THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

DIFFERENTIAL HOST ANTIBODY TARGETING OF MALE AND FEMALE GASTRIC PARASITES

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ABSTRACT

Parasites continue to pose a significant threat to human health around the world. The key to new and improved treatments is to improve our understanding of the interaction between parasite and host. This study examines the immune response of the European rabbit, *Oryctolagus cuniculus* to the gastric helminth, *Graphidium strigosum*. Host antibody response intensity to male and female parasite antigens was measured and the relative strengths of these sex-specific responses were compared. It was found that during certain points of the infection, female worms were disproportionately targeted by the host immune response. This targeting of female worms was found to correlate significantly with decreased female worm length and fecundity. These results indicate that the host immune system sees the parasites not as a homogenous population but as two distinct subpopulations of males and females.

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Chapter 1

Introduction

Helminth infections remain a large economic and health problem in low-income nations. Despite the large burden of parasitic diseases around the world, they remains understudied relative to diseases like malaria and human immunodeficiency virus (1). The key to the control, prevention, and treatment of helminth infection lies in our understanding of the underlying infection mechanisms. Of particular importance are the interactions between host and parasite. A better understanding of how the host responds to a parasite population could allow for improved drug design or the development of a vaccine.

Helminths are divided into two major groups, the nematodes or roundworms and the platyhelminths or flatworms (1). Nematodes are a major source of gastrointestinal infection and can cause diarrhea, abdominal pain, weakness, anemia and possibly death (2). *Graphidium strigosum* is a nematode that parasitizes the stomach of the European rabbit, *Oryctolagus cuniculus* (3). The nematode has a direct life cycle consisting of an egg, five larval stages and a dioecious adult stage. The first three larval stages, L1, L2 and L3, are free-living stages where L3 is the infective stage. The fourth larval stage, L4, develops from L3 in the host stomach and is the first stage where the two sexes noticeably vary phenotypically: L4 males have larger tails than females. When the L4 molts into the immature or fifth larval stage, L5, then the adult stage, the sexual dimorphism is significant. Externally, males possess bursal rays and a spicule (the reproductive organ), and an enlarged tail. Females are larger than their male counterparts with an exposed vulva, an ovejector and a blunted tail (4). The males and females reproduce sexually, generating eggs that are passed in the host feces. Eggs typically appear 42 days after infection by

the L3 stage (5). Once the eggs are released into the environment they will hatch into the L1 stage and begin the cycle again.

The host response to *G. strigosum* is predominately a Th2 type response characterized by a strong IL-4 and eosinophil response (5, 6). While in the host, the worms also provoke a strong IgA and IgG antibody response, though the host is unable to clear the infection (5–8). It has been shown in other gastro-intestinal parasite models that the IgA response is associated with reduced worm length (9–11). Hosts with stronger IgA responses harbor shorter worms and this elevated IgA response is heritable (9). In intestinal parasites, female worm length directly correlates to egg production thus reduced worm length has a strong effect on fecundity (12).

Previous research on host antibody response to helminthes has treated the parasite population as a single homogenous group, the same way one would study a bacterial or viral infection. However, parasite populations are composed of two physiologically distinct subpopulations, males and females. This study investigated whether there is a difference in the host antibody response to male and female *G. strigosum* over time and between infection types. The effect of antibodies on body size and female fecundity was also examined to detect the impact of sex-bias on parasite traits.

In this experiment, the strength of host antibody responses to male and female gastric parasites were measured and compared. I predicted that during some or all of the infection period, female worms would be targeted more heavily by the host antibody response than male worms. I also hypothesized that female targeting by the host antibody response would correlate with negative impacts on measures of parasite population health including average body size and egg production.

Chapter 2

Materials and Methods

Experimental Design

Previous experiments in our lab infected two-month old New Zealand White rabbits with four single and co-infections: *Graphidium strigosum* (GS), *Bordetella bronchiseptica* and *Graphidium strigosum* (BBGS), *Graphidium strigosum* and *Trichostrongylus retortaeformis* (GSTR), and *Bordetella bronchiseptica* and *Graphidium strigosum* and *Trichostrongylus retortaeformis* (BBGSTR). In each infection at 4, 7, 14, 30, 45, 60, 75, 90 and 120 days post infection (dpi), four infected and two control animals were euthanized and dissected. Of importance to this experiment are the mucus samples taken from the stomach. At each time point, the *G. strigosum* parasites present in the rabbit were sexed, counted and measured. The number of eggs in the female worms were also recorded (5, 6).

Whole parasite antigen preparation and antibody quantification

Graphidium strigosum worms collected from two male New Zealand White rabbits 120 dpi with *Bordetella bronchiseptica* and *Graphidium strigosum* and preserved in 10% neutral buffered formalin were sorted by sex. Approximately 140 males and 120 females were obtained. Worms were washed three times in PBS with protease inhibitor and homogenized with a tissue lyser in 2 ml of the PBS protease inhibitor solution. The male and female lysates were spun at 10,000g for 10 minutes. Protein concentration in the supernatant was estimated using a Bradford assay. Solutions were diluted to 1 µg/µl in the PBS protease inhibitor solution.

To quantify Immunoglobulin G (IgG) and Immunoglobulin A (IgA) antibody responses against adult male and female worm antigens an indirect ELISA was used (13). The male and female *G. strigosum* antigens were coated onto ELISA plates overnight at 4 C. Plates were washed in PBS plus 0.05% Tween 20 and blocked in 1% milk powder PBS solution at 37 C. Mucus samples from animals from four infections (GS, BBGS, GSTR and BBGSTR) and at four time points (30,60, 90, and 120 dpi) were added in a 2-fold serial dilution (1:5, 1:10 for IgA, 1:10, 1:20 for IgG). Each plate also included high, low and background controls. For each infection type, the same concentration of antigen, 1 µg/µl, was used to coat the plates. For each infection type, equal volumes of stomach mucus were used to probe the plates. Plates were then probed with anti-IgG and anti-IgA Horseradish peroxidase linked goat antibodies. 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) was used to develop the plates for 15 minutes. Immunosorbant optical density (OD) was measured with a spectrophotometer at 405 nm.

Excretory and secretory antigen preparation and antibody quantification

To measure the strength of the host immune response to adult male and female parasite 24 hr excretory and secretory products, indirect ELISAs were also performed using these antigens (8). Excretory and secretory products were obtained by live culturing 5 male or female worms in 1 ml of RPMI-1640 cell growth media for 24 hours at 37 C and 5% CO_2. The resulting culture supernatants containing worm excretory and secretory products were collected and concentrated by ultra-filtration using centrifugal concentrators with a cut-off of 5 kDa for 60 minutes at 3,000g and 4 C. Protein concentration was estimated by a Bradford assay. The male and female excretory and secretory *G. strigosum* antigens were coated on ELISA plates overnight at 4 C. Plates were washed in PBS supplemented with 0.05% Tween 20 and blocked in 5% milk powder PBS solution at room temperature. Mucus samples from two infections (GS, BBGS) and at four time

points (30, 60, 90, and 120 dpi) were added in a 2 fold serial dilution (1:5, 1:10 for IgA, 1:10,1:20 for IgG). Each plate also included high, low and background controls. For each infection type, the same concentration of antigen, 1 µg/µl, was used to coat the plates. For each infection type, equal volumes of stomach mucus were used to probe the plates. Plates were then probed with anti-IgG and anti-IgA horseradish peroxidase-linked goat antibodies. 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) was used to develop the plates for 15 minutes. Immunosorbant optical density (OD) was measured with a spectrophotometer at 405 nm.

Statistical Analysis

The ratio of antibody response to females to antibody response to males was calculated for each individual for both IgA and IgG using the following formula.

$$\text{F : M ratio} = \frac{\ln(1+\text{optical density of female response})}{\ln(1+\text{ optical density of male response})}$$

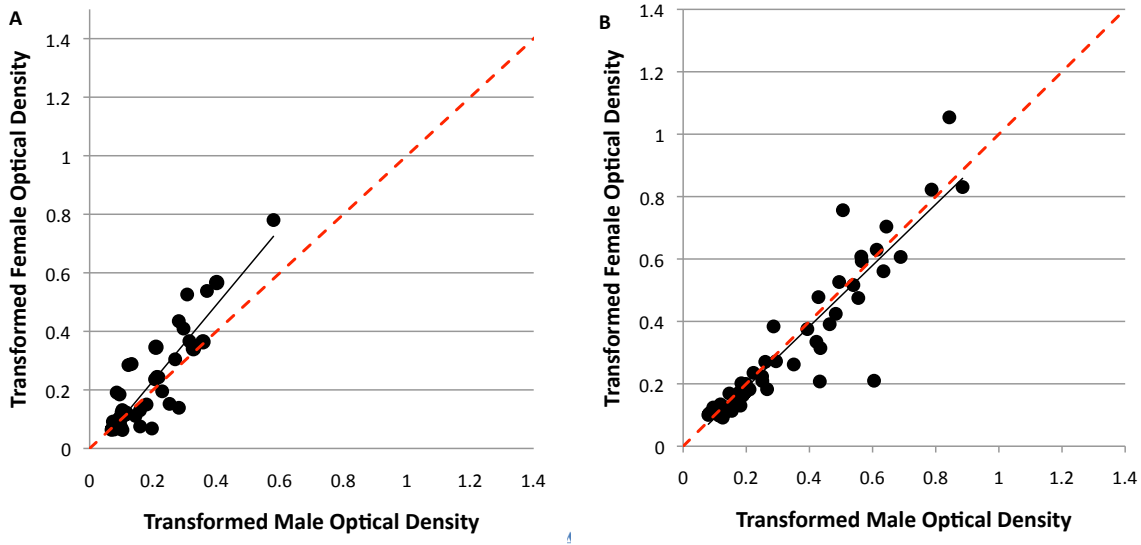
To investigate the relationship between the optical density of the immune response and parasite traits, generalized linear models were created in R. Male and female parasite population totals, male and female parasite lengths and female egg counts were regressed on the respective sex-specific antibody optical densities to determine if there was a significant correlation between antibody response and parasite biometrics. To test if the F:M ratios differed significantly from a 1:1 ratio, which represents an equal male and female antibody response, a Student's t-Test was used to compare the slope of the F:M ratio least squares regression line to a 1:1 ratio line in Minitab. Box plots of antibody optical densities and worm biometrics over infection type were created to compare the range of values by infection. This was used to justify the aggregation of data over the four single and co-infections.

Chapter 3

Results

Antibody responses to whole worm lysate

The goal of these analyses was to determine if and when there was a difference in the strength of the host antibody response to male and female parasites. The optical density of the stomach mucosa antibody binding to female *G. strigosum* whole worm lysate was plotted against the optical density of the antibody binding to male whole worm lysate. (Figure 3-1). For IgA the slope of this line skewed significantly toward a stronger response against females compared to a neutral 1:1 relationship ($p<0.001$). The slope of the IgG line did not significantly differ from a 1:1 relationship.



Optical densities of sex-specific antibody binding to whole worm lysate were plotted for IgA and IgG. Red line has a slope of 1 and represents a hypothetical situation of equal female and male responses for comparison purposes. The IgA regression line differs significantly from a slope of 1 ($p<.001$) while the IgG regression does not.

The ratio of the female-specific optical densities to the male-specific optical density was calculated for each individual rabbit and these ratios were averaged by time point for IgA and IgG (**Figure 3-2)**. For IgA, F:M ratios increase as the infection progresses and at d120 the average ratio is significantly higher than a neutral F:M ratio of 1 ($p=.007$). The earlier time points do not significantly differ from a neutral F:M ratio of 1. Whereas for IgG, F:M ratios start out skewing slightly toward the female response and decrease over the course of the infection, although this difference is not significant.

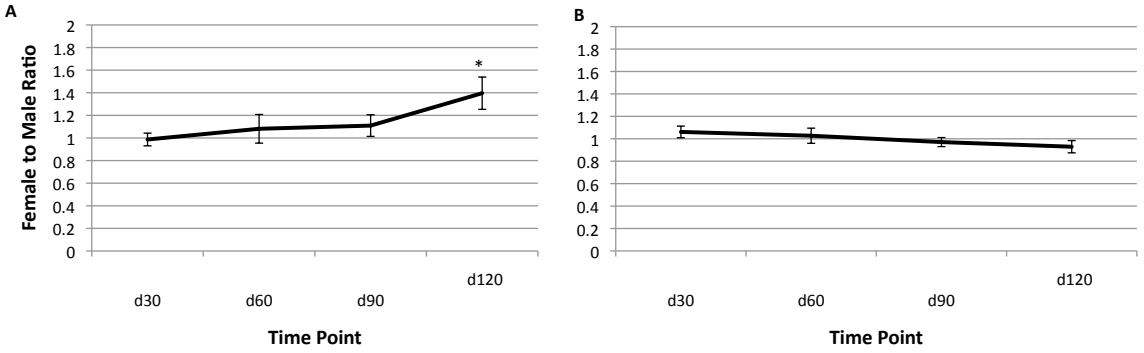


Figure 3-2 Female to Male antibody intensity ratio over time for IgA (A) and IgG (B).
F:M ratios were averaged by time point for IgA (A) and IgG (B). Asterisk indicates ratio is statistically significantly different from a neutral F:M ratio of 1.

Regression of biometric data on sex-specific host antibody response

Here, I examined the effect of sex-specific antibody responses on female and male average body size and female egg count. In generalized linear model (GLM) analysis, female-specific antibody responses correlated with reductions in female length. Conversely, male-specific antibody responses correlated with increased male length. These trends were significant for both IgA and IgG (**Table 3-1**).

Table 3-1, Coefficients from GLM analysis of parasite length regressed on sex-specific antibody intensity.
Male and female worm lengths were regressed on their respective sex-specific antibody responses using a generalized linear model for both IgA and IgG.

		Coefficient	p-value	Std. Error
IgG	Female	-0.234074	<0.001	0.016106
	Male	0.09470	<0.001	0.02251
IgA	Female	-0.917755	<0.001	0.029758
	Male	0.23556	<0.001	0.05262

Regarding the relationship between female-specific antibody response and eggs in utero, GLM analysis of egg count and female-specific antibody responses found that increased female responses significantly correlated with reduced egg numbers for both IgA and IgG (**Table 3-2**).

Table 3-2 Coefficients from GLM analysis of egg count regressed on female-specific antibody intensity.
Egg counts were regressed on female-specific antibody responses using a generalized linear model for IgA and IgG.

	Coefficient	p-value	Std. Error
IgG	-0.182602	<0.001	0.004245
IgA	-0.306280	<0.001	0.007012

Antibody responses to excretory and secretory product

To support the analyses of the antibody response to male and female whole worm lysate, antibody responses to female and male worm excretory and secretory products were graphed in a similar manner (**Figure 3-2**). For both IgA and IgG, the slope of the least squares regression line of antibody responses differed significantly from the neutral 1:1 relationship, showing a stronger response against female parasites in both cases ($p<0.001$ for both).

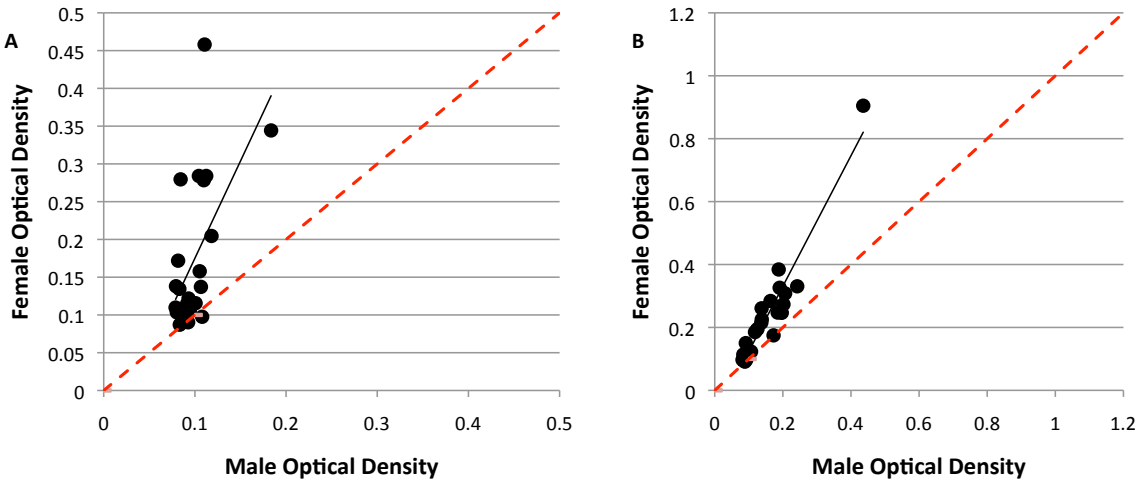


Figure 3-3 Female to Male ratio of antibody intensity to excretory and secretory products for IgA (A) and IgG (B)

Optical densities of sex-specific antibody binding to 24hr excretory and secretory products were plotted for IgA and IgG. Red line has a slope of 1 and represents a where female and male responses are exactly equal. Both the IgA and IgG regression line differs significantly from a slope of 1 ($p<0.001$)

Chapter 4

Discussion

Female worms are targeted by host immune system

By measuring the antibody response of *Oryctolagus cuniculus* to the gastric parasite, *G. strigosum*, and comparing the response elicited by male worms to that of female worms I was able to show that the host antibody response to male and female gastric worms in not consistently equal. When examining the female and male responses aggregated from multiple infection types and sampling points, the IgA responses to female whole worm lysate were stronger than the responses to male whole worm lysate. The host responses to female worm excretory and secretory products were also stronger than the response to male worm excretory and secretory products for both IgA and IgG. These results support the hypothesis that the host immune responses are not homogenous against this parasite population. There is evidence that two distinct subpopulations, males and females, are differentially targeted both by IgA and IgG.

When the responses to the whole worm lysates are broken down by time, in IgA we see that while only the 120 dpi average ratio statistically differs from a ratio of 1, there is a trend of increasing ratios of female response to male response increase over the course of the infection. This suggests that as the infection progresses and the antibody immune response undergoes affinity maturation, a stronger IgA antibody response is produced to females relative to the male antibody response. The IgG response, by comparison, showed roughly equal male- and female-specific antibody responses. These different patterns may be observed because IgA has been shown to reduce parasite length (9–11). A reduction in female parasite length is more beneficial to the host than a reduction in male length because female length directly correlates to fecundity

(12). Conversely, an effect between host IgG and parasite length or fecundity has not been observed.

Female worms are negatively affected by antibody targeting

I also examined biometric data of the worm populations in the rabbits including population size, length and egg count, and compared them to the male and female antibody responses. The goal was to find instances where the development of the female worms was impaired compared to that of the male worms as a consequence of the targeting of the female worms by the host immune response. I hypothesized that host targeting of female worms would be beneficial to the host by reducing the fitness of female worms thus I expected to see reduced egg production or body size for female worms and no reduction in body size of male worms.

The opposing relationships between sex-specific antibody response and male and female lengths supported this hypothesis. Increasing female-specific host antibody response correlated with reduced length, suggesting that the host targeting was negatively impacting female length and thus fecundity. Male worms did not appear to suffer reductions in length because of the host immune response. This is notable because, while IgA response has already been shown to reduce worm length and affect fecundity, when the worm population is divided by gender, these results suggest that the overall length reduction may not be experienced equally by males and females (8). Taken together, these results suggest that not only are female worms disproportionately targeted by the host immune system but they are also disproportionately affected by the antibody attack. Reducing female fitness would slow the population growth and reduce egg production. Both of these outcomes would benefit the host and the host's population by reducing the intensity of shedding and slowing the population growth of the parasite.

Conserving female targeted antibody response pattern is advantageous

Female worms are critical to controlling the size of the worm population since theoretically one male may fertilize many females but one female may only produce so many eggs. There is a clear benefit to the host in reducing or impairing the female population. Female length directly correlates with female fecundity and IgA has been shown to reduce worm length (10–12)**.** Additionally, IgA response patterns are heritable (9). A possible explanation for why one observes this female dominated response is that, while *G. strigosum* infections are not well controlled by the rabbit immune system, immune responses that target female worms could slow the growth of the parasite population and thus improve the fitness of the host (3). There is not evidence that the host is preferentially killing female worms but even minor reductions in female fecundity could have important implications for the growth of the parasite population. Thus reducing female length and fecundity could improve host fitness and make a female-targeted IgA response pattern evolutionarily valuable, thereby conserving it in the population.

Beyond population dynamics, female parasites are larger than their male counterparts and thus have the potential to cause more tissue damage. Reducing female length may have another benefit to the host of reducing damage to the stomach.

Strengths and Limitations

While this study is limited in the number of animals used, the F:M ratio trends are consistent when aggregated and when broken down by infection type. The results of the generalized linear model analysis draw from a significantly larger sample size because they are based on the number of worms. These models were thus significant with well-distributed errors. While it can be argued that our results are complicated by the various infections, it is important to

note that a comparison of the optical densities, parasite lengths and egg counts shows the range of values for each measurement are comparable over the infection types. The different infections also have the benefit of demonstrating that these trends hold in diverse biological contexts.

Chapter 5

Conclusion

These results show that there are more complex dynamics at play during parasitic infection than previously observed. The sexually dimorphic subpopulations of male and female worms are distinguished by the host immune response and at least part of the defensive response targets the female worm. This differential response has important ecological implications for the parasite population as female fecundity is impaired by reductions in female worm length. This targeting may benefit the host by slowing population growth more efficiently than targeting male and female worms equally. Additionally, reducing the size of female worms may benefit the host by reducing local tissue damage and inflammation. Moving forward, these results suggest that special care must be taken when studying diecious parasite infections. Compared to bacterial or viral infection, there is another layer of complexity introduced by the sexual dimorphism of helminthes.

What may seem like minor differences in parasite ecology has significant implications for treating parasitic infections in the future. In the coming years, it is possible that vaccines against parasitic worms may be developed. Their design may be improved by using the already present host immune response aggression against female worms to provoke a strong protective immune response from a vaccine.

Appendix A

Supplemental Data

Justification of Aggregation of Infections:

Box plots of optical density and parasite length values were examined by infection types. The range of values for each measurement was comparable across the infections so I felt comfortable aggregating the results into a single group for analysis.

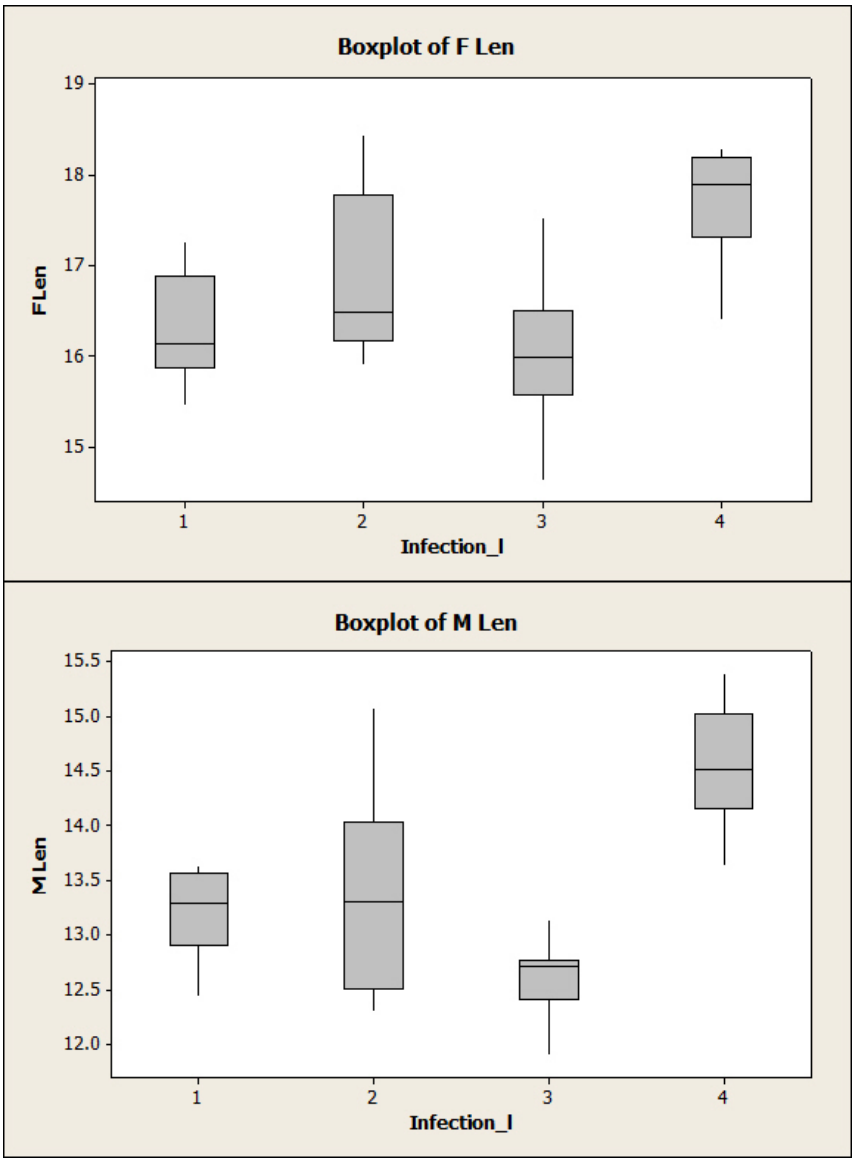


Figure 5-1 Box plots of Worm Length across infections

Worm length (mm) of males and females across infection types: GS (1), BBGS (2), GSTR (3), and BBGSTR (4) for females (top) and males (bottom).

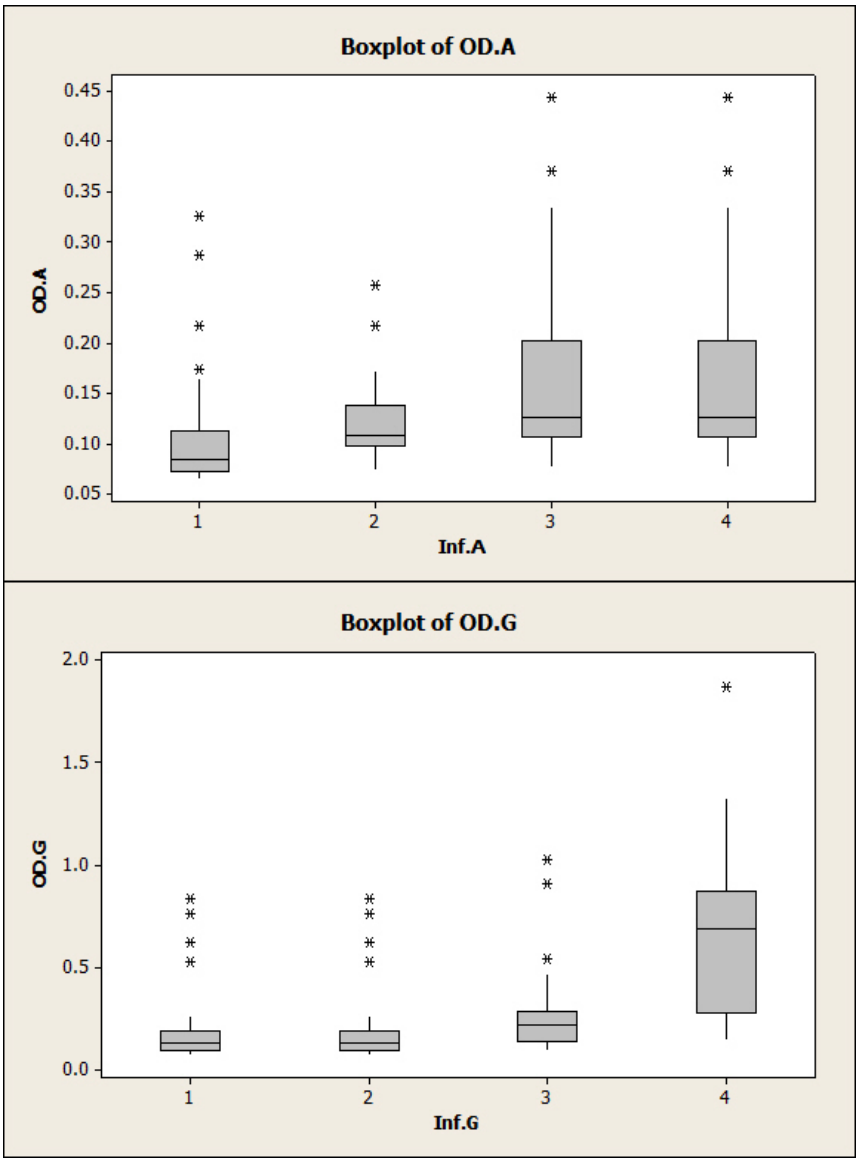


Figure 5-2 Box plots of optical density across infections:
Optical density of antibody response to parasites across infection types: GS (1), BBGS (2), GSTR (3), and BBGSTR (4) for IgA (top) and IgG (bottom). Note the optical densities include both the male- and female-specific responses.

Error distribution of generalized linear models

For the GLM analysis of both worm length and egg count regressed on sex-specific antibody response, the distribution of the errors was sufficiently normal and random.

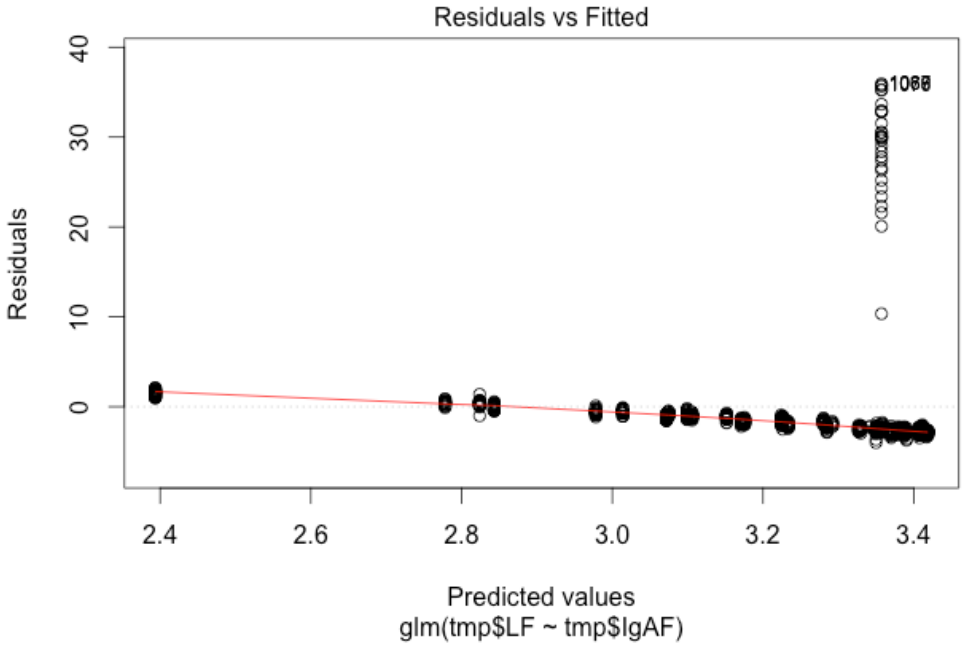


Figure 5-3 Residuals from GLM of female worm length regressed on female IgA optical density

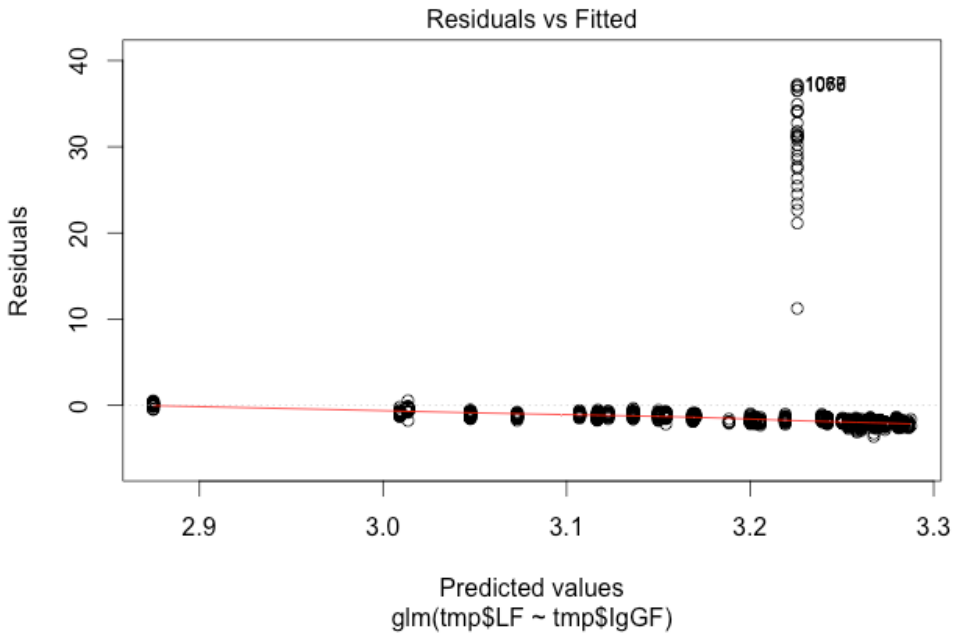


Figure 5-4 Residuals from GLM of female worm length regressed on female IgG optical density

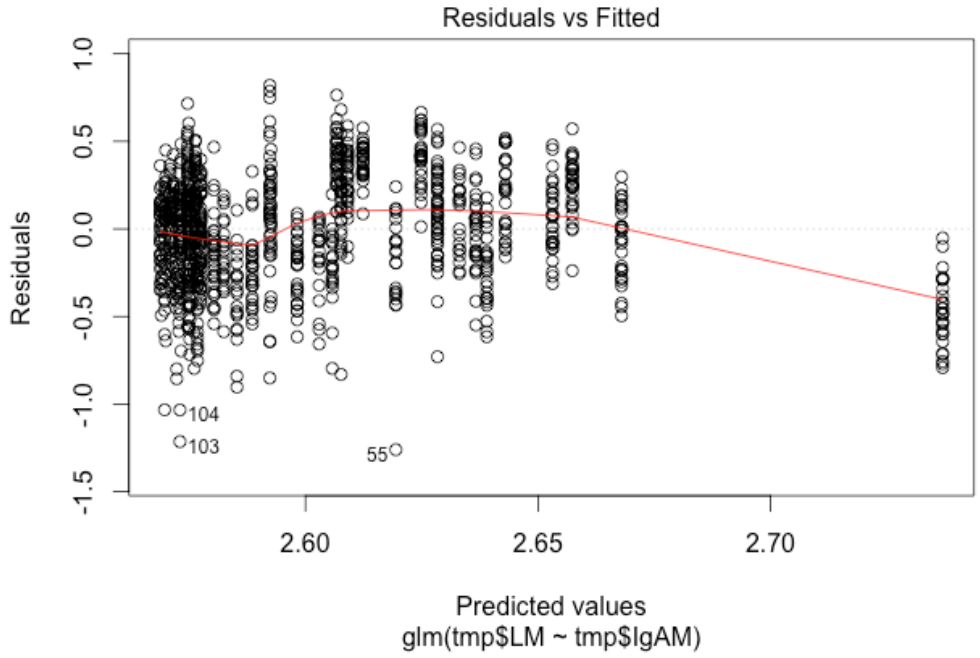


Figure 5-5 Residuals from GLM of male worm length regressed on male IgA optical density

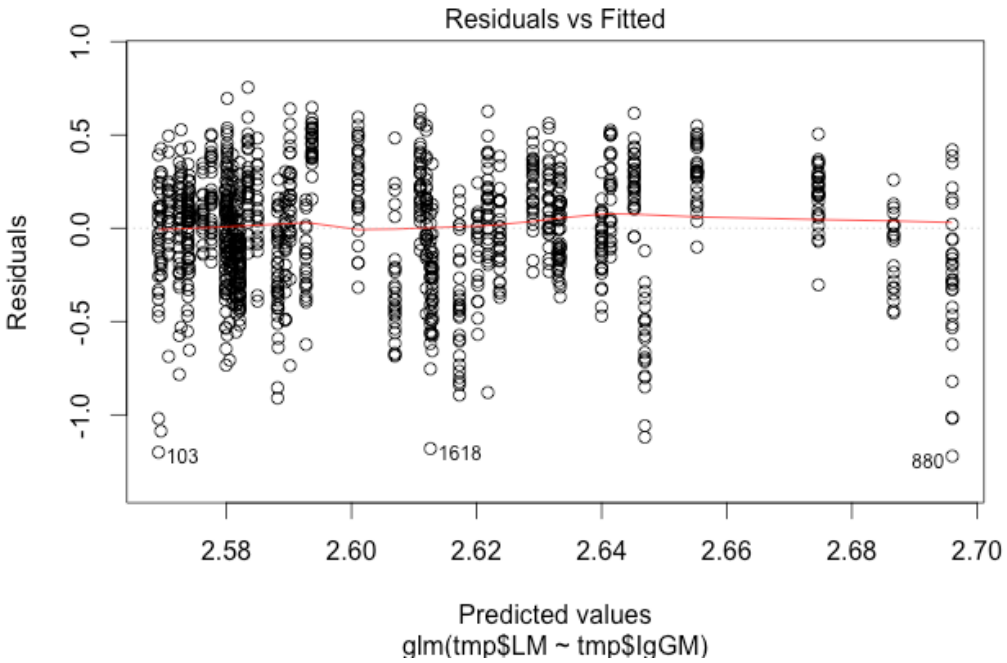


Figure 5-6 Residuals from GLM of male worm length regressed on male IgG optical density

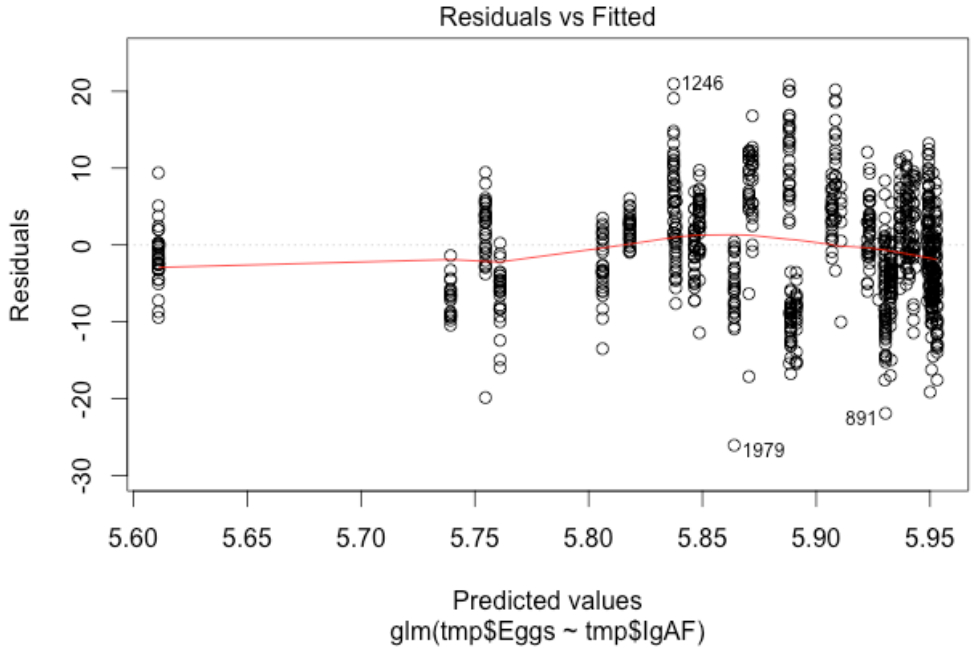


Figure 5-7 Residuals from GLM of egg count regressed on female IgA optical density

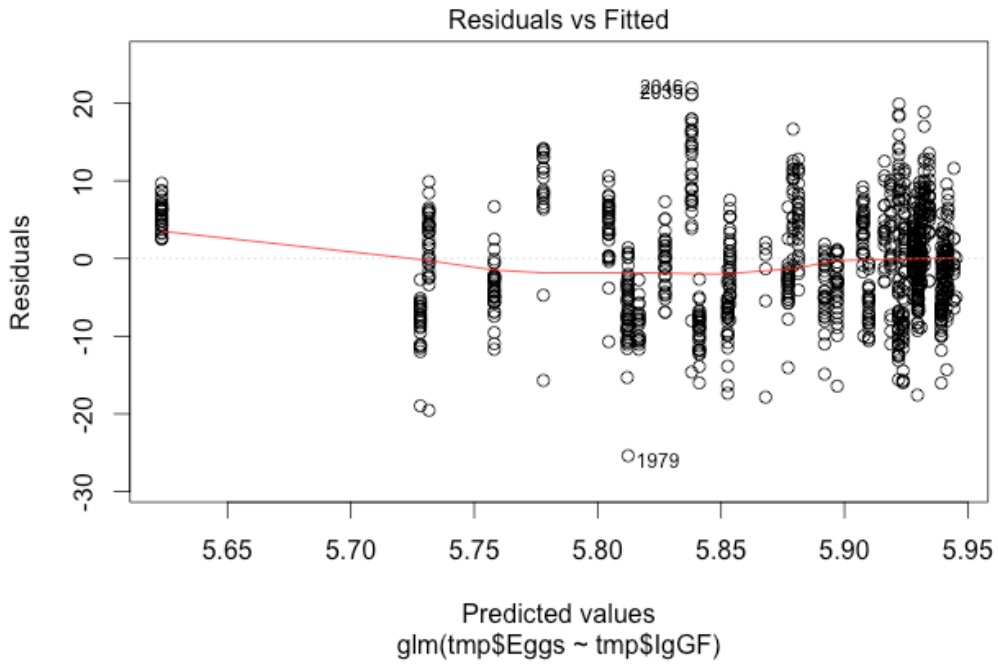


Figure 5-8 Residuals from GLM of egg count regressed on female IgG optical density

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John N. Adam Jr. Scholarship for Excellence in Agriculture, 2012

Theola F. Thevaos Honors Scholarship in the College of Agricultural Sciences, 2012
President Sparks Award, 2011
President's Freshman Award, 2010

MEMBERSHIPS
Mu Sigma Rho Statistics Honorary