Alma Mater Studiorum Università di Bologna

SCUOLA DI SCIENZE MATEMATICHE FISICHE E NATURALI
Corso di laurea magistrale in BIOLOGIA MARINA

Adattamenti degli animali all'ambiente marino

# Acute stress regulates vasotocinergic and isotocinergic systems in the gilthead sea bream (*Sparus aurata* L.)

**Relatore**
Prof.ssa ELENA FABBRI

**Tesi di Laurea di**:
MARCO BASTAROLI

**Correlatore**
Dott. JUAN ANTONIO MARTOS SITCHA
Prof. JUAN MIGUEL MANCERA ROMERO

Sessione III
Anno Accademico 2013/2014

## GENERAL INDEX

**FIGURES INDEX**

## TABLES INDEX

## List of Abbreviations

| | |
|---|---|
| **ACTH** | Adrenocorticotropic hormone |
| **ADH** | Antidiuretic hormone |
| **ANOVA** | Analysis of variance |
| **AVP** | Vasopressin |
| **AVT** | Arginine vasotocin |
| **AVTR** | AVT receptor |
| **BSA** | Bovine serum albumin |
| **CAs** | Catecholamines |
| **cDNA** | Complementary DNA |
| **gDNA** | Genomic DNA |
| **CLIP** | Corticotropin-like intermediate lobe peptide |
| **CNS** | Central nervous system |
| **CRH** | Corticotropin releasing hormone |
| **CRH-BP** | CRH binding protein |
| **DNA** | Deoxyribonucleic acid |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **FBPase** | Fructose 1,6-bisphosphatase |
| **G6Pase** | Glucose-6-phosphatase |
| **GK** | Glucokinase |
| **GnRH** | Gonadotropin-releasing hormone |
| **GPase** | Glycogen phosphorylase |
| **HPI axis** | Hypothalamic-pituitary-interrenal axis |
| **HK** | Hexokinase |
| **HSC axis** | Hypothalamic-sympathetic-chromaffin axis |
| **IGF-I** | Insulin-like growth factor 1 |
| **IT** | Isotocin |
| **ITR** | IT receptor |
| **LDH** | Lactate dehydrogense |
| **MCH** | Melanophores concentrating hormone |
| **mRNA** | Messenger RNA |
| **MSH** | Melanocyte-stimulating hormone |
| **NLT** | Nucleus lateralis tuberis |
| **NPO** | Nucleus preopticus |
| **NPY** | Neuropeptide Y |
| **NSO** | Nucleus supraoptic |
| **OXY** | Oxytocin |
| **PCR** | Polymerase chain reaction |
| **PDH** | Pyruvate dehydrogense |
| **PEPCK** | Phosphoenolpyruvate carboxykinase |
| **PFK-1** | Phosphofructo-1-kinase |
| **PK** | Pyruvate kinase |
| **PKA** | Protein kinase A |
| **PKC** | Protein kinase C |
| **PLC/IP3** | Inositol-1,4,5-triphosphate |

| | |
|---|---|
| **POMC** | Pro-opiomelanocortin |
| **PPB** | Potassium phosphate buffer |
| **PRL** | Prolactin |
| **pro-IT** | IT precursor |
| **pro-VT** | Vasotocin precursor |
| **PVN** | Nucleus paraventriculra |
| **qPCR** | Real-time PCR |
| **RIN** | RNA Integrity Number |
| **RNA** | Ribonucleic acid |
| **SW** | Seawater |
| **TAG** | Triacylglycerides |
| **TAG lipase** | Triacyglycerol lipase |
| **TRH** | Thyrotropin releasing hormone |

**ABSTRACT**

The hypothalamus-pituitary-interrenal axis is involved in stress response regulation. In addition, arginine vasotocin (AVT) and isotocin (IT) are also considered as important players in this stress regulation. The present study assessed, using the teleost gilthead sea bream (*Sparus aurata*) as a biological model, hypothalamic mRNA expression changes of AVT and IT and their receptors at hepatic level after an acute stress situation. Specimens were submitted to air for 3 min and place back in their respective tanks after that, being sampled at different times (15 min, 30 min, 1, 2, 4 and 8 hours post-stress) in order to study the time course response. Plasma cortisol values increased after few minutes post-exposure, decreasing during the experimental time while a metabolic reorganization occurred in both plasmatic and hepatic levels. At hypothalamic level, acute stress affects mRNA expression of AVT and IT precursors, as well as hepatic expression of their receptors, suggesting the involvement of both vasotocinergic and isotocinergic systems in the acute stress response. Our results demonstrate the activation and involvement of both endocrine pathways in the regulation of metabolic and stress systems of *Sparus aurata*, which is stated, at least, through changes in mRNA expression levels of these genes analysed.

# 1. INTRODUCTION

### 1.1. Endocrine system in teleost: general view

The endocrine system of teleosts involves a high number of glands, and its hormonal secretions control several physiological processes in the animal. The pituitary gland is considered in vertebrates, including teleost, the *master gland* involved in the control of different physiological processes. However, other peripheral glands (and their endocrine products) are also involved in these functions. Thus, we could cite the kidney (production of cortisol and angiotensin), liver (somatomedins or inslulin growth factor I, IGF-I), gonads (sex steroids), the urophysis (urotensins I and II), the heart (natriuretic peptides) or the corpuscles of Stannius (stanniocalcin), among others (Figure 1.1.), as other important endocrine players.

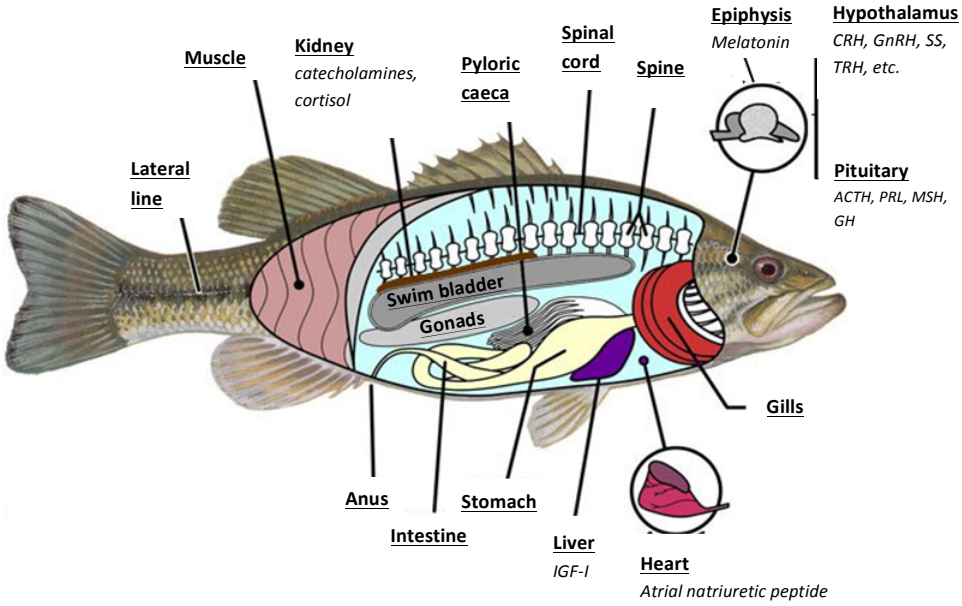


**Figure 1.1.** Scheme of the internal anatomy of a teleost fish (European sea bass, *Dicentrarchus labrax*) showing the main organs involved in different neuroendrocrine axes (modified from oqlarypt.wordpress.com).

### 1.2. Hypothalamic-pituitary axis

Hypothalamic-pituitary axis is one of the most important players in the regulation of various endocrine systems at central level.

Hypothalamus presents several nuclei formed by hypothalamic neurons that projecting their axons in different brain regions, including the pituitary gland. The hypothalamus receives information

from the internal and external environment of the animal. This region contains neurons specialized in:

i) Secretion of several hormones, named neurohormones, into the bloodstream.

ii) Secretion of different peptides or factors that stimulate or inhibit the release of different pituitary hormones. For this reason, hypothalamus is considered to be the region where the "endocrine cascade" begins (Bertok, 1998).

Pituitary is also considered an important gland for the hormonal control of a great variety of physiological functions, such as reproduction, development, growth, osmoregulation, metabolism, etc. This gland is composed by two different types of tissues from different sources: i) the neurohypophysis, derived from neural tissue, whose hormones are synthesized in hypothalamic neurons, and ii) the adenohypophysis, derived from primary endoderm (Gorbman et al., 1983; Norris, 1997; Bentley, 1998). The neurohypophysis is connected to the hypothalamus through the infundibular stalk, where neuropeptides are transported from different hypothalamic nuclei, as the *supraoptic nucleus* (SON) and the *nucleus lateralis tuberalis* (NLT), and then released from the neurohypophysis into the bloodstream (see section 1.3.). Different cell types of the adenohypophysis are located in certain parts of the gland, so that two or more cell types constitute a pituitary lobe. Thus, each type of cells integrated in different pituitary lobes are distinguished by i) morphology, ii) affinities to different immunocytochemical reactions, as well as iii) physiological function related to the hormone produced and secreted. The adenohypophysis of teleost presents three differentparts: i) *rostral pars distalis* (RPD), ii) *proximal pars distalis* (RPD), and iii) *pars intermedia* (PI), whose main synthesized hormones are described in Figure 1.2.

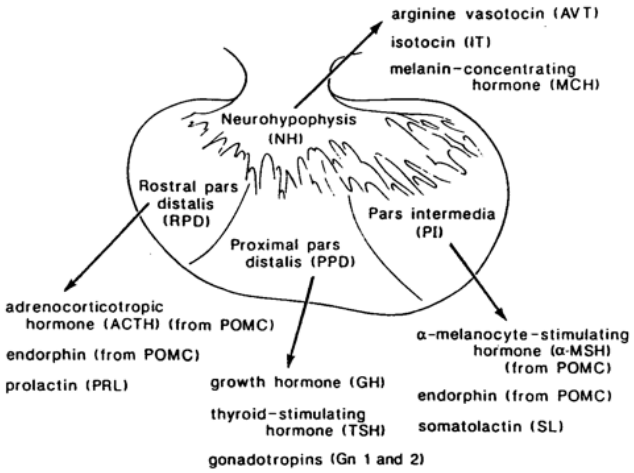


**Figure 1.2.** Scheme of the pituitary gland of teleost showing hormones released from neurohypophysis and different regions of adenohypophysis (RPD, PPD, PI) (aken from Bern and Madsen, 1992).

### 1.3. Vasotocinergic and Isotocinergic systems

A neuropeptide is defined as a peptide synthesized in neurons. The arginine vasotocin (AVT) and the isotocin (IT) are neuropeptides consisting of nine amino acids in its mature form (nonapeptide). These hormones are, respectively, homologs to the arginine vasopressin (AVP) and oxytocin (OXY) in mammals. However, the AVT differs from the AVP in the third amino acid of the mature protein, with isoleucine in the case of AVT and phenylalanine in the AVP. On the other hand, IT differs from OXY in the fourth amino acid, with glutamine for the IT and serine for OXY (Acher, 1996) (Figure 1.3).

**A**

**ARGININE VASOTOCIN**
**Cys** – *Tyr* – *Ile* – *Gln* – **Asn** – **Cys** – **Pro** – *Arg* – **Gly** – **NH$_2$**
**1** *2* *3* *4* **5** **6** **7** *8* **9**
**ARGININE VASOPRESSIN**
**Cys** – *Tyr* – *Phe* – *Gln* – **Asn** – **Cys** – **Pro** – *Arg* – **Gly** – **NH$_2$**
**LYSINE VASOPRESSIN**
**Cys** – *Tyr* – *Phe* – *Gln* – **Asn** – **Cys** – **Pro** – *Lys* – **Gly** – **NH$_2$**
**PHENYPRESSIN**
**Cys** – *Phe* –*Phe* – *Gln* – **Asn** – **Cys** – **Pro** – *Arg* – **Gly** – **NH$_2$**
**ISOTOCIN**
**Cys** – *Tyr* – *Ile* – *Ser* – **Asn** – **Cys** – **Pro** – *Ile* – **Gly** – **NH$_2$**
**OXITOCIN**
**Cys** – *Tyr* – *Ile* – *Gln* – **Asn** – **Cys** – **Pro** – *Leu* – **Gly** – **NH$_2$**
**MESOTOCIN**
**Cys** – *Tyr* – *Ile* – *Gln* – **Asn** – **Cys** – **Pro** – *Ile* – **Gly** – **NH$_2$**
**VALITOCIN**
**Cys** – *Tyr* – *Ile* – *Gln* – **Asn** – **Cys** – **Pro** – *Val* – **Gly** – **NH$_2$**
**GLUMITOCIN**
**Cys** – *Tyr* – *Ile* – *Ser* – **Asn** – **Cys** – **Pro** – *Gln* – **Gly** – **NH$_2$**
**ASPARGTOCIN**
**Cys** – *Tyr* – *Ile* – *Asn* – **Asn** – **Cys** – **Pro** – *Leu* – **Gly** – **NH$_2$**
**ASVATOCIN**
**Cys** – *Tyr* – *Ile* – *Asn* – **Asn** – **Cys** – **Pro** – *Val* – **Gly** – **NH$_2$**
**PHASVATOCIN**
**Cys** – *Tyr* – *Phe* – *Asn* – **Asn** – **Cys** – **Pro** – *Val* – **Gly** – **NH$_2$**

**B**

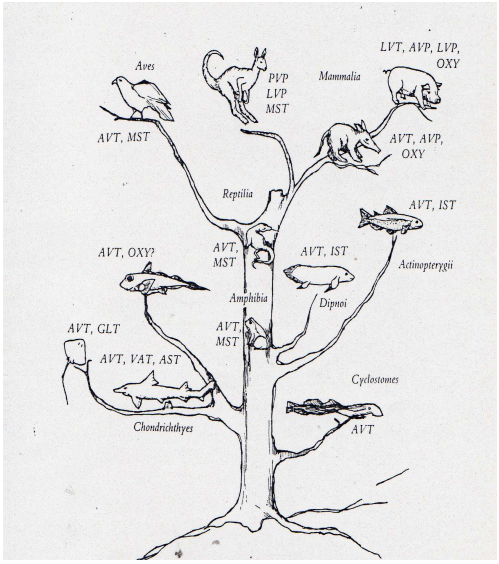


**Figure 1.3.** Neurohypophyseal hormones structure (A) and distribution in vertebrates (B). (Taken from *Vertebrate Endocrinology* by Norris, 1997).

The synthesis of the neuropeptides, starting from messenger RNA (mRNA) which is translated like pro-peptides in the cytoplasm of the neuronal soma, occurs at the level of neurons located in various hypothalamic nuclei. In teleost, the most important are the *nucleus lateralis tuberis* (NLT) and the *nucleus preopticus* (NPO). Subsequently, they are transported by axons through the pituitary stalk, where the mature peptide is stored in the neurohypophysis awaiting a suitable stimulus for their release into the bloodstream, through which it will reach the target tissues and perform its physiological action. Most fish show both AVT and IT neurohypophyseal hormones, excepting cyclostomes that only possess AVT. For this reason, it is thought that the IT hormone emerged from the third genome duplication occurred when cyclostomes appear (Norris, 1997).

In vertebrates both hormones (AVT/AVP and IT/OXY), given the tissue distribution of their receptors, have been associated to various physiological processes. In mammals, AVP (also called antidiuretic hormone or ADH) stimulates water reabsorption in the kidneys, thus maintaining blood osmolality within stable levels. In addition, AVP also regulates cardiovascular activity, being able in some cases to play the role of neurotransmitter (Balment et al., 2006; Kulcykowska, 2007). On the other hand, OXY plays a key role in reproduction, stimulating uterine contractions during birth and milk secretion during lactation (Gainer and Wray, 1994). However, in lower invertebrates like fish, these peptides have been associated with different physiological processes, such as osmoregulatory processes, control of blood pressure and cardiovascular activity, metabolism, stress, reproductive behavior, brain neurotransmission and pituitary endocrine activity (Balment et al., 2006; Kulczykowska, 2007). The presence of both AVT and IT receptors has been reported in a large variety of organs and tissues (Hausmann et al., 1995; Martos-Sitcha et al, 2014a).

The receptor structure presents a typical conformation of seven transmembrane helical domains, showing four intracellular and three extracellular domains (Bentley, 1998) (Figure 1.4.).

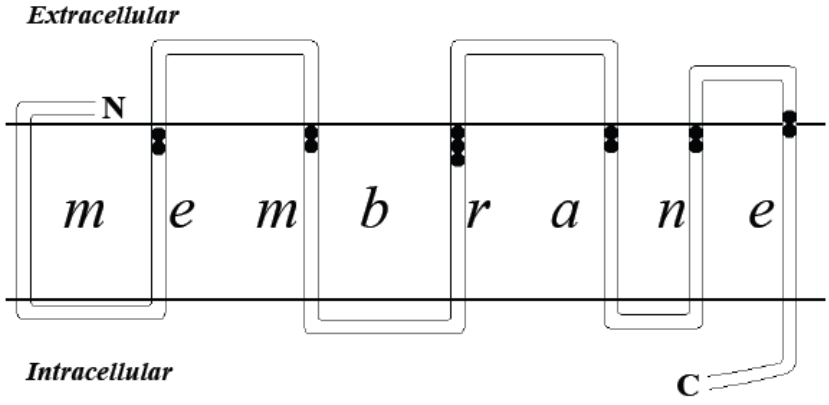


**Figure 1.4.** Topography and structure of AVT/AVP and IT/OXY receptors. Conserved regions, marked as black dots, are the putative interaction sites with the hormone. (Taken from Kulczykowska, 2007).

Currently, different types of AVT and IT receptors (AVTR and ITR, respectively) have been described in fishes. In the case of the AVT, three receptor types have been cloned, corresponding to two different types of V1 receptor (V1a1-type, and V1a2-type) and one of V2, depending on the species (Konno et al, 2009; Lema, 2010; other sequences available at http://www.ncbi.nlm.nih.gov/ website), including those of the gilthead sea bream *Sparus aurata* (Martos-Sitcha et al., 2014a). Meanwhile, two different types of ITR have been described in some species of fish (i.e., *Oryzias*

*orizyases*, GenBank access numbers: ITR1, AB646240; ITR2, AB646241.). However, the subfunctionalization of each of these types of receptors has not been addressed in detail.

Receptor of AVT and IT are widely distributed in various organs of teleosts, such as brain, pituitary, spleen, sideline, ovary, bladder, intestine, liver, heart, gill, kidney and skeletal muscle and smooth, suggesting a specific function of that nonapeptides in these organs (Mahlmann et al., 1994; Hausmann et al., 1995; Martos-Sitcha et al., 2014a). Anyway, and according to the mammalian model, each of these types of receptor have been described for their participation in different paracellular pathways. In this way, the AVT V1-type receptor depends on the Protein Kinase C (PKC), using the phospholipase C and the inositol-1,4,5-triphosphate (PLC/IP3) as intracellular pathways (Warne, 2001). On the other hand, AVTR V2-type does it primarily through phosphorylation sites of Protein Kinase A (PKA) using the cyclic AMP (cAMP) and the adenylate cyclase (Wargent et al., 1999). Meanwhile, the IT receptor performs its effect starting from the routes of inositol phosphate and $Ca^{2+}$ as second messengers (Hausmann et al., 1995). The importance of these paracellular route lies in the possible role of each of these receptors on the regulation of various hormones, enzymes, etc. which regulate different physiological functions (Martos-Sitcha et al., 2014a).

## 1.4. Stress system

### *1.4.1. Stress concept*

Stress can be defined as a complex set of adaptive responses to a stressor, which disturbs the normal or homeostatic state of the animal (Wendelaar Bonga, 1997; Pottinger, 2008). For their classification, we can consider different types of stress i) depending on the time that the stressor persists ("acute" short duration, or "chronic" long duration), or ii) depending on the source of stress (endogenous or exogenous). These factors threaten the homeostatic balance of the animals, and when they exceed a certain time or intensity threshold, trigger a series of coordinated physiological and behavioral changes in order to adapt the animal to disturbed conditions. These changes are globally called "*integrated stress response*". This response produces measurable hormonal changes responsible of stress reactions. The stress response not only depends on the intensity of the stressor, but also from the susceptibility of the organism. In this respect, the organism activates both the hypothalamic-sympathetic-chromaffin (HSC) axis and the hypothalamic-pituitary-interrenal (HPI) axis. For its functioning, the organism responds to a stressful situation with the, so called, "*general*

*adaptation syndrome*", in which both endocrine axes are involved. This syndrome defines the overall timing of response to stress and having different stages depending on the duration of the stress agent (Adams, 1990; Schreck, 1990): i) *alarm phase*, in which the body recognizes the stressor as a threat to their basal status; ii) *resistance stage*, in which resources, mainly hormonal and metabolic, are mobilized to meet the threat; and iii) *exhaustion phase*, in which despite of the responses activated the homeostasis stage is lost. These sets of responses have a number of common features that pass in a sequential manner and are jointly regulated by both the nervous system and the endocrine system.

### *1.4.2. Endocrinology of stress*

At endocrine level, a stressful situation involves two different types of endocrine responses: i) an adrenergic response through out the HSC axis, which increases the concentration of plasma catecholamines (adrenaline and noradrenaline), and ii) the HPI axis response, where a plasma cortisol concentration enhancement is observed (Figure 1.5.).

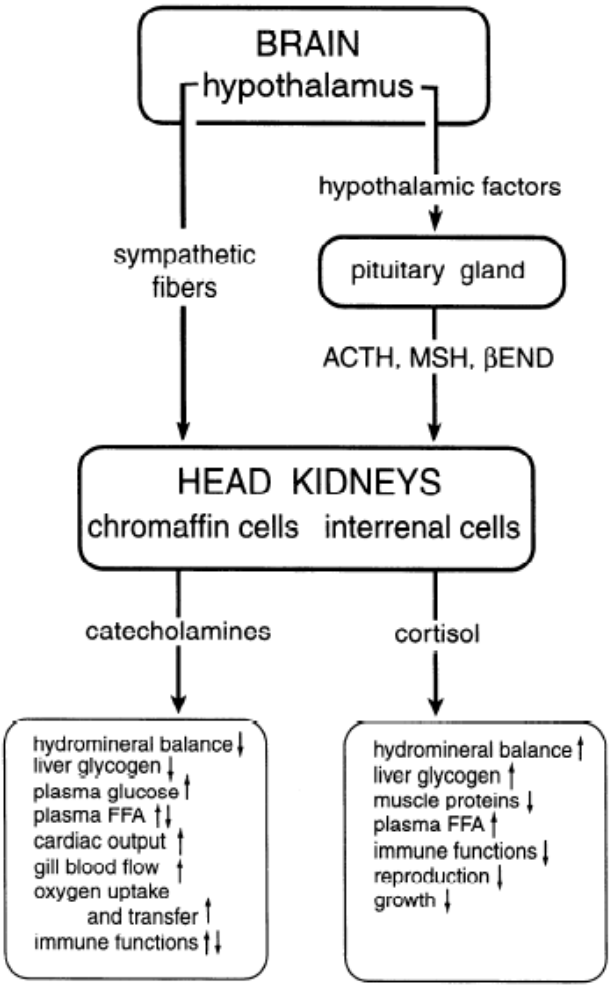


**Figure 1.5.** Scheme of the main factors involved in the neuroendocrine stress response system of teleost (taken from Wendelaar Bonga et al., 1997).

The HSC axis is mainly involved in situations of acute stress, with high intensity and short duration of the stressor. In the interrenal tissue, there are chromaffin cells that produce catecholamines (adrenaline and/or noradrenaline), releasing these hormones in response to stress after sympathetic stimulation. In the HSC axis the synthesis and release of catecholamines into the bloodstream is stimulated by cholinergic fibers of the sympathetic system, followed by a slightly delayed increase in the release of glucocorticoid hormones. In addition, catecholamines activate several cardiovascular, respiratory and metabolic responses aimed to reduce the adverse effects associated with acute stress (Perry et al., 2004).

However, the HPI axis is more related to chronic stress situations where the stressor is less intense and longer in time. However, activation of this axis is also seen during acute stress situations. The

starting point of HPI axis involves release of corticotropin releasing hormone (CRH), mainly from NPO and NLT. CRH is a 41 amino acid peptide with a structure highly conserved throughout phylogeny, showing a sequence identity of 93% between fish and humans (Flik et al., 2006). In teleosts, hypothalamic CRH-producing neurons directly innervate the corticotrophs cells of adenohypophysis, where they promote synthesis and secretion of adrenocorticotropic hormone (ACTH) (Wendelaar Bonga, 1997; Flik et al., 2006; Bernier et al., 2009). This hormone stimulated interrenal tissue to release adrenal steroids, mainly cortisol. ACTH is a hormone of 39 amino acids produced from the proteolytic processing of Pro-opiomelanocortin (POMC), which at its amino-terminal extreme in the anterior pituitary produces β-lipotrophin, instead of ACTH, and in the intermediate lobe produces α-MSH, β-MSH, γ-MSH, CLIP (corticotropin-like intermediate lobe peptide) and β-endorphin (Castro and Morrison, 1997). In addition, the peptide CRH binding protein (CRH-BP) is synthesized in the preoptic area of the hypothalamus, in a different cell group from those that synthesizes CRH, modulating the availability of active CRH in plasma and acting as a reserve of this peptide (Potter et al., 1991; Cortright et al., 1995; Huising et al., 2004; Flik et al., 2006). Thus, CRH-BP is considered an antagonist of CRH activity (McClennen et al., 1998).

In addition to the CRH function regarding the control of ACTH synthesis and secretion, AVT has been proposed to be one of the most important regulators of this complex cascade (see section 1.5.). Moreover, other molecules such as IT, thyrotropin releasing hormone (TRH) and neuropeptide Y (NPY) have also been reported as putative players stimulating ACTH secretion, whereas melanin-concentrating hormone (MCH) could inhibit its secretion (Wendelaar Bonga, 1997).

### *1.4.3. Physiological responses*

*a)* The physiological responses to stress can be grouped into three categories *Primary response or neuroendocrine response*: catecholamines are secreted from the chromaffin tissue (Reid et al., 1998) and HPI axis is stimulated to produce and release cortisol into the bloodstream (Wendelaar Bonga, 1997; Mommsen et al., 1999).

*b)* *Secondary response*: changes of electrolytes and different metabolites in plasma and tissue. These secondary responses are related to physiological adjustments of metabolism, respiratory function, acid-base regulation, osmoregulation, immune function and cellular responses (Iwama et al., 1997, 1998; Mommsen et al., 1999).

*c)* *Tertiary response:* individual-level variations such as changes in growth, condition, disease resistance, behavior, and finally compromising survival (Wedemeyer et al., 1990).

In this regard, an allostatic situation is defined as the level of the homeostatic stability (McEwen and Wingfield, 2003) after a sustained activity of agents causing primary stress response (catecholamines and/or cortisol), through which the body maintains its adequate homeostasis. The allostatic state is part of the primary response to stress, but can also indicate situations where certain changes of catecholamine/cortisol levels from baseline are required, without necessarily occur a stress situation.

### 1.5. Interactions between vasotocinergic/isotocinergic systems and stress system

Due to all functions mediated by AVT/IT (final products of vasotocinergic/isotocinergic systems) and cortisol (final product of the activation of HPI axis) in stress and metabolic processes, it has been suggested a possible interaction between these hormones. In fact, previous studies have demonstrated the existence of an interaction between both vasotocinegic/isotocinergic and stress systems in teleost (Gilchriest et al., 2000; Kulczykowska, 2001, 2007; Balment et al., 2006; Cádiz et al., 2015). Thus, hypothalamic neurons responsible for the synthesis and secretion of AVT innervate the corticotrophs cells of adenohypophysis to stimulate ACTH release into the bloodstream, controlling cortisol secretion in the interrenal tissue (Batten et al., 1990). In this way, it has been observed that AVT stimulates ACTH synthesis and secretion (Fryer et al., 1985; Baker et al., 1996), as well as regulates plasma cortisol levels in fish subjected to high stress situations (Fryer and Leung, 1982). These results suggested a cooperation between both endocrine systems. In *Oncorhynchuis mykiss*, concomitant administration of AVT and CRH in pituitaries incubated *in vitro*, stimulated ACTH secretion dependent on the concentration of both hormones (Baker et al., 1996). The same synergism has been previously observed in mammals treated with CRH and AVP (Rivier and Vale, 1983). For this reason, it has been postulated the existence of a greater plasticity of ACTH secretion regulated by at least two hormones (AVT and CRH) whose expression occurs independently.

Previous studies on metabolic and osmoregulatory changes in specimens of *S. aurata* treated with AVT shown that plasma cortisol levels increased after transfer from seawater (38 ppt salinity) to low salinity water (6 ppt) or high salinity water (55 ppt), but not to seawater (Sangiao-Alvarellos et al., 2006). This could be interpreted as a synergistic or additive effect between AVT and osmotic stimulation on plasma cortisol levels. In fact, such synergy may be due to:

a) The existence of AVT receptors in corticotrophs cells that producing ACTH in pituitary, thereby AVT stimulate the production of this hormone and consequently the cortisol release (Moons et al., 1989).
b) The existence of a cooperation between AVT and CRH to increase ACTH release, as it has been shown in other teleosts (Baker et al., 1996; Kulczykowska, 2001).
c) The regulation of cortisol synthesis *in situ* in the interrenal tissue by AVT (and IT) receptors stimulation without activating the typical endocrine cascade (Cadiz et al., 2015).

Results reported by other authors indicate the possible cooperation between exogenous AVT and endogenous CRH (stimulated by salinity transfer), so that the transfer to extreme salinities may be the necessary stimulus for this cooperation (Martos-Sitcha et al., 2013b), in addition to hormone treatment (Sangiao-Alvarellos et al., 2006). Consequently, changes in osmoregulatory and metabolic parameters in specimens of *S. aurata* treated with AVT and transferred to extreme salinity can be attributed to the action of cortisol. Considering that treatment with cortisol increases hepatic glycogenolysis and hyperglycemia in this species (Laiz-Carrión et al., 2002, 2003), and the increase observed in plasma cortisol levels in fish treated with AVT acclimatized to extreme salinities, the metabolic effects of AVT in *S. aurata* specimens transferred to extreme salinities can not be ruled out (Sangiao-Alvarellos et al., 2006). These results agree with the stimulation of hepatic glycogenolysis by AVT reported previously in other teleost species (Moon and Mommsen, 1990).

In addition, a role of IT has been proposed in the processes of stress produced by hyperosmotic salinities (Martos-Sitcha et al., 2013b), although the role of this hormone as a modulator of the stress system remains unclear. It has been demonstrated that osmotic stress do not affect IT plasma levels in *O. mykiss* (Kulczykowska, 2001), although this hormone seems to be involved in the response of female fish of the Gasterosteidae family subjected to different stressors (Kleszczyńska and Kulczykowska, 2006). In *S. aurata*, IT plasma levels significantly increased in response to high stocking density, while fasting stress has no effect on it (Mancera et al, 2008). In addition, it has been shown that the hypothalamic mRNA expression levels of the IT precursor (pro-IT) increase after changes in the environmental salinity (Martos-Sitcha et al., 2013b). These data suggest plasticity in the stress axis of the isotocinergic system depending on the type of stressor to which the animal is subjected.

### 1.6. Biology of gilthead sea bream (*Sparus aurata*)

The gilthead sea bream (*Sparus aurata*, Linnaeus 1758) is a marine teleost from the Class Actinopterygi, Order Perciformes and Family Sparidae (Figure 1.6.). This species grows to a maximum length of 70 cm and 17.2 Kg, living up to eleven years (www.fishbase.org).

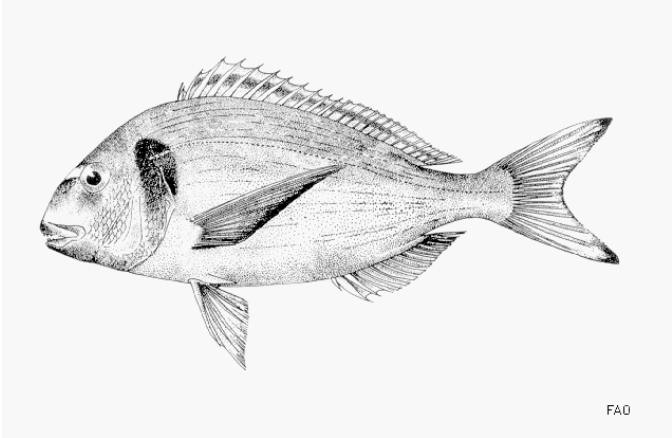


**Figure 1.6.** *Sparus aurata* (Linnaeus, 1758) (aken from Bauchet and Hureau,1986).

Gilthead sea bream is distributed through all over the Mediterranean Sea as well as in the Atlantic Ocean, from the British Islands to Cape Verde and the Canary Islands (Bauchet and Hureau, 1986) (Figure 1.7.). In addition, *S. aurata* is an eurythermic and euryhaline species, as it could tolerate a wide range of temperatures and environmental salinities. It is located mainly in coastal areas, but it could enter into brackish estuaries, hyperosmotic coastal lagoons or salt marshes (Arias, 1976).

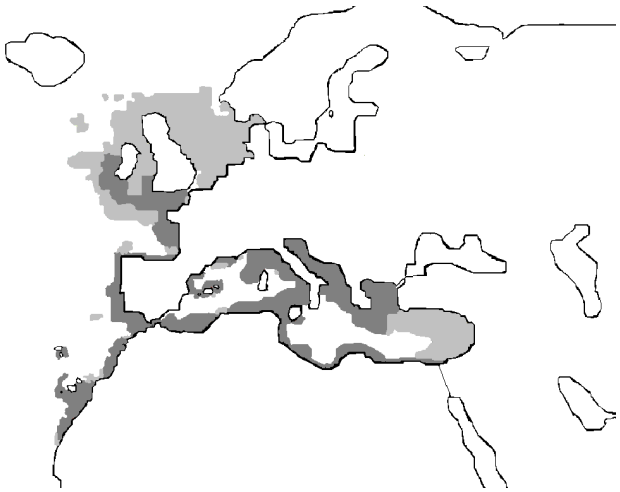

**Figure 1.7.** Geographic distribution of *S. aurata* (dark areas: highly present; light grey areas: present). Modified from the FishBase (available at www.fishbase.org).

Gilthead sea bream is a protandric species. This means that during its first reproduction season, which starts at the end of its second year of live, it is a male. Later on, when reaches 30 cm length, this species undergoes a process of sex reversal becoming a female (Moretti et al., 1999). Immature juveniles are normally find in coastal marshes during the months of spring and summer, moving to deeper waters as they become sexually mature at the end of autumn (Arias, 1976).

# 2. AIM OF THE STUDY

Marine aquaculture has expanded in Europe in recent decades, where the gilthead sea bream could be considered as one of the species with higher increase in its production. To capitalize this growth, it is necessary optimize the biological processes in its production to lead a good farm management. The high level of knowledge of this species in captivity reached in the last decades has enabled the development of an effective industrial exploitation. In this way, we actually have a deep knowledge about different processes related to growth, reproduction, feeding, larval development, etc. (Robaina et al., 1995; Rowlerson et al., 1995; Duguay et al., 1996; Moyano et al., 1996; Carnevali et al., 1999).

The stress system of the gilthead sea bream has been extensively studied both by our Research Group and others (Arends et al., 1999; Laiz-Carrion et al., 2002, 2005; Mancera et al., 2008, Rotlant et. al., 2000, 2001; Sangiao-Alvarellos et al., 2003, 2005, 2006). However, the possible role of vasotocinergic and/or isotocinergic system in the stress process of this species is less known (Sangiao-Alvarellos et al, 2004, 2006; Martos-Sitcha et al., 2013a, 2013b, 2014a, 2014b). Moreover, we also have a thorough knowledge about the functioning of the stress system and its interaction with osmoregulatory and metabolic processes (Laiz-Carrion et al., 2002, 2005; Sangiao-Alvarellos et al., 2003, 2005, 2006; Kleszczynska et al., 2006; Cádiz et al., 2015).

This Thesis is focused on the study of the stress process in *S. aurata*, analyzing an aspect previously not addressed: the response of vasotocinergic and isotocinergic systems after an acute stress situation (exposure to air during 3 minutes). For this aim, we will investigate the putative role of hypothalamic neurohormones (AVT and IT) and their receptors (AVTR type 1, AVTR type 2, ITR) at hepatic level. In addition, these results will be supported by evaluation of plasma cortisol levels, which are expected to increase during the stress response, as well as hepatic and plasma metabolites to investigate the hormonal regulation of fish energetic metabolism.

# 3. MATERIALS AND METHODS

### 3.1. Animals maintenance

Immature specimens of gilthead sea bream (*Sparus aurata*, Linnaeus 1758) (*n*= 64, 108.83 ± 1.47 g body mass, 17.79 ± 0.07 cm body length) were provided by "*Servicio Central de Investigación de Cultivos Marinos (SCI-CM)*" (CASEM, University of Cadiz, Puerto Real, Cádiz, Operational Code REGA ES11028000312). The specimens were randomly distributed and maintained in this installation in sixteen 80 L-tanks (*n* = 4, density 5 kg/$m^3$) containing seawater (SW, 38 ‰ salinity), in open circuit, at constant temperature (18-19 °C) and natural photoperiod for our latitude (36°31'44''N, February-March 2013). These conditions were maintained both during a previous acclimation period of 15 days and during the time that experiment lasted. Fish were fed every day with commercial pellets (Trow España S.A., Cojóbar, Burgos) with a daily ration of 1% weight, except the day before to the start of the experiment where the animals were fasted. The maintenance of the tanks during the acclimation period was carried out daily by siphoning to remove faces and possible remains of food.

### 3.2. Experimental design and sampling

After the acclimation period *S. aurata* specimens held in 12 of the 16 tanks were captured all together and moved to a rigid mesh where they were exposed to air conditions for 3 minutes. After this time of exposition, fish were returned to their respective tanks. No mortality was observed during the time that experiment lasted (Figure 3.1.).

The sampling was performed in the following experimental times: 0 hours (control group without stress), 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 8 hours after air exposition. For each experimental time 2 tanks (*n* = 4 per tank) were used, so a total of 8 specimens for experimental time were sampled. In addition, the other 4 tanks constituted, in duplicate, both control groups at i) 0 hours and ii) 8 hours without stress (Vera et al., 2014). The extra control group without stress was also sampled 8 hours after the start of the experiment. This last group served to identify and discard possible circadian rhythms in the levels of metabolites and expression patterns of those genes analysed that could mask our results (Vera et al., 2014).

Specimens of each tank were anesthetized, measured, weighed and killed by decapitation. Blood samples were collected from the caudal peduncle into 1-mL ammonia-heparinised syringes

(Heparin ammonium Sigma, H-6279, 25000 units/3 mL 0.9% NaCl saline) to prevent coagulation during extraction, and centrifuged (3 min at 13,000 g; ALC centrifuge, model 4204) to obtain plasma, snap-frozen in liquid nitrogen and stored at -80 ° C until use. To obtain liver samples, a longitudinal cut was made in the ventral area (from the anal opening to the base of the mandible) to leave the entire digestive tract exposed. After removing the viscera, the liver was removed, weighed and divided into 2 portions that were introduced in 1.5 mL Eppendorf tubes for subsequent measurement of metabolites. The freezing process was the same of the plasma. For studies of molecular biology, both hypothalamic lobes and a biopsy (~15 mg) of liver were deposited in Eppendorf tubes containing 500% (v/w) of *RNAlater* (Ambio®, Applied BioSystems). These samples were stored 24 hours at 4 °C and subsequently stored at -20 °C until further processing.

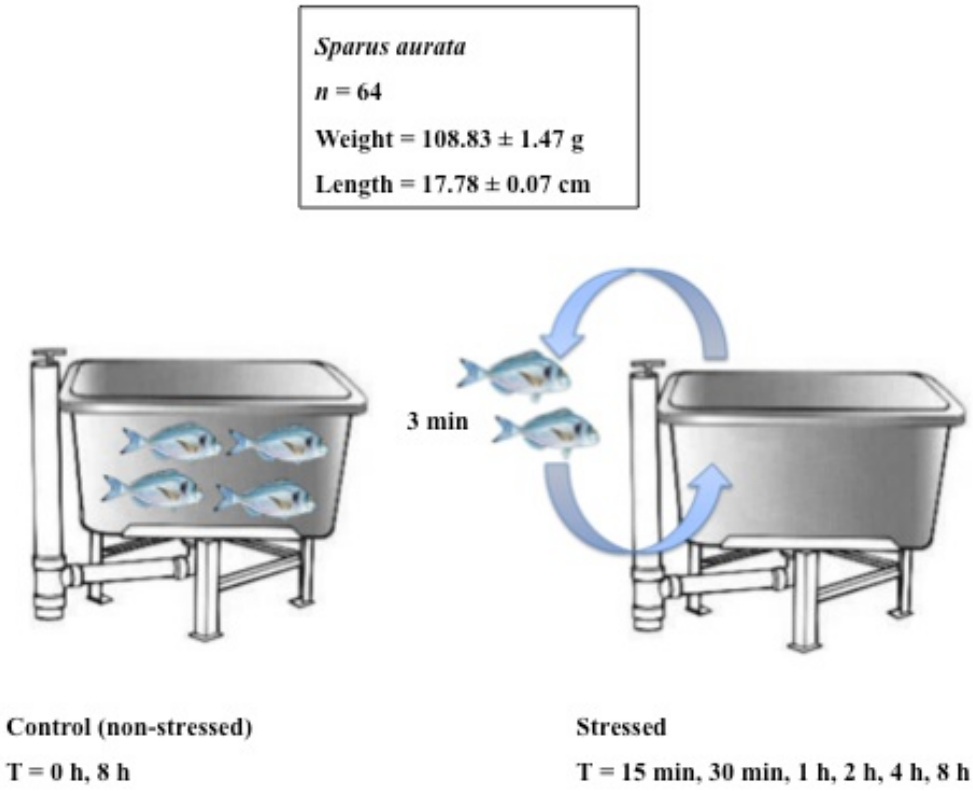


**Figure 3.1.** Scheme of the experimental design used in this study.

### 3.3. Analysis techniques

#### *3.3.1. Plasma metabolites*

The analyses of all plasma metabolites were performed with specific commercial kits (Table 3.1.) adapted to 96-well microplates. Each sample was measured in duplicate using 10 µL of plasma, or from those dilutions described in Table 3.1, according to the methodology described by the manufacturer. Samples were read using an automated microplate reader (*PowerWave 340*, BioTek Instrument Inc., Winooski, USA) controlled by the *KCjunior*$^{TM}$ software.

| Metabolites | Commercial Kit | Standard | Abs (nm) | Diluicion |
|---|---|---|---|---|
| **Glucose** | *Glucose-HK*<br>Spinreact, Ref. 1001200 | Glucose anhydrous Panreac, Cod. 131341 | 340 (read.) | 1/1 |
| **Lactate** | *Lactate*<br>Spinreact, Ref. 1001330 | 10 mg/dl<br>Spinreact, Ref. 1002460 | 340 (read.)<br>630 (ref.) | 1/6 |
| **Triglycerides** | *Triglycerides*<br>Spinreact, Ref. 1001311 | 200 mg/dl<br>Spinreact, Ref. 1002380 | 340 (read.)<br>550 (ref.) | 1/6 |
| **Proteins** | *BCA*$^{TM}$ *Protein Assay Kit*<br>Pierce P.O. #23225 | 2 mg/mL<br>BCA, Prod #23209 | 550 (read.)<br>630 (ref.) | 1/50 |

**Table 3.1.** References of the commercial kits and dilutions used for the measurement of different plasma metabolites. Absorbances (Abs, nm) used are marked as reading (read.) and reference (ref.) in each case.

#### *3.3.2. Liver metabolites*

Liver biopsies used for the assessment of metabolite levels were finely minced on an ice cold Petri dish and subsequently homogenized by mechanic disruption (Ultra-Turrax, T 25 basic, IKA®-WERKE) with 7.5 vol. (w/v) of ice-cooled 0.6 N perchloric acid (Merck KGaA, 1.00518.1001) and neutralized after the addition of the same volume of 1 M $KHCO_3$ (Merck KGaA, 1.04854.0500). This homogenization resulted in a dilution of 15 times the weight of the tissue. Previous to centrifugation, an aliquot of each homogenate was separated for triglycerides (TAG) measurements. After that, the homogenates were centrifuged (30 min, 13,000 g, 4 °C) and the supernatants

(aqueous phase) were recovered in different aliquots, which were stored at -80 °C until use in metabolite assays, following the manufacturer methodology described in Table 3.2. Samples were read using an automatic microplate reader (P*owerWave 340,* BioTek Instrumincanteent Inc., Winooski, USA) controlled by the *KCjunior*$^{TM}$ software.

| Metabolites | Commercial Kit | Standard | Abs (nm) | Diluicion |
|---|---|---|---|---|
| **Glucose / Glycogen(*)** | *Glucose-HK*<br>Spinreact, Ref. 1001200 | Glucose anhydrous<br>Panreac, Cod. 131341 | 340 (read.) | 1/330 |
| **Lactate** | *Lactate*<br>Spinreact, Ref. 1001330 | 10 mg/dl<br>Spinreact, Ref. 1002460 | 340 (read.)<br>630 (ref.) | 1/15 |
| **Triglycerides** | *Triglycerides*<br>Spinreact, Ref. 1001311 | 200 mg/dl<br>Spinreact, Ref. 1002380 | 340 (read.)<br>550 (ref.) | 1/15 |

**Table 3.2.** References of the commercial kits and dilutions used for the measurement of each of different hepatic metabolites. Absorbances (Abs, nm) used are marked as reading (read.) and reference (ref.) in each case. (*) The results of glycogen are obtained from the difference between samples measured with amyloglucosidase (Sigma, A7420-100mg), enzyme responsible for the breakdown of glucose-glucose bonds, and free glucose present in the liver.

### *3.3.3. Measurement of plasma cortisol*

Plasma cortisol levels were measured by using the ELISA (Enzyme-Linked Immunosorbent Assay) as previously described by Martos-Sitcha et al. (2014b) for this species. This technique is based on the use of antigens or antibodies with an enzyme, in a way that the resulting conjugates have both immunological and enzymatic activity. In this regards, being one of the components (antigen or antibody) coupled to an enzyme and insolubilized on a support (immunoadsorbent, 96-well microplate), the antigen-antibody reaction is immobilized and, therefore, is easily revealed by the action of a specific substrate that acting on the enzyme, which produces colour observable and measurable with the use of a spectrophotometer or a colorimeter at 405 nm in the case of cortisol.

The general steps followed for the development of the technique are:
1. Dilution of 5 µL of plasma in 100 µL of RB (100 µL 1 M PPB (Potassium Phosphate Buffer), 0.1 g NaN3, 0.37 g EDTA, 1 g BSA (Bovine Serum Albumin) buffer, screed to 1 L with water

distilled), and subsequent extraction of cortisol in three stages by the addition of 600, 300 and 300 µL of methanol, respectively.

2. Coating of the wells with the monoclonal antibody (Mouse Monoclonal Ab, Cat. 400003, Cayman Chemical Company).

3. Washing of the wells with a Wash Buffer, consisting in 10 mL of PPB 1 M, 0.5 mL of Tween 20 and screed up to 1 L with distilled water (scrubber ImmunoWash MODEL 1575, BIO-RAD), to remove the excess of unbound monoclonal antibody from each well.

4. Adding samples and standards to their respective wells, and mix with the second and specific antibody (Cortisol Express EIA Antiserum, Cat. # 10007334), producing the binding of this monoclonal antibody with its antigen (cortisol) during 1 h of incubation at 25ºC.

5. Washing of the well to remove excess of unbound enzyme.

6. Adding the substrate (Ellman reagent) to the enzyme, and binding of both of them.

7. Colour development by incubation with continuously shaking at 25 °C for 15-30 minutes and measurement at 405 nm in the microplate reader (Benchmark Microplate Reader, BIO-RAD).

Finally, from the absorbance obtained from standard curve, we are able to calculate the concentration of cortisol hormone in each plasma sample. Subsequently, a correction of the real concentration according to dilutions made from the initial 5 µL of plasma was necessary. In our case, this factor was between 50 and 150 times according to the period elapsed after air exposition as well as the experimental group (control or air-exposed) in each case.

### *3.3.4. Total RNA extraction*

Total RNA was isolated from complete hypothalamus, as well as from liver biopsies, using the NucleoSpin®RNA II kit (Macherey-Nagel, Cat. # 740955), which allows the maximum purification of 100 µg total RNA from less than 30 mg of tissue. Moreover, the on-column RNase-free DNase digestion was used for gDNA elimination. The manufacturer's instructions were followed in this procedure. Additionally, the amount of RNA was spectrophotometrically measured by the fluorimetric determination with *Qubit® 2.0 Fluorometer* (Invitrogen™, Life Technologies) using 3 µL of each of the samples and its quality was determined in a 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies). Subsequently, total RNA was stored at -80 °C until analysis. Only samples with a RNA Integrity Number (RIN) higher than 8.5 were used for real-time PCR (qPCR).

*3.3.5. Relative expression analysis by real-time PCR (qPCR)*

Total RNA (500 ng) was reverse-transcribed in a 20 µL reaction using the commercial kit *qScript™ cDNA Síntesis Kit* (Quanta Biosciences, Cat. # 95047-500), which is valid for the synthesis of cDNA from amounts of total RNA between 10 pg and 1 µg. Briefly, the reaction was performed using qScript Reaction Mix (1x final concentration) and qScript Reverse Transcriptase (2.5 x final concentration). The reverse transcription program was carried out using the *Mastercycler®ep Realplex 2S* (Eppendorf), and consisted in a reaction of 5 min at 22 °C, 30 min at 42 °C and 5 min at 85 °C.

For semi-quantitative (qPCR) mRNA expression of each of the genes of interest, primer oligonucleotide sequences and the final concentrations used in the qPCR reactions are shown in Table 3.3. and were as described by Martos-Sitcha et al. (2013b, 2014a). These conditions were used due to the previous validation test for each of the primer pairs, checking the optimal range of cDNA concentration to ensure efficiencies of reaction (E) near to E≈1, and regression fits ($r^2$) higher than $r^2$=0.99. Each reaction mixture (10 µL) contained 4 µL of cDNA (10 ng from total RNA input), 0.5 µL each specific forward and reverse primer, and 5 µL *PerferCTa™ SYBR® Green FastMix™* (Quanta Biosciences, Cat. # 95072-012) by using the *Mastercycler®epgradient S Realplex²* controlled by the software *Realplex* (Eppendorf, version 2.2). Reactions were conducted in semi-skirted twin.tec 96-well real-time PCR plates (Eppendorf) covered with adhesive Masterclear real-time PCR Film (Eppendorf). The thermocycling procedures were as previously described by Martos-Sitcha et al. (2013b, 2014a). In short, the PCR profile was as follow: (95 ºC, 10 min; [95 ºC, 20 s; 60 ºC, 35 s] X 40 cycles; melting curve [60 ºC to 95 ºC, 20 min], 95 ºC, 15 s). Melting curves were used to ensure that only a single PCR product was amplified and to verify the absence of primer-dimer artefacts. Each sample was run in triplicate. The results were normalised to β-actin (GenBank accession number X89920) levels, which was use like internal control (*housekeeping gene*) for to check the level of expression (in number of cycles); this is due to its low variability (less than 0.07 $C_T$ in hypothalamus and 0.09 $C_T$ in liver) under our experimental conditions. Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), based on:

$$2^{-\Delta\Delta C_T} = 2^{-(\Delta C_T \text{sample} - \Delta C_T \text{calibrador})}$$

Which is equal to:

$$2^{-[(C_T \text{sample} - C_T \text{housekeeping gene}) - (C_T \text{calibrator} - C_T \text{housekeeping gene})]}$$

| *Primers* | *Nucleotide sequence* | *Size amplified* | *Primer concentration* |
|---|---|---|---|
| **qPCR-proVTF** | 5'-AGAGGCTGGGATCAGACAGTGC -3' | 129 pb | 200 nM |
| **qPCR-proVTR** | 5'-TCCACACAGTGAGCTGTTTCCG-3' | | |
| **qPCR-proIT$_F$** | 5'-GGAGA TGACCAAAGCAGCCA-3' | 151 pb | 200 nM |
| **qPCR-proIT$_R$** | 5'-CAACCATGTGAACTACGACT-3' | | |
| **qPCR-A VTR-V1$_F$** | 5'-GACAGCCGCAAGTGA TCAAG-3' | 203 pb | 400 nM |
| **qPCR-A VTR-V1$_R$** | 5'-CCCGACCGCACACCCCCTGGCT-3' | | |
| **qPCR-A VTR-V2$_F$** | 5'-A TCACAGTCCTTGCA TTGGTG-3' | 120 pb | 600 nM |
| **qPCR-A VTR-V2$_R$** | 5'-GCACAGGTTGACCA TGAACAC-3' | | |
| **qPCR-ITR$_F$** | 5'-GGAGGATCGTTTTAAAGACATGG-3' | 120 pb | 400 nM |
| **qPCR-ITR$_R$** | 5'-TGTTGTCTCCCTGTCAGA TTTTC-3' | | |
| **qPCR-β-actin$_F$** | 5'-TCTTCCAGCCA TCCTTCCTCG-3' | 108 pb | 200 nM |
| **qPCR-β-actin$_R$** | 5'-TGTTGGCATACAGGTCCTTACGG-3' | | |

**Table 3.3.** Primer sequences of gilthead sea bream used for measuring expression. Take from Martos-Sitcha et al. (2013b, 2014a).

### 3.4. Statistical analysis

Significant differences between groups were analysed by one-way analysis of variance (one-way ANOVA), taking the experimental time from air exposition as the main factor (T = 0 h, 15 min, 30 min, 1 h, 2 h, 4 h and 8 h). When significant differences were detected, the Tukey's test for comparison of the groups was used. Prior to the execution of the analysis of variance, test for normality and homoscedasticity was run. In addition, for the comparison between groups non-stressed at times T = 0 h and T = 8 h, as well as between the groups with and without stress at time T = 8 h, a Student's *t* test analysis was used. In all analysis performed, statistical significance was accepted at $P<0.05$. All statistical analysis were performed using the GraphPad Prism® (v.5.0b) for Macintosh.

# 4. RESULTS

### 4.1. Plasma metabolites

Metabolic responses of *S. aurata* specimens submitted to air exposure (3 min) at plasma level are shown in Table 4.1. All plasma metabolites analysed did not show changes in specimens maintained under normal conditions (control, non-stressed) at 0 and 8 hours post-emersion. Plasma glucose rapidly increases its values, which being significantly higher 15 min after air exposure when compared with control group, reaching more than 2-fold increase at end of experimental time. In turn, lactate values in air-exposed fish enhanced more than 4-fold within 15 min compared with unstressed specimens, and then decrease to levels similar to those of departure. However, a second increase occurred 4 hours after air exposure, while at end of experimental time (8h) no differences between both groups sampled at 8 hours (with and without stress) were observed. Moreover, plasma proteins showed a gradual decrease in its values, which only was statistically different 4 hours after emersion. Triglyceride levels did not show significant differences during the time that experiment lasted. In addition, glucose and proteins levels were statistically different at the end of the experiment when compared both stressed and non-stressed specimens.

### 4.2. Hepatic metabolites

Table 4.2. showed changes in hepatic metabolites in *S. aurata* specimens subjected to an acute stress situation produced by air exposure during 3 min. Hepatic free glucose increased rapidly 15 min after air exposure, but at 30 min. showed similar values that control group. After that, enhanced its values until the end of experimental time. Moreover, significant differences were found between both groups maintained under normal conditions (control, non-stressed fish), with higher values in the group sampled after 8 hours compared to those of the group sampled at time 0 hours, as well as when compared with stressed fish at 8 hours. In turn, hepatic glycogen levels decreased clearly from the first 15 min post-stress until the end of experimental time, but that resulted in a non-statistical difference when compared with the control group at 0 hours. In addition, hepatic lactate presented a two-pick increase during the first 15 min after the emersion, as well as at the end of the trial, leading to a significant differences between both stressed and unstressed fish (control group) after 8 hours of experiment. Triglyceride levels did not show significant differences during the time that experiment lasted, in the same way that those observed in plasma.

| Plasma metabolites | 0 h | 15 min | 30 min | 1 h | 2 h | 4 h | 8 h | 8 h without stress |
|---|---|---|---|---|---|---|---|---|
| **Glucose (mM)** | $4.16 \pm 0.14^{a}$ | $6.18 \pm 0.62^{b}$ | $6.53 \pm 0.25^{bc}$ | $6.40 \pm 0.49^{bc}$ | $6.99 \pm 0.28^{bc}$ | $6.56 \pm 0.44^{bc}$ | $8.49 \pm 0.20^{c*}$ | $4.97 \pm 0.15^{a}$ |
| **Lactate (mM)** | $0.37 \pm 0.05^{a}$ | $1.71 \pm 0.27^{b}$ | $0.65 \pm 0.17^{ab}$ | $0.85 \pm 0.17^{ab}$ | $0.37 \pm 0.08^{a}$ | $1.47 \pm 0.28^{b}$ | $0.34 \pm 0.08^{a}$ | $0.26 \pm 0.02^{a}$ |
| **Triglyceride (mM)** | $1.16 \pm 0.13$ | $1.25 \pm 0.11$ | $1.33 \pm 0.18$ | $1.32 \pm 0.12$ | $1.21 \pm 0.11$ | $1.27 \pm 0.13$ | $1.138 \pm 0.08$ | $1.05 \pm 0.07$ |
| **Proteins (mg/mL)** | $45.89 \pm 1.87^{a}$ | $44.64 \pm 1.63^{ab}$ | $42.52 \pm 1.53^{ab}$ | $42.17 \pm 2.89^{ab}$ | $42.27 \pm 1.26^{ab}$ | $38.54 \pm 1.72^{b}$ | $44.09 \pm 2.24^{ab*}$ | $50.66 \pm 0.48^{a}$ |

**Table 4.1.** Time course changes in plasma metabolites (glucose, lactate, triglycerides and total proteins) levels in *S. aurata* exposed to air for 3 min. Values are represented as mean ± S.E.M. ($n$ = 8 fish per group). Significant differences within each group for different experimental times are identified with different letters; asterisks (*) indicate significant differences between groups at the same time ($P<0.05$, one-way ANOVA followed by Tukey's test).

| Liver metabolites | 0 h | 15 min | 30 min | 1 h | 2 h | 4 h | 8 h | 8 h without stress |
|---|---|---|---|---|---|---|---|---|
| **Glucose (µmol/g ww)** | $30.86 \pm 1.50^{a}$ | $37.95 \pm 2.57^{b}$ | $26.79 \pm 1.59^{a}$ | $31.91 \pm 1.10^{ab}$ | $34.97 \pm 1.92^{ab}$ | $37.06 \pm 1.74^{ab}$ | $39.56 \pm 3.50^{ab*}$ | $44.91 \pm 2.44^{b}$ |
| **Glycogen (µmol/g ww)** | $154.62 \pm 8.90^{a}$ | $138.56 \pm 2.89^{ab}$ | $139.10 \pm 5.73^{ab}$ | $143.72 \pm 4.76^{ab}$ | $136.69 \pm 3.55^{ab}$ | $134.46 \pm 7.05^{ab}$ | $136.05 \pm 4.51^{ab*}$ | $122.53 \pm 6.61^{b}$ |
| **Lactate (µmol/g ww)** | $0.011 \pm 0.001^{a}$ | $0.017 \pm 0.001^{b}$ | $0.012 \pm 0.001^{ab}$ | $0.013 \pm 0.001^{ab}$ | $0.012 \pm 0.001^{ab}$ | $0.012 \pm 0.001^{ab}$ | $0.016 \pm 0.001^{b*}$ | $0.009 \pm 0.003^{a}$ |
| **Triglyceride (µmol/g ww)** | $0.09 \pm 0.01$ | $0.05 \pm 0.02$ | $0.08 \pm 0.01$ | $0.10 \pm 0.01$ | $0.10 \pm 0.01$ | $0.5 \pm 0.02$ | $0.01 \pm 0.01$ | $0.09 \pm 0.01$ |

**Table 4.2.** Time course changes in hepatic metabolites (glucose, glycogen, lactate, triglycerides) levels in *S. aurata* exposed to air for 3 min. Values are represented as mean ± S.E.M. ($n$ = 8 fish per group). Significant differences within each group for different experimental times are identified with different letters; asterisks (*) indicate significant differences between groups at the same time ($P<0.05$, one-way ANOVA followed by Tukey's test).

### 4.3. Plasma cortisol levels

Specimens stressed by emersion for 3 min revealed significant differences in plasma cortisol levels throughout the experimental period (Figure 4.1.). Thus, a significant increase in hormonal values was observed at 15 and 30 min post-emergence, reaching the highest levels at 15 min post-stress with more than 6-fold enhancement respect to control group. Then, cortisol values decreased over time until reaching values similar to those found in unstressed fish, both at 0 hours and 8 hours of the experiment. In addition, control group at 8 hours showed no significant differences when compared with time 0 hours group, or even with animals from the stressed group after 8 hours.

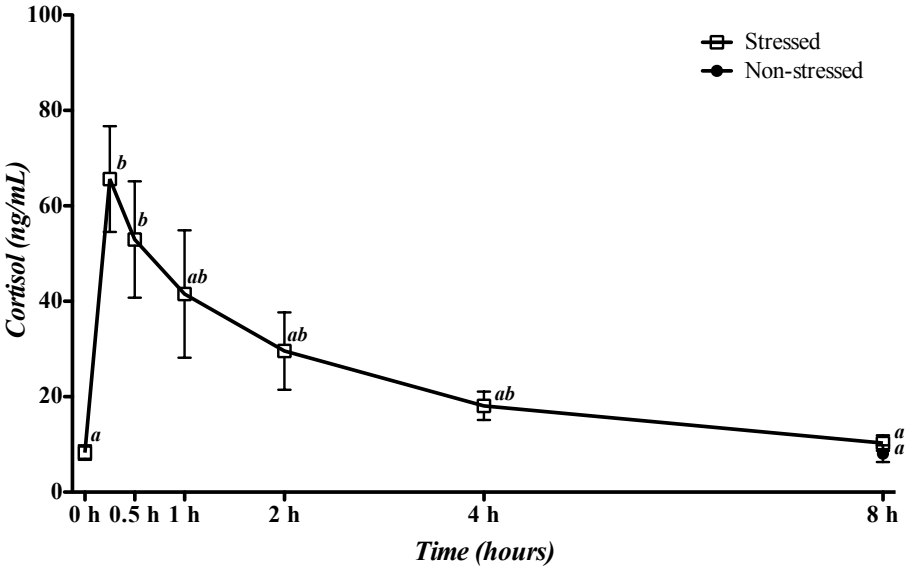


**Figure 4.1.** Time course changes in plasma cortisol levels in *S. aurata* exposed to air for 3 min. Values are represented as mean ± S.E.M. ($n$ = 8 fish per group). Significant differences within each group for different experimental times are identified with different letters ($P<0.05$, one-way ANOVA followed by Tukey's test).

### 4.4. Hypothalamic pro-VT and pro-IT mRNA expression

Specimens of the group exposed to air (3 min) significantly increased both pro-VT (Figure 4.2.A) and pro-IT (Figure 4.2.B) mRNA expression levels 1 hour after stress. Then, mRNA values gradually decreased back to baseline values after 4 hours, maintaining these expression levels until the end of experiment. Meanwhile, control group, not subjected to stress and sampled at 8 hours, showed no significant differences when compared to control group at 0 hours as well as to those found 8 hours after stress.

### 4.5. Hepatic AVT and IT receptors mRNA expression

Hepatic mRNA levels of the two AVT receptors, AVTR V1-type and AVTR V2-type, after exposure to air for 3 min showed a different trend in their mRNA expression. AVTR V1-type significantly increased its mRNA levels 15 min post-stress decreasing rapidly to similar values found in the control group (0 hours) 30 min after air exposure, and maintaining this basal values until the end of the experiment (Figure 4.3.A). However, AVTR V2-type mRNA expression levels revealed a two-phase pattern. Thus, there was an increase in the first 15 min post-stress, with a subsequent decrease presenting its lowest levels 1 hour after air exposure. In addition, a further increase in the mRNA expression of this receptor was observed 4 hours after the stress, with an ulterior decrease close to those values observed in control group (0 hours) (Figure 4.3.B). Finally, ITR gene expression showed a similar trend to that observed in AVTR 2-type, with a rapid increase of its mRNA levels during the first 30 min after emersion. Subsequently, a significant fall-down was observed 1 hour post-stress with a posterior and gradual recovery of its values close to those found at 0 hours (4.3.C). Moreover, no differences between mRNA expression levels of the three receptors studied was observed between the specimens sampled at time 8 hours post-stress and the two groups at normal conditions (0 hours and 8 hours without stress).

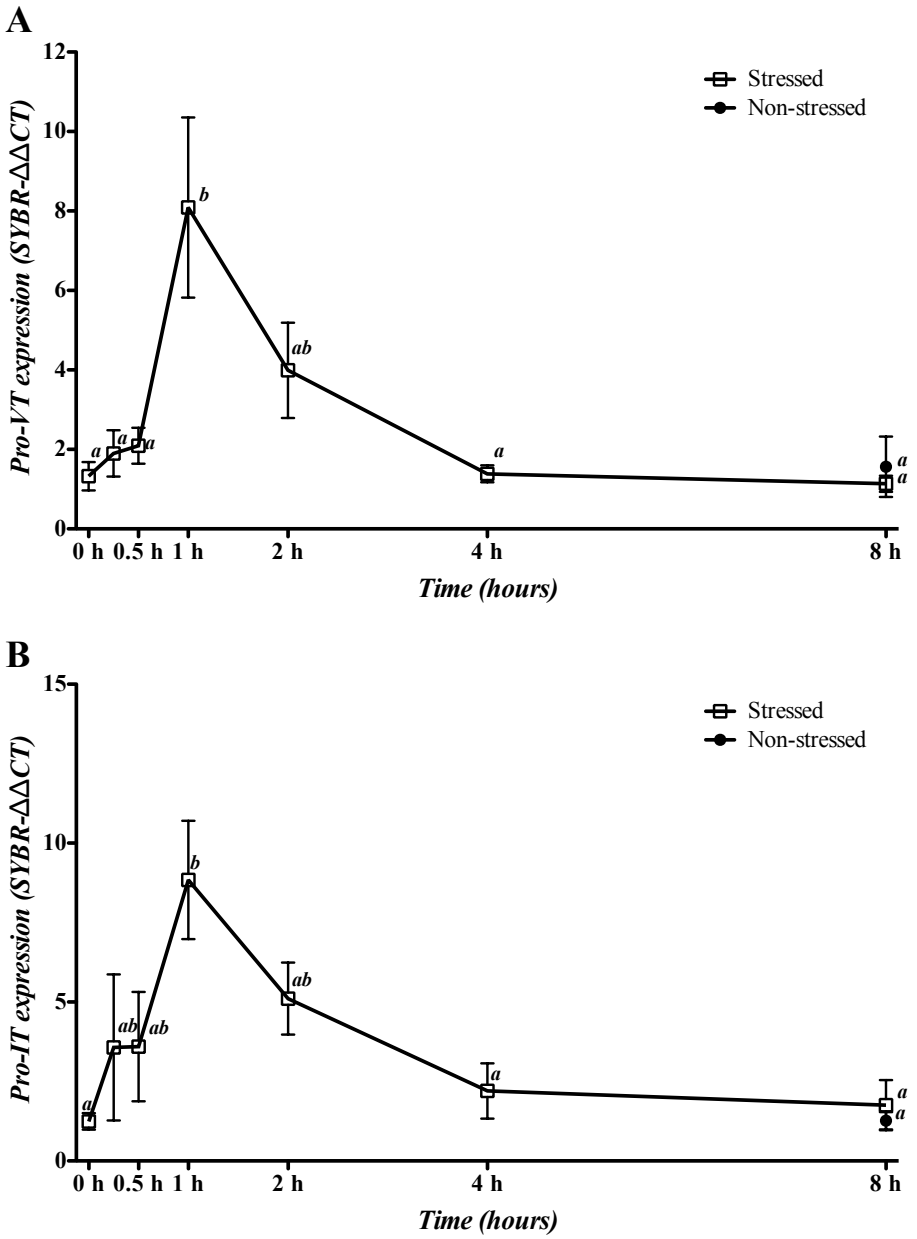


**Figure 4.2.** Time course changes in hypothalamic pro-VT (A) and pro-IT (B) mRNA expression levels (relative to β-actin) in *S. aurata* exposed to air for 3 min. Values are represented as mean ± S.E.M. ($n$ = 8 fish per group). Significant differences within each group for different experimental times are identified with different letters ($P<0.05$, one-way ANOVA followed by Tukey's test).

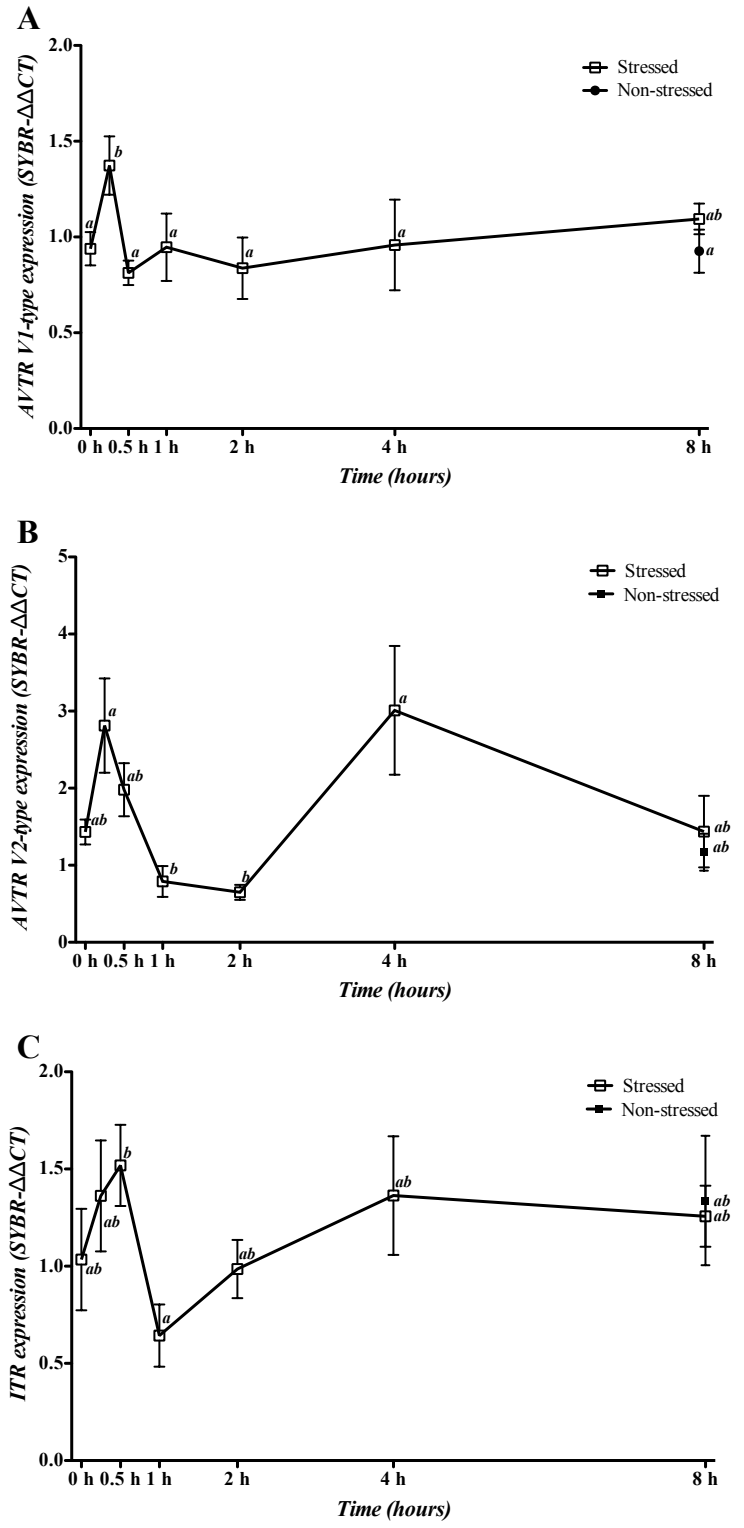


**Figure 4.3.** Time course changes in hepatic AVTR V1-type (A), AVTR V2-type (B) and ITR (C) mRNA expression levels (relative to β-actin) in *S. aurata* exposed to air for 3 min. Values are represented as mean ± S.E.M. (*n* = 8 fish per group). Significant differences within each group for different experimental times are identified with different letters (P<0.05, one-way ANOVA followed by Tukey's test).

# 5. DISCUSSION

In aquaculture, fish are continually subjected to different management practices such as handling, transport or confinement that cause different stress responses (Davis et al., 2002). In the literature there are numerous studies on the response of teleost fish to different typical stress situations in aquaculture. In addition, it has been addressed several aspects of stress system, from the metabolic effects to its regulation by different endocrine systems (Tort et al., 1996; Waring et al., 1996; Arends et al., 1999; Barton et al., 2002). Most of these studies have been carried out in the most important species for aquaculture, especially in salmonids and cyprinids. Moreover, the response of each species previously studied differs about the mechanisms that are triggered to maintain homeostasis after a stressful situation (Wedemeyer et. al., 1990). Physiological changes described after a stressful situation include variations at hematologic level (Dethloff et al., 1999), balance in the osmolyte concentration (McDonald and Milligan, 1997) and various changes in plasma hormones and metabolic pathways (Iwama and Barton, 1991; Carragher and Rees, 1994). One of the physiological responses in vertebrates, including fish, after exposure to a stressor is increased glucose and plasma cortisol (Pickering and Pottinger, 1989; Barton and Iwama, 1991; Barton, 2002; Ortuño et al., 2002). Thus, these parameters are considered as the most reliable methods for distinguish between a situation of stress and rest (Thompson et al., 1993; Biron and Benfey, 1994; Yin et al., 1995; Rotllant and Tort, 1997).

### 5.1. Cortisol

The variation of plasma cortisol levels in response to acute stressors has been defined within the range about 30 to 300 ng/mL (Wedemeyer et al, 1990; Barton and Iwama, 1991), although these variations depend not only on the source of stress but also on the species used as biological model. Barton et al. (1998, 2000) found that the highest levels of cortisol after an acute stress manipulation were lower in sturgeon (*Scaphirhynchus spp.*) and in paddlefish (*Polyodon spathula*). Their results suggest a trend toward lower stress responses in chondrichthyans compared with teleosts. However, Belanger et al. (2001) found that plasma cortisol in white sturgeon (*Acipenser transmontanus*) was about 40 ng/mL, indicating that lower stress responses cannot be a universal phenomenon in this group of fish. Among teleosts, some species also have low responses to acute stressors. In this way, Atlantic cod (*Gadus morhua*) peaks plasma cortisol levels with 15 ng/mL after handling (Hemre et al., 1991). However, Maule et al. (1988) demonstrated increases in the levels of this hormone near to 400 ng/mL in juveniles of Chinook salmon (*Oncorhynchus tshawytscha*) after an acute stress situation generated during and after transport processes. Furthermore, Mazik et al. (1991)

documented that cortisol levels increased up to 2000 ng/mL in striped bass (*Morone saxatilis*) during the recovery phase after 5 hours of transportation, which is one of the highest levels reported in teleosts.

The basal concentration of plasma cortisol obtained for *Sparus aurata* specimens in this study is comparable both to that described in other teleosts (Barton and Iwama, 1991) as well as to that previously reported for the same species (Arends et al., 1999; Ortuño et al., 2001; Sangiao-Alvarellos et al., 2006; Mancera et al., 2007). In our experiment, a significant ≈ 10-fold increase in cortisol levels following exposure to air indicates the activation of stress system with the existence of a primary stress response. This response can be considered temporary, since the plasma concentration of this hormone returned to baseline levels during the first hours post-stress. In a previous experiment with the same species and using the same experimental design an increase of ≈ 50 times in plasma cortisol values was observed (Arends et al., 1999), demonstrating that this experimental design induces an alteration in specimens, activating stress system aimed to restore the homeostatic condition of the animal. The results of our experiments also indicate that in *S. aurata* dynamic response depend on the type of stressor, and also that size of the animals can affect the magnitude of stress response even being the same pattern. However, Arends et al. (1999) did not find that the increase in plasma cortisol was accompanied by an increase in the plasmatic levels of adrenocorticotropic hormone (ACTH). This suggests a cortisol increase ACTH-independent, and hence the results obtained after exposure to air induces a significant increase in cortisol levels to address acute stress, this process must be regulated by different routes/endocrine systems (see below).

### 5.2. Metabolites

Liver is one of the main reservoirs of energy in vertebrates, whereas the brain is responsible for between 1.5% and 8.5% of the total body energy consumption in vertebrates (Van Ginneken et al., 1996). Previous studies have demonstrated that different stress situations such as osmotic challenges, hormonal injections, high stocking densities or fasting processes enhance hepatic glycogenolytic routes and free-glucose production processes in teleosts, including *S. aurata* (Janssens and Lowrey, 1987; Moon and Mommsen, 1990; Mommsen et al., 1999; Sangiao-Alvarellos et al., 2006; Mancera et al., 2008; Cádiz et al., 2015). Moreover, studies with this species have additionally demonstrated that cortisol injection enhanced metabolic rates (Laiz-Carrión et al.,

2003; Sangiao-Alvarellos et al., 2004, 2006; Cádiz et al., 2015). Our results showed a pattern of changes in metabolic parameters at both plasma and hepatic levels that agree also with the previously reported for *S. aurata* submitted to the same experimental design (Arends et al., 1999) or even to abrupt salinity challenges, a situation that also is considered as an acute stress for this specie (Sangiao-Alvarellos et al., 2005). Thus, the enhancement of plasma metabolites (glucose, lactate and protein) levels concomitantly with plasma cortisol values as well as a consumption of energy reserves identified by the lower liver glycogen storage, suggested the existence of a clear energetic reorganization due to stress situation.

Air exposure induces a strong and rapid increase of both glucose and lactate, which point to a sympathetic activation of the chromaffin cells in head kidney with a release of catecholamines (CAs). Thus, arterially infused CAs have been shown to induce hyperglycemia in carp (*Cyprinus carpio*), and increased plasma lactate levels are associated with elevated CA concentrations and hypoxia situation (van Raaji et al. 1995, 1996; Fabbri et al., 1998). As gas exchange is compromised in air-exposed fish, the resulting hypoxia will contribute to the production of high lactate levels (Vijayan et al., 1994; Maxime et al., 1995). Furthermore, it has been show that 30 s of handling in rainbow trout (*Oncorhynchus mykiss*) juveniles results in a rise of plasma glucose levels, even in fish fed cortisol-enriched food to downregulate their cortisol producing cells (Barton et al., 1987). Moreover, hepatic glycogenolysis has been shown to be a source for such a CA-mediated rise of plasma glucose (Vijayan et al., 1994). In addition, higher levels of plasma lactate could reflect the greater supply required by some important tissues (e.g. gills, kidney or brain), where the use of this metabolite in those organs has been demonstrated (Mommsen, 1984; Mommsen et al., 1985; Soengas et al., 1998). Furthermore, this metabolite is produced as a consequence of anaerobic metabolic pathways, so that its role after air exposure situation cannot be rule out as an important player.

In our experiment, plasma proteins values decreased constantly in specimens air exposed, suggesting the use of this metabolite to produce amino acids, which could be used as energy substrates in peripheral tissues via gluconeogenesis (Van den Thillart, 1986; Cowey and Walton, 1989; Mommsen and Walsh, 1992). In addition, those amino acids, product of the protein breakdown, will be also able to enter in gluconeogenic pathways and the Krebs cycle in order to cope with the energetic demands caused by the hypoxia status on the fish (Van Waarde, 1988). On the other hand, triglycerides levels, both in plasma and liver, did not show variations during the time that the experiment lasted, suggesting a lack of involvement of these metabolites (at least

during the first hours) in the short-term response to stress. However, *S. aurata* specimens subjected to a chronic stress situation like osmotic challenge showed an increase of this metabolite at plasma level (Martos-Sitcha et al., 2013), while juveniles of red porgy (*Pargus pargus*) subjected to high stocking density and food deprivation condition presented a decreased plasma triglyceride levels and enhanced hepatic triglycerides values that suggested a mobilization of hepatic lipids as energetic source when protein and glucidic reserves are consumed in this organ.

### 5.3. Hypothalamic mRNA pro-AVT and pro-IT expression

The vasotocinergic and isotocinergic systems present physiological functions (see Introduction), and its activity depends on many external/internal factors that regulate different processes at neuronal level (gene expression, synthesis of proteins, transport, maturation, storage and release) as well as plasmatic AVT/IT values (Martos-Sitcha et al., 2013). In addition, AVT/IT plasma levels decrease due to its elimination both by renal clearance processes and by binding to their specific receptors on target organs (e.g. liver, gill, kidney, etc.) (Ward et al., 1990; Kulczykowska, 2007; Martos-Sitcha et al., 2013). Furthermore, in both vasotocinergic and isotocinergic systems a negative-feedback can inhibit hormonal synthesis and release while maintaining AVT/IT circulating levels in certain limits. This process ensures the survival of the specimen in variable environments and against seasonal changes in the physiological demands (Balment et al., 2006).

In the hypothalamus-pituitary-interrenal axis (HPI), corticotrophs cells of adenohypophysis are responsible for the synthesis of adrenocorticotropic hormone (ACTH). This hormone stimulates the secretion of cortisol in the interrenal tissue (Wendelaar Bonga, 1997), although AVT hypothalamic neurons also innervate these adenohypophyseal cells (Batten et al., 1990). This supports a possible role of AVT in the activation of telesot stress system (Kulczykowska, 2001; Balment et al., 2006).

The effect of stress on AVT/IT synthesis and release depends on the type of stressor applied (Kulczykowska, 2001; Mancera et al., 2008). However, good correlation between stress indicators (e.g. cortisol) and AVT plasma levels allows considered plasma AVT as i) a hormonal marker of the internal status of specimens, ii) an important intermediate in the regulation of stress response, and therefore iii) seems to be a good candidate as indicator for animal welfare (Kulczykowska et al., 2010; Martos-Sitcha et al., 2013). In *S. aurata* it has been shown that AVT and cortisol plasma concentrations increased in specimens submitted to different stress situations, such as cortisol

injection (Cádiz et al., 2015), saline transfer (Kleszczynska et al., 2006; Martos-Sitcha et al, 2014th) or high stocking density (Mancera et al, 2008). In addition, AVT treatment is effective in increasing cortisol levels in *S. aurata* specimens submitted to further stress situation (which activate HPI system, with a clear CRH enhancement), suggesting a cooperation between exogenous AVT and endogenous CRH to activate ACTH cells and consequently interrenal tissue, for increasing plasma cortisol levels (Sangiao-Alvarellos et al., 2006). These dates suggests a possible role of AVT on HPI axis, specifically on ACTH cells stimulation in specimens subjected to chronic stress conditions (Batten et al., 1990; Wendelaar Bonga, 1997). Moreover, according to our results, in situations of acute stress (such as exposure to air for 3 minutes) high pro-AVT expression levels are seen in the first hour post-stress, suggesting a role of AVT also during the acute stress processes. This indicates the existence, from the beginning of the application of the stressor, of a regulation of the entire cascade endocrine triggered and mediated by different endocrine systems that are expressed independently.

Meanwhile, although hypothalamic mRNA expression appeared to be affected by endogenous environmental cycles as has been described in AVP secretion in mammals (Tominaga et al., 1992), the absence of variations in pro-VT expression between specimens not subjected to stress situation for emersion and sampled at different times (0 h and 8 h) suggest that this system, at least in the brain, does not appear to be subjected to circadian variations. However, previous studies in trout (*Oncorhynchus mykiss*) have demonstrated the existence of a circadian cycle in hypothalamic pro-VT expression and pituitary AVT secretion with a peak level at the end of the light phase (Rodriguez-Illamola et al., 2011). In addition, the confirmation of this hypothesis was evaluated by maintaining specimens of *O. mykiss* for 24 hours of continuous light, which showed attenuation of AVT expression levels (Rodríguez-Illamola et al., 2011), suggesting that this endogenous cycle could be species-specific.

The physiological functions of IT in teleosts are not well established, but different authors suggest a role of this hormone in the stress system, enhancing its plasmatic levels under stress situation such as dominance and subordination (*Oreochromis mossambicus*: Almeida et al., 2012), or changes in the reproductive and social status (*Gasterosteus aculeatus*: Kleszczynska et al., 2012).
The results obtained in the present Thesis show a pro-IT mRNA expression enhancement during the first hour after stress, suggesting a possible role of IT in acute stress situations (where an increase in plasma cortisol levels is observed). In *S. aurata*, the existence of a high mRNA pro-IT expression values, pituitary as well as plasma values IT levels after hypersaline changes, suggest that IT could

be involved in modulation of saline stress (Kleszczynska et al., 2006; Martos-Sitcha et al., 2013). However, environmental salinity challenges did not induce modifications in the pattern of IT receptor expression in different tissues, including hypothalamus (Martos-Sitcha et al., 2014a). Even so, treatment with cortisol (that emulated a chronic stress situation with high levels of this hormone) induces changes in both central (brain and pituitary) as well as peripheral (liver, gill and head kidney) players of isotocinergic system (pro-IT, IT and IT receptor) in *S. aurata*, suggesting the involvement of isotocinergic pathways in stress processes (Cádiz et al., 2015). In this regard it has been shown that *S. aurata* specimens under high stocking density and fasting (two situations that can be also considered as chronic stress situations) enhanced plasma cortisol and IT levels (Mancera et al., 2008).

Furthermore, the effect of AVT and/or IT on metabolism associated with stress processes cannot be ignored (Sangiao-Alvarellos et al., 2004; 2006. Martos-Sitcha et al., 2013, 2014b; Cádiz et al., 2015). Previous studies have shown that brain undergoes a metabolic reorganization in specimens subjected to different types of stress, such as food deprivation, exposure to toxic or hypoxia (Soengas and Aldegunde, 2002), hormonal treatments with catecholamines (Sangio-Alvarellos et al., 2003a) or cortisol (Laiz-Carrion et al., 2002, 2003), or in response to osmotic changes (Sangiao-Alvarellos et al., 2003b; Laiz-Carrion et al., 2005; Polakof et al., 2006; Martos-Sitcha et al., 2013). Thus, the experimental design used in this Thesis assumed a stress situation that would require metabolic activation at brain and hepatic levels, so that a stimulation of AVT and IT synthesis and release could be beneficial due to the putative metabolic action of these hormones.

### 5.4. Hepatic mRNA AVT and IT receptor expression

In teleost, the liver is the major organ responsible for energy storage (mainly in the form of glycogen/glucose) as well as for fatty acids synthesis and the processes of aminogenesis and gluconeogenesis (Peragón et al., 1998). The presence of AVT and IT receptors in liver has been described in several teleost species (Moon and Mommsen, 1990; Hausmann et al., 1995; Guibbolini et al., 2000; Konno et al., 2009; Lema, 2010; Martos-Sitcha et al, 2014a), suggesting a putative role of AVT and IT in the energy metabolism control due to the important metabolic role of this organ. So, while the physiological response to the administration of these hormones seems to be unclear in fish (Balment et al., 2006; Kulczykowska, 2007), in *S. aurata* it has been shown that AVT injection and the subsequent transfer to different environmental salinities induced variations in hepatic

metabolism, with a mobilization of glycogen together with a plasma glucose enhancement (Sangiao-Alvarellos et al., 2006). Furthermore, glycogenolytic processes are also stimulated by AVT in amphibian's hepatocytes (Janssens et al., 1983; Ade et al., 1995), and by AVP in mammal's hepatocytes (Smith et al., 2003), so their metabolic function of AVT in liver of *S. aurata* cannot be ruled out.

The increase in the expression levels of the three receptors analysed (AVTR V1a2-type, AVTR V2-type, and ITR) seems to suggest a role of all of them associated with stressful situations that involve high levels of cortisol, with the subsequent need of metabolic activation for energy release into the internal medium of the animal (Laiz-Carrión et al., 2002, 2003; Mommsen, 1999). In this way, the increase observed may indicate a stimulation of gluconeogenesis and glycogenolysis processes in liver. This stimulation of the gluconeogenic potential has been previously demonstrated by our research group in specimens of *S. aurata* treated with cortisol, where high concentrations of this hormone in plasma produces important changes in the activity of several enzymes as fructose-bisphosphatase 1-6 (FBPase) as well as pyruvate kinase (PKase) enzyme activities (Laiz-Carrion et al., 2003), together with an increase in the mRNA expression levels of AVT and IT receptors in liver (Cádiz et al., 2015). Most hepatic glucose production in specimens treated with cortisol is associated with the use of this metabolite in other tissues that require higher energy consumption (Soengas et al., 2007). So, it is known that the increase in plasma glucose is one of the most typical secondary responses to stress, being the result of the mobilization of hepatic glycogen stores, also described in our experiment, as a consequence of activation of glycogenolytic routes cause by the action of catecholamine and/or cortisol (Barton et al., 2002). In this way, it has been demonstrated that after injection of cortisol, hepatic glycogen levels decrease, inferring the actions of cortisol on various metabolic enzymes such as glycogen phosphorylase (GPase) (Laiz-Carrion et al., 2003).

Our results showed a clear and rapid mRNA up-regulation in the expression of both AVTRs, already in the first hour of the experiment and 4 hours post-stress in case of AVTR V2-type. Thus, AVTR V2-type function is mediated by PKA activation, suggesting that the metabolic actions of AVT are controlled by this receptor in hypoxia condition due to the higher energy requirement. Furthermore, air exposition stress also increased hepatic AVTR V1a2-type mRNA levels, the effect of which could be associated with the incorporation of inorganic phosphate in phosphatidyl inositol routes, acting as a substrate for phospholipase C, which is the intracellular pathway for this type of AVT receptor (Kirk et al., 1977, 1979). These results suggest that hepatic gene activation of V1a2-type and/or V2-type receptors is related to the control of the energy supply organs due to the higher

requirements produced by the air exposition challenge, both regulating those enzyme activities controlled by the same intracellular pathways (Martos-Sitcha et al., 2014a).

To our knowledge, only few data are available concerning the physiological role of IT, mainly about its osmoregulatory role (Hiraoka et al., 1996; Kulczykowska, 1997; Warne et al., 2000; Kleszczynska et al., 2006; Motohashi et al., 2009). However, hepatic ITR expression appears to be undisturbed by osmotic challenge, suggesting a minor role (if any) for IT in *S. aurata* osmoregulatory system during salinity challenge (Martos-Sitcha et al., 2014a). However, specimens of *S. aurata* treated with exogenous cortisol, which simulates a chronic stress situation, showed a late activation of the ITR, suggesting a regulatory action of IT on the liver (Cádiz, et al., 2015). Our results indicated a rapid ITR expression enhancement in the first hour post-emersion and a subsequent increase 4 hours after stress (in a way similar to what was seen for the AVT V2-type). These corroborate a role of this hormone in the regulation of the stress response, which may trigger processes of metabolic reorganization at hepatic level in *S. aurata*.

### 5.5. AVT, IT and stress: an integrative view

In this work we studied the response of vasotocinergic and isotocinergic systems after an acute stress situation caused by exposure of *S. aurata* specimens to air for 3 minutes (Figure 5.1.).

There is an increase in the hypothalamic pro-VT and pro-IT expression at 1 h post-emersion. At hepatic level, considering the changes observed in receptors gene expression (together with the metabolites modifications seen at hepatic and plasmatic levels), we propose that the both AVT and IT receptors may trigger a metabolic reorganization in the liver: with an initial regulation by all three receptors and later regulation by AVTR V2-type and ITR. In addition, the role of catecholamines and cortisol cannot be rule out (Mazeaud et al, 1981; Wendelaar Bonga, 1997).

Acute stress induces a rapid increase in plasma cortisol levels, showing high values of this hormone 15 minutes after air exposure. In specimens of *S. aurata* treated with exogenous cortisol, this hormone induced significant changes in energy metabolism in the liver, brain and gills (Laiz-Carrión et al., 2002, Cádiz et al., 2015). In our study, we correlated air exposition with plasma glucose and lactate levels enhancement together a decrease in hepatic glycogen and rise of glucose, all from the first 15 minutes post-emersion. This fact suggests that the increased energy requirements due to hypoxia condition are fulfilled both from glucose and lactate.

In specimens subjected to experimental situations that increased plasma concentration of AVT (e.g., hyperosmotic challenges, or AVT treatment) (Kleszczynska et al., 2006; Sangiao-Alvarellos et al., 2006), a plasma cortisol level enhancement has been reported, suggesting a strict relationship between the AVT and ACTH. In this way, it can be inferred that AVT receptors (one of them, at least) could be involved in stress processes (both acute and chronic). In fact, in specimens subjected to high stocking density (Mancera et al., 2008) or osmotic changes (Martos-Sitcha et al., 2013b, 2014a) showed a stimulation of hypothalamic pro-VT expression and its receptors, as well as plasma AVT levels in plasma, which reflect the physiological regulation performed by this endocrine system. In addition, in specimens of *S. aurata* treated with exogenous cortisol, which could be assimilate as a chronic stress situation, a decreased in the expression of AVT receptor V1-type has been reported, suggesting i) the existence of an inhibition of the expression due to the circulating levels of this hormone, and ii) a likely involvement of the vasotocinergic system in chronic stress processes for this species (Cádiz, et al., 2015). The results of this work also suggest a role of vasotocinergic system after an acute stress situation, as shown in the initial activation of

these receptor genes during the first moments of stress in which high levels of cortisol production and release are required.

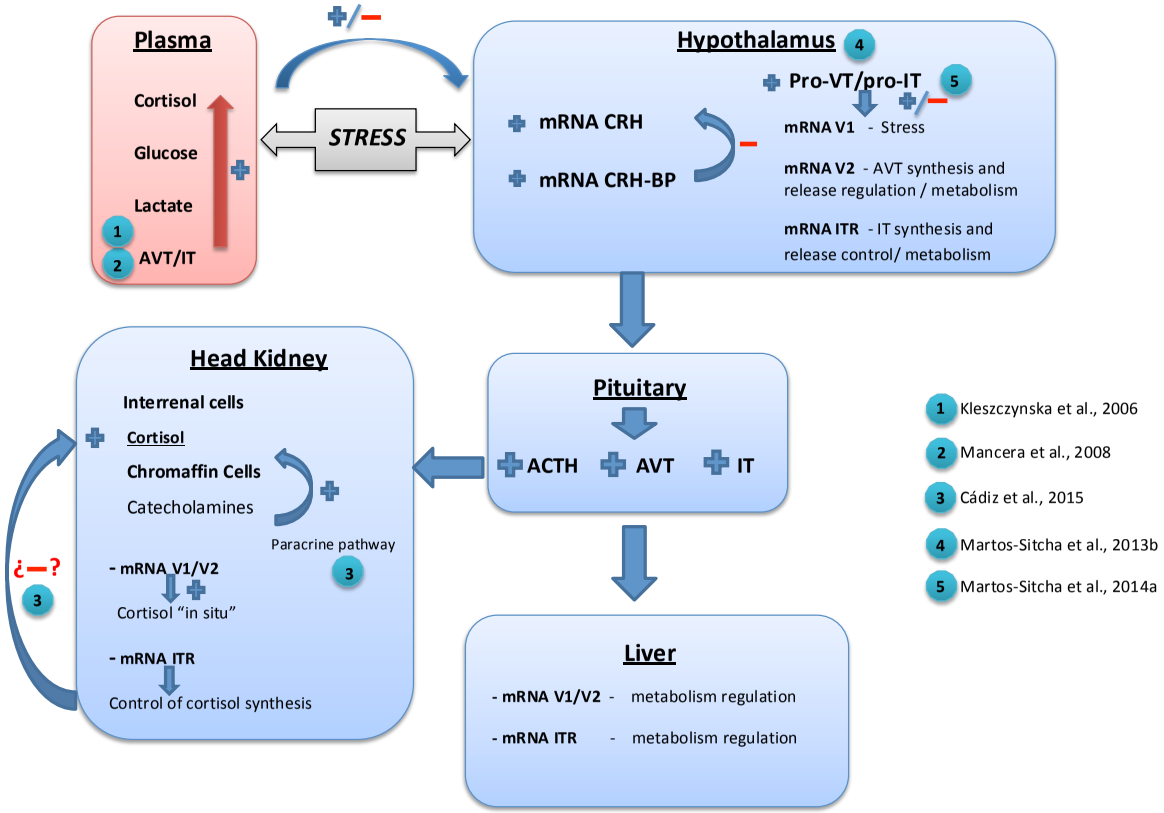


**Figure 5.1.** Scheme-summary of the observed effects on endocrine systems facing a situation of acute stress caused by air exposition.

# 6. CONCLUSIONS

1.- Plasma cortisol levels enhances in *S. aurata* specimens submitted to air exposure for 3 min, indicating that this acute stress situation is effective for inducing an activation of stress system.

2.- The acute stress due to air exposure triggers a metabolic reorganization in *S. aurata* specimens to cope with hypoxia condition induced. The metabolic changes involve i) an increase in hepatic glycogenolysis and lactate production, ii) high levels of glucose and lactate available in plasma, and iii) a decrease in free plasma proteins.

3.- The acute stress response involves changes in gene expression of both hypothalamic AVT and IT precursor levels as well as in their specific hepatic receptors, suggesting that the activation of both vasotocinergic and isotocinergic systems is important during the stress system answer induced by an acute stress situation.

4.- The high mRNA pro-VT and pro-IT expression levels registered one hour after acute stress suggest that both hormones may stimulate/regulate hypothalamic-pituitary-interrenal axis activity to cope with endocrine function and also the subsequent effect on metabolic pathways.

5.- Metabolic reorganization observed at hepatic level seems to be regulated firstly by both AVT (V1a2 and V2 types) and IT receptors, and later on by AVTR V2-type and ITR.

# 7. REFERENCES

Acher, R., 1996. Molecular evolution of fish neurohypophysial hormones: Neutral and selective evolutionary mechanisms. *General and Comparative Endocrinology* 102, 157–172.

Adams, S.M., 1990. Status and use of biological indicators for evaluating the effects of stress on fish. *American Fisheries Society Symposi*um 8, 1-18.

Ade, T., Segner, H., Hanke, W., 1995. Hormonal response of primary hepatocytes of the clawed toad, *Xenopus laevis*. *Experimental and Clinical Endocrinology & Diabetes: official journal, German Society of Endocrinology and German Diabetes Association* 103, 21-7.

Almeida, O., Gozdowska, M., Kulczykowska, E., Oliveira, R.F., 2012 Brain levels of arginine–vasotocin and isotocin in dominant and subordinate males of a cichlid fish. *Hormones and Behavior* 61, 212–217.

Arends, R.J., Mancera, J.M., Munoz, J.L., Wendelaar Bonga, S.E., Flik, G., 1999. The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *The Journal of Endocrinology* 163, 149-57.

Arias, A., 1976. Sobre la biología de la dorada, *Sparus aurata* L., de los esteros de la provincia de Cádiz. *Investigación Pesquera* 40, 201-222.

Baker, B.I., Bird, D.J., Buckingham, J.C., 1996. In the trout, CRH and AVT synergize to stimulate ACTH release. *Regulatory Peptides* 67, 207-210.

Balment, R.J., Lu, W., Weybourne, E., Warne, J.M., 2006. Arginine vasotocin a key hormone in fish physiology and behavior: A review with insights from mammalian models. *General and Comparative Endocrinology* 147, 9-16.

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Reviews of Fish Diseases* 1, 3-26.

Barton, B.A., Rahn, A.B., Feist, G., Bolling. H., Schreck, C.B., 1998. Physiological stress responses of the freshwater chondrostean paddlefish (*Polyodon spathula*) to acute physical disturbances. *Comparative Biochemistry and Physiology* 120A, 355-363.

Barton, B.A., Bolling, H., Hauskins, B.L., Jansen, C.R., 2000. Juvenile pallid (*Scaphirhynchus albus*) and hybrid pallid x shovelnose (*S. Albus* x *S. platorhynchus*) sturgeons exhibit low physiological responses to acute handling and severe confinement. *Comparative Biochemistry and Physiology* 103A, 445-450.

Barton, B.A., 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology* 42, 517-525

Batten T.F.C, Cambre M.L, Moons L., Vandesande F., 1990. Comparative distribution of neuropeptide- immunoreactive systems in the brain of the green molly, *Poecilia latipinna*. *Journal of Comparative Neurology* 302, 893-919.

Bauchet, M.L., Hureau, J.C., 1986. Fishes of the North-eastern Atlantic and the Mediterranean.

UNEASCO, Paris.

Belanger, J.M., Son, J.H., Laugero, K.D., Moberg, G.P., Doroshov, S.I., Lankford S.E., Cech, J.J., 2001. Effects of short-term management stress and ACTH injections on plasma cortisol levels in cultured white sturgeon, *Acipenser transmontanus*. *Aquaculture* 203, 165-176.

Bentley, P.J., 1998. *Comparative Vertebrates Endocrinology*. 3rd Edition. *Cambridge University Press. Cambridge*, pp. 67-176.

Bern H.A., Madsen S.S., 1992. A selective survey of the endocrine system of the rainbow trout (*Oncorhynchus mykiss*) with emphasis on the hormonal regulation of ion balance. *Aquaculture* 100, 237-262.

Bernier, N.J., Flik, G., Klaren, P.H.M., 2009. Regulation and contribution of the corticotropic, melanotropic and thyrotropic axes to the stress response in fishes. In: *Fish Neuroendocrinology, Fish Physiology*, Vol. 28 (ed. N.J. Bernier, G. Van Der Kraak, A.P. Farrell and C.J. Brauner), pp. 235-311. Burlington, MA: Academic Press.

Bertok, L., 1998. Endotoxins and Endocrine System. *Domestic Animal Endocrinology* 15(5), 305-308.

Biron, M., Benfey, T.J., 1994. Cortisol, glucose and hematocrit changes during acute stress, cohort sampling, and the diel cycle in diploid and triploid brook trout (*Salvelinus fontinalis* Mitchill). *Fish Physiology and Biochemistry* 13, 153–160.

Cádiz, L., Román-Padilla, J., Gozdowska, M., Kulczykowska, E., Martínez-Rodríguez, G., Mancera, J.M., Martos-Sitcha, J.A., 2015. Cortisol modulates vasotocinergic and isotocinergic pathways in the gilthead sea bream (*Sparus aurata*, Linnaeus 1758). *The Journal of Experimental Biology* 218 (2), 316-325.

Carnevalli, O., Carletta, R., Cambi, A., Vita, A., Bromage, N., 1999. Yolk formation and degradation during oocyte maturation in seabream *Sparus aurata*: Involvement of two lysosomal proteinases *Biology of Reproduc*tion 60, 140-146.

Carragher, J.F., Rees, C.M., 1994. Primary and secondary stress responses in golden perch, (*Macquaria ambigua*). *Comparative Biochemistry and Physiology* 107A, 49 – 56.

Castro, M.G., Morrison, E., 1997. Post-translational processing of pro-opiomelanocortin in the pituitary and in the brain. *Critical Reviews in Neurobiology* 11, 35–57.

Cortright, D.N., Nicoletti, A., Seasholtz, A.F., 1995. Molecular and biochemical characterization of the mouse brain corticotropin-releasing hormone-binding protein. *Molecular and Cellular Endocrinology* 111, 147– 157.

Cowey, C.B., Walton, M.J., 1989. Intermediary metabolism. *Fish nutrition* 2, 259-329.

Davis, J.M., Clay, H., Lewis, J.L., Ghori, N., Herbomel, P., Ramakrishnan, L., 2002. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma

formation in zebrafish embryos. *Immunity* 17(6), 693-702.

Dethloff, Gail M., Schlenk, Daniel, Hamm, Jonathan T., Bailey, Howard C., 1999. Alterations in Physiological Parameters of Rainbow Trout (*Oncorhynchus mykiss*) with Exposure to Copper and Copper/Zinc Mixtures. *Ecotoxicology and Environmental Safety* 42(2), 253-264.

Duguay, S.J., Lai-Zhang, J., Steiner, D.F., Funkenstein, B., Chan, S.J., 1996. Developmental and tissue regulated expression of IGF-I and IGF-II mRNAs in *Sparus aurata*. *Journal of Molecular Endocrinology* 16, 123-132.

Fabbri, E., Capuzzo, A., Moon, T.W., 1998. The role of catecholamines in the regulation of fish metabolism: an overview. *Comparative Biochemistry and Physiology* 120C, 177–192.

Fanjul, M.L., Hiriart M., 1998. Biología funcional de los animales. Siglo XXI, 01/gen/1998.

Fanouraki, E., Mylonas, C.C, Papandroulakis, N., Pavlidis M., 2011. Species specificity in the magnitude and duration of the acute stress response in Mediterranean marine fish in culture. *General and Comparative Endocrinology* 173, 313-322.

Flik, G., Klaren, P.H.M., Van den Burg, E.H., Metz, J.R., Huising, M.O., 2006. CRF and stress in fish. *General and Comparative Endocrinology* 146, 36-44.

Fryer, J.N., Leung, E., 1982. Neurohypophysial hormonal control of cortisol secretion in the teleost *Carassius auratus*. *General and Comparative Endocrinology* 48, 425-431.

Fryer, J., Lederis, K., Rivier, J., 1985. ACTH releasing activity of urotensin I and ovine CRF: Interactions with arginine vasotocin, isotocin and arginine vasopressin. *Regulatory Peptides* 11, 11-15.

Gainer, H., Wray, S., 1994. Cellular and molecular biology of oxytocin and vasopressin. In: Knobil, E., Neill, J.D. (Eds.), *The Physiology of Reproduction*. Raven Press, Ltd., New York, pp. 1099-1119.

Gilchriest, B.J., Tipping, D.R., Hake, L., Levy, A., Baker, B.I., 2000. The effects of acute and chronic stresses on vasotocin gene transcripts in the brain of the rainbow trout (*Oncorhynchus mykiss*). *Journal of Neuroendocrinology* 12, 795–801.

Gorbman, A., Dickhoff, W., Vigna, S., Clark, N., Ralph, C., 1983. *Comparative Endocrinology*. John Wiley & Sons, New York, NY.

Guibbolini, M.E., Pierson, P.M., Lahlou, B., 2000. Neurohypophysial hormone receptors and second messengers in trout hepatocytes. *The Journal of Endocrinology* 167, 137-44.

Hausmann, H., Myerhof, W., Zwiers, H., Lederis, K., Richter, D., 1995. Teleost isotocin receptor: Structure, functional expression, mRNA distribution and phylogeny. *FEBS Letters* 370, 227-230.

Hemre, G.I., Lambertsen, G., Lie, Ø., 1991. The effect of dietary carbohydrate on the stress response in cod (*Gadus morhua*). *Aquaculture* 95, 319–328.

Hiraoka, S., Hyodo, S., Kubokawa, K., Ando, H., Urano, A., 1996. Effects of osmotic stimu- lation on expression of neurohypophysial hormone genes in pre-spawning chum salmon. *Zoological Science* 13, 737–745.

Huising, M.O., Metz, J.R., van Schooten, C., Taverne-Thiele, A.J., Hermsen, T., Verburg-van Kemenade, B.M., Flik, G., 2004. Structural characterisation of a cyprinid (*Cyprinus carpio* L.) CRH, CRH-BP and CRH- R1, and the role of these proteins in the acute stress response. *Journal of Molecular Endocrinology* 32(3), 627–648.

Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B., 1997. Fish stress and health in aquaculture. *Cambridge University Press*, New York NY 42, 517-525.

Iwama, G.K., 1998. Stress in fish. *Annals of the New York Academy of Sciences* 851, 304-310.

Janssens, P.A., Caine, A.G., Dixon, J.E., 1983. Hormonal control of glycogenolysis and the mechanism of action of adrenaline in amphibian liver *in vitro*. *General and Comparative Endocrinology* 49, 477-84.

Janssens, P.A., Lowrey, P., 1987. Hormonal regulation of hepatic glycogenolysis in the carp, *Cyprinus carpio*. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 252, R653– 660.

Kirk, C.J., Rodrigues, L.M., Hems, D.A., 1979. The influence of vasopressin and related peptides on glycogen phosphorylase activity and phosphatidylinositol metabolism in hepatocytes. *Biochemical Journal* 178, 493–496.

Kirk, C.J., Verrinder, T.R., Hems, D.A., 1977. Rapid stimulation, by vasopressin and adrenaline, of inorganic phosphate incorporation into phosphatidyl inositol in isolated hepatocytes. *FEBS Letters* 83, 267–271.

Kleszczynska, A., Vargas-Chacoff, L., Gozdowska, M., Kalamarz, H., Martínez-Rodríguez, G., Mancera, J.M., Kulczykowska, E., 2006. Arginine vasotocin, isotocin and melatonin responses following acclimation of gilthead sea bream (*Sparus aurata*) to different environmental salinities. *Comparative Biochemistry and Physiology* 145A, 268-273.

Kleszczynska, A., Sokolowska, E., Kulczykowska, E., 2012. Variation in brain arginine vasotocin (AVT) and isotocin (IT) levels with reproductive stage and social status in males of threespined stickleback (*Gasterosteus aculeatus*). *General and Comparative Endocrinology* 175, 290-6.

Konno, N., Hyodo, S., Yamaguchi, Y., Kaiya, H., Miyazato, M., Matsuda, K., Uchiyama, M., 2009. African lungfish, *Protopterus annectens*, possess an arginine vasotocin receptor homologous to the tetrapod V2- type receptor. *The Journal of Experimental Biology* 212, 2183-2193.

Kulczykowska, E., 1997. Response of circulating arginine vasotocin and isotocin to rapid osmotic challenge in rainbow trout. *Comparative Biochemistry and Physiology* 118A, 773–778.

Kulczykowska, E., 2001. Responses of circulating arginine vasotocin, isotocin, and melatonin to osmotic and disturbance stress in rainbow trout (*Oncorhynchus mykiss*). *Fish Physiology and*

*Biochemistry* 24, 201-206.

Kulczykowska, E., 2007. Arginine Vasotocin and Isotocin: Towards Their Role in Fish Osmoregulation. In: *Fish Osmoregulation* B. Baldisserotto, J.M. Mancera Romero, B.G. Kapoor (Eds.). *Science Publisher, Endfield (NH)*, 151-176.

Kulczykowska, E., Gozdowska, M., Martos-Sitcha, J.A., Kalamarz, H., Nietrzeba, M., Martínez-Rodríguez, G. 2010. Melatonin, vasotocin and isotocin as biomarkers of the condition of fish. *Comparative Biochemistry and Physiology* 157:S18.

Laiz-Carrión, R., Sangiao-Alvarellos, S., Guzmán, J.M., Martin del Río, M.P., Míguez, J.M., Soengas, J.L., Mancera, J.M., 2002. Energy metabolism in fish tissues related to osmoregulation and cortisol action. *Fish Physiology and Biochemistry* 27, 179-188.

Laiz-Carrión, R., Martín del Río, M.P., Miguez, J.M., Mancera, J.M., Soengas, J.L., 2003. Influence of cortisol on osmoregulation and energy metabolism in gilthead seabream *Sparus aurata*. *Journal of Experimental Zoology A* 298, 105-118.

Laiz-Carrión, R., Guerreiro, P.M., Fuentes, J., Canario, A.V.M., Martín del Río, M.P., Mancera, J.M., 2005. Branchial osmoregulatory response to salinity in the gilthead sea bream, *Sparus aurata*. *Journal Experimental Zoology A* 303, 563-576.

Lema, S.C., 2010. Identification of multiple vasotocin receptor cDNAs in teleost fish: sequences, phylogenetic analysis, sites of expression, and regulation in the hypothalamus and gill in response to hyperosmotic challenge. *Molecular and Cellular Endocrinology* 321, 215-30.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 25, 402-408.

Mahlmann, S., Meyerhof, W., Hausmann, H., Heierhorst, J., Schönrock, C., Zwiers, H., Lederis, K., Richter, D., 1994. Structure, function, and phylogeny of [Arg8]vasotocin receptors from teleost fish and toad. *Proceedings of the National Academy of Sciences of the United States of America* 91, 1342-1345.

Mancera, J.M., McCormick, S.D., 2007. Role of prolactin, growth hormone, insuline-like growth factor I and cortisol in teleost osmoregulation. In: Baldisserotto, B., Mancera, J.M., Kapoor, B.G. (Eds.), *Fish osmoregulation*. *Science Publishers, Enfield NH*, pp. 497-515.

Mancera, J.M., Vargas-Chacoff, L., García-López, A., Kleszczyńska, A., Kalamarz, H., Martínez-Rodríguez, G., Kulczykowska, E., 2008. High density and food deprivation affect arginine vasotocin, isotocin and melatonin in gilthead sea bream (*Sparus aurata*). *Comparative Biochemistry and Physiology* 149A, 92-97.

Martos-Sitcha, J.A., Gregório, S.F., Carvalho, E.S.M., Canario, A.V.M., Power, D.M., Mancera, J. M., Martínez-Rodríguez, G., Fuentes, J., 2013a. AVT is involved in the regulation of ion transport in the intestine of the sea bream (*Sparus aurata*). *General and Comparative Endocrinology* 193, 221-228.

Martos-Sitcha, J.A., Wunderink, Y.S., Gozdowska, M., Kulczykowska, E., Mancera, J.M., Martínez- Rodríguez, G., 2013b. Vasotocinergic and isotocinergic systems in the gilthead sea bream (*Sparus aurata*): an osmoregulatory story. *Comparative Biochemistry and Physiology* 166A, 571-581.

Martos-Sitcha, J.A., Fuentes, J., Mancera, J.M., Martínez-Rodríguez, G., 2014a. Vasotocin and isotocin receptors in gilthead sea bream *Sparus aurata*: expression variations during different osmotic challenges. *General and Comparative Endocrinology* 197, 5-17.

Martos-Sitcha, J.A., Wunderink, Y.S., Straatjes, J., Skrzynska, A.K, Mancera, J.M., Martínez-Rodríguez, G., 2014b. Different stressors induce differential responses of the CRH-stress system in the gilthead sea bream (*Sparus aurata*). *Comparative Biochemistry and Physiology* 177A, 49-61.

Maule, A.G., Schreck, C.B., Bradford, C.S., Barton, B.A., 1988. Physiological effects of collecting and transporting emigrating juvenile chinook salmon past dams on the Columbia River. *Transactions of the American Fisheries Society* 117, 245-261.

Maxime V, Nonnotte G, Peyraud C, Williot P, Truchot JP 1995 Circulatory and respiratory effects of an hypoxic stress in the siberian sturgeon. *Respiratory Physiology* 100, 203–212.

Mazeaud, M.M., Mazeaud, F., 1981. Adrenergic responses to stress in fish. In: *Stress and Fish A.D.Pickering (Ed.). Academic Press, London*, 49-75.

Mazik, P.M., Simco, B.A., Parker, N.C., 1991. Influence of water hardness and salts on survival and physiological characteristics of striped bass during and after transport. *Transactions of the American Fisheries Society* 120, 121-126.

McClennen, S.J., Cortright, D.N., Seasholtz, A.F., 1998. Regulation of pituitary corticotropin-releasing hormone-binding protein messenger ribonucleic acid levels by restraint stress and adrenalectomy. *Endocrinology* 139, 4435–4441.

McDonald, D.G., Milligan, C.L., 1997. Ionic, Osmotic and Acid –Base Regulation in Stress. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P, Schreck, C.B. (Eds.), *Fish Stress and Health in Aquaculture*. Cambridge Univeristy Press, Cambridge, U.K., pp. 119–144.

McEwen, B.S., Wingfield, J.C., 2003. The concept of allostasis in biology and biomedicine. *Hormonesand Behavior* 43, 2-15.

Mommsen, T.P., 1984. Metabolism of the fish gill. *Fish Physiology* 10 B, 203–238.

Mommsen, T.P., Walsh, P.J. and Moon, T.W., 1985. Gluconeogenesis in hepatocytes and kidney of Atlantic salmon. *Molecular Physiology* 8, 89–100.

Mommsen, T. P., y Walsh, P. J., 1992. Biochemical and environmental perspectives on nitrogen metabolism in fishes. *Experientia*, 48(6), 583-593.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts, dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries* 9, 211-268.

Moon, T.W., Mommsen, T.P. 1990. Vasoactive peptides and phenylephrine actions in isolated teleost hepatocytes. *The American Journal of Physiology - Endocrinology and Metabolism* 259, E644-649.

Moons, L., Cambre, M., Batten, T.F.C., Vandesande, F., 1989. Autoradiographic localization of binding sites for vasotocin in the brain and pituitary of the sea bass (*Dicentrarchus labrax*). *Neuroscience Letters* 100, 11-16.

Moretti, A., Fernandez-Criado, M.P., Cittolin, G., Guidastri, R., 1999. Manual on hatchery production of seabass and gilthead seabream. FAO, Rome.

Motohashi, E., Hasegawa, S., Mishiro, K., Ando, H., 2009. Osmoregulatory responses of expression of vasotocin, isotocin, prolactin and growth hormone genes following hypoosmotic challenge in a stenohaline marine teleost, tiger puffer (*Takifugu rubripes*). *Comparative Biochemistry and Physiology* 154A, 353–359.

Moyano, F.J., Díaz, M., Alarcón, F.J., Sarasquete, M.C., 1996. Characterization of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*). *Fish Physiology and Biochemistry* 15, 121-130.

Norris, D.O., 1997. Vertebrate endocrinology, *Academic Press*, San Diego.

Ortuño, J., Esteban, M.A., Meseguer, J., 2001. Effects of short-term crowding stress on the gilthead seabream(*Sparus aurata* L.) innate immune response. *Fish & Shellfish Immunology* 11, 187-197.

Ortuño, J., Esteban, M.A., Meseguer, J., 2002. Effects of four anaesthetics on the innate immune response ofgilthead seabream (*Sparus aurata* L.). *Fish & Shellfish Imumunology* 12, 49-59.

Peragón, J., Barroso, J.B., de la Higuera, M., Lupiáñez, J.A., 1998. Relationship between growth and protein turnover rates and nucleic acids in the liver of rainbow trout (*Oncorhynchus mykiss*) during development. *Canadian Journal of Fisheries and Aquatic Sciences* 55, 649-657.

Perry, S.F., Reid, S.G., Gilmour, K.M., Boijink, C.L., Lopes, J.M., Milsom, W.K., Rantin, F.T., 2004. A comparison of adrenergic stress responses in three tropical teleosts exposed to acute hypoxia. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 287, R188-R197.

Pickering, A.D., Pottinger, T.G., 1989. Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. *Fish Physiology and Biochemestry* 7, 253-258.

Polakof, S., Arjona, F.J., Sangiao-Alvarellos, S., Martín del Río, M.P., Mancera, J.M., Soengas, J.L., 2006. Food deprivation alters osmoregulatory and metabolic responses to salinity acclimation in gilthead sea bream *Sparus aurata*. *Journal of Comparative Physiology* B 176, 441-452.

Potter, E., Behan, D.P., Fischer, W.H., Linton, E.A., Lowry, P.J., Vale, W.W., 1991 Cloning and characterization of the cDNAs for human and rat corticotropin releasing factor-binding proteins. *Nature* 349, 423-426.

Pottinger, T., 2008. The stress response in fish - mechanisms, effects and measurement. In: Branson EJ (Ed.). *Fish Welfare. Oxford: Blackwell Publishing*.

Reid, S., Bernier, N., Perry, S., 1998. The adrenergic stress response in fish: control of catecholamines storage and release. *Comparative Biochemistry and Physiology* 120C, 1-27.

Rivier, C., Vale, W., 1983. Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. *Nature* 305, 325-327.

Robaina, L., Izquierdo, M.S., Moyano, F.J., Socorro, J., Vergara, J.M., Montero, D., Fernández-Palacios, H., 1995. Soybean and lupin seed meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications. *Aquaculture* 130, 219-233.

Rotllant, J., Tort, L., 1997. Cortisol and glucose responses after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. *Journal of Fish Biology* 51, 21-28.

Rotllant, J., Balm, P.H., Ruane, N.M., Pérez-Sánchez, J., Wendelaar-Bonga, S.E., Tort, L., 2000. Pituitary Proopiomelanocortin-Derived Peptides and Hypothalamus–Pituitary–Interrenal Axis Activity in Gilthead Sea Bream (*Sparus aurata*) during Prolonged Crowding Stress: Differential Regulation of Adrenocorticotropin Hormone and α-Melanocyte-Stimulating Hormone Release by Corticotropin- Releasing Hormone and Thyrotropin-Releasing Hormone. *General and Comparative Endocrinology* 119, 152-163.

Rotllant, J., Balm, P.H., Pérez-Sánchez, J., Wendelaar-Bonga, S.E., Tort, L., 2001. Pituitary and interrenal function in gilthead sea bream (*Sparus aurata* L., Teleostei) after handling and confinement stress. *General and Comparative Endocrinology* 121, 333-342.

Rotllant, J., Worthington, G.P., Fuentes, J., Guerreiro, P.M., Teitsma, C.A., Ingleton, P.M., Balment, R.J., Canario, A.V.M., Power, D.M., 2003. Determination of tissue and plasma concentrations of PTHrP in fish: development and validation of a radioimmunoassay using a teleost 1-34 N-terminal peptide. *General and Comparative Endocrinology* 133, 146-153.

Rowlerson, A., Mascarello, F., Radaelli, G., Veggetti, A., 1995. Differentiation and growth of muscle in the fish *Sparus aurata* (L): II. Hyperplastic and hypertrophic growth of lateral muscle from hatching to adult. *Journal of Muscle Research and Cell Motility* 16, 223-236.

Sangiao-Alvarellos, S., Bouca, P., Miguez, J.M., Soengas, J.L., 2003a. Intracerebroventricular injections of noradrenaline affect brain energy metabolism of rainbow trout. *Physiological and Biochemical Zoology* 76, 663-71.

Sangiao-Alvarellos, S., Laiz-Carrión, R., Guzmán, J.M., Martín del Río, M.P., Miguez, J.M., Mancera, J.M., Soengas, J.L., 2003b. Acclimation of *S. aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 285, 897-907.

Sangiao-Alvarellos, S., Lapido, M., Miguez, J.M., Soengas, J.L., 2004. Effects of central administration of arginine vasotocin on monoaminergic neurotransmitters and energy metabolism

of rainbow trout brain. *Journal of Fish Biology* 64, 1313-1329.

Sangiao-Alvarellos, S., Arjona, F.J., Martín Del Río, M.P., Míguez, J.M., Mancera, J.M., Soengas, J.L., 2005. Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. *Journal of Experimental Biology* 208, 4291-4304.

Sangiao-Alvarellos, S., Polakof, S., Arjona, F.J., Kleszcynska, A., Martin del Río, M.P., Míguez, J.M., Soengas, J.L., Mancera, J.M., 2006. Osmoregulatory and metabolic changes in the gilthead sea bream *Sparus aurata* after arginine vasotocin (AVT) treatment. *General and Comparative Endocrinology* 148, 348-358.

Schreck, C.B., 1990. Physiological, behavioral, and performance indicators of stress. *American Fisheries Society Symposium* 8, 29-37.

Smith, R.F., French, N.P., Saphier, P.W., Lowry, P.J., Veldhuis, J.D., Dobson, H., 2003. Identification of stimulatory and inhibitory inputs to the hypothalamic-pituitary-adrenal axis during hypoglycaemia or transport in ewes. *Journal of Neuroendocrinology* 15, 572-85.

Soengas, J.L., Strong, E.F., Andres, M.D., 1998. Glucose, lactate, and beta-hydroxybutyrate utilization by rainbow trout brain: changes during food deprivation. *Physiological Zoology* 71, 285–293.

Soengas, J.L., Aldegunde, M., 2002. Energy metabolism of fish brain. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* 131, 271-296.

Soengas, J.L., Sangiao-Alvarellos, S., Laiz-Carrión, R., Mancera, J.M., 2007. Energy Metabolism and Osmotic Acclimation in Teleost Fish. In: *Fish Osmoregulation B. Baldisserotto, J. M. Mancera Romero and B.G. Kapoor (Eds.). Science Publisher, Enfield (NH)*, 277-307.

Sokolowska, E., Kleszczynska, A., Kalamarz-Kubiak, H., Arciszewski, B., Kulczykowska, E., 2013. Changes in brain arginine vasotocin, isotocin, plasma 11-ketotestosterone and cortisol in round goby, *Neogobius melanostomus*, males subjected to overcrowding stress during the breeding season. Comparative Biochemistry and Physiology 165B, 237–242.

Thompson, I., White, A., Fletcher, T.C., Houlihan, D.F., Secombes, C.J., 1993. The effect of stress on the immune system of Atlantic salmon (*Salmo salar* L.) fed diets containing different amounts of vitamin C. *Aquaculture* 114, 1–18.

Tominaga, K., Shinohara, K., Otori, Y., Fukuhara, C., Intuye, S.T., 1992. Circadian rhythms of vasopressin content in the suprachiasmatic nucleus of the rat. *Neuroreport* 3, 809-812.

Tort, L., Sunyer, J. O., Gómez, E., Molinero, A., 1996. Crowding stress induces changes in serum haemolytic and agglutinating activity in the gilthead sea bream *Sparus aurata*. *Veterinary Immunology and Immunopathology* 51(1–2), 179-188.

van den Thillart, G., 1986. Energy metabolism of swimming trout (*Salmo gairdneri*). *Journal of Comparative Physiology B*, 156(4), 511-520.

Van Ginneken, V., Nieveen, M., Van Eersel, R., Van den Thillart, G. and Addink, A., 1996. Neurotransmitter levels and energy status in brain of fish species with and without the survival strategy of metabolic depression. *Comparative Biochemistry and Physiology* 114A, 189–196.

van Raaij, M.T., Pit, D.S., Balm P.H.M., Steffens A.B., van den Thillart G.E., 1996 Behavioural strategy and the physiological stress response in rainbow trout exposed to severe hypoxia. *Hormones and Behaviour* 30, 85–92.

van Raaij, M.T., van den Thillart. G.E., Hallemeesch, M., Balm, P.H.M., Steffens, A.B., 1995 Effect of arterially infused catecholamines and insulin on plasma glucose and free fatty acids in carp. *American Journal of Physiology* 268, 1163–1170.

Van Waarde, A., 1988, Biochemistry of non-protein nitrogenous compounds in fish including the use of amino acids for anaerobic energy production. *Comparative Biochemistry and Physiology* 91B, 207-228.

Vera, L.M., Montoya, A., Pujante, I.M., Pérez-Sánchez, J., Calduch-Giner, J.A., Mancera, J.M., Moliner, J., Sánchez-Vázquez, F.J., 2014. Acute stress response in gilthead sea bream (*Sparus aurata* L.) is time-of- day dependent: Physiological and oxidative stress indicators. *Chronobiology International* 31(9), 1051-61.

Vijayan, M.M., Pereira, C., Moon, T.W., 1994 Hormonal stimulation of hepatocyte metabolism in rainbow trout following an acute handling stress. *Comparative Biochemistry and Physiology* 108C, 321-329.

Ward, P.E., Benter, I.F., Dick, L., Wilk, S., 1990. Metabolism of vasoactive peptides by plasma and purified renal aminopeptidase M. *Biochemical Pharmacology* 40, 1725-1732.

Wargent, E.T., Burgess, W.J., Laycock, J.F., Balment, R.J., 1999. Separate receptors mediate oxytocin and vasopressin stimulation of cAMP in rat inner medullary collecting duct cells. *Experimental Physiology* 84, 17-25.

Waring, C.P., Stagg, R.M., Poxton, M.G. 1996. Physiological responses to habdling in the turbot. *Journal of Fish Biology* 48, 161-173.

Warne, J.M., Hyodo, S., Harding, K., Balment, R.J., 2000. Cloning of pro-vasotocin and pro-isotocin cDNAs from the flounder *Platichthys flesus*; levels of hypothalamic mRNA following acute osmotic challenge. G*eneral and Comparative Endocrinology* 119, 77-84.

Warne, J.M., 2001. Cloning and characterization of an arginine vasotocin receptor from the euryhaline flounder *Platichthys flesus*. *General and Comparative Endocrinology* 122, 312-319.

Wedemeyer, G.A., Barton, B.A., McLeay, D.J., 1990. Stress and acclimation. In: Schreck, C.B., Moyle, P.B. (Eds.), *Methods for fish biology. American Fisheries Society, Bethesda* MD, pp. 451-489.

Wendelaar Bonga, S.E., 1997. The stress response in fish. *Physiological Reviews* 77, 591-625.

Yamase, K., Takahashi, S., Nomura, K., Haruta, K., Kawashima, S., 1991. Circadian changes in arginine vasopressin level in the suprachiasmatic nuclei in the rat. *Neuroscience Letters* 130, 255-258.

Yin, Z., Lam, T.J., Sin, Y.M., 1995. The effects of crowding stress on the nonspecific immune response in fancy carp (*Cyprinus carpio* L.). *Fish & Shellfish Immunology* 5, 519–529.

**Acknowledgements/Ringraziamenti**

In primo luogo volevo ringraziare la mia relatrice, la professoressa Elena Fabbri, che mi ha aiutato nei problematici momenti precedenti alla partenza per l'Erasmus. Senza il suo appoggio probabilmente non sarei mai partito e questo lavoro, assieme a questa bellissima esperienza, non sarebbero mai avvenuti.

Ahora empiezo a escribir en español ... Espero no cometer demasiados errores.
Quiero entonces agradecer a mis co-directores de Tesis Dr. Juan Antonio Martos-Sitcha y Dr. Juan Miguel Mancera Romero por darme la bienvenida en su laboratorio, por su consejo y por su disponibilidad durante toda esta temporada. Todavía me acurdo el primer día que llegué aquí y recibí su llamada que me preguntaba si todo estaba bien… y me acuerdo también de mi contesta en un ingles muy incierto.
Y si hablamos de ingles no puedo no pensar a Juan Antonio y a toda su infinita paciencia en el corregir mi trabajo sin enfadarse: siempre con el sonriso, mucha simpatía, conocimiento y rigurosidad académica. Gracias también para cocinar siempre una alternativa vegetariana en las comida de laboratorio.
Quiero agradecer a todo mis compañeros de laboratorio: Yinet, Etiane, David, Ismael, Nacho, Leandro y Ruben. Una mención especial para Arleta, que me ayudó en los primero periodo de mi estancia cuando hablaba un ingles muy malo y nada de español. Gracias a Isa, mi compañera italiana de experimento, por su ayudo mientras escribía este trabajo. Quiero también agradecer a el Dr. Gonzalo Martinez que me permitió trabajar en su laboratorio y a Ruchi y Juan que me ayudaron con las análisis. Agradezco también la Universidad de Cádiz, al Consejo Superior de Investigaciones Científicas (CSIC) (Instituto Ciencias Marinas Andalucía) y todo el personal del departamento de Biología Marina y Acuicultura y a todas las personas que han estado involucradas directamente e indirectamente contribuyendo a la realización de esta tesis.

Ahora hay la parte mas difícil de escribir porqué tengo que agradecer todos los amigos que he encontrado durante esta estancia. En dieces meses he conocido muchísima gente y si tuviera que enumerar todos esta sesión podría ser más larga de la tesis. Sabéis que esta fue una de la temporada mejor de mi vida y si pasó esto es solo por la gente maravillosa que he encontrado aquí.
Entonces gracias a todos por las estupendas noches, los días en la playa y los menos buenos días en la biblioteca trabajando. Gracias a mis amigos de todo el mundo, a mis compañeros de piso y a Cádiz, una ciudad de la cuál me enamoré.

Riprendo a scrivere in italiano per ringraziare i miei amici di sempre: Marco, Alessandro, Edoardo e Alberto che mi hanno accompagnato in questi anni di studio, chi fin dall'infanzia chi dal periodo universitario. Ringrazio anche tutti gli amici di Pavia e Ravenna. Anche in questo caso elencarvi tutti sarebbe impossibile.

Ringrazio infine la mia famiglia che mi è sempre stata vicina in questi cinque anni di studio. Grazie per avermi sempre supportato nella mia scelta di studiare Biologia Marina e per essermi stati vicino in quei momenti dove poco di quello che facevo sembrare avere un senso.
Grazie anche per avermi permesso di intraprendere questo viaggio e vivere questi dieci mesi bellissimi.