Acute stress response in gilthead sea bream (*Sparus aurata* L.) is time-of-day dependent: physiological and oxidative stress indicators

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ABSTRACT

Since fish show daily rhythms in most physiological functions, it should not be surprising that stressors may have different effects depending on the timing of exposure. Here we investigated the influence of time of day on the stress responses, at both physiological and cellular levels, in gilthead sea bream (*Sparus aurata* L.) submitted to air exposure for 30 s and then returned to their tank. One hour after air exposure, blood, hypothalamus and liver samples were taken. Six fish per experimental group (control and stressed) were sampled every 4 h during a 24-h cycle. Fish were fed in the middle of the light cycle (ML) and locomotor activity rhythms were recorded using infrared photocells to determine their daily activity pattern of behavior, which showed a peak around feeding time in all fish. In the control group cortisol levels did not show daily rhythmicity whereas in the stressed fish a daily rhythm of plasma cortisol was observed, being the average values higher than in the control group, with increased differences during the dark phase. Blood glucose showed daily rhythmicity in the control group but not in the stressed one which also showed higher values at all sampling points. In the hypothalamus of control fish a daily rhythm of *corticotropin-releasing hormone* (*crh*) gene expression was observed, with the acrophase at the beginning of the light phase. However, in the stressed fish, this rhythm was abolished. The expression of *corticotropin-releasing hormone binding protein* (*crhbp*) showed a peak at the end of the dark phase in the control group, whereas in the stressed sea bream this peak was found at ML. Regarding hepatic gene expression of oxidative stress biomarkers: i) *cytochrome c oxidase 4* (*coxIV*) showed daily rhythmicity in both control and stressed fish, with the acrophases located around ML, ii) *peroxiredoxin 3* (*prdx3*) and *5* (*prdx5*) only presented daily rhythmicity of expression in the stressed fish, with the acrophase located at the beginning of the light cycle, and iii) *uncoupling protein 1* (*ucp1*) showed

significant differences between sampling points only in the control group, with significantly higher expression at the beginning of the dark phase. Taken together these results indicate that stress response in gilthead sea bream is time-dependent as cortisol level rose higher at night, and that different rhythmic mechanisms interplay in the control of neuroendocrine and cellular stress responses.

Keywords: Daily rhythms, fish welfare, cortisol, glucose, *crh* expression, *crhbp* expression, oxidative stress biomarkers.

INTRODUCTION

Fish in the wild and in aquaculture facilities face a variety of challenges, such as attacks from predators, food competition, disturbance and exposure to poor water quality, which seriously compromise fish welfare (Huntingford et al., 2006). The specimens react to these adverse conditions through cellular, neuroendocrine and behavioral adjustments, although the circadian mechanisms controlling these responses are not fully understood (Kulkckzykowska & Sánchez-Vázquez, 2010).

The primary physiological response to stress in fish involves two major neuroendocrine pathways: i) the hypothalamic sympathetic chromaffin cells (HSC) axis, and ii) the hypothalamic-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997). In the HPI cascade, corticotropin-releasing hormone (CRH) is synthesized in the hypothalamus and activates the production and release of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn stimulates the production and release of cortisol in the interrenal cells. In the bloodstream, cortisol stimulates glycogenolysis to cope with the increased energy demand (Mommsen et al., 1999). At the hypothalamic level, a CRH binding protein (CRH-BP) with antagonistic roles to CRH has been also described in fish (Huising et al., 2004, Wunderink et al., 2011). In mammals, daily rhythms in the HPI axis have been reported (Haus, 2007), with cortisol levels rising at the beginning of the active phase of the animal. In fish, plasma cortisol daily rhythms have recently been reviewed (Ellis et al., 2012). In Senegalese sole (*Solea senegalensis* K.), a nocturnal flatfish, marked daily oscillations in cortisol appeared under light-dark (LD) conditions with a peak in the afternoon, which persisted under continuous light (LL) conditions with lower values (Oliveira et al., 2013). In this flatfish, a recent paper revealed that stress responses differed during day or night, so that higher cortisol was registered when the stressor was applied at “zeitgeber” time 1 (ZT 1, one hour after

lights on) than at ZT 13 (one hour after lights off) (López-Olmeda et al., 2013). In gilthead sea bream (*Sparus aurata*), plasma cortisol showed a postprandial peak in both fish fed in the middle of the day and in the middle of the night, indicating that feeding time influenced the daily rhythm of cortisol production (Montoya et al., 2010). In rainbow trout cortisol exhibited a diurnal pattern which also seemed to be correlated with feeding time, although additional changes associated with the scotophase were observed too (Holloway et al., 1994). However, despite the great interest of this teleost fish for the European aquaculture industry, little is known about the existence of daily rhythms in the HPI axis and the time-dependent response to acute stress.

The role of mitochondria as the first responders to various stress challenging homeostasis of the cell and organism has been extensively evidenced in many organisms (Manoli et al., 2007), including gilthead sea bream (Calduch-Giner et al., 2014; Pérez-Sánchez et al., 2013). The mitochondrial DNA of current vertebrates encodes 37 genes, such as c*ytochrome c oxidase 4* (*coxIV*), *peroxiredoxins* (*prdx*) or *uncoupling proteins* (*ucp*), with many of them being involved in the maintenance of balance between the oxidative and antioxidative processes that occur inside the cell (Brown, 2008). Indeed, recent studies have reported changes in the expression of these genes when sea bream were subjected to stress conditions (Bermejo-Nogales et al., 2010, 2014, Pérez-Sánchez et al., 2011, 2013), pointing that these mitochondrial genes could be used as biomarkers of health and welfare in this fish species (Pérez-Sánchez et al., 2011, 2013). However, there are no data about the time-dependent differences in their expression when fish are stressed at different times of the day. The aim of the present research was to investigate the possible existence of time-dependent stress response in gilthead sea bream. To this end, we recorded the locomotor activity of light-entrained fish and studied the effect of 30 s air exposure at different times of the day

and night on physiological stress indicators (cortisol and glucose), as well as hypothalamic expression of genes encoding hormones of HPI axis (*crh* and *crhbp*) and mitochondrial oxidative stress biomarkers (*coxIV, prdx3, prdx5* and *ucp1*).

MATERIALS AND METHODS

Animals & housing

A total of 72 gilthead sea bream (211 ± 6 g initial body weight) were obtained from a local farm (Culmarex S.A., Aguilas, Murcia) and reared at the marine facilities of the University of Murcia located at the Naval Base of Algameca (E.N.A., Cartagena, Spain). Fish were kept in 150-L tanks supplied with aeration and filtered seawater from an open system. The photoperiod was set at 12:12 h LD and water temperature at 18º C.

Experimental design

Experimental procedure complied with the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 53/2013 and law 32/2007) for the use of animals in research. 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).

Fish were divided into 12 tanks of 150 L (n=6/tank). Each tank was equipped with an automatic feeder (EHEIM, model 3581, Germany), which provided the fish with 1% of the biomass once a day (D-4 EXCELL 2-P, Skretting), in the middle of the light phase (ML), at ZT6. Locomotor activity was measured by means of infrared photocells (Omron, mod E3S-AD62, Kyoto Japan) immersed in each tank under the feeder and 3 cm from the water surface. A computer connected to the photocells counted and stored the number of light beam interruptions in 10-min intervals. This system has been previously used and validated in this species (Sánchez et al., 2009).

Gilthead sea bream were maintained under these experimental conditions for two weeks and, after one day of fasting, blood, hypothalamus and liver samples were collected. Six fish per treatment (stressed and control) were sampled every 4 h during a 24-h cycle, at ZT3, 7, 11, 15, 19 and 23. To this end, one hour before each sampling point (ZT2, 6, 10, 14, 18 and 22) 6 fish were removed from their tank and exposed to the air during 30 seconds. This experimental procedure has been previously reported to elicit an acute stress response in gilthead sea bream (Arends et al., 1999; 2000). Then, fish were returned to the tank and sampled one hour later (stressed group). Fish from the control group, in contrast, were sampled directly at each sampling time (Figure 1). Both groups of fish were anesthetized with eugenol (clove oil essence, Guinama, Valencia, Spain) dissolved in water at a concentration of 50 µL/L. Previously, eugenol was diluted in ethanol (1 eugenol: 9 ethanol) to facilitate dissolution in water (Cooke et al, 2004). Blood was collected by caudal puncture with heparinised sterile syringes. Blood samples were collected from all fish of each tank in less than 5 min, to avoid the increase of plasma cortisol and glucose levels originated by manipulation (Molinero et al., 1997). Blood was centrifuged at 3000 rpm for 15 min at 4ºC and plasma was separated and frozen at -80ºC until analysis. After blood collection, fish were sacrificed by decapitation and hypothalamus and liver samples were collected, snap frozen and stored at -80ºC until further analysis. During the dark phase a dim red light (λ>600 nm) was used for sampling.

Plasma cortisol and glucose analyses

Blood glucose concentration was measured immediately after extraction by means of a glucometer (Glucocard G meter, Menarini, Italy). Plasma cortisol levels were measured with a commercial ELISA kit (IBL Hamburg, Germany). Both

analytical techniques had been previously validated for gilthead sea bream (López-Olmeda et al., 2009a, b).

Gene expression analyses

Hypothalamus and liver samples were homogenised in Trizol reagent (Invitrogen, Carlsbad, CA, USA) using a tissue homogeniser (POLYTRON®, PT1200, Kinematica, Lucerne, Switzerland). For total RNA isolation the homogenized tissues were mixed with chloroform and separated by centrifugation. RNA was then precipitated from the aqueous phase with isopropanol. Total RNA concentration was determined by spectrometry (Nanodrop® ND-1000, Thermo Fisher Scientific Inc., Wilmington, DE, USA), and 1 µg was treated with DNase I amplification grade (1 unit/µg RNA, Invitrogen, Carlsbad, CA) to prevent genomic DNA contamination. cDNA synthesis was carried out with Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and Oligo $(dT)_{12-18}$ (Invitrogen, Carlsbad, CA) in a 20 µL reaction volume. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and ABI Prism 7500 apparatus (Applied Biosystems, Foster City, CA). The ABI Sequence Detection System 7000 software (Applied Biosystems, Foster City, CA) was programmed to perform the following protocol: 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. The final volume of the PCR reaction was 20 µL: 5 µL of cDNA, 10 µL of the qPCR Master Mix and 5 µL of forward and reverse primers (Table 1). All samples were run in triplicate. The primers used to amplify *prdx3* and *prdx5* genes were previously tested and validated for sea bream (Pérez-Sánchez et al., 2011), as well as those for *ucp1* (Bermejo-Nogales et al., 2010), *coxIV* (Pérez-Sánchez et al., 2013), *crh* and *crhbp* (GenBank accessions KC195964 and KC195965, respectively). The amplification efficiency, specificity of primers and the quantity of cDNA per sample were tested by

199 the standard-curve method. Moreover, melting curves were analysed to verify PCR
200 specificity. The relative expression of all genes was calculated by the $2^{-\Delta\Delta CT}$ method
201 (Livak & Schmittgen, 2001), using *S.aurata β-actin* as the endogenous reference.

202 **Data analysis**

203 Locomotor activity records were analysed and represented as mean waveforms,
204 for which chronobiology software *El Temps* was used (Version 1,228; Prof. Díez-
205 Noguera, University of Barcelona). Glucose, cortisol and gene expression data were
206 subjected to Cosinor analysis to test the existence of significant daily rhythmicity.
207 Cosinor analysis is based on least squares approximation of time series data with a
208 cosine function of known period of the type Y = Mesor + Amplitude * cos (2π(t-
209 Acrophase)/Period), where Mesor is the time series mean; Amplitude is a measure of
210 the amount of temporal variability explained by the rhythm; Period (τ) is the cycle
211 length of the rhythm, i.e., 24 h for circadian rhythms; and Acrophase is the time of the
212 peak value relative to the designated time scale. Cosinor analysis also provided a
213 statistical value for a null hypothesis of zero amplitude. Therefore, if for a statistical
214 significance of $p<0.05$, this null hypothesis was rejected, the amplitude could be
215 considered as differing from 0, thereby constituting evidence for the existence of a
216 statistically significant rhythm of the given period under consideration.

217 Statistical differences in cortisol, glucose and gene expression levels between
218 sampling points were analysed by a one-way ANOVA (ANOVA I). In addition, at each
219 sampling point, cortisol, glucose and target genes expression levels were compared
220 between treatments (control *vs* stressed) by means of a t-test, for which a Levene's test
221 was previously used to check for homogeneity of variances. A Univariate General
222 Linear Model (GLM) was carried out to analyze possible interactions between

223 experimental groups and time points. For this, the fixed factors were “ZT” and
224 “treatments”.
225 All statistical tests were carried out with the SPSS v19.0 program (SPSS Inc.,
226 USA), being the statistical threshold set at P values <0.05 in all tests. All values are
227 reported as the mean ± S.E.M.
228
229 **RESULTS**
230 **Locomotor activity rhythms**
231 Gilthead sea bream activity showed an arrhythmic daily pattern of locomotor
232 activity (57% of the total daily activity registered during the light phase), displaying
233 most of activity around meal time (food anticipatory activity, FAA), followed by a
234 gradual decrease after feeding. Fish increased significantly their activity levels 1.5 h
235 before meal time, reaching a peak just before meal time. (Figure 2). FAA was calculated
236 as the time span in which activity increased 50% over the baseline without subsequent
237 inflections until meal time.
238 **Circulating physiological stress indicators**
239 In the control fish, plasma cortisol values did not show significant differences
240 between sampling points. However, a significant daily rhythm was observed in fish
241 subjected to stress (Cosinor, $p<0.05$) with the acrophase being found around the middle
242 of the dark phase (ZT=18:24) (Table 2) (Figure 3). In addition, plasma cortisol levels in
243 the stressed fish were significantly higher than in the control group at all sampling
244 points, except at ZT3, with mean differences between groups being higher during the
245 scotophase (66.1 ± 9.0 ng/mL) than during the photophase (29.8 ± 8.2 ng/mL) (t-test
246 independent samples, $p<0.05$) (Figure 4).

A significant daily rhythm of blood glucose was observed in the control group with the acrophase located at ZT=16:17 (Cosinor, $p<0.05$), but not in the stress group (Table 2). Overall, blood glucose levels in the stressed fish were significantly higher than in the control ones, at all sampling points except at ZT19 (t-test independent samples, $p<0.05$) (Figure 5). However, in this case the average increase in the stressed group was similar during the photophase and scotophase (~1.1 mmol/L) (t-test independent samples, $p>0.05$).

Brain HPI axis

Hypothalamic *crh* showed a significant daily rhythm of expression in the control group (Cosinor, $p<0.05$), with the acrophase at the beginning of the light phase and the lowest levels during the first hours of the night (Table 2) (Figure 3). However, in the stressed fish no daily rhythmicity was observed, though a significant peak of expression was found at ZT23 (ANOVA I, $p<0.05$). Moreover, there was a statistically significant interaction between the effects of ZTs and treatments (control/stressed) (Univariate GLM, $p<0.01$) (Table 3) being *crh* expression at ZT3 in the control group significantly higher than in the stressed one (t-test independent samples, $p<0.05$) (Figure 6A).

As regards *crhbp* expression, significant differences were detected in both experimental groups: in the control group maximum expression was observed at ZT23 (ANOVA I, $p<0.05$) whereas in the stressed fish *crhbp* expression peaked at ZT7. Furthermore, the Univariate GLM revealed an interaction between treatments and sampling points ($p<0.01$) (Table 3). Simple main effects analysis showed that *crhbp* expression was significantly lower in the control sea bream at ZT3, whereas at ZT23 this expression was significantly higher than in the stressed group (t-test independent samples, $p<0.05$) (Figure 6B). However, the Cosinor analysis failed to reveal significant daily rhythms of *crhbp* expression in both groups.

Expression of mitochondrial oxidative stress biomarkers in liver

CoxIV expression displayed a significant daily rhythm in both control and stressed fish (Cosinor, $p<0.05$), with the acrophases located around the middle of the day (~1-1.5 h before feeding time) (Table 2) (Figure 3). In addition, there was a significant interaction between the effects of ZTs and treatments (Univariate GLM, $p<0.01$) (Table 3). Thus, in the control sea bream *coxIV* expression was significantly higher than in the stressed fish at ZT3 and ZT7 (t-test independent samples, $p<0.05$) (Figure 7A).

Ucp1 gene expression showed significant differences between sampling points in the control group, with a peak of expression at ZT15 (ANOVA I, $p<0.05$) (Figure 7B). However, significant daily rhythmicity was not detected using the Cosinor analysis. On the contrary, in the stressed group neither significant differences between sampling points nor daily rhythmicity was observed.

In the case of *prdx3*, no significant rhythmicity or daily differences between sampling points were observed in the control group. However, a significant daily rhythm was detected in the stressed fish (Cosinor, $p<0.05$) with the acrophase located ~2 h after lights on (Table 2), peaking at ZT3 (ANOVA I, $p<0.05$) (Figure 2). The Univariate GLM showed an interaction between the fixed factors (ZT and treatments) ($p<0.01$) (Table 3). Hence, expressionlevels at ZT3 and ZT23 were significantly lower in the control fish(t-test independent samples, $p<0.05$) (Figure 7C).

Prdx5 expression showed significant differences between sampling points in both control and stressed sea bream: in the control fish *prdx5* expression presented two peaks, at ZT7 and ZT15, whereas in the stressed group a peak of expression was observed at ZT7 (ANOVA I, $p<0.05$). Furthermore, a significant daily rhythm (Cosinor, $p<0.05$) was found in the stressed fish group, with the acrophase located during the day

297 at ZT = 4:30. In addition, there was a significant interaction between the effects of ZTs
298 and treatments (Univariate GLM, $p<0.01$) (Table 3), with *prdx5* expression being higher
299 in the control fish at ZT15, whereas at ZT18 this expression was down-regulated
300 compared with the stressed group (t-test independent samples, $p<0.05$) (Figure 7D).
301
302 **DISCUSSION**
303 In vertebrates, cortisol rhythms are tightly related to the species-specific
304 circadian rhythm of behavior. Thus, the acrophase of cortisol daily rhythm is usually
305 located in the transition from dark to light in diurnal species such as humans, while it is
306 located at the beginning of the dark phase in nocturnal animals, such as the rat
307 (Dickmeis, 2009). In teleosts, daily rhythms of plasma cortisol have been also reported
308 to be species-specific and related to the activity pattern (diurnal/nocturnal) (Ellis et al.,
309 2012).
310 In Senegalese sole, a nocturnal flatfish, cortisol levels peaked at the beginning of
311 the dark phase (López-Olmeda et al., 2013). In the present study the control sea bream
312 showed a cortisol increase during the first hours of the dark phase whereas the stressed
313 fish showed a daily rhythm of plasma cortisol with the acrophase at mid-darkness (MD)
314 . All gilthead sea bream (control and stressed) were fed at ML and fish actually showed
315 an activity peak around meal time, suggesting their synchronisation to the feeding cycle.
316 Indeed, feeding entrainment occurs when fish are presented with food on a daily basis
317 and they display locomotor activity in anticipation of the forthcoming meal (López-
318 Olmeda et al., 2009b). In gilthead sea bream, previous results pointed out the role of
319 feeding time in changing the diurnal/nocturnal behavioral pattern of fish and thus their
320 cortisol rhythms: fish fed at MD were nocturnal and had a cortisol peak at ZT23, while
321 fish fed at ML were mostly diurnal and showed a cortisol peak around ZT7 (Montoya et

al., 2010). In our trial, however, fish were not strictly diurnal despite displaying food anticipatory activity (FAA) at ML. Furthermore, gilthead sea bream has been reported to show dual behavioural patterns, with seasonal inversions from diurnal to nocturnal behaviour (Velázquez et al., 2004). The fact that our experiment was carried out in winter-early spring, when sea bream shows nocturnal behaviour (Velázquez et al., 2004), may explain the shifts in plasma cortisol rhythms.

Glucose levels in the control fish showed a daily rhythm with the acrophase located around 10 h later than mealtime (ZT6), which is consistent with a previous investigation reporting that in this species glucose concentration peaked 8 h after feeding, regardless of mealtime (Montoya et al., 2010). Variations in plasma cortisol and glucose levels are closely related, especially under stress conditions, since after exposure to an acute stressor, cortisol elevation acts as a gluconeogenic signal increasing blood glucose levels. Actually, in our experiment the stressed sea bream showed higher plasma cortisol and glucose levels than the control fish, which supports previous results obtained in gilthead sea bream subjected to air exposure (Arends et al., 1999). Interestingly, the stress-induced increase in plasma cortisol was higher during the dark phase, suggesting that stress response show daily rhythmicity in this species. Recent investigations carried out in Senegalese sole, a nocturnal fish, pointed also to the existence of daily rhythms in HPI-axis endocrine factors, as well as differences in the stress response depending on the time of day. However, contrasting with sea bream, Senegalese sole showed the highest cortisol and glucose levels when they were stressed in the middle of the light phase (López-Olmeda et al., 2013), indicating that response to acute stress (i.e. air exposure) is species-specific. Furthermore, previous investigations in rainbow trout have showed that the serotonergic system could play a role in triggering the initial steps of the activation of both HPI and HSC axis in fish (Gesto et

al., 2013) and that. increased cortisol synthesis in head kidney under stress conditions could be linked to the hyperglycaemia elicited by catecholamines (Gesto et al., 2014). Nevertheless, in the present study glucose levels in the stressed fish were higher than in the control ones at all sampling times and did not show daily rhythmicity whereas the increase of plasma cortisol was higher during the dark phase. In mammals, there is evidence of a rhythm in the sensitivity of the adrenal gland to ACTH, which might be controlled by neural mechanisms (Engeland & Arnhold, 2005). Furthermore, a peripheral clock in the adrenal gland itself also seems to be involved in the circadian control of glucocorticoid secretion (Dickmeis, 2009). So far, in fish species there are no studies reporting the existence of a daily rhythm in the sensitivity of adenohypophyseal ACTH cells to CRH or the interrenal gland to ACTH. Therefore, further studies will be necessary to fully understand rhythmicity of stress response in gilthead sea bream.

Regarding hypothalamic gene expression of *crh*, a significant daily rhythm was observed in the control sea bream, with the acrophase located two hours before meal time, coinciding with the peak of locomotor activity. Similarly, in Senegalese sole, *crh* expression peaked at the beginning of their activity phase -at night- (López-Olmeda et al., 2013). In the stressed sea bream, however, *crh* expression levels showed daily oscillations, but no daily rhythmicity. It seems there is a rhythm in the response of crh expression to air exposure (induction rhythm), but not under control conditions (basal rhythm). As for *crhbp*, a peak of expression was observed in the stressed fish at ZT7, coinciding with the acrophase of *crh* expression. Contrasting, at that time of the day plasma cortisol levels were lowest, suggesting that CRHBP might be binding and inactivating the ACTH releasing activity of CRH (Huising at al., 2004, Wunderink et al., 2011). Conversely, during the dark phase the average expression of *crhbp* decreased, whereas cortisol levels increased. Nonetheless, no differences in *crhbp*

372 expression were observed between control and stressed groups. Previous studies in
373 rainbow trout (*Oncorhynchus mykiss*) showed that stress-induced response by the CRH-
374 BP gene differs between brain regions and different stressors. Thus, after 24 h of
375 hypoxic stress, hypothalamic *crhbp* expression decreased in dominant fish and remained
376 at control levels in subordinate fish, whereas in telencephalon *crhbp* expression
377 increased significantly (Alderman et al., 2008). Therefore, further investigations on
378 *crhbp* expression in different sea bream brain regions would be needed to clarify its role
379 in the regulation of the HPI axis response to stress.

380 Cytochrome c oxidase (COX) is an oligomeric enzymatic complex located in the
381 inner membrane of mitochondria and it is considered to be a major site of regulation of
382 mitochondrial oxidative phosphorylation (Kadenbach et al., 2000). At high
383 intramitochondrial ATP/ADP ratio, COX IV is phosphorylated and therefore ATP
384 synthesis is inhibited. On the contrary, food intake increases the mitochondrial
385 $NADH/NAD^+$ ratio and the substrate pressure for COX IV, resulting in relief of ATP
386 inhibition (Arnold & Kadenbach, 1997). In the present study, in both control and
387 stressed sea bream a daily rhythm of *coxIV* expression was found, with the acrophases
388 located close to meal time, indicating that feeding cycles might entrain the expression of
389 this enzyme, which in turn would improve ATP synthesis following oxidation of
390 reducing equivalents of nutrients. Previous studies have reported changes in hepatic
391 cytochrome oxidase activity in fish exposed to toxic compounds and pathogens (Craig
392 et al., 2007, Sinha et al., 2012, Tiwari & Singh, 2006). In gilthead sea bream liver,
393 *coxIV* was transiently up-regulated after 24 h of confinement exposure, depending upon
394 the nutritional background (Pérez-Sánchez et al., 2013). Our present results indicate that
395 in sea bream liver, oxidative stress caused by air exposure would also affect *coxIV*
396 expression in a time-dependent manner.

Uncoupling proteins (UCP) are mitochondrial transporters that uncouple oxidative phosphorylation by net discharge of the proton gradient (Krauss et al., 2005). In gilthead sea bream, enhancement of metabolic rates after chronic confinement exposure significantly reduced hepatic *ucp1* expression (Bermejo-Nogales et al., 2010). In our trials significant differences between sampling points were found in the control group. Thus, *ucp1* expression peaked at the beginning of the night (ZT15) which would result in the uncoupling of oxidative phosphorylation and thereby inhibition of ATP synthesis, in accordance with hepatic *coxIV* rhythm of expression which showed lowest levels during the dark phase. However, in the stressed fish no significant differences in *ucp1* expression were observed between sampling points.

Peroxiredoxins are the most recently discovered family of antioxidant enzymes. Initially identified in yeast, they have been found in all kingdoms of life, playing a key role in the organisms defence against oxidative stress (Rhee et al., 2005). Furthermore, a recent study has reported that the oxidation-reduction cycles of peroxiredoxin proteins constitute a universal marker for circadian rhythms in all domains of life (Edgar et al., 2012). In this regard, it must be noted that the daily differences in *prdx5* expression in control fish are parallel to those in *ucp1*, showing two peaks (at ZT7 and ZT15) and suggesting the existence of temporal coordination between the antioxidant systems and mitochondrial respiration uncoupling to minimize the risk of oxidative stress. However, the physiological relevance of these daily differences remains unclear. In fish exposed to air a daily rhythm of hepatic *prdx3* and *prdx5* expression was found, with the acrophase at the beginning of the light phase in both cases and only two hours apart, suggesting a time-dependent response of *prdx3* and *prdx5* expression to oxidative stress induced by air exposure. As seen before for hypothalamic *crh* expression, there appears a daily rhythm in induction, but not in basal *prdx3-5* expression. In gilthead sea bream

previous investigations have reported that different stressors can exert an effect on peroxiredoxins gene expression in liver and head kidney (Pérez-Sánchez et al., 2011, 2013). In addition, our results point that stress response shows daily rhythmicity and therefore up- or down-regulation of *prdx3* and *prdx5* expression show differences between sampling points.

In summary, the present results indicate that stress response shows daily rhythmicity in gilthead sea bream, although the phase of the rhythm differs among stress indicators (neuroendocrine and mitochondrial oxidative markers). Hence, in the stressed fish the acrophase of the daily rhythm of plasma cortisol was located at MD whereas the acrophases of *coxIV*, *prdx3* and *prdx5* rhythms were located during the light phase, which suggests that different timing mechanisms may be involved in the control of specific stress response. Taken together, these results indicate that cortisol responses are species-dependent (diurnal/nocturnal behaviour). Therefore the time of day should be considered when submitting fish to stressful conditions.

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579

FIGURE LEGENDS

Figure 1. Schematic representation of experimental design.

Figure 2. Average diel profile of locomotor activity in gilthead sea bream (n=12 tanks) reared for 2 weeks under a 12: 12 h LD cycle and fed at mid-light (ML). The height of each point represents the mean of infrared lightbeam 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. The vertical arrow indicates the feeding time. Data represent the mean (black area) + S.E.M. (dashed line) of all tanks. ZT, zeitgeber time.

Figure 3. Acrophases map for the statistically significant parameters analyzed in the present research (Cosinor, $p<0.05$). The acrophase is indicated by a circle, black and white for stressed and control group, respectively. The confidence intervals (set at 95%) are indicated by the lateral bars. White and black bars above the graph represent light and darkness, respectively.

Figure 4. Daily profiles of plasma cortisol in control (white circles) and stressed sea bream (black circles). Values represent the mean ± S.E.M. (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 that time point (t-test independent samples, $p<0.05$). Superscript letters indicate statistically significant differences between sampling points (ZTs) in the stressed group (ANOVA I, $p<0.05$). The discontinuous black line represents the sinusoidal function determined by Cosinor analysis for the stressed group.

Figure 5. Daily profiles of blood glucose in control (white circles) and stressed sea bream (black circles). The continuous black line represents the sinusoidal function determined by Cosinor analysis for the control group. Further details as given in Figure 3.

Figure 6. Relative expression of *crh* (A) and *crhbp* (B) genes in hypothalamus of control (white circles) and stressed sea bream (black circles). The continuous black line represents

the sinusoidal function determined by Cosinor analysis for the control group. Further details as given in Figure 3.

Figure 7. Relative expression of *coxIV* (A), *ucp1* (B), *prdx5* (C) and *prdx3* (D) genes in liver of control (white circles) and stressed sea bream (black circles). The continuous and discontinuous black lines represent the sinusoidal function determined by Cosinor analysis for the control and stressed groups, respectively. Further details as given in Figure 3.

Table 1. Gilthead sea bream primer sequences used for real-time PCR

Gene	Accession number	F/R	Primer sequence (5'-3')	Amplicon size (bp)
crh	KC195964	F	CARTTYACMTTCACAGCAGA	718
		R	CARGAGCTRCAGRYGATYAA	
crhbp	KC195965	F	GTRTTYGAYTGGGTGATGAA	501
		R	ATGAARRTYGGYTGTGAYAAC	
coxIV	JQ308835	F	ACCCTGAGTCCAGAGCAGAAGTCC	187
		R	AGCCAGTGAAGCCGATGAGAAAGAAC	
prdx3	GQ252681	F	ATCAACACCCCACGCAAGACTG	150
		R	ACCGTTTGGATCAATGAGGAACAGACC	
prdx5	GQ252683	F	GAGCACGGAACAGATGGCAAGG	175
		R	TCCACATTGATCTTCTTCACGACTCC	
ucp1	FJ710211	F	GCACACTACCCAACATCACAAG	137
		R	CGCCGAACGCAGAAACAAAG	
β-actin	JN546630	F	TCCTGCGGAATCCATGAGA	51
		R	GACGTCGCACTTCATGATGCT	

Table 2. Parameters of the cosine function calculated by Cosinor analysis ($p<0.05$) for physiological and oxidative stress markers in liver of seabream under control conditions or subjected to stress by air exposure.

Biological parameters	Experimental group	Significance variance (%V)	Mesor	Amplitude	Acrophase (ZT hours)
Cortisol	Control	NS	-	-	-
	Stress	29.6	69.9 ± 12.8	31.2 ± 17.6	18:24 ± 2:54
Glucose	Control	18.1	2.6 ± 0.7	0.4 ± 0.2	16:17 ± 4:04
	Stress	NS	-	-	-
crh	Control	36.0	8.0 ± 1.8	4.2 ± 2.5	4:25 ± 3:35
	Stress	NS	-	-	-
crhbp	Control	NS	-	-	-
	Stress	NS	-	-	-
coxIV	Control	58.5	170.8 ± 59.2	316.6 ± 82.6	4:24 ± 1:03
	Stress	21.5	100.3 ± 49.7	98.7 ± 71.2	5:02 ± 4:06
prdx3	Control	NS	-	-	-
	Stress	27.4	21.9 ± 14.9	35.1 ± 20.1	2:21 ± 3:00
prdx5	Control	NS	-	-	-
	Stress	26.8	9.7 ± 3.5	7.3 ± 4.4	4:30 ± 3:32
ucp1	Control	NS	-	-	-
	Stress	NS	-	-	-

The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method. All parameters are expressed as the value ± standard error (SE). NS=nonsignificant.

Table 3. Effect of the ZT, treatments (control/stressed) and their interaction on cortisol, glucose and gene expression levels.

Biological parameters	**ZT**	**Treatment**	**ZT x T**
Cortisol	**	**	NS
Glucose	NS	**	NS
crh	**	NS	**
crhbp	**	NS	**
coxIV	**	**	**
prdx3	**	**	**
prdx5	**	NS	**
ucp1	**	NS	NS

Asterisks indicate significant differences as **P≤0.01. NS=nonsignificant.

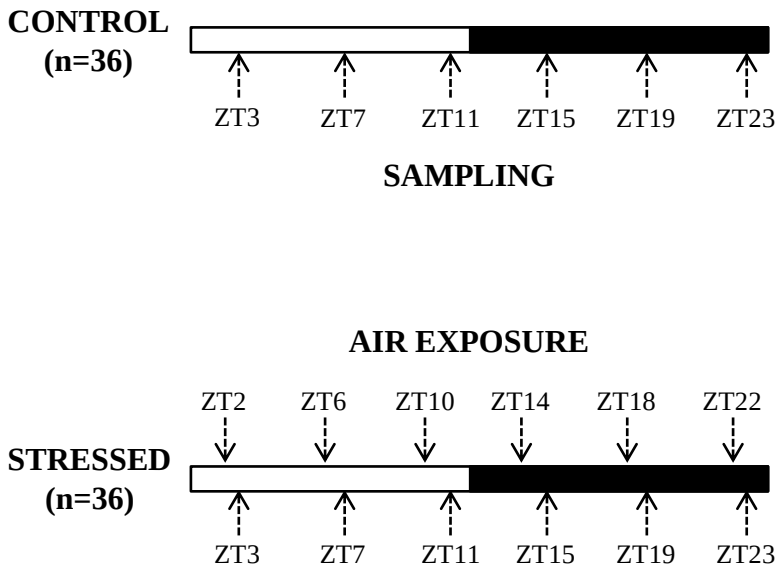
CONTROL
(n=36)
ZT3
ZT7
ZT11
ZT15
ZT19
ZT23
SAMPLING
AIR EXPOSURE
ZT2
ZT6
ZT10
ZT14
ZT18
ZT22
STRESSED
(n=36)
ZT3
ZT7
ZT11
ZT15
ZT19
ZT23
SAMPLING

Figure 1

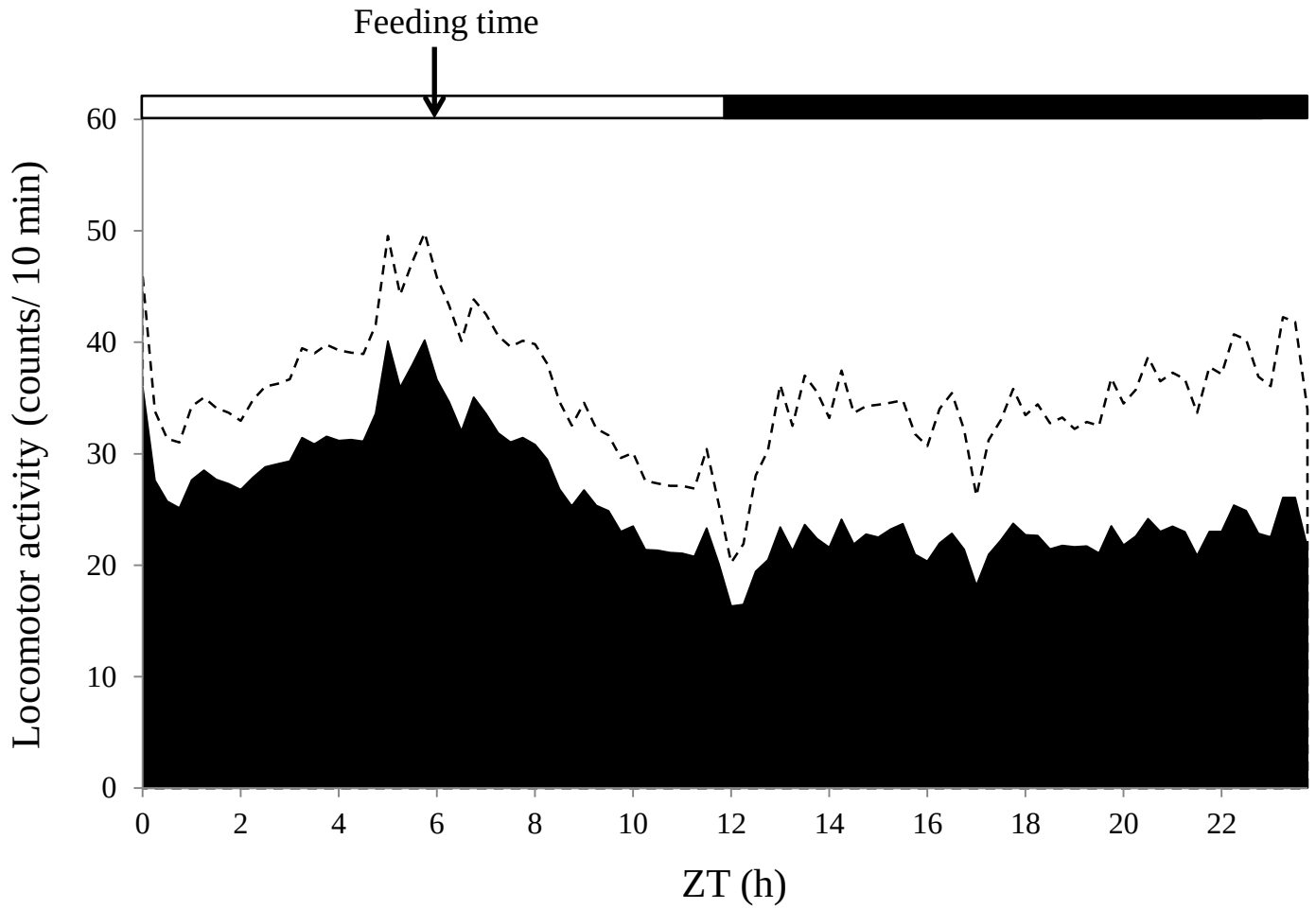
Feeding time
Locomotor activity (counts/ 10 min)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
ZT (h)

Figure 2

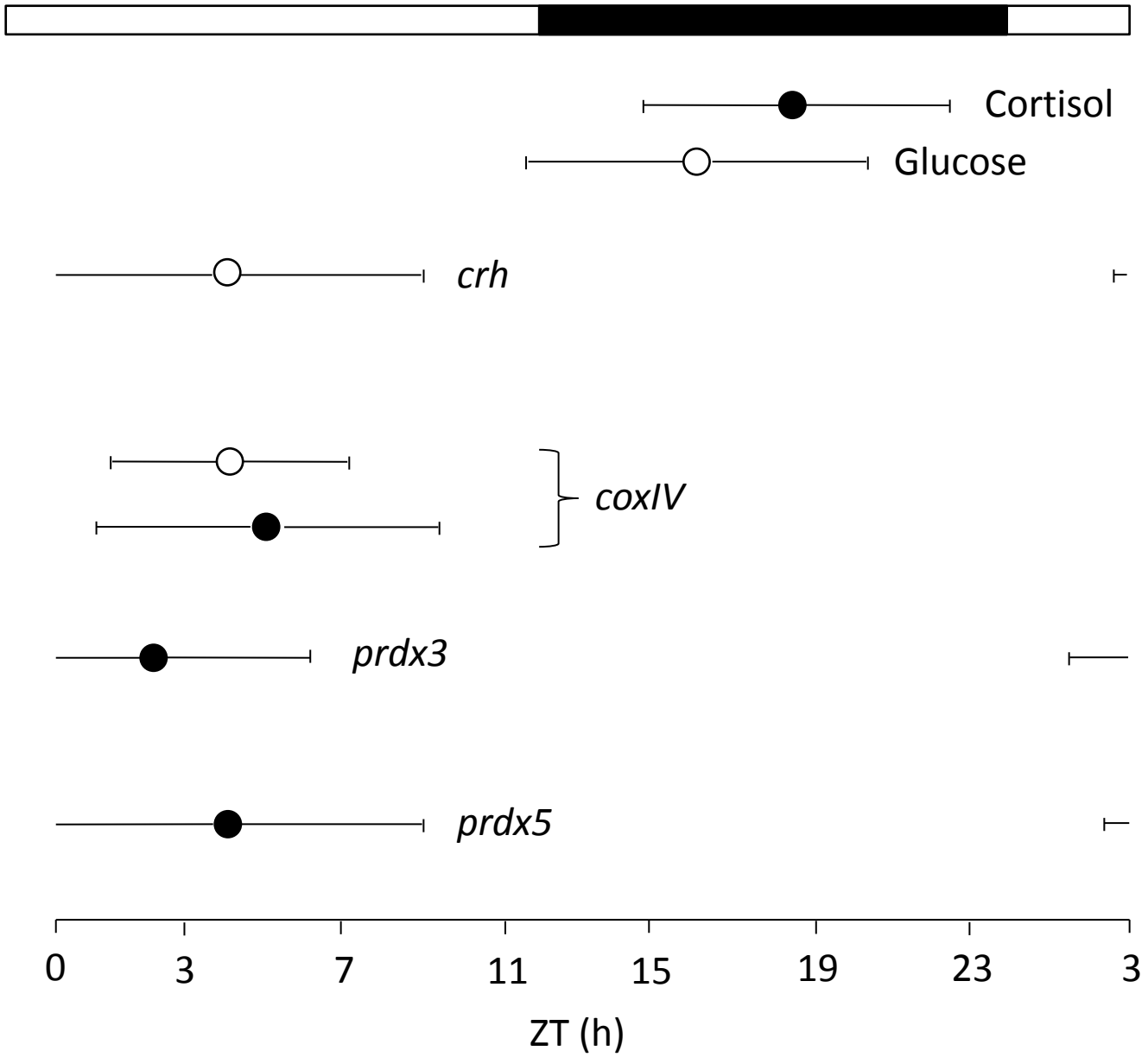

Figure 3

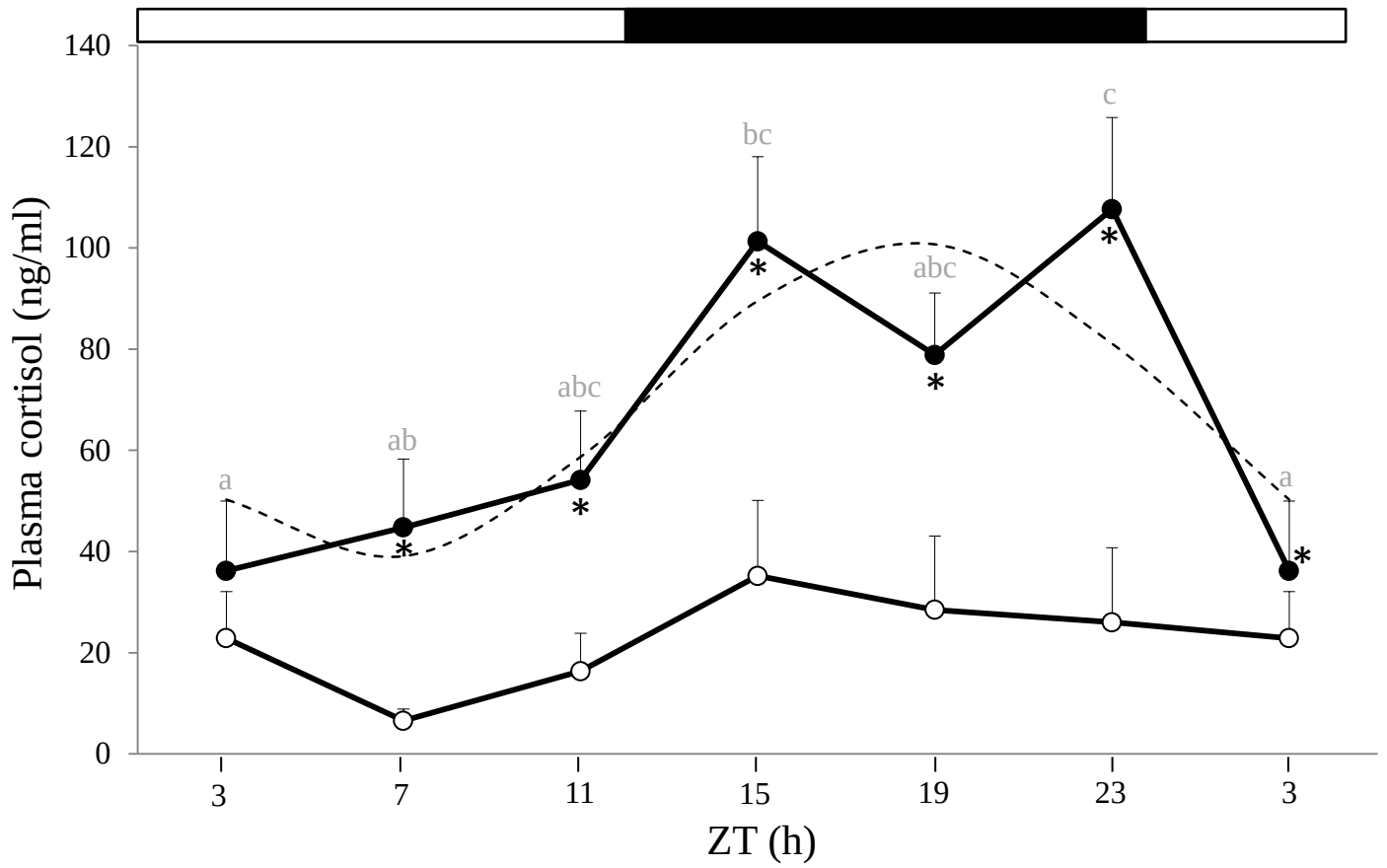
Plasma cortisol (ng/ml)
140
120
100
80
60
40
20
0
3
7
11
15
19
23
3
ZT (h)
a
ab
abc
bc
abc
c
a
*

Figure 4

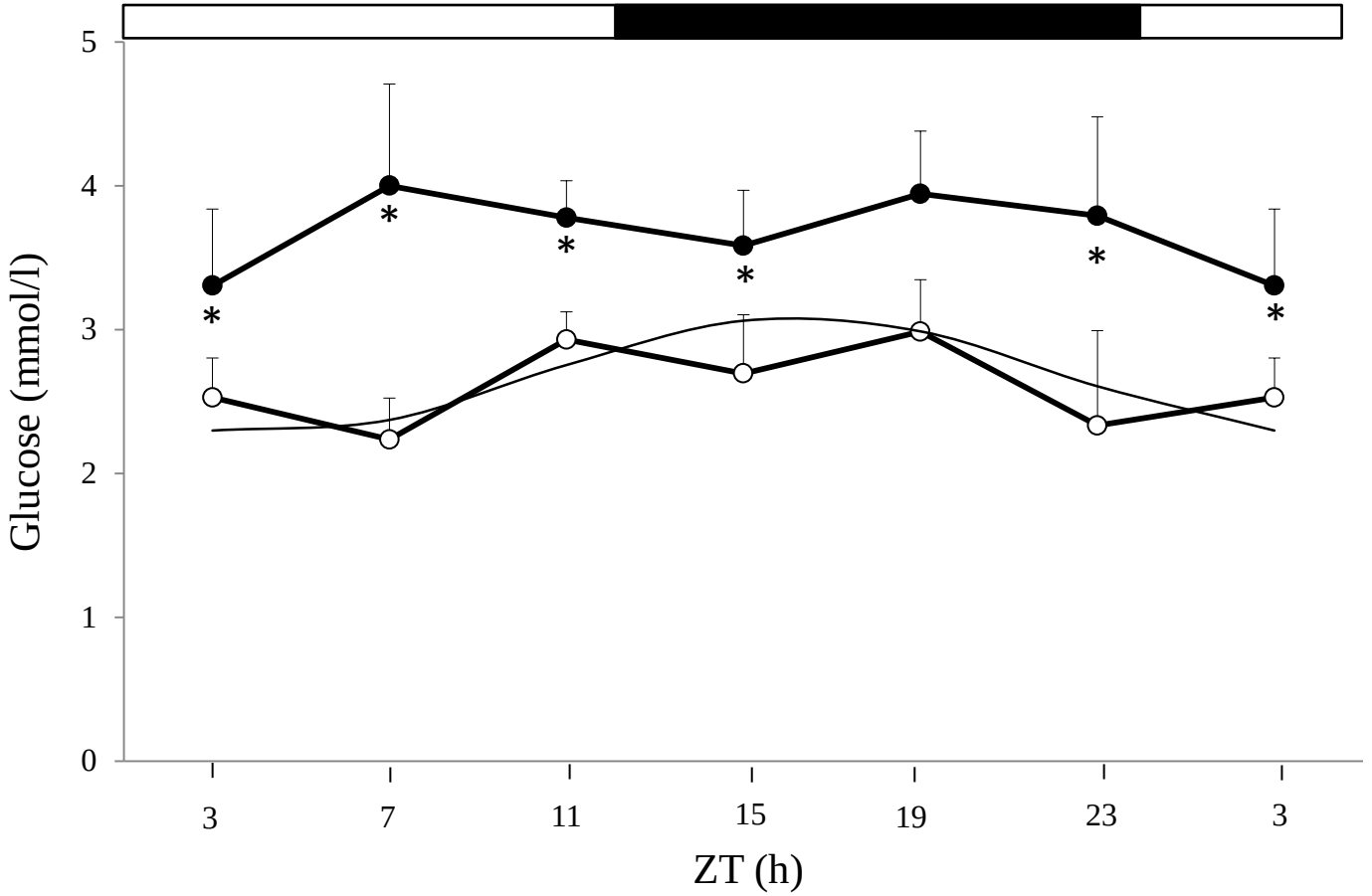

Figure 5

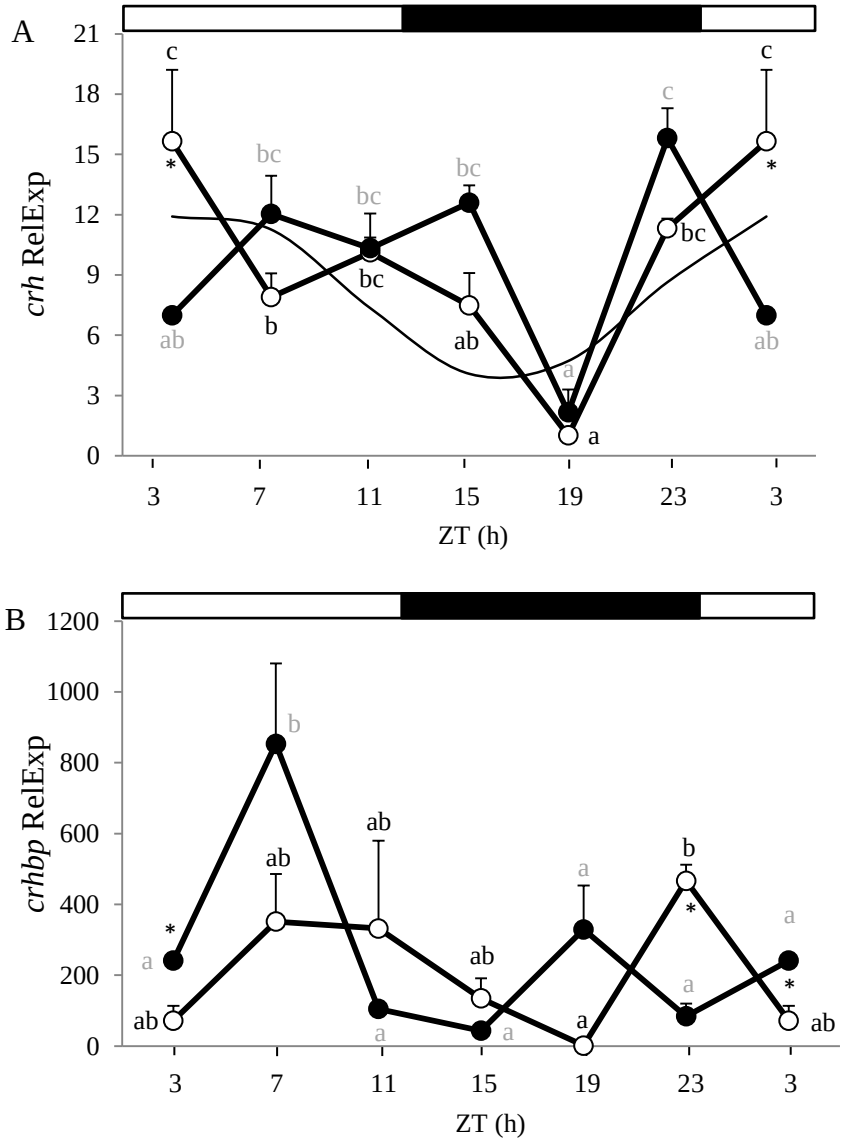
A
crh RelExp
21
18
15
12
9
6
3
0
c
bc
bc
bc
c
c
*
*
bc
b
bc
ab
ab
a
a
ab
3
7
11
15
19
23
3
ZT (h)
B
crhbp RelExp
1200
1000
800
600
400
200
0
b
ab
ab
a
b
a
*
*
*
a
a
a
a
a
ab
ab
3
7
11
15
19
23
3
ZT (h)

Figure 6

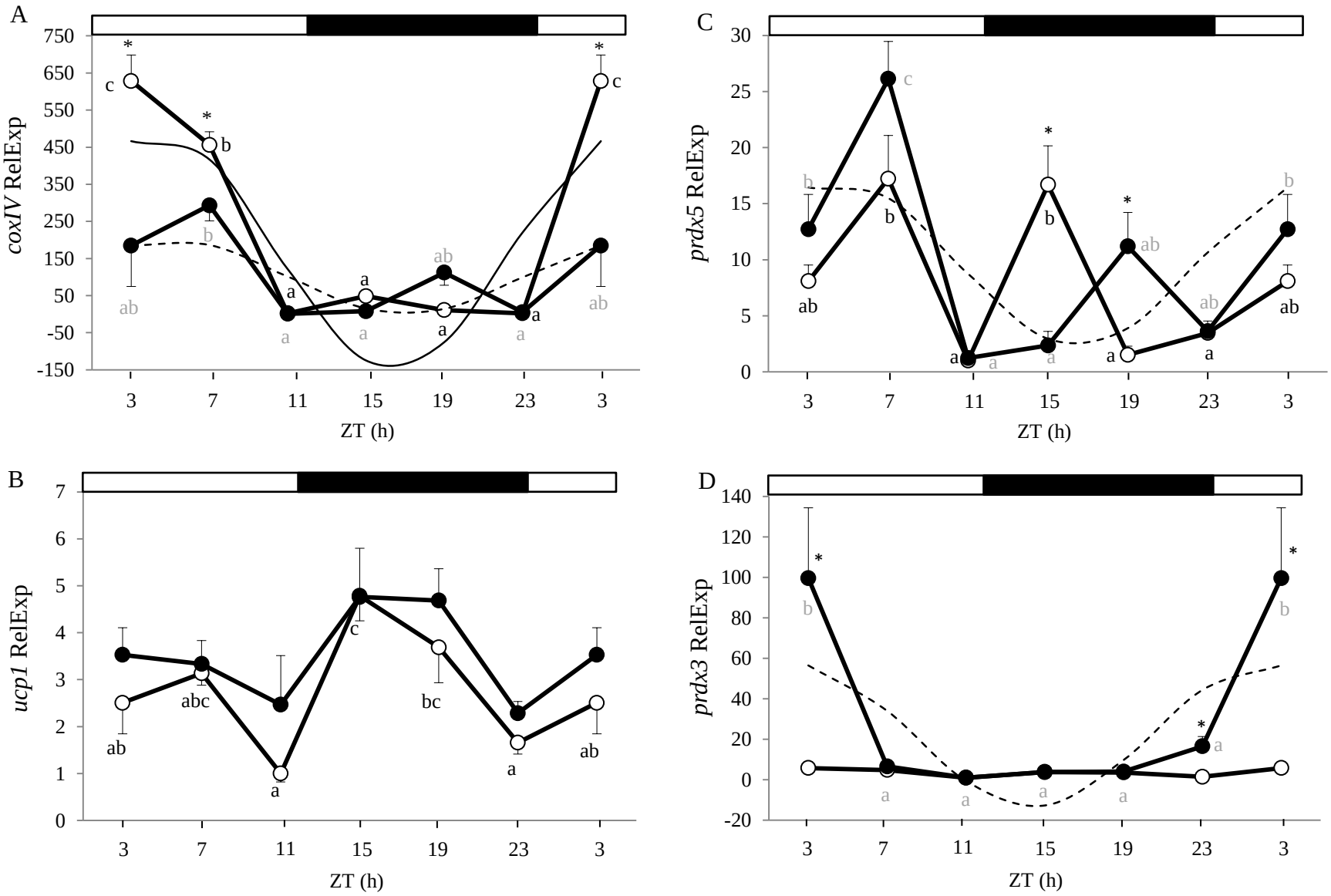
A
coxIV RelExp
ZT (h)
B
ucp1 RelExp
ZT (h)
C
prdx5 RelExp
ZT (h)
D
prdx3 RelExp
ZT (h)

Figure 7