Slide 1: Hello everyone! My name is Rachel Summers and the title of my project is “Embryonic Development of the Stress Hormone Axis in Two Model Teleost Species.”

Slide 2: So, first we’re going to start with the model organisms.

Slide 3: In this study I used zebrafish (*Danio rerio)* and Japanese medaka (*Oryzias latipes)* as the model organisms. Both belong to the extremely large aquatic vertebrate group known as teleosts. They are becoming increasingly useful in biomedical and comparative endocrinology research for their many advantages such as their relatively small sized compared to other teleosts, such as the tuna and salmon. Their development is possible to study due to their embryos developing externally and within a translucent membrane that can be easily observed under a microscope. Lastly, they have a short generation time of about 2-4 months which allows transgenerational studies to be completed in a shorter period of time than other aquatic vertebrates.

Slide 4: Zebrafish and medaka, though both teleosts, differ significantly in some aspects of embryonic development. The zebrafish develops at a much faster rate, hatching at about 48 hours post fertilization (hpf), whereas medaka take around 8 days post fertilization (dpf) to hatch. On this image, you can see the developmental timelines and similarities at timepoints between the two species.

Slide 5: Just in case you were curious, here is what the two species look like at different stages of their development under a microscope.

Slide 6: So now we’re going to talk about glucocorticoid hormones and the HPI axis.

Slide 7: Glucocorticoid hormones mediate the stress response in all vertebrates, from teleost fishes to mammals. In teleosts, the primary glucocorticoid, cortisol, is synthesized within interrenal tissue via a series of enzyme-mediated reactions regulated by the hypothalamic-pituitary-interrenal (HPI) axis in response to stressors. This is comparable to humans such that our primary glucocorticoid is also cortisol. However, in humans, cortisol is synthesized within the adrenal gland in response to the hypothalamic-pituitary-adrenal (HPA) axis.

Slide 8: This is a diagram representing the HPI axis in teleosts that I made. So you can see that in response to stressors, the hypothalamus releases the peptide corticotropin-releasing hormone (CRH) which stimulates the production and release of the pituitary protein adrenocorticotropin hormone (ACTH), which then stimulates cortisol production in the interrenal cells through a series of enzyme-mediated reactions. Once the cortisol is produced, it can be deactivated by the enzyme 11 b hydroxysteroid dehydrogenase type 2. Active cortisol then exerts its effects on target cells via two types of receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). The boxes that are highlighted in yellow represent the genes that I measured in this study, which I will talk about shortly.

Slide 9: Our lab has been working to address gaps in the knowledge of developing HPI axes. To add to this, the objectives of my study were to measure the expression of HPI axis genes and cortisol levels throughout embryonic development. Based on the known genetic similarities between these two teleosts, I hypothesized that both species would show similar trends for changes in gene expression and cortisol levels throughout their embryonic development, but at different magnitudes.

Slide 10: My first prediction for this experiment was that zebrafish and medaka would show similar activation in gene expression at comparative timepoints, with differences in magnitude. The second prediction was that the zebrafish cortisol levels would show a similar pattern of elevation as the medaka through embryogenesis.

Slide 11: Now getting into the experiment itself...

Slide 12: To compare HPI gene expression in medaka and zebrafish at similar developmental stages, I utilized quantitative polymerase chain reaction (qPCR). In order to measure the whole-embryo cortisol levels for zebrafish, I used a commercially available enzyme-linked immunosorbent assay (ELISA) kit.

Slide 13: I measured these key genes involved in the HPI axis including *crh*, melanocortin type 2 receptor (*mc2r*), *star*, 11 β hydroxysteroid dehydrogenase (*hsd2*), and *mr*.

Slide 14: As a reminder, these were the genes highlighted in yellow on the HPI axis.

Slide 15: Over the summer of 2019, pools of 10-50 embryos were collected at a lab in Norway. Thirteen developmental stages were collected from 12 hpf to the start of hatching at 8 dpf to be used for gene expression analysis.

Slide 16: During the Fall 2019 semester, zebrafish were bred by placing four female and two male adult zebrafish in 10 breeding cages in 2.5 L tanks the night before an intended breeding. This allows us to collect the embryos from the bottom of the tank the next morning after spawning. We collected the embryos at the 2- to 8-cell stage (or ~1 hpf) and incubated at 28°C in embryo water. Pools of 25-50 embryos at 5 developmental stages from 2 hpf to 8 hpf were collected for RNA extractions. Pools of 25 embryos at seven developmental stages from 2 hpf to the start of hatching at 48 hpf were collected for cortisol determination.

Slide 17: After collecting all the samples, I isolated the total RNA, synthesized cDNA, and used the cDNA to run qPCR analysis. For those of you who are unfamiliar with how qPCR works, it is a laboratory technique that binds fluorescent dye to double-stranded DNA during synthesis by polymerase chain reaction (PCR) in order to measure gene expression. The more cycles that it takes for the sample to cross the fluorescence threshold line, the less transcripts were present in the sample initially.

Slide 18: Next, I extracted cortisol using a technique previously described in a scientific paper and quantified it using an ELISA according to manufacturer's protocols.

Slide 19: Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. From this we were able to calculate the fold change relative to 2 hpf in zebrafish and 2 dpf in medaka. Using SPSS Statistics, the temporal pattern of cortisol and gene expression was tested for normality and the Levine's test was used for equality of variance. When $P < 0.05$ in each test, the non-parametric Kruskal-Wallis test was used. If $P > 0.05$, one-way ANOVA and the Tukey post-hoc analyses were used to test for significant differences ($P < 0.05$) in fold change of gene expression between each timepoint. The grouping variable was time, and the dependent variable was the fold change.

Slide 20: Now we can get into the results!

Slide 21: First beginning with crh, you can see that both species show the same general upward trend in expression throughout development. However, one thing to notice is the difference in magnitude of the y-axis between the species. Showing much larger changes in expression in medaka versus zebrafish. Data points with different letters are statistically significant as determined by SPSS.

Slide 22: Now looking at mc2r, you can see that both species have initially high levels of this transcript before dropping down. It is the acth receptor, so it makes sense that it has higher maternal deposit of RNA transcripts in order to handle the high cortisol that is also maternally deposited. It makes sense that the medaka then begin to transcribe on their own, however the zebrafish do not follow the same trend there.

Slide 23: Now, StAR is not a receptor but is involved in cortisol production and cholesterol transport across the mitochondrial membrane, so it makes sense that its transcript levels start low initially and then increase to prepare for hatch. Again, it is important to note the large difference in magnitude on the y-axis between the two species. There is a clear increase seen at 30 hpf in zebrafish whereas medaka doesn't have as clear of a starting point but is more gradual in increase.

Slide 24: Looking at hsd2 expression, you can see the large maternal deposit in medaka and then never rises again. Perhaps this could mean that they need all their cortisol that is being produced and don't want to inactive any? Zebrafish see that same general increasing trend at 30 hpf as was seen in crh and star. So, as you can see, these two species showed starkly different patterns in expression for this gene, so this raises the question as to whether maybe there are multiple versions of this gene? I could not find any evidence supporting this, but maybe it really is a possibility that I measured two different ones due to stark differences in results that are seen.

Slide 25: For mr expression, medaka has the clear increasing trend as seen with many other of its genes. The zebrafish is a bit noisy and hard to really conclude anything here.

Now I'm going to present this same data in a different way that will hopefully allow you to better compare the different expression patterns amongst the genes within the individual species. So first we're going to start with zebrafish.

Slide 26: Note that as I change slides here the y-axis will be changing in order allow for the new genes that are being added.

Slide 27-29: no words.

Slide 30: Adding star at the end really allows you to see how much greater its magnitude of change in expression was compared the other measured zebrafish genes. So next, I'm going to show you all of the medaka data again in this same way.

Slide 31: Make sure, again, to note the changing y-axis as I add the genes on here.

Slide 32-34: no words.

Slide 35: Adding mc2r at the end really allows you to see how much greater its magnitude of change in expression was compared the other measured medaka genes.

Slide 36: Now we see the zebrafish cortisol data. You can see there is a high value seen at 12 hpf before dropping significantly by 48 hpf. Looking at the changes in gene expression we would have expected to see an increase in cortisol near hatch, but perhaps the rise in hsd2 expression (which deactivates cortisol if you remember) is what is contributing to drop in cortisol levels.

Slide 37: Lastly, is the medaka cortisol data, which was obtained from other members in our lab Summer of 2019. Compared to the zebrafish, there is a significant increase in cortisol levels before hatch. However, what we don't see, is the initially high levels of cortisol that we would expect from maternal deposit. Perhaps this could be due to the initially high levels of medaka hsd2 that are deactivating the maternally deposited cortisol. Interesting to see the differences in the patterns of cortisol throughout embryogenesis in the two species. Overall, based on the expression profiles for the HPI axis genes examined in both species, it can be seen that all five genes were clearly expressed in both species during embryogenesis, suggesting potential roles of the stress axis in development.

Slide 38: I just want to say thank you to all of my lab members, especially Lauren Closs for helping me out since Summer Science 2018. Also, a huge thank you to Dr. Baker for initially getting me started in this research and helping in the lab ever since. Thank you everyone!