ANTHELMINTIC DRUG SENSITIVITY IN MALE AND FEMALE *PANAGRELLUS REDIVIVUS* NEMATODES

by

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A THESIS

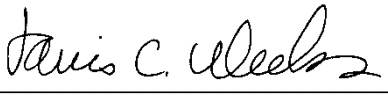
Presented to the Department of Biology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

June 2017

An Abstract of the Thesis of

Wanjiru Karanja-Senge for the degree of Bachelor of Arts
in the Department of Biology to be taken June 2017

Title: Anthelmintic Drug Sensitivity in Male and Female *Panagrellus redivivus* Nematodes

Approved: 

Dr. Janis C. Weeks

Caenorhabditis elegans is a well-studied model species of nematode worm that has been widely used for anthelmintic (anti-nematode) drug development as drug resistance has increased. The nematode *Panagrellus redivivus* presents itself as an alternative nematode to *C. elegans* that is easy to rear, making it useful for research in low-resource settings. In collaboration with the Gabon-Oregon Center, our laboratory aims to develop a *P. redivivus* screening system for natural products. Electropharyngeogram (EPG) recordings are similar to electrocardiograms of the human heart. In nematodes, EPG recordings show the electrical signals produced by rhythmic contractions of the pharynx. Pharyngeal contractions are used as a readout for electrical activity of neurons and muscles in nematodes. *C. elegans* is a hermaphroditic species, but *P. redivivus* is a gonochoristic species with males and females. My study tested the hypothesis that adult male and female *P. redivivus* have different sensitivity to anthelmintic drugs. I used EPG recordings to test this hypothesis, while applying

ivermectin, an anthelmintic drug. Dimethyl Sulfoxide (DMSO) was the ivermectin solvent, and was used as a control. EPG recordings from male and female *P. redivivus* were analyzed using IGOR Pro software. My results suggest that there is no sex difference in the sensitivity of *P. redivivus* to ivermectin, supporting the idea that mixed cultures containing both sexes can be used for drug screening experiments.

Acknowledgements

I would like to thank my Thesis committee: Dr. Janis Weeks, Dr. Mai-Lin Cheng, and Dr. Shawn Lockery. Additionally, I would like to thank Dr. William Roberts and Kristin Robinson for sharing their expertise and their patience with me. I want to thank to the University of Oregon Summer Program for Undergraduate Research (SPUR), Miriam Alexis Jordan and the Robert D. Clark Honors College.

I would also like to thank my family: Amina, who helped me move forward when I didn't believe in myself, and of course, I also want to thank my Mom and Dad for their support and encouragement since day one. Finally, I want to give thanks to Ruth Wanjiru Karanja; my Grandmother and my namesake, whose faith, love and hard work helped me and so many others.

Table of Contents

List of Figures

Introduction

Nematodes make up a diverse class of organisms in the animal kingdom. Nematodes as a phylum are over 500 million years old and have adapted to survive with a variety of life styles [1]. Nematoda has substantial genetic diversity, with 30% - 50% expressed sequences from 30 different species found to be unique to those species [2]. Most nematodes are not parasitic[1], but as a result of the diversity of this phylum, the parasitic species have broad impact on living things.

Parasitic worms affect humans, livestock, plants and pets[3]. The effects of parasites can be subtle compared to many other diseases. Parasite infections result in lower productivity from livestock and plants, as breeds of animals with higher productivity may not be able to survive in a region with high rates of parasitic disease [4]. Humans may also be forced to leave locations where parasitic diseases persist[5]. In many ways, the cost of parasitic disease can be seen as a loss of potential from animals, plant and human beings [4].

Parasitic worms are also referred to as helminths. Three soil-transmitted helminth infections are among the neglected tropical diseases caused by helminths[5]. These diseases are: Ascariasis, Trichuriasis, Lymphatic Filariasis, Schistosomiasis, Hookworm infection, and Onchocerciasis (river blindness) [5]. Onchocerciasis can cause blindness, Lymphatic Filariasis causes elephantiasis and hookworm infections and schistosomiasis can cause anemia [5]. Persistent soil-transmitted helminth infections in childhood are linked to a decrease in school performance, and stunted growth [6]. Parasitic worm infections and their combined impact on agriculture and human health

contribute to poverty. Treatment of these diseases can cause a substantial decrease in poverty [5].

Unfortunately, the reason that soil transmitted helminth infections fall under the category of neglected tropical diseases is because nations most impacted by these diseases do not have sufficient resources to invest in treatment or drug development to the extent needed [3]. Endemic helminth infections have been combated with help from organizations such as the World Health Organization and the World Bank in West Africa [7] or the Pacific Programme to Eliminate Lymphatic Filariasis (PacELF) in the Pacific[8]. Successful campaigns against helminth infections have been conducted in tandem with affected communities. Using local health professionals and staff for dosing and drug distribution of ivermectin, Onchocerciasis was almost completely eliminated in 10 West African countries [7]. Collaboration between local health services, communities and Japanese research and development of diethylcarbamazine hydrochloride (DEC) led to the eradication of Lymphatic Filariasis in Japan [8].

Most anthelmintic drugs used for human treatment were first developed for veterinary use [3,6]. More importantly, parasites that infect humans are not included in drug development for veterinary parasites [6], even though these drugs have been repurposed for human treatment. There are several approved classes of drugs for humans, which include the benzimidazoles, the nicotinic acetylcholine receptor agonists and the macrocyclic lactones. Ivermectin, a drug in the class of macrocyclic lactones, is less effective against hookworms and whipworms, and is used to treat filarial nematode infections [6]. In more developed parts of the world, the impact from parasitic worms is expressed as an increase in veterinary costs, especially in food production, where large

amounts of anthelmintic drugs are given to livestock [4]. As a result, anthelmintic resistance has been detected in livestock, and must be acknowledged as a future problem in anthelmintics targeted for human use [3–5].

The nematode *Caenorhabditis elegans* (*C. elegans*) has been the primary species used in the laboratory for the search for new anthelmintic compounds [3,9]. *C. elegans* is a well-studied species of nematode that is free-living, meaning that it is not parasitic. *C. elegans* has two sexes, hermaphrodites and males [1,3,10]. *C. elegans* is most easily cultured on the bacteria *Escherichia coli* (*E. coli)* in the laboratory [10]. However, while *C. elegans* is a useful nematode for the study of animal development and behavior, *C. elegans* alone cannot represent the diversity of parasitic nematodes for anthelmintic drug development. For example, the sheep parasite *Haemonchus contortus,* and the parasite responsible for Lymphatic Filarisis, *Brugia malayi,* exist as males and females [11]. Therefore, while *C. elegans* may be more popular as a model organism in the laboratory, it is not clear whether nematodes that exist as males and females show different sex-dependent responses to anthelmintic compounds.

The nematode *Panagrellus redivivus* (*P. redivivus*) can be traced back to Linneaus' description of an organism in the genus *Chaos* and identified as *Chaos redivivum* [12]. *P. redivivus* is also known as the beer mat nematode. The various names of *P. redivivus* reflect the locations of discovery of the worm. *P. redivivus* has been found in beer mats, insect frass, tree wound slime and wheat paste, but is always associated with yeast [12]. The genus name *Panagrellus* was assigned by Goodey in 1945, and in 1984 *P. silusiae* was designated as a synonym for *P. redivivus* [12].

P. redivivus has been used as a comparative species relative to *C. elegans* in various studies [1,2,13]. *P. redivivus* was the first free-living nematode to be sequenced outside of the *Caenorhabditis* genus [1]. In the lab, *P. redivivus* can be cultured on a water-based mixture of oatmeal and yeast, or flour and yeast [14,15]. Alternatively, *P. redivivus* can be maintained on *E. coli* and agar plates [1,2]. On a wheat paste mixture *P. redivivus* worms can grow to a population of 500,000 within 20 days and survive without replenishing the mixture for 40 days [14]. *P. redivivus* thrive at lower pH values, the population of worms declining with a decrease in acidity [14]. Unlike *C. elegans*, *P. redivivus* is a gonochoristic species, with males and females[1,15,16]. Larvae hatch within the adult female and exit through the vulva [1,15].

P. redivivus and *C. elegans* share many features. Compared to *C. elegans*, *P. redivivus* exhibits different locomotor behavior in response to food, but both species have similar staining patterns of dopaminergic and serotoninergic neurons[2]. Differences between nematode families can be evaluated most basically by placing individuals on an agar plate and observing their behavior [2,6,17]. However, for the purposes of anthelmintic drug development and the search for new anthelmintic compounds, it is important to note that many of the drugs used today affect ion channels and neurotransmitter receptors[18]. Observation of nematode motility on agar plates does not give data sensitive enough to distinguish the mechanism of action on a particular species [19]. A drug that affects nematode neurotransmitter receptors for example, ivermectin (IVM), can target multiple receptor subunits encoded by multiple genes in a species [20]. In *C. elegans* alone there are at least 5 genes that encode different types of chloride channel subunits, and mutations in these subunits have conferred IVM

resistance to *C. elegans* [20]. One advantage of drugs that target nematode neurotransmitter receptors or ion channels is the similarity of genes that encode for receptors between different nematode species [18].

To identify a compound that affects ion channels or neurotransmitter receptors, a more sensitive measure of the effect of anthelmintic drugs is required. Observing the behavior of the nematode pharynx is more sensitive in assessing neurotransmitter receptor activity than a motility assay. The pharynx is a muscular throat structure in the nematode head that is used to draw food into the body of the worm [13,19,21]. In *C. elegans* and similar nematodes, there are two types of pharyngeal behaviors: " pumping" and "peristalsis" [21,22]. Pumps refer to pharyngeal contractions that occur when the worm sucks in bacteria, and subsequently moves the bacteria to be chewed up in its grinder [21].The neuromuscular signal of the pharyngeal muscle in nematodes can be monitored via the electropharyngeogram (EPG) method [13,19,21,22]. In a manner similar to the electrocardiogram, an EPG recording measures the signal generated by the action of pharyngeal muscles and neurons [13,19,21]. Pharyngeal peristalsis does not generate EPG currents [21]. Therefore, the EPG method captures pumping modulated by neurotransmitters in all nematodes that produce this behavior. In EPG assays, the neuromodulator serotonin is used to stimulate pharyngeal pumping [13,19,21,22].The original EPG recording method [22], is not suited for large batches of recordings, because an experimenter must load individual worms into a pipette to begin recording. The microfluidic EPG chip method improves upon the conventional EPG recording method by automating the loading and recording of eight worms simultaneously [19].

As a nematode species with males and females, and with its ease in rearing, *P. redivivus* has the potential to be used as an alternative to parasitic nematode species for screening compounds with anthelmintic drug activity. To my knowledge, potential sex differences in the response to anthelmintic drugs have not been explored in *P. redivivus*. I decided to use the microfluidic EPG method to investigate potential sex-related differences in the response of *P. redivivus* to the anthelmintic drug IVM.

Materials and Methods

Panagrellus redivivus, strain Pr MT8872 were obtained from the *Caenorhabditis* Genetics Center (CGC; Minneapolis, MN). *P. redivivus* were maintained on an oatmeal and yeast mixture at room temperature. Twenty ml of water was added to approximately 9 g of Gerber Oatmeal Cereal single grain in a PI-750, 29.57 ml ($7^{1/2}$ fl oz.) tumbler from Plastics Inc., Coon Rapids, Minnesota. Two portions of the oatmeal and yeast mixture were prepared at the same time. Approximately 0.7 g of yeast was added to each water and oatmeal mix, and *P. redivivus* were added. The food mixture was replaced weekly.

A portion of oatmeal-yeast mix was placed on an agar plate and worms were picked into a glass well containing M9 buffer before each experiment[23]. Worms were first washed in the M9 solution to remove oatmeal particles then placed into glass wells containing a mixture of serotonin (5-hydroxytryptamine, 5HT) and M9 solution (see below). Males and females were identified via visual inspection according to Sternberg et. al. (1981) and Hechler (1970).

Microfluidics

Chips were fabricated using the replica molding technique, and standard lithographic methods as adapted by Lockery et. al. (2012) from Xia and Whitesides (1998). In brief, a silicon wafer master was created with 1 layer of 70 µm SU-8 2050 photoresist through a transparency mask of the 8-channel chip features. The wafer was developed via submersion in glycol monomethyl ether acetate (PGMEA). Chlorotrimethylsilane was applied after the development step to help release polydimethylsiloxane (PDMS) from the master. Chip masters were fabricated by K.L. Robinson.

A layer of PDMS (Dow Corning Sylgard 184, Corning, NY) was poured into a master with the 8-channel chip design. To cure, the PDMS and master were baked at 65˚C for 3 h. After curing, the PDMS was cut away from the master. Biopsy punches were used to complete the features of the chip: 1.5 mm for the ports and inlets, and 5 mm for the reservoirs. After work on the features of the chip, the PDMS layer was bonded to a glass slide after 30 s exposure to oxidizing air plasma. The PDMS layer was placed on a glass slide, feature side down and baked at 65˚C for 1 h to complete the bonding process. After bonding, the capacity of each fluid reservoir was increased by inserting a 1.5 cm length of 5 mm glass tubing.

Solution preparation

The serotonin (5-hydroxytryptamine) solution was prepared with 0.1 M Serotonin creatinine sulfate monohydrate powder (Sigma H7752-5G, St Louis, MO) and diluted to a stock solution of 40 mM in M9 buffer. Serotonin solution is abbreviated as 5HT. M9 buffer was composed of 3 g KH_2PO_4, 6 g $NaHPO_4$, 5g NaCl, 1 ml of 1M

$MgSO_4$ in 1 liter of water according to Sulston et. al (1988). Serotonin dissolved in M9 buffer is subsequently referred to as 5HT-M9. 40 mM aliquots of 1ml 5HT-M9 were stored at 20˚C.

Before an experiment, 40 mM of 1 ml 5HT-M9 were mixed with 3 ml of M9 to give 4 ml of a 10 mM 5HT-M9 solution that was filtered with a 25 mm Acrodisc Syringe Filter (0.2 µm HT Tuffryn Membrane Pall 4192; Ann Arbor, MI). Adult *P. redivivus* were incubated for 45 minutes in the 10mM 5HT-M9 solution. Previous experiments determined that a 45 minute pre-exposure to 10 mM 5HT-M9 was required to ensure that *P. redivivus* worms were pumping at maximum frequency (K.L. Robinson, unpublished data).

Two aliquots of 1 ml 5HT-M9 were mixed then filtered with 6 ml of M9 to create the 10 mM 5HT-M9 control solution. One aliquot of 1 ml 5HT-M9 was mixed with 2.6 ml of M9 and 0.4 ml of 0.05% fast green dye to create a 10 mM 5HT experimental dye solution. The IVM (Sigma I8898, St. Louis, MO) solution was stored as a 5 mM stock solution in 1 ml aliquots at -20˚C. IVM was dissolved in 0.06% Dimethyl Sulfoxide (DMSO). After the experimental solution was filtered, 1.8 µl of DMSO or IVM was added to give a 3 ml drug solution of 0.06% DMSO or 3 µM IVM.

Worm loading

After 45 min pre-exposure to 10 mM 5HT-M9, adult *P. redivivus* were loaded into an 8 channel EPG chip. Adult male and female *P. redivivus* were determined by visual observation of gonadal structures as described by Hechler (1970). A 3 cc syringe of freshly prepared 10 mM 5HT-M9 was connected to the perfusion inlet of the chip and the chip was filled. Pre-exposed adult *P. redivivus* were picked with platinum wire

into the inlet port and the worms allowed to settle to the bottom of the inlet port. After the worms settled, the syringe filled with control solution was reattached and used to gently push the worms into the channels of the chip. Waste reservoirs were inserted into the worm and drug vents of the chip.

EPG recording

After loading the chip, the syringe filled with control solution was left attached, and the chip placed on the rig. Without removing the syringe, 0.5 inch passivated 1 gauge steel tube electrodes (0.058 inch OD, 0.0475 inch ID, New England Small Tube, Litchfield, NH) were inserted into the electrode ports. EPGs were recorded via AC differential amplifiers (A-M Systems model 1700, Carlsborg, WA). Solutions were perfused through the chip at 6 µl / min. The control solution was perfused through the chip for 30 min then a switch was made to the experimental solution of 3 µM IVM or 0.06 % DMSO. The experimental solution was perfused through the chip for 1 hour.

Recordings were made at a voltage gain of 1000x with a bandwidth of 500 to 1 Hz, and a notch filter at 60 Hz. Data were recorded with an analog to digital converter [Micro1401-3, Cambridge Electronic Design (CED), Cambridge, UK]. Readouts were stored using Spike2 software (version 7.06a, CED). Data were down sampled to 500 Hz. A keystroke channel was used to keep track of when the dyed experimental solution reached the worms. Orientation and sex of each worm was recorded after the experiment under an upright scope.

Analysis

After experiments, recordings were viewed using Spike2. Noisy or empty channels were excluded before saving the data as text files. Text files were imported into the IGOR Pro software environment (Wavemetrics, Lake Oswego, OR) for analysis using a custom algorithm [24] that identified each pharyngeal pump in the data. To compare the time required for the drug solution to take effect, the algorithm computed a $t_{1/2}$ value for each worm. The algorithm defined $t_{1/2}$ as the time during the 60 minute observation period following addition of the drug at which half of the pumps during the 60 minutes had occurred. A frequency criterion, such as the time required for the pump frequency to fall to 50% of its baseline rate, was not used. Because pumping was often interrupted by brief pauses, causing the worm's pump frequency to be highly variable in time, a simple frequency criterion was too noisy. The $t_{1/2}$ was less impacted by interruptions in pump activity. A $t_{1/2}$ value was computed for each worm and the Mann-Whitney U-test [25] was used to compare $t_{1/2}$ values between groups of worms.

Results

Figure 1 shows the EPG chip with 8 electrodes attached; the common reference electrode was not inserted into the inlet port (top) in this view. Red dye was injected into the inlet port to highlight the branching channels within the chip. The electrodes were inserted into their respective ports at the bottom. Waste reservoirs were located beyond the electrodes at the bottom of the chip.

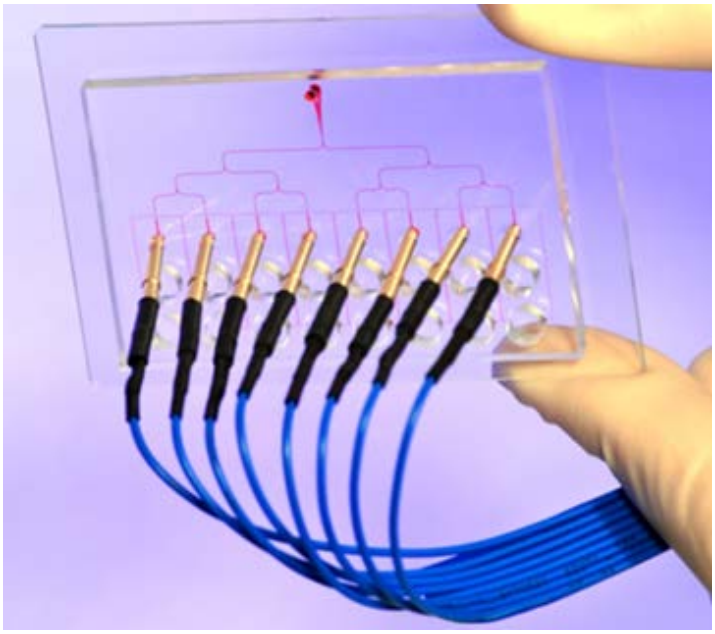


Figure 1: An EPG chip

This chip has been filled with red dye to highlight the micro-channels.

Photo credit: Derek Robinson

Figure 2 shows a schematic of the chip and the location of a worm in the worm trap. The arrows in the inset show the flow of solution through the chip. The transparent quality of the EPG chip allowed for clear observation of a worm in a channel. The best recordings were generated from worms firmly ensconced within the trap. Solutions could also be observed, and I used dye in my experimental solutions to create a visual marker of exactly when worms were exposed to 3 µM IVM or 0.06% DMSO.

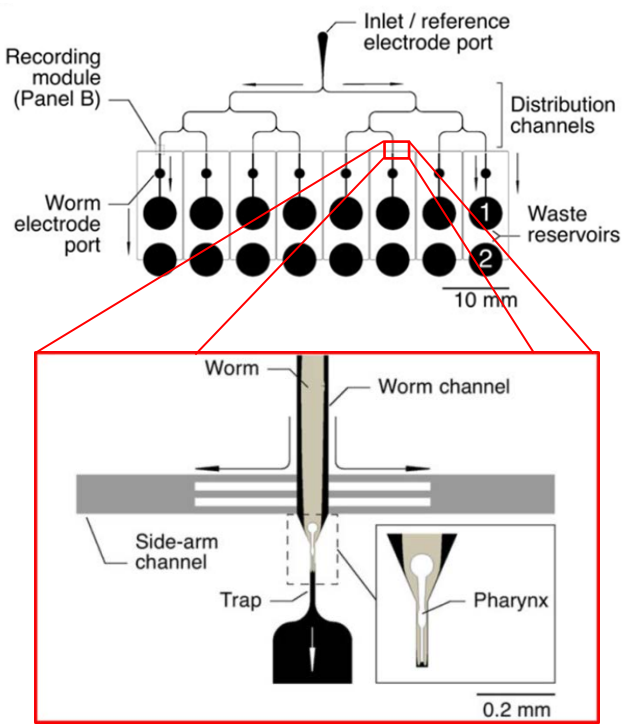


Figure 2. EPG chip design.

Top: PDMS fabrication design. Bottom: expanded view shows the recording module portion of one of the eight channels. Modified from Lockery et al. (2012).

Figure 3 shows representative EPG recordings from *P. redivivus* individuals under different experimental conditions. Each trace is an excerpt of the recording from an individual worm in four different experiments. The first two recordings (Fig. 3A, 3B) show a steady pump frequency in females and males that were switched from control solution to 0.06% DMSO. The recordings have been compressed in time so the EPG waveforms appear as a thick line. Comparison of pump frequency between males and females in 5HT-M9 showed no significant difference during the baseline period. Males had a mean pump frequency of 1.80 Hz (n=46, S.E.M ± 0.168 Hz), females had a mean

pump frequency of 2.01 Hz (n=46, S.E.M ± 0.158 Hz; U statistic 1002, p = 0.66). In Figures 3C and 3D, pumping in female and male worms became less frequent over time after switching the perfusion to 3 µM IVM.

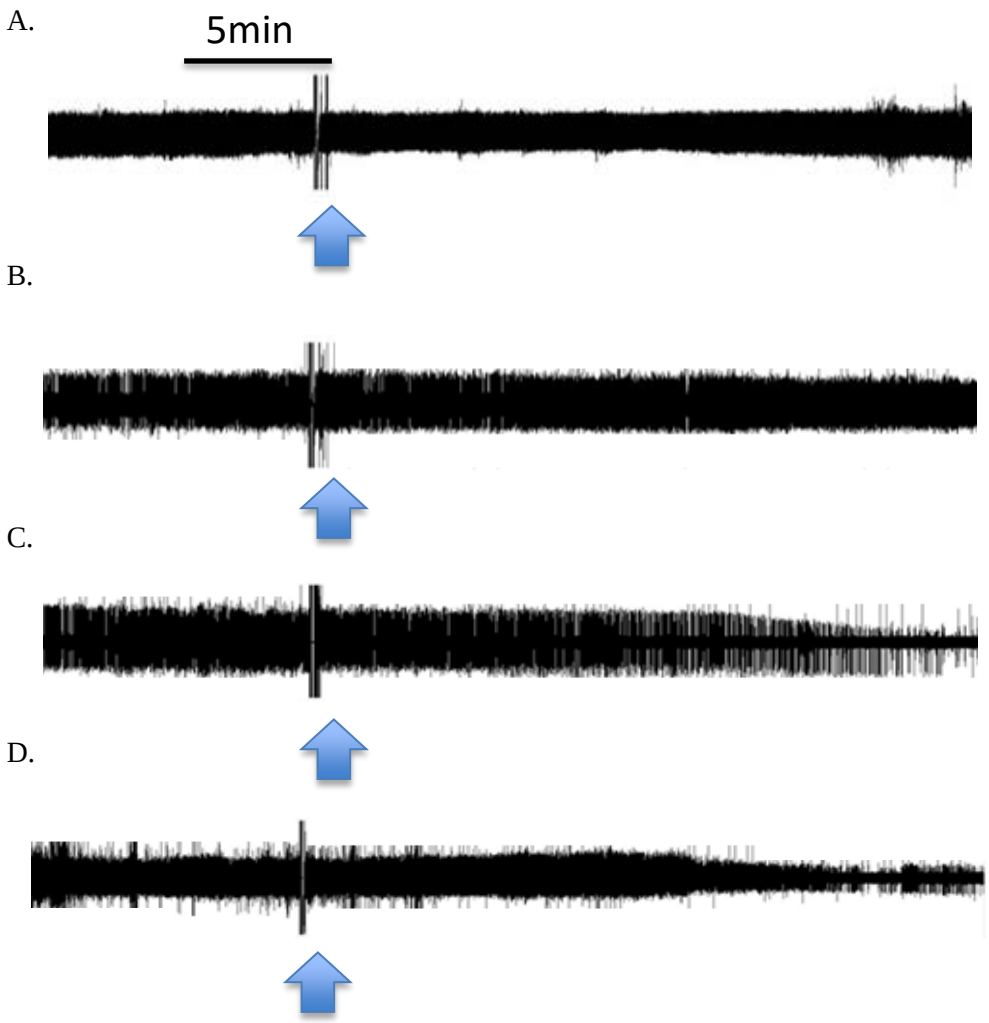


Figure 3. Sample EPG Traces

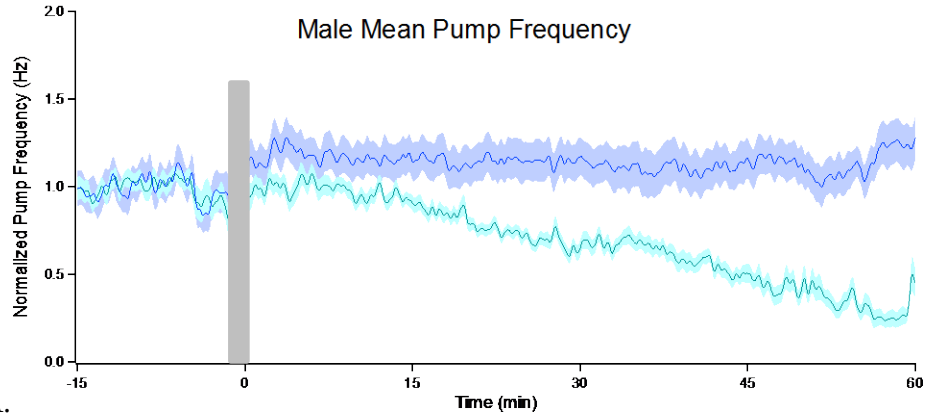
Traces show portions of the baseline (control) and experimental periods of EPG recordings from 4 different worms. The arrows mark the time of the switch of solution from control to experimental solution.

A. Female exposed to 0.06% DMSO. B. Male exposed to 0.06% DMSO. C. Female exposed to 3 µM IVM. D. Male exposed to 3 µM IVM.

Figure 4 shows data from males exposed to IVM or DMSO. Early in my work with *P. redivivus* I noticed that adult males exposed to 5HT-M9 exhibited a tail curling behavior (see Discussion). Males curled the posterior third of their bodies into a tight spiral, and maintained this shape even when loaded into the EPG chip. Females did not exhibit this curling behavior. In Fig. 4A, time is plotted on the x-axis and pump frequency is plotted on the y-axis. These data were normalized to the pump frequency of the last 2 minutes of the control period. The negative time period in part A corresponds to the 5HT-M9 solution. Time 0 marks the time when the solution switch occurred. Males exposed to 0.06% showed a steady pump frequency throughout. In contrast, males exposed to 3 µM IVM show a steady decrease in pump frequency.

In Fig. 4B, a pump frequency integration curve summarizes the data from Fig. 4A. The y-axis shows the mean fraction of total pumps taken by each worm over the course of the experimental period. The x-axis shows time starting at 0 min, the beginning of the experimental period. Males exposed to 0.06% DMSO achieved 50% of their total pumps at ~29 minutes (mean = 29.43 minutes, S.E.M ± 0.8215). Males exposed to 3 µM achieved 50% of the total pumps at 21 minutes (mean = 21.69 minutes, S.E.M ±0.836). U statistic 48, $p = 3.93 \times 10^{-8}$. Thus, exposure to 3 µM IVM caused a statistically significant reduction in pumping frequency compared to DMSO controls in adult male *P. redivivus.*

A.



B.

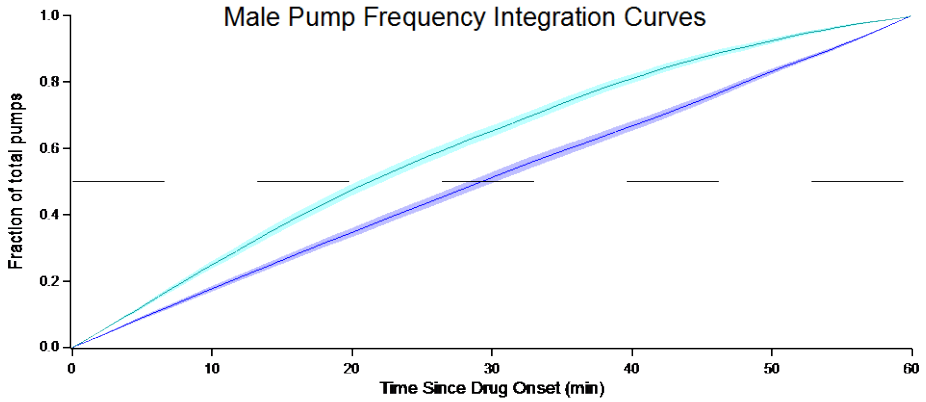
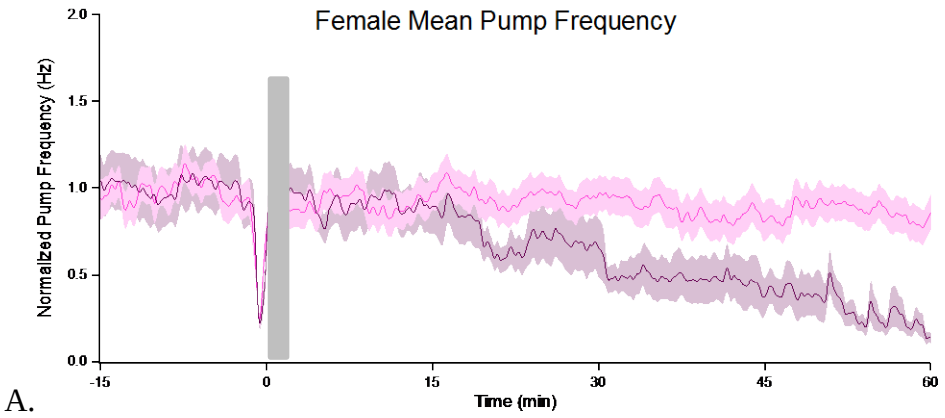


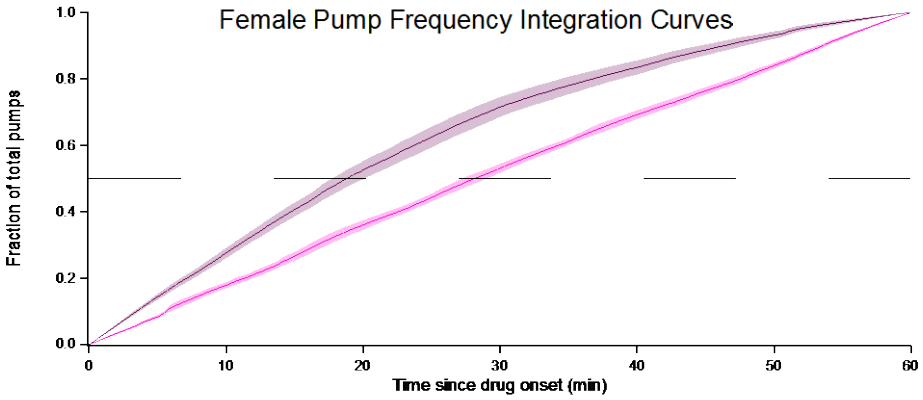
Figure 4. Ivermectin vs. DMSO: Mean pump frequency and integration curve data from males

A. The negative time period (-15 to 0 min) corresponded to the baseline recording period, while the worms are exposed to 5HT-M9. The first 15 min have been cut off. Blue line shows males exposed to 0.06% DMSO (n=26). Teal line shows males exposed to 3 µM IVM (n=20). The grey bar masks the electrical artifact produced by switching from the control to the experimental solution. Shading represents ± 1 S.E.M.

B. Male pump rate integration curves. Color scheme as in A. $T_{1/2}$ is apparent from the dotted line at 0.5 on the y-axis. Comparison of $t_{1/2}$ by the Mann-Whitney U-test showed a significant difference between the IVM and DMSO groups, $P< 0.0001$ ($3.93x10^{-8}$). The linear DMSO males curve corresponds to a steady pump frequency during the experimental period. Shading represents ± 1 S.E.M.



A.



B.

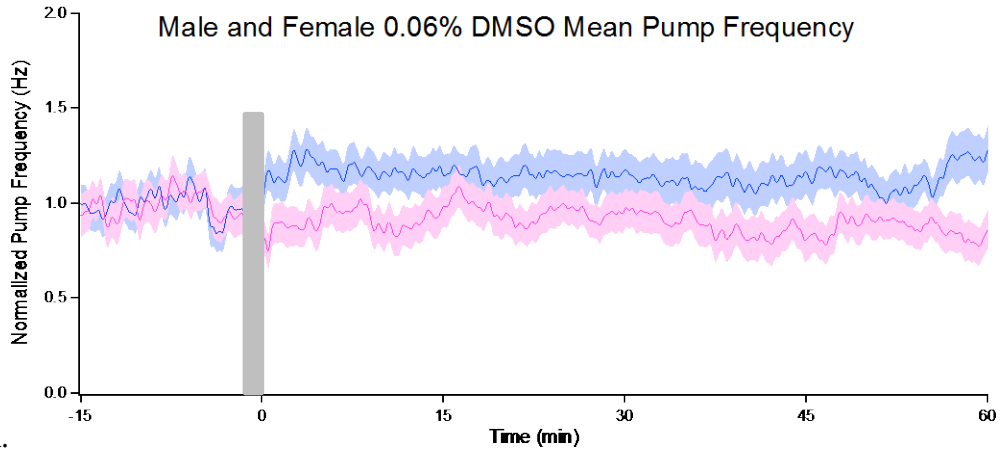
Figure 5. Ivermectin vs DMSO: Mean pump frequency and integration curve data from females

A. The negative time period (-15 to 0 min) corresponded to the baseline recording period, while the worms were exposed to 5HT- M9. The first 15 min have been cut off. Pink line shows females exposed to 0.06% DMSO (n=26). Purple shows females exposed to 3 µM IVM (n = 20). Shading represents ± one S.E.M. The grey bar masks the electrical artifact produced by switching from the control to the experimental solution.

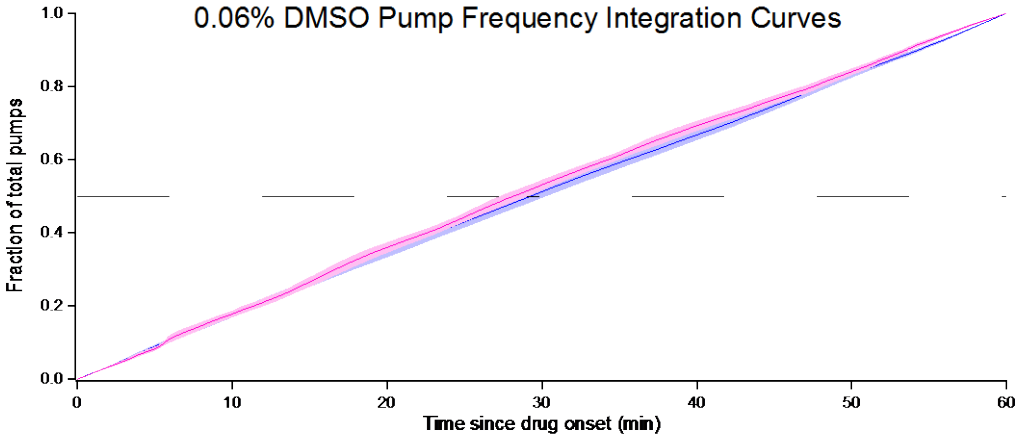
B. Female pump rate integration curves. Color scheme as in A. T1/2 is apparent from the dotted line at 0.5 on the y-axis. Comparison of t 1/2 by the Mann-Whitney U-test showed a significant difference between the IVM and DMSO groups, $P< 0.0001$ ($6.41x10^{-7}$). The linear DMSO females curve corresponds to a steady pump rate during the experimental period. Shading represents ± one S.E.M.

In Figure 5, females exposed to 3 µM IVM are compared to females exposed to 0.06% DMSO. As in Figure 4, the y-axis displays normalized pump frequency and the x-axis includes the last 15 minutes of the control period. Time 0 marks the time of the solution switch. Females exposed to 0.06% DMSO showed a steady pump frequency whereas females exposed to 3 µM show a decrease in pump frequency during the experimental period.

In Figure 5B, a pump frequency integration curve summarizes the data from Figure 5A. The y-axis shows the average fraction of total pumps taken by each worm over the course of the experimental period. The x-axis shows time starting at 0 min, at the beginning of the experimental period. Females exposed to 0.06% DMSO achieved 50% of their total pumps at 29 minutes (mean = 28.17 minutes, S.E.M. ± 0.842). Females exposed to 3 µM IVM achieved 50% of their total pumps at 20 minutes (mean = 20.17 minutes, S.E.M ±0.842). U statistic 467, $p = 6.41 \times 10^{-7}$. Thus, exposure to 3 µM IVM caused a statistically significant reduction in pumping compared to DMSO controls in adult female *P. redivivus*.



A.



B.

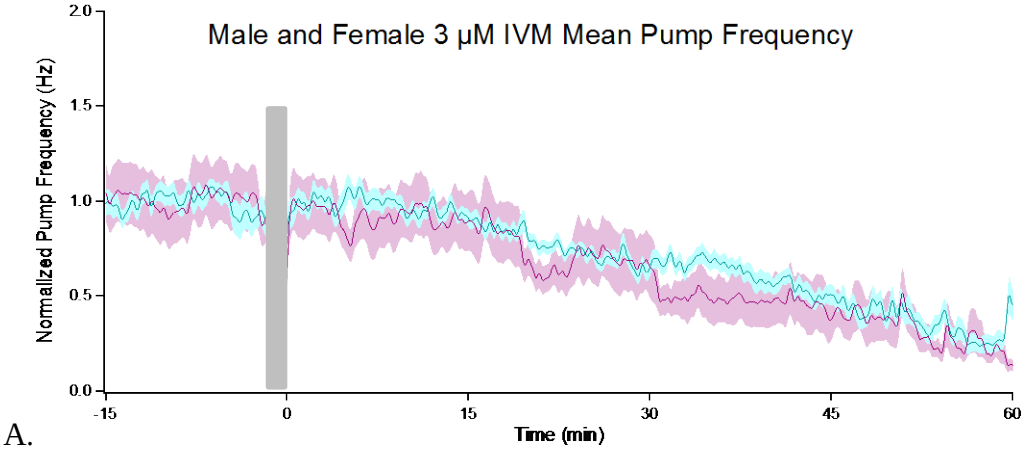
Figure 6. DMSO mean pump frequency and integration curve data, males and females.

A. The negative time period (-15 to 0 minutes) corresponded to the baseline recording period, while the worms are exposed to 5HT-M9. The first 15 minutes have been cut off. Blue line shows males exposed to 0.06% DMSO (n=26). Pink line shows females exposed to 0.06% DMSO (n=26). Shading represents ± one S.E.M. The grey bar masks the electrical artifact produced by switching from the control to the experimental solution.

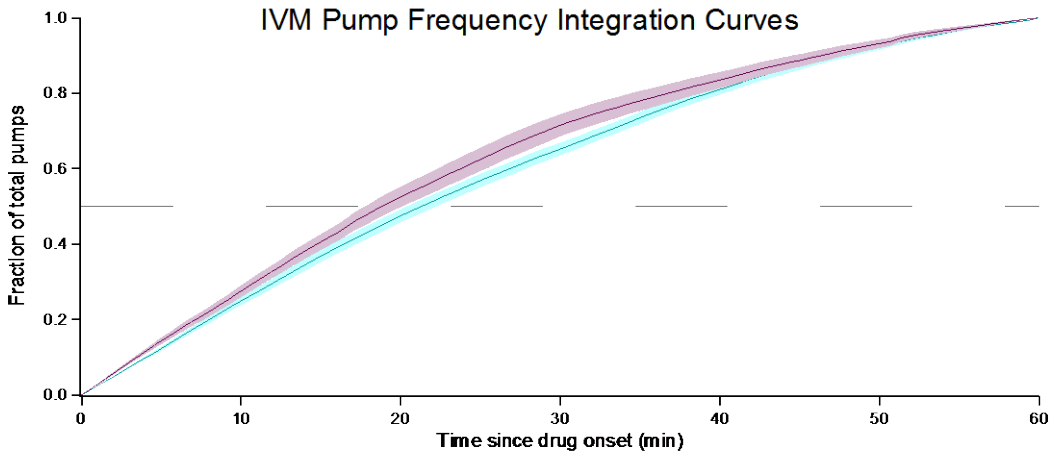
B. DMSO Male and female pump rate integration curves. $T_{1/2}$ is apparent from the dotted line at 0.5 on the y-axis. Comparison of $t_{1/2}$ by the Mann-Whitney U-test showed no significant difference between males and females, P= 0.365. The linear DMSO males curve corresponds to a steady pump rate during the experimental period. Shading represents ± one S.E.M.

Figure 6 compares males exposed to 0.06% DMSO to females exposed to 0.06% DMSO. As in Figures 4A and 5A, Figure 6A shows that males and female exposed to 0.06% DMSO displayed a steady pump frequency. Males had a normalized pump frequency slightly above baseline compared to females.

In Figure 6B, the pump frequency integration curve summarizes the data from Fig. 6A. Males and female exposed to 0.06% DMSO had a steady pump frequency. Males achieved 50% of total pumps at 29 minutes (mean =29.43 minutes, S.E.M ± 0.821). Females achieved 50% of total pumps at 28 minutes (mean = 28.17minutes, S.E.M.± 0.842). These values did not differ significantly (U statistic = 388, p = 0.365).



A.



B.

Figure 7. Ivermectin: mean pump frequency and integration curve data males, females.

A. The negative time period (-15 to 0 minutes) corresponded to the baseline recording period, while the worms were exposed to 5HT-M9. The first 15 minutes have been cut off. Teal line shows males exposed to 3 µM IVM (n=20). Purple line shows females exposed to 3 µM IVM (n = 20). Shading represents ± one S.E.M. The grey bar masks the electrical artifact produced by switching from the control to the experimental solution.

B. Ivermectin male and female pump rate integration curves. $T_{1/2}$ is apparent from the dotted line at 0.5 on the y-axis. Comparison of $t_{1/2}$ by the Mann-Whitney U-test showed, no significant difference between males and females, P = 0.409. The linear DMSO males curve corresponds to a steady pump rate during the experimental period. Shading represents ± one S.E.M.

In Figure 7, females exposed to 3 µM IVM were compared to males exposed to 3 µM. In Figure 7A, time is plotted on the x-axis, and pump frequency is plotted on the y-axis. These data have been normalized. The negative time period in panel A corresponds to the last 15 minutes of the 30 minute control period when the worms were exposed to 5HT-M9. Time 0 marks the time when the solution switch occurred. Both sexes displayed a decrease in pump frequency during the 60 min experimental period.

In Figure 7B, a pump frequency integration curve restates the data from Figure 7A. The y-axis shows fraction of total pumps. The x-axis shows time, starting at 0 minutes, the beginning of the experimental period. Females achieved 50% of total pumps at 20 minutes (mean = 20.10 minutes, S.E.M. ± 1.22). Males achieved 50% of total pumps at 21 minutes (mean = 21.69, S.E.M. ± 0.836). U statistic = 169, p-value 0.409. Both males and females reached 50% of total pumps after 20 minutes, with both sexes taking a decreasing fraction of their total pumps for the next 40 minutes.

Discussion

In this thesis I examined sex-related differences in pharyngeal pump frequencies in response to IVM using EPG chips. My hypothesis was that male and female *P. redivivus* have different sensitivity to anthelmintic drugs. To identify potential differences in males and females, I compared the electrical signals of male and female *P. redivivus* generated by the pumping of the pharynx during exposure to 3 μM IVM.

With each EPG experiment, I included a pre-exposure period where males and females were exposed to a 5HT-M9 solution for 45 mins before being placed in a chip. After the 45 minute pre-exposure period, the worms were loaded into the chip, and the first 30 minutes on the rig were a control period where the worms were exposed to 5HT-M9. The 45 min pre-exposure was necessary to elevate the baseline pump frequency, and maintain pharyngeal pumping at a steady state. In the results section of this thesis, I cut off the first 15 minutes of this control period. This control period was necessary to confirm that the worms were pumping at a steady frequency inside the chip. After the 30 minute control period, worms were exposed to DMSO or IVM for 1 hour.

During the pre-exposure period, I observed that males exclusively displayed a tail-curling/paralysis behavior. The male tail-curling appeared to be irreversible; with males displaying a tight curl even after the experiment was finished. In. *C. elegans*, tail curling is the physical mechanism by which *C. elegans* males search for the hermaphrodite vulva while wrapped around their prospective mate [17]. Male-specific tail curling has been observed in *C. elegans*, at 5HT concentrations of 10-20 mM, males are unable to uncurl their tails [17].

It has been observed that the *P. redivivus* pharynx produces a standard electrical signal that corresponds to the excitation, resting and repolarization phases during exposure to serotonin[13]. Serotonin immunoreactivity staining experiments on *C. elegans* and *P. redivivus* show strong staining of serotonergic neurosecretory motor neurons located in the pharynx [2]. The advantage of using the EPG method in my experiments is that it gives a readout of what is occurring as a worm is exposed to a drug or compound that affects the nervous or muscular systems. Ivermectin activates glutamate-gated chloride channels in nematodes, resulting in virtually irreversible paralysis of the pharynx and body-wall muscle [20]. My experiments confirm that *P. redivivus* displays a similar decrease in pump frequency over time as observed in *C. elegans* exposed to 3 µM IVM [19]. The effect of 3 µM IVM on male and female *P. redivivus* was not significantly different.

In conclusion, there is no sex difference in pharyngeal pumping between males and females when exposed to 10 mM 5HT or 3 µM IVM. The similarity of *P. redivivus* pharyngeal response to the *C. elegans* pharynx is a positive mark in favor of *P. redivivus* as an alternative to *C. elegans* as an experimental model nematode in the search for new anthelmintics. I observed a tail-curling response to 5HT that suggests new avenues for research with *P. redivivus*. Male adult *P. redivivus* displayed effectively permanent tail-curling when exposed to 10mM 5HT a response not seen in females. Therefore, *P.* redivivus does display sexually distinct behavior in response to the neuromodulator 5HT, but not to the anthelmintic IVM. Similar sex-linked mating behavior has been observed in *C. elegans* [26].

In *C. elegans*, neuronal circuits involved in mating and locomotion differ according to the sex of the individual [26]. My observation of male-specific tail curling suggests that *P. redivivus* may also have sexually distinct neuronal circuits. In the search for new anthelmintic drugs, sex associated genes, as well as neurotransmitter receptors may be targets for new anthelmintics. Li (2016) preformed the first genome-wide analysis of sex associated gene expression in *Brugia malayi*, one of the nematodes responsible for Lymphatic Filariasis. In *B. malayi*, metabolic, energy and cell-motility processes were more expressed in males, while cell-cycle, transcription and translation related pathways were more expressed in females [26]. Li (2016) also found sex associated homologues in *C. elegans*, genes that are differentially expressed according to sex.

Ivermectin's discovery was something of a fluke. In the 1970s, IVM's anthelmintic activity may have remained unknown without Dr. Satoshi Ōmura, who focused on investigating naturally occurring compounds and their potential for drug development [27,28]. Today, the Weeks lab, in collaboration with the Gabon-Oregon center, aims to make the discovery of new, naturally-occurring anthelmintic compounds less dependent on chance [29]. With an easy-to-raise nematode like *P. redivivus*, and the ability to use males and females, researchers studying the anthelmintic properties of plant extracts may have a powerful tool for identifying new naturally occurring anthelmintic compounds. With the EPG method, the mode-of-action of new anthelmintic compounds can be explored in different species of nematodes. More research needs to be done with *P. redivivus*, however, as IVM represents only one class of anthelmintic compound. In addition, *P. redivivus*, as a gonochoristic species, may

also be a useful tool in the search for compounds that affect the two sexes differently in nematodes. If I were to continue to investigate the effects of anthelmintic drugs on *P. redivivus*, I would investigate the effect of the major classes of anthelmintic drugs on *P. redivivus* males and females using the EPG chip method.

Glossary

Caenorhabditis elegans
-A non-parasitic nematode. The first multicellular organism to undergo complete genome sequencing.

Anthelmintic
-A type of anti-parasite drug that acts on nematodes and other parasitic worms.

Panagrellus redivivus
-A non-parasitic nematode that can be grown on a flour or oatmeal paste.

Electropharyngeograms (EPGs)
-A technique that records the signal from the action of the nematode pharynx (throat muscle).

Gonochoristic
-Also known as unisexualism; the state of only having one of at least two distinct sexes in an organism.

Ivermectin (IVM)
-An anthelmintic drug in the class of macrocyclic lactones. Causes hyperpolarization of pharynx and body wall muscle in nematodes, leading to paralysis.

DMSO
-Dimethyl Sulfoxide. A colorless liquid solvent.

Dopaminergic
-Related to dopamine. Dopamine is a neurotransmitter.

Serotonergic
-Related to serotonin. Serotonin is a neuromodulator.

Serotonin
-A neuromodulator and a chemical messenger used to transmit signals in the nervous system. Also called 5-hydroxytryptamine (5HT)

Experimental Protocol for EPGs: *P. redivivus*

Step 1: Pre-expose *P. redivivus* to 5HT

-Remove a 40 mM aliquot of 5HT-M9 from the -20˚C freezer.

-Mix with 3 ml of 1xM9 to make a 10 mM solution that is a total of 4 ml.

-Filter with a 10 cc syringe and pipet the amount into an empty glass well.

-Pick adult *P. redivivus* into the well; pre-expose adult *P. redivivus* to the 10 mM 5HT solution for at least 45 min before running the experiment.

Step 2: Mix up reagents

40 mM aliquots of 5HT are in the -20˚C freezer

-Remove 3 aliquots of 5HT from the -20˚C freezer.

- For the 5HT Control solution, add 6 ml of M9 to 2 ml of 40 mM 5HT in order to achieve a 10 mM solution of 5HT-M9.

-For the Experimental (drug) solution, take 1 ml of 40 mM 5HT and add 2.6 ml M9 and 400 µl (0.4 ml) 0.05% fast green dye to achieve a 10 mM solution (if you are using a large volume of drug subtract from M9 before mixing solution).

- Filter each solution into small petri dishes. The filter will hold some of the solution. This is not a problem for the Control solution as it is already at the correct concentration.

- For the Experimental (drug) solution only take 3 mls from the filtered volume in the petri dish.

-Calculate the amount of drug needed remove this amount from the filtered solution, then add drug. Subtracting the added amount will ensure molarity.

-Fill two 3 cc syringes with Control (baseline) solution- one for the rig, one for the loading syringe.

-Fill one 3 cc syringe with Experimental (drug) solution.

Step 3: Set up Rig

- Use the leur lock to connect syringes (Control soln. and Experimental soln.) with tubing, place snugly into syringe pump.
- Turn syringe pump on.
- Prime tubing by allowing the pump to perfuse at 6 ml/min into waste container. This will fill tubing and prevent air bubbles from occurring in the tubing.
- Once the solution has reached the electrode pump, speed should be turned down to 6 µl/min.
- Turn on amplifiers, CED, A/D board, computer and Spike2 software.

Step 4: Load Worms

-Take prepared 3 cc syringe with filter and fill syringe with Control solution-there should be plenty for rig syringe and loading syringe.
-Preload the chip by connecting the syringe via Leur lock and tubing and filling the chip until solution appears in the electrode ports.
- Select adults from the pre-exposure well and place them in the inlet port of the chip.
- Connect syringe and gently propel worms into individual channels.
- Keep syringe connected and insert glass reservoirs.
-Check worm placement and apply pressure to syringe if necessary for correct worm placement.
- Carry chip to rig and connect to already running perfusion electrode. Allow the perfusion electrode to run solution through the chip for about 1 min. This will allow the electrode ports to fill and prevent air bubbles from entering the chip when electrodes are inserted.
- Insert 8 individual recording electrodes.
-Check signals and adjust as needed-may require removal of air bubbles or re-insertion of recording electrodes to obtain a good signal.

Step 5: Recording

- Turn on Spike2 program.
- Select File-> New data document.
- Press start.
- Collect baseline recording for 30 min.
- Switch to drug solution.
    - Press “A” three times
        - “A” when pump is turned off.

        - "A" switch to drug and restart pump.
        - "A" observe the chip as drug is pumped, press when drug hits worm.
- Continue to record for 1 hr.
- Stop experiment after 1 hr of exposure to Experimental solution and save the file.

Step 6: Clean-up and Notation

- Turn off amplifiers: unplug CED, A/D board, power supply, syringe pump.
- Clean rig: rinse electrodes with de-ionized water in a petri dish, rinse tubing by attaching DI water, EtOH and air can to tubing, rinse reservoirs with de-ionized water and dry on bench.
- Look at the chip under the upright scope.
- Record orientation and sex.
- Discard chip in glass waste.

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