The role of nonapeptides in male reproduction in two cyprinid species, the zebrafish (*Danio rerio*) and the goldfish (*Carassius auratus*)

By
Zeinab Altmieme

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Abstract

Two distinct nonapeptide systems, consisting of the vasotocin- and oxytocin-related peptides have evolved in vertebrates, and their role in male reproduction is well-described in mammals. In contrast, their comparative role in reproduction in basal vertebrate species, and teleost fishes in particular, has not been investigated in great detail. Using two cyprinid species, the zebrafish (*D. rerio*) and the goldfish (*C. auratus*), I address the hypothesis that the teleost nonapeptides vasotocin and isotocin stimulate male cyprinid reproductive physiology by affecting central neuronal and/or peripheral endocrine pathways.

To test this hypothesis in zebrafish, an indeterminate breeder, I conducted pharmacological inhibition experiments employing vasotocin and isotocin-specific antagonists in males, a treatment predicted to inhibit reproductive success in mating trials. Because nonapeptides can act both as central peptide neuromodulators and as secreted hormone, I further quantified indices of male courtship behavior (nudging, circling and chasing) and major androgens (testosterone and 11-keto-testosterone) as key endocrine indices of the male reproductive axis. Together, these experiments revealed a dose-dependent, differential inhibition of spawning success, with significant reductions (-65%) in egg fertilization rate observed in pairs in which males had been i.p. injected with 5 ng/g vasotocin and significant reductions (-79%) observed at 500 ng/g i.p injected isotocin. In either case, these partial inhibitions of reproductive success were correlated with significant decreases in specific indices of male courtship behavior, but not endocrine indices, suggesting that individual nonapeptides mediate their effects via central modulation of behavioural neurocircuits. Interestingly, a co-administration of vasotocin and isotocin antagonists completely abolished reproductive success, however this effect was neither correlated with decreases in male courtship behavior, nor endocrine indices, suggesting a separate

mode of action, possibly at the level of male pheromone release. To further probe the role of nonapeptides in male zebrafish reproduction, I subsequently tested the hypothesis that nonapeptide systems are acutely activated by key reproductive cues, specifically the releaser pheromone $PGF_{2\alpha}$, which serves as a chemoattractant and acutely stimulates male reproductive behavior in male cyprinids. Using a chemoattractant choice assay in conjunction with immunohistochemistry and gene expression approaches, I determined whether male zebrafish are attracted to pheromonal cues and acutely activate isotocinergic neurons in the short term and/or regulate nonapeptide gene expression in the longer term. My results show that individual male zebrafish are attracted to $PGF_{2\alpha}$ in an acute choice test. Furthermore, an increase in p-ERK immunoreactivity, a marker of neuronal activation, was observed in the olfactory bulb 10 min following exposure, suggesting a specific response to the pheromone compared to EtOH vehicle. However, no co-localization of p-ERK and IT-positive perikarya was observed in the preoptic area (POA), refuting the hypothesis that $PGF_{2\alpha}$ exposure acutely activates isotocinergic neurons in zebrafish. Analysis of whole brain relative mRNA transcript abundance revealed that $PGF_{2\alpha}$ exposure time-dependently regulates whole brain isotocin, but not vasotocin transcript abundance, suggesting secondary longer-term effects of $PGF_{2\alpha}$ exposure on the isotocinergic system.

Using an analogous experimental approach, I further tested the hypothesis that nonapeptides stimulate male reproductive physiology in goldfish, a determinate breeder. Sexually mature male goldfish pretreated with saline or vasotocin or isotocin antagonists were exposed to saline or $PGF_{2\alpha}$-injected stimulus females and male courtship behavior (chasing, circling), endocrine indices (circulating testosterone) and milt release were quantified. Both nonapeptide antagonists reduced strippable male milt quantity in response to $PGF_{2\alpha}$-injected females, suggesting a neuronal or hormonal action of both nonapeptides on goldfish milt release.

Together, I show that nonapeptides contribute to male reproductive physiology in two species of cyprinids with different reproductive tactics. However, the mode of action may differ from one species to another, with evidence suggesting that nonapeptides play a role in the regulation of reproductive behavior and, possibly, male pheromone, release in zebrafish, while effects on male goldfish seem to be exclusively related to the release of milt. Future studies should compare other teleost species with specific reproductive biology and focus on the gonadal roles of nonapeptides in sperm maturation and / or release.

Résumé

Deux systèmes nonapeptides distincts, qui sont composés de peptides reliés à la vasotocine et à l'ocytocine, ont évolué chez les vertébrés, et leur rôle dans la reproduction masculine est bien décrit chez les mammifères. En revanche, leur rôle comparatif dans la reproduction chez les espèces de vertébrés basaux, et les poissons téléostéens en particulier, n'a pas été étudié en détail. En utilisant deux espèces de cyprinidés, le poisson zèbre (*D. rerio*) et le poisson rouge (*C. auratus*), je pose l'hypothèse selon laquelle les nonapeptides téléostiques, la vasotocine et l'isotocine, stimulent la physiologie de la reproduction des cyprinidés mâles en altérant les voies neuronales et/ou endocriniens.

Afin de tester cette hypothèse chez le poisson zèbre, un reproducteur indéterminé, j'ai realisé des inhibitions pharmacologiques en utilisant des antagonistes specifiques de la vasotocine et de l'isotocine chez les mâles et quantifié le taux de succès reproducteur. Étant donné que les nonapeptides peuvent agir à la fois comme neuromodulateurs peptidiques centraux et comme hormone sécrétée, j'ai quantifié d'avantage les indices du comportement sexuel des mâles (la poursuite, la poussée, l'encerclement) et des androgènes majeurs (testostérone et 11-kéto-testostérone) en tant qu'indices endocriniens essentiels de l'axe reproducteur masculin. Ensemble, ces expériences ont révélé une inhibition différentielle du succès de la ponte qui dépend de la dose, avec des réductions significatives du taux de fécondation des œufs observées chez les couples dans lesquels les mâles avaient été injecté intraperitonéalement avec 5 ng/g vasotocine (-65%) et des réductions significatives observées avec 500 ng/g d'isotocine (-79%). Dans les deux cas, ces inhibitions partielles du succès de la reproduction étaient corréliées à des baisses significatives d'indices spécifiques du comportement sexuel masculin, mais pas d'indices endocriniens, ce qui suggère que les nonapeptides individuels atténuent leurs effets par une modulation centrale de

neurocircuits comportementaux. Fait intéressant, une co-administration d'antagonistes de l'isotocine et de la vasotocine a complètement aboli le succès de la reproduction, mais cet effet n'a pas été corrélé à une diminution du comportement reproducteur masculine, ni à des indices endocriniens, suggérant un mode d'action séparé, possiblement au niveau de la libération des pheromones mâles. Pour approfondir le rôle des nonapeptides dans la reproduction chez les poissons zèbres males, j'ai par la suite testé l'hypothèse selon laquelle les systèmes des nonapeptides sont activés par des signaux de reproduction essentiels, en particulier la phéromone libératrice $PGF_{2\alpha}$, qui sert d'agent chimioattractant et qui stimule de manière aiguë le comportement reproducteur chez plusieurs cyprinidés mâles. En utilisant un test de choix conjointement avec des approches d'immunohistochimie et d'expression génique, j'ai déterminé si le poisson zèbre mâle était attiré par les signaux phéromonaux et activait les neurones isotocinergiques à court terme et / ou régulait l'expression génique du nonapeptide à long terme. Mes résultats montrent que le poisson-zèbre mâle individuel a été attiré par le $PGF_{2\alpha}$ dans un test de choix aiguë. En plus, une augmentation de l'immunoréactivité de p-ERK, marqueur de l'activation neuronale, a été observée dans le bulbe olfactif 10 min après l'exposition, suggérant une réponse spécifique à la phéromone par rapport au véhicule. Cependant, aucune co-localisation de p-ERK et de périkarya positif en isotocine dans la zone préoptique (POA) n'a été observée, réfutant l'hypothèse selon laquelle l'exposition à la $PGF_{2\alpha}$ active de manière aiguë les neurones isotocinergiques chez le poisson zèbre. L'analyse de l'abondance relative du transcrit d'ARNm dans le cerveau entier a révélé que l'exposition au $PGF_{2\alpha}$ régulait l'abondance du transcrit par l'isotocine, mais non par la vasotocine, suggérant des effets secondaires à long terme de l'exposition au $PGF_{2\alpha}$ sur le système isotocinergique.

En utilisant une approche expérimentale analogue, j'ai ensuite testé l'hypothèse selon laquelle les nonapeptides stimulent la physiologie de la reproduction chez le poisson rouge cyprinidé, un reproducteur déterminé. Des poissons rouges sexuellement matures, prétraités avec une solution saline ou des antagonistes de la vasotocine ou d'isotocine et, ont été exposés à des femelles stimulantes injectées avec saline ou $PGF_{2\alpha}$. Les deux antagonistes nonapeptidiques ont réduit la libération de laitance chez les mâles en réponse aux femelles injectées avec $PGF_{2\alpha}$, suggérant une action neuronale ou hormonale des deux nonapeptides sur la libération de laitance chez les poissons rouges.

Ensemble, je montre que les nonapeptides contribuent au succès de la reproduction chez les mâles de deux espèces de cyprinidés, qui se reproduisent de manière indéterminée et déterminée. Toutefois, le mode d'action peut différer d'une espèce à l'autre, certains éléments suggérant que les nonapeptides jouent un rôle dans la régulation du comportment reproducteur et, éventuellement, de la libération des pheromones chez le poisson zèbre, tandis que les effets sur les poissons rouges mâles semblent être exclusivement liés à la libération de la laitance. Les études à venir devraient comparer d'autres espèces de téléostéens présentant une biologie de la reproduction spécifique et se concentrer sur les rôles gonadiques des nonapeptides dans la maturation et / ou la libération du sperme.

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17,20P	17α,20β-dihydroxy-4-pregnen-3-one
AB	Antibody
AVP	Arginine vasopressin
AVT	Arginine vasotocin
C. auratus	*Carassius auratus*
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CNG	Cyclic nucleotide gated channels
CNS	Central nervous system
D. rerio	*Danio rerio*
DP	Dorsal posterior thalamic nucleus
EDTA	Ethylenediaminetetraacetic acid
EGME	Ethylene glycol monomethyl ether
ELISA	Enzyme-linked immunosorbent assay
End	Entopeduncular nucleus, dorsal part
EtOH	Ethanol
FSH	Follicle stimulating hormone
GL	Glomerular layer of the olfactory bulb
GnRH	Gonadotropin-releasing hormone
GSI	Gonadosomatic index
GVBD	Germinal vesicle breakdown
IHC	Immunohistochemistry
IT	Isotocin
ITa	Isotocin antagonist
KCl	Potassium chloride
KT	11-keto-testosterone
KP	Kisspeptin
LH	Luteinizing hormone
LOT	Lateral olfactory tract
MOT	Medial olfactory tract
MS-222	Tricaine mesylate
OB	Olfactory bulb
OR	Olfactory receptor
OT	Oxytocin
pERK	Phosphorylation of extracellular signal-regulated kinase
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
$PGF_{2\alpha}$	Prostaglandin F2 alpha
POA	Pre-optic area
POF	Primary olfactory fiber layer

Ppa	Parvocellular pre-optic nucleus, posterior part
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcriptase
T	Testosterone
VP	Vasopressin
Vp	Post-commissural nucleus of the ventral telencephalic area
VT	Vasotocin
VTa	Vasotocin antagonist
WT	Wild type

Chapter 1 - Introduction

1.1 Reproductive physiology in two cyprinid species, zebrafish and goldfish

Zebrafish (*Danio rerio*), and goldfish (*Carassius auratus*), belong to the family of *Cyprinidae*, which in turn is the most abundant family of the infraclass of teleost fishes, constituting 8% of the world's freshwater fishes (Nelson, 1994). As such, *Cyprinidae* are also considered to form the largest and most diverse vertebrate family (Nelson, 1994). Within the *Cyprinidae*, both species have diverged approximatively 60 million years ago (Ota and Abe, 2016). Native to streams of the Himalaya region, zebrafish have first been described at the end of 19th century (Spence et al., 2006), and have been used as laboratory model since the 1930s (Creaser, 1934). Zebrafish achieved vast popularity as a research model following studies pioneered by George Streisinger at the University of Oregon in the 1970 and 1980s (Streisinger, 1981). Because of well-described advantages including a rapid and easily observable development, small size, easy housing and breeding, a sequenced genome, and, more recently, the feasibility of genome-editing techniques, zebrafish have rapidly become an important fundamental and translational research model (Best et al., 2018). Zebrafish reproduction in sexually mature fish (~ 3 months post fertilization, 3 mpf), is indeterminate in laboratory conditions, and male and female fish are able to spawn approximately every 1-2 days, preferably within the first minutes after onset of light (Spence et al., 2006). In the wild, the situation is less clear, as both seasonal and extra-seasonal spawning has been described (Spence et al., 2006). It is believed that food availability and size rather than seasonal cues or biological age, are important determinants of reproductive success, leading to the designation of zebrafish as indeterminate breeders, especially in laboratory conditions (Spence et al., 2006). Differences in reproduction between wild and laboratory-housed zebrafish may be, at least to some extent, related to genetic selection within widely used strains

and artificial housing conditions (Spence et al., 2006). During a single spawning event, initiated by specific male courtship behaviours that include chasing, nudging, and encircling, female zebrafish ultimately periodically release 5-20 eggs, an event synchronized with male milt release to maximize external fertilization (Spence et al., 2006). Single clutches can result in variable egg numbers, which can reach total numbers as high as several 100 eggs per clutch. Female zebrafish egg deposition is dependent on mating with male zebrafish, and in addition to described male reproductive courtship behaviours, male gonadal pheromones have been linked to reproductive success in early studies revealing that male holding water, testis homogenates and testis fractions containing steroid glucuronides will induce ovulation in intact females, but not anosmic females with cauterized nasal epithelium (Eaton and Farley, 1974; van den Hurk and Lambert, 1983; Van Den Hurk et al., 1987). Conversely, male zebrafish courtship behaviour is triggered by female pheromones, as ovarian extract attracted male zebrafish and courtship behaviour in response to ovulating female zebrafish was only observed in control, but not anosmic zebrafish (Van Den Hurk and Lambert, 1983). Following spawning events, non-adhesive and demersal zebrafish eggs, with a diameter of approximately 0.7 mm, are released directly onto the substrate. Eggs become activated on contact with water and even in the absence of sperm, undergo a series of programmed developmental steps. Unfertilized eggs develop a perivitelline space but fail to develop beyond the first few cleavages, while fertilized eggs will continue development and hatch at 3 to 4 days post fertilization (2-4 dpf), as described by Lee et al. (1999).

Goldfish, domesticated more than a thousand years ago in China (Ota and Abe, 2016), have been used as a laboratory model teleost to study many aspects of vertebrate reproduction for decades (Trudeau, 1992; Kobayashi et al., 2002; Popesku et al., 2008; Chen et al., 2018). In goldfish, the integration of environmental signals via neuronal and endocrine circuits to coordinate physiology for determinate reproduction is comparatively well understood, owing to the fact that

species-specific assays for reproductive hormones are available (Trudeau et al., 1992). However, compared to zebrafish, goldfish research has suffered from the lack of a sequenced genome, a limitation which has only very recently been resolved (Chen et al., 2018). With regard to reproduction, male and female goldfish undergo highly seasonal development in their reproductive system, which is largely driven by photoperiod and temperature (Trudeau et al., 1992). This development is generally divided into three distinct phases in latitudes with clear seasonal climates: a somatic growth phase corresponding to long photoperiods (=sexually regressed fish), a preparatory phase characterized by increased investment into gonadal growth (=sexually recrudescent fish) in a period of short photoperiods, and a period of sexual reproduction (=sexually mature fish), which occurs at the transition from short to long photoperiod. Importantly, seasonal reproduction is maintained in captivity (Zhang et al., 2009), allowing to study a determinately breeding cyprinid in laboratory conditions. As described for zebrafish, goldfish exhibit reproduction characteristic of a many cyprinid species (Spence et al., 2006). Goldfish reproduction is characterized by male courtship behaviour that consists of male chasing nudging and circling of the female (Kawaguchi et al., 2014). Again, as in zebrafish, acute spawning events are mediated by pheromonal communication, with males detecting (conjugated) female lipid hormones excreted via urine at picomolar concentrations (reviewed by Kobayashi et al., 2002). In goldfish, the nature of these pheromones is well established. Male goldfish are able to 'spy' on female egg maturation status via 17,20 progesterone, a steroid involved in germinal vesicle breakdown (GVBD) in eggs, and acute egg release promoted by follicular rupture induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Both pheromones act in temporal sequences to prime the male endocrine system and promote sperm maturation (priming pheromone) and to promote acute spawning behaviour in response to $PGF_{2\alpha}$. Goldfish are substrate spawners, and green substrates are necessary in laboratory conditions for female spawning (Partridge, Liley, and Stacey, 1976), while male goldfish spawning behaviour

can reliably be induced by $PGF_{2\alpha}$-injected female goldfish regardless of female reproductive status (Kyle et al., 1985, Sørensen et al., 1986; Sørensen et al. 1988). In both indeterminately breeding zebrafish and determinately breeding goldfish, reproductive physiology and success is coordinated by the integration of described abiotic and biotic environmental factors by neuronal and endocrine pathways, which will be briefly reviewed for males in the following section.

1.2. Neuronal and endocrine pathways regulating male reproduction in zebrafish and goldfish

1.2.1 The preoptic area (POA)

A key part of the teleost brain involved in integrating environmental factors to coordinate reproductive physiology via neuronal and endocrine pathways is the preoptic area (POA), a forebrain region which forms a continuous structure with the hypothalamus and extends from the medio-ventral telencephalon to the anteriormost diencephalon, disappearing when it reaches the first hypothalamic nuclei (Peter and Gill, 1975; Wullimann, 1996;). Both early electric stimulation studies, as well as electrolytic lesioning studies suggested direct preoptic control of simple sexual motor systems in male teleost fishes (Peter, 1977). For example, low level (<100 μA), repeated electrical stimulation of the POA, but not telencephalic regions of male green sunfish (*Lepomis cyanellus*), evoked sperm release (Demski, Bauer and Gerald, 1975). Conversely, destruction of the POA, but not other forebrain areas by electrolytic lesioning reduced or abolished spawning reflex responses to exogenous pituitary hormones in the mummichog (*Fundus heteroclitus*), as described by Macey et al. (1974). Both electrical stimulation (Demski and Hornby, 1982; Demski and Sloan, 1985) and lesioning (Koyama et al., 1984) of the POA have also been reported in male goldfish, evoking and inhibiting sperm release, respectively. In addition to revealing a role in the

control of sperm release, electric stimulation and lesioning studies of the male POA also revealed a role in the control of reproductive behaviour. Specifically, electrical stimulation of the POA of male bluegill sunfish (*Lepomis macrochirus*) induced male courtship behaviour including circling of the female (Demski and Knigge, 1971), while lesioning studies of the POA in male goldfish almost completely abolished male chasing and nudging behaviours towards females (Koyama et al., 1984). Together, these early studies established the preoptic area as important site for the control male reproduction.

1.2.2. Efferent pathways from the preoptic area in teleost fish and their role in reproduction

Following the identification of the preoptic area as an important forebrain structure involved in male teleost reproduction, subsequent studies were directed at identifying efferent pathways regulating male reproductive physiology, as well as afferent pathways transmitting environmental and endogenous cues to regulate male reproduction. Among the efferent pathways emanating from the POA, both neuronal and endocrine pathways are well-described and will be briefly reviewed. Of the afferent pathways, the olfactory bulb link to the preoptic area is particularly well described and will be presented in more detail.

1.2.2.1 The preoptic area-spinal pathway

In vertebrates, the POA exerts an influence over other organ systems by way of its connections to the somatic and visceral motor system (Bass and Forlano, 2008). In goldfish, neuroanatomical studies provide evidence for efferent pathways from the POA to the hindbrain and spinal cord (Demski and Sloan, 1985). This neuroanatomical link, identified through retrograde labelling, is believed to be involved in mediating milt release via sperm duct contractions in response to POA stimulation. While the specific identity of the neurons labelled

through retrograde tracing has not been verified, they were morphologically identified as magno- and parvocellular neurons and speculated to be nonapetidergic in nature (Demski and Sloan, 1985).

1.2.2.2 The preoptico-hypophyseal pathway

The preoptico-hypophyseal pathway in fishes (Palay, 1945) has been a focal point of research in reproductive physiology of fishes, especially in the goldfish model. The teleost pituitary is directly innervated in teleost fish, which, in contrast to mammals, lacks a median eminence (Peter, 1990; Gorbman 1995). Retrograde tracing of PFA-fixed pituitaries in goldfish confirmed their direct innervation from the POA (Anglade et al., 1993), and subsequent studies have revealed that the preoptico-hypopheasal pathway is crucially involved in mediating many stimulatory and/or inhibitory neuronal control of the reproductive endocrine axis in teleost fish (reviewed by Trudeau, 1992). As in all vertebrates, the reproductive endocrine axis in teleost fish is stimulated by gonadotropin (GnRH), which in turn, results in the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the blood stream, from where they act on gonadal receptors to regulate gonadal growth, maturation and steroidogenesis. Sex steroids, in turn feedback to elicit central feedback on the reproductive endocrine axis (Trudeau et al., 1992). In addition to the teleost-specific direct innervation of the entire pituitary gland, the neurohypophysis in teleost fishes, as in other vertebrate species, is also directly innervated by nonapeptide neurons originating in the POA (Reaves and Hayward, 1979; Khan et al., 2010). From the teleost pituitary not only the gonadotropins LH and FSH (Trudeau et al., 1992), but also nonapeptides vasotocin and isotocin are released into circulation (Pierson et al., 1995) from where they may act on peripheral organs expressing gonadotropin and nonapeptide receptors, including gonads (Hausmann et al. 1995, Mennigen et al., 2017).

1.3. Afferent innervation of the POA and integration of reproductive cues in cyprinids

As critical component of teleost reproduction, the POA receives afferent input to integrate exogenous and endogenous environmental signals to regulate reproductive physiology. A particularly well characterized system in teleost fish is the olfactory bulb, which will be described in detail, while other afferent systems are mentioned for completeness.

1.3.1 The olfactory bulb and reproductive pheromones in zebrafish and goldfish

1.3.1.1 Neuroanatomical and functional evidence for olfactory-POA link in regulating fish reproduction

A link between the olfactory bulb and the POA has been described in several cyprinid species, including the carp (*Cyprinus carpio*), the goldfish (Fujita et al., 1984; Levine and Dethier, 1985); and the zebrafish (Miyasaka et al., 2009; Kermen et al., 2013). In the carp, projection areas from mitral cell axons of the lateral olfactory tract (LOT) and the medial olfactory tract (MOT) include areas in the bilateral telencephalon and the POA (Fujita et al., 1984). Similar neuroanatomical connections were subsequently identified in the goldfish, where both dorsolateral and ventromedial rami from the MOT innervate telencephalic targets, the POA as well as di- and possibly mesencephalic regions (Levine and Dethier, 1985). In zebrafish, GFP-reporter based tracing studies identified mitral cells project to multiple regions in the forebrain and hypothalamus (Miyasaka et al., 2009), and while evidence of exact targets of mitral cell terminals in the POA area is unknown, it has been speculated that nonapeptides may mediate reproductive olfactory cues in zebrafish (Kermen et al., 2013).

A functional role for the olfactory bulb in regulating reproduction in male cyprinids stems from early electric stimulation and lesioning studies. For example, electrical stimulation of 5mA

via suction electrodes evoked sperm release in male goldfish, an effect abolished by sectioning the medial, but not lateral olfactory bundle (Demski and Dulka, 1984). In line with this finding, sectioning of the MOT, but not LOT reduced male courtship behaviour in goldfish exposed to sexually mature female fish (Stacey and Kyle, 1983). Subsequently, more precise lesion studies not only confirmed the functional importance of this neuronal circuitry in transducing olfactory reproductive cues in goldfish to stimulate male courtship behaviour and reproductive physiology, but specified the lateral MOT bundles descending from olfactory bulb as crucial components (Weltzien et al., 2003). Because evidence from these studies are functionally similar to previously described functional studies in the POA, this suggests an important role of the olfactory POA pathway in male cyprinids.

1.3.1.2. Reproductive pheromones in cyprinid fish

Following described nasal occlusion studies in both zebrafish and goldfish models, as well as evidence from studies identifying a stimulatory role in male reproductive behaviour when exposed to water from spawning females, research focused on the chemical identification of suspected pheromones. Pheromones are chemical external signs that transmit information between conspecifics (Kobayashi, Sorensen, and Stacey, 2002). An organism's environment is a key feature, which determines the presence of pheromone activity. Various fish, and especially cyprinids, live in an aquatic environment that is characterized by dim light and murky water. These species rely heavily on pheromones, because they convey an abundance of chemical information that is independent of the level of light and vision (Kobayashi et al., 2002). In teleost fish, and cyprinids, especially goldfish, research efforts have identified specific pheromones involved in the regulation of reproductive behaviours (Sorensen and Stacey, 1999). Two key pheromones in particular, 17α,20β-dihydroxy-4-pregnen-3-one (17,20P) and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), have

been characterized, and their role in reproductive behaviour and physiology characterized (reviewed by Sorensen et al., 2002). More recently, genetic modulation of the zebrafish model (*Danio rerio*), has contributed to our understanding of reproductive pheromone function in male cyprinids through genetic approaches identifying and ablating an olfactory receptor specific to $PGF_{2\alpha}$ (Yabuki et al., 2016). Based on studies in both model species, the following section will highlight key elements of our understanding of the reproductive function of the two aforementioned pheromones, which can be functionally divided into primer and releaser pheromones.

1.3.1.3 The primer pheromone 17,20P

In seasonally breeding goldfish in our latitude, oocyte development occurs in the spring season (April to May), triggered by increasing length of photoperiods as an environmental cue (Trudeau et al., 1992, Zhang et al., 2009). In sexually mature females, increasing amounts of 17,20P are synthesized in response to increasing luteinizing hormone (LH), and act to induce oocyte maturation by activating germinal vesicle breakdown (GVBD), which terminates the oocytes meiosis I arrest and serves resumption of oocyte meiosis (Nagahama et al., 1983). In addition to these modes of action on female reproductive physiology, 17,20P is released into the water by female goldfish urinary excretions (also in the form of water soluble sulfated 17,20P) prior to ovulation ('preovulatory pheromone') at concentrations as low as 2 to 4 x 10^{-10} M (Dulka et al., 1987). Male goldfish, regardless of sexual status, are extremely sensitive to 17,20P, which induces electrical responses in the male goldfish olfactory bulb epithelium at 17,20P concentrations as low as 1 x 10^{-13} M (Sorensen et al., 1987). In exposed males, 17,20P stimulates male reproductive physiology largely via the endocrine reproductive axis: At a dose of 1 x 10^{-10} M, waterborne 17,20P increases circulating levels of luteinizing hormone (LH) in male goldfish

15 minutes after exposure, and this process appears to be under a positive feedback loop. The pheromone induced LH rise in male goldfish stimulates the testicular synthesis of 17,20P which then acts as a positive feedback on neuroendocrine controlled LH release, thus increasing circulating LH levels in males within minutes of 17,20P synthesis (Kobayashi et al., 2002). Following this immediate stimulation of LH release, milt release is significantly increased after 4-6h (Stacey and Sorensen, 1986). Several studies have demonstrated that this milt release in response to 17,20P is indeed dependent on the previous activation of the endocrine reproductive axis in male goldfish. In both hypophysectomized male fish and dopamine-agonist treated fish, (dopamine is a potent inhibitor of pituitary release of LH in goldfish - reviewed by Trudeau et al., 1998), 17,20P does not stimulate milt release (Zheng and Stacey, 1997). Therefore 17,20P is considered a primer pheromone in goldfish, which is defined as evoking changes in the endocrinology and physiological state of conspecifics (Kawai et al., 2015). In addition to this primary function, 17,20P exposure also stimulates reproductive behavior in exposed male goldfish, albeit moderately. For example, within approximately 6 hours after the LH surge, 17,20P becomes the dominant sex pheromone in male goldfish and triggers increased swimming and inspection behaviours among the male goldfish (Dulka et al., 1987). It is not known whether the behavioural effects of 17,20P are equally dependent on activation of the reproductive axis, however their timing coincides with increases in circulating androgens following 17,20P exposure (Mennigen et al., 2010), important for reproductive behavior in males (Stacey and Kobayashi, 1996). Comparatively, little is known regarding the role of 17,20P in zebrafish. A potential pheromonal role for 17,20P has only been described for its sulfated form in zebrafish (Friedrich and Korsching, 1998), and is reported to activate single clusters of glomerular cells in the olfactory bulb. However, whether 17,20S induces changes in male zebrafish behavior and reproductive physiology has not been investigated.

1.3.1.4 The releaser pheromone Prostaglandin $F_{2\alpha}$

Following 17,20P release during oocyte maturation, the inflammatory eicosanoid $PGF_{2\alpha}$ is acutely released in the process of follicle rupture leading to ovulation prior to egg deposition in goldfish. Upon its release, $PGF_{2\alpha}$ enters circulation in female fish and reaches the brain to acutely induce female reproductive behaviour in goldfish (Sorensen and Goetz, 1993; Kobayashi and Stacey, 1993). Additionally, $PGF_{2\alpha}$ is released by female goldfish in a pulsatile fashion through the urine in large quantities (>50ng/h) that coincide with male spawning activity (Appelt and Sorensen, 1999). Indeed, $PGF_{2\alpha}$ is detected by the male goldfish olfactory bulb at low concentrations of 10^{-10} M (Sorensen et al., 1999). $PGF_{2\alpha}$ principally acts to acutely stimulate reproductive behaviour (chasing and nudging), as well as sperm release, which is not dependent on hypophyseal function and LH release (Zheng and Stacey, 1997). Consequently, $PGF_{2\alpha}$ is considered a releaser pheromone in male goldfish, defined as inducing rapid behavioural responses in conspecifics. While not dependent on LH release from the pituitary, the nature of the pathways responsible for behavioural responses and milt release in response to $PGF_{2\alpha}$ remain unknown. In addition to the primary induction of reproductive behavioural responses and increases in milt release (and suppression of non-reproductive behaviours, such as feeding), $PGF_{2\alpha}$ does result in small, but significant additional increases in LH release into the circulation, and stimulates testicular androgen production after 6 hours (Mennigen et al., 2010). This effect appears, however, to occur only in groups of exposed males, but not in isolated males (Sorensen 1989, Kobayashi et al., 2002). It is therefore thought that the $PGF_{2\alpha}$-induced increases in LH are a consequence of socio-sexual interactions (Sorensen et al., 1989; Zheng and Stacey, 1996; Zheng and Stacey, 1997). A summary of the described timeline of release of the primer pheromone 17,20P and the

releaser pheromone $PGF_{2\alpha}$ by female goldfish, and the reproductive roles of both pheromones in male goldfish, are depicted in **Fig. 1.1.**

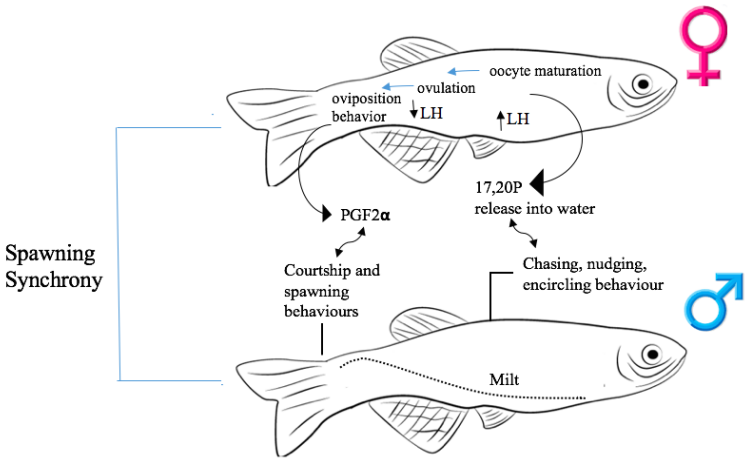


Figure 1.1. *Schematic model of female goldfish pheromones and their physiological and behavioural effects on male conspecifics. Adapted from Stacey et al., 2009.*

Recently, zebrafish have equally been shown to respond to $PGF_{2\alpha}$ (Yabuki et al., 2016), but not other prostaglandins (Pradhan and Olson, 2015). Taking advantage of genomic resources and tools (genetic ablation through knock-out) available in the zebrafish model, Yabuki and colleagues (2016) found that $PGF_{2\alpha}$ function is mediated by two specific odorant receptor paralogues, termed OR114-1 and OR114-2, which are expressed in the olfactory bulb. This provided, for the first time, evidence for a specific receptor of $PGF_{2\alpha}$ pheromones necessary for the initiation of signal transduction in the olfactory bulb to mediate male cyprinid chemo-attraction to this compound. The study also revealed that the signal is transduced to brain areas highly implicated in reproductive behavior and physiology, such as the ventral telencephalic area and parvocellular neurons in the preoptic area (Yabuki et al., 2016). However, the nature and function of these neurons in mediating $PGF_{2\alpha}$ reproductive effects in male zebrafish remain currently unknown.

1.4. Teleost nonapeptides

1.4.1. Evolutionary history of teleost nonapeptides

Nonapeptides are peptide chains that are made up of nine amino acid residues, which form ring structures through disulfide bridges between cysteine residues at position 1 and 6 of the peptide chains (Banerjee et al., 2017). Vertebrate nonapeptides are divided into two groups, which arose from ancestral gene duplication (Gwee et al., 2008; **Fig. 1.2.**). The two nonapeptide groups present in all vertebrates are determined by a single amino acid substitution at position 8 (arginine in the ancestral vasotocin lineage, isoleucine in oxytocin-like peptide lineage), dictating the specific function and activity of the nonapeptide (Banerjee et al., 2017). Additional substitutions within both groups give rise to specific nonapeptide forms in vertebrate classes. In the infraclass of teleost fish, the two nonapeptides present are the ancestral vasotocin and isotocin, which evolved from the ancestral vasotocin form by the aforementioned R->I substitution at position 8 characteristic of the oxytocin-like peptide lineage, and an additional Q->S substitution at position 4 (**Fig. 1.2**).

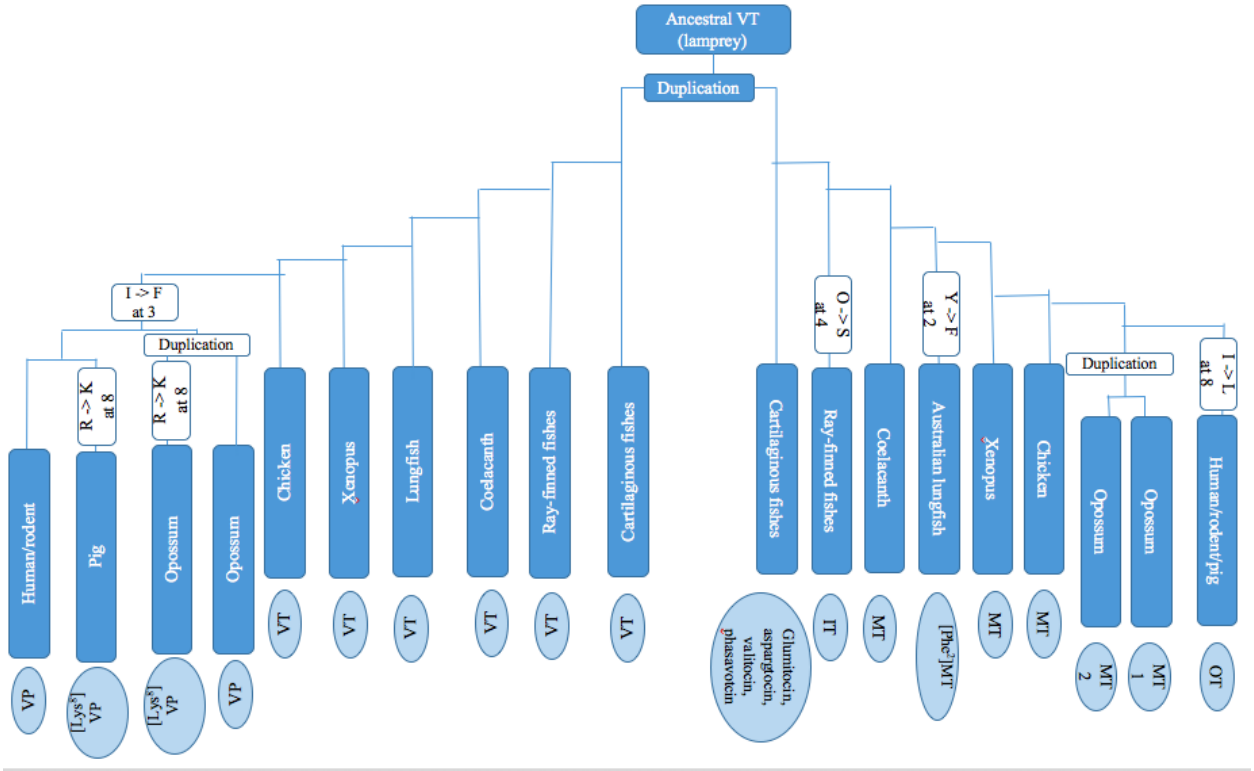


Figure 1.2. *Evolution of nonapeptides in vertebrates. D signifies gene duplication, while circles signify an amino acid substitution adapted from Gwee et al., 2008.*

1.4.2. Nonapeptide systems in teleost fish

The neuroanatomic organization of vasotocin and isotocin nonapeptide systems is relatively well-established in teleost fish, and their comparative organization compared to other vertebrates has recently been reviewed (Knobloch et al., 2014; Albers, 2015). Briefly, both nonapeptides are highly expressed in separate giganto-, magno- and parvocellular perikaya in the preoptic area in all teleost species investigated to-date, including zebrafish and goldfish (Reaves et al., 1979; Eaton et al., 2008; Khan et al., 2010; Canosa et al., 2011; Mennigen et al., 2017). Recent comparative neuroanatomical studies in cichlids with different social behaviour have revealed potential species-specific organization of nonapeptidergic perikarya in the POA (Reddon et al., 2017), reinforcing the notion that structural differences may contribute to differential roles

of nonpeptidergic systems between species (Goodson and Bass, 2011). However, it is worth pointing out that studies in teleost models, such as zebrafish (Eaton et al., 2008; Coffey et al., 2013) and the African cichlid fish (*Astatotilapia burtoni*), as described by Rodriguez-Santiago et al. (2017), have demonstrated small neuronal populations expressing nonapeptides in olfactory bulb, forebrain and the hypothalamus, thus beginning to challenge the long-held notion of a singular origin of vertebrate nonapeptide perikaryal restricted to the POA. In the POA, the separate neuronal populations are capable of synthesizing vasotocin and isotocin, are also characterized by different neuronal signaling dynamics, reinforcing the notion of separate functions. Indeed, while both magnocellular vasotocin and isotocin neurons in rainbow trout (*Onchorynchus mykiss*) form clusters of 2-5 cells which exhibit synchronization of periodic Ca^{2+} pulses, the anatomical connection and pattern of Ca^{2+} oscillation patterns differ (Saito and Urano, 2001), a fact potentially related to the presence of electrical coupling between different isotocin but not vasotocin clusters Saito et al., 2004). A recent study in zebrafish revealed that Cnga5, a novel cyclic nucleotide-gated and calcium permeable ion channel was specifically enriched in isotocin but not vasotocin neurons of the POA, providing a molecular correlate that may also underlie different Ca^{2+} dynamics between both cell populations.

From the teleost POA, both isotocin and vasotocinergic cells prominently project to the neuronal part of the pituitary gland, but also innervate diverse brain areas, which include the olfactory bulb, ventral telencephalon, ventral thalamus, and various mesencephalic areas (Saito et al., 2004; Holmqvist and Ekström, 1995; Godwin and Thompson, 2012). At least in rainbow trout, a single nonapeptide neuron can simultaneously innervate the pituitary gland and diverse brain areas (Saito et al., 2004), a unique report in vertebrates (Godwin and Thomson, 2012). This suggest that neuromodulatory and endocrine regulation may be coordinated by single nonapeptidergic neurons. Evidence for both isotocinergic and vasotocinergic innervation of the spinal cord from

immunohistochemistry studies in rainbow trout (Van den Dungen et al., 1982), while vasotocinergic innervation from the POA has been shown to extend to the hindbrain regions implicated in motor responses in goldfish (Thompson and Walton, 2009). In zebrafish, vasotocin receptor 1a expressing neurons in the hindbrain have been shown to be connected to neurons extending from the POA, suggesting motor output in response to POA signaling is mediated via a vasotocinergic POA-spinal pathway in this species as well (Iwasaki et al., 2011). In general, nonapeptidergic innervation of diverse brain areas, again at least in the rainbow trout brain, is denser for isotocin fibers compared to vasotocin fibers (Van den Dungen, 1982; Saito et al., 2004).

The nonapeptidergic innervation of the neuronal part of the pituitary gland has been well-described in several teleost species, and has been resolved at the ultrastructural level by electron microscopy in (Batten et al., 1999). This research provided evidence for possible modulatory roles of nonapeptide fibers on peptide hormone storing pituitary cell types via *puncta adherentia*, in addition to the quantified release of nonapeptides in the teleost blood stream (Pierson et al., 1995; Kleszczyńska et al., 2006). In the few teleost species investigated in detail, nonapeptide receptors are expressed in not only in the diverse innervated brain areas including olfactory bulb and hindbrain, but also in peripheral tissues that include gonads (Hausmann et al., 2004; Lema et al., 2010, 2015). Together, the structure of both teleost isotocin and vasotocin nonapeptide systems reveal that these nonpeptides may act both centrally as neuromodulator, or peripherally as hormone (reviewed by Banerjee et al. 2017), potentially allowing for neuronal and endocrine modulation of reproductive function.

1.4.3. Reproductive function of nonapeptides in teleost fish

Neuroanatomical evidence obtained from a few teleost species has implicated nonapeptides in reproductive neuronal circuits. For example, a co-localization of isotocin and GnRH has been described in the POA in the dwarf gourami, *Colisa lalia* (Maejima et al., 1994), and evidence in medaka reveals that the reproductive neuropeptide kisspeptin directly regulates vasotocin and isotocin neurons, as both neuronal populations in the POA abundantly express the kisspeptin receptor *gpr54-2*, (Khanda et al., 2013; Nakajo et al., 2018). Similarly, studies investigating hypophysiotrophic nonapeptidergic innervation in different teleost species (Goossens, 1977; Batten 1986; Batten et al., 1999) suggests contact of at least some nonapeptide fibers with gonadotrophs in goldfish, rainbow trout, European plaice (*Pleuronectes platessa*) and the sailfin molly (*Poecilia latipinna*).

Additional, albeit indirect, evidence for a reproductive role of nonapeptides in teleost fish also stems from studies investigating sexual dimorphisms in nonapeptide gene expression, as well as correlative studies investigating nonapeptide transcript abundance, and more recently, protein content, in brain and pituitary of teleost species in different reproductive contexts. For example, sexually dimorphic expression of both vasotocin and isotocin was identified in the POA of medaka, with higher expression in males compared to females (Ohya et al., 2006; Kawabata et al., 2012). Further evidence for sex-specific expression of nonapeptide systems in the POA stems from teleost fish with plastic reproductive phenotypes involving sex changes in response to environmental cues. For instance, in the blue-banded goby (*Lythrypnus dalli*), in which dominant females undergo sex change to become males, forebrain isotocin protein, more abundant in females in this species, decreased in the terminal phases of the female to male sex-change (Black et al., 2004). Similarly, IT immunoreactive cells were more numerous in the forebrain of a female blue-banded golby, than a dominant male, but decreased during the female to male sex change (Black et al., 2004). In the bluehead wrasse (*Thallosama bifasciatum*), a species characterized by high reproductive plasticity

with different reproductive tactics exhibited male morphs, reveal that sneaker males have a 'feminized' brain transcriptomic signature, which includes increased isotocin transcript abundance.

Finally, evidence from species with determinate breeding patterns often reveal increased seasonal expression of specific nonapeptides, which correlates with sexual maturity. For example, several studies investigating preoptic gene expression of nonapeptides in chum salmon (*Oncorhynchus keta*) demonstrated dynamic and complex sexually dimorphic nonapeptide transcript abundance changes during seasonal anadromous migration in the reproductive season, which have been postulated to correlate with sex steroids (Ota et al., 1996, Hiraoka et al., 1997, Ota et al., 1999). Indeed, a direct role for sex steroids in modulating sex specific nonapeptide gene expression has recently been described in the medaka brain, where gonadal androgen sex-specifically induced isotocin gene expression in males (Yamashita et al., 2017). Indirect evidence for gonadal steroid regulation of nonapeptides also stems from a recent study in goldfish, which identified membrane estrogen receptor *gpr30* as being expressed in isotocin neurons (Mangiamele et al., 2017). In a seasonal transcriptomic analysis of female goldfish forebrains across different reproductive stages, forebrain isotocin mRNA abundance in the hypothalamus was highest in sexually mature females (Zhang et al., 2009). Similarly, in stickleback (*Gasterosteus aculeatus*), whole brain protein levels of vasotocin and isotocin were highest in reproductive months in both males and females. Recently acute mating paradigms in stickleback revealed sex specific modulation of vasotocin and isotocin, which depended on the specific reproductive context: In female sticklebacks, the highest concentrations of female whole brain vasotocin has been reported in females maintained with non-courting males, while the highest concentrations of female whole brain isotocin were observed in dyads with courting males (Kulczykowska et al., 2014). Conversely, in male sticklebacks with nest, isotocin was significantly increased compared to males

without nest when exposed to a mirror image (Kleszczyńska et al., 2012). Together, several lines of evidence strongly suggest different reproductive roles for nonapeptides in several teleost species. While more limited, especially in male fish, direct functional evidence for reproductive roles of teleost nonapeptides, obtained from direct modulation of nonapeptide systems, will be reviewed in the following paragraph.

Central effects of nonapeptides in reproductive behavior have been characterized in some fish species. In a landmark study conducted in the midshipman fish (*Porichtys notatus*), the authors used pharmacological inhibition approaches to demonstrate that isotocin and vasotocin mediate sex-specific and morph specific reproductive vocalization patterns in males and female fish (Goodson and Bass, 2000). Specifically, vocalization patterns of females courting for males were vasotocin controlled, while females and sneak spawning males exhibited vocalization patterns controlled by isotocin. Subsequent pharmacological inhibition in a male monogamous convict cichlid (*Amatitlania nigrofasciata*), have since demonstrated that paternal behavior is also controlled by isotocin (O'Connell et al., 2012). In male goldfish, nonapeptides appear to regulate social approach behaviours in both non-reproductive (Thompson and Walton, 2004) and reproductive contexts (Mangiamele et al., 2013). In non-reproductive male-male encounters, isotocin stimulates, while vasotocin inhibits social approach behaviour (Thompson and Walton, 2004). Male goldfish exposed to the pheromone androstenedione avoided competing males, but not sexually receptive females, an effect mediated by vasotocin (Mangiamele et al., 2013). Finally, a recent study employing medaka KO models demonstrated that vasotocin mutant males exhibited decreased sexual motivation, but normal aggression in triadic settings consisting of 2 males and one receptive female (Yokoi et al., 2015). With regard to a central modulatory role of the endocrine reproductive axis in teleost fish, studies in goldfish have shown that isotocin stimulates LH release and estradiol concentrations in females *in vivo* (Mennigen et al., 2008; Popesku et al., 2011) and

in mixed sex primary pituitary cell cultures in *vitro* (Mennigen et al., 2017). In the sailfin molly, vasotocin stimulates LH release from gonadotrophs *in vitro*, with higher sensitivity in male gonadotrophs compared to female gonadotrophs (Groves and Batten, 1986).

Evidence for peripheral reproductive effects of nonapeptides largely stem from studies in female Asian stinging catfish (*Heteropneustis fossilis*), in which vasotocin stimulates ovarian steroidogenesis (Singh et al., 2009) and oocyte maturation (Joy and Chaube, 2015, Rawat et al. 2017). With regard to peripheral nonapeptide function on male gonads in teleost fish, evidence for a stimulatory role of vasotocin and, to a lesser degree, isotocin on testicular steroidogenesis has been reported using incubation of rainbow testes slices *in vitro* (Rodriguez et al., 1991). Studies in the mummichog, and the African catfish (*Clarias gariepinus*), equally suggest more prominent role of vasotocin compared to isotocin in regulating contraction and milt release *in vitro*, and via peripheral i.p. injection *in vivo*, suggesting endocrine (or potentially paracrine) roles for nonapeptides in mediating sperm release in the absence of central, neuronally transmitted signaling via the spinal cord (Pickford and Strecker, 1977, Viveiros et al., 2003). Overall, correlative and a few functional studies suggest roles for nonapeptides in reproductive physiology in diverse teleost species with different reproductive tactics. However, while roles of nonapeptides have recently begun to be investigated in zebrafish (Braida et al., 2012), potential roles on reproductive physiology in zebrafish are completely unknown, and remain currently limited in goldfish, especially in males. Thus, my research set out to fill this current gap in knowledge, as discussed in the following section.

1.5. Hypothesis and Objectives

As described previously, the POA in male cyprinids is important in reproductive behaviour and neuronal and endocrine mediated milt release on the one hand, and is directly innervated by the olfactory bulb on the other, it is a likely candidate to serve as a major relay node to integrate pheromonal signaling and well-characterized reproductive effects in male cyprinid fish. Indeed, recent studies in goldfish and zebrafish have revealed acute activation in neuronal perikaya in the POA area in response to the well characterized, important reproductive pheromone systems in response to both 17,20P and $PGF_{2\alpha}$ (Kawai et al., 2015; Yabuki et al., 2016). In a previous study, Mennigen et al. (2010) reported significant increases in both isotocin and vasotocin expression in micro-dissected telencephali containing the preoptic area in male goldfish exposed to $PGF_{2\alpha}$, but not 17,20P. This finding suggests that nonapeptide neurons may be acutely activated by $PGF_{2\alpha}$, and may be critically involved in regulating reproductive physiology in male goldfish and possibly other cyprinids. Given the potential the location of the nonapeptide system POA and its potential to affect male reproductive physiology and success via central neuronal regulation of courtship behaviour and sperm release, as well as peripherally via endocrine mechanism (**Fig. 1.3)**.

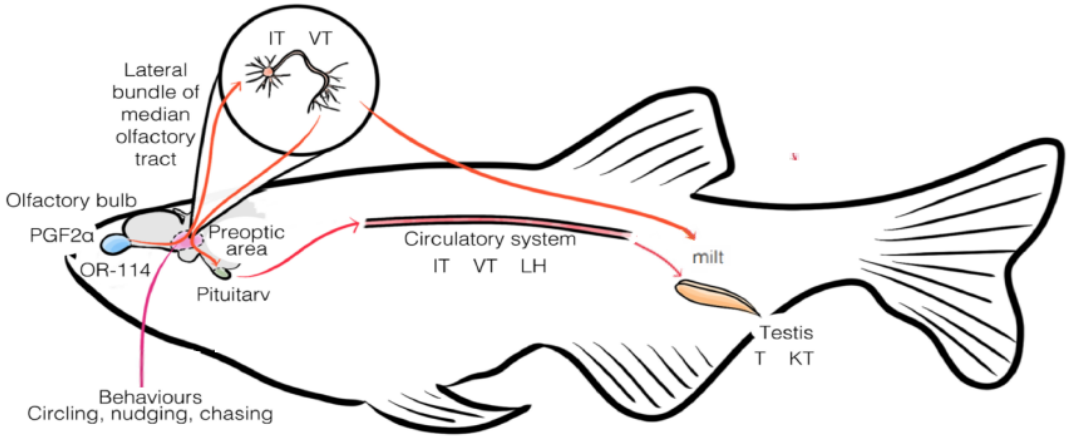


Figure 1.3. *Schematic illustration of the proposed pheromonal regulation of nonapetides and potential functional neuronal and endocrine pathways through which nonapeptides may regulate male reproduction.*

I here investigate the hypothesis that nonapeptides are important regulators of male cyprinid reproduction in two important models, indeterminately breeding zebrafish and determinately breeding goldfish. I addressed this hypothesis via two distinct objectives in zebrafish (**Chapter 2 and 3**). Firstly, I determined whether the nonapeptides individually and/or in combination affect male reproductive success in zebrafish, an indeterminate breeder, and whether changes in male reproductive success correlated with behavioural or endocrine effects. To address these questions, I employed a pharmacological inhibition approach in conjunction with behavioural assays and androgen quantification assays. Secondly, I determined whether components of the nonapeptide systems in zebrafish are capable of responding to the key reproductive pheromonal cue ($PGF_{2\alpha}$) either acutely (assessed by an immunohistochemistry approach) or in the longer-term (assessed by quantifying whole brain transcript abundance).

I subsequently tested the same hypothesis in goldfish, a determinate breeder, using an integrative approach that combined both objectives used in zebrafish to determine whether pharmacological inhibition of nonapeptides affects male reproductive endpoints via neuronal and/or endocrine mechanisms (courtship behaviour, circulating androgen concentration) at baseline or in response to $PGF_{2\alpha}$. Results are discussed and discussed in the context of the current state of knowledge in the field in **Chapter 5**.

Chapter 2 - Regulation and function of nonapeptides in male zebrafish reproduction

I would like to acknowledge the contributions of several undergraduate volunteers (Tess Julian, Georges Côté, Kenan Touma) and UROP student (Manuela Fonseca), who have contributed to the blind analysis of male zebrafish courtship recorded during the zebrafish mating assays.

2.1. Pharmacological inhibition studies to probe the role of nonapeptides in male reproductive physiology in zebrafish

In order to directly test the hypothesis that male nonapeptides, alone or in combination, are important contributors of male reproductive success, we used a systemic pharmacological inhibition approach. The specific predictions for this experiment were (1) that if nonapeptides mediate male reproductive success, systemic pharmacological inhibition should decrease fertilized egg counts, a measure of reproductive success, and (2) that if central and/or endocrine mechanisms are involved, a significant correlation between reproductive success and male courtship indices or whole-body androgen concentrations should be identifiable across treatment groups.

2.1.1. Materials and methods

2.1.1.1 Animal housing and experimental design

Wild type male and female zebrafish were obtained from the University of Ottawa Aquatics Facility. All fish used were between 10-14 months old and sexually mature. Fish were housed in individual 12 L glass tanks at a density of 2 fish/L and fed a mixed diet (Adult Zebrafish diet, Zeigler Bros Inc, Gardners, PA, USA; Larval AP-100, Zeigler Bros Inc, Gardners, PA, USA; Golden Pearls, Artemia International, Fairview, TX, USA) twice a day. The tank water was continuously filtered and changed once a week with 28 °C system water. The fish were housed in a room with a 12 h light cycle 8:00 h-23:00 h and at a room temperature maintained at 27 °C. Two weeks prior to the beginning of the experiments, male and female fish were separated by sex by visual inspection of pectoral fin breeding tubercles, body shape and coloring (Yossa et al., 2013; MacMillan et al., 2015). One day prior to the mating assay, pairs consisting of a single male and female zebrafish were set a 2 L breeding tank at noon, separated by a divider and left undisturbed

overnight. The following day, male zebrafish were removed from the tank and anesthetized using 0.24 mg/mL of tricaine (Syndel laboratories, Nanaimo, BC, Canada) as described by Westerfield (2000). Following the confirmation of deep anesthesia by gentle pinching of the tail, male fish then received intraperitoneal injections of either physiological saline and different concentrations of vasotocin antagonist (Experiment A), physiological saline and different concentrations of isotocin antagonist (Experiment B), or physiological saline and a combination of both antagonists at equal concentrations (Experiment C). In Experiment A, control fish were injected with 0.9% saline solution, while fish treated the selective vasopressin 1A receptor antagonist ($d(CH_2)_5^1$, $Tyr(Me)^2$, Arg^8) vasopressin, also termed Manning's compound (Tocris Bio-Techne, Oakville, ON, Canada), was i.p. injected with low (5 ng/g), medium (50ng/g) or high (500 ng/g) concentrations. This antagonist has previously been validated in injection studies in diverse vertebrate classes, including mammalian (Piet et al., 2015), avian (Barab et al., 2016) and, importantly, teleost fish models (Backström and Winberg, 2009; Soares et al., 2012, Huffmann et al., 2015) and shown to be biologically active in the ng/g to low µg/g concentration range. Similarly, in Experiment B, control fish received an i.p. injection of 0.9% saline, while treated fish received i.p. injection of the oxytocin antagonist L-368,899 hydrochloride (Tocris Bio-Techne, Oakville, ON, Canada) at low (5 ng/g), medium (50 ng/g) or high (500 ng/g) concentrations, respectively. Again, the antagonist had previously been validated in several vertebrate classes (Dölen et al., 2013; Yao et al., 2017), including teleost fish (Zimmerman et al., 2016). For the combined vasotocin and antagonist treatment, male fish were injected with either physiological saline control or a mixture of equal concentrations of both 50 ng/g vasotocin antagonist and 50 ng/g isotocin antagonist). These concentrations were based on observations from the previously conducted individual Experiments A and B. The injection volume in all Experiments (A-C) was 1 µl/g body weight. Following injections, the male fish was then placed in a recovery tank and allowed to recover for 5 minutes

before being placed back in the breeding tank. The divider was then removed and the male and female fish were allowed to interact for 2 h, of which the first 15 min were video recorded to quantify reproductive behaviour. Following the exposure, eggs, if present were quantified and categorized as described in the following section, and animals were anesthetized using a concentration of 0.72 mg/ml tricaine (Westfield, 2000). Following terminal anesthesia, a subset of animals (n=9 per group) were immediately flash frozen and stored at -80 °C for whole body androgen quantification. All experimental procedures are summarized in **Fig. 2.1.** and were approved by the University of Ottawa Animal Ethics under protocol #BL-2811.

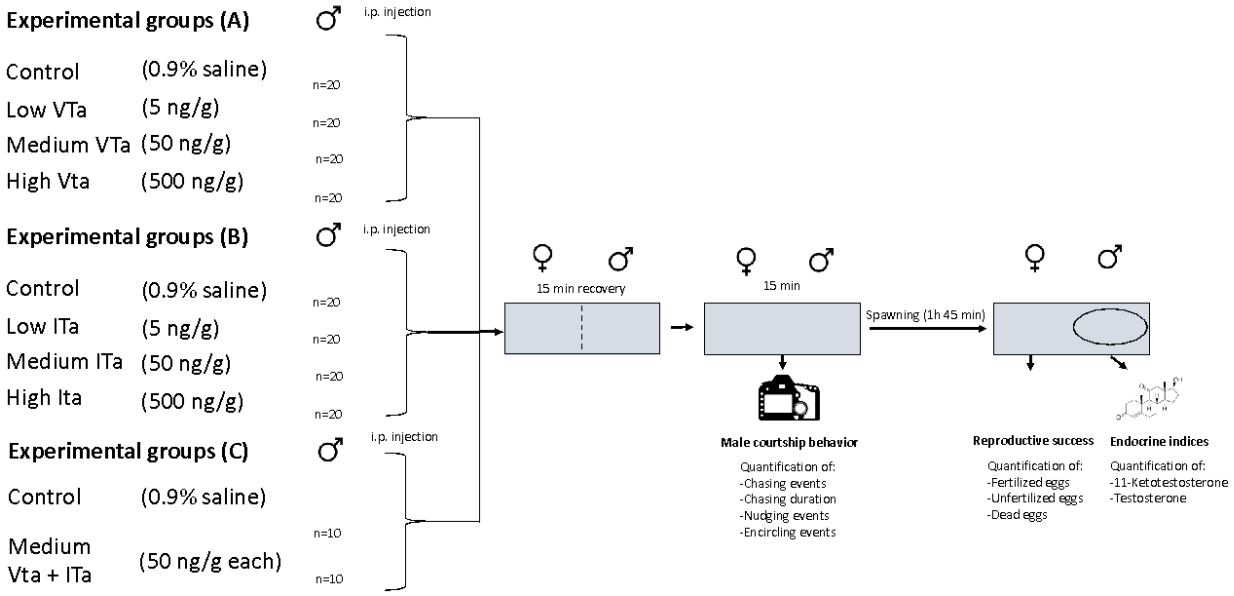


Figure 2.1. *Schematic illustration of the experimental design using pharmacological inhibition of vasotocin and isotocin to determine the role of nonapeptides on male reproductive success, male courtship behaviour and the male reproductive endocrine axis.*

2.1.1.2. Zebrafish reproductive success quantification

Following the 2 h interaction period, eggs, if present, were collected using a strainer and placed in a petri dish filled with system water. The total number of eggs, were then immediately counted under a dissecting microscope, and, in a recount, quantified as fertilized (translucent, symmetrical with increased perivitelline space), unfertilized (slightly yellowish, granular looking eggs) and dead eggs (whitish in color and often broken down).

2.1.1.3. Male zebrafish courtship behaviour analysis

The first 15 min of the zebrafish mating behaviour was recorded using the Zebrafish behaviour platform (Viewpoint Behaviour Technology, Montréal, QC, Canada), using a Dragonfly2 DR2-HIBW camera (Point Grey Research, Richmond, BC, Canada) with 30 frames/s. Following initial analyses of male zebrafish courtship behaviours to establish a high degree of correlation of behaviour scoring between different observers (>90%), the number of individual chasing events (**Fig. 2.1**), the total duration of chasing in seconds (**Fig. 2.2**), the number of nudging (**Fig. 2.3)** and encircling events (**Fig. 2.4**) were analyzed by observers blind to the treatment.

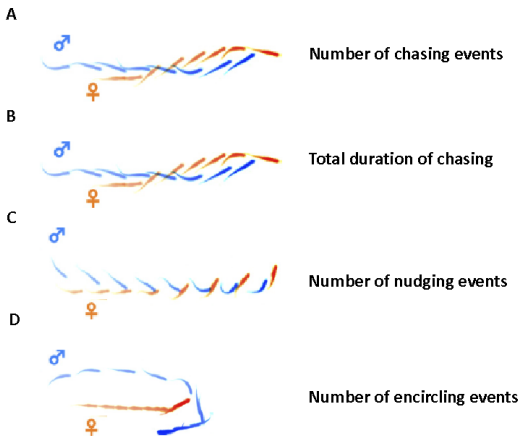


Figure 2.2. *Schematic illustration of quantified male zebrafish courtship behaviour. The male fish is indicated in blue, the female fish is indicated in orange, adapted from Yabuki et al., 2016.*

2.1.1.4. Male zebrafish whole body lipid extraction and androgen quantification

Frozen male fish stored at -80 °C were homogenized and lipids were extracted using an adapted Folch extraction procedure prior to enzyme-linked immunosorbent assay (ELISA)-based quantification of two key teleost androgens, 11-ketotestosterone and testosterone. Briefly, upon retrieval from storage, frozen fish samples were immediately immersed in liquid nitrogen and individually ground up to a fine powder using a mortar and pestle. Individual fish powder was then transferred into separate 50 mL Falcon tubes, to which 15 mL Folch solution (2:1 volume ratio of chloroform and methanol) was added. The mixture was then homogenized using a Sonic Dismembrator Model 100 (Fisher Scientific, Ottawa, ON, Canada) at a setting of 4, after which the mixture was allowed to sit for 15 min at room temperature. After the incubation period, 5 mL of a solution containing 2M KCl and 5mM EDTA was added to each tube. The tubes were then vortexed and again allowed to sit at room temperature for 30 min to allow for separation of aqueous and organic phases. The organic phase was extracted using a pipette and placed into clean test tubes. The test tubes were dried using a nitrogen stream for approximately 1 h, and dried constituents resuspended in a volume of 0.5 mL of EGME (Ethylene glycol monomethyl ether, Sigma-Aldrich, Oakville, ON, Canada). The reconstituted solution was stored in the -80°C freezer until measurement by ELISA assays. For quantification of testosterone, samples were 1:2 diluted in EGME prior to using the TEST96 ELISA kit (Teco diagnostics, Anaheim, CA, USA) according to the manufacturer's instructions. For the 11-ketotestosterone quantification, samples were prediluted 1:400 in assay buffer and analyzed using the 11-ketotestosterone ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The KT assay has previously been validated in teleost fish (O'Connell, 2012). In both assays, samples were run in duplicates and samples with intra-assay CVs of <10% were retained.

2.1.1.5. Statistical analysis

For each individual experiment and endpoint, raw data from each treatment group were tested for normality and homoscedasticity using the Shaprio-Wilk test and Bartlett's test, respectively. In normally distributed groups, Grubb's outlier test was used to identify and remove single outliers, if applicable. In cases where raw data or standard transformed (log, sqrt, inversion) datasets met both criteria, a one-way analysis of variance (Experiment A and Experiment B), or two-tailed t-tests (Experiment C) were used to identify significant differences between treatment groups which were subsequently resolved by Tukey's post-hoc test. In cases raw or transformed data did not meet ANOVA criteria, data were analyzed by a Kruskal-Wallis test and specific differences between groups resolved using Dunn's nonparametric comparison post-hoc test. In all cases, a *P*-value <0.05 was considered as cut-off for significance. These analyses were conducted and graphs plotted using Prism Version 7 (Graphpad Software, LaJolla, CA, USA). For the subset of individual animals for which male reproductive success data, courtship behaviour data and endocrine data was present, a principal component analysis was conducted to identify whether behavioural or endocrine indices correlated with reproductive success across treatment groups in individual experiments. These analyses were conducted and graphs plotted using XLSTAT software (Addinsoft, New Tork, NY, USA).

2.1.2. Results

2.1.2.1. Male reproductive success in mating trials is significantly decreased following pre-treatment with 50 ng/g vasotocin antagonist

The overall amount of fertilized eggs in response to different concentrations of i.p. injected vasotocin antagonist (Manning's compound) (**Fig. 2.3A**) was significantly different between treatment groups ($H_{3,79}$=8.17; P=0.04), with a significantly decreased amount (-65%) of fertilized eggs in fish administered the medium vasotocin antagonist concentration compared to saline control (P<0.05). Conversely, the number of unfertilized eggs in groups administered low or medium concentrations of vasotocin antagonist were not significantly different from either the saline control group or the group receiving the medium vasotocin antagonist concentration (P>0.05). Both unfertilized eggs ($H_{3,79}$=4.93; P=0.18) and dead eggs ($H_{3,79}$=3.57; P=0.31), did not reveal significant differences between treatment groups (**Fig. 2.3B-C**).

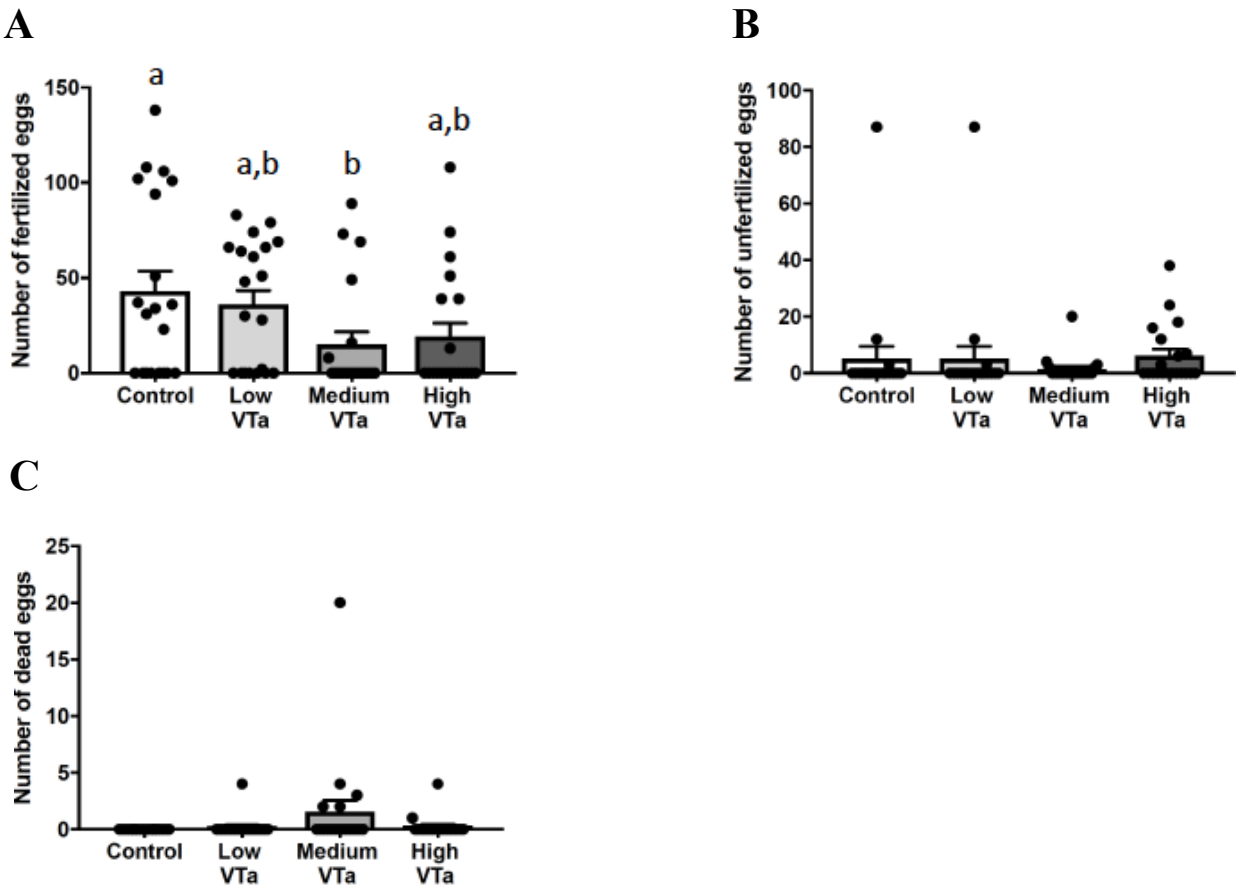


Figure 2.3. *Average number of fertilized eggs (**A**) unfertilized eggs (**B**) and dead eggs (**C**) in pairs in which males received either saline control injection or different doses of vasotocin antagonists (Manning's compound - 5,50,500 ng/g) by i.p. injection prior to 2 h mating trials. Individual data for n=20 trials per group are shown and average values S.E.M. indicated. Letters indicate significant differences between groups as determined by Dunn's post-hoc test following significant Kruskal-Wallis omnibus tests.*

2.1.2.2. Several indices of male courtship behaviour are significantly suppressed in males injected with 50 ng/g vasotocin antagonist prior to mating trials

All male courtship indices quantified were significantly changed between treatment groups. Both number (**Fig. 2.4A**; $F_{3,39}$=3.80; P=0.02) and duration (**Fig. 2.4B**; $H_{3,39}$=11.92; P=0.01) of chases were significantly reduced in fish injected with the medium dose of the vasotocin antagonist compared to control (P<0.05). Similarly, the number of nudges (**Fig. 2.4C**;

$H_{3,39}$=10.36; *P*=0.01) and encircling events (**Fig. 2.4D;** $F_{3,39}$=4.09; *P*=0.01) were significantly reduced in fish injected with medium concentration of the vasotocin antagonist compared to control (*P*<0.05). In the case of encircling behaviour (**Fig. 2.4D**), the fish administered a medium concentration of vasotocin antagonist also exhibited a reduced number of encirclements compared to fish administered a low dose of vasotocin antagonist (*P*<0.05)**.**

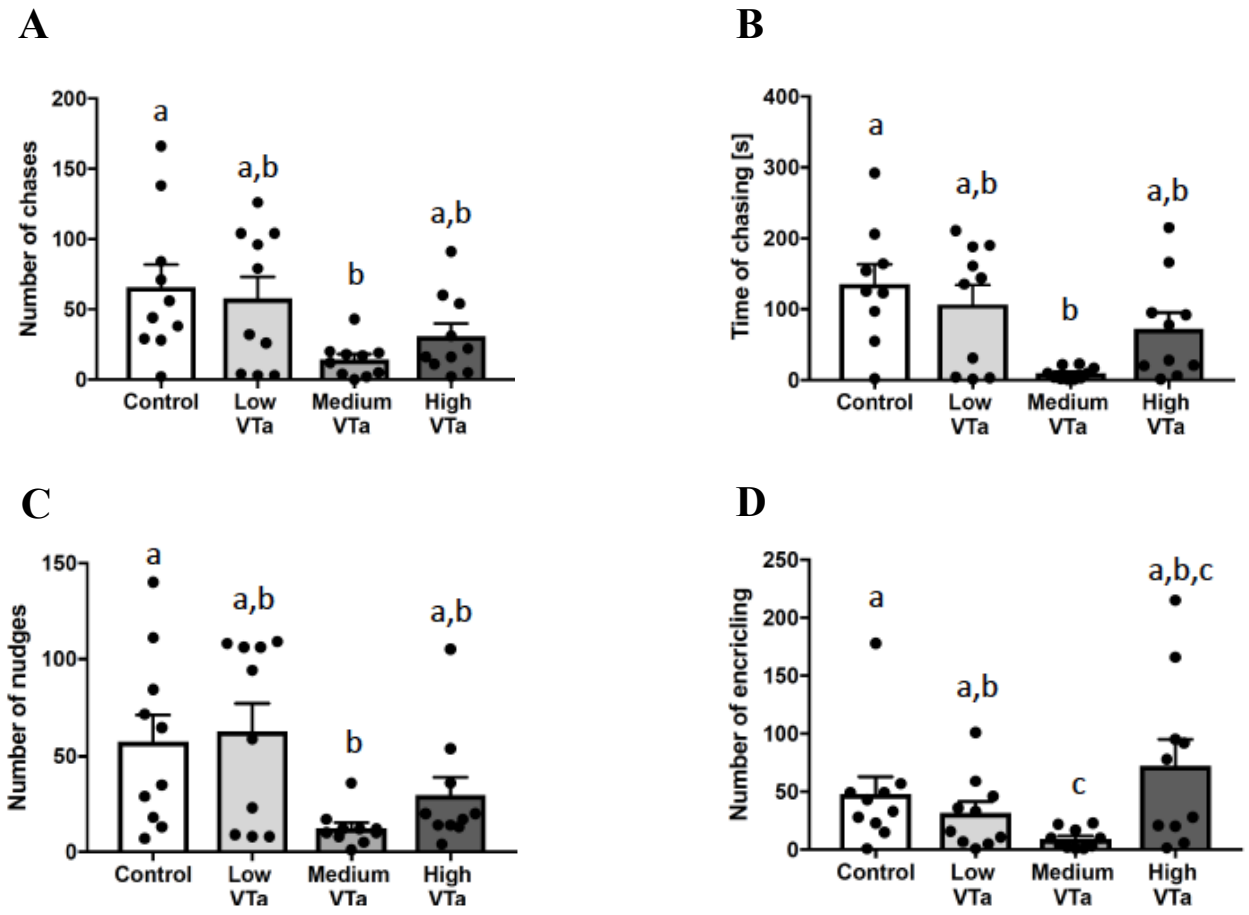


Figure 2.4. *Indices of male courtship behaviours in pairs in which males received either saline control injection or different doses of vasotocin antagonists (Manning's compound - 5,50,500 ng/g) by i.p. injection prior to 2 h mating trials. The data was obtained from n=10 trials, of which the first 15 min were recorded and analyzed. Quantified male courtship behaviours are number of chases (**A**), the duration of chasing time (**B**), the number of nudges (**C**) and the number of encircling behaviour (**D**). For each behaviour data from individual trials in each experimental group are shown and average values S.E.M. indicated. Letters indicate significant differences between groups as determined by Tukey's or Dunn's post-hoc analysis following significant one-way ANOVA or Kruskal-Wallis omnibus tests.*

2.1.2.3. Male whole-body androgen concentrations following mating trials are not affected by prior vasotocin antagonist treatment

No significant differences in whole body testosterone (**Fig. 2.5A**; $F_{3,35}$=1.92) or 11-ketotestosterone concentrations (**Fig. 2.5B**; $F_{3,35}$=1.03 *P*=0.39) were identified in male fish.

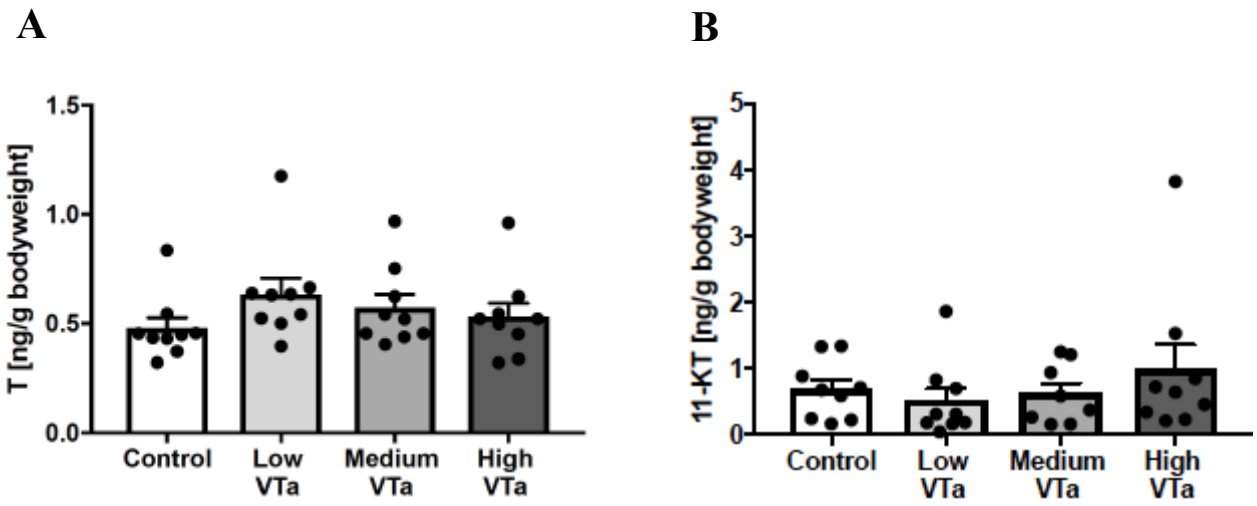


Figure 2.5. *Whole body androgen concentrations normalized to body weight in males from breeding pairs in which males received either saline control injection or different doses of vasotocin antagonists (Manning's compound - 5,50,500 ng/g) by i.p. injection prior to 2 h mating trials. The data was obtained from n=9 individual males per group are shown and average values S.E.M. indicated. Data were analyzed using one-way ANOVA omnibus tests.*

2.1.2.4. Principal component analysis reveals that male reproductive success correlates with male courtship behaviour, but not whole-body androgen concentration across groups in Experiment A

Principal component analysis of animals for which reproductive success data, behavioural data and endocrine data was collected *(*n=3-4 per group*)* was used to describe possible correlations between measured endpoints. As visualized in **Fig. 2.6**, male courtship behaviours co-vary along principal component axis F1, which explains 51% of the variation in the dataset, while male androgens co-vary along principal component axis F2, explaining 17% of the variation of the

dataset. Specific contribution of the variables to principal component axes are summarized in **Table 2.1**, and correlations between variables presented in **Table 2.2**. Throughout the analyzed subgroup of Experiment A, positive correlations between reproductive success and all indices of male courtship behaviour, but not male androgen concentrations exist that are significantly different from 0 (**Table 2.2**).

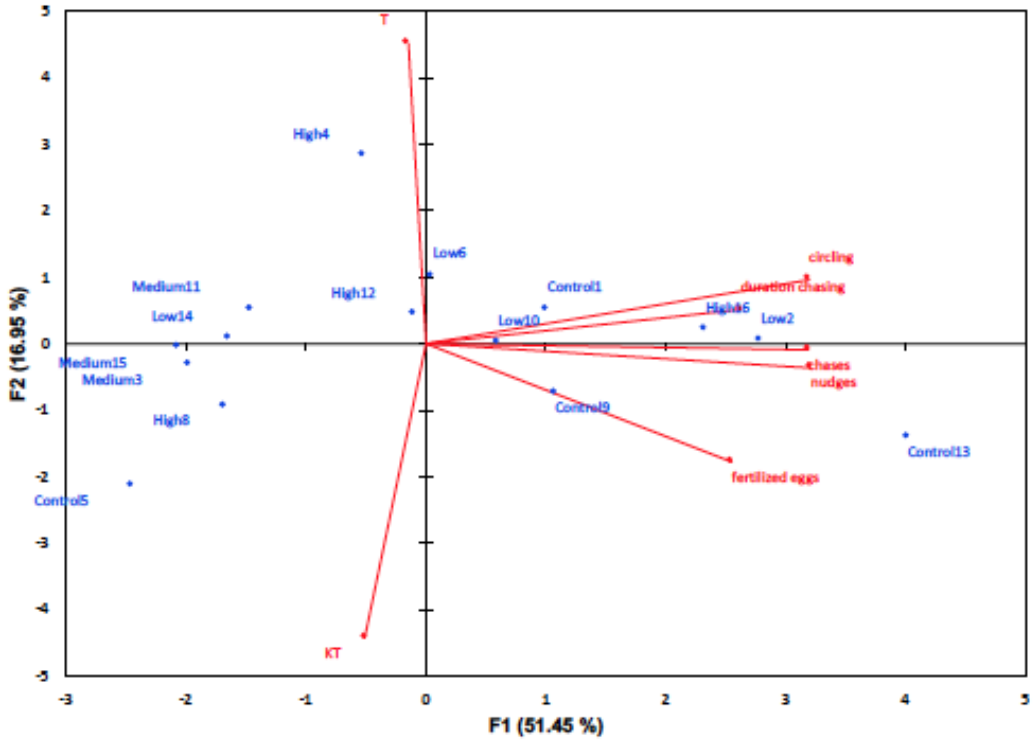


Figure 2.6. *Principal component analysis along axes F1 and axis F2 which explain 68.4% of the variance of datasets from individual male fish for which all measurements exist. Specific loadings of F1 and F2 axis are shown in* ***Table 1****, and correlations between variables in* ***Table 2****.*

Contribution of the variables (%):

	F1	F2	F3	F4	F5	F6	F7
Fertilized eggs	14.655	7.123	3.214	21.354	50.490	3.083	0.081
Chases	22.918	0.019	8.913	5.879	0.604	28.126	33.540
Nudges	23.262	0.303	3.844	16.022	0.590	0.516	55.463
Circling	23.005	2.066	0.731	3.800	8.526	53.959	7.913
Duration Chasing	15.522	0.592	2.681	39.519	31.008	8.066	2.611
T	0.048	45.784	38.802	9.521	3.665	1.827	0.353
KT	0.590	44.112	41.815	3.904	5.117	4.423	0.039

Table 2.1. *Contribution of specific variables to principal component axes loading in Experiment A.*

Correlation matrix (Pearson (n)):

Variables	fertilized eggs	chases	nudges	circling	duration chasing	T	KT
fertilized eggs	1	0.546	0.543	0.503	0.575	-0.196	-0.005
chases	0.546	1	0.955	0.770	0.530	0.074	0.008
nudges	0.543	0.955	1	0.840	0.465	-0.044	-0.032
circling	0.503	0.770	0.840	1	0.660	-0.024	-0.276
duration chasing	0.575	0.530	0.465	0.660	1	0.016	-0.144
T	-0.196	0.074	-0.044	-0.024	0.016	1	-0.113
KT	-0.005	0.008	-0.032	-0.276	-0.144	-0.113	1

Table 2.2. *Correlation matrix of variables derived from the subset of animals from Experiment A investigated in principal component analysis.*

2.1.3.1. Male reproductive success in mating trials is decreased in response to pre-administration of 500 ng/g isotocin antagonist

The number of fertilized eggs in Experiment B changed significantly between treatment groups (**Fig. 2.7A**; $H_{3,79}$=7.89; P=0.04), and was significantly reduced (-79%) in pairs in which the males had been pretreated with a concentration of 500 ng/g isotocin antagonist (L-368,899 hydrochloride) compared to pairs in which male fish pretreated with saline controls (P<0.05). Conversely, no significant differences in the number of unfertilized eggs (**Fig. 2.7B**; $H_{3,79}$=2.46; P=0.48) or dead eggs (**Fig. 2.7C**; $H_{3,79}$=5.61; P=0.13) was found between treatment groups.

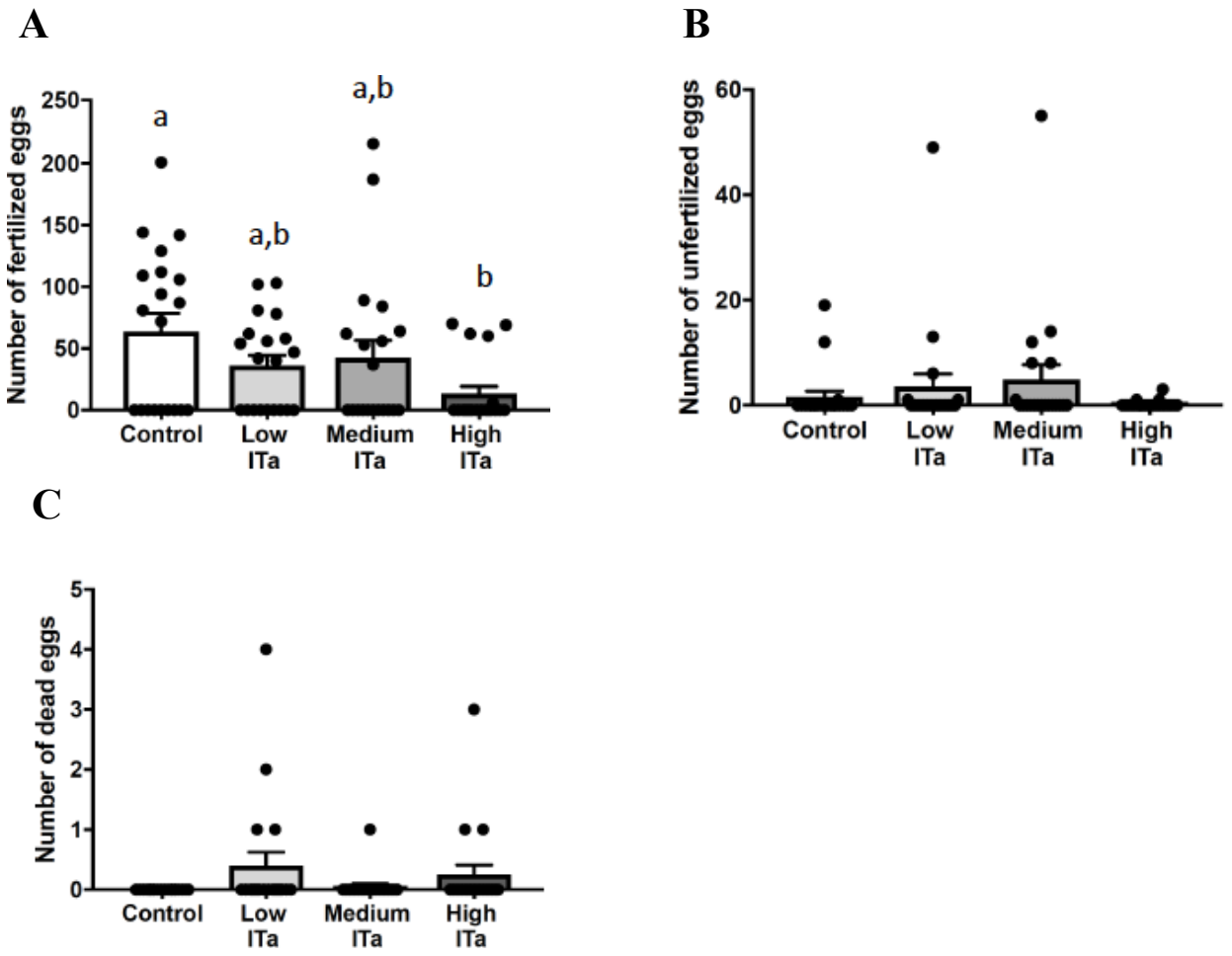


Figure 2.7. *Average number of fertilized eggs (**A**) unfertilized eggs (**B**) and dead eggs (**C**) in pairs in which males received either saline control injection or different doses of isotocin antagonists (L-368,899 hydrochloride - 5,50,500 ng/g) by i.p. injection prior to 2 h mating trials. Individual data for n=20 trials per group are shown and average values S.E.M. indicated. Letters indicate significant differences between groups as determined by Dunn's post-hoc analysis following significant Kruskal-Wallis omnibus tests.*

2.1.3.2 Encircling behaviour during mating trials is significantly reduced in male fish pre-administered 50 ng/g isotocin antagonist

While the number of chasing events (**Fig. 2.8A**; $F_{21,3}$=1.93; *P*=0.16), chasing duration (**Fig. 2.8B**; $F_{21,3}$=3.346; *P*=0.05), and nudging behaviours (**Fig. 2.8C**; $F_{21,3}$=2.02; *P*=0.15) did not significantly change between treatment groups, a significant reduction in encircling events (**Fig. 2.8D**; $H_{21,3}$=8.33; *P*=0.04) was observed in fish pairs in which males had been pre-injected with

50 ng/g isotocin antagonist when compared to control pairs in which males had been pre-injected with saline (P<0.05).

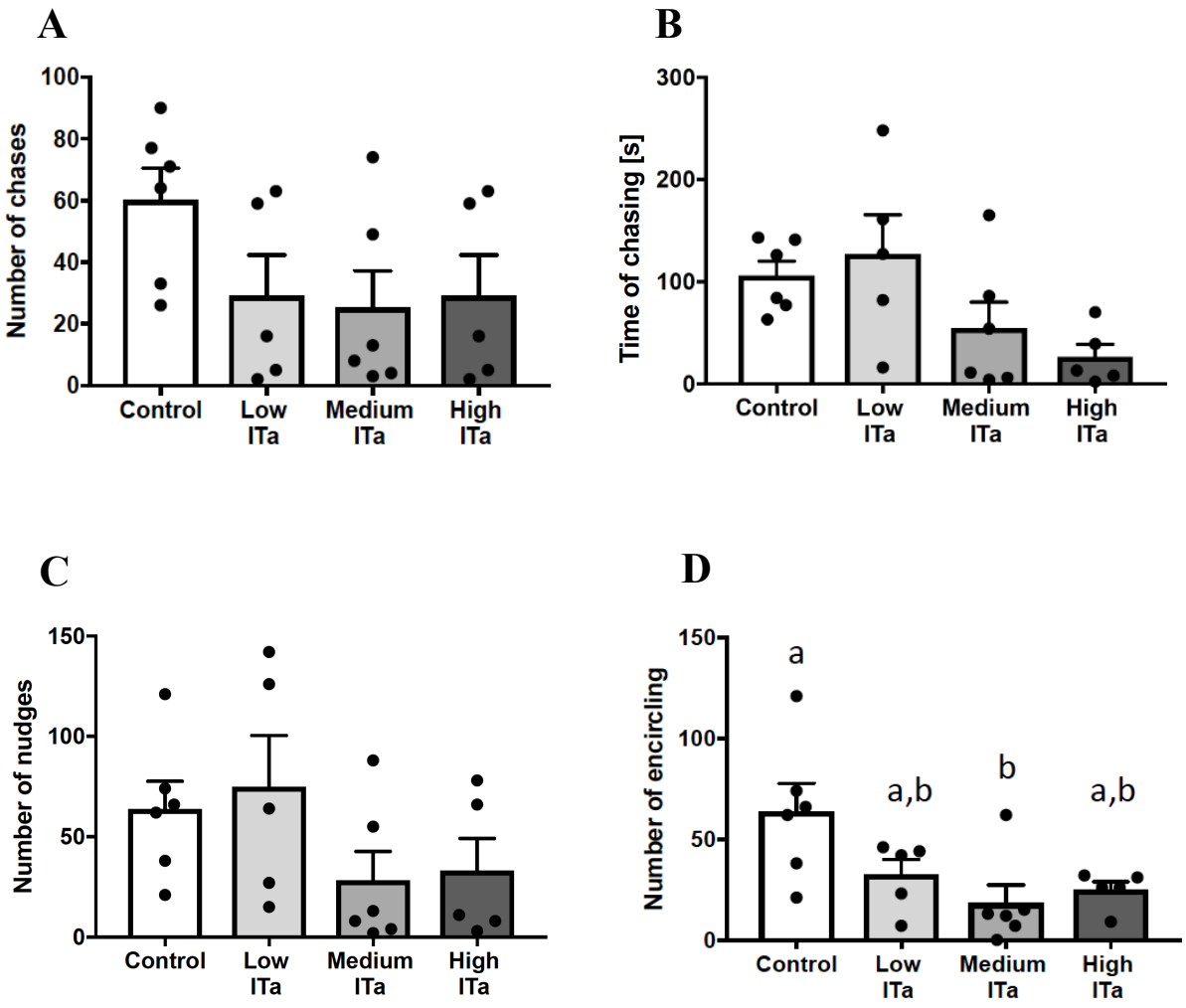


Figure 2.8. *Indices of male courtship behaviours in pairs in which males received either saline control injection or different concentrations of isotocin antagonists (L-368,899 hydrochloride - 5,50,500 ng/g) by i.p. injection prior to 2 h mating trials. The data was obtained from n=5-6 trials, of which the first 15 min were recorded and analyzed. Quantified male courtship behaviours are number of chases (**A**), the duration of chasing time (**B**), the number of nudges (**C**) and the number of encircling behaviour (**D**). For each behaviour data from individual trials in each experimental group are shown and average values S.E.M. indicated. Letters indicate significant differences between groups as determined by Tukey's or Dunn's post-hoc analysis following significant one-way ANOVA or Kruskal-Wallis omnibus tests.*

2.1.3.3. Male whole-body androgen concentrations following mating trials are not affected by prior isotocin antagonist treatment.

No significant changes in whole body testosterone (**Fig. 2.9A**; $F_{3,35}$=0.36; *P*=0.79) or 11-ketotestosterone (**Fig. 2.9B**; $F_{3,35}$=0.02; *P*=0.99) between treatment groups were identified.

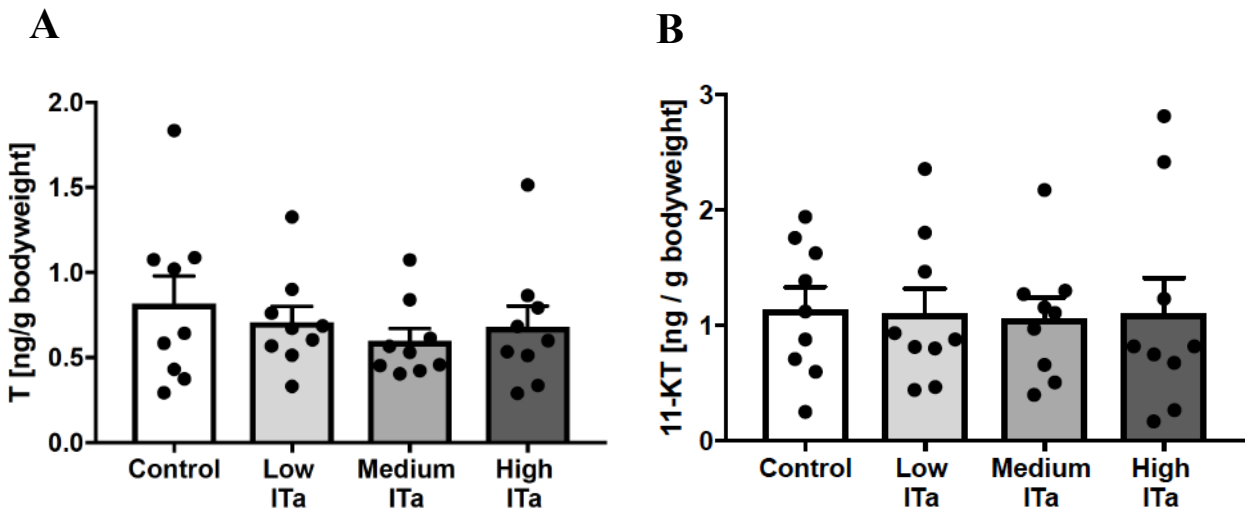


Figure 2.9. *Whole body androgen concentrations for testosterone (**A**) and 11-ketotestosterone (**B**) normalized to body weight in males from breeding pairs in which males received either saline control injection or different concentrations of isotocin antagonists (L-368,899 hydrochloride - 5,50,500 ng/g) by i.p. injection prior to 2 h mating trials. The data was obtained from n=9 individual males per group are shown and average values S.E.M. indicated. Data was analyzed using one-way ANOVA omnibus test.*

2.1.3.4. Principal component analysis reveals correlations of reproductive success with male encircling behaviour and androgen concentration in Experiment B

Principal component analysis of animals for which reproductive success data, behavioural data and endocrine data was collected *(n=3-4 per group)* was used to describe possible correlations between measured endpoints. As visualized in **Fig. 2.10**, male courtship behaviours co-vary along principal component axis F1, which explains 43% of the variance in the dataset, while male androgens co-vary along principal component axis F2, explaining 28% of the variance of the dataset. Specific contribution of the variables to principal component axes loading are summarized

in **Table 2.3**, and correlations between variables presented in **Table 2.4**. Throughout Experiment B, positive correlations between reproductive success and one index of male courtship male courtship behaviour, specifically the number of encircling events as well as male whole body androgen concentrations androgen concentrations exist that are significantly different from 0 (**Table 2.4**).

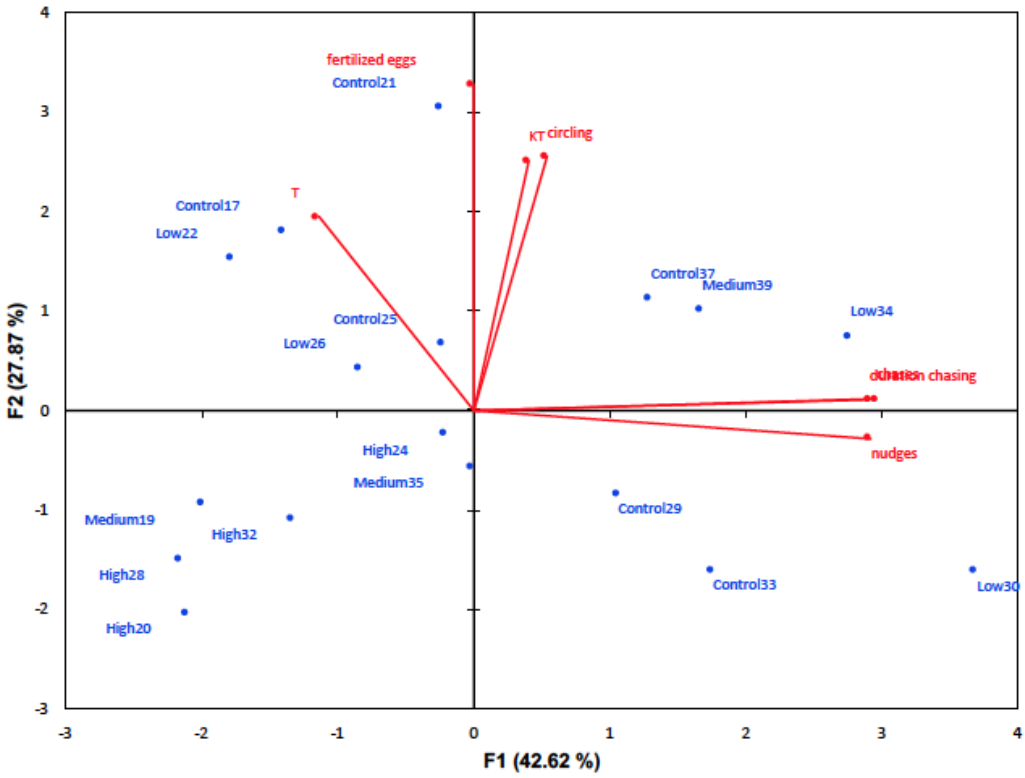


Figure 2.10. *Principal component analysis along axis F1 and axis F2 which explain 70.5% of the variance observed for individual male fish for which all measurements exist. Specific loadings of F1 and F2 axis are shown in* ***Table 2.3****, and correlations between variables in* ***Table 2.4****.*

Contribution of the variables (%):

	F1	F2	F3	F4	F5	F6	F7
fertilized eggs	0.000	39.084	1.076	0.454	54.941	0.483	3.962
chases	31.822	0.052	1.939	0.585	3.113	4.970	57.519
nudges	31.011	0.293	1.256	1.408	2.400	30.868	32.764
circling	1.054	23.716	47.442	1.993	24.102	0.185	1.509
duration chasing	30.791	0.044	0.163	1.674	0.001	63.354	3.973
T	4.728	13.862	32.241	41.179	7.675	0.044	0.272
KT	0.594	22.950	15.883	52.707	7.768	0.098	0.001

Table 2.3. *Contribution of specific variables to principal component loading in Experiment B.*

Correlation matrix (Pearson (n)):

Variables	fertilized eggs	chases	nudges	circling	duration chasing	T	KT
fertilized eggs	1	0.071	-0.111	0.511	0.022	0.352	0.431
chases	0.071	1	0.947	0.090	0.925	-0.269	0.137
nudges	-0.111	0.947	1	0.099	0.890	-0.278	0.070
circling	0.511	0.090	0.099	1	0.176	0.082	0.235
duration chasing	0.022	0.925	0.890	0.176	1	-0.265	0.097
T	0.352	-0.269	-0.278	0.082	-0.265	1	0.189
KT	0.431	0.137	0.070	0.235	0.097	0.189	1

Table 2.4. *Correlation matrix of variables investigated in Experiment B.*

2.1.4.1. Pre-administration of combined nonapeptide antagonists to male zebrafish prior to mating trials completely abolishes reproductive success

Co-administration of vasotocin (Manning's compound) and isotocin (L-368,899 hydrochloride) antagonists in males prior to mating trials completely abolished the presence of fertilized eggs, while saline-injected male control fish produced a median of 75 eggs (**Fig. 2.11A**; $P<0.01$). No significant differences in the number of unfertilized eggs (**Fig. 2.11B**; $P=0.97$) or dead eggs (**Fig. 2.11C**; $P=0.23$) were found.

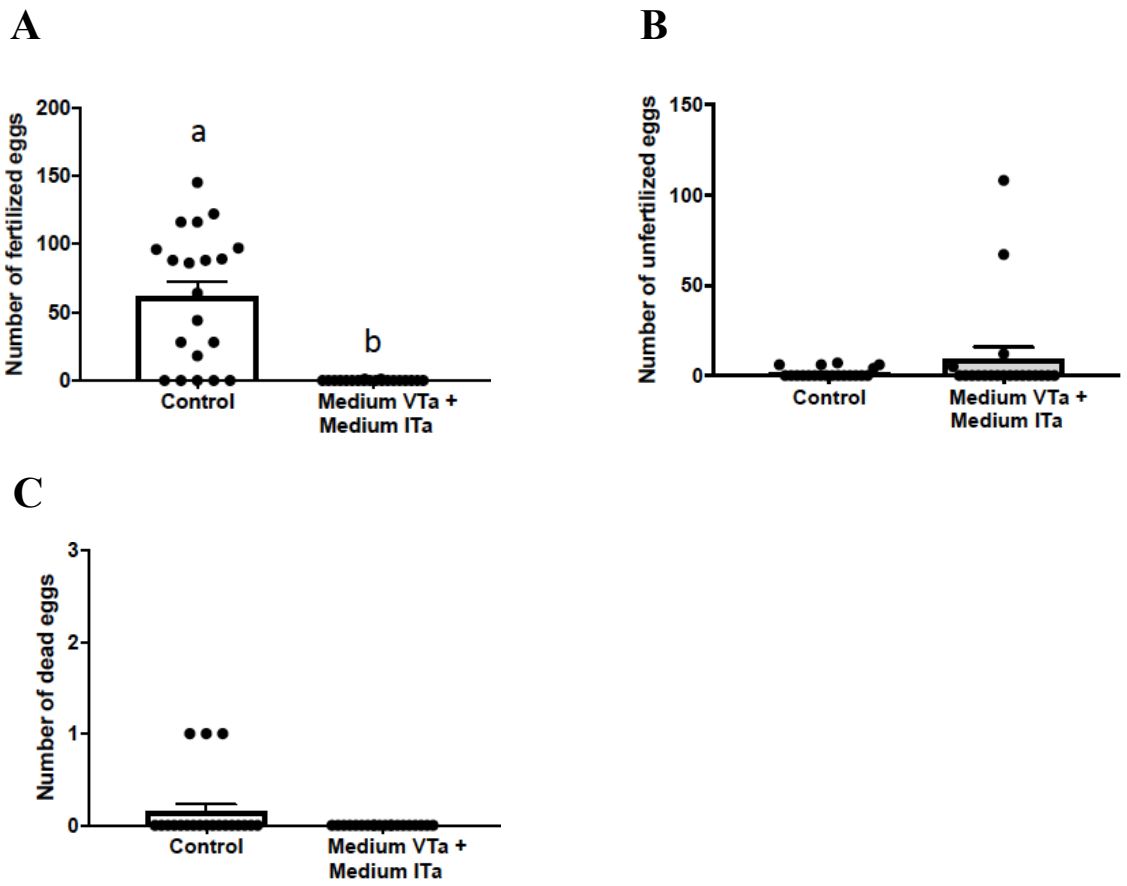


Figure 2.11. *Average number of fertilized eggs (**A**) unfertilized eggs (**B**) and dead eggs (**C**) in pairs in which males received i.p. injections of saline control or co-administration of vasotocin (Manning's compound) and isotocin antagonists (L-368,899 hydrochloride) (50 ng/g each) prior to 2 h mating trials. Individual data for n=20 trials per group are shown and average values S.E.M. indicated different letters indicate significant difference between control and treatment groups, determined by Mann-Whitney U test.*

2.1.4.2. Co-administration of nonapeptide antagonists does not affect male courtship behaviour in subsequent mating trials

The number of chasing events (**Fig. 2.13A**; df=18 t=0.26; *P*=0.80), the total duration of chasing (**Fig. 2.13B**; df=18 t=1.50; *P*=0.15), the number of nudges (**Fig. 2.13C**; df=18 t=1.45; *P*=0.89), and the number of encircling events (**Fig. 2.13D**; df=17 t=0.88; *P*=0.39) were not significantly different between control and treatment groups.

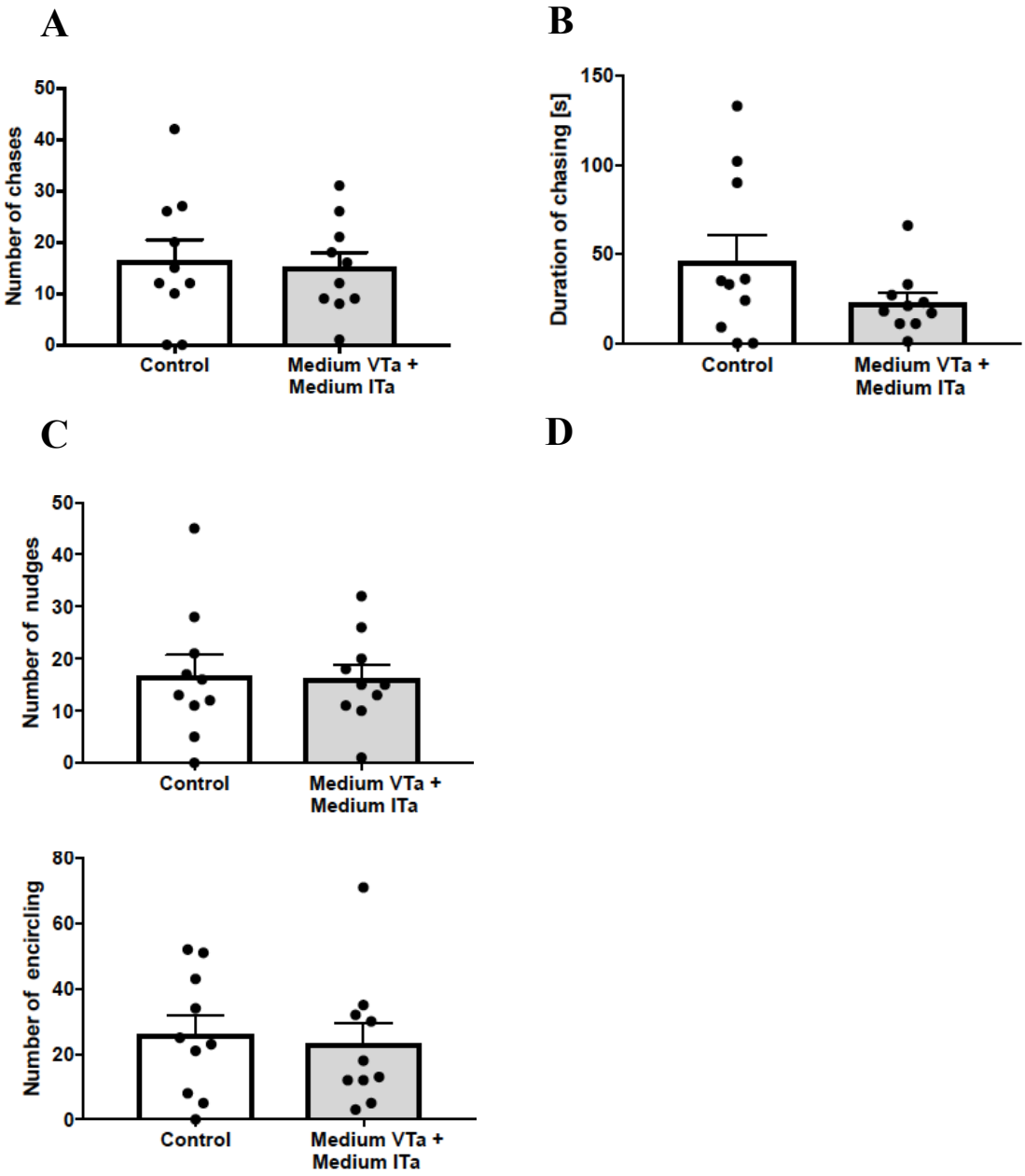


Figure 2.12. *Indices of male courtship behaviours in pairs in which males were administered either saline control injection or combined vasotocin (Manning's compound) and isotocin antagonists (L-368,899 hydrochloride) (50 ng/g each) by i.p. injection prior to 2 h mating trials. The data was obtained from n=10 trials, of which the first 15 min were recorded and analyzed. Quantified male courtship behaviours are the number of chases (**A**), the duration of chasing time (**B**), the number of nudges (**C**) and the number of encircling behaviour (**D**). For each behaviour data from individual trials in each experimental group are shown and average values S.E.M. indicated. All data were analyzed using two-tailed t-tests.*

2.1.4.3. Co-administration of nonapeptide antagonists does not affect male whole-body androgen concentration

No significant changes in male whole-body testosterone (**Fig. 2.13A**; df=18; t=0.10, *P*=0.92**)** and 11-ketotestosterone (**Fig. 2.13B**; df=18; t=0.003, *P*=0.99) were evident between males that had received saline control injections and males that had received co-administered nonapeptide antagonists.

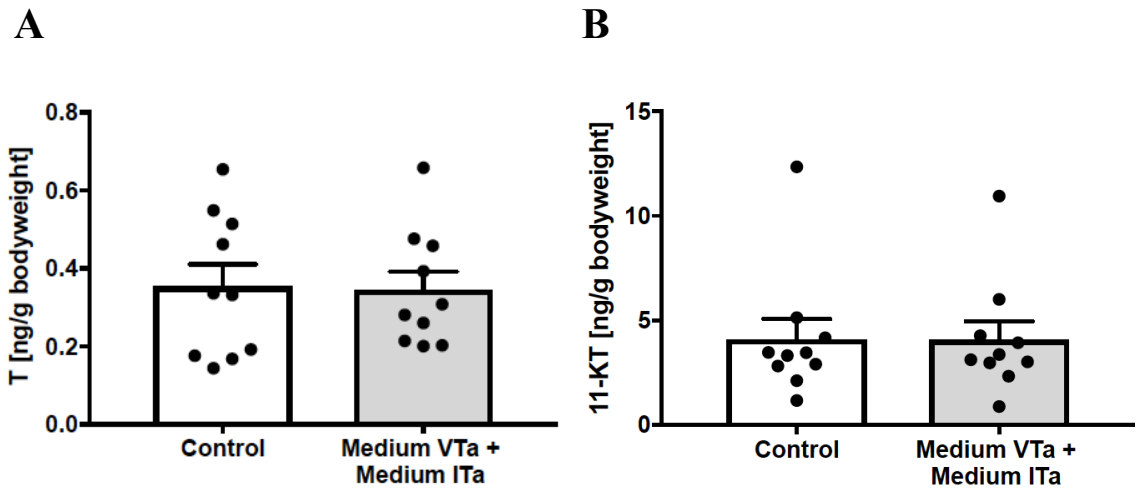


Figure 2.13. *Whole body androgen concentrations for testosterone (**A**) and 11-ketotestosterone (**B**) normalized to body weight in males from breeding pairs in which males received either saline control injection or a co-injection of nonapeptide antagonists (Manning's compound and L-368,899 hydrochloride - , 50ng/g each) by i.p. injection prior to 2 h mating trials. The data was obtained from n=10 individual males per group are shown and average values +/- S.E.M. indicated. Data were analyzed using a one-way ANOVA.*

2.2. Discussion

The pharmacological inhibition studies indicate differential, dose-dependent effects of vasotocin and isotocin antagonists alone and in combination. The number of fertilized eggs, used as a measure of reproductive success, was significantly reduced in pairs with males which had been injected with 50 ng/g Manning compound, a vasotocin receptor 1A (Avtr1A) antagonist, compared to pairs with males which had received saline control injections. Pre-treatment of males

with both lower and higher concentrations did not result in a significant reduction of fertilized eggs in breeding trials compared to trials with saline injected males. Non-monotonous dose-response relationships are well-described occurrence in endocrine research in general (Vandenberg et al., 2009), and for the effect of Manning's compound on aggression in the tropical damselfish (*Stegastes leucostictus*) in particular (Santangelo and Bass, 2006). While the lack of observable effects on 5 ng vasotocin on the production of fertilized eggs release may be attributed to subthreshold concentration of the antagonist, the lack of an effect of higher concentrations may reflect compensatory vasotocin receptor localization on cell membranes.

Pharmacological blocking of the vasotocin receptor 1a by i.p. injection of 50 ng/g Manning compound revealed a clear-cut reduction of all quantified indices of male reproductive behaviour. While not quantified, this reduction in indices of behavioural courtship did not seem to be related to reduced overall locomotion. At the same concentration, whole-body androgen concentrations were not significantly altered compared to control fish, suggesting through that central behavioural, but not endocrine pathways underlie this effect. Descriptive analysis of individual fish across treatment groups by principal component analysis revealed a clear separation of males injected with 50 ng/g vasotocin antagonist from other groups along principal component axis F1, which is loaded with positively correlated fertilized eggs and male courtship behaviours. This correlative evidence suggests that central vasotocin pathways are likely involved in male reproductive success in zebrafish. However, the postulated lack of involvement of endocrine axis derived androgens should be regarded with caution, as whole body analysis of androgen in small animals like zebrafish may not be reflective of more meaningful circulating androgen measurements obtainable in larger fish species, such as goldfish (Mennigen et al., 2010). In addition to direct control of central behavioural pathways, vasotocin may, via neuronal or endocrine mechanisms, also control milt liberation via smooth muscle contractions (**Fig. 1**). While

not directly measured in this study, the fact that the reduction of the number of fertilized eggs did not occur at the expense of an increase in unfertilized eggs, shows that sperm release is likely not inhibited in males pretreated with vasotocin antagonist, but that rather male-stimulated egg release of females is reduced, reinforcing the notion of impaired courtship behaviour. Therefore, pharmacological inhibition clearly provides evidence for a role of vasotocin in male zebrafish reproductive success via Vtr1a receptor signaling, and furthermore correlatively points to centrally regulated male reproductive behaviour pathways as likely underlying mechanism.

Indeed, a role for vasotocin in male teleost courtship has been described in the territorial male bluehead wrasse, where administration of vasotocin increased aggression and male courtship behaviour, while injection of Manning compound reduced both behaviours (Semsar et al., 2001). A role for vasotocin-dependent effects of male reproductive behaviour has recently been identified in medaka (Yokoi et al., 2015), where genetic ablation of vasotocin reduced male courtship and mate-guarding behaviour in a triad consisting of two males in competition for a receptive stimulus female. However, this effect in medaka was not mediated by the Avtr1a or Avt1rb receptors, as genetic ablation of *avtr1a* affected only male-male aggressive behaviour and mate-guarding, but not courtship behaviour, while genetic ablation of *avtr1b* ablation had no effect. A role for vasotocin in male plainfin midshipman vocal courtship behaviour has equally been demonstrated, while sneaker males and females exhibited vocalization patterns that were isotocin-sensitive (Goodson and Bass, 2000). Together, this study revealed that nonapeptides not only act differentially in this species, but also dissociate sex-specific reproductive behaviour from genetic and gonadal sex. In spite of this evidence, a strict and unified vasotocin-dependent modulation of reproductive behaviour in male teleost fishes is unlikely, as courtship behaviour of females and sneaker males, but not males, is sensitive to vasotocin in the peacock blenny, *Salaria pavo* (Carneiro et al., 2003), reflecting the need for detailed comparative studies with respect to

nonapeptides in teleost reproduction (Goodson, 2008; Goodwin and Thompson, 2012). While my study reveals that vasotocin modulates male zebrafish reproductive success via Avt1a receptors, likely via central behavioural pathways, future studies taking advantage of the utility of the zebrafish model for gene editing, should utilize KO models, reporter lines and optogentic tools to fully elucidate the role of the central vasotocin system in regulating male zebrafish courtship behaviour.

With regard to the isotocin antagonist, the experiment identified a differential, monotonic dose-dependent effect on male reproductive success. Pre-mating trial administration of only the highest isotocin antagonist concentration (500 ng/g) reduced male zebrafish mating success, assessed as number of fertilized eggs. In contrast to the vasotocin antagonist, the effective dose which significantly reduced male reproductive success did not result in significant changes in male courtship behaviour indices. Nevertheless, a significant reduction in the number of encircling behaviours was observed at a isotocin concentration of 50 ng/g. Similarly, to the vasotocin antagonist experiment, no changes where seen in whole body androgen concentrations and/or the number of unfertilized eggs, suggesting that the male endocrine reproductive axis (at least when assessed on the whole-body level), and/or milt release or viability were not correlated with reduced reproductive success. Principal component analysis of animals for which all endpoints were present revealed that animal separation by reproductive success occurs across principal component axis F2, which is also loaded with the positively correlated androgens and encircling behaviour. Altogether, the isotocin antagonist experiment, while revealing a role for the male isotocin system in mediating reproductive success, at least when inhibited with the highest tested concentration of the antagonist, does not appear to point to a single correlated endpoint indicative of potential underlying mechanisms. While the specific mechanisms through which isotocin reduces male reproductive success in zebrafish remain unknown, my study is the first to reveal a role for isotocin

in male reproductive success in a teleost fish. This finding questions the notion that isotocin is predominantly involved in the regulation of female reproductive function. Indeed, while a stimulatory role for isotocin on female endocrine axis has been established in goldfish (Popesku et al., 2011; Mennigen et al., 2017), limited functional evidence also suggests an involvement of isotocin in male reproduction-related behaviours, exemplified by its stimulation of paternal care in monogamous convict cichlids (O'Connell et al., 2012).

Given the differential modulation of male zebrafish reproductive success by vasotocin and isotocin, and in order to probe potential synergistic actions of both nonapeptide systems in this model, isotocin and vasotocin antagonists were co-administered at 50 ng/g each in the same experimental paradigm and compared to saline injected control animals. Surprisingly, co-administration resulted in a complete inhibition of male reproductive success, however neither male courtship behaviours, nor whole body androgen concentrations or the number of unfertilized eggs differed significantly between treatment groups. Together, this data is suggestive of an additive or synergistic function of nonapeptide systems, especially since individual antagonists at higher concentrations (500 ng/g) fail to induce a similarly drastic effect. Therefore, it is unlikely that the combinatorial effect of both antagonists is due to reduced receptor specificity at higher concentrations of antagonists used. This is important, because while both Manning compound and L-368,899 exhibit ~100-fold higher affinity to their V1a receptors and oxytocin receptors compared to oxytocin and V1a receptors in mammals (Manning et al., 2012; Williams et al., 1994), binding studies have never been directly conducted in teleost fish. However, similar to previous studies utilizing Manning compound and L-368,899 in teleost fish (Backström and Winberg, 2009; Soares et al., 2012; Huffmann et al., 2015; Zimmerman et al., 2016), our results reveal differential effects on male reproduction in zebrafish, suggesting that vasotocin and isotocin influence male reproductive success in zebrafish via distinct mechanisms.

The possibility for synergistic action of both nonapeptide systems has, in spite of their at least somewhat similar organization, not been formally investigated in teleost fishes. Interestingly, early studies in the rat provided evidence for a synergistic effect of vasotocin and oxytocin on gonadotropin action on gonadal growth in mice, albeit in females. The exact mechanisms underlying the complete abolition of male reproductive success by concurrent pharmacological inhibition of vasotocin (or at least Vtr1a-dependent vasotocin signaling) and isotocin in zebrafish clearly warrant future study. Overall, all three experiments clearly demonstrate a functional role of male zebrafish nonapeptide systems in mediating reproductive success. Evidence from the (partial) pharmacological inhibition of the vasotocin system via Manning compound, a selective Vtr1a antagonist, points to central neurocircuits involved in the control of male courtship behaviour as principal candidate in mediating these effects, while the effects of isotocin and additive/or synergistic effects of both nonapeptide systems warrant future study.

Chapter 3 – Acute and long-term response of the nonapeptide system to a key reproductive pheromonal cue (female pheromone) acutely and/or in the longer-term in zebrafish.

3.1. Responsiveness of the male nonapeptide system to olfactory reproductive cues

Environmental cues are important regulators of teleost reproduction. Among the most studied cues in male cyprinid fish are olfactory cues, and specifically the ability to detect and respond to $PGF_{2\alpha,}$ a releaser pheromone which stimulates male courtship behaviour following its release by ovulating females (Sorensen et al., 2004). Recent evidence showed that male zebrafish are not only attracted to $PGF_{2\alpha}$, but also activate neuronal perikarya in the posterior preoptic area and increase male courtship behaviour in response to $PGF_{2\alpha}$ (Yabuki et al., 2016). Knock-out studies have shown that these effects are dependent on an olfactory encoded by the *or114.1* gene, which is specifically activated by $PGF_{2\alpha}$ at picomolar concentrations (Yabuki et al., 2016). Based on this evidence, I further probed a reproductive role for nonapeptides by investigating the hypothesis that $PGF_{2\alpha}$ acutely stimulates nonapeptidergic neurons in the POA. I predicted that if nonapeptidergic neurons are involved in mediating reproductive success in zebrafish, acute or long-term exposure of zebrafish to $PGF_{2\alpha}$, a potent cyprinid reproductive pheromone, will either acutely stimulate markers of neuronal activity in nonapeptide neurons in the short-term (10 min), or induce nonapeptide gene expression in the brain in the longer-term (2-18 h).

3.1.1. Materials and Methods

3.1.1.1. Animals housing

Adult, sexually mature male zebrafish were obtained from the University of Ottawa Aquatics Facility. All fish were between 10-14 months old. Fish were housed in 12 L tanks at a

density of 2 fish/L and fed a mixed diet (Adult Zebrafish diet, Zeigler Bros Inc, Gardners, PA, USA; Larval AP-100, Zeigler Bros Inc, Gardners, PA, USA; Golden Pearls, Artemia International, Fairview, TX, USA) twice a day. The tank water was continuously filtered and changed once a week with 28 °C system water. The fish were housed in a room with a 12 h light cycle 8:00 h - 23:00 h and at a room temperature maintained at 27 °C.

3.1.1.2. Experimental design to probe acute effects of $PGF_{2\alpha}$ on the male zebrafish nonapeptide system

A single male fish was placed in a choice tank with a partial divider in the middle which allowed the fish to access both compartments freely (**Fig. 3.14**). The fish was allowed to acclimate to the entire tank for 30 min prior to the beginning of the experiment. After the 30 min period, the fish was segregated into the 'start' area of the tank with an additional removable physical barrier, and the fish was allowed to acclimate in this area for another 15 min (**Fig. 3.15**). Then, repeated rounds of two combinations of treatments (n=15 each) were administered by simultaneous pipette injection into the far end of the two areas separated by the partial divider, while maintaining the temporal barrier in place. The first combination of treatments consisted of a simultaneous injection of 10^{-3} M $PGF_{2\alpha}$ (Bachem, Torrance, CA, USA) and 95% EtOH vehicle control, with randomized application of treatments between the two sides (left and right) of the divider. The final $PGF_{2\alpha}$ concentration in the tank water was 10^{-7} M, an order of magnitude above the described threshold of 10^{-8} M $PGF_{2\alpha}$ sufficient to activate or114.1 (Yabuki et al., 2016). The second combination of treatments was 95% EtOH vehicle in both pipettes, which was employed to address possible side-preferences of experimental fish irrespective of pheromone treatment. Through repeated preliminary dye injection trials, the time of diffusion to the edge of the area in which male zebrafish were restricted for 15 min prior to the choice test was ~10 s. Following the injection of vehicle or

$PGF_{2\alpha}$ to the edges of the separate arms of the tank, the water divider was removed following exposure and the fish were allowed to swim freely in the tank for 10 minutes while being recorded for behavioural analysis. The fish was then terminally euthanized and the head separated. The head was then placed in 4% PFA in PBS overnight for subsequent fixation for immunohistochemistry. The 10-minute time point was chosen based on described timelines for p-ERK signaling as neuronal marker in general (Gao and Ji, 2009), and for p-ERK signaling used to visualize olfactory bulb stimulation patterns in zebrafish in particular (Yabuku et al., 2016; Dieris et al., 2017). A schematic overview of the experimental design for the experiment (Experiment A) is depicted in **Fig. 3.14A**.

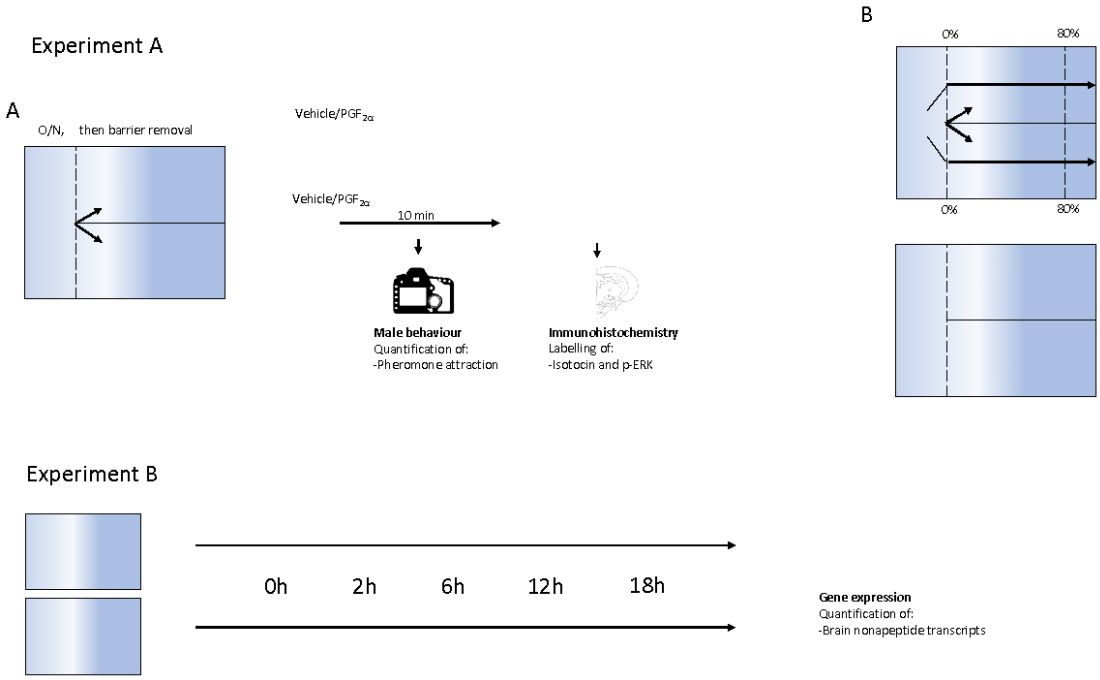


Figure 3.14 (A) & 3.15. (B). *Schematic illustration of the experimental design using pharmacological inhibition of vasotocin and isotocin to determine the role of nonapeptides on male reproductive success, male courtship behaviour and the male reproductive endocrine axis.*

3.1.1.3. Behavioural analysis

10 min of the individual experimental male zebrafish's movement (n=14 and n=15 for each treatment combination) following the removal of the temporary barrier were recorded with the Zebrafish Behaviour Platform (Viewpoint Behaviour Technology, Montréal, QC, Canada) using a Dragonfly2 DR2-HIBW camera (Point Grey Research, Richmond, BC, Canada) with 30 frames/s (**Fig. 3.14**). Full or partial movements along either the left or the right arm of the choice tank were quantified in two ways (**Fig. 3.15**): Firstly, zone crossings into the arms (crossing the 0%) line were scored as 'partial choice' when fish did not reach the 80% length crossing line (the area of treatment administration) before re-crossing the 0% line. Conversely, if following the initial crossing zebrafish also crossed the 80% length line prior to re-crossing the 0% line, a 'full choice' was noted. The complete time of the recordings (10 min) were analyzed. Because the scoring of crossed zones does not account for the possibility that experimental male zebrafish may choose to spend time in one area over the other without crossing zones, the total time spent in each area was also quantified for both time points (**Fig. 3.15**).

3.1.1.4. Immunohistochemistry

Following the placement of the collected zebrafish head samples in 4% PFA in PBS and incubation on an orbital shaker at room temperature overnight, the samples were washed for 3x20 min in PBS on the orbital shaker. The samples were subsequently placed in a Falcon tube containing Richard Allen's solution (active ingredient: hydrogen chloride) for decalcification on the orbital shaker and again incubated at room temperature overnight. The following day, the samples were once again washed twice in PBS on the orbital shaker for 15 minutes at room temperature. The samples were then successively dehydrated in 30%, 50%, 70%, 80%, and 99% EtOH on the orbital shaker at room temperature, with a duration of 30 min for each step. Samples

were then incubated for 30 min at room temperature in a series of solutions comprised of 50% Citrisolv + 50% EtOH, 100% Citrisolv I, and100% Citrisolv II, respectively. Samples were then incubated in paraffin for 1 h at 60 °C. The samples were transferred to new paraffin, and left overnight in 60 °C. Samples were then embedded and sectioned in the coronal plane using a the HM350 Microm motorized microtome (Heidelberg, BW, Germany), and serially cut 7.5 µm sections collected on Fisherbrand Superfrost Plus microscope slides (Thermo-Fisher Scientific, Ottawa, ON, Canada). Areas of interest included the olfactory bulb (OB), the preoptic area (POA) and the pituitary gland, which were identified under a light microscope following the descriptions in 'Neuroanatomy of the zebrafish brain' by Wulliman et al. (1996). An overview of relevant sections is presented in **Fig. 3.16**. Slides were successively rehydrated in xylene I and xylene II for 20 min, followed by immersion in 100% EtOH (twice), 95% EtOH (twice), 70% EtOH, and PBS for 10 min each, respectively. The slides were then incubated at 85 °C in 0.01 M sodium citrate (pH 6) for 1 h in order to promote antigen retrieval. The slides were cooled down, washed in PBS twice for 20 min each and incubated with blocking buffer (1.47 g skim milk powder, 250 µL triton, and 50 µL PBS) for 30 min. Slides were then incubated with 200 µL consisting of primary polyclonal antibody raised in guinea pig against ovine oxytocin (Thermo-Fisher Scientific, catalog # 4030890.0001) at a dilution of 1:200 and monoclonal anti-pERK1/2 raised against a human synthetic peptide in rabbits at a dilution of 1:400 dilution (Cell Signalling Technologies catalog #– T202/Y204, New England Biolabs, Danvers, MA, USA). Both antibodies were diluted in blocking buffer. The oxytocin antibody, while previously validated for isotocin in goldfish (Canosa et al., 2011; Mennigen et al., 2017) has not previously been validated in zebrafish, and a pre-adsorption control using 1 mg/mL isotocin (Bachem, catalog # PA1-18416) was conducted to control for the specificity of isotocin labeling. Conversely, p-ERK had previously been employed and validated as marker of neuronal activity in zebrafish (Randlett, 2015; Yabuki

et al. 2016; Dieris et al., 2017). All slides incubated with primary antibody mixture were covered with parafilm and left overnight at 4 °C. The following day, the slides were washed for 2x10 min with PBS and incubated with secondary, fluorescent dye-coupled anti-guinea pig IgG (Alexa Fluor 594; red, catalog #A11076) and anti-rabbit antibodies (Alexa Fluor-488; green, catalog # A11008) at a dilution of 1:200 (both Thermo-Fisher Scientific). Slides incubated with secondary antibodies were covered with parafilm and incubated for 1 h at room temperature. Following two final washing steps with PBS (10 min each), the coverslip was mounted using Prolong Gold Antifade mountant with DAPI (Thermo-Fisher Scientific, catalog #S36939). Slides were stored at 4 °C until imaging with the Olympus confocal microscope FV1000 (Olympus, Richmond Hill, ON, Canada) operating with the FV10-ASW- 4.0 software. Multiple slides (n=3-4, depending on the region) from n=2 animals of control and $PGF_{2\alpha}$ exposure group were used for quantification, as tissue staining and section quality was consistently high in these animals.

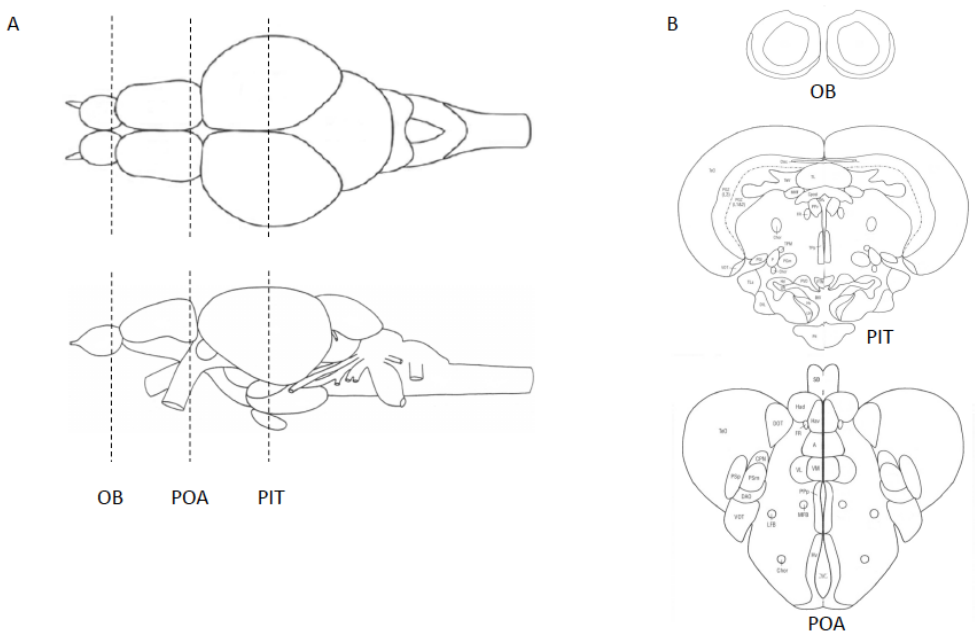


Figure 3.16. *Sections used to visualize neuronal activity marker p-ERK in relation to isotocin immuno-reactive areas in the zebrafish brain in male zebrafish exposed to EtOH vehicle or PGF_2. Investigated areas include the olfactory bulb (OB), pituitary gland (PIT), and preoptic area (POA), illustrated in horizontal and sagittal planes of view (**A**) and detailed coronal section (**B**) used in the current study. All images are adapted from 'Neuroanatomy of the zebrafish brain' by Wulliman et al. (1996).*

3.1.1.5. Quantification of p-ERK immunoreactivity

Intensity calculations of the entire olfactory bulb frontal sections were conducted using a basic intensity quantification test analyzing the mean gray value in the freely accessible Fiji software containing the relevant plug-ins (www.imagej.net/fiji). Intensity quantification protocol was used from Université de Genève (https://www.unige.ch/medecine/bioimaging/files/1914/1208/6000/Quantification.pdf)

3.1.1.6. Statistical analysis

Raw and transformed behavioural data was analyzed for parametric distribution using the Shapiro-Wilk test. In cases where data was normally distributed, individual data was analyzed

using paired, one-tailed t-tests, while a corresponding Mann-Whitney U test was used to determine significant differences in behavioural data not normally distributed. Normalized p-ERK intensity data was transformed to improve fit of a normal distribution, and following confirmation of normal distribution of values in each group by the Shapiro-Wilk test, analyzed by an unpaired one-tailed t-test.

3.1.2.1. Experimental design to probe long-term effects of PGF_2 on male zebrafish nonapeptide systems

A total of 25 male fish were separated into a 12 L tank housing the control group, and 25 male fish were separated in another 12 L tank housing the treatment group two weeks prior to the experiment. The morning of the experiment (immediately after lights turned on), fish were not fed and a subsample of n=5 fish was euthanized from each tank and brains collected, placed in Eppendorf tubes and immediately stored on dry ice. This pre-experimental sampling was used to determine potential *a priori* differences in relative whole brain nonapeptide mRNA abundance. Following this initial sampling, 10^{-3} M $PGF_{2\alpha}$ (dissolved in 95% EtOH vehicle) or 95% EtOH (vehicle control) was then injected into the treatment tank and the control tank, respectively. The the final nominal tank concentration of $PGF_{2\alpha}$ was 10^{-7} M, while final EtOH concentration (v/v) did not exceed 0.0001%, far below any EtOH dependent reported acute neuronal and/or behavioural effects in this species (Chaterjee and Gerlai et al., 2009). Water filtration was stopped in both tanks to and subsamples of n=5 fish were collected from each tank as previously described for various time-points (2 h, 6 h, 12 h, 24 h) following the exposure. All brains were then stored in the -80°C freezer for subsequent RNA extraction. A summary of the experimental design (Experiment B) is illustrated in **Fig. 3.15**.

3.1.2.2. Whole brain RNA extraction and cDNA synthesis

RNA was extracted from each brain. The entire brain was homogenized in 0.5 mL TRIzol reagent (Thermo-Fisher) and care was taken to avoid thawing of samples. The samples were then sonicated, ensuring the probe was cleaned with 75% EtOH and RNAse free water in between each sample. The samples were then incubated for 5 minutes at room temperature. Following the incubation step, 0.1 mL of chloroform was added to each sample and then the tubes were vigorously shaken by hand for 15 s, followed by another incubation at room temperature for 2 min. The samples were then centrifuged at 12000*g* for 15 min at 8 °C. The upper aqueous phase of each sample was transferred to a new RNAse-free 1.5 mL microtube. 0.25 mL of isopropyl alcohol was added to the aqueous phase, and the samples incubated for 15 min at room temperature. The samples were then centrifuged at 12000*g* for 10 min at 8 °C. The supernatant was poured out and the RNA pellet was washed twice in 0.5 mL of ice-cold 75% EtOH (kept at -20 °C), during which the pellet was initially loosened by vortexing and then precipitated again by centrifugation at 6.6*g* for 5 min at 8 °C. Using a pipette, the EtOH was removed from the tube, and left over EtOH was allowed to evaporate off from the inverted tubes placed on Kimwipe paper in the fume hood for 15 min. Finally, 30 µL of RNAse free molecular grade water (VWR, Mississauga, ON, Canada) was added to each tube in order to dissolve the pellet, and samples were stored at -80 °C. Extracted RNA was quantified using a NanoDrop® 2000c UV-Vis Spectrophotometer (Thermo Scientific). cDNA was then generated from 1 µg of total RNA per sample using a QuantiTech Reverse Transcription Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. Briefly, the volume corresponding to 1 µg total RNA was adjusted to a total volume of 12 µL for each reaction, and 2 µL of 7x gDNA WipeOut buffer were added to each sample. All samples were then incubated at 42 °C for 2 min, after which reaction were immediately quick chilled on ice. A total of 6 µL of Mastermix consisting of 1 µL Quantiscript RT, 4 µL of 5x Quantiscript RT buffer,

and 1 µL of RT primer mix was then added to each reaction for a final volume of 20 µL. The final reaction was finally incubated at 42 °C for 15 min, followed by an incubation for 3 min at 95 °C to deactivate reverse transcriptase. All incubation steps were conducted using an Eppendorf Mastercycler Gradient machine (Eppendorf, Hamburg, Germany). Following condensation on ice, tubes were spun down to collect the reaction volume at the bottom of the well and stored at -80 °C for subsequent semi-quantitative quantification of nonapeptide transcripts.

3.1.2.3. Semi-quantitative whole brain nonapeptide transcript analysis

Two-step, SYBR-green-based, semiquantitative *real-time* RT-PCR assays were used to measure relative fold-changes in steady-state isotocin and vasotocin mRNA abundance in whole zebrafish brain on a BioRad CFX96 machine (Bio-rad, Mississauga, ON, Canada). Briefly, a standard curve consisting of serial dilutions of pooled cDNA, a negative no-RT control consisting of cDNA generated in a reaction that did not include reverse transcriptase, and individual samples were run in duplicate for each experiment. For each individual reaction, the total volume was 20 µL, which consisted of 4 µL of diluted cDNA template, 0.5 µL of 10 nM specific forward and 0.5 µL of 10 nM specific reverse primer (**Table 3.7**), 10 µL of SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad), and 5 µL of RNAse-free H_2O (VWR). For each assay, cycling parameters were a 5 min activation step at 95 °C, followed by 40 cycles consisting of a 20 s denaturation step at 95 °C and a combined 30 s annealing and extension step at primer specific temperatures between 57-60 °C (**Table 3.5**). After each run, melting curves were produced by gradually increasing temperature and the final curves were monitored for single peaks to confirm the specificity of the reaction and the absence of primer dimers. The acceptable range for amplification efficiency calculated from serially diluted standard curves was 90 –110%, with R^2 values of 0.95. Assays were subsequently normalized using the ΔΔCT approach as described by

Pfaffl (2001). Finally, mRNA fold changes were calculated relative to the 0 h vehicle control time point group to determine relative fold-change across time. All primer pairs used in this study had previously been validated in zebrafish (Wong et al., 2013; Pavlidis et al., 2011).

Transcript ID	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)	Annealing T (°C)
vasotocin NM131327.1	CCCAGCCGGAGCCCATCAGA	CCATGCAGACCTGCGCCTCC	131	60
Isotocin NM178291.2	ATTCGACAGTGTATGCCGTG	TCACACGGAGAAGGGAGAAA	146	60
β-actin NM_131031.2	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	100	57

Table 3.5. *Primer sequences and reaction parameters for semi-quantitative, whole brain nonapeptide transcript quantification by SYBR Green real-time RT-PCR assays.*

3.1.2.4. Statistical analysis

Following the verification that raw or transformed data met normal distribution and homoscedasticity criteria, a two-way ANOVA was used to investigate effects of treatment, time and their interaction on whole brain nonapeptide gene expression. Tukey's posthoc test was used to delineate specific differences in cases of significant omnibus test analysis results.

3.2. Results

3.2.1. Male zebrafish are attracted to $PGF_{2\alpha}$

Full (**Fig. 3.17A**) and partial choices (**Fig. 3.17B**) were scored for $PGF_{2\alpha}$ and EtOH vehicle application to randomized arms of the choice tank, as well as double EtOH vehicle application to either arm of the tank. Results for overall 'full choices' (**Fig. 3.17A**) indicate a significant preference for pheromone over vehicle control (n=14; df=13 t=1.901 *P*=0.04). When investigating 'full choices' between pheromone and vehicle control in cases where pheromone was administered on the left side from the experimenter's perspective and vehicle on the right side (n=7), no significant difference was observed (df=6; t=1.229; P=0.13). Similarly, when considering 'full choices' between pheromone and vehicle control in cases where pheromone was administered on the right side from the experimenter's perspective and vehicle on the left (n=7), no significant difference was observed (df=6; t=1.52; *P*=0.09). When considering the second treatment condition in which EtOH was administered to both arms, no significant preference for either side was identified (df=14 t=0.8043 *P*=0.2173). No significant differences in 'partial choices' (**Fig. 3.17B)** were identified between pheromones and vehicle overall (df=13 t=0.5553 *P*=0.29), between pheromone administered to the right sight of the experimenter and vehicle administered to the left side of the experimenter (df=6 t=0.6348 *P*=0.27), towards pheromone administered on the left side of the experimenter and vehicle administered on the right side of the experimenter (df=6 t=1.023 *P*=0.730) or between arms when vehicle was administered to both sides (df=14 t=1.14 *P*=0.14). Total time spent in in specific arms **(Fig. 3.17C)** did not change between pheromone and vehicle treatment overall (df=13 t=1.715 *P*=0.06)**,** or when pheromone was applied on the left (df=6 t=1.236 P=0.13) or right side (df=6 t=0.034 *P*=0.97) of the experimenter. No difference between time spent in each arm was observed between two vehicle controls (df=14 t=0.9581 *P*=0.18).

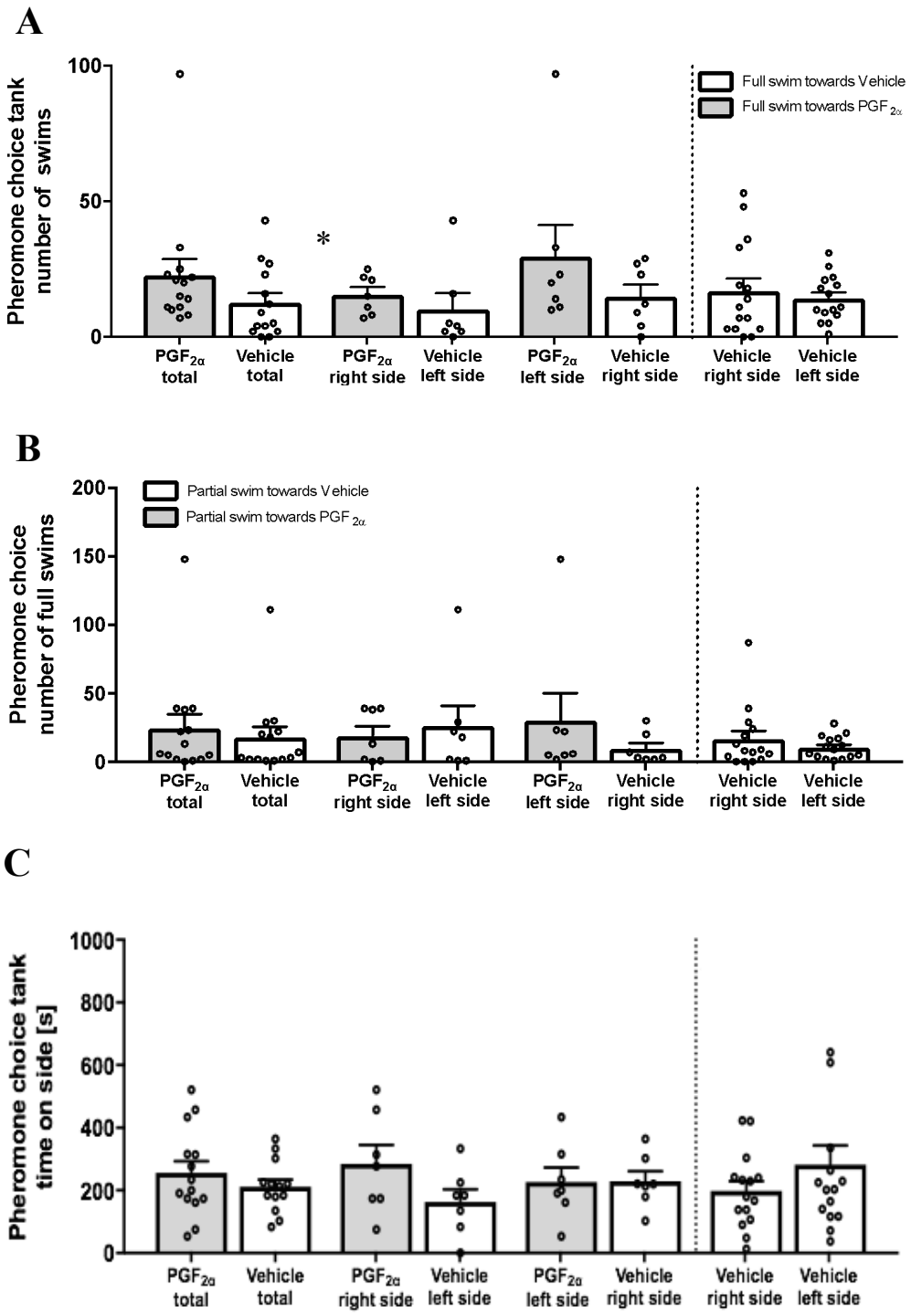


Figure 3.17. Full (**A**) and partial (**B**) swims of individual male zebrafish towards PGF_2 (grey bars) or EtOH vehicle (white bars) in a choice tank. Specific criteria are detailed in the text. Panel (**C**) depicts time spent in either arm of the choice tank. Individual and average data for overall swims and time. (S.E.M.) is presented. Asterisks indicate significant differences between groups as determined by paired one-tailed t-test.

3.2.2. Olfactory bulb response to acute $PGF_{2\alpha}$ in relation to the isotocinergic system

The olfactory bulb of zebrafish revealed an overall significant increase in p-ERK fluorescence (df=9 t=2.311 P=0.03) compared to vehicle exposed control zebrafish. Representative images are depicted in **Fig. 3.18**. Overall specific increases of p-ERK activity were identified in the ventromedial glomerulus of the male olfactory bulb and the olfactory nerve in response to of $PGF_{2\alpha}$ (**Fig. 3.18E**) compared to EtOH vehicle exposed control animals (**Fig. 3.18A)**. Isotocin-positive stained areas in the OB are largely restricted to the primary olfactory fiber layer and the glomerular layer (**Fig. 3.18B+F**). No significant amount of co-localization between p-ERK and isotocin signal is observed in the ventromedial glomerulus, in spite of spatial proximity (**Fig. 3.18H**).

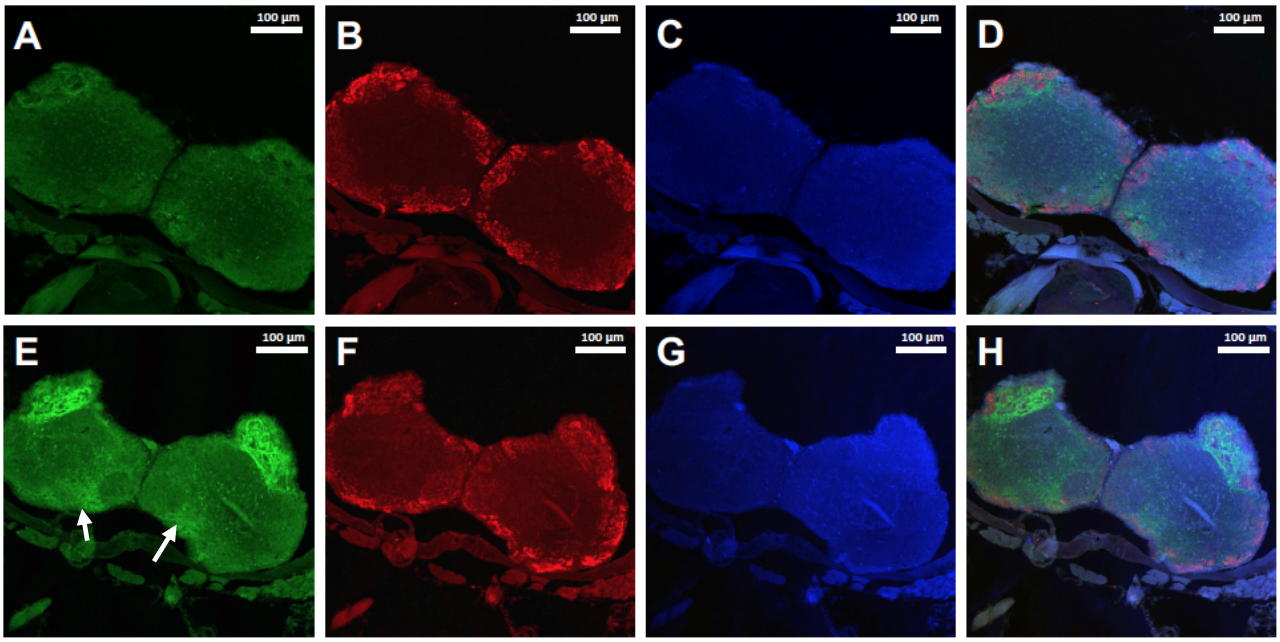


Figure 3.18. *Olfactory bulb immunoreactivity in male fish acutely exposed to EtOH vehicle control (**A-D**) and PGF_2 (**E-F**) for 10 min at 10x magnification. Panels **A** and **E** show green p-ERK immunoreactivity, panels **B** and **F** show red isotocin immunoreactivity, panels **C** and **G** blue DAPI stain, and panels **D** and **H** overlay of all channels. White arrows indicate p-ERK activation in the ventromedial glomerulus area.*

3.2.3. Preoptic area response to acute $PGF_{2\alpha}$ in relation to the isotocinergic system

Signal of p-ERK immunoreactivity was detected in the parvocellular POA of $PGF_{2\alpha}$ exposed male zebrafish in addition to an area corresponding to the ventrolateral optic tract (**Fig. 3.19A+E** and **3.20A+E**). Parvocellular isotocin perikarya and fibers were identified in the posterior part of the POA (**Fig. 3.19B+F** and **Fig. 3.20B+F)**, but did not co-localize with interspersed p-ERK positive cell bodies. (**Fig. 3.19D+H and Fig. 3.20D+H).**

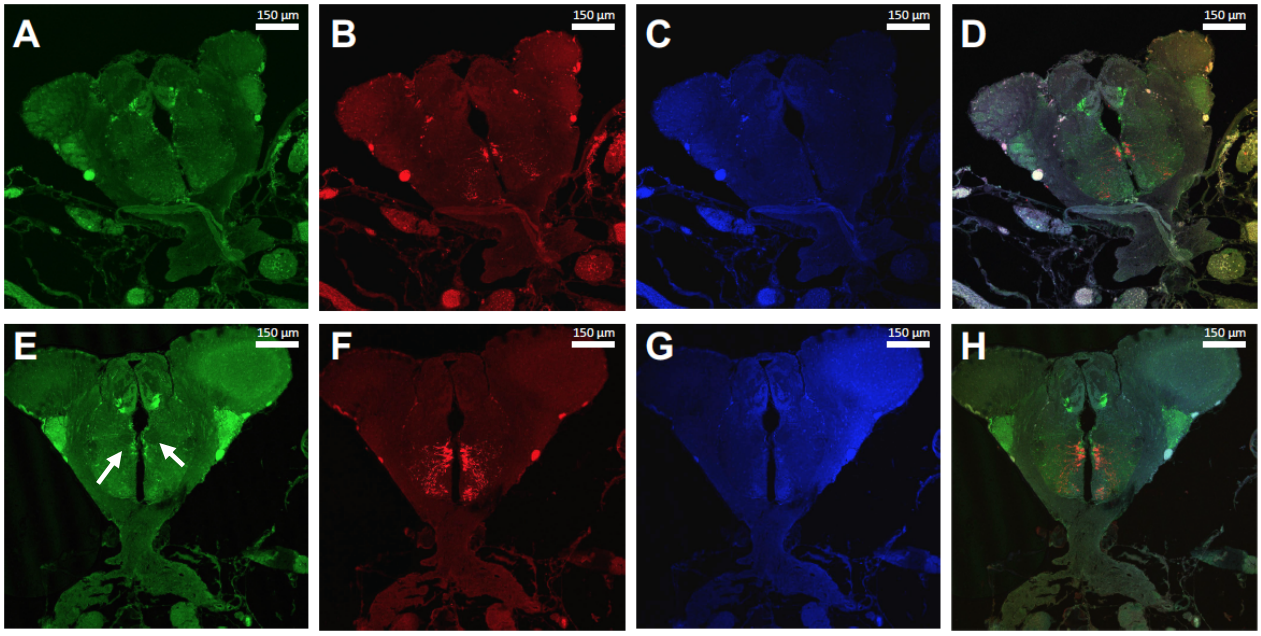


Figure 3.19. *Preoptic area immunoreactivity in male fish acutely exposed to EtOH vehicle control (**A-D**) and PGF_2 (**E-F**) for 10 min at 10x magnification. Panels **A** and **E** show green p-ERK immunoreactivity, panels **B** and **F** show red isotocin immunoreactivity, panels **C** and **G** blue DAPI stain, and panels **D** and **H** overlay of all channels. White arrows indicate p-ERK activation in the parvocellular POA area.*

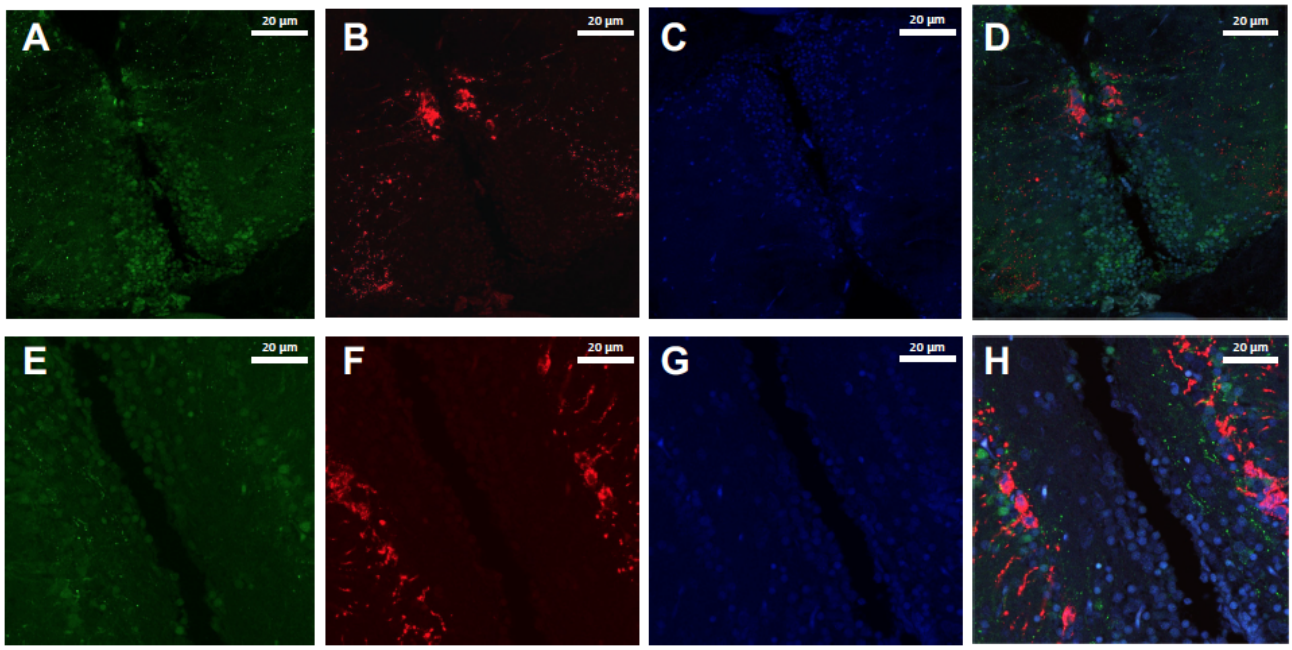


Figure 3.20. *Preoptic area immunoreactivity in male fish acutely exposed to EtOH vehicle control (**A-D**) and PGF_2 (**E-F**) for 10 min at 40x magnification. Panels **A** and **E** show green p-ERK immunoreactivity, panels **B** and **F** show red isotocin immunoreactivity, panels **C** and **G** blue DAPI stain, and panels **D** and **H** overlay of all channels.*

3.2.4. Pituitary area response to acute $PGF_{2\alpha}$ in relation to the isotocinergic system

Due to technical issues in tissue processing, slides for the pituitary gland were only available for $PGF_{2\alpha}$ exposed males. While no comparative information in relation to control can be obtained, the images are included for completeness at two different magnifications (**Fig. 3.21**). p-ERK immunoreactivity is evident in the mediobasal hypothalamus, as well as distinct cells and fibers, including the pars nervosa, of the pituitary gland (**Fig. 3.21A+E**). Strong isotocinergic immunoreactivity is present largely in the pars nervosa of the pituitary (**Fig. 3.21B+F**), but also in individual cases, in proximity to pituitary cells (**Fig.3.21C+G**). Some spatial colocalization of p-ERK and isotocin appears to exist in the pars nervosa of the pituitary gland (**Fig. 3.21D+H**).

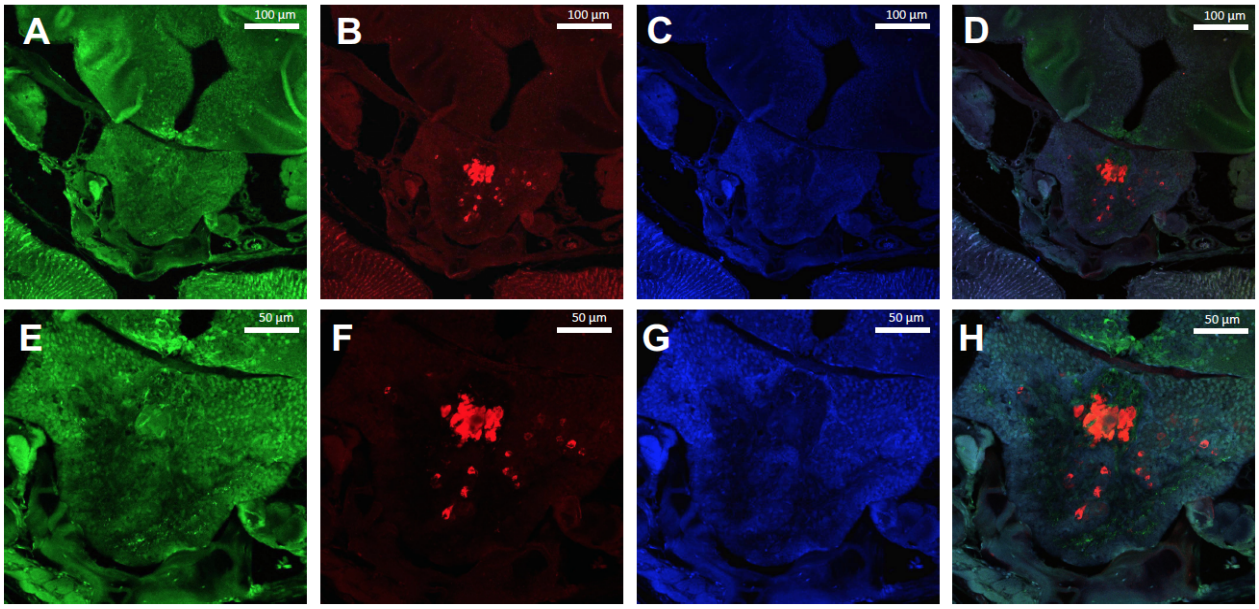


Figure 3.21. *Pituitary gland immunoreactivity in male fish acutely exposed to PGF_2 at magnification of 40x (**A-D**) and 40x (**E-F**) for 10 min at Panels **A** and **E** show green p-ERK immunoreactivity, panels **B** and **F** show red isotocin immunoreactivity, panels **C** and **G** blue DAPI stain, and panels **D** and **H** overlay of all channels.*

3.2.5. Preadsorption of oxytocin antibody with isotocin reveals specificity of isotocin labeling in the male zebrafish brain

While the signal for p-ERK (**Fig. 3.22A**) remains visible, the isotocin signal (**Fig. 3.22B**) was strongly reduced when antibody incubation was conducted with added isotocin, confirming specificity of isotocin labeling with the used experimental protocol.

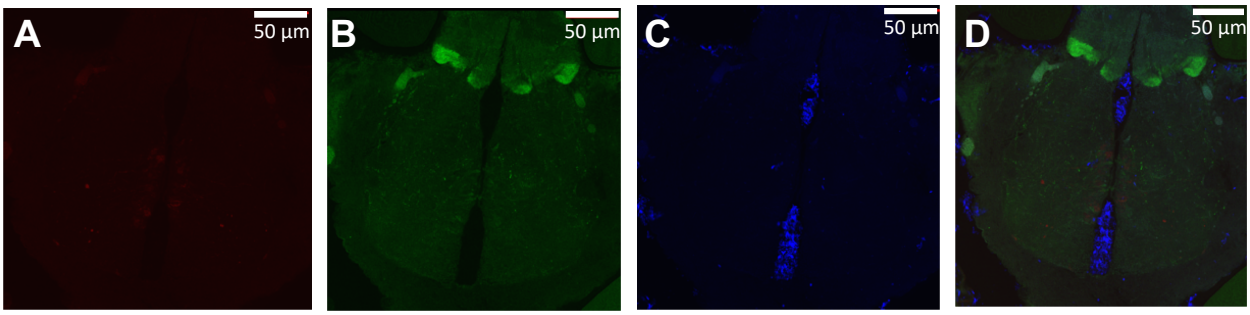
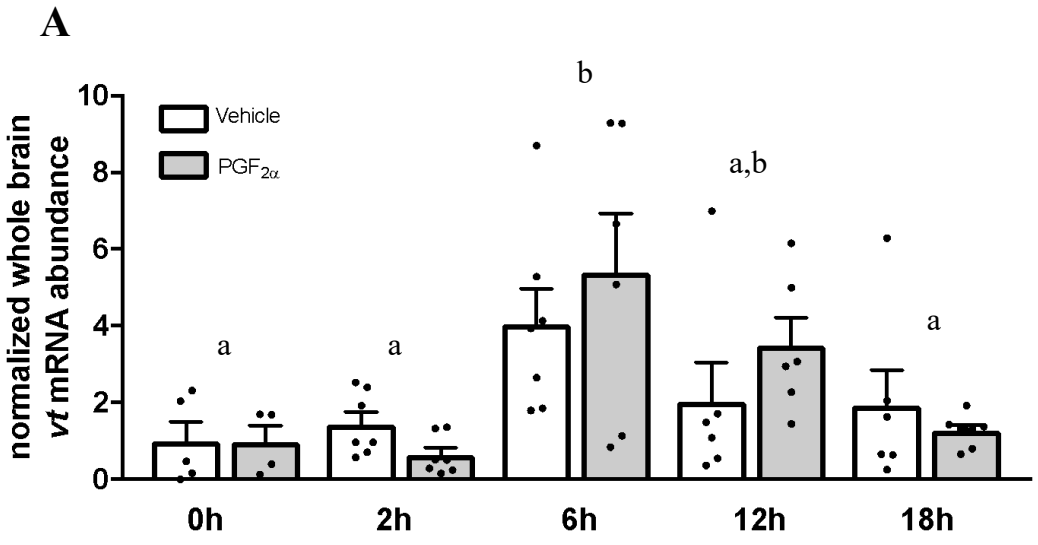


Figure 3.22. *Preoptic area immunoreactivity in male fish acutely exposed to EtOH vehicle for 10 min at a magnification of 20x (**A-D**). Panel **A** shows green p-ERK immunoreactivity, panel **B** show red isotocin immunoreactivity, panel **C** blue DAPI stain, and panel **D** overlay of all channels following pre-adsorption of antibody incubation mixture with 1 mg/ml isotocin.*

3.2.6. Long-term nonapeptide gene expression in response to $PGF_{2\alpha}$ exposure

Whole brain transcript abundance of vasotocin (**Fig. 3.22A**) did not change significantly in response to exposures to vehicle or pheromone ($F_{1,50}$= 0.262 *P*=0.61). Conversely, a significant effect of time abundance ($F_{4,50}$= 0.816 *P*<0.01), but no interaction between treatment and time ($F_{4,50}$=0.992 *P*=0.42) on vasotocin transcript abundance was identified. Compared to baseline values just prior to exposure, vasotocin transcript increased significantly at 6 h compared to all groups (p<0.05) except for 12 h (p>0.05), irrespective of treatment (**Fig. 3.22A**).

Whole brain transcript abundance of isotocin (**Fig. 3.22B**) did not exhibit global differential expression when considering treatment ($F_{1,51}$= 3.538 *P*=0.07) or time ($F_{1,51}$= 2.518 *P*=0.06) as main factors, however a time-dependent change in isotocin transcript abundance was identified by a significant treatment*time interaction ($F_{1,51}$= 3.433 *P*=0.02). Post-hoc analysis revealed significant increases in isotocin transcript abundance in pheromone exposed male zebrafish compared to vehicle control exposed zebrafish at 0 h and 6 h (*P*<0.05).



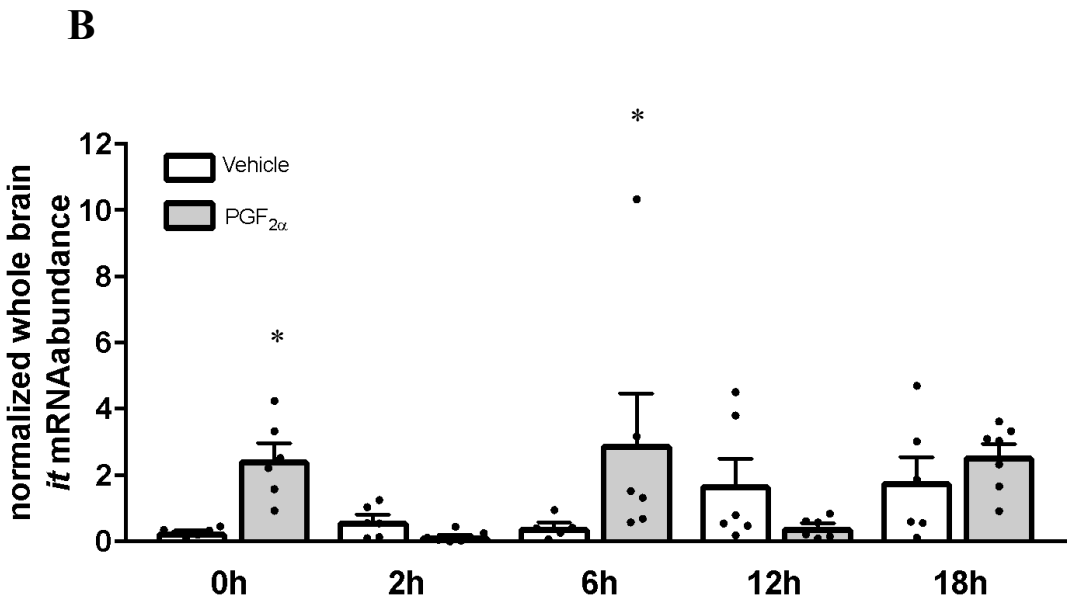


Figure 3.23. *Whole brain relative transcript abundance of vasotocin (**A**) and isotocin (**B**) in male fish acutely exposed to EtOH control (white bars) or PGF_2 (grey bars). An n=7 fish were sampled at different timepoints. Individual data points and average S.E.M. values are shown and were normalized to average control values at 0 h. Different letters indicate significant differences in transcript abundance over time irrespective of treatment, while asterisks indicate significant differences in pair-wise comparisons at specific timepoints.*

3.3. Discussion

3.3.1. Acute exposure to $PGF_{2\alpha}$ does not induce rapid activation of isotocin neurons in the POA

Acute exposure of male zebrafish to $PGF_{2\alpha}$ and vehicle control reveals a mild, but significant preference of 'full choices' towards $PGF_{2\alpha}$ compared to vehicle control. Furthermore, overall p-ERK activity increased in the olfactory bulb compared to male fish exposed to EtOH control, suggesting increased neuronal signaling in response to pheromone. More specifically, this activation is seen in the ventromedial glomerular cell layer, previously reported to be highly specific to $PGF_{2\alpha}$ activation in zebrafish (Friedrich and Korsching, 1998; Yabuki et al., 2016,

Dieris et al., 2017). Additionally, a strongly increased activation was evident in areas of the dorsal external cellular layers. Together, these data are indicative of an acute response of male zebrafish to pheromone, as previously reported (Friedrich and Korsching, 1998; Yabuki et al., 2016, Dieris et al., 2017). My work shows, for the first time in zebrafish, that isotocin is present in the male olfactory bulb, the primary processing center for olfactory information (Myasaka et al., 2014; Kermen et al., 2013). Isotocin was specifically detected in the primary olfactory fiber layer (POF) and glomerular layer (GL) of the olfactory bulb. In zebrafish, the olfactory bulb is organized into four concentric layers, consisting of the lateral POF, which is formed by olfactory sensory neuron axons (Sato et al., 2007), and progressing towards the centre of the bulb, the GL, which contains glomeruli (Braubach et al., 2012), the external cellular layer (ECL) containing mitral cells and ruffed cell somas (Fuller and Byrd, 2005; Fuller 2006), and the internal cellular layer, which contains cell bodies of interneurons such as juxtaglomerular and periglomerular and granular cells (Edwards and Michel, 2002; Bundschuh et al, 2012). In zebrafish, isotocinergic fibers have not previously been identified in zebrafish olfactory bulb, although detailed analysis of projection have, to date, only been analyzed in larval fish (Herget et al., 2017). Conversely, isotocin innervation in the olfactory bulb via ventral telencephalic regions has been demonstrated in the plainfin midshipman (Goodson 2003), suggesting that this feature may be widespread in teleost fishes, at least in adults.

The fact that little colocalization between p-ERK and pheromone signaling occurs in the male zebrafish olfactory bulb, in spite of spatial proximity, suggests that isotocin may play a modulatory, rather than transmissive role for olfactory stimuli in male zebrafish. This notion is further supported by studies in mammals. In rats, for example, oxyotocin enhances social recognition by modulating cortical control of early olfactory processing, at least in part by modulating long-term potentiation at the mitral to granule cell synapse (Dluzen et al., 1998; Fang

et al., 2008; Oettl et al., 2016). The identification of isotocinergic signal in the peri-glomerular and glomerular layers of the adult male zebrafish olfactory bulb raises the possibility that a top-down regulation of isotocin may regulate sensitivity to olfactory reproductive cues, and reproductive pheromones in particular. Future studies investigating possible enhancement of sensitivity to pheromones in intact and occluded fish by isotocin are warranted to test this possibility, and will provide insight as to whether regulation of sensitivity of (reproductive) olfactory stimuli may be a conserved feature in vertebrates.

In the preoptic area of males acutely exposed to $PGF_{2\alpha}$ and EtOH, p-ERK immunoreactive cell bodies were identified but did not reveal a significant difference with regard to the overall p-ERK signal intensity in the area. However, the distribution of p-ERK activation in response to $PGF2_{2\alpha}$ consistently appeared to occur further removed from the ventricular border, while p-ERK immunoreactivity in response to EtOH vehicle appeared to occur in cells immediately adjacent to the third ventricle. No colocalization of p-ERK and isotocin was evident in either case, thus refuting the hypothesis that $PGF_{2\alpha}$ acutely activates isotocinergic neurons in the POA of male zebrafish. While acute activation of isotocinergic perikarya was not observed in response to $PGF_{2\alpha}$ in my experiment, the identity of activated neurons in proximity of isotocin neurons in response to $PGF2_{2\alpha}$ warrants further study. Given its potent role in male zebrafish reproduction identified in pharmacological inhibition studies, and the immediate proximity of p-ERK positive cells to isotocin positive cells in response to $PGF2_{2\alpha}$, allow for the possibility that vasotocinergic neurons are acutely stimulated by $PGF_{2\alpha}$ in zebrafish. Unfortunately, colocalization studies of p-ERK and custom zebrafish-validated (described in Eaton et al., 2008) or commercial cichlid-validated vasotocin antibodies (Reddon et al., 2017) were not possible, as all antibodies were raised in rabbit. Future studies should therefore focus on the identification of vasotocin-specific antibodies raised in other species, or utilize emerging zebrafish nonapeptide reporter lines in conjunction with the

well described p-ERK antibodies used in the current study. However, to our knowledge, only isotocin, but not vasotocin gene expression GFP-based reporter lines have been described (Blechmann et al, 2011; Coffey et al., 2013). Our identification of spatial proximity of p-ERK and isotocin positive signals raises the possibility of $PGF_{2\alpha}$ stimulation of isotocin hormone release. However, in absence of an appropriate immunohistochemistry control from animals exposed to vehicle only, this possibility requires additional validation. Measurements of circulating hormones would be a more direct way to address this, although this possibility is limited in zebrafish compared to larger fish species, where this approach is feasible (Gozdowska et al., 2004).

Overall, while behavioral indices and p-ERK immunoreactivity patterns in the olfactory bulb confirm distinct responsiveness of male zebrafish to acute $PGF_{2\alpha}$ exposure, acute neuronal activation of neurons in the posterior POA in response to $PGF_{2\alpha}$ does not involve isotocin neurons, refuting the hypothesis that $PGF_{2\alpha}$ acutely activates at least part of the nonpapeptidergic population. Given activation of adjacent neurons, and the strong role of vasotocin in regulating male zebrafish courtship behaviour future studies validating non-rabbit vasotocin antibodies or using genetic advantages of the zebrafish model through knock outs, reporter lines or optogenetics are clearly warranted to determine whether $PGF_{2\alpha}$ acutely activates vasotocin neurons.

3.3.2. *Acute exposure to $PGF_{2\alpha}$ selectively and time-dependently modulates brain isotocin transcript abundance*

The possibility of longer-term consequences of pheromones on the noanpeptidergic systems in male zebrafish, assessed by whole brain relative nonapeptide transcript quantification, revealed that isotocin was time-dependently changed in response to pheromone, while vasotocin transcript change was not altered by pheromone exposure, but dependent on time. Previous studies had identified stimulating effects of acute $PGF_{2\alpha}$ on both isotocin and vasotocin in the telencephalon of goldfish at 6 h (Mennigen et al., 2010). While the results for isotocin appear to

confirm this regulation in male zebrafish, the current study reveals that this stimulatory effect is time-dependent, as evidenced by a lack of significant stimulation of whole brain isotocin mRNA abundance at different timepoints. The data should furthermore be interpreted with caution, given that the included a priori baseline control reveals significantly induced isotocin mRNA abundance in fish derived from the designated pheromone groups compared to the designated vehicle control groups immediately prior to exposure. The reason for these baseline differences is unknown, but speculatively may be related to differential hierarchies or interaction patterns between zebrafish groups. Indeed, in zebrafish whole brain nonapeptides abundance has been reported to change in response to social status (Larson et al., 2006), identifying a potential confounding factor in my study. Whether the difference observed in vasotocin mRNA abundance in response to $PGF_{2\alpha}$ between zebrafish and a previous study in goldfish (Mennigen et al., 2010) is reflective of different sampling technique (whole brain or telencephalon), or potential species-specific responses cannot be delineated in the current study, and would require utilization of techniques more sensitive to spatial distribution of transcript abundance, such as *in situ* hybridization techniques. Nevertheless, the current approach is indicative of the fact that the longer-term (6h) responsiveness of the isotocin system to $PGF_{2\alpha}$ may also be in zebrafish. Interestingly, a recent study in medaka (*Oryzias latipes*) reported a male-specific specific upregulation of isotocin mRNA in parvo- but not magno- or gigantocellular neurons which is dependent on androgens. While androgens were not quantified in this experiment, it is possible that $PGF_{2\alpha}$-induced changes in androgens may secondarily have affected isotocin transcript abundance, and whether such secondary regulation occurs across different (cyprinid) fish species is a question of interest. Indeed, acute $PGF_{2\alpha}$-administration in goldfish significantly increases circulating T and telencephalic isotocin mRNA abundance (Mennigen et al., 2010), suggesting that modulation of the isotocin system may indeed be a secondary effect dependent on circulating androgens.

With regard to the time-dependent, but not pheromone-dependent regulation of whole brain vasotocin mRNA abundance, it is interesting to note that clear circadian variations in central whole brain mRNA and circulating protein have been reported for vasotocin, but not isotocin, in several teleost species. For example, in both male and female pupfish (*Cyrpinodon nevadensis amargosae*), pro-vasotocin mRNA (Lema et al., 2010), but not pro-isotocin mRNA reveal diurnal expression patterns in whole brain. In rainbow trout, circulating vasotocin, but not isotocin (Kulczykowksa and Stolarski, 1999) exhibited significant diurnal concentration changes. Together, this finding may be indicative of wide-spread role of vastocin in circadian rhythms in teleost fish, similar to described roles in mammals (Balment et al, 2006).

In summary, I show evidence for a distinct regulation of both nonapeptide systems in zebrafish in response to $PGF_{2\alpha}$ exposure. While the hypothesis of an acute activation of the isotocin system is not supported, the possibility of an acute activation of vasotocin neurons remains to be addressed in future studies, especially given the evidence for vasotocin-dependent regulation of courtship in male zebrafish presented in this thesis. Conversely, I show evidence of a specific regulation of isotocin, but not vasotocin mRNA in response to $PGF_{2\alpha}$, indicating that this system may be sensitive to $PGF_{2\alpha}$ through longer-term and likely indirect mechanisms in multiple cyprinid species.

Chapter 4 – The role of nonapeptides in reproduction in sexually mature, male goldfish

I would like to acknowledge the contributions of several undergraduate students, particularly Sam Touma, Batoul Auf, and Kenan Touma, who have contributed to the blind analysis of male goldfish courtship behaviour recorded during mating assays. I would also like to acknowledge the assistance of Mais Jubouri who contributed to molecular work presented in this chapter.

4.1. Pharmacological inhibition studies to probe the role of nonapeptides in male reproductive physiology in goldfish

In order to probe a possible role for nonapeptides in $PGF_{2\alpha}$ mediated male reproduction in a determinate breeding cyprinid, I used goldfish to experimentally address the hypothesis that nonapeptide systems mediate reproductive effects of $PGF_{2\alpha}$ described in this species. Indeed, previous correlative evidence in male goldfish revealed that a group of males exposed to 6 h of waterborne $PGF_{2\alpha}$ at a concentration of $3x10^{-6}$M increased telencephalic abundance of vasotocin and isotocin mRNA by 3 -fold compared to vehicle exposed control fish. This effect correlated with a significant increase in strippable milt and circulating androgens, raising the possibility that nonapeptides mediate $PGF_{2\alpha}$ effects on the male reproductive endpoints (Mennigen et al., 2010). The specific predictions are that pharmacological inhibition of nonapeptide signaling will reduce baseline or PGF-stimulated of one or several male reproductive endpoints, specifically indices of male courtship behaviour, milt release, and circulating androgens.

4.1.1. Materials and Methods

4.1.1.1. Animal housing and experimental design

A total of n=24 male and n=24 female goldfish were obtained from Aleong's International (Mississauga, ON, Canada) and maintained at the University of Ottawa Aquatics Facility for 1 year prior to the experiment. Sexually mature males, identified by visual inspection of breeding tubercles and the capacity to release milt upon mild pressure application to the abdomen were

sorted and housed in groups of n=6 fish in 70 L tanks two weeks prior to experiments in May. All goldfish were fed once a day with a standard diet (Zeigler Bronze, Zeigler Bros Inc.) at approximately 2% bw per day and maintained in a flow through system at 12 °C under a photoperiod mimicking local seasonal Ottawa sunrise and sunset times, as described previously (Zhang et al., 2009). These photoperiod conditions have been shown to reliably induce sexual maturity in April and May (Zhang et al., 2009). For each trial, a pair consisting of one male and one female fish were allowed to acclimate in an aquarium with gravel and artificial green vegetation for a period of 10 min. Following this period, fish were retrieved and mildly anesthesized in MS-222, weighed, and finally i.p. injected with the following compounds: Male fish were treated with either saline control, 500 ng/g vasotocin antagonist (Manning compound, Tocris) or 500 ng/g isotocin antagonist (L-368,899 hydrochloride, Tocris) by injection with a volume of 1 μL per g bodyweight. Stimulus female fish received either an injection of saline control or $3x10^{-3}$ M $PGF_{2\alpha}$ (Bachem) at an injection volume of 1 μL per g bodyweight concentration of i.p. administered $PGF_{2\alpha}$ in female stimulus goldfish were based on concentrations in previous studies reported to result in an active female release of sufficient $PGF_{2\alpha}$ in the water to stimulate male courtship behaviour in goldfish, irrespective of female sexual maturation status (Sorensen et al., 1988) Following injections, fish were then placed in separate recovery tanks and allowed to recover for 5 min. Following recovery, both fish were placed back into the recording tank and behaviour was recorded for 2 h using a Panasonic HC V180K camcorder on a tripod. At 4 hours following injection, male fish were anesthetized in MS-222, weighed and strippable milt collected in one or multiple pre-weighed capillary tubes for quantification by weight. Following collection of strippable milt, blood was collected by caudal puncture and serum separated by centrifugation at 2200 rpm for 15 min. Serum was finally collected in separate Eppendorf tubes and stored at -80 °C until steroids were extracted for testosterone quantification. Fish were then

euthanized by separation of the spinal cord. Following the verification of sex by visual inspection of the gonads, testes were carefully dissected and weighed for the calculation of the gonadosomatic index. The testes, as well as microdissected telencephali were subsequently individually collected in Eppendorf tubes, quickly frozen on dry ice and stored at -80 °C for transcript quantification. The 4 h endpoint was chosen based on previous studies reporting significant induction of strippable milt in response to $PGF_{2\alpha}$ compared to vehicle control between (Zheng and Stacey, 1996) following exposure and reported responses of telencephalic gene expression (Chung Davidson et al., 2008). Observation of individual pairs following i.p. injection, rather than waterborne group exposures of male goldfish previously reported (Chung-Davidson et al., 2008; Mennigen et al., 2010) was used to allow quantification of male courtship behaviour in addition to strippable milt and circulating androgens. An overview of the experimental design is provided in **Fig. 4.1.**

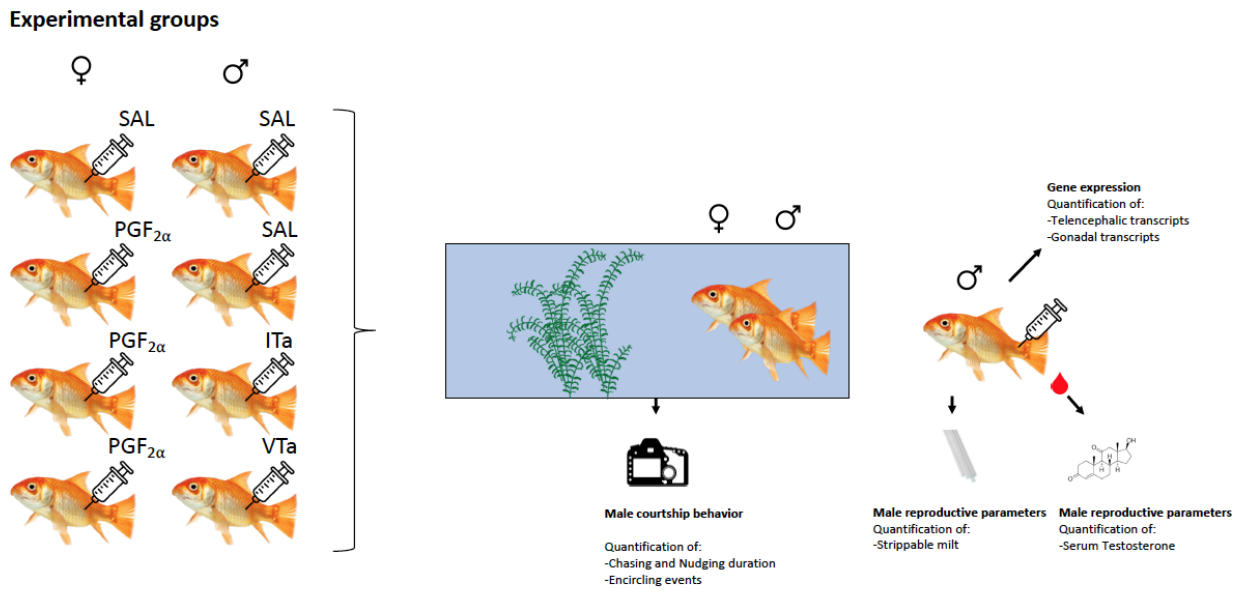


Figure 4.1. *Overview of the experimental design pharmacologically probing the role of nonapeptides in mediating PGF_2 dependent stimulation of reproductive physiology in sexually mature male goldfish.*

4.1.1.2. Courtship behaviour analysis

Behavioural analysis of male courtship behaviour included two quantified indices. Firstly, the time of chasing was measured 10 min prior to injection (baseline) and 10 min following injection. Because chasing occurs in very close proximity in goldfish, nudging behaviour that occurred during chasing could not be reliably be quantified independently, especially given that the videotaping was conducted form the side of the tank. The second quantified index was male circling behaviour. As previously described for zebrafish (Yabuki et al., 2016), these behaviours are well-characterized in other cyprinid fish, including goldfish (Sorensen et al., 1988; Sorensen et al., 1989; Kobayashi et al; 2002; Ghosal and Sorensen, 2016).

4.1.1.3. Steroid extraction serum testosterone quantification

Steroids were extracted from total plasma samples as described by McMaster et al. (1992), using 100 ml of serum as starting volume for repeated diethyl ether extraction (Thermo-Fisher), and a final reconstitution in 500 mL ELISA buffer. Isolated steroid fractions were used for quantification of testosterone in serum samples using the TEST96 ELISA kit (Teco Diagnostics) according to the manufacturers' instructions. Samples were pre-diluted to fall into assay sensitivity range based on expected seasonal ranges of circulating male sex steroids (Kobayashi et al. 1986, Mennigen et al., 2010) and assayed in duplicate. Serum steroid concentrations were calculated based on a regression formula obtained from standard curve absorbance readings fitted by four parameter logistic regression with R^2 values >0.99, taking into account additional pre-dilution of samples.

4.1.1.4. RNA extraction, cDNA synthesis

RNA was extracted from individual telencephalon and testis samples. Approximately 20 mg of tissue was homogenized in 0.5 mL TRIzol reagent (Thermo-Fisher) and care was taken to avoid thawing of samples. The samples were then sonicated, ensuring the probe was cleaned with 75% EtOH and RNAse free water in between each sample. The samples were then incubated for 5 min at room temperature. Following the incubation step, 0.1 mL of chloroform was added to each sample and then the tubes were vigorously shaken by hand for 15 s, followed by another incubation at room temperature for 2 min. The samples were then centrifuged at 12000*g* for 15 min at 8 °C. The upper aqueous phase of each sample was transferred to a new RNAse-free 1.5 mL microtube. 0.25 mL of isopropyl alcohol was added to the aqueous phase, and the samples incubated for 15 min at room temperature. The samples were then centrifuged at 12000*g* for 10 min at 8 °C. The supernatant was poured out and the RNA pellet was washed twice in 0.5 mL of ice-cold 75% EtOH (kept at -20 °C), during which the pellet was initially loosened by vortexing and then precipitated again by centrifugation at 6.6*g* for 5 min at 8 °C. Using a pipette, the EtOH was removed from the tube, and left over EtOH was allowed to evaporate off from the inverted tubes placed on Kimwipe paper in the fume hood for 15 min. Finally, 30 µL of RNAse free molecular grade water (VWR, Mississauga, ON, Canada) was added to each tube in order to dissolve the pellet, and samples were stored at -80 °C. Extracted RNA was quantified using a NanoDrop® 2000c UV-Vis Spectrophotometer (Thermo Scientific). cDNA was then generated from 1 µg of total RNA per sample using a QuantiTech Reverse Transcription Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. Briefly, the volume corresponding to 1 µg total RNA was adjusted to a total volume of 12 µL for each reaction, and 2 µL of 7x g DNA WipeOut buffer were added to each sample. All samples were then incubated at 42 °C for 2 min, after which reaction were immediately quick chilled on ice. A total of 6 µL of

Mastermix consisting of 1 µL Quantiscript RT, 4 µL of 5x Quantiscript RT buffer, and 1 µL of RT primer mix was then added to each reaction for a final volume of 20 µL. The final reaction was finally incubated at 42 °C for 15 min, followed by an incubation for 3 min at 95 °C to deactivate reverse transcriptase. All incubation steps were conducted using an Eppendorf Mastercycler Gradient machine (Eppendorf, Hamburg, Germany). Following condensation on ice, tubes were spun down to collect the reaction volume at the bottom of the well and stored at -80 °C for subsequent semi-quantitative quantification of nonapeptide transcripts.

4.1.1.5. Semi-quantitative quantification of telencephalic and gonadal transcripts

Two-step, SYBR-green-based, semiquantitative *real-time* RT-PCR assays were used to measure relative fold-changes in chicken-gonadotropin releasing hormone (*c-gnrh*), salmon-gonadotropin releasing hormone (*s-gnrh*), vasotocin (*vt*) and isotocin (*it*), mRNA abundance in the telencephalon and relative-fold changes in luteinizing hormone receptor (*lhr*), follicle stimulating hormone receptor (*fshr*), vasotocin 1a recptor (*vtr1a*), and isotocin receptor (*itr*) in the testes using a BioRad CFX96 machine (Bio-Rad). Specific transcripts associated with the endocrine reproductive axis in telencephalon (*c-gnrh* and *s-gnrh*), as well as testis (*lhr*, *fshr*) were profiled to assess potential effects of $PGF_{2\alpha}$ pheromone exposure and in conjunction with vasotocin and isotocin antagonist on this axis at the transcript level. Previous studies in sexually mature male goldfish exposed to $PGF_{2\alpha}$ (Chung Davidson et al., 2008; Lado et al., 2013) have reported differential regulation of *c-gnrh* and *s-gnrh* isoforms in response to $PGF_{2\alpha}$. Central (*vt*, *it*) and peripheral indices of nonapeptide systems (*vtr1a*, *itr*) were probed at the transcript level to confirm previously reported nonapeptide transctipt stimulation in response to $PGF2_{2\alpha}$ in male goldfish (Mennigen et al., 2010) and to assess potential changes in testicular response to circulating nonapeptides at the transcript level. Briefly, a standard curve consisting of serial dilutions of

pooled cDNA, a negative no-RT control consisting of cDNA generated in a reaction that did not include reverse transcriptase, and individual samples were run in duplicate for each experiment. For each individual reaction, the total volume was 20 µL, which consisted of 4 µL of diluted cDNA template, 0.5 µL of 10 nM specific forward and 0.5 µL of 10 nM specific reverse primer (**Table 4.1**), 10 µL of SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad), and 5 µL of RNAse-free H_2O (VWR). For each assay, cycling parameters were a 5 min activation step at 95 °C, followed by 40 cycles consisting of a 20 s denaturation step at 95 °C and a combined 30 s annealing and extension step at primer specific temperatures between 57-60 °C (**Table 4.1**). After each run, melting curves were produced by gradually increasing temperature and the final curves were monitored for single peaks to confirm the specificity of the reaction and the absence of primer dimers. The acceptable range for amplification efficiency calculated from serially diluted standard curves was 90 –110%, with R^2 values of 0.95. Assays were subsequently normalized using the ΔΔCT approach as described by Pfaffl (2001). Finally, mRNA fold changes were calculated relative to the saline injected control group values to determine relative fold-change between treatment groups. All primer pairs, with the exception of primers targeting the isotocin receptor transcript, had previously been validated in goldfish (Chung Davidson et al. 2008; Mennigen 2010; Walton et al., 2010). Isotocin receptor specificity was, in addition to monitoring dissociation curves from a single peak, validated by sequencing of purified PCR product at the Ottawa Hospital Research Institute (OHRI) sequencing facility followed by BLAST search to confirm sequence identity.

Transcript ID	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)	Annealing T (°C)
s-gnrh U30301	TGGTGAAGTGGAGGCAACA	TTCAGCGTCCACCTCACTCA	122	60
c-gnrh U30386	TGCAGGCTGTTTGTGGTGAT	TGCAGGCTGTTTGTGGTGAT	90	60
vt HM140792	TGCCTGCTACATCCAGAAC	GAGACCCGAGCAGACAA	117	60
it AF322651	GTATCTGCTGTGGTGAAGG	ATCTTGGCTACTGGCAGCTT	236	60
lhr HM347776	CCTCTGCATCGGTGTGTATC	AGAGCGTGTGTGATCGTTGT	206	60
fshr HM347775	AGCATCTGCCTGCCAATG	GAAGTCGGTGAAGATGAGCA	184	60
vtr1a XM_026227356	GCATCTCGTTTCCAAACCCAACCA	AGTGCATCCGTGAGCTCTTCTTCT	205	59
itr XM_026261054	TCAGCATTGCAGATCTGGTC	CGTAACAGCGGTCCTTTCTC	228	58
β-actin AB039726	ACTACTGGTATTGTGATGGACTCC	CGGTCAGGATCTTCATCAGGTAG	100	57

Table 4.1. *Primer sequences and reaction parameters for semi-quantitative transcript quantification in telencephalon and testes using SYBR Green real-time RT-PCR assays.*

4.1.1.6. Statistical analysis

In all cases, data were tested for normality and homoscedasticity using Shapiro-Wilk and Bartlett's test respectively. In cases where data did not meet either of these criteria, data was transformed using standard transformation procedures (log, sqrt, inversion). Raw or transformed datasets which met the criteria of a normal distribution and homoscedasticity were then analyzed using a one-way analysis of variance to identify significant differences between treatment groups. In cases of significance, Tukey's post-hoc test was used to delineate differences between specific treatment groups. In cases raw or transformed data did not meet ANOVA criteria, data was analyzed by a Kruskal-Wallis test and specific differences between groups resolved using Dunn's nonparametric comparison post-hoc test. In all cases, a P-value <0.05 was considered as cut-off for significance. All analyses were conducted and graphs plotted using Prism Version 7 (Graphpad Software, LaJolla, CA, USA).

4.2. Results

4.2.1. Pharmacological inhibition of isotocin and vasotocin signaling does not affect male courtship behaviour

Two quantified indices of male courtship behaviour, specifically the duration of chasing and the number of encircling events 10 min prior to treatment and 10 min post-treatment were not significantly different within any treatment groups (P>0.05 in all cases).

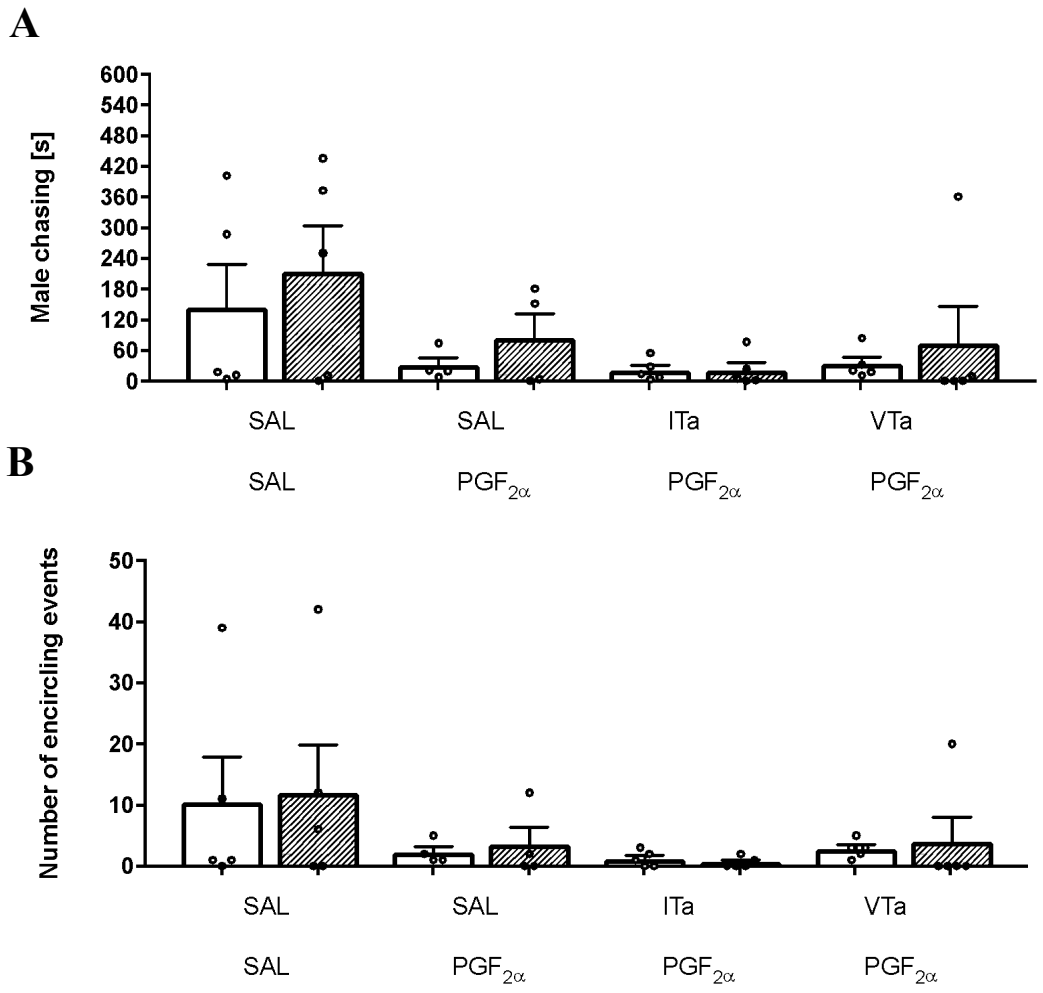
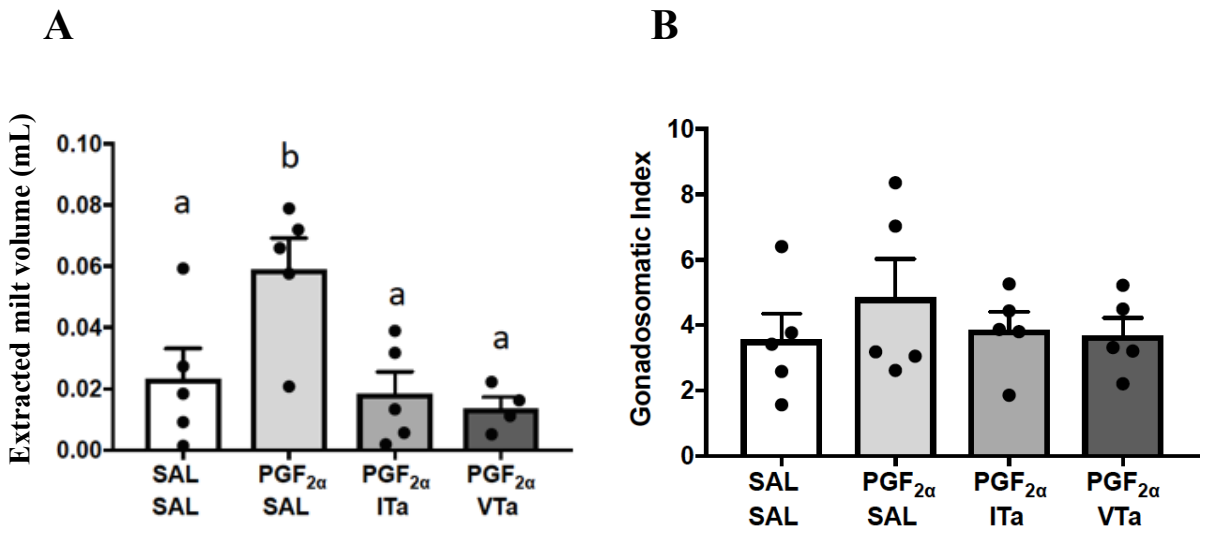


Fig. 4.2. *Courtship behaviour measured as chasing duration* (**A**) *and number of encircling events* (**B**) *in male goldfish pretreated with saline or specific nonapeptide antagonists in response to stimulus females injected with saline or PGF_2. Individual behaviours S.E.M. of n=4-5 male fish 10 min pre- and 10 min post-injection are shown and average are shown. Data were analyzed using paired t-test.*

4.2.2. Pharmacological inhibition of isotocin and vasotocin signaling reduces $PGF_{2\alpha}$ stimulated milt release

The quantity of milt exhibited significant differences between treatment groups ($F_{3,19}$=5.825 P<0.01) with significantly higher milt in saline injected male fish exposed to a $PGF_{2\alpha}$ injected females compared to other groups (P<0.05). No significant changes were identified in gonadosomatic index ($F_{3,19}$=0.5241 P=0.67). Given that a trend for higher gonadal weight was observed in the same group, I also analyzed strippable milt normalized to GSI between different treatment groups and identified significant differences between groups ($F_{3,19}$=4.42 P<0.02). While males pre-injected with ITa and VTa paired with $PGF_{2\alpha}$ injected females exhibited lower strippable milt volumes compared to saline injected male fish paired with a $PGF_{2\alpha}$ injected females (P<0.05), saline injected males paired with saline injected females exhibited strippable milt amounts not significantly different from any other group (P> 0.05).



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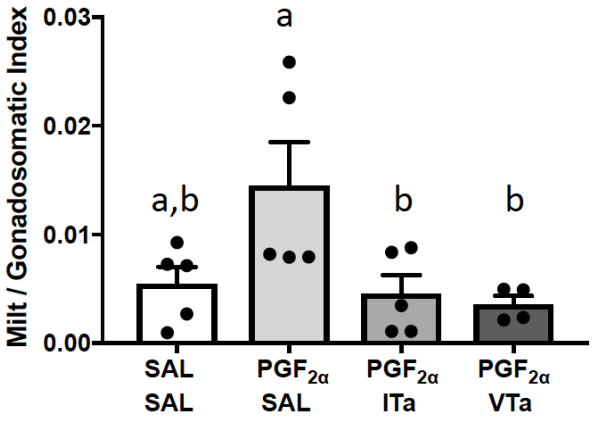

Fig. 4.3. *Male goldfish strippable milt* (**A**) *gonadosomatic index* (**B**) *and strippable milt normalized to gonadosomatic index* (**C**) *in male goldfish pretreated with saline or specific nonapeptide antagonists in response to stimulus females injected with saline or* PGF_2. *Individual data points and average S.E.M. are depicted. Individual data points and average S.E.M. of n=5 male fish are shown. Data were analyzed using a one-way ANOVA. Different letters indicate significantly different groups as determined by Tukey's post-hoc test.*

3.2.3. Pharmacological inhibition of isotocin and vasotocin signaling does not affect male serum testosterone concentrations

Male goldfish serum testosterone concentration was not significantly different between treatment groups ($F_{3,17}$=1.486 P=0.26).

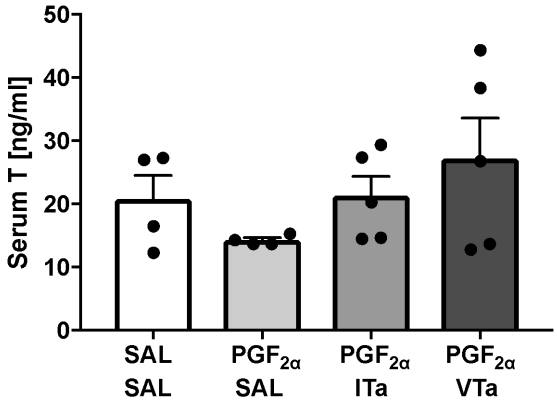

Figure 4.4. *Male goldfish strippable milt* (**A**) *gonadosomatic index* (**B**) *and strippable milt normalized to gonadosomatic index* (**C**) *in male goldfish pretreated with saline or specific nonapeptide antagonists in response to stimulus females injected with saline or* PGF_2. *Individual data points and average S.E.M. are depicted. Individual data points and average S.E.M. of n=4-5 male fish per group are shown. Data were analyzed using a one-way ANOVA. Different letters indicate significantly different groups as determined by Tukey's post-hoc test.*

4.2.4. Nonapeptide antagonists do not significantly alter telencephalic transcript abundance of genes associated with the endocrine reproductive axis and nonapeptides

No significant changes in telencephalic transcript abundance of *c-gnrh* ($F_{3,24}$=0.37 P=0.77), *s-gnrh* ($F_{3,24}$=0.76 P=0.53), *vt* ($F_{16,3}$=0.98 P=0.43), or *it* ($F_{16,3}$=1.74 P=0.21) were found between treatment groups.

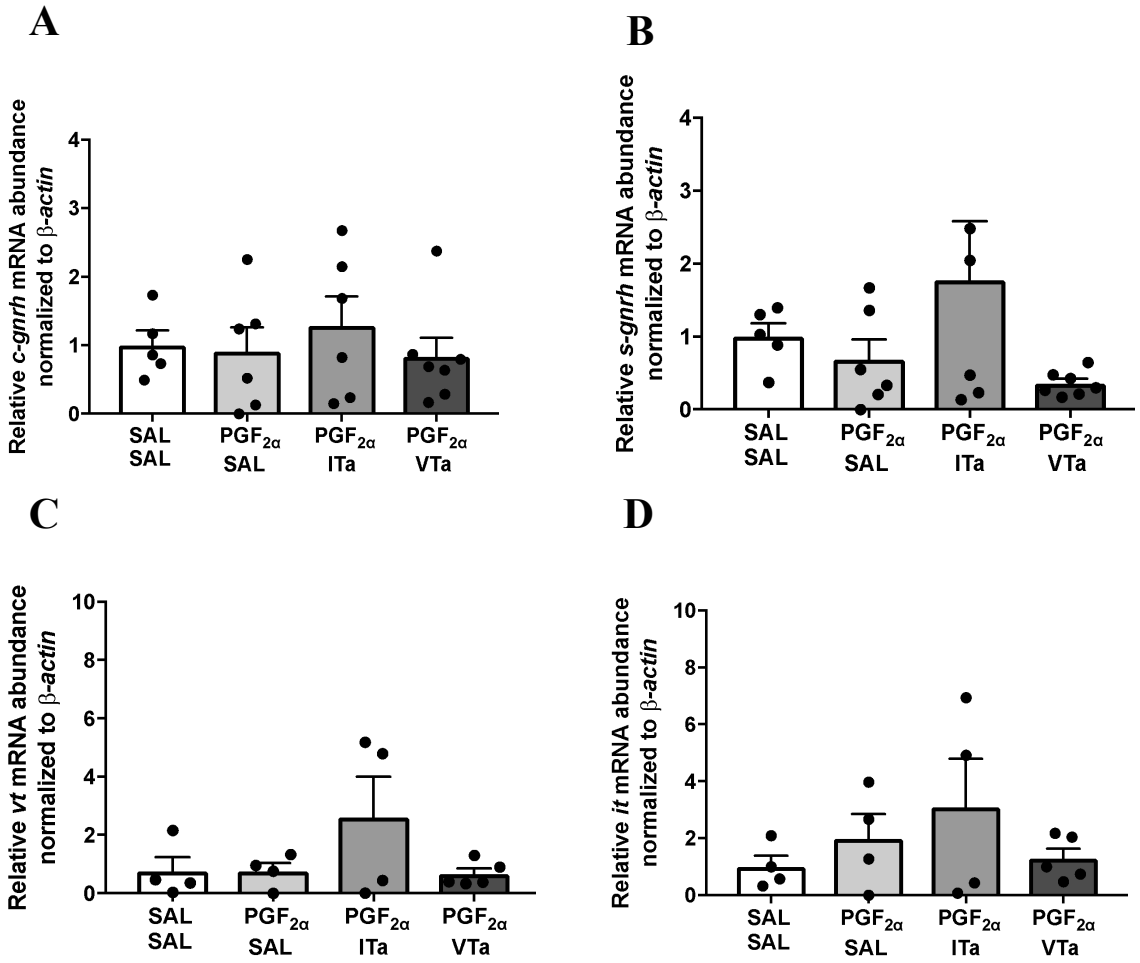

Figure 4.5. *Male goldfish testes transcript abundance of c-gnrh* (**A**) *s-gnrh* (**B**) *vt* (**C**) and *it* (**D**) *in male goldfish pretreated with saline or specific nonapeptide antagonists in response to stimulus females injected with saline or PGF_2. Individual data points and average S.E.M. are depicted. Individual data points and average S.E.M. of n=6 male fish are shown. Data were analyzed using a one-way ANOVA. Different letters indicate significantly different groups as determined by Tukey's post-hoc test.*

4.2.4. Nonapeptide antagonists do not significantly alter testes transcript abundance of genes associated with the endocrine reproductive axis and nonapeptides

The transcript abundance of *lhr* ($F_{3,22}$=0.8233 *P*=0.50), *fshr* ($F_{3,22}$=1.198 *P*=0.34), *vtr1a* ($F_{3,22}$=0.3412 *P*=0.80) and *itr* ($F_{3,22}$=0.38 *P*=0.77) was not significantly different between groups.

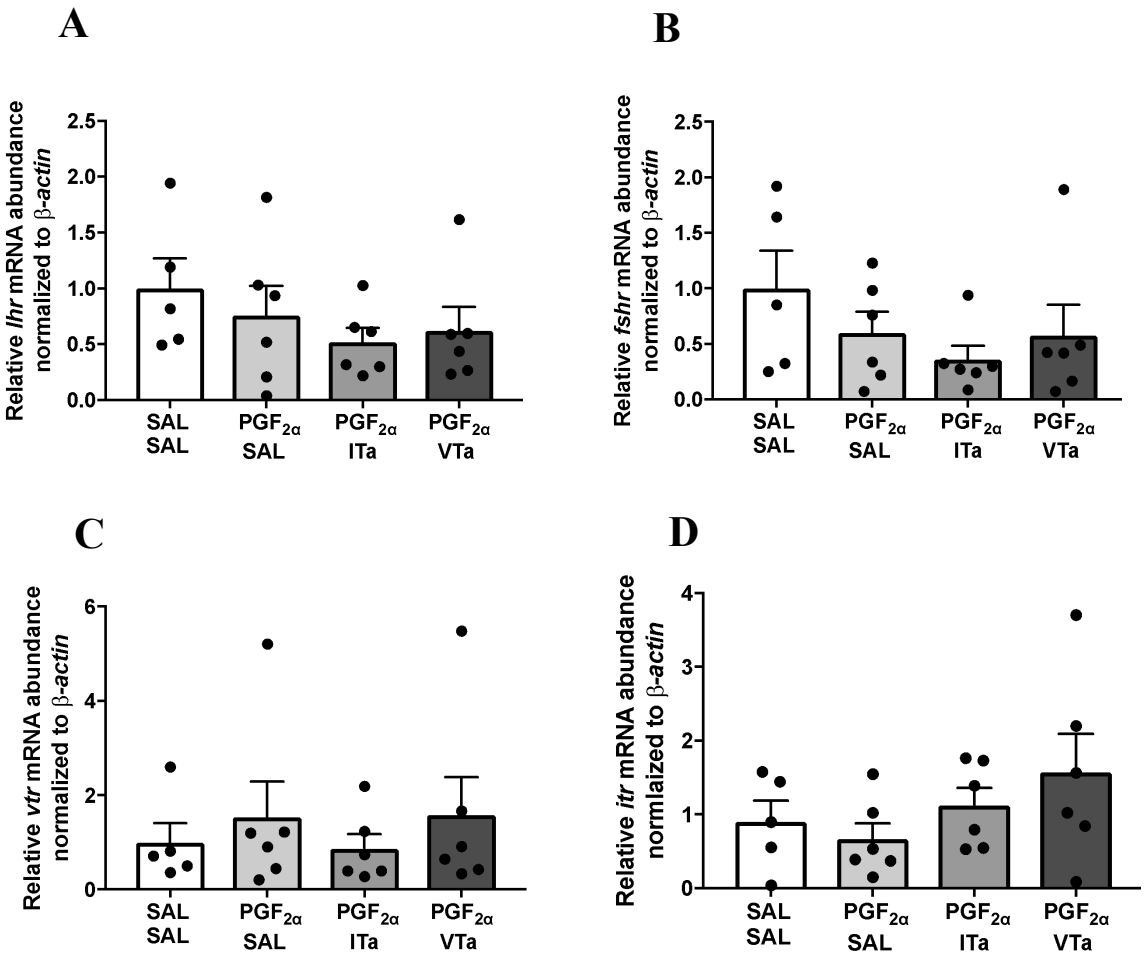

Figure 4.6. *Male goldfish testes transcript abundance of lhr* (**A**) *fshr* (**B**) *vtr1a* (**C**) and *itr* (**D**) *in male goldfish pretreated with saline or specific nonapeptide antagonists in response to stimulus females injected with saline or* $PGF_{2\alpha}$. *Individual data points and average S.E.M. are depicted. Individual data points and average S.E.M. of n=4-6 male fish are shown. Data were analyzed using a one-way ANOVA. Different letters indicate significantly different groups as determined by Tukey's post-hoc test.*

4.3. Discussion

When probing the role of nonapeptide systems in mediating $PGF_{2\alpha}$-dependent stimulation of male reproductive physiology at the level of male courtship behaviour, strippable milt and circulating testosterone using a design combining saline or $PGF_{2\alpha}$ stimulus females with saline or nonapeptide receptor antagonist-injected sexually mature male fish, a clear effect on strippable milt volume, but not endocrine or behavioural endpoints was observed in male fish. The identified significant $PGF_{2\alpha}$ stimulated milt release in this study is in line with previous reports identifying an increase in strippable milt in male goldfish exposed to $PGF2_{2\alpha}$ via direct waterborne exposure or waterborne exposure through $PGF2_{2\alpha}$ injected females (Sorensen et al., 1989; Mennigen et al., 2010). As indicated by the gonadosomatic index (Trudeau, 1992), the capacity to release milt upon stimulation as tested one week before the experiment, and the presence of breeding tubercles, all males used in the experiment were sexually mature, rendering them able to respond to $PGF2_{2\alpha}$ at a high sensitivity (Kobayashi et al., 2002). Because there was a slight trend for increased gonadosomatic index in the $PGF2_{2\alpha}$ group, we normalized strippable milt data to gonadosomatic index, which rendered the $PGF2_{2\alpha}$ mediated increase not significantly different between groups comprised of saline-injected males paired with saline injected females and saline injected males paired with $PGF2_{2\alpha}$ injected females. Importantly, however, a significant reduction in extractable milt was identified in males injected with either a vasotocin receptor 1a or isotocin receptor antagonist prior to pairing with $PGF2_{2\alpha}$ injected females when compared to saline injected males exposed to $PGF_{2\alpha}$-injected females. This result suggests that nonapeptide systems play a functional role in $PGF_{2\alpha}$-mediated increases in strippable milt which had been postulated based on correlative evidence (Mennigen et al., 2010). Indeed, nonapeptides have long been considered to be candidates of mediating milt release in sexually mature male goldfish, either based on neuroanatomical

evidence of a hypothalamic-spinal pathway (Demski and Sloan, 1985) or based on evidence that nonapeptides act as potent stimulators of strippable milt in other teleost species (Pickford & Strecker, 1977). However, early studies in goldfish investigating this possibility by intracerebroventricular administration of vasotocin in male goldfish reported only mild effects at high concentrations believed to be indicative of peripheral actions rather than centrally mediated effects (Peter, 1977). Our experimental design is not able to differentiate between central and/or peripheral endocrine effects, both of which can be hypothesized to play a role in increasing milt concentration in the sperm ducts. In mammals, both central and peripheral roles for nonapeptides in male reproductive function have been described (Nicholson et al, 1999; Thakare et al., 2006; Viero et al., 2010). Additional studies measuring circulating nonapeptides, *ex vitro* approaches investigating the role of nonapeptides on intact testes devoid of innervation, or given the recent advances on the goldfish genome sequence (Chen et al., 2018), future targeted KO studies are clearly warranted to distinguish between these possibilities in goldfish. With regard to the comparatively strong effect of antagonists in reducing strippable milt compared to the previous studies investigating central administration of at least vasotocin directly, differences in potency may depend on the experimental approach. Our study used $PGF2_{2\alpha}$ to increase baseline amount of strippable milt in sexually mature male goldfish and, to our knowledge for the first time in this species, used nonapeptide antagonists to probe the role of nonapeptides in this process. Therefore, it is possible that the nonapeptide systems are already strongly activated to promote milt release in sexually mature fish, and that antagonist approaches provide a better experimental approach to probe the role of nonapeptides on milt release in sexually mature male goldfish, especially when stimulated with the known releaser pheromone $PGF2_{2\alpha.}$ Interestingly, seasonal increases in brain nonapeptide transcripts and protein abundance or circulating nonapeptides been reported in several teleost fishes exhibiting determinate breeding and seasonal reproduction. In goldfish, while true

for female fish (Zhang et al., 2008) possible seasonal variations of nonapeptide systems remain unknown and await characterization in order to test whether activity of nonapeptide systems is indeed enhanced in sexually mature male goldfish.

In contrast to the identified effect on $PGF_{2\alpha}$ stimulated milt release, neither endocrine nor behavioural indices were affected by antagonist exposure. $PGF_{2\alpha}$-dependent increases in groups of sexually mature male goldfish have previously been reported after 6 h of exposure (Mennigen et al., 2010), likely reflecting prior activation of the endocrine axis as indicated by increases in circulating LH after 1 h in groups of males exposed to $1x\ 10^{-7}$ M $PGF_{2\alpha}$ via direct administration of the pheromone. While this effect is dependent on social context, as isolate males exposed to the same concentration of waterborne $PGF_{2\alpha}$ do not mount an acute LH response, single males paired with $PGF_{2\alpha}$ injected females do (Sorensen et al., 1989), suggesting that, in contrast to the reported stimulation of the male endocrine axis in response to the primer pheromone 17,20P, socio-sexual context is important in the $PGF_{2\alpha}$ dependent activation of the endocrine reproductive axis in sexually mature male goldfish. In light of this evidence, a stimulation of the endocrine axis was expected, however a possible explanation for the lack of observed effect on circulating testosterone in $PGF2_{2\alpha}$ females may be that the timepoint of 4 h was too early to detect alterations of circulating responses to increased LH. In order to test this possibility and further validate male goldfish responses to $PGF_{2\alpha}$, goldfish LH concentrations will be quantified by Dr. John Chang, a collaborator at the University of Alberta, using a cyprinid-specific LH assay (Mennigen et al., 2010). At least at the 4 h sampling time, which, as discussed above, may reflect baseline rather than $PGF_{2\alpha}$-stimulated testosterone concentrations, neither the vasotocin receptor 1a antagonist nor the isotocin receptor antagonist affected circulating testosterone concentrations. In the case of isotocin, sexually regressed female, but not male fish increased LH and sex steroids in a time-dependent manner in response to intraperitoneal injection of isotocin (Popesku et al., 2011;

Mennigen et al., 2017), suggesting that effects of nonapeptides on the endocrine axis in goldfish male be sexually dimorphic, with female-specific stimulation of the axis. Vasotocin stimulation of LH release has been reported to be sex-specific in another teleost, the sailfin molly, with higher responsiveness in male fish (Groves and Batten, 1986). In addition to modulating circulating testosterone via LH and hence pituitary-dependent effects, nonapeptides may modulate sex steroid synthesis at the level of the gonad via specific membrane-bound receptors, as indicated by the capacity of vasotocin and isotocin to stimulate testosterone release in *ex vivo* incubated rainbow trout testes. The lack of observable differences in circulating testosterone concentration in sexually mature goldfish pre-injected with saline or specific nonapeptide receptor antagonists following 4 h of exposure of females injected with $PGF_{2\alpha}$ suggests that nonapeptide-dependent peripheral modulation of testosterone release does not occur in sexually mature goldfish *in vivo*.

No significant differences between male courtship behaviour 10 minutes prior and 10 minutes after injections were observed within groups. The lack of stimulation of male reproductive behaviour in response to female s injected with $PGF_{2\alpha}$ compared to females injected with saline is surprising as robust effects of this treatment have been widely documented (Sorensen et al, 1988; Sorensen et al., 1989; Kobayashi et al., 2002). While large variability in pre-injection measurements between groups as well as small sample size may have been a contributing factor, the lack of difference between pre-and post-injection behavior in groups treated with nonapeptide antagonist cannot be adequately interpreted in the absence of this positive control. Future larger scales experiments are therefore warranted to conclusively address the role of nonapeptides in $PGF_{2\alpha}$ dependent stimulation of sexually mature male reproductive behaviour. Following the assessment of nonapeptide receptor inhibition on key components of male goldfish reproductive physiology (strippable milt, endocrine axis endpoints, male courtship behaviour) following a 4 h exposure to $PGF_{2\alpha}$ females, transcript abundance of key reproductive genes was measured in the

telencephalon and hypothalamus to identify possible molecular changes underlying the observed changes in strippable milt. In line with the observed lack of effect of either antagonist treatment on the endocrine axis, assessed by circulating testosterone, no transcript changes in *c-gnrh* or *s-gnrh* were identified in the male telencephalon between treatment groups. Previous studies have reported an effect of $PGF_{2\alpha}$ on *s-gnrh* but not *c-gnrh* in groups of sexually mature male goldfish exposed to $PGF_{2\alpha}$ for 4 h and 6 h (Chung Davidson et al. 2008, Lado et al., 2013). Our study is the first to investigate reproductive gene transcript abundance in male goldfish that are individually paired with $PGF_{2\alpha}$ females. The lack of observable changes in transcript abundance of *s-gnrh* in response to $PGF_{2\alpha}$ may therefore be reflective of a different sociosexual context, although this hypothesis will require further validation. In goldfish, *c-gnrh* II, but not s-gnrh is generally considered to be hypophysiotrophic, while *s-gnrh* has been linked to neuromodulatory function which affects female, but not male reproductive behaviour in goldfish (Volkoff and Peter, 1999). At the level of the testes, no alteration of *lhr* or *fshr* abundance was identified between treatment groups. A lack of change in transcript abundance of either receptor had previously been reported in a group of sexually mature goldfish exposed to $PGF_{2\alpha}$ compared to a vehicle exposed control group (Mennigen et al., 2010). Together, these results reveal firstly that peripheral components of the reproductive endocrine axis are, at least at the transcript level, not responsive to $PGF_{2\alpha}$ under different experimental conditions, and secondly, that they are not subject to modulation by vasotocin (at least via Vtr1a signaling) or isotocin in sexually mature male goldfish paired with $PGF_{2\alpha}$ injected female fish. In addition to transcript components of the hypothalamic pituitary gonadal (HPG) axis. I also probed transcript abundance of the nonapeptides isotocin and vasotocin in the telencephalon,, as well as their receptor in the testes. As for HPG axis components, no significant differences in transcript abundance were identified between groups. This is contrast to the reported increases in isotocin and vasotocin mRNA abundance in sexually mature male

goldfish after 6 h (Mennigen et al., 2010), and may be related to time and or experimental design differences.

Overall, results from my study show that isotocin and vasotocin signaling play a role in mediating stimulated milt release in sexually mature male goldfish paired with $PGF_{2\alpha}$ females for 4 h, but do not affect endocrine endpoints, as assessed by the lack of changes in circulating testosterone. A lack of action on the HPG axis is also confirmed at the transcript level by a lack of observable change in gonadotropin releasing hormone transcripts in the telencephalon, as well as gonadotropin receptors in the testes. While some of the effects of $PGF_{2\alpha}$ on sexually mature male goldfish reproductive physiology, specifically the stimulation of strippable milt and courtship behaviour, have been shown to be independent of the pituitary and HPG axis (Zheng and Stacey, 1997), different neuroendocrine pathways have recently been speculated to mediate specific effects of $PGF_{2\alpha}$ (Ghosal and Sorensen, 2016). Here we provide evidence that the reproductive effects of $PGF_{2\alpha}$ on milt release, but not courtship behaviour or the HPG axis are mediated by vasotocin and isotocin in sexually mature male goldfish.

Chapter 5 -- Conclusion

Through experiments in two male cyprinid species, the zebrafish and the goldfish, which are characterized by indeterminate and determinate reproductive strategies in a laboratory setting, respectively, I investigated the specific role of the two conserved nonapeptide systems in male cyprinid species. While specific discussions of the experimental results obtained in zebrafish and goldfish are contained within the relevant data chapters, this short conclusion section serves to place my experimental findings in the context firstly of my overall hypothesis, and secondly in the context of the current state of knowledge in the field. Finally, I will conclude by commenting on possible limitations of the experimental approaches and identify specific research questions for future investigation of the role of nonapeptides in male teleost fishes and non-mammalian vertebrates in general.

My experimental results clearly identify a role for both nonapeptide systems in male reproductive physiology and success. In zebrafish, vasotocin, by acting via the VTr1a receptor, significantly reduced male reproductive success, an effect correlated with significant inhibition of most indices of male courtship behaviour, suggesting that, at least in part, vasotocin contributes to male reproductive success in zebrafish via acute central activation of male courtship behaviour pathways. While isotocin also contributed to male zebrafish reproductive success, no clear correlation with behavioural or endocrine endpoints could be identified. Because females paired with isotocin antagonist pre-treated males did not release unfertilized eggs, indicative of altered sperm release in male fish, while maintaining adequate reproductive behaviour and signals to induce female egg release at the same time, the mechanisms remains unknown. However, because significant reductions in specific aspects of male courtship behaviour in mating trials were identified in isotocin antagonist pretreated males, albeit at a different concentration than the highest concentration associated with significant reduction in fertilized eggs, a role of isotocin in

regulating male zebrafish reproductive success via modulation of male courtship behavior cannot be fully excluded. Indeed, because of technical limitations related to the camera angle used, behaviour could only be reliably quantified for a subset of males pre-injected with isotocin. Therefore, additional studies are needed to test whether observable tendencies of reduced male courtship behaviour at the highest concentration of isotocin antagonist used may translate into significant reduction at similar sample sizes compared to the vasotocin antagonist used. Surprisingly, a complete inhibition of male reproductive success in mating trials was observed when nonapeptide antagonists were co-administered. This effect was not related to male courtship behaviour, endocrine reproductive axis activation, at least as assessed by whole body androgen concentrations or sperm release, as assessed by the lack of unfertilized eggs. Therefore, a different mechanism other than the ones addressed correlatively in zebrafish in this thesis must be responsible for this effect. I propose that male pheromone release associated with female egg release (Eaton and Farley, 1974; Van Den Hurk and Lambert, 1983; Van Den Hurk et al., 1987) may underlie this effect, however the nature of this pheromone is unknown in male zebrafish and can therefore not be easily quantified. Future experiments using water from successfully spawned couples could be used to see if exposure to a putative male pheromone may rescue the complete inhibition of male reproductive success in mating trials. Overall my pharmacological inhibition experiments clearly demonstrate a role for nonpeptides in male reproductive physiology and success, however increasingly used gene-editing methods in zebrafish (optogenetics, knock-out approaches) will be useful to confirm these effects in the absence of the utilization of mammalian based antagonists in this species. With regard to the hypothesized acute activation of male nonapeptide systems in response to the well characterized pheromone $PGF_{2\alpha,}$ my experiments refute and acute activation of isotocin system, while identifying a potential long-term effect on the system in the form of time dependent increases in isotocin transcript abundance in the brain. While

no long term effects on whole brain vasotocin transcript abundance were identified in male zebrafish, the possible acute activation of vasotocin neurons in response to $PGF2_{2\alpha}$ requires careful investigation, especially given that both $PGF_{2\alpha}$ (Yabuki et al., 2016) and vasotocin (this study) induce male courtship behaviour in this species. The $PGF_{2\alpha}$-dependent activation of neuronal perikarya in close proximity to isotocin neurons in response to $PGF_{2\alpha}$ further warrants testing the hypothesis that vasotocin neurons are acutely activated by $PGF_{2\alpha}$ in male zebrafish. Unfortunately, and in contrast to isotocin (Coffey et al., 2013), no reporter lines are currently available for vasotocin in zebrafish and future reporter lines, optogenetic or knock-out approaches (of the $PGF_{2\alpha}$ olfactory receptor or vasotocin) are needed to link $PGF_{2\alpha}$ to vasotocin dependent regulation of male courtship behaviour and ultimately male reproductive success. In the larger picture, male-male, and male-female interaction experiments using the same experimental approaches to study regulation and function of specific nonapeptide systems as described may be useful in teasing apart confounding social from purely reproductive effects of both nonapeptide systems.

With regards to experiments in goldfish, I identify either nonapeptide systems as a neuroendocrine mechanism underlying $PGF_{2\alpha}$ stimulated milt release in this species. This finding provides a functional link between previously reported correlation of $PGF_{2\alpha}$ induced activation of the nonapeptide systems and increased strippable milt volume (Mennigen et al., 2010). Indeed, a role for nonapeptides as neuroendocrine integrator stimulating male reproductive physiology has long been postulated in goldfish (Peter, 1977; Demski and Sloan et al., 1985, Stacey and Zheng, 1997), but never been formally investigated using antagonists and in conditions of $PGF_{2\alpha}$ stimulation of male reproduction. My experiment reveals that milt release, but not male courtship behaviour or activation of the endocrine reproductive axis are regulated by nonapeptides in sexually mature male goldfish, at least under the current experimental conditions. While these results confirm that different neuroendocrine mechanisms underlie different aspects of mature

male goldfish reproductive physiology (Ghosal and Sorensen, 2016), additional experiments including time courses and larger sample sizes are warranted to substantiate these findings. Unfortunately, recurrent issues in the goldfish facility across two yearly periods of sexual maturity in goldfish precluded me from conducting a larger experiment. Future experiments should also address whether the effect of male nonapeptides on milt release is mediated by neuronal or endocrine mechanisms. Approaches using hypophysectomy in conjunction with $PGF_{2\alpha}$ treatment and/or *ex vivo* incubation of testes devoid of functional innervation with nonapeptides should successfully allow to distinguish between both possibilities.

Overall, my experimental results support the hypothesis that nonapeptides are important regulators of male reproductive physiology and success in two cyprinid fishes, with different reproductive strategies, albeit via different mechanisms. Future studies should include comparative experiments in additional teleost species, as they exhibit a variety of reproductive strategies, and ultimately additional non-mammalian vertebrates. Indeed, while structurally conserved, nonapeptide systems appear to contribute differently to evolved reproductive phenotypes (Goodson 2008; Godwin and Thompson, 2012), making comparative functional studies especially relevant. Two additional novel areas of investigation arise from the work presented in my thesis. Firstly, the first neuroanatomical evidence locating nonapeptide in the teleost olfactory bulb serve as the basis for the hypothesis that nonapeptides may modulate sensitivity of pheromone signal integration and potential lead to habituation of aspects of male reproductive physiology, recently described in goldfish (Ghosal and Sorensen, 2016). In mammals, a role for afferent oxytocin signaling in the olfactory bulb has been linked to the recognition of pheromonal cues (Fang et al., 2008; Oettl et al., 2016). Secondly, the finding that co-administration of nonapeptides results in a strong phenotype in male reproductive success is suggestive of possible additive or synergistic effects of similarly organized, yet separate nonapeptide systems. Future studies should therefore

explore the possibility that both systems interact in their regulation of male reproductive physiology.

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