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

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Running head: Hydrogen peroxide chronotoxicity in salmon

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

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

Previous studies have also indicated that H_2O_2 exposure can increase the activity of catalase and glutathione in fish (Tort et al., 2005). In fact, changes in the expression of oxidative stress markers are considered to be informative biomarkers to identify and quantify the sublethal effects of environmental stressors in fish, including toxicant exposure. This has led to the rising of ecotoxicogenomics, a new discipline combining genomics and ecotoxicology to identify biomarkers at the transcriptome level that may be used as indicators for the exposure to contaminants and to quantify their effects on the organisms (Geist et al., 2007). In addition, it is now accepted that the expression of genes involved in the metabolism of xenobiotics is under circadian clock control, showing highest expression levels at certain times of the day (Hamby et al., 2013; Lin et al., 2014). However, such rhythmicity and its implications on fish toxicological response have not been yet investigated.

In the present study we examined the time-dependent effects of H_2O_2 exposure in Atlantic salmon. Physiological and molecular markers were investigated, including the expression of detoxification enzymes (*gpx1*, *cat*, *hsp70* and *Mn-sod*) involved in the cellular defence against oxidative stress caused by enhanced levels of ROS, as well as the expression of core clock genes (*bmal1* and *clock*) involved in the activation of transcription factors regulating metabolic processes in mammalian liver. The final objective of the present research was to determine the influence of the administration time of this aquaculture therapeutant on the physiological status of fish and potentially refine health management strategies.

MATERIALS AND METHODS

Animals & housing

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

Experimental design

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

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

The trial was performed in June 2014 with ambient water temperature of 12.3 ± 0.3 ºC and simulated natural photoperiod set at 18 h light: 6 h darkness (18L:6D). Fish were treated with a nominal concentration of 1500 mg/L for 20 min at six different times of the day (every 4 h during a 24-h cycle): "zeitgeber time" (ZT) 2 (two hour after lights on), ZT6, ZT10, ZT14, ZT18 and ZT22. At each time point, one of the 16 tanks (n=8 salmon) was treated with H_2O_2. During treatment, water was oxygenated ensuring that oxygen levels were above 7 mg/L and water temperature was monitored. In addition, at each sampling point, another tank containing 8 control salmon (not exposed to H_2O_2) were also sampled. Following exposure, fish were sacrificed by lethal anaesthesia (2-phenoxyethanol 1 mL/L, Sigma) and blood, liver and gills samples were obtained from each fish. Water samples were collected from each treated tank and H_2O_2 concentration was immediately measured by cerium sulphate titration method (Reichert et al., 1939) (provided by Solvay Interox Ltd.). Briefly, 5 mL of 5N sulfuric acid and 7.5 mL of cerium IV sulphate solution were mixed in a conical flask. Then, a burette was filled with 50 mL of water sample and was slowly dispensed into the cerium IV sulphate solution, swirling to mix until the solution went colourless. The reading of the burette was then recorded and H_2O_2 concentration (mg/L) calculated. The measured concentrations throughout the trial was 1419.6 ± 9.4 mg/L.

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

Glucose, lactate and cortisol analyses

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

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

Gene expression analyses

Liver and gill samples were homogenised in 1 mL of TRIzol® (Invitrogen, UK) and total RNA extracted in accordance with the manufacturer's instructions. RNA pellets were rehydrated in MilliQ water and total RNA concentration was determined using an ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK). RNA integrity was assessed by electrophoresis. In order to eliminate any genomic DNA contamination, total RNA was DNase treated following DNA-free™ kit (Applied Biosystems, Warrington, UK). cDNA was then reverse transcribed from 1 µg of DNase treated total RNA using random hexamer and Oligo $(dT)_{12\text{-}18}$ primers in a 20 µL total reaction volume (high capacity reverse transcription kit without RNase inhibitor, Applied Biosystems, UK). Real-time PCR was performed using Luminaris color Higreen qPCR Master mix (Thermo Fisher Scientific, MA, USA) and Mastercycler RealPlex 2 thermocycler (Eppendorf, UK) which was programmed to perform the following protocol: 50 ºC for 2 min, 95 ºC for 1 min, followed by 40 cycles at 95 ºC for 15 s, X ºC for 15 s and 72 ºC for 30 s (see Table 1 for target specific annealing temperature). This was followed by a temperature ramp from 70 to 90 ºC for melt-curve analysis to verify that no primer-dimer artefacts were present and only one product was generated from each qPCR assay. The final volume of the PCR reaction was 10 µL: 2.5 µL of cDNA, 5 µL of the qPCR Master Mix and 2.5 µL of forward and reverse primers (Table 1). The primers used to amplify *gpx1*, *cat*, *hsp70*, *Mn-sod*, *bmal1* and *clock* were previously tested and validated for Atlantic salmon (Betancor et al., 2014; McStay et al., 2014; Olsvik et al., 2014). All samples were run in duplicate. For both liver and gills samples, we verified the efficiency of the primers by doing standard curves for all genes investigated. Expression of the target genes was measured by absolute quantification with all samples being normalized with *β-actin* mRNA expression (McStay et al., 2014). Quantification was achieved by translating CT values of unknown samples from a parallel set of reactions containing a serial dilution of spectrophotometrically determined linearised plasmid containing partial cDNA sequences.

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: $Y = M + A * [\text{Cos}(\Omega t + \Phi)]$, 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

groups, with the acrophases located at ZT=9:25 (control) and ZT=7:23 (H_2O_2) (Cosinor, $p<0.05$) (Table 2, 3). Overall, lactate levels were significantly higher in the treated group during the day (5.1 ± 0.2 mmol/L) and lower at night (1.8 ± 0.2 mmol/L) (t-test independent samples, $p<0.05$) (Fig. 2B).

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

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

Expression of oxidative stress markers

In the liver, mRNA expression of all oxidative stress genes analysed in this experiment showed daily rhythmicity in both treated and control groups, with acrophases around ZT0-1 for the control fish and around ZT4-5 for the salmon treated with H_2O_2 (Cosinor, $p<0.05$) (Table 2, 3). In addition, for both *gpx1* and *Mn-sod*, expression was significantly higher at ZT6 in the treated fish (t-test independent samples, $p<0.05$), with fold-change (FC) differences in expression of 2.3 and 1.7, respectively. *Cat* and *hsp70* expression levels also appeared to be higher at ZT6 although differences between groups were not statistically significant. Gene expression of all enzymes was lower at ZT22 in the treated group (Fig. 3). Finally, there was a significant interaction between treatments and sampling points for *gpx1*, *hsp70* and *Mn-sod* (Univariate GLM, $p<0.05$).

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

influencing fish response to H_2O_2 exposure. Previous investigations have revealed an important correlation between daily activity patterns of fish and the toxicity of anaesthetics when these were administered in a bath. Thus, during the active phase of fish (i.e. during the day in diurnal fish), the toxicity and effectiveness of these compounds (MS-222 and eugenol) was found to be higher, possibly due to an increase in the ventilatory frequency when fish are active and consequently in the uptake of anaesthetics from the water (Sánchez-Vázquez et al., 2011; Vera et al., 2010; Vera et al., 2013a). In the present investigation no data about the activity patterns of the experimental fish could be obtained; therefore a correlation cannot be determined. However, as previously indicated above, Atlantic salmon has been reported to be a diurnal species which may explain, at least in part, the lower effects of hydrogen peroxide at night when we observed significantly lower levels of physiological stress markers and gene expression of antioxidant enzymes.

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

research suggested that higher activity could be due to either daily variation or *cry* deficiency while low activity would be caused by daily variation or *bmal1* and/or *clock* deficiency. Likewise, CLOCK/BMAL1 heterodimer activates the expression of *klf10* (*krüppel-like factor*), a transcription factor integrating both circadian and metabolic cues to regulate a crucial gene of hepatic gluconeogenesis in mice (Guillaumond et al., 2010). In our study, the hepatic expression of both *bmal1* and *clock* in the treated group peaked at around ZT8-9 but translation of the corresponding proteins would be expected to peak later in the day, giving support to our results showing lower effects of H_2O_2 at the end of the day and at night. Further studies focusing on the levels of CLOCK/BMAL1 complex are clearly needed to confirm this hypothesis.

To conclude, the physiological and molecular biomarkers used in this investigation showed that the sublethal toxic effect of H_2O_2 was time-dependent in Atlantic salmon, with both stress and toxicological responses being higher during the first half of the day. Although several mechanisms seem to be involved in the daily control of H_2O_2 toxicity, the present study provides evidence for the optimisation of the timing of treatment administration in aquaculture, opening the door to chronotherapy to treat fish diseases. Next steps will necessarily involve looking at the temporal variations of H_2O_2 on controlling salmon parasitic infections (e.g. sea lice, amoebic gill disease). So far, despite its successful application to treat human diseases (e.g. cancer) only a few studies have focused on the potential application of chronotherapy in veterinary medicine (Giuseppe & Giovanni, 2002; Giudice et al., 2006). Although future studies will be required to investigate the potential time-dependence efficacy of a range of aquaculture treatments, this paper presents first evidence of 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.

FIGURE LEGENDS

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

Figure 2. Daily profiles of blood glucose (A), lactate (B) and plasma cortisol (C) in control (black circles and solid line) and H_2O_2-treated salmon (white circles and dashed line). Values represent the mean ±SEM (n=6)/time point. White and black bars above the graph represent light and darkness, respectively. Asterisks indicate statistically significant differences between experimental groups at the same time point (t-test independent samples, $p<0.05$). Superscript letters indicate statistically significant differences between sampling points (ZTs) within the control (lower case letters) and treated (capital letters) groups. The continuous and dotted black lines represent the sinusoidal function determined by Cosinor analysis for the control and treated groups, respectively.

Figure 3. Gene expression of *gpx1* (A), *cat* (B), *hsp70* (C) and *Mn-sod* (D) genes in liver of control (black circles and solid line) and H_2O_2-treated salmon (white circles and dashed line). Full details given in Figure 2.

Figure 4. Gene expression of *gpx1* (A), *cat* (B), *hsp70* (C) and *Mn-sod* (D) genes in gills of control (black circles and solid line) and H_2O_2-treated salmon (white circles and dashed line). Full details given in Figure 2.

Figure 5. Gene expression of *bmal1* (A, B) and *clock* (C, D) genes in liver and gills of control (black circles and solid line) and H_2O_2-treated salmon (white circles and dashed line). Full details given in Figure 2.

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

Fig. 1

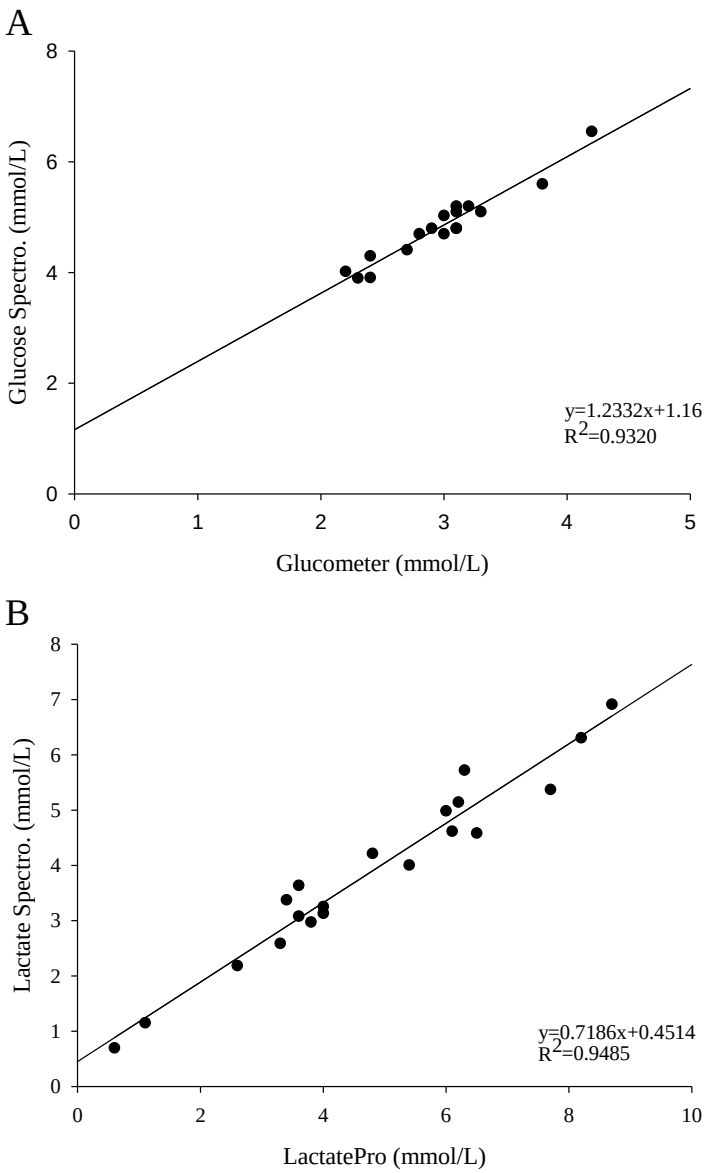

A
Glucose Spectro. (mmol/L)
Glucometer (mmol/L)
y=1.2332x+1.16
R2=0.9320
B
Lactate Spectro. (mmol/L)
LactatePro (mmol/L)
y=0.7186x+0.4514
R2=0.9485

Fig. 2

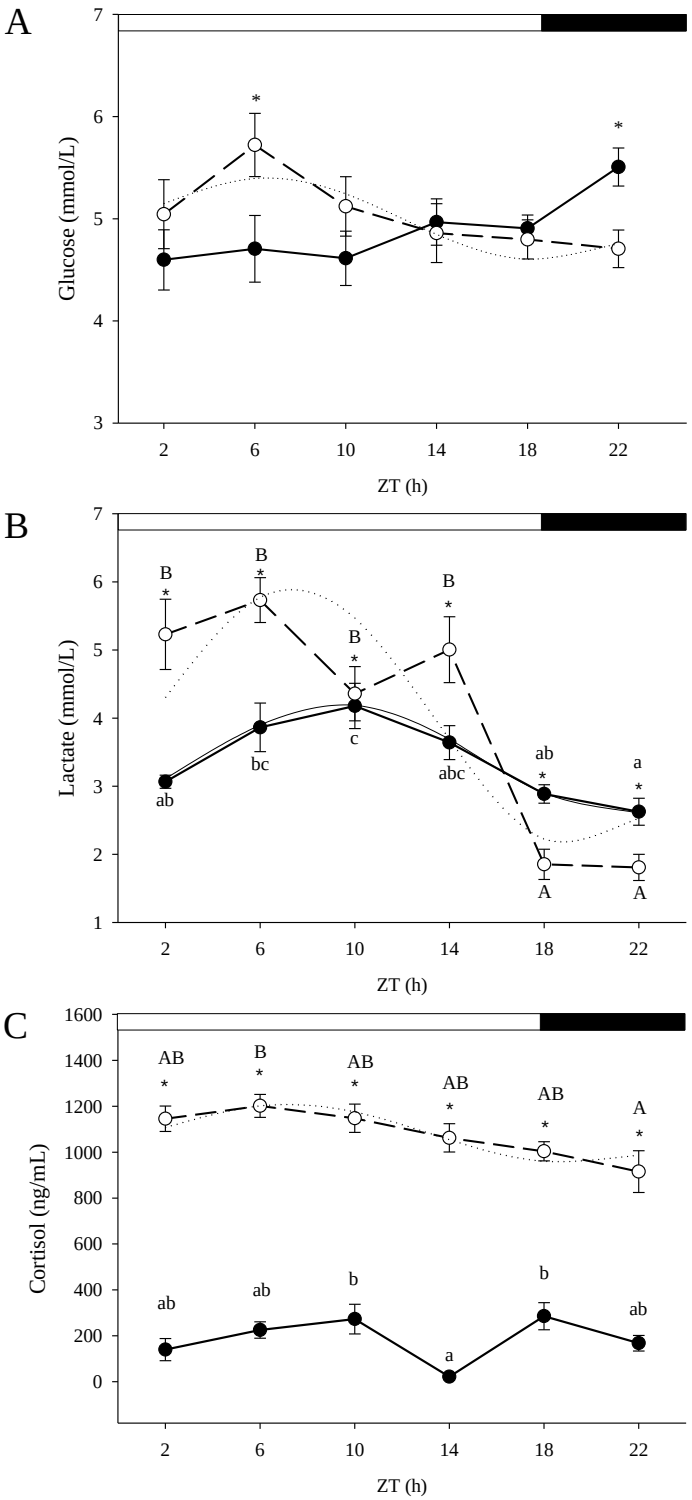


Fig. 3

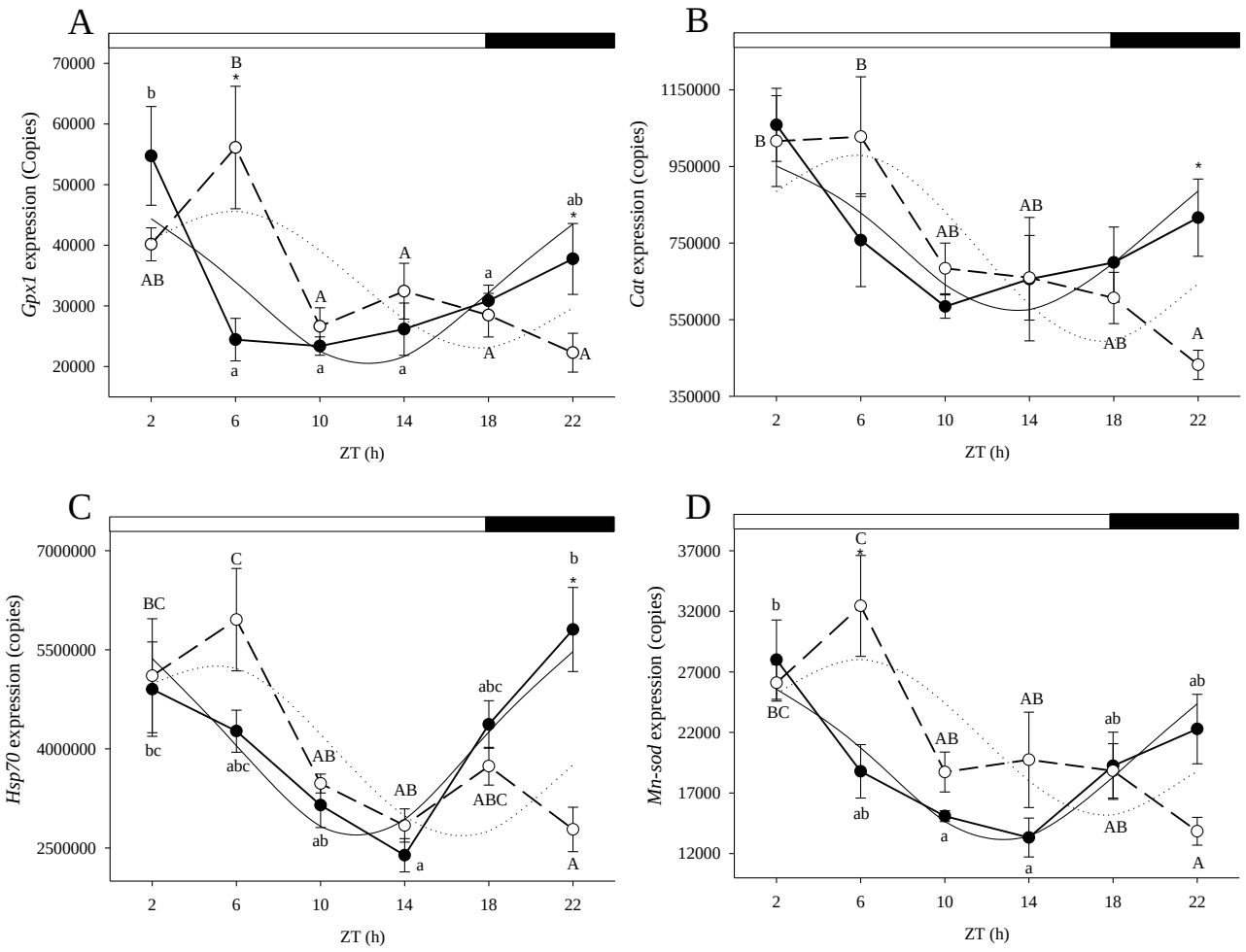
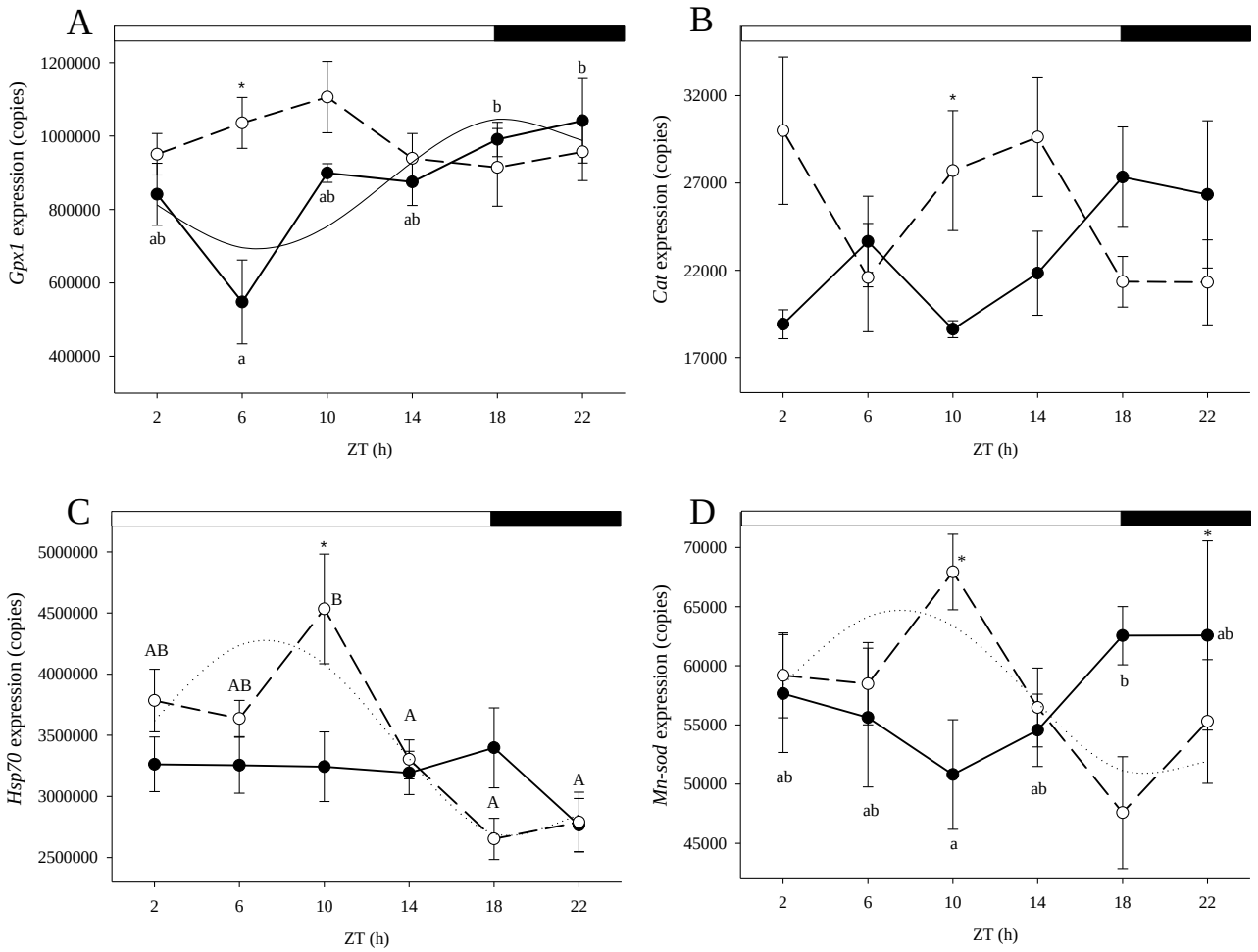

A
B
C
D
Gpx1 expression (Copies)
Cat expression (copies)
Hsp70 expression (copies)
Mn-sod expression (copies)
ZT (h)
2
6
10
14
18
22

Fig. 4


A
Gpx1 expression (copies)
1200000
1000000
800000
600000
400000
2
6
10
14
18
22
ZT (h)
*
b
b
ab
ab
ab
a
B
Cat expression (copies)
32000
27000
22000
17000
2
6
10
14
18
22
ZT (h)
*
C
Hsp70 expression (copies)
5000000
4500000
4000000
3500000
3000000
2500000
2
6
10
14
18
22
ZT (h)
*
AB
AB
B
A
A
A
D
Mn-sod expression (copies)
70000
65000
60000
55000
50000
45000
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18
22
ZT (h)
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ab
ab
a
ab
b
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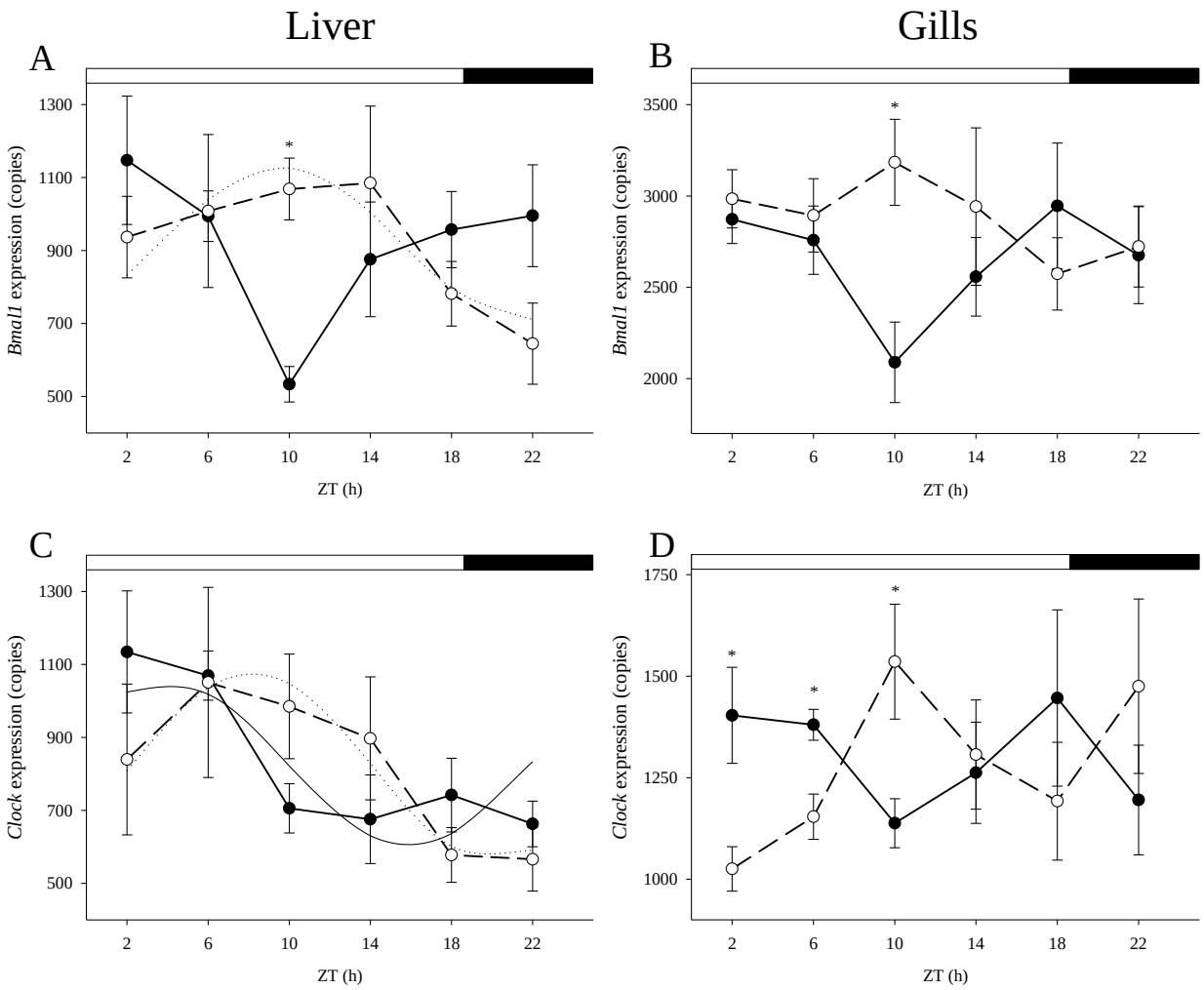
Fig. 5


Liver
Gills
A
B
C
D
Bmal1 expression (copies)
Clock expression (copies)
ZT (h)
1300
1100
900
700
500
3500
3000
2500
2000
1750
1500
1250
1000
2
6
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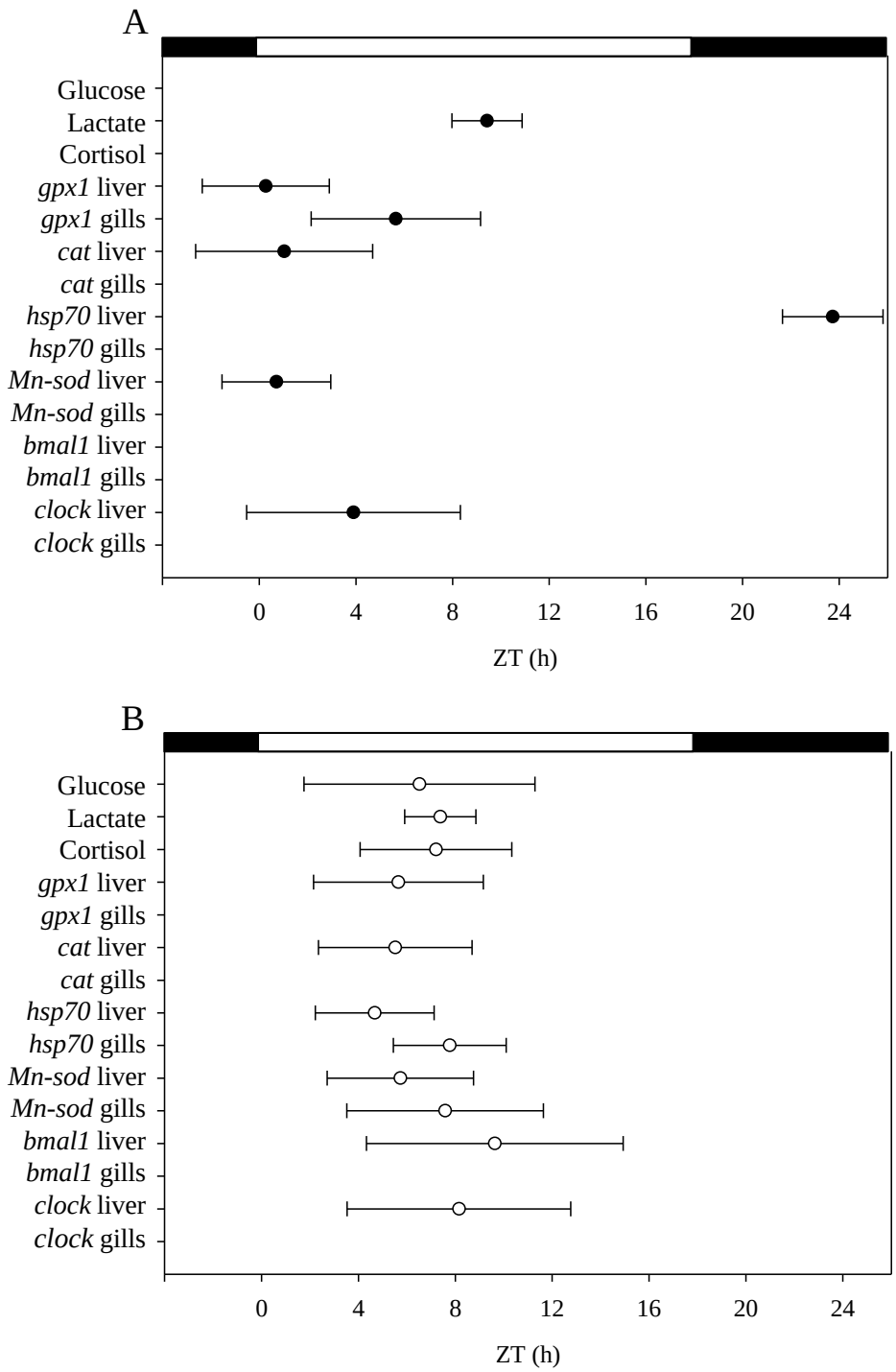
14
18
22
*

Fig. 6


A
Glucose
Lactate
Cortisol
gpx1 liver
gpx1 gills
cat liver
cat gills
hsp70 liver
hsp70 gills
Mn-sod liver
Mn-sod gills
bmal1 liver
bmal1 gills
clock liver
clock gills
0
4
8
12
16
20
24
ZT (h)
B
Glucose
Lactate
Cortisol
gpx1 liver
gpx1 gills
cat liver
cat gills
hsp70 liver
hsp70 gills
Mn-sod liver
Mn-sod gills
bmal1 liver
bmal1 gills
clock liver
clock gills
0
4
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24
ZT (h)