1 **HYDROGEN PEROXIDE TREATMENT IN ATLANTIC SALMON INDUCES**

2 **STRESS AND DETOXIFICATION RESPONSE IN A DAILY MANNER**

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4 L.M. Vera[1] and H. Migaud[1]

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

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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

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

18

ABSTRACT

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

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

INTRODUCTION

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

Cultured Atlantic salmon in intensive conditions are susceptible to outbreaks of infectious bacterial, viral and parasitic diseases. Hence, fish are routinely exposed to a number of therapeutic agents during a production cycle to guarantee their health and welfare (Burka et al., 1997; Grant, 2002). Most of these compounds are administered to fish by bath treatments, in-feed additives or through IP injection. In all cases, fish are exposed to the active ingredients at arbitrary times of the day dictated by time and operational constraints rather than scientific evidence. Sea lice are the most widespread pathogenic parasite in Atlantic salmon aquaculture (Costello, 2006; Torrissen et al., 2013), costing the world industry around €300m per year (Costello, 2009). Hydrogen peroxide (H_2O_2) has been used to treat fish ectoparasites for a long time including sea lice *Lepeophtheirus salmonis* and more recently amoebic gill disease caused by *Neoparamoeba perurans* (Adams et al., 2012). Currently H_2O_2 is commonly used by salmon farmers at concentrations between 1500 and 2000 mg/L as a bath treatment at ambient water temperatures (Kiemer & Black, 1997). Other uses of H_2O_2 in aquaculture include the disinfection of fish eggs and microbial control (Bozwell et al., 2009; Matthews et al., 2012). Moreover, despite being a powerful oxidizer, it is rapidly decomposed in water, leaving no toxic products (Arvin & Pedersen, 2015). However, although it has been considered a relatively low toxic compound there is a recent concern on 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 H_2O_2 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 H_2O_2 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 ℃ 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 H_2O_2.
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 H_2O_2) 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 H_2O_2 concentration was immediately measured by cerium sulphate titration
135 method (Reichert et al., 1939) (provided by Solvay Interox 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 H_2O_2 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 80º C until analysis. Liver and gills samples were preserved in RNAlater and kept on ice until
145 further storage at -20 ℃.
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 (Arkray 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 ℃ for 2 min, 95 ℃ for 1 min, followed by 40 cycles at 95 ℃ for

175 15 s, X ℃ for 15 s and 72 ℃ for 30 s (see Table 1 for target specific annealing temperature).

176 This was followed by a temperature ramp from 70 to 90 ℃ 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*, *bmal1* 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.

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Data analysis

Statistical differences in glucose, lactate, cortisol and gene expression levels between sampling points were analysed by a one-way ANOVA (ANOVA I). In addition, at each sampling point, glucose, lactate, cortisol and target genes expression levels were compared between treatments (control *versus* H_2O_2-treated) by means of a t-test, for which a Levene's test was previously used to check for homogeneity of variances. A Univariate General Linear Model (GLM) was carried out to analyse possible interactions between experimental groups and time points. For this, the fixed factors were ''ZT'' (ZT2-22) and ''treatments'' (control and treated). All statistical tests were carried out with the SPSS v19.0 program (SPSS Inc., Chicago, IL), with a statistical threshold set at *p* values <0.05 in all tests. All values are reported as the mean ± SEM.

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

RESULTS

Circulating physiological stress indicators

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

In the control salmon, glucose levels did not show significant differences between sampling points. However, a significant daily rhythm was observed in fish treated with H_2O_2 with the acrophase being found at around ZT6 (Cosinor, *p*<0.05) (Table 3). In addition, at ZT6, glucose levels in the treated group were significantly higher than in the control group. On the contrary glucose levels in the control group were higher at ZT22 (t-test independent samples, *p*<0.05) (Fig. 2A). A significant daily rhythm of lactate was observed in both experimental

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223   groups, with the acrophases located at ZT=9:25 (control) and ZT=7:23 (H_2O_2) (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 H_2O_2 (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,

with daily rhythmicity and higher expression in ZT10 in the treated salmon (FC=1.3). However, *Mn-sod* expression also showed significant differences between sampling points in the control group, peaking at ZT18 (ANOVA I, $p<0.05$) (Fig. 4D). Furthermore, for all enzymes, there was a statistically significant interaction between the effects of ZTs and treatments (control/H_2O_2-treated) (Univariate GLM, $p<0.05$).

Clock gene expression

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

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

DISCUSSION

In mammals, it is widely accepted that side effects caused by the exposure to drugs can vary significantly depending on the time of administration, a biological phenomenon referred to as chronotoxicity. However, this is a very new topic in fish with very few studies reported to date. Atlantic salmon is one of the leading aquaculture species in the world and as such, fish are exposed to several therapeutants during their farming cycle. In the present study, we showed significant differences in the stress and toxicological response of fish depending on the time of the day at which they were treated with H_2O_2. Previous investigations had reported that H_2O_2 can result in stress response in this species, increasing plasma glucose and cortisol following exposure, remaining high for around 12 h and then resuming to resting levels 24 h post treatment (Bowers et al., 2002). Likewise, increased plasma glucose, lactate and cortisol levels as well as hematocrit, albumin and several plasma electrolytes were shown in sea bass (*Dicentrarchus labrax*) following H_2O_2 exposure (Roque et al., 2010). In the present study, blood glucose and lactate levels were higher in fish treated during daytime whereas at night the differences between control and treated salmon were either not

significant or reversed (higher in the control group). It is difficult to explain such a response given the complexity of the mechanisms regulating glucose and lactate levels and the many factors involved. Stress hyperlactaemia has been suggested to be due to increased aerobic lactate production and related to adrenergic stimulation. In turn, lactate production provides substrate for gluconeogenesis and improves bioenergetics efficiency (Garcia-Alvarez et al., 2014). Moreover, stress response in fish has been reported to be time-dependent (López-Olmeda et al., 2013; Vera et al., 2014). Therefore, further studies are needed to elucidate the effects of hydrogen peroxide at night on glucose and lactate levels. Cortisol levels were also increased after all H_2O_2 treatments irrespective of the time of the day, although differences with control levels were higher during the day in comparison with the night. In all cases glucose and lactate levels ranged between 4.6-5.7 mmol/L and 0.7-10.6 mmol/L, respectively, which is in accordance with previous observations in Atlantic salmon (Lerfall et al., 2014; Hansen et al., 2015). Cortisol levels found in the control group were also similar to those reported for this species (1.9-540.6 ng/mL) (Bowers et al., 2002) although the concentrations measured in the H_2O_2 treated fish were higher in the present experiment (672.5-1357.6 ng/mL). Altogether, these results suggest that H_2O_2 elicited a higher physiological stress response when salmon were treated during the day and in particular around ZT6, indicating that stress response in this species may be under circadian regulation. The acrophase of glucocorticoids daily rhythm seems to vary depending on the activity phase of the animals, occurring in the early morning in diurnal species and at night in nocturnal ones (Dickmeis, 2009). Atlantic salmon has been reported to be a diurnal species (Guðjónsson et al., 2015) but its cortisol basal rhythm seems to show seasonal and life stage differences. Interestingly, similarly to our results in the control group, Ebbesson et al. (2008) described a cortisol profile showing two peaks in summer. The responsiveness of the hypothalamus-pituitary-adrenal (HPA) axis to stress has been shown to vary daily in rats. In fact, the nature of the stress influences these responses: psychological stress (e.g. new environment, confinement) results in the highest stress response during the rest phase whereas physical stress does at the onset of the activity (reviewed in Dickmeis (2009)). In fish, stress response showed daily rhythmicity in gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) exposed to air at different times of the day, showing higher cortisol levels when the stressor was applied during their resting phase (López-Olmeda et al., 2013; Vera et al., 2014). In contrast, green sturgeon (*Acipenser medirostris*) subjected to the same physiological stress showed higher blood cortisol and lactate levels during the active phase of this species, as observed in the present study. These contrasting results

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

It is known that H_2O_2 exposure induces oxidative stress in fish, increasing the activity of antioxidant enzymes (Tkachenko et al., 2015). In the present study, oxidative stress response in the liver was shown to be time-dependent. Indeed, gene expression of target antioxidant enzymes displayed daily rhythmicity in both experimental groups with an apparent higher expression in the H_2O_2-treated salmon at ZT6 (although difference was not statistically significant for *cat*). In addition, the acrophases of all enzymes studied were found at similar times of the day (around ZT0-1 for the control fish and around ZT4-5 for the treated fish). In gills, such rhythmic expression was only found for *gpx1* in control fish and for *hsp70* and *Mn-sod* in treated fish. In mice, previous studies have revealed that the expression patterns of detoxification genes is regulated by circadian mechanisms that control their daily rhythmicity (Zmrzljak & Rozman, 2012) suggesting that the analysis of daily gene expression of antioxidant enzymes may be used as a tool to predict chronotoxicity. This is further supported by Hamby et al. (2013) who investigated daily activity patterns and detoxification gene expression in *Drosophila suzukii* to determine susceptibility rhythms to insecticides (malathion and fenpropathrin). These authors found that the expression of five out of the six genes investigated (two cytochrome P450 genes, two glutathione S-transferases and one α-esterase) showed daily rhythmicity with increased expression at the time of the day when tolerance to malathion was the highest (ZT20). However, most of the target genes peaked at ZT0, when the flies' response to malathion was most variable. Furthermore, a key enzyme in the biotransformation of malathion (converting it to the more toxic malaoxon) peaked at ZT6, when malathion tolerance was the lowest. In our study, the expression of enzymes in the liver of control fish (basal levels) peaked between ZT22-ZT0 (around dawn) and reached minimum expression levels between ZT10-ZT14. However, H_2O_2 treatment induced increased gene expression and stress response of fish when administered at ZT6. In gills, results were less consistent and significant daily rhythmicity of gene expression in control salmon was only found for *gpx1*. However, in the treated fish, higher gene expression of all the analysed enzymes was observed when basal levels in control fish were lower (ZT6-10). In general, it may be expected that fish were more susceptible to toxicant exposure at those times of the day when the expression of detoxification genes in basal conditions was lower. However, in liver, the highest stress and toxicological response of treated fish did not occur at those times. Altogether these results suggest that although daily variations in detoxification in control conditions may affect the toxicity of xenobiotics, there may be other mechanisms

359 influencing fish response to H_2O_2 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 c*lock* 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 H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 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.

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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
gpx1	DW566563	F	GCCCACCCCTTGTTTGTGTA	103	61 ºC
		R	AGACAGGGCTCCACATGATGA		
cat	BT059457	F	CCCAAGTCTTCATCCAGAAACG	123	61 ºC
		R	CGTGGGCTCAGTGTTGTTGA		
hsp70	BG933934	F	CCCCTGTCCCTGGGTATTG	121	61 ºC
		R	CACCAGGCTGGTTGTCTGAGT		
Mn-sod	DY718412	F	GTTTCTCTCCAGCCTGCTCTAAG	227	61 ºC
		R	CCGCTCTCCTTGTCGAAGC		
bmal1	DY735402	F	GCCTACTTGCAACGCTATGTCC	110	64 ºC
		R	GCTGCGCCTCGTAATGTCTTCA		
clock	CA038738	F	AGAAATGCCTGCACAGTCGGAGTC	197	64 ºC
		R	CCACCAGGTCAGAAGGAAGATGTT		
β-actin	AF012125	F	ATCCTGACAGAGCGCGGTTACAGT	112	61 ºC
		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	-	-	-
gpx1	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
cat	Liver	<0.05	763,541±88,594	190,294±155,798	1:02±3:40
	Gills	NS	-	-	-
hsp70	Liver	<0.01	4,150,828±420,019	1,470,551±730,079	23:44±2:05
	Gills	NS	-	-	-
Mn-sod	Liver	<0.01	19,514±2,029	6,420±3,608	0:43±2:15
	Gills	NS	-	-	-
bmal1	Liver	NS	-	-	-
	Gills	NS	-	-	-
clock	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
gpx1	Liver	<0.01	34,366±5,108	11,295±9,053	5:39±3:30
	Gills	NS	-	-	-
cat	Liver	<0.01	737,607±92,341	243,719±164,522	5:31±3:10
	Gills	NS	-	-	-
hsp70	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
Mn-sod	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
bmal	Liver	<0.05	919±117	209±203	9:38±5:18
	Gills	NS	-	-	-
clock	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 H_2O_2-treated salmon (white circles and dashed line). Values

7   represent the mean ±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 H_2O_2-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 H_2O_2-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 H_2O_2-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.

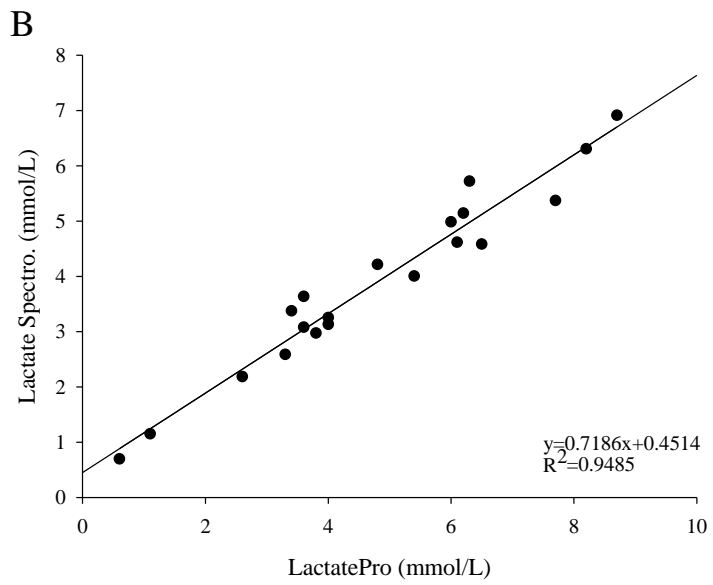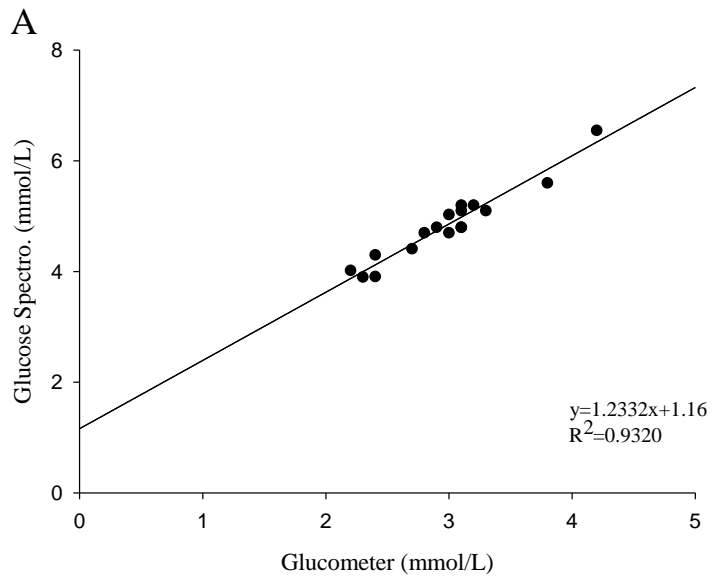25   White and black bars above the graph represent light and darkness, respectively.

Fig. 1

A
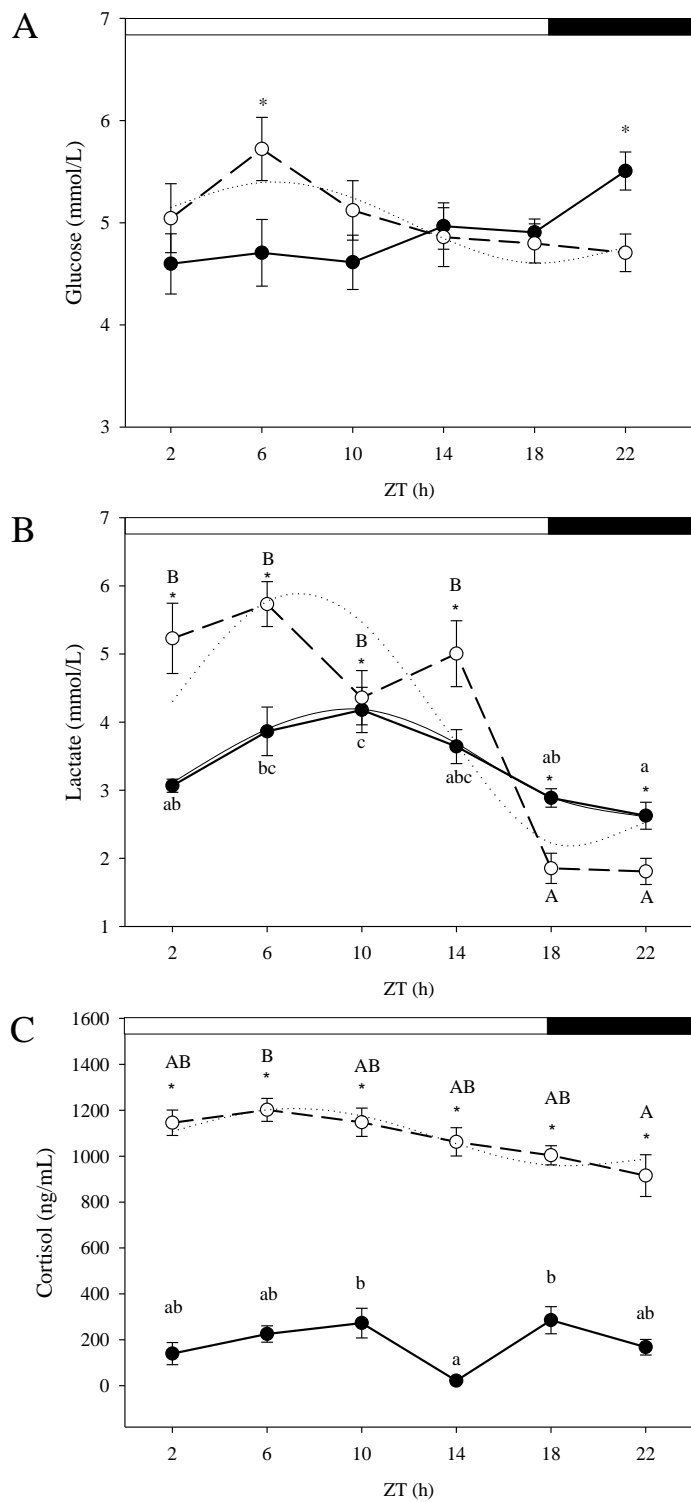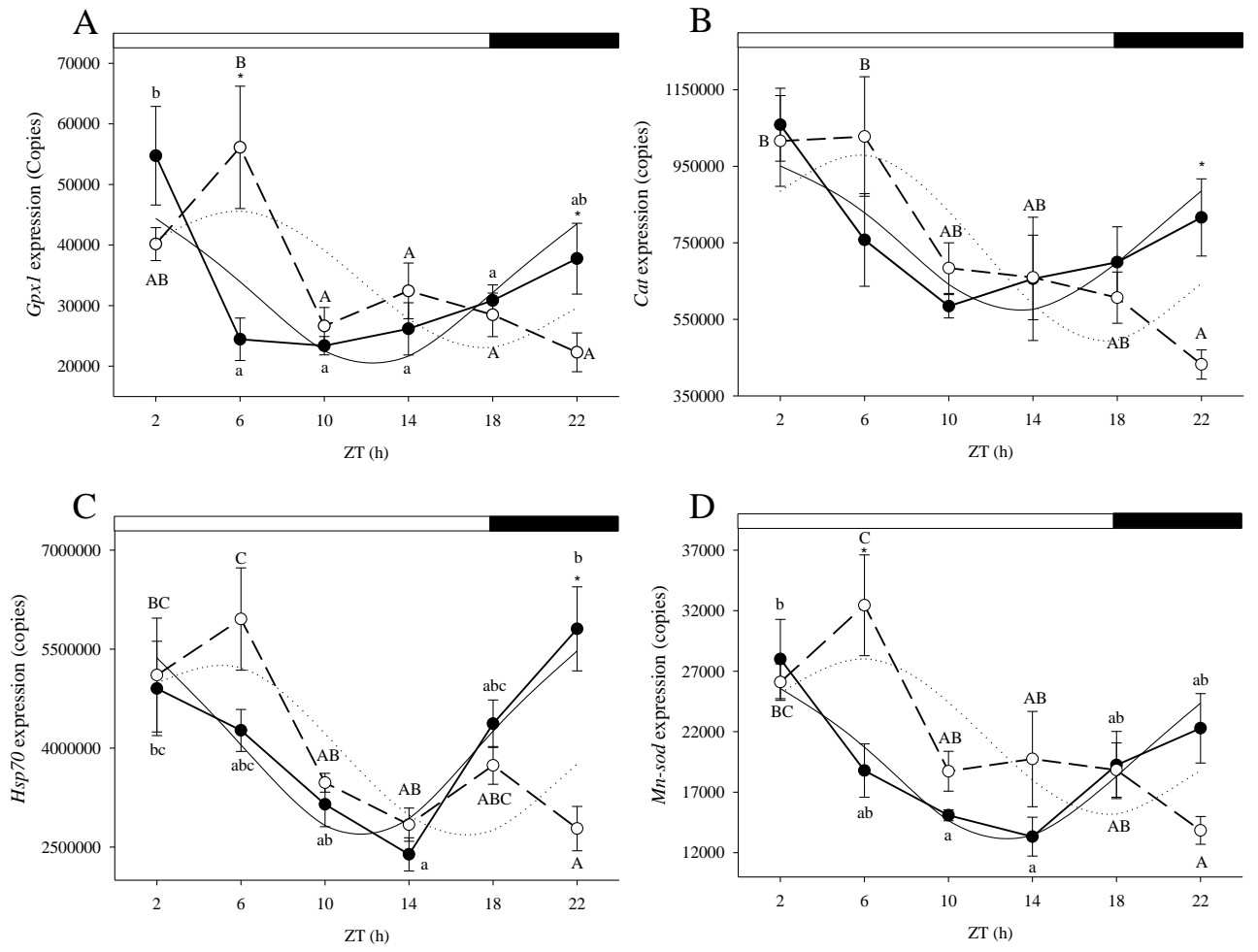

$y=1.2332x+1.16$
$R^2=0.9320$

B

$y=0.7186x+0.4514$
$R^2=0.9485$

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Liver   Gills

A   B

Fig. 6