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RESEARCH ARTICLE

Can the behaviour of threespine stickleback parasitized with *Schistocephalus solidus* be replicated by manipulating host physiology?

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ABSTRACT

Sticklebacks infected by the parasitic flatworm *Schistocephalus solidus* show dramatic changes in phenotype, including a loss of species-typical behavioural responses to predators. The timing of host behaviour change coincides with the development of infectivity of the parasite to the final host (a piscivorous bird), making it an ideal model for studying the mechanisms of infection-induced behavioural modification. However, whether the loss of host anti-predator behaviour results from direct manipulation by the parasite, or is a by-product (e.g. host immune response) or side effect of infection (e.g. energetic loss), remains controversial. To understand the physiological mechanisms that generate these behavioural changes, we quantified the behavioural profiles of experimentally infected fish and attempted to replicate these in non-parasitized fish by exposing them to treatments including immunity activation and fasting, or by pharmacologically inhibiting the stress axis. All fish were screened for the following behaviours: activity, water depth preference, sociability, phototaxis, anti-predator response and latency to feed. We were able to change individual behaviours with certain treatments. Our results suggest that the impact of *S. solidus* on the stickleback might be of a multifactorial nature. The behaviour changes observed in infected fish might result from the combined effects of modifying the serotonergic axis, lack of energy and activation of the immune system.

KEY WORDS: Manipulation, Parasite, *Gasterosteus aculeatus*, Serotonin, Fluoxetine, Oxazepam, Immunity, Fasting

INTRODUCTION

Parasites are often associated with phenotypic changes in their host (Poulin and Thomas, 1999; Thomas et al., 2011) and can be responsible for fitness-decreasing changes, including immunosuppression (Shi et al., 2015; Sitjà-Bobadilla, 2008), decreased reproductive success (Marzal et al., 2005) and reduced body size (Agnew et al., 2000). Host behaviour, personality and behavioural syndromes can also be modified by parasite presence (Wesenberg-Lund, 1931; Holmes and Bethel, 1972; Poulin, 2010, 2013; Koprivnikar et al., 2011). In many cases, host behavioural changes lower host fitness, and in some cases have been proposed or shown to increase the likelihood of parasite life cycle completion (Seppälä et al., 2006; Lagrue et al., 2007). For example, ants parasitized with trematodes show modified anti-predator responses and altered activity, which increase their risk of predation by grazers, the final hosts of the parasite (Carney, 1969). Three non-mutually exclusive hypotheses have been proposed to explain differences in behaviour between parasitized and non-parasitized animals (reviewed by Poulin, 2010). Host behavioural changes might be: (1) the result of host responses to pathology (Poulin, 2010; Adamo, 2013; Dantzer et al., 2008); (2) non-adaptive side effects of infection, such as the consequences of the energetic stress endured by the host owing to the presence of the parasite (Poulin, 1995); or (3) the result of direct host manipulation by parasites that obtain fitness benefits.

Although we often have a rich body of information on parasite-modified behaviours, the hypotheses explaining their origin are far less well understood. Testing several predictions in parallel using the same host–parasite system would strengthen our understanding of the evolutionary causes of these interactions among species. One approach to testing the predictions of each of these three hypotheses involves manipulating the physiology of non-parasitized individuals with the aim of recreating the behaviour of the parasitized host (e.g. rodents, Tan et al., 2015; gammarids, Perrot-Minnot et al., 2014). An experimental manipulation approach is appealing as it can potentially provide an insight into the nature of the connection between hosts and their parasites (Hébert and Aubin-Horth, 2014).

Our aim was to test whether any of the host-response, side effect or direct manipulation hypotheses could explain the suite of behavioural changes triggered by the internal cestode parasite, *Schistocephalus solidus* Müller 1776, in its threespine stickleback host (*Gasterosteus aculeatus* Linnaeus 1758). We attempted to use experimental treatments to induce the behavioural phenotype of parasitized sticklebacks in non-infected individuals. The parasite has a three-host life cycle, which includes a copepod, a fish and a bird (Smyth, 1946; Barber and Scharsack, 2010). Sticklebacks ingest the first intermediate host (a cyclopoid copepod) and act as the second intermediate hosts of the parasite, which grows to a large size in the body cavity of the fish. During this growth phase, the parasite shifts from being non-infective to attaining infectivity, when it is able to successfully establish and reproduce in its final host (Tierney and Crompton, 1992). The parasite life cycle is completed when a stickleback harbouring an infective worm is ingested by a suitable definitive host, typically a fish-eating bird or other endothermic vertebrate (Clarke, 1954). A suite of behaviours is changed in sticklebacks harbouring at least one parasite in the infective stage, forming a behavioural syndrome (Sih et al., 2004; Poulin, 2013). Infected sticklebacks spend less time shoaling than uninfected ones when satiated (Barber et al., 1995, 1998). They also recover more quickly after a frightening stimulus and forage at a higher rate, even under the risk of predation (Milinski, 1985; Giles,

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1987; Godin and Sproul, 1988; Tierney et al., 1993; Ness and Foster, 1999). Moreover, *S. solidus*-infected sticklebacks spend more time near the water surface, being overrepresented in surface trawls (Quinn et al., 2012). These behavioural modifications (Table 1) in the intermediate fish host can be used to test predictions that stem from each hypothesis about their cause (host response, side effect or direct manipulation). We used different treatments and predicted how each should affect behaviours with a suite of six different behaviours: activity, water depth preference, sociability, phototaxis, predator avoidance, and latency to feed (detailed in Table 1 and below).

First, we predicted that if sickness behaviour created by the host immune response plays a role in the behavioural changes, sticklebacks treated with purified lipopolysaccharides (LPS) found at the surface of gram-negative bacteria would exhibit behaviours similar to infected conspecifics (Table 1). In order to see if activation of the host immune system plays a role in the behavioural changes (i.e. sickness behaviour; Shakhar and Shakhar, 2015; Hart, 1988), we administered LPS (Pepels et al., 2004) to non-parasitized sticklebacks. LPS is known to create an *in vitro* immune response from stickleback head kidney leukocytes that is similar to the response measured by exposing the same cell line to *S. solidus* tissues (Scharsack et al., 2013). Moreover, sickness behaviours are often similar for different pathogen types (Hart, 1988; Sullivan et al., 2016).

Second, we predicted that if the side effect hypothesis is true, then fish that are subjected to nutritional stress should exhibit similar behaviours to infected fish (Table 1). Indeed, behaviour modification in a host might also arise as side effects from the energetic costs of being infected (Poulin, 1994, 2010; Thomas et al., 2011). The hypothesis that there is an energetic cost for the fish host in harbouring the *Schistocephalus* parasite is supported, for example, by significantly higher oxygen demands of infected individuals (Meakins and Walkey, 1975). In turn, this energetic deficit presumably results in behaviour changes such as avoidance to approach or reduction of predator vigilance (e.g. in zebrafish; Filosa et al., 2016), as found in food-deprived non-parasitized individuals.

Finally, we predicted that if behavioural changes in this system arise from adaptive manipulation, and that the parasites affect host behaviour through candidate physiological regulatory networks, manipulating serotonin levels and GABA receptors in non-infected fish by treating them acutely with fluoxetine or oxazepam might result in similar behaviours as infected fish (Table 1). Parasites can alter host neurochemistry, resulting in behavioural changes (Adamo, 2013). One obvious candidate in this host–parasite system is serotonin; elevated serotonin activity is found in the neural tissues (brainstem and hypothalamus) of wild sticklebacks harbouring infective *S. solidus* worms (Øverli et al., 2001). Serotonin is also known to be implicated in the variation of several behaviours in vertebrates, including geotaxis and scototaxis (Maximino et al., 2013), and feeding behaviour (Alanärä et al., 1998; Ortega et al., 2013). Furthermore, serotonin administration to non-infected amphipods (*Gammarus fossarum* and *Gammarus pulex*) is sufficient to recreate a typical infection syndrome usually caused by acanthocephalan parasites (*Pomphorhynchus laevis*, Perrot-Minnot et al., 2014). In fathead minnows, *Pimephales promelas*, selective serotonin re-uptake inhibitors (SSRIs) result in increased latency to the initiation of escape response (Painter et al., 2009) and decrease predator avoidance (Weinberger and Klaper, 2014). In zebrafish, the SSRI fluoxetine increases the time spent in the top half of the tank (Wong et al., 2013). We thus administrated fluoxetine to manipulate the serotonin physiological regulatory network. We were also interested in altering anxiety-related behaviours in host fish by targeting an entirely different mechanism using a benzodiazepine (oxazepam), which acts as an agonist of gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter (Skolnick and Paul, 1981). It increases activity and feeding rate and reduces sociability in the European perch *Perca fluviatilis* (Brodin et al., 2013).

Table 1. Summary of how the behaviours tested in the present study are affected by different treatments in previous studies of various species

Behaviour	INF	FLX	OXA	STRV	LPS
Activity	? Present study	+ Singer et al., 2016	+ Brodin et al., 2013	+ Sogard and Olla, 1997	– O'Connor et al., 2009
Water depth preference (swims at the surface)	+ Lobue and Bell, 1993	+ Singer et al., 2016; Ansai et al., 2016; Wong et al., 2013	? Present study	? Present study	? Present study
Sociability (schooling)	– Barber et al., 1995; Barber et al., 1998	– Ansai et al., 2016	– Brodin et al., 2013	– Symons, 1968	– Henry et al., 2008; Godbout et al., 2005
Phototaxis	? Present study	+ Hamilton et al., 2016	? Present study	+ Gibson and Keenleyside, 1966	? Present study
Predator response	– Milinski, 1985; Giles, 1987	– Painter et al., 2009; Sebire et al., 2015; Weinberger and Klaper, 2014	? Present study	– Croy and Hughes, 1991; Damsgird and Dill, 1998	? Present study
Feeding latency	– Giles, 1983, 1987	+ Kellner et al., 2015; Amodeo et al., 2015	– Brodin et al., 2013	– Croy and Hughes, 1991; Damsgird and Dill, 1998	+ Henry et al., 2008

INF, sticklebacks infected with *S. solidus*; FLX, fluoxetine; OXA, oxazepam; STRV, fasting; LPS, lipopolysaccharide.
The '?' symbol indicates that activity and phototaxis behaviours have never been measured before in threespine sticklebacks parasitized by *S. solidus* and that certain behaviours have not been measured in fish exposed to OXA, STRV or LPS treatments before this study. The '+' and '–' symbols indicate that behaviour is increased or decreased, respectively.

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MATERIALS AND METHODS

Model fish supply

We caught adult threespine sticklebacks from Llyn Frongoch, an upland lake (280 m altitude) in mid-Wales (52°21′N, 3°52′W) in May 2014 and brought them into breeding condition in laboratory aquaria. Numerous natural spawnings (between June and August) involving multiple males and females generated fry, which were reared in 100 l stock tanks. We fed these fish a sequential diet of Liquifry™, newly hatched *Artemia* nauplii and frozen boodworms *ad libitum* to satiation each day for 6 months, during which photoperiod and temperature conditions in the lab were adjusted each week to match natural environmental conditions. We then assigned 82 lab-bred fish (means±s.e.m.; mass 0.40±0.01 g, length 38.6±0.3 mm), randomly to five 20 l tanks (18 to 20 fish per tank), and held them at 15±1°C under a 12 h:12 h light:dark photoperiod for 3 months before testing.

Sex identification

We determined the sex of each individual using a genetic sex-linked marker (Peichel et al., 2004) prior to exposure, using a non-invasive skin swabbing and DNA extraction method (Breacker et al., 2016).

Experimental groups

In our study, we examined the behaviour of fish under seven conditions. The group of fish exposed to the parasite (EXP, n=20) resulted in two outcomes: exposed but not infected (E-NI, n=17) and infected (E-INF, n=3). Non-exposed fish were either held under constant conditions (no-treatment controls: CTRL, n=12) or under one of four treatments: treated with *E. coli* lipopolysaccharide (LPS, n=12), subjected to food witholding (STRV, n=13), treated with fluoxetine (FLX, n=13) or treated with oxazepam (OXA, n=12). Because infections established in only a portion of fish, sample sizes varied between treatments.

Experimental infections

Parasite culture

We collected *S. solidus* eggs following *in vitro* culture (Smyth, 1946; Wedekind, 1997) of a single infective plerocercoid (i.e. >50 mg; Tierney and Crompton, 1992) recovered from a naturally infected adult stickleback from Llyn Frongoch. We incubated eggs in the dark at 20°C for 6 months, then exposed them to natural daylight to stimulate hatching of the free-swimming larval stage (Scharsack et al., 2007). Laboratory-cultured copepods (*Cyclops strenuus abyssorum*) were each fed a single *S. solidus* coracidium (Smyth, 1990; and see Barber et al., 2001 for details of infection techniques). Remaining eggs that did not hatch were released into a batch culture of 100 copepods. Copepods are transparent, making it possible to view and measure the developing procercoid stage of the parasite *in vivo* (Wedekind et al., 2000; Benesh and Hafer, 2012). We screened each exposed copepod after 30 days to detect the presence or absence of a cercomer (caudal appendage of the procercoid). Although the function of the cercomer is unknown, it is a reliable proxy of infectivity to the fish host (Smyth and McManus, 2007; Hafer and Benesh, 2015). We identified 20 copepods harbouring infective procercoids: 17 were single-infected and three (from the batch exposure) harboured multiple infections.

Exposure of fish host to the parasite

We aimed for sample size of 10 infected fish. The success rate of infection is very unpredictable, even in the laboratory, and we thus exposed 20 randomly selected fish. Fish that were selected as hosts for the parasite had food withheld for 48 h prior to being exposed to infected copepods to increase the likelihood that fish ingested the infected copepod. On the day of exposure, we isolated individual fish in 1 litre plastic tanks. Each exposed fish (n=20) was fed one infected copepod and was left in its individual filtered tank for 1 week, with water changes every 48 h. Eight days after parasite exposure, we tagged fish with Visible Implant Elastomer (VIE) tags (Northwest Marine Technology, Inc.) to identify them once returned to mixed-exposure groups in the 20 l tank. Non-exposed fish were also VIE tagged. Parasites established in three of the 20 exposed fish (infection rate of 15%, see Results below).

Immersion protocol

Each day for 16 days we selected four to six fish, and one experimental treatment was randomly assigned to each fish. Individual fish were isolated for 72 h prior to the start of the behavioural experiments. We conducted static exposures in 1 litre (15 cm×8 cm×8 cm) plastic tanks. We added the appropriate treatment substance to each isolation tank on the first day of this period (CTRL, EXP and STRV fish did not receive drug treatment during this time, but were housed in otherwise identical conditions). Immersions were static-renewal with 100% water replacement at 48 h, with aeration provided by compressed air delivered through airstones.

Selection of treatment levels

Most of the treatments used in the present study have never been used in threespine sticklebacks in conjunction with behavioural trials (see Table 1). LPS exposure has been shown to significantly impact the expression of corticotropin-releasing hormone of juvenile Nile tilapia *Oreochromis niloticus* at a concentration of 12.5 mg l^{-1} over 10 days (Pepels et al., 2004). Based on these results, we chose an LPS treatment concentration of 10 mg l^{-1} (*Escherichia coli* O55:B5, L4005, Sigma Chemical Co., USA). Fluoxetine (FLX) exposure decreases fish swimming speed in medaka (*Oryzias latipes*) larvae and increases the time spent at the edges of aquaria at a concentration of 1 mg l^{-1} for 72 h (Chiffre et al., 2016). We undertook a pilot study to determine the fluoxetine exposure concentration that resulted in stickleback behaviour changes similar to those reported from other species. We tested the effects of 50, 100, 200, 1000 and 2000 µg l^{-1} of fluoxetine (fluoxetine HCl, BML-NS140, Enzo Life Sciences Inc., USA) (3 day exposure as in final experiment) and selected 1 mg l^{-1} for our main experiment. Oxazepam (OXA) exposure increases activity and decreases sociability and feeding rate of juvenile perch at the concentration of 1.8 and 910 µg l^{-1} (Brodin et al., 2013). In our pilot study involving oxazepam (oxazepam glucuronide solution, O-023, Sigma Chemical Co.) exposure on behaviour, we tested doses of 2 and 10 µg l^{-1} and selected 10 µg l^{-1} for the main experiment. Food-witheld fish (STRV) did not receive any food for 72 h. This duration was used to maintain the same treatment period as pharmacologically treated fish.

Behavioural screening

We recorded behavioural observations in an experimental aquarium (50×50×20 cm) that was wrapped in opaque, white self-adhesive foil with a small window (13×8 cm) on one side. A semi-transparent screen covering half of the aquarium area was placed 45 cm above the tank, a mini-projector (Philips PicoPix PPX2055) was placed above the screen to enable visual stimulation, as well as a webcam (Creative Live! Cam Sync HD) connected to a computer to track animals during experiments using Ethovision XT (Noldus) (see Fig. S1 for a schematic). When fish were tested, they had been food-

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deprived for ~24 h (except STRV fish, which had been subject to 72 h of food withholding).

Fish were screened sequentially for each behaviour. We transferred each fish from its treatment tank to the experimental aquarium and tested it for a specific behavioural response, before replacing the test fish in its treatment tank while the other fish were tested. When the last fish was tested, the second behaviour test started with the same order of fish, and so on until the last test (six tests in total). Each test lasted 150 s after 150 s of acclimatization (see below).

For the activity test, we recorded the time spent moving and total distance travelled. For the water depth preference test, fish were accustomed to a water depth of 15 cm prior to inserting a platform that covered half of the tank and generated a shallow area (water depth 7.5 cm). We recorded the time spent in the shallow and deep water. For the sociability test, we measured the time that sticklebacks spent within 10 cm of a laminated photograph of a conspecific shoal. To measure light intensity preferences (phototaxis), we introduced fish to a test environment where one side received direct light from a mini-projector (55 lumen), whereas the other remained in the shadow. Time spent in the brighter area and in the zone between the two areas was recorded. For the predator response test, the mini-projector projected a shadow onto a semi-opaque screen placed above the test aquarium (Yilmaz and Meister, 2013). We recorded the distance travelled before and after the 'attack', the time taken to freeze and the time spent frozen. To quantify latency to feed, we supplied food directly after the fish was transferred to the experimental aquarium. Three bloodworms were introduced with a plastic pipette into the same corner and the time taken to approach and ingest the food was recorded.

At the end of the behavioural screening tests, fish were immediately euthanized with an overdose of benzocaine anaesthetic (10 g l^{-1}). We measured the length and the mass of the fish. One fish (FLX treatment) died during the final 24 h of treatment.

Identification of worm life stage using transcription profiles

Plercocercoid infectivity is associated with behavioural alterations in the fish host. Infectivity is normally assessed on the basis of plerocercoid mass (Tierney and Crompton, 1992); however, the threshold mass for infectivity might vary between individuals and populations, as small infective worms have been reported (Dörücü et al., 2007). We thus used the transcriptome profile of individual worms to determine their life stage, instead of relying on mass. Previous transcriptomic analyses conducted on different developmental stages of *S. solidus* revealed strikingly different transcriptional signatures between non-infective and infective plerocercoids (Hébert et al., 2016a), which allows unambiguous classification of parasite infectivity. We extracted worms from the three infected sticklebacks and placed them in RNAlater (Ambion Inc., USA) at 4°C overnight, transferring them the next morning to −80°C until RNA extraction. We quantified the transcriptome of each individual worm using RNA-seq (Illumina Hi-Seq). Details of RNA extraction, sequencing library preparation and sequencing methods are described in previous work (Hébert et al., 2016b).

Statistical analysis

We performed all statistical analyses in R software version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). We assessed homoscedasticity (verified by visual assessment of fitted data against residuals) and normal distribution of behavioural data using Shapiro–Wilk and d'Agostino–Pearson tests. To compare the effect of *S. solidus* on host behaviour with the behavioural response observed in other treatments, we used a generalized linear mixed-effects model in the lme4 package (Bates et al., 2015). To account for variation between individual sticklebacks, we included mass, length and sex as fixed effects, and rearing tank as a random effect. Treatment effects are only reported when there were no effects of sex, length or mass. A linear mixed model (lmer) was used to analyze differences in distance and movement (squared transformation) during the activity test, as well as light preference. We used a generalized mixed-effects model (glmer) and simultaneous tests for general linear hypothesis (glht) for binomial distribution when we could not normalize the data to analyze the time spent in shallow water, near the shoal picture, the time before freezing, time spent frozen and feeding, as well as the distance before and after the attack. A multidimensional scaling analysis was performed on the parasite transcriptomes using the package limma-voom (Ritchie et al., 2015) to assess the infectivity status of *S. solidus* in each fish host. The analysis was based on transcriptome similarity with previously obtained data from worm plerocercoids sampled at different life stages (Hébert et al., 2016a,b).

Ethics statement

The experimental work was undertaken under the authority of a UK Home Office project license (PPL 70/8148, held by I.B.). The project was authorised by the Comité de Protection des Animaux de l'Université Laval (experimental animal use permit, certificate number 2014069-1).

RESULTS

Infection characteristics

Three out of the 20 fish (15%) exposed to the parasite became infected, with all infected fish harbouring single plerocercoids. Fish E-INF1, E-INF2 and E-INF3 were infected with plerocercoids with a respective mass of 31, 60 and 41 mg, and are represented throughout all figures with a purple triangle, a green square and a red diamond, respectively. Transcriptomes of the three parasites used in this study (infecting fish E-INF1, E-INF2 and E-INF3) revealed a typical gene expression signature associated with the infective stage (multi-dimensional scaling analysis, Fig. S2). This result suggests that all experimentally infected fish in this study harboured functionally infective plerocercoids.

Activity

There were no significant differences in the distance travelled or the time spent moving during the activity test across any of the treatments (Fig. S3A,B).

Water depth preference

The number of individuals that spent time in the shallow water varied between treatments, with 69% of fluoxetine-treated fish spending some time in shallow water, and only 8% of food-withheld fish doing so. Fish treated with fluoxetine (FLX) spent significantly more time in shallow water than E-NI (P=0.021), LPS (P=0.008) and STRV fish (P=0.007) (Fig. 1; Table S1).

Sociability

Fish spent significantly less time close to the shoal picture in the food-withheld (STRV, P=0.027), FLX-treated (P=0.041) and LPS-treated groups (P=0.028) compared with CTRL fish. E-INF fish spent more time near the picture than E-NI (P=0.031) and FLX fish (P=0.014). Most of the fish across all treatment groups avoided the shoal picture (Fig. 2; Table S1).

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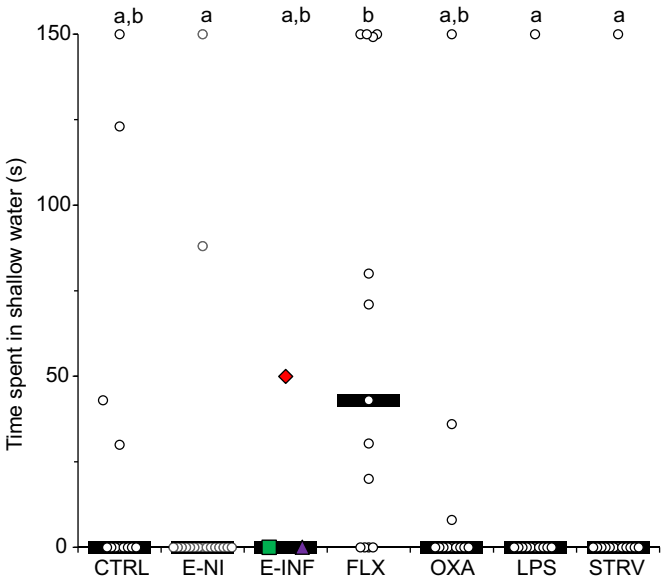

Fig. 1. Time spent in shallow water during a 150 s water depth preference test, depending on treatment. Each circle represents an individual and the black bar represents the median. Infected fish (E-INF) are shown as E-INF1 (purple triangle), E-INF2 (green square) and E-INF3 (red diamond). Significant differences are indicated by different letters (P<0.05 by glmer).

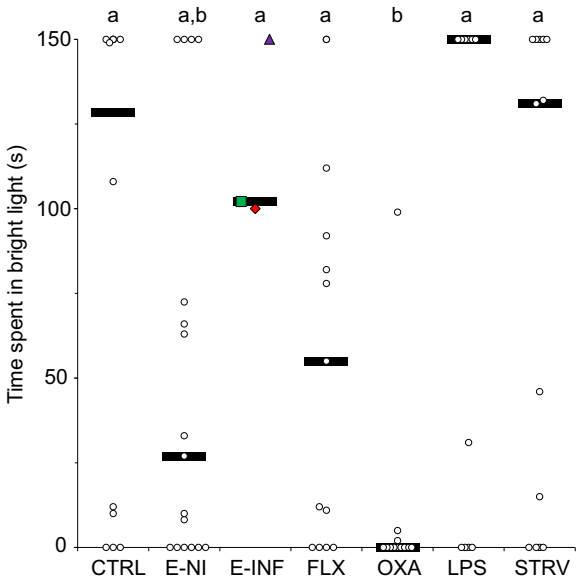

Fig. 3. Time spent in bright light during a 150 s light preference test, depending on treatment. Each circle represents an individual and the black bar represents the median. Infected fish (E-INF) are shown as E-INF1 (purple triangle), E-INF2 (green square) and E-INF3 (red diamond). Significant differences are represented by different letters (P<0.05 by glmer).

Phototaxis

The time spent on the brightly lit side of the tank was significantly higher for CTRL (P=0.002), E-INF (P=0.008), FLX (P=0.046), STRV (P=0.0007) and LPS (P=0.003) treatments than for fish treated with oxazepam (OXA) (Table S1, Fig. 3). Almost all OXA-treated fish stayed in the dark area, unlike fish in the other treatments. There were no differences between treatments in terms of time spent in the intermediate zone.

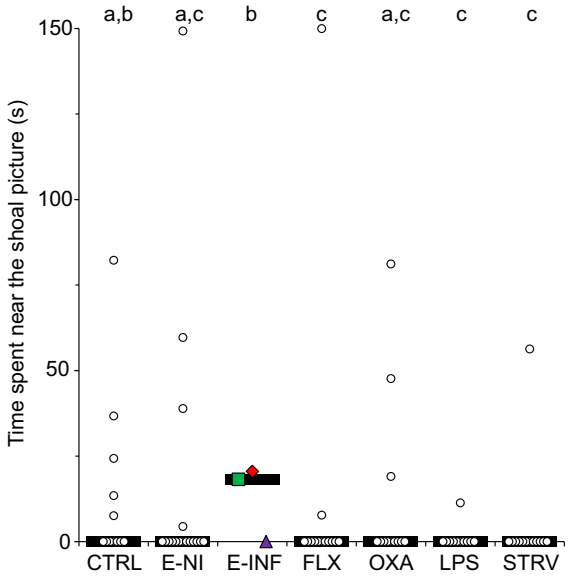

Fig. 2. Time spent near the shoal picture during a 150 s sociability test, depending on treatment. Each circle represents an individual and the black bar represents the median. Infected fish (E-INF) are shown as E-INF1 (purple triangle), E-INF2 (green square) and E-INF3 (red diamond). Significant differences are represented by different letters (P<0.05 by lmer).

Predator response

Almost all fish froze instantly after the 'attack', and there was no difference between treatments in latency to freeze (Fig. S4A). An exception was fish E-INF3, which froze 26 s after the attack. Once fish froze, most of them remained motionless; however E-NI fish and E-INF fish regained activity significantly sooner than OXA-treated fish (P=0.022 and P=0.016, respectively; Table S1). E-INF fish also stayed frozen a significantly shorter time than LPS-treated fish (P=0.033; Table S1, Fig. 4). The distance travelled before and after the 10 s attack was also analysed. Whereas there were no differences between treatments in distance travelled before the predator attack (Fig. S4B), the distance travelled by LPS-treated fish after the predator attack was significantly lower than in E-NI fish (P=0.008; Table S1), E-INF fish (P=0.018; Table S1) and FLX treatment (P=0.032; Table S1). Furthermore, OXA-treated fish travelled a significantly shorter distance than E-NI fish (P=0.031; Table S1, Fig. 5).

Feeding latency

There were no significant differences between groups in the latency to feed (Fig. S5).

The entire dataset of results is available as Table S2.

DISCUSSION

Infection-associated changes in host behaviour are often explained as parasite adaptations that increase transmission rates to final hosts and facilitate life cycle completion. However, there are plausible alternative explanations for these observations. Here, we tested predictions of three, non-mutually exclusive hypotheses that could explain changes in host behaviour in parasitized hosts: the activation of the host immune system ('host-response' hypothesis), energetic drain ('side effect' hypothesis) and the direct manipulation of behaviour by parasites. Although we were not able to replicate the behavioural syndrome characteristic of infected fish using any of the

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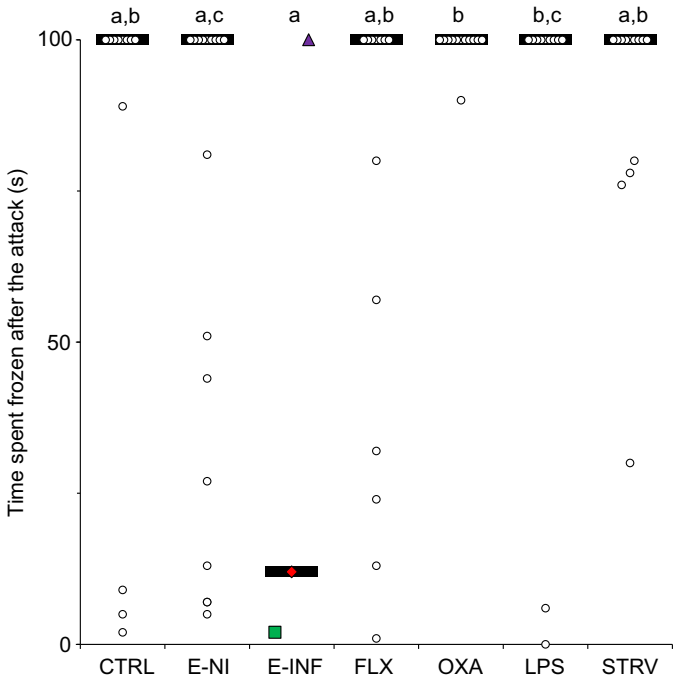

Fig. 4. Time spent frozen after an attack during the anti-predator test, depending on treatment. Each circle represents an individual and the black bar represents the median. Infected fish (E-INF) are shown as E-INF1 (purple triangle), E-INF2 (green square) and E-INF3 (red diamond). Significant differences are represented by different letters (P<0.05 by glmer).

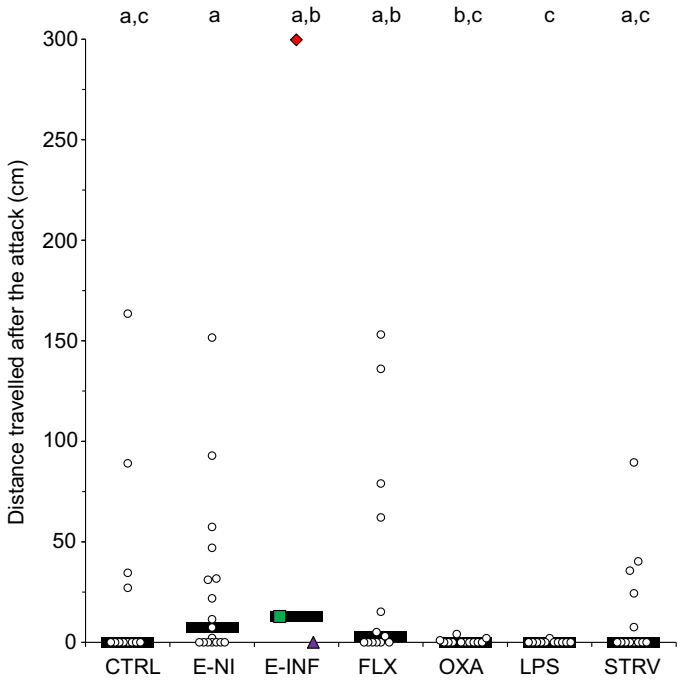

Fig. 5. Distance moved after an attack during the anti-predator test, depending on treatment. Each circle represents an individual. Infected fish (E-INF) are shown as E-INF1 (purple triangle), E-INF2 (green square) and E-INF3 (red diamond). Significant differences are represented by different letters (P<0.05 by glmer).

experimental manipulations, we were able to modify specific ecologically important behavioural axes, such as sociality.

The host immune response hypothesis

According to the host response hypothesis, behavioural changes in infected fish might arise as a consequence of immune activation. Changes in behaviour following experimental infections have been shown in many animals (Ezenwa et al., 2016; Barber et al., 2000) and might reflect 'sickness behaviours' including lethargy, depression and a reduction in maintenance behaviours, such as grooming (Hart, 1988). During its pre-infective early growth phase within its fish host, *S. solidus* presence does not induce a host leucocyte immune response (Scharsack et al., 2004) and is associated with decreased monocyte proliferation (Scharsack et al., 2007). Moreover, it has been proposed that the parasite is able to evade the innate host immune system in this early phase by adjusting its surface carbohydrate composition (Hammerschmidt and Kurtz, 2005). *Schistocephalus solidus* triggers the stickleback immune system only when it reaches an infective state (Scharsack et al., 2007), at which time behaviour is also modified. Although we found that LPS-treated fish spent significantly less time near a shoal than CTRL fish, we did not find any significant changes in the other behaviours. One possible explanation could be that fish immune systems are unable to recognize LPS (Seppola et al., 2015); however, both LPS and *S. solidus* antigens increase head kidney leucocyte activity of sticklebacks (Franke et al., 2014), suggesting that LPS does induce a response in this species. This result must nonetheless be viewed in the context of pathogen specificity of the immune response. Indeed, in mammals, a Th2 response is measured in response to a multicellular parasite, whereas a bacterium challenge predominantly results in a Th1 response (Constant and Bottomly, 1997; Mosmann and Coffman, 1989). If this is the case in the present system, LPS and *S. solidus* would not result in an entirely similar response. Therefore, whereas we expect the LPS treatment did activate the immune system in our experimental fish, our results suggest that it did not generate a sickness behaviour response that was similar to that observed among the *S. solidus*-infected fish. The type and/or the magnitude of the immune response activated by our LPS treatment was insufficient to replicate the behavioural syndrome that typifies *S. solidus*-infected fish; hence our results do not provide strong support for the host immune response hypothesis, but do not definitely invalidate it.

The energetic side effect hypothesis

Behavioural modifications that arise as side effects from the energetic costs of host infection have often been proposed (Poulin, 2010; Thomas et al., 2005; Vickery and Poulin, 2010). For example, under *ad libitum* feeding conditions, *S. solidus*-infected sticklebacks ingest more food than uninfected conspecifics (Walkey and Meakins, 1970) and the reduced liver size of infected sticklebacks strongly implies substantial energetic drain (Arme and Owen, 1967; Walkey and Meakins, 1970). Moreover, at low feeding levels, mortality is significantly higher for infected than non-infected sticklebacks (Pascoe and Mattey, 1977). We found that whereas STRV fish spent less time near the shoal than CTRL fish, they exhibited normal anti-predator responses and did not show enhanced feeding responses. It is possible that the fasting period we imposed was insufficient to generate more severe behavioural effects. The anti-predator response of STRV fish is in accordance with results from Giles (1987), who showed that non-infected fish starved for 96 h did not feed when facing a frightening stimulus when compared with infected individuals. However, starved Atlantic salmon (*Salmo salar*) increase their food intake and are bolder (Damsgird and Dill, 1998), whereas walleye pollock (*Gadus*

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chalcogrammus) increase their activity when hungry (Sogard and Olla, 1997). Because our treatment might have imposed only a mild nutritional stress, we are cautious in interpreting our results and cannot refute the side-effect hypothesis with the treatment we used.

The manipulation hypothesis

To test the explanation of a direct manipulation of host behaviour, we have to consider the potential proximal mechanisms that could be affected by the parasite (Adamo, 2013). For example, natural variation in monoamines found in the central nervous system (dopamine, serotonin, adrenaline and noradrenaline) has an influence on mood and behaviour in vertebrates, including fish. Our results show that, to some extent, manipulating serotonin levels using fluoxetine produces a similar effect to *S. solidus* on stickleback behaviour, by lowering sociability. However, this effect is not specific to manipulating serotonin levels. The treatments that activated the immune response and that increased energy deficit also resulted in a lower tendency to shoal with conspecifics. This suggests that all these causes could potentially act additively or synergistically to generate lower shoaling tendencies among infected fish and that we cannot single one out with our experiment. Furthermore, pharmacologically manipulating serotonin metabolism did not recreate the characteristic shift in anti-predator responses seen among infected individuals. Interestingly, fluoxetine did drastically increase risky behaviour in certain individuals (nine fish spent some time in shallow water in this treatment, compared with one to four fish in other treatments, see Fig. 1) but our results show that manipulating serotonin was not sufficient to replicate the behavioural syndrome that is typical of *S. solidus*-infected fish. Based on these results, we argue that modifying the stress axis through the serotonin pathway could be one of the mechanisms by which host behaviour is changed, thus supporting the direct manipulation hypothesis as one of the causes of behavioural modification in parasitized sticklebacks.

We found few differences in behaviours to be associated with OXA treatment, and no behaviours changed in the way we predicted. Although we expected to recreate the enhanced feeding rate characteristic of infected fish among OXA-treated fish – as this has been shown in previous studies in European perch *Perca fluviatilis* (Brodin et al., 2013) – this was not found. Oxazepam (Skolnick and Paul, 1981) and fluoxetine (Wong et al., 1995; Schafer, 1999) do not use the same molecular mechanism to act on the stress axis of vertebrates. Our results show that oxazepam, unlike fluoxetine, does not change any stickleback behaviour in the way in which *S. solidus* does. This suggests that if *S. solidus* does modify the stress axis of its host, it is more likely that it does this through the presence of serotonin in the brain and not by binding to g-aminobutyric acid (GABA) receptors.

Limitations and future approaches

This was a complex study, combining experimental parasitology, pharmacological exposure and behavioural testing, and a number of difficulties were encountered. Firstly, parasites only established in three of the 20 exposed sticklebacks, limiting statistical power. As a likely consequence, we did not record any significant differences in behaviours previously shown to differ between E-INF and CTRL fish (see Table 1). Comparing a group with three individuals is statistically unsatisfactory so to interpret the effects of treatments (STRV, FLX, OXA, LPS), we compared our results with what is known about behaviour changes in infected fish from the literature, rather than with the behaviour of infected fish in the current study. Secondly, we could not identify significant differences between treatments with the food intake test, even in the STRV group. One possibility is that the 72 h food withholding period might have been insufficient to elicit behavioural changes; previous studies investigating the effects of food withholding have starved fish for longer [zebrafish: 21 days (Drew et al., 2008), walleye pollock: 30–54 days (Sogard and Olla, 1997)], to see differences in transcriptomes and behaviour, respectively. Alternatively, as this was the final test, fish might have been stressed. Third, we set each treatment to the same duration (72 h), such that we had to choose drug concentrations that would give an effect during short exposures. Low concentrations of fluoxetine seem to cause effects that higher concentrations do not cause, hence the importance of the concentration–response relationship (Sumpter et al., 2014).

There are a number of points that must be considered when interpreting our results and designing future studies. First, non-specific effects could be at play in the behavioural effects seen in the fluoxetine treatment. The serotonin axis is implicated in numerous physiological responses, ranging from neuroendocrine stress response to gut contraction (Barnes and Sharp, 1999; Nichols and Nichols, 2008). Fluoxetine, for instance, causes *in vitro* contraction of gastric muscle through a cholinergic pathway (James et al., 2005) and slows gut movement in rats subjected to a force-swimming test (Xie et al., 2013). Behavioural effects of SSRI exposure have been shown in different fish species, although none of these previous studies have ruled out non-specific effects by using a separate treatment that combines exposure to a SSRI and to a serotonin antagonist to determine if it reverses the observed behavioural effects of SSRI completely (Gaworecki and Klaine, 2008; Wong et al., 2013; Hedgespeth et al., 2014; Sebire et al., 2015). Our results are thus consistent in methodology and findings with previous studies but do not rule out non-specific effects. Moreover, the parasite might act to modify behaviour (directly or indirectly) through other physiological regulatory networks than the serotonergic axis, which we did not assess. For example, evolutionary divergence between threespine stickleback populations in behaviour, including activity levels and the tendency to school, has been associated with changes in brain gene expression in the serotonergic physiological regulatory network, but also in the dopaminergic, adrenergic and glucocorticoid networks (Di Poi et al., 2016). Furthermore, candidate mimicry peptides have been identified in the *S. solidus* transcriptome, using bioinformatics analysis of sequence similarity with the fish host proteins, suggesting that these mimics could play a role in modulating physiological regulatory networks in the host, possibly resulting in phenotypic changes (Hébert et al., 2015). However, none of these mimicry candidates were included in the present study. Finally, another hypothesis that could explain some of the behavioural changes triggered by *S. solidus* infections has not yet been addressed; the possibility that the physical presence of a large parasitic mass inside the host is able to change host behaviour. Indeed, *S. solidus* can reach the same size as the host, and the parasite:host mass ratio can reach up to 94% (Clarke, 1954). Increase in parasite mass and the impact of this mass on internal organs could also be a cause of behaviour changes.

Non-mutually exclusive causes of behaviour modification

In the present study, we attempted to recreate the behavioural syndrome of *S. solidus*-infected sticklebacks by altering host physiology using different drugs or treatments. Our findings suggest that although individual behavioural components can be altered by some of the treatments, the behavioural syndrome that typically characterizes parasitized sticklebacks cannot be recreated

by the manipulation of a single variable. Behaviour is a complex phenotype. Our results indicate that the behaviours of an infected stickleback are not correlated and can be modified independently. The impact of *S. solidus* on the stickleback might therefore be of a multifactorial nature. It could be argued that the specific suite of behaviour changes observed in infected fish arises from a set of conditions: modification of the stress axis, in addition to the energetic drain and the activation of the immune system caused by the parasite. Now that we have been able to retrieve some parts of the behaviour modification with those different conditions, it would be of great interest to use these treatments in combination to attempt to recreate the entire behavioural syndrome of *S. solidus*-infected sticklebacks.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
L.G. designed the study with input from N.A.-H. and I.B. L.G. and C.S.B. pretested treatment concentrations. L.G. performed the manipulation experiments under the supervision of the licence holder (I.B.), carried out the statistical analysis and extracted RNA from the worms. F.-O.H. performed the transcriptomic analyses to confirm the infectivity status of the worms. I.B. provided experimental aquarium and laboratory facilities. L.G. and N.A.-H. drafted the manuscript with input from F.-O.H., C.S.B. and I.B.

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