

1 **HYDROGEN PEROXIDE TREATMENT IN ATLANTIC SALMON INDUCES**
2 **STRESS AND DETOXIFICATION RESPONSE IN A DAILY MANNER**

3

4 L.M. Vera¹ and H. Migaud¹

5

6 ¹Genetics and Reproduction Group, Institute of Aquaculture, University of Stirling, FK9 4LA, Stirling, UK

7

8

9 **Corresponding author:**

10 Dr. L.M. Vera

11 Institute of Aquaculture

12 University of Stirling, FK9 4LA, UK

13 Tel: + 44-1786-466573

14 E-mail:luisa.veraandujar@stir.ac.uk

15

16

17 **Running head:** Hydrogen peroxide chronotoxicity in salmon

18

19 **ABSTRACT**

20 Daily variation in the absorption, metabolism and excretion of toxic substances will
21 ultimately determine the actual concentration to which the cells and tissues are exposed. In
22 aquaculture, Atlantic salmon (*Salmo salar*) can be frequently exposed to hydrogen peroxide
23 (H_2O_2) to treat topical skin and gill infections, particularly in relation to parasitic infections
24 (e.g. sea lice *Lepeophtheirus salmonis* and amoebic gill disease caused by *Neoparamoeba*
25 *perurans*). It is well accepted that the time of administration influences pharmacodynamics
26 and pharmacokinetics of drugs which in turn affects their efficacy and toxicity. Consequently,
27 a better understanding of drug side effects as a function of time of day exposure would help
28 to improve treatment efficacy and fish welfare. To this end, salmon were exposed to H_2O_2
29 (1500 mg/L) for 20 min at six different times of the day during a 24-h cycle and we
30 investigated the time-dependent effects of exposure on physiological stress (glucose, lactate
31 and cortisol) and antioxidant enzyme expression (*gpx1*, *cat*, *Mn-sod* and *hsp70*) in liver and
32 gills. In addition, at each sampling point, 8 control fish were also sampled. Our results
33 revealed that the time of administration of H_2O_2 caused significant differences in the
34 induction of both physiological and oxidative stress responses. Glucose and lactate were
35 higher in the treated fish during daytime whereas cortisol levels appeared to be systematically
36 increased (>1000 ng/mL) after H_2O_2 treatment irrespective of exposure time, although
37 differences with control levels were higher during the day. In liver, gene expression of
38 antioxidant enzymes displayed daily rhythmicity in both treated and control groups and
39 showed higher mRNA expression levels in salmon treated with H_2O_2 at ZT6 (6 h after lights
40 onset). In gills, rhythmic expression was only found for *gpx1* in the control fish and for *hsp70*
41 and *Mn-sod* in the treated groups. However, in the treated salmon, higher gene expression
42 levels of all the investigated enzymes were also observed at ZT6-10. Clock gene expression
43 showed rhythmicity only in the liver in accordance with the daily rhythm of enzyme
44 expression observed in this tissue. Altogether, this study provides first evidence of
45 chronotoxicity in Atlantic salmon treated with H_2O_2 and suggests increased sublethal toxic
46 effect during the first half of the day. These results have direct relevance to the salmon and
47 broader aquaculture industry by optimising the timing of treatment administration, opening
48 the door to chronotherapy to treat fish diseases.

49

50

51 **Keywords:** Daily rhythms, Atlantic salmon, hydrogen peroxide, stress response, oxidative
52 stress, chronotoxicity, chronotherapy.

53 INTRODUCTION

54 The circadian clock of vertebrates is a key regulator controlling behavioural, physiological
55 and biochemical processes that provides organisms with anticipatory mechanisms to predict
56 cyclic events. The molecular mechanisms underlying circadian oscillations involve
57 transcriptional-translational feedback loops of clock genes and proteins that drive the
58 rhythmic expression of a wide range of genes, including transcription factors, which in turn
59 relays rhythmicity to a wider set of genes (Reppert & Weaver, 2001; Vatine et al., 2011).
60 Microarray experiments have recently provided analysis of 24-h transcriptome regulation in
61 multiple tissues of mammalian species, revealing that approximately 5-10 % of genes display
62 daily rhythms in expression, including genes encoding detoxification enzymes (Duffield,
63 2003; Johnston, 2012). It is widely accepted that drug efficacy and toxicity may also vary
64 with time of the day (Bruguerolle, 1998) and thus, daily rhythms in drug absorption,
65 distribution, metabolism, detoxification and excretion have been reported in mammalian
66 species (Smolensky & Peppas, 2007). However, the mechanisms underlying toxicity rhythms
67 are not fully understood, although drug pharmacokinetics is closely related to circadian
68 variations in physiology driven by clock genes.

69 Cultured Atlantic salmon in intensive conditions are susceptible to outbreaks of infectious
70 bacterial, viral and parasitic diseases. Hence, fish are routinely exposed to a number of
71 therapeutic agents during a production cycle to guarantee their health and welfare (Burka et
72 al., 1997; Grant, 2002). Most of these compounds are administered to fish by bath treatments,
73 in-feed additives or through IP injection. In all cases, fish are exposed to the active
74 ingredients at arbitrary times of the day dictated by time and operational constraints rather
75 than scientific evidence. Sea lice are the most widespread pathogenic parasite in Atlantic
76 salmon aquaculture (Costello, 2006; Torrissen et al., 2013), costing the world industry around
77 €300m per year (Costello, 2009). Hydrogen peroxide (H_2O_2) has been used to treat fish
78 ectoparasites for a long time including sea lice *Lepeophtheirus salmonis* and more recently
79 amoebic gill disease caused by *Neoparamoeba perurans* (Adams et al., 2012). Currently
80 H_2O_2 is commonly used by salmon farmers at concentrations between 1500 and 2000 mg/L
81 as a bath treatment at ambient water temperatures (Kierner & Black, 1997). Other uses of
82 H_2O_2 in aquaculture include the disinfection of fish eggs and microbial control (Bozwell et
83 al., 2009; Matthews et al., 2012). Moreover, despite being a powerful oxidizer, it is rapidly
84 decomposed in water, leaving no toxic products (Arvin & Pedersen, 2015). However,
85 although it has been considered a relatively low toxic compound there is a recent concern on
86 its effects on fish welfare (Papiol & Roque, 2013). Actually, H_2O_2 treatment has been

87 reported to be stressful to Atlantic salmon for the initial 24-h following exposure, increasing
88 plasma glucose, cortisol and electrolyte levels (Bowers et al., 2002). However, to date the
89 effect of time-of-administration on toxicity levels has not been investigated.

90 Previous studies have also indicated that H₂O₂ exposure can increase the activity of catalase
91 and glutathione in fish (Tort et al., 2005). In fact, changes in the expression of oxidative
92 stress markers are considered to be informative biomarkers to identify and quantify the
93 sublethal effects of environmental stressors in fish, including toxicant exposure. This has led
94 to the rising of ecotoxicogenomics, a new discipline combining genomics and ecotoxicology
95 to identify biomarkers at the transcriptome level that may be used as indicators for the
96 exposure to contaminants and to quantify their effects on the organisms (Geist et al., 2007).
97 In addition, it is now accepted that the expression of genes involved in the metabolism of
98 xenobiotics is under circadian clock control, showing highest expression levels at certain
99 times of the day (Hamby et al., 2013; Lin et al., 2014). However, such rhythmicity and its
100 implications on fish toxicological response have not been yet investigated.

101 In the present study we examined the time-dependent effects of H₂O₂ exposure in Atlantic
102 salmon. Physiological and molecular markers were investigated, including the expression of
103 detoxification enzymes (*gpx1*, *cat*, *hsp70* and *Mn-sod*) involved in the cellular defence
104 against oxidative stress caused by enhanced levels of ROS, as well as the expression of core
105 clock genes (*bmal1* and *clock*) involved in the activation of transcription factors regulating
106 metabolic processes in mammalian liver. The final objective of the present research was to
107 determine the influence of the administration time of this aquaculture therapeutant on the
108 physiological status of fish and potentially refine health management strategies.

109

110 **MATERIALS AND METHODS**

111 **Animals & housing**

112 For this study a total of 96 Atlantic salmon (110.3 ± 0.5 g and 23.3 ± 0.1 cm final body
113 weight and length, respectively) were kept at the Machrihanish Marine Environmental
114 Research Laboratory (Institute of Aquaculture, Stirling, Scotland). Experimental fish were
115 randomly allocated to 16 cylindrical tanks (n=8/tank; 397 L tank) within a flow-through
116 system supplied with natural sea water under a simulated natural photoperiod. Fish were
117 hand-fed to satiation twice a day over a two-week acclimation period.

118 **Experimental design**

119 Experimental procedure complied with the Guidelines of the European Union (2010/63/UE)
120 and the Animal (Scientific Procedures) Act 1986 UK under the approval of the local ethical

121 review board. In addition, the experimental design and methodology followed in this
122 investigation were in accordance with the international ethical standards of Chronobiology
123 International (Portaluppi et al., 2010).

124 The trial was performed in June 2014 with ambient water temperature of 12.3 ± 0.3 °C and
125 simulated natural photoperiod set at 18 h light: 6 h darkness (18L:6D). Fish were treated with
126 a nominal concentration of 1500 mg/L for 20 min at six different times of the day (every 4 h
127 during a 24-h cycle): “zeitgeber time” (ZT) 2 (two hour after lights on), ZT6, ZT10, ZT14,
128 ZT18 and ZT22. At each time point, one of the 16 tanks (n=8 salmon) was treated with H₂O₂.
129 During treatment, water was oxygenated ensuring that oxygen levels were above 7 mg/L and
130 water temperature was monitored. In addition, at each sampling point, another tank
131 containing 8 control salmon (not exposed to H₂O₂) were also sampled. Following exposure,
132 fish were sacrificed by lethal anaesthesia (2-phenoxyethanol 1 mL/L, Sigma) and blood, liver
133 and gills samples were obtained from each fish. Water samples were collected from each
134 treated tank and H₂O₂ concentration was immediately measured by cerium sulphate titration
135 method (Reichert et al., 1939) (provided by Solvay Interlox Ltd.). Briefly, 5 mL of 5N sulfuric
136 acid and 7.5 mL of cerium IV sulphate solution were mixed in a conical flask. Then, a burette
137 was filled with 50 mL of water sample and was slowly dispensed into the cerium IV sulphate
138 solution, swirling to mix until the solution went colourless. The reading of the burette was
139 then recorded and H₂O₂ concentration (mg/L) calculated. The measured concentrations
140 throughout the trial was 1419.6 ± 9.4 mg/L.

141 Blood was withdrawal by caudal puncture with heparinised sterile syringes. This procedure
142 was done in less than 5 min to avoid the increase of plasma cortisol and glucose levels due to
143 handling. Blood was centrifuged at 3000 rpm for 15 min and plasma removed and frozen at -
144 80° C until analysis. Liver and gills samples were preserved in RNAlater and kept on ice until
145 further storage at -20 °C.

146

147 **Glucose, lactate and cortisol analyses**

148 Blood glucose and lactate were measured immediately after extraction by means of handheld
149 meters: Contour® usb (Bayer HealthCare, UK) and LactatePro™ 2 (Arkay Europe, The
150 Netherlands). In addition, to validate these methods for Atlantic salmon, a total of 20 samples
151 were further assayed using enzymatic-colorimetric commercial test kits: Glucose (GO) Assay
152 Kit (Sigma) and Lactate Dry-Fast (Sentinel Diagnostics, Italy). The spectrophotometric
153 method and handheld metre for glucose and lactate analysis were compared by assessing the
154 strength (coefficient of determination; r^2) and direction (slope) of the linear relationship

155 between pairs of assay results, for which a regression analysis was performed. Plasma
156 cortisol levels were measured with a commercial ELISA kit (Calbiotech, USA) that was also
157 validated for Atlantic salmon. In all cases, serial dilution of pooled salmon plasma was used
158 to obtain an inhibition curve that, following logit-log transformation, was found to be linear
159 and parallel to the standard curves, indicating that sample and standard glucose, lactate and
160 cortisol were comparable (Soper, 2015).

161

162 **Gene expression analyses**

163 Liver and gill samples were homogenised in 1 mL of TRIzol® (Invitrogen, UK) and total
164 RNA extracted in accordance with the manufacturer's instructions. RNA pellets were
165 rehydrated in MilliQ water and total RNA concentration was determined using an ND-1000
166 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK). RNA integrity was assessed by
167 electrophoresis. In order to eliminate any genomic DNA contamination, total RNA was
168 DNase treated following DNA-free™ kit (Applied Biosystems, Warrington, UK). cDNA was
169 then reverse transcribed from 1 µg of DNase treated total RNA using random hexamer and
170 Oligo (dT)₁₂₋₁₈ primers in a 20 µL total reaction volume (high capacity reverse transcription
171 kit without RNase inhibitor, Applied Biosystems, UK). Real-time PCR was performed using
172 Luminaris color HiGreen qPCR Master mix (Thermo Fisher Scientific, MA, USA) and
173 Mastercycler RealPlex 2 thermocycler (Eppendorf, UK) which was programmed to perform
174 the following protocol: 50 °C for 2 min, 95 °C for 1 min, followed by 40 cycles at 95 °C for
175 15 s, X °C for 15 s and 72 °C for 30 s (see Table 1 for target specific annealing temperature).
176 This was followed by a temperature ramp from 70 to 90 °C for melt-curve analysis to verify
177 that no primer-dimer artefacts were present and only one product was generated from each
178 qPCR assay. The final volume of the PCR reaction was 10 µL: 2.5 µL of cDNA, 5 µL of the
179 qPCR Master Mix and 2.5 µL of forward and reverse primers (Table 1). The primers used to
180 amplify *gpx1*, *cat*, *hsp70*, *Mn-sod*, *bmall* and *clock* were previously tested and validated for
181 Atlantic salmon (Betancor et al., 2014; McStay et al., 2014; Olsvik et al., 2014). All samples
182 were run in duplicate. For both liver and gills samples, we verified the efficiency of the
183 primers by doing standard curves for all genes investigated. Expression of the target genes
184 was measured by absolute quantification with all samples being normalized with *β-actin*
185 mRNA expression (McStay et al., 2014). Quantification was achieved by translating CT
186 values of unknown samples from a parallel set of reactions containing a serial dilution of
187 spectrophotometrically determined linearised plasmid containing partial cDNA sequences.

188

189 **Data analysis**

190 Statistical differences in glucose, lactate, cortisol and gene expression levels between
191 sampling points were analysed by a one-way ANOVA (ANOVA I). In addition, at each
192 sampling point, glucose, lactate, cortisol and target genes expression levels were compared
193 between treatments (control *versus* H₂O₂-treated) by means of a t-test, for which a Levene's
194 test was previously used to check for homogeneity of variances. A Univariate General Linear
195 Model (GLM) was carried out to analyse possible interactions between experimental groups
196 and time points. For this, the fixed factors were "ZT" (ZT2-22) and "treatments" (control
197 and treated). All statistical tests were carried out with the SPSS v19.0 program (SPSS Inc.,
198 Chicago, IL), with a statistical threshold set at *p* values <0.05 in all tests. All values are
199 reported as the mean ± SEM.

200 Cosinor analysis was also performed using Ritme software (Antoni Díez-Noguera, University
201 of Barcelona, Spain) to determine whether the daily expression of the studied genes fitted the
202 cosine function: $Y = M + A * [\text{Cos}(\Omega t + \Phi)]$, where *M* is mesor, *A* is amplitude, Ω is angular
203 frequency (360°/24h for the daily and circadian rhythms) and Φ is acrophase. The
204 significance level was fixed at *p* < 0.05.

205

206 **RESULTS**

207 **Circulating physiological stress indicators**

208 For glucose and lactate, a subset of samples was assayed in parallel using both
209 spectrophotometric methods and handheld metres. Pairs of assay results showed that the
210 glucometer underestimated glucose levels by 29.8 ± 0.9 % on average and the LactatePro™ 2
211 overestimated lactate concentrations by 15.1 ± 0.2 %. For both parameters, the relationship
212 between both analytical methods was linear: $r^2 = 0.9320$ for glucose and $r^2 = 0.9485$ for
213 lactate (Fig. 1). Consequently, concentrations measured by the glucometer were transformed
214 to estimate the spectrophotometric equivalent using a linear correction according to the
215 equation $G_s = 1.2332 G_g + 1.16$, whereas data obtained with LactatePro™ 2 were transformed
216 with the equation $L_s = 0.7183 L_1 + 0.4514$.

217 In the control salmon, glucose levels did not show significant differences between sampling
218 points. However, a significant daily rhythm was observed in fish treated with H₂O₂ with the
219 acrophase being found at around ZT6 (Cosinor, *p*<0.05) (Table 3). In addition, at ZT6,
220 glucose levels in the treated group were significantly higher than in the control group. On the
221 contrary glucose levels in the control group were higher at ZT22 (t-test independent samples,
222 *p*<0.05) (Fig. 2A). A significant daily rhythm of lactate was observed in both experimental

223 groups, with the acrophases located at ZT=9:25 (control) and ZT=7:23 (H₂O₂) (Cosinor,
224 $p<0.05$) (Table 2, 3). Overall, lactate levels were significantly higher in the treated group
225 during the day (5.1 ± 0.2 mmol/L) and lower at night (1.8 ± 0.2 mmol/L) (t-test independent
226 samples, $p<0.05$) (Fig. 2B).

227 Plasma cortisol did not show daily rhythmicity in the control group, although levels showed
228 two peaks, at ZT10 (272.7 ± 64.4 ng/mL) and ZT18 (285.3 ± 58.9 ng/mL) (ANOVA I,
229 $p<0.05$). However, a significant daily rhythm was found in the treated salmon, being the
230 acrophase located at ZT=7:12 (Cosinor, $p<0.05$) (Table 3). Moreover, at all sampling points,
231 cortisol levels were significantly higher in the treated fish (t-test independent samples,
232 $p<0.05$), with the higher differences being observed during the day when the average cortisol
233 concentration was 1139.8 ± 2.3 ng/mL (Fig. 2C).

234 Furthermore, for the three stress indicators (glucose, lactate and cortisol), the Univariate
235 GLM revealed an interaction between treatments and sampling points ($p<0.05$).

236

237 **Expression of oxidative stress markers**

238 In the liver, mRNA expression of all oxidative stress genes analysed in this experiment
239 showed daily rhythmicity in both treated and control groups, with acrophases around ZT0-1
240 for the control fish and around ZT4-5 for the salmon treated with H₂O₂ (Cosinor, $p<0.05$)
241 (Table 2, 3). In addition, for both *gpx1* and *Mn-sod*, expression was significantly higher at
242 ZT6 in the treated fish (t-test independent samples, $p<0.05$), with fold-change (FC)
243 differences in expression of 2.3 and 1.7, respectively. *Cat* and *hsp70* expression levels also
244 appeared to be higher at ZT6 although differences between groups were not statistically
245 significant. Gene expression of all enzymes was lower at ZT22 in the treated group (Fig. 3).
246 Finally, there was a significant interaction between treatments and sampling points for *gpx1*,
247 *hsp70* and *Mn-sod* (Univariate GLM, $p<0.05$).

248 In gills, *gpx1* expression showed daily rhythmicity in the control fish, with the acrophase at
249 ZT=18:44 (Cosinor, $p<0.05$) (Table 2). However, no daily rhythms were observed in the
250 treated salmon. In addition, *gpx1* mRNA expression was significantly higher in the treated
251 group at ZT6 (t-test independent samples, $p<0.05$) (FC=1.9) (Fig. 4A). *Cat* expression did not
252 show daily rhythmicity in both treated and control groups. However, gene expression was
253 significantly higher in the treated fish at ZT10 (t-test independent samples, $p<0.05$) (FC=1.5)
254 (Fig. 4B). *Hsp70* expression profile displayed daily rhythmicity only in the treated group and
255 was significantly higher at ZT10 in comparison with the control fish (t-test independent
256 samples, $p<0.05$) (FC=1.4) (Fig. 4C). *Mn-sod* expression showed a similar temporal pattern,

257 with daily rhythmicity and higher expression in ZT10 in the treated salmon (FC=1.3).
258 However, *Mn-sod* expression also showed significant differences between sampling points in
259 the control group, peaking at ZT18 (ANOVA I, $p<0.05$) (Fig. 4D). Furthermore, for all
260 enzymes, there was a statistically significant interaction between the effects of ZTs and
261 treatments (control/H₂O₂-treated) (Univariate GLM, $p<0.05$).

262

263 **Clock gene expression**

264 In the liver, *bmal1* expression showed daily rhythmicity in the treated fish, with the acrophase
265 at ZT=9:38 (Cosinor, $p<0.05$) (Table 3). However, no daily variations in mRNA expression
266 were observed in the control group. In addition, at ZT6, *bmal1* expression was significantly
267 higher in the treated salmon (t-test independent samples, $p<0.05$) (FC=2) (Fig. 5A). *Clock*
268 expression was rhythmic in both experimental groups, with acrophases at ZT=3:54 (control)
269 and ZT=8:09 (treated) (Cosinor, $p<0.05$), respectively (Table 2, 3).

270 In gills, none of the studied clock genes showed a daily rhythm of expression. However, in
271 the treated salmon, *bmal1* and *clock* expression levels were significantly higher at ZT6 (t-test
272 independent samples, $p<0.05$) (FC=1.5 and 1.3, respectively) (Fig. 5C-D). In addition, for
273 *bmal1*, the Univariate GLM revealed an interaction between treatments and sampling points
274 ($p<0.05$).

275

276 **DISCUSSION**

277 In mammals, it is widely accepted that side effects caused by the exposure to drugs can vary
278 significantly depending on the time of administration, a biological phenomenon referred to as
279 chronotoxicity. However, this is a very new topic in fish with very few studies reported to
280 date. Atlantic salmon is one of the leading aquaculture species in the world and as such, fish
281 are exposed to several therapeutants during their farming cycle. In the present study, we
282 showed significant differences in the stress and toxicological response of fish depending on
283 the time of the day at which they were treated with H₂O₂. Previous investigations had
284 reported that H₂O₂ can result in stress response in this species, increasing plasma glucose and
285 cortisol following exposure, remaining high for around 12 h and then resuming to resting
286 levels 24 h post treatment (Bowers et al., 2002). Likewise, increased plasma glucose, lactate
287 and cortisol levels as well as hematocrit, albumin and several plasma electrolytes were shown
288 in sea bass (*Dicentrarchus labrax*) following H₂O₂ exposure (Roque et al., 2010). In the
289 present study, blood glucose and lactate levels were higher in fish treated during daytime
290 whereas at night the differences between control and treated salmon were either not

291 significant or reversed (higher in the control group). It is difficult to explain such a response
292 given the complexity of the mechanisms regulating glucose and lactate levels and the many
293 factors involved. Stress hyperlactaemia has been suggested to be due to increased aerobic
294 lactate production and related to adrenergic stimulation. In turn, lactate production provides
295 substrate for gluconeogenesis and improves bioenergetics efficiency (Garcia-Alvarez et al.,
296 2014). Moreover, stress response in fish has been reported to be time-dependent (López-
297 Olmeda et al., 2013; Vera et al., 2014). Therefore, further studies are needed to elucidate the
298 effects of hydrogen peroxide at night on glucose and lactate levels. Cortisol levels were also
299 increased after all H₂O₂ treatments irrespective of the time of the day, although differences
300 with control levels were higher during the day in comparison with the night. In all cases
301 glucose and lactate levels ranged between 4.6-5.7 mmol/L and 0.7-10.6 mmol/L,
302 respectively, which is in accordance with previous observations in Atlantic salmon (Lerfall et
303 al., 2014; Hansen et al., 2015). Cortisol levels found in the control group were also similar to
304 those reported for this species (1.9-540.6 ng/mL) (Bowers et al., 2002) although the
305 concentrations measured in the H₂O₂ treated fish were higher in the present experiment
306 (672.5-1357.6 ng/mL). Altogether, these results suggest that H₂O₂ elicited a higher
307 physiological stress response when salmon were treated during the day and in particular
308 around ZT6, indicating that stress response in this species may be under circadian regulation.
309 The acrophase of glucocorticoids daily rhythm seems to vary depending on the activity phase
310 of the animals, occurring in the early morning in diurnal species and at night in nocturnal
311 ones (Dickmeis, 2009). Atlantic salmon has been reported to be a diurnal species
312 (Guðjónsson et al., 2015) but its cortisol basal rhythm seems to show seasonal and life stage
313 differences. Interestingly, similarly to our results in the control group, Ebbesson et al. (2008)
314 described a cortisol profile showing two peaks in summer. The responsiveness of the
315 hypothalamus-pituitary-adrenal (HPA) axis to stress has been shown to vary daily in rats. In
316 fact, the nature of the stress influences these responses: psychological stress (e.g. new
317 environment, confinement) results in the highest stress response during the rest phase
318 whereas physical stress does at the onset of the activity (reviewed in Dickmeis (2009)). In
319 fish, stress response showed daily rhythmicity in gilthead seabream (*Sparus aurata*) and
320 Senegalese sole (*Solea senegalensis*) exposed to air at different times of the day, showing
321 higher cortisol levels when the stressor was applied during their resting phase (López-Olmeda
322 et al., 2013; Vera et al., 2014). In contrast, green sturgeon (*Acipenser medirostris*) subjected
323 to the same physiological stress showed higher blood cortisol and lactate levels during the
324 active phase of this species, as observed in the present study. These contrasting results

325 suggest that daily variations in the stress response are species-specific, and further research is
326 needed to clarify the underlying mechanisms regulating this process.

327 It is known that H₂O₂ exposure induces oxidative stress in fish, increasing the activity of
328 antioxidant enzymes (Tkachenko et al., 2015). In the present study, oxidative stress response
329 in the liver was shown to be time-dependent. Indeed, gene expression of target antioxidant
330 enzymes displayed daily rhythmicity in both experimental groups with an apparent higher
331 expression in the H₂O₂-treated salmon at ZT6 (although difference was not statistically
332 significant for *cat*). In addition, the acrophases of all enzymes studied were found at similar
333 times of the day (around ZT0-1 for the control fish and around ZT4-5 for the treated fish). In
334 gills, such rhythmic expression was only found for *gpx1* in control fish and for *hsp70* and
335 *Mn-sod* in treated fish. In mice, previous studies have revealed that the expression patterns of
336 detoxification genes is regulated by circadian mechanisms that control their daily rhythmicity
337 (Zmrzljak & Rozman, 2012) suggesting that the analysis of daily gene expression of
338 antioxidant enzymes may be used as a tool to predict chronotoxicity. This is further supported
339 by Hamby et al. (2013) who investigated daily activity patterns and detoxification gene
340 expression in *Drosophila suzukii* to determine susceptibility rhythms to insecticides
341 (malathion and fenprothrin). These authors found that the expression of five out of the six
342 genes investigated (two cytochrome P450 genes, two glutathione S-transferases and one α -
343 esterase) showed daily rhythmicity with increased expression at the time of the day when
344 tolerance to malathion was the highest (ZT20). However, most of the target genes peaked at
345 ZT0, when the flies' response to malathion was most variable. Furthermore, a key enzyme in
346 the biotransformation of malathion (converting it to the more toxic malaoxon) peaked at ZT6,
347 when malathion tolerance was the lowest. In our study, the expression of enzymes in the liver
348 of control fish (basal levels) peaked between ZT22-ZT0 (around dawn) and reached
349 minimum expression levels between ZT10-ZT14. However, H₂O₂ treatment induced
350 increased gene expression and stress response of fish when administered at ZT6. In gills,
351 results were less consistent and significant daily rhythmicity of gene expression in control
352 salmon was only found for *gpx1*. However, in the treated fish, higher gene expression of all
353 the analysed enzymes was observed when basal levels in control fish were lower (ZT6-10). In
354 general, it may be expected that fish were more susceptible to toxicant exposure at those
355 times of the day when the expression of detoxification genes in basal conditions was lower.
356 However, in liver, the highest stress and toxicological response of treated fish did not occur at
357 those times. Altogether these results suggest that although daily variations in detoxification in
358 control conditions may affect the toxicity of xenobiotics, there may be other mechanisms

359 influencing fish response to H₂O₂ exposure. Previous investigations have revealed an
360 important correlation between daily activity patterns of fish and the toxicity of anaesthetics
361 when these were administered in a bath. Thus, during the active phase of fish (i.e. during the
362 day in diurnal fish), the toxicity and effectiveness of these compounds (MS-222 and eugenol)
363 was found to be higher, possibly due to an increase in the ventilatory frequency when fish are
364 active and consequently in the uptake of anaesthetics from the water (Sánchez-Vázquez et al.,
365 2011; Vera et al., 2010; Vera et al., 2013a). In the present investigation no data about the
366 activity patterns of the experimental fish could be obtained; therefore a correlation cannot be
367 determined. However, as previously indicated above, Atlantic salmon has been reported to be
368 a diurnal species which may explain, at least in part, the lower effects of hydrogen peroxide
369 at night when we observed significantly lower levels of physiological stress markers and gene
370 expression of antioxidant enzymes.

371 Clock gene expression showed rhythmicity only in the liver, supporting the more robust
372 rhythmicity in the enzyme expression observed in this tissue. For both experimental groups,
373 *clock* expression in liver displayed a similar pattern to that observed in the antioxidant
374 enzymes but with a phase delay of 4 h, whereas *bmal1* expression matched the same pattern
375 only in the treated fish. Previous results in Atlantic salmon have reported that under long-day
376 conditions (as in the present experiment) *clock* and *bmal1* expression did not show daily
377 rhythmicity in the brain (Davie et al., 2009) whereas in liver only *bmal1* expression was
378 rhythmic, with the acrophase found at ZT13 (Betancor et al., 2014). In our study, *clock*
379 expression showed daily rhythmicity in both experimental groups, whereas *bmal1* only did in
380 the treated salmon, with the acrophase at ZT9:38. However, the feeding regime of salmon in
381 the present study differed from that described by Betancor et al. (2014) and this factor has
382 been shown to be a powerful signal able to shift the phase of the daily rhythm of clock gene
383 expression in the liver (Vera et al., 2013b). The expression of both clock genes in gills was
384 arrhythmic but was higher in the treated group at ZT10, as observed for most of the
385 antioxidant enzymes. In mice, Gorbacheva et al. (2005) found that circadian sensitivity to a
386 chemotherapeutant (cyclophosphamide) could not be attributed to variations in metabolic
387 activation or detoxification but it was modulated by the functional status of the
388 CLOCK/BMAL1 transactivation complex which regulates the survival of B cells, probably
389 through the existence of variations in plasma levels of growth factors and cytokines. In
390 particular, the time of the highest resistance (lowest toxicity) to the drug corresponded to the
391 daily peak in the activity of the CLOCK/BMAL1 transactivation complex whereas low-
392 complex activity was correlated with higher sensitivity. In addition, the authors of this

393 research suggested that higher activity could be due to either daily variation or *cry* deficiency
394 while low activity would be caused by daily variation or *bmal1* and/or *clock* deficiency.
395 Likewise, CLOCK/BMAL1 heterodimer activates the expression of *klf10* (*krüppel-like*
396 *factor*), a transcription factor integrating both circadian and metabolic cues to regulate a
397 crucial gene of hepatic gluconeogenesis in mice (Guillaumond et al., 2010). In our study, the
398 hepatic expression of both *bmal1* and *clock* in the treated group peaked at around ZT8-9 but
399 translation of the corresponding proteins would be expected to peak later in the day, giving
400 support to our results showing lower effects of H₂O₂ at the end of the day and at night.
401 Further studies focusing on the levels of CLOCK/BMAL1 complex are clearly needed to
402 confirm this hypothesis.

403 To conclude, the physiological and molecular biomarkers used in this investigation showed
404 that the sublethal toxic effect of H₂O₂ was time-dependent in Atlantic salmon, with both
405 stress and toxicological responses being higher during the first half of the day. Although
406 several mechanisms seem to be involved in the daily control of H₂O₂ toxicity, the present
407 study provides evidence for the optimisation of the timing of treatment administration in
408 aquaculture, opening the door to chronotherapy to treat fish diseases. Next steps will
409 necessarily involve looking at the temporal variations of H₂O₂ on controlling salmon parasitic
410 infections (e.g. sea lice, amoebic gill disease). So far, despite its successful application to
411 treat human diseases (e.g. cancer) only a few studies have focused on the potential
412 application of chronotherapy in veterinary medicine (Giuseppe & Giovanni, 2002; Giudice et
413 al., 2006). Although future studies will be required to investigate the potential time-
414 dependence efficacy of a range of aquaculture treatments, this paper presents first evidence of
415 chronotoxicity in salmon.

416

417 **Acknowledgments**

418 This research was supported by IMPACT Fellowship awarded to L.M. Vera (University of
419 Stirling). The authors wish to thank staff in MERL for their technical assistance and fish
420 management during the acclimatization of fish before the trial and to E. Leclercq and P.F.
421 Almaida-Pagán for their help during fish sampling.

422

423 **Declaration of interest:** The authors report no conflicts of interest. The authors alone are
424 responsible for the content and writing of the paper.

425

426 **REFERENCES**

- 427 Adams MB, Crosbie PBB, Nowak BF. (2012). Preliminary success using hydrogen peroxide
428 to treat Atlantic salmon, *Salmo salar* L., affected with experimentally induced amoebic gill
429 disease (AGD). J Fish Dis. 35:839-848.
- 430 Arvin E, Pedersen L-. (2015). Hydrogen peroxide decomposition kinetics in aquaculture
431 water. Aquac Eng. 64:1-7.
- 432 Betancor MB, McStay E, Minghetti M, Migaud H, Tocher DR, Davie A. (2014). Daily
433 rhythms in expression of genes of hepatic lipid metabolism in Atlantic salmon (*Salmo salar*
434 L.). PLoS ONE 9: e106739.
- 435 Bowers JM, Speare DJ, Burka JF. (2002). The effects of hydrogen peroxide on the stress
436 response of Atlantic salmon (*Salmo salar*). J Vet Pharmacol Ther. 25:311-313.
- 437 Bozwell JL, Clayton RD, Morris JE. (2009). Use of hydrogen peroxide to improve golden
438 shiner egg hatchability. North Am J Aquac. 71:238-241.
- 439 Bruguerolle B. (1998). Chronopharmacokinetics: Current status. Clin Pharmacokinet. 35:83-
440 94.
- 441 Burka JF, Hammell KL, Horsberg TE, Johnson GR, Rainnie DJ, Speare DJ. (1997). Drugs in
442 salmonid aquaculture - A review. J Vet Pharmacol Ther. 20:333-349.
- 443 Costello MJ. (2009). The global economic cost of sea lice to the salmonid farming industry. J
444 Fish Dis. 32:115-118.
- 445 Costello MJ. (2006). Ecology of sea lice parasitic on farmed and wild fish. Trends Parasitol.
446 22:475-483.
- 447 Davie A, Minghetti M, Migaud H. (2009). Seasonal variations in clock -gene expression in
448 atlantic salmon (*Salmo Salar*). Chronobiol Int. 26:379-395.
- 449 Dickmeis T. (2009). Glucocorticoids and the circadian clock. J Endocrinol. 200:3-22.
- 450 Duffield GE. (2003). DNA microarray analyses of circadian timing: The genomic basis of
451 biological time. J Neuroendocrinol. 15:991-1002.
- 452 Ebbesson LOE, Björnsson BT, Ekström P, Stefansson SO. (2008). Daily endocrine profiles in
453 parr and smolt Atlantic salmon. Comp Biochem Physiol A Mol Integr Physiol. 151:698-704.
- 454 García-Alvarez M, Marik P, Bellomo R. (2014). Stress hyperlactaemia: Present
455 understanding and controversy. Lancet Diabetes Endocrinol. 2:339-347.
- 456 Geist J, Werner I, Eder KJ, Leutenegger CM. (2007). Comparisons of tissue-specific
457 transcription of stress response genes with whole animal endpoints of adverse effect in
458 striped bass (*Morone saxatilis*) following treatment with copper and esfenvalerate. Aquatic
459 Toxicology 85:28-39.

- 460 Giudice E, Pugliese A, Piccione G. (2006). Chronotherapy in veterinary medicine. *Med*
461 *Weter.* 62:243-247.
- 462 Giuseppe P, Giovanni C. (2002). Biological rhythm in livestock. *J Vet Sci* 3:145-157.
- 463 Gorbacheva VY, Kondratov RV, Zhang R, Cherukuri S, Gudkov AV, Takahashi JS, Antoch
464 MP. (2005). Circadian sensitivity to the chemotherapeutic agent cyclophosphamide depends
465 on the functional status of the CLOCK/BMAL1 transactivation complex. *Proc Natl Acad Sci*
466 *USA.* 102:3407-3412.
- 467 Grant AN. (2002). Medicines for sea lice. *Pest Manage Sci.* 58:521-527.
- 468 Guðjónsson S, Einarsson SM, Jónsson IR, Guðbrandsson J. (2015). Marine feeding areas and
469 vertical movements of Atlantic salmon (*Salmo salar*) as inferred from recoveries of data
470 storage tags. *Can J Fish Aquatic Sci.* 72:1087-1098.
- 471 Guillaumond F, Gréchez-Cassiau A, Subramaniam M, Brangolo S, Peteri-Brünback B, Staels
472 B, Fiévet C, Spelsberg TC, Delaunay F, Teboul M. (2010). Krüppel-like factor KLF10 is a
473 link between the circadian clock and metabolism in liver. *Mol Cell Biol.* 30:3059-3070.
- 474 Hamby KA, Kwok RS, Zalom FG, Chiu JC. (2013). Integrating circadian activity and gene
475 expression profiles to predict chronotoxicity of drosophila *suzukii* response to insecticides.
476 *PLoS ONE* 8: e68472.
- 477 Hansen TJ, Olsen RE, Stien L, Oppedal F, Torgersen T, Breck O, Remen M, Vågseth T,
478 Fjellidal PG. (2015). Effect of water oxygen level on performance of diploid and triploid
479 Atlantic salmon post-smolts reared at high temperature. *Aquaculture* 435:354-360.
- 480 Johnston, JD. (2012). Adipose circadian rhythms: Translating cellular and animal studies to
481 human physiology. *Mol Cell Endocrinol.* 349:45-50.
- 482 Kierner MCB, Black KD. (1997). The effects of hydrogen peroxide on the gill tissues of
483 Atlantic salmon, *Salmo salar* L. *Aquaculture* 153:181-189.
- 484 Lerfall J, Roth B, Skare EF, Henriksen A, Betten T, Dziatkowiak-Stefaniak MA, Rotabakk
485 BT. (2014). Pre-mortem stress and the subsequent effect on flesh quality of pre-rigor filleted
486 Atlantic salmon (*Salmo salar* L.) during ice storage. *Food Chem.* 175:157-165.
- 487 Lin Y-, Lee C-, Huang J-, Lee H-. (2014). Circadian regulation of permethrin susceptibility
488 by glutathione S-transferase (BgGSTD1) in the German cockroach (*Blattella germanica*). *J*
489 *Insect Physiol.* 65:45-50.
- 490 López-Olmeda JF, Blanco-Vives B, Pujante IM, Wunderink YS, Mancera JM, Sánchez-
491 Vázquez FJ. (2013). Daily rhythms in the hypothalamus-pituitary-interrenal axis and acute
492 stress responses in a teleost flatfish, *Solea senegalensis*. *Chronobiol Int.* 30:530-539.
- 493 Matthews MD, Sakmar JC, Trippel N. (2012). Evaluation of hydrogen peroxide and
494 temperature to control mortality caused by saprolegniasis and to increase hatching success of
495 largemouth bass eggs. *North Am J Aquac.* 74:463-467.

- 496 McStay E, Migaud H, Vera LM, Sánchez-Vázquez FJ, Davie A. (2014). Comparative study
497 of pineal clock gene and AANAT2 expression in relation to melatonin synthesis in Atlantic
498 salmon (*Salmo salar*) and European seabass (*Dicentrarchus labrax*). *Comp Biochem Physiol*
499 *A Mol Integr Physiol.* 169:77-89.
- 500 Olsvik PA, Ørnsrud R, Lunestad BT, Steine N, Fredriksen BN. (2014). Transcriptional
501 responses in Atlantic salmon (*Salmo salar*) exposed to deltamethrin, alone or in combination
502 with azamethiphos. *Comp Biochem Physiol C Toxicol Pharmacol.* 162:23-33.
- 503 Papiol GG, Roque A. (2013). Uses of hydrogen peroxide in aquaculture. *Hydrogen Peroxide:*
504 *Detect, Appl and Health Implic.* 153-170.
- 505 Portaluppi F, Smolensky MH, Touitou Y. (2010). Ethics and methods for biological rhythm
506 research on animals and human beings. *Chronobiol Int.* 27:1911-1929.
- 507 Reichert JS, McNeight SA, Rudel HW. (1939). Determination of hydrogen peroxide and
508 some related peroxygen compounds. *Industrial and Engineering Chemistry* 11:194-197.
- 509 Reppert SM, Weaver DR. (2001). Molecular analysis of mammalian circadian rhythms.
510 *Annu Rev Physiol.* 63:647-676.
- 511 Roque A, Yildiz HY, Carazo I, Duncan, N. (2010). Physiological stress responses of sea bass
512 (*Dicentrarchus labrax*) to hydrogen peroxide (H₂O₂) exposure. *Aquaculture* 304:104-107.
- 513 Sánchez-Vázquez, FJ, Terry, MI, Felizardo, VO, Vera, LM. (2011). Daily rhythms of toxicity
514 and effectiveness of anesthetics (MS222 and eugenol) in zebrafish (*Danio rerio*). *Chronobiol*
515 *Int.* 28:109-117.
- 516 Smolensky MH, Peppas NA. (2007). Chronobiology, drug delivery, and chronotherapeutics.
517 *Adv Drug Deliv Rev.* 59:828-851.
- 518 Tkachenko H, Kurhaluk N, Grudniewska J. (2015). Biomarkers of oxidative stress and
519 antioxidant defences as indicators of different disinfectants exposure in the heart of rainbow
520 trout (*Oncorhynchus mykiss* Walbaum). *Aquac Res.* 46:679-689.
- 521 Torrissen O, Jones S, Asche F, Guttormsen A, Skilbrei OT, Nilsen F, Horsberg TE, Jackson
522 D. (2013). Salmon lice - impact on wild salmonids and salmon aquaculture. *J Fish Dis.*
523 36:171-194.
- 524 Tort MJ, Hurley D, Fernandez-Cobas C, Wooster GA, Bowser PR. (2005). Effects of
525 hydrogen peroxide treatments on catalase and glutathione activity in walleye *Sander vitreus*.
526 *J World Aquac Soc.* 36:577-586.
- 527 Vatine G, Vallone D, Gothilf Y, Foulkes NS. (2011). It's time to swim! Zebrafish and the
528 circadian clock. *FEBS Lett.* 585:1485-1494.
- 529 Vera LM, Montoya A, Sánchez-Vázquez FJ. (2013a). Effectiveness of the anaesthetic MS-
530 222 in gilthead seabream, *Sparus aurata*: Effect of feeding time and day-night variations in
531 plasma MS-222 concentration and GST activity. *Physiol. Behav.* 110-111:51-57.

- 532 Vera LM, Ros-Sánchez G, García-Mateos G, Sánchez-Vázquez FJ. (2010). MS-222 toxicity
533 in juvenile seabream correlates with diurnal activity, as measured by a novel video-tracking
534 method. *Aquaculture* 307:29-34.
- 535 Vera LM, Negrini P, Zagatti C, Frigato E, Sánchez-Vázquez FJ, Bertolucci C. (2013b). Light
536 and feeding entrainment of the molecular circadian clock in a marine teleost (*Sparus aurata*).
537 *Chronobiol Int.* 30:649-661.
- 538 Vera LM, Montoya A, Pujante IM, Pérez-Sánchez J, Caldach-Giner JA, Mancera JM,
539 Moliner J, Sánchez-Vázquez FJ. (2014). Acute stress response in gilthead sea bream (*Sparus*
540 *aurata* L.) is time-of-day dependent: Physiological and oxidative stress indicators.
541 *Chronobiol Int.* 31:1051-1061.
- 542 Zmrzljak UP, Rozman D. (2012). Circadian regulation of the hepatic endobiotic and
543 xenobiotic detoxification pathways: The time matters. *Chem Res Toxicol.* 25:811-824.
- 544

Table 1. Atlantic salmon primer sequences used for real-time PCR

Gene	Accession number	F/R	Primer sequence (5'-3')	Amplicon size (bp)	Anneal
<i>gpx1</i>	DW566563	F	GCCCACCCCTTGTTTGTGTA	103	61 °C
		R	AGACAGGGCTCCACATGATGA		
<i>cat</i>	BT059457	F	CCCAAGTCTTCATCCAGAAACG	123	61 °C
		R	CGTGGGCTCAGTGTTGTTGA		
<i>hsp70</i>	BG933934	F	CCCCTGTCCCTGGGTATTG	121	61 °C
		R	CACCAGGCTGGTTGTCTGAGT		
<i>Mn-sod</i>	DY718412	F	GTTTCTCTCCAGCCTGCTCTAAG	227	61 °C
		R	CCGCTCTCCTTGTCGAAGC		
<i>bmal1</i>	DY735402	F	GCCTACTTGCAACGCTATGTCC	110	64 °C
		R	GCTGCGCCTCGTAATGTCTTCA		
<i>clock</i>	CA038738	F	AGAAATGCCTGCACAGTCGGAGTC	197	64 °C
		R	CCACCAGGTCAGAAGGAAGATGTT		
<i>β-actin</i>	AF012125	F	ATCCTGACAGAGCGCGGTTACAGT	112	61 °C
		R	TGCCCATCTCCTGCTCAAAGTCCA		

Table 2. Parameters of the cosine function calculated by Cosinor analysis ($p < 0.05$) for physiological stress indicators, oxidative stress markers and clock genes of salmon under control conditions.

Biological parameters	Tissue	p value	Mesor	Amplitude	Acrophase (ZT hours)
Glucose	Blood	NS	-	-	-
Lactate	Blood	<0.01	3.4±0.2	0.8±0.4	9:25±1:27
Cortisol	Plasma	NS	-	-	-
<i>gpx1</i>	Liver	<0.01	33,025±4,578	12,644±8,116	0:16±2:38
	Gills	<0.01	34,366±5108	11,295±9053	5:39±3:30
<i>cat</i>	Liver	<0.05	763,541±88,594	190,294±155,798	1:02±3:40
	Gills	NS	-	-	-
<i>hsp70</i>	Liver	<0.01	4,150,828±420,019	1,470,551±730,079	23:44±2:05
	Gills	NS	-	-	-
<i>Mn-sod</i>	Liver	<0.01	19,514±2,029	6,420±3,608	0:43±2:15
	Gills	NS	-	-	-
<i>bmal1</i>	Liver	NS	-	-	-
	Gills	NS	-	-	-
<i>clock</i>	Liver	<0.05	827±114	224±207	3:54±4:25
	Gills	NS	-	-	-

All parameters are expressed as the value ± standard error (SE). NS=non significant.

Table 3. Parameters of the cosine function calculated by Cosinor analysis ($p < 0.05$) for physiological stress indicators, oxidative stress markers and clock genes of salmon after hydrogen peroxide treatment for 20 min.

Biological parameters	Tissue	p value	Mesor	Amplitude	Acrophase (ZT hours)
Glucose	Blood	<0.05	5.0±0.2	0.4±0.4	6:31±4:46
Lactate	Blood	<0.01	4.0±0.4	1.9±0.7	7:23±1:28
Cortisol	Plasma	<0.01	1081±51	127±94	7:12±3:08
<i>gpx1</i>	Liver	<0.01	34,366±5,108	11,295±9,053	5:39±3:30
	Gills	NS	-	-	-
<i>cat</i>	Liver	<0.01	737,607±92,341	243,719±164,522	5:31±3:10
	Gills	NS	-	-	-
<i>hsp70</i>	Liver	<0.01	3,987,115±496,032	1,307,700±885,973	4:40±2:27
	Gills	<0.01	3,465,693±234,801	815,854±422,044	7:46±2:02
<i>Mn-sod</i>	Liver	<0.01	21,615±2,565	6,429±4,568	5:44±3:01
	Gills	<0.05	57,650±3,440	7,108±6,189	7:35±4:04
<i>bmal</i>	Liver	<0.05	919±117	209±203	9:38±5:18
	Gills	NS	-	-	-
<i>clock</i>	Liver	<0.05	819±135	258±241	8:09±4:37
	Gills	NS	-	-	-

All parameters are expressed as the value ± standard error (SE). NS=non significant.

1 **FIGURE LEGENDS**

2 **Figure 1.** Linear relationship (line) between plasma glucose (A) and plasma lactate (B)
3 measured in parallel using handheld metres (x-axis) and spectrophotometric methods (y-
4 axis).

5 **Figure 2.** Daily profiles of blood glucose (A), lactate (B) and plasma cortisol (C) in control
6 (black circles and solid line) and H₂O₂-treated salmon (white circles and dashed line). Values
7 represent the mean \pm SEM (n=6)/time point. White and black bars above the graph represent
8 light and darkness, respectively. Asterisks indicate statistically significant differences
9 between experimental groups at the same time point (t-test independent samples, p<0.05).
10 Superscript letters indicate statistically significant differences between sampling points (ZTs)
11 within the control (lower case letters) and treated (capital letters) groups. The continuous and
12 dotted black lines represent the sinusoidal function determined by Cosinor analysis for the
13 control and treated groups, respectively.

14 **Figure 3.** Gene expression of *gpx1* (A), *cat* (B), *hsp70* (C) and *Mn-sod* (D) genes in liver of
15 control (black circles and solid line) and H₂O₂-treated salmon (white circles and dashed line).
16 Full details given in Figure 2.

17 **Figure 4.** Gene expression of *gpx1* (A), *cat* (B), *hsp70* (C) and *Mn-sod* (D) genes in gills of
18 control (black circles and solid line) and H₂O₂-treated salmon (white circles and dashed line).
19 Full details given in Figure 2.

20 **Figure 5.** Gene expression of *bmal1* (A, B) and *clock* (C, D) genes in liver and gills of
21 control (black circles and solid line) and H₂O₂-treated salmon (white circles and dashed line).
22 Full details given in Figure 2.

23 **Figure 6.** Acrophases map for the parameters analysed in control (A) treated (B) salmon
24 (Cosinor, p<0.05). The confidence intervals (set at 95%) are indicated by the lateral bars.
25 White and black bars above the graph represent light and darkness, respectively.

Fig. 1

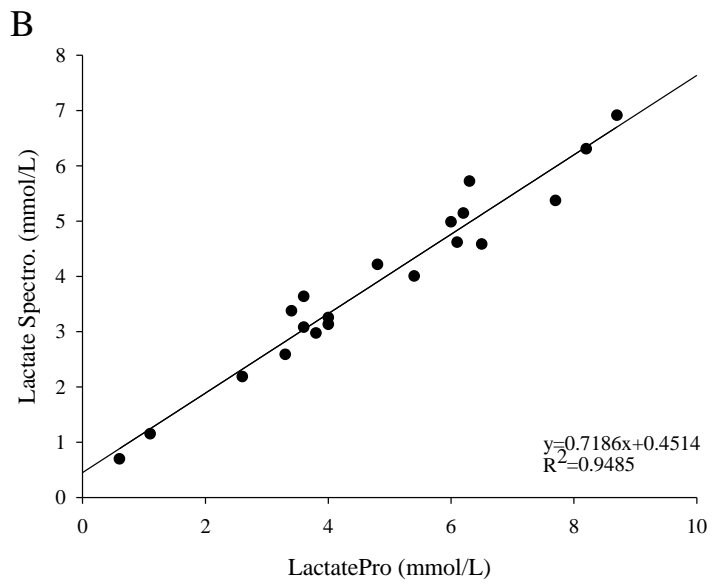
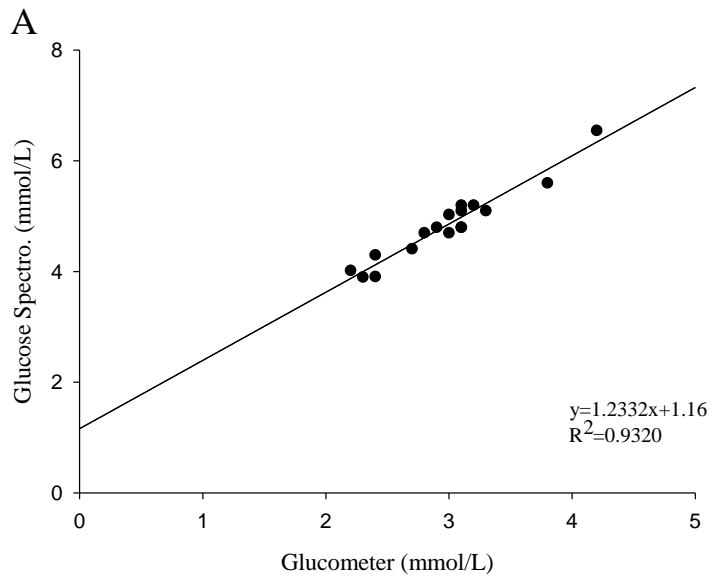


Fig. 2

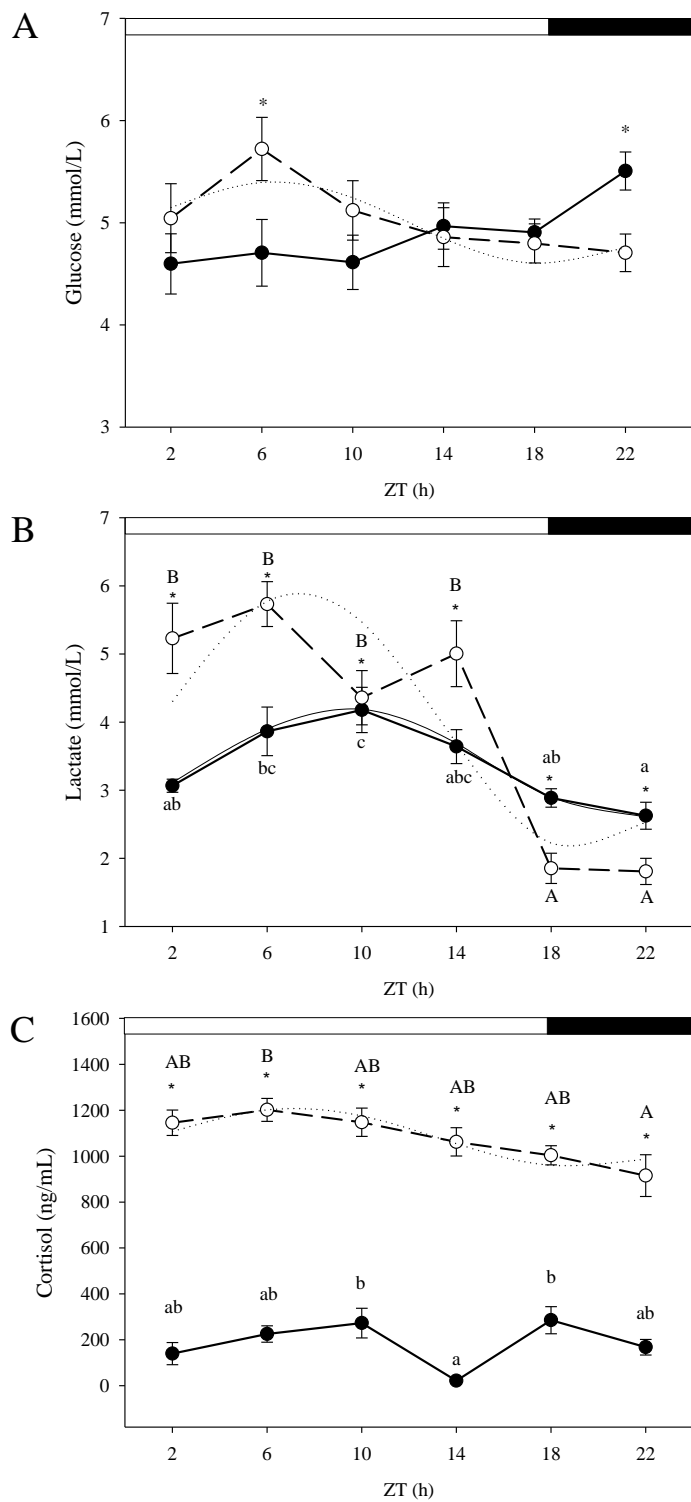


Fig. 3

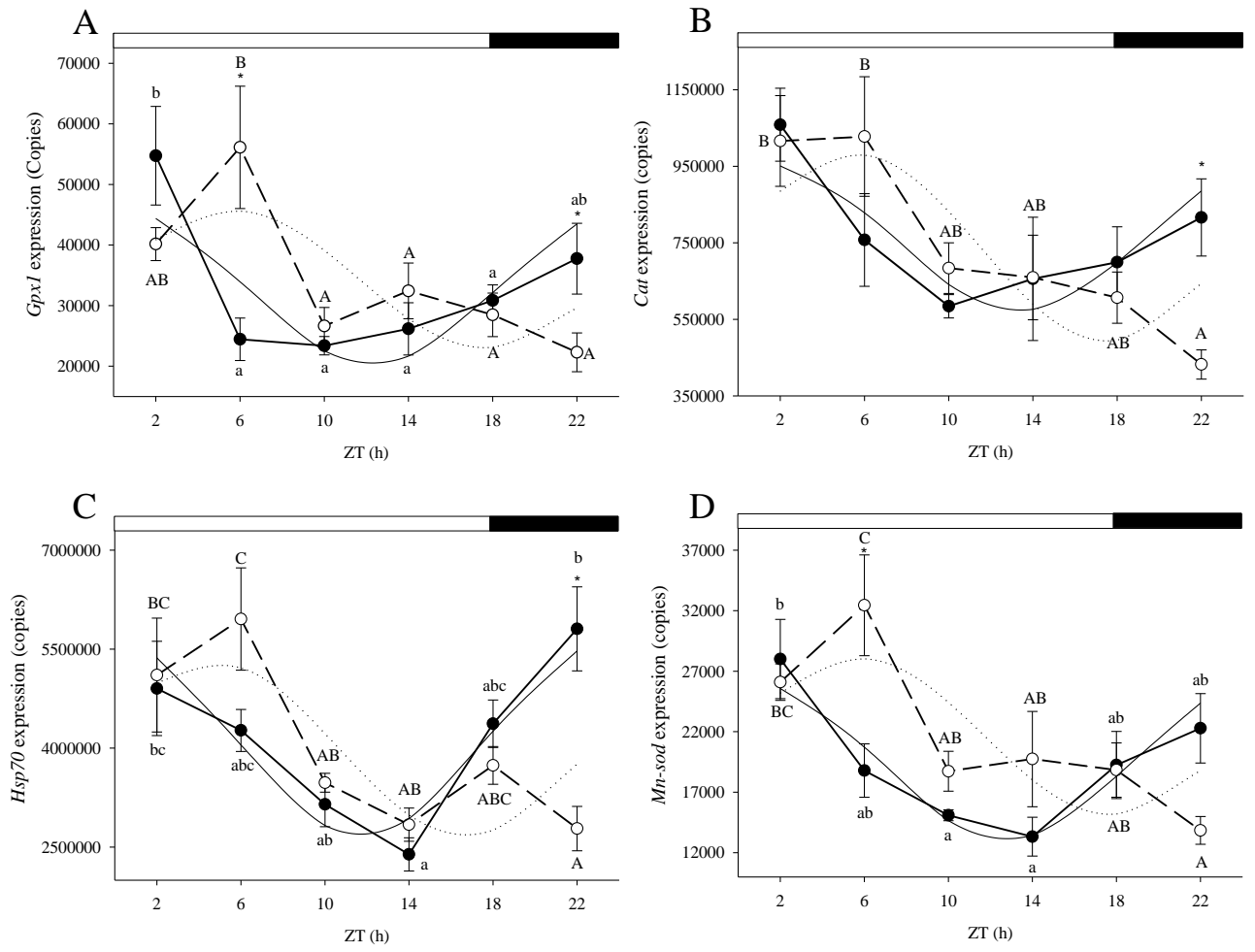


Fig. 4

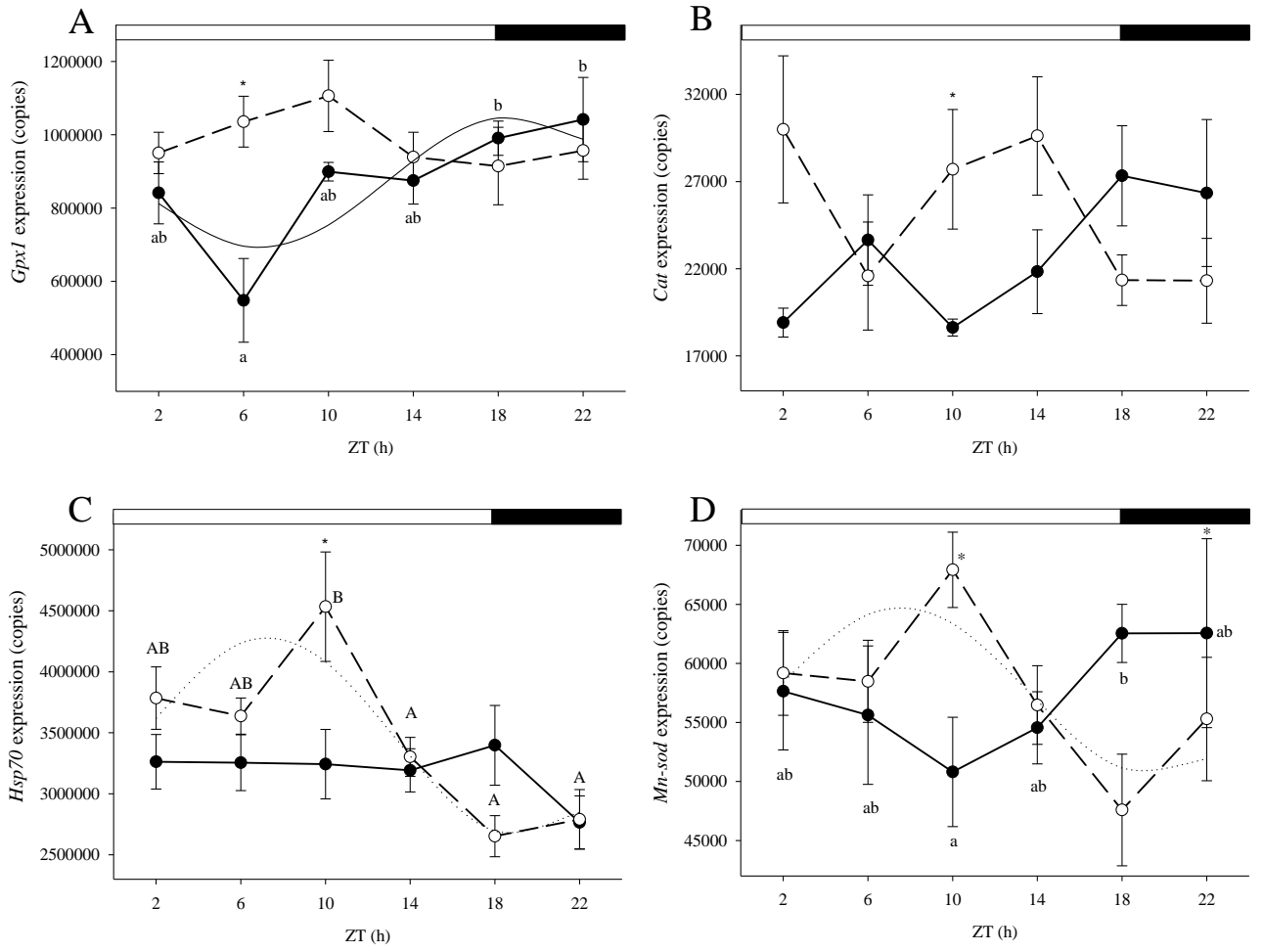


Fig. 5

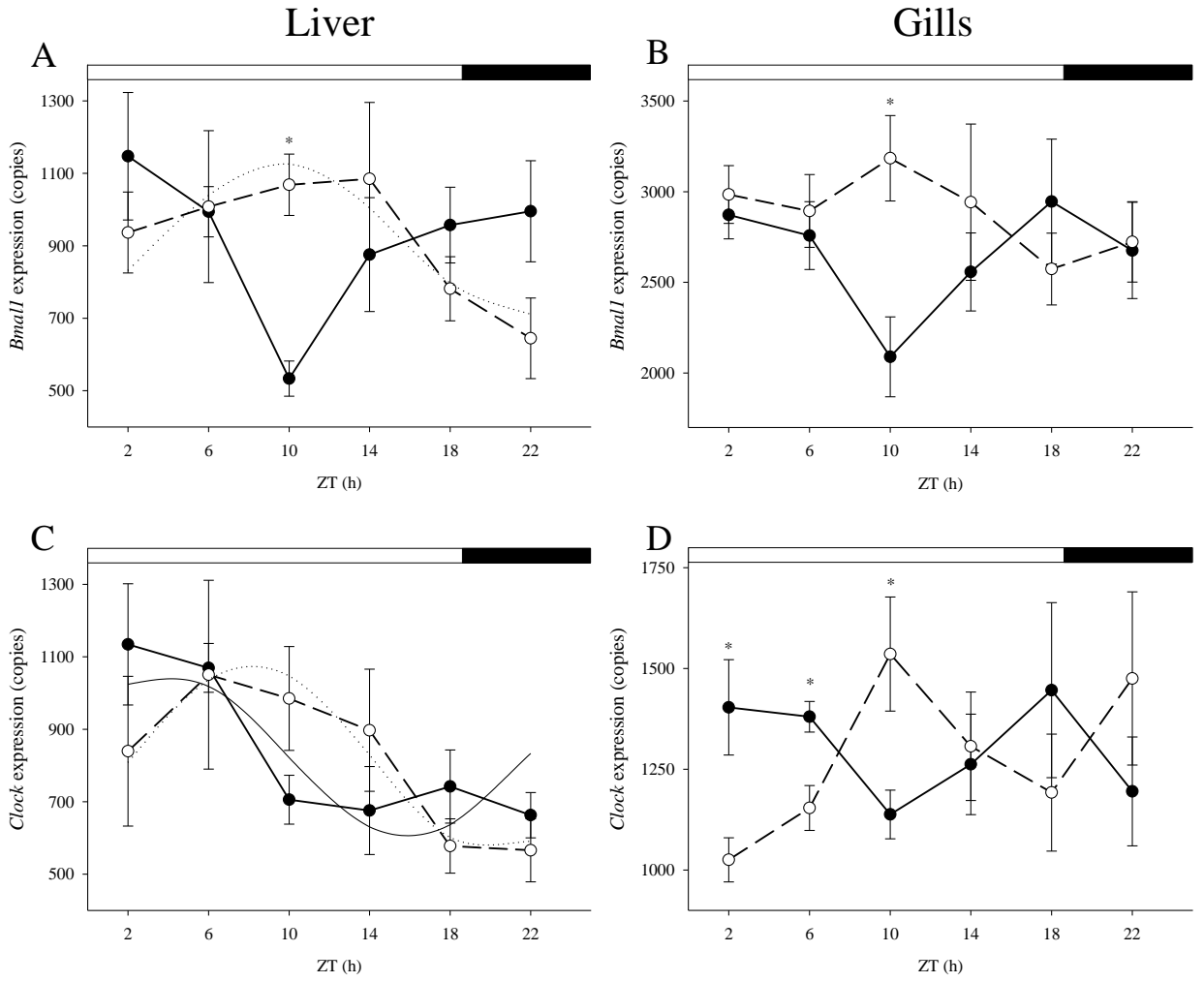


Fig. 6

