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A Histopathological Review of Immune Response in Largemouth Bass to Parasitic Infections of Soft Tissues

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A HISTOPATHOLOGICAL REVIEW OF IMMUNE RESPONSE IN LARGEMOUTH BASS TO PARASITIC INFECTIONS OF SOFT TISSUES

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ABSTRACT

The immune response can serve as a key indicator of a fish's overall health and the effect stressors have on the health of the fish. Anthropogenic factors can stress a fish's immune system and inhibit immune responses. This study investigated the response of eosinophilic cells and macrophage aggregates to parasites in the livers, spleens, and gonads of Largemouth Bass, *Micropterus salmoides.* Largemouth Bass were sampled from three bodies of water in the Chattahoochee Valley of varying levels of urbanization. Histopathology of the aforementioned organs was conducted to observe both the parasite density and immune response. Eosinophilic cells were shown to be the most robust indicator of immune response based on parasite density. Bass from more urbanized areas were shown to have significantly lower immune response and significantly higher parasite density. The ovaries had the strongest immune response of all organs observed, and the livers showed the weakest response.

INDEX WORDS: Histopathology, parasites, nematodes, flukes, tapeworms, immune response, urbanization, largemouth bass

DEDICATION

This research is dedicated to the people and institutions who have supported and aided me throughout my academic endeavors. I would like to thank my parents, Mary Beth and Victor Stephenson, and younger brother, Brian Stephenson, for always supporting me and fostering my love for science throughout my life. Without their love and support, I would not have made it far in academia. I would also like to thank my undergraduate academic advisor, Nancy Dalman, for encouraging me to continue my education past my undergraduate career. I would like to thank my thesis advisor, Dr. Harlan Hendricks, for his guidance on the parasitology portion of my research. I would like to thank my committee members, Elizabeth Klar and Michael Newbrey, for their guidance on the histology and ichthyology portions of my thesis. Without their help, this research would not have been possible. Lastly, I would like to thank my family, friends, past professors of the University of North Georgia and Columbus State University for their support and encouragement in my pursuit of knowledge.

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INTRODUCTION

Host-parasite relationships offer a unique insight into the health of the environment in which the host resides. The environment can apply pressure to the host causing its immune response to be inhibited and allow parasites to more easily infiltrate its host (Henrique et al. 2015), or apply pressure to the parasite, hindering its ability to infiltrate and infect the host (Lafferty and Kuris 1999). Aquatic ecosystems are a prime example to observe since they are constantly affected by climate change, pollution (Jobling & Tyler 2003), and human activity (Kight & Swaddle 2011). This study focused on how the effects of urbanization, pollution, and human activity affected the host-parasite relationship.

There were three primary objectives of this project. The first goal was to observe and quantify the immune response of Largemouth Bass to gut parasites infecting the livers, spleens, and gonads. Like many fish, Largemouth Bass have eosinophils, leukocytes dedicated to fighting parasitic infections. Macrophage aggregates form as a more general response to tissue damage, which parasites cause by burrowing into the tissue. Both forms of immune response can be observed through histological sectioning and staining. The histopathology of Largemouth Bass is commonly used to observe biochemical effects stressors have on the health of the fish (Teh et al. 1997). The second goal was to investigate parasite load and diversity between organs and sexes of the Largemouth Bass. Parasites commonly found in the gut of the fish, as well as evidence of parasite activity, such as shed cuticles, were observed and recorded to meet this goal.

The final goal of this project was to take the data from the previous two goals and summate them into a data set to be compared between the locations from which the Largemouth Bass were obtained. This was done to assess the different effects the environment has on the host-parasite relationship. I predicted that as the areas become more urbanized, there will be an

increase in parasite load and a reduction in eosinophilic cell and macrophage counts due to increased stress on the immune system from urbanized environmental factors.

Immune Response

Eosinophilic Cells

Eosinophils in fish are bilobed granulocytes with crystalline granules. Not all fish have granulocytes, but bass are known to contain neutrophils and eosinophils (Ainsworth 1992). The function of eosinophils in fish is similar to their function in mammals, to aid in the immune response to multicellular parasites. While scarce in the peripheral blood, eosinophils can be seen in the tissue of organs of most fish (Hine 1992).

During a parasitic infection, eosinophils can be seen aggregating in the tissue near the parasite (Esch and Huffines 1973). The eosinophils react to cytokines and leukotrienes, in addition to peptides on the surface of the parasite (Buchmann 1999). Once activated, the eosinophils will release the contents of their granules through degranulation. The granules are filled with enzymes that dissolve the outer layer and internal structures of the parasite, in addition to cytotoxic, bactericidal, helminthotoxic, and ribonucleolytic compounds that interfere with cellular activity within the parasite (Balla et al. 2010). In addition to directly attacking the parasite, eosinophils also play a role in activating other immune cells (Buchmann 1999). For example, rodlet cells are enigmatic cells that may play a role in immune response. They are seen in some species of teleost fish in few organs. While their function is not entirely known, they are thought to play a part in immune response. Particularly in helminthic infections, rodlet cells are shown to be at an increased number and could aid in activating eosinophil activity in addition to

actively defending the host from parasites (Reite and Evenson 2006). The direct attack on parasites by eosinophils, in addition to activating other parts of the immune response, such as fibroblasts and lymphocytes (Petchimuthu et al. 2018), can lead to the expulsion, encystment, or killing of the parasite (Makepeace et al. 2012).

Macrophage Aggregates

Macrophages aggregates, also known as melano-macrophage centers, are localized accumulations of macrophages, usually at the site of tissue damage or inflammation. They are commonly found in many vertebrates and are used as biomarkers due to their ubiquity (Wolke 1992). They are normally found in the liver, spleen, and kidney (Blazer 2002), but have also been seen in other tissues (Wolke 1992). They are a normal part of the reparative physiology of the fish, appearing at sites of damaged tissue (Dezfuli et al. 2016). Increases in the number, in the size, and changes in the color of the macrophage aggregates can be used to determine change in the health of the fish (Wolke 1992) and changes in characteristics of macrophage aggregates have been found to be related the environment (Fournie et al. 2001). Wolke's (1992) review examined how melano-macrophage aggregates are a response to inflammation, tissue damage, and coccidian parasites. Fournie et al. (2001) described the relationship between the density of macrophage aggregates of fish spleens to degraded environments. They found that fish with a macrophage aggregate density of $40/mm^2$ were strongly associated with hypoxic conditions and sediment contamination.

Parasites

Nematodes

Parasitic nematodes are roundworms that typically infect the intestines of fish. Largemouth bass can be used as either an intermediate, paratenic, or definitive host depending on the environment and the species of nematode (Yanong 2002). As intermediate hosts, the nematodes typically encyst themselves in the small intestines of the fish, but they have also been found in the liver (Chubb 1982), gonads, spleen (Blazer 2002), the body cavity, other internal organs, and external muscle tissue (Yanong 2002). The intestines are a suitable environment for the nematodes due to their access to nutrients and proximity to the bloodstream. When encysted, the parasites have an additional layer of protection against the immune system. In the case of the Largemouth Bass serving as intermediate hosts, predatory birds act as the definitive hosts (Leung and Koprivnikar 2016).

Parasites typically do not encyst when mature. This allows them to more easily move through organs and undergo sexual reproduction (Galaktionov and Dobrovolskij 2003). While burrowing through the organs, nematodes cause structural damage to the organs and impair their function (Yanong 2002; Dezfuli et al. 2009).

Tapeworms

Also known as cestodes, tapeworms are helminths that use Largemouth Bass as both intermediate hosts (Amin and Cowen 1990) and definitive hosts (Banks and Ashley 2000). The mature tapeworm is normally found within the intestines. Plerocercoids have been observed in the liver, gonads, and other tissues near the intestines (Banks and Ashley 2000). As the tapeworms burrow through tissue, they cause significant damage to the surrounding tissue, as

well as eliciting a strong immune response. In the gonads, the combination of structural damage and the immune response can lead to sterilization (Esch and Huffines 1973).

Flukes

Flukes, also known as trematodes, are species of parasitic flatworms that infect fish as their secondary intermediate host. Their first intermediate hosts are snails, where the flukes develop into cercariae. The cercariae are then released into the water from the snails. The flukes then either penetrate the fish's body wall. Once it has infiltrated the tissue, the fluke develops into metacercariae and waits for the fish to be eaten by its definitive host, a predatory bird, in which the flukes undergo sexual development. The mature flukes then lay eggs that are later excreted into the water (Lane and Morris 2010).

The flukes can infect a variety of tissues in fish, ranging from the gills to the gonads. A large load of trematodes can place a heavy burden on the organs they reside in. Fibrosis, inflammation, and necrosis are three symptoms of a trematode infection, especially at a high load. The fibrosis occurs due to the immune system trying to encyst the parasite, and necrosis occurs if the load is too high for the organ to withstand (Petrie-Hanson 2001).

Spiny Headed Worms

Acanthocephala, also known as thorny headed worms, are a parasitic helminth seen in many vertebrate groups. They infect their hosts through the ingestion of their intermediate hosts, typically arthropods and crustaceans. Once inside the vertebrate, the spiny headed worms use their namesake spiked proboscis to latch onto the intestines of its host. There the helminths will leech off the nutrients the host has consumed. If ingested by an unsuitable host, the larvae can

burrow out of the gut and encyst themselves elsewhere until the new host is eaten (O'Rourke and Lertpiriyapong 2015). In mammals, morbidity is typically present when infected, however fish show tolerance to high numbers of acanthocephala, despite the worms burrowing deep into the intestinal wall. Spiny headed worms also have the capability of acting as a bioindicator due to their high absorption rate of heavy metals (Taraschewski 2000).

MATERIALS & METHODS

Study Species & Study Sites

Largemouth bass (*Micropterus salmoides*) are predatory centrarchids that feed upon smaller fish, small crustaceans, large insects and arthropods, and some amphibians (Hoyle and Keast 1987). They reside in freshwater lakes, rivers, and creeks from southern Florida to southern Canada and have been introduced to bodies of water around the world (Becker 1983). They can begin spawning once they are a year old and have reached at least 25 centimeters in length (Davis and Lock 2007). The males are territorial and very aggressive, often biting at anything that moves. This makes them very popular among fishers due to their ability to fight (Davis and Lock 2007). The histopathology of Largemouth Bass tissue is often used to assess the health of aquatic ecosystems that they reside in due to their ubiquity and adaptability (Teh et al. 1997). The outward appearance of the Largemouth Bass alone would not be suitable for judging the health of the fish, but their histopathology allows for a greater understanding of how they respond to stressors from their environment.

Sites along the Chattahoochee River and its tributaries around Columbus, GA were sampled during varying times of year. The sites studied were Lake Oliver, Lindsey Creek, and Warm Springs National Fish Hatchery (WSFH) in Warm Springs, GA. The WSFH Largemouth

Bass were collected in October 2016 and July 2017. The fish from Lindsey Creek and Lake Oliver (Fig. 6A) were collected from winter through summer of 2017 and 2018. Lindsey Creek is impacted the most by urban factors given that it runs near a university, mall, airport, and major roads (Fig. 6B). Lindsey Creek empties into Bull Creek, a tributary of the Chattahoochee River. Lake Oliver is impacted moderately by urban factors as there are many lakefront residential and commercial properties, but it also has long stretches of undeveloped shore. The Warm Springs Fish Hatchery would be the least impacted by urban stressors due to its goal of keeping fish healthy and recovering endangered species. Largemouth Bass from Lindsey Creek (n=23) and Lake Oliver (n=26) were previously procured by electrofishing. Largemouth Bass from Warm Springs National Fish Hatchery (n=27) were reared in captivity collected and provided to the study by the hatchery. The liver, spleen, and gonads were collected used to quantify parasite infestation.

Field Procedure

Largemouth Bass were collected by electro-fishing on a boat in open water and along the shores of Lake Oliver. The Largemouth Bass collected from Lindsey Creek were collected by Smith-Root LR24 backpack electro-fishers. The fish were kept in aerated live-wells until they were transported to the lab. Largemouth bass were collected under the Georgia Department of Natural Resources (permit number: 29-WJH-15-181, 29-WJH-16-93) and IACUC protocol A050715K. All fish used in this project were collected and processed prior to the start of this study.

Lab procedure

Tissue Preparation

The Largemouth Bass were anesthetized using a 100 mg/L tricaine methanesulfonate solution. Fish length (cm) and weight (g) were recorded. The abdominal cavity was dissected and the spleens, livers, gonads, and intestines removed. The organs were placed in 10% buffered neutral formalin. Before fixation, the liver and gonads were weighed (g). Spleens were collected, but were not weighed. The organs were processed using normal histological techniques (Carson 1997). The tissues were embedded in paraffin, 5-micron sections prepared, and then stained using a standard hematoxylin and eosin staining procedure (Carson 1997). The gonadal tissues were sectioned as whole organs, except when they were too large these samples were divided into equal sections and their data combined for analysis. The tissues were serially sectioned longitudinally and sections were stained every 100 microns. The sixth slide prepared (600 micron deep) for each organ was used for histopathology and parasite counts. The slides for tissue from Lake Oliver and Lindsey Creek were cut and stained prior to the start of this study. As a result, some of the tissues were only cut in cross-section and not longitudinal. All tissue slides were still observed microscopically in the same fashion for data collection.

Histopathology

The two biomarkers used for histopathology are eosinophilic cell counts and macrophage aggregate counts. Eosinophils are visually distinct from other cells in the tissue due to their heavy eosin staining (Blazer 2002), granulation, and bilobed nucleus (Fig. 1). They can be used to observe how strong an immune response is elicited by the parasite infection. The macrophage

aggregates have a pale yellow to tan coloring and appear as a large group of cells (Blazer 2002), typically in a round grouping (Fig. 1). Macrophage aggregates are part of a more generalized immune response to tissue damage, in this study caused by fibrosis and burrowing of helminths. An increased number of macrophage aggregates can be attributed to the fish being in bad health. Both biomarkers were counted manually for each tissue slide using an Olympus microscope (CX31). The macrophage aggregates were observed under 100x magnification and the eosinophilic cells were counted under 400x magnification due to their smaller size. The entire section of the organ was examined for counts of biomarkers used in this study.

Parasite Identification

Live parasites were removed from the abdominal cavity of the Largemouth Bass and then identified before being placed into 10% neutral buffered formalin. Using normal histological procedures, the parasites were embedded in paraffin, cut into 5-micron sections, and stained with hematoxylin and eosin (Carson 1997). The parasites were sectioned longitudinally and, if able to, in cross section. Images of the parasite sections are then compiled into an identification sheet (Appendix 1) to use in identifying parasites embedded in the Largemouth Bass tissue. The histological images, cuticle, and internal organs of the parasites were used as the primary indicators of the four categories of parasites found: nematodes (Fig.2), tapeworms (Fig. 3), flukes (Fig. 4), and spiny headed worms.

Unknowns were defined as either evidence of parasitic activity or other parasites that were not identified in this study. Evidence of parasites is defined as burrows with incomplete parasites inside them, including the remaining cuticles or internal structures (Fig. 5). Empty

burrows were not counted as unknowns because they may have been present due to other causes such as the tissue tearing during histological processing.

All prepared organs slides were viewed microscopically at 100x. All parasites were manually counted and categorized over the whole section of the slide. These counts were used to determine the parasite density per organ. The density was calculated by taking the number of parasites divided by the weight of the organs (g). Densities were not calculated for spleens due to weight not being collected at the time.

Data Analysis

Least squares regression analysis and Pearson's Correlation coefficient were used to test for correlations between parasite activity and the two immune response biomarkers. Analysis of variance (ANOVA) was used to examine the effects the locations, organs, and gender have on both parasite density and the respective immune responses. All tests were performed with the statistical program Statistical Package for the Social Sciences (SPSS) using the Tukey pairwise comparisons. Alpha is set to 0.05.

The slopes from the linear regressions will be used to define the eosinophilic cell rate (ECR), macrophage aggregate rate (MAR), eosinophilic cells per parasite (ECPP), and macrophage aggregates per parasite (MAPP). The ECR is defined as the eosinophilic cell count over parasite density and the MAR is defined as the macrophage aggregate count over parasite density. When unable to calculate density, the ECPP and MAPP was used. The ECPP can be defined as the eosinophilic cell count over total number of parasites, and the MAPP can be defined as the macrophage aggregate count over the total number of parasites. These slopes were used to establish the patterns between comparable immune responses.

RESULTS

Quantification of immune response

Eosinophilic Cells

When comparing the number of eosinophilic cells to parasite density, the strength and direction of the correlation varied (Fig. 7). For Largemouth Bass from the Warm Springs National Fish Hatchery, the correlations were fairly strong and positively correlated for the livers ($p=0.011$), ovaries ($p=0.008$), and testes ($p<0.001$) that were examined. For the moderately urbanized Lake Oliver, the Largemouth Bass had a very strong and positive correlation for the ovaries ($p=0.005$) and testes ($p<0.001$). However, the livers of these fish showed no significant correlation between eosinophilic cells and parasite density. In the Largemouth Bass of the highly urbanized Lindsey Creek, there were no significant correlations in any of the organs.

Due to not having the weights to calculate parasite density in the spleen, least squares regressions were run using the total parasite counts. The correlations in the spleens, livers, and ovaries were not significant. The correlation in the testes was significantly ($p<0.001$) strong and positive ($r=0.70$).

The slopes for the linear regressions represent the eosinophilic cell rate (ECR) (Table 1). The ECR differed between ovaries, livers, and testes, and location of the Largemouth Bass subjected to urbanized environments. With the exception of the ECR in the ovaries of the bass in Lake Oliver, all of the other sampled organs had a lower ECR than the Warm Springs National Fish Hatchery Largemouth Bass. The ECR in the ovaries of the Lake Oliver bass was much higher than the ECR of the ovaries in the Warm Springs National Fish Hatchery Largemouth Bass.

When comparing the organs of the Lake Oliver Largemouth Bass, the total parasite count was used instead of parasitic density to determine the eosinophilic cells per parasite (ECPP) slope (Table 2). The ECPP of the liver was the only organ to have a negative slope at -1.57 ECPP. In the spleen, the ECPP slope was very small at only 0.53 ECPP. The gonads had higher ECPP slopes with the testes having a slope of 52.37 ECPP and the ovaries having a much higher slope at 114.09 ECPP.

Macrophage Aggregates

When comparing macrophage aggregates to parasite density, the strength of the correlations remained consistent while the direction varied. There were no significant correlations in any organ at any location between macrophage aggregates and parasite density.

The slopes of the regressions for macrophage aggregate counts over parasite density represent the macrophage aggregate rate (MAR). The correlations showed no significant relationships ($p>0.05$; Table 3). The only organs showing a positive MAR were the ovaries from Lake Oliver and Lindsey Creek, and the livers from Lindsey Creek. All other organs in all locations showed a negative MAR.

For the Lake Oliver Largemouth Bass, the macrophage aggregate counts were compared against the total parasite counts to get the macrophage aggregates per parasite (MAPP) slopes (Table 4). The correlations for the livers, ovaries, spleens ($p=0.001$) and testes ($p=0.006$) were all significantly positive ($0.56<r<0.87$). The spleen had a higher MAPP slope at 2.24 over all other organs. The MAPP slopes of the livers, ovaries, and testes were all relatively the same, ranging from 0.35 to 0.80.

Eosinophilic Cells and Macrophage Aggregates in the Spleen

The spleen data showed a negative relationship between the eosinophilic cell count and the macrophage aggregate count regression (m=-0.56, r=-0.35, p=0.0026, Fig. 8). This is a fairly strong and negative correlation (Fig. 8) between the two immune response biomarkers.

Parasite Diversity

Spiny headed worms were not found in any of the organs observed in this study. Of the four categories of parasites observed, the mean number of nematodes (8.93) was significantly greater than the tapeworms (1.04), flukes (3.29), and unknowns (4.64) (1-way ANOVA, $F_{4,855}=29.99$, $p<0.001$) (Fig. 9). When comparing parasite counts from the specific organs within all locations (Fig. 10) the mean number of nematodes was greater than the other parasitic categories in the testes from Lake Oliver and Lindsey Creek, and the spleens from Lake Oliver. In the spleens from Lake Oliver, the mean number of unknowns was greater than flukes and tapeworms. Only in the livers from Lake Oliver did the nematodes and the flukes outnumber the other parasites (Fig. 10), with the average number of flukes (16.60) being greater than the average number of nematodes (10.30). In the Lindsey Creek livers, the averages for nematodes, tapeworms, flukes, and unknowns were not significantly different. For the fish of WSFH, there was no significant difference between any of the parasite groups.

Effects on Immune Response

Location

The geographic location and organs of the fish had a significant effect on the parasite density (2-way ANOVA, $F_{4,128} = 8.05$, $p<0.001$, Fig. 11). A pairwise comparison among locations shows that Lindsey Creek ($p<0.001$) and Lake Oliver ($p=0.025$) have higher parasite densities than Warm Springs National Fish Hatchery, and that Lindsey Creek has a higher parasite density than Lake Oliver ($p=0.002$). A pairwise comparison for the organs also shows that the testes have a higher parasite density than the liver and ovaries ($p<0.001$) while there is no significant difference between the livers and ovaries ($p=0.554$).

The effects of geographic location and organ on ECPP were compared (Fig. 12). There was a significant effect between the location and organs of the fish on ECPP (2-way ANOVA, $F_{4,75}= 2.55$, $p=0.046$). A pairwise comparison showed that the ovaries have a higher ECPP over testes and livers ($p<0.001$). It also showed that Lake Oliver has a higher ECPP than Lindsey Creek ($p=0.001$), but does not have a significant difference with WSFH ECPP ($p=0.891$). A comparison of the effects of sex and location on ECPP was also conducted, but did not find a significant interaction (2-way ANOVA, $F_{2,131}=1.47$, $p=0.236$).

Lastly, the effects of location and organ on MAPP were compared. There was no significant interaction found between location and organ (2-way ANOVA, $F_{4,78}=1.64$, $p=0.172$). A comparison of the effects of location and sex on MAPP also yielded no significant interaction (2-way ANOVA, $F_{2,80}=0.27$, $p=0.763$).

Gender

A comparison of the effect of sex on parasite density, ECPP, and MAPP were run (Fig. 13). The ANOVAs showed that males had a higher parasite density (24.62) than females (4.82) (1-way ANOVA, $F_{1,135}$= 7.83, p=0.006), but no significant difference for ECPP (p=0.45) or MAPP (p=0.51) between sexes overall. The interaction of the gender of the fish and the geographic location had a significant effect on the parasite density (2-Way ANOVA, $F_{2,131}$= 5.79, p=0.004). A comparison of the effects of sex and organs on total parasite count in the Largemouth Bass from Lake Oliver showed no significant interaction between sex and organs (p=0.222).

Spleen

A comparison of the effects of organs on ECPP (Fig. 14A) and MAPP (Fig. 14B) showed the spleen and liver had significantly lower ECPP than the gonads (1-way ANOVA, $F_{3,71}$= 23.36, p<0.001) and that the spleen had a significantly higher MAPP than the liver or gonads (1-way ANOVA, $F_{3,71}$=11.39, p<0.001). Another comparison of the differences in total parasite count among the organs showed that the spleen and liver had significantly higher total parasite counts than the ovaries (1-way ANOVA, $F_{3,76}$=5.26, p=0.002).

DISCUSSION

Immune Responses

The organs with the highest eosinophilic cell rate (ECR) also had the highest correlation. This would indicate a healthy immune response given that the correlations were both strong and

positive. The organs with extremely low slopes also had a very weak correlation, indicating a weak immune response. The variation in the poorly responding fish could be attributed to a weakened immune system. From the given data, the organs of the fish from Lindsey Creek had the weakest immune response. This could be attributed to Lindsey Creek being surrounded by a heavily urbanized area. Numerous urban factors could be contributing to the suppression of the immune system including chemicals, such as endocrine disrupting compounds (Jobling & Tyler 2003) entering the creek and anthropogenic noise (Kight & Swaddle 2011). These previous studies show the disruption of the endocrine system can cause negative downstream effects on the immune system, such as lowered humoral immune response. The cause may not be solely anthropogenic. The parasites have also evolved ways to either evade or suppress the immune system. They can evade the immune system by masking their antigens, burrowing to other tissues where the immune response has not reached yet, or simply developing quickly (Sitjà-Bobadilla 2008). The parasites can also suppress the immune system by inducing immunodepression and immunomodulation. For example, liver flukes have been observed to secrete a proteinase that prevents or deters the attachment of eosinophils to the fluke. This protects the flukes from being affected by the degranulation of the eosinophils (Carmona et al. 1993). Lastly, time could also play a role in the immune response, but the time of year that specimens were collected was not examined by this study. The immune systems of fish do vary seasonally due to changes in temperature, photoperiods (Bowden et al. 2007), and feeding habits (Anderson et al. 2015).

Normally, the gonads are immune-privileged tissues, meaning the organs are not usually subject to the immune system so the organ can function properly. This is ideal for parasites as they would not have to expend energy to evade or suppress the immune system in these organs

(Sitjà-Bobadilla 2008). When the immune system is exposed to these once immune-privileged tissues, the immune system will attack the tissue as gametes are immunogenic. The higher ECR seen in the ovaries and testes of Lake Oliver specimens compared to the liver could be explained by the removal of immune privilege.

Despite being an immune-privileged organ, the ovaries had a significantly lower parasite density than the testes. There are two possible explanations for the phenomenon. First, the ovary is covered by a thick outer layer of connective tissue known as the tunica albuginea (Esch and Huffines 1973). This thick tissue may deter parasites from burrowing into the ovaries. The other option is that testosterone is negatively affecting the immune system. It has been shown that testosterone can have negative effects on immune response in gazelles (Ezenwa et al. 2012), humans (Hamano et al. 1998), and chinook salmon (Slater & Schrek 1993). Given that testes are immune-privileged and are the producers of testosterone, it would be a good environment for parasites to invade and mature without trouble from the immune system.

The macrophage aggregate rates (MAR) varied across location and organs with most being negative and lacking a significant correlation. However, the macrophage aggregates counts showed a strong positive correlation when compared with the total parasite count. The spleen had a significantly higher macrophage aggregate per parasite (MAPP) than the liver and gonads. This is to be expected as the spleen acts like a large lymph node and hosts many macrophages that normally function as antigen-presenting cells. When the parasite burrows into the tissue, the macrophages would not only swarm the parasite, but also the tissue around the burrow that has been damaged by the parasite. While splenic macrophage aggregates have been used to show if the fish's environment has become degraded (Fournie et al. 2001), the data suggests it would be a strong indicator of parasite load as well.

Parasitic Diversity

Nematodes were the most numerous parasites in the organs of all the fish, but particularly more common in the testes. The flukes were most numerous in the liver, and in some cases being as numerous or more numerous than nematodes in the liver. Flukes were not observed in any ovaries, possibly due to their comparatively smaller size and need to burrow past the thick tunica albuginea of the ovaries to successfully invade the organ. Tapeworms were only found in the liver and were not observed in any gonads. Spiny-headed worms were not observed in any organ as these parasites prefer their niche of hooking into the intestinal wall while they mature and sexually reproduce (Taraschewski 2000).

Parasites were found in Largemouth Bass from Warm Springs National Fish Hatchery, but only in a very small number of fish. Helminths are what usually infect Largemouth Bass (de Melo Souza et al. 2019) and nematodes are most commonly found in the infected tissues. The parasites were low in number and the immune response measured by the ECR that they elicited appeared very strong. Some fish showed eosinophilic cells in their gonadal tissue without any parasites present, which could indicate that the parasites are elsewhere in the organ, or just had begun to invade the organ.

Health Assessment Between Locations

The Largemouth Bass of Lake Oliver are faring better than Lindsey Creek bass. The immune response in their gonads are still strong, but the immune response in their livers and spleens are weakened. This could be attributed to other environmental factors outside of a moderately urbanized residential area. The Largemouth Bass of Warm Springs National Fish

Hatchery are the healthiest, having been raised in an aquaculture environment. They have the lowest parasite load, having little to no parasites in the population, and their immune response is still fairly robust. The differences in parasite load between these bodies of water supports the notion that parasite abundance can be a good indicator of the effects of urbanization on fish populations. As seen in a previous study in the Columbus, GA area observing bluegill and redbreast sunfish from urban and rural creeks, the parasite abundance was higher in fish from urban creeks (Anderson et al. 2015).

Limitations and Future Studies

This study was not able to survey all of the immune responses to parasitic infection, nor cover every aspect of parasite activity. Future studies should examine other types of immune response, such as rodlet cells or antibodies. The duration of the parasitic infection in the fish prior to grossing was not determined during this study, so the timing of infection versus immune response was not assessed. As previously mentioned, the season in which the specimens were collected was not examined in this study either and could play a role in how the immune system is modulated at different times of the year. Lastly, future studies should look at a wider range of parasites outside of what is commonly found in the intestines. In some organs, the “unknown” parasite counts were fairly large, so expanding the types of parasites studied could reduce these numbers.

CONCLUSIONS

The immune response to parasitic infection can best be described by the eosinophilic cell rate (ECR) because of the high levels of correlation as opposed to the lack of correlation in the

macrophage aggregate rate (MAR). The ECR can serve as a biomarker for assessing the health of the fish. The higher the ECR with a significant correlation, the healthier the fish.

While there was no overall difference in parasite load and immune response between the sexes, the testes showed a much higher parasite density and a much lower ECR than the ovaries. This suggests that the gonads of different sexes have physiological differences that affect their ability to resist parasitic infection. Testosterone and other androgens or different tissue composition of the gonads could account for this difference.

Lastly, urbanization around a body of water has shown to have negative effects on the immune systems of Largemouth Bass in the Chattahoochee Valley area. Immune response was observed to be heavily and negatively impacted, while the parasite's ability to infect the hosts did not seem to be affected by the urbanized environment.

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FIGURES AND TABLES

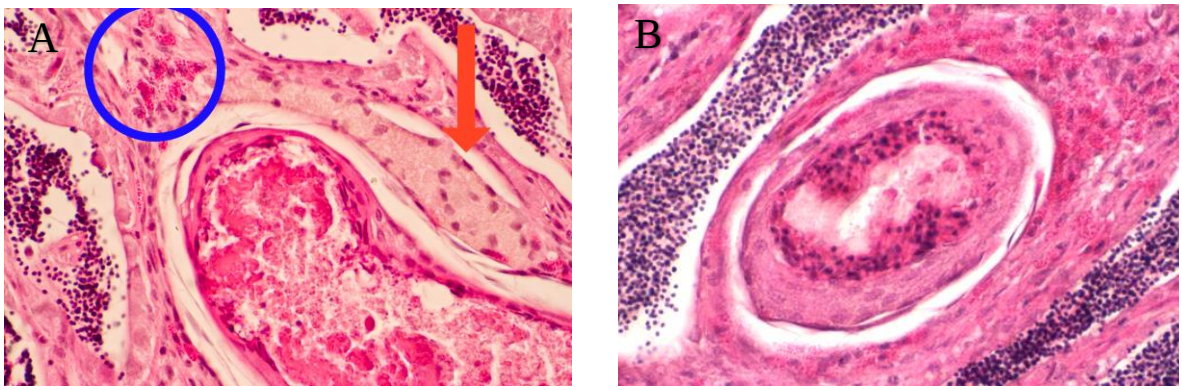


Fig 1. (A) A nematode in testicular tissue. Stained in H&E and magnified 400x. Eosinophilic cells are in the blue circle and a macrophage aggregate is indicated by the red arrow. (B) Eosinophilic cells surrounding a tapeworm stained in H&E and magnified 400x.

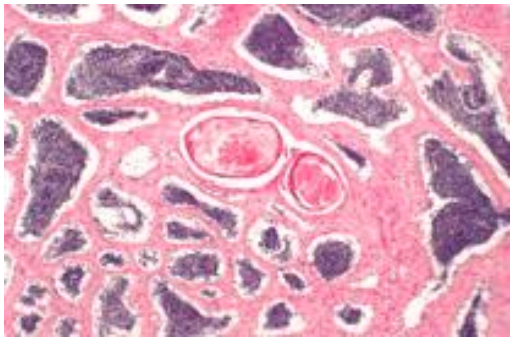


Fig 2. A nematode in testicular tissue. Stained in H&E and magnified 40x.

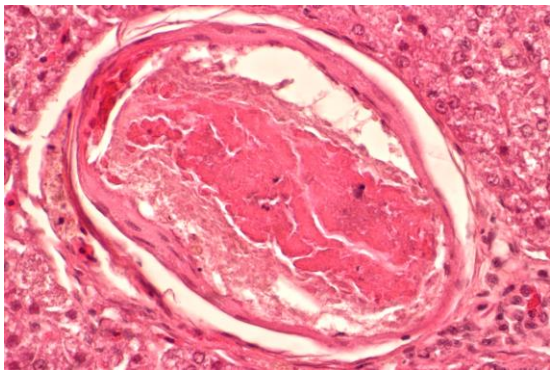


Fig 3. A tapeworm in liver tissue. Stained with H&E and magnified 400x.

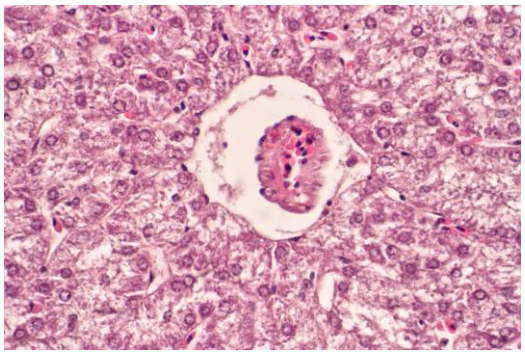


Fig 4. A fluke in liver tissue. Stained with H&E. Magnified 400x.

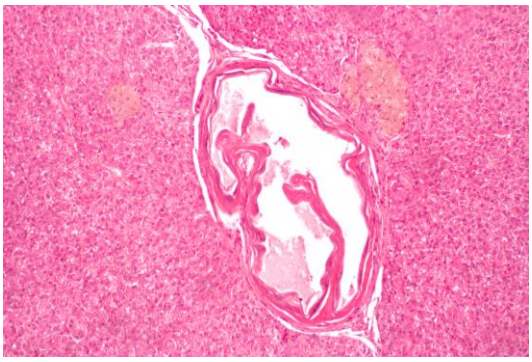


Fig 5. An example of an unknown parasite in liver tissue. Stained in H&E and magnified 100x. The cuticle remains, but it lacks any identifiable internal structures.

Fig 6. (A) A map depicting the areas where the fish were sampled from, Lake Oliver on the left, and Lindsey Creek on the bottom right. (B) An outline of the path Lindsey Creek flows through Columbus, GA. It notably passes through the Columbus Airport, Peachtree Mall, and Columbus State University.

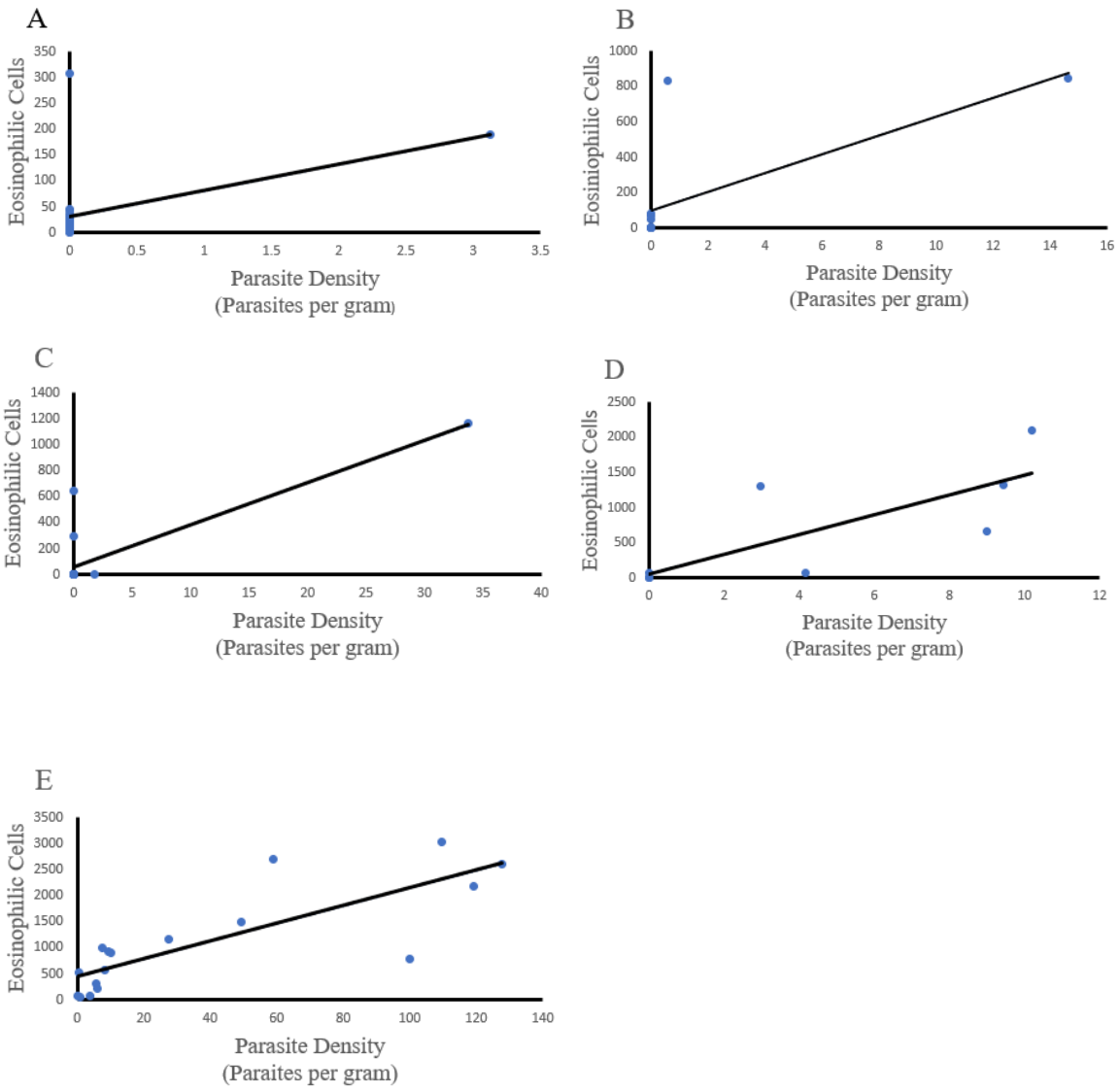


Fig 7. A comparison of graphs depicting the significant effect of parasite density on eosinophilic cell counts. The Warm Springs National Fish Hatchery livers (A), ovaries (B), and testes (C) all show positive trends with strong correlation. The Lake Oliver ovaries (E) and testes (F) also show significant positive trends with strong correlations.

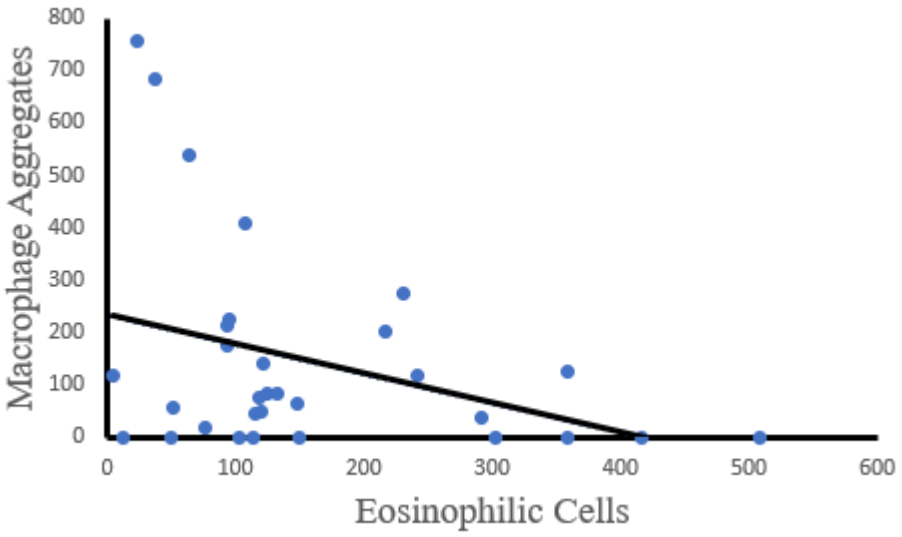


Fig 8. The number of macrophage aggregates compared to the number of eosinophilic cells counted. The data suggests a significant inverse relationship between the two immune responses with a slope of -0.56 and a correlation of -0.35 ($p=0.026$).

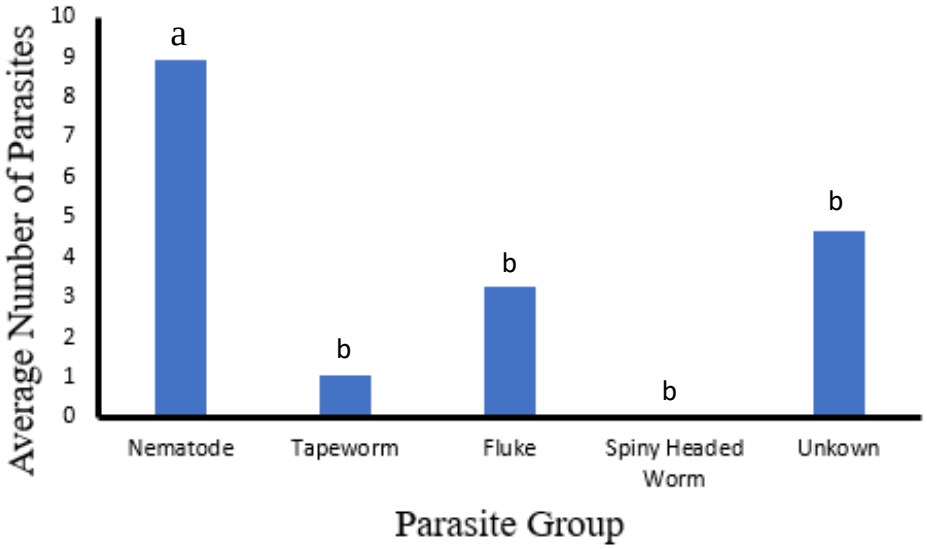


Fig 9. The average number of each parasite group over all locations and organs. The average number of nematodes was significantly higher than all other groups of parasites ($^{a}P<0.001$).

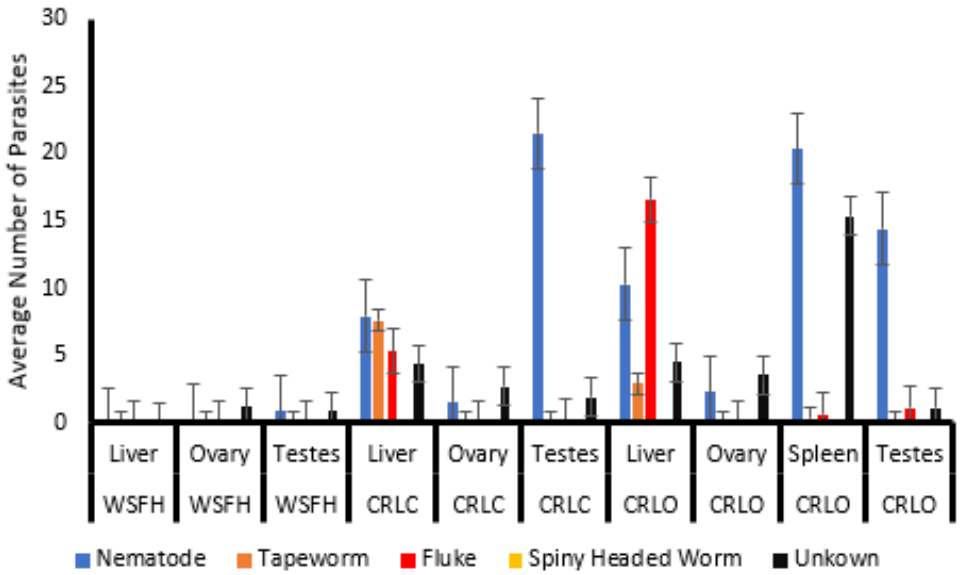


Fig 10. The average number of parasites in each organ by location. Nematodes were the most numerous with the except of the Lake Oliver livers where the flukes had the highest average. The error bars represent 95% confidence intervals.

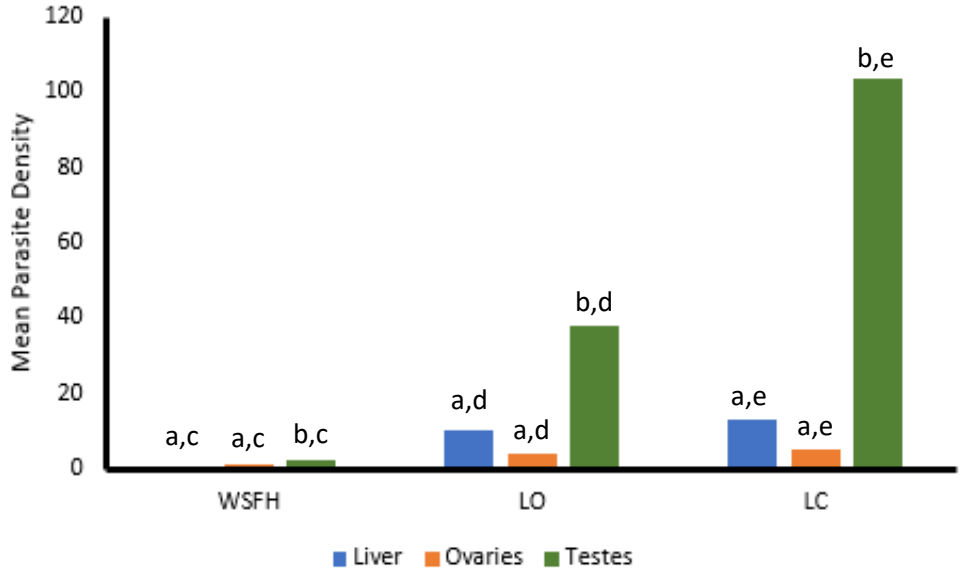


Fig 11. The mean parasite density by the organ and location of origin for the Largemouth Bass. The testes had the highest parasite density of all the organs ($P<0.001$), and the bass from Lindsey Creek (LC) ($P<0.001$) and Lake Oliver (LO) ($P=0.028$) had significantly higher parasite densities than the bass from Warm Springs National Fish Hatchery (WSFH). Lindsey Creek has a higher parasite density than Lake Oliver ($P=0.002$). Differences between organs are represented by the letters a and b, and the differences between locations are represented by the letters c, d, and e.

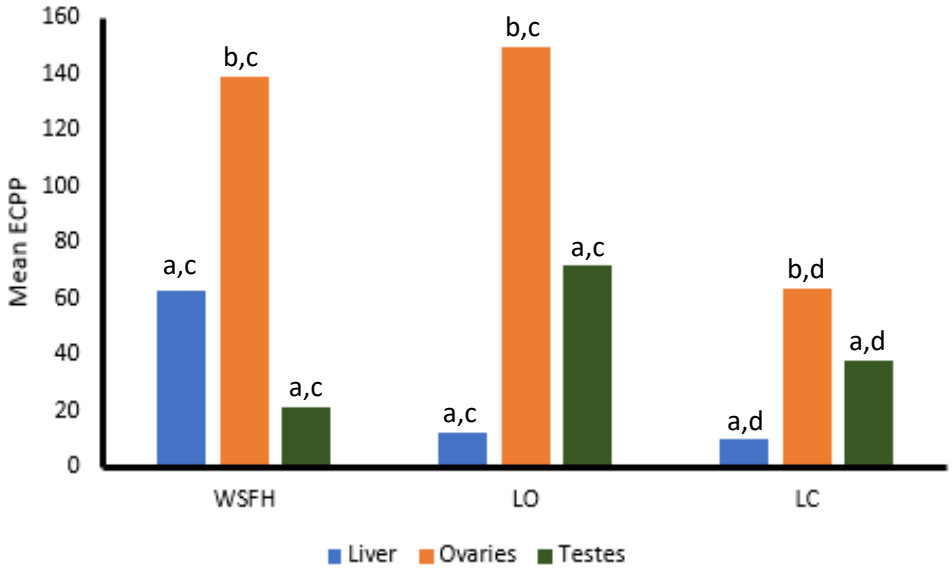


Fig 12. The mean ECPP between the organs and the location of origin for the Largemouth Bass. The ovaries had the highest ECPP ($P<0.001$) and the organs from Lindsey Creek (LC) had a lower ECPP ($P=0.001$) than Lake Oliver (LO) and Warm Springs National Fish Hatchery (WSFH). The differences between organs are represented by the letters a and b, and the differences between locations are represented by the letters c and d.

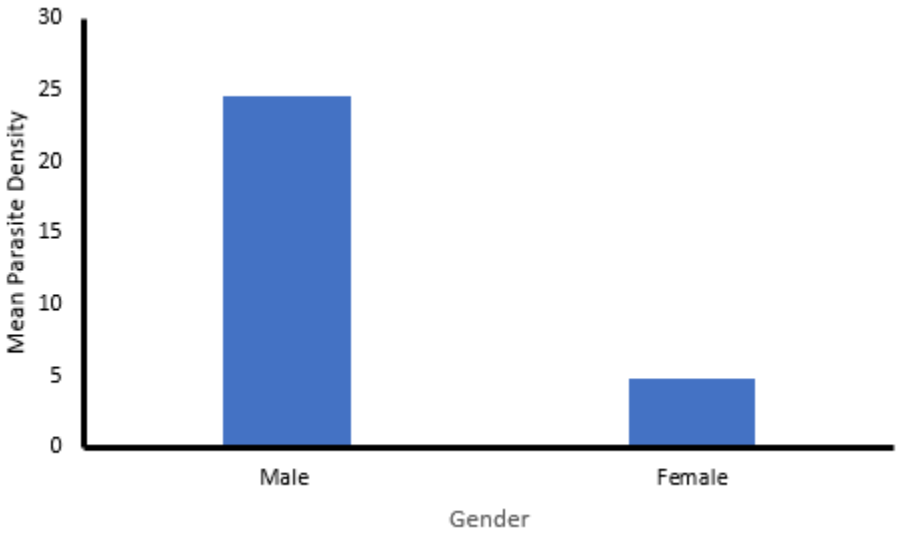


Fig 13. The mean parasite density between the sexes. Males had a higher parasite density than females (P=0.006).

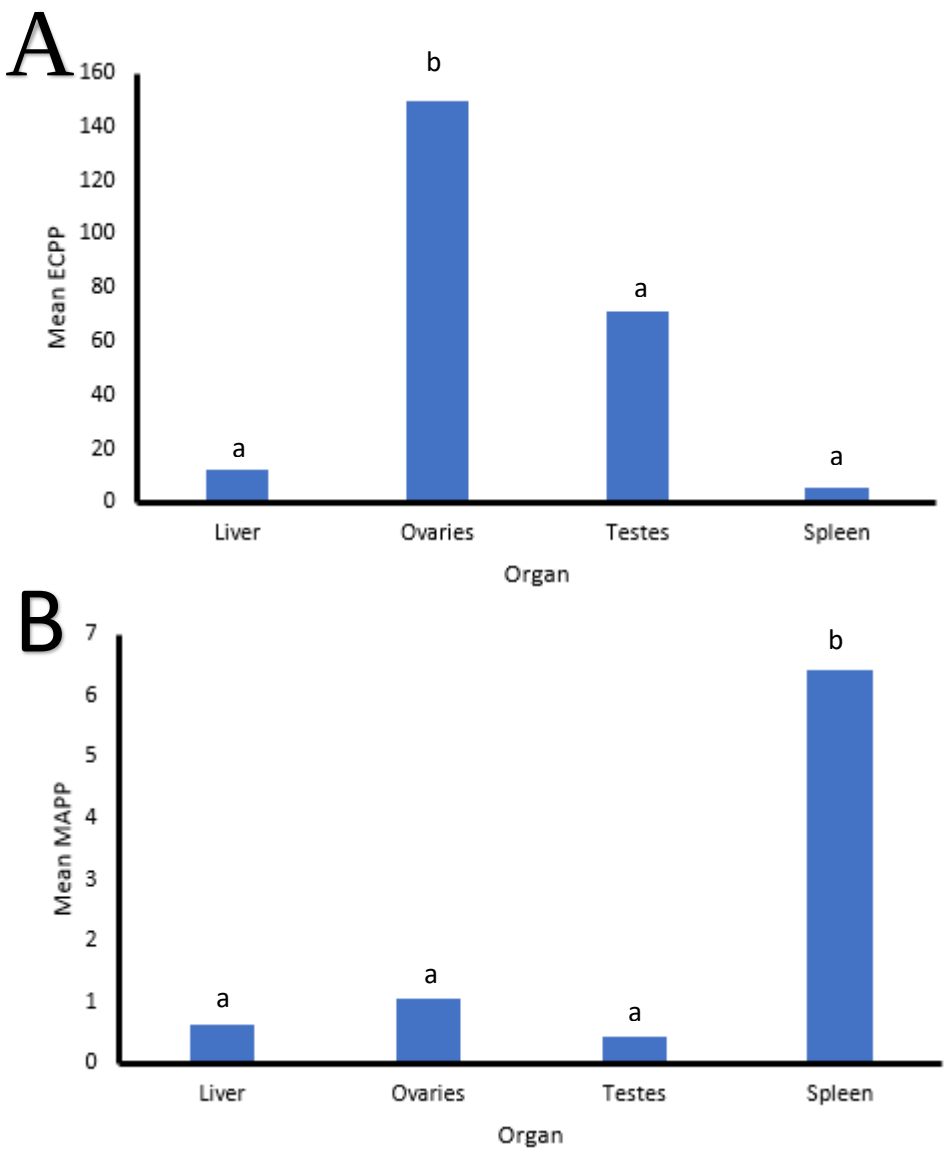


Fig 14. (A) The mean ECPPs by organ for fish collected from Lake Oliver. The ovaries had the highest ECPP count ($P<0.001$). (B) The mean MAPP by organ for fish collected from Lake Oliver. The spleens had the highest MAPP count ($P<0.001$).

Table 1. The eosinophilic cell rate in different organs at each location.

Location	Organ	Number (n)	ECR	PCC	P
WSFH	Liver	23	50.68	0.47	0.011
WSFH	Ovaries	11	53.22	0.71	0.008
WSFH	Testes	16	32.48	0.84	<0.001
Lake Oliver	Liver	23	0.28	0.00	0.489
Lake Oliver	Ovaries	9	139.99	0.79	0.005
Lake Oliver	Testes	17	17.03	0.82	<0.001
Lindsey Creek	Liver	18	0.75	0.05	0.419
Lindsey Creek	Ovaries	13	1.22	0.03	0.467
Lindsey Creek	Testes	10	0.85	0.45	0.114

Table 2. A comparison of ECPP in the organs of Largemouth Bass collected from Lake Oliver.

Organ	ECPP	Number (n)	PCC	P
Liver	-1.57	23	-0.17	0.29
Ovary	114.09	9	0.48	0.09
Testes	52.37	17	0.70	0.001
Spleen	0.53	31	0.08	0.33

Table 3. The macrophage aggregate rate in the organs of the Largemouth Bass in each location.

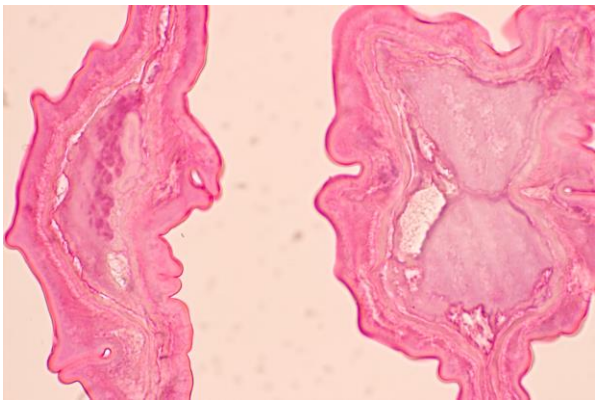
Location	Organ	Number (n)	MAR	PCC	P
WSFH	Liver	23	-1.69	-0.12	0.295
WSFH	Ovaries	11	-0.008	-0.16	0.317
WSFH	Testes	16	-0.17	-0.09	0.369
Lake Oliver	Liver	23	-0.80	-0.23	0.151
Lake Oliver	Ovaries	13	0.62	0.37	0.116
Lake Oliver	Testes	17	-0.03	-0.17	0.256
Lindsey Creek	Liver	18	0.25	0.11	0.337
Lindsey Creek	Ovaries	13	0.08	0.11	0.359
Lindsey Creek	Testes	10	-0.01	-0.29	0.222

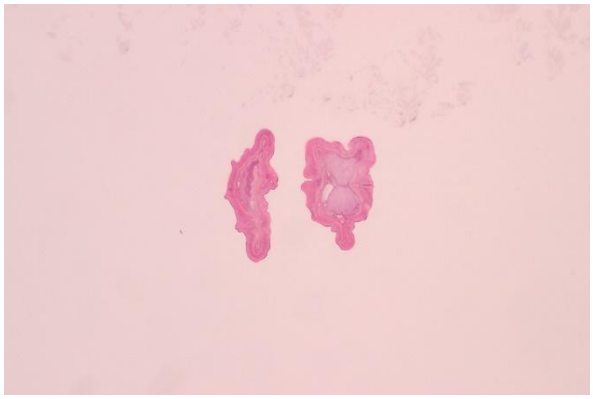
Table 4. A comparison of MAPP of the organs collected from Largemouth Bass in Lake Oliver.

Organ	Number (n)	MAPP	PCC	P
Liver	23	0.44	0.63	0.001
Ovary	9	0.80	0.87	0.001
Testes	17	0.35	0.60	0.005
Spleen	31	2.24	0.56	0.001

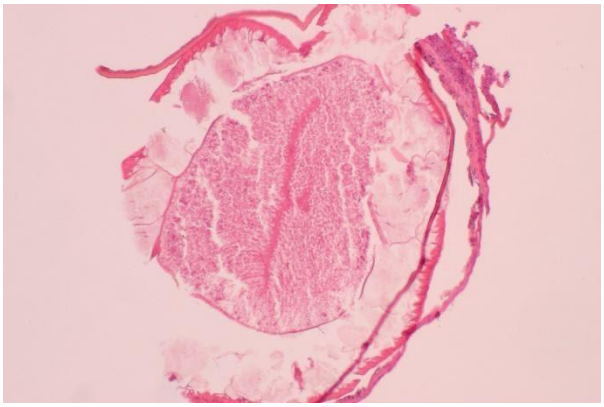
APPENDIX - Parasite Identification Guide

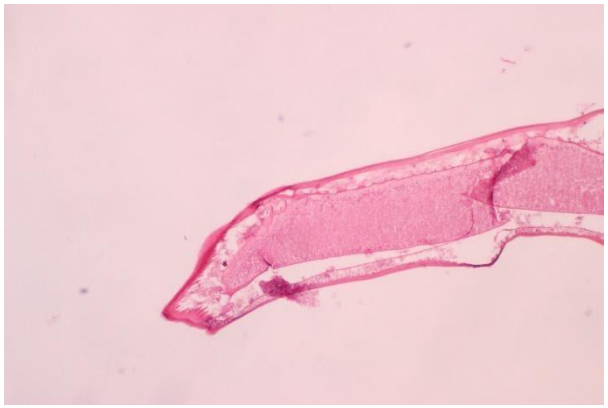
Fluke

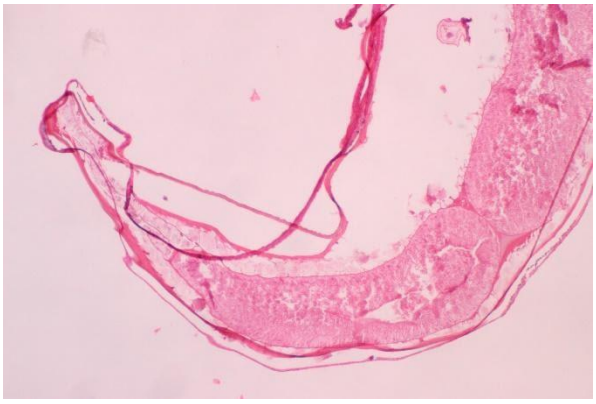


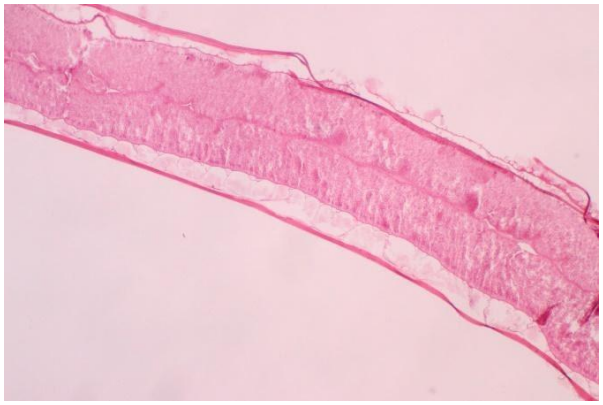


Nematode

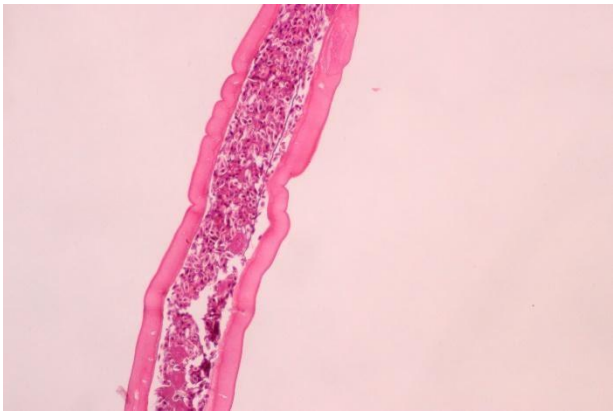
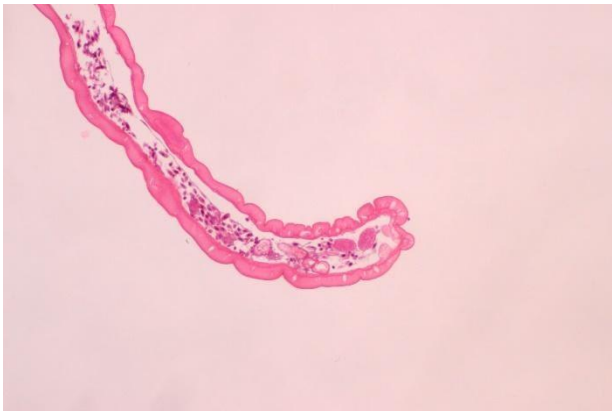
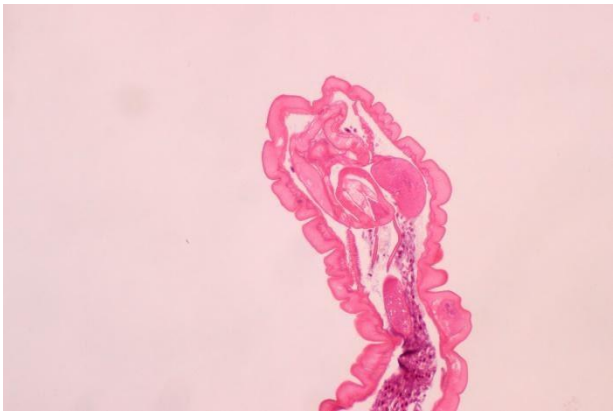
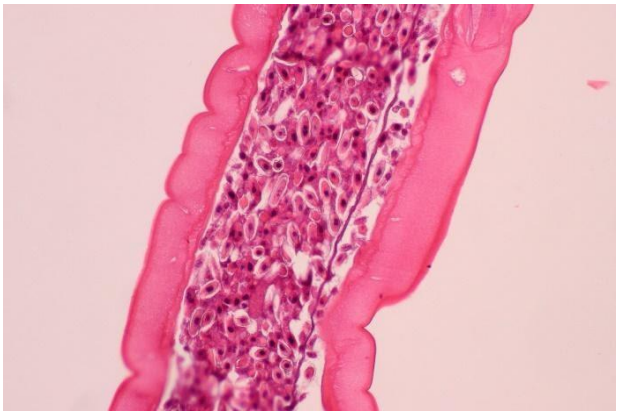




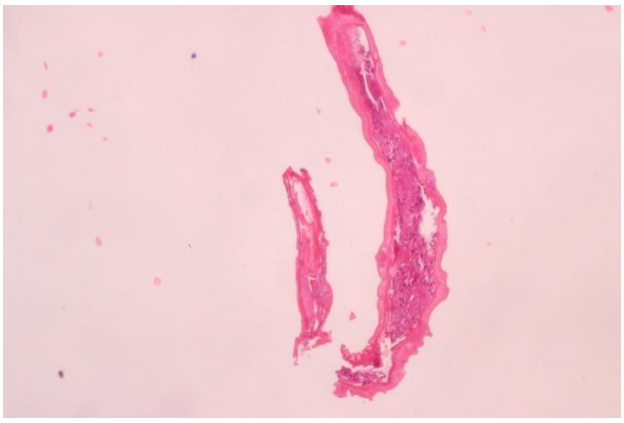
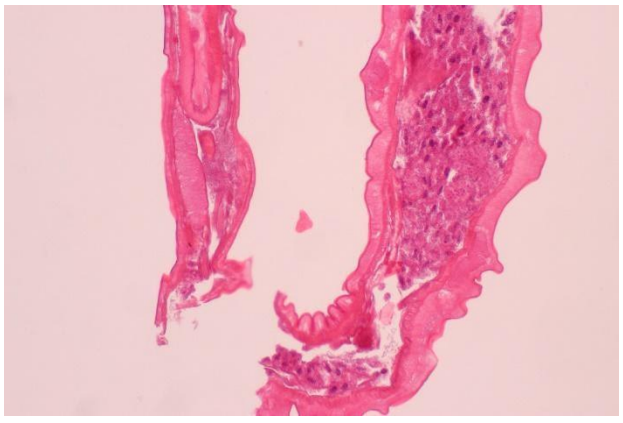




Tapeworm

Spiny Headed Worm

Spiny Headed Worm Continued