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A nematode that can manipulate the behaviour of slugs

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Abstract

      The ability of parasites to manipulate the behaviour of their hosts has evolved multiple times, and has a clear fitness benefit to the parasite in terms of facilitating growth, reproduction and transfer to suitable hosts. The mechanisms by which these behavioural changes are induced are poorly understood, but in many cases parasite manipulation of serotonergic signalling in the host brain is implicated. Here we report that *Phasmarhabditis hermaphrodita*, a parasite of terrestrial gastropod molluscs, can alter the behaviour of slugs. Uninfected slugs (*Deroceras panormitanum, Arion subfuscus* and *Arion hortensis*) avoid areas where *P. hermaphrodita* is present, but slugs infected with *P. hermaphrodita* are more likely to be found where the nematodes are present. This ability is specific to *P. hermaphrodita* and other nematodes (*Steinernema carpocapsae* and *Heterorhabditis bacteriophora*) do not induce this behavioural change. To investigate how *P. hermaphrodita* changes slug behaviour we exposed slugs to fluoxetine (a selective serotonin reuptake inhibitor) and cyproheptadine (a serotonin receptor antagonist). Uninfected slugs fed fluoxetine no longer avoided areas where *P. hermaphrodita* was present; and conversely, infected slugs fed cyproheptadine showed no increased attraction to areas with nematodes. These findings suggest that a possible mechanism by which *P. hermaphrodita* is able to manipulate parasite avoidance behaviour in host slugs is by manipulating serotonergic signalling in the brain, and that increased serotonin levels are potentially associated with a reduction in parasite avoidance.

Running head: Zombie slugs on drugs

1. Introduction

The ability of parasites to manipulate the behaviour of their hosts is surprisingly common, and has been documented for fungal, protozoan and helminth parasites, and a wide range of host animals (Hughes et al., 2012; Moore, 2002). A classic example of this phenomenon is the fungal parasite of the genus *Ophiocordyceps* (Araújo et al., 2015), which manipulates the behaviour and circadian rhythms of host ants in a manner which enhances dispersal of fungal spores and therefore facilitates parasite transmission (de Bekker et al., 2014). Another well-known example of host manipulation is the protozoan *Toxoplasma*, which changes the behaviour of infected intermediate host rodents so they actively seek out the feline predators which are the parasite's definite host (Webster, 1994). There are also many examples of helminth parasites manipulating the behaviour of their hosts; trematodes in particular are renowned for their ability to manipulate the behaviour of their intermediate hosts in ways that facilitate transmission to the definitive host where sexual reproduction can occur. The trematode *Leucochloridium* induces the intermediate molluscan host *Succinia* to climb high in the tree canopy where it is likely to be predated by definitive host birds (Wesolowska and Wesolowski, 2013); and there are similar examples of acanthocephalan infected amphipods (Jaquin et al., 2014) and trematode infected fish (Lafferty and Morris, 1996) behaving in a conspicuous manner likely to attract their definitive host predators.

Though the fitness benefits to the parasite in terms of enhanced transmission or reproduction are clear, the neurobiological mechanisms by which parasites cause these behaviour changes in their animal hosts are not completely understood. However a few studies that have been conducted suggest alterations in biogenic amine signalling, for example changes in serotonin, dopamine or octopamine levels in the host brain, are a potential causative mechanism. The parasitoid jewel wasp controls the brain and behaviour of its cockroach host in this way, first raising dopamine levels to induce obsessive grooming, and then blocking octopamine signalling to induce a lethargic state (Libersat and Gal, 2014). Acanthocephalan and trematode parasites of gammarid amphipods (small crustaceans) use serotonergic modulation to alter phototaxis and geotaxis behaviour in a way which increases the chance of the amphipod being consumed by a definitive host (Tain et al. 2006, Helluy 2013). Perhaps the most compelling evidence of this proposed mechanism of host mind

control comes from the example of the trematode *Euhaplorchis californiensis* infecting the killifish *Fundulus parvipinnis*. The parasite forms cysts on the surface of the host's brain which increase dopamine signalling and suppress serotonin signalling, resulting in a conspicuous behavioural phenotype which greatly increases predation by definitive host herons and egrets (Shaw et al. 2009; Lafferty and Morris 1996).

Apart from investigations into trematodes such as *Leucochloridium* (Wesolowska and Wesolowski 2013), there is little information on the ability of other parasites able to manipulate the behaviour of molluscan hosts. Terrestrial gastropods such as slugs and snails are infected with many parasites including viruses, bacteria, trematodes and nematodes (Barker, 2004). Of these, the nematodes are most abundant with over 108 species, from 4 out of 5 clades of the Nematoda infecting slugs that are used as paratenic, definitive or intermediate hosts (Blaxter et al., 1998; Grewal et al., 2003a). Some species use slugs for transport e.g. *Caenorhabditis elegans* (Petersen et al., 2015) but some species e.g. *Phasmarhabditis hermaphrodita* are lethal parasites of several slug and snails species (Wilson et al., 1993; Rae et al., 2009). This nematode has been developed as a biological control agent (Nemaslug® from BASF-Becker Underwood Agricultural Specialities) for use against slugs and snails in farms and gardens (Rae et al., 2007). Nematodes are applied to soil where they then search for slugs, enter through the back of the mantle and kill the slug 4-21 days later and reproduce on the cadaver making more infective stage nematodes that go and search for more slugs (Wilson et al., 1993; Tan and Grewal, 2001). *Phasmarhabditis hermaphrodita* is a facultative parasite that can reproduce on rotting matter e.g. leaf litter, dead earthworms (MacMillan et al., 2009; Tan et al., 2001; Rae et al., 2009) and can infect larger resistant slug species e.g. *Arion ater* and wait for them to die where it then reproduces on the decaying cadaver (termed 'necromeny' by Schulte (1989). The commercial strain of *P. hermaphrodita* (DMG00001) has been shown to provide protection against slug damage in many agriculturally important crops (Rae et al., 2007).

Slugs are able to detect and avoid areas where *P. hermaphrodita* has been applied (Wilson et al, 1999; Wynne et al., 2016). Snail species tend not to avoid *P. hermaphrodita* as they are resistant and are able to encapsulate and kill nematodes using their shell (Williams and Rae, 2016). Although several slug species can detect and avoid *P. hermaphrodita* there have been no experiments investigating whether the behaviour of slugs infected with *P. hermaphrodita* could change. Therefore, we

1 decided to investigate whether *P. hermaphrodita* could control the behaviour of

2 several slug species. We also investigated whether the ability to control the behaviour

3 of slugs was species specific to *P. hermaphrodita* or other distantly related nematodes

4 (*Steinernema carpocapsae* and *Heterorhabditis bacteriophora*). Finally, we examined

5 indirectly the potential mechanism of how nematodes change slug behaviour by

6 taking a pharmacological approach using a serotonin reuptake inhibitor (fluoxetine)

7 and a serotonin receptor antagonist (cyproheptadine).

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10 **2. Materials and Methods**

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12 *2.1 Source of invertebrates*

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14   The commercial strain of *P. hermaphrodita* (DMG0001) (Fig. 1A), *S.*

15 *carpocapsae* and *H. bacteriophora* were purchased from Becker Underwood BASF

16 Agricultural Specialities and stored at 15°C until use. Slugs (*Deroceras*

17 *panormitanum*, *Arion hortensis*, *Arion subfuscus, Milax sowerbyi* and *Lehmannia*

18 *valentiana*) (Fig. 1B-D) were collected from Liverpool John Moores University

19 (LJMU) greenhouses and stored in clear non-airtight plastic containers and fed lettuce

20 *ad libitum*. This location was chosen for slug collection as the populations of each

21 slug species have never yielded *P. hermaphrodita* from over three years of dissections

22 and experiments.

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24 *2.2 Behavioural assay investigating slug avoidance of nematodes*

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26   The behavioural assay used to assess whether slugs avoided nematodes was

27 based on Wilson et al. (1999) and Wynne et al. (2016). Briefly, three non-airtight

28 plastic boxes (9 x 24 x 6 cm) were filled with 50 g peat soil. Each box had 2 cm of

29 copper tape added to the top of each box, which prevented slugs from moving to the

30 lid. Each section (9 x 12 cm) was labelled either as the "control side" or the

31 "nematode side". Nematodes were applied at a rate of 120 per cm^2 in 6 ml of tap

32 water and applied evenly over the soil surface (Wilson et al., 1999; Wynne et al.,

33 2016) to the "nematode side" of each box. Water was added to the other side and

34 acted as the control. *P. hermaphrodita* does not move from the point of application

(Wilson et al., 2000; Wynne et al., 2016) hence it was unnecessary to confine their movement to the "nematode" side. Five slugs (*D. panormitanum*, *A. hortensis*, *A. subfuscus, M. sowerbyi* or *L. valentiana*) were added to the middle of each box and each day for 4 days the side the slugs were found on was recorded. After each recording they were placed back in the middle of the box. Three discs of lettuce (diameter 3.5 cm) were added to each side and replaced every 48 hours. The boxes were stored at 18°C with 12 hour light and dark cycles. Three replicate boxes were used for each slug species and the experiment was repeated three times (N = 9 replicate boxes; n = 45 slugs).

In order to understand if the ability of slugs to detect and avoid areas where *P. hermaphrodita* was present was specific to this nematode or if slugs avoided nematodes in general, additional experiments were performed using nematodes we expected to have little interaction with gastropods. Hence, we also exposed slugs to entomopathogenic nematodes (*S. carpocapsae* and *H. bacteriophora*) which utilise symbiotic bacteria to infect and kill insect hosts (Forst et al., 1997); although there have been reports claiming that these parasites can also infect slugs (Jaworska, 1993; Kaya, 2000).

This assay was used to investigate the following: 1. Whether slugs (*D. panormitanum*, *A. hortensis*, *A. subfuscus, M. sowerbyi* or *L. valentiana*) avoided *P. hermaphrodita* 2. Whether slugs (*D. panormitanum, A. hortensis*, *A. subfuscus, M. sowerbyi* or *L. valentiana*) infected with *P. hermaphrodita* avoided *P. hermaphrodita* 3. Whether *D. panormitanum* avoided entomopathogenic nematodes (*S. carpocapsae* and *H. bacteriophora*) 4. Whether *D. panormitanum* avoided entomopathogenic nematodes (*S. carpocapsae* and *H. bacteriophora*) when infected with *S. carpocapsae* or *H. bacteriophora*.

2.3 Infection of slugs with molluscicidal and entomopathogenic nematodes

In a separate experiment examining the behaviour of infected slugs, *D. panormitanum* were exposed to each nematode species: *P. hermaphrodita*, *S. carpocapsae* and *H. bacteriophora* (30 nematodes per cm^2) for 96 hours (a suitable time for infection (Fig 1E); Wilson et al., 1993; Tan and Grewal, 2001) before being used in the behaviour assay as previously described.

1 *2.4 Oral administration of pharmacological compounds to slugs*

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3   To investigate the potential role of a serotonergic mechanism in influencing

4 slug behaviour towards nematodes, boxes were set up as previously described but two

5 discs of bread (4 cm in diameter) were soaked in 2.5 ml of 10 µM fluoxetine and

6 placed at each end of the boxes (no lettuce was added). We have found in previous

7 experiments that slugs and snails will readily feed on bread supplemented with

8 compounds that affect serotonergic and dopaminergic signalling (Williamson,

9 unpublished observation). Numbers of slugs found on each side were recorded daily

10 and the experiment was repeated 5 times with uninfected and infected *D.*

11 *panormitanum*. The infected and uninfected slugs were also exposed to

12 cyproheptadine (10 µM) in separate experiments using the same procedures and was

13 repeated 3 times.

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15 *2.5 Examining the effects of P. hermaphrodita, fluoxetine and cyproheptadine on*

16 *feeding, movement and behaviour of D. panormitanum*

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18   We investigated whether treatment of fluoxetine and cyproheptadine would

19 affect locomotion and feeding behaviour of *D. panormitanum*. We fed *D.*

20 *panormitanum* (non-infected and infected with *P. hermaphrodita*) either water, 10

21 µM fluoxetine or 10 µM cyproheptadine on 4 cm bread discs for 48 hours (as

22 described above). We then removed 5 *D. panormitanum* from each treatment and

23 filmed their movement and behaviour for 10 mins. Individual slugs were placed in 10

24 cm Petri dishes filled with 1.2% technical agar and were allowed to acclimatise for

25 several minutes before filming began. The speed, distance and time spent immobile of

26 30 *D. panormitanum* (5 non-infected or infected animals for each treatment) was

27 recorded in each experiment simultaneously using idTracker (Pérez-Escudero et al.,

28 2014) then analysed in MATLAB® using the script in Supplementary File 1.

29   We also assessed the effects of fluoxetine and cyproheptadine on the food

30 consumption of *D. panormitanum*. Therefore, after exposure to bread discs with

31 water, 10 µM fluoxetine or 10 µM cyproheptadine (as described above) for 48 hours

32 we removed 5 slugs (non-infected and infected with *P. hermaphrodita*) and then

33 individually placed them in a 5 cm Petri dish, with pre-moistened filter paper with a 1

34 cm disc of lettuce. The following day the amount of lettuce that each slug had eaten

was then quantified. Lettuce was chosen as a substrate to quantify the amount the slugs ate, as it was difficult to reliably quantify the amount of bread eaten over this time due to evaporation of moisture.

2.6 The effects of fluoxetine and cyproheptadine on survival and behaviour of P. hermaphrodita.

To understand whether fluoxetine or cyproheptadine would affect the survival of *P. hermaphrodita,* 100 µl of 10 µM fluoxetine, 10 µM cyproheptadine or water (control) was added to 10 separate wells in a 96 well plate. To each well a single dauer juvenile of *P. hermaphrodita* was added. A lid was placed on the 96 well plate, sealed with Parafilm® and then incubated at 20°C for 4 days after which the numbers of alive nematodes were counted. The experiment was repeated twice.

To examine if fluoxetine or cyproheptadine affected the behaviour of *P. hermaphrodita* a thrashing assay was carried using methods described for *C. elegans* (Sleigh, 2010). Thrashing has been used to understand the effect drugs, chemicals and mutations have on motility of *C. elegans* (Buckingham and Sattelle, 2009). Approximately 400 *P. hermaphrodita* were added to 2.5 ml of water, 10 µM fluoxetine or 10 µM cyproheptadine and stored at 20°C for 4 days. On days 1, 2, 3 and 4 the number of thrashes 5 separate animals performed were counted per minute, and was repeated three times for each animal. A single thrash is defined as a complete change in direction of bending at the mid-body (Sleigh, 2010; Miller et al., 1996).

2.7 Data analysis

The mean number of slugs found on the nematode and control side over 4 days was compared using a Student's t test. As multiple comparisons were carried out the Bonferroni correction was applied and an adjusted P value of 0.01 was used instead of 0.05. The amount of lettuce eaten by *D. panormitanum* after exposure to water, fluoxetine or cyproheptadine when uninfected or infected with *P. hermaphrodita* was compared using a One way ANOVA with Tukey's post hoc test. Number of thrashes per minute by individual *P. hermaphrodita* exposed to water, fluoxetine or cyproheptadine for 1, 2, 3 and 4 days was compared using a Two-way ANOVA. Average speed, distance travelled and time immobile of infected and non-infected *D.*

panormitanum fed water, fluoxetine or cyproheptadine was compared using a One-Way ANOVA with Tukey's post hoc test. Multiple comparisons were carried out so the Bonferroni correction was applied and an adjusted P value of 0.01 was used instead of 0.05. Effect Size was calculated using Cohen's d for Student's t test comparisons and Partial Eta squared (η_p^2) when One or Two Way ANOVAs were used. Statistical analysis was carried out using SPSS.

3. Results

3.1 Infection of P. hermaphrodita can change the behaviour of slugs

Deroceras panormitanum ($p < 0.0001$; $d = 1.78$; Fig 2A)*, A. hortensis* ($p < 0.0001$; $d = 1.14$; Fig 2C) and *A. subfuscus* ($p < 0.0001$; $d = 1.82$; Fig 2E) spent significantly more time on the control side and avoided *P. hermaphrodita*. In contrast, *M. sowerbyi* spent more time on the nematode side ($p < 0.0001$; $d = 1.25$; Fig 2G) and *L. valentiana* spent equal amounts of time on each side ($p = 1$; $d = 0$; Fig 2I). However, when *D. panormitanum* ($p < 0.0001$; $d = 1.89$; Fig 2B)*, A. hortensis* ($p < 0.0001$; $d = 1.20$; Fig 2D), *A. subfuscus* ($p < 0.0001$; $d = 2.10$; Fig 2F), and *L. valentiana* ($p = 0.010$; $d = 0.59$; Fig 2J) were infected with *P. hermaphrodita* they were found significantly more on the nematode side compared to the control side. However, *M. sowerbyi* spent more time on the control side avoiding the nematodes ($p = 0.004$; $d = 0.66$; Fig 2H).

Deroceras panormitanum did not avoid *H. bacteriophora* ($p = 0.37$; $d = 0.21$; Fig 3B) but did avoid *S. carpocapsae* ($p = 0.001$; $d = 0.20$; Fig 3A) (for reasons unknown) however, when *D. panormitanum* were infected with *H. bacteriophora* ($p = 0.09$; $d = 0.50$) or *S. carpocapsae* ($p = 0.47$; $d = 0.82$) they did not change the behaviour of the slugs and they were found equally on each side (Fig 3C,D).

3.2 Behaviour of slugs is altered after treatment with fluoxetine and cyproheptadine

Surprisingly, just like infected *D. panormitanum*, uninfected *D. panormitanum* that were fed fluoxetine spent significantly more time on the side with *P. hermaphrodita* present ($p < 0.0001$; $d = 0.50$; Fig 4A). More individuals of *D. panormitanum* infected with *P. hermaphrodita* then fed fluoxetine were found on the

side with *P. hermaphrodita* present ($p < 0.0001$; $d = 0.69$; Fig 4B), though this was significantly less than recorded in the previous experiment using *P. hermaphrodita* infected *D. panormitanum* without fluoxetine treatment ($p = 0.01$; $d = 0.61$).

When uninfected *D. panormitanum* were fed cyproheptadine they avoided areas where *P. hermaphrodita* was present (just like untreated *D. panormitanum*) ($p < 0.0001$; $d = 1.32$; Fig 4C). However, when *D. panormitanum* were previously infected with *P. hermaphrodita* and fed cyproheptadine this treatment abrogated their attraction to the side with the nematodes present and they spent an equal amount of time on both sides ($p = 0.08$; $d = 0.50$; Fig 4D).

3.3 The effects of fluoxetine and cyproheptadine on survival and behaviour of P. hermaphrodita.

When single *P. hermaphrodita* were exposed to 10 µM fluoxetine or 10 µM cyproheptadine there was little effect on survival or thrashing behaviour. After 4 days, 13 out of 20 nematodes were alive when exposed to water (acting as the control), 15 out of 20 nematodes were alive when exposed to 10 µM fluoxetine and 18 out of 20 nematodes were alive when exposed to 10 µM cyproheptadine. There was no significant difference in the number of thrashes by individual *P. hermaphrodita* exposed to water, fluoxetine or cyproheptadine on day 1, 2, 3 or 4 ($p = 0.56$; $\eta_p^2 = 0.03$; Supplementary Fig 1).

3.4 Examining the effects of P. hermaphrodita, fluoxetine and cyproheptadine on feeding, movement and behaviour of D. panormitanum

There was no effect of cyproheptadine or fluoxetine on the average speed or distance travelled by non-infected ($P = 0.06$; $\eta_p^2 = 0.04$) or infected *D. panormitanum* ($P = 0.04$; $\eta_p^2 = 0.04$) (Supplementary Fig 2A,B; Supplementary Video 1 and 2) but both non-infected *D. panormitanum* ($P = 0.002$; $\eta_p^2 = 0.08$), and infected *D. panormitanum* ($P = 0.001$; $\eta_p^2 = 0.13$) fed fluoxetine spent more time mobile than those fed water or cyproheptadine (Supplementary Fig 2C). Neither drug affected the appetite of *D. panormitanum* as there was no significant difference between the amount of lettuce that was eaten by nematode infected and non-infected *D.*

1 *panormitanum* when treated with fluoxetine and cyproheptadine ($P = 0.13$; $\eta_p^2 = 0.28$;

2 Supplementary Fig 2D).

3

4 **4. Discussion**

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6   Although it may not seem surprising that sick animals behave differently than

7 non-infected ones (Hart, 1988), it is perhaps more unusual to find that infected

8 animals behave in a manner that increases their exposure to the pathogen causing their

9 illness. When several slug species were infected with the nematode *P. hermaphrodita*,

10 they consistently preferred to remain in an area where additional parasites of this

11 species were found. Manipulation of behaviour by parasites is largely split into those

12 that change behaviour of hosts to get to intermediate and definitive hosts to complete

13 their lifecycle or those that need to get to more suitable environments for growth

14 (Hughes et al., 2012; Moore, 2002). In terms of an adaptive benefit of changing the

15 behaviour of slugs, we presume that the nematode is driving the host towards areas

16 where the same species of pathogenic nematode that would allow more nematodes to

17 penetrate into the slugs to expedite host mortality. *Phasmarhabditis hermaphrodita* is

18 able to kill slugs in 4-21 days (Tan and Grewal, 2001) with increasing numbers of

19 nematodes increasing the mortality rate (Wilson et al., 1993, Glen et al., 2000; Rae et

20 al., 2009). *P. hermaphrodita* is a facultative parasite that can reproduce on organic

21 substrates such as slug faeces, leaf litter and compost (Tan and Grewal, 2001;

22 MacMillan et al., 2009). However, the most ideal substrate is a dead slug host which

23 can provide sufficient resources to support hundreds of thousands of *P.*

24 *hermaphrodita* offspring (Rae et al., 2009), and can increase the chance of finding

25 males to increase genetic variation (even though they are produced in low amounts,

26 Maupas, 1900). It might be thought that co-infection of a host with additional

27 conspecifics would reduce parasite fitness by increasing competition for resources

28 (Poulin, 1998); which has been shown specifically in *P. hermaphrodita* where high

29 doses feeding on rotting slugs can lead to intraspecific competition and reduced yield

30 of offspring (Nermut et al., 2012). Yet as many as fifty *P. hermaphrodita* growing and

31 feeding on a single slug carcass can result in 15,000 - 40,000 viable infectious

32 offspring (Rae et al., 2009) that are highly virulent towards gastropod hosts such as

33 the grey field slug (*Deroceras reticulatum*) (Rae et al., 2010). Another potential

34 adaptive reason for controlling the behaviour of gastropods may be to manoeuvre the

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1   host to an environment that is preferential for the reproduction, survival or dispersal

2   of the nematodes. For example, *P. hermaphrodita* can disperse easily in leaf litter and

3   peat but not in mineral soils (MacMillan et al., 2009) and sandy loam soil provides a

4   suitable environment for the nematodes whereas clay loam decreases survival

5   (MacMillan et al., 2006). As these soil parameters can substantially affect *P.*

6   *hermaphrodita* it is of great interest to the nematode to find an optimal environment

7   that would increase the chances of locating another slug host.

8   *Phasmarhabditis hermaphrodita* is lethal to *D. panormitanum* (Wilson et al.,

9   1993; Rae et al., 2009) though does not cause mortality to *A. subfuscus*, *A. hortensis*

10 or *L. valentiana* (Grewal et al., 2003b; Dankowska, 2006); yet it was able to change

11 the behaviour of all these species. It seems that members of the Arionidae,

12 Agriolimacidae and Limacidae are particularly susceptible to behavioural changes

13 induced by *P. hermaphrodita*, while infected *M. sowerbyi* did not display these

14 behavioural changes. The differential ability of the nematode parasite to affect the

15 behaviour of slugs may relate to different co-evolutionary relationships with disparate

16 host species, regarding the fitness benefit to the parasite gained by host behavioural

17 manipulation. The ability to affect host slug behaviour is specific to *P.*

18 *hermaphrodita*: parasitic nematodes which infect arthropod hosts (*S. carpocapsae* and

19 *H. bacteriophora*) are unable to induce behavioural changes in slugs. This also

20 suggests that a degree of co-evolution between parasite and host, resulting in

21 enhanced reproductive fitness for the parasite, is a major factor influencing the ability

22 to manipulate host behaviour.

23   The genetic mechanisms by which parasites are able to exert control over the

24 behaviour of their hosts are unclear, but one potential route is through the

25 manipulation of neurotransmitters, specifically biogenic amines. The protozoan

26 parasite *Toxoplasma gondii* has the ability to change the behaviour of rats (Webster,

27 1994) and analysis of the genome found tyrosine hydroxylase genes that are used for

28 making L-DOPA, the precursor for dopamine synthesis (Gaskall et al., 2009).

29 Experimental evidence shows that the dopamine antagonist haloperidol reduces the

30 characteristic behaviours of infected rats (Webster et al., 2006). Amphipods living in

31 freshwater usually avoid the water surface to avoid predation by birds or fish, but

32 when infected by acanthocephalan worms (*Pomphorhynchus spp.*) their phototaxis

33 response is reversed, increasing predation by the acanthocephalan's definitive hosts

(Jacquin et al. 2014, Tain et al. 2006). Infected amphipods showed increased levels of serotoninergic brain activity, and the effects of parasite infection on behaviour could also be replicated using injections of serotonin (Tain et al. 2006). The trematode parasite *Euhaplorchis californiensis* also manipulates biogenic amine signalling in the brain to induce behavioural changes in its intermediate fish host: increased dopamine signalling in the hypothalamus, and reduced serotonin signalling in the hippocampus and raphe nucleus, cause the fish to behave boldly and suppress the natural fear response (Shaw et al. 2007).

We found that pharmacological treatment of infected and uninfected *D. panormitanum* with drugs which affect serotonergic brain signalling had striking effects on their behaviour. Increasing serotonin levels in uninfected slugs with fluoxetine drove them towards nematodes; but suppression of serotonin signalling in infected slugs (*D. panormitanum*) using cyproheptadine stopped their attraction to the nematode parasites. This research suggests that *P. hermaphrodita* is potentially secreting serotonin in the slug, or inducing its production in the host, which drives it towards *P. hermaphrodita*. It should be noted that other nematodes from the Rhabditidae, such as *C. elegans*, can produce biogenic amines such as serotonin, dopamine, tyramine and octopamine (Chase and Koelle, 2007). An alternative mechanism of biogenic amine upregulation may be for the parasite to speed up a rate-limiting step in monoamine synthesis, like the parasite *T. gondii* increases host dopamine levels by secreting the enzyme tyrosine hydroxylase (Gaskell et al., 2009).

In general there are only a few examples of nematodes that can change the behaviour of their hosts. Mice infected with *Trichinella spiralis* have decreased exploratory behaviour and make them more likely to be eaten by bird hosts (Rau, 1983). Ants parasitised by nematodes (*Myrmeconema neotropicum*) cause both behavioural and morphological changes (Poinar and Yanoviak, 2008). Infected ants are less aggressive, do not bite and exhibit increased gaster-flagging (where gaster is held in air whilst walking), which also turns a rich red hue and due to this they are more likely to be eaten by birds (Yanoviak et al., 2008). Our research is the first example of nematode parasites that can change the behaviour of gastropods.

Phasmarhabditis hermaphrodita infected slugs have previously been observed to behave in atypical ways: showing reduced feeding (Glen et al., 2000) and reduced locomotion (Bailey et al., 2003), and moving down into soil to die (Pechova and Foltan, 2008). *Phasmarhabditis hermaphrodita* infected freshwater snails also move

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out of water and onto soil, potentially to get to a more nematode favourable environment (Morley and Morrit, 2006). It appears that *P. hermaphrodita* infection also deters predators, such as beetles, from nematode infected rotting slug carcasses (Foltan and Puza, 2009).

5. Conclusions

In summary, we have shown that *P. hermaphrodita* can control the behaviour of infected slugs and makes them move towards areas where nematodes are present. We have also shown indirectly that the manipulation of biogenic amines may be a plausible mechanism by which the nematode controls the behaviour of slugs, as the effects of infection on host behaviour can be both induced using fluoxetine, and reversed by cyproheptadine. As this nematode is being developed as a model to study the evolution of parasitism (Rae, 2017), the *P. hermaphrodita*-host slug system shows great potential for further investigation of the neurobiological and genetic mechanisms involved in mind control by parasites.

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Figure legends

Fig 1: The slug parasitic nematode *P. hermaphrodita* (A) is a parasite of *D. panormitanum* (B), *A. subfuscus* (C) and *M. sowerbyi* (D). *Phasmarhabditis hermaphrodita* infects slugs by entering through the back of the mantle causing a characteristic swelling that can lead to rupture and shedding of the internal shell (E). Scale bars in A represent 100 μm and in B-E represent 1 cm.

Fig 2: Mean number of uninfected *D. panormitanum* (A), *P. hermaphrodita* infected *D. panormitanum* (B), uninfected *A. hortensis* (C), *P. hermaphrodita* infected *A. hortensis* (D), uninfected *A. subfuscus* (E), *P. hermaphrodita* infected *A. subfuscus* (F), uninfected *M. sowerbyi* (G), *P. hermaphrodita* infected *M. sowerbyi* (H), uninfected *L. valentiana* (I) and *P. hermaphrodita* infected *L. valentiana* (J) found on control side (blue) or *P. hermaphrodita* side (red) over 4 days. Bars represent ± one standard error.

Fig 3: Mean number of uninfected *D. panormitanum* exposed to *S. carpocapsae* (A) or *H. bacteriophora* (B) and *D. panormitanum* infected with *S. carpocapsae* (C) and exposed to *S. carpocapsae* and *D. panormitanum* infected with *H. bacteriophora* and exposed *H. bacteriophora* (D) found on control side (blue) or nematode side (red) over 4 days. Bars represent ± one standard error.

Fig 4: Mean number of uninfected *D. panormitanum* (A) or *D. panormitanum* infected with *P. hermaphrodita* (B) fed 10 μM fluoxetine or uninfected *D. panormitanum* (C) or *D. panormitanum* infected with *P. hermaphrodita* (D) fed 10 μM cyproheptadine found on control side (blue) or *P. hermaphrodita* side (red) over 4 days. Bars represent ± one standard error.

Supplementary material

Supplementary File 1: Script that was used to analyse videos of *D. panormitanum* using idTracker and analysed in MATLAB®.

1   **Supplementary Figure 1**: Mean number of thrashes that *P. hermaphrodita*

2   performed when exposed to water (blue bars), 10 µM fluoxetine (red bars) or 10 µM

3   cyproheptadine (orange bars) in 1 min on days 1, 2, 3 and 4. Bars represent ± one

4   standard error.

5

6   **Supplementary Figure 2**: The mean total distance (cm) (A), average speed (cm per

7   second) (B), mean time (secs) spent immobile (C) and the mean percentage of lettuce

8   discs that were eaten (D) by *D. panormitanum* uninfected (U) or infected (I) with *P.*

9   *hermaphrodita* were fed water (control), 10 µM fluoxetine or 10 µM cyproheptadine.

10   Significant differences ($p < 0.01$) are shown as * between treatments. Bars represent ±

11   one standard error.

12

13   **Supplementary Video 1**: The behaviour of non-infected *D. panormitanum* fed on

14   water (control) (bottom row), 10 µM fluoxetine (middle row) or 10 µM

15   cyproheptadine (top row) filmed for 10 mins.

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17   **Supplementary Video 2**: The behaviour of *D. panormitanum* infected with *P.*

18   *hermaphrodita* fed on water (control) (bottom row), 10 µM fluoxetine (middle row)

19   or 10 µM cyproheptadine (top row) filmed for 10 mins.

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