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# Retrospective study of the prevalence of *Pseudoloma neurophilia* shows male sex bias in zebrafish *Danio rerio* (Hamilton-Buchanan)

**F W Chow**[1], **L Xue**[2], and **M L Kent**[1]

[1]Department of Microbiology, Oregon State University, Corvallis, Oregon, USA

[2]Department of Statistics, Oregon State University, Corvallis, Oregon, USA

[3]Department of Biomedical Sciences, Oregon State University, Corvallis, Oregon, USA

The use of zebrafish *Danio rerio* ((Hamilton-Buchanan) is a model in biomedical research has greatly expanded in the last 20 years (Phillips & Westerfield 2014). As with any laboratory animal, underlying infections or diseases in zebrafish may impact research beyond merely causing morbidity or mortality (Kent, Wolf and Harper 2013). The microsporidium, *Pseudoloma neurophilia*, (Microsporidia) is the most commonly detected pathogen in zebrafish research colonies (Sanders, Watral & Kent 2012), probably associated with its ability to be transmitted both horizontally and vertically (Sanders & Kent 2013). *Pseudoloma neurophilia* infections are associated with reduced growth, emaciation, spinal deformation, but subclinical infections are common (Sanders et al. 2012). Ramsay, Watral, Schreck & Kent (2009) showed that crowding induced more severe infections, and we are interested in other factors that may influence severity or prevalence of *P. neurophilia* infections. Zebrafish exhibit considerable variation in sex distribution with amongst populations, which may be influenced by both parental genetics and epigenetic factors, such as temperature (Liew, Bartfai, Lim, Sreenivasan, Siegfried & Orban 2012).

The National Institutes of Health (NIH) Zebrafish International Resource Center (ZIRC) at the University of Oregon, Eugene, Oregon has been providing a diagnostic service to the zebrafish research community since 1999. The service uses histopathology of whole fish as its primary diagnostic method. One of us (M.K.) is a member of the service, and hence we had available some 10,000 fish in histological slides to conduct a retrospective study on the role of sex in this common infection.

The first step in the 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 database is displayed in a FileMaker Pro (FileMaker Inc., Santa Clara, California.) format and required re-entering the data into a Microsoft Excel spreadsheet for proper calculation and statistical analysis. Factors noted include 1) case number, 2) facility location, 3) total fish in group, 4) fish identification number, 5) date

Correspondence: M.L. Kent, Department of Microbiology, 220 Nash Hall, Corvallis, Oregon, 97331. USA. Michael.Kent@oregonstate.edu.

received, 6) case type – clinical or routine 7) sex and 8) presence of infection by *P. neurophilia*. For the latter, terms such as "microsporidia", "xenoma" or "*Pseudoloma*" were used to identify infected fish. The database was closely examined to assure that positive fish were not scored more than once. After all data were consolidated into the excel spreadsheet, all three search categories were combined and duplicate case entries were removed. A few fish were infected with *Pleistophora hyphessobryconis*, rather than *P. neurophilia*, and these were removed from positive categories as well. Zebrafish from sentinel programs or healthy-appearing fish that were submitted from other tanks within systems for pathogen screening were classified as routine cases. The clinical category included fish submitted with outward signs of disease.

The total number of fish that we included in our analysis was 9,469 (Table 1). The sex ratio was close to 50:50, with 4,726 males and 4,743 females. One intersex fish and a few fish without gonads were not included in the study. Regarding the "routine" fish (n = 7,520), there were slightly more males than females (50.44%) in this category. Total number of clinical submissions, regardless if they were infected or not with *P. neurophilia*, in which sex could be determined was 1,959 and 52.13% of these were females.

About 1,100 fish were positive for *P. neurophilia*, representing 12.5% of the fish. Associations between sex and prevalence of infection in the fish submitted as routine or clinical and combined data were evaluated with Pearson's Chi-squared test (Agresti 2012). In order to properly compare the two genders, it was necessary to adjust the expected values to a percentage value that reflected the gender distribution within the overall data set. 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 Pearson's Chi-squared test. Based on the sex ratios in the total was 69 more infected males than expected (Table 1; Fig 1). These results were statistically significant from the expected values with a two-tailed value with the Chi-square test ($p = 0.0045$).

Further evaluation of the infected fish by health status showed that there were about 4–5 times more infected fish in routine cases than in those submitted with clinical disease (Table 1), consistent with the overall numbers of fish in these two categories. As seen in the combining both categories, about a 12.4% and 7.5% higher prevalence in males were observed in routine and clinical cases, respectively, than was predicted. Chi-square tests of routine and clinical cases reveal contrasting results; there was a statistically significant increase in infection in males with in the routine category ($p = 0.0003$), but not in the clinical category ($p = 0.0738$).

The fish in the database came from 109 laboratories. We evaluated the possibility of a laboratory source as a confounding factor using the Cochran-Mantel-Haenszel test (Agresti 2012) – e.g., some laboratories with high prevalence of infections may have had biased sex ratios. Analysis included the five laboratories that submitted the most fish (n = 672 infected fish). We found no statistical influence of laboratory ($p = 0.284$), and source of submission were therefore not considered a confounding variable.

With a data base of over 9,500 fish samples and over 1,000 infected individuals, we clearly showed here that there was a bias of infection in males. Previous studies have demonstrated sex bias relating to prevalence of parasite infections. Schalk & Forbes (1997) summarized the findings of numerous examples in which there was a male bias to infection in mammals, and Poulin (1996) extended this review to birds and fish. Potential causes of male sex bias due to parasitism include hormone differences (Schalk and Forbes 1997), as androgens may depress immunity, while estrogen may stimulate humoral and cell-mediated immunity (Schuurs and Verheul 1990). Fitness-related difference between sexes may also be important (Nunn, Lindenfors, Pursall, & Rolff 2009).

As with *Loma salmonae*, a common microsporidium of salmonid fishes (Lovey, Speare, Stryhn, & Wright 2008), we previously showed that crowding stress and associated elevated cortisol were associated with more severe *P. neurophilia* infections in zebrafish (Ramsay et al. 2009). Perhaps males experience greater levels of cortisol when exposed to stressors in comparison to females, which may ultimately lead to more infections by *P. neurophilia*. Brown & Grunberg (1995) showed that male rats experienced a greater increase in stress from crowded environments in comparison to females, but we did not see gender differences in cortisol levels in crowded zebrafish (Ramsay, Feist, Varga, Westerfield, Kent, & Schreck 2006). A common characteristic in many males in many species is that they 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 & Grunberg 1995). Recent studies have demonstrated that the zebrafish in aquaria establish hierarchies, which is more pronounced in males (Spence, Gerlach, Lawrence & Smith. 2008; Paull, Filby, Giddins, Coe, Hamilton & Tyler 2010). Filby, Paull, Barltett, Van Look, & Tyler (2010) showed that subordinate males exhibited a greater rise in cortisol than subordinate females, providing one possible explanation of the higher prevalence in males.

In conclusion, evaluation of a large number of zebrafish infected with *P. neurophilia* showed that males are more susceptible to *P. neurophilia* than females. The exact reason behind this increase in prevalence has yet to be precisely elucidated, but differences in response to stress and hierarchy structures between males and females is a plausible explanation.

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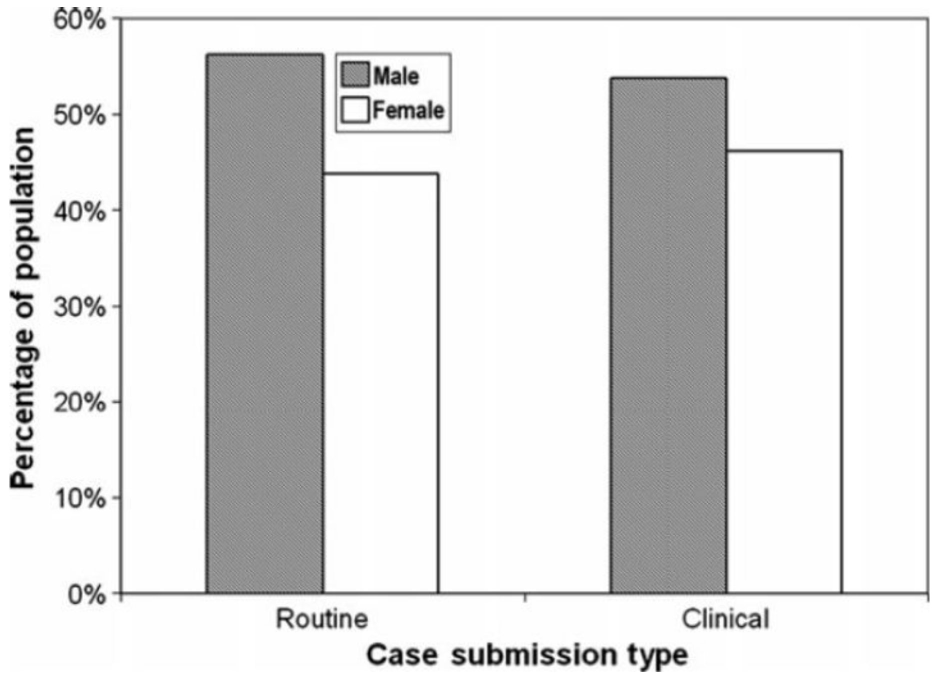


**Figure 1.**

Distribution of *Pseudoloma neurophilia* in routine and clinical cases from zebrafish laboratories based on sex.

**Table 1**

Distribution of *Pseudoloma neurophilia* in zebrafish from 109 research facilities from 2000–2013. () = expected values based on ratio of male to female in infected and total populations.

| Case Type | Sex | *Pseudoloma neurophilia* | |
|---|---|---|---|
| | | Positive | Negative |
| Total | Male | 659 (590) | 4,067 |
| | Female | 523 (592) | 4,220 |
| Routine | Male | 545 (489) | 3,248 |
| | Female | 425 (481) | 3,302 |
| Clinical | Male | 114 (101) | 819 |
| | Female | 98 (111) | 918 |