Serotonin as a regulator of the hypothalamic-pituitary-interrenal axis in rainbow trout (*Oncorhynchus mykiss)*

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DEDICATION

I dedicate this thesis to my grandfather Brian Wilson who sadly passed away on May 9th, 2010. Although he is not here to share this accomplishment with me, Gramps was a firm believer in the value of learning and education at all levels. His compassion and dedication to his community as an educator, as well as his wit and humour are sorely missed.

ABSTRACT

Although empirical evidence suggests that interactions occur between serotonin (5-HT) and the hypothalamic-pituitary-interrenal (HPI) axis teleost fish, the mechanisms of serotonergic regulation of the HPI axis require elucidation. The hypothesis that 5-HT regulates the stress response in rainbow trout (*Oncorhynchus mykiss*) by acting at multiple levels in the HPI axis through tissue-specific 5-HT receptor subtypes was examined. Messenger RNA for 5-HT_{1A}, 5-HT_2 and 5-HT_4 receptor subtypes was expressed in all HPI axis tissues. Administration of 5-HT *in vivo* in cannulated trout caused significant increases in plasma cortisol and glucose concentrations. *In vitro* head kidney preparations revealed a stimulatory effect of 5-HT, acting through the 5-HT_4 receptor, on cortisol production. Collectively, these data suggest that 5-HT plays a role in HPI axis activation in rainbow trout, and that at the head kidney level, these effects likely are mediated by the 5-HT_4 receptor.

RÉSUMÉ

Des recherches antérieures suggèrent qu'il y a des intéractions entre la sérotonine (5-HT) et l'axe hypothalamo-hypophyso-interrénale (HPI) chez les téléostéens. Cependant, les mécanismes qui régulent ces interactions demeurent incertains. L'hypothèse que la 5-HT régule la réponse au stress chez les truites arc-en-ciel (*Oncorhynchus mykiss*) en activant multiples niveaux de l'axe HPI, par de sous-types de récepteurs 5-HT spécifiques aux tissus de l'axe, a été examinée. De l'ARN messager pour les récepteurs 5-HT_{1A}, 5-HT_2 and 5-HT_4 était exprimé dans tous les tissus de l'axe HPI. L'injection *in vivo* de 5-HT dans des truites cannulées a causé une augmentation significative des concentrations de cortisol et de glucose dans le plasma. Des préparations *in vitro* de rein antérieur ont révélées un effet stimulant de la 5-HT, agissant par le récepteur 5-HT_4. Ensemble, ces données suggèrent que la 5-HT joue un rôle dans l'activation de l'axe HPI chez la truite arc-en-ciel. Au niveau du rein antérieur, ces effets sont probablement régulés par le récepteur 5-HT_4.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
α-methyl 5-HT	α-methyl-5-hydroxytryptamine
µg	microgram
µL	microliter
µmol	micromole
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin; 5-hydroxytryptamine
8-OH-DPAT	8-hydroxy-2-dipropylaminotetralin hydrobromide
AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
BBB	blood-brain barrier
°C	degrees Celsius
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CRF	corticotropin releasing factor
CRF-BP	corticotropin releasing factor binding protein
CRF-R	corticotropin releasing factor receptor
Ct	threshold cycle
DAG	diacyl glycerol
DEPC	diethylpyrocarbonate
DNase	deoxyribonuclease
dNTPs	deoxyribonucleotides
EDTA	Ethylenediaminetetraacetic acid
EtOH	ethanol
g	gram
g	gravity
GI	gastrointestinal
GPCR	g-protein coupled receptor

GR	glucocorticoid receptor
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	hypothalamic-pituitary-adrenal
HPI	hypothalamic-pituitary-interrenal
IP_3	inositol 1,4,5-triphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
IU	international unit
kg	kilogram
L	Liter
LB	lysogeny broth
M	Molar
MC2R	melanocortin 2 receptor
MEM	minimum essential medium
min	minute
mL	milliliter
mmol	millimole
mol	mole
MR	mineralocorticoid receptor
N	sample size
nm	nanometer
nmol	nanomole
P450 scc	cytochrome p450 side cleavage chain enzyme
p	p-value
PCR	polymerase chain reaction
PE	polyethylene
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
POA	preoptic area
RIA	radioimmunoassay

RM	repeated measure
mRNA	messenger ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
s	seconds
SEM	standard error of the mean
SERT	serotonin transporter
s-qPCR	semi-quantitative real-time reverse transcriptase
StAR	steroidogenic acute regulatory protein
TPH	tryptophan hydroxylase

1. INTRODUCTION

The stress axis activates a suite of physiological and whole-animal changes, collectively termed a stress response, that serve to increase the likelihood of an individual's survival during and after exposure to a challenging event (reviewed by Wendelaar Bonga, 1997). A key component of the stress axis is the synthesis and release of glucocorticoid hormones (reviewed by Wendelaar Bonga, 1997). Stress axis activity is regulated by a variety of mechanisms, with negative feedback regulation being perhaps most obvious (Barton, 2002). The monoamine serotonin (5-hydroxytryptamine, 5-HT) also is thought to play a role in regulating the stress axis; this relationship has been well described in mammals (reviewed by Chaouloff, 1993; Dinan, 1996). The serotonergic system is comprised of 5-HT produced in the in the central nervous system (CNS) as well as in the gastro-intestinal (GI) tract (reviewed by Mück-Šeler and Pivac, 2011). Centrally produced 5-HT acts as a neurotransmitter, whereas 5-HT produced peripherally is mainly involved in regulating digestion and cardiac function (reviewed by Jonnakuty and Gragnoli, 2008).

The serotonergic system regulates the mammalian stress axis by acting on multiple 5-HT receptor subtypes (reviewed by Chaouloff, 1993; Dinan, 1996). A growing body of evidence suggests that a relationship between these two systems also exists in teleost fish (Winberg et al., 1997; Medeiros et al., 2010; Lim et al., 2013); however, the mechanisms involved in serotonergic regulation of the piscine stress axis, the hypothalamic-pituitary-interrenal (HPI) axis, require further study. Thus, the present thesis tested the hypothesis that 5-HT activates the HPI axis at

multiple levels in rainbow trout (*Oncorhynchus mykiss*). A secondary hypothesis, that the 5-HT receptors involved in serotonergic regulation of the HPI axis are tissue specific, also was examined. This introduction will briefly review concepts central to the thesis, such as the HPI axis, the serotonergic system and the interaction between 5-HT and the stress axis, to provide context for the experiments that were carried out.

1.1 The HPI axis

Teleost fish may be exposed to a variety of stressors (e.g. predation, social stress or toxicant exposure) that can disrupt the animal's homeostasis (reviewed by Wendelaar Bonga, 1997). Exposure to a stressor elicits a stress response, which can be described as a series of behavioural and physiological responses that help the fish to overcome the effects of the stressor (reviewed by Wendelaar Bonga, 1997). The HPI axis is a key component of the stress response, and is responsible for the synthesis and release of glucocorticoid hormones in teleost fish, with cortisol being the main end product (reviewed by Mommsen et al*.,* 1999; Barton, 2002). The effects of cortisol are thought to be adaptive, at least in the short term (reviewed by Wendelaar Bonga, 1997), and include changes in lipid, protein and carbohydrate metabolism that are designed to mobilize energy reserves (reviewed by Wendelaar Bonga, 1997; Mommsen et al., 1999). Plasma cortisol and glucose concentrations both have been shown to increase within minutes post-stress (Barton et al., 1987; Gesto et al., 2013; reviewed by Mommsen et al., 1999) and can stay elevated for up to 6 h for glucose (Barton et al., 1987; Gesto et al., 2013) and 24 h for cortisol

(reviewed by Mommsen et al., 1999). Plasma glucose and cortisol concentrations often are used to characterize activation of the stress axis in teleosts (Gesto et al., 2013).

Activation of the HPI axis leads to the release of corticotropin releasing factor (CRF) (reviewed by Barton, 2002), a well-conserved 41 amino acid neuropeptide (reviewed by Lovejoy and Balment, 1999; Flik et al., 2006) that induces the release of adrenocorticotropic hormone (ACTH) from the pituitary (reviewed by Fryer, 1989; Wendelaar Bonga, 1997). High levels of CRF messenger RNA (mRNA) are found in the preoptic area (POA) of the hypothalamus (Ando et al., 1999; Bernier et al., 2008), and CRF-containing neurons that originate in this region directly innervate the pituitary (Pepels et al., 2002; reviewed by Wendelaar Bonga, 1997; Flik et al., 2006). Corticotropin releasing factor exerts its effects by binding to CRF receptors, which are G protein coupled receptors (GPCRs) (reviewed by Chalmers et al., 1996; Flik et al., 2006). Two main CRF receptors are found in teleost fish (CRF-R1 and CRF-R2), although a third CRF receptor was detected in the brown bullhead, *Ameiurus nebulosus* (Arai et al., 2001; Pohl et al., 2001; reviewed by Flik et al., 2006). These receptors bind CRF as well as other members of the CRF family such as urotensin I and sauvagine (Pohl et al., 2001). The activity of CRF may be modulated by its binding protein (CRF-BP) (Alderman et al., 2008), which is expressed throughout the central nervous system in rainbow trout, and likely is involved in regulation of the HPI axis (Alderman et al., 2008).

Activation of CRF receptors causes ACTH to be released from the anterior pituitary (reviewed by Wendelaar Bonga, 1997). Cleavage of pro-opiomelanocortin

by prohormone convertase 1 in the corticotropes of the anterior pituitary leads to the formation of ACTH and β-lipotropic hormone (reviewed by Raffin-Sanson et al., 2003; Dores and Baron, 2011). Once ACTH is released into the circulation, it binds to the melanocortin 2 receptors (MC2R) of the interrenal cells of the head kidney to stimulate the synthesis and release of cortisol (Aluru and Vijayan, 2008).

The teleost head kidney is homologous to the mammalian adrenal gland (reviewed by Gallo and Civinini, 2003); however, rather than being localized to distinct regions within a separate gland as is the case in mammals, the catecholamine-producing chromaffin cells and the cortisol-producing interrenal cells in teleost fish are integrated within the anterior region of the kidney, i.e. the head kidney (reviewed by Gallo and Civinini, 2003). The MC2R is a GPCR that activates adenylyl cyclase (AC) and exerts its effects via the cyclic adenosine monophosphate (cAMP) signalling pathway. One action of cAMP, effected through protein kinase A, is to mobilize cholesterol within the interrenal cell through its influence on steroidogenic acute regulatory (StAR) protein (Aluru and Vijayan, 2008). Cholesterol is the starting material for the formation of steroidogenic hormones such as cortisol; its movement from the outer to the inner mitochondrial membrane is facilitated by StAR (reviewed by Stocco, 2000). Once at the inner mitochondrial membrane, cholesterol is converted to pregnenolone in a reaction catalyzed by cytochrome p450 side chain cleavage enzyme (p450scc) (reviewed by Mommsen et al., 1999; Payne and Hales, 2004) and pregnenolone is then converted to cortisol through a series of enzymatic reactions (reviewed by Mommsen et al., 1999). The rate-limiting steps in cortisol synthesis are considered to be movement

of cholesterol to the inner mitochondrial membrane facilitated by StAR, and the conversion of cholesterol to pregnenolone catalyzed by p450scc (Bernier et al., 2008; Mommsen et al., 1999).

Cortisol produced by the interrenal cells is carried by the circulation to target tissues where it binds to corticosteroid receptors, which are of two types, glucocorticoid (GR) and mineralocorticoid (MR) (reviewed by Mommsen et al., 1999). Cortisol binds to both GRs and MRs in teleost fish; however GRs are thought to be key in mediating the actions of cortisol during the stress response (reviewed by Prunet et al., 2006). The GRs are intracellular receptors that elicit cellular responses mainly by acting as transcription factors of genes that include a glucocorticoid response element in their promoter region (reviewed by Mommsen et al., 1999). There are two main GR isoforms in teleost fish, GR1 and GR2 (Bury et al., 2003; Greenwood et al., 2003; reviewed by Prunet et al., 2006), and in rainbow trout, both GRs are expressed in many tissues including the brain, liver, gill and white muscle (Alderman et al., 2012; Jeffrey et al., 2012).

Once activated, the HPI axis can be down-regulated by negative feedback. For example, elevated plasma cortisol levels are associated with decreased binding affinity of GRs, presumably as a mechanism to dampen the effects of cortisol (reviewed by Mommsen et al., 1999). Similarly, Jeffrey et al. (2012) reported that cortisol-treated rainbow trout exhibited low plasma ACTH concentrations. Evidence of negative feedback effects of cortisol directly on the head kidney also has been reported, whereby high levels of cortisol in the head kidney could serve as a signal to decrease cortisol production (Bradford et al., 1992). Negative feedback

provides for regulation within the HPI axis itself, but there is also evidence of HPI axis modulation by a number of other factors, both internal and external (Barton, 2002). Physiological processes such as cross-talk between the HPI axis and the reproductive (reviewed by Fuzzen et al., 2011) and thyroid (Geven et al., 2009) axes or the serotonergic system and the HPI axis (reviewed by Contesse et al., 2000) can modulate HPI axis function. Beyond physiological mechanisms, external factors including exposure to chronic social stress (reviewed by Gilmour et al., 2005) or environmental toxicants (reviewed by Hontela, 2005) also can regulate stress axis activity. This thesis aimed to assess whether and how 5-HT acts as a regulator of the HPI axis in rainbow trout.

1.2 5-HT as a regulator of the stress axis

1.2.1 The serotonergic system

Serotonin is a monoamine neurotransmitter and circulating hormone that is involved in regulation of the cardiovascular system, the immune system, and the GI tract, and also in regulating mood and behaviour (reviewed by Khan and Deschaux, 1997; Jonnakuty and Gragnoli, 2008; Mück-Šeler and Pivac, 2011). In mammals, approximately 95% of 5-HT is synthesized in the enterochromaffin cells (a population of neuroendocrine cells) of the GI tract, with the remaining 5% being synthesized by serotonergic neurons in the brain (reviewed by Jonnakuty and Gragnoli, 2008; Mück-Šeler and Pivac, 2011). Although 5-HT synthesis has been studied less extensively in teleosts, the conserved nature of the serotonergic system

and the presence of key enzymes for 5-HT biosynthesis (Johnston et al., 1990; Algedunde et al., 1998) and degradation (Olcese and De Vlaming, 1979; Khan and Joy, 1988) indicate that the pathways responsible for 5-HT synthesis and degradation in teleosts likely are similar to those in mammals.

Synthesis of 5-HT, both centrally and peripherally, begins with conversion of the essential amino acid L-tryptophan into 5-hydroxytryptophan catalyzed by the enzyme tryptophan hydroxylase (TPH) (reviewed by Jonnakuty and Gragnoli, 2008; Mück-Šeler and Pivac, 2011). Hydroxylation of L-tryptophan by TPH is considered to be the rate-limiting step in 5-HT synthesis (reviewed by Jonnakuty and Gragnoli, 2008). Recent studies have demonstrated the presence of two isoforms of TPH (TPH1 and TPH2) in mammals (Walther *et al.,* 2003; Sakowski *et al.,* 2006) and in a variety of fish species including the medaka (*Oryzias latipes*) and Atlantic croaker (*Micropogonias undulates)* (Rahman and Thomas, 2009; Kawabata *et al.,* 2012). In mammals, the distribution of the TPH isoforms seems to be tissue specific, with TPH1 being found primarily in the GI tract and TPH2 being restricted to the brain (Walther *et al.,* 2003; Sakowski *et al.,* 2006; reviewed by Chen *et al.,* 2013). The final step in 5-HT synthesis involves the decarboxylation of 5-hydroxytryptophan to 5-HT, catalyzed by the enzyme aromatic L-amino acid decarboxylase (reviewed by Mück-Šeler and Pivac, 2011; Fidalgo et al., 2013).

Once synthesis of peripheral 5-HT is complete, enterochromaffin cells release 5-HT into the circulation where the bulk of it is taken up via the serotonin transporter (SERT) into platelets and stored in dense granules (reviewed by Mercado and Kilic, 2010; Fidalgo et al., 2013). Once sequestered in platelets, 5-HT is

released when platelets are activated to exert vasoactive or immunological effects (reviewed by Berger et al., 2009; Mercado and Kilic, 2010; Ahern, 2011). To our knowledge, there is no evidence of 5-HT storage in the platelets of teleost fish. In mammals, 5-HT cannot cross the blood-brain barrier (BBB) [although this is not the case in teleost fish (Khan and Deschaux, 1997)] and thus production of 5-HT in the central nervous system requires the movement of either L-tryptophan or 5-hydroxytryptophan across the BBB (Gomes and Soares-da-Silva, 1999). Whereas 5-hydroxytryptophan moves easily across the BBB without requiring a transport protein, movement of tryptophan relies on specific transporters (reviewed by Jonnakuty and Gragnoli, 2008). Serotonin produced in the central nervous system is stored in secretory granules of the pre-synaptic neuron until it is released into the synaptic cleft (reviewed by Jonnakuty and Gragnoli, 2008). After exerting its effects on the post-synaptic neuron, 5-HT is the subject of reuptake by SERT located on the pre-synaptic neuron (reviewed by Jonnakuty and Gragnoli, 2008). Serotonin taken back up into the pre-synaptic neuron is degraded; 5-HT is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) in a series of reactions catalyzed by monoamine oxidase and aldehyde dehydrogenase (reviewed by Jonnakuty and Gragnoli, 2008; Mück-Šeler and Pivac, 2011).

1.2.2 5-HT receptors

The effects of 5-HT are mediated by well-conserved receptors that are thought to have appeared in single-celled organisms some 700-800 million years ago (reviewed by Hannon and Hoyer, 2008). There are seven 5-HT receptor families

(5-HT_{1-7}) encompassing 14 different receptor subtypes (reviewed by Barnes and Sharp, 1999). The 5-HT receptors all belong to the 7-transmembrane domain GPCR superfamily with the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel (reviewed by Barnes and Sharp, 1999; Contesse et al., 2000; Mück-Šeler and Pivac, 2011). The molecular structure and receptor function of mammalian 5-HT receptors have been well established (reviewed by Barnes and Sharp, 1999; Hannon and Hoyer, 2008), and several 5-HT receptors have been identified and sequenced in teleost fish (Yamaguchi et al., 1997; Wang and Tsai, 2006; Norton et al., 2008; Medeiros et al., 2010; Mager et al., 2012; Schneider et al., 2012; Lim et al., 2013). Mammalian serotonin receptors are distributed throughout the CNS on both the pre- and post-synaptic membranes of neurons as well as outside of the CNS, in peripheral tissues (reviewed by Hannon and Hoyer, 2008; Mück-Šeler and Pivac, 2011). For the purpose of this thesis, four 5-HT receptor subtypes, 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_4 receptors are of particular interest, because these subtypes most often have been implicated in serotonergic regulation of the stress axis in mammals, amphibians and teleosts (see Figure 1.1; sections 1.2.3 and 1.2.4).

The 5-HT_{1A} receptor is a GPCR that preferentially acts through an inhibitory G protein, Gαi (reviewed by Shpakov, 1999; Pucadyil et al., 2005). Activation of Gαi inhibits cAMP formation by decreasing levels of AC (reviewed by Barnes and Sharp, 1999; Shpakov, 1999). The mammalian 5-HT_{1A} receptor gene is intronless and codes for a 421-422 amino acid-long protein (reviewed by Pucadyil et al., 2005; Hannon and Hoyer, 2008). The 5-HT_1 receptor family was initially characterized by its high affinity for 5-HT (reviewed by Barnes and Sharp, 1999) and the 5-HT_{1A}

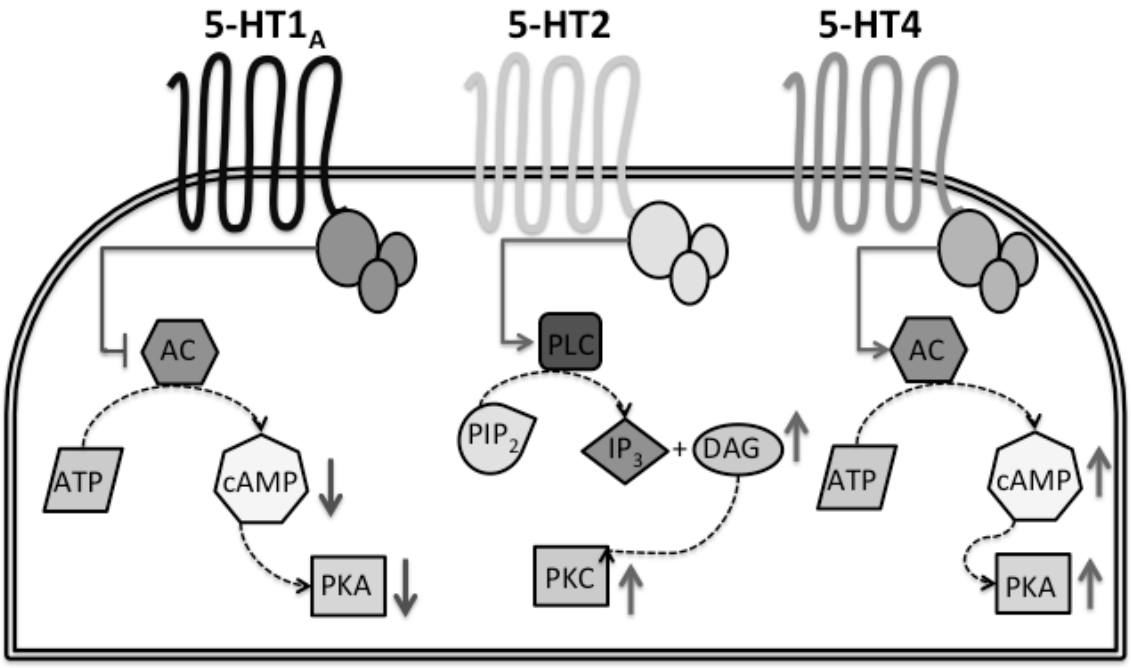


Figure 1.1. Schematic of 5-HT$_{1A}$, 5-HT$_2$ and 5-HT$_4$ receptor signalling pathways.

See text for detailed descriptions of pathways. *DAG,* diacyl glycerol, *IP$_3$*, inositol 1,4,5-triphosphate, *PIP$_2$,* phosphatidylinositol 4,5-bisphosphate, *PKA*, protein kinase A, *PKC,* protein kinase C, *PLC,* phospholipase C.

receptor has been reported to have a binding affinity of approximately 8 (pKi values; where pKi refers to the negative logarithm of the affinity constant Ki) for 5-HT (Watson et al., 2000). This receptor is located throughout the CNS and is thought to be present on both pre- and post-synaptic neurons (reviewed by Barnes and Sharp, 1999). The 5-HT_{1A} receptor also is located peripherally in tissues such as the liver (Zhou et al., 1999), ovaries (Henriksen et al., 2012) and GI tract (Kirchgessner et al., 1993). This receptor has been implicated in neural development (Del Olmo et al., 1998) and anxiety-linked behaviours (Heisler et al., 1998) as well as learning and memory (Ögren et al., 2008).

The 5-HT_2 receptors include 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} subtypes (reviewed by Barnes and Sharp, 1999; Hanon and Hoyer, 2008). The different subtypes share similar molecular characteristics, as well as cell signalling pathways and pharmacology (reviewed by Barnes and Sharp, 1999). These GPCRs are coupled to the stimulatory Gq protein, which activates the phosphatidylinositol signalling cascade to increase cytosolic Ca^{2+} concentrations (reviewed by Barnes and Sharp, 1999; Raymond et al., 2001). Generally, the 5-HT_2 receptor family is thought to have lower affinity for 5-HT compared to the 5-HT_1 receptor family (reviewed by Barnes and Sharp, 1999). However the 5-HT_{2C} receptor shows higher affinity for 5-HT than most 5-HT_2 receptors, which is why this receptor initially was categorized as a 5-HT_1 receptor (reviewed by Barnes and Sharp, 1999). In humans, the 5-HT_{2A} receptor is 471 amino acids in length and shows a high degree of amino acid sequence identity with the 5-HT_{2C} receptor that is approximately 460 amino acids in length (reviewed by Barnes and Sharp, 1999; Raymond et al., 2001). However, a

unique feature of the 5-HT_{2C} receptor is that it undergoes post-transcriptional mRNA editing to yield multiple isoforms (Burns et al., 1997). These 5-HT_{2C} receptor isoforms exhibit different levels of RNA expression throughout the brain (Burns et al., 1997) and the authors suggested that the presence of multiple isoforms could lead to functional changes in 5-HT signal transduction.

The 5-HT_{2A} and 5-HT_{2C} receptors are found throughout the central nervous system and in the spleen, liver, pancreas, kidney and heart of mammals (Bonhaus et al., 1995; Yanaboglu et al., 2009). The 5-HT_{2A} receptor also is found in smooth muscle of the small intestine of mice (Fiorica-Howells et al., 2002), where it is thought to regulate smooth muscle contraction (reviewed by Hannon and Hoyer, 2008). Indeed, 5-HT_{2A} receptors were found to regulate muscle contractions in the stomach (Janssen et al., 2002), trachea (Baez et al., 1994) and uterus (Minosyan et al., 2007) of various mammals. Additionally, activation of 5-HT_{2A} receptors has been implicated in mediating a variety of cardiovascular functions such as the regulation of cardiac fibroblasts and bradycardia (Brattelid et al., 2007; Yanaboglu et al., 2009; reviewed by Nagatomo et al., 2004). The 5-HT_{2C} receptor acts primarily within the central serotonergic system and has been implicated in mood disorders, feeding and even sleep (reviewed by Barnes and Sharp, 1999; Giorgetti and Tecott, 2004; Seretti et al., 2004).

The 5-HT_4 receptor is a GPCR that activates Gs proteins to stimulate cAMP formation (reviewed by Langlois and Fischmeister, 2003). This receptor subtype, which is 388 amino acids in length in humans, is found in the CNS, heart, intestine, adrenal cortex, ovaries and bladder (Henriksen et al., 2012; reviewed by Raymond

et al., 2001; Langlois and Fischmeister, 2003). Ten different 5-HT_4 receptor splice variants were identified and found to have mRNA that was differentially expressed in various tissues in the pig (Maeyer et al., 2008). A similar situation applies to the expression patterns of 5-HT_4 receptor splice variants in humans (Bender et al., 2000). Although numerous splice variants of this receptor have been identified, the functionality of these splice variants remains to be confirmed and may be species-specific (Bender et al., 2000; reviewed by Langlois and Fischmeister, 2003). However, affinity of the 5-HT_4 receptor for 5-HT has been reported to be similar across some splice variants (Claeysen et al., 1999; Bender et al., 2000). The main function of this receptor subtype is regulation of the GI tract (reviewed by Tonini et al., 1991; McLean et al., 2006), although it may also be involved in regulating cardiac function (Brattelid et al., 2007).

1.2.3 5-HT as a regulator of the hypothalamic-pituitary adrenal (HPA) axis

Interactions between the HPA axis (the mammalian and amphibian homologue of the teleost HPI axis) and the serotonergic system have been the focus of extensive research (reviewed by Chaouloff, 1993; Dinan, 1996; Contesse et al., 2000). Neuronal 5-HT of the central serotonergic system can regulate the HPA axis at the hypothalamus and pituitary, whereas regulation of the HPA axis at the adrenal gland likely occurs via locally produced 5-HT acting in a paracrine manner (reviewed by Chaouloff, 1993; Dinan, 1996; Contesse et al., 2000). At the whole-animal level, administration of 5-HT_{1A} or 5-HT_2 receptor agonists to rats and humans led to increased plasma ACTH and corticosterone (rat)/cortisol (human)

concentrations (Bagdy et al., 1989; Van de Kar et al., 2001; reviewed by Chaouloff, 1993). Such results support a role for 5-HT in regulating HPA axis activity without specifically identifying the level(s) within the HPA axis at which 5-HT acts. Insight into the actions of 5-HT within the HPA axis has come from using reduced *in vitro* preparations. Within the hypothalamus, 5-HT-containing neurons form synapses with CRF-positive neurons (Liposits et al, 1987). Also, 5-HT can induce CRF release from hypothalamic explants or cell culture systems (Holmes et al., 1982; reviewed by Chaouloff, 1993; Dinan, 1996). Incubation of rat hypothalamic tissue *in vitro* with 5-HT as well as selective 5-HT_1 and 5-HT_2 receptor agonists had stimulatory effects on CRF production, suggesting involvement of these two receptor types in the regulation of HPA axis function (Calogero et al., 1989). In the pituitary, 5-HT-like immunoreactivity was detected in corticotropes (reviewed by Contesse et al., 2000), and rat pituitary cells incubated with 5-HT showed increased ACTH release (Spinedi and Negro-Vilar, 1983). Furthermore, rats treated with selective 5-HT_{1A} and 5-HT_2 receptor agonists exhibited CRF-independent ACTH release, suggesting that 5-HT may exert direct effects on the pituitary (Calogero et al., 1990).

In adrenocortical tissue, 5-HT is thought to act in a paracrine fashion, although the cell type responsible for 5-HT production and the 5-HT receptors involved seem to be species specific (reviewed by Chaouloff, 1993; Contesse et al., 2000). Both 5-HT and its main metabolite, 5-HIAA, were detected in human, frog and rat adrenal glands, suggesting the occurrence of local 5-HT production and metabolism (Delarue, 1988b; Lefebvre et al., 1992). In humans, 5-HT was located in mast cells of the adrenal gland (Lefebvre et al., 1992; reviewed by Dinan, 1996;

Contesse et al., 2000). However, in frogs and rats, 5-HT was detected in the chromaffin cells of the adrenal gland (Brownfield et al., 1985; Delarue et al., 1988a). Human and frog adrenal glands incubated *in vitro* with 5-HT or a selective 5-HT_4 receptor agonist showed increased cortisol (human)/corticosterone (frog) production, implicating this receptor subtype in the synthesis and release of glucocorticoids from the adrenal gland (Delarue et al., 1988b; Idres et al., 1991; Lefebvre et al., 1992).

While much research has focussed on serotonergic regulation of the HPA axis as outlined above, it is important to point out that the HPA axis can, in turn, regulate the serotonergic system. For instance, GRs were detected in brain regions known to contain 5-HT neurons (Härfstrand, et al., 1986; reviewed by Chaouloff, 1993). Further, adrenalectomized rats showed decreased hippocampal serotonergic turnover that was restored with corticosterone treatment (De Kloet et al., 1982). Adrenalectomy also increased 5-HT_{1A} receptor binding and mRNA expression levels in the rat hippocampus (Chalmers et al., 1993). Conversely, corticosterone treatment decreased 5-HT_{1A} receptor mRNA expression in the rat hippocampus (Meijer and De Kloet, 1994). These studies suggest that interactions between the HPA axis and the serotonergic system via corticosteroid receptors are complex (reviewed by Chaouloff 1993; 1995).

1.2.4 5-HT as a regulator of the HPI axis

A growing body of research supports the existence of links between the serotonergic system and the HPI axis in teleost fish. *In vivo* injection of (unstressed)

rainbow trout with the selective 5-HT_{1A} receptor agonist 8-hydroxy-2-dipropylaminotetralin (8-OH-DPAT) led to increased plasma cortisol concentrations (Winberg et al., 1997), and similar effects of 8-OH-DPAT administration were found in Gulf toadfish (*Opsanus beta)* (Medeiros et al., 2010) and goldfish (*Carassius auratus*) (Lim et al., 2013). Indeed, Winberg et al. (1996) found multiple binding sites for 5-HT in the brain of Arctic charr (*Salvelinus alpinus*), including a binding site with a pharmacological profile similar to that of the 5-HT_{1A} receptor, and this receptor has since been cloned and sequenced in several fish species (Yamaguchi et al., 1997; Wang et al., 2006; Norton et al., 2008, Medeiros et al., 2010; Lim et al., 2013). Treatment of Gulf toadfish with 8-OH-DPAT led to significant increases in plasma cortisol and ACTH concentrations as well as POA expression of 5-HT_{1A} mRNA (Medeiros et al., 2014). Furthermore, *in vitro* stimulation of both goldfish (Lim et al., 2013) and Gulf toadfish (Medeiros et al., 2012) head kidney tissue with 5-HT led to significant increases in cortisol production. These results imply a role for 5-HT in stimulating HPI axis function at multiple levels in teleost fish, as in mammals, and implicate the 5-HT_{1A} receptor in this action.

In a second line of evidence supporting interactions between 5-HT and the stress axis in teleost fish, rainbow trout exposed to a confinement stressor exhibited increased plasma cortisol concentrations that were accompanied by an increased 5-HIAA/5-HT ratio (a measurement of 5-HT turnover) in the brain stem and telencephalon (Schjolden et al., 2006). Additionally, chased rainbow trout had significantly increased hypothalamic and telencephalic 5-HIAA/5-HT ratios that were accompanied by increased plasma cortisol concentrations (Gesto et al., 2013).

Interestingly, however, when 8-OH-DPAT was administered to stressed rainbow trout, stress-induced increases in circulating cortisol concentrations were diminished (Höglund et al., 2002), suggesting a context-specific role for 5-HT in modulating HPI axis function. Along similar lines, supplementation with dietary L-tryptophan (a 5-HT precursor) led to higher baseline cortisol concentrations in rainbow trout (Lepage et al., 2002). However, the L-tryptophan-fed fish exhibited an attenuated cortisol response when exposed to a stressor (Lepage et al., 2002). Overall, these studies suggest that in teleost fish, as in mammals, there is regulation of the stress axis via the serotonergic system. However, the levels at which 5-HT is exerting its effects, the 5-HT receptor families involved and the role of 5-HT regulation *in vivo* require elucidation, particularly in rainbow trout.

1.3 Hypotheses and study species

Although a substantial body of research has identified and characterized interactions between the HPA axis and the serotonergic system in mammals and amphibians, our knowledge of comparable interactions in teleost fish is much less complete. Mechanistic studies have been carried out in only two species, goldfish (Lim et al., 2013) and Gulf toadfish (Medeiros et al., 2010; Medeiros and McDonald, 2012; Medeiros et al., 2014), with Gulf toadfish being unusual with respect to its high levels of circulating cortisol under control conditions and the involvement of serotonin in the pulsatile urea excretion that appears to be unique to this species (McDonald et al., 2004; McDonald and Walsh, 2004). By contrast, the mechanisms behind serotonergic regulation of the HPI axis in rainbow trout still require

elucidation. Thus, this thesis set out to test two broad hypotheses. First, 5-HT was hypothesized to activate the HPI axis by exerting effects on multiple levels within the stress axis. Based on this hypothesis, 5-HT receptor mRNA was predicted to be detectable in all three tissues of the HPI axis. Additionally, *in vivo* administration of 5-HT to rainbow trout was predicted to lead to increased plasma cortisol, ACTH and glucose concentrations as well as increased CRF mRNA abundance post-injection. Abundance of CRF mRNA was used as a proxy for CRF release, owing to the difficulties associated with measuring CRF release directly. The second hypothesis was that the 5-HT receptors involved in regulation of the HPI axis would be tissue-specific. As such, 5-HT_{1A}, 5-HT_2 and 5-HT_4 mRNAs would be predicted to be differentially expressed in the tissues of the HPI axis. Moreover, differential cortisol production by head kidney tissue incubated *in vitro* would be predicted with agonists selective for specific 5-HT receptor subtypes.

Juvenile rainbow trout (*Oncorhynchus mykiss*) were chosen as the study species for the reason outlined above; namely that 8-OH-DPAT injection was found to have different effects on circulating cortisol concentrations depending on the state of activation of the stress axis, and because the mechanisms underlying the effects of serotonin on the HPI axis had not been investigated in this species. Moreover, rainbow trout were also of interest because of the possibility that 5-HT may regulate HPI axis function during social interactions. Juvenile rainbow trout readily form social hierarchies consisting of fish of dominant, subdominant and subordinate status, with fish of each social category displaying a suite of specific behaviours and physiological responses (reviewed by Gilmour et al., 2005).

Subordinate social status is thought to act as a chronic stressor and is associated with prolonged activation of HPI axis and correspondingly elevated circulating cortisol concentrations (reviewed by Gilmour et al., 2005). Interestingly, socially subordinate rainbow trout also exhibit elevated brain serotonergic activity (Winberg et al., 1998). Thus, the present thesis was designed to acquire a better understanding of the mechanisms underlying serotonergic regulation of the HPI axis in rainbow trout, with the ultimate aim of applying this knowledge to the study of chronic stress in socially subordinate rainbow trout.

2. MATERIALS AND METHODS

2.1 Experimental animals

Female juvenile rainbow trout (*Oncorhynchus mykiss*) (*N*=146, 245.8 ± 7.6 g; mean ± SEM) obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) were used for all experiments. Fish were held at the University of Ottawa Aquatic Facility in 1275 L fiberglass tanks under a 12L:12D photoperiod. Tanks were supplied with flowing, aerated 13°C dechloraminated tap water from the city of Ottawa. Fish were fed to satiation with commercial fish pellets once every two days.

2.2 Experimental protocols

Three broad experimental approaches were used for the experiments presented in this thesis. To evaluate the potential for 5-HT to act at different levels of the HPI axis, the distribution of 5-HT_{1A}, 5-HT_2 and 5-HT_4 receptor mRNA within the tissues of the HPI axis was determined using semi-quantitative real-time RT-PCR (s-qPCR). The ability of 5-HT, as well as various agonists and antagonists selective for specific 5-HT receptors, to stimulate (agonists) or block (antagonists) cortisol production was assessed using an *in vitro* head kidney preparation. These experiments served to determine whether 5-HT can act directly at the level of the cortisol-producing interrenal cells to regulate cortisol production. Finally, the potential for 5-HT to regulate cortisol production at the whole-animal level was evaluated using a series of *in vivo* experiments. In addition to circulating cortisol concentrations, plasma glucose concentrations were measured as an index of HPI

axis activity. Circulating ACTH levels and mRNA abundance of CRF in the POA also were measured, to assess activity at the level of the pituitary and/or hypothalamus.

2.3 Tissue distribution of 5-HT receptor transcripts

2.3.1 Tissue sampling, RNA extraction and cDNA synthesis

Fish (*N*=14, 285.0 ± 20.9 g; mean ± SEM) were removed from the holding tanks and terminally anaesthetized in a solution of benzocaine (0.5 g L^{-1}, Sigma Aldrich). Head kidney, pituitary and POA tissues were dissected on ice and immediately flash frozen in liquid N_2. Tissues were dissected according to methods described by Jeffrey et al. (2012) and tissue sampled for the POA was located posterior to the telencephalon and consisted of the first quarter of the optic lobe (Bernier et al., 2008). Tissues were stored at -80°C until further analysis.

Extraction of RNA from approximately 50 mg tissue was carried out using TRIzol® reagent (Life Technologies) according to the manufacturer's protocol. Tissue was homogenized by pulling tissue and TRIzol® reagent through 18G and then 23 G needles into a syringe until the homogenate passed smoothly through the needle. For head kidney tissue, an additional centrifugation step at 4°C for 10 min at 12,000 *g* was performed after homogonization to remove excess cellular debris. After phase separation, RNA was precipitated overnight at -20°C. The resultant RNA was washed with an additional 100 µL of ice-cold 75% EtOH to ensure RNA purity, and then was suspended in diethylpyrocarbonate (DEPC) water and quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer. Purity was assessed using

absorbance measurements at 260/280 nm and 260/230 nm. Samples were stored at -80°C until used for cDNA synthesis.

An aliquot of RNA (1 μg) was treated with DNase 1, Amplification grade (Life Technologies) according to the manufacturer's protocol. Samples that had been DNase-treated then were reverse transcribed using M-MLV Reverse Transcriptase (Life Technologies) according to the manufacturer's protocol. The resultant cDNA samples were stored at -20°C until further use. Omission of reverse transcriptase was used to generate 'no RT control' samples for the purpose of checking for genomic DNA contamination.

2.3.2 Cloning and sequencing of 5-HT receptors

Primers were designed for the rainbow trout 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_4 receptors using regions of these receptors conserved across various teleost fish species (see Table 2.1). Primer Quest® (IDT) was used for primer design and primer quality (i.e. presence of hairpin structures, unwanted dimerization, etc.) was assessed using OligoAnalyzer 3.1 (IDT). The resulting primers (Table 2.1) were used for polymerase chain reactions (PCR), which were carried out on a Bio-Rad S1000 Thermal Cycler using the following cycling conditions: 95°C (30 s), 60°C (30 s) and 72°C (30 s) for 37 cycles. Template (2 μL of cDNA derived from rainbow trout POA) was added to 2.5 μL of 10x PCR buffer (Denville), 0.5 μL of 10 mmol L^{-1} dNTPs (Life Technologies), 0.5 μL each of forward and reverse primers (10 μmol L^{-1} stock concentration), 18.75 μL of autoclaved H_2O and 0.25 μL of Choice *Taq* DNA polymerase (Denville). The sizes of PCR products were determined by gel

electrophoresis and bands of the expected product size were isolated. The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. Gel extracted-products were used as template DNA for direct cloning.

Cloning was performed using half-reactions of a PCR cloning kit (Qiagen) according to the manufacturer's protocol. The ligation reaction was incubated at 4°C for one hour before ligation products were transformed in Subcloning Efficiency™ DH5α™ Competent Cells (Life technologies) and then incubated at 37°C for 1.5 h while being shaken at 200 rpm. Transformed cells were spread onto LB plates containing 50 μg mL^{-1} ampicillin (Sigma-Aldrich), 15 μg mL^{-1} kanamycin (Acros), 50 μM IPTG (Bioshop) and 80 μg mL^{-1} X-Gal (Bioshop). Plates were incubated at 37°C for 17 h and then at 4°C for 6 h to allow colony colouration to develop. White colonies were isolated from the plates and allowed to grow in 5 mL of LB media (50 μg mL^{-1} ampicillin and 15 μg mL^{-1} kanamycin) with shaking (200 rpm) at 37°C for 17 h. Finally, a plasmid preparation and restriction digest were used to verify the presence and size of the insert. An aliquot of colony growth (3 mL) was centrifuged at 7,000 *g* for 3 min and plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's protocol. Once plasmids had been isolated, 3 μL of plasmid were added to 1.5 μL of 10x React3 buffer (Life Technologies), 0.75 μL of EcoRI enzyme (Life Technologies) and 9.75 μL of autoclaved water for the restriction digest. Samples were incubated at 37°C for 60 min. Restriction digest products were sequenced by Genscript USA Inc.

Sequences obtained for the rainbow trout 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_4 receptors were translated with the ExPasY translate tool (ExPasY Bioinformatics Research Portal). Both nucleotide and predicted amino acid sequences were aligned with sequences for other teleost fish, amphibian, reptile, avian, and mammalian species (obtained from the Ensembl database; see Table 2.2) using ClustalX 2.1 (Clustal). Phylogenetic trees were generated using a Bayesian analysis (Mr. Bayes; 10^6 generations, sampling frequency =100, burnin=250) with *Ciona intestinalis* as an outgroup.

2.3.3 Semi-quantitative real-time RT-PCR

Semi-quantitative real-time RT-PCR was used to determine the relative mRNA abundances of various 5-HT receptors in POA, pituitary and head kidney tissues. Partial sequences for rainbow trout 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_4 receptors were used to design gene-specific primers for s-qPCR using Primer Quest® (IDT) (see Table 2.3). However, owing to a high degree of nucleotide sequence identity between the 5-HT_{2A} and 5-HT_{2C} receptor sequences, 'generic' 5-HT_2 primers (i.e. primers that would detect multiple 5-HT_2 subtypes) were used for further analysis. Primer specificity was verified by the presence of a single amplicon of the expected size; gel extracts were then used to clone and sequence the amplicon (as described above). The ribosomal subunit 18S was used as a reference gene for all s-qPCR experiments.

All s-qPCR reactions were carried out on a Biorad CFX96™ Realtime system using a SYBR green mastermix kit (Agilent Technologies). Reactions were carried

out according to the manufacturer's protocol with the exception that the total reaction volume was halved. All s-qPCR reactions were performed using the following cycling conditions: 95°C (10 s) and 60°C (6 s) for 39 cycles. Standard curves for each primer set (a separate standard curve was created for 18S for use with 5-HT_{1A}) were created using pooled cDNA from head kidney, POA and pituitary tissue (5-HT_{1A} and $18S_{1A}$) or from POA tissue (5-HT_2, 5-HT_4 and 18S). Additionally, samples for which template had been omitted were used as no-template controls. Dissociation curves were used to verify the presence of a single amplicon. Dissociation curves for the 5-HT_2 receptor indicated the presence of a second, smaller amplicon corresponding to primer-dimerization in the no-template controls. Samples that contained primer-dimers (based on the dissociation curves) were eliminated from further analysis. The 5-HT_4 receptor dissociation curves showed the presence of a secondary amplicon, but this amplicon was not present in the negative controls. This secondary amplicon may have been a splice variant of the 5-HT_4 receptor as this receptor is known (at least in mammals) to have multiple splice variants (reviewed by Hannon and Hoyer, 2008). The absence of genomic DNA contamination was confirmed by incorporating into each s-qPCR trial samples from which reverse transcriptase had been omitted during cDNA synthesis (no RT controls). The mRNA abundances of the different genes in the three tissues were expressed relative to that of the head kidney using a modified delta-delta Ct method (Pfaffl, 2001) with 18S being used as the normalizing gene.

2.4 *In vitro* head kidney preparations

Female juvenile rainbow trout (*N*=44; 264.7 ± 13.7 g; mass ± SEM) were euthanized by immersion in a solution of benzocaine (0.5 g L^{-1}, Sigma Aldrich). A ventral midline incision was made to expose the posterior cardinal vein, which was perfused with 40 mL of chilled (4°C) modified Hanks' solution [92.56 mmol L^{-1} NaCl, 3.63 mmol L^{-1} KCl, 0.55 mmol L^{-1} $MgSO_4$, 0.40 mmol L^{-1} KH_2PO_4, 0.23 Na_2HPO_4, 7.50 mmol L^{-1} HEPES, 2.81 mmol L^{-1} $NaHCO_3$, 0.85 mmol L^{-1} $CaCl_2$, 0.03 % (w/v) bovine albumin serum; final pH adjusted to 7.4] to remove the majority of blood found in the head kidney tissue. Head kidney tissue then was removed for incubation *in vitro* using protocols described by Jeffrey et al. (2014), Conde-Sieira et al. (2013) and Aluru et al. (2005). In brief, head kidney tissue was weighed, minced with a razor blade and incubated on a shaker for 1.5 h at 13°C in 75 mL g^{-1} tissue of incubation medium [modified Hanks' solution supplemented with minimum essential medium (MEM) amino acid solution (50x; 2 mL per 100 mL medium; Sigma Aldrich), MEM non-essential amino acid solution (100x; 1 mL per 100 mL medium; Sigma Aldrich), and antibiotic-antimycotic (100x; 1 mL per 100 mL medium; Sigma Aldrich)]. The tissue was then distributed evenly into the wells of a 48-well microplate (~25 mg of tissue per well) and incubated in duplicate with 250 µL of one of the following solutions; fresh medium (blank; negative control), 1 IU mL^{-1} of porcine ACTH (Sigma Aldrich) (positive control), or an experimental treatment. In the first experimental series, one of three 5-HT concentrations was used; 10^{-8} mol L^{-1}, 10^{-7} mol L^{-1} or 10^{-6} mol L^{-1} 5-HT (Sigma Aldrich). The second experimental series used the 5-$HT_{1A/7}$ receptor agonist 8-OH-DPAT (10^{-7} mol L^{-1}) or

10^{-7} mol L^{-1} 8-OH-DPAT in combination with the 5-HT_{1A} receptor antagonist WAY100635 (2 x 10^{-6} mol L^{-1}; Sigma Aldrich). The third experimental series used the 5-HT_2 receptor agonist α-methyl-5-hydroxytryptamine maleate (10^{-7} mol L^{-1}; α-methyl-5-HT; Tocris Bioscience) or 10^{-7} mol L^{-1} α-methyl-5-HT in combination with 2 x 10^{-6} mol L^{-1} of ketanserin (Sigma Aldrich), a 5-HT_2 receptor antagonist. The final experimental series used 10^{-7} mol L^{-1} cisapride monohydrate (cisapride; Sigma Aldrich), a 5-HT_4 receptor agonist, or 10^{-7} mol L^{-1} cisapride in combination with 2 x 10^{-6} mol L^{-1} GR-125487 (Tocris Bioscience), a 5-HT_4 receptor antagonist. Treatments and doses were selected based on previous experiments involving superfused head kidney preparations in goldfish (Lim *et al.,* 2013).

Tissue was incubated with shaking for 1 h at 13°C. After incubation, tissue suspensions were transferred to microcentrifuge tubes and centrifuged at 14,000 *g* for 10 min. Supernatants were flash frozen in liquid N_2 and stored at -80°C for later analysis of cortisol concentration, and the head kidney tissue was weighed. Cortisol concentrations were measured using a commercially available radioimmunoassay (ImmuChem™ Coated Tube Cortisol ^{125}I RIA Kit; MP Biomedicals) according to the manufacturer's protocol. Use of this kit with rainbow trout plasma samples was validated in previous studies (Jeffrey et al.., 2012; reviewed by Gamperl et al.., 1994). Inter-assay and intra-assay coefficients of variation were 7.3% and 6.9%, respectively. Cortisol production was normalized to mg wet tissue weight and then expressed as a percent increase over the corresponding unstimulated (blank) value.

2.5 Serotonergic stimulation of the HPI axis *in vivo*

2.5.1 Experimental protocol

Rainbow trout (*N*=61; 200.1 ± 5.9 g; mass ± SEM) were anaesthetized by immersion in an aerated solution of benzocaine (0.05 g L^{-1}, Sigma Aldrich) and placed on a surgery table that allowed constant irrigation of the gills with the anaesthetic solution. An indwelling cannula (PE-50 polyethylene tubing, Clay-Adams) was implanted into the dorsal aorta using the methods described by Soivo et al. (1975), and fish were allowed to recover from the anaesthetic by irrigating the gills with aerated water. Fish were then allowed to recover for 48 h in individual experimental chambers supplied with flowing, dechloraminated city of Ottawa tap water at 13°C, and cannulae were flushed periodically with saline. The basic experimental protocol involved withdrawal of a baseline blood sample (0.2-0.4 mL, depending on the experiment). Either saline or 5-HT (0.8 mL kg^{-1}) was then administered via the dorsal aortic cannula, and blood samples (0.2-0.4 mL) were subsequently withdrawn at times determined by the experimental series (see below). Blood samples were collected using heparin (5 IU mL^{-1} of blood; for samples to be analyzed for cortisol and glucose concentrations) or EDTA (1.5 mg mL^{-1} of blood; for samples to be analyzed for ACTH concentrations) and centrifuged for 2 min at 10,000 *g*. Plasma was flash frozen in liquid N_2 and stored at -80°C for later analysis. After the final blood sample was withdrawn, fish were euthanized by immersion in a solution of benzocaine (0.5 g L^{-1}). The POA was quickly dissected out on ice, flash frozen in liquid N_2 and stored at -80°C for later analysis of CRF mRNA abundance.

In the first experiment, fish were injected (after collection of a baseline blood sample) with 375 mmol L^{-1} 5-HT to achieve a nominal circulating concentration of 1000 nmol L^{-1}; matched controls received an equivalent volume of 0.9% NaCl. This circulating concentration of 5-HT was chosen because it reflected circulating plasma 5-HT concentrations reported in Gulf toadfish (McDonald and Walsh, 2004). Blood samples (0.2 mL) were withdrawn at 15, 30, 60, 120 and 240 min post-injection and plasma was used for quantification of cortisol and glucose concentrations. Three independent trials were carried out in an attempt to determine optimal sampling times for ACTH and CRF. After collection of the baseline blood sample, either 5-HT or saline was administered as described above and blood samples (0.4 μL) were collected for analysis of ACTH concentrations following which fish were euthanized and POA was collected for analysis of CRF mRNA abundance. In the first trial, a blood sample was collected at 15 min post-injection and fish were euthanized for collection of POA; the second trial involved collection of blood samples at 15 and 30 min post-injection following which fish were euthanized at 240 min post-injection for collection of POA; and in the final trial, blood samples were collected at 3 and 60 min post-injection following which fish were euthanized for collection of POA. In a final experiment, a set of fish was treated (after collection of a baseline blood sample) with 37.5 mmol L^{-1} 5-HT to achieve a nominal circulating concentration of 100 nmol L^{-1} 5-HT; control fish were injected with a corresponding volume of saline solution. This 5-HT dose was chosen because it was the most effective concentration at eliciting cortisol production in *in vitro* head kidney experiments (see section 2.4). Blood samples for cortisol analysis were collected from these fish

at 60 and 120 min post-injection; in separate fish, blood samples were collected for ACTH analysis at 15, 60 and 120 min post-injection following which fish were terminally sampled for POA tissue for analysis of CRF mRNA abundance.

2.5.2 Cortisol quantification

Plasma cortisol concentrations were measured using a commercially-available RIA kit (MP Biomedicals) according to the protocols described above.

2.5.3 Glucose quantification

Plasma glucose concentration was analyzed spectrophotometrically according to Bergmeyer (1974). Intra-assay variation for glucose analyses was 14.3%.

2.5.4 ACTH quantification

Plasma ACTH concentrations were measured using a commercially available radioimmunoassay (hACTH Double Antibody RIA Kit; MP Biomedicals) according to the manufacturer's protocol. Use of this kit with rainbow trout plasma samples was validated in previous studies (Jeffrey et al., 2014; Jeffrey et al., 2012). Samples were run in duplicate, and inter-assay and intra-assay coefficients of variation were 6.3% and 8.2%, respectively.

2.5.5 Quantification of CRF mRNA abundance

The methods described above were used to extract RNA from POA tissue, to synthesize cDNA, and to carry out s-qPCR reactions. A standard curve for CRF was developed using a previously published primer set for rainbow trout CRF (Table 2.2). The mRNA abundance for fish treated with 5-HT was expressed relative to the value for the corresponding saline treatment using a modified version of the delta-delta Ct method (Pfaffl, 2001) with 18S being used as the normalizing gene.

2.6 Statistical analyses

Statistical analyses were performed using SigmaPlot (11.0, Systat Software) and data are presented as mean values ± one standard error of the mean (SEM). The level of statistical significance (α) was set to $p<0.05$ for all analyses. One-way analysis of variance (ANOVA) was used to evaluate the effects of HPI axis tissue on relative mRNA abundance for each 5-HT receptor type. One-way repeated-measures (RM) ANOVA was used to test for effects of treatments on *in vitro* cortisol production by head kidney tissue. Two-way RM ANOVA was used to test for effects of treatment (5-HT vs. saline) and sampling time on circulating cortisol, glucose and ACTH concentrations for *in vivo* experiments on serotonergic stimulation of the HPI axis. Finally, one-sample Student's *t*-tests were used to assess changes in CRF mRNA expression in the different treatment groups (5-HT vs. saline).

Table 2.1. Oligonucleotide primer pairs used for identification of 5-HT receptors by RT-PCR in rainbow trout (*Oncorhynchus mykiss*)

Gene	Primer Pair	Product length (bp)	Accession number
5-HT$_{1A}$	F CTGGTACTACCCATGGCAGC	891	KP334154
	R AATAGCCCAGCCAGTTGATG		
5HT$_{2A}$	F ATGCCGGTCTCCATGGTG	575	KP334155
	R GATGGACTGCATGGTACGG		
5HT$_{2C}$	F GTGTCCCTGGAGAAGAAGCT	757	KP334156
	R GTGATGAAAAACGGACACCACAT		
5HT$_{4}$	F CTCAGGAAAATCAAGACCAACT	765	KP334157
	R CGGAAGGACTTGTTGAGGAA		

Primer sequences are given in the 5’ to 3’ direction.

Table 2.2. Ensembl database accession numbers for amino acid sequences used for phylogenetic analysis of rainbow trout 5-HT receptors

Species/5-HT receptor	**5-HT$_{1A}$**	**5-HT$_{2A}$**	**5-HT$_{2C}$**	**5-HT$_{4}$**
Anole lizard (*Anolis carolinensis*)		ENSACAG00000014570	ENSACAG00000004275	ENSACAG00000015764
Chinese soft-shell turtle (*Pelodiscus sinensis*)	ENSPSIG00000000377	ENSPSIG00000006940	ENSPSIG00000011128	ENSPSIG00000006016
Xenopus(*Xenopus tropicalis*)	ENSXETG00000014812	ENSXETG00000032623		ENSXETG00000019373
Chicken (*Gallus gallus)*	ENSGALG00000014742	ENSGALG00000016992	ENSGALG00000005853	ENSGALG00000001383
Duck (*Anas platyrhynchos*)			ENSAPLG00000012164	ENSAPLG00000009505
Flycatcher (*Ficedula albicollis*)	ENSFALG00000014512	ENSFALG00000008251		
Human (*Homo sapiens*)	ENSG00000178394	ENSG00000102468	ENSG00000147246	ENSG00000164270
Mouse (*Mus musculus*)	ENSMUSG00000021721	ENSMUSG00000034997	ENSMUSG00000041380	ENSMUSG00000026322
Nile tilapia (*Oreochromis niloticus*)	ENSONIG00000020994 ENSONIG00000021056	ENSONIG00000016184	ENSONIG00000006962	ENSONIG00000004225
Coelocanth (*Latimeria chalumnae*)	ENSLACG00000002267	ENSLACG00000001141	ENSLACG00000002608	
Medaka (*Oryzias latipes*)		ENSORLG00000013998	ENSORLG00000008442	ENSORLG00000011251
Molly (*Poecilia formosa*)	ENSPFOG00000020826 ENSPFOG00000020353			
Nile tilapia (*Oreochromis niloticus*)	ENSONIG00000020994 ENSONIG00000021056	ENSONIG00000016184	ENSONIG00000006962	ENSONIG00000004225
Platyfish (*Xiphophorus maculatus*;)	ENSXMAG00000020325 ENSXMAG00000016395	ENSXMAG00000009303	ENSXMAG00000015068	
Spotted gar (*Lepisosteus oculatus*)		ENSLOCG00000010438	ENSLOCG00000013705	ENSLOCT00000015003
Stickleback (*Gasterosteus aculeatus*)				ENSGACG00000016819
Tetraodon (*Tetraodon nigroviridis*)	ENSTNIG00000005134 ENSTNIG00000004036			
Zebrafish (*Danio rerio*)	ENSDARG00000093745 ENSDARG00000057098	ENSDARG00000058165 ENSDARG00000057029	ENSDARG00000018228 ENSDARG00000013210	ENSDARG00000061940

Table 2.3. Oligonucleotide primer pairs used for semi-quantitative real-time RT-PCR quantification of 5-HT receptor and CRF mRNA abundance in rainbow trout (*Oncorhynchus mykiss*)

Gene	Primer Pair	Product length (bp)	Efficiency (%)	Accession number	Reference
5-HT$_{1A}$	F CTGGTACTACCCATGGCAGC	120	92.4	KP334154	
	R AGATGCAGAATGGACGACG				
5HT$_2$	F ACAGGGATGGGCATGGAGAT	165	100.7	KP334155	
	R GTTCTCCACAGCCTCCATCA				
5HT$_4$	F TGGAGAAACCTTCTGCCTGG	112	107.7	KP334157 KP334156	
	R TGGCAGCAGATGGCATAGTA				
CRF	F ACAACGACTCAACTGAAGATCTCG	54	97.7	AF296672.1	Bernier *et al.,* 2008
	R AGGAAATTGAGCTTCATGTCAGG				
18S	F GGCGGCGTTATTCCCATGACC	115	91.7	AF309412.1	Jeffrey *et al.,* 2012
	R GGTGGTGCCCTTCCGTCAATTC				
18S$_{1A}$	F GGCGGCGTTATTCCCATGACC	115	96.9	AF309412.1	Jeffrey *et al.,* 2012
	R GGTGGTGCCCTTCCGTCAATTC			AF309412.1	Jeffrey *et al.,* 2012
	R GGTGGTGCCCTTCCGTCAATTC			AF309412.1	Jeffrey *et al.,* 2012

Primer sequences are given in the 5' to 3' direction. *CRF*, corticotropin releasing factor

3. RESULTS

3.1 Tissue distribution of 5-HT receptors

Partial nucleotide sequences were obtained for the rainbow trout 5-HT_{1A} (765 nucleotides), 5-HT_{2A} (575 nucleotides), 5-HT_{2C} (756 nucleotides) and 5-HT_4 (765 nucleotides) receptors. For additional details concerning the regions of the rainbow trout 5-HT receptor that were cloned as well as nucleotide and amino acid alignments of the 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_4 receptors, refer to the Appendices. Alignment of these nucleotide sequences using ClustalW2 (EMBL-European Bioinformatics Institute) revealed identity ranging from 40.3 to 65.4% among the trout 5-HT receptors (Table 3.1). Nucleotide sequence indentity of (partial) rainbow trout 5-HT receptors with the corresponding human 5-HT receptors were 67.1%, 64.6%, 55.7% and 81.6% for the 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_4 receptors, respectively. Phylogenetic analysis of 66 5-HT receptor predicted amino acid sequences across a variety of vertebrates placed the rainbow trout 5-HT receptor partial sequences within their respective receptor clades (Fig. 3.1). Posterior probabilities for 5-HT receptor nodes were 0.99 (5-HT_{1A}), 0.52 (5-HT_{2A}), 0.77 (5-HT_{2C}) and 0.89 (5-HT_4) and placed the rainbow trout 5-HT receptors in their respective clades. For this analysis, posterior probabilities represented the likelihood of the placement of rainbow trout 5-HT receptors in their respective clades, given an a priori model of evolution (in this case a mixed model).

The relative mRNA abundance in HPI axis tissues of the 5-HT_{1A}, 5-HT_2 and 5-HT_4 receptors was assessed (Fig. 3.2). All three receptor subtypes were detected in

all three tissues of the HPI axis. The relative mRNA abundance of the 5-HT_{1A} receptor was significantly higher in the POA than in the pituitary or head kidney (Fig. 3.2A, one-way ANOVA on ranks, p=0.01). Similarly, the POA exhibited significantly higher relative mRNA abundance for the 5-HT_2 receptor than the pituitary or head kidney (Fig. 3.2B, one-way ANOVA, p=0.004). For the 5-HT_4 receptor, relative mRNA abundance was highest in the POA and lowest in the head kidney with intermediate levels in the pituitary (Fig. 3.2C, one-way ANOVA on ranks, p=0.002).

3.2 Serotonergic stimulation of head kidney tissue *in vitro*

The ability of different concentrations of 5-HT (10, 100 and 1000 nmol L^{-1}) to stimulate cortisol production by head kidney preparations *in vitro* was assessed in separate experimental trials. All three 5-HT concentrations significantly increased cortisol production over that of unstimulated tissue (rank sum tests, p=0.015 for 10 nmol L^{-1} 5-HT and p=0.0445 for 1000 nmol L^{-1} dose; Student's t-test, p<0.001 for 100 nmol L^{-1} 5-HT). Although 5-HT caused significant increases in cortisol production, it was substantially less effective than ACTH in terms of stimulating cortisol production. To compare effects on cortisol production across 5-HT concentrations, cortisol production for each 5-HT concentration was expressed as a percentage of the corresponding unstimulated value. This analysis revealed that cortisol production by head kidney tissue was highest in tissue incubated with 100 nmol L^{-1} 5-HT (Fig. 3.3, one-way ANOVA on ranks, p=0.02).

To test for the involvement of specific 5-HT receptors subtypes in stimulating cortisol production by head kidney tissue *in vitro*, agonist and antagonist combinations selective for specific 5-HT receptor subtypes were used. No significant effect of incubation with 8-OH-DPAT (a 5-$HT_{1A/7}$ receptor agonist) or a combination of 8-OH-DPAT and WAY100635 (a 5-HT_{1A} receptor antagonist) on cortisol production by head kidney tissue was detected (Fig. 3.4A, one-way RM ANOVA, p=0.108). Similarly, there was no significant change in cortisol production by head kidney tissue incubated with α-methyl 5-HT (a 5-HT_2 receptor agonist) or a combination of α-methyl 5-HT and ketanserin (a 5-HT_2 receptor antagonist) (Fig. 3.4B, one-way RM ANOVA, p=0.261). However, cortisol production was significantly increased over the blank value in tissue incubated with cisapride, a 5-HT_4 receptor agonist (Fig. 3.4C, RM ANOVA on ranks, p=0.016). Cortisol production did not differ significantly between unstimulated tissue and tissue incubated with a combination of cisapride and GR125487 (a 5-HT_4 antagonist).

3.3 Serotonergic stimulation of the HPI axis *in vivo*

The potential for 5-HT to activate the HPI axis was assessed by treating rainbow trout with saline or one of two different doses of 5-HT. Baseline circulating cortisol concentrations were elevated above the value considered typical of unstressed fish (<10 ng mL^{-1}) (Gamperl et al., 1994), likely reflecting stress associated with cannulation and/or isolation of the fish in individual experimental chambers. In fish exposed to 1000 nmol L^{-1} 5-HT, plasma cortisol concentrations at 30 and 60 min post-injection were significantly elevated over values in their saline

treated counterparts (Fig. 3.5A, two-way RM ANOVA, p= 0.001 for treatment x sample time). Within the 5-HT treatment group, plasma cortisol concentrations were significantly higher than baseline levels at 15, 30 and 60 min post-injection. Changes in circulating cortisol concentrations in saline-injected fish were not significant. Although a significant effect of sampling time on circulating cortisol concentrations was detected in fish exposed to a lower dose of 5-HT, 100 nmol L^{-1}, neither the effect of treatment nor the interaction of treatment and sampling time was significant; both 5-HT- and saline-treated fish exhibited a significant increase in cortisol concentrations 60 min post-injection (Fig. 3.5B, two-way RM ANOVA, p=0.937 for treatment, p=0.024 for sample time and p=0.210 for treatment x sample time).

Changes in plasma glucose concentrations (Fig. 3.6) were measured as a downstream index of HPI axis activation. In fish exposed to 1000 nmol L^{-1} 5-HT, plasma glucose concentrations were significantly elevated at 240 min post-injection over values in the saline-treated fish (Fig. 3.6A, two-way RM ANOVA, p= 0.014 for treatment x sample time). Within treatments, changes in circulating glucose concentrations in both 5-HT and saline-injected fish were not significant. Circulating glucose concentrations were unaffected by time or saline/5-HT administration in trout exposed to 100 nmol L^{-1} 5-HT (Fig. 3.6B, two-way RM ANOVA, p=0.123 for treatment, p=0.375 for sample time and p=0.605 for treatment x sample time).

Changes in plasma ACTH concentrations (Fig. 3.7) and POA CRF relative mRNA abundance (Fig. 3.8) also were measured after administration of 5-HT.

Statistical analysis of individual trials in which fish were exposed to 1000 nmol L^{-1} 5-HT and sampled at 15 min (Figure 3.7A, two-way RM ANOVA, p=0.401 for treatment, p=0.015 for sample time and p=0.391 for treatment x sample time) or at 15 and 30 min (Figure 3.7A, two-way RM ANOVA, p=0.367 for treatment, p=0.001 for sample time and p=0.651 for treatment x sample time) post-injection revealed a significant effect of sampling time in both treatment groups, i.e. a sampling artefact as opposed to an effect of 5-HT *per se*. No significant changes in plasma ACTH concentrations were detected in fish sampled 3 and 60 min post-injection (Figure 3.7A, two-way RM ANOVA, p=0.741 for treatment, p=0.225 for sample time and p=0.077 for treatment x sample time); for presentation purposes, data for all three trials were combined (Fig. 3.7A). No significant differences in plasma ACTH concentrations were detected in fish exposed to the lower dose of 5-HT (Fig. 3.7B, two-way RM ANOVA p= 0.728 for treatment, p=0.062 for sample time and p=0.207 for treatment x sample time). One-sample Student's t-tests were used to determine whether changes in CRF relative mRNA levels in POA tissue sampled from 5-HT-treated fish were significantly different from the control value of one. No significant effects of 5-HT administration on POA CRF relative mRNA abundance were detected for fish exposed to 1000 nmol L^{-1} 5-HT (Fig. 3.8A, Student's t-test, p=0.42 at 15 min, p=0.58 at 60 min and p=0.28 at 240 min). However, CRF relative mRNA levels were significantly lower than 1 in trout exposed to 100 nmol L^{-1} 5-HT (Fig. 3.8B, Student's t-test, $p<0.00001$).

Table 3.1. Serotonin receptor nucleotide sequence identities for partially sequenced 5-HT receptors in rainbow trout (*Oncorhynchus mykiss*).

Receptor Family/Subtype	5-HT1A	5-HT2A	5-HT2C	5-HT4
5-HT1A	-	50.3%	41.4%	40.3%
5-HT2A		-	65.4%	49.0%
5-HT2C			-	44.6%
5-HT4				-

Figure 3.1. Phylogenetic analysis of 66 5-HT receptor sequences. Sequences were aligned using ClustalX 2.1, and the tree constructed using Bayesian analysis (10^6 generations, sampling frequency =100, burnin=250). Arrows indicate the locations of cloned partial sequences for the rainbow trout (*Oncorhynchus mykiss*) 5-HT receptors. The scale bar represents the number of amino acid substitutions per branch length. Nodes are labeled with an abbreviated species name followed by 5-HT receptor subtype (e.g. Meda4 represents the 5-HT_4 receptor of the medaka, *Oryzias latipes*). *Anol*, anole lizard, *Anolis carolinensis*; *Ciona*, *Ciona instestinalis*; *Chick*, chicken, *Gallus gallus*; *Cod*, Atlantic cod, *Gadus morhua*; *Coelo*, Coelocanth, *Latimeria chalumnae*; *Danio*, zebrafish, *Danio rerio*; *Duck*, duck, *Anas platyrhynchos*; *Gar*, spotted gar, *Lepisosteus oculatus*; *Meda*, medaka; *Mol*, molly, *Poecilia formosa*; *Plat*, platyfish, *Xiphophorus maculatus*; *Stick*, stickleback, *Gasterosteus aculeatus*; *Tetr*, green spotted pufferfish, *Tetraodon nigroviridis*; *Tila*, Nile tilapia, *Oreochromis niloticus*; *Trout*, rainbow trout; *Turt,* Chinese soft shell turtle, *Pelodiscus sinensis*; *Xeno*, *Xenopus tropicalis*. Nodes labeled *tetrapod* indicate a grouping of tetrapod species, including duck, chicken, anole lizard, fly-catcher (*Ficedula albicollis*), *Xenopus*, turtle, mouse (*Mus musculus*) and human (*Homo sapiens*).

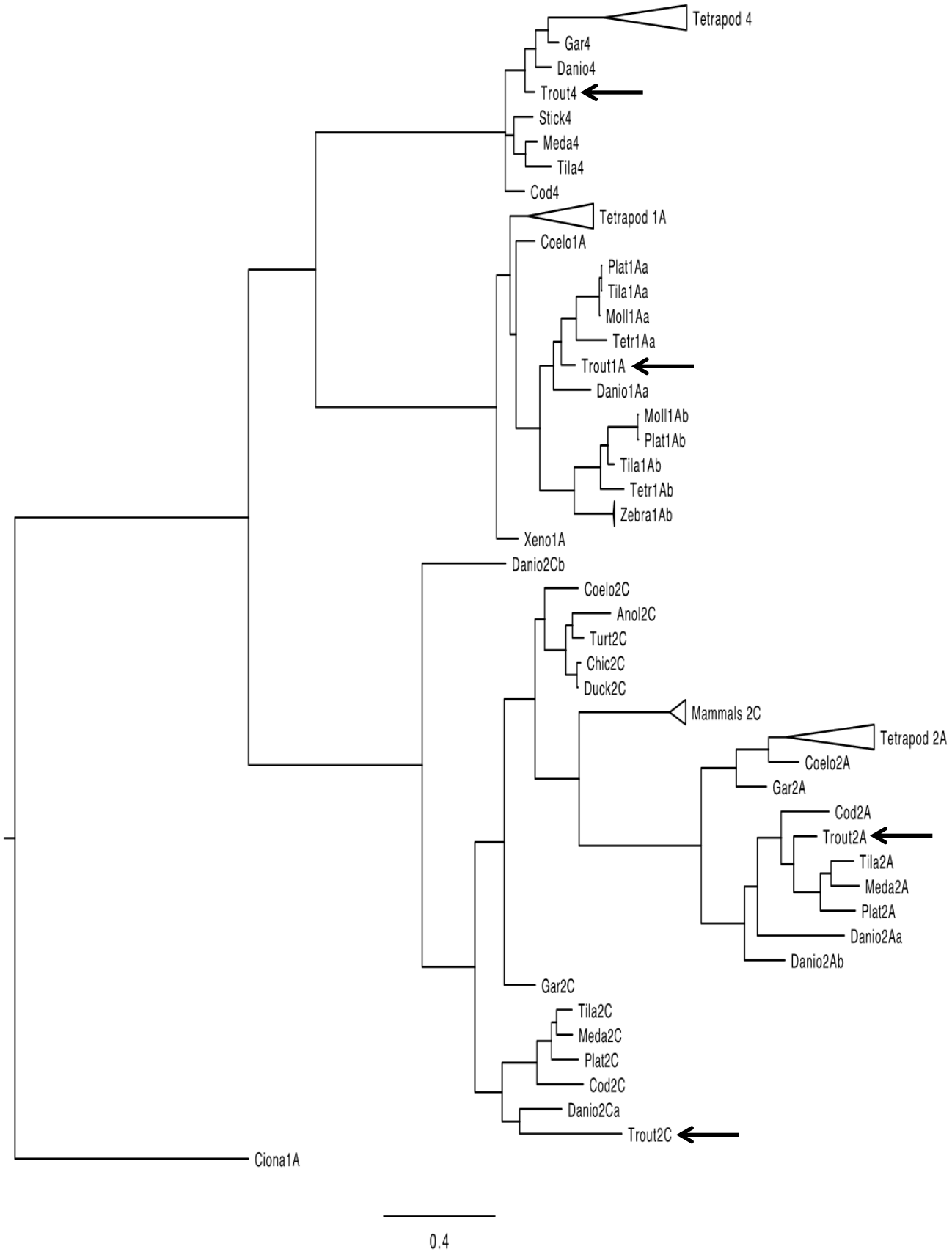

Tetrapod 4
Gar4
Danio4
Trout4
Stick4
Meda4
Tila4
Cod4
Tetrapod 1A
Coelo1A
Plat1Aa
Tila1Aa
Moll1Aa
Tetr1Aa
Trout1A
Danio1Aa
Moll1Ab
Plat1Ab
Tila1Ab
Tetr1Ab
Zebra1Ab
Xeno1A
Danio2Cb
Coelo2C
Anol2C
Turt2C
Chic2C
Duck2C
Mammals 2C
Tetrapod 2A
Coelo2A
Gar2A
Cod2A
Trout2A
Tila2A
Meda2A
Plat2A
Danio2Aa
Danio2Ab
Gar2C
Tila2C
Meda2C
Plat2C
Cod2C
Danio2Ca
Trout2C
Ciona1A
0.4

Figure 3.2. Relative mRNA abundance of 5-HT$_{1A}$ (A), 5-HT$_2$ (B) and 5-HT$_4$ (C) receptors in preoptic area, head kidney and pituitary tissue of rainbow trout (*Oncorhynchus mykiss*). All data are expressed relative to the mRNA abundance of 18S, and to the value in head kidney tissue for the particular receptor subtype. Values are presented as means + SEM, *N*=5 for preoptic area and pituitary, *N*=6 for head kidney. Data were analyzed by one-way ANOVA and treatment groups that share a letter do not significantly differ from one another (see text for details).

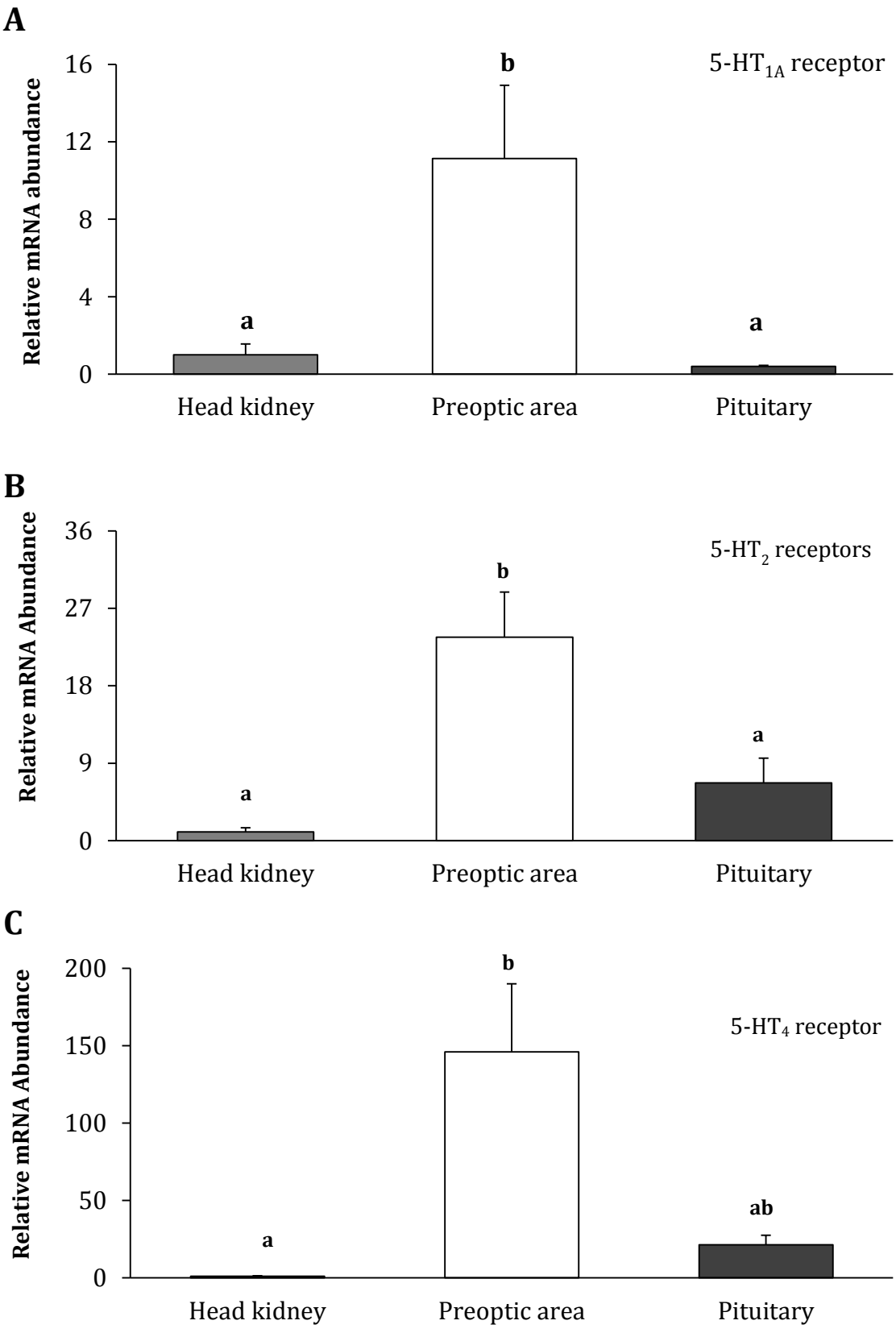

A
Relative mRNA abundance
16
12
8
4
0
b
a
a
5-HT1A receptor
Head kidney
Preoptic area
Pituitary
B
Relative mRNA Abundance
36
27
18
9
0
b
a
a
5-HT2 receptors
Head kidney
Preoptic area
Pituitary
C
Relative mRNA Abundance
200
150
100
50
0
b
a
ab
5-HT4 receptor
Head kidney
Preoptic area
Pituitary

Figure 3.3. Cortisol production by rainbow trout (*Oncorhynchus mykiss*) head kidney tissue incubated *in vitro* with different concentrations of 5-HT. Cortisol production in the presence of 5-HT is presented as a percentage of cortisol production by unstimulated tissue. Values are means + SEM; *N*=6 for 10 and 100 nmol L^{-1} 5-HT and *N*=12 for 1000 nmol L^{-1} 5-HT. Data were analyzed by one-way ANOVA and treatment groups that share a letter do not differ significantly from one another (see text for details).

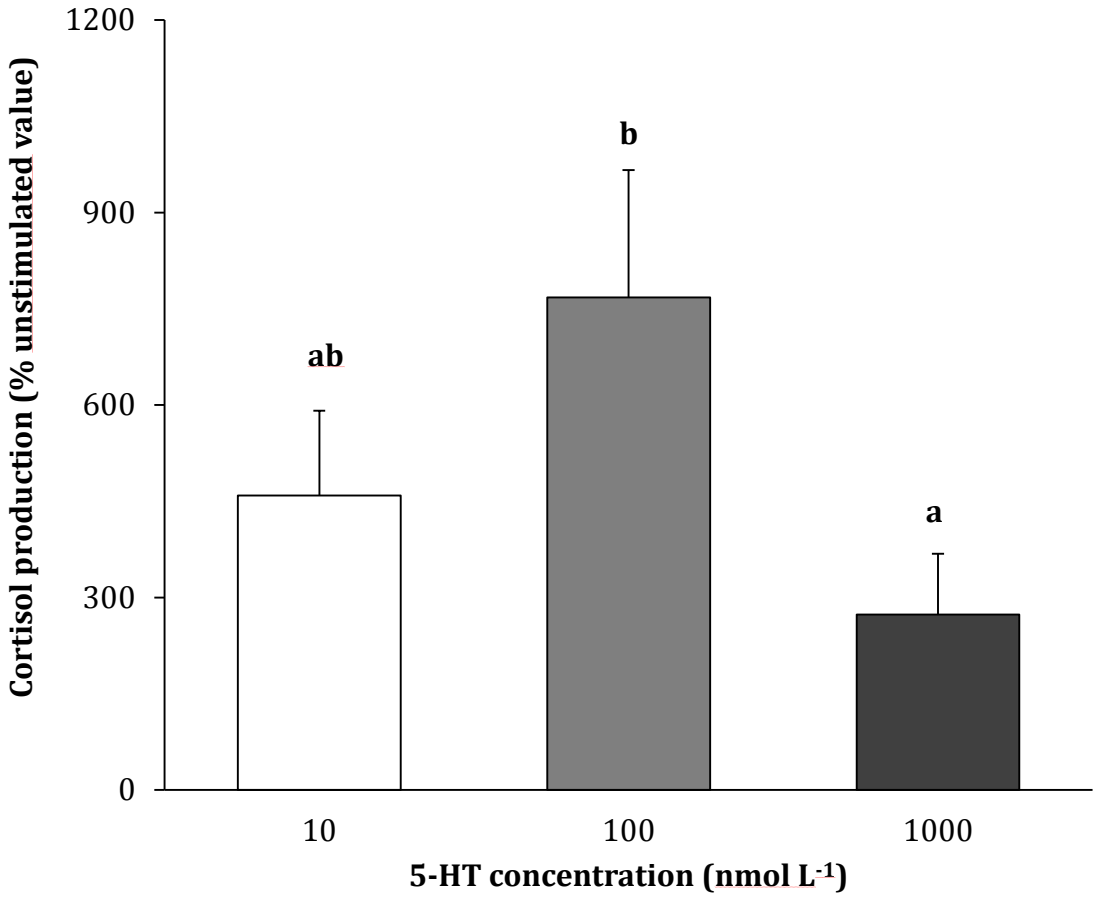
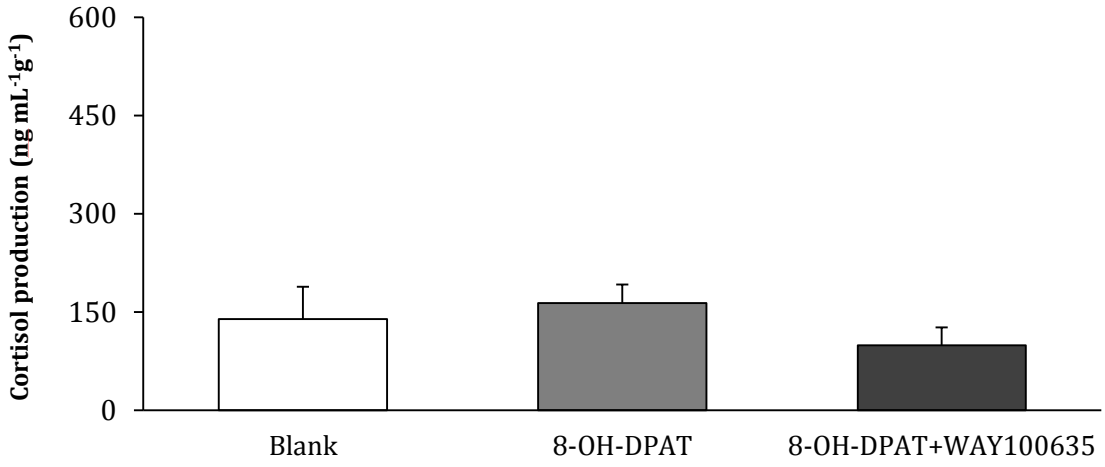
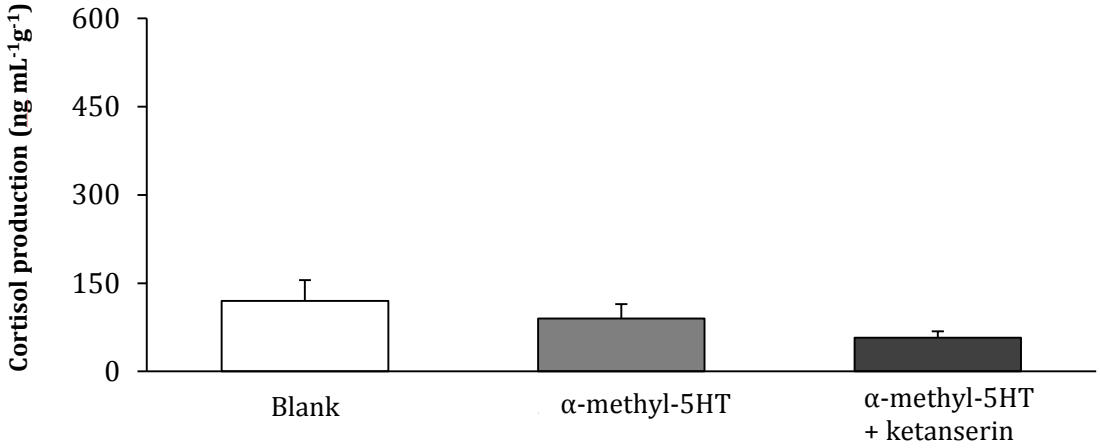

1200
900
600
300
0
Cortisol production (% unstimulated value)
ab
b
a
10
100
1000
5-HT concentration (nmol L-1)

Figure 3.4. Cortisol production by rainbow trout (*Oncorhynchus mykiss*) head kidney tissue incubated *in vitro* with agonists and antagonists specific for selected 5-HT receptors. Panel A presents data for head kidney tissue (*N*=6) incubated with 8-OH-DPAT (5-$HT_{1A/7}$ receptor agonist) or 8-OH-DPAT and WAY100635 (5-HT_{1A} receptor antagonist). Panel B presents data for head kidney tissue (*N*=5) incubated with α-methyl-5HT (5-HT_2 receptor agonist) or α-methyl-5HT and ketanserin (5-HT_2 receptor antagonist). Panel C presents data for head kidney tissue (*N*=9) incubated with cisapride (5-HT_4 receptor agonist) or cisapride and GR125487 (5-HT_4 receptor antagonist). Values are means + SEM. Data were analyzed by one-way RM ANOVA and treatment groups that share a letter do not differ significantly from one another (see text for details).

A


Cortisol production (ng mL-1g-1)
600
450
300
150
0
Blank
8-OH-DPAT
8-OH-DPAT+WAY100635

B


Cortisol production (ng mL-1g-1)
600
450
300
150
0
Blank
α-methyl-5HT
α-methyl-5HT
+ ketanserin

C

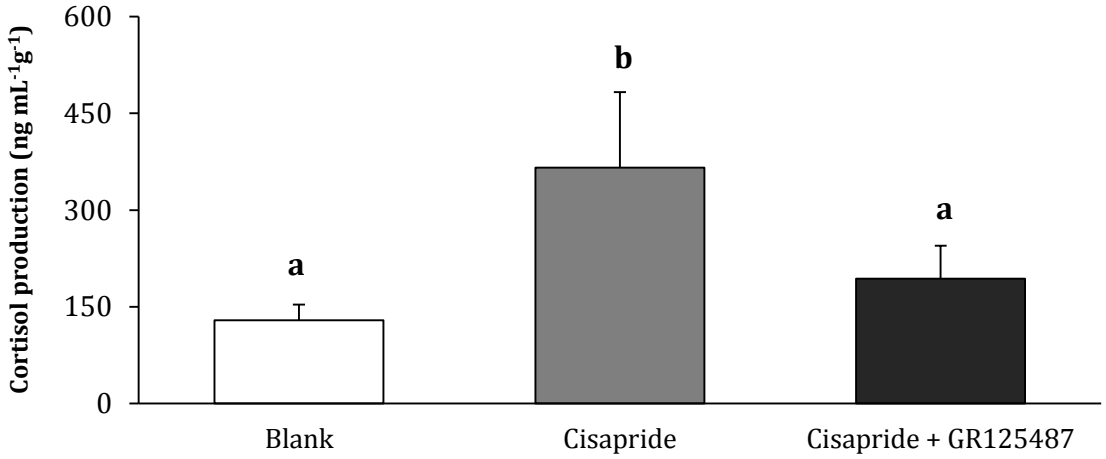
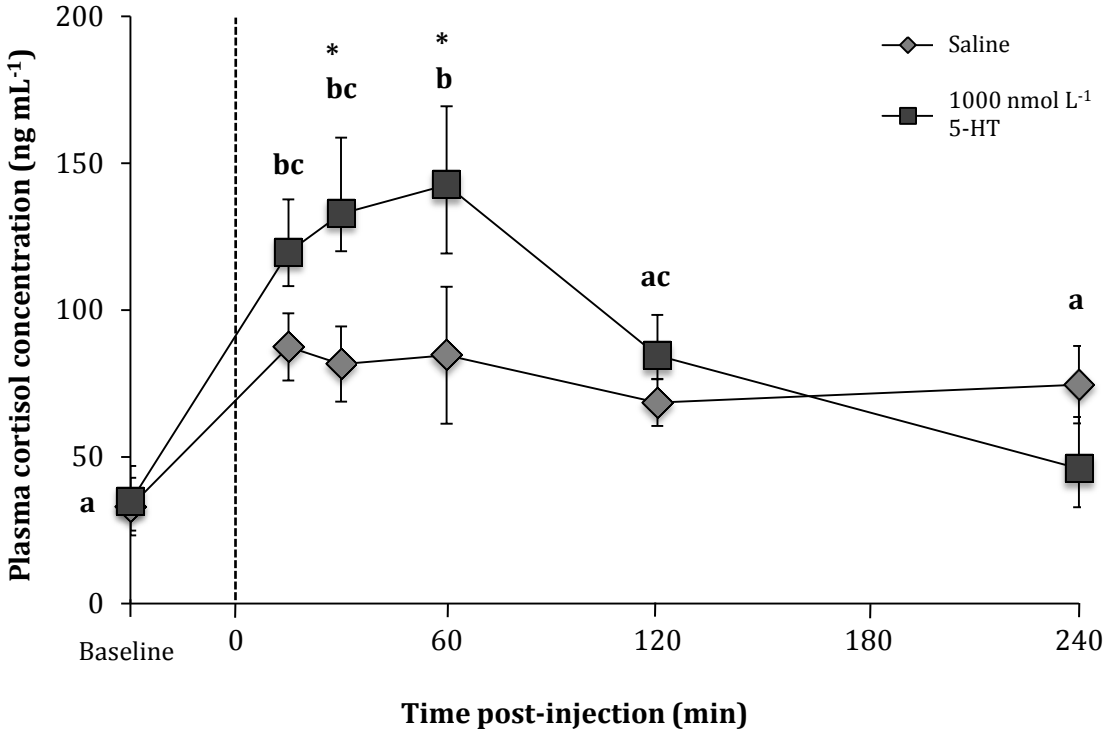

Cortisol production (ng mL-1g-1)
600
450
300
150
0
a
b
a
Blank
Cisapride
Cisapride + GR125487

Figure 3.5. Plasma cortisol concentrations in rainbow trout (*Oncorhynchus mykiss*) treated with either saline or 5-HT. Panel A presents data for fish that were treated with 0.8 mL kg^{-1} of saline (*N*=6) or 375 mmol L^{-1} 5-HT to achieve a nominal circulating concentration of 1000 nmol L^{-1} (*N*=7). Panel B presents data for fish that were treated with 0.8 mL kg^{-1} of saline (*N*=5) or 37.5 mmol L^{-1} 5-HT to achieve a circulating dose of 100 nmol L^{-1} (*N*=9). Values are presented as means ± SEM. The dashed line indicates the point of saline or 5-HT administration. Data were analyzed by two-way RM ANOVA and time points within a treatment that share a letter do not differ significantly from one another (see text for details). Significant differences between treatments at a specific time are indicated by an asterisk.

A


Plasma cortisol concentration (ng mL-1)
200
150
100
50
0
Saline
1000 nmol L-1 5-HT
*
*
bc
bc
b
ac
a
a
Baseline
0
60
120
180
240
Time post-injection (min)

B

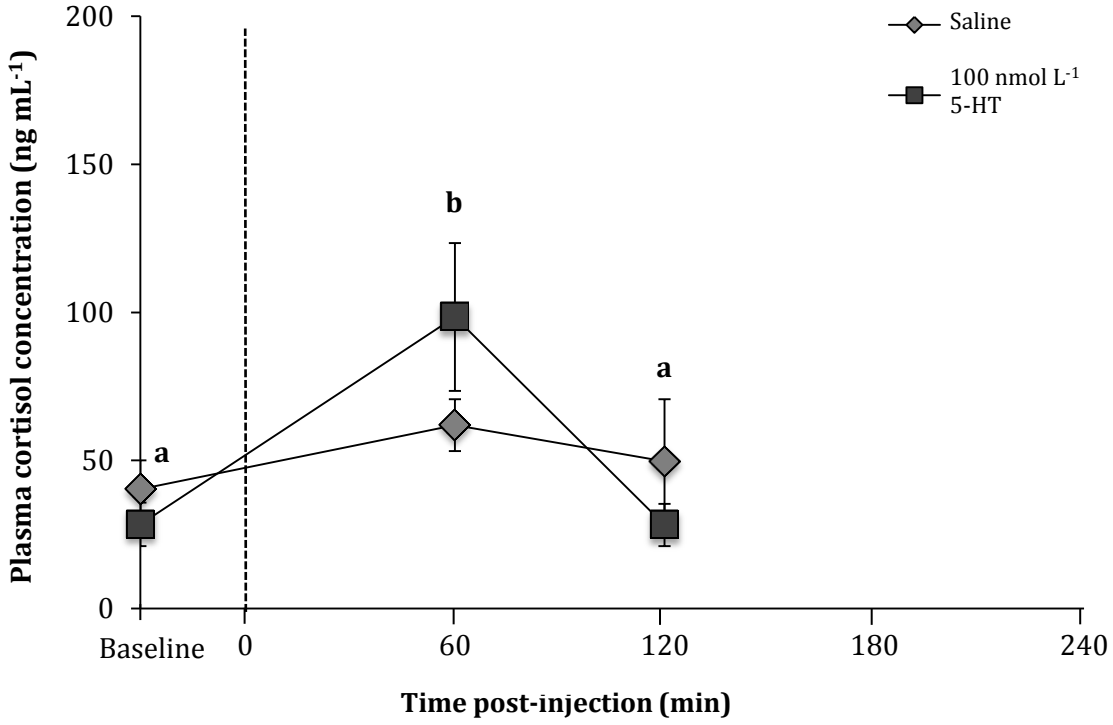
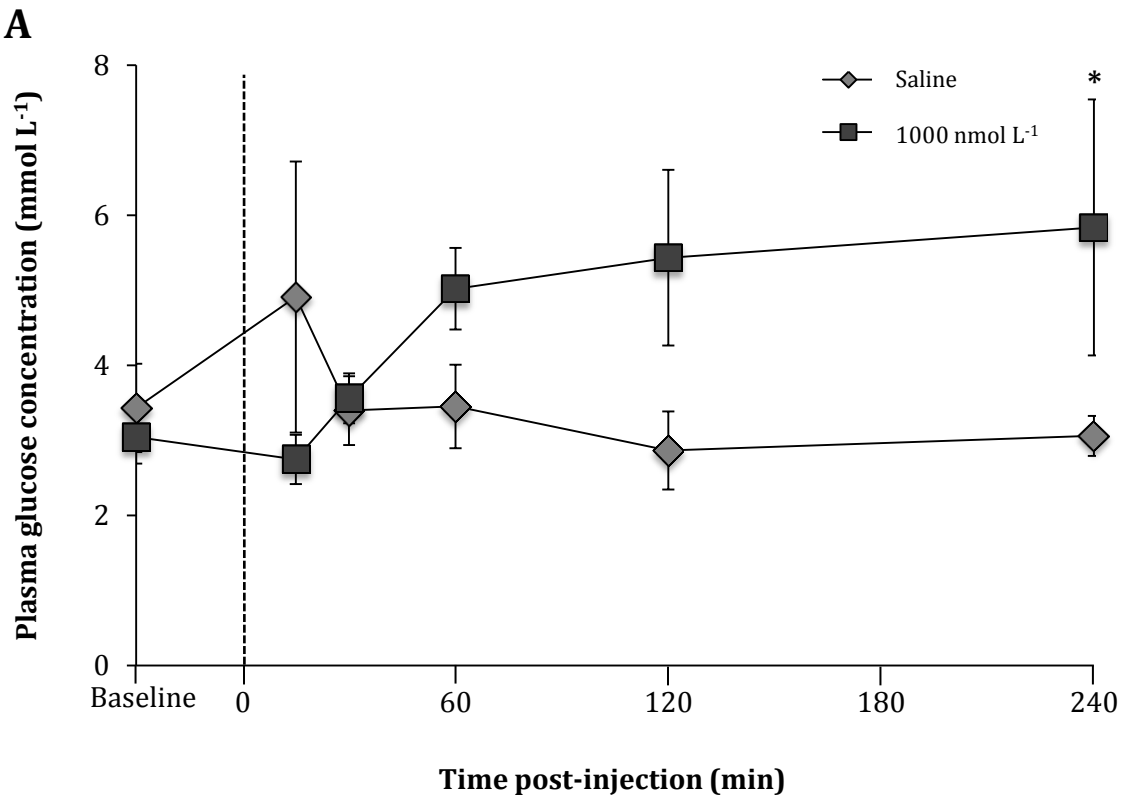

Plasma cortisol concentration (ng mL-1)
200
150
100
50
0
Saline
100 nmol L-1 5-HT
b
a
a
Baseline
0
60
120
180
240
Time post-injection (min)

Figure 3.6. Plasma glucose concentrations in rainbow trout (*Oncorhynchus mykiss*) treated with either saline or 5-HT. Panel A presents data for fish that were treated with 0.8 mL kg^{-1} of saline (*N*=6) or 375 mmol L^{-1} 5-HT to achieve a nominal circulating concentration of 1000 nmol L^{-1} (*N*=6-7). Panel B presents data for fish that were treated with 0.8 mL kg^{-1} of saline (*N*=5) or 37.5 mmol L^{-1} 5-HT to achieve a circulating dose of 100 nmol L^{-1} (*N*=9). Values are presented as means ± SEM. The dashed line indicates the point of saline or 5-HT administration. Data were analyzed by two-way RM ANOVA and significant differences between treatments at a specific time are indicated by an asterisk (see text for details).


A
Plasma glucose concentration (mmol L-1)
8
6
4
2
0
Saline
1000 nmol L-1
*
Baseline
0
60
120
180
240
Time post-injection (min)

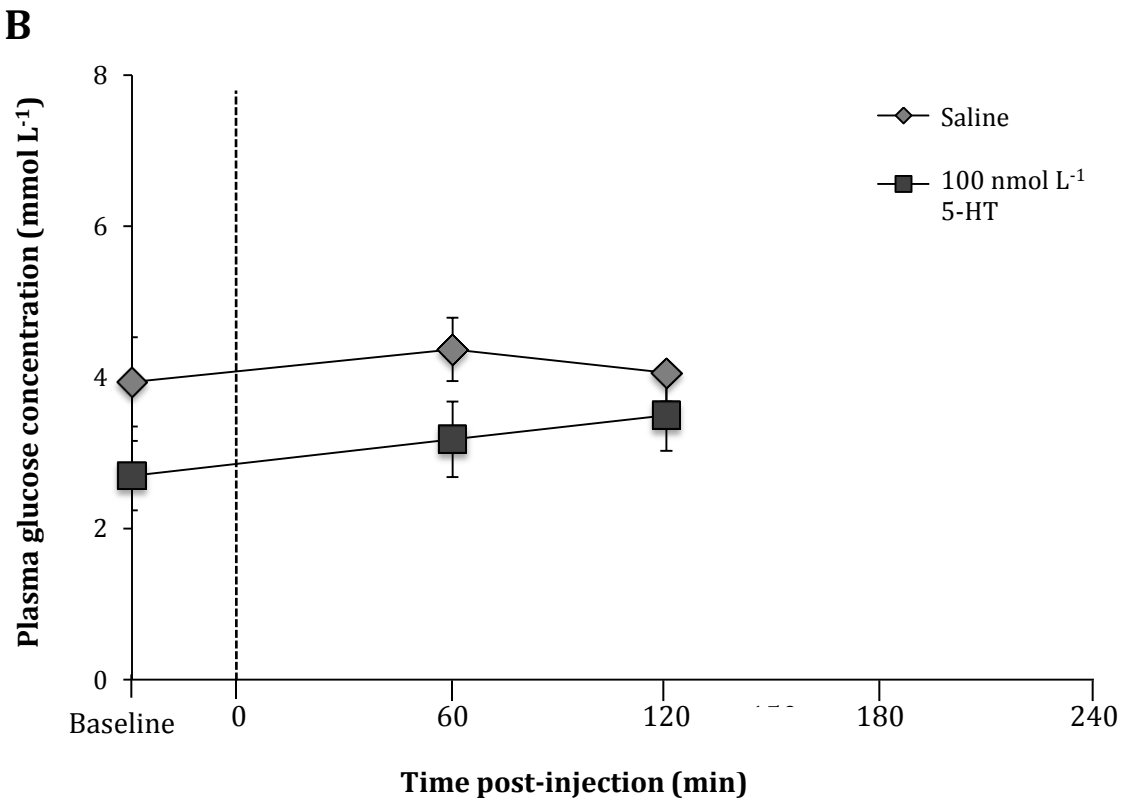
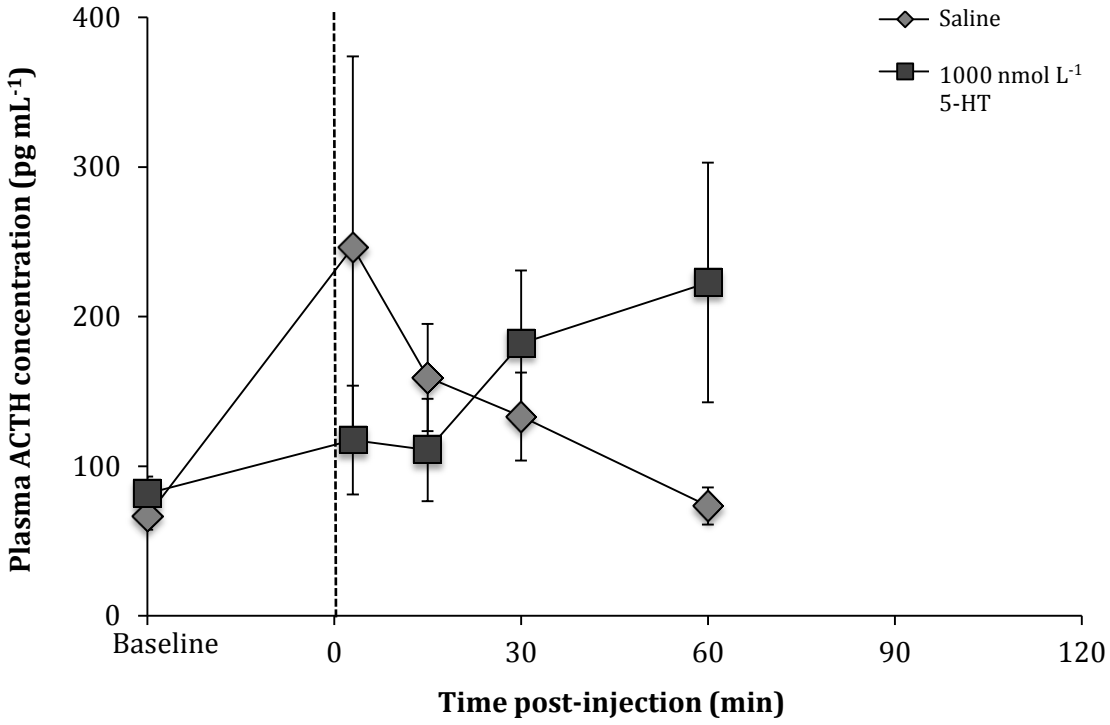

B
Plasma glucose concentration (mmol L-1)
8
6
4
2
0
Saline
100 nmol L-1
5-HT
Baseline
0
60
120
180
240
Time post-injection (min)

Figure 3.7. Plasma ACTH concentrations in rainbow trout (*Oncorhynchus mykiss*) treated with saline or 5-HT. Panel A presents data for fish injected with either 0.8 mL kg^{-1} of saline (*N*=5-11) or an equivalent volume of 375 mmol L^{-1} dose 5-HT (to achieve a nominal circulating concentration of 1000 nmol L^{-1} 5-HT; *N*=5-14). Panel B presents data for fish injected with either 0.8 mL kg^{-1} of saline (N=5) or an equivalent volume of 37.5 mmol L^{-1} dose 5-HT (to achieve a nominal circulating concentration of 100 nmol L^{-1}; *N*=7). Values are means ± SEM. The dashed line indicates the point of saline or 5-HT administration. Data were analyzed by two-way RM ANOVA (see text for details).

A


Saline
1000 nmol L-1 5-HT
Plasma ACTH concentration (pg mL-1)
400
300
200
100
0
Baseline
0
30
60
90
120
Time post-injection (min)

B

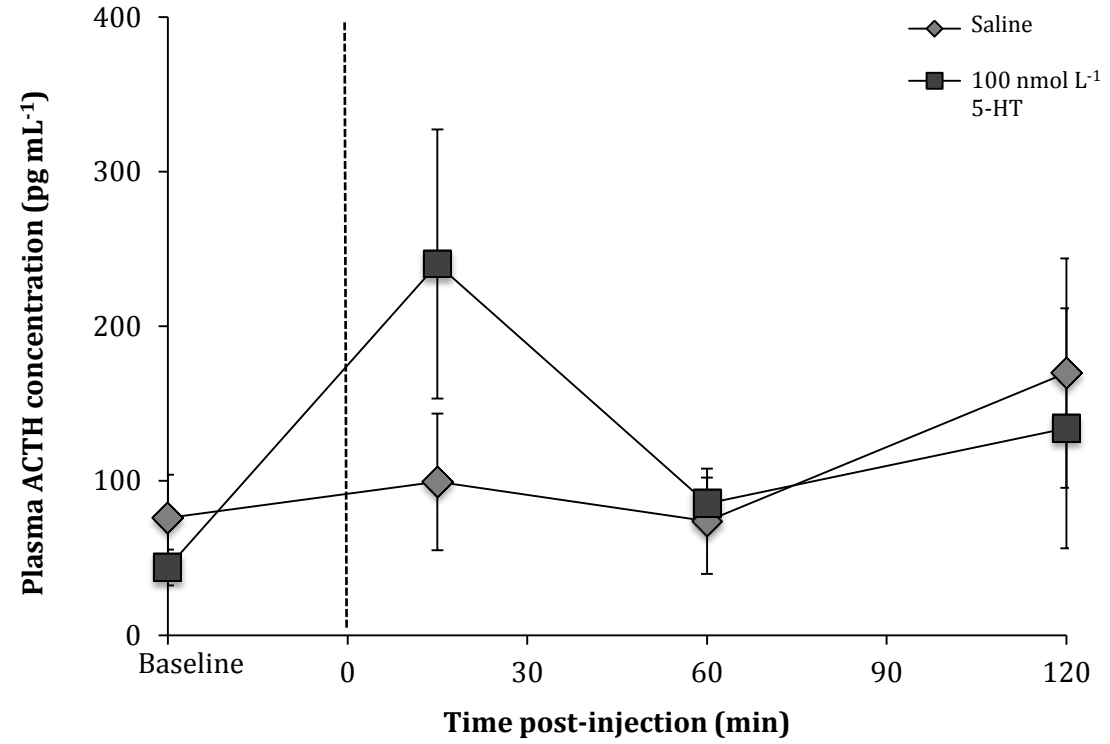
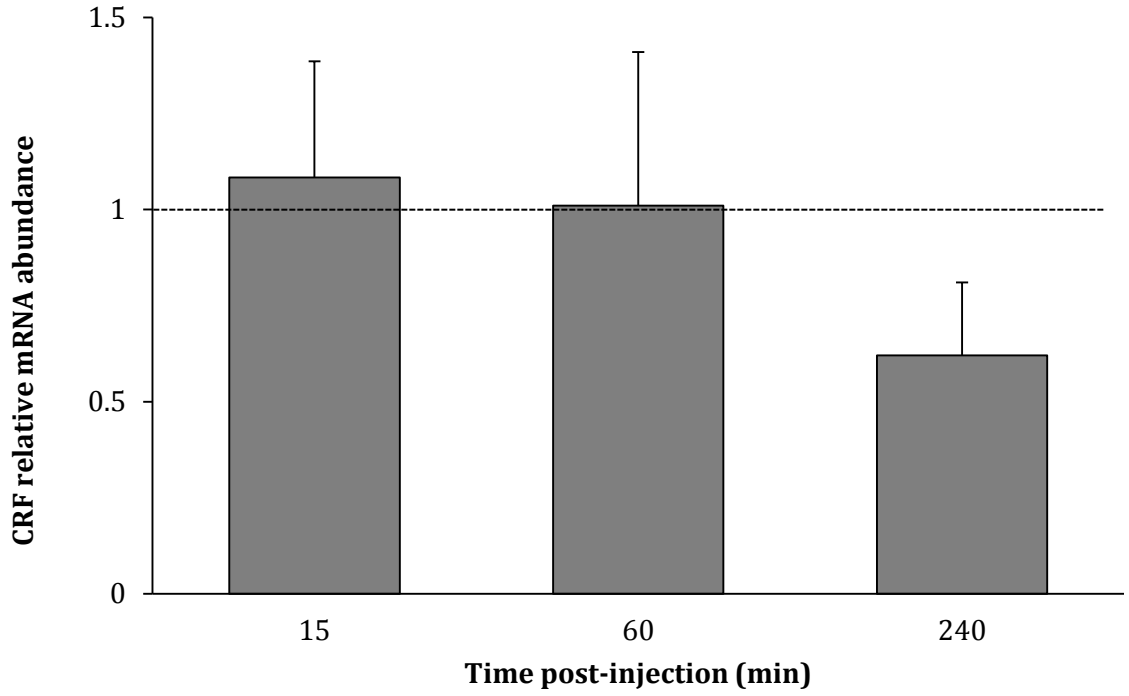
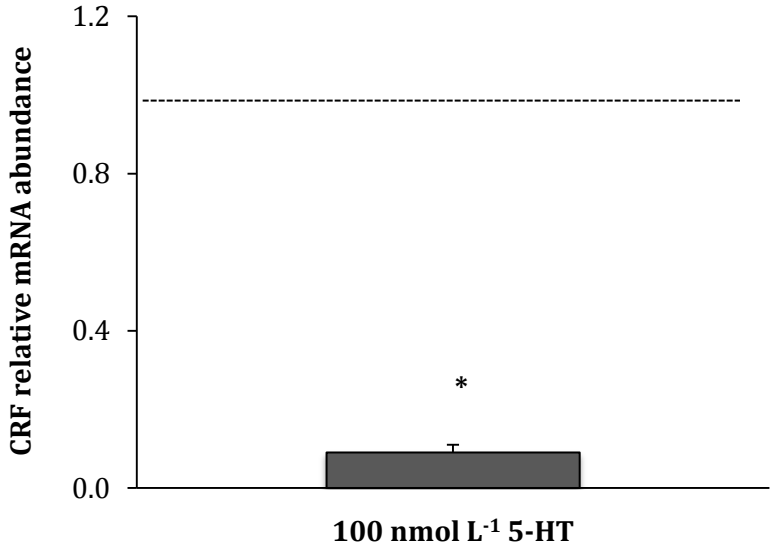

Saline
100 nmol L-1 5-HT
Plasma ACTH concentration (pg mL-1)
400
300
200
100
0
Baseline
0
30
60
90
120
Time post-injection (min)

Figure 3.8. Relative mRNA abundance of corticotropin releasing factor (CRF) in preoptic area tissue of rainbow trout (*Oncorhynchus mykiss*) injected with 5-HT. Panel A presents fish injected with 0.8 mL kg^{-1} of 375 mmol L^{-1} 5-HT (to achieve a circulating dose of 1000 nmol L^{-1}) and sampled at 15 (*N*=7), 60 (*N*=5-7) or 240 (*N*=7) min post-injection. Panel B presents fish injected with 0.8 mL kg^{-1} of 37.5 mmol L^{-1} 5-HT (to achieve a circulating dose of 100 nmol L^{-1}) and sampled at 120 min post-injection (*N* = 5-9). All data are expressed relative to mRNA expression of the normalizing 18S gene, and to the respective saline group for each time point. Values are presented as means + SEM. Data were analyzed by Student's t-tests and an asterisk indicates a value that is significantly different from the control value of 1, which is indicated by the dashed line (see text for details).

A


1.5
1
0.5
0
CRF relative mRNA abundance
15
60
240
Time post-injection (min)

B


1.2
0.8
0.4
0.0
CRF relative mRNA abundance
*
100 nmol L-1 5-HT

4. DISCUSSION

Previous studies in unstressed rainbow trout suggested that cortisol production could be stimulated by activation of the 5-HT_{1A} receptor (Winberg et al., 1997). The present thesis adopted a mechanistic approach to investigate whether and how 5-HT regulates stress axis activity in rainbow trout. The results of this thesis indicate that at least three subtypes of 5-HT receptors are expressed throughout the HPI axis in rainbow trout. Further, 5-HT can increase plasma cortisol concentrations *in vivo* and can directly stimulate cortisol production from head kidney tissue, likely via the 5-HT_4 receptor. The following discussion will expand upon three key themes; expression of 5-HT receptors in the HPI axis, 5-HT-induced stimulation of cortisol production by head kidney tissue, and 5-HT-induced stimulation of the HPI axis as a whole. Comparisons will be made with current literature in this field and experimental limitations will be considered. Additionally, unanswered questions and future directions stemming from this research will be discussed.

4.1 5-HT receptors are expressed throughout the HPI axis

Of the many different 5-HT receptor subtypes that have been identified to date in mammals (reviewed by Hannon and Hoyer, 2008), this thesis focussed on the 5-HT_{1A}, 5-HT_2 and 5-HT_4 receptors, because previous work in fish (Winberg et al., 1997; Medeiros et al., 2010; Mager et al., 2012; Lim et al., 2013) and mammals (Bagdy, 1989; Calogero et al., 1989; Lefebvre et al., 1992) suggested that these are

the receptors most likely to regulate the HPI axis in teleost fish. Phylogenetic analysis of predicted amino acid sequences for the partial sequences obtained for these three 5-HT receptors placed each receptor in its respective clade, suggesting that the molecular structure, and perhaps the function, of these receptors may be conserved in rainbow trout. Detailed molecular characterizations have been published for the 5-HT_{1A} receptor in toadfish (Medeiros et al., 2010), zebrafish (Norton et al., 2008), tilapia (Wang et al., 2006) and fugu (Yamaguchi et al., 1997). Teleost 5-HT_{1A} receptors, including the partial sequence for the rainbow trout 5-HT_{1A} receptor, share nucleotide sequence identity ranging from 67.5 to 76% with the human 5-HT_{1A} receptor (Medeiros et al., 2010; Norton et al., 2008; Wang et al., 2006 and Yamaguchi et al., 1997) supporting the notion that this receptor is well conserved. Although other 5-HT receptors in teleost fish have received less research effort to date than the 5-HT_{1A} receptor, the toadfish 5-HT_{2A} receptor (Mager et al., 2012) as well as the zebrafish 5-HT_{2C} receptor (Schneider et al., 2012) have been sequenced and characterized. The toadfish (Mager et al., 2012) and partial sequence for the rainbow trout 5-HT_{2A} receptors, as well as the zebrafish (Schneider et al., 2012) and partial sequence for the rainbow trout 5-HT_{2c} receptors, have sequence homologies of over 54% with their respective human sequences, indicating that these receptors also are likely well conserved.

A fairly high degree of nucleotide sequence identity (57%) between the 5-HT_{2A} and 5-HT_{2C} receptor subtypes has been reported in humans (reviewed by Hoyer et al., 2002). A similarly high level of nucleotide sequence identity (65%) was found between these receptor subtypes in rainbow trout. The conserved nature of

the 5-HT_{2A} and 5-HT_{2C} receptors made it difficult to design primers that were specific to each subtype, and therefore, relative mRNA levels were evaluated for 5-HT_2 receptors as a family, rather than for individual subtypes. It is likely that there are tissue-specific differences between these receptor subtypes in rainbow trout, as is the case in humans (reviewed by Baxter et al., 1995).

The partial sequence for the rainbow trout 5-HT_4 receptor exhibited a high degree of nucleotide sequence identity (82%) with its human counterpart, suggesting that this receptor also is well conserved. However, the mammalian 5-HT_4 receptor gene is complex and is known to encode at least 10 unique splice variants (reviewed by Bender et al., 2000; Hannon and Hoyer, 2008). These splice variants are differentially expressed among mammalian tissues and the function of each splice variant remains unclear (reviewed by Bender et al., 2000; Hannon and Hoyer, 2008). The primers used in the present study for s-qPCR on the rainbow trout 5-HT_4 receptor may also have detected more than one splice variant, suggesting that the rainbow trout 5-HT_4 gene, like its mammalian equivalent, gives rise to multiple splice variants; these splice variants may be differentially expressed within HPI axis tissues. The likely presence of splice variants in the s-qPCR data for the 5-HT_4 receptor does not detract from the importance of detecting the mRNA for this receptor throughout the HPI axis, but it does underline the need to combine results from mRNA expression studies with physiological experiments in order to determine the function of 5-HT receptors in the HPI axis.

Most of the 5-HT receptor sequences available for teleost fish were obtained as a part of whole-genome sequencing projects, with only a handful of studies having detailed the tissue distribution and function of these receptors (Norton et al., 2008; Medeiros et al., 2010; Mager et al., 2012; Lim et al., 2013). In this thesis, mRNA abundance of 5-HT_{1A}, 5-HT_2 and 5-HT_4 receptors was detected among all tissues of the rainbow trout HPI axis with the highest relative levels of receptor mRNA being detected in the POA for all three receptors. Similar mRNA patterns were reported for the 5-HT_{1A} receptor in Gulf toadfish (Medeiros et al., 2010) and goldfish (Lim et al., 2013). Interestingly, 5-HT_{1A} receptor mRNA levels were 12-fold higher in the head kidney than the pituitary of goldfish, possibly reflecting involvement of this receptor subtype in mediating cortisol release from the head kidney (Lim et al., 2013). Mager et al. (2012) reported the presence of 5-HT_{2A} receptor mRNA in the toadfish HPI axis, with higher expression levels found in the midbrain/diencephalon (a brain region containing the POA) as compared to the pituitary. Abundance of 5-HT_{2A} receptor mRNA in the toadfish kidney was significantly lower than that of the whole brain (Mager et al., 2012). To date, mRNA expression of 5-HT_{2C} and 5-HT_4 receptors does not appear to have been reported in HPI axis tissues of fish species other than the rainbow trout data of the present thesis.

It is important to point out that the presence of 5-HT receptor mRNA within POA, pituitary and head kidney tissues does not automatically implicate these receptors in HPI axis regulation. Given the heterogeneity of HPI axis tissues, cell types having functions other than HPI axis regulation could express mRNA for one

or more 5-HT receptors. For instance, the preoptic area of the rainbow trout brain is composed of complex bundles of neuronal fibres (Folgueira et al., 2004) while the teleost pituitary is composed of different clusters of cells, each responsible for the release of specific hormones (e.g. corticotropes that release ACTH or thyrotropes that release thyroid-stimulating hormone) (reviewed by Schreibman et al., 1973). The teleost head kidney is composed of multiple cell types including chromaffin cells (responsible for the production and release of adrenaline and noradrenaline), interrenal cells (responsible for cortisol production) and immune cells (such as macrophages and reticular cells) (reviewed by Press and Evensen, 1999; Gallo and Civinini, 2003). Furthermore, while mRNA expression often is used as a proxy for protein expression, the presence of receptor mRNA does not necessarily correlate with protein abundance. Generally, positive correlations between mRNA and protein expression are observed, but post-transcriptional and translational modifications can contribute to differences between mRNA and protein expression levels (reviewed by de Sousa Abreu et al., 2009). Additionally, regulation of 5-HT receptors through desensitization and internal trafficking (reviewed by Gray and Roth, 2001) or receptor down-regulation (Medeiros et al., 2013) also should be considered. In brief, the detection of 5-HT receptor mRNA within the rainbow trout HPI axis provides a strong basis for further investigation but offers little information on the role(s) played by these receptors in HPI axis function.

4.2 5-HT directly stimulates cortisol production from head kidney tissue

The use of *in vitro* head kidney preparations exposed to receptor-specific agonists and antagonists provided means of evaluating the involvement of specific 5-HT receptor subtypes in cortisol production. Although direct stimulation by 5-HT of cortisol secretion from head kidney tissue has been reported in the goldfish (Lim et al., 2013) and toadfish (Medeiros and McDonald, 2012), the present study appears to be the first that has demonstrated 5-HT-mediated cortisol production by the head kidney in rainbow trout. All 5-HT concentrations tested on rainbow trout head kidney, between 10 and 1000 nmol L^{-1}, were effective in stimulating cortisol production, with the 100 nmol L^{-1} dose generating the maximal effect. This range of effective doses is similar to those reported for goldfish head kidney (Lim et al., 2013), or frog (Delarue et al., 1988a) and human (Lefebvre et al., 1992) adrenal *in vitro* preparations. By contrast, Medeiros and McDonald (2012) reported that the lowest effective concentration of 5-HT to elicit significant cortisol production by toadfish head kidney tissue was 10 000 nmol L^{-1}. This unusually high minimum effective concentration may reflect the involvement of 5-HT in pulsatile urea release, a phenomenon that is unique to this species. The diminished effectiveness of 1000 nmol L^{-1} 5-HT (relative to 100 nmol L^{-1}) in rainbow trout head kidney preparations of the present study could reflect a variety of possibilities including ultra-short negative feedback occurring within the head kidney tissue itself (Bradford et al., 1992), receptor desensitization (reviewed by Chuang et al., 1996; Gray and Roth, 2001), or tissue damage associated with the preparation; these issues require further investigation.

The effects of 5-HT on cortisol production by the rainbow trout head kidney likely are mediated by the 5-HT_4 receptor. Increased cortisol production was detected in head kidney tissue incubated with a 5-HT_4 receptor agonist (cisapride), an effect that was abolished when a combination of 5-HT_4 receptor agonist and antagonist (GR125487) was used. Conversely, there was little evidence to suggest a stimulatory role for the 5-HT_{1A} and 5-HT_2 receptors in the head kidney, despite the presence in head kidney tissue of receptor mRNA for all three of these 5-HT receptor subtypes. A possible explanation for this apparent contradiction is that the 5-HT_{1A} and 5-HT_2 receptors may be located in cell types found in the head kidney other than interrenal cells, such as chromaffin cells (reviewed by Gallo and Civinini, 2003). Information concerning which cell types express specific 5-HT receptor subtypes clearly is needed; in the meantime, the function of the 5-HT_{1A} and 5-HT_2 receptors in the rainbow trout head kidney remains unclear.

The available data for mammals and amphibians also suggest that 5-HT_4 receptors, rather than 5-HT_1 or 5-HT_2 receptors, are involved in regulating cortisol production by adrenal tissue (Idres et al., 1991; Lefebvre et al., 1992). However, the data for teleost fish are less clear-cut, and the 5-HT receptors involved in cortisol production by the head kidney may be species-specific. For example, the findings of this thesis suggest a stimulatory role for the 5-HT_4 receptor, with no involvement of the 5-HT_{1A} and 5-HT_2 receptors, in cortisol production in rainbow trout. On the other hand, perfused goldfish head kidneys exhibited significant increases in cortisol production in response to both 5-HT_{1A} and 5-HT_4 receptor agonists (Lim et al., 2013), and these increases were eliminated when the tissue was treated with the

respective receptor antagonists (Lim et al., 2013). For *in vitro* preparations of Gulf toadfish head kidney, Medeiros and McDonald (2012) reported significant stimulatory effects of 5-HT_2 and 5-HT_4 but not 5-HT_{1A} receptor agonists. It is worth noting that although the affinity and specificity of 5-HT receptor agonists/antagonists have been well established in mammals (Fletcher et al., 1996; Wood et al., 2000; Allee et al., 2008), studies of this nature generally are lacking in teleosts. However, specificity of 8-OH-DPAT in toadfish was confirmed by demonstrating specific binding in *Xenopus* oocytes expressing the toadfish 5-HT_{1A} receptor (Medeiros et al., 2010). Thus, collectively, these studies emphasize the species-specific nature of 5-HT receptors in teleosts. The observation that the 5-HT_4 receptor seems to have a stimulatory effect on cortisol production by the head kidney/adrenal tissue in teleost, amphibian and mammalian preparations suggests that there may be a conserved role for this receptor among vertebrates.

Whereas 5-HT seems to be able to directly act on the head kidney, the *in vivo* source of 5-HT mediating these effects remain unclear. Effects of 5-HT on cortisol production in the head kidney suggest that paracrine regulatory mechanisms may be at play. In support of this possibility, 5-HT immunoreactivity was detected in the head kidney of goldfish (Lim et al., 2013), as well as in the adrenal tissue of frogs (Delarue et al., 1988b) and humans (Lefebvre et al., 1992). In teleost fish, it has been hypothesized that locally-produced 5-HT released by chromaffin cells together with catecholamines could stimulate cortisol production from interrenal cells (Lim et al., 2013). Further, immunohistochemical approaches have localized 5-HT to the chromaffin cells of Atlantic cod (*Gadus morhua*), European eel (*Anguilla anguilla*)

and goldfish (Reid et al., 1995; Lim et al., 2013) but not rainbow trout (Reid et al., 1995). Although 5-HT was not detected in rainbow trout chromaffin cells, it was present in head kidney tissue and was found to be capable of inducing the release of catecholamines (Fristche et al., 1993; Reid et al., 1995).

The role of 5-HT in regulation of cortisol production from head kidney tissue, via paracrine mechanisms or otherwise, warrants further study. Overall, this thesis provided evidence that 5-HT may regulate the HPI axis at the head kidney level. Given the effectiveness of 8-OH-DPAT at increasing plasma cortisol concentrations *in vivo* (Winberg et al., 1997) combined with the lack of stimulatory effect of this agonist at the head kidney level, it is likely that 5-HT also acts on the HPI axis further upstream. Thus, while a tissue-specific approach provided detail on the 5-HT receptors involved in the head kidney, *in vivo* experiments were used to investigate potential upstream mechanisms of 5-HT regulation of the HPI axis at the whole-animal level.

4.3 5-HT activates the HPI axis *in vivo*

To evaluate the capacity of 5-HT to activate the HPI axis, *in vivo* intra-arterial injections of 5-HT were used. The doses chosen corresponded to those used for *in vitro* head kidney experiments as well as baseline circulating concentrations of 5-HT reported in Gulf toadfish (100-1000 nmol L^{-1}; McDonald and Walsh, 2004). Baseline levels of circulating 5-HT in rainbow trout have yet to be determined and therefore, the physiological relevance of the chosen doses remains uncertain. Although 100

nmol L^{-1} 5-HT was most effective at eliciting increases in cortisol production for head kidney preparations, a comparable dose *in vivo* failed to significantly raise plasma cortisol concentrations in comparison to saline-treated fish. The higher dose of 5-HT, chosen to achieve a nominal circulating concentration of 1000 nmol L^{-1}, significantly increased plasma cortisol concentrations post-injection, providing evidence that 5-HT can activate the HPI axis in rainbow trout. The significantly higher plasma glucose concentrations observed in 5-HT over saline-treated fish following the cortisol response provided further confirmation of HPI axis activation. Although 5-HT can cross the BBB in fish (Khan and Deschaux, 1997), it is not necessarily the case that equivalent concentrations of 5-HT were present in the general circulation and in the brain given the time frame of the experiment. A slow rate of 5-HT movement across the blood-brain barrier could have resulted in 5-HT concentrations in the brain that were lower than the nominal value, which could account for the higher 5-HT doses needed to stimulate cortisol production *in vivo versus in vitro*. Alternatively, it is also possible that the pituitary and brain simply need higher levels of 5-HT than the head kidney to activate the mechanisms regulating HPI axis activity.

Activation of the teleost HPI axis likely is mediated in part by the 5-HT_{1A} receptor. Indeed, toadfish (Medeiros et al., 2010) and goldfish (Lim et al., 2013), like trout (Winberg et al., 1997), showed a significant cortisol response after injection with 8-OH-DPAT. The cortisol response to 8-OH-DPAT injection in goldfish (Lim et al., 2013) was comparable with that observed in 5-HT-injected rainbow trout (present study). However, the cortisol response in 8-OH-DPAT-treated Gulf toadfish

(Medeiros et al., 2010) was almost double that of 5-HT-injected trout. Injection of 8-OH-DPAT into rainbow trout led to significant increases in plasma cortisol concentrations (Winberg et al., 1997) that were similar to the values reported in this thesis for 5-HT-injected rainbow trout. The 8-OH-DPAT-induced increases in *in vivo* plasma cortisol concentrations (Winberg et al., 1997), coupled with a lack of stimulatory effect of this agonist at the head kidney level (present thesis), suggests that the rainbow trout 5-HT_{1A} receptor is likely involved in HPI axis activation at the hypothalamus and/or the pituitary.

Despite the likely involvement of 5-HT receptors in activating the HPI axis upstream of the head kidney, no convincing changes in plasma ACTH concentrations or hypothalamic CRF mRNA abundance were detected in the present study following 5-HT injection. Similarly, no significant increase in plasma ACTH concentrations was detected in goldfish treated with 8-OH-DPAT (Lim et al., 2013). Lim et al. (2013) concluded that the sampling time used in their study may not have been optimal for the detection of changes in ACTH levels, and a similar situation may apply to the present study, despite the use of multiple sampling times. A second challenge in the present study was the use of cannulated fish. Cannulation, injection of compounds and blood withdrawal can elicit stress responses. For example, McDonald and Walsh (2004) reported injection-induced increases of cortisol in both control and treated toadfish. However, 5-HT-mediated increases in ACTH were detected in toadfish (Medeiros et al., 2014). It seems likely that an ACTH response to 5-HT administration also occurs in rainbow trout, but documenting such a response has proven challenging.

No convincing changes in CRF mRNA abundance were observed in rainbow trout exposed to 1000 nmol L^{-1} 5-HT, and the significant decrease in CRF mRNA abundance observed in fish exposed to 100 nmol L^{-1} 5-HT was unexpected. In a similar experiment conducted in goldfish injected with 8-OH-DPAT, no changes in CRF mRNA abundance were detected (Lim et al., 2013) and in toadfish, only a minor (1.4 fold), albeit significant, increase in CRF mRNA was detected (Medeiros et al., 2014). Again, selection of an appropriate sampling time may be difficult. For example, changes in CRF mRNA expression in toadfish were detected 3 min after 8-OH-DPAT administration (Medeiros et al., 2014). It has been suggested that prolonged and/or severe stressors such as chronic social stress are required to induce changes in CRF mRNA expression (Bernier et al., 2008; Jeffrey et al., 2014). Thus, the short-term exposure to 5-HT used in this thesis may not have been sufficient to cause distinct changes in CRF mRNA abundance. It is also the case that measurement of CRF mRNA abundance serves as a proxy for CRF release from the POA, likely reflecting replenishment of CRF mRNA rather than actual CRF release.

The involvement of 5-HT in the regulation of CRF release remains likely, despite the lack of effect of 5-HT injection on POA CRF mRNA levels. A substantial body of evidence suggests that crosstalk occurs between 5-HT and CRF in the teleost hypothalamus. For instance, 5-HT neurons were found to project to the POA (the main site of CRF release) of zebrafish (Lillesaar et al., 2009). Serotonin-like immunoreactivity was localized to the POA of both goldfish (Kah and Chambolle, 1993) and three-spined stickleback (Ekström and Van Veen, 1984). In rainbow trout, both 5-HT and its main metabolite 5-HIAA have been detected in the POA

using high performance liquid chromatography (Rozas et al., 1990; Saligaut et al., 1990). The presence of 5-HT in the teleost POA supports the possibility of interaction between 5-HT and CRF. Thus, despite the absence in the present study of convincing ACTH and CRF responses to 5-HT administration, it remains likely that 5-HT exerts direct effects on CRF release via 5-HT_{1A} receptors and direct and/or indirect (i.e. via CRF) effects on ACTH release.

4.4 Unanswered questions and future directions

The findings of this thesis provide a starting point for elucidating the mechanisms underlying serotonergic regulation of the HPI axis in rainbow trout, and identify avenues for future research. For example, although mRNA for 5-HT_{1A}, 5-HT_2 and 5-HT_4 receptors was found in all HPI axis tissues, these tissues are composed of multiple cell types, not all of which are involved in HPI axis function. A combination of immunohistochemistry and *in situ* hybridization could be used to determine the nature and location of cell types that express specific 5-HT receptor subtypes within the HPI axis. In turn, this information would allow for a better understanding of the role of 5-HT at each level of the HPI axis. It would also be useful to localize 5-HT immunoreactivity to specific cell type(s) within the head kidney, to better assess the potential role of paracrine 5-HT in regulating cortisol production by interrenal cells.

Although convincing evidence for effects of 5-HT on cortisol production at the head kidney level was obtained in this study, empirical support for actions of 5-

HT further upstream in the HPI axis remains equivocal. Moreover, the relative importance of upstream *versus* downstream actions of 5-HT remains to be explored. In addition, the present study placed emphasis on potential stimulatory effects of 5-HT, but interactions between this neurotransmitter and the HPI axis may be complex, and modulatory or inhibitory roles of 5-HT also should be considered.

4.5 Conclusions

Collectively, the results of this thesis provide evidence of a direct, stimulatory action of 5-HT on the HPI axis in rainbow trout, identifying 5-HT_4 receptors as important at the head kidney level and, when considered in the context of previous work (Winberg et al., 1997; Hoglund et al., 2002), suggesting that 5-HT_{1A} receptors play roles at the hypothalamus and/or pituitary. Certain elements of the interaction between the serotonergic system and the stress axis seem well conserved among vertebrates (e.g. stimulatory effect of the 5-HT_4 receptor at the adrenal/interrenal level), whereas teleost fish exhibit species-specific variation in other aspects (e.g. differential involvement of the 5-HT_{1A} receptor at the interrenal level). Such species-specific variation provides a strong rationale for examining serotonergic regulation of the stress axis in a diversity of teleost species. The findings of the present study complement similarly mechanistic studies that have been carried out in goldfish (Lim et al., 2013) and toadfish (Medeiros et al., 2010; Medeiros et al., 2012), but focus on a species that has been studied extensively in the context of social interactions and chronic social stress, a situation in which both central

serotonergic activity (Winberg et al., 1998; DiBattista et al., 2005) and HPI axis activity (Jeffrey et al., 2012; Jeffrey et al., 2014; reviewed by Gilmour et al., 2005) are affected.

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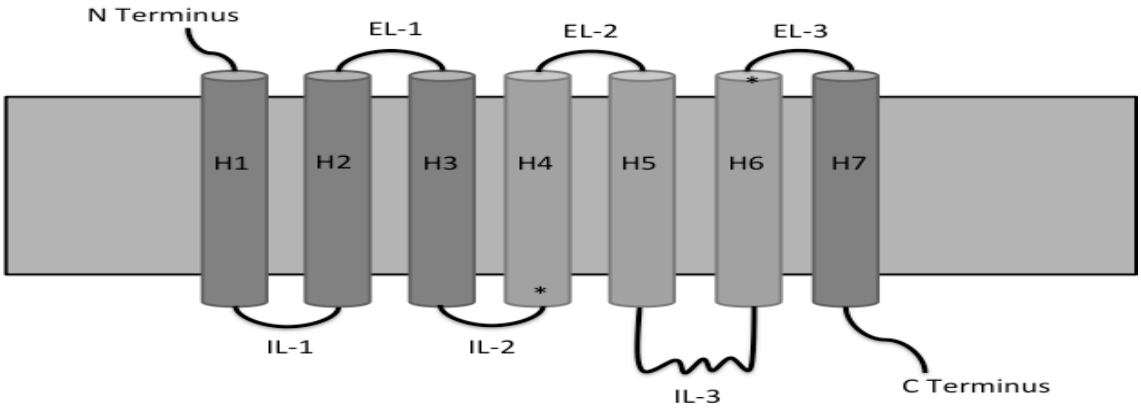
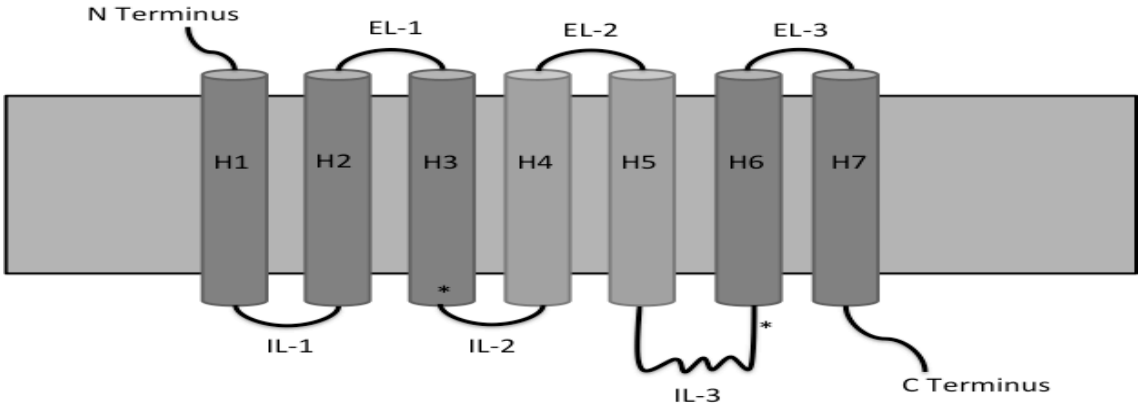
APPENDICES

Appendix 1. Schematic representation of sequenced regions of the rainbow trout 5-HT1A, 5-HT2A and 5-HT2C receptors. Sequenced regions of the 5-HT1A receptor (panel A) are predicted to include transmembrane domains 4, 5 and 6, the second extracellular loop and the third intracellular loop. Sequenced regions of the 5-HT2A receptor (panel B) are predicted to include transmembrane domains 4 and 5, the second extracellular loop as well as both the second and third intracellular loop. Sequenced regions of the 5-HT2C receptor (panel C) are predicted to include transmembrane domains 2 through 5, the first and second extracellular loop as well as the second and third intracellular loop. Due to the complex nature of the 5-HT4 receptor (this receptor is known to have multiple splice variants [Bender et al., 2000]), no predictions were made as to the location of the sequenced regions in the rainbow trout 5-HT4 receptor. Predictions as to the location of sequenced regions of the rainbow trout 5-HT receptors were made based on amino acid alignment of these receptors (see Appendices 2 to 5). An asterisk denotes the beginning and end of rainbow trout 5-HT receptor sequences. Light grey transmembrane domains denote sequenced regions, while dark grey transmembrane domains denote regions that have not been sequenced in rainbow trout 5-HT receptors. H, transmembrane domain; EL, extracellular loop; IL, intracellular loop.

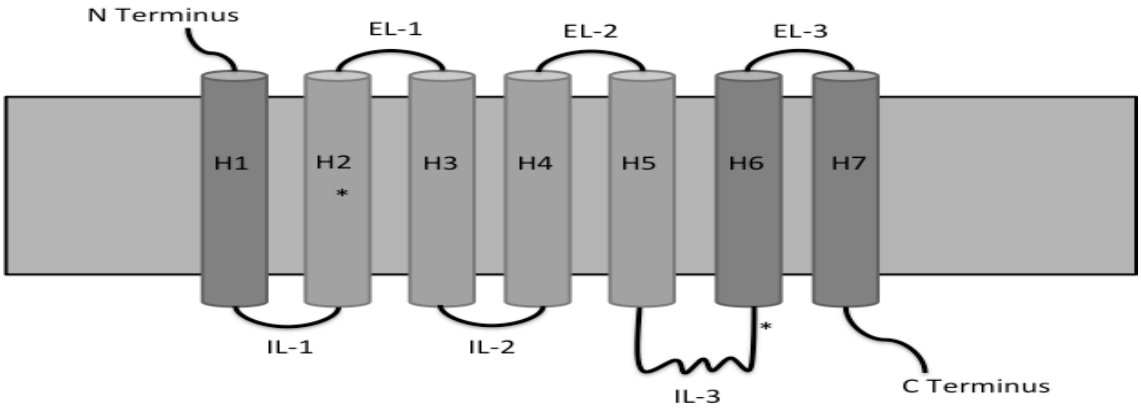
A


N Terminus
EL-1
EL-2
EL-3
H1
H2
H3
H4
H5
H6
H7
IL-1
IL-2
IL-3
C Terminus

B


N Terminus
EL-1
EL-2
EL-3
H1
H2
H3
H4
H5
H6
H7
IL-1
IL-2
IL-3
C Terminus

C


N Terminus
EL-1
EL-2
EL-3
H1
H2
H3
H4
H5
H6
H7
IL-1
IL-2
IL-3
C Terminus

Appendix 2. Nucleotide and amino acid alignment of 5-HT$_{1A}$ receptor sequences from human (*Homo sapiens*), ENSG00000178394; mouse (*Mus musculus*), ENSMUS00000021721; zebrafish (*Danio rerio*), ENSDARG00000093745; Nile tilapia (*Oreochromis niloticus*), ENSONIG00000020994 and rainbow trout (*Oncorhynchus mykiss*), KP334154.

5-HT$_{1A}$ receptor nucleotide alignment

```
Mouse          ATGGATATGTTCAGTCTTGGCCAGGGCAACAACACCACAACGTCCCTG---------- 48
Human          ATGGATGTGCTCAGCCCTGGTCAGGGCAACAACACCACATCACCACCG---------- 48
Zebrafish      ----------ATGGAGAGCTA---CAACAACACCACAGAAAGCCAA---------- 33
Tilapia        ATGGATTTTATAACCACAAGCTA---CAACGACAGCAACGCGACCAGTGTTTACCCCAGT 57
Trout          ---------------------------------------------------------- 0

Mouse          ----------GAGCCCTTCGGGACAGGCGGC---AACGATACT-------GGCCTC-- 84
Human          ----------GCTCCCTTTGAGACCGGCGGC---AACACTACT-------GGTATC-- 84
Zebrafish      ----------GATTGGAGCGGG------AACGCGACG-------AGTGTC-- 60
Tilapia        GGGGGGGACGTGGTTGCCGACTGGAACGGGGGTGAGAATGGCACGGGGTCCGGGTCTCTT 117
Trout          ---------------------------------------------------------- 0

Mouse          TCCAACGTGACCTTCAGCTACCAAGTGATCACCTCTCTTTTGCTGGGGACGCTCATTTTC 144
Human          TCCGACGTGACCGTCAGCTACCAAGTGATCACCTCTCTGCTGCTGGGCACGCTCATCTTC 144
Zebrafish      AGCGAAGTTGCTCTGAGTTACCAAATCATCGGCTCGCTTTTCCTGGCCGCGTTAATTCTG 120
Tilapia        CCAGATCTGGTGCTGAGTTACCAGATTATTACCTCAATGCTCCTGGGGGCCCTCATCCTC 177
Trout          ---------------------------------------------------------- 0
```

```
Mouse          TGCGCGGTGCTCGGCAATGCCTGCGTGGTTGCTGCCATCGCCCTGGAGCGCTCCCTTCAG 204
Human          TGCGCGGTGCTGGGCAATGCGTGCGTGGTGGCTGCCATCGCCTTGGAGCGCTCCCTGCAG 204
Zebrafish      TTTGCCATTTTGGGAAACGCGTGTGTCATCGCTGCTATCGCCTTGGAGAGATCGCTTCAG 180
Tilapia        TGCTCCATATTTGGCAATGCGTGCGTCGTGGCAGCCATCGCCCTGGAGAGATCTCTCCAG 237

Trout          ------------------------------------------------------------ 0

Mouse          AATGTTGCCAACTATCTCATCGGCTCCTTGGCGGTCACCGATCTCATGGTGTCAGTGCTG 264
Human          AACGTGGCCAATTATCTTATTGGCTCTTTGGCGGTCACCGACCTCATGGTGTCGGTGTTG 264
Zebrafish      AATGTGGCCAACTATCTCATCGGCTCCCTGGCCGTTACAGACCTTATGGTGTCGGTGCTG 240
Tilapia        AATGTGGCCAACTATCTGATCGGATCCCTGGCCGTGACAGACCTCATGGTTTCTGTGCTG 297
Trout          ------------------------------------------------------------ 0

Mouse          GTGCTGCCCATGGCTGCTCTGTACCAGGTGCTCAACAAGTGGACTCTGGGTCAGGTCACC 324
Human          GTGCTGCCCATGGCCGCGCTGTATCAGGTGCTCAACAAGTGGACACTGGGCCAGGTAACC 324
Zebrafish      GTGCTCCCCATGGCAGCCCTGTATCAGGTTTTGAATAAATGGACTTTGGGACAGGAGATG 300
Tilapia        GTCCTGCCAATGGCGGCCCTGTATCAGGTTTTAAACAAGTGGACTCTAGGGCAGGAGATC 357
Trout          ----------------------------------------------GACAGGAGATA 11
                                                              * ****  *
Mouse          TGTGACCTGTTTATCGCCCTGGATGTGCTGTGCTGCACTTCGTCCATCCTGCACCTGTGC 384
Human          TGCGACCTGTTCATCGCCCTCGACGTGCTGTGCTGCACCTCATCCATCTTGCACCTGTGC 384
Zebrafish      TGCGATATTTTCATCTCTCTAGACGTGCTGTGCTGCACTTCTTCCATCCTGCACCTGTGC 360
Tilapia        TGTGATTTATTCATCTCTCTGGATGTACTGTGTTGTACATCATCCATCCTGCATCTGTGC 417
Trout          TGTGATATATTCATCTCCCTGGACGTGTTGTGCTGCACGTCGTCCATTCTGCATCTGTGC 71
               ** **  * ** *** * ** ** **  **** ** ** ** *****  **** ******
```

```
Mouse          GCCATCGCGCTAGACAGGTACTGGGCAATCACCGACCCTATAGACTACGTGAACAAGAGG 444
Human          GCCATCGCGCTGGACAGGTACTGGGCCATCACGGACCCCATCGACTACGTGAACAAGAGG 444
Zebrafish      GCAATCGCTTTGGATAGGTACTGGGCCATCACCGATCCCATAGACTACGTGAATAAAAGG 420
Tilapia        GCAATTGCTTTGGACAGGTACTGGGCCATAACGGACCCTATTGACTATGTAAATAAACGG 477
Trout          GCAATTGCCCTGGACAGGTACTGGGCTATCACGGACCCCATAGACTATGTGAACAAAAGG 131
               ** ** **  * ** ********** ** ** ** ** ** ***** ** ** **  **

Mouse          ACGCCCCGGCGCGCCGCTGCGCTGATCTCGCTCACTTGGCTCATTGGCTTTCTCATCTCC 504
Human          ACGCCCCGGCGCGCCGCTGCGCTCATCTCGCTCACTTGGCTTATTGGCTTCCTCATCTCT 504
Zebrafish      ACGCCCAGACGAGCTGCTATCTTGATCAGTCTCACTTGGCTAATAGGATTTTCCATTTCC 480
Tilapia        ACACCAAGGAGAGCTGCGTTCTTGATTAGCGTCACATGGCTAATTGGTTTCTCAATTTCT 537
Trout          ACACCCAGGCGAGCAGTGATGTTAATCAGCGTGACTTGGCTGATTGGGTTCTCAATCTCC 191
               ** **  *  * ** *     * **    * ** ***** ** ** **   ** **

Mouse          ATCCCGCCTATGCTGGGCTGGCGCACCCCGGAAGACCGCTCGAACCCCAACGAGTGCACC 564
Human          ATCCCGCCCATGCTGGGCTGGCGCACCCCGGAAGACCGCTCGGACCCCGACGCATGCACC 564
Zebrafish      ATTCCGCCCATGTTGGGTTGGAGGAAACCGGAGGACCGGGCAGATCCCGACGCGTGCACA 540
Tilapia        ATTCCGCCTATGTTAGGCTGGAGAAGCGCCGAAGACAGGGCAGACCCCGACGCCTGCATG 597
Trout          ATCCCTCCAATGTTAGGCTGGAGGAAAGCCGAGGACAGGGCGAACCCGGACGCCTGCACC 251
               ** ** ** *** * ** *** * *   * ** *** *  *  * **  ***  ****

Mouse          ATCAGCAAGGACCACGGGTACACCATCTACTCCACTTTCGGCGCTTTCTATATTCCGCTG 624
Human          ATTAGCAAGGATCATGGCTACACTATCTATTCCACCTTTGGAGCTTTCTACATCCCGCTG 624
Zebrafish      ATCAGCCAGGACCACGGGTACACCATCTACTCAACTTTCGGGGCGTTCTACATCCCGCTC 600
Tilapia        ATCAGCCAGGACCCGGGCTACACCATCTACTCCACGTTTGGAGCTTTTTACATTCCTCTT 657
Trout          ATCAGTCAGGACCCGGGTTATACCATCTACTCCACGTTCGGAGCATTTTACATCCCACTT 311
               ** **  **** *  ** ** ** ***** ** ** ** ** ** ** ** ** ** **
```

```
Mouse          CTGCTCATGCTGGTCCTCTATGGGCGCATCTTCAGAGCCGCGCGCTTCCGAATCCGCAAG 684
Human          CTGCTCATGCTGGTTCTCTATGGGCGCATATTCCGAGCTGCGCGCTTCCGCATCCGCAAG 684
Zebrafish      ATCCTCATGCTGGTCCTTTACGGACGGATATTCCGAGCGGCGAGGTTTCGCATAAGGAAA 660
Tilapia        ATCCTCATGTTGGTTCTGTACGGACGAATATTCAGAGCTGCTCGGTTTCGGATTCGAAAG 717
Trout          ATCCTCATGCTGGTCCTCTACGGGCGGATATTCAAGGCAGCCAGGTTCCAGGTTTGGAAA 371
                * ****** **** ** ** ** ** ** ***   ** **  * ** *   *  * **

Mouse          ACGGTCAAGAAGGTGGAAAAGAA---GGGAGCGGGCACCAGCTTCGGAACATCGTCGGCC 741
Human          ACGGTCAAAAAGGTGGAGAAGAC---CGGAGCGGACACCCGCCATGGAGCATCTCCCGCC 741
Zebrafish      ACGGTAAAGAAAACGGAGAAAGCGAAAATCGCGGATAAATGCTTGGCGGTGTCTCCAGCG 720
Tilapia        ACAGTGAAGAAAGCAGAGAAAGCAAAAGTGTCAGAAAAGTGCTTGAGTGTGTCTCCGGCC 777
Trout          ACTGTGAAGAAATCGGAAAAGGTAAAAGTGTCGGACAAGTGCTTGGCCGTGTCGCCGGCT 431
               ** ** ** **    ** **           * *  *   **         **  * **

Mouse          CCGCCCCCCAAGAAGAGCCTGAATGGTCAGCCAGGTAGTGGGGACTGCAGGCGCAGTGCT 801
Human          CCGCAGCCCAAGAAGAGTGTGAATGGAGAGTCGGGGAGCAGGAACTGGAGGCTGGGCGTG 801
Zebrafish      CTCTTCCCGAGGAA---AGCGAACGGAGAGGTG---GGTAAAACTTGGAGGCGAAGCGTG 774
Tilapia        ATCTTTCACAAGAA---AACCAACGGAGAGGCCGGCGGCAAAGGCTGGAAGCGCGGGGAT 834
Trout          ATTTTCCACAAGAA---GATTAATGGAGAGGCGGGGGGCAAGAATTGGAAGCGCAGTGTG 488
                     *  * ***       ** **  **       *       ** * **   * *

Mouse          GAGAACAGGGCGGTGGGGACTCCGTGCGCTAATGGGGCGGTGAGACAGGGTGAGGACGAC 861
Human          GAGAGCAAGGCTGGGGGTGCTCTGTGCGCCAATGGCGCGGTGAGGCAAGGTGACGATGGC 861
Zebrafish      GAG------------------CCGTGCGCAAACGGCGCGCTGAAAAACTCGGACGACGGA 816
Tilapia        GAGTCTAAATC---CAGCTCTCCGTGCGTAAATGGCGCGGTGAAGCATGGAGACGAGGGC 891
Trout          GAACCTACATT---CAACCCCCCGTGCGTAAACGGCTCGGTGAAGCACGGGGAGGACGGC 545
               **                   * *****  ** **  ** ***   *    ** ** *
```

```
Mouse          GCCACCCTGGAGGTGATCGAGGTGCATCGAGTGGGCAACTCCAAAGGGCACCTTCCTCTG 921
Human          GCCGCCCTGGAGGTGATCGAGGTGCACCGAGTGGGCAACTCCAAAGAGCACTTGCCTCTG 921
Zebrafish      GAGTCGTTCGAGATTACAGAAGTTCAA------AGCATCTCCAAAAATCACCTGAGCCTG 870
Tilapia        GAATCATTGGAGATCATAGAAGTTATC------AGCAACTCAAAGACGCACTTGCCTCTG 945
Trout          GAGTCGTTGGAGATCATAGAAGTGACC------AACAATTCTAAGAACCACCTTCCTCTC 599
               *   *  * *** * *  ** **            **  ** **    *** *    **

Mouse          CCCAGCGAATCAGGAGCTACCTCCTATGTCCCCGCC-TGCTTGGAGAGAAAAAATGAGCG 980
Human          CCCAGCGAGGCTGGTCCTACCCCTTGTGCCCCCGCC-TCTTTCGAGAGGAAAAATGAGCG 980
Zebrafish      CCAAACAACCCTCAGCCG----------T---------GCTTCGAGAACCGAAATGAGAA 911
Tilapia        CCCAACACTCCTCAGTCC----------TCCTCGCATGGCTATGAAAACATGAATGAAAA 995
Trout          CCCAACACTCCACAGTCC-------------TCACAAGGGTTTGAGAACAGGAACGAAAA 646
               ** * *    *     *                      *  ** *     ** **

Mouse          CACTGCTGAGGCAAAGCGCAAGATGGCCTTGGCCCGTGAGAGGAAGACAGTGAAGACACT 1040
Human          CAACGCCGAGGCGAAGCGCAAGATGGCCCTGGCCCGAGAGAGGAAGACAGTGAAGACGCT 1040
Zebrafish      AAACACGGAAGCGAAGCGCAAAGTGGCTCTGGCCAGAGAACGCAAAACAGTCAAGACTCT 971
Tilapia        GAACTCGGGGGCGAAGAGAAAGATCGCGCTGGCCAGGGAACGTAAAACGGTGAAAACACT 1055
Trout          GAACACGGAGGCTAAGAGGAAAATAGCTCTGTCCAGGGAGAGGAAAACGGTAAAGACGCT 706
                *   * *  ** *** * **  * **  ** ** * **  * ** ** ** ** ** **

Mouse          GGGCATCATCATGGGCACCTTCATCCTCTGCTGGCTGCCCTTTTTCATTGTGGCCCTGGT 1100
Human          GGGCATCATCATGGGCACCTTCATCCTCTGCTGGCTGCCCTTCTTCATCGTGGCTCTTGT 1100
Zebrafish      GGGAATAATCATGGGCACGTTTATTTTCTGCTGGCTGCCCTTCTTCATCGTAGCGCTCGT 1031
Tilapia        CGGCATCATTATGGGAACTTTCATCTTCTGCTGGCTGCCCTTTTTCATCGTTGCACTAGT 1115
Trout          CGGTATCATCATGGGCACTTTTATTTTCTGCTGGCTGCCCTTTTTCATCGTGGCGCTGGT 766
                ** ** ** ***** ** ** **  *************** ***** ** ** ** **
```

```
Mouse          CCTACCTTTCTGTGAGAGCAGTTGCCACATGCCTGAGTTGTTGGGTGCCATAATTAACTG 1160
Human          TCTGCCCTTCTGCGAGAGCAGCTGCCACATGCCCACCCTGTTGGGCGCCATAATCAATTG 1160
Zebrafish      GTTGCCTTTTTGTCAGGA---CTGTTTTATGCCCGAATGGTTGGGCGCTGTCATAAACTG 1088
Tilapia        ACTACCTTTCTGTGCAGAGAGCTGCTACATGCCCGAGTGGCTGGGCGCAGTCATAAACTG 1175
Trout          CTTGCCTTTCTGTGCGGAGAGT------------------------------------ 788
                 * ** ** **

Mouse          GCTAGGCTACTCCAACTCCCTGCTCAACCCAGTTATTTATGCTTATTTCAACAAAGACTT 1220
Human          GCTGGGCTACTCCAACTCTCTGCTTAACCCCGTCATTTACGCATACTTCAACAAGGACTT 1220
Zebrafish      GCTCGGATACTCCAACTCACTTTTGAATCCTGTCATATATGCGTACTTCAATAAAGACTT 1148
Tilapia        GCTGGGCTATTCAAACTCTCTCCTCAACCCCATCATATATGCCTACTTCAACAAAGACTT 1235
Trout          ------------------------------------------------------------ 788

Mouse          TCAAAACGCTTTTAAGAAGATCATCAAGTGCAAGTTCTGCCGCTGA---     1266
Human          TCAAAACGCGTTTAAGAAGATCATTAAGTGTAAGTTCTGCCGCCAGTGA     1269
Zebrafish      TCAGAACGCATTCAAGAAGATTTTGAAATGCAAATGTATTAGACAGTGA     1197
Tilapia        CCAAAACGCTTTTAAGAAGATTATAAAATGCAAATTCCACAGACCATAA     1284
Trout          ---------------------------------------------     788
```

5-HT$_{1A}$ receptor amino acid alignment

```
Mouse          MDMFSLGQGNNTT-------TSLEPFG---TGGNDTGLSNVTFSYQVITSLLLGTLIFCA 50
Human          MDVLSPGQGNNTT-------SPPAPFE---TGGNTTGISDVTVSYQVITSLLLGTLIFCA 50
Zebrafish      ------------MESYN-NTTESQDWSGNAT-----SVSEVALSYQIIGSLFLAALILFA 42
Tilapia        MDFLTSTNASNATGGYPEGMDVVADWIGGNNATESRFQPDLVLAYQIITSLLLVALILCS 60
Trout          ------------------------------------------------------------ 0

Mouse          VLGNACVVAAIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCD 110
Human          VLGNACVVAAIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCD 110
Zebrafish      ILGNACVIAAIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQEMCD 102
Tilapia        IFGNACVVAAIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQEICD 120
Trout          ------------------------------------------------------------ 0

Mouse          LFIALDVLCCTSSILHLCAIALDRYWAITDPIDYVNKRTPRRAAALISLTWLIGFLISIP 170
Human          LFIALDVLCCTSSILHLCAIALDRYWAITDPIDYVNKRTPRRAAALISLTWLIGFLISIP 170
Zebrafish      IFISLDVLCCTSSILHLCAIALDRYWAITDPIDYVNKRTPRRAAILISLTWLIGFSISIP 162
Tilapia        LFISLDVLCCTSSILHLCAIALDRYWAITDPIDYVNKRTPKRAALLISATWLIGFSISIP 180
Trout          -------------------------------------------MLISVTWLIGFSISIP 16
                                                           *** ****** ****

Mouse          PMLGWRTPEDRSNPNECTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTV 230
Human          PMLGWRTPEDRSDPDACTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTV 230
Zebrafish      PMLGWRKPEDRADPDACTISQDHGYTIYSTFGAFYIPLILMLVLYGRIFRAARFRIRKTV 222
Tilapia        PMLGWRSAEDRANPDACMISQDPGYTIYSTFGAFYIPLILMLVLYGRIFRAARFRIRKTV 240
Trout          PMLGWRKAEDRANPDACTISQDPGYTIYSTFGAFYIPLILMLVLYGRIFKAARFQVWKTV 76
               ******  ***  *  * ** * *************** ********** ****   ***
```

```
Mouse          KKVEKKGAGTSFGTSSAPPPKKSLNGQPGSGDCRRSAENRAVGTPCANGAVRQGEDDATL 290
Human          KKVEKTGADTRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQGDDGAAL 290
Zebrafish      KKTEKAKIADKCLAVSPALFPRKANGEV-GKTWRRSVE------PCANGALKNSDDGESF 275
Tilapia        KKTETAKVSDKCLAVSPAIFHKKSNGETRGKGWRRCDESK-SSSPCVNGAVKHGEEGESL 299
Trout          KKSEKVKVSDKCLAVSPAIFHKKINGEAGGKNWKRSVEPT-FNPPCVNGSVKHGEDGESL 135
               ** *                    **          *       * **

Mouse          EVIEVHRVGNSKGHLPLPSESGATSYVPACLERKNERTAEAKRKMALARERKTVKTLGII 350
Human          EVIEVHRVGNSKEHLPLPSEAGPTPCAPASFERKNERNAEAKRKMALARERKTVKTLGII 350
Zebrafish      EITEVQ--SISKNHLSLPNNPQP------CFENRNEKNTEAKRKVALARERKTVKTLGII 327
Tilapia        EIIEVI--SNSKTHLPLPNTPQSS---SHGYENMNERNSGAKRKLALSRERKTVKTLGII 354
Trout          EIIEVT--NNSKNHLPLPNTPQS----SQGFENRNEKNTEAKRKIALSRERKTVKTLGII 189
               *  **     ** ** **             *  **    **** ** ************

Mouse          MGTFILCWLPFFIVALVLPFCESSCHMPELLGAIINWLGYSNSLLNPVIYAYFNKDFQNA 410
Human          MGTFILCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNA 410
Zebrafish      MGTFIFCWLPFFIVALVLPFCQ-DCFMPEWLGAVINWLGYSNSLLNPVIYAYFNKDFQNA 386
Tilapia        MGTFIVCWLPFFIVALVLPFCAESCYMPDWLGAVINWLGYSNSLLNPIIYAYFNKDFQSA 414
Trout          MGTFIFCWLPFFIVALVLPFCAES---------------------------------- 213
               ***** ***************

Mouse          FKKIIKCKFCR-    421
Human          FKKIIKCKFCRQ    422
Zebrafish      FKKILKCKCIRQ    398
Tilapia        FKKIIKCKFHRP    426
Trout          ------------    213
```

Appendix 3. Nucleotide and amino acid alignment of 5-HT$_{2A}$ receptor sequences from human (*Homo sapiens*), ENSG00000102468; mouse (*Mus musculus*), ENSMUS00000034997; zebrafish (*Danio rerio*), ENSDARG00000058165; Nile tilapia (*Oreochromis niloticus*), ENSONIG00000016184 and rainbow trout (*Oncorhynchus mykiss*), KP334155.

5-HT$_{2A}$ receptor nucleotide alignment

```
Mouse          ATGGAAATTCTCTGTGAAGACAATATCTCCCTGAGCTCAATTCCAA---------ACTCC  51
Human          ATGGATATTCTTTGTGAAGAAAATACTTCTTTGAGCTCAACTACGA---------ACTCC  51
Tilapia        ATGAACCTGCATGGT---GGCAATGT--GTCACAGTTGATCTCTAACACTGTCACAGCCG  55
Zebrafish      ATGGCCCTGAATGGG---ACGGCT----------GCTGAACTCAG-----------GTCC  36
Trout          ------------------------------------------------------------  0

Mouse          TTAATGCAATTAGGTGACGACTCGAGGCTCTACCCTAATGACTTCAACTCCAGGGATGCT  111
Human          CTAATGCAATTAAATGATGACACCAGGCTCTACAGTAATGACTTTAACTCCGGAGAAGCT  111
Tilapia        TTACCACTCTGGGGTCTAACCCCTGGCCTCCACACGAGGAAGGTGGCCTCCAAAG-----  110
Zebrafish      TTA-------GGGATTTCTGC--------------------G---------GAGAT-----  56
Trout          ------------------------------------------------------------  0

Mouse          AACACTTCCGAAGCCTCGAAC-------------------TGGACAATTG----------  142
Human          AACACTTCTGATGCATTTAAC-------------------TGGACAGTCG----------  142
Tilapia        -----TGGCAATGCCACGAACCTGGGCTGCCAGA------GGAACAATGTGGTTCATAAT  159
Zebrafish      -----TTCCAAAGACACCAGCATGGAGCAGAAGGTGTCTTTGGGTAATGCAGTTCTCAAT  111
Trout          ------------------------------------------------------------  0
```

```
Mouse          -------ATGCT-------GAAAACAGAACCAACCTCTCCTGC--GAAGGGTACCTCCCA 186
Human          -------ACTCT-------GAAAATCGAACCAACCTTTCCTGT--GAAGGGTGCCTCTCA 186
Tilapia        GGCAATGACGCACAGCTGGGGAACTGGTCTCACCATAACGTGGCGGGAGTCTACGGGT-- 217
Zebrafish      A------ACTCTATGGATTGCAATGGGAG---------CGAAG--AGA-GAAACCGGT-- 151
Trout          ------------------------------------------------------------ 0

Mouse          CCGACATGCCTCTCCATTCTTCATCTCCAGGAAAAAAACTGGTCTGCTTTATTGACAACT 246
Human          CCGTCGTGTCTCTCCTTACTTCATCTCCAGGAAAAAAACTGGTCTGCTTTACTGACAGCC 246
Tilapia        -CTCAGTGCCCTCCCTTGGAGCAGCTGATGCAGAAAAACTGGGCAGCCCTGCTGATTTTA 276
Zebrafish      -CGGAGTTTTCGTCCTCTGATTA---TGTCCAGAAGAACTGGGTGGCACTTTTGATTTCG 207
Trout          ------------------------------------------------------------ 0

Mouse          GTCGTGATTATTCTCACCATTGCGGGAAACATACTGGTCATCATGGCAGTGTCCCTAGAG 306
Human          GTAGTGATTATTCTAACTATTGCTGGAAACATACTCGTCATCATGGCAGTGTCCCTAGAG 306
Tilapia        GTGGTCATCGCTGTCACGGTCATGGGCAACATTCTGGTGATCCTGGCCGTCTCCCTGGAG 336
Zebrafish      CTGGTCATAATCATCACCGTGACGGGCAACATCCTGGTCATCATGGCGGTGAGTTTGGAG 267
Trout          ------------------------------------------------------------ 0

Mouse          AAAAAGCTGCAGAATGCCACCAACTATTTCCTGATGTCACTTGCCATAGCTGATATGCTG 366
Human          AAAAAGCTGCAGAATGCCACCAACTATTTCCTGATGTCACTTGCCATAGCTGATATGCTG 366
Tilapia        AAGAAGCTGCAGAACGCCACAAACTATTTCTTGATGTCACTGGCAGTAGCTGACATGCTT 396
Zebrafish      AGGAAGTTACAGAACGCCACCAACTACTTCTTGAGGTCTTTGGCCATTACAGACATGCTT 327
Trout          ------------------------------------------------------------ 0
```

```
Mouse          CTGGGTTTCCTTGTCATGCCCGTGTCCATGTTAACCATCCTGTATGGGTACCGGTGGCCT 426
Human          CTGGGTTTCCTTGTCATGCCCGTGTCCATGTTAACCATCCTGTATGGGTACCGGTGGCCT 426
Tilapia        CTCGGCATCTTGGTCATGCCGGTTTCCATGGTGACCATTCTCTATGATTACGGCTGGCCC 456
Zebrafish      TTGGGAATCCTTGTTATGCCGGTAGCAATGGTGACAATCCTCTACGGTTACACCTGGCCT 387
Trout          ---------------------------------ACAATTCTCTATGACCACTGGTGGCCC 27
                                                ** ** ** ** *   **   *****

Mouse          TTGCCCAGCAAGCTCTGTGCCGTCTGGATTTACCTGGATGTGCTCTTCTCCACGGCGTCC 486
Human          CTGCCGAGCAAGCTTTGTGCAGTCTGGATTTACCTGGACGTGCTCTTCTCCACGGCCTCC 486
Tilapia        CTGCCCTCTGATCTTTGCCCCATCTGGATCTATCTGGACGTCCTGTTCTCCACAGCATCC 516
Zebrafish      CTCCCTCGCACCCTGTGTCCGATCTGGATCTACCTGGACGTTCTCTTCTCCACCGCCTCC 447
Trout          CTGCCCTCGGACCTGTGCCCTATCTGGATCTACCTGGATGTGCTGTTCTCCACAGCCTCC 87
                * **       ** **  *  ******* ** ***** ** ** ******** ** ***

Mouse          ATCATGCACCTCTGCGCCATCTCCCTGGACCGCTACGTGGCTATCCAGAACCCCATTCAC 546
Human          ATCATGCACCTCTGCGCCATCTCGCTGGACCGCTACGTCGCCATCCAGAATCCCATCCAC 546
Tilapia        ATCATGCACCTTTGTGCCATCTCTCTGGATAGATACATCGCCATACGCAACCCAATCCAC 576
Zebrafish      ATCATGCACCTGTGCGCCATATCTCTGGACAGATACATCGCCATCCGCAACCCCATCCAC 507
Trout          ATCATGCACCTGTGTGCCATCTCTCTGGACCGCTACATCGCCATCAGAAACCCCCTCCAC 147
               *********** ** ***** ** *****  * *** * ** **    ** **  * ***

Mouse          CATAGCCGCTTCAACTCCAGAACCAAAGCCTTCCTGAAAATCATTGCGGTGTGGACCATA 606
Human          CACAGCCGCTTCAACTCCAGAACTAAGGCATTTCTGAAAATCATTGCTGTTTGGACCATA 606
Tilapia        CACAGCCGCTTCAATTCTCACACCAAGGCCCGCATCAAGATCATGGCAGTGTGGACCATT 636
Zebrafish      CACAGCCGCTCCAACTCCCTAACAAAAGCCCGCGTCAAAATCATCGCAGCCTGGACCATC 567
Trout          CACAGCCGCTTCAACTCCCGAACCAAGGCTCGCCTCAAGATCATGGCTGTCTGGACCATC 207
               ** ******* *** **    ** ** **     * ** ***** ** *  ********
```

```
Mouse          TCCGTAGGTATATCCATGCCAATCCCAGTCTTCGGGCTACAGGATGATTCGAAGGTCTTT 666
Human          TCAGTAGGTATATCCATGCCAATACCAGTCTTTGGGCTACAGGACGATTCGAAGGTCTTT 666
Tilapia        TCAGTGGGGATCTCAATGCCGGTTCCTGTGTTTGGTCTCAGAGACCACTCCAAGGTCTTC 696
Zebrafish      TCTGTGGTTATCTCCATGCCTGTTCCAGTCCTCGGCCTTCATGACCACTCCAAAGTCTTC 627
Trout          TCTGTGGGTATCTCCATGCCCATCCCTGTCCTAGGCCTGCGGGACCACACAAAGGTATTT 267
               ** ** *  ** ** *****  * ** **  * ** **    **  *  * ** ** **

Mouse          AAGGAAGGGAGCTGCCTGCTCGCCGATGACAACTTTGTCCTCATAGGCTCTTTTGTGGCA 726
Human          AAGGAGGGGAGTTGCTTACTCGCCGATGATAACTTTGTCCTGATCGGCTCTTTTGTGTCA 726
Tilapia        AAAGATGGCAGCTGTCAGCTGACAGATAACAATTTCGTGCTGGTCGGCTCCTTCGTGGCC 756
Zebrafish      CGCAACGAAAGCTGCCAACTTACGGACAACAATTTCGTCCTGATTGGCTCGTTTGTGGCG 687
Trout          AAAGATGGCAGCTGCCTGCTGACAGATGACAGTTTCGTCCTGGTAGGCTCCTTCGTGGCC 327
                   * *  ** **    **  * **  * *  ** ** **  * ***** ** *** *

Mouse          TTTTTCATCCCCCTAACCATCATGGTGATCACCTACTTCCTGACTATCAAGTCACTTCAG 786
Human          TTTTTCATTCCCTTAACCATCATGGTGATCACCTACTTTCTAACTATCAAGTCACTCCAG 786
Tilapia        TTCTTCATCCCCCTCACCATCATGGTCGTCACCTATTTCCTGACCATCAACGCCCTGCAG 816
Zebrafish      TTTTTCGTCCCGCTCATCATCATGGTCGTCACATACTTTCTCACCATCAGTGCTCTACAA 747
Trout          TTCTTCGTCCCCCTCACCATCATGGTGGTCACCTACTTCCTCACCATCAGCGCCCTGCAG 387
               ** *** * **  * * *********  **** ** ** ** ** ****   * ** **

Mouse          AAAGAAGCCACCTTGTGTGTGAGTGACCTCAGCACTCGGGCCAAATTATC---------C 837
Human          AAAGAAGCTACTTTGTGTGTAAGTGATCTTGGCACACGGGCCAAATTAGC---------T 837
Tilapia        AACGAAGCCACCCTCTGCTTGGACCAGCTGGTCCCACGGCCCAAATGGAGCACTACCTTC 876
Zebrafish      AGCGAAGCGACTTTGTGCCTGGACCAACTCATCGTGCGTCCAACATGGTCGTC------C 801
Trout          AATGAGGCTACACTCTGCCTGGACCAGCTGGTGCCCAGGCCCAAGTGGAGTGCCACCCTG 447
               *  ** ** **  * **  *    * **       *  * *  *
```

```
Mouse          TCCTTCAGCTTCCTCCCTCAGAGTTCTCTGTCATCAGAAAAGCTCTTCC---AGCGGTCC  894
Human          TCTTTCAGCTTCCTCCCTCAGAGTTCTTTGTCTTCAGAAAAGCTCTTCC---AGCGGTCG  894
Tilapia        ACCTTGAGCTTCTTTCCACAAACGTCTTTGTCCTCCGAGAAACTC---CTCAGGCGATCG  933
Zebrafish      ACCATTGGACTCCTCCCTCGAGGTTCAGTTTCCTCTGAGCGTCTCTTCTCACGCTCATCA  861
Trout          ACCCTGGGATTCTTCCCACAGTCCTCGCTCTCCTCAGAGAAGCTCTTCTTCAGGCGTTCG  507
                *  *  *  ** * ** *     **  * ** ** **    ***           **

Mouse          ATCCACA-----------------GAGAGCCAGGCT-------------------CC---  915
Human          ATCCATA-----------------GGGAGCCAGGGT-------------------CC---  915
Tilapia        ATCAGCCGTGACACCACAGGCAGAGGGAGCACCGGC-----GGGCTGGGTGGTGCCCGTG  988
Zebrafish      ATCTGCCGAGAA-------------GGAG------CTTC----------------C----  882
Trout          CTCAGCCGTGACACGAGAGGGGT-GGGACGAGGGGTTGGCGGGGCTGGGGTGGACCCGCG  566
                **                       **                           *

Mouse          -----------------TACGCAGGCCGAAGGACGATGCAGTCCATCAGCAACGAGCAAA  958
Human          -----------------TACACAGGCAGGAGGACTATGCAGTCCATCAGCAATGAGCAAA  958
Tilapia        GGAGCATATCTGGCTCTCTCTTTGGGCGCCGCACCATGCAGTCGATCAGCAACGAGCAGA  1048
Zebrafish      AGAGGA--------------TCCGGACGCCGATCCATGCAGTCCATCAGCAATGAACAGA  928
Trout          GTA-------------------CGGGCG--------------------------------  575
                                     **  *

Mouse          AAGCATGCAAGGTGCTGGGCATCGTGTTCTTTCTGTTTGTTGTAATGTGGTGCCCATTCT  1018
Human          AGGCATGCAAGGTGCTGGGCATCGTCTTCTTCCTGTTTGTGGTGATGTGGTGCCCTTTCT  1018
Tilapia        AGGCTTCAAAGGTCCTTGGGGTGGTGTTTTTTCTCTTTGTGGTTATGTGGTGCCCGTTTT  1108
Zebrafish      AGGCATCCAAAGTGCTCGGCGTGGTTTTCCTACTTTTTGTGATCATGTGGTGTCCGTTTT  988
Trout          ------------------------------------------------------------  575
```

```
Mouse          TCATCACCAATATCATGGCCGTCATCTGCAAAGA---ATCCTGCAATGAAAATGTCATTG 1075
Human          TCATCACAAACATCATGGCCGTCATCTGCAAAGA---GTCCTGCAATGAGGATGTCATTG 1075
Tilapia        TCATCACCAACGTGCTGGTGGTGGTGTGTGAACCTGACGTTTGCGACCCGGGAGTCATGG 1168
Zebrafish      TTGTGACGAACGTGATGGCGGTGGTGTGCGGCTC---CGTGTGCGATGAAGATTTGGTAG 1045
Trout          ------------------------------------------------------------ 575

Mouse          GAGCCCTGCTCAATGTGTTTGTCTGGATTGGTTATCTCTCCTCAGCCGTCAACCCACTGG 1135
Human          GGGCCCTGCTCAATGTGTTTGTTTGGATCGGTTATCTCTCTTCAGCAGTCAACCCACTAG 1135
Tilapia        GAGGTCTGCTGAATGTTTTCGTCTGGGTAGGCTACTTGTCCTCTGCAGTCAACCCACTGG 1228
Zebrafish      GCGGGTTGATGAATGTGTTTGTTTGGGTTGGATATTTATCATCGGCGGTCAACCCGTTCA 1105
Trout          ------------------------------------------------------------ 575

Mouse          TATATACGTTGTTCAATAAAACTTATAGGTCCGCCTTCTCACGGTACATTCAGTGCCAGT 1195
Human          TCTACACACTGTTCAACAAGACCTATAGGTCAGCCTTTTCACGGTATATTCAGTGTCAGT 1195
Tilapia        TCTACACGTTGTTCAATAAGACGTACCGCGCCGCGTTCCTGCGATACGTTCGCTGCCAGT 1288
Zebrafish      TCTACACACTCTTCAACAAGACTTACCGTGCCGCGTTCGCCCGGTACATGCAGTGCCGCT 1165
Trout          ------------------------------------------------------------ 575


Mouse          ACAAGGAGAACAGAAAGCCGCTGCAGTTAATTTTAGTGAACACTATACCAACATTGGCCT 1255
Human          ACAAGGAAAACAAAAAACCATTGCAGTTAATTTTAGTGAACACAATACCGGCTTTGGCCT 1255
Tilapia        ACCAGCCAGAGAAGAAACCTCTTCAGCTGATACTGGTGAACACCATCCCCCCACTGGCTT 1348
Zebrafish      ACCATGAGGAGAGGAGACCCCTGCAGCTGATACTGGTCAACACAATCCCCCCTCTGGCCT 1225
Trout          ------------------------------------------------------------ 575
```

```
Mouse          ACAAGTCTAGTCAGCTCCAGGTGGGACAAAAAAAGAACTCACAGGAAG---ATGCTGA-G 1311
Human          ACAAGTCTAGCCAACTTCAAATGGGACAAAAAAAGAATTCAAAGCAAG---ATGCCAA-G 1311
Tilapia        ACAGCTCCACCCAGCTACCTTTGGGAGACATCGGGAAGTTAGGCAATGGAGTGGCGCACT 1408
Zebrafish      ACAGCTCCTCTGGGCTTCCCCTGAAG---GTGGAGAACTCATT--ACGGA--------GG 1272
Trout          ------------------------------------------------------------ 575

Mouse          CCGACA-----GCTAATGACTGCTCCATGGTTACACTAGGGAACCAACACTCGGAAGAGA 1366
Human          ACAACA-----GATAATGACTGCTCAATGGTTGCTCTAGGAAAGCAGCATTCTGAAGAGG 1366
Tilapia        GTAGCAGCGGGGCGAAGGAGTTCT-----CTT-CTCCTGGG---CAGGAGACGGAAAAAT 1459
Zebrafish      AAAGCAGAGGGCAG---CCGGTCTGGGAGCTT-CACCAACA----CTGAGAGGTCAA-TC 1323
Trout          ------------------------------------------------------------ 575

Mouse          TGTGTACAGACAATATTGAAAC----CGTGAATGAAAAGGTTAGCTGTGTGTGA 1416
Human          CTTCTAAAGACAAT---------------------------------------- 1380
Tilapia        CCAGGCAGGACAAAAGCCACGGAG-------ACAAAGACGAGAGCTGTGTGTGA 1506
Zebrafish      TGTGGAAGCACACAAAACAAGCAGGAACGGGATGAGGTAGTAAGCCATTTGTAA 1377
Trout          ------------------------------------------------------ 575
```

5-HT$_{2A}$ receptor amino acid alignment

```
Mouse          MEI--------LCEDNIS---LSSIPN-----SLMQLGDDSRLYPN-DFNSRDANTSEAS  43
Human          MDI--------LCEENTS---LSSTTN-----SLMQLNDDTRLYSN-DFNSGEANTSDAF  43
Tilapia        MNLHGGNVSQLISNTVTAVTTLGSNPWPPHEEGGLQSGNATNLGCQRNNVVHNGNDAQLG  60
Zebrafish      MALNGTA---------AELRSLGISAEISKD-------------------TSMEQKVSLG  32
Trout          ------------------------------------------------------------  0

Mouse          NWTIDAENRTNLSCEG--YLPPTCLSILHLQEKNWSALLTTVVIILTIAGNILVIMAVSL  101
Human          NWTVDSENRTNLSCEG--CLSPSCLSLLHLQEKNWSALLTAVVIILTIAGNILVIMAVSL  101
Tilapia        NWSHHNV----AGVYG-----SQCPPLEQLMQKNWAALLILVVIAVTVMGNILVILAVSL  111
Zebrafish      NAVLNNS----MDCNGSEERNRSEFSSSDYVQKNWVALLISLVIIITVTGNILVIMAVSL  88
Trout          ------------------------------------------------------------  0

Mouse          EKKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLFSTA  161
Human          EKKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLFSTA  161
Tilapia        EKKLQNATNYFLMSLAVADMLLGILVMPVSMVTILYDYGWPLPSDLCPIWIYLDVLFSTA  171
Zebrafish      ERKLQNATNYFLRSLAITDMLLGILVMPVAMVTILYGYTWPLPRTLCPIWIYLDVLFSTA  148
Trout          ------------------------------------------------------------  0

Mouse          SIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDDSKV  221
Human          SIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDDSKV  221
Tilapia        SIMHLCAISLDRYIAIRNPIHHSRFNSHTKARIKIMAVWTISVGISMPVPVFGLRDHSKV  231
Zebrafish      SIMHLCAISLDRYIAIRNPIHHSRSNSLTKARVKIIAAWTISVVISMPVPVLGLHDHSKV  208
Trout          --MHLCAISLDRYIAIRNPLHHSRFNSRTKARLKIMAVWTISVGISMPIPVLGLRDHTKV  58
                 *********** ** ** **** ** ***  ** * ***** **** ** ** *  **
```

```
Mouse          FKEGSCLLADDNFVLIGSFVAFFIPLTIMVITYFLTIKSLQKEATLCVSDLSTRAKLSS- 280
Human          FKEGSCLLADDNFVLIGSFVSFFIPLTIMVITYFLTIKSLQKEATLCVSDLGTRAKLAS- 280
Tilapia        FKDGSCQLTDNNFVLVGSFVAFFIPLTIMVVTYFLTINALQNEATLCLDQLVPRPKWSTT 291
Zebrafish      FRNESCQLTDNNFVLIGSFVAFFVPLIIMVVTYFLTISALQSEATLCLDQLIVRPTWSST 268
Trout          FKDGSCLLTDDSFVLVGSFVAFFVPLTIMVVTYFLTISALQNEATLCLDQLVPRPKWSAT 118
               *   ** * *  *** **** ** ** *** ******  ** *****   *  *

Mouse          --FSFLPQSSLSSEKLFQRS-IHRE-----------------PGSYAGRRTMQSISNEQK 320
Human          --FSFLPQSSLSSEKLFQRS-IHRE-----------------PGSYTGRRTMQSISNEQK 320
Tilapia        FTLSFFPQTSLSSEKLL-RRSISRDTTGRGSTGGLGGARGSISGSLFGRRTMQSISNEQK 350
Zebrafish      --IGLLPRGSVSSERLFSRSSICREGASRG---------------SGRRSMQSISNEQK 310
Trout          LTLGFFPQSSLSSEKLFFRRSLSRDTRGVGRGVGGAGVDPR-----YG------------ 161
                     *  * *** *  *    *                       *

Mouse          ACKVLGIVFFLFVVMWCPFFITNIMAVICK-ESCNENVIGALLNVFVWIGYLSSAVNPLV 379
Human          ACKVLGIVFFLFVVMWCPFFITNIMAVICK-ESCNEDVIGALLNVFVWIGYLSSAVNPLV 379
Tilapia        ASKVLGVVFFLFVVMWCPFFITNVLVVVCEPDVCDPGVMGGLLNVFVWVGYLSSAVNPLV 410
Zebrafish      ASKVLGVVFLLFVIMWCPFFVTNVMAVVCG-SVCDEDLVGGLMNVFVWVGYLSSAVNPFI 369
Trout          ------------------------------------------------------------ 161

Mouse          YTLFNKTYRSAFSRYIQCQYKENRKPLQLILVNTIPTLAYKSSQLQVGQKKNSQEDAEPT 439
Human          YTLFNKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYKSSQLQMGQKKNSKQDAKTT 439
Tilapia        YTLFNKTYRAAFLRYVRCQYQPEKKPLQLILVNTIPPLAYSSTQLPLGDIGKLGNGVAHC 470
Zebrafish      YTLFNKTYRAAFARYMQCRYHEERRPLQLILVNTIPPLAYSSSGLPLKVENSLRRKAEGS 429
Trout          ------------------------------------------------------------ 161
```

```
Mouse          ANDCSMVTLGNQHSEEMCTDNIETVNEKVSCV    471
Human          DNDCSMVALGKQHSEEASKDNSDGVNEKVSCV    471
Tilapia        SSGAKEFSSPGQE-TEKSRQDKSHGDKDESCV    501
Zebrafish      RSGS--FTNTERS-ICGSTQNKQERDEVVSHL    458
Trout          --------------------------------    161
```

Appendix 4. Nucleotide and amino acid alignment of 5-HT$_{2C}$ receptor sequences from human (*Homo sapiens*), ENSG00000147246; mouse (*Mus musculus*), ENSMUS00000041380; zebrafish (*Danio rerio*), ENSDARG00000018228; Nile tilapia (*Oreochromis niloticus*), ENSONIG00000006962 and rainbow trout (*Oncorhynchus mykiss*), KP334156.

5-HT$_{2C}$ receptor nucleotide alignment

```
Mouse          ---ATGGTG-AACCTGG----GCAC-----------TGCGGTGCGCT--CACTCCTTGTG  39
Human          ---ATGGTG-AACCTGA----GGAA-----------TGCGGTGCATT--CATTCCTTGTG  39
Tilapia        GAAATGGGCGGCCCAGCAAGGGCTCTTCTTGGCGGGTTCGGGACAACCACATCCTCCTTG  60
Zebrafish      ---ATGGGGGCACCTGGTGGGCCAGTTCTGGGTGGGTTTAGTTCCACCACATCCTCCTTA  57
Trout          ------------------------------------------------------------  0

Mouse          CACCTAATTGGCCTATTGGTTTGGCAGTTCGATATTTCCATAAGTCCA-GT---AGCAGC  95
Human          CACCTAATTGGCCTATTGGTTTGGCAATCTGATATTTCTGTGAGCCCA-GT---AGCAGC  95
Tilapia        GAGGTCGGGGGTCGAATGGCTTGGCCGAGCAACAACCCCTTAGATCTCAATCAAACCCTC  120
Zebrafish      GACCTCGTTGGCTGGATGATCTGGCCTGGGAACACCACGGTCAGCCTGAACCAGAGCTTC  117
Trout          ------------------------------------------------------------  0

Mouse          T---ATAGTAACTGACACTTTTAATTCCTCC-GATGGTGGACGCTTGTTTCAATTCCCG-  150
Human          T---ATAGTAACTGACATTTTCAATACCTCC-GATGGTGGACGCTT---CAAATTCCCA-  147
Tilapia        CCGTGGGGGATAGGA--CTTGGAAGCAGCAGTGGAGGTGGCGG-------TGCTTCCGGC  171
Zebrafish      T-TTGGGACAGACTACAGCTTCAATCTGTCGTCCTCCTCTTCGTCT--TCTCCTCATGGG  174
Trout          ------------------------------------------------------------  0
```

```
Mouse          --------GACGG-------GGTACAAAACTGGCCAGCACTTTCAATAGTCGTGATTATA 195
Human          --------GACGG-------GGTACAAAACTGGCCAGCACTTTCAATCGTCATCATAATA 192
Tilapia        ATGAGCAGGGCGGTGATCAAAGAGAAGAACTGGCCAGCGCTGCTCATACTCGTCATCATT 231
Zebrafish      GTGAAAAGGGAGGAGATGAAGGAGAAGAACTGGCCGGCCCTGCTGATTCTAGTGATTATC 234
Trout          ------------------------------------------------------------ 0

Mouse          ATCATGACAATAGGGGGCAACATTCTCGTTATCATGGCAGTAAGCATGGAGAAGAAACTG 255
Human          ATCATGACAATAGGTGGCAACATCCTTGTGATCATGGCAGTAAGCATGGAAAAGAAACTG 252
Tilapia        GCGCTGACGATCGGCGGAAACATTCTGGTCATCCTCGCGGTGTCGCTGGAAAAGAAGCTC 291
Zebrafish      ATTCTGACCATAGGAGGGAATATTTTGGTTATTTTGGCCGTGTCGCTGGAGAAAAAGCTG 294
Trout          ------------------------------------------------------------G 1

Mouse          CACAATGCTACCAATTATTTCTTAATGTCCCTAGCCATTGCTGATATGCTGGTGGGACTA 315
Human          CACAATGCCACCAATTACTTCTTAATGTCCCTAGCCATTGCTGATATGCTAGTGGGACTA 312
Tilapia        CAGAATGCCACCAACTTCTTTCTGAGGTCACTGGCTGTAGCGGACATGCTCGTAGGGATT 351
Zebrafish      CAAAATGCAACAAACTTCTTCCTCCGCTCACTCGCAGTGGCAGACATGCTGGTTGGCATC 354
Trout          CAGAATGCCACCAGCTTCAT-CTGCGCTCCCTGGCTGTGGCAGACATGCTCGTGGGCTTT 60
               ** ***** ** *  *   *  *    ** ** **  * ** ** ***** ** **  *

Mouse          CTTGTCATGCCCCTGTCTCTGCTTGCAATTCTTTATGATTATGTCTGGCCTTTACCTAGA 375
Human          CTTGTCATGCCCCTGTCTCTCCTGGCAATCCTTTATGATTATGTCTGGCCACTACCTAGA 372
Tilapia        CTGGTTATGCCGATCTCTCTTATCAACATCCTCTATGACTACGCCTGGCCCCTCCCCAGC 411
Zebrafish      TTGGTCATGCCAATATCTCTCATAAACATATTATATGATTATGCCTGGCCACTGCCCAGT 414
Trout          CTCGTCATGCCGGTCTCACTGATTAACATTCTCTATGACTACCTGTGGCCCTTCCCCAGA 120
                * ** *****  * ** **  *    **  * ***** **    *****  * ** **
```

```
Mouse          TATTTGTGCCCCGTCTGGATTTCACTAGATGTGCTATTTTCAACTGCGTCCATCATGCAC 435
Human          TATTTGTGCCCCGTCTGGATTTCTTTAGATGTTTTATTTTCAACAGCGTCCATCATGCAC 432
Tilapia        GCTCTGTGTCCGATCTGGATCTACCTGGATGTGCTGTTCTCCACTGCCTCCATCATGCAC 471
Zebrafish      GCTCTCTGTCCTATTTGGATATACCTGGATGTGCTTTTCTCAACTGCTTCCATCATGCAC 474
Trout          CCCCTTTGTCCCATCTGGATCTATCTAGACGTGCTGTTCTCCACAGCATCCATCATGCAC 180
                   * ** **  * ***** *   * ** **  * ** ** ** ** ***********

Mouse          CTCTGCGCCATATCGCTGGACCGGTATGTAGCAATACGTAATCCTATTGAGCATAGCCGG 495
Human          CTCTGCGCTATATCGCTGGATCGGTATGTAGCAATACGTAATCCTATTGAGCATAGCCGT 492
Tilapia        CTCTGCGCCATCTCCCTCGACCGATACGTCGCCATCCGCAACCCTATAGAGCATAGCCGC 531
Zebrafish      CTGTGTGCCATTTCTCTCGACCGCTACGTGGCCATACGCAATCCAATTGAGCACAGTCGC 534
Trout          CTGTGTACCATTTCACTGGACCGCTATGTGGGCATCTGTAACCCCATCAAACACAGCCGC 240
               ** **  * ** ** ** ** ** ** ** *  **  * ** ** **  * ** ** **

Mouse          TTCAATTCGCGGACTAAGGCCATCATGAAGATTGCCATCGTTTGGGCAATATCAATAG-- 553
Human          TTCAATTCGCGGACTAAGGCCATCATGAAGATTGCTATTGTTTGGGCAATTTCTATAG-- 550
Tilapia        TTCAACTCTAGAACCAAAGCCATGATGAAGATCGCTGCAGTGTGGACCATATCTATAGTA 591
Zebrafish      TTCAACTCCCGCACCAAGGCCATGCTGAAGATCGCTGCCGTTTGGACCATTTCCATAG-- 592
Trout          TCCAGCTCCCTCTCCAAAGCCAAGGCTAAGATCGCCCTCGTCTGGAGCATCTCCATAG-- 298
               * **  **     * ** ****     ***** **    ** ***   ** ** ****

Mouse          -GAGTTTCAGTTCCTATCCCTGTGATTGGACTGAGGGACGAAAGCAAAGTGTTCGTGAAT 612
Human          -GTGTATCAGTTCCTATCCCTGTGATTGGACTGAGGGACGAAGAAAAGGTGTTCGTGAAC 609
Tilapia        GGCGTGTCAATGCCCATTCCAGTGATTGGCCTCCACAACAAGGACAAGGTCTTCGTCAAC 651
Zebrafish      -GTATCTCAATGCCCATTCCAGTGATTGGACTGAGTAACAGGGAAAAGGTCTTGAAGAAT 651
Trout          -TGATCTCCATGCCCATCCCTGTGATTGGCCTGTATGATGAGGGGAAGGTGTTTGTCAAT 357
                   * **  * ** ** ** ******** **     *       ** ** **    **
```

```
Mouse          AATACTACCTGCGTGCTCAATGACCCGAACTTCGTTCTCATCGGGTCCTTCGTGGCATTC  672
Human          AACACGACGTGCGTGCTCAACGACCCAAATTTCGTTCTTATTGGGTCCTTCGTAGCTTTC  669
Tilapia        ---GGCAGCTGTGTCCTCAATGAGGAGCGCTTCGTGCTGATTGGCTCCTTTGTGGCCTTT  708
Zebrafish      ---GGCATCTGTGCTCTAAACGAGGAACTCTTCATACTGGTTGGCTCTTTTGTTGCCTTC  708
Trout          ---GGGATCTGTGTACTGAACGAGCACAGCTTCGTCCTGATTGGCTCCTTCGTAGCCTTC  414
                     *  ** *  ** ** **       *** * **  * ** ** ** ** ** **

Mouse          TTCATCCCGTTGACAATTATGGTGATCACCTACTTCTTAACGATCTACGTCCTACGCCGT  732
Human          TTCATACCGCTGACGATTATGGTGATTACGTATTGCCTGACCATCTACGTTCTGCGCCGA  729
Tilapia        TTCATCCCGCTCGTAATCATGGTGGTGACATACTGCCTCACCATACAGGTGCTTCAGAGG  768
Zebrafish      TTTATTCCTCTGGTCATCATGGTGGTCACATACTGTCTCACCGTCCAAGCACTTCAACGC  768
Trout          TTCGTCCCTCTGGTCGTCATGGTGGTCAGCTACTGTTTGACCGTGCGCGTGCTGCAGTGC  474
               **  * **  *     * ****** * *  ** *   * **  *    *  ** *   *

Mouse          CAAACCCTGATGTTACTTCGA-------------------------------------  753
Human          CAAGCTTTGATGTTACTGCAC-------------------------------------  750
Tilapia        CAGGCCACAGTCTTCCTGTATGAGGCAAAGACTTCTTCCCAGCAGCCTTTGCACACACCA  828
Zebrafish      CAGGCCACAGTCTTCCTCTATGAGGGCAAAGCTTCTTCCCAGCAGCCTTTGCAGCCTCCA  828
Trout          CAAGCCTCTGATTACCTGCACGGAGGCATGGCTTCGATCGACCGGCCT------------  522
               **  *       *  **

Mouse          ------------------------GGTCACACCGAGGAGGAACTGCGTA---------A  779
Human          ------------------------GGCCACACCGAG---GAACCGCCTG---------G  773
Tilapia        GCAATGGCCCCTTGCTCAGAGTTGAAG----------GTCCCGCCTCCTCAAAGCAGACG  878
Zebrafish      GCT---CCACCTTCTTCCCAGTTGGTCCCAC-CCTTGGCTCCATCTCGTC---GCA--G-  878
Trout          --------ACCTTACTC------GGGATCACCCCAAGGCTCC------CC---GCA--GG  557
```

```
Mouse          TATCAGCCTGAACTTTCTAAAGTGCTGCTGCAAGAAGGG----------TGATGAGGAA-  828
Human          ACTAAGTCTGGATTTCCTGAAGTGCTGCAAGAGGAATAC----------GGCCGAGGAA-  822
Tilapia        AAACACCTTGAGCTGCCTGAAGGGCACAGA--GCCC----A--GCATTCTGCTCAGCGCC  930
Zebrafish      ---CAGTCTGAACTGCTTACGGATCAGCAACAGCGATGGAAATACACTCGGTTT--GACC  933
Trout          GGACAGTCTTAGCCTCCT--------CAAACAGG---------ACC-------------C  587
                   *   *        *

Mouse          GAGAACGCTCCCAACCC--------CA--ATCCAGATCAGAAGCCACGTCGA--------  870
Human          GAGAACTCTGCAAACCC--------TA--ACCAAGACCAGAACGCACGCCGAAGA-----  867
Tilapia        TCCA---CCTCAGATAGCATCAGCATCATTCCCAGCTCTGAGGTGGCGTCCCAGCTCAGC  987
Zebrafish      GTGC---CCCCAGACACTATCTCAATAATCCCAAGTTCAGAAGCGCCATCTCAAATGAAT  990
Trout          TAGT---CCCGAGACC----TTCAATATTTCCG-TCTCTCAAGCCTCCTCGCAACCCGTC  639
                      *    *                *    *  *    *  *

Mouse          --------AAGAAGA------AAGAAAAG-------------CGGCCTAGAGGCACCATG  903
Human          --------AAGAAGA------AGGAGAGA-------------CGTCCTAGGGGCACCATG  900
Tilapia        TCGCCGGCAGCTGGACCGGGCCGGAGCGACGCGTCAAGTCACGGTCGCCGGGGGATGATG  1047
Zebrafish      TCACCCGCAGGA---C-----GGGACCCAG-CTGGAGCTCATGGACGACGGGGGATGATG  1041
Trout          TCTCCAGCAGGGAGGC-----AGGAGCCAT-GGGGACCTGGTGGGAAACCTGGTGTGGCT  693
                       *             **                 *      **

Mouse          CAAGCTATCAACAATGAGAAGAAAGCTTCCAAAGTCCTTGGCATTGTATTCTTTGTGTTT  963
Human          CAGGCTATCAACAATGAAAGAAAAGCTTCGAAAGTCCTTGGGATTGTTTTCTTTGTGTTT  960
Tilapia        CAGGCCATAAAAAATGAGAGGCGGGCCTCAAAGGTTCTGGGAATCGTCTTCTTCCTCTTC  1107
Zebrafish      CAGGCCATCAAGAACGAGCGGCGAGCATCCAAAGTGTTGGGTGTAGTGTTCTTCCTCTTT  1101
Trout          CAATCAATCAAAAATGAGAGGAGAGCCTCCAAGGTCCTGGGTGTGGTGTTTTTCCTCTTC  753
               **  * ** ** ** **       ** ** ** **  * **  * ** ** **  * **
```

```
Mouse          CTGATCATGTGGTGCCCGTTTTTCATCACCAATATCCTGTCGGTGCTTTGTGGGAAGGCC 1023
Human          CTGATCATGTGGTGCCCATTTTTCATTACCAATATTCTGTCTGTTCTTTGTGAGAAGTCC 1020
Tilapia        CTCGTCATGTGGTGTCCCTTCTTTATCACCAACGTCACCTTCGTCTTGTGCAGCAACTCC 1167
Zebrafish      CTATTCATGTGGTGTCCCTTTTTCATCACTAACGTACTGTACGTCCTCTGCCATAGAACC 1161
Trout          CTG---------------------------------------------------------- 756
               **

Mouse          TGTAACCAAAAGCTAATGGAGAAACTTCTCAATGTGTTTGTTTGGATTGGCTATGTGTGT 1083
Human          TGTAACCAAAAGCTCATGGAAAAGCTTCTGAATGTGTTTGTTTGGATTGGCTATGTTTGT 1080
Tilapia        TGCAACGAGTCACTGCTCCACGACCTCCTCAACGTCTTTGTCTGGGTGGGTTACATCTCC 1227
Zebrafish      TGTAAGGAAACTCTACTCACGGAACTGCTCAACGTGTTTGTTTGGGTGGGTTACATCTCA 1221
Trout          ---------------------------------------------------------- 756

Mouse          TCAGGCATCAATCCTCTGGTGTACACTCTCTTCAACAAAATTTACCGAAGGGCTTTCTCT 1143
Human          TCAGGAATCAATCCTCTGGTGTATACTCTGTTCAACAAAATTTACCGAAGGGCATTCTCC 1140
Tilapia        TCCGGAGTCAACCCTCTGGTCTACACCCTCTTCAATAAGACCTACAGGAGAGCGTTCTCC 1287
Zebrafish      TCAGGGGTCAACCCTTTAGTGTACACTTTGTTTAACAAGACCTACCGAAGGGCGTTTTCA 1281
Trout          ---------------------------------------------------------- 756

Mouse          AAATATTTGCGCTGCGATTATAAGCCAGAC----------------------AAAAAGC 1180
Human          AACTATTTGCGTTGCAATTATAAGGTAGAG----------------------AAAAAGC 1177
Tilapia        AGCTACATCAGGTGCCAGTACAAAGTCGGGGCCAACGCAGCCGGGCAGGGTTGCAAAACC 1347
Zebrafish      AACTATATGCACTGCCAATACAGACACGT-----------------AGGGCTT-AAACCC 1323
Trout          ---------------------------------------------------------- 756
```

```
Mouse          CTCCTG-TTCG--------ACAGATTCCTAGGGTTGCTGCCACTGCTTTGTCTGGGAGGG 1231
Human          CTCCTG-TCAG--------GCAGATTCCAAGAGTTGCCGCCACTGCTTTGTCTGGGAGGG 1228
Tilapia        CTCCTTGTCCCGCCGCCGTGCCCTTCACATGCCG---TGACTCCTCTTCTTATGGGCGGT 1404
Zebrafish      ATCACTATAAACGTTCCATGTCCATCCCATGCGGTAGTCACACCTATTCTGATGTGTGAC 1383
Trout          ------------------------------------------------------------ 756

Mouse          -AGCTCAATGTTAACATTTATCGGCATACCA--------------------ATGAA-CG 1268
Human          -AGCTTAATGTTAACATTTATCGGCATACCA--------------------ATGAA-CC 1265
Tilapia        CACGGGAAGGCAGGTGTGGACCGCAACAGTAACTGTCGCAATGGCGGTAGGGGGGATAAC 1464
Zebrafish      ------AAAGTTTCCATTGACCGGAATAGTAACTGCCGCAATGGGGATGGGAATGGAATC 1437
Trout          ------------------------------------------------------------ 756


Mouse          TGTAG-----------TTAGGAAAGCTAATGACACAGAGCCTGGCATAGAGATGCAGGTA 1317
Human          GGTGA-----------TCGAGAAAGCCAGTGACAATGAGCCCGGTATAGAGATGCAAGTT 1314
Tilapia        -------GGG--AGGCTTGCAGTGGACCCGGAAACAGAGCCGGAAACGGAGCTGGAGCCG 1515
Zebrafish      CGAAATCTGGAGACTCTTGATATAGACACTGACA------CCGGACTGGAGTTGAAACCT 1491
Trout          ------------------------------------------------------------ 756

Mouse          GAGAATTTAGAGCTGCCGGTCAATCCCTCTAATGTGGTCAGCGAGAGGATTAGTAGTGTG 1377
Human          GAGAATTTAGAGTTACCAGTAAATCCCTCCAGTGTGGTTAGCGAAAGGATTAGCAGTGTG 1374
Tilapia        GAGCTGTCACTCAT------CAGCTACAGCCCTGCCCCGAGAGAACACACAAGCAGCGTG 1569
Zebrafish      GGAATATCAGAGATCTCTATAAGCAGCGGTCACAGTCACACAGAACACACCAGCAGTGTC 1551
Trout          ------------------------------------------------------------ 756
```

Mouse	TAA	1380
Human	TGA	1377
Tilapia	TGA	1572
Zebrafish	TGA	1554
Trout	---	756

5-HT$_{2C}$ receptor amino acid alignment

```
Mouse          -------MVNLGTAVRSLLVHLIGLLVWQFDISISPVAAIVTDTFN-----SSDGG---R  45
Human          -------MVNLRNAVHSFLVHLIGLLVWQSDISVSPVAAIVTDIFN-----TSDGG---R  45
Tilapia        EMGGPARALLGGFGTTTSSLEVGGRMAWPSNNPLDLNQTLP---WGIGLGSSSGGGGASG  57
Zebrafish      -MGAPGGPVLGGFSSTTSSLDLVGWMIWPGNTTVSLNQSFFGTDYSFNLS-SSSSSSPHG  58
Trout          ------------------------------------------------------------  0

Mouse          LFQFPDGVQNWPALSIVVIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADMLVGL  105
Human          -FKFPDGVQNWPALSIVIIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADMLVGL  104
Tilapia        MSRAVIKEKNWPALLILVIIALTIGGNILVILAVSLEKKLQNATNFFLRSLAVADMLVGI  117
Zebrafish      VKREEMKEKNWPALLILVIIILTIGGNILVILAVSLEKKLQNATNFFLRSLAVADMLVGI  118
Trout          ------------------------------------------------------MLVGF  5
                                                                      ****

Mouse          LVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNPIEHSR  165
Human          LVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNPIEHSR  164
Tilapia        LVMPISLINILYDYAWPLPSALCPIWIYLDVLFSTASIMHLCAISLDRYVAIRNPIEHSR  177
Zebrafish      LVMPISLINILYDYAWPLPSALCPIWIYLDVLFSTASIMHLCAISLDRYVAIRNPIEHSR  178
Trout          LVMPVSLINILYDYLWPFPRPLCPIWIYLDVLFSTASIMHLCTISLDRYVGICNPIKHSR  65
               **** **  ***** ** *  *** ** ************** ******* * *** ***

Mouse          FNSRTKAIMKIAIVWAIS-IGVSVPIPVIGLRDESKVFVNNTTCVLNDPNFVLIGSFVAF  224
Human          FNSRTKAIMKIAIVWAIS-IGVSVPIPVIGLRDEEKVFVNNTTCVLNDPNFVLIGSFVAF  223
Tilapia        FNSRTKAMMKIAAVWTISIVGVSMPIPVIGLHNKDKVFVN-GSCVLNEERFVLIGSFVAF  236
Zebrafish      FNSRTKAMLKIAAVWTIS-IGISMPIPVIGLSNREKVLKN-GICALNEELFILVGSFVAF  236
Trout          SSSLSKAKAKIALVWSIS-IVISMPIPVIGLYDEGKVFVN-GICVLNEHSFVLIGSFVAF  123
                 *  **  *** ** **    * *******    **  *   * **   * * ******
```

```
Mouse          FIPLTIMVITYFLTIYVLRRQTLMLLRGHTEEELRNI-----------------SLNFL 266
Human          FIPLTIMVITYCLTIYVLRRQALMLLHGHTEEP-PGL-----------------SLDFL 264
Tilapia        FIPLVIMVVTYCLTIQVLQRQATVFLYEAKTSSQQPLHTPAMAPCSELKVPPPQSRRNTL 296
Zebrafish      FIPLVIMVVTYCLTVQALQRQATVFLYEGKASSQQPLQPPA-PPSSQLVPPLAPSRRSSL 295
Trout          FVPLVVMVVSYCLTVRVLQCQASDYLHGGMASIDRPTLLGITP-------RLP--AGDSL 174
               * **  **  * **   *  *    *                                 *

Mouse          KCCCKKGDE-------------------EENAPNPNPDQKPR-RKKKEKRPRGTMQAINN 306
Human          KCCKRNTAE-------------------EENSANPNQDQNARRRKKKERRPRGTMQAINN 305
Tilapia        SCLKGTEP--SILLSASTSDSISIIPSSEVASQLSSPAAGPGRSDASSHGRRGMMQAIKN 354
Zebrafish      NCLRISNSDGNTLGLTVPPDTISIIPSSEAPSQMNSPA---GRDPAGAHGRRGMMQAIKN 352
Trout          SLLKQD----------PSPETFN-ISVSQASSQPVSPAG--RQEPWGPGGKPGVAQSIKN 221
                                                                *  * * *

Mouse          EKKASKVLGIVFFVFLIMWCPFFITNILSVLCGKACNQKLMEKLLNVFVWIGYVCSGINP 366
Human          ERKASKVLGIVFFVFLIMWCPFFITNILSVLCEKSCNQKLMEKLLNVFVWIGYVCSGINP 365
Tilapia        ERRASKVLGIVFFLFLVMWCPFFITNVTFVLCSNSCNESLLHDLLNVFVWVGYISSGVNP 414
Zebrafish      ERRASKVLGVVFFLFLFMWCPFFITNVLYVLCHRTCKETLLTELLNVFVWVGYISSGVNP 412
Trout          ERRASKVLGVVFFLFL-------------------------------------------- 237
               *  ****** *** **

Mouse          LVYTLFNKIYRRAFSKYLRCDYKPDKKPPVRQIP-----------RVAATALSGRELNVN 415
Human          LVYTLFNKIYRRAFSNYLRCNYKVEKKPPVRQIP-----------RVAATALSGRELNVN 414
Tilapia        LVYTLFNKTYRRAFSSYIRCQYKVGANAAGQGCKTLLVPPPCPSHAVT-PLLMGGHGKAG 473
Zebrafish      LVYTLFNKTYRRAFSNYMHCQYRH------VGLKPITINVPCPSHAVVTPILM--CDKVS 464
Trout          ------------------------------------------------------------ 237

Mouse          IYRHTNE---------RVVRKANDTEPGIEMQVENLELPVNPSNVVSERISSV  459
Human          IYRHTNE---------PVIEKASDNEPGIEMQVENLELPVNPSSVVSERISSV  458
Tilapia        VDRNSNCRNGGRGDNG-RLAVDPETEPETELEPELSLISYSPA--PREHTSSV  523
Zebrafish      IDRNSNCRNGDGNGIRNLETLDIDTDTGLELKPGISEISISSGHSHTEHTSSV  517
Trout          ----------------------------------------------------  237
```

Appendix 5. Nucleotide and amino acid alignment of 5-HT$_4$ receptor sequences from human (*Homo sapiens*), ENSG00000164270; mouse (*Mus musculus*), ENSMUS00000026322; zebrafish (*Danio rerio*), ENSDARG00000061940; Nile tilapia (*Oreochromis niloticus*), ENSONIG00000004225 and rainbow trout (*Oncorhynchus mykiss*), KP334157.

5-HT$_4$ receptor nucleotide alignment

```
Mouse          ATGGA-----------CAAACTTGATGCTA---ATGT--GAGTTCCAA--CGAGG---GT  39
Human          ATGGA-----------CAAACTTGATGCTA---ATGT--GAGTTCTGA--GGAGG---GT  39
Tilapia        ATGAATGTCAGCGCTGCAGGCCAGATGCCGGCGATGGCGGAGCTCGGCGGTGAGAGCGAT  60
Zebrafish      ------------------------------------------------------------  0
Trout          ------------------------------------------------------------  0

Mouse          TTCAGGTCCGTGGAGAAGGTCGTGCTGCTCACGTTCCTTGCAGTGGTTATCCTGATGGCC  99
Human          TTCGGGTCAGTGGAGAAGGTGGTGCTGCTCACGTTTCTCTCGACGGTTATCCTGATGGCC  99
Tilapia        AGCATGCC---AAAGCGGATGGCCCTGATCTGCTTCCTGTCTCTGGTCATGCTGATGAGC  117
Zebrafish      ------------------------------------------------------------  0
Trout          ------------------------------------------------------------  0

Mouse          ATCTTGGGCAACCTGCTGGTGATGGTGGCTGTGTGCAGGGACAGGCAGCTCAGGAAAATA  159
Human          ATCTTGGGGAACCTGCTGGTGATGGTGGCTGTGTGCTGGGACAGGCAGCTCAGGAAAATA  159
Tilapia        ATCTTTGGAAACCTGCTGGTCATGGTGGCCGTCTGCAAGGACAGACAACTCAGGAAAATC  177
Zebrafish      ------------------------------------------------------------  0
Trout          ------------------------------------------------------------  0

Mouse          AAAACCAACTATTTCATTGTGTCTCTCGCCTTTGCTGACCTGCTGGTTTCGGTGCTGGTG  219
Human          AAAACAAATTATTTCATTGTATCTCTTGCTTTTGCGGATCTGCTGGTTTCGGTGCTGGTG  219
Tilapia        AAGACCAACTACTTCATCGTGTCGCTGGCGTTTGCCGACTTGCTCGTGTCGGTGCTGGTG  237
Zebrafish      ------------------------------------------------------------  0
Trout          ------------TTCATAGTATCGCTGGCCTTTGCAGACCTGATGGTGTCGGTGCTGGTG  48
```

```
Mouse          ATGCCCTTTGGTGCCATTGAGCTGGTCCAAGACATCTGGGCTTATGGGGAGATGTTCTGC 279
Human          ATGCCCTTTGGTGCCATTGAGCTGGTTCAAGACATCTGGATTTATGGGGAGGTGTTTTGT 279
Tilapia        ATGCCGTTCGGCGCCATTGAGCTGGTCCACCAGCACTGGATCTACGGCGAGACCTTCTGT 297
Zebrafish      ------------------------------------------------------------ 0
Trout          ATGCCGTTTGGTGCCATCGAGCTGATCCACCAGAACTGGATATATGGAGAAACCTTCTGC 108

Mouse          CTGGTCCGGACCTCTCTGGATGTCCTACTTACCACAGCATCGATCTTTCACCTGTGCTGT 339
Human          CTTGTTCGGACATCTCTGGACGTCCTGCTCACAACGGCATCGATTTTTCACCTGTGCTGC 339
Tilapia        TTGGTGAGGACTTCTCTGGACGTTCTGCTCACCACCGCGTCGATACTACACCTGTGCTGC 357
Zebrafish      ------------------------------------------------------------ 0
Trout          CTGGTCCGAACATCTCTAGATGTTCTGTTGACAACAGCCTCCATATTACACCTGTGTTGC 168

Mouse          ATTTCCCTGGACAGGTATTACGCCATCTGCTGCCAGCCTTTGGTTTATAGGAACAAGATG 399
Human          ATTTCTCTGGATAGGTATTACGCCATCTGCTGCCAGCCTTTGGTCTATAGGAACAAGATG 399
Tilapia        ATCGCCCTGGACAGATACTACGCTATCTGCTGCCAGCCTCTGGTCTACCGGAACAAAATG 417
Zebrafish      ------------------------------------------------------------ATG 3
Trout          ATAGCCTTGGACAGGTACTATGCCATCTGCTGCCAGCCGCTGGTCTACAGGAAGAAGATG 228
                                                                        ***

Human          ACCCCTCTGCGCATCGCATTAATGCTGGGAGGCTGCTGGGTCATCCCCACGTTTATTTCT 459
Tilapia        ACGCCCATGCGAGTGGCTTTAATGATCGGCGGCTGCTGGGTCATTCCCACTTTCATCTCC 477
Zebrafish      ACACCGCTGAGGGTGACTCTAATGATTGGAGGATGCTGGATTATCCCAACTGTCATTTCC 63
Trout          ACATCACTGAGAGTGGCTCTAATGTTAGGAGGATGCTGGGTAATCCCCACCTTTATATCC 288
               **  *  *  *  *  *  ***** * ** ** ****** *  * ** *   * ** **

Mouse          TTTCTCCCCATAATGCAAGGCTGGAACAACATCGGCATAGTTGATGTGATAGAGAAAAGG 519
Human          TTTCTCCCTATAATGCAAGGCTGGAATAACATTGGCATAATTGATTTGATAGAAAAGAGG 519
Tilapia        TTCCTGCCCATCATGCAGGGCTGGAACAGCATCGGCATCGACCACCTGATTAACGAGCGG 537
Zebrafish      TTCCTACCCATAATGCAAGGCTGGAACAGTATAGGAATAAAGGACTTGATAGACAAGCGG 123
Trout          TTTCTACCCATTATGCAAGGATGGAACAGTATTGGAATAGACCATTTGATAGAGGAACGG 348
               ** ** ** ** ***** ** ***** *  ** ** **     *  ****  *  *  **
```

```
Mouse          AAATTCAGCCA---CAACTCTAACTCCACGTGGTGTGTCTTCATGGTCAACAAGCCCTAT 576
Human          AAGTTCAACCA---GAACTCTAACTCTACGTACTGTGTCTTCATGGTCAACAAGCCCTAC 576
Tilapia        CGCTTCAACAA---CAGCAGCAACACCACGTCCTGCGTCTTCATGGTCAACAAGCCGTAT 594
Zebrafish      AAGATCT---------CCGGCAACTCTACAGTATGTGTGTTCATGGTGAACAAGCCATAC 174
Trout          AAGATCAGCGGTGATGCAGCCAACGCGACGGCGTGTGTCTTCATGGTCAATAAGCCCTAC 408
                   **              *** * **    ** ** ******** ** ***** **

Mouse          GCTATCACCTGCTCTGTGGTGGCCTTCTACATCCCGTTTCTCCTCATGGTGCTGGCCTAT 636
Human          GCCATCACCTGCTCTGTGGTGGCCTTCTACATCCCATTTCTCCTCATGGTGCTGGCCTAT 636
Tilapia        GCGCTCACCTGCTCCTTGGTGGCCTTCTACATCCCTCTGGTCCTCATGGTGCTGGCCTAC 654
Zebrafish      GCGTTGACGTGCTCAGTAGTGGCCTTCTACTTACCGCTGGTCCTGATGGTCCTGGCGTAC 234
Trout          GCTCTGACGTGTTCCGTGGTGGCCTTCTACATCCCACTGGGGTTGATGGTGCTGGCCTAC 468
               **  * ** ** **  * *********** * **  *     * ***** ***** **

Mouse          TACCGAATCTATGTCACTGCTAAGGAGCATGCCCAGCAGATACAGATGTTACAACGGGCA 696
Human          TACCGCATCTATGTCACAGCTAAGGAGCATGCCCATCAGATCCAGATGTTACAACGGGCA 696
Tilapia        CAGAGGATCTACATTACAGCCCGGGCGCACGCCCTGCAGATCAGCATGCTGCAGCGGGCC 714
Zebrafish      CAACGCATCTACGTCACTGCACGGGAACACGCTCGCCAGATCAGCATGCTGCAGCGCGCT 294
Trout          CAGCGCATCTATGTGACTGCACGGGAGCACGCACGTCAAATCAGTATGCTGCAACGGGCG 528
                *  * *****  * ** **   **  ** ** *  ** **    *** * ** ** **

Mouse          GGAGCCACCTCTGAAAG--------------------------------CAGGCCCCAG 723
Human          GGAGCCTCCTCCGAGAG--------------------------------CAGGCCTCAG 723
Tilapia        GGAGGAGCTGGTACCAGTGCCGCCGGGACTTCTGGCGGGGTCAGCGCTGGTGCGCCGGAT 774
Zebrafish      GGAGGAGCGGG---------------------------------------GAATGCGGAC 315
Trout          GGGGCCGC------------------------------------------CTCGGCAGAC 546
               ** *   *                                             *  *
```

```
Mouse          CCAGCTGACCAGCACAGCACACATCGCATGAGGACAGAGACCAAGGCAGCCAAGACTTTA 783
Human          TCGGCAGACCAGCATAGCACTCATCGCATGAGGACAGAGACCAAAGCAGCCAAGACCCTG 783
Tilapia        TCTGCCGACCATCAGCGCAACCACCGCATGCGAACAGAAACTAAGGCGGCAAAGACGCTG 834
Zebrafish      AGCGCAGACCACCAGCGCAACCACCGGATGCGCACCGAGACCAAGGCAGCCAAGACTCTG 375
Trout          AGCGCCGACCACCAGCGCAACCACCGCATGCGTACAGAGACCAAGGCGGCCAAGACTCTG 606
                  ** ***** **  ***  ** ** *** * ** ** ** ** ** ** *****  *

Mouse          TGTGTCATCATGGGCTGCTTCTGTTTCTGCTGGGCCCCCTTCTTTGTCACCAATATTGTG 843
Human          TGCATCATCATGGGTTGCTTCTGCCTCTGCTGGGCACCATTCTTTGTCACCAATATTGTG 843
Tilapia        TGCATCATCATGGGCTGCTTTTGCCTGTGCTGGGCGCCGTTCTTCGTTACCAATGTGGTG 894
Zebrafish      TGCATCATCATGGGCTGCTTCTGCCTATGCTGGGCCCCGTTCTTCATCACAAACGTGGTG 435
Trout          TGCATCATCATGGGCTGTTTCTGCCTGTGCTGGGCCCCTTTTTTTATCACCAACGTGGTG 666
               **  ********** ** ** **  * ******** ** ** **  * ** **  * ***

Mouse          GACCCTTTCATAGACTACACTGTCCCCGAGCAGGTGTGGACTGCTTTCCTCTGGCTTGGC 903
Human          GATCCTTTCATAGACTACACTGTCCCTGGGCAGGTGTGGACTGCTTTCCTCTGGCTCGGC 903
Tilapia        GACCCCTTTATAGACTATACCGTGCCCGAACAGCTGTGGGCTGCCTGCCTGTGGCTGGGC 954
Zebrafish      GACCCCTTCATAGACTACAGTGTTCCTGAGCAGCTGTGGGCCGCCTGCCTCTGGCTGGGC 495
Trout          GACCCCTTTATAAACTACACAGTGCCGGGCCAGCTGTGGGCAGCATGCCTGTGGCTGGGC 726
               ** ** ** *** **** *  ** ** *  *** ***** * ** * *** ***** ***

Mouse          TATATCAATTCGGGGTTGAACCCTTTTCTCTATGCCTTCTTGAATAAGTCTTTCAGACGT 963
Human          TATATCAATTCCGGGTTGAACCCTTTTCTCTACGCCTTCTTGAATAAGTCTTTTAGACGT 963
Tilapia        TACATCAACTCCATGCTGAACCCCATCCTCTACGCGTTCCTCAACAAGTCCTTCCGCCGT 1014
Zebrafish      TACATCAACTCCATGCTCAACCCCATCCTCTACGCCTTCCTCAACAAGTCCTTCCGTCGT 555
Trout          TACATTAACTCCATGCTTAATCCCATTCTCTATGCCTTC-------------------- 765
               ** ** ** **   * * ** **  * ***** ** ***
```

```
Mouse          GCCTTCCTCATCATCCTCTGCTGTGATGATGAGCGCTACAAAAGACCCCCCATTCTGGGC 1023
Human          GCCTTCCTCATCATCCTCTGCTGTGATGATGAGCGCTACCGAAGACCTTCCATTCTGGGC 1023
Tilapia        GCCTTCCTCATCATCCTGTGTTGCGGGAGGCAGAGGTACCGCAGACCGTCCATCCTCGGT 1074
Zebrafish      GCCTTCCTTATCATTCTCTGCTGCGGACACAAGCGATACCGCCGACCCTCCATCCTTGGC 615
Trout          ------------------------------------------------------------ 765

Mouse          CAGACTGTCCCCTGTTCAACCACAACCATTAATGGATCCACCCATGTACTAAGGGATACA 1083
Human          CAGACTGTCCCTTGTTCAACCACAACCATTAATGGATCCACACATGTACTAAGGGATGCA 1083
Tilapia        TCTGGCGCTCCGTGCACGGCCACGCAGATCAACGGCTCCACGCACGTGCTCAAGTATTGC 1134
Zebrafish      CCTGGAACGACCTGCACGGCCACACAGATTAATGGATCTACACATGTACTGAACGGCTGC 675
Trout          ------------------------------------------------------------ 765


Mouse          GT----G-GAA---TGTGGTGGCCAGTGG----GAGAGTCGGT--GTCACCTCACAGCAA 1129
Human          GT----G-GAG---TGTGGTGGCCAGTGG----GAGAGTCAGT--GTCACCCGCCAGCAA 1129
Tilapia        GT----G-CTCCACAATGGGAACCACTCAGAGCAGGAGAAGAA--GTCCCTGCACACCCA 1187
Zebrafish      TCCTCTGCCTCCAAACTGCTCCTCTGGTTCTGCAACACTAGGCCTGTTCCTG-------- 727
Trout          ------------------------------------------------------------ 765

Mouse          CTTCTCCTTTGGTGGCTGCTCAGCCCAGTGATACTTAA    1167
Human          CTTCTCCTTTGGTGGCTGCTCAGCCCAGTGACACTTAG    1167
Tilapia        CATAGAGTCTCACGAGTCC---TGCTTGTGA-------    1215
Zebrafish      -------TC----------------TAG----------    732
Trout          ----------------------------------    765
```

5-HT$_4$ receptor amino acid alignment

```
Mouse          ----------MDKLDANVSSNEGFRSVEKVVLLTFLAVVILMAILGNLLVMVAVCRDRQ 49
Human          ----------MDKLDANVSSEEGFGSVEKVVLLTFLSTVILMAILGNLLVMVAVCWDRQ 49
Tilapia        MNVSAAGQMPAMAELG-----GESDSMPKRMALICFLSLVMLMSIFGNLLVMVAVCKDRQ 55
Zebrafish      ------------------------------------------------------------ 0
Trout          ------------------------------------------------------------ 0

Mouse          LRKIKTNYFIVSLAFADLLVSVLVMPFGAIELVQDIWAYGEMFCLVRTSLDVLLTTASIF 109
Human          LRKIKTNYFIVSLAFADLLVSVLVMPFGAIELVQDIWIYGEVFCLVRTSLDVLLTTASIF 109
Tilapia        LRKIKTNYFIVSLAFADLLVSVLVMPFGAIELVHQHWIYGETFCLVRTSLDVLLTTASIL 115
Zebrafish      ------------------------------------------------------------ 0
Trout          ------------------MVSVLVMPFGAIELIHQNWIYGETFCLVRTSLDVLLTTASIL 42

Mouse          HLCCISLDRYYAICCQPLVYRNKMTPLRIALMLGGCWVLPMFISFLPIMQGWNNIGIVDV 169
Human          HLCCISLDRYYAICCQPLVYRNKMTPLRIALMLGGCWVIPTFISFLPIMQGWNNIGIIDL 169
Tilapia        HLCCIALDRYYAICCQPLVYRNKMTPMRVALMIGGCWVIPTFISFLPIMQGWNSIGIDHL 175
Zebrafish      ----------------------MTPLRVTLMIGGCWIIPTVISFLPIMQGWNSIGIKDL 37
Trout          HLCCIALDRYYAICCQPLVYRKKMTSLRVALMLGGCWVIPTFISFLPIMQGWNSIGIDHL 102
                                     **  *  ** ****  *  *********** ***

Mouse          IEKRKFSHN-SNSTWCVFMVNKPYAITCSVVAFYIPFLLMVLAYYRIYVTAKEHAQQIQM 228
Human          IEKRKFNQN-SNSTYCVFMVNKPYAITCSVVAFYIPFLLMVLAYYRIYVTAKEHAHQIQM 228
Tilapia        INERRFNN-SSNTTSCVFMVNKPYALTCSLVAFYIPLVLMVLAYQRIYITARAHALQISM 234
Zebrafish      IDKRKISG---NSTVCVFMVNKPYALTCSVVAFYLPLVLMVLAYQRIYVTAREHARQISM 94
Trout          IEERKISGDAANATACVFMVNKPYALTCSVVAFYIPLGLMVLAYQRIYVTAREHARQISM 162
               *  *       * * ********** *** **** *  ****** *** **  ** ** *
```

```
Mouse          LQRAGATSES-----------RPQPADQHSTHRMRTETKAAKTLCVIMGCFCFCWAPFFV 277
Human          LQRAGASSES-----------RPQSADQHSTHRMRTETKAAKTLCIIMGCFCLCWAPFFV 277
Tilapia        LQRAGGAGTSAAGTSGGVSAGAPDSADHQRNHRMRTETKAAKTLCIIMGCFCLCWAPFFV 294
Zebrafish      LQRAGGAG-------------NADSADHQRNHRMRTETKAAKTLCIIMGCFCLCWAPFFI 141
Trout          LQRAGAA--------------SADSADHQRNHRMRTETKAAKTLCIIMGCFCLCWAPFFI 208
               *****                    **    ************** ****** ******

Mouse          TNIVDPFIDYTVPEQVWTAFLWLGYINSGLNPFLYAFLNKSFRRAFLIILCCDDERYKRP 337
Human          TNIVDPFIDYTVPGQVWTAFLWLGYINSGLNPFLYAFLNKSFRRAFLIILCCDDERYRRP 337
Tilapia        TNVVDPFIDYTVPEQLWAACLWLGYINSMLNPILYAFLNKSFRRAFLIILCCGRQRYRRP 354
Zebrafish      TNVVDPFIDYSVPEQLWAACLWLGYINSMLNPILYAFLNKSFRRAFLIILCCGHKRYRRP 201
Trout          TNVVDPFINYTVPGQLWAACLWLGYINSMLNPILYAF---------------------- 245
               ** ***** * ** * * * ******** *** ****

Mouse          PILGQTVPCSTTTINGSTHVLRDTVECGGQWESRCHLTATSPLVAAQPSDT    388
Human          SILGQTVPCSTTTINGSTHVLRDAVECGGQWESQCHPPATSPLVAAQPSDT    388
Tilapia        SILGSGAPCTATQINGSTHVLKYCVLHNGNHSEQE-KKSLHTHIESHESCL    404
Zebrafish      SILGPGTTCTATQINGSTHVLNGCSSASKLLLWFCNTRPVPV---------    243
Trout          ----------------------------------------------------    245
```