

Neuroanatomy of The Blackspotted Rockskipper, *Entomacrodus striatus*

Pooja Dayal

Department of Biology

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Approved: Dr. Richard Londraville

Date:

Honors Project Sponsor (signed)

Honors Project Sponsor (printed)

Dr. Jordan Renna

Date:

Reader (signed)

Reader (printed)

Dr. Qin Liu

Date:

Reader (signed)

Reader (printed)

Accepted: Dr. Brian Bagatto

Date:

Honors Department Advisor (signed)

Honors Department Advisor (printed)

Date:

Department Chair (signed)

Department Chair (printed)

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Abstract

Here I characterized the central neuroanatomy of the Blackspotted Rockskipper, *Entomacrodus striatus*, native to French Polynesia. The neuroanatomy of *E. striatus* has not been studied prior to this paper. I used several histology and antibody staining techniques to accomplish this, including Crystal Violet, immunohistochemistry, immunofluorescence, and Bielschowsky's Silver Nitrate staining. This paper describes the most successful techniques used, identifies major structures in the species' neuroanatomy, and also explains why studying *E. striatus* is important in the future of vertebrate research.

Introduction

The Blackspotted Rockskipper, *Entomacrodus striatus* is an amphibious fish species that inhabits crevices of wet rocks along ocean shores in the tropics, including French Polynesia (Aronson 1951). This habitat is common to other fish in the blenny family, *Blenniidae* (Myers 1991). This species appears to have well-developed memory, visual perception, and locomotion capabilities using its jumping and flipping locomotion from crevice to crevice. When startled, the fish appear to jump to a specific, rather than random, locations. This pattern has intrigued researchers, who studied behavioral implications of similar gobies, *Bathygobius soporator*, and their specific jumps (Aronson 1951).

Recently, I studied *Entomacrodus striatus* while traveling to Mo'orea, French Polynesia for Tropical Vertebrate Zoology. Our goal was to test hypotheses related to Aronson's conclusions by studying the extent of rockskippers' memory capabilities. Researchers spent days

catching fish from White House Reef and Temae (17.49668 S 149.756 W, Moorea, French Polynesia), and then setting up artificial habitats using rocks and water tables in an outdoor research lab at CRIOBE Research Facility aquaria. Here, fish were kept safely while daily trials were run to test their jumping behavior. This was done by trapping fish in a net, placing them on the rock, and then prodding the fish until it jumped. These trials were analyzed with directional analyses. Overall, we found that the fish had a statistically significant preference for jumping in a South direction. Following this, we injected the fish with *N*_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), a nitric oxide synthase inhibitor, to disrupt the fish's memory and induce retrograde amnesia (Xu 2001). Soon after, trials were repeated, and the fish no longer had a preferable direction of jumping after injections with L-NAME. The study suggested that *E. striatus* use spatial mapping mechanisms in their brain to navigate through tide pools and wet rocks rather than simple visual cues, suggesting the use of long-term memory of their environment (unpublished observation by Buo).

The Blackspotted Rockskipper is not well studied, and its brain structure is not fully described. The brains of fish in similar families, such as *Rhinogobius flumineus* (a goby endemic to Japan), have acted as a model in studies of the brains of other teleosts, like Blackspotted Rockskippers (Kawaguchi 1960). Encephalization (formation of brain during development) of other fish, such as *T. chatareus* (Karoubi 2016) and *Gobiodei teleosti* (Bauchot 1989), has been compared to the well-studied and understood brain of zebrafish larvae (Marquart 2017). Comparative brain structures can give insight to how different species allocate sensory resources. For example, fish with relatively large olfactory bulbs likely rely on smell for evolutionary fitness (Chiasson and Radke 1991). Similarly, knowledge on the gross anatomy of the Blackspotted Rockskipper brain may give insight to how they navigate their environment.

I used previous research on zebrafish in describing rockskipper neuroanatomy. I paid attention to the optic lobe, which accounts for a major portion of fish visual processes, as well as the corpus cerebelli, which controls aspects of fish movement. Since rockskippers are a highly visual species and are motivated by memory, these aspects of its brain may play significant roles and were studied closely (Chiasson and Radke 1991).

In addition to the gross anatomy description, I used histology, immunohistochemistry, and immunofluorescence techniques to describe the brains. Staining for astrocytes, axons, neurofibrillary aggregates, senile plaques, and whole brain cell nuclei all took place. Astrocytes (cells that play a role in the brain's blood-brain barrier), bring insight regarding the brain's internal structure, signaling, and function (Blackburn 2009). When blood perfuses into the brain, the blood-brain barrier is selective on what is allowed into and out of the brain in order to prevent harm. Astrocytes are glial cells that play a role in this mechanism. When astrocytes are to damaged or degenerated, homeostasis is disrupted and diseases such as Alzheimers' Disease, stroke, and epilepsy may occur (Weiss 2009). Axons are a substructure of neurons that conduct action potentials from one neuron to the next, while neurofibrillary aggregates and plaques are a characteristic condition in Alzheimer's disease (Trambauer 2020).

Methodology

Once completing our behavioral study in Mo'orea (unpublished observation by Buo), the fish were sacrificed with a solution consisting of 10% clove oil, 20% isopropyl alcohol, and 70% seawater. They were then preserved in 10% paraformaldehyde and phosphate-buffered saline (PBS) and transported from French Polynesia back to Akron, Ohio. Once arriving, rockskipper brains were extracted from five of eight fish for sectioning. This process involved freezing and then slicing the brains. Buffered paraformaldehyde (2.5%) was used for the initial preservation at

4°C. The following day, fish were rinsed three times for ten minutes each using PBS and then incubated at 4°C in 20% sucrose and PBS (Davis 2018). The day after, optimal cutting temperature (OCT) compound with 20% sucrose at room temperature was used to embed the tissue samples (Fischer 1970). Samples were placed in an aluminum foil cup, which was then briefly placed but not submerged into a bath of crushed dry ice and ethanol. Once frozen, the brain was removed and stored in a -80°C environment. Three fish were left whole and preserved in PBS. These were used later for dissections and imaging of the whole brain under an Olympus 5261 microscope (Davis 2018).

With the five frozen brains, cryosectioning took place by first lowering the temperature of OTF5000 Bright cryostat by 5°C increments until it reached -20°C (Davis 2018). In a deep freeze shelf, the brains remained while the mounting stud of the cryostat was coated with OTC compound. This compound acted as a framework that supported the brain samples on the cryostat (Fischer 1970). Once mounted, a blade was attached to the cryostat and equilibrated at 15° until it was in a position that allowed it to sit closely to the brain sample, but not directly touching it. By pressing on a foot pedal, the cryostat moved to axially slice the brain into 16 µm slices. Once the sample was cut, white Fisherbrand Superfrost Plus Microscope Slides were used to collect the brain slice samples and were saved for later analysis. Approximately eight brain slice samples fit onto one microscope slide. The amount of samples taken varied by brain, but continued to be taken until the brain sample depleted (Davis 2018).

Once sectioned, brain slices on microscope slides were stored in a freezer. Prior to staining, a wax pencil was used to circle each sample on the slides. The first staining process that took place was Crystal Violet. The process for these steps matched closely with OpenWetWare's Crystal Violet Staining protocol. The staining method targeted the nuclei of brain cells, allowing

researchers to view and study brain tissue. The process began with washing of samples over ice with cold PBS and 100% methanol for 10 minutes. The brain slices were then coated in 50% glycerol and PBS, and then stored in parafilm. Samples were then removed and incubated with 0.5% Crystal Violet and 25% methanol for ten minutes. I then covered the samples in Crystal Violet and washed them until no traces of staining agent were left (Crystal Violet Staining). Samples dried before being viewed for analysis under an Olympus BX51 Microscope. This microscope was equipped with SPOT Insight Digital Camera (Diagnostic Instruments Inc., Sterling Heights, Michigan).

The next staining technique that took place was immunohistochemistry using Rabbit-Anti-Zebrafish LeptinA #1382 Primary Antibody Application (Genscript, Piscataway, NJ) as the primary antibody and Donkey Anti-Goat IgG #ab205723 (Abcam, Cambridge, MA) as the secondary antibody to stain for astrocytes. On Day 1 of staining, the samples were blocked in a solution of 10% donkey normal serum (DNS) and 5% bovine serum albumin (BSA) in 0.1 M PBS at room temperature. In some trials, goat normal serum (GNS) was substituted for a blocker. Then, blocker solution was aspirated and Anti-GFAP primary antibody diluted in 1% BSA/PBS with Tween 20 at 1:50 concentration was applied. This antibody incubated at 4°C overnight (Santos 2018).

On Day 2, the remaining antibody solution was aspirated, and the samples were washed with PBS with Tween 20 four times. Each wash lasted fifteen minutes. After the washes, donkey anti-goat IgG secondary antibody diluted in 1% BSA / PBST at 1:2000 concentration was applied. This antibody also incubated overnight at 4°C. On Day 3, the remaining antibody was aspirated and samples were again washed four times with PBSTw. Each wash lasted twenty minutes. Then, the samples were washed with PBS. Each wash lasted five minutes. Final washes

then took place using glycerol/PBS solution. Three washes took place using 25%, 50%, and 75% glycerol to PBS. Each wash lasted twenty minutes. This step was only used once and was later removed from my procedure. After washes were complete, DAB stain was added and incubated for five minutes. We later incubated for a longer time period (10, 20, then 30 minutes) in other staining trials to ensure staining took place. Stain was then aspirated and washed with PBS to stop the reaction with brain tissue. Liquid was aspirated and samples were imaged with coverslips and without a mounting medium. Slides were imaged using Olympus BX51 Microscope at 4X, 10X, 100X, and 200X (Santos 2018).

The same procedures were completed on zebrafish brains to ensure efficiency of the antibody and technique used. When performing immunofluorescence, the same procedures as the immunohistochemistry protocol took place, with the addition of fluorescent molecules at the end to induce immunolabeling. Slides were also more protected from light when performing immunofluorescence. Between staining steps, samples were kept in the StainTray box with the black lid closed to prevent light from entering (Tse 2018).

The final procedure used was Silver Nitrate staining. The protocol matched closely with Bielschowsky's Silver Staining procedures (Bielschowsky's Silver Staining Protocol). In the initial three trials, I placed my slides in a container, pouring the solutions over the slides, and draining them after each step. In the final two trials, I used 40-200 μ L Finnpiptette to stain each brain slice individually. 10% Silver nitrate was pre-warmed using Benchmark Incu-Shaker 10LR oven set at 40 °C. Once warmed, the solution was used to stain brain sections for fifteen minutes. The slides were covered during this step to prevent evaporation of silver nitrate. The sections became light to dark brown in color. Solution was drained or aspirated, and then washed three times with distilled water. Each wash lasted one minute. Ammonium hydroxide was added to the

silver nitrate solution using a dropper. While dropping, the solution was swirled to ensure mixing. A precipitate formed after dropping sufficient amounts, and after adding more drops, the solution cleared (Bielschowsky's Silver Staining Protocol).

Sections were then placed in this ammonium silver solution. Slides stained in this solution for 30 minutes in Benchmark Incu-Shaker 10LR oven set at 40°C. Slides became dark brown. After sitting in ammonium silver, they were placed in developer solution for thirty seconds. Slides then were placed in 1% ammonium hydroxide to cease silver reaction. Then, they were washed three times in distilled water, placed in 5% thiosulfate for five minutes, washed again three times in distilled water and then dehydrated. Dehydration took place using 95% ethanol, absolute alcohol, and xylene. This step was only used for the first trial and was later removed. Lastly, slides were mounted using Permount Histological Mounting Medium and coverslips. Slides were left to dry overnight and then imaged the following day using Olympus BX51 Microscope at 4X, 10X, 100X, and 200X (Bielschowsky's Silver Staining Protocol).

In the remaining three whole preserved rockskippers, brain dissections took place by cutting from the mouth, into the nasal cavity, and through the skull using scissors. The brain samples were viewed under Olympus 5261 microscope. This device is connected to a screen so details can be analyzed and images can be taken. The brain images will be taken from several angles and will be used to label general neuroanatomy.

Results



Figure 1. *Entomacrodus striatus* species.



Figure 2. *E. striatus* taken out of fixative, prior to dissection.



Figures 3 and 4. *E. striatus* after cut through the mouth, into the nasal cavity, and through the skull during dissection. Some brain tissue is visible here, as shown by the yellow arrows.

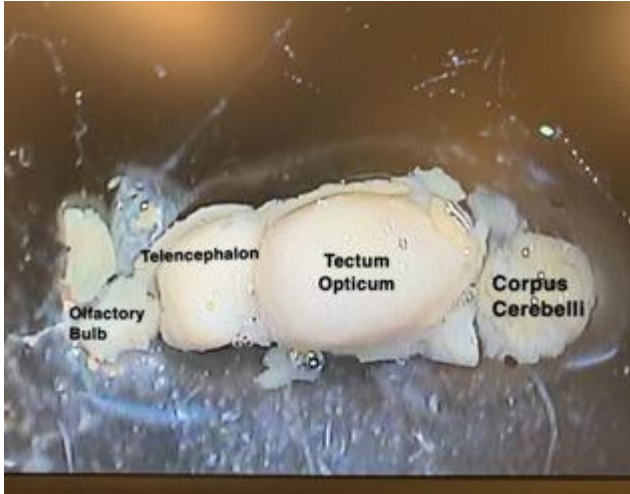


Figure 5. Lateral view of *E. striatus* brain. Image shows each structure labeled. Since this is a lateral view, this assumes that it is the same on both sides of the fish brain. Some of this tissue was lost due to over-preservation and during dissection.



Figure 6. Close-up of *E. striatus* Tectum Opticum (right) and Corpus Cerebelli (left).



Figure 7. Close-up of *E. striatus* Tectum Opticum (bottom) and Corpus Cerebelli (top).

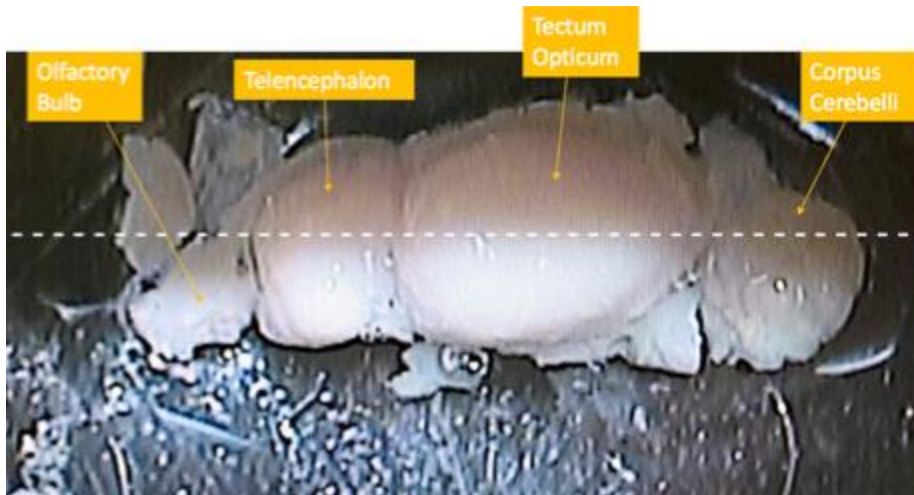


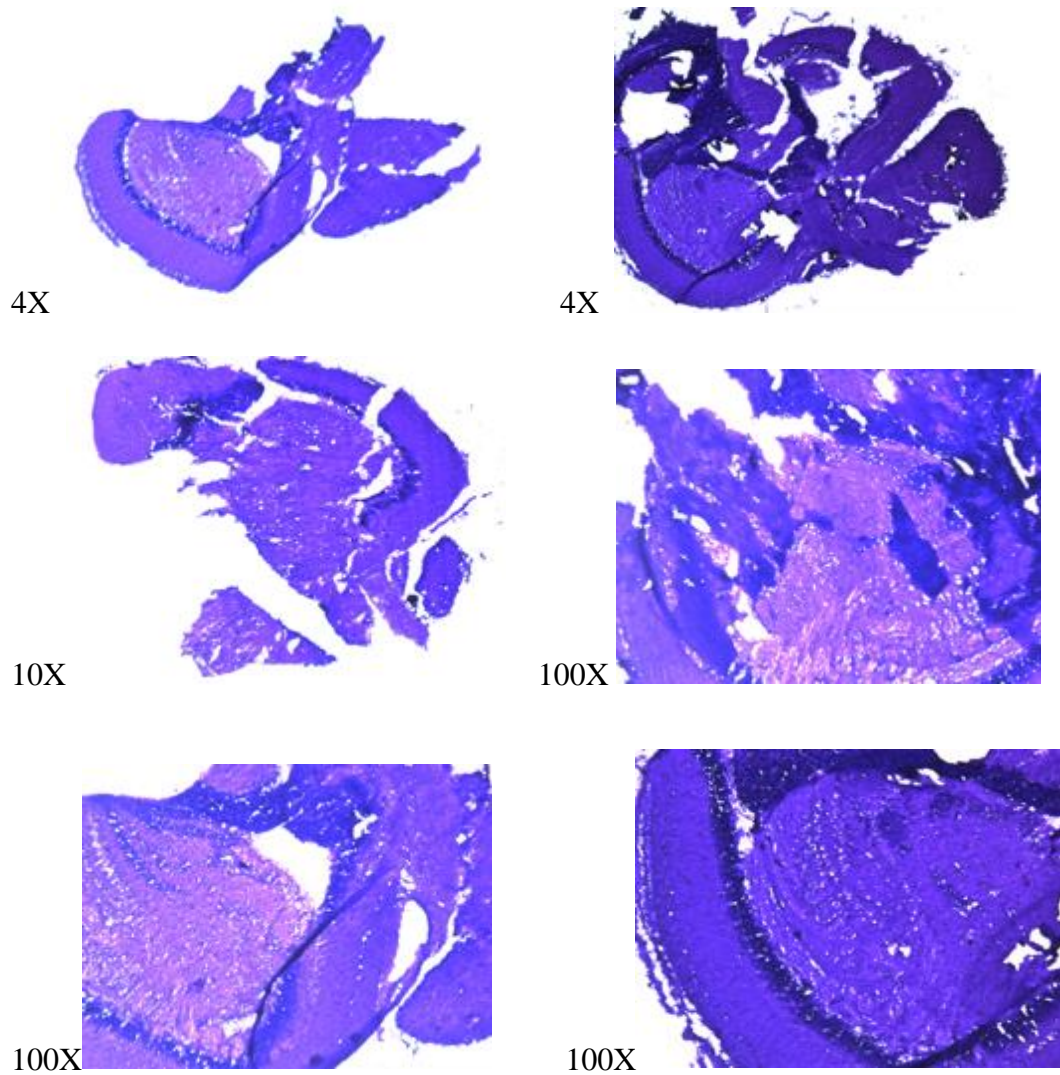
Figure 8. Image shows how brain samples were sectioned axially. Lateral view of *E. striatus* brain as shown, as in in **Figure 5**. The white dashed line represents an axial cut. This cut moved in this direction dorsal to ventral on *E. striatus* brain samples.



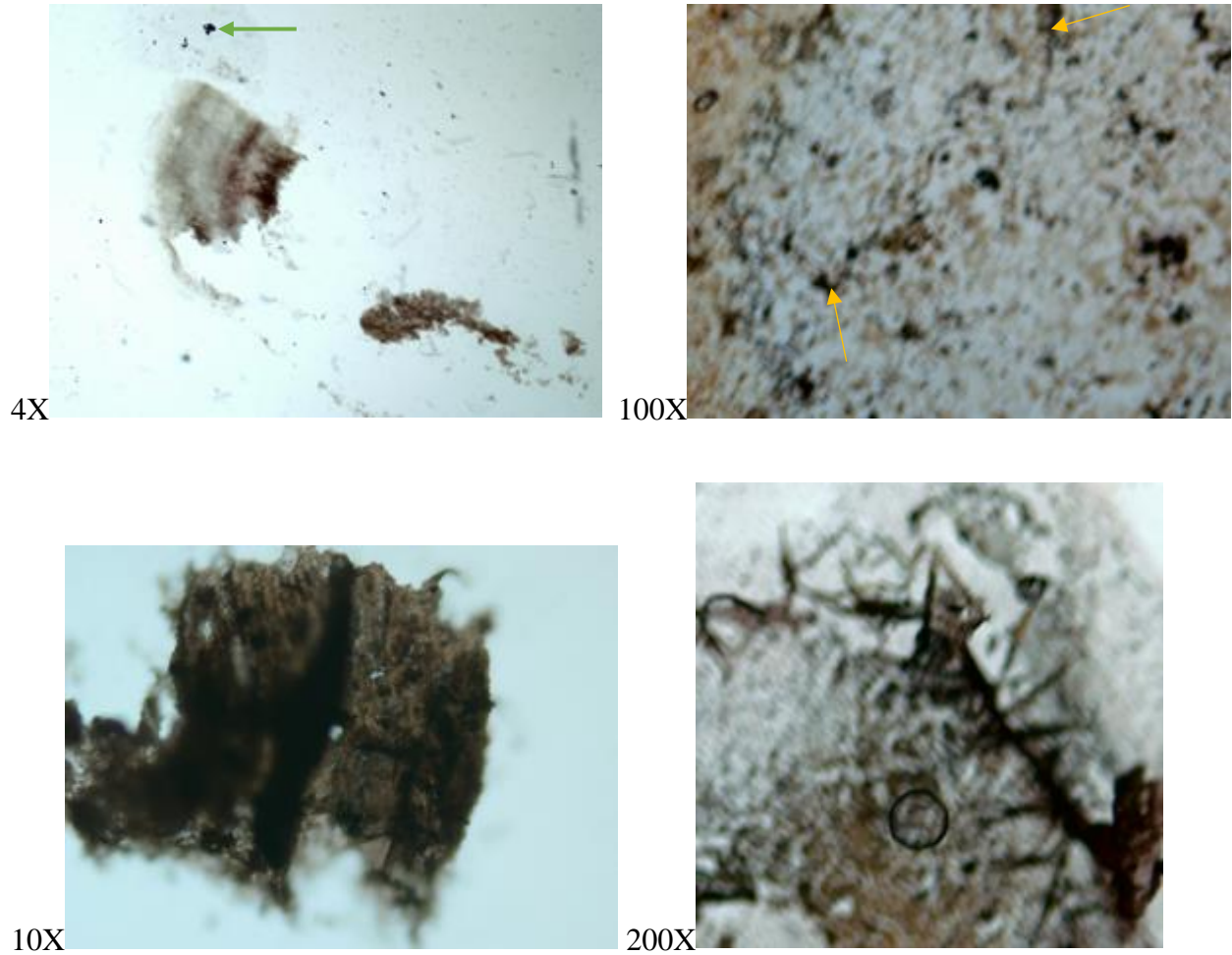
Figure 9. Example of tissue slides. Image shows sectioned *E. striatus* brain tissue and wax pencil circling each section. The yellow arrow is pointing at brain tissue.



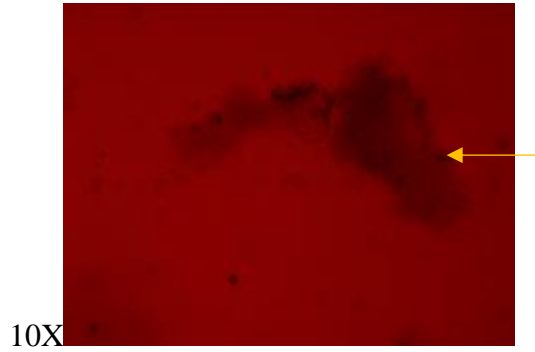
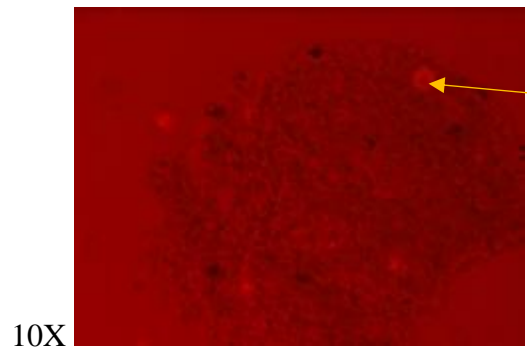
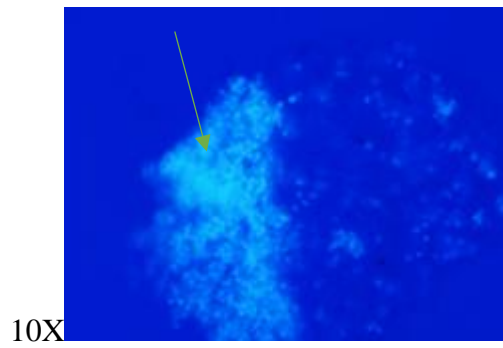
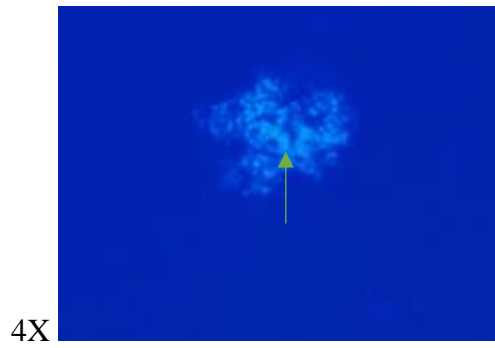
Figure 10. Example of how *E. striatus* tissue slides were treated during staining procedures. This specific image is from a trial of Bielschowsky's Silver Nitrate staining method. The yellow arrow is pointing at brain tissue staining brown.



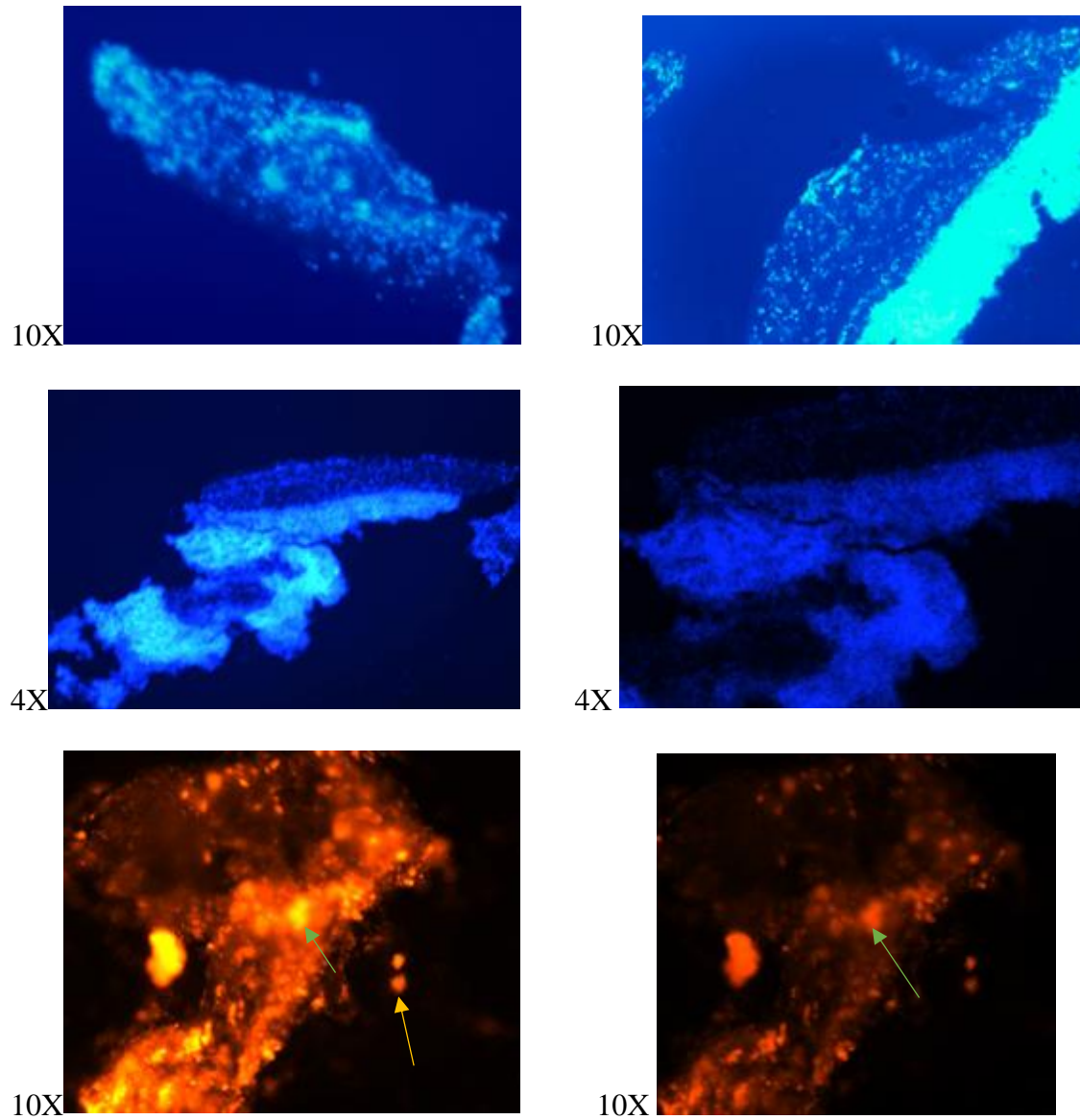
Figures 11-16. Axial cuts and Crystal Violet staining of *E. striatus* brain. The whole axial cut is not shown in 4X images because some tissue was lost in the process of cryosectioning.



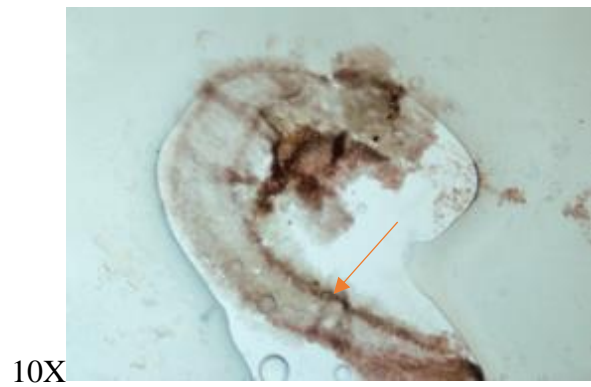
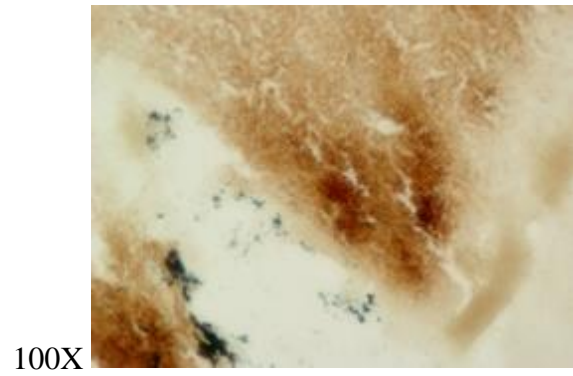
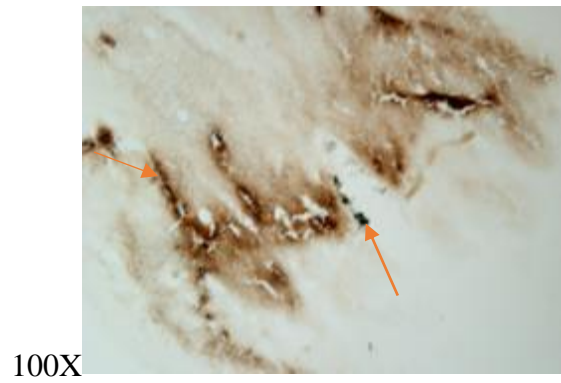
Figures 17-20. Immunohistochemistry antibody staining for general brain tissue (brown to gray) and astrocyte staining (black) on *E. striatus*. Overstaining was sometimes present in tissues. Staining also took place outside of brain tissue sample (green arrow), and black astrocytes (yellow arrow) stains gave a bubbly, unclear appearance. This made it difficult to interpret.



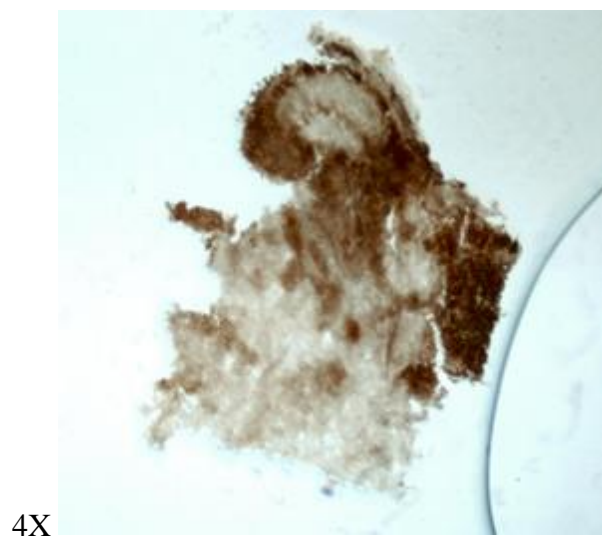
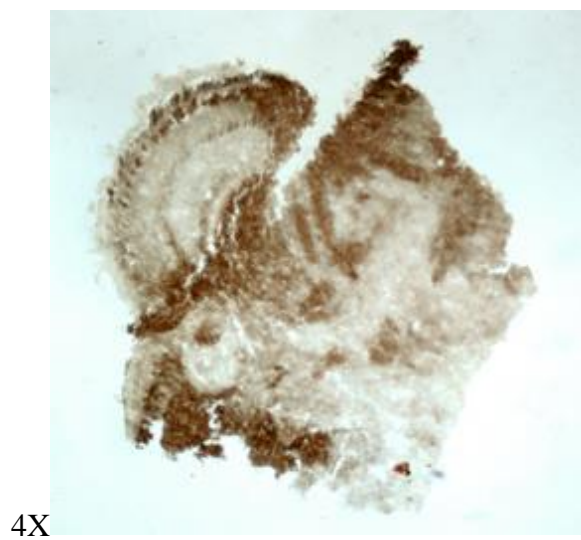
Figures 21-24. Immunofluorescence antibody staining for general brain tissue cells (blue) and astrocyte staining (red) on *E. striatus*. Green arrows represent general brain tissue cells, and yellow arrows represent possible astrocytes.



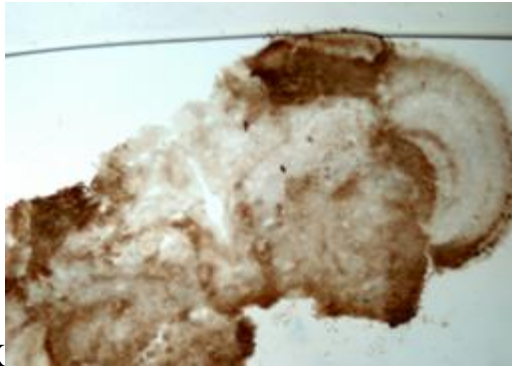
Figures 25-30. Verification of antibody using Immunofluorescence staining for general brain tissue cells (blue) and astrocyte staining (red) on Zebrafish. Green arrows point at possible astrocytes. Yellow arrow points at staining that took place outside of the tissue sample.



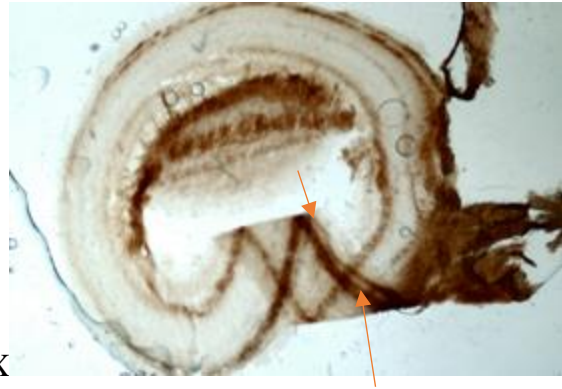
Figures 31-33. Bielschowsky's Silver Nitrate images from staining for axons, nerve fibers, neurofibrillary tangles, and senile plaques in Zebrafish. Black structures (orange arrows) represent axons, nerve fibers, neurofibrillary tangles and senile plaques. Yellow/beige to brown structures represent remaining brain tissue.



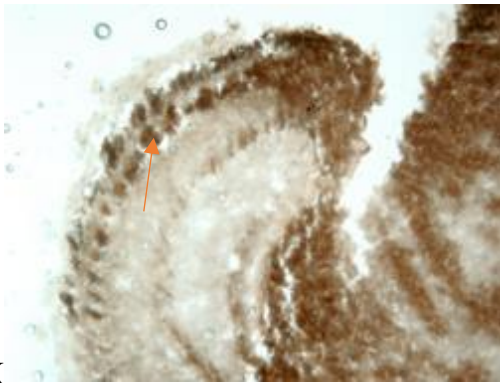
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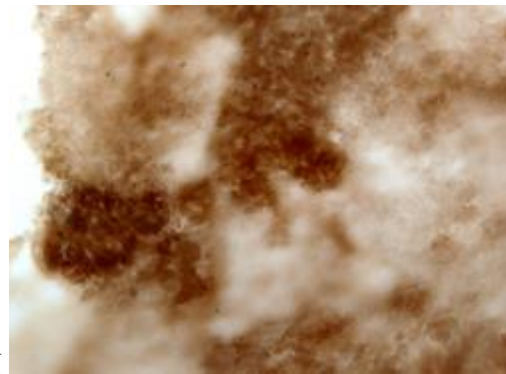
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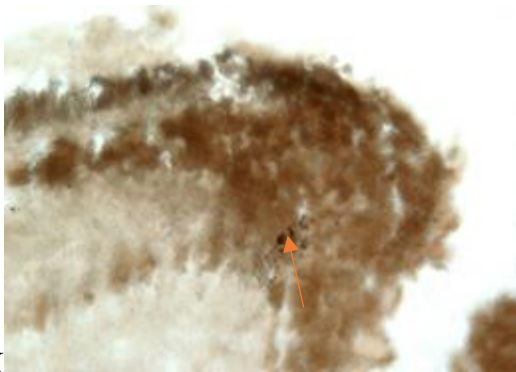
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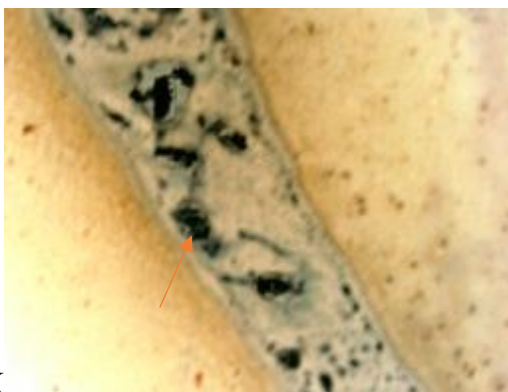
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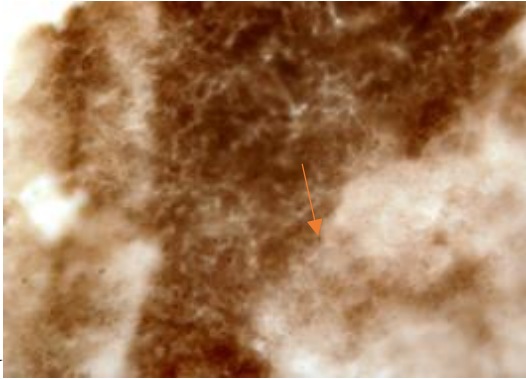
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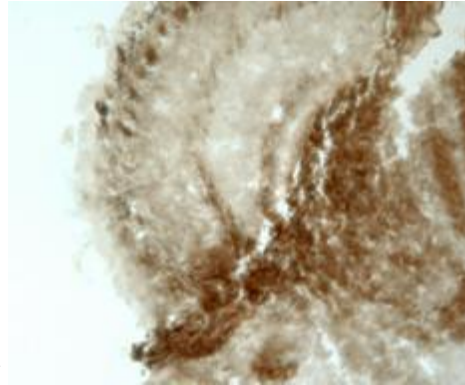
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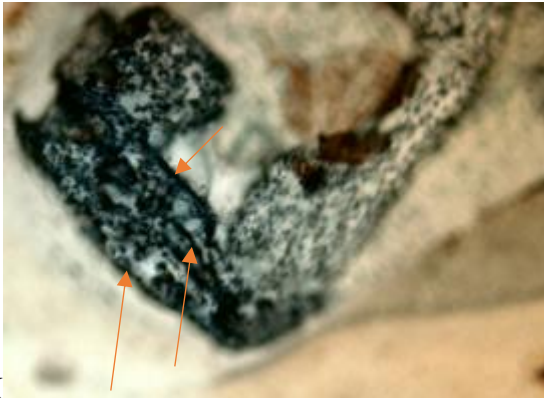
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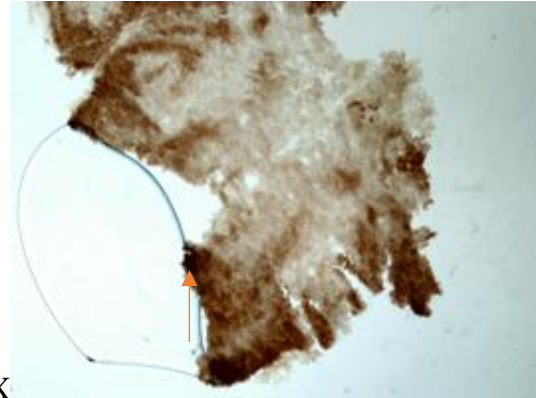
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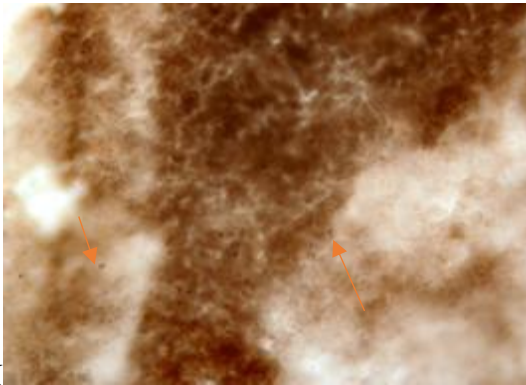
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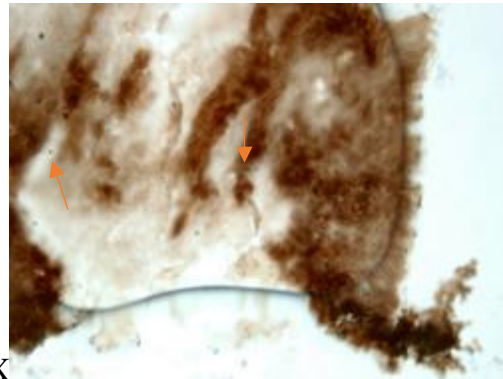
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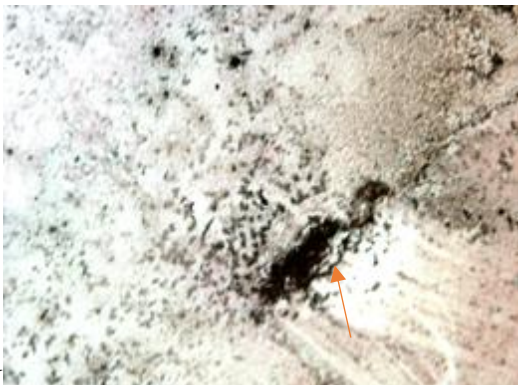
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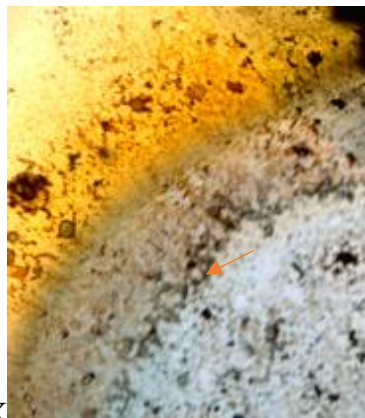
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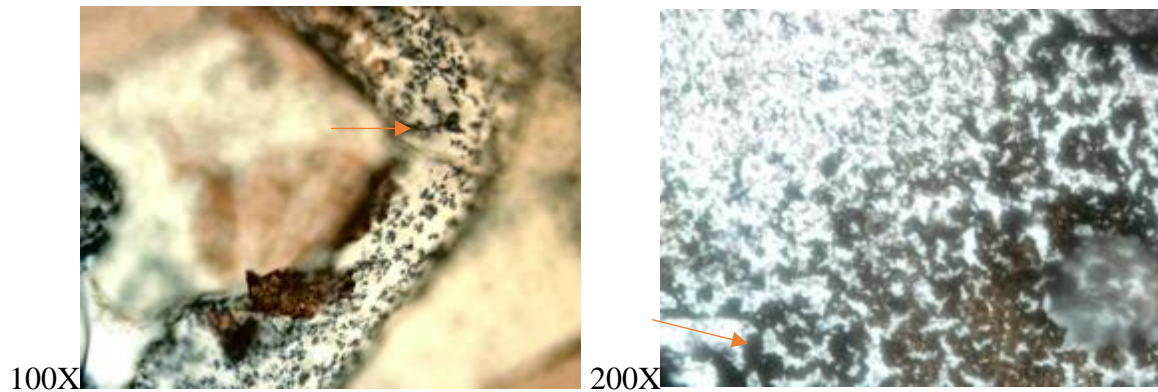


100X



200X





Figures 34-53. Bielschowsky's Silver Nitrate staining images for axons, nerve fibers, neurofibrillary tangles, and senile plaques in *E. striatus*. Black structures (orange arrows) represent axons, nerve fibers, neurofibrillary tangles and senile plaques. Yellow/beige to brown structures represent remaining brain tissue.

Discussion

The collective six months working on *Entomacrodus striatus* brains has brought a better understanding of their neuroanatomy. I expected the research process to go from dissection, to cryosectioning, to staining, and then finally viewing and interpreting results. Many prior research papers have worked in this fashion, such as studies on the telencephalon of *Rhinogobius flumineus*, and my plan was to model these previous experiments (Kawaguchi 1960). Instead, the process became one of extensive trial and error. As shown in **Figures 17-20**, antibody staining with immunohistochemistry gave blurry, unclear results. Black staining gave the appearance of bubbles rather than astrocytes, and staining also was present outside of the tissue samples. This raised the question on whether the antibody used was effective or not. As seen in **Figures 21-24** depicting *E. striatus*, results were dim and not visibly present. In **Figures 25-30** depicting Zebrafish, results were more vivid, but still did not show the typical pattern of astrocytes. Here, it

also seemed as though the tissue was staining for other structures in addition to astrocytes, making the images difficult to interpret.

With more time, money, and resources, I would have taken the initiative to understand why the antibody did not work as expected. I would have tested the antibody on a mouse brain to understand whether the antibody was the problem, or if the *E. striatus* samples were too over-preserved to ensure quality testing. It is also a possibility that *E. striatus* neuroanatomy is not analogous to Zebrafish. Staining with a different antibody would have been ideal, as well as having fresher *E. striatus* brain samples, rather than ones that were preserved for two months prior to staining. With extra time, I would have searched different methods to stain for more structures to better understand *E. striatus* neuroanatomy, since it has not been studied in depth prior to this experiment.

Despite uncertainty in results of antibody staining with immunohistochemistry and immunofluorescence, Crystal Violet and Bielschowsky's Silver Nitrate methods were successful. Crystal Violet, as shown in **Figures 11-16**, gave a clear depiction of an axial cut of *E. striatus* structure and imaging of general neuroanatomical regions. Bielschowsky's Silver Nitrate (**Figures 34-53**) gave the clearest and most informative results. The distinctions made between specific structures and remaining tissue was easily visible. The procedure itself was timely and efficient, with little waiting periods and all methods taking place in one day, rather than a three-day protocol like with antibody staining. Each test showed similar light brown/beige to dark black features. All black features were localized in a specific area, which is characteristic of axons in typical fish neuroanatomy.

This study has served a way to gain a better understanding of the neuroanatomy of *E. striatus*. Since this is an understudied species, this study can be used as a model when future

studies take place on *E. striatus*, as it shows axial cross sections and imaging of its major neuroanatomical regions. Another point to consider is the initial experiment in French Polynesia that took place on *E. striatus* prior to sacrificing and preserving. After injection with L-NAME, a nitric oxide synthase inhibitor, retrograde amnesia and a decrease in spatial awareness occurred. It is possible that the black structures from Bielschowsky's Silver Nitrate stain are also depicting neurofibrillary tangles and senile plaques, which are both hypotheses for the neurodegeneration that occurs during Alzheimer's Disease. Alzheimer's Disease is typically characterized by retrograde amnesia, which was the result L-NAME injections on *E. striatus*. It is possible that L-NAME could have led to the formation of these plaques and tangles in the *E. striatus* samples used by inhibiting nitric oxide synthase functioning, but future studies would have to be performed to explore this idea further (Trambauer 2020).

If this experiment is to be repeated, researchers should avoid over-preservation of brain tissue by sectioning and staining tissue soon after sacrificing and dissecting. Waiting too long could result in a crumbly-like appearance of brain tissue, leading to trouble when staining and imaging, and loss of tissue. Often, since tissue was not intact, it would aspirate with fluid in between staining methods. The brain was also difficult to keep whole when dissecting. Another precaution is to ensure tissue sections are not too thick. This could result in folding of the tissue, making it difficult to have clear imaging results after staining.

Future researchers can also explore other ways to study this species, as its spatial awareness is an interesting region of study. While this study can be used as a basic understanding of *E. striatus* brain, there is scope for more learning, and even a possible relationship to Alzheimer's Disease. For this reason, it is important that the study of this fish species continues hereafter.

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