Extrasynaptic volume transmission: A novel route for neuropeptide signaling in nematodes

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Abstract

Neural circuit synaptic connectivities (the connectome) provide the anatomical foundation for our understanding of nematode nervous system function. However, other non-synaptic routes of communication are known in invertebrates including extrasynaptic volume transmission (EVT), which enables short- and/or long-range communication in the absence of synaptic connections. Although EVT has been highlighted as a facet of *Caenorhabditis elegans* neurosignaling, no experimental evidence identifies body cavity fluid (pseudocoelomic fluid; PCF) as a vehicle for either neuropeptide or biogenic amine transmission. In the parasitic nematode *Ascaris suum* FMRFamide-like peptides encoded on *flp*-18 potently stimulate female reproductive organs but are only expressed in cells that are anatomically distant from the reproductive organ, with no known synaptic connections to this tissue. Here we report a new non-synaptic mode of signaling in nematodes mediated by neuropeptides within the PCF. Our data show that: (i) *A. suum* PCF (As-PCF) contains a catalogue of neuropeptides including FMRFamide-like peptides and neuropeptide-like proteins; (ii) the *A. suum* FMRFamide-like peptide As-FLP-18A dominates the As-PCF peptidome; (iii) As-PCF potently modulates nematode reproductive muscle function *ex vivo,* mirroring the effects of synthetic FLP-18 peptides; (iv) As-PCF activates the *C. elegans* FLP-18 receptors NPR-4 and -5; (v) As-PCF alters *C. elegans* behavior and, (vi) FLP-18 and FLP-18 receptors display pan-phylum distribution in nematodes. Here we provide the first direct experimental evidence that supports an extrasynaptic volume route for neuropeptide transmission in nematodes. These data demonstrate non-synaptic signaling within the nematode functional connectome and are pertinent to receptor deorphanisation approaches underpinning drug discovery programs for nematode pathogens.

Introduction

Our current understanding of nematode neuronal circuitry is based on comprehensive *Caenorhabditis elegans* synaptic connectome data [1-3]. The *C. elegans* blueprint underpins fundamental parasitic nematode neurobiology driving anatomical and functional connectomics

studies in model parasites such as *Ascaris*, in which the simple neuronal architecture described in *C. elegans* appears to be highly conserved [see 4]. Whilst not experimentally demonstrated in nematodes to date, the existence and significance of additional forms of non-synaptic neuronal communication have been recognized among invertebrates [5, 6]. For example, in crustaceans, extrasynaptic volume transmission (EVT) operates beyond the synaptic connectome, mediating long-range hormonal communication in the absence of neuronal synapses [7, 8]. Notably, EVT has been implicated in nematode neuronal signaling [9-14]. Indeed, the *C. elegans* wired connectome does not always support receptor-ligand interactions that have been functionally linked; there are examples of signaling pathways where receptors are not located co-synaptically with neurons in both monoamine and neuropeptide systems [15-18], supporting a role for EVT in neurotransmission. Further, EVT has been putatively linked to nematode neuropeptide signaling via *in silico* approaches [19]; however, no experimental data are available which test these hypotheses.

Neuropeptides are well known neuromodulators of a wide array of essential neuronal functions in nematodes, including locomotion, reproduction and feeding [see 20 for review]. Despite biological importance, nematode neuropeptide signaling beyond the synaptic connectome is poorly understood. Indeed, the capability and characteristics of neuropeptide EVT in nematodes remain unreported, likely due to the lack of appropriate experimental and analytical tools for the investigation of extracellular communication via body fluid in nematodes including *C. elegans*.

The zoonotic gastrointestinal parasite *A. suum* offers a unique opportunity to probe extrasynaptic communication in nematodes. Adult *A. suum,* which are up to 30 cm in length, contain body cavity fluid (pseudocoelomic fluid, As-PCF) that maintains hydrostatic pressure, bathes internal organs and muscle systems, and provides an ideal vehicle for non-neuronal communication. In contrast to *C. elegans*, the size of adult *A. suum* facilitates routine collection of relatively large volumes (~500 µl/nematode) of As-PCF for bioanalysis. This, in conjunction with the experimental tractability of *Ascaris,* where tissues and organs can be readily dissected for physiology studies [see 20], offers a unique opportunity to investigate EVT in nematodes.

This study exploits peptidomics, nematode physiology, and behavioral bioassays, heterologous expression and bioinformatic approaches to determine the role of nematode As-PCF in EVT of neuropeptides. We show that As-PCF contains a complex array of neuropeptides which modulate nematode behavior. Specifically, we demonstrate that: (i) neuropeptides in As-PCF include FMRFamide-like peptides (FLPs) and neuropeptide-like proteins (NLPs); (ii) As-FLP-18A (GFGDEMSMPGVLRF) is the dominant neuropeptide in As-PCF; (iii) As-PCF is bioactive on the reproductive muscle, altering activity *ex vivo* in a manner similar to that of synthetic As-FLP-18A; (iv) As-PCF activates the *C. elegans* FLP-18 receptors NPR-4 and -5 heterologously expressed in mammalian cells; (v) As-PCF and synthetic As-FLP-18A impact *C. elegans* behavior; and (vi) FLP-18 and the cognate receptors NPR-4 and -5 display pan-distribution across phylum Nematoda. This study provides the first direct experimental evidence that neuropeptide signaling can be mediated non-neuronally by EVT in nematodes. These novel data highlight that non-neuronal, long-range communication is a key facet of neuropeptide signaling in nematodes, adding a new dimension to the interpretation of functional data pertaining to the nematode nervous system.

Results and discussion

Ascaris PCF contains a rich peptide library

Liquid chromatography coupled with mass spectrometric (LC-MS/MS) analyses of As-PCF (n=24 LC-MS/MS runs representing 67 individual worms; see Supplementary Data S1) reveals that multiple peptide families are present in nematode body cavity fluid. We queried the As-PCF peptidome with *A. suum in silico* peptide libraries (unpublished, constructed in-house) for the major nematode neuropeptide families [FLPs and neuropeptide like proteins (NLPs)], as well as antimicrobial peptides (AMPs). We detected 76 peptides in As-PCF of adult nematodes [41 neuropeptides (6 FLPs, 35 NLPs and 35 AMPs; see Fig 1A and Supplementary Data 1 (S1))]. High-confidence peptide spectrum matches were confirmed for 10 peptides [1% FDR; 21; see Table 1].

Our experimental approach involved peptidome analyses of single (n=20 worms; 20 LC-MS/MS runs) and pooled adult female (n=17 worms; one LC-MS/MS run) and pooled adult male As-PCF (3 LC-MS/MS runs: n=18, n=6, n=6, worms respectively). This strategy enabled comparison of both pooled and single-worm As-PCF samples, in addition to sex differences. Pooled As-PCF revealed a richer peptide complement than single As-PCF samples; 54 peptides were detected in the pooled sample (n=17 female worms) relative to 17.75 ± 1.2 peptides detected in single worm samples (n=20 worms) (Fig 1B). Six peptides were detected in the pooled sample that were not identified from single worm samples (As-FLP-18D, As-FLP-18E, As-NLP-14N, As-NLP-66B, As-GRSP-2 and As-NLP-14K; see S1). These data indicate that, whilst pooled samples reveal As-PCF peptidome complexity, single worm As-PCF characterization also has utility as an experimental platform for the interrogation of key peptides which are consistently detected in individual worms (see 1% FDR peptides, Table 1 and S1). Male and female As-PCF comparisons revealed two peptides that were male As-PCF specific: As-NLP-43C and a novel FLP, As-FLP-35 (ANTATASWSIEWLMRLNH$_2$; see S1). As-FLP-35 is a novel FLP recently predicted from the *A. suum* genome [data unpublished; 22].

Antimicrobial Peptides were the most abundant family of peptides detected in the As-PCF (97% of AMP library predictions); As-Cecropin-1 and 2 [also known as P1 and P2 respectively; 23] were the most highly detected peptides in >96% of samples at >97% coverage (Fig 1A, C and Table 1). FLPs and NLPs displayed greater variability in both the frequency of detection and overall peptide coverage. However, most strikingly, As-FLP-18A equaled the frequency and coverage of the AMPs, dominating the As-PCF peptidome (detected in 92% of samples at >93% coverage; Fig 1A, C and S1). Whilst the number of NLPs detected was greater than the number of FLPs, the majority were low frequency (where approximately half of NLPs were detected only once) with the exception of As-NLP-21G and As-NLP-42C (detected in 42% and 46% of samples, respectively; see Fig 1A-D and S1).

This is the first experimental demonstration of neuropeptides in nematode body cavity fluid. This discovery represents a step-change in the understanding of nematode neuronal signaling and

requires that EVT should be considered in the interpretation and comprehension of nematode neurobiology.

The As-PCF neuropeptidome is compelling in the context of *Caenorhabditis elegans* neurobiology

Neuropeptides are released into the body cavity fluid of invertebrates where they act as neurohormones to regulate many physiological functions [24]. A number of the peptides consistently detected in As-PCF in this study (FLP-18, NLP-21, -37, -40) have been reported in *C. elegans* in cells that link them to the PCF. For example NLP-21, -37 and -40 have been localized to *C. elegans* coelomocytes, which have roles in the endocytosis of secreted proteins from the PCF [11, 12, 25, 26]; this has also been observed for additional neuropeptides, e.g. INS-22 [25], that were not included in the custom peptide library used to probe the As-PCF peptidome in this study. Due to their localization in coelomocyte scavenger cells, it has been postulated that these peptides are released from dense core vesicles and endocytosed. However, until now, their presence in nematode PCF has not been substantiated. In addition, *C. elegans* NLP-37 (PDF-2) has been assigned a long-range neuromodulatory function via release into the PCF as observed for the *Drosophila* homologue [27-30]. The detection of NLP-37 in As-PCF provides evidence for a conserved neuromodulatory role in nematodes. Significantly, a neuromodulatory/neurohormonal role for FLP-18 has also previously been suggested [31]; localization data revealed a physical disconnect between neurons expressing FLP-18 and the location of FLP-18 cognate receptors. The consistent presence of As-FLP-18A in the As-PCF peptidome confirms the potential for FLP-18 peptides to modulate neurobiology via EVT in nematodes.

Based on indirect evidence, a role for EVT of neuropeptides in body cavity fluid has been hypothesised for both *A. suum* and *C. elegans* [9, 14, 19, 31]. However, the data presented here provide the first direct evidence for EVT by neuropeptides in As-PCF. Notably, As-PCF has a restricted peptidome relative to the predicted (*in silico*) whole worm peptidome, consistent with selective release of bioactive peptides into this compartment.

As-FLP-18A dominates the *Ascaris* PCF neuropeptidome

As-flp-18 encodes six peptides that share a common C-terminal **PGVLRF-NH$_2$** motif (As-FLP-18A, GFGDEMSM**PGVLRF-NH$_2$**; As-FLP-18B, GM**PGVLRF-NH$_2$**; As-FLP-18C, AV**PGVLRF-NH$_2$**; As-FLP-18D, GDV**PGVLRF-NH$_2$**; As-FLP-18E, SDM**PGVLRF-NH$_2$**; As-FLP-18F, SM**PGVLRF-NH$_2$**). As-FLP-18A is the most frequently detected neuropeptide in As-PCF (92% of samples, >93% coverage; Fig 1C), which leads us to hypothesize that As-FLP-18 peptides play an important role in the *A. suum* PCF.

In our LC/MS analysis, we consistently detected a truncated form of As-FLP-18A (GFGDEMSMPGVLR; see Fig 1D) lacking the C-terminal phenylalanine-amide motif required for biological activity (see Fig 2B). Whilst the detection of a truncated peptide could be an artefact of sample processing, we attempted to minimize this possibility as As-PCF was collected and processed rapidly and shipped under temperature-controlled conditions to limit proteolytic degradation. Enhanced detection of truncated As-FLP-18A could be a result of more-ready ionisation of the arginine residue (position -1) in comparison to the intact As-FLP-18A (C-terminal phenylalanine-amide motif). Interestingly, in the pooled As-PCF samples we also detected As-FLP-18D and As-FLP-18E in addition to As-FLP-18A. In these samples, As-FLP-18D and E were also detected in the truncated form without the expected C-terminal phenylalanine-amide motif. Detection of these additional As-FLP peptides in pooled samples, but not in individual worm As-PCF, suggests that these peptides are present at lower concentrations, nearing the LC/MS limit of detection. An alternative hypothesis is that the truncated forms of As-FLP-18A, D and E are produced post-interaction with As-FLP-18 receptors in a signal termination event. Indeed, the E/S products and PCF of nematodes including *A. suum* are known to be peptidase-rich and include a range of cysteine proteases [32-35]. These peptidases may be selectively released at the target receptor to spatiotemporally control peptide interactions and mediate neuropeptide action [36]. In this scenario, peptidase action could manifest in an elevation, and thus an enhanced detection, of truncated versus intact As-FLP-18 peptides. This is consistent with our observations and would contribute to the plasticity of nematode neuronal signaling via EVT. The biological reason for the predominance of As-FLP-18A over the additional five peptides encoded by *As-flp-18* is unclear.

Indeed, *As-flp-18* is not predicted to be differentially spliced in *A. suum* as revealed by both genome-level analyses and molecular (PCR) investigation (data not shown). Regardless, truncated As-FLP-18A (synthetic) lacks biological activity in *A. suum* muscle physiology assays in contrast to intact As-FLP-18A (synthetic) and As-PCF, which both induce characteristic FLP-18 responses (see subsequent discussion and Fig 2A and B). These data strongly suggest that both the intact and truncated form of As-FLP-18A are present in As-PCF.

The bioactivity of *Ascaris* PCF on *Ascaris* reproductive tissue mirrors that of synthetic As-FLP-18A

Neuropeptides potently modulate the intrinsic muscle activity of *Ascaris* reproductive organs [ovijector; 37, 38-42] defined by five distinct response types (RT-1-RT5), ranging from inhibition of muscle contraction (flaccid paralysis, e.g., RT-1) to increased contraction frequency [e.g. RT5; 38]. As-PCF also potently modulates *Ascaris* reproductive muscle activity. Exogenously applied As-PCF induces two distinct myoactivity profiles on the ovijector that are characteristic of either an RT-1- or RT-2 like response [38; see Figure 2B]. At low concentrations [0.015X As-PCF (where 1X As-PCF represents the native biological sample)], As-PCF consistently inhibits muscle activity in an RT-1 like response [Fig 2A; Supplementary Table 1 (S1 Table)]. At higher concentrations (≥0.15X As-PCF), the response profile included both RT-1 (inhibitory; 50% of preparations) and RT-2 like responses (excitatory; 50% of preparations) (see Fig 2A; S1 Table). All effects were reversible upon As-PCF washout.

Interestingly, in this study we present myoactivity profiles for As-PCF that are consistent with the myoactivity profile of synthetic As-FLP-18A which is also the dominant As-PCF peptide. Synthetic As-FLP-18A induces consistent excitatory effects (10 nM-1 μM) that include transient muscle contraction followed by muscle paralysis [1 μM; RT-2 like; Fig 2B; Supplementary Table 2 (S2 Table)] [38]. At high concentrations (10 μM) synthetic As-FLP-18A induces two distinct myoexcitatory profiles that resemble those observed with high concentrations of As-PCF (RT-1 like, 64.2% of preparations; RT-2 like, 35.7% of preparations; see Fig 2B and S2 Table). These

responses were corroborated in synthetic peptide positive controls subjected to the same experimental manipulations as As-PCF (Fig 2B; S2 Table).

As-FLP-18 peptides ($PGVLRFNH_2$) have been implicated in locomotion, feeding and reproductive function via whole worm, pharyngeal and ovijector tissue bioassays [see 20]. However, although *As-flp-18* encoded peptides are potent stimulators of the *Ascaris* ovijector, their expression pattern is limited to neurons that are anatomically distinct from, and have no known synaptic connections to, reproductive tissue [as demonstrated by in situ hybridization and MS/MS; 43]. The ovijector receives input from two parallel neuronal cell bodies situated in close proximity to the body wall and the ventral nerve cord (similar positions to *C. elegans* VC4 and VC5) [37]. In this study, RNAseq analysis was conducted on two distinct tissue types: (i) the gonopore region, an area of body wall tissue which contains the neuronal cell bodies which innervate the ovijector and; (ii) the ovijector itself which expresses the post-synaptic receptors. Our tissue-specific differential expression analyses of *As-flp*-18 and the As-FLP-18 cognate GPCR sequelogs *As-npr*-4 and *As-npr*-5 [13, 31, 44], revealed that the neuronal cell bodies innervating the ovijector do not express *As-flp*-18 despite the presence of both cognate receptors in the ovijector tissue (unpublished data manuscript in preparation). These data support the hypothesis that As-PCF-circulating neuropeptides modulate *A. suum* reproductive function. Whilst our data point towards a significant role for As-FLP-18A in modulating reproductive function via EVT, the contribution of other peptides detected in the As-PCF will require further investigation to unravel EVT dynamics.

Ascaris PCF activates heterologously expressed *Caenorhabditis elegans* NPR-4 and NPR-5

To characterize the interaction of As-PCF with the cognate FLP-18 receptors NPR-4 and -5, and to provide further evidence for As-FLP-18 signaling via EVT, we measured the response of Ce-NPR-4 and -5 (expressed in CHO cells) to As-PCF and synthetic As-FLP-18 peptides. Note that attempts to express As-NPR-4 and -5 in the heterologous CHO system were unsuccessful in this study [consistent with difficulties in parasitic nematode GPCR heterologous expression; see 20 for review], requiring the use of the *C. elegans* receptors as surrogates. As-PCF and synthetic As-

242 FLP-18 peptides potently activated Ce-NPR-4 and -5 (synthetic Ce-FLP-18 peptides also elicited
243 the expected responses; see Figs 3A-C). Synthetic As-FLP-18A-E elicited significant Ca^{2+}
244 responses in cells transfected with *C. elegans* NPR-4 and cells transfected with NPR-5, compared
245 to the negative control (cell medium without peptide; see Fig 3A). Synthetic As-FLP-18 peptides
246 did not evoke significant Ca^{2+} responses in CHO cells transfected with an empty vector, confirming
247 that Ce-NPR-4 and -5 mediate the observed physiological responses (Fig 3A). As-PCF also
248 evoked a significant Ca^{2+} response in Ce-NPR-4 or Ce-NPR-5 transfected cells compared to
249 negative controls (see Figs 3B and C). A low-level Ca^{2+} response was observed in cells transfected
250 with an empty control vector as well, indicating that some component(s) of As-PCF interact with
251 receptors endogenously expressed in CHO cells (see Figs 3B and C). Despite this, As-PCF elicited
252 a significantly higher response in cells expressing Ce-NPR-4 or -5 than the negative control (empty
253 vector transfected CHO cells), indicating that a significant component of the As-PCF response is
254 specific to Ce-NPR-4 and -5 activation (see Fig 3B and C respectively).
255 The activation of heterologously expressed Ce-NPR-4 and -5 by As-PCF and synthetic As-FLP-18
256 peptides provides data that verifies conservation of the FLP-18 interaction with NPR-4 and -5 [31,
257 44] in *Ascaris* and supports our hypothesis that As-PCF peptides interact with putative FLP-18
258 receptors *in vivo* via EVT.
259

260 *Ascaris* PCF impacts *Caenorhabditis elegans* growth, reproduction and
261 locomotion

262 To further investigate the biological activity of As-PCF in nematodes we examined the effects of
263 As-PCF and synthetic As-FLP-18A in *C. elegans* using three behavioral assays which assess
264 nematode growth (body length), reproduction (progeny) and motility (locomotory activity) [45, 46]
265 in *Ce-acs*-20 [cuticle defective mutant to facilitate peptide uptake; 47]. *Caenorhabditis elegans*
266 growth was potently inhibited in a dose-dependent manner by 2% and 5% As-PCF; this effect was
267 mirrored by treatment of *C. elegans* with the dominant As-PCF neuropeptide As-FLP-18A (100 μM,
268 synthetic peptide) (see Fig 4A). In addition, As-PCF dose-dependent effects on reproduction were
269 also evident, where progeny was reduced in the presence of 1, 2 and 5% As-PCF; these effects

were also evident in nematodes treated with 100 μM synthetic As-FLP-18A (see Fig 4B). *Caenorhabditis elegans* motility was significantly impaired in the presence of 5, 7.5 and 10% As-PCF as measured by the wMicroTracker (InVivo Biosystems, Oregon, USA); synthetic As-FLP-18A (10 μM) also produced significant inhibition of locomotion (see Fig 4C). In all assays the As-PCF induced modulation of *C. elegans* behavior was consistent with that observed for the synthetic As-FLP-18A peptide (see Fig 4A-C) and corresponds to published literature which demonstrates the modulatory activity of FLP-18 in a number of *C. elegans* behaviors and biological processes [see 48 for review].

flp-18 and the cognate receptors, *npr*-4 and *npr*-5, display pan-phylum conservation in nematodes

In silico analyses of 134 available nematode genomes, representing 109 species, 7 Clades and 3 distinct lifestyles [free-living, animal parasitic and plant parasitic; 49, 50, 51], demonstrates striking *flp-18* pan-phylum conservation in 99% of nematode species [see Fig 5 Supplementary Data 2 (S2)]. *flp*-18 is the only neuropeptide gene which displays this level of conservation in phylum Nematoda (unpublished data). These data drive the hypothesis that FLP-18 peptides are fundamental to nematode biology and support the potential for FLP-18 signaling via EVT to be a pan-phylum trait.

Broad conservation was also noted for the putative FLP-18 receptor encoding genes (*npr*-4 in 93% of species and *npr*-5 in 84% of species; see Fig 5), highlighting their importance across phylum Nematoda. It is interesting to note that, for most of the species examined (98%), the presence of the FLP-18-encoding gene was indicative of the presence of either putative cognate receptor or both and *vice versa* (Fig 5). These data suggest that the FLP-18-NPR-4/5 interaction, which has been functionally validated in *C. elegans*, *Brugia malayi* [31, 44] and, in this study, in *A. suum*, is highly conserved across nematodes; consequently these receptors hold significant appeal as broad-spectrum drug targets in parasitic nematodes.

Conclusions

This study reveals a rich neuropeptide profile in the body cavity fluid of a parasitic nematode and, for the first time, provides direct evidence that EVT is an integral part of the nematode functional connectome. These data demonstrate that nematode signaling systems operate within a connectome comprising both wired and wireless components, adding complexity and plasticity to nematode communication frameworks. The biological significance and exploitation of the nematode connectome is significantly enhanced by the wireless data presented here. The combined network infrastructure revealed the experimental tractability of *A. suum* in this study provides a unique and powerful tool to unravel the intricacies of information flow within the nervous system of nematodes and higher organisms.

Materials and methods

Collection and maintenance of *Ascaris suum*

Adult *A. suum* were collected at a local abattoir (Karro Food Group Ltd, Cookstown, Northern Ireland), transported to the laboratory in saline (0.9% NaCl), and were maintained in *Ascaris* Ringers Solution (ARS: 13.14 mM NaCl, 9.47 mM $CaCl_2$, 7.83 mM $MgCl_2$, 12.09 mM $C_4H_{11}NO_3$/Tris, 99.96 mM $NaC_2H_3O_2$,19.64 mM KCl, pH 7.8) at 37 °C until use.

Maintenance of *Caenorhabditis elegans*.

The *C. elegans* cuticle defective mutant strain *acs*-20 [tm3232; 47] was obtained from the National BioResource Project of the Tokyo Women's Medical University (Tokyo, Japan). *Ce-acs*-20 have defects in the cuticle barrier which increase permeability to small molecules [47]. *Ce-acs*-20 were maintained on Nematode Growth Media (NGM) plates seeded with *Escherichia coli* OP50 (CGC) and synchronized using established methods [52].

Ascaris suum pseudocoelomic fluid collection

As-PCF collection from adult *A. suum* was carried out within 3 hrs of parasite collection. Female worms >20 cm were used in experiments. Between 400 and 1000 µl of As-PCF was collected from each female worm and between 100 and 500 µl of As-PCF was collected from each male worm. As-PCF was obtained from blot-dried (Kimwipes, Fisher Scientific, UK) female worms by holding the specimen vertically above a 2 ml low binding microcentrifuge tube (Fisher Scientific, UK) and carefully snipping <3 mm tissue from the tail (posterior to the anal pore and the major ganglia) using a pair of sterile scissors. AS-PCF was discharged from the worm into the collection tube with the aid of internal turgor pressure and gentle manual compression. Male As-PCF was collected by drying the cuticle and making a 1 cm incision with a sharp scalpel approximately 5 cm posterior to the head (mid body region) and allowing turgor pressure to discharge As-PCF into a 2 ml collection tube; male worms are not amenable to female worm As-PCF collection processes due to the close proximity of male reproductive organs at the posterior tip of the tail which prevents access to the pseudocoelomic cavity. As-PCF was placed on ice immediately after collection. Single worm As-PCF (n=24) and pooled As-PCF analyses (n=17 worms pooled) were conducted for female worms. Male single worm As-PCF analysis was not possible due to the low volume of As-PCF retrieved from individual male worms; As-PCF collected from 30 males was used in pooled analyses

LC-MS/MS

Peptide extraction: Peptide extraction was carried out in two stages. Phase one was conducted at Queen's University Belfast immediately following As-PCF collection; phase two was conducted at the University of Wisconsin-Madison, WI, USA. *Phase one*: As-PCF was spiked with an equal volume of acidified methanol (90% MeOH, 9% ddH_2O, 1% glacial CH_3COOH) immediately following collection (to extract peptides and precipitate large proteins), vortexed and centrifuged for 15 min at 20,000 *g*. The supernatant was retained in a 2 ml low binding microcentrifuge tube (Fisher Scientific, UK). The pellet was resuspended in 500 µl acidified methanol using a sterile plastic pestle. This was repeated twice more and the supernatant from each extraction was retained as above in addition to the final pellet. 250 µl ddH_2O was added to the supernatant solutions from extractions 2 and 3 to decrease MeOH concentration to 60%. All three supernatant solutions and

the final pelleted solids remaining after the third extraction were snap frozen in liquid N_2 and stored at -80 °C prior to shipping. *Phase two*: upon receipt (University of Wisconsin, Madison), frozen supernatant was thawed and loaded immediately onto a 10 kDa molecular weight cut-off membrane (Sigma-Aldrich, St. Louis, MO) which was rinsed with 0.1 M NaOH and 50/50 MeOH/H_2O, (v/v) prior to sample loading. The tube holding the membrane was centrifuged at 14,000 *g* for 10 min and the filtrate was dried in a SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA). The purified sample was resuspended in 150 µl 0.1% formic acid (FA) in H_2O, desalted by OMIX C18 pipette tips (Agilent Technologies, Santa Clara, CA) and finally eluted into 0.1% FA in 50/50 ACN/H_2O (v/v). The eluate was concentrated until dry and stored at -80 °C. *LC-MS/MS analysis:* Stored samples were dissolved in 10 µl 0.1% formic acid (FA). Nano LC-MS/MS/MS analysis was carried out using a Waters Nano-Acquity Ultra Performance LC system (Waters Corp, Milford, MA, USA) coupled to a Q-Exactive Quadrupole-Orbitrap mass spectrometer (Thermo Scientific Bremen, Germany). A self-packed column (150 mm length of 1.7 µm C18 with a 3 µm C18 cap) was used for chromatographic separation. The mobile phase involved in online separation was: A, 0.1% FA in H_2O and B, 0.1% FA in ACN. A 120-min gradient was applied at flow rate of 0.3 µl/min, starting from 100% A. Mobile phase B increased to 10% in 1 min, 35% at 90 min, 95% at 92 min. The gradient remained at 5% A for 10 min, then recovered to 100% A at 105 min. Data were collected under positive electrospray ionization data-dependent mode (DDA), with the top 15 abundant precursor ions selected for HCD fragmentation with listed settings: full-MS, resolution, 70,000; automatic gain control (AGC), 1e6; scan range, m/z 200-2000; dd-MS2, resolution 17,500; AGC, 2e5; isolation window, m/z 2.0; fixed first mass, m/z 100.0; normalized collision energy, 30. Other typical MS parameters were: spray voltage, 2.1 kV; no sheath and auxiliary gas flow; heated capillary temperature, 275 °C. *MS data analysis:* Parent mass error tolerance was 10 ppm and fragment mass error tolerance was 0.02 Da. C-terminal amidation, methionine oxidation and pyro-glutamation were specified as variable post-translational modification (PTMs). No enzyme cleavage was selected within the de novo sequencing. A custom neuropeptide database representing predicted *A. suum* FLPs, NLPs and AMPs was used in the search process. A threshold of false discovery rate (FDR) of 1% was used for data filtration and

379 validation. NanoLC-ESI-MS raw data were analyzed by PEAKS Studio 7 (Bioinformatics Solution
380 Inc., Waterloo, ON, Canada).
381
382 ## *Ascaris suum* ovijector bioassays
383 *Tissue excision and data collection:* Adult female *A. suum* ovijector tissue and data collection was
384 carried out as previously described [41]. Briefly, freshly excised *A. suum* ovijector tissue was
385 attached to the recording apparatus and synthetic peptide or As-PCF were added directly to the
386 chamber following tissue equilibration. Muscle activity was recorded for 10 min. Media in the
387 recording chamber was then replaced with fresh HBSS (37°C; Sigma-Aldrich), and the muscle
388 allowed to recover for a further 10 min. If regular baseline activity was achieved following washout
389 further experiments with the specimen were conducted. *FLP-18 bioassays: A. suum* synthetic FLP-
390 18 peptide [As-FLP-18A, GFGDEMSMPGVLRFNH$_2$; Genosphere Biotechnologies (France)] was
391 tested for bioactivity on ovijector tissue. Peptide stock solutions (10 mM – 1 µM) were prepared
392 using ddH_2O such that 4 µl peptide stock solution was added to 4 ml HBSS in the recording
393 chamber to achieve the desired final peptide concentration (10 - 0.001 µM). ddH_2O was used as
394 a negative control. *As-PCF bioassays:* 3 ml fresh As-PCF was collected from female worms (as
395 described above) and passed through a Sep-Pak® Classic C18 cartridge (Waters, Ireland). Sep-
396 Pak® Classic C18 cartridges were activated with 3 ml activation solution (3 ml Ultrapure Acetonitrile,
397 0.1 % HPLC grade trifluoroacetic acid) and washed with 6 ml wash solution (6 ml ddH_2O, 0.1 %
398 HPLC grade trifluoroacetic acid). Following initial flow through, As-PCF effluent was passed
399 through the C18 cartridge twice more to ensure optimal peptide binding. C18 cartridges were then
400 washed using 6 ml wash solution and peptides were eluted in 3 ml activation solution. All samples
401 were dried overnight using a Genevac™ MiVac concentrator at 37°C and stored at 4°C until use.
402 Samples were resuspended in 200 µl ddH_2O immediately before use. Controls included
403 phosphate-buffered saline (PBS; 150 mM NaCl, 0.025 M $NaH_2PO_4.2H_2O$, 0.075 M Na_2HPO_4, pH
404 7.4) spiked with synthetic As-FLP-18A (PBS-As-FLP-18 SB; 100 µM) before C18 perfusion, PBS
405 spiked with synthetic As-FLP-18A after C18 perfusion (PBS-As-FLP-18 SA; 100 µM), and perfused
406 activation solution only (resuspended in ddH_2O; negative control). 4 or 40 µl As-PCF [15X

concentrated; final concentration in recording chamber 0.015X and 0.15X As-PCF respectively (where 1X As-PCF represents the native biological sample)], PBS-As-FLP-18 SB (final concentration in recording chamber 0.1 µM or 1 µM), or PBS-As-FLP-18 SA (final concentration in recording chamber 0.1 µM or 1 µM) were added to the recording chamber (n=4) and effects on ovijector muscle recorded. All data were analyzed as described [41]. Statistical analyses included repeated measures ANOVA and Student's *t*-test to assess the significance of test substance/peptide effects and effect reversal after washout, respectively (see Fig 2).

Cell-culture activation assays for *Caenorhabditis elegans* receptors

GPCR activation assay was performed as previously described [17, 53]. Briefly, mammalian CHO K1 cells stably overexpressing apo-aequorin and human $G\alpha_{16}$ (ES-000-A24, PerkinElmer) were transiently transfected with *Ce-npr*-4a/pcDNA3.1, *Ce-npr*-5a/pcDNA3.1 or empty pcDNA3.1 (negative control) plasmid using Lipofectamine LTX and Plus reagent (Invitrogen, UK). After transfection, cells were grown in culture flasks overnight at 37 °C, after which they were transferred to 28 °C and allowed to incubate for 24 h. On the day of the assay, CHO cells were harvested and loaded with Coelenterazine H (Invitrogen) for 4 h at room temperature, which elicits the formation of the Ca^{2+}-sensitive photoprotein aequorin. As-PCF or synthetic peptides (As-FLP-18 or Ce-FLP-18) dissolved in DMEM/BSA medium were added to cells and luminescence measured for 30 s at 496 nm using a Mithras LB940 luminometer (Berthold Technologies, Germany). After 30 s of readout, 0.1% Triton X-100 was added to lyse the cells, resulting in a maximal Ca^{2+} response that was measured for 10 s. Note that *C. elegans* synthetic peptides were synthesized by Thermo Scientific and *A. suum* synthetic peptides were synthesized by Genosphere Biotechnologies (France), all peptides were tested at a final concentration of 10 µM. The final concentration of As-PCF was half the concentration of the undiluted sample. In addition, a 10-fold dilution series ranging from 1/20 to 1/2000 of the undiluted As-PCF sample was tested. Assays were performed in triplicate on at least two different days.

Caenorhabditis elegans bioassays

Body length assay: C. elegans body size assays were carried out as previously described [45]. Nematodes were treated with either 1, 2 or 5% As-PCF [diluted in S-medium, 1 L S Basal, 10 ml 1 M potassium citrate pH 6.0, 10 ml trace metals solution, 3 ml 1 M $CaCl_2$, 3 ml 1 M $MgSO_4$) [52]],10 µM synthetic As-FLP-18A [positive control; Genosphere Biotechnologies (France)] or S-medium (negative control; n>9 nematodes per treatment). Following incubation at 20˚C for 96 hr nematodes were immobilized in M9 buffer containing 50 mM sodium azide (NaN_3) and body length was calculated using a Leica MZ 12.5 stereomicroscope, Unibrain Fire-i digital camera and ImageJ software [54]. *Brood size assay:* Five L4 *C. elegans* were transferred into one well of a 24-well plate containing either 1, 2 or 5% As-PCF (diluted in S-medium), 10 µM synthetic As-FLP-18A (positive control) or S-medium (negative control) and supplemented with *E. coli* OP50 to a final volume of 1 ml (n=4 wells/treatment). Brood size was calculated by counting the number of progeny in each well after 96 hr at 21˚C [46]. *wMicroTracker motility assay:* 50 L4 *C. elegans* in M9 buffer were added to each well of a 96-well plate along with *E. coli* OP50 (OD_{600nm} of 1) to a final volume of 90 µl. Basal locomotory activity was recorded for 1 hr using the wMicroTracker (InVivo Biosystems, Oregon, USA) with a bin size of 30 mins. After 1 hr each well was supplemented with 10 µl 1, 2, 5, 7.5 or 10% As-PCF (diluted in M9), M9 buffer (negative control) or 10 uM synthetic As-FLP-18A (positive control) (n>4 wells/treatment). Nematode locomotory activity was recorded for a further 12 hrs. Data were analyzed using GraphPad Prism version 8. Statistical significance was determined by one-way ANOVA and Dunnett's multiple comparisons test or two-way ANOVA and Tukey's multiple comparisons test.

In silico* identification of *flp-18*, *npr-4* and *-5

Putative nematode *flp-18*, *npr-4* and *npr-5* orthologs were identified from WormBase ParaSite version 14 [https://parasite.wormbase.org/index.html; 49, 50, 51] and aligned using CLUSTAL Omega [default settings; 55]. Alignments were subsequently used to construct Hidden Markov Models (HMM) using HMMER v3.2 [default hmmbuild parameters; 56] based on methods previously described [57]. *hmmsearch* (default settings) was employed to identify potential *flp-18*, *npr-4* and *npr-5* sequences within the predicted protein datasets of 109 nematode species [134

463 genomes, see Supplementary Data 2 (SD2); 51]. The putative *npr-4* and *-5* sequences identified
464 via *hmmsearch* were then used as queries in BLASTp searches in the NCBI non-redundant *C.*
465 *elegans* predicted protein database (https://blast.ncbi.nlm.nih.gov; default settings). The putative
466 *flp-18* sequences identified via *hmmsearch* were confirmed visually via the presence of the
467 conserved PGXXRFG C-terminal motif. Queries that failed to return a putative target gene as the
468 highest scoring pair/top hit were excluded from downstream analyses. Genomes lacking putative
469 target gene hits were subjected to manual BLASTp and tBLASTn using WormBase ParaSite
470 version 14 and *C. elegans flp-18*, *npr-4* and *npr-5* as query sequences. Additionally, orthologs from
471 the most closely related nematode species were also used as queries in BLASTp/ tBLASTn
472 searches.
473

474 ### Statistical analysis

475 All statistical analyses were performed using GraphPad Prism Version 8. Statistical tests included
476 one-way ANOVA (Dunnett's multiple comparisons test), two-way ANOVA (Tukey's multiple
477 comparisons test), repeated measures ANOVA and Student's *t*-test. LC-MS/MS data were
478 analyzed using PEAKS Studio 7 (Bioinformatics Solution Inc., Waterloo, ON, Canada)
479

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Figure Legends

Fig 1. *Ascaris suum* pseudocoelomic fluid (As-PCF) contains a rich neuropeptide library. (A) 41 neuropeptides, including 6 FMRFamide like peptides (FLPs) and 35 neuropeptide like proteins (NLPs), and 35 antimicrobial peptides (AMPs) are present in As-PCF (n=24 LC-MS/MS runs representing 67 individual worms; combined data from single worm, pooled female and male samples). This represents 9.09%, 14.89% and 97.20% of FLP, NLP and AMP peptides (respectively) predicted from the *in silico* libraries used to query the As-PCF LC-MS/MS data. (B) Pooled As-PCF (P As-PCF) derived from female nematodes has a richer neuropeptide complement than single worm As-PCF samples (SW As-PCF) [54 peptides detected in pooled As-PCF (n=17) vs an average of 17.75 ±1.2 peptides across single worm samples (n=20)]. (C) The frequency of detection of individual peptides is variable in As-PCF. Dotted line represents detection rate in 50% of samples. Nine peptides are detected consistently in >50% of As-PCF samples [As-Cecropin P1, As-Cecropin P2, As-FLP-18A, As-Cecropin P3, As-Defensin 2, As-Defensin 1, As-Nemapore 1, As-GRSP-16, As-Cecropin 6]. See Supplementary Data 1 (S1) for all peptide sequences detected. (D) Mass spectra of (i) FLP-18A detected in As-PCF (GFGDEMSMPGVLR) and (ii) As-FLP-18A isotopic standard (GFGDEMSMPGVLRFNH$_2$). Amino acid alignments indicate similar fragmentation patterns for As-FLP-18A detected in As-PCF and As-FLP-18A isotopic standard but that As-PCF-derived As-FLP-18A is truncated at the C-terminus. All data are represented as mean ± SEM.

Fig 2. *Ascaris suum* pseudocoelomic fluid (As-PCF) is bioactive on *Ascaris* ovijector tissue and mirrors the synthetic As-FLP-18A peptide response. (A) Representative muscle tension recordings showing the effects of As-PCF on *A. suum* ovijector tissue: (i) As-PCF induces two distinct, concentration dependent, myoactivity profiles (known as response types; RT) on the *A. suum* ovijector; 40 µl As-PCF (equivalent to ≥0.15X As-PCF where 1X is representative of the

737 biological sample) induces either a transient muscle contraction followed by muscle paralysis (50%
738 of preparations; RT-2), or immediate muscle relaxation (50% of preparations; RT-1). 4 µl As-PCF
739 (equivalent to 0.015X As-PCF where 1X is representative of the biological sample) induces an RT-
740 1 response only; (ii) Positive controls [phosphate buffered saline spiked with synthetic As-FLP-18A
741 (GFGDEMSMPGVLRFNH$_2$) before C18 peptide purification; SB] induce two distinct, concentration
742 dependent, myoactivity profiles on the *A. suum* ovijector. 40 µl SB (equivalent to final concentration
743 of 1 µM synthetic As-FLP-18A) induces either a RT-2 (50% of preparations), or RT-1 (50% of
744 preparations) response. 4 µl SB (equivalent to final concentration of 0.1 µM synthetic As-FLP-18A)
745 induces an inhibitory RT-1 response only; (iii) Positive controls [phosphate buffered saline spiked
746 with synthetic As-FLP-18A (GFGDEMSMPGVLRFNH$_2$) after C18 peptide purification (SA) also
747 induce a concentration dependent myoactivity profile on the *A. suum* ovijector. 40 µl and 4 µl SA
748 controls (equivalent to final concentration of 1 µM and 100 nM synthetic As-FLP-18A, respectively)
749 induce an RT-2 response in 100% of preparations. 40 µl and 4 µl SA controls (equivalent to final
750 concentration of 1 µM and 100 nM synthetic As-FLP-18A, respectively) induce an RT-2 response
751 in 100% of preparations; (iv) Negative control (perfused activation solution only resuspended in
752 ddH_2O) does not modulate intrinsic ovijector contractility. Test compounds (As-PCF, SB, SA.
753 negative control), were present during the period indicated by arrows. Vertical scales represent 2
754 mg and horizontal scales represent 2 min. (B) The effects of As-PCF and positive controls (SB and
755 SA) on: (i) contraction frequency, (ii) contraction amplitude and, (iii) muscle tension of the *A. suum*
756 ovijector. Test compounds (As-PCF, SB or SA) were added at 0 min and washed out at 10 min.
757 Data are presented as mean ± SEM [see Supplementary Table 1 (S1 Table)]. (C) Representative
758 muscle tension recordings showing the effects of synthetic As-FLP-18A on *A. suum* ovijector tissue.
759 1 nM-10 µM synthetic As-FLP-18A induce concentration dependent myoexcitatory effects; 10 µM
760 synthetic As-FLP-18A induces two distinct myoexcitatory profiles consisting with RT-2 (35.7% of
761 preparations), or RT-1 (64.2% of preparations); 1 µM-10 nM synthetic As-FLP-18A induce RT-2
762 responses only. The truncated form of synthetic As-FLP-18A (GFGDEMSMPGVLR; 10 µM) does
763 not modulate ovijector contractility. Negative control (ddH_2O) does not modulate intrinsic ovijector
764 contractility. Peptide was present during the period indicated by the arrows. Vertical scales

represent 2 mg and horizontal scales represent 2 min. (D) The concentration dependent effects of 1 nM-10 μM synthetic As-FLP-18A on (i) contraction frequency, (ii) contraction amplitude and, (iii) muscle tension of the *A. suum* ovijector. Peptide was added at 0 min and washed out at 10 min. All data are presented as mean ± SEM [see Supplementary Table 2 (S2 Table)].

Fig 3. *Ascaris* PCF activates heterologously expressed *Caenorhabditis elegans* neuropeptide receptors NPR-4 and NPR-5 in mammalian cell lines. (A) *C. elegans* NPR-4 (Ce-NPR-4) and -5 (Ce-NPR-5) expressed in CHO cells are activated by synthetic As-FLP-18 peptides (10 μM; As-FLP-18A: GFGDEMSMPGVLRFNH$_2$; As-FLP-18B: GMPGVLRFNH$_2$; As-FLP-18C: AVPGVLRFNH$_2$; As-FLP-18D: GDVPGVLRFNH$_2$; As-FLP-18E: SDMPGVLRFNH$_2$; As-FLP-18F: SMPGVLRFNH$_2$) compared to controls transfected with an empty vector (pcDNA3.1). (B) Ce-NPR-4 is activated by As-PCF in a concentration dependent manner compared to both peptide-free cell medium (BSA) and empty vector (pcDNA3.1) negative controls. The cognate Ce-NPR-4 peptide Ce-FLP-18A (DFDGAMPGVLRFNH$_2$) and As-FLP-18A were used as positive controls. (C) Ce-NPR-5 is also activated by As-PCF in a concentration dependent manner compared to both peptide-free cell medium (BSA) and empty vector (pcDNA3.1) negative controls. The cognate Ce-NPR-5 peptide Ce-FLP-18A and As-FLP-18A were used as positive controls. In all cases data are shown as the ratio of peptide/As-PCF response to total calcium response. Error bars represent mean ± SEM., n>3 replicates. Statistical significance of peptide or As-PCF-evoked responses compared with BSA or empty vector controls was determined by two-way ANOVA and Tukey's multiple comparisons test. *P*-values are denoted by: ***<0.001 and ****<0.0001.

Fig 4. Exogenous application of *Ascaris suum* PCF (As-PCF) and synthetic As-FLP-18A influences *Caenorhabditis elegans* growth, reproduction and motility. (A) *C. elegans* growth (as measured by changes in body length) is significantly reduced in nematodes exposed to 2% and 5% As-PCF and 100 μM synthetic As-FLP-18A (GFGDEMSMPGVLRFNH$_2$) compared to the negative control (nematodes exposed to S-medium only). (B) *C. elegans* reproduction (as measured by progeny) is significantly reduced in nematodes exposed to 1%, 2%, 5% As-PCF and

100 μM synthetic As-FLP-18A relative to the negative control (nematodes treated with S-medium only). (C) *C. elegans* motility (as measured locomotory activity over time) is significantly reduced in nematodes exposed to 5%, 7.5%, 10% As-PCF and 10 μM synthetic As-FLP-18A compared to the negative control (nematodes exposed to M9 only). Statistical significance determined by one-way ANOVA and Dunnett's multiple comparisons test (A and B) or two-way ANOVA and Tukey's multiple comparisons test (C). *P*-values are denoted by: * <0.05, **<0.01, ***<0.001 and ****<0.0001.

Fig 5. *Caenorhabditis elegans flp*-18, and the cognate FLP-GPCR encoding gene homologues *npr*-4 and *npr*-5 display pan-phylum conservation in nematodes. Pan-phylum HMM analysis of 134 nematode genomes (109 species) demonstrates that *C. elegans flp*-18, *npr-4* and *npr-5* encoding gene homologues are highly conserved (99%, 93%, 84% respectively) in nematodes. Black boxes indicate the presence of a putative gene homologue. Presence/absence identified based on motif conservation and reciprocal BLAST. Nematode species are arranged based on 12 clade designation [58]. Asterisk denotes that multiple genomes were mined per species. Genome quality is indicated by CEGMA and BUSCO scores obtained from Wormbase Parasite version 14 [49-51, 59, 60]. A list of genomes mined, HMM query sequences returned and retrieved gene homologues are detailed in Supplementary Data 2 (S2).

Table 1. High confidence peptides (>1% FDR) detected by LC-MS/MS in *Ascaris suum* PCF.

Peptide Name	Peptide Sequence Predicted	Peptide Sequence Detected	% Peptide Coverage Detected	Confidence (-10lgP)	% Samples Peptide Detected	Sex
As-FLP-18A	GFGDEMSMPGVLRF	(A) GFGDEMSMPGVLR*$ (B) (GFGDEM(+15.99)SMPGVLR)*	93	88.71*$	92	FM, M
As-NLP-20D1	EMQFAFEEPSVDAERFARFA	(A) AFEEPSVDAER* (B) FEEPSVDAER$	55	92.71* 60.76$	21	FM, M
As-NLP-40C	SVVSTKEQVLQAIE	SVVSTKEQVLQ	79	45.32	21	FM
As-NLP-42C	ALGGSVSPQWESSGWTWGEQTFPLQESHTQRALRVAMT	E(-18.01)QTFP(-.98)	13	71.58	46	FM
As-Cecropin-1 (P1)	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR*$	100	309.6* 182.2$	100	FM, M
As-Cecropin-2 (P2)	SWLSKTYKKLENSAKKRISEGIAIAIQGGPR	SWLSKTYKKLENSAKKRISEGIAIAIQGGPR*$	100	290.1* 154.2$	96	FM, M
As-Cecropin-3 (P3)	SWLSKTAKKLENSAKKRISEGIAIAIKGGSR	SWLSKTAKKLENSAKKRISEGIAIAIKGGS*$	97	210.5* 125.0$	87.5	FM, M
As-Defensin-1 (ASABF-6Cysa)	NPQSTMDNSEDHNVKRDLCNGRCKRMKCVLGASCKQRSGQWVCVCKRPKDV MIKN	(A) P.Q(-17.03)STMDNSEDHNVK* (B (P.Q(-17.03)STM(+15.99)DNSEDHNVK)* (C) Q(-17.03)STMDNSEDHNVK$	24	191.6* 134.8$	79	FM, M
As-Defensin-2 (ASABF-a)	AVDFSSCARMDVPGLSKVAQGLCISSCKFQNCGTGHCEKRGGRPTCVCDRCG RGGGEWPSVPMPKGRSSRG	(A) GGGEWPSVPMPKGRSSRG (B) (Q(-17.03)GLCISS(-.98)(+79.96))	35	271.4	88	FM
As-Defensin-14	GCPQHHVCNKHCLKLHGSRGYCGGEDSRSCTCGMLFDY	LHGSRGY	18	67.96	17	FM

*Denotes data derived from female As-PCF samples; $Denotes data derived from male As-PCF samples.

Supplementary information

S1 File. As-PCF peptides detected in this study. High confidence peptides (>1% FDR) detected across all samples (Table 1; Tab 1); All peptides detected in single female samples (Table 2; Tab 2); All peptides detected in pooled female samples (Table 3; Tab 3); All peptides detected in pooled male samples (Table 4; Tab 4). In all cases -10lgP denotes P-value [-10*log10(P-value)] as converted by PEAKS software.

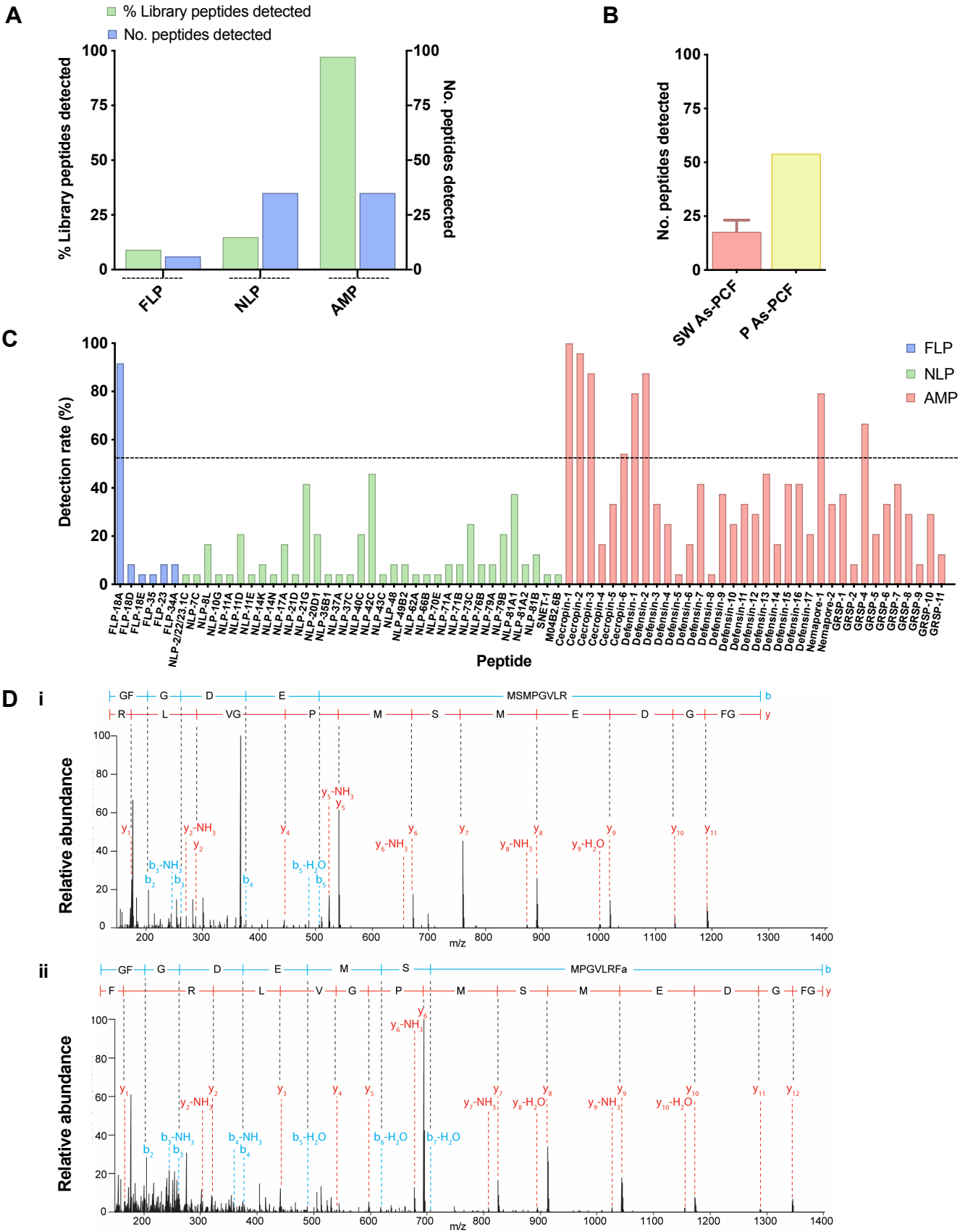
S2 File. Nematode genomes mined for gene sequelogues of *flp*-18, *npr*-4 and *npr*-5. Wormbase ParaSite [https://parasite.wormbase.org/index.html; 49, 50, 51] gene IDs are provided for each identified sequelogue. Gene IDs highlighted in red indicate sequences that were used to build Hidden Markov Models. Genomic locations for representative partial sequences from TBLASTN hits are detailed where appropriate.

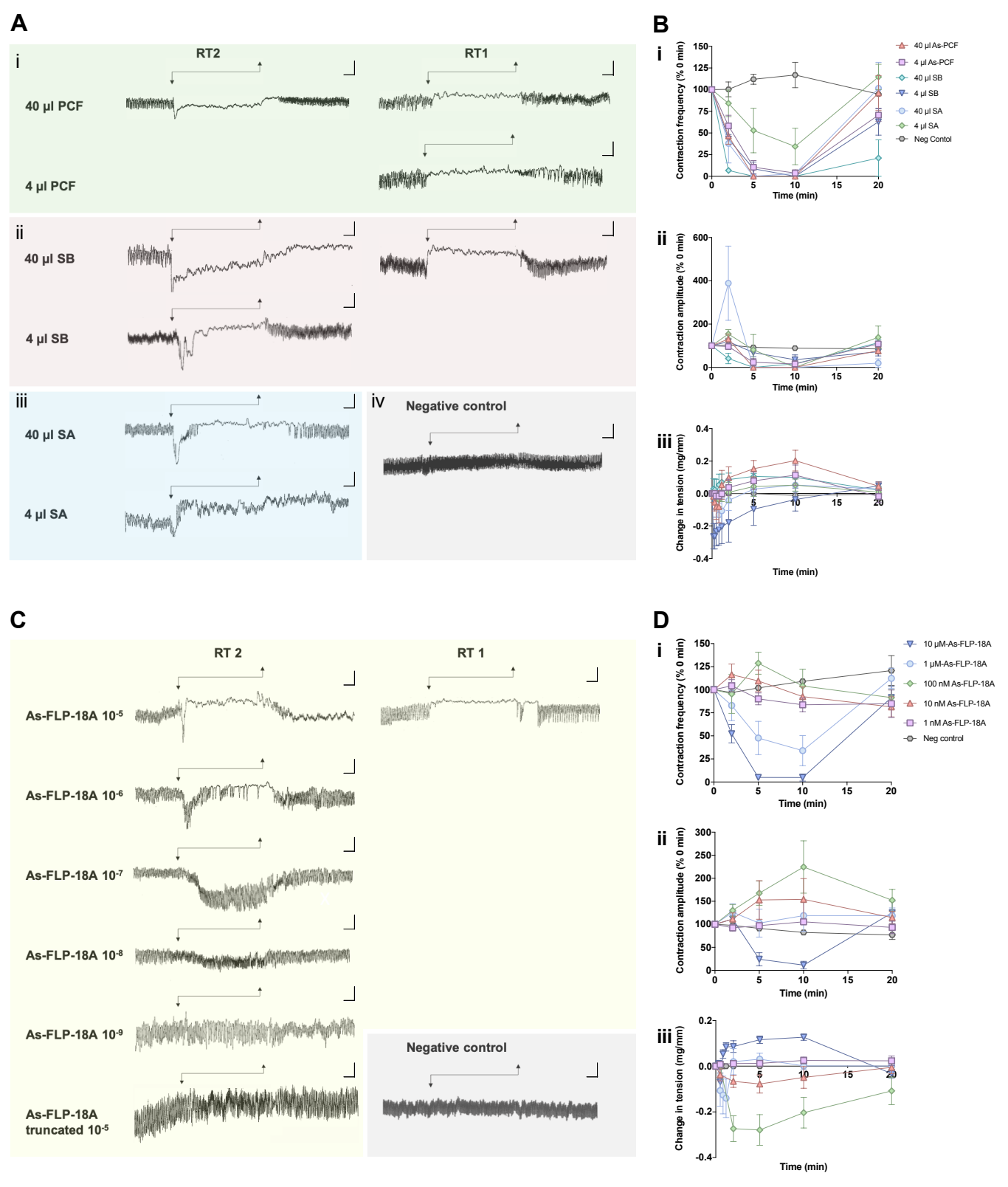
S1 Table. The modulatory effects of As-PCF on *Ascaris suum* ovijector tissue preparations.

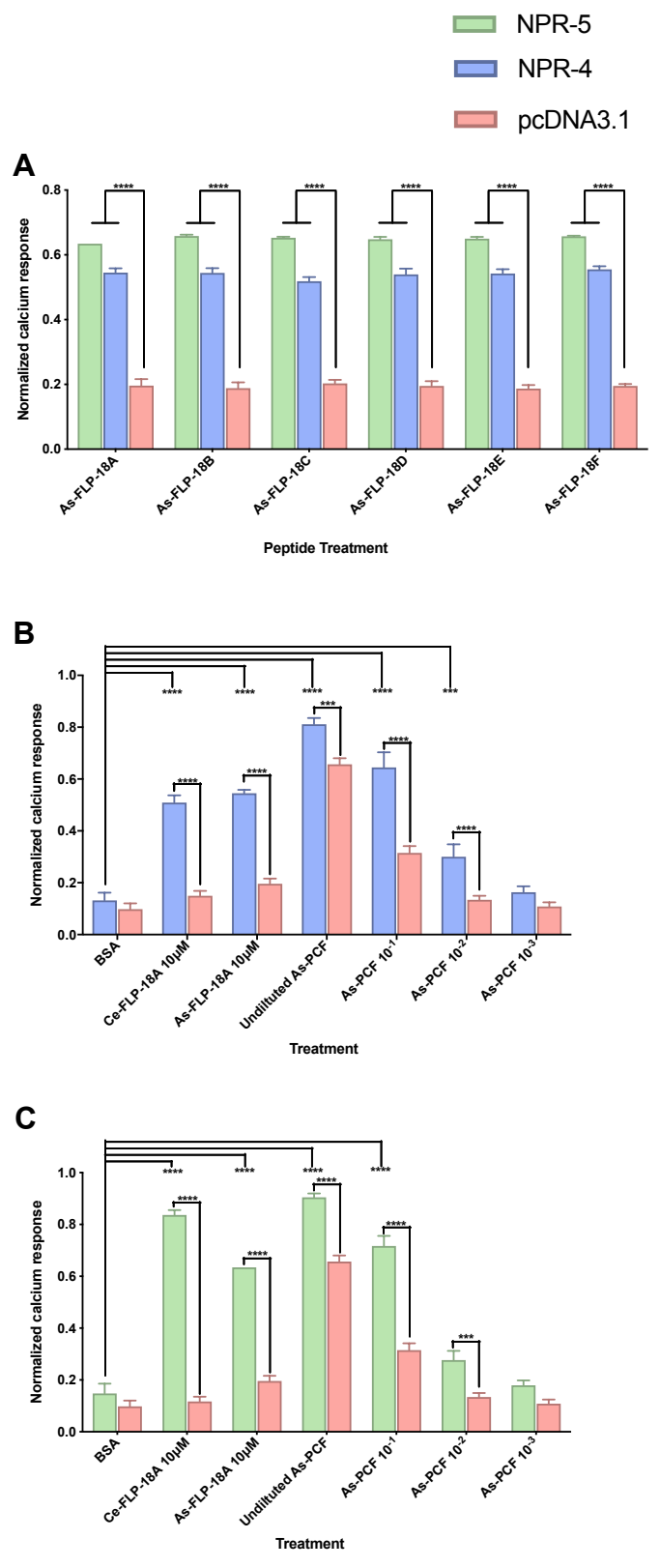
832 **S2 Table. The modulatory effects of synthetic As-FLP-18A on *Ascaris suum* ovijector**

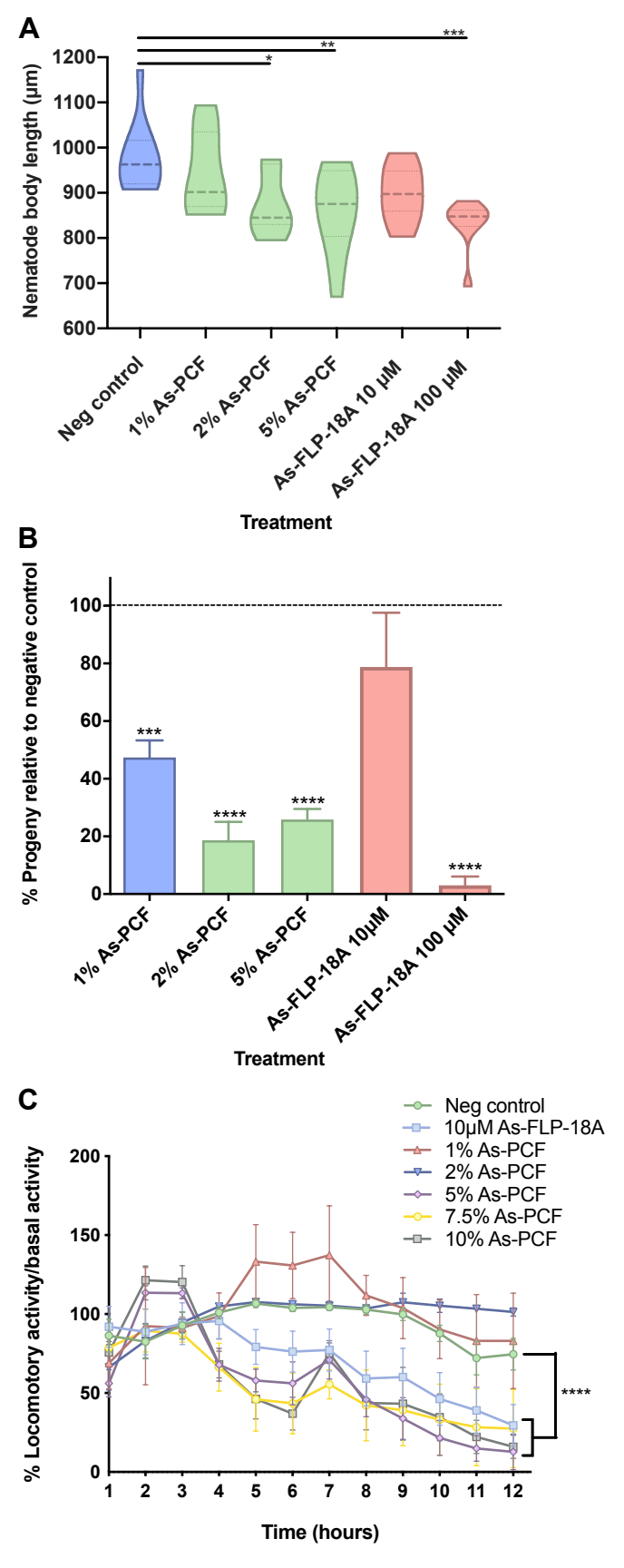
833 **tissue preparations.**

834


A
% Library peptides detected
No. peptides detected
% Library peptides detected
No. peptides detected
FLP
NLP
AMP
B
No. peptides detected
SW As-PCF
P As-PCF
C
Detection rate (%)
FLP
NLP
AMP
Peptide
D
i
MSMPGVLR
MPGVLRFa
Relative abundance
m/z
ii
Relative abundance
m/z


A
i
RT2
RT1
40 µl PCF
4 µl PCF
ii
40 µl SB
4 µl SB
iii
40 µl SA
4 µl SA
iv
Negative control
B
i
Contraction frequency (% 0 min)
Time (min)
40 µl As-PCF
4 µl As-PCF
40 µl SB
4 µl SB
40 µl SA
4 µl SA
Neg Contol
ii
Contraction amplitude (% 0 min)
Time (min)
iii
Change in tension (mg/mm)
Time (min)
C
RT 2
RT 1
As-FLP-18A 10-5
As-FLP-18A 10-6
As-FLP-18A 10-7
As-FLP-18A 10-8
As-FLP-18A 10-9
As-FLP-18A truncated 10-5
Negative control
D
i
Contraction frequency (% 0 min)
Time (min)
10 µM-As-FLP-18A
1 µM-As-FLP-18A
100 nM As-FLP-18A
10 nM As-FLP-18A
1 nM As-FLP-18A
Neg control
ii
Contraction amplitude (% 0 min)
Time (min)
iii
Change in tension (mg/mm)
Time (min)


NPR-5
NPR-4
pcDNA3.1
A
Normalized calcium response
0.0
0.2
0.4
0.6
0.8
****
As-FLP-18A
As-FLP-18B
As-FLP-18C
As-FLP-18D
As-FLP-18E
As-FLP-18F
Peptide Treatment
B
Normalized calcium response
0.0
0.2
0.4
0.6
0.8
1.0
***
****
BSA
Ce-FLP-18A 10μM
As-FLP-18A 10μM
Undiluted As-PCF
As-PCF 10-1
As-PCF 10-2
As-PCF 10-3
Treatment
C
Normalized calcium response
0.0
0.2
0.4
0.6
0.8
1.0
***
****
BSA
Ce-FLP-18A 10μM
As-FLP-18A 10μM
Undiluted As-PCF
As-PCF 10-1
As-PCF 10-2
As-PCF 10-3
Treatment


A
Nematode body length (µm)
1200
1100
1000
900
800
700
600
*
**
***
Neg control
1% As-PCF
2% As-PCF
5% As-PCF
As-FLP-18A 10 µM
As-FLP-18A 100 µM
Treatment
B
% Progeny relative to negative control
100
80
60
40
20
0
***
****
****
****
1% As-PCF
2% As-PCF
5% As-PCF
As-FLP-18A 10µM
As-FLP-18A 100 µM
Treatment
C
% Locomotory activity/basal activity
200
150
100
50
0
Neg control
10µM As-FLP-18A
1% As-PCF
2% As-PCF
5% As-PCF
7.5% As-PCF
10% As-PCF
****
1
2
3
4
5
6
7
8
9
10
11
12
Time (hours)

Clade	Species	CEGMA %	BUSCO %	*flp* -18	*npr* -4	*npr* -5
		0 50 100	0 50 100			
2	*Romanomermis culicivorax*					
	Soboliphyme baturini					
	Trichinella britovi					
	Trichinella murrelli					
	*Trichinella nativa**					
	Trichinella nelsoni					
	Trichinella papuae					
	Trichinella patagoniensis					
	*Trichinella pseudospiralis**					
	*Trichinella spiralis**					
	Trichinella sp. T6					
	Trichinella sp. T8					
	Trichinella sp. T9					
	Trichinella zimbabwensis					
	Trichuris muris					
	*Trichuris suis**					
	Trichuris trichiura					
6	*Plectus sambesii*					
8	*Acanthocheilonema viteae*					
	Anisakis simplex					
	Ascaris lumbricoides					
	*Ascaris suum**					
	Brugia malayi					
	Brugia pahangi					
	Brugia timori					
	Dirofilaria immitis					
	Dracunculus medinensis					
	Elaeophora elaphi					
	Enterobius vermicularis					
	Gongylonema pulchrum					
	Litomosoides sigmodontis					
	*Loa loa**					
	*Onchocerca flexuosa**					
	*Onchocerca ochengi**					
	Onchocerca volvulus					
	Parascaris equorum					
	Parascaris univalens					
	Syphacia muris					
	Thelazia callipaeda					
	*Toxocara canis**					
	*Wuchereria bancrofti**					
9	*Ancylostoma caninum*					
	*Ancylostoma ceylanicum**					
	Ancylostoma duodenale					
	Angiostrongylus cantonensis					
	Angiostrongylus costaricensis					
	Caenorhabditis angaria					
	Caenorhabditis brenneri					
	Caenorhabditis briggsae					
	Caenorhabditis elegans					
	Caenorhabditis inopinata					
	Caenorhabditis japonica					
	Caenorhabditis latens					
	Caenorhabditis nigoni					
	*Caenorhabditis remanei**					
	Caenorhabditis sinica					
	Caenorhabditis tropicalis					
	Cylicostephanus goldi					
	*Dictyocaulus viviparus**					
	Diploscapter coronatus					
	Diploscapter pachys					
	*Haemonchus contortus**					
	Haemonchus placei					
	*Heligmosomoides polygyrus**					
	Heterorhabditis bacteriophora					
	Mesorhabditis belari					
	Micoletzkya japonica					
	Necator americanus					
	Nippostrongylus brasiliensis					
	Oesophagostomum dentatum					
	Oscheius tipulae					
	Parapristionchus giblindavisi					
	Pristionchus arcanus					
	Pristionchus entomophagus					
	Pristionchus expectatus					
	Pristionchus fissidentatus					
	Pristionchus japonicus					
	Pristionchus maxplanckі					
	Pristionchus mayeri					
	Pristionchus pacificus					
	Strongylus vulgaris					
	Teladorsagia circumcincta					
10	*Bursaphelenchus xylophilus*					
	Halicephalobus mephisto					
	Panagrellus redivivus					
	Parastrongyloides trichosuri					
	Rhabditophanes sp. KR3021					
	Steinernema carpocapsae					
	Steinernema feltiae					
	Steinernema glaseri					
	Steinernema monticolum					
	Steinernema scapterisci					
	Strongyloides papillosus					
	Strongyloides ratti					
	Strongyloides stercoralis					
	Strongyloides venezuelensis					
11	*Acrobeloides nanus*					
12	*Ditylenchus destructor*					
	Ditylenchus dipsaci					
	Globodera pallida					
	Globodera rostochiensis					
	Heterodera glycines					
	*Meloidogyne arenaria**					
	Meloidogyne enterolobii					
	*Meloidogyne floridensis**					
	Meloidogyne graminicola					
	Meloidogyne hapla					
	*Meloidogyne incognita**					
	*Meloidogyne javanica**					