

Peyton J. Murin graduated *magna cum laude* with University Honors with Thesis from the University of Memphis Department of Biology in the spring of 2018. For his work in the lab and in the classroom, Peyton has been awarded the Department of Biology Faculty Award and been nominated to the College of Arts and Sciences Dean's Award (honoree TBD). Outside the classroom, Peyton served as a research assistant in the McKenna Laboratory, a tutor for the Educational Support Program and the Center for Athletic Academic Success, a volunteer soccer coach at Arlington Soccer Association, and a hospital volunteer at St. Francis-Bartlett. In the coming year, Peyton will be attending medical school where he will continue to pursue his passion for advancing knowledge and helping others.

Peyton Murin

Relationships Among Ovary, Secondary Sex Characteristics, and
Embryo Output in the Transparent *Casper* Zebrafish

Faculty Sponsor

Dr. Charles Lessman

Abstract

Casper zebrafish, a mutant zebrafish strain showing both melanocyte and iridophore loss resulting in a body that is largely transparent, provide a unique model organism to gain insight into normal and cancerous cells as well as general zebrafish reproduction (White et al., 2008). Despite this utility, little is known with regard to the factors influencing the level of reproductive activity in these fish. In our experiment, the *Casper* zebrafish were housed as pairs, checked for spawning Monday through Friday, and imaged one-to-two times per week. The images were analyzed in ImageJ; data were analyzed using Excel. The results indicate a statistically significant relationship between average diameter of the ten largest oocytes and the vent area in reproductively active females. At the level of individuals, it was seen that the average oocyte diameter versus time and the vent area versus time graphs tended to mirror one another. This indicates varied reproductive success amongst female *Casper* zebrafish based on measurable characteristics, which may allow for more efficient pairing in future experimentation using zebrafish.

Introduction

The zebrafish is an increasingly important vertebrate model organism, best known for its suitability for reverse and forward genetic procedures, including mutagenesis and morpholino knockdown of gene expression. In addition, zebrafish provide relatively large, translucent oocytes with great frequency in laboratory conditions (26°C). They have short generational time spans and are especially responsive to a number of insults (mutagens, teratogens, toxins) (Selman et al., 1993).

Despite its utility as a model organism, rather little is known about the basic reproductive biology of zebrafish. While the embryos and larvae are transparent, the wild-type juveniles and adults are not. The internal organs, including the gonads, are obscured in the juvenile and adult by different pigment cells that provide the zebrafish with its name (denoting horizontal dark stripes on a mirror-like iridescent background). Thus, direct visualization of the developing oocytes in the female wild-type fish to assess embryonic development is not possible. In 2008, a new line of mutant zebrafish called *Casper* became available. This double-mutant fish is translucent, allowing direct visualization of the oocytes and their development in vivo. The *Casper* double-mutant is the result of a cross between two pigmentation mutants (White et al., 2008). The *roy* mutant is the result of a spontaneous mutation resulting in a “lack of iridophores, uniformly pigmented eyes, sparse melanocytes, and translucency of the skin” (White et al., 2008). The *nacre* mutant has a mutation in the gene encoding the *mitf* (Microphthalmia-Associated Transcription Factor) gene resulting in a complete lack of melanocytes (Lister et al., 1999). A cross between *roy* and *nacre* yields *casper* (i.e. *roy*^{-/-} and *nacre*^{-/-}) that lacks both functional melanophores and iridiophores (White et al., 2008) and allows visualization of the oocytes in the living animal without dissection.

Oocyte development is characterized by five phases. The first phase is the primary growth phase (pre-follicle). In this phase the oocyte is in a pre-follicle cell-surrounded nest. The oocytes are 7-20µm in diameter. The second phase is called the primary growth phase (follicle phase). The oocytes are in transparent follicles. The vitelline envelope begins to form a unilaminar layer. The oocytes are 20-40µm in size. The third phase is called the cortical alveolus phase. The follicles remain translucent and the cytoplasm takes on a foamy appearance. The vitelline envelope reaches two, then three layers. The oocytes are 140-340µm in size. The fourth

phase is called the maturation phase. The follicle is opaque until after the germinal vessel breakdown. The vitelline envelope begins to thin. The follicles are 690-730 μ m in size. The final phase is called the egg phase. The follicles are now translucent. The vitelline layer remains tripartite and some pore canals retain oocyte microvilli. The follicles are 730-750 μ m in size (Selman et al., 1993).

Though little is still known about the reproductive endocrinology of zebrafish, there is information known with regard to the incorporation of vitellogenin into the developing follicles. As with other non-mammalian species, the transfer of vitellogenin from the blood into the vitellogenic ovaries is enhanced by the pituitary gonado tropins. This occurs via extensive stimulation of micropinocytotic activity in the surface region of the oocytes. "Steroids believed to take on this role are Estradiol-17 β and 17 α , 20 β -Dihydroxy-4-pregnen-3-one." (Nagahama & Yamashita, 2008)

Another chemical linked with the maturation of zebrafish oocytes is Activin B. In this study (Pang & Ge, 1999) the Activin B from goldfish created an increase in oocyte maturation when injected into zebrafish. This relationship was further confirmed by the addition of human follistatin abolishing the effect. There are no current studies dealing with the effect of Activin B on the vent or ovipositor area. The present study takes advantage of the transparent nature of adult *Casper* zebrafish to observe the production and diameter of the oocytes, which will then be related to other measureable reproductive factors.

Materials and Methods

Fish

Casper zebrafish (White et al., 2008) were created by mating *nacre* mutants with *roy orbison* (*roy*) mutants. The *nacre* mutant has a mutation in the gene encoding the *mitfa* gene resulting in a complete lack of melanocytes (Lister et al., 1999). The *roy* mutant is the result of a spontaneous mutation resulting in a "lack of iridophores, uniformly pigmented eyes, sparse melanocytes, and translucency of the skin" (White et al., 2008). The seven female and seven male *Casper* zebrafish were housed, in male-female pairs, in plastic containers (Aquatic Habitats, Inc.) filled with 1 L de-chlorinated water and held in a thermostatically controlled incubator (28°C) with a 14-hr. light: 10-hr. dark photoperiod. All animals in this research were used in accordance with an approved IACUC protocol

(#0714). Fish were fed once daily with a diet of flaked fish food (<http://www.aquaticceco.com>). The water in the plastic containers was changed daily.

Imaging

The fish were imaged by removing them from their plastic tank via net. The fish were then transferred, along with a small amount of water, to small transparent plastic bags. The bags with the fish were then placed on a microscope with an attached camera. Pictures of both the right and left side of the fish were taken using a Zeiss STEMI stereomicroscope and captured with a 9 Mp Amscope eyepiece camera running Toupview image software.

Checking for Spawning

The fish were checked for spawning every weekday. When embryos were found they were transferred to a 100mm petri dish containing 10ml de-chlorinated water and imaged. The images and the containers were labeled with the box number and the date.

Data Collection

Image analysis and image processing were carried out with ImageJ software (<https://imagej.nih.gov/ij/download.html>). Images of a stage micrometer, taken at the same resolution, were used to calibrate ImageJ for oocyte diameter and vent (also called the ovipositor or genital tubercle) area measurements. The line measuring tool was dragged across the oocytes and used to determine the diameter. The polygon measuring tool was used to encapsulate the vent, and again using the scale from the 8x micrometer, the vent area was attained. All the measurements were then transferred to an Excel file and sorted by individual and date. The images of the embryo plates were also transferred to ImageJ. Using the counting tool, the number of embryos was ascertained and exported to Excel then sorted by individual and date.

Analysis

Using the data attained from ImageJ, the average oocyte diameter versus time (Figure 1), vent area versus time (Figure 2), and embryo number versus time were graphed for each individual. Using the entirety of the data, the relationship between vent area and average oocyte diameter was graphed (Figure 4). This was then also graphed using only the average of

the ten largest oocytes, and then only the ten largest oocytes in reproductively active animals (i.e. those that spawned). The resolution obtained from images captured at 8x did not allow measurements of follicles <100um.

Results

Vent area and average oocyte diameter of the ten largest follicles positively correlate in reproductively active *Casper* zebrafish.

From the graphs of oocyte diameter vs. time and vent area vs. time in individual fish it could be seen that a correlation between vent area and average oocyte diameter of some degree was present (Figures 1 and 2).

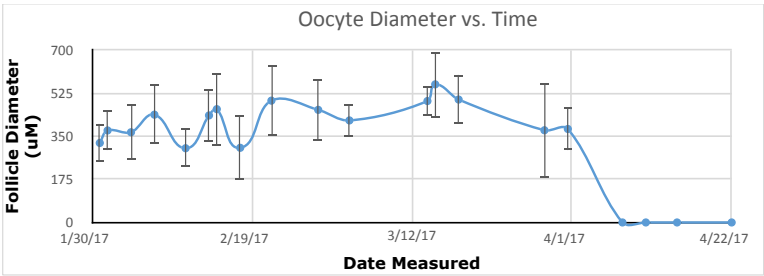


Figure 1. The positive correlation between oocyte diameter in uM and time in the reproductively active females

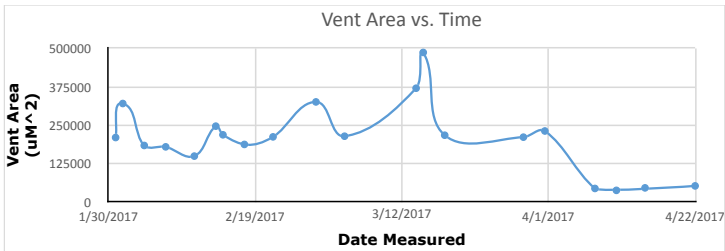
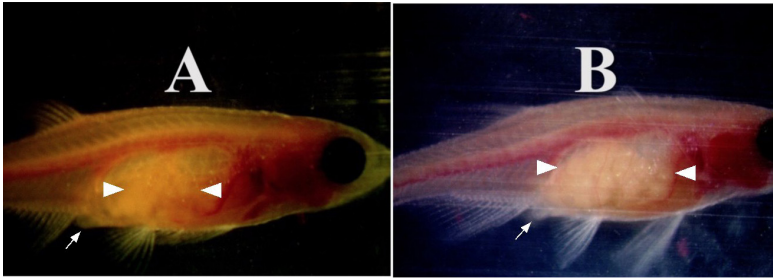


Figure 2. Vent area in uM² compared against time for female 11, one of the most representative and reproductively active females

This relationship was also visible, albeit to a lesser degree, when looking at representative images of the fish (Figure 3a and 3b).



Figures 3A & 3B. The change in vent area and corresponding change in oocyte diameter in female 11 from 03/03/2017 (left) to 03/13/2017 (right)

As a result of these observations the decision was made to compare these two data points (oocyte diameter and vent area) against one another to determine if there was in fact a relationship. The decision to use the ten largest rather than just a straight average was made in order to account for the formation of new oocytes which coincided with the growth of previous oocytes.

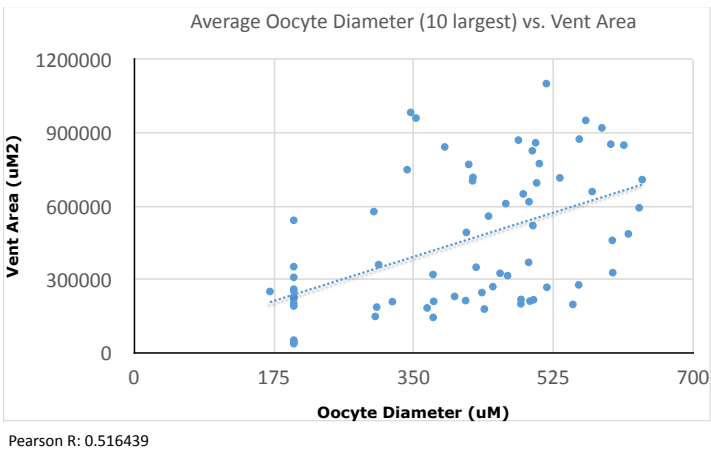


Figure 4. The vent area in μM^2 (y-axis) compared to the average oocyte diameter of the ten largest oocytes in μM (x-axis)

To determine correlation amongst average diameter of the ten largest oocytes and vent area in reproductively active *Casper* zebrafish it was necessary that we determine degree of freedom. We decided to use $n=73$ (number of data points) rather than $n=7$ (number of individuals), since the individual data points were distinct measures collected on an individual over time. Thus, using a Pearson R table and taking into account our r of 0.516439 and n of 73, $df = N-2=71$. For a df of 70, the critical r value is 0.232. Given that our r value is greater than 0.232, since the relationship has a p value less than 0.05 it was concluded to be statistically significant. This led to the decision to compare the two variables against one another as seen in Figure 1. This relationship was also visible by comparing the vent area and oocytes in the images of the fish (Figure 3).

Discussion

The results indicate with a high degree of certainty that there is a biological relationship between the oocyte diameter and the vent area. This significant relationship between oocyte diameter and vent area leaves the door open for further study in a number of ways. First, the frequency of our measurements made it hard to determine the more volatile relationships, such as that between changes in vent area and changes in oocyte diameter. Second, the frequency of our measure impaired our ability to draw conclusions with regards to spawning. More frequent measures would have allowed data on both variables to be observed immediately before and after spawning, something that would be extremely helpful given the quick-changing nature of the variables. Third, further study into potential common causation of this correlation could potentially illuminate the underlying mechanisms involved. Finally, in this study we were limited by the number of reproductively active females. Future studies may benefit from increasing the number of fish, thereby allowing for stronger evidence of correlation between the various reproductive factors. The study by Pang and Ge (1999) brought forward one chemical, Activin B, known to play a role in oocyte maturation. Activin B, a growth factor, is thought to play a mediating role leading to the maturation of the oocytes in zebrafish. Conducting further study to determine if this chemical also plays a role in vent area increase would shed light onto this relationship. Guanesekera et al. (1999) showed that there was a relationship between oocyte diameter, amongst other factors and dietary protein in the fish, *Oreochromis niloticus*. It is possible that something of this sort could also be implicated in

impacting oocyte growth in *Casper* zebrafish as well.

Acknowledgements

The Department of Biological Sciences at the University of Memphis for funds for fish husbandry and supplies used in this study.

Dr. Charles A. Lessman for outstanding support and mentorship.

Citations

- Gunasekera, R., Shim, K., and Lam, T. "Effect of dietary protein level on puberty, oocyte growth and egg chemical composition in the tilapia, *oreochromis niloticus* (L.)." *Aquaculture*, vol. 134, no. 1-2, 1999, pp. 169-183.
- Lister, J., Robertson, C., Lepage, T., Johnson, S., and Raible D. "Nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate." *Development*, vol. 126, 5 Aug. 1999, pp. 3757–3767.
- Nagahama, Y., and Yamashita, M. "Regulation of oocyte maturation in fish." *Development, Growth & Differentiation*, vol. 50, 2008, doi:10.1111/j.1440-169x.2008.01019.x.
- Pang, Y., and Ge, W. "Activin stimulation of zebrafish oocyte maturation in vitro and its potential role in mediating gonadotropin-induced oocyte maturation." *Biology of Reproduction*, vol. 61, no. 4, Jan. 1999, pp. 987–992.
- Selman, K., Wallace, R., Sarka, A., and Xiaoping, Q. "Stages of oocyte development in the zebrafish, *brachydanio rerio*." *Journal of Morphology*, vol. 218, no. 2, 1993, pp. 203–224.
- White, R., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., ... and Zon, L. "Transparent adult zebrafish as a tool for in vivo transplantation analysis." *Cell Stem Cell*, vol. 2, no. 2, 2008, pp. 183-189.