Impacts of a newly identified behaviour-altering trematode on its host amphipod: from the level of gene expression to population

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SUMMARY

Changes to host behaviour induced by some trematode species, as a means of increased trophic transmission, represents one of the seminal examples of host manipulation by a parasite. The amphipod *Echinogammarus marinu*s (Leach, 1815) is infected with a previously undescribed parasite, with infected individuals displaying positive phototaxic and negative geotaxic behaviour. This study reveals that the unknown parasite encysts in the brain, nerve cord and the body cavity of *E. marinus*, and belongs to the Microphallidae family. An 18 month population study revealed that host abundance significantly and negatively correlated with parasite prevalence. Investigation of the trematode's influence at the transcriptomic level revealed genes with putative neurological functions, such as serotonin receptor 1A, an inebriated-like neurotransmitter, tryptophan hydroxylase and amino acid decarboxylase, present consistent altered expression in infected animals. Therefore, this study provides one of the first transcriptomic insights into the neuronal gene pathways altered in amphipods infected with a trematode parasite associated with changes to its host's behaviour and population structure.

Key words: parasitic-manipulation, host behaviour, neurological genes, serotonin, amphipods.

INTRODUCTION

Certain parasite species are known to induce changes to host behaviour as a means of increased trophic transmission, a process that represents one of the seminal examples of parasite-induced behavioural manipulation (Pearson, 1972; Bethel and Holmes, 1973; Camp and Huizinga, 1979; Helluy, 1983*a*, *b*; Combes, 1991; Combes *et al*. 1994; Haas, 1994; Esch *et al*. 2002; Mouritsen and Poulin, 2002). Among intertidal animals, trematodes are one of the most common metazoan parasites (Mouritsen and Poulin, 2002) and consist of an estimated 25 000 species, many of which are yet to be described (Esch *et al*. 2002). Trematodes are obligate parasites that have complex life cycles, infecting up to four hosts, taking many distinct forms and infecting their hosts in a variety of ways (Esch *et al*. 2002); with amphipod crustaceans generally often acting as second intermediate hosts (Pearson, 1972). It is known that for some trematode species, the metacercarial stage is capable of manipulating its intermediate host's behaviour to increase its chance of trophic transmission to the definitive host (Helluy, 1983*a*, *b*; Combes *et al*. 1994; Haas, 1994; Esch *et al*. 2002; Mouritsen and Poulin, 2002).

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The widespread marine amphipod *Echinogammarus marinus* has been used to understand strategies employed by a diverse range of parasites capable of manipulating the reproductive biology of its host (Short *et al*. 2012). However, the discovery that *E. marinus* can also be infected with a parasite apparently capable of manipulating host behaviour (Guler and Ford, 2010) has never been further investigated. In this case, infected specimens of the normally evasive *E. marinus* were found to spend significantly more time in the light and higher in the water column than uninfected individuals, a behaviour consistent with a parasitic strategy to increase trophic transmission. Such manipulative parasites are not unknown in amphipods, as the behaviour of infected *E. marinus* is similar to that observed in *Gammarus insensibilis* infected by the trematode *Microphallus papillorobustus* (Helluy, 1983*a*, *b*). It has been suggested that such parasites alter host behaviour by influencing pathways in a similar manner to neuropharmcological methods. For example, it appears that biogenic amines are frequently targeted by behaviour manipulating parasites (Nolan, 1998; Pryon and Elizee, 2000; Klein, 2003; Adamo, 2013; Helluy, 2013). Furthermore, components of the serotonin pathway have been implicated by experiments demonstrating that injection of serotonin, a monoamine neurotransmitter, into gammarids causes behaviours similar to that induced by manipulating parasites (Helluy and Holmes, 1990; Tain *et al*. 2006;

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Perrot-Minnot *et al*. 2014), while other neurotransmitters at similar concentrations, failed to induce a comparable response (Helluy and Holmes, 1990). In addition, it has been shown that once *M. papillorobustus* metacercariae encyst in the cerebral ganglia, specifically the protocerebrum responsible for all visual sensory input (Thomas *et al*. 2000; Helluy and Thomas, 2003; Kostadinova and Mavrodieva, 2005), serotonin levels become altered in specific regions, with a decrease of 62% observed in the optic neuropils (Ponton *et al*. 2006), a decrease thought to be due to the degeneration of discrete sets of serotonergic neurons. Consistent with studies implicating serotonin related pathways, positive phototaxic and negative geotaxic behavioural changes have been observed in *E. marinus* exposed to serotonin and the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Guler and Ford, 2010). Uncovering the neurological pathways modulated by cerebral encysting behaviour-manipulating parasites promises to reveal profound insights into arthropod neurobiology (Poulin and Mouritsen, 2006; Shaw *et al*. 2009; Helluy and Thomas, 2010) and although our understanding of the molecular host–parasite interactions are still limited (Biron and Loxdale, 2013), the existing investigations make it possible to credibly hypothesize which molecular pathways are altered in infected *E. marinus*. This opens the possibility of using the available *E. marinus* genomic resources (Short *et al*. 2014) to investigate the molecular biology underlying parasite-induced behavioural-manipulation at a transcriptomic level.

This study aims to identify the parasite species infecting *E. marinus* by comparing the parasite ribosomal RNA gene sequences with sequences deposited in public databases and those isolated from *M. papillorobustus*. Secondly, this study investigates the potential impact of the *E. marinus* parasite at the population level by comparing parasite prevalence with host abundance. Finally, we investigate the molecular pathways being altered in infected animals by attempting to identify expression changes in genes with plausible links to neurological pathways.

MATERIAL AND METHODS

Trematode harvesting and DNA isolation

Echinogammarus marinus were collected from beneath seaweed in the intertidal zone during low tide. Infected *E. marinus* individuals were taken from Langstone Harbour, Portsmouth, UK (50°47′23·13N 1°02′37·25W). Sixty adult males and females (n = 120) were collected during June and July 2011 and the location of the trematode metacercaria were recorded within the body cavity and head. *Gammarus insensibilis* infected by *M. papillorobustus* were collected from Étang de Thau, France (43°25′N, 3°35′E) and were kindly donated by Dr Frédéric Thomas (National Center of Scientific Research (CNRS) in Montpellier, France). Twenty cysts from each amphipod population were stored in 70% ethanol at −80 °C. DNA was extracted using the DNAeasy kit (Qiagen, UK) following the manufacturers' protocol and quantified using a spectrophotometer (NanoDrop 1000).

Amplification of trematode ribosomal RNA genes

Primers were used to amplify the 18S (537F, 1133R, 1073F, 18SR, 18SF and 549R) (Near *et al*. 1998) and 28S (LSU-5, 1500R) (Olson *et al*. 2003) ribosomal RNA genes. Primers (PITSF, PITSR, Table 1) used to amplify the Internal Transcribed Spacer (ITS) region were designed using the 3′ end of the sequenced 18S region and the 5′ end of the 28S region. All primers were synthesized by Eurofins MWG Operon, Germany. Polymerase chain reaction (PCR) reactions were performed in 25 μL reactions containing 1·25 mM $MgCl_2$, 1x PCR Buffer, 0·2 mM of each dNTP, 0·25 mM of each primer, 1 U Taq DNA polymerase (Promega, UK), 10 ng of genomic DNA. Reactions were carried out using the following thermal cycling conditions: 94 °C for 4 min followed by 32 cycles of 94 °C (45 s), 59 °C (45 s) and 72 °C (1·5 min), and a final incubation of 5 min at 72 °C. PCR products were analysed by agarose gel electrophoresis and were eluted and purified using the QIAquick Gel Extraction and purification Kit (Qiagen, UK) following the manufacturer's protocol. The purified PCR products were subsequently sequenced by Source Bioscience, UK.

Phylogenetic analysis

The isolated parasite ribosomal sequences were used to perform a basic local alignment search tool (BLAST) analysis against the non-redundant sequences deposited in GenBank National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nlh.gov) to reveal closely related sequences. The sequences obtained were aligned multiple sequence comparison by log-expectation (MUSCLE) and trimmed, before a phylogenetic tree was constructed using the maximum likelihood method implemented by the MEGA (v5·0) program (Hall, 2013).

Population sampling. *Echinogammarus marinus* were collected from Langstone Harbour, Portsmouth, UK (50°47′23·13N 1°02′37·25W) during low tide by selecting five 1 m^2 quadrats (total area = 5 m^2) in the intertidal zone each month over an 18 month period (January 2010–June 2011). All algae and surface sediment (approximately 2 cm in depth) was retrieved and stored in polythene bags. In the laboratory, samples were washed and decanted through a 0·7 mm sieve and all algae were scraped to ensure all *E. marinus* individuals were collected. Individuals were separated into males, females and juveniles, and counted to assess relative abundances. To allow for

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Table 1. Primers designed using Primer-3 software [65] and synthesized by Eurofins MWG Operon

Primer name	Sequence 5′ end to 3′
PITSF	GTTTCGACTGCTCGAGTGGTG
PITSR	AACAACCTGAACACCACATTG
RHOD1F	CCCGCCAACATGCTGCCTGA
RHOD1R	CGGGTGACCGCAGGCTCTTG
RHOD2F	CGCGGTGTCCACCAACCCAT
RHOD2R	ATTGGCGGGTGATCGCAGGC
Ine1F	CGTGGAGGAGCCGTTGCCTG
Ine1R	CCTGTGCGGCATCCCTCTGC
Ine2F	CCGGAGGGCAATCTTGCCGG
Ine2R	CGAGACGAAGCTGGGCCGTC
ATFF	AACTGGCGATGGCTTGGGCG
ATFR	CCGAACCATGTGGGATCGGCC
Ty1F	GCGCGGTCTGAAATGCAGCC
Ty1R	GCTCCGTTTGGCCGGCTATGA
ADF	TAGCGGAGGCTGCGTCTGGT
ADR	TATCAATGCGTCCGGGCGGC
PHF	GGTCAAGACCTGGAGCGCGG
PHR	GGTGCTGTGGAACACGCGGA
5HT1F	CAACGCAGAGTACGGGGTTGGT
5HT1R	GCAAAACGGCGAAATCGAACGGG
AKF	GGAGGCTTAAGCAGTCA
AKR	GACGGGTTTTTGCCAAAGT

later assessment of parasite prevalence and seasonality, 20 adults of each sex were selected from each monthly sample and stored in 70% ethanol at −80 °C.

PCR parasite screen. The DNA was isolated from the 20 adults of each sex set aside following the monthly sampling. The gonads and muscle tissue were dissected from the animal and washed with distilled water. DNA was extracted using the CHELEX© DNA extraction followed by a phenol-chloroform step and ethanol precipitation. Extracted DNA was quantified using a spectrophotometer (NanoDrop 1000). The samples were screened for evidence of infection using PCR. All PCR reactions were performed in 25 μL reactions containing 2·5 mM $MgCl_2$, 0·25 mM of each dNTP, 0·5 mM of each primer (18SF 5-GATTAAGCCATGCATG CGTAAG-3 and Trem18SR1 5-GCCGCGG TAATTCCAGCTC-3), 1x PCR buffer, 1 U Taq DNA polymerase (Promega, UK) and 10 ng of template DNA. Reactions were carried out using the following thermal cycling conditions: 94 °C for 4 min followed by 32 cycles of 94 °C (45 s), 59 °C (45 s) and 72 °C (1·5 min), and a final incubation of 5 min at 72 °C. To check the quality of all DNA samples, amplification of the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was performed (Yang *et al*. 2011). PCR product size was visualized on a 1·2% agarose gel under a UV transilluminator following electrophoresis.

Statistical analysis

Relationships between two biological parameters, such as host abundance and parasite prevalence, were statistically analysed using Pearson's correlation coefficients. Relationships between parasite prevalence and environmental parameters were conducted using regression analysis performed with a statistical software package (SPSS® statistics v17·0·0).

Candidate gene selection and primer development

Genes with functional links to neurological and serotonin pathways in *Drosophilia melanogaster* were retrieved from FlyBase (flybase.org) and were used to perform a local BLAST search against the *E. marinus* transcriptome database (Short *et al*. 2014). Sequences sharing a high level of sequence identity (*E*-value $\leqslant$1E-5) were taken and additional BLAST analyses (BLASTn) were performed against GenBank sequences (NCBI) to confirm the annotation. Gene candidates were selected on the basis of their direct involvement in the serotonin pathway or within processes that might plausibly influence behavioural responses in *E. marinus* (see Table 2). The only exception to this was a predicted Arginine Kinase, chosen due to its involvement in a specific immune response induced by the behaviour-manipulating trematode *M. papillorobustus* (Ponton *et al*. 2006). Primers were designed (Rozen and Skaletsky, 2000) against the selected *E. marinus* contiguous sequence (Table 1) and synthesized by Eurofins MWG Operon, Germany. All primers were tested for their quantitative real-time PCR (qPCR) suitability using PCR carried out in 25 μL volume containing 1·5 mM $MgCl_2$, 1x PCR buffer, 0·25 mM each dNTP, 1 U Taq DNA polymerase (Promega GoTaq®) and 10 ng cDNA (see section

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Table 2. Identifying *Echinogammarus marinus* genes with putative neurological roles. *Drosopholia* genes with functional links to serotonin and neurological pathways were used to BLAST search the *E. marinus* transcriptome database to identify orthologous sequences. The retrieved *E. marinus* sequences were then compared with annotated sequences in GenBank (NCBI) database to confirm the putative annotation

Primer	Primer name	Sequence 5′ end to 3′	Target Gene	GenBank ID	Ref. species	Query Coverage (%)	Max identity (%)	*E*-value
1	Ine1F	CGTGGAGGAGCCGTTGCCTG	Neurotransmitter	NM057664·5	*Culex quinquefasciatus*	37	74	4·00E-05
	Ine1R	CCTGTGCGGCATCCCTCTGC						
2	Ine2F	CCGGAGGGCAATCTTGCCGG	Inebriated neurotransmitter	NM_001169400·1	*Drosophila melanogaster*	79	70	8·00E-09
	Ine2R	CGAGACGAAGCTGGGCCGTC						
3	RHOD1F	CCCGCCAACATGCTGCCTGA	Rhodopsin	DQ852593·1	*Neomysis americana*	67	74	4·00E-74
	RHOD1R	CGGGTGACCGCAGGCTCTTG						
4	RHOD2F	CGCGGTGTCCACCAACCCAT	Rhodopsin	HM044848·1	*Lysiosquillina maculata*	62	75	6·00E-98
	RHOD2R	ATTGGCGGGTGATCGCAGGC						
5	ATFF	AACTGGCGATGGCTTGGGCG	Nutrient Amino acid transporter	NM_131991·2	*Drosophila melanogaster*	31	75	2·00E-21
	ATFR	CCGAACCATGTGGGATCGGCC						
6	Ty3F	GCGCGGTCTGAAATGCAGCC	Trytophan 5-monooxygenase activation protein	NM_013011·3	*Rattus norvegicus*	82	71	2·00E-35
	Ty3R	GCTCCGTTTGGCCGGCTATGA						
7	ADF	TAGCGGAGGCTGCGTCTGGT	Amino acid decarboxylase	XM002403644·1	*Ixodes scapularis*	57	79	2·00E-34
	ADR	TATCAATGCGTCCGGGCGGC						
8	PHF	GGTCAAGACCTGGAGCGCGG	Trytophan hydroxylase	AY099427·1	*Aedes aegypti*	49	72	6·00E-142
	PHR	GGTGCTGTGGAACACGCGGA						
9	5HT1F	CAACGCAGAGTACGGGGTTGGT	Serotonin receptor 1	NM_057454·3	*Drosophila melanogaster*	No sequence found primers designed directly from sequence		
	5HT1R	GCAAAACGGCGAAATCGAACGGG						
10	AKF	GGAGGCTTAAGCAGTCA	Arginine Kinase	GQ246164·1	*Penaeus monodon*	38	86	0·0E
	AKR	GACGGGTTTTTTGCCAAAGT						

PCR parasite screen). A primer concentration 10 mM was determined to be suitable for all primers except *ine1*, *2* and *5-HT$_{1A}$*, the concentrations were reduced to 5 mM to eliminate primer-dimer formation. Reactions were carried out using the following thermal cycling conditions: 95 °C (4 min) followed by 35 cycles of 95 °C (30 s), 60 °C (45 s), 72 °C (45 s) with a final incubation of 5 min at 72 °C. PCR products were analysed by agarose-gel electrophoresis to establish primer specificity.

RNA isolation and cDNA synthesis

The heads were removed from the bodies of infected and uninfected males ($n = 18$ in each group), before the first pereon and antennae were amputated. Heads were immediately snap frozen using liquid nitrogen following dissection and crushed in TRI Reagent® (Ambion, UK). The samples were divided to give three biological replicates for uninfected and infected groups (each replicate containing pooled head tissue from six animals). The total RNA was extracted using TRI Reagent® (Ambion, UK) according to manufacturer's instructions and cleaned using RNA Clean and Concentrator™−5 columns (Zymo Research, Orange, CA, USA). The RNA quantity and quality was assessed using a spectrophotometer (NanoDrop 1000) and agarose-gel electrophoresis, respectively. From the extracted RNA, 250 ng was reverse transcribed into cDNA using reverse transcriptase (Promega, UK) following the manufacturer's guidelines using OligoDT$_{15}$ primers and RNasin Ribonuclease Inhibitor (NEB, UK). The quality of the resulting cDNA was tested by PCR amplification of the constitutively expressed GAPDH gene as previously described (Yang *et al*. 2011).

Q-PCR analysis

SYBR green-based qPCR was performed using a real-time PCR cycler (Eco Illumina). Each reaction was performed in triplicate and the absence of genomic DNA was confirmed by performing minus reverse transcription (RT) reactions for all samples. Reactions were carried out using GoTaq qPCR Master Mix (Promega, UK) in a 15 μL volume containing 1 μL of cDNA, 0·4 μL of the forward and reverse primer taken from a 10 μM stock (note: 0·2 μL of forward and reverse primer was used for *ine1*, *2* and *5-HT$_{1A}$* genes), 5·7 μL ultra-pure water and 7·5 μL of 2X GoTaq qPCR Master Mix. Reactions were carried out with Rox normalization and underwent an initial incubation step of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s, completed with 1 cycle of 60 °C for 95 s. Melt curve analysis was performed to confirm the specificity of the PCR product in each reaction. The amplification data were analysed by plotting the fluorescence signal ΔRn against the cycle number. An arbitrary threshold was selected within the linear phase of the log ΔRn against cycle number plot. The quantification cycle (Cq value) was determined to be the cycle number at which ΔRn crossed this threshold. A relative expression of a gene was determined using the ΔΔCq method, with normalization to the expression of the GAPDH reference gene, using the dedicated Eco (v3·0) software package. Statistical differences in the expression of infected *vs* unifected samples were compared using an independent *t*-test (α0·05).

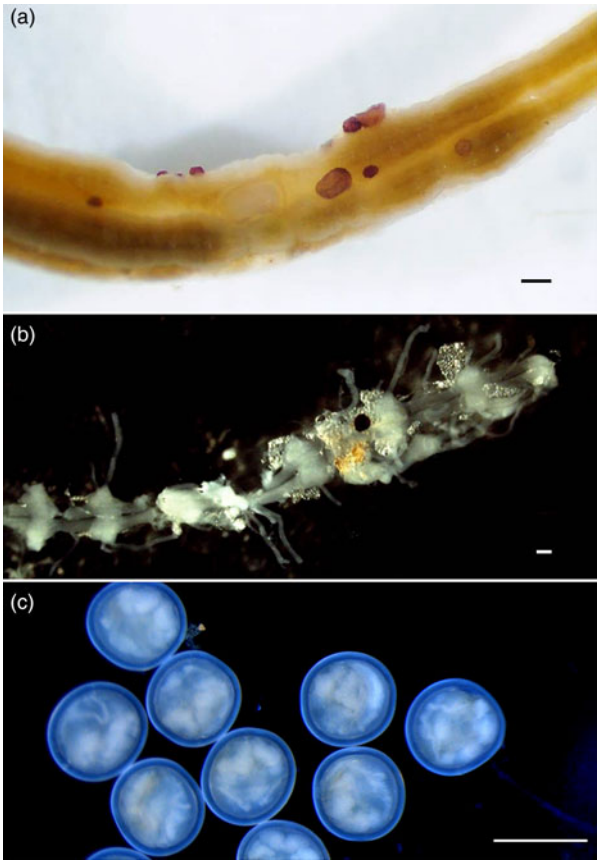

Fig. 1. Metacercariae of trematodes that have encysted and melanized in the (a) hepatopancreas (scale bar = 200 μM) and (b) ventral nerve cord of *Echinogammarus marinus* (scale bar = 100 μM) and (c) unmelanized metacariae from *E. marinus* body cavity (scale bar = 400 μM).

RESULTS

Trematode characterization and identification

The parasite residing in *E. marinus* was found to encyst within the brain, hepatopancreas and abdomen, as well being attached to nerves in the thorax (Fig. 1). A survey of infection prevalence and intensity in 120 specimens during June and July 2011 revealed that approximately half the population were infected by one or more encysted trematodes (55·7% males and 50·7% females). The mean intensity (9·8) and abundance (4·9) of infection was greater for females than for males (4·8 and 2·7,

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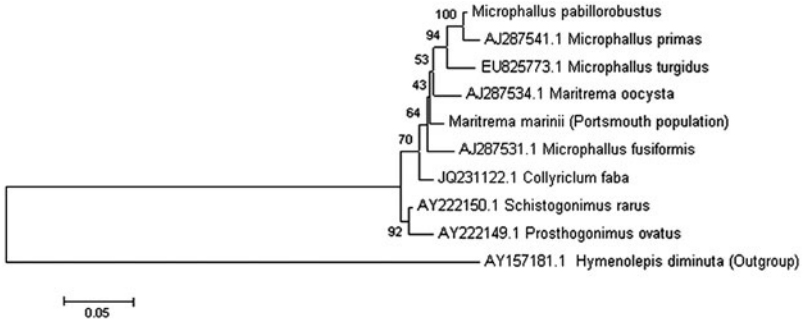

Fig. 2. Identification of unknown trematode species infecting *Echinogammarus marinus* at Langstone Harbour, UK. Molecular characterization of the well-studied trematode *Microphallus pabillorobustus*. A representative phylogenetic tree was generated using available rDNA sequences of digenean trematodes from NCBI. Sequences were aligned using MUSCLE and a phylogenetic tree was constructed using the maximum likelihood method implemented by the MEGA (Version 5) program. Bootstrap values ($n = 100$) for branches are shown as percentages. All branches are drawn to scale as indicated by the scale bar representing sequence divergence. The phylogenetic tree was generated as described above using trematode small subunit rDNA sequences (18S); scale bar represents 5% sequence divergence.

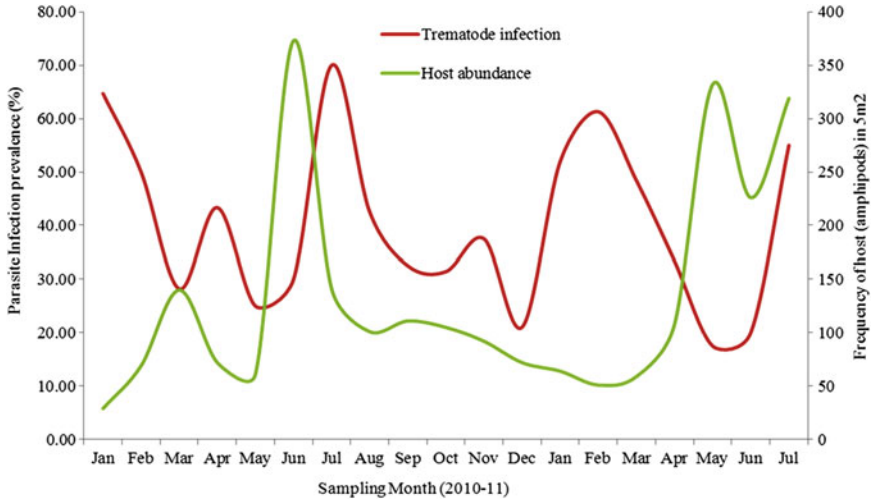

Fig. 3. Overall monthly infections prevalence of trematode sp. infecting *Echinogammarus marinus* population at Langstone Harbour (Portsmouth, UK) between January 2010 and July 2011.

respectively). Those that were found infecting the brain were found to make up approximately 7–9% of the total population (~20% of the infected portion) with similar infection between males and females The 18S, 28S and ITS regions of the parasite ribosomal RNA gene was obtained and used in conjunction with previously published sequences to perform a phylogenetic analysis. This reveals that the unknown parasite within the *E. marinus* population at Langstone Harbour is a trematode belonging to the family Microphallidae (Fig. 2). On the basis of rRNA gene sequence comparisons, the *E. marinus* parasite represents a new species of trematode falling between the branches of the genus *Maritrema* and *Microphallus* (Tkach *et al.* 2003).

Determination of trematode seasonal prevalence

To assess whether the trematode infecting *E. marinus* may cause population level effects, an 18-month field study (January 2010–June 2011) was undertaken to determine if parasite prevalence significantly correlates with host abundance. The overall mean infection prevalence over the study period was 40·2 ± 3·6% and ranged from 17·5 to 70·0%. Infection prevalence peaked in February and July for both 2010 and 2011, with a crash in infection rates in October 2010 (Fig. 3). There was no correlation observed between the trematode prevalence and host abundance when directly comparing the months (Pearson's correlation coefficient; $R = -0{\cdot}330$, $P = 0{\cdot}168$). However, as trematode prevalence can have a delayed effect on host abundance (due to the time required to produce a development stage capable of definitive host infection), the trematode prevalence was aligned +1 month. This adjustment resulted in a significant negative relationship between host abundance and parasite prevalence (Pearson's correlation coefficient; $R = -0{\cdot}461$, $P = 0{\cdot}047$) (Fig. 4). Regression analysis failed to detect

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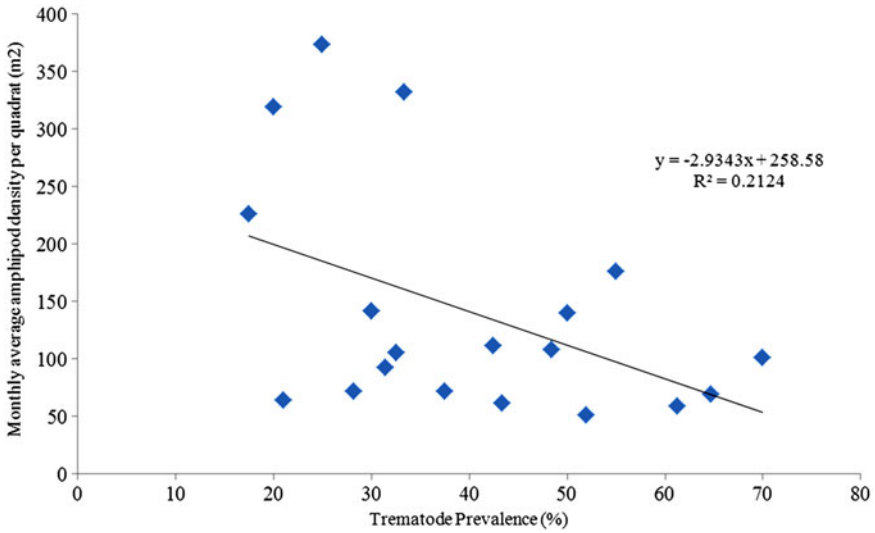

Fig. 4. Linear relationship between *Echinogammarus marinus* density from Langstone Harbour, Portsmouth (UK) and prevalence of a trematode parasite. Data obtained from field study during 2009–2011.

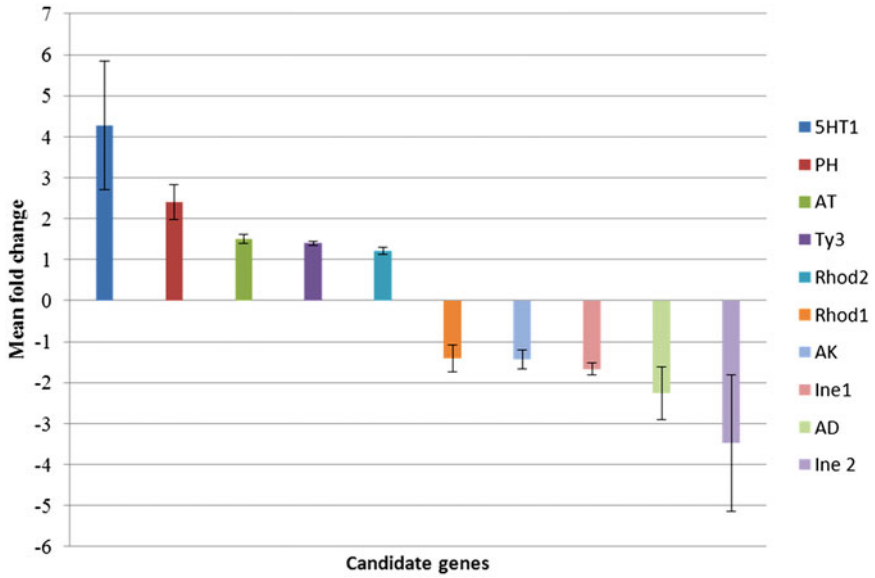

Fig. 5. Gene expression changes in *Echinogammarus marinus* induced by trematode infection in three independent trials with a mean and standard error (±1 S.E.). qPCR analysis of possible serotonin related genes using *E. marinus* head cDNA pooled ($n = 6$) control (uninfected) and infected trematode for each trial. Abbreviation: qPCR, quantitative real-time polymerase chain reaction. Bars represent mean fold change and error bars ±1 S.E.

a relationship between trematode prevalence and environmental parameters such as sea temperature ($P = 0{\cdot}135$, $R = 0{\cdot}582$, df = 1, $F = 0{\cdot}315$) or salinity ($P = 0{\cdot}282$, $R = 0{\cdot}260$, df = 1, $F = 1{\cdot}236$).

Gene expression analysis

Following candidate gene selection, ten sequences, representing genes with plausible links to neuronal pathways (Table 2), were chosen from the *E. marinus* transcriptome (Short *et al*. 2014) for expression analysis in infected and uninfected animals. Three biologically independent repeats indicated several genes present altered expression in infected animals (Fig. 5; Table 3). The expression of putative Tryptophan 5-monooxygenase activation protein gene (*Ty3*), two putative Rhodopsin genes (Rhod 1 and Rhod 2), the putative amino acid nutrient transporter gene (*AT*), the putative Arginine Kinase gene (*AK*) and the inebriated-like neurotransmitter gene 1 (*Ine1*) present no altered expression in trematode infected *E. marinus*. However, the putative serotonin receptor gene ($5\text{-}HT_{1A}$) and tryptophan hydroxylase gene (*PH*) present consistently increased expression in infected animals in each biologically independent trial, with $5\text{-}HT_{1A}$ showing the highest overall mean fold change. In contrast, the inebriated-like neurotransmitter gene 2 (*Ine2*) and the putative amino

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Table 3. Fold change in expression of putative neurological genes in trematode infected normalized *Echinogammarus marinus* to uninfected individuals

Trial	1	2	3	Mean	S.E.
5HT1	7·362	3·135	2·297	4·265	1·567
PH	2·042	3·249	1·932	2·408	0·422
AT	1·301	1·683	1·537	1·507	0·111
Ty3	1·292	1·414	1·485	1·397	0·056
Rhod2	1·301	1·277	1·050	1·209	0·080
Rhod1	−2·085	−1·094	−1·050	−1·409	0·338
AK	−1·190	−1·214	−1·892	−1·432	0·230
Ine1	−1·434	−1·611	−1·953	−1·666	0·152
AD	−3·387	−2·204	−1·182	−2·258	0·637
Ine 2	−6·727	−2·477	−1·223	−3·475	1·666

AD, acid decarboxylase gene; AK, Arginine Kinase gene; AT, acid nutrient transporter gene; Ine1 and Ine 2, inebriated-like neurotransmitter gene 1 and 2; Ty3, tryptophan 5-monooxygenase activation protein gene; Rhod2 and Rhod1, two putative Rhodopsin genes; PH, tryptophan hydroxylase gene; 5HT1, 5-hydroxytryptamine.

acid decarboxylase gene (*AD*) present reduced expression in infected animals. For the two genes that present the greatest overall fold change in infected animals ($5\text{-}HT_{1A}$ and *Ine2*), the greatest change in expression was observed in experimental repeat 1 (Fig. 5). Despite some consistency in the up or downregulation of genes, no significant differences were observed in the mean expression values between infected and uninfected samples ($P > 0{\cdot}05$).

DISCUSSION

Parasites account for a substantial portion of an ecosystem's total biomass (Kuris *et al*. 2008) and play pivotal roles in community structures and ecosystem dynamics (Holmes, 1996; Horwitz and Wilcox, 2005; Hudson *et al*. 2006; Wood *et al*. 2007; Lefevre *et al*. 2009). Parasites that are capable of manipulating their host's behaviour to enhance transmission are of particular interest to both ecologists and parasitologists (Poulin and Mouritsen, 2006; Lefevre *et al*. 2009). This study investigates one such parasite infecting a population of the amphipod *E. marinus*. The rRNA gene subunit sequences revealed the parasite to be a trematode within the Microphallidae family. Our findings suggest this newly described species can dramatically influence its *E. marinus* host population. The parasite prevalence reached almost 70% at some points of the year and was found to significantly correlate with host abundance in a negative fashion. It is thought that populations of *Corophium* amphipods infected with trematodes are prone to local collapse due to the capacity of its parasites to induce behavioural change (Damsgaard *et al*. 2005), and our findings are certainly consistent with the hypothesis that trematodes can have a major impact on amphipod populations. In addition, we found no significant relationship between trematode abundance and any recorded environmental parameter, a finding also consistent with other trematode and host population studies (Lagrue and Poulin, 2008). The large fluctuations in the prevalence of trematode infection possibly reflect a build-up of individuals infected with trematode cysts undergoing a period of development prior to reaching the infective stage, after which the parasite manipulates its host in an attempt to complete its life cycle. If successful, this manipulative strategy would cause an increase in mortality biased towards infected individuals, leading to a reduced prevalence of infection within the population. This scenario is consistent with the observed infection fluctuations in *E. marinus*. Further investigation is needed to determine the extent to which mortality results from increased predation facilitated by behavioural manipulation or the pathogenic effects of infection. However, mortality as a result of increased predation is in congruence with laboratory experiments revealing this parasite has the capacity to significantly influence *E. marinus* behaviour (Guler and Ford, 2010). In combination with other studies, our findings emphasize the potential importance of a parasite to the population dynamics of its host (Poulin and Mouritsen, 2006).

This study attempts to link the altered *E. marinus* behaviour observed in infected individuals (Guler and Ford, 2010) with the altered expression of genes with putative neurological functions. The majority of metacercariae were not found in the head region, but heavily encysted and melanized throughout the body cavity, including the hepatopancreas and encysting the ventral nerve cord. Whether this parasite can influence host behaviour from elsewhere in the hosts body (i.e. not within the head) is an interesting avenue to pursue especially given the production of serotonin throughout the ventral nerve cord (Harzsch and Waloszek, 2000) The 'neurological' sequences retrieved from the *E. marinus* transcriptome (Short *et al*. 2014) were annotated by comparison with publicly available gene sequences possessing varying levels of functional annotation. The evolutionary divergence between *E. marinus* and other arthropod species with well-

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annotated genomes means the extent of functional overlap between orthologous genes is uncertain. However, the degree of sequence similarity suggests that, even if some functional divergence has occurred, the *E. marinus* sequences likely represent genes with closely related neurological roles.

Several putative neurological genes were consistently up or downregulated across the three biologically independent repeats (e.g. putative *5-HT$_{1A}$* and *Ine2*, respectively) but with a notable extent of variation between the biological repeats. This variation may be due to several factors. Previously it has been shown that parasite load (Thomas and Poulin, 1998), age and size of parasite (Benesh *et al*. 2008), age of host (Poulin, 1993), infection by multiple parasite species (Cezilly *et al*. 2000; Haine *et al*. 2005) and seasonality (Brodeur and McNeil, 1989) can all affect the intensity of parasitic manipulation. In addition, these genes can be putatively linked to serotonin pathways. Serotonin is critical to many biological processes in invertebrates, including feeding, metabolism, moulting and reproduction (Yeoman *et al*. 1994; Fong, 1998, 2001), and the variation may reflect the involvement of these other processes. Overall, despite the variation, there is broad consistency in gene expression patterns between repeats, suggesting we have revealed the reliable alteration of 'neurological' gene expression associated with infection of *M. marinnii*. Of course, even assuming the chosen sequences represent genes critical to the regulation of host behaviour, it would still be uncertain whether the extent of altered regulation (e.g. ~4-fold in the case of *5-HT$_{1A}$*) is sufficient to induce the observed behavioural changes (Guler and Ford, 2010). However, it is known that subtle differences in gene expression can lead to substantial neurological dysfunction (Tudor *et al*. 2002) and therefore the observed expression changes in *E. marinus* may, to some extent, account for the observed behavioural phenotype.

A trend in the expression of several genes with putative links to the serotonin pathway was found in infected *E. marinus*. In all three repeats, the gene encoding a putative *5-HT$_{1A}$* receptor gene and a tryptophan hydroxylase gene (*PH*) was upregulated in the infected groups. The 5-HT$_{1A}$ receptor is a transmembrane, G-protein coupled, somatodendritic autoreceptor within the dorsal raphe neurons and mediates inhibitory neurotransmission (Hall and Wedel, 1985). The activation of serotonin 1A receptors blocks subsequent serotonin release at the axon terminal, therefore significantly influences serotonin regulation in the brain (Riad *et al*. 2000). Interestingly, the 5-HT$_{1A}$ receptor is believed to play a pivotal role in desensitization following chronic administration of SSRIs and restraints serotonin elevation (Hjorth *et al*. 2000). This raises the hypothesis that the increased expression of this gene is an attempt by the host to counter balance the chronic elevation of serotonin induced by parasite infection. The 5-HT$_{1A}$ receptor represents just a single receptor 5-HT subtype. Therefore, it would be of interest, given that various subtypes play different roles in modulating serotonin levels (Hjorth *et al*. 2000), to investigate the expression patterns of a larger range of receptor subtypes once a more complete *E. marinus* genomic resource is available. Such investigations have been attempted in the amphipod *Gammarus pulex* and suggest the 5-HT$_2$ receptor plays an important role in photic behaviour. In addition, the same study linked the histaminergic system with behavioural modulation, revealing the molecular interactions may be more complex than originally believed and suggesting potential future directions of study (Perrot-Minnot *et al*. 2013). Tryptophan hydroxylase (*PH*) is a rate-limiting enzyme that catalyses serotonin biosynthesis in the serotonergic nerves (Kim *et al*. 2002). Therefore, the upregulation of this gene could potentially increase the biosynthesis of serotonin within the host brain. This hypothesis is supported by studies that have shown upregulation of a tryptophan hydroxylase gene in rats exposed to SSRIs (Kim *et al*. 2002; Shishkina *et al*. 2007). A recent study that measured gene expression in *E. marinus* exposed to the SSRIs sertraline and fluoxetine found no significant change in the expression of *PH* (Bossus *et al*. 2014), however, gene expression levels were measured following an 8 day exposure and infecting trematode parasites have considerably longer to modulate their hosts' gene expression. Furthermore, we do not currently have a detailed mechanistic understanding of the gene pathways influenced, it is possible that SSRIs and manipulating parasites influence serotonin related pathways in different ways and therefore cause distinct gene expression profiles. Whether the upregulation of the *5-HT$_{1A}$* gene expression is in response to elevated PH levels is unclear at this stage but is a plausible hypothesis.

A slight trend in downregulation of the putative amino acid decarboxylase (*AD*) was observed in all experimental repeats. This was unexpected, as past work on gammarids infected with behaviour manipulating parasites have demonstrated increased expression of aromatic L-amino acid decarboxylase proteins (Kostadinova and Mavrodieva, 2005). Although, it should also be noted that the differential expression of aromatic L-amino acid decarboxylase was shown in gammarid species that display only altered phototaxis (Kostadinova and Mavrodieva, 2005), rather than combined phototaxic and geotaxic behavioural responses (Guler and Ford, 2010). However, as this study also finds upregulation of a putative tryptophan hydroxylase (*PH*), the downregulation, albeit small, of the *AD* gene is somewhat incongruous, as both enzymes are involved in serotonin synthesis, and requires further investigation.

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A study investigating the proteomic response of amphipod hosts to infection by behaviour manipulating parasites revealed that Arginine Kinase is induced in infected animals (Ponton *et al*. 2006). However, our study found no altered expression of the putative Arginine Kinase gene in trematode infected *E. marinus*. This difference may reflect divergent molecular strategies employed by various parasite species or a differential response by different host species. Of course, as our study measured mRNA levels, it is also possible that Arginine Kinase is post-transcriptionally regulated, leading to changes in proteins levels in infected animals that are not reflected by changes to transcript abundance.

The putative *E. marinus* inebriated-like neurotransmitter gene 2 (*Ine2*) presented consistent trend in downregulation in infected groups. *Ine2* is a neurotransmitter in *Drosophila* that resembles Na^+/Cl^--dependent neurotransmitters, a family of transporters responsible for catalysing the rapid reuptake and release of neurotransmitters, such as serotonin, dopamine and norepinephrine, into the synapse (Soehnge *et al*. 1996). These neurotransmitters appear to play important roles in regulating behaviour. Dopamine deficiency in *Drosophila* can lead to several changes in behavioural traits including phototaxis, activity levels, negative geotaxis and olfactory learning (Riemensperger *et al*. 2011). It has also been shown that inebriated mutants present defective reuptake of neurotransmitters causing over stimulation of motor neurons and oscillations of the light-induced photoreceptor potential (Huang *et al*. 2002). The extent to which the *Drosophila* and putative *E. marinus* inebriated genes share a function is uncertain, however, given the sequence similarity, it is likely the putative *E. marinus Ine2* represents a closely related neurotransmitter. Although considerable work is required to better understand the function of this gene in *E. marinus*, it is plausible that its downregulation could lead to elevated neurotransmitter levels that could conceivably be associated with the behavioural traits observed in infected individuals.

New technologies applied to the study of animal behaviour are revealing links between the underlying genome and behavioural phenotypes (Bell and Robinson, 2011). Such investigations will elucidate the genetic basis of novel behaviours and help answer important evolutionary questions about the life histories of parasites and their hosts. Furthermore, a better understanding of the pathways from genes to phenotypes will increase our knowledge of the mechanistic basis of animal behaviour. Amphipods clearly represent one of the best animal groups for the study of parasite-induced behavioural manipulation (Bethel and Holmes, 1973; Helluy, 1983*a*, *b*, 2013; Helluy and Holmes, 1990; Cezilly *et al*. 2000; Helluy and Thomas, 2003; Damsgaard *et al*. 2005; Kostadinova and Mavrodieva, 2005; Ponton *et al*. 2005, 2006; Leung and Poulin, 2006; Tain *et al*. 2007). However, in comparison with well-established model arthropod species, investigations using amphipods have been hampered by a lack of genomic resources and an inability to apply transgenic technologies. However, the progress currently being made in both areas (Rehm *et al*. 2009; Zeng *et al*. 2011; Hook *et al*. 2013; Christie, 2014; Short *et al*. 2014) will greatly facilitate research uncovering the molecular biology underlying parasite-induced behavioural manipulation.

In summary, this study found the seasonal prevalence of a newly identified species of parasite infecting an amphipod population significantly correlated with host abundance in a negative fashion. When taken together with behavioural assays (Guler and Ford, 2010) and the observed transcriptomic alterations, our findings are consistent with the hypothesis that this trematode species can alter the behaviour of its host by modulating neuronal processes, and this influence increases the likelihood of predation to the extent of causing population level effects. These findings add to the mounting evidence that parasites alter their host's behaviour in ways that promote transmission and represent some of the first links between parasite-induced behavioural manipulation of amphipods and changes in gene expression. Although the data we present here are clearly of a preliminary nature, this study has produced a foundation for a deeper understanding of the consequences of trematode infection the amphipod *E. marinus*.

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