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# A molecular war: convergent and ontogenetic evidence for adaptive host manipulation in related parasites infecting divergent hosts

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Mermithids (phylum Nematoda) and hairworms (phylum Nematomorpha) somehow drive their arthropod hosts into water, which is essential for the worms' survival after egression. The mechanisms behind this behavioural change have been investigated in hairworms, but not in mermithids. Establishing a similar mechanistic basis for host behavioural change between these two distantly related parasitic groups would provide strong convergent evidence for adaptive manipulation and insight into how these parasites modify and/or create behaviour. Here, we search for this convergence, and also contrast changes in physiology between hosts infected with immature and mature mermithids to provide the first ontogenetic evidence for adaptive manipulation by disentangling host response and pathology from the parasite's apparent manipulative effects. We used SWATH-mass spectrometry on brains of *Forficula auricularia* (earwig) and *Bellorchestia quoyana* (sandhopper), infected with the mermithids *Mermis nigrescens* and *Thaumamermis zealandica*, respectively, at both immature and mature stages of infection, to quantify proteomic changes resulting from mermithid infection. Across both hosts (and hairworm-infected hosts, from earlier studies), the general function of dysregulated proteins was conserved. Proteins involved in energy generation/mobilization were dysregulated, corroborating reports of erratic/hyperactive behaviour in infected hosts. Dysregulated proteins involved in axon/dendrite and synapse modulation were also common to all hosts, suggesting neuronal manipulation is involved in inducing positive hydrotaxis. Furthermore, downregulation of CamKII and associated proteins suggest manipulation of memory also contributes to the behavioural shift.

## 1. Introduction

A molecular war has been raging for millions of years between two factions: parasites and their hosts [1]. Much of the molecular fallout is the result of parasites defending against host immunological attacks. Fascinatingly, in rare cases, natural selection appears to have morphed the parasites' immunological defence mechanisms into an indirect attack on the hosts' central nervous system (CNS) to alter host behaviour [2–4]. Parasitic 'mind control' is particularly common in parasites trophically transmitted from intermediate to definitive host or parasitoids that rely on their host being in a specific location before they egress from it [5–9]. For example, *Toxoplasma gondii* can lure rats into close proximity of their natural cat predators (definitive host for the parasite) [10], while parasitoid wasps can induce their insect host to die in concealed locations, where the parasitoid can safely complete its development [11].

Transmission success rate, optimal egression location and other factors can result in evolutionary pressures so strong that parasites can seemingly develop absolute control of their hosts, leading to some behavioural alterations being

labelled as extended phenotypes of the manipulating parasite [9,12,13]. While many behavioural and ecological studies have deftly answered *why* parasites would manipulate host behaviour [3,14–16], *how* parasites manipulate behaviour is the question that has yet to be definitively answered [17,18].

The positive hydrotaxis induced by aquatic endoparasitic hairworms (phylum Nematomorpha) in a wide range of terrestrial insect hosts globally is a textbook example of the extended phenotype [4,19–23] and an ideal system to begin elucidating the mechanisms of host manipulation. Hairworms are prone to desiccation and must lay their eggs in water [20]. These two factors combine into a strong evolutionary pressure to force their normally hydrophobic host into water [19]. The mechanisms the parasite uses to switch the host's behaviour from hydrophobia to hydrophilia were investigated by Biron *et al*. [4,21] in hairworms infecting grasshoppers and crickets. They found a range of proteins involved in geotactic behaviour, neurogenesis, neurotransmitter/signalling and CNS development that were expressed in the brains of both infected crickets and grasshoppers during behavioural manipulation. While this is a promising result, the source of these proteomic changes could not empirically be found, meaning this evidence only establishes a correlation between infection and the physiological changes in the host.

Correlative evidence for host manipulation dominates the neuroparasitology literature [17]. By itself, this type of evidence cannot disentangle the host's adaptive (immune) and non-adaptive (side-effects) response to infection from the parasite's own manipulative efforts [18], leading researchers to construct narratives around their results [24] and ultimately question whether parasites actually manipulate behaviour adaptively [14,25–27]. Establishing causation between parasite infection and changes in host physiology is a major challenge in most systems. However, correlative evidence for host manipulation can be strengthened in many ways, making the argument in favour of adaptation very compelling.

First, finding similar physiological changes and behavioural modifications induced in disparate host species when infected with different parasite species would provide strong evidence for convergent, adaptive evolution. Across a broad phylogenetic array of host species, it is very unlikely that host responses to infection would have identical side effects. The most parsimonious explanation would be that convergent evolution shaped the parasites' manipulative mechanisms, resulting in similar adaptive physiological impacts on the host [16,28,29].

Second, evidence based on the ontogenetic component of host manipulation would be invaluable for supporting adaptive manipulation. Parasites often go through large physiological and morphological changes during development within their host. The parasite's mechanisms of manipulation probably only manifest once its development is complete within the host, as premature manipulation would jeopardize further growth in the nutrient-rich and safe host environment [30]. Physiological and biochemical changes occurring in the host early post-infection may represent immune or other responses from the host, or other infection-related changes not connected to manipulation. Contrasting these early changes with physiological changes in the host appearing later in the parasite's development, or during the manipulative event, would be a powerful approach to identify more precisely the mechanisms used to manipulate host behaviour.

Evidence for both convergent evolution and ontogenetic changes in host physiology coinciding with the timing of host manipulation is rare in neuroparasitology [16]. However, mermithid nematodes (phylum Nematoda) that, like hairworms, induce hydrophilic behaviour in their hosts are perfect models to generate both these forms of evidence [31–34]. From an ontogenetic perspective, both hairworms and mermithids are easy to study (relative to other parasites) as their length and external appearance are good indicators of maturity. In terms of convergent evidence, similar proteomic results across these two taxa of worms would establish convergence in mechanism at the phylum level (Nematoda versus Nematomorpha). Furthermore, mermithids infect a wide range of terrestrial arthropod hosts [32,35–37]. By selecting model parasite species infecting an insect host in one case and a crustacean in the other, convergence in mechanism could also be examined from the perspective of different host taxa.

Overall, this study uses the similar hydrophilic behaviour induced in two distantly related host species infected with mermithid parasites to test for convergent and ontogenetic evidence for host manipulation. Specifically, we conducted a proteomic analysis of the brains of earwigs, *Forficula auricularia*, and sandhoppers, *Bellorchestia quoyana*, infected with the mermithids *Mermis nigrescens* and *Thaumamermis zealandica*, respectively. These host–parasite systems were selected to test for similar physiological effects across phylogenetically distant hosts to seek evidence for convergence in manipulative mechanism across mermithids. We then compared our findings with the proteomic results from Biron *et al*.'s [4,21] studies on hairworms infecting grasshoppers and crickets, to test for convergent mechanisms of manipulation between the nematode and hairworm phyla. Furthermore, we contrast the proteomic profiles of selected hosts infected with young versus mature worms, in order to disentangle pathological side-effects and host immune responses from the parasite's own manipulative efforts, which should only be detected in hosts harbouring large/mature worms.

## 2. Methods

Sandhoppers were collected haphazardly by hand under patches of kelp from Smails Beach (45.9076° S, 170.5589° E, Dunedin, New Zealand) in late spring (November–December) 2016. Earwigs were collected from flowers in the Dunedin Botanical (45.8553° S, 170.5191°) and Mercy Hospital (45.8611° S, 170.4992°) gardens and two private gardens close by in early summer (January–February) 2017. Upon capture all hosts were immediately placed into an Eppendorf tube and immersed in liquid nitrogen. Frozen arthropods were transferred to a −70°C freezer, and subsequently freeze-dried and dissected under a microscope on a dissection stage cooled by dry ice. The sex of each earwig and sandhopper was determined by the shape of the cerci and absence/presence of gnathopods, respectively. Worms found were photographed with a microscope-mounted camera and length measured using ImageJ (1.52i). Infected hosts were decapitated, exterior appendages (antennae, etc.) were removed from their head, and the latter was immediately placed back in the −70°C freezer. Uninfected controls of the same sex and size as infected hosts were randomly selected, and their heads were dissected and stored as above.

Brains from four hosts with the longest worm infections (sandhopper: 60.7–119 mm; earwig: 129–263 mm) and brains from four hosts with the shortest single worm infections (sandhopper: 10.4–15.3 mm; earwig: 5–7.6 mm) were chosen in both earwig and sandhoppers, along with four brains from non-infected hosts (total of 12 brains selected for both earwigs and sandhoppers). Two female and two male brains were selected in each

category. Brains were processed separately in batches of 6 (two from each category).

Therefore, brain tissues were homogenized and proteins extracted in lysis buffer containing 0.2% (m/v) SDS (sodium dodecyl sulfate), 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), 5 mM TCEP (tris(2-carboxyethyl)phosphine) in 40 mM Tris-base (tris(hydroxymethyl)aminomethane). Proteins were then purified and digested with trypsin following the filter-aided sample preparation (FASP) protocol [38]. Tryptic peptides were further purified by solid phase extraction on C18 Sep-Pak cartridges (Waters) and dried using a centrifugal vacuum concentrator. One earwig control was contaminated and had to be excluded from further analysis. Peptides were then reconstituted in 0.1% formic acid in 5% aqueous acetonitrile in preparation for liquid chromatography–coupled-tandem mass spectrometry (LC-MS/MS). LC-MS/MS was performed in data-dependent acquisition mode for protein identification (build of spectral library) and data-independent acquisition mode using sequential windowed acquisition of all theoretical fragment ion spectra (SWATH)—mass spectrometry (MS) for protein quantification. Both approaches were performed on a nanoflow uHPLC-coupled TripleTOF 5500+ LC-MS/MS system. In brief, SWATH-MS acquires fragment ion intensities of all peptide precursor ions within a defined mass range (here *m*/*z* 400–1250) without intensity-based precursor ion selection. The fragment ion intensities provide accurate relative quantities of the respective peptide precursor ions after matching the spectral information against a spectral library for identification. SWATH-MS is therefore a label-free method for accurate global profiling of relative protein quantities between multiple samples (66). Library matching, peak picking and intensity extraction was performed using the SWATH 2.0 application in PeakView software v. 2.2 (ABSciex). The extracted peak information was then exported to MarkerView software (AB SCIEX) where principal component analysis (PCA) and Student's *t*-test were used to compare the quantitative protein profiles of the different brain samples. Only proteins with a log10 fold change greater than ±0.2 (*t*-test $p < 0.01$) relative to other sample groups were included in the analysis. For further details, see methods in electronic supplementary material.

For the earwigs, GenInfo identifiers (gi) of target proteins (derived from MarkerView) were then cross-referenced with the National Centre for Biotechnology Information (NCBI) database, while TrEMBLE coded peaks in sandhoppers were cross-referenced with the UniProtKB database. The STRING functional protein association networks (version 10.5; https://string-db.org/) was then used to perform network and functional enrichment analysis of differentially regulated proteins. The resulting networks were analysed clustered using the Markov clustering algorithm (MCA) with an inflation parameter of 3 and tested for function enrichments. Because sequenced data could not be used, *Drosophila melanogaster* was used as the model organism for converging the earwig and sandhopper peak data into a uniform format, allowing the establishment of relationships and convergence in proteins between sandhoppers and earwigs. Each identified protein from MarkerView was cross-referenced with the match identified by STRING (relative to *D. melanogaster*) against the UniprotKB database for name and function. Any earwig/sandhopper protein which did not match *D. melanogaster* proteins (less than 1%) was excluded from further analysis. To contrast the earwig/sandhopper data against the proteomic data from Biron *et al.*'s [4,21] studies on hairworm-infected grasshopper and cricket hosts, *D. melanogaster* was used as a convergence platform as for the earwig and sandhopper data above.

Proteins identified in the above analyses (see electronic supplementary material, tables S1 and S2) were given a general function based on the average specific functions as per the UniprotKB database. Each list of significantly up/downregulated proteins generated from a comparison between two groups was compared with all other protein lists to identify overlapping proteins (see colour coding of protein lists in electronic supplementary material, tables S1–S3).

## 3. Results

### (a) Prevalence of infection and worm length range

In total, 2538 sandhoppers and 1302 earwigs were dissected, with 23 and 46 individuals found to be infected with mermithids, respectively. Single worms infecting earwigs ranged from 1.7 to 263.5 mm in length, with a mean (±s.d.) of 56.1 ± 62.7 mm, while single worms infecting sandhoppers ranged from 3.6 to 119.1 mm, with a mean of 44 ± 33.7 mm.

### (b) PCA of the individual sandhopper protein profiles and protein regulation

The PCA of the protein profiles of individual sandhopper brain samples indicated differences between control (uninfected) brains and those from both short-worm-infected hosts (SWIH), excluding individual F 10.6, and long-worm-infected hosts (LWIH), with controls and infected hosts grouping apart (figure 1*a*). The two LWIH individuals with the shortest worms (F 78.8 and M 60.7) clustered with SWIH, while the two with the longest worms diverged in different directions, one being a female and the other a male. Despite this discrepancy, when LWIH are compared against all other hosts for significantly down or upregulated proteins, all 4 LWIH show similar high levels of downregulation of specific proteins relative to SWIH (figure 2*a*2). This difference is even more pronounced versus control brains.

### (c) PCA of the individual earwig protein profiles and protein regulation

The PCA of the earwigs protein profiles indicates some variation within the LWIH group, irrespective of worm length or sex. The two individuals M 173 and F 172 group with the SWIH while the other two (M 263 and F 129) are distinct from any other grouping (figure 1*b*). In fact, the samples M 173 and F 172 were found to have very few significantly different up/downregulated proteins relative to SWIH individuals (see electronic supplementary material, table S2), whereas M 263 and F 129 showed some strongly upregulated proteins relative to all other hosts (figure 2*b*1). The opposite is true for the downregulated proteins, with the two isolated hosts being the least downregulated relative to all other hosts (figure 2*b*2).

Based on these results, hereafter hosts M 263 and F 129 were placed into their own group now referred to as aberrant, while the other two LWIH hosts (M 173 and F 172) were grouped with SWIH, hereafter called infected (see circles in figure 1*b*). The re-arrangement, purely based on proteomic differences, ensured the full suite of proteins distinguishing brains M 263 and F 129 from the others could be analysed without the other LWIH brains masking them.

### (d) Functional trends in proteomic data and STRING analysis

To establish the earwig and sandhopper basal physiological response to infection, controls were compared against the

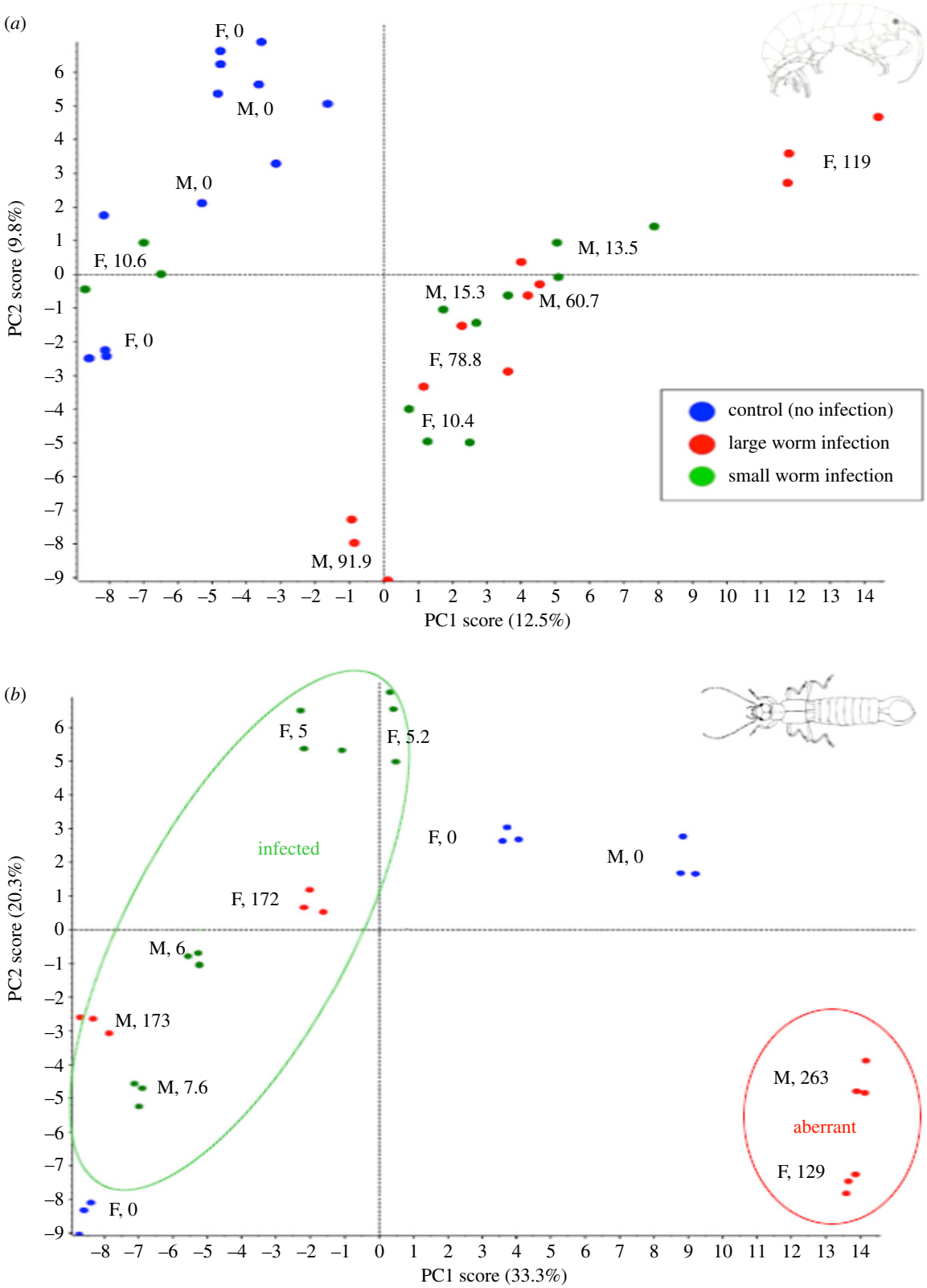


**Figure 1.** Unsupervised PCA of both (*a*) sandhopper (*Bellorchestia quoyana*, $n = 12$) and (*b*) earwig (*Forficula auricularia*, $n = 11$) brains analysed by SWATH-MS. Brains are grouped by similarity/difference in their quantitative protein profiles, with PC1 and PC2 accounting for 33.9%/20.3% and 12.5%/9.8% (earwig/sandhopper) of the variation, respectively. Individuals infected with long/mature worms (*Thaumamermis zealandica* in the sandhopper; *Mermis nigrescens* in the earwig), short/immature worms and uninfected controls are represented by different colours; there were four individuals in each of these categories, but only three for uninfected earwigs. Each brain was analysed in three technical replicates, hence the triplicate points. The label near each group of three points indicates the sex of the host (M or F) and the length of the worm (mm). Red and green circles indicate the grouping of hosts for analysis.

SWIH/infected grouping (sandhopper/earwig), based on the outcome of the PCA (figure 1) and protein regulation trends (figure 2). To then establish the host's response to late stages of infection and potentially the effects of the mermithids' manipulative effort, LWIH were compared against controls in sandhoppers. In earwigs, the aberrant grouping was compared to the infected grouping. Specific proteins found to be up/downregulated in the basal physiological response to infection

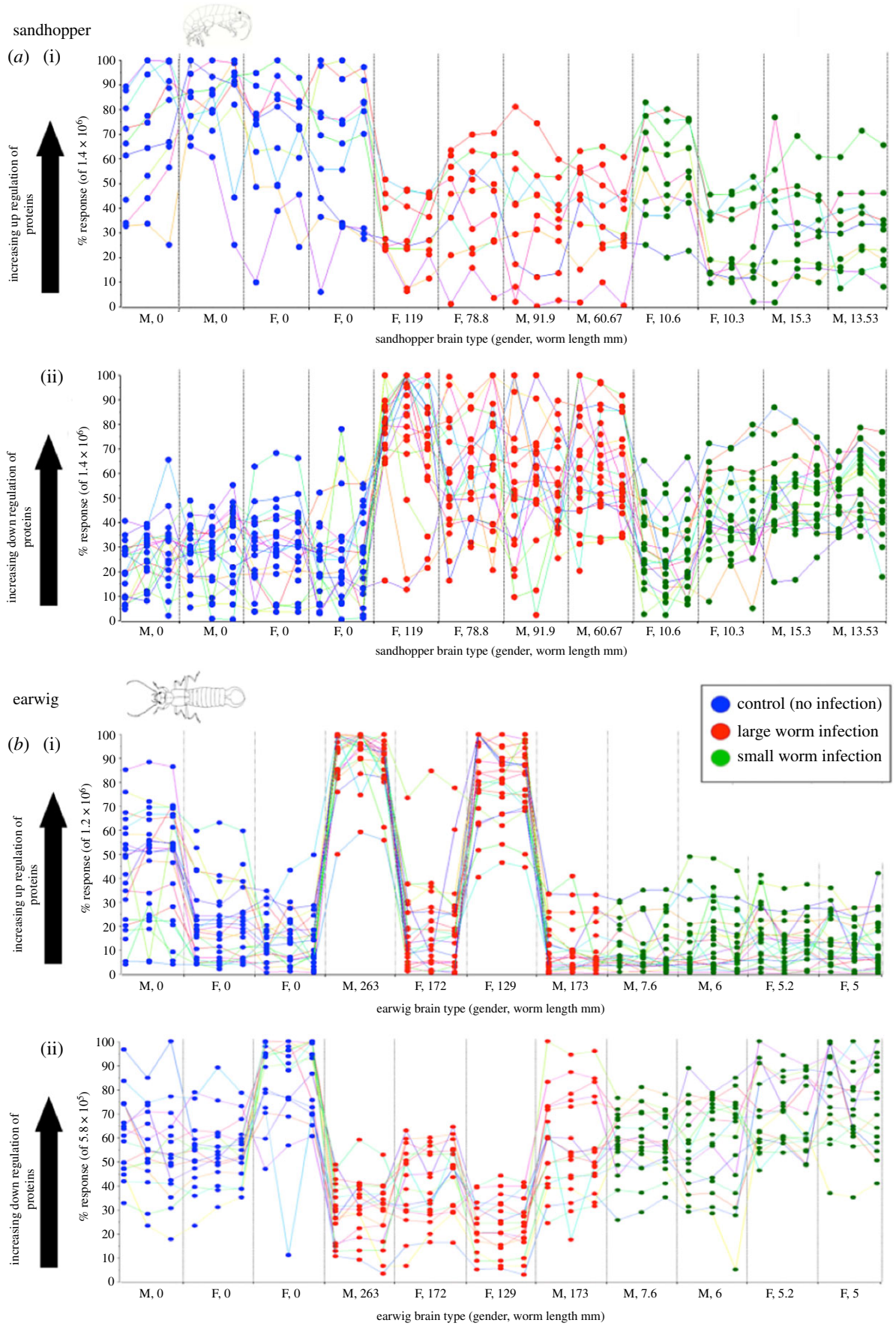


**Figure 2.** Profiles of the relative abundance of significantly up or downregulated ($p < 0.01$, log fold change ±0.2) proteins between (*a*) sandhoppers (*Bellorchestia quoyana)* and (*b*) earwigs (*Forficula auricularia*) infected by a large mermithid (*Thaumamermis zealandica* in the sandhopper; *Mermis nigrescens* in the earwig) against hosts infected by a small worm and control hosts with no infection. The top graphs (A1 and B1) show proteins with significantly greater abundance (upregulation), while the bottom graphs (A2 and B2) show proteins with a significantly lower abundance, relative to each host (downregulation). Each differently coloured line represents a distinct protein. The *y*-axis is presented as a percentage response relative to the greatest peak, i.e. the protein with greatest or lowest abundance, against the different brain types (see details on *x*-axis). Each brain was analysed in three technical replicates, hence the triplicate points. The label for each group of three points indicates the sex of the host (M or F) and the length of the worm (mm).

(controls versus SWIH/infected grouping) were then cross-referenced for overlap in the specific proteins found to be regulated in late stages of infection (sandhopper: LWIH versus controls; earwig: aberrant versus infected grouping).

Across both sandhoppers and earwigs, the general functions of up or downregulated proteins found between group comparisons fell into six major categories: apoptosis, axon/dendrite and synapse modulation, development, DNA/protein modification, energy generation/metabolism and muscle growth/repair/activity. However, the number of unique proteins and their specific identity in each category varied among host groups.

Significantly regulated proteins found in the sandhopper comparisons between LWIH versus Control and SWIH versus Control were biased towards downregulation, with few proteins found to be upregulated (see electronic supplementary material, table S1). Compared to earwigs, much of the protein regulation in the LWIH versus Control comparison was focused around DNA/protein modification. Proteins involved in axon/dendrite and synapse modulation were rare relative to other major categories (see electronic supplementary material, table S1). STRING network analysis of the protein lists generated from the comparisons between groups found very little functional enrichment (statistically confirmed relationship between proteins relative to a specific physiological process) in the networks, excluding the upregulated protein network from the LWIH versus Control comparison (see electronic supplementary material, figure S1). In this network, functional enrichment was found in relation to energy generation/metabolism and muscle growth/repair/activity.

Conversely, for the earwig, protein hits were biased towards upregulation (see electronic supplementary material, table S2). A large portion of proteins were involved in energy generation/metabolism, with specific functions of proteins centred around manipulation of ATP generation and mobilization of various forms of energy cycling from glycolysis to the citric acid cycle. Dysregulation of proteins involved in axon/dendrite and synapse modulation was much more common as well, specifically around synaptic vesicle endo/exocytosis, axonogenesis and long-term potentiation (see electronic supplementary material, table S2). Over 70 proteins were significantly upregulated in the aberrant versus infected comparison, while only four were upregulated in the infected versus control comparison. The upregulated protein network obtained from the aberrant versus infected comparison was the only earwig network with functional enrichments. Some of the enrichments in the network were very similar to the ones found in the sandhopper upregulated network. Electronic supplementary material, figure S2 shows a large network of strongly interrelated proteins involved in energy generation/metabolism, with enrichments specific to carbon metabolism, glycolysis, pyruvate metabolism, citrate cycling and ATP metabolic process. Smaller networks are enriched in muscle cell differentiation and ribosome processes. Cytoskeleton organization is also enriched within these smaller networks.

## 4. Discussion

Although host manipulation by parasites is a widespread phenomenon essential for parasite transmission, the underlying mechanisms remain poorly studied. Here, by contrasting different species of mermithid nematodes using phylogenetically distant host species, we confirmed that alterations in host brain proteins appearing late in the infection process differ from those observed early after infection. More importantly, we reveal both similarities and differences in the set of proteins up/downregulated by the parasites in their different host species (figure 3), but also some apparent convergence between proteomic changes induced by mermithids and those induced by hairworms, which belong to a different phylum but cause remarkably similar behavioural changes in their arthropod hosts.

### (a) Sandhoppers

Mermithids force their sandhopper host to burrow deeper into the sand down to water-saturated layers [32]. A previous study suggested that a parasite-induced increase in haemolymph osmolality could be the mechanism driving sandhoppers to seek water-saturated sand [39]. Our present findings suggest another mechanism reflected in the proteomic data. Exclusive to LWIH, proteins involved in ATP mobilization (Oscp, Vps4, CG4769, sea and porin) the citric acid cycle (Mtpalpha, Acon, L(1)G0255), glycogen processing (Pgi, Thiolase) and muscle repair/growth (bt, Mlp84B, slgA, Prm, Zasp52, rhea) were dysregulated (electronic supplementary material, table S1). This dual impact on energy generation/metabolism and muscle activity was reflected in the small upregulated protein network between LWIH versus control groups (electronic supplementary material, figure S1), and the aberrant versus infected earwig network (electronic supplementary material, figure S2).

Furthermore, three proteins found to be involved in axon/dendrite and synapse modulation (Chc, ben and CalpB) were exclusive to the LWIH, with Clathrin (Chc) a key factor in chemical synaptic transmission, being found to be dysregulated across the mermithid and hairworm hosts (discussed later). Collectively, these proteomic changes suggest that mature mermithids may elevate energy mobilization and alter muscle activity in the sandhopper, inducing hyperactive/erratic behaviour and thus increased locomotion/burrowing. The protein changes associated with neuronal functioning may signify an effort to induce hydrophila in the host in tandem with energy mobilization/hyperactivity.

However, many downregulated proteins found in LWIH were also found in SWIH, and protein regulation showed a uniform increase across all hosts from SWIH to LWIH (figure 2*a*2). Therefore, hosts' responses may be driving the changes seen here, not the parasite manipulative effort. The proteomic impacts may be proportional to the size of the worm, as greater worm length increases the energetic and physical demands on the host. However, worms in sandhoppers were found to egress from their host after the 105 mm mark [40]. As discussed, two worms were found relatively close to this size in this study, and both diverged significantly from all the other worms which grouped together in the PCA (figure 1*a*). If manipulation only takes place once the worm has matured (as discussed previously), the PCA spread might suggest that the hosts infected with the longest worms diverge due to parasite manipulation, whereas the other infected hosts have proteomic profiles associated with pathology or host responses. If this is the case it may explain the overlap in protein lists between SWIH and LWIH. If similar worm lengths as in earwigs were obtained in the sandhoppers, we may see more comparable results between the two systems.

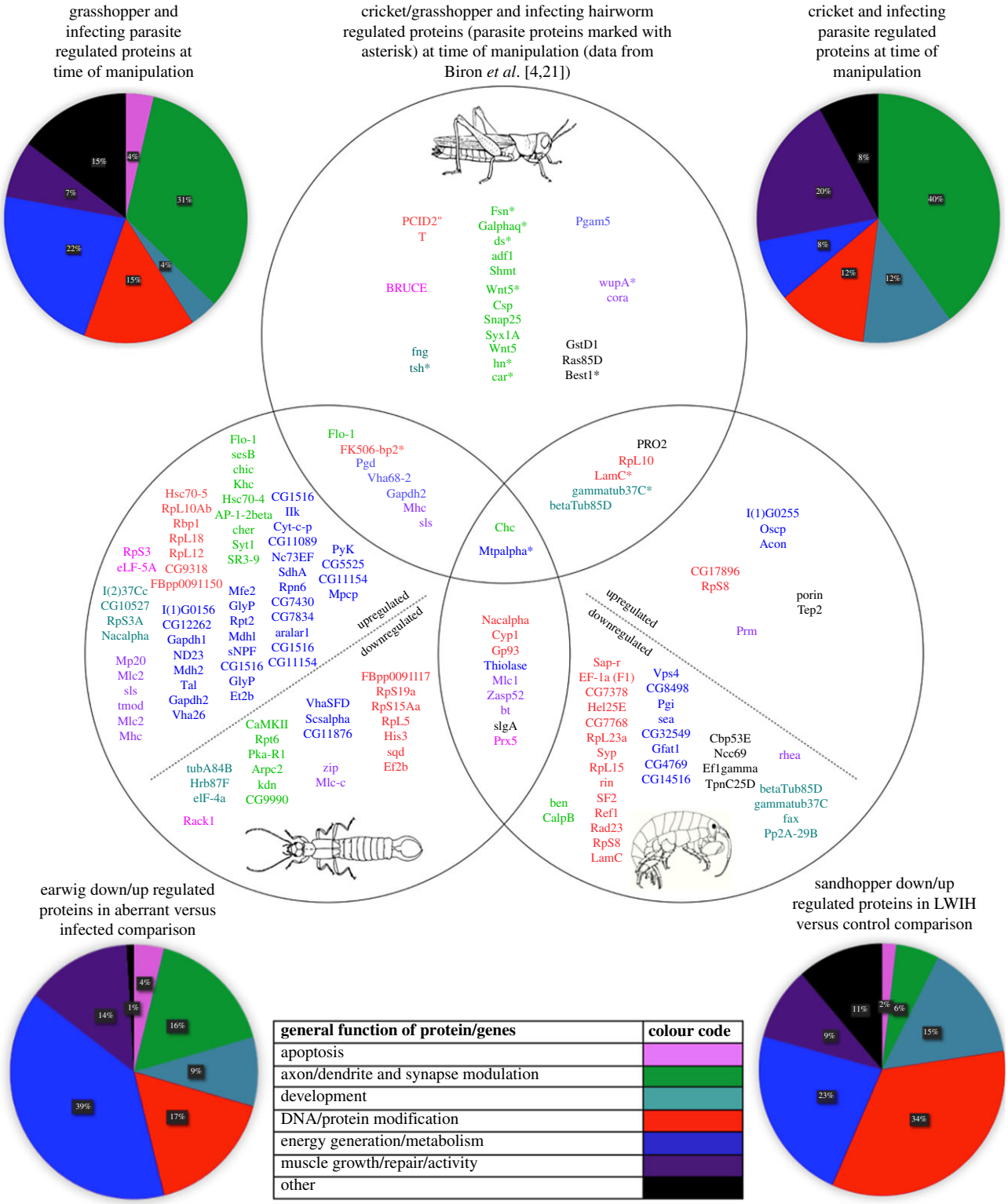


**Figure 3.** Venn diagram of regulated proteins (cross-referenced with *Drosophila melanogaster*) found across mermithid-infected sandhoppers (infected with long worm versus Control)/earwigs (infected but aberrant versus infected others) and hairworm-infected cricket/grasshopper hosts. Note: grasshopper, cricket and infecting hairworm data were combined into one grouping (top circle) for simplicity. Proteins marked with an asterisk indicate they are from hairworms. Proteins are represented as the abbreviation given by the UniprotKB database. Proteins found in the overlapping parts of the circles are shared between host–parasite systems. Pie charts indicate the per cent composition of the proteomic data relative to the six major general functions of the proteins found. Colour of the proteins in the Venn diagram also align with their function. Data from Cricket/grasshopper-hairworm systems come from Biron *et al*. [4,21].

## (b) Earwigs

If proteomic changes in infected earwigs reflect energy depletion or another pathological response due to a large worm, it should be seen in *all* earwigs infected by long worms. The spike in regulation of proteins in earwigs was primarily restricted to 2 'aberrant' hosts infected with long worms (figure 2*b*). Therefore, parsimony may favour adaptive manipulation over pathology/host response as the best explanation for the changes seen in these aberrant hosts. Once the worms reach maturity, they may not immediately induce manipulation but instead wait for an environmental or physiological cue that signals the opportune time to

begin manipulation. Furthermore, unlike the sandhoppers, there is no direct overlap in significantly regulated proteins between the aberrant, infected and control groups (see electronic supplementary material, table S2).

Holistically, specific functions of the up and downregulated proteins are very similar between the earwig and sandhopper (see electronic supplementary material, figures S1, S2 and figure 3). However, the energy generation in aberrant earwigs extends to more processes than the citric acid cycle, with enrichment relative to glycolysis and pyruvate metabolism being present also. Proteins involved in muscle repair and activity are also enriched similar to the sandhopper, with both systems sharing upregulation of Mlc1, Zasp52 and bt. Overall, this implies either both the sandhopper and earwig respond similarly to mermithid infection, or the different mermithid species induce hyperactive/erratic behaviour in both hosts through a similar mechanism to get the host to water. Considering the observed hyperactivity in hairworm- and mermithid-infected hosts [19] (S.E. 2017, personal observation), the latter explanation may be more parsimonious.

A far greater proportion of proteins were found to be involved in axon/dendrite and synapse modulation in the aberrant earwig group, relative to sandhoppers infected with long worms. Proteins involved in synaptic transmission, specifically vesicle transport of neurotransmitters (Khc, sesB, AP-1-2beta, Syt1, Chc, CaMK11, Rpt6 Hsc70-4 and Pka-R1) were dysregulated exclusively in the aberrant earwigs' brains, as were proteins involved in axonogenesis, dendrite/axon guidance and general neuronal activity/modelling (CaMkII, Arpc2, kdn, khc, Hsc70-4, cher, SR3-9, Flo-1 and chic) (electronic supplementary material, table S2). The upregulated proteins AP-1–2beta and Synaptotagmin-1 (Syt1) are part of Clathrin-dependent synaptic vesicle endo- and exocytosis, which is crucial in neurotransmitter release in the synaptic cleft [41]. Given that Clathrin (Chc) is found in the earwig, sandhopper and the hosts of hairworms, this is a possible key component in the mermithid's manipulative process. Also, Syt1 by itself is a critical $Ca^{2+}$ sensor for neurotransmitter release [42,43].

$Ca^{2+}$/calmodulin-dependent protein kinase II (CaMkII), downregulated in aberrant earwig brains, is a key regulator in synaptic plasticity and behaviour [44–46]. Specifically, CaMkII is required in long-term potentiation, and therefore plays a crucial role in long-term memory and learning, as do the proteins cheerio (cher) and Pka-R1 also found in aberrant earwigs' brains [45,47–49]. Modulation of CaMkII, cher and Pka-R1 in the brains of aberrant earwigs may suggest alteration of host memory is a key part of the manipulative process. Perhaps removing the innate fear/avoidance of water by disassociating neurons that collectively function to keep the host away from water may be key. Taken together, the specific functions of the proteins involved in axon/dendrite and synapse modulation suggest that mermithid infection has the potential to manipulate where axon connections form and what neurotransmitters are released at those synapses. These two aspects of neuronal functioning are crucial in generating behaviour [45,50].

## (c) Convergence across hairworm and mermithid-infected hosts

When the proteomic data set from Biron *et al.*'s [4,21] studies on hairworm-infected crickets and grasshoppers is compared to the proteomic data from this study (figure 3), two things are immediately noticeable. First, the general function of dysregulated proteins across both parasite taxa and all hosts is conserved (excluding apoptosis in hairworm-infected crickets). Second, only two proteins were found in common across data sets: clathrin and Mtpalpha.

As discussed, clathrin is a key component in neurotransmitter release and, considering its ubiquitous dysregulation across all these different systems, it may be a fundamental target for parasites in influencing behaviour. In a similar vein, mitochondrial trifunctional protein alpha (Mtpalpha) is a crucial catalyst in beta oxidation in mitochondria [51]. This process is a rate-limiting step in metabolizing energy sources, including pyruvate and glycogen metabolism [52,53], and in the citric acid cycle for producing energy [54,55], all of which were enriched in upregulated protein networks (see electronic supplementary material, S1 and S2). This suggests alteration of Mtpalpha levels may be the initial cause of most of the changes in energy-related proteins found in infected hosts.

It is also important to note the huge proportion of proteins dedicated to axon/dendrite and synapse modulation in the hairworm–host systems relative to the sandhopper and earwig proteomic data (figure 3). This crucially establishes that the alteration of the CNS is a key component in forcing the host to water. Furthermore, proteins involved in synaptic vesicle endo–exocytosis, axonogenesis and also proteins specific to memory and learning (adf1 and hn) are modulated during the manipulative event in the hairworm-cricket/grasshopper systems (electronic supplementary material, table S3). This convergence in the regulation of proteins related to supposed CNS manipulation, across both parasite taxa and all host species, is strongly indicative of adaptive manipulation.

In Biron *et al.*'s [4,21] discussion of the hairworm–host proteomic data, proteins belonging to the Wnt family, found in both the hairworm and its host, are posited as a key manipulative agent behind the positive hydrotaxis, dictating axon/dendrite guidance and neuron differentiation. In the present study, Wnt was not found directly, although proteins were found that indirectly modify Wnt signalling. Specifically, Flotillin and Rack 1 (electronic supplementary material, tables S1–S3) upregulate and downregulate Wnt levels, respectively [56–58]. A methodological aspect of our study may have influenced the chance of finding matching proteins such as Wnt. Hairworm hosts are observed to exhibit the hydrophilic behaviour nocturnally [19,21], however, in our study mermithid hosts were collected and snap frozen during the day. If both hairworms and mermithids induce manipulation in a short burst at night, rather than over an extended period of time, this may be significantly limiting protein matches.

In the broader context of host manipulation, similar protein functions, particularly around energy generation/metabolism, were found in trypanosomes and malaria that increase the frequency of feeding behaviour (hyperactivity) in their vector host [59–61], suggesting a potential convergence in mechanisms. In gammarid amphipods infected with manipulative acanthocephalans, cestodes or trematodes, proteomic investigation found very different protein functions compared to the mermithid-sandhopper/earwig systems, centring around neuro-inflammation and serotonin modulation [62]. Similarly, other proteomic studies on different forms of manipulation found different protein functions to be affected [63–66]. Ultimately, this suggests the general functions of the proteins found in the present study are specific to the type of

manipulation being performed and are not part of a generic response to any form of infection.

## 5. Conclusion

Given the protein regulation seen in host brains, the restriction of most proteomic changes to hosts infected by large worms and the convergence in general function of proteins regulated across mermithid and hairworm-infected hosts, particularly in relation to axon/dendrite and synapse modulation, adaptive manipulation of host behaviour is likely to be the most parsimonious explanation. It appears the parasites are capable of inducing erratic or hyperactive behaviour, potentially via initial modulation of Mtpalpha, increasing the likelihood the hydrophobic host comes across water. By manipulating neuronal connections, and altering neurotransmitter release, the parasites may then induce a hydrophilic state, forcing the host into water. Clathrin may be a key protein in this aspect of manipulation as well as alteration of memory.

This study builds upon the foundation laid by Biron *et al*.'s [4,21] studies, shedding further light on the mechanisms used by manipulative parasites. The next step will involve exploration of the genomes, epigenomes and transcriptomes of both hosts and parasites, combined with experimental infection of hosts [65]. This will allow following any genomic shifts in expression over the parasite's life cycle within the host, and thus open a very effective avenue for finding manipulation factors, and establishing causation [18].

Data accessibility. The data for this paper have been uploaded to MassIVE: https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp Code for finding the data set is MSV000084448.

Authors' contributions. R.H. designed and conceptualized study, wrote manuscript, carried out fieldwork and proteomic component of study to completion. S.E. advised and assisted fieldwork, critically revised manuscript. J.F.D. critically revised manuscript. M.A. assisted in proteomic component, critically revised manuscript. T.K. constructed and assisted in carrying out the proteomic methodology. R.P. assisted in conceptualization and critically revised manuscript.

Competing interests. We declare we have no competing interests.

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