Stress inhibition of melatonin synthesis in the pineal organ of rainbow trout (*Oncorhynchus mykiss*) is mediated by cortisol.

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SUMMARY

Cortisol has been suggested to mediate the effect of stress on pineal melatonin synthesis in fish. Therefore, we aimed to determine how pineal melatonin synthesis is affected by exposing rainbow trout to different stressors, such as hypoxia, chasing and high stocking density. In addition, to test the hypothesis of cortisol as mediator of such stress-induced effects, a set of animals were IP implanted with coconut oil alone or containing cortisol (50 $mg.kg^{-1}$ bw) and sampled 5 h or 48 h post injection at mid-day and mid-night. The specificity of such effect was also assessed in cultured pineal organs exposed to cortisol alone or with the general glucocorticoid receptor antagonist, mifepristone (RU486). The patterns of plasma and pineal organ melatonin content displaying highest values at night were affected by stressors (in particular chasing and high stocking density), resulting in decreased plasma and pineal organ melatonin content in both time periods, but with the most robust effect being found at night. The decrease in nocturnal melatonin levels in the pineal organ of stressed fish was accompanied by increased serotonin content and decreased AANAT2 enzymatic activity and mRNA abundance. Similar effects on pineal melatonin synthesis to those elicited by stress were observed in trout implanted with cortisol for either 5 h or 48 h. These data indicate that stress influences negatively the synthesis of melatonin in the pineal organ, thus attenuating the day-night variations of circulating melatonin. The effect might be be mediated by increased cortisol levels which bind to trout pineal organ specific glucocorticoid receptors to modulate melatonin rhythms. Our results in cultured pineal organs are on its support. Considering the relevant role of melatonin conveying photoperiodical information to the synchronization of daily and annual rhythms, the results suggest that stress-induced alterations in melatonin synthesis could affect the availability of fish to integrate rhythmic environmental information.

INTRODUCTION

In teleost fish the pineal organ perceives and transduces the light-dark signal (Bromage et al., 2001) into neural and humoral signals from which the hormone melatonin is well recognized. Melatonin is rhythmically synthesized mainly from the pineal organ and released into the blood, showing highest plasma levels at night and basal melatonin values occurring at day-time. The penultimate step of melatonin synthesis in the pineal organ is carried out by the enzyme arylalkylamine *N*-acetyltransferase (AANAT), which is considered as the rate-limiting enzyme based on its daily variations of activity that parallel those of melatonin (Klein, 2007). Once released into blood, melatonin rhythmic profile conveys photic information to the organism (see rev. Falcón et al., 2010) and acts as synchronizer of a variety of processes including larval development, locomotor activity, sedation, skin pigmentation, oxygen consumption, thermoregulation and food intake behavior (Ekstrom and Meissl, 1997; Reebs, 2002; Falcón et al., 2010; Zhdanova and Reebs, 2006). In addition, annual rhythms of reproduction, growth, immune response and migration, are also timed by melatonin in different fish species (Bromage et al., 2001; Oliveira and Sánchez-Vázquez, 2010). The daily melatonin profile persists even after exposing fish to constant darkness as described for most teleost species (Cahill, 2002; Migaud et al., 2007). This is due to the fact that pineal organ hosts a true circadian light sensitive pacemaker which drives melatonin rhythms. Only salmonids, including rainbow trout, represent an exception to this rule. In all salmonid species investigated to date it has been demonstrated that pineal melatonin synthesis does not involve an endogenous clock, so that lack of melatonin oscillation has been described under constant conditions (Thibault et al., 1993; Gern and Greenhouse, 1988; Mizusawa et al., 2000; Migaud et al., 2007). However even under constant darkness several core circadian genes continue to cycle in other trout neural regions (retina and hypothalamus) (López-Patiño et al., 2011a), which are involved in the regulation of daily rhythms of several parameters such as feeding behavior and locomotor activity (Cuenca and De la Higuera, 1994; Sánchez-Vázquez and Tabata, 1998).

In addition to external factors and the circadian influence, several internal factors modulate melatonin synthesis in fish (Ekström and Meissl, 1997). Among those some studies suggested a role for prolactin (De Vlaming and Olcese, 1981), estrogens (Bégay et al., 1994; Forlano et al., 2005), glucocorticoids (Falcón, 1999; Benyassi et al.,

2001) and catecholamines (Martin and Meissl, 1992; Samejima et al., 1994; Ekström and Meissl, 1997). Cortisol is a glucocorticoid synthesized in the interrenal tissue of fish which plays an important role in several aspects of fish physiology, including energy metabolism, ionic and osmotic regulation, growth, immune function, and stress response (Henderson and Garland, 1980; McCormick, 1995; Wendelaar Bonga, 1997; Mommsen et al., 1999). Plasma cortisol levels display a circadian rhythm in a teleost species like common dentex, *Dentex dentex* (Pavlidis et al., 1999), but such daily pattern appears to depend on the fish species (Garcia and Meier, 1973; Pickering and Pottinger, 1983; Pavlidis et al., 1999; Saito et al., 2004; Ebbesson et al., 2008). Many studies have reported for rainbow trout increased plasma cortisol at night-time, peaking before the light onset, then falling down and remaining low during the day (Rance et al., 1982; Boujard and Leatherland, 1992). Such daily profile is also influenced by feeding time (Boujard and Leatherland, 1992), in support of a rhythmic cortisol secretion being synchronized by both photoperiod and feeding activity, with differences among seasons.

Based on findings describing that *i)* cortisol levels appear to show daily variations, *ii)* the interaction within several neurohumoral signals and melatonin production, *iii)* cortisol circulating levels increase right after fish stress exposure (Wendelaar Bonga, 1997; Mommsen et al., 1999), and *iv)* the inhibitory effect exerted by glucocorticoids on AANAT activity of cultured pineal organs in trout (Benyassi et al., 2001; Yanthan and Gupta, 2007), we hypothesized that, in the same way than that previously described for other teleost species such as tipalia, *Oreochromis mossambicus* (Nikaido et al., 2010), and that previously suggested for trout (Larson et al., 2004), stress negatively affect melatonin synthesis in pineal organ of rainbow trout with cortisol mediating such inhibitory effect.

The aim of the present study was therefore to evaluate the impact of stress on melatonin synthesis in rainbow trout pineal organ, and to evaluate the role of cortisol on such effect. Thus, we evaluated day-night variations of plasma cortisol and melatonin levels, pineal content of melatonin, serotonin (immediate melatonin precursor) and its main oxidative metabolite, 5-hydroxyindoleacetic acid, as well as AANAT2 enzyme activity and mRNA abundance in fish kept under normal housing conditions, or exposed to different stressors, or receiving cortisol implants. An *in vitro* assay of pineal organs was also performed in order to corroborate the specificity of the effect.

RESULTS

Stress affects plasma cortisol and melatonin levels

The effect of exposing trout to different stressors on plasma cortisol and melatonin content is shown in Figure 1. Plasma cortisol displayed a significant (P=0.004) day-night variation in control fish (higher levels observed at night) and those under high stocking density (lower levels at night). Exposing animals to different stress condition significantly increased cortisol levels at both mid-day and mid-night, relative to the respective control non-stressed group. The increase of cortisol levels was more notorious in animals exposed to acute stress, i.e., hypoxia and chasing.

Melatonin levels in control group showed a day-night variation with higher levels being observed during the night (P< 0.001). The same trend was observed for all the stressed groups. However, a significant decrease of plasma melatonin levels was noticed after stressing animals at mid-day-time (P=0.047; P=0.006 and P=0.012 for hypoxia, chasing and high stocking, compared to controls), whereas at mid-night-time it happened under chasing and high stock conditions (P=0.041 and P=0.008 respectively).

Effect of stress on melatonin content, AANAT2 activity and mRNA abundance in trout pineal organ

Melatonin content, AANAT2 enzyme activity and mRNA abundance in pineal organ of trout exposed to different stressors are shown in Figure 2. Similarly to that found for plasma melatonin, a day-night variation of melatonin content in pineal organ was observed, with higher values occurring at night (P<0.001 relative to day-time). No effect of stress was noticed at mid-day, whereas melatonin levels significantly decreased in fish exposed to high stocking density at mid-night (P=0.042 relative to control).

AANAT2 enzyme activity in trout pineal organ showed a clear day-night variation in control, hypoxia and high stocking groups, with higher activity noticed at mid-night. Stress did not affect AANAT2 activity at day-time, but significantly decreased it at night (P=0.002; P<0.001; P<0.001 relative to control at night for hypoxia, chasing and high stocking). This effect was more robust in fish exposed to chasing, in which the day-night variation disappeared.

A significant day-night variation of *aanat2* mRNA abundance was observed in pineal organ of control and high stocking fish, with higher values at mid-night (P=0.002

158 and *P*=0.029 respectively). Decreased nocturnal *aanat2* mRNA abundance, relative to
159 control group, was found in fish exposed to acute stress (hypoxia and chasing), leading
160 the day-night variation of *aanat2* expression to disappear in both groups.
161
162 **5-HT and 5-HIAA contents in pineal organ of stressed trout**
163 Day-night variations of serotonin and its main metabolite, 5-HIAA, and the
164 ratios 5-HIAA/5-HT and melatonin/5-HT are shown in Figure 3. Serotonin content in
165 pineal organ was significantly lower at mid-night in all the experimental groups. Stress
166 did not significantly affect 5-HT levels at day-time, whereas a significant increase was
167 observed at mid-night only in fish exposed to high stocking (*P*=0.001; *P*=0.002; and
168 *P*=0.008, relative to the other groups).
169 Similarly to that described for serotonin, 5-HIAA day-night changes were found
170 in all the experimental groups, with significantly higher levels at mid-day. During the
171 night, a significant decrease of 5-HIAA content was found in trout exposed to hypoxia,
172 relative to that of control (*P*=0.034) and high stocking groups at this time period.
173 The 5-HIAA/5-HT ratio did only show day-night variations in the
174 hypoxia-exposed group, which was higher at night (*P*=0.040). In addition, exposing fish
175 to high stocking density tended to decrease the 5-HIAA/5-HT ratio at night, but this
176 effect did not reach significance when compared to control at night.
177 A clear day-night variation of the ratio Melatonin/5-HT was found in all the
178 experimental groups, with higher values occurring at night. During the day, hypoxia and
179 high stocking density significantly decreased the Melatonin/5-HT ratio, compared to
180 control and chasing groups. In contrast, only the high stocking density significantly
181 decreased the ratio at mid-night, relative to control, hypoxia and chasing groups.
182
183 **Plasma cortisol and melatonin levels after cortisol IP administration**
184 Plasma cortisol and melatonin levels after coconut oil administration alone or
185 with cortisol are shown in Figure 4. Data obtained from the control non-implanted
186 group are not shown but remained quite similar to those of the control implanted fish.
187 Though no significant day-night variation of plasma cortisol levels was found in control
188 group, a tendency to higher nocturnal hormone levels persisted (*P*=0.082) in the same
189 way than that observed in the previous experiment (see Figure 1). As expected, a

significant increase of cortisol levels was found in trout sampled 5-h or 48-h after the IP administration, compared to control group at both time periods. Only a significant day-night variation was found in trout IP injected with cortisol at 5-h post injection, with higher levels at night, relative to the same group at day-time.

Plasma melatonin levels in control group showed the same day-night variation as above described, with higher levels at night ($P<0.001$). The administration of cortisol enhanced levels of melatonin after 48-h at day and decreased after 48-h at night, in both cases relative to control group ($P=0.016$ and $P=0.038$ respectively), then making the amplitude of the day-night variation to decrease in those 48-h cortisol implanted trout.

Pineal content of melatonin and AANAT2 activity and mRNA abundance in cortisol implanted trout

Figure 5 shows melatonin content, AANAT2 enzyme activity, and mRNA abundance in pineal organ of implanted trout. Data in non-implanted fish (not shown) were consistent with those observed in control-implanted trout for the three parameters assessed. A significant day-night variation was found for melatonin content in all the experimental groups with higher levels occurring at mid-night. The IP cortisol administration significantly reduced nocturnal melatonin levels relative to that found in controls at night ($P=0.011$ and $P=0.027$ for 5-h and 48-h respectively). No effects of cortisol administration were found during the day.

AANAT2 activity displayed a significant day-night variation in control trout, with higher levels at mid-night. In contrast, cortisol significantly inhibited the enzyme activity only at night ($P=0.018$ and $P=0.022$ for 5-h and 48-h respectively), leading the typical day-night variation of the enzyme activity to disappear at both 5-h and 48-h post injection. This inhibitory effect of cortisol was not observed at mid-day.

The analysis of *aanat2* mRNA abundance in pineal organ of rainbow trout revealed significantly higher expression at night-time in control trout, relative to that measured during mid-day. Cortisol administration showed a time-dependent effect. Thus, *aanat2* expression was significantly enhanced during day-time at 5-h post-cortisol injection ($P=0.041$), but decreased after 5-h and 48-h at night-time ($P=0.042$ and $P=0.006$ for 5-h and 48-h respectively), with the effect being more effective after 48-h.

This inhibitory effect of cortisol on mRNA expression did lead the day-night variation of *aanat2* expression to disappear in both cortisol-implanted groups.

5-HT and 5-HIAA contents in pineal organ of implanted animals

Figure 6 shows the daily variation of serotonin and 5-HIAA levels, and the ratios 5-HIAA/5-HT and melatonin/5-HT. Control group displayed a significant day-night variation for 5-HT content in the pineal organ, with higher levels at mid-day. Whereas cortisol administration for 5-hours had no effect on 5-HT content (relative to control group) the 48-h administration significantly decreased the diurnal 5-HT (P=0.024 and P=0.038 relative to control and 5-h at day-time) and increased the nocturnal 5-HT content (P=0.005 and P=0.011 relative to control and 5-h at day-time). Thus, the day-night variation of pineal 5-HT content displayed higher levels at night and lower levels during the day, which was the opposite profile than that observed in control.

No daily variations of 5-HIAA pineal content were observed in control group and that IP administered with cortisol for 5 hours. However, a nocturnal significant increase of the metabolite was observed in animals administered with cortisol for 48 hours (P=0.049 relative to control). Then 5-HIAA content in trout pineal organ displayed a significant day-night variation only in animals IP implanted with cortisol for 48 hours, with higher 5-HIAA levels occurring at night (P=0.004 relative to day-time).

The ratio 5-HIAA/5-HT did not significantly change among groups at both day- and night-time. Day-night significant variations of the ratio were found only in trout IP administered with cortisol for 5-h, with higher values at night (P=0.007 relative to day).

All the experimental groups displayed a significant day-night variation in the ratio Melatonin/5-HT, with higher values at mid-night. Cortisol administration significantly decreased the ratio only at night compared to control group (P<0.001 and P=0.007 for 5-h and 48-h relative to control), with the more important effect being observed after 48-h. No such effect was found in animals IP injected during the day at both 5-h and 48-h post injection.

Effect of cortisol treatment on melatonin production *in vitro*

Melatonin production in cultured pineal organ in the presence/absence of light was compared among experimental groups (Figure 7). Melatonin was detected in the culture medium of samples collected under each lighting condition. There were no

group-specific differences in basal melatonin release at both light and dark. However, the addition of medium containing cortisol resulted in a significant decrease of melatonin production in darkness, relative to control (P=0.037) at the same lighting condition and to that observed before cortisol addition within the same group. This inhibitory effect of cortisol was prevented by RU486 (P=0.032, relative to cortisol) when both chemicals were added together.

DISCUSSION

In the present study, different stressors were evaluated, i.e., hypoxia, chasing and high stocking density, mimicking those potentially stressing situations to which fish can be exposed when reared. The response to stress in fish involves the activation of the hypothalamus-sympathetic nervous system-chromaffin tissue, and the hypothalamus-pituitary-interrenal tissue axes, followed by a fast increase of catecholamine and cortisol levels in plasma, which in fact induce metabolic and functional alterations (Iwama et al., 2006), and affect fish physiology (Barton et al., 2002). Little is known regarding the effects of those hormones at the trout pineal organ, but previous studies describe that catecholamines appear not to have any effect at this tissue location, in contrast to that in other teleost for which a regulatory role has been proposed (Falcón et al., 1991). Regarding cortisol, melatonin synthesis in trout pineal organ was reported to be influenced by glucocorticoid hormones (Benyassi et al., 2001), with cortisol being a serious candidate as mediator of such effect.

Day-night variations of cortisol and melatonin levels in plasma, the pineal organ content of 5-HT, 5-HIAA and melatonin, and the AANAT2 activity and mRNA abundance at the pineal level were evaluated in non-stressed, stressed and cortisol-implanted trout. Those fish reared under normal housing conditions showed significant (Experiment 1) or a tendency (Experiment 2) to day-night variations of plasma cortisol with night values being higher than those measured during the day, in concordance with previous studies in the same species (Rance et al., 1982; Boujard and Leatherland, 1992) and others such as the brown trout (Pickering and Pottinger, 1983) and tilapia (Martínez-Chavez et al., 2008; Nikaido et al., 2010). Our results show that cortisol levels were higher in those acutely stressed groups (hypoxia and chasing) whereas such increase was lower in high-stocked fish in particular during the night. Also, the increase in cortisol was higher in those fish stressed at day-time compared

with the same groups at night, independently of the stress condition. This result suggests that the integrated response to stress could be influenced by the time of the day in which the stressor is present. Also the fact that the higher cortisol increase was coincident with the time of day in which trout are more active suggest that a relationship between behavioural components and the response to stress might exist. Further research should be carried out.

Plasma melatonin levels were negatively affected by stress in a way that the highest reduction of day-night variation of hormone levels was observed in fish exposed to long-term stress (high stocking), rather than those acutely stressed (chasing, hypoxia), which in fact also showed decreased plasma melatonin levels, with those of the pineal organ remaining unaltered. This indicates that time periods longer than 1-5 minutes (hypoxia and chasing, respectively) might be required for a significant inhibition of melatonin content to be observed in pineal organ. In contrast, changes affecting AANAT2 enzyme activity and mRNA abundance immediately occur as our results indicate. However, one might hypothesize the presence of a correlation between stress duration and the magnitude of the inhibition of the night-time melatonin production in the pineal organ, which is supported by the existence of similar effects in both pineal organ melatonin content and plasma values. On the contrary, day-time changes in pineal melatonin after any stressor were minor, reflecting that reduced levels of the hormone in blood of stressed trout during day-time might involve alterations in melatonin clearance rates or, alternatively, that hormone synthesis in other tissues was also defective, i.e., retina and the gastrointestinal tract that were suggested to contribute to blood melatonin levels during the day (Lepage et al,. 2005; Muñoz et al., 2009). Further research is needed to discard any explanation.

The effect of stress on the day-night melatonin secretion pattern has been studied in several fish species and shows contradictory results. Then, Larson et al (2004) reported higher night-time melatonin and cortisol levels in socially subordinated rainbow trout relative to the dominant fish. Similar results were observed in our laboratory in trout exposed to increased salinity (López-Patiño et al., 2011b), but that was in contrast to that reported for European sea bass (López-Olmeda et al., 2009). Increased circulating melatonin levels were also found in gilthead sea bream subjected to high stocking density, with such effect being prevented by fasting (Mancera et al., 2008). Similar results have been also described for rainbow trout in which fasting

decreases pineal organ melatonin synthesis at night (Ceinos et al., 2008), and disturbance stress negatively affect several parameters including melatonin (Kulczykowska, 2001), which is in consistency with our data herein reported for trout exposed to different stressors. Since different species appear to specifically respond to any stressor, we might speculate with the idea of a species-specific stress effect on melatonin synthesis at the pineal organ. Taking in mind that *i)* pineal melatonin synthesis is differentially regulated among teleost in relation with the environmental signals, i.e., light/dark cycle (trout melatonin system generating rhythm seems to lack a functional clock in contrast to that of most non-salmonids), and *ii)* the different social behaviours and physiological adaptations to the aquatic environments in which fish inhabit, it might not be surprising a different species-specific response to stress. In addition, the nature and the duration of the stressor appear to also influence the response of the pineal organ, as revealed by our study.

In spite of that above mentioned for melatonin, stress also diminished the AANAT2 enzymatic activity and mRNA abundance in the pineal organ at night. It is generally accepted that the nocturnal increase in AANAT2 enzyme activity is the main responsible of the daily rhythm of melatonin synthesis. In trout, light by directly acting on pineal photoreceptors, exerts an inhibitory influence on both AANAT2 gene expression (López-Patiño et al., 2011b) and enzyme activity (Falcón, 1999; Ceinos et al., 2005), with immediate consequences on pineal melatonin content (Ceinos et al., 2005). This is in contrast to that previously reported for AANAT2 activity and gene expression in trout pineal organ, in which the *aanat2* expression daily profile was not observed (Bégay et al., 1998; Coon et al., 1998; Falcón et al., 2001). The reasons for these discrepancies are not known, but methodological differences or different trout strains might be the most plausible explanations as we previously reported (López-Patiño et al., 2011b). Further research needs to be carried out in order to understand the nature of. Thus according to our previous data and that describing a light effect, our present results indicate that melatonin synthesis in trout pineal organ is inhibited by stress by specifically affecting AANAT2 activity, which is probably a consequence of the inhibition observed in *aanat2* mRNA expression. In addition, our data showing increased nocturnal levels of 5-HT, but not its main oxidative metabolite 5-HIAA, in pineal organs of stressed trout also support an inhibitory role for stress on melatonin synthesis. Therefore, it is likely that, when stressed, the N-acetylation

pathway of 5-HT is inhibited due to decreased of AANAT2 enzyme activity and expression, leading to decreased melatonin levels and intracellular accumulation of 5-HT, which is oxidized to 5-HIAA.

Previous *in vitro* studies on the hormonal regulation of fish pineal melatonin synthesis revealed the presence of glucocorticoid receptors in trout pineal organ, and when *in vitro* assayed with the glucocorticoid analogue dexamethasone decreased AANAT2 activity was observed (Benyassi et al., 2001), which is in consistency with our results from the pineal organ *in vitro* assay showing decreased melatonin release in pineal organs exposed to cortisol in darkness. Also Yanthan and Gupta (2007) found decreased AANAT activity in cultured pineal organ of the North African catfish (*Clarias gariepinus*) after milimolar corticoid treatment, an effect similar to that reported recently in cultured pineal organs of tilapia with nanomolar cortisol concentrations (Nikaido et al., 2010). Therefore a glucocorticoid regulation of pineal melatonin production is possible under physiological conditions, and this might occur when cortisol levels are elevated due to a stressful condition. On the basis of these studies, an *in vivo* experiment was conducted in trout to evaluate whether or not cortisol treatment might result in changes in plasma and pineal parameters similarly to those elicited by stress. Our results show that cortisol implants for 48 hours increase plasma melatonin levels at day-time, whereas both plasma (at 48-h) and pineal melatonin (5-h and 48-h) levels decreased during the night in cortisol-implanted trout. This differential effect of cortisol on melatonin secretion seems to be independent of the cortisol increase (i.e., cortisol levels in implanted fish were higher than those of controls any time). Thus, we might speculate with the idea that increased cortisol could differentially affect the photoreceptor cell properties at day and night (i.e., by altering membrane permeability or light transduction pathways) with melatonin synthesis being affected. In agreement with this hypothesis, we previously reported for rainbow trout that exposure to a chemical stressor (i.e., polycyclic aromatic hydrocarbons; Gesto et al., 2009) elicited a similar effect than that herein reported for pineal organ melatonin synthesis. In addition, cortisol implants also decrease AANAT2 enzyme activity and mRNA abundance in the pineal organ at night. These results clearly demonstrate the negative effect exerted by cortisol on AANAT2 abundance and activity. In addition, the specificity of the negative effect of cortisol on pineal organ melatonin synthesis has been proved, as our results from the *in vitro* assay with cortisol and its antagonist (RU486) clearly demonstrate. In

support of that, we also observed that trout receiving cortisol implants for 48-h displayed increased 5-HT levels at night. This effect was similar to that observed for 5-HIAA, which suggests that the inhibitory effect of cortisol on melatonin synthesis leads to increased 5-HT content, which is then oxidized. A similar effect of cortisol was not seen at mid-day, which may suggest that those variations observed during the night might be mainly due to decreased 5-HT utilization through the N-acetylation pathway to synthesize melatonin at this time period. Thus we may discard any possible specific effect of 5-HT synthesis in trout pineal organ. Taken together, we conclude that cortisol, through the activation of specific glucocorticoid receptors, might be the main responsible of the influence of stress on teleost pineal organ physiology, especially in those species in which catecholamines might play a minor role (Falcón et al., 1991).

Therefore, the effects observed in both stressed, cortisol-implanted trout, and cultured pineal organs tended to decrease the day-night variation of both pineal content and plasma melatonin levels. It is likely that cortisol effect on pineal melatonin is meditated by either specific glucocorticoid receptors as indicated previously and for other teleost species (Benyassi et al., 2001; Nikaido et al., 2010), which might activate glucocorticoid-responsive elements at the AANAT gene promoter (Benyassi et al., 2001), and/or other non-genomic actions at the cell-surface (Mommsen et al., 1999). Our results from cultured pineal organs are in support of this hypothesis. In fish, plasma cortisol levels are known to cycle diurnally (Holloway et al. 1994; Reddy and Leatherland 1995) and to change through the seasons (McLeese et al. 1994), although cortisol profile exhibits species-specific daily and seasonal patterns affected by several factors like photoperiod (Pickering and Pottinger, 1983) or feeding time (Boujard and Leatherland, 1992), among others. In some species, such as common dentex, daily fluctuations of plasma cortisol levels show endogenous rhythmic characteristics (Pavlidis et al., 1999), and this hormone is taking increased relevance as an important output of the circadian clock system in vertebrates (Lilley et al., 2012). However, a temporal relationship between physiological clocks and plasma levels of cortisol and melatonin has not been established in fish. Taking into account that trout appears to lack a pineal circadian signalling that controls melatonin synthesis, one might not discard the possibility for daily changes in cortisol to play a modulatory role in the light-dark regulation of trout melatonin rhythm. In fact, cortisol values were shown to be especially high at the end of the night and in the early morning, a time in which pineal

melatonin synthesis has decreased (Ceinos et al., 2005). On the contrary, high melatonin levels have been reported to reduce cortisol secretion in goldfish (Azpeleta et al., 2010) and to counteract the stress-induced cortisol increase in Senegalese sole (López-Patiño et al., 2013). This hormonal correlation is in support of our data showing in trout an inverse relationship between high cortisol levels and melatonin levels in stressed, cortisol-implanted fish and in cultured pineal organs. Then, digging into cortisol rhythms and its interaction with melatonin is a promising topic to work with in order to understand the regulation of pineal rhythmic physiology and the circadian organization which is believed to exist in trout (Sánchez-Vázquez and Tabata, 1998).

In summary, our results provide evidence for the inhibitory effect exerted by cortisol on melatonin synthesis by the pineal organ of rainbow trout. This steroid likely mediates the effects of different stressful situations on the pineal organ by activating specific glucocorticoid receptors. Such activation seems to directly influence AANAT2 enzyme activity and expression which are normally increased at night, allowing the melatonin secretory peak. Our results also indicate that cortisol, either in non-stressful or stressful conditions, might have a modulatory role of the pineal rhythms, in particular those related to melatonin synthesis. Considering the pivotal role of melatonin in synchronizing rhythmic physiological events to the cyclic environmental changes (mainly light-dark cycle), the effect of stress on melatonin synthesis might be translated into a process that can jeopardize the availability of the animal to respond to such fluctuations and in consequence, to compromise its physiological integration.

MATERIALS AND METHODS

Animals

Immature rainbow trout (7.0±0.5 months old; 100±5 g body mass) were obtained from a fish farm (Soutorredondo, Noia, Spain) and transferred to our facilities at the Vigo University. Animals were acclimated for 15 days in 120 L tanks under our laboratory conditions: 12:12 light (L):dark (D) photoperiod (lights on at 08:00 h, 400 lux intensity at the water surface), 14±1ºC water temperature, and continuously renovated and aerated water. During acclimation fish were fed daily to satiety (10:00 h) with commercial dry pellets (Dibaq-diproteg, Segovia, Spain). All the experimental procedures and animal manipulation were designed according to the European Union Council (2010/63/EU), and the Spanish Government (RD 53/2013) legal requirements.

Sample collection

Animals were deeply anesthetized by fast immersion in MS-222 (50 mg/L) buffered to pH 7.4 with sodium bicarbonate and blood was collected from the caudal vein, using 1 ml heparinized syringes. Then, fish were sacrificed by decapitation and pineal organs were removed with sterilized material and placed into RNase-free 1.5 ml Eppendorf tubes. Samples were immediately frozen in liquid nitrogen, and stored at -80°C until assayed. Plasma was obtained by centrifuging blood at 9,000 rpm for 10 min at 4°C. Aliquots were immediately frozen and stored at -80°C until analyzed.

Experimental designs

Three experiments were designed to evaluate the influence of stress on melatonin production in rainbow trout pineal organ. In a first experiment, fish were randomly distributed in four groups: control, or stressed by hypoxia, chasing, and high stocking density (n=20 animals each). Following acclimation, a quantity of water was removed from those tanks hosting the "high stocking density" group until reaching a stressful high density (70 kg fish mass·m^{-3}), where fish remained for 4 days before sacrificed. Stocking density conditions remained unaltered for the other groups (10 kg fish mass·m^{-3}). On the day of sacrifice, animals from the other stressed groups were exposed to different manipulations and then sacrificed at mid-day and mid-night as follows. Fish from the "hypoxia" group were normally handled and netted but remained in the net for 60 seconds before being deeply anesthetized in a MS-222 solution, blood sampled and sacrificed. "Chasing" group was subjected to a standardized handling disturbance consisting on 5 min of repeated chasing followed by 15 min of recovery. Then trout were netted and anesthetized before blood collection and fish sacrifice for sample collection. The "high stocking" and the control groups were exposed to normal handling procedures, then netted and rapidly transferred into new tanks where they were deeply anesthetized. Once anesthetized, blood was individually collected and fish were immediately sacrificed. When sacrificed at mid-night, all the manipulations and sample collection were done under low intensity (< 0.4 lux) dim red light. In each group (n=20), blood was collected from 8 animals for plasma cortisol and melatonin quantifications, and their pineal organs were assayed for indole content and AANAT2

activity by HPLC. Addition pools (n=4/group) of pineal organs (n=3 organs/pool) were processed for mRNA quantification, with the remaining 12 fish from each group.

In a second experiment the effect of cortisol on pineal organ metabolism was evaluated at day and night. Four groups of trout were divided into two 120 L tanks each (n=20 animals/tank). Following acclimation, fish were anesthetized, weighed and intraperitoneally administered with slow-release coconut oil implants (control group included) following procedures previously described (Soengas et al., 1992). Another extra control group was not implanted controls but also injected. Implants consisted on coconut oil alone (10 $\mu g.g^{-1}$ body weight) in controls, or containing a dose of cortisol (50 $mg.kg^{-1}$ b. w.) according to previous studies in this species (Vijayan et al., 2003). After implanted, fish were placed back to their respective tank. Fish from each tank were sacrificed and sampled at 5-h or 48-h post-implant administration, at mid-day (the first tank of each group) or mid-night (the second tank). No mortality was observed during the experiment. Animals sacrificed at night were manipulated as above. Samples were processed as indicated (experiment 1).

A third experiment was performed to corroborate that pineal organ melatonin synthesis is inhibited by a specific action exerted by cortisol. Thus, individual pineal organs were taken out from fish and immediately placed in 96-well culture plates each containing 250 µl of modified Hanks' medium according to Yañez and Meissl (1995), but supplemented with 0.1 $mmol \cdot L^{-1}$ tryptophan. Assays were carried out under controlled temperature (16ºC) and in the presence or absence of light. After 3-h incubation the culture medium was removed and stored at -80ºC, and replaced with 250 µl modified Hanks' medium alone (control group) or containing cortisol (100 $ng \cdot mL^{-1}$), the general glucocorticoid receptors antagonist, mifepristone (RU486; 1.0 $\mu g \cdot mL^{-1}$), or RU486+cortisol (n=8 organs/group). After 3 hours, medium was removed and stored at -80ºC until assayed. Melatonin content was assessed on each medium fraction.

Hormones and metabolites quantification

Plasma cortisol levels were measured using a commercially available Enzyme Immunoassay Kit (Cayman, Ann Harbor, MI, USA), according to manufacturer´s indications. Plasma melatonin levels were assayed by HPLC with fluorimetrical detection as described (Muñoz et al, 2009). The resultant residue after the extraction procedure was dissolved in 100 µl mobile phase and filtered (0.5 µm filter). An aliquot

(50 µl) of the filtrate was injected into the HPLC system. Data from the analysis are expressed as pg·mL^{-1} of plasma.

Melatonin content in culture medium was assayed by directly injecting 20 µl from each collected fraction (*n*=8 per group) into the HPLC system. For pineal melatonin quantification, each organ (*n*=8 per group) was homogenized by ultrasonic disruption in 100 µl of 0.2 mol·L^{-1} phosphate buffer (pH 6.7) and centrifuged at 16,000 *g* for 10 min. A 60 µl aliquot was assayed for melatonin and pineal indole contents. The resulting 40 µl aliquot was immediately assayed for AANAT2 enzyme activity. From the 60 µl aliquot, pineal melatonin content was measured by direct injection of a 20 µl volume into the HPLC system, similarly to that described for plasma melatonin assessment. Other conditions were as previously described (Ceinos et al., 2008).

Pineal 5-HT and its acidic metabolite, 5-hydroxindoleacetic acid (5-HIAA) were assayed by HPLC with electrochemical detection from a 10 µl aliquot of the pineal homogenates as described by Ceinos et al. (2005).

AANAT2 enzyme activity was assayed by *in vitro* incubation of a 40 µl of the above mentioned sample homogenates with the substrate, 40 µl of 27 mmol·L^{-1} tryptamine and 40 µl of 1.0 mmol·L^{-1} acetyl-CoA (final concentrations in assay: 9 mmol·L^{-1} tryptamine and 0.5 mmol·L^{-1} acetyl-CoA). Assay conditions were as previously described and performed (Ceinos et al., 2008) with a modification consisting of a 60 min incubation period at 16°C (temperature within the optimal range for trout). 20 µl of the final solution were directly injected into the HPLC system in order to quantify the reaction product (*N*-acetyl tryptamine; NAT) formed. The system was a HPLC pump (Gilson M101) with an Ultrasphere Beckman column (3 µm particles, 75 mm and 4.6 mm i.d.) and a Jasco FP-1520 fluorescence detector set at 285/360 nm excitation/emission wavelengths. All analyses were performed at room temperature and 1 mL·min^{-1} flow rate. Sample peak areas were quantified relative to that of suitable standards.

Analysis of pineal organ *aanat2* mRNA abundance

After dissection, pineal organs from fish receiving the same treatment were pooled (*n*=3 organs/pool). Total mRNA was extracted from each pool (*n*=4 pools/group) using the TRIzol method (Gibco BRL, Gaithersburg, MD, USA) according

to manufacturer's instructions. The isolated RNA quality and quantity were spectrophotometrically determined. From each sample, 2 μg RNA were converted into cDNA as described (López-Patiño et al., 2011a). A negative control of each sample was assessed without reverse transcriptase in order to discard any genomic contamination.

Real-time quantitative RT-PCR (qPCR) was performed using a MaximaTM SYBR Green qPCR Master Mix (K0252, Fermentas, Burlington, ON, Canada) and a Bio-Rad (Hercules, CA, USA) MyIQ real-time PCR system. The primers and probes were based on previously reported sequences of rainbow trout genes and obtained from Sigma-Genosys (St Louis, MO, USA), including: *aanat2* (accession number AF106006.1) forward 5′-CATTCGTCTCTGTGTCTGGT-3′, reverse 5′-TTTCTGGGA TATGCTGGGT-3′; and *β-actin* (AJ438158) forward 5′-GATGGGCCAGAAAGACA GCTA-3′, reverse 5′-TCGTCCCAGTTGGTGACGAT-3′. Gene expression was normalized to that of *β-actin* for each sample. Relative mRNA expression was calculated according to the comparative ΔCt method. For each gene, samples collected at the same time point were processed in parallel and the expression was measured in triplicate.

Statistical analysis

Differences were evaluated by Two-Way ANOVA analyses of variance, with treatment and time of day as main factors. When a significant effect was identified within a factor, *post hoc* comparisons were carried out within that factor using a Student-Newman-Keuls test. Significance level was set at $P < 0.05$.

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804

805 **Figure legends**

806 **Figure 1.** Day-night variations of plasma (A) cortisol and (B) melatonin levels in
807 rainbow trout adapted to normal housing conditions or exposed to different stressors,
808 hypoxia, chasing and high stock density. Data represent the average ± S.E.M. of animals
809 sampled at either mid-day (*n*=8-10) or mid-night (*n*=8-10). * Significantly affected ($P <$
810 0.05) compared with day at the same treatment within the same group. Different letters
811 indicate significant differences ($P < 0.05$) among groups at the same time point.

812

813 **Figure 2.** (A) Melatonin content, (B) AANAT2 enzyme activity and (C) *aanat2* mRNA
814 abundance in the pineal organ of stressed or non-stressed trout sacrificed both at
815 mid-day or mid-night. Data represent the average ± S.E.M. of animals sampled at either
816 mid-day (*n*=8-10) or mid-night (*n*=8-10). For mRNA abundance measurements, each
817 value is the mean ± S.E.M. of 4 pools of pineal organs (*N*=3 pineals/pool) and data
818 show relative fold change to that measured in control group at day-time. * Significantly
819 affected ($P < 0.05$) compared with day at the same treatment within the same group.
820 Different letters indicate significant differences ($P < 0.05$) among groups at the same
821 time point.

822

823 **Figure 3.** Day-night variations in the content of (A) 5-HT, (B) 5-HIAA, and the ratios
824 (C) 5-HIAA/5-HT and (D) Mel/5-HT obtained from pineal organ of stressed or
825 non-stressed rainbow trout. Data represent the average ± S.E.M. of animals sampled at
826 either mid-day (*n*=8-10) or mid-night (*n*=8-10). * Significantly affected ($P < 0.05$)
827 compared with day at the same treatment within the same group. Different letters
828 indicate significant differences ($P < 0.05$) among groups at the same time point.

829

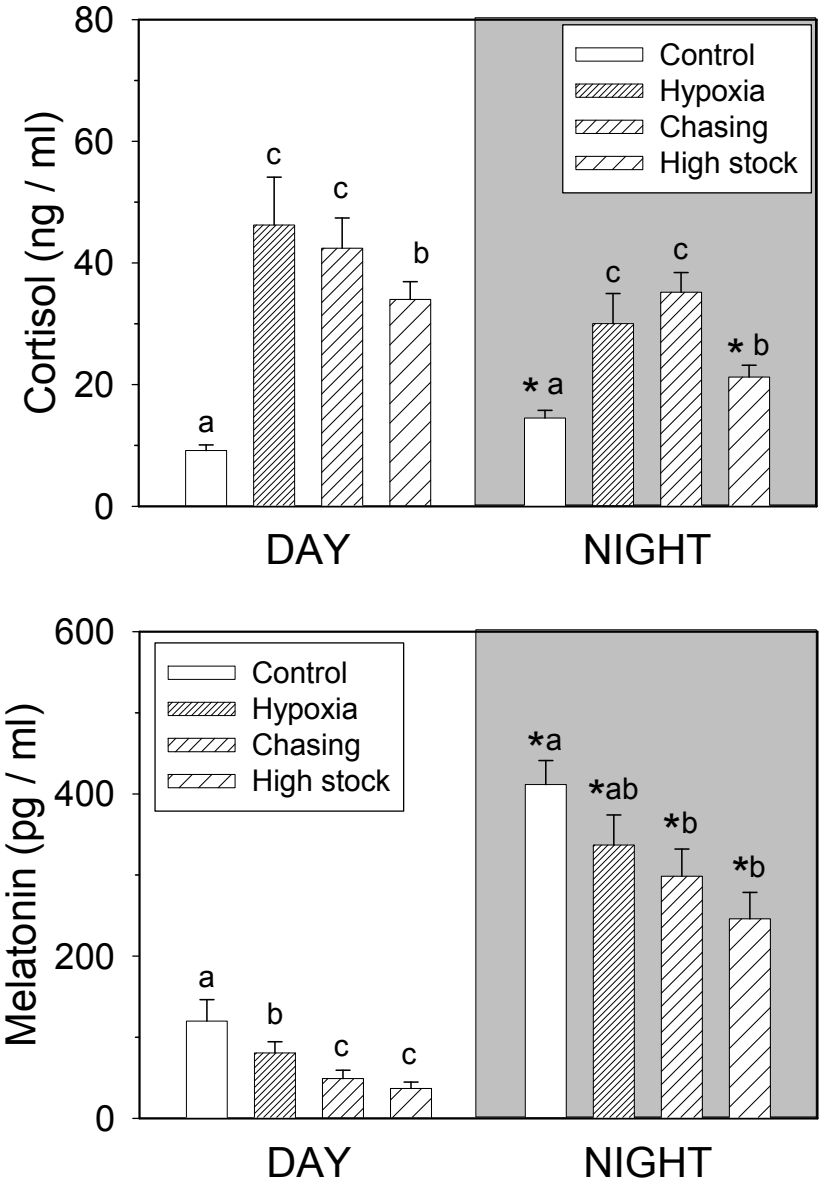
830 **Figure 4.** Day-night variations of plasma levels of (A) cortisol and (B) melatonin in
831 rainbow trout implanted with coconut oil alone or containing cortisol (50 $mg.kg^{-1}$ body
832 weight), and sampled after 5-h or 48-h post injection. Data represent the
833 average ± S.E.M. of animals sampled at either mid-day (*n*=8-10) or mid-night (*n*=8-10).
834 * Significantly affected ($P < 0.05$) compared with day at the same treatment within the
835 same group. Different letters indicate significant differences ($P < 0.05$) among groups at
836 the same time point.

Figure 5. Melatonin content, (B) AANAT2 enzyme activity and (C) *aanat2* mRNA abundance in the pineal organ of trout implanted with coconut oil alone or containing cortisol (50 $mg.kg^{-1}$ body weight), and sampled after 5-h or 48-h post injection. Data represent the average ± S.E.M. of animals sampled at either mid-day (*n*=8-10) or mid-night (*n*=8-10). For mRNA abundance measurements each value is the mean ± S.E.M. of 4 pools of pineal organs (*n* = 3 pineals/pool) and data represent the relative fold change to that measured in control group at day-time. * Significantly affected ($P < 0.05$) compared with day at the same treatment within the same group. Different letters indicate significant differences ($P < 0.05$) among groups at the same time point.

Figure 6. Day-night variations in the content of (A) 5-HT, (B) 5-HIAA, and the ratios (C) 5-HIAA/5-HT and (D) Mel/5-HT obtained from pineal organ of trout implanted with coconut oil alone or containing cortisol (50 $mg.kg^{-1}$ body weight), and sampled after 5-h or 48-h post injection. Data represent the average ± S.E.M. of animals sampled at either mid-day (*n*=8-10) or mid-night (*n*=8-10). * Significantly affected ($P < 0.05$) compared with day at the same treatment within the same group. Different letters indicate significant differences ($P < 0.05$) among groups at the same time point.

Figure 7. The inhibitory effect of cortisol on melatonin release by cultured pineal organ of rainbow trout is prevented by the glucocorticoid receptors general antagonist, RU486. After 3 hours of culture in the presence or absence of light (respectively: *N*=4 groups; *n*=8 pineals/group), drugs (1.0 µg $\cdot mL^{-1}$ RU486, 100 $ng \cdot mL^{-1}$ cortisol, or RU486+cortisol) or vehicle were added by replacing culture medium with fresh medium containing each drug. Incubations were pursued in light or dark for the following 3 hours. Melatonin content in culture medium is also provided (Before treatment) in order to demonstrate the absence of any difference in basal (Before treatment) melatonin release among groups. * Significantly affected ($P < 0.05$) compared to basal within the same group. Different letters indicate significant differences ($P < 0.05$) among groups at the same time point.

Figure 1.



852

853 **Figure 2.**

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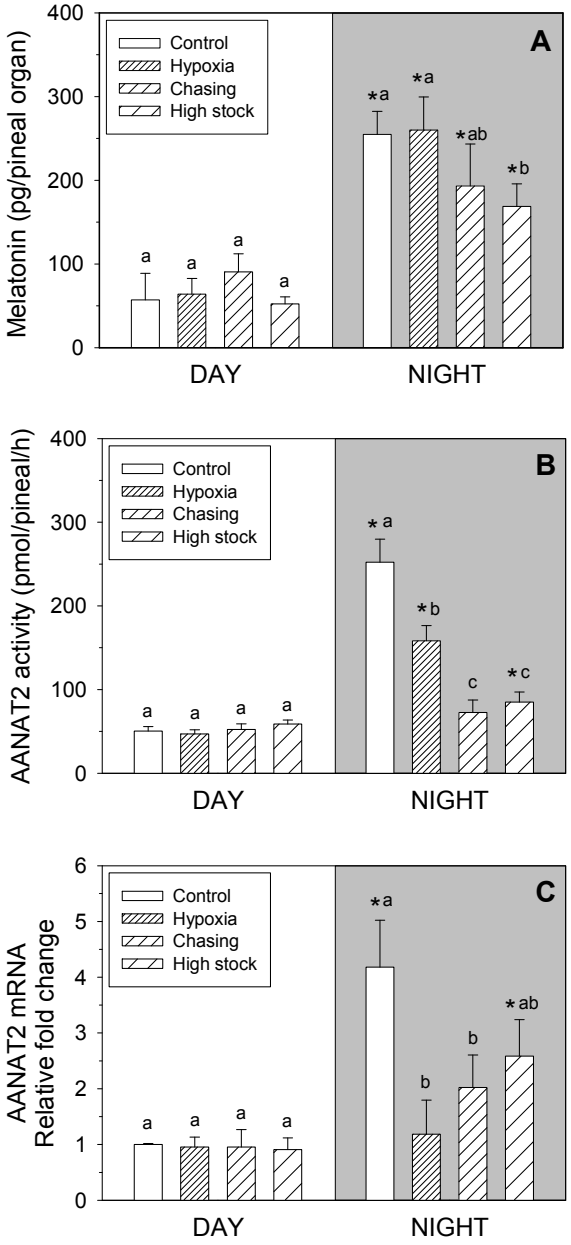

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Figure 3.

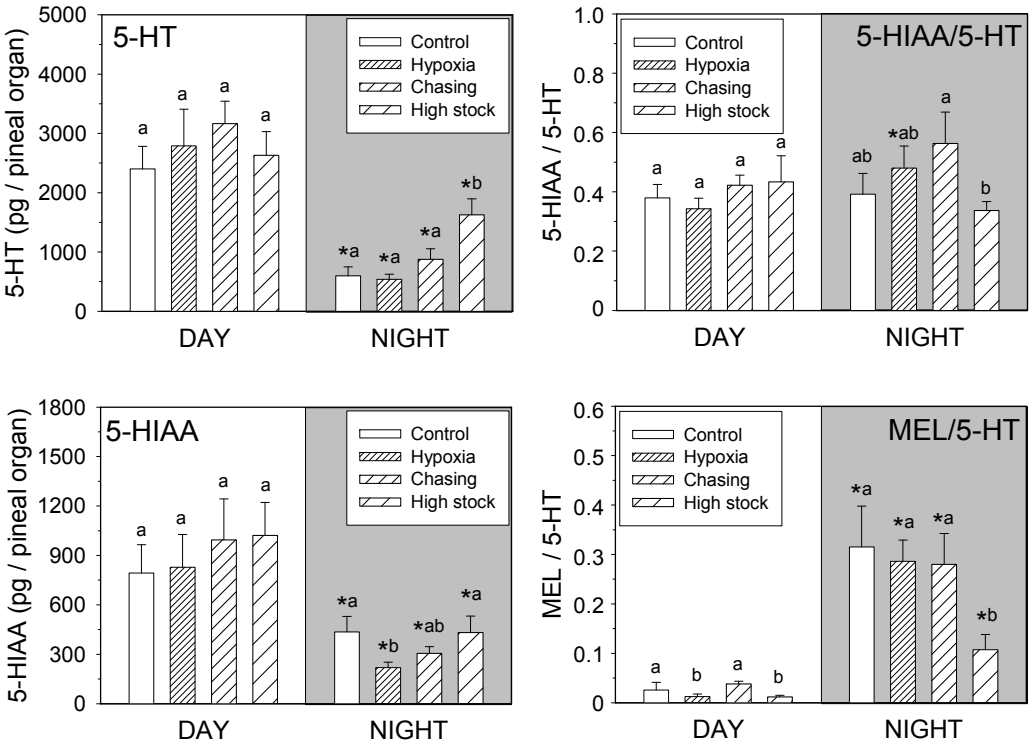

Figure 4.

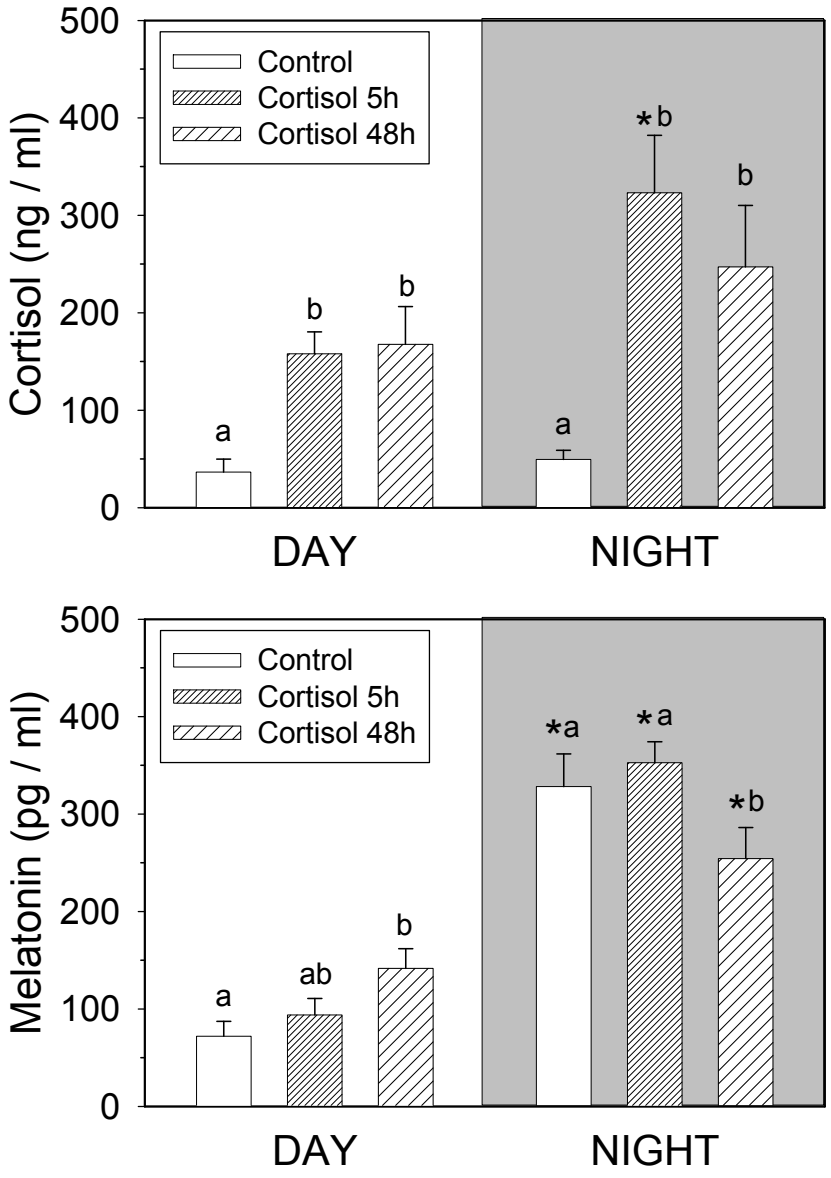

Figure 5.

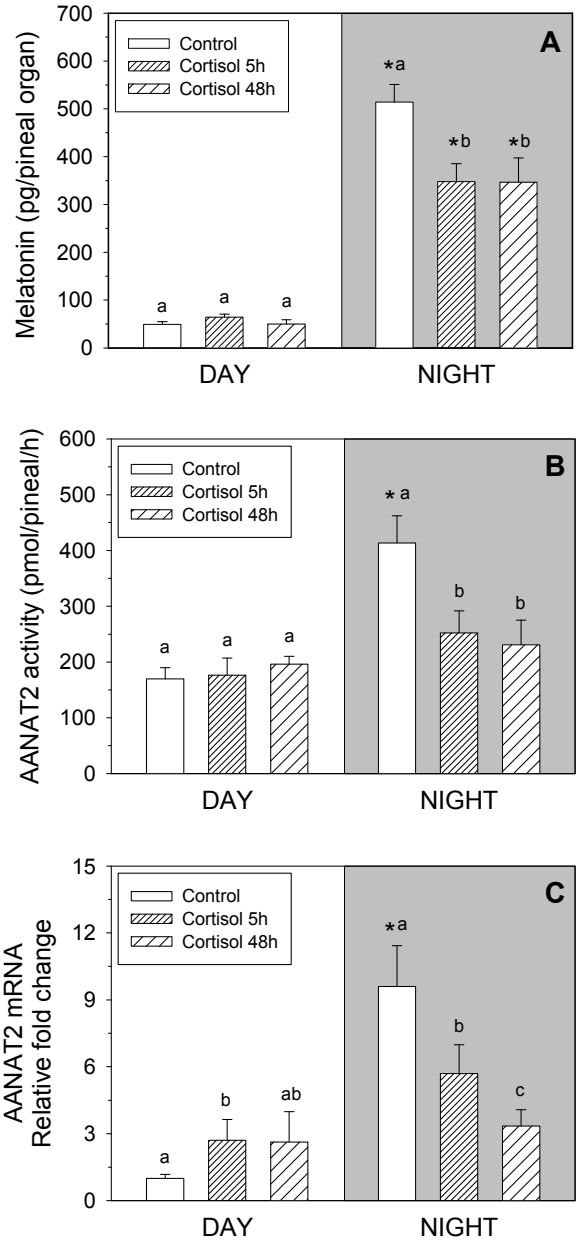

Figure 6.

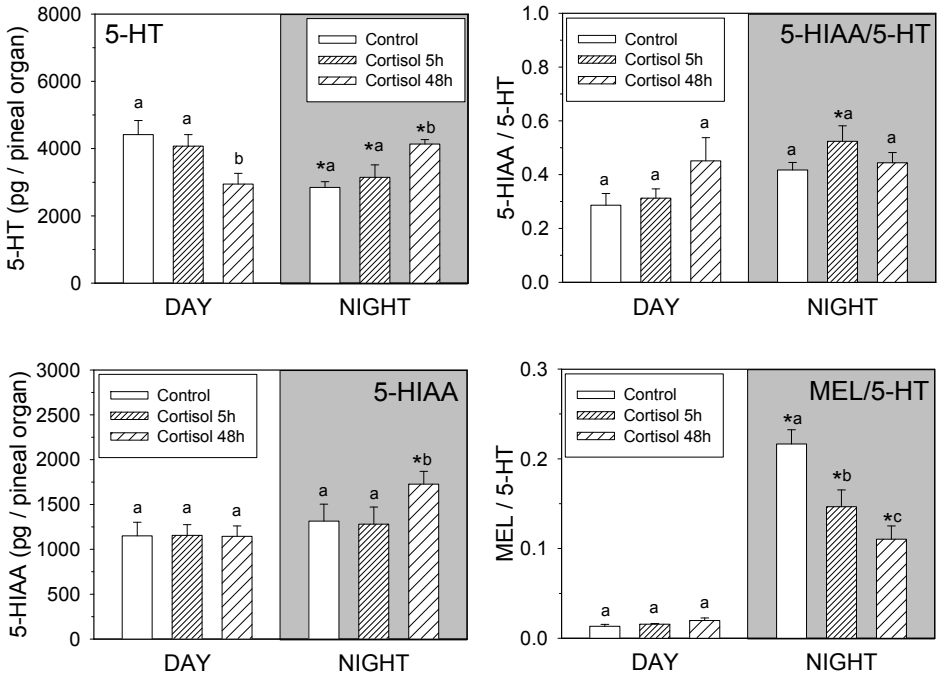

Figure 7.