PROJECT INFORMATION SUMMARY

1. Title of Proposed Project: Extraction and Quantification of RNA from Zebrafish (*Danio rerio*) Olfactory Tissue

2. Primary Director

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5. Thesis/Dissertation or Faculty Advisor: Todd Hibbard

6. Dates of Entire Project Period: (From) January 2016 (To) April 2017

	Signature	**Date**
Principal Investigator Associate Investigator(s):	Ana L. Barajas	4/27/17
	Steven Chang	4/27/17
Thesis/Dissertation or Faculty Advisor:		4/27/17

Objective: The aim of the proposed study is to characterize and quantify expression of specific olfactory receptors (*oras*) and create a transcriptome library of gene (messenger RNA) expression. This library is to be used to look at changes in olfactory-related genes and also to determine broad

changes or patterns in gene expression between control, 11-ketotestosterone-treated zebrafish (*Danio* rerio) in addition to adult male and female zebrafish.

Introduction:

Sensory information is obtained from both our external and internal world through sensory receptors. All of our sensory organs receive sensory stimulation, via specialized receptor cells. Animals have specialized sensory receptors that are used to detect various types of chemical stimuli including olfactory and gustatory information. Neurons transmit messages when stimulated by chemical signals (or ligands) from our chemical senses and in response, a neuron generates an action potential. Then these sensory receptors deliver the neural information to the brain. In interpreting the world around us, our brain integrates their inputs. Furthermore, the thalamus receives information from the senses and routes it to the higher brain regions where it is integrated with other sensory information (1). Chemoreception was first discovered in bacteria. They are the first organisms believe to have communicated chemically (2). In humans, the chemical senses are located in the nose and mouth and are involved in detecting the chemical stimuli located in the air via the nose and on the tongue, dissolved in saliva.

Gustatory receptor cells (or taste cells) are found on the tongue in the taste buds that are on the papillae of the tongue. However, other taste buds have been found on the palate, larynx, and pharynx. There are individual taste cells, or microvilli, recognize chemical signals (or tastants) in saliva. Tastants are taste-provoking chemical molecules that are dissolved in ingested liquids or saliva. Tastants are ligands which activate G-protein coupled receptors and lead to the production of a second messenger. Binding of the ligand to the receptor can result in action potential production in afferent nerves that project to the brain. Various regions of the tongue are innervated by cranial nerves that carry the signal to the thalamus, from which it is transmitted to other regions

of the brain, ultimately to the sensory cortex (1). There are four fundamental taste receptors types for all the tastes classified all over the tongue. Tastants have been classified as: sweet, bitter, salty, sour, and umami (savory). The center of the tongue doesn't get stimulated due to lack of the receptors in that area. Thus, one might respond better to certain tastes because there are more receptors concentrated in that area (1, 2).

There are hundreds of receptors found at top of the nasal cavity in humans (1). The sense of smell (or olfaction) in humans is poorly developed compared to other mammals. Furthermore, higher organisms, such as invertebrates and vertebrates, have been able to develop specific chemoreceptors that detect odorants; we classify these as olfactory receptors. The olfactory system contains hundreds of receptors and olfactory sensory neurons (OSNs) located in the nasal cavity, along with supporting basal cells. Basal cells differentiate to continuously replace OSNs. The cilium of OSNs extends into the nasal mucosa where it binds odor molecules to G proteins. Binding activates a signal transduction mechanism that opens channels (1, 2). Moreover, olfactory fatigue is experienced when a series of stimuli flood nerve receptors, then nerves become accustomed to stimulus. It happens because rate of change within nerve's membrane is inadequate to keep up with continuous simulation. Furthermore, there are also trigeminal nerve endings, perceived by cranial nerve number five, located throughout the nasal cavity that are very sensitive to strong chemicals that are not only irritating, but sometimes painful (3). This serves as an alert mechanism for the body.

The anatomy of the peripheral olfactory organ (or nose) is quite simple. The olfactory organs are located in the nasal cavity on either side of the nasal septum. Olfactory mucosa covers the olfactory epithelium, which is on the posterior side of the nasal cavity. The axons of OSNs project to the olfactory bulb, which is located under the frontal lobe. These OSNs are bipolar

neurons because they have an axon and a single dendrite. The olfactory cilia contains olfactory receptors that detect chemical stimuli. The OSN axons project to the olfactory bulb via the olfactory nerve (cranial nerve I). Furthermore, there are supporting cells that allow the passage of substances from surface to basal layer and basal cells precursors for new OSNs (for review see (2)).

The physiology of the nose is simple in one way, complex in another. One can only get so far up in the nostril because we have shell like bony structures called concha. Their function is make the air more turbulent, since the air we breathe does not have a nice laminar flow. Turbulent air flow is good for many reasons, not just because it's good for the respiratory system. Because one does not want to breathe in cold air, the air is mix up and heated to body temperature. Water vapor is added to prevent dry air from drying up the surface of lungs. If there was no concha, one would hit the bony plate, cribiform plate, which is part of ethmoid bone (bone that sits in middle) and separates olfactory bulbs. The cribiform plate is a bony plate with lots of holes going through it because there are axons of bipolar neurons. These neurons are like inter-neurons, which go from point A to point B. For example, one end of the axon, dendrite, sits inside the air craft while the axon synapse to the olfactory bulb. The olfactory bulb is where the axons of OSNs (or the olfactory nerve- cranial nerve one) synapse (for review see (2)).

The physiology of the human nose is similar to fish. The only difference is that instead of having sheets in the nose like humans, fish have "folds" or lamellae (2). Also, humans respond to odorants in the air, but fish respond to odorants molecules that are dissolved in water. Based on previous research, we know odors bind to receptors, receptors open stimulus gated sodium channels, which change the membrane potential, depolarizing it. Likewise, olfaction is caused when a receptor binds an odor molecule, which are organic molecules (aliphatic, aromatic, esters,

ketones, alcohols, etc.) (2). This odorant bind to OSNs which transmit information to higher order areas like the olfactory bulb, pyriform cortex, to the orbital frontal cortex where it activates three different parts of the brain: thalamus, hypothalamus, and hippocampus. This can bring about motor, visceral, and emotional reactions to olfactory stimuli (2). Likewise, since the hippocampus is the center for learning and memory, there is the idea that when you smell something that you have smelled before memory helps you remember that smell and identify it. Thus, the sense of olfaction has been linked to memory loss. Also, olfaction can play a major role in reproductive, courtship, and territorial behavior. Nonetheless, hormonal effects can be rapid, and memories, sometimes facilitated by local neurochemistry, can be long-lasting (4).

Chemical senses of olfaction and taste are the oldest, shared by all organisms including bacteria, so animals are pre-adapted to detect chemical signals in the environment. Chemical senses are very different from other senses since the chemical senses rely on the physical perception of odorant molecules from the environment. Chemical information from the environment is used by animals to 1) locate potential food sources, 2) find shelters, 3) detect predators 4) respond to alarm cues or pheromones, and 5) respond to mating odors or pheromones, released from conspecifics (2). During chemical communication, odorants must be sent from a signaler and subsequently detected by a receiver. Olfaction is important because it provides reliable information once the pheromone/odor reaches the OSN and stimulates the CNS. Furthermore, most animal olfactory systems have a large range of relatively non-specific olfactory receptors which means that almost any chemical in the rich chemical world of animals will stimulate some OSN. If detection of a particular chemical cue leads to greater reproductive success or survival, there can be selection for receptors more sensitive to it or expressed in greater numbers (4).

There is a variation in the ability to detect odors, due to changes in olfaction. These changes can be: environmental, genetic, and/or developmental/hormonal. For example, environmental changes in olfaction can occur when a person is around a person who smokes or when a person's home is around factories that cause pollution. Some of these people usually experience hyposmia, which is the diminished sense of smell. Similarity, the cues from the environmental may cause anatomical changes in fishes' nose. Hamdani et al. (5) demonstrated that environmental seasonal cues lead to proliferation of crypt cells in the olfactory epithelium of the crucian carps (*Carassius carassius*). Results show crypt cells to be scattered during the winter, in the spring they are deep in the epithelium and not yet exposed to the environment; however, in the summer crypt cells are on the epithelium surface. Morphological changes in the nose are expected in response to environmental changes and this seems to be cyclical to the thickening of nose tissue as breeding season approaches (5). Thus, fish have a higher sensitivity to pheromones in the spring and this leads to changes in behavior, which may make it easier for them to find a mate (5). Barth et al. examined differential *ora* expression in zebrafish (*Danio rerio*) at the embryonic stage where there was up-regulation at different times. This is important because there can be changes based on development of olfactory receptors (6).

Development, on the other hand, can either be natural or induced. In the lab, developmental changes can be induced by exposing organisms like zebrafish, to sex hormones (7). Several studies have shown that hormones change behavior and olfaction. For example, in one experiment, induced changes in olfactory responses in zebrafish and three other cyprinids are seen after exposure to testosterone. Belanger et al. (7) exposed zebrafish to 17 mg/L of 17α-methyltestosterone and this lead to increases in their ability to detect odorants, which are putative pheromones. Specifically, they found that androgen exposure lead to changes in olfactory

sensitivity to 15 keto-prostaglandin $F_{2\alpha}$ (15-keto $PGF_{2\alpha}$) and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (7). Furthermore, there were behavioral changes between control and androgen-treated redtail sharkminnows (*Epalzeorhynchos bicolor*). Androgen treatment in juvenile redtail sharkminnows increased a display of behaviors including nuzzling and quivering, this are usually seen in spawning adults. Likewise, treated juvenile redtail sharkminnows displayed more courtship behaviors towards PGF-injected partners than saline-injected partners. This indicates that androgen-treated fish can respond to specific odorants, possibly through the accumulation of hormonal androgens acting on androgen receptors located in the olfactory epithelium of the nose (7). Thus, this study shows that hormones change olfactory physiology and behavior when exposing animals to androgens; however, all four different species of fish show the same response.

Another experiment on cyprinids examined both female and male goldfish (*Carassius auratus)*. Ghosal and Sorenson (8) showed how androgens lead to changes in hormone levels leading to differences that are seen in reproductive behaviors, as well as changes in anatomy and physiology. It only took a few weeks for goldfish to become fully masculinized by the exposure of natural and artificial androgens, and thus have olfactory sensitivity to sex pheromone. Courtship behavior took a full eighteen weeks; however, mating and time-to-court behavior could not be induced after thirty weeks in females. Findings suggest that courtship behavior in the male goldfish is enhanced by previous sexual experience and not mating. Thus, neuroendocrine mechanism(s), not yet identified, are associated with different types of androgens that drive the expression of male reproductive behaviors (8).

Furthermore, an observation in South-East Asian Cyprinids (*Puntius schwanenfeldi*) led researchers to investigate the possibilities of using androgen implants in order to prove the relationship between an androgen and peripheral olfactory receptor response (9). After juveniles

were treated with androgen, results demonstrate a plasticity, in peripheral olfactory receptors, that is hormonally induced (9). These experiments show that that hormones can affect the olfaction system, since males increase testosterone as they reach sexual maturity.

Changes in detection/sensitivity to specific odorants during induced sexual maturity were seen in many previous research studies in Cyprinids. This work is part of a larger project that seeks to understand how changes in gene expression in zebrafish olfactory epithelium are affected by treatment with the androgen 11-ketotestosterone (11-KT), a steroid hormone. Previous work has shown that exposure of juvenile zebrafish to androgens results in olfactory responses and courtship behaviors of redtail sharkminnows that mimic those of mature male zebrafish (7). What is not known is the effect of 11-KT on olfactory receptor (OR) expression. Given the changes olfactory detection after androgen exposure, we hypothesize that expression of olfactory related genes is affected and that 11-KT likely up-regulates expression of genes related to pheromone detection, odorant processing and sexual behavior. In this project we want to examine receptor level changes following treatment with the androgen 11-KT. Because zebrafish, treated with testosterone, have an increase in sensitivity to female pheromones (7), we are interested in investigating why this is happening when we treat with testosterone and what is happening at the molecular level of the nose that is allowing for this change to occur. We hypothesize that there will be an up regulation of specific olfactory receptors in treated zebrafish compared to control, or an increase number of OSNs and specific receptors that respond to putative pheromones. Also, receptors of treated fish will have similar expression as we see with adult zebrafish. Saraiva and Korsching (10) have identified and characterized a novel family of ORs that show some similarity to vomeronasal receptors of mammals. Expression patterns of these genes have not been shown in zebrafish. Our interest lies in characterizing the expression of a novel family of olfactory receptors

(*oras*) that have been shown in teleost fish, and have sequence similarity to pheromone receptors. We are interested in investigating if there are changes in OR expression. If so, which of the six *oras* known in zebrafish are causing this. This study is important because if the genes that up regulate or down regulate receptors are found, then in the future those genes can be found in humans and may be helpful with treating anosmia. Further, treating anosmia may alleviate the symptoms of Alzheimer's disease, since anosmia has been linked to memory loss, changes in central processing of information, and behavior (11).

Methods:

***Steroid treatment*:**

Juvenile zebrafish (approximately 60 days old) were obtained from Wayne State University (School of Medicine) and kept in 4 glass tanks (30 cm long · 20 cm wide · 22 cm tall) that contained 19 fish per aquarium and 4 L of dechlorinated water. During treatment, juveniles were held in 2 control (ethanol-treated) and 2 experimental (11-ketotestosterone (11-KT)-treated) aquaria. Methods for treatment of juvenile test fish were adopted from Belanger et al. (7). Test fish were treated with 60 µl of 11-KT and controls were treated with ethanol in aquaria since the main route of uptake of lipophilic compounds is via the gills (7). On each of the treatment days, for 14 consecutive days, water was completely emptied every morning and freshwater was added to the tanks (removing the 11-KT). The 11-KT was added in evening (~1700 h) and 1 mL of 11-KT stock solution (0.2 mg/mL dissolved in 95% ethanol) was delivered to each aquarium near the air stone creating an aquarium water concentration of approximately 10^{-8} M; control fish were treated with the same volume of 95% ethanol. Control and 11-KT-treated changes in receptor and gene expression were examined for 0, 7, and 14 days post-treatment. About ten fish were removed and

on each of the days the treated fish were removed; however, at day fourteen, seven fish were removed because some died. Fish were initially classified based on size and age. Size of control and 11-KT-treated fish were initially classified as immature based on size and age. While gonads of immature fish were examined in order to identify males and female fish. At the termination of experiments, weight and standard length of juveniles were as follows: Weight and standard length of control zebrafish (N = 30) was 0.13 g ± 0.08 g, 2.25 cm ± 0.46 cm ; treated zebrafish (N = 27) was 0.12 g ± 0.07 g, 2.28 cm ± 0.42 cm; female zebrafish (N = 11) was 0.5 g ± 0.23g, 3.6 cm ± 0.43 cm ; male zebrafish (N = 10) was 0.44 g ± 0.11 g, 3.51 cm ± 0.33 cm. Weights and lengths of control and 11-KT-treated fish were not significantly different (t -tests, $P > 0.05$). Peripheral olfactory organs were then collected from all zebrafish and immediately place in a microcentrifuge tube and frozen in liquid nitrogen. Tissues were stored at -80°C until they could be processed.

***Total RNA Extraction for Quantitative PCR*:**

RNA was extracted from treated and control zebrafish using an organic extraction method (12). Tissues were removed from -80°C freezer and kept on ice. TRIzol reagent (400 µl) was immediately added to each tube to prevent RNA degradation. Tissues were homogenized until no large chunks were visible. The volume was brought up to 1 ml with TRIzol reagent (usually 1 ml TRIzol/50-100 mg of tissue). The sample volume did not exceed 10% of the volume of TRIzol reagent used for homogenization. Eppendorf tubes were inverted several times to mix the homogenates. To avoid genomic DNA contamination, the sample was centrifuged at 12,000 x g for 10 min at 4°C and supernatant was transferred to a clean tube. Chloroform (200µl) was added per 1 ml TRIzol. The eppendorf tube was then inverted vigorously for 15 seconds and samples were incubated at room temperature (~25°C) for 3 min. Samples were spun again (12,000 x g) for 15 min at 4°C. The upper aqueous layer was transferred into a clean tube. Protein (middle white

layer) and DNA (bottom pink layer) were saved and stored at -80°C.The RNA was precipitated using 500 µl of isopropyl alcohol (per 1 ml TRIzol) and samples were incubated overnight at -20°C. The next day samples were centrifuged again (12,000 x g for 10 min at 4°C). Following this, the supernatant was discarded and the RNA pellet was washed with 1 ml 75 % ETOH in 0.1% diethylpyrocarbonate (DEPC) H_2O and spun at 7,500 x g for 10 min at 4°C. Supernatant was discarded and pellet washing steps were repeated. 88 µl 0.1% DEPC H_2O was added to the tube and vortexed to dissolve the pellet. Turbo DNase treatment was done immediately by adding 10 µl of 10x Turbo DNase buffer and 2 µL of Turbo DNase enzyme. Samples were vortexed and incubated at 37°C for 30 min with 50 rpm rotation. Following this step, 10 µl DNase inactivation reagent was added. Samples were incubated at 25°C for 2 min and centrifuged at 10,000 x g for 2 min. Supernatant was transferred to a new tube. Samples were placed on ice and RNA concentration was immediately measured using a NanoDrop (Thermo Scientific NanoDrop Lite Spectrophotometer, Wilmington, DE) (12, 13).

***Sample preparation*:**

RNA was combined (three olfactory organ pairs per sample) and sent out to Cofactor (San Francisco, California) for sequencing and bioinformatics analyses.

***Cofactor & Blast Data Analysis*:**

The Cofactor genomics database was chosen because it's an industry that provides sequencing in just 3 weeks and data is supplied as a webpage with graphical user interface, which makes it convenient to use. To find the ora genes between databases, minimum fold change was set to 10x. Fold change was used to measure change in the expression level of genes, for this the threshold was set to 2, 5, 10, 50, 100-300, and 100. After candidate olfactory genes were selected based on known biological, physiological, and function relevance to the olfactory system, their

cDNAs were downloaded from Cofactor and compared to the 6 cDNAs from Saraiva and Korsching (10) in BLAST (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi) and ZFIN (www.zfin.org) :

Nucleotide sequences of the cloned ORA cDNA fragments	
NAME	cDNA fragment sequence
Dr ORA1	ATGGACCTGTGTGTCACCATCAAAGGCGTCTCCTTCCTGCTGCAGGCTGGTCTGGGGATACTGGCC AACGCACTGGTTCTGCTGGCGTATGCCCACATCCGTCTGGCGGAGGCGCGACTGCAGCCTGTGGAT GCCATCCTGTGCCACCTGGCGCTGGTGGACCTGCTGCTGCTGCTGACTCGCGGTGTCCCGCAGACTA TGACTGTGTTTGGCATGAGGAACCTGCTGGACGACACGGGCTGTAAGGTGGTGATCTACACGTACC GTATCGCCCGAGCGCTGTCCGTCTGCATCACCTGCATGCTGAGCGTGTTCCAGGCAGTGACGGTGG CGCCCGCGGCAGGACCGCTGCTATCCGGGGTGAAGGCCCGGCTCCCACAGCTGCTCGCGCCTACAT TTGCAGCACTGTGGTTCATCAATATGGCCGTCTGCATAGCTGCCCCATTCTTCTCAGTGGCCCCTCG CAACGGCACCGTCCCACCATTCACGCTCAACCTGGGCTTCTGCCATGTGGACTTCCATGA
Dr ORA2	TGCTAACGGGGCGGCGCAGGTGGCCAGGGATGTGGTGCCGATGGTGCTGATGGCGGCAGGCAGCC TGGTGCTGTTGGTGTACCTGGTGCGGCAGCGGCGGCGAGTACAGGGTCTGCGGGGTACGGCAGGG GGGGCAGCAGAGCGGAGGGCTGCGGTCACAGTGGTCACACTGGTCTCGCTATACCTGCTGGTCTTT GGGTTGGACAATGGGCTGTGGGTGTACACGCTCACTGTTTCACATACGCTGAGCTCCGCGCTGATC ACTGACCTCCGGCTCTTCTTCACCTCACTGTACACTGCCGTCAGCCCACTCCTGATCCTCGTCTCCA ACACACGGCTGCGCTGCGGGAAACAGCCAGAGACCATGCACTGA
Dr ORA3	TGGCGCCTCAAAAGAAACCCGTGAACATCAGTCAGCGTATCACTTCATCTCCACTCCACATCATGC TGTACGTGGTTCTGGTGCTGCTGGGGAACGCGGGGAACACTACGGTGATCGCGGTGGTCGGACAGA GCCTTCTGCAGGAGACGGGAACCGTGCGGAGCTCGGATGTCATTCTGGTCAACATGGCTTTCTCAA ACCTGATGGTGTCGTTGTTGAGGAACACAGTCCTGATGGTGTCCGACCTGGGAGTGGAGATTTTCC TCAGTAGAGATATGTGTCAGTTCATGATGGGCCTGTGGGTTTGGGTCCGCTCTGCTAATGTCTGGTC GACGTTCTTTCTGAGCGCGTTTCACTTCCAAACCTTGCGTCGGGTCGCTCCTCCTGTCATCAACCTG CACGGCCCTCGCGGACCCCCACTATCCCTCATCCTGGGCTTCTGCCTCATCTGGAGCCTCAACCTGA TCTACTCCATCCCTGCCTTCATCT
Dr ORA4	ATGTCTGAGGTCCTGACGGTGGACGCGGTTCTCTTCGGCCTGCTGGTGTTCTCTGGTATCATTGGAA ACATCATGGTCATCTATGTGGTGTTTGACTGTGCTAAATTGTGCGCCTCTCGCCACCTGCCGCCGTC TGACACCATCCTGGTGCACCTGTGTCTGGCTAACCTGCTGACGTCAGTGTTCCGCACGGTGCCGATC TTCGTGTCGGACCTGGGCCTGCAGGTGTGGCTGACGGCGGGCTGGTGCCGCGTCTTCATGCTGCTGT GGGTGTGGTGGCGGGCGGTGGGCTGCTGGGTCACCTTGGCTCTCAGCGCCTTCCACTGCGCCACCC TGCGTCGCCAGCATGTCTCCATGGGGCCGCTGGGTCACTCGCGGGAGCGTCGCCGCGTCTGGGTCG TCCTGGCGGTGGTGTGGGCTGCAAACCTGCTGTTCTCGCTGCCGGCGCTGGTCTACACCACACAGG TGCGTGGGAACGCTACCGTGGAGCTGATGGTGATTAGCTGCACCAC
Dr ORA5	ATGCAGCTCCAAGACTGGGTTGAATCCTCAATCCGAGCCTTTTTCTGCGTTACAGGCATCACTGGGA ACTTCTGGCTGGCTCTGCGCTCTCTCCCTAGATCCAGATCCCGCTTGAGGCCGAATGACGTGCTCTT CATCAATCTGGCCGTCTCCAACCTCATCACCAACTGCATGGTGGACCTGCCGGACACTTTAGCGCA GTTTCTGAACAGCTGGCTGTTAAGCAGAAACTACTGCAGCGTGCTCCAGTTTTCATCAGACCTCTCG GAGACCAGCAGCATCTTCTCCACCATGTTCATCACCCTGTACTGGCACCAGAAGCTGGTGGGCTCC GTCCGGCGCGGTGGAGCCCCGGTGCAGCTGGACAATCTCCGGCTGGTGCTCTGGCTGCTGCTCGGG AGCTGGATGGTGGCGTTAACATTCAGCGTCCCCCACTTCTTCATAGCTGAGCACGACGGAAATGAC AGTTTGGAGGTTTGTGAGGAAAAATTCCCAACTCC
Dr ORA6	ATGGTGATGGAGCAGATACAGGTGAATCTCCTCTCTCTGCGCCTCTTCATCTCCATCATTGGTGTTG TGGGAAACACACTGCTCCTGGTCTCCATCCTGCACACACACACACACCTGAAGTCGTTCGAGCTGT TCCTGCTGGCTCTGTGCTCCGCCAACCTGCAGCAGCTGGTGATGGTGGATGTGTATGATGTTCTGCT GCTGTGTTCTCCGTCCTGCATCGGTGTGTGTTCCTGCCGCGCGCTGCGCTTCCTGACGGTGTTCGGG GAGGTCTGCAGCGTGCTCTTCACCGCACTCATCAGCATCTACCGTCACCAGAAGCTCCACGACGTG TTCTCGCATGTGAACGTGCCCGTGCTGCTGGACAGCCTGCGCTGGGCGGTGTGTATGTGTGTGCTGT GTGTGTGTGTGGCGCTCGCCTTCGGTCTGCCCACGCTCCTGGTTAACACACACTGGAGTGTCTCCAA CTCTTCGCTCGAGCGCTGCCCGGTGGACTTCTTCCAGTGCCCGTCCTCCTCCCCGTGTCTCACACAC ATCTACAAGTACGTGTTCCTGCTGGTGTGTGTGGTGCTGCCGCTGCTGGTCGTCACGGTGACGAGTG TGCT

This allowed us to find regions of similarity between biological sequences. In the blast database (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi), protein blast, Blastn, and highly similar sequences tab were selected. BLAST permitted direct comparison of the nucleotide sequences from Saraiva and Korsching (10) and the candidate *ora* gene sequences from our transcriptome database. After the percentage similarity was obtained from compared genes, individual olfaction genes were analyzed to accept and reject candidates. Olfaction receptor genes allowed us to determine if there were any changes in olfactory genes based on increase and decrease in expression found in individual genes. Furthermore, the ENSDART number for *oras* 1-5 was found in www.zfin.org and put into Cofactor in order to compare expression levels and fold changes for all oras for each of the treatments. For our data analysis, descriptive statistics were used to summarize data from samples using the mean and standard error.

Results:

Bioinformatics analysis data for females, males, control, and 11-KT treated juvenile male zebrafish was analyzed and compared. Table 1 was provided by Cofactor and indicates the variability of the samples within their respective treatments. This information is graphically depicted in Figure 1 A-F. For the majority of them there is almost a 1 to 1 relationship; however, C shows that on treated day 7 there was not as much correlation as the other samples. Table 1 shows that control 7 days was 0.91 and its correlation to treated 7 days decreased dramatically to 0.65. This could be because day 7 is a time of huge developmental change for juvenile males where some fish are responding more to the treatment than others. After figure 1C, figure 1 D-F show almost 1 to 1 relationship again. Thus, it can be concluded that the replicates for each one of our treatments gene expression is similar.

Table 1: Correlation Matrix for treatment replicates.

Treatments	Correlation
Control 7 Days	***0.91***
7 Days	***0.65***
Control 14 Days	***0.88***
14 Days	***0.88***
Female	***0.89***
Male	***0.81***

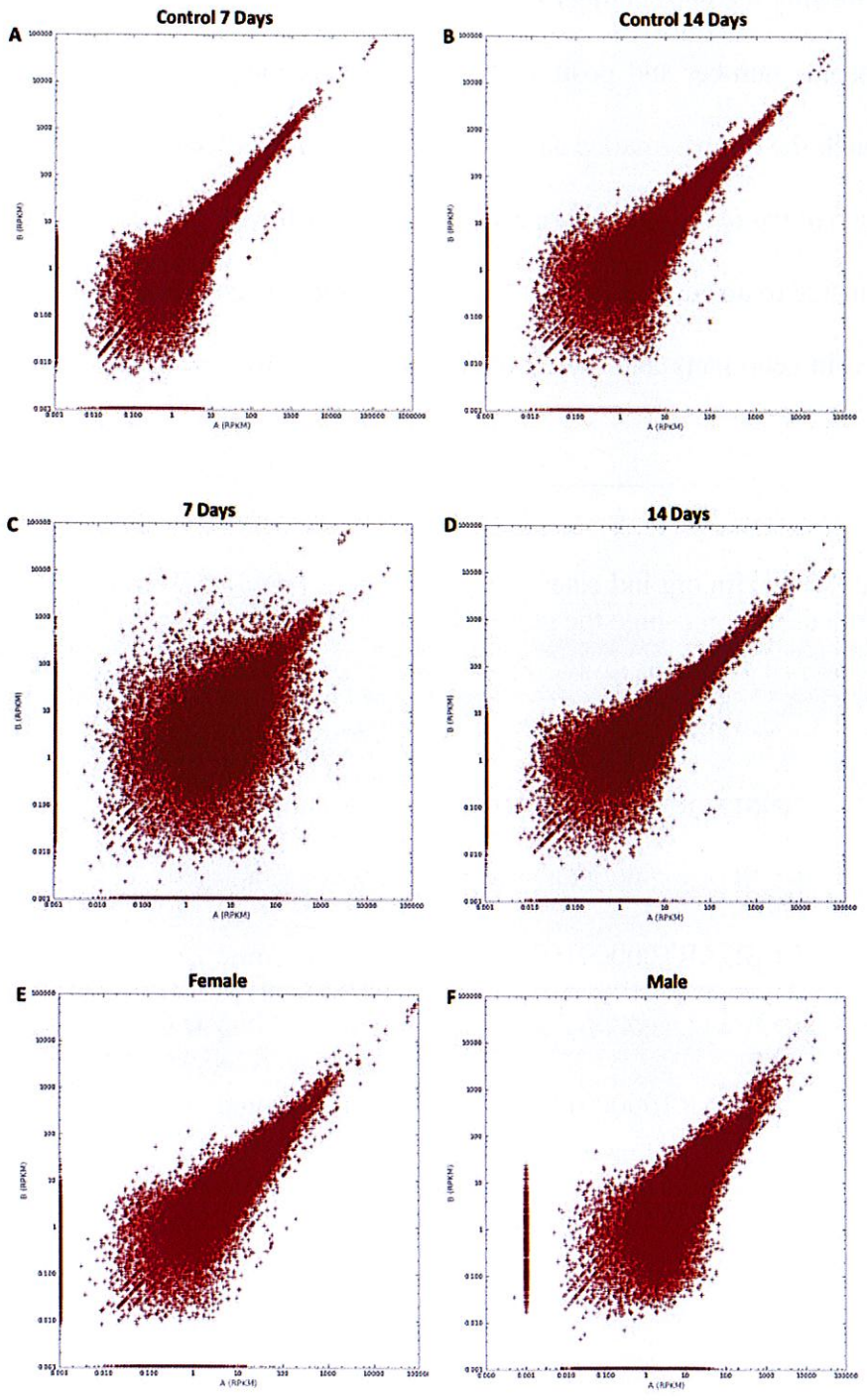

Figure 1: Gene expression/correlation plot for treatment replicates. A is for control group 1 for week 1 (7 days). B is for control group 2 for week 2 (14 days). C is for 11-KT-treated juvenile fish (N=3 fish) for 7 days. D is for 11-KT-treated juvenile fish (N=3 fish) for 14 days. E is for adult females (N =11) and F is for adult males (N=10).

We used zfin.org to identify the gene number for each of the six olfactory receptors (*ora*'s) in addition to their chromosome number and position on the chromosome (see Table 2). This information was used to search the bioinformatics database obtained from Cofactor to determine the level of expression of each of the *ora*'s (see figure 2). Treating the fish with 11-KT, may have caused over expression compared to an adult as seen in figure 2D. It should also be noted that *ora* 6 has not yet been annotated in gene bank and therefore expression data for *ora* 6 could not be obtained at this time.

Table 2: Data obtained from www.zfin.org indicates the gene number (ENSDART number) for each of the *ora*'s in addition to the chromosome the gene is found on and the position.

ora	ENSDART number	Chromosome # and position
***ora* 1**	ENSDART00000171954.1	Chromosome 22: 222,601-223,698
ora 2	ENSDART00000050910.4	Chromosome 5: 60,871,325-60,886,924
ora 3	ENSDART00000082406.4	Chromosome 22: 376,322-378,506
ora 4	ENSDART00000160884.1	Chromosome 22: 369,734-375,538
ora 5	ENSDART00000114666.2	Chromosome 10: 1,562,858-1,563,357
***ora* 6**	ENSDART00000110558	Chromosome 20: 54,754,854-54,755,756

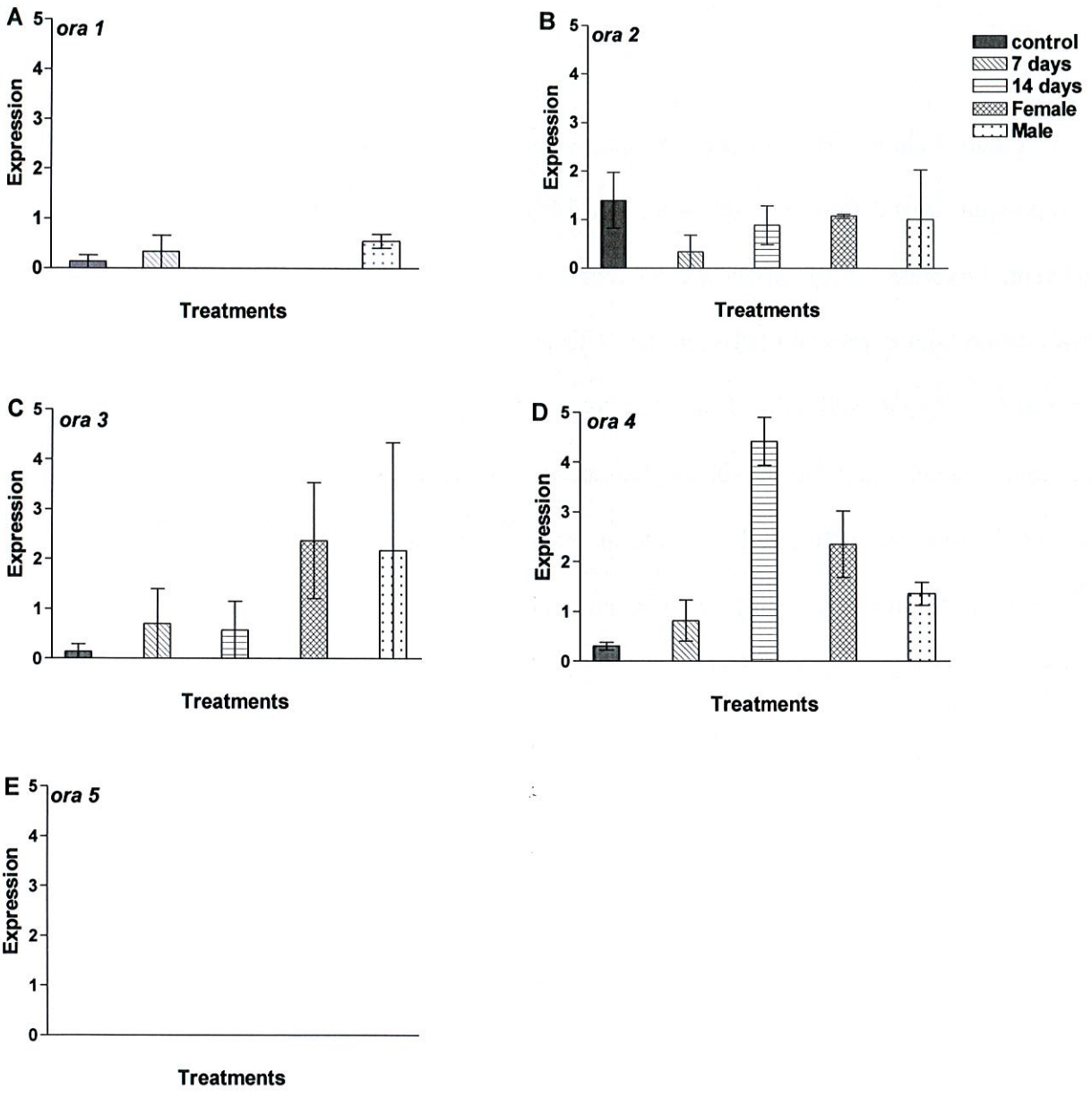

Figure 2: Gene expression (± standard error) is shown for each of the *ora*'s. A-E.
A shows gene expression for control group, 11-KT-treated juvenile fish (N=3 fish) for 7 days, adult females (N =11) and adult males (N=10). B-D show gene expression for control group, 11-KT-treated juvenile fish for 7 days, 11-KT-treated juvenile fish for 14 days, adult females and adult males. E shows no gene expression for control, 11-KT-treated juvenile fish for 7 or 14 days, adult females, and adult males.

Figure 3 shows differential expression when comparing control and treatments samples in the following: *ora* 2 for day 7, *ora* 4 for day 14, female, and male, *ora* 3 for female and male. Differential expression was also seen between treatments and samples for the following: *ora* 2 shows differential expression between day 7 to female and male. *Ora* 3 shows that treated are different from female and male. Thus, there is a differential expression between day 7 and 14 to male and female. Likewise, C shows that this might be an adult receptor. *Ora* 4 shows no deferential expression between day 14 and male. Also, there is no differential expression for day 7 to females. D shows that 14 days, female, and male are different from the control.

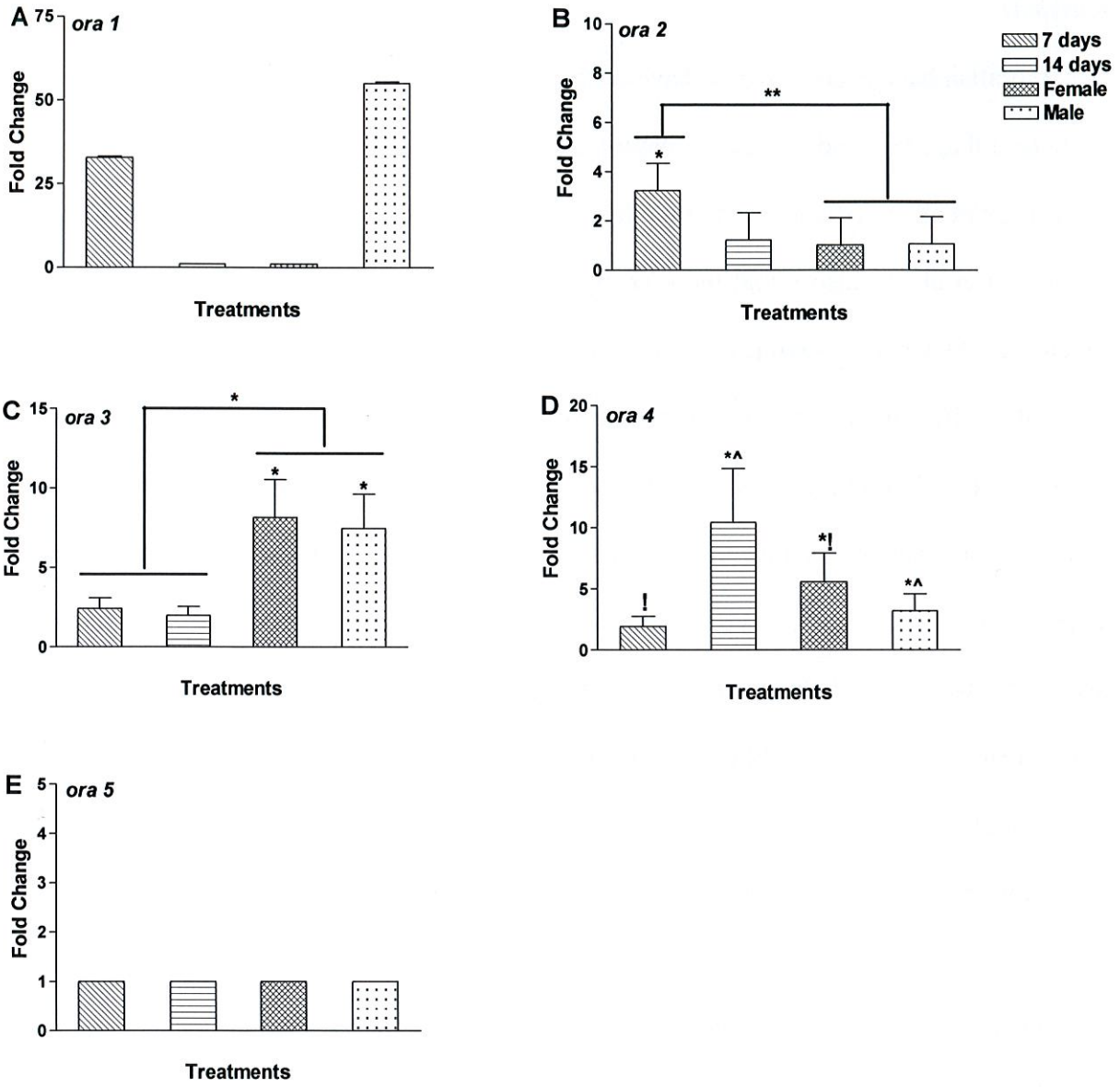

Figure 3: Bar graphs A-E show fold change data. B-D show a star* above the bar, this represents deferential expression from the control. B shows two stars above bar ** to indicate differential expression between groups. B and C have bars that represent groups that are differentially expressed from each another. D shows a variety of symbols. The star * and hat ^ above the bar show no difference between the groups that have a star and hat. Exclamation mark ! above bar show no difference between the groups with that symbol. 14 days, female, and male have no fold changes that are significantly different than the control. If there is no symbol above bar this indicates that there was no differential expression from control or between groups (A, E).

Discussion:

Zebrafish have been shown to have differential olfactory responses to odorants (putative pheromones) that are based on sexual maturity. Furthermore, Belanger et al. demonstrated that these changes in olfactory sensitivity could be induced by treatment with 17α methyltestosterone (7). Cardwell et al. (9) also found these changes occurred in other Cyprinids after 12 days of treatment with 11-ketotestosterone (11-KT). It was therefore hypothesized that exposing juveniles would lead to differential expression of one or more of the olfactory receptors (*ora*'s) following treatment of juvenile zebrafish with 11-ketotestosterone. Bioinformatics analysis of the olfactory epithelia of control and treated juvenile zebrafish and adult male and female zebrafish do not match the hypothesis (Figures 2 and 3). We hypothesized that the expression levels of the *ora*'s that may be responsible for differential detection of putative pheromones should be significantly increased in 11-KT-treated juveniles after 14 days of treatment. Further, expression levels of fish treated for 14 days should be similar to adult males. Barth et al. (6) showed that *ora* expression is asynchronous and highly regulated by cues during development in larval zebrafish. We did not find any *ora*'s where the treated juveniles expressed significant increases in expression when compared to control fish and where the expression levels match that of adult males.

When examining *ora* expression levels, we were not able to present data for all of the *ora*'s as *ora* 6 has not yet been annotated in the zebrafish database and is still reported as a suspected *ora* in the online zebrafish genomics bank (www.zfin.org). Saraiva and Korsching (10) suggest that *ora* 6 may have been overlooked in the past due its low sequence identity. It is possible that *ora* 6 may be differentially expressed in 11-KT-treated juvenile fish; however, we are unable to confirm that based on the current data available. For the *ora*s (*ora* 1-5) that we were able to examine expression level show that there was no single *ora* expression pattern that matched our

hypothesis (see Figure 2). After treatment with 11-KT, expression levels did not seem to show an increase; however, *ora* 4 displayed an increase in the 14 day treatment when compared to controls (Figure 2D). This increase in expression for *ora* 4 in the 14 day 11-KT treatment was more than the expression levels for both males and females which seemed similar to one another. Overall, when examining the *ora* expression levels, we did not find an *ora* expression pattern that matched our hypothesis.

Statistical analysis was available for the cofactor data for fold change increases (Figure 3). When compared to the control, there was a significant increase in fold change for *ora* 2 when 11-KT-treated juveniles (7days) were compared to control fish and both adult males a female zebrafish (Figure 3B). Furthermore, *ora* 3 showed an increase in fold change expression when compared to the controls and both the 7 and 14 day 11-KT-treated juveniles (Figure 3C). When fold change expression was examined for *ora* 4, zebrafish treated with 11-KT for 14 days and adult female and male displayed significant increases when compared to control zebrafish. There was no difference in fold change when female fish were compared to juveniles treated for 7 days and between juveniles treated for 14 days and adult male zebrafish. Lastly, there were no difference in fold change expression for ora 1 and 5 (Figure 3A,E). None of these comparisons matched our predictions; however, *ora* 4 did display some differential fold change expression patterns that warrant further investigation.

Overall, our results do not show any changes in expression of any of the *ora*'s investigated. This may be due to many factors including the type of androgen used. Belanger et al. (7) used 17α methyltestosterone in their study and we used 11-KT in this study. Although Cardwell et al. (9) showed that similar increases in olfactory sensitivity are seen following treatment with 11-KT in Cyprinids, followed up electroolfactogram experiments need to be performed on juvenile zebrafish

using 11-KT. Another reason for not detecting changes may be due to RNA quality. As seen in Table 1 and Figure 1D, there is considerable variation in expression data for all genes for zebrafish treated for 7 days with 11-KT. During the analysis, we had to send more RNA to Cofactor because the quality of the samples initially sent was not good. We should have converted mRNA to cDNA right away because cDNA is more stable, whereas mRNA degrades more quickly. This might have affected some of the results. Sample size could have also affected our results too, since the larger the sample size the smaller the variability. We were only able to have two samples analyzed for each treatment due to cost of this analysis. Unfortunately, we were unable to examine data for *ora* 6. Further research may show that *ora* 6 was the receptor being affected by our treatment of 11-KT, but because *ora* 6 has not been annotated we cannot find the expression of it yet. Nonetheless, research doesn't always work out the way we think it will work out and it might be that it's not 1 receptor, but 2 receptor types working together. Barth et al. (6) showed there can be changes based on development of olfactory receptors that may have in waves following embryonic development. Future experiments to look at protein analysis using a Western blot may be combined with quantitative PCR to determine if changes in *ora* expression may be happening in post-transcription.

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