LIGHT- AND CLOCK-CONTROL OF GENES INVOLVED IN DETOXIFICATION

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Running head: Detoxification rhythms in zebrafish

ABSTRACT

Circadian regulation of hepatic detoxification seems to be amongst the key roles of the biological clock. The liver is the major site for biotransformation, and in mammals, it contains several clock-controlled transcription factors such as PAR basic leucine zipper proteins (bZIP) and basic-helix-loop-helix (bHLH)-PAS family that act as circadian regulators of detoxification genes. This investigation explored the existence of daily and circadian expression of transcription factors involved in detoxification, as well as the temporal profile of a set of their target genes in zebrafish liver. In our study, zebrafish were able to synchronize to a light-dark (LD) cycle and displayed a diurnal pattern of activity. In addition, the expression of clock genes presented daily and circadian rhythmicity in liver. Apart from *hlfa*, the expression of PAR bZIP transcription factors also displayed daily rhythms, which appeared to be both light-dependent and clock-controlled, as circadian rhythms free-ran under constant conditions (continuous darkness, DD). Under LD, *tefb*, *dbpa* and *dbpb* expression peaked at the end of the darkness period whereas *tefa* showed peak levels of expression at the onset of the photophase. In addition, these four genes exhibited circadian expression under DD, with higher expression levels at the end of the subjective night. The expression of the bHLH-PAS transcription factor *arh2* also showed circadian rhythmicity in zebrafish liver, peaking in the middle of the subjective night and approximately 3-4 hours before peak expression of the PAR bZIP genes. Regarding the detoxification genes, the major target gene of AhR, *cyp1a*, showed daily and circadian expression with an acrophase 2 hours after *ahr2*. Under LD, *abcb4* also showed daily rhythmicity, with an acrophase 1-2 h after that of PAR bZIP factors during the transition between darkness and light phases, when zebrafish become active. However, the expression of six detoxification genes showed circadian rhythmicity under DD, including *cyp1a* and *abcb4* as well as *gstr1*, *mgst3a*, *abcg2* and *sult2_st2*. In all cases, the acrophases of these

genes were found during the second half of the subjective night, in phase with the PAR bZIP transcription factors. This suggested that their expression is clock-controlled, either directly by core clock genes or through transcription factors. This study presents new data demonstrating that the process of detoxification is under circadian control in fish. Results showed that time of day should be considered when designing toxicological studies or administering drugs to fish.

Keywords: Zebrafish, liver, circadian, PAR bZIP, bHLH-PAS, detoxification genes.

INTRODUCTION

All vertebrates show rhythmic regulation of most of their biological functions, which enable them to adapt to daily environmental cycles generated by the Earth's rotation (e.g. day/night alternation, temperature) and food availability. The existence of endogenous clocks allows animals to anticipate cyclic events and consequently perform biological activities at the most suitable times throughout the day or the year (Mazzoccoli et al., 2012). As in other vertebrates, the molecular circadian clock in fish involves two interlocked negative feedback loops of clock genes and proteins that drive the rhythmic expression of a wide set of genes, leading to overt cycles in physiology and behavior (Dibner & Schibler, 2015). Natural toxins and other harmful compounds are mainly found in food, so circadian regulation of xenobiotic detoxification is a key function of the biological clock (Claudel et al., 2007). Previous investigations in mice have suggested that the circadian clock is involved in the time-dependent drug toxicity, as an anticancer agent (cyclophosphamide) was found to be more toxic to mice with a null allele of *Bmal1* or a mutation of the *Clock* gene, whereas animals lacking both copies of *Cry* were more resistant (Gorbacheva et al., 2005).

The liver is the core organ involved in nutrient metabolism and detoxification, processes that are adjusted in a timely manner, allowing the organisms to adapt and meet the demands of changing environmental conditions. In mammals, detoxification seems to be subjected to circadian regulation (de Wit et al., 2014). Hepatic detoxification includes multiple biochemical processes that convert lipophilic toxins into water-soluble metabolites that can be efficiently eliminated from the body via the urine (Grant, 1991). This protective ability stems from the expression of a variety of xenobiotic biotransforming enzymes with the ability to catalyze the oxidation, reduction and hydrolysis (Phase I) and/or conjugation (Phase II) to make them hydrophilic and excretable by transporter proteins in phase III (Reinke & Asher, 2016). Phase I oxidative enzymes are mainly microsomal cytochromes P450 (CYPs), alcohol

and aldehyde dehydrogenases, which in mice are regulated by the circadian clock and show peak levels of expression during the active phase of the animals, when they are more likely to be exposed to xenobiotics (i.e. at night) (Zhang et al., 2009). Previous research has also found diurnal rhythmicity in phase II conjugating glutathione-S-transferases and phase III transporters, including ATP-binding cassette transporter (ABC transporters) and metallothionein, although daily patterns vary between protein families (Pedrini-Martha et al., 2016; Zhang et al., 2009). Phase II enzymes also include other transferases such as sulfotransferases and nonconjugation enzymes, i.e. quinone reductase and epoxide hydrolase (Chen, 2012). In addition, biotransformation of xenobiotics may increase the production of reactive oxygen species (ROS), which are neutralized by antioxidant enzymes such as catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase (Ribalta et al., 2015).

In mammals, the liver-specific PAR basic leucine zipper proteins (bZIP), thyrotroph embryonic factor (TEF), albumin D box-binding protein (DBP) and hepatic leukemia factor (HLF) act as circadian regulators of numerous genes involved in the metabolism of endobiotic and xenobiotic substances and, in turn, are transcriptionally regulated by core oscillator components (Gachon et al., 2006). Once activated, hepatic transcription factors trigger target gene expression by binding to response elements within regulatory regions of detoxification enzymes and nuclear receptors. This is the case for the constitutive androstane receptor (CAR) and the aryl hydrocarbon receptor (AhR), which are known to be xenobiotic sensors directly involved in the transcriptional regulation of numerous phase I and II enzymes, as well as transporter proteins that play a key role in the elimination of toxicants (Košir et al., 2013; Nakata et al., 2006). The AhR, which forms part of the basic-helix-loop-helix (bHLH)-PAS family, dimerizes with the Ahr nuclear translocator (ARNT) thereby

triggering toxicological response upon activation by halogenated and polycyclic aromatic hydrocarbons (i.e. dioxins) (Claudel et al., 2007).

In zebrafish, clock systems have been reported to be directly photosensitive and entrainable by the light-dark (LD) cycle (Whitmore et al., 2000) and, in fact, comparison of the promoter regions of these light-inducible genes has revealed the existence of D-box enhancer elements that are activated by the PAR bZIP family (Idda et al., 2012; Vatine et al., 2009). Previous research has investigated the spatial expression pattern of PAR bZIP genes in zebrafish embryo, showing higher expression in cranial areas. In addition, rhythmic gene expression was detected in the pineal gland, including both clock-controlled and light-dependent expression patterns (Ben-Moshe et al., 2010). However, the daily (under an LD cycle) and circadian (in the absence of environmental cues) rhythmicity of this family of transcription factors and the detoxification enzymes regulated by them have not yet been investigated in the liver of zebrafish, although clear evidence is pointing at their key role in the circadian regulation of xenobiotic detoxification in mammals, including the metabolism of therapeutic drugs (Gachon, 2007; Gachon & Firsov, 2011). Therefore, the administration time can affect the tolerance and efficiency of such drugs in vertebrates, including fish species.

Despite studies showing daily rhythms of toxicity in zebrafish (Sánchez-Vázquez et al., 2011), insight into the molecular mechanisms driving this rhythmicity was still lacking. Thus, the aim of the present study was to demonstrate light- and clock-controlled expression patterns of transcription factors mediating the circadian regulation of detoxification together with the temporal profile of a set of their target genes in zebrafish liver.

MATERIALS AND METHODS

Animals & housing

A total of 84 3-month old wild-type AB mixed-sex zebrafish (0.42 ± 0.13 g body weight) were obtained from the University College of London Fish Facility (London, UK) and housed in an isolated fish laboratory at the Institute of Aquaculture of the University of Stirling (Stirling, UK). Experimental fish were randomly allocated to twelve 11 L plastic tanks (35.6 x 23.4 x 22.8 cm) (Geo Extra Large Tank, Ferplast, Italy) (n=7 fish/tank), each one equipped with an individual filter (PF Mini Internal Power Filter, Interpet, UK) and supplied with filtered and dechlorinated tap water. During the acclimation period, the photoperiod was set at 12 h: 12 h light-dark (LD) and temperature was kept constant at 25 ºC throughout the trial using water heaters (H2 Therm 15W Micro Aquarium Heater, Tropical Marine Centre, UK). Fish were hand-fed once a day *ad libitum* a commercial diet (Otohime B2 360-650 µM, Marubeni Nisshin Feed Co., Ltd., Japan) at random times during daytime over a two-week acclimation period and during the trial. The walls of all aquaria were covered with black plastic sheets to prevent different groups of animals from seeing each other.

Experimental design

The 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 Animal Welfare and Ethical Review Body (AWERB) of the University of Stirling. 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).

To investigate daily and circadian rhythms of locomotor activity and gene expression, zebrafish were initially kept under a 12:12 h LD cycle. Throughout the experiment, the existence of a daily activity rhythm and its synchronization to the LD cycle was monitored. To this end, locomotor activity was recorded by an infrared photocell (E3Z-D67, Omron,

Kyoto, Japan) placed in each tank, 11 cm away from the bottom and 7 cm away from the lateral wall. The photocells were connected to a computer, and every time a fish interrupted the infrared light beam, it produced an output signal that was recorded and stored in 10 minutes bins using specialized software (DIO98USB, University of Murcia, Spain).

To investigate daily rhythms of gene expression in LD, after a two-week period, 42 fish (n=7/tank, 6 tanks) were fasted for one day and then sacrificed by lethal anaesthesia (2-phenoxyethanol, 1 mL/L, Sigma) every 4 h during a 24 h period, at "Zeitgeber Times" (ZT) 2, 6, 10, 14, 18 and 22 (1 tank/ZT). Liver samples were obtained from each fish and preserved in RNALater® (Sigma-Aldrich, Poole, UK). In darkness conditions, sampling was performed using dim red light attached to the dissecting microscope.

To determine the existence of circadian rhythms of gene expression, the remaining 42 experimental fish (n=7/tank, 6 tanks) were kept under an LD cycle for an additional week and then lights were switched off at ZT0. Fish were fasted and kept in continuous darkness (DD) for 24 h and then sampled, starting at circadian time (CT) 2 (onset of the subjective day). Samples were obtained every 4 h during a 24 h cycle (at CT2, 6, 10, 14, 18 and 22). From each fish, liver samples were also collected in RNALater®.

Gene expression

Liver samples were homogenized in 1 mL of TRIzol® (Invitrogen, UK) and total RNA extracted in accordance with the manufacturer's instructions. RNA pellets were rehydrated in DNase RNase-free distilled water (Merck Millipore) and total RNA concentration determined using an ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK). RNA integrity was assessed by agarose gel electrophoresis.

The relative expression of 26 genes was determined in liver from fish of all treatments: 4 clock genes (*bmal1*, *clock1a*, *cry1a*, *per2*), 6 transcription factors (*hlfa*, *tefa*, *tefb*, *dbpa*, *dbpb*,

198 *arh2*) and 16 detoxification genes (*smtb*, *mt2*, *sod1*, *cyp1a*, *cyp1d1*, *cat*, *gpx7*, *gsr*, *gstt2*,
199 *gstt1a*, *gstr1*, *mgst3a*, *abcb4*, *abcc2*, *abcg2*, *sult2_st2*) (Table 1) (S1 Table). The software
200 PRIMER3 (Untergasser et al., 2012) was used to design new sets of primers and their target
201 specificity was checked *in silico* using Blast (NCBI) (Table 1). cDNA was reverse
202 transcribed from 1 µg of total RNA using QuantiTect Reverse Transcription kit (Qiagen Ltd.,
203 Manchester, UK). The resulting cDNA was diluted 20-fold with Milli Q water and 2.5 µL of
204 each sample was used in combination with 300 nM of each primer and 5 µL of Luminaris
205 Color HiGreen qPCR Master mix (Thermo Fisher Scientific, MA, USA) to reach a final PCR
206 volume of 10 µL. Reactions were run in a Mastercycler RealPlex 2 thermocycler (Eppendorf,
207 UK) programmed to perform the following protocol: UDG pre-treatment at 50 ºC for 2 min
208 preceded thermal cycling, which was initiated at 95 ºC for 10 min, followed by 40 cycles
209 with a denaturing step at 95 ºC for 15 s, annealing for 30 s at Ta according to Table 1 and
210 extension at 72 ºC for 15 s. The amplification cycle was followed by a temperature ramp with
211 0.5 ºC increments ranging between 60 ºC and 90 ºC for melt-curve analysis to verify that no
212 primer-dimer artefacts were present and only one product was generated from each qPCR
213 assay. Amplifications were carried out including systematic negative controls containing no
214 cDNA (NTC, no template control) and omitting reverse transcriptase enzyme (-RT) to check
215 for DNA contamination. In addition, the qPCR product sizes were checked by agarose gel
216 electrophoresis and the identity of random samples was confirmed by sequencing (GATC
217 Biotech, Germany). No primer-dimer occurred in the NTC. Gene expression quantification
218 was achieved by including a parallel set of reactions containing serial dilutions from all
219 pooled cDNA experimental samples and assigning each dilution the appropriate value of
220 relative units (RUs). As a result, an estimated number of relative copies, corrected for the
221 efficiency of the reaction, was automatically calculated for each sample.
222

223 **Data analysis**

224 The locomotor activity displayed by fish during the experiment was analyzed by a
225 chronobiology software (El Temps© v.1.179, Dr. Díez-Noguera, Barcelona, Spain).
226 The normalized expression values were generated by the ΔCt method (Pfaffl, 2001) and the
227 results expressed as mean normalized ratios (±SE) between the RUs of target genes and a
228 reference gene index calculated from the geometric mean of the two most stable reference
229 genes (i.e. ribosomal protein L3, *rpl13* and solute carrier family 25a, *slc25a*). Housekeeping
230 gene stability (S2 Table) was determined applying a correction for efficiency to the raw Ct
231 standard deviation (Pfaffl, 2004) using RefFinder (Xie et al., 2012).
232 Statistical differences in gene expression between different sampling times were analyzed by
233 one-way ANOVA (ANOVA I), followed by Tukey's post hoc test, using SPSS v.19 software
234 (IBM, Armonk, NY). Cosinor analysis was performed using Ritme software (Antoni Díez-
235 Noguera, University of Barcelona, Spain) to determine whether the daily expression of the
236 studied genes fitted the cosine function: $Y = M + A * [\mathrm{Cos}\ (\Omega t + \Phi)]$, where M is mesor, A is
237 amplitude, Ω is angular frequency (360°/24h for the circadian rhythms) and Φ is acrophase.
238 The significance level was fixed at $p < 0.05$ for all the statistical analysis.
239

240 **RESULTS**

241 **Locomotor activity**

242 Zebrafish kept under an LD cycle showed a diurnal activity pattern, with 80-95 % of their
243 total swimming activity displayed during the day. Activity levels sharply increased at the
244 beginning of the day and in most experimental tanks continued to increase gradually during
245 the first 4 h of the photophase. In other cases activity levels were sustained during the
246 photophase after lights onset. When lights switched off, activity decreased abruptly in all
247 tanks (Fig. 1).

Clock genes

Under LD, the expression of all clock genes investigated in the liver of zebrafish showed significant differences during the 24 h cycle (ANOVA I, $p<0.05$) and daily rhythmicity, as revealed by cosinor analysis ($p<0.05$). The daily rhythms of *bmal1a* and *clock1a* were in phase with their acrophases only two hours apart, at ZT=11:11 and ZT=13:31 (Table 2), respectively, whereas the expression of *cry1a* and *per2* peaked during the first half of the day, in antiphase to *bmal1a* and *clock1a* (Fig. 2A).

In the absence of external zeitgebers (DD), the expression of all clock genes showed circadian rhythmicity (Fig. 2B), with acrophases within two hours apart from those observed under LD (Table 2).

Transcription factors

All PAR bZIP transcription factors except *hlfa* showed daily and circadian rhythmicity in zebrafish liver (Fig. 3). Under LD, the acrophases of *tefb*, *dbpa* and *dbpb* expression were located in the second half of the night phase (ZT between 21:32-22:58) whereas *tefa* expression peaked at the beginning of the day (ZT=01:19). Under DD, the acrophases of all the rhythmic PAR bZIP transcription factors were located around CT23 (Table 3).

No daily rhythmicity of *ahr2* expression or significant differences between time points were observed in zebrafish exposed to LD. However, cosinor analysis revealed the existence of circadian rhythmicity in fish exposed to DD (acrophase at CT19:23; Table 3).

Detoxification genes

Under LD, two detoxification genes showed a daily rhythm of expression (cosinor, $p<0.05$), *cyp1a* and *abcb4*, with their acrophases located at ZT=21:30 and ZT=00:03, respectively

(Table 4). In addition, *smtb* expression displayed significant statistical differences between time points, peaking at ZT2 (ANOVA I, $p<0.05$) (Fig. 4), although a daily rhythm could not be fitted neither in LD nor DD. On the other hand, cosinor analysis in DD revealed that the transcript expression of six detoxification genes followed circadian rhythmicity ($p<0.05$), including *cyp1a* and *abcb4* (as in LD), as well as *gstr1*, *mgst3a*, *abcg2* and *sult2_st2* (Fig. 5 and 6). In all cases, the acrophases of these genes peaked during the second half of the night, between ~CT20-23, in phase with the PAR bZIP transcription factors (Table 4, Fig. 7). Gene expression was also determined from detoxification genes including *sod1*, *cyp1d1*, *cat*, *gpx7*, *gsr*, *gstt2*, *gstt1a*, *abcc2* and *mt2*; however, no circadian rhythmicity or circadian-control were detected (S3 Fig.).

DISCUSSION

The present study showed that zebrafish displayed a diurnal pattern of activity and were able to synchronize to the LD cycle, in accordance with previous behavioural studies carried out in this species (Del Pozo et al., 2011; Hurd et al., 1998). Overall, the expression of clock genes showed daily and circadian rhythms in agreement with results previously reported in zebrafish and other teleost species (Boyle et al., 2017; Cahill, 2002; Li et al., 2013; Vera et al., 2013). Only the results obtained for *per2* differed from previous research (Cahill, 2002; Vatine et al., 2009), which showed that the expression of this gene was exclusively regulated by light. In the present study, *per2* displayed circadian rhythmicity in DD, as observed in *Sparus aurata* (Vera et al., 2013), suggesting that the expression of this clock gene in liver may not be exclusively light-regulated, although the amplitude of the rhythm in DD was much lower than under LD. In addition, the present study provided new evidence on the circadian regulation of detoxification mechanisms in zebrafish, revealing that both detoxification genes and key transcription factors regulating their expression displayed

rhythmicity. Recent research in fish demonstrated the chronotoxicity of anaesthetics (Sánchez-Vázquez et al., 2011; Vera et al., 2010; Vera et al., 2013b) and aquaculture medicines (Vera & Migaud, 2016). However, the molecular mechanisms underlying the temporal variations in toxicity and/or effectiveness of these compounds remained unclear.

In the last few decades, the circadian regulation of xenobiotic and endobiotic detoxification has been widely investigated in mammals, with studies revealing that the circadian clock regulates daily differences in toxicity, either directly or through clock-controlled transcription factors, such as PAR bZIP proteins and nuclear receptors which drive the expression of many detoxifying enzymes. In zebrafish liver, the expression of most PAR bZIP transcription factors showed daily rhythmicity, in tune with the clock genes, which appeared to be both light-dependent (under an LD cycle) and clock-controlled, as circadian rhythms persisted in the absence of external cues (DD) for 26-44 h (CT2-CT22, respectively). The hepatic expression profiles of *tefb*, *dbpa* and *dbpb* were in phase and peaked at the end of the darkness period in LD and at the end of the subjective night in DD, which is in accordance with results obtained in the pineal organ of zebrafish embryo (Ben-Moshe et al., 2010). On the other hand, microarray analysis in zebrafish liver identified the acrophase of *tefb* and *dbpb* at the beginning of the light phase, altough in this case fish had been kept in a 14h:10h LD cycle (Boyle et al., 2017). In addition, *dbpa* expression was found arrythmic, in contrast with our results. Regarding *tefa*, the present study identified maximum levels of expression in the beginning of the light phase in LD, two hours before the acrophase reported by Boyle et al. (2017). With respects to *hlfa* expression, we did not observe time-of-day variation in contrast with the study by Ben-Moshe et al. (2010) but in accordance to results by Boyle et al. (2017). Vatine et al. (2009) reported an increase of *tef* mRNA levels following exposure to light under LD and at the beginning of the subjective day in DD and concluded that *tef* is up-regulated predominantly by light and partially by the circadian clock. Moreover, Li et al.

(2013) found that *tefa* is a fast light-induced transcription factor in zebrafish, showing a peak of expression around 4 h after light onset. Other studies have also observed that the acrophase of *tef* expression preceded that of *per2*, another fast light-induced gene, which suggested that TEF may be a regulatory factor contributing to the light-driven expression of *per2*. In order to test this hypothesis, knock-down experiments were carried out, demonstrating that TEF mediates *per2* light-induction. In our study, the daily rhythm of *tefa* expression peaked around 4 h before *per2*, which is in accordance with previous findings (Vatine et al., 2009). In fact, regulation of PAR bZIP factors by the core clock has been demonstrated in mammals, showing that CLOCK/BMAL1 heterodimer regulates the rhythmic expression of *Dbp* by E-box-mediated transcription (Ripperger et al., 2000; Ripperger & Schibler, 2006). In rat, a nocturnal species, *Dpb* expression is barely detectable during the early morning hours; however, its expression increases during the afternoon and reaches maximal levels at the end of the day, just before the active phase of the animal (Wuarin & Schibler, 1990). Similarly, in diurnal zebrafish, a peak of expression for both *dbpa* and *dbpb* was observed at the end of the night period, just preceding their active diurnal phase. According to Li et al. (2013), the time-lag between the acrophases of transcription factors involved in circadian regulation and that of their target genes can vary from immediate up to 12 hours. Furthermore, additional phases can be generated by transcription factors regulated by core circadian genes. Indeed, the generation of diverse circadian phases in gene expression is critical as different metabolic processes require activation at different times to optimize physiological functions (Dibner & Schibler, 2015). In our study, the hepatic expression of PAR bZIP genes showed the acrophase between 11 h and 14 h later than *bmal1a* and *clock1a* in both LD and DD, which is in accordance with the circadian gene regulatory cascade described before in zebrafish larvae (Li et al., 2013).

Under DD, the expression of the nuclear receptor *arh2* also showed circadian rhythmicity in zebrafish liver, with peak levels detected in the middle of the subjective night, corresponding to the resting phase of zebrafish, and 3-4 hours before the PAR bZIP acrophases. Similarly, the expression of *AhR* showed a daily rhythm in rat liver, showing a peak in the middle of the day, during the resting phase of this nocturnal species (Richardson et al., 1998). AhR is located in the cytoplasm, but after binding to a ligand (i.e. xenobiotic), it is activated and translocated to the nucleus where it dimerizes with ARNT. This complex binds to the xenobiotic responsive element (XRE) region of a number of detoxification genes and activates their transcription, including phase I and II enzymes. In particular, the major target gene of AhR is *Cyp1a* which also displays circadian rhythmicity in rat (Huang et al., 2002). In our study, *cyp1a* showed daily and circadian expression in zebrafish liver, with the acrophases located at ZT21:30 and CT21:58, respectively in LD and DD, approximately 2 hours later than *arh2*. This suggests that this gene may also be involved in the activation of *cyp1a* expression in this species. In addition, as in the PAR bZIP genes, an E-box element has been identified in the mouse *AhR* promoter (Garrison & Denison, 2000). Since this region is a consensus-binding site for CLOCK/BMAL1 heterodimer, it is plausible to assume that *Ahr* expression may be regulated by core clock genes. In zebrafish liver, *ahr2* expression peaked 5-9 hours later than *clock1* and *bmal1*, which is in accordance with this hypothesis, but further studies are required to explore the exact mechanisms involved in the activation of *ahr2* transcription.

Regarding detoxification genes, only *abcb4* and *cyp1a* showed daily rhythmicity in LD, with the acrophase detected 1-3 h after that of PAR bZIP factors and in the interphase between night and day, just before or at the onset of the active period of zebrafish. Therefore, these detoxification proteins would be more expressed when the risk of exposure to toxicants or the production of metabolic byproducts is higher in this species. Likewise, in mouse liver, *Abcb4*

372 is involved in the metabolism of cholesterol and shows a peak of expression in the interphase
373 day-night. In addition, further analysis of the hepatic transcriptome of mouse has revealed
374 that a large number of detoxification genes are expressed according to a circadian pattern,
375 with expression peaking just before the active phase of the animal indicating that, as for other
376 physiological functions, the circadian clock allows the organisms to anticipate cyclic
377 environmental events (Gachon & Firsov, 2011). The ATP-binding cassette (ABC) family is
378 primarily involved in the transport of molecules across the cellular membrane, while only
379 some specific families are responsible to eliminate chemicals from the hepatocytes into bile
380 or blood (Fischer et al., 2013). Thus, rhythmic expression of membrane transporters may play
381 a role in the diurnal transport of nutrients, metabolic substances or toxins present in food
382 (Zhang et al., 2009).
383 In the present study, six detoxification genes showed circadian variation in expression under
384 DD indicating that their expression is clock-controlled, either directly by core clock genes or
385 through transcription factors, although further studies would be required to determine the
386 robustness of such rhythmicity when zebrafish are kept in constant conditions for a longer
387 period of time. The expression of all the detoxification genes was in phase or slightly
388 advanced to PAR bZIP genes. Nevertheless, in all cases, gene expression reached peak levels
389 at the end of the subjective night, in anticipation to the active period of zebrafish. According
390 to Gachon et al. (2006), PAR bZIP proteins regulate the expression of Phase II and Phase III
391 detoxification genes that include *GSTt1*, *GSTa3* and *Abcg2*. In zebrafish no significant
392 rhythmicity of *gstt1a* was detected in LD or DD although *mgst3a* and *abcg2* showed
393 circadian expression in DD, with acrophases 2 h earlier than PAR bZIP genes in agreement
394 with previous results reported in zebrafish liver (Boyle et al., 2017). The fact that the
395 acrophase of detoxification genes occurred earlier than that of PAR bZIP genes could be
396 explained by gene-specific differences in free-running periods in the absence of external

cues, since multiple independent oscillators have been described in zebrafish (Idda et al., 2012). In addition, *gstr1*, another gene belonging to the GST (glutathione S-transferase) family, also displayed circadian expression in DD, with the acrophase in phase with *mgst3a*. The expression of detoxification enzymes such as Cyp1a is regulated by AhR, as mentioned above, whereas other genes involved in xenobiotic metabolism are mostly controlled by CAR, whose circadian expression is driven by PAR bZIP proteins (Gachon et al., 2006).

Previous research in fish species revealed that the time of exposure to hydrogen peroxide caused time-dependent differences in the induction of detoxification genes, being higher in the middle of the light phase (Vera & Migaud, 2016). On the other hand, the exposure of zebrafish to anaesthetics (MS-222 and eugenol) resulted in higher toxicity to fish when trials were carried out in the middle of the light phase (ML) in comparison to mid-darkness (MD) (Sánchez-Vázquez et al., 2011). MS-222 detoxification route involves N-acetylation whereas eugenol metabolism comprises glucuronidation and sulfate conjugation. In the present investigation we did not measure the expression of N-acetylases or glucuronidases; however, the expression of *sult2_st2* (a sulfotransferase gene) peaked around the middle of the subjective night in DD which is in accordance with the lower toxicity of eugenol around this time of the day. In the case of bath exposures, as routinely done in the fish industry to treat against parasitic infections, it is important to take into account that the toxic uptake from the water will also vary depending on the activity pattern of the fish and will be higher during the day in diurnal species, thus inducing a more noxious effect at that time, as reported by Vera et al. (2013b).

In conclusion, this study demonstrated that the expression of PAR bZIP and bHLH-PAS transcription factors as well as a number of detoxification genes is under circadian regulation in zebrafish liver. Our findings suggest that core circadian genes, such as *bmal1a* and *clock1a* may control the activation of *tefa, tefb*, *dbpa*, *dbpb* and *ahr2* which in turn would be involved

in the transcriptional activation of detoxification genes. Previous research in fish had provided evidence of how toxicological response and the effect of xenobiotics can be influenced by the time of administration. Insight into the basic molecular mechanisms involved in detoxification suggests that such differences are clock-controlled and highlights the importance of considering the time of day when designing toxicological studies or administering drugs to vertebrates. In particular, the zebrafish model is extensively used in the field of toxicology and pharmacology, to test the toxicity of a wide range of chemicals, and has also been established as a model to investigate key aspects of the vertebrate circadian clock. In addition, the use of diurnal zebrafish in biomedical research offers an advantage over nocturnal rodents, making zebrafish a practical and useful model organism when extrapolating results and making comparisons to humans. Therefore, the present investigation contributes to increase our knowledge about circadian regulation of detoxification, a topic that has been scarcely addressed in zebrafish but with strong potential impact on the use of drug therapies in vertebrates, including fish species.

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548 **Table 1**. Primers used for RT-qPCR.

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Amplicon	Ta	Accession number	Reference
bmal1a	GTCACAGACAAGTGCTACAGATGCG	TCCCTCCGCCATCTCCTGA	261 bp	60 ºC	AF144690.1	Amaral & Johnston (2012)
clock1a	GGTTCAAGGACAGGGTTTACAGATG	GGTCGACCTCTGAGACTGCTGG	280 bp	60 ºC	XM_017352431	Amaral & Johnston (2012)
cry1a	CTACAGGAAGGTCAAAAAGAACAGC	CTCCTCGAACACCTTCATGCC	334 bp	60 ºC	AB042248.1	Amaral & Johnston (2012)
per2	GTGGAGAAAGCGGGCAGC	GCTCTTGTTGCTGCTTTCAGTTCT	252 bp	60 ºC	XM_017357611.1	Amaral & Johnston (2012)
hlfa	GCAGCTCTCACAATGGGATG	ATGGAGTCTGGGTCAATGGG	106 bp	57 ºC	NM_001077334.2	New design
tefa	TGTCTGTCAAAGCAAGCCTG	GAAAAGGGCGAACTCCATCC	73 bp	56 ºC	NM_131400.1	New design
tefb	GCTGTTGTTTTGGCTTGCTC	CGTGTCCAGGCATCATTAGC	108 bp	56 ºC	NM_001020661.1	New design
dbpa	TGGAGGAGTTCTTGACGGAG	CTGTGTGCTCTGAGATGGGA	92 bp	57 ºC	NM_001197060.1	New design
dbpb	AGATGCTCGTCCCTGAAGAC	GTTCTTACAACGCCGACTCC	59 bp	57 ºC	NM_001197062.1	New design
ahr2	CAGATGCCTCCTTGACAACTCG	TCCAAGATCGAGGGTGGCTG	165 bp	60 ºC	BC163711.1	Li et al. (2013)
smtb	TGCTCCAAATCTGGATCTTG	GCAGTCCTTCTTGCCCTTAC	218 bp	55 ºC	EU918132.1	Wu et al. (2012)
mt2	AGACTGGAACTTGCAACTGTGGT	CAGCTGGAGCCACAGGAATT	474 bp	55 ºC	NM_001131053.2	Wu et al. (2012)
sod1	GGTAATGTGACCGCTGATGC	ACTTTCCTCATTGCCACCCT	150 bp	55 ºC	NM_131294.1	New design
cyp1a	AAACCAGTGGCAAGTCAACC	AAAACCAACACCTTCTCGCC	126 bp	56 ºC	NM_131879.1	New design
cyp1d1	TCGACCTGAACGGTTCCTCA	ACAACATTGCCCGTCTGGAG	118 bp	60 ºC	NM_001007310.1	New design
cat	CCTGTTGAAGAAGCGGATCG	GGATGGGAAGTTGCCATTGG	93 bp	57 ºC	AJ007505.1	New design
gpx7	ACGGAGATGGTTCGGAAGTT	AGGTCTGAGTGTCAACAGGG	85 bp	56 ºC	NM_001020501.1	New design
gsr	GGGGTCATATCGTGGTGGAT	ATCAGGTGTCAGAAGGGCTC	95 bp	57 ºC	NM_001020554.1	New design
gstt2	GCTGTCCGACTCCTTTGATG	AATTTGTCCCTCAGGCGGTA	57 bp	56 ºC	NM_200521.1	New design
gstt1a	ATCTCATGGCTCAAAGGTCT	AAGACATGTTGAGATCCTCCA	110 bp	60 ºC	NM_001327762.1	Glisic et al. (2015)
gstr1	TAAAGAGAGATGTCCCAGACT	ACCGGCTTCTCCAGCCACT	99 bp	60 ºC	NM_001045060.2	Glisic et al. (2015)
mgst3a	TGTGTTGGGGATGATCTGGA	ACTCTCCCGGTGTCCACTGT	144 bp	60 ºC	NM_213427.1	New design
abcb4	TACTGATGATGCTTGGCTTAATC	TCTCTGGAAAGGTGAAGTTAGG	159 bp	60 ºC	JQ014001	Fisher et al. (2013)
abcc2	TCTGGACCCGTTTCAGACCT	CCTCCGACACCTCATGTTCA	116 bp	60 ºC	BC056740.1	New design
abcg2	TCCAGCAGACACACGCTGAT	TGAGCACCCAGTGGAACTGA	120 bp	60 ºC	NM_001042775	New design
sult2_st2	TGCTGCTCCTCTGATCATCT	CACACCTTTATGCACCGAAT	101 bp	60 ºC	BC142761.1	New design
bactin1	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	102 bp	56 ºC	AF057040	McCurley et al. (2008)
slc25a5	AAGCGACACCTCTCCAAGAA	TAGCATGTTGCACCTGAAGC	153 bp	56 ºC	NM_173247	New design
b2m	AGGATTGTCTGCTTGGCTCTCT	GGAGTGGAGACTTTCCCCTGTAC	110 bp	56 ºC	NM_131163	Tang et al. (2007)
elf1a	CCTCTTGGTCGCTTTGCTGT	CTTGGTCTTGGCAGCCTTCT	129 bp	57 ºC	AY422992.1	New design
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	148 bp	56 ºC	NM_212784	Tang et al. (2007)

549 **Table 2**. Parameters of the cosine function calculated by Cosinor analysis ($p<0.05$) for diel
550 expression of clock genes in zebrafish exposed to a light-dark (LD) cycle or continuous
551 darkness (DD).

Gene	Light regime	p value	Mesor	Amplitude	Acrophase (ZT/CT hours)
bmal1	LD	<0.01	50.7±2.8	41.9±4.9	11:11±00:23
	DD	<0.05	72.4±7.0	28.9±12.5	10:12±01:59
clock1	LD	<0.01	81.6±11.0	87.4±19.6	13:31±00:54
	DD	<0.05	85.1±7.8	34.5±13.7	14:01±01:52
cry1a	LD	<0.01	111.0±7.3	105.0±13.1	04:00±00:15
	DD	<0.01	91.5±8.3	64.6±14.6	02:16±00:55
per2	LD	<0.01	104.6±10.4	94.7±18.6	05:55±00:46
	DD	<0.05	41.4±5.3	19.41±9.4	05:10±02:03

552

553 All parameters are expressed as the mean value ± standard error (SE).

554

Table 3. Parameters of the cosine function calculated by Cosinor analysis ($p<0.05$) for transcription factors in zebrafish exposed to a light-dark (LD) cycle or continuous darkness (DD).

Gene	Light regime	p value	Mesor	Amplitude	Acrophase (ZT/CT hours)
hlfa	LD	NS	-	-	-
	DD	NS	-	-	-
tefa	LD	<0.01	108.1±11.7	98.5±20.6	01:19±01:40
	DD	<0.01	71.0±4.7	50.0±8.4	23:09±00:58
tefb	LD	<0.01	55.4±5.9	49.1±10.4	22:17±00:51
	DD	<0.01	99.9±9.3	62.3±16.3	23:56±01:05
dbpa	LD	<0.01	73.6±9.6	84.2±16.9	22:58±00:48
	DD	<0.01	95.9±8.4	95.6±14.9	23:06±00:40
dbpb	LD	<0.01	67.4±9.6	94.6±16.9	21:32±00:42
	DD	<0.01	77.5±8.2	96.2±14.6	22:39±00:34
ahr2	LD	NS	-	-	-
	DD	<0.05	721.3±56.4	249.5±100.7	19:23±01:50

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

561 **Table 4**. Parameters of the cosine function calculated by Cosinor analysis (p<0.05) for genes
562 coding detoxification enzymes and transporter proteins in zebrafish exposed to a light-dark
563 (LD) cycle or continuous darkness (DD).

Gene	Light regime	p value	Mesor	Amplitude	Acrophase (ZT/CT hours)
smtb	LD	NS	-	-	-
	DD	NS	-	-	-
mt1	LD	NS	-	-	-
	DD	NS	-	-	-
mt2	LD	NS	-	-	-
	DD	NS	-	-	-
sod1	LD	NS	-	-	-
	DD	NS	-	-	-
cyp1a	LD	<0.01	51.5±9.7	25.0±8.6	21:30±01:26
	DD	<0.01	63.5±5.1	29.1±9.3	21:58±01:03
cyp1d1	LD	NS	-	-	-
	DD	NS	-	-	-
cat	LD	NS	-	-	-
	DD	NS	-	-	-
gpx7	LD	NS	-	-	-
	DD	NS	-	-	-
gsr	LD	NS	-	-	-
	DD	NS	-	-	-
gstt2	LD	NS	-	-	-
	DD	NS	-	-	-
gstt1a	LD	NS	-	-	-
	DD	NS	-	-	-
gstr1	LD	NS	-	-	-
	DD	<0.01	658.7±55.6	334.7±100.3	20:11±01:13
mgst3a	LD	NS	-	-	-
	DD	<0.01	489.0±32.6	166.2±59.1	20:19±01:31
abcb4	LD	<0.01	692.0±62.9	354.0±110.5	00:03±01:19
	DD	<0.01	533.8±28.2	170.7±50.6	23:10±01:11
abcb5	LD	NS	-	-	-
	DD	NS	-	-	-
abcc2	LD	NS	-	-	-
	DD	NS	-	-	-
abcg2	LD	NS	-	-	-
	DD	<0.05	152.4±17.3	71.1±31.2	21:13±02:05
sult2_st2	LD	NS	-	-	-
	DD	<0.05	438.0±42.0	183.7±74.6	19:50±01:52

564

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

566

FIGURE LEGENDS

Figure 1. Mean waveforms representing the average diel profile of locomotor activity for each experimental tank (n=7 fish) under an LD cycle The height of each point in the waveform represents the mean of infrared light beam interruptions for each period of 10 min during the 24 h cycle. The white and black bars at the top of the graph indicate the light and dark periods, respectively. Data represent the mean (black area) + SE (dashed line). ZT, zeitgeber time.

Figure 2. Relative expression of clock genes in the liver of zebrafish kept in LD (A) and DD (B). The white, grey and black bars at the top of the graph indicate the light, subjective day and dark periods, respectively. Data are shown as the mean ± SE. (n=7). Superscript letters indicate statistically significant differences (ANOVA I, $p<0.05$). The continuous black line represents the sinusoidal function determined by Cosinor analysis. ZT, zeitgeber time. CT, circadian time.

Figure 3. Relative expression of PAR bZIP and (bHLH)-PAS transcription factors in the liver of zebrafish kept in LD. The white and black bars at the top of the graph indicate the light and dark periods, respectively. Graph definitions as given in Figure 2.

Figure 4. Relative expression of PAR bZIP and (bHLH)-PAS transcription factors in the liver of zebrafish kept in DD. The grey and black bars at the top of the graph indicate the subjective day and night, respectively. Graph definitions as given in Figure 2.

Figure 5. Relative expression of Phase I detoxification enzymes in the liver of zebrafish kept in LD (A) and DD (B). Graph definitions as given in Figure 2.

Figure 6. Relative expression of Phase II detoxification enzymes and ABC transporters in the liver of zebrafish kept in LD (A) and DD (B). Graph definitions as given in Figure 2.

Figure 7. Acrophase map for the statistically significant parameters analyzed in LD (A) and DD (B) (Cosinor, $p<0.05$). The acrophase is indicated by a circle, black and white for LD and

DD, respectively. The SE is indicated by the lateral bars. White and black bars above the graph represent light and darkness, respectively.

Supplementary Figure 1. Relative expression of detoxification enzymes not showing daily or circadian rhythmicity in the liver of zebrafish kept in LD (A) and DD (B). Data are shown as the mean ± SE. (n=7). Superscript letters indicate statistically significant differences (ANOVA I, $p<0.05$). The continuous black line represents the sinusoidal function determined by Cosinor analysis. ZT, zeitgeber time. CT, circadian time.

Figure1

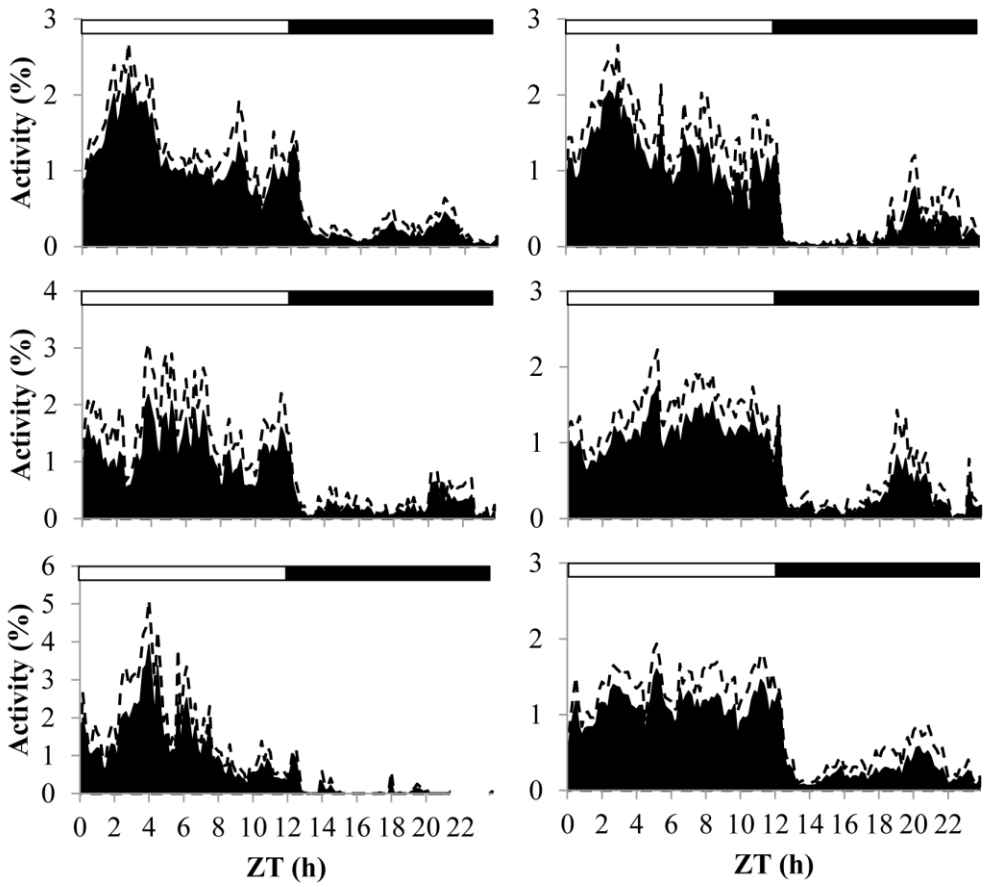

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Figure 2

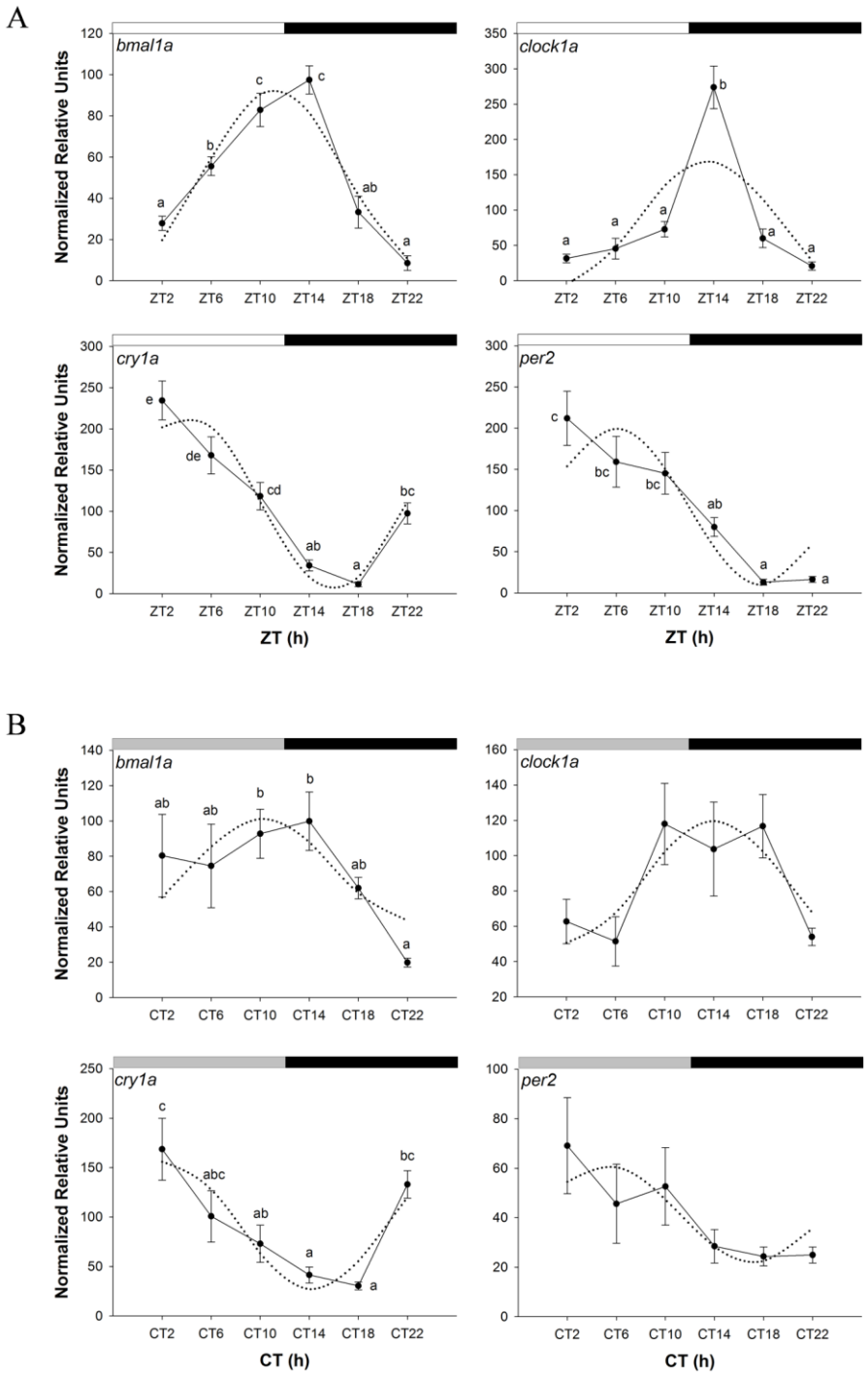

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Figure 3

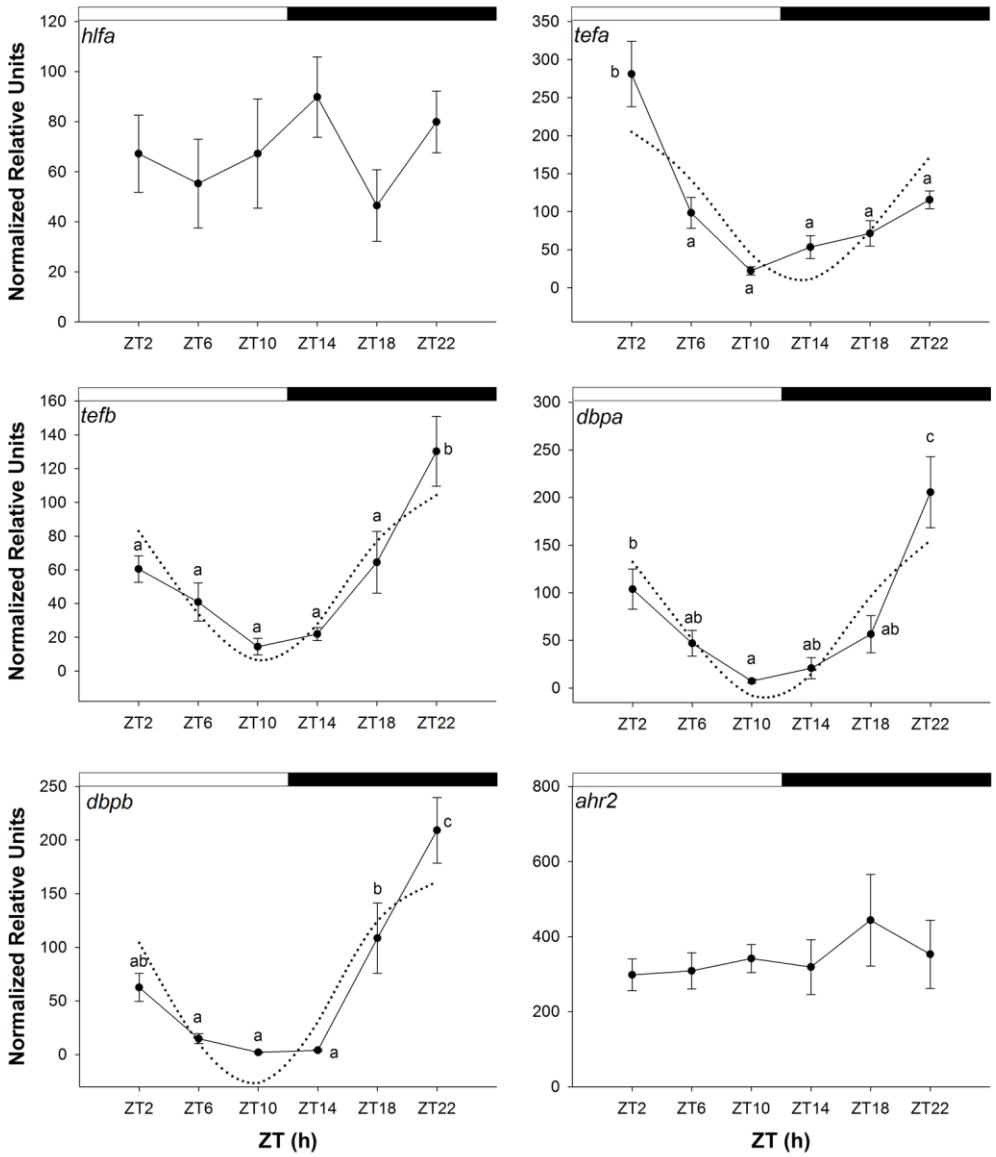

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Figure 4

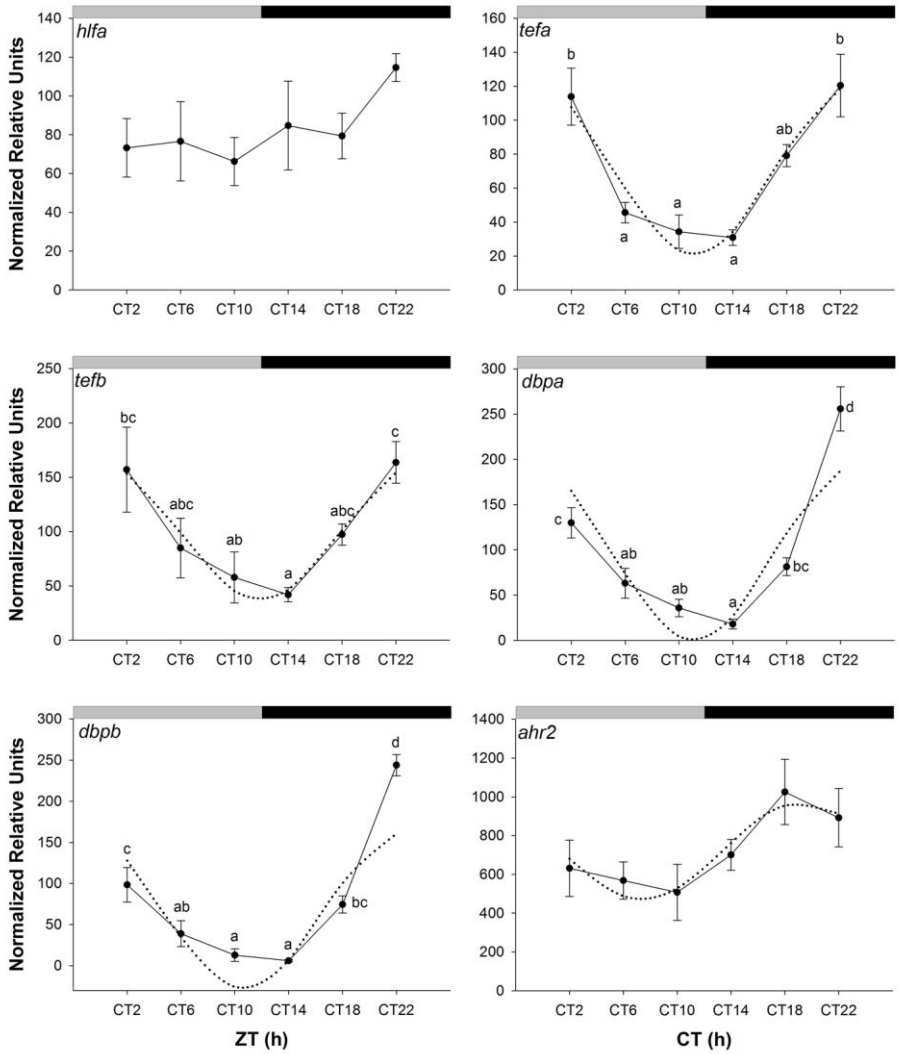

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Figure 5

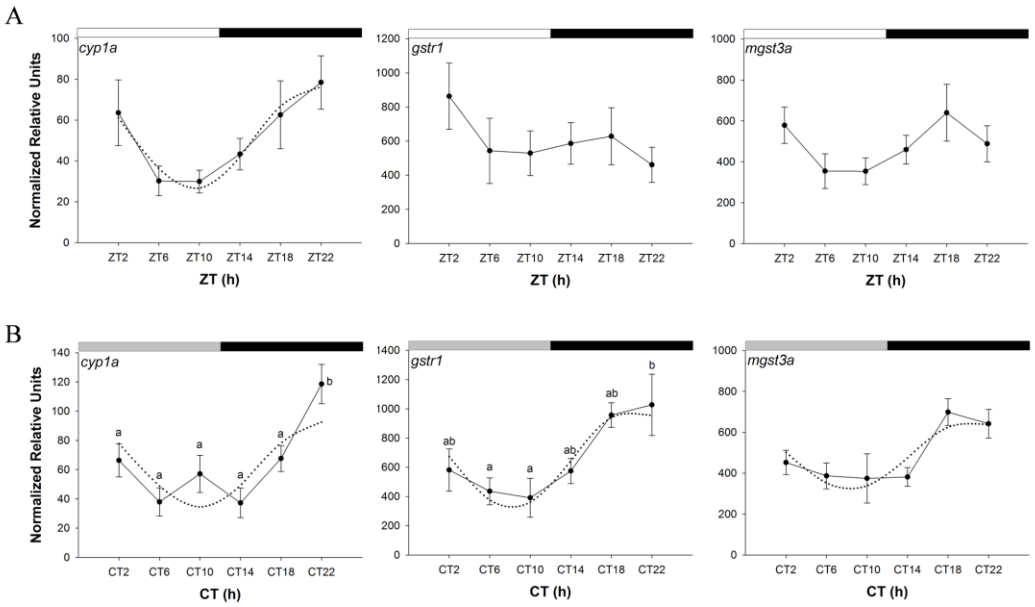

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Figure 6

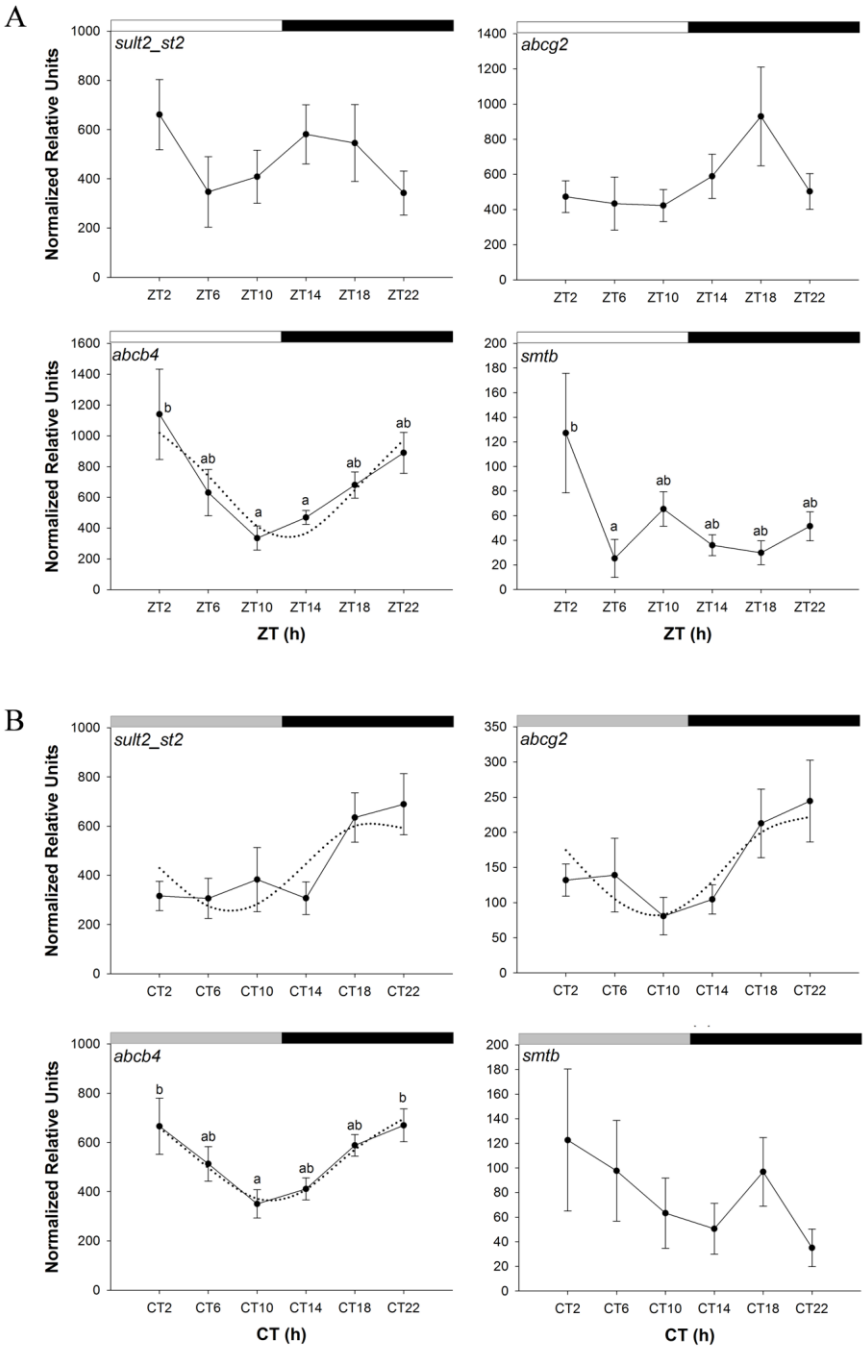

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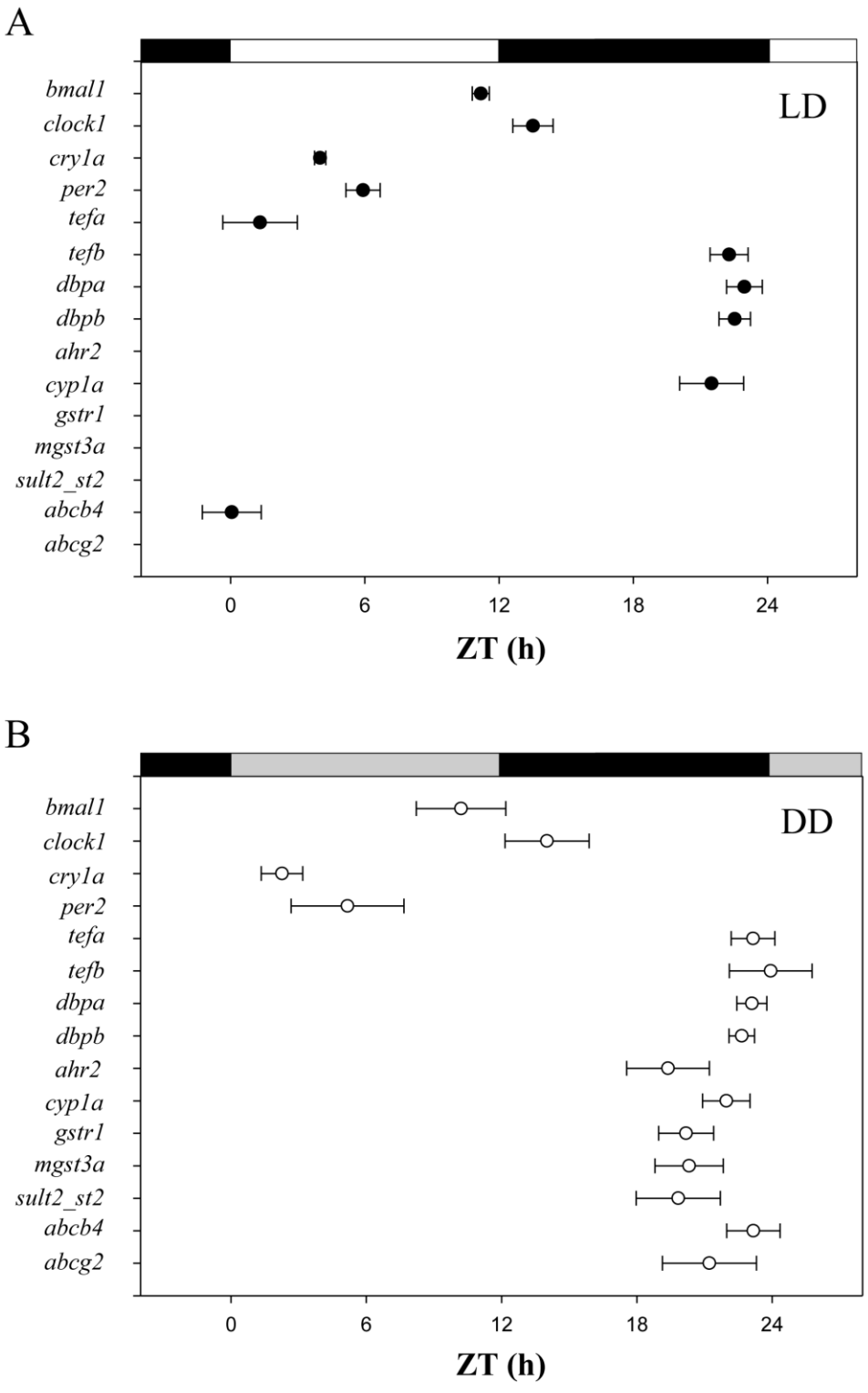
Figure 7


A
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cry1a
per2
tefa
tefb
dbpa
dbpb
ahr2
cyp1a
gstr1
mgst3a
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abcb4
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LD
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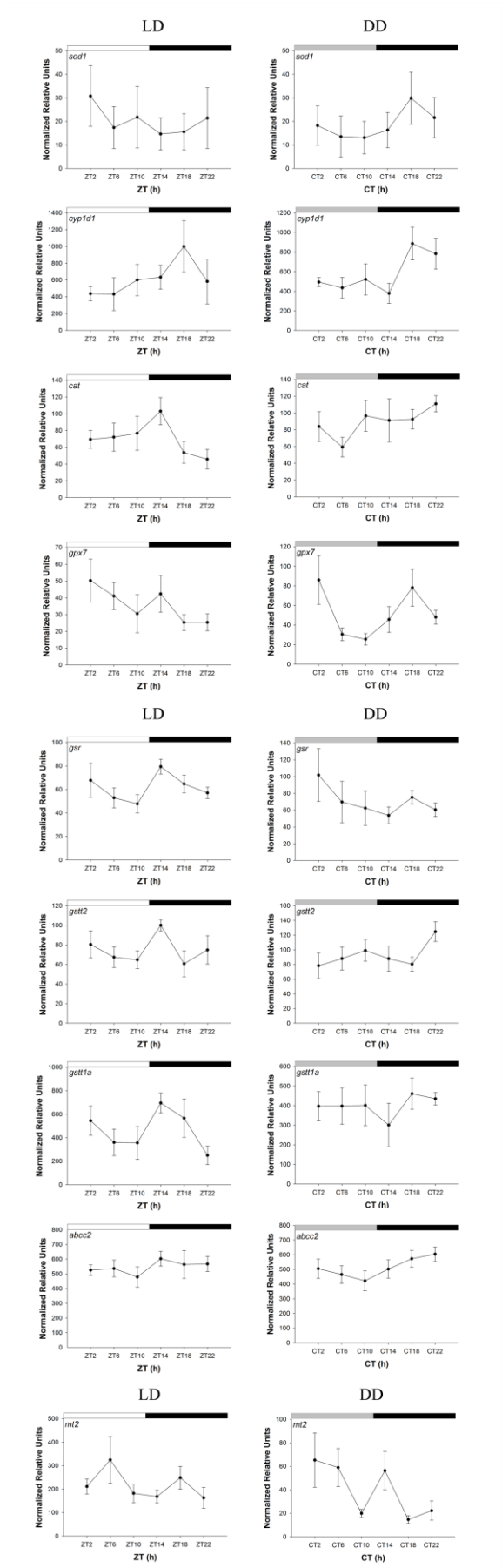
617 **Supplementary Figure 1**

618



S1 Table

Detoxification genes				
Gene name	**Gene abbreviation**	**Function**	**Gene Ontology (GO) Terms**	**Source**
metallothionein-B-like	*smtb*	Binds heavy metals	Molecular function - metal ion binding	UniProtKB-KW
			Biological process - angiogenesis	ZFIN
metallothionein 2	*mt2*	Binds heavy metals	Molecular function - metal ion binding	ZFIN
superoxide dismutase 1	*sod1*	Major antioxidant defense against the superoxide anion, catalysing	Molecular function - copper ion binding	GO_Central
			Molecular function - superoxide dismutase activity	ZFIN
			Molecular function - zinc ion binding	GO_Central
			Biological process - neuron cellular homeostasis	ZFIN
			Biological process - response to metal ion	ZFIN
			Biological process - response to methylmercury	ZFIN
			Biological process - response to xenobiotic stimulus	ZFIN
cytochrome P450, family 1, subfamily A	*cyp1a*	Oxidation of hydrophobic substrates in phase I metabolism	Molecular function - aromatase activity	UniProtKB-EC
			Molecular function - heme binding	InterPro
			Molecular function - iron ion binding	InterPro
			Molecular function - monooxygenase activity	GO_Central
			Biological process - cellular aromatic compound metal	ZFIN
			Biological process - cellular response to organic cyclic	ZFIN
			Biological process - cellular response to xenobiotic stir	ZFIN
			Biological process - response to xenobiotic stimulus	ZFIN
cytochrome P450, family 1, subfamily D, polypept	*cyp1d1*	Oxidation of hydrophobic substrates in phase I metabolism	Molecular function - aromatase activity	UniProtKB-EC
			Molecular function - heme binding	InterPro
			Molecular function - iron ion binding	InterPro
			Molecular function - testosterone 6-beta-hydroxylase a	ZFIN
catalase	*cat*	Protects cells from the toxic effects of hydrogen peroxide, catalysir	Molecular function - catalase activity	GO_Central
			Molecular function - heme binding	GO_Central
			Molecular function - metal ion binding	UniProtKB-KW
			Biological process - hydrogen peroxide catabolic proce	GO_Central
			Biological process - response to copper ion	ZFIN
			Biological process - response to hydrogen peroxide	GO_Central
glutathione peroxidase 7	*gpx7*	Cell protection from oxidative damage by reducing hydroperoxides	Molecular function - glutathione peroxidase activity	InterPro
			Biological process - response to oxidative stress	InterPro
glutathione reductase	*gsr*	Cell protection against oxidative damage, increasing the level of re	Molecular function - flavin adenine dinucleotide binding	InterPro
			Molecular function - glutathione-disulfide reductase act	InterPro
			Molecular function - NADP binding	InterPro
			Biological process - cell redox homeostasis	InterPro
			Biological process - glutathione metabolic process	InterPro
glutathione S-transferase theta 2	*gstt2*	Xenobiotic detoxification by catalysis of the nucleophilic attack of th	Molecular function - glutathione transferase activity	UniProt-GOA
glutathione S-transferase theta 1a	*gstt1a*	Xenobiotic detoxification by catalysis of the nucleophilic attack of th	Molecular function - glutathione transferase activity	ZFIN
glutathione S-transferase rho	*gstr1*	Xenobiotic detoxification by catalysis of the nucleophilic attack of th	Molecular function - glutathione transferase activity	ZFIN
microsomal glutathione S-transferase 3a	*mgst3a*	Xenobiotic detoxification by catalysis of the nucleophilic attack of th	Molecular function - glutathione peroxidase activity	GO_Central
			Molecular function - glutathione transferase activity	GO_Central
ATP-binding cassette, sub-family B, member 4	*abcb4*	Cellular toxicant transporter	Molecular function - ATPase-coupled protein transmen	ZFIN
			Molecular function - ATP binding	UniprotKB-KW
			Molecular function - efflux transmembrane transporter	ZFIN
			Molecular function - toxin transporter activity	ZFIN
			Biological process - response to toxic substance	ZFIN
ATP-binding cassette, sub-family C, member 2	*abcc2*	Cellular toxicant transporter	Molecular function - ATPase activity, coupled to transm	GO_Central
			Molecular function - ATP binding	UniprotKB-KW
			Molecular function - organic anion transmembrane trar	InterPro
			Biological process - transmembrane transport	GO_Central
ATP-binding cassette, sub-family G, member 2	*abcg2*	Cellular toxicant transporter	Molecular function - ATPase activity, coupled to transm	GO_Central
			Molecular function - ATP binding	UniprotKB-KW
			Biological process - cholesterol efflux	GO_Central
			Biological process - drug transmembrane transport	GO_Central
sulfotransferase family 2, cytosolic sulfotransfera	*sult2_st2*	Phase II detoxifying enzyme mediating sulfate conjugation of hydro	Molecular function - sulfotransferase activity	ZFIN

S2 Table

Details of reference genes used for qPCR. Expression stability was assessed according to BestKeeper (Pfaffl *et al*., 2004) calculated on corrected Ct values. *, genes used to normalise expression.

Data of candidate reference genes (n = 40)									
		Zeitgeber Time				**Circadian Time**			
Genes	***Efficiency***	**GeoMean** ***[Ct]***	**Ct Range** ***[Min, Max]***	**SD** ***[± Ct]***	**SD** ***[± corrected Ct]***	**GeoMean** ***[Ct]***	**Ct Range** ***[Min, Max]***	**SD** ***[± Ct]***	**SD** ***[± corrected Ct]***
b2m	1.99	22.00	[20.4, 23.7]	1.02	1.79	21.26	[18.4, 23.6]	1.21	3.61
elf-1a	1.96	20.12	[18.7, 22.7]	0.84	2.99	20.14	[18.3, 23.0]	0.92	3.39
rpl13*	1.97	16.66	[16.0, 18.6]	0.53	0.33	16.61	[15.6, 18.0]	0.48	0.35
slc25a*	1.91	16.83	[15.4, 18.7]	0.72	0.74	16.60	[15.4, 19.8]	0.90	0.99
b-actin	1.97	24.09	[20.0, 26.6]	1.24	4.55	23.61	[19.1, 25.8]	1.51	7.97
BestKeeper gene index calculated on corrected Ct values from the most stable reference genes									
Normalisation factor (n=2)		**1.71**	**[0.46, 2.97]**	**-**	**0.58**	**1.88**	**[0.66, 3.29]**	**-**	**0.70**