The secretome of a parasite is sufficient to alter its host's behaviour

Running title: Behavioural effects of a parasite secretome

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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 external environment (*i.e.* the secretome), but this has rarely been assessed. Here, 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 when the worm is ready to reproduce in its final avian host. We tested the effects of secretome exposure on behaviour by using secretions from the early and late infection of *S. solidus* and by injecting it 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 in sticklebacks, but secretome from early infection parasite resulted in more cautious host, 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.

INTRODUCTION

Parasites have strong impacts on the phenotype of their hosts, including modifications of the host morphology (1), physiology (2), immunity (3) and behaviour (4). Parasites with complex life cycles have been proposed to manipulate the behaviour of their hosts, in order to increase their transmission rates to their final host (5). For example, ants infected by the fungus *Ophiocordyceps* leave their normal activities and climb into the vegetation, which facilitates the propagation of the parasite's spores (6). If parasites are manipulative, they should disturb the functioning of their host central nervous system and physiological systems (hormonal and immune systems) by the production, storage, and release of molecules called manipulation factors (7-9). To demonstrate that behavioural changes in the host are the result of direct parasitic manipulation, it is crucial to find the manipulation factors produced by the parasite and their source, and to test how they impact the physiology and behaviour of the host. Such a link has almost never been established in previous studies of parasitic manipulation (9). Parasites release a wide variety of molecules into their external environment, including lipids (10), nucleic acids (11) and proteins (12), which are sometimes included in extracellular vesicles (13). These molecules are collectively referred to as the secretome. While the effects of exposing hosts to the secretome of their parasite on their immune response has been demonstrated in some host-parasite pairs (14, 15), there is currently no experimental evidence that the secretome content from a parasite directly affects behaviour in the host in the same way that the whole parasite infection does (16).

We studied the effect of the parasite secretome on host behaviour using the interaction between the threespine stickleback and the parasite *Schistocephalus solidus*. *Schistocephalus solidus* is a freshwater cestode that is trophically transmitted to three hosts (17). It enters the body cavity of its first host, a copepod, after being ingested, and then negatively affects its growth and reproduction (18). In the copepod, the expression of risky behaviours shows a pattern of "protection then facilitation" (19): infected copepods exhibit reduced activity in the early phase of infection, then increased activity and a

63 changed swimming pattern, leading to higher detection and ingestion by the second host,
64 the threespine stickleback (20-22). Infection in the second host results in changes in
65 morphology, physiology, immune response and behaviour (23, 24). The worm life cycle
66 is completed when it reaches its final host, usually a bird (23).

67

68 The parasite's life stage within the threespine stickleback lasts 7 to 16 weeks, is spent
69 within the body cavity of its host, and is characterised by two phases with distinct
70 phenotypes in both the parasite and its host. During the early days of infection (the non-
71 infective phase), the worm shows high growth and is too small to mature if switched to its
72 final bird host (25). In this first phase of infection, the host does not show the expected
73 adaptive immune response (26) and no behavioural changes are known (24, 27). At the
74 infective phase, the worm is ready to reproduce in its final avian host (24, 25). The
75 transition from the non-infective to the infective phase is characterized by major genome-
76 wide reprogramming events in the worm, including a shift from growth related genes to
77 the activation of genes predicted to be involved in neural pathways and sensory
78 perception (28). In this infective phase, drastic behavioural changes that result in a loss
79 of the anti-predator response appear in sticklebacks (24, 27). They are more exploratory
80 (29), less anxious (30) bolder in the presence of a predator (31), they recover more
81 quickly from a frightening overhead stimulus than non-infected individuals, and they feed
82 following the stimulus, while non-parasitized fish do not (31). These behavioural
83 perturbations could result from the combined effects of modifying the serotonin pathway
84 and the immune responses in infected fish (32, 33). The infective phase also corresponds
85 to the activation of the immune response in the host, when up-regulation of the respiratory
86 burst activity occurs (26). At this phase, secretome collected from *S. solidus* has been
87 shown to modulate the stickleback immune response *in vitro* (34). Whether this
88 modulation of the immune system is also involved in behavioural changes is unclear (32,
89 33).

90

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 (35). However, whether the worm secretes any molecule that can be classified as manipulation factors that are able to alter fish behaviour is unknown (9, 36). 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 and it dies rapidly when ingested by other fish species (37). *S. solidus* infects freshwater sticklebacks (24, 38) and a combination of studies of wild populations and of experimental reciprocal cross infection data suggests local adaptation between sympatric (“native”) host-parasite pairs (39-43). For example, in distinct host-parasite population pairs, the ratio of worm to host weight, an indicator of parasite virulence, is highly similar, suggesting an optimal virulence and local adaptation (40). On the other hand, marine sticklebacks are rarely exposed to *S. solidus*, as the worm does not tolerate high salinity levels when its hatches (44). Potentially reflecting the lower probability of co-evolution between these allopatric (“naïve”) marine fish and *S. solidus*, infection success of experimental infections are highly variable, ranging from 1.4% to 99% depending on the population (43, 45). Marine fish infected by *S. solidus* show morphological changes (46) and the immune response of allopatric fish *in vitro* exposed to *S. solidus* antigens at the infective phase differs from the one of sympatric sticklebacks (47). 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 (48). 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 (43). If allopatric sticklebacks do not have adaptive mechanisms to fight against the effects of secretome on fish physiology and behaviour because they did not co-evolve with the parasite (49), we predicted that they would be more sensitive to the secretome from the infective phase than the sympatric population and that they would show stronger behavioural changes. Conversely, if allopatric fish have an early activation of their immune response that would reduce the effects of secretome on fish physiology and behaviour (50), which sympatric hosts did not show because of the coevolution arms race ongoing with its parasite, 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. For both results, 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 determine if a parasite located outside the brain can manipulate the behaviour of its vertebrate host by releasing manipulation factors through its secretome. We tested if the secretome could be sufficient to induce behavioural changes in the vertebrate host, by injecting secretions from the non-infective and infective phases of *S. solidus* in non-infected sticklebacks from a sympatric (native) population and an allopatric (naïve) population.

148 # MATERIALS AND METHODS

149 **Collecting secretome from the non-infective and infective phases**

150 The collection of the secretome from *S. solidus* was performed according to a protocol
151 adapted from (51). We caught threespine stickleback fish from Lake Témiscouata
152 (Québec, 47°80' N 68°87' O), in June and July 2016 using minnow traps. One day after
153 fishing, fish (n=41) were euthanized with an overdose of MS-222 (75 mg/L mg/kg) and
154 dissected to determine if they were infected by *S. solidus*. Fish sex, size and mass, and
155 *S. solidus* mass and number in each fish were noted. Twenty-eight fish were infected by
156 worms at the non-infective (worm mass ≤ 50 mg; 8 fish) and infective (worm mass > 50
157 mg; 20 fish) phases (see "experimental treatments" for details). Following dissection, we
158 put each *S. solidus* worm in a 2 mL tube of PBS (Phosphate Buffered Saline, pH 7.4 Life
159 Technologies) after removing fish blood by washing the worm with 1 mL of PBS when
160 necessary. The tube was covered with aluminium foil to protect the worm from light and
161 placed in a water recipient from the lake for 2 hours. We then removed the worm from the
162 tube. The secretome tube obtained was stored at -20°C.

163

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

165 We performed the injections in two populations of threespine sticklebacks. The first
166 population, from Lake Témiscouata, Québec, was composed of fish that lived in sympatry
167 with the worms used to obtain secretome samples. The second population was composed
168 of fish from a salt march at Isle-Verte, Québec (48°00' N 69°40' O; salinity 22-26 ppt) and
169 that thus lived in allopatry with the worms used to obtain the secretome. Fish in this
170 population are anadromous, meaning that they live in saltwater and migrate to the lower
171 salinities of the salt marsh to reproduce. Sticklebacks in this population are not expected
172 to be infected by *S. solidus* because the worm does not tolerate high levels of salinity
173 (52).

174

175 We caught sticklebacks from Lake Témiscouata using a seine in August 2016. Fish from
176 the anadromous Isle-Verte population were sampled in tide pools in July 2016 using hand
177 nets. Fish from both populations were juveniles at the time of capture. We brought them
178 to the "Laboratoire Aquatique de Recherche en Sciences Environnementales et
179 Médicales" at Université Laval. Sticklebacks from both populations were maintained in
180 separate 80 L tanks under a Light : Dark photoperiod of 12 h:12 h and a temperature of
181 15°C reflecting their natural environment conditions (Québec, Canada). For anadromous
182 fish, salinity was at 28 ppt. We fed fish daily with brine shrimps. All sticklebacks were
183 mature at the start of the injections.

184

185 **Experimental treatments**

186 We used four treatments for each population: an injection control treatment, an "infective
187 secretome" treatment, a "non-infective secretome" treatment and a "denatured infective
188 secretome" treatment. For these four treatments, we used healthy non-infected fish from
189 the two populations. In addition, in the Témiscouata population, we added a group of fish
190 infected by *S. solidus* that were not injected as a positive control, to validate that the
191 behavioural test could discriminate between healthy and *S. solidus* infected fish (n=7).
192 The control treatment involved fish injected with phosphate-buffered saline (PBS), to test
193 handling effects on fish behaviour. The three other treatments involved injections of
194 secretome collected in Lake Témiscouata. For each of these three treatments, we
195 prepared a mixture of secretome coming from 7 worms in order to remove individual
196 effects from a specific worm and to insure sufficient volume for injections. The mixture
197 was made of 1600 µL of secretome from each worm. The infective secretome treatment
198 included fish injected with secretome from worms whose weight was above 50 mg (the
199 mass at which the worm becomes infective, i.e. it can reproduce in its final host and it
200 induces behavioural perturbation in fish (24)). Mass of the worms ranged from 80 mg to
201 700 mg with a mean of 368.6 mg. The non-infective secretome treatment used fish
202 injected with secretome from worms whose weight was equal to or under 50 mg (the mass
203 at which the worm is non-infective, i.e. it does not induce behavioural changes in fish).

204 Worms had a mass around 10 mg (minimal value detected by the balance) to 50 mg with
205 a mean of 27.1 mg. This treatment was used as a control for which behavioural changes
206 were not expected. The denatured secretome treatment used fish injected with secretome
207 from worms whose weight was above 50 mg, but for which the proteins included in the
208 secretome were denatured by heat before each injection. This treatment was also added
209 as a negative control, as proteins lose their secondary and tertiary structures following
210 heat treatment, and consequently their biological activities. If we found differences in
211 behaviours between fish injected with the intact and the denatured secretome from large
212 worms, this would suggest that the activity of proteins included in the secretome is
213 involved in the behavioural alteration. Worm mass for this last treatment was comprised
214 between 170 mg and 730 mg with a mean of 464.3 mg. Each treatment was composed
215 of n=11 fish in each population, except for the infective secretome treatment in the
216 Témiscouata population (n=10).

217

218 **Protein content of secretome**

219 Prior to injections, we determined the protein concentration of each secretome mixture
220 using a Pierce Coomassie (Bradford) Protein Assay Kit (Fisher Scientific). Bovine Serum
221 Albumin (BSA) (2 mg/mL) was used as standard. The concentrations obtained for the
222 infective secretome, the non-infective secretome and the denatured secretome were
223 respectively 24.08 µg/mL, 6.47 µg/mL and 40.48 µg/mL. We then diluted the denatured
224 secretome to the concentration of the infective secretome in order to remove potential
225 concentration effects. We visualized proteins for each secretome mixture on a SDS-
226 PAGE (resolving gel 12%) using a Pierce Silver Stain Kit (Fisher Scientific). Before
227 running a gel, each mixture (100 µL) was precipitated with Trichloroacetic Acid (TCA) (53)
228 to facilitate protein visualization at low concentration. A yeast protein extract was used as
229 a positive control and a Blue pre-stained protein standard (BioLabs) was added on the
230 gel. A negative control including the SDS-PAGE loading buffer and water was used.
231 Migration was performed during 60 min at 175 V followed by silver staining. For the three
232 mixtures, proteins of various sizes were effectively separated on gel (Supp Figure 1).

233

234 **Injection protocol**

235 At the beginning of the protocol, we isolated 51 fish from the Témiscouata population and
236 44 fish from the Isle-Verte population in 2 L tanks and assigned an identification number
237 (ID). We applied the environmental DNA method developed to non-invasively detect the
238 infection by *S. solidus* (54). On 2 consecutive days following isolation, we injected 100 µL
239 of PBS in the abdominal cavity of an individual and we immediately aspirated it back into
240 the syringe, to sample the eDNA present in the abdominal cavity fluids of each fish. Forty-
241 four fish from the Témiscouata population were detected as non-infected and could be
242 used for injections, while 7 fish were infected by *S. solidus* and were used as controlled
243 infected, non-injected fish. As expected, none of the fish from the anadromous population
244 was infected by *S. solidus* and all could be used for injections.

245

246 Following the environmental DNA injection protocol, we randomly assigned each fish to
247 an injection treatment (PBS, “infective secretome”, “non-infective secretome” or
248 “denatured infective secretome”). During the first week (control week), we inserted a
249 needle into the abdominal cavity of each fish (no injection), once a day for four days. On
250 4th day, we tested behaviour of each fish between 30 and 45 minutes after the last needle
251 insertion. During the second week (injection week), we injected each fish in the abdominal
252 cavity with 100 µL of the appropriate treatment (PBS, “infective secretome”, “non-infective
253 secretome” or “denatured secretome”), once a day for four days (total of 400 µL injected
254 per fish). On the 4th day, we tested behaviour of each fish between 30 and 45 minutes
255 after the last injection. Denatured secretome was heated at 95°C for 20 minutes and was
256 allowed to be cooled before each injection. Infected fish used as a positive control were
257 exposed to the same protocol, except that during the injection week the needle was only
258 inserted as during the control week. We fed fish daily with brine shrimps following needle
259 insertion or injection, except on day 4. Each fish was given 3 days of rest between the
260 control week and the injection week. One fish for the infective secretome treatment of the

Témiscouata population died between the control week and the injection week and was not taken into account in the analysis.

Behavioural tests and analysis

We performed behavioural tests in an aquarium (30 Width x 30 Depth x 60 Length cm) inside which a white box (25 W x 36 D x 57 L cm) was inserted in order to prevent fish reflections on the glass of the aquarium and to optimize tracking during analyses. We filled the box with 25 cm of water depth and water was changed between each fish. The box was opened on the top to allow recording by a camera. The day before the behavioural test of a fish, we enclosed the 2L individual tank with a small white box (14 W x 17 D x 30 L cm) opened on top, to acclimatize the fish to a white environment. From this point the fish was not fed. The day of the test, at the start of the experiment, we put the fish in the acclimation zone inside the white box of the aquarium for 600 seconds. The acclimation zone, which was delimited by a white wall, represented one third of the white box. After 600 seconds, the wall was removed, and 3 behaviours were measured.

<u>Exploration</u>

Exploration of a novel environment was measured using 2 variables: 1) Latency to leave the acclimation zone (sec). As soon as the wall was removed, the fish was given 600 seconds to leave the acclimation zone. If the fish never left the acclimation zone, it was given the worst score for exploration (*i.e.* 600 sec). 2) Time spent swimming (sec). As soon as the fish left the acclimation zone (or after 600 sec), time spent swimming by the fish was recorded for 300 seconds.

<u>Thigmotaxis</u>

286 Wall hugging was measured with 1 variable according to a previously developed protocol:
287 time spent in the centre of the aquarium (sec) (30). We quantified thigmotaxis as soon as
288 the fish left the acclimation zone (or after 600 sec) and measured for 300 seconds. The
289 centre of the aquarium was defined as a zone at more than 5 cm of the edge of the white
290 box.

291

292 Boldness

293 The inclination to take risks was measured using 4 variables: 1) Latency to feed before a
294 predator attack (sec). We deposited brine shrimps with a pipette in front of the fish so that
295 it could see it. The fish was given 300 seconds to feed. 2) Time spent fleeing after a
296 predator attack (sec). 3) Time spent frozen after a predator attack (sec). 4) Latency to
297 feed after a predator attack (sec). For these last three variables, as soon as the fish fed
298 after food deposition, or 300 seconds after food deposition, we simulated a predator
299 attack using a heron head model (beak of the model was going back and forth into the
300 water during 5 seconds near the fish head). Time spent fleeing corresponds to the time
301 during which the fish escapes just after the heron head model was removed. Time spent
302 frozen indicates the time during which the fish stays motionless (*i.e.* does not swim a
303 distance greater than its body length) just after the heron head model was removed.
304 Latency to feed after an attack was also measured just after the heron head model was
305 removed. These three variables were determined for 300 seconds.

306

307 Following the behavioural tests, we euthanized fish on day 4 of the injection week with an
308 overdose of MS-222 (75 mg/L) and dissected them to confirm that they were infected by
309 *S. solidus* or not. Fish sex, size and mass, and *S. solidus* mass and number in each fish
310 were noted.

311

312 Two behaviours were automatically analysed using a tracking module with the Ethovision
313 software (Ethovision XT 11.5, Noldus Information Technology) (55): time spent swimming
314 and time spent in the centre of the aquarium. All the other behaviours were analysed
315 manually. All the videos were analysed blinded by C.S.B.

316

317 **Statistical analyses**

318 Statistical analyses were performed using the R software version 3.5.0 (56). We used the
319 package lme4 (57) to create a linear mixed model for each population separately including
320 the week (control or injection), the treatment (PBS, “infective secretome”, “non-infective
321 secretome”, “denatured secretome” or infected) and their interaction (week*treatment),
322 the mass, the size and the sex of the fish as fixed effects and the ID of the fish as random
323 effect. For each analysis, we checked homoscedasticity by plotting residuals and fitted
324 data. We checked for homogeneity of variances using a Levene Test. We tested normality
325 of residuals using graphical assessment (QQ-plot and normal distribution histogram) and
326 a Shapiro-Wilk test. When the data were not normally distributed, we used the Boxcox
327 test from the package MASS (58) to find the best transformation to fit normality (log,
328 square root, square or no transformation). To obtain p-values, we performed pairwise
329 comparisons between weeks and treatments using the package lmerTest (59). In the
330 sympatric Témiscouata population, the first analysis was performed with two reference
331 treatments (infected and PBS) to confirm that there was no difference in behaviour before
332 (PBS-control week) and after (PBS-injection week) control injections, and to determine if
333 infected fish (infected-control week) and non-infected fish (PBS-control week) showed
334 significantly different behaviours as expected. The second analysis aimed to test our main
335 predictions and was performed with the secretome treatments only (“infective secretome”,
336 ‘non-infective secretome” and “denatured secretome”) to determine if the secretome
337 injections were able to reproduce the behaviours of infected fish, using each individual as
338 their own control. In the allopatric anadromous population, fish were not infected by *S.*
339 *solidus*. The first analysis was thereby performed with all reference (PBS) and secretome
340 treatments, to test if PBS injections changed behaviours. To test our predictions about

341 the effects of secretome on behaviour, we performed a second analysis using fish from
342 the three secretome treatments ("infective secretome", "non-infective secretome" and
343 "denatured secretome"), using each individual as their own control.

344

345 # RESULTS

346 **Behaviours that differentiated infected and non-infected fish**

347 We measured seven behaviours in order to quantify exploration, thigmotaxis and
348 boldness in infected and non-infected fish. We found that in the sympatric population,
349 infected and non-infected fish differed significantly in two behaviours associated with
350 boldness: latency to feed before a predator attack and time spent frozen after a predator
351 attack. Infected fish ate two times more rapidly before a predator attack (average for
352 infected fish, measured in control week: 113 sec) than non-infected fish (average for non-
353 infected fish, measured in control week: 224 sec) ($p = 0.049$) (Figure 1 panel 1.A).
354 Infected fish almost did not freeze after the predator attack (average measured in control
355 week: 6 sec) while non-infected fish froze for a longer time (average measured in control
356 week: 107 sec) ($p = 0.009$) (Figure 1 panel 1.B). For the other five behaviours, infected
357 fish did not differ significantly from non-infected fish, although trends were apparent.
358 Infected fish took 12 seconds on average to leave the acclimation zone, while non-
359 infected fish took 98 seconds ($p = 0.958$) (Figure 1 panel 2.A) and spent 30% more time
360 swimming (average measured in control week: 152 sec) than non-infected fish (average
361 measured in control week: 116 sec) ($p = 0.596$) (Figure 1 panel 2.B). On average, infected
362 fish spent 57% more time in the centre of the aquarium compared to non-infected fish
363 (average of 179 sec versus 114 sec) ($p = 0.998$) (Figure 1 panel 2.C). Infected fish spent
364 6.1 seconds fleeing on average after the predator attack, while non-infected fish spent
365 3.6 seconds ($p = 0.543$) (Figure 1 panel 2.D). Infected fish took 176 seconds before
366 resuming feeding on average after the predator attack, while non-infected fish mostly
367 forewent eating, with an average feeding latency close to the maximum given time of 300
368 seconds (average: 274 sec) ($p = 0.214$) (Figure 1 panel 2.E). To confirm these results,
369 we performed additional statistical analyses to validate that our model was not overfitted

370 and that the obtained p-values were accurate. We found that mass of fish was correlated
371 with behaviour, but p-values were not changed when removing this effect from the model.

372

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

375 Sympatric population: Lake Témiscouata

376 We tested behaviour twice in each individual: during the control week and during the
377 injection week. We first tested control non-infected individuals in which we injected only
378 a saline solution, in order to quantify any changes related to the disturbance of the
379 experiment in these two trials. For all behaviours assessed, we did not find any significant
380 differences between the two weeks in these control fish, suggesting that handling did not
381 significantly affect them (Supp Tables 1 to 7).For the latency to feed before a predator
382 attack, infected and healthy individuals differed in their behaviour on both the control ($p =$
383 0.049) and injection week ($p<0.001$), as stated above (Supp Table 4). During the first
384 week, infected fish took 113 seconds on average before eating, and started eating almost
385 twice as fast on the second week (average: 60 sec), although there were no statistically
386 significant differences between these two tests (average between weeks, $p = 0.619$, Supp
387 Table 4) (Figure 2 panel 1.A). Non-infected fish showed much greater latency to eat than
388 infected fish and did not change their behaviour between the first and second tests
389 (average: 224 sec vs 256 sec) (average between weeks, $p = 0.781$, Supp Table 4) (Figure
390 2 panel 1.B).

391

392 We were not able to reproduce the typical behaviours of infected fish using secretome
393 injections from the infective phase when injecting healthy individuals from the
394 Témiscouata freshwater population (Supp Tables 1 to 7). For example, in this sympatric
395 population, none of the secretome injections had significant effects on feeding latency
396 before a predator attack. Fish injected with the infective secretome did not significantly
397 change the time they took before eating between the two trials. Their average latency in

398 the control week was 191 seconds, then 222 seconds in the injection week (p = 0.975,
399 Supp Table 4) (Figure 2 panel 1.C). The same was observed in fish injected with the non-
400 infective secretome (average: 154 sec vs 201 sec) (p = 0.852, Supp Table 4) (Figure 2
401 panel 1.D) and following injection of denatured secretome (average: 206 sec vs 276 sec)
402 (p = 0.526, Supp Table 4) (Figure 2 panel 1.E). None of the other behaviours showed
403 changes after injections in the Témiscouata population (Supp Figures 2 to 7).

404

405 Allopatric population: The Isle-Verte salt marsh

406 In the allopatric population, we first tested control individuals in which we injected only a
407 saline solution, in order to quantify any changes related to the disturbance of the
408 experiment in these two trials. For all behaviours assessed, we did not find any significant
409 differences between the two weeks in these control fish, suggesting that handling did not
410 significantly affect them (Supp Tables 8 to 14). For example, the control non-infected fish
411 had similar latency to eat before the predator attack in the first and second weeks
412 (average: 194 sec vs 243 sec) (p = 0.762, Supp Table 11) (Figure 2 panel 2.A).

413

414 We found that behaviour was changed after secretome injections in the allopatric
415 population in some treatments. The latency to eat before a predator attack was not
416 changed after injection of the infective secretome in healthy individuals. Their latency to
417 feed on the control week was 145 seconds and 189 seconds after the secretome injection
418 (p = 0.642, Supp Table 11) (Figure 2 panel 2.B). However, fish injected with the non-
419 infective secretome significantly increased their latency to approach food, taking more
420 than twice longer before resuming feeding (average: 101 sec vs 269 sec) (p < 0.001,
421 Supp Table 11) (Figure 2 panel 2.C). After injection of denatured secretome, fish
422 significantly delayed or refrained entirely from eating before the predator attack compared
423 to the control week (average: 165 sec vs 281 sec) (p=0.025, Supp Table 11) (Figure 2
424 panel 2.D). We also found that anadromous fish took significantly longer to resume
425 feeding after a predator attack following injection of the non-infective secretome (p=0.037,

426 Supp Table 14). After a predator attack, their latency to feed was 227 seconds during the
427 control week, while they stopped feeding entirely during the injection week, with an
428 average feeding latency of 300 seconds, the maximum possible value (Supp Figure 7
429 panel 2.C). This behaviour was not significantly affected in the two other secretome
430 treatments nor in the control fish injected with PBS (Supp Table 14). Additionally, we
431 found that anadromous fish significantly spent more time swimming after injection of
432 denatured secretome (average: 128 sec vs 176 sec) (p=0.016, Supp Table 9) while other
433 secretome treatments did not affect this behaviour (Supp Figure 3 panel 2.D). None of
434 the other behaviours showed changes after injections of secretome in this population
435 (Supp Tables 8, 10, 12 and 13 and Supp Figures 2 to 7).

436

437 **DISCUSSION**

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

449

450 The lack of behavioural change in fish injected with infective secretome can be interpreted
451 in two ways: either the secretome is not sufficient to alter behaviour, or our protocol did
452 not recreate the biological alterations that happen in the host during the weeks of
453 infection. In both population, fish injected with the infective secretome did not differ in

454 behaviour from the control non-infected fish that were injected with PBS, despite the fact
455 that proteins were detected in the secretome. Clearly, it is possible that *S. solidus* does
456 not act on the behaviour of its host through its secretome, and if such is the case, the
457 probability that this host behaviour alteration is the result of active manipulation by the
458 parasite would be low. If this is the correct interpretation, behavioural changes would be
459 only the result of a side effect of infection, such as the effect of the parasite mass burden,
460 or the result of a host response (30). However, if this was the case, it would be improbable
461 to observe the significant changes in behaviour we quantified when injecting the non-
462 infective secretome. An alternative explanation would be that our exposure protocol was
463 not sufficient to test our prediction. First, it is possible that we did not reach a threshold
464 level of manipulation factors, as it has been proposed that they may be accumulated
465 slowly and stored by the parasite (9, 60). Because secretomes were sampled from the
466 wild, the amount of secretome collected, and thus available for injection, was limited by
467 the number of caught fish that were effectively infected by *S. solidus* at the infective
468 phase. We consequently performed injections for 4 days, with a small dose injected every
469 24h. In contrast, if manipulative, *S. solidus* probably secretes molecules continuously in
470 the abdominal cavity of the threespine stickleback. Increasing the dose and the duration
471 of exposure in non-infected fish should definitely be tested in order to better understand
472 the role, if any, of the parasitic secretome in host manipulation. Second, a potential
473 explanation is that *S. solidus* may secrete molecules during the non-infective phase,
474 which lasts several weeks, that would act to “prime” the host (16). The importance of the
475 interaction between the effects of molecules of the non-infective phase and of the infective
476 phase to induce behavioural changes should be tested by sequentially injecting the non-
477 infective secretome and the infective secretome in non-infected fish. Finally, it is also
478 possible that the molecules of the secretome at the infective phase act in combination
479 with molecules of the stress response, which was previously proposed to be involved in
480 the behavioural changes of the threespine stickleback (32, 33), to induce the complex
481 behavioural changes. A next step will require to adopt an integrative functional approach
482 to simultaneously test the effects of the secretome and of the activation of the stress
483 response on the behaviour of the host.

484

485 The significant increase in the fish latency to eat before and after a predator attack
486 induced by the secretome from worms at the non-infective phase is similar to the altered
487 behaviour seen in infected copepods, the first intermediate host of *S. solidus.* The
488 copepod host exhibits a behaviour pattern of "protection then facilitation", where the
489 copepod first shows reduced activity in the early phase of infection by *S. solidus*,
490 presumably reducing detection by predators, then an increased activity, which leads to
491 higher detection by the threespine stickleback (19-22). The induced behavioural
492 perturbations in sticklebacks are surprising, as behavioural changes have not been
493 reported in infected sticklebacks when *S. solidus* is in the early non-infective phase (24,
494 27). It is possible that the same shift in behaviour exists in the fish host but has gone
495 undetected. Studying the possible behavioural sequence of "protection then facilitation"
496 in threespine sticklebacks infected by *S. solidus* thus represents a promising opportunity
497 to better understand behavioural manipulation. Furthermore, the divergence in
498 behavioural effects between the non-infective and the infective secretome suggests that
499 the secretome content between these two phases is different. In accordance with our
500 results, the analysis of the transcriptome of *S. solidus* previously demonstrated that the
501 genes expressed by the worm are significantly different between non-infective and
502 infective phases, with thousands of genes differing in activity (28). Prior to injections,
503 proteins were detected in secretomes from both phases. Analysing the nature of the
504 protein content of the *S. solidus* secretome would provide functional information that could
505 shed light on the nature of the molecular interaction between the threespine stickleback
506 and *S. solidus*.

507

508 Studies in several host-parasite pairs from different branches of the tree of life have
509 shown that behavioural alteration in the host affects specific behaviours rather than
510 disrupting the host response to all cues. (5, 7, 23, 36, 61). Not all behaviours were altered
511 by the non-infective secretome in our study. The specificity of effects on cautiousness in
512 presence of a predator, but not on exploration of a novel and risky environment, or of
513 anxiety-like behaviour such as wall hugging, is in accordance with previous studies that
514 have shown that not all behaviours are modified in infected sticklebacks (61) and that not

515 all behaviours are changed when healthy sticklebacks are experimentally manipulated to
516 behave like infected ones (32). Indeed, manipulating serotonin levels in healthy
517 sticklebacks to mimic the changes observed in the brain of infected ones lead to changes
518 in schooling and the tendency to swim close to the surface, but not the response to a
519 predator attack, which was significantly altered (in the opposite direction to an infected
520 fish) in the present study (32). This result points to a probable multifactorial nature of host
521 altered behaviours, as no experimental manipulation leads to changes in all the
522 behaviours observed in *Schistocephalus* infected fish.Here, the non-infective secretome
523 exposure resulted in more cautious fish that took longer to feed under risk (or refrained
524 for feeding). Identifying the content of the secretome will potentially uncover molecules
525 that affect directly or indirectly feeding, as found in another host-parasite system where
526 feeding is affected (16).

527

528 Our results show that the secretome of *S. solidus* had significant effects on fish risky
529 behaviour only in an allopatric (naïve) population and that allopatric fish are therefore
530 more sensitive to the secretome. These results are in accordance with the hypothesis
531 that since allopatric sticklebacks did not co-evolve with *S. solidus*, they did not develop
532 the adaptive "generalised response" against *S. solidus* and its effects on fish physiology
533 and behaviour, as proposed for this parasite and others (43, 49, 62, 63). Local adaptation
534 and potential co-evolution between the threespine stickleback and *S. solidus* was
535 previously suggested with cross-infection studies (40, 43, 64). We provide here some new
536 insights that point to the need for further studies that include exposure of fish that have
537 evolved in the presence of another worm genotype, *i.e.* sticklebacks of freshwater origin
538 that can recognize the parasite but did not co-evolve specifically with that genotype, to
539 reveal further information on local adaptation in this host-parasite pair (43, 64).

540

541 The significant increase in the latency to eat before an attack, which was reported after
542 the injection of the non-infective secretome in the allopatric population, was also found in
543 fish injected with heat-denatured secretome, suggesting that these impacts on behaviour

544 were not related solely to the activity of proteins. Indeed, since these secretome samples
545 were denatured by heat in order to lose the secondary and tertiary structures of proteins,
546 and consequently their biological activities, it is possible that this behavioural change
547 might be induced by other molecules included in the secretome that were not as impacted
548 by the heat treatment. It is likely that *Schistocephalus* secretes and/or excretes other
549 molecules, such as extracellular vesicles including micro-RNA (65, 66). To confirm this
550 hypothesis in the future, we could replace the heat treatment of secretome by a protease
551 exposure, in order to specifically break down and inactivate proteins in the secretome.

552

553 Our findings demonstrate that the secretome of *S. solidus* is an important component of
554 the molecular cross-talk between the parasite and its threespine stickleback host.
555 Secretome alone is not able to reproduce the behavioural changes of infected
556 sticklebacks in non-infected sticklebacks. Yet, its effects on boldness in the allopatric
557 population suggest that the secretome may act in combination with other processes, like
558 the activation of the stress response in fish, to induce the complex behaviour of infected
559 sticklebacks. Future studies could explore how the secretome of *S. solidus*, in
560 combination with other factors linked to the activation of the stress response in
561 sticklebacks, could induce this complex set of behavioural changes.

562

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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. analyzed the data with input from N.A.-H.. C.S.B. and N.A-H. wrote the manuscript.

Data availability

The complete dataset is available as a supplementary tab-delimited supp_dataset_final.txt file.

Ethics

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

586 # REFERENCES

587 1. Sandland GJ, Goater CP. Parasite-induced variation in host morphology: Brain-
588 encysting trematodes in fathead minnows. *The Journal of Parasitology*. 2001**;87**(2):267-
589 72.
590 2. Garvin MC, Szell CC, Moore FR. Blood parasites of nearctic-neotropical migrant
591 passerine birds during spring trans-gulf migration: Impact on host body condition. *The*
592 *Journal of Parasitology*. 2006;**92**(5):990-6.
593 3. Rigano R, Profumo E, Bruschi F, Carulli G, Azzara A, Ioppolo S, et al. Modulation
594 of human immune response by *Echinococcus granulosus* antigen B and its possible role
595 in evading host defenses. *Infection and Immunity*. 2001;**69**(1):288.
596 4. Watson DW, Mullens BA, Petersen JJ. Behavioral fever response of *Musca*
597 *domestica* (Diptera: Muscidae) to infection by *Entomophthora muscae* (Zygomycetes:
598 Entomophthorales). *Journal of Invertebrate Pathology*. 1993;**61**(1):10-6.
599 5. Lafferty K, Shaw J. Comparing mechanisms of host manipulation across host and
600 parasite taxa. *The Journal of Experimental Biology*. 2013;**216**(1):56-66.
601 6. De Bekker C, Merrow M, Hughes DP. From behavior to mechanisms: An
602 integrative approach to the manipulation by a parasitic fungus (*Ophiocordyceps*
603 *unilateralis* s.l.) of its host ants (*Camponotus* spp .). *Integrative and Comparative*
604 *Biology*. 2014;**54**(2):166-76.
605 7. Adamo SA. Parasites: evolution's neurobiologists. *The Journal of Experimental*
606 *Biology*. 2013;**216**(1):3-10.
607 8. Biron DG, Loxdale HD. Host-parasite molecular cross-talk during the manipulative
608 process of a host by its parasite. *The Journal of Experimental Biology*. 2013;**216**(1):148-
609 60.
610 9. Herbison R, Lagrue C, Poulin R. The missing link in parasite manipulation of host
611 behaviour. *Parasites & Vectors*. 2018;**11**(1): 222.
612 10. Coppens I, Vielemeyer O. Insights into unique physiological features of neutral
613 lipids in *Apicomplexa*: from storage to potential mediation in parasite metabolic activities.
614 *International journal for parasitology*. 2005;**35**(6):597-615.

615 11. Fromm B, Ovchinnikov V, Høye E, Bernal D, Hackenberg M, Marcilla A. On the
616 presence and immunoregulatory functions of extracellular micro RNA s in the trematode
617 *Fasciola hepatica*. *Parasite immunology*. 2017;**39**(2):e12399.
618 12. Grimwood J, Smith JE. *Toxoplasma gondii*: the role of parasite surface and
619 secreted proteins in host cell invasion. *International journal for parasitology*.
620 1996;**26**(2):169-73.
621 13. Coakley G, Maizels RM, Buck AH. Exosomes and other extracellular vesicles: the
622 new communicators in parasite infections. *Trends in parasitology*. 2015;**31**(10):477-89.
623 14. Harnett W. Secretory products of helminth parasites as immunomodulators.
624 *Molecular & Biochemical Parasitology*. 2014;**195**(2):130-6.
625 15. Hewitson JP, Grainger JR, Maizels RM. Helminth immunoregulation: The role of
626 parasite secreted proteins in modulating host immunity. *Molecular & Biochemical*
627 *Parasitology*. 2009;**167**(1):1-11.
628 16. Adamo SA. Turning your victim into a collaborator: Exploitation of insect
629 behavioural control systems by parasitic manipulators. *Current opinion in insect science*.
630 2019;**33**:25-29.
631 17. Hammerschmidt K, Kurtz J. Chapter 5 Ecological immunology of a tapeworms'
632 interaction with its two consecutive hosts. *Advances in Parasitology*. 2009;**68**:111-37.
633 18. Wedekind C. The infectivity, growth, and virulence of the cestode *Schistocephalus*
634 *solidus* in its first intermediate host, the copepod *Macrocyclops albidus*. *Parasitology*.
635 1997;**115**(3):317-24.
636 19. Dianne L, Perrot-Minnot MJ, Bauer A, Gaillard M, Léger E, Rigaud T. Protection
637 first then facilitation: a manipulative parasite modulates the vulnerability to predation of
638 its intermediate host according to its own developmental stage. *Evolution: International*
639 *Journal of Organic Evolution.* 2011;**65**(9):2692-8.
640 20. Hafer-Hahmann N. Experimental evolution of parasitic host manipulation.
641 *Proceedings of the Royal Society B: Biological Sciences.* 2019;**286**(1895):20182413.
642 21. Wedekind C, Milinski M. Do three-spined sticklebacks avoid consuming copepods,
643 the first intermediate host of *Schistocephalus solidus*? An experimental analysis of
644 behavioural resistance. *Parasitology*. 1996;**112**(4):371-83.

645 22. Weinreich F, Benesh D, Milinski M. Suppression of predation on the intermediate
646 host by two trophically-transmitted parasites when uninfective. *Parasitology*.
647 2013;**140**(1):129-35.
648 23. Barber I. Sticklebacks as model hosts in ecological and evolutionary parasitology.
649 *Trends in Parasitology*. 2013;**29**(11):556-66.
650 24. Barber I, Scharsack JP. The three-spined stickleback- *Schistocephalus solidus*
651 system: an experimental model for investigating host-parasite interactions in fish.
652 *Parasitology*. 2010;**137**(3):411-24.
653 25. Tierney JF, Crompton DWT. Infectivity of plerocercoids of *Schistocephalus solidus*
654 (Cestoda: Ligulidae) and fecundity of the adults in an experimental definitive host, *Gallus*
655 *gallus*. *The Journal of Parasitology*. 1992;**78**(6):1049-54.
656 26. Scharsack JP, Koch K, Hammerschmidt K. Who is in control of the stickleback
657 immune system: interactions between *Schistocephalus solidus* and its specific vertebrate
658 host. *Proceedings of the Royal Society B: Biological Sciences.* 2007**;274**(1629):3151-8.
659 27. Barber J, Walker P, Svensson PA. Behavioural responses to simulated avian
660 predation in female three spined sticklebacks: The effect of experimental *Schistocephalus*
661 *solidus* infections. *Behaviour*. 2004;**141**:1425-40.
662 28. Hebert FO, Grambauer S, Barber I, Landry CR, Aubin-Horth N. Major host
663 transitions are modulated through transcriptome-wide reprogramming events in
664 *Schistocephalus solidus*, a threespine stickleback parasite. *Molecular ecology*.
665 2017;**26**(4):1118-30.
666 29. Arber B, Huntingford FA, Crompton DWT. The effect of hunger and cestode
667 parasitism on the shoaling decisions of small freshwater fish. *Journal of Fish Biology*.
668 1995;**47**(3):524-36.
669 30. Grécias L, Valentin J, Aubin-Horth N. Testing the parasite mass burden effect on
670 alteration of host behaviour in the -stickleback system. *The Journal of Experimental*
671 *Biology*. 2018;**221**: jeb174748.
672 31. Giles N. Behavioural effects of the parasite *Schistocephalus solidus* (Cestoda) on
673 an intermediate host, the three-spined stickleback, *Gasterosteus aculeatus* L. Animal
674 *Behaviour*. 1983;**31**(4):1192-4.

675 32. Grécias L, Hébert F-O, Berger CS, Barber I, Aubin-Horth N. Can the behaviour of
676 threespine stickleback parasitized with *Schistocephalus solidus* be replicated by
677 manipulating host physiology? *The Journal of Experimental Biology*. 2016;**220**(2):237-46.
678 33. Overli O, Pll M, Borg B, Jobling M, Winberg S. Effects of *Schistocephalus solidus*
679 infection on brain monoaminergic activity in female three-spined sticklebacks
680 *Gasterosteus aculeatus*. *Proceedings of the Royal Society B: Biological Sciences*.
681 2001;**268**(1474):1411-5.
682 34. Scharsack JP, Gossens A, Franke F, Kurtz J. Excretory products of the cestode,
683 *Schistocephalus solidus*, modulate in vitro responses of leukocytes from its specific host,
684 the three-spined stickleback (*Gasterosteus aculeatus*). *Fish & shellfish immunology*.
685 2013;**35**(6):1779-87.
686 35. Yoneva A, Scholz T, Kuchta R. Comparative morphology of surface ultrastructure
687 of diphyllobothriidean tapeworms (Cestoda: Diphyllobothriidea). *Invertebrate biology*.
688 2018;**137**(1):38-48.
689 36. Poulin R. Parasite manipulation of host behavior-Chapter 5: An update and
690 frequently asked questions. *Advances in the Study of Behavior*. 2010;**41**(C):151-86.
691 37. Orr TSC, Hopkins CA, Charles GH. Host specificity and rejection of
692 *Schistocephalus solidus*. *Parasitology*. 1969;**59**(3):683-90.
693 38. Kennedy C. A checklist of British and Irish freshwater fish parasites with notes on
694 their distribution. *Journal of Fish Biology*. 1974;**6**(5):613-44.
695 39. Hamley M, Franke F, Kurtz J, Scharsack JP. An experimental approach to the
696 immuno-modulatory basis of host-parasite local adaptation in tapeworm-infected
697 sticklebacks. *Experimental parasitology*. 2017;**180**:119-32.
698 40. Kalbe M, Eizaguirre C, Scharsack JP, Jakobsen PJ. Reciprocal cross infection of
699 sticklebacks with the diphyllobothriidean cestode *Schistocephalus solidus* reveals
700 consistent population differences in parasite growth and host resistance. *Parasites &*
701 *vectors*. 2016;**9**(1):130.
702 41. Nishimura N, Heins DC, Andersen RO, Barber I, Cresko WA. Distinct lineages of
703 *Schistocephalus* parasites in threespine and ninespine stickleback hosts revealed by
704 DNA sequence analysis. *PLoS One*. 2011;**6**(7):e22505.

705 42. Piecyk A, Ritter M, Kalbe M. The right response at the right time: Exploring
706 helminth immune modulation in sticklebacks by experimental coinfection. *Molecular*
707 *ecology*. 2019;**28**(10): 2668-2680.
708 43. Weber JN, Kalbe M, Shim KC, Erin NI, Steinel NC, Ma L, et al. Resist Globally,
709 Infect Locally: A transcontinental test of adaptation by stickleback and their tapeworm
710 parasite. *The American naturalist*. 2017;**189**(1):43.
711 44. Simmonds NE, Barber I. The effect of salinity on egg development and viability of
712 *Schistocephalus solidus* (Cestoda: Diphyllobothriidea). *Journal of Parasitology*.
713 2016;**102**(1):42-7.
714 45. Confer A, Vu V, Drevecky C, Aguirre W. Occurrence of *Schistocephalus solidus* in
715 anadromous threespine stickleback. *Journal of Parasitology*. 2012;**98**(3):676-8.
716 46. Macat Z, Bednarik A, Rulik M. The three-spined stickleback (*Gasterosteus*
717 *aculeatus*) infection with *Schistocephalus solidus* in Hel marina (Puck Bay, Baltic Sea,
718 Poland). *Oceanological and hydrobiological studies*. 2015;**44**(1):11-7.
719 47. Franke F, Rahn AK, Dittmar J, Erin N, Rieger JK, Haase D, et al. In vitro leukocyte
720 response of three-spined sticklebacks (*Gasterosteus aculeatus*) to helminth parasite
721 antigens. *Fish & shellfish immunology*. 2014;**36**(1):130-40.
722 48. De Bekker C, Quevillon LE, Smith PB, Fleming KR, Ghosh D, Patterson AD, et al.
723 Species-specific ant brain manipulation by a specialized fungal parasite. *BMC*
724 *evolutionary biology*. 2014;**14**(1):166.
725 49. Thompson JN. The role of coevolution. *Science*. 2012;**335**(6067):410-1.
726 50. Franceschi N, Cornet S, Bollache L, Dechaume-Moncharmont FX, Bauer A,
727 Motreuil S, et al. Variation between populations and local adaptation in acanthocephalan-
728 induced parasite manipulation. *Evolution*. 2010;**64**(8):2417-30.
729 51. Cao X, Fu Z, Zhang M, Han Y, Han Q, Lu K, et al. Excretory/secretory proteome
730 of 14-day schistosomula, *Schistosoma japonicum*. *Journal of Proteomics*.
731 2016;**130**(C):221-30.
732 52. Poulin R, FitzGerald GJ. Risk of parasitism and microhabitat selection in juvenile
733 sticklebacks. *Canadian Journal of Zoology*. 1989;**67**(1):14-8.
734 53. Link AJ, Labaer J. Trichloroacetic acid (TCA) precipitation of proteins. *Cold Spring*
735 *Harbor protocols*. 2011;**2011**(8):993.

736 54. Berger CS, Aubin-Horth N. An eDNA-qPCR assay to detect the presence of the
737 parasite *Schistocephalus solidus* inside its threespine stickleback host. *The Journal of*
738 *Experimental Biology*. 2018; **221**: jeb178137.
739 55. Noldus L, Spink A, Tegelenbosch R. EthoVision: A versatile video tracking system
740 for automation of behavioral experiments. *Behavior Research Methods, Instruments, &*
741 *Computers*. 2001;**33**(3):398-414.
742 56. RCoreTeam. R: A language and environment for statistical computing. *R*
743 *Foundation for Statistical Computing*, Vienna, Austria. 2018.
744 57. Bates D, Maechler M, Bolker B, Walker S. Fitting linear mixed-effects models using
745 lme4. *Journal of Statistical Software*. 2015;**67**(1):1-48.
746 58. Venables WN, Ripley BD. Modern Applied Statistics with S. Fourth Edition.
747 *Springer, New York*. 2002.
748 59. Kuznetsova A, Brockhoff P, Christensen R. lmerTest package: Tests in linear
749 mixed effects models. *Journal of Statistical Software*. 2017;**82**(13):1–26.
750 60. Ditgen D, Anandarajah EM, Meissner KA, Brattig N, Wrenger C, Liebau E.
751 Harnessing the helminth secretome for therapeutic immunomodulators. *BioMed research*
752 *international*. 2014;**2014**: 964350.
753 61. Talarico M, Seifert F, Lange J, Sachser N, Kurtz J, Scharsack JP. Specific
754 manipulation or systemic impairment? Behavioural changes of three-spined sticklebacks
755 (*Gasterosteus aculeatus*) infected with the tapeworm *Schistocephalus solidus*.
756 *Behavioral ecology and sociobiology*. 2017;**71**(2):36.
757 62. Fayard M, Cezilly F, Perrot-Minnot M-J. Inter-population variation in the intensity
758 of host manipulation by the fish acanthocephalan *Pomphorhynchus tereticollis*: are
759 differences driven by predation risk? *Parasitology*. 2019;**146**(10):1296-304.
760 63. Rosenkranz M, Poulin R, Selbach C. Behavioural impacts of trematodes on their
761 snail host: Species-specific effects or generalised response? *Ethology*.
762 2018;**124**(11):790-5.
763 64. Piecyk A, Roth O, Kalbe M. Specificity of resistance and geographic patterns of
764 virulence in a vertebrate host-parasite system. *BMC evolutionary biology*. 2019;**19**(1):80.
765 65. Twu O, Johnson PJ. Parasite extracellular vesicles: Mediators of intercellular
766 communication. *PLoS Pathogens*. 2014;**10**(8):e1004289.

767 66. Zaborowski MP, Balaj L, Breakefield XO, Lai CP. Extracellular vesicles:
768 Composition, biological relevance, and methods of study. *BioScience*. 2015;**65**(8):783-
769 97.
770

771

FIGURES

Figure 1. Two boldness-related behaviours differ between *S. solidus*-infected and non-infected sticklebacks. 1. Behaviours that were significantly different between infected and non-infected sticklebacks ($p < 0.05$). A. latency to feed before a predator attack (sec). B. time spent frozen after a predator attack (sec). **2.** Behaviours that were non-significantly different between infected and non-infected sticklebacks. A. latency to leave the acclimation zone (sec). B. time spent swimming (sec). C. time in the center (sec). D. time spent fleeing after a predator attack (sec). E. latency to feed after a predator attack (sec). Each comparison was performed between infected fish and non-infected fish (from the PBS group) of the control week. For each boxplot, the black line represents the median and the green triangle represents the average. Each dot stands for one individual.

Figure 2. Injections of non-infective secretome significantly lowers the expression of boldness-related behaviours, only in fish from the allopatric population. 1. Latency to feed before a predator attack (sec) in the Témiscouata population. A. Infected fish. B. Fish injected with control PBS. C. Fish injected with “infective secretome”. D. Fish injected with “non-infective secretome”. E. Fish injected with “denatured secretome”. **2.** Latency to feed before a predator attack (sec) in the anadromous population. A. Fish injected with control PBS. B. Fish injected with “infective secretome”. C. Fish injected with “non-infective secretome”. D. Fish injected with “denatured secretome”. For each boxplot, the black line represents the median and the green triangle represents the average. Each dot stands for one individual. Each grey line is the reaction norm of one individual between the control week and the injection week.

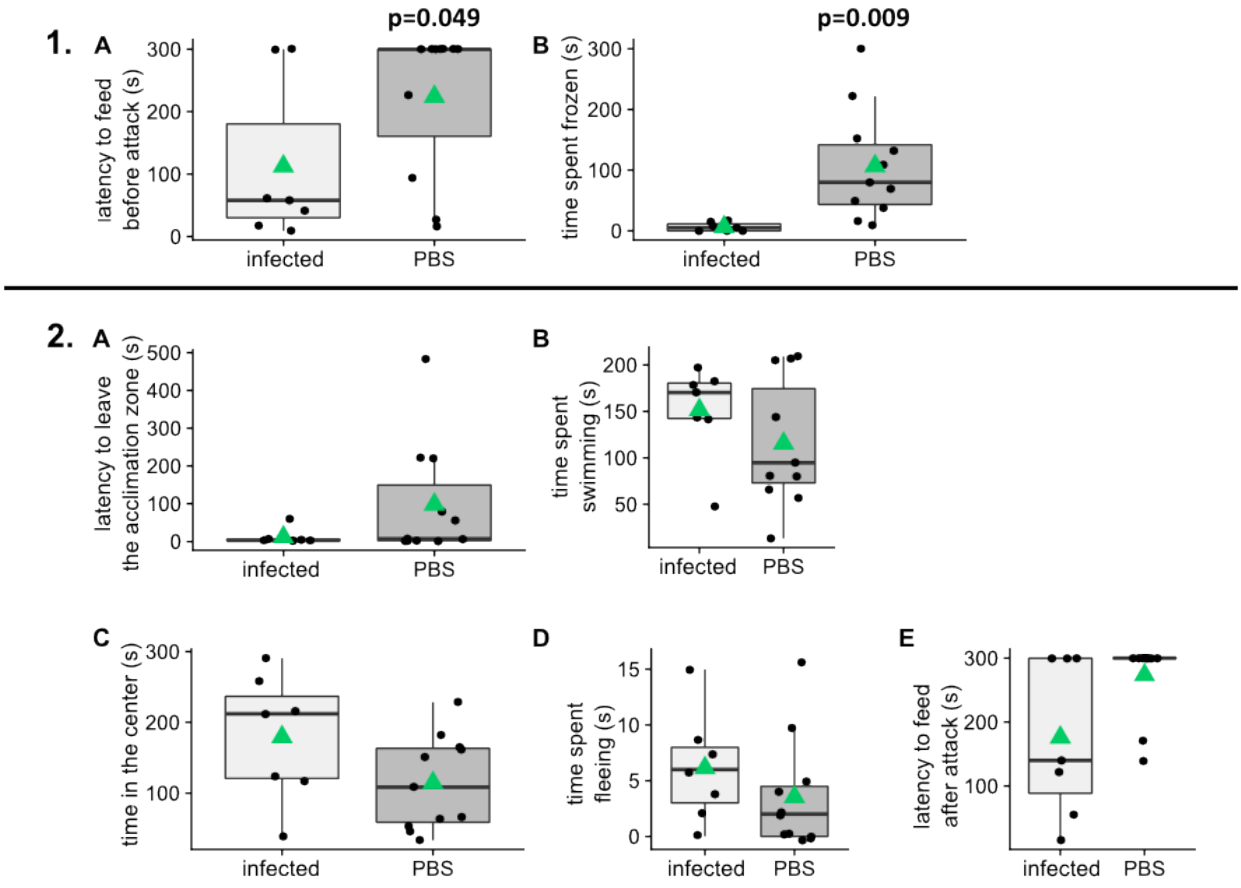

1.
A
p=0.049
latency to feed before attack (s)
300
200
100
0
infected
PBS
B
p=0.009
time spent frozen (s)
300
200
100
0
infected
PBS
2.
A
latency to leave the acclimation zone (s)
500
400
300
200
100
0
infected
PBS
B
time spent swimming (s)
200
150
100
50
infected
PBS
C
time in the center (s)
300
200
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

Figure 1.

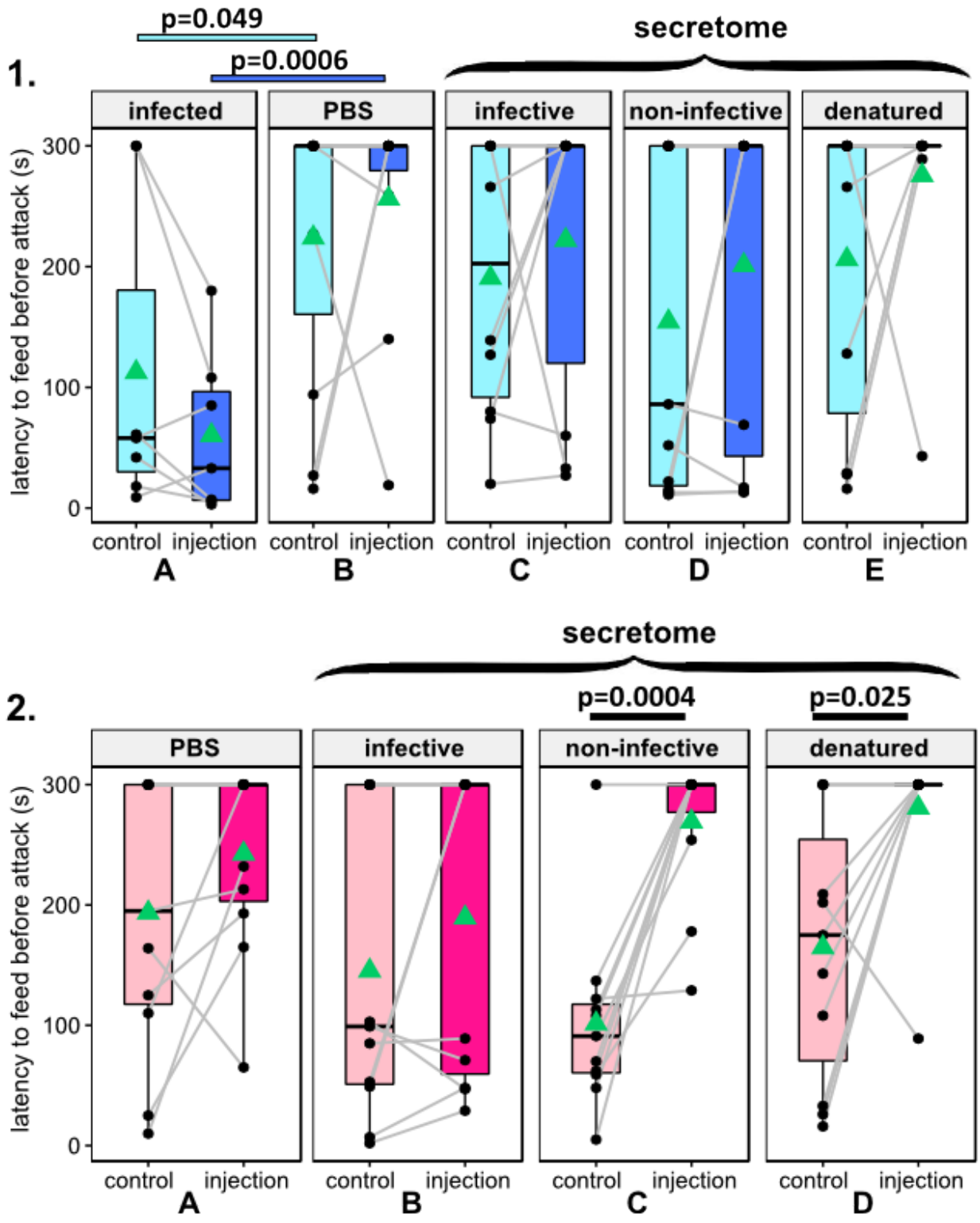
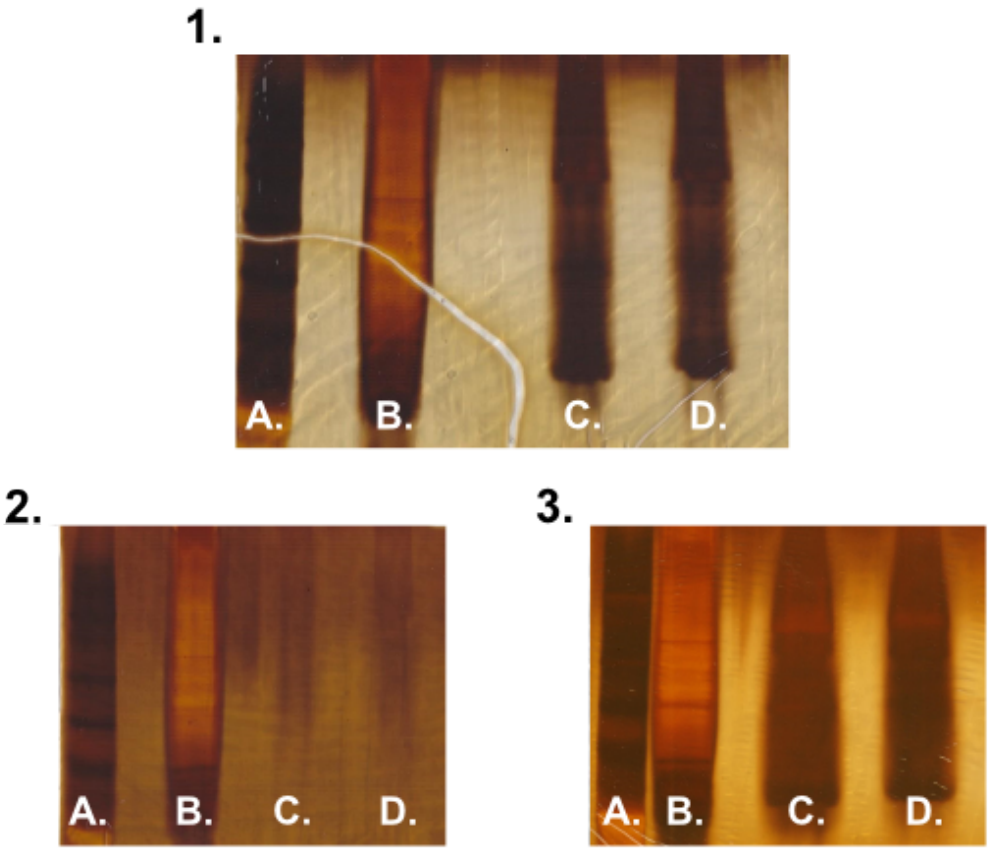
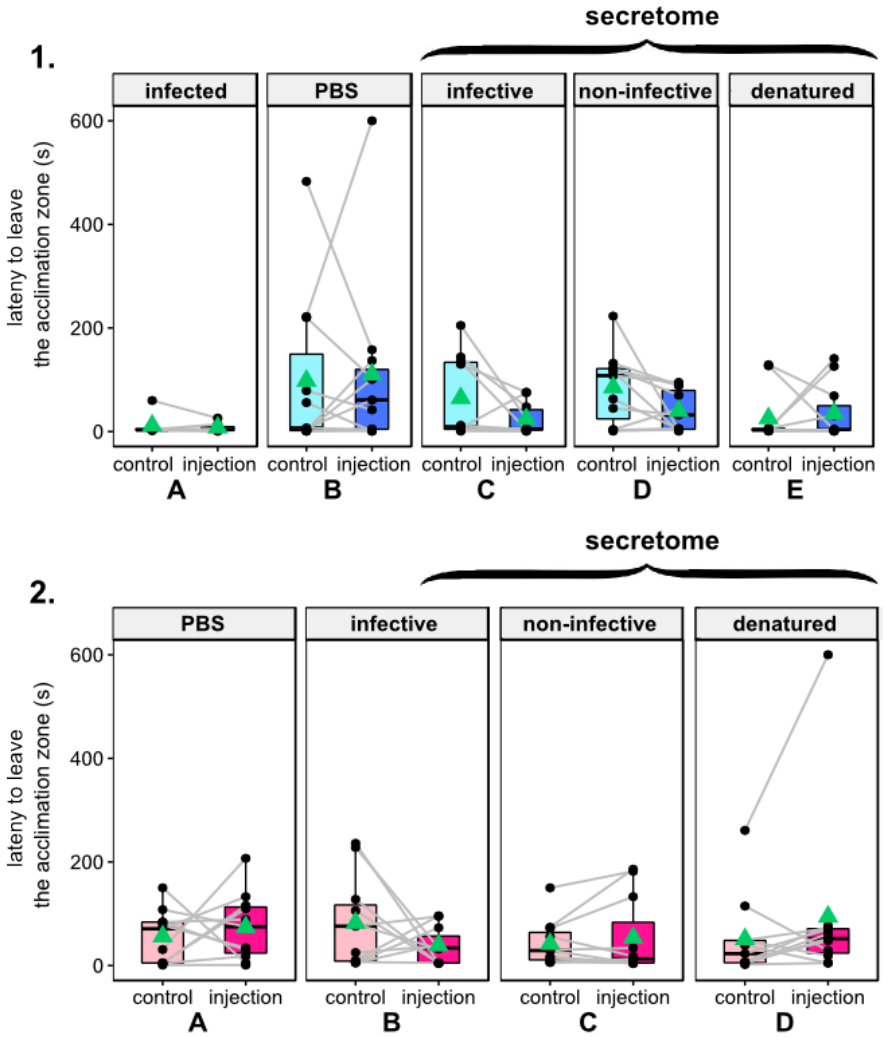

1.
p=0.049
p=0.0006
secretome
infected
PBS
infective
non-infective
denatured
latency to feed before attack (s)
300
200
100
0
control
injection
A
B
C
D
E
2.
secretome
p=0.0004
p=0.025
PBS
infective
non-infective
denatured
latency to feed before attack (s)
300
200
100
0
control
injection
A
B
C
D

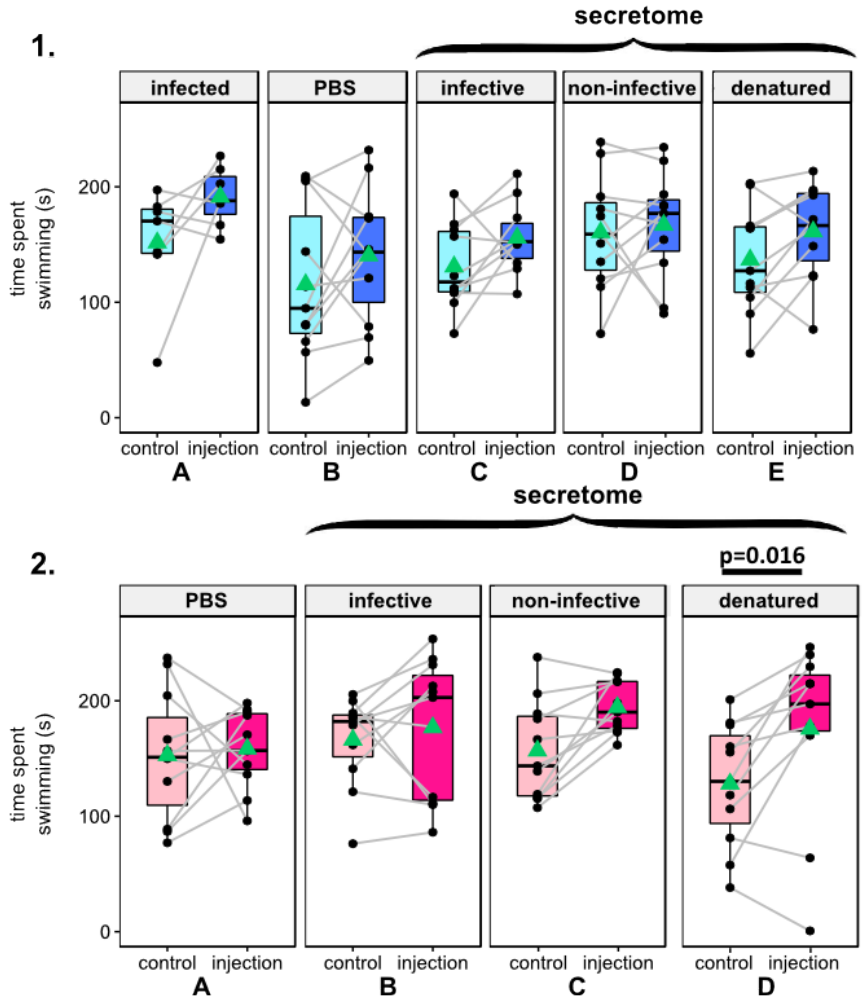
Figure 2.



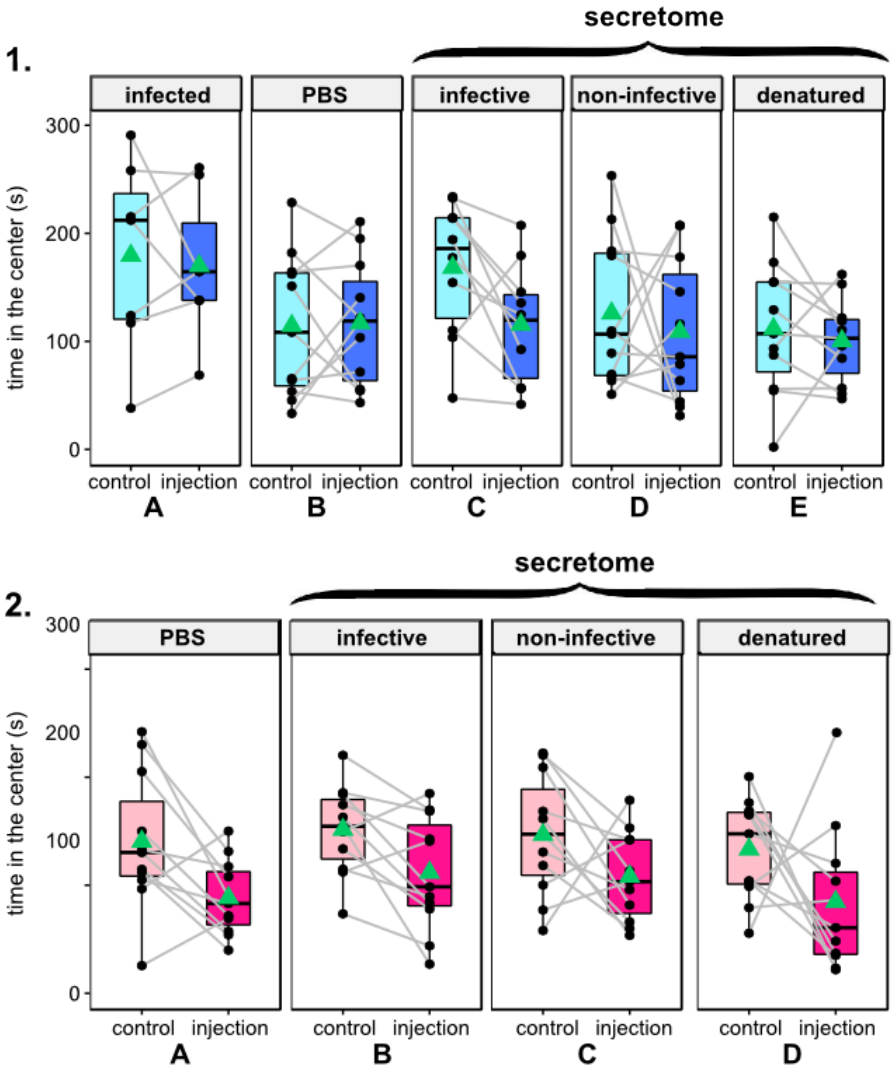
Supplementary Figure 1. Visualization of protein content of secretome on SDS-PAGE gel. 1. "Infective secretome". **2.** "Non-infective secretome". **3.** "Denatured secretome". A. Ladder. B. Yeast extract. C. and D. Secretome**.**



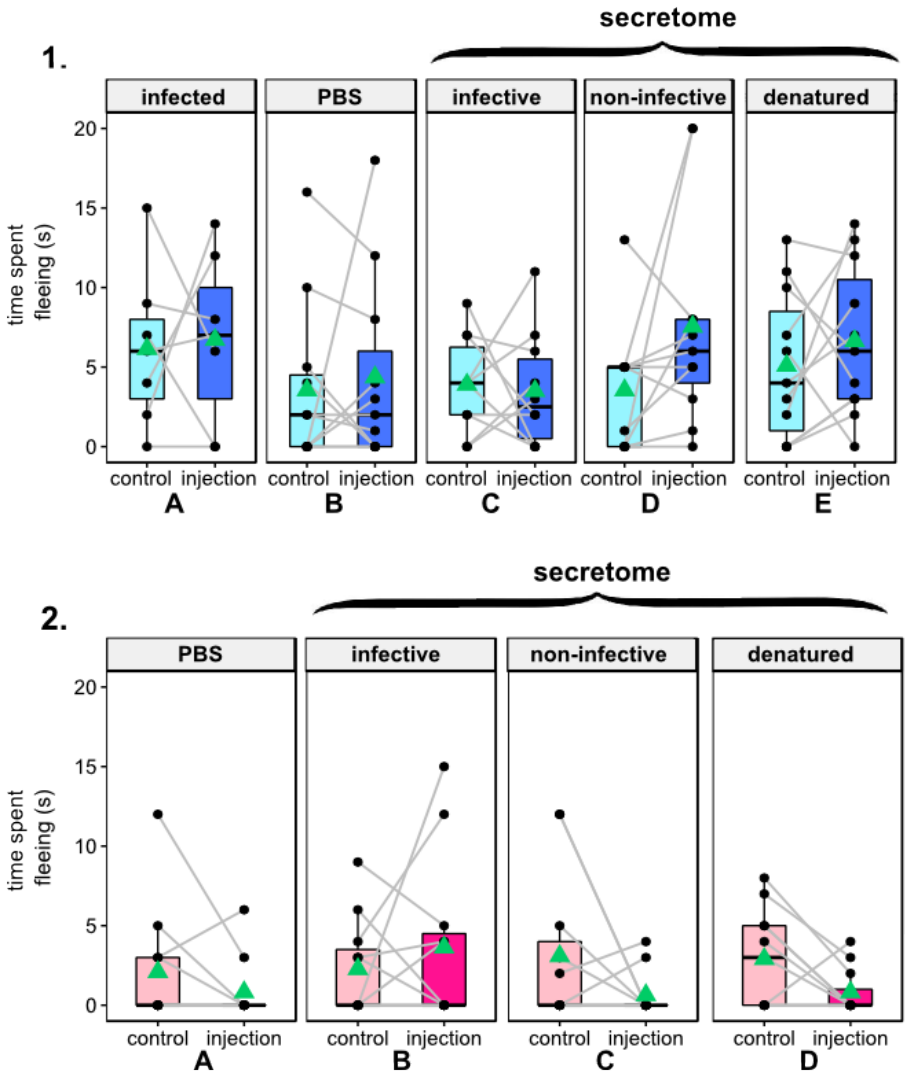
Supplementary Figure 2. Latency to leave the acclimation zone (sec) 1. in the Témiscouata population. A. Infected fish. B. Fish injected with control PBS. C. Fish injected with "infective secretome". D. Fish injected with "non-infective secretome". E. Fish injected with "denatured secretome". **2.** in the anadromous population. A. Fish injected with control PBS. B. Fish injected with "infective secretome". C. Fish injected with "non-infective secretome". D. Fish injected with "denatured secretome". For each boxplot, the black line represents the median and the green triangle represents the average. Each dot stands for one individual. Each grey line is the reaction norm of one individual between the control week and the injection week.



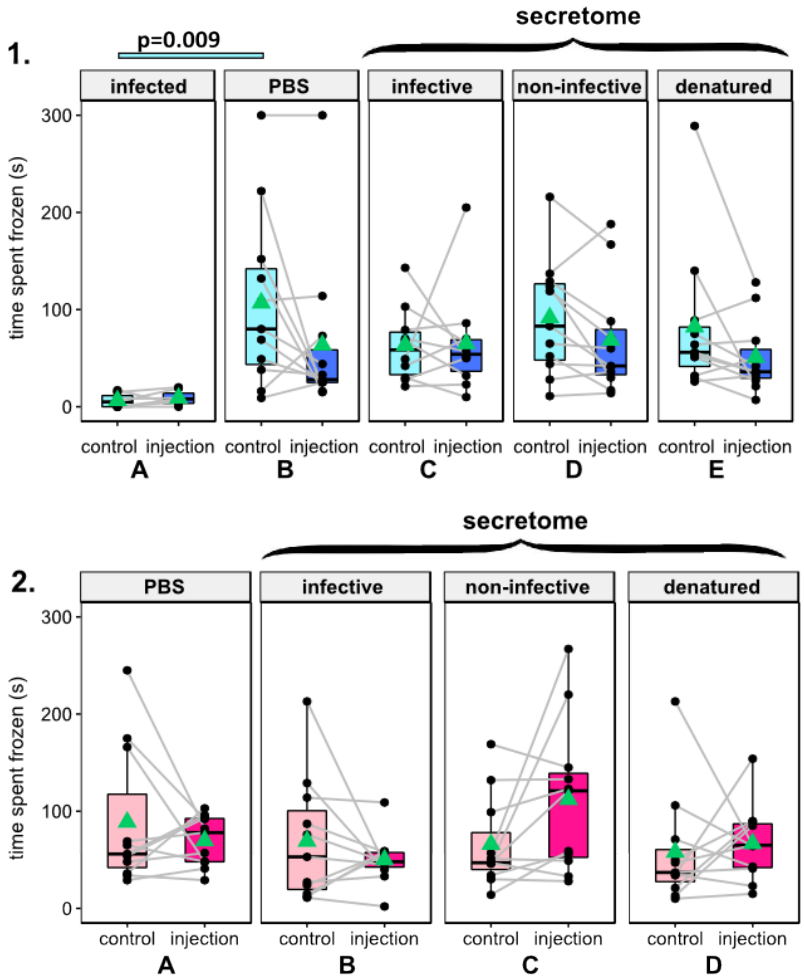
Supplementary Figure 3. Time spent swimming (sec) 1. in the Témiscouata population. A. Infected fish. B. Fish injected with control PBS. C. Fish injected with "infective secretome". D. Fish injected with "non-infective secretome". E. Fish injected with "denatured secretome". **2.** in the anadromous population. A. Fish injected with control PBS. B. Fish injected with "infective secretome". C. Fish injected with "non-infective secretome". D. Fish injected with "denatured secretome". For each boxplot, the black line represents the median and the green triangle represents the average. Each dot stands for one individual. Each grey line is the reaction norm of one individual between the control week and the injection week.



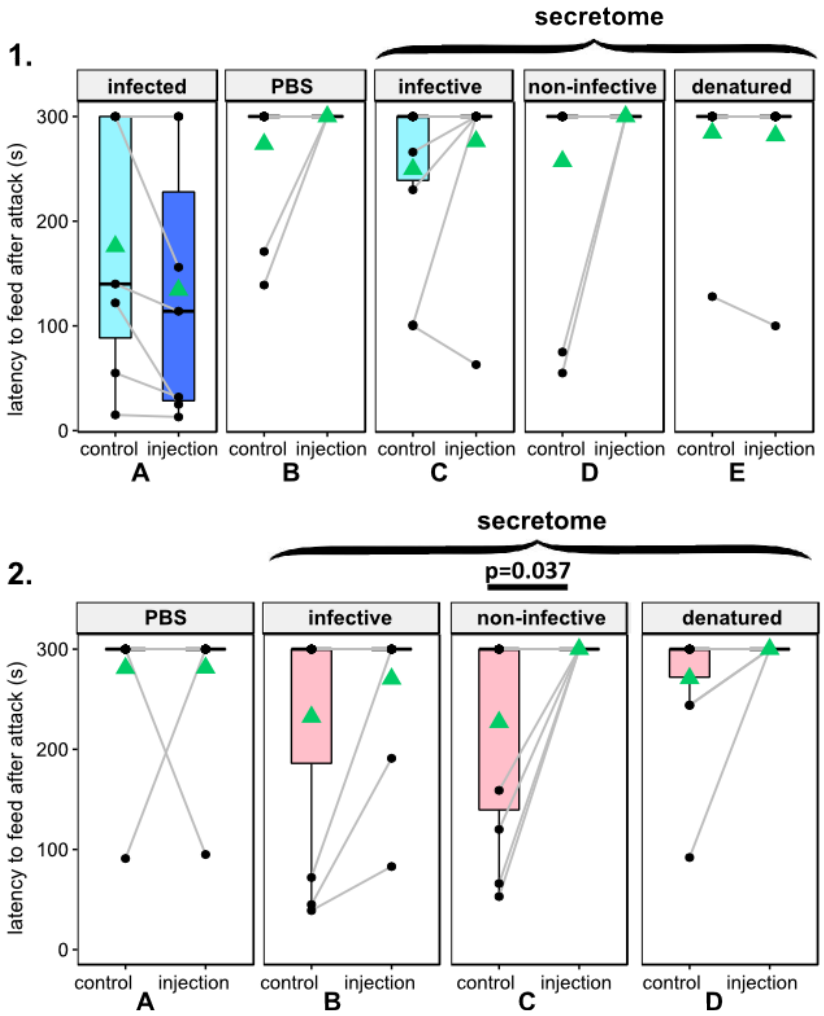
Supplementary Figure 4. Time spent in the center of the aquarium (sec) 1. in the Témiscouata population. A. Infected fish. B. Fish injected with control PBS. C. Fish injected with “infective secretome”. D. Fish injected with “non-infective secretome”. E. Fish injected with “denatured secretome”. **2.** in the anadromous population. A. Fish injected with control PBS. B. Fish injected with “infective secretome”. C. Fish injected with “non-infective secretome”. D. Fish injected with “denatured secretome”. For each boxplot, the black line represents the median and the green triangle represents the average. Each dot stands for one individual. Each grey line is the reaction norm of one individual between the control week and the injection week.



Supplementary Figure 5. Time spent fleeing after a predator attack (sec) 1. in the Témiscouata population. A. Infected fish. B. Fish injected with control PBS. C. Fish injected with “infective secretome”. D. Fish injected with “non-infective secretome”. E. Fish injected with “denatured secretome”. **2.** in the anadromous population. A. Fish injected with control PBS. B. Fish injected with “infective secretome”. C. Fish injected with “non-infective secretome”. D. Fish injected with “denatured secretome”. For each boxplot, the black line represents the median and the green triangle represents the average. Each dot stands for one individual. Each grey line is the reaction norm of one individual between the control week and the injection week.



Supplementary Figure 6. Time spent frozen after a predator attack (sec) 1. in the Témiscouata population. A. Infected fish. B. Fish injected with control PBS. C. Fish injected with “infective secretome”. D. Fish injected with “non-infective secretome”. E. Fish injected with “denatured secretome”. **2.** in the anadromous population. A. Fish injected with control PBS. B. Fish injected with “infective secretome”. C. Fish injected with “non-infective secretome”. D. Fish injected with “denatured secretome”. For each boxplot, the black line represents the median and the green triangle represents the average. Each dot stands for one individual. Each grey line is the reaction norm of one individual between the control week and the injection week.



Supplementary Figure 7. Latency to feed after a predator attack (sec) 1. in the Témiscouata population. A. Infected fish. B. Fish injected with control PBS. C. Fish injected with "infective secretome". D. Fish injected with "non-infective secretome". E. Fish injected with "denatured secretome". **2.** in the anadromous population. A. Fish injected with control PBS. B. Fish injected with "infective secretome". C. Fish injected with "non-infective secretome". D. Fish injected with "denatured secretome". For each boxplot, the black line represents the median and the green triangle represents the average. Each dot stands for one individual. Each grey line is the reaction norm of one individual between the control week and the injection week.

855 **SUPPLEMENTARY MATERIAL**

856

857 **Supplementary tables in PDF format. Results of all the statistical comparisons**
858 **performed with the lmer function to determine if the secretome injections change**
859 **the behaviour of non-infected fish of the Témiscouata population and of the**
860 **anadromous population.** Results are reported in separate tables for each of the seven
861 behaviours and for each population (supplementary tables 1 to 14).

862

863 **Supplementary dataset in tab-delimited format. Complete dataset of behavioural**
864 **and phenotypic results for the Témiscouata and the anadromous populations of**
865 **threespine stickleback.** Data on seven behaviours measured during the control and
866 treatment weeks, mass (g), size (mm) and sex of each fish are reported, as well as the
867 infection status (no=0; yes=1), the number and the total mass of *S. solidus* (g) for
868 individual fish in each treatment.

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