powder spread), either alone or in interaction with temperature, is particularly interesting in that it contrasts with prior work in mammalian systems showing that LPS injection reduces behavioural interactions with conspecifics that are likely important for pathogen spread (Lopes et al., 2016; Stockmaier et al., 2018). One possibility is that we did not have sufficiently high sample sizes to uncover the complex interactions that may be occurring between temperature and pseudo-infection. For example, at subthermoneutral temperatures, where a key sickness behaviour (immobility) was expressed in pseudo-infected birds, powder spread is relatively lower from pseudo-infected versus control index birds. Nonetheless, we did not have strong statistical evidence for any effect of pseudo-infection on powder spread. Another caveat to our study is the use of non-pathogenic models for both pseudo-infection and powder spread. Non-pathogenic models are useful tools for studying the basic drivers shaping contact rates (Hoyt et al., 2018) and the expression of sickness behaviours (Burness et al., 2010; Love et al., 2023; Moyers et al., 2015), but pathogenic models are important for understanding the complex relationships between these factors and should be included in future research. Further, the behaviours expressed by MG-infected house finches (Adelman et al., 2015, 2017; Bouwman & Hawley, 2010), which have largely only been characterised at thermoneutral temperatures, may respond differently to temperature, and at different magnitudes, compared to those expressed by pseudo-infected individuals in this study. Although LPS-induced sickness behaviours in house finches (Moyers et al., 2015) appear to be broadly similar to those produced from MG-infection, in order to extrapolate our results to MG transmission dynamics, future studies should directly compare temperature effects on MG-induced sickness behaviours to those detected here in response to LPS.

To our knowledge, this is the first study to investigate how ambient temperature and pseudo-infection interact to influence host behaviours and subsequent pathogen transmission

potential. Despite the importance of the external environment to disease dynamics (Altizer et al., 2006; Grimaudo et al., 2022; Kirk et al., 2022; Sipari et al., 2022), few studies examine the complex relationships between components of transmission (environment, infected host, susceptible host) through a behavioural lens. Here, we combined an inert, non-infectious assay of transmission potential with a non-pathogenic infection model (LPS injection) to isolate the role of temperature-induced behaviour on the potential for disease spread. By doing so, we demonstrate the non-uniform effect of ambient temperature on sickness behaviours, and how effects of ambient temperatures on host behaviours such as immobility can have potential downstream consequences for transmission potential in the house finch-MG system and beyond.

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Tables

Table 1. Number of male-female pairs per temperature and pseudo-infection treatment. The sex of the index bird in each pair is indicated in parentheses (F = female, M = male). Sample sizes are unequal across temperatures for the pseudo-infected group because four pairs in this treatment were eliminated from analyses due to improper LPS preparation (see Methods). Because each pair contained one treated index bird (pseudo-infected or control) and one untreated cagemate, total sample sizes (N = 37) were identical for index birds and cagemates.

	Subthermoneutral	Thermoneutral
Pseudo-infected	8 (4F, 4M)	11 (6F, 5M)
Control	9 (6F, 3M)	9 (6F, 3M)

Table 2. Ethogram of behaviours recorded during focal sampling (modified from Moyers et al., 2015), definitions of these behaviours, and whether or not a behaviour could have been expressed when the subject was out of view of the camera. Since activity and immobility could be expressed out of view of the camera, proportion of time spent was adjusted to exclude total time out of view.

Behaviour	Description	Possible out of view?
Immobile	Focal bird shows complete lack of movement for >3 seconds	Yes
Time spent at the food dish	Focal bird is within pecking distance of food dish, regardless of whether it is eating	No
Activity	Any movement that involves the focal bird traveling at least one body width in distance	Yes
Out of view	Focal bird disappears from view of camera for >3 seconds; any behaviours expressed out of view could not be measured	N/A

Figures

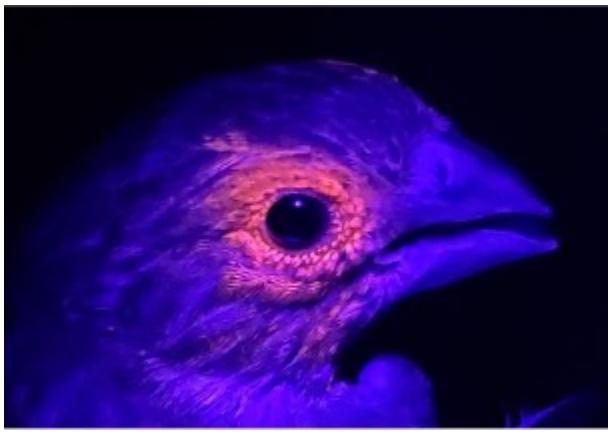

Figure 1. Index bird with fluorescent powder around its conjunctiva. Image taken under UV light. Photo credit: Sara Teemer

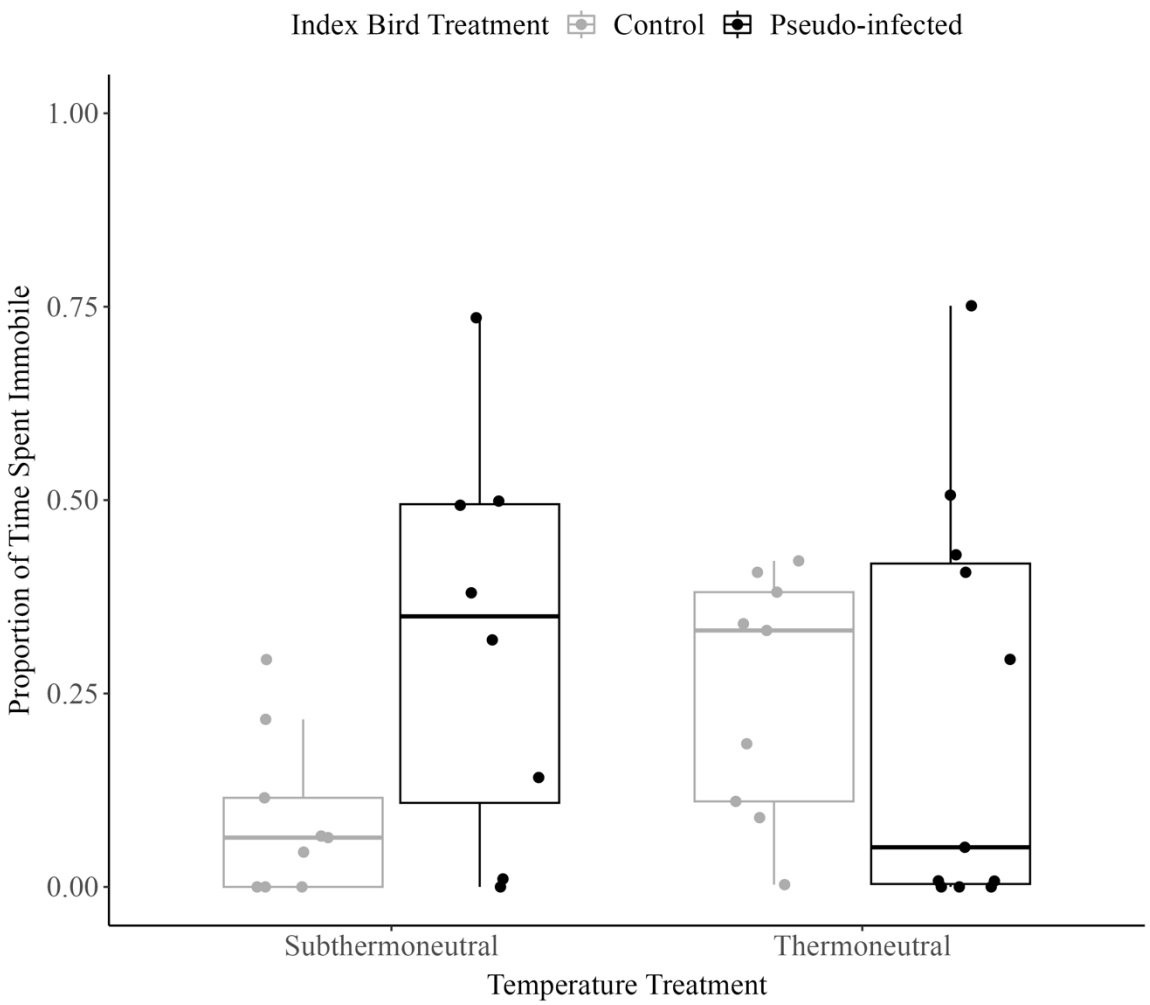

Figure 2. Proportion of time spent immobile by index house finches across temperature treatments (subthermoneutral versus thermoneutral) and pseudo-infection status (grey = control birds, black = LPS-injected birds). Immobility was measured as the proportion of time the index bird showed complete lack of movement for >3 seconds.

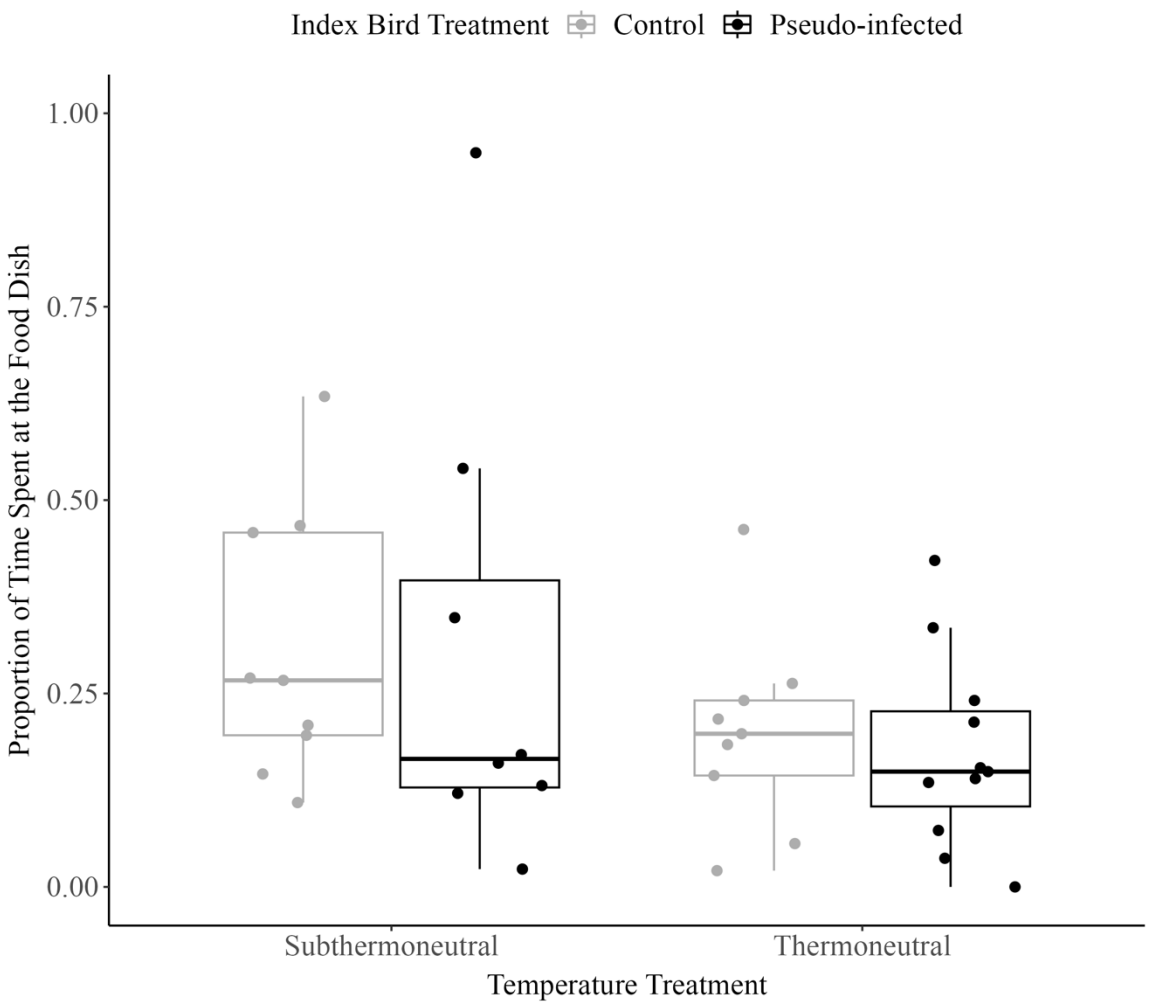

Figure 3. Proportion of time spent at the food dish by index house finches across ambient temperature treatments (subthermoneutral versus thermoneutral) and pseudo-infection status (grey = control birds, black = LPS-injected birds). Time spent at the food dish was measured as the proportion of time the index bird spent within pecking distance of the feeder, regardless of whether it is eating.

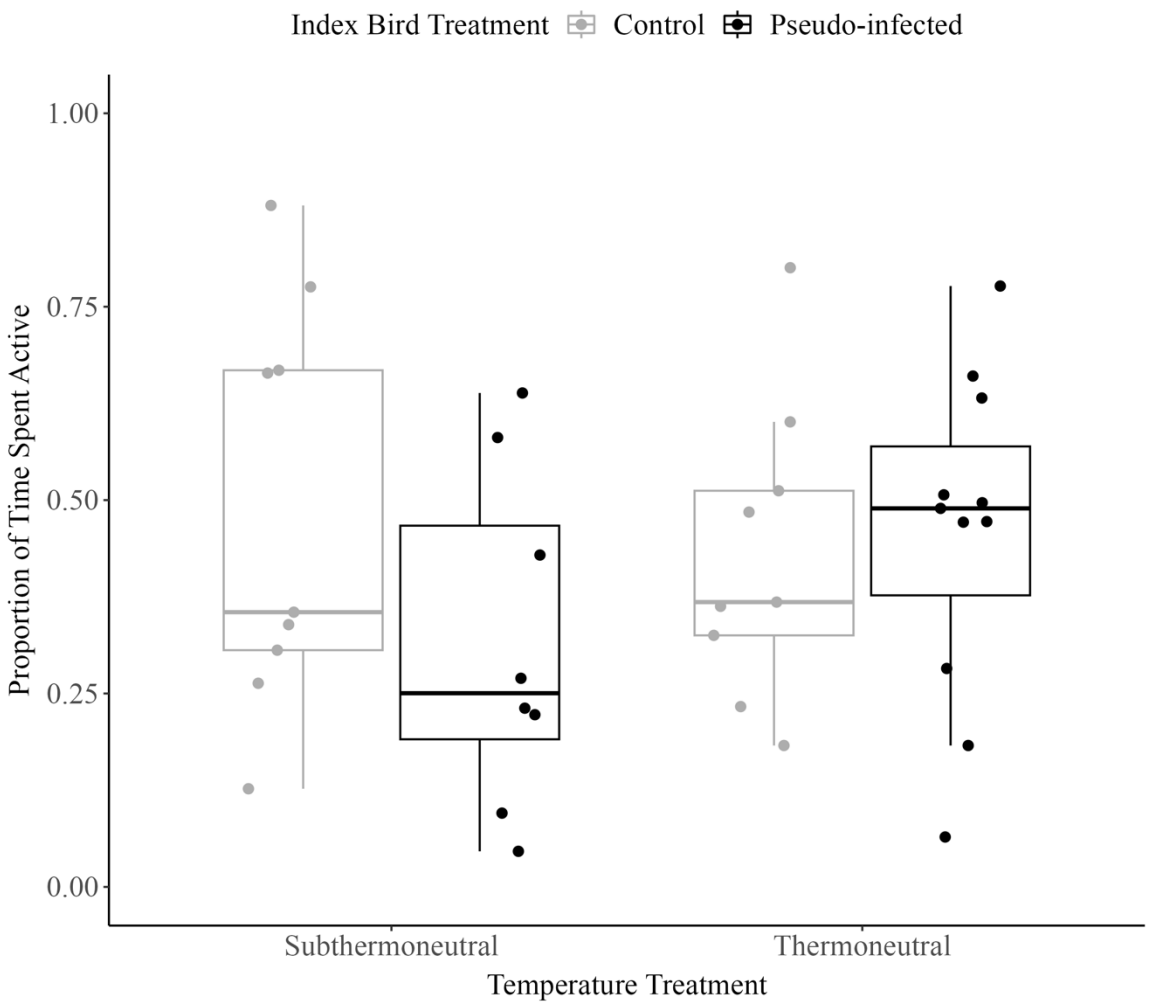

Figure 4. Proportion of time spent active by index house finches across temperature treatments (subthermoneutral versus thermoneutral) and pseudo-infection status (grey = control birds, black = LPS-injected birds). Activity was measured as the proportion of time the index bird showed any movement involved traveling at least one body width in distance.

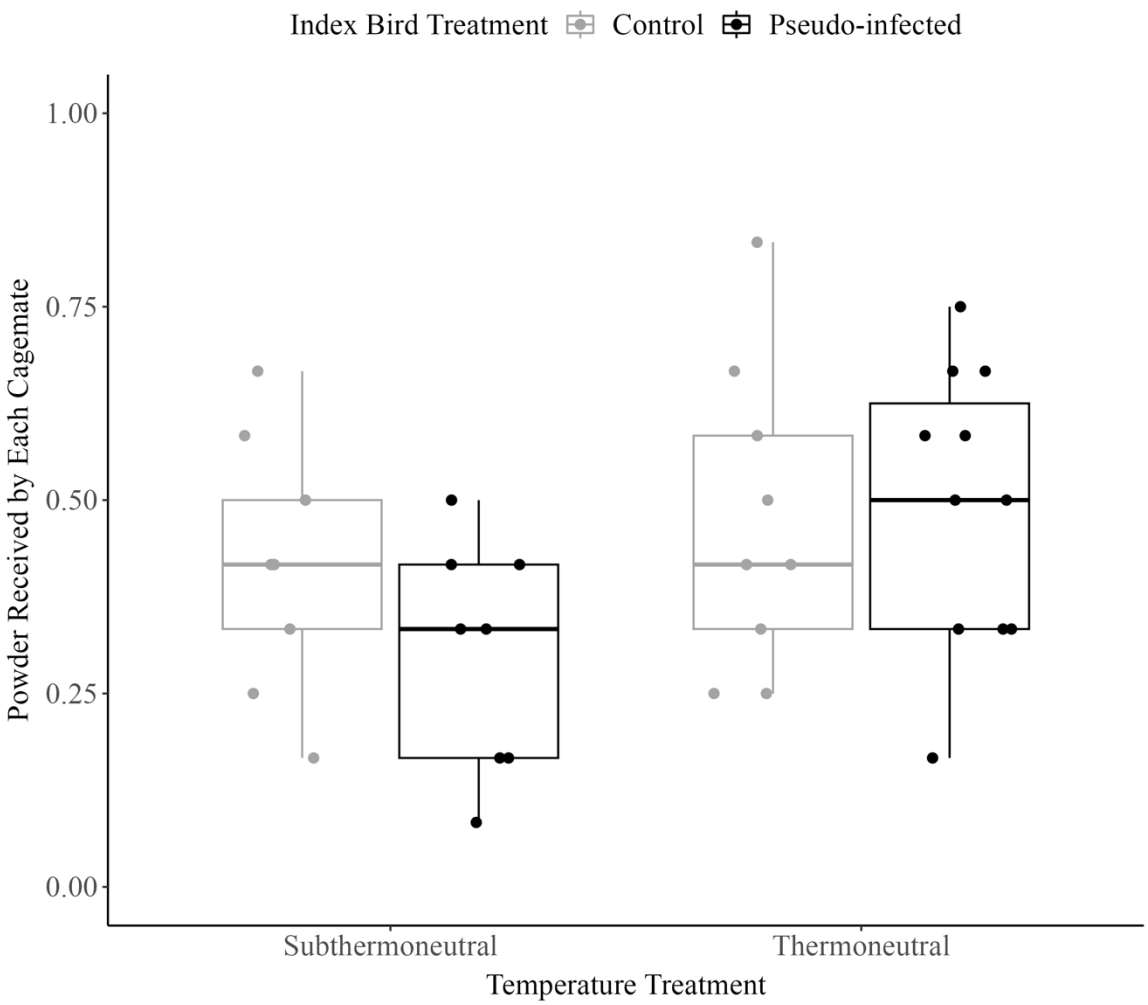

Figure 5. Transmission potential (amount of powder spread to untreated cagemate; see Methods for calculation) was higher at warm ambient temperatures relative to cooler ambient temperatures. Although this difference appears driven by pseudo-infected index birds, the interaction between temperature and index bird pseudo-infection status was not statistically significant (see Results).

Supplementary Materials

Time Spent at the Food Dish by Cagemates

GLM parameter estimates showed that cagemates in subthermoneutral conditions spent more time at the food dish relative to thermoneutral cagemates, regardless of index bird status (Figure S1; intercept: subthermoneutral, control index bird: $\beta = -0.55 \pm 0.26$; temperature, $\beta = -0.95 \pm 0.33$, $T = -2.9$, $N = 37$; pseudo-infected index bird, $\beta = -0.20 \pm 0.33$, $T = -0.60$, $N = 37$). Likelihood ratio tests comparing models for time spent at the food dish by cagemates indicated more support for the additive model compared to the interactive model (cagemate time spent at feeder: $\chi^2 = 0.15$, $df = 1$, $P = 0.36$).

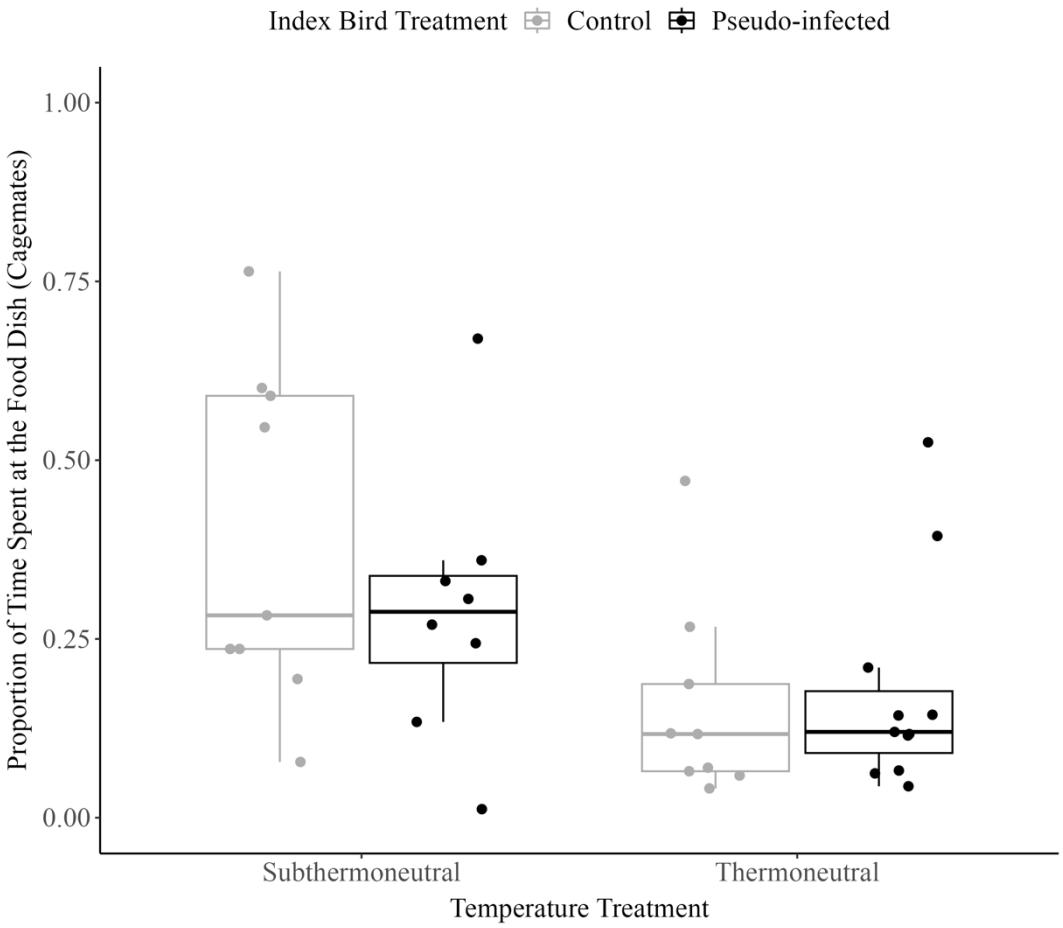

Figure S1. Proportion of time spent at the food dish by cagemates (which were all untreated with LPS) across ambient temperature treatments (subthermoneutral versus thermoneutral) and the pseudo-infection status of the index birds they were paired with (grey = control index birds, black = LPS-injected index birds). Time spent at the food dish was measured as the proportion of time the cagemate spent within pecking distance of the feeder, regardless of whether it is eating.

Cagemate Activity

GLM parameter estimates showed that cagemates in thermoneutral conditions spent slightly more time active compared to thermoneutral birds when paired with a control (non-LPS) index bird (Figure S2; intercept: subthermoneutral, control index bird: $\beta = -0.12 \pm 0.29$; temperature, $\beta = 0.27 \pm 0.34$, $T = 0.78$, $N = 37$; pseudo-infected index bird, $\beta = 0.45 \pm 0.34$, $T = 1.32$, $N = 37$). Likelihood ratio tests comparing additive and interactive for cagemate activity indicated more support for the additive model compared to the interactive model (cagemate activity: $\chi^2 = 0.51$, $df = 1$, $P = 0.15$).

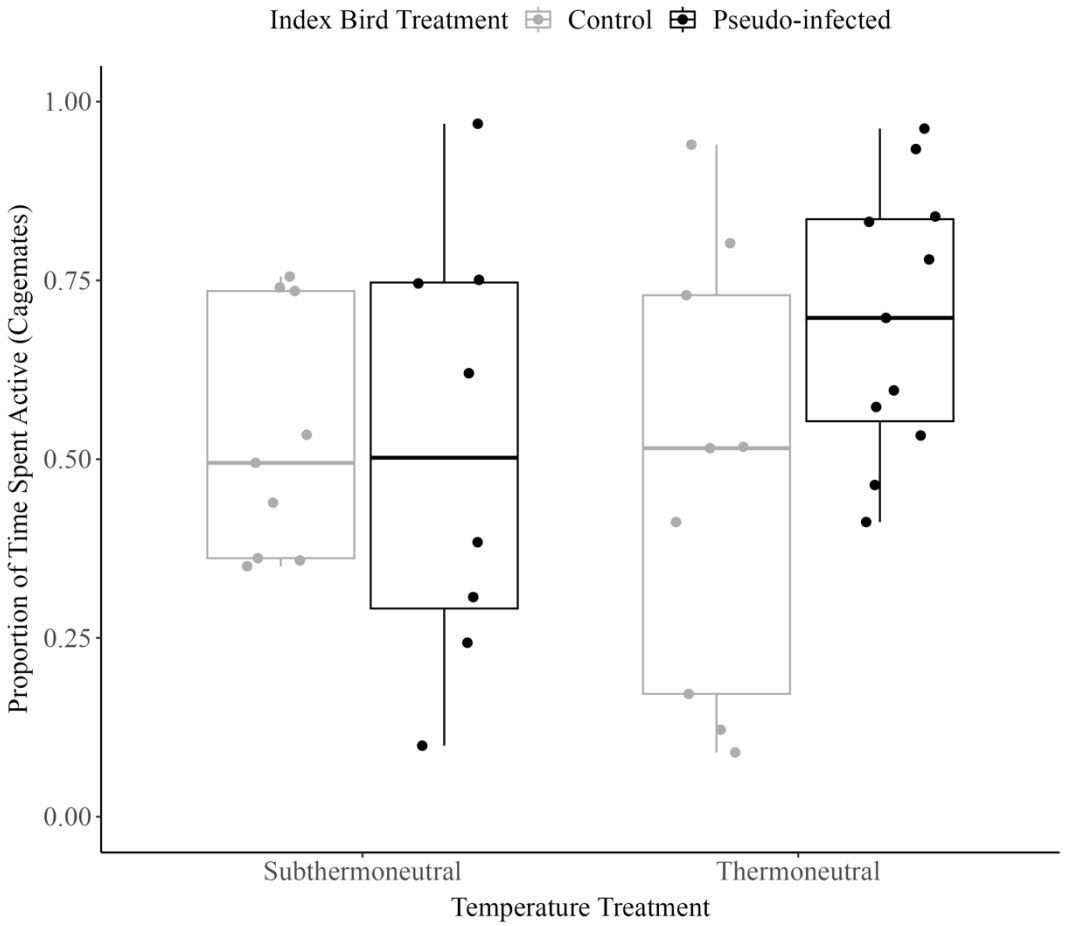

Figure S2. Proportion of time spent active by cagemates (which were all untreated with LPS) across temperature treatments (subthermoneutral versus thermoneutral) and the pseudo-infection status of the index birds they were paired with (grey = control index birds, black = LPS-injected index birds). Activity was measured as the proportion of time the cagemate showed any movement involved traveling at least one body width in distance.

Chapter 3: Colder temperatures augment viability of an indirectly transmitted songbird pathogen on bird feeders

Sara R. Teemer, Edan R. Tulman, Alicia G. Arneson, Steven J. Geary, Dana M. Hawley

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Abstract

Inanimate surfaces that are contaminated with infectious pathogens are common sources of spread for many communicable diseases. Understanding how ambient temperature alters the ability of pathogens to remain viable on these surfaces is critical for understanding how fomites can contribute to seasonal patterns of disease outbreaks. House finches (*Haemorhous mexicanus*) experience fall and winter outbreaks of mycoplasmal conjunctivitis, caused by the bacterial pathogen *Mycoplasma gallisepticum* (MG). Although bird feeder surfaces serve as an indirect route of MG transmission between sick and healthy individuals, the contributions of feeders to MG transmission in the wild will depend on how ambient temperature affects viability and pathogenicity of MG on feeder surfaces over time. Here, we used two experiments, with identical initial design, to assess such temperature effects. For both experiments, we pipetted equal amounts of MG onto replicate feeder ports held at night-day temperatures representing summer (22-27 °C) or winter (4-9 °C). We allowed MG to incubate on feeders at either temperature, and swabbed remaining inocula from surfaces at 0, 1, 2, 4, or 7 days post-inoculation of the feeder, with each replicate feeder port only swabbed at a single time point. In the first study, we analyzed swabs using a culture-based assay and found that MG incubated at colder versus warmer temperatures maintained higher viability on feeder surfaces over time. In the second study, we replicated the same experimental design but used MG swabs from feeder surfaces to inoculate wild-caught, pathogen-naïve birds, and measured resulting disease severity and pathogen loads to determine pathogenicity. We found that MG remained pathogenic on feeder surfaces at cold ambient temperatures for up to one week, much longer than previously documented. Further, MG was significantly more pathogenic when incubated on feeders in colder versus warmer temperatures, with the strongest effects of temperature present after at least four days of incubation on feeder surfaces. Overall, cold ambient temperatures appear to alter the role of fomites in the MG transmission process, and temperature likely contributes to seasonal disease dynamics in this system and many others.

Introduction

Environmental transmission is a key route of pathogen spread for numerous infectious diseases in both humans and wildlife (Lange, Kramer-Schadt, and Thulke 2016; Stephens et al. 2019). Even directly-transmitted pathogens (i.e., respiratory viruses) can show notable transmission through indirect pathways when such pathogens survive for sufficiently long on the surfaces of objects, known as fomites (Kraay et al. 2018; Kramer, Schwebke, and Kampf 2006). The relative contributions of fomite transmission to pathogen spread rely on the pathogen's ability to survive long enough on a given surface to encounter and then establish in a susceptible host (Breban 2013). However, despite the importance of abiotic factors such as temperature to such pathogen survival on fomite surfaces (Riddell et al. 2020), we lack empirical studies of how factors such as temperature influence pathogen survival on fomites for the majority of host-pathogen systems (Lange, Kramer-Schadt, and Thulke 2016). Such studies are critical for understanding the role of indirect transmission for pathogen spread and for predicting effects of seasonal and long-term changes in temperature on host-pathogen dynamics (Altizer et al. 2006; McCallum et al. 2017; Pascual, Bouma, and Dobson 2002; Wißmann et al. 2021).

Abiotic factors such as temperature are particularly interesting to study for pathogens characterized by seasonal outbreaks because temperature may contribute to seasonality by altering environmental persistence of pathogens. For example, colder temperatures have been associated with increased viability on inanimate surfaces for several pathogens, including SARS-CoV-2 (Riddell et al. 2020), *Escherichia coli* (Wilks, Michels, and Keevil 2005; Williams et al. 2005), and *Salmonella typhimurium* (Helke and Wong 1994). Further, Fine et al. (2011) found that persistence of *Mycobacterium bovis,* the pathogen responsible for bovine tuberculosis, was significantly longer in fall and winter months, and suggested that care should be taken during this period to reduce cross contamination between infected and susceptible individuals. Thus, understanding the effects of ambient temperature on pathogen viability outside of the host can give insight into the role of abiotic factors on transmission dynamics and potential management strategies to reduce disease spread.

Fomite transmission is most likely to occur from commonly-used surfaces, where infected and susceptible individuals can interact indirectly (McCallum, Barlow, and Hone 2001). For wildlife, supplemental feeding stations serve as key fomites for infectious pathogens because they attract large numbers of individuals that then interact with common surfaces to feed (Becker

and Hall 2014; Murray et al. 2016). However, the role of ambient temperature in driving pathogen survival on supplemental feeding surfaces, such as bird feeders, remains unknown. While there is evidence that bird feeding contributes to transmission of diverse pathogens including avian pox, salmonellosis, and trichomoniasis (Lawson et al. 2018; McBurney et al. 2017; Robb et al. 2008; Sorensen, van Beest, and Brook 2014; Wilcoxen et al. 2015), the extent to which feeders facilitate environmental transmission will vary strongly with abiotic conditions (Lawson et al. 2018). Therefore, understanding how temperature affects pathogen survival on feeder surfaces can provide insight into the complex relationship between abiotic factors and fomite transmission dynamics.

Here, we examine the relationship between temperature and pathogen viability on fomites using a feeder-transmitted disease system. House finches (*Haemorhous mexicanus*) are common songbirds in the Eastern United States that often experience fall and winter outbreaks of mycoplasmal conjunctivitis, a disease caused by the bacterium *Mycoplasma gallisepticum* ([MG], Dhondt et al. 2005; Ley, Berkhoff, and McLaren 1996). Although this pathogen originally emerged in songbirds from poultry, the bacterial strains currently circulating in house finches represent a distinct, monophyletic clade from poultry strains (Hochachka et al. 2013). House finches are highly gregarious and often rely on bird feeders in winter to meet increased energy demands (Bonter et al. 2013; Hawley et al. 2012). House finches often visit tube-style bird feeders (Hartup et al. 1998) which contain small circular ports that birds use to access seed inside. Fomite transmission is thought to occur largely when infected birds feed at ports and deposit MG from infected tissues around the eye as they feed, which susceptible individuals can then acquire during a subsequent feeding visit. Consistent with this mechanism, prior work shows that infected birds deposit MG onto port feeder surfaces at loads proportional to the amount of pathogen harbored in their conjunctivae (Adelman et al. 2013), and susceptible individuals exposed to a MG-contaminated feeders can become infected (Dhondt et al. 2007).

MG has been shown to survive on and transmit from contaminated feeders for up to 24 hours at standard room temperatures (Dhondt et al. 2007), but little is known as to how ambient temperatures affect MG viability on feeder surfaces despite the importance of feeder-transmission for this system (Adelman et al. 2015; Moyers et al. 2018; Ruden and Adelman 2021). To date, the direct effects of temperature on MG survival outside of the host have only been examined in culture for chicken MG strains (Christensen et al. 1994; Nagatomo et al. 2001;

Woode and McMartin 1974) and not for MG strains affecting songbirds. For instance, poultry MG has been shown to have higher viability in colder environments outside of the host (Zain and Bradbury 1996). This is likely due to the slowing of metabolic activity, which allows limited resources within the external environment to last longer (Chandiramani, Van Roekel, and Olesiuk 1966). Due to similarities in pathogen biology between poultry and songbird strains of MG, we expect to see similar increases in viability at colder temperatures outside of the host, but it is possible that house finch strains differ in their response to environmental factors given the distinct selective pressures operating on poultry versus house finch pathogen strains (e.g., Mugunthan et al. 2023; Reinoso-Pérez et al. 2022).

We tested how ambient temperatures broadly representative of winter versus summer conditions influence the viability of house finch MG strains on feeders using two separate experiments. For both studies, we inoculated replicate feeder ports housed at one of two temperature regimes and sampled ports at 0, 1, 2, 4, and 7 days later to quantify pathogen viability at each temperature. To isolate potential effects of variable ambient temperature on MG viability while on feeder surfaces only, temperature was held constant once swabs were collected from experimental feeders and then used in two downstream viability assays. In the first experiment, we measured viability of MG swabbed from feeder ports at a given temperature and time point using culture-based methods. In the second experiment, we measured MG viability with a bioassay by inoculating birds (all housed at identical room temperatures) with swab samples from feeder ports. We predicted that colder ambient temperatures would augment the ability of MG to survive on feeder surfaces, as measured by both cell viability and pathogenicity to wild birds.

Methods

Experimental Design

To test whether ambient temperature influences pathogen viability on feeder surfaces, we conducted two separate experiments using an identical sampling design to incubate MG on feeder surfaces for distinct time periods at distinct ambient temperatures. The experiments differed only in the endpoint assays used to quantify resulting pathogen viability at each temperature-time point combination: 1) we quantified cell viability of MG *in vitro* (Experiment

1, October 2022), and 2) we measured *in vivo* pathogenicity of feeder-incubated MG in wild-caught but pathogen-naive house finches (Experiment 2, January 2023).

For both studies, we placed several tube-style bird feeders in walk-in environmental chambers and set chambers to one of two fluctuating day-night temperature regimes representing summer (22 ℃ [night]-27 ℃ [day]) or winter (4 ℃ [night]-9 ℃ [day]) conditions. We then pipetted equal amounts of MG (or sterile media for control ports) onto replicate bird feeder ports, the most relevant feeder surface for birds to interact with while feeding. We allowed MG to incubate on feeder port surfaces for 0 (swabbing within 30 seconds after inoculation), 1, 2, 4, or 7 days at their respective temperatures, with each individual feeder port only swabbed once at its assigned time point, such that all feeder ports represent independent samples. At each sample time point, we used a sterile swab to collect any remaining inocula from relevant feeder ports while allowing remaining feeders to continue incubating. The swabs of each port were then used for one of two viability assays, which differed between the two studies. All temperature-time point combinations were replicated across 2-5 independent feeder ports for Experiment 1 (Table 1) and 4-6 independent feeder ports for Experiment 2 (Table 2). For both experiments, we used fewer replicates for day 0 versus later time points because we anticipated lower variability from samples that were only exposed to ambient environmental conditions for a brief period (<30 seconds).

In the first experiment, conducted October 2022, we used the feeder port swabs to conduct a viable cell count *in vitro* to quantify the number of living MG cells in each sample collected from feeder surfaces. In the second experiment, with identical initial design and conducted in January 2023, we assessed *in vivo* viability and pathogenicity of the MG collected from feeder surfaces by using a bioassay, whereby feeder port swabs were used to directly inoculate house finches by placing the swabs directly into the conjunctival sacs of wild-caught but MG-naive birds. We then measured the ability of MG to produce infection in the host by quantifying disease severity and pathogen load in each bird post-inoculation. Importantly, all birds were housed at room temperature during the bioassay, such that temperatures only differed during pathogen incubation on bird feeders.

Temperature Selection

The experimental temperatures were selected to represent ambient temperatures that bird feeders in many parts of the United States are exposed to in summer versus winter. Because MG is directly deposited onto feeder surfaces (Adelman et al. 2013; Dhondt et al. 2007), MG cells are inherently subject to the same ambient temperatures that the feeders are exposed to. Thus, we ensured that experimental winter temperatures chosen for this study fell within the minimum range of winter temperatures for Southwestern Virginia, USA where the birds were captured (-10 to 9 ℃ from January to February 2023, National Centers for Environmental Information 2024a), as well as Durham, North Carolina (-3 to 16 ℃ from January to February 2023, National Centers for Environmental Information 2024b) where the MG strain used was originally isolated from a bird submitted to the Piedmont Wildlife Center (Ley et al. 2016).

Feeder Inoculation and Incubation

Tube-style bird feeders (Droll Yankees; Plainfield, Connecticut), each with two feeding ports, were placed in separate environmental chambers set to summer or winter temperature conditions (see *Temperature Selection*). All feeders were disinfected, sanitized, and stored until one week prior to the experiment. Immediately before inoculation, feeder ports were wiped down with a dry KimWipe to ensure the inoculation surface was free of debris. We then inoculated replicate feeder ports with MG inoculum (one 5 µL droplet for culture assay in Experiment 1, two separate 5 µL droplets for bioassay in Experiment 2) or sterile Frey's media as a control.

Droplets were then left to incubate on the feeder port surface for 0, 1, 2, 4, or 7 days, depending on the assigned treatment for a given port. For both experiments, we used an MG strain that was first isolated from a male house finch with conjunctivitis near Durham, North Carolina in 2006, and has since been passaged four total times, but otherwise remained frozen at -80C (NC2006, 2006.080-5 [4P] 7/26/12). The inoculum used in this experiment contained an initial concentration of 2.49×10^8 color-changing units per mL, and prior to use, was thawed, divided into five separate aliquots, and refrozen before feeder inoculation. This allowed us to control for freeze-thaw while also varying the calendar day of inoculation for each feeder time point (0, 1, 2, 4, or 7) such that we could collect all feeder swabs and inoculate birds with those swabs on the same day, regardless of whether the MG had incubated on the feeder for 0 or 7 days.

Experiment 1: Viable Cell Count Assay

At each incubation time point, a sterile flocked swab (Copan FloqSWABS, Copan Diagnostics) was moistened with two drops of sterile eye lubricating drops (CVS Health, propylene glycol 0.6%) to aid in collecting remaining inocula from each feeder port surface. Using a small LED light, we located the MG droplet on each port surface and swabbed the area for 5 seconds (5 turns) to collect any remaining MG inocula or Frey's media. The flocked swab was then swirled in 300 µL triptose phosphate broth and wrung on the side of the tube before freezing at -80 ℃.

MG swab samples were sterilely titrated in 96-well sterile microtiter plates (Costar 3370) as follows: For each sample, a plate was loaded with 180 µL completed Frey's medium per well (Frey, Hanson, and Anderson 1968). Frozen MG swab samples were individually thawed from ultralow freezers at time of titration, vortexed, and collected quickly at the bottom of the tube. We inoculated 20 µL/well in column one of the plate, and all eight replicates were then serially titrated ten-fold across the plate using a multichannel micropipette. Plates were sealed with tape, incubated at 37 °C, and observed over seven weeks for pH-induced color change indicating growth. Viable count, as color-changing units per mL (CCU), were calculated using a most-probable number (MPN) table (Meynell and Meynell 1970).

Experiment 2: Bioassay

Bird Capture and Housing

Hatch-year house finches (identified following Pyle 2022) were captured using mist nets or feeder traps in Montgomery County, Virginia and near the campus of Virginia Tech and housed at standard room temperatures (20-22 ℃). Birds were quarantined for two weeks, during which time they were captured every 3-4 days to visually assess whether clinical signs of mycoplasmal conjunctivitis had developed. Any birds with visible conjunctivitis were isolated along with their cagemates and not used in this experiment. Following the 2-week quarantine, we took blood samples between 14-18 days post capture for all birds that did not develop signs of conjunctivitis to screen for MG exposure via seropositivity. To confirm seronegativity for these birds, blood samples were run using an ELISA via a commercial IDEXX kit (IDEXX 99-06729; Westbrook, Maine) following published methods (Hawley et al. 2011). All birds used in this study were MG seronegative at the beginning of the experiment.

Birds were then single-housed at room temperature for the duration of the bioassay. We randomly distributed birds by sex into treatment or sham groups across temperature treatments (Summer [22-27 ℃] or Winter [4-9 ℃]), and incubation time on the feeder (0, 1, 2, 4, or 7 days). All birds were equally divided across treatments by sex, except for one MG treatment group (5 males:1 female) and one sham treatment group (2 females), due to the sex ratios of birds available (Table 2). Birds were given water and food (80% Roudybush Maintenance Diet and 20% sunflower hearts) *ad libitum* throughout the course of the experiment. Following capture from the wild and up through completion of the experiment, birds were treated with prophylactic medications, Cankerex Plus (Dimetridazole B,P (Vet) 40% m/m powder) and Endocox (2.5% Toltrazuril), to prevent Trichomoniasis and coccidiosis, respectively, which can be fatal to captive finches. Details of medication doses for Cankerex and Endocox can be found in the Supplement of Hawley et al. (2024).

Bioassay inoculation

At each incubation time point, and for each 5 µL droplet (two per port for Experiment 2), a new flocked swab was moistened with two drops of sterile eye lubricating drops to aid in collecting dry inocula from the feeder and to prevent abrasion in the bird's eye during inoculation. After swabbing each feeder port, birds were inoculated by gently pulling open the lower eyelid of each eye using sterile forceps and inserting one of the two flocked swabs (a swab of each droplet was used for each eye) for 5 seconds (5 turns). Although indirect transmission typically occurs between birds and contaminated feeders, by inoculating the bird with the swab directly in the conjunctiva (instead of allowing it to occur through natural interactions with the feeder), we ensured that each bird had equivalent exposure to the inoculum regardless of how much MG was still viable on the feeder surface. Birds were then immediately returned to their cages at room temperatures.

We assessed viability of the MG inoculum by quantifying both disease severity and pathogen load in birds over the course of infection. We scored disease severity for each bird on days 2-7 and 10, 14, 21, and 28 days post-inoculation (PID). Following Hawley et al. (2011), each eye of each bird was scored on a scale of 0-3 per eye in 0.5 point increments, where 0 = no swelling, 1 = minor swelling around eye, 2 = moderate swelling, and 3 = severe swelling around the eye ring. Scores for both eyes were then summed for a maximum of 6 for each bird, per

sampling day. All scoring was done by a single individual while blind to the specific temperature and time treatment of the MG swab used to inoculate each bird. We also quantified pathogen load by taking conjunctival swabs on days 7, 14, and 21 post-inoculation of the bird. Using sterile forceps, the lower eyelid of each eye was gently pulled back and a cotton swab (Fisherbrand 22-363-167), moistened with 2 drops of sterile lubricating eye drops, was inserted into the conjunctiva for 5 seconds (5 turns). The swab was then swirled in 300 µL triptose phosphate broth and rung on the side of the tube prior to being discarded. All samples were then frozen at -20 °C.

Conjunctival swab samples were then thawed and 300 µL were used for genomic DNA extraction using Qiagen DNeasy 96 Blood and Tissue kits (Qiagen; Valencia, California), with a final elution volume of 100 µL. To quantify MG load in conjunctival samples, each 15 µL reaction contained 7.5 µL QuantiNova Probe Master Mix, 2.9 µL DNase-free water, 0.075 µL ROX (1:200), 3 µL extracted DNA sample, and 0.3 µL of 0.20 µM probe and 0.6 µL each of 0.4 µM forward and reverse primers specific to the *mgc2 gene* in *M. gallisepticum* (Grodio et al. 2008). We then used QuantStudio5 to cycle reactions, with 1 cycle at 95 ℃ for 2 min, and 40 cycles of 95 ℃ for 5 seconds and 60 ℃ for 30 seconds. Standard curves were generated for each run, based on 10-fold serial dilutions of plasmid that contained a 303-bp *mgc2* insert (Grodio et al. 2008). Serial dilutions consisted of $3.9x10^1$ - $3.9x10^8$ copies of plasmid. Reactions were then analyzed using Design & Analysis Software v2.6.0.

Statistical Analyses

All statistical analyses were conducted using RStudio Version 4.3.1 (R Core Team 2023), using the 'stats' (R Core Team 2023) and 'tidyverse' (Wickham et al. 2019) packages. Data were visualized using the 'ggplot2' (Wickham 2016) package. For our viable cell count assay, we used an exponential decay model (Brouwer et al. 2017) of the form $y = (a + c \cdot ind)e^{(b + d \cdot ind)t}$, where *y* is the number of color changing units per mL (CCU/mL) remaining at time *t* in days, *a* is the parameter for the initial concentration of MG, *b* is the parameter for the rate of decay, and *c* and *d* are offsets that were allowed to change each parameter according to temperature treatment. The variable, *ind*, represents a binary indicator variable that was set to 0 for the summer condition and 1 for the winter condition. This allowed for different parameters to be fit to each temperature condition. It also allowed for direct statistical comparisons of parameters between

groups, since when *c* or *d* were statistically different from 0, it could be inferred that the initial conditions or decay rates differed between the temperature treatments, respectively. Starting parameter values for the model were set such that $a = 5$ CCU/mL (log MG load+1) and *b, c,* and *d* were all set to 0. Parameter estimation was performed using the 'nlme' package in Rstudio (Pinheiro and Bates 2000). Non-linear regression models carry the same assumptions around the model residuals as linear models. Therefore, the assumptions of the non-linear model were checked graphically and we found no major deviations from the assumptions. The full set of diagnostics used to assess model assumptions can be found in the supporting material.

For our bioassay, we used a linear mixed model to examine the additive or interactive effects of temperature (summer or winter), incubation time on feeder (0, 1, 2, 4, or 7 days), and the number of days post-inoculation of the host (PID 7, 14, 21) on disease severity or pathogen load as continuous variables, with bird ID as a random effect since disease metrics of individual birds were evaluated multiple times throughout the experiment. The sex of each bird was initially included as a covariate in each model but had no significant effect on response variables and was thus removed from final models. All final models contained the main variables of temperature, incubation time on feeder, and PID because all had significant effects on response variables. Although we collected data across the entire course of infection, only three post-inoculation time points (days 7, 14, and 21) were used for analysis because 1) data for both disease severity and pathogen load were available at these points, 2) this period represents peak infection for this MG isolate in this study (Appendix S1: Fig. S1) and other studies (Grodio et al. 2012; Dhondt et al. 2017), and 3) given that host responses to this acute infection are inherently non-linear over time post-inoculation, limiting our analysis to peak timepoints allowed for more robust interpretation of effects of interest (temperature and incubation time on feeder) in interaction with post-inoculation timepoint. We tested for and confirmed the absence of overdispersion of linear model residuals using the 'DHARMa' package (Hartig 2022). In the case of disease severity, the assumption of a continuous response is technically violated given the bounded nature of the pathology score used in this analysis. However, our use of the linear model in this case is supported by previous literature (Harpe 2015). Linear mixed models were conducted using the 'lme4' package (Bates et al. 2015). We then compared additive and interactive models using likelihood ratio tests in the 'stats' package (R Core Team 2023) to identify the model with the most support. Following each analysis, we used a Type III ANOVA Wald Chi-square Test in the

'car' package (Fox and Weisberg 2019) to determine the overall significance of fixed effects on each response variable (disease severity and pathogen load). We then computed the estimated marginal means for each model using the 'emmeans' package (Lenth 2023) and obtained un-adjusted *p*-values from post-hoc contrasts between interacting predictors. Lastly, we determined the significance of each pairwise contrast using the Bonferroni correction for alpha by dividing $\alpha = 0.05$ by the relevant number of comparisons.

Results

Viable Cell Count Assay

MG incubated on feeder surfaces at colder ambient temperatures had a significantly lower rate of decay (difference = 0.7792, $SE = 0.1689$, $p < 0.0001$) over time compared to MG incubated on feeder surfaces at warm ambient temperatures (Figure 1, exponential decay: winter, $b = -0.23 \pm 0.03$ CCU/mL log[MG load+1], summer, $b = -1.01 \pm 0.17$ CCU/mL log[MG load+1]; $df = 36$). As expected, given that equivalent doses of MG were inoculated onto feeder surfaces at each temperature, we did not detect a statistical difference in the number of MG cells swabbed from ports within 30 seconds of inoculation at time 0 (difference = 0.1177, $SE = 0.4980$, $p = 0.814$; exponential decay: winter, $a = 4.24 \pm 0.30$ CCU/mL log[MG load+1]; summer, $a = 4.12 \pm 0.40$ CCU/mL log[MG load+1]; $df = 36$). Sham-control ports did not have any viable MG detected at any time point or temperature.

Disease Severity

Overall, MG incubated on feeders at colder ambient temperatures caused higher disease severity in birds compared to MG incubated at warmer temperatures, but this relationship is also influenced by the total incubation time of MG on feeders as well the time course of host infection (post-inoculation day, or PID of the host, Figure 2). Specifically, our model indicated a significant three-way interaction between temperature treatment, incubation time of MG on the feeder, and PID (ANOVA; $\chi^2 = 23.04$, $df = 8$, $p = 0.003$), with post-hoc contrasts indicating that across 15 comparisons, peak disease severity in birds (on PID 14) differed by temperature only when MG had incubated on feeder ports for four (post-hoc contrasts; $M = 3.5$, $SE = 0.99$, $df = 80.2$, $t = 3.55$, $p = 0.0006$, adjusted $\alpha = 0.003$) and seven days (post-hoc contrasts; $M = 3.67$, $SE = 0.99$, $df = 80.2$, $t = 3.72$, $p = 0.0004$, adjusted $\alpha = 0.003$). Specifically, our model estimated

that birds inoculated with MG incubated for 4 days on feeders at colder temperatures caused 15 times higher disease severity on day 14 post-inoculation than MG incubated for the same length at warm temperatures (estimated marginal means for disease severity at PID 14: winter = 3.75, summer = 0.25). All relevant post-hoc contrasts and estimated marginal mean comparisons for disease severity can be found in the supporting material (Appendix S1: Table S1, Table S2). Likelihood ratio tests indicated more support for the interactive model compared to the additive model (likelihood ratio test: $\chi^2 = 18.7$, $df = 22$, $p = 0.008$), and no additional main effects or interactions between parameters were significant (all $p > 0.05$). Non-zero eye scores were not detected in any birds inoculated with swabs from sham-control ports.

Pathogen Load

MG incubated on feeders at colder temperatures produced higher pathogen loads in inoculated birds compared to MG incubated on feeders at warmer temperatures, but the strength of this effect depended on the total length of time that MG incubated on feeder surfaces (Figure 3). Specifically, there was a significant interaction between temperature treatment and incubation time of MG on the feeder surface (ANOVA; $\chi^2 = 11.58$, $df = 4$, $p = 0.020$), with post-hoc contrasts indicating that across five comparisons, pathogen load in birds only differed by temperature when MG had incubated on feeder ports for four (estimated marginal means; $M = 2.08$, $SE = 0.64$, $df = 42.2$, $t = 3.27$, $p = 0.002$, adjusted $\alpha = 0.01$) or seven days (estimated marginal means; $M = 2.37$, $SE = 0.64$, $df = 42.2$, $t = 3.73$, $p = 0.0006$, adjusted $\alpha = 0.01$). All relevant post-hoc contrasts for pathogen load can be found in the supporting material (Appendix S1: Table S3). There were no significant interactions between other parameters (all $p > 0.05$). Fixed effects for the LMM showed a significant main effect of post-inoculation day on pathogen load ($\chi^2 = 62.44$, $df = 2$, $p < 0.0001$), but no individual effects of temperature ($\chi^2 = 0.39$, $df = 1$, $p = 0.53$) or pathogen incubation time on feeder ($\chi^2 = 3.75$, $df = 4$, $p = 0.44$). Likelihood ratio tests confirmed more support for the model that contained a pairwise interaction between temperature and pathogen incubation time, with PID as an additive variable, compared to the 3-way interactive model (likelihood ratio test: $\chi^2 = 30.88$, $df = 14$, $p = 0.006$). Birds inoculated with swabs from sham-control ports either had no MG detected ($n = 30$ total samples) or were below the maximum detected from background contamination in our sensitive qPCR assay (0.83-17.5 total copies of MG in $n = 6$ samples; see Leon and Hawley 2017).

Discussion

Fomites are a common and potentially underappreciated route of transmission for many infectious diseases (Murray et al. 2016). However, the relative importance of fomites to transmission often relies heavily on abiotic conditions (Breban 2013; Satterfield et al. 2017) such as temperature, which affect the persistence of pathogens in the environment. Here, we show that ambient temperature had strong effects on the ability of MG to remain viable and pathogenic on bird feeder surfaces, a key fomite in this system. Overall, our results suggest that colder ambient temperatures, at least within the temperature range examined here, augment the role of fomites in the MG transmission process as a whole (McCallum et al. 2017), with temperature effects on fomite survival likely contributing to seasonal disease dynamics in this system (Altizer, Hochachka, and Dhondt 2004) and others (Altizer et al. 2006; Wang et al. 2012).

First, using a culture-based assay of viability, we found that MG can remain viable on feeder surfaces for at least seven days in colder ambient temperatures, versus only up to two days in warmer ambient temperatures. For many bacterial pathogens that can survive outside of the host, persistence is often greater at colder versus warmer ambient temperatures (e.g., Brown et al. 2009; Kramer, Schwebke, and Kampf 2006). This may be due to cold temperature conditions facilitating changes in microbial cellular processes that promote greater survival, such as increased cell size (Wiebe, Sheldon, and Pomeroy 1992) and slowed metabolism (Chattopadhyay 2006). The mechanisms allowing MG to remain viable longer on feeder surfaces at colder versus warmer temperatures are outside of the scope of this study but represent an area for future research. For pathogenic mycoplasmas in poultry (Catania et al. 2024) and dairy cattle (Justice-Allen et al. 2010), increased viability in the environment may be facilitated by the formation of biofilms, which can offer protection from extreme environments and abiotic conditions (Yin et al. 2019). However, the ability of mycoplasmas to form biofilms can be highly diverse and strain dependent (H. Chen et al. 2012). For instance, Bekő et al. (2022) found that 19 of 32 strains of *Mycoplasma anserisalpingitidis* were able to form biofilms, and that those strains were more resistant to heat and desiccation relative to strains that did not form biofilms. While some strains of *Mycoplasma gallisepticum* have been shown to form biofilms (H. Chen et al. 2012), whether the strain used in this study has biofilm forming abilities that may contribute to increased survival on bird feeders remains unknown.

In our bioassay experiment, we found that MG on bird feeders remained pathogenic for significantly longer when incubated in colder versus warmer ambient temperatures, producing high levels of disease severity and pathogen load in birds after incubation on feeders of up to seven days at cold temperatures. Interestingly, temperature effects on pathogenicity were not present until MG had incubated on bird feeder surfaces for at least four days, suggesting that both temperature and the length of time that MG has been present on a feeder surface are important factors influencing pathogenicity to visiting birds. Notably, ambient temperature did not have detectable effects on pathogenicity of MG that incubated on feeder surfaces for only a short time window (1-2 days), at least within the range of experimental temperatures used here. However, expanding the range of temperatures examined to include extremes such as sub-freezing winter temperatures is critical for determining the upper and lower limits of pathogen viability, and for predicting how temperature effects on pathogen persistence scale up to influence transmission dynamics (Satterfield et al. 2017) and seasonality in the system (Altizer, Hochachka, and Dhondt 2004). Further, determining how the detected interactive effects of incubation time on feeders and temperature ultimately play out in for free-living birds requires a better understanding of the temporal and spatial heterogeneity in host contacts with feeders (S. Chen et al. 2013; Scherer et al. 2020; White, Forester, and Craft 2018), as well as an understanding of how much MG is removed from a feeder surface at each visit by a feeding bird. Overall, our results suggest that colder ambient temperatures within the range examined here could allow MG outbreaks to persist at some sites where time intervals of up to one week occur between house finch visits. This spatial heterogeneity may ultimately allow longer population-level pathogen persistence (Swinton et al. 2002), particularly that of virulent strains (Wood and Thomas 1996), at colder temperatures.

To our knowledge, this is the first study to demonstrate how ambient temperature affects the viability and pathogenicity of MG on bird feeders, which are commonly used resources for backyard birds as well as fomites for pathogen transmission (Hartup et al. 1998). Many studies of microbial survival on fomites use either culture or experimental infection to examine the effects of abiotic factors on viability, such as in cattle (Fine et al. 2011) and rabbit pathogens (Henning et al. 2005). Here, we integrated both culture-based and bioassay approaches, using identical sampling designs, allowing us to make broad comparisons between them. While incubation on fomites at colder temperatures resulted in longer culture-based viability and higher

pathogenicity to birds after several days of incubation, there were some interesting qualitative differences in the patterns observed over time for the two assays. In particular, our culture assay detected a steady decline of MG viability on feeder surfaces in cold temperatures, while our bioassay indicated that MG remained almost equivalently pathogenic to live birds after seven days of incubation on feeder surfaces. This discrepancy can result from several possibilities, including a potential dose threshold effect for pathogenic disease, whereby severe disease results whenever birds are exposed to a threshold minimum amount of viable MG (Leon and Hawley 2017). Further, the minimum dose threshold for infecting live birds may be lower than the minimum color change threshold in the culture assay, resulting in the detected qualitative differences in viability patterns. Lastly, if the strain used in this study has biofilm forming abilities, it may have also had a short-term impact on the discrepancy between MG viable titer and its ability to induce conjunctivitis when sampled from feeders. Regardless of the underlying mechanism, the detected discrepancy suggests that the results of culture-based assays alone must be interpreted with some caution, as such assays may not capture the biologically meaningful effect of variables such as temperature on hosts.

One caveat of our study design is that we only varied ambient temperature in our experiments. Diverse abiotic conditions in addition to temperature may also influence our understanding of when and where bird feeders contribute most to disease transmission. For instance, abiotic factors such as humidity (Qiu et al. 2022; Wright, Bailey, and Hatch 1968) and increased ultraviolet radiation (Oppezzo, Costa, and Pizarro 2011) have been shown to negatively affect bacterial cell growth and survival. Because these abiotic factors are often correlated with temperature (e.g., Häder et al. 2015; Mbithi, Springthorpe, and Sattar 1991), it is particularly important to examine how covariation in ambient conditions, such as temperature and daylight, influence pathogen viability on feeder surfaces in more natural conditions. Further, our study used a single, well-characterized strain of MG (NC2006) that is known to be virulent in house finches, but future studies should examine temperature effects for other MG strains of variable geographic origin and virulence, including strains directly isolated from wild birds with conjunctivitis. Previous studies show a rapid increase in virulence among songbird strains of MG since emergence in house finches (e.g., Bonneaud et al. 2018; Hawley et al. 2013). Work on other pathogenic microbes hypothesizes that virulence can relate to pathogen persistence in the environment (Walther and Ewald 2004) or to pathogen thermal tolerance. For example, Ashrafi

et al. (2018) found that the fish pathogen, *Flavobacterium columnare*, has increased in virulence since emergence, but these increases were associated with marked decreases in pathogen thermal tolerance. In addition to variation in virulence, MG strains isolated from states with diverse winter conditions (with at least 227 isolates of MG collected from 17 states to date; Ley et al. 2016), may also differ in thermal tolerance as an adaptation to survive outside the host in varying ambient conditions. For instance, Tian et al. (2022) determined that constant warming and temperature fluctuations in the environment were associated with thermal adaptation in widely distributed soil bacteria. Thus, future studies of MG strains that differ in both virulence and geographic origin are needed to determine whether temperature effects on fomite transmission covary with aspects of strain biology in this system.

Overall, our study indicates that cold ambient temperatures strongly influence the ability of MG to remain viable and pathogenic over time on feeder surfaces. Importantly, under natural conditions, the effects of cold temperatures on MG outside the host shown here may be further compounded by effects of cold temperatures on other aspects of the transmission process. Winter conditions are particularly challenging for small-bodied endotherms, such as birds, since they must maintain high metabolic demands at a time when natural food sources are scarce. As a result, house finches increase their time spent at supplemental feeding stations (Bonter et al. 2013; Teemer and Hawley 2024) and increase food intake (Adelman et al. 2013) in colder ambient temperatures. Given that cold temperatures also lengthen pathogen survival on feeder surfaces, as demonstrated here, this could provide longer windows of time for susceptible individuals to encounter pathogens (Murray et al. 2016) and for infected individuals to deposit MG onto feeder surfaces (Adelman et al. 2015) at times of the year when feeders are heavily used to meet thermoregulatory demands. Therefore, future studies should consider how temperature-induced pathogen survival on fomites may interact with other aspects of the transmission process, including feeder use by healthy (Adelman et al. 2013; Altizer, Hochachka, and Dhondt 2004) and infected hosts (Hawley et al. 2012; Teemer and Hawley 2024).

We show that variation in temperature could have key downstream implications for transmission in this system and many others (Sipari et al. 2022). Additionally, we provide insight into one of many links underlying the complex relationship between annual environmental variation and seasonal disease dynamics (Altizer et al. 2006). However, wide scale climate warming has already altered the current patterns of seasonality of several infectious diseases and

is expected to continue rapidly (Harvell et al. 2009; Lindgren et al. 2012). Thus, understanding the mechanisms that shape seasonality is not only important for our ability to predict seasonal outbreaks, but is also urgent in the face of global climate change (Lafferty 2009).

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Tables

Table 1. Number of replicate feeder port inoculations used in Experiment 1.

	Summer (22-27 ℃)		Winter (4-9 ℃)	
Incubation Time on Feeder	MG	Sham control	MG	Sham control
0 Days	2	1	2	1
1 Day	4	1	4	1
2 Days	5	1	5	1
4 Days	5	1	5	1
7 Days	4	1	4	1

Equal volumes of *Mycoplasma gallisepticum* (MG) or control sterile media were incubated on feeder ports for variable lengths of time at one of two night-day temperature regimes representing summer and winter. Swabs from each feeder port were used to measure culture viability of MG *in vitro*.

Table 2. Sample sizes of birds per temperature and incubation treatment (n = 68 birds) for Experiment 2.

	Summer (22-27 ℃)		Winter (4-9 ℃)	
Incubation Time on Feeder	MG	Sham control	MG	Sham control
0 Days	4	2	4	2
1 Day	6		6	
2 Days	6	2	6	2
4 Days	6		6	
7 Days	6	2	6 (5M, 1F)	2 (2F)

All treatments had equal male:female sex ratios, except where indicated (sex ratios in parentheses: M = males, F = females).

Figures

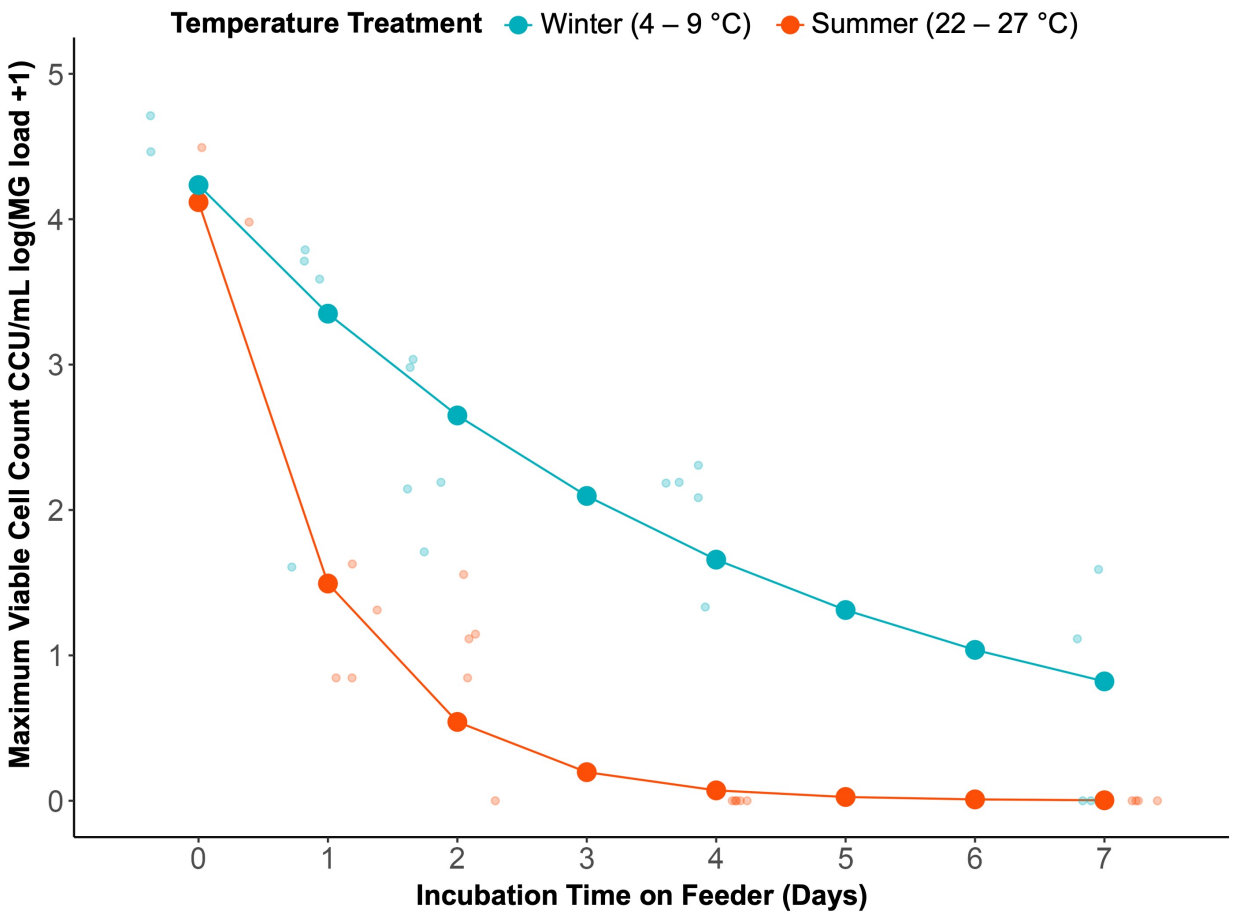

Figure 1. *Mycoplasma gallisepticum* (MG) had higher viability on feeder surfaces over time in colder temperatures compared to MG incubated on feeder surfaces held in warmer conditions. Predicted values from the exponential decay model are shown as connected circles and replicate feeder ports are shown as smaller circles. Sham control ports, which did not have viable MG detected at any time point or temperature, are not shown.

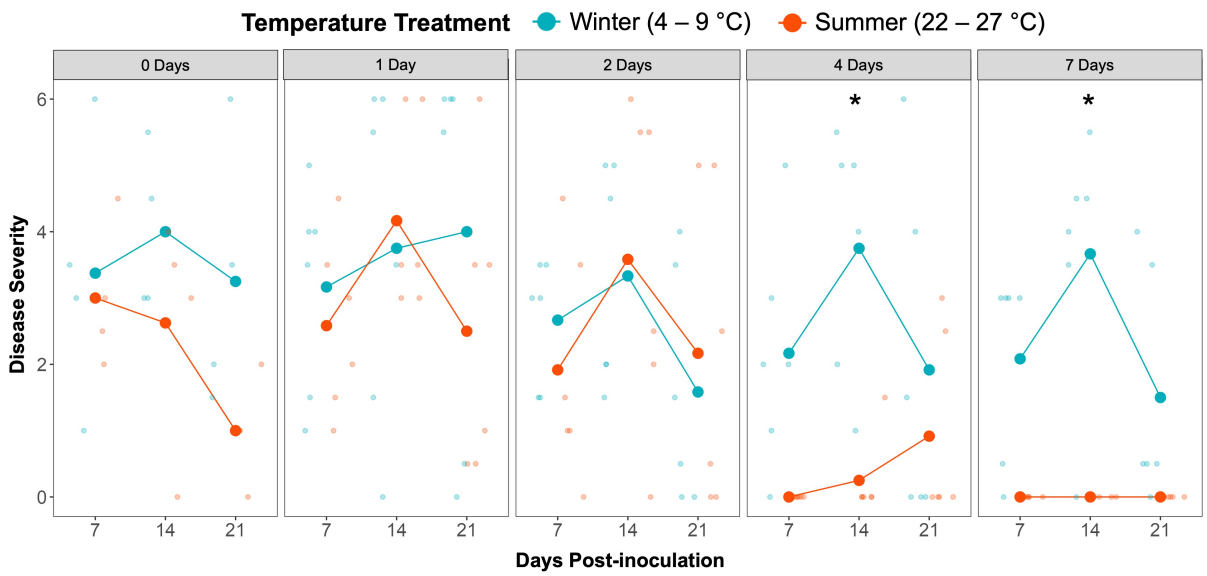

Figure 2. After longer incubation times (4 or 7 days) on feeder surfaces, *Mycoplasma gallisepticum* (MG) incubated in colder temperatures produced higher disease severity in pathogen-naive birds over the course of peak infection (days 7, 14, and 21 post-inoculation) compared to MG incubated on feeders in warmer temperatures. Facet labels show the length of incubation for MG on feeder surfaces (days 0, 1 2, 4, or 7), and the feeder incubation temperature for each MG sample is denoted by color. Predicted values are shown as connected circles and raw data is shown as smaller circles. Significant post-hoc pairwise comparisons across temperature treatment, PID, and incubation time are indicated by asterisks. Birds inoculated with swabs from sham control ports are not shown to better visualize temperature effects, but these birds did not have detectable disease. Note that we converted the predicted value to zero in the one instance when the model predicted value for disease severity was negative (Summer, Incubation Day 4, PID 7, predicted value = -8.88×10^{-16}).

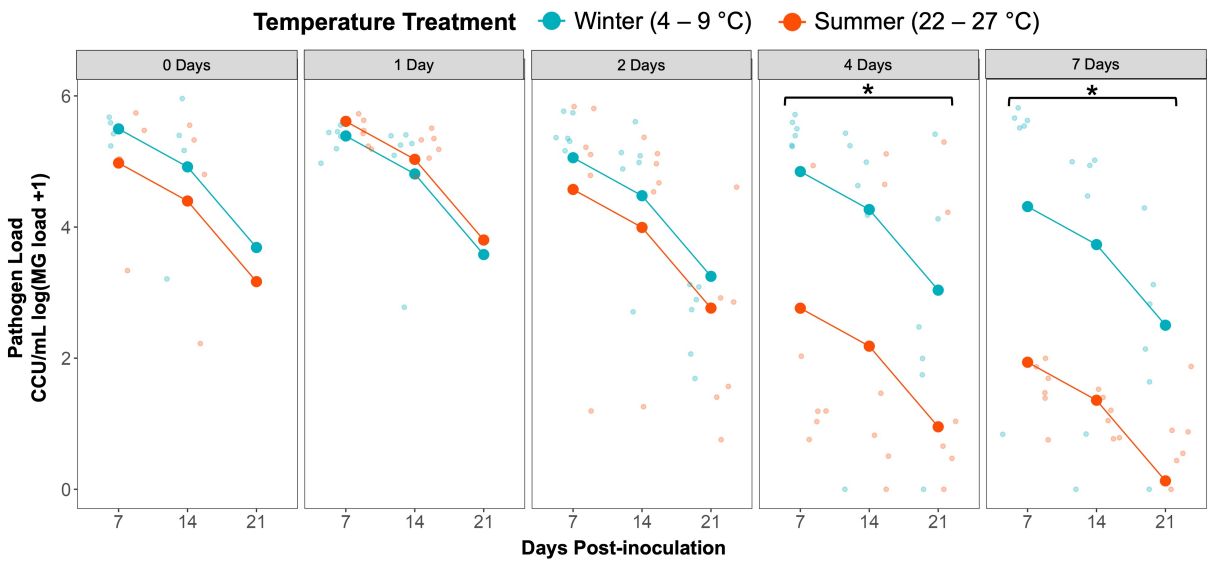

Figure 3. After longer incubation times (4 or 7 days) on feeder surfaces, *Mycoplasma gallisepticum* (MG) incubated on feeders at colder temperatures was associated with higher pathogen loads in birds at peak infection (days 7, 14, and 21), compared to MG incubated on feeders at warmer temperatures. Facet labels show the length of incubation for MG on feeder surfaces (days 0, 1 2, 4, or 7), and feeder incubation temperature for each MG sample is denoted by color. Significant post-hoc pairwise comparisons across temperature treatment and incubation time are indicated by asterisks. Predicted values are shown as connected circles and raw data is shown as smaller circles. Birds inoculated with swabs from sham control ports are not shown here to better visualize temperature effects on MG treatments.

Appendix

Disease Severity

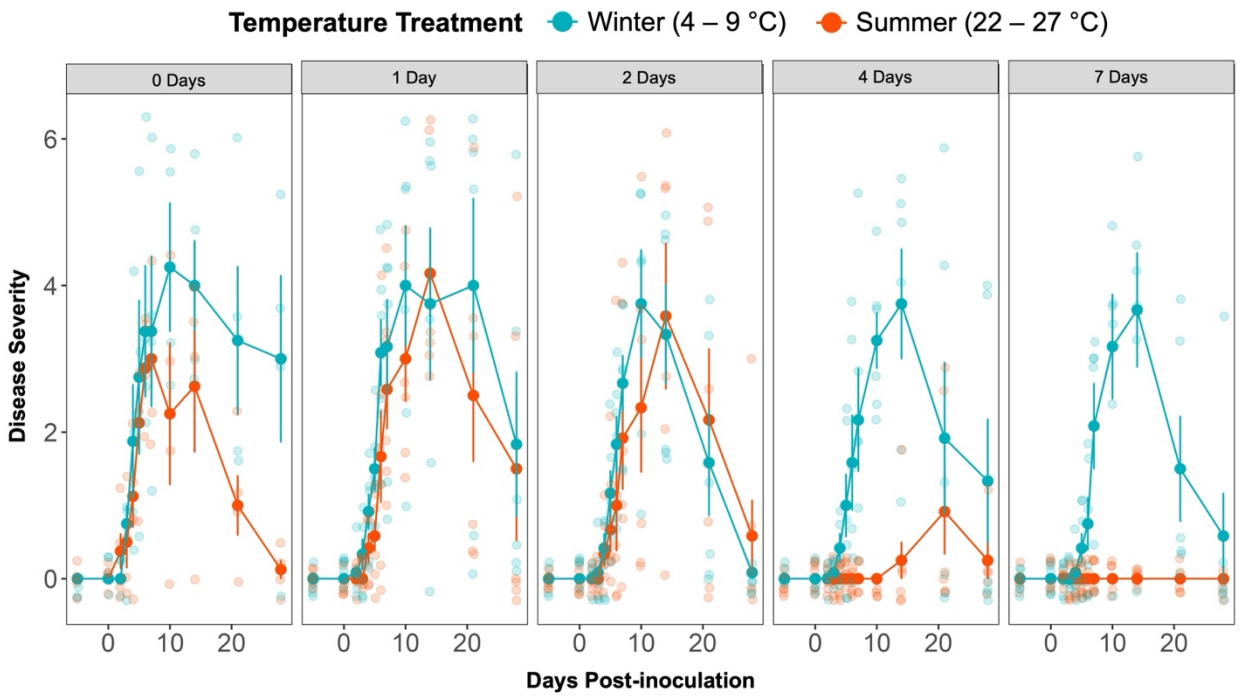

Figure S1. Disease severity in house finches, inoculated with *Mycoplasma gallisepticum* (MG) incubated on bird feeders, was highest 7-21 days after inoculation in this experiment. Facet labels show the amount of time MG incubated on feeder surfaces, and feeder incubation temperature (summer = red, winter = blue) is denoted by color. Mean and standard error are shown as solid points connected with a line, with raw values shown as translucent points.

Table S1. Estimates for pairwise comparisons between interacting predictors (temperature*incubation day*post-inoculation day [PID]) influencing disease severity, with standard errors (*SE*), degrees of freedom (*df*), t-values (*t*), and p-values (*p*). All contrasts are comparisons between winter and summer treatments. Significance was assessed using the Bonferroni adjustment for alpha across 15 comparisons (new alpha = 0.003) and indicated in bold.

Incubation Day	PID	Estimate	*SE*	*df*	*t*	*p*
Day 0	7	0.3750	1.207	80.2	0.311	0.7568
	14	1.3750	1.207	80.2	1.139	0.2580
	21	2.2500	1.207	80.2	1.864	0.0659
Day 1	7	0.5833	0.985	80.2	0.592	0.5556
	14	-0.4167	0.985	80.2	-0.423	0.6736
	21	1.5000	0.985	80.2	1.522	0.1319
Day 2	7	0.7500	0.985	80.2	0.761	0.4488
	14	-0.2500	0.985	80.2	-0.254	0.8004
	21	-0.5833	0.985	80.2	-0.592	0.5556
Day 4	7	2.1667	0.985	80.2	2.199	0.0308
	14	**3.5000**	**0.985**	**80.2**	**3.552**	**0.0006**
	21	1.0000	0.985	80.2	1.015	0.3133
Day 7	7	2.0833	0.985	80.2	2.114	0.0376
	14	**3.6667**	**0.985**	**80.2**	**3.721**	**0.0004**
	21	1.5000	0.985	80.2	1.522	0.1319

Table S2. Comparison of estimated marginal means (winter vs. summer) between interacting predictors (temperature*incubation day*post-inoculation day [PID]) influencing disease severity, with lower and upper 95% confidence intervals (CI).

Incubation Day	PID	Temperature	Mean	*SE*	*df*	Lower CI	Upper CI
Day 0	7	Winter	3.3750	0.8534	80.2	1.6767	5.0733
		Summer	3.0000	0.8534	80.2	1.3017	4.6983
	14	Winter	4.0000	0.8534	80.2	2.3017	5.6983
		Summer	2.6250	0.8534	80.2	0.9267	4.3233
	21	Winter	3.2500	0.8534	80.2	1.5517	4.9483
		Summer	1.0000	0.8534	80.2	-0.6983	2.6983
Day 1	7	Winter	3.1667	0.6968	80.2	1.7800	4.5533
		Summer	2.5833	0.6968	80.2	1.1967	3.9700
	14	Winter	3.7500	0.6968	80.2	2.3633	5.1367
		Summer	4.1667	0.6968	80.2	2.7800	5.5533
	21	Winter	4.0000	0.6968	80.2	2.6133	5.3867
		Summer	2.5000	0.6968	80.2	1.1133	3.8867
Day 2	7	Winter	2.6667	0.6968	80.2	1.2800	4.0533
		Summer	1.9167	0.6968	80.2	0.5300	3.3033
	14	Winter	3.3333	0.6968	80.2	1.9467	4.7200
		Summer	3.5833	0.6968	80.2	2.1967	4.9700
	21	Winter	1.5833	0.6968	80.2	0.1967	2.9700
		Summer	2.1667	0.6968	80.2	0.7800	3.5533
Day 4	7	Winter	2.1667	0.6968	80.2	0.7800	3.5533
		Summer	0.0000	0.6968	80.2	-1.3867	1.3867
	14	Winter	3.7500	0.6968	80.2	2.3633	5.1367
		Summer	0.2500	0.6968	80.2	-1.1367	1.6367
	21	Winter	1.9167	0.6968	80.2	0.5300	3.3033
		Summer	0.9167	0.6968	80.2	-0.4700	2.3033
Day 7	7	Winter	2.0833	0.6968	80.2	0.6967	3.4700
		Summer	0.0000	0.6968	80.2	-1.3867	1.3867
	14	Winter	3.6667	0.6968	80.2	2.2800	5.0533
		Summer	0.0000	0.6968	80.2	-1.3867	1.3867
	21	Winter	1.5000	0.6968	80.2	0.1133	2.8867
		Summer	0.0000	0.6968	80.2	-1.3867	1.3867

Pathogen Load

Table S3. Estimates for pairwise comparisons between interacting predictors (temperature*incubation day) influencing pathogen load, with standard error (*SE*), degrees of freedom (*df*), t-value (*t*), and p-values (*p*) for each comparison. Significance was assessed using the Bonferroni adjustment for alpha across 5 comparisons (new alpha = 0.01) and indicated in bold.

Incubation Day	Estimate	*SE*	*df*	*t*	*p*
Day 0	0.5205	0.834	54.9	0.624	0.5349
Day 1	-0.2224	0.681	54.9	-0.327	0.7450
Day 2	0.4842	0.637	42.2	0.760	0.4513
Day 4	**2.0843**	**0.637**	**42.2**	**3.273**	**0.0021**
Day 7	**2.3744**	**0.637**	**42.2**	**3.729**	**0.0006**

Chapter 4: Colder temperatures augment some but not all mechanisms of pathogen transmission in house finches

Sara R. Teemer and Dana M. Hawley

Prepared for submission to *Functional Ecology*

Abstract

Seasonal variation in ambient temperature can augment diverse mechanisms of pathogen transmission. However, mechanistic studies examining effects of temperature on transmission remain limited in vertebrate disease systems, particularly for endotherms, which often simultaneously balance the energetic costs of thermoregulation and infection in colder temperatures. Here, we test the effects of temperature on transmission in wild-caught, but captive-held house finches (*Haemorhous mexicanus*). These small songbirds are hosts for the pathogen *Mycoplasma gallisepticum* (MG), which causes fall and winter outbreaks of mycoplasmal conjunctivitis. We pair-housed wild-caught birds in environmental chambers set to night-day thermoneutral (22-27 °C) or subthermoneutral temperatures (4-9 °C). The male bird in each mixed-sex pair, termed the "donor", was experimentally inoculated with MG (or sham media), and transmission to the female "receiver" bird in each pair was monitored over the course of infection. First, because colder temperatures can influence infectiousness and recovery in endotherms, we measured the degree of disease severity and pathogen load in donor males. We found that donor males in colder temperatures were slower to recover from infection relative to birds in warmer temperatures, resulting in birds remaining infectious for longer at colder ambient temperatures. Second, we quantified feeding behavior as a transmission-relevant contact in this system that is likely to increase at colder temperatures due to thermoregulatory demands. We found that receiver birds, but not donor birds, spent more time at the feeder in colder temperatures, suggesting that contact rates of susceptible birds with infectious fomites are more likely at colder versus warmer temperatures. We then used both the timing of infection for receiver birds at each temperature and donor bird pathogen loads to estimate the maximum exposure dose of MG for receiver birds at the time of initial exposure. We estimated that receiver birds housed at colder temperatures were exposed to significantly higher exposure doses of MG relative to birds housed at warmer temperatures. Despite the detected temperature effects on

infectiousness, behavior, and exposure dose, we did not find support for temperature effects on the likelihood and speed of transmission, or the proliferation of MG within receiver birds. This suggests that, at least in our captive experimental system where donor and receiver birds were in continuous close contact, the detected effects of temperature on various mechanisms of spread did not notably influence transmission. However, the way in which the detected effects of temperature on infectious period length and contact rates play out in the wild remain unknown. Our results support the need for more mechanistic studies of the often complex effects of temperature on transmission in wildlife disease systems.

Introduction

Infectious diseases are shaped by the transmission events that occur as a result of dynamic interactions between hosts, pathogens, and abiotic factors (reviewed in Dowell, 2001). Ambient temperature, in particular, has been shown to affect diverse aspects of pathogen transmission, such as host or vector infectiousness (Bayoh & Lindsay, 2003), host behavior (Shocket et al., 2019), and environmental pathogen survival (Pascual et al., 2002; Riddell et al., 2020), often in ways that alter pathogen success (Lafferty & Kuris, 1999; McCallum et al., 2017; VanderWaal & Ezenwa, 2016). Experimental studies of how temperature influences the mechanistic components of the transmission process (e.g., Paull & Johnson, 2011; Shapiro et al., 2017; Shocket et al., 2019) are thus necessary for understanding how temperature shapes both the strength and timing of transmission in ways that could scale up to influence seasonal disease dynamics. To date, mechanistic studies of temperature effects on transmission have largely been limited to invertebrate and vector-borne disease systems (e.g., Dallas & Drake, 2016; Johnson et al., 2015; Mordecai et al., 2013). However, because temperature also has the potential to shape transmission-relevant traits in vertebrate systems, a group facing conservation threats from disease (e.g., white-nose syndrome in bats, Langwig et al., 2015; Verant et al., 2012) and serving as key reservoir hosts for many zoonotic pathogens (Dearing & Dizney, 2010; Plowright et al., 2017), mechanistic studies in these systems are critical for our understanding of the broader implications of temperature on transmission dynamics.

Temperature has the potential to alter certain aspects of transmission differently for endothermic vertebrate systems because of the metabolic costs associated with thermoregulation. At colder ambient temperatures below the thermoneutral zone (i.e., within the subthermoneutral

zone), endotherms implement energy-expensive methods to maintain internal body temperatures, which can influence both host immunity and behavior. For example, Demas and Nelson (1998) found that in deer mice, *Peromyscus maniculatus,* colder ambient temperatures and food restriction were associated with reduced immune function, increasing the potential for pathogen success during winter. As a result, the strength of the immune response may vary with temperature and season (e.g., Kokolus et al., 2013; Martin et al., 2010; Sandmeier et al., 2016), leading to potential differences in pathogen loads or disease severity for infected hosts (J. F. W. Chan et al., 2022). Similarly, ambient temperature can influence host body temperature (D. P. Anderson & Hanson, 1965), fever responses to infection (Owen-Ashley & Wingfield, 2007), and pathology (Hawley et al., 2012), which could affect the ability of hosts to successfully recover and the subsequent length of the infectious period. Overall, any temperature-induced heterogeneity in the strength or duration of infectiousness among hosts will ultimately influence the amount of pathogen shed by an infected host (e.g., Hershberger et al., 2013; Kang et al., 2024), and thus the amount of pathogen that susceptible hosts are potentially exposed to.

The energetic costs of thermoregulation for endotherms also lead to key changes in the behavior of susceptible hosts. To compensate for such energy costs, endotherms often respond through modifying behavior, such as huddling (Beal, 1978), moving to warmer areas (Milling et al., 2017), or changing foraging patterns (LaRocque et al., 2024; Parikh et al., 2021). These compensatory mechanisms, however, may also increase opportunities for contact between hosts or with pathogens in the environment. While the importance of temperature for host behaviors relevant to pathogen spread has been well-documented for invertebrates (e.g., Elderd & Reilly, 2014; Shocket et al., 2019), few studies have examined how temperature alters transmission-relevant behaviors in vertebrate systems (but see Roznik & Alford, 2015), particularly behavior in small endothermic vertebrates. Meeting high metabolic demands in cold temperatures is especially challenging for small songbirds since a high surface area-to-volume ratio increases rates of heat loss (Dawson et al., 1985). As a result, colder ambient temperatures lead some songbird species to increase food intake and spend more time at backyard bird feeders, which provide easy access to nutrient rich food year-round (Adelman et al., 2013; Bonter et al., 2013). Increased feeder use at colder temperatures, however, could potentially increase contact rates between individuals at the feeder, and both the deposition and acquisition of pathogens from feeder surfaces (Adelman et al., 2013, 2015; Murray et al., 2016). In contrast, contact rates could

remain unaffected by temperature if infected birds express sickness behaviors, such as lethargy and anorexia, and spend less time at bird feeders as a result (Ruden & Adelman, 2021). The interactions between temperature and the behaviors of healthy (Abram et al., 2017; Bateson & Laland, 2013; Bro-Jørgensen et al., 2019; Shocket et al., 2019) and sick individuals (Aubert et al., 1997; Martin et al., 2010; Teemer & Hawley, 2024) are challenging to study and thus are often overlooked in mechanistic studies, despite the potential for such behavioral differences to have downstream implications for the transmission process (Stockmaier et al., 2021).

One approach to understanding how the complex relationships between temperature, hosts, and pathogens contribute to transmission is by examining these interactions in a wild-caught, but controlled, captive system (Altizer et al., 2006). House finches (*Haemorhous mexicanus*) are highly social songbirds that experience fall and winter outbreaks of mycoplasmal conjunctivitis, a disease caused by the bacterial pathogen *Mycoplasma gallisepticum* ([MG], Dhondt et al., 2005; Ley et al., 1996). Infection with MG is typically characterized by severe conjunctival lesions (Kollias et al., 2004; Ley et al., 1996) that correlate with pathogen loads (Hawley et al., 2013) and pathogen deposition onto bird feeders (Adelman et al., 2013), which are an important source of spread for this pathogen (Adelman et al., 2015). Recent work documented strong effects of ambient temperature on the ability of MG to remain viable and pathogenic to house finches while on bird feeder surfaces (Teemer et al., 2024), suggesting that temperature is important for pathogen survival outside of the host.

Temperature is likely to be important for several other aspects of transmission in this system (Figure 1). First, for sick birds, who experience additive metabolic costs of thermoregulation and infection (Hawley et al., 2012), cold ambient temperatures could influence immune responses in ways that alter the duration of shedding or amount of MG they shed to conspecifics or onto common surfaces (Figure 1A). Second, at colder temperatures, house finches often alter transmission-relevant behaviors (Figure 1B) by eating more food (Adelman et al., 2013) and spending more time at bird feeders (Bonter et al., 2013), which could increase opportunities for direct interactions between susceptible and infected conspecifics (Murray et al., 2016; Ruden & Adelman, 2021). Increased feeding at colder temperatures could also facilitate contacts with bird feeders, increasing exposure of susceptible birds to contaminated feeder surfaces under temperature conditions where MG is shown to better maintain viability outside of the host for longer periods (Teemer et al., 2024). Together, the infectiousness and behavior of

sick “donor” birds under distinct temperatures, and the timing of exposure of susceptible “receiver” birds (which is potentially a complex result of susceptible bird behavior, donor bird infectiousness, and receiver host susceptibility) will determine the maximum dose of pathogen that a susceptible bird was likely exposed to at a given temperature (Figure 1C).

To test these relationships, we performed pairwise transmission trials at ambient temperatures either below or within the thermoneutral zone of house finches. We evaluated how temperature influenced diverse aspects (donor infectiousness, behavior, and estimated exposure dose) of the transmission process, and then determined whether such effects scaled up to influence overall transmission success of MG (Figure 1D and Figure 1E). Because of prior work indicating house finches increase their feeding behavior and that MG survives for longer on feeder surfaces (Teemer et al., 2024; Teemer & Hawley, 2024) at colder versus warmer temperatures, we predicted that colder temperatures would influence both the process and outcomes of transmission, with implications for the seasonal patterns observed in the wild.

Methods

Experimental Design

We tested effects of ambient temperature on components of MG transmission using experimental pairs of finches housed at distinct ambient temperature conditions. We inoculated one bird per pair with MG or control media (n = 19 pairs in each temperature treatment) and tracked transmission to the other member of the pair. Birds were housed in male-female pairs in walk-in environmental chambers within (thermoneutral: 22°C [night] – 27°C [day]) or below the thermoneutral zone (subthermoneutral treatment: 4°C [night] – 9°C [day]). We inoculated the male bird in each pair (the “donor” index bird) with MG (thermoneutral, n = 15 pairs; subthermoneutral, n = 14 pairs) or sham control media (thermoneutral, n = 5 pairs; subthermoneutral, n = 4 pairs), selecting males as the donors because epidemics appear to spread faster when initiated by male house finches (Adelman et al., 2015; Sauer et al., 2024). We then monitored MG transmission to female receiver birds for 32 days after initial inoculation of the male donor bird.

We determined how ambient temperature affected several steps of the pairwise transmission process (Figure 1). First, we measured disease severity and pathogen load in the donor bird to estimate how ambient temperature influenced several proxies of MG infectiousness

(conjunctival pathogen loads, disease severity, and recovery probability) of donor birds (Figure 1A). We then measured how temperature influenced the amount of time that both donor birds and receiver birds spent at the bird feeder (Figure 1B), an important source of transmission in the house finch-MG system (Adelman et al., 2015; Dhondt et al., 2007). Third, we estimated how temperature affected a proxy for the maximum MG exposure dose of receiver birds by quantifying the pathogen load of the donor bird on the day MG was estimated to have spread to the receiver bird (Figure 1C). We then measured effects of temperature on several outcomes of the above pathways, including overall transmission success across treatments (Figure 1D), and resulting disease severity and pathogen load of the receiver bird (Figure 1E).

Bird Capture and Housing

We captured 78 hatch-year house finches (aged by plumage following Pyle, 2022) using wire feeder traps and mist nets around Blacksburg, Virginia between June and August 2021. The use of hatch-year birds was important to minimize any prior exposure to MG in the wild. Birds were quarantined for the first two weeks following capture and housed in indoor animal rooms on the campus of Virginia Tech in groups of one or two at standard vivarium temperatures (20-22°C). During quarantine and throughout the duration of the experiment, birds were treated with prophylactic medications in their water to prevent Trichomoniasis (Endocox, 2.5% Toltrazuril) and coccidiosis (Dimetridazole B,P (vet) 40% m/m powder), diseases known to cause mortality in captive finch colonies. Details of this treatment can be found in Gregory et al. (2024). Every 3-4 days during quarantine, birds were monitored for signs of conjunctivitis. On days 14-18 after capture, birds that did not develop clinical signs of MG infection were bled to confirm seronegativity via Enzyme Linked Immunosorbent Assays (ELISA commercial IDEXX kit 99-06729) using established methods (Hawley et al. 2011). Only birds that were seronegative and showed no signs of conjunctivitis were used in the experiment. All capture, handling, and housing of birds was conducted with permission from the United States Fish and Wildlife Service (MB158404), Virginia Department of Game and Inland Fisheries (066646), and under approved Institutional Animal Care and Use Committee protocols.

Temperature Selection

Because we were interested in how ambient temperature alters host energetics and behavior in ways relevant to MG transmission, we selected experimental temperatures shown to be either within or below the thermoneutral zone of house finches (Hawley et al. 2012; Dawson et al. 1985; Root et al. 1991). In subthermoneutral versus thermoneutral ambient temperatures, house finches experience changes in several variables relevant to MG transmission, including food consumption (Adelman et al. 2013), time spent at feeders (Bonter et al. 2013; Teemer and Hawley 2024), and metabolic rate, MG-induced pathology, and circulating plasma levels of IL-6 (Hawley et al. 2012). Additionally, the subthermoneutral ambient temperatures used here increase the survival of MG on contaminated bird feeders, relative to thermoneutral temperatures (Teemer et al. 2024), which has key implications for fomite-based transmission in this system. Finally, the subthermoneutral temperatures used here fall within the minimum range of temperatures that house finches in southwest Virginia experience in the autumn and winter, which is important for extrapolating observed effects to transmission in the wild (3.3 to 16.7 °C in October 2021, National Centers for Environmental Information, 2024).

At least 18 days prior to the start of the experiment, male-female pairs were transferred to and housed in one of two walk-in environmental chambers based on their randomly assigned temperature treatment. Pairs were housed in identical wire cages (76 x 46 x 46 cm) under a 12h dark:12h light cycle. Birds were provided with water and a 1:4 mixture of sunflower hearts and Roudybush Maintenance Diet (Roudybush Inc., Woodland, CA, USA) *ad libitum* in tube-style bird feeders (with only one port accessible) in each cage. We chose male-female pairings for the experiment because the study was conducted in autumn, a period when house finches associate in mixed-sex flocks (Altizer et al., 2004) and are still photorefractory (Hamner, 1968), such that seasonal reproductive behavior should not influence pairwise interactions.

To facilitate gradual acclimation to experimental temperatures, birds in the subthermoneutral treatment group were initially housed at 26 °C (day/night) and birds in the thermoneutral treatment group were housed at 27 °C (day/night). Every 1-2 days, nighttime and daytime temperatures were incrementally lowered by 1-3 °C until all birds reached experimental temperatures by 3 days prior to inoculation at latest. Birds were housed at experimental temperatures for the remainder of the experiment (thermoneutral: 22 °C [night] – 27 °C [day]; subthermoneutral: 4 °C [night] – 9 °C [day]).

Inoculation

On inoculation day (Post-inoculation Day [PID] 0), we inoculated the male house finch in each pair with either a high dose of MG (n = 14 subthermoneutral, n = 15 thermoneutral) or an equal volume of sterile media as a sham control (n = 5 subthermoneutral, n = 4 thermoneutral). Each conjunctiva of the donor bird was inoculated via droplet instillation with ~35 µL (70 µL total) of 5 x 10^4 color changing units/mL MG (NC2006 2006.080-5 4P 7/26/12) or sterile Frey's media. Following inoculation, males were immediately returned to their cages with their female cagemate.

Disease Severity

We scored disease severity of donor and receiver birds at numerous points throughout the course of the experiment (PIDs -4, 4, 5, 7, 9-18, 21, 23, 25, 28, 30, 32), but focused our analyses on PIDs 4-28 for donor birds since it best captured peak infection and potential recovery (Dhondt et al., 2017; Grodio et al., 2012). Similarly, we shifted the analyses to PIDs 7-32 for receiver birds since these birds were exposed to MG through the donor bird, and thus we expected a delay in the peak infection period. At each sampling point, each eye was given a score from 0-3 in 0.5 increments, following Hawley et. al (Hawley et al., 2011). An eye score of 0 = no swelling around the eye ring, 1 = minor swelling, 2 = moderate swelling, and 3 = severe swelling, for a maximum score of 6 per bird, per sampling day. While it was not possible to be blind to temperature treatment, all eye scoring was conducted by one individual who was blind to the MG or sham treatment of each bird. To minimize incidental transmission between birds due to handling, the receiver bird in each cage was always caught with clean gloves prior to the donor bird.

Pathogen Load

On PIDs 4, 7, 14, and 21 we also took conjunctival swabs from donor and receiver birds to quantify pathogen load. Using sterile forceps, we gently pulled back the lower eyelid of each eye and inserted a saline-moistened cotton swab (Fisherbrand, Pittsburgh, PA, USA) into the conjunctiva for 8 seconds (5 turns). Each swab was swirled in 300 µL of tryptose phosphate broth, rung on the side of the tube, and discarded. All samples were frozen at -20 °C until use.

All birds were also swabbed with a saline-moistened flocked swab on PIDs -1 and 9 for microbiome characterization. These swabs (from the thermoneutral treatment) were used in an adjacent study examining effects of the ocular microbiome on pathogen transmission. Results from that experiment can be found in Gregory et al. (2024).

Conjunctival swab samples were later thawed and used for genomic DNA extraction (Qiagen DNeasy 96 Blood and Tissue Kit; Qiagen, Valencia, California). We used 15 µL quantitative polymerase chain reactions (qPCR) to determine the MG load within each conjunctival sample, using methods described in Gregory et al. (2024). Each reaction contained 3 µL extracted DNA sample, 2.9 µL DNase-free water, 7.5 µL QuantiNova Probe Master Mix, 0.075 µL ROX (1:200), 0.3 µL of 0.20 µM probe, and 0.6 µL of both 0.4 µM forward and reverse primers specific to the *M. gallisepticum* gene, *mgc2* (Grodio et al., 2008). We then cycled reactions using QuantStudio5 at 95 ℃ for 2 minutes, followed by 40 cycles at 95 ℃ for 5 seconds, with the final cycle at 60 ℃ for 30 seconds. We generated standard curves for each run using 10-fold serial dilutions containing 3.9×10^1 - 3.9×10^8 copies of plasmid with a 303-bp *mcg2* insert (Grodio et al., 2008). Design & Analysis software v2.6.0 was then used to analyze the reactions.

Behavioral Assessment

Since the behavior of both sick and healthy individuals can be influenced by temperature, and in turn, play a key role in transmission potential (Teemer & Hawley, 2024), we recorded 1 hour of behavior on PID 10, during the peak transmission period (PID 7-14, Dhondt et al., 2008). We analyzed 30 minutes of this recording, beginning 15 min after cameras were powered on and human observers left the room, until 15 minutes prior to cameras being turned off. We focused on the amount of time each bird spent at the bird feeder because this common food source has been shown to be an important source of transmission, either indirectly (though MG deposited on to the feeder surface) or directly between birds (Adelman et al., 2015; Dhondt et al., 2007; Hawley et al., 2023). Following Moyers et al. (2015), we measured the amount of time each focal bird (donor or receiver) was within pecking distance from the exposed feeder port regardless of whether it was eating, with a maximum possible duration of 1800s (the length of the observation). All behavioral videos were analyzed using time budget behavioral analysis software (BORIS, Friard & Gamba, 2016).

Statistical Analyses

All statistical analyses were performed using R version 4.4.1 (R Core Team, 2024). Data were visualized using the 'ggplot' package (Wickham, 2016). When appropriate to test for overall significance of fixed effects, we used Type III ANOVA Wald Chi-Square Tests in the 'MASS' package (Fox & Weisberg, 2019). We then used the *AIC()* function in base R to calculate Akaike Information Criterion (AIC) values, which were then used to select the final model (R Core Team, 2024). All sham control donor birds and their cagemates had no detectable eye scores and were below the maximum amount of background contamination detected in our sensitive qPCR assays (log10 0.82-2.2 copies in $n = 9$ samples; see Leon and Hawley [2017]) and were thus not included in subsequent analyses (but see *Behavioral Analysis*). Finally, we used the 'DHARMa' package (Hartig, 2022) to confirm the absence of overdispersion in all our linear models.

I. Donor Bird Infectiousness

Because past work has shown that both conjunctival pathogen load and disease severity predict the relative amount of MG deposited onto bird feeders (Adelman et al., 2013) and the likelihood of spread to flockmates (Hawley et al., 2023; Ruden & Adelman, 2021), we analyzed how temperature affected pathogen loads and disease severity of donor birds, as well as the resolution of disease over time, a proxy for the length of the infectious period under different ambient temperatures. We used a linear mixed model in the 'lme4' package (Bates et al., 2015) to determine how temperature affects these proxies for the amount of MG that a donor bird may contribute to its cagemate, either directly to the receiver bird or indirectly on feeder surfaces. Because pathogen loads and disease change over time for this acute infection, our model examined the additive or interactive effects of temperature and post-inoculation day (PID) on donor bird disease severity and pathogen load, respectively, with bird ID as a random effect. Given the bounded nature of the pathology score used in this study, our linear approach for disease severity in this objective (and also in *IV. Pathogen Success – Established Load*) technically violates the assumption of a continuous response. However, the use of linear models has been supported by previous literature (Harpe, 2015).

We additionally examined whether temperature predicted the probability of donor bird recovery over time, which will influence the length of the infectious period. Because all donor

birds were confirmed to have been diseased at some point after inoculation (i.e., to have eye scores > 0), we conservatively quantified recovery via complete resolution of disease symptoms (eye score = 0). We used disease symptoms (eye scores) to quantify recovery because we had high temporal sampling resolution for disease severity, and while birds may still harbor residual pathogen loads following resolution of disease, such loads are typically below the infectious threshold (Dhondt et al., 2008). To address whether temperature influenced recovery, we used a mixed logistic regression in the 'lme4' package (Bates et al., 2015) to determine how temperature treatment (subthermoneutral vs. thermoneutral) and time since inoculation (PID) affected the probability of recovery (1 = not recovered [eye score greater than zero]; 0 = recovered [eye score equal to zero]). Finally, to best distinguish if ambient temperature influenced average disease severity of donor birds by altering the speed of recovery, the extent of disease severity when sick, or both, we used a linear mixed model to determine how temperature and time since inoculation affected disease severity in birds with detectable eye scores only. In both mixed models, we included bird ID as a random effect to account for multiple disease severity observations per bird.

II. Behavioral Assessment

Since temperature can affect the behavior of both healthy and sick individuals, we used separate linear models to estimate how time spent on the feeder (in seconds) by donor and receiver birds varied in response to temperature and MG treatment (infected versus sham control) of donor birds. The treatment status of donor birds (infected versus sham control) was included in the model of receiver bird feeding behavior, because the behavior of healthy hosts is influenced by the infection status of conspecifics in many systems (Lopes et al., 2021).

III. Estimated Receiver Exposure Dose

To estimate the amount of MG that receiver birds were exposed to, we used the average pathogen load on each potential day of transmission as a proxy for the maximum amount of MG spread from the donor. To be conservative in estimating likely transmission day, we assumed several potential incubation periods for each receiver bird (incubation period = number of days between exposure and first detectable eye score in receiver birds), with lengths of 3, 4, 5, 6, or 7 days (determined from past studies in this system, Adelman et al., 2015; Farmer et al., 2002;

Kollias et al., 2004; Sauer et al., 2024; Sydenstricker et al., 2006). We used a range of lengths because incubation periods are often dose-dependent, such as in infected cattle (Wells et al., 2007), skunks (Charlton et al., 1987), and other birds (Aguiar De Souza Penha et al., 2023), and the exposure doses of the receiver birds in our study were unknown. In the case of MG infection, incubation period may also vary by sex (Sauer et al., 2024), but importantly, all receiver birds in our study were female. Using this range of potential incubation periods, we back-calculated the range of days that receiver birds were most likely exposed to MG based on the day of first eye score in receiver birds. For example, if we first detected eye score in a bird on PID 14, we considered PID 11 (3-day incubation period), PID 10 (4-day incubation period), PID 9 (5-day incubation period), and so forth, such that each receiver bird had a unique 5-day window during which they may have been exposed to MG from their donor bird cagemate. Determining this window was important because donor bird pathogen load (our proxy for estimated exposure dose) varied with time across the experiment, such that the amount of MG receiver birds were exposed to may have depended on the pathogen load of the donor bird on a given day.

To determine the range of estimated exposure doses for each receiver bird across temperature treatments, we modified the original donor bird linear mixed model for pathogen load (see *Statistical Analysis: Donor Bird Infectiousness*) by first removing donor birds that did not transmit to their cagemates. We then used the 'glmmTMB' package (Brooks et al., 2017) to create a model using the raw (non-log transformed) pathogen load values and a squared PID term to create a sigmoidal fit, allowing for more precise pathogen load estimates over time. Although the model was overdispersed when evaluated using dHARMA, diagnostic residual plots (R Core Team, 2024) indicated that assumptions for the model were otherwise met. Using predicted values from the modified model, we manually bootstrapped (with resampling) 10,000 potential mean donor bird pathogen loads for each potential day of transmission at each temperature (Efron & Tibshirani, 1998; R Core Team, 2024). We then calculated the average pathogen load for each potential day of transmission, so that each receiver bird had five potential exposure doses of MG based on a range of incubation periods and when they first showed eye score.

Finally, we used a linear mixed model in the 'lme4' package (Bates et al., 2015) to determine how ambient temperature (subthermoneutral vs. thermoneutral) and incubation period (3, 4, 5, 6, or 7 categorical days) affected the average estimated dose acquired by receiver birds, with bird ID as a random effect. Note that since some receiver birds did not ever develop

detectable eye scores (n = 6 thermoneutral, n = 4 subthermoneutral), we were unable to estimate their day of transmission and thus they are not included in the analyses.

IV. Pathogen Success - Transmission

We conducted a survival analysis to examine the effect of temperature (subthermoneutral or thermoneutral) on transmission speed and success in receiver birds over time (32 days). We considered transmission successful if receiver birds developed a non-zero eye score at any point during the experiment (score = 1) or unsuccessful if the receiver bird did not harbor a detectable eye score by the end of the experiment (score = 0). For instance, a receiver bird that developed a 0.5 total eye score on PID 12 would be censored as a success (score = 1) on day 12, regardless of the severity of the pathology. We analyzed survival probabilities using the log-rank test in the 'survival' package (Therneau & Grambsch, 2000) and generated Kaplan-Meier survival curves using the 'survminer' (Kassambara et al., 2021) and 'ggplot2' (Wickham, 2016) packages in R. Tracking transmission success over time requires numerous observations at short time intervals. Thus, we chose to use disease severity as our only metric for transmission success because sampling for eye score can be done frequently and causes minimal stress to birds, whereas swabbing for pathogen load is more invasive and the inherent disturbance of swabbing has the potential to influence the course of infection if done too frequently. Additionally, eye score and pathogen load are correlated (Hawley et al., 2013), and all receiver birds that had detectable eye score in this study had pathogen loads above background contamination levels at some point during the experiment.

V. Pathogen Success – Established Load

We used a linear model (R Core Team, 2024) to determine how temperature treatment (subthermoneutral vs. thermoneutral) affected the maximum disease severity and maximum pathogen load of the receiver birds, our proxies for pathogen success. Because the timing of infection for receiver birds was variable and potentially influenced by temperature treatment, our analysis removed temporal confounds by analyzing the maximum value of each proxy for pathogen success (disease severity, pathogen load) in each receiver bird. Further, we only included receiver birds that were sick for at least seven days or had recovered before the end of

the experiment to ensure we only included birds who likely reached peak disease severity or pathogen load during our period of sampling (n = 6 birds excluded).

Results

I. Donor Bird Infectiousness

Donor birds experimentally infected at colder temperatures had significantly higher levels of disease severity (ANOVA; temperature*PID: $X^2 = 12.0$, $df = 3$, $p = 0.007$, Figure 2A) than those infected at warmer ambient temperatures, but these effects depended on relative timing post-infection, with the largest differences detected days 14-21. Model comparison using Akaike's Information Criterion indicated more support for the interactive model versus the additive model (AIC; disease severity: $df = 10$, AIC = 403.7). When considering whether these overall temperature effects were driven by slower recovery at colder temperatures or higher disease severity when infected, we found that donor birds in thermoneutral temperatures were significantly more likely to recover from MG relative to donor birds in subthermoneutral conditions, with these differences most prominent later in infection (ANOVA; temperature*PID: $X^2 = 19.9$, $df = 1$, $p > 0.0001$). Further, after excluding birds that had recovered from the disease severity analysis, we found that disease severity of donor birds was no longer significantly shaped by temperature, though time post-infection still predicted disease severity (ANOVA; PID: $X^2 = 63.9$, $df = 1$, $p > 0.0001$; temperature: $X^2 = 0.03$, $df = 1$, $p = 0.87$, Figure 2B).

We also found that pathogen loads of donor birds were significantly higher at colder versus warmer ambient temperatures, but these effects depended on relative timing post-infection, with the largest differences detected days 14-21 (ANOVA; temperature*PID: $X^2 = 22.2$, $df = 3$, $p < 0.0001$, Figure 3). Model comparison using Akaike's Information Criterion indicated more support for the interactive model versus the additive model (AIC; disease severity: $df = 10$, AIC = 403.7; pathogen load: $df = 10$, AIC = 354.4).

II. Time Spent at the Feeder

Ambient temperature had the greatest effect on the amount of time receiver birds spent at the bird feeder (Figure 4), with receiver birds at colder temperatures spending significantly longer time at feeders than receiver birds at warmer temperatures, regardless of the MG

treatment status (control or MG) of the receiver bird's cagemate (ANOVA; temperature: $df = 1$, $F = 5.0$, $p = 0.03$; MG treatment: $df = 1$, $F = 0.3$, $p = 0.6$; MG*temperature treatment: $df = 1$, $F = 2$, $p = 0.2$). In contrast, donor birds spent similar amounts of time on the feeder, regardless of their ambient temperature or MG treatment (MG inoculated or sham; ANOVA; temperature: $df = 1$, $F = 0.8$, $p = 0.4$; MG treatment: $df = 1$, $F = 0.4$, $p = 0.5$; Figure 5). Receiver bird behavior was best described using the interactive model (AIC; $df = 5$, AIC = 563.2), whereas AIC model selection tests on donor bird behavior models indicated more support for the additive model (AIC; $df = 4$, AIC = 525.9).

III. Estimated Receiver Exposure Dose

Our model results indicated that receiver birds in colder ambient temperatures were exposed to significantly higher estimated doses of MG relative to receiver birds housed in warmer ambient temperatures, and that estimated exposure doses were higher when incubation periods were longer (ANOVA; temperature*incubation period: $X^2 = 12.7$, $df = 4$, $p = 0.01$, Figure 6). There were also significant individual effects of temperature (ANOVA; temperature: $X^2 = 6.0$, $df = 1$, $p = 0.01$) and incubation period (ANOVA; incubation period: $X^2 = 29.5$, $df = 4$, $p > 0.0001$). Model comparison using Akaike's Information Criterion indicated more support for the interactive model versus the additive model (AIC; $df = 12$, AIC = 2254.5).

IV. Pathogen Success - Transmission

We found that MG successfully transmitted (produced a non-zero eye score) in 79% of receiver birds in subthermoneutral conditions (11 out of 14 birds) and 60% of receiver birds in thermoneutral (9 out of 15 birds). However, both the speed and number of transmission events were not significantly influenced by temperature (Log-rank Test; $X^2 = 12.0$, $p = 0.4$, Figure 7).

V. Pathogen Success - Established Load

Ambient temperature did not have a significant effect on the maximum disease severity or maximum pathogen loads of receiver birds, our proxies for pathogen success (ANOVA; disease severity: $F = 2.35$, $df = 1$, $p = 0.14$, Figure 8; pathogen load: $F = 1.2$, $df = 1$, $p = 0.29$, Figure 9).

Discussion

Host infectiousness, recovery, and behavior are considered vital components of the transmission process because variation at any of these stages can have profound and non-uniform effects on host-parasite dynamics (McCallum et al., 2017). Ambient temperature has been shown to be an important source of variation for diverse individual components of transmission and is thus predicted to be an important driver of seasonal outbreaks (Schmid-Hempel, 2011; Thomas & Blanford, 2003). Numerous mechanistic studies have demonstrated the importance of temperature to pathogen transmission but have largely focused on invertebrate, plant, and vector-borne disease systems (M. Chan & Johansson, 2012; Jones & Barbetti, 2012; Shocket et al., 2019). In contrast, empirical studies examining temperature and transmission dynamics remain limited in vertebrates (Berger et al., 2004; Roznik & Alford, 2015), particularly for small endotherms, with even fewer studies testing temperature-related changes in behavior as a mechanism for transmission (Sipari et al., 2022). Here, we show that colder ambient temperatures have strong effects on several components of the MG-house finch transmission process, but overall, we were not able to detect significant differences in transmission success across temperature with our experimental design. This study highlights that temperature can affect numerous aspects of the transmission process, but that the relative contribution of these effects to overall pathogen spread is complex in this system and likely in others (Altizer et al., 2006).

When infectious diseases co-occur with colder ambient temperatures, endotherms often pay high costs of infection and thermoregulation simultaneously (Hawley et al., 2012; Schmid-Hempel, 2011), and the way organisms respond to both challenges could have important implications for pathogen transmission. For instance, energy that is prioritized for thermoregulation can no longer be used towards mounting an immune response, which in turn may diminish the host's ability to clear infection (Owen-Ashley & Wingfield, 2007). Consistent with these patterns, we found that donor birds housed at colder ambient temperatures had higher disease severity and pathogen loads later in infection, such that disease severity and pathogen load remained high for subthermoneutral birds but appeared to decline rapidly for thermoneutral birds after peaking at seven days post-inoculation. Because both disease severity and pathogen loads are associated with increased pathogen deposition onto feeder surfaces (Adelman et al., 2015), and pairwise transmission probability in this system (Leon et al., 2024), these results

indicate that donor males remained infectious for longer at subthermoneutral versus thermoneutral temperatures. When we incorporated the effects of temperature on recovery probability into our analysis, the detected differences in disease severity later in infection appeared to be driven by donor birds in thermoneutral temperatures recovering more rapidly relative to birds at colder temperatures. While we did not explicitly test the effects of temperature on immune parameters in this experiment, our results suggest that pathogen clearance responses were likely suppressed in donor males in colder temperatures, thus lowering the host's ability to recover and prolonging the infectious period as a result (Altizer et al., 2006). Given the broader importance of infectious period length in driving pathogen fitness and epidemic size (R. M. Anderson & May, 1991), the detected effect of temperature on a proxy for infectious period length in donor males likely has key implications for epidemic dynamics. However, because receiver birds were housed alongside donor birds for the entire course of infection, any downstream effects of an extended infectious period on transmission success were difficult to detect in our experiment.

We also found key effects of ambient temperature on transmission-relevant behaviors. As expected, receiver birds spent more time at the feeder in colder ambient temperatures, likely because of the documented energetic costs of thermoregulation in this system (Hawley et al., 2012). This increase in time spent feeding can have important implications for transmission because the amount of time a susceptible individual spends on a bird feeder is positively associated with risk of conjunctivitis (Adelman et al., 2015), and fomites have been shown to be important drivers of indirect transmission of MG in this system (Dhondt et al., 2007). However, while receiver birds spent more time feeding at colder temperatures, the amount of time donor birds spent at the feeder did not vary with temperature. At the time that behavior was recorded in this study (PID 10), donor birds were at peak disease severity, whereas most receiver birds were early in infection or had not yet been exposed to MG. Together, this suggests that effects of acute MG infection on donor bird behavior, which include reduced food intake (Adelman et al., 2013) and activity levels (Love et al., 2016) in this system, may outweigh effects of temperature on behavior that were detectable in receiver birds, offering unique insight to the importance of sickness behaviors to transmission in this system and potentially other endothermic animals. If donor birds in colder temperatures were prioritizing expression of sickness behaviors over increased foraging, this would be consistent with prior work showing that the expression of

sickness behaviors is highly-context dependent and possibly represents an energy allocation strategy that maximizes the benefits of expression over the costs (e.g., Lopes et al., 2014, 2021; Stockmaier et al., 2018).

Given our study design (which used all males as donor birds and all females as receiver birds), we cannot eliminate the possibility that the distinct effects of temperature on time at the feeder for donor versus receiver birds reflect sex differences rather than effects of sickness behaviors on donor bird behavior. While we did not have a sufficiently large sample size of sham-inoculated donor males to statistically separate effects of experimental infection versus sex on time spent at the feeder, the sham-inoculated males showed patterns of time at the feeder that fell in alignment with the receiver birds, providing tentative support that the distinct patterns of time at the feeder in donors versus receiver birds were driven largely by infection rather than sex. Nonetheless, the behaviors of male and female house finches vary in ways that could influence transmission. For example, female house finches are generally more dominant to males in aggressive interactions over scarce resources in winter (Brown & Brown, 1988), and healthy male house finches prefer to feed near other, less competitive males with visible pathology, while females show no preference (Bouwman & Hawley, 2010). Overall, future studies with donor and susceptible receiver birds of both sexes are critical for detecting potential sex-biases in behavior and transmission patterns which may be present in this system (Sauer et al., 2024).

Based on the timing of when each receiver bird first displayed clinical signs of MG and the differences in donor bird infectiousness, we also estimated that receiver birds in colder temperatures were exposed to higher doses of MG relative to receiver birds in warmer temperatures. It is important to note, however, that the estimated exposure dose likely represents the maximum pathogen dose that receiver birds were exposed to through both direct and indirect interactions, which tend to be brief and often cryptic (Hoyt et al., 2018; Moreno-Torres et al., 2018). Thus, while we cannot determine from our data whether MG exposure of receiver birds occurred largely via deposition onto bird feeder surfaces versus direct contacts, the increased time that receiver birds spent feeding at colder temperatures, alongside prior work showing that MG can survive longer on feeder surfaces at colder ambient temperatures (Teemer et al., 2024), points to the possibility that indirect contacts through contaminated bird feeders are a particularly important source of spread in colder versus warmer ambient temperatures. We also found that the estimated exposure dose was higher when our model assumed longer incubation period lengths,

which may simply reflect the early peak in pathogen loads of donor birds (days 4-7 in our study). Given that incubation period length is also a key epidemiological trait for disease dynamics in other systems (e.g., vector-borne diseases, M. Chan & Johansson, 2012; Johansson et al., 2010; plants, Mersha et al., 2014), future studies should explicitly examine how ambient temperature influences the length of the incubation period in this system.

To our knowledge, this is the first study to investigate how the individual effects of temperature on donor infectiousness, host behavior, and estimated exposure dose scale up to influence the process of MG transmission. Although we found that colder temperatures were associated with longer donor bird infectiousness, increased time spent at the bird feeder by receiver birds, and higher estimated exposure doses, we found that overall pathogen success did not vary with temperature treatment. One possibility is that our sample sizes were too small to detect transmission differences across temperature. For example, while not significant, the likelihood and speed of transmission appeared to potentially vary with temperature, with transmission occurring quickly in thermoneutral temperatures and then slowing down, while the reverse occurred in subthermoneutral temperatures. Interestingly, these patterns align with the finding of faster recovery of donor birds in thermoneutral temperatures, and with prior work showing that MG cannot survive for long on feeder surfaces at warmer temperatures (Teemer et al., 2024). However, because such effects are complex and the donor and receiver birds were housed in close proximity for the entirety of the donor infection in this study, our experimental design was unlikely to detect dynamic differences in infection timing. Future studies that examine infectiousness of donor birds at specific time periods post-infection, as was done in Dhondt et al. (2008) at a single temperature, would allow better determination of whether transmission timing differs across ambient temperature in this system.

We also did not detect differences in pathogen success across temperature using our second proxy, maximum disease severity and pathogen load in receiver birds. Our sample size was even more limited for this analysis because three receiver birds in subthermoneutral and six receiver birds in thermoneutral never developed detectable eye score during the experiment, and thus we were unable to determine maximum disease severity for those birds. Further, our experimental design allowed for receivers to become ‘naturally’ infected by their cagemates which likely introduced several potential sources of variation. Disease responses in receiver birds could have then been shaped by temporal differences in exposure, differences in exposure dose,

and long incubation periods that did not allow for the full progression of disease, which can typically last for weeks in nature (Love et al., 2016). Finally, the receiver birds in our study were all females, and given documented sex-biases in transmission patterns in this system (Sauer et al., 2024), it is possible that sex differences contributed to the lack of detected effects of temperature on pathogen success in receiver birds.

Overall, our results show that temperature has a significant impact on many aspects of the transmission process for house finches and MG and supports the growing body of evidence that the relationship between temperature and transmission is complex (Lafferty, 2009; Teemer & Hawley, 2024; Thomas & Blanford, 2003). While our experimental design varied ambient temperature alone to isolate its effects on different components of transmission, other abiotic factors such as rainfall (Sipari et al., 2022), day-length (Demas & Nelson, 1998), and season (Owen-Ashley & Wingfield, 2006), can also affect numerous aspects of the transmission process, and should thus be considered in future studies, either individually or in conjunction with temperature. Further, while our experimental design intentionally simplified the transmission process to elucidate specific mechanisms, the effects of temperature on host (recovery probability, contact rates) and pathogen (environmental survival) traits relevant to transmission dynamics are important to examine in a modeling context to understand how such individual-level effects likely scale up to influence the timing and probability of outbreaks at the population level. Overall, because temperature effects on disease dynamics will likely complicate further in the face of changing global temperatures (Harvell et al., 2009; Lafferty, 2009; Lindgren et al., 2012; Zhang et al., 2024), mechanistic studies that examine how temperature alters diverse host and pathogen traits relevant to pathogen spread are needed more than ever.

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Figures

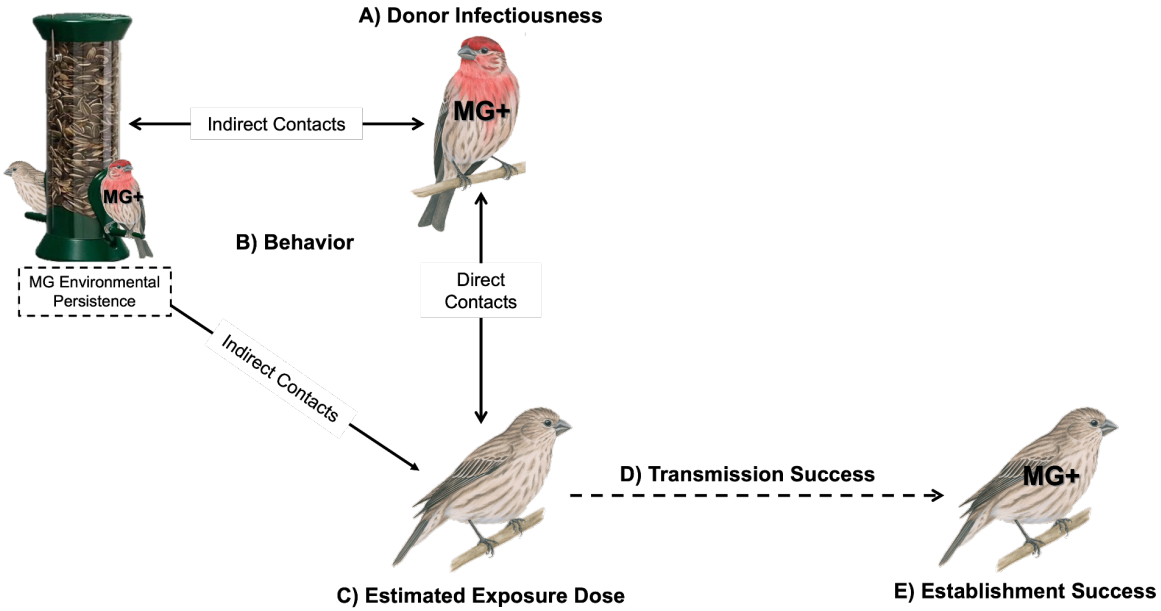

Figure 1. Potential routes by which ambient temperature may influence transmission in the house finch-MG system, modified from McCallum et al. (2017). A) Temperature can affect the infectiousness of donor birds which are experimentally inoculated with identical doses of MG. B) Behavioral interactions between donor and receiver birds, either direct or indirect, can be influenced by temperature. C) Temperature can influence the amount of MG that receiver birds are exposed to as a product of the pathogen burden in donor birds, as well as behavioral interactions between donors and receivers. D) Temperature may influence the probability that MG successfully transmits to receiver birds, and E) the subsequent pathogen burden harbored by receiver birds. Environmental persistence (dashed box) of MG is also influenced by ambient temperature in this system (Teemer et al. 2024) but was not directly quantified in this study.

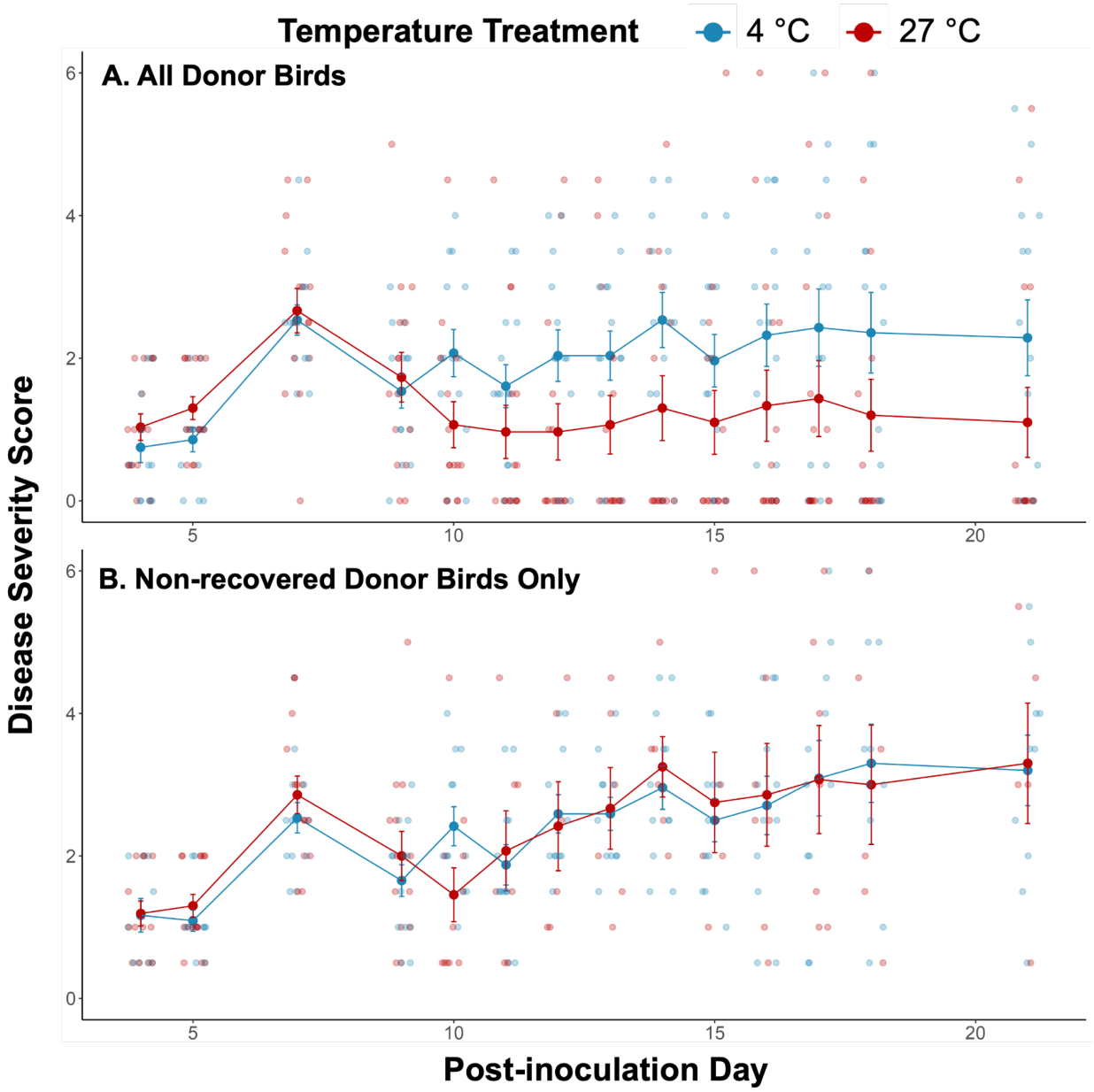

Figure 2. Donor birds housed in colder ambient temperatures had higher disease severity relative to donor birds in warm ambient temperatures, but this effect varied over time post-inoculation (Figure 2A). When non-recovered donor birds (eye scores > 0) were subsetted from the analysis, disease severity no longer differed with temperature (Figure 2B). Large points show mean disease severity with standard error bars. Raw data is shown as small points.

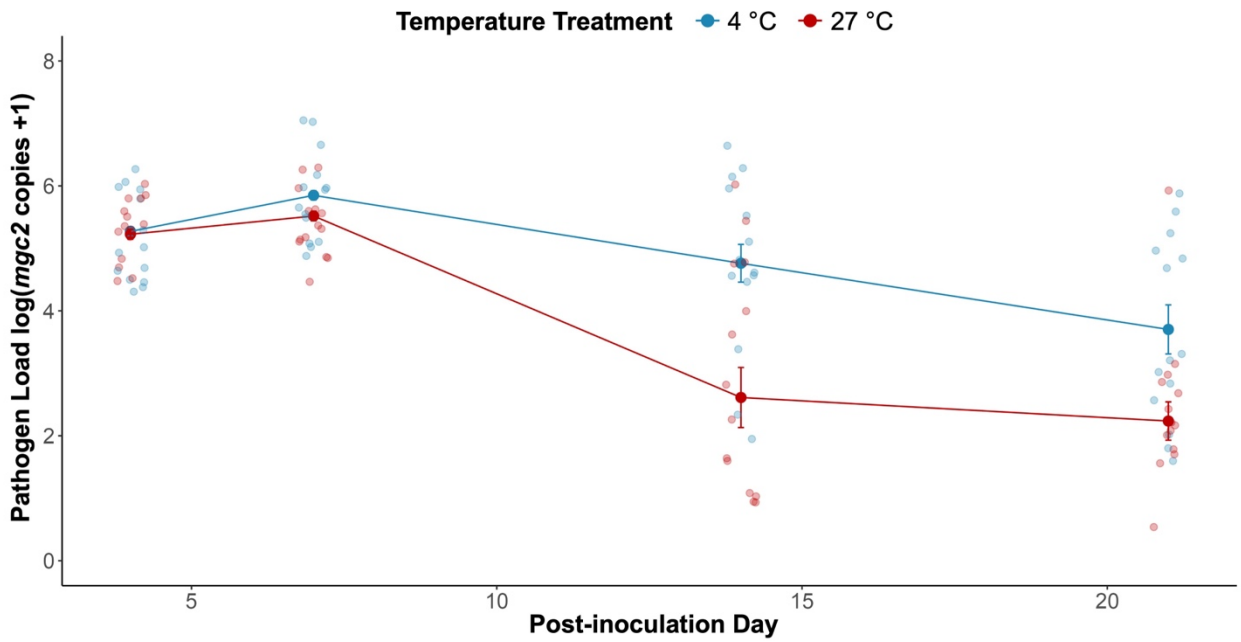

Figure 3. Donor birds housed in colder ambient temperatures had higher pathogen loads relative to donor birds in warm ambient temperatures, but this effect varied over time post-inoculation. Large points show mean pathogen load with standard error bars. Raw data is shown as small points.

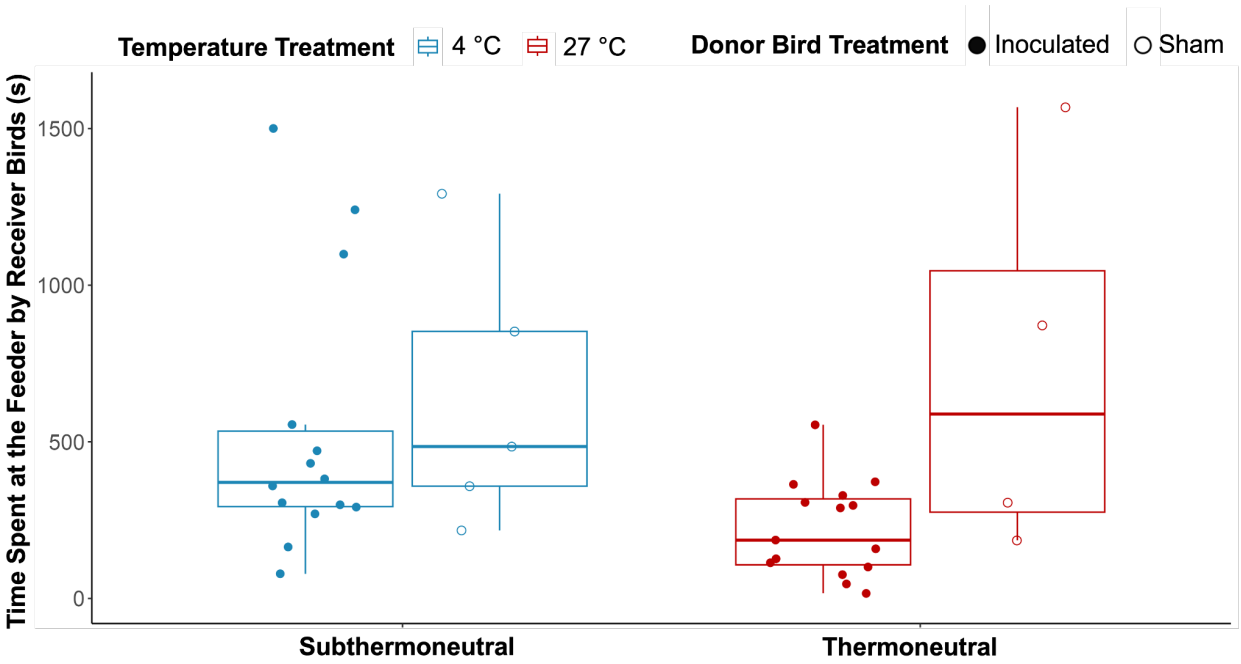

Figure 4. Receiver house finches in cold ambient temperatures spent significantly more time at the bird feeder compared to receiver birds in warm ambient temperatures ($p = 0.03$), regardless of whether the donor bird cagemate was inoculated with *Mycoplasma gallisepticum* (MG) or sham inoculum. Box plots are bordered by 25% and 75% quartiles, and a line within the box indicates median time spent at the bird feeder. Whiskers show maximum and minimum values. Raw data is shown as points, with circle type indicating donor bird MG status (MG inoculated = filled circle, sham = open circle). Temperature treatment is indicated by color (subthermoneutral = blue, thermoneutral = red).

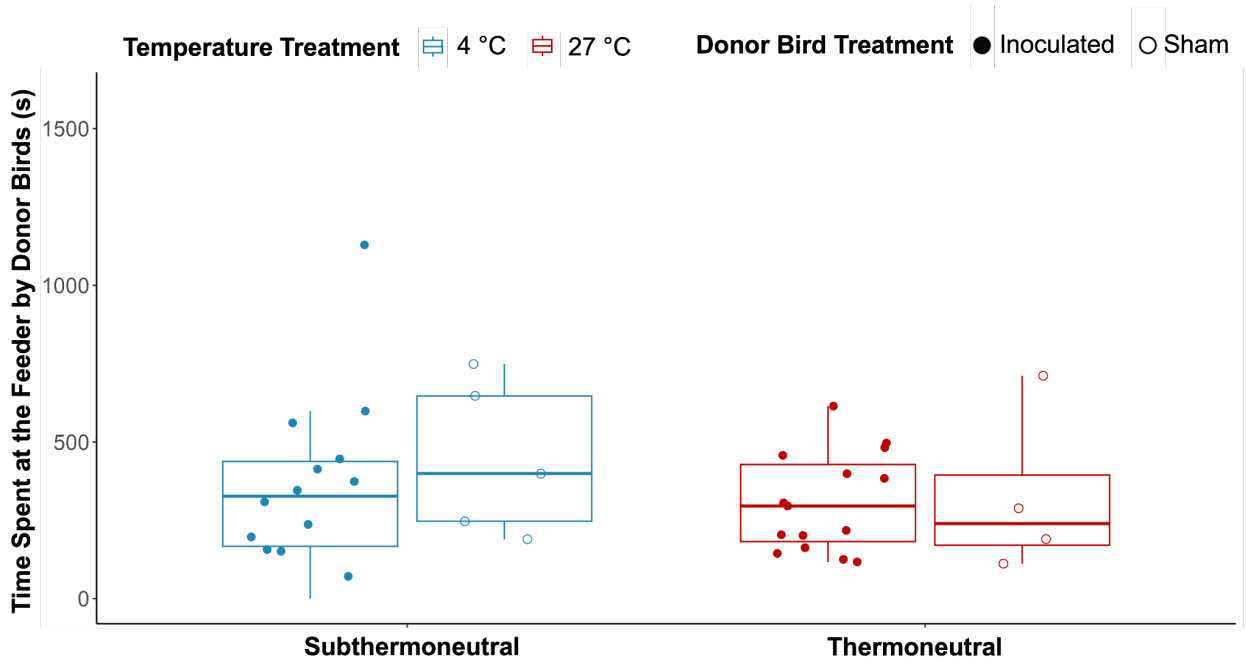

Figure 5. Donor house finches spent similar amounts of time at the feeder regardless of temperature treatment ($p = 0.4$) or inoculation with *Mycoplasma gallisepticum* ($p = 0.5$). Box plots are bordered by 25% and 75% quartiles, and a line within the box indicates median time spent at the bird feeder. Whiskers show maximum and minimum values. Raw data is shown as points, with circle type indicating donor bird MG status (MG inoculated = filled circle, sham = open circle). Temperature treatment is indicated by color (subthermoneutral = blue, thermoneutral = red).

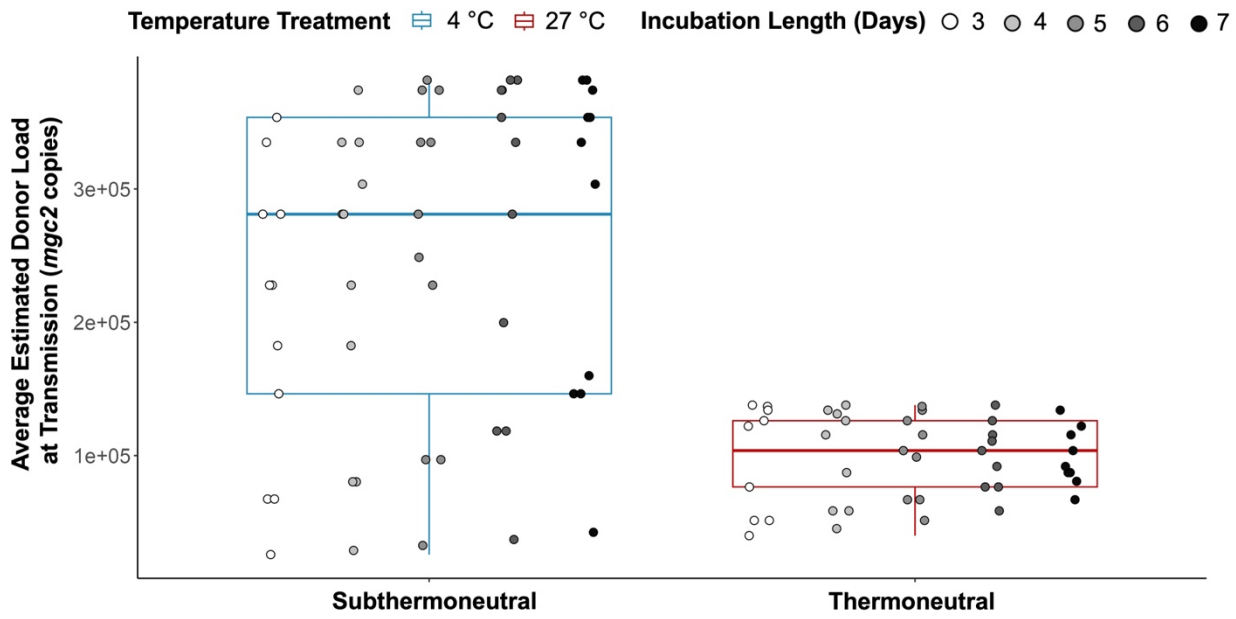

Figure 6. Receiver birds in subthermoneutral conditions were, on average, estimated to have been exposed to higher doses of MG compared to receiver birds in thermoneutral conditions. Box plots contain average estimated exposure doses as points, with the darkest points representing longer assumed incubation periods. Box plots are bordered by 25% and 75% quartiles, and a line within the box indicates average estimated exposure dose. Box plot colors indicate temperature treatment (subthermoneutral = blue, thermoneutral = red). Whiskers show maximum and minimum values.

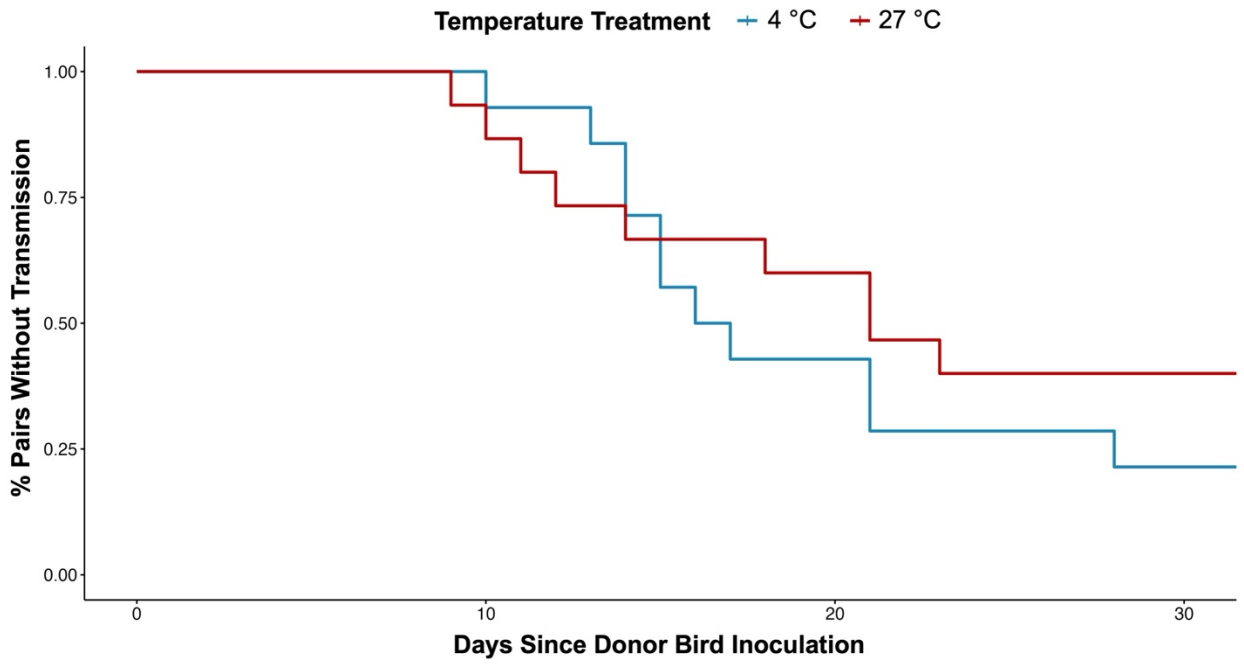

Figure 7. The likelihood and speed of transmission of *Mycoplasma gallisepticum* (MG) from donor to receiver house finches over time did not differ with temperature treatment (subthermoneutral = blue, thermoneutral = red). Each Kaplan-Meier curve shows PID on the x-axis as time since the donor bird was inoculated (0-32 days) and the percent of donor-receiver bird pairs with successful transmission of MG on the y-axis. Successful transmission events were censored so that 1 = non-zero eye score detected and 0 = no eye score detected.

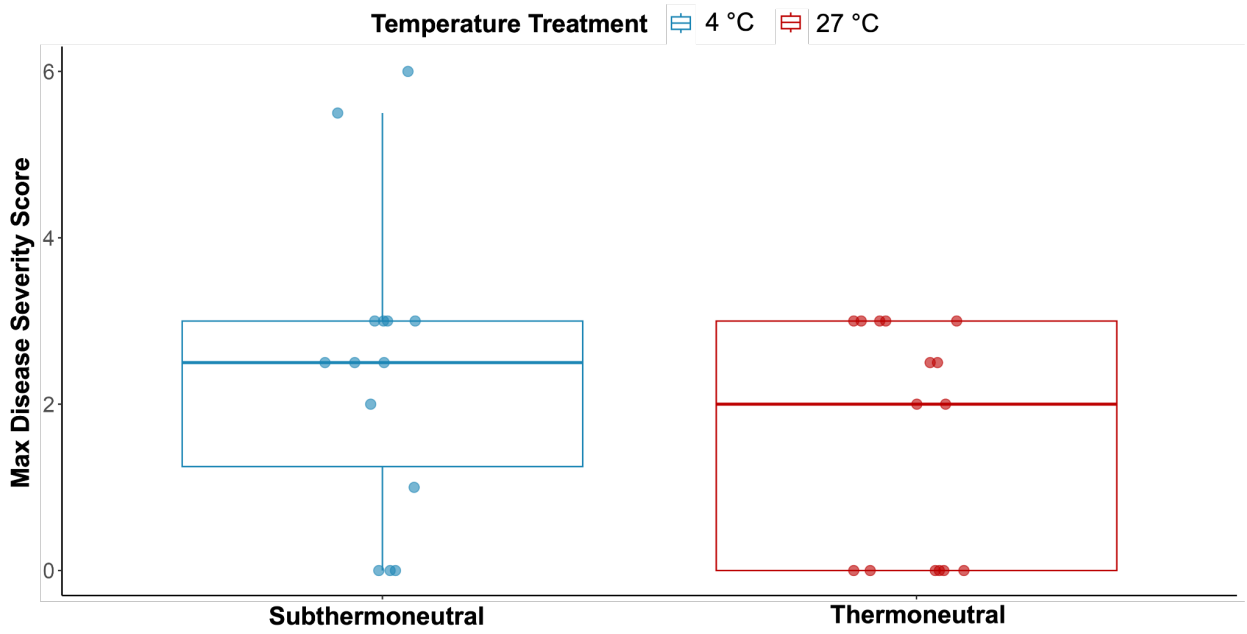

Figure 8. Receiver birds exposed to *Mycoplasma gallisepticum* (MG) via housing with experimentally-infected donor birds did not differ in maximum disease severity with ambient temperature treatment. Raw data is indicated by points, and temperature treatment is indicated by color (subthermoneutral = blue, thermoneutral = red). Box plots are bordered by 25% and 75% quartiles, and a line within the box indicates average estimated exposure dose. Whiskers show maximum and minimum values.

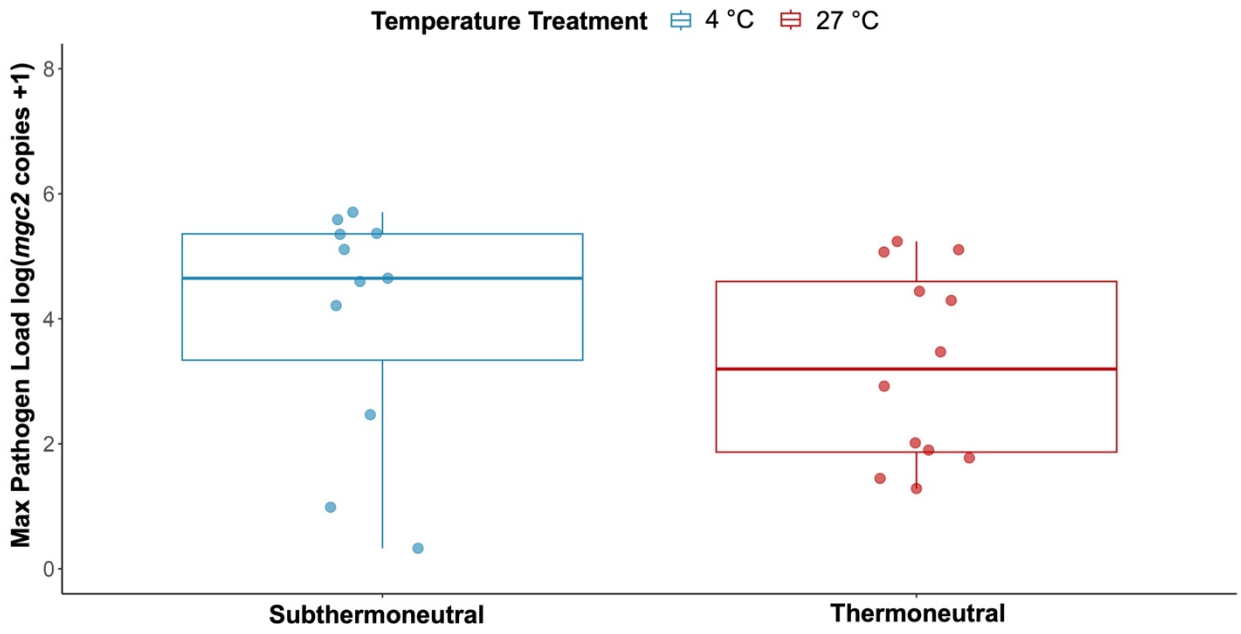

Figure 9. Receiver birds exposed to *Mycoplasma gallisepticum* (MG) via housing with experimentally-infected donor birds did not differ in maximum pathogen load with ambient temperature treatment. Raw data is indicated by points, and temperature treatment is indicated by color (subthermoneutral = blue, thermoneutral = red). Box plots are bordered by 25% and 75% quartiles, and a line within the box indicates average estimated exposure dose. Whiskers show maximum and minimum values.

Chapter 5: Synthesis

Dynamic interactions between hosts, pathogens, and the environment play an important role in the transmission of infectious diseases. However, these interactions are becoming increasingly difficult to predict in a rapidly changing climate (Harvell et al., 2009; Lafferty, 2009), especially because the effects of climate change are not uniform (Arnell et al., 2019) and result in some geographical areas growing warmer while others grow colder (Karl et al., 1996). Ambient temperature, in particular, is predicted to be one of the most important abiotic factors shaping disease dynamics because of its effects on numerous components of the transmission process (Schmid-Hempel, 2011), including host infectiousness (Chan et al., 2022) and pathogen environmental survival (Riddell et al., 2020). Thus, mechanistic studies that explore how ambient temperatures impact disease dynamics provide important information underlying the dynamics of infectious diseases around the world (Martinez & Merino, 2011). Because of the complexities of the transmission process, studies examining the effects of temperature on transmission mechanisms are generally correlative (Antolin, 2008; Pascual & Dobson, 2005), and most comprehensive temperature and transmission studies are in ectotherm and plant systems (Dallas & Drake, 2016; e.g., Jones & Barbetti, 2012). Notably fewer temperature and transmission studies are conducted in endothermic vertebrates, despite this group serving as important reservoirs of zoonotic pathogens (e.g., Dearing & Dizney, 2010; Paz, 2015). Further, ambient temperatures have the potential to alter disease-relevant traits in these systems, such as behavior (Teemer & Hawley, 2024) and energy budgets (Owen-Ashley & Wingfield, 2006) and should thus be studied for a fuller understanding of the factors shaping seasonal pathogen transmission. The simultaneous variation of numerous factors in nature, however, can make it difficult to examine direct effects of the abiotic environment on the transmission process, and often require a controlled approach (Altizer et al., 2006). In this dissertation, I address this knowledge gap using three laboratory experiments examining temperature and transmission dynamics in wild house finches (*Haemorhous mexicanus*), which are hosts for the bacterial pathogen *Mycoplasma gallisepticum* (MG) and experience fall and winter outbreaks of mycoplasmal conjunctivitis (Dhondt et al., 2005; Ley et al., 1996).

Behavior

The behaviors of both sick and healthy hosts have been well-studied as an important mechanism of transmission for many disease systems (e.g., Moore, 2002). However, it is important to consider that behavior itself can be shaped by abiotic factors (Beal, 1978), which is necessary for understanding the underlying factors shaping the process of pathogen transmission. I examined this relationship in my first data chapter (Chapter 2), where I tested how ambient temperature and pseudo-infection affected sickness behaviors in donor (index) birds. I found that the effects of temperature varied with the type of behavior being studied, and in some—but not all—cases, the pseudo-infection status of each bird. For instance, temperature did not affect activity levels but did significantly affect the amount of time donor birds spent at the feeder and, in conjunction with pseudo-infection, immobility. The detected temperature-related differences in behavior are consistent with other studies that show the expression of sickness behaviors is highly-context dependent (Lopes, 2014; Owen-Ashley & Wingfield, 2006) and can vary in ways that minimize costs of expression while maximizing its benefits. For instance, Lopes et al. (2012) found that immune-challenged zebra finches (*Taeniopygia guttata*), who benefit from social interactions, had significantly reduced activity in isolation, but not in colony settings. For house finches in this study, the results could point to an energy allocation strategy that minimizes the costs of thermoregulation (Hawley et al., 2012) and maximizes the benefits of sickness behaviors by conserving energy through immobility (Hart, 1990). By using pseudo-infection approaches in this study, I was able to isolate how individual birds respond to varying infection and environmental contexts in a controlled manner. Additionally, this study gave important insights to how variation in sickness behavior expression could shape transmission potential. However, given that temperature can affect other mechanisms of MG transmission in addition to behavior, such as MG survival in the environment (Chandiramani et al., 1966; Teemer et al., 2024), and pathology (Hawley et al., 2012), it was important to examine the relationship between temperature and behavior again using experimental infection with MG in Chapter 4.

Pathogen Survival

Environmental transmission depends heavily on the ability of pathogens to survive outside of the host, such as on the surfaces of inanimate objects known as fomites (e.g., Kraay et

al., 2018). As a result, any alteration to pathogen viability on fomites can affect the likelihood of a pathogen successfully transmitting to the next host. Previous studies in the house finch-MG system have shown that bird feeders are important sources of fomite transmission (Adelman et al., 2015; Dhondt et al., 2007), but the extent to which they influence seasonal disease dynamics was largely unknown. In my second data chapter (Chapter 3), I showed that MG remained significantly more viable and pathogenic over time when incubated on feeder surfaces in colder versus warmer temperatures. I also found that temperature effects on pathogenicity were strongest after at least four days of incubation on feeder surfaces, suggesting that both temperature and the timing of when birds interact with the feeder may also be important for shaping this route of transmission (Adelman et al., 2015; Ruden & Adelman, 2021). For instance, because cold temperatures prolong the survival of pathogens on bird feeders, susceptible birds visiting the feeder during winter could encounter viable pathogens on surfaces and become infected for up to one week after the initial deposition of the pathogen, versus up to two days in warmer seasons. Studies on temperature in conjunction with other factors shaping fomite transmission, such as the behavior of sick and healthy birds (Adelman et al., 2013; Teemer & Hawley, 2024), bird feeder type (Hartup et al., 1998), other abiotic factors (e.g., Qiu et al., 2022), and human intervention (Feliciano et al., 2018; Horn & Johansen, 2013), will thus be an important area for future research.

Because I used a single MG strain in this study, more work is needed in other MG strains that vary in virulence and geographical origin (e.g., Bonneaud 2013; Hawley et al. 2013). These traits could contribute to differences in thermal tolerance (Ashrafi et al. 2018) and pathogen persistence (Walther and Ewald 2004) as seen in other microbial pathogens, and are thus important for determining how temperature could influence fomite transmission across diverse environmental conditions and locations.

Transmission

In Chapter 4, I used a pairwise transmission experiment to test how temperature affected multiple transmission mechanisms simultaneously, which is likely the case in nature (VanderWaal & Ezenwa, 2016). First, I examined how temperature affected infectiousness and recovery, since outbreaks are driven by interactions over space and time between infected and susceptible hosts. As a result, any change in the host's response to infection can determine the

amount of pathogen shed into the environment, and ultimately, the dose that susceptible individuals are exposed to. I found that temperature played an important role in shaping host infectiousness and recovery. Specifically, I found that birds in colder temperatures maintained high disease severity and pathogen loads for longer, compared to birds in warmer temperatures. This suggests that house finches remain infectious for longer in colder ambient temperatures, because disease severity and pathogen loads in house finches have been directly linked to metrics of infectiousness, specifically the degree of pathogen deposition on to bird feeders (Adelman et al., 2015) and pairwise transmission (Leon et al., 2024). Further, I found that temperature effects on the length of infectiousness appear to be driven by differences in recovery speeds, where donor birds in colder temperatures recovered more slowly compared to birds in warmer temperatures. This is consistent with previous work showing that temperature can play an important role in shaping the immune response to infection (Martin et al., 2010; Ward et al., 2007), likely through the energetic trade-offs that occur between thermoregulation and immunity (Hawley et al., 2012; Kusumoto & Saitoh, 2008). These results could have important implications for seasonal transmission dynamics because reduced immune function in favor of thermoregulation in colder temperatures (e.g., Demas & Nelson, 1998; Xu et al., 2017) could increase the length of the infectious period (Anderson & May, 1991) and the rate of pathogen shedding into the environment (Hershberger et al., 2013; Kang et al., 2024). For instance, Chan et al. (2022) found that golden Syrian hamsters infected with the SARS-CoV-2 virus had a suppressed immune response, increased viral shedding and disease severity, and delayed recovery in colder versus warmer temperatures. If donor birds shed more MG on to feeder surfaces in colder temperatures as a result of such energetic trade-offs, this would also support my results suggesting that receiver birds at colder temperatures were likely exposed to higher MG doses from feeder surfaces compared to receiver birds in warmer temperatures. Future studies that examine how temperature interacts with other seasonal factors to influence host infectiousness, such as day-length (Nelson, 2004) and food restriction (Kusumoto, 2009), are needed to fully understand the implications of how variation in host infectiousness could affect transmission under different conditions.

I then examined how temperature affected the contact-relevant behaviors of MG-inoculated donor birds and their untreated receiver cagemates in Chapter 4. This was important because the expression of behavior may differ across contexts in ways that alter pathogen spread

across biotic and abiotic environments (Lopes et al., 2016; Love et al., 2023; Stockmaier et al., 2021). In sick house finches at thermoneutral temperatures, severe pathology can impact vision (Ley et al., 1996), predator avoidance (Adelman et al., 2017), and food preferences (Tillman & Adelman, 2025). Additionally, infection with MG can signal cues to healthy birds, which can affect social interactions at the feeder (Bouwman & Hawley, 2010). As a result, any temperature-induced effects on pathology of the donor bird, and on the behavior of both donor and receiver birds, could influence transmission. In Chapter 4, I found that MG-inoculated donor birds spent the same amount of time at the feeder regardless of temperature treatment. However, untreated receiver birds spent more time at the feeder at colder temperatures, similar to receiver birds in Chapter 2 (see Chapter 2: Appendix). This suggests that contacts with the feeder were likely higher for susceptible birds in colder temperatures, which could result in increased transmission under colder conditions.

Although I found that colder temperatures were associated with slower recovery, and thus prolonged infectiousness in donor birds, and higher contacts with the feeder and higher estimated MG exposure doses in receiver birds, overall pairwise transmission success did not differ with temperature treatment. While it is possible that components of the experimental design, including small samples sizes and co-housing of birds for the entire course of the study, limited my ability to detect transmission differences across temperatures, this study nonetheless provided important insights to the role of temperature in shaping mechanisms of pathogen transmission in this system. Future studies should explore other transmission mechanisms (described in McCallum et al. [2017]), and their potential to interact with ambient temperatures, to fully understand how abiotic factors could shape the transmission process.

Conclusion

In this dissertation, I show that ambient temperatures affect several transmission mechanisms in ways that could influence seasonal disease dynamics in the house finch-MG system. By using laboratory experiments, I provide a framework for isolating the effects of temperature, and other abiotic factors, on specific transmission mechanisms, which is especially needed in endotherm disease systems (Sipari et al., 2022). Incorporating mathematical modeling into future studies will be useful for testing how the dynamic relationships among temperature and transmission mechanisms scale up to influence population-level processes, which are

otherwise difficult to study in both field and captive settings (Altizer et al., 2006). Future studies should also incorporate a wider range of temperatures similar to those seen in nature to test the potential for non-linear relationships between temperature and transmission, as demonstrated in other host-pathogen systems (e.g., Mordecai et al., 2013). The findings in this dissertation inform our current understanding of the factors shaping pathogen transmission, which are key for predicting and navigating current, and future, impacts of global climate change on disease dynamics.

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