MEASURING SEX-BIAS GENE EXPRESSION IN GENES INVOLVED IN THE SEX DETERMINATION PROCESS IN THE BRAIN OF BROOK TROUT

by

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Submitted in partial fulfillment of the

requirements for Departmental Honors in

the Department of Biology

Texas Christian University

Fort Worth, Texas

May 8, 2017

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ABSTRACT

Many species from fruit flies to humans exhibit sexual dimorphism. Much of this phenotypic variation can be attributed to differential expression of genes between males and females, also known as sex-bias gene expression. The salmonid family, which includes salmon, trout, and charr, are known to exhibit a high degree of sexual dimorphism in many phenotypes. However, previous sex-bias gene expression studies have been limited to either gonadal tissues, or whole embryos at early stages of development. This project aims to expand our knowledge of sex-bias gene expression by identifying and quantifying gene expression within the brain tissue of two-year old brook trout (*Salvelinius fontinalis*). A special emphasis was placed on the *sdY* gene, a possible master sex-determining gene in salmonids. Expression of *sdY* has been characterized in the gonads of developing salmonids, but its involvement in processes other than sex determination is unknown. This project looked for similar patterns of expression between *sdY* and other genes to try and reveal the role of *sdY* in brain tissue. Using RNA-seq data and qRT-PCR validation, 12 genes were measured for sex-bias gene expression. Of these genes, only *sdY* showed a significant difference in expression between male and female brook trout ($p =< 0.05$). Two other genes (*sox9, ar-alpha*) were upregulated in male brook trout although this difference in expression was not significant ($p>0.05$). These results indicate that *sdY* is being expressed in the brains of mature male brook trout far past the point of sex determination and that the upregulation of *sdY* in males could be connected to androgen receptivity and chondrogenesis pathways.

INTRODUCTION

Many species, from fruit flies to humans, exhibit phenotypic differences between the sexes (sexual dimorphism: Cheng and Kirkpatrick, 2016). These differences have fascinated biologists since Darwin first discussed them in 1871 (Darwin, 1871). Since then, theories explaining the underlying reason for sexual dimorphism, such as sexual selection, have been well documented and studied (Lande, 1980; Korkman, 1957). Sexual selection (the process of natural selection arising through certain physical traits that may grant the possessors of these traits greater success in obtaining mates) is believed to be a driving force behind the evolution of sexually dimorphic traits. Sexual selection can give rise to sexual dimorphism through two separate mechanisms: intrasexual competition for mates (members of the same sex competing with each other in order to mate with the opposite sex) and intersexual mating preferences (one sex choosing members of the opposite sex to mate with) (Darwin, 1871). Despite the large degree of phenotypic difference between males and females of many species, the genome of males and females are essentially identical (apart from the protein coding genes located on the heterogametic sex chromosome). Therefore, many differences in phenotype must be due to alternative expression of genes on the autosomes (Rinn and Snyder, 2005; Ranz et al., 2003). This alternative expression is often referred to as sex-bias gene expression (Grath and Parsch, 2016). Discovering and measuring both the identity of genes exhibiting sex-bias gene expression, and the level of sex-bias gene expression can help explain phenotypic differences between males and females.

Although sex-bias gene expression is well documented in mammals and birds, there is limited information about sex-bias gene expression in fish (Naurin et al., 2011; Itoh et al.,2010; Rinn and Snyder, 2005; Brawand et al., 2011). However, fish also exhibit many differences in phenotypes between the sexes. These phenotypic differences represent a potential product of sex-bias gene expression and provide a wide range of targets for gene expression studies. For example, male and female guppies exhibit drastic differences in coloration, with the males having a wide variety of bright colors and patterns while the females are much less colorful (Endler 1978). These differences eventually led to the discovery of genes involved in pigmentation pathways that have high levels of sex-bias gene expression (Endler, 1978; Sharma et al., 2014).

The salmonids (salmon, trout, and charr) are known to exhibit sexual dimorphism in many morphological and behavioral traits, such as color, nose and jaw shape, adipose fin size, and aggression (Kazyak et al., 2013; Beacham and Murray, 1986; Johnsson and Akerman, 1998).

Fig 1. Male and female coaster brook trout

These differences strongly suggest the occurrence of sex-bias in gene expression. However, most gene expression studies on salmonid fish have focused on well-known commercial species such as rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar)* (Hale et al., 2011; Lubieniecki et al., 2015; Baron et al., 2005; Cavileer et al., 2009). In addition, most previous studies have also been limited to measuring sex-bias gene expression in gonadal tissues during embryonic or juvenile life stages (Hale et al., 2011; Lubieniecki et al., 2015; Baron et al., 2005; Cavileer et al., 2009). Sex-bias in gene expression is known to be tissue specific; and therefore, patterns of gene expression in the gonads are unlikely to be typical of other organs.

All salmon, trout, and charr have a male heterogametic sex determining system, where males have a XY sex karyotype, and females have a XX karyotype (Baron et al., 2005). The gene *sdY* is believed to be (or be linked to) the master sex-determining gene. Male specific amplification has been found in 15 salmonid species and it is found on the Y-chromosome (Yano et al., 2013). The discovery of *sdY* in 15 different species suggests that sex determination is conserved between these different salmon and trout (Yano et al., 2013). It is known that *sdY* is highly expressed in the testis of male fish during testicular differentiation (Yano et al., 2012; Lubieniecki et al., 2015). However, patterns of expression for *sdY* are otherwise unknown. Given its identity as a male-specific master sex-determining gene, it is possible that *sdY* is similar in function to *SRY*, the master sex-determining gene in mammals (Yano et al., 2012; Kashimada and Koopman, 2010). In humans, *SRY* has been shown to play a role in multiple pathways including the regulation

of catecholamine synthesis and metabolism in mature male brains (Czech et al., 2012). Whether *sdY* is similarly involved in other pathways is unknown, as far as we know.

The goal of this project is to identify and quantify sex-bias gene expression within the brains of mature, two-year-old brook trout with a special emphasis on the *sdY* gene and other candidate genes possibly related to *sdY* expression in the brain. Using RNA-seq data and qRT-PCR validation, genes that exhibit sex-bias gene expression can be identified (Marioni et al., 2008; Fang and Cui, 2011). These genes can then be annotated and searched for genes with functions related to catecholamine synthesis/metabolism or other pathways/functions hypothesized to be related to *sdY*. If these candidate genes are identified and found to have similar patterns of expression as *sdY*, it could help reveal the role of *sdY* in tissues other than the gonads after sexual maturation.

MATERIALS AND METHODS

Samples

Samples for this study were selected from 32 two-year old brook trout from Tobin Harbor, Michigan. Samples used to collect data came from two different lines of brook trout — one produced by crossing two resident trout and one produced by crossing two migratory coaster trout. The coaster trout is a potamodromous ecotype that migrates from small streams to Lake Superior for up to three years before returning to their natal streams to spawn (Huckins et al., 2008). Fish were euthanized with a lethal dose of MS222 and measured and weighed. The whole brain was extracted and placed in 500 ul

of RNAlater® for preservation. Samples were kept at -80 °C until RNA extraction which followed standard Trizol procedure.

Gene expression and candidate gene selection

RNA-seq data were collected previously from the 32 samples. These data were first trimmed to remove primers and poor quality sequence and then assembled into contigs using Trimmomatic and Trinity respectively (Bolger et al., 2014; Grabherr et al., 2011). Annotation of the assembly was performed using BLAST methods against the zebrafish transcriptome (Altschul et al., 1990). RSEM was used to align raw reads back to the assembly for estimating gene expression. Gene expression analyses were performed to look for sex-bias genes within resident and coaster lines. Each differential gene expression analysis was performed on these read-count data using edgeR (Robinson et al., 2009). A cutoff of 1 count per million in at least three samples was applied and any gene that did not meet this requirement was removed from the analysis. A trimmed mean of m-values (TMM) normalization was then applied to account for library differences (Robinson and Oshlack, 2010). Likelihood ratio tests were used to identify differentially expressed genes between the sexes. A false discovery rate correction of 0.05 was set to account for multiple comparisons (Benjamini and Hochberg, 1995). These genes were annotated using blastx (Altschul et al., 1990). Four total genes were selected for validation by qRT-PCR (*sdY, POMC, somatolactin, FSH)*. These candidate genes were selected based on RNA-seq data and annotations that suggested important functions within brain tissue. Eight more genes were analyzed using qRT-PCR based on their hypothesized participation in pathways involving *sdY* (*cyp19b, AR-α, AR-β, sox9, Th,*

MAO A, amh, D2R). These genes were either involved in pathways connected to male vs female brain development in mammals (*Th, MAO A, D2R)* or involved in sexual determination and differentiation in salmonids (*cyp19b, AR-α, AR-β, sox9, amh)*.

cDNA production

Extracted RNA was converted to DNA using the Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR. The manufacturer's procedure for First-Strand cDNA synthesis was followed, using random hexamers as the primer. For each sample, 2 µL of RNA was denatured at 65 °C for 5 minutes with 6 µL water, 1 µL of 50 ng/µL random hexamers, and 1 µL of 10 mM dNTP mix. Following denaturation, 10 µL of the cDNA Synthesis Mix described by kit was added to each sample. These mixtures were incubated for 10 min at 25 °C followed by 60 min at 50 °C. The reactions were terminated at 85 °C for 5 min. Samples were diluted with 30 µL of nuclease-free water and stored in a -20 °C freezer.

Primer synthesis and optimization

All primers were designed using transcriptome data from either rainbow trout (*Oncorhynchus mykiss)* or Atlantic salmon (*Salmo salar)*. For the four genes selected for validation (*sdY, POMC, somatolactin, FSH)*, the sequence data that were used came from the EST (expressed sequence tag) that produced the strongest hit in BLAST based on e-value and bit score. For the eight genes hypothesized to be involved in *sdY*-related pathways, sequence data were obtained from NCBI's GenBank. Two or three primer pairs per candidate gene were designed in Primer3 (Untergasser et al., 2012). PCR

amplification and gel electrophoresis were used to determine the optimal primer pair for each candidate gene. PCR reactions consisted of 0.2 mM of the forward primer, 0.2 mM of the reverse primer, 5.0 μL of GoTaq polymerase, 3.6 μL of water, and 1 μL of cDNA for a total of 10 μL. PCR products were run out using gel electrophoresis and viewed under UV light. Multiple primer pairs were compared and the optimal pair was selected through comparison of gels.

Table 1. Optimal primer pairs used in qRT-PCR

Gene	Reference Genome	Forward Primer Sequence	Reverse Primer Sequence
sdY	*Salmo salar*	TGCGAAGAGGAGGTGCTTAT	GCTTTGGGAGAGAGATGACG
POMC	*Salmo salar*	TCCCCTCCACTGTTTACTGG	TTTTTCCAAGTTAAGGCTTCC
Somatolactin	*Salmo salar*	CAGGAGAGCCTGACTGTTCC	TTTTGCCATGTCCTATGTGTG
FSH	*Oncorhynchus mykiss*	ACACCGACTGTGATCGCATA	GCACATCAACAATGGAAACG
cyp19b	*Oncorhynchus mykiss*	GAACCCTGAGGTGGAGATGA	CAGGATGGAACCTCATGGAC
AR-α	*Oncorhynchus mykiss*	CTGGAGCTGGAGAAAACGTC	GCTGTATGGTCGCCACTTTT
AR-β	*Oncorhynchus mykiss*	CACGCTCTGCATCTTGTCAT	CACAGACATGAGCTGGGATG
Sox9	*Oncorhynchus mykiss*	CGAGTTCAAGAAGGCTGACC	CTTGACATGGGGCTTGTTTT
Th	*Salmo salar*	GTCCCACTCTGTGCCAATTT	GTCGCCACTTAGCAAACACA
MAO A	*Salmo salar*	TGTGGTGGTGGGTGAGACTA	TTCTGACGGCACTGTATGACTT
AMH	*Salmo salar*	ACCTCAAGGTGGTGGAGTTG	ATCCACACTCCTTGGACACC
D2R	*Salmo salar*	AAAACGGGTGAACACAAAGC	TTGACAGGAAAGCTCCCATT

EF1-α[*]	*Oncorhynchus mykiss*	ACTCCAATGGGGTGACTCTG	GCCCACAGGTACAGTTCCAA
60S[*]	*Oncorhynchus mykiss*	ATTGGCATCTCGGTTGACTC	TGAGCTTGGAGCGGTACTCT
Bactin[*]	*Oncorhynchus mykiss*	ACTGGGACGACATGGAGAAG	GGGGTGTTGAAGGTCTCAAA

*Housekeeping/reference gene used to normalize expression of candidate genes

qRT-PCR

Initially, eight samples were run for each candidate gene: five males and three females from the coaster group. Eight additional samples, three males and five females, were run for several of the more promising candidate genes, including *sdY*. These samples were also taken from the coaster group. For each gene, every sample was run in triplicate along with a negative control well that lacked candidate gene cDNA. Each qRT-PCR reaction consisted of 5 μL of SYBR Green, 0.36 mM of the forward primer, 0.36 mM of the reverse primer, 2.28 μL of water, and 2 μL of candidate gene cDNA for a total of 10 μL. All qRT-PCR plates were run using the StepOnePlus machine. qRT-PCR data was also collected for three housekeeping genes: beta-actin, 60S rRNA, and EF1-alpha. Only beta-actin was used in the final ΔΔcT calculations because the other two genes did not show equal expression between males and females ($p < 0.05$). The thermal cycling protocol for each genes was as follows: a holding stage of 95 °C for 10 min, a cycling stage of 40 cycles composed of 95 °C for 15 sec followed by the annealing stage for 1 min (temperature varied between 56-62 °C depending on the gene), and a melt curve stage composed 95 °C for 15 sec, 60 °C for 1 min, and then a step and hold increase to 95

°C in increments of +0.3 °C. The melt curve stage was used to ensure that no non-specific products or primer dimers were being amplified. A fluorescence threshold of 2.5 relative fluorescent units (RFU) was applied at the end of each run to provide consistency in cT scores across multiple plates. To account for PCR efficiency, serial dilutions were performed for each candidate and housekeeping gene (Lalam, 2006). Five μL of cDNA for each sample was pooled and then four 1:5 dilutions were performed. For all genes, each dilution was run in triplicate with a negative control that lacked cDNA. Each qRT-PCR reaction consisted of 5 μL of SYBR Green, 0.36 mM of the forward primer, 0.36 mM of the reverse primer, 3.28 μL of water, and 1 μL of diluted stock. The thermal cycling protocol was identical to the original qRT-PCR reactions.

ΔΔcT analysis

PCR efficiency (E) for each gene was determined using the serial dilutions explained above (Lalam, 2006). These efficiencies can be used in an improved ΔΔcT method to provide more accurate estimates of gene expression than the traditional method that assumes all efficiencies are 2 (Rao et al., 2013). E was calculated for both the candidate genes and the house-keeping genes. The average cT score for each dilution was plotted on the y-axis and the log10 of the arbitrary 5-fold dilution number was plotted on the x-axis. The slope of this line was used to help calculate E through this equation (Pfaffl, 2001):

Eq. 1

$$E = 10^{(-\frac{1}{slope})}$$

Using E for both the candidate gene and the house-keeping gene, the ratio of the expression of the candidate gene in male brook trout versus female brook trout in comparison to the house-keeping gene could be determined. The mathematical model to determine this relative expression ratio or relative quantification is shown in equation 2 (Pfaffl, 2001):

Eq. 2

$$ratio\ or\ RQ = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

In equation 2, E_{target} refers to the PCR efficiency of the candidate gene and E_{ref} to the PCR efficiency of the house-keeping gene. ΔCP_{target} represents the difference between the cT score of the female control sample for the candidate gene – the cT score of the sample in question for the candidate gene. ΔCP_{ref} represents the difference between the cT score of the female control sample – the cT score of the sample in question for the house-keeping gene. For each candidate gene, ratios were calculated for each sample. A log transformation was applied and then an ANOVA was used to test for statistical significance between the expression of male and female brook trout.

RESULTS

After expression analysis of the 12 candidate genes, only one gene, *sdY*, exhibited significant differential expression ($p<0.05$) between the brain tissue of male and female brook trout (Fig 2A). It was shown to be upregulated in males with a fold change of greater than 2600 compared to female brook trout. Out of the 11 other genes that were examined, six exhibited upregulation (*sdY, ar-beta, sox9, ar-alpha, D2R, Th, MAO*) and five exhibited downregulation (*cyp19b, POMC, amh, FSH, somatolactin)* in male brook

trout compared to female. However, the differences in expression for the majority of these genes was minimal and none showed statistically significant differential expression between males and females ($p \geq 0.05$). Nine out of these 11 genes exhibited a fold change of <1.5 between the brain tissue of males and females (*ar-beta, D2R, Th, MAO, cyp19b, POMC, amh, FSH, somatolactin)*. *MAO* was one of these genes and offers a good visual representation of the relative expression of these nine genes between males and females (Fig 2B). Two genes, *sox9* and *ar-alpha*, showed upregulation in males compared to females with fold changes 1.55 and 2.02 respectively (Fig 2C-D).

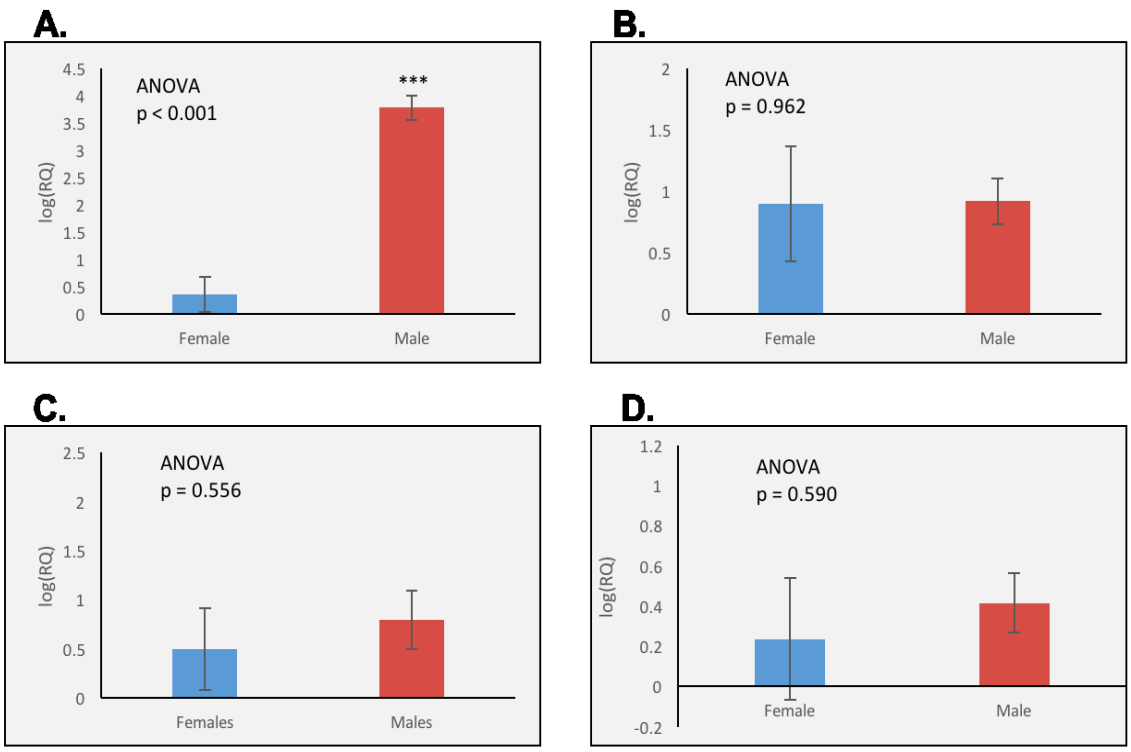

Fig 2A-D. (A) Relative expression of *sdY* in the brain tissue of brook trout. (B) Relative expression of *MAO* in the brain tissue of brook trout. (C) Relative expression of *ar-alpha* in the brain tissue of brook trout. (D) Relative expression of *sox9* in the brain tissue of brook trout.

DISCUSSION

The main finding of this study was the expression of *sdY* in the brain tissue of male brook trout. This shows that expression of *sdY* is not limited to the gonads and the processes of sexual determination and differentiation. Previous studies concerning the expression of *sdY* confine it solely to the gonads (Yano et al., 2012; Lubieniecki et al., 2015). One potential reason for the lack of differential expression in tissues other than the gonads in these previous studies is the age of the samples. They focused on early post hatch stages whereas this study looked at two year old fish at the point of sexual maturation. The results of this study reveal that, much like *SRY, sdY* has a role to play other than sexual determination and differentiation. With that known, the question then becomes what exactly is the function of *sdY* in the brain tissue of mature, male brook trout?

The ability to measure the expression of many genes in a relatively short period of time for a low cost using qRT-PCR offers one avenue to determine the function of a gene. This is possible through the principle of co-expression, which involves developing hypotheses for gene function based on similarities in expression patterns between the gene in question and genes of known function (Uygun et al., 2016). *sdY* exhibits male-specific expression so finding other genes that are upregulated in male brook trout could provide some clue to what the function of *sdY* is in the brain.

Two genes from this study, *ar-alpha* and *sox9*, were shown to be upregulated in males with fold changes >1.5. Previous gene expression studies have shown that a fold

change of 1.5 or greater is a reliable predictor of differentially expressed genes (Peart et al., 2005; Raouf et al., 2008; Bhargava et al., 2013). So despite the lack of statistically significant differential expression of these two genes between male and female brook trout, these fold changes indicate that they still could be playing a role biologically.

Based on the similar patterns of expression between these two genes and *sdY*, two biological pathways were selected that could possibly be linked to *sdY* expression in the brain tissue of male brook trout. The first of these pathways is androgen receptivity. This selection was based on the male-specific upregulation of *ar-alpha*, which codes for an androgen receptor. In a previous study looking at Arctic charr, it was shown that more aggressive males tend to have higher levels of circulating androgens (Elofsson et al., 2000). Aggression is also a sexual dimorphic trait in salmonids with the males being more aggressive in order to compete with other males for the rights to spawning grounds (Johnsson, 2001; Johnsson and Akerman 1998). Therefore, it is possible that *sdY* is helping contribute to these patterns of sexually dimorphic aggression. One possible pathway is that the expression of *sdY* in the brain tissue of male brook trout and other salmonids leads to the male-specific enhancement of *ar-alpha*, which in turn contributes to the male-specific aggression that has already been observed in many salmonid species.

The second hypothesized pathway is chondrogenesis or the production of cartilage and chondroid bone. This pathway was selected based on the male-specific upregulation of *sox9. Sox9* is a transcription factor that plays a role in many different biological processes with one of those roles being the master regulator of chondrogenesis

(Hino et al., 2014). Chondrogenesis is important in male brook trout and other male salmonids because it is required for the development of the kype (Witten et al., 2003). A kype is a hook that develops on the lower jaw of many male salmonids before spawning season. It is a secondary sexual characteristic that the male fish use to fight off other males for the rights to spawning grounds (Witten et al., 2003). Coaster brook trout tend to spawn around 2-3 years of age, meaning that kype development will also take place around this same age (Huckins et al., 2008). The samples used in this study were all around two years of age. Therefore, it is possible that expression of *sdY* is helping promote the male-specific production of cartilage and chondroid bone needed for kype formation. One possible pathway is that the expression of *sdY* leads to male-specific enhancement of *sox9*, which in turns leads to the activation of chondrogenesis needed for kype formation.

It is worth mentioning that these pathways are simply hypothesized pathways based on similar patterns of gene expression. Determining the function of a gene is difficult, especially in non-model organisms. In an ideal world, some sort of gene modification is the most direct and effective method (Alberts et al., 2002). This allows for observation of the downstream effects on different cellular processes that the gene modification produces and helps provide physical evidence for the function of the gene. Techniques of this nature include gene knockouts, RNA interference, and gene expression modifications using bacterial plasmids (Alberts et al., 2002). However, most of these are time and resource intensive. For example, it takes about a year and over $12,000 to create a single knockout mouse (Hall et al., 2009). RNA interference has

become a popular technique in recent years, but still requires cell transfection, which is both costly and labor intensive (Mocellin and Provenzano, 2004). Gene expression studies offer a cost and time-effective alternative to these methods.

While gene expression studies are very helpful in providing possible functions of a gene, ultimately some sort of gene expression modification is necessary to confirm function definitively. However, there are steps that can be taken to improve this study's ability to detect sex-bias gene expression and therefore its ability to reveal the function of *sdY*. One such step would be to use samples of individual brain structures rather than whole brain samples. The use of whole brain samples in this study could have served to mask expression profiles of individual regions of the brain (Yang et al., 2006). The brain is not a homogenous organ. It contains many different regions that perform different functions and therefore could be expressing genes differently. By looking at the expression of *sdY* and other possible sex-biased genes in multiple structures of the brain, one could achieve a much clearer picture of what the role of *sdY* is.

Similarly, it would be helpful to look at the expression profile of *sdY* in tissues other than the brain. Doing so could reveal if the expression of *sdY* past the point of sexual determination and differentiation is confined to the brain or if it has a role to play throughout multiple tissue types in mature male brook trout. In addition, different salmonid species could be observed for expression of *sdY*. This would be important in determining if this brain-specific expression is unique to brook trout or if it is involved in pathways shared throughout the salmonid family. Finally, the function of *sdY* could be

narrowed down even further by looking at its expression during different developmental time points. The samples used in this study were mature adults, but it is possible that expression of *sdY* could be important much earlier, such as in setting up the sexual differentiation of the brain seen in many vertebrates, including salmonids (Vizziano-Cantonnet et al., 2011).

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