

University of Alabama in Huntsville

LOUIS

Honors Capstone Projects and Theses

Honors College

8-25-2010

Differential Expression of N-Methyl-D-Aspartate Receptors of Cynprinidae

Taito Eguchi

Follow this and additional works at: <https://louis.uah.edu/honors-capstones>

Recommended Citation

Eguchi, Taito, "Differential Expression of N-Methyl-D-Aspartate Receptors of Cynprinidae" (2010). *Honors Capstone Projects and Theses*. 324.

<https://louis.uah.edu/honors-capstones/324>

This Thesis is brought to you for free and open access by the Honors College at LOUIS. It has been accepted for inclusion in Honors Capstone Projects and Theses by an authorized administrator of LOUIS.

2010 - 10

Differential Expression of N-methyl-D-aspartate Receptors of Cyprinidae

Taito Eguchi

Advisor: Dr. Bruce Stallsmith

August 25, 2010

Abstract

The plasticity of N-methyl-D-aspartate glutamate receptor (NMDAR) of Cyprinidae is relatively unknown. This study compared the relative amounts of NMDAR levels found in sexually dimorphic *Lythrurus fasciolaris* and non-sexually dimorphic *Notropis telescopus*. The male fish of the *L. fasciolaris* were expected to have higher levels of NMDAR than the female fish during the breeding season; however, the differences between sexes were insignificant for both *L. fasciolaris* and *N. telescopus*. Comparing the NMDAR levels across the species showed that the month of May is a critical time for the sexually dimorphic *L. fasciolaris*; during May *L. fasciolaris* had significantly higher levels of NMDAR compared to the *N. telescopus*. This modulation of NMDAR may suggest that sexually dimorphic fish regulate the presence of glutamate receptors differently from non-sexually dimorphic fish.

Introduction

N-methyl-D-aspartate receptors (NMDAR) are found in the postsynaptic membrane that lines the central nervous system, including the brain tissue. These glutamate receptors are known to be vital for memory and learning, both of which are essential to the reproductive success of fish [3]. Via dot blot, the abundance of NMDAR can be measured by tagging the glutamate receptor with a protein specific antibody. In this experiment, the NMDA receptors were tagged at the subunit NMDAR1. The NMDAR1 antibody was tagged with second antibody and detected with a colorimetric dye.

In this experiment, NMDAR levels of the brain tissue were compared between sexually dimorphic *Lythrurus fasciolaris* and non-sexually dimorphic *Notropis telescopus*. During breeding season when sexual dimorphism of fish is at its peak, male *L. fasciolaris* tend to become more colorful and larger [4]. *Notropis telescopus* also live in similar environments of the *L. fasciolaris*, yet they do not exhibit this kind of sexual dimorphism. This physiological difference between the sexes may have profound effects in the expression of NMDA receptors [4].

In addition, the level of NMDAR was also examined in the muscle tissue of the *L. fasciolaris*. In other studies, NMDAR was not found in the gonadal tissue of animals, so it was also unexpected to find NMDA receptors in the muscle tissue of the *L. fasciolaris* [2].

Materials and Methods

Fish used in this study were collected from Estill Fork, Limestone Creek, and Sipsey Fork, Alabama. The fish were collected from January 2010 to July 2010, with the exception of February 2010. For each month the relative amount of NMDAR present in the fish brains was measured via the dot blot, which uses antibodies to tag the NMDAR1 subunit.

After the fish were collected, they were euthanized with tricaine mesylate (MS-222), which is a white powder commonly used in the sedation and euthanasia of fishes. During the

transport back to the lab, the fish were stored in an ice cooler at 4°C and later transferred to the -80°C freezer for storage until proceeding with the dot blots.

Brain Lysates

Fish were removed from the -80°C freezer and thawed in a glass bowl chilled over ice. Biometric information was obtained for each fish including standard length and mass before brain dissections. The brain of each fish was dissected and placed into a separate pre-weighed and labeled micro-tube. Micro-tubes were chilled over ice to keep brains tissue cool. The micro-tubes were once again weighed to determine the overall mass of the brain tissue.

After the mass of the brain tissue was determined in milligrams, the mass of the brain tissue was multiplied by a factor of 10 to calculate the amount of lysis cocktail in micro-liters to add to the brain tissue. The lysis cocktail was a mixture of protease inhibitor and lysis buffer at 0.03 mL of lysis buffer per 1 mL of lysis buffer. The brains of the fish were dissolved into the lysis cocktail using a glass stirring rod to mix the inner contents of the micro-tube in a mortar and pestle like fashion. The brain tissue was mixed into the lysis cocktail until the solution became homogeneous.

Protein Assay

The protein concentrations of the fish brain lysates were determined using a spectrophotometer and reagents from the Bio-Rad Protein Assay Kit 500-116. This kit comes with three reagents: A, B and S, which were used to determine the protein concentrations in detergent buffers. Reagent A' was made by combining A and S in the following ratio:

$A' = 40 \mu\text{L S} + 2 \text{ mL A reagent.}$

A new set of micro-tubes was labeled for the protein assay. The stock fish brain lysates were then diluted to a 1:10 ratio of brain lysate to lysis buffer. A total of 20 μL was made for

each fish brain lysate. Into a 96 well plate, 5 μ L of fish brain lysate was mixed with 25 μ L of A' reagent and 200 μ L of B reagent. A set of protein standards were made using BSA dissolved in lysis buffer at concentrations of 0 mg/mL, 0.1875 mg/mL, 0.375 mg/mL, 0.75 mg/mL, and 1.5 mg/mL. The 96 well plate was then left to sit for 15 minutes and then read at 750 nm with the spectrophotometer using KC Junior software. A standard protein curve was made using the known protein concentrations of the standards and the corresponding absorbance values to determine the protein concentrations of each fish brain lysate. The concentrations were then multiplied by the dilution factor to calculate the actual concentrations of the stock fish brain lysates.

Dot Blot

Another set of micro-tubes were labeled and used for standardizing all fish brain lysates to the lowest stock concentration. Preferably, the fish brains were standardized to a concentration of 5 mg/mL. A total of 40 μ L of each sample was made using the necessary amount of fish brain lysate and lysis buffer to make the chosen concentration. For negative controls, BSA and pure lysis buffer was prepared; as a positive control, rat brain lysate was prepared.

Using a pre-made 1.5 cm grid to line the blots of each standardized fish brain lysate, triplicate 5 μ L blots of each fish brain lysate was plotted onto a 8 x 11 cm² nitrocellulose membrane. A staggered formation was made to fit the maximum amount of blots onto a single membrane. The nitrocellulose membrane was then left to dry for 30 minutes.

To block the nitrocellulose membrane, 25 mL of a 3% BSA solution was poured into a plastic container containing the membrane. The plastic container was then placed onto a rotator for 1.5 hours at room temperature. Meanwhile, a primary antibody was made by mixing 40 μ L

of a NMDAR polyclonal primary antibody in 10 mL of 1% BSA. After the 1.5 hours, the membrane was washed three times at 5 minute intervals with 25 mL of PBST on a rotator. The primary antibody solution was then placed into the plastic container with the membrane and set onto a rotator for 2 hours at room temperature.

After the 2 hours, the primary antibody was stored into a 15 mL conical tube and placed into a 4°C fridge. The membrane was washed three times with 25mL of PBST at 5 minute intervals. The secondary antibody was then added to the plastic container and set on a rotator for 1.5 hours at room temperature. The secondary antibody solution was prepared by mixing 3.3 μ L of secondary antibody in 10mL of 1% BSA.

After the 1.5 hours, the secondary solution was discarded and the membrane was washed three times with 25 mL of PBST at 5 minute intervals on a rotator. A colorimetric detection was done using the Opti 4CN kit to stain the blots. For one membrane 16.875 mL of DI H₂O, 1.8 mL Opti 4CN Diluent, and 0.75 mL substrate was mixed into a flask during the last wash after the secondary primary. This colorimetric detection solution was then added to the plastic container on a rotator for 5-30 minutes. Once the protein blots were sufficiently dark, the colorimetric detection solution was discarded and 25 mL of DI H₂O was added for approximately 15 minutes to stop the detection. The DI H₂O was then discarded and the membrane was left to air-dry for a few minutes. After drying to completion, the membrane was scanned and stored at 4°C wrapped in aluminum foil. Pixel densitometry was then determined using UN-SCAN-IT.

Statistical analysis

Since each fish had triplicate blots on the membrane, an average of the three pixel densities was calculated for each fish. To measure any differences between the different species of fish, average pixel counts were obtained for each species and sex for each species along with

their respective 95% standard error values. The averages were plotted into a bar graph using the 95% standard error values to see any difference between the species or sexes of the fish. In addition, two-tailed Mann-Whitney tests were performed to determine if there were significant differences between the fish (p -value). A value of $p < 0.01$ was marked as significant. All statistical analysis was performed with an online Mann-Whitney U Test calculator [1].

Results

Below are the results obtained for the dot blots from April 2010 to July 2010. The pixel densities were used to compare the relative amount of NMDAR found in the different species of fish and by gender.

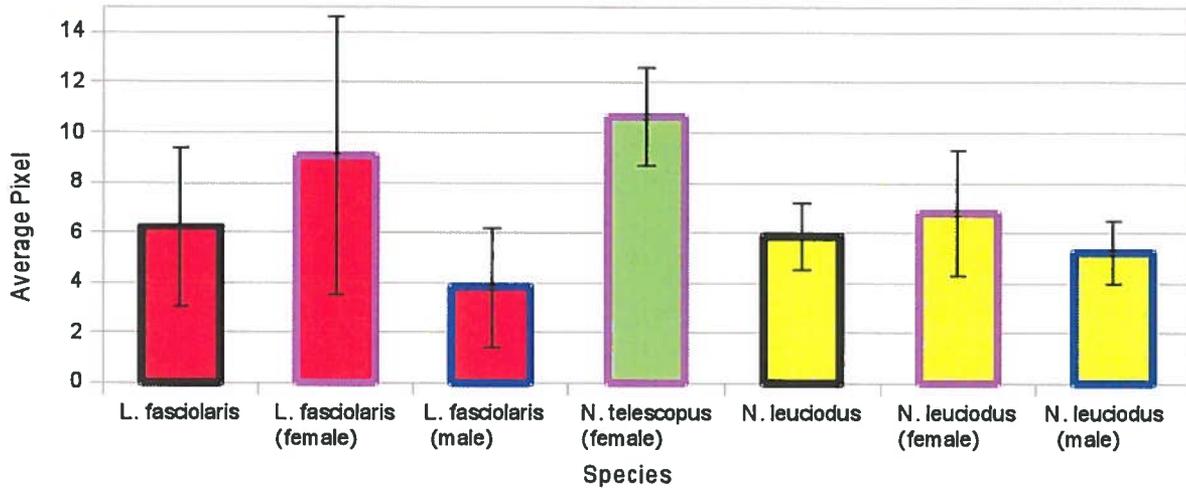


Figure 1. April 2010 NMDAR comparisons

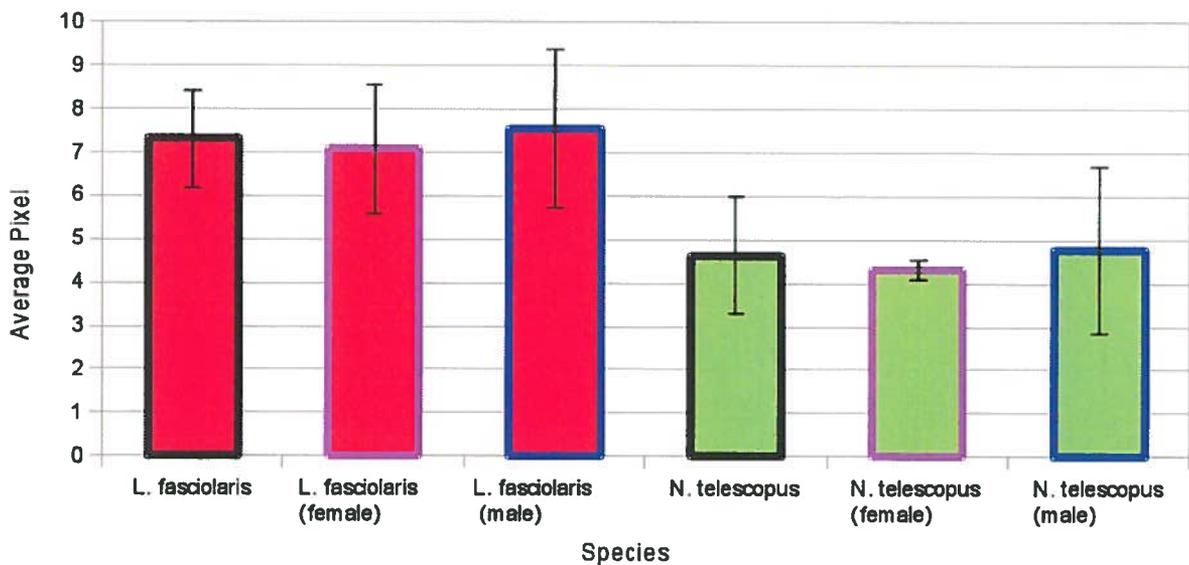


Figure 2. May 2010 NMDAR comparisons

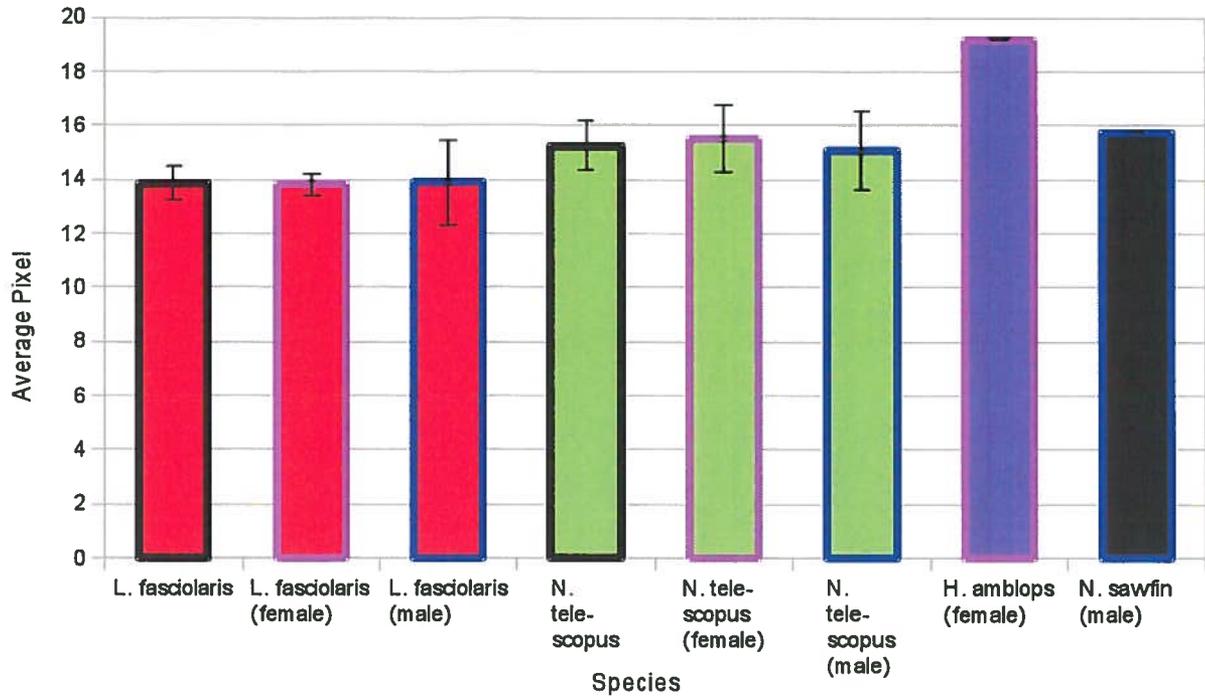


Figure 3. June 2010 NMDAR comparisons

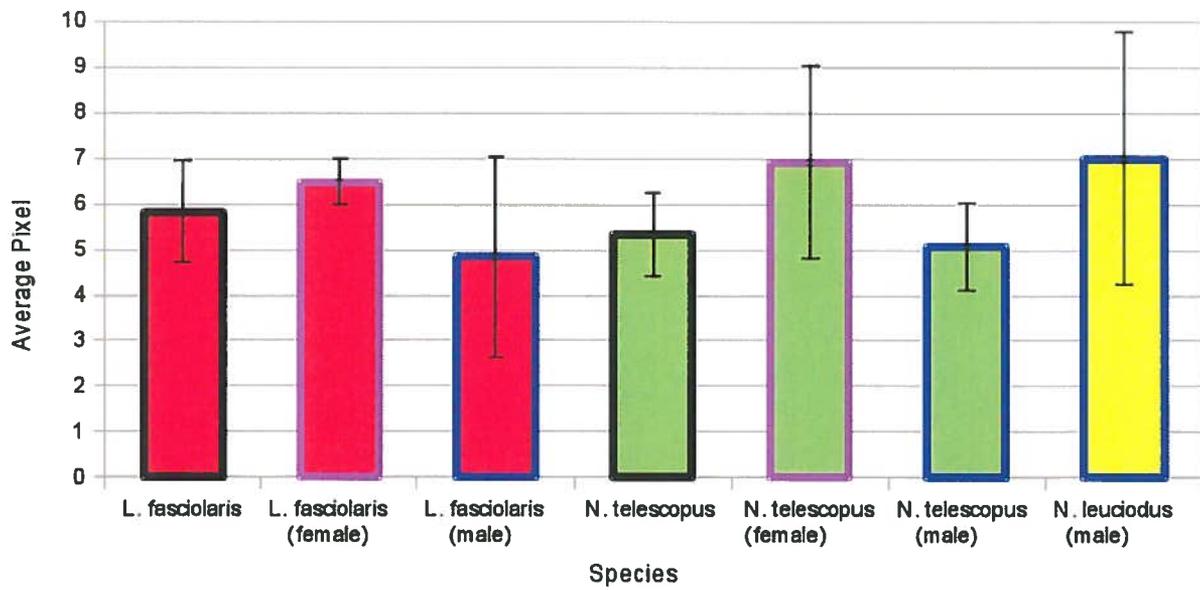


Figure 4. July 2010 NMDAR comparisons

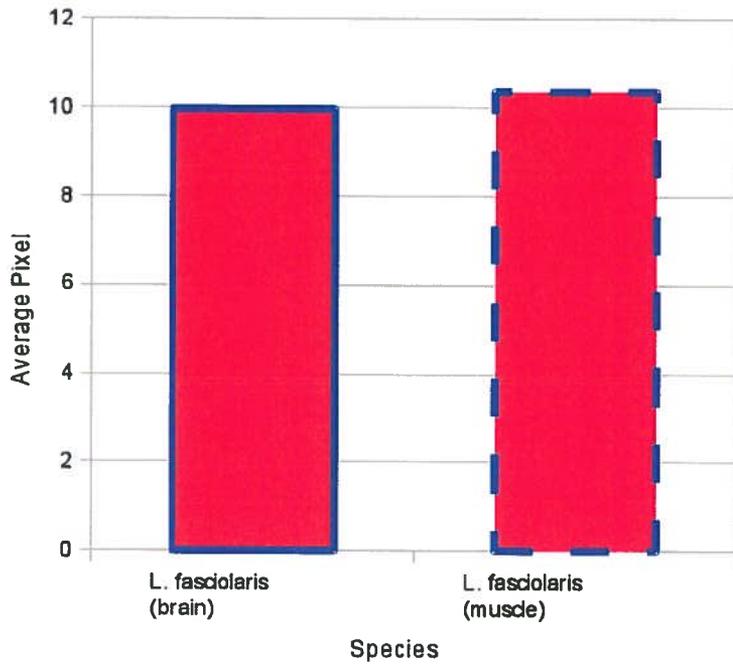


Figure 5. May 2010 *L. fasciolaris* NMDAR comparisons (brain vs. muscle)

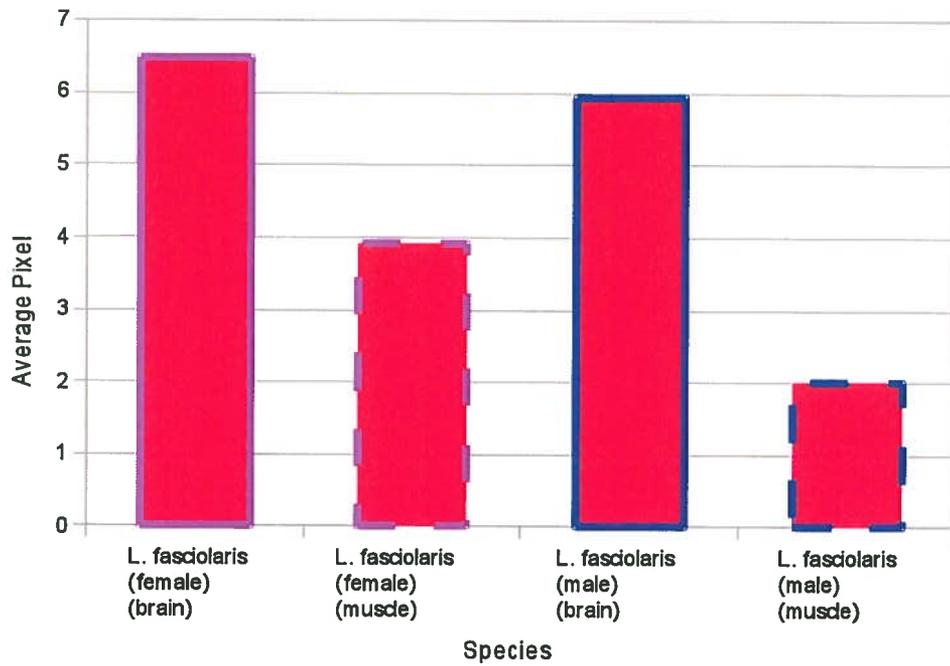


Figure 6. July 2010 *L. fasciolaris* NMDAR comparisons (brain vs. muscle)

Month	Gender	<i>Lythrurus fasciolaris</i>			<i>Notropis telescopus</i>			U	Significance
		n	Average Pixel	Standard Deviation	n	Average Pixel	Standard Deviation		
April 10'	mixed	11	6.21	5.22	6	-	-	-	-
	female	5	9.08	6.21	6	10.60	2.39	23	P>0.05
	male	6	3.81	2.91	0	-	-	-	-
May 10'	mixed	13	7.30	2.01	7	4.64	1.77	80	P=0.005
	female	7	7.08	1.96	2	4.29	0.17	14	P>0.05
	male	6	7.56	2.23	5	4.78	2.15	26	P>0.05
June 10'	mixed	7	13.84	0.83	9	15.25	1.41	52	P=0.03
	female	4	13.80	0.38	4	15.49	1.25	16	P=0.03
	male	3	13.89	1.35	5	15.07	1.64	11	P>0.05
July 10'	mixed	5	5.82	1.22	15	5.31	1.76	47	P>0.05
	female	3	6.48	0.42	2	6.91	1.49	3	P>0.05
	male	2	4.84	1.55	13	5.07	1.72	15	P>0.05

Table 1 *L. fasciolaris* vs. *N. telescopus* NMDAR comparisons

Since sample sizes were not equal, nonparametric Mann-Whitney tests were performed to compare NMDAR levels across species. For April 2010, there are no statistics reported for mixed and male fish since male *N. telescopus* were not captured for analysis during that month. A value of $p < 0.05$ was marked as marginally significant and a value of $p < 0.01$ was marked as significant.

Discussion

The comparison of NMDAR levels across species was interesting. During the months April to July, only May showed a significant difference between *L. fasciolaris* and *N. telescopus* ($U=80$, $n[L. fasciolaris]=13$, $n[N. telescopus]=7$, $p=0.005$). Thus, May must specifically be a key month for the expression of NMDAR in *L. fasciolaris*. Otherwise, the differences in the levels of NMDAR present in the brain tissue of *N. telescopus* in comparison to *L. fasciolaris* were insignificant. There seems to be plasticity in the amount of NMDAR present in the brain tissue,

which may be caused by the sexually dimorphic character of the *L. fasciolaris*. This pattern may suggest that during May, *L. fasciolaris* significantly increase their levels of NMDAR found in the central nervous system, while non-sexually dimorphic fish such as *N. telescopus* simply try to maintain high levels of NMDAR to compete with sexually dimorphic fish.

When separating the fish according to sex and comparing levels of NMDAR across species, there was no clear distinction between *L. fasciolaris* and *N. telescopus*. During June, the comparison of the female fish showed marginal significance ($p < 0.05$). The female *N. telescopus* had slightly higher levels of NMDAR than the female *L. fasciolaris* as seen with the combined male and female NMDAR levels in June.

In addition, the insignificant differences between male and female levels of NMDAR within each species were unexpected. Pilot studies have indicated that in sexually dimorphic *L. fasciolaris*, the male fish had higher levels of NMDAR than female fish. Even in the key month of May, this difference was not seen with the relative NMDAR levels for *L. fasciolaris* and *N. telescopus*.

The presence of NMDA receptors in muscle tissue was highly interesting as well. Initially, high levels of NMDAR were not expected to be found in the muscle tissue and therefore used as a negative control [3]. However, the data suggests a remarkable fluctuation in the level of NMDAR found in the muscle tissue of *L. fasciolaris*. During May the amount of NMDAR found in the muscle tissue of a male *L. fasciolaris* seemed equally abundant in its brain tissue. In July, however, the amount of NMDAR found in the muscle tissue was greatly reduced to almost a third of the NMDAR found in the brain tissue. In both cases, the muscle tissue was extracted from an alpha male *L. fasciolaris*. Once again, May seems to be a highly critical month in the expression of NMDAR for *L. fasciolaris*.

In June, the single female *Hybopsis amblops* showed an extremely high level of NMDAR. Extending research on the *Hybopsis amblops* could possibly reveal more in depth knowledge about the plasticity of NMDAR and its presence throughout the central nervous system of fish.

Conclusion

The plasticity of NMDA receptors seems evident among sexually dimorphic fish. Though speculation is limited to the relative amount of NMDAR, it seems that *N. telescopus* tend to have a more stable amount of NMDAR. *L. fasciolaris* on the other hand, increase their levels of NMDA receptors quite drastically during May and decrease in following months. Whether this is the case for all sexually dimorphic fish is not conclusive, the *N. leuciodus*, also sexually dimorphic, seemed to mirror the *L. fasciolaris*. There appears to be no difference between the levels of NMDAR between the sexes of the fish. The finding of NMDAR in the muscle tissue of *L. fasciolaris* was also noteworthy and seemed to show the same kind of NMDAR fluctuation as the brain tissue. To fully understand the modulation of NMDA receptors in the tissues of fish, larger sample sizes would need to be analyzed to measure differences more accurately.

Acknowledgement

I sincerely thank Dr. Bruce Stallsmith for his guidance and mentoring over the semesters. His role as my PI and advisor is most greatly appreciated. I am also truly grateful for the dot blot method suggested by Dr. Ernie Curto. His ability to think creatively made it possible to run dot blots that were time and material efficient. The Neuro Lab was vital to the sponsoring of this project. Asuka Eguchi was most helpful explaining many of concepts and techniques behind the Western Blot, which was tremendously useful in understanding the dot blot. I must also thank members of the lab, most especially Andrew Adrian and Brittany Holmes. Andrew Adrian started this pilot NMDAR project and helped me to learn the dot blot protocol. Brittany Holmes was involved in just about every aspect of this project, from collecting fish at local streams to running statistics to find significant differences between the fish. This research project would not have been possible without her help.

References

- [1] Avery, Leon. 2007. Mann-Whitney U Test. Accessed on July 21, 2010.
<<http://elegans.swmed.edu/~leon/stats/utest.cgi>>.
- [2] Flynn, K.M., S.A. Miller, S.A. Sower, and M.P. Schreibman. 2002. Sexually dimorphic effects of NMDA receptor antagonism on brain-pituitary-gonad axis development in the platyfish. *Comparative Biochemistry and Physiology Part C* 131: 9-18.
- [3] Flynn, K.M., M.P. Schreibman, E. Yabalonsky-Alter, and S.P. Bannerjee. 1999. Sexually dimorphic development and binding characteristics of NMDA receptors in the brain of platyfish. *Gen. Comp. Endocrin.* 115: 282-91.
- [4] Stallsmith, B.W., A.B. Adrian, E. Sosa, A. Burgos, B. Holmes, A. Eguchi, A. Bishop. 2009. Sexual Dimorphism in the Teleost Central Nervous System Varies With Spawning Strategy.