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Sex differences in host resistance and tolerance to the common avian pathogen Mycoplasma

gallisepticum

Honors Thesis for Chloe Connelly

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Biological Sciences

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Abstract

As we face the threat of global pandemics, one thing becomes clear: biological research is not just about the pursuit of the unknown, but about protecting our future. Understanding disease transmission and predicting pathogen epidemics is more important than ever. Prior studies have indicated that in populations where one sex engages in more social behaviors and movement that sex may drive disease transmission. This supports sex as a factor to consider in the study of epidemic dynamics. Host physiology and immune strategies are another factor that can influence epidemics. Two commonly examined strategies are tolerance and resistance. Tolerance is the ability to endure pathogen loads with minimal sickness symptoms while resistance is the strategy of fighting off infection and minimizing the pathogen. However, the effect of sex on host immune strategies in wildlife is under researched and poorly understood. In this experiment, I use a common avian pathogen to determine if differential infection response strategies exist between sexes. I hypothesized that both males and females will favor a tolerance strategy. Males may prioritize tolerance because they have higher levels of testosterone, which is immunosuppressive. While females may also employ a tolerance strategy as a way to protect their ova by minimizing self-damage from strong immune responses. By using domesticated canaries in the laboratory setting with identical *Mycoplasma gallisepticum* exposure, I was able to observe strictly the physiological effects of sex on infection defense strategies. We observed that after exposure, MG-exposed male canaries increased their relative monocytes and eosinophils levels while MG-exposed female canaries decreased relative eosinophils levels and maintained monocyte levels. Further, MG-exposure males produced more MG-specific antibodies, had greater pathology, and greater pathogen growth when compared to MG-exposed females. Thus, males have less effective MG resistance while females tolerate infection and keep MG loads lower while producing fewer antibodies. This contradicts my initial hypothesis that differential immune strategies do not exist between sexes in the avian conjunctivitis system. In conclusion, sex differential immune response exists in the avian conjunctivitis system. Since sex is a factor of disease dynamics and immune response, further research is necessary to explore this perspective.

Introduction

Epidemics are becoming more frequent and harder to control as a result of the changing climate, ecological landscape, and global connectivity (Bedford and Ihekweazu 2019). To face this increased threat to public health and conservation, it is critical to understand and predict pathogen epidemics. Understanding how individuals vary in their contribution to pathogen transmission is crucial for predicting transmission. Heterogeneity in individual characteristics, such as migratory behavior, sociality, or age, can influence transmission (Sands 2016; Morse 1995; Davies 2020). For example, male yellow-necked mice (*Apodemus flavicollis*) with severe infection drove the spread of disease throughout a population while their female counterparts proved to be an insignificant driver of infection (Ferrari et al. 2004).

Sex as a determinant for transmission could provide understanding on the way that diseases spread in an epidemic, thus I focused my research on whether host sex influences infection immune response. I tested the hypothesis that there are not differential immune responses between sexes in the avian conjunctivitis system. There are two different infection defense strategies that we are observing through these measurements and samples in my experiment: tolerance and resistance. Tolerance is the ability to endure pathogen loads with minimal sickness symptoms while resistance is the strategy of fighting off infection and minimizing the pathogen (Scheider and Ayres 2008). Males may take a tolerant approach in response to an infection because their hormones, specifically testosterone, can be immunosuppressive (Nolan 1998). Males in the wild by way of their increased social encounters dull their immune response from consistent exposure to the same antigen; this phenomenon is known as tolerance (Medawar 1960). Females on the other hand may use a tolerance strategy as a way to protect their ova by minimizing damage and enduring the pathogen (Marcenac et al. 2020). Thus, I expect to see both MG infected females and males utilize the tolerant strategy. If this were to be true, it may be possible in future studies to compare the significance of ova and testosterone in the tolerance of infection.

Wild passerines experience epidemics of conjunctivitis each year throughout North America following its initial detection in 1994, which spilled into wildlife populations from poultry farms (Ley et. al 2010). Ten years after the initial introduction of conjunctivitis, the Eastern United States population of house finches (*Haemorhous mexicanus*) was reduced by forty percent (Hochachka & Dhondt 2000). This mass mortality event shifted the sex ratio of some house finch populations to favor females over males (Nolan et. al 1998). Several studies have analyzed the potential sex differences in infection intensity with free-range, wild house finches that have been caught and released across a season (Altizer et. al 2004). However, surveys of these wild birds are likely affected by sex-associated, social behaviors that change exposure patterns between males and females. Thus, there is not a clear understanding of how sexes differ in their resistance and tolerance to MG given identical exposure to the pathogen.

Methods

Experimental Design

Domestic canaries were used as a model for house finches during this study since they have a similar response to MG (Dana M. Hawley, Jessica Grodio, Salvatore Frasca Jr, Laila Kirkpatrick & David H. Ley 2011). A sample of 25 mixed sex domestic canaries were used during this study of which there were 6 control and 6 MG females and 7 control and 6 MG males. The canaries had no prior exposure to conjunctivitis and tested negative for MG and MG-antibodies prior to exposure. On day 0, the birds were inoculated in the palpebral conjunctiva of both eyes with either 0.025 mL of MG suspended in Frey's media (7.415x103 CCU/mL; VA1994, stock ID 2009.799-1-7P) or with a sham of Frey's media alone. The birds were housed individually with the sexes randomly distributed among the cage racks. An opaque room divider served to separate the exposed birds from the control birds.

Data Collection- Assessing Disease Pathology Host Response

The data collected includes fat reserves, mass change (g), hematocrit, relative white blood cell count, pathogen load, antibody assay and eye score. Eye scores were recorded on days 0, 1, 3, 5, 7, then twice a week until day 35. Body mass and fat scores were recorded on days 0, 7, 14, 21, and 35. Blood samples and eye swabs were collected on days 0, 7, 14, and 21. White blood cell count was recorded based on the first 100 white blood cells observed (basophil, eosinophil, heterophil, lymphocyte, and monocyte). The JorVert Dip Quick Stain Kit, Jorgensen Labs, Loveland, CO was used to stain the blood smears.

Eye score ranges from zero to three in which zero would indicate no sign of eye inflammation or lesions while three would indicate eyes swollen nearly shut (Roberts 2001). Both eye's eye scores are added together to yield an overall eye score from 0 to 6 for each individual. Furcular fat score ranges from 0 to 3 where little to no fat reserves scores a 0 and readily apparent fat reserves earn a 3. MG-specific IgY titers were quantified from blood plasma using an enzyme-linked immunosorbent assay (ELISA) (FlockChek M. gallisepticum ELISA kit, IDEXX, Westbrook, ME) (Love et al. 2021). To measure pathogen growth, the conjunctiva of each eye was swabbed for 5 seconds, then DNA was extracted from the swabs using the Qiagen DNeasy Blood and Tissue Protocol and qPCR was used to quantify pathogen load based on Adelman et al. (2013) with gBlock ® plasmid-based standards.

Statistical Analysis

The data collected above were analyzed using R version 4.1.0 in R Studio. Linear regression models were conducted to determine the effects of sex, treatment, and their interaction on changes in furcular fat score, mass, and hematocrit. Changes in fat score and mass were calculated as the difference between days 35 and 0 values because they take longer to change and happen over the entire course of infection. Change in hematocrit was calculated as the difference between days 7 and 0 because hematocrit changes quickly and it is expected that peak difference will align with peak infection.

Linear mixed effects regressions were conducted to determine the effects of sex, days since exposure, and their interaction on MG-specific antibody production and log-transformed

pathogen load in MG-exposed birds only. A random intercept for bird ID was included in both models to deal with the non-independence of the repeated measures.

A MANOVA was conducted to determine the effect of sex, treatment, and days since exposure on the change in relative white blood cell counts (eosinophil, heterophil, lymphocyte, and monocyte). For this analysis, changes in relative white blood cell counts were calculated as the difference between days 7 and 0 because day 7 is closest to peak symptomatic illness which is typically between days 3 through 7.

A generalized additive mixed (GAM) model was used to determine the effect of sex and time since exposure on the eye scores in MG-exposed birds only. There was a random effect for bird ID and a smoothing spline to deal with the non-linear effect of time on eye score.

Results

There was a significant main effect of sex on fat stores (main effect of sex: $\beta = 0.750$, t=2.096, p=0.051; Table 1 and Figure 1). Males gained significantly more fat stores over time than females, regardless of exposure status. Female fat stores generally stayed the same throughout the experiment while males started off with lower fat stores initially and mostly caught up to females by the end of the experiment. There was no significant main effect of treatment or its interaction with sex on change in fat score. MG-exposed birds lost significantly more mass than unexposed birds (main effect of treatment: $\chi^2=20.265$, df=1, p=0.038; Table 2 and Figure 2). There was no significant main effect of sex or its interaction with treatment on change in mass. There were no significant effects of sex, treatment, or their interaction on change in hematocrit % (p > 0.100 in all instances, Table 3 and Figure 3).

There were no significant main effects of sex, treatment, or their interaction on the overall change in relative white blood cell counts. There was an overall significant effect of sex

on eosinophil counts (sex: χ^2 =284.73, df=1, p=0.024; Table 4 and Figure 4a). Males increased their relative eosinophil levels significantly more than females, regardless of MG exposure. There was no significant main effect of treatment or its interaction with sex on change in eosinophil levels. There were no significant main effects of sex, treatment, or their interaction on the overall change in heterophil or lymphocyte counts (Figures 4b and 4c). There was a significant interaction between sex and treatment on monocyte counts (sex*treatment: χ^2 =99.986, df=1, p=0.007; Table 4 and Figure 4d). MG-exposed male canaries had a significant increase in monocyte levels while all other groups stayed largely the same. There were no significant main effects of sex or treatment on the change in monocyte levels.

There was a significant main effect of sex and a significant negative effect of day on pathogen load (sex: β = 1.343, t=0.659; χ^2 =3.677, df=1, p=0.055; day: β =-0.422, t=-4.963; χ^2 =39.816, df=1, p=2.790e-10; Table 5 and Figure 5). Females had significantly lower MG-loads than males. There was no significant interaction between sex and time on MG-load. There was a significant interaction between sex and day on the antibody levels (sex* day: β =0.003, t=-2.891; χ^2 =8.358, df=1, p=0.004; Table 6 and Figure 6). Male birds had greater levels of MG-specific antibodies and increased those levels more rapidly than females. There was a significant effect of sex on eye score (sex: β = 1.845, t=8.486, p<0.001; Table 7 and Figure 7).

Figures

Figure 1| Differential effect of canary sex and exposure to *Mycoplasma gallisepticum* (MG) on furcular fat score over time. Gray shading represents the associated 95% confidence bands.



Figure 2| Differential effect of canary sex and exposure to *Mycoplasma gallisepticum* (MG) on body mass over time. Gray shading represents the associated 95% confidence bands.



Figure 3| Differential effect of canary sex and exposure to *Mycoplasma gallisepticum* (MG) on hematocrit % over time. Gray shading represents the associated 95% confidence bands.



Figure 4| Box plots modeling the difference in relative quantities of various white blood cell types in male and female canaries from Day 0 to Day 7 after exposure to MG or sham (control)



The Difference in Eosinophil Levels from Day 0 to Day 7



The Difference in Heterophil Levels from Day 0 to Day 7

Figure 4b: Heterophil



The Difference in Lymphocyte Levels from Day 0 to Day 7

Figure 4c: Lymphocyte



The Difference in Monocyte Levels from Day 0 to Day 7

Figure 5| Differential effect of canary sex on log transformed pathogen load when exposed to MG pathogen. Gray shading represents the associated 95% confidence bands.



Figure 6| Differential effect of canary sex on antibody count when exposed to MG pathogen. Gray shading represents the associated 95% confidence bands.



Figure 7| Differential effect of canary sex on eye score when exposed to MG pathogen. Gray shading represents the associated 95% confidence bands.



Discussion

Here we set out to determine whether immune strategies differ between sexes in the avian conjunctivitis system. We exposed canaries in the laboratory setting to the same pathogen load at initial exposure separating controls from MG-exposed to identify potential sex differences in infection defense mechanisms without the influence of differential exposure between sexes. By taking away the factor of differential exposure, the significance of sex based physiology in immune response can be observed. The two immune response strategies that I observed were tolerance and resistance. Tolerance is the ability to inhibit self harm in this case having a weak immune response (low antibody and white blood cell count), as well as low eye scores (Schneider, David S, and Janelle S Ayres 2008). Resistance is the ability to use immune response (high

antibody and white blood cell count), as well as high eye inflammation (Schneider, David S, and Janelle S Ayres 2008).

I hypothesized that differential infection strategies would not exist between sexes in the avian conjunctivitis system due to male's immunosuppressive hormones and female prioritization of oval health that I assumed would indicate tolerant immune strategies in both sexes (Nolan 1998). The results of my research contradict this hypothesis. MG-exposed male canaries had less effective tolerance because they displayed severe pathology (eye score) and created strong immune responses (high eosinophils, monocytes, and antibodies) as well as less effective resistance as evident by their higher pathogen load in comparison to their female counterparts. However, female canaries exposed to MG showed more effective tolerance through less severe pathology (eye score) and weaker immune response (lower eosinophils, monocytes, and antibodies) as well as more effective resistance as evident by a lower pathogen load than MG-exposed males.

These results are consistent with literature in which mature male vertebrates endure high burdens and are often more vulnerable to disease (Zuk and McKean 1996). In many species, male sexual selection encourages characteristics that hinder their survivability and increase their mating success (ie: long tail feathers) (Zuk and McKean 1996). Male territoriality in many species also contributes to increased encounters with disease as they are more highly mobile and may encounter more diseased individuals (Craft, Volz, Packer, and Meyers 2010). Stress could also play a role in male susceptibility as stress can inhibit the capacity to resist pathogens (Stein & Schleifer, 1985).

As the body of research dedicated to the study of disease transmission and epidemics grows, our ability to curb outbreaks and save biodiversity will strengthen. Sex as a factor needs to be further explored as there is extensive literature citing interest in the potential effects of sex on immune response and transmission dynamics with most of the emphasis on sex based behavioral influence (Zuk and McKean 1996). Still the physiological implications of sex as a direct effector of disease transmission is unexplored.

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Tables

Table 1: Linear model and ANOVA analyzing the potential effect of sex, treatment of Mycoplasma gallisepticum, and their interaction in Serinus canaria domestica on the furcular fat reserves from prior to exposure compared to 35 days after exposure. Linear Model Coefficients SE t value p value 0.750 0.358 2.096 0.051 Sex Treatment -0.133 0.375 -0.355 0.727 Sex:Treatment -0.575 0.549 -1.048 0.309 Residuals 0.620 ANOVA

	Chi Sq	df	p value
Sex	1.334	1	0.080
Treatment	0.831	1	0.160
Sex:Treatment	0.422	1	0.309
Residuals	6.529	17	

Table 2: Linear model and ANOVA analyzing the potential effect of sex, treatment							
of Mycoplasma	of Mycoplasma gallisepticum, and their interaction in Serinus canaria domestica on						
the body ma	ass from prior to	exposure compar	ed to 35 days afte	er exposure.			
		Linear Model					
	Coefficients SE t value p value						
Sex	1.539	1.153	1.335	0.200			
Treatment	-0.747	1.210	-0.618	0.545			
Sex:Treatment	-2.651	1.768	-1.500	0.152			
Residuals 1.997							
	ANOVA						
Chi Sq df p value							
Sex		0.883	1	0.644			
Treatment	20.265 1 0.038						
Sex:Treatment	Sex:Treatment 8.974 1 0.152						
Residuals		67.830	17				

Table 3: Linear model and ANOVA analyzing the potential effect of sex, treatment of <i>Mycoplasma gallisepticum</i> , and their interaction in <i>Serinus canaria domestica</i> on						
the hemato	crit from prior to	exposure compa	red to / days afte	r exposure.		
		Linear Model				
	Coefficients SE t value p value					
Sex	-0.833	7.134	-0.117	0.908		
Treatment	eatment -0.333 7.976 -0.042 0.967					
Sex:Treatment	ex:Treatment -18.667 10.937 -1.707 0.106					
Residuals 12.360						
ANOVA						

	Chi Sq	df	p value
Sex	402.260	1	0.123
Treatment	539.990	1	0.077
Sex:Treatment	444.820	1	0.106
Residuals	2595.830	17	

Table 4: MANC <i>gallisepticum</i> , a	OVA analyzing th nd their interaction	e potential effect on in Serinus can	of sex, treatment aria domestica or	of <i>Mycoplasma</i> n the quantity of			
white blo	od cells prior to e	exposure compare	ed to / days after	exposure.			
		MANOVA					
	Pillai's trace	df	F value	p value			
Sex	0.369	1,4	2.049	0.142			
Treatment	0.264	1,4	1.258	0.332			
Sex:Treatment	0.441	1,4	2.759	0.070			
Residuals		17					
		Eosinophil					
Chi Sq df F value p value							
Sex	284.730	1	6.133	0.024			
Treatment	19.650	1	0.423	0.524			
Sex:Treatment	4.140	1	0.089	0.769			
Residuals	789.300	17					
	Heterophil						
	Chi Sq	df	F value	p value			
Sex	0.390	1	0.004	0.951			
Treatment	338.110	1	3.341	0.085			
Sex:Treatment	9.540	1	0.094	0.763			
Residuals	1720.530	17					
Lymphocyte							
	Chi Sq	df	F value	p value			
Sex	230.690	1	1.613	0.221			
Treatment	357.580	1	2.500	0.132			
Sex:Treatment	133.250	1	0.932	0.348			

Residuals	2431.720	17		
Monocyte				
	Chi Sq	df	F value	p value
Sex	3.744	1	0.351	0.561
Treatment	21.391	1	2.004	0.175
Sex:Treatment	99.986	1	9.368	0.007
Residuals	181.450	17		

Table 5: Linear mixed effects model and ANOVA analyzing the potential effect of sex, days since exposure to *Mycoplasma gallisepticum*, and their interaction in *Serinus canaria domestica* on the log10-transformed MG pathogen load.

Linear Model							
	Estimate SE t value						
Sex	1.343	2.039	0.659				
Day	-0.442	0.085	-4.963				
Sex:Day	0.061	0.125	0.490				
	ANG	OVA					
	Chi Sq df p value						
Sex	3.677	1	0.055				
Day	39.816	1	2.79E-10				
Sex:Day	0.240	1	0.624				

Table 6: Linear mixed effects model and ANOVA analyzing the potential effect of sex, days since exposure to <i>Mycoplasma</i> gallisepticum, and their interaction in <i>Serinus canaria domestica</i> on MG-specific antibody levels						
Linear Model						
	Estimate SE t value					
Sex	0.003 0.015 0.182					
Day	Day 0.001 0.001 1.916					
Sex:Day	0.003	0.001	2.891			

ANOVA					
Chi Sq df p value					
Sex	6.436	1	0.011		
Day	26.584	1	2.52E-07		
Sex:Day	8.358	1	0.004		

Table 7 | Generalized additive mixed model (GAMM) analyzing the potential effect of sex since exposure to *Mycoplasma gallisepticum*, and their interaction in *Serinus canaria domestica* on eye score.

Parametric coefficients				
	Coefficient	SE	t value	p value
Sex:Male	1.8499	0.218	8.486	< 0.001
Approximate significance of smooth terms:				
		edf	F statistic	p value
s(Day)		2.883	10.72	< 0.001