Wild guppies from populations exposed to higher predation risk exhibit greater vasotocin brain gene expression

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Abstract

Intraspecific variation in social behaviour is often observed among animal populations. Local predation risk can be a key driver of these differences, with populations that are exposed to greater threat typically showing greater aggregation and reduced intraspecific aggression. The Trinidadian guppy, *Poecilia reticulata*, is found in populations that vary dramatically in predation risk and show greater grouping and reduced agonism in high predation populations compared to low predation populations. The neurohormonal mechanisms that underpin these differences in behaviour across populations remain unknown and elucidating these mechanisms may help us to understand the evolution of behavioural diversity in this species. We predicted that guppies naturally exposed to higher predation risk would show greater expression of the isotocin system and reduced expression of the vasotocin system when compared to the low predation fish, because these peptides are thought to promote gregariousness and aggressivity respectively. We collected guppies of both sexes from high and low predation sites, replicated in two different Trinidadian rivers, and measured the brain-gene expression of isotocin and vasotocin along with their central receptors. Contrary to our prediction, we found that high predation guppies showed greater expression of vasotocin, while we did not find evidence that the populations differed in isotocin expression, nor in the expression of the receptors. These results support the hypothesis that vasotocin may act as a neural substrate for social variation in fishes but call into question generalisations about its specific role across species.

Keywords

AVT, isotocin, nonapeptide, *Poecilia reticulata,* population differences

Introduction

Forming groups is widespread throughout the animal kingdom (Krause & Ruxton, 2002) and is a prerequisite for more complex social systems including cooperative breeding and eusociality (Bourke, 2011). Living within a group confers several advantages, many of which center around reducing predation risk (Ioannou *et al.*, 2012). However, living in a group can also have drawbacks, including increased intraspecific conflict over resources leading to aggressive interactions which can impose substantial costs (Earley & Dugatkin, 2010; Lacasse & Aubin-Horth, 2014; Balshine *et al.*, 2017). Depending on relative strength of these costs and benefits, there is considerable variation in grouping tendencies both between and within species (Lott, 1991; Krause & Ruxton, 2002). To fully understand the causes and consequences of social variation, it is crucial that we grasp the mechanisms underlying social behaviour as these may shape or constrain the expression of sociality (Soares *et al.*, 2010; Monaghan, 2014; Aubin-Horth, 2016).

Prime candidates for the proximate control of social behaviour in vertebrates are the nonapeptide hormones (Goodson *et al.*, 2012; Goodson, 2013), including oxytocin and vasopressin in mammals, which are important for social recognition, pair bonding, and mating behaviour (Donaldson & Young, 2008; Lee *et al.*, 2009). The nonapeptide hormones are well conserved throughout the vertebrate lineage and homologous molecules can be found in all vertebrates (Hoyle, 1999). The role of nonapeptides in regulating social behaviour also appears to be conserved across taxonomic lines (Goodson & Bass, 2001; Goodson & Thompson, 2010; Goodson, 2013). In birds, for example, the tendency to flock is tied to the activity of the nonapeptide circuitry and can be manipulated by perturbing the nonapeptide systems (Goodson *et al.*, 2009, 2012). To date, many insights about the role of the nonapeptides in regulating social behaviour have been gleaned by comparing related species that differ in their social system as well as the expression and function of the nonapeptide circuits in their brains (e.g., Insel & Shapiro, 1992; Insel *et al.*, 1994; Goodson *et al.*, 2009, 2012). This comparative approach suggests that the observed differences in social behaviour are mediated, at least in part, by the observed differences in nonapeptide circuit function (Goodson *et al.*, 2012). However, comparing species, even closely related ones, is not without caveats. It can be difficult to confidently ascribe differences in neurobiology to selection on the behaviour of interest when other factors will inevitably differ among even closely related species (Pozzi *et al.*, 2014). This challenge to the comparative approach can be at least partially overcome using multiple replicate species pairs and by controlling comparisons phylogenetically (MacLean *et al*., 2012). Another valuable tool for understanding how evolution acts upon the proximate bases of social behaviour is the study of a single species that shows social variation across populations, which can mitigate some of the problems with cross-species comparisons (Pavosudov & Clayton, 2002; Aubin-Horth, 2016; Lacasse & Aubin-Horth, 2019).

The Trinidadian guppy, *Poecilia reticulata*, is a model species in the study of intraspecific variation in social behaviour. Guppies are small, live-bearing, freshwater fish found throughout Trinidad (Magurran, 2005). In the mountainous terrain of Northern Trinidad, waterfalls subdivide much of the guppy habitat, creating populations exposed to higher or lower predation risk (high- and low predation populations). Large fish predators are abundant below the waterfalls, whereas above them, adult guppies are relatively safe from aquatic predation (Endler, 1978). These high and low predation populations differ in their social behaviour (Magurran & Seghers, 1991, 1994). In high predation areas, guppies show a stronger tendency to aggregate because shoaling is an effective strategy to avoid predation from large, active aquatic predators (Magurran, 1990). Where predation pressure is reduced, guppies show a weaker tendency to shoal (Magurran & Seghers, 1991). Low predation guppies also tend to be more aggressive to conspecifics than their high predation counterparts (Magurran & Seghers, 1991), presumably because competition for resources tends to be stronger in low predation populations (Endler, 1995) and the need to shoal is decreased. These population differences are likely to be at least partially due to genetic divergence among guppy populations (Magurran *et al*., 1993), though plasticity may also play a role (Houslay *et al*., 2018).

The nonapeptide hormones oxytocin and vasopressin have homologues in teleost fishes known as isotocin and vasotocin, respectively (Hoyle, 1999). Considerably less research attention has been directed towards understanding the role of the nonapeptides in fishes than in mammals or birds, but the existing literature strongly suggests that nonapeptides are key regulators of social behaviour in fishes (Godwin & Thompson, 2012). For example, in the Amargosa pupfish, *Cyprinodon nevadensis amargosae*, hypothalamic vasotocin gene expression correlates positively with aggressive behaviour (Lema *et al*. 2015). Peripheral injections of vasotocin also increase aggression in the beaugregory damselfish, *Stegastes leucostictus* (Santangelo and Bass, 2006), while in the cooperatively breeding daffodil cichlid, *Neolamprologus pulcher,* injections of isotocin increase submissive behaviour (Reddon *et al*., 2012; Hellmann *et al*., 2015), which may facilitate group living in this species (Reddon *et al*., 2019; Ruberto *et al*., 2020). Similarly, in the daffodil cichlid, expression levels of the isotocin gene correlate positively with submission and social affiliation (O'Connor *et al*., 2016). Populations of stickleback that show lower levels of aggression exhibit greater expression of their lone isotocin receptor (Lacasse & Aubin-Horth, 2019). In zebrafish, *Danio rerio* (Lindeyer *et al*., 2015), and goldfish, *Carassius auratus* (Thompson & Walton, 2004), administration of exogenous vasotocin reduces social approach and shoaling tendency. In guppies, central administrations of isotocin increases shoaling behaviour, while vasotocin administrations decrease it (Cabrera-Álvarez, 2018). A recent study in guppies also found more shoaling behaviour in fish given a peripheral injection of isotocin compared to those given a non-specific nonapeptide antagonist (Mehr *et al*. 2020). Together, these pharmacological manipulations suggest that the endogenous vasotocin and isotocin systems could differ between high and low predation populations of guppies, but the expression levels of these nonapeptides and their receptors across populations remain unknown. Recent studies of behavioural divergence in fishes have shown that ligands may be the target of evolutionary change (Kitano & Lema, 2013; Reddon *et al*., 2017), while other studies have found that receptors are more likely to diverge between populations (Di Poi *et al*., 2016; Lacasse & Aubin-Horth, 2019).

In the current study, we compared brain gene expression of the isotocin and vasotocin coding genes along with the genes that code for their central receptors between guppies from high and low predation populations in Northern Trinidad using quantitative PCR (qPCR). We predicted that the high predation populations would show greater expression of isotocin and the isotocin receptors that have been linked to prosocial behaviour (O'Connor *et al*. 2016; Cabrera-Álvarez, 2018) compared to the low predation fish. Vasotocin, by contrast, has been implicated in social withdrawal (Thompson & Walton, 2004) and the expression of aggressive behaviour (Santangelo & Bass, 2006; Dewan & Tricas, 2011; Silva & Pandolfi, 2019), which may interfere the formation of cohesive social groups (Lacasse & Aubin-Horth, 2014). Therefore, we predicted that the low predation guppy populations would show increased expression of vasotocin and its central receptor, compared to high predation fish.

Materials and methods

Sampling

We captured 151 (n = 79 males and n = 72 females) adult guppies in March 2016 using butterfly nets from 4 collection sites, one high predation and one low predation site in each of two rivers (Aripo and Marianne) in Northern Trinidad. We chose to sample from these sites based on their use in previous studies (Millar *et al*., 2006; Millar & Hendry, 2012; Gotanda *et al*., 2013). The high and low predation sites were differentiated by the presence or absence of large piscivorous fishes (as reported in Gotanda *et al*. 2013). For further details of the sample collections, see Reddon *et al*. (2018).

Following collection, fish were transported to the William Beebe Research Station near Arima, Trinidad where we euthanised them with an overdose of pH buffered MS222 (Argent Chemical Laboratories) approximately 24 hours after capture. This timing was necessary to ensure consistency between collection sites in the delay between capture and euthanasia, given that some sites were remote, meaning that not all fish could be collected and processed on the same day. We measured the standard length (SL, taken from the tip of the snout to the end of the caudal peduncle) in mm of each fish using a pair of dial callipers. We then dissected out their brains using a stereomicroscope. Samples were incubated in RNAlater (Sigma-Aldrich) for 24 hours at room temperature and then frozen at -20°C. Following our return to McGill University (Quebec, Canada), we weighed each whole brain to the nearest 0.1 mg using a Mettler-Toledo ME104E balance (see Reddon *et al*. 2018 for details) and then placed them into fresh RNAlater and returned them to -20°C.

Analysis of gene expression

We transported the samples to Université Laval (Quebec, Canada) where we homogenised each brain and extracted total whole brain RNA using Qiagen RNeasy mini kits, following the manufacturers protocol. The concentration and purity of each sample was then evaluated using a nanodrop spectrophotometer (Thermo Fisher Scientific). Samples with total RNA concentrations below 100ng/ul and/or 260/280 ratios below 1.8, indicating possible RNA degradation, were removed from the analyses, resulting in a final sample size of 115 fish (low predation males n = 24, low predation females n = 26, high predation males n = 37, high predation females n = 28). A haphazardly selected subset of 12 samples was further checked for RNA integrity using a 2100 Bioanalyzer instrument (Agilent Technologies). All tested samples had an RNA Integrity Number (RIN) > 8.0, and were therefore acceptable for qPCR (Fleige *et al*., 2006).

Before cDNA synthesis, we treated 2000ng aliquots of RNA with DNase I (Invitrogen) to eliminate DNA contamination. First strand cDNA synthesis was then conducted using SuperScript II Reverse Transcriptase (Invitrogen) with a mix of random hexamer (Invitrogen) and oligo dt primers (Invitrogen). We checked the success of our cDNA synthesis reaction with PCR followed by a 1.2% agarose electrophoresis gel using SyberSafe (Life Technologies).

We designed primers for the nonapeptide genes and their receptors in silico using Primer 3 (Rozen & Skaletzky, 2000) and Amplify 3 (Engels, 2005) based on guppy sequences retrieved from the NCBI database. We created primers for both nonapeptide ligands, vasotocin (AVT) and isotocin (IT), along with the central receptors for each. Fishes possess multiple receptors for vasotocin (Lema, 2010; Lema *et al*., 2015) and we chose to focus on AVTv1a2 (following the naming convention in Lema *et al*. 2019; hereafter referred to as AVTr) because it is the central receptor which has been most consistently implicated in the regulation of social behaviour in fishes (Lema, 2010; Kline et al. 2011; Oldfield et al. 2013). There are two known isotocin receptors (ITr1, ITr2) in guppies, and while studies have not yet been conducted to fully determine their binding affinities (e.g., one or both may also bind with vasotocin; Lyu *et al*., 2021), it can be assumed that both receptors bind isotocin with high affinity. Little is known about the individual function of these isotocin receptors but divergent expression patterns within species suggest that they may have different functions (O'Connor *et al*., 2015, 2016). Therefore, we chose to examine both isotocin receptors in the current study. Here we follow the naming conventions for the isotocin receptors (ITr1, ITr2) found in Lema *et al*. (2019), which contrast with those in another recent report (Lyu *et al*., 2021). We also designed primers for the metabolic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is known for strong constitutive expression across individuals and tissues (Livak & Schmittgen, 2001), and therefore is often used as a control (housekeeping) gene in qPCR studies in fishes (Rui-Xue *et al*., 2010). Following primer design, each primer pair was tested by amplifying guppy cDNA using PCR and examining the output of a 1.2% agarose electrophoresis gel using SyberSafe for the presence of a single well-defined band of the appropriate size. To determine amplification efficiency, the absence of primer dimers and the specificity of amplification for each primer pair, qPCR experiments and melting curves (50 to 90°C) were run using standard curves consisting of 5 x 10-fold dilutions (of pooled samples) in duplicates. Information on the primers used can be found in Supplemental Table 1.

We measured the expression of our 5 target genes (AVT, IT, AVTr, ITr1, ITr2) and our control gene (GAPDH) in a 384-well plate qPCR machine (Roche Light Cycler). Each gene for each individual fish was assayed in triplicate on 384-well plates (Axygen) prepared using an EpMotion liquid handler (Eppendorf), following the scaled-down version of the

Quantitect SYBRGreen PCR kit manufacturer's protocol (Qiagen) including no-primer and no-template controls. To verify that only a single amplified product was present and that no primer dimers were produced, a melting curve (50 to 90°C) was also performed for each gene. The mean Cq value across the three replicates for each gene in each fish was used for analysis.

Statistical analysis

We compared the expression of our control gene, GAPDH, between the sexes and predation regimes using a using a linear mixed model including river as a random effect. We rank transformed GAPDH Cq prior to analysis to conform to the assumption of homogeneity of variance. We examined the expression of each of our five target genes (AVT, IT, AVTr, ITr1, ITr2) relative to the expression of the reference gene GAPDH (Pfaffl, 2001). For purposes of comparison, expression of each gene was calculated relative to the mean expression of the high predation males from the Aripo river. We ran a linear mixed model for each gene including sex, predation regime, and the sex*predation interaction as fixed effects. We included the river of collection (Aripo, Marianne) as a random effect in each model. Because we were interested in the relative expression of each gene between sexes and populations rather than the magnitude of these differences, and to conform to the assumption of homogeneity of variance between groups, we rank transformed the response measure prior to analysis and present the rank transformed data graphically. In our sample, we had previously reported sex and predation regime differences in both body length and brain mass (Reddon *et al*. 2018). Differences in brain mass could have affected transcript abundance in our samples. Therefore, we included brain mass as a covariate in all of our analyses, although this had no qualitative effect on the pattern of results we observed. The data required to recreate our analyses and figures are available in the supplementary materials. Analysis was conducted in SPSS v.27 (IBM) for Mac OS 11.4 and the figures were made using ggplot2 v3.3.5 in R v.4.1.0 for Mac OS 11.4.

Ethical note

Sampling methods were approved by the McGill University Animal Care Committee (2015-7708) and followed the ABS/ASAB guidelines. Animal collection was approved by the Ministry of Agriculture, Land and Marine Resources of the Republic of Trinidad and Tobago. Guppies are not threatened and were abundant at all collection sites.

Results

We found that GAPDH expression did not differ significantly between high and low predation populations ($F_{1,110.58} = 0.81$, $p = 0.37$), however females did have lower average GAPDH expression than did males ($F_{1,110} = 16.72$, $p < 0.001$). There was no statistically significant interaction between sex and predation regime on GAPDH expression ($F_{1,100.02} = 0.005$; $p = 0.94$) and the brain mass covariate was not statistically significant ($F_{1,109.93} = 0.08$; $p = 0.79$). All subsequent results refer to gene expression relative to the expression of GAPDH.

The expression of the vasotocin gene was greater in the high predation populations than in the low predation populations (Fig. 1, $p = 0.02$, Table 1). This result was qualitatively similar in each of the two sampled rivers, Aripo and Marianne (Fig. 1). Males had greater expression of vasotocin than females, but this difference did not reach statistical significance (Fig. 1, $p = 0.07$, Table 1). We did not find evidence that expression of the isotocin gene differed between populations (Fig. 2, $p = 0.79$, Table 1), but females showed greater expression of isotocin than did males (Fig. 2, $p = 0.04$, Table 1). This sex difference in isotocin expression seems to be primarily driven by the Aripo fish (Fig. 2) and may also reflect the fact that GAPDH expression was lower in females than males. We did not detect evidence that any of the three receptor genes (AVTr, ITr1, Itr2) that we examined showed a difference between the high and low predation populations, or between the sexes (all $p \geq 0.18$, Table 1). We did not detect any statistically significant interactions between population and sex on the expression of any of the examined genes (all $p \geq 0.47$, Table 1). The brain mass covariate was not statistically significant in any of our analyses (all $p \geq 0.08$; Table 1).

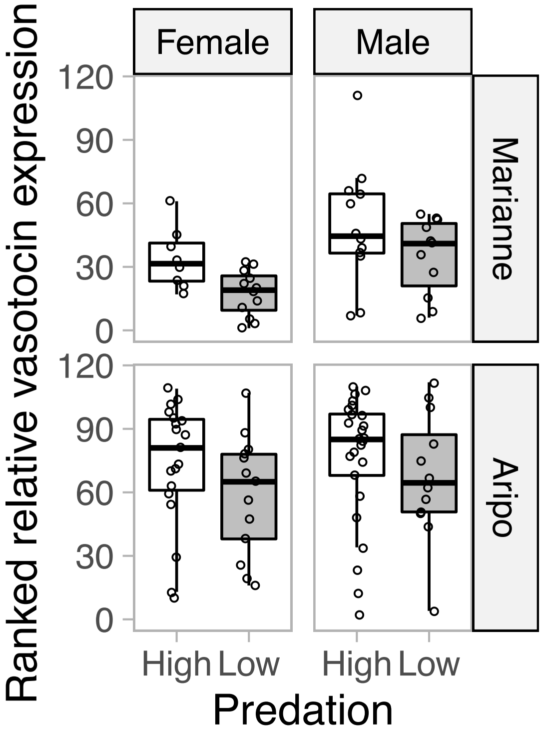

Fig. 1 – Ranked relative vasotocin expression by sex and predation regime for the Marianne and Aripo rivers. Guppies collected from high predation sites (white boxes) showed higher relative brain gene expression of vasotocin than did guppies from low predation sites (grey boxes; p = 0.01). Males had higher vasotocin expression than females, but this difference did not reach statistical significance (p = 0.07). Boxes indicate the interquartile range with the median represented by the horizontal line. Circles represent the individual data points.

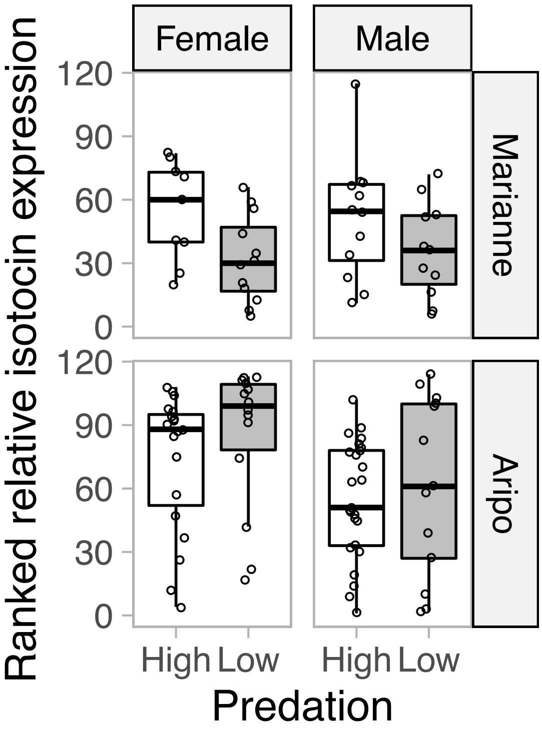

Fig. 2 – Ranked relative isotocin expression by sex and predation regime for the Marianne and Aripo rivers. There was no statistically significant difference in isotocin brain gene expression between guppies collected at high (white boxes) and low predation sites (grey boxes; p = 0.73). Female guppies had higher isotocin expression than males (p = 0.04). Boxes indicate the interquartile range with the median represented by the horizontal line. Circles represent the individual data points.

Table 1 – Summary statistics for linear mixed models examining the effects of sex, predation regime, and their interaction, with brain mass as a covariate on the ranked expression of each nonapeptide or receptor gene relative to GAPDH. Statistically significant effects ($p < 0.05$) are bolded. River (Aripo, Marianne) was included as a random effect in all models.

Gene	Effect	Denominator df	F	p
AVT	**predation**	**104.13**	**5.62**	**0.02**
	sex	104.02	3.41	0.07
	predation*sex	104.00	0.52	0.47
	brain mass	104.18	0.47	0.50
IT	predation	107.24	0.08	0.79
	sex	**107.04**	**4.38**	**0.04**
	predation*sex	107.00	0.17	0.68
	brain mass	107.52	0.26	0.61
AVTr	predation	106.21	1.29	0.26
	sex	106.04	0.62	0.43
	predation*sex	106.00	0.08	0.78
	brain mass	106.44	2.32	0.13
ITr1	predation	107.14	1.81	0.18
	sex	107.02	0.14	0.71
	predation*sex	107.00	0.08	0.78
	brain mass	107.32	2.69	0.10
ITr2	predation	107.10	0.01	0.91
	sex	107.02	0.05	0.82
	predation*sex	107.00	0.01	0.96
	brain mass	107.24	3.13	0.08

Discussion

Guppies from high predation populations exhibit greater shoaling behaviour and lower aggression than those from lower predation environments (Magurran & Seghers, 1991). In several species of fish, isotocin administration promotes shoaling behaviour (Thompson & Walton, 2004; Braida *et al*., 2012; Cabrera-Álvarez, 2018), while vasotocin administration inhibits shoaling (Lindeyer *et al*. 2015; Thompson & Walton, 2004; Cabrera-Álvarez 2018) and increases aggression (Santangelo & Bass, 2006). We therefore predicted that the isotocin system would be upregulated in the high predation populations and the vasotocin system downregulated. Unexpectedly, in two replicated river systems we found that high predation guppies showed greater brain gene expression of vasotocin than did low predation guppies and did not find evidence for a difference between predation regimes in the expression of isotocin. We did not find evidence supporting a difference between predation regimes for any of the receptors we examined. We also detected a sex difference in the expression of isotocin, with females having greater expression than males, and some evidence for the opposite pattern in vasotocin, but found no evidence for a sex difference in the expression of any of the receptors. Our results suggest that variation in vasotocin expression may be related to variation in behaviour among wild guppy populations, though the direction of the population difference in vasotocin expression ran counter to our prediction.

A potential explanation for the greater expression of vasotocin in the high predation fish may lie in the fact that vasotocin is involved in the stress response and tends to be positively correlated with both acute and chronic stress (de Kloet, 2010; Sokołowska *et al*., 2020). For example, acute stress leads to an increase in vasotocin expression in the rainbow trout, *Oncorhynchus mykiss* (Gilchriest *et al*., 2000). Central administration of vasotocin in the same species induces a stress response (Gesto *et al*., 2014), suggesting vasotocin has anxiogenic effects in rainbow trout. Chronic osmotic stress in medaka, *Ozyzias latipes*, leads to an increase in pituitary vasotocin and alterations in the size and number of vasotocin producing neurons in the preoptic area (Haruta *et al*., 1991). High predation guppies are likely to be living under more chronically stressful conditions and/or show greater reactivity to acute stressors (Fischer *et al*. 2014; Chouinard-Thuly *et al*. 2018). Given that the capture and transport procedures were likely at least somewhat stressful for the fish, and acute stress responses have been found to affect nonapeptide gene expression in other fishes over similar timeframes (Lema *et al*., 2010; Skrzynska *et al*., 2018), either chronic stress in the high predation environment, or a larger acute stress effect on vasotocin expression in the high predation fish could explain the greater levels of vasotocin brain gene expression that we observed.

The relationship between nonapeptide gene expression and behavioural phenotype is complicated by the fact that nonapeptide synthesis is a multistage process and nonapeptides have multiple sites of action (Sokołowska *et al*., 2020), therefore, different measurement approaches may yield different results. For example, in the daffodil cichlid, dominant breeding individuals have greater expression of the vasotocin gene than do subordinates (Aubin-Horth *et al*., 2007), whereas when measuring free bioactive peptide in the brain, the subordinate individuals exhibit higher levels (Reddon *et al*., 2015). Similarly, cooperatively breeding and closely related non-cooperative cichlid species show a consistent pattern of parvocellular isotocin neuronal phenotypes, with cooperative species having fewer of these cells (Reddon *et al*., 2017), however when comparing brain gene expression, no consistent difference in isotocin was apparent between social systems (O'Connor *et al*., 2015). This may reflect a difference in production versus storage of the peptide (Ota *et al*., 1999; Grober *et al*., 2002). Species differences in the apparent behavioural functions of the nonapeptides are also common (Goodson, 2008; Goodson & Thompson, 2010), especially in fishes (Godwin & Thompson, 2012), for example, exogenous isotocin may either reduce shoaling motivation or have no effect (e.g., Reddon *et al*., 2014; Lindeyer *et al*., 2015) in contrast to its prosocial effects in other species (e.g., Thompson & Walton, 2004; Braida *et al*., 2012). Given the variation between fish species and between different approaches for studying the effects of nonapeptides on social behaviour, our initial predictions of greater isotocin system gene expression in the high predation guppies and greater vasotocin expression in the less social low predation guppies may have been overly simplistic.

Oxytocin and the related non-mammalian peptides are often associated with the regulation of female behaviour, and correspondingly, the oxytocin signalling system is typically upregulated in females relative to males (Dumais & Veenema, 2016), which is consistent with our results, albeit driven predominantly by fish from Aripo river. It should be noted however that this sex difference is not always observed in fishes (Reddon *et al*., 2015; Cunha-Saraiva *et al*. 2019) and the unexpected sex difference in mRNA transcript abundance for our chosen control gene does complicate the interpretation of this difference. Lower expression of GAPDH in females may exaggerate the expression of isotocin in females relative to males. This sex difference in isotocin gene expression should be confirmed using an alternative control gene. The vasopressin/vasotocin system by contrast has been associated with regulating social behaviour predominantly in males (De Vries & Panzica, 2006; Albers, 2015; Dumais & Veenema, 2016), though again this finding may be absent or reversed in fishes (Aubin-Horth *et al*., 2007; Reddon *et al*., 2015; Cunha-Saraiva *et al*., 2019). We did find a notable, although non-significant, trend for greater expression of vasotocin in male relative to female guppies. Collectively our results suggest that guppies may show sex differences in the expression of the nonapeptides broadly reminiscent of those often observed in birds and mammals (De Vries & Panzica, 2006). If they do prove reliable, the functional significance of these sex differences in guppies would be an interesting area for future work given the sex differences in behaviour, cognition, and physiology exhibited in this species (e.g., Griffiths & Magurran, 1998; Harris *et al.*, 2010; Lucon-Xiccato *et al.*, 2016, 2020; Lucon-Xiccato & Bisazza, 2017; Chouinard-Thuly *et al.*, 2018).

In our analyses, we examined gene expression across the entire brains of our sampled animals which may have obscured differences between the populations in particular regions of the brain. In fishes, nonapeptides are synthesised in three separate nuclei in the preoptic era of the hypothalamus (Ramallo *et al.* 2012; Silva & Pandolfi, 2019), the parvocellular, magnocellular, and gigantocellular regions, each of which has distinct cell morphologies, projections, and apparent roles in the regulation of social behaviour (Godwin & Thompson, 2012). For example, in the African cichlid fish, *Astatotilapia burtoni*, vasotocin activity in the parvocellular region is associated with fleeing and submission while vasotocin activity in the magnocellular region is associated with aggression and dominance (Greenwood *et al.*, 2008). In contrast to the nonapeptide synthesising neuronal populations, the nonapeptide receptors are widely dispersed throughout the brain (Godwin & Thompson, 2012), including in several regions that have been associated with distinct social behaviours and responses (Goodson, 2005). Our whole brain approach may therefore be less sensitive to population differences in region specific expression of nonapeptide receptors. The nonapeptides are integrated into the social decision-making network, which controls social behaviour via relative activity across a network of brain areas (Goodson 2005; Goodson & Thompson, 2010; O'Connell & Hofmann, 2012; Nunes *et al.*, 2020). Therefore, we might predict both up- and down-regulation of nonapeptide receptors among distinct nodes of the network across social phenotypes and thus we may not expect a whole brain change in the expression of these receptors between guppy populations. Future work should examine nonapeptide ligand expression separately in each of the preoptic nuclei, and receptor expression independently in each node of the social decision-making network across populations of guppies exposed to different predation regimes. Future studies should also attempt to link social phenotypes directly to nonapeptide brain gene expression at the individual level, as population level correlations offer only indirect evidence of a causal relationship between nonapeptide circuity and behaviour.

We sampled adult fish from the wild for this study, therefore, we cannot distinguish between the possible influences of genetic differences among populations, developmental organisation of the nonapeptide circuitry influenced by early life experiences (e.g., Baran, 2017), or acute variation in the expression of vasotocin in response to recent predation threat or current conditions. Lema (2006) found both genetic and plastic differences in AVT immunoreactivity between populations of Amargosa pupfish that differed in social behaviour. Future work should compare guppies from high and low predation populations raised under common garden conditions, and experimentally expose guppies to cues of predation threat in the laboratory to distinguish between genetic differences among populations and plastic responses to predation threat (e.g., Lema 2006; Gosline & Rodd, 2008; Fischer *et al*., 2014; Ghalambor *et al*., 2015; Chouinard-Thuly *et al*., 2018; Reddon *et al*., 2018).

Wild guppy populations are exposed to variance in ecological conditions beyond predation risk, for example collection sites may also vary in resource availability (Grether *et al*., 2001; Reznick *et al*., 2001; Millar *et al*., 2006; Schwartz & Hendry, 2010), which can affect levels of competition among guppies (Potter *et al*. 2018) and therefore also drive differences in social behaviour (Magurran & Seghers, 1991; Endler, 1995). Moreover, Lema (2006) found that under laboratory conditions a complex interaction between population of origin, water temperature, and salinity determined vasotocin neuronal phenotypes in Amargosa pupfish. Such results suggest that additional unmeasured ecological parameters may also have affected the differences we observed in addition to variance in predation threat. We replicated our sampling in two rivers (four total populations), but further replication across additional independent river basins to confirm the generality of our findings would also be valuable. Experimental laboratory studies could also help to distinguish the specific effects of predation risk from other correlated ecological factors in the generation of social variation between guppy populations and the neural substrates that underpin that variation.

Conclusions

We found that guppy populations exposed to differing levels of predation risk showed distinct patterns of vasotocin brain gene expression and this result was replicated in two independent river drainages. Contrary to prediction, the high predation populations showed greater expression of vasotocin than the low predation populations. Our results add to a growing literature implicating vasotocin as a proximate mediator of social behaviour and suggest that it may serve as a proximate substrate for intraspecies variation in social behaviour.

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Author contributions

All authors jointly conceived of and designed the study. N.A.H. and S.M.R. secured funding for the project. A.R.R. conducted the field sampling and performed the laboratory analyses under the supervision of N.A.H. A.R.R. analysed the data and wrote the first draft of the manuscript. All authors contributed comments on the final version.

Conflict of interest

The authors declare no conflicts of interest.

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