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IMMUNOREACTIVITY OF THE SEROTONIN-LIKE AND FMRF-AMIDE-LIKE NERVOUS SYSTEMS OF FOUR GNESIOTROCHAN ROTIFERS

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Robert Neil Walsmith II

Dedication

To my boyfriend Javier Camacho and best friend Eric Ray Alba.
For showing me that light can exist in even the darkest of times.

IMMUNOREACTIVITY OF THE SEROTONIN-LIKE AND FMRF-AMIDE-LIKE NERVOUS SYSTEMS OF FOUR GNESIOTROCHAN ROTIFERS

BY

ROBERT N. WALSMITH

B.S., ENVIRONMENTAL SCIENCE

Thesis

Presented to the Faculty of the Graduate School of

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Abstract

Reorganization of the nervous system during metamorphosis is a common phenomenon across taxa (species through phyla). In phylum Rotifera, sessile species belonging to the superorder Gnesiotrocha metamorphose from free-swimming larvae into sessile adults. Once mature, gnesiotrochan rotifers display an intricate corona or infundibulum associated with lifestyle modalities and feeding behaviors. Here, I examine serotonin-like immunoreactivity (SLIR) and FMRF-amide-like immunoreactivity (FLIR) of the gnesiotrochans *Lacinularia flosculosa*, *Collotheca ferox,* and *Acyclus inquietus* with a focus on life stage and sex.

Based on previous research, I hypothesize that 1) innervation of the larval nervous system will vary according to the lifestyle constraints observed in each species, and 2) innervation of the nervous system in male rotifers will vary from the adult female and will be similar to the female larvae. Neural features were observed using immunohistochemistry and confocal laser scanning microscopy to provide renditions of SLIR and FMRF-amide-like structures. The SLIR and FLIR nervous systems of *L. flosculosa* larvae showed a reduction of brain perikarya as larvae mature into adults. The nervous system of adult *A. inquietus* showed an absence of two SLIR varicosities, and the absence of two FLIR longitudinal neurites extending posteriorly into the larval foot. Contrastingly, the number FLIR brain perikarya increased in the nervous system of adult *C. ferox*. This may result from species-specific lifestyle adaptations (i.e., coloniality (*L. flosculosa*) versus feeding behaviors exhibited by predatory rotifers (*C. ferox*)), or lifestyle constraints associated with sessility.

To further understand if the variation of SLIR/FLIR nervous system elements occurred because of metamorphosis, the direct developing gnesiotrochan rotifer *Filinia longiseta* was observed (neonate/adult and male/female). As expected, the reorganization of nervous system elements was not observed. However, similarities between the nervous system of males and

females were observed. Similar to results of previous studies, SLIR structures comprising the nervous system are species-specific.

The nervous system of male *L. flosculosa*, *C. ferox,* and *A. inquietus* was comparable to the larval stage, while the nervous system of male *F. longiseta* was comparable to the female. This may correlate with their morphologies or behaviors associated with motility. This phenomenon aligns with the concept of progenesis and suggests that the lifestyle constraints associated with the male life history may influence the development and evolution of their nervous system. This study represents the first examination of the relationships between sexual dimorphism and the nervous system in gnesiotrochan rotifers. The knowledge collected here enhances our comprehension of specific nervous system characteristics that may be essential to the diverse lifestyle demands associated with adulthood and sexes within Gnesiotrocha.

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Introduction

Metamorphosis in metazoans may be advantageous, especially where larval life stages occupy different ecological niches and display different lifestyle adaptations than their adult counterparts (Wilbur, 1980). This process allows for the development of specialized structures or capabilities that may be necessary for survival and reproduction. In sessile aquatic invertebrates, metamorphosis often occurs as a rapid transition involving free-swimming or planktonic larvae attaching to a substrate where the metamorphic process takes place (Morse, 1991). Though specific to each taxonomic group, the process analogously involves drastic changes to the external and internal anatomy that has been described in numerous aquatic taxa such as brachiopods (Zhang et al., 2018), bryozoans (Wendt, 2000), annelids (Meyer et al., 2015), and mollusks (Couper and Leise, 1996).

Modifications made to the morphology, behavior, and internal anatomy of organisms may involve the restructuring of the nervous system, innervating newly developed regions that were once absent (Martina, 2000. Remodeling of the nervous system during metamorphosis is apparent in many lophotrochozoans as they mature from larval to adult life stages (Temereva and Tsitrin, 2014). Rotifers provide a unique model to investigate this process because variation of their head or “corona” may be specific to their lifestyle. For instance, function and shape of the rotifer corona is similar in larvae across sessile species prior to metamorphosis, and is associated with swimming behaviors, and is likely innervated by specific neurotransmitters.

After metamorphosis, the structural components of the corona are modified to match their lifestyle (e.g., coloniality, predatory, tube living, etc.) once metamorphosis has taken place (Wallace, 2002). This change can either consist of the corona elongating and further developing, or the replacement of the corona with the adult infundibulum, which is typical in sessile

predatory rotifers (Hochberg and Hochberg, 2015). Regardless, documentation of nervous system development during metamorphosis, and between sexes are lacking for most rotifers.

The overall structure of the rotifer nervous system exhibits bilateral symmetry, featuring a cerebral ganglion, mastax ganglion, and pedal ganglion, all interconnected by a network of neurite connections (Leasi and Ricci, 2011). The cerebral ganglion is positioned dorsal to the mastax (jaw muscles) and forms connections with chemo-, mechano-, and photo- receptors found throughout the body. Brain neurons account for roughly 20% of the total cell count (Kotikova et al., 2005). The bilateral distribution of neuronal cell types forms distinct, species-specific patterns, shaping the brain into one of three forms: X-shaped, arch-shaped, or ring-shaped (Kotikova, 1998). Nerve cords originating from the brain, may be laterally or ventrolaterally paired, and extend caudally throughout the trunk of the animal. Together, the brain and nerve cords are vital in the transmission of signals throughout the body.

Neuromodulation through a variety of neurotransmitters has been investigated in Rotifera and may be subject to reorganizational or phenotypic modifications in species that undergo metamorphosis. Previous studies have yielded identification of serotonin-like (Kotikova et al., 2005), cholinergic-like (Pineda-Rosas et al., 2005), GABA-like (Gallardo et al., 2000), and FMRF-amide-like (Hochberg, 2007) histological structures in the nervous systems of species belonging to the orders Ploima (Sørensen et al., 2003), Bdelloidae, (Leasi et al., 2009) and Gnesiotrocha (Table 1).

Indirect metamorphosis is a trait commonly associated with the sessile lifestyles in Rotifera as seen in the families Collothecidae, Flosculariidae, and Atrochidae. Metamorphosis takes place after larval attachment to a substrate or colony, where development of the adult corona/infundibulum begins. In collothecid and atrochid rotifers, the larval corona is thought to

be replaced entirely by tissues arising from the larval foregut, forming the adult infundibulum (Hochberg et al., 2019). This process involves the loss of cilia surrounding the larval corona, and reorganization of musculature surrounding the head and mouth to from the bowl-shaped infundibulum (Hochberg et al., 2010). This type of metamorphosis distinguishes itself from the metamorphic process observed in many flosculariid rotifers where adulthood is achieved after their corona extends, and fully matures as a more intricate, lobate, and ciliated structure (Fontaneto et al., 2003).

Previous investigation of metamorphosis and the transition of the larval corona to the adult infundibulum in the rotifer *Stephanocerus fimbriatus* (Goldfuß, 1820) found minimal distinction of the nervous system before and after metamorphosis; except for perikaryal structures found in larvae and adult (Hochberg and Hochberg, 2015). Contrastingly, in the rotifer *Cupelopagis vorax*, (Leidy, 1857) the presence of the coronal neurite ring was seen in larvae, but not in adults after development of the infundibulum. This may be caused by a series of apoptotic events leading to the loss of neurites that previously innervated the structure (Preza et al. 2020). Structural rearrangement during ontogeny of these species derives primarily from the morphological requirements of the corona/infundibulum during each life stage, larva (swimming) and adult (feeding and reproducing). Similarly, the rotifer species *Collotheca ferox* (Penard, 1914), *Acyclus inquietus* Leidy, 1882, and *Lacinularia flosculosa* (Müller, 1773) all have common features in their life cycle development. As non-feeding motile larvae, these species eventually mature into predatory adults (*C. ferox*; *A. inquietus*) or into filter feeding colonies (*L. flosculosa*) with an altered corona (*L. flosculosa*) or infundibulum (*C. ferox*, *A. inquietus*) that fit each lifestyle modality.

Though the morphology of direct developers typically remains the same throughout their life cycle, changes in neurophysiology have been documented at sexual maturity (Croll and Chiasson, 1989). Thus, examining the nervous system of the planktonic rotifer *Filinia longiseta* (Ehrenberg, 1834) at different life stages (neonate and adult) could provide valuable insights into changes within the nervous system throughout ontogeny. In addition, examining variation in nervous systems associated with sexual maturity provides an opportunity to identify nervous system features that may be associated with reproductive strategies in male and female rotifers.

Morphological distinctions between the sexes in rotifers are often species-specific but typically they have comparable traits. Characteristics of male rotifers include gut reduction/absence, dwarfism, and anatomical variation of the reproductive system (i.e., the single testes and copulatory organ (Ricci and Melone, 1998)). In species undergoing indirect development and metamorphosis, dwarf males are morphologically similar to the female larval life stages, which result from the lifestyle requirements needed to successfully reproduce (e.g., swimming and finding mates) (Rico- Martínez and Walsh, 2013). Aside from variations in morphology, nervous systems of male rotifers remain understudied and overlooked except for one investigation of *Epiphanes senta* (Müller, 1773) (Gąsiorowski et al., 2019). Findings included a reduced stomatogastric nervous system in dwarf males and variation in some nervous system elements (e.g., innervation of longitudinal nerve cords, commissures, foot ganglion, etc.) that may apply to other monogonont rotifers. Males belonging to Gnesiotrocha do not undergo metamorphosis, express the trait of dwarfism, and may lack structural components associated with the female anatomy (defensive spines, antenna, lorica, etc.). However, the neuroanatomy of males has not been previously explored for gnesiotrochans.

This investigation also focused on the role of neurotransmitters, which innervate specific regions of the body through the nervous system to achieve an array of functions. For instance, serotonin is a highly conserved neurotransmitter found in most metazoans. In invertebrates, serotonin has functional roles that influence behavior, mobility, and circadian activity (Lucki, 1998). In Rotifera, neuronal innervation of serotonin depends on the taxonomic order and species. In the bdelloid rotifer *Macrotrachela quadricornifera* De Koning 1929, serotonin is involved in the regulation of locomotion, feeding behavior, reproduction, and has been proposed to have roles in egg laying and development (Leasi and Ricci, 2011). In Ploima, serotonin innervates regions of the cerebral ganglion, ventrolateral nerve cords, and stomatogastric nervous system in *Notommata copeus* Ehrenberg, 1838 (Hochberg, 2007). Serotonin has also been proposed to have sensory function in the corona of the rotifer *Asplanchna brightwellii* Gosse, 1850 (Hochberg, 2009). However, the rotifer *Asplanchna herricki* Guerne, 1888 lacks serotonergic innervation in the foot (Kotikova et al., 2005). In the rotifer *Brachionus plicatilis* Müller, 1786 serotonin is associated with mixis induction, which is crucial to the life cycle of monogonont rotifers alternating between sexual and asexual reproduction (Gallardo et al., 2000). In gnesiotrochans, serotonin innervates parts of the larval corona in *Cupelopagis vorax* until it reaches maturity when the coronal neurites in the adult infundibulum are lost (Preza et al., 2020).

Similarly, Phe-Met-Arg-Phe-NH_2 (FMRF-amide) also relates to locomotion, feeding behavior, and reproduction and its functionality likely varies across taxa. For instance, FMRFamide and FaRPs (FMRF-amide related proteins) have roles in feeding, development, and circadian activity (Lopez-Vera et al., 2008). In mollusks, where FaRPs were first discovered, they play critical roles in neuromodulation, neurotransmission, and act as neurohormones

(Krajniak, 2013). FMRF-amide differs from serotonin in major aspects of male reproduction and innervates the male reproductive tract of the pond snail *Helix aspersa* (Müller, 1774) (Lehman and Greenberg, 1987). In Rotifera, FMRF-amide like modulation differs between males and females in *E. senta*, primarily in the gut where males lack stomatogastric features, and varies in areas of the reproductive system (Gąsiorowski et al., 2019).

In brachiopod larvae, FaRPs have also been associated with defense behavior and may also influence ciliary beating and locomotion (Thiel et al., 2017). Neuromodulation of cilia control in rotifers is likely to vary across species, and further analysis is needed to fully understand ciliary function. Further identifying potential neuromodulators innervating the portion of the larval corona before the development of the adult corona or infundibulum may provide the framework for understanding these concepts. This would further strengthen our understanding on how the change in innervation of neurotransmitters/neuropeptides may influence adaptations specific to each life stage, larva, and adult. Evaluating distinct differences in FMRF-amide like neural modulation in rotifer males and females belonging to Gnesiotrocha may clarify key aspects of FaRPs roles in behavior and reproduction.

Here, variation in nervous system morphology was observed with a focus on life stage and sex in the rotifer species *L. flosculosa*, *C. ferox, A. inquietus*, and *F. longiseta*. Each species exhibits a different lifestyle (colonial, sessile, parasitic, and planktonic) with morphological adaptations associated with their life history that may be influenced differently by the nervous system. Based on the differences in overall morphology, locomotory capacities, and reproductive anatomies of the different adult females, female larvae, and dwarf males, I investigated the following hypotheses:

1) innervation of the larval nervous system will vary according to the lifestyle constraints observed of each species.

2) innervation of the nervous system in male rotifers will differ from the adult female and will be more similar to the female larvae because of their resemblance in morphology and locomotion.

Studying these traits in gnesiotrochan rotifers enhances our understanding of their nervous system. The unique life histories of each rotifer species highlighted in this study provide insights into nervous system changes that may be linked to the biochemical, physiological, and morphological shifts required by each life stage and sex. This investigation yields a deeper understanding of nervous system reconstruction that may be linked to adaptive strategies employed by rotifers throughout ontogeny.

Table 1. Neurotransmitter distribution in rotifer taxa. Ntr, type of neurotransmitter (5HT, serotonin; catechol, catecholamine; acetylc, acetylcholine; Fra, FRMFamide; SCPb; small cardioactive peptide b, Tyr-tub; tyrosinated tubulin). Met, method of analysis (IHC, immunohistochemical; HC, histochemical). CNS, central nervous system; PNS, peripheral nervous system; Ma, mastax; Or, other organs. Modified from Leasi et al., 2009.

Taxa	**Ntr**	**Met**	**CNS**	**PNS**	**Ma**	**Or**	**Reference**
Gnesiotrocha							
Conochilus coenobasis	5HT	IHC	Y	Y	N	N	Hochberg (2006)
Conochilus dossuarius	5HT	IHC	Y	Y	N	N	Hochberg (2006)
Sinantherina socialis	5HT	IHC	Y	N	Y	N	Hochberg and Lilley (2010)
Stephanocerus fimbriatus	5HT	IHC	Y	N	Y	Y	Hochberg and Hochberg (2015)
Cupelopagis vorax	5HT	IHC	Y	Y	Y	Y	Preza et al. (2020)
Lacinularia flosculosa	5HT Fra	IHC	Y	Y	Y	N	present study
Collotheca ferox	5HT Fra	IHC	Y	Y	N	N	present study
Acyclus inquietus	5HT Fra	IHC	Y	Y	N	N	present study
Filinia longiseta	5HT Fra	IHC	Y	Y	N	N	present study
Ploima							

Asplanchna herrickii	5HT	IHC	Y	N	Y	N	Kotikova (1998)
	FMRF	IHC	Y	Y	Y	N	Kotikova et al. (2005)
Asplanchna herrickii	Catechol	HC	Y	Y	Y	N	Kotikova (1998)
Asplanchna brightwelli	5HT	IHC	Y	Y	Y	Y	Hochberg (2009)
Brachionous plicatilis	Catechol	HC	Y	Y	Y	Y	Keshmirian and Nogrady (1988)
Brachionous quadridentatus	Catechol	HC	Y	Y	Y	N	Kotikova (1998)
Dicranophorus forcipatus	Catechol	HC	Y	Y	Y	N	Kotikova (1998)
Euchlanis dilatata	5HT	IHC	Y	Y	Y	N	Kotikova (1998)
	Fra	IHC	Y	Y	Y	N	Kotikova et al. (2005)
	Catechol	HC	Y	?	?	N	
Lecane arcuata	Catechol	HC	Y	Y	Y	N	Kotikova (1998)
Manfredium eudactylotum	Catechol	HC	Y	?	?	N	Kotikova (1998)
Notommata copeus	5HT	IHC	Y	Y	Y	N	Hochberg (2007)
	Fra	IHC	Y	Y	Y	N	
	SCPb	IHC	Y	Y	Y	N	
Notommata sp.	Catechol	HC	Y	?	Y	N	Kotikova (1998)
	5HT	IHC	Y	Y	Y	N	
	Fra	IHC	Y	Y	Y	N	
	Catechol	HC	Y	?	Y	N	Kotikova (1998)
	5HT	IHC	Y	Y	Y	N	
	Fra	IHC	Y	Y	Y	N	
Platyias quadricornis	Catechol	HC	Y	Y	?	N	Kotikova (1998)
Epiphanes senta	5HT	IHC	Y	?	Y	Y	Gąsiorowski et al. (2019)
	Fra	IHC	Y	?	Y	Y	
	Tyr-tub	IHC	Y	?	Y	Y	
Bdelloidea							
M*acrotrachela quadricornifera*	5HT	IHC	Y	Y	N	N	Leasi et al. (2009)
Dissotrocha macrostla tubercolata	Acctylc	HC	Y	Y	Y	Y	Raineri (1984)
Philodina roseola	Acctylc	HC	Y	Y	Y	Y	Raineri (1984)

Philodina sp.	Catechol	HC	Y	Y	?	N	Kotikova (1998)
Rotaria rotatoria	Acctylc	HC	Y	Y	Y	Y	Raineri (1984)

Methods

1. Animal Culture

Lacinularia flosculosa, *Collotheca ferox*, and *Acyclus inequitus* were isolated from aquatic vegetation samples collected from Hueco Tanks State Park and Historic Site, El Paso Co., TX (31.92°, -106.04°) (*L. flosculosa*), Cuatrocienegas, Coahuila, MX (29.9226666°, 102.1226333°) (*C. ferox*), or Moon Lake, WI (43.806367°, -89.366509°) (*A. inquietus*). Once isolated, rotifers were cultured in modified MBL media (Stemberger, 1971) and fed a mixture of algae (*Chlamydomonas reinhardtii* (Daneard, 1888) (The Culture Collection of Algae at the University of Texas at Austin (UTEX #90), *Chlorella vulgaris* (Beyernick, 1890) (UTEX #30), and *Cryptomonas erosa* (Ehrenberg, 1831).

2. Antibody preparation and labelling

All specimens (larvae, adults) were detached from colonies or walls of the culture dish using pins, while motile larvae and males were pipetted from cultures. Adults and larvae were placed into glass nine-well plates in MBL media. Adult rotifers were removed from colonies/tubes using pins and were transferred through each well to remove impurities that may interfere with the immunohistochemical process. Adult/larvae rotifers were anesthetized using 100-250 μL of 0.37M $MgCL_2$ solution. Once the corona or infundibulum was fully extended, specimens were fixed using 4% paraformaldehyde solution.

Adults and larvae remained in PFA solution on a slow rotator for 30-60 min at room temperature. Following fixation, specimens were rinsed consecutively every 20 min in 1% PBS (Phosphate Buffer Solution) using 400 μL exchanges for a total of 4 washes and stored at 4 °C until antibody preparation. Antibody preparation began by placing specimens in 0.5% PBT (PBS + 0.1% Triton X-100) for 1 hr, and the placing them in 1% BSA solution (0.5 g Bovine Serum

Albumin + 50 mL) for approximately 1 hr. Specimens were then placed in 400 µL of 10X PBT and diluted primary polyclonal antibody anti- serotonin produced in rabbit (Table 2). Anti-5HT primary antibodies were diluted in PBT at 1:800 µL ratio while anti-FMRF primary antibodies were diluted at 1:400 µL. Incubation for primary antibody reactivity were done at 4°C for 24-48 hrs.

Application of the secondary antibody was done after the specimens had been washed using four exchanges of 400 µL PBT for 30 min each at room temperature. Secondary polyclonal antibody anti-rabbit Igg Alexa fluor 568 (for 5HT) or polyclonal anti-rabbit Igg Alexa fluor 488 (for FMRF-amide) was diluted in PBT and was reacted with specimens at 4 °C for 24 hrs. The chemical stain Alexa fluor 488 phalloidin (20 µL) was applied on the last day of the IHC process in 400 µL of PBT for 2 hrs at room temperature or 24 hrs at 4 °C on slow rotation. Once the IHC protocol was complete, animals were mounted to slides in one drop of Invitrogen™ ProLong™ gold anti-fade mounting media with DAPI solution and examined using Zeiss LSM confocal microscopy.

Table 2. Primary and secondary antibodies used for the immunohistochemical protocol. Each antigen was diluted as indicated and placed on slow rotation at 4ºC or room temperature (≈23 ºC) for time needed for each reagent to react. Dilutions of antibodies used for each species [*L. flosculosa* (LF), *C. ferox* (CF), and *Acyclus inquietus* (AI), *Filinia longiseta* (FL)] are indicated.

	Antigen	**Type**	**Source**	**Dilution (µL)**	**Incubation**
Primary Antibody	Anti-Serotonin	Polyclonal	Sigma-Aldrich	1:1000 (LF, FL) 1:800 (CF) 1:500(AI)	24 h, 4 ºC

	Anti-FMRF-amide	Polyclonal	Sigma-Aldrich	1:1000 (LF, FL) 1:1000(CF) 1:1000 (AI)	
Secondary Antibody	Anti-Rabbit IgG	Polyclonal	Invitrogen	1:1200 (AI)	24 h, 4 ºC
Chemical stain	Phalloidin		Invitrogen	20	2 h, ≈24 ºC

3. Microscopy

Observations of antibody reactivity, f-actin reactivity, and nuclear stains were done using a Zeiss LSM confocal microscope located in the Cytometry Screening and Imaging Core Facility at the University of Texas at El Paso. Confocal stacks were generated at 1 airy unit (AU) at high resolution 8-pixel, 0.1 μm z-plane. Renditions of the nervous system were prepared using a combination of Zeiss 2009 Zen Digital imaging for Light Microscopy (RRID:SCR_013672) and Image J software (Rasband, 1997-2018). Schematics were produced using a combination of confocal z-stacks and Adobe Photoshop 2019 software.

4. Results

Lacinularia flosculosa

Some components of the nervous system of female *L. flosculosa* are variable between life stages but more generally it is comprised of a cerebral ganglion (CG) that varies in brain perikaryal number, a coronal neurite ring innervating the corona, and two longitudinal nerve cords that extend downwards from the trunk and into the foot (Figs.1-4). Differential Interference Contrast (DIC), DAPI nuclear stain, Alexa fluor-488 Phalloidin, and serotonin-like immunoreactivity (SLIR) of larval and adult nervous systems revealed the most prominent

variation of the cerebral ganglion between life stages was the number of brains perikarya (BP) and anatomical position of the coronal neurite ring in larvae and adults (Figs. 1, 2, 4).

The SLIR cerebral ganglion of the larvae (n=8) is composed of six brain perikarya (Figs. 2, 4). Neurites extend laterally from these brain perikarya in the cerebral ganglion forming the larval coronal neurite ring (CNR) that extends entirely throughout the larval corona. In adult *L. flosculosa* (n=7) the SLIR cerebral ganglion is composed of two BP tethered by a laterally extending commissure (C). The neurites of this commissure extend anteriorly, then posteriorly into the lower lobes of the adult's corona forming the coronal neurite ring of the adult. Innervation of only the lower lobes of the corona contrasts findings from the colonial rotifer *Sinantherina socialis* (Linnæus, 1758) where the entirety of the *S. socialis* corona are innervated by the CNR (Hochberg and Lilley, 2010).

The male nervous (n=5) system is similar in structure and shape to the larval female, and also contains a cerebral ganglion, and two longitudinal nerve cords that extend posteriorly into the male foot (Figs. 2-4). Unlike the females, the male SLIR cerebral ganglion is comprised of four SLIR brain perikarya and two varicosities. Two neurites extend from the cerebral ganglion anteriorly into two varicosities located in the upper lobes of the male corona, contrasting with the coronal neurite ring found only in the female larvae and adults.

FMRF-amide-like immunoreactivity (FLIR) of larval (n=10) and adult (n=8) nervous systems revealed many similarities in structure and shape, with some structures that vary between life stages. The cerebral ganglion of larval *L. flosculosa* is composed of eight FLIR brain perikarya connected by a neuropil in the center (Figs. 5, 6). Two neurites descend inferiorly from the FLIR cerebral ganglion, into two peripheral perikarya (PP) located just above the larval

vitellarium; no other FLIR was observed below this region. In adult females, the FLIR cerebral ganglion is comprised of six brain perikarya. Retention of the two peripheral perikarya innervating the upper portions of the vitellarium was also apparent. Lastly, nerve cords extend into the adult mastax (MN) surrounding the trophi, which was not observed in the larva.

Contrasting to SLIR, FLIR results for male *L. flosculosa* (n=6) revealed a nervous system that is similar in structure and shape to the adult female. The cerebral ganglion is composed of four neurons, and four ganglia which are positioned similarly to the mastax neurite (MN) that was observed in female adults. In contrast to females, males also had two longitudinal nerve cords that extended posteriorly into two peripheral perikarya located in the penis.

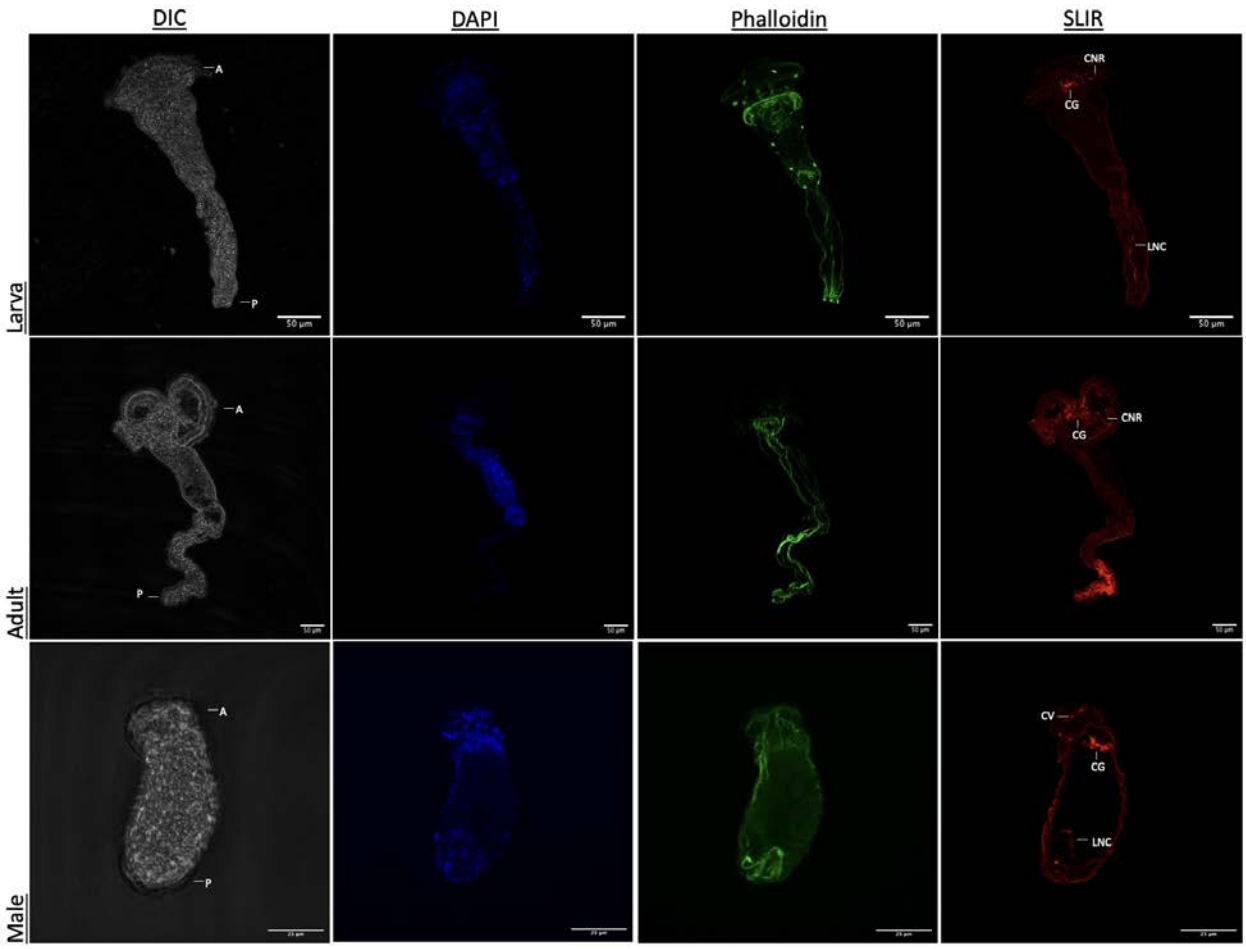

Figure 1. Differential Interference Contrast (DIC), DAPI nuclear stain, Phalloidin, and Serotonin-like immunoreactivity (SLIR) of larval, adult, and male *Lacinularia flosculosa* visualized using 20x confocal magnification (larva/adult) and 68x magnification (male). All panels viewed dorso-ventrally, with coronae positioned near the top. Abbreviations: anterior (A), posterior (P), cerebral ganglion (CG), coronal neurite ring (CNR), longitudinal nerve cord (LNC), coronal varicosity (CV).

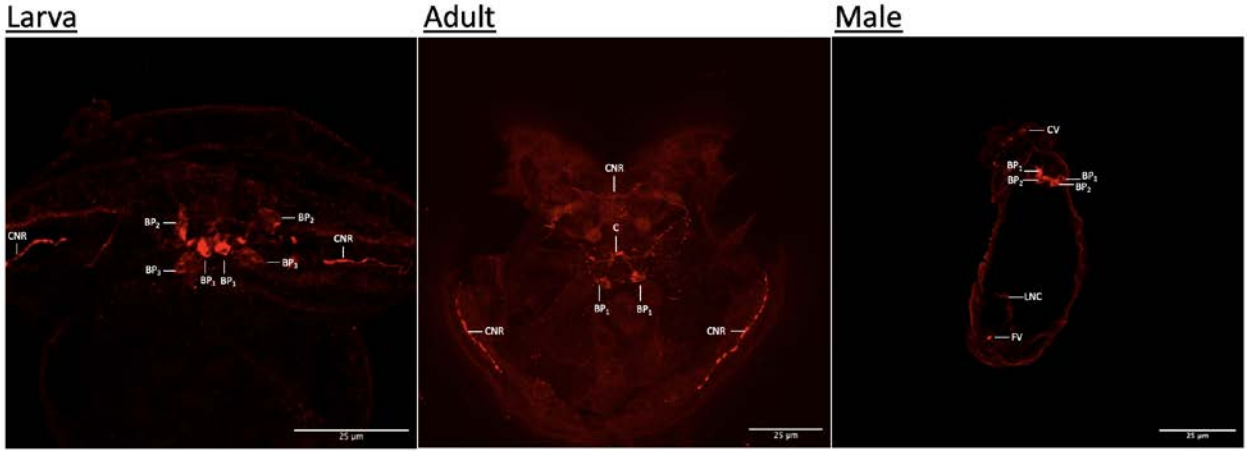

Figure 2. Z-projections of the serotonin-like immunoreactivity (SLIR) of female larva, adult, and male *Lacinularia flosculosa* visualized using 68x magnification. All panels viewed dorso-ventrally, with coronae positioned near the top. Abbreviations: brain perikarya (BP), coronal neurite ring (CNR), commissure (C), longitudinal nerve cord (LNC), varicosity (V).

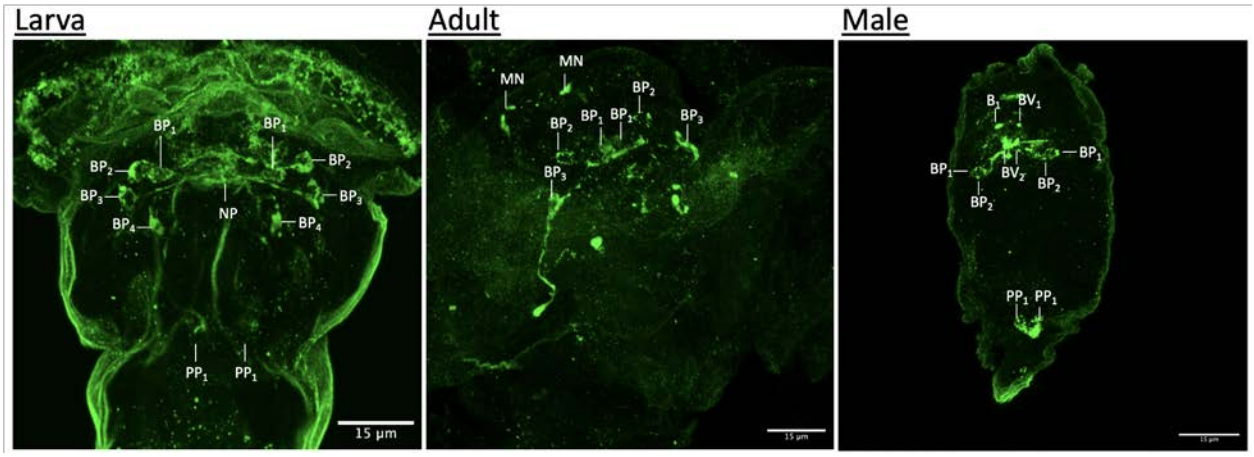

Figure 3. Z-projections of the FMRF-amide-like immunoreactivity (FLIR) of female larva, adult, and male *Lacinularia flosculosa* visualized using 100x magnification. All panels viewed dorso-ventrally, with coronae positioned near the top. Abbreviations: brain perikarya (BP), neuropil (NP), peripheral perikarya (PP), varicosity (V), mastax neurite (MN).

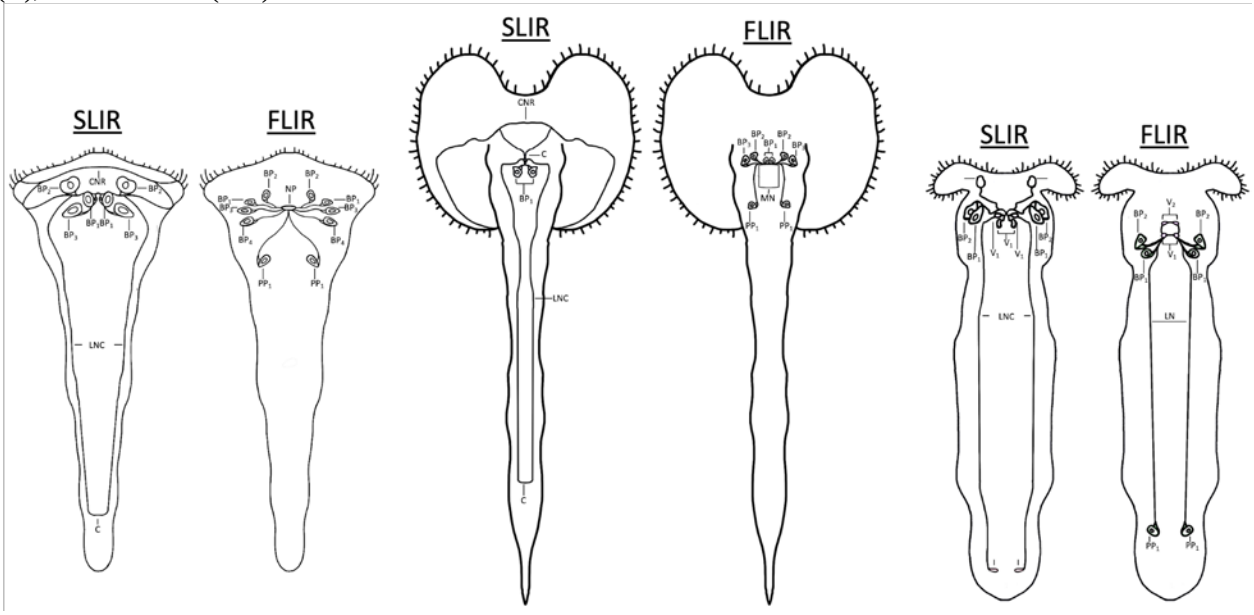

Figure 4. Schematic representation of nervous system derived from serotonin-like immunoreactivity (SLIR) and FMRF-amide-like immunoreactivity (FLIR) in larva (left), adult (middle), and male (right) *Lacinularia flosculosa*. Abbreviations: brain perikarya (BP), coronal neurite ring (CNR), commissure (C), longitudinal nerve cord (LNC), varicosity (V), neuropil (NP), peripheral perikarya (PP), mastax neurite (MN).

Table 3. Summary of nervous system elements throughout each life stage of *Lacinularia flosculosa* using Serotoninlike Immunoreactivity (SLIR) and FMRF-amide-like immunoreactivity (FLIR). Key: (+) indicates structure is present (-) indicates structure is absent.

SLIR | FLIR Element	*L. flosculosa* Larva		*L. flosculosa* Adult		*L. flosculosa* Male	
	SLIR	FLIR	SLIR	FLIR	SLIR	FLIR
Brain Perikarya	6	8	2	6	4	4
Brain Varicosity	-	-	-	-	4	4
Coronal Neurite Ring	+	-	+	-	-	-
Mastax Neurite	-	-	-	+	-	+
Commissure	+	-	+	-	-	-
Longitudinal Nerve Cords	+	-	+	-	+	-
Foot Varicosity	-	-	-	-	2	-
Coronal Varicosity	-	-	-	-	2	-
Coronal Neurite Ring	+	-	+	-	-	-
Peripheral Perikarya	-	2	-	2	-	2

Collotheca ferox

Nervous system morphology of larva and adult *C. ferox* revealed that the larval nervous system varied in overall structure and brain perikaryal number. The SLIR nervous system of larvae (n=4) is composed of a cerebral ganglion (CG) containing four brain perikarya (BP) each with lateral neurites extending posteriorly forming two longitudinal nerve cords that extend through the larval trunk forming a commissure in the foot. Contrastingly, the adult nervous system did not appear to retain SLIR longitudinal nerve cords in the adult nervous system (n=9). Instead, the only neurite projections observed in the adult extended anteriorly from the cerebral ganglion forming varicosities near the upper lobe of the infundibulum. This was the only structure variable across life stages as the adult cerebral ganglion also contained four brain perikarya (Fig. 6, 8).

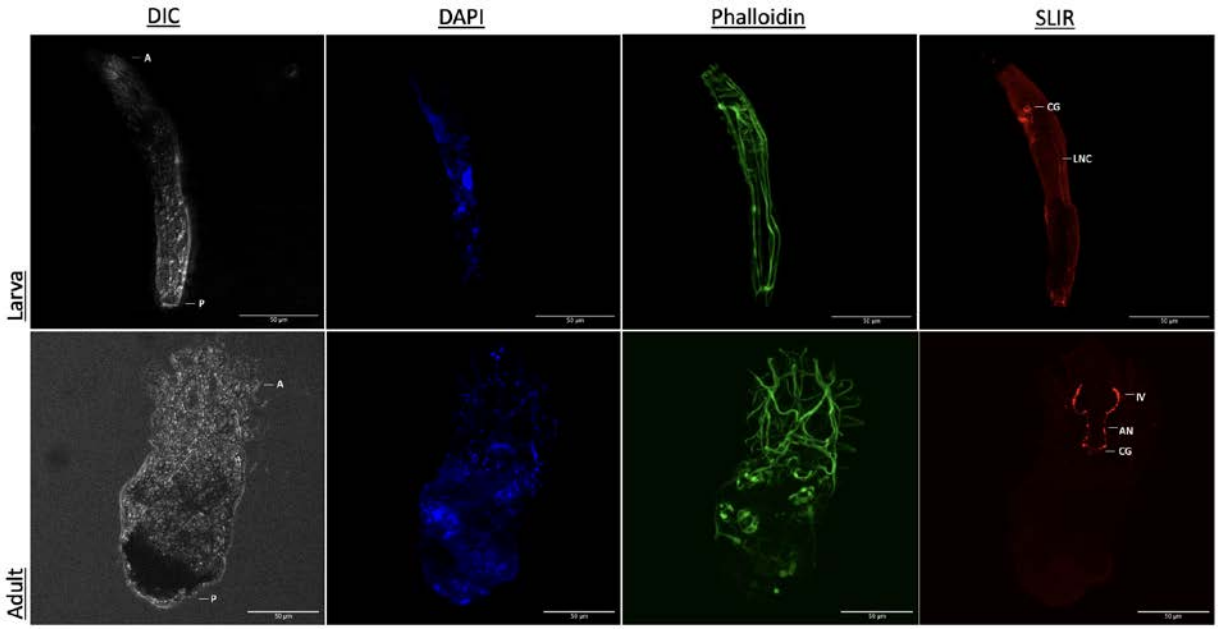

Figure 5. Differential Interference Contrast (DIC), DAPI nuclear stain, Phalloidin, and Serotonin-like immunoreactivity (SLIR) of larval and adult *Collotheca ferox* visualized using 20x confocal magnification. All panels viewed dorso-ventrally, with corona/infundibulum positioned near the top. Abbreviations: anterior (A), posterior (P), cerebral ganglion (CG), longitudinal nerve cord (LNC), infundibular varicosity (IV), anterior neurite (AN).

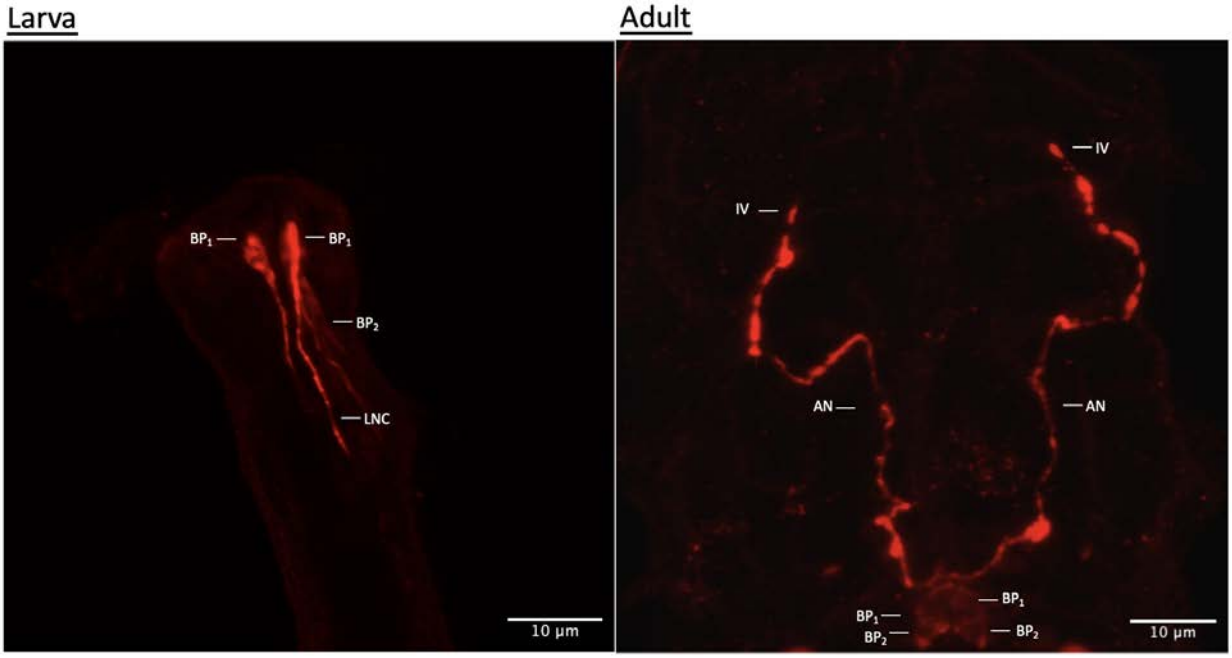

Figure 6. Z-projections of the serotonin-like immunoreactivity (SLIR) of female larva and adult *Collotheca ferox* visualized using 100x magnification. All panels viewed dorso-ventrally, with corona/infundibulum positioned near the top. Abbreviations: brain perikarya (BP), longitudinal nerve cord (LNC), infundibular varicosity (IV), anterior neurite (AN).

FLIR positive structures in larva (n=6) and adult (n=4) *C. ferox* appeared to be more variable in shape and brain perikaryal number. The FLIR cerebral ganglion of larval *C. ferox* is X- shaped and is composed of a total of eight FLIR positive brain perikarya. Two neurite bundles (anterior neurite projections-ANP) extend from the cerebral ganglion anteriorly toward the larval corona. After metamorphosis, the adult cerebral ganglion appears more arch-shaped and is composed of 14 brain perikarya. Although no neurite bundles were observed, two lightly positive neurites partially innervate the lower portion of adult infundibulum. Surprisingly, no peripheral perikarya were observed in either life stage of *C. ferox*, contrasting to all rotifers in this study.

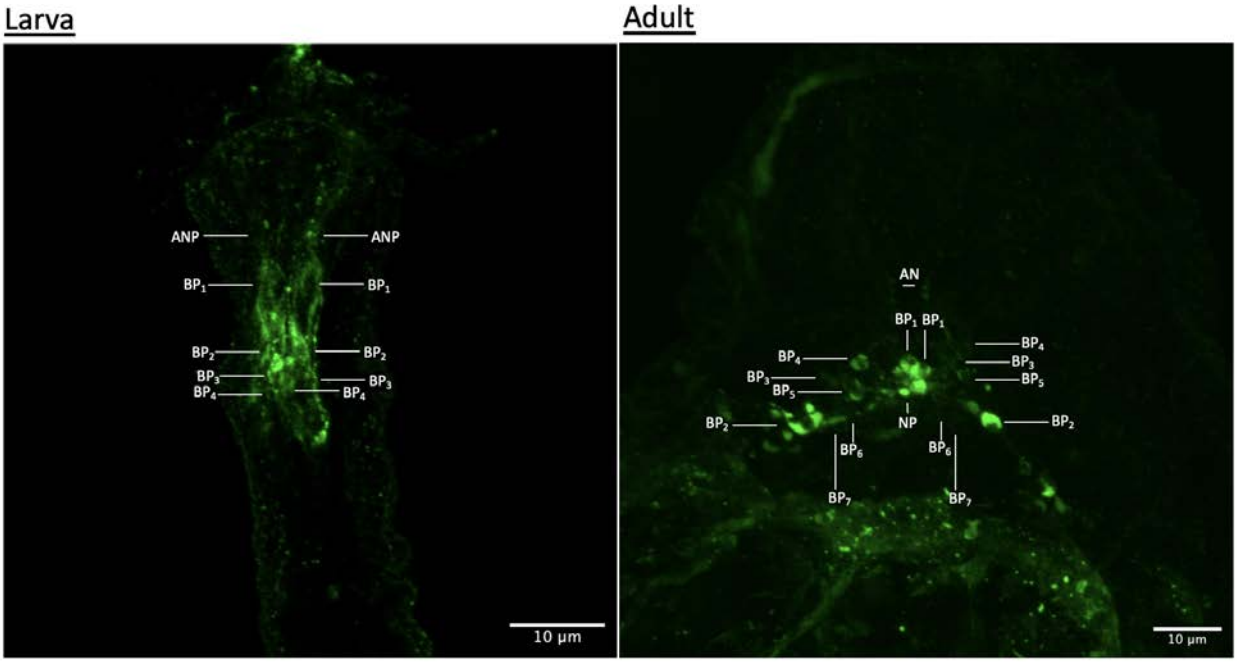

Figure 7. Z-projections of the serotonin-like immunoreactivity (SLIR) of female larva and adult *Collotheca ferox* visualized using 100x magnification. All panels viewed dorso-ventrally, with corona/infundibulum positioned near the top. Abbreviations: brain perikarya (BP), anterior neurite projections (ANP), longitudinal nerve cord (LNC), varicosity (V), anterior neurite (AN), neuropil (NP).

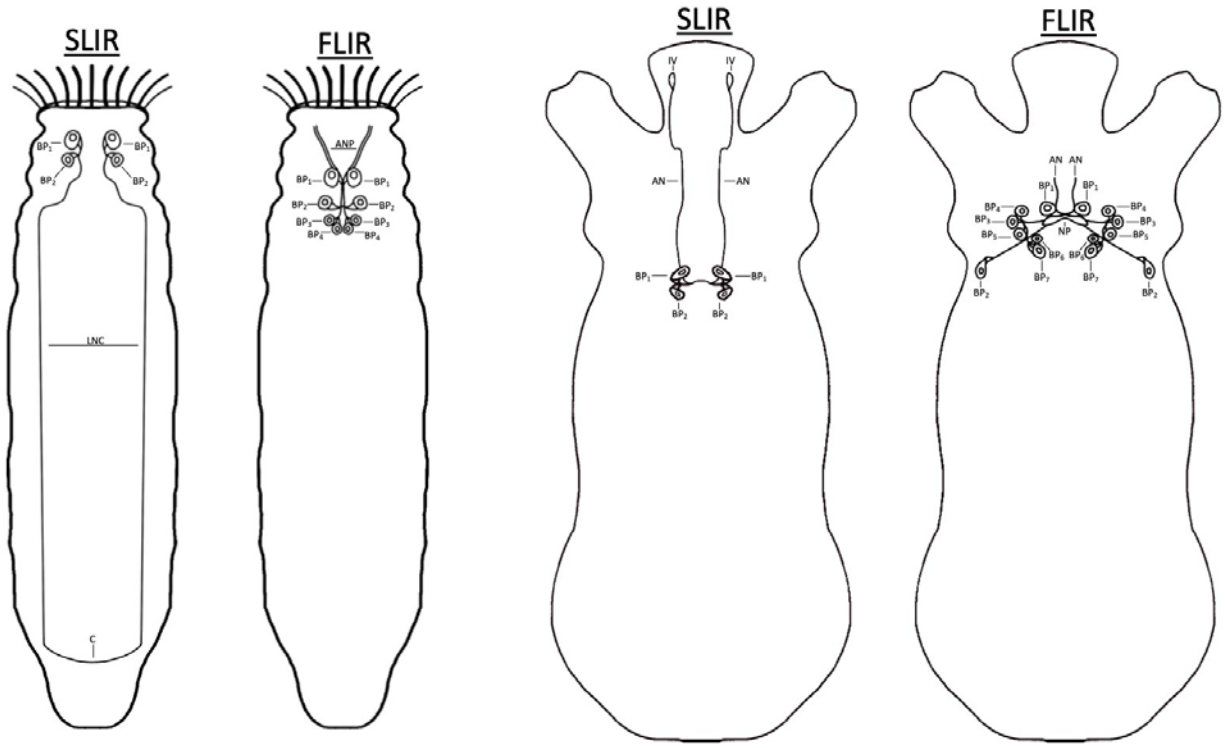

Figure 8. Schematic representation of nervous system derived from serotonin-like immunoreactivity (SLIR) and FMRF-amide-like immunoreactivity (FLIR) in larva (left) and adult (right) *Collotheca ferox.* Abbreviations: brain perikarya (BP), anterior neurite projections (ANP), commissure (C), infundibular varicosity (V,) anterior neurite (AN), neuropil (NP).

Table 4. Summary of nervous system elements throughout each life stage in *Collotheca ferox* using Serotonin-like Immunoreactivity (SLIR) and FMRF-amide-like immunoreactivity (FLIR). Key: (+) indicates structure is present (-) indicates structure is absent.

SLIR | FLIR Element	*C. ferox* Larva		*C. ferox* Adult	
	SLIR	FLIR	SLIR	FLIR
Brain Perikarya	4	8	4	14
Infundibular Varicosity	-	-	2	-
Anterior Neurite Projections	-	2	-	-
Anterior Neurites	-	+	2	2
Longitudinal Nerve Cords	+	-	-	-
Commissure	+	-	-	-

Acyclus inquietus

The SLIR renditions of the larva (n=7) of *A. inquietus* revealed the larval cerebral ganglion is comprised of two brain perikarya and is structurally similar to larval *C. ferox.* In the center of the brain perikarya, there are two varicosities that form a heart shape. Four neurites project from

the larval cerebral ganglion and into the corona. Two longitudinal nerve cords descend posteriorly through the larval trunk and form a commissure in the foot (Figs. 8, 9).

The FLIR structures observed in the larvae (n=2) indicated the larval brain is composed of four brain perikarya. Two lateral neurites extend inferior to the cerebral ganglion into two peripheral perikarya located just above the upper portion of the larval vitellarium. Two lateral neurites extend inferior to the cerebral ganglion and into the mid portion of the trunk where they are connected by a commissure. They continue to extend posteriorly to the commissure into two peripheral perikarya located near the foot.

Structurally, the SLIR nervous system of adults (n=4) retains its morphological structure and is composed of two brain perikarya. From the cerebral ganglion, two lateral nerve cords extend, and then descend downward into two varicosities located in the lower portion of the adult infundibulum. The longitudinal nerve cords seen in larval *A. inquietus* are retained and descend from the cerebral ganglion maintaining the commissure in the foot. The heart shaped varicosities observed in larval *A. inquietus* were not present after metamorphosis into adulthood.

The adult cerebral ganglion consists of four brain perikarya connected by a commissure. Two lateral neurites project inferiorly from the cerebral ganglion into two peripheral perikarya located in the upper portions of the adult vitellarium. No FLIR innervation was observed anywhere in the animal below this point, contrasting the innervation of the foot by SLIR neurites and peripheral perikarya.

SLIR nervous system in male *A. inquietus* (n=7) was nearly identical to the larvae, with only slight variation in the number of brain perikarya. The male SLIR nervous system was

composed of six brain perikarya (four more than the larva and adults). Like the larvae, two varicosities are in the center of the cerebral ganglion that form a heart shape. Two longitudinal nerve cords descend inferiorly from the cerebral ganglion and form a commissure in the foot.

FLIR structures observed in a male *A. inquietus* (n=1) showed the cerebral ganglion which is comprised of six brain perikarya (Figs. 11,12). Aside from this, no neurite projections associated with the peripheral nervous system were observed which contrasts the peripheral perikarya found above the larval and adult vitellarium. This structure varies from the larvae, which had two peripheral neurons extending into the foot. This also contrasts the female adult, which had no FLIR innervation beyond the vitellarium.

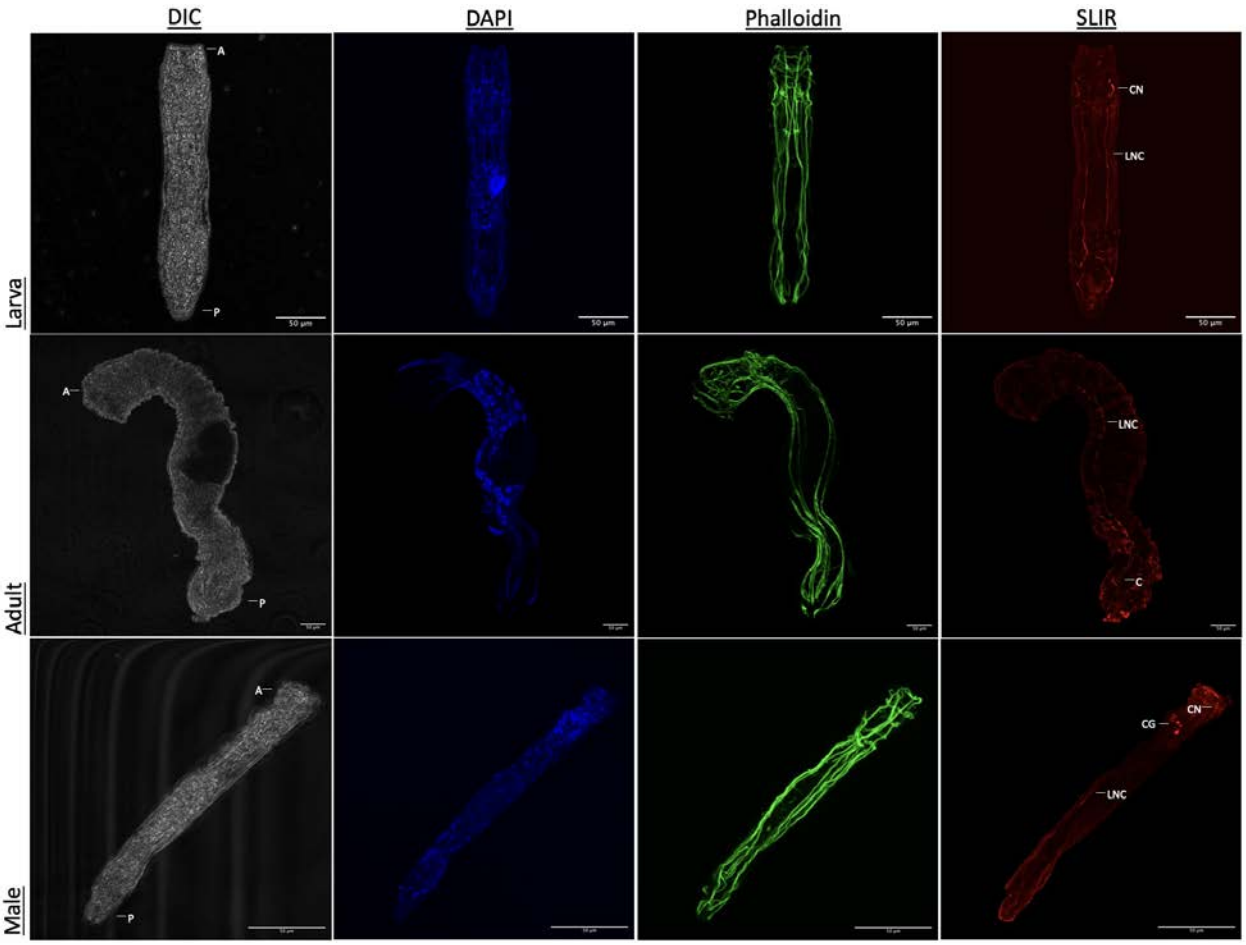

Figure 9. Differential Interference Contrast (DIC), DAPI nuclear stain, Phalloidin, and Serotonin-like immunoreactivity (SLIR) of larva, adult, and male, *Acyclus inquietus* visualized using 20x confocal magnification (female adult) and 40x confocal magnification (larva and male). All panels viewed dorso-ventrally, with coronae positioned near the top. Abbreviations: anterior (A), posterior (P), cerebral ganglion (CG), coronal neurite (CN), longitudinal nerve cord (LNC), coronal neurite (CN).

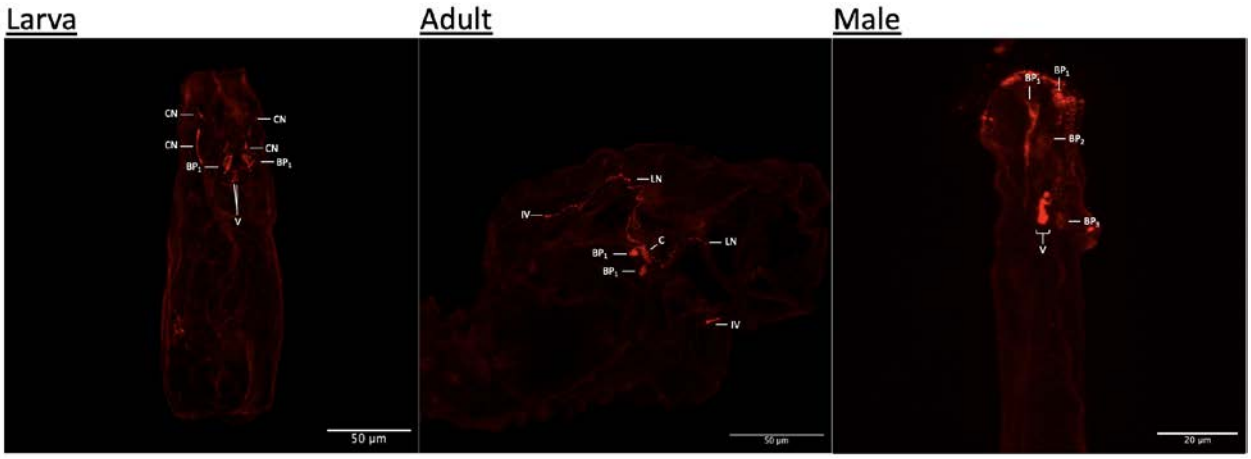

Figure 10. Serotonin-like immunoreactivity (SLIR) *Acyclus inquietus* (left) visualized using 68x confocal magnification. All panels viewed dorso-ventrally, with coronae positioned near the top. Abbreviations: brain perikarya (BP), coronal neurite (CN), lateral neurite (LN), infundibular varicosity (IV), commissure (C), longitudinal nerve cord (LNC), varicosity (V).

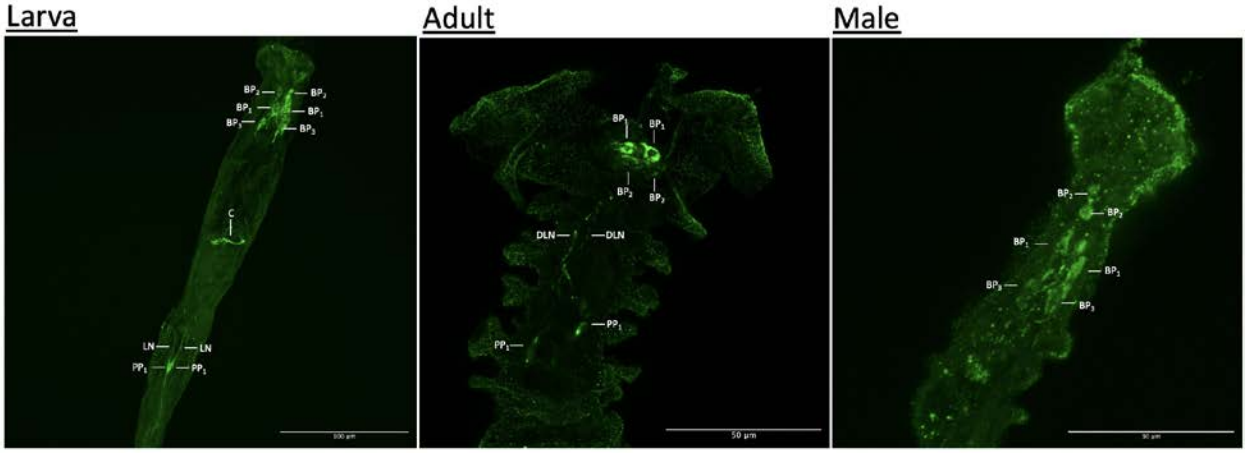

Figure 11. FMRF-amide-like immunoreactivity (FLIR) of larva *Acyclus inquietus* (left) visualized using 20x confocal magnification. All panels viewed dorso-ventrally, with coronae positioned near the top. Abbreviations: brain perikarya (BP), longitudinal neurite (LN), commissure (C), longitudinal nerve cord (LNC), peripheral perikarya (PP).

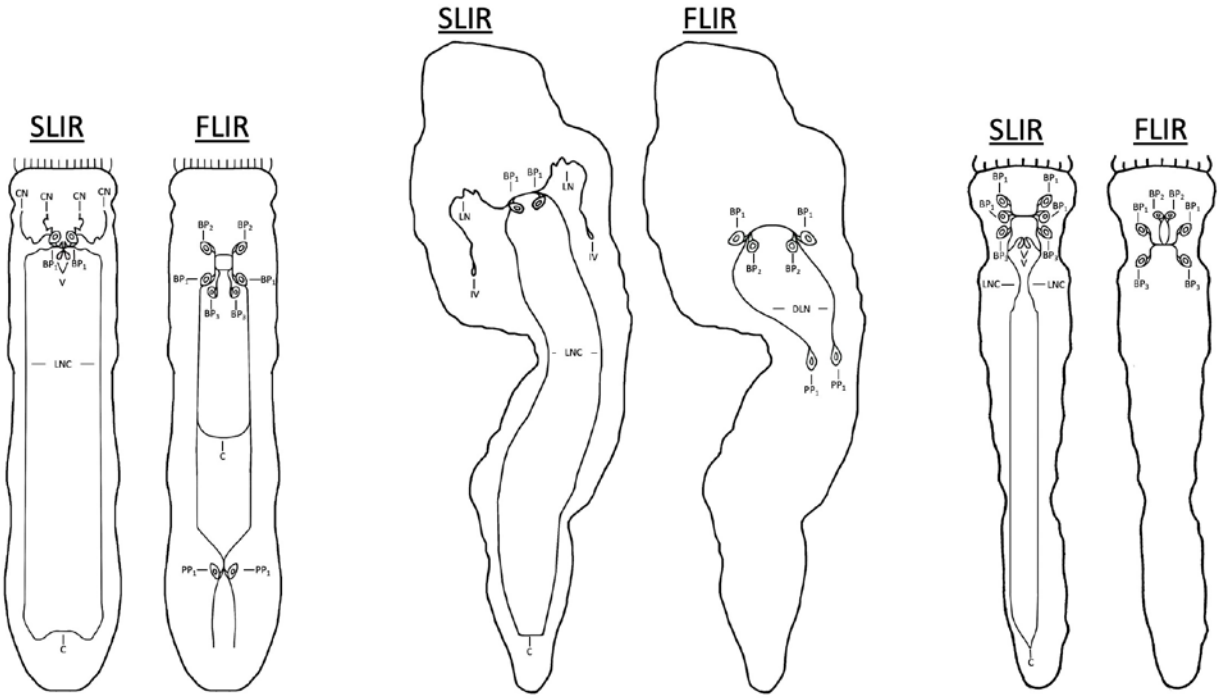

Figure 12. Schematic representation of nervous system derived from serotonin-like immunoreactivity (SLIR) and FMRF-amide-like immunoreactivity (FLIR) in larva (left), adult (middle), and male (right) *Acyclus inquietus.* Abbreviations: brain perikarya (BP), coronal neurite ring (CNR), commissure (C), longitudinal nerve cord (LNC), varicosity (V), neuropil (NP), peripheral perikarya (PP).

Table 5. Summary of nervous system elements throughout each life stage and sex in *Acyclus inquietus* using Serotonin-like Immunoreactivity (SLIR) and FMRF-amide-like immunoreactivity (FLIR). Key: (+) indicates structure is present (-) indicates structure is absent.

SLIR | FLIR Element	*A. inquietus* Larva		*A. inquietus* Adult		*A. inquietus* Male		*Filinia*
	SLIR	FLIR	SLIR	FLIR	SLIR	FLIR	
Brain Perikarya	2	4	2	4	6	6	
Brain Varicosity	2	-	-	-	2	-	
Coronal Neurites	4	-	-	-	-	-	
Commissure	+	+	+	-	+	-	
Longitudinal Nerve Cords	+	+	+	-	+	-	
Infundibular Varicosity	-	-	2	-	-	-	
Peripheral Perikarya	-	2	-	2	-	-	

longiseta

The nervous system of *Filinia longiseta* (n=13) was not variable in adults and neonates as expected and is morphologically consistent with that of other rotifers. The SLIR nervous system consists of six brain perikarya and four neurite projections. Two neurites project from the

cerebral ganglion anteriorly into the corona, while two extend posteriorly towards the trunk. These neurites seemed to end midway through the trunk of each specimen and did not extend downward into their caudal appendage.

The FLIR nervous system yielded similar results. The FLIR nervous system of female *F. longiseta* (n=7) consists of twelve brain perikarya, and two peripheral perikarya that descend from the cerebral ganglion into the upper regions of the vitellarium. FLIR neurites from the brain descend towards the caudal appendage innervating two additional peripheral perikarya (PP) near the vitellarium. Two additional neurite projections descend from the brain downward, near the caudal appendage into four peripheral perikarya and a commissure. Lastly, neurite projections originating from the brain extend anteriorly to form the coronal neurite ring.

The body plan of male *F. longiseta* is highly reduced when compared to the female. SLIR renditions of the nervous system (n=9) showed variation in the overall shape and complexity. The cerebral ganglion of male *F. longiseta* is comprised of four brain perikarya and two laterally extending neurites. These neurites then extend anteriorly into the male corona forming the coronal neurite ring. Like the females, no longitudinal nerve cords were observed descending into the posterior region of the animal, nor into the penis.

The FLIR renditions of male *F. longiseta* (n=9) were similar in shape and morphology to SLIR. The FLIR cerebral ganglion is comprised of four brain perikarya. Two FLIR longitudinal nerve cords extend laterally from the cerebral ganglion then posteriorly form a commissure near the male penis.

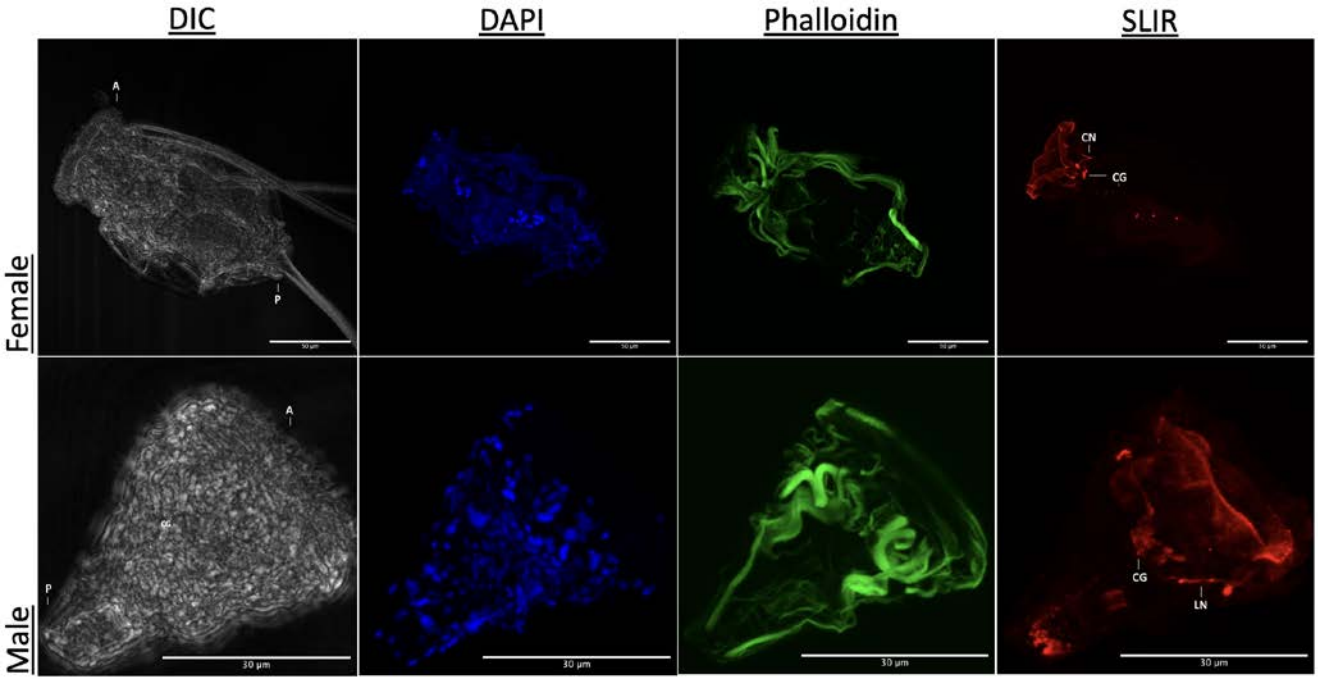

Figure 13. Differential Interference Contrast (DIC), DAPI nuclear stain, Phalloidin, and Serotonin-like immunoreactivity (SLIR) of *Filinia longiseta* visualized using 20X confocal magnification (female) and 100X confocal magnification (male). All panels viewed dorso-ventrally, with coronae positioned near the top. Abbreviations: anterior (A), posterior (P), cerebral ganglion (CG), coronal neurite (CN), lateral neurite (LN).

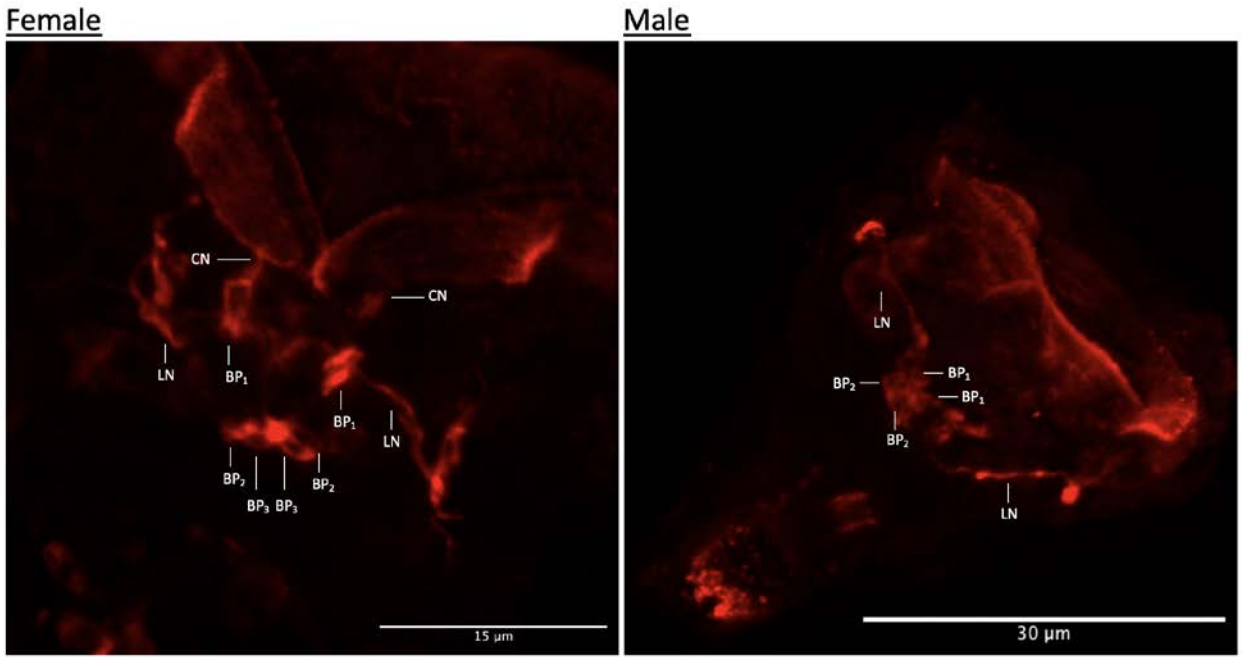

Figure 14. Serotonin-like immunoreactivity (SLIR) of female (left) and male (right) *Filinia longiseta* visualized using 100X confocal magnification. All panels viewed dorso-ventrally, with coronae positioned near the top. Abbreviations: brain perikarya (BP), coronal neurite (CN), lateral neurite (LN).

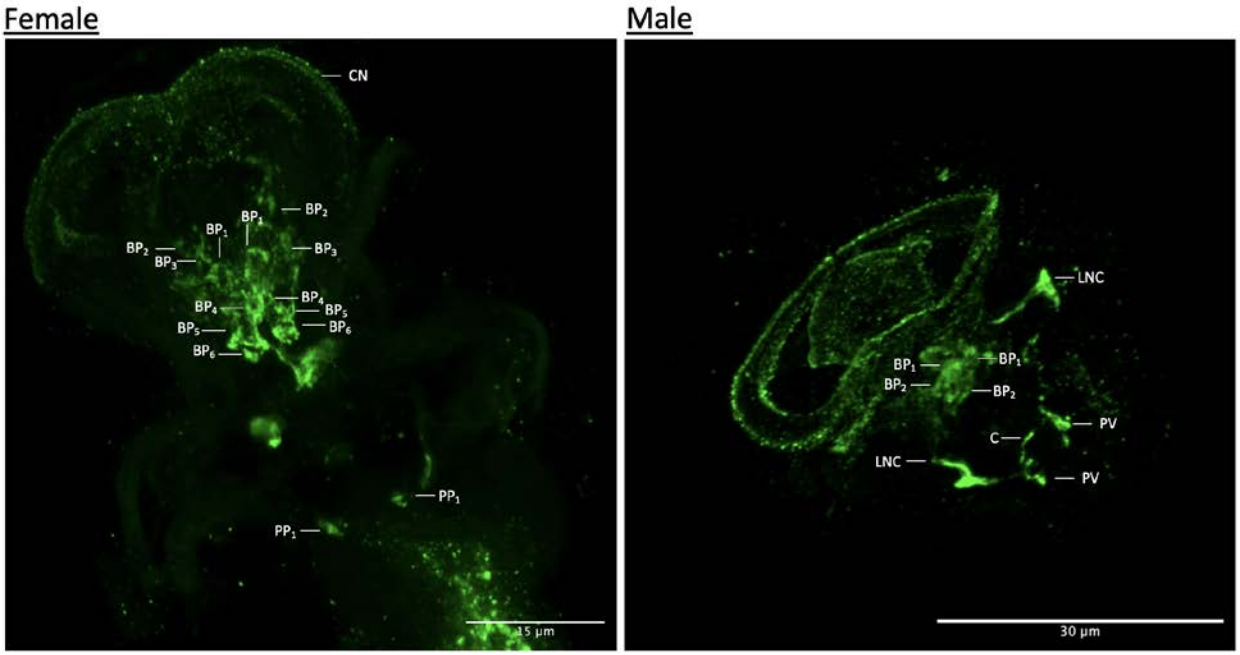

Figure 15. FRMF-amide-like immunoreactivity (FLIR) of female (left) and male (right) *Filinia longiseta* visualized using 100X confocal magnification. All panels viewed dorso-ventrally, with coronae positioned near the top. Abbreviations: brain perikarya (BP), coronal neurite ring (CNR), longitudinal nerve cord (LN), commissure (C), varicosity (V).

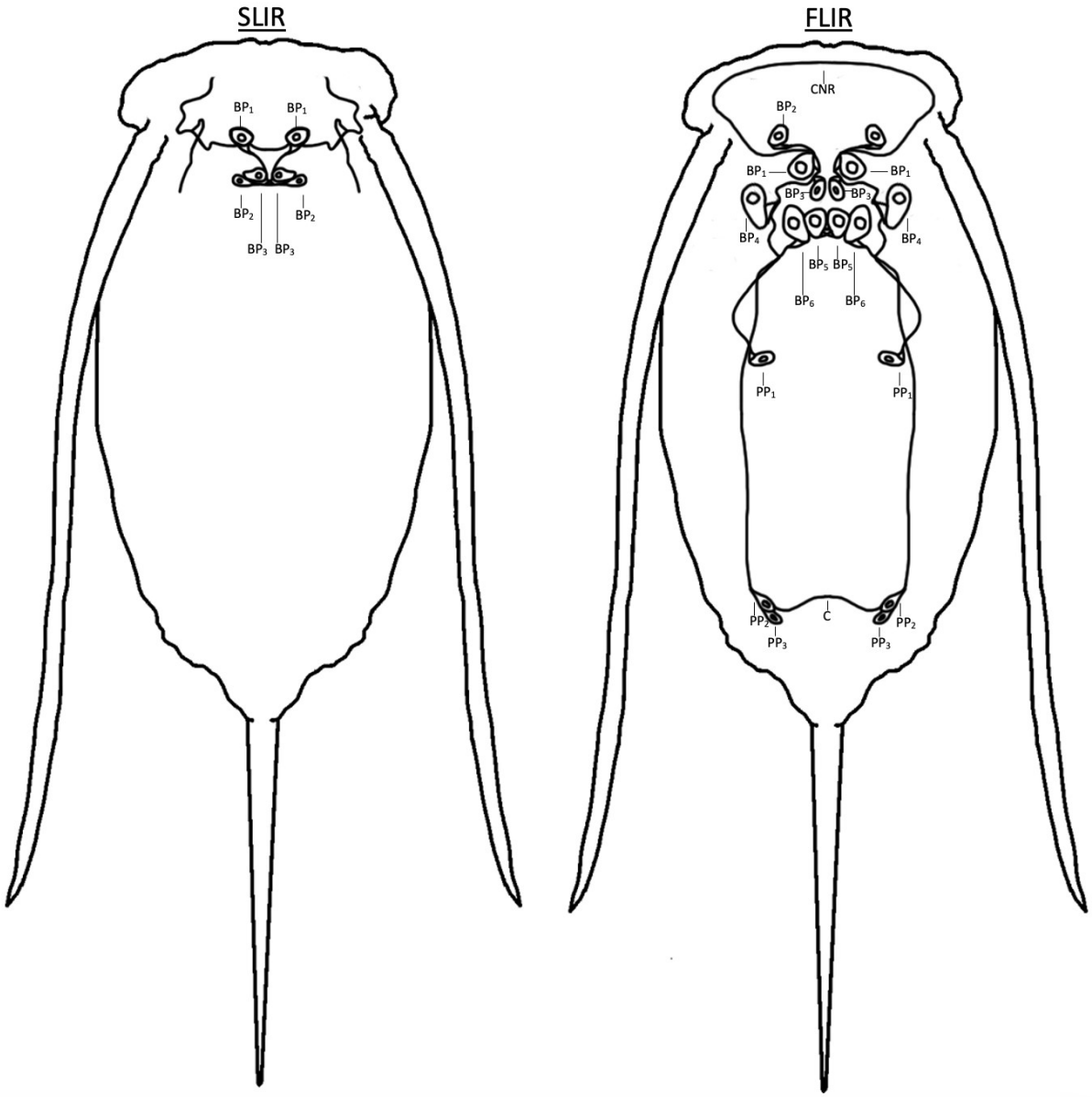

Figure 16. Schematic representation of nervous system derived from serotonin-like immunoreactivity (SLIR) and FMRF-amide-like immunoreactivity (FLIR) adult for *Filinia longiseta.* Abbreviations: brain perikarya (BP), coronal neurite ring (CNR), peripheral perikarya (PP). (Caudal spine not to scale).

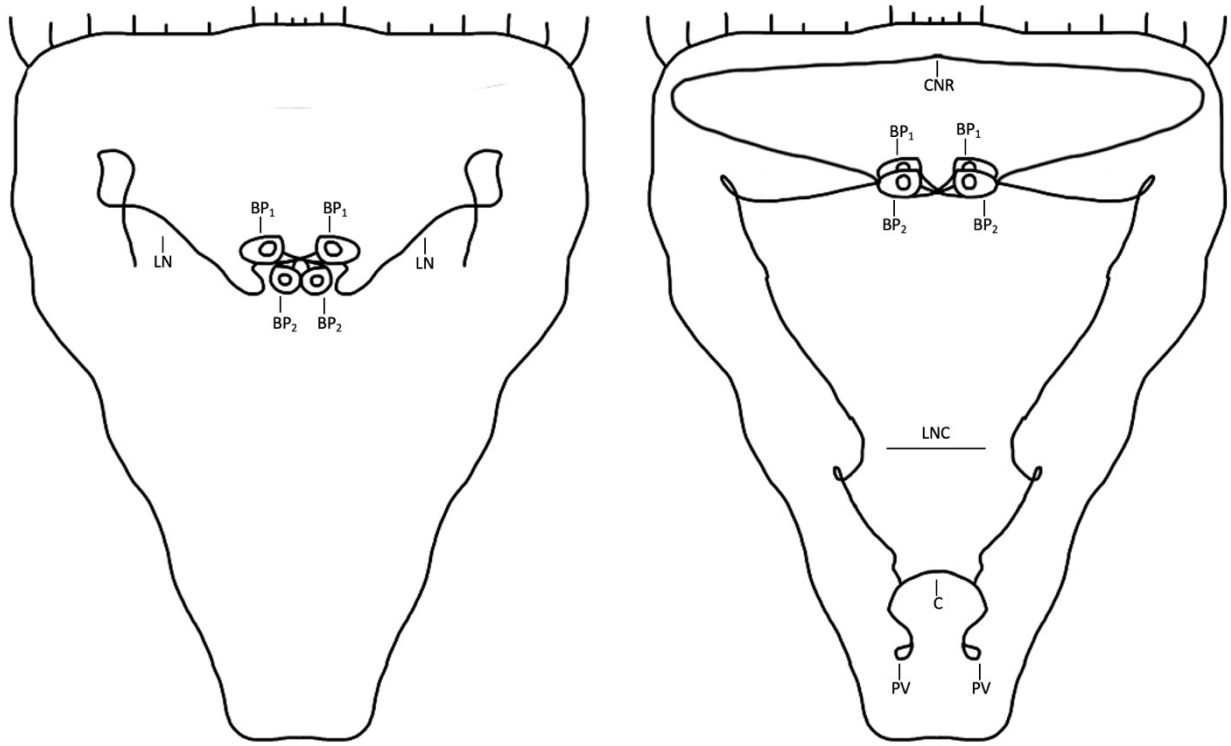

Figure 17. Schematic representation of nervous system derived from serotonin-like immunoreactivity (SLIR) and FMRF-amide-like immunoreactivity (FLIR) for male *Filinia longiseta.* Abbreviations: brain perikarya (BP), lateral neurite (LN), peripheral perikarya (PP), longitudinal nerve cord (LNC), commissure (C), penis varicosity (PV).

Table 6. Summary of nervous system elements in each sex of *Filinia longiseta* using Serotonin-like Immunoreactivity (SLIR) and FMRF-amide-like immunoreactivity (FLIR). Key: (+) indicates structure is present (-) indicates structure is absent.

SLIR | FLIR	*F. longiseta*		*F. longiseta*	
Element	Adult		Male	
Brain Perikarya	6		4	4
Varicosity Commissure	-	-	2	-
	-	+	-	+
Lateral Neurites	+	-	+	-
Longitudinal Neurite Cord	-	+	-	+
Coronal Neurite Ring	-	+	-	+
Peripheral Perikarya	-	6	-	-
	SLIR	FLIR	SLIR	FLIR

For comparison, neuronal elements of all species and life stages are highlighted in Tables 3-5. Nomenclature of nervous system components followed Richter et al. (2010). Variation of SLIR positive brain perikaryal number was evident in *L. flosculosa*, where larvae begin their life cycle with six SLIR positive brain perikarya that reduces to two after metamorphosis has taken

place. Retention of the larval coronal neurite ring in adult *L. flosculosa* was seen and is a common characteristic amongst other lophotrochozoan larvae with ciliary bands (Wanninger, 2008), while the incorporation or retention of some aspects of the larval nervous system is also apparent in these species, consistent with previous findings in actinotroch larvae (Santagata and Zimmer, 2002). Contrastingly, the coronal neurite ring observed in female *F. longiseta* appeared to be innervated by FLIR rather than SLIR. In many invertebrates, ciliary control is often associated with serotonin (Hay-Schmidt, 2000). However, this finding is consistent with similar findings in other rotifers, such as *Notommata copeus* and *Asplanchna brightwellii* (Daday, 1898) (Hochberg 2007, 2009). The lack of the coronal neurite ring in *C. ferox* and *A. inquietus* could suggest alternative neuromodulation, or the modulation of cilia through musculature. There is possibility that SLIR neurites extend into the lobes of the corona since anteriorly projecting neurites are present.

The number of SLIR neurons remained constant throughout each life stage of *C. ferox* and *A. inquietus*, with the only variation being in neurites that innervated the larval corona (*C. ferox/A. inquietus*) and trunk (*C. ferox*). The possible loss of structures in the nervous system may also be associated with metamorphic changes, which is also observed in other lophotrochozoans (e.g., Mollusca (Wanninger and Haszprunar, 2003) and Ectoprocta (Wanninger, et al., 2005)). Specifically, the absence of SLIR/FLIR coronal neurites observed in *C. ferox* (FLIR) and *A. inquietus* (SLIR/FLIR) may be consistent with the absence of the SLIR positive coronal neurite ring observed in *Cupelopagis vorax* (Preza et al. 2020). Overall shape of the nervous system appeared to change in all species that underwent metamorphosis, where the larval life stage appeared X – shape, and the adults arch- shaped. Lastly, SLIR structures remained the same in neonate and adult *F. longiseta*.

Differences in FLIR positive brain perikaryal number was associated with *L. flosculosa* and *A. inquiteus,* where the cerebral ganglion of both species reduced by a total of two brain perikarya after metamorphosis from their larval life stage (brain perikaryal positive structures reduced by two). In *C. ferox* however, the opposite occurs, where the cerebral ganglion of adults had six additional FLIR positive brain perikarya. Though the mechanisms behind the possibility of perikaryal gain and loss after metamorphosis is currently unknown in Rotifera, it is inferred that apoptotic events or changes in neuronal phenotype may occur. Regardless, the shape of the FLIR nervous systems in all metamorphosing appeared to change from X to arch-shaped. No changes in nervous system morphology were observed in *F. longiseta*.

The nervous system of male *L. flosculosa* and *A. inquietus* closely resembled the larval life stage. Male *L. flosculosa* had fewer SLIR brain perikarya (bp=4) than the larval (bp=6) stage, but more than the adult (bp=2). Contrastingly, *A. inquietus* males had more SLIR brain perikarya (bp=4) than the larval and adult life stage (bp=2). FLIR positive structures also revealed that the cerebral ganglion of *A. inquiteus* closely resembled the larval life stage in morphology and brain perikaryal number (bp=6). Interestingly, the FLIR nervous system of *L. flosculosa* males resembled the female adult, containing a square-like neurite structure in the center of the cerebral ganglion, reminiscent of the adult FLIR mastax neurite that was only observed in adult *L. flosculosa*. Difference in brain perikaryal number was also apparent in *L. flosculosa* where males had fewer brain perikarya (bp=4) than larva (bp=8) and adults (bp=6). SLIR/FLIR nervous system elements varied in *F. longiseta* appeared reduced in all aspects but appeared similar to the females in terms of neurite morphology with the only variable structure being FLIR-like longitudinal nerve cords extending into the male copulatory organ.

Discussion

An understanding of the gnesiotrochan nervous system throughout metamorphosis is limited to two studies conducted on the species *Collotheca vorax* and *Stephanocerus fimbriatus*, which documented nervous system variation between life stages (Preza et al., 2020; Hochberg and Hochberg, 2015). These two species represent only a fraction of the diversity in this specialized clade. Also, a lack of information on male gnesiotrochans leaves significant gaps in understanding the relationship between sexual dimorphism and nervous system morphology. My study bridges these gaps in knowledge by examining not only differences in the nervous system of female and male rotifers, but also how the nervous system changes across ontogeny during female metamorphosis.

In my study, I tested two hypotheses on the effects of sexual dimorphism and ontogeny on nervous system morphology in four gnesiotrochan rotifers. These four species represent a range of lifestyles (sessile, colonial, planktonic, parasitic) and developmental strategies (indirect, direct). My hypotheses were tested through confocal analyses of SLIR/FLIR elements of the nervous system of each species. The protocols for immunohistochemistry are well established and permit observations in this study to be compared to observations made on other species in previously published works.

Based on observations of the four gnesiotrochans in this study, I found that the life stages of each species exhibited a comparable level of nervous system complexity, with variation mostly present in their shape and some neuronal elements. The most pronounced variation of the nervous system was observed in *Lacinularia flosculosa*, where a difference of brain perikaryal number was evident after metamorphosis. Additionally, the complexity of the male nervous system in indirect developing species was comparable in shape and complexity with the larval

females. In *Filinia longiseta,* neonates showed no variation of the nervous system compared to adults, and variability of the nervous system was only apparent between sexes. Overall, these findings supported both hypotheses. Firstly, nervous systems of larval rotifers did vary in shape, nervous system elements, and in some cases brain perikarya number. Secondly, the nervous system of male rotifer was comparable to the larval female, varying in few neuronal elements and brain perikarya number.

In *L. flosculosa, Collotheca ferox,* and *Acyclus inquietus,* vermiform larvae and male adults exhibit variation in both SLIR and FLIR expressivity in components of the nervous system when compared to adult females. *Filinia longiseta* had no nervous system variation between neonate and adult, and some similarity between the female and male nervous system (Tables 36). During the metamorphosis of *L. flosculosa,* cellular events appear to reduce the amount of SLIR positive structures in adult females, mostly involving brain perikaryal number in larvae (bp=6) and adults (bp=2) and the no SLIR observed in the upper lobes of the adult corona by the coronal neurite ring. This decrease was also observed using FLIR to view brain perikarya in *L. flosculosa* larvae (bp=8) and adults (bp=6). Two FLIR positive brain perikarya that were observed in *A. inquietus* larvae (bp=6) were not observed after they metamorphose into adults (bp=4).

In the case of *L. flosculosa*, larvae metamorphose to fit the adaptations associated with a sessile/colonial lifestyle. Interestingly, the SLIR/FLIR larval nervous system appears to become more simplified in adulthood. Synaptic pruning events could occur during metamorphosis as has been observed in other lophotrochozoans, specifically phoronids (Santagata and Zimmer, 2002). In phoronids, the process of structural remodeling within the nervous system culminates the establishment of a definitive architecture, integrating parts of the larval nervous system it into

adulthood. This process of neural development may also extend to rotifers, in the form of apoptotic events since a reduction of brain perikarya was evident. One possible explanation for this in *L. flosculosa* could be attributed to their shift from a free-swimming, solitary lifestyle to a sessile, colonial lifestyle. Such a transition could lead to a reduced need for extensive coronal modulation of the adult cilia, and a re-allocation of neurotransmission resources towards other crucial functions (e.g., reproduction) associated with adulthood. It has been previously suggested that the role of serotonin in invertebrates is related to ciliary beating and feeding (Hay-Schmidt, 2000) while the role of FMRF-amide is associated with functions of the peripheral nervous system associated with integrative processing, motion, digestion, and sensory functioning (Serova et al., 2016). In the case of adult *L. flosculosa*, their colonial lifestyle may necessitate less energy allocation for feeding since stronger water currents can be generated by other members of the colony (Wallace, 1987).

Although the reduction of SLIR positive brain perikarya was not observed in *C. ferox* and *A. inquiteus*, the absence of SLIR positive structural components of the nervous system was observed after metamorphosis such as the longitudinal nerve cords in *C. ferox*, and the absence of SLIR positive coronal neurites in *A. inquietus*. In *C. ferox,* the possible loss of the SLIR positive longitudinal nerve cords may be attributed to the limited range of adult movement, which is generally confined to a gelatinous tube where only forward and backward motion is attributed to the contraction of the adult infundibulum rather than the trunk and foot (Meksuwan et al., 2013), which may be associated with the development of the adult infundibular neurites. In *A. inquietus* the retention of the longitudinal nerve cords may be necessary for their colonial/parasitic lifestyle, where predation on *S. socialis* relies on the circular motion of their

infundibulum and trunk to capture prey (Hochberg and Lilley, 2010). Both of which could derive from neuromodulation of serotonin in each anatomical region.

Lastly, changes or reduction of SLIR/FLIR elements in *C. ferox* and *A. inquietus* may have a strong association with the cell types that make up the adult infundibulum. Currently, it is believed the adult infundibulum is derived from tissues in the larval foregut (Hochberg et al., 2019). In rotifers, innervation of the gastrointestinal system is species-specific. For instance, innervation of partial regions of the stomatogastric nervous system by FLIR, SLIR, and -SCPb (small cardioactive peptide b) has been noted in *Notommata copeus* (Hochberg, 2007). Since this is the case, it may be interesting to observe the possible innervation of other neurotransmitters in future research using larval and adult gnesiotrochans.

Overall changes of nervous system shape remained consistent (X-shaped in larvae and arch-shaped in adults) and may be associated with the vermiform larval morphology of gnesiotrochans exhibiting indirect lifestyles. In *L. flosculosa*, *C. ferox*, and *A. inquietus*, the nervous system appeared X- shaped, which was observed in previously studied gnesiotrochans (*S. fimbriatus* and *C. vorax*; Hochberg and Hochberg, 2015; Preza et al., 2020). In all instances, the shape of the nervous system after metamorphosis appeared arched. The overall morphology of this may be driven by factors associated with the retraction of the corona/infundibulum, since invertebrate nervous systems must remain flexible in order to maintain their structure and function in bodies that do not have definitive structural support.

Changes in nervous system morphologies also result from the growth or migration of cells during their metamorphosis. Unlike *A. inquietus* and *L. flosculosa*, *C. ferox* adults express more FLIR positive brain perikarya (bp=14) than their larval life stage (bp=8). While the precise mechanism behind this increase remains uncertain, several potential explanations for these

additional neuronal cell bodies exist. For instance, *C. ferox* may require additional FLIR structures as a result in the change in body plan from larvae to adult. If this is the case, an increase in neurophysiological innervation of FMRF-amide in adulthood may be required for behaviors associated with mating and feeding. Though the mechanisms of their feeding and reproduction are not considered complex, the loss of the corona and eye spots during metamorphosis may require additional neuronal circuits to facilitate sensory adaptation as an adult lacking these features (Hochberg et al., 2019). Regardless, the enigma behind the increase of brain perikarya remains.

Since rotifers are eutelic (i.e., cell number does not increase after hatching), the exact mechanisms that underlie reorganization of cells during metamorphosis remain incompletely understood but is not likely a result of mitotic division (Clément, 1980). Cell ablation may provide insight into the metamorphic process. For instance, in gastropod veliger larvae destruction of the apical sensory organ via irradiated bleaching produce larvae that were not able to respond to metamorphic cues and attain competency (Ruiz-Jones and Hadfield, 2011). Laser cell ablation was also used to destroy the apical sensory organ of the larval polychaetae *Hydroides elegans* but did not impact the metamorphosis of the larvae (Nedved et al., 2021). Currently, no cell ablation studies have been conducted in Rotifera but they may provide interesting results regarding metamorphic strategy and larval competency.

Regeneration of somatic cells in rotifers has not been supported as seen in removal of *S. fimbriatus* tentacles (Van Cleave, 1932). Regeneration of the body is often associated with cell proliferation after tissue damage to repair impaired systems (Bergmann and Stellar, 2010). However, cellular damage does not always constitute mitotic divisions and repair in cells, instead bodily repair has been documented in other phyla such as Ctenophora, where wound healing

occurs as cells migrate to reduce the size of damaged areas (Ramon-Mateu et al., 2019). Furthermore, the overall cellular fate of the larval corona after the development of the adult infundibulum is unknown. In other metamorphic taxa, cells may migrate to other anatomical regions (Karaiskou et al., 2015) or the corona could be reabsorbed similar to velar lobes in veliger larvae (Kriegstein et al., 1974). Ablation of the larval corona or larval nervous system components may provide insight to not only the overall fate of cells during metamorphosis but also the function since some components of the nervous system do not appear to be retained in adulthood. Moreover, focusing specifically on the male nervous system, an examination of the consequences resulting from the ablation of the nervous system components may offer a deeper understanding of the functions affected.

Regardless, the investigation of the evolutionary patterns in the male nervous system's morphology seems to align with the concept of heterochrony which was also proposed for *E. senta* (Gąsiorowski et al., 2019) Notably, numerous similarities can be observed between the overall morphology of the male nervous system in *L. flosculosa* and *A. inquietus* when compared to larval females. In both species examined, the SLIR/FLIR nervous system revealed an Xshaped pattern. The only deviation from this was observed using FLIR in *L. flosculosa* males, which displayed a ringlike structure similar to the mastax neurite found only in adult females.

The shared morphological characteristics of the body plan and nervous system of male and larval *A. inquietus* could imply a conserved developmental pathway, with species-specific modifications and adaptations. For instance, the SLIR nervous system in *A. inquietus* males was nearly identical to larval females, but this was not the case with FLIR which varied in nervous system innervation outside the regions of the cerebral ganglion despite having the same number of brain perikarya. In *F. longiseta,* the male nervous system was structurally similar to the female

for SLIR but varied in innervation associated with FLIR. Despite the drastic variation in morphology of *F. longiseta* dwarf males from females, the shape and structure of the male nervous system appears to be similar, with the only variations being the FLIR longitudinal nerve cords, commissure, and penis varicosities.

Nonetheless, the observation that the male nervous system and its morphology could be linked to the concept of progenesis may hold intriguing implications. Progenesis refers to a process where organisms attain sexual maturity at a faster pace (Westheide, 1987). The gnesiotrochan life history in indirect developing species conforms to this definition, where males are sexually mature upon hatching, while females achieve sexual maturity after metamorphosis (Ricci and Melone,1998). In phylum Rotifera, this could be considered an evolutionary trade off that could enhance reproductive success for male rotifers.

Reproductive success in male rotifers may be attributed to several factors involving their life history and general morphologies. Haploidy serves as a sex determining mechanism, selecting against deleterious mutations that could persist in parthenogenetic populations (Gatto et al, 1992). Additionally, size difference in male rotifers also accounts for an energetic tradeoff between mother and offspring, granted that the male egg is smaller, and requires less yolk partitioning per egg (Gilbert and Schröder, 2004). The co-occurrence of gut vestigialization or reduction also enhances male reproductive success, since they do not need to feed in high density populations that offer increased mating opportunities (Ricci and Melone, 1998).

However, significant gaps in the understanding male rotifer nervous systems still exist, primarily in the development of the embryo. Based on previous investigation, dwarfism does not result from haplodiploidy (Ricci and Melone, 1998). Additionally, the loss of the digestive system and dwarfism were both traits that were more recently proposed to be reversible in

monogonont rotifers (Gąsiorowski et al., 2019). Evolutionary reversal from dwarf progenetic males has also been documented in *Osedax priapus* (Vrijenhoek, 2002) providing further evidence that male dwarfism may be evolutionarily labile and is likely a result of selective pressure (Rouse et al., 2015).

In this context, the implications of reverting dwarfism of male gnesiotrochans may yield interesting results and could shed light on the embryonic development, evolution, and phylogenetic placement of rotifers overall. The investigation of rotiferan HOX genes have been conducted on a single rotifer species, *Brachionous manjavacas*, Fontaneto, Giordane, Melone and Serra, 2007 and are likely conserved throughout the phylum (Fröbius and Funch, 2017*)*. Given this, the exploration of rotiferan HOX genes is essential in understanding the phylogenetic placement of Rotifera within Lophotrochozoa, while understanding their modes of development through cell lineage studies could further establish their relationship with Spiralia. HOX gene expression may be differentially expressed in each sex, since males lack many features associated with females (e.g., trophi, defensive spines, complete digestive tracts, etc.), and could help us understand aspects of organogenesis/neurogenesis, and further our understanding of metamorphosis in Rotifera.

Dwarfism, gut reduction, and faster swimming speed in male rotifers combined with the anatomy and nervous system complexity of male gnesiotrochans could be advantageous since their energy allocation can be strictly associated with increasing their reproductive output (RicoMartínez and Snell, 1997). The association of the male nervous system, dimorphism, and progenesis suggests that the neural development and maturation of the male reproductive system could play a crucial role in facilitating reproductive strategies (e.g., pre copulatory mate guarding (Schröder, 2003), circling (Rico-Martínez and Walsh, 2013), and pheromonal detection (Snell

and Rico- Martínez, 1996)). Lastly, it is important to note that progenesis, gut reduction/absence, or simplification of the male nervous system are likely shaped by a combination of genetic factors and environmental pressures. Understanding the mechanisms underlying this evolutionary trade-off can provide valuable insights into the reproductive strategies and dynamics of rotifer populations.

Conclusions

The neuroanatomy of *L. flosculosa*, *C. ferox*, and *A. inquietus* females exhibited variability in some nervous system structures that comprise the larval and adult nervous system but show similar levels of complexity. This could indicate a divergence in neurophysiology and function necessary for each life stage. *Filinia longiseta* females showed no variation in the nervous system during different life stages (neonate versus adult). The nervous system of male *L. flosculosa* and *A. inquietus* was comparable to the larval females and may be associated with the heterochrony and progenesis. Similarly, the nervous system of male *F. longiseta* was reduced, but comparable to the adult female. These findings imply that the male nervous system in these species share similarities with the female based on different lifestyle strategies (direct versus indirect development). The observed similarities between larval, adult, and male rotifers provide valuable insights into the neuroanatomy, life stage, sex, and reproductive strategy. Further investigation is needed to elucidate the developmental mechanisms, neurophysiology, and function, of these neural adaptations to shed light on the evolutionary history and complex behaviors of Rotifera.

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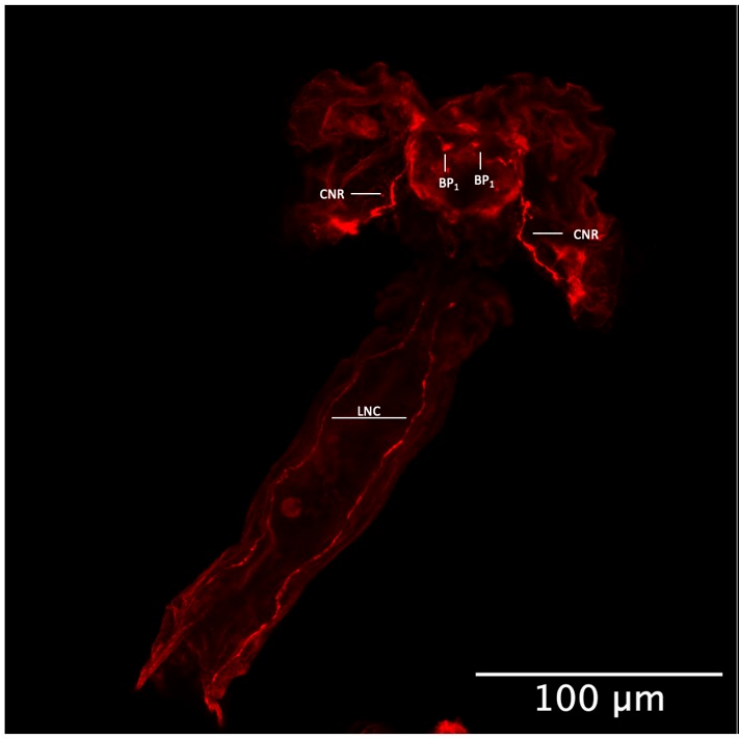
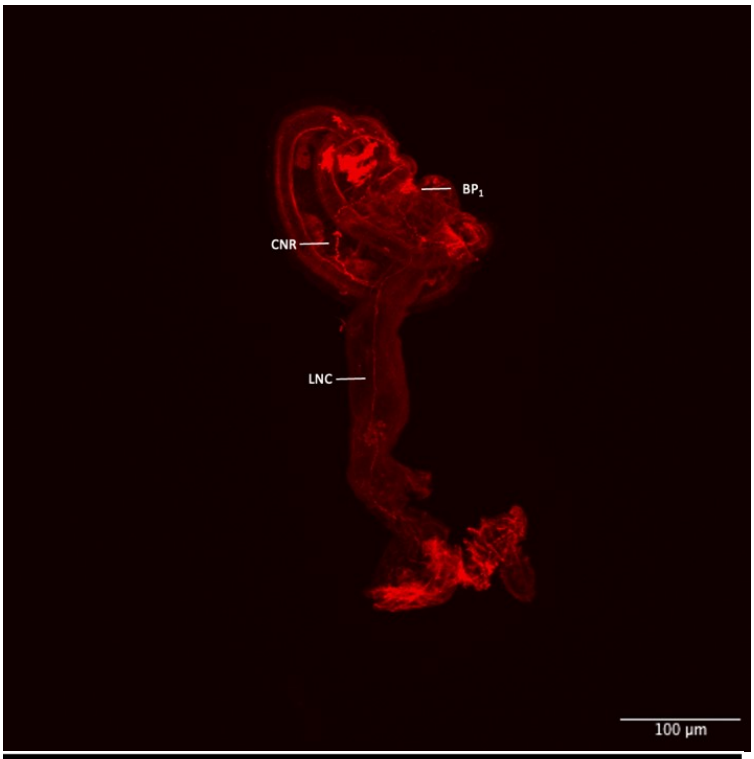
APPENDIX

This appendix contains a collection of additional confocal images highlighting features of the nervous system in the following species: *L. flosculosa*, *C. ferox*, *A. inquietus*, and *F. longiseta*. The inclusion of these images serves to establish that the structures presented in the results were not isolated occurrences and observed in multiple instances. The images provided further support the presence of morphological characteristics of the nervous system in each of the mentioned species.

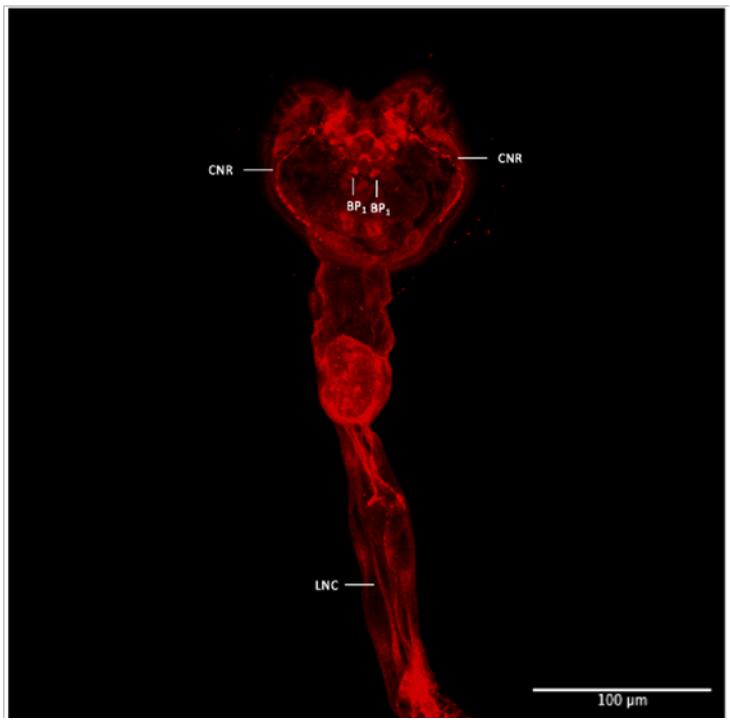
Abbreviations: brain perikarya (BP), coronal neurite ring (CNR), longitudinal neurite cord (LNC), coronal neurite (CN), mastax neurite (MN), lateral neurite (LN), anterior neurite (AN), anterior neurite projections (ANP), varicosity (V), peripheral perikarya (PP), longitudinal nerve cord (LNC), commissure (C), penis varicosity (PV), foot varicosity (FV), infundibular varicosity (IV), coronal varicosity (CV), neuropil (NP).

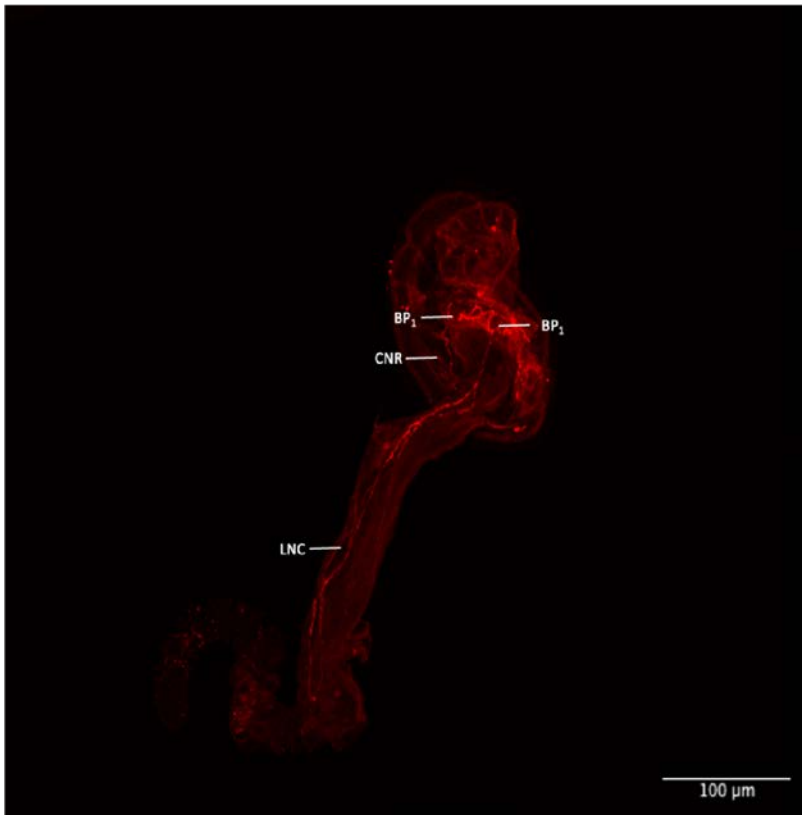
The images below show SLIR positive structures of adult female *Lacinularia flosculosa*

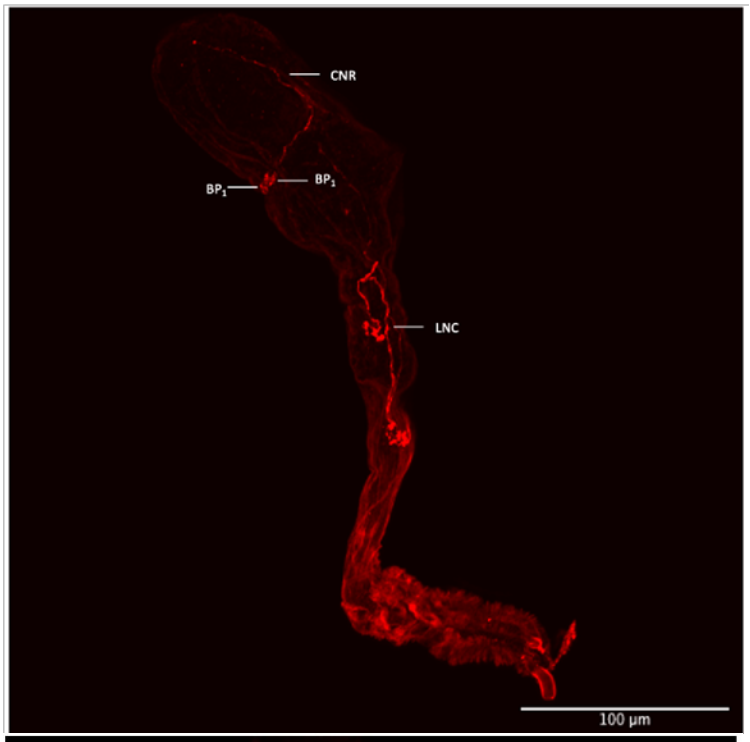

CNR
BP1
BP1
CNR
LNC
100 µm

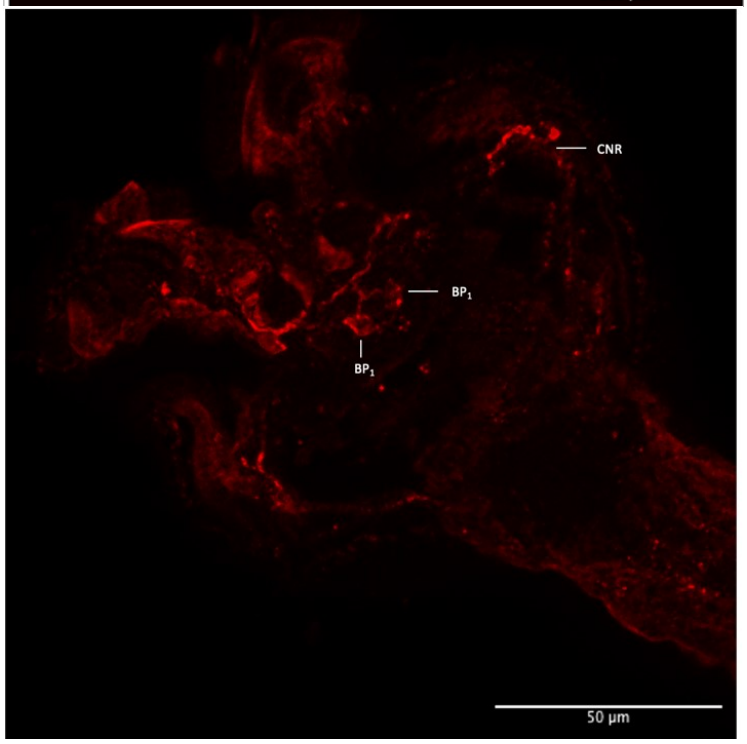

BP1
CNR
LNC
100 µm


BP1
BP1
CNR
CNR
100 µm

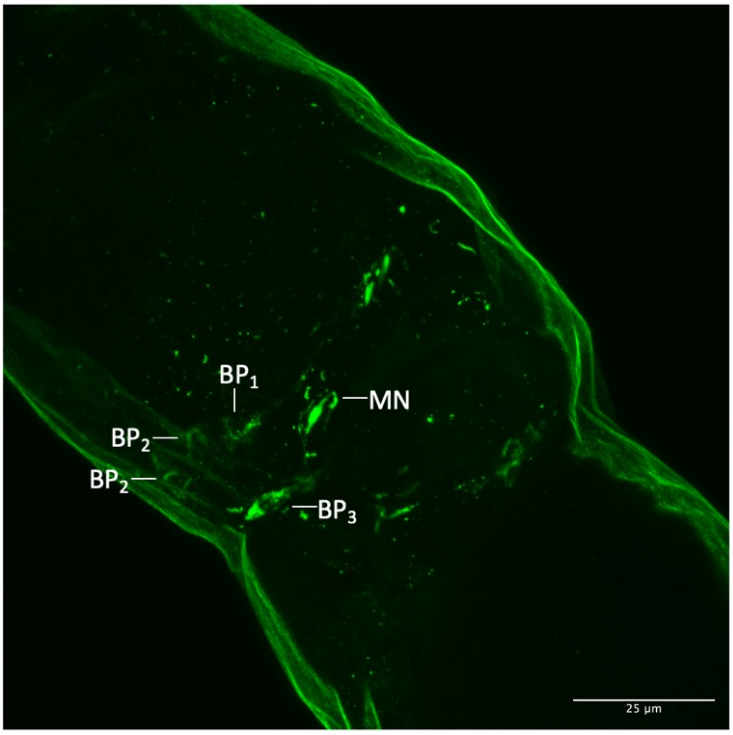

CNR
CNR
BP1 BP1
LNC
100 µm

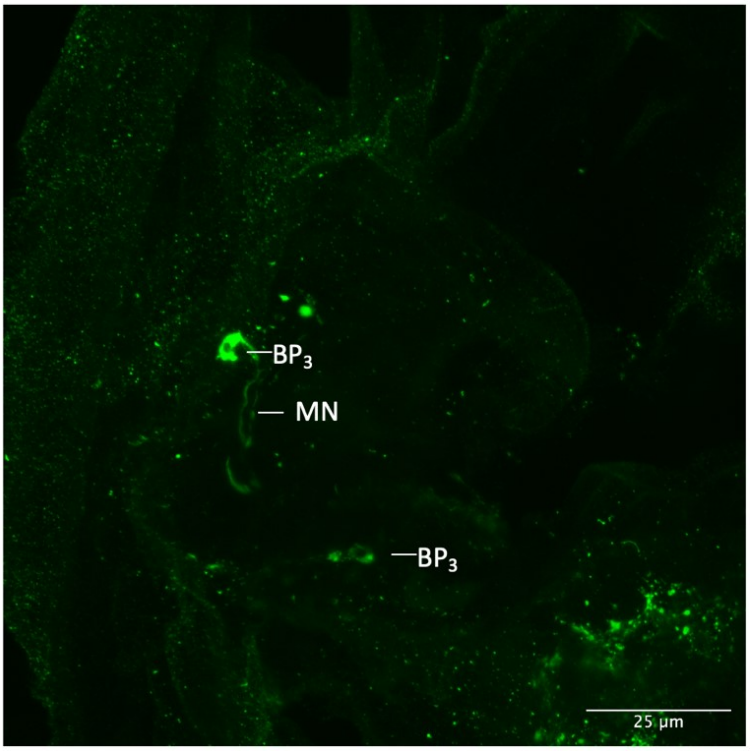

BP1
BP1
CNR
LNC
100 µm

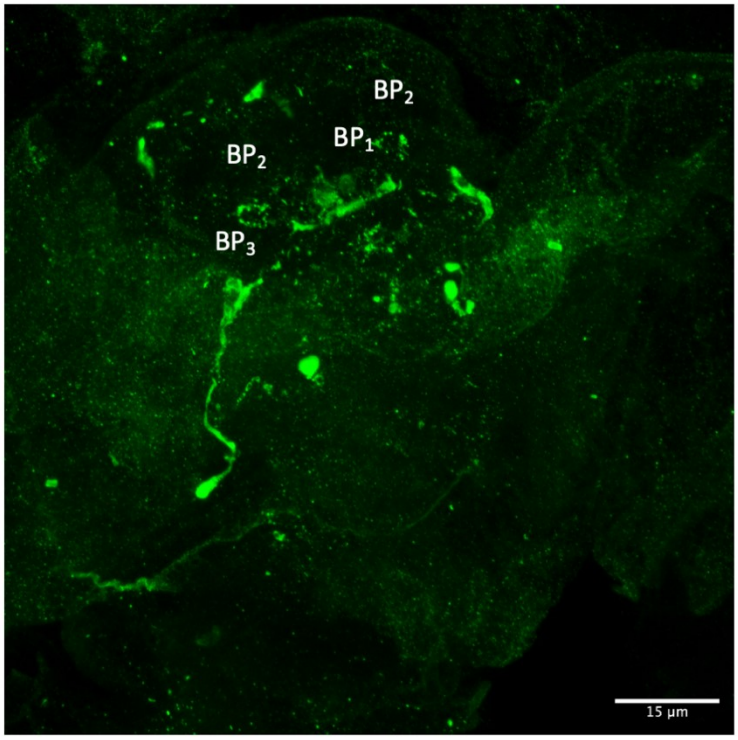


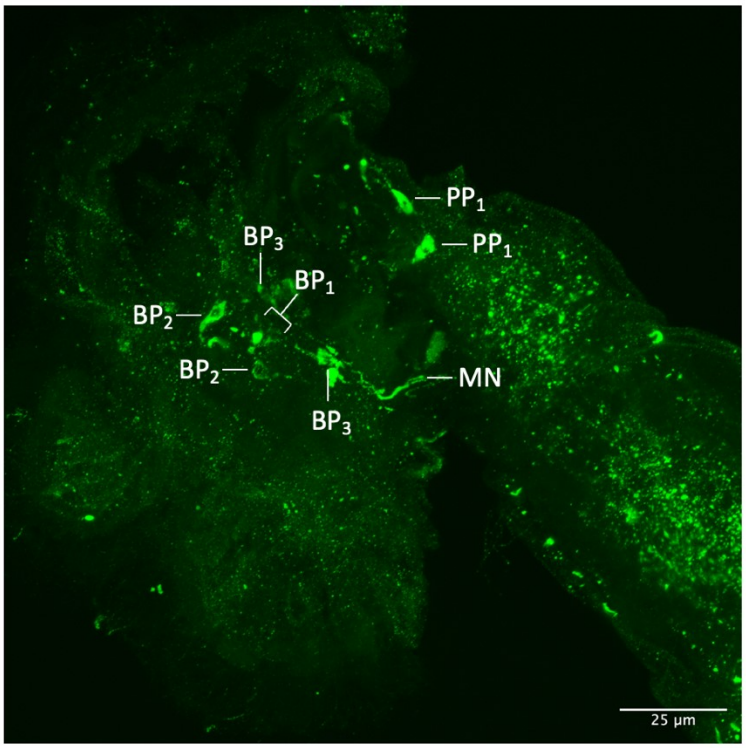


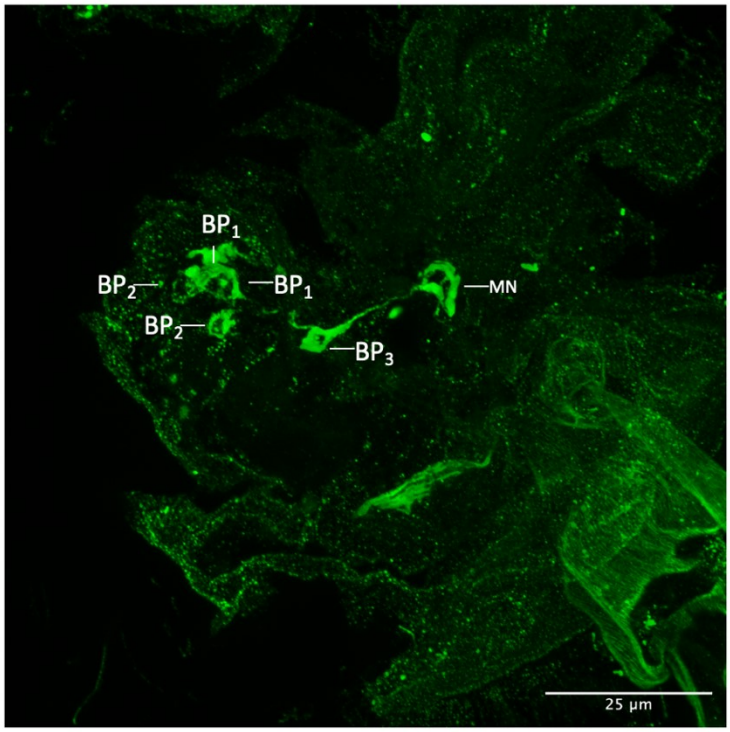
The images below show FLIR positive structures observed in adult female *Lacinularia flosculosa.*

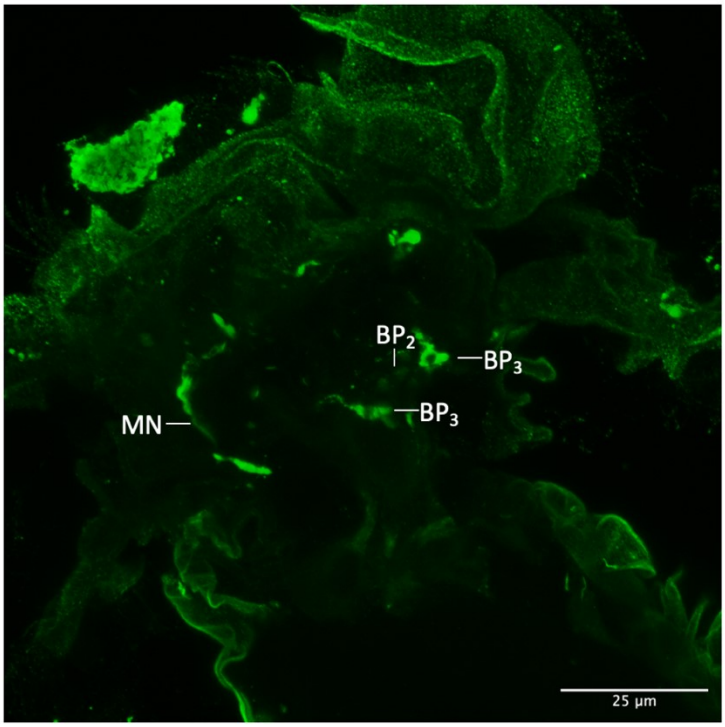

BP1
MN
BP2
BP2
BP3
25 µm

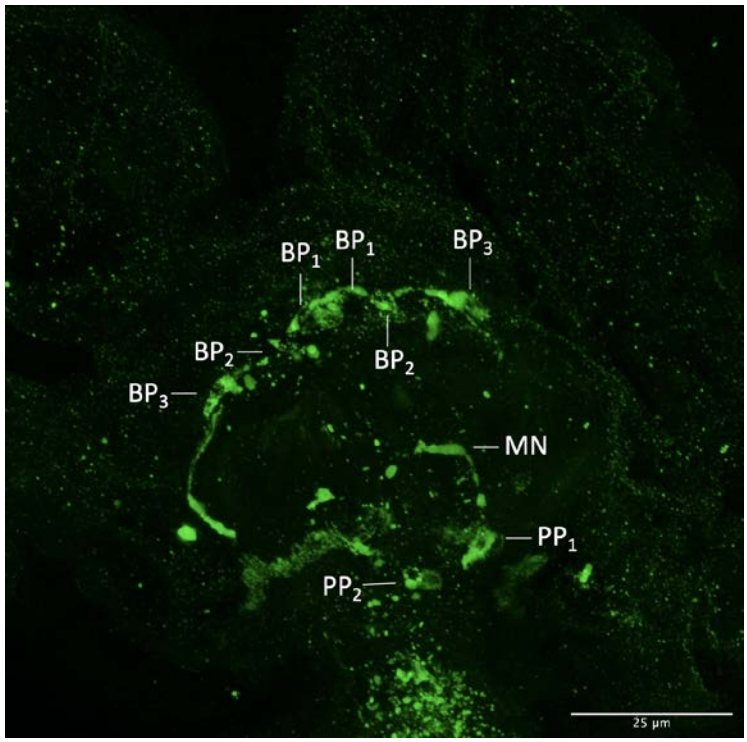

BP3
MN
BP3
25 µm


BP2
BP1
BP2
BP3
15 µm

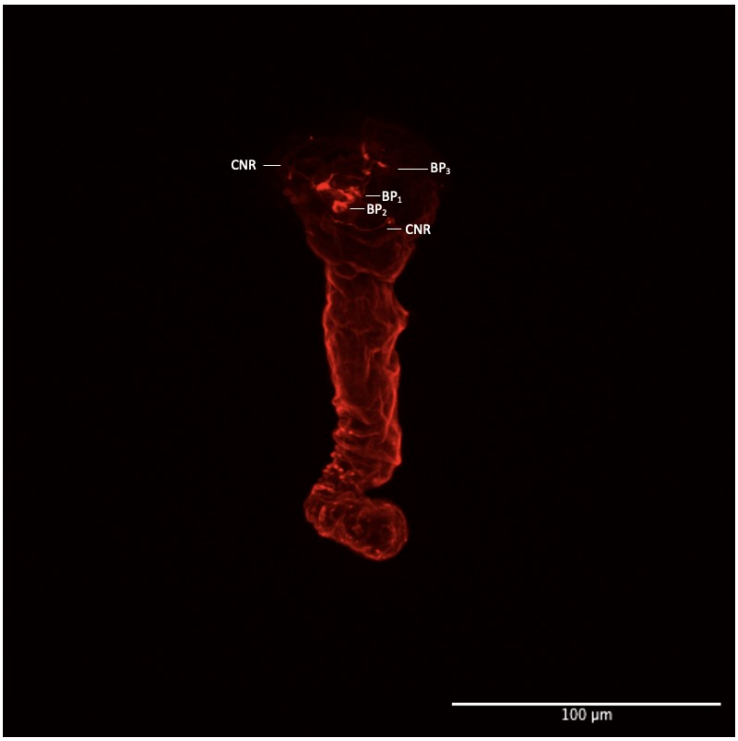

PP1
PP1
BP3
BP1
BP2
BP2
MN
BP3
25 µm

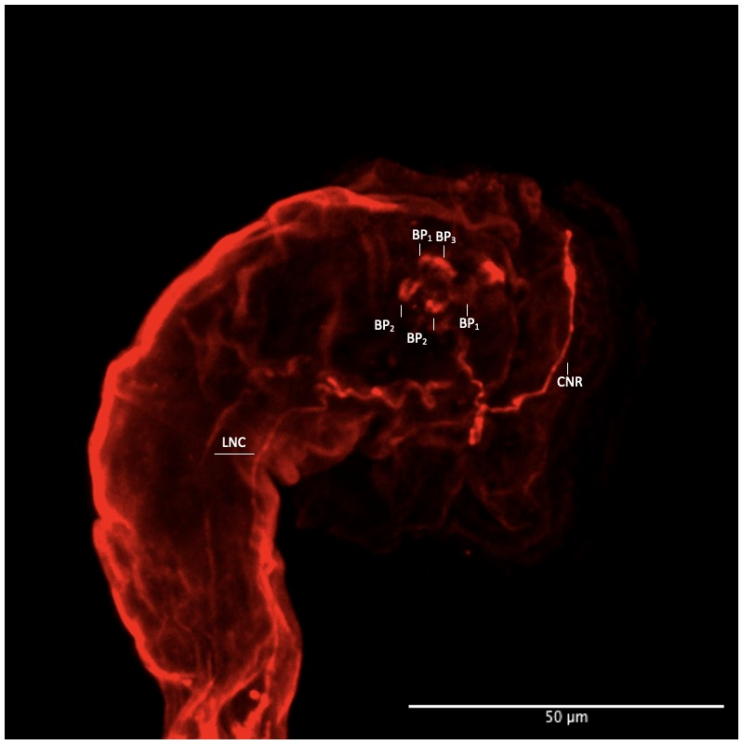

BP1
BP2
BP1
MN
BP2
BP3
25 µm

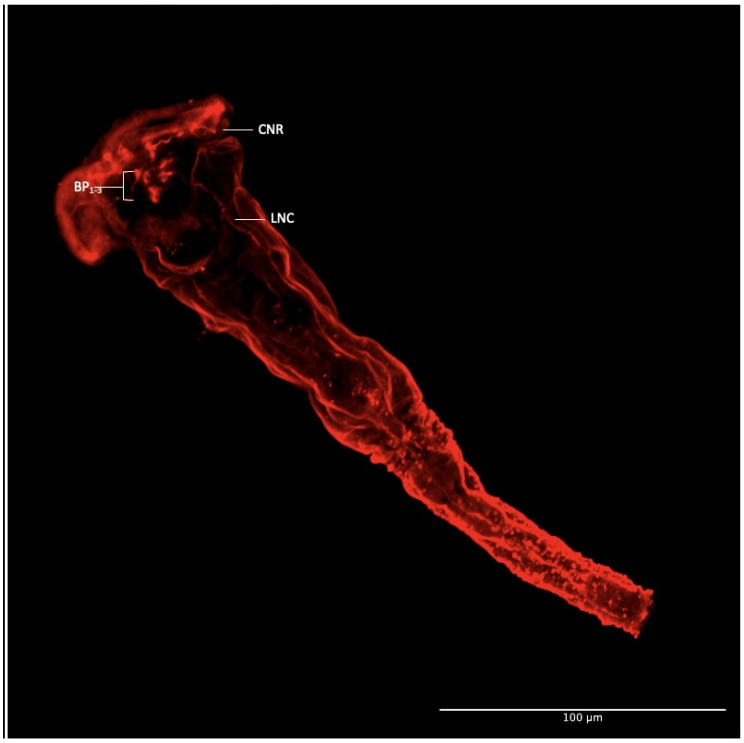

BP2
BP3
BP3
MN
25 µm

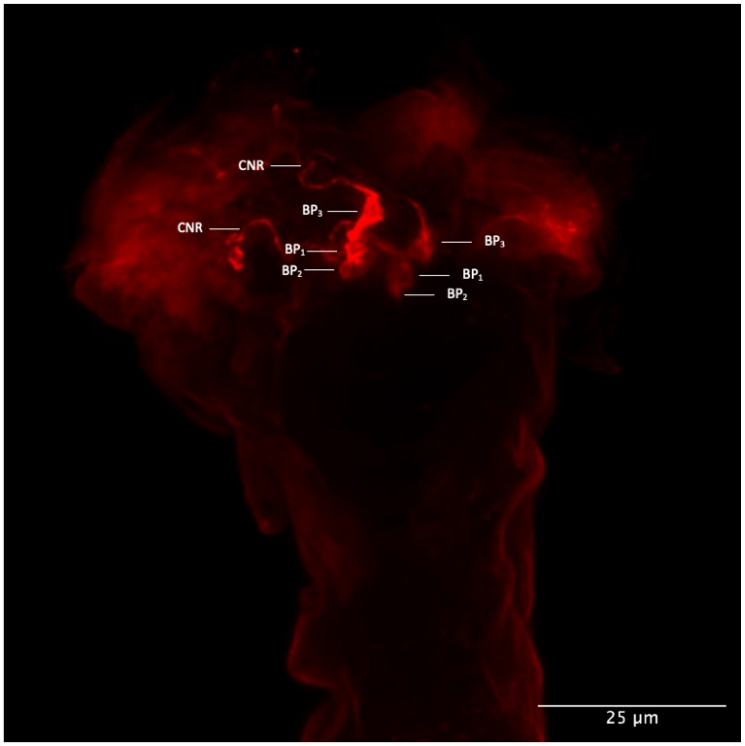


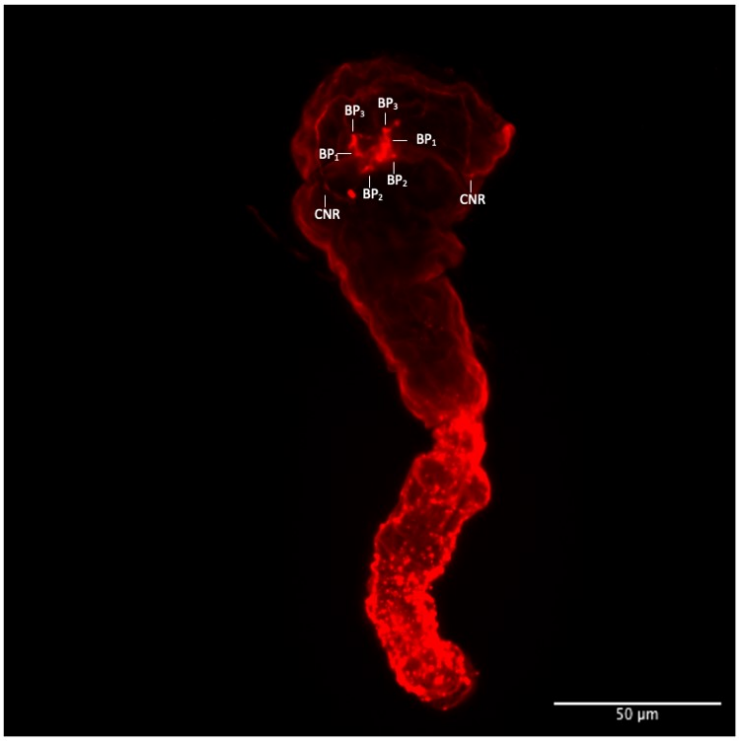
The images below show SLIR positive structures observed in larval *Lacinularia flosculosa.*

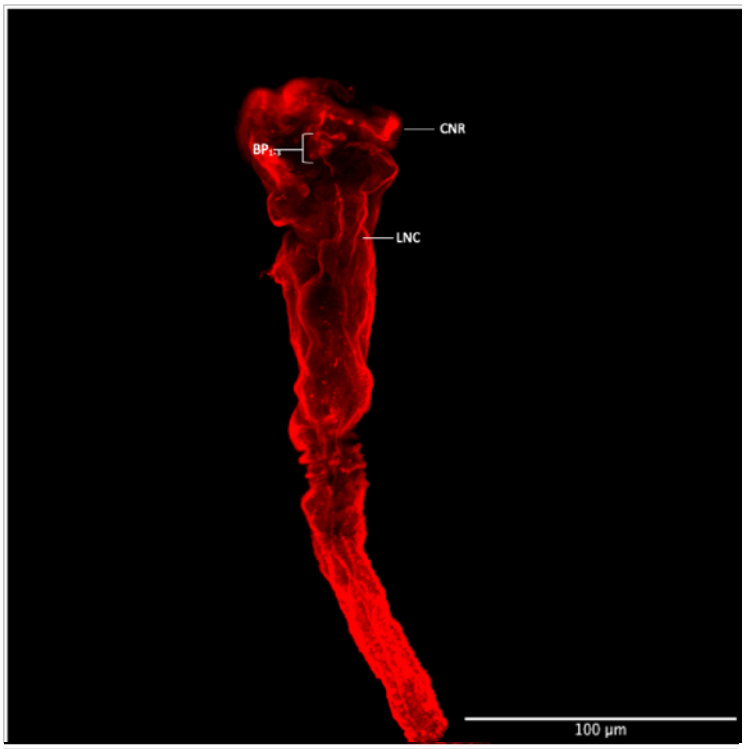


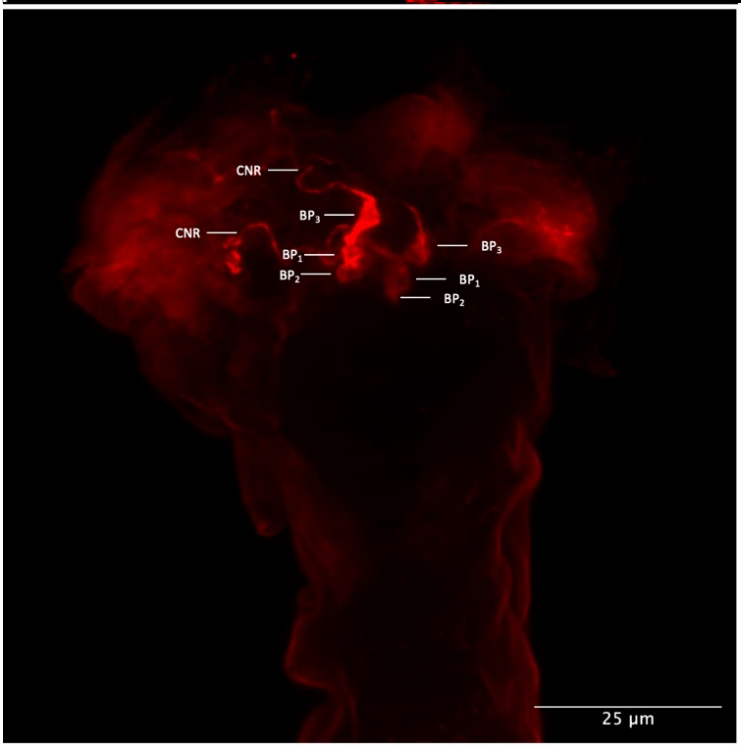

BP1 BP3
BP2
BP2
BP1
CNR
LNC
50 µm

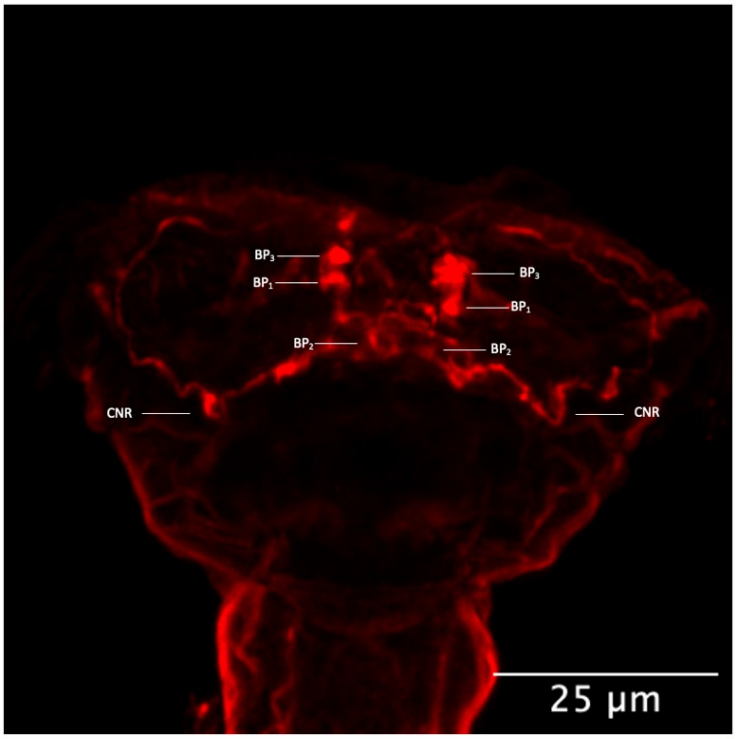

CNR
BP1-3
LNC
100 µm

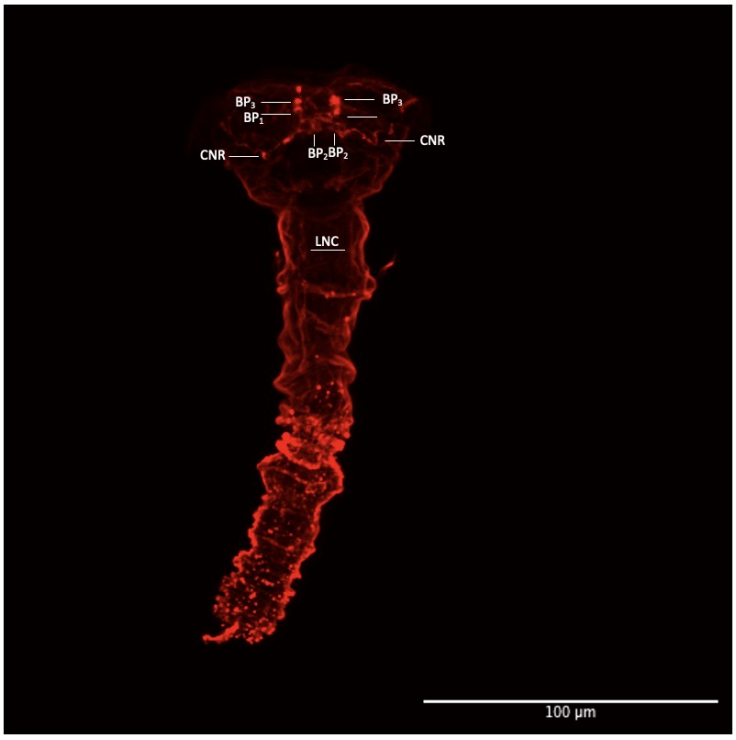

CNR
BP3
CNR
BP1
BP2
BP3
BP1
BP2
25 µm

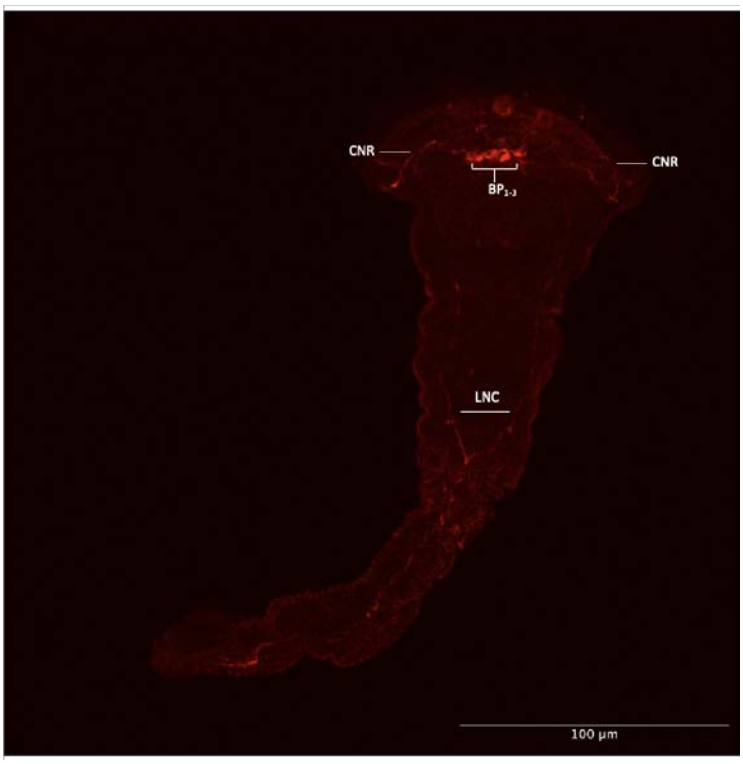

BP3
BP3
BP1
BP2
BP2
BP2
CNR
CNR
50 µm


CNR
BP1-3
LNC
100 µm

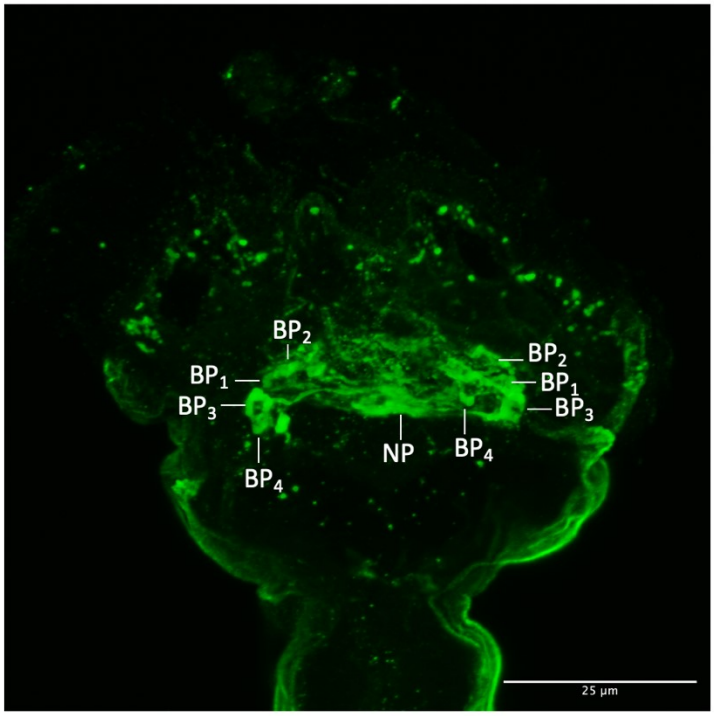

CNR
BP3
CNR
BP1
BP2
BP3
BP1
BP2
25 µm

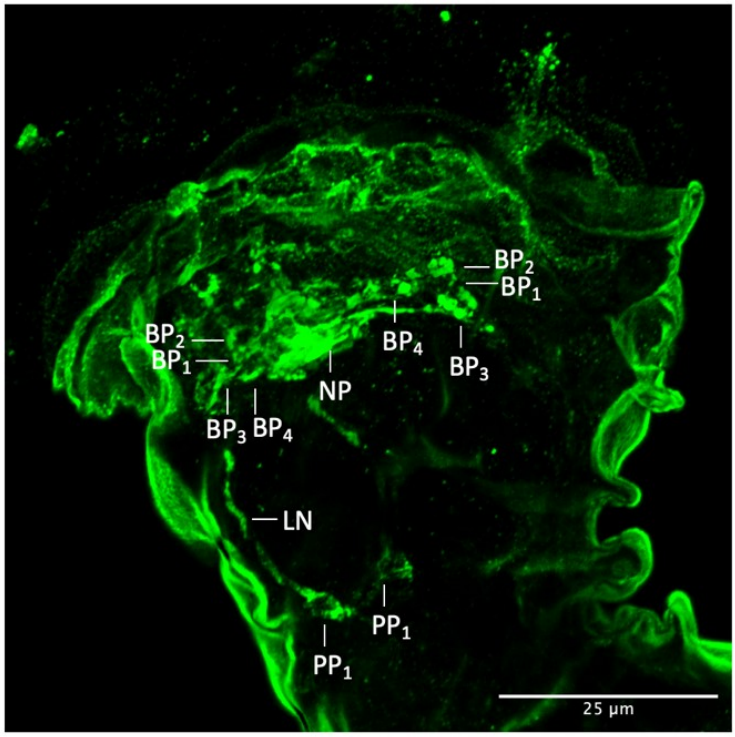

BP3
BP1
BP3
BP1
BP2
BP2
CNR
CNR
25 µm

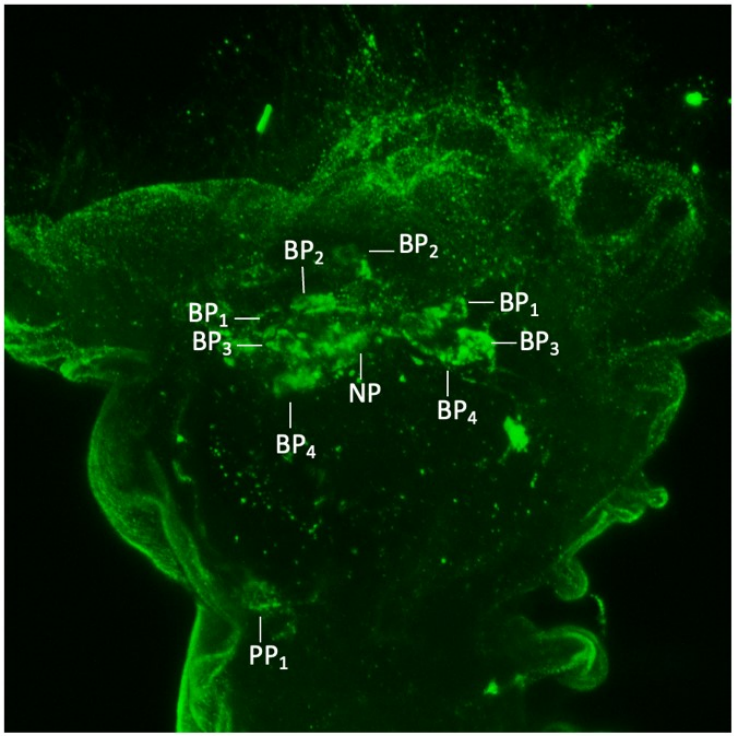

BP3
BP1
BP3
CNR
CNR
BP2BP2
LNC
100 µm

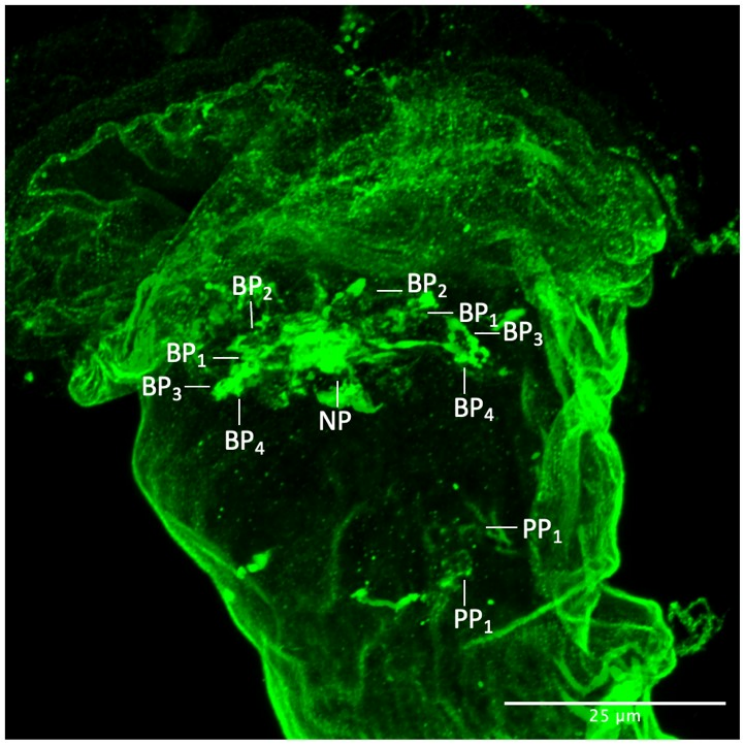


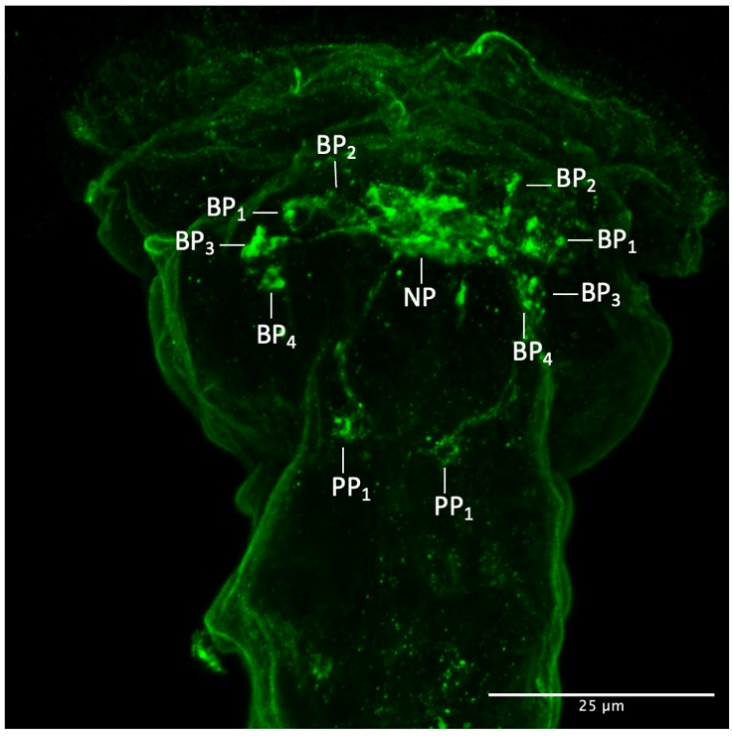
The images below show FLIR positive structures observed in larval *Lacinularia flosculosa.*

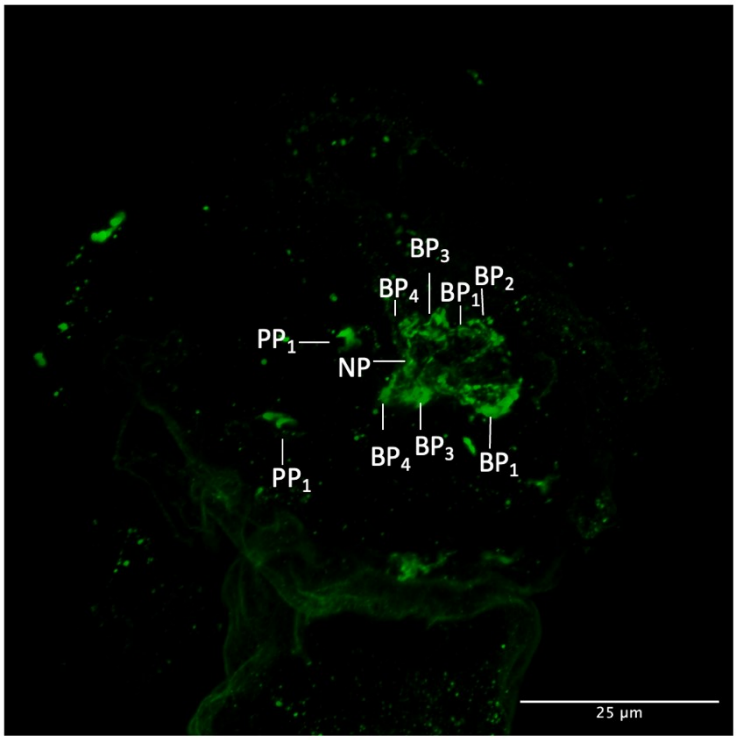


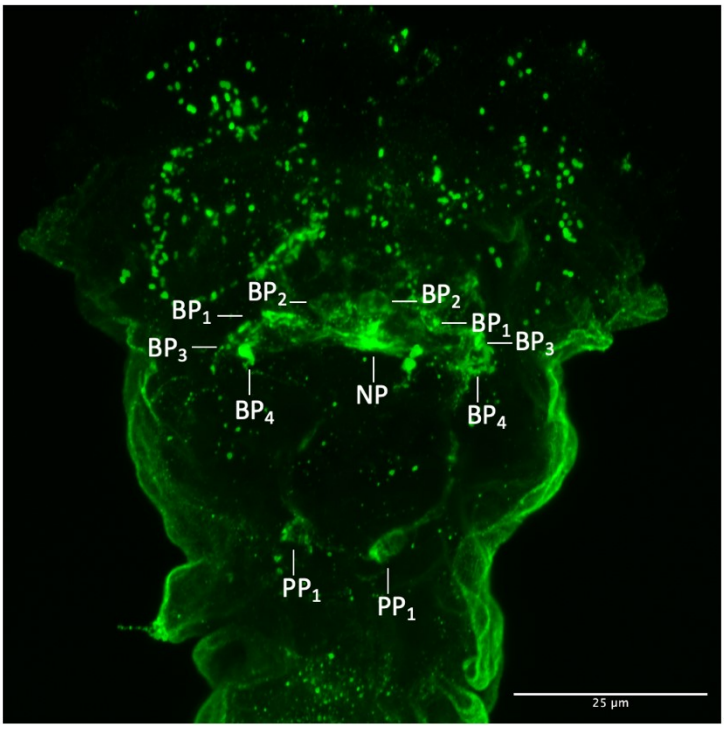

BP2
BP1
BP2
BP1
BP4
BP3
NP
BP3
BP4
LN
PP1
PP1
25 µm

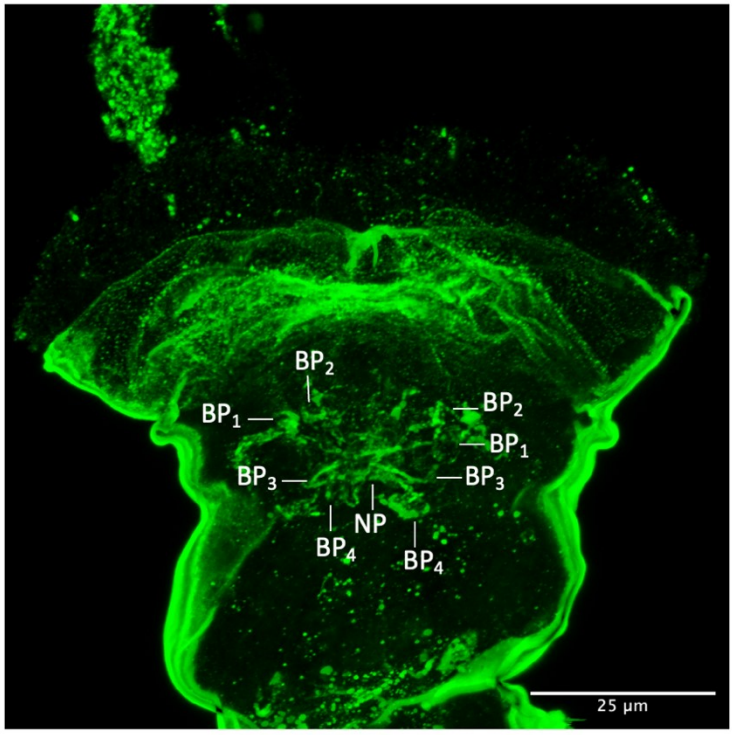

BP2
BP2
BP1
BP1
BP3
BP3
NP
BP4
BP4
PP1

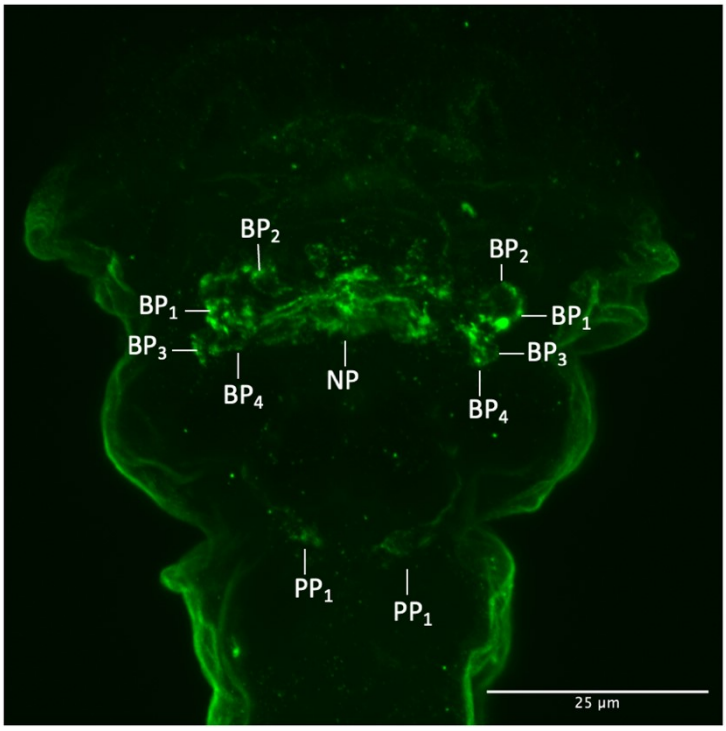

BP2
BP2
BP1
BP3
BP1
BP3
NP
BP4
BP4
PP1
PP1
25 µm


BP2
BP2
BP1
BP3
BP1
NP
BP3
BP4
BP4
PP1
PP1
25 µm

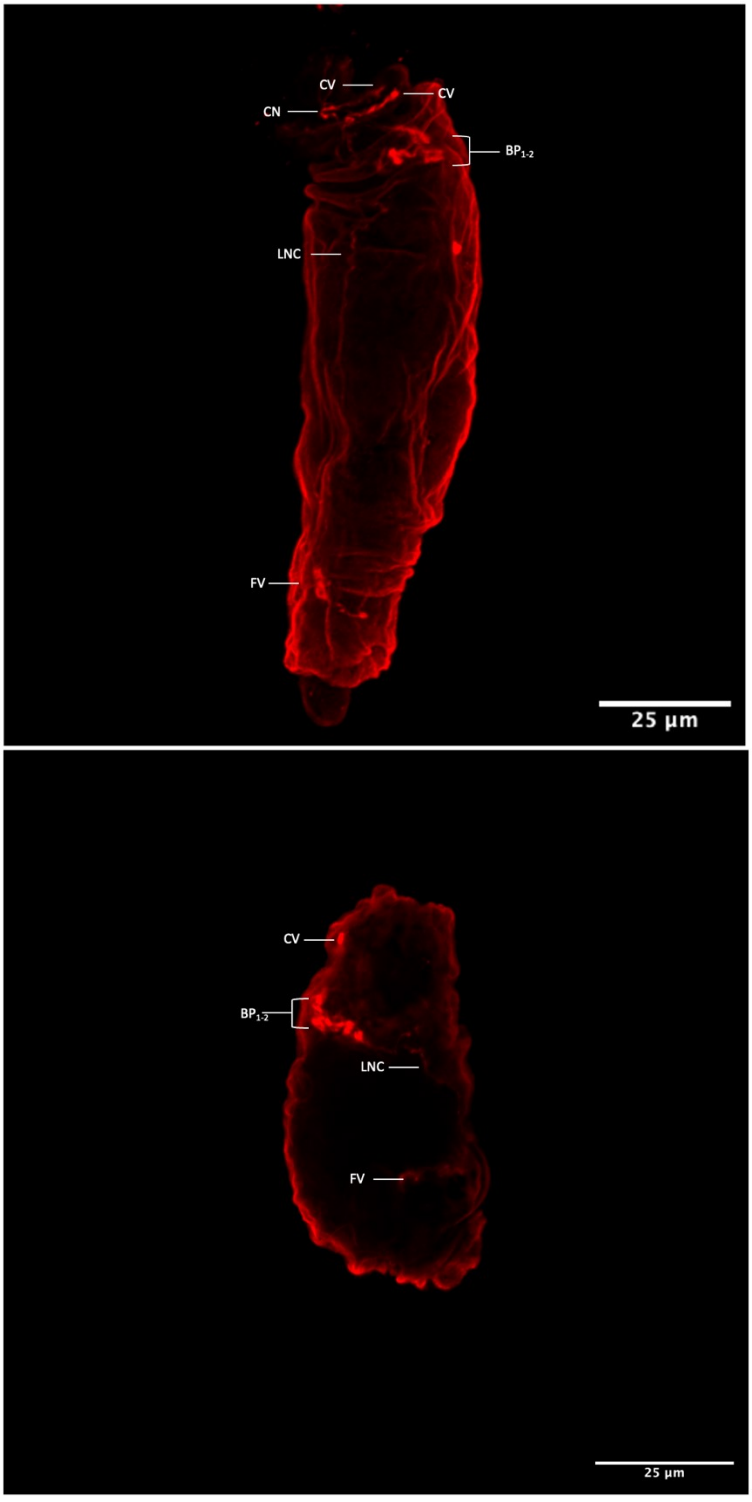

BP3
BP4
BP1
BP2
PP1
NP
BP4
BP3
BP1
PP1
25 µm

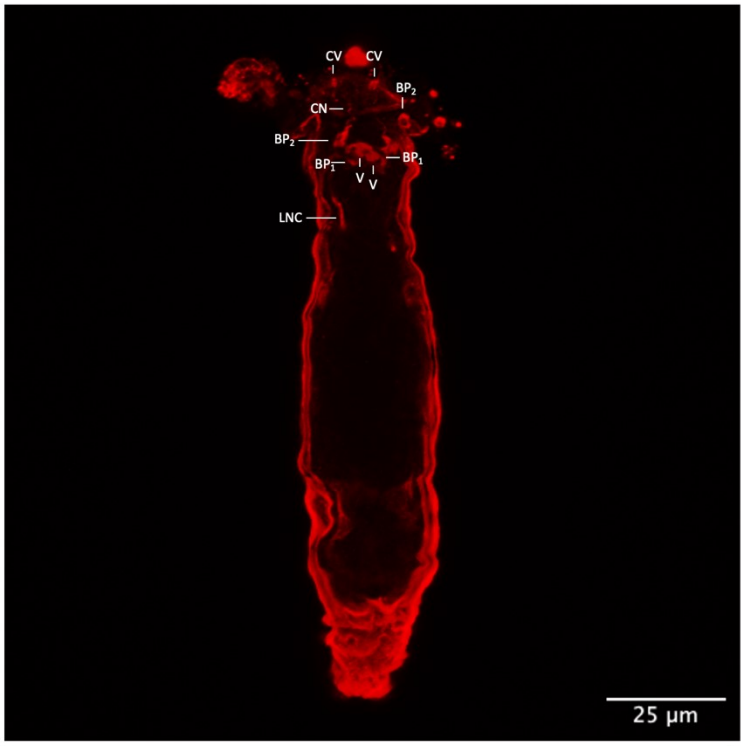

BP1
BP2
BP2
BP1
BP3
BP3
NP
BP4
BP4
PP1
PP1
25 µm

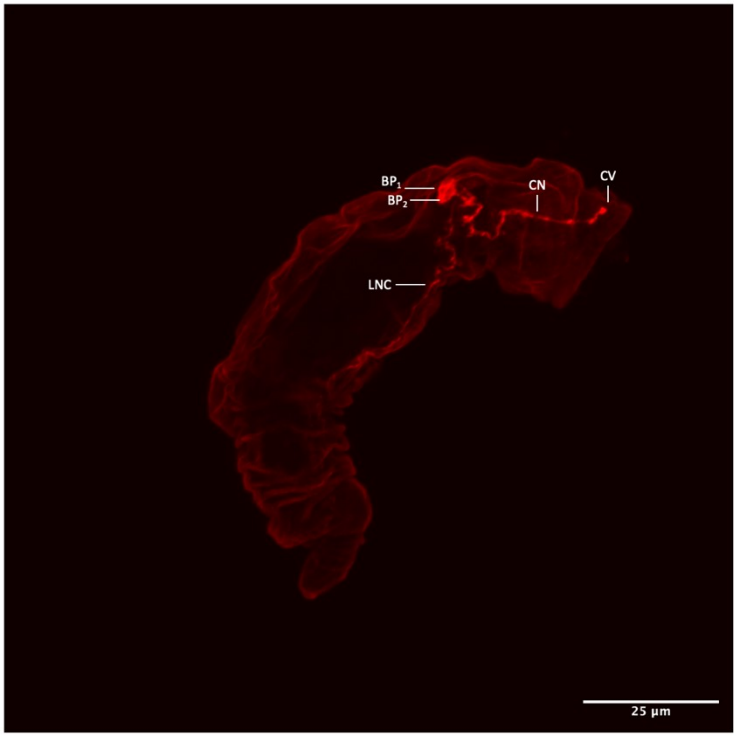

BP2
BP1
BP2
BP1
BP3
BP3
NP
BP4
BP4
25 µm

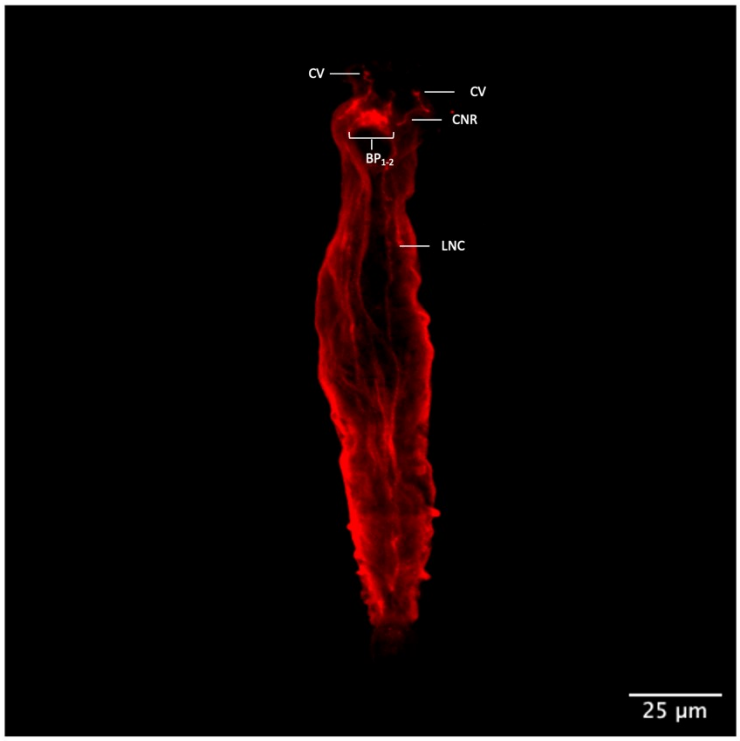

BP2
BP2
BP1
BP1
BP3
BP3
NP
BP4
BP4
PP1
PP1
25 µm

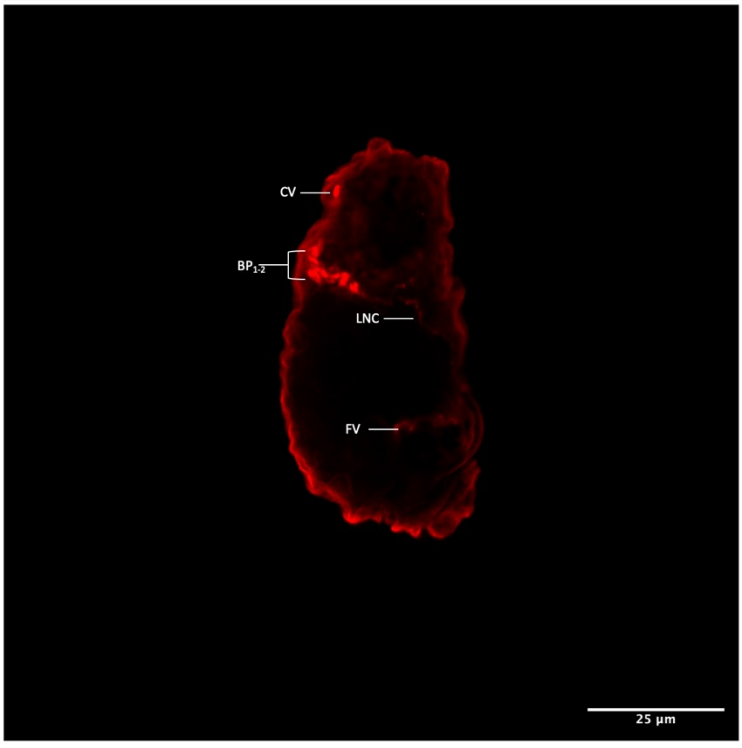
The images below show SLIR positive structures observed in male *Lacinularia flosculosa.*

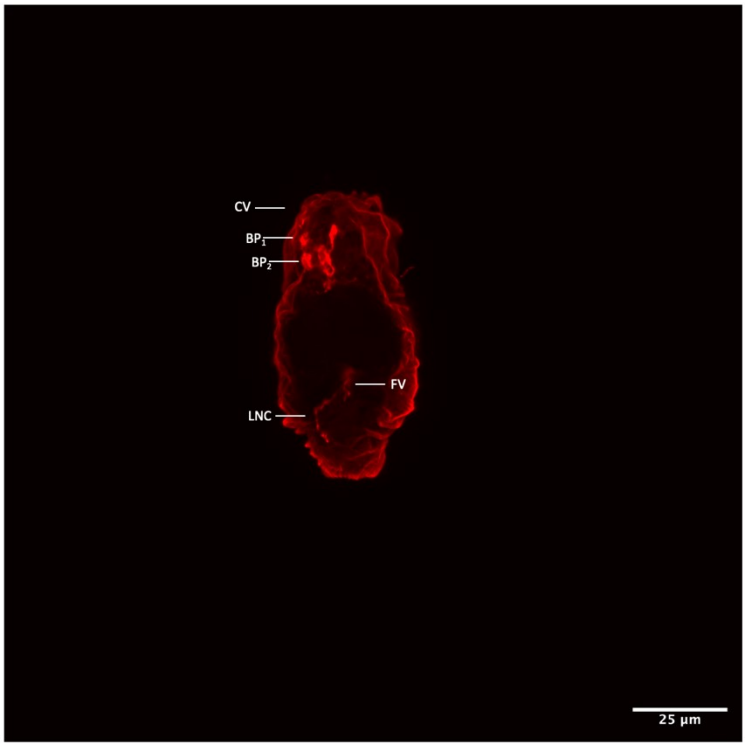



CV
CV
BP2
CN
BP2
BP1
BP1
V
V
LNC
25 µm

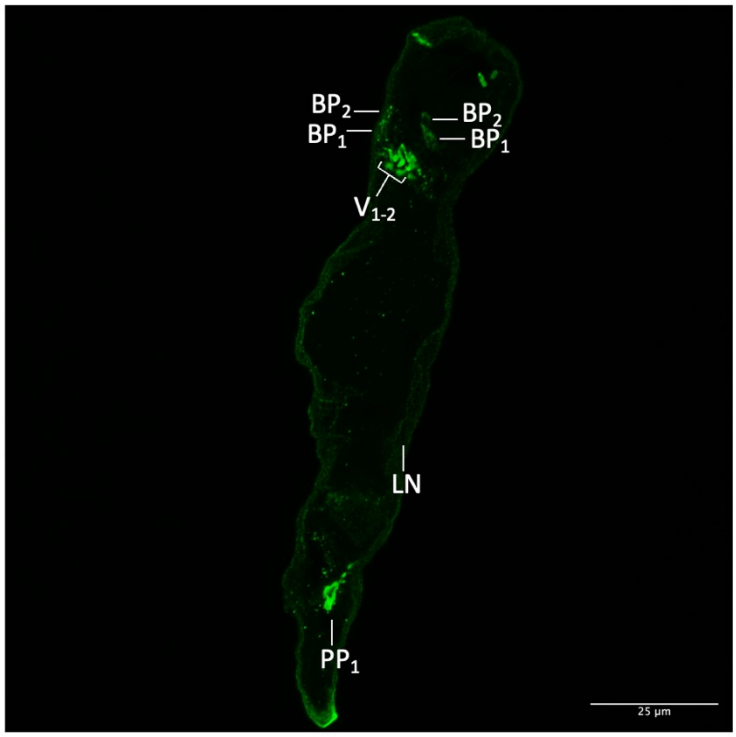

BP1
BP2
CN
CV
LNC
25 µm

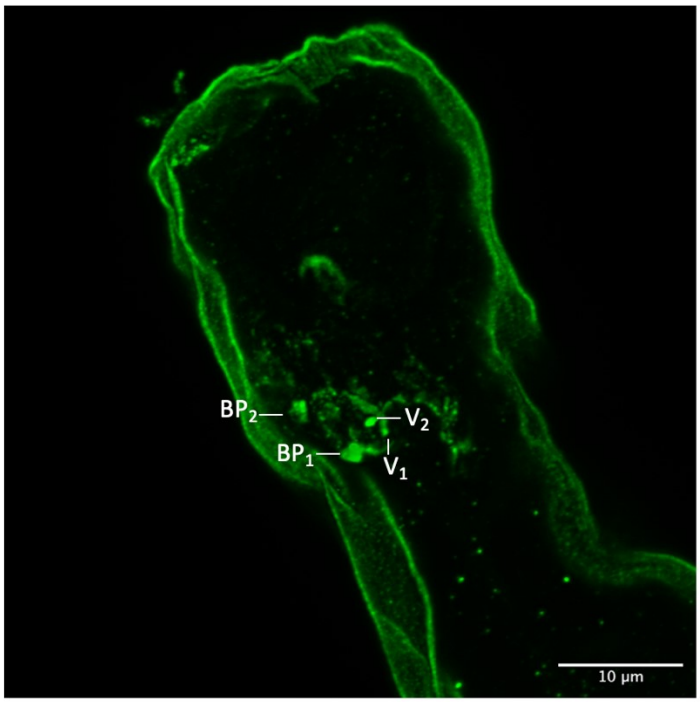

CV
CV
CNR
BP1-2
LNC
25 µm

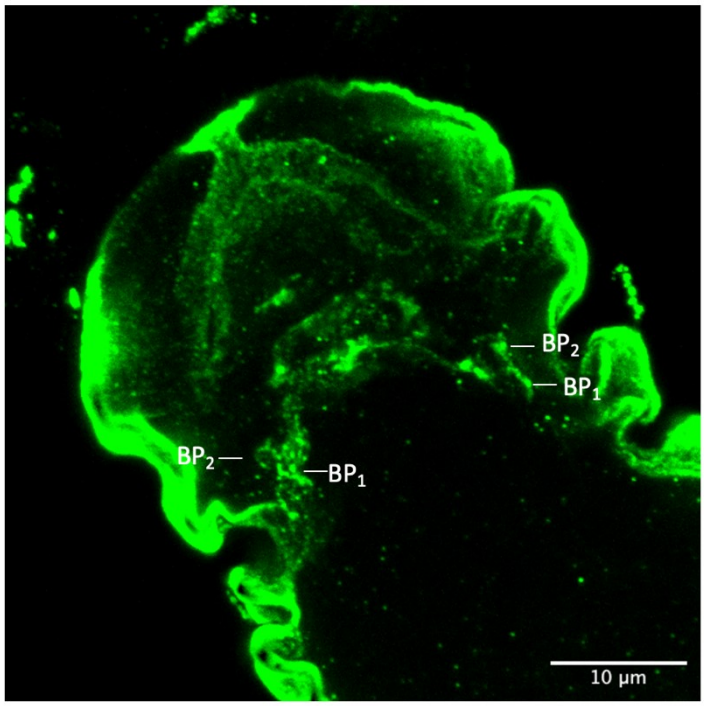

CV
BP1-2
LNC
FV
25 µm

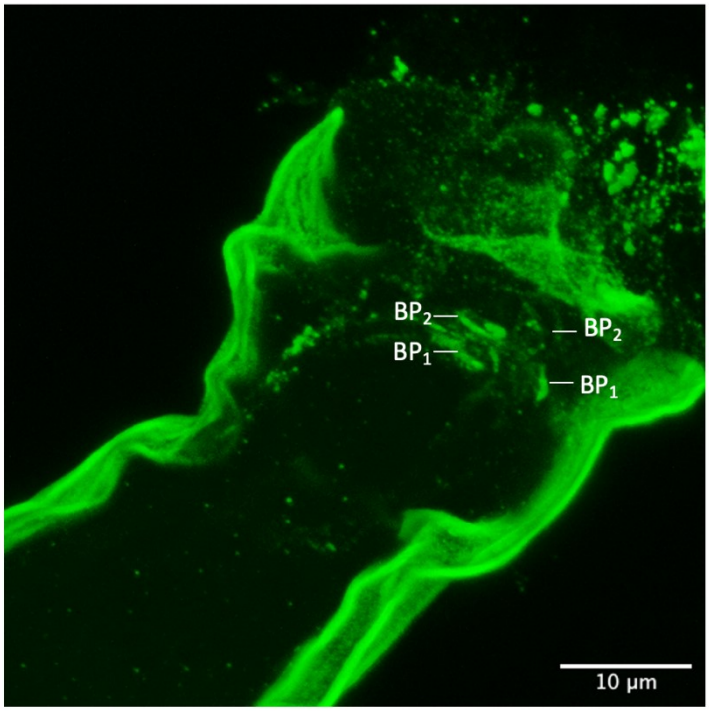


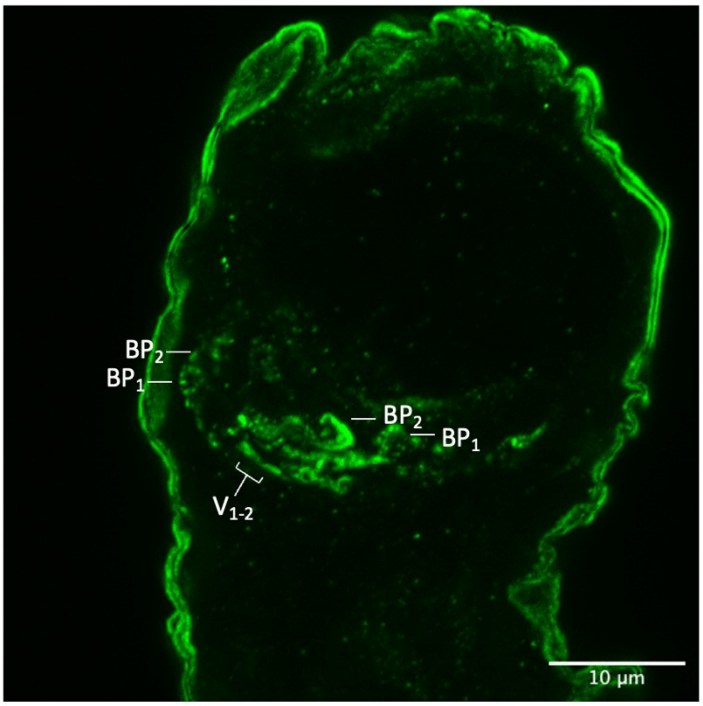
The images below show FLIR positive structures observed in male *Lacinularia flosculosa.*

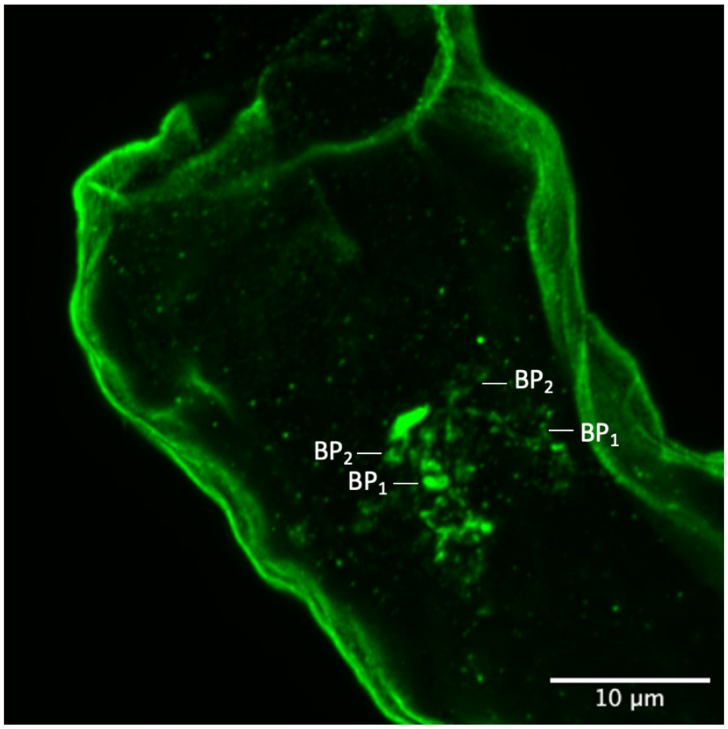



BP2
V2
BP1
V1
10 µm

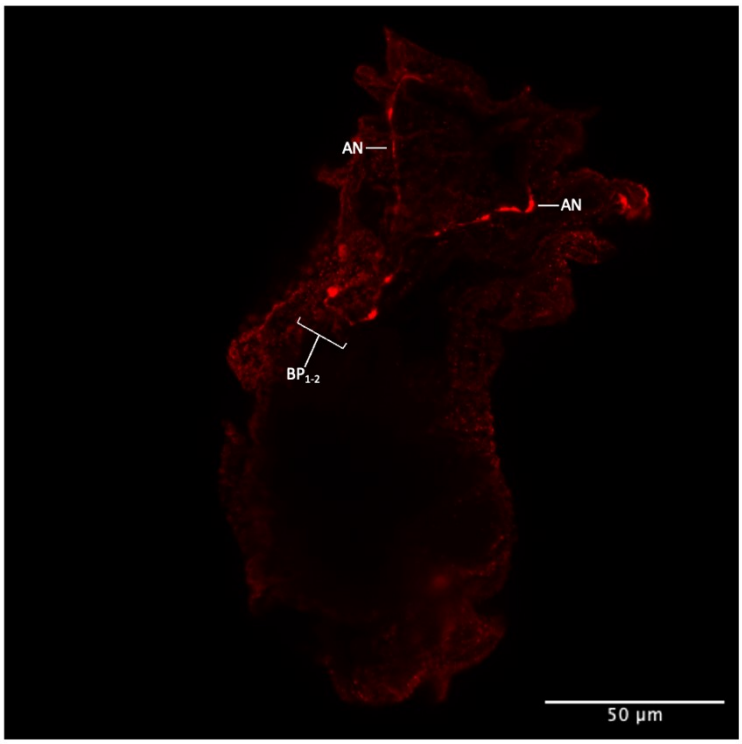

BP2
BP1
BP2
BP1
10 µm

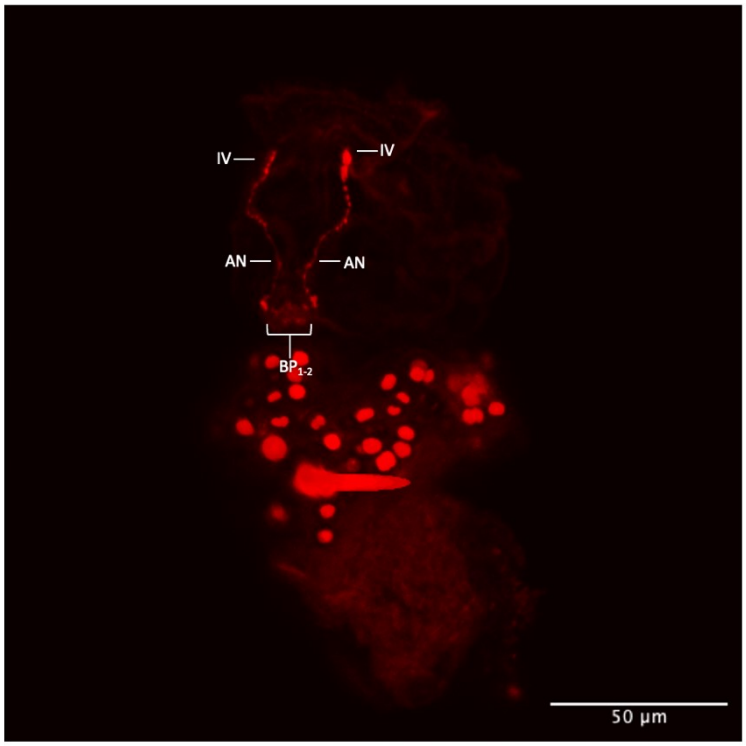

BP2
BP1
BP2
BP1
10 µm

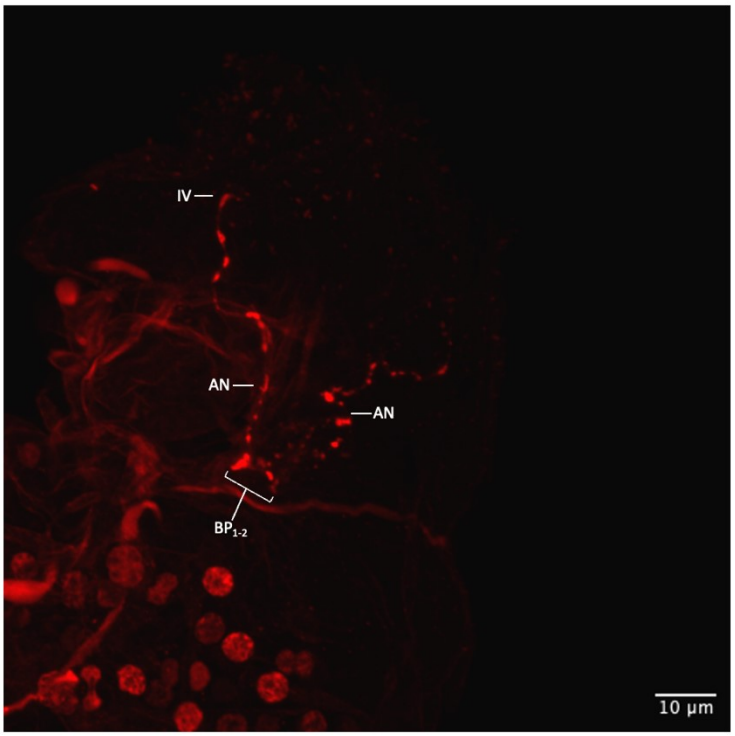

BP2
BP1
BP2
BP1
V1-2
10 µm

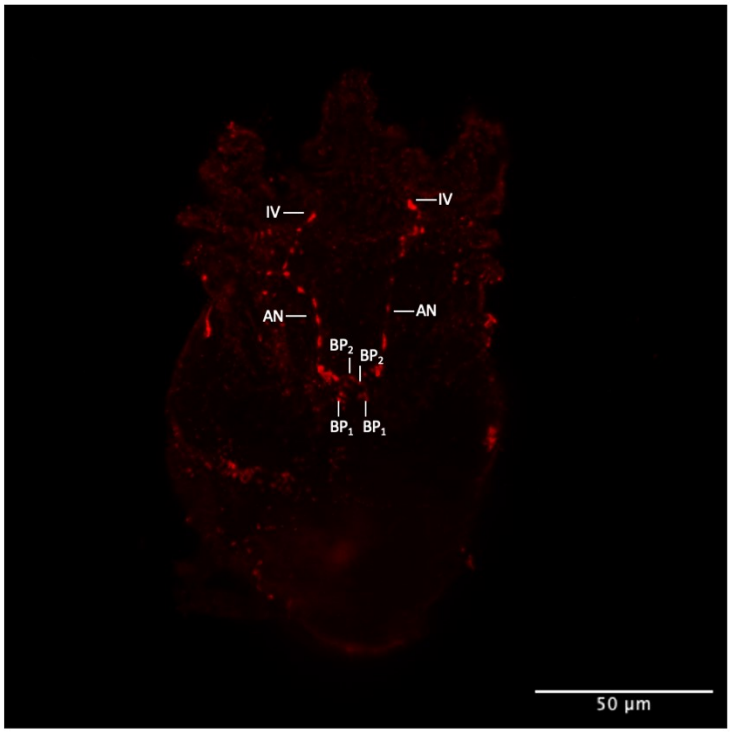


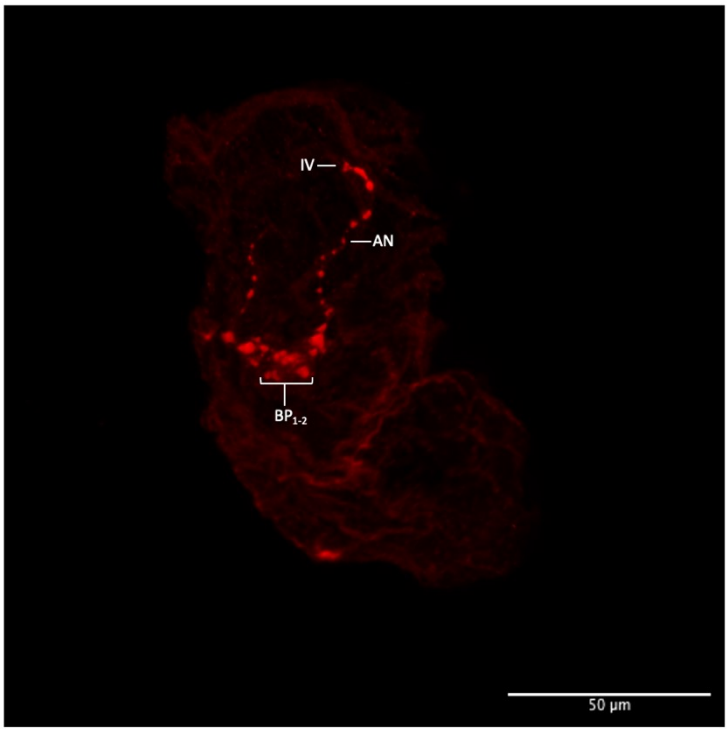
The images below show SLIR positive structures observed in adult female *Collotheca ferox.*

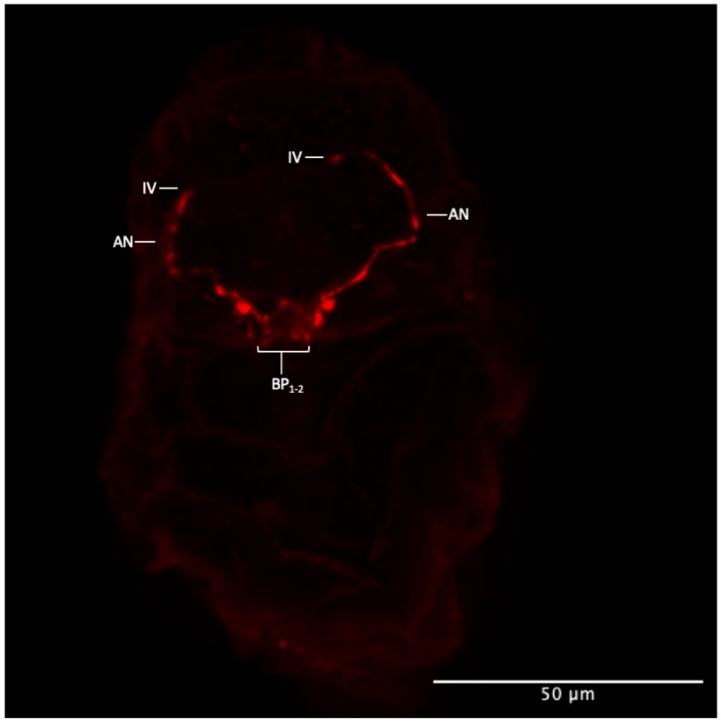


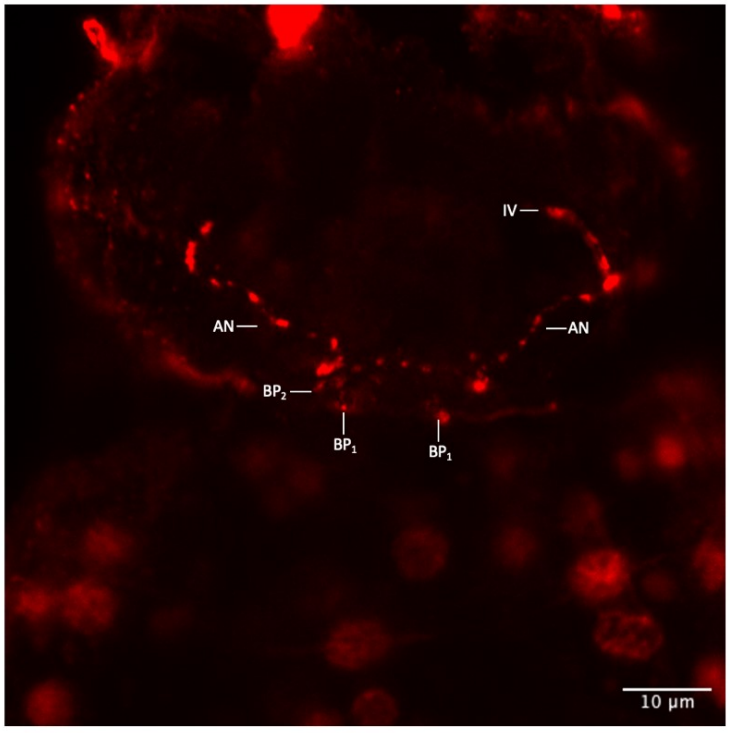

IV
IV
AN
AN
BP1-2
50 µm


IV
AN
AN
BP1-2
10 µm

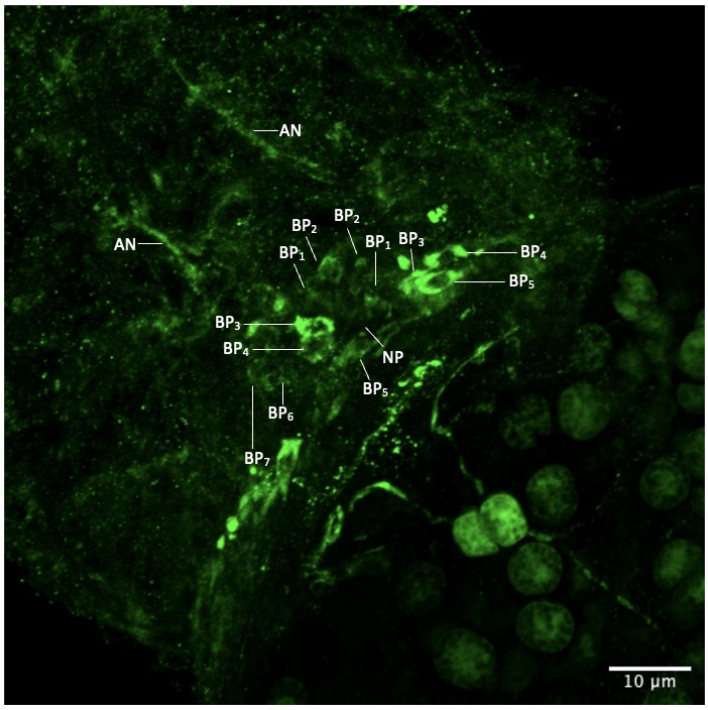

IV
IV
AN
AN
BP2
BP2
BP1
BP1
50 µm

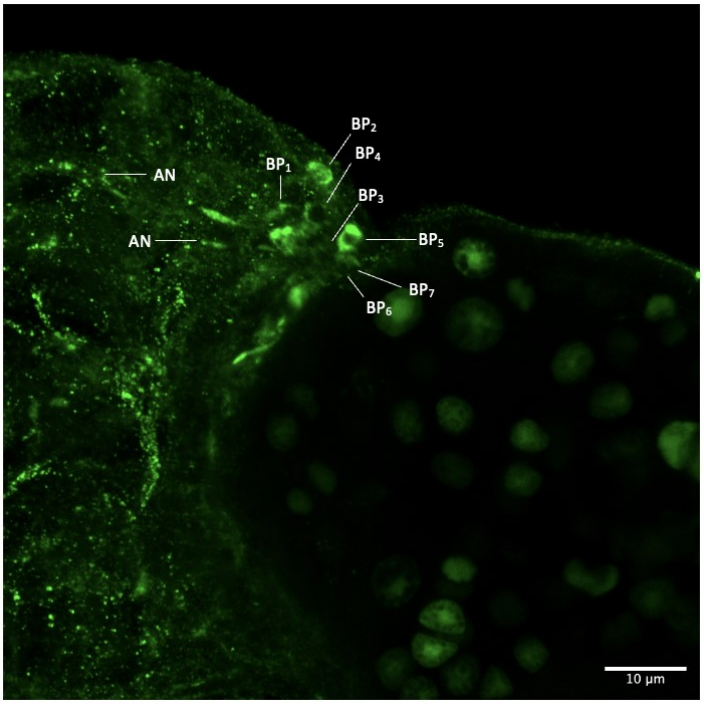

IV
AN
BP1-2
50 µm

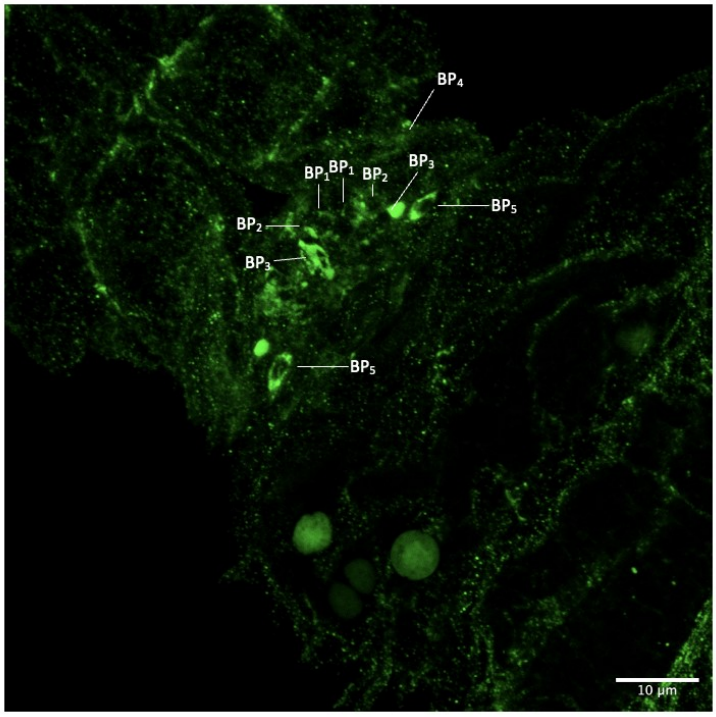




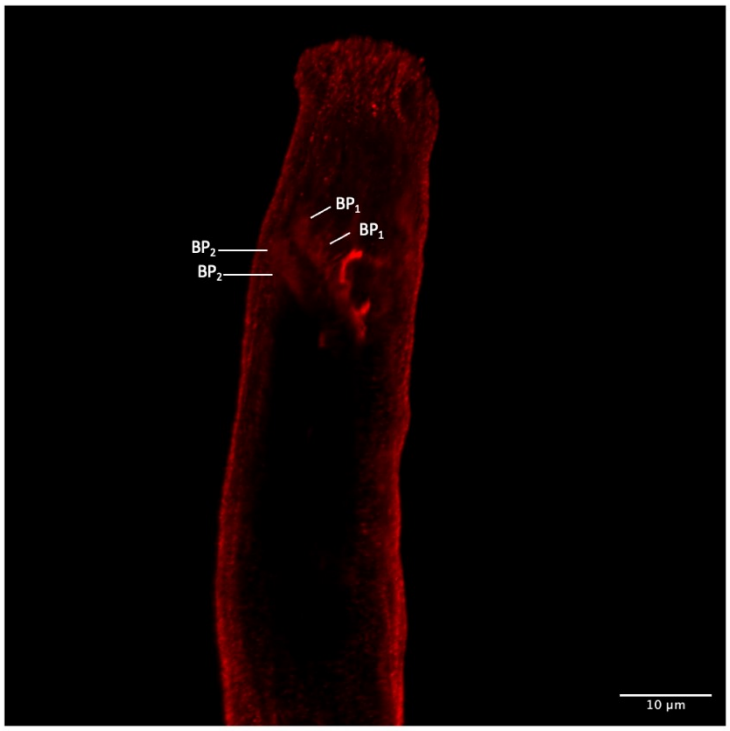
The images below show FLIR positive structures observed in adult female *Collotheca ferox.*

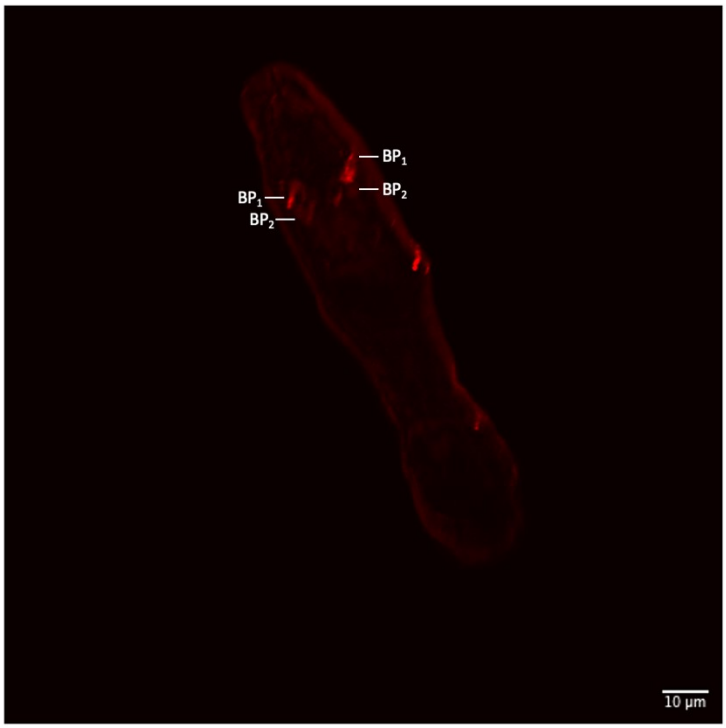

AN
AN
BP2
BP1
BP2
BP1
BP3
BP4
BP5
BP3
BP4
NP
BP5
BP6
BP7
10 µm

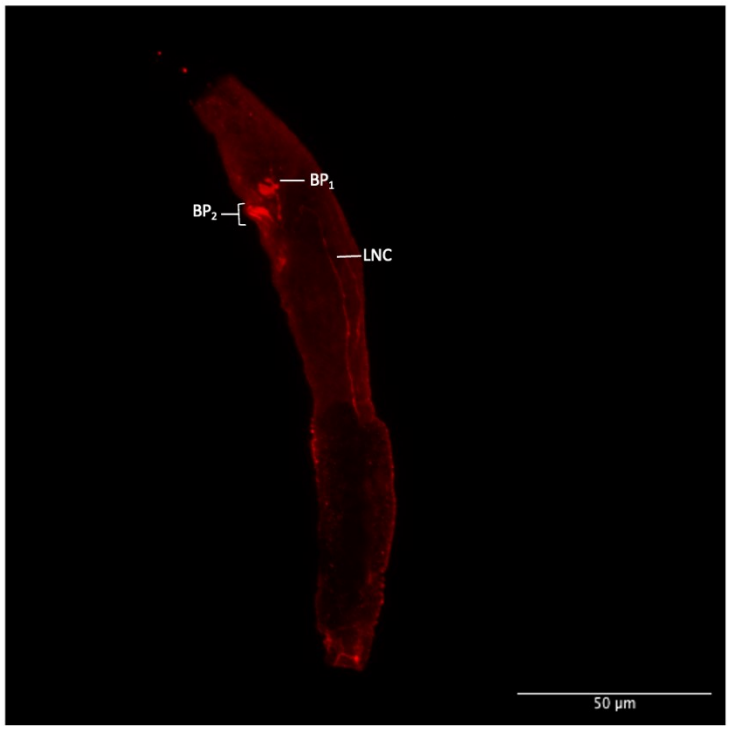

BP2
BP4
AN
BP1
BP3
AN
BP5
BP7
BP6
10 µm


BP4
BP1
BP1
BP2
BP3
BP5
BP2
BP3
BP5
10 µm

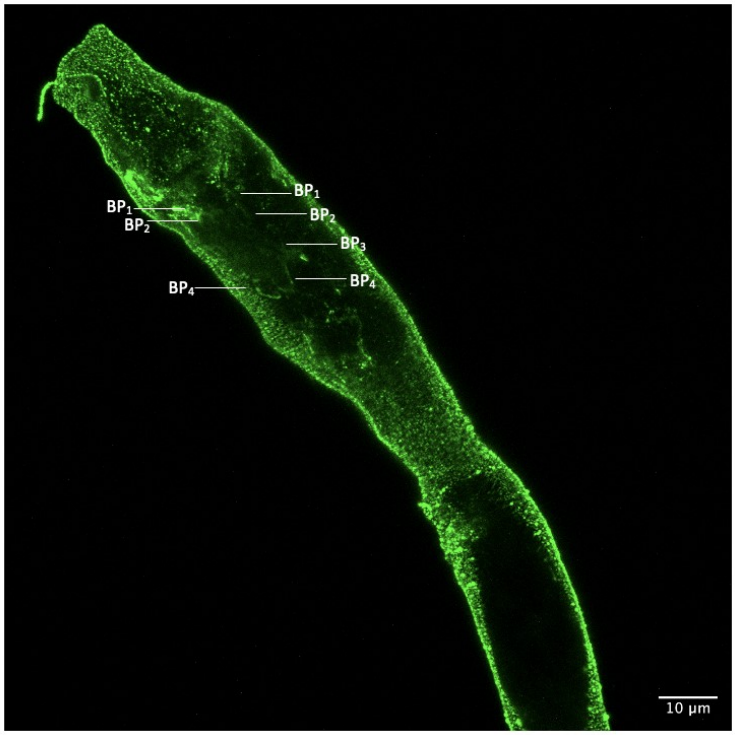
The images below show SLIR positive structures observed in larval female *Collotheca ferox.*

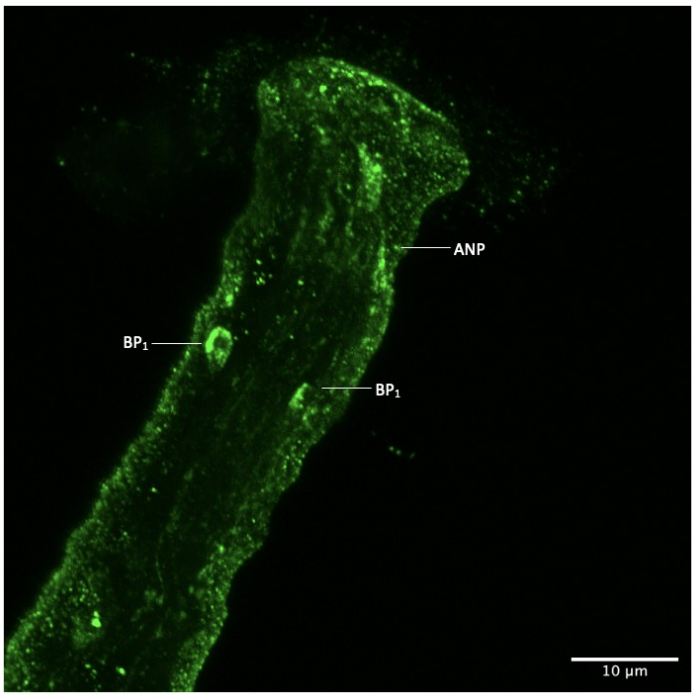


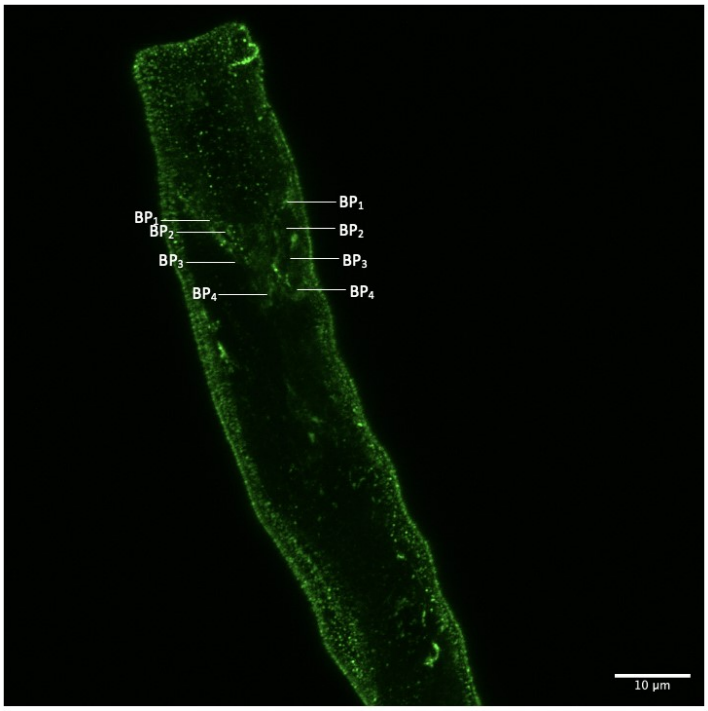


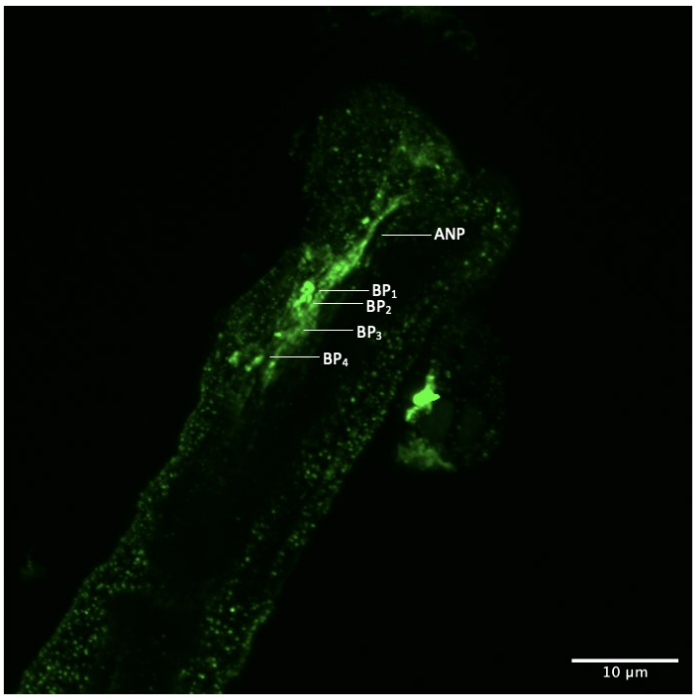


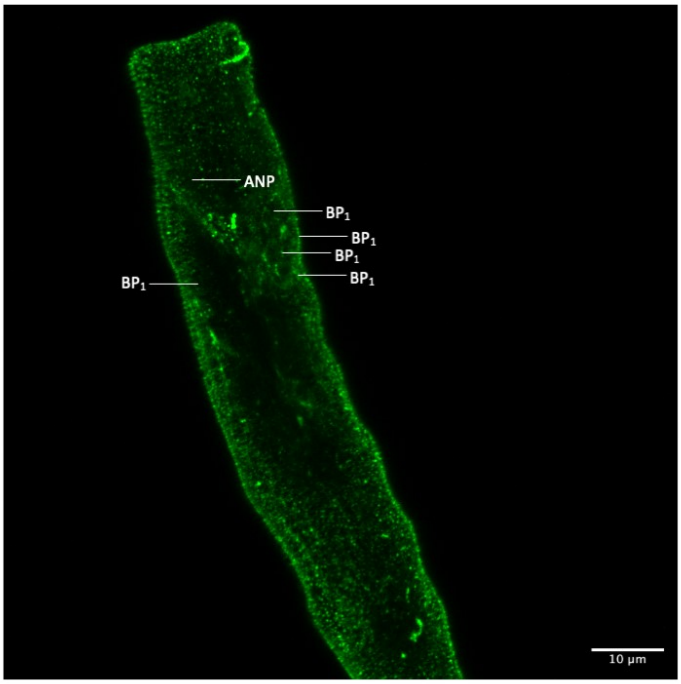
The images below show FLIR positive structures observed in larval female *Collotheca ferox.*

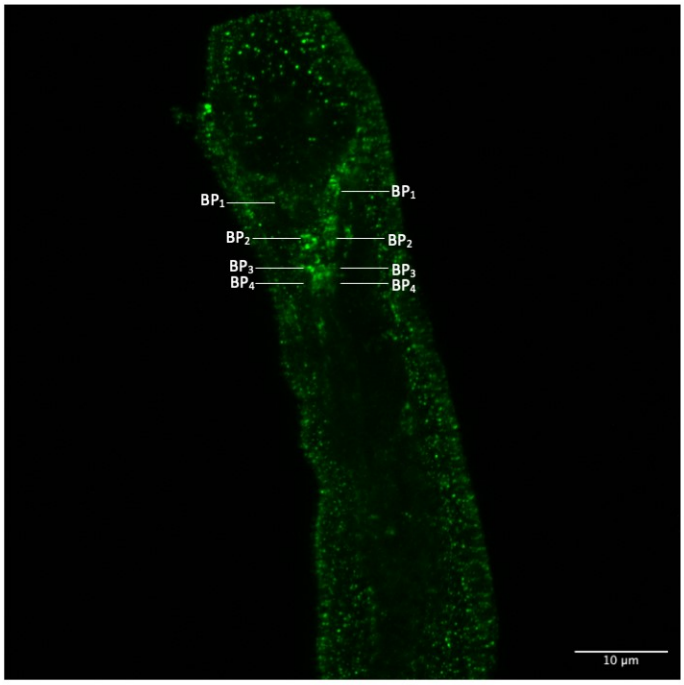



ANP
BP1
BP1
10 µm

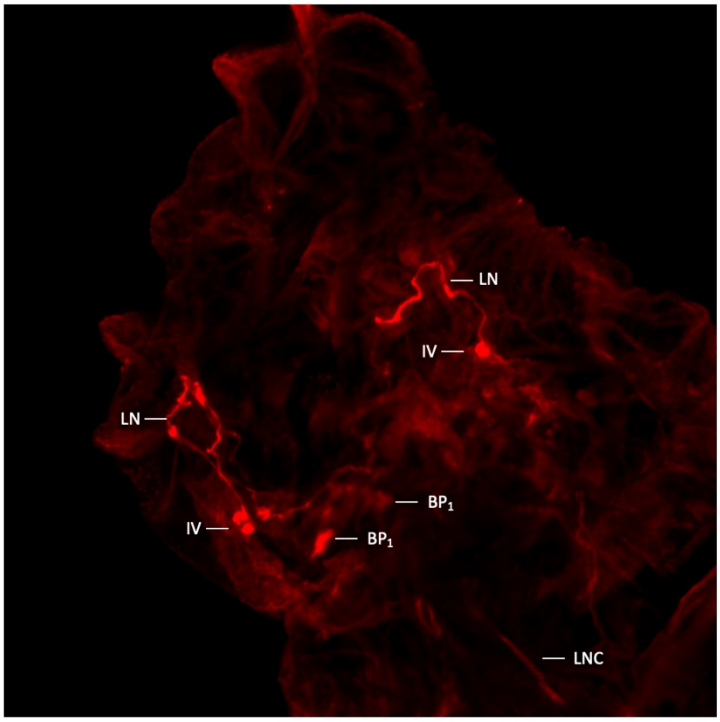

BP1
BP2
BP3
BP4
BP1
BP2
BP3
BP4
10 µm

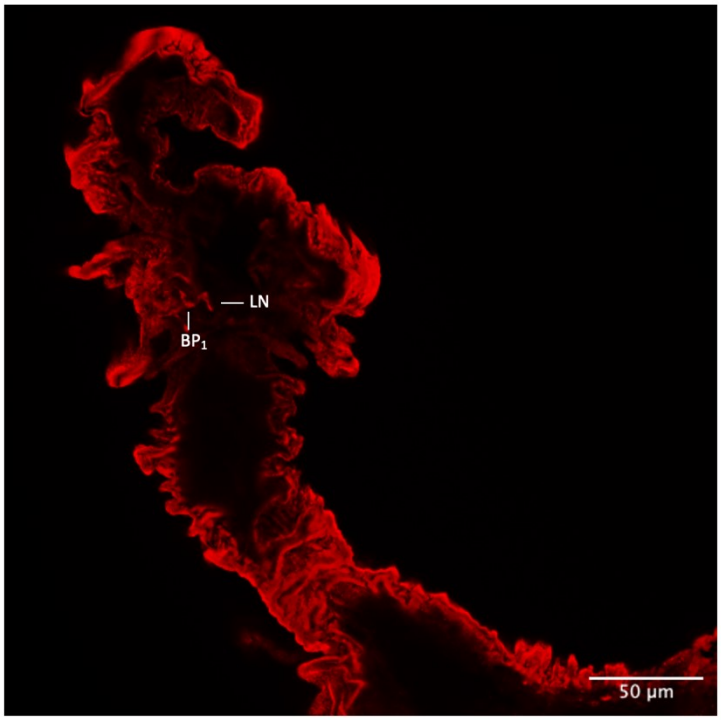

ANP
BP1
BP2
BP3
BP4
10 µm

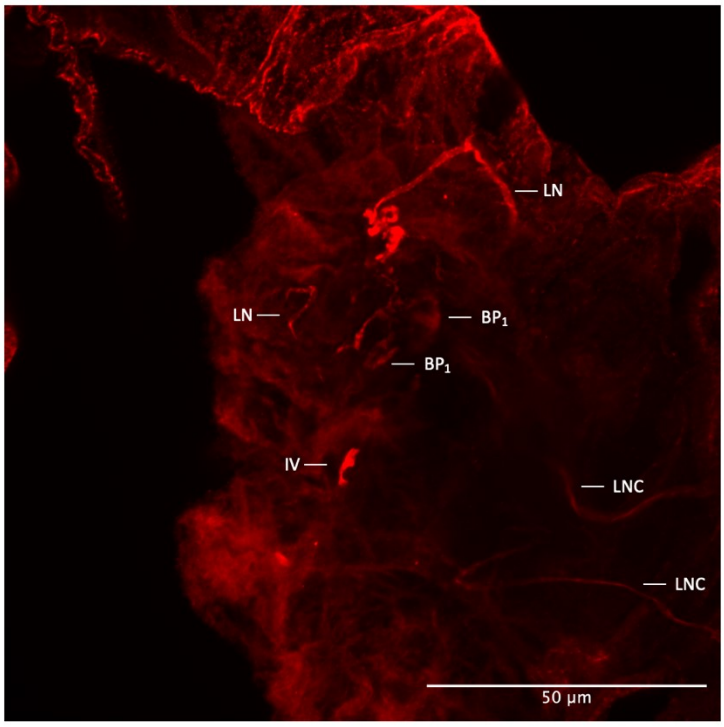

ANP
BP1
BP1
BP1
BP1
BP1
10 µm



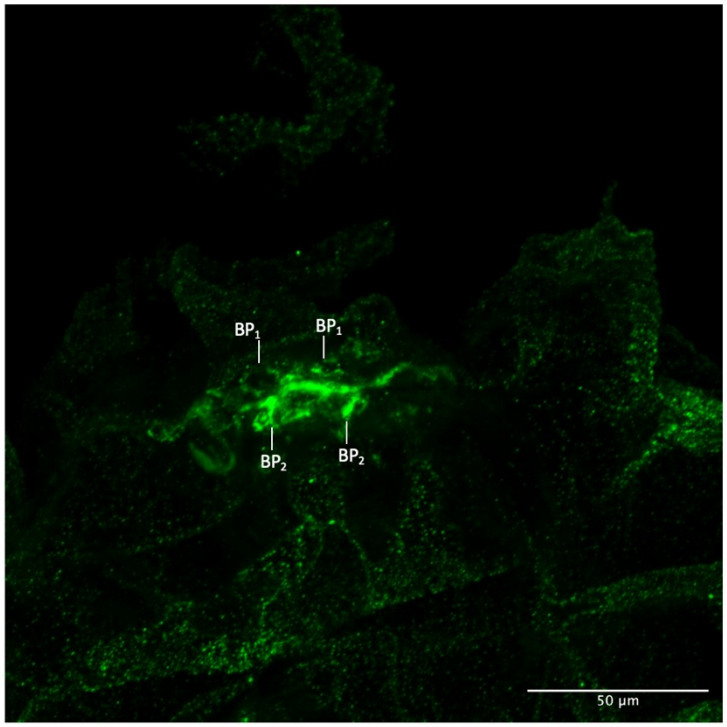
The images below show SLIR positive structures observed in adult female *Acyclus inquietus*.

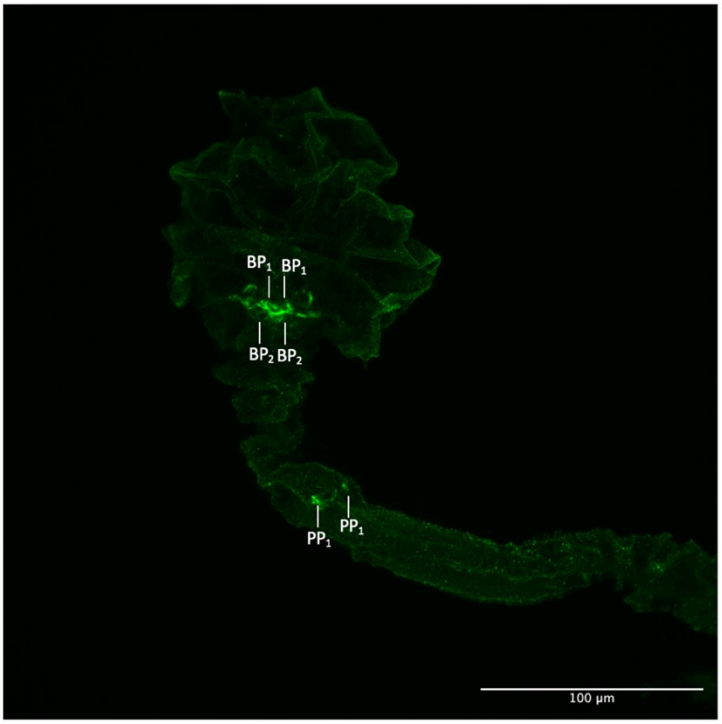



LN
BP1
50 µm

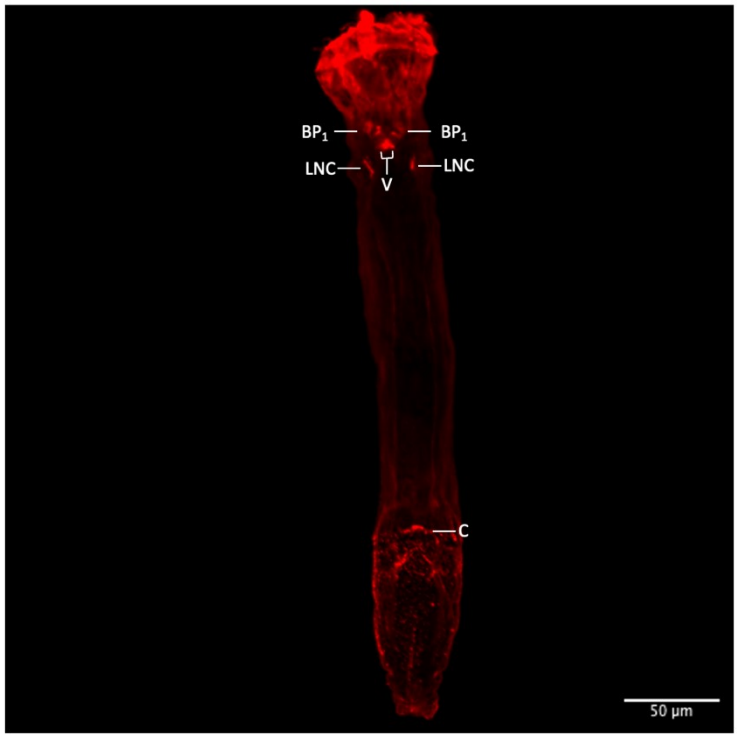

LN
LN
BP1
BP1
IV
LNC
LNC
50 µm

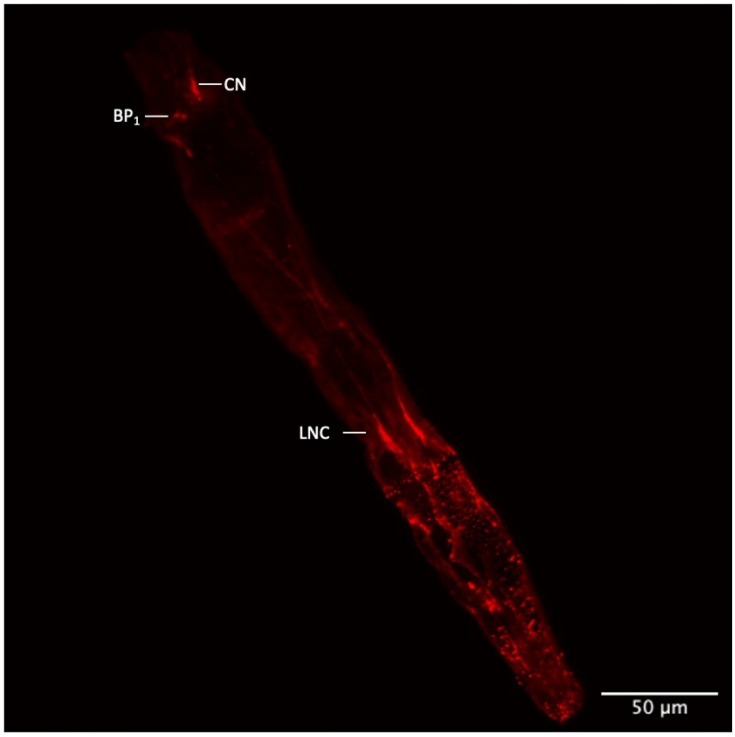
The images below show FLIR positive structures observed in adult female *Acyclus inquietus*.

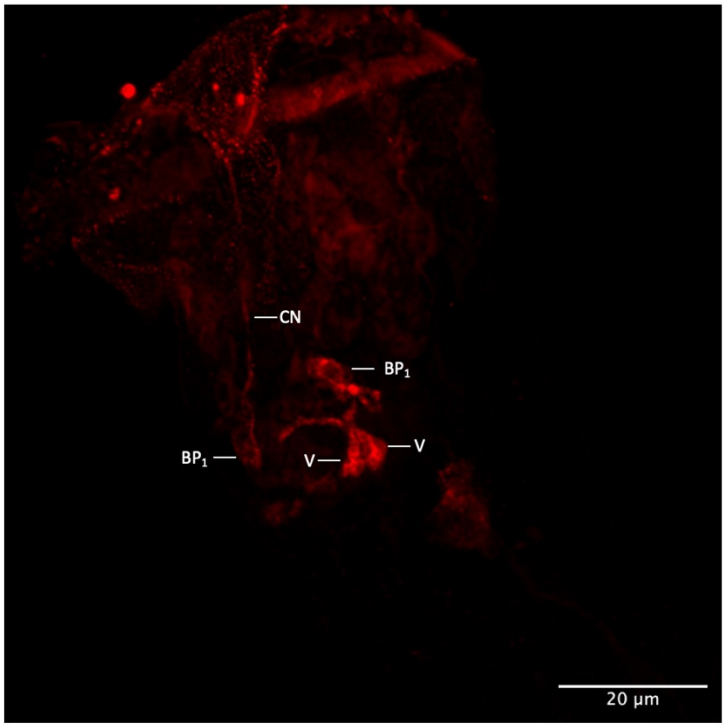


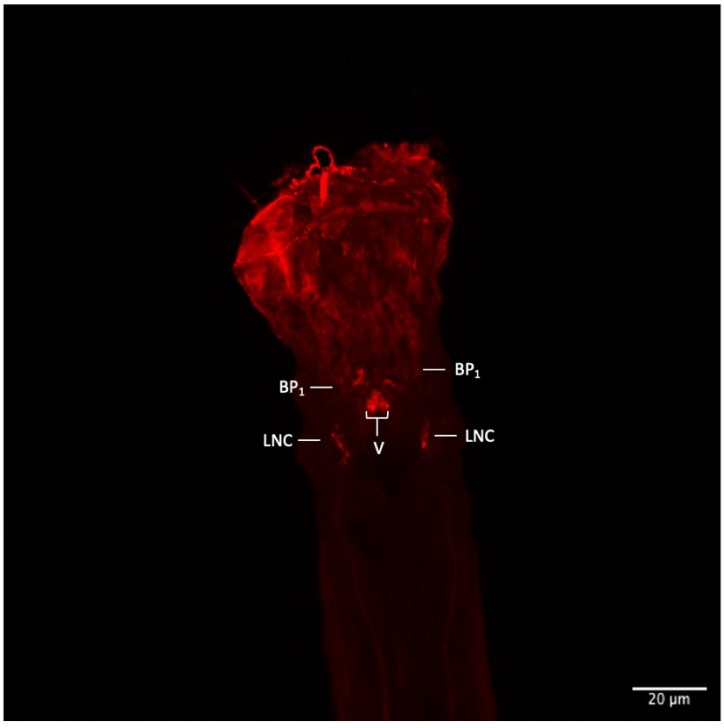


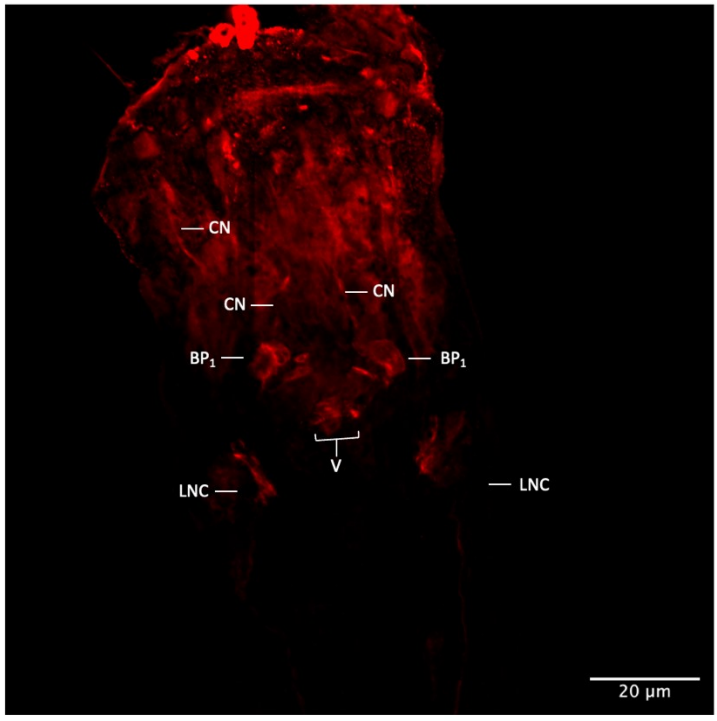
The images below show SLIR positive structures observed in larval female *Acyclus inquietus*.

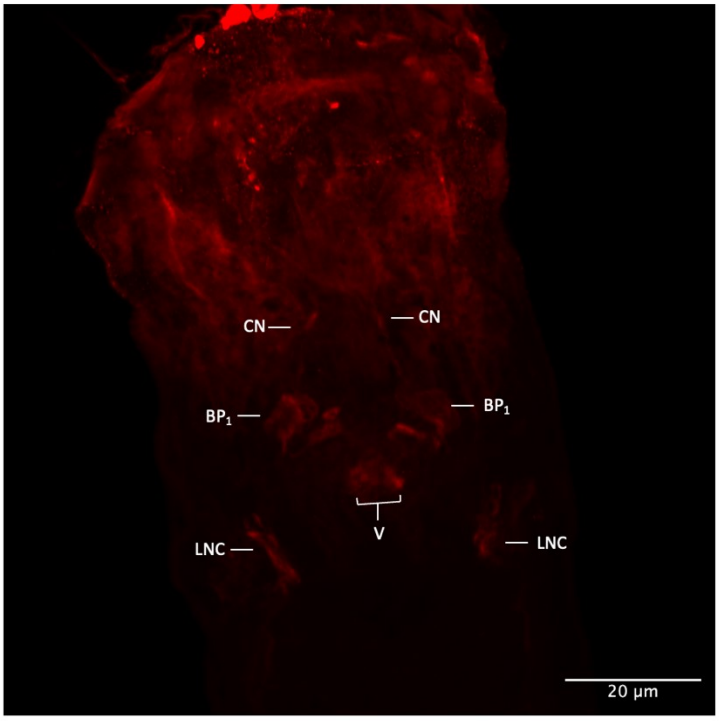


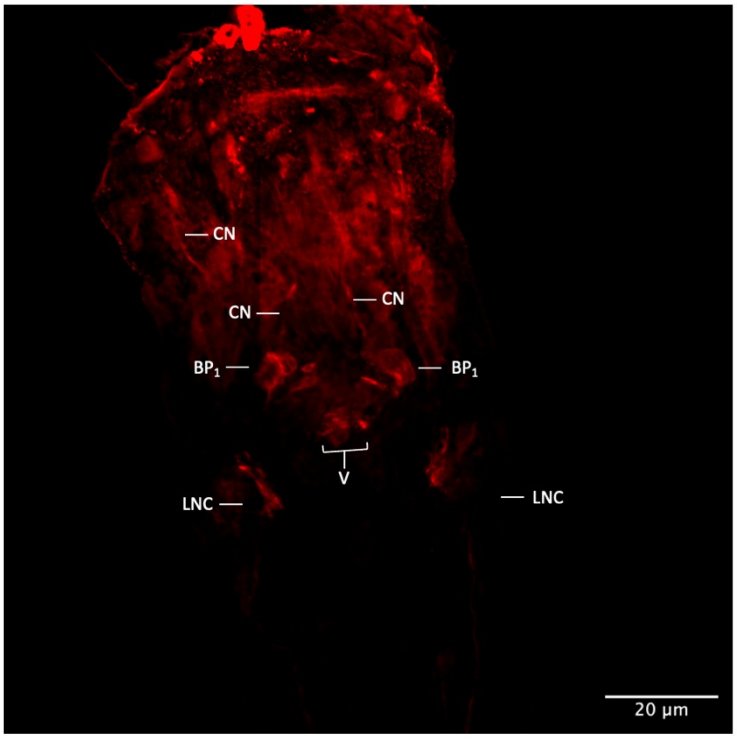



CN
BP1
BP1
V
V
20 µm

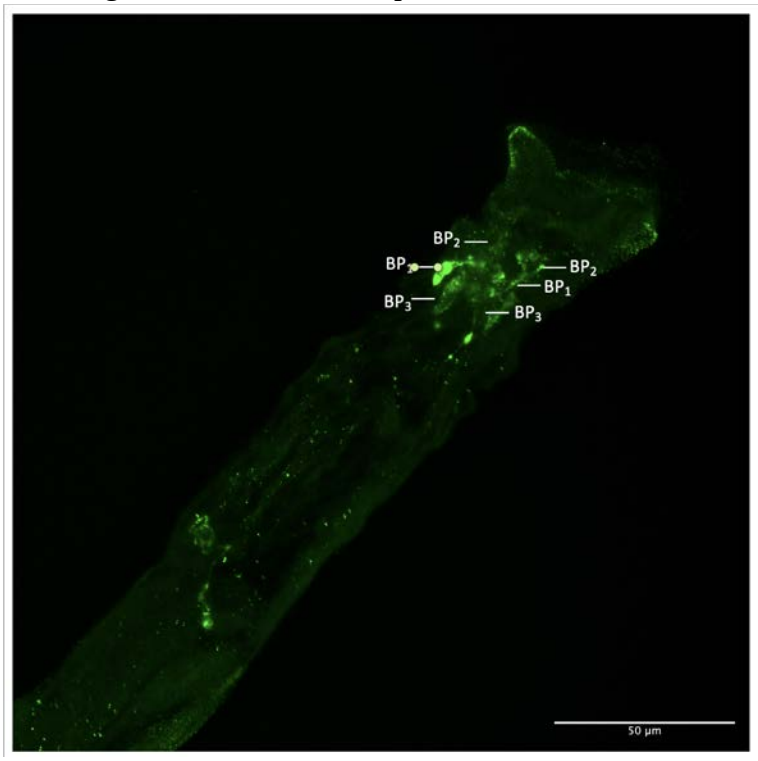

BP1
BP1
LNC
V
LNC
20 µm


CN
CN
CN
BP1
BP1
V
LNC
LNC
20 µm

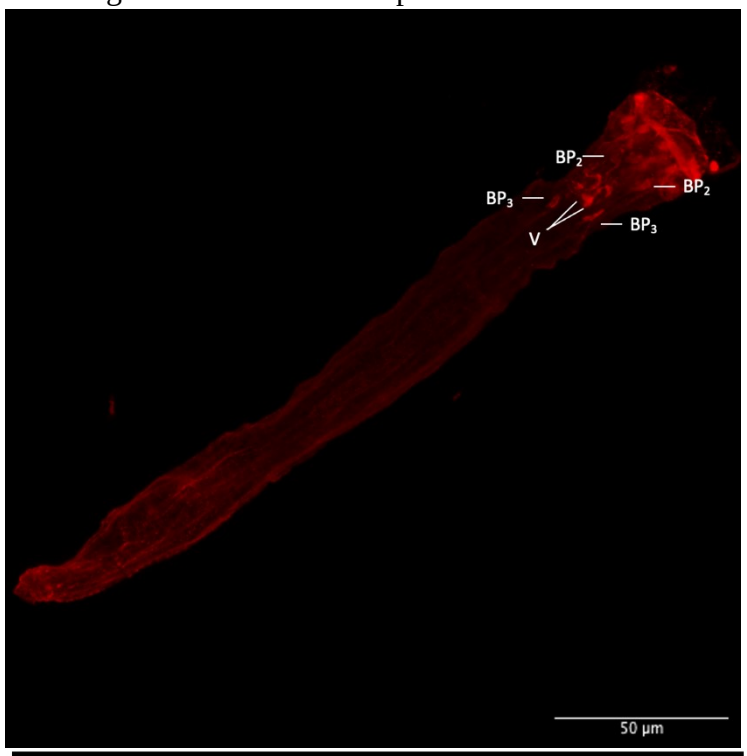

CN
CN
BP1
BP1
V
LNC
LNC
20 µm

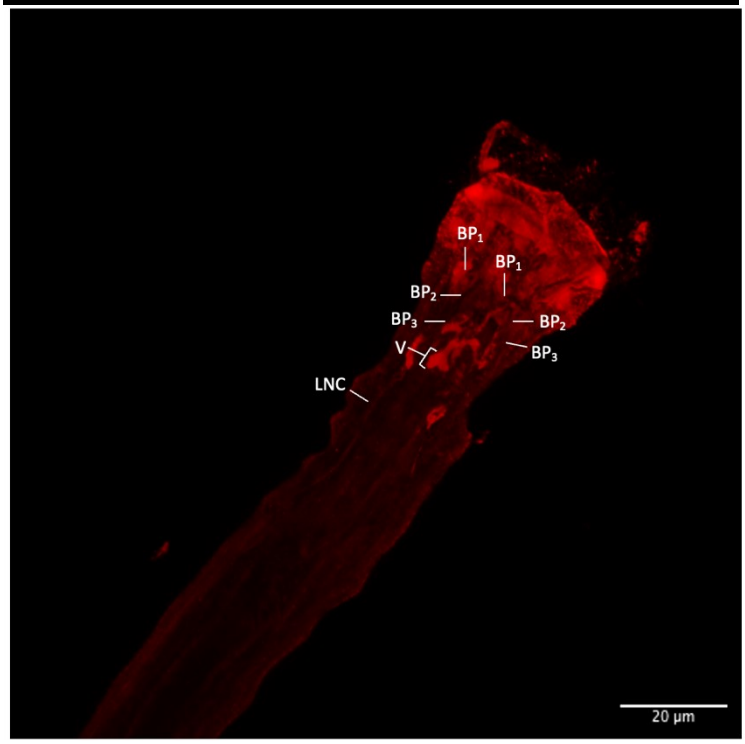


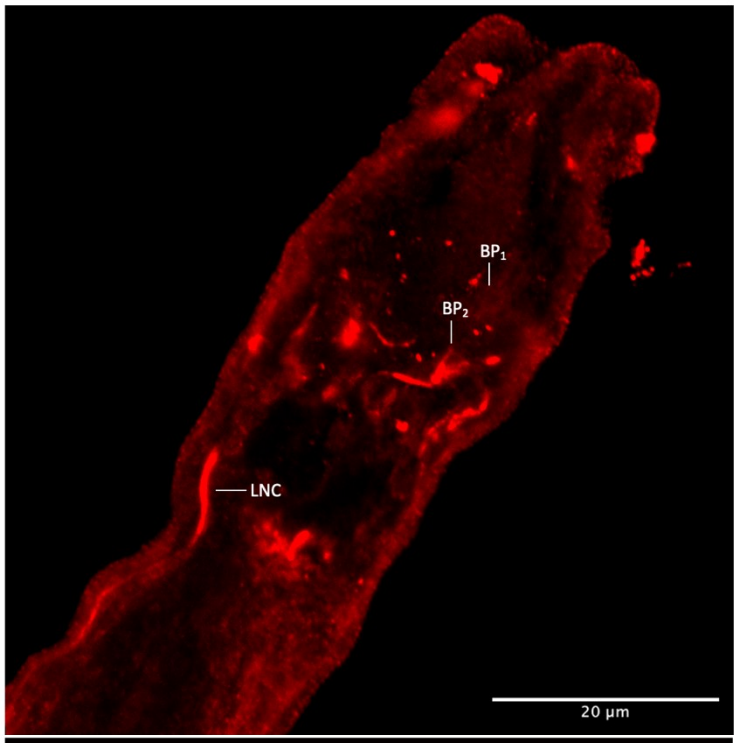
The images below show FLIR positive structures observed in adult female *Acyclus inquietus*.

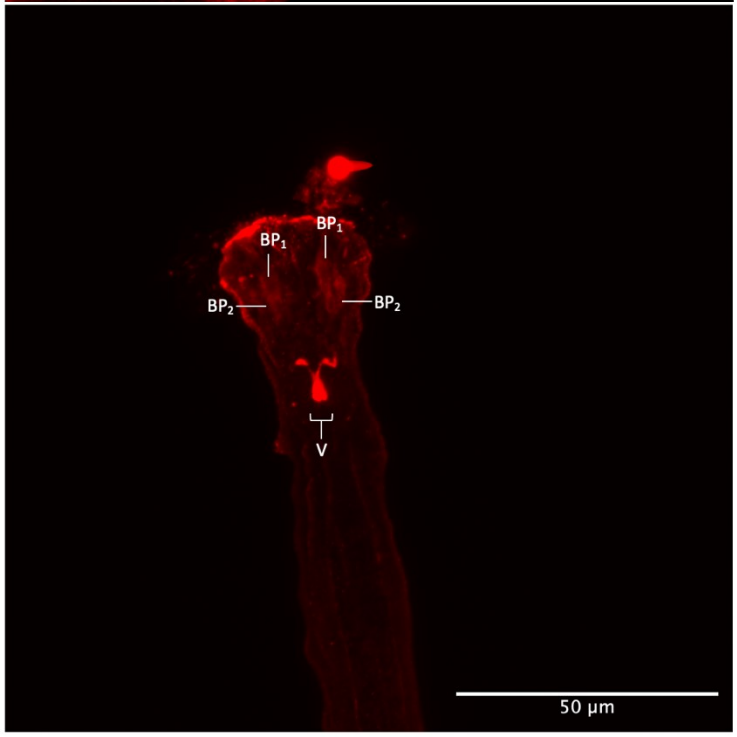


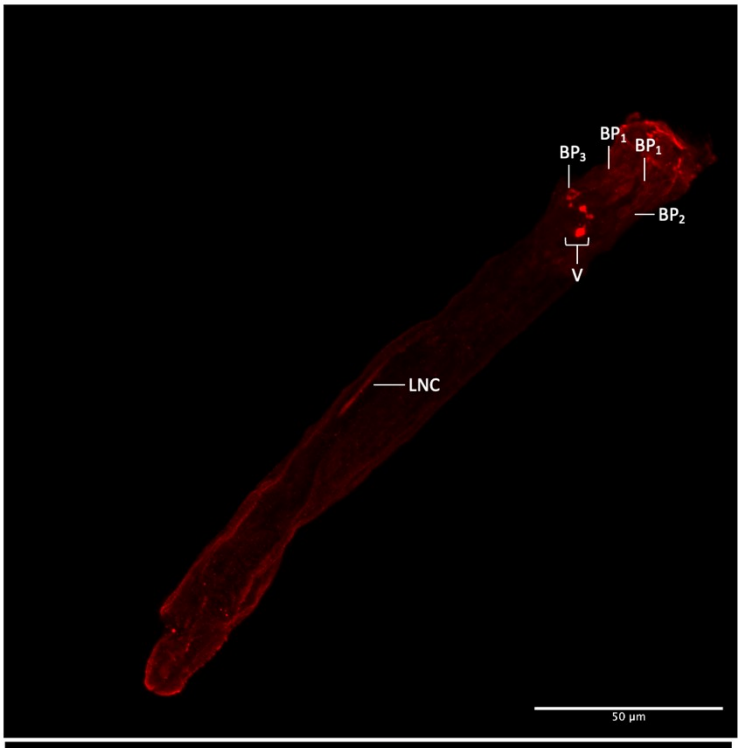
The images below show SLIR positive structures observed in male *Acyclus inquietus*.

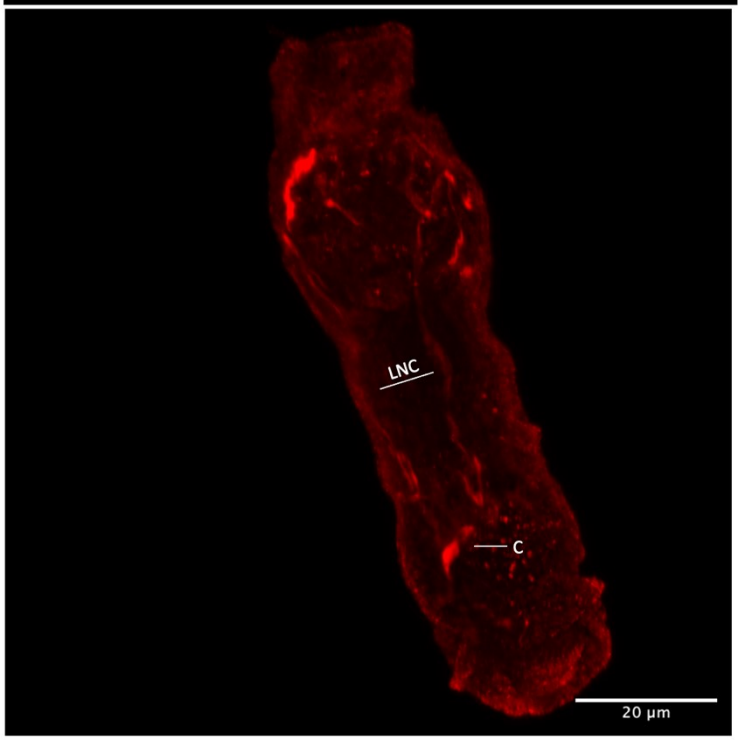




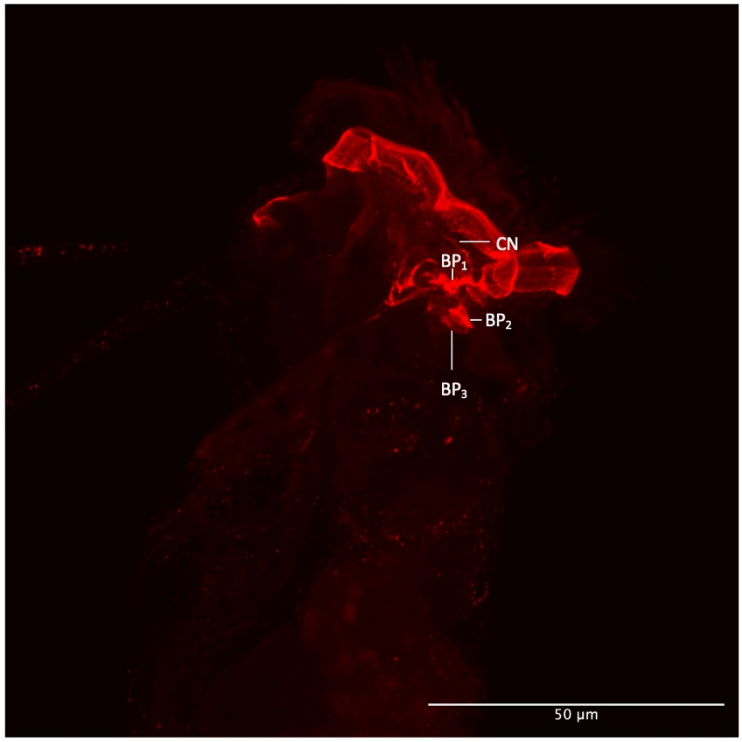

BP1
BP2
LNC
20 µm

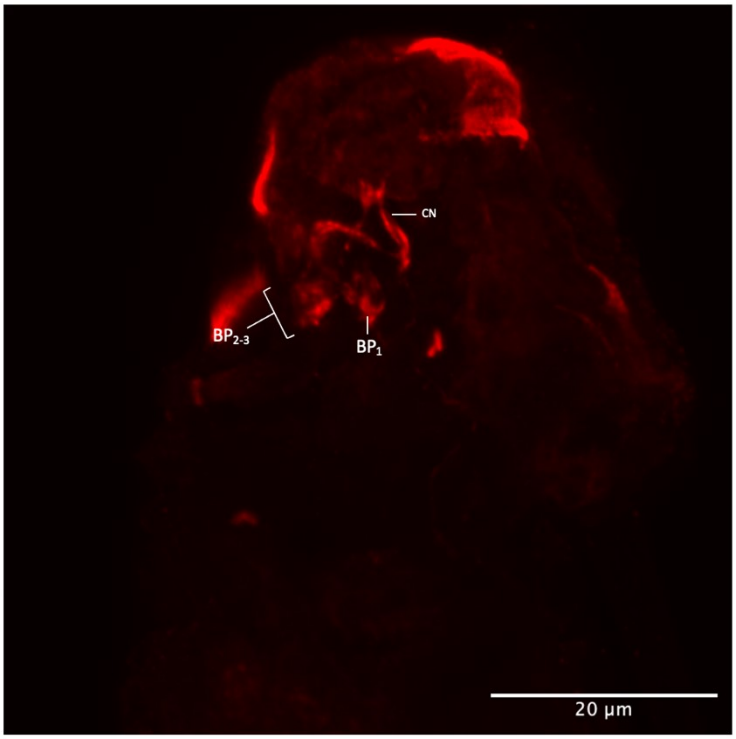

BP1
BP1
BP2
BP2
V
50 µm

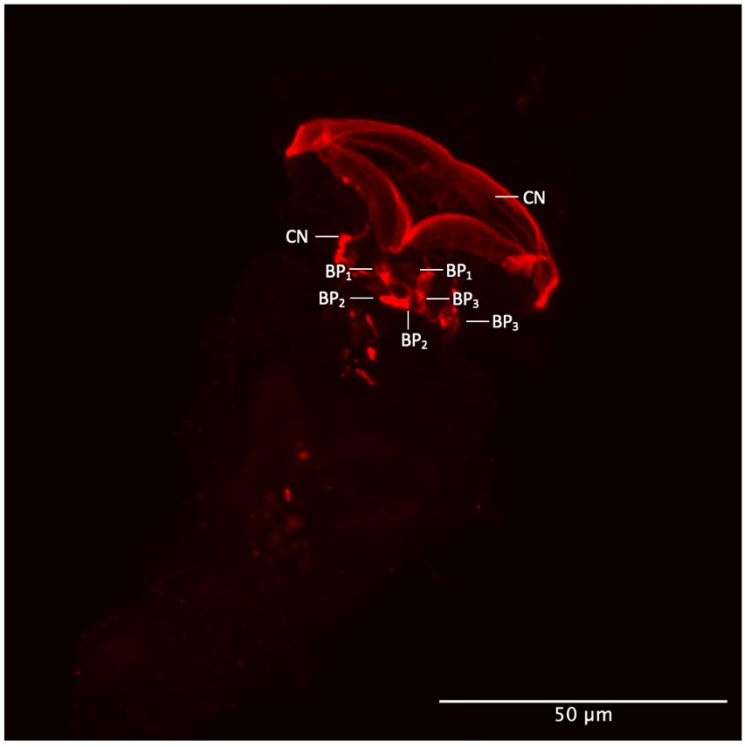

BP3
BP1
BP1
BP2
V
LNC
50 µm

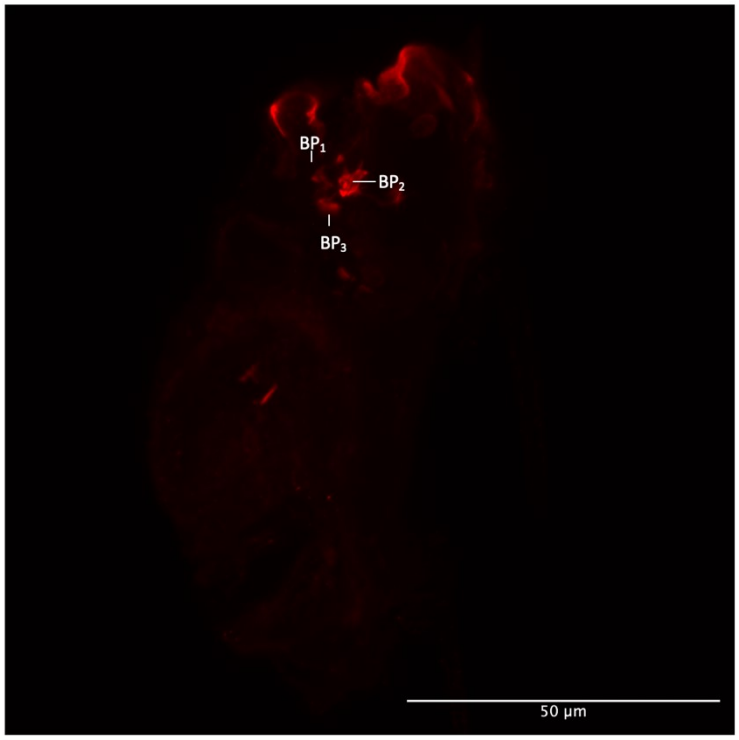

LNC
C
20 µm

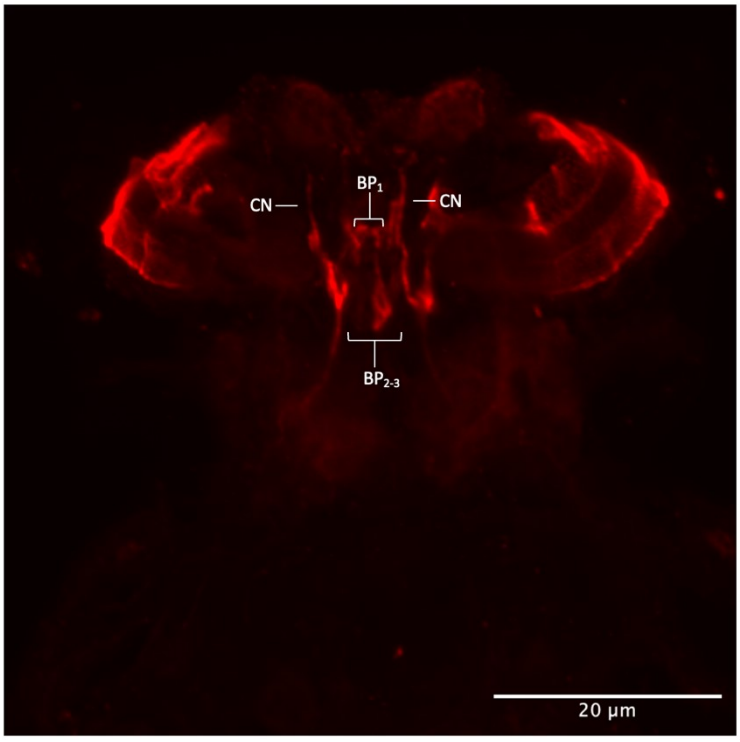
The images below show SLIR positive structures observed in female *Filinia longiseta*

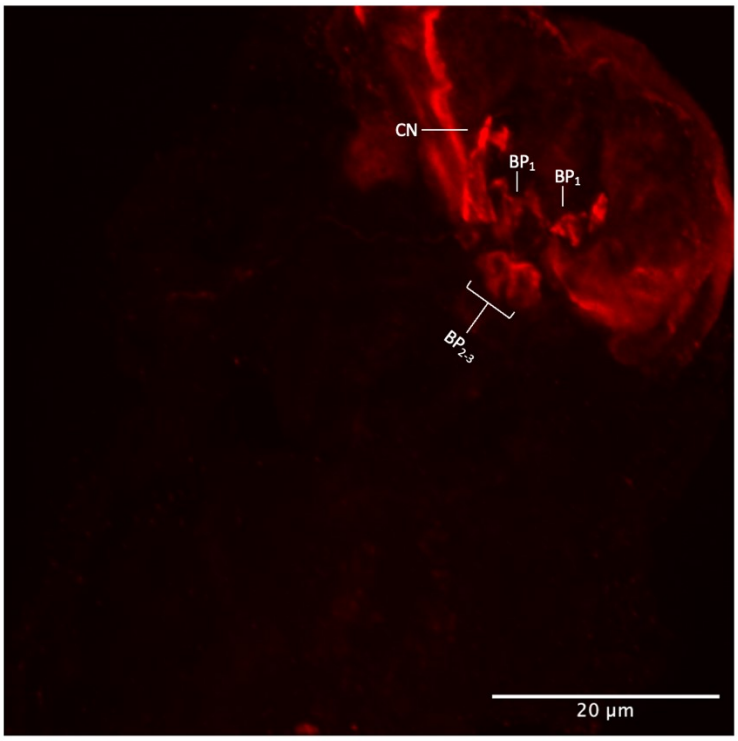


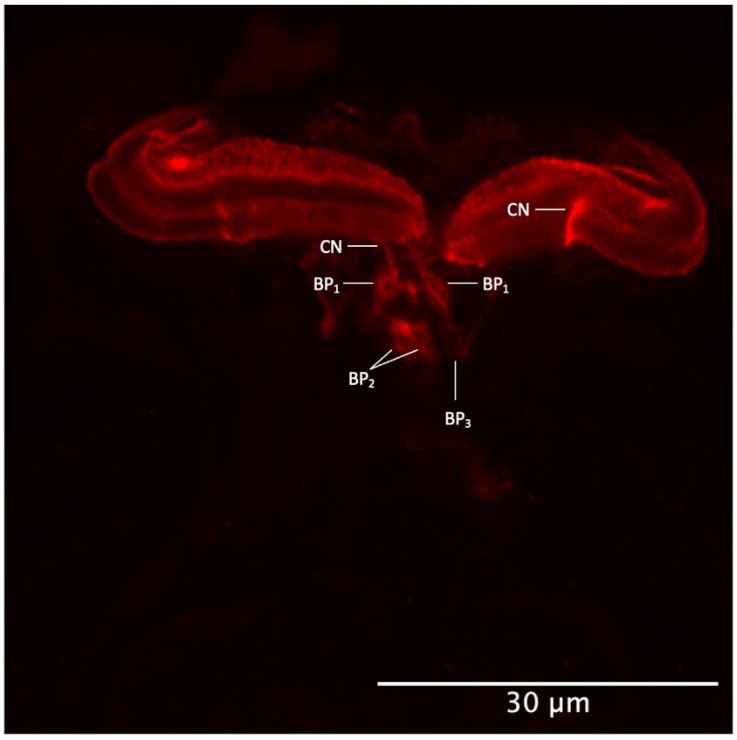

CN
BP2-3
BP1
20 µm

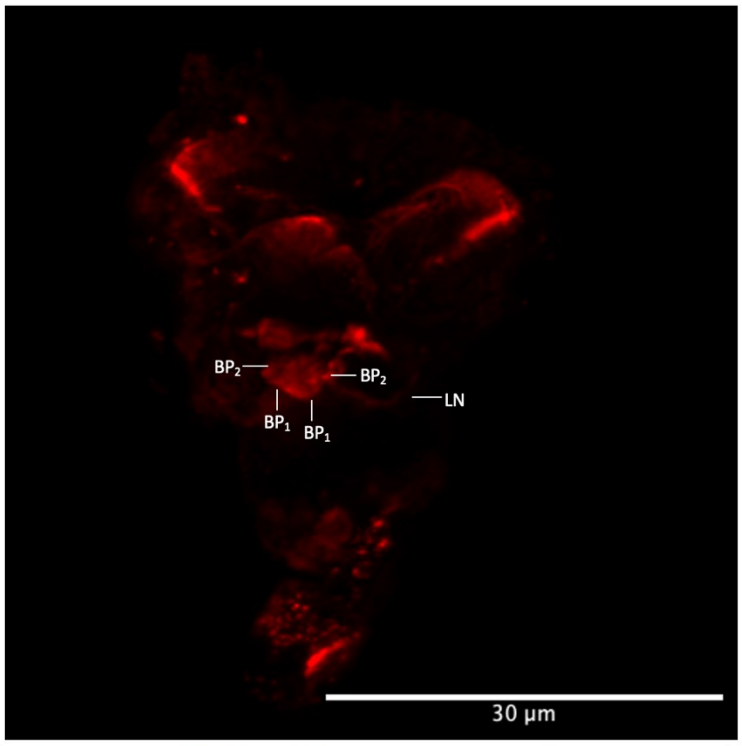

CN
CN
BP1
BP1
BP2
BP3
BP3
BP2
50 µm

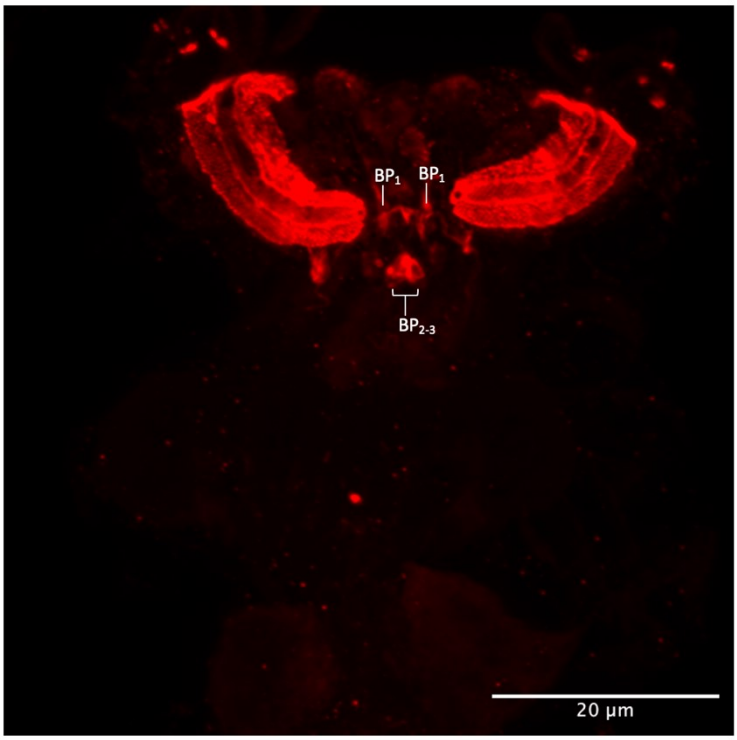

BP1
BP2
BP3
50 µm


CN
BP1
CN
BP2-3
20 µm

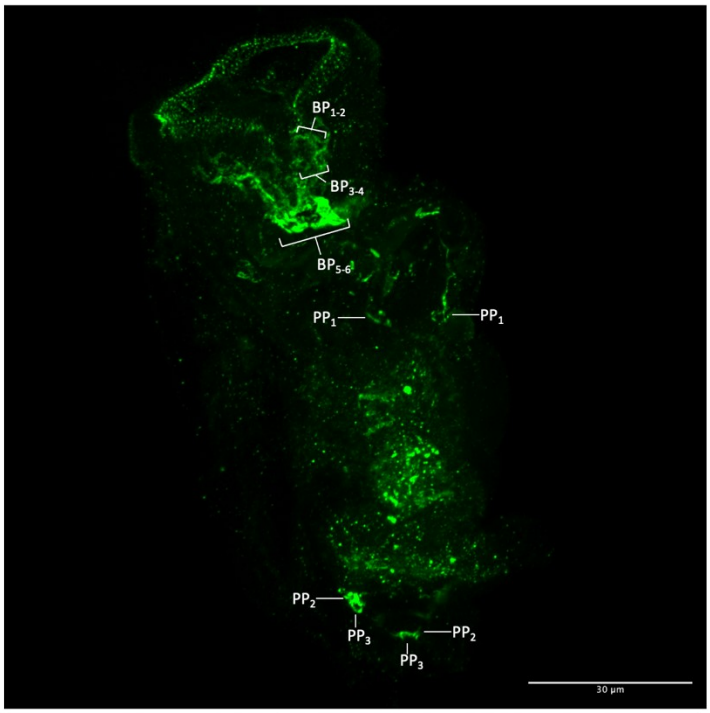

CN
BP1
BP1
BP2-3
20 µm

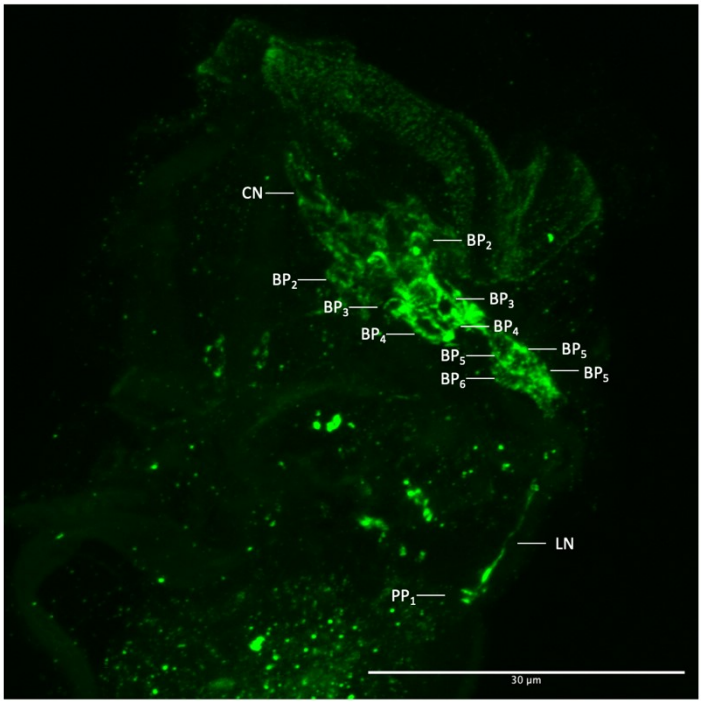

CN
CN
BP1
BP1
BP2
BP3
30 µm

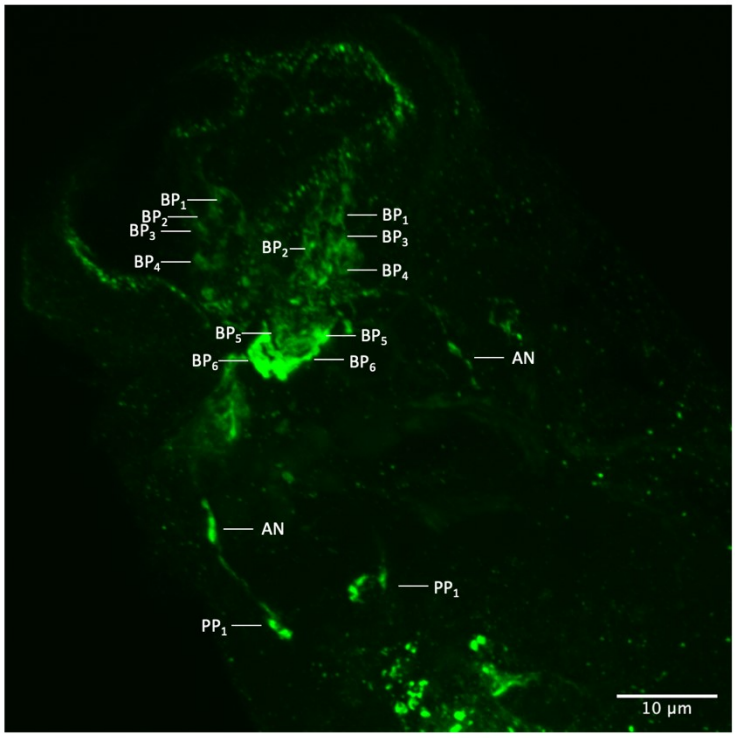

BP2
BP2
LN
BP1
BP1
30 µm

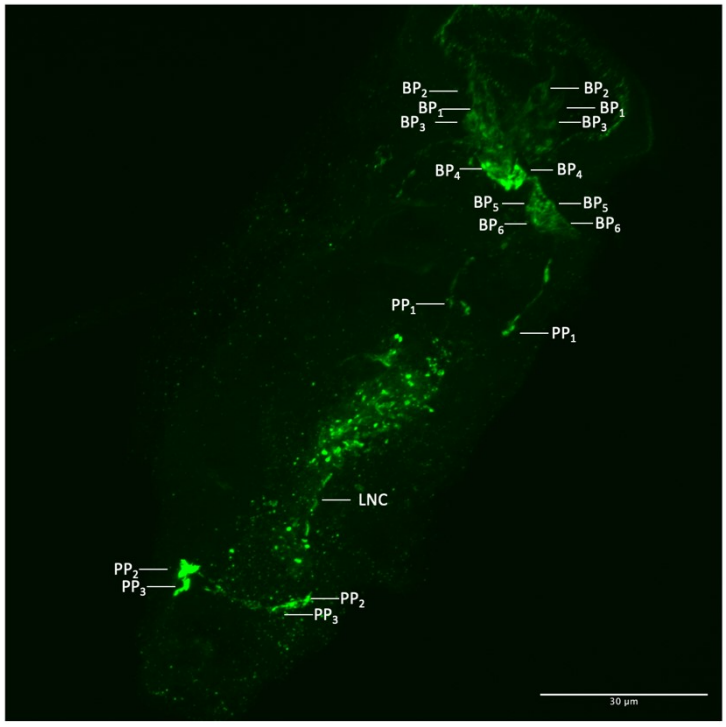

BP1
BP1
BP2-3
20 µm

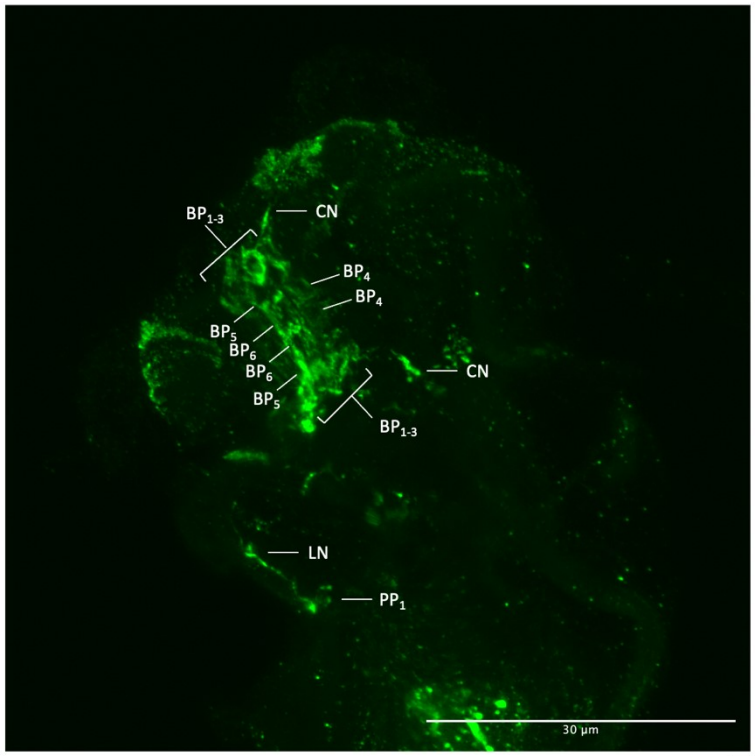
The images below show FLIR positive structures observed in female *Filinia longiseta*

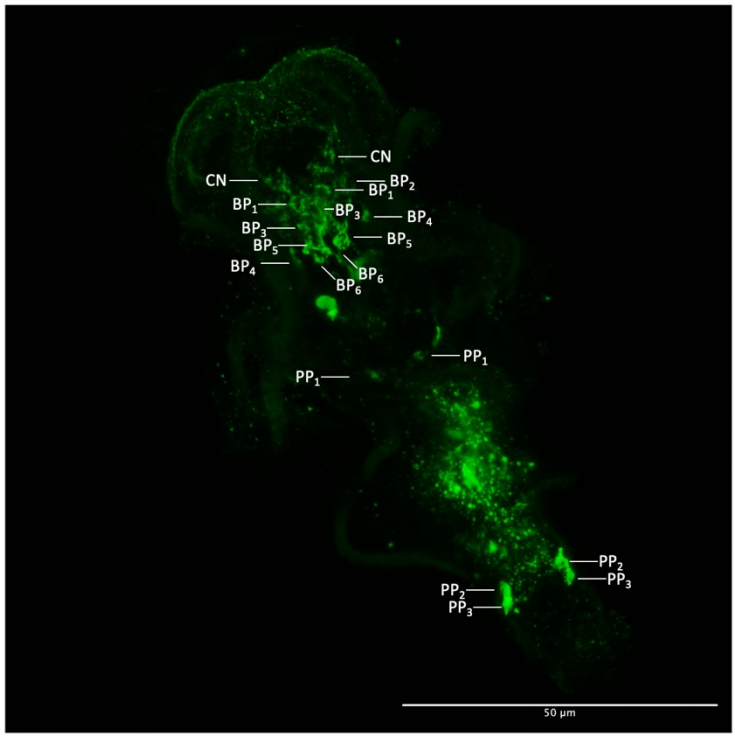




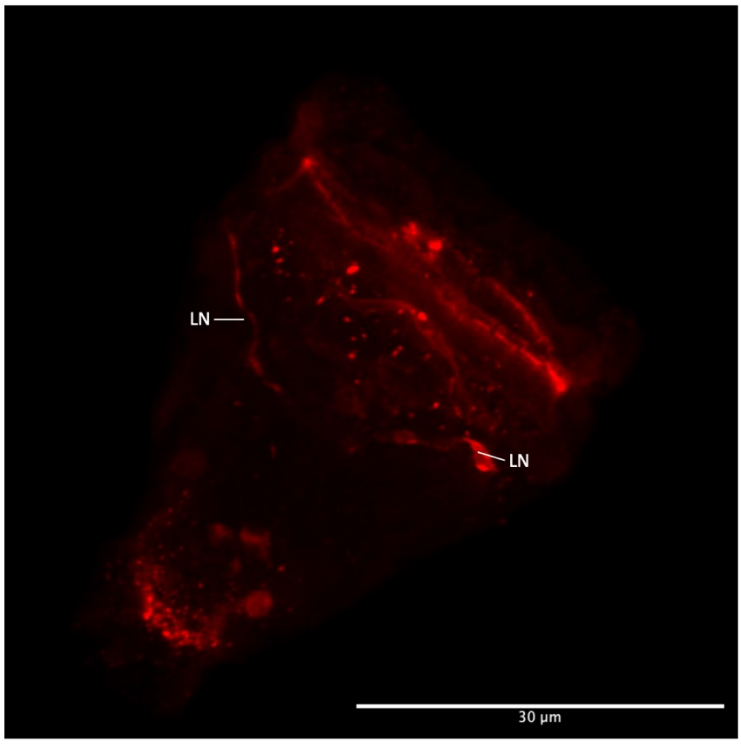

BP1
BP2
BP3
BP4
BP5
BP6
AN
PP1
10 µm

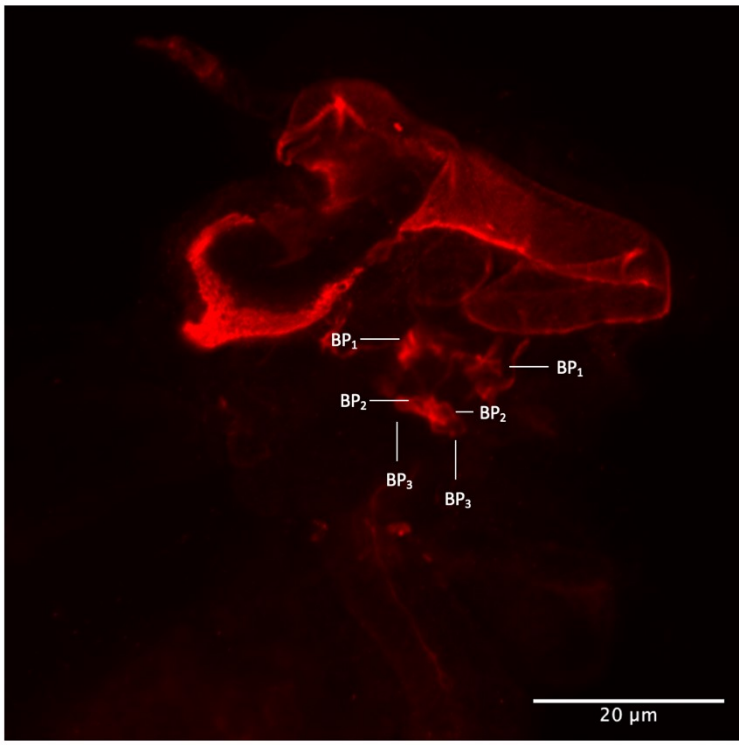

BP2
BP1
BP3
BP4
BP5
BP6
PP1
LNC
PP2
PP3
30 µm

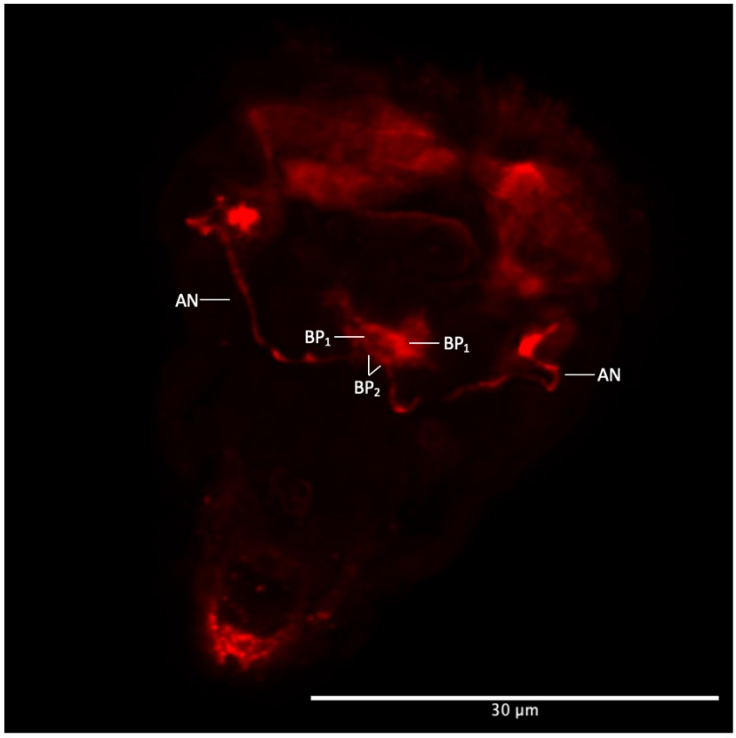

BP1-3
CN
BP4
BP4
BP5
BP6
BP6
BP5
CN
BP1-3
LN
PP1
30 µm

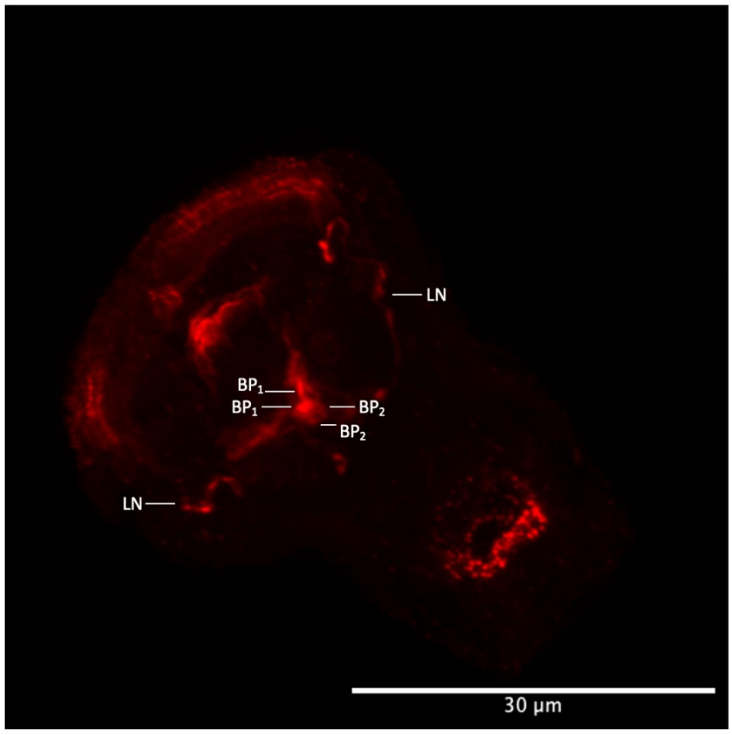

CN
CN
BP1
BP2
BP1
BP3
BP4
BP3
BP5
BP5
BP4
BP6
BP6
PP1
PP1
PP2
PP3
PP2
PP3
50 µm

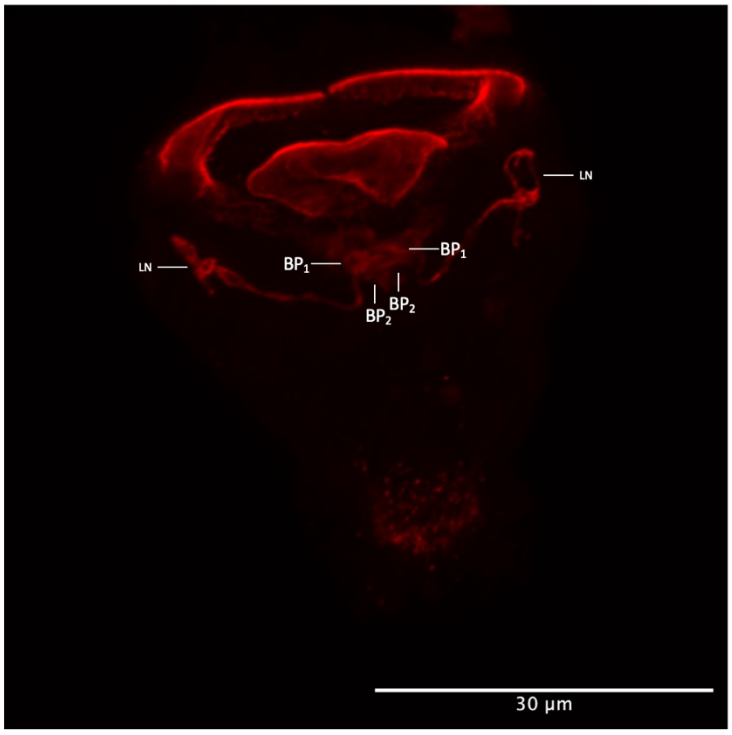
The images below show SLIR positive structures observed in male *Filinia longiseta*

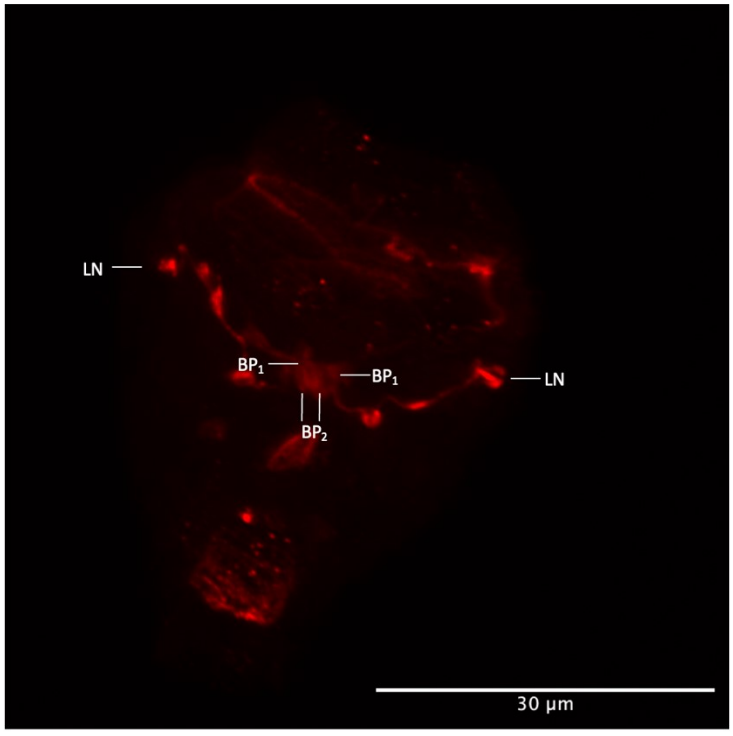


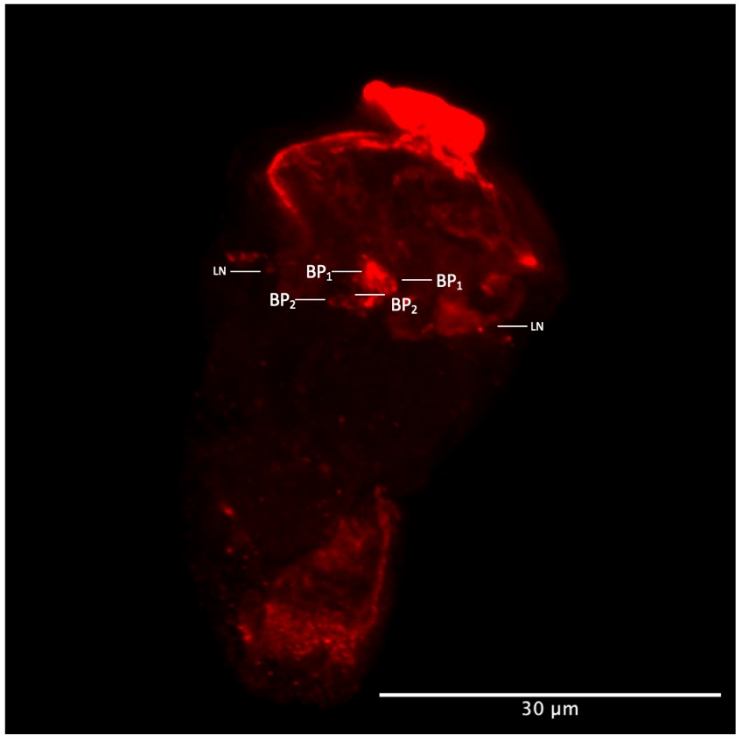


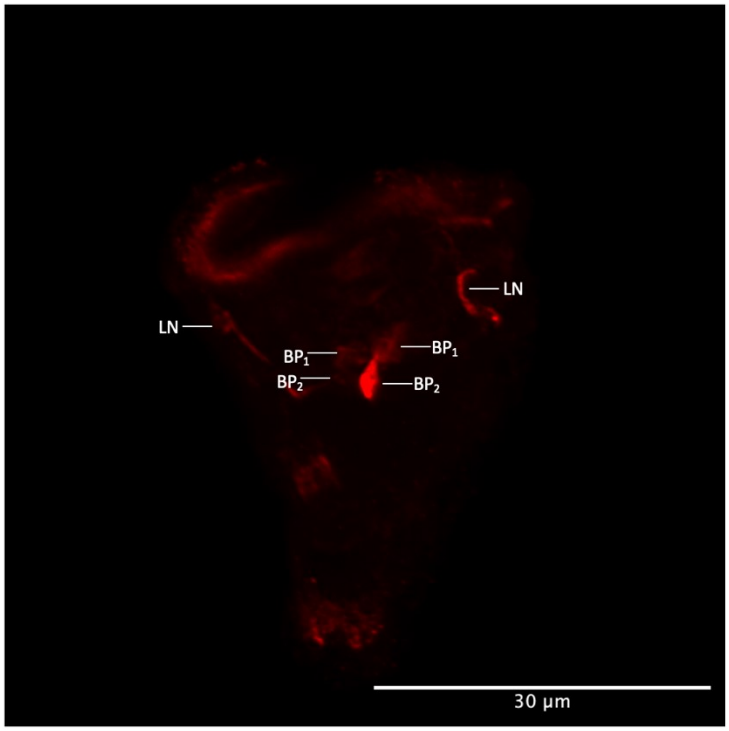

AN
BP1
BP1
BP2
AN
30 µm


LN
BP1
BP1
BP2
BP2
LN
30 µm

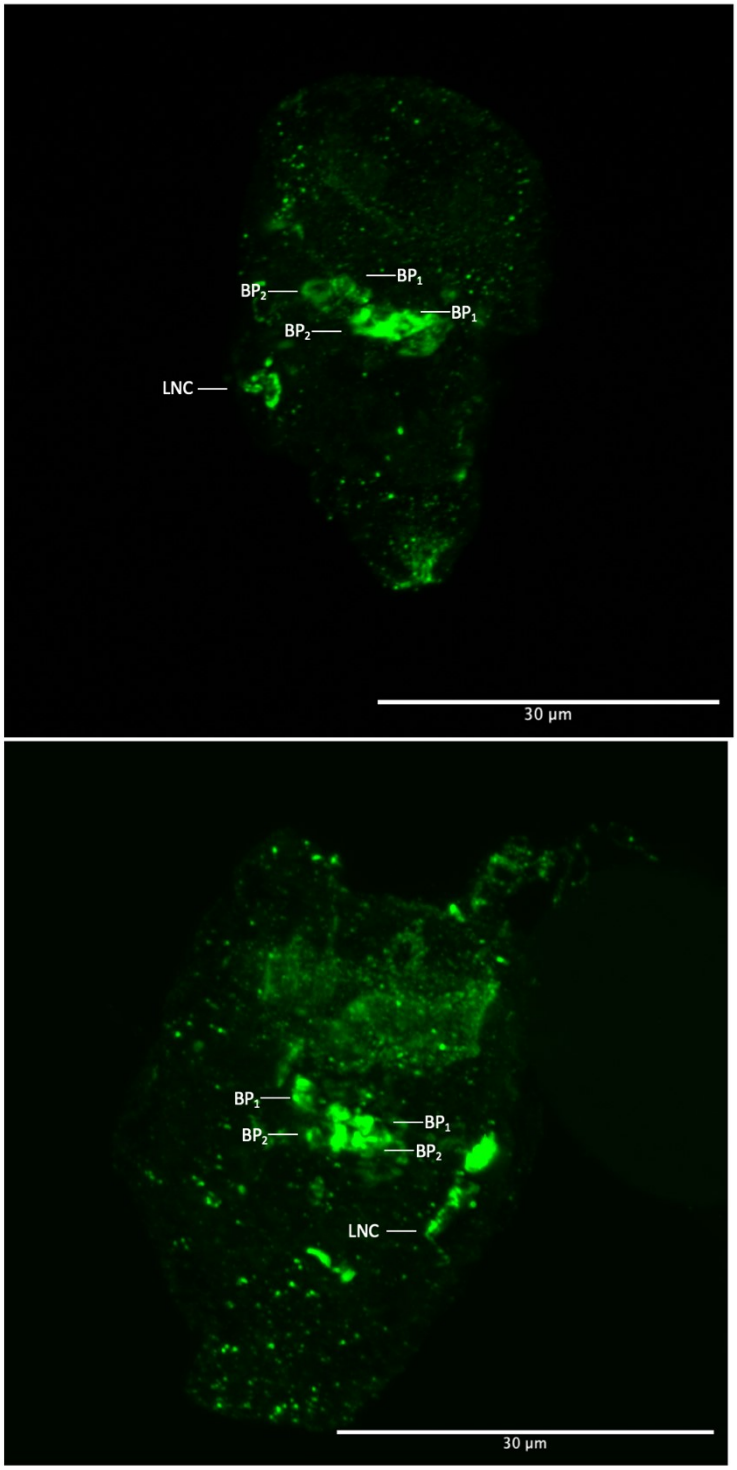

LN
BP1
BP1
LN
BP2
BP2
30 µm

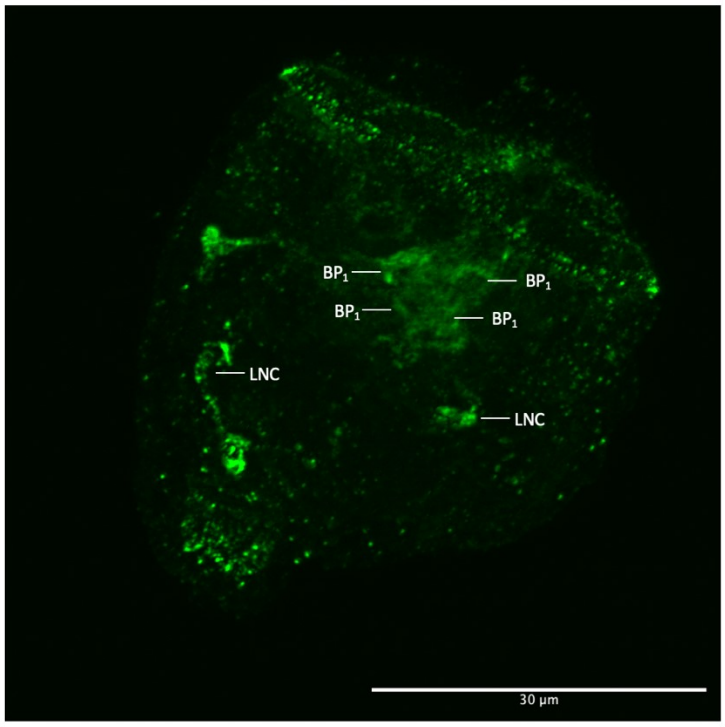

LN
BP1
BP1
LN
BP2
30 µm

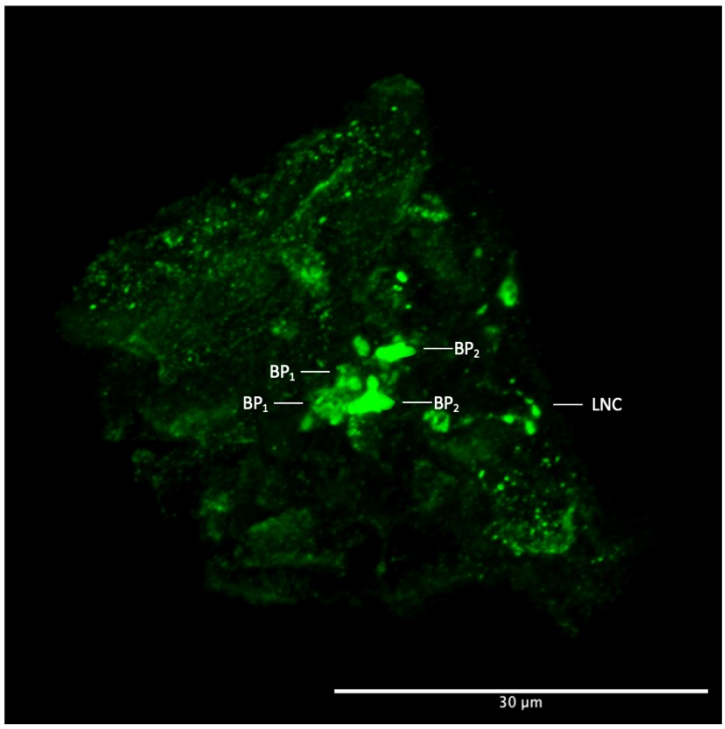

LN
BP1
BP1
BP2
BP2
LN
30 µm

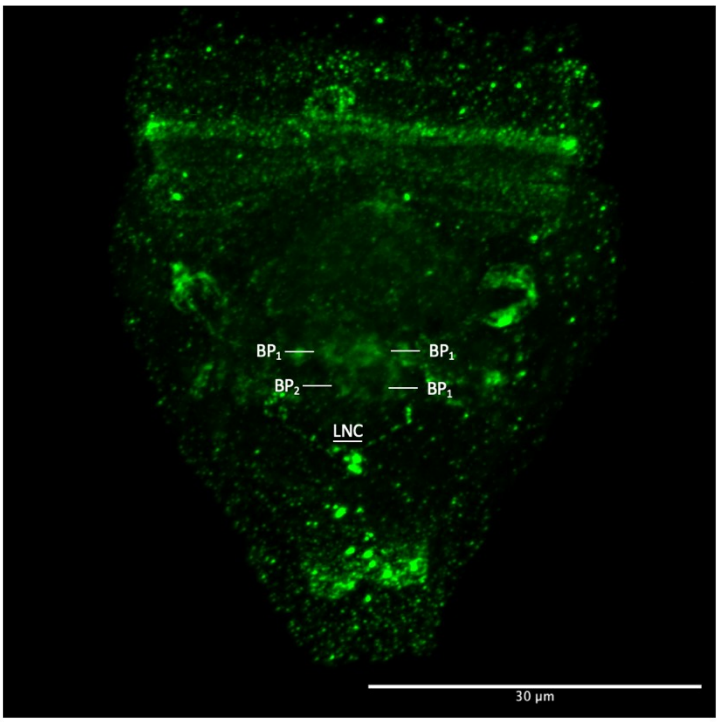

LN
LN
BP1
BP1
BP2
BP2
30 µm

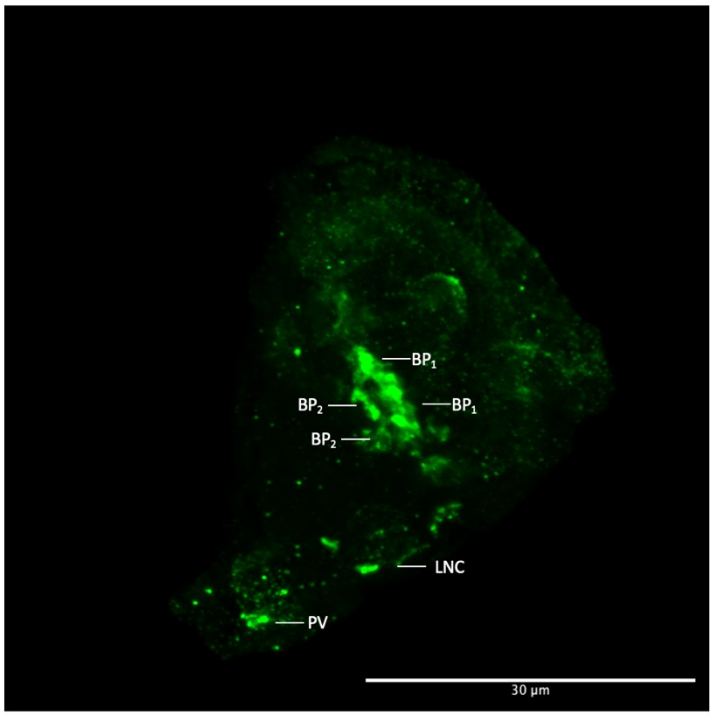
The images below show FLIR positive structures observed in male *Filinia longiseta*

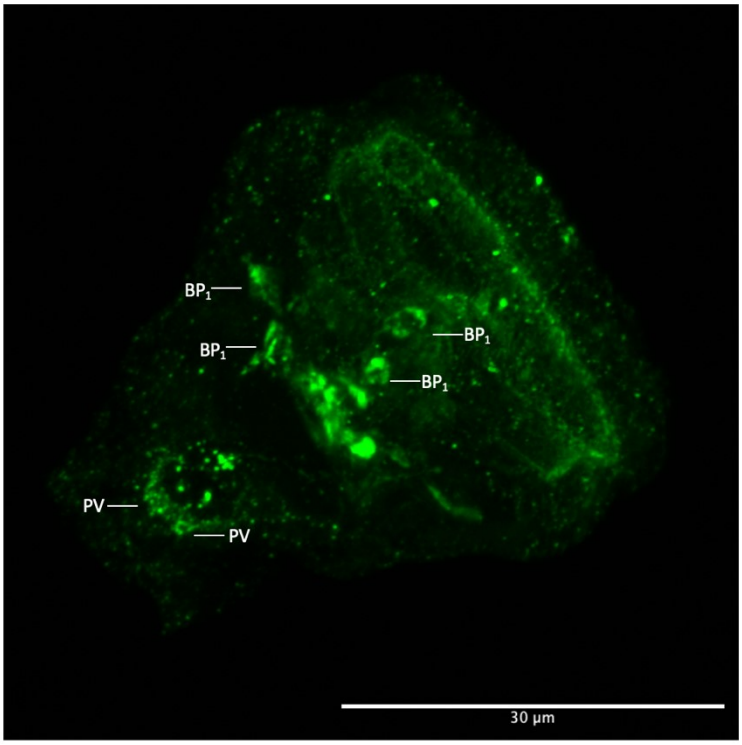



BP1
BP1
BP1
BP1
LNC
LNC
30 µm


BP2
BP1
BP1
BP2
LNC
30 µm


BP1
BP1
BP2
BP1
LNC
30 µm


BP1
BP2
BP1
BP2
LNC
PV
30 µm


BP1
BP1
BP1
BP1
PV
PV
30 µm

Vita

Robert Walsmith (Bobbie) graduated from the University of Texas at El Paso with a Bachelor of Science in Environmental Science in Spring of 2017. He never gave up his pursuit of knowledge of invertebrate systems and continued his education in the master's Program in Biological Sciences. During his graduate career he worked as both a teaching assistant and research assistant for Dr. Walsh funded by the National Science Foundation from 2020 – 2023. He was also awarded the Summer Research Funding Program (SRFP) in 2021.

Bobbie presented his research internationally at the XVI International Rotifer Symposium in Zagreb, Croatia, and nationally at the Society of Comparative and Integrative Biology (SICB) Conference in Austin, Texas. Additionally, he presented the research of colleague Patrick Brown at the SICB conference in Austin, TX. Bobbie was accepted into the Evolutionary Development of Marine Invertebrates course in Friday Harbor Washington instructed by Dr. Billie Swalla and Dr. Andreas Heyland. To attend, Bobbie was awarded the Robert L. Fernald Endowment Fellowship and Anne H. Blinks Fellowship in marine Biology. Bobbie will be continuing his studies under the Genetics and Genomics Scholars PhD fellowship awarded to him through the genetics and genomics doctoral program at North Carolina State University.