Control of the Endocrine Stress Response in Goldfish

(\textit{Carassius auratus})

by

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ABSTRACT

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The purpose of this study was to obtain a deeper understanding of the serotonergic control of the hypothalamic-pituitary-interrenal (HPI) stress axis and characterize a neuronal and behavioural response to a known stressor, using goldfish (*Carassius auratus*) as a model. The first part of this study investigated the serotonergic control of the HPI stress axis. *In vivo* injections of the serotonin (5-HT$_{1A/7}$) receptor agonist 8-OH-DPAT resulted in significant and sustained increases in plasma cortisol without any changes in the mRNA abundance of brain corticotropin-releasing factor (CRF) and pituitary pro-opiomelanocortin or plasma adrenocorticotropic hormone levels. *In vitro* superfusion experiments of head kidney tissue showed that 5-HT can directly stimulate cortisol synthesis from the interrenals via several 5-HT receptor subtypes. Finally, double-labeling with anti-tyrosine hydroxylase and anti-5-HT revealed that the chromaffin cells of the head kidney contain 5-HT. The second part of the study investigated the goldfish response to a known stressor, the alarm pheromone. Alarm pheromone exposure resulted in an inhibition of movement and exploration and in suppression of neuronal activity in the *pars parvocellularis* of the nucleus preopticus, a brain region with extensive CRF immunolabelling. Overall, the results from this thesis suggest that in goldfish 5-HT may be involved in the paracrine regulation of cortisol secretion and that the CRF neurons of the *pars parvocellularis* play a role in mediating the stress response to alarm pheromones.
STATEMENT OF AUTHORSHIP: All of the work presented in this thesis was carried out by the author with the exception of the data presented in Figures 2 and 6 of Chapter 2. The data presented in Figures 2 and 6 is to be credited to Dr. Nick Bernier and Dr. Cosima Porteus, respectively.
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Chapter 1

General Introduction

Organisms maintain an immensely complex dynamic and harmonious equilibrium, or homeostasis, that is constantly being challenged by extrinsic or intrinsic disturbances or stressors (Chrousos and Gold 1992). The stressors produce effects that disturb homeostasis, and organisms employ behavioural and physiological responses that enable the maintenance of homeostasis (Wendelaar Bonga 1997). Maintenance of homeostasis is necessary for an organism as constant chronic stress may lead to the stress response to lose its adaptive value and become dysfunctional, which may result in inhibition of growth, reproductive failure, and reduced resistance to pathogens (Wendelaar Bonga 1997). The hypothalamic-pituitary-adrenal (HPA) axis, and the teleost homologue, the hypothalamic-pituitary-interrenal (HPI) axis regulates the endocrine stress response and is highly conserved across vertebrate species (Alsop and Vijayan 2008). While we do have an understanding on the stress response in fish (Bernier et al. 2009), there is still much that we do not know. The purpose of my thesis was to expand on our knowledge of the control of the HPI axis. My objectives were to examine the control of the HPI axis by the neurotransmitter serotonin or 5-hydroxytryptamine (5-HT) and the alarm pheromone (Fig.1). This chapter provides insight into the control of the endocrine stress response through the serotonergic system and the alarm pheromone.

The Endocrine Stress Response

During stressful situations, animals shift metabolic energy away from growth and reproduction towards activities that help restore homeostasis (Wendelaar Bonga 1997). One way the brain coordinates the stress response is through the activation of the
hypothalamic-pituitary-interrenal axis (HPI) (Chrousos and Gold 1992; Wendelaar Bonga 1997). The end product of the HPI axis in teleost is cortisol and it is widely used as an indicator of stress (Wendelaar Bonga 1997). Adrenocorticotrophic hormone (ACTH) has been shown to be a potent stimulator of interrenal cortisol synthesis, with the pituitary gland dominating the endocrine control of cortisol secretion (Sumpter et al. 1986; Schreck et al. 1989; Young 1993). However, even though ACTH has been considered the primary secretagogue of cortisol, others have been identified (Schreck et al. 1989; Arnold-Reed and Balment 1994). A major regulator of ACTH secretion in teleost fishes is hypothalamic corticotropin-releasing factor (CRF) (Fryer and Peter 1977; Sullivan Hanley and Van de Kar 2003; Flik et al. 2006). The brain serotonergic system, in turn, plays a key role in regulating the HPA/HPI stress axis by regulating CRF output. However, there is evidence of 5-HT stimulating targets other than the hypothalamus. The actions of 5-HT on these alternate sites within the stress axis are poorly understood (Dinan 1996; Winberg et al. 1997; Contesse et al. 1998).

**Serotonin and the Stress Response**

5-HT is a neurotransmitter that is widely synthesized in the central nervous system. It has been suggested to have an early evolutionary significance as neurons containing 5-HT have been found in multiple metazoan clades and is responsible for numerous behavioural and physiological functions (Lillesaar 2011). One notable physiological function is the stress response, as the serotonergic system is thought to have a close relationship with the HPA axis in mammals (Dinan 1996; Sullivan Hanley and Van de Kar 2003). It has been shown that 5-HT can directly stimulate the HPA axis at the hypothalamic, pituitary and adrenal gland levels (Dinan 1996). There is also
immunohistological evidence of 5-HT being stored in secretory vesicles within the chromaffin cells of rats (Brownfield et al. 1985).

Serotonin acts on multiple receptor subtypes that vary in localisation and function (Jørgensen 2007). In rats, the 5-HT receptor subtypes 5-HT$_{1A}$ and 5-HT$_{2A}$ are found to be the main receptors in regulating the HPA axis (Sullivan Hanley and Van de Kar 2003). In amphibians, *in vitro* studies have shown that 5-HT can stimulate corticosteroid secretion directly at the level of the adrenal gland through the 5-HT$_4$ receptor (Indres et al. 1991).

In fish, it has been suggested that there is a relationship between the serotonergic system and the HPI stress axis (Winberg and Nilsson 1993). Multiple high affinity binding sites for 5-HT, in particular one that is pharmacologically similar to the mammalian 5-HT$_{1A}$ receptor have been found in the brain of the arctic charr (Winberg and Nilsson 1996). Elevation of plasma cortisol in catheterized rainbow trout injected with 5-HT$_{1A}$ receptor agonist, 8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT) suggested an activation of the HPI axis via the 5-HT$_{1A}$ receptor (Winberg et al. 1997). However, intraperitoneal (i.p.) injections and permanent i.p. implanted catheters delivering 8-OH-DPAT to juvenile arctic charr provided equivocal results (Höglund et al. 2002).

**Alarm Pheromones and the Stress Response**

Animals respond to perceived threats by employing adaptive behavioural and physiological changes (Brönmark and Lwin 1997; Lowry and Moore 2006; Fraker et al. 2009). One major threat that animals in the wild face is the threat of predation. To minimize the risk of predation, animals exhibit anti-predatory responses when exposed to chemical cues of predation (Apfelbach et al. 2005; Wyatt 2005). Predatory chemical cues
can be released by vertebrate prey through injury or predation (Chivers and Smith 1998; Døving and Lastein 2009). An example of anti-predatory behaviour exhibited by fishes when exposed to predatory chemical cues is the alarm response (Höglund et al. 2005; Døving and Lastein 2009). The alarm response is a typical avoidance behaviour exhibited when exposed to olfactory cues from injured skin of conspecifics (Pfeiffer 1962; Höglund et al. 2005; Døving and Lastein 2009). Goldfish (*Carassius auratus*) exposed to conspecific scale extract displays agitated, non-directional, jerky movements with fins fully erected (Kimbrell et al. 1970). Other cyprinids, such as the crucian carp (*Carassius carassius*), have been found to swim against the bottom of their tanks – a behaviour that is beneficial in their natural habitat as it disturbs the mud and debris on the bottom (Pfeiffer 1962). The alarm response is believed to be due to the alarm pheromones found in the club cells in the epidermis and released by epidermal damage (Kimbrell et al. 1970; Pfeiffer 1977). For this thesis, pheromones will be defined as a mixture of odourous substances, released by an individual (sender) and evoking in conspecifics (the receiver) adaptive, specific, and species-specific response(s), the expression which need not require prior experience or learning (Sorensen and Stacey 2004).

The alarm response is developed early in many cyprinids – with sensitivity comparable to adults at 3-4 months of development (Kasumyan 2004). The ability of larvae to detect predators also appears at about the same developmental period as they become sensitive to alarm pheromones (Paschenko and Kasumyan 1986; Døving and Lastein 2009). Studies have shown that fish display phenotypic changes that make them less vulnerable to predation when exposed to predators that have recently eaten a conspecific. These findings suggest that the determining factor for the phenotypical
Detection of alarm pheromones occurs via olfaction (Døving and Lastein 2009). Electrophysiological studies have shown that the medial part of the medial olfactory tracts (mMOTs) with somas in the dorsomedial part of the olfactory bulb (OB) respond specifically to skin extracts, suggesting their role as alarm neurons (Hamdani and Døving 2002; Døving and Lastein 2009). The mMOTs have also been implicated in defensive behaviour patterns, such as the alarm response (Døving and Selset 1980, Hamdani et al. 2000). While there is strong evidence for a specific alarm pathway in the olfactory organ of fishes, the central nuclei and the neuronal pathways that are implicated in the alarm response are not yet known (Hamdani and Døving 2002; Døving and Lastein 2009).

Exposure to alarm pheromones causes rapid changes in behaviour, but not much is known about the neural and physiological mechanisms underlying the behavioural response. In a number of vertebrate species, corticotropin-releasing factor (CRF) has been found to modulate diverse behavioural responses (Lowry and Moore 2006). CRF also plays a key role in regulating the secretory activity of the hypothalamic-pituitary-interrenal (HPI) stress axis in teleost fish (Baker et al. 1996; Winberg et al. 1997). In mammals, exposure to predator odors results in an increase in plasma corticosteroid levels suggesting an activation of the hypothalamic-pituitary-adrenal (HPA) stress axis (Sullivan and Gratton 1998; Apfelbach et al. 2005; Masini et al. 2005).

**Thesis Objective and Hypothesis**

The purpose of this study was to gain a deeper understanding of the control of the HPI axis in teleost fish, using goldfish (*Carassius auratus*) as a model. I explored the
control of the HPI axis through two different avenues: control through the traditional neurotransmitter, 5-HT and through conspecific exposure to alarm pheromone, a known stressor. I hypothesized that the stimulatory role of 5-HT in stress axis regulation is similar to goldfish as it is to mammals as it conserved throughout vertebrates, and predicted that injections of the serotonin receptor agonist 8-OH-DPAT will stimulate the HPI axis, eliciting an increase in plasma cortisol and increases in the gene expression of key effectors of the HPI axis. Also, in accordance with other vertebrates (Dinan 1996), I predicted that 5-HT would stimulate the HPI axis directly at the level of the interrenal cells. I also hypothesized that exposure to alarm pheromones is a stressor in goldfish; therefore, neural pathways should be activated in response to alarm pheromones. I predicted that differences in the immunohistological staining of c-Fos, an indirect marker of neuronal activity, would be apparent between control and treated fish, particularly in the hypothalamic preoptic area (POA). As CRF has also been implicated in modulating behaviour (Lowry and Moore 2006), I also predicted apparent differences in CRF staining between control and treated fish.

Towards this goal, I determined the effects of an intra-peritoneal injection of the 5-HT$_{1A/7}$ receptor agonist 8-OH-DPAT at all levels of the HPI axis. I further explored the direct control of cortisol secretion at the level of the interrenals through the use of an in vitro superfusion system. To determine whether the nucleus preopticus (NPO) and the telencephalic area ventralis lateralis (VI) are activated in response to alarm pheromone exposure, I used c-Fos immunohistochemistry as proxy of neuronal activation. The NPO contains key nuclei in the hypophysiotropic regulation of the HPI axis (Bernier et al. 2009) and the VI is a region potentially implicated in avoidance behavioural response
(Portavella et al. 2004; Portavella and Vargas 2005). I also used CRF immunohistochemistry to identify the phenotype of the neurons in the NPO. Finally, I characterized the alarm response behaviour to be able to implicate behavioural parameters with specific regions of the brain.
**Figure 1.** Schematic diagram of the tissues and primary signals of the hypothalamic-pituitary-interrenal stress axis in fish. Upon encountering a stressor, NPO CRF containing neurons release their product into the pituitary. CRF stimulates the expression of POMC and induces the cleavage of POMC into ACTH from the corticotropes in the pituitary. ACTH in turn stimulates the biosynthesis and secretion of cortisol from the interrenal cells. However in fish, the sites of serotonergic action on other components of the HPI axis are not fully understood. Alternatively, specific stressors, such as detection of alarm pheromones through the olfactory bulbs are known to activate the HPI axis, but still needs to be fully elucidated. Abbreviations: Serotonin (5-HT), corticotropin-releasing factor (CRF), pro-opiomelanocortin (POMC), adrenocorticotropic hormone (ACTH).
Figure 1.
Chapter 2

Serotonin directly stimulates cortisol secretion from the interrenals in goldfish

Introduction

The serotoninergic system plays a pivotal role in regulating the activity of the hypothalamic-pituitary-interrenal (HPA) stress axis (Chaouloff, 1993; Dinan, 1996; Jørgensen, 2007). Serotonin or 5-hydroxytryptamine (5-HT)-immunoreactive (ir) fibers make direct and indirect synaptic connections with the corticotropic-releasing factor (CRF) neurons of the hypothalamic paraventricular nucleus (PVN) in mammals (Herman et al., 2003; Liposits et al., 1987) and stimulate CRF gene expression through several 5-HT receptors including the 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A/2C} receptor subtypes (Heisler et al., 2007; Jørgensen et al. 2002). 5-HT also stimulates the secretion of the pro-opiomelanocortin (POMC)-derived peptide adrenocorticotropic hormone (ACTH) from the anterior pituitary gland but this effect is predominantly indirect and mediated via the activation of PVN CRF neurons (Jørgensen et al., 2002). At the level of the adrenal gland, locally produced 5-HT can directly stimulate corticosteroid secretion from the adrenocortical cells (Contesse et al., 2000). Notably, specific stressors can increase serotonergic activity in the CNS (Linthorst and Reul, 2008) and pre-treatment with 5-HT receptor antagonists reduces stress-induced ACTH release (Jørgensen et al., 1998; Osei-Owusu et al., 2005). Despite the importance of 5-HT in the regulation of the HPA axis under normal physiological conditions and pathological states (Sullivan Hanley and Van de Kar, 2003), the mechanisms underlying 5-HT-mediated activation of the HPA axis in other vertebrates, including fish, are poorly understood.
In general, the anatomical organization of the serotonergic system in the brain of teleosts is highly similar to that of other vertebrates (Lillesaar, 2011). As in mammals, serotonergic neurons from the raphe nuclei in the hindbrain innervate the hypothalamic preoptic area (POA) in several fish species (Ekstrom and van Veen, 1984; Kah and Chambolle, 1983; Lillesaar et al. 2009), a region homologous to the PVN. Moreover, 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor subtypes are expressed in the POA of zebrafish (Danio rerio; Norton et al., 2008). Since the POA is the primary site of CRF expression in teleosts (Alderman and Bernier, 2007; Bernier et al., 2009), the occurrence of 5-HT-containing nerve terminals and 5-HT receptors in this brain region supports an involvement of 5-HT in the regulation of CRF release. Similarly, in rainbow trout (Oncorhynchus mykiss), the close correlation between hypothalamic serotonergic activity and plasma cortisol levels in socially subordinate (Winberg and Lepage, 1998) or hyperammonemic (Ortega et al., 2005) fish suggest an important role for 5-HT in the regulation of the hypothalamic-pituitary-interrenal (HPI) axis, the homolog to the HPA axis.

Studies involving the administration of 5-HT receptor agonists also provide evidence that 5-HT can affect the activity of the HPI axis in teleosts. For example, intra-arterial injection of rainbow trout with the 5-HT$_{1A/7}$ receptor agonist 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) elicited a dose-dependent increase in plasma cortisol (Winberg et al., 1997). Similarly, 8-OH-DPAT given to non-stressed Arctic char (Salvelinus alpinus) induced an increase in plasma cortisol but had no effect on plasma ACTH levels (Höglund et al., 2002). In contrast, 8-OH-DPAT given to stressed Arctic char dampened the rise in plasma cortisol induced by handling and injection (Höglund et al., 2002). In experiments performed with cannulated Gulf toadfish (Opsanus beta) that
have high circulating cortisol levels, intra-arterial injections of physiological doses of either 8-OH-DPAT or α-methyl-5-HT (a 5-HT₂ receptor agonist) have no effect on plasma cortisol (McDonald and Walsh, 2004). However, pharmacological doses of 8-OH-DPAT caused an increase in circulating cortisol levels (Meideros et al., 2010). Moreover, while 5-HT, α-methyl-5-HT and RS67506 (a 5-HT4 receptor agonist) can directly stimulate cortisol secretion from toadfish kidney pieces in vitro, 8-OH-DPAT does not (Medeiros and McDonald, 2012). Therefore, while 5-HT can stimulate the HPI axis in teleosts, these effects appear to depend on the state of the fish and many questions remain with regards to the receptors involved and the specific site(s) of 5-HT action.

In this study, to identify the potential site(s) of 5-HT action within the HPI axis and to determine whether the 5-HT₁₅ receptor may be involved, we quantified the expression of the 5-HT₁₅ receptor in the POA, pituitary and head kidney, and determined the effects of 8-OH-DPAT injections on plasma cortisol and ACTH and on the expression of POA CRF and pituitary POMC in goldfish (Carassius auratus). Since the results from our in vivo experiments suggested that 8-OH-DPAT may have a direct stimulatory effect on cortisol secretion, we also investigated whether 5-HT and various 5-HT receptor agonists can stimulate the secretion of cortisol from the interrenals using in vitro superfusions. Lastly, we used immunohistochemistry to localize 5-HT in the head kidney of goldfish.

Materials and methods

Animals

Mixed sex goldfish (Carassius auratus) of the common variety were obtained from Straits Aqualife (Toronto, ON, Canada) and transported to the Hagen Aqualab at the University of Guelph (Guelph, ON, Canada). Fish were acclimated for at least 4 weeks
in a common holding tank (800 l) that was supplied with well water (pH 7.9; water hardness 411 mg/l as CaCO$_3$; Ca$^{2+}$, 10.5 mmol/l; Cl$^-$, 1.5 mmol/l; Mg$^{2+}$, 3.0 mmol/l; K$^+$, 0.06 mmol/l; Na$^+$ 1.1 mmol/l), maintained at 20°C and exposed to a 12h:12h light:dark photoperiod regime. Fish were fed daily with commercially prepared fish food (3PT Classic Sinking, Martin Mills, Elmira, Canada). All procedures were approved by the University of Guelph Animal Care Committee and conform to the principles of the Canadian Council for Animal Care.

**Experimental protocol**

**Experiment 1: Effects of 8-OH-DPAT on the HPI axis in vivo**

Three weeks prior to the start of experimentation, fish (73.0 ± 2.3g, mean ± SEM; n=80) were removed from a common holding tank and placed in 16 separate 30 l aquaria. Fish of different sizes were distributed evenly so that each aquarium had a similar total mass of fish (362.2g ± 5.7g , mean ± SEM; n = 16 aquarium). Water temperature was maintained at 17.6°C throughout the experimental period and the water inflow for each tank was kept at 720 ml/min and dissolved oxygen maintained at ≥6.6 mg/L.

The experiment consisted of four experimental treatments: a) an undisturbed control group, b) fish injected with saline (0.9% NaCl), c) fish injected with the 5-HT$_{1A/7}$ receptor agonist 8-OH-DPAT (Sigma-Aldrich, St. Louis, MI, USA) at a dose of 100 µg/kg body weight (BW), and d) fish injected with 8-OH-DPAT at a dose of 400 µg/kg BW. Fish were anaesthetized with buffered (NaHCO$_3$; 1.0 g/l) tricaine methanesulfanate (0.5 g/l; MS-222; Syndel, Vancouver, BC, Canada), weighed and given an intraperitoneal (ip) injection of saline or 8-OH-DPAT. Each treatment was replicated in duplicate tanks and the experiment lasted either 1.5 h or 8 h. The fish were terminally anaesthetized (5
ml/l 2-phenoxyethanol; Sigma-Aldrich) within a minute of tank removal. Blood was collected from the caudal vein using EDTA-treated syringes and centrifuged (15000g x 5 min) to collect plasma for quantification of plasma cortisol and ACTH. The brain was regionally dissected as per Bernier et al. (2008) to recover the preoptic area (POA) and pituitary and quantify the mRNA levels of POA CRF and pituitary POMC.

**Experiment 2: Gene expression of the 5-HT1A receptor subtype within the HPI axis**

To determine the relative mRNA levels of the 5-HT1A receptor subtype within the HPI axis of control goldfish, fish (52.1 ± 4.1 g, mean ± SEM; n=5) were randomly sampled from a common holding tank and terminally anesthetized as in Experiment 1 above. The POA of the brain, the pituitary and head kidney tissue adjacent to the midline were immediately dissected, flash frozen in liquid nitrogen and stored at -80°C until RNA extraction.

**Experiment 3: Effects of 5-HT receptor agonists on cortisol secretion in vitro**

To determine whether 5-HT can directly stimulate cortisol secretion in goldfish and to assess the potential 5-HT receptor subtypes that may be involved in mediating this response, a series of in vitro superfusions was performed. Fish (67.3 ± 3.2g, mean ± SEM; n=68) were randomly sampled from a common holding tank and terminally anesthetized as in Experiment 1 above. To assess cortisol release in vitro, head kidney tissue immediately adjacent to the midline (100-150 mg) was carefully dissected and immediately placed in 5 ml of 0.015 M HEPES/Tris-buffered superfusion medium (128 mM NaCl, 2 mM KCl, 2 mM CaCl2•2H2O, 0.25% w/v glucose, 0.03% w/v bovine serum albumin, 0.1 mM ascorbic acid, pH = 7.4) for 30 min to flush residual endogenous
cortisol. Individual samples were then finely diced, placed on a fine-meshed filter in a chamber and superfused at room temperature (20°C) using carbogen (95% O₂, 5% CO₂)-saturated medium at a flow-through rate of 30 µl/min via a multichannel peristaltic pump (Miniplus 3; Gilson, Middleton WI, USA). Following a 240 min equilibration period (t=0 min), the head kidney preparations were superfused for 20 min with medium containing one of eight treatments: 1) 10⁻⁷ M serotonin hydrochloride (5-HT; Sigma-Aldrich) (n=10), 2) 10⁻⁷ M human ACTH₁₋₃₉ (hACTH, American Peptide, Sunnyvale, CA, USA) (n=10), 3) 10⁻⁷ M of both 5-HT and hACTH (n=7), 4) 10⁻⁷ M 8-OH-DPAT (a 5-HT₁A/7 receptor agonist; Sigma-Aldrich) (n=10), 5) 10⁻⁷ M 8-OH-DPAT and 2x10⁻⁶ M (S)-WAY-100135 dihydrochloride (WAY-100135, a 5-HT₁A receptor antagonist; Tocris Bioscience, Park Ellisville, MO, USA) (n=6), 6) 10⁻⁷ M 8-OH-DPAT and 2x10⁻⁶ M SB-258719 hydrochloride (SB-258719, a 5-HT₇ receptor antagonist; Tocris Bioscience) (n=5), 7) 10⁻⁷ M cisapride monohydrate (cisapride, a 5-HT₄ receptor agonist; Sigma-Aldrich) (n=10), and 8) 10⁻⁷ M cisapride and 2x10⁻⁶ M GR-125487 sulfamate (GR-125487, a 5-HT₄ receptor antagonist; Tocris Bioscience) (n=8). In all superfusions, with the exception of those in the hACTH treatment, a 20 min hACTH pulse was applied at t=160 min to assess the responsiveness of the interrenal tissue post 5-HT agonist treatment. Fractions were collected at 10 min intervals after the addition of each secretagogue for a 60 min period and at 20 min intervals at any other time point. All fractions were analyzed for cortisol levels. Within a treatment, basal un-stimulated cortisol release at t=0 min was normalized to a value of 1 and stimulation was expressed as a fold change from basal release. Also calculated from each perifusion experiment in the 5-HT, ACTH and 5-HT/AICTH treatments was the maximal fold change in cortisol.
release rate, the total amount of cortisol released over the duration of the superfusion
experiment and the time to peak cortisol release rate. The area under the curve was used
to calculate the total amount of cortisol released and was determined using ImageJ
(version 1.46; National Institute of Mental Health, Bethesda, MA, USA).

**Plasma analysis**

Plasma and superfusion medium cortisol concentration were measured in triplicate
using a radioimmunoassay (RIA) as per Bernier et al. (2008). Briefly, 200 µl of standard,
diluted plasma or superfusion medium was combined with 200 µl ³H-cortisol (5500-6000
cpm/tube, 70-100 Ci/mmol, PerkinElmer, Boston, MA, USA) and 200 µl of diluted rabbit
anti-cortisol antibody (R4866, Clinical Endocrinology Laboratory, University of
California, Davis, CA, USA). Samples were incubated at 4°C for 16 h then chilled on ice
for 10 min prior to the addition of 200 µl of dextran-coated charcoal suspension in
phosgel buffer (5.75 g dibasic sodium phosphate, 1.28 g monobasic sodium phosphate, 1
g gelatin and 0.1 g thimerosal per litre of dH₂O, pH = 7.6). Samples were then vortexed
and centrifuged at 3000 rpm at 4°C for 12 min. The resultant supernatant was decanted
directly into scintillation vials containing 5 ml of scintillation fluid (667 ml toluene, 333
ml Triton X-100, 4 g of 2,5-diphenyloxazole (PPO) and 0.2 g of 1,4-bis[5-phenyl-3-
oxazolyl]-benzene; 2,2’-p-phenylene-bis[5-phenyloxazole] (POPOP)) and counted in a
multipurpose scintillation counter (LS 6500, Beckman, Fullerton, CA, USA). The
antibody concentration used was adjusted so that 40% of the radiolabeled cortisol was
bound in the absence of a competitor. Plasma cortisol concentrations were determined
using a three-parameter sigmoidal curve regression equation (SigmaPlot 10.0, SPSS,
Chicago, IL, USA) obtained from the standard curve. Cortisol measurement in plasma
and medium were diluted to fall within the 20-80% range of the standard curve. A serial dilution of goldfish plasma gave a displacement curve that was parallel to the standard curve and the lower detection limit of the assay was 16 pg/ml. All plasma cortisol samples were measured in a single assay with an intra-assay variability of 4.4% (n=3), while inter- and intra-assay variation of superfusion medium cortisol concentrations were 5.3% (n=8) and 2.8% (n=6), respectively.

Plasma ACTH levels were measured in duplicate using a radioimmunoassay. The reagents were provided and the procedure followed as outlined in the ImmuChem Double Antibody hACTH kit (cat. # 07-106101; MP Biomedicals, Orangeburg, NY, USA). All samples were analyzed in one assay that had a lower detection limit of 10 pg/mL and an intra-assay variation of 5.1% (n=4). Furthermore, a serial dilution of goldfish plasma gave a displacement curve parallel to the ACTH standard curve.

**Quantification of gene expression**

Total RNA from the POA, pituitary and head kidney were extracted using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Life Technologies Inc., Burlington, ON, Canada). One microgram of total RNA was treated with DNase I (DNase I amplification grade, Invitrogen) and reverse transcribed to cDNA using SuperScript II Rnase H− reverse transcriptase (Invitrogen). The cDNA product was amplified using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl SYBR Green PCR Master Mix (Applied Biosystems), 5 µl cDNA template and 2.5 µl each of forward and reverse primers (0.4 µM). Default cycling conditions were used: 10 min at 95°C followed by 40 cycles of 15s at 95°C and 1 min at 60°C. This protocol was followed by a melting curve
analysis to verify the specificity of the PCR products. To account for differences in amplification efficiency, a standard curve was constructed for each target using serial dilutions of cDNA. Input values were obtained by fitting the average threshold cycle value to the antilog of the standard curve. To correct for minor variations in template input and transcriptional efficiency, each sample was normalized to the expression level of the housekeeping gene elongation factor 1-α (EF1α). All samples were assayed in triplicate and only one target was assayed per well. Finally, non-reverse transcribed RNA and water controls were run to ensure that no genomic DNA was amplified and that the reagents were not contaminated. The primers used were as follows: CRF forward 5’-CTG GCA AAG TTC AAA AAC CA-3’ and reverse 5’-AAG TGG CGA GGA GAA TCT GT-3’ (GenBank accession no. AF098629), POMC forward 5’-AGC GCA GGC CTA TCA AGG T-3’ and reverse 5’-GAG AGT CTC GGC GGA TTC GCT G-3’ (GenBank accession no. AJ431209), 5-HT₁A receptor forward 5’-GGC GAG GTT TCG CAT TAG G-3’ and reverse 5’-TTG GCC ATT TTA GCT TTC -3’ (GenBank accession no. EF493019) and EF1α forward 5’-GGC TGC CAA GAC CAA ATG A-3’ and reverse 5’-GGG AAG AGC ATA CCA CAA CCA CCT T-3’ (GenBank accession no. AB056104). Primers were designed using Primer Express (Applied Biosystems).

**Immunohistochemistry**

To determine whether the chromaffin cells of the head kidney were a source of 5-HT, immunohistochemistry was performed on sections of goldfish head kidney tissue. Fish (n=5, 1.5-2.5 g) of either sex were terminally anaesthetized and fixed by immersion in 4% paraformaldehyde in PBS (containing in mM: NaCl, 137; Na₂HPO₄, 15.2; KCl, 2.7; KH₂PO₄, 1.5; buffered to pH 7.8 with 1M NaOH) at 4°C overnight (Jonz and Nurse,
Tissues were then rinsed in PBS, cryoprotected in a 30% sucrose solution, and frozen in Tissue-Tek® (Sakura Finetek, Fisher Scientific) at -50°C and stored at -80°C. Blocks were sectioned cross sectional in the area of the head kidney at 12 μm using a cryostat (Leica CM3050 S, Leica Microsystems, Germany) and mounted on Superfrost® plus slides (VWR International, Mississauga, ON, Canada).

Slides containing frozen tissue were washed in PBS and blocked in 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for one hour. Primary antibodies (tyrosine hydroxylase raised in mouse, 1:500 dilution, Immunostar; serotonin raised in rabbit, 1:400, Sigma-Aldrich) were diluted in a permeabilizing solution (PBS/0.1% Triton X-100/ 3% normal goat serum) and set on the slides to incubate overnight at room temperature. Following incubation with primary antibodies, the slides were again washed in PBS. The slides were then incubated with fluorescently labeled secondary antibodies (Alexa 488 goat anti-rabbit, 1:500 dilution, Molecular Probes, Invitrogen; Alexa 594 goat anti-mouse, Molecular Probes, Invitrogen) diluted in PBS containing 0.1% Triton-X and 3% normal goat serum and left to incubate at room temperature for 2 hours in darkness. Following a final wash with PBS, slides were mounted with coverslips and Vectashield with DAPI (Vector Laboratories, Burlington, Ontario) to prevent photobleaching and to label cell nuclei. Slide boxes were stored at 4°C until viewed under a fluorescent light microscope. Control experiments were performed in which the primary antibodies were excluded to control for effects of the secondary antibody. Normal serum (IgG) from primary antibody host species was also used (at an equivalent protein concentration) to test for adverse staining due to reaction with the primary hosts.
Immunolabelled tissue sections were viewed and captured using a confocal scanning system (Olympus Fluoview FV10i, Tokyo, Japan) equipped with solid state lasers emitting at 405 nm, 473 nm and 559 nm. Z-stacks of 6-13 optical sections and 0.68-0.82 µm apart were captured using the 60x objective.

Statistical Analysis

Results are presented as mean ± SEM. Differences among values in Experiment 1 were assessed by a two-way ANOVA followed by a Holm-Sidak post-hoc test for multiple comparisons to determine differences between treatments groups and between sampling times. For the superfusion data in Experiment 3, a repeated measures one-way ANOVA followed by Dunnett’s multiple comparison post-hoc test was used to determine differences in cortisol release rate from the time 0 value within a given treatment. Differences between treatments in maximal cortisol release rate, total cortisol release and response time to maximal cortisol release rate were determined by one-way ANOVAs and by pairwise Student-Newman-Keuls multiple comparison tests. Non-parametric data was log-transformed prior to analysis. All analyses were performed using SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA) and P < 0.05 was considered statistically significant for all tests.

Results

Experiment 1: Effects of 8-OH-DPAT on the HPI axis in vivo

Relative to undisturbed controls, injection of saline elicited a seven-fold increase in plasma cortisol 1.5 h post-injection (Fig. 1). Similarly, the 100 µg/kg 8-OH-DPAT treatment was characterized by a five-fold increase in plasma cortisol 1.5 h post-injection. By 8 h post-injection, plasma cortisol levels in the saline and 100 µg/kg 8-OH-DPAT
treatments had returned to basal levels and did not differ from those in the undisturbed control group. In contrast, the 400 μg/kg 8-OH-DPAT treatment elicited a sustained and significantly larger increase in plasma cortisol.

Despite their effects on plasma cortisol levels, neither the saline nor the two 8-OH-DPAT treatments had an effect at any sampling time on the gene expression of POA CRF and pituitary POMC or on the circulating levels of plasma ACTH (Table 1).

Experiment 2: Gene expression of the 5-HT<sub>1A</sub> receptor subtype within the HPI axis

While the 5-HT<sub>1A</sub> receptor subtype is expressed at all levels of the HPI axis in goldfish, the POA of the brain and the head kidney have 5-HT<sub>1A</sub> receptor mRNA levels that are 16- and 12-fold higher than in the pituitary, respectively (Fig. 2).

Experiment 3: Effects of 5-HT receptor agonists on cortisol secretion in vitro

In vitro, 5-HT increased cortisol release rate from the head kidney for 60 min post-addition before returning to basal levels (Fig. 3A). Comparatively, hACTH increased cortisol release rate for 140 min post-addition (Fig. 3B) and the combined 5-HT/hACTH treatment increased cortisol release rate for 100 min (Fig. 3C). Overall, the hACTH and combined 5-HT/hACTH treatments were characterized by higher maximal increases in cortisol release rate (Fig. 3D) and total cortisol release (Fig. 3E) than in the 5-HT treatment. However, the response time to peak cortisol release was shorter in the combined 5-HT/hACTH treatment than in either the 5-HT or hACTH treatments (Fig. 3F).

The 5-HT<sub>1A/7</sub> receptor subtype agonist, 8-OH-DPAT, elicited a complex dual peak cortisol release profile from the head kidney tissue. Following exposure to 8-OH-DPAT, cortisol release rate increased for 30 min, transiently returned to basal levels and
increased once more for 20 min (Fig. 4A). The selective 5-HT_{1A} receptor antagonist, WAY-100135, inhibited and nearly abolished the initial and delayed stimulatory effects of 8-OH-DPAT on cortisol secretion, respectively (Fig. 4B). In contrast, while the selective 5-HT_{7} receptor antagonist, SB-258719, inhibited the initial stimulatory effects of 8-OH-DPAT, it had no effect on the delayed increase in cortisol release (Fig. 4C).

The 5-HT_{4} receptor subtype agonist, cisapride, increased cortisol release rate from the head kidney for 50 min post-addition before returning to basal levels (Fig. 5A). The selective 5-HT_{4} receptor antagonist, GR-125487, completely abolished the stimulatory effects of cisapride on cortisol secretion (Fig 5B).

In all superfusions, independent of the initial response to the 5-HT agonist/antagonist treatment, the head kidney predictably responded with an increase in cortisol secretion rate to the stimulation with hACTH confirming the responsiveness of the tissues (data not shown).

**Immunohistochemistry**

Immunohistochemistry with antisera raised against TH, an enzyme of the catecholamine-synthesizing Blaschko pathway, identified the presence of chromaffin cells in the immediate vicinity of the walls of the posterior cardinal vein (Fig 6). Double-labelling these sections with antisera raised against 5-HT revealed that a large number of the TH-positive chromaffin cells contained 5-HT. Control tissue section treated with the standard histochemical techniques except for the omission of the primary antibodies showed only negligible staining with no specific immunolabelling. Similarly, the use of normal serum from primary antibody host species did not produce any specific labeling (data not shown).
Discussion

Our investigation of the potential site(s) of 5-HT action within the HPI axis of goldfish has provided novel in vivo and in vitro evidence that 5-HT agonists can stimulate cortisol secretion in this species. Using in vitro head kidney superfusions, we demonstrated that 5-HT can directly stimulate interrenal steroidogenesis and provided pharmacological evidence suggesting that the stimulatory effects of 5-HT on cortisol secretion is mediated through 5-HT$_{1A}$ and 5-HT$_{4}$ receptor subtypes. In addition, immunohistochemistry showed that the chromaffin cells in goldfish contain 5-HT. Together these results suggest that 5-HT localized within the chromaffin cells of the head kidney in goldfish can stimulate the secretory activity of interrenal cells via paracrine actions.

Effects of 8-OH-DPAT on the HPI axis in vivo

The stimulatory effects of i.p. injections of 8-OH-DPAT on plasma cortisol in goldfish further support a role for the serotonergic system in the regulation of the HPI axis (Winberg and Nilsson, 1993) and are consistent with the well documented ability of 5-HT$_{1A}$ receptor agonists to stimulate the HPA axis (Sullivan Hanley and Van de Kar, 2003). However, although the binding properties of the 5-HT$_{1A}$ receptor appear to be highly conserved (Meideros et al., 2010; Winberg and Nilsson, 1996), the 8-OH-DPAT dose needed to stimulate the HPI axis varies considerably among studies. While the very steep binding curve of the 5-HT$_{1A}$ receptor for 8-OH-DPAT (Medeiros et al. 2010) may explain the marked difference in potency between the 100 and 400 ug/kg treatments in this study, it is also known that the ability of 8-OH-DPAT to increase plasma cortisol levels depends on the stress status of the fish (Hoglund et al. 2002). For example,
whereas ip injections of 8-OH-DPAT (62.5-500 ug/kg) in Arctic char without anesthesia inhibited a handling-induced increase in plasma cortisol (Hoglund et al. 2002), ip injections of 8-OH-DPAT (400 ug/kg) in this study with anesthesia elicited increases in plasma cortisol that were well above those induced by the injection procedure. Similarly, in catheterized fish, 8-OH-DPAT at a dose of 40 ug/kg elicited a ~12-fold increase in plasma cortisol in non-stressed rainbow trout (Winberg et al., 1997), but a dose of 16250 ug/kg was needed to increase cortisol levels by ~2-fold in chronically stressed toadfish (Medeiros et al., 2010). In both mammals and fish, chronically elevated levels of glucocorticoids can inhibit 5-HT<sub>1A</sub> receptor gene expression and binding capacity (Lanfumey et al., 2008; Medeiros and McDonald, 2013). Moreover, acute applications of corticosterone in rats can attenuate 5-HT<sub>1A</sub> receptor function via both direct and indirect actions (Chaouloff, 1995). Therefore, the ability of 8-OH-DPAT to stimulate (or in some case inhibit; Hoglund et al. 2002) the HPI axis may depend on the intensity, the duration and the type of stressor a fish is experiencing at the time of 5-HT<sub>1A</sub> receptor activation.

Despite having a marked and sustained stimulatory effect on circulating cortisol levels, the 8-OH-DPAT treatment in this study did not change the mRNA levels of POA CRF and pituitary POMC or the plasma levels of ACTH. These results were unexpected given the stimulatory effects of i.c.v. 8-OH-DPAT in rats on PVN CRF and anterior pituitary POMC gene expression (Jørgensen et al., 2002), as well as the direct and indirect stimulatory effects of 8-OH-DPAT on ACTH secretion (Calogero et al., 1990; Jørgensen et al., 2001, 2002; Zhang et al., 2004). Moreover, stimulation of the serotonergic system in goldfish with the 5-HT reuptake inhibitor fluoxetine is associated with an increase in hypothalamic CRF gene expression (Mennigen et al. 2010). Since 8-
OH-DPAT can cross the blood-brain barrier (Scroggin, 2002) administration of the drug by i.p. injection should not prevent it from stimulating the HPI axis. However, 8-OH-DPAT does not affect POMC gene expression in the intermediate pituitary lobe (Jørgensen et al., 2002) and quantification of whole pituitary POMC gene expression in this study may have masked the effect of 8-OH-DPAT on POMC gene expression in the anterior pituitary. Also, by optimizing our sampling protocol to quantify the changes in plasma cortisol, transient changes in plasma ACTH may have been missed.

The expression of 5-HT$_1$A receptors in the POA, pituitary and head kidney of goldfish suggest a potential involvement of this receptor in mediating the actions of the serotonergic system at all levels of the HPI axis. In the POA, our results are consistent with the mRNA expression of 5-HT$_1$A receptors in the parvocellular POA of zebrafish (Norton et al., 2008) and the co-localization of 5-HT$_1$A receptors in the PVN CRF neurons of rats (Zhang et al., 2004). Interestingly, however, in both mice and rats, recent evidence suggests that 5-HT$_2$C receptors make a more important direct contribution to 5-HT activation of PVN CRF neurons than 5-HT$_1$A receptors (Heisler et al., 2007; Lanfumey et al., 2008). At the pituitary level, the expression of 5-HT$_1$A receptors in goldfish and toadfish (Medeiros et al. 2010) is consistent with the stimulatory effects of 8-OH-DPAT on ACTH release from rat anterior pituitary cell cultures (Calogero et al., 1993). Alternatively, 5-HT$_1$A receptors may be involved in mediating the stimulatory effects of 5-HT on luteinizing hormone secretion or its inhibitory effects on growth hormone release from goldfish pituitary glands (Somoza and Peter, 1991). In contrast to toadfish where 5-HT$_1$A receptor expression is 50-times higher in the brain than in the whole kidney (Medeiros et al., 2010), goldfish in this study were characterized by similar
5-HT$_{1A}$ receptor mRNA levels in the POA and the head kidney. In addition to potential differences in the 5-HT receptor subtypes expressed by the interrenal cells of goldfish and toadfish (Medeiros and McDonald, 2012), the presence of additional cell types in the head kidney that express 5-HT$_{1A}$ receptors such as lymphocytes and chromaffin cells (Brennan and Littleton, 1991; Ferriere et al., 1996) may explain the difference in 5-HT$_{1A}$ expression patterns between studies.

*Effects of 5-HT agonists on cortisol secretion in vitro*

The sustained stimulatory effects of i.p. injections of 8-OH-DPAT on plasma cortisol without any evidence of central HPI axis activation led us to assess whether 5-HT receptor agonists can directly stimulate the synthesis of cortisol at the level of the interrenals in goldfish. As previously observed in humans (Lefebvre et al., 1992), rats (Hinson et al., 1989), mice (Yang et al., 1995), frogs (*Rana ridibunda*; Delarue et al., 1988) and recently in toadfish (Meideros and McDonald, 2012), our results show that 5-HT exerts a direct stimulatory effect on corticosteroid secretion in goldfish. Consistent with the potency of 5-HT in goldfish, previous studies in mammals and frogs have observed that the lowest concentration of 5-HT which induced a significant stimulation of corticosteroids was in the $10^{-8}$-$10^{-7}$ M range, and maximum stimulation was obtained with 5-HT doses of $10^{-7}$-$10^{-6}$ M (Delarue et al., 1988; Lefebvre et al., 1992; Yang et al., 1995). In contrast, the lowest effective concentration of 5-HT that stimulated cortisol secretion in toadfish was $10^{-5}$ M, suggesting a reduced sensitivity of the interrenals to 5-HT in this species (Medeiros and McDonald, 2012).

In addition to 5-HT directly stimulating cortisol secretion, the results of this work indicate that 5-HT can modulate ACTH stimulation of cortisol secretion. Although 5-HT
was less potent than ACTH at stimulating cortisol secretion when given at equivalent molar concentrations, combined with ACTH, 5-HT significantly reduced the response time to peak cortisol secretion from 49 to 16 min. This novel finding suggests differences in the mechanism of action by which 5-HT and ACTH stimulate steroidogenesis in the interrenal cells of goldfish. In contrast, unlike the potentiating effects of urotensin I (UI) and UII on ACTH-stimulated cortisol secretion in fish (Arnold-Reed and Balment, 1994; Kelsall and Balment, 1998), or the synergistic effects of 5-HT and angiotensin II in stimulating aldosterone secretion in rat adrenal glomerulosa cells (Rocco et al., 1990), combined 5-HT and ACTH had no effect on the maximal fold change in cortisol release rate or on the total amount of cortisol secreted throughout the in vitro superfusions. Although the 10^−7 M 5-HT concentration used was sub-maximal (data not shown), the lack of additive effects of 5-HT on peak and total ACTH-stimulated cortisol release suggests that the ACTH concentration used in this study was too high to fully characterize the nature of the interaction between 5-HT and ACTH in cortisol secretion.

Results from our in vitro superfusion experiments indicate that more than one 5-HT receptor subtype may be involved in the mechanism of action of 5-HT on cortisol secretion in goldfish. Consistent with our in vivo results, the 5-HT_{1A/7} receptor agonist 8-OH-DPAT stimulated cortisol secretion from superfused head kidney tissue. Given their affinities for the different 5-HT receptor subtypes (see Supplementary Table 1), our observation that the antagonists WAY-100135 and SB-258719 nearly abolished and only delayed, respectively, the stimulatory effects of 8-OH-DPAT on cortisol secretion suggest that the actions of 8-OH-DPAT in the head kidney of goldfish are mediated by 5-
HT$_{1A}$ but not by 5-HT$_7$ receptors. However, 8-OH-DPAT does not stimulate cortisol secretion from the kidney of toadfish (Medeiros and McDonald, 2012) or from the interrenals of frogs (Idres et al., 1991), and the stimulatory effects of 8-OH-DPAT on adrenal steroidogenesis in rats is mediated via activation of 5-HT$_7$ receptors (Contesse et al., 2000). Moreover, although an increase in cAMP formation appears to be a key step in the transduction mechanism of adrenal serotonergic receptors (Contesse et al., 2000), 5-HT$_{1A}$ receptors are negatively coupled to adenyl cyclase (Hoyer et al., 2002). In contrast, the stimulatory effects of cisapride on cortisol secretion in goldfish and the ability of the antagonist GR-125487 to completely abolish this response are consistent with the observation that 5-HT-induced corticosteroid production in toadfish (Medeiros and McDonald, 2012), frogs (Idres et al., 1991) and humans (Lefebvre et al., 1992) is mediated through activation of the 5-HT$_4$ receptor subtype. Interestingly, neither 8-OH-DPAT nor cisapride mimicked the actions of 5-HT on cortisol secretion from the head kidney in vitro. Therefore, although the pharmacological agents used in vitro identified the 5HT$_{1A}$ and 5-HT$_4$ receptor subtypes as potential mediators of the actions of 5-HT on cortisol secretion in goldfish, we suggest that complementary histological studies are needed to confirm the identity and localization of 5-HT receptor subtypes in the head kidney of fish.

**Immunohistochemistry**

The clear co-localization of TH- and 5-HT-immunoreactive cells in the head kidney shows that the chromaffin cells in goldfish contain 5-HT. Similarly, 5-HT has been localized in the chromaffin cells of Atlantic cod (Gadus morhua) and European eel (Anguilla anguilla; Reid et al., 1995), amphibians (Delarue et al., 1992), reptiles
The presence of 5-HT in the chromaffin cells along with the findings that 5-HT can directly and potently stimulate corticosteroid secretion from the interrenals, suggests that 5-HT can play a paracrine role in the regulation of cortisol secretion in goldfish. In the head kidney of teleosts, the catecholamine-containing chromaffin cells are closely intermingled with the interrenal cells (Gallo and Civinini, 2003). The fact that sympathetic-chromaffin cell axis activation and catecholamine release primarily occurs in response to acute severe stressors in fish (Perry and Bernier, 1999; Reid et al., 1998;) suggests that paracrine interactions between the chromaffin and interrenal cells likely occur during such conditions. As corticosteroids must be synthesized de novo and the neuroendocrine stimulation of cortisol synthesis via the HPI axis takes much longer than the activation of the sympathetic-chromaffin cell axis (Wendelaar Bonga, 1997), faster stimulation of cortisol synthesis via 5-HT during acute severe stressors could be advantageous. Finally, the stimulatory effects of 5-HT on catecholamine secretion in teleosts and cyclostomes (Bernier and Perry, 1996; Fritsche et al., 1993;) also suggest that 5-HT secretion in response to acute severe stressors may generate a positive feedback loop that helps to locally sustain the contribution of the chromaffin and interrenal cells to the endocrine stress response.

**Perspective**

While the results do not provide any evidence to substantiate the proposed role of 5-HT$_{1A}$ receptors in the central regulation of CRF and ACTH secretion in fish, they suggest that 5-HT contained within the chromaffin cells of the head kidney may participate in the
paracrine regulation of cortisol secretion. Therefore, in addition to the endocrine regulation of cortisol secretion by ACTH and several other peptide hormones (Arnold-Reed and Balment, 1994; Schreck et al., 1989), 5-HT stored within the head kidney may provide a means by which the sympathetic nervous system can rapidly influence corticosteroid secretion during stress. To determine the functional significance of this novel paracrine interaction, future studies should elucidate the specific mechanism of action of 5-HT on the interrenals and the physiological conditions under which local 5-HT regulates cortisol secretion.
Table 1. The ratio of brain preoptic area corticotropin-releasing factor (CRF) to elongation factor 1α (EF1α) mRNA levels, pituitary pro-opiomelanocortin (POMC) to EF1α mRNA levels and plasma adrenocorticotropic hormone (ACTH) concentrations of goldfish either left undisturbed (Control) or given an intraperitoneal injection of saline, 100 µg/kg body weight (BW) 8-OH-DPAT or 400 µg/kg BW 8-OH-DPAT, and sampled 1.5 or 8 h post-injection.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time (h)</th>
<th>Control</th>
<th>Saline</th>
<th>8-OH-DPAT (100 µg/kg)</th>
<th>8-OH-DPAT (400 µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRF</td>
<td>1.5</td>
<td>1.14 ± 0.11</td>
<td>0.82 ± 0.15</td>
<td>1.39 ± 0.23</td>
<td>1.03 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.44 ± 0.16</td>
<td>1.09 ± 0.07</td>
<td>1.26 ± 0.17</td>
<td>0.93 ± 0.13</td>
</tr>
<tr>
<td>POMC</td>
<td>1.5</td>
<td>0.17 ± 0.03</td>
<td>0.24 ± 0.05</td>
<td>0.17 ± 0.04</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.22 ± 0.07</td>
<td>0.22 ± 0.07</td>
<td>0.24 ± 0.04</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>ACTH (pg/ml)</td>
<td>1.5</td>
<td>21.1 ± 2.0</td>
<td>27.0 ± 3.4</td>
<td>30.0 ± 5.2</td>
<td>36.4 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>36.4 ± 9.9</td>
<td>36.1 ± 2.4</td>
<td>29.9 ± 4.8</td>
<td>36.0 ± 3.4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n=10).
Figure 1. Plasma cortisol concentration of goldfish either left undisturbed (control) or injected with saline, 100 µg/kg body weight (BW) 8-OH-DPAT or 400 µg/kg BW 8-OH-DPAT and sampled 1.5 or 8 h post-injection. Values are mean ± S.E.M. (n=10). Bars that do not share a common letter are significantly different from each other as determined by two-way ANOVA and by pairwise Holm-Sidak post-hoc test (P<0.05).
Figure 1.
**Figure 2.** The ratio of brain preoptic area, pituitary and head kidney 5-HT$_{1A}$ receptor to elongation factor 1α (EF1α) mRNA levels in undisturbed goldfish. For comparative purposes, the expression ratios are presented relative to the preoptic area value. Bars that do not share a common letter are significantly different from each other as determined by one-way ANOVA and by pairwise Student-Newman-Keuls multiple comparison test. Values are means ± S.E.M. (n=5). The significance level for all statistical tests was $P < 0.05$. 
Figure 2.
Figure 3. *In vitro* cortisol release rate from goldfish head kidney tissue in a superfusion setup. Following an equilibration period of 240 min (t=0 min), the head kidney preparations were superfused for 20 min (shaded box) with medium containing (A) $10^{-7}$ M 5-HT (n=10), (B) $10^{-7}$ M human ACTH (n=10) and (C) $10^{-7}$ M 5-HT and ACTH (n=7). Asterisks indicate a statistical difference from time 0 value within a treatment as determined by one way repeated measure ANOVA and by Dunnett’s post-hoc test. Also determined from the data presented in (A-C) are (D) the maximal cortisol release rate, (E) the total cortisol release and (F) the time to maximal cortisol release rate in response to 5-HT, ACTH and 5-HT/ACTH combined. Bars that do not share a common letter are significantly different from each other as determined by one-way ANOVA and by pairwise Student-Newman-Keuls multiple comparison test. Values are means ± S.E.M. The significance level for all statistical tests was $P < 0.05$. 
Figure 3.
Figure 4. *In vitro* cortisol release rate from goldfish head kidney tissue in a superfusion setup. Following an equilibration period of 240 min (t=0 min), the head kidney preparations were superfused for 20 min (shaded box) with medium containing (A) $10^{-7}$ M 8-OH-DPAT (a 5-HT$_{1A/7}$ receptor subtype agonist) (n=10), (B) $10^{-7}$ M 8-OH-DPAT with $2 \times 10^{-6}$ M (S)-WAY 100135 dihydrochloride (a 5-HT$_{1A}$ receptor antagonist) (n=6) and (C) $10^{-7}$ M 8-OH-DPAT with $2 \times 10^{-6}$ M SB 258719 hydrochloride (a 5-HT$_{7}$ receptor antagonist) (n=5). Asterisks indicate a statistical difference from time 0 value within a treatment as determined by one way repeated measure ANOVA and by Dunnett’s *post-hoc* test. Values are mean ± S.E.M. The significance level for all statistical tests was $P < 0.05$. 
Figure 4.
**Figure 5.** *In vitro* cortisol release rate from goldfish head kidney tissue in a superfusion setup. Following an equilibration period of 240 min (t=0 min), the head kidney preparations were superfused for 20 min (shaded box) with medium containing (A) $10^{-7}$ M cisapride (a 5-HT$_4$ receptor subtype agonist) (n=10) and (B) $10^{-7}$ M cisapride with $2\times10^{-6}$ M GR 125487 sulfamate (a selective 5-HT$_4$ receptor antagonist). Asterisks indicate a statistical difference from time 0 value within a treatment as determined by one way repeated measure ANOVA and by Dunnett’s *post-hoc* test. Values are mean ± S.E.M. The significance level for all statistical tests was $P < 0.05$. 
Figure 5.
Figure 6. (a) Z-stack compressions of triple fluorescent immunolabelling of tyrosine hydroxylase (TH, magenta), serotonin (5-HT, green) and cell nuclei (DAPI, blue) in a transverse section of goldfish head kidney tissue surrounding the posterior cardinal vein and (b) an expansion of the area bounded by the box. Magnification 60x (a, b). Scale bars, 50 µm (a) and 10 µm (b).
Figure 6.
Chapter 3

Correlation of the behavioural and neuronal response of goldfish to alarm pheromones

Introduction

Chemical cues have long been demonstrated to elicit behavioural and physiological responses in fish (Liley 1982). One of the most intensively studied chemical cues that induce such responses is the alarm pheromone (Døving and Lastein 2011). Released from club cells as a result of damage to the epidermis (Smith 1992), alarm pheromones induce what is described as an anti-predatory alarm response that is observed in various taxa, from mammals to insects (Blum 1969; Chivers et al. 1996). In fish, behavioural responses to alarm pheromones seem to be species specific (Pfeiffer 1962). Fathead minnows exposed to alarm pheromones (Pimephales promelas) display a combination of dose- and time-dependent behaviours such as dashing, freezing, slowing and exploring (Lawrence and Smith 1989). A recent study in zebrafish exposed to alarm pheromones (Danio rerio) shows that they exhibit erratic movement, changes in shoal cohesion, but no freezing behaviour (Speedie and Gerlai 2008). In goldfish, the alarm response is characterized by a very rapid and transient display of agitated, non-directional, jerky movements with fully erected fins, followed by more sustained freezing behaviour (Kimbrell et al. 1970; Zhao and Chivers 2005). While exposure to alarm pheromones causes rapid changes in behaviour, little is known about the neural and physiological changes underlying these behavioural responses.

In a number of vertebrate species, corticotropin-releasing factor (CRF) has been found to modulate diverse behavioural responses (Lowry and Moore 2006). CRF neurons
are involved in extra-hypophysiotropic roles (Alderman and Bernier 2007; Lastein et al. 2008). One of the noticeable effects of central CRF injections is an increase in locomotion (Deak et al. 1999; Clements and Schreck 2002; Lowry and Moore 2006; Carpenter et al. 2007; Lastein et al. 2008). One mechanism through which CRF could modify behavioural responses is by interacting with the brainstem monoaminergic systems such as the adrenergic, dopaminergic or serotonergic systems (Clements et al. 2003). Studies with Chinook salmon (*Oncorhynchus tshawytscha*) suggest a potential role of the serotonergic system in CRF-induced locomotion (Clements et al. 2003). CRF has been found to alter serotonergic and dopaminergic activity along with inducing anxiogenic locomotion in fish (Carpenter et al. 2007). In general, CRF does not appear to drive specific motor patterns but to modify behavioural responses that are elicited by environmental cues (Lowry and Moore, 2006).

Evidence also suggests that CRF may mediate the behavioural changes associated with alarm pheromone exposure. In crucian carp (*Carassius carassius*), it has been found that the CRF-R1 receptor plays a role in mediating behavioural responses to fear and stress (Lastein et al. 2008). Crucian carp injected with the CRF-R1 receptor antagonist antalarmin, showed no behavioural response to alarm pheromones (Lastein et al. 2008). In rats, the primarily inhibitory effects of alarm pheromones on sexual behaviour were also blocked by a CRF-R1 receptor antagonist (Kobayashi et al. 2011; Kobayashi et al. 2012).

Along with the immediate behavioural changes that characterize exposure to alarm pheromone, studies have suggested an effect on the neuroendocrine stress response. CRF also plays an important part in the regulation of the stress axis (Wendalaar
Bonga 1997). In fish, elevations in plasma cortisol and glucose have been observed after alarm pheromone detection (Rehnberg and Smith 1987; Tierney et al. 2006; Lastein et al. 2008). In rats, exposure to alarm pheromone has been shown to even enhance behavioural and physiological stress response of con-specific animals (Kikusiu et al. 2001). However, other studies have suggested that alarm pheromones cause a suppression of the neuroendocrine stress axis (Fraker et al. 2009) or that corticosteroid do not mediate alarm pheromone effects (Abel et al. 1991). As CRF is implicated in multiple behavioural and physiological effects, CRF is considered to be a neurohormone that integrates behavioral and physiological responses to physical and emotional stressors (Crespi and Denver 2004). However, the source of CRF-related peptides involved in the extra-hypophysiotropic actions of the CRF system is not known (Alderman and Bernier 2007).

One important brain region that has been implicated in mediating the neuroendocrine stress response is the nucleus preopticus (NPO), the teleost homologue of the mammalian paraventricular nucleus (PVN) (Charmandari et al 2005; Bernier et al. 2009). Aside from its hypophysiotropic roles, the PVN and the NPO have been shown to have roles in mediating behaviour. Neurons in the PVN project to a number of structures in the limbic system that are responsible for behavioural regulation (Swanson and Sawchenko 1983). Microinjections of CRF in the PVN of mice have been shown to induce dose-related behavioural profiles in rats (Mönnikes et al. 1992). The NPO also contains other neurohormones that have been implicated in behavioural responses such as arginine vasotocin (AVT) and isotocin (IST) (Goodson and Bass 2001; Goodson et al. 2003; Balment et al. 2006). AVT and CRF are also co-localized in the NPO, which
suggests that the peptide may play a role along with the activation of the stress axis (Olivereau et al. 1988).

Another brain region that could be important in mediating the response to alarm pheromones is the telencephalic area ventralis lateralis (VI), an area that is homologous to the cholinergic activating system of the pallium (Rink and Wulliman 2004). As the pallium has been implicated in avoidance and spatial learning (Portavella et al. 2004; Portavella and Vargas 2005) and the cholinergic system in awareness (Smythies 1997), the VI might be an important region for the alarm response. Other studies have suggested that the area ventralis telencephali pars lateralis is homologous to the olfactory tubercle in mammals, which receives direct input from the olfactory bulb, an important site of alarm pheromone detection (Northcutt 1981; Murakami et al. 1983; Døving and Lastein 2009).

In this study, the behavioural response of goldfish to alarm pheromones is correlated with the neuronal response. In order to do this, I identified key robust behavioural responses of goldfish to alarm pheromone and determined their timing of each. I then used c-Fos immunolabelling as a proxy of neuronal activation (Kovács 1998) to determine whether the NPO and the VI are activated in the behavioural response to alarm pheromone exposure. Lastly, I also used CRF-immunolabelling to determine whether the CRF neurons of the NPO are implicated. Given, the role of the NPO in the hypophysiotropic regulation of the HPI axis, potential behavioural modulation of CRF, and the stimulatory effects of alarm pheromones on the HPI axis in several fish species, I predicted that post alarm pheromone exposure, NPO c-Fos immunoreactivity will increase, and NPO CRF immunoreactivity will decrease (Suemaru et al. 1985). Similarly,
given the role of the Vl in behavioural awareness, I predicted that Vl c-Fos immunoreactivity will increase after alarm pheromone exposure.

**Materials and methods**

**Animals**

Mixed sex goldfish (*Carassius auratus*) of the common variety were obtained from Straits Aqualife (Toronto, ON, Canada) and transported to the Hagen Aqualab at the University of Guelph (Guelph, ON, Canada). Fish were acclimated for at least 4 weeks in a common holding tank (800 l) that was supplied with well water (pH 7.9; water hardness 411 mg/l as CaCO₃; Ca²⁺, 10.5 mmol/l; Cl⁻, 1.5 mmol/l; Mg²⁺, 3.0 mmol/l; K⁺, 0.06 mmol/l; Na⁺ 1.1 mmol/l), maintained at 19°C and exposed to a 12h:12h light:dark photoperiod regime. Fish were fed daily with commercially prepared fish food (3PT Classic Sinking, Martin Mills, Elmira, Canada). All procedures were approved by the local Animal Care Committee and conform to the principles of the Canadian Council for Animal Care.

**Experimental Design**

Three weeks prior to the start of experimentation, fish (32.9 ± 1.3g, mean ± SEM; n=12) were removed from a common holding tank and individually placed in aerated recirculating 21 l aquaria (41cm × 20cm × 25cm, length × width × height). Water temperature was kept at 19°C throughout the experimental period. Water quality was maintained with individual clip-on aquarium filters and water quality was checked weekly.

The experiment consisted of two experimental treatments. Fish were either exposed to distilled water (control) or to conspecific skin extract. Within a given trial,
the activity of a fish was recorded using a video camera for a period of 60 min before and after the addition of distilled water or skin extract. The fish were then quickly netted and terminally anaesthetized with 2-phenoxyethanol (5 ml/l; Sigma-Aldrich; St. Louis, MI, USA). Within 3 min of tank removal, the brain was exposed by cutting and removing a three-sided flap of the frontal bone. The fish were then decapitated and the head placed in 4% phosphate-buffered paraformaldehyde. The brains left in 4% phosphate-buffered paraformaldehyde for a minimum of 24 h before being dissected and then processed for c-Fos and CRF immunohistochemistry.

To study the time-course of c-Fos immunolabeling and neuronal activation in response to a known stressor (Singley and Chavin 1975), a preliminary experiment was carried out where goldfish (n=4) were exposed to an osmotic disturbance. Three goldfish were netted, directly transferred from freshwater to 10 ppt seawater for 15 minutes, and terminally anaesthetized as above 30, 60 and 120 min post-transfer. One fish was kept in freshwater and used as control. The brains were removed and processed for c-Fos immunoreactivity. Qualitative assessment of the micrographs revealed that the strongest c-Fos labelling was at 60 min post-transfer. Therefore the 60 min time period was determined to be adequate to detect alarm pheromone-induced c-Fos immunolabelling in this study.

**Skin extract preparation**

The skin extract was prepared as per Mirza et al. (2003) with the following modifications. Four goldfish (45.6 ± 2.6g, mean ± SEM) were sacrificed by percussive stunning. Approximately 25 cm² of skin was removed from both sides of each fish and was homogenized in 100 ml of distilled water using a Teflon homogenizer attached to a
drill. The homogenate was filtered with a 300 micron filter and distilled water was added to bring the final volume to 500 ml. The skin extract was then divided into 20 ml aliquots and stored at –80°C until required. Prior to the start of the experiments, a preliminary trial was performed to determine the lowest skin extract concentration needed to consistently elicit an alarm response from goldfish. Dilutions of 1:1, 1:10, 1:50, 1:100 and 1:200 were tested. Only the 1:1 and 1:10 dilutions were able to elicit an alarm response with >90% success rate. Therefore, immediately before a series of experiments, a frozen aliquot of skin extract was diluted to 200 ml (making a 1:10 dilution) with distilled water and kept on ice until delivered to the test aquarium.

Videotaping procedures and administration of skin extract

To provide visual isolation between tanks and minimize disturbances, the aquaria were separated from each other by white acrylic plexiglass boards and the backdrop of the aquaria rack was covered with a white canvas sheet. One hour before videotaping and throughout each experiment, the aquaria were indirectly illuminated using two 100 W halogen floodlights. Videotaping occurred between 10:00 h and 17:00 h and a maximum of 3 fish were recorded on a given day. The timing of the control and skin extract treatments was randomized to achieve a balanced sampling procedure. The fish were videotaped for 60 min before and after the addition of either 50 ml of distilled water or 50 ml of the 1:10 skin extract. The water and skin extract were delivered over a 30 sec interval using a 10 mm diameter aquarium tubing. While one end of the tubing was attached to the rack and positioned in the aquarium directly above the air stone to maximize mixing of the injected solution with the aquarium water, the other end was hidden from the fish and positioned behind the rack. To ensure that the aquaria received
all 50 ml of the treatment, the lines were flushed with an additional 50 ml of distilled water over an additional 30 sec period. The locomotor activity of each animal was recorded using a SONY HDD video recorder (DCR-SR68) mounted on a stationary tripod.

Quantification of behavioural responses

The duration of time moving and time spent at the bottom of the tank was quantified using the tracking software EthoVision XT (version 8.0; Noldus Information Technology, Wageningen, The Netherlands). Recorded videos were transferred to a computer for replay and analysis. A still image of the aquarium was digitally outlined to set an arena in which the tracking took place. The arena was then divided into three horizontal zones (top, middle and bottom) that separated the aquarium into 3 equal parts. The arena was also calibrated to the actual dimensions of the aquarium so that the parameters were calculated in cm instead of pixels. The 2 h videos were analyzed at a rate of 25 Hz. Fish that moved less than 1.5 cm/min were considered immobile. Since the contrast between the subject and background within the arena was variable, a differencing detection method was used as it was found to be the most accurate and precise way to detect the goldfish. Differencing detection method is an automated method in the software that takes into account the variable contrast between the subject and the background.

The values obtained from EthoVision XT were compared to a manual analysis. Randomly selected videos (full footage duration, 5 fish) was watched with a stopwatch in order to quantify the duration of time spent moving and the duration of time spent in the bottom zone of the tank. Results from the manual analysis were then compared to those
obtained by the software to verify precision and accuracy of the automated behavioural analysis.

The two behaviours quantified (duration of movement, time spent at the bottom) were very consistent in the 60 min prior to the addition of the test substance in both treatments. Therefore, for clarity of presentation, only the behavioural results from the last 30 min pre-injection are reported. Moreover, since the duration of time spent moving and the position of the fish in the tank changed rapidly initially after addition of the skin extract, the behavioural data was averaged over 1 min intervals for the first 10 min post-injection and over 2 min intervals for the remainder of the trial. Time of injection was considered as time 0. All behavioural data was converted to percent duration for each time point interval.

c-Fos and CRF immunohistochemistry

The brains were embedded in 4.4% agar-agar and 50 μm-thick transverse sections were cut on a vibratome (VT 1200S, Leica Microsystems, Wetzlar, Germany) and transferred to phosphate buffer (PB; 0.08 M Na₂HPO₄, 0.02 M KH₂PO₄, pH 7.4). Sections were alternately transferred to two different wells; one for c-Fos and one for CRF-labelling. Sections were incubated in 0.5% hydrogen peroxide PB for 15 min, then washed 3 ×10 min in PB, incubated 60 min in 3% BSA PB followed by 60 min in 1.5% normal goat serum (NGS), 0.1% Triton X-100 PB. The sections were then incubated overnight at room temperature in a solution containing a 1:4000 dilution of the primary antibody rabbit anti-c-Fos (sc-253, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 1:4000 dilution of the primary antibody rabbit anti-ovine CRF (20084, Immunostar, Hudson, Wisconsin, USA) and 3% BSA, 0.5% NGS, and 0.5% Triton X-100. The next
day, sections were washed 3 × 10 min in PB before a 90 min incubation in a solution containing a 1:200 dilution of the biotinylated secondary antibody goat anti-rabbit IgG (Vectastain Rabbit PK-4001 kit, Vector Laboratories, Burlingame, CA, USA), 0.5% NGS, and 0.1% Triton X-100. The sections were washed again 3 × 10 min in PB before a 90 min incubation in an avidin-biotin-peroxidase complex diluted in 0.1% Triton X-100 PB, then washed again in 3 × 10 min in PB and mounted on Superfrost® Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). The antibody-peroxidase complex was visualized using diaminobenzidine (Sigma-Aldrich, St. Louis, MI, USA) as chromogen with heavy metal intensification (Adams, 1981) in the presence of 0.0009% hydrogen peroxide. Sections were dehydrated in ascending ethanol concentrations, cleared in xylene and coverslipped with Cytoseal 60 (Richard Allan Scientific, MI, USA). Control and treated sections were run in parallel with each other to account for any variation between processing days. Control immunocytochemical procedures were performed in parallel to the normal c-fos and CRF assays in ~5% of the brain sections in selected animals by omission of the primary antibody. Also, to control for the use of heterologous antibodies, the primary antibodies were pre-absorbed prior to use. The c-Fos antibody was pre-absorbed with a 1:400 dilution of c-Fos blocking peptide (100μg/ml; sc-253 P, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 4 h, and the CRF antibody was incubated with 100 nM ovine CRF peptide (C3167, Sigma-Aldrich) overnight.

Immunolabeled tissue sections were visualized using a light microscope (Leica DMLA, Leica Microsystems, Wetzlar, Germany) and photomicrographs were captured using the Andor iQ software (Version 2.6; Andor Bioimaging Division, Belfast, UK).
Quantification of c-fos and CRF immunolabeling

The c-Fos and CRF-immunoreactive cells (Fig. 1) were quantified in a semi-automated fashion using ImageJ (version 1.46; National Institute of Mental Health, MA, USA). To increase contrast between labelled and non-labelled tissue, the relative background was subtracted in each photomicrograph using a minimum pixel intensity threshold of 220 and 200 for c-Fos and CRF-labelled sections, respectively. The relative background was the background that needed to be subtracted to ensure that the pixel intensities were below the maximum pixel intensity measured by the software. The selected thresholds were the minimum settings to detect the faintest c-Fos- and CRF-immunolabeled cells, evaluated visually. A rectangle was drawn to include all the labelled cells within the region of interest and the total intensity detected by the software was measured. The total intensity is the product of area and pixel intensity. In addition, the average pixel intensity of the total area detected by the software between tissues of the same fish within a given region of interest was measured. Average pixel intensity was assessed to determine if the difference between the control and treatment groups, if any, were in the strength of the signal or the number of cells affected. Brain sections between fish were matched for rostrocaudal levels as closely as possible using the goldfish atlas of Peter and Gill (1975) and the zebrafish atlas of Wulliman et al. (1996). Determining total pixel intensity, as opposed to counting the number of c-Fos and CRF-positive cells, proved best for three reasons: 1) the CRF and c-Fos labeling in the NPO were cytoplasmic making visual cell counts less accurate; 2) the semi-automated quantification removed biases; 3) there was no difference in average intensity between and within treatment groups. Some brain sections were missing, damaged or used as antibody
controls. Thus, specific rules were followed to quantify labeling intensity: 1) Missing or damaged hemisections were attributed the value obtained on the opposite side. Sections with both hemisections intact were averaged. 2) The labeling intensity in a given brain region that had one or more missing whole sections was calculated according to the formula: \[ X = \frac{\text{total labeling intensity}}{\text{number of brain sections}} \times \frac{\text{average number of brain sections per brain region across all animals}}{1}. \]

Nomenclature of the goldfish brain is according to Peter and Gill (1975). For quantification purposes the NPO was separated into two distinct areas: 1) the \textit{pars parvocellularis} (PP) that contained small cells located anteroventrally, and 2) the \textit{pars magnocellularis} (PM) that contained large cells located posterodorsally. Labelled cells in the NPO were counted on eight transverse brain sections over a distance of 400 μm in the rostrocaudal direction. Alternate sections were used for c-Fos and CRF labelling quantification. The c-Fos labelled cells were counted on the 5 most rostral sections of the \textit{area ventralis telencephali pars lateralis} (VI) starting 300 μm rostral of the anterior commissure (up to 500 μm; alternate sections were also used to CRF labelling).

Representative transverse micrographs of the regions targeted for c-Fos and CRF immunolabelling quantification are shown in Figure 1.

\textit{Statistics}

Results are presented as mean ± SEM. Differences in behaviour across 90 min of the recorded sessions (30 min prior to and 60 min after the addition of the test solution) were analyzed using a repeated measures one-way ANOVA followed by Dunnett’s multiple comparison \textit{post-hoc} test to identify differences from the time 0 value. Differences in c-Fos and CRF labeling intensity between the control and experimental
treatments were assessed by two-tailed Student’s t-test. All analyses were performed using SigmaStat 3.0 (SPSS Inc., IL, USA) and $P < 0.05$ was considered statistically significant for all tests.

**Results**

*Effects of alarm pheromone on goldfish behaviour*

In the control treatment, the addition of distilled water did not have any effect on the percent of time spent moving (Fig. 2A). In contrast, goldfish exposed to alarm pheromones stopped moving within 2 min of skin extract exposure and remained in this state until approximately 6 min post-exposure when movement resumed (Fig. 2B). Movement recovery post alarm pheromone exposure was very gradual and still significantly depressed by 25% relative to the time 0 value after one hour.

The addition of distilled water in the control treatment did not have any effect on the percent of time bottom dwelling and on average control fish spent approximately 30% of their time in the bottom third of the aquarium (Fig. 3A). In sharp contrast, goldfish that were exposed to skin extract all moved to the bottom third of the tank within 2 min and remained in this portion of the aquarium before gradually starting to explore once again at around 6 min after alarm pheromone exposure (Fig. 3B). Interestingly, recovery from bottom dwelling after alarm pheromone exposure was much slower than movement recovery. Even one hour after skin extract exposure, once swimming activity had mostly resumed, goldfish were still spending ~70% of their time bottom dwelling. Manual analysis of the videotapes also revealed that exposure to skin extract was associated with a very rapid and transient dashing behaviour immediately after addition of the skin extract; however, this behaviour was too quick and variable to be quantified. As all the
fish that were exposed to alarm pheromone exhibited dashing behavior but at different time intervals, it was difficult for the software to quantify the behaviour objectively.

**Effect of alarm pheromone in c-Fos immunolabelling in goldfish brain**

The total pixel intensity of the c-Fos immunolabelled cells in the *pars parvocellularis* region of the NPO was significantly lower in the fish exposed to conspecific skin extract than in the control fish (Fig. 4A, 4D and 5A). In contrast, there was no significant difference between treatments in the total c-Fos signal detected in either the *pars magnocellularis* of the NPO (Fig. 4B, 4E and 5B) or the *area ventralis telencephali pars lateralis* (Fig. 4C, 4F and 5C). Average c-Fos labelling intensity did not differ between treatments in all three brain regions quantified (data not shown).

**Effect of alarm pheromone in CRF immunolabelling in goldfish brain**

The total pixel intensity of the CRF immunolabelled cells in either the *pars parvocellularis* (Fig. 6A, 6C and 7A) or the *pars magnocellularis* (Fig. 6B, 6D and 7B) regions of the NPO did not differ between the control and skin extract-exposed fish. In addition, average CRF labelling intensity did not differ between treatments in either region of the NPO (data not shown). CRF-immunolabelling was not detected in the Vl.

**Antibody controls**

There was no specific immunostaining after the omission of the primary antibodies (Fig. 8A). Similarly, immunostaining was abolished after pre-absorption of the c-Fos primary antibody with the c-Fos blocking peptide (Fig. 8B), as well as after pre-absorption of the CRF primary antibody with ovine CRF (Fig. 8C).
**Discussion**

Exposure of goldfish to skin extract in this study resulted in a potent inhibition of locomotor activity and exploration. These alarm-pheromone induced changes in behaviour were associated with a marked suppression of neuronal activity in the parvocellular preoptic nucleus, a brain region with extensive CRF immunolabelling.

Detailed behavioural analysis showed that goldfish performed three general types of behaviour in response to alarm pheromone exposure: rapid dashing, freezing (the term freezing was used in analogy with rodent fear behaviour) and increased duration spent at the bottom of the tank. This is consistent with other behavioural responses to alarm pheromones in fish species (Pfeiffer 1962; Rehnberg et al. 1987; Rehnberg et al.1989) and suggests that while the specifics of the response may vary from species to species, alarm pheromones induce a fright reaction in most fish. While previous studies have described the response of goldfish to conspecific skin extract and its components (Kimbrell et al. 1970; Zhao and Chivers 2005), a detailed time-course analysis had not been previously performed. Although rapid dashing movements had previously been implicated as one of the key response to alarm pheromone in goldfish (Kimbrell et al. 1970; Zhao and Chivers 2005), this behavioural response was very transient and inconsistent in this study. In contrast, the freezing behaviour and increased duration of bottom dwelling appears to be more robust behavioural responses. However, the previous studies on the behavioural responses of goldfish to alarm pheromones were carried out with groups of fish (Kimbrell et al. 1970; Zhao and Chivers 2005) and that the behavioural response of isolated goldfish may differ (Laudien et al. 1986). Differences between studies in the properties of the transient rapid dashing behaviour could be also
attributed to the constraints of the confinement in the aquarium (Ide et al. 2003). In a larger aquarium, fish may be able to avoid the alarm pheromone and in return this may alter the duration of the rapid movement phase as the timing of alarm pheromone detection will vary. Similarly, the concentration of the alarm pheromone has been found to alter the response of fathead minnows from darting to freezing (Lawrence and Smith 1989). Speedie and Gerlai (2008) suggested that elements of their experimental tank, such as lighting set up could have resulted in their unexpected behavioural response (i.e. lack of freezing and increased bottom dwell time) to alarm pheromone in zebrafish.

Future experiments should look at the possible effects of tank size variation and skin extract concentration on the detailed behavioural and physiological responses to alarm pheromones.

The results from this study also suggest that the alarm response in goldfish involves the pars parvocellularis of the NPO. Moreover, the results suggest that exposure of goldfish to alarm pheromone suppresses neuronal activity in this specific region of the brain. This is an unexpected result since exposure to alarm pheromones in rats results in an increase in c-Fos labelling in the PVN (Kiyokawa et al 2005; Kobayashi et al 2013), a region homologous to the NPO of fish. As alarm pheromones have been shown to be a stressor in fish (Rehnberg and Smith 1987; Scott et al. 2003; Tierney et al. 2006; Lastein et al. 2008), c-Fos expression in the NPO might be predicted to increase as it is known as an important nuclei for the hypophysiotropic regulation of the neuroendocrine stress response (Bernier et al. 2009). c-Fos mapping experiments in rats suggested that perception of alarm pheromones is related to stress-responsive brain structures, such as the hypothalamus (Kiyokawa 2005). Mudskippers (Periophthalmus...
cantonensis) stressed with agitation (using a stirring rod) are also characterized with very prominent c-Fos labelling in the hypothalamus (Wai et al. 2006). In contrast, exposure of wood frogs (Rana sylvatica) and green frogs (Rana clamitans) tadpoles to alarm pheromones caused a rapid and strong suppression of the neuroendocrine stress axis and was associated with a behaviorally quiescent state (Fraker et al. 2009). This suggests that in some species alarm pheromones that result in behavioural suppression may be correlated along with the suppression of the neuroendocrine stress axis.

Contrary to the results observed in the pars parvocellularis of the NPO, alarm pheromone exposure did not elicit any change in c-Fos immunolabelling in the pars magnocellularis. This suggests that alarm pheromone exposure in goldfish has a differential effect on the parvocellular and magnocellular preoptic nucleus. While the NPO projects to several different motor nuclei in the brainstem (Rao et al. 1993), it is not known whether the parvocellular and magnocellular preoptic nucleus are differently involved in regulating the motor nuclei of the brainstem in fish. However in rats, after staining with ovine CRF-like antibody, there seems to be evidence of neural impulse interaction between the pars parvocellularis and pars magnocellularis of the PVN (Lérañth et al. 1983); cells in the paraventricular nucleus were found to be in synaptic-like contact with protrusions of magnocellular neurons. The timing of neuronal activation in the pars magnocellularis neurons could also differ from the response in the pars parvocellularis neurons after alarm pheromone exposure. It is also a possibility that timing of the experiment missed any c-Fos immunolabelling in pars magnocellularis. Previous studies have found that a 30 min difference in sampling time is sufficient to miss c-fos expression in the olfactory bulb (Kikusui et al. 2001; Kiyokawa 2005).
However, this is highly unlikely as c-Fos is maximally expressed 1 – 3 h after induction (Kovács 1998) and we sampled an hour after exposure to skin extract, a time where I found that c-Fos is strongly expressed in both regions of the NPO after preliminary trials with an osmotic stressor.

I predicted that there would be an increase in c-Fos labelling in the telencephalic area ventralis pars lateralis as the pallium, a region implicated for avoidance response in goldfish, receives efferent projection from this region (Murakami et al. 1983; Portavella et al. 2004a). It also receives direct input from the olfactory bulb, where alarm pheromones are detected (Northcutt 1981; Murakami et al 1983; Døving and Lastein 2009). However, the lack of change in c-Fos labelling suggests that the cholinergic activation of the pallium is not involved in the response to alarm pheromones. However, the pallium is also where the pathways of both visual and olfactory inputs converge prior to the motor system (Wisenden et al., 2003; Jesuthasan and Mathuru 2008). As fish display a different set of behaviour when they are isolated compared to when conspecifics are present (Hoare et al. 2004), this region might be worth revisiting to compare the two different response to determine the social effect in alarm response.

Although conspecific skin extract exposure was not associated with a change in CRF immunolabelling, the considerable overlap in c-Fos and CRF signal in the pars parvocellularis suggests that the alarm pheromone in goldfish may inhibit this specific subset of CRF neurons. This suggestion is not consistent with a recent study on rats where exposure to alarm pheromone displayed an increase in neurons that are both CRF and c-fos-positive (Kobayashi et al. 2013). Rats however exhibit an increase in movement and general activity after exposure to alarm pheromones (Zalaquett and
Thiessen 1991; Kikusui et al. 2001). As goldfish display a different behavioural response to alarm pheromones, different neuronal circuits might be involved. Mice who display freezing behaviour have different neuronal activity than mice who display flight responses against aversive stimulus (Mongeau et al. 2003). The reduction in neuronal activity of CRF neurons could be part of the freezing behaviour in goldfish. This supports the hypothesis that CRF is involved in locomotion and that neurons in the PVN/NPO are involved in the modulation of this behaviour. Several fish studies have shown that CRF stimulates locomotor activity independently of the pituitary (Clements et al. 2002; Carpenter et al. 2007) and this is also been consistent with mammals and amphibians (Clements et al. 2002; Lowry and Moore 2006). CRF also has anxiogenic properties that result in a decrease in exploratory behaviour (Butler et al. 1990). As CRF in the NPO is an important neuropeptide in the regulation of the HPI axis (Bernier et al. 2009), the inhibition of this specific subset of CRF neurons in the *pars parvocellularis* may inhibit the HPI axis in goldfish. Future studies need to look at the changes in plasma cortisol and ACTH after skin extract exposure, as well as CRF mRNA changes in the POA and POMC changes in the pituitary, to further elucidate the effect of alarm pheromones on the endocrine stress response.

Besides CRF, the parvocellular preoptic neurons express several neuropeptides that have known behavioural effects (Cerdá-Reverter and Canosa 2009). AVT and IST are neurohormones that are both produced in the *pars parvocellularis* and *pars magnocellularis*, which are both implicated as having key roles in wide physiological and behavioural processes (Goodson and Bass 2001; Goodson et al. 2003; Balment et al. 2006). Interestingly, differences in behaviour in fish, such as being dominant or
subordinate are dependent on AVT expression on the two distinct areas of the NPO; aggressive males have a higher AVT expression in the magnocellular cells, and subordinate individuals have a higher AVT expression in the parvocellular cells (Larson et al. 2006; Greenwood et al. 2008). The specific phenotype of the pars parvocellularis neurons affected by alarm pheromone exposure in goldfish remains to be determined.

In summary, this study provides original evidence that the neural circuit of alarm pheromone signalling in goldfish involves the pars parvocellularis of the NPO. The data suggests that there is a suppression of neuronal activity and it is likely to have a CRF-phenotype. However, it is a possibility that it could be several other neuropeptides in the region that have known behavioural effects. I have also shown a robust behavioural response of goldfish to alarm pheromones and correlated it to neuronal responses in the brain. This is an important novel contribution as to fully understand neurophysiological processes, a full understanding of behaviour is necessary to elucidate the neural basis of behaviour (Eaton et al. 2001)
Figure 1. Sagittal overview of the brain of goldfish and representative transverse micrographs of the regions targeted for c-Fos and CRF immunolabelling quantification (Cytoplasmic labelling). Areas within boxes are region of interest: (A) *area ventralis telencephali pars lateralis*, (B) NPO *pars parvocellularis*, and (C) NPO *pars magnocellularis*. The letters A-C in the sagittal overview of the brain correspond to the rostrocaudal position of the transverse sections shown in panels A-C. Scale bar = 300 μm.
Figure 1.
**Figure 2.** Effects of exposure to conspecific alarm pheromone on the duration of time spent moving in goldfish. Following a control period of 30 min, goldfish were exposed at time 0 min to either (A) 50 ml of distilled water or (B) 50 ml of a 1:10 dilution of skin extract. The duration of time spent moving is reported as a percentage. The behaviour was averaged over 1 min intervals for the 10 min of the exposure, and over 2 min intervals for the remainder of the trial. Videos were analyzed using Ethovision XT (Version 8); fish moving less than 1.5 cm/min were considered immobile. Asterisks indicate the time interval within a treatment where a statistical difference from the time 0 value was identified as determined by one way repeated measure ANOVA and by Dunnett’s *post-hoc* test. Values are mean ± SEM (n=6). The significance level for all statistical tests was $P < 0.05$. 
Figure 2.
Figure 3. Effects of exposure to conspecific alarm pheromone on the duration of time spent at the bottom of the tank. Following a control period of 30 min, goldfish were exposed at time 0 min to either (A) 50 ml of distilled water or (B) 50 ml of a 1:10 dilution of skin extract. The duration of time spent at the bottom of the tank is reported as a percentage. The behaviour was averaged over 1 min intervals for the 10 min of the exposure, and over 2 min intervals for the remainder of the trial. Videos were analyzed using Ethovision XT (Version 8). Asterisks indicate the time interval within a treatment where a statistical difference from the time 0 value was identified as determined by one way repeated measure ANOVA and by Dunnett’s post-hoc test. Values are mean ± SEM (n=6). The significance level for all statistical tests was $P < 0.05$. 
Figure 3.
Figure 4. Transverse micrographs showing the distribution of c-Fos immunolabelling in the brains of control (A-C) and skin extract-exposed (D-F) goldfish. The labelling shown is found in (A,D) the *pars parvocellularis* of the NPO, (B,E) the *pars magnocellularis* of the NPO, and (C,F) the *area ventralis telencephali pars lateralis*. Scale bar = 150 μm.
Figure 4.
**Figure 5.** Total pixel intensity of c-Fos labelled cells in (A) the *pars parvocellularis* of the NPO, (B) the *pars magnocellularis* of the NPO, and (C) the *area ventralis telencephali pars lateralis* in the brains of distilled water-exposed (control) and skin extract-exposed (treated) goldfish. Asterisks indicate a statistical difference between treatments as determined by two-tailed Student’s t-test. Values are mean ± SEM (n=4, except 5A control where n = 3). The significance level of statistical tests was $P < 0.05$. 
Figure 5.
Figure 6. Transverse micrographs showing the distribution of CRF immunolabelling in the brains of control (A, B) and skin extract-exposed (C, D) goldfish. The CRF labelling is found in the *pars parvocellularis* (A, C) and in the *pars magnocellularis* (B, D) of the NPO. Scale bar = 150 μm.
Figure 7. Total pixel intensity of CRF labelled cells in (A) the *pars parvocellularis* of the NPO and (B) the *pars magnocellularis* of the NPO in the brains of distilled water-exposed (control) and skin extract-exposed (treated) goldfish. No statistical difference was identified as determined by two-tailed Student’s t-test. Values are mean ± SEM (n=4). The significance level of statistical tests was $P < 0.05$. 
Figure 7.
**Figure 8.** Transverse micrographs showing the *pars parvocellularis* of the NPO in the goldfish brain and results from the immunohistochemistry antibody controls. Exclusion of the primary antibodies showed no staining (A). Similarly, immunostaining was abolished after absorption of the c-Fos antibody with c-Fos blocking peptide (B), and after absorption of the CRF antibody with ovine CRF (C). Scale bar = 150 μm.
Figure 8.
Chapter 4

General Discussion

While numerous studies have expanded our understanding of the endocrine stress response in fish, our knowledge of the control of the HPI axis is still fairly limited. This study provided insight into the control of the endocrine stress response through the serotonergic system and alarm pheromones. I found that the serotonergic control of the HPI axis is complex and that 5-HT appears to be involved in the paracrine regulation of cortisol secretion at the level of the head kidney. I also observed that the response to alarm pheromone exposure in goldfish is associated with a reduction in neuronal activity in the POA. However, the role of CRF in the neuro-behavioural response to alarm pheromones is still contentious. Collectively, the results of this thesis further our understanding of the control of the endocrine stress response in fish.

Serotonergic control of the HPI axis

The present studies have demonstrated the complexity of the serotonergic control of the HPI axis. In vivo injection of 8-OH-DPAT elicited increases in cortisol secretion but this was not accompanied by any changes in the mRNA levels of key effectors of the HPI axis at the level of the hypothalamus and the pituitary. However, in vitro superfusion of the head kidney with various 5-HT agonists did elicit cortisol secretion. While the results did not find any evidence to support the role of 5-HT$_{1A}$ receptors in the regulation of CRF and ACTH secretion, my results suggest that the serotonergic control of the stress response may involve paracrine regulation aside from the classical endocrine regulation. Immunohistochemistry work showing that the chromaffin cells in the head kidney is a
source of 5-HT for the nearby interrenal cells suggests that cortisol biosynthesis through 5-HT might occur during acute stress response along with the release of catecholamines.

As the different 5-HT agonists elicited cortisol secretion from the head kidney with varying potency, it is also important to figure out the relative importance of endogenous neurotransmitter / hormone 5-HT at the other levels of the HPI axis. An antibody against the goldfish 5-HT_1A receptor could be developed and used to specifically determine whether the CRF-producing neurons of the POA, the corticotropes of the pituitary and the interrenal cells of the head kidney express 5-HT_1A receptors. Superfusion of the pituitary with 5-HT could help elucidate the role of 5-HT in the secretion of pituitary ACTH. The use of various 5-HT agonist and antagonist could also help identify the potential 5-HT receptor subtypes involved in ACTH secretion. Future studies would benefit from determining the relative importance of the 5-HT_1A receptor relative to other 5-HT receptor subtypes to further our knowledge of the serotonergic control of the HPI axis.

**Effects of Alarm Pheromones**

The current study has shown that exposure of goldfish to alarm pheromones elicited freezing behaviour and increased bottom dwelling duration. Along with the robust behavioural changes, I have shown that exposures to alarm pheromones suppresses the neuronal activity of the pars parvocellularis neurons. The immunohistochemistry data suggests that the suppression of the neuronal activity in the pars parvocellularis of the NPO, is likely to have a CRF phenotype but it is not definitive.

Further work is necessary to elucidate how these behavioural and neuronal responses to alarm pheromone exposure are integrated. The suppression of neuronal
activity in the *pars parvocellularis* leads to a variety of questions related to the identity and functions of these specific neurons as mediators of the alarm stress response. Double-labelling of c-Fos and CRF or c-Fos and UI would be important as a next step to verify the identity of the neurons that were suppressed. Double labelling of c-Fos and AVT or IST, which are also expressed in the *pars parvocellularis*, would provide a greater understanding of whether these neurohormones play a role. Determining changes in key components of the HPI axis, such as plasma cortisol and ACTH, CRF mRNA expression in the POA and POMC mRNA expression in the pituitary would help us understand whether alarm pheromones elicit a stress response in goldfish. Developing an antibody that would provide robust c-Fos and CRF immunolabelling in brain regions other than the ones presented would help visualize neurons that are implicated in the overall response. In rats, key brain regions involved in fear response have been identified using lesion studies (Jesuthasan and Mathuru 2008). Lesion studies in fish might also help us isolate important key brain regions that are important for the alarm response. A greater understanding of the processes that mediate the alarm response, in particular, the key role that the CRF system plays in integrating the behavioural and physiological responses could help to further our understanding of the complexities of the stress axis.

**Conclusion**

Collectively, the results of these experiments further our understanding of the endocrine stress response and the control of the HPI axis (Fig. 1). This included the demonstration that the serotonergic system might play a paracrine role in regulating the HPI axis. While I managed to describe the robust behavioural response of goldfish to alarm pheromones, the neuronal activity associated with the alarm response still needs to
be further investigated. However, I have shown that there is a suppression of neuronal activity in a region that is usually implicated with the stress response. Future research needs to be carried out, especially to determine the neuronal phenotypes that are suppressed during the response. This will help further our understanding of the integration of behavioural, endocrine and neuronal response to stressors.
**Figure 1.** Schematic diagram of the tissues and primary signals of the hypothalamic-pituitary-interrenal stress axis in fish. Upon encountering a stressor, NPO CRF containing neurons release their product into the pituitary. CRF stimulates the expression of POMC and induces the cleavage of POMC into ACTH from the corticotropes in the pituitary. ACTH in turn stimulates the biosynthesis and secretion of cortisol from the interrenal cells. This study has demonstrated that 5-HT can directly stimulate the biosynthesis of cortisol at the level of the interrenal cells; the nearby chromaffin cells in the head kidney could be a potential paracrine source. The sites of serotonergic action on other components of the HPI axis are still not fully understood. Detection of the alarm pheromones through the olfactory bulbs has been shown to decrease fos-labelled cells in the *pars parvocellularis* of the NPO. The effect of alarm pheromones on the stress response still needs to be further investigated.

Abbreviations: Serotonin (5-HT), corticotropin-releasing factor (CRF), pro-opiomelanocortin (POMC), adrenocorticotropic hormone (ACTH).
Figure 1.
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