

USING RNA-SEQ TO STUDY THE SEX-ROLE REVERSED GULF PIPEFISH:
ARE PATTERNS OF SEX-BIAS IN GENE EXPRESSION DIFFERENT
WHEN WE ARE DEALING WITH MR. MOM?

by

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I. Introduction

The family of fishes known as Syngnathidae (seahorses, pipefish, and seadragons) are a charismatic group of teleost fish that can be found in saltwater and brackish water ecosystems throughout temperate and tropical areas. This family of fishes exhibits male brooding where males carry the fertilized eggs either attached to their tail or within a pouch located on the abdomen. In addition, syngnathids exhibit a large range of mating systems which includes monogamy (Rose et al., 2014; Woodall et al., 2011), polygynandry (both sexes taking on multiple partners; Jones and Avise, 1997b; Hübner et al., 2013), polygyny, and polyandry. This wide range of mating systems creates an equally large range of sex-roles. A sex-role is based upon parental contribution and the limiting of one sex's reproductive success by the other sex through sexual selection (Darwin, 1871; Andersson, 1994; Clutton-Brock and Parker, 1992). A traditional sex role is where males compete for access to mates and females provide most of the parental care (Bateman, 1948; Emlen and Oring, 1977; Trivers, 1972). The opposite scenario, i.e. that males limit the reproductive success of females through mate choice, would cause female-female competition for mates (Trivers, 1972; Eens and Pinxten, 2000) which is known as sex-role reversal and is usually accompanied by increased male parental investment (Trivers, 1972). Sex-role reversal is generally very rare in nature but has been described in insects, fish, amphibians, and birds (Eens and Pinxten, 2000).

In Syngnathids, sex roles range from female choice seen in *Hippocampus fuscus* and *Hippichthys penicillus* (Vincent, 1994; Watanabe et al., 1997) to sex-role reversal in *Nerophis ophidion*, *Syngnathus typhle*, and *Stigmatopora nigra*, (Vincent et al., 1992; Wilson et al., 2003), as well as more complex situations than a clear conventional or reversed role (Rosenqvist and

Berglund, 2011). In Syngnathids, sex-roles and mating systems have been seen to have an effect on the amount of secondary sexual characteristics that a species will exhibit along with which sex will be exhibiting them (Vanpé et al., 2008; Björcklund, 1991; Jones and Avise, 2001; Berglund and Rosenqvist, 2003; Sogabe and Yanagisawa, 2008; Vincent, 1994). The role of sex-bias in gene expression in the evolution and development of sexually dimorphic traits has been studied in a number of different organisms (e.g. Mank et al., 2008; Huby et al., 2014; Sharma et al., 2014; Small et al., 2009; Prince et al., 2010), however, most studies of sex-bias in gene expression come from species where male-male competition for females was abundant. There is a general trend for most species to have more male sex-biased genes than female sex-biased genes (Ellegren and Parsch, 2007; Small et al., 2009; Zhao et al., 2011). It has been hypothesized that sexual selection upon males is mediating the evolution of these sex-biased genes (Swanson and Vacquier, 2002; Ranz et al., 2003; Connallon and Knowles, 2005; Small et al., 2009; Artieri and Singh, 2010). Therefore, a logical extension in a species exhibiting sex-role reversal would be more female-biased genes than male biased genes, especially if these patterns are influenced by sexual selection, where selection is directly connected to the acquisition of mates. The Gulf pipefish (*Syngnathus scovelli*), is a polyandrous species that exhibits sex-role reversal with male choice and is sexually dimorphic with females exhibiting bright stripes and a deeply keeled abdomen that is lacking in the males (Jones and Avise, 1997a; Brown, 1972). This species presents us with an ideal opportunity to study sex-bias gene expression in a sex-role reversed species.

Over the years, sex-bias in gene expression studies have uncovered several genes connected to different functions, including hormone biosynthesis, metabolism, circadian

rhythms, and immunity (Melo and Ramsdell, 2001; Roselli, 1991; Cai et al., 2016; Bur et al., 2009; Barna et al., 1996; Oertelt-Prigione, 2012). In addition, studies of sex-bias in gene expression have found patterns of gene expression to be tissue specific (Yang et al., 2006; Mank et al., 2008; Kawabata et al., 2012; Sharma et al., 2014). Here, we wanted to use next-generation sequencing approaches to study the transcriptome of the Gulf pipefish to look at sex-bias expression differences in the brain and gill. Next-generation sequencing approaches produce a lot of data, therefore, we decided to focus on three areas of interest in the sex-bias field of study: Sex steroids/hormones in the brain, the expression of genes connected to circadian rhythms in the brain, and immunity. The brain is a major component of the hypothalamus-pituitary-gonadal axis and the hypothalamus-pituitary-interrenal axis, both of which control the biosynthesis of sex steroids (Löhr and Hammerschmidt, 2011; Liley and Stacey, 1983; Liu et al., 2011). As such, differences in gene expression in the brain may have a direct link with the development of sexually dimorphic characteristics, and indeed previous studies have found patterns of sex-bias in gene expression in the brain for genes connected to sex steroids (Mank et al., 2009; Sharma et al., 2014; Liu et al., 2015). The brain also houses the central oscillator or “Master clock” for biological oscillations which controls biochemical, physiological, and behavioral changes that occur within a 24 hour time span in most living organisms (Reppert, 1998; Bell-Pedersen et al., 2005; Mistlberger and Antle, 2011). This biological clock is entrained to the solar day by photoreceptors that receive light and synchronize the central oscillator (Takahashi et al., 1993). Sexually dimorphic traits have been linked to circadian rhythms and genes connected to circadian rhythms have been found to show sex-bias expression (Bur et al., 2009; Lin et al., 2011; Honnen et al., 2016). Differences in

immuno-competence exist between males and females across several species (Hamilton, 1948; Falagas et al., 2007; Janele et al., 2006). The gill is the first organ to encounter pathogens from the environment and therefore is an ideal place to investigate this difference in a sex-role reversed species. There have been no sex-bias studies (to the best of our knowledge) that have looked at the gill, due in large part to its perceived lack of influence in the development of sexually dimorphic traits (compared to e.g. the brain, or gonads) which makes this study a unique exploration into uncharted territory.

Therefore, the objectives of my master's thesis were to build the first transcriptome of the brain and gills for the Gulf pipefish and then look at sex-bias gene expression differences. Some specific questions I wanted to answer were: 1. Is there sex-biased gene expression differences in the Gulf pipefish in the brain and/or gill? 2. If there is differential expression, do any genes pertain to sex steroids/hormones in the brain, circadian rhythms in the brain, or immunity? and 3. Are there differential functional pathways enriched in the brain and gill for the sex-biased genes?

II. Methods

Sampling

Samples of *S. scovelli* were collected from Port Lavaca Bay, Texas using hand nets on May 7 and 8, 2015. There were a total of 7 samples taken (4 males, 3 females). Males were identified by the visible pouch on the ventral surface of the body and females were identified by the bright stripes on the sides of the body and lack of a pouch on the ventral surface. All males were currently pregnant with eggs and one female discharged eggs while being

dissected. All fish were first held in a bucket of seawater from their environment for less than 10 minutes and then placed in a bucket with 1 gram Tricaine Methanesulfonate (MS-222) to approximately 1.5 liters of seawater until all movement had stopped. Dissections were performed directly after. All surgical equipment used for dissections was sterilized and treated with RNase-ZAP (Thermo Fisher Scientific) before dissection. Brain and both sets of gills were removed from each specimen and placed in their own tubes (i.e. brain in one tube, left and right gill in one tube) containing 500 μ L of RNA later (Ambion). The rest of the bodies for the first 7 samples were put into the University of Texas at Austin's collection. The sample IDs are TNHC-FT-847 through -853. The tubes containing the brain and gills were then placed on dry ice/ ice mixture for less than 48 hours until transferred to a -80°C freezer where they were held until extraction.

RNA Extraction and RNA-seq Library Preparation

RNA extractions followed the protocol suggested by Maxwell[®]16 simplyRNA kit. Tissues (brain and gills) were removed from RNAlater and placed in 200 μ L of cold homogenization solution. A sonicator was used to homogenize the tissues. All of the homogenate was transferred into a tube that held 200 μ L of lysis buffer held on ice. Samples were then loaded into the Maxwell[®]16 automated RNA purifier. Total RNA of each sample was checked by Nanodrop and gel electrophoresis for quantity and quality before being sent to UT Southwestern center in Dallas TX for Next generation sequencing on an Illumina Hi-Seq 2000 (Illumina Inc.). Samples were quality checked using a BIO-RAD (Experion). Any sample with a RNA integrity number (RIN) less than 8 was not sequenced. Each sample was given a unique

four base bar code, and then an equimolar pool was made. The pool was sequenced on two lanes using paired-end 100 base pair Illumina sequence chemistry.

Transcriptome Assembly

The returned sequences were matched and combined from the two lanes of sequencing. Using TRIMMOMATIC v.0.32 (Bogler et al., 2014), sequences had adapters removed, sequence ends with quality scores <33 trimmed (i.e. phred score of 33), and sequences <30 nucleotides in length removed. We constructed a de novo assembly from both gill and brain transcripts. The samples with the highest number of quality trimmed reads for both male and female from each tissue were combined (samples 1B and 6G for males, 3B and 8G for females) for *de novo* assembly using Trinity v.r.2012-10-05 with default parameters (Grabherr *et al.*, 2011). All quality-filtered sequences were then mapped back to the transcriptome using RNA-seq by Expectation Maximization (RSEM v.1.2.1 (Li and Dewey, 2011)). We used the number of quality-filtered sequences as a proxy for gene expression, with samples with more reads being more highly expressed than samples with a low number of reads. However, using the raw number of reads that mapped is subject to bias in both the total number of reads and contig length, longer contigs will naturally have more reads mapped to them than shorter contigs (Conesa et al., 2016). Therefore, we used transcripts per million (TPM) which normalizes raw counts to both variance in the number of reads per individual and contig length to determine gene expression (Li and Dewey, 2011; Marinov et al., 2014). We chose TPM over Fragments per kilobase of transcripts per million (FPKM, also known as RPKM) because it makes all contigs comparable without further calculations when it comes to comparing the relative amount of gene expression because the denominator is calculated the

same for all samples instead of individually for each sample (Li and Dewey, 2011). Lastly, we removed contigs that were lowly expressed. Although this may result in removing transcripts that are important in the development of sex-specific phenotypes, it is likely that such contigs represent rare alternative splices, and /or errors in the assembly. Therefore, we removed contigs that had a TPM of less than one in at least three of the sequence samples. This was done separately for both the gill and brain assemblies.

Annotation

The remaining sequences were annotated against the uniprot database (Accessed January 2016, <http://www.uniprot.org/>) and the Zebrafish database (Accessed January 2016, <http://zfin.org/>) using NCBI BLASTX v.2.3.0+. A maximum e-value for successful annotation of 1.0^{-10} was used for both BLASTs. All BLAST analyses were performed on the Mason server at Indiana University. XML output from BLAST was uploaded into Blast2Go v 3. 2 (Conesa et al., 2005). Gene Ontology (GO) terms associated with the BLAST hits were downloaded, and used for a GO enrichment analysis (Fisher's Exact Test) to determine if any GO terms that had DEGs were enriched compared to GO terms associated with non-enriched genes. We also compared our assembly with a recently published brood-pouch transcriptome from the big-bellied seahorse (*Hippocampus abdominalis*: Whittington et al., 2015). NCBI BLAST software was used for this comparison as well with a maximum e-value of 1.0^{-20} , a minimum sequence similarity of 90%, and a one-to-one match between contigs (i.e. each contig did not produce more than one alignment to another contig).

Statistics

Differential gene expression was calculated using EdgeR (Robinson et al., 2010), which is a pre-designed software package in R format. EdgeR fits a negative binomial distribution to the TPM data in order to account for differences in the number of sequences between samples. In EdgeR, samples are further filtered by converting the TPM to counts per million where they are further filtered to at least one CPM in at least 3 samples. A Fisher's exact test was used to determine differential expression separately for the brain and gill samples, a Benjamini-Hochberg false discovery test with alpha set to 0.05 was used to correct for multiple testing (Benjamini and Hochberg, 1995). We only used contigs that were annotated in edgeR, however, we did run edgeR three different ways. We ran both a swissprot annotated set of contigs in edgeR and a zebrafish annotated set of contigs. We then ran a combined annotation set for swissprot and zebrafish with duplicates removed, which is the set that we used to further analyze DEGs. We chose to use this set for further analysis for simplicity and because of time restraints.

Validation

We had intended to validate our results using quantitative real time reverse transcription PCR (qRT-PCR) like many other papers have done in the past, however, the next generation sequencing used up most of our RNA and so there were only 2 male brain samples left, 1 female gill sample, and 3 male gill samples left after RNA-seq. Although next generation sequencing is still a fairly new molecular method, it has been used extensively since its introduction, and time after time it has been proven to work (Ramayo-Caldas et al., 2012; Dunning et al., 2013; Castellanos-Martinez et al., 2014; Seaman et al., 2015; Nie et al., 2016;

Kelly et al., 2016; Zorrilla-Fontanesi et al., 2016). Due to all the previous validation done with next generation sequencing we feel that our results are still valid despite not being able to do qPCR. There are a number of papers that have been published without qPCR validation for the same fact that NGS has been proven to work (Small et al., 2013; Perry et al., 2014; Liu et al., 2015; Rose et al., 2015; Whittington et al., 2015).

III. Results

Transcriptome assembly and filtering results

To allow for quantitative comparisons, each sample was individually barcoded and the average number of raw reads for individuals was about 25 million raw reads (see Table 2 for all individuals). The average number of trimmed raw reads for brain was 22, 877, 838 with an average of 73% mapping back to the assembly and the average number of trimmed raw reads for the gills was 27, 934, 575 with the average percent of reads mapping back at 74%. After quality filtering there was a combined (i.e. brain and gill) total of 168, 264 contigs that mapped back to the assembly with a total length of about 178 megabase pairs. After further filtering and removal of lowly expressed contigs, there were 65, 552 contigs left for the brain and 57,564 contigs left for the gill with 41, 757 of these contigs overlapping between brain and gill leaving a total of 81,361 unique contigs (Figure 1) with a total length of about 126.9 megabase pairs.

TABLE 1: TABLE OF READS AND MAPPING. List of individuals and which tissue (B=brain and G=Gill). Reads before trim refers to raw reads from sequencing, % reads after trim refers to trimming done by Trimmomatic, and % mapped back to assembly refers to reads mapped back to assembly by RSEM.

Sample ID	Sex	number of reads before trim	% reads after trim	% reads mapped back to assembly
1B	M	21,782,575	99.78	73.80
1G	M	26,751,544	99.76	74.61
2B	M	20,703,070	99.75	72.18
2G	M	27,152,903	99.77	74.77
3B	F	20,939,856	99.77	73.05
3G	F	30,704,805	99.77	74.84
4B	F	27,690,177	99.76	73.56
4G	F	27,140,208	99.75	71.47
5B	M	21,720,826	99.77	73.39
5G	M	26,240,651	99.77	73.75
6B	M	20,675,807	99.77	72.53
6G	M	27,383,524	99.78	75.13
8B	F	26,997,584	99.77	73.28
8G	F	30,617,729	99.77	75.21

Annotation and alignment to seahorse assembly

There were more unique contigs annotated from the Zebrafish database, we believe this was most likely due to pipefish being more closely related to the Zebrafish than many of the species recorded in Swissprot. Blasting the remaining filtered contigs against the Swissprot database (Uniprot) yielded a total of 519,247 hits with 22,466 unique contigs annotated. Of these unique contigs annotated, there were 22,338 contigs associated with GO terms and a total of 306,631 GO terms for these contigs. When blasted against the Zebrafish database a

total of 407,109 hits with 34,943 unique contigs annotated. Of those contigs annotated there were 31,686 associated with GO terms and a total of 217,918 GO terms for these contigs. We used a comprehensive combined list of annotated contigs to do our DEG analysis in R. There was a total of 77,132 contigs annotated from both databases when combined. Of those, 37,480 were duplicate contigs leaving a total of 39,652 unique annotated contigs to be used in analysis.

TABLE 2: TABLE OF BLAST RESULTS FOR ANNOTATION AND GO TERMS. Showing number of blast hits for contigs for each database, Swissprot and Zebrafish, and number of GO terms for each database.

Database	# of blast hits	# of unique contigs annotated	# of contigs annotated with GO	Total GO terms for annotated contigs
Swissprot	519,247	22,466	22,338	306,631
Zebrafish	407,109	34,242	34,943	31,686

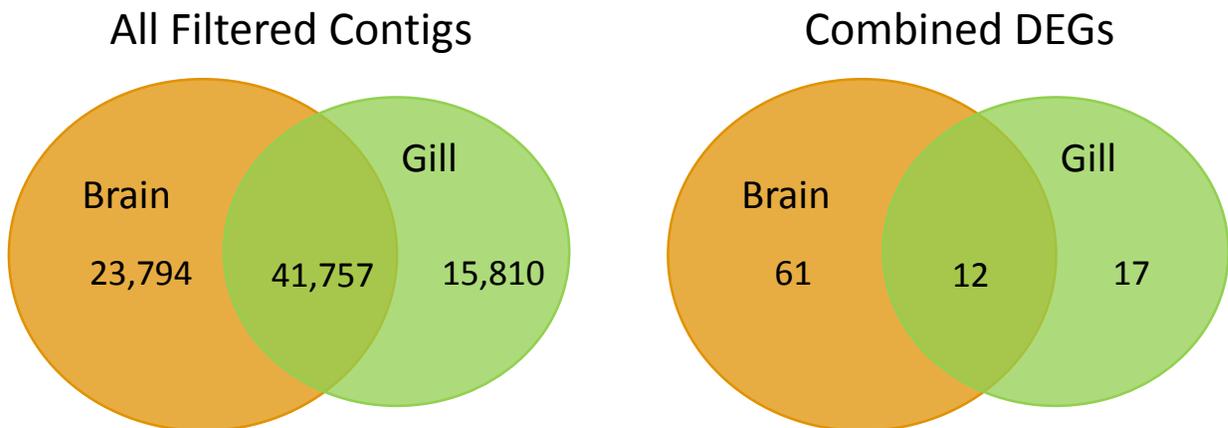


FIGURE 1: VENN DIAGRAMS SHOWING TOTAL CONTIGS AND DEGs. Venn diagram on the left shows break down of filtered contigs left after trimming and filtering through Trimmomatic and RSEM. Total contigs for brain is 65,551 and for the gill is 57,567 with 41,757 contigs that overlapped between the two tissues. There was a total of 81,361 unique contigs. Venn diagram on the right shows total number of differentially expressed genes found using a combined set of annotated genes from both databases in edgeR. Total for brain was 73 and total for gill was 29 with 12 upregulated in both tissues.

We were given the opportunity to use the seahorse database created by Whittington et al. (2015) to blast our transcriptome against. The seahorse database was not annotated but

produced 41,023 direct matches (nucleotide matches) without duplicates with a minimum of 85% match and 250 bp length alignment.

Differentially expressed genes and GO term enrichment analysis

More differentially expressed genes (DEGs) were found in the brain (73 for the combined swissprot and zebrafish annotated gene list) in comparison to the gills (29; see figure 1). There were also more DEGs with male up regulation found overall (66 in total for males for brain and gill vs 36 in total for females for brain and gill; see figure 4). Cluster analysis shows a very complex grouping of DEGs based on their relative abundances for both the brain and gills (see figure 4 and 5). All DEGs reported from here on out are based on an FDR of <0.05 . We acknowledge that some papers determine DE using a log fold of ≥ 2 , however, due to the low number of DEGs returned for both brain and gill and the fact that we are using whole brain and our signal to noise ratio was most likely low, we decided to use all genes determined DE by an FDR of < 0.05 . When we conducted the DEG analysis using the Zebrafish only annotated list, the analysis returned 301 DEGs in the brain and 226 DEGs in the gill. The Swissprot only analysis returned 36 DEGs in the brain and 21 DEGs in the gill. In the combined annotation for the brain, there was a total of 73 DEGs (Figure 1). Of these DEGs, 26 were up-regulated in females and 47 were up regulated in males (Figure 4). In the gills we found 29 DEGs from the total combined swissprot and zebrafish annotated genes list (Figure 1). Ten of these genes were up-regulated in the females and 19 were up-regulated in males (Figure 4).

We did an enrichment analysis on our GO terms using a Fischer's exact test which returned 52 enriched GO terms in the brain and 25 enriched GO terms in the Gill (see tables 4

and 5). In the brain 41 of the enriched GO terms were over represented, meaning that they appeared more than expected by chance in the DEGs, and 11 were under represented, meaning that they appear less than expected by chance in the DEGs. In the Gill, there were 22 GO terms over represented and 3 under represented.

TABLE 3: ENRICHED GO TERMS FOR DIFFERENTIALLY EXPRESSED GENES IN THE BRAIN. GO terms are grouped by broad categories: C= cellular component, F= molecular function, and P= biological process. Test= the number of times a DEG was annotated with a specific GO term, Ref= the number of times a gene that was not differentially expressed was annotated with a specific GO term, Over/Under= if the GO term is present more times in the DEG than expected by chance (over), or fewer times than expected by chance (under).

GO-ID	Term	Category	P-Value	#Test	#Ref	Over/Under
GO:1990904	ribonucleoprotein complex	C	2.04E-04	7	609	OVER
GO:0030529	intracellular ribonucleoprotein complex	C	2.04E-04	7	609	OVER
GO:0005840	ribosome	C	2.04E-04	7	609	OVER
GO:0005829	cytosol	C	2.82E-02	31	1137 6	OVER
GO:0005730	nucleolus	C	3.89E-02	12	3386	OVER
GO:0005739	mitochondrion	C	4.95E-02	18	5835	OVER
GO:0003735	structural constituent of ribosome	F	3.18E-06	7	313	OVER
GO:0016740	transferase activity	F	4.50E-04	1	5022	UNDER
GO:0005198	structural molecule activity	F	1.04E-03	11	1874	OVER
GO:0016772	transferase activity, transferring phosphorus-containing groups	F	2.88E-02	1	2986	UNDER
GO:0019843	rRNA binding	F	2.90E-02	2	133	OVER
GO:0016301	kinase activity	F	4.23E-02	1	2745	UNDER
GO:0001071	nucleic acid binding transcription factor activity	F	4.24E-02	1	2731	UNDER
GO:0042254	ribosome biogenesis	P	7.38E-04	7	757	OVER
GO:1901361	organic cyclic compound catabolic process	P	2.15E-03	9	1453	OVER
GO:0046700	heterocycle catabolic process	P	2.15E-03	9	1453	OVER
GO:0034655	nucleobase-containing compound catabolic process	P	2.15E-03	9	1453	OVER
GO:1901575	organic substance catabolic process	P	2.15E-03	9	1453	OVER
GO:0019439	aromatic compound catabolic process	P	2.15E-03	9	1453	OVER
GO:0044248	cellular catabolic process	P	2.15E-03	9	1453	OVER
GO:0044270	cellular nitrogen compound catabolic process	P	2.15E-03	9	1453	OVER
GO:0034622	cellular macromolecular complex assembly	P	3.22E-03	6	731	OVER

GO:0071826	ribonucleoprotein complex subunit organization	P	3.22E-03	6	731	OVER
GO:0022618	ribonucleoprotein complex assembly	P	3.22E-03	6	731	OVER
GO:0022613	ribonucleoprotein complex biogenesis	P	4.31E-03	8	1318	OVER
GO:1901564	organonitrogen compound metabolic process	P	1.04E-02	12	2737	OVER
GO:0044403	symbiosis, encompassing mutualism through parasitism	P	1.23E-02	12	2863	OVER
GO:0044419	interspecies interaction between organisms	P	1.23E-02	12	2863	OVER
GO:0051704	multi-organism process	P	1.23E-02	12	2863	OVER
GO:0006518	peptide metabolic process	P	1.36E-02	8	1610	OVER
GO:0006412	translation	P	1.36E-02	8	1610	OVER
GO:1901566	organonitrogen compound biosynthetic process	P	1.36E-02	8	1610	OVER
GO:0034645	cellular macromolecule biosynthetic process	P	1.36E-02	8	1610	OVER
GO:1901576	organic substance biosynthetic process	P	1.36E-02	8	1610	OVER
GO:0044249	cellular biosynthetic process	P	1.36E-02	8	1610	OVER
GO:0043043	peptide biosynthetic process	P	1.36E-02	8	1610	OVER
GO:0043604	amide biosynthetic process	P	1.36E-02	8	1610	OVER
GO:0043603	cellular amide metabolic process	P	1.36E-02	8	1610	OVER
GO:0009059	macromolecule biosynthetic process	P	1.36E-02	8	1610	OVER
GO:0044271	cellular nitrogen compound biosynthetic process	P	1.36E-02	8	1610	OVER
GO:1902578	single-organism localization	P	1.53E-02	18	5161	OVER
GO:0044765	single-organism transport	P	1.53E-02	18	5161	OVER
GO:0051276	chromosome organization	P	3.10E-02	2	3841	UNDER
GO:0000902	cell morphogenesis	P	4.02E-02	8	7632	UNDER
GO:0032989	cellular component morphogenesis	P	4.02E-02	8	7632	UNDER
GO:0006810	transport	P	4.59E-02	34	1318 3	OVER
GO:0051234	establishment of localization	P	4.59E-02	34	1318 3	OVER
GO:0006996	organelle organization	P	4.73E-02	13	1063 7	UNDER
GO:0044085	cellular component biogenesis	P	4.76E-02	28	1038 7	OVER
GO:0051674	localization of cell	P	4.79E-02	5	5737	UNDER
GO:0006928	movement of cell or subcellular component	P	4.79E-02	5	5737	UNDER
GO:0048870	cell motility	P	4.79E-02	5	5737	UNDER

TABLE 4: ENRICHED GO TERMS FOR DIFFERENTIALLY EXPRESSED GENES IN THE GILL. GO terms are grouped by broad categories: C= cellular component, F= molecular function, and P= biological process. Test= the number of times a DEG was annotated with a specific GO term, Ref= the number of times a gene that was not differentially expressed was annotated with a specific GO term, Over/Under= if the GO term is present more times in the DEG than expected by chance (over), or fewer times than expected by chance (under).

GO-ID	Term	Category	P-Value	#Test	#Ref	Over/Under
GO:0044421	extracellular region part	C	6.02E-05	10	2867	OVER
GO:0005615	extracellular space	C	1.34E-04	9	2520	OVER
GO:0005886	plasma membrane	C	1.04E-03	18	11478	OVER
GO:0016020	membrane	C	1.04E-03	18	11478	OVER
GO:0071944	cell periphery	C	1.85E-03	18	11555	OVER
GO:0005576	extracellular region	C	3.44E-03	17	11059	OVER
GO:0005654	nucleoplasm	C	1.00E-02	2	9787	UNDER
GO:0005764	lysosome	C	1.63E-02	5	1829	OVER
GO:0000323	lytic vacuole	C	1.63E-02	5	1829	OVER
GO:0005739	mitochondrion	C	1.78E-02	10	5652	OVER
GO:0044444	cytoplasmic part	C	4.57E-02	22	20106	OVER
GO:0005737	cytoplasm	C	4.79E-02	26	26056	OVER
GO:0016491	oxidoreductase activity	F	1.75E-03	6	1600	OVER
GO:0016740	transferase activity	F	2.59E-02	0	4823	UNDER
GO:0003735	structural constituent of ribosome	F	3.21E-02	2	337	OVER
GO:0005488	binding	F	4.33E-02	12	20808	UNDER
GO:0016853	isomerase activity	F	4.44E-02	2	403	OVER
GO:0044281	small molecule metabolic process	P	1.03E-02	12	7285	OVER
GO:0006790	sulfur compound metabolic process	P	1.23E-02	4	1095	OVER
GO:0051186	cofactor metabolic process	P	2.50E-02	4	1357	OVER
GO:0030198	extracellular matrix organization	P	3.14E-02	4	1458	OVER
GO:0043062	extracellular structure organization	P	3.14E-02	4	1458	OVER
GO:0000003	reproduction	P	3.54E-02	9	5481	OVER
GO:0044710	single-organism metabolic process	P	4.36E-02	12	8311	OVER

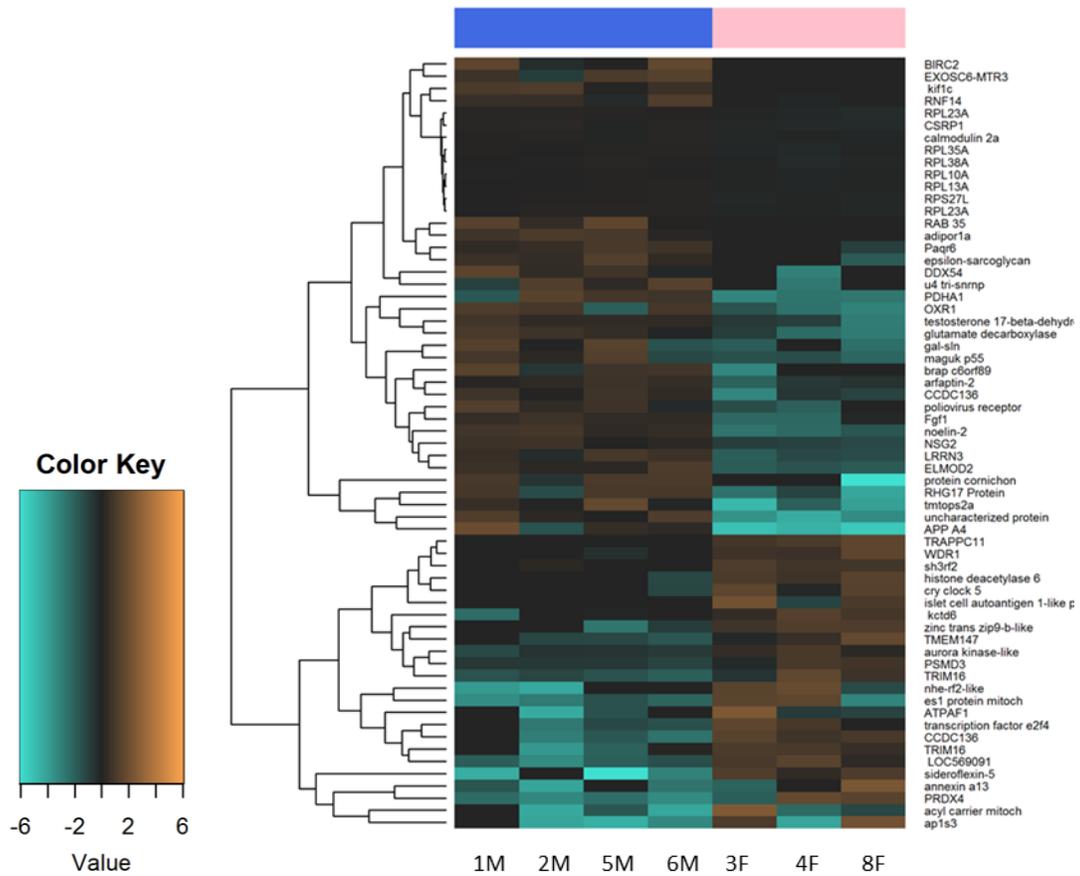


FIGURE 2: HEAT MAP FOR DEGs FOUND IN THE BRAIN. Map was created with the Log₂ of the TPM for each individual minus the Log₂ of the average TPM for each gene.

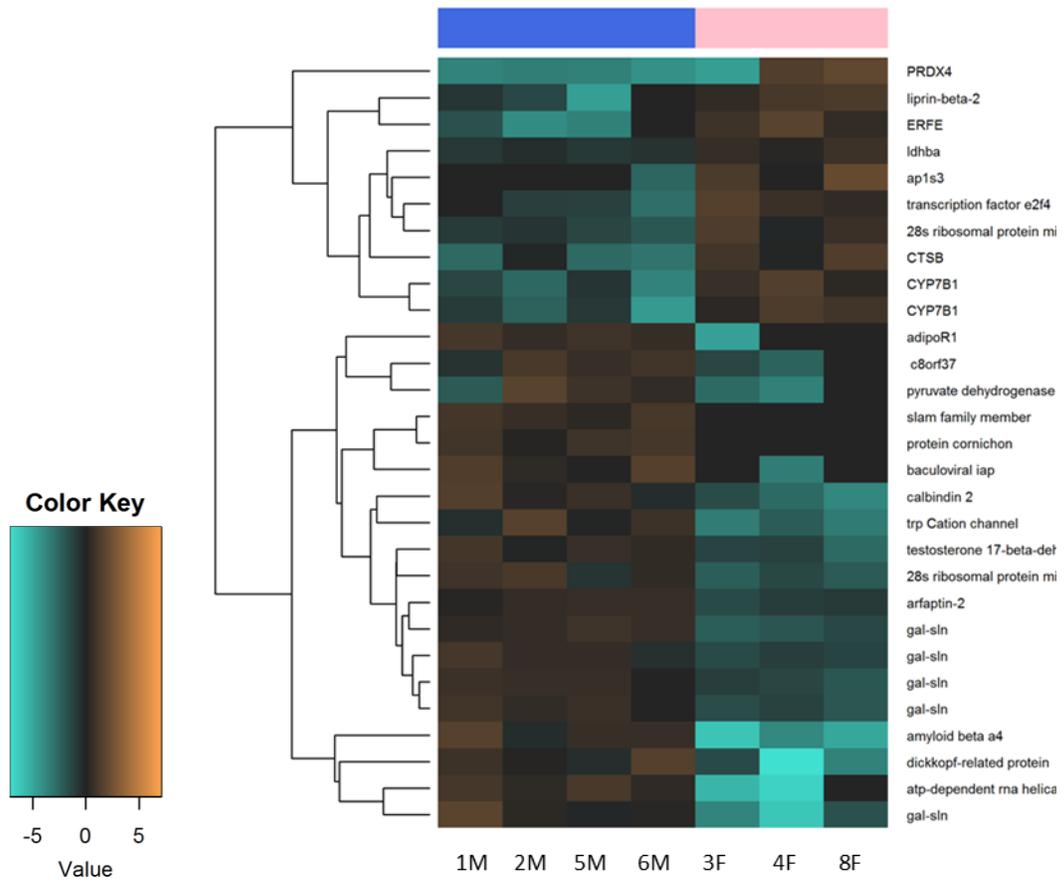


FIGURE 3: HEAT MAP FOR DEGS FOUND IN THE GILLS. Map was created using Log2 of TPM for each individual minus the Log2 of the average for each gene.

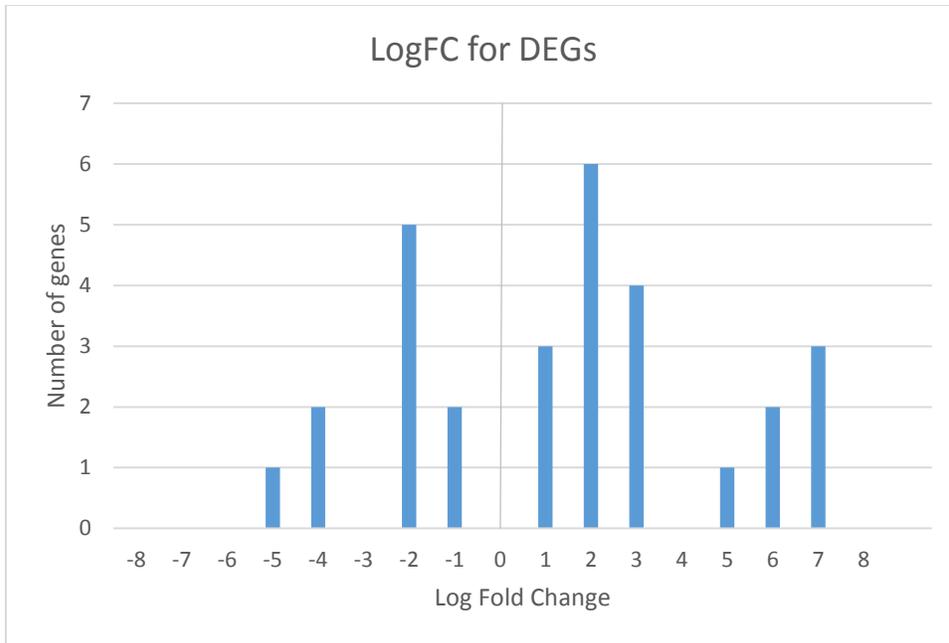


FIGURE 4: LOG FOLD CHANGE FOR DIFFERENTIALLY EXPRESSED GENES. Negative numbers represent fold change in females and positive numbers represent fold change in males.

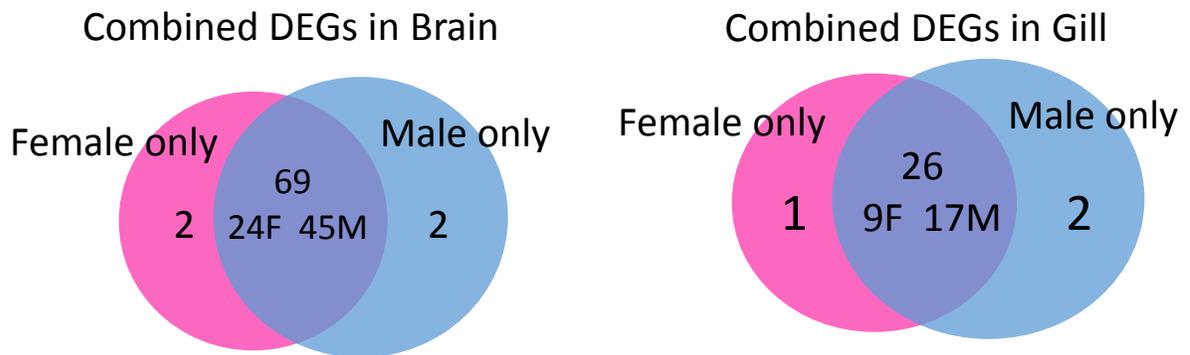


FIGURE 5: VENN DIAGRAMS FOR DEGs. Venn diagram on right shows differentially expressed genes found in the brain and whether they were male or female upregulated. Venn diagram on left shows differentially expressed genes found in the gill and whether they were male or female upregulated. In the center of both venn diagrams F and M stand for Female-biased and Male-biased respectively.

Sex-specific and sex-biased gene expression

Of the 26 genes found up-regulated in the brain of females (Figure 2), only two genes (islet cell autoantigen 1-like protein, Ica1l and trafficking protein particle complex subunit 11, Trappc11; See Appendix; Figure 5) were found to be female specific. Ica1l is involved in

immunity and Trappc11 is involved with Golgi transport. There were several female-biased genes involved in energy specifically involving the mitochondria and several other contigs possibly involved in immunity and stress response (Figure 6). In males, there were two contigs that were male-specific (exosome complex component mtr3 Exosc6, and kinesin-like protein kif1c; See Appendix) out of the 47 contigs up-regulated in male brains. The Exosc6 gene is involved in RNA processing and degradation whereas the kif1c gene is involved in Golgi to ER transport. There were two contigs that were expressed in males and lowly in only one female (baculoviral iap repeat-containing protein 2, ras-related protein rab-35-like). Baculoviral iap repeat-containing protein 2 is involved in regulating apoptosis and also functions in regulating the inflammatory signal and immunity. The ras-related protein rab-35-like is involved in GTPase activity (contributing to energy functions). Just a reminder, in figure 6 you will see the functional groups that I created to try and relay a “big picture” function for the genes, GO terms were used in creating these categories, however, these are not GO terms. There were several male-biased contigs involved in immunity, metabolism (specifically involving glycolysis), and cell signaling (Figure 6).

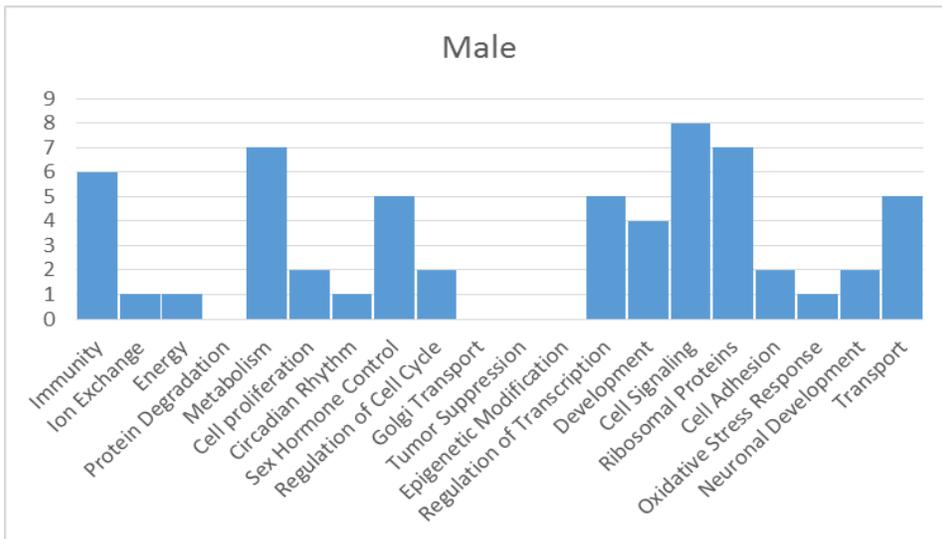
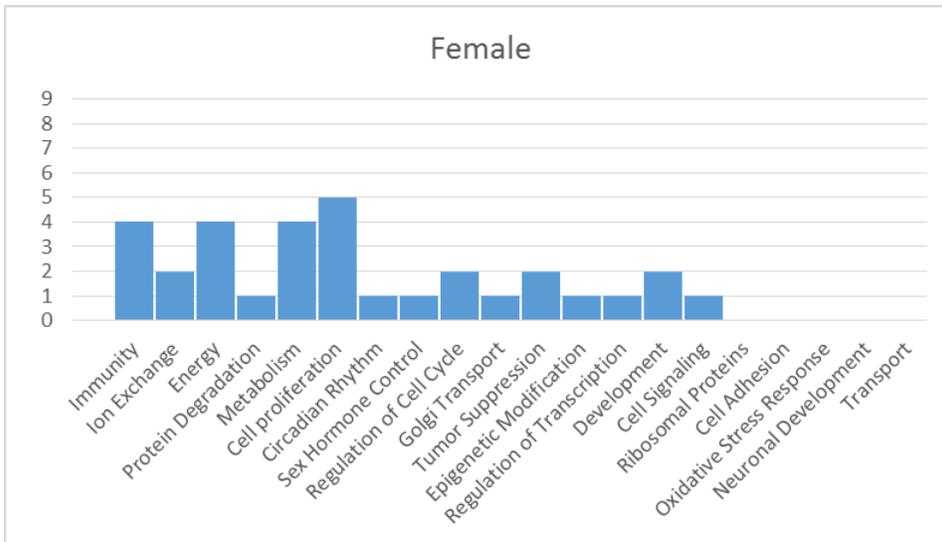


FIGURE 6: FUNCTIONAL CATEGORIES FOR BRAIN DEGs. Graphs show what functional categories DEGs fall into for the brain, top graph for females and bottom graph for males. These categories are not GO terms.

In the gills, there were 29 DEGs, ten of these were up regulated in females. Of the ten, only one was exclusively expressed in females which was ap-1 complex subunit sigma-3(AP1S3) (See Appendix; Figure 5) which functions in immunity. There were several female-biased contigs involved in immunity and then all other up-regulated genes had different functions such

as cell proliferation and tumor suppression (Figure 7). Out of the 19 DEGs up-regulated in males, two were male-specific (slam family member 9-like SLAMF9 and protein cornichon homolog 2 CNIH2; See Appendix). SLAMF9 functions in immunity and CNIH2 functions in cell signaling (Cruz-Munoz et al., 2009; Figure 7). Two other contigs were expressed in males and only in one female (baculoviral iap repeat-containing protein 2 and adiponectin receptor 1a). There were several male-biased contigs involved in immunity and then all other upregulated genes fell into only one category except for cell signaling and metabolism which each had two genes upregulated (Figure 7).

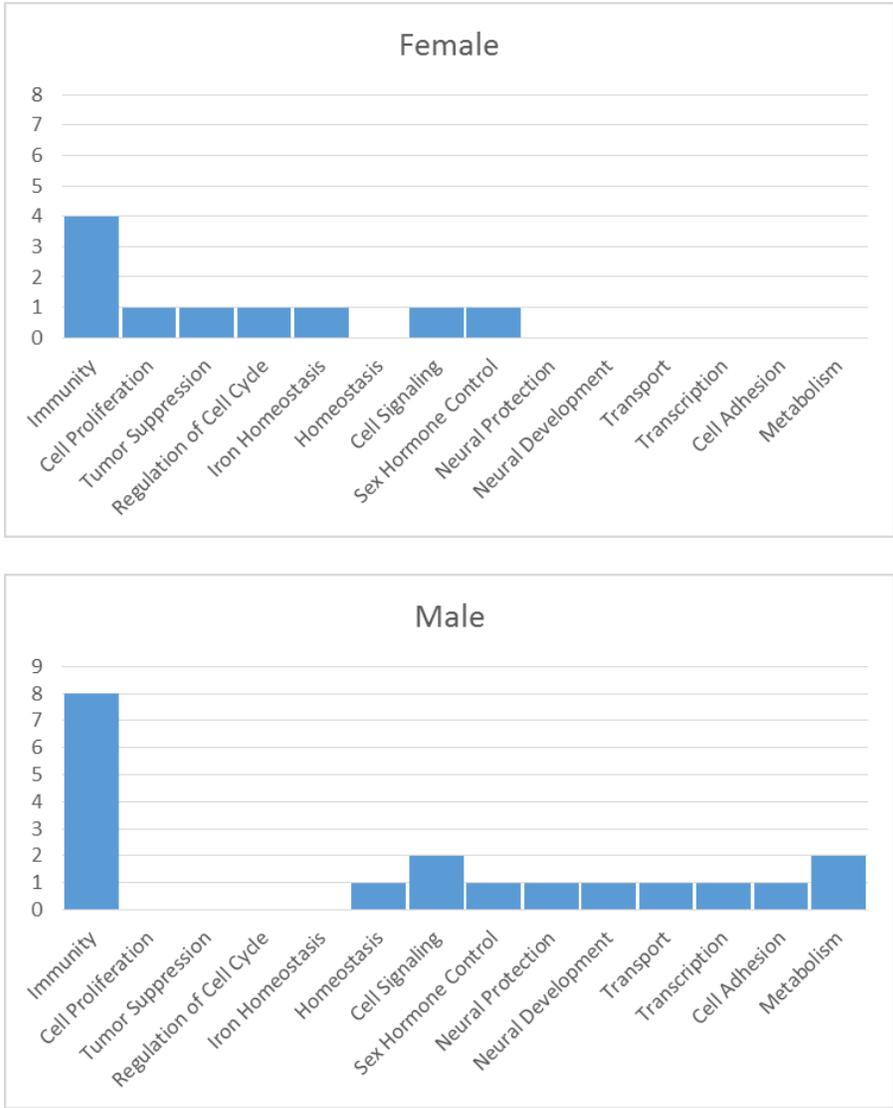


FIGURE 7: FUNCTIONAL CATEGORIES FOR GILL DEGs. Graphs show what functional categories DEGs fall into for the Gills, top graph for females and bottom graph for males. These are not GO terms.

IV. Discussion

In this study of the sex-role reversed Gulf pipefish, we found sex-bias in gene expression in both the brain and gills. The brain had more sex-biased gene expression (73 DEGs) than the gills (29 DEGs) along with more enriched GO terms (52 enriched GO terms in brain, 25 in Gill). Although there were more DEGs in the brain, only 0.18 percent of annotated genes (73 out of

39, 507) for the brain were found DE. In Liu et al. (2015), they looked at forebrain and midbrain and out of 115, 259 genes only 8 ($6.9 \times 10^{-5}\%$) were found DE in the blue headed wrasse (*Thalassoma bifasciatum*) vs. 20,183 out of 89,022 for the gonads (22.6%). In a study on mice, there were thousands of genes sex-biased in the liver, adipose, and muscle tissue and only hundreds differentially expressed in brain (Yang et al., 2006). It is also very likely that we did not detect all the DEGs in the brain due to our use of the whole brain. The brain is made up of several areas that specialize in different functions and since we were unable to isolate specific regions of the brain and it is known that specific areas of the brain will have sex-biased expression (Melo and Ramsdell, 2001; Huby et al., 2014; Roselli, 1991) it is likely that the number of DEGs reported is far fewer than if we were able to isolate the different compartments of the brain. In the brain, some of the top enriched GO terms included: Structural constituent of Ribosome, ribonucleoprotein complex, ribosome biogenesis, structural molecular activity, intracellular ribonucleoprotein complex, ribosome, transferase activity, organic cyclic compound catabolic process, and heterocyclic catabolic process were over represented except transferase activity which was under represented (for full table of enriched GO terms see table 3). This broadly suggests that the DEGs in the brain are involved in a wide range of functions and processes. GO terms can be useful when trying to determine the functions of differentially expressed genes in a particular tissue but they are not always intuitive when looked at alone. In order to relay a more intuitive “big picture” description for the roles these DEGs are playing, I have sorted the DEGs into “functional categories” which should be more familiar, however, do keep in mind that these are not “enriched” categories but just a likely function for the specific DEG. In the brain, there were several genes that fell into

energy, cell proliferation, and immunity for females and ribosomal proteins, transcription, cell signaling, and metabolism in males (see figure 6). This broadly suggests that there may be functional differences between male and female brains and the specific genes that are being used in these functions.

In the gills, top enriched GO terms included extracellular region part, extracellular space, plasma membrane, oxidoreductase activity, transferase activity, structural constituent of ribosome, small molecular metabolic process, sulfur compound metabolic process, and cofactor metabolic process. These enriched GO terms suggest that the DEGs in the gill are also involved in a broad amount of functions and processes particular to the functioning of the gills. For my “functional categories”, in the gills there were several genes that fell into immunity for both males and females, with all other upregulated genes falling into their own categories by themselves except for cell signaling which had two DEGs in males (see figure 7). This may suggest that males and females utilize different specific genes in protecting the body from pathogens, however, controlled studies need to be conducted.

There were 12 sex-biased genes that were found in both tissues. All of these genes showed the same pattern of expression in both tissues. Three genes were upregulated in females (Peroxiredoxin-4 precursor, transcription factor e2f4, and ap-1 complex subunit sigma-3) and two of these genes fell into the “functional category” of immunity (Peroxiredoxin-4 precursor and ap-1 complex subunit sigma-3) and the other one fell into the category of regulation of transcription (transcription factor e2f4). The other nine genes were upregulated in males and two were associated with immunity (baculoviral iap repeat-containing protein 2 and galactose-specific lectin natectin), two were involved with sex hormones (Testosterone 17-

beta-dehydrogenase 3 and atp-dependent rna helicase ddx54), two functioned in metabolism (pyruvate dehydrogenase e1 alpha 1 and adiponectin receptor 1a), two in transport (arfaptin-2 and protein cornichon homolog 2), one in development(galactose-specific lectin nattectin), one in regulation of transcription (probable atp-dependent rna helicase ddx5) and one in cell adhesion and neuron plasticity(amyloid beta a4 protein precursor). This suggests that patterns of sex-bias in expression for these 12 genes are shared between these two tissues.

Most studies of sex-bias gene expression have occurred in species that exhibit traditional sex roles (Mank et al., 2008; Small et al., 2009; Huby et al., 2014; Sharma et al., 2014; Prince et al., 2010). Our results have interesting implications for our understanding of how patterns of sex-bias in gene expression vary in a species with sex-role reversal. The genes and molecular pathways that are altered between the sexes may be associated with the development of phenotypes that differ between the sexes as well as unusual behaviors such as male brooding. Next generation sequencing methods take a transcriptome wide approach and are an excellent method to detect differentially expressed genes. However, the volume of data generated from these approaches is immense. Therefore I will limit my discussion to those sex-biased genes that have connections to known sex-biased phenotypes that I have introduced previously: sex steroids/hormones in the brain, genes related to circadian rhythms in the brain, and immunity in the brain and gill.

Sex steroids/hormones in the brain

The karyotype of five different Syngnathids has been completed and none have been found to possess heterogametic sex chromosomes (Vitturi et al., 1988). Although the karyotype of the Gulf pipefish has not yet been investigated (to the best of our knowledge), the lack of

heterogametic sex chromosomes in this family thus far strongly suggests their absence in the Gulf pipefish. Therefore, sex must be determined by another factor, whether it be temperature, sex-ratio presence, or some other environmental factor (Devlin and Nagahama, 2002; Black et al., 2005; Baroiller et al., 2009). Steroid hormones are involved in many physiological and behavioral aspects that differ between the sexes, from embryonic development, sex differentiation, and reproduction to metabolism, immune responses, and circadian rhythms (Bailey et al., 2012; Bell-Pedersen et al., 2005; Black et al., 2005; Boden and Kennaway, 2006; Borg, 1994; Godwin, 1996). Like tetrapods, teleost fish steroid biogenesis is controlled by the hypothalamus-pituitary-interrenal hormonal axis and the hypothalamus-pituitary-gonadal hormonal axis (Löhr and Hammerschmidt, 2011; Liley and Stacey, 1983; Liu et al., 2011). This makes the brain an essential component in the production of many sex hormones. Altogether, there were six DEGs that were connected to sex steroids and sex hormones. Of these, five genes were upregulated in the brain of males (testosterone 17-beta-dehydrogenase 3, 17 β -hsd3 or HSD17b3; atp-dependent rna helicase ddx54, ddx54; tetratricopeptide repeat protein 9a, TTC9; e3 ubiquitin-protein ligase rnf14-like, RNF14; and probable e3 ubiquitin-protein ligase mgrn1, mgrn1). The most notable of these four genes is testosterone 17-beta-dehydrogenase 3 (17 β -hsd3 or HSD17b3) which catalyzes the chemical reaction between testosterone and androst-4-ene-3,17-dione (Mindnich et al., 2004). This specific type (i.e. 3) of HSD17b favors the synthesis of testosterone via the reduction of androstenedione (Geissler et al., 1994; Mindnich et al., 2004), however, it does participate in both directions of the reaction. In many fish, testosterone is highly expressed by both males and females and is not considered a male specific sex hormone (Borg, 1994). Two androgens that are associated with male specific fish

hormones are 11-ketotestosterone (11-KT) and 11 β -hydroxytestosterone (11 β -OHT) which may mediate androgen actions in males depending on the species (Lokman et al., 2002; Mayer et al., 1993) and are more effective than testosterone at stimulating secondary sexual characteristics, reproductive behavior and spermatogenesis (Borg, 1994). Testosterone is a precursor to these active forms of male androgens and HSD17b3 is essential for the synthesis of testosterone (Mindnich et al., 2005). However, it has been seen that male pipefish decrease androgen production when pregnant (Mayer et al., 1993). Testosterone is also necessary in the synthesis of estrogen through its aromatization via aromatase, it could be possible that this testosterone is being used for estrogen production, but more studies need to be conducted to see if this could be the case. Another possible scenario could be that our pipefish had very recently become pregnant and so testosterone production was still active as it is required for both spermatogenesis and the formation and maintenance of the male brood pouch prior to pregnancy (Boisseau, 1967; Scobell and MacKenzie, 2011). However, it is important to keep in mind that this was found in the brain and not the gonads which may be the primary source of testosterone for these male phenotypes including the brood pouch. Future studies should compare brain and gonad transcripts of both pregnant and non-pregnant pipefish to further distinguish what role this gene may be playing.

The other four genes found to associate with sex hormones in males were less clear on their exact contribution to the male phenotype. The *ddx54* gene is a member of the DEAD box family of RNA helicases which are known to be involved in RNA metabolism, transcription, and translation (Linder and Jankowsky, 2011). However, it was also suggested that this gene may function in an estrogen receptor pathway (Rossow and Janknecht, 2003). The ubiquitin E3 ligase

RNF6 was found to be an androgen receptor associated protein which has implications in human prostate cancer (Xu et al., 2009) and it is suggested that RNF14-like may function in a similar manner due to sequence similarity according to the Uniprot database. The mgrn 1 gene is found to mediate Tumor susceptibility gene 101 (TSG101). TSG101 was found to repress transcriptional activation of various nuclear receptors including androgen receptors and estrogen receptors (Sun et al., 1999). It is unclear if/how these genes are functioning in regard to sex hormones in the Gulf pipefish. It is interesting that all these genes are up-regulated in males, could it be possible that the male pregnant phenotype requires more hormone control than the female phenotype since all of our males were pregnant? It has been found in several species of teleost that males that exhibit male brooding in some form (i.e. from actually carrying the eggs as in syngnathids to simply being the primary care parent for the young as in stickleback) show a decrease in androgens during pregnancy/the parental phase (Mayer et al., 1993; Oliveira et al., 2002; Mayer et al., 2004) which could be one possible explanation for the upregulation of genes connected to hormone control in the male Gulf pipefish, however, comparisons to non-pregnant male Gulf pipefish need to be made.

Only one DEG was upregulated in females and was connected to sex steroids/hormones in the brain. This gene is tripartite motif-containing protein 16 (TRIM 16) which is regulated by both estrogen and anti-estrogen in epithelial cells that stably express estrogen receptors (Reymond et al., 2001), however, this description was in reference to humans and other mammals. TRIM genes are known to play a role in viral immunity for both mammals and fish (Van der Aa et al., 2009) and due to the strong selection pressure that viruses impose on immune systems, antiviral TRIM genes have diversified greatly in humans and even more so in

teleosts due to the genome wide duplication (Glasauer and Neuhauss, 2014). TRIM 16 however is a conserved orthologue of TRIMs that is seen in mammals and teleosts (van der Aa et al., 2009). Given the wide range of functions that TRIM genes are involved in, it is impossible to know for sure what the expression of TRIM 16 signifies in the brain.

Circadian rhythms

Circadian rhythms, also known as biological clocks, are oscillatory biochemical, behavioral, and physiological processes that are usually entrained (synchronized) to the 24 hour day by cues from the environment such as the daily light-dark cycle, the daily temperature cycle, and food availability (Reppert, 1998; Bell-Pedersen et al., 2005; Mistlberger and Antle, 2011). Not only do circadian rhythms mediate when an animal sleeps versus being awake (Emery et al., 1998; Stanewsky et al., 1998), they also mediate other behaviors such as eating/metabolism and reproduction (Boden and Kennaway, 2006; Brubaker and Gil-Lozano, 2016; Karlsson et al., 2016; Bur et al., 2009). Additionally, there is mounting evidence that clock-controlled processes show a sexual dimorphism and even mediate sexually dimorphic phenotypes (Feillet et al., 2016; Yang et al., 2014; Bur et al., 2009). There are three main parts to the internal clock: 1. sensors for input signals- this is mainly photoreceptor proteins that entrain the central oscillator (Takahashi et al., 1993), 2. a central oscillator- this is made up of an autoregulatory transcriptional feedback loop which maintains a rhythmic output even in the absence of input signals (Wenderoth and Bock, 1999), and 3. the output pathways- transferred circadian rhythms to downstream genes (Lakin-Thomas, 2000). The central oscillator is controlled through transcription activators such as *clock* and *Bmal1* and negative regulatory factors such as *Periods* (*Per1* and *Per2*) and *cryptochromes* (Dunlap, 1999; Liu et al., 2007). The

hypothalamic suprachiasmatic nucleus (SCN) in mammals, reptiles, and birds is known as the biological “Master” clock that is entrained by light-dark cycles and relays this information to peripheral tissues (Bertolucci et al., 2000; Welsh et al., 2010). In teleost fish, the pineal gland is thought of as its equivalent (Cahill, 1996), with studies done by Ziv et al. (2005) and Watanabe et al. 2012) in Zebrafish and Medaka supporting this claim that the pineal and not the SCN is a molecular clock. However, it was found that in flounder (*Paralichthys olivaeus*) and amberjack (*Perciformes*) that a molecular clock does exist in the SCN due to its periodic expression of *per2* (Watanabe et al., 2012; Mogi et al., 2015). In Watanabe et al. (2012), they hypothesized that the importance of the SCN as a pacemaker may lie in the lifestyle of the fish, with marine fish relying more on the SCN as a pacemaker than small freshwater fish. Circadian rhythms have not been explored yet, to best of our knowledge, in any member of Syngnathidae so we do not know whether the pineal or SCN is the master oscillator, however, since we sampled the whole brain, we did obtain both of these regions. Furthermore, in teleost fish, the central oscillator is greatly decentralized and peripheral tissues show a response to light as well as several parts of the brain rather than just the SCN or pineal gland (Whitmore et al., 2000; Tamai et al., 2007; Moore and Whitmore, 2014).

There was one gene found up-regulated in our female pipefish that is directly connected to the central oscillation system. This gene was annotated as Cryptochrome circadian clock 5 by the Zebrafish database and identified as Cry1 (Cryptochrome) by the Swissprot database. Cry1 and Cry2 in mammals (teleost fish have more orthologues due to the teleost specific genome duplication) are part of the photolyase/cryptochrome family and are flavoproteins that detect blue-light and entrain the internal clock to solar day (Thompson and Sancar, 2002).

Cryptochromes were found to be a key element of the circadian system for vertebrates (Cashmore, 2003; Van der Horst et al., 1999), however, they are not required for the operation of the internal clock (Dunlap, 1999). In mice, Cry1 and Cry2 mutants with no expression of these genes were found to affect male liver metabolism with little effect on females. The study also showed that these affects could be due to an effect on the pulsation of secretion of growth hormone (GH) from the pituitary gland which also had an effect on growth size therefore showing how the time-keeping mechanism can affect sexually dimorphic traits (Bur et al., 2009). Honnen et al. (2016) found differential expression in the cryptochrome genes of male and female mosquitoes (*Culex pipiens*) exposed to artificial light at night (ALAN). This study suggests that males and females may perceive and/or react to light differently.

There was one gene, teleost multiple tissue opsin 2a (*tmt opsin 2a*), which was found upregulated in male Gulf pipefish that may play a role as a sensor for the peripheral oscillating clock. As mentioned earlier, the internal clock of teleosts is highly decentralized and several peripheral tissues show light entrainment separate from the “Master” clock area of the brain. This gene (*tmt opsin*) was identified by Moutsaki et al. (2003) and is homologous to Opsin 3 in mammals (Blackshaw and Snyder, 1999; Arendt et al., 2004; Velarde et al., 2005). The information we have about *tmt* opsins is very limited, as with our overall knowledge of circadian rhythms, but *tmt* opsins have been found in several peripheral tissues including other regions of the brain aside from SCN and the pineal gland (Whitmore et al., 1998; Whitmore et al., 2000; Koyanagi et al., 2012; Koyanagi et al., 2013; Moutsaki et al., 2003) and have been shown to have photosensitivity in tissues such as the heart and kidney as demonstrated by Whitmore et al. (2000). Since we found this *tmt opsin 2a* in the brain of males, perhaps there

are several ways in which pipefish entrain their physiological oscillations? With our limited knowledge on these genes that we found differentially expressed between our males and females, it is hard to speculate why these genes are differentially expressed and their exact role in circadian rhythms and entrainment of the oscillatory clocks between the sexes, however, it could be that male and female circadian clocks do show differences in expression and/or perhaps the mechanism/way that entrainment of oscillations occurs is different between the sexes. Studies that pin-point more specific regions of the brain are necessary to deduct what this expression difference really means for the Gulf pipefish.

Immunity

Gills are analogous to lungs in that they both function in respiration and the exchange of molecules into and out of the body. They are also both mucus membranes that are susceptible to letting pathogens into the body. Like other animals that respire, the gills show an immediate immune response to pathogens entering the body like the respiratory organs do as the body's first line of defense. When it comes to the immune response in general, it has been documented that males across several species are more susceptible to various pathogens and have higher death rates than their female counterparts (Hamilton, 1948; Falagas et al., 2007; Goble and Konopka, 1973, Klein, 2000; Zuk, 1990; Møller et al., 1998; Zuk and McKean, 1996; Kurtz et al., 2000; Roth et al., 2008). Roth et al. (2011), looked at immune differences between the sexes in a sex-role reversed pipefish, *Syngnathus typhle*, and found that in this species males actually had a more active immune system than females determined by the higher lymphocyte-to-monocyte ratio found in males compared to females. Their data consisted of both field data from four different populations and a lab experiment with a fully factorial design

all confirming that males had a more efficient immune system than females. This study suggests that life history traits such as parental contribution, rather than sex per se, is a determining factor for which sex will have the better immune system (Rolff, 2002).

In our data set immunity was not an enriched GO term however there were several immune pathway genes found differentially expressed between males and females for both sexes in the gill and also in the brain. There were a similar number of differentially expressed immune genes found in the gill than in the brain (12 and 10 transcripts respectively) but considering the total number of DEGs, the gill had more DEGs in immunity by proportion of DEGs (16% brain, 34% gill). If you take into consideration that in the gill five of the immune genes were annotated as the same gene (see Table in appendix for all DEGs) and only count that as one, the % of immune genes out of all up-regulated genes found in each tissue is still larger in the gill than in the brain (16% in brain and 20% in gill). This makes sense when thinking about the gill as the bodies first line of defense against all impending pathogens found in the environment. However, this is still a small number of immune genes found DE overall and immunity was not a GO term found enriched.

In the gills of males, the five isocontigs found upregulated were galactose-specific lectin natectin. This gene has been found to induce a pro-inflammatory state in mice and is thought to act as a chemoattractant for neutrophils and play a role in the innate and the adaptive immune response (Lopes-Ferreira et al., 2011). Other immune related genes that were upregulated by males in the gills included baculoviral iap repeat-containing protein 2 which functions in regulating caspases and apoptosis including inflammatory signaling which contributes to innate immunity and the Slam family member 9-like which is part of the SLAM-

family receptors that function on immune cells and can either promote or inhibit activating receptors which operates in the innate and adaptive immune response (Cruz-Munoz et al., 2009; Dong et al., 2009).

In the gills of females, there were four genes found up-regulated that may contribute to the immune response in some way. These genes were ap-1 complex subunit sigma-3 working in the innate and adaptive immune response (Setta-Kafetzi et al., 2014), peroxiredoxin-4 precursor which operates in the innate response and is well known for its protective effects against oxidative stress (Butterfield et al., 2008), 28s ribosomal protein mitochondrial which helps in vesicle formation in the innate immune response (Kissil et al., 1995), and the liprin-beta-2 gene whose function in immunity is unknown and most likely innate (Serra-Pagés et al., 1998). There are more genes up-regulated by males and there are more genes in males that may be functioning in adaptive immunity. Without going into as much detail, in the brain there were six genes that contribute to the immune response up-regulated in males and four up-regulated in females. Judging by numbers alone we see that male Gulf pipefish have more immune pathway genes up-regulated in both gill and brain than females. We would expect this based on Roth et al.'s (2011) study that found males in a sex-role reversed pipefish to have the more active immune response than their female counter parts, however, several of these genes have multiple roles they may be playing and are not necessarily contributing to immunity per se in their upregulation. Overall, there was a small number of immune genes found differentially expressed and the fact that males had more individual DEGs related to immunity does not necessarily mean that these male Gulf pipefish have a better immune system than the females. A parallel study to Roth et al. (2011) would be a good way to see if the pattern holds for this

sex-role reversed pipefish. Furthermore, our male pipefish were pregnant at the time of sampling and this too may have an effect on the status of the male immune system which may possibly be weakened due to the pregnancy.

Other uses for the Gulf pipefish transcriptome

In addition to allowing us to explore sex-bias in expression differences between male and female Gulf pipefish, this transcriptome also helps spark several questions that need further exploration. This transcriptome can now be used in more specific studies to try to characterize what role these genes are playing in sexual dimorphisms and can even be used in studies other than sex-bias in gene expression. Within the family of Syngnathidae, there are several endangered species. The top threats to syngnathids appears to be over harvesting for the pet trade and Chinese medicine, habitat modification, and climate change (Vincent et al., 2011; Birrer et al., 2012). This transcriptome can be used in making biological markers necessary to monitoring health, population dynamics (such as movement), and population genetics that are crucial to the conservation of these species. Additionally, this unique family of teleost fish tends to draw sympathy and support from people for their conservation and in 2009, a study provided evidence that by creating marine protected areas (MPAs) to conserve syngnathids they would also be helping several other endangered species that utilize similar habitats (Shokri et al., 2009; Bowen-Jones and Entwistle, 2002).

V. Conclusion

In conclusion, it is clear that there are sex-biased genes in the Gulf pipefish in the brain and gills. In addition, these DEGs that we found were from our annotated set of genes using BLAST approaches. However, it should be noted that we were somewhat limited in our ability to annotate the transcriptome because of the lack of closely related genome sequence in GenBank. Using blast2Go software, we were able to connect GO terms to our differentially expressed genes and other annotated genes and then determine which functional pathways were enriched for Gulf pipefish in the brain and gills. Lastly, males had more DEGs upregulated in both the brain and gills than females. This appears consistent with other studies done on traditional sex-role species that males typically have more DEGs than females, however, most sex-bias studies were looking at the number of DEGs in the gonads and with the brain showing DE within its specific compartments it is hard to say for sure that this pattern exists for brain tissue as well. Furthermore, if we had sampled different tissues, such as the gonads, would we still have seen this result? Instead of sexual selection mediating the increase in DEGs in males, could it be that the male phenotype, regardless of sex-role, requires more upregulated genes than the female phenotype? More studies on this subject are clearly needed. Also, we must keep in mind that this is just a snap shot of the time and place that we sampled this population and at different times of the year or even different times of development we may see a different result. These are all questions that warrant proper study in the near future.

DEGs in the Brain		
Sequence ID	Gene Description	GO Names list
Female DEGs		
c38126_g1_i2	islet cell autoantigen 1-like protein	F:protein domain specific binding; C:intracellular membrane-bounded organelle; C:bounding membrane of organelle; C:endomembrane system; C:cytoplasmic part; C:intracellular organelle part
c44691_g1_i3	na(+) h(+) exchange regulatory cofactor nhe-rf2-like	P:regulation of biological quality; C:early endosome; F:L27 domain binding; P:maintenance of epithelial cell apical/basal polarity; P:retrograde transport, endosome to plasma membrane; P:signal transduction; C:membrane; P:protein localization to membrane; F:phosphatidylinositol-3-phosphate binding; P:intracellular protein transport
c44291_g3_i4	trafficking protein particle complex subunit 11	P:retina development in camera-type eye; P:liver development
c35679_g1_i2	ap-1 complex subunit sigma-3	C:perinuclear region of cytoplasm; P:vesicle-mediated transport; P:nervous system development; P:extracellular matrix organization; F:protein transporter activity; C:AP-2 adaptor complex; P:intracellular protein transport
c31587_g1_i1	acyl carrier mitochondrial	P:fatty acid metabolic process
c33249_g2_i1 3	atp synthase mitochondrial f1 complex assembly factor 1	P:mitochondrial proton-transporting ATP synthase complex assembly; C:mitochondrion
c36375_g2_i8	histone deacetylase 6	P:regulation of neural crest formation; P:generation of neurons; P:gland development; P:epithelium development; P:central nervous system morphogenesis; P:dorsal/ventral pattern formation; P:angiogenesis; P:digestive system development; P:response to stimulus; P:tissue morphogenesis; P:tube development; P:protein deacetylation; P:embryonic cranial skeleton morphogenesis; F:cysteine-type peptidase activity; C:protein complex; P:negative regulation of cellular process; P:developmental growth; F:binding; P:organelle organization; C:intracellular part; P:cell cycle; P:neural crest cell migration; P:proteolysis; P:regulation of gene expression;

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		P:regulation of primary metabolic process; P:regulation of cellular metabolic process
c42072_g3_i2	abcd3 atp-binding cassette sub-family d member 3	F:ATPase activity; F:transmembrane transporter activity; P:catabolic process; F:ion binding; P:biosynthetic process; C:plastid; C:plasma membrane; P:response to stress; P:small molecule metabolic process; C:peroxisome; C:mitochondrion; P:lipid metabolic process; P:cofactor metabolic process; C:cytosol; F:enzyme binding
c36770_g1_i5	annexin a13	C:cytoplasm; P:plasma membrane repair; F:calcium ion binding; P:fin regeneration; P:regulation of cell motility; F:calcium-dependent phospholipid binding
c33914_g1_i2	cryptochrome circadian clock 5	P:DNA repair; P:response to temperature stimulus; F:photoreceptor activity; P:signal transduction; P:response to hydrogen peroxide; P:response to light stimulus; C:nucleus; C:transcription factor complex; F:protein binding; P:entrainment of circadian clock; P:negative regulation of transcription, DNA-templated; C:cytoplasm; F:DNA (6-4) photolyase activity
c31854_g1_i5	peroxiredoxin-4 precursor	P:oxidation-reduction process; F:peroxiredoxin activity
c40347_g6_i3	coiled-coil domain-containing protein 136-like	
c39525_g1_i5	wd repeat-containing protein 1	
c39220_g2_i3	transcription factor e2f4	C:nucleus; C:transcription factor complex; F:DNA binding; F:transcription factor activity, sequence-specific DNA binding; P:transcription, DNA-templated; P:regulation of transcription, DNA-templated; P:regulation of cell cycle; P:mitotic cell cycle
c41072_g3_i5	es1 protein mitochondrial	F:ubiquitin protein ligase activity; C:mitochondrion; P:SCF-dependent proteasomal ubiquitin-dependent protein catabolic process; C:SCF ubiquitin ligase complex; P:protein ubiquitination
c35395_g1_i5	zinc transporter zip9-b-like	P:single-organism transport
c31351_g1_i3	PREDICTED: uncharacterized protein LOC569091	
c34080_g1_i1	btb poz domain-containing protein kctd6	P:negative regulation of canonical Wnt signaling pathway; P:regulation of RNA biosynthetic process; P:protein homooligomerization; P:habenula

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		development; P:canonical Wnt signaling pathway involved in neural crest cell differentiation; P:pharyngeal system development; P:regulation of dendrite morphogenesis
c44212_g1_i3	sideroflexin-5	P:ion transport; C:mitochondrial membrane; P:transmembrane transport; F:ion transmembrane transporter activity; C:integral component of membrane
c43794_g4_i5	integral membrane protein (dgcr2)	P:anatomical structure development; C:cellular_component; P:neurological system process
c40530_g5_i1	e3 ubiquitin-protein ligase sh3rf2	F:acid-amino acid ligase activity
c40066_g1_i3	transmembrane protein 147	F:protein binding; C:integral component of membrane
c41511_g2_i4	tripartite motif-containing protein 16	F:metal ion binding; P:regulation of transforming growth factor beta receptor signaling pathway
c40467_g4_i1	tripartite motif-containing protein 16	F:zinc ion binding; F:metal ion binding; P:intracellular signal transduction; C:intracellular
c38311_g1_i6	26s proteasome non-atpase regulatory subunit 3	P:regulation of protein catabolic process; C:proteasome regulatory particle, lid subcomplex; F:enzyme regulator activity; P:ubiquitin-dependent protein catabolic process
c45275_g1_i4	aurora kinase-like	F:metal ion binding
Male DEGs		
c8919_g1_i2	60s ribosomal protein l10a	F:RNA binding; F:structural constituent of ribosome; C:large ribosomal subunit; P:translation
c28200_g1_i2	60s ribosomal protein l13a	F:structural constituent of ribosome; C:cytosol; F:mRNA binding; C:ribosome; P:translation
c41643_g1_i1	calmodulin 2a	F:calcium ion binding; P:cilium assembly; P:microtubule cytoskeleton organization; P:interkinetic nuclear migration
c3421_g1_i1	60s ribosomal protein l23a	F:nucleotide binding; F:structural constituent of ribosome; C:cytosolic large ribosomal subunit; P:regulation of cell cycle; P:ribosomal large subunit assembly; F:poly(A) RNA binding; P:translation; P:pancreas development
c33657_g2_i1	40s ribosomal protein s27-like	P:erythrocyte differentiation; F:structural constituent of ribosome; C:cytosolic small ribosomal subunit; F:metal ion binding; P:ribosomal small subunit assembly; F:poly(A) RNA binding; P:translation

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c27734_g1_i2	60s ribosomal protein l35	P:embryo development; F:structural constituent of ribosome; P:homeostatic process; P:cell differentiation; P:ribosome biogenesis; C:cytosol; F:mRNA binding; C:ribosome; P:immune system process; P:cell cycle; P:translation
c31853_g2_i2	60s ribosomal protein l38	P:chordate embryonic development; F:RNA binding; F:structural constituent of ribosome; P:ribonucleoprotein complex assembly; C:cytosolic large ribosomal subunit; P:translation
c28098_g2_i1	60s ribosomal protein l23	F:structural constituent of ribosome; C:cytosol; C:ribosome; F:rRNA binding; P:translation
c37928_g2_i1	cysteine and glycine-rich protein 1	F:zinc ion binding; P:heart morphogenesis; P:heart contraction; P:cell migration involved in gastrulation; P:sarcomere organization; P:Wnt signaling pathway; P:neuron projection regeneration; P:JNK cascade; P:convergent extension involved in gastrulation; C:intracellular
c39916_g1_i4	arfaptin-2	F:phosphatidylinositol-4-phosphate binding; F:protein domain specific binding; P:regulation of protein secretion; P:ruffle organization; C:trans-Golgi network membrane; P:regulation of Arp2/3 complex-mediated actin nucleation
c42440_g1_i2	bovinprotein	C:nucleus; P:catabolic process; P:signal transduction; C:cytosol; P:mitotic nuclear division; C:protein complex; C:chromosome; P:symbiosis, encompassing mutualism through parasitism; F:molecular_function; P:response to stress; P:immune system process
c33102_g1_i1	neuron-specific protein family member 2	P:cellular process
c42327_g3_i7	fibroblast growth factor 11	F:growth factor activity; P:nervous system development; P:fibroblast growth factor receptor signaling pathway; C:extracellular region
c42029_g1_i3	isovaleryl- mitochondrial	P:sulfur compound metabolic process; P:catabolic process; F:oxidoreductase activity; P:homeostatic process; C:mitochondrion; P:lipid metabolic process; P:cofactor metabolic process; F:ion binding; P:cellular amino acid metabolic process; P:cellular nitrogen compound metabolic process; C:nucleoplasm
c38055_g1_i3	testosterone 17-beta-dehydrogenase 3	C:mitochondrion; F:steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor; P:cellular hormone metabolic process; C:endoplasmic reticulum; P:steroid biosynthetic process; P:single-

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		organism cellular process; P:cellular biosynthetic process; P:hormone biosynthetic process
c40347_g6_i8	coiled-coil domain-containing protein 136-like	
c43260_g1_i3	tetratricopeptide repeat protein 9a	C:cytoplasm; P:cellular protein modification process; C:endoplasmic reticulum; P:protein folding; F:isomerase activity
c31076_g2_i2	leucine-rich repeat neuronal protein 3-like	F:protein binding; C:membrane; P:olfactory nerve formation; P:olfactory placode morphogenesis
c38329_g2_i1	maguk p53 subfamily member 3-like	C:cell junction
c40106_g2_i6	elmo domain-containing protein 2 precursor	F:GTPase activator activity; P:positive regulation of GTPase activity
c38829_g1_i2	e3 ubiquitin-protein ligase rnf14-like	F:ubiquitin-protein transferase activity; F:zinc ion binding; F:transcription coactivator activity; P:regulation of canonical Wnt signaling pathway; F:acid-amino acid ligase activity; P:regulation of transcription from RNA polymerase II promoter; P:protein ubiquitination
c44420_g1_i2	glutamate decarboxylase 1-like	P:heart development; F:pyridoxal phosphate binding; F:glutamate decarboxylase activity; P:neurotransmitter biosynthetic process; P:gamma-aminobutyric acid biosynthetic process; P:taurine biosynthetic process
c42231_g1_i1	afadin	P:cell proliferation; P:signal transduction; P:cell junction organization; P:cellular component assembly; P:cell-cell signaling; P:anatomical structure development; P:cell adhesion; C:plasma membrane; C:nucleoplasm; P:homeostatic process; P:cell differentiation; C:cytosol; F:enzyme binding
c41372_g2_i1	protein disulfide-isomerase a6 precursor	F:protein disulfide isomerase activity; P:cell redox homeostasis; P:protein folding; P:response to endoplasmic reticulum stress
c41691_g5_i2	rho gtpase-activating protein 17	C:cytoplasm; P:signal transduction; P:regulation of GTPase activity
c45162_g4_i1	poliovirus receptor	C:integral component of plasma membrane; F:protein homodimerization activity; C:cell-cell adherens junction; F:cell adhesion molecule binding; P:heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules; P:cell recognition; P:homophilic cell adhesion via plasma membrane adhesion molecules; F:receptor activity; F:receptor binding

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c34690_g1_i1	noelin-2	P:locomotory behavior; P:neural crest cell development; P:retina development in camera-type eye; P:positive regulation of neural retina development; P:axonogenesis; P:cellular response to estrogen stimulus
c33704_g1_i2	progesterin and adipog receptor family member 6	P:regulation of cellular process; P:oogenesis; C:integral component of membrane; F:lipid binding; F:receptor activity; P:response to stimulus
c41032_g2_i6	oxidation resistance protein 1	P:biological_process; C:cellular_component; F:molecular_function
c37252_g1_i3	pyruvate dehydrogenase e1 alpha 1	C:intracellular membrane-bounded organelle; F:pyruvate dehydrogenase (acetyl-transferring) activity; P:glycolytic process; P:oxidation-reduction process
c40779_g1_i1	galactose-specific lectin natectin	P:biological_process; C:cellular_component
c36708_g2_i3	epsilon-sarcoglycan	F:calcium ion binding; C:sarcoglycan complex
c42299_g1_i1	atp-dependent rna helicase ddx54	C:nucleus; F:ATP binding; F:RNA binding; P:RNA secondary structure unwinding; F:ATP-dependent RNA helicase activity
c34763_g2_i2	PREDICTED: uncharacterized protein	F:WW domain binding
c33772_g1_i4	teleost multiple tissue opsin 2a	F:G-protein coupled receptor activity; P:G-protein coupled receptor signaling pathway; F:photoreceptor activity; C:integral component of membrane; P:protein-chromophore linkage; P:visual perception; P:phototransduction
c40061_g1_i1	u4 tri-snRNP-associated protein 1	P:mRNA cis splicing, via spliceosome; P:maturation of 5S rRNA; C:U4/U6 x U5 tri-snRNP complex; P:spliceosomal snRNP assembly
c41597_g1_i7	probable e3 ubiquitin-protein ligase (mgrn1)	F:zinc ion binding; F:protein-glycine ligase activity, elongating; F:tubulin-glycine ligase activity; F:ribosomal S6-glutamic acid ligase activity; F:coenzyme F420-2 alpha-glutamyl ligase activity; F:tubulin-glutamic acid ligase activity; F:coenzyme F420-0 gamma-glutamyl ligase activity; F:protein-glycine ligase activity, initiating; F:UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-alanine ligase activity; P:protein ubiquitination
c39224_g1_i1	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6	
c40806_g1_i3	kinesin-like protein kif1c	F:nucleotide binding; P:posterior lateral line nerve development; P:posterior lateral line ganglion development; P:vesicle-mediated transport; P:intracellular mRNA localization; P:cytoskeleton-dependent intracellular

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		transport; P:axon extension; P:neuron projection maintenance; C:kinesin complex; F:ATP-dependent microtubule motor activity, plus-end-directed; P:microtubule-based movement; P:macrophage differentiation
c38543_g2_i3	bombesin receptor-activated protein c6orf89 homolog	C:cytoplasm; C:membrane
c34013_g1_i1	exosome complex component (mtr3)	C:cytoplasmic exosome (RNase complex); C:nuclear exosome (RNase complex); P:polyadenylation-dependent snoRNA 3'-end processing; F:RNA binding; P:U4 snRNA 3'-end processing; P:rRNA catabolic process; P:nuclear mRNA surveillance; C:nucleolus; P:nuclear-transcribed mRNA catabolic process, exonucleolytic, 3'-5'; P:rRNA 3'-end processing
c43051_g1_i4	amyloid beta a4 protein precursor	F:serine-type endopeptidase inhibitor activity; F:transition metal ion binding; F:protein binding; P:central nervous system development; P:axon extension; P:vasculature development; C:integral component of membrane; F:heparin binding; P:convergent extension
c39841_g1_i2	adiponectin receptor 1a	C:integral component of membrane; F:hormone binding; P:adiponectin-activated signaling pathway; F:receptor activity; C:plasma membrane
c41634_g1_i1	baculoviral iap repeat-containing protein 2	P:inhibition of cysteine-type endopeptidase activity involved in apoptotic process; C:nucleus; C:spindle microtubule; F:ubiquitin-protein transferase activity; F:zinc ion binding; C:cytoplasm; F:cysteine-type endopeptidase inhibitor activity involved in apoptotic process; P:regulation of signal transduction; P:protein ubiquitination; P:mitotic spindle assembly
c43341_g4_i3	ras-related protein rab-35-like	P:single-organism membrane organization; P:single-organism cellular localization; P:cellular component assembly; P:Rab protein signal transduction; C:Golgi apparatus; P:intracellular protein transport; P:organelle organization; C:intracellular organelle part; F:GTP binding; C:nucleus; C:endosome; P:vesicle-mediated transport; P:angiogenesis; C:bounding membrane of organelle; F:GTPase activity; P:cytoplasmic transport; P:endosomal transport; C:cytoplasmic, membrane-bounded vesicle; C:whole membrane; P:secretion by cell
c43420_g1_i4	atp-dependent rna helicase (dbp2)	F:ATPase activity; F:RNA binding; C:mitochondrion; P:ribonucleoprotein complex assembly; F:ion binding; P:nucleobase-containing compound

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		catabolic process; P:anatomical structure development; C:cytosol; P:ribosome biogenesis; F:helicase activity; C:nucleolus
c31966_g1_i3	protein cornichon homolog 2	C:membrane; P:intracellular signal transduction

DEGs in the Gill		
SeqName	Description	GO Names list
Female DEGs		
c35679_g1_i2	ap-1 complex subunit sigma-3	C:perinuclear region of cytoplasm; P:vesicle-mediated transport; P:nervous system development; P:extracellular matrix organization; F:protein transporter activity; C:AP-2 adaptor complex; P:intracellular protein transport
c31854_g1_i5	peroxiredoxin-4 precursor	P:oxidation-reduction process; F:peroxiredoxin activity
c43089_g4_i4	PREDICTED: erythroferrone	F:phosphatase activity
c42308_g1_i7	liprin-beta-2	P:biological_process; C:cellular_component; F:molecular_function
c39220_g2_i3	transcription factor e2f4	C:nucleus; C:transcription factor complex; F:DNA binding; F:transcription factor activity, sequence-specific DNA binding; P:transcription, DNA-templated; P:regulation of transcription, DNA-templated; P:regulation of cell cycle; P:mitotic cell cycle
c43089_g4_i7	25-hydroxycholesterol 7-alpha-hydroxylase	P:small molecule metabolic process; F:phosphoprotein phosphatase activity; F:oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen; F:steroid hydroxylase activity; P:lipid metabolic process; C:endoplasmic reticulum; P:single-organism cellular process; F:binding; P:dephosphorylation; P:regulation of biological process
c43089_g4_i8	25-hydroxycholesterol 7-alpha-hydroxylase	P:small molecule metabolic process; F:oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen; F:steroid hydroxylase activity; P:protein dephosphorylation; P:lipid metabolic process; C:endoplasmic reticulum; P:single-organism cellular

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		process; F:binding; P:regulation of biological process; F:protein tyrosine/serine/threonine phosphatase activity
c34541_g3_i2	cathepsin b precursor	F:peptidase regulator activity; C:extracellular space; P:regulation of catalytic activity; F:cysteine-type endopeptidase activity; P:fin regeneration; P:proteolysis involved in cellular protein catabolic process; C:lysosome
c33990_g1_i2	28s ribosomal protein mitochondrial	C:mitochondrion; C:ribosome
c20699_g1_i2	l-lactate dehydrogenase b-a chain	P:small molecule metabolic process; P:carbohydrate metabolic process; C:cytoplasm; F:oxidoreductase activity; P:response to stress
Male DEGs		
c39916_g1_i4	arfaptin-2	F:phosphatidylinositol-4-phosphate binding; F:protein domain specific binding; P:regulation of protein secretion; P:ruffle organization; C:trans-Golgi network membrane; P:regulation of Arp2/3 complex-mediated actin nucleation
c32569_g2_i1	galactose-specific lectin nattectin	P:system development
c33910_g2_i1	galactose-specific lectin nattectin	P:biological_process; P:skeletal system development; P:central nervous system development; P:cell adhesion; F:hyaluronic acid binding; C:cellular_component; C:proteinaceous extracellular matrix
c38055_g1_i3	testosterone 17-beta-dehydrogenase 3	C:mitochondrion; F:steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor; P:cellular hormone metabolic process; C:endoplasmic reticulum; P:steroid biosynthetic process; P:single-organism cellular process; P:cellular biosynthetic process; P:hormone biosynthetic process
c22170_g1_i2	galactose-specific lectin nattectin	P:biological_process; C:cellular_component
c40779_g1_i1	galactose-specific lectin nattectin	P:biological_process; C:cellular_component
c39419_g1_i2	28s ribosomal protein mitochondrial	F:structural constituent of ribosome; C:mitochondrial small ribosomal subunit
c43665_g1_i1	protein c8orf37 homolog	
c34034_g1_i3	calbindin 2	C:nucleus; F:calcium ion binding; P:regulation of cytosolic calcium ion concentration; C:cytosol; C:synapse; C:neuron projection

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c37969_g2_i2	transient receptor potential cation channel subfamily m member 2	F:hydrolase activity
c36992_g1_i2	dickkopf-related protein 2 precursor	P:posterior lateral line neuromast development; P:forebrain neuron differentiation; P:hepatic duct development; P:endocardial cushion development; P:cell dedifferentiation; P:ectoderm development; C:extracellular region; P:negative regulation of canonical Wnt signaling pathway; F:protein binding; P:notochord development; C:cytoplasm; P:cell migration involved in gastrulation; P:liver development; P:sensory epithelium regeneration; P:regulation of cell migration
c29643_g1_i1	galactose-specific lectin natectin	F:DNA binding; P:biological_process; C:nucleus; C:BAF-type complex; P:chromatin remodeling; P:skeletal system development; P:central nervous system development; F:calcium ion binding; P:cell adhesion; F:hyaluronic acid binding; C:cellular_component; C:proteinaceous extracellular matrix
c37252_g1_i3	pyruvate dehydrogenase e1 alpha 1	C:intracellular membrane-bounded organelle; F:pyruvate dehydrogenase (acetyl-transferring) activity; P:glycolytic process; P:oxidation-reduction process
c41634_g1_i1	baculoviral iap repeat-containing protein 2	P:inhibition of cysteine-type endopeptidase activity involved in apoptotic process; C:nucleus; C:spindle microtubule; F:ubiquitin-protein transferase activity; F:zinc ion binding; C:cytoplasm; F:cysteine-type endopeptidase inhibitor activity involved in apoptotic process; P:regulation of signal transduction; P:protein ubiquitination; P:mitotic spindle assembly
c44201_g1_i1 4	slam family member 9-like	
c43051_g1_i4	amyloid beta a4 protein precursor	F:serine-type endopeptidase inhibitor activity; F:transition metal ion binding; F:protein binding; P:central nervous system development; P:axon extension; P:vasculature development; C:integral component of membrane; F:heparin binding; P:convergent extension
c39841_g1_i2	adiponectin receptor 1a	C:integral component of membrane; F:hormone binding; P:adiponectin-activated signaling pathway; F:receptor activity; C:plasma membrane
c43420_g1_i4	probable atp-dependent rna helicase ddx5	F:nucleic acid binding; C:intracellular organelle; F:ATP binding; C:intracellular ribonucleoprotein complex; P:positive regulation of transcription, DNA-

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		templated; P:RNA secondary structure unwinding; P:rRNA processing; F:ATP-dependent RNA helicase activity
c31966_g1_i3	protein cornichon homolog 2	C:membrane; P:intracellular signal transduction

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ABSTRACT

USING RNA-SEQ TO STUDY THE SEX-ROLE REVERSED GULF PIPEFISH:
ARE PATTERNS OF SEX-BIAS IN GENE EXPRESSION DIFFERENT
WHEN WE ARE DEALING WITH MR. MOM?

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Syngnathid fishes (sea horses, pipefish, and sea dragons) are a group of organisms that exhibit several unusual behaviors such as male brooding and sex-role reversal. Most sex-bias gene expression studies have been done on species that exhibit traditional sex-roles (male-male competition) where females invest more parental time than males. Here we use the Gulf pipefish (*Syngnathus scovelli*) which exhibits female-female competition for mates along with male brooding to look at sex-biased expression. We used RNA-seq technology to build the first transcriptome of the brain and gills of this species using four mature male and three mature female pipefish from Port Lavaca Bay, Texas. Differential gene expression analysis returned 73 differentially expressed genes (DEGs) in the brain and 29 DEGs in the gill. In the brain, there were 26 DEGs upregulated in females and 47 upregulated in males. In the gills, there were 10 DEGs upregulated in females and 19 upregulated in males. Gene Ontology analysis found many pathways altered between the brain and gill transcriptomes. We focused on three areas of interest in the sex-bias gene expression field which included sex steroids/hormones in the brain, circadian rhythms, and immunity. We found a few genes differentially expressed for all these categories. In addition, this novel transcriptome will help in conservation efforts of

threatened species from this family via its use in identifying polymorphic markers. Such markers may prove valuable in surveying population structure and genetic variability.