AN ABSTRACT OF THE THESIS OF

Fidelis Chow for the degree of Honors Baccalaureate of Science with an option in Pre-pharmacy presented on May 28th, 2014. Title: Retrospective study of the prevalence of *Pseudoloma neurophilia* between zebrafish sexes.

Abstract Approved By: ____

Michael L. Kent

As a major biological model for biomedical research, maintaining healthy zebrafish (*Danio rerio*) is imperative for accurate and consistent lab results. This retrospective study focuses on *Pseudoloma neurophilia*, a microsporidium, because it is the most common pathogen found in laboratory zebrafish. An online database maintained by the Zebrafish International Resource Center (ZIRC) contains data on thousands of zebrafish submitted to their diagnostic laboratory since 2000. Zebrafish samples were examined in order to find sex related patterns, which may point to potential factors that contribute to increased chances of having *P. neurophilia*. Sex, clinical status (sick or apparently healthy), and presence of the infection based on histopathology were analyzed for any potential correlation between sex, *P. neurophilia*, with comparisons between sick and healthy fish. Data compiling resulted in a chi-square test p-value of 0.0045, indicating that there exists a strong correlation that male zebrafish are more often infected with *P. neurophilia*. References suggest a wide variety of causes. However, a main contributor to increased infection may be an increased level of stress in males compared to females. This study serves as an analysis into ZIRC submissions of zebrafish infected with *P. neurophilia* and determining the potential variation in infection rates and degree of severities between zebrafish sexes.

Keywords: Zebrafish, *Pseudoloma neurophilia*, Microsporidia, Xenoma, Sex, Stress, Cortisol
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Retrospective study of the prevalence of

Pseudoloma neurophilia between zebrafish sexes

by

Fidelis Chow

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Fidelis Chow, Author

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Table of Contents

List of Figures

List of Equations

1. Introduction

Zebrafish (*Danio rerio*) offer a wide array of advantages that allow it to become an ideal model organism for laboratory research. Specifically, zebrafish develop a vertebrate structure that bears resemblance to human. In addition, eggs laid by zebrafish appear transparent and exist outside of the female for easy non-intrusive observations of the embryo (Badman et al., 1995). Furthermore, genetic experiments can be applied with relative ease on zebrafish (Grunwald & Eisen, 2002). Zebrafish also possess a quick maturation development cycle (Badman et al., 1995). Thus, the zebrafish acts as an ideal model for many researchers to use for the study of disease and vertebrate development.

The male zebrafish typically have a skinnier appearance along with a more golden ventral side (Schilling, 2002). Female zebrafish tend to be much larger and rounder around the stomach area due to pregnancy, and in addition they also possess silvery-blue stripes on their lateral sides (Schilling, 2002).

Clinical signs of *Pseudoloma neurophilia* include “reduced growth, emaciation, spinal deformation, or low-level mortalities with no grossly visible lesions” (Sanders et al., 2012). Signs may appear only after the zebrafish become subjected to acute stress events such as crowding or shipping (Ramsay et al., 2009). Crowding involves placing zebrafish into a large population environment with little room to allow movement. Shipping involves the handling and transferring of zebrafish into new environments, which may expose the fish to open air and other harsh conditions. Note, that clinical signs of *P. neurophilia* “are not pathognomonic” and may potentially be an indicator of other common diseases of zebrafish, such as mycobacteriosis or capillarid nematode infections (Sanders et al., 2012). *P. neurophilia* may also appear as an asymptomatic and chronic condition that would otherwise be undetectable from an external perspective (Sanders et al., 2012).

Pseudoloma neurophilia possess a high infection rate that can be easily passed to other zebrafish either through infected tissue or spores located in the water (Sanders et al., 2011). Infection can occur through a variety of ways including close proximity and maternal transmission (Sanders et al., 2011). As a result, transmission of *P. neurophilia* is considered the highest among other microsporidia infections with a prevalence rate that could quickly increase from 6% to 77% in a year (Murray et al., 2011). Hence, more than 50% of zebrafish laboratories contain infected fish (Murray et al., 2011). This poses a major problem for laboratory facilities conducting clinical research involving human disease development as *P. neurophilia* can occur asymptomatically in zebrafish and result in skewed lab results.

As a microsporidium, *P. neurophilia* can be very resistant to most sterilization methods such as chlorine egg sterilization (Ferguson et al., 2007). This poses a major issue as laboratories often employ egg sterilization as their main method of retaining healthy zebrafish groups. Currently, the most effective method of handling *P. neurophilia* infections is to use avoidance methods to isolate and contain infected zebrafish from exposing other healthy fish to the microsporidia (Sanders et al., 2011). Early and routine methods of detection also assist in keeping the damage to a minimum in laboratory settings. Thus, a goal of this study is to understand the possible difference in sex when exposed to *P. neurophilia* in order to help further develop other additional methods to reducing infection.

2. Methods

2.1 Collection

The first step in the retrospective study was to extract all the necessary data from the ZIRC database concerning zebrafish with a histopathology result that indicated infections of *P. neurophilia*, microsporidia, and xenoma. The ZIRC database is displayed in a Filemaker Pro format and required re-entering the data into a Microsoft Excel spreadsheet for proper calculation and statistical analysis. Factors noted include case number, facility location, total fish in group, fish ID number, date received, case type, sex, and whether or not histopathology indicated *P. neurophilia*, microsporidia, or xenoma in the case. Case type refers to whether or not the fish were submitted as a routine check or a clinical case. Routine checks or sentinel surveillance involves consistent submissions of zebrafish regardless of health status. Routine monitoring allows for not only timely detection of *P. neurophilia*, but also includes “the detection of other pathogens and the monitoring of the overall health of the colony” (Kent et al., 2009). Histological examinations of routine zebrafish submissions allow for detection of novel pathogens that otherwise could not be specifically found through specific PCR-based assays (Kent et al., 2009). Sentinel samples acted as “a very effective means to monitor microsporidian infections in laboratory colonies” (Sanders et al., 2012). The process involves exposing two populations of zebrafish to each other: a known healthy group and an unknown group. If an infection occurs during or after the exposure, then the results would indicate that there exists an infected fish within the group (Sanders et al., 2012). A more specific submission method in *P. neurophilia* detection is the clinical submissions.

Zebrafish that appeared sickly or originated from tanks that have experienced high mortalities were specifically submitted for examination as a clinical case. Furthermore, three specific searches using the key words *Pseudoloma*, microsporidia, and xenoma were used, which resulted in three different spreadsheets. These words were chosen for their related properties to *P. neurophilia*. Specifically, *P. neurophilia* is classified as a "microsporidia" (Sanders et al., 2012). Furthermore, xenoma is a condition caused by microsporidia where "hypertrophic growth of the invaded cell of the host animal" can occur (Lom et al., 2005). Therefore, the term "xenoma" in the database was an indication of *P. neurophilia* infection.

2.2 Analysis

Once all the data was consolidated into the excel spreadsheet, all three search categories were combined and duplicate case entries were removed. Each factor column was totaled and converted to percentages for comparison between the different searches. The results were compared and analyzed for any potential patterns that may lead to an understanding of how to decrease cases of *P. neurophilia* and improve zebrafish husbandry. Associations of types of infection (neural or somatic) muscle and occurrence of clinical disease were evaluated for comparison between sex using statistical analyses as recommended by Dr. Tom Sharpton and Dr. Lan Xue of the Statistics department. Statistical methods include using the chi-square test and Z-test for evaluation to examine the potential statistical significance and correlation of the results. For comparison and statistical calculations, data from microbiology graduate, Benjaporn Somridhivej, was used to calculate the total percentage of all case type and sex in the ZIRC database. Expected values were adjusted according to the percentage of male and female zebrafish in the entire database (including zebrafish without *P. neurophilia* infections) in order to prevent a skewed comparison of the positive cases of *P. neurophilia* due to unequal distributions of male and female zebrafish. Conclusions were drawn based on the results and literature relevant to zebrafish treatment and *P. neurophilia* pathology.

3. Results

3.1 Total population of zebrafish in ZIRC database

Calculation of the total population of zebrafish in the ZIRC database revealed a nearly 1:1 ratio of male zebrafish to female zebrafish. Male zebrafish held the majority in the routine group category and female zebrafish outnumbered the male zebrafish in the clinical group category. However, the overall difference between each group was very small with male zebrafish claiming 50.44% of the total routine population and female zebrafish consisting of 52.13% of the clinical population.

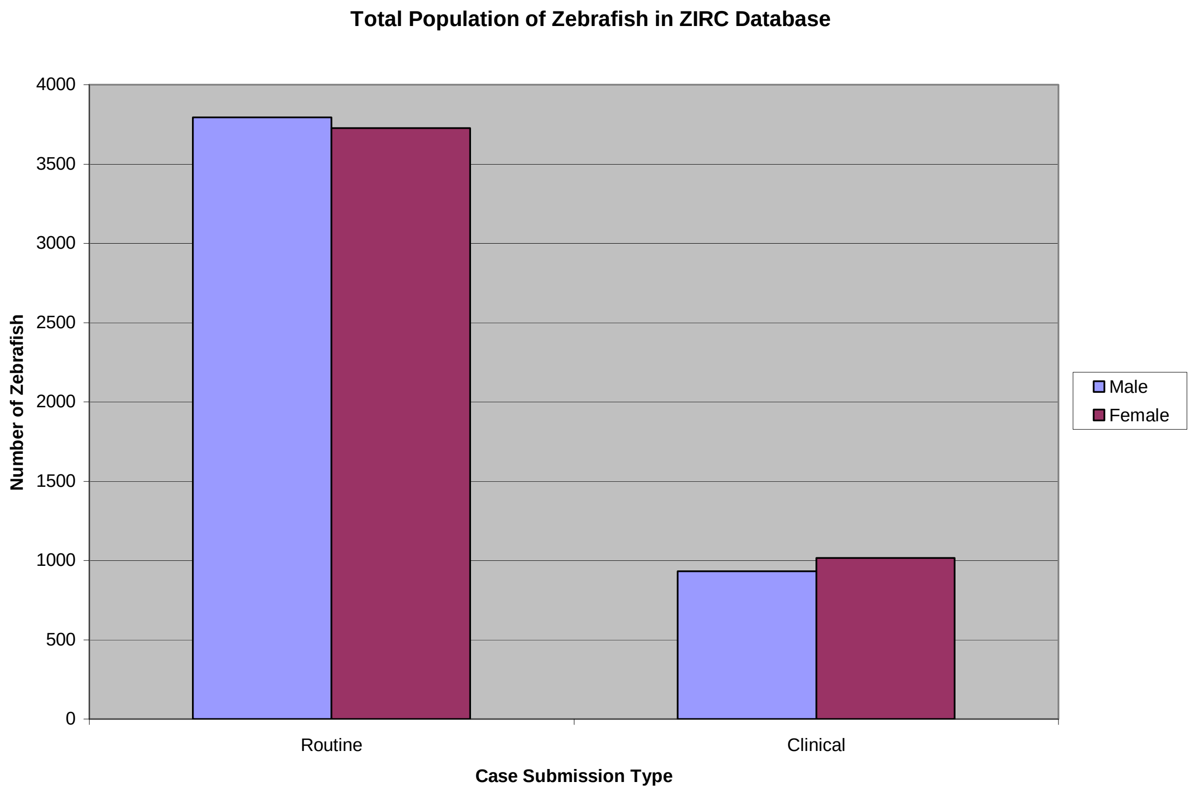

Figure 1: *Danio rerio.* Population data represented the total amount of submissions overall that existed within the ZIRC database. Total population was compiled based on the results and findings of Benjaporn Somridhivej. Cases were split between routine monitored zebrafish and clinically diseased zebrafish in addition to further separating them into male and female subsections. The following numbers of zebrafish were found: male routine was 3793, female routine was 3727, male clinical was 933, and female clinical was 1016.

3.2 Adjusting values for fair comparison

In order to properly compare the two sexes, it was necessary to scale the expected values to a percentage value that reflected the general trend of male to female zebrafish submissions.

$$Adjusted\ expected\ value = \frac{Total\ specific\ routine + Total\ specific\ clinical}{Total\ population} \times Total\ positive\ cases$$

Equation 1: Values were adjusted according to the total population ratios of male and female zebrafish before calculation of the p-value. Total routine values and total clinical values for male or female were added, and then they were divided by the total population for a percentage. They were then scaled to the number of actual positive cases and became the expected values for the chi-square test and Z-test.

3.3 Calculation values used in chi-square test

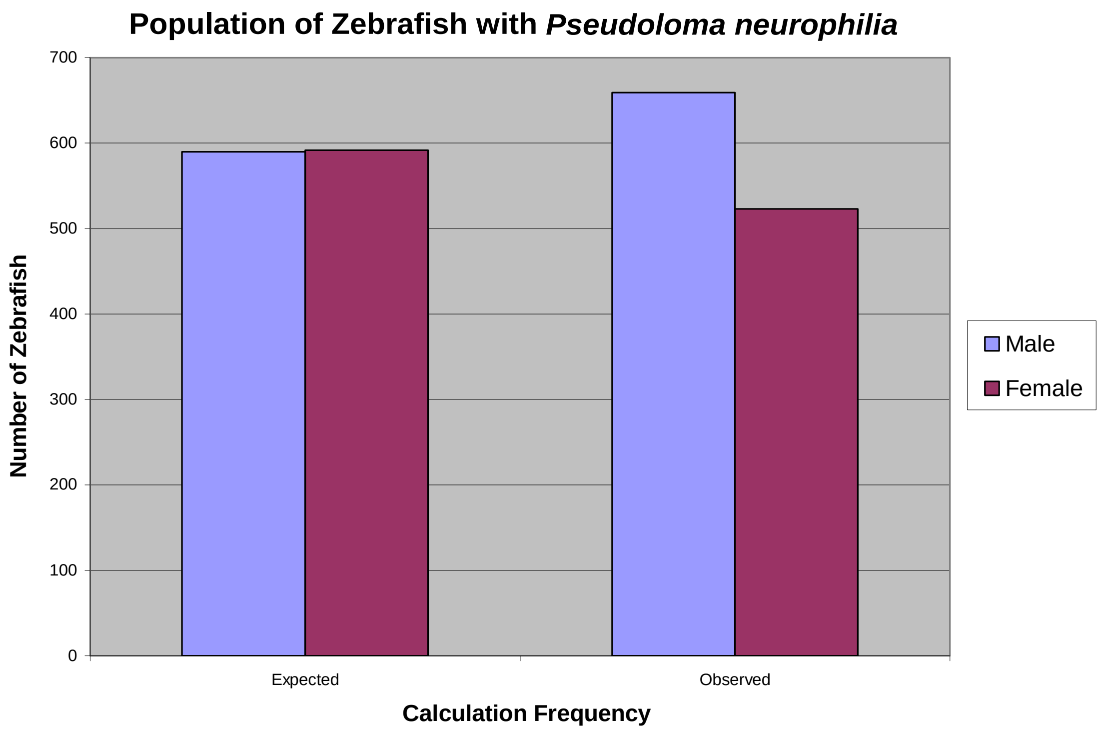

Figure 2: Calculated values used in the chi-square test and Z-test. The expected values contained 590 males and 592 females noted to be positive for *P. neurophilia*. The observed values contained 659 males and 523 females. Total positive cases of *P. neurophilia* were 1,182 fish out of 9,469 zebrafish submissions.

3.4 Calculating Chi-square Test

$$X^2 = \sum \frac{(observed - expected)^2}{expected}$$

Equation 2: Chi-square test used in calculation of p-value for determination of significance with the use of data from Figure 2. P-value calculation was 0.0045 with a two-tailed value.

3.5 Calculating Z-test

$$Z_{observed} = \frac{\hat{P}_m - P_{m,e}}{\sqrt{\frac{P_{m,e}(1 - P_{m,e})}{n}}}$$

Equation 3: Z-test used in calculation of p-value for determination of significance between observed and expected male zebrafish data from Figure 2. P-value calculation was 5.97×10^{-5} with a two-tailed value.

3.6 Percentage comparison of positive Pseudoloma neurophilia sex

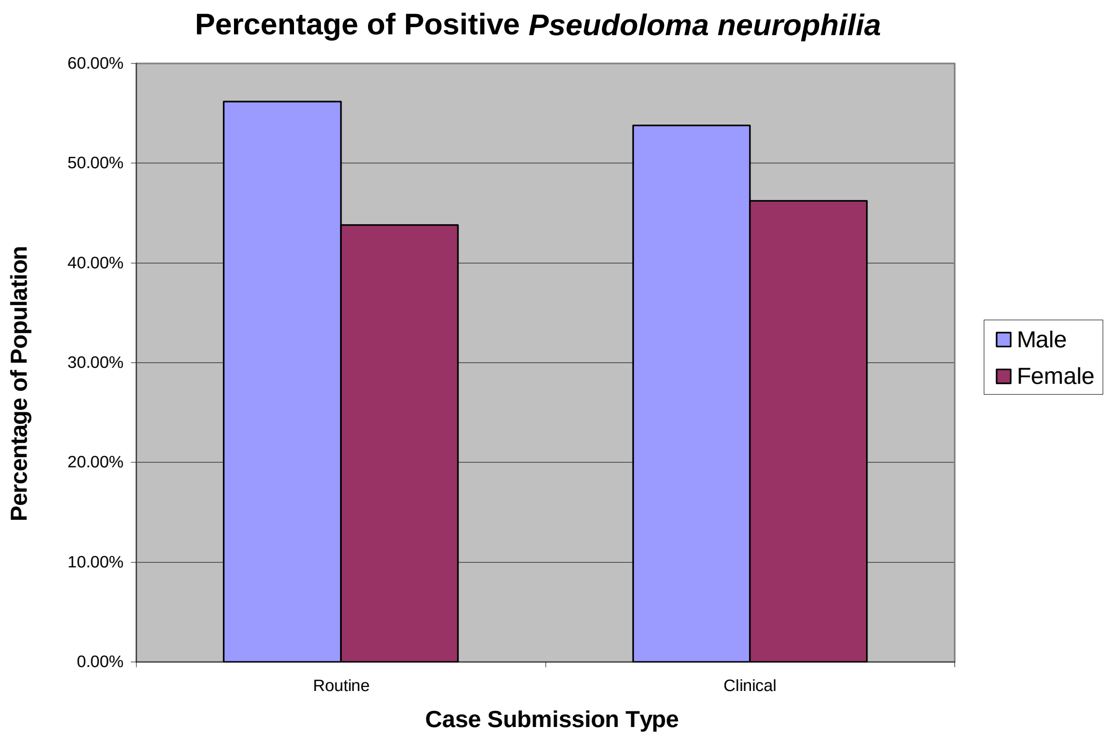

Figure 3: Percentage of positive *P. neurophilia* cases showed male zebrafish having greater number of *P. neurophilia* infection. Male routine was 56.19%, female routine was 43.81%, male clinical was 53.77%, and female clinical was 46.23%.

4. Discussion (Explanation & Interpretation)

Based on the results, male zebrafish had a higher tendency to be infected with *P. neurophilia* than female zebrafish. In the routine and clinical cases, male zebrafish held the majority of the population for positive cases of *P. neurophilia* with the percentages 56.91% and 53.77%, respectively. The chi-square test indicated a strong correlation between male zebrafish having a greater rate of infection for *P. neurophilia* than female zebrafish with a p-value of 0.0045. In addition, the Z-test result indicated that, statistically, infected populations were more likely to have an unequal distribution of *P. neurophilia* between sexes based on a p-value of 5.97×10^{-5}. Furthermore, a total population submission of nearly ten thousand fish provided a very large and reliable data set for conducting statistical analyses. An issue that was considered during this research is the potential of having an unequal distribution of zebrafish sex for comparison. However, due to the data provided by Benjaporn Somridhivej in the Kent Laboratory, the average sex population in the ZIRC database was nearly equal between male and female. The equal distribution of male and female among the general population of zebrafish in ZIRC has shown that the results of the calculation were done fairly with no bias due to unbalanced sex ratios in the overall data set. In addition, calculations were purposely scaled to reflect any potential difference between male and female zebrafish submissions.

4.1 Stress and cortisol leads to Pseudoloma neurophilia

Pseudoloma neurophilia, like many other microsporidia, can infect a host through a variety of ways. Coming into close contact with infected tissue can lead to infection of *P. neurophilia*, but many fish experience asymptomatic infections (Ramsey et al., 2009).

Inducing stress on a living organism has been known to help facilitate a more accessible environment for microsporidia. Stress plays a key role in controlling the physiological balance of organisms when exposed to threatening environments (Pottinger et al., 1995). The result of being exposed to stress causes cortisol to be released from the adrenal tissues. Excessive cortisol suppresses the immune system, which increases the likelihood of *P. neurophilia* infecting the host (Pottinger et al., 1999; Ramsay et al. 2009). Thus, the results of the study may indicate that males experience greater levels of cortisol when exposed to a stressful event, such as crowding or shipping, in comparison to females. This may ultimately lead to infection of *P. neurophilia*.

As noted, zebrafish in laboratory settings are often subjected to environments that result in stress responses. Crowding and shipping in large numbers can cause many internal physiological changes in zebrafish as a response to the environment, mainly in the form of releasing cortisol into the bloodstream. Several reports indicate that increasing stress and cortisol levels lead to increased severity of infections in fish (Kent & Hedrick, 1987, Maule et al., 1989, Saeij et al., 2003). Essentially, exposure to stressful events has a positive correlation with increases in degree of severity of microsporidian infections such as *P. neurophilia*. Most importantly, Ramsey et al. (2009) showed that crowding stress, leading to increased cortisol, increased the severity of *P. neurophilia* infections in zebrafish.

4.2 Stress levels of adult males to adolescents

Cortisol levels can vary based on a variety of physical conditions in the organism, which include age and sex. Based on age and maturity factors, organisms such as mature male rainbow trout experience lower levels of cortisol compared to immature rainbow trout when exposed to a stress event (Pottinger et al., 1995). While the cause is still unknown, evidence shows that a change in pituitary response and reaction may be the true cause of the phenomenon (Pottinger et al., 1995). As zebrafish age and develop into adults, many internal changes are occurring in their bodies and that may result in similar effects as the rainbow trout in Pottinger's experiment. The increase in cortisol levels of immature fish would result in a stress response that will lower the immune system's capabilities. Although, the age of the zebrafish was not noted during this retrospective study, based on general observations, the zebrafish that were submitted had an age range of around half a year to a few years old, and thus were all sexually mature. Pottinger's use of rainbow trout as the model organism prevents direct age comparison of when stress response differences occur between young and adult male zebrafish. However, this coincides with another research that suggests "young zebrafish are particularly susceptible to *P. neurophilia*" (Ferguson et al., 2007).

4.3 Crowding, dominance hierarchies, modes of transmission and effects of sex

In terms of direct sex differences in stress response, there have been studies that support the hypothesis that male and female organisms respond to stress and environments differently. An experiment conducted by Brown and Grunberg (1995) showed that male rats experienced a greater increase in stress from crowded environments in comparison to female rats. A common characteristic in many male species is that many display aggressive behavior toward other males. In a crowded environment with many other similar animals, such as zebrafish held in research laboratories, this could result in an increased level of stress for a male in comparison to the more social female (Brown and Grunberg, 1995). Conversely, female rats were shown to experience increased stress when isolated in comparison to crowded environments (Brown and Grunberg, 1995). Zebrafish used in research are held in relatively small aquaria (Harper and Lawrence, 2011). This variation in stress response between male and female rats provides one explanation why male zebrafish are more susceptible to *P. neurophilia* than females. If an increase in stress reduces the capabilities of the immune system, then an increase in *P. neurophilia* would occur. Dominance hierarchies (Spence et al., 2008), and subordination in zebrafish communities would also more likely cause stress and show higher cortisol levels. However, this hierarchy includes both males and females, although studies have suggested some differences in dominance behavior between sexes (Paull et al., 2010). Female zebrafish, for example, have been noted to be less aggressive overall towards subordinates and prefer to display their dominance through non-physical methods such as waterborne pheromones (Paul et al., 2010). However, established dominance in zebrafish hierarchies is not determined by only sex traits (Paul et al., 2010). Thus, it is not known if subordinate males have particularly higher cortisol than subordinate females. Indeed, Ramsay et al. (2006) found no differences in elevated cortisol between males and females in a crowding experiment.

Pseudoloma neurophilia is transmitted by various routes. Horizontal transmission occurs by feeding on spores or infected tissues, and it is also vertically transmitted within the egg (Sanders et al., 2013). Pathogens which are transmitted vertically are generally less virulent than those which are transmitted horizontally as it is important for the infected female to live to reproductive age and pass the infection on to progeny (Ewald, 1987). As *P. neurophilia* is vertically transmitted only in females, one would suspect that the parasite is more virulent in males, in which only horizontal transmission occurs. This did not appear to be the case in my study because the ratio of infection between males and females that were healthy was very similar to that in fish with clinical infections.

4.4 Odors of death effects

Whereas zebrafish may not possess advance cognitive traits compared to mammals, they are still capable of enacting defensive behavior when presented with a threatening or hostile situation. In an experiment conducted by Oliveira (2014), several zebrafish were gathered and examined for whole-body cortisol measurements after introduction of dead conspecifics. The experiment theorizes that the chemical breakdown of decaying flesh in a crowded environment will give rise to a stress response and switch to defensive behavior from the zebrafish placed in the same environment (Oliveira et al., 2014). Oliveira's experiment proves that zebrafish exhibit a defensive behavior and state of alertness when exposed to a nearby dead zebrafish, which also leads to an increase in stress and release of cortisol into the body (Oliveira et al., 2014). This concept may play an adaptive role in zebrafish because detection of a decomposing corpse relays important information that the area may pose a threat whether a predator or an environmental hazard is present (Yao et al., 2009). It should be noted that dead zebrafish are rapidly torn apart and eaten by tank mates. Thus, the presence of dead or moribund zebrafish may result in increased levels of cortisol in tank mates, enhancing or allowing *P. neurophilia* and other pathogens to infect, especially if the deceased subject carries the microsporidium.

4.5 Source of errors/Confounding factors to consider:

During the data extraction, some traits were not logged into the spreadsheet. Specifically, fish age, laboratory name, and behavior were not noted in the study. As stress can be attributed to a variety of causes, it becomes important to note a wide variety of traits that may be confounding variables. However, due to time constraints and placing emphasis and focus on the role sex plays in *P. neurophilia* infection, the study required a scale down of the data collection process. Another issue to consider was the lack of documentation and details in many of the categories in the ZIRC submission forms. Many of the earlier submissions to ZIRC often lack pertinent information such as age, sex, and noting the various different treatments and environmental details of that particular zebrafish group. Different facilities have different methods toward zebrafish husbandry which may have affected the behavior and health of the zebrafish along with incomplete documentation. As a result, some of these entries had to be removed from the final count and calculation of the data, although the number of case submissions that lack full documentation consisted only of a very small portion of the overall submissions. Moreover, some large facilities submitted significantly more fish than others, and the role of facilities as a confounding factor was not investigated in this study. For example, although the overall database was about 50:50 ratio for males and females, a particular large facility, with an overrepresentation of samples, could have had a particularly high percentage of males and a high incidence of the infection. However, a colleague from Kent lab, Dr. Sean Spagnoli, is conducting a retrospective pathology description using essentially the same data set, and has identified source laboratories as a confounding variable. This is conceivable, because many non-genetic factors, such as temperature, can affect the phenotypic expression of sex in zebrafish.

5. Conclusion

Evaluation of a large number of zebrafish infected with *P. neurophilia* has shown that males are more susceptible to *P. neurophilia* than females, and results were statistically significant at $p<0.05$. The exact reason behind this increase in prevalence has yet to be precisely elucidated. It can still be attributed to a variety of causes. Specifically, increased stress likely acts as one of the major factors that allow for an increased spread of *P. neurophilia*, and it is possible that male zebrafish experience greater levels of stress, resulting in some fish with higher cortisol levels, and consequently have greater vulnerability to *P. neurophilia*.

A major issue with *P. neurophilia* is that "there is currently no effective treatment for *Pseudoloma neurophilia* infections in zebrafish" (Ramsey et al., 2009). Thus, the most effective treatment method for *P. neurophilia* is to use avoidance and select zebrafish strains that bear low stress responsive characteristics in order to reduce the chance of possible *P. neurophilia* infection. Crowding conditions can also bring about a stressful environment and the number of zebrafish placed in a single aquarium space should be considered to allow the zebrafish to experience a less hostile and confined environment. Finally, regular checks and maintaining the condition of the zebrafish through constant monitoring for infection and removal of dead material from the environment will assist in reducing the number of *P. neurophilia* cases.

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