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3 Temporal control of responses to chemically induced 4 oxidative stress in the gill mucosa of Atlantic salmon (*Salmo* 5 *salar*)

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20 **Abstract**

21 Molecular clocks are known to mediate cellular responses during oxidative stress. This
22 important interplay is less understood in fish, particularly at mucosal surfaces. Here we report
23 the coordinated modulation of the molecular clocks and antioxidant defence following
24 chemically induced oxidative stress in the gill mucosa of Atlantic salmon (*Salmo salar*). A short-
25 term gill explant (GE) culture was used as a model in a series of experiments aiming to
26 demonstrate how photoperiod during culture, levels of environmental reactive oxygen
27 species (ROS), time of oxidative stress induction, and the daily light-dark cycle affect the
28 expression of molecular clocks and antioxidant genes in the gills. Photoperiod (either 12
29 light:12 dark cycle, LD or 0 light:24 dark cycle, DD) during explant culture affected the
30 transcription of two clock genes, *circadian locomotor output cycles kaput* (*clk*) and *period 1*
31 (*per1*), as well as one antioxidant gene, *glutathione peroxidase* (*gpx*). When the GEs were
32 exposed to two ROS-generating oxidants (*i.e.*, peracetic acid, PAA and hydrogen peroxide,
33 H_2O_2), photoperiod condition was demonstrated to have a significant impact on the
34 transcription of the core genes. PAA significantly downregulated the expression of *reverb*
35 *alpha* (*reverbα*) under LD, while *per1* and *per2* expression were significantly upregulated
36 under DD. Nevertheless, there was no distinct pattern in the oxidant-induced expression of
37 clock genes. On the other hand, photoperiod was shown to influence the antioxidant defence
38 under increased ROS level, where significant transcriptional upregulation was a hallmark
39 response under LD. Interestingly, no changes were identified under DD. Induction of oxidative
40 stress either at ZT2 (2 h after lights on) or at ZT14 (2 h after lights off) revealed striking
41 differences that highlighted the temporal sensitivity of the oxidative defence repertoire. *Per1*
42 was significantly modulated following time-dependent induction of oxidative stress amongst
43 the clock genes. Inducing oxidative stress at ZT2 resulted in a significant upregulation of

antioxidant genes; but when the same stimuli were given at ZT14, all antioxidant genes exhibited downregulation. It was further revealed that neither of the genes demonstrated daily rhythmicity in their expression in the GE cultures. Collectively, the study revealed the coordinated expression of the core elements in the molecular clock and antioxidant systems in the gill mucosa following oxidative stress. Furthermore, the results reveal that the time of day plays a crucial influence on how defences are mobilised during oxidative stress, adding new insights into the rhythms of oxidative stress response in mucosal tissues in fish.

51 # 1. Introduction

52 Oxygen is essential to life but can also be toxic in its partially reduced forms [1]. Reactive 53 oxygen species (ROS), such as superoxide radicals (O_2^-), peroxides (ROOR), and hydroxyl 54 radicals (OH^-), are by-products of normal cellular metabolism, mainly in the mitochondria [2], 55 and their inherent chemical properties affect many biological targets [3]. One important 56 function is their participation as second messengers by transducing extracellular signals in a 57 variety of biological and physiological processes. Tight regulation is required for this process 58 because ROS imbalances might have serious consequences for lipid metabolism, protein 59 synthesis, and DNA, among others [4, 5]. ROS imbalance and the inability of the organism to 60 quench excessive radicals is collectively called oxidative stress. Cells have evolved intricate 61 machinery for the check and balance of ROS. Anti-oxidative reactions are activated to 62 decrease the ROS levels and to maintain homeostasis [4]. This includes the production of 63 protective enzymes (e.g., catalases, glutathione peroxidases, and superoxide dismutases) and 64 small-molecule antioxidants (e.g., Vitamins C and E, glutathione, and uric acid) that can 65 neutralise ROS [2, 6].

66 Organisms exhibit an adaptive response to the 24-h cycle on Earth that is largely 67 entrained by the daily light-dark cycle. The internal timekeeping system, called the molecular 68 clocks, provides the regulatory control on how patterns of physical, mental, and behavioural 69 changes in living organisms vary within the 24-h timescale, which are termed circadian 70 rhythms [7, 8]. At the core of this mechanism are the autoregulatory transcriptional and 71 translational feedback loops of clock genes and their corresponding proteins, which provides 72 temporal order to many clock-controlled genes [9]. External cues or *Zeitgebers* entrain the

73 rhythm of expression of circadian proteins and other rhythmic elements, and light is
74 presumably the strongest environmental signal [2].

75 ROS production and scavenging potential have been documented to exhibit circadian
76 rhythms. The dynamics of DNA damage, lipid peroxidation, and protein oxidation at different
77 times of the day emphasises the pervasive circadian control to oxidative stress responses
78 (reviewed in [2]). In mice, the direct role of clock genes on redox balance has been
79 demonstrated by the global deletion of Bmal1, a key clock gene, which resulted in increased
80 oxidative stress, thereby advancing the ageing process [10]. Loss of function of the same clock
81 gene dysregulated the redox homeostasis resulting in oxidative stress-induced death of β-cells
82 in the pancreas [11] and stress-induced neurodegeneration and astrogliosis in the brain [12].
83 Moreover, global transcriptomic profiles in murine models remarkably exposed the breadth
84 of circadian control as approximately 5–10% of genes demonstrated daily rhythmic
85 expression, including those with an essential role in oxidative stress response [13, 14].

86 In fish, the role of circadian rhythms on oxidative stress is not well understood,
87 although some evidence suggests that such an interplay is likely present [15, 16]. The activity
88 of *Glutathione peroxidase* in serum and skin mucus of permit (*Trachinotus falcatus*) exhibited
89 daily oscillation with contrasting peaks of activity, suggesting that there might be differences
90 on the circadian control of mucosal and systemic antioxidant defence [16]. The differential
91 regulation of genes coding for antioxidative defence revealed that the magnitude of responses
92 to ROS, such as elevated environmental H_2O_2 in Atlantic salmon (*Salmo salar*) [17] and
93 cadmium-induced oxidative stress in zebrafish (*Danio rerio*) [18], are largely controlled by the
94 time of the day. Moreover, recent studies have revealed that the sensitivity of fish defences

95 to external stimuli is time-dependent, indicating the significant influence of circadian rhythms
96 on mounting an adaptive response to challenging conditions [18-21].

97 In this study, we explored how the transcription of key genes governing the molecular
98 clock and antioxidant defence systems were impacted by photoperiodic changes and further
99 investigated whether there was temporal order in engaging a response to an increased level
100 of ROS in the gill mucosa of Atlantic salmon. Mucosal organs, such as the gills, provide the first
101 line of defence in fish. The gill surface area is estimated to be 0.1–0.4 m^2/kg body weight,
102 representing the largest organ-specific surface interacting with the external environment [22],
103 and is an excellent model to study the interaction of host and oxidative stress induced by
104 exogenous ROS. Induction of oxidative stress was achieved using two oxidants commonly used
105 in aquaculture, peracetic acid (PAA), and hydrogen peroxide (H_2O_2). PAA is mainly degraded
106 by chemical oxidation, while H_2O_2 is generally by microbial breakdown facilitated by catalase
107 activity [23]. The oxidative potential of both compounds contributes to their effectiveness as
108 disinfectants, with PAA considered to be far more potent antimicrobial agent than H_2O_2
109 because of its fat solubility.

110

111 ## 2. Materials and methods

112 ### 2.1. Ethics statement

113 All fish handling procedures complied with the Guidelines of the European Union
114 (2010/63/UE), as well as with national legislation.

115 ### 2.2. Fish

116 The fish used in the study were from the culture stocks of the Freshwater Fish Lab at the
117 Norwegian University of Life Sciences, Ås, Norway. They were cultured in a 300-L tank in a

118 flow-through system with water temperature maintained at 13°C and photoperiod set at 24
119 light:0 dark daily cycle. Constant illumination was the standard light regime in the facility. Fish
120 were fed with a commercial diet (Skretting Nutra, 2mm) at a daily ration of 3% biomass.
121 Feeding was ceased 24 h prior to each explant culture experiment.

122 **2.3. Short-term gill explant culture**

123 Upon arrival at Nofima's Biotechnology Laboratory, fish (50–80 g) were humanely
124 euthanised with an overdose of Aqui-S® (Scanvacc, Norway). The weight of each individual
125 fish was recorded. The gill explant culture (hereafter referred to as GE culture) was established
126 as described earlier [24, 25], but with modifications. Briefly, blood was withdrawn from the
127 caudal artery with a heparinised vacutainer. The operculum was cut, and the entire gills were
128 dissected out and immediately placed in chilled wash medium (i.e., Leibovitz's L-15
129 GlutaMax™ Supplement (Gibco, USA) with 5% v/v fetal bovine serum [FBS, Sigma-Aldrich,
130 USA], 1% 100× Antibiotic Antimycotic Solution (AA, Sigma-Aldrich), 1% 1M 4-(2-hydroxyethyl)-
131 1-piperazineethanesulfonic acid [Hepes, Sigma-Aldrich] and 0,2% 5000 IU/mL heparin
132 [Biochrom, Germany]). Both the left and right gills were collected. Sterile 1× phosphate
133 buffered saline (PBS, Gibco) was injected into the collected gill tissue through the gill arch. PBS
134 perfusion was performed 3–4 times until the gill tissue was almost blanched, indicating the
135 significant elimination of blood. Perfused gill tissues were gently washed with wash medium
136 and then transferred to chilled growth medium (i.e., Leibovitz's L-15 GlutaMax™ Supplement
137 with 10% FBS, 1% AA, 1% Hepes and 1% 100× Non-essential amino acids solution [Sigma-
138 Aldrich]), where they were cut into small pieces of approximately 1–2 mm in size. Gill
139 fragments (eight to ten pieces) were placed onto each well of a 24-well CellBIND™ (Corning,
140 USA) plate earlier seeded with 100 µl of the growth medium. The plates with the gill fragments
141 were placed in an incubator set at 13°C overnight to allow adherence. After 24 h, each well

142 was supplemented with an additional 200 µl of the growth medium as gently as possible to
143 avoid disturbing the fragments. A series of preliminary trials on culturing and maintaining the
144 explants were conducted prior to performing the series of experiments in Section 2.4. Daily
145 microscopic evaluation of the explants and their outgrowths were performed under a light
146 microscope during a 7-day period. All gill explants for tissue culture characterisation (i.e.
147 growth, morphology, development) and that were not used in the experiments in Section 2.4
148 were cultured under total darkness, which is the conventional protocol in fish cell and tissue
149 culture.

150 **2.4. Light manipulation and induction of oxidative stress**

151 *2.4.1. Experiment 1: Effects of photoperiod during explant culture*

152 The first experiment investigated the impact of photoperiod during explant culture on
153 the expression of clock and antioxidant genes. The GE culture plates were prepared, as
154 described in Section 2.3. The explants were collected from the same fish stock used in Section
155 2.3. One group was cultured under 12 light:12 dark (LD) cycle, with lights on at 6000 and lights
156 off at 1800, while the other group was under 0 light:24 (DD) dark cycle. Illumination in the
157 incubator was provided by an LED light connected to a timer. The GE cultures were maintained
158 under these photoperiod conditions for 7 days. About 80% of the medium was replaced on
159 the 3rd and 5th day. When the plates under DD regime were outside the incubator, they were
160 covered in aluminium foil and placed in a light-impermeable black container. Moreover, the
161 plates were exposed to ambient light no longer than 3 mins, in which preliminary trials
162 indicated no significant impact on the parameters studied. The room was in dim light and the
163 light inside the biological hood was not on during the whole process. On the 7th day, 90% of
164 the growth medium was pipetted out and replaced with Lysis Buffer (kit information in Section
165 2.5). The tissue fragments were collected, together with the explant outgrowths that were

166 thoroughly scraped off. The samples in lysis buffer were immediately stored in -80°C until RNA
167 extraction.

168 *2.4.2. Experiment 2: Effects of varying levels of environmental reactive oxygen species*

169 We then asked whether photoperiod history could influence the responses of
170 molecular clocks and antioxidant genes with the increased level of environmental ROS. GE
171 cultures were prepared as in Section 2.3 and kept under LD and DD for 7 days, as detailed in
172 Section 2.4.1. On the 7th day, the GE cultures were exposed to ROS-generating compounds,
173 namely peracetic acid (Divosan Forte™, Lilleborg AS, Norway) and hydrogen peroxide (Sigma-
174 Aldrich) at concentrations 10 ppm (low) and 100 ppm (high) at 0900 in the morning. The
175 unexposed group served as control. During the ROS exposure period, all plates were inside
176 their respective incubators. Handling of plates under DD was performed similarly as described
177 in section 2.4.1. The exposure duration lasted for 30 min and, thereafter, all of the medium
178 was pipetted out, the culture was gently washed with the growth medium once, and then a
179 new growth medium was added. This step was also performed with the control group. The
180 plates were returned to the incubator corresponding to their photoperiod condition prior to
181 exposure. Samples for RNA were similarly collected 24 h after, as in Section 2.4.1.

182 *2.4.3. Experiment 3: Time-dependent chemical induction of oxidative stress*

183 The third experiment investigated the temporal sensitivity of the gill mucosa to oxidative
184 stress. To this end, GE cultures were prepared as described above and cultured for 7 days
185 under 12L:12D photoperiod (i.e. lights on at 0600, lights off at 1800). Oxidative stress was
186 induced at day 7 by exposing the GE cultures to 100 ppm of either PAA or H_2O_2 for 30 min, as
187 in Section 2.4.2. One group was exposed to these oxidative stressors at *Zeitgeber time* ZT2 (2
188 h after lights on; day) while the other group was subjected to the same stressors at ZT14 (2 h
189 after lights off; night). The samples for the night period were handled similarly as with the

190 samples for DD group in section 2.4.1. A control group was included, handled similarly as
191 described in Section 2.4.2, at each occasion. Samples for RNA were collected 4 and 24 h post-
192 exposure in the same way as in Section 2.4.1.

193 *2.4.4. Experiment 4: Daily rhythm of the clock and antioxidant gene expression in the GE*
194 *culture*

195 The last experiment was aimed at identifying whether the expression of clock and
196 antioxidants genes in the GE culture exhibited daily rhythmicity by employing a 12L:12D
197 entraining signal. GE cultures were prepared as described above and cultured for 7 days under
198 LD photoperiod regime. At day 7, a time series sampling was performed every 4 h (ZT1, 5, 9,
199 13, 17, 21) during a 24 h cycle. Each sampling point had been dedicated to a single culture
200 plate to avoid disturbance. Sampling was performed as in the previous experiments.

201 **2.5. RNA extraction, cDNA synthesis, and real-time quantitative PCR**

202 The explants and their outgrowths suspended in lysis buffer were homogenised with
203 zirconium beads using a tissue homogenizer. Next, the total RNA from the resulting
204 supernatant was isolated following the manufacturer's protocol (Quick-RNA™ Microprep kit,
205 CA, USA). The RNA quantity was measured with NanoDrop 1000 Spectrophotometer
206 (ThermoFisher Scientific, DE, USA) and the quality was further assessed using an Agilent® 2100
207 Bioanalyzer™ RNA 6000 Nano kit (Agilent Technology Inc., Santa Clara, CA, USA). All samples
208 had an RNA integrity number greater than 8.8.

209 Complementary DNA was synthesised from 300 ng input RNA as a reaction template
210 using a High Capacity RNA-to-cDNA Reverse Transcription kit (Applied Biosystems, CA, USA).
211 First strand synthesis was carried out following this thermocycling protocol: 25°C for 10 min,
212 followed by 37°C for 120 min, and then the reaction ends after 5 min at 85°C.

213 The quantification of the transcript levels of the clock and antioxidant genes was
214 carried out using the PowerUp™ SYBR™ Green master chemistry (Applied Biosystems, CA USA)
215 in a QuantStudio5 real-time quantitative PCR system (Applied Biosystems). The qPCR reaction
216 mixture included 4 µL 1:10 dilution of cDNA, 5 µL SYBR™ Green Master, and 1 µL of the forward
217 and reverse primer. All samples were run in duplicate, including minus reverse transcriptase
218 and no template controls. The thermocycling protocol included a pre-incubation at 95°C for 2
219 min, amplification with 40 cycles at 95°C for 1 s, and 60°C for 30 min, and a dissociation step
220 series of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The primers used in the study are
221 given in Table 1. The primer sets were verified *in silico* and crossed intron/exon borders,
222 thereby avoiding the amplification of contaminating genomic DNA. A melting point analysis
223 was likewise performed to confirm the specificity of the primers. A five-point standard curve
224 of 2-fold dilution series was prepared from pooled cDNA to calculate the amplification
225 efficiencies.

226 Four reference genes were tested for their suitability for normalisation of the expression
227 data. *Ribosomal protein L13* (*rpl13*), *elongation factor 1a* (*eef1a*), *acidic ribosomal protein*
228 (*arp*), and *β-actin* (*actb*) were evaluated. The expression of *arp* and *eef1a* was identified as
229 stable across samples and under different experimental procedures, and thus, their geometric
230 average was used to normalise the expression of the target genes.

231 **2.6. Statistics**

232 All statistical tests were performed in SigmaPlot 14.0 Statistical Software (Systat
233 Software Inc., London, UK). Student's *t*-test was used to identify significant differences in the
234 expression following culture, either under LD or DD. The expression data from oxidative stress
235 induction and the daily rhythm experiments were analysed by one-way ANOVA after

236 complying with the requirements for normal distribution and equal variance. Differences
237 between treatment groups/ZTs were further identified by Tukey's multiple comparison test.
238 The level of significance was set at $P < 0.05$ in all statistical tests performed. Kruskal-Wallis
239 one-way ANOVA on ranks followed by Dunn's multiple comparison test were used for any data
240 sets that did not comply with the requirements of ANOVA.

241 COSINOR was employed to determine the parameters defining the rhythmicity and the
242 significance of oscillation of the gene expression. Analysis was performed by fitting a periodic
243 sinusoidal function to the gene expression values across the six ZTs, using the formula: $ƒ(t) =$
244 $M + A\cos(t/pi/12 - \phi)$, where $ƒ(t)$ is the transcript level at given time, mesor (M) is the mean
245 value, A is the sinusoidal amplitude of oscillation, t is time in hours, and f is the acrophase. For
246 gene expression to be characterised with a significant daily rhythm, it has to pass the level of
247 significance set for both ANOVA ($P < 0.05$) and COSINOR ($p < 0.05$) [19].

248

249 ## 3. Results and discussion

250 Detoxification of ROS is an evolutionarily conserved regulatory mechanism to maintain
251 redox homeostasis. The increase in ROS production and/or dysregulation in the level of
252 radicals and the inability of the organism to quench and neutralise them might lead to
253 oxidative-stress related pathologies. Though several factors have been demonstrated to
254 influence the scavenging potential of the antioxidant system [26, 27], the role of circadian
255 rhythms has gained significant interest in recent years and has been shown to be a crucial
256 determinant for a robust anti-oxidative response [2, 6]. ROS can be produced endogenously
257 (i.e., cellular metabolism) or come from exogenous sources. In fish farming, exogenous
258 sources of ROS include chemotherapeutics (*e.g.*, H_2O_2) and water disinfectant (*e.g.*, PAA),

259 among others. In this study, we employed H_2O_2 and PAA (degrades into H_2O_2 and O_2) as
260 exogenous sources of ROS because they are used in salmon aquaculture. They also exhibit
261 different degradation profiles: microbial breakdown through catalase for H_2O_2 vs. chemical
262 oxidation for PAA [28]. This feature might dictate the rate at which these oxidants could trigger
263 antioxidative responses.

264 Mucosal barriers of fish are in intimate contact with the water matrix, and
265 environmental ROS might likely target them. Because of their less complicated structure and
266 large surface contact area with the water, the gills mount a robust adaptive response to
267 varying ROS levels in the environment [17, 27, 29]. A short-term gill explant culture was
268 successfully established (Figure 1) to study the interactions of molecular clock and antioxidant
269 genes in a mucosal tissue under different environmental ROS conditions. A day after
270 explantation, new cells surrounded the tissue fragments (Figure 1B). More cell outgrowths
271 were observed in the next 5 days (Figure 1C), and some cells started to differentiate (Figure
272 1D) and acquire a typical morphology of gill epithelial cells [25]. Mucus secretion was noted
273 to be prominent at day 7 (Figure 1E) and about 80–90% of the explants exhibited the feature
274 by visual inspection. These manifestations indicate that the tissue explants were
275 physiologically and metabolically active under culture conditions, supporting the use of such
276 a model to study key processes in the gill mucosa [25, 30].

277 3.1. Photoperiod affects the expression of the circadian oscillators and 278 antioxidant genes in the gill mucosa

279 Photoperiod is a powerful external cue for molecular clocks in fish [8, 9, 31], and earlier
280 studies suggest that photoperiodic changes could trigger differential modulation in the
281 expression of several oxidative stress markers [16, 32]. We first explored whether light
282 conditions during culture influence the transcription of the clock and antioxidant genes in the

283 GE. Constant darkness was selected together with the equal day:night entraining signal
284 because, in typical cell/tissue culture experiments, DD is the standard light condition in the
285 incubator. Light conditions during explant culture affected the expression of two clock genes,
286 *clk* and *per1* (Figure 2). Culturing the explants under DD (i.e., constant darkness) resulted in
287 significantly elevated transcript levels of these clock genes. Expression of *clk* under DD was
288 20% higher than the explant cultured under LD. The relative increase was even higher for *per1*,
289 in which a 54% increase was observed under DD compared with LD. Though there was a clear
290 tendency that the expression of antioxidant genes was higher in explants cultured under LD
291 than DD, only the expression of *gpx* displayed a significant difference. The transcript level of
292 *gpx* for the LD group was 60% higher compared with the group under DD. It is important to
293 emphasise that samples were only collected at a single time point (i.e., 0900, for both LD and
294 DD). These differences can likely be attributed to the apparent variations in the expression of
295 these genes during the LD cycle (Figure 6). Moreover, there is a possibility that there were
296 physiological alterations in the explant in response to photoperiodic changes, thus accounting
297 for the differential regulation of the genes. This implication was supported by the next trial
298 that revealed the impact of photoperiod history in the oxidative stress responses.

299 3.2. Increased ROS regulates the expression of clock and antioxidant 300 genes, and the LD cycle is essential for mounting an adaptive response to 301 the radicals in the gill mucosa

302 The changes in the transcription of the target genes either under LD or DD led us to
303 hypothesise that photoperiod condition impacts the regulation of clock and antioxidant gene
304 expression when environmental ROS level increases. It was indeed apparent that photoperiod
305 history played a vital function in how gills responded to the two oxidants (Figures 3 and 4).
306 The expression of *rev-erbα* was significantly downregulated by at least 70% in PAA-exposed

307 GE compared with the control group under LD, regardless of the concentration (Figure 3). This
308 transcriptional response was not identified in GE under DD. *Rev-erbα* represents an essential
309 link between the positive and negative loops of the circadian clock by repressing *Bmal1* gene
310 expression and ensures the stability of the transcriptional-translational loop [8, 9]. More
311 importantly, REV-ERBα mediates responses during oxidative stress [33]. As a mediator of the
312 crosstalk in the circadian clock system, the regulation of *reverbα* suggests that it serves a
313 crucial function in maintaining the homeostasis and fidelity of the clocks in the gills under
314 increased environmental ROS, though this might be influenced by the timing of oxidative
315 stress induction (Figure 5). The stable expression of the rest of the clock genes, regardless of
316 the oxidant and dose, lends support to such an implication. Interestingly, the expression of
317 the two period genes (i.e., *per1* and *per2*) was significantly upregulated (by at least 30%) in
318 PAA-exposed GE under DD compared with the unexposed group. The role of Period genes
319 during oxidative stress has been elucidated to some extent in murine models [2], though
320 evidence in fish is limited. The upregulation of *per* expression indicates that they probably
321 protected the GE from the deleterious effects of increased ROS level and dysregulated
322 antioxidant defence due to the absence of an LD signal (absence of antioxidant response under
323 DD as shown in Figure 4), as Period has been implicated in protecting cells during oxidative
324 damage [34].

325 The transcriptional responses of the antioxidant genes revealed a striking influence of
326 photoperiod condition on how responses were mobilised when oxidative stress was triggered
327 (Figure 4). An elevated level of radicals from both sources resulted in significant increases of
328 *gpx*, *gr*, *mnsod*, and cu/*znsod* expression, demonstrating a classical adaptive response to
329 exogenously induced oxidative stress, predominantly a heightened activity [2, 3, 32]. These

330 four genes represent both the glutathione and superoxide dismutase families of the
331 antioxidant system that are known to have an active role in ROS scavenging [6]. The
332 upregulation of these genes suggests an increased scavenging potential to protect the GE from
333 oxidative damage. It appeared that in most cases, the high dose (100 ppm) elicited more
334 significant responses. Unlike in clock genes, where it was evident that PAA impacted the
335 expression more than H_2O_2, both of the ROS-generating oxidants provoked similar responses,
336 especially in the expression of *gr* and *cu/znsod*. We can speculate that the two oxidants likely
337 have the same capacity to trigger anti-oxidative responses in the gills, though the time of
338 induction might influence the responses, as discussed in the next section. It is interesting to
339 observe that oxidative response was only observed when GE was cultured under LD but not
340 in DD. It seems plausible that the LD cycle maintains the ability of the antioxidant repertoire
341 to mount responses, and the absence of this external cue might have dysregulated the
342 antioxidant system. Therefore, an appropriate response to increased ROS was not exhibited.
343 A recent human study might shed insight on this ostensible difference. Teixeira and colleagues
344 [35] showed that night workers have a lower antioxidant defence; hence, they are more prone
345 to oxidative stress damage.

346 ### 3.3. Chemically induced oxidative stress elicits a time-dependent
347 ### response from the antioxidant repertoire

348 The striking difference identified in the oxidant exposure experiment, particularly with
349 the transcriptional changes in antioxidant defence, prompted us to explore whether the time
350 of the day might impart a significant influence on the magnitude and type of response to
351 increased ROS levels. To this end, we induced oxidative stress in the LD-cultured GE either at
352 ZT2, representing responses when oxidative stress was experienced during the day, or at ZT14,
353 representing responses when the same stimulus was given at night. Interestingly, only the

354 transcription of *per1* was affected amongst the clock genes (Figure 5). The expression was
355 significantly downregulated 4 h after exposure to H_2O_2 at ZT2 and 24 h after exposure to the
356 same oxidant at ZT14. Downregulation 24 h after exposure was also identified when GE was
357 stimulated with PAA at ZT14. This profile was in contrast with the expression data for *per1* in
358 Experiment 2; hence, emphasising the importance of the timing of oxidative stress induction
359 played in the magnitude and type of responses. Some of the clock genes also tended to vary
360 according to the time-dependent induction of oxidative stress. However, the changes relative
361 to the unexposed group were not statistically different. We are unsure of why it appeared that
362 the clocks were not dramatically affected, such as for the antioxidant genes by PAA and/or
363 H_2O_2, as shown in Experiments 2 and 3 (Figures 3–5), and as demonstrated in other species [4,
364 33, 34]. Nonetheless, we can speculate that the stable expression is related to the
365 maintenance of physiological homeostasis, where the clock has a ubiquitous function, in the
366 period when the antioxidant system is actively quenching excessive radicals in the
367 environment, which can be a metabolically demanding process [36]. There was a marked
368 pattern in the transcriptional responses of the antioxidant genes to the two oxidants: When
369 oxidative stress was induced during the day, the post-exposure profile of the antioxidant gene
370 expression demonstrated significant upregulation. This was identified in the transcription of
371 *gr*, *gsta,* and *mnsod*. It also appeared that an antioxidative response was already mobilised 4
372 h after exposure to H_2O_2, unlike in PAA, for which significant alterations were only observed
373 24 h post exposure. When the same stimuli were given at night, *gr*, *gsta*, *mnsod,* and *cu/znsod*
374 were significantly downregulated 24 h after exposure. This response pattern was particularly
375 striking when GE was exposed to H_2O_2 as the transcript level of four genes (i.e., *gr*, *gsta*,
376 *mnsod*, and *cu/znsod*) was reduced by at least a fold compared with the unexposed group.
377 Collectively, the differential regulation of the antioxidant genes to increased ROS level

378 highlights the temporal sensitivity of antioxidant defence in the gills, which dictates the time-
379 wise regulation of the magnitude and type of response to oxidative stress. The ROS scavenging
380 potential in the gills, as indicated by heightened antioxidative state, was likely more efficient
381 when oxidative stress or increased ROS level is encountered during the day than at night.
382 Encountering increased ROS at night might limit the ability of the gills to quench the radicals
383 and hence the downregulation of expression in most of the genes. This is likely due to the
384 normal daily fluctuations of antioxidant defence which have been documented in many
385 organisms, including fish [2, 6, 16, 17]. Salmon is a diurnal species (active during the day). This
386 might explain, at least in part, why active responses were generated when oxidative stress
387 was induced at ZT2.

388 3.4. The expression of clock and antioxidant genes in the GE culture 389 does not exhibit a daily rhythm

390 Lastly, we asked whether the expression of clock and antioxidant genes oscillate during
391 the daily cycle under an LD entraining signal. Though there were tendencies in the expression
392 to vary during the LD cycle, such as with *reverbα*, *clk*, and *cu/znsod*, all core genes did not
393 exhibit significant daily rhythm (Figure 6). The absence of rhythmicity in the expression of clock
394 genes in GE is in line with an earlier report in the gills of seawater-adapted salmon [17].
395 Despite a direct LD signal being provided in this study, this did not entrain the expression *in*
396 *vitro*. The COSINOR algorithm provided some critical parameters on how the gene expression
397 behaved during the LD cycle despite being arrhythmic (Table 2). Two of the clock genes (i.e.,
398 *bmal1* and *reverbα*) displayed peak expression (acrophase) in the early hours of the dark
399 phase, while *clk* and *per2* were identified to peak at mid hours of the dark phase. Only *per1*
400 displayed an elevated transcript level at early hours of the light phase. For the antioxidant
401 genes, the expression of *gpx*, *mnsod*, and *cu/znsod* had peak expression during the early to

402 mid hours of the light phase, while *gr* and *gsta* displayed elevated transcript levels at the
403 beginning of the dark phase. There was an interesting pattern in the expression of *mnsod*,
404 concerning how it responded to time-dependent oxidative stress induction. *Mnsod* expression
405 peaked in the early hours of the dark phase (Table 2) and was likewise demonstrated to be
406 highly responsive to induced oxidative stress at ZT2 (Figure 5). This might partially explain why
407 a strong *mnsod* transcriptional response was observed when oxidative stress was induced in
408 the early hours of the light phase and can perhaps be attributed to the heightened state of
409 *mnsod* activity at this period. This similar association has been demonstrated earlier in
410 rainbow trout (*Oncorhynchus mykiss*), where a strong serum-mediated bacterial killing activity
411 was identified in the period where most of the potent molecules were at elevated status [19].
412 It is also interesting to highlight that the experimental peak of *mnsod* expression in this study
413 was similar to the identified peak of *mnsod* expression in the gills of salmon post-smolts *in*
414 *vivo* [17].

415 **3.5. Conclusions**

416 The current study supports the strong link between circadian rhythms and antioxidant
417 defence. To our knowledge, this is the first report that shows how the key molecular regulators
418 of these two important mechanisms are mediated under different photoperiod and oxidative
419 states in a mucosal tissue of a teleost fish. Our results reveal that a daily LD cycle is needed
420 for the antioxidant molecules to mount an effective response to increased environmental
421 ROS. The differential response to time-dependent induction of oxidative stress revealed that
422 the ROS scavenging potential – as indicated by the heightened antioxidative state in the gills
423 – was higher during the day. It is interesting to explore in the future the role of photoperiod

424 condition (i.e. LD, DD, LL) prior to tissue explantation on the distinct responses observed in
425 the gill explant model to the different manipulations *ex vivo*.

426 Endogenous ROS in aquaculture is often from peroxide-based treatments. The results
427 of the study have implications on the timing of treatment using these peroxides, taking into
428 account the time of the day in the application as well as the impact of photoperiod history.

429

430 ## Acknowledgements

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437 specific information and does not imply recommendation or endorsement by Nofima.

438 ## References

439 [1] R. Gerschman, D.L. Gilbert, S.W. Nye, P. Dwyer, W.O. Fenn, Oxygen poisoning and x-
440 irradiation: a mechanism in common, Science (New York, N.Y.) 119(3097) (1954) 623-6.

441 [2] M. Wilking, M. Ndiaye, H. Mukhtar, N. Ahmad, Circadian rhythm connections to oxidative
442 stress: implications for human health, Antioxid Redox Signal 19(2) (2013) 192-208.

443 [3] M. Schieber, N.S. Chandel, ROS function in redox signaling and oxidative stress, Curr Biol
444 24(10) (2014) R453-R462.

445 [4] Y. Tahara, A. Yokota, T. Shiraishi, S. Yamada, A. Haraguchi, A. Shinozaki, S. Shibata, *In vitro*
446 and *in vivo* phase changes of the mouse circadian clock by oxidative stress, J Circadian Rhythms
447 14(1) (2016) 4.

448 [5] C.E. Cross, B. Halliwell, E.T. Borish, W.A. Pryor, B.N. Ames, R.L. Saul, J.M. McCord, D.
449 Harman, Oxygen radicals and human disease, Annals of internal medicine 107(4) (1987) 526-
450 45.

451 [6] R. Hardeland, A. Coto-Montes, B. Poeggeler, Circadian Rhythms, Oxidative Stress, and
452 Antioxidative Defense Mechanisms, Chronobiology international 20(6) (2003) 921-962.

453 [7] J.O. Early, D. Menon, C.A. Wyse, M.P. Cervantes-Silva, Z. Zaslona, R.G. Carroll, E.M. Palsson-
454 McDermott, S. Angiari, D.G. Ryan, S.E. Corcoran, G. Timmons, S.S. Geiger, D.J. Fitzpatrick, D.
455 O'Connell, R.J. Xavier, K. Hokamp, L.A.J. O'Neill, A.M. Curtis, Circadian clock protein BMAL1
456 regulates IL-1β in macrophages via NRF2, Proceedings of the National Academy of Sciences of
457 the United States of America 115(36) (2018) E8460-E8468.

458 [8] C.C. Lazado, H.P. Kumaratunga, K. Nagasawa, I. Babiak, C.M.A. Caipang, J.M. Fernandes, In
459 vitro and ex vivo models indicate that the molecular clock in fast skeletal muscle of Atlantic
460 cod is not autonomous, Molecular Biology Reports 41(10) (2014) 6679-6689.

461 [9] C.C. Lazado, H.P. Kumaratunga, K. Nagasawa, I. Babiak, A. Giannetto, J.M. Fernandes, Daily
462 rhythmicity of clock gene transcripts in Atlantic cod fast skeletal muscle, PLoS ONE 9(6) (2014)
463 e99172.

464 [10] R.V. Kondratov, O. Vykhovanets, A.A. Kondratova, M.P. Antoch, Antioxidant N-acetyl-L-
465 cysteine ameliorates symptoms of premature aging associated with the deficiency of the
466 circadian protein BMAL1, Aging (Albany NY) 1(12) (2009) 979-987.

467 [11] J. Lee, M. Moulik, Z. Fang, P. Saha, F. Zou, Y. Xu, D.L. Nelson, K. Ma, D.D. Moore, V.K.
468 Yechoor, Bmal1 and beta-cell clock are required for adaptation to circadian disruption, and
469 their loss of function leads to oxidative stress-induced beta-cell failure in mice, Molecular and
470 cellular biology 33(11) (2013) 2327-38.

471 [12] E.S. Musiek, M.M. Lim, G. Yang, A.Q. Bauer, L. Qi, Y. Lee, J.H. Roh, X. Ortiz-Gonzalez, J.T.
472 Dearborn, J.P. Culver, E.D. Herzog, J.B. Hogenesch, D.F. Wozniak, K. Dikranian, B.I. Giasson,
473 D.R. Weaver, D.M. Holtzman, G.A. Fitzgerald, Circadian clock proteins regulate neuronal redox
474 homeostasis and neurodegeneration, Journal of Clinical Investigation 123(12) (2013) 5389-
475 400.

476 [13] G.E. Duffield, DNA microarray analyses of circadian timing: the genomic basis of biological
477 time, Journal of neuroendocrinology 15(10) (2003) 991-1002.

478 [14] J.D. Johnston, Adipose circadian rhythms: translating cellular and animal studies to human
479 physiology, Molecular and cellular endocrinology 349(1) (2012) 45-50.

480 [15] G. Carmona-Antoñanzas, M. Santi, H. Migaud, L.M. Vera, Light- and clock-control of genes
481 involved in detoxification, Chronobiology international 34(8) (2017) 1026-1041.

482 [16] C.C. Lazado, I. Lund, P.B. Pedersen, H.Q. Nguyen, Humoral and mucosal defense
483 molecules rhythmically oscillate during a light–dark cycle in permit, *Trachinotus falcatus*, Fish
484 & Shellfish Immunology 47(2) (2015) 902-912.

485 [17] L.M. Vera, H. Migaud, Hydrogen peroxide treatment in Atlantic salmon induces stress and
486 detoxification response in a daily manner, Chronobiol International 33(5) (2016) 530-42.

487 [18] J.-L. Zheng, S.-S. Yuan, C.-W. Wu, Z.-M. Lv, A.-Y. Zhu, Circadian time-dependent
488 antioxidant and inflammatory responses to acute cadmium exposure in the brain of zebrafish,
489 Aquatic Toxicology 182 (2017) 113-119.

490 [19] C.C. Lazado, M. Gesto, L. Madsen, A. Jokumsen, Interplay between daily rhythmic serum-
491 mediated bacterial killing activity and immune defence factors in rainbow trout
492 (*Oncorhynchus mykiss*), Fish & Shellfish Immunology 72 (2018) 418-425.

493 [20] C.C. Lazado, P.V. Skov, P.B. Pedersen, Innate immune defenses exhibit circadian
494 rhythmicity and differential temporal sensitivity to a bacterial endotoxin in Nile tilapia
495 (*Oreochromis niloticus*), Fish & Shellfish Immunology 55 (2016) 613-622.

496 [21] C. Qin, J. Sun, Y. He, J. Wang, Y. Han, H. Li, X. Liao, Diurnal rhythm and pathogens induced
497 expression of toll-like receptor 9 (TLR9) in Pelteobagrus vachellii, Fish & Shellfish Immunology
498 87 (2019) 879-885.

499 [22] E.O. Koppang, A. Kvellestad, U. Fischer, 5 - Fish mucosal immunity: gill in: E. Peatman, B.
500 Beck (Eds.), Mucosal Health in Aquaculture, Academic Press, San Diego, 2015, pp. 93-133.

501 [23] S.S. Block, Peroxygen compounds. , Lippincott Williams & Wilkins, Philadelphia, ,
502 Pennsylvania, USA., 1991.

503 [24] K.S. Sobhana, K.C. George, G. Venkat Ravi, G. Ittoop, R. Paulraj, Development of a cell
504 culture system from gill explants of the grouper, *Epinephelus malabaricus* (Bloch and
505 Shneider), Asian Fisheries Science 22(2) (2009) 541-547.

506 [25] M.C. Gjessing, M. Aamelfot, W.N. Batts, S.L. Benestad, O.B. Dale, E. Thoen, S.C. Weli, J.R.
507 Winton, Development and characterization of two cell lines from gills of Atlantic salmon, PLOS
508 ONE 13(2) (2018) e0191792.

509 [26] V. Lobo, A. Patil, A. Phatak, N. Chandra, Free radicals, antioxidants and functional foods:
510 Impact on human health, Pharmacogn Rev 4(8) (2010) 118-126.

511 [27] R. Martínez-Alvarez, A.E. Morales, A. Sanz, Antioxidant Defenses in Fish: Biotic and Abiotic
512 Factors, 2005.

513 [28] D.L. Straus, T. Meinelt, D. Liu, L.-F. Pedersen, Toxicity of Peracetic Acid to Fish: Variation
514 among Species and Impact of Water Chemistry, Journal of the World Aquaculture Society
515 49(4) (2018) 715-724.

516 [29] Z. Mozhdeganloo, M. Heidarpour, Oxidative stress in the gill tissues of goldfishes
517 (*Carassius auratus*) parasitized by *Dactylogyrus* spp, J Parasit Dis 38(3) (2014) 269-272.

518 [30] Z. Xu, F. Takizawa, D. Parra, D. Gómez, L. von Gersdorff Jørgensen, S.E. LaPatra, J.O.
519 Sunyer, Mucosal immunoglobulins at respiratory surfaces mark an ancient association that
520 predates the emergence of tetrapods, Nature Communications 7 (2016) 10728.

521 [31] N.S. Foulkes, D. Whitmore, D. Vallone, C. Bertolucci, Studying the Evolution of the
522 Vertebrate Circadian Clock: The Power of Fish as Comparative Models, Advances in genetics
523 95 (2016) 1-30.

524 [32] A. Giannetto, J.M. Fernandes, K. Nagasawa, A. Mauceri, M. Maisano, E. De Domenico, T.
525 Cappello, S. Oliva, S. Fasulo, Influence of continuous light treatment on expression of stress
526 biomarkers in Atlantic cod, Developmental and comparative immunology 44(1) (2014) 30-4.

527 [33] G. Yang, C.J. Wright, M.D. Hinson, A.P. Fernando, S. Sengupta, C. Biswas, P. La, P.A.
528 Dennery, Oxidative stress and inflammation modulate Rev-erbα signaling in the neonatal lung
529 and affect circadian rhythmicity, Antioxid Redox Signal 21(1) (2014) 17-32.

530 [34] M.C. Magnone, S. Langmesser, A.C. Bezdek, T. Tallone, S. Rusconi, U. Albrecht, The
531 mammalian circadian clock gene Per2 modulates cell death in response to oxidative stress,
532 Frontiers in Neurology 5(289) (2015).

533 [35] K.R.C. Teixeira, C.P. dos Santos, L.A. de Medeiros, J.A. Mendes, T.M. Cunha, K. De Angelis,
534 N. Penha-Silva, E.P. de Oliveira, C.A. Crispim, Night workers have lower levels of antioxidant
535 defenses and higher levels of oxidative stress damage when compared to day workers,
536 Scientific Reports 9(1) (2019) 4455.

537 [36] M. Frisard, E. Ravussin, Energy metabolism and oxidative stress: impact on the metabolic
538 syndrome and the aging process, Endocrine 29(1) (2006) 27-32.

539 [37] M.B. Betancor, E. McStay, M. Minghetti, H. Migaud, D.R. Tocher, A. Davie, Daily rhythms
540 in expression of genes of hepatic lipid metabolism in Atlantic salmon (*Salmo salar* l.), PLOS
541 ONE 9(9) (2014) e106739.

542 [38] M.F. Solberg, B.O. Kvamme, F. Nilsen, K.A. Glover, Effects of environmental stress on
543 mRNA expression levels of seven genes related to oxidative stress and growth in Atlantic
544 salmon Salmo salar L. of farmed, hybrid and wild origin, BMC Res Notes 5 (2012) 672-672.

545 [39] D. Garcia de la serrana, I.A. Johnston, Expression of heat shock protein (hsp90) paralogues
546 is regulated by amino acids in skeletal muscle of Atlantic salmon, PLOS ONE 8(9) (2013)
547 e74295.

548 [40] M. Sanden, P.A. Olsvik, Intestinal cellular localization of PCNA protein and CYP1A mRNA
549 in Atlantic salmon Salmo salar L. exposed to a model toxicant, BMC Physiology 9(1) (2009) 3.

550

551

552 Table 1. Primers used in the present study.

Gene name	Abbreviation	Sequence (5'→3')	Reference
Brain and Muscle ARNT-Like 1	*bmal1*	F: GCCTACTTGCAACGCTATGTCC R: GCTGCGCCTCGTAATGTCTTCA	[37]
Circadian Locomotor Output Cycles Kaput	*clk*	F: AGAAATGCCTGCACAGTCGGAGTC R: CCACCAGGTCAGAAGGAAGATGTT	[37]
Period 1	*per1*	F: AGGGGGTCATGCGGAAGGGGAAGT R: TGGGCCACCTGCATGGGCTCTGT	[37]
Period 2	*per2*	F: GCTCCCAGAATTCCTAGTGACAAG R: GAACAGCCCTCTCGTCCACATC	[37]
Rev-ErbA alpha	*reverbα*	F: CCCCCAAGACGAACCCAACAAGAC R: AGAGGGAGGCAAAGCGCACCATTA	[37]
Glutathione peroxidase	*gpx*	F: GATTCGTTCCAAACTTCCTGCTA R: GCTCCCAGAACAGCCTGTTG	[38]
Glutathione reductase	*gr*	F: CCAGTGATGGCTTTTTTGAACTT R: CCGGCCCCCACTATGAC	[38]
Glutathione S-transferase	*gsta*	F: AGGGCACAAGTCTAAAGAAGTC R: GTCTCCGTGTTTGAAAGCAG	This study*
Manganese superoxide dismutase	*mnsod*	F: GTTTCTCTCCAGCCTGCTCTAAG R: CCGCTCTCCTTGTCGAAGC	[38]
Copper/Zinc superoxide dismutase	*cu/znsod*	F: CCACGTCCATGCCTTTGG R: TCAGCTGCTGCAGTCACGTT	[38]
Elongation factor alpha-1	*ef1a*	F: GAATCGGCTATGCCTGGTGAC R: GGATGATGACCTGAGCGGTG	[39]
Acidic ribosomal protein	*arp*	F: TCATCCAATTGCTGGATGACTATC R: CTTCCCACGCAAGGACAGA	[40]
B-actin	*actb*	F: CCAAAGCCAACAGGGAGAA R: AGGGACAACACTGCCTGGAT	[40]
Ribosomal protein L13	*rpl13*	F: CGCTCCAAGCTCATCCTCTTCCC R: CCATCTTGAGTTCCTCCTCAGTGC	[39]

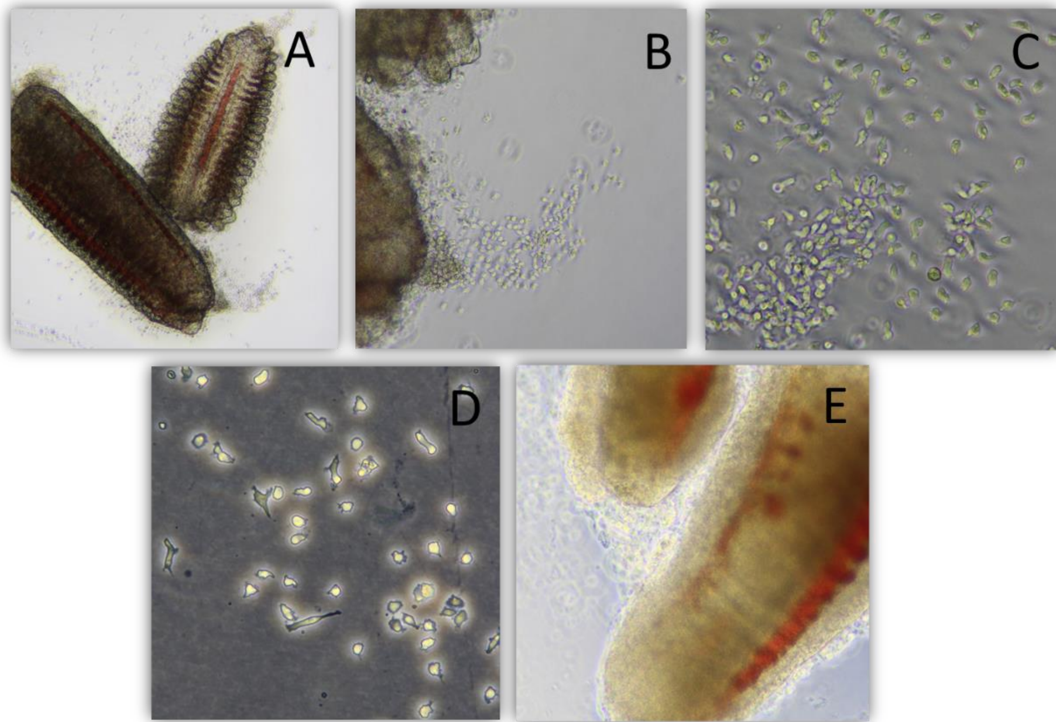
553 * NM_001141492.2

554 Table 2. Rhythmicity parameters identified by COSINOR.

Gene	Mesor	Amplitude	Acrophase (h)	P value[1]	% V[2]
bmal1	1.49	0.19	14.56	0.34	49.62
clk	1.42	0.34	17.14	0.14	72.62
per1	1.22	0.08	1.26	0.74	17.57
per2	1.69	0.26	20.17	0.57	31.25
reverbα	1.94	0.26	13.18	0.61	27.83
gpx	1.56	0.21	0.19	0.33	52.64
gr	1.42	0.07	15.22	0.89	7.70
gsta	1.43	0.25	14.43	0.14	73.19
mnsod	1.30	0.12	5.40	0.71	20.17
cu/znsod	1.09	0.29	1.07	0.19	66.76

555 [1] Significance of rhythmicity is set at $P>0.05$. [2] Percentage of variance

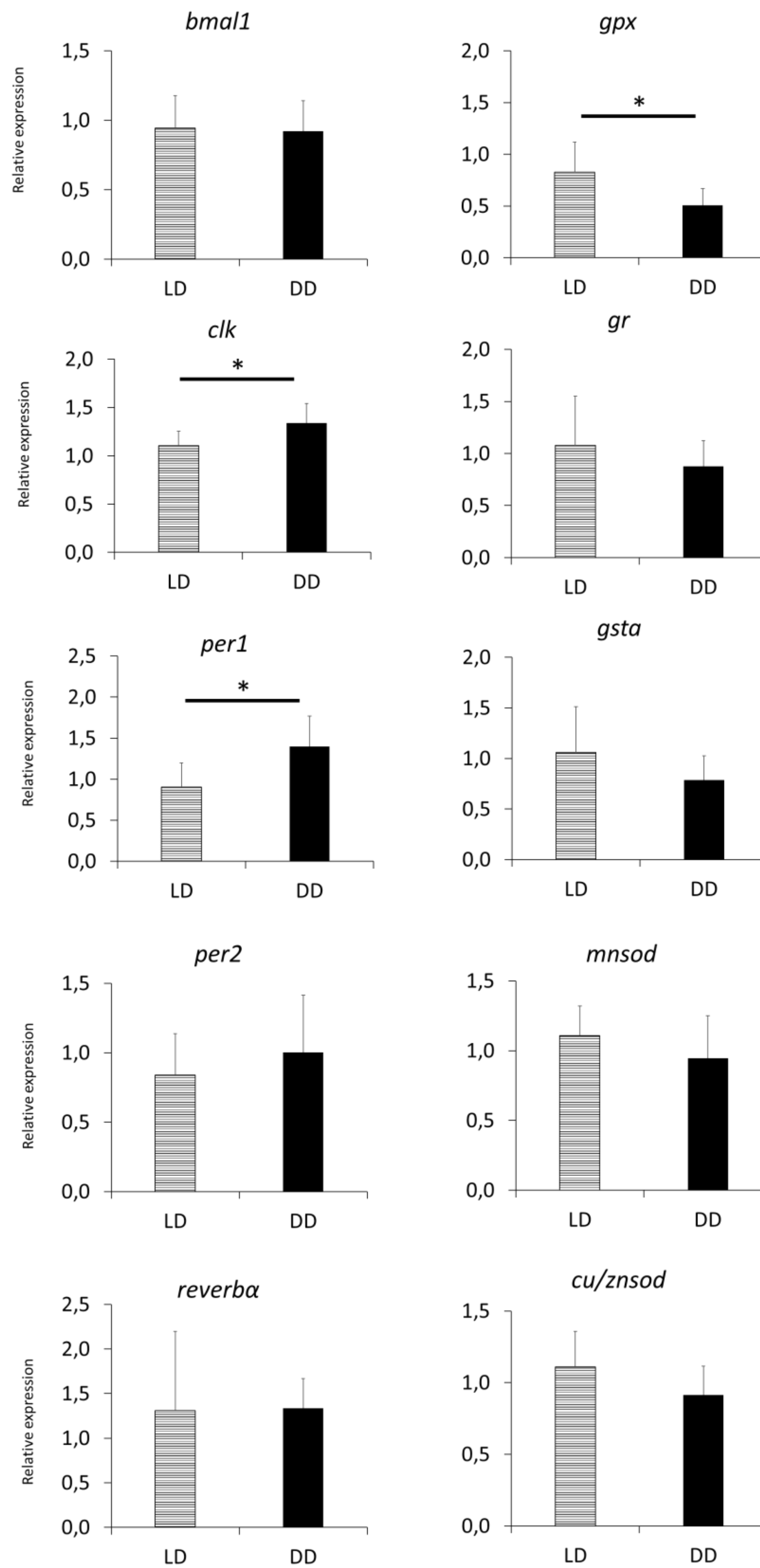
556



557

558 **Figure 1.** Gill explants and their outgrowths. A) Gill epithelial cell outgrowths surrounded the tissue fragment 1 day after
559 explantation (4×). B) Cells coming out of the explant, magnified (10×). C) and D) The cells outgrowths were proliferating and
560 differentiating between day 2–6. E) Explant appeared to excrete mucus profusely at 7 days after explantation.

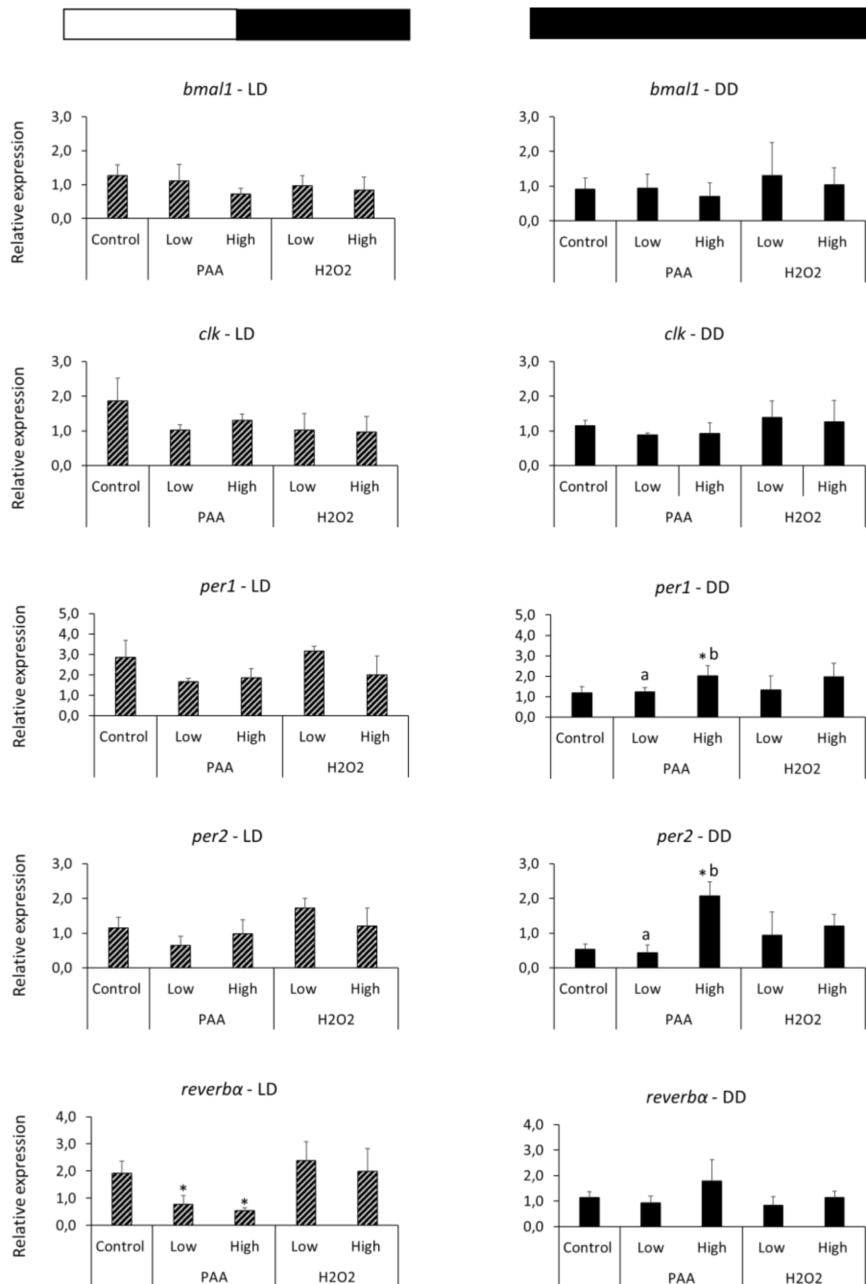
561



562

563 **Figure 2.** Transcript levels of the clock and antioxidants genes in gill explants cultured either under equal length of day and night
564 (LD) or total darkness (DD). Expression value represents mean ± SD. N = 4 wells, where each well has gill tissue fragments from
565 three individual fish. Asterisk (*) indicates that expression between LD and DD displayed a significant difference at P = 0.05.

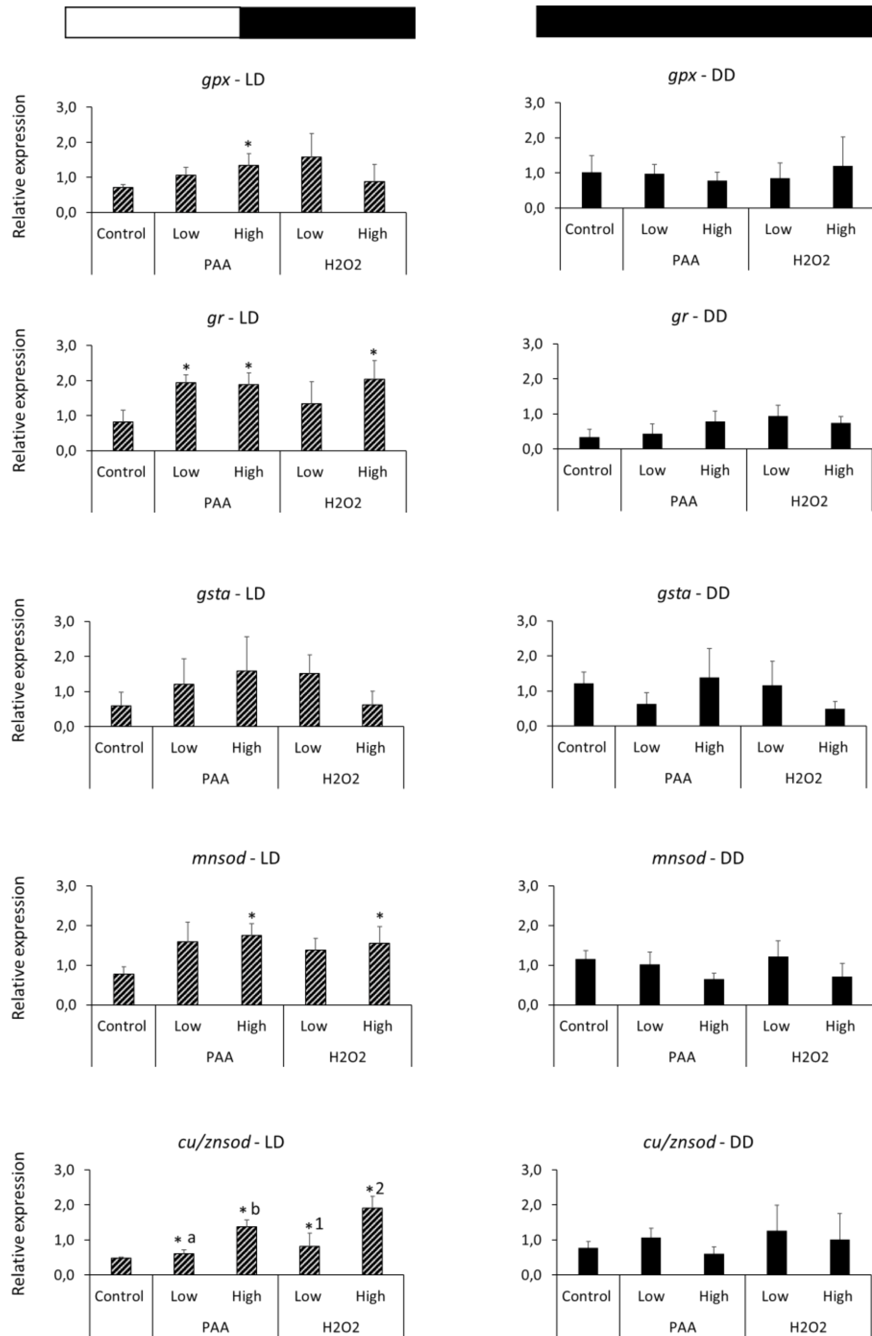
566



567

568 **Figure 3.** Regulation of clock gene expression following increased environmental ROS level in gill explants cultured either under
569 equal length of day and night (LD) or total darkness (DD). GE cultures were exposed either to low (10 ppm) or high (100 ppm)
570 concentrations of PAA or H_2O_2. Expression value represents mean ± SD. N = 3 wells, where each well had gill tissue fragments
571 from three individual fish. Asterisk (*) indicates that expression displayed a significant difference from the control group.
572 Different letters denote that a significant difference exists between the low and high group within a particular oxidant. The level
573 of statistical difference was set at P = 0.05.

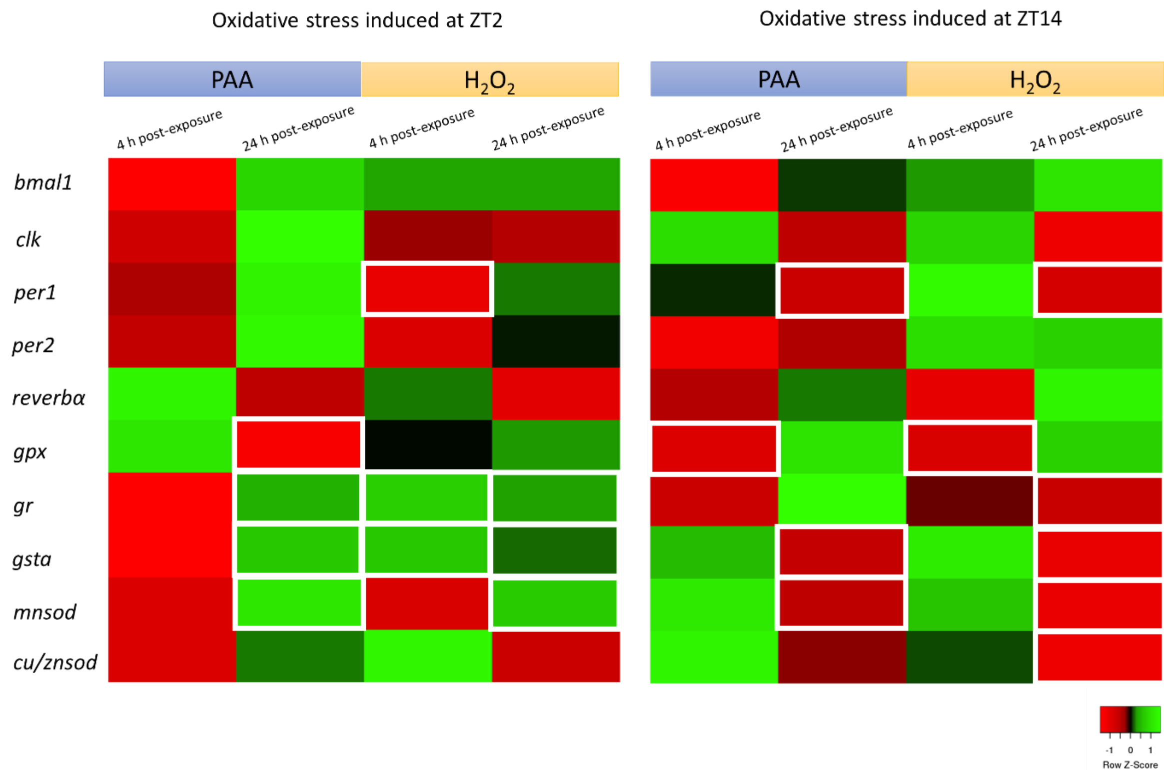
574



575

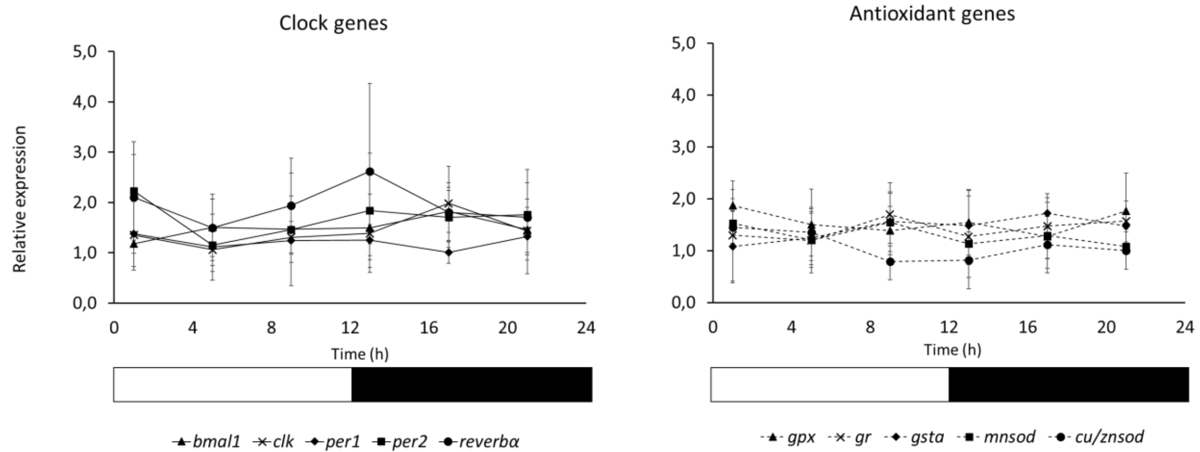
576 **Figure 4.** Regulation of clock gene expression following increased environmental ROS level in gill explants cultured either under
577 equal length of day and night (LD) or total darkness (DD). GE cultures were exposed either to low (10 ppm) or high (100 ppm)
578 concentrations of PAA or H_2O_2. The expression value represents mean ± SD. N = 3 wells, where each well had gill tissue fragments
579 from three individual fish. Asterisk (*) indicates that expression displayed a significant difference from the control group.
580 Different letters/numbers denote that a significant difference exists between the low and high group within a particular oxidant.
581 The level of statistical difference was set at P = 0.05.

582



583

584 **Figure 5.** Temporal sensitivity in the expression of clock and antioxidant genes in the LD-cultured gill explant exposed to oxidants
585 either at ZT2 (day) or at ZT14 (night). Samples were collected 4 and 24 h after exposure. Expression values were expressed as
586 the ratio between the transcript level in the treated group relative to the transcript level in the control group at that timepoint.
587 Spectral panels enclosed in white outline indicate that the response was significantly different ($P < 0.05$) from the
588 control/unexposed group at that particular time point. Expression value represents mean ± SD. N = 4 wells, where each well had
589 gill tissue fragments from three individual fish.
590



591

592 **Figure 6.** Expression of the clock and antioxidant genes during a complete 24-h LD cycle in GE cultures. None of the genes
593 displayed significant daily oscillation. Expression value represents mean ± SD. N = 4 wells, where each well had gill tissue
594 fragments from three individual fish.

595