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1 **Title: Shedding Light on the Threespine Stickleback Circadian Clock**

2

3 Running title: Stickleback circadian clock and activity

4

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6

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11

12 Key words: circadian rhythms, threespine stickleback, clock genes, locomotor activity,
13 natural population, interindividual variation

14

15 Summary statement: We found that in wild-caught threespine sticklebacks, the circadian
16 clock does not control locomotor activity in most, but not all, individuals. Sticklebacks are
17 mostly nocturnal, although interindividual variation exists.

18

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19 **ABSTRACT**

20 The circadian clock is an internal timekeeping system shared by most organisms, and
21 knowledge about its functional importance and evolution in natural environments is still
22 needed. Here, we investigated the circadian clock of wild-caught threespine sticklebacks
23 (*Gasterosteus aculeatus*) at the behavioural and molecular levels. While their behaviour,
24 ecology, and evolution are well studied, information on their circadian rhythms are scarce.
25 We quantified the daily locomotor activity rhythm under a light-dark cycle (LD) and under
26 constant darkness (DD). Under LD, all fish exhibited significant daily rhythmicity, while under
27 DD, only 18% of individuals remained rhythmic. This interindividual variation suggests that
28 the circadian clock controls activity only in certain individuals. Moreover, under LD, some
29 fish were almost exclusively nocturnal, while others were active around the clock.
30 Furthermore, the most nocturnal fish were also the least active. These results suggest that
31 light masks activity more strongly in some individuals than others. Finally, we quantified the
32 expression of five clock genes in the brain of sticklebacks under DD using qPCR. We did
33 not detect circadian rhythmicity, which could either indicate that the clock molecular
34 oscillator is highly light-dependent, or that there was an oscillation but that we were unable
35 to detect it. Overall, our study suggests that a strong circadian control on behavioural
36 rhythms may not necessarily be advantageous in a natural population of sticklebacks and
37 that the daily phase of activity varies greatly between individuals because of a differential
38 masking effect of light.

39

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40 **INTRODUCTION**

41 Many behaviours and physiological processes in living organisms exhibit daily rhythmicity,
42 for example: locomotor and feeding activity, hormone secretion, and metabolism (Refinetti,
43 2016). Some of these rhythms persist in the absence of external cues, because they are
44 driven by an endogenous mechanism called the circadian clock (Kumar, 2017). Found in
45 almost all life forms, this internal clock usually has an intrinsic period of approximately 24 h
46 and is entrained by temporal signals such as the light-dark cycle, so that the phase of
47 circadian rhythms is synchronized with relevant environmental variables (ex.: being awake
48 when feeding or mating opportunities are present). The circadian clock thus allows the
49 anticipation of daily environmental changes and the coordination of biological functions, and
50 can have fitness consequences (Vaze and Sharma, 2013; Dominoni et al., 2017). The heart
51 of the circadian clock is a cell-autonomous molecular oscillator made up of a transcription-
52 translation feedback loop that involves positive and negative elements (Bell-Pedersen et al.,
53 2005). In mammals, BMAL and CLOCK are positive elements that induce the transcription
54 of *period* (*per*) and *cryptochrome* (*cry*). PER and CRY are negative elements that inhibit
55 their own transcription by down-regulating the activity of BMAL and CLOCK (Rosensweig
56 and Green, 2020). Generally speaking, the expression level of *bmal* and *clock* is in antiphase
57 with that of *per* and *cry* (Takahashi, 2017). These four clock genes are highly conserved in
58 animals, but, because of genome duplication events, several of them retain two paralogous
59 copies in the different vertebrate lineages (Bell-Pedersen et al., 2005).
60
61 In the last decade, our knowledge of the organization and functioning of circadian rhythms
62 in animals has expanded with the study of various wild species, building on the work mostly
63 acquired in laboratory settings with model organisms (*Mus musculus*, *Danio rerio*,
64 *Drosophila melanogaster*) (Kronfeld-Schor et al., 2013; Schwartz et al., 2017). This growing
65 body of research shows that the implication of the circadian clock in driving biological
66 rhythms can vary greatly depending on a species' biology (reviewed in Bloch et al., 2013;
67 Hazlerigg and Tyler, 2019) and that the opportunities, threats and challenges that organisms
68 face in natural environments can influence their circadian rhythms (Hut et al., 2012; Helm et
69 al., 2017). For example, some species adjust the phase of their circadian activity rhythm in
70 response to light intensity (Chiesa et al., 2010), food availability (Lopez-Olmeda et al., 2010;
71 Ware et al., 2012), predation risk (Pellman et al., 2015) and social interactions (Fuchikawa
72 et al., 2016). In order to improve our understanding of the functional importance of the
73 circadian clock in nature (i.e. the benefits it provides to an individual in a given environment)

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74 and which selection pressures can shape the evolution of circadian rhythms, we must
75 continue to investigate a diversity of species that have evolved in various ecological contexts
76 and that are amenable to experimental and physiological studies (Kronfeld-Schor et al.,
77 2013; Schwartz et al., 2017). The threespine stickleback (*Gasterosteus aculeatus*) is well
78 studied in ecology and evolution (McKinnon et al., 2019). This small fish is also well suited
79 to answer questions about the ecological and evolutionary implications of the circadian clock
80 through the study of its natural phenotypic variation, which can be combined with
81 experimental work. Stickleback ecotypes are found in diverse habitats (marine waters, salt
82 marshes, streams, rivers, lakes, etc.) and display morphological, physiological, and
83 behavioural adaptations to these environments (Bell and Foster, 1994; Ostlund-Nilsson et
84 al., 2007; Kitano et al., 2010; Di-Poi et al., 2014; Di Poi et al., 2016; Ishikawa et al., 2019).
85 Many of the environmental pressures that differ between ecotypes such as the presence of
86 predators and parasites, prey availability, light intensity and social interactions (Ostlund-
87 Nilsson et al., 2007) have the potential to influence circadian rhythms (Helm et al., 2017).
88 This could be achieved either through selective pressure resulting in genetic divergence, or
89 through phenotypic plasticity, i.e. the effects of the environment on the development of a
90 phenotype, here the circadian rhythm itself. As sticklebacks are also known for their
91 interindividual variation in behaviour, called personality (activity, boldness, sociality, etc.
92 (Huntingford, 1976; Bell, 2005; Wark et al., 2011)), it is also possible that they exhibit
93 interindividual variation in circadian rhythms. So far, it has been suggested that circadian
94 molecular mechanisms may vary between ecotypes similarly to traits at other levels of
95 biological organization, although the functional impact of this difference is not known. For
96 example, using common garden-raised sticklebacks from two lake-stream pairs, a previous
97 study reported that a gene that is part of the molecular oscillator (*cry1ab*) was upregulated
98 in the liver of stream sticklebacks compared to lake ones (Hanson et al., 2017). Studying
99 circadian rhythms in sticklebacks will help us to better understand the functional importance
100 and the evolution of the circadian clock in natural environments.
101
102 In comparison to what is known about the ecology and evolution of sticklebacks, very little
103 knowledge is available on their circadian rhythms and clock. In fact, the existence of a
104 circadian clock has never been demonstrated in this species. At the behavioural level,
105 sticklebacks have, to our knowledge, only been studied once under constant light conditions.
106 This study showed that the frequency with which males visited their nests (in the hope of
107 finding eggs deposited by a female) did not display circadian rhythmicity in constant light

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108 (Sevenster et al., 1995). Regarding the daily activity rhythm (i.e. under a light-dark cycle),
109 some evidence suggests that sticklebacks are diurnal. For instance, stickleback visual
110 opsins (Rennison et al., 2012) correspond to those of diurnal fish (Carleton et al., 2020).
111 Moreover, previous studies reported that sticklebacks were mostly captured during the day
112 in the wild (Worgan and FitzGerald, 1981; Sjoberg, 1985; Reebs et al., 1995). On the other
113 hand, night activity (Reebs et al., 1984; Quinn et al., 2012) and night feeding (Mussen and
114 Peeke, 2001) have been observed in some sticklebacks. At the physiological level, we know
115 that melatonin levels (a hormone that plays a key role in the regulation of circadian rhythms)
116 are higher during the night than during the day in sticklebacks (Mayer et al., 1997;
117 Kulczykowska et al., 2017; Pomianowski et al., 2020) as in most vertebrates (Challet, 2007;
118 Falcón et al., 2009), but we do not know if this rhythm is driven by the clock or solely by light
119 (Falcón et al., 2009). At the molecular level, time-of-day variation in the expression of *per1b*
120 and *clock1b* has been observed in the liver of sticklebacks, but since this was measured
121 under a light-dark cycle, we do not know if this rhythm is self-sustained (Prokkola et al.,
122 2015).
123
124 In this study, using wild-caught threespine sticklebacks, we investigated the circadian clock
125 of this species at the behavioural and molecular levels. Our first objective was to determine
126 if the daily rhythm of locomotor activity is under circadian clock control, and we hypothesized
127 that it is indeed the case. Our prediction was that sticklebacks would show a significant
128 rhythm of locomotor activity under constant darkness (DD). Our second objective was to
129 determine the phase of activity of sticklebacks under LD. We hypothesized that sticklebacks
130 are diurnal. Our prediction was that the daily activity would be mainly performed during the
131 light phase. Our third objective was to quantify the molecular oscillation of five clock genes
132 (*bmal1a*, *clock1b, clock2, per1b* and *cry1b*) in the brain, an organ that is potentially
133 implicated in the control of circadian rhythms. We hypothesized that clock gene expression
134 shows circadian rhythmicity under DD. Our prediction was that the expression level of
135 *bmal1a*, *clock1b* and *clock2* would be in antiphase with that of *per1b* and *cry1b*.
136

137 **MATERIALS AND METHODS**

138 **Fish sampling and housing**

139 We collected threespine sticklebacks (*Gasterosteus aculeatus*) from the wild population of
140 the lac Témiscouata (47°48’37.1"N 68°51’56.6"W, Québec, Canada) in June 2019. We did
141 not have specific information on the daily activity patterns of this species in the lac

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142 Témiscouata. We thus sampled fish with a beach seine so that we could collect all
143 individuals in the water column no matter if they were resting at the bottom of the lake or
144 swimming at the surface. We sampled fish in the morning (around 8:00), in the afternoon
145 (around 15:00) and in the evening (around 19:00) to account for the possibility that some
146 individuals migrate daily between different parts of the lake. Sticklebacks were brought back
147 to the Laboratoire de Recherches en Sciences Environnementales et Médicales (LARSEM)
148 at Université Laval (Québec, Canada). In the animal facility, fish were held in two 1000 L
149 water tanks (n=140/tank) and were fed brine shrimp and nutritious flakes twice a day
150 (morning and late afternoon). They were exposed to non-breeding environmental conditions,
151 a water temperature of 14°C and a 12 h light:12 h dark cycle with lights on at 6:00 and lights
152 off at 18:00.
153
154 **Activity monitoring system**
155 To monitor locomotor activity, 18 fish were transferred in an adjacent room and individually
156 placed in 2 L experimental tanks. A white plexiglass separated each tank to prevent fish
157 from seeing each other. Lightning was provided by three full-spectrum LED light bars (Plant
158 3.0, Fluval) mounted above the tanks. Illuminance was measured by a lux meter (LX1330B,
159 Dr.meter) and was around 500 lux at the water surface. We chose this illuminance value
160 based on previous studies in other fish species (Iigo and Tabata, 1996; Whitmore et al.,
161 2000; Bayarri et al., 2004; Lopez-Olmeda et al., 2010). A dark plastic curtain was hanging
162 in front of the tanks to ensure a constant illumination (or darkness) when we needed to enter
163 the room for maintenance.
164
165 Each experimental tank was equipped with an infrared photoelectric sensor (E3Z-D67,
166 Omron) placed in the lower third of the front wall (Fig. S1). We had previously established
167 that this position was optimal to record stickleback movements (Fig. S2). Every time a fish
168 interrupted the infrared light beam that was emitted by the sensor, an output signal was sent
169 to a controller (ILC 131 ETH, Phoenix Contact). Each interruption was counted as one
170 movement. Data was retrieved by connecting a computer to the controller.
171
172 **Experimental design**
173 All experimental procedures were approved by the Comité de Protection des Animaux de
174 l'Université Laval (CPAUL 2018066-2). Since we could monitor 18 fish at a time, we divided
175 individuals into three groups (Fig. 1). Individuals were allowed to acclimate to the

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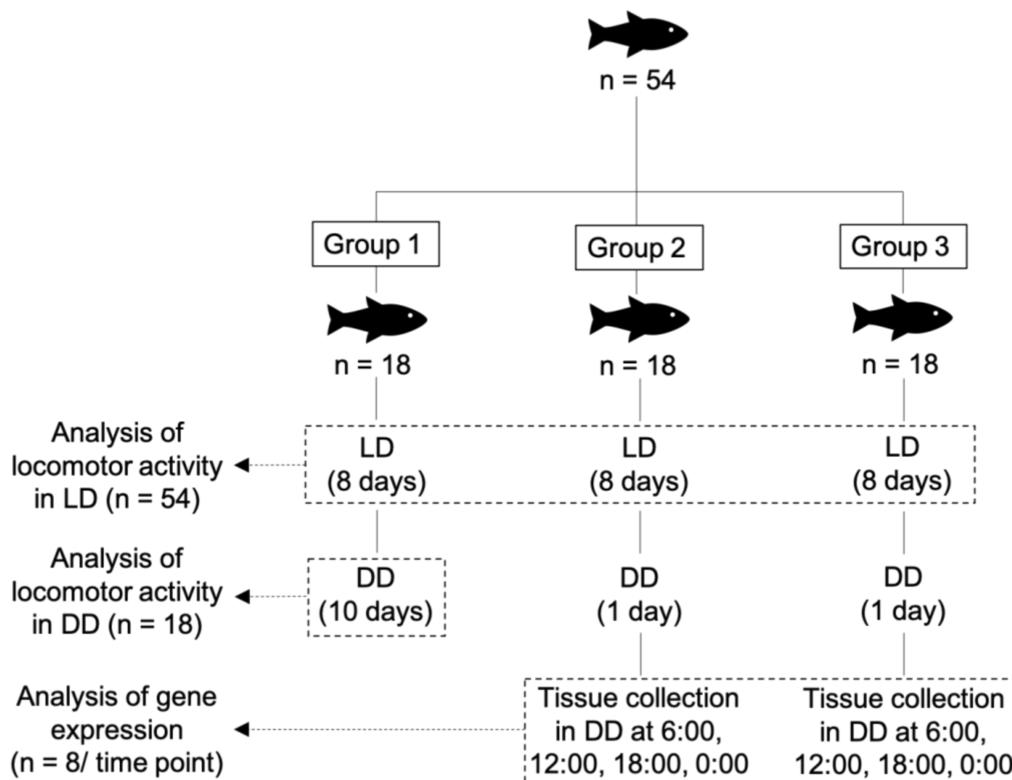
176 experimental tanks for at least three days before the start of the experiment. For all three
177 groups, food was provided by hand once a day at random time (previously determined using
178 the RAND() function in Excel software). We used a dim red light when food was provided
179 during the dark phase.
180
181 Group 1 was exposed to a 12 h light:12 h dark (LD) cycle for eight days (lights on at 6:00
182 and lights off at 18:00) followed by ten days of constant darkness (DD). Group 1 was used
183 to quantify locomotor activity under LD and DD. Groups 2 and 3 were also exposed to LD
184 for eight days and used to quantify locomotor activity under LD. On the ninth day of the
185 experiment with groups 2 and 3, lights were not turned on at 6:00 so all fish were exposed
186 to DD for at least 24 h. On the tenth day, we sampled the brain and the caudal fin of four
187 randomly selected individuals every 6 h throughout a 24-h cycle (6:00, 12:00, 18:00, 0:00),
188 see Fig. 1. Tissue collection was performed in darkness with the help of a dim red light and
189 took less than 3 minutes per fish. After dissection, brains and caudal fins were immediately
190 stabilized in RNA*later* (Ambion) and stored at -20°C. We used caudal fins to determine sex
191 with the IDH genetic sex marker (Peichel et al., 2004).



192
193 **Fig. 1. Experimental design.** Group 1 was used to quantify locomotor activity under a 12 h
194 light:12 h dark cycle (LD, lights on at 6:00 and lights off at 18:00) and under constant

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195 darkness (DD). To that end, group 1 was exposed to LD for 8 days, then to DD for 10 days.
196 Groups 2 and 3 were used to quantify locomotor activity under LD and brain gene expression
197 under DD. Groups 2 and 3 were thus exposed to LD for 8 days, then to DD for 1 day (day
198 9). The day following the switch to DD (day 10), we sampled the brain of four randomly
199 selected individuals every 6 h throughout a 24-h cycle (6:00, 12:00, 18:00, 0:00).
200

201 **Choice of genes**

202 We chose to quantify the expression of *bmal1a*, *clock1b, clock2, per1b* and *cry1b* although
203 sticklebacks have several other clock genes (Table 1). We chose these five genes for three
204 reasons. First, we wanted to quantify positive (*bmal, clock*) and negative (*per, cry*) elements
205 to have an overall view of the transcription-translation feedback loop. Second, we chose
206 genes that have an ortholog in the zebrafish to compare our results with what is known from
207 this model organism (Table 1). Third, we avoided quantifying *per2a* and *cry1aa* because
208 these two genes are mainly light-induced (in opposition to being clock controlled) in the
209 zebrafish (Pando et al., 2001; Tamai et al., 2007; Vatine et al., 2009), so their expression
210 rhythm rapidly loses its amplitude under DD (ex.: Beale et al., 2013) and thus would not be
211 informative in our study in DD.
212

213 **Table 1.** The four core genes of the transcription-translation feedback loop of the clock
214 molecular oscillator in mammals, zebrafish and sticklebacks. The five stickleback genes that
215 we investigated in this study are in bold in the table.

Gene	Mammals	Zebrafish	Stickleback	Reference for the phylogenetic analysis
bmal	*bmal1*	*bmal1a*	***bmal1a***	Wang (2009)
		bmal1b	-	
	bmal2	*bmal2a*	-	
		-	*bmal2b*	
clock	*clock*	*clock1a*	-	Wang (2008b)
		clock1b	***clock1b***	
	npas2	*clock2*	***clock2***	
period (per)	*per1*	*per1a*	-	Wang (2008a)
		per1b	***per1b***	
	per2	*per2a*	*per2a*	
		-	*per2b*	
	per3	*per3*	-	
cryptochrome (cry)	*cry1*	*cry1aa*	*cry1aa*	Liu et al. (2015)
		cry1ab	*cry1ab*	
		cry1ba	***cry1ba***	
		cry1bb	-	
	cry2	*cry2*	*cry2*	
	-	*cry3*	-	

216

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217 **Gene expression in the brain**

218 We studied clock gene expression using a quantitative real-time PCR (qPCR) approach. We
219 extracted total RNA in the brain of sticklebacks and performed a DNase digestion using the
220 miRNeasy Mini Kit (Qiagen) combined with the RNase-Free DNase Set (Qiagen). We stored
221 RNA at -70°C. We quantified RNA using the Quant-iT RiboGreen RNA Assay Kit (Invitrogen)
222 and assessed RNA quality and integrity with the RNA 6000 Nano Kit (Agilent). All samples
223 showed RNA integrity numbers (RIN) greater than 9.0. For all samples we reverse-
224 transcribed 10 µL of RNA at 100 ng µL^{-1} with 4 µL of the 5X qScript cDNA SuperMix
225 (Quantabio) and 6 µL of RNase-free water in a final volume of 20 µL. Following the
226 manufacturer's protocol, thermocycling parameters were 25 °C for 5 min, 42°C for 30 min
227 and 85°C for 5 min.

228

229 We obtained cDNA sequences of *bmal1a*, *clock1b, clock2, per1b* and *cry1b* from the
230 *Ensembl Genome Browser* (version 98) and designed primers using Primer3 (Table 2). We
231 did *in silico* specificity screen with the Amplify4 software to ensure that primers for a given
232 gene were not amplifying any paralogs. We also verified specificity of primers and absence
233 of primer dimers with melting curves (60-95°C). To further guarantee that the primers were
234 amplifying the targeted genes, we analyzed amplicons by Sanger sequencing. We assessed
235 PCR amplification efficiency of each primer pair with a qPCR experiment using a four or five-
236 point standard curve made of a fivefold dilution series of pooled cDNA samples. Efficiency
237 is reported in Table 2.

238

239 **Table 2.** Characteristics of the primers used to quantify clock gene expression. Efficiency
240 (E) was calculated using E = $(10^{-1/slope} - 1)*100$ (Pfaffl, 2001).

Gene	Ensembl Transcript ID	Forward primer	Reverse primer	Amplicon size (bp)	Efficiency (%)
bmal1a	ENSGACT00000003205	ACGGCTCGTTCATCACTCTG	AGTCCGATCCCTCCATCACA	123	98.7
clock1b	ENSGACT00000021080	GATCGACAGATCCGGTTCCC	GTCTGGGTTTGACCTCCCTG	164	98.5
clock2	ENSGACT00000026929	GCACTCACACTGTTGTCAGC	CCTTCACTGAAGAGGGAGCG	105	96.4
per1b	ENSGACT00000025573	CTACCAGCTCACCATCAGAG	ACGAGGAGTTTCGTATCCAG	94	109.8
cry1b	ENSGACT00000017852	GAGACAGAAGGCCTGACCAC	CTCAAAGTTTGCCACCCACG	105	107.9

241

242 We performed qPCR experiments in the 7500 Fast Real-Time PCR System (Applied
243 Biosystems) using 5 µL of cDNA at 1 ng µL^{-1}, 10 µL of the 2X PerfeCTa SYBR Green FastMix

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244 (Quantabio), 1 µL of primer pairs at 10 µM (final concentration of 250 nM for each primer)
245 and 4 µL of nuclease-free water for a total volume of 20 µL. All samples were run in triplicate
246 on a single 96-well plate for a given gene. We included no-template and no-reverse
247 transcription controls. The thermocycling protocol was 95°C for 3 min (initial denaturation),
248 followed by 40 cycles of 15 s at 95°C (denaturation) and 45 s at 60°C (annealing).
249
250 We used the NormFinder software (Andersen et al., 2004) to identify the optimal reference
251 gene (or combination of reference genes) for our experiment between *ubc*, *hprt1*, *rpl13a*,
252 *gapdh* and *β-actin* (Table 3). We did the analysis on 12 cDNA samples that were previously
253 obtained in the same conditions as experimental samples during a pilot study. The
254 NormFinder algorithm identified *ubc* as the most stable gene between time points. We thus
255 calculated the relative expression of target genes using the **ΔΔ**Cq method adjusted for
256 efficiency of each primer pairs (Pfaffl, 2001) with *ubc* as the reference gene.
257
258 **Table 3.** Characteristics of the primers used in the search for the optimal reference gene.
259 Using the NormFinder software, *ubc* was identified as the most stable gene between time
260 points and was used as the reference gene (shown in bold). Efficiency (E) was calculated
261 using E = $(10^{-1/slope} -1)*100$ (Pfaffl, 2001).

Gene	Ensembl Transcript ID	Forward primer	Reverse primer	Amplicon size (bp)	Efficiency (%)	Reference for primer sequences
ubc	**ENSGACT00000010662**	**AGACGGGCATAGCACTTGC**	**CAGGACAAGGAAGGCATCC**	**218**	**102.0**	**Hibbeler et al. (2008)**
hprt1	ENSGACT00000024687	TCTCCTCCGTTAGAAGACTGCAT	TTCAGGTCATACCCTTGCTCATC	92	108.3	*This study*
rpl13a	ENSGACT00000012382	CACCTTGGTCAACTTGAACAGTG	TCCCTCCGCCCTACGAC	218	95.4	Hibbeler et al. (2008)
gapdh	ENSGACT00000007902	CAAACCGTTGGTGACAGTATTTG	GCACTGAGCATAAGGACACATCTAA	71	100.4	Sanogo et al. (2011)
β-actin	ENSGACT00000010474	ACATCAGGGAGTGATGGTGG	CAGGATACCTCTCTTGCTCTG	79	108.2	Gao et al. (2011)

262
263 **Data analysis**
264 *Locomotor activity rhythm*
265 Of the 54 individuals that we used in our experiments, six were discarded from analyses
266 because they died during experiments (n=3) or were parasitized (n=3). For the 48 remaining
267 individuals, we gathered locomotor activity data in 10-min bins for analysis purposes.
268 Actograms, activity profiles and $\mathcal{X}^2$ periodograms were produced using the ActogramJ plugin

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269 in ImageJ (Schmid et al., 2011) for each fish under LD (n=48) and under DD (n=17). The $\mathcal{X}^2$
270 periodogram analysis calculates Qp values for multiple periods within a fixed range. The
271 period with the highest Qp value corresponds to the estimated period of the rhythm. Since
272 Qp has a probability distribution of $\mathcal{X}^2$ (with a P-1 degree of freedom, where P is the period),
273 we can determine if the Qp value for the estimated period is significant with α=0.05
274 (Sokolove and Bushell, 1978). In other words, the periodogram analysis lets us know if the
275 rhythm is significant and, if so, what is the period of this rhythm. We first did the periodogram
276 analysis using periods ranging between 0 h and 32 h, but we did not find any significant
277 ultradian endogenous rhythms (i.e. rhythms with periods shorter than circadian rhythms).
278 Thus, we show periodograms with periods ranging between 16 h and 32 h to facilitate
279 visualization. We performed all other statistical analyses using R software version 4.0.1 (R
280 Core Team, 2020). When needed, we evaluated normal distribution of data using Q-Q plots
281 and Shapiro-Wilk test and we verified homogeneity of variances using Levene's test.
282
283 *Masking effect of light*
284 We evaluated the difference in the average activity level (movements/10 min) during the
285 light phase in LD and the subjective light phase in DD using a paired t-test. This comparison
286 allows us to assess the importance of the masking effect of light, which can suppress or
287 enhance activity without entraining the internal clock (Mrosovsky, 1999). We also verified if
288 the difference in the average activity level during the light phase in LD and the subjective
289 light phase in DD differs between sexes using a t-test.
290
291 *Phase of activity*
292 Although our hypothesis was that sticklebacks are diurnal, we rather observed a tendency
293 towards nocturnality under LD. Thus, to quantify the phase of activity in each fish, we
294 calculated the percentage of the total daily activity performed during the dark phase (also
295 referred as the night activity). By assessing the night activity, we observed large
296 interindividual variation for the phase of activity, but we also noticed large interindividual
297 variation in total daily activity. We thus assessed sex differences in night activity and in total
298 daily activity using t-tests and we evaluated the correlation between these two variables
299 using Pearson's correlation test. Data are represented as mean ± standard error of the
300 mean.
301
302

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303 *Clock gene expression rhythms in the brain*
304 Among the six individuals that were discarded from the analysis, five were from groups 2
305 and 3, so there were 31 individuals left for the brain gene expression analysis. We thus
306 sampled eight individuals at 6:00, 18:00 and 0:00 (n=8) and seven individuals at 12:00 (n=7).
307 Moreover, one individual was removed from the 18:00 time point for *clock2* because it was
308 identified as an extreme outlier using the identify_outliers() function from the rstatix package
309 in R (Kassambara, 2020). We evaluated differences in relative gene expression between
310 time points using one-way ANOVA. Relative gene expression was also subjected to cosinor
311 analysis using the cosinor2 package (Mutak, 2018). The cosinor analysis fits a cosine
312 function with a known period (24 h) to the expression values so that we can estimate the
313 amplitude, the acrophase (peak time) and the mesor (mean of all expression values) of the
314 rhythm (Refinetti et al., 2007). This procedure also calculates the probability that the
315 amplitude is significantly different from zero using the *F*-distribution. When the p-value is
316 <0.05, we can consider that gene expression shows significant circadian rhythmicity.
317
318 **RESULTS**
319 **Locomotor activity rhythm**
320 Under a 12 h light:12 h dark cycle (LD), a significant daily rhythmicity of 24.0 h ($\mathcal{X}^2$
321 periodogram analysis, p<0.05) was observed for all fish (Fig. 2). Under constant darkness
322 (DD), most individuals were arrhythmic (Fig. 3A, C, E) and only three out of seventeen
323 sticklebacks (18%) showed significant circadian rhythmicity ($\mathcal{X}^2$ periodogram analysis,
324 p<0.05, Fig. 3B, D, F) with periods of 24.8 h, 25.0 h and 26.3 h.
325

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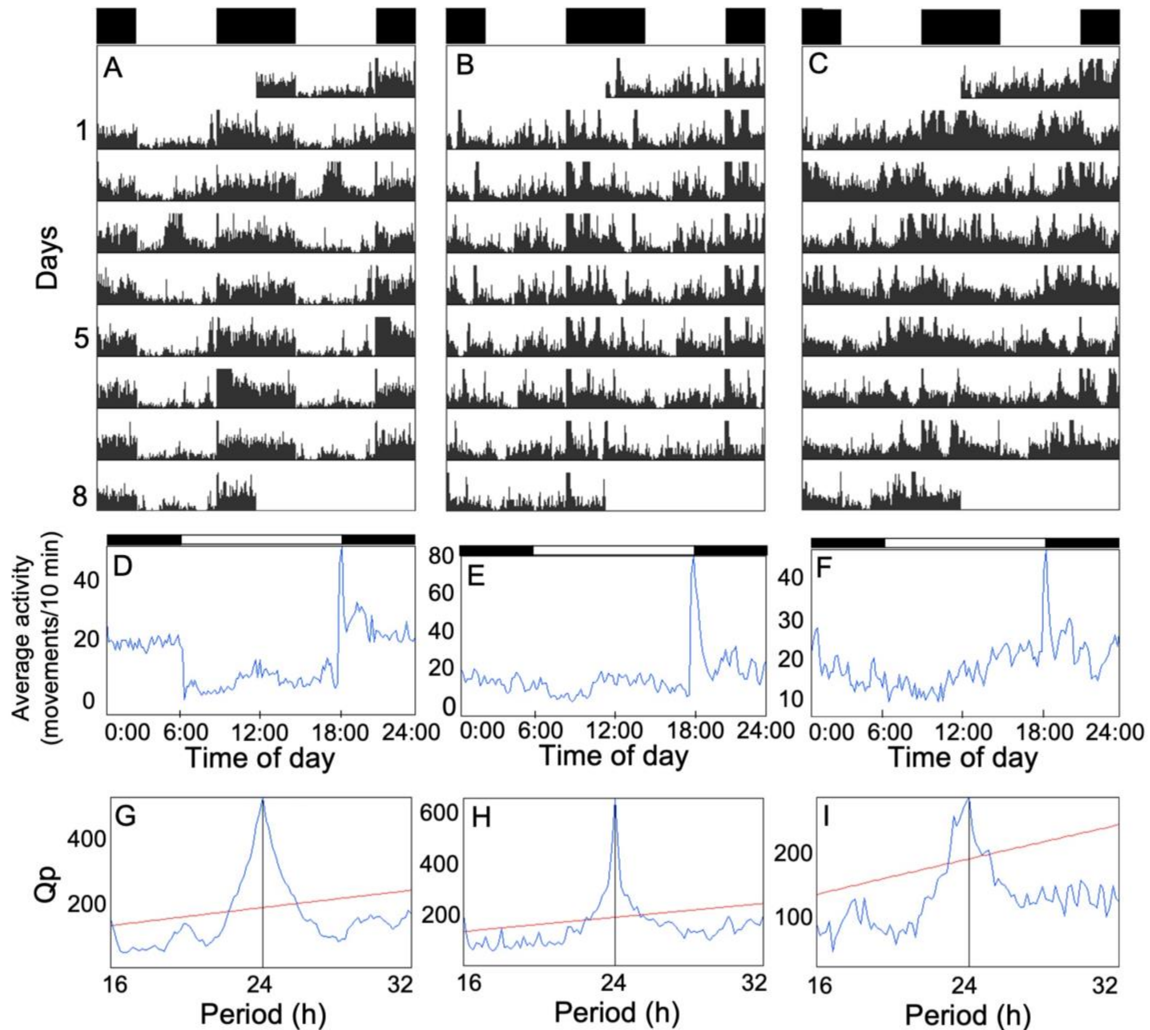

326

327 **Fig. 2. Under LD, sticklebacks display significant daily rhythmicity, but show variable**
328 **activity patterns.** Double-plotted actograms (A-C) of three representative individuals under
329 a 12h light:12h dark cycle (LD) and their corresponding activity profile (D-F) and $\mathcal{X}^2$
330 periodogram (G-I). The white and black bars at the top of the actograms and the activity
331 profiles represent the light and dark phases, respectively. From left to right, individuals
332 display respectively 77%, 65% and 55% of their daily activity during the dark phase. Activity
333 profiles show the average locomotor activity (number of movements) for each 10-min bin
334 over the 8 days in LD. Qp values on the $\mathcal{X}^2$ periodograms quantify the rhythmic component
335 of the activity and the red horizontal line indicates the significance threshold (set at p=0.05).

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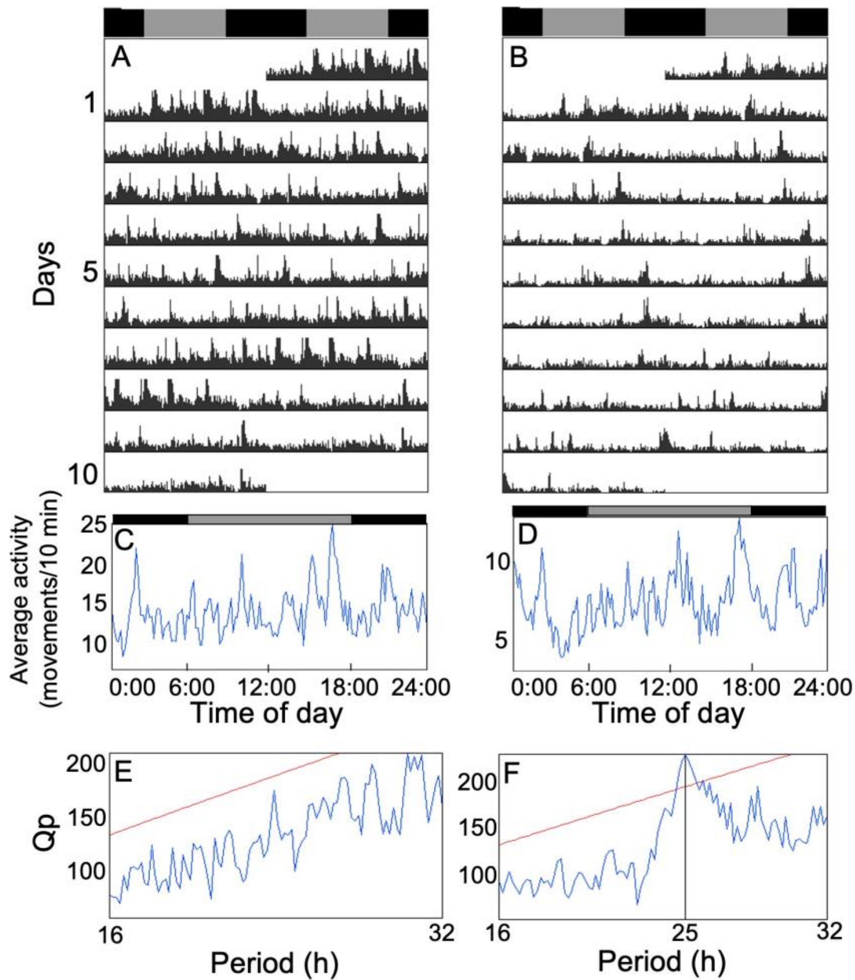

336

337 **Fig. 3. Under DD, most individuals are arrhythmic and only a few individuals show**
338 **circadian rhythmicity.** Double-plotted actograms (A-B) of two representative individuals
339 under constant darkness (DD) and their corresponding activity profile (C-D) and $\mathcal{X}^2$
340 periodogram (E-F). The gray and black bars at the top of the actograms and activity profiles
341 represent the subjective light and dark phases, respectively. Under DD, most sticklebacks
342 do not display circadian rhythmicity, as represented by the individual on the left of the figure.
343 On the right, we show one of the three individuals who exhibit significant circadian
344 rhythmicity. Activity profiles show the average locomotor activity (number of movements) for
345 each 10-min bin over the 10 days. Qp values on the $\mathcal{X}^2$ periodograms quantify the rhythmic
346 component of the activity and the red horizontal line indicates the significance threshold (set
347 at p=0.05).

348

349

350

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351 **Masking effect of light**

352 The average activity level (movements/10 min) was significantly lower during the light phase
353 in LD than during the subjective light phase in DD (paired t-test, p<0.001, n=17, Fig. S3).
354 The difference in the average activity level during the light phase in LD and the subjective
355 light phase in DD was not significantly different between males (n=7) and females (n=10) (t-
356 test, p=0.3).

357

358 **Phase of activity**

359 Under LD, a few sticklebacks showed a well-defined phase of activity and were almost
360 strictly nocturnal (Fig. 2A, D). However, most individuals displayed an unclear phase of
361 activity and were just slightly more active during the night than during the day (Fig. 2B, C,
362 E, F). On average, sticklebacks displayed 61.8±1.3% (n=48) of their daily activity during the
363 dark phase. There was interindividual variation in the phase of activity, as measured by the
364 percentage of the total daily activity displayed during the dark phase (also referred as the
365 night activity, Fig. 4A), with individuals spending 46.5% to 87.5% of their active time at night.
366 Of note, the three fish that were rhythmic in DD (described above) were not among the most
367 nocturnal fish, as they displayed on average 53.0%, 52.9% and 57.0% of their daily activity
368 during the night under LD. There was no significant difference between males (n=22) and
369 females (n=26) in night activity (t-test, p=0.3). Under LD, sticklebacks also showed large
370 interindividual variation in the total daily activity ranging from around 550 to 2750
371 movements/day (Fig. 4B). Males (1655±99 movements/day, n=22) were significantly more
372 active than females (1357±95 movements/day, n=26) (t-test, p=0.04). There was also a
373 significant negative correlation between night activity and total daily activity (Pearson's
374 correlation test, r=-0.3, p=0.04, n=48) so that the most nocturnal fish were also the least
375 active (Fig. 4C).

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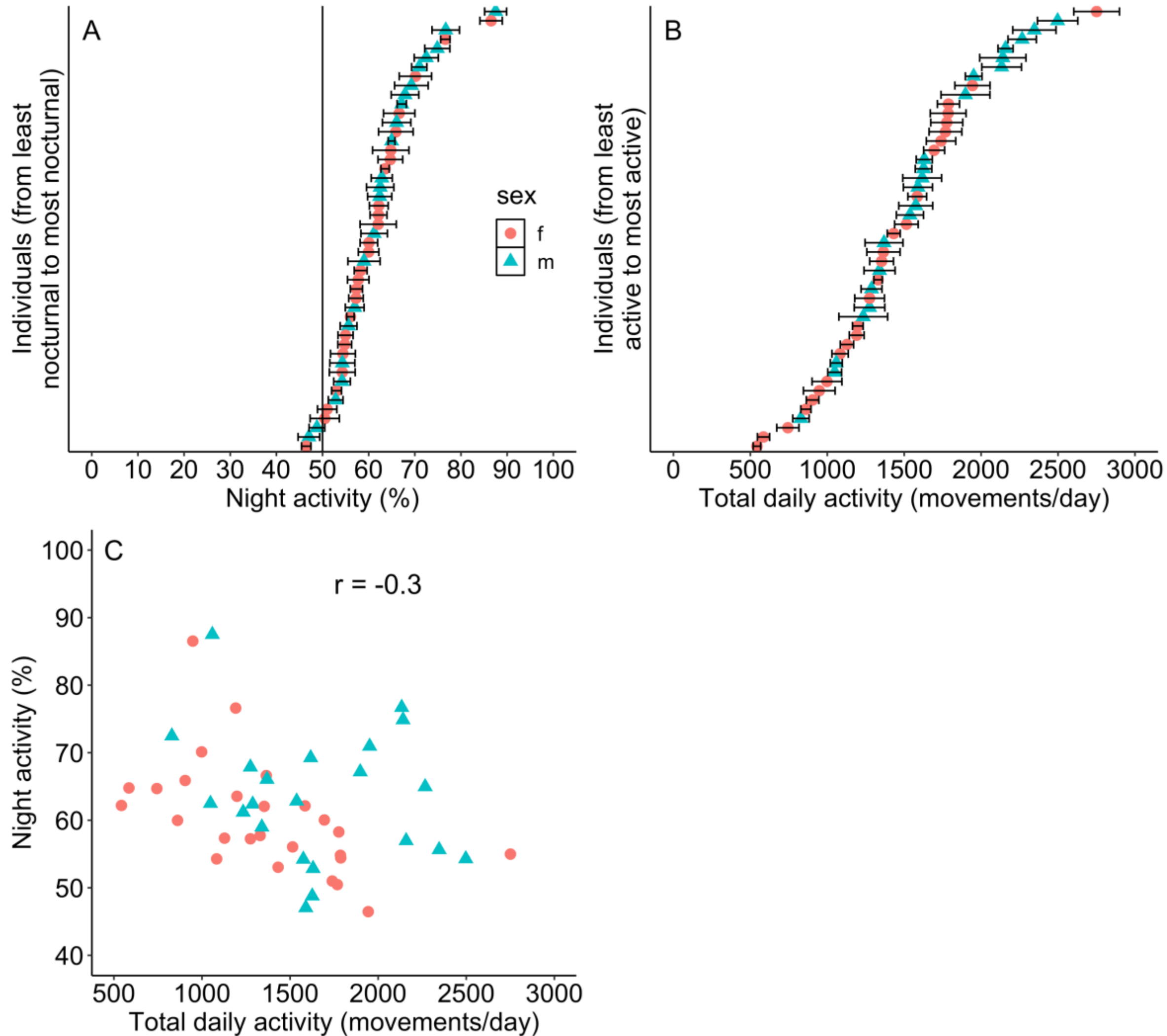

376

377 **Fig. 4. Under LD, sticklebacks are mostly nocturnal, but show large interindividual**
378 **variation in the phase of activity and in the total daily activity. The most nocturnal fish**
379 **are also the least active.** (A) Average night activity of each individual under a 12 h light:12
380 h dark cycle (LD) for 8 days. Night activity corresponds to the percentage of the total daily
381 activity displayed during the dark phase. Error bars represent the standard error of the mean.
382 (B) Average total daily activity (movements/day) of each individual under LD for 8 days. (C)
383 Correlation between the night activity (%) and the total daily activity (movements/day) under
384 LD (average for 8 days). Note that axes are not starting at zero. Pearson's correlation test,
385 $r=-0.3$, $p=0.04$, $n=48$.

386

387 **Clock gene expression rhythms in the brain**

388 We did not find any significant time-of-day variation in the relative expression of *bmal1a*,
389 *clock1b, clock2, per1b* and *cry1b* in the brain of sticklebacks (one-way ANOVA, $p>0.05$, Fig.

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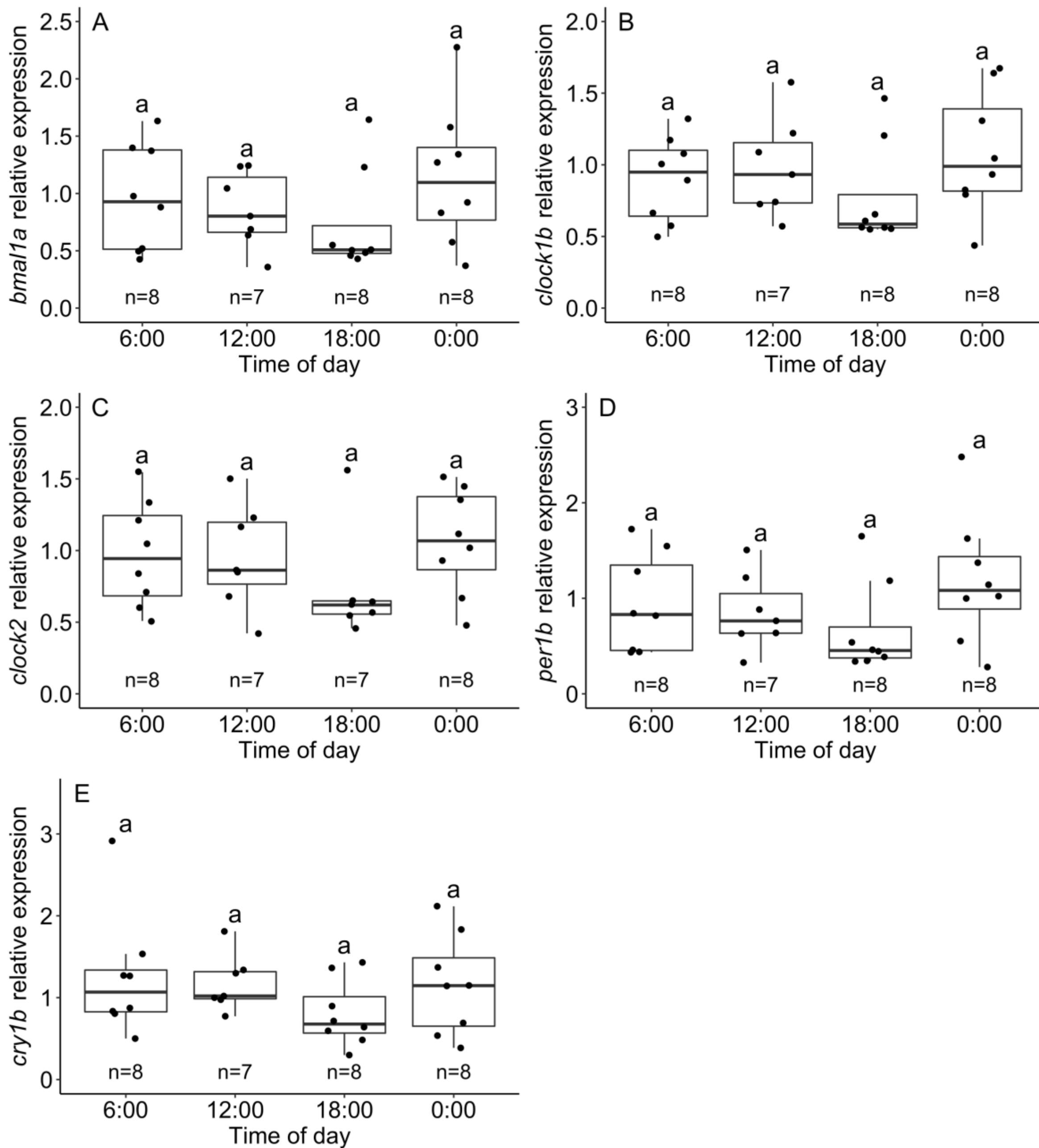
390 5). In addition, the cosinor analysis did not detect any significant circadian rhythmicity in the
391 relative expression of the five genes (cosinor analysis, p>0.05).
392



393
394 **Fig. 5. The expression of clock genes does not significantly vary during a 24-hour**
395 **period in the brain of sticklebacks in DD.** Time-of-day-dependent relative expression of
396 *bmal1a* (A), *clock1b* (B), *clock2* (C), *per1b* (D) and *cry1b* (E) measured by qPCR in the brain
397 of sticklebacks after one day in constant darkness (DD). The letter "a" denotes the absence
398 of significant difference between time points for the five genes (one-way ANOVA, p>0.05).

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399 The black line in the middle of each boxplot indicates the median and each dot represents
400 an individual. Sample size is shown for each time point.
401

402 DISCUSSION

403 The circadian clock is an internal timekeeping system shared by almost all living organisms
404 and has been mostly studied in model organisms. While knowledge about the functional
405 importance and the evolution of circadian rhythms in natural environments is mounting,
406 combining studies at the behavioural and molecular levels in individuals from natural
407 populations but in controlled experimental settings is still in its early phase. In this study,
408 using wild-caught sticklebacks, we investigated the circadian clock of this species at the
409 behavioural and molecular levels. Our first objective was to determine if the daily rhythm of
410 locomotor activity is under circadian clock control using a manipulation of the photoperiod.
411 Under LD, all fish exhibited significant daily rhythmicity, while under DD, only a few
412 individuals remained rhythmic. This result indicates that the circadian clock controls the
413 locomotor activity rhythm in only a few sticklebacks, revealing a noteworthy interindividual
414 variation. Our second objective was to determine the phase of activity of sticklebacks under
415 LD. Contrary to our hypothesis, sticklebacks were mostly nocturnal. However, we observed
416 again large interindividual variation: some fish were almost exclusively nocturnal while
417 others were just slightly more active during the night than during the day. This variation was
418 negatively correlated with the total daily activity, meaning that the most nocturnal fish were
419 also the least active. This result suggests that light suppresses activity more strongly in
420 some individuals, making them the most nocturnal fish. Our third objective was to describe
421 the molecular oscillation of five clock genes (*bmal1a*, *clock1b, clock2, per1b* and *cry1b*) in
422 the brain of sticklebacks under DD. Contrary to our hypothesis, we reported a lack of
423 circadian rhythmicity for the five genes in the brain, which could either indicate that clock
424 gene expression is not endogenously controlled, or that there was a significant oscillation
425 but that we were unable to detect it, as a result of the large biological variation observed
426 among individuals or because of technical issues.
427

428 Locomotor activity rhythm under constant darkness

429 We found striking interindividual variation in circadian rhythms of activity in threespine
430 sticklebacks. Our finding that not all individuals display a significant circadian rhythm of
431 locomotor activity has been reported previously in other fish species. For instance, under
432 constant conditions (constant darkness or light), the percentage of rhythmic individuals was

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433 57% in goldfish (*Carassius auratus*, Iigo and Tabata, 1996), 50% in Nile tilapia (*Oreochromis*
434 *niloticus*, Vera et al., 2009), 42% in tench (*Tinca tinca*, Herrero et al., 2003) and 30% in
435 sharpsnout seabream (*Diplodus puntazzo*, Vera et al., 2006). In our experiment, 18% of
436 sticklebacks were rhythmic in DD. Thus, a lack of circadian control on the locomotor activity
437 rhythm seems common in fish. An advantage of not being under the strict control of the
438 circadian clock could be that it allows the fish to rapidly adjust their phase of activity when
439 critical changes occur in the environment, such as a shift in food availability, predation risk,
440 mating opportunities, presence of parasites, etc. This is demonstrated by jet lag in animals
441 that are strongly influenced by their internal clock, such as humans: it takes several days to
442 adjust the phase of activity to a new environment and this re-entrainment is associated with
443 many negative effects on health and cognitive performance (Waterhouse et al., 2007). Thus,
444 in fish populations facing a particularly fluctuating environment, the individuals may benefit
445 from being flexible and able to adjust their phase of activity, rather than their activity being
446 rigidly controlled by their internal timekeeping system. For instance, the stickleback
447 population in lac Témiscouata has to cope with several aquatic and avian predators
448 (Reimchen, 1994; Tessier et al., 2008). All these fish and birds likely forage at various
449 moments during the day and might themselves change their phase of activity according to
450 various environmental factors or throughout the year. Sticklebacks thus probably must deal
451 with many conflictual – and sometimes unpredictable – daily patterns in predation risk.
452 Indeed, the lac Témiscouata population shows strong anti-predator morphology and
453 behaviour, even when laboratory-reared (Lacasse and Aubin-Horth, 2012). Having a flexible
454 daily schedule could further help sticklebacks to deal with several types of predators. On the
455 other hand, the fact that some individuals kept an activity rhythm in constant darkness
456 highlights that the extensive interindividual variation seen in many traits in sticklebacks, such
457 as personality (Huntingford, 1976; Bell, 2005; Aubin-Horth et al., 2012), is also present in
458 their circadian rhythms. Whether the variation quantified in these wild individuals arises from
459 genetic variation or developmental plasticity in their early environment will need to be tested
460 using common-environment experiments (Greenwood et al., 2011; Di-Poi et al., 2014). This
461 interindividual variation suggests the hypothesis that there is more than one successful way
462 to regulate its daily activities in that environment.
463
464 For the majority of the individuals that were not rhythmic in constant darkness, a lack of
465 circadian regulation does not mean that they do not have a functional clock. It is possible
466 that the clock molecular oscillator is partially uncoupled from the effectors, e.g. the locomotor

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467 system. For instance, uncoupling between clock gene expression rhythm and behavioural
468 rhythm have been reported in the Mexican blind cavefish (*Astyanax mexicanus*, Beale et al.,
469 2013). Similarly, the neuronal activity in the suprachiasmatic nucleus (the clock master
470 oscillator in mammals) of guinea pigs (*Cavia porcellus*) shows robust circadian rhythmicity,
471 but the animals express very unclear and weak activity rhythm (Kurumiya and Kawamura,
472 1988). It is thus possible that daily activities are not regulated by the clock molecular
473 oscillator in sticklebacks as well. If this is the case, other behaviours, or physiological
474 processes – such as the daily variation in the melatonin level – would be expected to be
475 controlled by the circadian clock. It is also possible that other environmental factors entrain
476 the circadian clock of sticklebacks. For instance, food availability was shown to entrain
477 circadian locomotor activity rhythms in goldfish (*Carassius auratus*, Sánchez-Vázquez et al.,
478 1997), tench (*Tinca tinca*, Herrero et al., 2005) and zebrafish (*Danio rerio*, Lopez-Olmeda et
479 al., 2010). In our study, sticklebacks could only be entrained by the light-dark cycle since
480 they were fed at random time and all other environmental cues were held constant. In future
481 studies, asking whether other environmental factors can entrain circadian rhythms in
482 sticklebacks would help us to understand what temporal cues are important for these fish in
483 their natural environment. Alternatively, the photoperiodic conditions we used might have
484 been inadequate and it could have led us to mistakenly think that the light-dark cycle could
485 entrain the circadian clock in only a few sticklebacks. For instance, under LD, transitions
486 between the light and the dark phases were very sudden, which is obviously not the case in
487 nature since the sun sets and rises progressively. The sharp increase in activity observed
488 every day just after the lights were turned off might indicate that this event was stressful for
489 the fish. In future experiments, using a light gradient at sunrise and at sunset could help to
490 better reproduce natural conditions (ex.: Lazado et al., 2014).
491
492 **Masking effect of light and phase of activity under light-dark cycle**
493 Having established that the locomotor activity rhythm of sticklebacks is not controlled by the
494 internal clock in most individuals, our results suggest that the masking effect of light
495 contributes to the significant daily rhythm that we observed for all fish under LD. The masking
496 effect of light refers to the direct influence of the photic signal on an organism's behaviour,
497 that is to say without the entrainment of its internal clock (Mrosovsky, 1999). As sticklebacks
498 were generally more nocturnal under LD, the masking effect of light should suppress activity
499 in this species (Mrosovsky, 1999). This is exactly what we observed: sticklebacks were less
500 active during the light phase in LD than during the subjective light phase in DD (same hours

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501 of the day but different lighting conditions). This result indicates that light suppresses activity
502 in sticklebacks, the definition of a masking effect.
503
504 We had hypothesized that sticklebacks are diurnal based on the fact that their visual opsins
505 (Rennison et al., 2012) correspond to those of diurnal fish (Carleton et al., 2020) and that
506 they are mostly captured during the day in the wild (Worgan and FitzGerald, 1981; Sjoberg,
507 1985; Reebs et al., 1995). However, we found that sticklebacks were, on average, mostly
508 nocturnal under LD. The fact that some fish were almost strictly nocturnal suggests that
509 sticklebacks can find food at night, either using visual or chemical cues (which has already
510 been suggested by Mussen and Peeke, 2001). Whether sticklebacks do perform night
511 activity in the wild is not known and might depend on several factors. In the laboratory, some
512 sticklebacks might have chosen to be active during the night because they did not need to
513 extensively rely on their visual system to find food (as their tank was quite small) and
514 because they perceived the dark phase as safer. It has been previously shown in some
515 species that there can be differences between the phase of daily activity in the laboratory
516 and in the natural environment (reviewed in Calisi and Bentley, 2009). For instance, while
517 mice (*Mus musculus*) are known for their nocturnal behaviour in the lab, they show variable
518 phases of activity and are sometimes even exclusively diurnal when they are held in a semi-
519 natural environment (Daan et al., 2011). Therefore, it is possible that sticklebacks are
520 nocturnal in the lab and diurnal in their natural environment, and this could be verified using
521 acoustic telemetry (March et al., 2010; Hussey et al., 2015; Alós et al., 2017).
522
523 We reported large interindividual variation in the phase of activity under LD. Interindividual
524 differences in the phase of activity have often been reported in fish (reviewed in Reebs,
525 2002). For example, under LD, some Nile tilapia (*Oreochromis niloticus*) are diurnal, others
526 are nocturnal, and some are active around the clock (Vera et al., 2009). Similar behaviours
527 have been reported in goldfish (*Carassius auratus*, Iigo and Tabata, 1996) and in Atlantic
528 salmon (*Salmo salar*, Richardson and McCleave, 1974). In line with these results, we
529 showed that some sticklebacks were clearly nocturnal under LD, while others displayed an
530 unclear phase of activity and were just slightly more active during the night than during the
531 day. Large interindividual variation in the phase of daily activity thus also seems common in
532 fish. In this study, we also observed that sticklebacks who restricted their daily activity to the
533 dark phase were also the least active. The masking effect of light could thus be involved:
534 some fish were more nocturnal because light suppressed their activity more strongly than

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535 that of the other fish. Observation of less active individuals in wild populations has already
536 been reported in other fish species (Slavík and Horký, 2012; Závorka et al., 2016; Alós et
537 al., 2017). Moreover, in accordance with our results, it has been shown that the less active
538 fish react more to variations in light intensity than the more active individuals in wild brown
539 trout juveniles (*Salmo trutta*) (Závorka et al., 2016). The ultimate cause of this interindividual
540 variation is not known, but it could be that some fish have less energy to invest in activity
541 and need to optimize the timing of their daily activity. They would thus benefit from being
542 strongly affected by the light signal because it would allow them to only be active at the most
543 optimal time of the day, which seems to be during the night for sticklebacks in our
544 experiment. We must also consider the fact that our activity measure may be affected by a
545 technical issue. The photoelectric sensors used only covered a portion of the tanks.
546 Thigmotaxis or "wall-hugging" is a stress-related behaviour found in fish as in mammals
547 (Maximino et al., 2010). If some individuals were more anxious than others in our study, they
548 might have swum very close to the wall of their tank and been less detected by the sensor.
549 Moreover, if some fish perceived the light phase as riskier, their thigmotaxis behaviour might
550 have been more pronounced during the day than during the night. Therefore, the fish that
551 we detected as less active and more nocturnal might have been as active as the other fish
552 and active around the clock like the other fish, the only difference being that they would have
553 spent more time swimming close to the wall of their tank during the day. In future
554 experiments, this bias could be avoided by using more than one photoelectric sensor on
555 each tank. Under LD, we also observed a significant sex difference in the total daily activity:
556 males were more active than females. We reported that light did not suppress activity
557 differently between sexes, so the masking effect of light is not in cause. One potential
558 explanation is that males invest more energy in their daily activities because they have a
559 higher energetic demand (Chmura et al., 2020) and forage more than females to find food
560 in their tank. Another potential explanation is that if males were less anxious than females
561 in our study, our activity monitoring system might have detected them more (as explained
562 above). Lower anxiety levels in males than in females have been reported, for instance, in
563 humans (Donner and Lowry, 2013) and in fish (Fontana et al., 2020). In summary, our results
564 suggest that circadian and daily locomotor activity rhythms display large interindividual
565 variation in sticklebacks, which seems to be a common feature in fish (Reebs, 2002). As
566 mammals tend to exhibit more robust circadian behavioural rhythms (although there are
567 exceptions: Bloch et al., 2013; Hazlerigg and Tyler, 2019), our study highlights the

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568 importance of investigating a wide diversity of species to better understand the evolution of
569 circadian clocks.
570
571 **Clock gene expression rhythms in the brain**
572 We did not detect any significant circadian rhythmicity in the relative expression of core clock
573 genes in the brain of sticklebacks under constant darkness (DD), which suggests that either
574 the molecular oscillator is highly light-dependent or that there was a significant oscillation
575 but we were unable to detect it. The first interpretation implies that clock gene expression
576 rhythms are not endogenously controlled, which contrasts with what has been observed in
577 the brain or neural tissues of many other fish species (Whitmore et al., 1998; Cermakian et
578 al., 2000; Patiño et al., 2011; Vera et al., 2013; Moore and Whitmore, 2014; Costa et al.,
579 2016; Ceinos et al., 2019). A more parsimonious explanation is that a biological or technical
580 effect prevented us from detecting any significant rhythmicity. First, it is possible that
581 sticklebacks displayed interindividual variation in their acrophases (i.e. different peak times)
582 of clock gene expression, so that the variation at each time point was too great to allow
583 detection of a significant rhythm. Interestingly, interindividual variation in peak times of clock
584 gene expression is often reported in natural populations, for example in humans (Teboul et
585 al., 2005; Nováková et al., 2013; Ferrante et al., 2015; Takahashi et al., 2018). In fish, clock
586 gene expression has not been quantified often in wild-caught populations, with the notable
587 exception of the Mexican cavefish (*Astyanax mexicanus*) (Beale et al., 2013). Without
588 surprise, it was shown that a wild population of Mexican cavefish displayed greater
589 interindividual variation in clock gene expression than a laboratory population, a result that
590 could be explained by greater genetic variation in the wild population (Beale et al., 2013).
591 To demonstrate that wild sticklebacks display different peak times of clock gene expression,
592 the same fish would have to be sampled multiple times over a 24-h period. As the sampling
593 would need not to be lethal, using fin samples could be considered (Cavallari et al., 2011;
594 Beale et al., 2013; Mogi et al., 2017).
595
596 Technical issues could also explain the fact that we did not detect significant circadian
597 rhythmicity. We quantified clock gene expression in the whole brain, but if different regions
598 of the stickleback brain host independent molecular oscillators that display different
599 circadian rhythms or if some brain tissues are arrhythmic, using the whole brain might have
600 drowned the rhythmic signal. For instance, previous studies in mammals reported that the
601 same clock gene can have various peak times of expression in different brain regions (Abe

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602 et al., 2002; Mure et al., 2018). In fish, few studies have quantified clock gene expression in
603 different brain regions, the size of this organ often being limiting. Among those who did,
604 some reported distinct expression peaks between brain regions (Cermakian et al., 2000;
605 Huang et al., 2010), but several others rather observed similar expression peaks throughout
606 the brain (Whitmore et al., 1998; Weger et al., 2013; Moore and Whitmore, 2014; Costa et
607 al., 2016). Besides, whole brains have often been used successfully to quantify clock gene
608 expression rhythms in fish, both under LD (Lopez-Olmeda et al., 2010; Sánchez et al., 2010;
609 Wang et al., 2015; Tudorache et al., 2018) and under DD (Whitmore et al., 1998; Cermakian
610 et al., 2000; Vera et al., 2013; Moore and Whitmore, 2014). It thus seems that we could have
611 detected significant rhythmicity using the whole brain of sticklebacks. That being said, in
612 future studies, it would be possible to sample specific regions of the stickleback brain such
613 as the diencephalon (which contains the hypothalamus) and the midbrain (which contains
614 the optic tectum) (Sanogo et al., 2012; Greenwood and Peichel, 2015; Bell et al., 2016).
615 These regions have been used a few times to quantify clock gene expression rhythms in
616 other fish species (Feliciano et al., 2011; Martín-Robles et al., 2012; Moore and Whitmore,
617 2014; Costa et al., 2016). Another possibility would be to sample other organs such as the
618 heart and the liver, which are commonly used to study the clock molecular oscillator in fish
619 (Sánchez et al., 2010; Cavallari et al., 2011; Wang et al., 2015).
620
621 In this study, we showed that there is interindividual variation in the circadian rhythm of
622 locomotor activity in wild sticklebacks, with most individuals exhibiting activity not controlled
623 by their clock. In addition, we found that sticklebacks were mostly nocturnal under LD, but
624 we observed large interindividual variation that could be due to a differential response to the
625 masking effect of light among individuals. In future studies, asking whether a lack of
626 circadian control is common in wild populations of sticklebacks or if it is driven by specific
627 environmental challenges (such as high predation risk) will allow to better understand what
628 selection pressures can shape the evolution of circadian rhythms. Moreover, assessing
629 whether other biological rhythms are more strongly controlled by the clock and if the
630 stickleback circadian system can be entrained by other environmental factors (such as food
631 availability) will inform us about the functional importance of the circadian clock in this
632 species. In parallel, studying the molecular oscillator will tell us what clock mechanisms
633 underlie potential differences in circadian rhythms between populations and individuals.
634 Importantly, in the study of gene expression, interindividual variation will need to be
635 addressed and the choice of target organs used to quantify clock gene expression will affect

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636 the capacity to detect significant rhythmicity. Overall, our study suggests that a strong
637 circadian control on behavioural rhythms is not necessarily advantageous in a natural
638 population of sticklebacks and that the masking effect of light is potentially responsible for
639 the large interindividual variation in the daily phase of activity.
640

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650 **Competing interests**

651 The authors declare no competing or financial interests.
652

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995 **Supplementary information**

996

997

998 **Fig. S1. Position of the infrared photoelectric sensor on an experimental tank.** Each
999 experimental tank was equipped with one infrared photoelectric sensor placed in the lower
1000 third of the front wall. Every interruption of the infrared light beam by the fish was counted
1001 as one movement.

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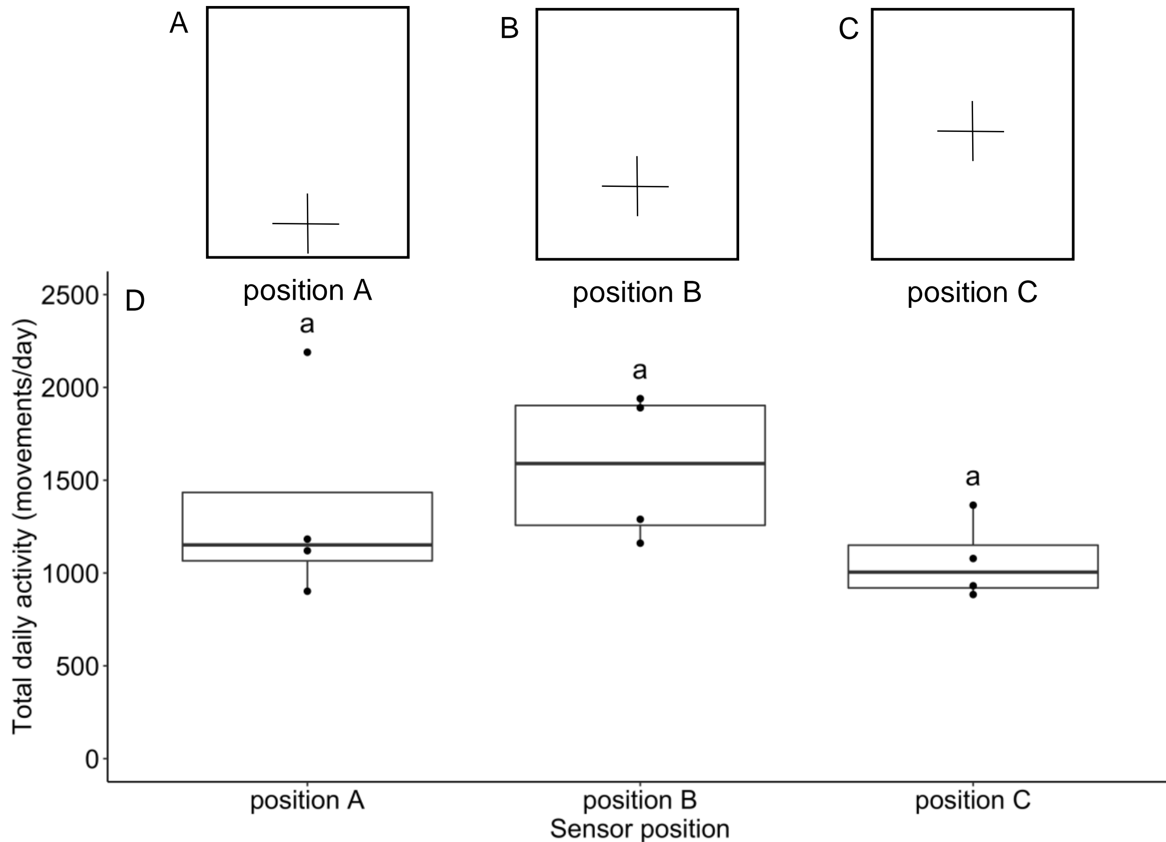

1002

1003 **Fig. S2. Sensors position optimization.** (A, B, C) In order to determine in which position
1004 the sensors detect the most movements, we did a pilot study using 12 fish. We put each fish
1005 in an experimental tank equipped with a sensor that was either placed at the very bottom
1006 (position A), in the lower third (position B) or in the middle of the front wall (position C), so
1007 that there were 4 fish per position (n=4/position). We monitored locomotor activity for 8 days
1008 under a 12 h light:12 h dark cycle. The rectangle and the cross represent the front wall of
1009 the tank and the position of the sensor, respectively. (D) Average of the total daily activity
1010 (movements/day, average for 8 days) depending on the position of the sensor. Although
1011 there is no significant difference between positions (as indicated by the letter "a", one-way
1012 ANOVA, p>0.05), the sensors in position B detect slightly more movements. We thus chose
1013 position B for our experiments (see Fig. S1). The black line in the middle of each boxplot
1014 indicates the median and each dot represents an individual.

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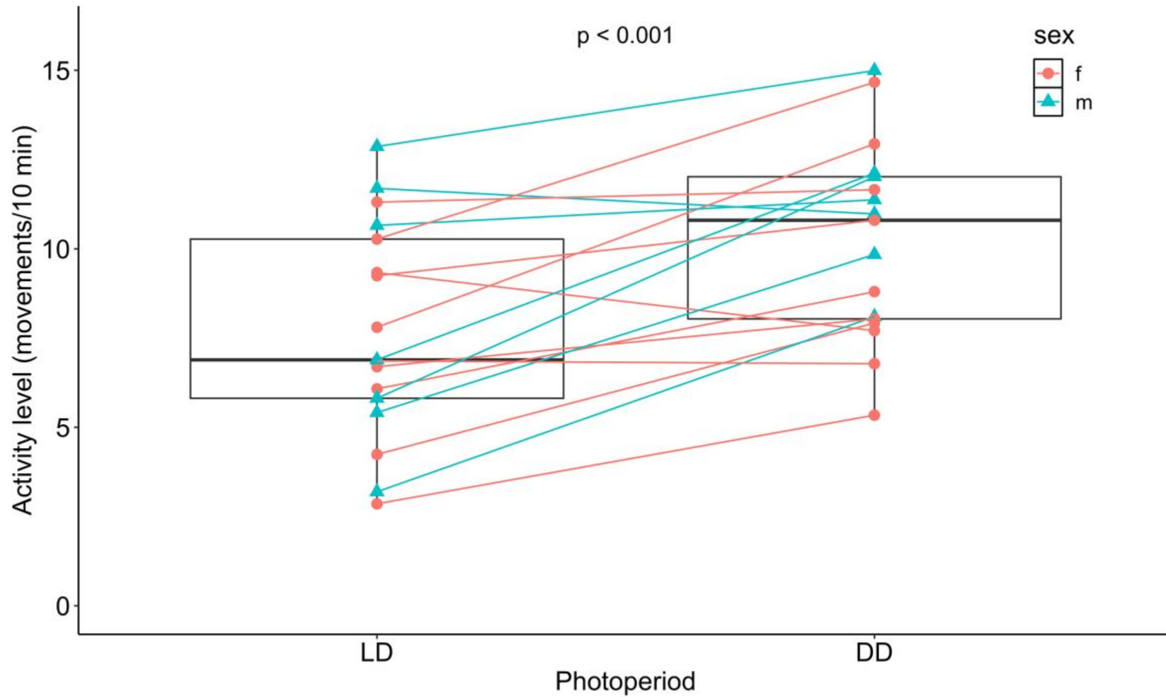

1015

1016 **Fig. S3. Sticklebacks are less active during the light phase in LD than during the**
1017 **subjective light phase in DD.** Average activity level (movements/10 min) for each
1018 individual during the light phase of a 12 h light:12 h dark cycle (LD, average for 8 days) and
1019 during the subjective light phase in constant darkness (DD, average for 10 days). Paired t-
1020 test, $p<0.001$, $n=17$.