

## **The response of brain serotonergic and dopaminergic systems to an acute stressor in rainbow trout: a time-course study**

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### **Summary**

The brain monoaminergic neurotransmitter systems are known to be involved in the integrated response to stress in vertebrates. However, the present knowledge about the timing of their actions as well as their specific roles in the regulation of the endocrine axes that drive the stress response is incomplete. This is partially because of the complexity of the reciprocal interactions among the monoaminergic systems and other biochemical actors of the stress response such as CRF, AVT, ACTH or corticosteroids. In this study, we show for the first time in teleost fish, the short- and mid-term time-course of the response of the forebrain serotonergic and dopaminergic activities after the exposure to an acute stressor in rainbow trout. Other stress markers like the plasma levels of cortisol, glucose and lactate were also monitored, providing a context to precisely locate the monoaminergic activation within the fish acute stress response. Our results show that the acute stress induced a rapid increase in the forebrain serotonergic activity, which became elevated after only 15 seconds of chasing. Several hours after stress, the serotonergic activity recovered its basal levels, in parallel to the recovery of other stress markers such as plasma catecholamines and cortisol. The dopaminergic activity was also increased after stress, but only in the telencephalon and only after 20 minutes post-stress. The increase in serotonergic activity happened before the elevation of plasma catecholamines, suggesting that this monoamine system could have a key role in triggering the initial steps of the activation of not only the hypothalamus-pituitary-interrenal axis, but also the brain-sympathetic-chromaffin axis in fish.

Key index words: stress, catecholamines, dynamics, serotonin, dopamine, trout.

Short title: Central monoamines after stress in trout.

## Introduction

When exposed to a stressor, animals respond with a complex series of behavioral and biochemical mechanisms at different levels in order to be prepared to cope with any potential threat (Barton, 2002). The vertebrate stress response is initiated by the activation of several biochemical pathways leading to the increase of plasma levels of catecholamines and corticosteroids, which are known as the primary responses to the stress stimulus. The catecholamines and the corticosteroids are the respective main end-products of the two major pathways coordinating the stress response in mammals: The brain-sympathetic-adrenal medulla axis and the hypothalamus-pituitary-adrenal (HPA) axis. In teleost fish, the brain-sympathetic-chromaffin cells (BSC) and the hypothalamus-pituitary-interrenal cells (HPI) axes are equivalent to those mammalian systems (Wendelaar Bonga, 1997).

Several biochemical actors participate in the normal function and regulation of these axes in fish, including, among others, the adrenocorticotrophic hormone (ACTH) (the main secretagogue of corticosteroids), the corticotropin-releasing factor (CRF) (the major regulator of ACTH secretion by the hypothalamus), the hypophyseal nonapeptide arginine vasotocin (AVT) and the brain monoaminergic neurotransmitters, including the catecholamines dopamine (DA) and noradrenaline (NA) and the indoleamine serotonin (5HT) (Balment, 2006; Wendelaar Bonga, 1997; Winberg and Nilsson, 1993). The role of most of those elements is well known, whereas the function of some others, such as the brain monoaminergic neurotransmitters is not yet clear. For instance, the activity of central monoaminergic systems increased after exposure to different types of stressors, such as handling, isolation, predator exposure, pollutant exposure or crowding (Gesto et al., 2008; Gesto et al., 2009; Schjolden et al., 2006; Weber et al., 2012; Winberg and Nilsson, 1993), with the response of the serotonergic activity being especially consistent. However, the causes and consequences of those increased activities within the stress response along with the dynamics of the monoaminergic response to stress are not well known. In this regard, the brain serotonergic system shows complex reciprocal interactions with the HPI axis, affecting and being affected by other elements of the stress response (Chaouloff, 2000; Chaouloff et al., 1999; Heisler et al., 2007; Pottinger, 2008; Winberg et al., 1997). In fact, it seems that the serotonergic system could have a dual role in the stress response, acting as an early signal during the initial steps of the response but also as a late response in situations of maintained stress. For instance, high

and sustained levels of serotonergic activity are considered as output signals in chronic stress situations (Browne et al., 2011; Øverli et al., 2007; Summers et al., 2003). On the other hand, very rapid increases in serotonergic activity have been observed in lizards after exposure to a stressor (Emerson et al., 2000; Matter et al., 1998) suggesting that the enhanced serotonergic neurotransmission could also act as an early signal during the initial steps of the stress response or during the recognition of the stressor by the central nervous system.

Although brain monoaminergic systems are activated by stressors, the temporal sequence of activation of brain monoaminergic activity within the first moments of the stress response is still unknown. Therefore, we carried out the present study to try to obtain a clear picture of the sequential changes in several stress biomarkers during the acute stress response in fish. We paid special attention to the brain monoaminergic response and its temporal relationship with other widely used stress markers such as plasma levels of catecholamines, cortisol, glucose, and lactate.

## Materials and methods

### Animals

Rainbow trout (*Oncorhynchus mykiss*) juveniles were obtained from a local farm (A Estrada, Pontevedra, Spain) and transported to the facilities of the Faculty of Biology of the University of Vigo (Spain). Fish were acclimated for at least two weeks before the experiments in well-aerated 100 L tanks, with continuous freshwater supply at a stocking density of 20 kg fish·m<sup>-3</sup>. The tanks were maintained under controlled photoperiod (12:12 day:night) and temperature (13-15°C). Fish were daily fed (1% body mass) commercial dry pellets (Dibaq-Diprotg S.A., Segovia, Spain). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 55/2013) for the use of animals in research.

### Experimental design

Experiment 1. Short-term acute stress response. One hundred and forty-four trout (80.6 ± 13.9 g body mass) were distributed (12 fish per tank) among 12 experimental tanks (80 liter tanks). After an acclimation period of 5 days, each of the following

protocols was randomly assigned to two replicate tanks: No chasing (controls) and chasing for 15 seconds, 2 min, 5 min or 15 min. The fish were chased in their tanks with a small net during the stipulated time in each case. After the chasing protocol, fish were immediately anesthetized in-tank by adding 0.2% of 2-phenoxyethanol to the water. After circa 1 min of anesthetic exposure, five fish were removed from the tank for sampling, while the remaining fish were immediately transferred to recovery tanks and were not used in the study. During sampling, 1 mL of blood of each fish was obtained by puncturing the caudal peduncle with a 1 mL disposable syringe. After blood extraction, the fish were sacrificed by spinal transection and hypothalamus and telencephalon were dissected out and stored in dry ice. Plasma was obtained after centrifugation of blood (6000 x g, 10 min, 4°C). All fish were sampled within 3 min after the end of the chasing protocol.

Experiment 2. Recovery after acute stress. A total of 144 fish ( $90.7 \pm 13.5$  g body mass) were distributed (12 fish per tank) among 12 experimental tanks. After an acclimation period of 5 days, each of the following chasing protocols was assigned to two replicate tanks: No chasing (controls) or chasing for 5 min followed by a recovery period of 15 min, 45 min, 2 h, 4 h or 8 h. After the corresponding recovery period, fish were anesthetized, sacrificed and sampled as described for experiment 1.

To avoid strong interactions of social hierarchies with the stress response, all fish tanks were inspected several times a day during the acclimation period. The tanks contained more fish than required for reducing a possible development of strong dominance hierarchies, which are known to occur more often in this species when the stocking density is low. Two tanks of fish, where a clear dominant specimen was present (usually swimming in the center of the tank, with other fish swimming near the surface) were not used in the experiment. Since we had extra tanks before beginning the experiments, we could avoid the use of those tanks, and the final number of experimental fish was not affected.

## Biochemical analyses

### *Plasma glucose, lactate and cortisol*

The plasma levels of cortisol were analyzed using an enzyme linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, USA). The plasma

glucose and lactate levels were measured using commercial kits from Spinreact (Girona, Spain).

#### *Plasma catecholamines*

The plasma adrenaline (A) and NA levels were quantified by HPLC with electrochemical detection after purification of plasma by deproteinization followed by solid-phase extraction (SPE). Samples were processed as follows: A 100  $\mu$ L plasma aliquot was deproteinized with 25  $\mu$ L of 0.6 M perchloric acid. After centrifugation (14000 x g, 4 min, 4°C), the supernatant was neutralized with 25  $\mu$ L of 1M  $\text{KHCO}_3$ . After centrifugation (14000 x g, 1 min, 4 °C), the obtained supernatants were diluted to 1 mL in ultrapure water to be used for the SPE procedure. The SPE cartridges (1 mL-100 mg tubes, Discovery® DSC-WCX, Supelco) were conditioned with 1.5 mL of ultrapure water at a flow rate of 5 mL/min. The samples were then applied to the conditioned columns at 1 mL/min. After that, the columns were washed twice with 1 mL of ultrapure water (5 mL/min). Finally, the catecholamines were eluted from the columns with two 400  $\mu$ L aliquots of 0.3 M  $\text{HClO}_4$  at 1 mL/min. Recoveries for NA and A were above 97%. Aliquots of 20  $\mu$ L of those eluates were directly injected in the HPLC system, which was equipped with a Jasco PU-2080 Plus pump, a 5  $\mu$ m analytical column (Phenomenex, Nucleosil C18, 150 mm length x 4,6 mm diameter) and a ESA Coulochem II detector. The detection system included a double analytical cell (M5011) with oxidation potentials set at + 40 mV (first electrode) and +400 mV (second electrode). The mobile phase was composed of 25 mM citric acid (Panreac), 25 mM  $\text{Na}_2\text{HPO}_4$  (Merck), 25  $\mu$ M  $\text{Na}_2\text{EDTA}$  (Sigma), 0.21 mM sodium 1-octanesulfonate (Fluka) and 1 % (v/v) methanol (VWR-Prolabo); its pH was adjusted to 3.4 with ortho-phosphoric acid (before the addition of methanol), and was filtered (0.20  $\mu$ m filter, Millipore, Bedford, USA) and degasified at vacuum before use. Analytical run time was 10 minutes at an isocratic flow rate of 1.3  $\text{mL} \cdot \text{min}^{-1}$  at room temperature. The sample peaks were quantified by comparing peak areas to those of appropriate external standards. The detection limits for the catecholamines were 3 pg of NA and 5 pg of A per injection, with a signal-to-noise ratio of 3. Acquisition and integration of chromatograms were performed using the ChromNAV version 1.12 software (Jasco Corp.).

#### *Telencephalic and hypothalamic monoamines*

The tissues were weighed and then homogenized by ultrasonic disruption in 0.5 mL of HPLC mobile phase. The homogenates were then centrifuged (16000 x g, 10 min) and supernatants further diluted 1:2 (supernatant: mobile phase) prior to the HPLC analysis. Data were normalized by protein content of the tissues, which was measured with the bicinchoninic acid method (Smith et al., 1985). The contents of NA, dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC, a major DA oxidative metabolite), serotonin (5-hydroxytryptamine, 5HT), 5-hydroxyindole-3-acetic acid (5HIAA, a major 5HT oxidative metabolite) and 5-hydroxytryptophan (5HTP, immediate 5HT precursor) were analyzed in hypothalamus and telencephalon by high performance liquid chromatography with electrochemical detection (HPLC-EC), as previously described (Gesto et al., 2006) with some modifications. The HPLC system was equipped with a Jasco PU-2080 Plus pump, a 5  $\mu$ m analytical column (Phenomenex, Nucleosil C18, 150 mm length x 4.6 mm diameter), a Jasco AS-2057 autosampler and a ESA Coulochem II detector. The detection system included a double analytical cell (M5011) with oxidation potentials set at + 40 mV (first electrode) and +340 mV (second electrode). The mobile phase was composed of 63.9 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1 mM Na<sub>2</sub>EDTA, 0.80 mM sodium 1-octanesulfonate and 15.3 % (v/v) methanol; its pH was adjusted to 2.95 with ortho-phosphoric acid, and was filtered (0.20  $\mu$ m filter, Millipore, Bedford, USA) and degasified at vacuum before use. Analytical run time was 15 minutes at an isocratic flow rate of 1.0 mL·min<sup>-1</sup> at room temperature. The detection limit for the amines and their metabolites were between 0.5 and 1.5 pg per injection, with a signal-to-noise ratio of 3. Acquisition and integration of chromatograms were performed using the ChromNAV version 1.12 software (Jasco Corp.).

## Statistics

Data were analyzed by ANOVA followed by Tukey's *post hoc* test. The data corresponding to fish of replicate tanks were pooled together after confirming that there were no statistical differences between replicates for any of the parameters assessed. Differences were considered statistically significant at  $P \leq 0.05$ .

## Results

### Plasma catecholamines

In the short term, catecholamines increased very fast, since fish chased for 15 seconds showed increased levels of both A and NA (Fig. 1). After that, both catecholamines further increased until reaching a plateau at 2 min after the start of the chasing. In the second experiment, both catecholamines showed signals of recovery at 45 min after chasing (Fig. 1). In the next sampling point, two hours after the stress stimulus, both A and NA had returned to unstressed levels. Although both plasma catecholamines showed very similar trends of variation after the acute stress, A was clearly more important quantitatively than NA.

#### Plasma cortisol, glucose and lactate

Cortisol levels showed a steep increase with time after stress (Fig. 2). A maximum in cortisol levels was observed in the group exposed to 15 min of chasing, in which cortisol levels were about 16 times higher than in control group. A slighter (1.9 times) significant increase in cortisol levels was already observed after only 2 min of chasing. At longer times, cortisol levels showed a maximum at 45 min after the stress stimulus and decayed after that, returning to control values 8 h after the chasing protocol (Fig. 2).

At early sampling times, the plasma levels of glucose increased with time after stress, reaching a maximum in the group exposed to 15 min of chasing (Fig. 2). Similarly to cortisol, the glucose levels were already increased after 2 min of stress, with respect to control fish. In the second experiment, the glucose levels reached maximum values at 2 h and 4 h after stress, and returned to control values 8 h after chasing (Fig. 2).

The lactate levels showed a pattern of changes quite similar to that of cortisol (Fig. 2) and increased with chasing duration until the end of the first experiment (15 min chasing). However, in this case the increase resulted statistically significant even in the group exposed to 15 seconds of chasing. In the second experiment, the lactate levels reached a maximum 15 min after chasing and returned to control levels after 4 hours (Fig. 2).

#### Brain serotonergic and dopaminergic activity

The serotonergic activity rapidly increased in both hypothalamus and telencephalon, as shown in 5HIAA/5HT ratio (Fig. 3). The increase was obvious after only 15 seconds of stress. After that initial increase, the serotonergic activity remained over control levels without important changes until the end of experiment in both brain regions. At

longer times, the serotonergic activity showed a maximum in both regions 45 min after stress. After that, the values started to decrease, returning to control values after 2 hours in the hypothalamus and 8 hours in the telencephalon (Fig. 3). In both regions, the increases in the serotonergic ratio were parallel to those in the levels of the metabolite 5HIAA whereas 5HT levels remained unaltered in all groups (Figs S1-S4). Also, stress did not induce any effect on the levels of the precursor 5HTP (Figs S1-S4).

The alterations in the dopaminergic system were minor. In the first experiment no significant changes in the dopaminergic activity index (the DOPAC/DA ratio) were observed in the hypothalamus or in the telencephalon (Fig. 4), although the DOPAC levels were increased after 15 minutes of chasing in the hypothalamus (Fig. S1) and after 2 minutes of chasing in the telencephalon (Fig. S3). In the second experiment, no changes were detected in the hypothalamus (Figs 4 and S2), whereas the telencephalic dopaminergic activity was increased (Fig. 4), with no changes in the levels of DA or DOPAC (Fig. S4). A maximum was reached in the telencephalic dopaminergic ratio 45 min after stress, and the dopaminergic activity returned to control levels 4 hours after chasing (Fig. 4).

Throughout the experiments there were no stress effects on the levels of NA, neither in the hypothalamus, nor in the telencephalon (Figs S1-S4).

## Discussion

When fish are exposed to an acute stressor, both BSC and HPI axes become activated. As a result, large quantities of the catecholamines NA and A, and then cortisol, are released to plasma (Wendelaar Bonga, 1997). Both plasma catecholamines and cortisol act on multiple targets within fish organism to aid the fish to cope with stress. Catecholamines enhance respiration and cardiac output as well as the mobilization of energy substrates such as glucose (mainly by increased liver glycogenolysis) or free fatty acids (Wendelaar Bonga, 1997). Cortisol actions contribute to the increase in the plasma availability of glucose (mainly by raised gluconeogenesis in liver), and free fatty acids (Mommensen, 1999; Sheridan, 1994), and also induce an inhibition in reproductive and immune functions (Mommensen et al., 1999). In this context, the role and timing of action of brain monoaminergic neurotransmission is not fully understood. Therefore, we carried out our study to get a view of the pattern of

activity of brain monoamines in the context of the simultaneous activation of both BSC and HPI axes during an acute stress response.

The dynamics of the response of several stress markers such as plasma cortisol or glucose after exposure to an acute stressor have been studied in fish (Aluru and Vijayan, 2006; Arends et al., 1999; Cockrem, 2013; Fast et al., 2008; Pickering et al., 1982; Saera-Vila et al., 2009; Small, 2004; Wilson et al., 1998). However, the first sampling time was usually performed at 15 minutes or more post-stress. Studies monitoring the activation of those markers in a more detailed fashion during the first minutes of the stress response are very scarce (Biron and Benfey, 1994; Pepels et al., 2004). In agreement with the study of Biron and Benfey (1994), we demonstrate here that the typical increase of plasma cortisol, glucose and lactate to a handling challenge takes place in rainbow trout at short times, with all the three parameters being increased within the initial 2 minutes of handling. In the available studies the levels of those parameters usually returned to control levels in a few hours, although recovery times strongly depend on species, kind of stressor and exposure time (Aluru and Vijayan, 2006; Arends et al., 1999; Biron and Benfey, 1994; Fast et al., 2008; Pickering et al., 1982; Small, 2004; Wilson et al., 1998). In this study, the plasma lactate values returned to control levels within 4 hours after the acute handling, whereas plasma cortisol and glucose returned to control levels 8 hours after exposure. In both cases, the time periods were similar to those already observed in other studies in salmonid fish (Fast et al., 2008; Pickering et al., 1982; Wilson et al., 1998). It is noteworthy that the levels of plasma glucose increased in two phases. An initial fast increase occurred in less than 2 min, and is most probably the result of increased glycogenolytic potential in liver elicited by enhanced levels of plasma catecholamines. A further increase was observed between 45 min and 2 h, which could be related to the secondary effects of cortisol promoting gluconeogenesis in liver (Mommensen et al., 1999). The plasma cortisol levels increased slowly at first and the acute increase occurred between 2 and 15 minutes after stress, when the plasma levels of glucose were already high. These changes are in agreement with previous findings suggesting that plasma glucose could have a role in promoting the release of cortisol by the interrenal cells in fish under ACTH stimulation (Conde-Sieira et al., 2013).

The time-course of the response of plasma catecholamines to acute stress has been scarcely studied to date because of the difficulty of sampling and measuring basal levels

of catecholamines in unstressed fish. In this study, the anesthetic was administered surreptitiously to the fish home tanks. Following this method, the first contact with the fish took place when they were already anesthetized, thus highly reducing the impact of capturing them from the tanks (Caamaño-Tubío et al., 2010; Gerwick et al., 1999), and therefore minimizing sympathetic activation. The plasma catecholamines increased very fast as expected, and both NA and A went back to control levels within 2 h after acute stress. Recovery time is in agreement with previous studies in rainbow trout, which described recovery times for plasma catecholamines between 1 and 4 hours after stress (Milligan and Wood, 1987; Wood, 1991; Wood et al., 1990).

Central 5HT seems to be an output signal of the integrated stress response, since chronically stressed fish, including socially subordinate individuals, usually display elevated serotonergic activity (Øverli et al., 2005; Winberg et al., 1992). Moreover, at the same time, 5HT activity seems to have a key role in the activation of the hormonal stress response and it is known that serotonergic activity acts as a stimulator of the HPA/HPI axis in both mammals and fish (Dinan et al., 1996; Le Fauvre et al., 1991; Winberg et al., 1997). In fact, the involvement of serotonergic activity on the stress response is very complex. For instance, it has been shown in mammals that 5HT stimulates the CRF release by the hypothalamus (Boisvert et al., 2011; Calogero et al., 1989), but also the ACTH release by the pituitary (Calogero et al., 1993). However, at the same time, serotonergic activity is affected by other elements of the stress response such as cortisol, CRF or AVT (DiBattista et al., 2005; Dinan et al., 1996; Price and Lucki, 2001; Sangiao-Alvarellos et al., 2004; Summers et al., 2005). Therefore, we consider that studying the temporal pattern of activation of the serotonergic signaling after exposure to a stressor is of vital importance to understand the participation of the brain serotonergic system in the integrated stress response. However, such type of studies is very infrequent in the literature. Several studies about the dynamics of the monoaminergic response have been carried out with lizards (Summers, 2002; Summers et al. 2003) after social interaction, which is known to be a potent stressor. Those studies show that the central serotonergic response to social stress seems to be biphasic, with an early increase in all animals and a second increase that is delayed in time in subordinated fish with respect to dominant individuals (Summers et al., 2003). In our study, the acute stress protocol induced a very rapid change in hypothalamic and telencephalic 5HIAA/5HT ratio, an indicator of serotonergic activity, which increases in

seconds, something that was observed before in lizards displaying elevated serotonergic activity only 30 seconds after a stressful social confrontation (Emerson et al., 2000; Matter et al. 1998). The serotonergic ratio reached a plateau very rapidly but, at longer times, it was further increased reaching a maximum around 45 minutes post-stress. The promoting effect of the acute stress on the serotonergic activity was transient, and serotonergic ratios went back to control levels in a few hours in a very similar way to plasma cortisol and plasma catecholamines. Our results suggest that the response of 5HT activity occurs previously or at least concomitantly with the rise of catecholamine levels in plasma. Therefore, central 5HT activity could play a role in triggering the BSC axis, leading to the massive release of chromaffin cell catecholamines to the blood. The initial activation of the brain monoaminergic neurons could be part of the stressor recognition mechanisms, taking part in the central integration of the stress signals to initiate the neuroendocrine response. The observed peaks in both hypothalamic and telencephalic serotonergic activities 45 min after stress could be the result of the indirect effects of other participants in the stress response such as cortisol, which is known to promote serotonergic activity in fish (Summers and Winberg, 2006; Weber et al., 2012). It is known that the regulation of the serotonergic neuronal system is different in cases of chronic stress, where the activation seems to be more permanent (Øverli et al., 2005, 2007). Furthermore, a reorganization of brain serotonergic receptors seems to take place after stress and is probably different in acute than in chronic stress (Chaouloff et al., 1999; McKittrick et al., 1995). The differential regulation of the serotonergic response after acute or chronic stress deserves further investigation.

The central dopaminergic system seems to be involved in the regulation of activities like aggression, social status, motor activity, learning and motivation/reward (Summers and Winberg, 2006). It has been reported that both serotonergic and dopaminergic activities are antagonically involved in the neural circuitry controlling aggression, which is known to overlap with that regulating stress responses (Summers and Winberg, 2006). At this respect, it has been shown in lizards (but also in fish) that, after social interactions, dominant individuals had higher levels of dopaminergic activity than subordinated fish, just opposite to what occurs with serotonergic activity (Winberg and Nilsson, 1993). Since increased serotonergic activity is considered as a behavioural inhibitor, Höglund et al. (2001) suggested that an initial activation of brain DA systems could serve to counteract the effects of a stress-induced elevation in brain 5HT activity

in dominant fish. Despite all that, the involvement of the central dopaminergic activity in the regulation of the HPA/HPI axis is unclear. In mammals, both stimulatory and inhibitory effects of stress on dopaminergic activity have been reported (Höglund et al., 2001; Waters et al., 2005). In fish, although several studies have reported the stimulatory action of different kinds of stressors on central dopaminergic system (Backström et al., 2011; Gesto et al., 2008; Øverli et al., 1999; Weber et al., 2012), those stimulations did not always take place (our own unpublished observations). The very limited data about central dopaminergic response to stress deserves further attention. In our study, dopaminergic activity remained unaffected in the hypothalamus and increased in the telencephalon after the acute stress protocol, supporting the idea that the activation of the dopaminergic tone is strongly dependent on stressor type and severity, and on the brain region considered.

Brain noradrenergic system seems to be also involved in the stress response in mammals (Dunn et al., 2004) and probably in fish (Øverli et al., 2001). NE could have a role in triggering the release of CRF, leading to the activation of the HPA axis in mammals (Dunn et al., 2004). In our experiments, we did not observe any alteration of NE levels in hypothalamus or telencephalon but with no data of the NE metabolization the effects on NA levels alone are difficult to interpret.

The simultaneous assessment of different stress markers allowed locating in time the serotonergic activity increase within the context of an integrated stress response. The transient increase in the forebrain serotonergic activity after an acute stress took place very fast and its initial increase was previous or at least concomitant with the increase in plasma catecholamines. Thus, besides its role in the regulation of the HPI axis, it is also possible that an increase in the forebrain serotonergic activity participates in the activation of the BSC axis leading to the massive release of NA and A into circulation. Regarding that, it is already known that peripheral serotonin promotes the release of catecholamines in mammals and fish through a cholinergic innervation-independent mechanism (Winberg et al., 1997; Reid et al., 1998). The rapid serotonergic response after the exposure to an acute stressor suggests that the serotonergic tone could be important in the recognition of potential stressors by the central nervous system and act as a mediator between the perception of the stressor by the sensory organs and the primary release of catecholamines and corticosteroids to plasma. Furthermore, the activity of the serotonergic system after stress seems to be tightly linked to other

elements of the BSC or HPI axis, since the transient increase observed after stress is driven back to resting levels in a very similar fashion than other elements of the stress response such as the catecholamines or cortisol. Whether or not there is a direct cause-effect relationship between forebrain serotonergic activity and other stress markers should be confirmed in future studies.

### **List of abbreviations**

5HIAA: 5-hydroxyindole-3-acetic acid

5HT: 5-hydroxytryptamine, serotonin

5HTP: 5-hydroxytryptophan

A: adrenaline

ACTH: adrenocorticotrophic hormone

AVT: arginine vasotocin

BSC: brain-sympathetic-chromaffin axis

CRF: corticotropin-releasing factor

DA: dopamine

DOPAC: 3,4-dihydroxyphenylacetic acid

HPA: hypothalamus-pituitary-adrenal axis

HPI: hypothalamus-pituitary-interrenal axis

HPLC: high performance liquid chromatography

NA: noradrenaline

SPE: solid-phase extraction

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## Figure captions

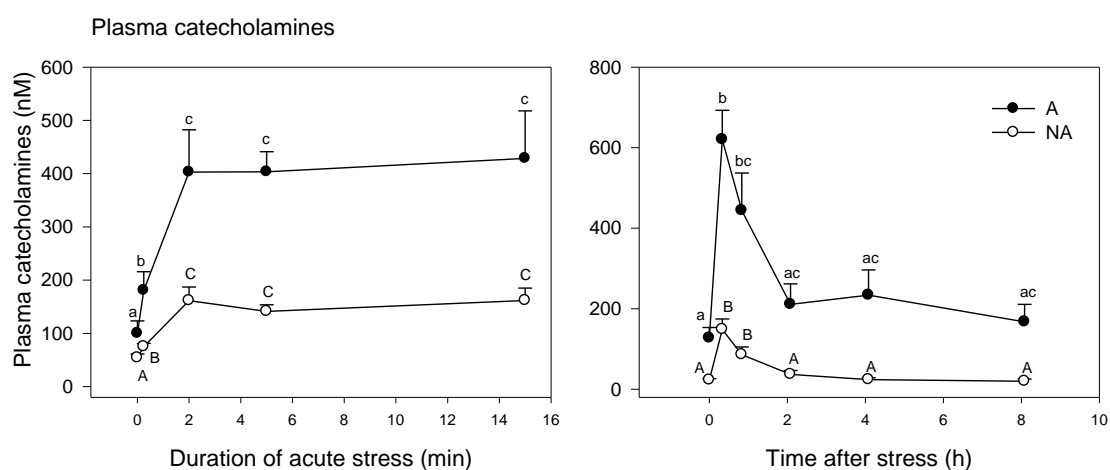
**Figure 1.** Changes in the plasma levels of the catecholamines NA (○) and A (●) in rainbow trout. Left panel: Fish exposed to acute handling stress for 15 s, 2 min, 5 min or 15 min and sacrificed immediately afterwards. Right panel: Fish exposed to 5 min of handling stress and sacrificed 15 min, 45 min, 2 h, 4 h or 8 h later. Values are mean  $\pm$  s.e.m. (n=10). Different letters indicate statistically significant differences among groups.

**Figure 2.** Changes in the plasma levels of cortisol (●), glucose (○) and lactate (□) in rainbow trout. Left panels: fish exposed to acute handling stress for 15 s, 2 min, 5 min or 15 min and sacrificed immediately afterwards. Right panels: fish exposed to 5 min of handling stress and sacrificed 15 min, 45 min, 2 h, 4 h or 8 h later. Values are mean  $\pm$  s.e.m. (n=10). Different letters indicate statistically significant differences among groups.

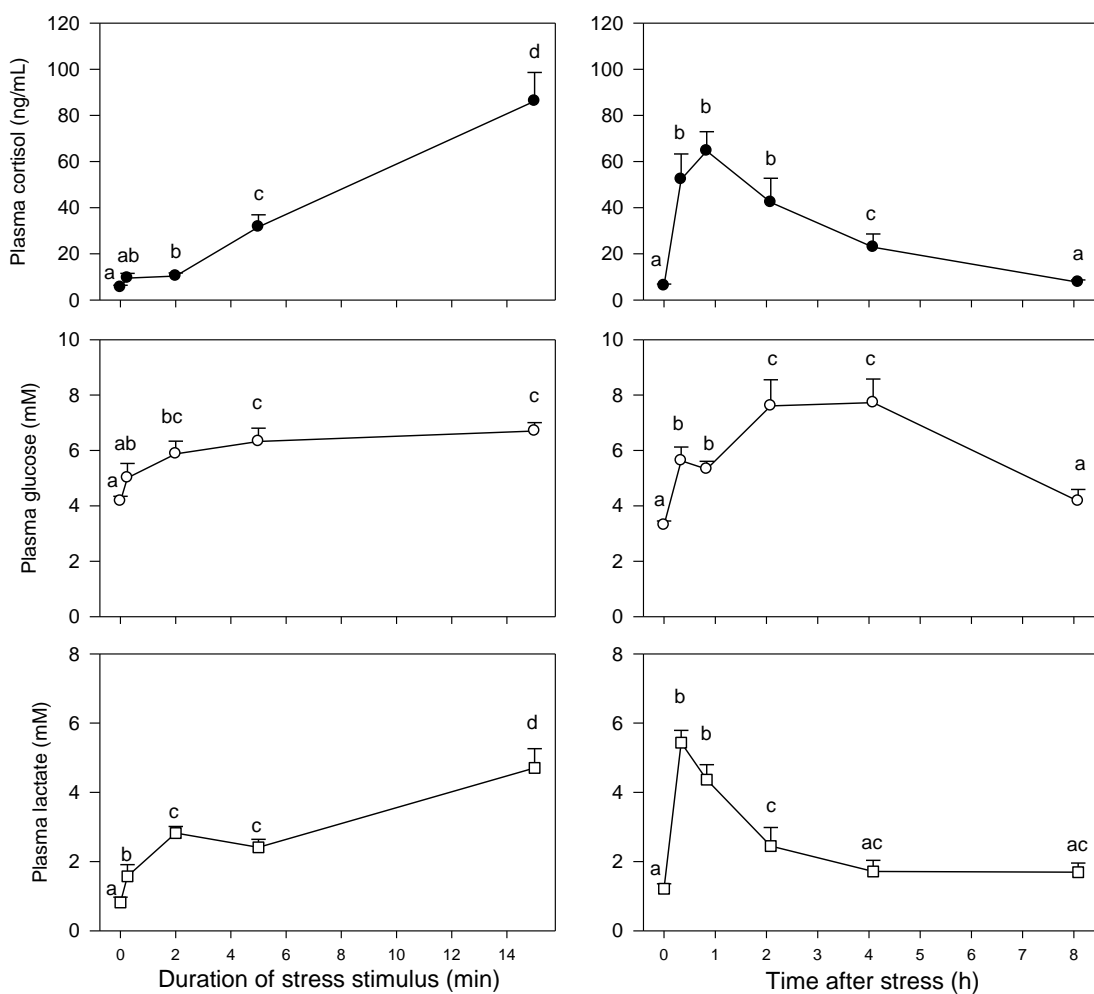
**Figure 3.** Changes in the serotonergic activity (estimated by the 5HIAA/5HT ratio) in the hypothalamus and the telencephalon of rainbow trout. Left panels: fish exposed to acute handling stress for 15 s, 2 min, 5 min or 15 min and sacrificed immediately afterwards. Right panels: fish exposed to 5 min of handling stress and sacrificed 15 min, 45 min, 2 h, 4 h or 8 h later. Values are mean  $\pm$  s.e.m. (n=10). Different letters indicate statistically significant differences among groups.

**Figure 4.** Changes in the dopaminergic activity (estimated by the DOPAC/DA ratio) in the hypothalamus and the telencephalon of rainbow trout. Left panels: fish exposed to acute handling stress for 15 s, 2 min, 5 min or 15 min and sacrificed immediately afterwards. Right panels: fish exposed to 5 min of handling stress and sacrificed 15 min, 45 min, 2 h, 4 h or 8 h later. Values are mean  $\pm$  s.e.m. (n=10). Different letters indicate statistically significant differences among groups.

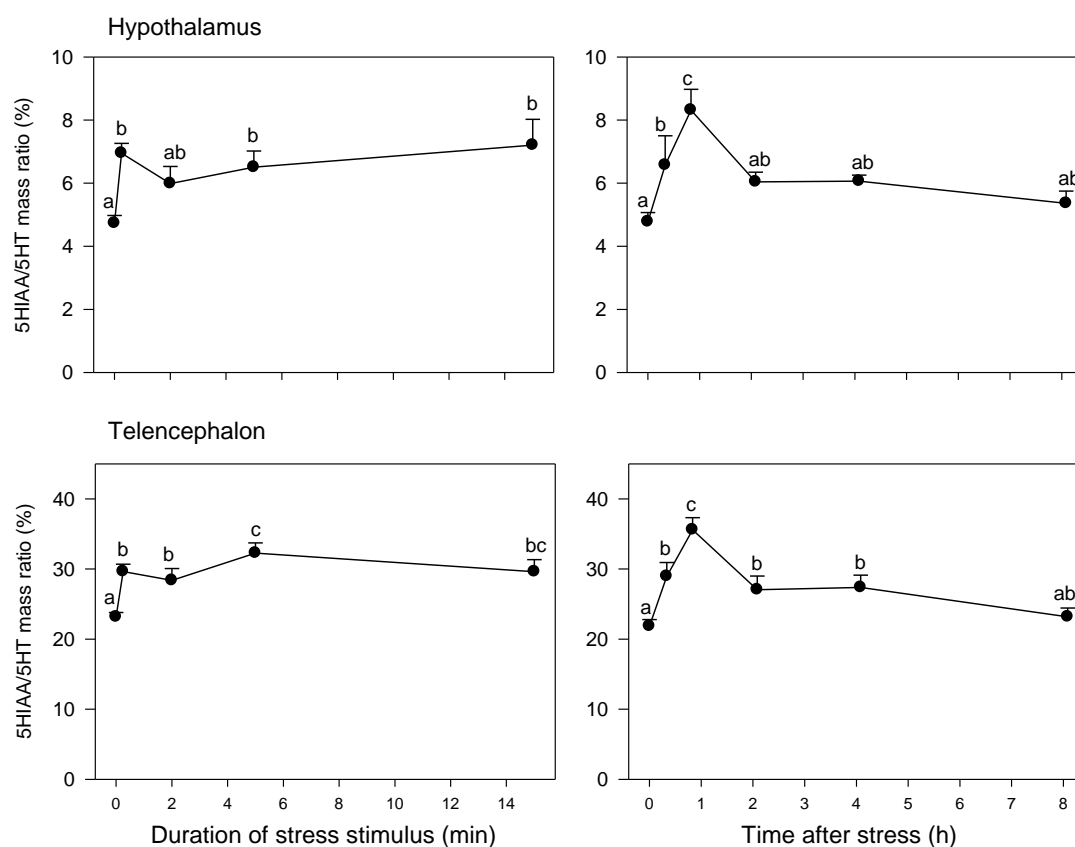
**Figure 1**



**Figure 2**



**Figure 3**



**Figure 4**

