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RESEARCH ARTICLE

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

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ABSTRACT

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 that cortisol is a mediator of such stress-induced effects, a set of animals were intraperitoneally implanted with coconut oil alone or containing cortisol (50 mg kg^{-1} body mass) and sampled 5 or 48 h post-injection at midday and midnight. 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). Stress (in particular chasing and high stocking density) affected the patterns of plasma and pineal organ melatonin content during both day and night, with the greatest reduction occurring 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 or 48 h. These data indicate that stress negatively influences the synthesis of melatonin in the pineal organ, thus attenuating the day–night variations of circulating melatonin. The effect might be mediated by increased cortisol, which binds to trout pineal organ-specific glucocorticoid receptors to modulate melatonin rhythms. Our results in cultured pineal organs support this. Considering the role of melatonin in 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.

KEY WORDS: Melatonin, Pineal organ, AANAT2, Cortisol, Rainbow trout, Stress

INTRODUCTION

In teleost fish, the pineal organ perceives and transduces the light–dark signal (Bromage et al., 2001) into neural and humoral signals, one of which is the hormone melatonin. Melatonin is rhythmically synthesized mainly from the pineal organ and released into the blood. Highest plasma levels occur at night and basal melatonin values occur during the day. 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 melatonin is released into the blood, its rhythmic profile conveys photic information to the organism (reviewed by 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 (Ekström 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 because the pineal organ hosts a true circadian light sensitive pacemaker which drives melatonin rhythms. Only salmonids, including rainbow trout, are 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) that 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). Roles have been suggested 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. It 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 teleost species such as 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). For rainbow trout, many studies have reported increased plasma cortisol at night, peaking before the light onset, then falling and remaining low during the day (Rance et al., 1982; Boujard and Leatherland, 1992). Such a daily profile is also influenced by feeding time (Boujard and Leatherland, 1992), and supports rhythmic cortisol secretion being synchronized by both photoperiod and feeding activity, with differences among seasons.

Based on findings that: (1) cortisol levels show daily variations; (2) there is interaction within several neurohumoral signals and melatonin production; (3) cortisol circulating levels increase right

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List of abbreviations

AANAT	arylalkylamine *N*-acetyltransferase
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)

after fish are exposed to stress (Wendelaar Bonga, 1997; Mommsen et al., 1999); and (4) glucocorticoids exert an inhibitory effect on AANAT activity of cultured pineal organs in trout (Benyassi et al., 2001; Yanthan and Gupta, 2007), we hypothesized that stress negatively affects melatonin synthesis in pineal organ of rainbow trout with cortisol mediating such inhibitory effect, in the same way as has been described for other teleost species such as tipalia, *Oreochromis mossambicus* (Nikaido et al., 2010), and that previously suggested for trout (Larson et al., 2004).

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 (5-HT; an immediate melatonin precursor) and its main oxidative metabolite, 5-hydroxyindoleacetic acid (5-HIAA), as well as AANAT2 enzyme activity and mRNA abundance in fish kept under normal housing conditions or exposed to different stressors or having received 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 on plasma cortisol and melatonin content of exposing trout to different stressors (hypoxia, chasing and high stocking density; see Materials and methods) is shown in Fig. 1. Plasma cortisol varied significantly (P=0.004) in the day and night in control fish (higher levels observed at night) and in fish under high stocking density (lower levels at night). Exposing animals to different stress conditions significantly increased cortisol levels at both midday and midnight, relative to the respective control non-stressed group. The increase of cortisol levels was greater in animals exposed to acute stress, i.e. hypoxia and chasing.

Melatonin levels in the 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 around midday (P=0.047; P=0.006 and P=0.012 for hypoxia, chasing and high stocking, compared with controls), whereas around midnight there was a decrease in melatonin in the chasing and high stocking density groups (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 the pineal organ of trout exposed to different stressors are shown in Fig. 2. Similarly to that found for plasma melatonin, a day–night variation of melatonin content in the pineal organ was observed, with higher values occurring at night (P<0.001 relative to daytime). No effect of stress was noticed at midday, whereas melatonin levels significantly decreased at midnight in fish exposed to high stocking density (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 midnight. Stress did not affect AANAT2 activity during the day, but significantly decreased it at night (P=0.002, P<0.001 and 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.

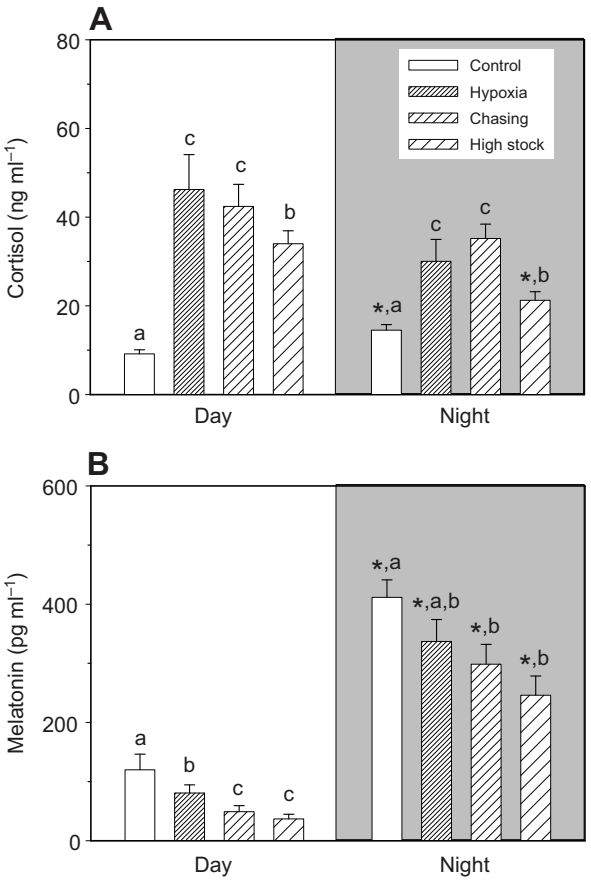

Fig. 1. Day–night variations of plasma cortisol and melatonin in trout subjected to various stressors. (A) Cortisol and (B) melatonin levels in rainbow trout adapted to normal housing conditions or exposed to different stressors: hypoxia, chasing and high stock density. Animals were sampled at either midday (N=8–10) or midnight (N=8–10). Data are means ± s.e.m. *Significantly affected (P<0.05) compared with the day values for the same treatment within the same group. Different letters indicate significant differences (P<0.05) between groups at the same time point.

A significant day–night variation of *aanat2* mRNA abundance was observed in the pineal organ of control fish and those exposed to high stocking density, with higher values at midnight (P=0.002 and P=0.029, respectively). Decreased nocturnal *aanat2* mRNA abundance, relative to the control group, was found in fish exposed to acute stress (hypoxia and chasing), such that the day–night variation of *aanat2* expression disappeared in both groups.

5-HT and 5-HIAA content in the pineal organ of stressed trout

Day–night variations of 5-HT and its main metabolite, 5-HIAA, and the ratios of 5-HIAA to 5-HT and melatonin to 5-HT are shown in Fig. 3. Serotonin content in the pineal organ was significantly lower at midnight in all the experimental groups. Stress did not significantly affect 5-HT levels during the day, whereas a significant increase was observed at midnight, only in fish exposed to high stocking (P=0.001, P=0.002 and P=0.008, relative to the other groups).

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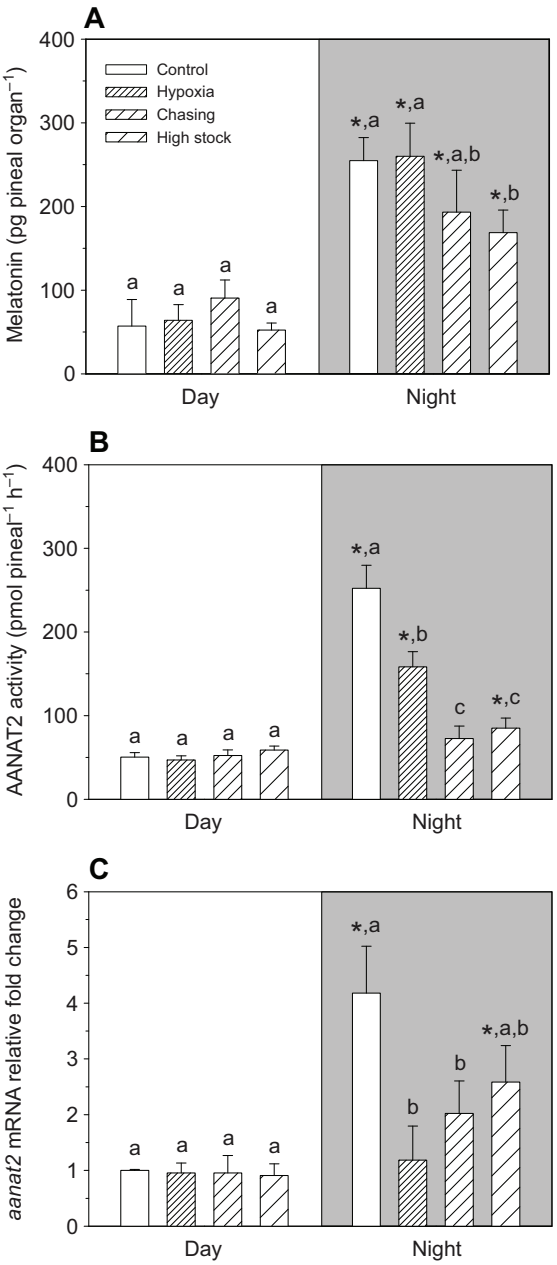

Fig. 2. Melatonin content, AANAT2 enzyme activity and *aanat2* mRNA abundance in the pineal organs of stressed or non-stressed trout at midday or midnight. (A) Melatonin content, (B) AANAT2 enzyme activity and (C) *aanat2* mRNA. Data are means ± s.e.m. of samples taken at either midday (*N*=8–10) or midnight (*N*=8–10). For mRNA abundance measurements, each value is the mean ± s.e.m. of four pools of pineal organs (*n*=3 pineals/pool) and data show relative fold change to that measured in control group during the day. *Significantly affected (*P*<0.05) compared with daytime values for the same treatment within the same group. Different letters indicate significant differences (*P*<0.05) among groups at the same time point.

Similarly to that described for serotonin, 5-HIAA day–night changes were found in all the experimental groups, with significantly higher levels at midday. During the night, a significant decrease of 5-HIAA content was found in trout exposed to hypoxia, relative to that of control (*P*=0.034) and high stocking groups at this time period.

The 5-HIAA/5-HT ratio only showed day–night variations in the hypoxia-exposed group, and was higher at night (*P*=0.040). In addition, exposing fish to high stocking density tended to decrease the 5-HIAA/5-HT ratio at night, but this effect did not reach significance when compared with the control at night.

A clear day–night variation of the melatonin/5-HT ratio was found in all the experimental groups, with higher values occurring at night. During the day, hypoxia and high stocking density significantly decreased the melatonin/5-HT ratio, compared with control and chasing groups. In contrast, only the high stocking density significantly decreased the ratio at midnight, relative to control, hypoxia and chasing groups.

Plasma cortisol and melatonin levels after cortisol intraperitoneal administration

Plasma cortisol and melatonin levels were measured after intraperitoneal administration of cortisol in coconut oil or coconut oil alone (Fig. 4). Data obtained from the control non-implanted group are not shown but remained quite similar to those of the control implanted fish. Although no significant day–night variation of plasma cortisol levels was found in the control group, a tendency to higher nocturnal hormone levels persisted (*P*=0.082) in the same way as that observed in the previous experiment (see Fig. 1). As expected, a significant increase of cortisol levels was found in trout sampled 5 h or 48 h after the intraperitoneal administration, compared with the control group at both times. The only significant day–night variation was found at 5 h after cortisol injection, with higher levels at night, relative to the same treatment group during the day.

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

Melatonin content, AANAT2 activity and mRNA abundance in the pineal organ of cortisol-implanted trout

Fig. 5 shows melatonin content, AANAT2 enzyme activity and mRNA abundance in the pineal organ of implanted trout. Data for non-implanted fish (not shown) were consistent with those taken from 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 midnight. The intraperitoneal 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 midnight. 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 midday.

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

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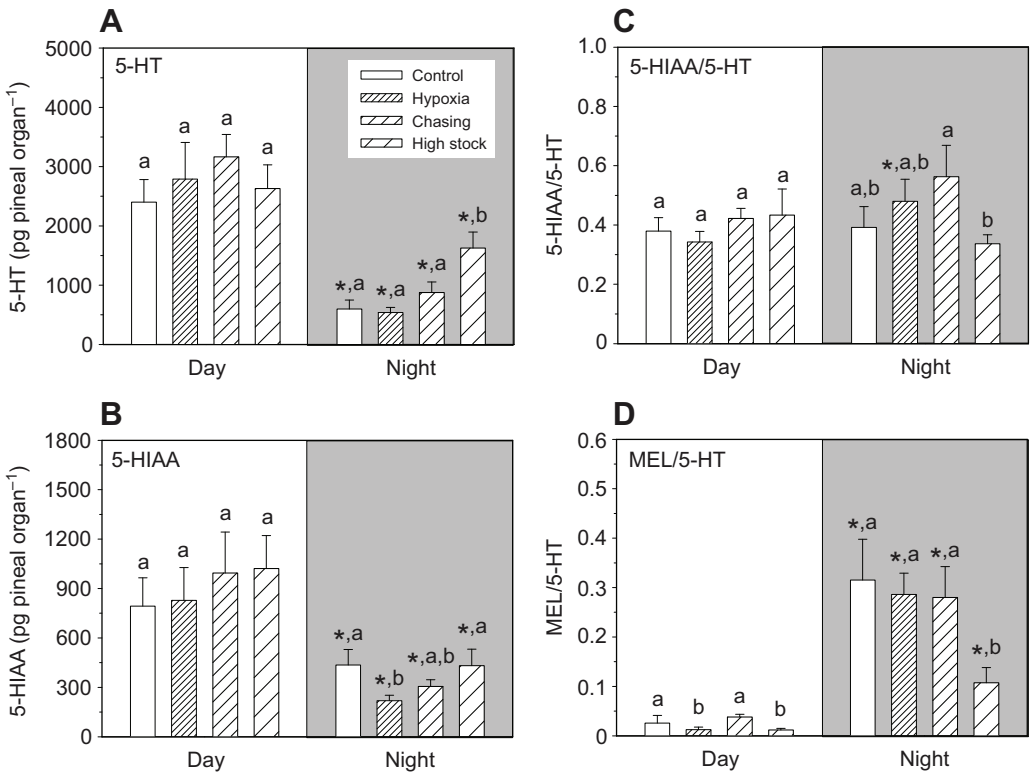

Fig. 3. Day–night variations in 5-HT and 5-HIAA in the pineal organs of stressed or non-stressed trout. (A–D) 5-HT (A) and 5-HIAA (B) levels, and the ratios of (C) 5-HIAA/5-HT and (D) Mel/5-HT obtained from pineal organ of stressed and non-stressed rainbow trout. Data are means ± s.e.m. for animals sampled at midday (N=8–10) or midnight (N=8–10). *Significantly affected (P<0.05) compared with daytime values for the same treatment within the same group. Different letters indicate significant differences (P<0.05) between groups at the same time point.

on mRNA expression did lead to the disappearance of the day–night variation in *aanat2* expression in both cortisol-implanted groups.

5-HT and 5-HIAA content in the pineal organ of implanted animals

Fig. 6 shows the daily variation of serotonin and 5-HIAA levels, and the 5-HIAA/5-HT and melatonin/5-HT ratios. The control group showed a significant day–night variation in 5-HT content in the pineal organ, with higher levels at midday. Whereas cortisol administration for 5 h 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 daytime sample) and increased the nocturnal 5-HT content (P=0.005 and P=0.011 relative to control and 5 h daytime sample). Thus, the pineal 5-HT content was higher at night and lower during the day, which was the opposite profile than that observed in the control.

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

The 5-HIAA/5-HT ratio did not significantly change within groups during the day and the night. Day–night significant variations of the ratio were found only in trout intraperitoneally administered 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 melatonin/5-HT ratio, with higher values at midnight. Cortisol administration significantly decreased the ratio only at night, compared with control group (P<0.001 and P=0.007 for 5 and 48 h relative to control), with the more important effect being observed after 48 h. No such effect was found during the day, at both 5 and 48 h post-injection.

Effect of cortisol treatment on melatonin production *in vitro*

Melatonin production in cultured pineal organs in the presence or absence of light was compared among experimental groups (Fig. 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 in both light and dark. However, the addition of medium containing cortisol resulted in a significant decrease in melatonin production in darkness, relative to the dark control (P=0.037) and to that observed before cortisol addition within the same group. This inhibitory effect of cortisol was prevented by the cortisol antagonist 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, that mimic those potentially stressful 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 axis and the hypothalamus–pituitary–interrenal tissue axis, followed by a rapid increase in catecholamine and cortisol levels in plasma, which 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 in the trout pineal organ, but previous studies have not found any effect of catecholamines in this tissue location, in contrast to that in other teleosts for which a regulatory role has been proposed (Falcón et al., 1991). 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 a mediator of such an effect.

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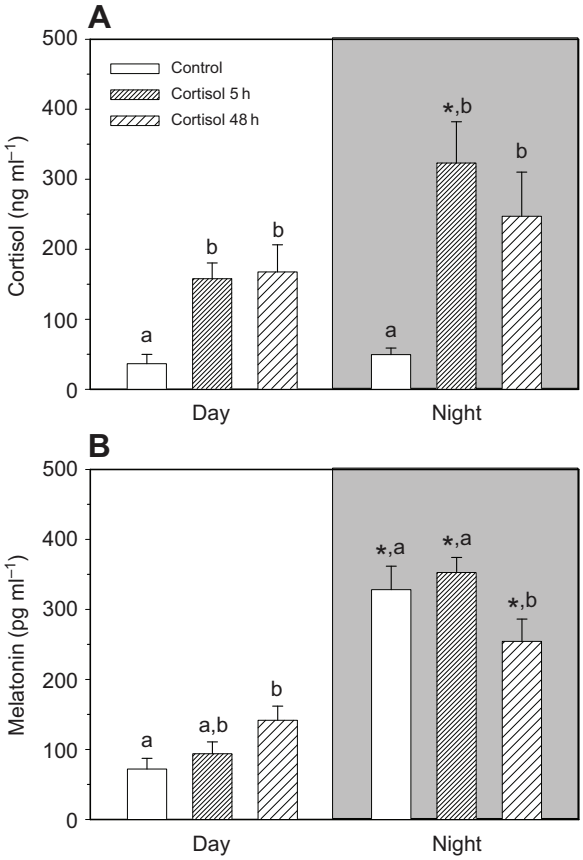

Fig. 4. Day–night variations of plasma levels of cortisol and melatonin in trout implanted with additional cortisol. (A) Cortisol and (B) melatonin levels in rainbow trout implanted with coconut oil alone or containing cortisol (50 mg kg^{-1} body mass), and sampled 5 or 48 h post-injection. Data are means ± s.e.m. for animals sampled at either midday (N=8–10) or midnight (N=8–10). *Significantly affected (P<0.05) compared with daytime values for the same treatment within the same group. Different letters indicate significant differences (P<0.05) between groups at the same time point.

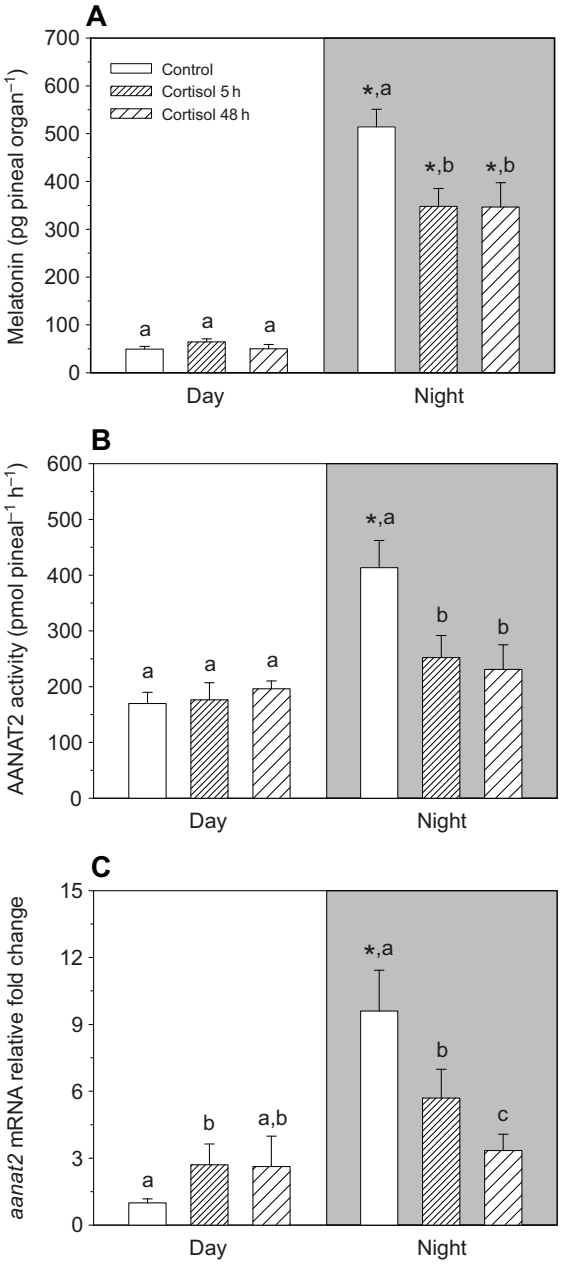

Fig. 5. Melatonin content, AANAT2 enzyme activity and *aanat2* mRNA abundance in the pineal organ of trout implanted with additional cortisol. (A) Melatonin content, (B) AANAT2 enzyme activity and (C) *aanat2* mRNA abundance in trout implanted with coconut oil alone or containing cortisol (50 mg kg^{-1} body mass), and sampled 5 or 48 h post-injection. Data are means ± s.e.m. for animals sampled at either midday (N=8–10) or midnight (N=8–10). For mRNA abundance each value is the mean ± s.e.m. of four pools of pineal organs (n=3 pineals/pool) and is the relative fold change to that in the control group at midday. *Significantly affected (P<0.05) compared with daytime values for the same treatment within the same group. Different letters indicate significant differences (P<0.05) between groups at the same time point.

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 apparent (experiment 2) day–night variations of plasma cortisol with night values being higher than those measured during the day, in agreement 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 they were lower in the high-density group in particular during the night. The increase in cortisol was higher in those fish stressed during the day compared with the same groups at night, independent 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 suggests that a relationship between behavioral components and the response to stress might exist. Further research should be carried out.

Plasma melatonin levels were negatively affected by stress, in that the highest reduction of day–night variation of hormone levels was observed in fish exposed to long-term stress (high stocking density), rather than those acutely stressed (chasing, hypoxia), which in fact also showed decreased plasma melatonin levels, whereas pineal organ melatonin levels remained unaltered. This

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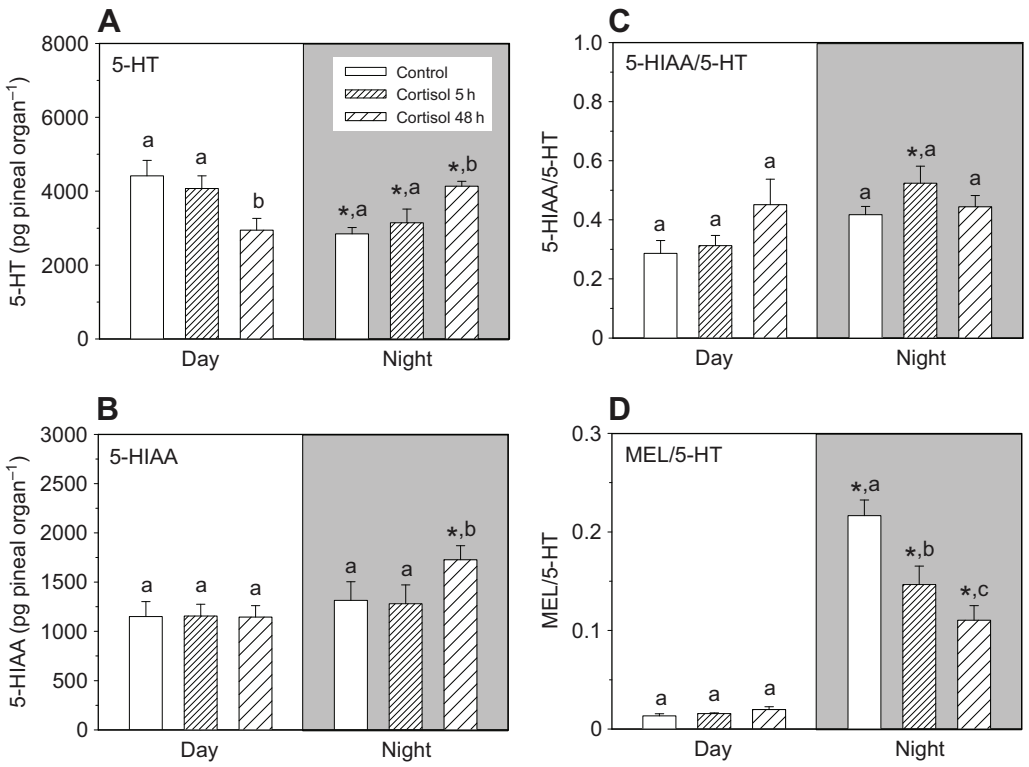

Fig. 6. Day–night variations in 5-HT and 5-HIAA in the pineal organs of trout implanted with additional cortisol. (A–D) 5-HT (A), 5-HIAA (B), and the ratios of 5-HIAA/5-HT (C) and Mel/5-HT (D) obtained from pineal organ of trout implanted with coconut oil alone or containing cortisol (50 mg kg^{-1} body mass), and sampled 5 or 48 h post-injection. Data are means ± s.e.m. for animals sampled at either midday (*N*=8–10) or midnight (*N*=8–10). For mRNA abundance, each value is the mean ± s.e.m. of animals sampled at either midday (*N*=8–10) or midnight (*N*=8–10). *Significantly affected (*P*<0.05) compared with daytime values for the same treatment within the same group. Different letters indicate significant differences (*P*<0.05) between groups at the same time point.

indicates that time periods longer than 1–5 min (of hypoxia and chasing) might be required for a significant reduction in melatonin content to be observed in the pineal organ. In contrast, changes affecting AANAT2 enzyme activity and mRNA abundance occur immediately, as our results indicate. However, one might hypothesize a correlation between stress duration and the magnitude of the inhibition of the nighttime melatonin production in the pineal organ, which is supported by the existence of similar effects in both pineal organ and plasma melatonin content. In contrast, daytime changes in pineal melatonin after any stress were minor, reflecting the reduced levels of the hormone in blood of stressed trout during the day, which 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, which have been suggested to contribute to blood melatonin levels during the day (Lepage et al., 2005; Muñoz et al., 2009). Further research is needed to clarify this.

The effect of stress on the day–night melatonin secretion pattern has been studied in several fish species with contradictory results. Higher nighttime melatonin and cortisol levels in socially subordinated rainbow trout relative to the dominant fish have been reported previously (Larson et al., 2004). Similar results were observed in our laboratory in trout exposed to increased salinity (López-Patiño et al., 2011b), but this 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 also been described for rainbow trout in which fasting decreases pineal organ melatonin synthesis at night (Ceinos et al., 2008), and disturbance stress negatively affects several parameters including melatonin (Kulczykowska, 2001), which is consistent with our data herein reported for trout exposed to different stressors. Because different species appear to respond specifically to any stressor, we might

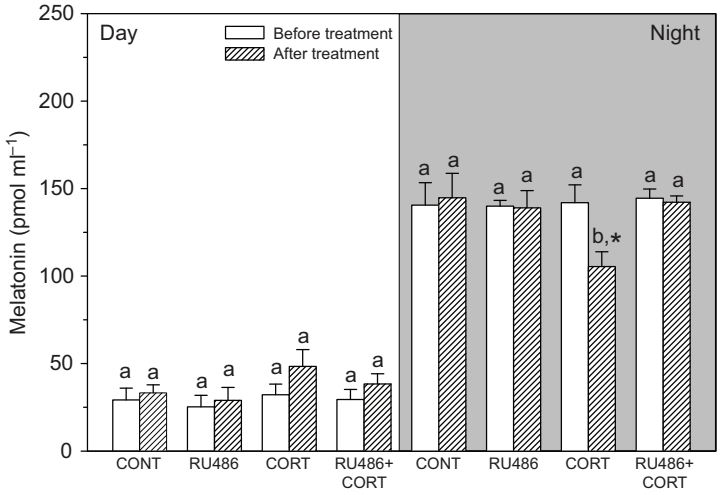

Fig. 7. The inhibitory effect of cortisol on melatonin release by cultured pineal organs of rainbow trout is prevented by the glucocorticoid receptor general antagonist, RU486. After 3 h in culture in the presence or absence of light (respectively: *N*=4 groups; *n*=8 pineals/group), drugs (1.0 µg ml^{-1}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

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speculate that there is a species-specific effect of stress on melatonin synthesis in the pineal organ. Bearing in mind that: (1) pineal melatonin synthesis is differentially regulated in teleosts in relation to the environmental signals, i.e. light–dark cycle (the trout melatonin rhythm-generating system seems to lack a functional clock, in contrast to that of most non-salmonids); and (2) the different social behaviors and physiological adaptations to the aquatic environments in which the fish live, it is probably not surprising that there are species-specific responses 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.

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 mainly responsible for 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). 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 is needed. Thus according to our previous data (López-Patiño et al., 2011b) and that describing a light effect (Ceinos et al., 2005), 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 because of decreased AANAT2 enzyme activity and expression, leading to decreased melatonin levels and intracellular accumulation of 5-HT, which is oxidized to 5-HIAA.

A previous *in vitro* study on the hormonal regulation of fish pineal melatonin synthesis revealed the presence of glucocorticoid receptors in trout pineal organ, and when assayed *in vitro* with the glucocorticoid analogue dexamethasone, decreased AANAT2 activity was observed (Benyassi et al., 2001), which is consistent with our results from the pineal organ *in vitro* assay showing decreased melatonin release in pineal organs exposed to cortisol in darkness. Also, previous research found decreased AANAT activity in cultured pineal organ of the North African catfish (*Clarias gariepinus*) after millimolar corticoid treatment, an effect similar to that reported recently in cultured pineal organs of tilapia with nanomolar cortisol concentrations (Nikaido et al., 2010; Yanthan and Gupta, 2007). Therefore, a glucocorticoid regulation of pineal melatonin production is possible under physiological conditions, and this might occur when cortisol levels are elevated as a result of stress. 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 h increase plasma melatonin levels during the day, whereas both plasma (at 48 h) and pineal melatonin (5 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 that increased cortisol differentially affects the photoreceptor cell properties during the 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 to 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 this, we also observed that trout receiving cortisol implants for 48 h had 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 midday, which suggests that those variations observed during the night might be mainly due to decreased 5-HT utilization through the *N*-acetylation pathway to synthesize melatonin during 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 mainly responsible for 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 stressed, cortisol-implanted trout and cultured pineal organs tended to decrease the day–night variation of both pineal and plasma melatonin levels. It is likely that the 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 in turn activate glucocorticoid-responsive elements at the AANAT2 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 support 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 the cortisol profile exhibits species-specific daily and seasonal patterns affected by several factors such as photoperiod (Pickering and Pottinger, 1983) or feeding time (Boujard and Leatherland, 1992), among others. In some species, for example common dentex, daily fluctuations of plasma cortisol levels show endogenous rhythmic characteristics (Pavlidis et al., 1999). This hormone is thought to be important in the 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 signal that controls melatonin synthesis, one might not discard the possibility that daily changes in cortisol 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, when pineal melatonin synthesis has decreased (Ceinos et al., 2005). In contrast, 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

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Senegalese sole (López-Patiño et al., 2013). This hormonal correlation is in support of our data in trout showing an inverse relationship between high cortisol levels and melatonin levels in stressed, cortisol-implanted fish and in cultured pineal organs. Therefore, further investigation into fluctuations in cortisol and its interaction with melatonin is likely to increase our understanding of 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 an inhibitory effect exerted by cortisol on melatonin synthesis in the pineal organ of rainbow trout. This steroid probably 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 on pineal rhythms, in particular those related to melatonin synthesis. Considering the pivotal role of melatonin in synchronizing rhythmic physiological events to cyclical environmental changes (mainly the light–dark cycle), the effect of stress on melatonin synthesis might jeopardize the response of the animal to such fluctuations and in consequence, to compromise its physiological integration.

MATERIALS AND METHODS

Animals

Immature rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792); 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 h:12 h light:dark photoperiod (lights on at 08:00 h, 400 lx intensity at the water surface), 14±1°C 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^{-1}) 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 implements 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 9000 ***g*** 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 the tanks containing the 'high stocking density' group until a stressful high density was reached (70 kg fish mass m^{-3}), where fish remained for 4 days before being killed. Stocking density conditions remained unaltered for the other groups (10 kg fish mass m^{-3}). On the day of sampling, animals from the other stressed groups were exposed to different manipulations and then killed at midday or midnight as follows. Fish from the 'hypoxia' group were normally handled and netted but remained in the net for 60 s before being deeply anesthetized in a MS-222 solution, blood sampled and killed. The 'chasing' group was subjected to a standardized handling disturbance consisting on 5 min of repeated chasing around the tank followed by 15 min recovery. Then trout were netted and anesthetized before blood collection and killing. 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 killed. All the manipulations and sample collections at midnight were done under low intensity (<0.4 lx) dim red light. In each group (n=20), blood was collected from eight animals for plasma cortisol and melatonin quantifications, and their pineal organs were assayed for indole content and AANAT2 activity by high-pressure liquid chromatography (HPLC). Additionally, pools (N=4 per group) of pineal organs (n=3 organs per pool) from the remaining 12 fish in each group were processed for mRNA quantification.

In a second experiment, the effect of cortisol on pineal organ metabolism was evaluated during the day and night. Four groups of trout were divided between two 120 l tanks (20 animals per 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). An additional control group was not implanted, but was subjected to the same manipulation (sham-implanted). Implants consisted of coconut oil alone (10 µg g^{-1} body mass) in controls, or the oil plus cortisol (50 mg kg^{-1} body mass) according to a previous study in this species (Vijayan et al., 2003). After implantation, fish were placed back into their respective tanks. Fish from each tank were killed and sampled at 5 h or 48 h post-implantation, at midday (the first tank of each group) or midnight (the second tank). No mortality was observed during the experiment. Animals killed 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 cortisol. Thus, individual pineal organs were removed from fish and immediately placed in 96-well culture plates each containing 250 µl modified Hanks' medium, according to Yañez and Meissl (Yañez and Meissl, 1995), but supplemented with 0.1 mmol l^{-1} tryptophan. Assays were performed under controlled temperature (16°C) and in the presence or absence of light. After 3 h incubation the culture medium was removed and stored at −80°C, and replaced with 250 µl modified Hanks' medium alone (control group) or containing cortisol (100 ng ml^{-1}), the general glucocorticoid receptor antagonist, mifepristone (RU486; 1.0 µg ml^{-1}), or RU486+cortisol (n=8 organs per group). After 3 h, medium was removed and stored at −80°C until assayed. Melatonin content was assessed on each medium fraction.

Hormone and metabolite quantification

Plasma cortisol levels were measured using a commercially available Enzyme Immunoassay Kit (Cayman, Ann Harbor, MI, USA), according to the manufacturer's instructions. Plasma melatonin levels were assayed by HPLC with fluorimetrical detection as described previously (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} plasma.

Melatonin content in the 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 content. 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. (Ceinos et al., 2005).

AANAT2 enzyme activity was assayed by *in vitro* incubation of 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). A 20 µl sample 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 and 4.6 mm internal diameter) 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 per pool). Total mRNA was extracted from each pool (n=4 pools per 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 previously (López-Patiño et al., 2011a). A negative control for each sample was assessed without reverse transcriptase in order to be able to disregard 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′-TTTCTGGGATATGCTGGGT-3′; and *β-actin* (AJ438158) forward 5′-GATGGGCCAGAAAGACAGCTA-3′, reverse 5′-TCGTCCCAGTTGGT-GACGAT-3′. Gene expression was normalized to that of *β-actin* for each sample. Relative mRNA expression was calculated according to the comparative ΔC_t 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, 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. The significance level was set at P<0.05.

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Competing interests

The authors declare no competing financial interests.

Author contributions

Conception and experiment design: M.A.L.-P., J.L.S. and J.M.M. Execution and analysis: M.A.L.-P., M.C.-S. and M.G. Data interpretation: M.A.L.-P., M.C.-S., M.G., J.L.S. and J.M.M. Writing and revisions: M.A.L.-P., M.G., J.L.S. and J.M.M.

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