

PERCEPTION OF INFECTION: BEHAVIORAL AND
PHYSIOLOGICAL RESPONSES TO ILLNESS-
RELATED SOCIAL CUES

By

ASHLEY CAROLINE LOVE

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Dissertation Approved:

Dr. Sarah DuRant

Dissertation Adviser

Dr. Jennifer Grindstaff

Dr. Matthew Lovern

Dr. Erika Lutter

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Abstract: Individuals vary in how they respond to and transmit infection. Both pre- and post-infection shifts in behavior, diet, and physiology can contribute to variation in disease susceptibility and disease transmission potential. However, little is known about how social information about infection risk shapes individual-level characteristics that contribute to the spread of disease through vertebrate populations. Animals can detect and respond to sick individuals, most commonly through avoidance behaviors that reduce the risk of infection. While the social effects of infection are rarely explored outside the context of avoidance behaviors, social information about disease could have prominent effects on reproductive and social behaviors, as organisms must weigh the benefit of engaging in social interactions with the risk of becoming sick. Work in humans and insects suggests that organisms are also capable of mounting immune responses to visual cues indicative of heightened infection risk, however, the effects of visual social cues on immune responses are not well understood. My dissertation work was focused on understanding the strategies that social vertebrates use to respond to and prepare for infection by investigating how infection and visual cues of disease alter behavioral, nutritional, and physiological responses relevant to disease susceptibility and transmission in songbirds. Birds make an excellent model for addressing questions about visual cues of disease because they are social animals that rely primarily on vision for detecting immune threats and carry diseases relevant to wildlife, domestic animal, and human health. The work in this dissertation emphasizes that social context and social cues indicative of heightened infection risk can influence behavioral and physiological responses relevant to disease susceptibility and transmission. In addition, my work indicates that birds have diverse, and likely integrative, behavioral, physiological, and nutritional strategies to respond to and prevent infection.

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CHAPTER I

INTRODUCTION

Understanding the factors that contribute to individual variation in disease susceptibility and disease transmission is a critical first step in understanding how diseases spread through populations. For most pathogens, 20% of the population is responsible for spreading 80% of disease (Woolhouse et al. 1997). Presumably, this phenomenon is driven by individual variation in disease susceptibility and transmission, traits inextricably linked with behavior and physiology. Studies have explored how pre- and post-infection behaviors and immune physiology can contribute to these patterns (Lopes et al. 2016, Temime et al. 2009, Ezenwa 2004), however, little information exists on how social information can shape individual-level characteristics that contribute to the spread of disease through vertebrate populations.

Animals have developed multiple ways to detect and avoid pathogens and parasites, and much of this ability involves processing social information (Sarabian et al. 2018). Sick conspecifics often provide visible cues that they are infected—through behaviors such as lethargy, and physical signs, such as inflammation and lesions. One of the most common mechanisms for avoiding infection is to detect and avoid sick

conspecifics. Behavioral avoidance of parasites and pathogens is a crucial defense against becoming infected and occurs in a diverse array of animals, ranging from insects to humans (Curtis 2014). Despite the apparent importance of behavioral shifts in response to perceived infection risk, the social effects of infection in conspecifics are rarely explored outside the context of avoidance behaviors. However, social information about disease may also have prominent effects on reproductive and social behaviors, as organisms must weigh the benefit of engaging in social interactions with the risk of becoming infected. Further, because nutrition can influence immune function, organisms may also respond to social cues of disease by altering their feeding behavior in a way that primes the immune system to fight off infection (Huffman and Seifu 1989, Hutchings et al. 2003, Povey et al. 2013). Additionally, recent work suggests that shifts in behavior are not the only defensive strategy that animals use to respond to a perceived immune threat. Work in humans indicates that visual cues of infection (e.g., seeing images of sick individuals) can alter immune function (Schaller et al. 2010), however, the effects of visual social cues on immune responses are not well understood.

The goal of this dissertation was to enhance our understanding of the strategies that social organisms use to respond to and prepare for infection, and investigate whether these strategies differ between individuals either experiencing an immune challenge or a social cue indicating a heightened risk of disease. Birds make an excellent model for addressing questions about visual cues of infection because they are social animals that rely primarily on vision for detecting immune threats. Further, birds are highly mobile organisms that carry several diseases relevant to wildlife, domestic animal, and human health. Thus, identifying and understanding the factors that contribute to variation in

avian responses to infection is of broad interest and integral to improving our understanding of avian health and epidemiology.

To address how social cues of disease influence avian behavior and physiology, I first examined how a simulated infection and perceived infection risk influence individual and cooperative behaviors in pair-bonded songbirds (Chapter 1). Additionally, because nutrition can influence immune responses and organisms can shift feeding behaviors in response to infection, I investigated how an immune challenge alters feeding behaviors and macronutrient selection. Because shifts in feeding behavior could indicate sickness, and because separate lines of evidence indicate that social cues can influence feeding behavior and alter physiological responses relevant to immune function (Cornelius et al. 2018, Schaller et al. 2010, Stevenson et al. 2011, 2012), I also explored how perceived infection risk alters feeding behaviors and diet selection (Chapter 2). Finally, I examined how social cues of heightened infection risk altered physiological responses relevant to responding to an immune threat using cues of infection that varied in signal strength: an immune-challenge with lipopolysaccharide (Chapter 2) and an infection with the avian bacterium, *Mycoplasma gallisepticum* (Chapter 3).

My research suggests that social information about disease can alter behavioral strategies beyond avoidance behavior and that visual cues of disease are capable of stimulating the immune system, but this may depend on signal strength. Because individual variation in behavior and physiology can lead to differential pathogen exposure and influence how likely an individual is to spread infection (Lloyd-Smith et al. 2005), social information about disease could have important consequences for disease dynamics.

CHAPTER II

SIMULATED INFECTION SHAPES INDIVIDUAL AND SOCIAL BEHAVIORS IN PAIR-BONDED SONGBIRDS AND THEIR HEALTHY NEIGHBORS

ABSTRACT: While infection and perceived infection risk are known to influence social and reproductive behavior in several taxa, relatively little is known about how infection affects pair bond behaviors. Some pair bond maintenance behaviors may be costly to maintain during infection, and infection could promote avoidance behaviors within an established pair. Many species exhibiting pair bonds are part of larger social groups, and behavioral shifts in established pairs can result in altered extra-pair contact rates that could also shape disease transmission. Yet, the reproductive and social effects of infection in conspecifics have rarely been explored outside the context of avoidance behaviors. Thus, infection-induced changes in pair maintenance are a likely, but largely unexplored, route through which infection could shape social and reproductive decisions and potentially influence disease transmission dynamics. Using captive zebra finches (*Taeniopygia guttata*), we examined how an immune challenge with lipopolysaccharide (LPS) influences activity, social behavior, and pair bond maintenance behaviors in established pairs and their healthy neighbors. We observed shifts in individual and pair maintenance behaviors in both immune-challenged pairs and healthy pairs exposed to a

social cue of infection (sick conspecifics). Specifically, LPS-challenged birds decreased activity and social interaction attempts relative to control birds, consistent with LPS-induced sickness behavior. Despite reduced activity, immune activation increased the frequency of clumping between individuals within a pair. While clumping is considered a pair maintenance behavior, it could serve an additional role during immune activation by reducing the thermoregulatory costs associated with maintaining a fever. Additionally, healthy pairs altered their behavior in response to a social cue of heightened infection risk. Specifically, birds exposed to a cue of infection decreased flight activity, which could function to limit contact rates within social groups when a threat of disease is present. Healthy birds seeing immune-challenged conspecifics also increased self-preening and allopreening behavior. These data indicate that healthy pairs respond to social cues elicited by immune-challenged pairs associated with heightened risk of infection through both individual and cooperative pair behaviors geared towards reducing infection risk. Changes in the behavior of both sick and healthy individuals could influence social dynamics and disease transmission, thus understanding how infection and the perceived risk of infection shape behaviors within and among paired individuals will increase our understanding of the role of social behaviors in shaping disease dynamics.

INTRODUCTION

Infection can result in shifts in behavior, including reduced activity and social withdrawal (Hart 1988, Kelley et al. 2003), an array of behavioral symptoms collectively

referred to as sickness behavior. Although thought to promote recovery from an infection (Hart 1988, Dantzer 2001), sickness behaviors can influence rates of disease transmission by influencing how sick individuals interact with members of their social group (Hart 1988, Dantzer 2004, Lopez et al. 2016). Additionally, sickness behaviors can act as a social cue signifying increased infection risk and induce behavioral changes in healthy individuals, such as avoidance behavior (Behringer et al. 2006, Kiesecker et al. 1999, Zylberberg et al. 2012). Indeed, avoidance of sick individuals is widespread across taxa (Behringer et al. 2006, Kiesecker et al. 1999, Zylberberg et al. 2012). However, there are several studies documenting cases in which social animals do not avoid sick conspecifics, and even some instances in which healthy individuals preferentially feed near visibly sick individuals (Willette et al. 2007, Fairbanks et al. 2015, Zala et al. 2015, Bouwman and Hawley 2010). Lack of avoidance behavior could occur if the benefits of social interactions outweigh the costs associated with the risk of infection. For example, one hypothesis postulates that the benefits of avoiding sick conspecifics might be lower for individuals that live in stable social groups, because pathogen exposure may be unavoidable in these situations (Loehle 1995). Thus, social context may influence how individuals respond to an immune threat.

Life history stage and reproductive status can also modulate behavioral responses to infection, such as individuals suppressing sickness behaviors during the breeding season or when in the presence of a conspecific (Owen-Ashley et al. 2006, Lopes et al. 2012). While immune activation is known to influence mate choice and reproductive investment in several taxa (Beltran-Bech and Richard 2014), most work to date has focused on how an immune challenge in one individual shapes that individual's behavior

or the behavior of a potential mate. For example, female mice can differentiate between immune-challenged males and control males and prefer to associate with control individuals (Beltran-Bech and Richard 2014, Lopes and König 2016). However, because social animals are often in close contact with one another, it seems likely that individuals with close bonds, such as those between mates, would share a similar pathogen environment—similar to findings that cohabitating family members and their pets share microbiota (Song et al. 2013). Thus, if one member of a pair becomes infected with a pathogen, their mate likely will also become infected, particularly in individuals that are pair-bonded and have frequent and close associations with one another. Despite this, relatively little is known about how infection and heightened perceived risk of infection influence social interactions in species that form pair bonds, and no studies to date have investigated how pair-bonded mates respond to an immune challenge or perceived immune threat when both members of the pair are challenged simultaneously.

In species that form pair bonds, infection may disrupt behaviors that affect the strength or duration of the bond, and ultimately influence reproductive decisions. A pair bond often consists of maintenance behaviors such as repeated and cooperative displays that serve to strengthen and lengthen the bond between paired individuals. Some pair bond behaviors may be costly to maintain during infection, and infection could promote avoidance behaviors within an established pair. It is not known whether an immune challenge within an established pair disrupts normal pair maintenance behaviors, however this could have important fitness consequences as remaining within an established pair bond can increase reproductive success (Griggio and Hoi 2011). Thus, infection-induced changes in pair maintenance and social behavior are a potential route

through which infection could shape reproductive decisions. Further, because cues indicative of heightened infection risk (*e.g.*, seeing sick conspecifics) can also play a prominent role in shaping behavior (Behringer et al. 2006, Kiesecker et al. 1999, Zylberberg et al. 2012), healthy individuals may invest differentially in self-maintenance and pair-maintenance behaviors and reduce engagement with other members of a social group when a cue of infection is present. Changes in these social associations could alter contact rates and ultimately influence disease transmission and disease dynamics (Hart 1988, Dantzer 2001, Lopez et al. 2016).

Here we investigate how an immune challenge and the perception of increased infection risk via cues from sick conspecifics shape individual, social, and pair-maintenance behaviors within established pairs of a socially monogamous passerine, the zebra finch (*Taeniopygia guttata*). Zebra finches are highly social and form pair bonds in which mates engage in pair-maintenance behaviors that include clumping (perching in bodily contact) and allopreening (Silcox and Evans 1982, Zann 1996). Wild zebra finches are also social beyond their pair, and live in colonies that range in size from small groups to hundreds of individuals (Kikkawa 1980, Zann and Straw 1984, Zann 1996, Zann et al. 1995). Within these colonies, zebra finches spend a majority of their time foraging and associating in mixed-sex pairs or small mixed-sex groups, making them an ecologically relevant model for assessing how infection influences pair behaviors and extra-pair social interactions (Birkhead et al. 1988, McCowan et al. 2015). In this study, we simulate an infection in established zebra finch pairs using the bacterial endotoxin lipopolysaccharide (LPS), which can reduce social interactions, social grooming, and interest in mates in several species (Fishkin and Winslow 1997, Lopes et al. 2016, Lopes and König 2016,

Stockmaier et al. 2018). We also test how social cues from immune-challenged individuals shape the behavior of healthy pair-bonded birds by housing established pairs near either a control pair (no immune threat), or a pair given an immune challenge with LPS (social cue of heightened infection risk). This study aims to enhance our understanding of how behaviors relevant to reproduction and disease transmission are shaped by both immune activation and perceived infection risk in a pair-bonding species that operates within a larger social unit.

MATERIALS AND METHODS

Bird Housing, Experimental Design and Timeline

All birds were kept on a 14 L: 10 D light cycle and housed in 24"x16"x16" cages that were divided down the center into two separate 12"x16"x16" cage sections that each housed one pair of birds. Birds were housed in previously established pairs with one female and one male per cage section. Each cage section had two wooden perches, a water dish, and two food dishes in which birds were fed an *ad libitum* diet of hulled millet mixed into a seed cake with agar and supplemented with hard-boiled chicken eggs and vegetable oil (Love, *unpublished data*). Pairs housed on one side of the cage were injected with either LPS or Saline (injected pairs; LPS-injected or Saline-injected), whereas pairs housed on the other side of the cage were unmanipulated (focal pairs; LPS-focal or Saline-focal). Injected pairs provided social cues to the focal pair. Solid opaque dividers were placed on both sides of each 24 x 16 x 16 cage to ensure that birds housed in each double cage could only see one another (Figure 1). To assess how an immune

challenge and social cue of infection shape behavior, we injected previously established pairs with either lipopolysaccharide (LPS), a non-replicating antigen that activates the immune system and induces sickness behaviors, or a saline solution (control).

Specifically, we injected stimulus birds intra-abdominally with either 50 μ L of 2 mg/kg LPS (Sigma-Aldrich #L7261, *Salmonella enterica* serotype typhimurium) or 50 μ L of phosphate-buffered saline (sham control, Sigma-Aldrich #P3813). We recorded the behaviors of stimulus pairs and focal pairs one day prior to and for 5 days following injection of the stimulus birds. All research protocols were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Behavioral Scoring

Cameras were stationed so that there was one camera pointed directly at each cage. Videos were recorded from 0600 to 0700 CDT, which was immediately after the lights came on in the room each day. Cameras were set to record automatically on a timer each morning in order to minimize the disruption of normal behaviors that would be caused by entering the room for manual camera setup or bird care maintenance which occurred every afternoon. Videos were analyzed by watching three different five-minute increments for each hour-long video recording (the 15-20 min, 30-35 min, and 45-50 min intervals of each hour long video were scored). These times were selected to best represent the entire recording period. Videos were observed for sickness behavior, self-maintenance behaviors, and pair maintenance behaviors. To determine whether birds were engaging in social withdrawal or avoidance behaviors, we recorded the time that

each member of the pair spent on the side of the cage closest to the neighboring pair and counted the number of times each bird attempted to interact with the neighboring pair. Details on the specific behaviors monitored are in Table 1. All behaviors for each individual at each time point were recorded and then tallied. The number of times each of the behaviors was observed for each bird was then used for statistical analyses. All videos were scored while blind to treatment. Due to logistical constraints, all stimulus bird (LPS-injected and Saline-injected) videos were scored by ACS, and all focal bird (LPS-focal and Saline-focal) videos were scored by AN. A subset of videos from both datasets (stimulus and focal) was watched by ACL to ensure accuracy in behavioral scoring.

Statistical Analyses

To meet parametric requirements for normality and homoscedasticity, all behavior counts were square root transformed. To test whether an immune challenge influenced individual behaviors we ran separate repeated-measures ANOVAs for each behavior with cage number included as a blocking factor to account for random behavioral variation between the birds housed in each cage. All models were run using PROC MIXED in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) and included treatment, days post-treatment, sex, and all pairwise interactions. Because pair-maintenance behaviors (clumping, allopreening) require the involvement of both members in a pair, these behavioral endpoints were analyzed per pair, rather than by individual. Thus, sex was not included in these models. Additionally, we assessed whether an immune challenge or perceived immune threat altered the overall behavioral profiles of birds using the FactorMineR

package in R (Version 1.1.456). For the multifactor analysis, we selected four behaviors relevant to responding to an immune threat and/or with implications for disease transmission through changes in rates of contact (Flight Activity, Eating, Social interactions, Self-Preening). All behaviors for each individual were grouped across days 1, 2, 3, 4, and 5 post-treatment to create a composite behavioral profile across timepoints for each individual. We also ran a separate multifactor analysis to assess whether immune activation or a perceived immune threat influenced pair maintenance behavioral profiles by using pair values for clumping and allopreening behavior to create a composite pair-maintenance profile. To compare treatment groups, we tested whether the mean for each treatment cluster was significantly different from zero ($p < 0.05$) using the test value criterion included in the FactoMineR package (Lê et al. 2008). The test values come from the transformation of a p-value into a quantile of the normal distribution, in which p-values less than 0.05 correspond with an absolute test value greater than 1.96 and the sign of the test value indicates whether the coordinate value is less than or greater than zero (Lê et al. 2008).

RESULTS

Behavioral Responses to an Immune Challenge

Activity and Social Behavior

Exposure to LPS resulted in decreased flight activity (Figure 2A, day*treatment: $F_{5,159} = 14.70$, $p < 0.0001$). The number of times a bird walked along the bottom of the cage and feeding behavior fluctuated over time (Figure 2B, walking: day: $F_{5,157} = 4.25$, p

= 0.001; Figure 2C, feeding: day: $F_{5,153} = 4.97, p = 0.0003$), but did not significantly differ between LPS- and saline-injected individuals (all $p \geq 0.563$). LPS-injection also increased self-preening behavior following the immune challenge (Figure 3A, day*treatment: $F_{5,146} = 2.71, p = 0.023$) and significantly decreased the number of times that birds beak-wiped (Figure 3B, day*treatment: $F_{5,152} = 2.86, p = 0.017$).

Exposure to LPS also resulted in a reduction in the number of times that birds interacted with their neighbors (Figure 4A, day*treatment: $F_{5,150} = 2.43, p = 0.037$), consistent with the lethargy and social withdrawal typically associated with LPS-induced sickness behavior. The decrease in social attempts in LPS-challenged birds was most prominent in LPS-injected females (Figure 4B), as LPS-challenged males had low numbers of social attempts prior to the immune-challenge and throughout the experiment. Additionally, saline-males had significantly higher levels of social interaction attempts over the course of the experiment when compared with LPS-injected males and females in both treatments (Figure 4B, sex*treatment: $F_{1,60.9} = 25.25, p < 0.0001$). Regardless of sex, we found that LPS-injected birds spent significantly less time on the side of the cage nearest the neighboring pair following LPS-injection (day*treatment: $F_{5,151} = 3.89, p = 0.0002$).

For the multifactor analysis, the first two dimensions had eigenvalues ≥ 1.0 that explained 51.3% of the variance (for the remaining 18 dimensions: eigenvalues < 0.80 , percentages of variance $< 10\%$; Table 2). The coordinates, contributions, and correlations for each behavior included in the multifactor analysis are shown in Table 3. LPS-injected and saline-injected birds had significantly ($p < 0.05$) different behavioral profiles for dimension 2 of the multifactor analysis (test value: 3.538). To visualize differences

between treatment groups, we plotted dimension 2 against dimension 1 and found separation in the 95% confidence intervals between the behavioral profiles of control saline-injected pairs and LPS-exposed pairs and these differences were largely driven by shifts in behavior on the first two days following treatment (Figure 6A).

Pair Maintenance Behavior

When we examined how LPS influenced pair maintenance behaviors, we found that LPS-challenged pairs significantly increased clumping behavior the day following LPS injection (Figure 5A, day*treatment: $F_{5,45.3} = 3.67$, $p = 0.007$). Allopreening was not affected by treatment ($p = 0.682$), however, LPS-challenged pairs appeared to increase allopreening behavior following the immune challenge while saline-injected pairs had consistent levels of allopreening over the course of the experiment (Figure 5B). For the pair-maintenance multifactor analysis, the first dimension had an eigenvalue of 3.225 that explained 56.7% of the variance, while dimension 2 had an eigenvalue of 0.85 that explained 14.9% of the variance (for the remaining 8 dimensions: eigenvalues<0.50, percentages of variance<9%; Table 4). The coordinates, contributions, and correlations for each pair maintenance behavior included in the multifactor analysis are shown in Table 5. LPS-injected and saline-injected birds had a significantly ($p<0.05$) different pair-maintenance profile for dimension 2 of the multifactor analysis (test value: 2.847) and we found separation in the 95% confidence intervals between control pairs and LPS-exposed pairs, where day 1 post-treatment had the greatest contribution to separation between LPS and saline pairs (Figure 6B).

Behavioral Responses to a Social Cue of Infection

Activity and Social Behavior

Birds housed next to LPS-challenged conspecifics decreased their flight activity (Figure 2D, day*treatment: $F_{5,149} = 2.85$, $p = 0.017$), but did not differ from saline-focal birds in the number of times they walked or fed (Figure 2E and 2F, all $F < 1.00$, all $p > 0.330$). Additionally, exposure to a social cue of infection significantly increased self-preening behavior (Figure 3C, treatment: $F_{1,18.1} = 5.30$, $p = 0.033$). Beak-wiping behavior also differed between treatments with saline-focal birds having higher bouts of beak wiping, but this was apparent prior to treatment and driven by the almost complete absence of beak wiping in LPS-focal birds (Figure 3D, treatment: $F_{1,18} = 4.59$, $p = 0.046$).

There was a non-significant trend for LPS-focal birds to decrease the number of social interactions they attempted following a cue of infection (Figure 4C, day*treatment: $F_{5,138} = 2.00$, $p = 0.082$). The number of social interactions each bird had was influenced by treatment in a sex-specific manner, where saline-focal males engaged in significantly more interaction attempts with the neighboring birds than LPS-focal males or females in either treatment (Figure 4D, treatment*sex: $F_{1,72.8} = 8.54$, $p = 0.005$). However, treatment did not alter the amount of time that birds spent on the side of the cage nearest their neighbors ($p = 0.537$).

To examine differences in behavioral profiles between LPS-focal and saline-focal birds, we ran a multifactor analysis and found that the first two dimensions had eigenvalues ≥ 1.0 that explained 57.6% of the variance (for the remaining 18 dimensions:

eigenvalues < 0.76, percentages of variance < 9%; Table 6). The coordinates, contributions, and correlations for each behavior included in the multifactor analysis are shown in Table 7. LPS-focal and saline-focal birds had a significantly different behavioral profile for dimension 2 of the multifactor analysis (test value: 3.538). We plotted dimension 2 against dimension 1 to visualize differences between treatment groups and found separation in the 95% confidence intervals between the behavioral profiles of birds exposed to either healthy conspecifics or a social cue of infection (Figure 7A).

Pair Maintenance Behavior

Clumping behavior changed over time (Figure 5C, day: $F_{5,51} = 2.99$, $p = 0.019$) but did not differ between pairs exposed to a cue of infection or pairs exposed to healthy conspecifics ($p = 0.548$). Specifically, clumping behavior increased over time but returned to baseline levels by day 5 post-cue. Allopreening behavior increased in LPS-focal individuals post cue then returned to baseline levels (Figure 5D, day*treatment: $F_{5,55.2} = 2.36$, $p = 0.052$). Saline-focal pairs had slight shifts in allopreening behavior over time that seemed to fluctuate around an average allopreening level for this treatment group (Figure 5D). For the pair-maintenance multifactor analysis, the first two dimensions had eigenvalues ≥ 1.0 that explained 73.5% of the variance (for the remaining 8 dimensions: eigenvalues < 0.70, percentages of variance < 12%; Table 8). The coordinates, contributions, and correlations for each pair maintenance behavior included in the multifactor analysis are shown in Table 9. LPS-focal and saline-focal birds did not significantly differ in pair-maintenance profiles (Figure 7B, all test values ≤ 0.942).

DISCUSSION

Many social species form pair bonds, and these close associations between mates can shape how individuals interact with one another as well as other members of their social group. Despite this, little is known about how infection influences the behaviors needed to maintain pair bonds. However, infection-induced shifts in activity and sociality could alter these relationships and potentially influence processes such as reproduction and disease transmission in pair-bonding species. This study sought to address this knowledge gap by investigating how an immune challenge and perceived risk of infection influence individual, social, and pair maintenance behaviors in established pairs of a socially monogamous songbird, the zebra finch. We found that an immune challenge shaped individual and social behaviors in pair-bonded zebra finches and their healthy neighbors. Shifts in these behaviors could influence rates of disease transmission by influencing how sick individuals interact with members of their social group and through behavioral changes in healthy individuals that modulate their risk of infection (Hart 1988, Dantzer 2001, Lopez et al. 2016, Behringer et al. 2006, Kiesecker et al. 1999, Zylberberg et al. 2012).

A multifactor analysis revealed that LPS-injected birds had behavioral profiles that were distinct from saline-injected control birds, and that differences in behavior between the two treatments were most prominent within the first two days following the immune challenge. This finding is consistent with other studies examining sickness behaviors in zebra finches challenged with LPS (Sköld-Chiriac et al. 2014), where

activity was significantly reduced 2 days following LPS-injection, but did not differ from baseline levels 4 days after the immune challenge. A separate multifactor analysis revealed that pair-maintenance behavioral profiles also differed between LPS and saline-injected birds, suggesting that an immune challenge can shape both individual behaviors and cooperative pair behaviors. These infection-induced shifts in behavior could play an important role in shaping social and reproductive decisions as well as having implications for disease dynamics. For example, LPS-induced shifts in behavior cause immune-challenged mice to reduce their social connectivity and this ultimately reduces the potential for disease outbreaks in their social group (Lopes et al. 2016).

Immune challenged individuals exhibited behaviors consistent with LPS-induced sickness behaviors including decreased flight activity and a reduction in social interactions (Owen-Ashley 2006, Sköld-Chiriac et al. 2014, Lopes et al. 2016). Interestingly, the observed decrease in social interactions in LPS-challenged birds was driven primarily by females decreasing their social attempts, as LPS-challenged males had consistently low numbers of social attempts prior to the immune-challenge and throughout the experiment. Unexpectedly, immune challenged pairs increased pair-maintenance behaviors, including a significant increase in pair clumping. While clumping is considered a pair maintenance behavior, clumping may also function to reduce the thermoregulatory costs associated with mounting and maintaining a fever during an immune challenge (Hart 1988). Indeed, the energetic benefits of huddling to conserve or maintain body heat have been illustrated in multiple bird species, ranging from penguins to small passerines (Gilbert et al. 2006, Hatchwell et al. 2009, Wojciechowski et al. 2011). Clumping was significantly elevated the morning following treatment with LPS,

and this timeline is consistent with when LPS-induced fever would occur (Harden et al. 2006). If this is the case, paired individuals may be using coordinated behaviors to modulate their responses to infection. Conversely, increased clumping may serve as a comfort behavior for sick individuals. In support of this idea, studies on domestic animals indicate that social support in the form of being housed in contact with a familiar conspecific can alleviate the behavioral and physiological effects associated with a stressor (Rault 2012). While we were not able to identify the function of increased clumping behavior in this scenario, future work could elucidate these relationships by investigating how clumping behavior, fever, and the costs associated with immunity and thermoregulation interact during an immune challenge.

We also observed a non-significant trend of increased allopreening behavior in LPS-immune challenged individuals. As a pair maintenance behavior, allopreening functions to strengthen and maintain social bonds through social grooming. Increased allopreening during an immune challenge may help birds and their mates combat ectoparasites and pathogens, as has been illustrated by studies of self-preening behavior. Indeed, immune challenged individuals in the present study also increased self-preening following injection. Preen oil from the uropygial (preen) gland can contain compounds harmful to bacteria, fungi, and ectoparasites (Moyer et al. 2003, Clayton et al. 2010), which could help birds combat potential sources of infection such as ectoparasites or pathogens living on the feathers. LPS-immune challenged individuals also decreased beak-wiping behavior. Beak-wiping has a diversity of functions, ranging from beak maintenance to courtship behavior (Clark 1970). Regardless of function, beak-wiping is considered an energetically expensive behavior, as it involves a series of movements involving the head, neck, trunk, and legs

(Clark 1970). Thus, beak-wiping may have declined in LPS-individuals due to lethargy and the energetic constraints associated with an immune challenge.

For birds receiving cues of heightened infection risk, multifactor analyses revealed that LPS-focal and saline-focal birds did not significantly differ in pair-maintenance behavior profiles but did have distinct behavioral profiles when considering individual and social behaviors relevant to responding to infection. These results provide support that healthy birds can detect and behaviorally respond to social cues of infection. The observed shifts in activity and preening behavior in birds exposed to a social cue of infection could alter how susceptible individuals and their partners are to becoming infected and shape disease transmission in social groups of pair-bonding species (Dizney & Dearing 2013, Sih et al. 2018). Specifically, healthy individuals housed next to immune-challenged neighbors decreased flight activity, increased preening behavior, and reduced social interactions. Because LPS-challenged birds also decreased flight activity, had altered social interactions, and increased preening behavior, it is possible that some of the observed shifts in LPS-focal birds could be a result of social learning, in which focal birds are mirroring the behavioral patterns of their neighbors (Galef 1988, Kavaliers and Choleris 2018). Decreased flight activity in LPS-focal birds could function to limit contact rates within social groups when a threat of disease is present. However, contrary to studies in other species (Behringer et al. 2006, Kiesecker et al. 1999), we did not find any evidence for avoidance behavior, as focal individuals did not alter how much time they spent on the side of the cage nearest their immune-challenged neighbors. However, because LPS-challenged birds significantly reduced social interaction attempts and spent less time near their neighbors during the day following the immune challenge, avoidance

behavior in focal individuals may not be necessary. This finding is consistent with a study in which mice challenged with LPS reduced their social connectivity, but healthy individuals within the same social group did not exhibit any avoidance behaviors or avoid areas with sick conspecifics (Lopes et al. 2016). Interestingly, LPS-focal individuals increased self-preening and allopreening behavior following a social cue of infection, implying that preening may be a generalized defense against perceived cues of disease. Indeed, preening functions to clean feathers and could help remove potential pathogens and parasites, ultimately reducing the risk of infection to that individual or that individual's mate (Jacob et al. 1997, Moyer et al. 2003, Clayton et al. 2010).

Conclusion

While there are many benefits associated with sociality, social organisms are thought to be at a higher risk of infection with pathogens and parasites (Altizer et al. 2003, Møller et al. 1993). Thus, individuals should benefit from distinguishing healthy social partners from sick individuals and be able to adjust their behavior according to social cues that indicate an immune threat is present. In this study, we found shifts in individual and pair maintenance behaviors in both immune-challenged zebra finch pairs and their healthy neighbors. Changes in the behavior of both sick and healthy individuals could influence social dynamics, disease susceptibility, and disease transmission (Lopes et al. 2016, Disney & Dearing 2013). Traditionally models of disease have assumed homogeneity of behavior and social contacts within a population, however in species that form pair bonds this is likely not the case. Indeed, even in the non-breeding season some

birds travel and forage together in pairs, suggesting that individuals associate closely with their pair-bonded mates year round (McCowan et al. 2015). Thus, infection-induced shifts in behavior could either facilitate or mitigate the spread of disease in a manner that is likely dependent on the primary social unit operating in the breeding system.

Understanding pair-bond associations and how infection influences these relationships may be a key factor in accurately predicting how diseases spread in social and pair-bonding species.

Table 1. Behaviors scored during video analysis.

Behaviors Recorded	Type of Behavior
Eating	Activity
Flying	Activity
Walking	Activity
Self-preening	Self-maintenance
Beak-wiping	Self-maintenance
Time spent near neighbors	Social
Social interaction attempts	Social
Clumping	Pair-maintenance
Allopreening	Pair-maintenance

Table 2. Eigenvalues and explained variance for the 20 dimensions of the stimulus bird behavioral multifactor analysis.

<i>Dimensions</i>	<i>Eigenvalue</i>	<i>Percentage of variance (%)</i>	<i>Cumulative percentage of variance (%)</i>
1	3.370	38.094	38.094
2	1.166	13.182	51.276
3	0.802	9.068	60.344
4	0.754	8.524	68.868
5	0.640	7.232	76.100
6	0.505	5.714	81.814
7	0.304	3.440	85.253
8	0.258	2.916	88.170
9	0.241	2.721	90.891
10	0.173	1.961	92.852
11	0.134	1.516	94.368
12	0.129	1.458	95.827
13	0.101	1.141	96.968
14	0.075	0.849	97.817
15	0.059	0.664	98.481
16	0.051	0.572	99.053
17	0.029	0.328	99.381
18	0.027	0.301	99.682
19	0.021	0.237	99.919
20	0.007	0.081	100.000

Table 3. Stimulus bird behavioral multifactor analysis. Coordinates, contributions, and correlations of each behavior (eating, flight activity, social interactions, self-preening) from days 1-5 post-injection for the first two dimensions of the multifactor analysis.

Behavior	Day	<i>Dimension 1</i>			<i>Dimension 2</i>		
		Coord.	Contrib.	Correl.	Coord.	Contrib.	Correl.
<i>Eating</i>	1	0.069	0.021	0.056	0.506	3.250	0.413
<i>Eating</i>	2	-0.036	0.006	-0.037	0.199	0.497	0.205
<i>Eating</i>	3	-0.328	0.542	-0.238	0.366	1.952	0.265
<i>Eating</i>	4	-0.344	0.674	-0.309	-0.333	1.827	-0.299
<i>Eating</i>	5	-0.244	0.391	-0.182	-0.082	0.126	-0.061
<i>Flight Activity</i>	1	1.845	14.979	0.763	1.171	17.450	0.484
<i>Flight Activity</i>	2	2.072	18.612	0.860	0.923	10.682	0.383
<i>Flight Activity</i>	3	2.065	21.473	0.911	-0.393	2.247	-0.173
<i>Flight Activity</i>	4	1.684	16.185	0.823	-0.623	6.396	-0.304
<i>Flight Activity</i>	5	1.600	16.832	0.809	-0.438	3.639	-0.221
<i>Social Interactions</i>	1	0.030	0.004	0.031	0.518	3.412	0.540
<i>Social Interactions</i>	2	-0.053	0.012	-0.054	0.623	4.859	0.632
<i>Social Interactions</i>	3	-0.034	0.006	-0.051	0.434	2.741	0.645
<i>Social Interactions</i>	4	-0.155	0.137	-0.218	0.180	0.535	0.253
<i>Social Interactions</i>	5	-0.066	0.028	-0.073	0.582	6.438	0.649
<i>Self-preening</i>	1	-0.560	1.378	-0.263	-0.481	2.949	-0.226
<i>Self-preening</i>	2	-0.749	2.435	-0.454	-0.521	3.404	-0.316
<i>Self-preening</i>	3	-0.669	2.254	-0.394	-0.024	0.008	-0.014
<i>Self-preening</i>	4	-0.599	2.049	-0.353	1.176	22.819	0.692
<i>Self-preening</i>	5	-0.549	1.980	-0.356	0.501	4.768	0.325

Table 4. Eigenvalues and explained variance for the 10 dimensions of the stimulus bird pair maintenance behavior multifactor analysis.

<i>Dimensions</i>	<i>Eigenvalue</i>	<i>Percentage of variance (%)</i>	<i>Cumulative percentage of variance (%)</i>
1	3.225	56.687	56.687
2	0.850	14.944	71.631
3	0.490	8.611	80.243
4	0.412	7.234	87.477
5	0.398	6.990	94.466
6	0.117	2.062	96.529
7	0.108	1.900	98.429
8	0.061	1.068	99.497
9	0.015	0.258	99.755
10	0.014	0.245	100.000

Table 5. Stimulus bird pair maintenance behavior multifactor analysis. Coordinates, contributions, and correlations of each pair maintenance behavior (allopreening, clumping) from days 1-5 post-injection for the first two dimensions of the multifactor analysis.

Behavior	Day	<i>Dimension 1</i>			<i>Dimension 2</i>		
		Coord.	Contrib.	Correl.	Coord.	Contrib.	Correl.
<i>Allopreening</i>	1	0.909	3.013	0.678	0.100	0.140	0.075
<i>Allopreening</i>	2	0.829	3.009	0.563	0.201	0.670	0.136
<i>Allopreening</i>	3	0.488	1.300	0.440	0.372	2.864	0.336
<i>Allopreening</i>	4	0.859	8.303	0.719	0.312	4.159	0.261
<i>Allopreening</i>	5	0.670	5.834	0.701	0.446	9.803	0.466
<i>Clumping</i>	1	1.554	8.812	0.543	-2.282	72.023	-0.797
<i>Clumping</i>	2	2.019	17.857	0.843	-0.024	0.010	-0.010
<i>Clumping</i>	3	1.992	21.677	0.857	-0.335	2.328	-0.144
<i>Clumping</i>	4	1.139	14.596	0.875	0.353	5.322	0.271
<i>Clumping</i>	5	1.096	15.599	0.841	0.233	2.681	0.179

Table 6. Eigenvalues and explained variance for the 20 dimensions of the focal bird behavioral multifactor analysis.

<i>Dimensions</i>	<i>Eigenvalue</i>	<i>Percentage of variance (%)</i>	<i>Cumulative percentage of variance (%)</i>
1	3.717	43.323	43.323
2	1.226	14.285	57.608
3	0.757	8.828	66.436
4	0.489	5.700	72.136
5	0.416	4.854	76.990
6	0.376	4.376	81.366
7	0.354	4.130	85.496
8	0.245	2.852	88.348
9	0.214	2.500	90.848
10	0.157	1.824	92.672
11	0.130	1.512	94.184
12	0.121	1.416	95.600
13	0.090	1.044	96.644
14	0.086	0.997	97.640
15	0.064	0.748	98.388
16	0.049	0.570	99.958
17	0.040	0.469	99.427
18	0.025	0.286	99.713
19	0.015	0.179	99.893
20	0.009	0.107	100.000

Table 7. Focal bird behavioral multifactor analysis. Coordinates, contributions, and correlations of each behavior (eating, flight activity, social interactions, self-preening) from days 1-5 post-cue for the first two dimensions of the multifactor analysis.

Behavior	Day	<i>Dimension 1</i>			<i>Dimension 2</i>		
		Coord.	Contrib.	Correl.	Coord.	Contrib.	Correl.
<i>Eating</i>	1	-0.002	0.000	-0.002	-0.435	3.121	-0.320
<i>Eating</i>	2	0.527	0.951	0.431	0.563	3.283	0.460
<i>Eating</i>	3	0.444	0.984	0.351	-0.154	0.359	-0.122
<i>Eating</i>	4	0.337	0.424	0.304	0.246	0.686	0.222
<i>Eating</i>	5	0.104	0.056	0.092	-0.296	1.372	-0.260
<i>Flight Activity</i>	1	1.894	19.531	0.893	0.441	3.218	0.208
<i>Flight Activity</i>	2	2.169	16.074	0.852	0.650	4.384	0.256
<i>Flight Activity</i>	3	1.884	17.721	0.890	-0.229	0.791	-0.108
<i>Flight Activity</i>	4	2.005	15.000	0.804	0.056	0.036	0.023
<i>Flight Activity</i>	5	1.820	17.144	0.859	0.355	1.974	0.167
<i>Social Interactions</i>	1	-0.057	0.017	-0.068	-0.436	3.139	-0.526
<i>Social Interactions</i>	2	-0.232	0.185	-0.219	-0.647	4.342	-0.609
<i>Social Interactions</i>	3	-0.027	0.004	-0.064	0.046	0.032	0.111
<i>Social Interactions</i>	4	-0.113	0.048	-0.139	-0.274	0.852	-0.337
<i>Social Interactions</i>	5	0.082	0.035	0.177	-0.060	0.057	-0.130
<i>Self-preening</i>	1	-0.617	2.075	-0.359	1.038	17.792	0.604
<i>Self-preening</i>	2	-0.763	1.991	-0.451	-0.173	0.311	-0.102
<i>Self-preening</i>	3	-0.670	2.243	-0.417	0.830	10.435	0.517
<i>Self-preening</i>	4	-0.977	3.564	-0.578	0.423	2.020	0.250
<i>Self-preening</i>	5	-0.614	1.953	-0.316	1.632	41.798	0.839

Table 8. Eigenvalues and explained variance for the 10 dimensions of the focal bird pair maintenance behavior multifactor analysis.

<i>Dimensions</i>	<i>Eigenvalue</i>	<i>Percentage of variance (%)</i>	<i>Cumulative percentage of variance (%)</i>
1	3.088	49.968	49.968
2	1.457	23.568	73.536
3	0.696	11.264	84.800
4	0.481	7.786	92.586
5	0.235	3.804	96.390
6	0.105	1.704	98.094
7	0.072	1.163	99.257
8	0.036	0.583	99.840
9	0.007	0.109	99.949
10	0.003	0.051	100.000

Table 9. Focal bird pair maintenance behavior multifactor analysis. Coordinates, contributions, and correlations of each pair maintenance behavior (allopreening, clumping) from days 1-5 post-cue for the first two dimensions of the multifactor analysis.

Behavior	Day	<i>Dimension 1</i>			<i>Dimension 2</i>		
		Coord.	Contrib.	Correl.	Coord.	Contrib.	Correl.
<i>Allopreening</i>	1	0.690	12.515	0.623	-0.814	36.978	-0.735
<i>Allopreening</i>	2	1.133	12.446	0.826	-0.213	0.930	-0.155
<i>Allopreening</i>	3	0.506	1.704	0.460	0.082	0.096	0.075
<i>Allopreening</i>	4	0.633	4.792	0.616	0.609	9.408	0.593
<i>Allopreening</i>	5	0.410	6.267	0.575	0.302	7.210	0.424
<i>Clumping</i>	1	0.497	6.506	0.702	0.343	6.573	0.485
<i>Clumping</i>	2	0.980	9.320	0.685	-0.883	16.039	-0.617
<i>Clumping</i>	3	1.581	16.627	0.744	-0.069	0.067	-0.032
<i>Clumping</i>	4	1.296	20.067	0.898	0.137	0.476	0.095
<i>Clumping</i>	5	0.511	9.756	0.664	0.530	22.224	0.689

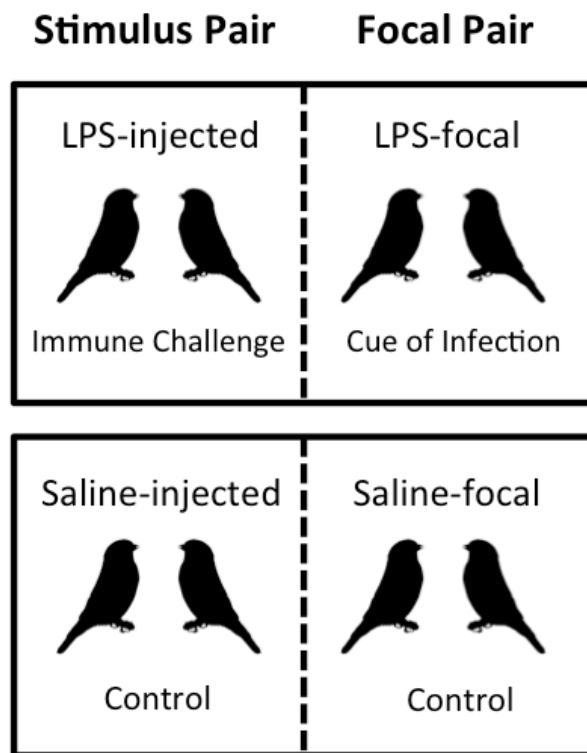


Figure 1. Experimental design and treatment groups. Stimulus pairs were either given an immune challenge with lipopolysaccharide (LPS-injected, N=9 pairs) or a sham injection with phosphate buffered saline (Saline-injected, N=11 pairs). Focal pairs were housed next to LPS-challenged pairs (LPS-focal, N=9 pairs) or healthy conspecifics (Saline-focal, N=11 pairs).

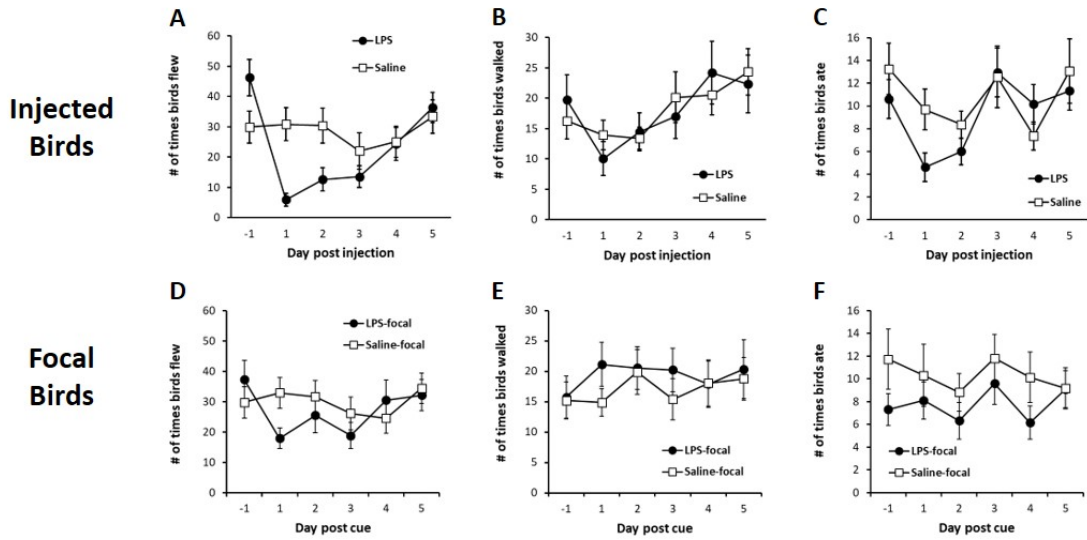


Figure 2. Behavioral counts for activity behaviors (flying, walking, eating) in injected stimulus birds (A, B, C) and focal birds (D, E, F). Stimulus birds were injected with either lipopolysaccharide (LPS: N=18) or phosphate buffered saline (saline: N=22). Focal birds were either housed next to sick-conspecifics (LPS-focal: N=19) or healthy conspecifics (Saline-focal: N=22). Data are reported as means \pm standard error.

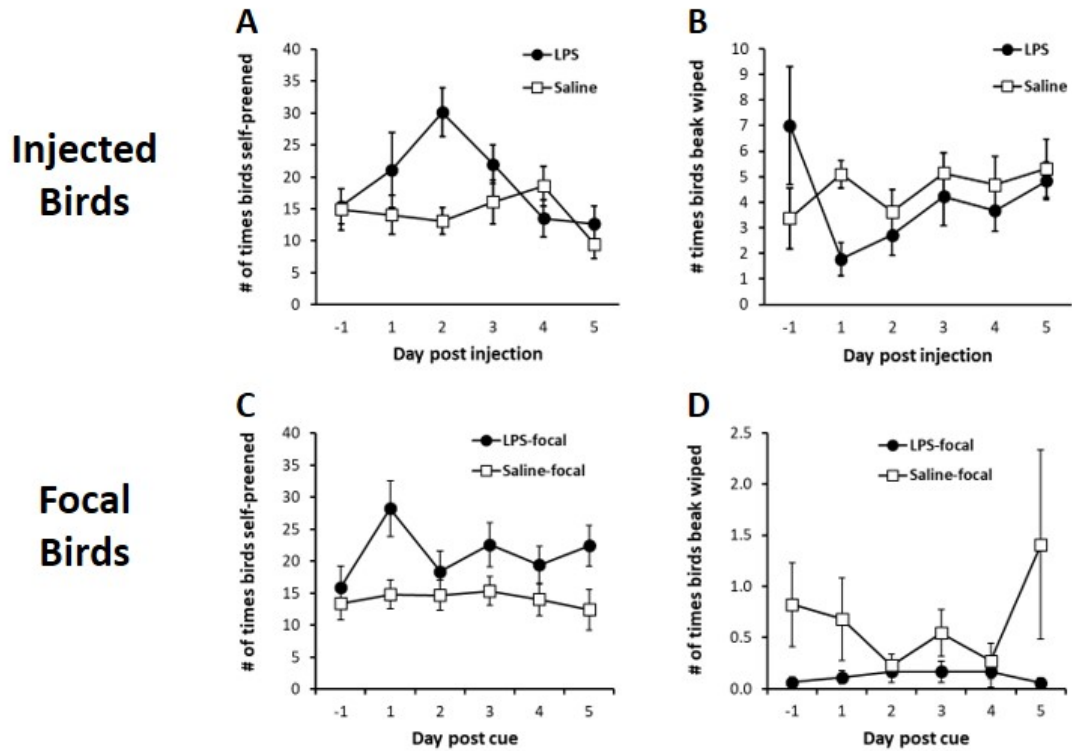


Figure 3. Behavioral counts for self-preening and beak wiping in injected stimulus birds (A, B) and focal birds (C, D). Stimulus birds were injected with either lipopolysaccharide (LPS: N=18) or phosphate buffered saline (Saline: N=22) and focal birds were either housed next to sick-conspecifics (LPS-focal: N=19) or healthy conspecifics (Saline-focal: N=22). Data are reported as means \pm standard error.

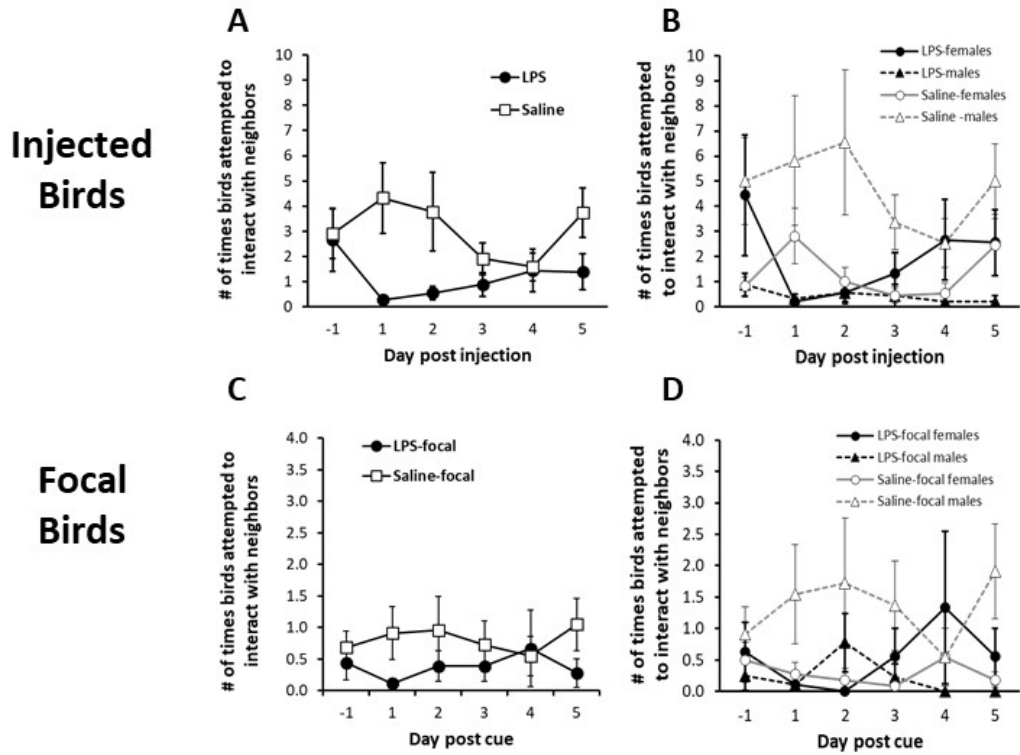


Figure 4. Behavioral counts for the number of social interaction attempts by stimulus injected birds (A, B) and focal birds (C, D), separated by treatment and sex. Data are reported as means \pm standard error.

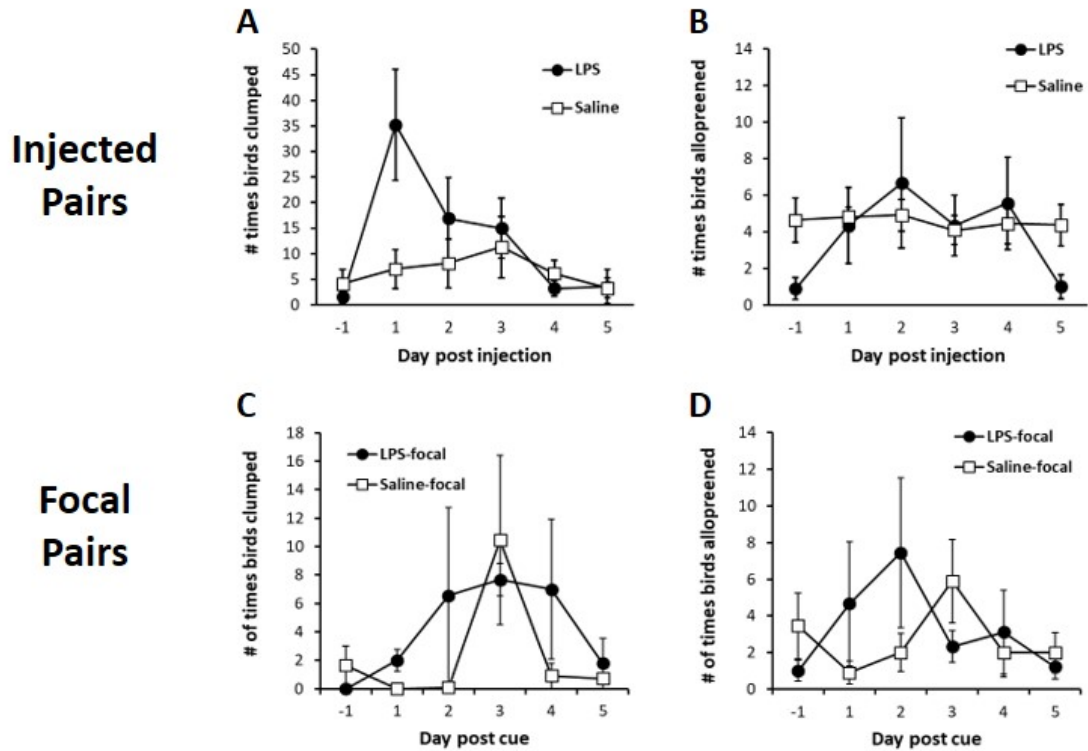


Figure 5. Behavioral counts for pair-maintenance behaviors including clumping (A) and allopreening (B) in birds injected with either lipopolysaccharide (LPS: N=9 pairs) or saline (Saline: N=11 pairs), and behavioral counts for clumping (C) and allopreening (D) in birds exposed to either sick conspecifics (LPS-focal: N=9 pairs) or healthy conspecifics (Saline-focal: N=11 pairs). Data are reported as means \pm standard error.

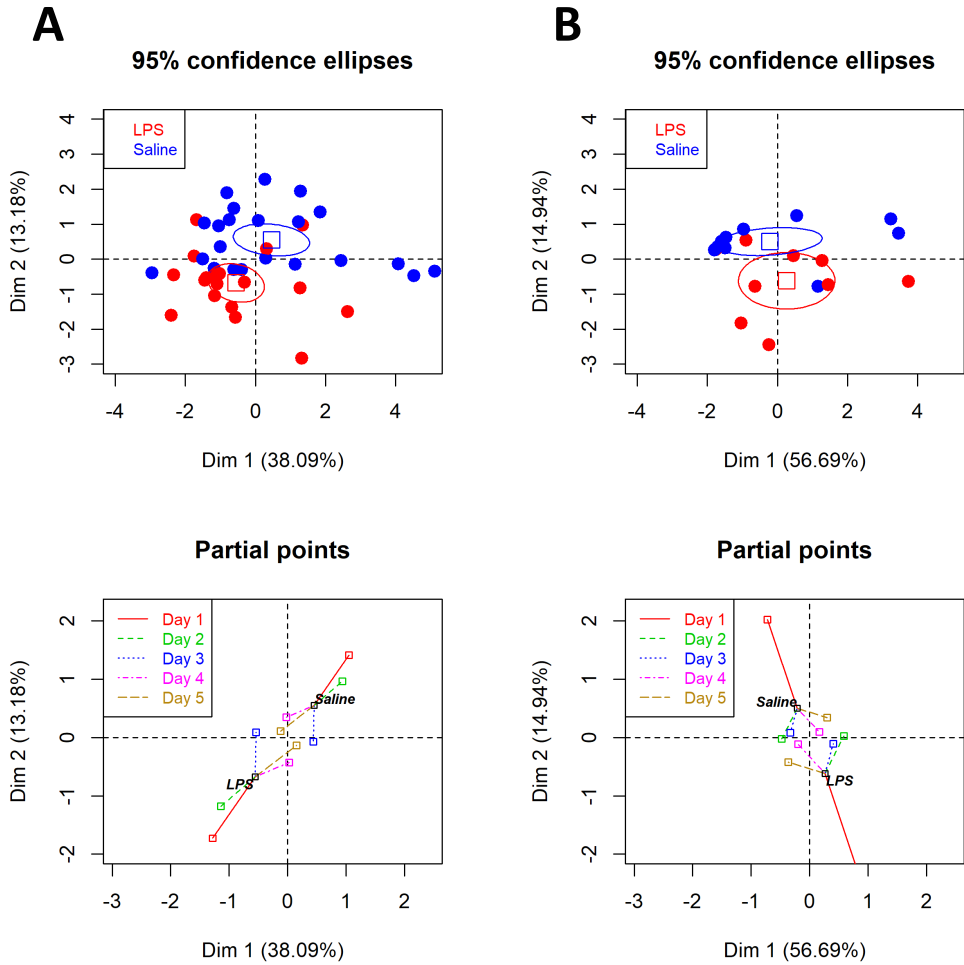


Figure 6. (A) Multifactor analysis plots of dimensions 1 and 2 for behaviors with implications for disease transmission through changes in rates of contact (flight activity, social interactions, eating, preening) in birds injected with either lipopolysaccharide (LPS: N=18) or saline (control: N=22). (B) Multifactor analysis plots of dimensions 1 and 2 for pair-maintenance behaviors (clumping, allopreening) in birds injected with either lipopolysaccharide (LPS: N=9 pairs) or saline (control: N=11 pairs). Individual points represent individuals (A) or pairs (B) respectively, and ellipses represent 95% confidence intervals for each treatment. Partial points plots show the contributions of each timepoint to the divergence in behavioral profiles between treatments.

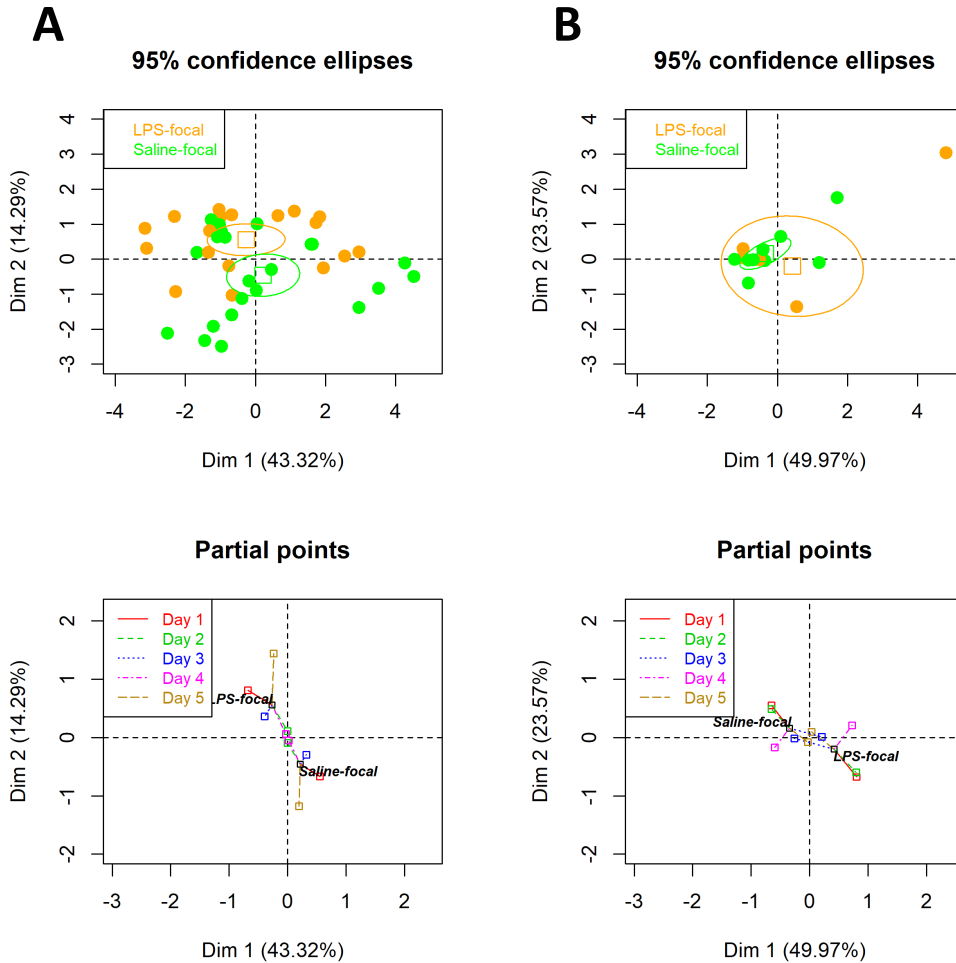


Figure 7. (A) Multifactor analysis plots of dimensions 1 and 2 for behaviors relevant to responding to an immune threat (flight activity, social Interactions, eating, preening) in birds exposed to sick conspecifics (LPS-focal: N=18) or healthy conspecifics (Saline-focal: N=22). (B) Multifactor analysis plots of dimensions 1 and 2 for pair-maintenance behaviors (clumping, allopreening) in birds exposed to sick conspecifics (LPS-focal: N=9 pairs) or healthy conspecifics (Saline-focal: N=11 pairs). Individual points represent individuals (A) or pairs (B) respectively, and ellipses represent 95% confidence intervals for each treatment. Partial points plots show the contributions of each timepoint to the divergence in behavioral profiles between treatments.

CHAPTER III

IMMUNE ACTIVATION, BUT NOT PERCEIVED INFECTION RISK, ALTERS MACRONUTRIENT-SPECIFIC CALORIC INTAKE

ABSTRACT: Pathogens and parasites are ubiquitous in nature, and hosts have evolved a suite of physiological and behavioral defenses to counter the fitness costs associated with infection. Nutritional resources play a vital role in host immunity and can influence host-pathogen dynamics as responding to infection is metabolically expensive and both hosts and pathogens utilize host resources. Macronutrient content of the diet can alter immune processes and offset the costs of mounting immune responses, thus, animals may shift feeding behaviors and selectively feed on foods with desirable macronutrient composition in response to an immune threat and in response to social cues of an impending threat, a phenomenon documented in insects, but understudied in vertebrates. This study sought to enhance our understanding of how feeding behavior and macronutrient selection are influenced by immune activation and perceived infection risk. To do so, we simulated an infection in zebra finches using the bacterial endotoxin lipopolysaccharide (LPS), and quantified feeding behavior in immune challenged and control individuals, as well as birds housed near either a control pair (no immune threat), or birds housed near a pair

given an immune challenge with LPS (social cue of heightened infection risk). Additionally, because social information can shape physiology, we also examined whether social cues of infection alter physiological responses relevant to responding to an immune threat, an effect that could be mediated through shifts in feeding behavior. Contrary to our predictions, we found no evidence for socially induced shifts in feeding behavior, complement activity, or corticosterone and testosterone concentrations. While social cues of infection did not shift feeding behavior in the present study, birds challenged with LPS altered feeding in a manner consistent with sickness-induced anorexia, and this reduction in caloric intake was driven by a decrease in protein, but not lipid consumption. This finding carries implications for host health and epidemiology, as sickness-induced anorexia may enhance or diminish infection severity depending on dietary context, and could shape disease transmission dynamics through nutritionally driven shifts in host-pathogen interactions.

INTRODUCTION

Nutrition is critical to the immune system and can influence how hosts respond to infection (Cunningham-Rundles et al. 2005, Ponton et al. 2011, Amar et al. 2007). Both under-nutrition and over-nutrition can disrupt normal immune processes and contribute to morbidity and mortality rates due to infectious disease (Cunningham-Rundles et al. 2005, Amar et al. 2007). Among ecological studies, the effect of the availability of resources on immune processes and disease outcomes has received significant attention (Becker et al. 2018, Strandin et al. 2018, Moyers et al. 2018), but the quality of those resources and

their nutritional make-up (e.g., macronutrient content) are also important (Povey et al. 2013, Cotter et al. 2011). Macronutrients like lipids, carbohydrates, and protein vary in their biological availability and can influence processes ranging from cellular function to whole organism performance (Warne 2014). Diet macronutrient content can alter immune processes and mitigate the costs of mounting immune responses. For example, restriction of dietary protein can limit immune activity (Lee et al. 2006, Povey et al. 2009) and high lipid diets can increase mortality rates during infection (Adamo 2008, Adamo et al. 2010). The nutritional composition of diets can also differentially affect different components of the immune system. For example in insects, the optimal nutritional composition of diets varies for different immunological parameters (Cotter et al. 2011). Thus, optimal diet selection may vary based on the type of immune threat that organisms are experiencing, and organisms may be able to optimize responses to infection through shifts in feeding behavior.

Despite the apparent need for nutritional resources to mount an immune response, some organisms respond to infection with sickness-induced anorexia (Adamo et al. 2007, Povey et al. 2013). This reduction in food intake following an immune challenge has been hypothesized to reduce the risk of ingesting additional infectious agents, or may function to starve pathogens and parasites of key nutrients (Kyriazakis et al. 1998, Adamo et al. 2007, Adelman and Martin 2009). Further, caloric restriction during illness can actually improve host health and recovery (Cheng et al. 2017; Wang et al. 2016). In addition to influencing the quantity of food that animals consume, infection can also alter diet selection in terms of diet quality. In caterpillars challenged with a viral infection, infected individuals select diets with a higher protein to carbohydrate ratio when

compared with control individuals, and infected individuals placed on a high protein diet are more likely to survive infection (Povey et al. 2013). Shifts in diet preference during infection that optimize recovery and survival are referred to as “self-medication” behaviors and have been documented in several taxa (Huffman and Seifu 1989, Hutchings et al. 2003, Povey et al. 2013). Thus, animals can shift feeding behaviors and selectively feed on foods with desirable macronutrient composition in response to an immune threat.

Because nutrition can influence immune responses, organisms may also respond to social cues of disease by altering their feeding behavior in a way that optimizes or primes the immune system to fight off infection. Such prophylactic behavior has been documented in ants that collect pine resin, which in turn provides anti-pathogen resistance to the ant colony (Castella et al. 2008). Thus, it is possible that social information about the disease environment could alter immune function and disease susceptibility through shifts in individual feeding behavior and macronutrient selection. Further, recent research in social organisms indicates that physiological changes can occur in response to public information (Cornelius et al. 2018; Schaller, et al. 2010, Stevenson et al. 2011, 2012). Because nutritional state is known to greatly impact disease outcomes (Murray et al. 1998, Chandra 1996, Lochmiller & Deerenberg 2000), prophylactic behaviors involving macronutrient selection in response to cues of disease could help prepare organisms for an impending immune threat.

This study aims to enhance our understanding of how feeding behavior and macronutrient selection are influenced by immune activation and perceived infection risk and provide insight into whether socially-induced shifts in feeding behavior alter

physiological processes relevant to disease susceptibility and transmission. To date, most work examining the interactions between macronutrient selection, immunity, and disease has been conducted in invertebrates. The goal of this study was to explore these relationships in a vertebrate by investigating how both an immune threat and the perceived risk of infection shape feeding behavior and diet macronutrient selection in birds. Because birds are hosts for diseases relevant to wildlife, domestic animal, and human health, identifying and understanding the factors that contribute to variation in avian responses to infection is of broad interest and integral to improving our understanding of avian epidemiology. Specifically, we wanted to test whether birds given an immune challenge shift their feeding behavior and macronutrient preferences. Finally, because recent work indicates that social cues can shape physiology (Cornelius et al. 2018; Schaller et al. 2010, Stevenson et al. 2011, 2012), we also wanted to investigate whether a social cue of infection (seeing immune-challenged conspecifics) alters physiological responses pertinent to immune function and examine whether shifts in feeding behavior are responsible for physiological responses to social cues of disease. Specifically, we wanted to examine whether social cues of infection alter complement activity (part of the innate immune system that contributes to the lysis of foreign cells) and concentrations of the steroid hormones corticosterone and testosterone, as both of these hormones change in response to environmental and social cues and can influence immunity (Roberts et al. 2007, Da Silva 1999). To explore these relationships, we simulated an infection in established zebra finch pairs using the bacterial endotoxin lipopolysaccharide (LPS), and quantified feeding behavior in immune challenged and control individuals, as well as birds housed near either a control pair (no immune threat),

or birds housed near a pair given an immune challenge with LPS (social cue of heightened infection risk). To investigate how an immune challenge and perceived immune threat shape macronutrient preference, we created artificial diets with varied lipid and protein ratios. We predicted that birds given an immune challenge and birds with a social cue of heightened infection risk would increase protein consumption, as studies in insects indicate that high protein diets are associated with increased immune capabilities and high lipid diets are associated with increased mortality during infection (Povey et al. 2013, Adamo 2008, Adamo et al. 2010).

MATERIALS AND METHODS

Bird Housing and Diets

Birds were kept on a 14 L: 10 D light cycle and housed in 24"x16"x16" cages that were divided down the center into two separate 12"x16"x16" cage sections that each housed one pair of birds. Birds were housed in previously established pairs with one female and one male per cage section. Each cage section had two perches, a water dish, and two food dishes in which birds were fed *ad libitum*. Diets consisted of hulled millet, egg white, egg yolk, vegetable oil, and sorbic acid (preservative) in agar blocks. All diets were isocaloric and only varied in the ratio of protein and lipid content. Birds were placed on a choice diet where each cage contained one high lipid diet (25% lipid, 13% protein) and one high protein diet (13% lipid, 25% protein). Diet type was randomly assigned to either the left or right food dish to avoid confounding effects of cage side preference. To assess whether an immune threat altered feeding behavior and diet preference, birds were

provided with the choice diet for seven days prior to experimental treatment and for seven days following experimental treatment. Diets were weighed daily and replaced every other day. Three desiccation controls for each diet type were also weighed daily and the average desiccation values for each diet type (high lipid, high protein) were subtracted from feeding values to account for daily changes in food mass due to desiccation.

Immune Challenge and Experimental Design

Pairs housed on one side of the cage were injected with either LPS or Saline (LPS-injected: N=24 birds or Saline-injected: N=24 birds), whereas pairs housed on the other side of the cage were unmanipulated (focal pairs; LPS-focal: N=24 birds or Saline-focal: N=24 birds). Injected pairs provided social cues to the focal pair. Solid opaque dividers were placed on both sides of each 24 x 16 x 16 cage to ensure that birds housed in each double cage could only see one another. To assess how an immune challenge and social cue of heightened infection risk shape feeding behavior, we injected previously established pairs with either lipopolysaccharide (LPS), a non-replicating antigen that activates the immune system and induces sickness behaviors, or a saline solution (control). Specifically, we injected stimulus birds intra-abdominally with either 50 μ L of 2 mg/kg LPS (Sigma-Aldrich #L7261, *Salmonella enterica* serotype typhimurium) or 50 μ L of phosphate-buffered saline (sham control, Sigma-Aldrich #P3813). To assess whether heightened infection risk altered complement activity or baseline corticosterone concentrations, we collected blood samples from all focal birds 3 days prior to stimulus

bird injections and 1, 2, and 5 days following stimulus bird injections. All blood samples for baseline corticosterone were collected within 3 minutes of entering the bird room. We collected additional blood samples from all focal males to assess changes in plasma testosterone concentrations in response to a heightened cue of infection 4 days prior to and 3 days following injection of the stimulus pairs. Immediately after collection, blood samples were centrifuged and blood plasma was separated and frozen at -20°C. Body mass and fat score data were collected on all stimulus and focal birds prior to treatment and on days 1, 2, and 5 post treatment. Research protocols were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Complement Activity Assay

To assess if social cues of disease influenced the complement pathway, we conducted a CH50 complement assay that measures the ability of proteins in the plasma to lyse sheep red blood cells (MP Biomedicals, Cat#55876). We measured complement activity following the methods outlined in Sinclair and Lochmiller (2000). Briefly, we ran duplicate 80 µl samples of 1:20 and 1:40 plasma dilutions and averaged each set of duplicates prior to analyses. Hemolytic complement activity was expressed as CH50 units/ml plasma, where one CH50 unit signifies the reciprocal of the dilution of plasma needed to lyse 50% of the sheep red blood cells (French et al. 2010).

Corticosterone Assay

Plasma corticosterone concentrations were measured in duplicate following standard radioimmunoassay techniques (Wingfield et al., 1992). To determine the coefficient of intra- and inter-assay variation, four standard samples were prepared with 200 pg of corticosterone and plasma and standard sample tubes were prepared with 500 μ l ddH₂O and 2000 dpm of tritiated corticosterone (NET-399) from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). Samples were equilibrated overnight at 4 °C. Corticosterone was extracted from plasma using 4 ml of diethyl ether and dried in a 37 °C water bath with the aid of nitrogen gas. Following extractions, samples were suspended in 500 μ l of phosphate buffered saline and refrigerated overnight at 4 °C. The following day, we determined individual extraction efficiency using 50 μ l of each sample. Corticosterone concentrations were corrected for individual extraction efficiency (mean recoveries were 83%). For the assay, each sample was allocated into two duplicates, each consisting of 200 μ l. 100 μ l of corticosterone antibody (B3-163; Endocrine μ l Sciences, Calabasas, CA, USA) and 100 μ l of tritiated corticosterone were added to each sample and standard tube. We compared the mean value of the duplicates for each sample to a standard curve (also run in duplicate) that contained known amounts of corticosterone (C2505 corticosterone standard, Sigma-Aldrich, St. Louis, MO, USA). The intra-assay coefficient of variation and inter-assay coefficient of variation were 11.4% and 13.3% respectively.

Testosterone Assay

Plasma testosterone concentrations were measured using an Enzo Testosterone ELISA Kit (Cat#: ADI-900-065). Samples were run in duplicate at a 1:20 dilution following treatment with 1% steroid displacement buffer. Each plate contained a standard curve run in triplicate. Absorbance was measured at 405 nm using a SpectraMAX 190 spectrophotometer (Molecular Devices). The intra-assay coefficient of variation and inter-assay coefficient of variation were 12.8% and 14.0% respectively. Cross-reactivity of the testosterone antibody was as follows: androstenedione 7.2%, estradiol 1%, dehydroepiandrosterone 1%, dihydrotestosterone 1%, and progesterone 1% (Enzo Life Sciences).

Statistical Analyses

All data were checked for normality and homoscedasticity. To meet parametric requirements for normality and homoscedasticity, complement activity and corticosterone and testosterone concentrations were square root transformed. To examine how feeding behavior and diet preference changed over time with treatment, we ran separate repeated measures ANOVAs to test for differences in the stimulus (LPS-injected, saline-injected) and focal (LPS-focal, saline-focal) groups, respectively. Because birds were housed in pairs, feeding behavior was analyzed by cage rather than by individual. Cage number was included as a blocking factor to account for random behavioral variation between the birds housed in each cage. To examine how baseline corticosterone concentrations and hemolytic complement activity varied in the focal birds, we ran separate repeated

measures ANOVAs, which included treatment (LPS-focal or saline-focal), time, sex, and all pairwise interactions as predictors. Testosterone samples were collected from males only, so sex was not included in this model. For all analyses, all non-significant pairwise interactions were removed from each model. For all models, we used the Satterthwaite approximation to calculate denominator degrees of freedom. All models were run using PROC MIXED in SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Stimulus Birds

Birds given an LPS immune challenge significantly reduced the total grams of food that they consumed post-injection (Figure 1A, Day*Treatment: $F_{6,102} = 2.80$ $p = 0.015$). This reduction in overall food intake was also macronutrient specific. Specifically, LPS-challenged birds did not alter their consumption of the high lipid diet (Figure 2A, all $F \leq 0.33$, all $p \geq 0.574$), but significantly decreased consumption of the high protein diet (Figure 2B Day*Treatment: $F_{1,22} = 4.97$, $p = 0.036$). Body mass differed over time in the two treatments (3A, Day*Treatment: $F_{4,160} = 11.76$ $p < 0.0001$), where LPS-injected birds lost weight in the days following the immune challenge. Regardless of treatment, body mass also varied by sex (Sex: $F_{1,47.4} = 7.83$ $p = 0.007$), where female birds were typically heavier than males. Fat score did not differ between the two treatments but did vary over the course of the experiment (3B, Day: $F_{4,105} = 2.92$ $p = 0.025$).

Focal Birds

LPS-focal and Saline-focal birds did not differ in diet intake in terms of quantity or macronutrient composition (Figure 2C and 2D, all $F \leq 0.46$, all $p \geq 0.506$), but the total grams consumed did vary over time for focal birds in both treatments (Figure 1B, Day: $F_{6,86.3} = 2.37$ $p = 0.037$). In focal birds, body mass did not differ between treatments or over time (Figure 3C, all $F \leq 1.18$, all $p \geq 0.322$), and furcular fat score varied over time but was not influenced by treatment (Figure 3D, Day: $F_{4,105} = 3.57$ $p = 0.009$). Focal bird physiology was also not influenced by a cue of infection. Specifically, complement activity (Figure 4), baseline corticosterone concentrations, and testosterone concentrations did not vary by treatment (all $F \leq 1.54$, all $p \geq 0.228$). Regardless of treatment, baseline corticosterone concentrations increased 2 days after experimental treatment in males, but not females (Figure 5, Day*Sex: $F_{3,99.4} = 2.91$ $p = 0.038$) and testosterone concentrations (collected from males only) decreased over time (Figure 6, Day: $F_{1,22.8} = 17.47$ $p = 0.0004$).

DISCUSSION

The goal of this study was to 1) investigate how an immune threat influences feeding behavior and macronutrient selection and 2) explore if social cues of disease can alter feeding behavior and immune and endocrine responses relevant to disease susceptibility. Based on research in invertebrates, we predicted that birds given an immune challenge would either increase protein intake or maintain consistent levels of protein consumption while reducing lipid intake (Adamo 2008, Adamo et al. 2010, Povey

et al. 2013, Cotter et al. 2010). Conversely, we found that birds given an immune challenge with LPS engaged in macronutrient-specific sickness-induced anorexia, by maintaining consumption of the high lipid diet while significantly reducing consumption of the high protein diet. Consistent with sickness-induced anorexia, immune challenged individuals lost weight, but did not have any detectable changes in furcular fat stores. Caloric restriction during illness can improve host health and recovery in some cases (Cheng et al. 2017; Wang et al. 2016); thus, the observed reduction in caloric intake in LPS-challenged birds may be an adaptive response to an immune threat.

The finding that sickness-induced anorexia in LPS-injected birds was driven by a macronutrient-specific reduction in protein intake is particularly interesting given the apparent importance of protein to immune function and responding to and surviving infection (Lee et al. 2006, Povey et al. 2013). Prior research in insects suggests that individuals should benefit from reducing lipid intake during infection, however we saw no change in lipid intake in birds given an immune challenge. For example, infected caterpillars assigned to a high-lipid diet have higher mortality rates than infected individuals feeding on water or sucrose (Adamo et al. 2007). Further, research in crickets identified a trade off between immunity and lipid-transport, suggesting that reducing lipid consumption can maximize immune responses (Adamo et al. 2010). It is unknown whether a trade off between lipid-transport and immunity exists in vertebrates (Demas and Nelson 2012), however our finding that LPS-challenged birds reduce protein consumption but not lipid consumption challenges this idea and warrants further investigation in avian and other vertebrate systems. Although studies examining the relationship between infection and dietary macronutrient preference are uncommon in

vertebrates, one study in mammals found a similar reduction in protein intake following LPS immune-challenge. Specifically, rats injected with LPS voluntarily decreased protein intake while lipid intake remained unchanged, however this study also observed a significant increase in carbohydrate consumption in LPS-treated individuals (Aubert et al. 1995). Coupled with our finding that LPS-challenged birds selectively reduce protein but not lipid intake, this suggests that reduced protein consumption may be a common behavioral response to an immune threat in vertebrate species, although the function of this shift in macronutrient preference is still unclear. It is possible that a reduction in protein consumption occurs in response to an immune challenge because protein is more likely to contain iron than other macronutrients. Although iron is essential to host immune function, it is also utilized by pathogens. Thus, limiting iron intake could interfere with pathogen growth and help limit infection (Soyano and Gómez 1999, Kluger and Rothernberg 1979). Further work is needed to determine if reduced protein consumption during infection is adaptive for hosts in terms of responding to and overcoming infection, and whether these effects are mediated through shifts in micronutrients such as iron.

Because shifts in behavior and pathology associated with infection could act as social cues of heightened infection risk to uninfected conspecifics, we also examined if perceived risk of infection (seeing sick conspecifics) could alter feeding behavior and macronutrient selection. Separate lines of evidence indicate that social cues can influence feeding behavior and alter physiological responses relevant to immune function (Cornelius et al. 2018, Schaller et al. 2010, Stevenson et al. 2011, 2012). Thus, we predicted that birds exposed to a heightened risk of infection would have physiological

responses relevant to responding to an immune threat and shift feeding behavior in a way that maximizes immunity. Contrary to our predictions, we found no evidence for shifts in feeding behavior, macronutrient intake, or physiological responses in birds exposed to a cue of heightened infection risk. Because we used a simulated infection (injection with LPS) in this study, it is possible that the cue of infection was not sufficient to stimulate physiological changes or alter the feeding behavior of focal birds. The behavioral effects of LPS typically only last between 2-4 days following injection (Sköld-Chiriac et al. 2014, Love *unpublished data*), thus the cue of infection elicited by LPS-injection is temporally limited. Future work should explore whether an immune challenge or infection that elicits stronger and longer-lasting behavioral and physiological signs of disease is capable of influencing feeding behavior and physiological responses in healthy individuals, as this could have implications for host disease susceptibility and disease transmission potential.

Although we did not detect an effect of perceived infection risk on any of the physiological parameters examined in this study, we did observe changes in corticosterone and testosterone concentrations in male zebra finches over the course of the experiment. Specifically, we saw an increase in corticosterone concentrations in males from both focal treatments 2 days after injection of the stimulus birds, but did not observe a similar increase in corticosterone concentrations in focal females. The observed difference in corticosterone concentrations between males and females could occur because males and females differ in endocrine responses to handling stress (Kudielka and Kirschbaum 2005). Further, we observed a decrease in testosterone levels in males 3 days after the injection of stimulus birds, and this decrease occurred in both LPS-focal and

saline-focal males. Corticosterone is known to have inhibitory effects on the release of testosterone (Da Silva 1999), so it is possible that the observed decrease in testosterone concentrations in males is related to the increase in circulating corticosterone. Although heightened infection risk did not affect baseline levels of corticosterone or testosterone in the present study, it is possible that the effects of social cues of disease could be more prominent when examining stress-induced levels of these hormones. Finally, because both of these hormones respond to social cues and are effective modulators of immunity (Roberts et al. 2007, Da Silva 1999), whether cues of infection can shape hormonal and immunological responses in birds deserves further investigation.

The present study extends our understanding of how immune activation can influence feeding behavior and diet selection in vertebrates. We were not able to detect any shifts in feeding behavior, nor any immune or endocrine changes in response to social cues of infection in the present study. However, we did detect macronutrient-specific illness-induced anorexia in LPS-challenged birds, where birds decreased protein but not lipid intake. Shifts in feeding behavior in sick individuals can affect both host and parasite fitness and ultimately influence disease severity, which is inherently related to disease transmission (Hite and Cressler 2019, Povey et al. 2013). Models indicate that sickness-induced anorexia, like the reduction in caloric intake observed in LPS-birds in the present study, is capable of enhancing or diminishing disease severity depending on dietary context (Hite and Cressler 2019), suggesting that the interactions between infection, resource availability, and host macronutrient selection can have important consequences for disease dynamics and deserve further attention.

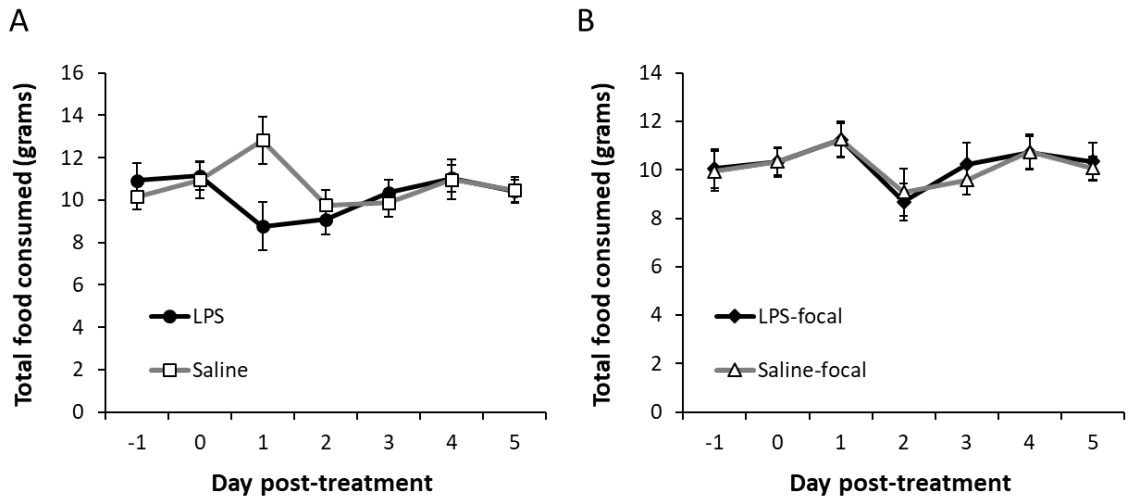


Figure 1. Total grams of food consumed per day in response to A) injection with the bacterial endotoxin lipopolysaccharide (LPS) or saline (control) and in B) focal birds that were either housed next to sick-conspecifics (cue of infection, LPS-focal) or healthy conspecifics (Saline-focal). Data are reported as means \pm standard error.

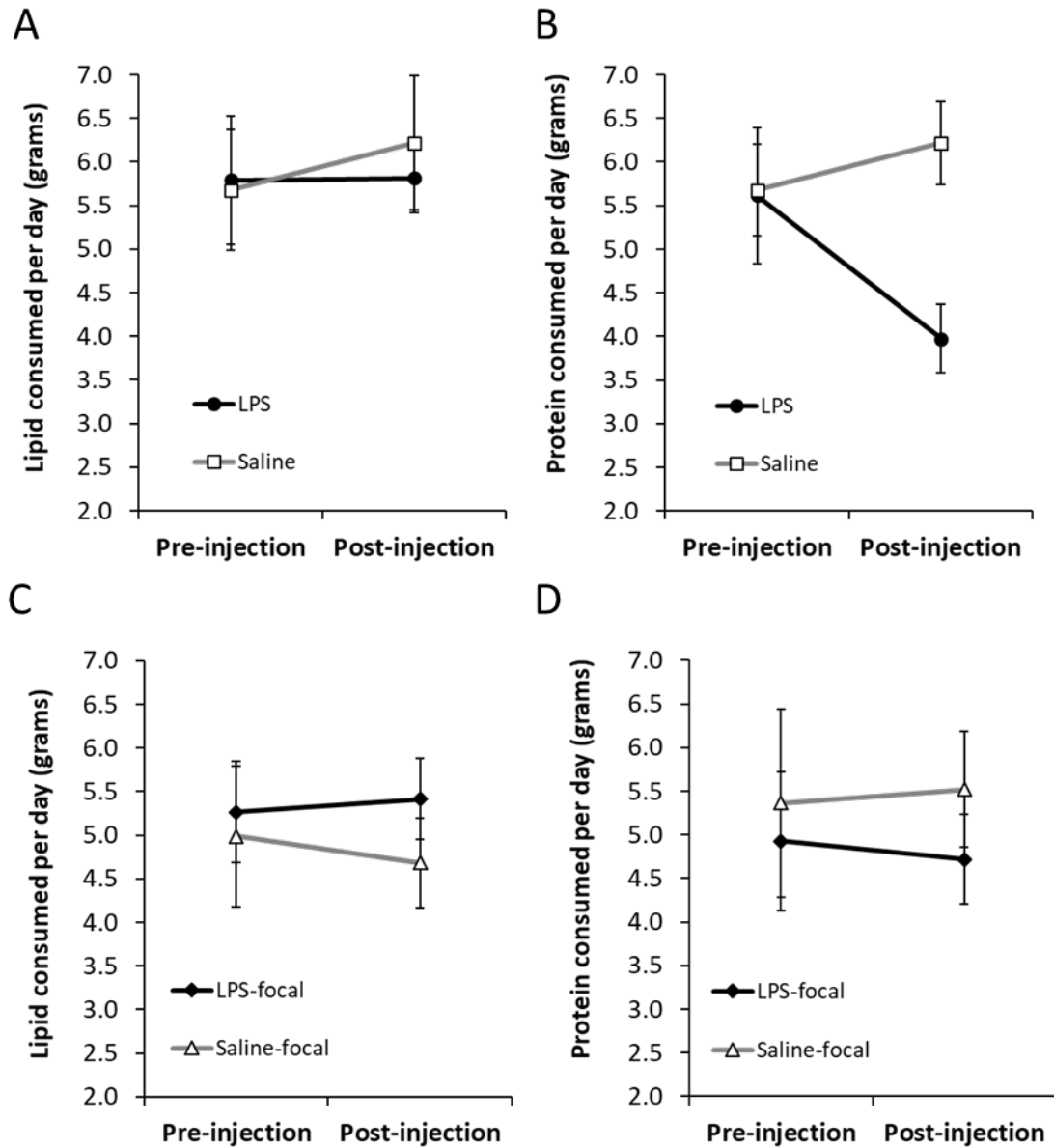


Figure 2. Macronutrient specific diet consumption. Grams of high lipid and high protein diet consumed per day in birds challenged with lipopolysaccharide (LPS) or saline (A, B) and in focal birds that were either housed next to sick-conspecifics (LPS-focal) or healthy conspecifics (Saline-focal) (C, D). Data are reported as means \pm standard error.

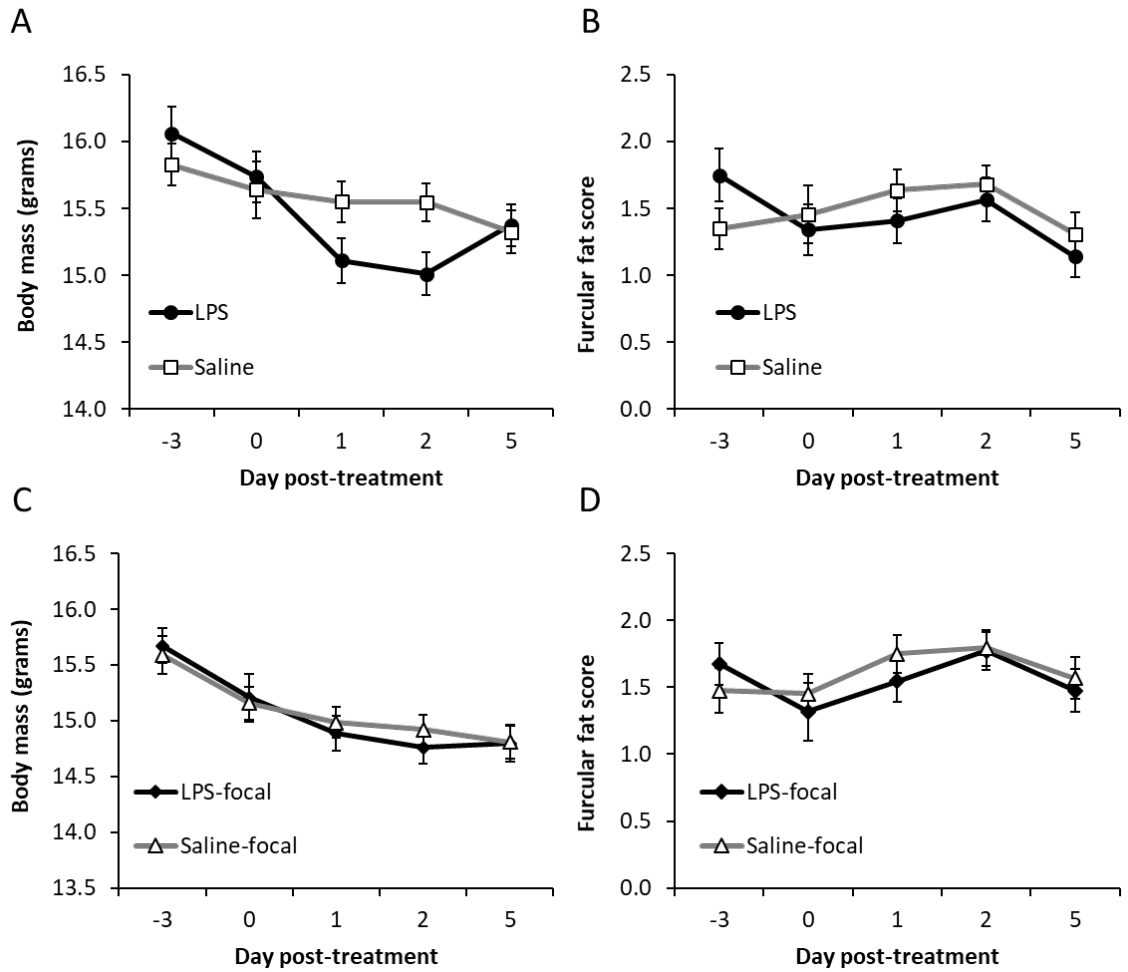


Figure 3. Body mass and furcular fat score in in birds challenged with lipopolysaccharide (LPS) or saline (A, B), and in focal birds that were either housed next to sick conspecifics (LPS-focal) or healthy conspecifics (Saline-focal) (C, D). Data are reported as means \pm standard error.

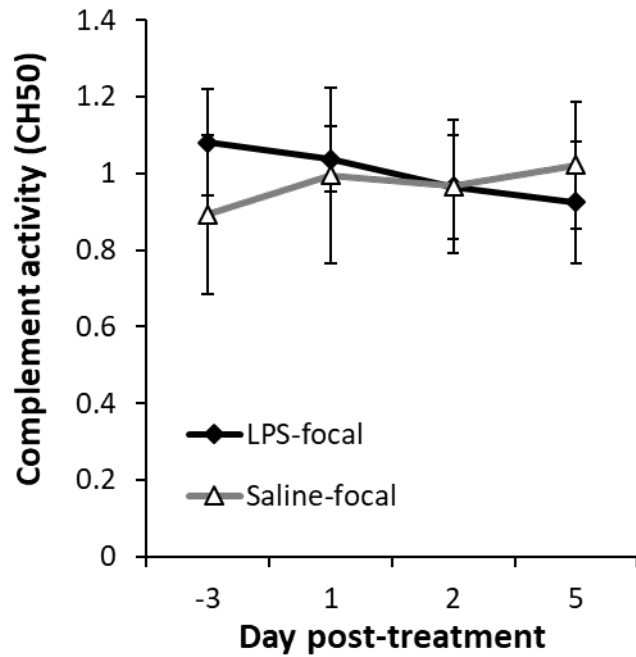


Figure 4. Hemolytic complement activity (CH50) in focal birds that were housed next to sick-conspecifics (cue of infection, LPS-focal) or healthy conspecifics (no cue of infection, Saline-focal). Data are reported as means \pm standard error.

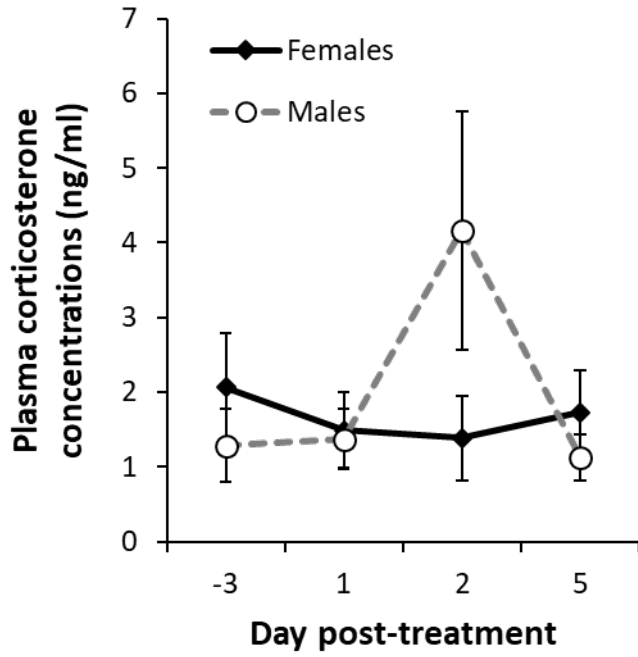


Figure 5. Sex differences in plasma corticosterone concentrations in focal birds. Data are reported as means \pm standard error.

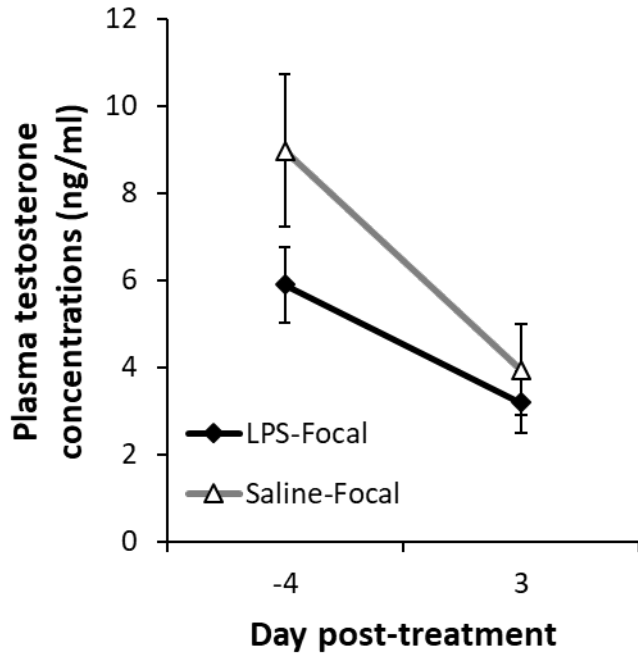


Figure 6. Plasma testosterone concentrations in male zebra finches that were housed next to sick-conspecifics (cue of infection, LPS-focal) or healthy conspecifics (no cue of infection, Saline-focal). Data are reported as means \pm standard error.

CHAPTER IV

BIRDS HOUSED IN VISUAL CONTACT WITH SICK NEIGHBORS HAVE ALTERED IMMUNE PROFILES

ABSTRACT: The detection and avoidance of sick conspecifics is common among animals, but less is known about how viewing diseased conspecifics influences an organism's physiological state. While immune activation in response to a perceived immune threat is a relatively new concept in the field of disease ecology, it is well established that shifts in physiology occur in response to other external cues such as perceived predation risk. Indeed, recent work in humans suggests that visual cues of infection are capable of stimulating the immune system, presumably to help the body prepare for an impending immune threat. Whether visual cues of disease can also induce changes in immunity in non-human organisms is not well understood, however understanding how social cues impact immunity in wildlife may be particularly important for highly mobile species like bats and birds that are known to transmit diseases to both domestic animals and humans. Using an avian host-pathogen system in a social bird, we examined if cues elicited by infected conspecifics affect complement activity and white blood cell differentials in uninfected neighboring individuals. Uninfected birds did not have physical contact with infected birds to prevent pathogen transmission, which

primarily occurs through fomites. Immune activation occurred in birds visually exposed to infected individuals around 6-12 days post-inoculation, which is also when infected stimulus birds exhibited the greatest degree of disease pathology and lethargy. These data suggest that social cues of infection are capable of altering immune responses in healthy individuals and could play a role in shaping individual variation in disease susceptibility and disease transmission.

INTRODUCTION

Animals have developed multiple ways to detect and avoid pathogens and parasites, and much of this ability involves processing social information (Sarabian et al. 2018). Sick conspecifics often provide visible cues that they are infected through behaviors such as lethargy, and physical signs, such as inflammation and lesions. Healthy individuals can detect these changes in sick individuals and adjust their behavior according to this social information. One of the most common mechanisms for avoiding infection is to detect and avoid sick conspecifics. Behavioral avoidance of parasites and pathogens is a crucial defense against becoming infected and occurs in a diverse array of animals, ranging from insects to humans (Kiesecker et al. 1999, de Roode and Lefèvre 2012, Curtis 2014). While perceiving a sick conspecific can result in behavioral changes such as avoidance behavior, behavioral shifts may not be the only defense mechanism available to organisms when faced with an immune threat.

Work in humans suggests that social cues of infection are also capable of stimulating the immune system, presumably to help the body prepare for an impending

immune threat. For example, people have greater IL-6 responses to LPS stimulation after viewing images of sick individuals (Schaller et al. 2010). Similarly, viewing images of “disgust” such as roaches crawling on food, dead animals, and vomit can elevate body temperature and alter oral immunity in human subjects (Stevenson et al. 2011, Stevenson et al. 2012). In insects, visual cues indicative of heightened infection risk, such as population density and crowding can also alter immune function (Wilson and Reeson 1998, Ruiz-González et al. 2009, Cotter et al. 2004). These studies imply that organisms can adjust investment in immune defenses to match the probability of exposure to an immune threat.

Many wildlife diseases are capable of causing obvious external pathology (e.g., Devil Facial Tumor Disease in Tasmanian devils, Mycoplasmal conjunctivitis in Fringillidae songbirds) that could be detected by other organisms and act as a signal of increased infection risk. Despite this, we know relatively little about how the perceived risk of infection shapes physiological responses in wildlife. It is well established that social information about disease can shift host behavior, and these cues of infection have even been implicated in shaping individual and population-level differences in group structure and behavior (Patterson and Ruckstuhl 2013, Barber and Dingemanse 2010, Buck et al. 2018). Moreover, behavioral changes following the perception of disease can influence the capacity of pathogens to successfully invade and persist in a population (Curtis 2014, Moore 2002, Lloyd-Smith et al. 2004). If the perception of an immune threat can also shift host immune responses, then the population-level consequences of these shifts in immune physiology could play a critical role in disease dynamics.

The goal of this study was to address whether the visual perception of an immune threat can shape immune physiology in birds. Birds are highly mobile and harbor diseases relevant to wildlife, domestic animal, and human health, including infectious diseases such as West Nile virus and avian influenza. Understanding how social cues shape immunity in birds will likely enhance our understanding of how diseases spread through avian populations. To date, most studies examining how organisms respond to visual cues of disease employ an artificial immune threat as the source of experimental manipulation, such as images of sick people or conspecifics injected with a non-replicating antigen (Schaller et al. 2010, Stevenson et al. 2011, Stevenson et al. 2012). Using a live pathogen to elicit a cue of disease is more ecologically relevant and can cause stronger and longer-lasting signs of disease than the transient effects caused by non-replicating antigens. The avian pathogen *Mycoplasma gallisepticum* (MG) is an ideal tool for investigating how the perception of social cues of disease shape how healthy individuals respond to infection, as infection with this bacterium causes obvious visual signs, including lethargy and conjunctivitis (Hawley et al. 2011, Love et al. 2016). Moreover, birds make an excellent model for assessing questions about visual cues of infection because they are social animals that rely primarily on vision for detecting immune threats. Here, we test whether social information transmitted by infected individuals can stimulate innate immune responses in domestic canaries (*Serinus canaria domestica*) housed in visual contact with either healthy or *Mycoplasma gallisepticum*-infected conspecifics.

MATERIALS AND METHODS

Bird Housing

All birds were housed individually in 15”x18”x18” cages on a 14 L: 10 D light cycle. Cages had two plastic perches, a water dish, and a food dish in which birds were fed an *ad libitum* mixed seed diet (Canary Food/European Blend, ABBA 3700).

Individual racks only housed birds of the same treatment to minimize cross-contamination of MG. An opaque divider was placed between infected and control birds to block visual cues of infection and visually isolate the stimulus and focal groups. Thus, control and control-focal birds were housed on one side of the divider, while MG-infected and MG-focal birds were housed on the opposite side of the divider (Figure 1). All research protocols were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Experimental Timeline

In order to assess how visual cues of disease shape immune function, we inoculated stimulus birds with either *M. gallisepticum* (MG-infected) or a control media (Control). We characterized the strength of the cue of infection by assessing disease pathology. Specifically, we recorded eye score (conjunctival inflammation), body mass, and fat score in the MG-infected and control stimulus birds before and at several time points post-inoculation (Figure 2). Focal birds were sampled on different days than stimulus birds due to logistical constraints on the number of birds that could be processed and blood sampled in one sampling period. To determine if seeing sick conspecifics can

activate immune responses, we collected blood samples from all focal birds (MG-focal (N=10): seeing cue of disease, Control-focal (N=9): not seeing cue of disease) prior to experimental treatment and on days 2, 6, 12, and 24 post exposure to the cue (Figure 2). Specifically, blood samples were used to assess treatment differences in white blood cell profiles and hemolytic complement activity. Additionally, we recorded eye score, body mass, and fat score for each focal-bird prior to inoculation of the stimulus birds and at multiple time points post-inoculation.

Experimental Inoculation

We inoculated stimulus birds bilaterally in their prebril conjunctiva with either 25 uL of Frey's media (sham control) or 25 uL of *M. gallisepticum* (VA1994; stock ID 2009.7994-1-7P; D. H. Ley, North Carolina State University, College of Veterinary Medicine, Raleigh, NC). Inoculum was stored at -80°C prior to use, and was thawed immediately prior to experimental inoculation. All birds in the MG-infected group showed clinical signs (conjunctival inflammation) of Mycoplasmal conjunctivitis.

Serology

To monitor immune responses to infection in birds infected with MG (collected for a separate study), and to ensure that none of our control birds or focal birds became infected with MG, we collected blood samples from all individuals to measure MG-specific antibody levels before inoculation of the stimulus birds and at multiple time

points post-inoculation. To assess MG-specific antibody titers, plasma was separated and frozen at -20°C. Serum antibodies were quantified using the IDEXX *M. gallisepticum* antibody enzyme-linked immunosorbent assay test kit (IDEXX, Cat#99-06729) following the manufacturer's instructions with some minor modifications outlined in Hawley et al. 2011 and Adelman et al. 2013. Specifically, a blocking step was added to the protocol in which 300 uL of 1% bovine serum albumin (Pierce 10X BSA; Thermo Fisher Scientific) in phosphate-buffered saline was added to room temperature plates and incubated for 40 minutes. For all washing steps, plates were washed three times with phosphate-buffered saline containing 0.05% Tween 20 using an ELx50 plate washer (BioTek). Serum samples were diluted 1:50 in sample buffer before plating and all samples were run in duplicate. Absorbance was measured at 630 nm using a SpectraMAX 190 spectrophotometer (Molecular Devices) and an ELISA value was calculated with the following equation: $(\text{sample mean} - \text{negative control}) / (\text{positive control} - \text{negative control})$. All control and focal birds were seronegative for MG throughout the experiment.

Eye Lesion Scoring

Eyes were scored for conjunctival inflammation on a 0-3 scale following the methods outlined in Hawley et al. (2011). Specifically, eye scores of 0 showed no amount of swelling around the eye. Eye scores of 1 showed minor swelling around the eye, while a score of 2 was given to birds with moderate swelling. A score of three was given to birds in which the conjunctiva was almost or completely swollen shut. Eye scores for

both eyes were summed to get a total eye score (ranging from 0-6) for each individual within each time point. Eyes were always scored by the same individual (ACL). Control and focal individuals never developed any signs of conjunctival swelling.

Blood Cell Differentials

Differential leukocyte counts provide a description of cellular immunity. To quantify leukocyte counts, blood was collected in microhematocrit tubes and smeared across a glass slide using the edge of another glass slide. Blood smears were stained with the JorVet Dip Quick Stain Kit (Jorgensen Labs, Loveland, CO). Differential counts were conducted by counting 100 white blood cells within the feathered edge portion of each smear and cells were classified as lymphocytes, heterophils, monocytes, eosinophils, or basophils. All cell counts were conducted by one individual (KG) while blind to treatment.

Hemolytic Complement Activity Assay

The complement pathway is part of the innate immune system and involves a series of proteins present in the plasma that contribute to the lysis of foreign cells (Janeway et al. 2005). To assess how social cues of disease influence the complement pathway, we conducted a CH50 complement assay that measures the ability of proteins in the plasma to lyse sheep red blood cells (MP Biomedicals, Cat#55876). We measured complement activity following the methods outlined in Sinclair and Lochmiller (2000)

with some minor modifications. Briefly, we ran duplicate 80 µl samples of 1:20 and 1:40 plasma dilutions and averaged each duplicate prior to analysis. Hemolytic complement activity was expressed as CH50 units/ml plasma, where one CH50 unit signifies the reciprocal of the dilution of plasma needed to lyse 50% of the sheep red blood cells (French et al. 2010).

Statistical Analyses

All statistical analyses were run in SAS 9.1 (SAS Institute Inc., Cary, NC, USA). To meet parametric requirements for normality and homoscedasticity, leukocyte counts, body mass, fat score, and eye score data were log transformed and complement activity data were square root transformed. To examine the effect of visual cues of disease on the type of white blood cells present, we used a doubly multivariate repeated measures design (SAS PROC GLM, MANOVA). We ran separate mixed models (PROC MIXED) to test whether exposure to a visual cue of disease influenced body mass, fat score, complement activity, and heterophil/lymphocyte ratios. Each model included treatment (control-focal or MG-focal), time post-cue, and a treatment by time interaction. Bird identity was included as a random effect in all models since individuals were repeatedly sampled over time. Additionally, to gain insight into the complex relationships among immune parameters, we performed a multifactor analysis to assess how a perceived immune threat altered the overall immune profiles of birds. Multifactor analyses were performed using the FactorMineR package in R (Version 1.1.456). For the multifactor analysis, the heterophil/lymphocyte ratio and hemolytic complement activity (CH50) for

each individual were grouped across days 2, 6, 12, and 24 post-treatment to create a composite immune profile for each individual. To compare treatment groups, we tested whether the mean for each treatment cluster was significantly different from zero ($p < 0.05$) using the test value criterion included in the FactoMineR package (Lê et al. 2008). Specifically, the test values come from the transformation of a p-value into a quantile of the normal distribution, in which p-values less than 0.05 correspond with an absolute test value greater than 1.96 and the sign of the test value indicates whether the coordinate value is less than or greater than zero (Lê et al. 2008).

RESULTS

Characterization of Visual Cue of Disease

The visual symptoms of stimulus birds varied based on treatment and with time post-infection (day*treatment: $F_{7,111} = 51.07, p < 0.0001$). Specifically, control stimulus birds never developed conjunctivitis, while peak conjunctival swelling occurred around days 5 – 10 post inoculation in MG-infected stimulus birds (Figure 3). This timeframe was also when peak lethargy occurred for MG-infected birds (personal observation, ACL) and coincides with peak sickness behavior reported in other studies of passerines infected with MG (Adelman et al. 2013, Love et al. 2016). Lethargic behavior was never observed in control individuals.

Physiological Responses to Visual Cue of Disease

Body mass of focal birds was not affected by whether birds were seeing a cue of infection and did not differ over time during the course of the experiment (all $F \leq 2.41$, all $p \geq 0.139$). Fat score did not vary by treatment ($F_{1,17} = 0.05$, $p = 0.826$) but did fluctuate over time in both control-focal and cue of disease birds (day: $F_{4,72} = 6.67$, $p = 0.0001$). Leukocyte counts differed between birds seeing a diseased conspecific and birds seeing healthy conspecifics (Table 1; treatment: $F_{5,85} = 2.64$, $p = 0.029$). Specifically, birds seeing a cue of disease had more heterophils (Figure 4A; treatment: $F_{1,18} = 4.55$, $p = 0.047$) and fewer lymphocytes (Figure 4B; treatment: $F_{1,18} = 4.10$, $p = 0.058$). Consequently, birds viewing a diseased conspecific had an increased heterophil/lymphocyte ratio (Figure 4C; treatment: $F_{1,18} = 4.56$, $p = 0.047$). Monocyte counts decreased on day 6 post-cue of disease in MG-focal birds and decreased in control individuals on day 12 (Figure 4D; day*treatment: $F_{4,67} = 3.49$, $p = 0.012$). Eosinophils and basophils were rarely observed in blood smears and did not differ by treatment or over time (all $F \leq 1.14$, all $p \geq 0.344$). Finally, there was a non-significant trend for birds viewing a sick conspecific to temporarily increase CH50 complement activity that eventually returned to baseline levels as pathology in the stimulus birds began to subside (Figure 5; day*treatment: $F_{4,68} = 2.33$, $p = 0.065$).

For the multifactor analysis, the first two dimensions had eigenvalues ≥ 1.0 that explained 63.6% of the variance (for the remaining 8 dimensions: eigenvalues < 0.70 , percentages of variance $< 12\%$; Table 2). The coordinates, contributions, and correlations

for each immune parameter (heterophil/lymphocyte ratio and complement activity) in the multifactor analysis are shown in Table 3. MG-focal and control-focal birds had significantly different immune profiles for dimension 1 of the multifactor analysis (test value: 2.166). To visualize differences between treatment groups, we plotted dimension 1 against dimension 2 and found separation in the 95% confidence intervals between the immune profiles of MG-focal and control-focal birds and these differences were largely driven by shifts in immunity on days 6 and 12 following treatment (Figure 6).

DISCUSSION

In this study, we examined whether social cues of infection can stimulate the immune system by housing birds within visual and auditory—but not direct—contact with either healthy or sick conspecifics. We found that birds housed in visual contact with MG-infected conspecifics had immune profiles that were distinct from birds housed across from healthy conspecifics and that the strength of this immune activation depended on the severity of the cue of infection that birds were detecting. Specifically, we found a shift in white blood cell profiles and an increase in complement activity in MG-focal birds that was concomitant with peak disease severity in the stimulus birds infected with MG. This suggests that healthy individuals can detect sick conspecifics infected with MG and that the perception of diseased conspecifics can alter physiological responses relevant to disease susceptibility and disease transmission.

Notably, the immune activation observed in MG-focal individuals occurred when the MG-infected stimulus birds were noticeably symptomatic, suggesting that the focal

birds detected the cue of disease when pathology and lethargy were noticeably present. Specifically, MG-infected individuals had the most severe conjunctivitis and lethargy between 5-10 days post-infection, and MG-focal birds had increased heterophil/lymphocyte ratios and complement activity beginning on day 6 and through day 12 post-cue of disease. This suggests that the MG-focal birds were able to detect alterations in their sick neighbors, most likely the visual signs of conjunctival swelling and lethargy. It is worth noting that we did not characterize auditory or olfactory changes in MG-infected stimulus birds, so it is possible that infection-induced changes in call/song rate and odor could have also served as cues of infection to the MG-focal individuals. For example, in many mammals olfactory cues can provide information about the infection status of conspecifics (Penn and Potts 1998). Traditionally, olfactory cues were not thought to play a prominent role in birds, however recent research is challenging this assumption (Balthazart and Taziaux 2009). Birds rely heavily on auditory cues to detect predators, communicate, and attract mates (Marler 2004), and infection is known to influence acoustic communication in some avian species, including canaries (Buchanan et al. 1999, Spencer et al. 2005). However, because all treatments were housed in the same room and were in auditory contact with one another in the present study, it seems unlikely that auditory cues played a prominent role in driving the observed shift in immune responses in MG-focal birds. Regardless of what cue birds were detecting in infected conspecifics, the finding that immune activation in healthy individuals corresponds temporally with increasing disease severity of sick conspecifics could have interesting implications for the spread of disease. For example, immune activation in individuals exposed to a social cue of infection may depend on cue strength,

in which some cues have greater effects on immunity than others. In support of this view, zebra finches housed near conspecifics challenged with lipopolysaccharide (LPS) did not have detectable changes in immune or endocrine physiology (Love, *unpublished data*), possibly because LPS does not elicit signs of infection as severe as those caused by MG-infection. Conversely, some infected individuals may be infectious but not symptomatic, in which case the potentially protective immune activation in healthy individuals would not occur. Because immune activation can be energetically expensive and shifts resources away from other important processes such as reproduction (Demas et al. 1997, Martin et al. 2003, Lochmiller and Deerenberg 2000), organisms may benefit from selectively responding to cues of infection based on the level of risk indicated by the cue.

The finding that birds housed in visual contact with MG-infected conspecifics had immune profiles that were distinct from birds housed across from healthy conspecifics suggests that visual cues of disease could influence disease susceptibility. More specifically, birds exposed to a cue of infection had increased heterophils and an increased heterophil/lymphocyte ratio. Heterophils are phagocytic cells that play an important role in inflammation and shaping host resistance and susceptibility to pathogens (Genovese et al. 2013). For example, chickens with heterophils that are less functionally active are more susceptible to infections than chickens with highly functional heterophils (Ferro et al. 2004, Genovese et al. 2013). Thus, an increase in heterophils following exposure to a cue of disease could help provide protection against a perceived immune threat. Conversely, higher heterophil/lymphocyte ratios are associated with decreased resistance to pathogens in some poultry (Al-Murrani et al. 2010), and great tits with higher heterophil/lymphocyte ratios have weaker antibody responses to an

immune challenge (Krams et al. 2012). Thus, an increased heterophil/lymphocyte ratio following a cue of infection could be beneficial or costly depending on context and likely has varied implications for disease susceptibility depending on host-pathogen interactions as well as social and abiotic environmental factors. We also detected a significant time by treatment interaction for the relative number of monocytes present in the blood stream. We found that MG-focal birds had decreased monocyte levels 6 days after the inoculation of MG-infected birds. Unexpectedly, we detected a decrease in monocyte levels in control-focal birds that occurred on day 12 post-cue. While it is unclear what caused these shifts in monocyte levels in control birds, it is possible that because monocytes are less common, the observed changes are simply due to the random chance of encountering a monocyte on a blood smear and not necessarily biologically meaningful trends. In addition to observing shifts in white blood cell profiles, we also observed an upregulation of CH50 hemolytic complement activity in birds housed near MG-infected conspecifics. An increase in CH50 indicates activity of the complement system, suggesting that immune activation and inflammation are present. The complement system is responsible for the opsonization of pathogens and inducing inflammatory responses that help fight infection (Janeway et al. 2001), and complement deficiencies are associated with increased susceptibility to bacterial infections (Abeles et al. 2015). Thus, an increase in complement in response to social cues indicative of a heightened risk of infection could help prime individuals to respond more quickly to infection.

Unexpectedly, we had three mortalities occur during the experiment, and all of these individuals were MG-infected stimulus birds. Because mortality is likely a strong social cue indicative of an immune threat, it is worth discussing when these mortalities

occurred. Two individuals died on day 9 post-infection and an additional bird died on day 19 post-infection. While a dead conspecific presumably acts as a strong cue indicating that a threat like infection or predation is present (Swift et al. 2015), we started to see shifts in immunity in MG-focal birds before these mortalities occurred (around day 6 post-infection), suggesting that conspecific mortality was not the primary cue driving the initial shifts in immune physiology.

While immune activation in response to a perceived immune threat is a relatively new concept in disease ecology, it is well established that shifts in physiology occur in response to other external cues such as predation risk. For example, manipulating perceived predation risk alters corticosterone and testosterone concentrations in common blackbird nestlings (Ibáñez-Álamo et al. 2011) and conspecific alarm call playbacks alter glucocorticoid concentrations in Belding's ground squirrels (Mateo 2010). It is possible that the perception of diseased conspecifics could alter immunity through a shift in hormones such as testosterone and glucocorticoids, as both of these hormones are known to influence immune function (Dhabhar 2009, Martin 2009, Da Silva 1999). Future work should explore what mechanisms underlie changes in immunity following exposure to social cues of disease, particularly the neural regulation of innate immune responses and neuroendocrine crosstalk (Sternberg 2006, Demas et al. 2011).

Our results demonstrate that social cues of infection can alter immunity in birds, and that the observed physiological changes in healthy individuals seeing a sick conspecific coincide with the severity of visual signs of infection in infected conspecifics. Immune activation following perception of sick conspecifics could prime individuals to respond to an impending immune threat, and better enable them to fight off and recover

from infection. Clearly, the ability to effectively detect and appropriately respond to an immune threat has implications for disease dynamics. Well-studied behavioral responses to cues of infection, such as avoidance behavior have already been implicated in shaping individual and population-level differences in group structure and behaviors often correlated with infection risk, such as sociality, territoriality, and personality (Patterson and Ruckstuhl 2013, Barber and Dingemanse 2010, Buck et al. 2018). This suggests that changes in behavior following the perception of sick conspecifics could influence how organisms interact with their environment and other individuals, and shifts in physiology may mediate the likelihood of infection or disease severity. Thus, investigating the interactions between behavioral and physiological responses of healthy individuals to sick conspecifics could increase our understanding of how diseases spread through populations. Now that it has been established that social cues of disease can alter immunity in birds, future research should investigate whether immune activation following a social cue of infection confers any protection against infection, such as decreased recovery time or reduced disease severity, and whether this alters avian disease transmission potential. Finally, this research has important implications for experimental design and animal housing practices, as sick animals could influence the physiology of their neighbors.

Table 1. White blood cell differentials for birds viewing healthy conspecifics (Control-focal) and birds viewing a cue of infection (MG-focal).

Treatment	Day	Lymphocytes (%)	Heterophils (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
Control-focal	-5	84.6	11.4	3.7	0.3	0.0
	2	82.1	14.6	3.1	0.2	0.0
	6	78.78	17.1	3.9	0.2	0.0
	12	82.9	15.6	1.4	0.0	0.0
	24	76.8	19.7	2.8	0.7	0.1
MG-focal	-5	80.9	17.3	1.6	0.0	0.1
	2	75.3	21.9	2.5	0.1	0.0
	6	71.0	28.0	0.7	0.1	0.0
	12	70.4	27.9	1.5	0.1	0.0
	24	72.3	25.7	1.8	0.1	0.0

Table 2. Eigenvalues and explained variance for the eight dimensions of the immune profile multifactor analysis.

<i>Dimensions</i>	<i>Eigenvalue</i>	<i>Percentage of variance (%)</i>	<i>Cumulative percentage of variance (%)</i>
1	2.611	45.299	45.299
2	1.077	18.340	63.639
3	0.699	11.898	75.537
4	0.478	8.140	83.677
5	0.458	7.804	91.481
6	0.275	4.684	96.165
7	0.150	2.552	98.717
8	0.075	1.283	100.000

Table 3. Coordinates, contributions, and correlations of immune parameters from days 2, 6, 12, and 24 for the first two dimensions of the immune profile multifactor analysis.

<i>Parameter</i>	<i>Day</i>	<i>Dimension 1</i>			<i>Dimension 2</i>		
		<i>Coord.</i>	<i>Contrib.</i>	<i>Correl.</i>	<i>Coord.</i>	<i>Contrib.</i>	<i>Correl.</i>
<i>H:L Ratio</i>	2	0.050	14.819	0.756	-0.018	4.772	0.099
<i>H:L Ratio</i>	6	0.063	20.805	0.626	-0.039	20.338	0.248
<i>H:L Ratio</i>	12	0.056	16.978	0.531	0.025	8.140	0.103
<i>H:L Ratio</i>	24	0.072	22.929	0.646	-0.011	1.351	0.015
<i>Complement</i>	2	0.042	10.619	0.355	-0.016	3.593	0.049
<i>Complement</i>	6	0.041	8.959	0.255	0.058	44.662	0.515
<i>Complement</i>	12	0.029	4.427	0.238	0.031	12.913	0.281
<i>Complement</i>	24	0.010	0.464	0.028	0.020	4.231	0.102

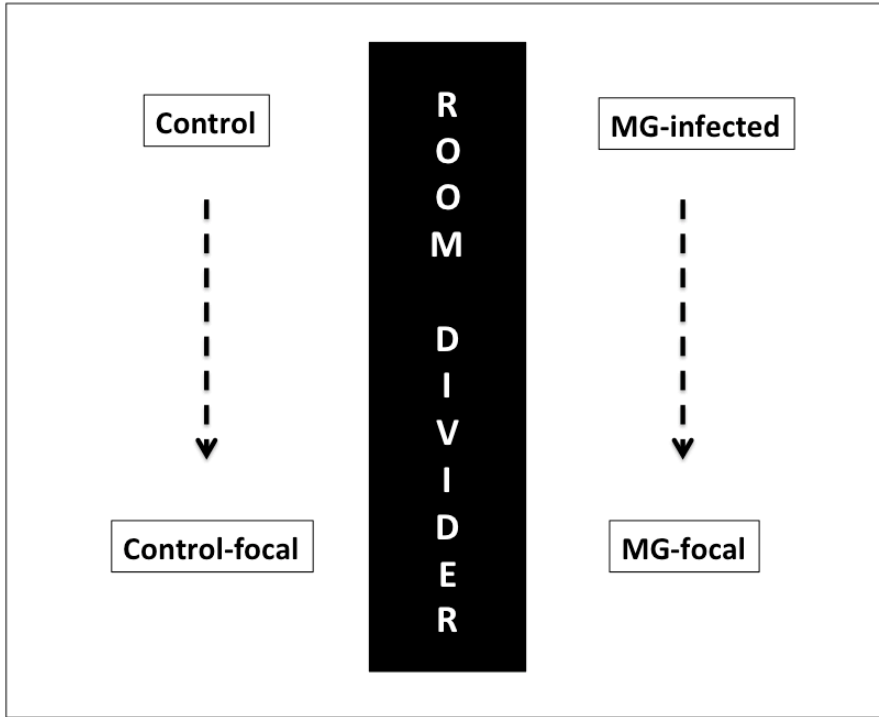


Figure 1. Birds were housed in a single room and separated from visual contact with other treatment groups using an opaque room divider. 2.5 meters of distance separated the racks containing stimulus birds (Control, MG-infected) from the racks housing the focal birds.

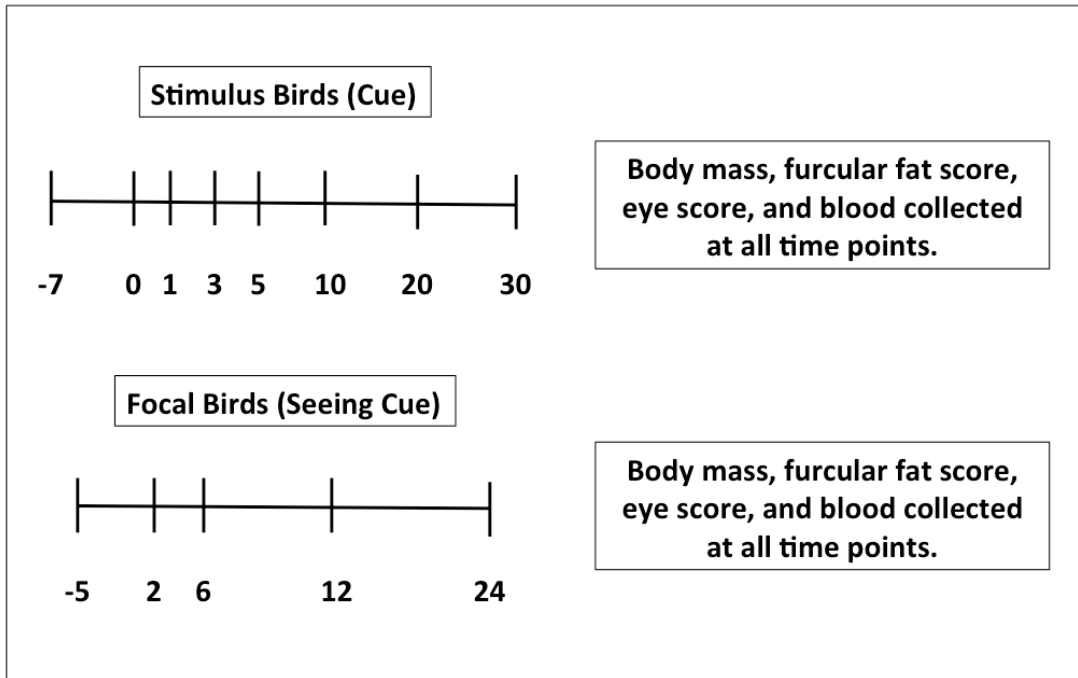


Figure 2. Experimental timeline for stimulus (MG-infected and control) and focal birds (MG-focal and control-focal).

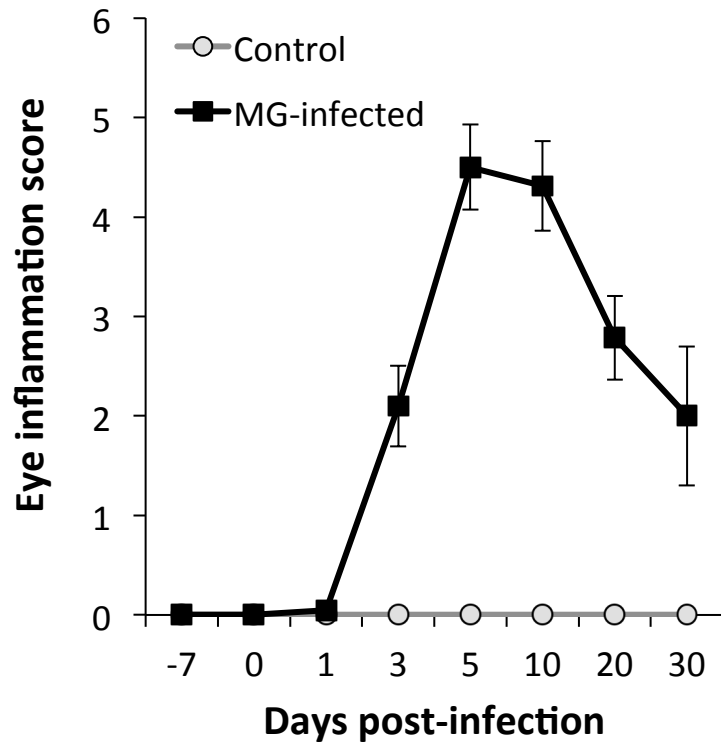


Figure 3. Characterization of disease severity in stimulus birds exposed to *Mycoplasma gallisepticum* (MG-infected, cue of disease) or a control media (Control, no cue of disease). The points denote the average eye inflammation score (\pm SE) of birds in the control (n=9) and infected (n=7-10) treatments.

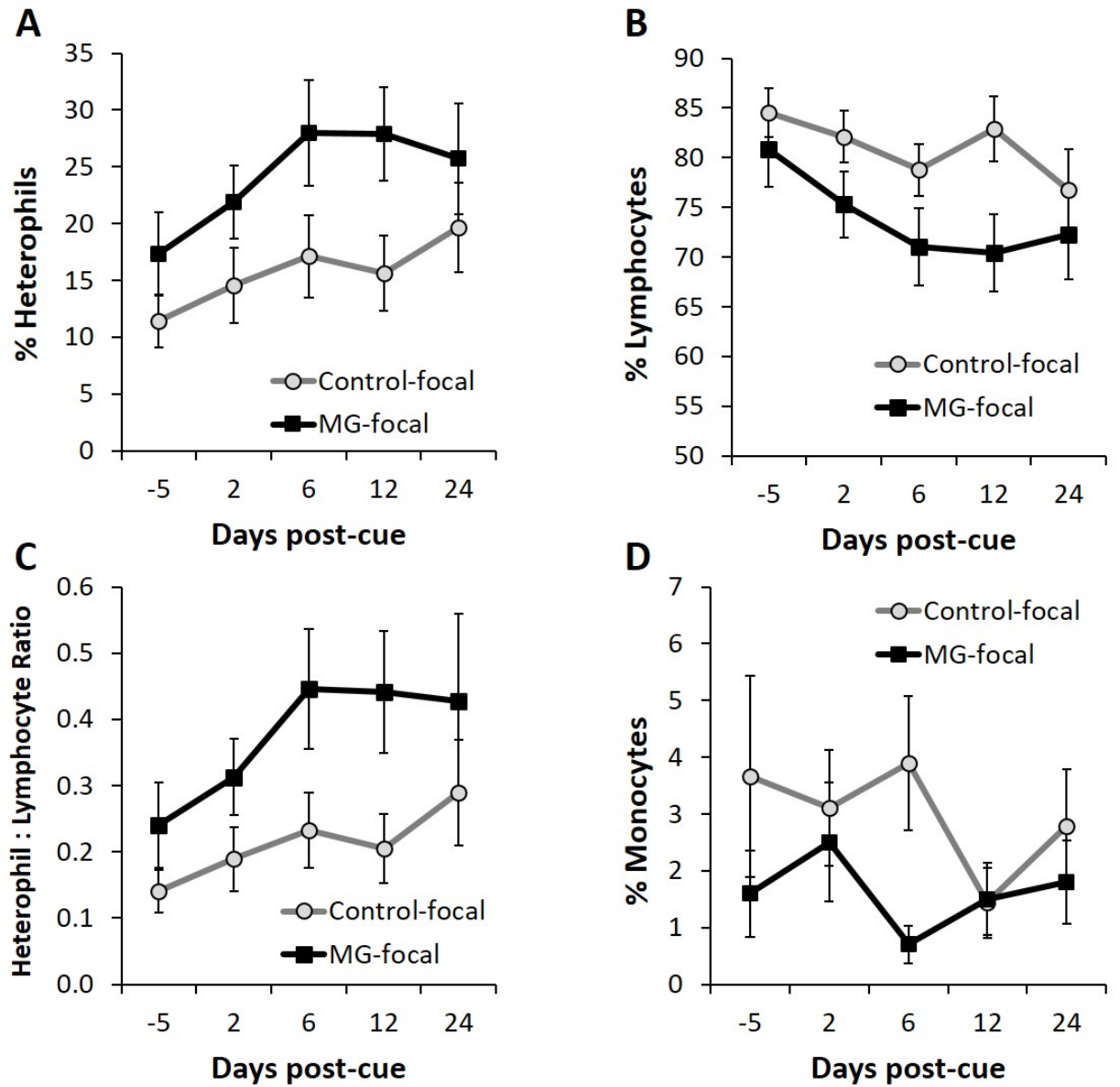


Figure 4. Percentage of circulating (A) heterophils, (B) lymphocytes, (C) heterophil/lymphocyte ratio, and circulating (D) monocytes in birds exposed to a cue of infection (MG-focal) or healthy conspecifics (Control-focal). The points denote average counts (\pm SE) of birds in the control-focal (n=9) and MG-focal (n=10) treatments.

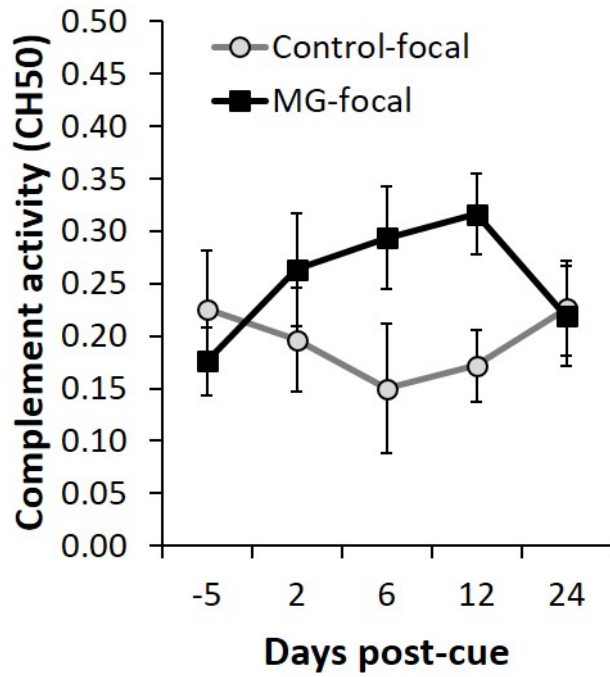


Figure 5. Hemolytic complement activity (CH50) in birds exposed to a cue of infection (MG-focal) or healthy conspecifics (Control-focal). The points denote average CH50 (\pm SE) of birds in the control-focal (n=9) and MG-focal (n=10) treatments.

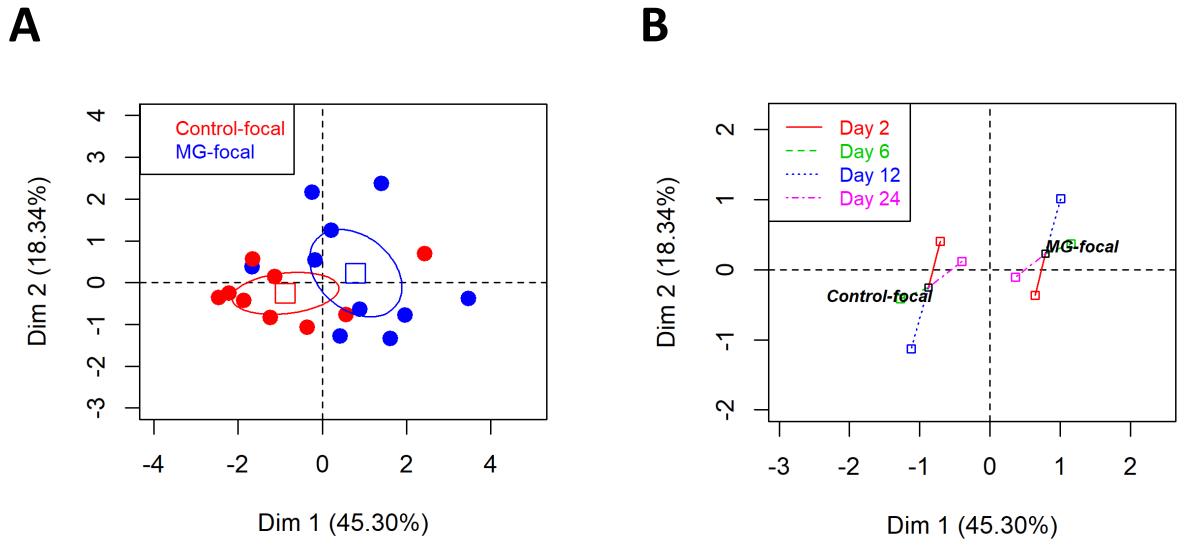


Figure 6. (A) Multifactor analysis plot of dimensions 1 and 2 for immune profiles (heterophil/lymphocyte ratio, complement activity) in birds viewing sick conspecifics (MG-focal: N=10) or healthy conspecifics (control-focal: N=9). Individual points represent individual birds and ellipses represent 95% confidence intervals for each treatment. (B) Partial points plot showing the contributions of each timepoint (Days 2, 6, 12, and 24) to the divergence in behavioral profiles between each treatment.

CHAPTER V

CONCLUSIONS

Traditionally models of disease have assumed homogeneity of behavior and social contacts within a population, however in most social species this is likely not the case (Lopes et al. 2012). In support of this idea, I found that zebra finches given an immune challenge with lipopolysaccharide (LPS) had altered social and pair maintenance behaviors, including increased preening and clumping between established mates (Chapter 1). Both sick birds and healthy birds seeing a cue of heightened infection risk engaged in more pair-maintenance behaviors (clumping and allopreening, respectively). This suggests that birds are able to maintain pair bonds and respond to an immune threat by engaging in cooperative pair behaviors that could modulate infection severity or infection risk (Hart 1988, Gilbert et al. 2006, Moyer et al. 2003, Clayton et al. 2010). Zebra finches challenged with LPS also had altered feeding behavior, exhibiting sickness-induced anorexia that was macronutrient-specific (Chapter 2). These results carry implications for host health and epidemiology, as shifts in social behavior following infection can alter contact rates and disease transmission (Lopes et al. 2016), and sickness-induced anorexia can alter infection severity and result in nutritionally driven shifts in host-pathogen interactions (Hite and Cressler 2019).

Social organisms are thought to be at a higher risk of infection with pathogens and parasites (Altizer et al 2003, Møller et al. 1993). Thus, individuals should benefit from distinguishing healthy social partners from sick individuals and adjust their behavior according to social cues that indicate an immune threat is present. Indeed, I observed behavioral changes in zebra finches exposed to a cue of heightened infection risk. Birds seeing sick conspecifics did not exhibit avoidance behaviors in response to sick conspecifics, but did reduce their activity levels and engage in increased maintenance behaviors such as preening and allopreening when housed in visual contact with sick neighbors. These data indicate that healthy pairs respond to social cues associated with heightened risk of infection through both individual and cooperative social behaviors geared towards reducing infection risk.

Finally, I examined whether cues of disease could alter physiological responses relevant to responding to an immune threat in zebra finches exposed to LPS-challenged conspecifics (Chapter 2) and in domestic canaries exposed to conspecifics infected with the bacterium *Mycoplasma gallisepticum* (MG) (Chapter 3). While we did not observe any shifts in physiology in zebra finches housed next to LPS-challenged neighbors, we found that canaries housed in visual contact with MG-infected conspecifics had altered immune profiles, with higher heterophil/lymphocyte ratios and higher hemolytic complement activity. The differences between these two studies likely results from the strength of the signal elicited by sick individuals, as the behavioral effects of LPS-injection typically only last a few days, while infection with the bacterium *Mycoplasma gallisepticum* results in lethargy and obvious visual pathology (conjunctivitis) that can last for weeks. Further, the observed physiological changes in healthy canaries seeing

sick neighbors coincided with the severity of visual signs of infection in their MG-infected conspecifics. Together, these results suggest that immune activation in individuals exposed to a social cue of infection may depend on cue strength, in which some cues have greater effects on immunity than others. This idea makes sense in the context of disease risk, as in many cases disease severity (cue strength) is related to disease transmission potential (Adelman et al. 2013). Additionally, because immune activation can be energetically demanding and shift resources away from processes such as reproduction (Demas et al. 1997, Martin et al. 2003, Lochmiller and Deerenberg 2000), organisms may benefit from selectively responding to cues of infection based on the level of risk indicated by the cue.

Together, this work demonstrates that infection and perceived infection risk can alter several behaviors and physiological responses relevant to disease susceptibility and disease transmission. Responding to an immune threat involves more than just the immune system or avoidance behavior, as I found that several behaviors respond to both infection and perceived infection risk (Chapter 1). Further, infection can alter feeding behaviors known to shape immune responses (Povey et al. 2013, Cotter et al. 2011), potentially providing infected organisms with a way to “self-medicate” and influence within-host pathogen dynamics and disease outcomes (Hite and Cressler 2019). Perceived infection risk can also influence behaviors (Chapter 1) and physiological responses (Chapter 3) and these shifts presumably occur in order to prepare birds for an impending immune threat. This research indicates that birds have diverse responses to infection that are likely integrative in nature. For example, birds may employ a combination of behavioral, nutritional, and immunological strategies simultaneously to

combat an immune threat or these strategies may trade-off or be balanced with one another as has been demonstrated with behavioral and immunological strategies in house finches (Zylberberg et al. 2012). Additionally, strategies may vary temporally depending on the degree of infection or infection risk (Chapters 2 and 3). Future work should explore the integrative nature of behavioral, nutritional, and physiological defenses against infection, as the ability to effectively detect and appropriately respond to an immune threat has clear implications for disease dynamics.

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VITA

Ashley Caroline Love

Candidate for the Degree of

Doctor of Philosophy

Dissertation: PERCEPTION OF INFECTION: BEHAVIORAL AND
PHYSIOLOGICAL RESPONSES TO ILLNESS-RELATED SOCIAL
CUES

Major Field: Integrative Biology

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Integrative Biology at Oklahoma State University, Stillwater, Oklahoma in July, 2019.

Completed the requirements for the Bachelor of Science in Biological Sciences and Wildlife Science at Virginia Polytechnic Institute and State University, Blacksburg, Virginia in 2013.

Experience:

Oklahoma State Graduate College Summer Dissertation Fellow (2018)

National Science Foundation GRFP Fellow (2014 – 2019)

USFWS Eider Research Field Assistant (Utqiagvik, Alaska, Summer 2013)

VA Tech Undergraduate Researcher (Hawley Disease Ecology Lab, 2011-2013)

VA Tech Research Technician (Hopkins Wildlife Physiology Lab, 2010-2013)

Oklahoma State University Teaching Assistant

Introductory Biology Fall 2013, Spring 2014

Animal Biology Fall 2014, Spring 2015, Fall 2018

Human Heredity Spring 2019

Professional Memberships:

Society for Integrative and Comparative Biology, Animal Behavior Society,

American Ornithologists' Union, American Society of Parasitologists