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# **The Effects of Sex on Zebrafish Bone Metabolism**

Simon Bagatto

## **Introduction**

Zebrafish, *Danio rerio*, are used as model vertebrates in research because they have a short maturation time and can easily breed in a controlled environment. Zebrafish have clear embryos that are easy to manipulate genetically. The zebrafish genome has been sequenced and has significant similarities to the human genome, which allows researchers to study zebrafish with the goal of understanding human diseases (Howe et al. 2013). Specifically, this project investigated bone metabolism of zebrafish between males and females. This project occurred alongside a project testing for bone metabolism differences with a high-calorie diet or a low-calorie diet.

Zebrafish are bony fishes, that descended from the same fish ancestor as tetrapods (Vollf et al. 2005). Through evolutionary relatedness and many genomic similarities, zebrafish can be studied in many research topics concerning humans, such as immunity or metabolism. Zebrafish scales are composed of much of the same material as their skeleton, including a significant calcium content (de Vrieze et al. 2014). Once a scale is removed, the zebrafish will grow another scale to replace it, and the removed scale can be analyzed for its metabolism without sacrificing the fish (de Vrieze et al. 2014).

To measure bone metabolism in zebrafish scales, two similar methods are used. One procedure is the Tartrate-Resistant Acid Phosphatase (TRAP) assay. The TRAP assay measures enzyme activity of osteoclasts, cells that destroy or resorb bone (Yang et al. 2018). Another method, the alkaline phosphatase (ALP) assay, measures enzyme activity of cells that build bone,

known as osteoblasts (Sabokbar et al. 1994). These two assays were used to determine the bone metabolic activity in zebrafish scales and analyzed for any difference based on sex. Additionally, these two assays were used for the high-calorie and low-calorie diet experiment. This project determined if zebrafish sex interacts with diet effects on bone metabolism.

In human males and females, sex hormones can have significant effects on bone metabolism. Human females produce higher levels of estrogen, while human males produce higher levels of testosterone. Estrogen has a strong association with inhibiting bone resorption (Syed and Khosla 2004). Human females typically have rapid bone loss once they reach menopause because the sex hormone estrogen is produced at a much lower rate (Syed and Khosla 2004). Human males, however, produce smaller amounts of estrogen compared to females, however estrogen is still integral to male bone metabolism due to its inhibition of bone resorption, which prevents bone loss (Syed and Khosla 2004). Thus, human females are much more susceptible to a net bone loss after menopause. To further assess bone metabolism differences, this experiment tested the bone metabolism of adult male and female zebrafish to provide implications for bone metabolism conditions in humans.

## **Methods**

At the start of the experiment, 40 adult zebrafish at 3 months post-fertilization were sorted by sex (20 male and 20 female zebrafish). Ten of each sex were given either a high-calorie diet or a low-calorie diet. The high-calorie diet fish were fed three times daily while the low-calorie diet fish were fed two times per week. All fish were fed with Ziegler Adult Zebrafish Diet

(Ziegler Bros., Gardner, PA). The fish received this diet for a 5-week period prior to my conducting either the TRAP scale assay or the ALP scale assay.

At the conclusion of the diet treatment, fish were anesthetized by individually submerging them in 0.02% tricaine methane-sulfonate (MS-222), and their length and weight were recorded. Ten scales were removed from each fish above the pectoral fin using forceps, and the scales were placed in a phosphate-buffered saline (PBS, pH = 7.4) solution to prevent the scales from drying (Pasqualetti et al. 2012). After the scale removal, the fish were sacrificed and dissected to confirm sex. Shortly after removing the scales from the fish, the scales were individually separated under a dissecting microscope, and added to a well plate. A fluorescent substrate, 4-Methylumbelliferyl phosphate (MUP), was used as an indicator of enzyme activity in the scales. MUP was measured and added to the ALP and TRAP buffers to create a 5 millimolar final concentration. PBS was removed from each well and replaced with either the ALP or TRAP buffers with the substrate. The TRAP buffer was used for detecting the enzyme tartrate-resistant acid phosphatase, whereas the ALP buffer was used to detect the enzyme alkaline phosphatase (Sabokbar et al. 1994; Yang et al. 2018). The MUP substrate was converted to a fluorescent product that has an exciting wavelength at 360 nm and an emitting wavelength at 440 nm. After adding the buffers and the substrate, the scales were homogenized with a Turrax homogenizer (Biospec Products Tissue Tearor, Bartlesville, OK). The scale solutions were incubated at 28°C for two hours, a temperature that is optimal for zebrafish physiological functions (Pasqualetti et al. 2012). At the end of the incubation, a fluorescence spectrometer was used to record fluorescence in each scale. These data provided an indication of any differences based on sex and diet, and whether more activity is occurring with osteoblasts or osteoclasts.

After measuring each scale's area through imaging software, standard curves were created for TRAP and ALP to normalize the varying areas of the scales. This was done by mixing shrimp alkaline phosphatase enzyme with MUP and either the TRAP or the ALP buffer. This solution started at a 7.8 mM concentration and was subsequently diluted by 10 mM six times, with the final dilution having a concentration of 0.0000078 mM. The dilutions were performed twice for each of the enzyme buffers to obtain two solutions for each concentration. The average of the fluorescence data at each concentration was used for the creation of the standard curves, which normalized the scale area variation.

Lastly, the number of resorption lacunae (products of osteoclast activity) in the high-calorie and low-calorie groups were recorded via calcein staining to provide further indication of significant differences in osteoclast activity. All of the data were compared using a repeated measures ANOVA statistical test to first determine if there is any difference between the experimental groups, then if there is a difference between the sex or diet experimental groups, and finally if sex and diet interact. The repeated measures ANOVA test was performed separately for the TRAP assay and the ALP assay.

## **Results**

The 5-week diet resulted in an increase in the average zebrafish weight for the high-calorie diet group and a decrease in the average weight for the low-calorie diet group. The high-calorie diet zebrafish had an average weight increase of approximately 0.2 g while the low-calorie diet zebrafish had an average weight decrease of approximately 0.1 g (**Figure 1**). In both

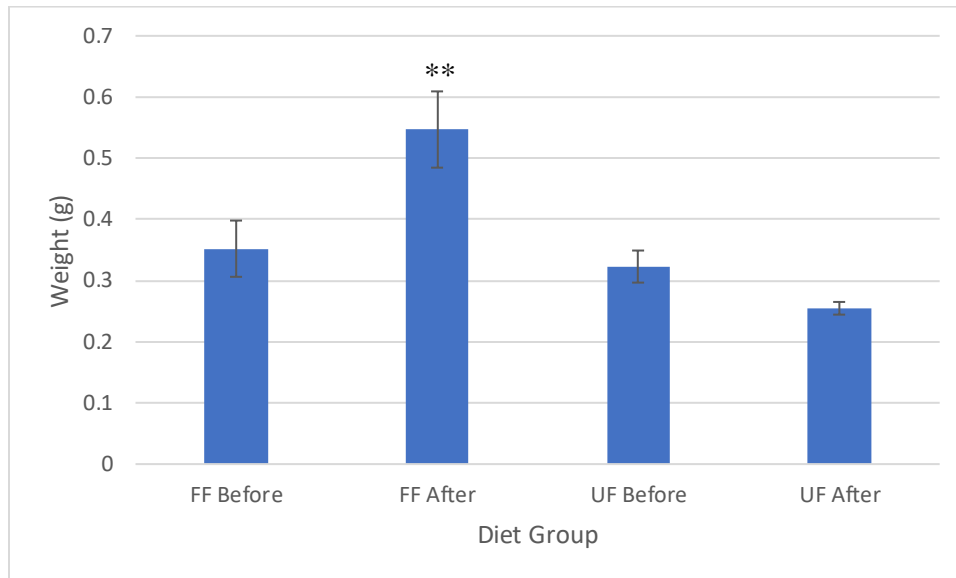
diet groups, the average length of the zebrafish increased by approximately 7 mm after the 3-week diet (**Figure 2**).

The results of the TRAP and ALP scale assays and the repeated measures ANOVA showed that the TRAP activity per square mm of scale was not significantly different for males and females (**Figure 3**). However, TRAP had a significantly higher activity per square mm of scale in the low-calorie diet fish ( $p = 0.014$ , **Figure 4**). This indicates that the low-calorie diet causes an increased osteoclast activity. Additionally, the ALP activity per square mm of scale had no significant differences for both the sex and diet groups of the zebrafish (**Figures 5, 6**). This indicates that osteoblast activity was not significantly affected by the high-calorie diet or the low-calorie diet, and neither sex was significantly affected.

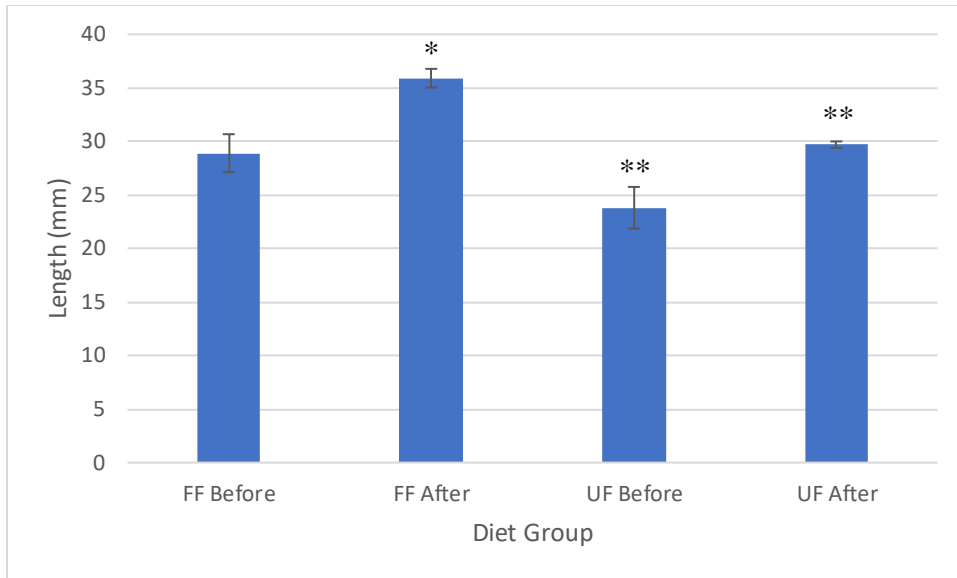
Many of the scale assays were performed using frozen scales, which had decreased enzymatic activity (**Figure 7**). The enzymatic activity between fresh scales and scales frozen for one day was not significantly different. While there was a significant decline in enzyme activity between one day of freezing and one week of freezing, the enzymatic activity of the scales was not significantly different between one, three, and four weeks of freezing. Since a large number of scales were used in the assay, this allowed the scales to be used at later time periods, rather than immediately upon removal. The enzymatic activity of the fresh and frozen scales was measured by the ALP assay only.

Resorption lacunae of the fish scales were also measured as another indicator of osteoclast activity. The lacunae were measured both by the number of lacunae per scale and the number of lacunae per area of scale after calcein staining. The number of lacunae per scale was significantly higher in the low-calorie diet group ( $p = 0.0191$ , **Figure 8**). Thus, osteoclast activity

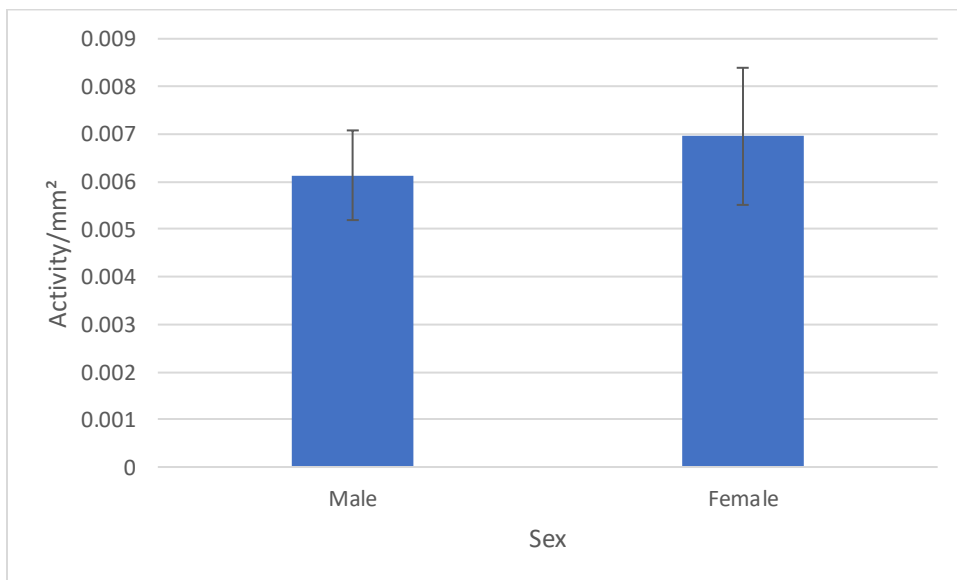
and the number of lacunae are higher in the low-calorie diet group while there were no significant differences between the sexes in osteoblast activity.



**Figure 1.** The average weight of both sexes in each diet group before and after the 5-week feeding experiment. There was no significant difference between the total weight of the fish before the experiment ( $p = 0.5823$ ). After the feeding experiment, the male fed fish had significantly more weight ( $p = 0.0011$ ). The females in the fed fish group had significantly more weight after feeding ( $p = 0.0387$ ). FF = Fed Fish (High-calorie diet), UF = Unfed Fish (Low-calorie diet).

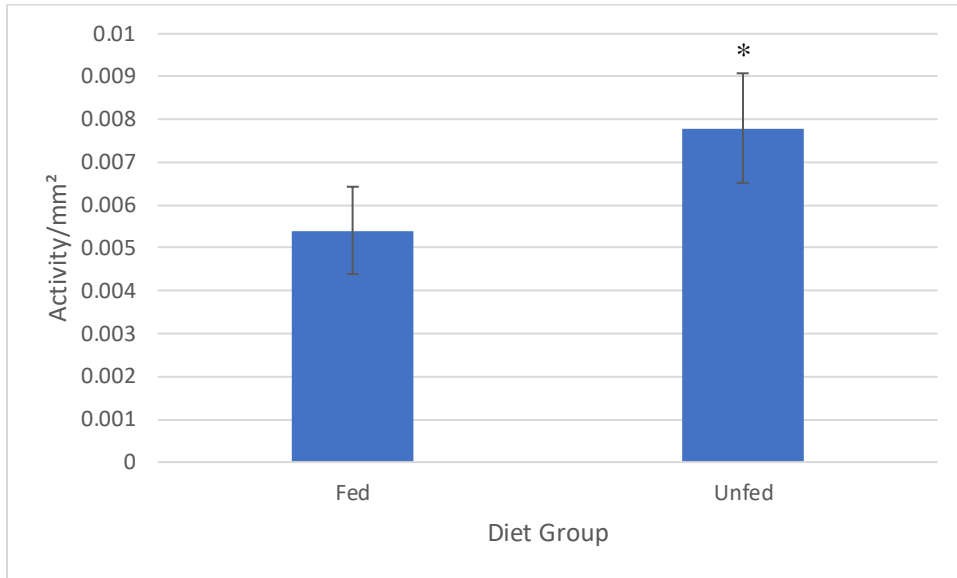


**Figure 2.** The average length of both sexes in each diet group before and after the 5-week feeding experiment. The fed fish were longer before the feeding regimen ( $p = 0.0064$ ). Males were longer in the fed group after the experiment ( $p = 0.0011$ ). Females were longer in the fed group after the feeding experiment ( $p = 0.0134$ ). FF = Fed Fish (High-calorie diet), UF = Unfed Fish (Low-calorie diet).

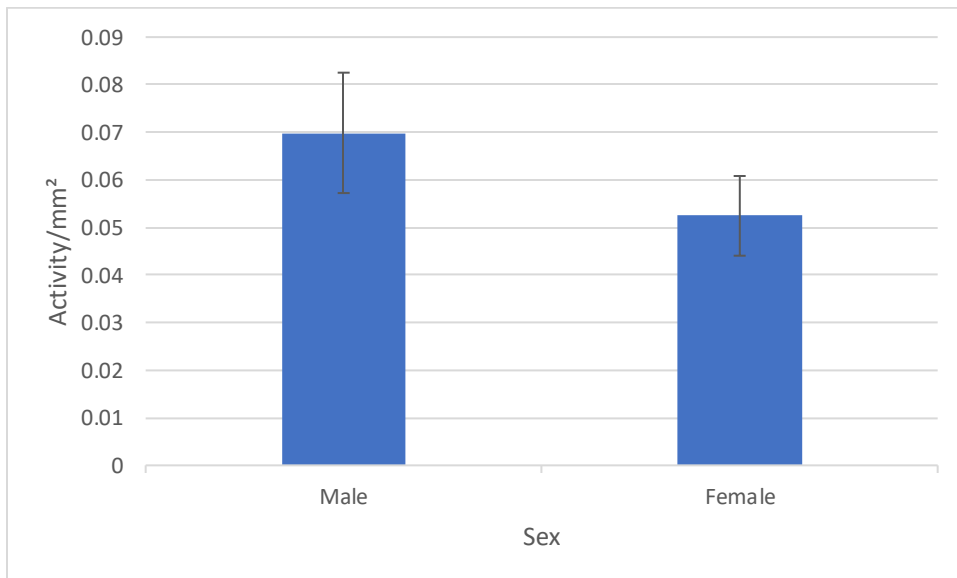




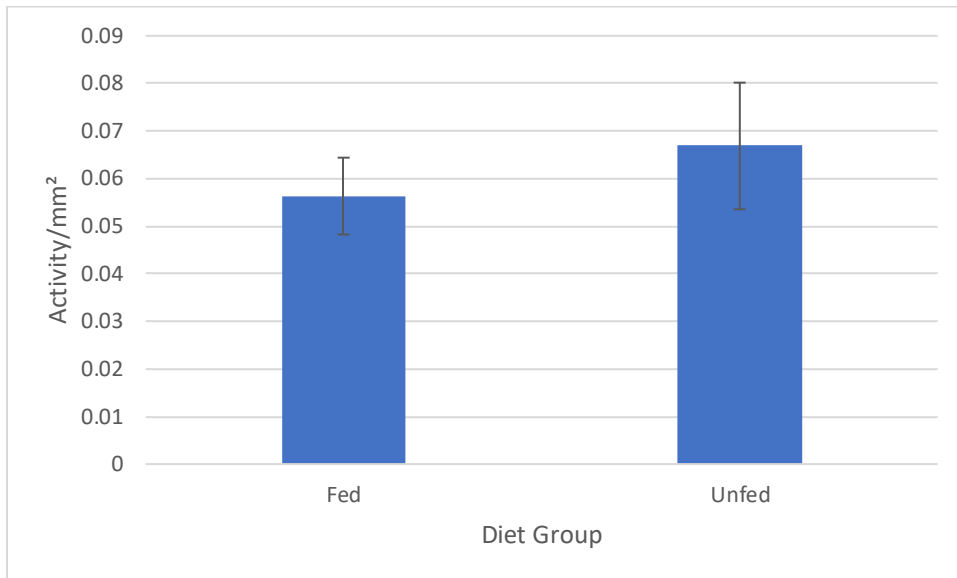
**Figure 3.** The average TRAP enzyme activity per square mm per scale for all zebrafish based on sex. There was no significant difference detected for osteoclast activity ( $p = 0.5506$ ).



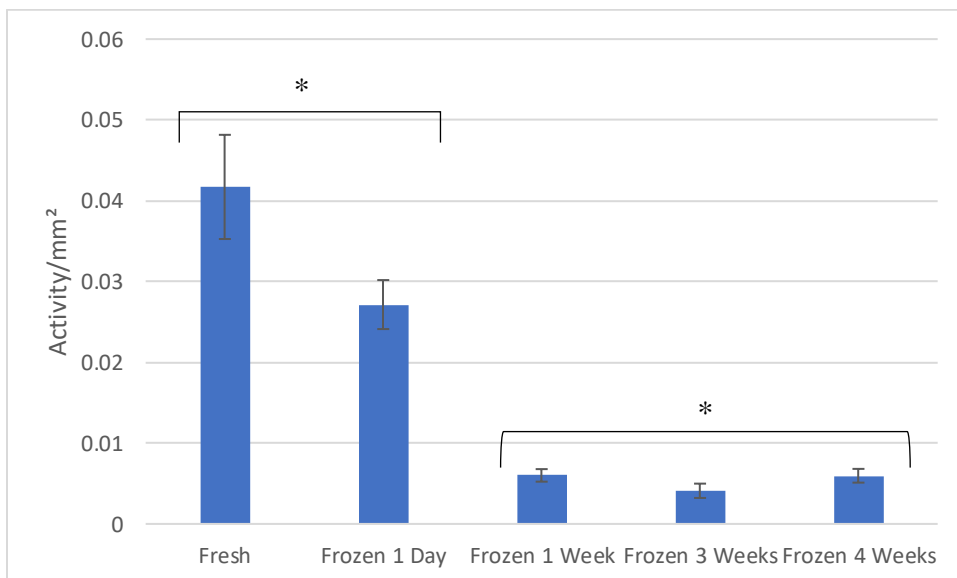
**Figure 4.** The average TRAP enzyme activity per square mm per scale for all zebrafish in each diet group. Fed indicates the high-calorie diet group and Unfed indicates the low-calorie diet group. The unfed group had significantly more osteoclast activity ( $p = 0.0503$ ).



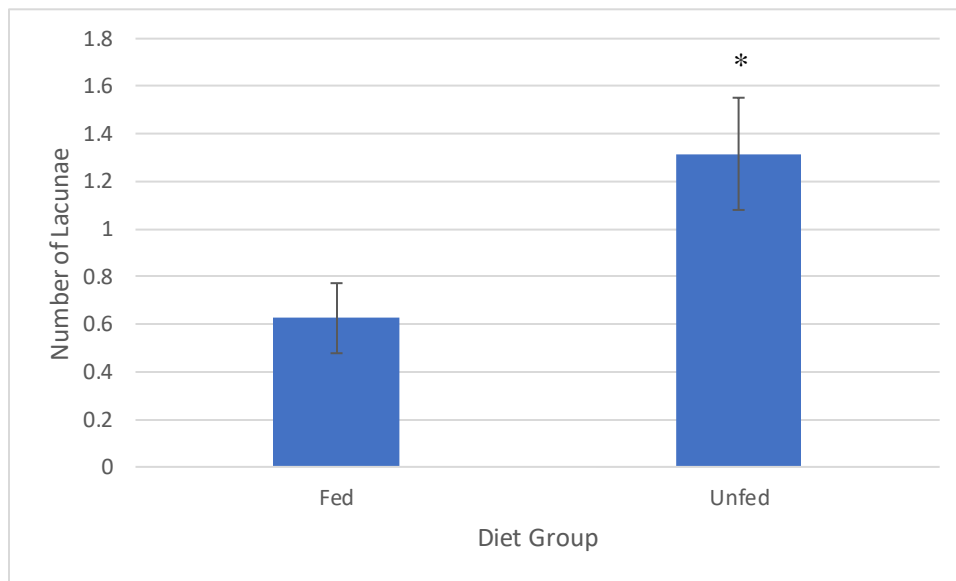
**Figure 5.** The average ALP enzyme activity per square mm per scale for all zebrafish based on sex. There was no significant difference detected for osteoblast activity ( $p = 0.3231$ ).



**Figure 6.** The average ALP enzyme activity per square mm per scale for all zebrafish based on diet. There was no significant difference detected for osteoblast activity ( $p = 0.5404$ ). Fed indicates the high-calorie diet group and Unfed indicates the low-calorie diet group.



**Figure 7.** The average ALP enzyme activity per square mm per scale in fresh scales and scales frozen for different times. There was a significant decline in ALP activity between 1 day of freezing and 1 week of freezing ( $p = 0.05$ ). There was no significant difference detected between fresh scales and scales frozen for 1 day. There were no significant differences between scales frozen for 1 week, 3 weeks, and 4 weeks.



**Figure 8.** The average number of resorption lacunae per scale for both sexes based on diet group. The unfed group has significantly more lacunae than the fed group ( $p = 0.0191$ ). Fed indicates the high-calorie diet group and Unfed indicates the low-calorie diet group.

## Discussion

I found that a low-calorie treatment increased the osteoclast activity regardless of sex. This was further confirmed by my finding that the number of lacunae was significantly higher in the low-calorie diet group, indicating increased bone turnover. The mechanisms causing this increased bone turnover are unknown, however one possible mechanism could be the inhibition

of osteoblasts (Bourrin et al., 2009). One experiment found that aged male rats that lack protein in their diet have an uncoupling of their osteoclast and osteoblast activities which results in the inhibition of osteoblast activity and allows the osteoclast activity to increase (Bourrin et al., 2009). From a hormonal perspective, age is an important factor for bone mineral density in both men and women (Khosla et al., 1998). One such study found that aged men with an estrogen deficiency experience a decrease in bone mineral density, similar to post-menopausal women (Khosla et al., 1998). While the current experiment did not find a significant difference between the ALP or TRAP activity between sexes, an area for further study could include a sample of zebrafish of various ages.

The experiment encountered some complications such as a reduction in the sample size and a dysfunctional spectrometer during data collection. During the feeding experiment, one of the automatic fish feeders was not functioning properly, which caused the sample size to be reduced to 20 fish. The sample still had equal numbers of male and female fish, but any repetition of this experiment should focus on a larger sample size. Additionally, the dysfunctional spectrometer caused the data collection to occur across two spectrometers, so future experiments should focus on the use of the same spectrometer throughout data collection.

The protocol for this project previously used para-Nitrophenylphosphate (PNPP) as the substrate, but it produced variable results with less sensitivity in detecting enzyme activity. The project builds on previous zebrafish bone research performed on larvae due to osteoblasts and osteoclasts being more visible in larvae (Mackay et al. 2013). However, since little research has been performed on adult zebrafish bones, this project looks at the metabolic differences between the bone building tissue between sexes.

Since the specific causes for the increased bone turnover are unknown, there are many areas for future study. As established by previous studies, fish of various ages could be studied to determine the bone turnover effects over time. This could include whether the older fish display the same response to the high-calorie or low-calorie diets, or an investigation of their bone turnover in relation to their hormonal levels. Additional areas of study could investigate if any genetic control of bone turnover occurs as a result of the low-calorie diet.

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