Study explanation:
Multiple species of nemertean were collected from multiple different study sites around the San Juan Islands. These worms were photographed, their pH levels were taken, some stylets were photographed, and tissue samples of different sections of the worm were taken both for EDNA purposes and the ELISA assay. The ELISA assay was used to quantify the tetrodotoxin content between species and between different portions of the same species.

Resources used or referenced:
See: Lit Review - Nemerteans

Hypothesis:
Nemerteans which possess vibrant colors and abundance of tint (such as very neon greens, yellows, reds, blues, or purples) will be more likely to have high concentrations of TTX than those without, due to the fact that aposematism is often an indicator of toxicity within nature.

Null Hypothesis:
Nemerteans which possess vibrant colors and abundance of tint (such as very neon greens, yellows, reds, blues, or purples) will not likely have higher concentrations of TTX than those without, due to the fact that aposematism is often an indicator of toxicity within nature, but not always

Species used:
Tubulanus ruber - Red ribbon worm. Originally known as T. polymorphus.
Kulikova montgomeryi - Montgomery ribbon worm.
Tubulanus sexlineatus - Lined ribbon worm
Emplectonema burgeri - Mottled ribbon worm
Amphiporus formidabilis - White ribbon worm
Paranemertes peregrina - Restless ribbon worm
Emplectonema viride - Green ribbon worm
Cerebratulus cf marginatus - Milky ribbon worm
Lineus rubescens - Red-ish ribbon worm

Procedure:
1. Measure the pH level of the nemertean before sectioning.
2. Remove a small sample for homogenization with EtOH.
3. Sample homogenate to measure pH level.
4. Adjust pH of homogenization to 6.5 - 7 if the supernatant does not possess such a range naturally.
5. Add antibodies. Let samples incubate at 37 C.
6. Wash samples.

7. Add substrate to trigger coloration, and let incubate in the dark.
8. Measure absorbance value and optical density values.
9. Graph values using a four-parameter logistic function.
See full procedures from “Procedure for Assay of Nemertean Tetrodotoxins

Safety needs:
General biosafety training
BSL 2 Training

Conclusion (s):
1. There seems to be a trend of worms possessing aposematism exhibiting higher TTX-antibody binding.
2. There seems to be a trend of worms which have TTX-bearing sister species to also have high TTX-antibody binding.
3. There seems to be a trend that could indicate TTX is generally more concentrated in the proboscis, midbody, and mucus.
4. We could not definitively reject the null hypothesis.

Presentation:
See: “Cutting Ribbons”

Procedure for Assay of Nemertean tetrodotoxins

Materials

- Microplate reader equipped with 450 nm wavelength measurement, with a correction wavelength between 600-680 nm.
- Micropipettes and tips
- 100-1000 mL graduated cylinder.
- Multichannel pipette or automatic microplate washer.
- Orbital microplate shaker (speed of 450-550 rpm)
- Test tube x7
- Tissue homogenizer
- 0.1% acetic acid
- Hotplate with stirrer
- Rapid qualitative filter paper
- 1 M NaOH
- Microplate
- Standard
- 100x primary antibody
- 100x enzyme labeled antibody
- 20x concentrated diluent
- Substrate solution a
- Substrate solution b
- Stop solution
- 20x concentrated wash solution
- Plate sealing film
- (Optional for serum testing) Serum separation tube
- (Optional for plasma testing) Heparin or ethylenediaminetetraacetic acid (EDTA)

Cautionary Statements

1. Washing process is critical to assay performance. Incorrect washing invokes inaccuracy and elevated optical density (OD) values.

2. All reagents must be brought to room temperature before use in the assay.

3. All microcentrifuge tubes, tips, and plate sealers are single use.

4. Keep reagents A and B in the dark (line with foil when brought to light).

5. Every addition to a sample should be done using a pipette.

6. When calculating the final concentration of tetrodotoxin in an assay well, multiply the final result by the total dilution factor.

7. Long storage of samples can result in protein degradation or denaturing, which can change the observed results. Fresh samples are most effective.

8. Dilution factor is based on analyte concentration in the sample. If it exceeds the highest standard value (800 ppb) then you will need to increase the dilution factor. Prepare the standard stock solution and the 100x enzyme labeled antibody solution based on the number of wells required for the assay, and store remaining solution at -20℃.

9. Avoid repeated freeze-thaw cycles.

Procedure

1. Remove all reagents from storage and allow to warm to room temperature. ***Keep reagents A and B in the dark, or cover with light-blocking material.***

2. Weigh and homogenize 5g of worm tissue. (or, x grams of tissue). Multiply the mass by 5, and add that much 0.1% acetic acid to the tissue sample. (i.e: If you have 5 grams of tissue, you will add 25 mL of 0.1% acetic acid to the sample). Homogenize, then boil and stir for 10 minutes. ***Do steps 3 and 4 while the sample is waiting to boil. You need at least 50 grams of the initial sample.***

3. Prepare a wash solution using the 20x concentrated wash solution. Dilute with distilled water at a ratio of 1:20. (i.e: if you use 1mL of wash solution, add 19mL of diluted water) ***If there are crystals present within the solution, heat at 37***℃ ***until all crystals are dissolved.***

4. Prepare the standards (or control) for the test. Label 7 test tubes as a preparation for a dilution series, in parts per billion (ppb). Dilute the 1,0000 ppb standard with 1x dilution solution to make 800ppb, as needed for sample. (i.e: to make a 1,000 uL of 800 ppb standard, mix 80 uL of the stock solution with 920 uL of the 1x dilution solution). Then, add 500 uL to each of the 6 other test tubes. Make the following serial dilutions: 800 ppb, 400ppb, 200 ppb, 100ppb, 50ppb, 25ppb, and 12.5 ppb. You can do so by transferring 500 uL from the highest concentration standard solution to the very next tube, and repeating the process for each tube. Mix gently. Use 1x dilution solution as your 0 ppb standard.

5. Allow the homogenized solution to cool to room temperature, then centrifuge the tube for 10 minutes at 3,000 RPM. ***5 minutes in, move to step 6 and begin preparing the antibody working solution and enzyme labeled secondary antibody working solution.***

6. Prepare the primary antibody working solution by diluting the given 100x primary antibody working solution to 1x using the 1x dilution solution. Prepare according to the required volume. Dilute the 100x enzyme-labeled antibody solution to 1x using the 1x dilution solution, and prepare according to the required volume.

7. Filter the resulting supernatant using rapid qualitative filter paper into a test tube. Label and cover.

8. Take 1mL of extract. Measure the resulting pH. If the pH range is not within the 6.5-7.0 range, adjust it dropwise with 1M NaOH until it is so.

9. Add 50 uL of the different concentrations of standards to each well. At the same time, add 50 uL if anti-reagent per well. Cover with sealing film and incubate at 37℃ for 30 minutes, then add 300 uL of the 1x wash buffer to each well, shaking gently for 30 seconds. Remove the liquid by flicking the plate and blotting with paper. Wash 3 times with this method.

10. Add 100 uL of enzyme-labeled antibody working solution to each well and gently mix. Cover with sealing film and incubate at 37℃ in the dark for 30 minutes.

11. Repeat the wash procedure from step 9 four times.

12. Add 50 uL of substrate solution A to each well, followed immediately by 50 uL of substrate solution B, then gently mix. Cover with sealing film and incubate in the dark at 37℃ for 15 minutes. ***During the incubation time, pre-warm and calibrate the 450nm microplate reader.***

13. After the color development reaction is complete, add 50 uL of stop solution to each well and gently mix. Measure the absorbance value at 450 nm using a microplate reader.

14. To calculate final results, plot a standard curve using the logarithm of the concentration on the x-axis and the optical density value on the y-axis, following a four-parameter logistic function. (4-P). If the sample optical density value exceeds the upper limit of the standard curve, dilute the sample and retest, multiplying the calculated sample concentration by the corresponding dilution factor. God help me when we get to this part. Who here doesn't have dyscalculia and would like to be noted in the paper?

Sample Addendums

15A. If sampling serum, collect a whole blood sample in a serum separation tube and allow it to stand at room temp for 2 hours or overnight at 4℃. Centrifuge the sample at 3000 rpm for 10 minutes, and then use the supernatant for testing.

15B. If sampling plasma, collect using ethylenediaminetetraacetic acid (EDTA) or heparin as an anti-coagulating agent. Centrifuge at 3000 rpm for 10 minutes, then use the supernatant for testing.

Relevant documentation and training

BIOSAFETY LEVEL 2 (BSL-2) LABORATORY PRACTICES

Biosafety Training Online | UW Environmental Health & Safety

Nemertean overview:

Highly Toxic Ribbon Worm Cephalothrix simula Containing Tetrodotoxin in Hiroshima Bay, Hiroshima Prefecture, Japan - Manabu Asakawa, Katsutoshi Ito, and Hiroshi Kajihara (2013)

*Maximum toxicity in terms of TTX in C. simula was 25,590 Mouse units per gram for the whole worm.

*Highest toxicity of C. simula exceeded human lethal dose per a single worm.

*Seasonal variation in toxin potency was observed.

*C. Simula food items were annelids, crustaceans, and mollusks

*Amphiporus, Cerebratulus, Lineus and Tetrastemma have been found to contain neurotoxic polypeptides and pyridyl alkaloids.

*No relationship was observed between body weight and toxicity

* Lethal amount of TTX for humans is 2mg

*Toxicity was measured by standard bioassay method for TTX.

***Pure crystals of TTX were obtained in this study. The method is described on page 382.**

*Chromatography and IR, HNMR graphs are shown.

*TTX in the C. simula species was found in vesicles of the epidermal cells, vesicles in the basal position of intestinal epithelial cells near blood vessels, and glandular cells in the probiscus's epithelium.

*Study suggests that portion of the toxins may come from sediment or bacteria.

*Vibrio alginolyticus was found in multiple nemerteans

*Suggests TTX is exogenous, as nemerteans are carnivorous.

New Invasive Nemertean Species (Cephalothrix Simula) in England with High Levels of Tetrodotoxin and a Microbiome Linked to Toxin Metabolism - Andrew D.Turner, David Fenwick, Andy Powell , Monika Dhanji-Rapkova, Charlotte Ford, Robert G. Hatfield, Andres Santos, Jaime Martinez-Urtaza, Tim P. Bean, Craig Baker-Austin, Paul Stebbing (2018)

*Gene sequence of microbiome (C.simula) - Alyrtomonas, Vibrio, and pseudomonas found.

*multiple TTX analogues found.

* P.luteola and V.alginolyticus were cultured at low temperatures and both contained TTX.

*claims proboscis produces a "range of chemicals"

*Defines tetrodotoxins as having low molecular weights and being water soluble.

* Suggests possible connection with phytoplankton in production with TTX. **Cites paper. Study further.**

*Bacillus sp produces TTX, found in C. simula.

*tested toxins using liquid chromatography and tandem mass spectrometry.

* specimen storage and transfer is notated.

* **methods for culturing species of bacilli, P.lutiola, V. alginolyticus on petri dishges and growing pure cultures is written.**

* Chromatographs of sampled TTX and anaolgues is shown.

*Argues non-native nemerteans still possess TTX producing bacteria, complicating the relationship between micro and megafauna.

*Acute toxicity and lethal dosage of TTX in humans is theorized.

Nemertean Toxin Genes Revealed through Transcriptome Sequencing - Nathan V. Whelan, Kevin M. Kocot, Scott R. Santos, and Kenneth M. Halanych (2014)

* a toxin like gene was found in all analyzed nemerteans which had a high sequence similarity to a DNase 2 hepatotoxin. Authors suggest the acidic body walls of some nemerteans could work to enhance the efficiency of protein toxins.

* Study claims that toxic proteins with similar form and function show convergent evolution in differing animal groups.

* tetrodotoxin is a non-peptide toxin.

* dictates methods of transcriptome extraction and sequencing.

* suggests toxin gene expression may be dependent on severalfactors, such as life habits, gender, physiological state, and whether venom glands were active could affect expression.

Tetrodotoxins:

Paralytic toxicity in the ribbon worm Cephalothrix species (Nemertea) in Hiroshima Bay, Hiroshima Prefecture, Japan and the isolation of tetrodotoxin as a main component of its toxins - Manabu Asakawaa, Tadayoshi Toyoshimaa, Katsutoshi Itoa, Kentaro Besshoa, Chisato Yamaguchia, Shogo Tsunetsugua, Yasuo Shidab, Hiroshi Kajiharac, Shunsuke F. Mawataric, Tamao Noguchid, Keisuke Miyazawaa (2003)

*Details methods by which an unknown toxin from a nemertean specimen was quantified and proven to be TTX. **Keep and review for use. Details can be found under 2.3 isolation of ribbon worm toxins.**

* Lists lethal dose of TTX in a human as 10,000 MU.

Tetrodotoxins in Larval Development of Ribbon Worm Cephalothrix cf. simula (Palaeonemertea, Nemertea) - Grigorii V. Malykin, Peter V. Velansky, Daria I. Melnikova, Timur Yu. Magarlamov (2023)

*TTX has been found in the eggs of C.simula

*TTx labeling through larval development was measured and noted both to particular developmental layers and cells.

*Five TTX toxins were identified in the eggs and larvae

*A paper is cited which studied the pharmacokinetics of similar TTX development and presence in the maturation of flatworms

*details egg hatching method

*4 days post fertilization (after hatching) strong TTX signals could be observed

*Photos with fluorescent tagging detailing TTX concentration in tissues is shown.

* TTX content and location is also visualized for adult nemerteans.

* In depth descriptions of nemertean formation, development, and organ use is given. May be useful for further studies.

Intrabody Tetrodotoxin Distribution and Possible Hypothesis for Its Migration in Ribbon Worms Cephalothrix cf. simula (Palaeonemertea, Nemertea) - Grigorii V. Malykin, Alexei V. Chernyshev and Timur Yu. Magarlamov (2021)

*Cell types of positive TTX tissues in C.simula were identified.

*findings indicate toxin moves from the digestive system to key organs, and that it is excreted through nephridia and mucus of epidermal cells.

*TTX nis found in all classes of nemertean, and is the only shared toxin between the three classes.

*photos illustrating immunoreactivity in different sections of the nemertean body are shown.

***Very useful identifying image presented in figure 3. Cross reference material when observing nemerteans.**

*Nemerteans possess pseudocnidae cells

*TTX was found in the cytoplasm and phagosomes of enterocytes.

*Toxin was detected in the cytoplasm and yolk granules of oocytes

***Details cellular metabolization of food**

*Posits that the foregut is the nain region of toxin absorption

*Suggests blood is involved in the transfer of TTX, with extra being removed bt the protonephridial system

The Toxins of Nemertean Worms - Ulf Göransson, Erik Jacobsson, Malin Strand, and Håkan S.Andersson (2019)

*Overview of research into nemertean toxins

*Details pharmacologically active compounds found in nemerteans

****Toxins produced by nemerteans (pyridine alkaloids) are illustrated. (chemical structure, figure 5)***

*Spiny lobsters ate nemerteans when the mucus was removed. This may indicate the TTX in the mucus also functions as a pheromonal deterrent for predators.

*pyridyl alkaloids seemed to prevent barnacle settlement

*a systematic study of anabaseine lifecycle and transformation

An Updated Review of Tetrodotoxin and Its Peculiarities - Panagiota Katikou, Cengiz Gokbulut, Ali Rıza Kosker, Mònica Campàs, and Fatih Ozogul (2022)

A tetrodotoxin-producing marine pathogen - Kim B. Ritchie, Ivan Nagelkerken, Sara James, Garriet W. Smith (2000)

Stable Tetrodotoxin Production by Bacillus sp. Strain 1839 - Daria I. Melnikova, Anna E. Vlasenko, and Timur Yu. Magarlamov (2019)
On the origins and biosynthesis of tetrodotoxin - Rocky Chau, John A. Kalaitzis, Brett A. Neilan (2011)

Tetrodotoxin-Producing Bacteria: Detection, Distribution and Migration of the Toxin in Aquatic Systems - Timur Yu. Magarlamov, Daria I. Melnikova, and Alexey V. Chernyshev (2017)

Evolution, Expression Patterns, and Distribution of Novel Ribbon Worm Predatory and Defensive Toxins - Aida Verdes, Sergi Taboada, Brett R. Hamilton, Eivind A.B. Undheim, Gabriel G. Sonoda, Sonia C.S. Andrade, Esperanza Morato, Ana Isabel Marina, César A. Cárdenas, and Ana Riesgo (2022)

Venomous Noodles: The Evolution of Toxins in Nemertea through Positive Selection and Gene Duplication - Gabriel Gonzalez Sonoda, Eric de Castro Tobaruela , Jon Norenburg, and Sónia C. S. Andrade, João Paulo Fabi (2023)

On the Origins and Biosynthesis of tetrodotoxin - Rocky Chau, John A, Kalaitzis, Brett A. Neilan (2011)

- *Chemical structures of marine toxins are illustrated
-

Vibrio:

Differential specificity of selective culture media for enumeration of pathogenic vibrios: Advantages and limitations of multi-plating methods - Olivia D. Nigro, Grieg F. Steward (2015)

A modified culture medium for improved isolation of marine vibrios - Marcello Tagliavia, Angela Cuttitta, Monica Salamone,Carmelo Bennici,| Paola Quatrini (2019)

Vibrio - Steven L. Percival, David W. Williams (2014)

Intersectional:

The Microbial Community of Tetrodotoxin-Bearing and Non-Tetrodotoxin-Bearing Ribbon Worms (Nemertea) from the Sea of Japan - Daria I. Melnikova and Timur Yu. Magarlamov (2020)

*Compared 16S rRNA gene data from eight species of marine ribbon worm, 4 TTX-bearing and 4 non-bearing.

*Showed different nemertean species harbor distinct bacterial communities, though members of the same species largely share similar microbiomes.

*Suggests nemerteans do not just accumulate TTX but also produce and metabolize it.

<u>*Categorizes bacteria found within the microbiomes of each nemertean species as well as how prevalent it was in each.</u>

*Microflora typical of toxic nemerteans were from orders Alteromondales, Pseudomondales, Oceanospirillales, and Vibrionales.

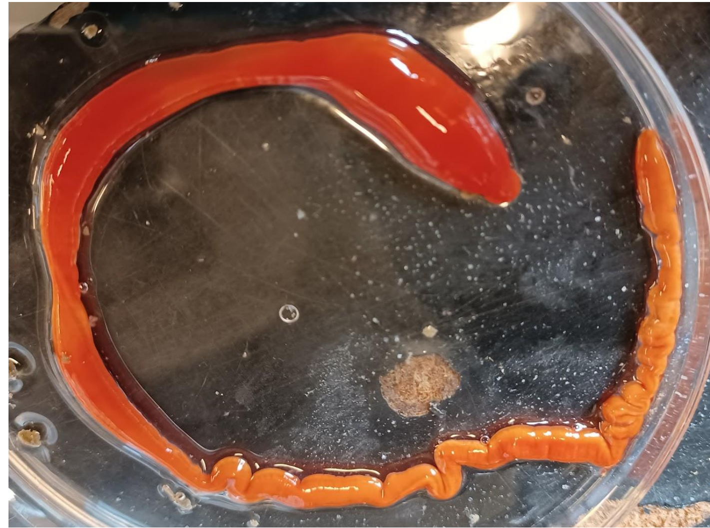
*Vibrio anguillarum was found in all TTX producing nemerteans.

*Statement indicating using liquid chromatography-mass spectrometry and bioassay for TTX detection in bacterial and nemertean samples can lead to a false-positive result. It is suggested to supplement with tandem mass spectrometry.

The production of tetrodotoxin-like substances by nemertean worms in conjunction with bacteria - Stuart Carroll, Eric G. McEvoy, Ray Gibson (2002)

Cutting Ribbons:

An investigation into nemertean worms and their tetrodotoxin content



Presented by Chayse Bono, student of Marine Invertebrate Zoology, FHL 432
Summer 2025. Submitted 7/17/25.

Important werms (worm terms)

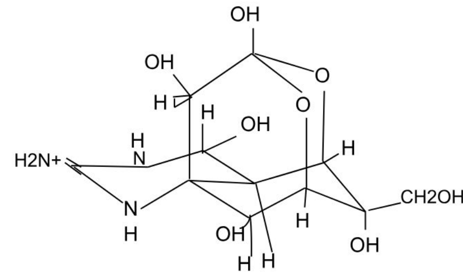
- Aposematism - The phenomenon by which an animal presents to predators via coloration that it may be unpleasant or harmful to eat.
- Tint - A color which is mixed with white, increasing its lightness.
- Gonochoric - Beings are either male or female, and do not change sex throughout their lives.
- Vermiform - Worm like

What are Nemerteans?

- Also known as Ribbon worms
- Possess eversible probosci - retractable at will
- Many exhibit aposematism
- Can be found in freshwater, saltwater, and terrestrially.
- Abundance of connective tissue (important later)
- Gonochoric
- Three classes: Pilidiophora, Hoplonemertea, Paleonemertea
- Use peristalsis to move, burrow, and swim

What is tetrodotoxin?

- Neurotoxin, most commonly known from pufferfish, specifically from Fugu.
- Currently irreversible and cumulative
- Understudied in many species
- Comprised only of organic molecules
- Capable of inducing paralysis via blocking of sodium gated receptors in nerve cells



Tetrodotoxin isolated from C. simula in the study conducted by Asakawa et al. 2013. Molecule structure pulled from figure eight within the study

How can you differentiate the three classes?

Hoplonemertea:

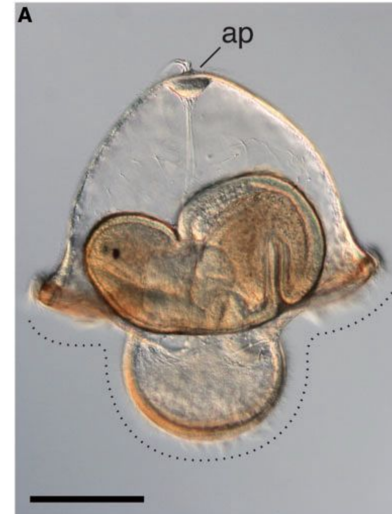
* Proboscis has stylets with calcified extensions or barbs.

Paleonemertea:

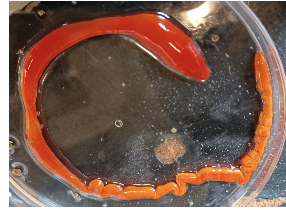
* “Unarmed” or unadorned stylets

Pilidiophora:

* Have pilidium larva



Species of import



- Tubulanus ruber - Red ribbon worm. Originally known as T. polymorphus.
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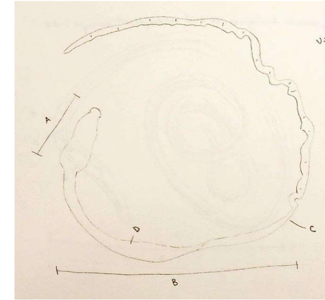
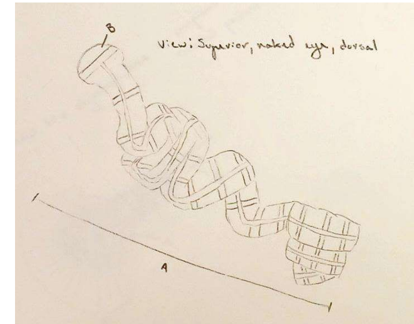




Hypothesis

Nemerteans which possess vibrant colors and abundance of tint (such as very neon greens, yellows, reds, blues, or purples) will be more likely to have high concentrations of TTX than those without, due to the fact that aposematism is often an indicator of toxicity within nature.

Null Hypothesis

Nemerteans which possess vibrant colors and abundance of tint (such as very neon greens, yellows, reds, blues, or purples) will not likely have higher concentrations of TTX than those without, due to the fact that aposematism is often an indicator of toxicity within nature, but not always.

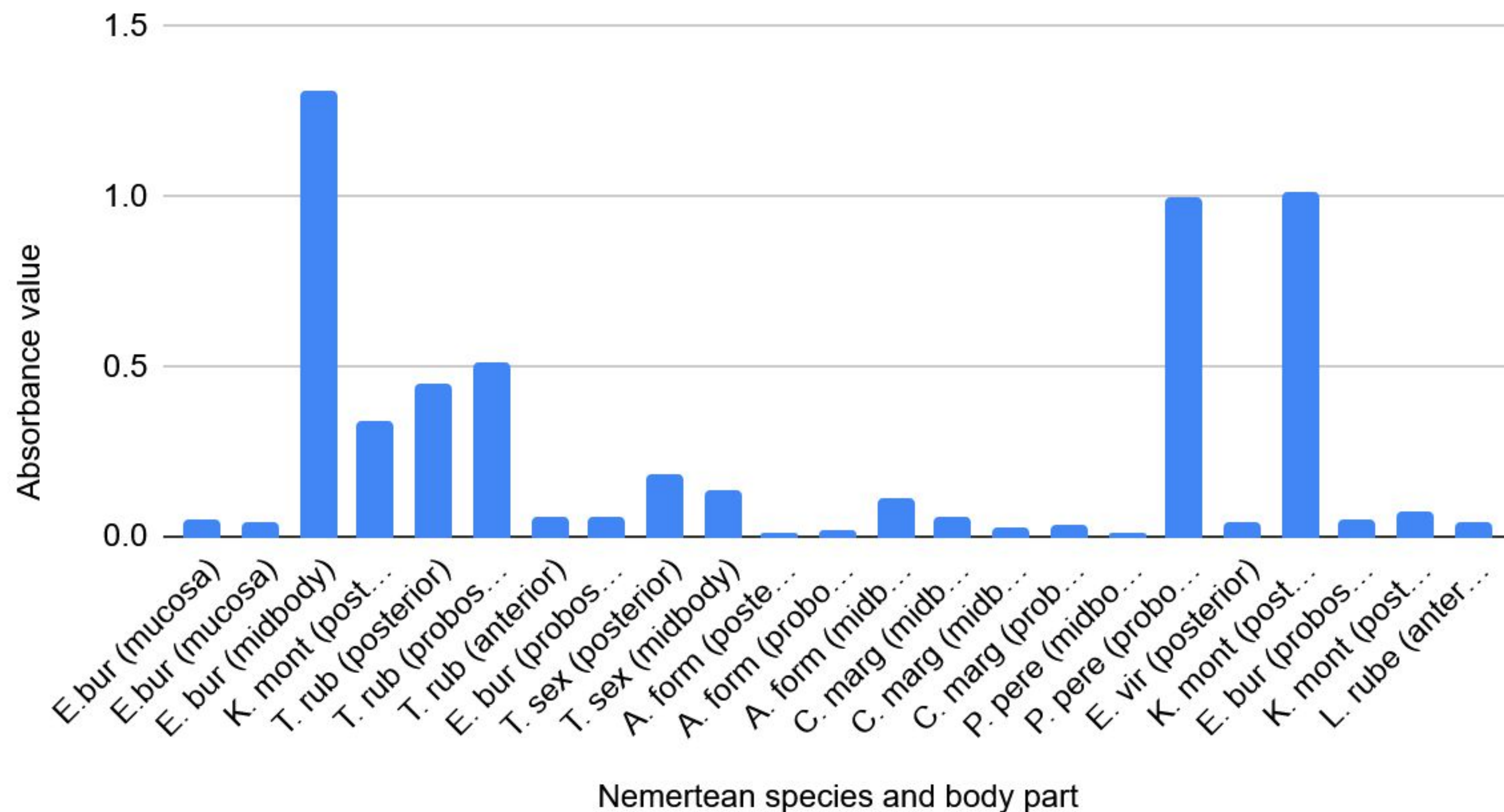
Making worm whiskey

- Slice ~0.5g or less of worm (small but potent)
- Place into sealable container with ethanol (and a little acetic acid)
- Blend
- Blend some more
- Actually more
- Yeah no like, more
- Okay so that was rough
- Centrifuge to hell (or I guess hades since we’re praying to Poseidon here)
- Wow it smells like orange
- Do not drink the fruit-scented worms of your labor.
- Make yourself some worms in dirt and cry in preparation for the next steps.

Actual procedure

1. Measure the pH level of the nemertean before sectioning.
2. Remove a small sample for homogenization with EtOH.
3. Sample homogenate to measure pH level.
4. Adjust pH of homogenization to 6.5 - 7 if the supernatant does not possess such a range naturally.
5. Add antibodies. Let samples incubate at 37 C.
6. Wash samples.
7. Add substrate to trigger coloration, and let incubate in the dark.
8. Measure absorbance value and optical density values.
9. Graph values using a four-parameter logistic function.

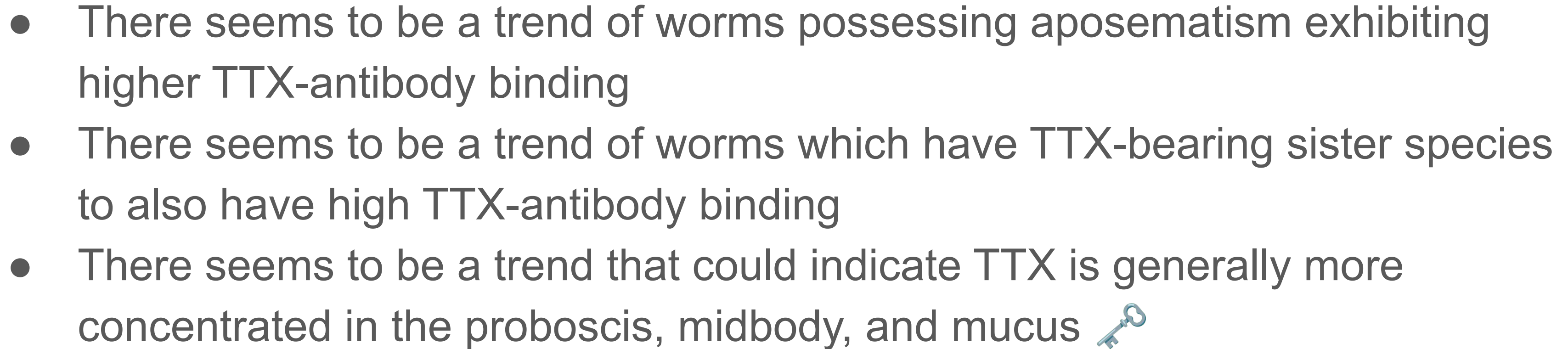
Results



Why you should never trust a graph

- Every tissue sample taken, save for sample 4 (K. mont posterior) was less than 0.5 grams.
- We could not reliably quantify below 0.5 grams as we did not possess an analytical scale.

What can our data tell us?



- There seems to be a trend of worms possessing aposematism exhibiting higher TTX-antibody binding
- There seems to be a trend of worms which have TTX-bearing sister species to also have high TTX-antibody binding
- There seems to be a trend that could indicate TTX is generally more concentrated in the proboscis, midbody, and mucus 🗝️

Continuing research

- Use an analytical scale to standardize measurements and get more accurate readings.
- Run more ELIZA assays with more body parts (include one of each for every species).
- Invest in / prepare use of multichannel pipette.
- Confirm species identities via DNA analysis.
- Find more efficient ways to create worm shots.

This research was made possible by:

- Friday Harbor Labs - gave me the opportunity to collect needed specimens.
- Billly Swalla - Donated an abundance of pipette tips and microcentrifuge tubes to the cause.
- Dr. Megan Schwartz - Made me aware of the existence of the program, introduced me to nemerteans, catalogued, sampled, collected, and actively participated in lab work for the experiment.
- Jim Truman - Helped double check dilution calculations.
- Family - Made it possible for me to travel to and stay on the island for study.
- Cassy, Siddharth, Sam, Caroline, Rachael, and Amanda - Made sure I stayed on track, visited in lab, and provided photos for this slide.

The gut and snot theory

Nemerteans may not innately make all of the TTX they possess

They may use microbiota they ingest or possess on their epidermis to produce TTX

Original research proposal
Procedure used for Assay
Nemertean literature review

Vermicedure

Worm around. Find out.