The secretome of a parasite alters its host's behaviour but does not recapitulate the behavioural response to infection

Running head: Secretome behavioural effects

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ABSTRACT

Parasites with complex life cycles have been proposed to manipulate the behaviour of their intermediate hosts to increase the probability of reaching their final host. The cause of these drastic behavioural changes could be manipulation factors released by the parasite in its environment (the secretome), but this has rarely been assessed. We studied a non-cerebral parasite, the cestode *Schistocephalus solidus*, and its intermediate host, the threespine stickleback (*Gasterosteus aculeatus*), whose response to danger becomes significantly diminished when infected. These altered behaviours appear only during late infection, when the worm is ready to reproduce in its final avian host. Sympatric host-parasite pairs show higher infection success for parasites, suggesting that the secretome effects could differ for allopatric host-parasite pairs with independent evolutionary histories. We tested the effects of secretome exposure on behaviour by using secretions from the early and late infection of *S. solidus* and by injecting them in healthy sticklebacks from a sympatric and allopatric population. Contrary to our prediction, secretome from late infection worms did not result in more risky behaviours, but secretome from early infection resulted in more cautious hosts, only in fish from the allopatric population. Our results suggest that the secretome of *Schistocephalus solidus* contains molecules that can affect host behaviour, that the causes underlying the behavioural changes in infected sticklebacks are multifactorial, and that local adaptation between host-parasite pairs may extend to the response to the parasite's secretome content.

37 # INTRODUCTION

38 Parasites have strong impacts on the phenotype of their hosts, including modifications of
39 the host morphology (1), physiology (2), immunity (3) and behaviour (4). Parasites with
40 complex life cycles have been proposed to manipulate the behaviour of their hosts, in
41 order to increase their transmission rates to their final host (5). If parasites are
42 manipulative, they should disturb the functioning of their host central nervous system and
43 physiological systems (hormonal and immune systems) by the production, storage, and
44 release of molecules called manipulation factors (6-7). Parasites release a wide variety
45 of molecules into their external environment, including nucleic acids (8) and proteins (9),
46 which are sometimes included in extracellular vesicles (10). These molecules are
47 collectively referred to as the secretome. While the effects of exposing hosts to the
48 secretome of their parasite on their immune response has been demonstrated in some
49 host-parasite pairs (11, 12), there is currently no experimental evidence that the
50 secretome content from a parasite directly affects behaviour in the host in the same way
51 that the whole parasite infection does (13).

52

53 We studied the effect of the parasite secretome on host behaviour using the interaction
54 between the threespine stickleback and the parasite *Schistocephalus solidus*.
55 *Schistocephalus solidus* is a freshwater cestode that is trophically transmitted to three
56 hosts (14). It enters the body cavity of its first host, a copepod, after being ingested, and
57 then negatively affects its growth and reproduction (15). In the copepod, the expression
58 of risky behaviours shows a pattern of “protection then facilitation” (16-17): infected
59 copepods exhibit reduced activity in the early phase of infection, then increased activity
60 and a changed swimming pattern, leading to higher detection and ingestion by the second
61 host, the threespine stickleback (18). Infection in this host results in changes in
62 morphology, physiology, immune response and behaviour (19). The worm life cycle is
63 completed when it reaches its final avian host. (19).

64

The parasite's life stage within the body cavity of the threespine stickleback is characterised by two phases with distinct phenotypes in both the parasite and its host. During the early days of infection (the non-infective phase), the worm shows high growth and is too small to mature if switched to its final bird host (20). The host does not show the expected adaptive immune response (21) and no behavioural changes are known (22). At the infective phase, the worm is ready to reproduce in its final avian host (20). The transition from the non-infective to the infective phase is characterized by major genome-wide reprogramming events in the worm, including the activation of genes predicted to be involved in neural pathways and sensory perception (23). In this infective phase, drastic behavioural changes that result in a loss of the anti-predator response appear in sticklebacks (22). Compared to non-infected individuals, they are more exploratory (24), less anxious (25) bolder in the presence of a predator (26), they recover more quickly from a frightening overhead stimulus, and feed following the stimulus (26). These behavioural perturbations could result in part from the combined effects of modifying the serotonin pathway and the immune response in infected fish (21, 27, 28). At the infective phase, secretome collected from *S. solidus* has been shown to modulate the stickleback immune response *in vitro* (29). Whether this modulation of the immune system is also involved in behavioural changes is unclear (27, 28).

Altogether, the timing and the nature of these changes in both host and parasite support the hypothesis of a manipulation of the threespine stickleback by *Schistocephalus solidus* to reach its final avian host. The tegument of *S. solidus* is known to include vesicles (30). However, whether the worm secretes any molecule that can be classified as manipulation factors that are able to alter fish behaviour is unknown (7). This system offers a unique opportunity to functionally test if the secretome is responsible for the behavioural changes. We predict that only exposure to secretome from worms at the late, infective phase should induce behavioural perturbations similar to what is seen in infected sticklebacks, and not exposure to secretome from worms at the early, non-infective phase.

The interaction between *S. solidus* and the threespine stickleback is highly specific, since it is the only fish host that this parasite can infect (31). *S. solidus* infects freshwater sticklebacks (19) and a combination of studies of wild populations and of experimental reciprocal cross infection data suggests local adaptation between sympatric ("native") host-parasite pairs (32-34). On the other hand, marine sticklebacks are rarely exposed to *S. solidus*, as the worm does not tolerate high salinity levels when its hatches (35). Potentially reflecting the lower probability of co-evolution between these allopatric ("naïve") marine fish and *S. solidus*, infection success of experimental infections is highly variable, ranging from 1.4% to 99% depending on the population (34, 36). Marine fish infected by *S. solidus* show morphological changes (37) and the immune response of allopatric fish exposed *in vitro* to *S. solidus* antigens at the infective phase differs from the one of sympatric sticklebacks (38). However, the effects on behaviour of infecting an allopatric stickleback with *S. solidus* have never been studied.

The occurrence of threespine stickleback populations from freshwater and marine environments and their variation in infection susceptibility allow us to functionally test if *S. solidus* and its secretome co-evolved with its sympatric freshwater host to specifically modify its behaviour. Strong co-evolution between a manipulative parasite and its sympatric host was previously reported in the *Ophiocordyceps*-ant system, where *ex vivo* essays demonstrated that the fungus *Ophiocordyceps* secretes a specific array of metabolites only in presence of the host brain it has specifically evolved to manipulate (39). We had two opposite but plausible predictions according to the manipulation hypothesis: the first one was based on the observation that marine fish can be highly susceptible to *S. solidus* infection (34). If allopatric sticklebacks do not have adaptive mechanisms to fight against the secretome effects because they did not evolve with the parasite, we predicted that they would be more sensitive to the secretome than the sympatric population and would show stronger behavioural changes. The second prediction was that marine fish would activate their immune response earlier than

sympatric fish following infection, which would reduce the effects of secretome on fish physiology and behaviour (40). In this case, we predicted that the allopatric population would be less sensitive to the secretome from the infective phase than sympatric fish and that they would show reduced or no behavioural changes. In both cases, we predicted that the injection of secretome from the non-infective phase would not modify behaviour of allopatric fish.

The objective of this study was to test if the secretome of a parasite located outside the brain is sufficient to induce behavioural changes in its vertebrate host. To reach this objective, we used a functional approach (Figure 1).

MATERIALS AND METHODS

Collecting secretome from the non-infective and infective phases

The collection of the secretome from *S. solidus* was performed according to a protocol adapted from (41). We caught threespine stickleback fish from Lake Témiscouata (Québec, 47°80' N 68°87' O), in June and July 2016 using minnow traps. One day after fishing, fish (n=41) were euthanized with an overdose of MS-222 (75 mg/L mg/kg) and dissected to determine if they were infected by *S. solidus*. Fish sex, size and mass, and *S. solidus* mass and number in each fish were noted. Twenty-eight fish were infected by worms, 8 at the non-infective phase (worm mass ≤ 50 mg) and 20 at the infective phase (worm mass > 50 mg;). Following dissection, each *S. solidus* worm was washed to remove fish blood and placed in a saline solution protected from light to collect its secretome for 2 hours (see Supplementary material). The secretome was stored at -20°C. For subsequent injections, we only used secretomes sampled from fish harbouring a single worm (n=21, including 7 non-infective secretomes and 14 infective secretomes) in order to remove potential effects of multiple infection on secretome content and function.

150 **Sampling of sympatric and allopatric fish hosts**

151 We performed the injections in two populations of threespine sticklebacks. The first
152 population, from Lake Témiscouata, Québec, was composed of fish that lived in sympatry
153 with the worms used to obtain secretome samples. The second population was composed
154 of fish from a salt march at Isle-Verte, Québec (48°00' N 69°40' O; salinity 22-26 ppt),
155 which thus lived in allopatry with the worms used to obtain the secretome. Fish in this
156 population are anadromous, meaning that they live in saltwater and migrate to the lower
157 salinities of the salt marsh to reproduce. Sticklebacks in this population are not expected
158 to be infected by *S. solidus,* because the worm does not tolerate high salinity (42).
159 Detailed sampling in Supplementary material.

160

161 **Experimental treatments**

162 We used four treatments for each population: an injection control treatment, an "infective
163 secretome" treatment, a "non-infective secretome" treatment and a "denatured infective
164 secretome" treatment. For these four treatments, we used healthy non-infected fish from
165 the two populations. In addition, in the Témiscouata population, we added a group of fish
166 infected by *S. solidus* that were not injected as a positive control, to validate that the
167 behavioural test could discriminate between healthy and *S. solidus* infected fish (n=7).
168 The control treatment involved fish injected with phosphate-buffered saline (PBS), to test
169 handling effects on fish behaviour. The three other treatments involved injections of
170 secretome collected in Lake Témiscouata. For each of these three treatments, we
171 prepared a mixture of secretome that insured sufficient volume for injections, for a total
172 of 3 mixtures ("infective secretome", "non-infective secretome" and "denatured infective
173 secretome"). For each treatment, we used one secretome mixture from 7 worms to
174 remove potential individual effects from a specific worm: if a worm secreted proteins with
175 aberrant effects on fish behaviour, then their effects would be more likely to be buffered
176 by the proteins secreted by the other six worms. For each treatment, the same mixture
177 was injected in fish from the Témiscouata and the Isle-verte populations, which allowed
178 to compare behavioural effects between them. Each mixture was made of 1600 µL of

179 secretome from each worm. The infective secretome treatment included fish injected with
180 secretome from worms whose weight was above 50 mg (the mass at which the worm
181 becomes infective (19)). Mass of the worms ranged from 80 mg to 700 mg with a mean
182 of 368.6 mg. The non-infective secretome treatment used fish injected with secretome
183 from worms whose weight was equal to or under 50 mg. Worms had a mass around 10
184 mg (minimal value detected by the balance) to 50 mg with a mean of 27.1 mg. This
185 treatment was used as a control for which behavioural changes were not expected. The
186 denatured secretome treatment used fish injected with secretome from worms whose
187 weight was above 50 mg, but for which the proteins included in the secretome were
188 denatured by heat at 95°C for 20 minutes before each injection. This treatment was also
189 added as a negative control, as proteins lose their secondary and tertiary structures
190 following heat treatment, and consequently their biological activities. If we found
191 differences in behaviours between fish injected with the intact and the denatured
192 secretome from large worms, this would suggest that the activity of proteins included in
193 the secretome is involved in the behavioural alteration. Worm mass for this last treatment
194 was comprised between 170 mg and 730 mg with a mean of 464.3 mg. Each treatment
195 was composed of n=11 fish in each population, except for the infective secretome
196 treatment in the Témiscouata population (n=10). We verified protein concentration and
197 content of the secretome mixtures using a Bradford assay and SDS-Page
198 (Supplementary material, supp. Fig 1).

199

200 **Injection protocol**

201 At the beginning of the protocol, we isolated 51 fish from the Témiscouata population and
202 44 fish from the Isle-Verte population in 2-L tanks and assigned an identification number
203 (ID). The first day following isolation, we verified infection by *S. solidus* using the
204 environmental DNA method (43). Forty-four fish from the Témiscouata population were
205 detected as non-infected and could be used for injections, while 7 fish were infected by
206 *S. solidus* and were used as control infected fish. As expected, none of the fish from the
207 allopatric population was infected by *S. solidus* and all could be used for injections.

We randomly assigned each fish to an injection treatment (PBS, “infective secretome”, “non-infective secretome” or “denatured infective secretome”). During the first week (control week), we inserted a needle into the abdominal cavity of each fish (no liquid injected), once a day for four days. On the 4th day, we tested behaviour of each fish between 30 and 45 minutes after manipulation. During the second week (injection week), we injected each fish in the abdominal cavity with 100 µL of the appropriate treatment, once a day for four days (total of 400 µL injected per fish). On the 4th day, we tested behaviour of each fish between 30 and 45 minutes after the last injection. Denatured secretome was heated at 95°C for 20 minutes and was cooled before each injection. Infected fish used as a positive control were exposed to the same protocol, except that during the injection week the needle was only inserted. Fish were not anesthetized, as manipulation took less than 1 minute and induced minor stress to fish. We fed fish daily with brine shrimps following needle insertion or injection, except on day 4. Each fish was given 3 days of rest between the control and the injection weeks. One fish for the infective secretome treatment of the Témiscouata population died between the two weeks and was removed from analysis.

Behavioural tests and analysis

The behaviours of each fish were measured in a 45 L test aquarium using standard tests to measure exploration, thigmotaxis and boldness in threespine sticklebacks (22, 25, 27) (see Supplementary material). Statistical analyses were performed using the R software version 3.5.0 (44). See supplementary material for additional information on statistical tests.

RESULTS

Behaviours that differentiated infected and non-infected fish

235 We measured seven behaviours in order to quantify exploration, thigmotaxis (wall-
236 hugging tendency (25)) and boldness in infected and non-infected fish. We found that in
237 the sympatric population, infected and non-infected fish differed significantly in two
238 behaviours associated with boldness: latency to feed before a predator attack and time
239 spent frozen after a predator attack. Infected fish ate two times more rapidly before a
240 predator attack (average measured in control week: 113 sec) than non-infected fish
241 (average measured in control week: 224 sec) ($p = 0.049$) (Figure 2 panel 1.A). Infected
242 fish almost did not freeze after the predator attack (average measured in control week: 6
243 sec) while non-infected fish froze for a longer time (average measured in control week:
244 107 sec) ($p = 0.009$) (Figure 2 panel 1.B). For the other five behaviours, infected fish did
245 not differ significantly from non-infected fish, although trends were apparent (Figure 2,
246 panel 2).

247

248 **Behavioural effects of secretome injections in non-infected fish from two**
249 **populations of threespine sticklebacks**

250 Sympatric population: Lake Témiscouata

251 We tested behaviour twice in each individual: during the control week and during the
252 injection week. We first tested control non-infected individuals in which we injected only
253 a saline solution, in order to quantify any changes related to the disturbance of the
254 experiment in these two weeks. For all behaviours assessed, we did not find any
255 significant differences between the two weeks in these control fish, suggesting that
256 handling and injections did not significantly affect them. Similarly, infected fish did not
257 change their behaviour between the first and second tests (Supp Tables 1 to 7).

258

259 We were not able to reproduce the typical behaviours of infected fish using secretome
260 injections from the infective phase when injecting healthy individuals from the
261 Témiscouata freshwater population (Supp Tables 1 to 7, Supp Figures 2 to 7). For
262 example, in this sympatric population, none of the secretome injections had significant

263 effects on feeding latency before a predator attack, while it did affect fish from the
264 allopatric population (see below). Sympatric fish injected with the infective secretome did
265 not significantly change the time they took before eating between the two trials ($p = 0.975$,
266 Supp Table 4, figure 3 panel 1.C). The same was observed in fish injected with the non-
267 infective secretome ($p = 0.852$, Supp Table 4, figure 3 panel 1.D) and following injection
268 of denatured secretome ($p = 0.526$, Supp Table 4, figure 3 panel 1.E).

269

270 Allopatric population: The Isle-Verte salt marsh

271 In the allopatric population, we first tested control individuals in which we injected only a
272 saline solution, in order to quantify any changes related to the disturbance of the
273 experiment in these two trials. For all behaviours assessed, we did not find any significant
274 differences between the two weeks in these control fish, suggesting that handling and
275 injections did not significantly affect them (Supp Tables 8 to 14). For example, the control
276 non-infected fish had similar latency to eat before the predator attack in the first and
277 second weeks ($p = 0.762$, Supp Table 11) (Figure 3 panel 2.A).

278

279 We found that behaviour was changed after secretome injections in the allopatric
280 population in some treatments. The latency to eat before a predator attack was not
281 changed after injection of the infective secretome in healthy individuals ($p = 0.642$, Supp
282 Table 11, figure 3 panel 2.B). However, fish injected with the non-infective secretome
283 significantly increased their latency to approach food, taking more than twice longer
284 before resuming feeding ($p < 0.001$, Supp Table 11, figure 3 panel 2.C). After injection of
285 denatured secretome, fish significantly delayed or refrained entirely from eating before
286 the predator attack compared to the control week ($p=0.025$, Supp Table 11, figure 3 panel
287 2.D). We also found that allopatric fish took significantly longer to resume feeding after a
288 predator attack following injection of the non-infective secretome ($p=0.037$, Supp Table
289 14). After a predator attack, they stopped feeding entirely during the injection week (Supp
290 Figure 7 panel 2.C). This behaviour was not significantly affected in any other treatments

291 (Supp Table 14, supp figure 7 panel 2). Additionally, we found that allopatric fish
292 significantly spent more time swimming after injection of denatured secretome (p=0.016,
293 Supp Table 9), while other secretome treatments did not affect this behaviour (Supp
294 Figure 3 panel 2.D). None of the other behaviours showed changes after injections of
295 secretome in this population (Supp Tables 8, 10, 12 and 13 and Supp Figures 2 to 7).

296

297 **DISCUSSION**

298 It has been proposed that parasites manipulate the behaviour of their host by secreting
299 molecules (*i.e.* manipulation factors) that ultimately impact the physiology and the
300 behaviour of their host. Here, we tested if the secreted molecules of *S. solidus* could be
301 responsible (entirely or in part) for the behavioural changes in infected sticklebacks, by
302 injecting secretome in fish from a native, sympatric population and from a naïve, allopatric
303 one. We were not able to recreate the typical risky behaviours of infected fish in healthy
304 individuals using solely the secretome of *S. solidus* from the infective phase. However,
305 the secretome of the parasite was sufficient to significantly alter two risk-taking
306 behaviours, the latency to feed before and after a predator attack, in fish injected with the
307 secretome from early infection worms, but only in the allopatric fish population that did
308 not evolve in presence of the parasite.

309

310 The lack of behavioural change in fish injected with infective secretome can be interpreted
311 in two ways: either the secretome is not sufficient to alter behaviour, or our protocol did
312 not recreate the biological alterations that happen in the host during the weeks of
313 infection. In both populations, fish injected with the infective secretome did not differ in
314 behaviour from the control non-infected fish, despite the fact that proteins were detected
315 in the secretome. It is possible that *S. solidus* does not act on the behaviour of its host
316 through its secretome. If such is the case, the probability that this host behaviour alteration
317 is the result of active manipulation by the parasite would be low. If this is the correct
318 interpretation, behavioural changes would be the result of a side effect of infection, such

319 as the parasite mass burden, or the result of a host response (25). However, it would be
320 improbable to observe the significant changes in behaviour we quantified when injecting
321 the non-infective secretome. Moreover, as our behavioural assay could detect significant
322 differences in latency to feed before a predator attack between infected and non-infected
323 fish, we expect that we also had the statistical power to detect differences in the infective
324 secretome treatment.

325

326 An alternative explanation would be that our exposure protocol was not sufficient to test
327 our prediction. First, it is possible that we did not reach a threshold level of manipulation
328 factors, as it has been proposed that they may be accumulated slowly and stored by the
329 parasite (7). Because secretomes were sampled from the wild, the amount of secretome
330 collected was limited by the number of fish caught that were parasitized by infective *S.*
331 *solidus*. We thus injected a small dose every 24 hours for 4 days. In contrast, if
332 manipulative, *S. solidus* probably secretes molecules continuously within its host.
333 Increasing the dose and exposure duration should definitely be tested in order to better
334 understand the role, if any, of the parasitic secretome in host manipulation. Second, a
335 potential explanation is that *S. solidus* may secrete molecules during the non-infective
336 phase that would act to "prime" the host (13). One could test the importance of the
337 interaction between the effects of molecules secreted in the non-infective and infective
338 phases in inducing behavioural changes by sequentially injecting a fish with the non-
339 infective and infective secretomes. Third, our protocol did not include the potential
340 combined effects on behaviour of the secretome and of the physical deformations induced
341 by the worm in the fish abdominal cavity. This could be tested by simultaneously injecting
342 the infective secretome with silicone, which was previously used to re-create the parasitic
343 mass burden in non-infected fish (25). Finally, it is also possible that the infective
344 secretome acts in combination with molecules of the stress response, which was
345 previously proposed to be involved in the host's behavioural changes (27, 28). A next
346 step will require to adopt an integrative functional approach to simultaneously test the
347 effects of the secretome, of the activation of the stress response, and of the parasitic
348 mass burden on host behaviour.

349

350 The significant increase in latency to eat before and after a predator attack induced by
351 the secretome from worms at the non-infective phase is similar to the altered behaviour
352 seen in infected copepods, the first intermediate host of *S. solidus.* The copepod host
353 exhibits a behaviour pattern of "protection then facilitation", first showing reduced activity
354 in the early phase of infection, presumably reducing detection by predators, then an
355 increased activity, which leads to higher detection by threespine sticklebacks (16-18). The
356 induced behavioural perturbations in sticklebacks are surprising, as behavioural changes
357 have not been reported in infected sticklebacks when *S. solidus* is in the early non-
358 infective phase (22). It is possible that the same shift in behaviour exists in the fish host
359 but has gone undetected. Using single experimental infection in 5 female sticklebacks,
360 Barber et al. tracked changes in their antipredator behaviour for 16 weeks post-parasite
361 exposure and reported significant behaviour changes only when the worm reached 50
362 mg (22). This could be explained in part by their low sample size, and testing more fish
363 infected by worms at the non-infective stage could provide a better understanding of the
364 potential behavioural sequence of "protection then facilitation". We can hypothesize that
365 the secretome contributes to the worm protection at the non-infective stage, while other
366 mechanisms (including the host stress response and the parasitic mass burden) could
367 act independently or in combination with the infective secretome to alter behaviours that
368 facilitate worm transfer to the final host. Also, it is important to keep in mind that
369 secretomes were sampled from naturally infected fish, which prevented to precisely
370 estimate the developmental stage of each worm. For example, small worms (≤ 50 mg),
371 which we considered non-infective, might have grown more slowly because they invested
372 more resources into molecules responsible for behavioural changes. Finally, the
373 divergence in behavioural effects between the non-infective and the infective secretomes
374 suggests that the secretome content between these two phases is different. In
375 accordance with our results, the analysis of the transcriptome of *S. solidus* previously
376 demonstrated that the genes expressed by the worm in the non-infective and infective
377 phases were associated with contrasting biological functions, with thousands of genes
378 differing in activity (23). However, it is also possible that behavioural differences result
379 solely from concentration effects, if non-infective worms produced less concentrated

secretomes compared to infective worms when their secretions were collected in a saline solution of constant volume. Analysing the nature of the protein content of the *S. solidus* secretome at each stage would provide functional information that could shed light on the nature of the molecular interaction between the threespine stickleback and *S. solidus*.

Studies in several host-parasite pairs from different branches of the tree of life have shown that behavioural alteration in the host affects specific behaviours rather than disrupting the host response to all cues (5, 7, 19, 45). Not all behaviours were altered by the non-infective secretome in our study. The specificity of effects on cautiousness in presence of a predator, but not on exploration of a novel and risky environment, or of anxiety-like behaviour such as wall hugging, is in accordance with previous studies that have shown that not all behaviours are modified in infected sticklebacks (45) and that not all behaviours are changed when healthy sticklebacks are experimentally manipulated to behave like infected ones (27). Indeed, manipulating serotonin levels in healthy sticklebacks to mimic the changes observed in the brain of infected ones lead to changes in schooling and the tendency to swim close to the surface, but not the response to a predator attack, which was significantly altered in the opposite direction to an infected fish in the present study (27). This result points to a probable multifactorial nature of host altered behaviours, as no experimental manipulation leads to changes in all the behaviours observed in *Schistocephalus* infected fish. Here, the non-infective secretome exposure resulted in more cautious fish that took longer to feed under risk. Identifying the content of the secretome will potentially uncover molecules that affect feeding directly or indirectly, as found in another host-parasite system where feeding is affected (13).

Our results show that the secretome of *S. solidus* had significant effects on fish risky behaviour only in an allopatric (naïve) population. This result is in accordance with the hypothesis that since allopatric sticklebacks did not evolve with *S. solidus*, they do not possess the adaptive “generalised response” against *S. solidus* and its effects on fish physiology and behaviour, as proposed for this parasite and others (34, 46). Local

409 adaptation and potential co-evolution between the threespine stickleback and *S. solidus*
410 was previously suggested with cross-infection studies (33, 43, 63). Our results point to
411 the need for further studies on local adaptation in this host-parasite pair, by including
412 secretome exposure of fish that have evolved in the presence of another worm genotype,
413 *i.e.* sticklebacks of freshwater origin that can recognize the parasite but did not co-evolve
414 specifically with that genotype (34, 47). For instance, non-infected freshwater sticklebacks
415 from the west Coast of Canada (known to be infected by *S. solidus* (34)) could be exposed
416 to the secretome from Lake Temiscouata worms.

417

418 The significant increase in the latency to eat before an attack, which was reported after
419 the injection of the non-infective secretome in the allopatric population, was also found in
420 fish injected with heat-denatured secretome, suggesting that these impacts on behaviour
421 were not related solely to the activity of proteins. Indeed, since these secretome samples
422 were denatured by heat in order to lose the secondary and tertiary structures of proteins,
423 and consequently their biological activities, it is possible that this behavioural change
424 might be induced by other molecules included in the secretome that were not impacted
425 by the heat treatment. It is likely that *Schistocephalus* secretes and/or excretes other
426 molecules, such as extracellular vesicles including micro-RNAs (11). These molecules
427 could have their biological activities reduced in the presence of the active secretome
428 proteins, and heat treatment possibly revealed their actions. To confirm this hypothesis
429 in the future, we could replace the heat treatment of secretome by a protease exposure,
430 in order to specifically break down and inactivate proteins in the secretome. Heat
431 treatment and protease exposure could also be applied to the non-infective secretome to
432 better understand the role of proteins and other molecules in the reported behavioural
433 change.

434

435 In this study, secretome alone did not recapitulate the behavioural changes of infected
436 sticklebacks in non-infected sticklebacks. Yet, the effects of secretome from the non-
437 infective phase on boldness in the allopatric population suggest that there may be a

438 “protection then facilitation” effect at play in the threespine stickleback host, as seen in
439 the first copepod host. Our findings demonstrate that the secretome of *S. solidus* is an
440 important component of the molecular cross-talk between the parasite and its threespine
441 stickleback host.

442

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Author contributions

C.S.B. designed the study with input from N.A.H.. C.S.B. performed the experiments. C.S.B. analysed the data with input from N.A.H.. C.S.B. and N.AH. wrote the manuscript.

Ethics

This study was approved by the Comité de Protection des Animaux de l'Université Laval (CPAUL 2014069-3).

Data availability

The complete dataset is available as a supplementary tab-delimited .txt file. Data on seven behaviours measured during the control and treatment weeks, mass (g), size (mm) and sex of each fish are reported, as well as the infection status (no=0; yes=1), the number and the total mass of *S. solidus* (g) for individual fish in each treatment.

Electronic supplementary material

A) Supplementary tables. Results of all the statistical comparisons performed with the lmer function to determine if the secretome injections change the behaviour of non-infected fish of the Témiscouata population and of the allopatric population are presented. Results are reported in separate tables for each of the seven behaviours and for each population (supplementary tables 1 to 14). B) Supplementary figures. Seven supplementary figures are presented. C) Supplementary information on methods.

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605

606

607 # FIGURES

608

609 **Figure 1. Experimental design used to determine if the secretome of a parasite is**
610 **sufficient to induce behavioural changes in its vertebrate host.** Secretomes from the
611 non-infective and infective phases of *S. solidus* were injected in non-infected sticklebacks
612 from a sympatric and an allopatric population. Only injection of the infective secretome
613 was predicted to induce behavioural changes. Saline (PBS), non-infective secretome, and
614 denatured secretome treatments were used as controls for which no behavioural changes
615 were expected. In the sympatric population, infected fish were also tested to validate that
616 the behavioural tests could discriminate between healthy and *S. solidus* infected fish.

617

618 **Figure 2. Two boldness-related behaviours differ between *S. solidus*-infected and**
619 **non-infected sticklebacks. 1.** Behaviours that were significantly different between
620 infected and non-infected sticklebacks ($p < 0.05$). A. latency to feed before a predator
621 attack (sec). B. time spent frozen after a predator attack (sec). **2.** Behaviours that were
622 non-significantly different between infected and non-infected sticklebacks. A. latency to
623 leave the acclimation zone (sec). B. time spent swimming (sec). C. time in the centre
624 (sec). D. time spent fleeing after a predator attack (sec). E. latency to feed after a predator
625 attack (sec). Each comparison was performed between infected fish and non-infected fish
626 (from the saline (PBS) group) of the control week. Black lines represent the median, the
627 green triangles the average, and each dot one individual.

628

629 **Figure 3. Injections of non-infective secretome significantly lower the expression**
630 **of boldness-related behaviours, only in fish from the allopatric population. 1.**
631 Latency to feed before a predator attack (sec) in the Témiscouata population. A. Infected
632 fish. B. Fish injected with control saline (PBS). C. Fish injected with infective secretome.
633 D. Fish injected with non-infective secretome. E. Fish injected with denatured secretome.
634 **2.** Latency to feed before a predator attack (sec) in the allopatric population. A. Fish

635 injected with control saline (PBS). B. Fish injected with infective secretome. C. Fish
636 injected with non-infective secretome. D. Fish injected with denatured secretome. Black
637 lines represent the median, the green triangles the average, each dot one individual, and
638 grey lines the reaction norm of one individual between the control and injection weeks.

639

Secretome collection:

Fish infected by *Schistocephalus solidus*
Lake Témiscouata

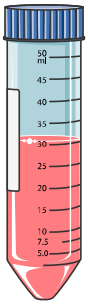
In saline for 2 hours

Experimental treatments:

Saline

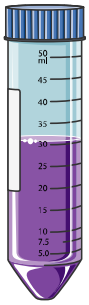
Effects of handling?

Infective secretome
from 7 worms > 50 mg



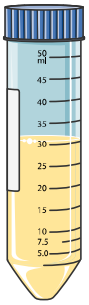
Effects of secretome?

Non-infective secretome
from 7 worms ≤ 50 mg



Effects of size of worm?

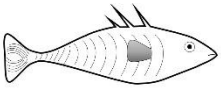
Denatured secretome
from 7 worms > 50 mg
95°C for 20 min before injection



Effects of reducing activity of proteins?

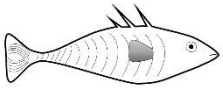
Behavioural tests:

Sympatric population:
Lake Témiscouata



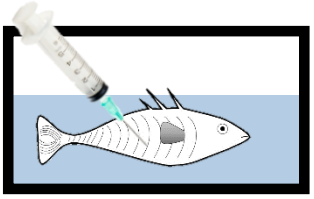
n=11 fish per treatment
(except infective secretome n=10)
+ n=7 infected fish

Allopatric population:
The Isle-Verte salt marsh

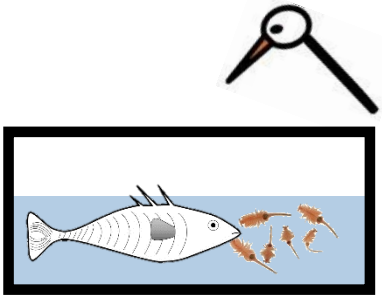


n=11 fish per treatment

First week: Control

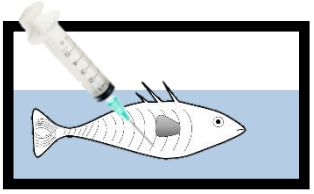


Needle insertion
during 4 days

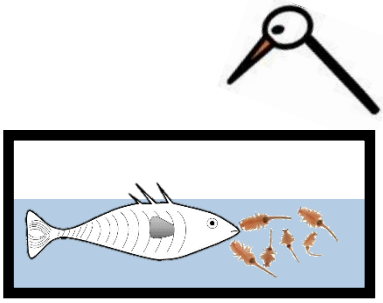


Day 4: Behavioural tests

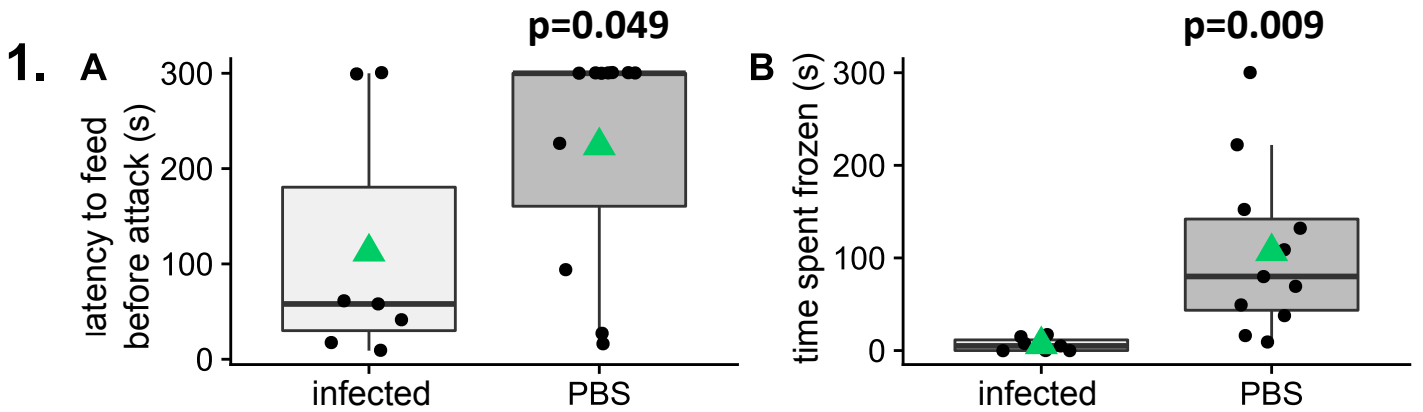
Second week: Injection

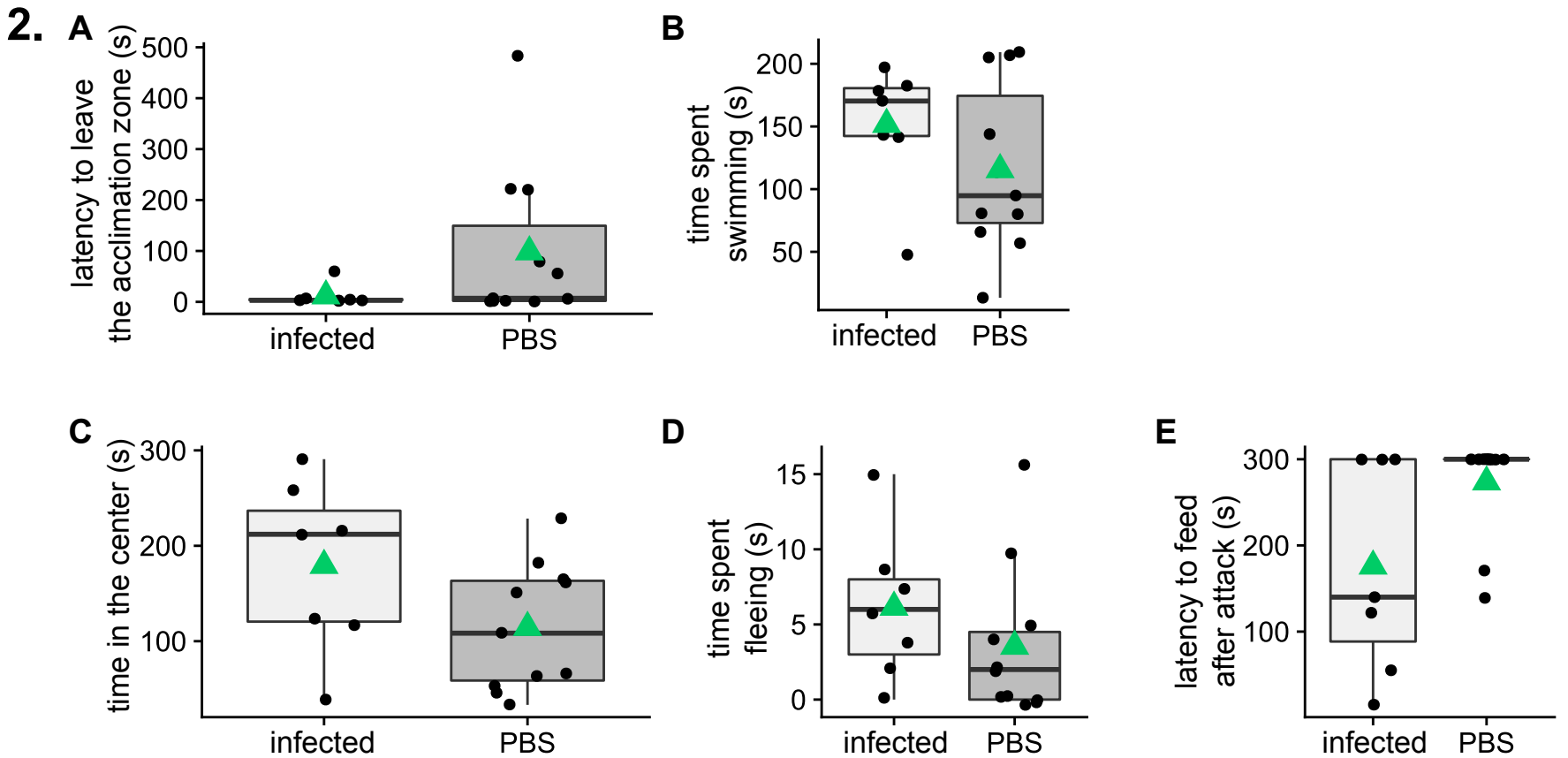


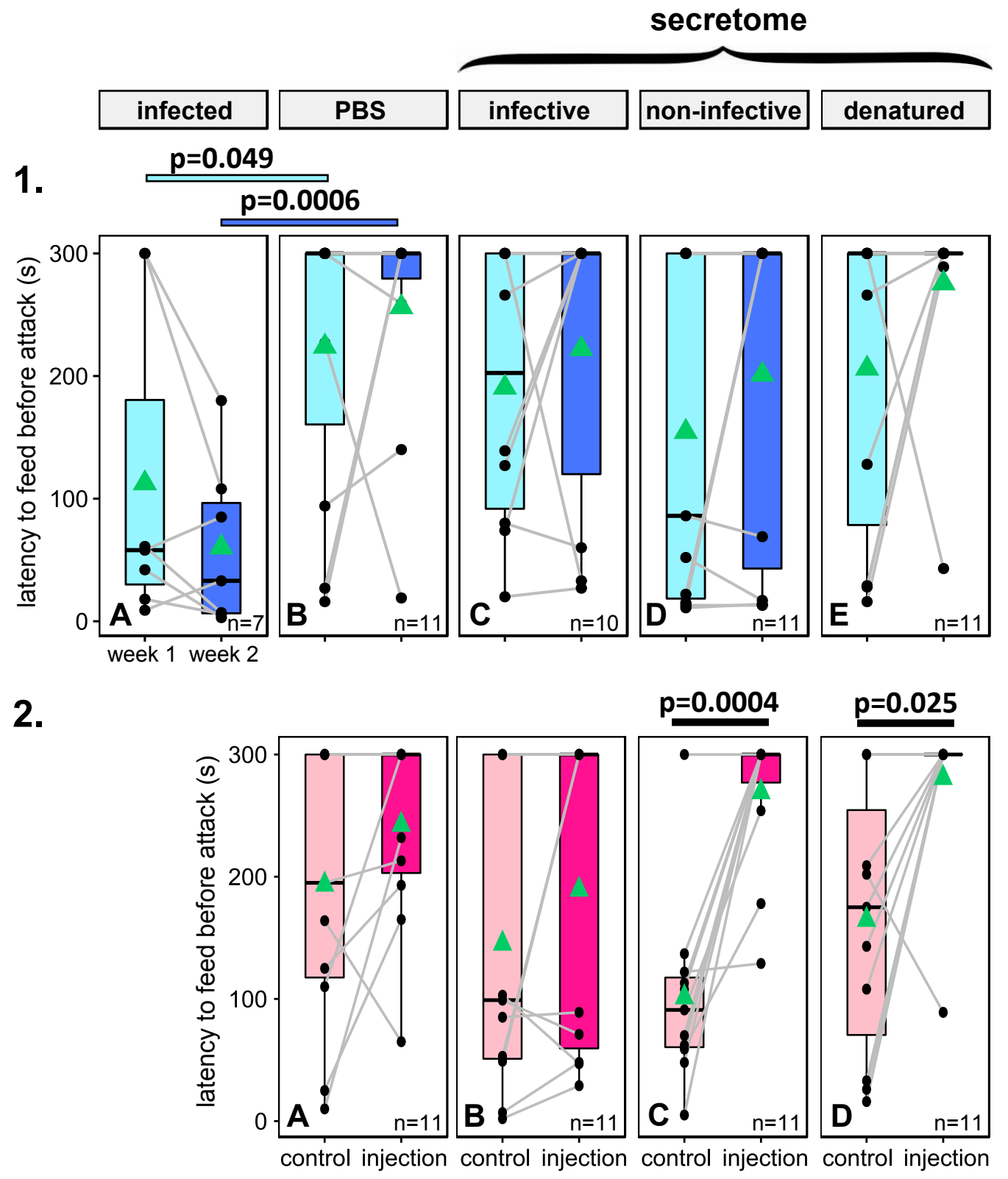
Injection (100 µL)
of the appropriate treatment
during 4 days



Day 4: Behavioural tests


1.
A
p=0.049
latency to feed before attack (s)
300
200
100
0
infected
PBS
B
p=0.009
time spent frozen (s)
300
200
100
0
infected
PBS


2.
A
latency to leave the acclimation zone (s)
500
400
300
200
100
0
infected
PBS
B
time spent swimming (s)
200
150
100
50
infected
PBS
C
time in the center (s)
300
200
100
infected
PBS
D
time spent fleeing (s)
15
10
5
0
infected
PBS
E
latency to feed after attack (s)
300
200
100
infected
PBS


secretome
infected
PBS
infective
non-infective
denatured
1.
p=0.049
p=0.0006
latency to feed before attack (s)
300
200
100
0
A
n=7
B
n=11
C
n=10
D
n=11
E
n=11
week 1
week 2
2.
p=0.0004
p=0.025
latency to feed before attack (s)
300
200
100
0
A
n=11
B
n=11
C
n=11
D
n=11
control
injection
control
injection
control
injection
control
injection