10-13-2022 12:30 PM

Effects of the Brain Nonapeptides Arginine-Vasotocin and Isotocin on Shoaling Behaviour in the Guppy (Poecilia reticulata)

Babak Ataei Mehr, *The University of Western Ontario*

Supervisor: Neff, Bryan D, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

Abstract

Brain nonapeptides have been suggested to regulate social behaviours. However, the contribution of Arginine-Vasotocin (AVT) and Isotocin (IT) to social behaviour in fishes is not well-characterized. Using the guppy (*Poecilia reticulata*), I first measured association preference for conspecifics in individuals injected with either AVT, an AVT-antagonist, or saline. The time spent associating with conspecifics did not differ significantly among the injection treatments. However, individuals injected with AVT performed more movement among areas of the tank than individuals injected with either the AVT-antagonist or saline, consistent with an effect of AVT on anxiety-related behaviours (i.e. hyperactivity). Second, I measured association preference for conspecifics in individuals injected with IT and an IT-antagonist. Individuals injected with IT spent more time associating with conspecifics than individuals injected with an IT-antagonist, consistent with a positive relationship between IT on shoaling. Third, I compared shoaling behaviour between a high- and a low-predation population and between sexes. Individuals from a high-predation population spent more time associating with conspecifics than individuals from a low-predation population, and females spent more time than males associating with conspecifics. Movement did not differ significantly between populations and sexes. Brain AVT and IT immunoreactivity measurements showed that AVT intensity in the gigantocellular neurons in the preoptic area was higher in individuals from the high-predation population. I found no difference in IT intensity between the two populations and no difference between the sexes in AVT and IT intensity. Finally, I examined the distribution of AVT receptors in the brain of individuals of mixed populations and sexes showing potential sites of action for AVT in the telencephalon, diencephalon, mesencephalon, and rhombencephalon. Overall, my study suggests a role of IT in shoaling behaviour, albeit IT intensity in the preoptic area was not associated with shoaling differences across populations and sexes. I did not observe an effect of AVT on shoaling, but instead showed a positive relationship between AVT and an anxiety-related behaviour, as well as greater AVT intensity in a population with high predation, which suggests the potential of AVT-associated fear as an important response to differences in predation.

Keywords: Social behaviour, Shoaling, Neuron, Vasotocin, Isotocin, Guppy

Summary for Lay Audience

Many animals form social groups, but little is known about how the brain regulates the formation of these groups. Nonapeptides are hormones found in the brain that have emerged as potential candidates for regulating social behaviours. In my study, I examined the effect of two nonapeptides—arginine-vasotocin (AVT) and isotocin (IT)—on social behaviour in the guppy (*Poecilia reticulata*). First, I measured the effect of experimental injections of AVT, IT and inhibitors of those hormones (antagonists) on the amount of time that individuals spent associating with another group of guppies. Next, I measured differences in association behaviour between two populations and sexes and looked at the relationship between behaviour and AVT and IT levels in the brain. Finally, I examined the distribution of AVT receptors in the brain. I found that IT injections led to more association behaviour, while AVT injections had no effect on association. Interestingly, AVT injections were associated with hyperactivity, consistent with a link between AVT and anxiety. Association behaviour was higher for a population where predators were present than a population where predators were absent, and the association was higher for females than males. AVT levels were higher in the population where predators were present and AVT receptor mapping in the brain revealed several areas that may be involved in the effects of this nonapeptide. Overall, my study is one of the first comprehensive studies on the contribution of brain nonapeptide to social behaviours in fish and offered new insights into the mechanisms underlying social behaviours.

Co-Authorship

Chapter 2

Babak Ataei Mehr: Collected samples, analyzed data, and drafted the manuscript

Kaitlyn Magyar: Collected samples

Kevin Adeli: Collected samples

Shawn Garner: Offered suggestions for experimental design, analyzed data, and revised the manuscript

Bryan Neff: Offered suggestions for experimental design and revised the manuscript

Chapter 3

Babak Ataei Mehr: Collected samples, analyzed data, and drafted the manuscript

Shawn Garner: Offered suggestions for experimental design, analyzed data, and revised the manuscript

Bryan Neff: Offered suggestions for experimental design and revised the manuscript

Chapter 4

Babak Ataei Mehr: Collected samples, analyzed data, and drafted the manuscript

Bryan Neff: Provided major input into experimental design and the manuscript

Chapter 5

Babak Ataei Mehr: Collected samples, analyzed data, and drafted the manuscript

Bryan Neff: Offered suggestions for experimental design and revised the manuscript

Acknowledgements

I thank my supervisor, Bryan Neff, for his guidance and support. Bryan has been an ideal mentor and thesis supervisor, offering advice and encouragement with a perfect blend of insight, patience, and humor. I have benefited greatly from his wealth of knowledge, professional attitude, and nice personality. I am extremely grateful that he took me on as a student and continued to have faith in me over the years. My sincere gratitude goes to my advisory committee members, Scott MacDougall-Shackleton and Graham Thompson. Wealth of knowledge and valuable contribution of Scott and Graham to my PhD project via their thoughtful and practical advice, supportive and encouraging words have been precious to me and made my research possible. I gratefully recognize the help of Shawn Garner for his great ideas and productive insights as well as his nice personality and patience, facilitating the experimental design, running experiments, and drafting of the manuscripts and writing the thesis. Without Shawn, this research would not have been possible. I also thank Caroline O'Neil and Zengxuan Nong (Robarts Research Institute, Western University) for assistance with histology, and Karen Nygard (Biotron, Western University) for assistance with microscopy imaging. I thank Timothy Hain for sharing his experience and suggestions for the behavioural trial design. My thank goes to my research-study students Nicole Carfagnini, Yu-Jen Chen, Sara Chenier, Laura Chiavegato, Ray Jia, Hyunjun Kim, Junhee Lee, Paneet Pandher, Parsa Raissi, Gerrit Stuivenberg, and Isuri Vidyarathne for assistance with the behavioural trials. I also thank Simone Miklosi and Kevin Adeli for feedback on some parts of this thesis. This research was supported by an NSERC Discovery Grant to Bryan Neff.

Table of Contents

List of Tables

List of Figures

List of Appendices

Chapter 1. General Introduction

Social behaviour

The idea that behaviour represents a key element of adaptation can be traced back to “On the Origin of Species by Means of Natural Selection” (1859), in which Darwin describes the evolution of behavior via selection, noting that the same mechanisms enabling morphological evolution could also lead to behavioural adaptations. This framework would then form the foundation of behavioral research for the next century. Another major milestone in the study of behaviour was marked by the 1973 Nobel Prize in Physiology or Medicine, which was jointly awarded to Karl von Frisch, Konrad Lorenz and Nikolaas Tinbergen "for their discoveries concerning organization and elicitation of individual and social behaviour patterns." Although this award recognized many ground-breaking advances in the study of behaviour, one of the most enduring contributions has been Tinbergen’s four levels of analysis (Tinbergen 1963). This framework noted that behaviour can have evolutionary explanations (adaptive function and phylogenetic distribution) or proximate explanations (physiological mechanisms and developmental onset).

Social behaviours are a major class of behaviour that involves interactions among individuals of the same species (Rubenstein and Rubenstein 2013). Examples of social behaviour include affiliation, aggression, reproduction, and communication (Insel and Young 2000). Affiliative behaviours and the formation of social groups are widespread among invertebrates and vertebrates (Ward and Webster 2016).

The adaptive value of social groups has been widely studied in vertebrates (for review see Silk 2007 and Majolo and Huang 2018). Some examples of the benefits of group formation include greater ability to hunt large prey in gray wolves (*Canis lupus*) (Zimen 1976), higher hunting success for black-headed gulls (*Latus ridibulldus*) (Götmark et al. 1986), reduced starvation risk in greenfinches (*Carduelis chloris*) (Ekman and Hake 1988) and increased reproductive success in Caribbean flamingos (*Phoenicopterus ruber*) (Pickering et al. 1992). Living in social groups, however, may

increase the risk of competition for food resources, living space, and heightens the risk of parasite infections (Altizer et al. 2003). For example, in mammals and birds, aggression and foraging interference among individuals often rise in larger groups leading to reduced net food intake due to within-group competition (Dunbar 1988; Beauchamp 1998). Thus, there may be a trade-off between the costs and benefits of group living (Majolo et al. 2008).

Many fishes readily form social groups, referred to as shoals. Shoals may provide a range of fitness benefits to their members. For example, individual bluntnose minnows (*Pimephales notatus*) form social groups with conspecifics, which increases their safety due to increased group vigilance for predators and their ability to obtain food due to social facilitation (Morgan and Colgan 1987). In common roach (*Rutilus rutilus*), living in a group enhances the ability to find mating partners (Wedekind 1996). In banded killifish (*Fundulus diuphunus)* individuals prefer to join larger shoals under predatory attacks because individuals are safer in larger groups (Krause and Godin 1994). In goldfish (*Carassius auratus*) and minnows (*Phoxinus phoxinus*), individuals find food faster when they live in large groups (Pitcher et al. 1982). In guppies (*Poecilia reticulata*) the risk of being attacked by the blue acara cichlid fish (*Aequidens pulcher*) decreases when the guppies live in large groups (Krause and Godin 1995). In daffodil cichlid (*Neolamprologus pulcher*), living in large groups provides more feeding efficiency and high reproductive success for breeders (Balshine et al. 2001).

Sex and predation are important factors influencing the formation of social groups. Differences in predation pressure can affect the motivation of individuals to form social groups (Ioannou 2017). In white-nosed coatis (*Nasua narica*), predation risk affects their grouping behaviour, vigilance, and alarm calling (Hass and Valenzuela 2002). Social group formation may also show variation between the sexes. Females in mammals and males in birds generally show greater fidelity to their groups and disperse less than the opposite sex (for reviews see Greenwood 1980; Pusey 1987). In fishes, predation risk as well as avoidance of sexual harassment are two factors that determine whether shoaling with conspecifics is sex-assortative or not. Indeed, the risk of being captured by a predator is often lower for an individual surrounded by same-sex conspecifics than opposite-sex conspecifics, presumably due to reduced mating activity

and more vigilance [e.g. mosquitofish (*Gambusia holbrooki*), Pilastro et al. 2003 and Agrillo et al. 2006; zebrafish (*Danio rerio*), Etinger et al. 2009; threespine stickleback (*Gasterosteus aculeatus*), Rystrom et al. 2018].

At the proximate level, the expression of social behaviour has been linked to interconnected neural circuits that form a network across brain regions (O'Connell and Hofmann 2011). During their lifetime, animals face many different environmental situations including a variety of social contexts. To produce a proper response, environmental stimuli are received via the sensory system, processed into informative signals, and transferred to the brain. The brain then integrates these signals to produce behavioural responses. The nervous system and brain thus have important roles in such a "stimulation-response" procedure, for processing and transferring the signal and acting as the central neural circuit upon which the system depends. There are two interactive neural networks in the brain that have homology across taxa: the social behaviour network and the mesolimbic reward system. The networks act as an integrated social decision-making mechanism that regulates and implements responses to stimuli in vertebrates (O'Connell and Hofmann 2011). The social behaviour network (including the preoptic area, anterior hypothalamus, ventromedial hypothalamus, and periaqueductal gray/central gray) is involved in the regulation of many social behaviours such as sexual behaviour, affiliative/aggressive behaviours, and parental care (O'Connell and Hofmann 2011; Ogawa et al. 2021). The mesolimbic reward system influences behaviour by generating motivation to seek reward and avoid negative stimuli and includes the striatum, nucleus accumbens, ventral pallidum, basolateral amygdala, hippocampus, and ventral tegmental area that shares overlapping nodes (lateral septum and bed nucleus of the stria terminalis/medial amygdala) (O'Connell and Hofmann 2011; Berridge 2012).

Neuroanatomical structure of the brain

The comparative neurobiological study of social behaviour, particularly in closely related species, may reveal important insights into brain-behaviour relationships (Insel and Young 2000). The brain is the main center for controlling neural circuits in vertebrates. Most vertebrates have similar structures and number of brain divisions, with the main difference coming in the sizes of the specific regions (Northcutt 2002;

Kawakami and Murakami 2017). There are three major neuroanatomical regions of the brain: forebrain (telencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon) (Figure 1.1). The forebrain has changed much more through the course of evolution than the midbrain and hindbrain (Pessoa et al. 2019). The telencephalon contains olfactory bulbs and cerebrum. The diencephalon connects the forebrain to the midbrain and contains epithalamus, thalamus and hypothalamus. The mesencephalon contains two connected optic lobs (tectum) and optic tegmentum. The rhombencephalon includes the cerebellum (metencephalon), pons (that are absent in non-mammalian brain) and medulla oblongata (myelencephalon) (for review see Hussein and Cao 2018).

It has been shown that each region of the brain has a specific or shared function in the regulation of behaviours. Fishes use olfactory and visual cues for recognition of both their environment and fellow conspecifics (Crapon de Caprona and Ryan 1990; Martin et al. 2010). The olfactory bulbs that are located at the anterior-most part of the telencephalon, are used for the processing of chemical information related to odorants and may be involved in regulating a range of odour-sensitive behaviours (Laberge and Hara 2001). The fish olfactory system processes odour signals and mediates olfactory-driven behaviours including feeding, reproduction, migration, kin recognition and predator avoidance (Kermen et al. 2013).

The telencephalon is in the forebrain, and it has been shown that it has a role in cognition in fish (Cheng et al. 2014). The anterior part of the telencephalon (above the olfactory bulb) is the frontal cortex and is key for decision-making behaviours (Sato et al. 2017). Parts of the telencephalon are related to the mesolimbic reward system and some components are shared between the social brain network and the mesolimbic reward system (Bshary et al. 2014). In fishes the telencephalon influences cognitive functions (Cheng et al. 2014). Telencephalon forms two solid hemispheres and is divided into the dorsal and ventral regions. The dorsal telencephalon (pallium, an equivalent to the mammalian hippocampus and amygdala) is a homologue of mammalian cortical region (Cheng et al. 2014). The medial and lateral telencephalic pallium is involved in emotional and temporal behaviours (Portavella et al. 2014). The pallium is involved in avoidance learning, spatial learning, and temporal aspects of the learning processes (Vargas et al.

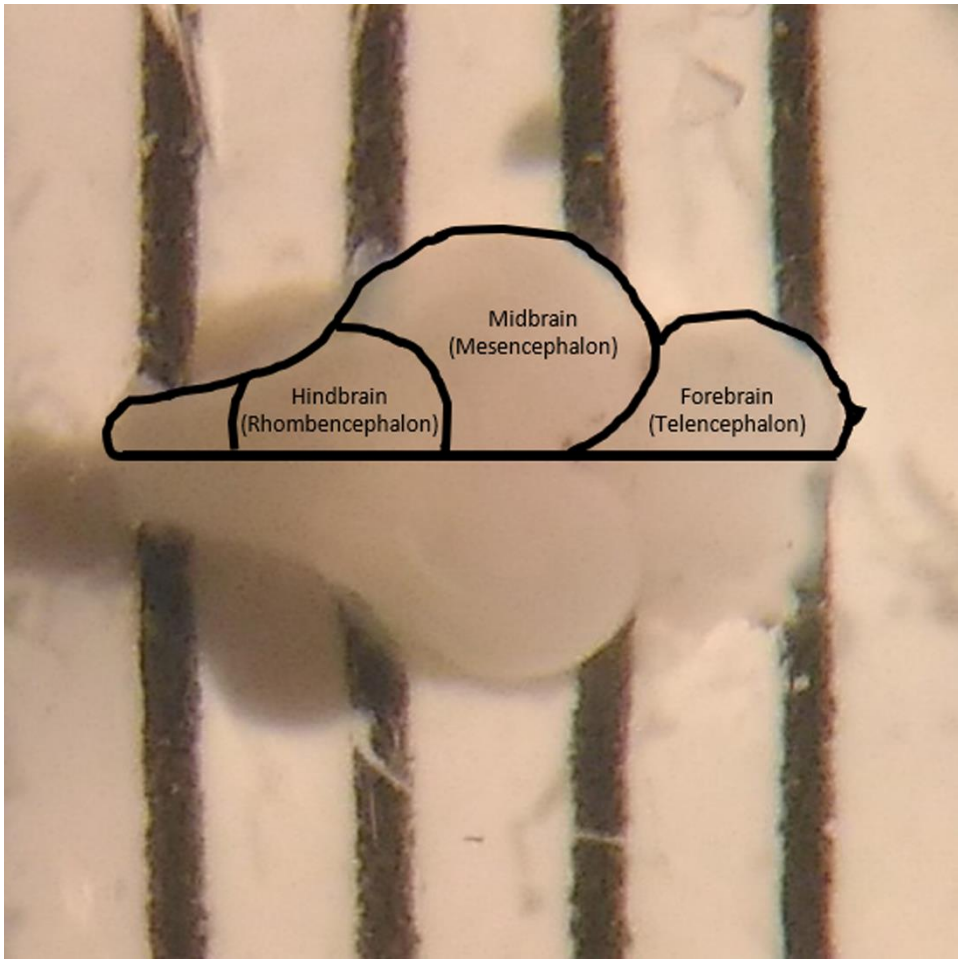


Figure 1.1. A dorsal view of the brain of a female guppy (*Poecilia reticulata*) from the Lower Aripo population. The upper half shows a line diagram identifying the major neuroanatomical regions of the brain. The distance between each vertical solid line is 1mm

2009). The ventral telencephalon (subpallium, an equivalent to mammalian basal ganglia) (Cheng et al. 2014), is a homologue of the mammalian sub-cortical region and influences motor control, learning, and reward reinforcement in fish (Ganz et al. 2012).

The diencephalon is a part of the social brain network with its important neuro-anatomical structures (such as the hypothalamus, thalamus and epithalamus) and it functions to connect the forebrain to the midbrain. Diencephalon has a role in the regulation of aggressive behaviour (Teles et al. 2013) and risk-taking behaviour in fish (Otsuka et al. 2020). In zebrafish, functions such as attention, alertness, circadian behaviours, and cognition are all regulated by the diencephalon (Mueller 2012, Lin and Jesuthasan 2017; Cheng et al. 2014). In fish, the preoptic area is a part of the hypothalamus that is horizontally located at the end of the telencephalon and the beginning of the diencephalon (Rincón et al. 2017). This region is the primary production site of vasotocin and isotocin which contribute to the regulation of many social behaviours such as affiliation, aggression, reproduction, and parental care (for review see Goodson and Thompson 2010; Godwin and Thompson 2012).

Some parts of the mesencephalon are related to the social behaviour network and are shared between the social brain network and the mesolimbic reward system (Bshary et al. 2014). In fishes, the mesencephalon is involved in visual processing and control of body movement (Roberts 1992). It has also been shown that mesencephalon has a role in functions like saccadic eye movements (Ángeles Luque et al. 2005) and acoustic circuitry in vocalizing fish (Bass et al. 2000). The center of visual processing is the optic tectum of the mesencephalon. Additionally, the mesencephalon is important in the control of approach, escape and rhythmic swimming-like body movements in fish (Roberts 1992).

The rhombencephalon is involved in different cognitive and emotional functions, from associative learning and emotional conditioning to more complex relational memory processes such as spatial cognition (Rodríguez et al. 2005). For example, the cerebellum is traditionally associated with motor control, cognition, and emotional functions. It is also involved in learning processes ranging from simple forms of associative sensory-motor learning and emotional conditioning to complex processes such as spatial cognition (Rodríguez et al. 2005). The hindbrain also contains a diverse set of sensory-motor networks that control movements required for vision, respiration, mastication, and

locomotion in vertebrates (Kinkhabwala et al. 2011). It has been shown that swimming, which is needed in most social behavioural contexts, is regulated in the rhombencephalon of fish (zebrafish, Kinkhabwala et al. 2011).

Nonapeptides

Research into neuro-behavioural mechanisms underlying social behaviour in vertebrates has suggested an important role for an evolutionary conserved family of nine-amino-acid nonapeptides (Goodson 2008; Goodson and Thompson 2010; Caldwell 2017). In vertebrates, nonapeptides can be divided into vasopressin- and oxytocin-related families. Arginine-vasopressin (AVP) is expressed in mammals, while non-mammalians express its homologue arginine–vasotocin (AVT). Oxytocin (OT) is expressed in mammals, and its homologue mesotocin (MT) in birds, reptiles and amphibians, and its homologue isotocin (IT) in fishes (Insel and Young 2000; Goodson 2008). The structure of nonapeptide hormones has been evolutionary well-conserved, with only three of nine amino acid positions showing variation across taxa (Lindeijer 2012) (Table 1.1).

In vertebrates, nonapeptide-producing sites can be found in different brain regions but they are produced predominantly in the neurosecretory cells of the hypothalamic area (for review see Moore and Lowry 1998). The preoptic area is one of the neuroanatomical parts of the social behaviour network that is in the hypothalamic region of the brain (O'Connell and Hofmann 2011). In teleost fish, neurosecretory cells of the preoptic area form a pair of thin layers along the walls of the third ventricle located at the ventral part of the preoptic area and it has been shown that they project to the pituitary and other regions of the brain (Godwin and Thompson 2012) (Figure 1.2). In the preoptic area of fish, vasotocin neurons are found intermingled with isotocin neurons but each specific cell produces only isotocin or vasotocin (Thompson and Walton 2013).

In all vertebrates, parvocellular and magnocellular neural somas are involved in the production of nonapeptides (Goodson and Thompson 2010). In fish, the parvocellular preoptic soma (PP) includes many small spherical cells (parvocellular cells), which form compact groups of neurons at the ventral part of the preoptic area and the surrounding area of the third ventricle. The magnocellular preoptic soma (PM) includes fewer large spherical cells which lay dorsal to the parvocellular preoptic soma (Rincon et al. 2017).

Table 1.1 Amino acid sequences of nonapeptides compared to oxytocin. Asterisks denote identical amino acid (Modified from Lindeijer 2012)

Nonapeptide	**Amino acid sequence**								
Oxytocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly
Isotocin	*	*	*	Ser	*	*	*	Ile	*
Mesotocin	*	*	*	*	*	*	*	Ile	*
Vasopressin	*	*	*	*	*	*	*	Arg	*
Vasotocin	*	*	Phe	*	*	*	*	Arg	*

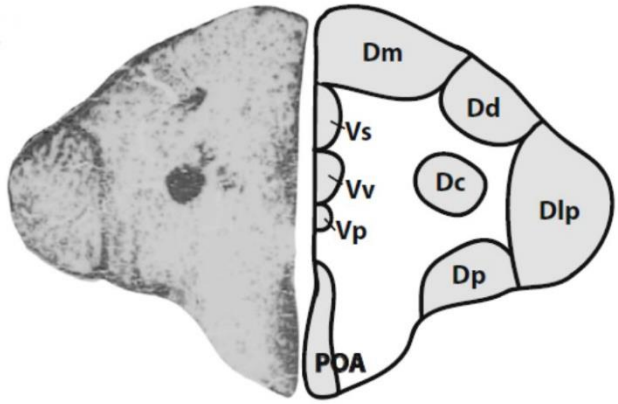


Figure 1.2. The coronal section of the telencephalon of the brain of the guppy (*Poecilia reticulata*) showing the location of the preoptic area. Landmarks denote POA: preoptic area, Vp: posterior part of ventral telencephalon; Vv: ventral part of ventral telencephalon; Vs: supracommissural nucleus of ventral telencephalon; Dm: medial part of dorsal telencephalon; Dd: dorsal part of dorsal telencephalon; Dlp: posterior part of the dorsolateral telencephalon; Dc: central part of dorsal telencephalon; Dp: posterior part of the dorsal telencephalon (Modified from Fischer et al. 2018)

The magnocellular soma contains two subtypes of cells: a group of larger cells that are less numerous than the parvocellular cells that lay on the dorsal part of the parvocellular preoptic soma (magnocellular cells), and a group of the largest cells with the fewest number in the preoptic area that are dorsal to the magnocellular cells (gigantocellular cells) (Almeida and Oliveira 2015). Nonapeptides are produced and stored in large dense vesicles located in the cell bodies and neural fibers of nonapeptide secretory neurons. They are then exocytosed in response to environmental stimuli (for review see Johnson and Young 2017). The neurosecretory soma of the preoptic area has homology across vertebrates. For example, in fish, the magnocellular soma and the parvocellular soma of the preoptic area are homologous to the supraoptic nucleus and the paraventricular nucleus in reptiles, birds and mammals respectively (for review see Moore and Lowry 1998). Considering the homology in the structure, it is likely that the preoptic area shares a common function in regulating social behaviour across vertebrates (for review see O'Connell and Hofmann 2011).

Effects of preoptic area nonapeptides on social behaviours

Vasopressin acts as a hormone in the peripheral blood circulation with a role in the regulation of blood pressure and osmoregulation. In the brain, vasopressin acts as a neuromodulator with a role in the regulation of social behaviours (Dumais and Veenema 2016). Oxytocin, when released in general blood circulation, acts as a hormone involved in the regulation of sex-specific behaviours. In females, it influences maternal responses such as milk production and uterine contractions whereas in males it affects reproductive functions such as ejaculation and cardiovascular homeostasis. When oxytocin is centrally released in the brain it acts as a neuromodulator with a role in the regulation of social behaviour (Dumais and Veenema 2016). In vertebrates, males generally have higher vasopressin expression and more vasopressin receptors in the brain than females. Sex differences in the oxytocin system, however, are not always in the same direction with females generally showing higher oxytocin expression but fewer oxytocin receptors in the brain than males (for review see Dumais and Veenema 2016). The general pattern emerging is that vasopressin and its homologues have anti-social effects whereas oxytocin and its homologues have pro-social effects (Goodson and Bass 2001; Heinrichs

and Domes 2008). Nevertheless, the specific effects of these nonapeptides may vary among species and sexes (for review see Goodson 2013).

Stimulation or lesion of the preoptic area has helped to reveal its regulatory function on social behaviours. For example, lesion of the preoptic area decreased aggression in male rats (*Rattus rattus*) (Albert et al. 1986), reduced contact with the sexual partner, mounting and ejaculation in male rhesus monkeys (*Rhesus macaque*) (Slimp et al. 1978), and disrupted parental care in both female and male California mice (*Peromyscus californicus*) (Lee and Brown 2007). In birds, stimulation of the preoptic area increased aggression in male pigeons (*Columba livia*) (Akerman et al. 1960) and lesion of the preoptic area disrupted parental care in both female and male ring doves (*Streptopelia risoria*) (Slawski and Buntin 1995). In reptiles, stimulation of the preoptic area increased aggression in both male and female iguana (*Iguana iguana*) (Distel 1978) and lesion of the preoptic area impaired courtship and copulatory behaviour in male whiptail lizards (*Cnemidophorus inornatus*) (Kingston and Crews 1994). In amphibians, stimulation of the preoptic area induced mate calling in male leopard frogs (*Rana pipiens*) (Wada and Gorbman 1977). In male green sunfish (*Lepomis cyanellus*), stimulation of the preoptic area increased sperm release (Demski et al. 1975), and increased feeding, aggressive and reproductive behaviour of both sexes in bluegills (*Lepomis macrochirus*) (Demski and Knigge 1971). In oyster toadfish (*Opsanus tau*), the agonistic grunt and the courtship sound are evoked by stimulation of the preoptic area (Fine and Perini 1994). In killifish (*Fundulus heteroclitus*) when the preoptic area was lesioned, fish showed impaired spawning reflex responses (Macey et al. 1974). These studies suggest that the preoptic area regulates many affiliative and aggressive behaviours across taxa.

The role of vasotocin neurons in the regulation of social behaviours has been investigated in several studies using immunocytochemistry and *in situ* hybridization techniques (see Godwin and Thompson 2012; Greenwood et al. 2008). These studies generally suggest that vasotocin parvocellular neurons regulate subordinate behaviours [e.g. peacock blenny (*Salaria pavo*), Grober et al. 2002; zebrafish, Larson et al. 2006; African cichlid fish (*Astatotilapia burtoni*), Greenwood et al. 2008; beaugregory damselfish (*Stegastes leucostictus*), Santagelo and Bass 2010; multiband butterflyfish

(*Chaetodon multicinctus*), Dewan and Tricas 2011] whereas vasotocin magnocellular and gigantocellular neurons regulate dominance and territorial behaviours [zebrafish, Larson et al. 2006; African cichlid fish, Greenwood et al. 2008; butterflyfish (*Chaetodon* sp), Dewan et al. 2008, Dewan and Tricas 2011 and Dewan et al. 2011]. Additionally, it has been shown that vasotocin and its receptors are found in brain regions related to the fear response [rock hind (*Epinephelus adscensionis*), Kline et al. 2011; African cichlid fish, Huffman et al. 2012 and Rodriguez-Santiago et al. 2017], suggesting that vasotocin could influence shoaling behaviour if shoaling is driven by the fear of predation.

Administration of vasopressin and vasotocin has been shown to influence social behaviours. For example, vasopressin administration reduced territorial aggression between residents and intruders in mice (*Mus musculus*) (Tan et al. 2019). Vasotocin administration likewise decreased aggression in field sparrows (*Spizella pusilla*) (Goodson 1998), zebrafish (Filby et al. 2010), pupfish (*Cyprinodon nevadensis amargosae*) (Lema and Nevitt 2004), and rainbow trout (*Oncorhynchus mykiss*) (Backström and Winberg 2009). In contrast, vasopressin administration had no effect on territorial aggression in deer mice (*Peromyscus* sp.) (Bester-Meredith et al. 2005), vasotocin administration increased aggressive territorial singing in white-crowned sparrows (*Zonotrichia leucophrys gambelii*) (Maney et al. 1997), and vasotocin administration increased territorial aggression in beaugregory damselfish (Santangelo and Bass 2006). Vasotocin has shown mixed effects on direct measures of association behaviour. For example, when vasotocin was administered to goldfish it led to reduced time spent in proximity with a same-sex conspecific (Thompson and Walton 2004; Thompson et al. 2008). In female zebrafish, administration of either vasotocin or a vasotocin-antagonist reduced the proportion of time spent shoaling with a female conspecific relative to the administration of saline (Lindeyer et al. 2015). In another study of zebrafish, vasotocin administration increased the proportion of time spent associating with a phenotypically similar strain relative to a phenotypically dissimilar strain, whereas an AVT-antagonist had the opposite effect (Braida et al. 2012).

Isotocin neurons have also been linked to social behaviour in fishes (Godwin and Thompson 2012; Reddon et al. 2017). In a sex-changing species, blue-banded goby (*Lythrypnus dalli*), Black et al. (2004) found that females have more isotocin

immunoreactive neurons in their preoptic area than males and that the number of isotocin neurons decreases when females change their sex. Parvocellular isotocin neurons are positively associated with paternal care in a cichlid (*Amatitlania nigrofasciata*) (O'Connell et al. 2012). Across species of lamprologine cichlids, Reddon et al. (2017) found that the number of parvocellular isotocin neurons in the preoptic area was lower in species characterized by cooperative breeding than in species without cooperative breeding. The number of magnocellular and gigantocellular isotocin cells was not related to the breeding system.

Oxytocin and mesotocin administration are generally associated with pro-social effects, while there is less support for a pro-social effect of isotocin. For example, in mammals, oxytocin administration increases maternal behaviour (Pedersen et al. 1982; McCarthy 1990), cooperative behaviour (Harmon et al. 2002; Madden and Clutton-Brock 2011) and social preferences for conspecifics (Smith et al. 2010; Lukas et al. 2011). In birds, time spent with a group of conspecifics (Goodson et al. 2009) and altruistic behaviour (Duque et al. 2018) increases after the administration of mesotocin. In goldfish, isotocin administration increases the time spent associating with another conspecific, but only when the authors examined the subset of the subjects (n = 6 of 13) that had the lowest baseline association values (Thompson and Walton 2004). Other studies in fishes have shown no effect of isotocin on social behaviour or even an anti-social effect. In the daffodil cichlid, isotocin administration led to reduced association with conspecifics, whereas administration of an isotocin antagonist increased association with conspecifics (Reddon et al. 2014). In zebrafish, Lindeyer et al. (2015) found no effect of isotocin or its antagonist on shoaling behaviour, and Braida et al. (2012) found that while isotocin administration at intermediate doses increases social preference for phenotypically similar conspecifics, isotocin at low or high doses have negative effects on this behaviour.

For nonapeptides to have regulatory effects on social behaviour, the expression of their receptors in different brain regions is critical. Importantly, vasotocin and isotocin function in the brain is intrinsically dependent on their receptors in the brain (Carter 2017). To understand the function of vasotocin and isotocin production and function in the brain, a closer look at vasotocin and isotocin receptors is needed. For example,

understanding the distribution of the receptors sites across multiple brain regions could be a key step to determine what regions are involved and what behaviours might be regulated by nonapeptides.

In mammals, three types of membrane-bound G protein-coupled receptors have been identified for vasopressin (V1a, V1b, V2) whereas fish have two V1a receptors (V1a1 and V1a2) and a V2 receptor (Godwin and Thompson 2012). In mammals, birds, amphibians, and fish the behavioural effects of vasopressin/vasotocin are mediated via V1a receptors (Goodson and Bass 2001; Huffman et al. 2012). V1a receptors are highly similar in fishes and mammals (> 60% amino acid identity; Iwasaki et al. 2013), and within fishes the V1a-type receptors (V1a1 and V1a2), show high similarity to each other (>70% amino acid identity; Loveland and Fernald 2017). The V1b receptor modulates the effects of vasopressin on adrenocorticotropic hormone in the pituitary to control the stress response whereas the V2 receptor is expressed in the kidney where it affects osmoregulation (Huffman et al. 2012). In contrast, only a single receptor is typically observed for oxytocin in mammals, mesotocin in birds, and isotocin in fishes (Huffman et al. 2012; Godwin and Thompson 2012). However, in zebrafish local gene duplication has produced two orthologous receptor genes for isotocin, although both are thought to have a similar role in the regulation of social behaviours (Landin et al. 2020).

A few studies have investigated the distribution of vasotocin and isotocin receptors in the brains of fishes. In a rock hind, Kline et al. (2011) found vasotocin receptor (V1a2) protein and mRNA were expressed in the brain regions that are involved in regulation of behaviour, olfaction, vision, learning, reproduction, and lateral line signals processing including the internal cellular layer of olfactory bulbs, torus longitudinalis, valvula of the cerebellum, the corpus cerebellum, the lateral and posterior recesses, and granular eminence. They also found a high vasotocin receptor (V1a2) protein and mRNA expression in the preoptic area, anterior hypothalamus, and habenula. In African cichlid, Huffman et al. (2012) found that vasotocin receptor (V1a2) and isotocin receptor (ITR) protein and mRNA generally show a similar pattern of expression throughout the forebrain to midbrain including the teleost homologue of mammalian amygdala, hypothalamus, striatum, and ventral tegmentum suggesting the expression of these receptors in the regions that are related to mediate social transitions and behaviour.

In the same fish, Loveland and Fernald (2017) found the overlapping areas of mRNA expression of vasotocin receptor V1a subtypes (V1a1 and V1a2) in regions that have a key role in vasotocin signalling related to aggression and courtship such as ventral telencephalon, hypothalamus, and thalamus. In bluehead wrasse (*Thalassoma bifasciatum*), Lema et al. (2012) found that mRNA transcripts of both V1a subtypes (V1a1 and V1a2) were highly prevalent in regions related to the regulation of reproduction (preoptic area, ventral hypothalamus) and social and sexual behaviours (preoptic area, ventral telencephalon).

The guppy as a model to study social behaviours

The guppy is a small live-bearing fish with internal fertilization and a non-resource-based promiscuous mating system that lives in tropical rivers and often forms social groups known as shoals (Houde 1997). The lifespan of the guppy is 2–3 years and the adult stage begins at approximately 3 months of age (Houde 1997). Adult males have a colourful body and rod-shaped anal fin, whereas adult females are larger and have drab colouration (Houde 1997). Guppies use both olfactory and visual cues for making decisions about forming shoals (Santacà et al. 2021). In their natural habitats, barriers such as waterfalls restrict predator access to upstream locations and produce a predation regime dichotomy that is associated with predictable differences in social behaviour (Endler 1995; Houde 1997; Magurran 2005). Shoaling behaviour differs among guppy populations and between sexes with the predation regime as a driving force. For example, high-predation guppy populations (such as the Lower Aripo population) have a greater propensity for shoaling than low-predation populations (such as the Paria population) (Seghers 1974; Magurran and Seghers 1994a). Female guppies usually show a higher tendency to shoal with conspecifics than males, independent of predation regime. Females spend more time with other females and engage more in antipredator behaviours than males. Females form the core of shoals and benefit from shoaling with other females due to both reduced predation and reduced sexual harassment from males (Magurran et al. 1992; Magurran and Seghers 1994b; Griffiths and Magurran 1998). Male guppies benefit by encountering new females, which motivates them to explore the environment actively,

frequently joining and splitting different shoals to find new mating opportunities (Croft et al. 2003).

The neural mechanism underlying the formation of shoals in fish is not well-characterized. Although it has been shown that brain neural activation occurs during social exposure in guppies (Cabrera-Álvarez et al. 2017), the role of vasotocin and isotocin in the regulation of approach to conspecifics in shoaling is unresolved. Indeed, a recent study showed that vasotocin transcripts were more abundant in the brains of guppies from high-predation populations than from low-predation populations whereas isotocin transcript levels did not differ between these two types of populations (Reddon et al. 2022). Isotocin transcript levels in the brain have been shown to respond to predator cues in guppies from high-predation populations but not low-predation populations (Dimitriadou et al. 2022). The difference in shoaling propensity across populations and between the sexes in guppies provides an excellent opportunity to examine the role of vasotocin and isotocin neural activity in regulating social behaviour.

The objectives of this study

My thesis uses guppies from different populations and sexes to examine the role of brain nonapeptides in regulating shoaling behaviour. I had four objectives. First, to determine whether the administration of vasotocin and its antagonist influence shoaling behaviour. I predict vasotocin will induce shoaling behaviour if shoaling is driven by the fear of predation and that a vasotocin antagonist will have the opposite effect. Second, to determine whether the administration of isotocin and its antagonist influence shoaling behaviour. I predict isotocin will induce and its antagonist will inhibit shoaling behaviour. Third, to characterize differences in shoaling behaviour across populations and sexes and examine the relationships to vasotocin and isotocin immunoreactivity in the brain. I predict that shoaling will be higher in a high-predation population (Lower Aripo) than a low-predation population (Paria) and I predict that females will shoal more than males in both populations. I also predict that there will be more isotocin and vasotocin in the brains of guppies from a high predation population compared to a low-predation population. Fourth, to examine the distribution of vasotocin and isotocin immunoreactive receptors in the brain. I predict that vasotocin and isotocin receptors will

be distributed widely across multiple brain regions from forebrain to hindbrain. Following Tinbergen's four levels of analysis (1963), my thesis mostly contributes to understanding the proximate mechanisms underlying shoaling behaviour.

Related to the research objectives, in chapter 2 of my study, I examined the effect of the administration of vasotocin and its receptor antagonist on shoaling behaviour. First, I injected 124 adult guppies of both sexes from Paria population with either vasotocin, a vasotocin-antagonist, or with saline as a control. Then, a standard dichotomous choice behavioural trial protocol was used to obtain shoaling behaviour measurements in both sexes including association preference for conspecifics and transitions among zones of the tank.

In chapter 3, I examined the effect of the administration of isotocin and its receptor antagonist on shoaling behaviour. First, a standard dichotomous choice behavioural trial protocol was used to obtain baseline shoaling behaviour measurements prior to the experimental manipulation of nonapeptide levels and association preference for conspecifics in 92 adult guppies from Paria population (46 females and 46 males) were measured. Then, half of the subjects were injected with isotocin and the other half with an isotocin antagonist and the post-injection shoaling behaviour was measured to identify differences in shoaling behaviour measurements before and after nonapeptide injection.

In chapter 4, I examined the contribution of the preoptic area vasotocin and isotocin neuronal phenotypes to differences in shoaling behaviour of guppies of different populations and sexes using immunocytochemistry. I first measured differences in shoaling behaviour between two populations (the Lower Aripo and the Paria) and sexes using a standard dichotomous choice behavioural trial protocol. Behavioural trials were conducted for 46 individuals from the Lower Aripo population and 122 individuals from the Paria population, with an equal number of adult females and adult males used within each population. Then, a subset of 20 test fish (including 10 individuals from the Lower Aripo population and 10 individuals from the Paria population, with 5 females and 5 males for each population) was used to measure the fluorescence intensity of vasotocin and isotocin containing neurons in the preoptic area.

In chapter 5, I examined the distribution of vasotocin and isotocin receptors in the brain of four adult guppies of mixed sex, originally from the Lower Aripo and Paria populations. The five regions of interest that I examined were: the anterior-telencephalon and the olfactory bulbs; the mid-telencephalon; the diencephalon; the mesencephalon; and the rhombencephalon. These regions were subjected to immunostaining of vasotocin and isotocin receptors and intensity of receptors staining was then compared qualitatively among regions.

In chapter 6, I discuss the significance of my thesis as a comprehensive examination of the role of brain nonapeptides in the regulation of a social behaviour in fish, and some of the first evidence on the contribution of nonapeptides to behavioural variation across populations and sexes. My thesis will illuminate some of the unexamined aspects of social behaviour in the guppy by exploring the mechanistic of shoaling behaviour in this species.

1.1 References

Agrillo C, Dadda M, Bisazza A (2006) Sexual harassment influences group choice in female mosquitofish. Ethology 112:592–598

Akerman B, Andersson B, Fabricius E, Svensson L (1960) Observations on central regulation of body: temperature and of food and water intake in the pigeon (*Columba livia*). Acta Physiologica Scandinavica 50:328–336

Albert DJ, Walsh ML, Gorzalka BB, Mendelson S, Zalys C (1986) Intermale social aggression: suppression by medial preoptic area lesions. Physiology & Behavior 38:169–173

Almeida O, Oliveira RF (2015) Social status and arginine vasotocin neuronal phenotypes in a cichlid fish. Brain, Behavior and Evolution 85:203–213

Altizer S, Nunn CL, Thrall PH, Gittleman JL, Antonovics J, Cunningham AA, Dobson AP, Ezenwa V, Jones, KE, Pedersen, AB, Poss M, Pulliam JRC (2003) Social organization and parasite risk in mammals: integrating theory and empirical studies. Annual Review of Ecology, Evolution, and Systematics 34:517–547

Ángeles Luque M, Pérez-Pérez MP, Herrero L, Torres B (2005) Involvement of the optic tectum and mesencephalic reticular formation in the generation of saccadic eye movements in goldfish. Brain Research Reviews 49:388–397

Backström T, Winberg S (2009) Arginine–vasotocin influence on aggressive behavior and dominance in rainbow trout. Physiology & Behavior 96:470–475

Balshine S, Leach B, Neat F, Reid H, Taborsky M, Werner N (2001) Correlates of group size in a cooperatively breeding cichlid fish (*Neolamprologus pulcher*). Behavioral Ecology and Sociobiology 50:134–140

Bass AH, Bodnar DA, Marchaterre MA (2000) Midbrain acoustic circuitry in a vocalizing fish. The Journal of Comparative Neurology 419:505–531

Beauchamp G (1998) The effect of group size on mean food intake rate in birds. Biological Reviews 73:449–472

Berridge KC (2012) From prediction error to incentive salience: mesolimbic computation of reward motivation. European Journal of Neuroscience 35:1124–1143

Bester-Meredith JK, Martin PA, Marler CA (2005) Manipulations of vasopressin alter aggression differently across testing conditions in monogamous and non-monogamous *Peromyscus* mice. Aggressive Behavior 31:189–199

Black MP, Reavis RH, Grober MS (2004) Socially induced sex change regulates forebrain isotocin in *Lythrypnus dalli*. Neuroreport 15:185–189

Braida D, Donzelli A, Martucci R, Capurro V, Busnelli M, Chini B, Sala M (2012) Neurohypophyseal hormones manipulation modulate social and anxiety-related behavior in zebrafish. Psychopharmacology 220:319–330

Bshary R, Gingins S, Vail AL (2014) Social cognition in fishes. Trends in Cognitive Sciences 18:465–471

Cabrera-Álvarez MJ, Swaney WT, Reader SM (2017) Forebrain activation during social exposure in wild-type guppies. Physiology & Behavior 182:107–113

Caldwell HK (2017) Oxytocin and vasopressin: powerful regulators of social behavior. The Neuroscientist 23:517–528

Carter CS (2017) The oxytocin–vasopressin pathway in the context of love and fear. Frontiers in Endocrinology 8:356

Cheng R-K, Jesuthasan SJ, Penney TB (2014) Zebrafish forebrain and temporal conditioning. Philosophical Transactions of the Royal Society B 369:20120462

Crapon de Caprona, MD, Ryan MJ (1990). Conspecific mate recognition in swordtails, *Xiphophorus nigrensis* and *X. pygmaeus* (Poeciliidae): Olfactory and visual cues. Animal Behaviour 39:290–296

Croft DP, Arrowsmith BJ, Bielby J, Skinner K, White E, Couzin ID, Magurran AE, Ramnarine I, Krause J (2003) Mechanisms underlying shoal composition in the Trinidadian guppy, *Poecilia reticulata*. OIKOS 100:429–438

Darwin C. (1859) On the origin of species by means of natural selection, or, the preservation of favoured races in the struggle for life. D. Appleton and Company, New York

Demski LS, Bauer DH, Gerald JW (1975) Sperm release evoked by electrical stimulation of the fish brain: a functional-anatomical study. Journal of Experimental Zoology 191:215–232

Demski LS, Knigge KM (1971) The telencephalon and hypothalamus of the bluegill (*Lepomis macrochirus*): evoked feeding, aggressive and reproductive behavior with representative frontal sections. The Journal of Comparative Neurology 143:1–16

Dewan AK, Maruska KP, Tricas TC (2008) Arginine vasotocin neuronal phenotypes among congeneric territorial and shoaling reef butterflyfishes: species, sex and reproductive season comparisons. Journal of Neuroendocrinology 20:1382–1394

Dewan AK, Ramey ML, Tricas TC (2011) Arginine vasotocin neuronal phenotypes, telencephalic fiber varicosities, and social behavior in butterflyfishes (Chaetodontidae): potential similarities to birds and mammals. Hormones and Behavior 59:56–66

Dewan AK, Tricas TC (2011) Arginine vasotocin neuronal phenotypes and their relationship to aggressive behavior in the territorial monogamous multiband butterflyfish, *Chaetodon multicinctus*. Brain Research 1401:74–84

Dimitriadou S, Santos EM, Croft DP, van Aerle R, Ramnarine IW, Filby AL, Darden SK (2022) Social partner cooperativeness influences brain oxytocin transcription in

Trinidadian guppies (*Poecilia reticulata*). Behavioural Brain Research 423:113643

Distel H (1978) Behavior and electrical brain stimulation in the green iguana, *Iguana iguana* L. II. stimulation effects. Experimental Brain Research 31:353–367

Dumais KM, Veenema AH (2016) Vasopressin and oxytocin receptor systems in the brain: sex differences and sex-specific regulation of social behavior. Frontiers in Neuroendocrinology 40:1–23

Dunbar RIM (1988) Primate social systems. Croom Helm, London

Duque JF, Leichner W, Ahmann H, Stevens JR (2018) Mesotocin influences pinyon jay prosociality. Biology Letters 14:20180105

Ekman J, Hake M (1988) Avian flocking reduces starvation risk: an experimental demonstration. Behavioral Ecology and Sociobiology 22:91–94

Endler JA (1995) Multiple-trait coevolution and environmental gradients in guppies. Trends in Ecology & Evolution 10:22–29

Etinger A, Lebron J, Palestis BG (2009) Sex-assortative shoaling in zebrafish (*Danio rerio*). BIOS 80:153–158

Filby AL, Paull GC, Hickmore TFA, Tyler CR (2010) Unravelling the neurophysiological basis of aggression in a fish model. BMC Genomics 11:498

Fine ML, Perini MA (1994) Sound production evoked by electrical stimulation of the forebrain in the oyster toadfish. Journal of Comparative Physiology A 174:173–85

Fischer EK, Westrick SE, Hartsough L, Hoke KL (2018) Differences in neural activity, but not behavior, across social contexts in guppies, *Poecilia reticulata*. Behavioral Ecology and Sociobiology 72:131

Ganz J, Kaslin J, Freudenreich D, Machate A, Geffarth M, Brand M (2012) Subdivisions of the adult zebrafish subpallium by molecular marker analysis. The Journal of Comparative Neurology 520:633–655

Godwin J, Thompson R (2012) Nonapeptides and social behavior in fishes. Hormones and Behavior 61:230–238

Goodson JL (1998) Territorial aggression and dawn song are modulated by septal vasotocin and vasoactive intestinal polypeptide in male field sparrows (*Spizella pusilla*). Hormones and Behavior 34:67–77

Goodson JL (2008) Nonapeptides and the evolutionary patterning of sociality. Progress in Brain Research 170:3–15

Goodson JL (2013) Deconstructing sociality, social evolution and relevant nonapeptide functions. Psychoneuroendocrinology 38:465–478

Goodson JL, Bass AH (2001) Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain Research Reviews 35:246–265

Goodson JL, Schrock SE, Klatt JD, Kabelik D, Kingsbury MA (2009) Mesotocin and nonapeptide receptors promote estrildid behavior. Science 325:862–866

Goodson JL, Thompson RR (2010) Nonapeptide mechanisms of social cognition, behavior and species-specific social systems. Current Opinion in Neurobiology 20:784–794

Götmark F, Winkle DW, Andersson M (1986) Flock-feeding on fish schools increases individual success in gulls. Nature 319:589–591

Greenwood AK, Wark AR, Fernald RD, Hofmann HA (2008) Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. Proceedings of the Royal Society B 275:2393–2402

Greenwood PJ (1980) Mating systems, philopatry and dispersal in birds and mammals. Animal Behaviour 28:1140–1162

Griffiths SW, Magurran AE (1998) Sex and schooling behaviour in the Trinidadian guppy. Animal Behaviour 56:689–693

Grober MS, George, AA, Watkins KK, Carneiro LA, Oliveira RF (2002) Forebrain AVT and courtship in a fish with male alternative reproductive tactics. Brain Research Bulletin 57:423–425

Harmon AC, Huhman KL, Moore TO, Albers HE (2002). Oxytocin inhibits aggression in female Syrian hamsters. Journal of Neuroendocrinology 14:963–969

Hass CC, Valenzuela D (2002) Anti-predator benefits of group living in white-nosed coatis (*Nasua narica*). Behavioral Ecology and Sociobiology 51:570–578

Heinrichs M, Domes G (2008) Neuropeptides and social behaviour: effects of oxytocin and vasopressin in humans. Progress in Brain Research 170:337–350

Houde AE (1997) Sex, color, and mate choice in guppies. Princeton University Press, Princeton

Huffman LS, O'Connell LA, Kenkel CD, Kline RJ, Khan IA, Hofmann HA (2012) Distribution of nonapeptide systems in the forebrain of an African cichlid fish, *Astatotilapia burtoni*. Journal of Chemical Neuroanatomy 44:86–97

Hussein MNA, Cao X (2018) Brain anatomy and histology in teleosts. Benha Veterinary Medical Journal 35:446–463

Insel TR, Young LJ (2000) Neuropeptides and the evolution of social behavior. Current Opinion in Neurobiology 10:784–789

Ioannou CC (2017) Grouping and predation. In: Shackelford TK, Weekes-Shackelford VA (eds) Encyclopedia of Evolutionary Psychological Science. Springer, Cham, pp 1–6

Iwasaki K, Taguchi M, Bonkowsky JL, Kuwada JY (2013) Expression of arginine vasotocin receptors in the developing zebrafish CNS. Gene Expression Patterns 13:335–342

Johnson ZV, Young LJ (2017) Oxytocin and vasopressin neural networks: implications for social behavioral diversity and translational neuroscience. Neuroscience and Biobehavioral Reviews 76:87–98

Kawakami K, Murakami Y (2017) Preface to Vertebrate Brains: evolution, structures and functions. Development, Growth & Differentiation 59:160–162

Kermen F, Franco LM, Wyatt C, Yaksi E (2013) Neural circuits mediating olfactory-driven behavior in fish. Frontiers in Neural Circuits 7:62

Kingston PA, Crews D (1994) Effects of hypothalamic lesions on courtship and copulatory behavior in sexual and unisexual whiptail lizards. Brain Research 643:349–351

Kinkhabwala A, Riley M, Koyama M, Monen J, Satou C, Kimura Y, Higashijima S, Fetcho J (2011) A structural and functional ground plan for neurons in the hindbrain of zebrafish. PNAS 108:1164–1169

Kline RJ, O'Connell LA, Hofmann HA, Joan Holt G, Khan IA (2011) The distribution of an AVT V1a receptor in the brain of a sex changing fish, *Epinephelus adscensionis*. Journal of Chemical Neuroanatomy 42:72–88

Krause J, Godin JJ (1994) Shoal choice in the banded killifish (*Fundulus diaphanus*, Teleostei, Cyprinodontidae): effects of predation risk, fish size, species composition and size of shoals. Ethology 98:128–136

Krause J, Godin JJ (1995) Predator preferences for attacking particular prey group sizes: consequences for predator hunting success and prey predation risk. Animal Behaviour 50:465–473

Laberge F, Hara TJ (2001) Neurobiology of fish olfaction: a review. Brain Research Reviews 36:46–59

Landin J, Hovey D, Xu B. Lagman D, Zettergren A, Larhammar D, Kettunen P, Westberg L (2020) Oxytocin receptors regulate social preference in zebrafish. Scientific Reports 10:5435

Larson ET, O'Malley DM, Melloni, RH (2006) Aggression and vasotocin are associated with dominant–subordinate relationships in zebrafish. Behavioural Brain Research 167:94–102

Lee AW, Brown RE (2007) Comparison of medial preoptic, amygdala, and nucleus accumbens lesions on parental behavior in California mice (*Peromyscus californicus*). Physiology & Behavior 92:617–628

Lema SC, Nevitt GA (2004) Exogenous vasotocin alters aggression during agonistic exchanges in male Amargosa river pupfish (*Cyprinodon nevadensis amargosae*). Hormones and Behavior 46:628– 637

Lema SC, Slane MA, Salvesen KE, Godwin J (2012) Variation in gene transcript profiles of two V1a-type arginine vasotocin receptors among sexual phases of bluehead wrasse (*Thalassoma bifasciatum*). General and Comparative Endocrinology 179:451–464

Lin Q, Jesuthasan S (2017) Masking of a circadian behavior in larval zebrafish involves the thalamo-habenula pathway. Scientific Reports 7:4104

Lindeijer CM (2012) A neurobehavioural analysis of social behaviour and learning in fish and mammals. PhD-Dissertation, Utrecht University, pp 27–28

Lindeyer CM, Langen EMA, Swaney WT, Reader SM (2015) Nonapeptide influences on social behaviour: effects of vasotocin and isotocin on shoaling and interaction in zebrafish. Behaviour 152:897–915

Loveland JL, Fernald RD (2017) Differential activation of vasotocin neurons in contexts that elicit aggression and courtship. Behavioural Brain Research 317:188–203

Lukas M, Toth I, Reber SO, Slattery DA, Veenema AH, Neumann ID (2011) The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rats and mice. Neuropsychopharmacology 36:2159–2168

Macey MJ, Pickford GE, Peter RE (1974) Forebrain localization of the spawning reflex response to exogenous neurohypophysial hormones in the killfish, *Fundulus heteroclitus*. Journal of Experimental Zoology 190:269–280

Madden JR, Clutton-Brock TH (2011) Experimental peripheral administration of oxytocin elevates a suite of cooperative behaviours in a wild social mammal. Proceedings of the Royal Society B: Biological Sciences 278:1189–1194

Magurran AE (2005) Evolutionary ecology: the Trinidadian guppy. Oxford University Press, Oxford

Magurran AE, Seghers BH (1994a) Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*, populations in Trinidad. Behaviour 128:121–134

Magurran AE, Seghers BH (1994b) Sexual conflict as a consequence of ecology: evidence from guppy, *Poecilia reticulata*, populations in Trinidad. Proceedings of the Royal Society B 255:31–36

Magurran AE, Seghers BH, Carvalho GR, Shaw PW (1992) Behavioural consequences of an artificial introduction of guppies (*Poecilia reticulata*) in N. Trinidad: evidence for the evolution of anti-predator behaviour in the wild. Proceedings of the Royal Society B 248:117–122

Majolo B, de Bortoli Vizioli A, Schino G (2008) Costs and benefits of group living in primates: group size effects on behaviour and demography. Animal Behaviour 76:1235–1247

Majolo B, Huang, P (2018) Group living. In: Vonk J, Shackelford TK (eds) Encyclopedia of Animal Cognition and Behavior. Springer, Cham, pp 1–12

Maney DL, Goode CT, Wingfield JC (1997) Intraventricular infusion of arginine vasotocin induces singing in a female songbird. Journal of Neuroendocrinology 9:487–491

Martin CW, Fodrie FJ, Heck KL, Mattila J (2010) Differential habitat use and antipredator response of juvenile roach (*Rutilus rutilus*) to olfactory and visual cues from multiple predators. Oecologia 162:893–902

McCarthy MM (1990) Oxytocin inhibits infanticide in female house mice (*Mus domesticus*). Hormones and Behavior 24:365–375

Moore FL, Lowry CA (1998) Comparative neuroanatomy of vasotocin and vasopressin in amphibians and other vertebrates. Comparative Biochemistry and Physiology Part C 119:251–260

Morgan MJ, Colgan PW (1987) The effects of predator presence and shoal size on foraging in bluntnose minnows, *Pimephales notatus*. 20:105–111

Mueller T (2012) What is the thalamus in zebrafish?. Frontiers in Neuroscience 6:64

Northcutt RG (2002) Understanding vertebrate brain evolution. Integrative and Comparative Biology 42:743–756

O'Connell LA, Hofmann, HA (2011) The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. The Journal of Comparative Neurology 519:3599–3639

O'Connell LA, Matthews BJ, Hofmann HA (2012) Isotocin regulates paternal care in a monogamous cichlid fish. Hormones and Behavior 61:725–733

Ogawa S, Pfaff DW, Parhar IS (2021) Fish as a model in social neuroscience: conservation and diversity in the social brain network. Biological reviews of the Cambridge Philosophical Society 96:999–1020

Otsuka A, Shimomura K, Niwa H, Kagawa N (2020) The presence of a conspecific induces risk-taking behaviour and enlargement of somata size of dopaminergic neurons in the brain of male medaka fish. The journal of Fish Biology 96:1014–1023

Pedersen CA, Ascher JA, Monroe YL, Prange, AJJ (1982) Oxytocin induces maternal behavior in virgin female rats. Science 216:648–650

Pessoa L, Medina L, Hof PR, Desfilis E (2019) Neural architecture of the vertebrate brain: implications for the interaction between emotion and cognition. Neuroscience and Biobehavioral Reviews 107:296–312

Pickering S, Creighton E, Stevens-Wood B (1992) Flock size and breeding success in flamingos. Zoo Biology 11:229–234

Pilastro A, Benetton S, Bisazza A (2003) Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. Animal Behaviour 65:1161–1167

Pitcher TJ, Magurran AE, Winfield IJ (1982) Fish in larger shoals find food faster. Behavioral Ecology and Sociobiology 10:149–151

Portavella M, Torres B, Salas C (2004) Avoidance response in goldfish: emotional and temporal involvement of medial and lateral telencephalic pallium. The Journal of Neuroscience 24:2335–2342

Pusey AE (1987) Sex-biased dispersal and inbreeding avoidance in birds and mammals. Trends in Ecology & Evolution 2:295–299

Reddon AR, Aubin-Horth N, Reader SM (2022) Wild guppies from populations exposed to higher predation risk exhibit greater vasotocin brain gene expression. Journal of Zoology 316:118–127

Reddon AR, O'Connor CM, Nesjan E, Cameron J, Hellmann JK, Ligocki IY, Marsh-Rollo SE, Hamilton IM, Wylie DR, Hurd PL, Balshine S (2017) Isotocin neuronal phenotypes differ among social systems in cichlid fishes. Royal Society Open Science 4:170350

Reddon AR, Voisin MR, O'Connor CM, Balshine S (2014) Isotocin and sociality in the cooperatively breeding cichlid fish, *Neolamprologus pulcher*. Behaviour 151:1389–1411

Rincón L, Obando MJ, Tovar MO, Pandolfi M, Hurtado H (2017) Topological and histological description of preoptic area and hypothalamus in cardinal tetra *Paracheirodon axelrodi* (Characiformes: Characidae). Neotropical Ichthyology 15:e160145

Roberts BL (1992) Neural mechanisms underlying escape behaviour in fishes. Reviews in Fish Biology and Fisheries 2:243–266

Rodríguez F, Durán E, Gómez A, Ocaña FM, Álvarez E, Jiménez-Moya F, Broglio C, Salas C (2005) Cognitive and emotional functions of the teleost fish cerebellum. Brain Research Bulletin 66:365–370

Rodriguez-Santiago M, Nguyen J, Winton LS, Weitekamp CA, Hofmann HA (2017). Arginine vasotocin preprohormone is expressed in surprising regions of the teleost forebrain. Frontiers in Endocrinology 8:195

Rubenstein DI, Rubenstein DR (2013) Social Behavior. In: Levin SA (ed) Encyclopedia of biodiversity. Academic Press, pp 571–579

Rystrom TL, Clement VF, Rick IP, Bakker TCM, Mehlis M (2018) Shoal sex composition and predation risk influence sub-adult threespine stickleback shoaling decisions. Behavioural Processes 157:495–501

Santacà M, Dadda M, Bisazza A (2021) The role of visual and olfactory cues in social decisions of guppies and zebrafish. Animal Behaviour 180: 209–217

Santangelo N, Bass AH (2006) New insights into neuropeptide modulation of aggression: field studies of arginine vasotocin in a territorial tropical damselfish. Proceedings of the Royal Society B: Biological Sciences 273:3085–3092

Santangelo N, Bass AH (2010) Individual behavioral and neuronal phenotypes for arginine vasotocin mediated courtship and aggression in a territorial teleost. Brain, Behavior and Evolution 75:282–291

Sato T, Kikkawa T, Saito T, Itoi K, Osumi N (2017) Organizing activity of Fgf8 on the anterior telencephalon. Development, Growth & Differentiation 59:701–712

Seghers BH (1974) Schooling behavior in the guppy (*Poecilia reticulata*): an evolutionary response to predation. Evolution 28:486–489

Silk JB (2007) The adaptive value of sociality in mammalian groups. Philosophical Transactions of the Royal Society B 362:539–559

Slawski BA, Buntin JD (1995) Preoptic area lesions disrupt prolactin-induced parental feeding behavior in ring doves. Hormones and Behavior 29:248–266

Slimp JC, Hart BL, Goy RW (1978) Heterosexual, autosexual and social behavior of adult male rhesus monkeys with medial preoptic-anterior hypothalamic lesions. Brain Research 142:105–122

Smith AS, Agmo A, Birnie AK, French JA (2010) Manipulation of the oxytocin system alters social behavior and attraction in pair-bonding primates, *Callithrix penicillata*. Hormones and Behavior 57:255–262

Tan O, Musullulu H, Raymond JS, Wilson B, Langguth M, Bowen MT (2019) Oxytocin and vasopressin inhibit hyper-aggressive behaviour in socially isolated mice. Neuropharmacology 156:107573

Teles MC, Dahlbom SJ, Winberg S, Oliveira RF (2013) Social modulation of brain monoamine levels in zebrafish. Behavioural Brain Research 253:17–24

Thompson RR, Walton JC (2004) Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*). Behavioral Neuroscience 118:620–626

Thompson RR, Walton JC (2013) Social regulatory functions of vasotocin and isotocin in fish. In: Choleris E, Pfaff DW, Kavaliers M (eds) Oxytocin, vasopressin, and related peptides in the regulation of behavior. Cambridge University Press, Cambridge, pp 75–96

Thompson RR, Walton JC, Bhalla R, George KC, Beth EH (2008) A primitive social circuit: vasotocin substance P interactions modulate social behavior through a peripheral feedback mechanism in goldfish. European Journal of Neuroscience 27:2285–2293

Tinbergen N (1963) On aims and methods of ethology. Zeitschrift für Tierpsychologie 20:410–433

Vargas JP, López JC, Portavella M (2009) What are the functions of fish brain pallium?. Brain Research Bulletin 79:436–440

Wada M, Gorbman A (1977). Mate calling induced by electrical stimulation in freely moving leopard frogs, *Rana pipiens*. Hormones and Behavior 9:141–149

Ward A, Webster M (2016) Sociality: The behaviour of group-living animals. Springer, Cham, p 276

Wedekind C (1996) Lek-like spawning behaviour and different female mate preferences in roach (*Rutilus rutilus*). Behaviour 133:681–695

Zimen E (1976) On the regulation of pack size in wolves. Zeitschrift für Tierpsychologie 40:300–341

Chapter 2. Effect of Arginine Vasotocin on Shoaling Behaviour of the Guppy (*Poecilia reticulata*)[*]

2.1 Introduction

Arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin (AVP) are nonapeptide hormones that have central effects on the regulation of social behaviours (Goodson 2013). Administration of AVT and AVP has generally been shown to reduce aggression towards conspecifics. For example, AVP administration reduced territorial aggression between residents and intruders in mice (*Mus musculus*) (Tan et al. 2019). AVT administration likewise decreased aggression in field sparrows (*Spizella pusilla*) (Goodson 1998), zebrafish (*Danio rerio*) (Filby et al. 2010), pupfish (*Cyprinodon nevadensis*) (Lema and Nevitt 2004), and rainbow trout (*Oncorhynchus mykiss*) (Backström and Winberg 2009). In contrast, AVP administration had no effect on territorial aggression in deer mice (*Peromyscus* sp.) (Bester-Meredith et al. 2005), AVT administration increased aggressive territorial singing in white-crowned sparrows (*Zonotrichia leucophrys gambelii*) (Maney et al. 1997), and AVT administration increased territorial aggression in a tropical damselfish (*Stegastes leucostictus*) (Santangelo and Bass 2006). AVT may regulate the formation of social groups through these effects on aggression, as reduced aggression has been associated with the formation of more cohesive social groups (Magurran and Seghers 1991; Estevez et al. 2003; Plath and Strecker, 2008). However, AVT has shown mixed effects on direct measures of social grouping behaviour. For example, when AVT was administered to goldfish (*Carassius auratus*) it led to reduced time spent in proximity with a same-sex conspecific (Thompson and Walton 2004; Thompson et al. 2008). In female zebrafish, administration of either AVT or an AVT-antagonist reduced the proportion of time spent shoaling with a female conspecific relative to the administration of saline (Lindeyer et al. 2015). In another study of zebrafish, AVT administration increased the proportion of time spent

[*] A version of this chapter has been submitted for publication: Ataei Mehr B, Magyar K, Adeli K, Garner SR, Neff BD. Effect of Arginine Vasotocin on Shoaling Behaviour of the Guppy (*Poecilia reticulata*). Acta Ethologica. **Submitted**

associating with a phenotypically similar strain relative to a phenotypically dissimilar strain, whereas an AVT-antagonist had the opposite effect (Braida et al. 2012). Overall there is still considerable uncertainty about the relationship between AVT and grouping behaviours.

AVP and AVT may also induce greater expression of behaviours related to stress and anxiety (for review see Zelena et al. 2015). For example, AVP administration induced excessive grooming, scratching, and hyper-activity in mice (Lumley et al. 2001). In goldfish, AVT administration reduced food intake but did not affect locomotor activity (Araishi et al. 2019) or the frequency of transitions among zones during a two-choice trial (Thomson and Walton 2004). In zebrafish, AVT administration was associated with more frequent transitions among zones during a two-choice trial in one study (Lindeyer et al. 2015) but not in another (Braida et al. 2012). Due to the limited studies and the mixed results, it is unclear how AVT influences behavioural measures of stress and anxiety.

AVT and AVP appear to be involved in the expression of some sex-specific behaviours. For example, in plainfin midshipman (*Porichthys notatus*), AVT decreases vocalizations in territorial males that acoustically court females but not in females and non-territorial males that do not engage in acoustic courtship (Goodson and Bass 2000). In bluehead wrasse (*Thalassoma bifasciatum*), AVT administration increased territory defence in territorial males but not in females and non-territorial males (Semsar and Godwin 2004). It is less clear if the effect of AVT differs between sexes for behaviours that are not sex-specific, with for example a study of goldfish showing that AVT administration was associated with a similar reduction in the time spent in proximity with a same-sex conspecific in both males and females (Thompson et al. 2008).

The guppy (*Poecilia reticulata*) is a small live-bearing fish (Houde 1997). Guppies often form social groups referred to as shoals, with the propensity to shoal showing differences among populations and between the sexes (Magurran and Seghers 1994a; Magurran and Seghers 1994b). Guppies from populations living in high-predation regimes show a higher propensity to shoal with conspecifics than guppies from populations living in low-predation regimes (Magurran and Seghers 1994a). Adult females also show a greater preference for shoaling than males (Magurran and Seghers 1994b). Guppies offer an excellent system to examine the effect of nonapeptides on

social behaviours in a fish. It has recently been shown that guppies from high-shoaling populations have greater expression of AVT in the brain than guppies from low-shoaling populations, with no significant difference in the expression of AVT between sexes (Reddon et al. 2022). This result suggests that AVT may have a positive effect on the expression of shoaling behaviour in guppies. Here I used experimental administration of AVT, an AVT-antagonist, and a control to test the effect of AVT on shoaling behaviour in guppies. I further examined transitions among sections of the experimental apparatus as a measure of anxiety.

2.2 Materials and Methods

Experimental design

The experimental animals were drawn from a laboratory-reared population of guppies which were originally collected from the Paria River in Trinidad (for additional collection details see Hain et al. 2016). This population has been maintained in 200 L aquariums that contain approximately 100 adult guppies and similar numbers of males and females. Fish were kept at 25±1°C and on a 12:12 h light:dark cycle. Fish were fed *ad libitum* daily with TetraMin tropical fish flake food (TETRA Werke Melle, Germany). Mature fish were haphazardly collected from stock tanks for use in trials and assigned to treatments in an alternating sequence. Relative to the STRANGE framework (Webster and Rutz 2020), this design has limited potential for sampling biases as all subjects had the same origin, subjects had similar prior experience and no prior experimental history, and there was no opportunity for self-selection to different treatments.

Test fish received one of three injection treatments: AVT (Arg8-vasotocin acetate; Sigma-Aldrich, Oakville, Canada), an AVT-antagonist ([β-Mercapto-β,β-cyclopentamethylenepropionyl^{1}, O-me-Tyr^{2}, Arg^{8}]-Vasopressin; Sigma-Aldrich, Oakville, Canada; Soares et al. 2012), or a control injection (0.9% saline). AVT and AVT-antagonist were dissolved in sterile 0.9% saline at a concentration of 0.5 mg/ml and stored at -20°C until the day of injection. For injection, the test fish was weighed by placing them in a pre-weighed container of water. Fish were then given an intraperitoneal injection using a 5µl Hamilton Neuros-Syringe equipped with a 33G needle (Hamilton Company, Canada) at a dose of 2.5 µg/g fish body mass. This dosage follows similar

studies in fishes, which showed that effects on behaviour occurred following peripheral injection of AVT or AVT-antagonist at doses in the range of 1 to 10 µg/g fish body mass (Lindeyer et al. 2015; Semsar and Godwin 2004; Soares et al. 2012). Following injection, fish were placed individually in a post-injection recovery tank for 5 minutes to provide time for the injected substances to cross the blood-brain barrier and reach the brain (Lindeyer et al. 2015; Ramsey et al. 2019; Ataei Mehr et al. 2020). AVT was administered to 21 females and 21 males, AVT-antagonist to 20 females and 21 males, and saline to 20 females and 21 males.

To examine shoaling behaviour, a standard dichotomous choice behavioural trial protocol was used. Following Ataei Mehr et al. (2020), a test tank (34 cm length × 19 cm width × 15 cm water depth) was divided into three chambers by adding two plastic barriers 8 cm from each end of the tank. The barriers were clear and permeable to odours. On the center chamber (18 cm in length), vertical lines were drawn 5 cm from each plastic barrier to indicate association preference zones for each end of the tank. For each behavioural trial, a group of six stimulus fish were placed in one end-chamber whereas no fish were placed in the other end-chamber. The stimulus fish were all adult fish of the same sex as the test fish. The stimulus fish were placed in the stimulus chamber of the test tank at least 15 minutes before the test fish. Shoal location (left or right side) was randomized across trials. For each trial, a single test fish was released in the center chamber and its behaviour was recorded for 20 minutes using a digital camcorder placed in front of the tank.

Statistical analysis

Body mass was compared between sexes using a *t*-test. As in Ataei Mehr et al. (2020), a test fish was considered to be shoaling when its head was in the association zone associated with the group of six stimulus fish. For each trial, the time spent in the association zone with the group of six stimulus fish was calculated as a proportion of the total duration of the trial (i.e., time spent in the association zone / 20 minutes). The number of transitions was calculated as the number of times a test fish entered or left the association zone on either side of the test tank. The proportion of time spent in the association zone and number of transitions were analyzed using linear models that

included sex, treatment, and sex × treatment interaction as factors. Statistical analysis was conducted using JMP statistical software version 4.0.2.

Ethical Note

Experimental methods used in this study were approved by the Western University Animal Care Committee (Protocol 2018-084).

2.3 Results

As expected, body mass was significantly higher for females (mean ± SD; 0.39 ± 0.16 g) than for males (0.11 ± 0.04 g; $t_{122} = 13.5$, $p < 0.001$).

After the experimental injections, time in the association zone did not differ significantly between males and females ($F_{1,118} = 1.68$, $p = 0.20$; Figure 2.1a). Time in the association zone also did not differ significantly among the three injection treatments ($F_{2,118} = 0.47$, $p = 0.63$; Figure 2.1a). The interaction between treatment and sex also showed no significant association with the amount of time in the association zone ($F_{2,118} = 0.05$, $p = 0.95$; Figure 2.1a).

The number of transitions between different zones showed a near-significant association with sex, with females performing more transitions than males ($F_{1,118} = 3.86$, $p = 0.052$; Figure 2.1b). There was a significant difference in the number of transitions among treatments; fish injected with AVT performed more transitions than fish injected with either the AVT-antagonist or saline ($F_{2,118} = 3.81$, $p = 0.025$; Figure 2.1b). The interaction between treatment and sex showed no significant association with the number of transitions ($F_{2,118} = 1.62$, $p = 0.20$; Figure 2.1b).

2.4 Discussion

AVP and AVT have been hypothesized to modulate social interactions via inhibiting effect on grouping behaviours, albeit previous studies have reported inconsistent results including inhibiting, enhancing, and no effects (for review see Goodson 2013). In guppies, I show that the time spent associating with conspecifics did not differ among fish that were administered AVT, an AVT-antagonist, or a saline

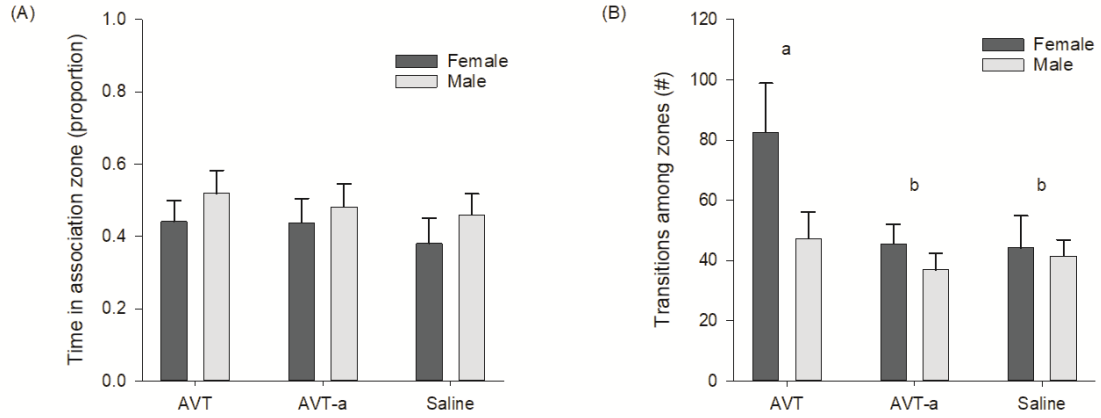


Figure 2.1. Behaviour of guppies (*Poecilia reticulata*) during a two-choice trial following injection with arginine vasotocin (AVT), an arginine vasotocin antagonist (AVT-a) or saline. Plots show mean ± SE for females (dark bars) and males (light bars). Time spent in the association zone with the group of six fish is presented in panel (A) and the number of transitions among zones are presented in panel (B).

control. In contrast to my findings, in zebrafish both AVT and an AVT-antagonist reduced the proportion of time spent shoaling with conspecifics relative to the administration of saline (Lindeyer et al. 2015). However, that study administered doses four times higher than in my study, and the similar effects of both AVT and its antagonist suggests that these higher doses may have caused a pharmacological inhibition of normal social behaviours, independent of the typical effects of AVT on social behaviour. Indeed another study of zebrafish that used much lower doses of AVT and its antagonist found that AVT administration increased the proportion of time spent associating with a phenotypically similar strain relative to a phenotypically dissimilar strain in a dose-dependent manner, whereas an AVT-antagonist had the opposite effect (Braida et al. 2012). In goldfish, AVT administration was consistently associated with reduced time spent in social proximity to a conspecific across a range of doses and injection conditions, suggesting that AVT may have a negative effect on association preference in this species (Thompson et al. 2008). The route of AVT administration may contribute to differences among studies, if for example AVT does not effectively cross the blood-brain barrier. However, effects of AVT on social behaviour have been observed following intracerebroventricular administration (Thompson et al. 2008), intramuscular administration (Braida et al. 2012) and intraperitoneal administration (Lindeyer et al. 2015), so intracerebroventricular administration does not appear to be necessary for AVT to influence behaviour. Given my results and the variation observed across previous studies, there is limited evidence that AVT is a key mediator of grouping behaviours in fishes, although further study, particularly using intracerebroventricular administration, may clarify the role of AVT in these behaviours.

AVP and AVT have previously been linked to an increase in the expression of behaviours related to stress and anxiety (for review see Goodson and Bass 2001; Zelena et al. 2015). I found that guppies injected with AVT performed more transitions across zones than fish injected with either the AVT-antagonist or saline. My finding suggests that stress and anxiety-related behaviours may be triggered by AVT in guppies, as hyperactivity is frequently associated with stress and anxiety in fishes (Schreck et al. 1997). A similar increase in the frequency of transitions among zones during a two-choice trial after AVT administration was observed in a study of zebrafish (Lindeyer et

al. 2015), although an effect of AVT administration on movement was not observed in another study of zebrafish (Braida et al. 2012) or in studies of goldfish (Araishi et al. 2019; Thomson and Walton 2004). AVT is also known to have peripheral effects on osmoregulation (Pang 1977; Babiker and Rankin 1980) and cardiovascular function (Pang et al. 1983; Le Mevel et al. 1993). Because I cannot separate peripheral from central effects of AVT in my study, it is possible that the hyperactivity I observed after AVT administration could be due to peripheral effects such as increased heart rate and blood pressure (rainbow trout, Le Mevel et al. 1993), osmoregulatory changes (catfish, *Clarias lazera*, Babiker and Rankin 1980), or the stress response (rainbow trout, Gesto et al. 2014). Further studies will be needed to distinguish between peripheral and central effects of AVT on activity. Regardless, my study adds to growing evidence that AVT is associated with hyperactivity in some fishes, possibly because of its relationship to stress and anxiety.

AVT and AVP may have similar effects on behaviour in both sexes or have sex-specific effects. I found limited evidence for sex-specific effects of AVT on either the time spent associating with conspecifics or the frequency of transitions among experimental zones. Although AVT appeared to have a larger influence on the number of transitions among experimental zones in females than males, the interaction term was not significant and both sexes showed a trend in the same direction. It is likely that there is no strong sex-specific effect because in guppies both association with conspecifics and exploratory behaviours are expressed in both sexes. For example, in goldfish both males and females associate with conspecifics, and in both sexes AVT administration was associated with a similar reduction in the time spent in proximity with a same-sex conspecific (Thompson et al. 2008). Instead, sex-specific responses to AVT have been shown most frequently for behaviours that are expressed by only one sex or otherwise have strong sex-specific differences in expression (e.g., fictive vocalization in plainfin midshipman, Goodson and Bass 2000; aggression in bluehead wrasse, Semsar and Godwin 2004). Overall, my data are consistent with the emerging pattern that sex-specific differences in the behavioural effects of AVT occur only when AVT mediates behaviours with strong sex-specific differences in expression.

AVT was hypothesized to influence shoaling behaviour in guppies in part due to the recent observation that AVT transcripts were more abundant in the brains of guppies from high-shoaling populations than from low-shoaling populations (Reddon et al. 2022). Although it is possible that AVT has a direct role in mediating population differences in shoaling, my results provide limited support for this hypothesis, as neither AVT nor its antagonist influenced shoaling when administered prior to a two-choice trial. Instead, because population differences in shoaling have previously been associated with predation risk (Magurran and Seghers 1994a), it may be that population differences in AVT are related to the stress and anxiety functions of this hormone. Indeed, AVT action is linked to fear-based conditioning such as learning to avoid predation (Rodriguez-Santiago et al. 2017; Soares et al. 2017). Differences in shoaling behaviour in guppies may instead be influenced by isotocin. Isotocin administration has previously been associated with a positive effect on shoaling in guppies (Ataei Mehr et al. 2020), and isotocin transcript levels in the brain have been shown to respond to predator cues in guppies from high-predation populations but not low-predation populations (Dimitriadou et al. 2022). However, isotocin transcript levels did not differ between high-predation and low-predation populations in a study of guppies (Reddon et al. 2022), so the nature of the mechanistic link between isotocin and shoaling behaviour also remains unresolved. Following the STRANGE framework, my design had minimal potential biases that would limit the generalisability of the reported findings.

2.5 References

Araishi K, Watanabe K, Yamazaki T, Nakamachi T, Matsuda K (2019) Intracerebroventricular administration of arginine vasotocin (AVT) induces anorexigenesis and anxiety-like behavior in goldfish. Peptides 119:170118

Ataei Mehr B, Garner SR, Neff BD (2020) Effect of isotocin on shoaling behaviour of the guppy (*Poecilia reticulata*). Animal Cognition 23:827–831

Babiker MM, Rankin JC (1980) Diuretic and antidiuretic actions of AVT and isotocin in the teleost fish *Clarias lazera*. General and Comparative Endocrinology 40:337

Backström T, Winberg S (2009) Arginine–vasotocin influence on aggressive behavior and dominance in rainbow trout. Physiology & Behavior 96:470–475

Bester-Meredith JK, Martin PA, Marler CA (2005) Manipulations of vasopressin alter aggression differently across testing conditions in monogamous and non-monogamous *Peromyscus* mice. Aggressive Behavior 31:189–199

Braida D, Donzelli A, Martucci R, Capurro V, Busnelli M, Chini B, Sala M (2012) Neurohypophyseal hormones manipulation modulate social and anxiety-related behavior in zebrafish. Psychopharmacology 220:319–330

Dimitriadou S, Santos EM, Croft DP, van Aerle R, Ramnarine IW, Filby AL, Darden SK (2022) Social partner cooperativeness influences brain oxytocin transcription in Trinidadian guppies (*Poecilia reticulata*). Behavioural Brain Research 423:113643

Estevez I, Keeling LJ, Newberry RC (2003) Decreasing aggression with increasing group size in young domestic fowl. Applied Animal Behaviour Science 84:213–218

Filby AL, Paull GC, Hickmore TFA, Tyler CR (2010) Unravelling the neurophysiological basis of aggression in a fish model. BMC Genomics 11:498

Gesto M, Soengas JL, Rodríguez-Illamola A, Míguez JM (2014) Arginine vasotocin treatment induces a stress response and exerts a potent anorexigenic effect in rainbow trout, *Oncorhynchus mykiss*. Journal of Neuroendocrinology 26:89–99

Goodson JL (1998) Territorial aggression and dawn song are modulated by septal vasotocin and vasoactive intestinal polypeptide in male field sparrows (*Spizella pusilla*). Hormones and Behavior 34:67–77

Goodson JL (2013) Deconstructing sociality, social evolution and relevant nonapeptide functions. Psychoneuroendocrinology 38:465–478

Goodson JL, Bass AH (2000) Forebrain peptides modulate sexually polymorphic vocal circuitry. Nature 403:769–772

Goodson JL, Bass AH (2001) Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain Research Reviews 35:246–265

Hain TJA, Garner SR, Ramnarine IW, Neff BD (2016) Multiple mating predicts intensity but not mechanism of kin recognition. Behavioral Ecology 27:93–100

Houde AE (1997) Sex, color, and mate choice in guppies. Princeton University Press, Princeton

Le Mevel JC, Pamantung TF, Mabin D, Vaudry H (1993) Effects of central and peripheral administration of arginine vasotocin and related neuropeptides on blood pressure and heart rate in the conscious trout. Brain Research 610:82–89

Lema SC, Nevitt GA (2004) Exogenous vasotocin alters aggression during agonistic exchanges in male Amargosa river pupfish (*Cyprinodon nevadensis amargosae*). Hormones and Behavior 46:628–637

Lindeyer CM, Langen EMA, Swaney WT, Reader SM (2015) Nonapeptide influences on social behaviour: effects of vasotocin and isotocin on shoaling and interaction in zebrafish. Behaviour 152:897–915

Lumley LA, Robison CL, Chen WK, Mark B, Meyerhoff JL (2001) Vasopressin into the preoptic area increases grooming behavior in mice. Physiology & Behavior 73:451–455

Magurran AE, Seghers BH (1991) Variation in schooling and aggression amongst guppy (*Poecilia reticulata*) populations in Trinidad. Behaviour 118:214–234

Magurran AE, Seghers BH (1994a) Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*, populations in Trinidad. Behaviour 128:121–134

Magurran AE, Seghers BH (1994b) Sexual conflict as a consequence of ecology: evidence from guppy, *Poecilia reticulata*, populations in Trinidad. Proceedings of the Royal Society B 255:31–36

Maney DL, Goode CT, Wingfield JC (1997) Intraventricular infusion of arginine vasotocin induces singing in a female songbird. Journal of Neuroendocrinology 9:487–491

Pang PKT (1977) Osmoregulatory functions of neurohypophysial hormones in fishes and amphibians. American Zoologist 17:739–749

Pang PKT, Furspan PB, Sawyer WH (1983) Evolution of neurohypophysial hormone actions in vertebrates. American Zoologist 23:655–662

Plath M, Strecker U (2008) Behavioral diversification in a young species flock of pupfish (*Cyprionodon* spp.): shoaling and aggressive behavior. Behavioral Ecology and Sociobiology 62:1727–1737

Ramsey ME, Fry D, Cummings ME (2019) Isotocin increases female avoidance of males in a coercive mating system: assessing the social salience hypothesis of oxytocin in a fish species. Hormones and Behavior 112:1–9

Reddon AR, Aubin-Horth N, Reader SM (2022) Wild guppies from populations exposed to higher predation risk exhibit greater vasotocin brain gene expression. Journal of Zoology 316:118–127

Rodriguez-Santiago M, Nguyen J, Winton LS, Weitekamp CL, Hofmann HA (2017) Arginine vasotocin preprohormone is expressed in surprising regions of the teleost forebrain. Frontiers in Endocrinology 8:195

Santangelo N, Bass AH (2006) New insights into neuropeptide modulation of aggression: field studies of arginine vasotocin in a territorial tropical damselfish. Proceedings of the Royal Society B: Biological Sciences 273:3085–3092

Schreck CB, Olla BL, Davis MW (1997) Behavioral responses to stress. In: Iwama GK, Pickering AD, Sumpter JP, Schreck CB (eds). Fish stress and health in aquaculture. Cambridge University Press, Cambridge

Semsar K, Godwin J (2004) Multiple mechanisms of phenotype development in the bluehead wrasse. Hormones and Behavior 45:345–353

Soares MC, Bshary R, Mendonça R, Grutter AS, Oliveira RF (2012) Arginine vasotocin regulation of interspecific cooperative behaviour in a cleaner fish. PLoS ONE 7:e39583

Soares MC, Cardoso SC, Mazzei R, André GI, Morais M, Gozdowska M, Kalamarz-Kubiak H, Kulczykowska E (2017) Region specific changes in nonapeptide levels during client fish interactions with allopatric and sympatric cleaner fish. PLoS ONE 12:e0180290

Tan O, Musullulu H, Raymond JS, Wilson B, Langguth M, Bowen MT (2019) Oxytocin and vasopressin inhibit hyper-aggressive behaviour in socially isolated mice. Neuropharmacology 156:107573

Thompson RR, Walton JC (2004) Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*). Behavioral Neuroscience 118:620–626

Thompson RR, Walton JC, Bhalla R, George KC, Beth EH (2008) A primitive social circuit: vasotocin substance P interactions modulate social behavior through a peripheral feedback mechanism in goldfish. European Journal of Neuroscience 27:2285–2293

Webster MM, Rutz C (2020) How STRANGE are your study animals? Nature 582:337–340

Zelena D, Pintér O, Balázsfi DG, Langnaese K, Richter K, Landgraf R, Makara GB, Engelmann M (2015) Vasopressin signaling at brain level controls stress hormone release: the vasopressin-deficient Brattleboro rat as a model. Amino Acids 47:2245–2253

Chapter 3. Effect of Isotocin on Shoaling Behaviour of the Guppy (*Poecilia reticulata*)[*]

3.1 Introduction

Oxytocin and its homologues generally have pro-social effects (for review see Goodson 2013). For example, in mammals, oxytocin administration increases maternal behaviour (Pedersen et al. 1982; McCarthy 1990), cooperative behaviour (Harmon et al. 2002; Madden and Clutton-Brock 2011) and the amount of time spent in proximity with a conspecific (Smith et al. 2010). In birds, administration of mesotocin, the avian homologue of oxytocin, increases time spent with a group of conspecifics (Goodson et al. 2009) and altruistic behaviour (Duque et al. 2018).

The effect of oxytocin may differ between the sexes at least for some social behaviours (for review see Dumais and Veenema 2016). For example, in the prairie vole (*Microtus ochrogaster*) oxytocin administration increases the amount of time that females spend in proximity to an adult of the opposite sex, but has no effect on the amount of time that males spend in proximity to an adult of the opposite sex (Cushing and Carter 2000). In humans, oxytocin administration improves kinship recognition in females but not in males, and improves competition recognition in males but not females (Fischer-Shofty et al. 2013). These sex-specific effects may occur because females are more sensitive to oxytocin, as the sex-steroid estrogen has been linked to the up-regulation of the expression of oxytocin receptors (Larcher et al. 1995; Carter 2007).

Although the pro-social effects of oxytocin and its homologues on behaviour are well-studied in birds and mammals, studies in fishes have provided only weak evidence for a pro-social effect of isotocin, the fish homologue of oxytocin. In goldfish (*Carassius auratus*), isotocin administration increases the time spent associating with another conspecific, but only when the authors examined the subset of the subjects (n = 6 of 13) that had the lowest baseline association values (Thompson and Walton 2004). Some other studies in fishes have shown no effect of isotocin on social behaviour or even an anti-

[*] A version of this chapter has been published: Ataei Mehr B, Garner SR, Neff BD (2020) Effect of isotocin on shoaling behaviour of the guppy (*Poecilia reticulata*). Animal Cognition 23(4):827-831

social effect. In daffodil cichlid (*Neolamprologus pulcher*), for example, the level of isotocin in the brain is negatively correlated with the expression of affiliative behaviour (Reddon et al. 2015). Furthermore, in this cichlid, isotocin administration led to reduced association with conspecifics, whereas administration of an isotocin antagonist increased association with conspecifics (Reddon et al. 2014). In zebrafish (*Danio rerio*), Lindeyer et al. (2015) found no effect of isotocin or its antagonist on shoaling behaviour, and Braida et al. (2012) found that, whereas isotocin administration at intermediate doses increases social preference for phenotypically similar conspecifics, isotocin administration at low or high doses has negative effects on this behaviour.

It is also unclear if the effect of isotocin on social behaviour is sex-biased in fishes. In mosquitofish (*Gambusia affinis*), isotocin administration decreases association time with conspecifics in males but not females (Ramsey et al. 2019). In plainfin midshipman (*Porichthys notatus*), Goodson and Bass (2000) showed that isotocin administration decreases vocalization in females but did not affect vocalization in males that acoustically court females. These initial studies suggest that there could be differences in the effect of isotocin between the sexes.

The guppy (*Poecilia reticulata*) is a small live-bearing fish with internal fertilization that is abundant in Trinidad (Houde 1997). Guppies often form social groups referred to as shoals, but the propensity to shoal differs among populations and between the sexes. The variation in shoaling behaviour among populations has been linked to predation regime, with guppies from populations with high levels of predation showing a higher propensity to shoal with conspecifics than guppies from populations with low levels of predation (Magurran and Seghers 1994a). At sexual maturity, female guppies are about three times larger than males, and females also show a greater preference for shoaling than males (Magurran and Seghers 1994b). Guppies offer an excellent system to examine the effect of isotocin on shoaling behaviour, with the opportunity to test whether isotocin administration leads to increase in shoaling behaviour, and whether these effects differ between the sexes with females shoal more than males.

3.2 Materials and Methods

Experimental design

The experimental animals were drawn from a laboratory-reared population of guppies that were originally collected from the low-predation Paria River in Trinidad (for additional collection details see Hain et al. 2016). This population has been maintained in 200 L aquariums that contain approximately 100 adult guppies and similar numbers of males and females. Fish were kept at 25±1°C and on a 12:12 h light:dark cycle. Fish were fed *ad libitum* daily with live brine shrimp nauplii (Canadian Aqua Farm, Canada) and TetraMin tropical fish flake food (TETRA Werke Melle, Germany).

To obtain baseline shoaling behaviour measurements prior to the experimental manipulation of isotocin levels, a standard dichotomous choice behavioural trial protocol was used. Following Hain and Neff (2007), a test tank (34 cm length × 19 cm width × 15 cm water depth) was divided into three chambers by adding two plastic barriers 8 cm from each end of the tank. The barriers were clear and permeable to odours. On the center chamber (18 cm in length), vertical lines were drawn 5 cm from each plastic barrier to indicate association preference zones for each end of the tank. For each behavioural trial, a group of six stimulus fish were placed in one end-chamber whereas no fish were placed in the other end-chamber. The stimulus fish were all adult fish of the same sex as the test fish, with a total of 30 males and 30 females used as stimulus fish. Males were identified by their colourful body and rod-shaped anal fin, whereas females were identified by their large body and the absence of male colouration (Houde 1997). Females may have mated prior to the start of trials, and their pregnancy status was not assessed. The stimulus fish were placed in the stimulus chamber of the test tank 15 minutes before the test fish to let them acclimate to the test tank and to allow any chemical cues to accumulate in the water (following Hain et al. 2016). Shoal location (left or right side) was randomized across trials. For each trial, a single test fish was released in the center chamber and its behaviour was recorded for 20 minutes using a digital camcorder placed in front of the tank. A total of 92 adult individuals (46 females and 46 males) were used as test fish. Test fish were never previously used as stimulus fish, nor were stimulus fish previously used as test fish. Stimulus fish were reused across multiple trials.

Isotocin (Bachem, Torrance, USA) and atosiban (an oxytocin/isotocin receptor antagonist; Cardoso et al. 2015) (Sigma-Aldrich, Oakville, Canada) were dissolved in sterile 0.9% saline at a concentration of 1 mg/ml and stored at -20°C until the day of

injection. Immediately following the baseline shoaling behaviour assessment, half of the test fish were injected with isotocin and the other half with isotocin antagonist. For injection, the test fish was removed from the test tank and weighed by placing them in a pre-weighed container of water. Fish were then immobilized using a pair of cloth-covered forceps and given an intraperitoneal injection using a 5µl Hamilton Neuros-Syringe equipped with a 33G needle (Hamilton Company, Canada) at a dose of 10 µg/g fish body mass. The dosage follows a similar study on zebrafish (Lindeyer et al. 2015). Injections were performed without anesthesia (see Lindeyer et al. 2015) to avoid the possible effect of anesthetic on subsequent behaviour. Following injection, fish were placed individually in a post-injection recovery tank for 5 minutes to allow the injected substance to reach the brain (Lindeyer et al. 2015; Ramsey et al. 2019). The test fish was then returned to the center chamber of its test tank and the post-injection shoaling behaviour was recorded for 20 minutes. The greatest effect of injection was seen within the first 20 minutes, consistent with rapid action and with the timeframes used in other studies (Lindeyer et al. 2015) (Appendix 1). The water of the test tank was replaced with fresh water after each trial.

Statistical analysis

Body mass was compared between sexes using a *t*-test. As in Hain and Neff (2007), a test fish was considered to be shoaling when its head was in the association zone associated with the group of six stimulus fish. For each trial, the time spent in the association zone with the group of six stimulus fish was calculated as a proportion of the total duration of the trial (i.e., time spent in association zone / 20 minutes). The proportion of time spent in the association zone was analyzed separately for the pre- and post-injection behavioural observations using linear models that included sex, treatment, and sex × treatment interaction as factors. The location of the group of stimulus fish (left vs right) was included as a random effect but was not significant (pre-injection $p = 1.00$; post-injection $p = 0.22$) and was removed from the final models. Quantile plots indicated that the time spent in the association zone was well-represented by a normal distribution. Statistical analysis was conducted using JMP statistical software version 4.0.2.

Ethical Note

Experimental methods used in this study were approved by the Western University Animal Care Committee (Protocol 2010-214).

3.3 Results

As expected, body mass was significantly higher for females (mean ± SD; 0.41 ± 0.11 g) than for males (0.13 ± 0.04 g; $t_{90} = 15.4$, $p < 0.001$).

Before the isotocin and isotocin antagonist injections, females spent significantly more time in the association zone adjacent to the six stimulus fish (hereafter referred to as the "association zone") than males ($F_{1,88} = 5.86$, $p = 0.02$; Figure 3.1a). Before the injections, there was no significant difference in the time spent in the association zone for fish assigned to the different treatments ($F_{1,88} = 0.03$, $p = 0.87$; Figure 3.1a), and no significant interaction between treatment and sex ($F_{1,88} = 0.33$, $p = 0.57$; Figure 3.1a).

After the isotocin and isotocin antagonist injections, females again spent more time in the association zone than males ($F_{1,88} = 11.37$, $p < 0.001$; Figure 3.1b). Individuals injected with isotocin spent significantly more time in the association zone than individuals injected with isotocin antagonist ($F_{1,88} = 6.93$, $p = 0.01$; Figure 3.1b). The amount of time in the association zone differed about twice as much between the isotocin and isotocin antagonist groups in males compared to females, albeit both sexes spent more time in the association zone after injection with isotocin than the isotocin antagonist, and the interaction between treatment and sex was not significant ($F_{1,88} = 1.15$, $p = 0.29$; Figure 3.1b).

3.4 Discussion

Oxytocin and its homologues have been reported to regulate social behaviour, acting as a pro-social modulator in mammals and birds (for review see Goodson 2013). Studies in fishes, on the other hand, have provided little support for pro-social effects of isotocin and instead often show an inhibition of affiliative behaviours (e.g., Braida et al. 2012; Reddon et al. 2014; Lindeyer et al. 2015; Ramsey et al. 2019). Here I provide some of the first evidence of pro-social effects of isotocin in a fish, showing that the time spent

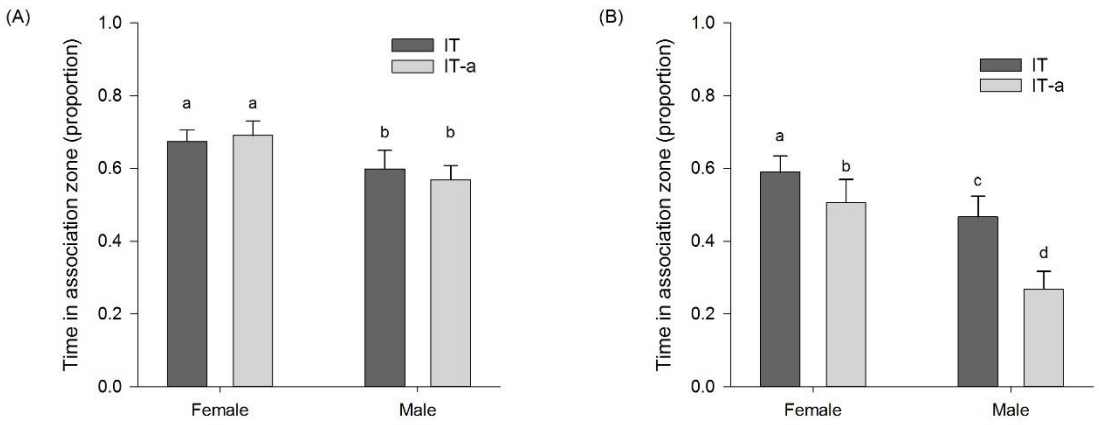


Figure 3.1. The time test fish spent in the association zone with a group of six stimulus fish during a dichotomous trial in guppies (*Poecilia reticulata*). Plots show mean ± SE for females (dark bars) and males (light bars). Behaviour prior to isotocin (IT) and isotocin antagonist (IT-a) injection are presented in panel (A) and behaviour after IT and IT-a injection are presented in panel (B).

in proximity to conspecifics was 29% higher in guppies that received isotocin than in guppies that received an isotocin antagonist. Several factors might explain why the pro-social effect of isotocin found in my study contrast with earlier studies in fishes. First, the pro-social effects of isotocin might depend on the dosage of isotocin administered. Indeed, there is some evidence in zebrafish that the effects of isotocin on social behaviour have a non-linear relationship with dosage (Braida et al. 2012). However, all the dosages used by Braida et al. (2012) were much lower than my study, and another study also in zebrafish that matched my dosage found no effect of isotocin on association behaviour (Lindeyer et al. 2015). Nevertheless, it is possible that only a specific dosage range will elicit social behaviour in a particular species of fish. Second, the differences among studies might reflect the social behaviour being measured and its context. Several of the studies that found no pro-social effect of isotocin compared association with groups of different sizes (Thompson and Walton 2004; Reddon et al. 2014) or groups that differed in body colouration (Braida et al. 2012), such that the studies were not clearly comparing a pro-social to non-social behaviour. In contrast, my study compared association with a group of six versus no conspecifics, providing a clear contrast between a pro-social and non-social choice. Regardless, I present some of the first evidence that isotocin has a homologous role to oxytocin in promoting social behaviour in a fish. More research is needed to better understand dosage and context-dependent effects.

Social behaviours may be expressed similarly in both sexes or may be sex-biased. In my study, before and after isotocin and isotocin antagonist administration, females spent more time with a group of same-sex conspecifics than males. Importantly, the effects of isotocin and its antagonist did not differ between sexes, with both male and female guppies showing greater shoaling after administration of isotocin than administration of the isotocin antagonist, albeit there was a non-significant trend towards a greater magnitude of effect in males than in females. This result suggests that both sexes in guppies share a common relationship between isotocin and shoaling behaviour and it is unlikely that isotocin regulates social behaviour in only one sex. In contrast, in mosquitofish the effects of isotocin administration differed between sexes, with males having reduced association with conspecifics following isotocin administration and females having no difference in association with conspecifics (Ramsey et al. 2019). Sex-

specific responses to isotocin were also described in plainfin midshipman in which isotocin suppresses vocalizations in females but not males that acoustically court females (Goodson and Bass 2000). It remains unknown what leads to the differences among species and behaviours in how the response to isotocin differs between sexes.

One potential limitation in the interpretation of my data is the absence of a control group that received an injection without an active compound (i.e., saline alone). Instead I used the pre-injection behaviour for a baseline measurement. However, I saw an overall decrease in the time spent in the association zone from the pre-injection to the post-injection observations across all groups. This decline might reflect a depressive effect of the handling and injection on social behaviour, or a decline in social interest as the test fish became acclimated to the test conditions. Regardless of its cause, the decline in association following injection means that it is not possible to confidently distinguish between three explanations that are consistent with my observations: 1) isotocin injections increased association and the isotocin antagonist had no effect, 2) isotocin injections increased association and the isotocin antagonist decreased association, or 3) isotocin injections had no effect and the isotocin antagonist decreased association. Although distinguishing between these explanations is an interesting question that is worthy of further investigation, this limitation does not alter my core conclusion that isotocin is positively associated with social behaviour in guppies.

In conclusion, although it has been shown that oxytocin and its homologues act as a pro-social modulator in mammals and birds, studies in fishes have provided mixed results. My study provides some of the first evidence of a pro-social effect of isotocin and suggests that isotocin and its antagonist affect the behaviour of both females and males similarly. My findings about shoaling in guppies contrast with some earlier studies on the effect of isotocin in fishes. Several factors might explain this contrast, including dosage and behaviour or context-specific effects of isotocin.

3.5 References

Braida D, Donzelli A, Martucci R, Capurro V, Busnelli M, Chini B, Sala M (2012) Neurohypophyseal hormones manipulation modulate social and anxiety-related behavior in zebrafish. Psychopharmacology 220:319–330

Cardoso SC, Paitio JR, Oliveira RF, Bshary R, Soares MC (2015) Arginine vasotocin reduces levels of cooperative behaviour in a cleaner fish. Physiology & Behavior 139:314–320

Carter CS (2007) Sex differences in oxytocin and vasopressin: implications for autism spectrum disorders?. Behavioural Brain Research 176:170–186

Cushing BS, Carter CS (2000) Peripheral pulses of oxytocin increase partner preferences in female, but not male, prairie voles. Hormones and Behavior 37:49–56

Dumais KM, Veenema AH (2016) Vasopressin and oxytocin receptor systems in the brain: sex differences and sex-specific regulation of social behavior. Frontiers in Neuroendocrinology 40:1–23

Duque JF, Leichner W, Ahmann H, Stevens JR (2018) Mesotocin influences pinyon jay prosociality. Biology Letters 14:20180105

Fischer-Shofty M, Levkovitz Y, Shamay-Tsoory SG (2013) Oxytocin facilitates accurate perception of competition in men and kinship in women. Social Cognitive and Affective Neuroscience 8:313–317

Goodson JL (2013) Deconstructing sociality, social evolution and relevant nonapeptide functions. Psychoneuroendocrinology 38:465–478

Goodson JL, Bass AH (2000) Forebrain peptides modulate sexually polymorphic vocal circuitry. Nature 403:769–772

Goodson JL, Schrock SE, Klatt JD, Kabelik D, Kingsbury MA (2009) Mesotocin and nonapeptide receptors promote estrildid behavior. Science 325:862–866

Hain TJA, Garner SR, Ramnarine IW, Neff BD (2016) Multiple mating predicts intensity but not mechanism of kin recognition. Behavioral Ecology 27:93–100

Hain TJA, Neff BD (2007) Multiple paternity and kin recognition mechanisms in a guppy population. Molecular Ecology 16:3938–3946

Harmon AC, Huhman KL, Moore TO, Albers HE (2002). Oxytocin inhibits aggression in female Syrian hamsters. Journal of Neuroendocrinology 14:963–969

Houde AE (1997) Sex, color, and mate choice in guppies. Princeton University Press, Princeton

Larcher A, Neculcea J, Breton C, Arslan A, Rozen F, Russo C, Zingg HH (1995) Oxytocin receptor gene expression in the rat uterus during pregnancy and the

estrous cycle and in response to gonadal steroid treatment. Endocrinology 136:5350–5356

Lindeyer CM, Langen EMA, Swaney WT, Reader SM (2015) Nonapeptide influences on social behaviour: effects of vasotocin and isotocin on shoaling and interaction in zebrafish. Behaviour 152:897–915

Madden JR, Clutton-Brock TH (2011) Experimental peripheral administration of oxytocin elevates a suite of cooperative behaviours in a wild social mammal. Proceedings of the Royal Society B: Biological Sciences 278:1189–1194

Magurran AE, Seghers BH (1994a) Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*, populations in Trinidad. Behaviour 128:121–134

Magurran AE, Seghers BH (1994b) Sexual conflict as a consequence of ecology: evidence from guppy, *Poecilia reticulata*, populations in Trinidad. Proceedings of the Royal Society B 255:31–36

McCarthy MM (1990) Oxytocin inhibits infanticide in female house mice (*Mus domesticus*). Hormones and Behavior 24:365–375

Pedersen CA, Ascher JA, Monroe YL, Prange, AJJ (1982) Oxytocin induces maternal behavior in virgin female rats. Science 216:648–650

Ramsey ME, Fryb D, Cummings ME (2019) Isotocin increases female avoidance of males in a coercive mating system: assessing the social salience hypothesis of oxytocin in a fish species. Hormones and Behavior 112:1–9

Reddon AR, O'Connor CM, Marsh-Rollo SE, Balshine S, Gozdowska M, Kulczykowska E (2015) Brain nonapeptide levels are related to social status and affiliative behaviour in a cooperatively breeding cichlid fish. Royal Society Open Science 2:140072

Reddon AR, Voisin MR, O'Connor CM, Balshine S (2014) Isotocin and sociality in the cooperatively breeding cichlid fish, *Neolamprologus pulcher*. Behaviour 151:1389–1411

Smith AS, Agmo A, Birnie AK, French JA (2010) Manipulation of the oxytocin system alters social behavior and attraction in pair-bonding primates, *Callithrix penicillata*. Hormones and Behavior 57:255–262

Thompson RR, Walton JC (2004) Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*). Behavioral Neuroscience 118:620–626

Chapter 4. Relationship between Social Behaviour and Brain Nonapeptides in Two Populations of Guppies (*Poecilia reticulata*)[*]

4.1 Introduction

Living in a group can provide many fitness benefits for group members (for review see Majolo and Huang 2018). Predation is one important factor influencing the formation of social groups and it has been shown that differences in predation pressure can affect the motivation of individuals to form social groups (Ioannou 2017). For example, predation risk shapes social behaviour of white-nosed coatis (*Nasua narica*) by affecting their grouping behaviour, vigilance, and alarm calling (Hass and Valenzuela 2002). Social group formation may also show variation between the sexes. Females in mammals and males in birds generally show greater fidelity to their groups and disperse less than the opposite sex (for reviews see Greenwood 1980; Pusey 1987). In fishes, predation risk as well as avoidance of sexual harassment are two factors that determine whether shoaling with conspecifics is sex-assortative or not. Indeed, the risk of being captured by a predator is often lower for an individual surrounded by same-sex conspecifics than opposite-sex conspecifics, presumably due to reduced mating activity and more vigilance [e.g. mosquitofish (*Gambusia holbrooki*), Pilastro et al. 2003 and Agrillo et al. 2006; zebrafish (*Danio rerio*), Etinger et al. 2009; threespine stickleback (*Gasterosteus aculeatus*), Rystrom et al. 2018]. Despite the attention that social groups have received in the literature from an adaptive perspective, the neuro-behavioural mechanism underlying such behaviour is less well understood.

Studies on the populations that differ in predation intensity have shown mixed results on individual exploration and activity. For example, a previous study in guppies (*Poecilia reticulata*) found that movement among areas of the experimental apparatus was higher in individuals from low-predation populations than high-predation populations (Ioannou et al. 2017) whereas Archard and Braithwaite (2011) in another

[*] A version of this chapter has been submitted for publication: Ataei Mehr B, Neff BD. Relationship between social behaviour and brain nonapeptides in two populations of guppies (*Poecilia reticulata*). Journal of Ethology. **Under revision**

study on a tropical poeciliid fish, the panamanian bishop (*Brachyrhaphis episcopi*) found that individuals from high-predation population were more explorative and active. They believed these differences were not related to the sex or size of the fish. Altogether, the relation between shoaling under different predation regimes and the individual's activity remains to be understood.

Expression of social behaviour has been linked to interconnected neural circuits that form a network across brain regions (O'Connell and Hofmann 2011). Nonapeptides are part of this neuronal network and are an evolutionary conserved family of peptides that are nine amino acids long (Caldwell 2017). These nonapeptides have been shown to modulate multiple social behaviours that include scent marking, vocalization, parental behaviour, sexual behaviour, pair-bonding, mate choice, aggression and social recognition (for reviews see Goodson and Bass 2001; Goodson 2013). The two types of nonapeptides most closely linked to social behaviour in vertebrates are the vasopressin-like (VP) and oxytocin-like (OT) peptides (Heinrichs and Domes 2008). These nonapeptides are produced and stored in large dense vesicles located in the cell bodies. The nonapeptides are then transferred to multiple brain regions via neural projections (Goodson and Thompson 2010; O'Connell and Hofmann 2011; Johnson and Young 2017). The effects of nonapeptides on social behaviour in vertebrates have been studied using methods that include administration of exogenous nonapeptides as well as measurement of neural structure and function by immunocytochemistry. The general pattern that is emerging is that vasopressin and its homologues have anti-social effects whereas oxytocin and its homologues have pro-social effects (Goodson and Bass 2001; Heinrichs and Domes 2008). However, the specific effects of these nonapeptides may vary among species and sexes (Goodson 2013), so more research is needed to better understand these patterns.

In fishes, expression of social behaviour has been linked to the vasotocin and isotocin neurosecretory cells that are found intermingled in the preoptic area of the brain (Thompson and Walton 2013). There are three subtypes of neurons involved in production of vasotocin and isotocin in the preoptic area, which differ in both number (parvocellular > magnocellular > gigantocellular) and size (gigantocellular > magnocellular > parvocellular) (Bradford and Northcutt 1983). The role of vasotocin

neurons in the regulation of social behaviours has been investigated in a number of studies using immunocytochemistry and *in situ* hybridization techniques. These studies have variation related to methodology and behavioural context (see Greenwood et al. 2008; Godwin and Thompson 2012) but generally suggest that vasotocin parvocellular neurons regulate subordination and submissive behaviours [e.g. peacock blenny (*Salaria pavo*), Grober et al. 2002; zebrafish, Larson et al. 2006; African cichlid fish (*Astatotilapia burtoni*), Greenwood et al. 2008; beaugregory damselfish (*Stegastes leucostictus*), Santangelo and Bass 2010; multiband butterflyfish (*Chaetodon multicinctus*), Dewan and Tricas 2011] whereas vasotocin magnocellular and gigantocellular neurons regulate dominance and territorial behaviours [zebrafish, Larson et al. 2006; African cichlid fish, Greenwood et al. 2008; butterflyfish (*Chaetodon* sp), Dewan et al. 2008, Dewan and Tricas 2011 and Dewan et al. 2011]. Interestingly, it has also been shown that vasotocin and its receptors are expressed in brain regions related to the fear response [rock hind (*Epinephelus adscensionis*), Kline et al. 2011; African cichlid fish, Huffman et al. 2012 and Rodriguez-Santiago et al. 2017], suggesting that vasotocin could influence shoaling behaviour if shoaling is driven by the fear of predation.

Despite a number of studies investigating the role of vasotocin neurons in the regulation of social behaviour, there are few studies examining the contribution of isotocin neurons to social behaviour in fishes (Godwin and Thompson 2012; Reddon et al. 2017). In a sex-changing species, blue-banded goby (*Lythrypnus dalli*), Black et al. (2004) found that females have more isotocin immunoreactive neurons in their preoptic area than males and that the number of isotocin neurons decreases when females change their sex. Parvocellular isotocin neurons are positively associated with paternal care in a cichlid (*Amatitlania nigrofasciata*) (O'Connell et al. 2012). Across species of lamprologine cichlids, Reddon et al. (2017) found that the number of parvocellular isotocin neurons in the preoptic was lower in species characterized by cooperative breeding than in species without cooperative breeding. The number of magnocellular and gigantocellular isotocin cells was not related to breeding system.

The guppy is a small live-bearing fish native to freshwater streams of Trinidad. Barriers such as waterfalls restrict predator access to upstream locations and produce a

predation regime dichotomy that is associated with predictable differences in social behaviour (Endler 1995; Houde 1997; Magurran 2005). For example, shoaling behaviour differs among populations of guppies, with a greater propensity for shoaling in high-predation populations than in low-predation populations (Seghers 1974; Magurran and Seghers 1994a). Independent of predation regime, female guppies show a higher propensity to shoal than males. Females spend more time with other females and engage more in antipredator behaviour than males. Females may benefit from shoaling with other females not only via reduced predation but also via reduced sexual harassment from males (Magurran et al. 1992; Magurran and Seghers 1994b; Griffiths and Magurran 1998). This difference in shoaling propensity both across populations and between the sexes affords an excellent opportunity to examine the role of vasotocin and isotocin neurons in regulating social behaviour.

Here I used guppies to examine the contribution of vasotocin and isotocin neurons in shoaling behaviour and provide some of the first evidence on neuro-behavioural mechanisms underlying this affiliative behaviour in fishes. I first measured shoaling behaviour in females and males from the high-predation Lower Aripo population and the low-predation Paria population to confirm the findings of previous studies that show shoaling propensity differs among these populations (Seghers 1974; Magurran and Seghers 1994a). A subset of these fish was then used to measure fluorescence intensity of vasotocin and isotocin immunoreactive neurons in the preoptic area of the brain to test the hypothesis that vasotocin and isotocin neurons mediate shoaling behaviour in guppies.

4.2 Materials and Methods

The experimental animals were from two laboratory-reared populations of guppies that were originally collected from the Lower Aripo River and the Paria River in Trinidad (for additional collection details see Hain et al. 2016). These populations have been maintained in replicate 200 L aquariums in groups of approximately 100 adults of mixed sex and kept at 25±1°C and on a 12:12 h light:dark cycle. Fish were fed ad libitum daily with live brine shrimp nauplii (Canadian Aqua Farm, Canada) and TetraMin tropical fish flake food (TETRA Werke Melle, Germany). Behavioural trials were

conducted for 46 individuals from the Lower Aripo population and 122 individuals from the Paria population, with an equal number of adult females and adult males used within each population. The behavioural data for 92 individuals from the Paria population were previously reported in Ataei Mehr et al. (2020). Adult males were identified by a colourful body and rod-shaped anal fin, whereas adult females have larger body size and drab colouration (Houde 1997). A standard dichotomous choice behavioural trial protocol was used to measure shoaling behaviour. Following Hain and Neff (2007) and Ataei Mehr et al. (2020), a test tank (34 cm length × 19 cm width × 15 cm water depth) was divided into three chambers by adding two clear and odour-permeable plastic barriers 8 cm from each end of the tank. On the 18 cm long central chamber, vertical lines were drawn 5 cm from each plastic barrier to indicate association (preference) zones for each end of the tank. The outer side of the test tank was covered with black plastic to minimize external visual disturbances. For each behavioural assay, a group of six stimulus fish were placed in one end chamber and zero fish in the other end chamber. The stimulus fish were adults from the same population and the same sex as the test fish. The stimulus fish were released inside the stimulus chamber of the test tank 15 minutes before the test fish to let them acclimate to the tank arena and allow their chemical cues to accumulate in the water. Shoal location (left or right side) was randomized across trials. For each trial, the test fish was released in the center chamber and its behaviour was recorded by a video camera for 20 minutes. After each behavioral trial, the test fish was removed from the test tank and its total body length was measured. As in Hain and Neff (2007), a test fish was considered to be shoaling when its head was in the association zone associated with the group of stimulus fish. The time spent in the association zone with the group of six stimulus fish was calculated for each trial and it was expressed as a proportion of the total duration of the trial. The number of transitions among zones was calculated as the number of times a test fish entered or left the association zone on either side of the test tank.

To examine vasotocin and isotocin immunoreactivity levels in the guppy brain, 5 females and 5 males from each population (n=20 total), were euthanized immediately after the behavioural trials by immersion in a buffered tricaine methanesulfonate (MS222) bath and then fixed in 10% formalin. After 48 hours in the formalin solution,

samples were transferred to 70% ethanol. The whole heads were then decalcified for 12 hours with an EDTA solution (pH 7.0) and embedded in paraffin. The entire brain was sectioned coronally at a thickness of 5µm per section. Every 20 sections (100 µm), a slide containing four adjacent sections was prepared and stained with hematoxylin and eosin (H and E) (Feldman and Wolfe 2014). The H and E slides were observed under a Zeiss StereoLumar V12 microscope (Carl Zeiss, Canada) to determine which sections contained the preoptic area, which is located between the middle of the ventral telencephalon and the beginning of the diencephalon and was identified based on morphological similarity to the images presented in Rincón et al. (2017) and Fischer et al. (2018). A slide containing four sections that were immediately posterior to the anterior-most preoptic area slide was then selected for immunostaining.

Two sections out of four per slide were used for vasotocin and isotocin immunostaining (VT^+ and IT^+) on which the primary and the secondary antibodies were applied. The other two sections were used for non-specific binding controls (VT^- and IT^-), which had the secondary antibody but not the primary antibody applied. Mounted sections were deparaffinized and heat-mediated antigen retrieval with 10mM citrate buffer (pH 6.0) was performed. Sections were then incubated with 5% donkey serum for 1 hour to block non-specific binding of the antibodies. The polyclonal Rabbit anti-[Arg8]-vasopressin (Peninsula Laboratories International Inc., catalogue number: T-4563, USA) and Guinea Pig anti-oxytocin (Peninsula Laboratories International Inc., catalogue number: T-5021, USA) primary antibodies were applied to the VT^+ and IT^+ sections at a 1:100 dilution and incubated at 4°C overnight. These antibodies have been successfully used in a cichlid fish, in which the vasopressin antibody binds to vasotocin, while the IT antibody binds to both isotocin and vasotocin (Reddon et al. 2017). Alexa Fluor 488 AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., catalogue number: 711-545-152, USA) was then applied to the VT^+ and VT^- sections, and Alexa Fluor 594 AffiniPure donkey anti-guinea pig IgG (Jackson ImmunoResearch Laboratories Inc., catalogue number: 706-585-148, USA) was applied to the IT^+ and IT^- sections at a dilution of 1:200 for 2 hours. Finally, 0.2µg/ml DAPI (Sigma-Aldrich, catalogue number: D9564, USA) was applied on the sections for 3 minutes. The sections were washed with 0.1M PBS 3 times for 5 minutes between steps and after the last round

of washing, slides were coverslipped with mounting medium (Thermo Scientific, catalogue number: TA-030-FM, USA). Immunofluorescence imaging was performed using an AxioImager Z1 microscope (Carl Zeiss, Canada) with a 63× oil immersion objective. For each section the preoptic area was divided coronally into three non-overlapping regions of interest. The dorsal region predominantly contains gigantocellular neurons, the central region predominantly contains magnocellular neurons and the ventral region predominantly contains parvocellular neurons (Bradford and Northcutt 1983; Reddon et al. 2017). The IT^+ section of one female from the Paria population was damaged during staining and was excluded from the analysis. Representative grayscale fluorescent images showing the VT^+ and IT^+ sections from one individual are provided in Figure 4.1. For the display images, the contrast was increased by 40% to visually highlight differences in intensity.

All image analysis was performed using Image Pro Premier 9.2 software (Media Cybernetics Inc, Rockville, MD). The fluorescence intensity was used to measure the immunoreactivity of vasotocin and isotocin containing neurons in the preoptic area following similar studies on catfish (*Clarias batrachus*) (Singh et al. 2012; Singh et al. 2016). First, nuclei were outlined on the DAPI channel, and the nuclear outlines thus generated were grown by 18 pixels, and converted to a cellular outline area, which was stored and reapplied at the identical location onto the matching vasotocin or isotocin channel as a region of interest. Enlarging by 18 pixels was predetermined to encompass neural cell bodies while limiting the inclusion of surrounding neuropil. The average fluorescence intensity of the region of interest in VT^+ and IT^+ sections was then measured on a scale of 0 to 65535. The negative controls had negligible fluorescence.

Statistical analysis

Total body length was compared between populations and sexes using linear models that included population and sex. The proportion of time spent in the association zone and the number of transitions among zones were analyzed using linear models that included population, sex, and population × sex interaction as factors. The fluorescence intensity of neurons in the dorsal, central, and ventral parts of the preoptic area were analyzed using a repeated measure model that included population and sex as factors and

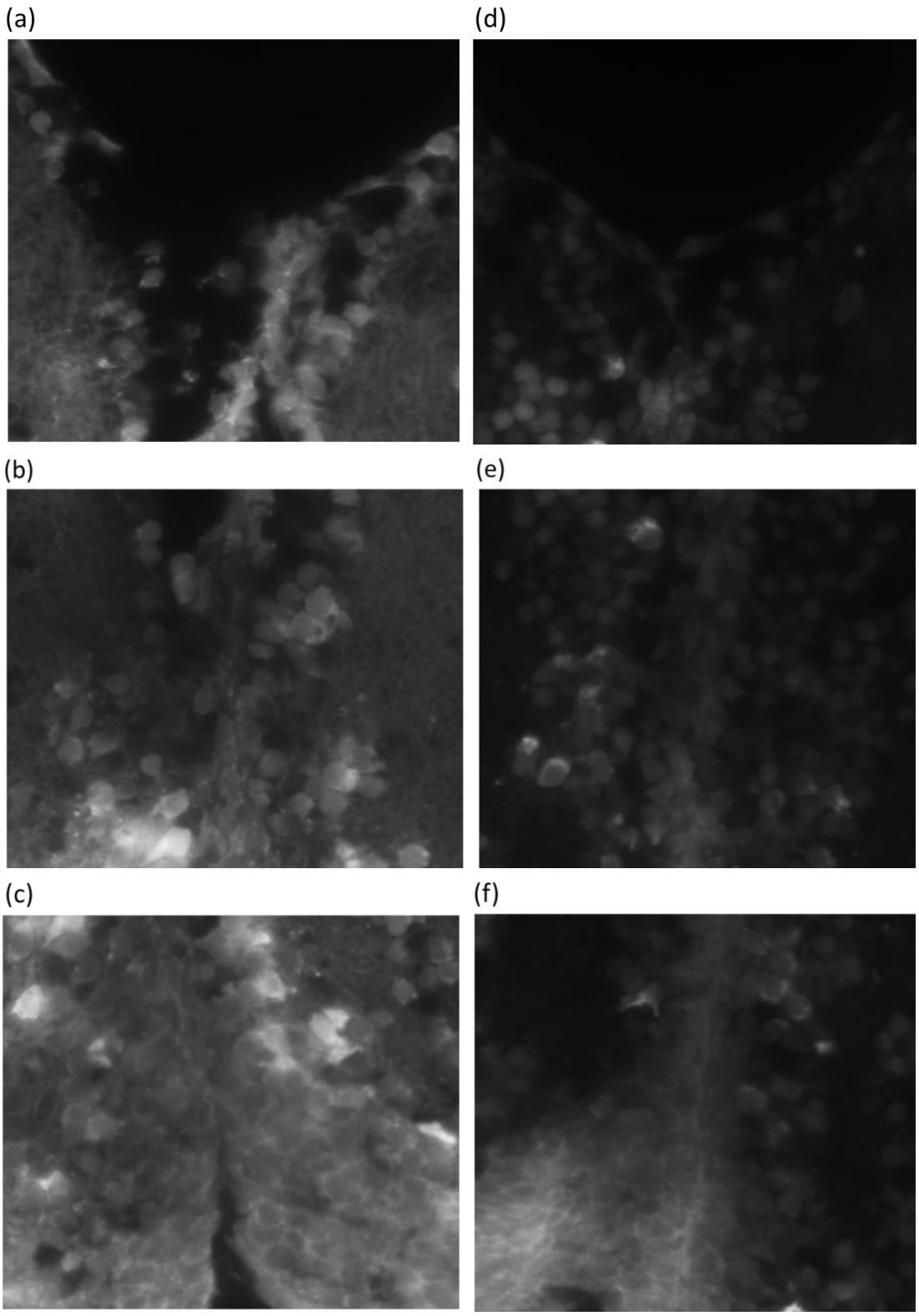

(a)
(d)
(b)
(e)
(c)
(f)

Figure 4.1. Representative grayscale fluorescent images from the preoptic area of a guppy (*Poecilia reticulata*). Sections were from a female guppy from the Paria population. Vasotocin-stained sections are shown for the dorsal (a), central (b), and ventral (c) regions and isotocin-stained sections are shown for the dorsal (d), central (e), and ventral (f) regions.

brain region as the repeated measure. Separate models were used for vasotocin-stained and isotocin-stained neurons. Where a significant interaction with the repeated measure (region) was observed, I used pairwise *t*-tests for each region after first dividing the intensity values by the average intensity across the three regions for that individual (i.e., I compared relative intensity to account for individual-level variation associated with the repeated measure). Pairwise correlations were used to analyse the relationship between the proportion of time spent in the association zone and the number of transitions and the vasotocin and isotocin fluorescence intensity. All statistical analysis was conducted using JMP (v4.0.2, SAS Institute Inc., Cary, NC, USA).

Ethical Note

Experimental methods and protocols used in this study were approved by the Animal Care Committee of Western University (Protocol 2018-084).

4.3 Results

There was a significant difference in total body length between the populations ($F_{1,165} = 14.35$, $p < 0.001$, Table 4.1); individuals from Paria were larger than individuals from Lower Aripo. There was also a significant difference in total body length between sexes ($F_{1,165} = 273.83$, $p < 0.001$, Table 4.1); females were larger than males. Examining the subset of fish used for brain sectioning, there was no significant difference in total body length between the populations ($F_{1,17} = 0.86$, $p = 0.37$, Table 4.1), but there was a significant difference in total body length between sexes ($F_{1,17} = 26.04$, $p < 0.001$, Table 4.1); females were larger than males.

Time spent in the association zone adjacent to the six stimulus fish, hereafter referred to as the “association zone”, was significantly different between populations ($F_{1,164} = 11.30$, $p = 0.001$); individuals from Lower Aripo spent more time than individuals from Paria in the association zone (Figure 4.2a). The effect of sex on the time spent in the association zone was also significant ($F_{1,164} = 6.43$, $p = 0.012$); females spent more time than males in the association zone (Figure 4.2a). No significant interaction was observed between population and sex in this analysis ($F_{1,164} = 0.36$, $p = 0.55$).

Table 4.1. Sample sizes and total body length of female and male guppies (*Poecilia reticulata*) from the Lower Aripo and Paria populations. Data are presented for all fish used in the behavioural trials and for the subset of the fish that were used for brain sectioning. Superscript letters denote significant differences among groups.

Population	Sex	Number of fish used for behavioural trials	Body length (mm)	Number of fish used for brain sectioning	Body length (mm)
Lower Aripo	Female	23	29.5 ± 3.0^{a}	5	27.6 ± 2.3^{a}
	Male	23	22.7 ± 0.8^{b}	5	22.8 ± 0.8^{b}
Paria	Female	61	33.6 ± 5.4^{c}	5	26.4 ± 3.0^{a}
	Male	61	23.3 ± 1.7^{d}	5	22.4 ± 0.5^{b}

NB. means are expressed ± SD

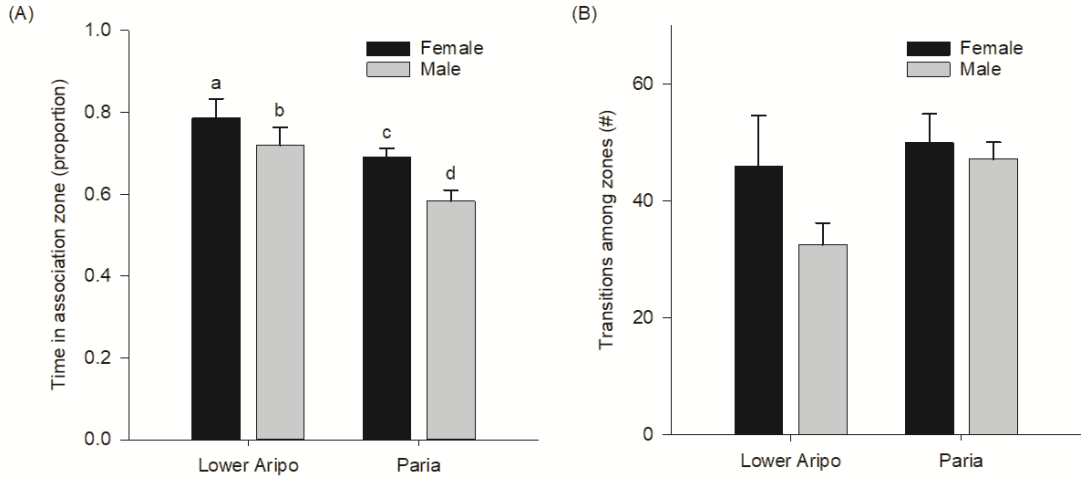


Figure 4.2. Behaviour of guppies (*Poecilia reticulata*) from Lower Aripo and Paria populations during a two-choice trial. Plots show mean ± SE for females (dark bars) and males (light bars). Time spent in the association zone with the group of six fish is presented in panel (A) and the number of transitions among zones are presented in panel (B). Different letters above the boxes indicate significant differences among groups.

The number of transitions among zones did not differ significantly between populations ($F_{1,164}$ = 2.93, p = 0.089; Figure 4.2b). The number of transitions among zones also did not differ significantly between sexes ($F_{1,164}$ = 2.20, p = 0.14). No significant interaction was observed between population and sex in this analysis ($F_{1,164}$ = 0.94, p = 0.33).

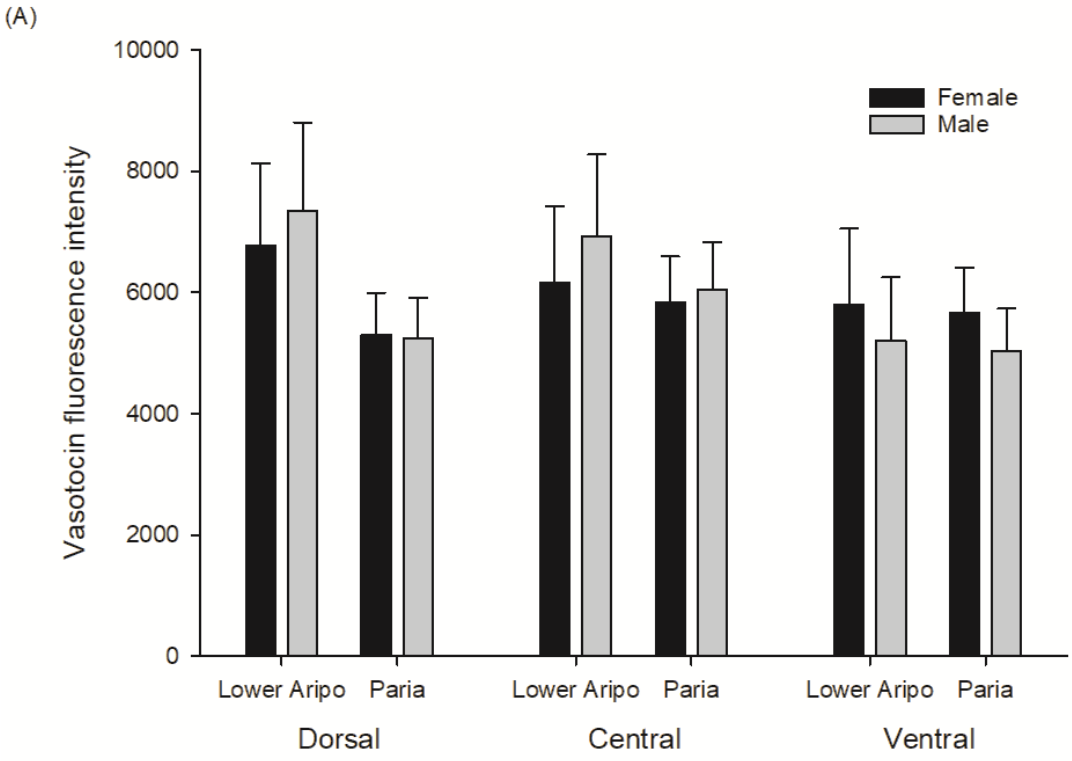
Examining the fluorescence intensity of vasotocin neurons across the three regions of the preoptic area, there was no significant effect of population ($F_{1,17}$ = 0.85, p = 0.37) or sex ($F_{1,17}$ = 0.002, p = 0.96). There was a trend showing that the fluorescence intensity of vasotocin neurons differed between the three regions ($F_{2,16}$ = 3.36, p = 0.06) with the intensity being lowest in the ventral region and similar in the dorsal and central regions (Figure 4.3). There was no interaction between region and sex ($F_{2,16}$ = 1.91, p = 0.18). There was a significant interaction between region and population ($F_{2,16}$ = 6.57, p = 0.008); the relative fluorescence intensity of vasotocin neurons was significantly higher in the Lower Aripo population than the Paria population in the dorsal preoptic area (t_{18} = 2.56, p = 0.020, Figure 4.3) but did not differ significantly between populations in the central (t_{18} = 0.89, p = 0.39) or ventral preoptic areas (t_{18} = 1.19, p = 0.25).

Examining the fluorescence intensity of isotocin neurons across the three regions of the preoptic area, there was no significant effect of population ($F_{1,16}$ = 0.99, p = 0.34) or sex ($F_{1,16}$ = 0.66, p = 0.43). The fluorescence intensity of isotocin neurons also did not differ significantly between the three regions ($F_{2,15}$ = 0.75, p = 0.49) and there was no interaction between region and sex ($F_{2,15}$ = 0.04, p = 0.96), or between region and population ($F_{2,15}$ = 0.35, p = 0.71).

The time spent in the association zone and the number of transitions among zones were not significantly correlated with the vasotocin or isotocin fluorescence intensity in any of the three regions (Table 4.2).

4.4 Discussion

Consistent differences in predation intensity have been hypothesized to lead to evolved differences in anti-predator responses across populations (Magurran et al. 1993). Consistent with this hypothesis, I found that guppies from the high-predation Lower Aripo population spent more time shoaling with conspecifics than guppies from the low-


(A)
Vasotocin fluorescence intensity
10000
8000
6000
4000
2000
0
Female
Male
Lower Aripo
Paria
Dorsal
Central
Ventral

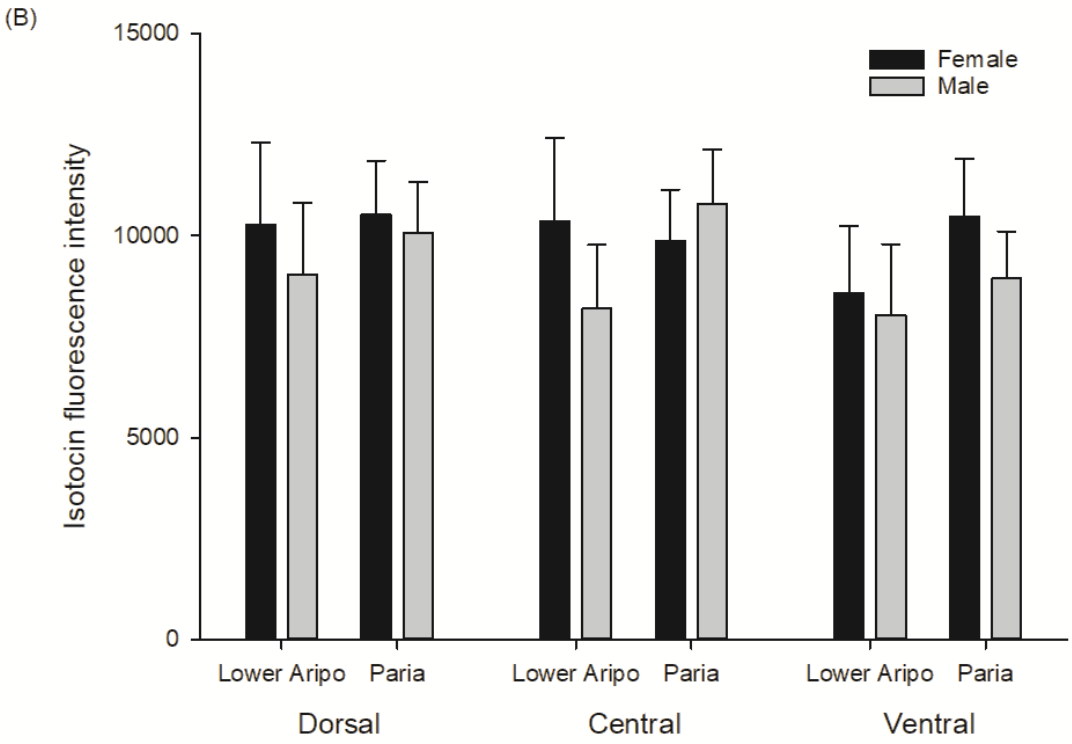

(B)
Isotocin fluorescence intensity
15000
10000
5000
0
Female
Male
Lower Aripo
Paria
Dorsal
Central
Ventral

Figure 4.3. The fluorescence intensity of vasotocin and isotocin neurons in guppies (*Poecilia reticulata*) from the Lower Aripo and Paria populations. Plots show mean ± SE for females (dark bars) and males (light bars) in the dorsal, central and ventral regions of the preoptic area. The fluorescence intensity of vasotocin neurons is presented in panel (A) and the fluorescence intensity of isotocin neurons is presented in panel (B).

Table 4.2. Pairwise correlations between time spent in the association zone and number of transitions and the fluorescence intensity of vasotocin- and isotocin-stained neurons in the preoptic area of the brain of guppies (*Poecilia reticulata*).

		Association time		**Number of transitions**	
	Region	r	p-value	r	p-value
Vasotocin	Dorsal	-0.02	0.94	-0.21	0.38
	Central	-0.13	0.58	0.03	0.91
	Ventral	-0.02	0.94	-0.01	0.96
Isotocin	Dorsal	0.15	0.55	-0.08	0.75
	Central	0.11	0.67	0.00	1.00
	Ventral	-0.08	0.75	0.11	0.67

predation Paria population. Shoaling behaviour is an anti-predator response in this fish (Magurran et al. 1993). My result matches previous comparisons of shoaling behaviour in this species (Seghers 1974; Magurran and Seghers 1994a) as well as in other fishes such as the banded killifish (*Fundulus diaphanus*) (Krause and Godin 1994). In contrast, the number of transitions among association zones, a measure that may be associated with anxiety or exploratory behaviour, did not differ between the Lower Aripo and Paria populations. Previous comparisons of populations that differ in predation intensity have shown mixed results, with high predation associated with both increased activity (Archard and Braithwaite 2011) and decreased activity (Ioannou et al. 2017). Given the many methodological differences in how exploration and activity have been measured, this behavioural variation might relate to the specifics of the experimental apparatus, which may alternatively favour either curiosity to explore the test environment or anxiety to escape the test environment. Regardless of the source of activity differences among studies, because I used a common garden-type experiment, my study confirms that these differences in association preferences have a genetic component, so may have evolved as an adaptation to increase survival (also see Seghers 1974; Magurran et al. 1993; Huizinga et al. 2009).

Many behaviours show sex-specific differences in expression. Females in mammals and males in birds generally have more fidelity to their groups and disperse less than their opposite sex (see Greenwood 1980). It has been suggested that the sex-bias in these taxa is driven at least partly by an asymmetry in the fitness benefits derived from group fidelity in which female mammals and male birds gain a greater fitness benefit from grouping behaviour than their opposite sexes (Greenwood 1980; Pusey 1987). My results showed that females from both the Lower Aripo and Paria populations spent more time with conspecifics than males. Similar results have been shown in guppies by Magurran et al. (1992) and Magurran and Seghers (1994b) as well as in other poeciliid species (mosquitofish and *Girardinus falcatus*; see Dadda 2015). It has been suggested that female guppies gain a greater benefit from shoaling than males because of reduced predation threat by forming the core of a shoal and reduced sexual harassment from males by forming shoals predominantly with other females (Magurran and Seghers 1994b; Griffiths and Magurran 1998). Furthermore, male guppies may also pay a higher

opportunity cost to shoaling than females through reduced mating opportunities (Griffiths and Magurran 1998; Croft et al. 2003). Male guppies benefit by encountering new females, which motivates them to explore the environment actively, frequently joining and splitting different shoals to find new mating opportunities (Croft et al. 2003). Regardless, my study confirms previous observations that female guppies have a greater propensity to form shoals than male guppies.

Differences in behaviour have been linked to differences in vasotocin-containing neurons in several species (see Goodson and Bass 2001; Goodson and Thompson 2010; O'Connell and Hofmann 2011). Given that my study observed differences in shoaling behaviour between populations and sexes, in the next step I investigated the potential contribution of vasotocin neurons in the preoptic area of the brain to these behavioural differences. I found that the relative fluorescence intensity of vasotocin was higher in the dorsal preoptic area (gigantocellular neurons) of the individuals from the Lower Aripo population (higher shoaling) than the Paria population (lower shoaling), suggesting a possible link between these neurons and differences in the affiliative shoaling behaviour. Indeed, a recent study showed findings consistent with my findings that vasotocin transcripts were more abundant in the brains of guppies from high-predation populations than from low-predation populations (Reddon et al. 2022). However, in my study there were no differences in the fluorescence intensity of vasotocin between sexes despite higher shoaling in females than males, and at the level of individual fish there was no correlation between the fluorescence intensity of vasotocin and shoaling behaviour. To resolve this apparent inconsistency, I propose that population differences in vasotocin levels may be unrelated to affiliative behaviour, but instead may relate to fear and anxiety aspects of anti-predatory behaviour. Studies in fishes have shown high vasotocin expression associated with fear conditioning [African cichlid fish, Rodriguez-Santiago et al. 2017; Indo-Pacific blonde naso tang (*Naso elegans*), Soares et al. 2017]. In guppies, experimental injections of vasotocin were not associated with a difference in affiliative behaviour but did lead to hyperactivity that was inferred to be associated with fear and anxiety (Ataei Mehr et al. submitted for publication). Although more research is needed, taken together, these data suggest that vasotocin levels in the brain are higher in high-predation populations, consistent with fear as the underlying motivation.

Previous research on isotocin neurons and social behaviour has suggested that parvocellular isotocin neurons affect affiliation in fishes (e.g., O'Connell et al. 2012; Reddon et al. 2017). Indeed, in my own previous work I found a pro-social effect of isotocin injection on shoaling behaviour in guppies (Ataei Mehr et al. 2020). In the current study, I tested the potential contribution of isotocin neurons in the preoptic area of the brain and found no significant difference in isotocin intensity between the two populations across any of the three regions of the preoptic area. I also found no difference between the sexes. Although I found weak specific antibody binding in my isotocin sections that might hinder my ability to observe significant differences; overall there is weak evidence of isotocin involvement in population and sex differences in guppies.

Consistent with my results, it has recently been shown that isotocin transcript levels did not differ between high-predation and low-predation populations in another study of guppies (Reddon et al. 2022). It is conceivable that isotocin neurons have an effect in other regions of the brain. For example, the isotocin neurons have projections from the preoptic area into the extra-hypothalamic regions such as the hindbrain in other fishes (Goodson et al. 2003; Saito et al. 2004). The hindbrain has sensory-motor functions such as regulating swimming (Kinkhabwala et al. 2011) and swimming is involved in predator avoidance and moving to be proximate to conspecifics. Future studies should aim to examine the interaction of vasotocin and isotocin and their receptors to fully characterize the contributions of nonapeptide neurons to shoaling behaviour in guppies.

In conclusion, my study tested the contribution of vasotocin and isotocin neurons in the preoptic area to a social behaviour (shoaling) in guppies. My data confirmed the findings of previous studies on the variation between populations and the sexes in shoaling behaviour and further implicates the gigantocellular vasotocin neurons in the dorsal preoptic area of the brain in regulating shoaling behaviour. My study provides supporting evidence on the abundance of vasotocin expression in the brain of guppies from a high-predation population. I speculate that the motivation underlying affiliative shoaling behaviour in guppies may involve fear of predation as opposed to being strictly pro-social attraction. My study also provides supporting evidence on the weak involvement of isotocin in population and sex differences in guppies, however, suggests

that isotocin neurons located in regions of the brain other than the preoptic area or perhaps interactions between vasotocin and isotocin peptides and receptors might be involved in regulating shoaling behaviour in guppies.

4.5 References

Agrillo C, Dadda M, Bisazza A (2006) Sexual harassment influences group choice in female mosquitofish. Ethology 112:592–598

Archard GA, Braithwaite VA (2011) Increased exposure to predators increases both exploration and activity level in *Brachyrhaphis episcopi*. Journal of Fish Biology 78:593–601

Ataei Mehr B, Garner SR, Neff BD (2020) Effect of isotocin on shoaling behaviour of the guppy (*Poecilia reticulata*). Animal Cognition 23:827–831

Black MP, Reavis RH, Grober MS (2004) Socially induced sex change regulates forebrain isotocin in *Lythrypnus dalli*. NeuroReport 15:185–189

Bradford MR, Northcutt RG (1983) Organization of the diencephalon and pretectum of the ray-finned fishes. In: Davis RE, Northcutt RG (eds) Fish neurobiology (vol. 2). Ann Arbor, University of Michigan Press, MI, pp 117–164

Caldwell HK (2017) Oxytocin and vasopressin: powerful regulators of social behavior. The Neuroscientist 23:517–528

Croft DP, Arrowsmith BJ, Bielby J, Skinner K, White E, Couzin ID, Magurran AE, Ramnarine I, Krause J (2003) Mechanisms underlying shoal composition in the Trinidadian guppy, *Poecilia reticulata*. OIKOS 100:429–438

Dadda M (2015) Female social response to male sexual harassment in poeciliid fish: a comparison of six species. Frontiers in Psychology 6:1453

Dewan AK, Maruska KP, Tricas TC (2008) Arginine vasotocin neuronal phenotypes among congeneric territorial and shoaling reef butterflyfishes: species, sex and reproductive season comparisons. Journal of Neuroendocrinology 20:1382–1394

Dewan AK, Ramey ML, Tricas TC (2011) Arginine vasotocin neuronal phenotypes, telencephalic fiber varicosities, and social behavior in butterflyfishes (Chaetodontidae): potential similarities to birds and mammals. Hormones and Behavior 59:56–66

Dewan AK, Tricas TC (2011) Arginine vasotocin neuronal phenotypes and their relationship to aggressive behavior in the territorial monogamous multiband butterflyfish, *Chaetodon multicinctus*. Brain Research 1401:74–84

Endler JA (1995) Multiple-trait coevolution and environmental gradients in guppies. Trends in Ecology & Evolution 10:22–29

Etinger A, Lebron J, Palestis BG (2009) Sex-assortative shoaling in zebrafish (*Danio rerio*). BIOS 80:153–158

Feldman AT, Wolfe D (2014) Tissue processing and hematoxylin and eosin staining. Methods in Molecular Biology 1180:31–43

Fischer EK, Westrick SE, Hartsough L, Hoke KL (2018) Differences in neural activity, but not behavior, across social contexts in guppies, *Poecilia reticulata*. Behavioral Ecology and Sociobiology 72:131

Godwin J, Thompson R (2012) Nonapeptides and social behavior in fishes. Hormones and Behavior 61:230–238

Goodson JL (2013) Deconstructing sociality, social evolution and relevant nonapeptide functions. Psychoneuroendocrinology 38:465–478

Goodson JL, Bass AH (2001) Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain Research Reviews 35:246–265

Goodson JL, Evans AK, Bass AH (2003) Putative isotocin distributions in sonic fish: relation to vasotocin and vocal-acoustic circuitry. The Journal of Comparative Neurology 462:1–14

Goodson JL, Thompson RR (2010) Nonapeptide mechanisms of social cognition, behavior and species-specific social systems. Current Opinion in Neurobiology 20:784–794

Greenwood AK, Wark AR, Fernald RD, Hofmann HA (2008) Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. Proceedings of the Royal Society B 275:2393–2402

Greenwood PJ (1980) Mating systems, philopatry and dispersal in birds and mammals. Animal Behaviour 28:1140–1162

Griffiths SW, Magurran AE (1998) Sex and schooling behaviour in the Trinidadian guppy. Animal Behaviour 56:689–693

Grober MS, George, AA, Watkins KK, Carneiro LA, Oliveira RF (2002) Forebrain AVT and courtship in a fish with male alternative reproductive tactics. Brain Research Bulletin 57:423–425

Hain TJA, Garner SR, Ramnarine IW, Neff BD (2016) Multiple mating predicts intensity but not mechanism of kin recognition. Behavioral Ecology 27:93–100

Hain TJA, Neff BD (2007) Multiple paternity and kin recognition mechanisms in a guppy population. Molecular Ecology 16:3938–3946

Hass CC, Valenzuela D (2002) Anti-predator benefits of group living in white-nosed coatis (*Nasua narica*). Behavioral Ecology and Sociobiology 51:570–578

Heinrichs M, Domes G (2008) Neuropeptides and social behaviour: effects of oxytocin and vasopressin in humans. Progress in Brain Research 170:337–350

Houde AE (1997) Sex, color, and mate choice in guppies. Princeton University Press, Princeton

Huffman LS, O'Connell LA, Kenkel CD, Kline RJ, Khan IA, Hofmann HA (2012) Distribution of nonapeptide systems in the forebrain of an African cichlid fish, *Astatotilapia burtoni*. Journal of Chemical Neuroanatomy 44:86–97

Huizinga M, Ghalambor CK, Reznick DN (2009) The genetic and environmental basis of adaptive differences in shoaling behaviour among populations of Trinidadian guppies, *Poecilia reticulata.* Journal of Evolutionary Biology 22:1860–1866

Ioannou CC (2017) Grouping and predation. In: Shackelford TK, Weekes-Shackelford VA (eds) Encyclopedia of Evolutionary Psychological Science. Springer, Cham, pp 1–6

Ioannou CC, Ramnarine IW, Torney, CJ (2017) High-predation habitats affect the social dynamics of collective exploration in a shoaling fish. Science Advances 3:e1602682

Johnson ZV, Young LJ (2017) Oxytocin and vasopressin neural networks: implications for social behavioral diversity and translational neuroscience. Neuroscience and Biobehavioral Reviews 76:87–98

Kinkhabwala A, Riley M, Koyama M, Monen J, Satou C, Kimura Y, Higashijima S, Fetcho J (2011) A structural and functional ground plan for neurons in the hindbrain of zebrafish. PNAS 108:1164–1169

Kline RJ, O'Connell LA, Hofmann HA, Joan Holt G, Khan IA (2011) The distribution of an AVT V1a receptor in the brain of a sex changing fish, *Epinephelus adscensionis*. Journal of Chemical Neuroanatomy 42:72–88

Krause J, Godin JJ (1994) Shoal choice in the banded killifish (*Fundulus diaphanus*, Teleostei, Cyprinodontidae): effects of predation risk, fish size, species composition and size of shoals. Ethology 98:128–136

Larson ET, O'Malley DM, Melloni, RH (2006) Aggression and vasotocin are associated with dominant–subordinate relationships in zebrafish. Behavioural Brain Research 167:94–102

Magurran AE (2005) Evolutionary ecology: the Trinidadian guppy. Oxford University Press, Oxford

Magurran AE, Seghers BH (1994a) Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*, populations in Trinidad. Behaviour 128:121–134

Magurran AE, Seghers BH (1994b) Sexual conflict as a consequence of ecology: evidence from guppy, *Poecilia reticulata*, populations in Trinidad. Proceedings of the Royal Society B 255:31–36

Magurran AE, Seghers BH, Carvalho GR, Shaw PW (1992) Behavioural consequences of an artificial introduction of guppies (*Poecilia reticulata*) in N. Trinidad: evidence for the evolution of anti-predator behaviour in the wild. Proceedings of the Royal Society B 248:117–122

Magurran AE, Seghers BH, Carvalho GR, Shaw PW (1993) Evolution of adaptive variation in antipredator behaviour. Marine Behaviour and Physiology 23:29–44

Majolo B, Huang, P (2018) Group living. In: Vonk J, Shackelford TK (eds) Encyclopedia of Animal Cognition and Behavior. Springer, Cham, pp 1–12

O'Connell LA, Hofmann, HA (2011) The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. The Journal of Comparative Neurology 519:3599–3639

O'Connell LA, Matthews BJ, Hofmann HA (2012) Isotocin regulates paternal care in a monogamous cichlid fish. Hormones and Behavior 61:725–733

Pilastro A, Benetton S, Bisazza A (2003) Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. Animal Behaviour 65:1161–1167

Pusey AE (1987) Sex-biased dispersal and inbreeding avoidance in birds and mammals. Trends in Ecology & Evolution 2:295–299

Reddon AR, Aubin-Horth N, Reader SM (2022) Wild guppies from populations exposed to higher predation risk exhibit greater vasotocin brain gene expression. Journal of Zoology 316:118–127

Reddon AR, O'Connor CM, Nesjan E, Cameron J, Hellmann JK, Ligocki IY, Marsh-Rollo SE, Hamilton IM, Wylie DR, Hurd PL, Balshine S (2017) Isotocin neuronal phenotypes differ among social systems in cichlid fishes. Royal Society Open Science 4:170350

Rincón L, Obando MJ, Tovar MO, Pandolfi M, Hurtado H (2017) Topological and histological description of preoptic area and hypothalamus in cardinal tetra *Paracheirodon axelrodi* (Characiformes: Characidae). Neotropical Ichthyology 15:e160145

Rodriguez-Santiago M, Nguyen J, Winton LS, Weitekamp CA, Hofmann HA (2017). Arginine vasotocin preprohormone is expressed in surprising regions of the teleost forebrain. Frontiers in Endocrinology 8:195

Rystrom TL, Clement VF, Rick IP, Bakker TCM, Mehlis M (2018) Shoal sex composition and predation risk influence sub-adult threespine stickleback shoaling decisions. Behavioural Processes 157:495–501

Saito D, Komatsuda M, Urano A (2004) Functional organization of preoptic vasotocin and isotocin neurons in the brain of rainbow trout: central and neurohypophysial projections of single neurons. Neuroscience 124:973–984

Santangelo N, Bass AH (2010) Individual behavioral and neuronal phenotypes for arginine vasotocin mediated courtship and aggression in a territorial teleost. Brain, Behavior and Evolution 75:282–291

Seghers BH (1974) Schooling behavior in the guppy (*Poecilia reticulata*): an evolutionary response to predation. Evolution 28:486–489

Singh O, Kumar S, Singh U, Bhute Y, Singru PS (2016) Role of isotocin in the regulation of the hypophysiotropic dopamine neurones in the preoptic area of the catfish, *Clarias batrachus*. Journal of Neuroendocrinology 28(12)

Singh U, Kumar S, Singru PS (2012) Interaction between dopamine- and isotocin containing neurones in the preoptic area of the catfish, *Clarias batrachus*: role in the regulation of luteinising hormone cells. Journal of Neuroendocrinology 24:1398–1411

Soares MC, Cardoso SC, Mazzei R, André GI, Morais M, Gozdowska M, Kalamarz-Kubiak H, Kulczykowska E (2017) Region specific changes in nonapeptide levels during client fish interactions with allopatric and sympatric cleaner fish. PLoS ONE 12:e0180290

Thompson RR, Walton JC (2013) Social regulatory functions of vasotocin and isotocin in fish. In: Choleris E, Pfaff DW, Kavaliers M (eds) Oxytocin, vasopressin, and related peptides in the regulation of behavior. Cambridge University Press, Cambridge, pp 75–96

Chapter 5. Distribution of Vasotocin and Isotocin Receptors in the Brain of the Guppy (*Poecilia reticulata*)

5.1 Introduction

Nonapeptides are an evolutionarily conserved family of hormones that have been shown to regulate many social behaviours in animals (Goodson 2008; Goodson and Thompson 2010). For example, vasopressin-like nonapeptides (vasopressin, vasotocin) have been linked to pro-anxiety behaviours like decreasing sexual behaviour [rabbit (*Oryctolagus cuniculus*), Kihlström and Agmo 1974], increasing the aggressive competition for mates [zebra finch (*Taeniopygia guttata*), Goodson et al. 2004], and decreasing social approach [goldfish (*Carassius auratus*), Thompson and Walton 2004]. In contrast, oxytocin-like nonapeptides (oxytocin, mesotocin, isotocin) have been linked to pro-social behaviours like increasing maternal care [Prairie vole (*Microtus ochrogaster*), Olazábal and Young 2006), increasing gregariousness (zebra finch, Goodson et al. 2009), and increasing social approach (goldfish, Thompson and Walton 2004). Although the neural distribution of vasopressin-like and oxytocin-like nonapeptides has been well-described across animals (Goodson and Bass 2001; Goodson and Thompson 2010), much less is known about the distribution of their receptors, especially in fishes. Understanding the distribution of these receptors is important because the effects of nonapeptides are determined via the interactions with their receptors in target tissues (Stoop 2012).

The effects of vasotocin in the brain are mediated via V1a receptors (Huffman et al. 2012), and the effects of isotocin via an isotocin receptor (Godwin and Thompson 2012). Projections of vasotocin and isotocin neurons enable these nonapeptides to affect receptors across multiple brain regions. For example, vasotocin and isotocin producing neurons project to regions that include the telencephalon, diencephalon, and mesencephalon [see Saito et al. 2004 for rainbow trout (*Oncorhynchus mykiss*) and Goodson and Bass 2000 for plainfin midshipman (*Porichthys notatus*)]. Nonapeptide receptors have similarly been observed to span across multiple brain regions [see Kline et al. 2011 for rock hind (*Epinephelus adscensionis*); Huffman et al. 2012 and Loveland and

Fernald 2017 for African cichlid (*Astatotilapia burtoni*); and Lema et al. 2012 for bluehead wrasse (*Thalassoma bifasciatum*)]. However, we know relatively little about the distribution of nonapeptide receptors throughout the brains in fishes (Huffman et al. 2012). Thus, we should expand our knowledge on the nonapeptide receptor sites in the brain to better understand the mechanisms through which nonapeptides may regulate social behaviours in fishes.

Previous studies have shown a potential linkage between the distribution of nonapeptides receptors in the connected brain regions and social behaviours in fishes. In a study on the distribution of vasotocin receptors in rock hind, Kline et al. (2011) found vasotocin receptor (V1a2) protein and mRNA were expressed in the brain regions that are involved in regulation of behaviour, olfaction, vision, learning, reproduction, and lateral line signals processing including the internal cellular layer of olfactory bulbs, torus longitudinalis, valvula of the cerebellum, the corpus cerebellum, the lateral and posterior recesses, and granular eminence. They also found a high vasotocin receptor (V1a2) protein and mRNA expression in the preoptic area, anterior hypothalamus, and habenula. In African cichlid, Huffman et al. (2012) found that vasotocin receptor (V1a2) and isotocin receptor (ITR) protein and mRNA generally show a similar pattern of expression throughout the forebrain to midbrain including the teleost homologue of mammalian amygdala, hypothalamus, striatum, and ventral tegmentum suggesting the expression of these receptors in the regions that are related to mediate social transitions and behaviour. In the same species, Loveland and Fernald (2017) found the overlapping areas of mRNA expression of vasotocin receptor V1a subtypes (V1a1 and V1a2) in the regions that have a role in aggression and courtship indicating the ventral telencephalon, hypothalamus, and thalamus. In bluehead wrasse, Lema et al. (2012) found that mRNA transcripts of both V1a subtypes (V1a1 and V1a2) were expressed in the regions related to the regulation of reproduction (preoptic area, ventral hypothalamus) and social and sexual behaviours (preoptic area, ventral telencephalon).

The guppy (*Poecilia reticulata*) is a small live-bearing fish with internal fertilization that lives in the tropical freshwaters of Trinidad (Houde 1997). In their natural habitats, barriers such as waterfalls restrict predator access to upstream locations and produce a predation regime dichotomy that is associated with predictable differences

in social behaviours, particularly shoaling behaviour (Endler 1995; Houde 1997; Magurran 2005). Guppies thereby can afford an excellent opportunity to examine the role of vasotocin and isotocin neural activity in regulating social behaviour in a fish. It has been shown that brain neural activation occurs during social exposure in guppies (Cabrera-Álvarez et al., 2017). More recently, it has been shown that vasotocin transcripts (Reddon et al., 2022) and neuronal vasotocin level (Ataei Mehr and Neff, unpublished) were more abundant in the brains of guppies from high-shoaling populations than from low-shoaling populations. Furthermore, isotocin transcript levels in the brain have been shown to respond to predator cues in guppies from high-predation populations but not low-predation populations (Dimitriadou et al. 2022). The functionality of the nonapeptides neurons can be confirmed by the presence of their receptors across the connected brain regions. However, the distribution of vasotocin and isotocin receptors has been investigated in only a few species. Here, I used immunocytochemistry trying to provide some of the first evidence of the distribution of vasotocin and isotocin receptors in the brain of the guppy.

5.2 Materials and Methods

The distribution of vasotocin and isotocin receptors in the brain were examined using four adult guppies of mixed sex (two males and two females), originally collected from the Lower Aripo and Paria rivers in northern Trinidad and subsequently maintained at Western University. The fish were first euthanized by immersion in a buffered tricaine methanesulfonate (MS222) bath. The whole head was removed and fixed in 10% formalin for 48 hours before storage in 70% ethanol. The heads were later decalcified with an EDTA solution (pH 7.0) for 12 hours and embedded in paraffin. The brain was sectioned coronally at a thickness of 5µm per section. Every 20 sections (100 µm), a slide containing four adjacent sections was prepared and stained with hematoxylin and eosin (H and E) (Feldman and Wolfe 2014). The H and E slides were observed under a Zeiss StereoLumar V12 microscope (Carl Zeiss, Canada) to identify the sections corresponding to five regions of interest: 1) the anterior-telencephalon, 2) the mid-telencephalon, 3) the diencephalon, 4) the mesencephalon, and 5) the rhombencephalon. These regions were identified through morphological similarity to the images of the brain presented in

previous studies (the brain atlas of the turquoise killifish, *Nothobranchius furzeri*, developed by D'Angelo, 2013; the brain atlas of the guppy developed by Fischer et al., 2018; see Figure 1a-5a). Sections adjacent to the H and E sections on the adjacent slide from the desired regions were then selected for immunostaining.

Two sections out of four per slide were used for vasotocin and isotocin receptor immunostaining (VTR+ and ITR+) on which the primary and the secondary antibodies were applied. The other two sections were used for non-specific binding controls (VTR- and ITR-), which had the secondary antibody but not the primary antibody applied. Mounted sections were deparaffinized and heat-mediated antigen retrieval with 10mM citrate buffer (pH 6.0) was performed. Sections were then incubated with 5% donkey serum for 1 hour to block non-specific binding of the antibodies. The polyclonal rabbit anti-arginine vasopressin receptor 1a (AVPR1A) (Invitrogen, catalogue number: 711640, Canada) and polyclonal goat anti-oxytocin receptor (OXTR) (Thermo Fisher Scientific, catalogue number: PA5-19038, USA) primary antibodies were applied to the VTR+ sections at a 1:100 dilution and ITR+ sections at a 1:50 dilution and incubated at 4°C overnight. The AVTR antibody for fish was unavailable. I tried a mammalian AVPR antibody because of high identity (> 60%) between mammalian AVPR1 and fish AVTR1 (Iwasaki et al. 2013). The mammalian OTR antibody has been previously used in a study in fish (Huffman et al. 2012) which was not available to use for my study at the time of the experiment. Using the same approach, I tried a mammalian OTR antibody because mammalian oxytocin receptors and fish isotocin receptors are closely related to one another with a high similarity (Hausmann et al. 1995). Alexa Fluor 488 AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., catalogue number: 711-545-152, USA) was then applied to the VTR+ and VTR- sections at a dilution of 1:200 for 2 hours, and Alexa Fluor 488 donkey anti-goat IgG (Invitrogen, catalogue number: A-11055, USA) was applied to the ITR+ and ITR- sections at a dilution of 1:200 for 2 hours. Finally, 0.2µg/ml DAPI (Sigma-Aldrich, catalogue number: D9564, USA) was applied on the sections for 3 minutes. The sections were washed with 0.1M PBS 3 times for 5 minutes between steps and after the last round of washing, slides were coverslipped with mounting medium (Thermo Scientific, catalogue number: TA-030-FM, USA). Immunofluorescence imaging was performed using an AxioImager Z1 microscope

(Carl Zeiss, Canada) with a 20× objective. The specificity of the immunocytochemical procedure was validated by negative controls to ensure that the labeling method accurately identifies the antibody bound to the tissue. The negative controls had a negligible fluorescence signal in the tissue. The distribution and intensity of receptor staining was then compared qualitatively among regions using the grayscale fluorescent images. The brightness of the VTR and ITR images increased by 50% to make the positive areas more visible. I also tried to examine VTR and ITR levels using qPCR, but primer sets did not amplify the genes (Appendix 2).

5.3 Results

In the anterior telencephalon, clusters of vasotocin receptors were distributed in the ventral and lateral part of the olfactory bulbs [the external cell layer (ECL) and glomerular layer (GL)] and in the margin of the medial part of the dorsal telencephalon (Dm) (Figure 5.1b).

In the mid-telencephalon, clusters of vasotocin receptors were distributed in the supracommissural nucleus of ventral telencephalon (Vs), and in the margins of the medial part of the dorsal telencephalon (Dm), dorsal part of the dorsal telencephalon (Dd), and dorsal part of the lateral part of the dorsal telencephalon (Dld) (Figure 5.2b).

In the diencephalon, the clusters of vasotocin receptors were distributed in the suprachiasmatic nucleus (SC), the preoptic area (POA), the ventromedial thalamic nucleus (VM), and the ventral part of the habenula (Ha) (Figure 5.3b).

In the mesencephalon, the clusters of vasotocin receptors were distributed in the lateral valvular nucleus (LV) and the dorsal hypothalamus (DH) at the midbrain tegmentum whereas no clusters of these receptors were found in the midbrain optic tectum (OT) (Figure 5.4b).

In the rhombencephalon, the clusters of vasotocin receptors were distributed in the central griseum (CG) of the ventro-caudal part of the rhombencephalon, whereas no clusters of these receptors were found in the dorso-cranial part of the rhombencephalon (Figure 5.5b).

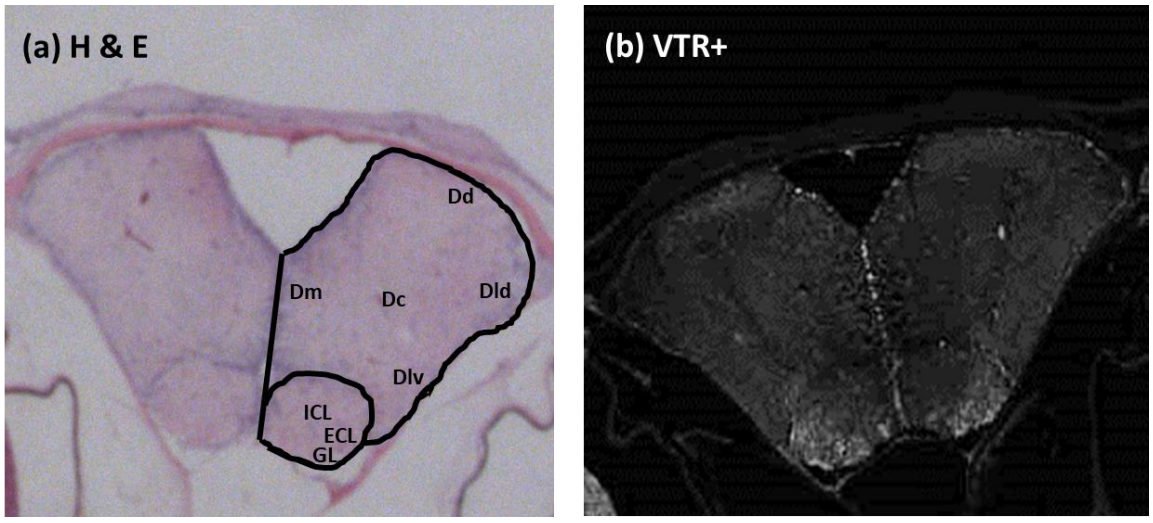


Figure 5.1. The coronal section of the anterior telencephalon of the brain of the guppy (*Poecilia reticulata*). (a) Hematoxylin and Eosin-stained section. The right half shows a line diagram identifying the major regions of interest, and (b) grayscale image of the vasotocin receptor fluorescent positive section. Legend: ICL: internal cell layer; ECL: external cell layer; GL: glomerular layer; Dm: medial part of dorsal telencephalon; Dd: dorsal part of dorsal telencephalon; Dld: dorsal part of lateral part of dorsal telencephalon; Dlv: ventral part of lateral part of dorsal telencephalon; Dc: central part of dorsal telencephalon

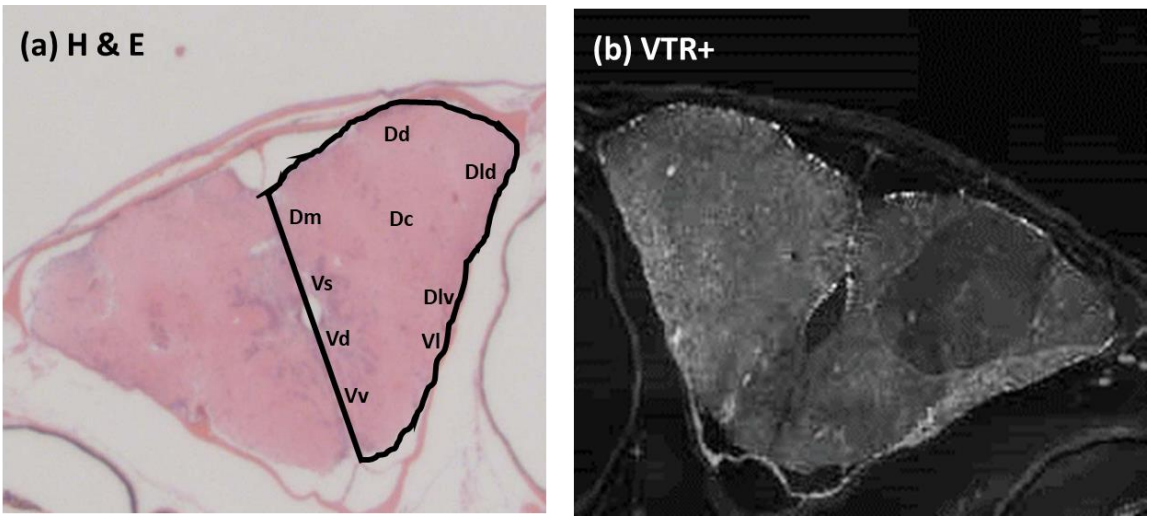


Figure 5.2. The coronal section of the mid telencephalon of the brain of the guppy (*Poecilia reticulata*). (a) Hematoxylin and Eosin-stained section. The right half shows a line diagram identifying the major regions of interest, and (b) grayscale image of the vasotocin receptor fluorescent positive section. Legend: Vv: ventral part of ventral telencephalon; Vd: dorsal nucleus of ventral telencephalon; Vs: supracommissural nucleus of ventral telencephalon; Dm: medial part of dorsal telencephalon; Dd: dorsal part of dorsal telencephalon; Dld: dorsal part of lateral part of dorsal telencephalon; Dlv: ventral part of lateral part of dorsal telencephalon; Dc: central part of dorsal telencephalon; Vl: lateral part of ventral telencephalon

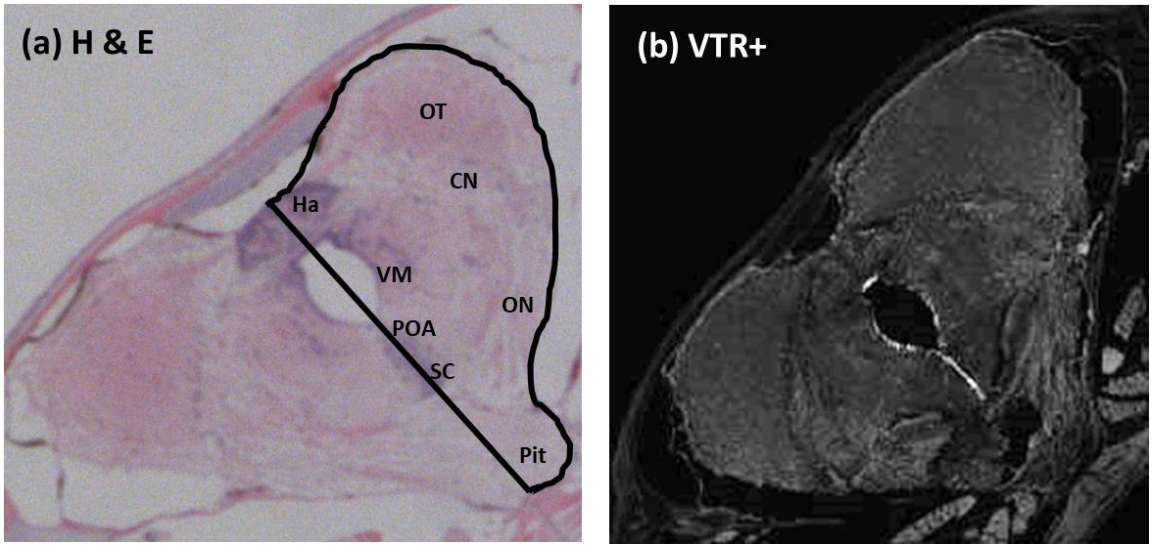


Figure 5.3. The coronal section of the diencephalon of the brain of the guppy (*Poecilia reticulata*). (a) Hematoxylin and Eosin-stained section. The right half shows a line diagram identifying the major regions of interest, and (b) grayscale image of the vasotocin receptor fluorescent positive section. Legend: Pit: pituitary (hypophysis); SC: suprachiasmatic nucleus; POA: preoptic area; VM: ventro-medial thalamic nucleus; Ha: habenula; OT: optic tectum; CN: cortical nucleus; ON: optic nerve

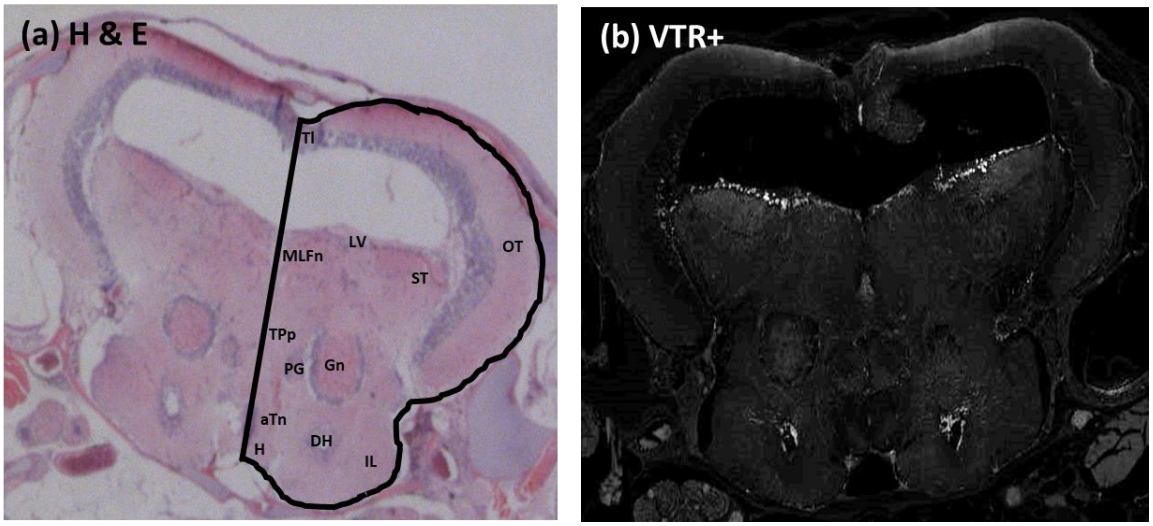


Figure 5.4. The coronal section of the mesencephalon of the brain of the guppy (*Poecilia reticulata*). (a) Hematoxylin and Eosin-stained section. The right half shows a line diagram identifying the major regions of interest, and (b) grayscale image of the vasotocin receptor fluorescent positive section. Legend: H: hypothalamus; aTNn: anterior tuberal nucleus; TPp: periventricular nucleus of the posterior tuberculum; MLFn: nucleus of the medial longitudinal fascicle; LV: lateral valvular nucleus; Tl: longitudinal tori; OT: optic tectum; ST: semicircular torus; Gn: glomerular nucleus; PG: preglomerular nucleus; DH: dorsal hypothalamus; IL: inferior lobe of hypothalamus

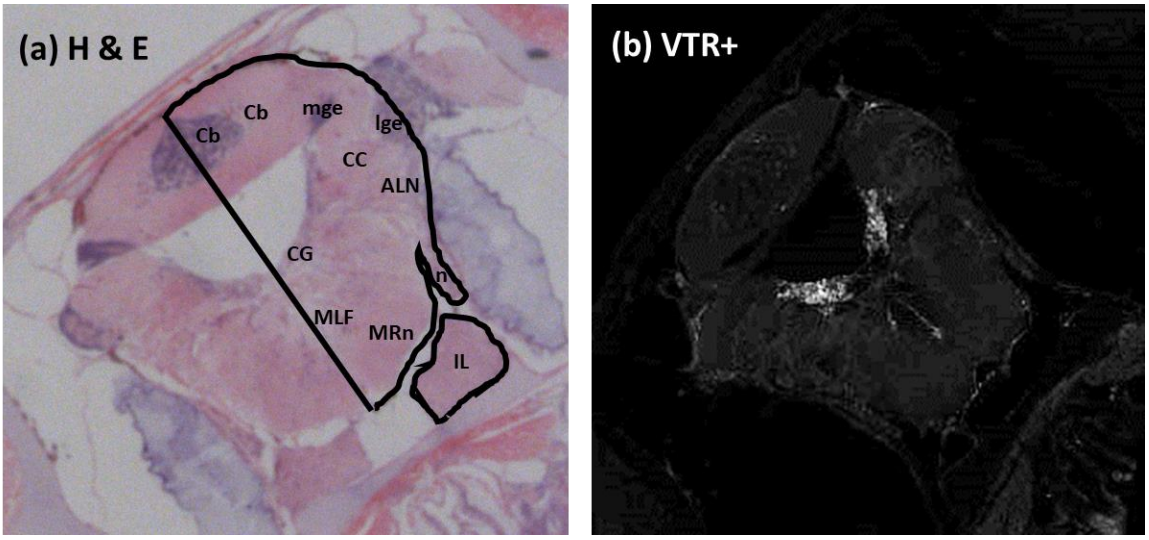


Figure 5.5. The coronal section of the rhombencephalon of the brain of the guppy (*Poecilia reticulata*). (a) Hematoxylin and Eosin-stained section. The right half shows a line diagram identifying the major regions of interest, and (b) grayscale image of the vasotocin receptor fluorescent positive section. Legend: MRn: medial reticular nucleus; MLF: medial longitudinal fascicle; CG: central griseum; Cb: cerebellum; mge: medial granular eminence; CC: cerebellar crest; lge: lateral granular eminence; ALN: anterior lateral line nerve; n: nerve; IL: inferior lobe of hypothalamus

In contrast to VTR+ sections, which showed clear clusters of fluorescent receptors in multiple regions, the ITR+ sections did not show clear clusters of fluorescent receptors in any region (Figure 5.6a and 5.6b). Consequently, I conclude that there was limited binding of the antibody to isotocin receptors and are not able to compare the distribution of these receptors among brain regions.

5.4 Discussion

My study used immunohistochemistry to examine the distribution of vasotocin and isotocin receptors in the brain of the guppy. Whereas vasotocin receptor sections showed strong specific binding in multiple regions, I did not observe strong specific binding for isotocin receptor sections. This result might partly be related to the region of the receptor that my OTR antibody recognized. Huffman et al. (2012) used an OTR antibody – which was no longer available at the time of my experiment – whose antigenic sequence comprises amino acids 244–259 in the third intracellular loop of the human OTR, whereas the OTR antibody that I used corresponds to the C-terminus amino acids of the human and rat oxytocin receptor. Regardless of the cause, it seems OTR antibodies are not interchangeable, and in the absence of commercially available ITR antibodies OTR antibodies may need to be chosen carefully for use in fishes.

Guppies show considerable homology in the distribution of vasopressin-like receptors relative to other taxa. Although fine-scale brain homology is difficult to determine in distantly related taxa, vasopressin-like receptors are widely distributed in the brain of the guppy, similar to the pattern observed in birds [white-throated sparrow (*Zonotrichia albicollis*) and zebra finch, Leung et al. 2011) and mammals [rat (*Rattus rattus*), Ostrowski et al. 1994]. Looking at other fishes, the V1a receptor distribution in the guppy shows considerable similarity across regions and sub-regions to the rock hind (Kline et al. 2011), African cichlid (Huffman et al. 2012; Loveland and Fernald 2017), and bluehead wrasse (Lema et al. 2012). I found that the clusters of vasotocin receptors were distributed in the external cell layer and glomerular layer of the olfactory bulbs. Vasotocin receptors were found in the external cell layer of the olfactory bulbs in African cichlid (V1a1, Loveland and Fernald 2017). In the anterior telencephalon, I also found

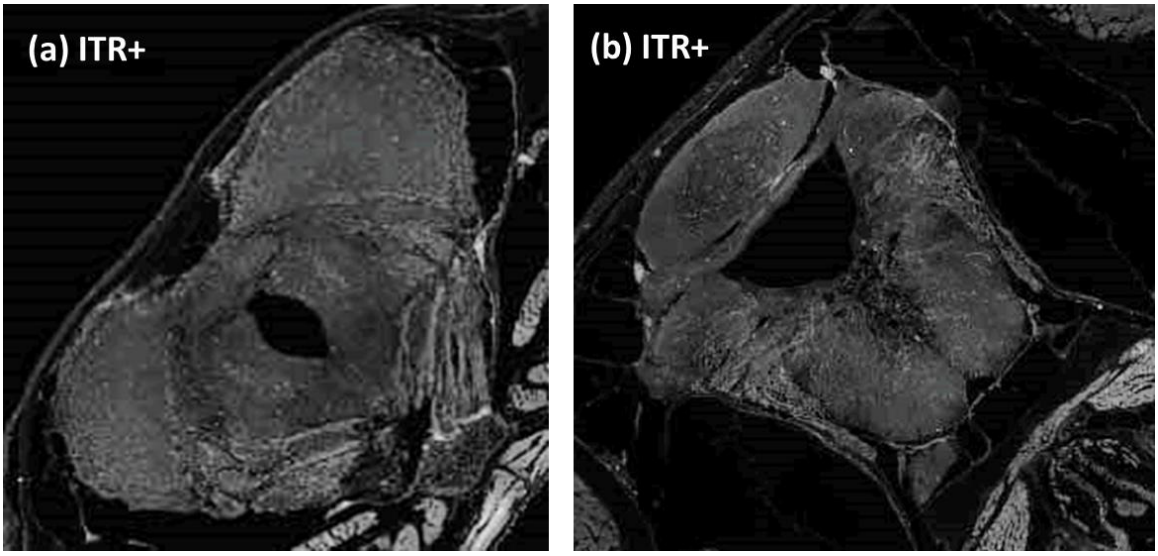


Figure 5.6. The grayscale images of the coronal section of the isotocin receptor fluorescent positive areas in the (a) diencephalon, and (b) rhombencephalon of the brain of the guppy (*Poecilia reticulata*).

that vasotocin receptors were distributed in the margin of the medial part of the dorsal telencephalon similar to the findings in rock hind (V1a2, Kline et al. 2011) and African cichlid (V1a2, Huffman et al. 2012). In the mid-telencephalon, I found that vasotocin receptors were expressed in the supracommissural nucleus of ventral telencephalon similar to the results from rock hind (V1a2, Kline et al. 2011), African cichlid (V1a2, Huffman et al. 2012), and bluehead wrasse (V1a1 and V1a2, Lema et al. 2012). I also found that vasotocin receptors were expressed in the medial, dorsal, and lateral part of the dorsal telencephalon. Similarly, vasotocin receptors were found in the medial and dorsal part of the dorsal telencephalon in rock hind (V1a2, Kline et al. 2011), African cichlid (V1a2, Huffman et al. 2012), and bluehead wrasse (V1a1, Lema et al. 2012). Vasotocin receptors were also found in the lateral part of the dorsal telencephalon in rock hind (V1a2, Kline et al. 2011) and African cichlid (V1a2, Huffman et al. 2012). In the diencephalon, I found that the clusters of vasotocin receptors were distributed in the suprachiasmatic nucleus, preoptic area, ventromedial thalamic nucleus, and habenula. Vasotocin receptors were found in the suprachiasmatic nucleus in bluehead wrasse (V1a1 and V1a2, Lema et al. 2012); in the preoptic area in rock hind (V1a2, Kline et al. 2011), African cichlid (V1a2, Huffman et al. 2012; V1a1 and V1a2, Loveland and Fernald 2017), and bluehead wrasse (V1a1 and V1a2, Lema et al. 2012); in the ventromedial thalamic nucleus in rock hind (V1a2, Kline et al. 2011), and African cichlid (V1a2, Huffman et al. 2012; V1a1, Loveland and Fernald 2017); and in the habenula in rock hind (V1a2, Kline et al. 2011), and African cichlid (V1a2, Huffman et al. 2012). In the mesencephalon, I found that the clusters of vasotocin receptors were distributed in the lateral valvular nucleus and the dorsal hypothalamus. Similarly, vasotocin receptors were found in the lateral valvular nucleus in rock hind (V1a2, Kline et al. 2011) and African cichlid (V1a2, Huffman et al. 2012); and in the dorsal hypothalamus in African cichlid (V1a2, Huffman et al. 2012). In the rhombencephalon, I found that the clusters of vasotocin receptors were distributed in the central griseum of the ventro-caudal region. Similar to my findings, it has been shown that vasotocin receptors were found in the central griseum in rock hind (V1a2, Kline et al. 2011). Together the considerable homology of vasotocin and vasopressin distribution across taxa suggests the potential for homology in function.

In the anterior telencephalon, the high level of vasotocin in the olfactory bulb reported in other species [Mozambique tilapia, *Oreochromis mossambicus*, Almeida et al. 2012] suggests the expression of vasotocin receptors I found in the olfactory bulbs in guppy might be linked to the regulation of odour-related behaviours via vasotocin hormone in the front telencephalon. In fishes, the medial part of the dorsal telencephalon is essential for the retention of the conditioned avoidance responses [zebrafish (*Danio rerio*), Lal et al. 2018]. The distribution of vasotocin receptors in this region in guppy suggests the potential role of vasotocin in the regulation of avoidance behaviour in the front part of the telencephalon.

It has been shown that the supracommissural nucleus of ventral telencephalon has a role in sexual behaviour in fish [hime salmon (*Oncorhynchus nerka*), Satou et al. 1984]. Robust vasotocin expression reported in the supracommissural nucleus of ventral telencephalon in other species (African cichlid, Rodriguez-Santiago et al. 2017) and the expression of vasotocin receptor in the same region suggests a potential role for vasotocin to regulate behaviours in this region. In fish, the medial part of the dorsal telencephalon plays an important role in the retention of the conditioned avoidance responses (zebrafish, Lal et al. 2018). Subdivisions of the lateral part of the dorsal telencephalon mediate cognitive and emotional behaviours (for review see Demski 2013) and it has been shown that lesions in this area abolish geometric spatial learning (goldfish, Vargas et al. 2006). Vasotocin expression reported in the dorsal subdivisions of the telencephalon in other species (African cichlid, Rodriguez-Santiago et al. 2017) and the expression of vasotocin receptors in the dorsal parts of the telencephalon I found in the guppy, suggests the potential involvement of vasotocin receptors in mediating behaviours via vasotocin signalling in the telencephalon.

In the diencephalon, the suprachiasmatic nucleus has a role in circadian rhythms in fish and vasotocin is highly expressed in this region (zebrafish, Noche et al. 2011). It also has been shown that the preoptic area has an important role in mediating many social behaviours in fish and vasotocin is highly expressed in this area (for review see Goodson and Bass, 2001; African cichlid, Greenwood et al. 2008). For example, vasotocin receptors were expressed in the preoptic area and hypothalamus where cells producing vasotocin and gonadotropin hormone-releasing hormone have been localized in other

species [for review see Foran and Bass, 1999; bluehead wrasse, Godwin et al. 2000; halfspotted goby (*Asterropteryx semipunctata*), Maruska et al. 2007; African cichlid, Greenwood et al. 2008]. With vasotocin receptor expression in this region, there is a potential for feedback on neuropeptide hormone-producing neurons such as gonadotropin hormone-releasing hormone and vasotocin via vasotocin receptor within the preoptic area. In fish, the habenula has a role in reproduction and socio-reproductive behaviours (for review see Ogawa and Parhar 2022) and experience-dependent modification of fear responses (zebrafish, Agetsuma et al. 2010) and the ventromedial thalamic nucleus has a role in the modulation of social learning (zebrafish, Pinho et al. 2021). However, there is a lack of knowledge on whether these two regions receive input from the vasotocin system (Robinson et al. 2019). The expression of vasotocin and its receptors in the diencephalon may indicate the regulatory role of vasotocin in this region of the brain in the guppy.

In the mesencephalon of fishes, the lateral valvular nucleus receives lateral line inputs as well as retinotopic and somatotopic inputs from trigeminal nuclei and relay the sensory information to the cerebellum (Toscano-Márquez et al. 2021). The role of the dorsal hypothalamus in fish is not well-documented. It is not known whether the vasotocin system has activity in these two regions. However, it has been shown that vasotocin fibres are present in wide regions of the brain including the hypothalamus and mesencephalon and that vasotocin neurons particularly project to the mesencephalon from the preoptic area [medaka (*Oryzias latipes*), Kagawa et al. 2016]. It is likely that the distribution of vasotocin receptors in the mesencephalon provides a linkage between the vasotocin neurons and the target areas to facilitate the regulation of behaviours via vasotocin in the mesencephalon of the brain in the guppy.

In the rhombencephalon of fishes, the central griseum is involved in social communication (for review see O'Connell and Hofmann 2011) and fear-related behaviours [lamprey (*Lampetra fluviatilis*), Olson et al. 2017]. It has been shown that gonadotropin hormone-releasing hormone and vasotocin cell populations have prominent projections to midbrain and hindbrain regions including the central griseum, but their functions remain to be clearly defined but it is possible that gonadotropin hormone-releasing hormone and vasotocin influence peripheral and central sensory and

sensorimotor processing (half-spotted goby, Maruska et al. 2007). The hindbrain has a role in regulating movements which may be involved in social approach or avoidance of stressful situations (zebrafish, Kinkhabwala et al., 2011). It has also been shown that changes in behavioural responsiveness to vasotocin are associated with changes in the expression of a V1a receptor in the hindbrain (goldfish, Walton et al. 2010). The presence of vasotocin neurons and the distribution of its receptors in the hindbrain may suggest the role of the vasotocin system in regulating motor-related behaviours in this region.

In conclusion, I examined the distribution of vasotocin receptors in the brain of guppies and found that vasotocin receptors were widely distrusted in the forebrain, midbrain, and hindbrain, including the major neuro-anatomical regions (telencephalon, diencephalon, mesencephalon, and rhombencephalon). My findings are consistent with considerable homology of vasotocin receptor expression among teleosts and suggest that the regulatory functions of vasotocin on social behaviour may be linked to the expression of their receptors in different regions of the brain. I could not characterize the distribution of isotocin receptors due to the technical limitations related to the non-specific binding of the antibody was used. I suggest considering the characterization of isotocin receptors in the brain of the guppy as a potential future study.

5.5 References

Agetsuma M, Aizawa H, Aoki T, Nakayama R, Takahoko M, Goto M, Sassa T, Amo R, Shiraki T, Kawakami K, Hosoya T, Higashijima S-i, Okamoto H (2010) The habenula is crucial for experience-dependent modification of fear responses in zebrafish. Nature Neuroscience 13:1354–1356

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

Cabrera-Álvarez MJ, Swaney WT, Reader SM (2017) Forebrain activation during social exposure in wild-type guppies. Physiology & Behavior 182:107–113

D'angelo L (2013) Brain atlas of an emerging teleostean model: *Nothobranchius furzeri*. The Anatomical Record 296:681–91

Demski LS (2013) The pallium and mind/behavior relationships in teleost fishes. Brain, Behavior and Evolution 82:31–44

Dimitriadou S, Santos EM, Croft DP, van Aerle R, Ramnarine IW, Filby AL, Darden SK (2022) Social partner cooperativeness influences brain oxytocin transcription in Trinidadian guppies (*Poecilia reticulata*). Behavioural Brain Research 423:113643

Endler JA (1995) Multiple-trait coevolution and environmental gradients in guppies. Trends in Ecology & Evolution 10:22–29

Feldman AT, Wolfe D (2014) Tissue processing and hematoxylin and eosin staining. Methods in Molecular Biology 1180:31–43

Fischer EK, Westrick SE, Hartsough L, Hoke KL (2018) Differences in neural activity, but not behavior, across social contexts in guppies, *Poecilia reticulata*. Behavioral Ecology and Sociobiology 72:131

Foran CM, Bass AH (1999) Preoptic GnRH and AVT: axes for sexual plasticity in teleost fish. General and Comparative Endocrinology 116:141-52

Godwin J, Sawby R, Warner RR, Crews D, Grober MS (2000) Hypothalamic arginine vasotocin mRNA abundance variation across sexes and with sex change in a coral reef fish. Brain, Behavior and Evolution 55:77–84

Godwin J, Thompson R (2012) Nonapeptides and social behavior in fishes. Hormones and Behavior 61:230–238

Goodson JL (2008) Nonapeptides and the evolutionary patterning of sociality. Progress in Brain Research 170:3–15

Goodson JL, Bass AH (2000) Forebrain peptides modulate sexually polymorphic vocal circuitry. Nature 403:769–772

Goodson JL, Bass AH (2001) Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain Research Reviews 35:246–265

Goodson JL, Lindberg L, Johnson P (2004) Effects of central vasotocin and mesotocin manipulations on social behavior in male and female zebra finches. Hormones and Behavior 45:136–143

Goodson JL, Schrock SE, Klatt JD, Kabelik D, Kingsbury MA (2009) Mesotocin and nonapeptide receptors promote estrildid flocking behavior. Science 325:862–866

Goodson JL, Thompson RR (2010) Nonapeptide mechanisms of social cognition, behavior and species-specific social systems. Current Opinion in Neurobiology 20:784–794

Greenwood AK, Wark AR, Fernald RD, Hofmann HA (2008) Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. Proceedings of the Royal Society B 275:2393–2402

Hausmann H, Meyerhof W, Zwiers H, Lederis K, Richter D (1995) Teleost isotocin receptor: structure, functional expression, mRNA distribution and phylogeny. FEBS Letters 370:227–230

Houde AE (1997) Sex, color, and mate choice in guppies. Princeton University Press, Princeton

Huffman LS, O'Connell LA, Kenkel CD, Kline RJ, Khan IA, Hofmann HA (2012) Distribution of nonapeptide systems in the forebrain of an African cichlid fish, *Astatotilapia burtoni*. Journal of Chemical Neuroanatomy 44:86–97

Iwasaki K, Taguchi M, Bonkowsky JL, Kuwada JY (2013) Expression of arginine vasotocin receptors in the developing zebrafish CNS. Gene Expression Patterns 13:335–342

Kagawa N, Honda A, Zenno A, Omoto R, Imanaka S, Takehana Y, Naruse K (2016) Arginine vasotocin neuronal development and its projection in the adult brain of the medaka. Neuroscience Letters 613:47–53

Kihlström JE, Agmo A (1974) Some effects of vasopressin on sexual behaviour and seminal characteristics in intact and castrated rabbits. Journal of Endocrinology 60:445–453

Kinkhabwala A, Riley M, Koyama M, Monen J, Satou C, Kimura Y, Higashijima S, Fetcho J (2011) A structural and functional ground plan for neurons in the hindbrain of zebrafish. PNAS 108:1164–1169

Kline RJ, O'Connell LA, Hofmann HA, Joan Holt G, Khan IA (2011) The distribution of an AVT V1a receptor in the brain of a sex changing fish, *Epinephelus adscensionis*. Journal of Chemical Neuroanatomy 42:72–88

Lal P, Tanabe H, Suster ML, Ailani D, Kotani Y, Muto A, Itoh M, Iwasaki M, Wada H, Yaksi E, Kawakami K (2018) Identification of a neuronal population in the telencephalon essential for fear conditioning in zebrafish. BMC Biology 16:45

Lema SC, Slane MA, Salvesen KE, Godwin J (2012) Variation in gene transcript profiles of two V1a-type arginine vasotocin receptors among sexual phases of bluehead wrasse (*Thalassoma bifasciatum*). General and Comparative Endocrinology 179:451–464

Leung CH, Abebe DF, Earp SE, Goode CT, Grozhik AV, Mididoddi P, Maney DL (2011) Neural distribution of vasotocin receptor mRNA in two species of songbird. Endocrinology 152:4865–4881

Loveland JL, Fernald RD (2017) Differential activation of vasotocin neurons in contexts that elicit aggression and courtship. Behavioural Brain Research 317:188–203

Magurran AE (2005) Evolutionary ecology: the Trinidadian guppy. Oxford University Press, Oxford

Maruska KP, Mizobe MH, Tricas TC (2007) Sex and seasonal co-variation of arginine vasotocin (AVT) and gonadotropin-releasing hormone (GnRH) neurons in the brain of the halfspotted goby. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 147:129–144

Noche RR, Lu P-N, Goldstein-Kral L, Glasgow E, Liang JO (2011) Circadian rhythms in the pineal organ persist in zebrafish larvae that lack ventral brain. BMC Neuroscience 12:7

O'Connell LA, Hofmann, HA (2011) The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. The Journal of Comparative Neurology 519:3599–3639

Ogawa S, Parhar IS (2022) Functions of habenula in reproduction and socio-reproductive behaviours. Frontiers in Neuroendocrinology 64:100964

Olazábal DE, Young LJ (2006) Oxytocin receptors in the nucleus accumbens facilitate "spontaneous" maternal behavior in adult female prairie voles. Neuroscience 141:559–568

Olson I, Suryanarayana SM, Robertson B, Grillner S (2017) *Griseum centrale*, a homologue of the periaqueductal gray in the lamprey. IBRO Reports 2:24–30

Ostrowski NL, Lolait SJ, Young WS III (1994) Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. Endocrinology 135:1511–1528

Pinho J, Cunliffe V, Petri G, Oliveira RF (2021) Social and asocial learning in zebrafish are encoded by a shared brain network that is differentially modulated by local activation. Preprint version: https://doi.org/10.1101/2021.10.12.464038

Reddon AR, Aubin-Horth N, Reader SM (2022) Wild guppies from populations exposed to higher predation risk exhibit greater vasotocin brain gene expression. Journal of Zoology 316:118–127

Robinson KJ, Bosch OJ, Levkowitz G, Busch KE, Jarman AP, Ludwig M (2019) Social creatures: model animal systems for studying the neuroendocrine mechanisms of social behaviour. Journal of Neuroendocrinology 31:e12807

Rodriguez-Santiago M, Nguyen J, Winton LS, Weitekamp CA, Hofmann HA (2017). Arginine vasotocin preprohormone is expressed in surprising regions of the teleost forebrain. Frontiers in Endocrinology 8:195

Saito D, Komatsuda M, Urano A (2004) Functional organization of preoptic vasotocin and isotocin neurons in the brain of rainbow trout: central and neurohypophysial projections of single neurons. Neuroscience 124:973–984

Satou M, Oka Y, Kusunoki M, Matsushima T, Kato M, Fujita I, Ueda K (1984) Telencephalic and preoptic areas integrate sexual behavior in hime salmon (landlocked red salmon, *Oncorhynchus nerka*): results of electrical brain stimulation experiments. Physiology & Behavior 33:441–447

Stoop R (2012) Neuromodulation by oxytocin and vasopressin. Neuron 76:142–159

Thompson RR, Walton JC (2004) Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*). Behavioral Neuroscience 118:620–626

Toscano-Márquez B, Oboti L, Harvey-Girard E, Maler L, Krahe R. (2021) Distribution of the cholinergic nuclei in the brain of the weakly electric fish, *Apteronotus leptorhynchus*: implications for sensory processing. Journal of Comparative Neurology 529:1810–1829

Vargas J P, Bingman VP, Portavella M, López JC (2006) Telencephalon and geometric space in goldfish. European Journal of Neuroscience 24:2870–2878

Walton JC, Waxman B, Hoffbuhr K, Kennedy M, Beth E, Scangos J, Thompson RR (2010) Behavioral effects of hindbrain vasotocin in goldfish are seasonally variable but not sexually dimorphic. Neuropharmacology 58:126–134

Chapter 6. General Discussion

The evolution of sociality represents one of the most important questions in behavioural biology (Székely et al. 2010). For example, why do some species show a complex social association with conspecifics, while other closely related species or even populations of the same species do not? To answer this question, it is crucial that we develop a comprehensive understanding of the mechanisms underlying social behaviours. To date, there is no precise and convincing answer to this question due to the complexity of social behaviours, and the unknown proximate mechanisms that generate such behaviours. Having a comprehensive research approach using probable neurological candidates involved in regulation of social behaviours along with an excellent model organism, could put light on some dark aspects of social behaviours. Here I provide such an approach, investigating the role of brain nonapeptides in the regulation of shoaling behaviour across populations and sexes. This was done using the administration of vasotocin and isotocin, the examination of vasotocin and isotocin immunoreactivity in the brain, and the characterization of the distribution of vasotocin and isotocin immunoreactive receptors in the brain.

Shoaling behaviour

Shoaling fishes benefit from antipredator advantages through several group defensive mechanisms such as enhanced vigilance, risk dilution, predator confusion and coordinated evasive maneuvers (Dugatkin and Godin 1992). Differences in predation intensity have been hypothesized to cause evolutionary differences in anti-predator responses across populations (Magurran et al. 1993). Consistent with this hypothesis, I found that guppies (*Poecilia reticulata*) from the high-predation Lower Aripo population spent more time shoaling with conspecifics than guppies from the low-predation Paria population. My result matches previous comparisons of shoaling behaviour in guppy (Magurran and Seghers 1994a; Seghers 1974) as well as in other fishes such as the banded killifish (*Fundulus diaphanus*) (Krause and Godin 1994). Furthermore, because I used a common garden-type experiment, my study provides evidence to support previous

findings that these differences in anti-predator response have a genetic component, so may have evolved as an adaptation to increase survival (see Huizinga et al. 2009; Magurran et al. 1993; Seghers 1974). A future study on the genetics of shoaling behaviour would provide an opportunity to confirm my explanation about the genetic basis of shoaling behaviour in this species as well as identify specific genes underlying this behaviour.

Stress could induce changes in shoaling behaviour (Kleinhappe et al. 2019). In my study, the number of transitions among association zones, a measure that may be associated with anxiety or exploratory behaviour, did not differ between the Lower Aripo and Paria populations. Previous comparisons of populations that differ in predation intensity have shown mixed results, with high predation associated with both increased activity (Archard and Braithwaite 2011) and decreased activity (Ioannou et al. 2017). Given the many methodological differences in how exploration and activity have been measured, this behavioural variation might relate to the specifics of the experimental apparatus, which may alternatively favour either curiosity to explore the test environment or anxiety to escape the test environment.

Grouping behaviour is sex-biased in many species. For example, female mammals and male birds have been shown to form more stable groups that disperse less than the opposite sex (see Greenwood 1980). It has been suggested that the sex-bias in these taxa is driven at least partly by an asymmetry in the fitness benefits derived from group fidelity in which female mammals and male birds gain a greater fitness benefit from grouping behaviour than their opposite sexes (Greenwood 1980; Pusey 1987). In fish, one important aspect of shoaling behaviour that has been less well studied is the effect of sex. Many studies disregard sex, either by examining only one sex, or by ignoring the sex of the fish in the behavioural trials (Snekser et al. 2010). Sexually dimorphic behaviours across species appear to be driven in part by sexual conflict and associated with sex-specific variation in cognition. For instance, poeciliid females have evolved sets of behaviours that reduce male harassment, including greater shoaling tendencies and risk sensitivities than males (Cummings 2018). My results showed that females from both the Lower Aripo and Paria populations spent more time with conspecifics than males. Similar results have been shown in guppies (Magurran and Seghers 1994b; Magurran et

al. 1992) as well as in other poeciliid species [mosquitofish (*Gambusia holbrooki*) and *Girardinus falcatus*; see Dadda 2015]. It has been suggested that female guppies gain a greater benefit from shoaling than males because of reduced predation threat by forming the core of a shoal and reduced sexual harassment from males by forming shoals predominantly with other females (Griffiths and Magurran 1998; Magurran and Seghers 1994b). Furthermore, male guppies may also pay a higher opportunity cost to shoaling than females through reduced mating opportunities (Croft et al. 2003; Griffiths and Magurran 1998). Male guppies benefit by encountering new females, which motivates them to explore the environment actively, frequently joining and splitting different shoals to find new mating opportunities (Croft et al. 2003). Regardless, my study confirms previous observations that female guppies have a greater propensity to form shoals than male guppies. As this study was conducted on individuals bred under captive conditions for many generations, the generality of these results for wild populations remains to be established.

Vasotocin and shoaling behaviour across populations

Vasopressin and vasotocin have been hypothesized to influence social interactions and grouping behaviours within and between individuals, between the sexes, and between species across taxa, however, previous studies have reported inconsistent results including enhancing, inhibiting, or no effect (for review see Godwin and Thompson 2012 and Goodson 2013). I used three experimental approaches to understand the contribution of vasotocin to variation in shoaling behaviour among different populations and sexes in guppies including administration of vasotocin and its antagonist to guppies in order to manipulate the nonapeptide levels, characterization of vasotocin neurons and its receptors using immunocytochemistry to understand the distribution of vasotocin and its receptors in the brain as part of neuronal pathways in regulating shoaling behaviour in the guppy.

First, I administered vasotocin, a vasotocin antagonist, and saline as control to a population of guppy (Paria). I showed that the time spent associating with conspecifics did not differ among fish that were administered vasotocin, a vasotocin antagonist, or a saline control. I found that guppies injected with vasotocin performed more transitions across zones than fish injected with either the vasotocin antagonist or saline. In contrast

to my findings, other studies found that both vasotocin and a vasotocin antagonist reduced the proportion of time spent shoaling with conspecifics [zebrafish (*Danio rerio*), Lindeyer et al. 2015], vasotocin administration increased the proportion of time spent associating with conspecifics, whereas a vasotocin antagonist had the opposite effect (zebrafish, Braida et al. 2012), and vasotocin administration was consistently associated with reduced time spent in social proximity to a conspecific [goldfish (*Carassius auratus*), Thompson et al. 2008]. This variation of effects suggests that the inconsistency in the results among studies might be related to the different dosage of vasotocin and its antagonist being administered, the route of vasotocin administration, or could be related to species-specific characteristics. Given my results and the variation observed across previous studies, there is limited evidence that vasotocin is a key mediator of grouping behaviours in fishes. Considering a wider range of dosage than used in previous studies, using other routes of administration such as intracerebroventricular administration, and designing a comparative study would help test this hypothesis. Another possible explanation for the absence of an effect of vasotocin on shoaling, could be related to the variation in vasotocin receptor subtypes that are expressed in the guppy brain. Different vasotocin receptor subtypes could have different actions in the neuronal pathway involved in regulating social behaviour in fish and the interaction of injected vasotocin with each of these subtypes or number of them could be the origin of mixed results in studies. Across studies, there is limited evidence that vasotocin influences shoaling behaviour. Maybe vasotocin is not involved in the regulating process of shoaling behaviour.

Since vasopressin and vasotocin enhance the corticotropin-releasing hormone (CRH) activation of the hypothalamus-pituitary–adrenocortical (HPA), they have previously been linked to an increase in the expression of behaviours related to stress and anxiety (for review see de Kloet 2010; Zelena et al. 2015). My findings on the increase of transitions across zones of two choice test apparatus suggest that stress and anxiety-related behaviours may be triggered by vasotocin in guppies, as hyperactivity is frequently associated with stress and anxiety in fishes (Schreck et al. 1997). My finding was consistent with a study in another fish (zebrafish, Lindeyer et al. 2015) but was different from other studies in fishes (zebrafish, Braida et al. 2012; goldfish, Araishi et al.

2019; Thomson and Walton 2004). I suggest this variation among studies may reflect methodological variation in how exploration and activity have been measured and the specifics of the experimental apparatus. For example, the study that similarly showed an effect of vasotocin on activity (Lindeyer et al. 2015) also compared activity in the presence of a group of six versus no conspecifics, providing a clear contrast between a group and non-group choice which may alternatively favour either curiosity to explore the test environment to find conspecifics or anxiety to escape the test environment. In contrast, studies that did not find a relationship between vasotocin and activity examined activity in the presence of groups that differed in body colouration (Braida et al. 2012), or groups of different sizes (Thompson and Walton 2004), or used a different experimental method and apparatus (Araishi et al. 2019). It is also possible that the hyperactivity I observed after vasotocin administration could be due to peripheral effects of vasotocin such as increased heart rate and blood pressure (Le Mevel et al. 1993), osmoregulatory changes (Babiker and Rankin 1980), or the stress response (Gesto et al. 2014). Because I cannot separate peripheral from central effects of vasotocin in my study, I can not confirm this hypothesis. Further studies will be needed to distinguish between peripheral and central effects of vasotocin on activity. Regardless, my vasotocin administration study adds to growing evidence that vasotocin can be associated with hyperactivity in fishes, which is likely a consequence of its relationship to stress and anxiety. I suggest a future study to investigate the interaction between vasotocin and hypothalamic–pituitary–adrenal axis to clarify the anxiolytic effect of vasotocin in guppy and my explanations for the results of my injection study.

Second, I examined the relative fluorescence intensity of vasotocin neurons in the preoptic area to examine the contribution of vasotocin neurons to shoaling behaviour in two population of guppies, one from a high-predation regime (Lower Aripo) and the other from a low-predation regime (Paria). I found that the relative fluorescence intensity of vasotocin was higher in the dorsal preoptic area (gigantocellular neurons) of the individuals from the Lower Aripo population than the Paria population, suggesting a possible link between these neurons and differences in the affiliative shoaling behaviour. Indeed, a recent study showed findings consistent with my findings that vasotocin transcripts were more abundant in the brains of guppies from high-predation populations

than from low-predation populations (Reddon et al. 2022). However, in my study there were no differences in the fluorescence intensity of vasotocin between sexes despite higher shoaling in females than males, and at the level of individual fish there was no correlation between the fluorescence intensity of vasotocin and shoaling behaviour. To resolve this apparent inconsistency, I propose that population differences in vasotocin levels may be unrelated to affiliative behaviour, but instead may relate to fear and anxiety aspects of anti-predatory behaviour. Studies in fishes have shown high vasotocin expression associated with fear conditioning [African cichlid fish (*Astatotilapia burtoni*), Rodriguez-Santiago et al. 2017; Indo-Pacific blonde naso tang (*Naso elegans*), Soares et al. 2017]. In my study, experimental injections of vasotocin were not associated with a difference in affiliative behaviour but did lead to hyperactivity that was inferred to be associated with fear and anxiety. Although more research is needed, taken together, these data suggest that vasotocin levels in the brain are higher in high-predation populations, consistent with fear as the underlying motivation.

The cause-and-effect relationship between neuronal phenotypes and social behaviour is not necessarily straightforward and there are still many more that have not been identified nor studied (Chen and Hong 2018). For example, preoptic neuronal phenotype function may vary among different life stages (Foran and Bass 1998), or early life experience in a social group or presence of predators could have organizational interactions with neuronal system (Antunes et al. 2021). Controlled developmental experiments in the laboratory will be required to disentangle these possibilities and conclusively rule out plastic differences in nonapeptide neurons in guppies.

Finally, I examined the distribution of vasotocin immunoreactive receptors in the main neuroanatomical regions of the brain. Apart from some variation was observed in the distributions of the receptors within regions, my findings on the distribution of vasotocin receptors (V1a1) in the brain of guppies, were consistent with the distribution of vasotocin receptors in the similar regions of the brain in rock hind (*Epinephelus adscensionis*) (V1a2, Kline et al., 2011), African cichlid (V1a2, Huffman et al., 2012; V1a1 and V1a2, Loveland and Fernald, 2017), and bluehead wrasse (*Thalassoma bifasciatum*) (V1a1 and V1a2, Lema et al., 2012). The considerable homology of vasotocin receptors distribution across species suggests the potential for homology in

function. For example, the expression of vasotocin in the olfactory bulb that has been previously reported in other species [Mozambique tilapia (*Oreochromis mossambicus*), Almeida et al. 2012] suggests the expression of vasotocin receptors that I found in the olfactory bulbs in guppy might be linked to the regulation of odour-related behaviours via vasotocin in the olfactory bulbs. The similarity of vasotocin receptor distribution in the telencephalon across species also may suggest a potential role for vasotocin in mediating behaviours such as regulation of cognition that usually occurs in the telencephalon (Cheng et al. 2014). A future study is needed to examine the vasotocin levels in the connected brain regions to fill the gap between vasotocin receptor distribution and vasotocin levels in each region in guppies.

Given that vasotocin administration to different species showed mixed results, I propose that behavioural effects of vasotocin could be partly mediated via different vasotocin receptor subtypes that could affect variety of pathways within the brain activating or suppressing the behavioural responses. Indeed, it has been shown that the administration of vasotocin in fish to alter behaviour has differing effects with differing reproductive strategies. Vasotocin increases courtship behaviour and aggression in the bluehead wrasse and white perch (*Morone americana*) (Salek et al. 2002; Semsar et al. 2001), whereas inhibits male-specific behaviour in damselfish (*Stegastes leucostictus*) and pupfish (*Cyprinodon nevadensis*) (Lema and Nevitt 2004; Santangelo and Bass 2006). Vasotocin has also shown mixed effects on direct measures of association behaviour. For example, when vasotocin was administered to goldfish it led to reduced time spent in proximity with a same-sex conspecific (Thompson and Walton 2004; Thompson et al. 2008). In zebrafish, administration of either vasotocin or a vasotocin-antagonist reduced the proportion of time spent shoaling with a female conspecific relative to the administration of saline (Lindeyer et al. 2015). In another study of zebrafish, vasotocin administration increased the proportion of time spent associating with a phenotypically similar strain relative to a phenotypically dissimilar strain, whereas a vasotocin antagonist had the opposite effect (Braida et al. 2012). In my vasotocin administration experiment, I also found vasotocin and a vasotocin antagonist administration had no significant effect on time spent associating with conspecifics in the guppy. A study on the expression of mRNA and protein of different vasotocin receptor

subtypes in the guppy brain would be beneficial to understand the mechanism underlying the plasticity of the vasotocin system in this species.

Isotocin and shoaling behaviour across populations

A growing body of research has linked oxytocin and its receptor to non-reproductive social behaviours, including affiliation and the suppression of social anxiety (MacDonald and MacDonald 2010). Oxytocin and its homologues have been reported to regulate social behaviour, generally acting as a pro-social modulator in mammals and birds (for review see Goodson 2013). Studies in fish, on the other hand, have provided little support for pro-social effects of isotocin (e.g., Braida et al. 2012; Reddon et al. 2014; Lindeyer et al. 2015; Ramsey et al. 2019). To understand he contribution of isotocin to variation in shoaling behaviour among different populations and sexes, I used three experimental approaches including administration of isotocin and its antagonist to guppies in order to manipulate isotocin levels, characterization of isotocin neurons and its receptors using immunocytochemistry to understand the distribution of isotocin and its receptors in the brain as part of neuronal pathways in regulating shoaling behaviour in guppy.

First, I injected the guppies form the population Paria with isotocin and an isotocin antagonist. I found that the time spent in proximity to conspecifics was higher in guppies that received isotocin than in guppies that received an isotocin antagonist. Several factors might explain why my results contrast with earlier studies: 1- the pro-social effects of isotocin may be dose-dependent. In zebrafish, Braida et al. (2012) using dosage that were much lower than my study found that the effects of isotocin on social behaviour have a non-linear relationship with dosage. In another study on the same fish, Lindeyer et al. (2015) used the dosage similar to my dosage and found no effect of isotocin on association behaviour. Nevertheless, I propose that only a specific dosage range will elicit social behaviour in a particular species of fish. 2- the variation in social behaviour being measured and its context might be another reason underlying the differences between my study and other studies. Indeed, in contrast to my study, previous studies that found no pro-social effect of isotocin were not directly differentiating a pro-social to non-social behaviour. For example, they compared association with groups of

different sizes (Thompson and Walton 2004; Reddon et al. 2014) or groups that differed in phenotype such as body colouration (Braida et al. 2012). My study instead provided a clear contrast between a pro-social and non-social choice using a group of six versus no conspecifics to compare association. Regardless, using administration approach, I presented some of the first evidence on pro-social effect of isotocin and suggest that isotocin has a homologous role to oxytocin in promoting social behaviour. However, I suggest future studies using a wider range of isotocin dosage and different antagonists to find a specific effective dosage of this nonapeptide. I also suggest using different experimental designs to assess context-dependent measurements to evaluate effects of isotocin on social behaviour in guppies.

Second, I examined the relative fluorescence intensity of isotocin neurons in the preoptic area to examine the potential contribution of isotocin neurons in the preoptic area of the brain to shoaling behaviour in two population of guppies (Lower Aripo and Paria). I found no significant difference in isotocin intensity between the two populations across any of the three regions of the preoptic area. Previous research on isotocin neurons and social behaviour has suggested that parvocellular isotocin neurons affect affiliation in fishes (e.g., O'Connell et al. 2012; Reddon et al. 2017). Although I found weak specific antibody binding in the isotocin sections that might hinder my ability to observe significant differences, overall there is weak evidence of isotocin involvement in population and sex differences in guppies. Consistent with my results, it has recently been shown that isotocin transcript levels did not differ between high-predation and low-predation populations in another study of guppies (Reddon et al. 2022). As I described in my injection work, I found a pro-social effect of isotocin injection on shoaling behaviour in guppies. Indeed, it is conceivable that isotocin neurons have an effect in other regions of the brain. For example, the isotocin neurons have projections from the preoptic area into the extra-hypothalamic regions in other fishes (Goodson et al. 2003; Saito et al. 2004). The hindbrain, for example, has sensory-motor functions such as regulating swimming (Kinkhabwala et al. 2011) and swimming is involved in predator avoidance and moving to be proximate to conspecifics. I suggest a future study to characterize the distribution of isotocin neurons in the extra-hypothalamic regions from forebrain to hindbrain to clarify this hypothesis in guppies.

Finally, I tried to examine the distribution of isotocin immunoreactive receptors in the main neuroanatomical regions of the brain. I used a mammalian OTR antibody since mammalian oxytocin receptors and fish isotocin receptors are closely related to one another with a high similarity (Hausmann et al. 1995). However, I did not observe strong specific binding for isotocin receptor sections due to the technical limitation. This result might partly be related to the region of the receptor that my OTR antibody recognized. Regardless of the cause, it seems OTR antibodies are not interchangeable, and in the absence of commercially available ITR antibodies OTR antibodies may need to be chosen carefully for use in fishes. I suggest considering the characterization of isotocin receptors in the brain of the guppy as a potential future study.

Sex-specific effects of nonapeptides

In my study, I found limited evidence for sex-specific effects of vasotocin on either the time spent associating with conspecifics or the frequency of transitions among experimental zones. Although vasotocin appeared to have a larger influence on the number of transitions among experimental zones in females than males, the interaction term was not significant and both sexes showed a trend in the same direction. It is likely that vasotocin effect is no sex-specific in guppies because both association with conspecifics and exploratory behaviours are expressed in both sexes. Similarly, Thompson et al. (2008) found that both males and females in goldfish associate with conspecifics, and vasotocin effect on the time spent in proximity with a same-sex conspecific was not sex-specific. The sex-specific responses to vasotocin have been shown generally for behaviours that are expressed by only one sex or otherwise have strong sex-specific differences in expression [e.g., fictive vocalization in plainfin midshipman (*Porichthys notatus*), Goodson and Bass 2000; aggression in bluehead wrasse, Semsar and Godwin 2004]. My results suggest that sex-specific differences in the behavioural effects of vasotocin occur only when vasotocin mediates behaviours with strong sex-specific differences in expression.

I also found the pro-social effects of isotocin administration did not differ between sexes, with both male and female guppies showing greater shoaling after administration of isotocin than administration of the isotocin antagonist. In contrast to my

findings, mosquitofish (*Gambusia affinis*) showed sex-specific differences following isotocin administration, with males having reduced association with conspecifics following isotocin administration and females having no difference in association with conspecifics (Ramsey et al. 2019). Sex-specific responses to isotocin were also described in plainfin midshipman in which isotocin suppresses vocalizations in females but not males that acoustically court females (Goodson and Bass 2000). It remains unknown how the response to isotocin differs between sexes.

Finally, I found no difference between the sexes in either vasotocin or isotocin relative fluorescence intensity, regardless of region of preoptic area. It seems neuronal phenotypes in the preoptic area show sex-specific pattern dependent to the social context. For example, Almeida and Oliveira (2015) showed that preoptic vasotocin neuronal phenotypes shows sex-biased pattern in a cichlid (Mozambique tilapia) with dominant-subordinate social status.

Given vasotocin and isotocin functions, the pattern of sex differences in the central expression of these nonapeptides are likely to vary depending on the species and the social context of the behaviour being measured. In mammals, vasopressin and oxytocin systems often modulate social behaviors in sex-specific manner. In general, vasopressin/vasotocin is more extensively associated with male behaviour than with female behaviour. In contrast, females are behaviorally less sensitive to vasopressin/vasotocin but sensitive to oxytocin-like peptides (for review see Goodson and Bass 2001). This pattern arises in part because vasopressin and its V1aR in the brain are typically higher in males than in females whereas oxytocin is higher in females while its receptor is higher in males across species (for review see Dumais and Veenema 2016). This pattern is not confirmed yet and needs to be studied in both sexes in guppies. The results of such studies will help better understanding of vasotocin- and isotocin-driven mechanisms underlying social behaviours.

Conclusion

My results confirmed the findings of previous studies on the variation between populations and sexes in shoaling behaviour in guppy with individuals from high-predation regime show more tendency to shoal with their same-sex conspecifics than

individuals from low-predation regime and that females show high propensity to shoal with conspecifics than males. My study also confirmed the contribution of vasotocin and isotocin to the variations between populations and sexes.

My results suggest that vasotocin is unlikely to directly mediate the population differences in shoaling, as neither vasotocin nor its antagonist influenced shoaling when administered. Instead, because population differences in shoaling have previously been associated with predation risk (Magurran and Seghers 1994a), it may be that population differences in vasotocin are related to the stress and anxiety functions of this hormone. Indeed, vasotocin action is linked to fear-based conditioning such as learning to avoid predation (Rodriguez-Santiago et al. 2017; Soares et al. 2017).

Isotocin is a more likely to contribute to the differences in shoaling behaviour in guppies as I showed that isotocin administration had a positive effect on shoaling in guppies, and isotocin transcript levels in the brain have been shown to respond to predator cues in guppies from high-predation populations but not low-predation populations (Dimitriadou et al. 2022). However, isotocin transcript levels did not differ between high-predation and low-predation populations in another study of guppies (Reddon et al. 2022), so the precise nature of the mechanistic link between isotocin and shoaling behaviour also remains unresolved. My study provides some of the first evidence of a pro-social effect of isotocin in a fish, and also suggests that isotocin and its antagonist affect the behaviour of both females and males similarly. My findings about isotocin administration and shoaling in guppies contrast with some earlier studies on the effect of isotocin in fishes. Several factors might explain this contrast, including differences in dosage, observed behaviour, or the context-specific effects of isotocin.

Finally, I examined the distribution of vasotocin receptors in the brain of guppies and found that vasotocin receptors were widely distrusted in the major neuro-anatomical regions of the brain including telencephalon, diencephalon, mesencephalon, and rhombencephalon. Consistent with previous studies in other species (Kline et al., 2011; Huffman et al., 2012; Lema et al., 2012; Loveland and Fernald, 2017), my findings suggest that the expression of vasotocin receptors in the regions that are involved in sensory information process (e.g., olfaction or vision) might indicate the involvement of vasotocin system in regulating behaviours and that vasotocin effects on behaviours might

be linked partially to the expression of its receptors in the brain. Future studies should aim to examine the characterization of isotocin receptors distribution in the brain regions in guppies since my study could not find evidence on contribution of isotocin receptors to social behaviours due to the technical limitations.

Current knowledge concerning vasopressin and oxytocin and their receptors indicate that they are parts of an integrated regulating pathway. Carter (2017) termed this pattern as "vasopressin-oxytocin" pathway and hypothesized that vasopressin and isotocin interact with their receptors to influence affiliative and aggressive behaviours. Oxytocin, in particular, rarely acts alone but acts with vasopressin via the vasopressin receptors or through effects on both the oxytocin receptor and vasopressin receptors. In safety, oxytocin predominately acts on the oxytocin receptor facilitating affiliation and social engagement whereas under stress oxytocin may function primarily through effects on the vasopressin receptors promoting aggression and social avoidance. That said, the results of my study suggesting a possibility of vasotocin-isotocin interactions rather than vasotocin or isotocin action alone in regulating shoaling behaviour in guppies. Overall, for interpretation of the results on the effects of vasotocin and isotocin on social behaviours, other factors such as species-specific conditions or experimental situations under that the shoaling behaviour is being measured should be considered. Future studies should examine the interaction of vasotocin and isotocin and their receptors to fully characterize the contributions of nonapeptide to shoaling behaviour in guppies.

6.1 References

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

Almeida O, Oliveira RF (2015) Social status and arginine vasotocin neuronal phenotypes in a cichlid fish. Brain, Behavior and Evolution 85:203–213

Antunes DF, Teles MC, Zuelling M, Friesen CN, Oliveira RF, Aubin-Horth N, Taborsky B (2021) Early social deprivation shapes neuronal programming of the social decision-making network in a cooperatively breeding fish. Molecular Ecology 30:4118–4132

Araishi K, Watanabe K, Yamazaki T, Nakamachi T, Matsuda K (2019) Intracerebroventricular administration of arginine vasotocin (AVT) induces anorexigenesis and anxiety-like behavior in goldfish. Peptides 119:170118

Archard GA, Braithwaite VA (2011) Increased exposure to predators increases both exploration and activity level in *Brachyrhaphis episcopi*. Journal of Fish Biology 78:593–601

Babiker MM, Rankin JC (1980) Diuretic and antidiuretic actions of AVT and isotocin in the teleost fish *Clarias lazera*. General and Comparative Endocrinology 40:337

Braida D, Donzelli A, Martucci R, Capurro V, Busnelli M, Chini B, Sala M (2012) Neurohypophyseal hormones manipulation modulate social and anxiety-related behavior in zebrafish. Psychopharmacology 220:319–330

Carter CS (2017) The oxytocin–vasopressin pathway in the context of love and fear. Frontiers in Endocrinology 8:356

Chen P, Hong W (2018) Neural circuit mechanisms of social behavior. Neuron 98:16–30

Cheng R-K, Jesuthasan SJ, Penney TB (2014) Zebrafish forebrain and temporal conditioning. Philosophical Transactions of the Royal Society B 369:20120462

Croft DP, Arrowsmith BJ, Bielby J, Skinner K, White E, Couzin ID, Magurran AE, Ramnarine I, Krause J (2003) Mechanisms underlying shoal composition in the Trinidadian guppy, *Poecilia reticulata*. OIKOS 100:429–438

Cummings ME (2018) Sexual conflict and sexually dimorphic cognition - reviewing their relationship in poeciliid fishes. Behavioral Ecology and Sociobiology 72:73

Dadda M (2015) Female social response to male sexual harassment in poeciliid fish: a comparison of six species. Frontiers in Psychology 6:1453

de Kloet ER (2010) From vasotocin to stress and cognition. European Journal of Pharmacology 626:18–26

Dimitriadou S, Santos EM, Croft DP, van Aerle R, Ramnarine IW, Filby AL, Darden SK (2022) Social partner cooperativeness influences brain oxytocin transcription in Trinidadian guppies (*Poecilia reticulata*). Behavioural Brain Research 423:113643

Dugatkin LA, Godin JGJ. (1992) Predator inspection, shoaling and foraging under predation hazard in the Trinidadian guppy, *Poecilia reticulata*. Environmental Biology of Fishes 34:265–276

Dumais KM, Veenema AH (2016) Vasopressin and oxytocin receptor systems in the brain: Sex differences and sex-specific regulation of social behavior. Frontiers in Neuroendocrinology 40:1–23

Foran CM, Bass AH (1998) Preoptic AVT immunoreactive neurons of a teleost fish with alternative reproductive tactics. General and Comparative Endocrinology 111:271–282

Gesto M, Soengas JL, Rodríguez-Illamola A, Míguez JM (2014) Arginine vasotocin treatment induces a stress response and exerts a potent anorexigenic effect in rainbow trout, *Oncorhynchus mykiss*. Journal of Neuroendocrinology 26:89–99

Godwin J, Thompson R (2012) Nonapeptides and social behavior in fishes. Hormones and Behavior 61:230–238

Goodson JL (2013) Deconstructing sociality, social evolution and relevant nonapeptide functions. Psychoneuroendocrinology 38:465–478

Goodson JL, Bass AH (2000) Forebrain peptides modulate sexually polymorphic vocal circuitry. Nature 403:769–772

Goodson JL, Bass AH (2001) Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain Research Reviews 35:246–265

Goodson JL, Evans AK, Bass AH (2003) Putative isotocin distributions in sonic fish: relation to vasotocin and vocal-acoustic circuitry. The Journal of Comparative Neurology 462:1–14

Greenwood PJ (1980) Mating systems, philopatry and dispersal in birds and mammals. Animal Behaviour 28:1140–1162

Griffiths SW, Magurran AE (1998) Sex and schooling behaviour in the Trinidadian guppy. Animal Behaviour 56:689–693

Hausmann H, Meyerhof W, Zwiers H, Lederis K, Richter D (1995) Teleost isotocin receptor: structure, functional expression, mRNA distribution and phylogeny. FEBS Letters 370:227–230

Huffman LS, O'Connell LA, Kenkel CD, Kline RJ, Khan IA, Hofmann HA (2012) Distribution of nonapeptide systems in the forebrain of an African cichlid fish, *Astatotilapia burtoni*. Journal of Chemical Neuroanatomy 44:86–97

Huizinga M, Ghalambor CK, Reznick DN (2009) The genetic and environmental basis of adaptive differences in shoaling behaviour among populations of Trinidadian guppies, *Poecilia reticulata*. Journal of Evolutionary Biology 22:1860–1866

Ioannou CC, Ramnarine IW, Torney, CJ (2017) High-predation habitats affect the social dynamics of collective exploration in a shoaling fish. Science Advances 3:e1602682

Kinkhabwala A, Riley M, Koyama M, Monen J, Satou C, Kimura Y, Higashijima S, Fetcho J (2011) A structural and functional ground plan for neurons in the hindbrain of zebrafish. PNAS 108:1164–1169

Kleinhappe TK, Pike TW, Burman OHP (2019) Stress-induced changes in group behaviour. Scientific Reports 9:17200

Kline RJ, O'Connell LA, Hofmann HA, Joan Holt G, Khan IA (2011) The distribution of an AVT V1a receptor in the brain of a sex changing fish, *Epinephelus adscensionis*. Journal of Chemical Neuroanatomy 42:72–88

Krause J, Godin JGJ (1994) Shoal choice in the banded killifish (*Fundulus diaphanus*, Teleostei, Cyprinodontidae): effects of predation risk, fish size, species composition and size of shoals. Ethology 98:128–136

Le Mevel JC, Pamantung TF, Mabin D, Vaudry H (1993) Effects of central and peripheral administration of arginine vasotocin and related neuropeptides on blood pressure and heart rate in the conscious trout. Brain Research 610:82–89

Lema SC, Nevitt GA (2004) Exogenous vasotocin alters aggression during agonistic exchanges in male Amargosa river pupfish (*Cyprinodon nevadensis amargosae*). Hormones and Behavior 46:628–637

Lema SC, Slane MA, Salvesen KE, Godwin J (2012) Variation in gene transcript profiles of two V1a-type arginine vasotocin receptors among sexual phases of bluehead wrasse (*Thalassoma bifasciatum*). General and Comparative Endocrinology 179:451–464

Lindeyer CM, Langen EMA, Swaney WT, Reader SM (2015) Nonapeptide influences on social behaviour: effects of vasotocin and isotocin on shoaling and interaction in zebrafish. Behaviour 152:897–915

Loveland JL, Fernald RD (2017) Differential activation of vasotocin neurons in contexts that elicit aggression and courtship. Behavioural Brain Research 317:188–203

MacDonald K, MacDonald TM (2010) The peptide that binds: a systematic review of oxytocin and its prosocial effects in humans. Harvard Review of Psychiatry 18:1–21

Magurran AE, Seghers BH (1994a) Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*, populations in Trinidad. Behaviour 128:121–134

Magurran AE, Seghers BH (1994b) Sexual conflict as a consequence of ecology: evidence from guppy, *Poecilia reticulata*, populations in Trinidad. Proceedings of the Royal Society B 255:31–36

Magurran AE, Seghers BH, Carvalho GR, Shaw PW (1992) Behavioural consequences of an artificial introduction of guppies (*Poecilia reticulata*) in N. Trinidad: evidence for the evolution of anti-predator behaviour in the wild. Proceedings of the Royal Society B 248:117–122

Magurran AE, Seghers BH, Carvalho GR, Shaw PW (1993) Evolution of adaptive variation in antipredator behaviour. Marine Behaviour and Physiology 23:29–44

O'Connell LA, Matthews BJ, Hofmann HA (2012) Isotocin regulates paternal care in a monogamous cichlid fish. Hormones and Behavior 61:725–733

Pusey AE (1987) Sex-biased dispersal and inbreeding avoidance in birds and mammals. Trends in Ecology & Evolution 2:295–299

Ramsey ME, Fryb D, Cummings ME (2019) Isotocin increases female avoidance of males in a coercive mating system: assessing the social salience hypothesis of oxytocin in a fish species. Hormones and Behavior 112:1–9

Reddon AR, Aubin-Horth N, Reader SM (2022) Wild guppies from populations exposed to higher predation risk exhibit greater vasotocin brain gene expression. Journal of Zoology 316:118–127

Reddon AR, O'Connor CM, Nesjan E, Cameron J, Hellmann JK, Ligocki IY, Marsh-Rollo SE, Hamilton IM, Wylie DR, Hurd PL, Balshine S (2017) Isotocin neuronal phenotypes differ among social systems in cichlid fishes. Royal Society Open Science 4:170350

Reddon AR, Voisin MR, O'Connor CM, Balshine S (2014) Isotocin and sociality in the cooperatively breeding cichlid fish, *Neolamprologus pulcher*. Behaviour 151:1389–1411

Rodriguez-Santiago M, Nguyen J, Winton LS, Weitekamp CA, Hofmann HA (2017). Arginine vasotocin preprohormone is expressed in surprising regions of the teleost forebrain. Frontiers in Endocrinology 8:195

Saito D, Komatsuda M, Urano A (2004) Functional organization of preoptic vasotocin and isotocin neurons in the brain of rainbow trout: central and neurohypophysial projections of single neurons. Neuroscience 124:973–984

Salek SJ, Sullivan CV, Godwin J (2002) Arginine vasotocin effects on courtship behavior in male white perch (*Morone americana*). Behavioural Brain Research 133:177–183

Santangelo N, Bass AH (2006) New insights into neuropeptide modulation of aggression: field studies of arginine vasotocin in a territorial tropical damselfish. Proceedings of the Royal Society B: Biological Sciences 273:3085–3092

Schreck CB, Olla BL, Davis MW (1997) Behavioral responses to stress. In: Iwama GK, Pickering AD, Sumpter JP, Schreck CB (eds). Fish stress and health in aquaculture. Cambridge University Press, Cambridge

Seghers BH (1974) Schooling behavior in the guppy (*Poecilia reticulata*): an evolutionary response to predation. Evolution 28:486–489

Semsar K, Godwin J (2004) Multiple mechanisms of phenotype development in the bluehead wrasse. Hormones and Behavior 45:345–353

Semsar K, Kandel FLM, Godwin J (2001) Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. Hormones and Behavior 40:21–31

Snekser JL, Ruhl N, Bauer K, McRobert SP (2010) The influence of sex and phenotype on shoaling decisions in zebrafish. International Journal of Comparative Psychology 23:70–81

Soares MC, Cardoso SC, Mazzei R, André GI, Morais M, Gozdowska M, Kalamarz-Kubiak H, Kulczykowska E (2017) Region specific changes in nonapeptide levels during client fish interactions with allopatric and sympatric cleaner fish. PLoS ONE 12:e0180290

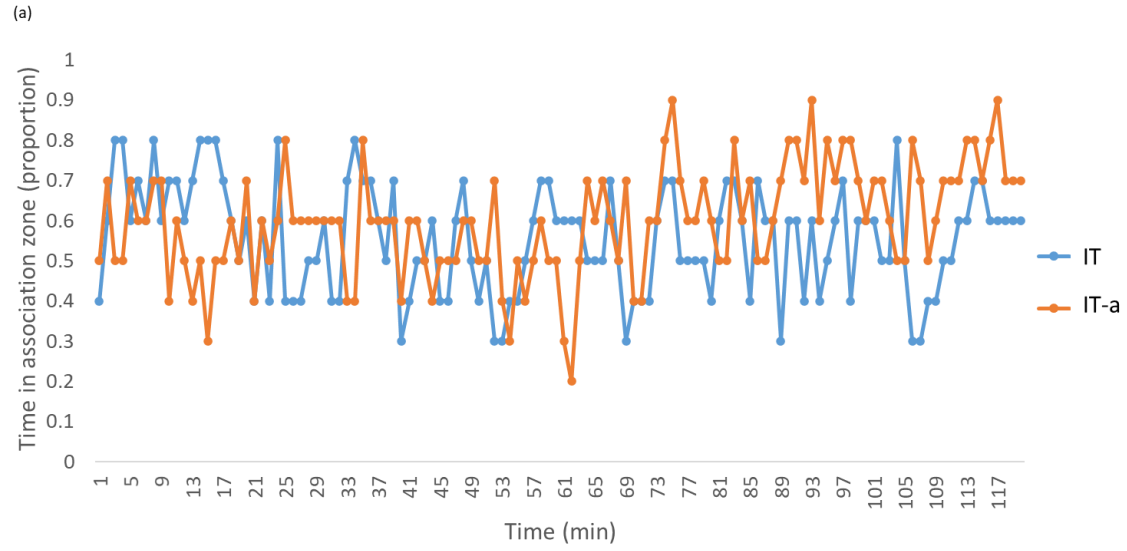
Székely T, Moore AJ, Komdeur J (2010) Social behaviour: genes, ecology and evolution. Cambridge University Press, Cambridge

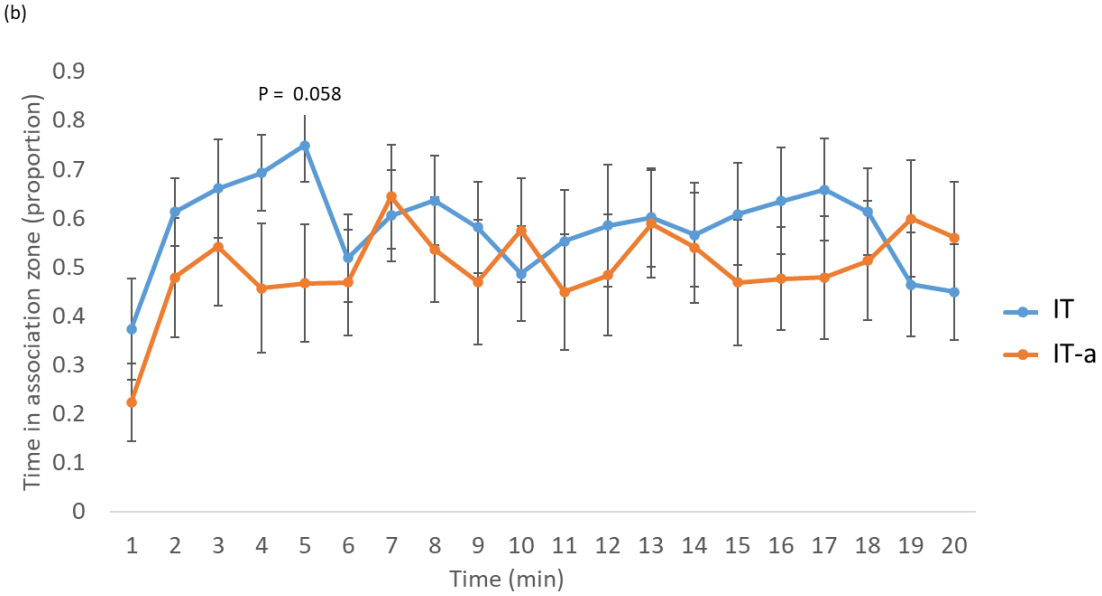
Thompson RR, Walton JC (2004) Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*). Behavioral Neuroscience 118:620–626

Thompson RR, Walton JC, Bhalla R, George KC, Beth EH (2008) A primitive social circuit: vasotocin substance P interactions modulate social behavior through a peripheral feedback mechanism in goldfish. European Journal of Neuroscience 27:2285–2293

Zelena D, Pintér O, Balázsfi DG, Langnaese K, Richter K, Landgraf R, Makara GB, Engelmann M (2015) Vasopressin signaling at brain level controls stress hormone release: the vasopressin-deficient Brattleboro rat as a model. Amino Acids 47:2245–2253

Appendix 1. The time test fish spent in the association zone with a group of six stimulus fish in a dichotomous trial in female guppies (*Poecilia reticulata*) from Paria population. Behaviour after isotocin (IT) (blue line) and isotocin antagonist (IT-a) (orange line) injection for 120 minutes (a) and 20 minutes (b) trials.





Appendix 2. The forward and reverse primers designed for IT and VT receptors in the guppy (*Poecilia reticulata*) using the sequence from pupfish (*Cyprinodon nevadensis*) as template. The underlined letters show differences in sequences between the two species.

IT-Receptor - forward
Guppy – CACCTGAGCATCGCAGATCTTGTC
Pupfish – CACCTGAGCATCGCAGATCTTGTT

IT-Receptor - reverse
Guppy – GGCTGGCAGATCGCTAAGCATCTA
Pupfish – GGCTGGCAGACTGCTAAACATCTA

AVT-Receptor - forward
Guppy – GGTGCAGATGTGGTCGGTGTGGGACC
Pupfish – GGTGCAAATGTGGTCAGTGTGGGATA

AVT-Receptor - reverse
Guppy – GGAGGTGGCCACTGAAGATCATGT
Pupfish – GAAGGTGGCCGCTAAAGATCATGT

Appendix 3. Ethics approval

eSirius3G

Western

PI :	Neff, Bryan
Protocol #	2018-084
Status :	Approved (w/o Stipulation)
Approved :	07/01/2018
Expires :	07/01/2022
Title :	Behavioural and molecular ecology of fishes

Table of Contents

Protocol Introduction

The questions on this page activate specific sections within the AUP form.

Note that species selection is part of this introductory page

Does this AUP involve teaching?

Yes ○ No ◉

Is the animal work on this project shared by another Animal Care Committee?

Yes ◉ No ○

Will you be using hazards?

Yes ◉ No ○

Will live animals be moved outside of their housing facility?

Yes ◉ No ○

Will field studies be conducted?

Yes ◉ No ○

Add/Update/Remove Species Used on this Protocol

Species	Agents	Drugs	Restraint	Breeding
Fish	Yes	Yes	Yes	Yes

Curriculum Vitae

Babak Ataei Mehr

Department of Biology, University of Western Ontario

1151 Richmond St., London, Ontario N6A 5B7

Education

2014 – 2022

PhD Candidate, Department of Biology, University of Western Ontario, London, Ontario

2005 – 2010

PhD, Department of Fisheries, University of Tehran, Tehran, Iran

Publications

Ataei Mehr B, Garner SR, Neff BD (2020) Effect of isotocin on shoaling behaviour of the Guppy (*Poecilia reticulata*). Animal Cognition 23:827–831

Ataei Mehr B, Garner SR, Neff BD. Effect of vasotocin on shoaling behaviour of the Guppy (*Poecilia reticulata*). Acta Ethologica (Submitted)

Ataei Mehr B, Neff BD. Relationship between social behaviour and brain nonapeptides in two populations of guppies (*Poecilia reticulata*). Journal of Ethology (Under revision)

Ataei Mehr B, Neff BD. Distribution of vasotocin and isotocin receptors in the brain of the Guppy (*Poecilia reticulata*) (in preparation)

Conference Presentations

Ataei Mehr B, Garner SR, Neff BD (2016) Effects of the Brain Nonapeptides Arginine-Vasotocin and Isotocin on Shoaling Behaviour in the Guppy (*Poecilia reticulata*).

20th Annual Meeting of the Society for Behavioral Neuroendocrinology, Montreal, Canada