Sex Determination in *Xenopus laevis* Tadpoles and Effects of Sexual Dimorphism on Traumatic Brain Injury

An Abstract of a Senior Honors Thesis

Presented to

the Faculty of the Department of Biology and Biochemistry at University of Houston

In Partial Fulfillment

of the Requirements for the Degree Bachelor of Science

in Biology

by Dania Khan

May 2021

Sex Determination in *Xenopus laevis* Tadpoles and Effects of Sexual Dimorphism on Traumatic Brain Injury

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Abstract

This paper aims to create a standard sex determination method of *Xenopus laevis* tadpoles at a stage in which gonadogenesis is not yet complete however, sexual differentiation is ongoing. The methods are comprised of using tadpole tail snips, isolating DNA, conducting PCR based on six different sex-related gene identified in this paper, and then using gel electrophoresis to observe the presence of each gene within the *Xenopus laevis* tadpoles by observing presence of correlating band patterns on the gel. This project is meant to be utilized in terms of identifying the sex of the tadpole subjects later used in Traumatic Brain Injury experiments (courtesy of Sydnee Spruiell) to be able to observe sexual dimorphism of tadpoles upon experiencing traumatic brain injuries. Of the six sex related genes: *dmrt1.L*, *dmrt1.S*, *dmw*, *capn5Z*, *ccdc69W*, and *scanW*, the results convey that *ccdc69W* and *scanW* are the most reliable indicators of sex determination. Both genes are highly likely (r=0.80) to be present together and if expression is tested and presence is observed then the corresponding tadpole can be identified as a female. The Edema Accumulation Experiment is conducted by measuring brain water content of tadpoles after a traumatic brain injury. The results implied that while both male and female tadpoles had undergone similar percentages of water content gain, the male tadpoles had a higher level of water accumulation because their water content levels at baseline are higher to begin with than in females.

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Introduction:

Traumatic brain injuries (TBIs) are known as events that have the potential to alter brain activity and function from its uninjured, homeostatic state. These events can occur as a result of injuries that are encountered in sports, vehicle accidents, and essentially any time the head experiences forceful impact or acceleration-deceleration forces. TBIs are the leading cause of morbidity and mortality in both males and females worldwide (Caplan et al 2016). Historically, men are reported to be more prone to fatal TBIs however, males are also represented at higher rates in clinical studies regarding TBI than their female counterparts which may act as a confounding variable (Caplan et al 2016). This theory is a leading cause of why it is important to study both sexes in research that focuses on TBIs. It is essential to fill the gap in knowledge about how TBIs are sexually dimorphic, caused by a lack of inclusion of females in modern neuroscience research.

The molecular biology perspective of TBIs revolves around inflammatory responses induced by specialized cells in the brain called microglia and astrocytes. Microglia are the "first responders" to the injury site when a TBI is induced. Microglia are macrophages that propagate the inflammation of neuronal cells that neighbor the site of injury. They can achieve this by producing inflammatory mediators such as cytokines and chemokines (Villapol et al 2017). Despite the beneficial response of microglial cells to TBIs, there is a dichotomy with the induction of inflammation and its effects on TBIs. On the one hand, inflammation is required because it helps the brain keep out parasites and maintain tissue homeostasis upon impact of injury (Cederberg and Siesjo 2009). On the other hand, copious or sustained levels of inflammation can lead to glial cells playing a large role in the activation of degenerative diseases linked to the central nervous system (CNS) in both acute and chronic aspects (Sandhir et al 2008). Astrocytes are glial

cells that help maintain a border between the non-neural cells and the neural cells, called the blood brain barrier. They are also thought to have a similar dichotomy as microglia do. Astrocytes were originally studied for the role they play in restricting cytotoxic inflammation in the brain; however, astrocytes have recently been discovered to have proinflammatory functions as well, that may contribute to the cellular death of neurons (Sofroniew 2017).

With the knowledge of conflicting effects of inflammation, it is important to consider the extent of inflammation in terms of cerebral edema. Cerebral edema is the influx of water content in brain tissue as a result of a TBI, causing the loss of integrity in the blood brain barrier, which allows a larger than normal of water to permeate into the brain. (Mcbride et al 2014). The glial cells, themselves, begin to swell as extracellular pH and concentration of ions such as potassium, sodium, and chloride changes begin to change (Mcbride et al 2014). The effect of a bloating, swelling brain can be fatal due to the increase of intracranial pressure and the decrease of cerebral perfusion pressure which can limit cerebral oxygenation (Zusman et al 2020). There are two types of cerebral edema that can occur due to a TBI. Vasogenic edema refers to the disruption of the blood brain barrier (BBB). The BBB is specific to the homeostatic functions of the brain and regulates what molecules can enter and exit the brain. Cytotoxic edema refers to the water accumulation that occurs intracellularly in astrocytes and neurons. Characteristics such as increased permeability of sodium and potassium ions and failure of water and ion pumps at the cell surface play a role in sustaining cytotoxic edema (Unterberg et al 2004).

One of the more difficult aspects of studying TBI is that there aren't many ways of measuring a TBI that can also be quantified, either in-vivo or in-vitro. In a medical aspect, the severity of TBIs in general can only be determined based on self-reported symptoms (from the person sustaining the injury). For the purposes of this study, we will focus on a way to implement

an in-vitro approach to understand what physical changes an organism's brain goes through after a TBI. Cerebral edema can be quantified based on wet-brain weight in contrast to dry-brain weight. The equation for determining difference in cerebral edema is as shown: $\boldsymbol{\%\, edema} = [((\boldsymbol{wet\ brain - dry\ brain\ weight})/\boldsymbol{wet\ brain\ weight})\ \boldsymbol{x}\ \mathbf{100}]$. Theoretically, the difference between the wet and dry weights can be an indicator of the severity of the TBI. Furthermore, behavioral assays of post-TBI tadpoles are a very important indicator of damage inflicted by TBIs interfering with brain functions. When comparing edema accumulation to an organism's behavior (post-TBI), we have the potential to correlate high levels of edema with lower behavioral responses than the standard.

The sex of an organism is a very important characteristic to consider due to the developmental differences that all male and females have in the majority of organisms. As an organism develops from its embryonic stage, the primordial germs cells, known as precursors of gametes, begin to differentiate into the destined sex of each individual organism. This process is known as sex differentiation which, prior to complete gonadogenesis, can play a confounding role because the physiology of male and female organisms begins to change in ways so subtle that it becomes hard to observe the change visibly. In the model organism *Xenopus laevis*, also known as the African clawed frog, the life cycle includes various stages in which metamorphosis occurs. The four basic stages are: egg, tadpole, froglet, and then the adult frog. According to Nieuwkoop and Faber, there are certain anatomical differences in each part of the life cycle that can be referred to as Nieuwkoop and Faber (NF) stages (Niewkoop and Faber 1994). At NF stage 50, undifferentiated gonads begin to develop. This is when the organism develops gonads that are identical for both sexes at this stage and have the ability to form into either testes or ovaries (Piprek et al 2019). At NF stage 53, *X. laevis* tadpoles are known to undergo the onset of sexual

differentiation where the expression of genes corresponding to each sex begins to occur (Piprek et al 2019). The completion of this process occurs at NF stage 56 where sexual differentiation is fully achieved through expression of genes that correspond to one of the two sex determining chromosomes. Between NF stages 50-56, it is important to understand that although sexual differentiation is progressing to completion, the gonads are still not fully developed in the tadpole stages of *Xenopus laevis*. This study will focus on determining the sex of tadpoles at NF stage 55 in order to study the effects of TBIs on brain water content, while controlling for the sex of each animal being studied. For further context, tadpoles at NF stage 55 do not have skull caps. This is important because research shows that simply the act of removing the brain from under the skull cap has the potential to inflict brain injuries that interfere with the controlled TBI state of the brain (Cole et al 2011).

Like many organisms, *Xenopus laevis* is known to have two different sex-linked loci that carry different genes that correspond to each sex. This is similar to many other organisms however, *Xenopus laevis* is unique because its two sex determinant chromosomes, Z and W, are homomorphic chromosomes making them karyotypically indistinguishable (Mawaribuchi et al 2017). As a result, they have small differences in size and gene content, which in the case of Z and W gives rise to two different sexes. For the purposes of this study, the two basic genotypes are ZZ and ZW although other combinations of these two chromosomes may exist in a trisomy or genetic mutation (Mawaribuchi et al 2017). In amphibians, the heterogametic sex is the female means that any frog with the ZW genotype will develop ovaries and express W-linked genes. For males, the genotype of ZZ would induce expression of genes that correlate with testis development (Yoshimoto and Ito 2011). The Z-specific sex-linked gene is determined to be *dmrt1* whereas the W-specific gene is *dmw*. According to current published literature, *dmw* diverged from *dmrt1* as a

result of a mutation, making the two genes paralogs. Furthermore, the DNA-binding domains of both *dmw* and *dmrt1* have shown high similarity in sequence identity although the sequence of transactivation domains, located in the C-terminus, differ between the two genes (Yoshimoto et al 2010). Although research is currently limited on how exactly the genes *dmw* and *dmrt1* function, it has been established that *dmw* is able to trigger ovarian development by blocking the activation of *dmrt1* which would have originally induced testis development (Sun et al 2017). It was also found that *dmw* acts as an inhibitor to *dmrt1* because transgenic ZW tadpoles were introduced to a *dmw* knockout vector which allowed the transgenic tadpoles to develop testicular-appearing gonads or functioning testicular gonads (Yoshimoto et al 2010).

In addition to *dmrt1* and *dmw*, research has indicated the presence of three more sex-linked genes that are able to be expressed in early sexual differentiation in *X. laevi*s. The two genes located in the W-specific loci are known as *scanw* and *ccdc69w*. In contrast there is one gene found in the Z-specific loci known as *capn5z* (Mawaribuchi et al 2017). What makes these particular genes useful in this study is that aside from high expression levels in gonads, all three have substantial expression rates in the somatic cells of tadpoles between NF stages 50-56. As previously discussed, these stages represent the initiation to completion of sexual differentiation, respectively (Mawaribuchi et al 2017). To ensure the successful sex determination of sample, all five sex-determining genes would be tested to determine patterns of gene segregation and correlation between genes that correspond to each sex-specific locus.

Methods and Materials:

Sample Collection: Various samples from *Xenopus laevis* were collected at two different stages in the life cycle. Starting from the adult phase of their life cycle, *Xenopus laevis* tissues such

as testes, ovaries, and skin samples were collected after euthanasia of adult frogs. The tadpole samples consisted only of tail snip samples that were obtained by snipping the tail of each respective *Xenopus laevis* tadpole at Nieuwkoop and Faber (NF) stage 55. In order to designate the samples for genomic DNA isolation, the samples must be directly placed in the Homogenization Buffer. A 10 milliliter (ml) sample of Homogenization Buffer can be prepared by adding 1 ml of 10% SDS (sodium dodecyl sulfate), 20 microliters (µl) of 0.5M Ethylenediaminetetraacetic acid (EDTA), 200 µl of 1M Tris buffer at 7.5 pH, 200 µl of 5M NaCl (sodium chloride), and 8.56 ml of autoclaved water. Each individual tissue sample should be reconstituted in 0.5 ml of Homogenization Buffer. Proteinase K is to be added immediately before starting the genomic DNA isolation protocol whereas the Homogenization Buffer can be prepared beforehand. After adding the sample in Homogenization Buffer, 20 µl of the Proteinase K needs to be added to the mixture. This entire process needs to occur directly after obtaining the samples fresh from the organisms.

Genomic DNA Isolation: The reconstitution of tissue in Homogenization Buffer and Proteinase K is the first step of genomic DNA isolation. This mixture needs to be left overnight at 55°C in a heating incubator. After the overnight step, the samples are removed and 1 µl RNAse A at a concentration of 20 mg/ml is added to each sample. The samples are then intubated at 37°C for one hour. Majority of the tissue sample should be reconstituted into the solution after completion of this step. Each sample is then mixed into equal volumes of phenol/chloroform under the fume hood. Equal volumes refer to adding 1000 µl of phenol/chloroform in a sample mixture of 1000 µl. Then place the samples on a nutator at room temperature for 5 minutes to allow samples to gently rock back and forth. Then use a centrifuge to spin down contents for 5 minutes at 12,000 revolutions per minute (rpm) at room temperature. Carefully remove the aqueous (top) layer into

a new labelled tube and precipitate the sample with 2.5X volume of 100% isopropyl alcohol (IPA). Next add 10% 3M sodium acetate (NaOAc). For reference, the centrifuged sample would be of 500 µl therefore, 1250 µl of IPA and 50 µl of NaOAc would be added. This mixture is placed at -20°C for an hour or overnight to precipitate the DNA. Proceed to spin the samples down at 12,000 rpm for 15 minutes at room temperature. Discard the supernatant (liquid on top of pellet at bottom of tube). Clean the pellet with two ethanol washes using 75% EtOH and spin down the samples between each wash for 5 minutes at 12,000 rpm at room temperature. After the final wash, dry the pellets of any ethanol by placing the tubes upside down on a paper towel and leave for 10 minutes. After the pellets are effectively dried, resuspend the pellets in 30 µl of nuclease-free water (NFW). The DNA can now be nano-dropped using the Thermo-Fisher Nanodrop. The nano-drop should be set to detect double-stranded DNA. Make sure to use 1 µl of NFW to blank the instrument which serves as a control. Then analyze samples using 1 µl and record data into a chart labelled with ng/µl, 260/280, and 260/230.

PCR Protocol: The real-time PCR Protocol has three parts. The first part consists of creating the master mix which is constant across all the samples hence it can be made altogether and then redistributed. The master mix consists of a 10X reaction buffer, 10 mM (millimolar) dNTPs, 50 mM $MgCl_2$ (magnesium chloride), and NFW. The concentration of each component for one individual sample is as follows: 2.5 µl of 10X reaction buffer, 0.5 µl of 10 mM dNTPs, 1 µl of 50 mM $MgCl_2$, and then add NFW up to a total volume of 24.8 µl. Each concentration listed needs to be multiplied by the number of samples plus one more. For example, if 9 samples were being prepared then a master mix for 10 samples needs to be prepared out of precaution. Part two of this protocol requires prepping the individual samples. The components needed for part two are 10 µM forward/reverse primer mix, template DNA, and Taq polymerase. First, 24.8 µl of the

master mix must be distributed in labelled tubes. For each tissue sample, 9 primers were used: *hisH4*, *odc*, *zw*, *dmrt1.L*, *dmrt1.S*, *dmw*, *capnZ*, *ccdc69W*, and *scanW* (Mawaribuchi et al 2017). For this set of 9 samples, each one would have the same template DNA. The concentration is determined based on the DNA concentration verified by the nanodrop. The DNA concentration should contain 1000 ng/µl. Of these 9 samples, each one has a different 10 µM forward/reverse primer mix corresponding to the primers mentioned above. The only time the template DNA changes is when a new tissue sample is being tested. For example, Tadpole A has 9 samples for each primer with the same template DNA. For Tadpole B, the template DNA would change for the same 9 primers. After the two components are added, 0.25 µl of Taq polymerase is added to each sample. The Taq polymerase is heat sensitive so it needs to be kept on an ice block and should not be kept out of the fridge for too long. The samples should be vortexed before being placed in the thermocycler. Part three of the PCR process requires setting up the thermocycler specific to the samples undergoing PCR. The starting time and melting time should be at a duration of 30 seconds at 95°C, annealing time should be 30 seconds at 55°C, elongation time should be 30 seconds at 72°C, and holding temperature should be at 4°C.

Gel Electrophoresis + Imaging: Gel electrophoresis requires making/setting an agarose gel with ethidium bromide, preparing samples with dye, and setting up the power supply equipment. In order to make the gel, 1.4 grams (g) of agarose powder is mixed into 70 ml of 1X Tris-acetate-EDTA (TAE) buffer in a flask. This particular concentration will make a 2% agarose gel. The mixture is then heated in the microwave in increments of 15 seconds to prevent boiling over. After the agarose powder is completely dissolved in the TAE buffer, the solution needs to be cooled. While the solution is set aside to be cooled, the gel tray needs to be balanced to achieve an equally thick gel. After the solution is reasonably cool, 3.5 µl of ethidium bromide needs to be

added to the 70 ml solution under the fume hood. Ethidium bromide is a carcinogen and needs to be handled with care. Pour the solution into the gel tray right after adding the ethidium bromide. Place two 10 well combs in the correct position of the gel tray. Let the gel set for 30 minutes and allow it to fully solidify before removing the combs carefully. Fill the gel chamber with 1X TAE buffer till it touches the conductor points. Undo the gel tray from the balancing apparatus and place into the gel chamber, making sure that the buffer covers the entire surface of the gel and into the wells. The samples need to be prepared by adding a 6X Blue/Orange loading dye buffer. For 10 µl of the sample, vortex 1.6 µl of the loading dye buffer into the solution. Load the first well with 1.6 µl of a 100 base pair ladder. Load the next nine wells with the nine samples. After loading the samples, the gel needs to be run at 100 volts and 2 amps for a duration of 30 minutes. The final step is to image the gel. The gel tray needs to be placed into the imager. The settings need to be at auto exposure for ethidium bromide for DNA which will allow the gel to be exposed for efficient observation of presence of bands.

TBI Data Accumulation: All TBI related data such as the cerebral edema accumulation data was conducted and analyzed by Ph.D. candidate, Sydnee Spruiell. The incorporation of TBI-related data into sex determination data serves as further evidence of the advantages that arise from being able to successfully determine the sex of *Xenopus laevis* tadpoles.

Results:

DNA Genotyping of Adult Skin *Xenopus laevis*:

The first gel electrophoresis of interest for this experiment tested what sex determination genes out of *dmrt1.L*, *dmrt1.S*, *dmw*, *capn5Z*, *ccdc69W*, and *scanW* were present in adult *Xenopus laevis* frogs. By using frogs whose sexes were already able to be determined by visibly observing

physical reproductive organs and sexually dimorphic secondary sex characteristics, the data was able to confirm which genes was correlated to which sex. When conducting the electrophoresis gels, each sample was run with a PCR for the following genes: *hisH4*, *odc*, *zw*, *dmrt1.L*, *dmrt1.S*, *dmw*, *capn5Z*, *ccdc69W*, and *scanW*. By observing expression in the form of bands on the gels, the data was able to confirm that adult skin tissue was a potent source for DNA isolation. Furthermore, breaking down the expression of each gene, all three control genes that were tested (*hisH4*, *odc*, *zw*) showed expression of bands in both the "Adult Male Skin" and the "Adult 1 Female Skin B" tissue samples. When observing the sex determinant genes, the "Adult Male Skin" expressed bands for *dmrt1.L*, *dmrt1.S*, and *capn5Z*. There is also an unexpected band that is present in the well where *dmw* was being tested for in the "Adult Male Skin". Similarly, "Adult 1 Female Skin B" tissue also expressed bands for all three Z-locus specific genes, but this sample contrasted with the "Adult Male Skin" sample by also expressing bands for *dmw*, *ccdc69W* and *scanW* (Figure 1).

The 100 base pair ladder present in the first well of the gel (left-most side of Figure 1) is indicative of each genes' approximate fragment size. Going from left to right, it can be observed that *odc* has an approximated fragment size of 100 base pairs, *hisH4* is observed to have 200 base pairs, and *zw* is observed to have 150 base pairs. Aside from the control genes, variable genes such as *dmrt1.L* are observed at 500 base pairs, *dmrt1.S* is observed at 350 base pairs, and *dmw* is observed at 300 base pairs. The "Adult Male Skin" sample also has what this paper will from now on refer to as the pseudo-*dmw* band at approximately between 50 to 100 base pairs in the well that was being tested for *dmw* gene expression. Furthermore, the *capn5Z* is observed to have 450 base pairs, *ccdc69W* is observed to have 500 base pairs, and *scanW* is observed to have 300 base pairs (Figure 1).

DNA Genotyping of Stage 55 Tadpoles Tail Snips:

Using tail snip samples from previously euthanized tadpoles, DNA was isolated and then was prepared through the PCR process to determine the unknown sex of each tadpole. Figure 2 displays the DNA gel electrophoresis for the Edema 8 Tail Snips dataset. The tail snips used throughout this experiment originate from NF stage 55 tadpoles which experienced a controlled focal impact traumatic brain injury in the midbrain, had behavioral analyses conducted, and then wet and dry brain was measured to quantify edema accumulation. In this particular dataset, tadpoles labelled at control 1, control 2, and injured 1 are being tested with sex determinant gene primers to identify each sex. For the control 1 tadpole, bands were expressed for the following genes: *hisH4*, *zw*, *dmrt1.L*, *dmrt1.S*, and *dmw* at the appropriate fragment sizes. The control 2 tadpole expressed *odc*, *hisH4*, *zw*, *dmrt1.L*, *dmrt1.S*, and *dmw*. Lastly, the injured 1 tadpole expressed *zw*, *dmrt1.L*, *dmrt1.S*, and *dmw* (Figure 2).

To provide a more informative and consistent gel electrophoresis, the gel shown in Figure 3 represents how twenty-five other sample gels were also conducted in the same exact layout with three control genes and six variable genes. The top row labelled as "Edema 6 Control 2" shows bands expressing the genes: *hisH4*, *odc*, *zw*, *dmrt1.L*, *dmrt1.S*, *dmw*, and *capn5Z*. The bottom row labelled as "Edema 6 Injured 6" shows bands expressing the genes: *hisH4*, *odc*, *zw*, *dmrt1.L*, *dmrt1.S*, *dmw*, *capn5Z*, *ccdc69W*, and *scanW*. Furthermore, the pseudo-*dmw* band is evident in both samples which will be explained in detail later on in the paper. Overall, prominent bands are expressed in respect to the approximate fragments sizes that were observed from the adult *X. laevis* frogs' gel mentioned above.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
hisH4	CGGGATAACATTCAGGGTA	TCCATGGCGGTAACTGTC
odc	GCCATTGTGAAGACTCTCTCCATTC	TTCGGGTGATTCCTTGCCAC
zw	GAAATGGACGTGTAGCAGGG	TCGCCTTCAGGTAAAACGTG
dmrt1.L	ATCACAGAAACCATCCAGCTG	AAGTGTGCTTTCTCCACCCA
dmrt1.S	CCCGATGCAGAAATCATGGAT	GCTTATTCCTAGCTCCTCTTCC
dmw	TTTTCGCACCTCCACCATCA	TCAGTGCATACAACATAGGGCA
capn5Z	CAGGTGGAGTTGAACTGCAC	CGTGTCACCAGGATTAAACA
ccdc69W	ATGGAGAGAAATCTGGTG	TGCTTAGCAGCTCCTCAGAT
scanW	TAACCCGACAGCAGTCCATAC	GCCGCATCAAAGTCTCGACT

Table 1: This real-time PCR primer table outlines the forward and reverse primers for each gene that was used in this experiment. The three control/ housekeeping genes are *hisH4*, *odc*, and *zw*-common. The genes being tested are: *dmrt1.L*, *dmrt1.S*, *dmw*, *capn5Z*, *ccdc69W*, and *scanW*. The importance of PCR primers and getting the correct sequence is to ensure that the primers are able to flank the target region during amplification in the polymerase chain reaction. The primer sequences were obtained from previously established literature (Mawaribuchi et al 2017).

Correlational Analysis of Gene Segregation:

After observing the difference in gene expression in male versus female *X. laevis* tadpoles using PCR and gel electrophoresis, a correlational analysis was conducted to find which genes pairs had a strong or weak rate of segregation. By successfully sexing a sample size of n=25 tadpoles, the focus was shifted to individually observing which sex determinant genes were present or absent in reference to one another. The gene pairs that were analyzed are: *ccdc69W* vs. *scanW*, *dmrt1.L* vs. *dmw*, *dmrt1.S* vs. *dmw*, *dmrt1.L* vs. pseudo-*dmw*, *dmrt1.S* vs. pseudo-*dmw*, pseudo-*dmw* vs. *dmw*, *capn5Z* vs. *ccdc69W*, and *capn5Z* vs. *scanW*. The correlation coefficient (r) for each of the eight pairs of genes is as follows: 0.80, .66, .66, 0.60, 0.60, 0.58, 0.21, and -0.23, respectively (Figure 4).

In order to efficiently label each correlation efficient (r), a range of coefficients designated to indicate the level of correlation will be utilized for interpretation of results. For example, values between 0 to 0.30 will indicate a weak linear relationship, values between 0.30 to 0.70 will indicate a moderately linear relationship, and values between 0.70 to 1.00 will indicate a strong linear

relationship. This applies to both positive and negative correlation coefficients however a positive value indicates that the two samples being compared are directly related whereas a negative value would indicate that the two samples being compared are inversely related to one another (Ratner 2009).

Edema Accumulation in Male vs. Female Tadpoles:

As previously mentioned, the purpose of learning to properly identify the sex of tadpoles at NF stage 55 is meant to understand sexual dimorphism between the physiology of males in females in terms of TBIs and edema accumulation. The edema results in this study were derived from a separate experiment, courtesy of Sydnee Spruiell who conducted and cross-analyzed the data shown in Figure 5. Brain water content is determined by difference in wet versus dry weight of excised brains from the same tadpoles in which a traumatic brain injury was induced and tail snips were collected to sex each particular tadpole. The edema water content categories were broken up by "hours post-injury" that the brain was collected and measured for weight. The four "hours post-injury" categories are: 3 hours, 24 hours, 48 hours, and 72 hours post-injury with one control category for each time point. Due to 18 out of the 25 tadpoles that were sexed being collected at 72 hours post-injury, this data will focus only on that male versus female edema accumulation at 72 hours post-injury. All tadpoles are either controls or 72 hours post-injury. The overall sample has a skewed sex ratio with only 4 male and 14 female tadpoles. The male controls have an average of 87.3% of brain water content. The injured males average at 89.4% of brain water content. The control females average at 86.2% brain water content. The injured females average at 88.1% brain water content. The overall control brain water content is averaged to be 86.9 whereas the overall injured tadpoles are averaged to be 88.1% (Figure 5).

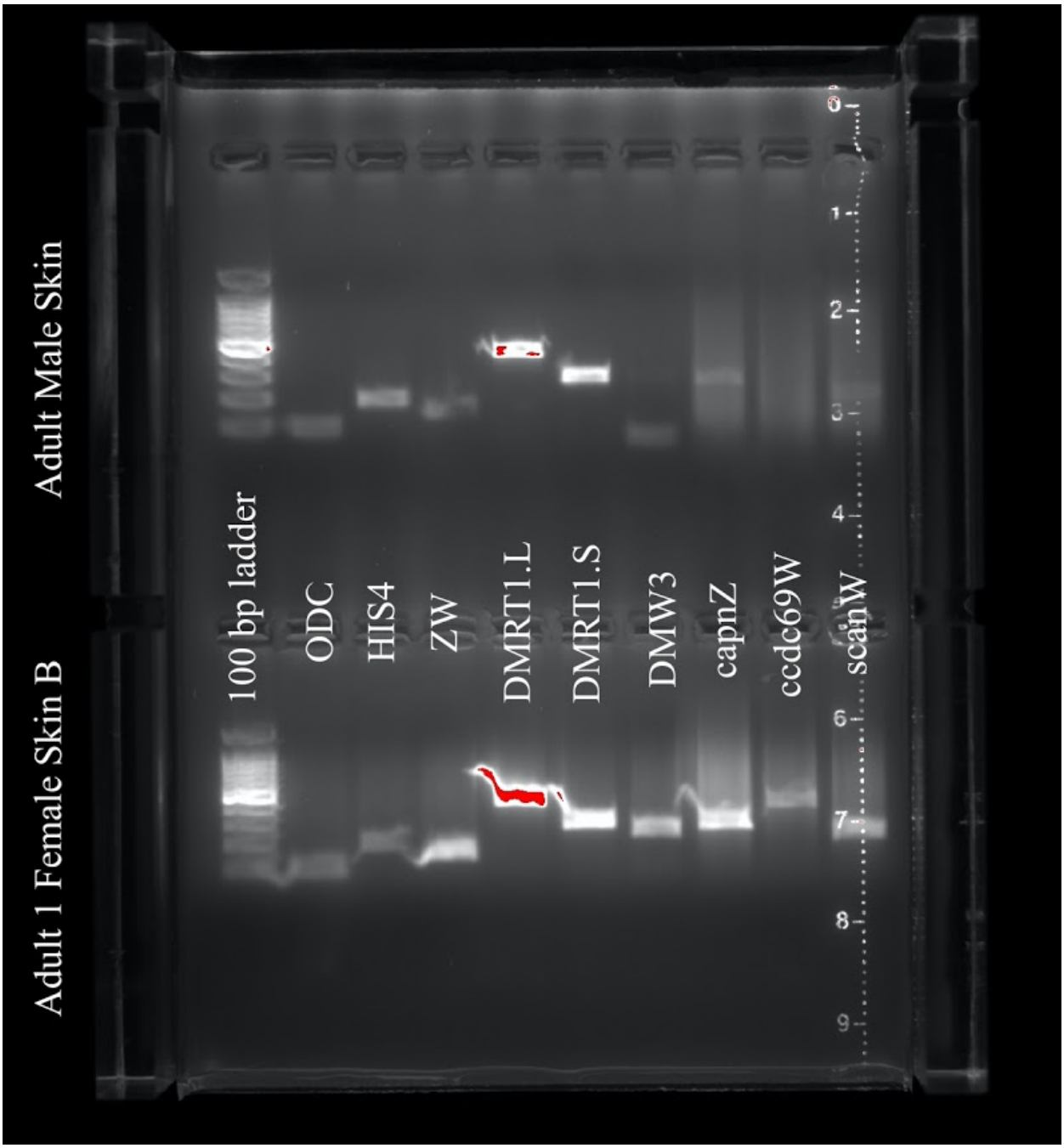

Figure 1: This gel electrophoresis contains DNA samples of previously PCR'd samples that contain DNA from Adult Male Skin and Adult 1 Female Skin B. Well 1 is the left-most well in the gel which has the 100 base pair ladder for reference. The nine following wells contain: *odc*, *hisH4*, *zw*, *dmrt1.L*, *dmrt1.S*, *dmw*, *capnZ*, *ccdc69W*, and *scanW* in the order that they are listed.

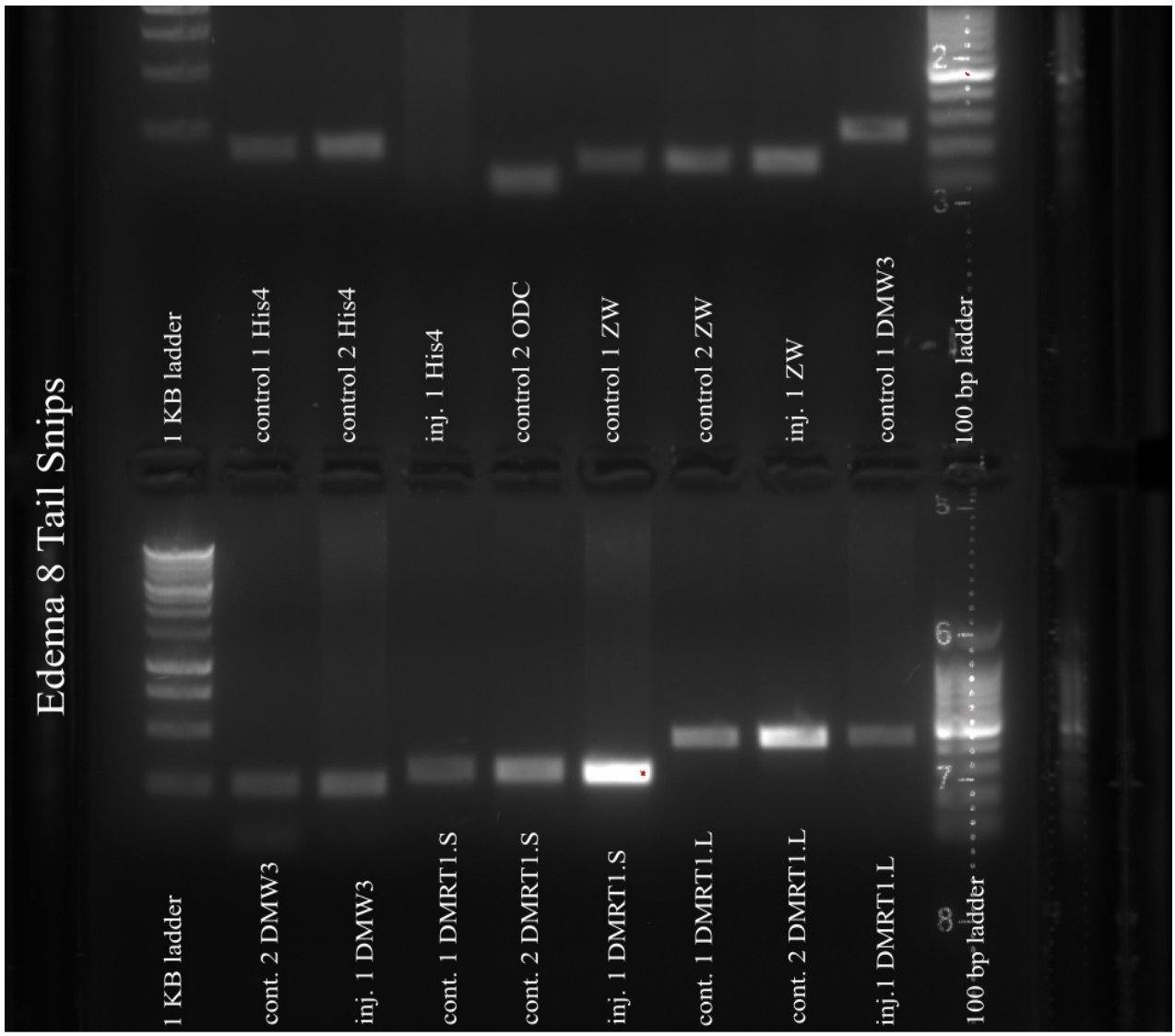

Figure 2: This gel electrophoresis shows DNA samples of the Edema 8 Tail Snips which refers to three different tadpoles labelled as control, control 2, and injured 1. This gel consists of the *odc*, *hisH4*, *zw*, *dmrt1.L*, *dmrt1.S*, and *dmw* genes. The significance of this gel is that it shows the contradictive presence of both *dmrt1* and *dmw* within the same tadpole. This led to adding more sex determinant primers to the project.

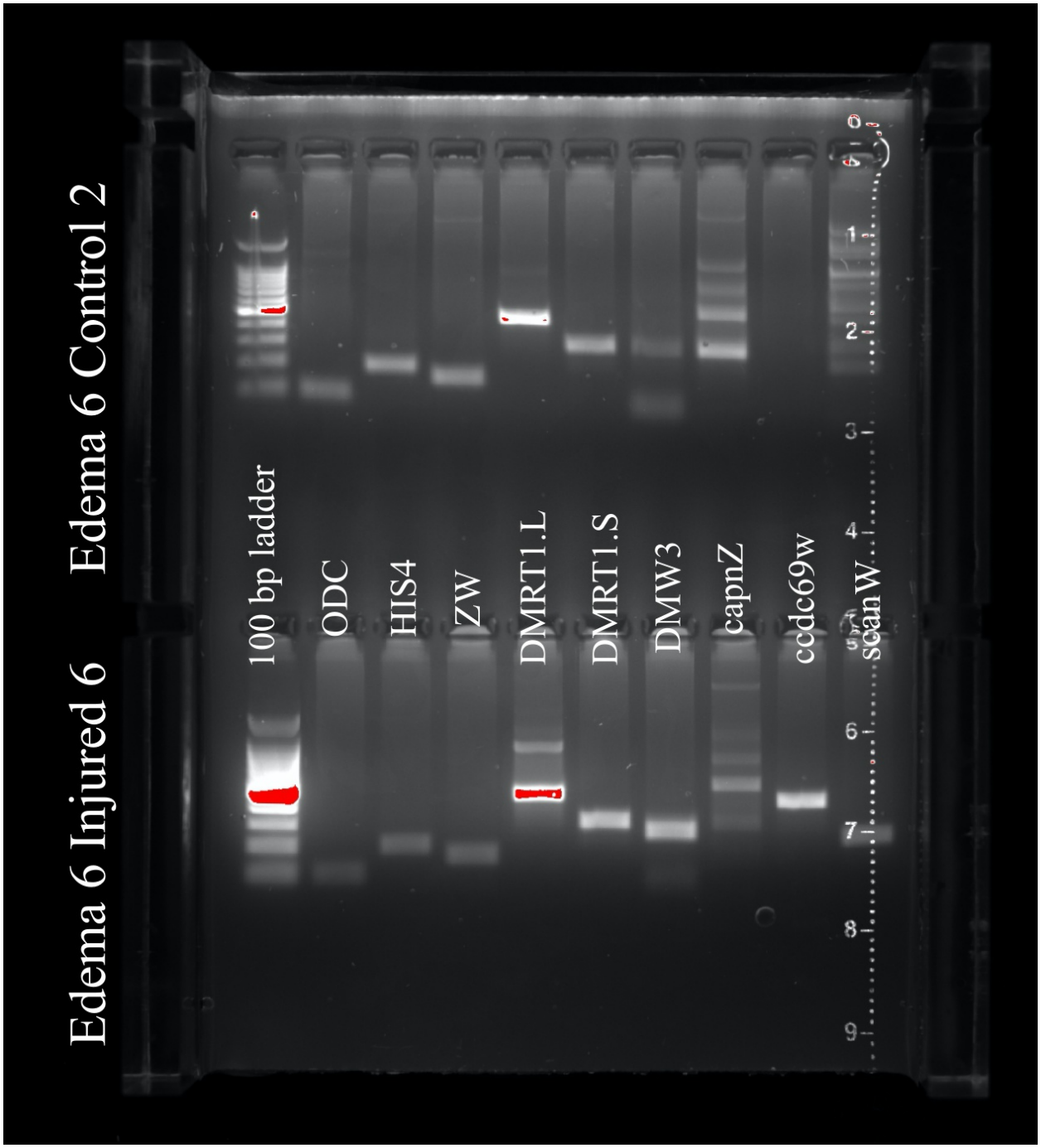

Figure 3: This gel electrophoresis conveys the tail snip DNA samples of Edema 6 tadpoles. The layout of this gel is the final testing method that was chosen because it contains three control genes and six variable genes to ensure proper sexing of each tadpole. This gel almost mimics the presence of bands that were observed in Figure 1 where the adult *X. laevis* frogs were tested to define which genes correlated to which sex. After analysis of data, it can be confirmed that the Control 2 tadpole (top row) is a male whereas the Injured 6 tadpole (bottom row) is a female.

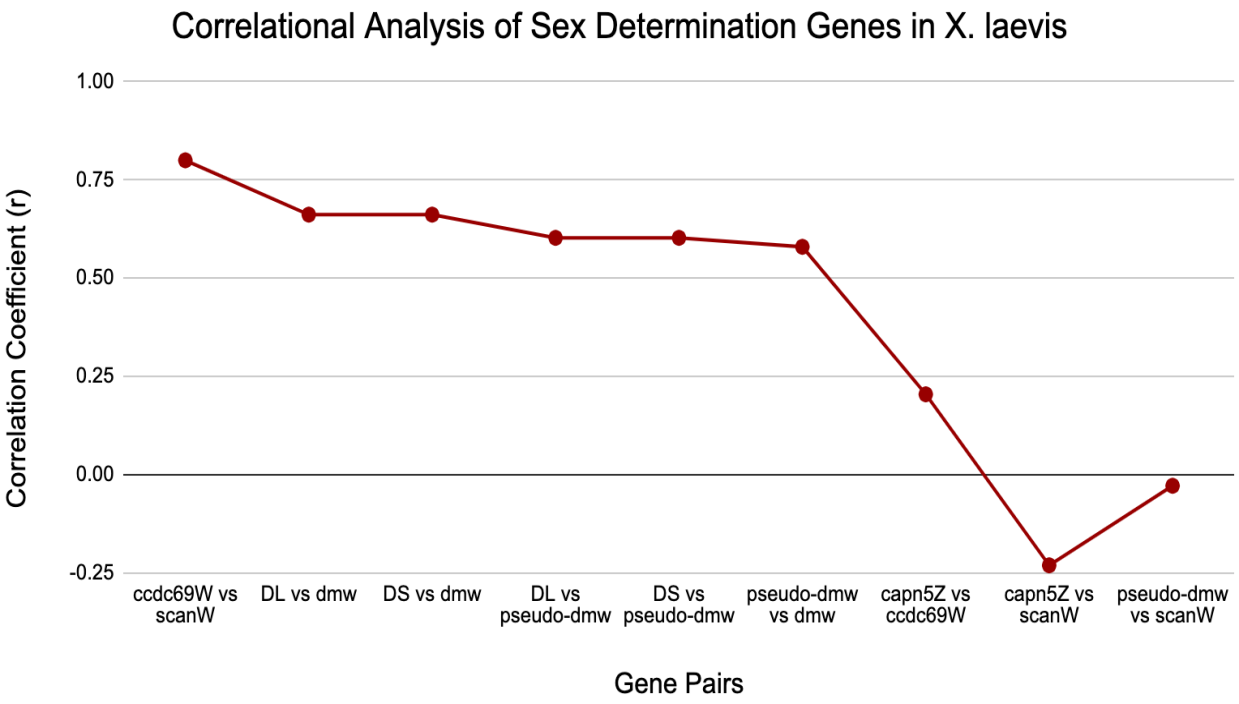

Figure 4: The correlational analysis conducted for the gene pairs in this experiment serves the purpose to understand gene correlation and gene segregation. The strongest correlation that can be observed from this line plot is the one between *ccdc69W* and *scanW*. While the other gene pairs also have great importance to the relevance of this sex determination experiment, the high correlation coefficient between *ccdc69W* and *scanW* concluded that either gene is sufficient to test alone in order to sex a tadpole using DNA isolated from tail snips.

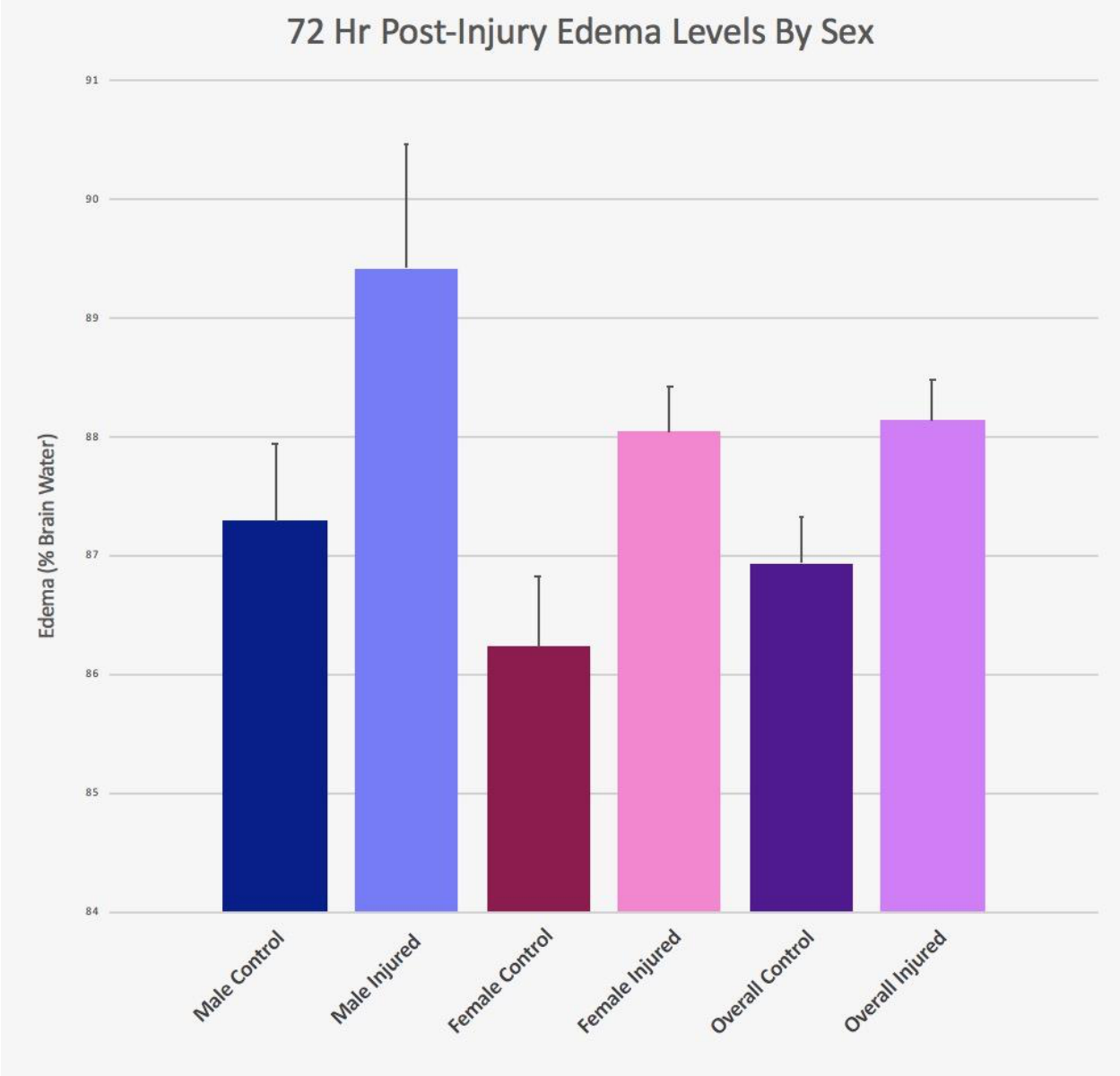

Figure 5: The brain water content (y-axis) is a percentage of edema accumulation that was measured in various male and female tadpoles within categories of control versus injured (x-axis). The sample size is n=18 with a skewed sex ratio containing 4 male tadpoles and 14 female tadpoles. The most notable observation from this graph is that male tadpoles contain higher brain water content even as controls when compared to their female counterparts. The lower rates of brain water content in females suggest lower morbidity and poses a theory of high rates of recovery in female tadpoles than males. This graph was created and cross-analyzed by Sydnee Spruiell.

Discussion:

Sex Determinant Genes:

The purpose of this research is to be able to successfully identify the sex of *Xenopus laevis* tadpoles prior to NF stage 56, when primordial gonads can be visibly observed. This study focuses on NF stage 55 tadpoles for the reason that the skull cap has not been formed and in order to surgically remove a tadpole's brain with minimal inflammation that isn't induced by the quantified TBI, one must surgically remove the brain when the skull cap has not yet formed. The same tadpoles undergoing TBIs are the ones being sexed through gene expression and accounted for in terms of edema accumulation.

The genes of interest that this experiment focuses on are *dmrt1.L*, *dmrt1.S*, *dmw*, *capn5Z*, *ccdc69W*, and *scanW*; they have been previously validated by literature (Mawaribuchi et al 2017). The initial goal of this experiment was to utilize only the *dmrt1.L*, *dmrt1.S*, and *dmw* genes which is why Figure 2 only has these genes and the control genes being tested for expression. After further research, it became apparent that *dmrt1* and *dmw* share homology to a larger extent than what was previously believed, possibly as a result of poor primer design or other potential issues with males expressing the *dmw* gene. This is why the need for gene expression in adult *Xenopus laevis* frogs became necessary in order to confirm which combination of genes are required to conclude that "x" organism is a male or female in the *Xenopus laevis* species. It is important to distinguish that two different versions of the *dmrt1* gene were tested for reasons such as the theory that *dmrt1* has different mRNA variant transcripts that are a result of alternative splicing (Mawaribuchi et al 2016). Furthermore, *X. laevis* frogs are allotetraploids which implies that these organisms can undergo polyploidy and therefore undergo genome doubling allowing one organism to carry both or just one type of either the *dmrt1.L* or *dmrt1.S* variant (Session et al. 2016).

From the adult frogs' gene expression (Figure 1), it can be concluded that the *dmrt1.L*, *dmrt1.S*, and *dmw* genes will express bands for both sexes at 500, 350, and 300 base pairs, respectively. In order to understand the extent of the homology between these three genes, a comparative BLASTn tool was utilized to find the sequence similarity rates between base pairs. Between variant *dmrt1.L* and *dmw*, the similarity was found to be 87% (Figure 6). Likewise, *dmrt1.S* and *dmw* were found to have a similarity rate of 89% (Figure 7). With the theory of both genes being paralogs and the high sequence similarity rates between both genes, it can be concluded that the gene sequences are too similar to be identified as different by simple methods of PCR and gel electrophoresis therefore, only testing the expression of *dmrt1* and *dmw* in order to determine the sex of *X. laevis* would not be sufficient. This sequence homology likely explains the presence of pseudo-*dmw* in the male tissues at an approximate length of 50 to 100 base pairs. The only logical reason that *dmw* is expressing a band at the appropriate fragment size in males is because its homology is too similar to create a fully *dmw*-specific PCR primer that won't match its paralog, *dmrt1*.

According to recently published literature, there are three other sex determinant genes that have been identified known as: *capn5Z*, *ccdc69W*, and *scanW* (Mawaribuchi et al 2016). As previously mentioned, the *capn5Z* gene is located on the Z-locus, which will be present in both males and females of this species. In contrast, *ccdc69W* and *scanW* are both located on the W-locus and are female-specific. With this background knowledge, the new method of testing expression went from testing three controls and three potential sex determinants genes to testing three controls and six potential sex determinant genes as is shown in Figure 3. After conducting this testing method in a total of n=25 sample size, there is a visible pattern of *capn5Z* showing expression for majority of both male and female tadpoles at approximately 350 base pairs. The

only difference that can be accounted for in expression can be of *ccdc69W* and *scanW* because both genes are W-specific genes showing bands at 500 and 250 base pairs, respectively. If a male frog has the genotype of ZZ then it is not possible for W-loci specific genes to be present in that frog. The only logical reason that *dmw* is expressing a band at the appropriate fragment size in males is because its homology is too close to create a fully *dmw*-specific PCR primer that won't match its paralog, *dmrt1*. In contrast, a female *X. laevis* is known to have a genotype of ZW or even WW however the latter was not present in the experiments conducted. With a genotype of ZW, a real-time PCR protocol is able to detect the presence of both Z and W-loci specific genes regardless of whether those genes were being actively expressed or not. Therefore, the majority of the tadpoles categorized as females have bands present in the PCR gels for all nine of the genes being tested. In the future, this work would benefit from implementing the use of qRT-PCR or another technique that can test for gene expression levels in different tissues at specific time points.

Gene Correlation and Segregation:

The correlational analysis conducted creates a standardized scale to understand the correlation of genes and their presence in reference to one another. In other words, one can identify which genes are commonly present together and which genes have an inverse relationship when a real-time PCR is conducted. The first pair of genes are *dmrt1.L* vs. *dmw* in which the correlation coefficient is determined to be 0.66. This is also the case of the gene pair *dmrt1.S* vs. *dmw* (Figure 4). From this value, it can be concluded that there is a moderately linear relationship between the two variant *dmrt1* and *dmw* genes. This evidence goes hand in hand with theory that these two genes are paralogs and that their gene sequences are very similar inducing the presence of the band in the gel electrophoresis regardless of actual expression or not. Moving onto the interesting case of the pseudo-*dmw* band, it was observed that pseudo-*dmw* vs. either *dmrt1* variant has a

correlation coefficient of 0.60 suggesting a moderate linear relationship. The emergence of this pseudo-*dmw* band in the majority of males led to the theory that this band is somehow male-specific although that is not the focus of this paper. Similarly, the gene pairs pseudo-*dmw* and *dmw* revealed a correlation coefficient of 0.58. This moderately linear relationship did not really emphasize on the difference between the bands as was hoped.

The most interesting part of this correlational analysis comes from the last three sex-specific genes: *capn5Z*, *ccdc69W*, and *scanW*. The correlation coefficient for *ccdc69W* vs. *scanW* came out to be 0.80 which can be characterized as a strong linear relationship and from this dataset, is the strongest positive relationship observed. This conveys that both W-loci specific genes are very likely to be present together in order to categorize a tadpole as female. While comparing *capn5Z* and *ccdc69W*, the correlation coefficient was observed to be 0.21 indicating that the relationship between the two genes is very weak. Furthermore, comparing *capn5Z* and *scanW* genes, the correlation coefficient was observed to be -0.23. With the negative value indicating that *capn5Z* and *scanW* are inversely related, it can be concluded that if *capn5Z* is present then *scanW* is less likely to be present although the correlation is weak.

Overall, attempting to identify the sex of tadpoles using PCR primers and gel electrophoresis can't be done only by testing *dmrt1* and *dmw* genes only. Moreover, since the female sex is the heterogametic sex in *X. laevis*, the Z-loci will still be present in females allowing *capn5Z* to have a band present in a genomic DNA PCR regardless of the fact that Z-loci specific genes wouldn't be actively expressed in female tadpoles. The most definitive gene pattern can be determined by the presence of either *ccdc69W* or *scanW*. The data concludes that if either of these genes are detected in a PCR and gel electrophoresis method then the corresponding *X. laevis* tadpole can be successfully defined as a female.

Difference of Edema Accumulation in Male and Female Tadpoles

The induction of TBIs to produce tadpoles that are undergoing edema accumulation is an ideal experiment in which knowing the sex of the tadpoles can introduce more information as to how the difference in physiology between sexes can affect the way the organism reacts to TBIs during a certain recovery period. The data shown in Figure 5 conveys the noticeable difference in brain water content, represented as a percentage, between male and female counterparts. The average male tadpoles seemed to have a naturally higher brain water content when comparing to the control male averages. Furthermore, both the male and female injured brain water content raises by approximately 2% when compared to their controlled counterpart, therefore the increase caused by the receipt of a TBI is proportional in both sexes. The equal gain in water content implies that the controlled TBI was induced in a similar manner however the distinctively high edema accumulation in male tadpoles serves to imply that male tadpoles may suffer a higher rate of morbidity in comparison to female tadpoles. This is consistent when observing the control male tadpoles in comparison to the control female tadpoles as the former has elevated water content at a baseline level. This theory is similar to previously published literature in which a mammalian model organism such as mice were also tested for edema accumulation based on sex. The study concluded that "female mice have less of the negative effects of neuroinflammation such as neuron cell death" and that "female mice have more protection against brain trauma in the first week after TB" (Villapol et al 2017). Similar studies indicate that males have a more aggressive inflammatory response that resolves quicker but may also lead to higher rates of neuronal cell death. With recovery periods and time post-injury creating a difference in how both sexes respond to TBIs, it is important to distinguish that this study only focuses on a recovery period of 72 hours. Overall, the male tadpoles have significantly higher brain water content than female tadpoles which can

conclude that at 72 hours post-injury, the male tadpoles may experience higher rates of morbidity due to difference in physiologies that has yet to be identified. While this data is preliminary, it suggests a possibility of sexual dimorphism in the injured brain of *Xenopus laevis*, synonymous with what has been suggested in mammals, further supporting its usefulness as a model organism for traumatic brain injuries.

Conclusion:

The method of sex determination of *Xenopus laevis* tadpoles as they undergo onset of sexual differentiation is important due to the possible sexual dimorphism that may arise as a result. By taking tail snips of tadpoles at N.F. stage 55, researchers can isolate the DNA, perform a polymerase chain reaction, and then observe results via gel electrophoresis. This project identifies that *ccdc69W* and *scanW* as the most reliable genes that serve as indicators of sexual determination. If these genes are to be present in the genome of a tadpole being tested, then that tadpole can be identified as a female and if not then it would be a male. The Edema Accumulation Experiment further proves the importance of understanding why it is important to study both male and female sexes, with distinction, in terms of how they react to a traumatic brain injury. The results convey that although both sexes experience approximately equal levels of increase in water content, the male tadpoles had higher levels of water content at baseline even before the TBI was induced. This observation was consistent in the injured and control male tadpoles in comparison to their female counterparts. This gives more insight to further investigate TBIs in male versus females to see whether male counterparts may potentially suffer higher levels of neurological damage in comparison to females.

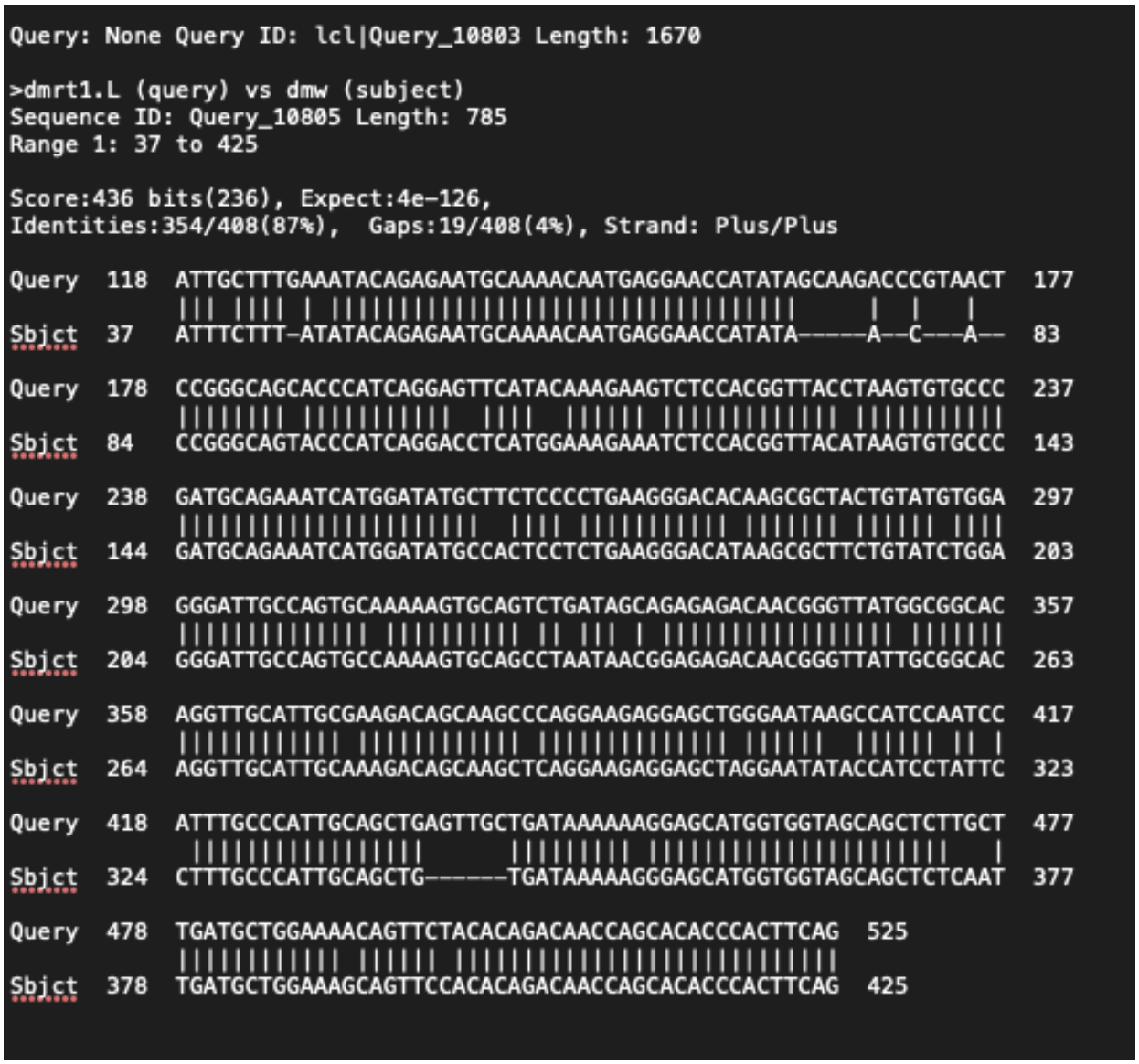

Figure 6: The BLASTn tool from NCBI was used to do a base by base comparison between the *dmrt1* and *dmw* genes. This particular figure shows *dmrt1.L* as the query and *dmw* as the subject. The sequence similarity rate was calculated to be 87% which provides evidence to the theory of *dmrt1* and *dmw* being paralogs with similar homology.

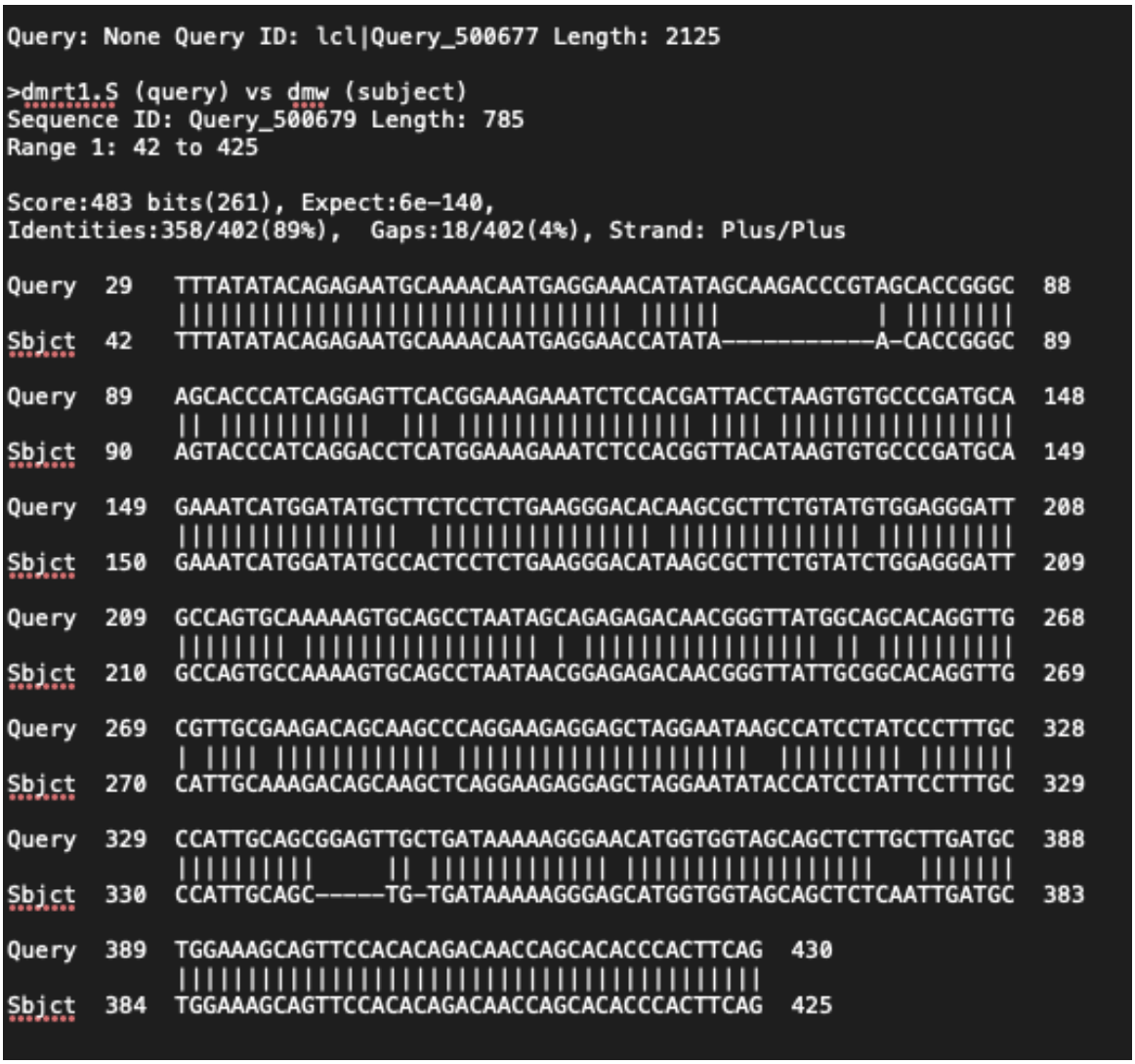

Figure 7: To ensure that both variants of *dmrt1* have high rates of similarity to *dmw*, the BLASTn tool was also used to conduct a base-by-base comparison. The sequence similarity for *dmrt1.S* (query) versus *dmw* (subject) came out to be 89% therefore providing further evidence of the similar homology of *dmrt1* and *dmw*.

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