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Evolutionary divergence in transcription levels of neuropeptide receptors linked to both social and stress-response behaviour between two threespine stickleback populations exhibiting distinct behavioural types

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ABSTRACT

Organism and background: Threespine stickleback (*Gasterosteus aculeatus*) from freshwater populations in Lake Témiscouata and Rond Lake (Québec, Canada) differ in predator defence morphology and behaviour. Individuals from Lake Témiscouata have more lateral plates, longer pelvic and dorsal spines, and a longer pelvic girdle than fish from Rond Lake. When raised in a common environment, Lake Témiscouata fish are also significantly less aggressive and more limited in their locomotor activity than those from Rond Lake.

Neurological background: Several neuropeptides and their receptors are known to be key players in both the molecular networks that underlie variation in social behaviour, and those that govern the physiological response to stress. These molecules include arginine vasotocin (AVT), isotocin (IT), corticotropin-releasing factor (CRF), and their receptors. Thus individuals that differ in social (aggression, sociality) and stress-response behaviours (locomotor activity, exploration, response to predators) might also differ in the activity of these neuropeptides and their receptors.

Question: Do the juveniles of Lake Témiscouata and Rond Lake diverge in the expression of these neuropeptides and their receptors, particularly in the context of a response to an acute stressor.

Methods: We quantified the genomic reaction norm of common-environment-reared juveniles from each population by measuring expression in the brain of genes coding for AVT, IT, CRF, and receptor subtypes (AVTR1a, ITR, CRFR1, respectively) before and after an acute stress using quantitative PCR.

Results: We found no significant effect of population of origin, stress treatment, or their interaction on the expression of the three neuropeptides studied (AVT, IT, CRF) or of the

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AVTR1a receptor. We found a significant difference in expression of the ITR receptor between the two populations, with Témiscouata fish exhibiting higher expression of that gene, both before and after a stress. We observed a tendency for Témiscouata fish to show a larger transcriptional stress response for the CRFR1 receptor. Thus receptors in these neuropeptide networks have evolved divergently in these two populations and might be functionally implicated in behavioural divergence.

Keywords: behaviour, corticotropin-releasing factor, evolutionary divergence, *Gasterosteus aculeatus*, gene expression, genetic variation, isotocin, personality, stress, threespine stickleback, vasotocin.

INTRODUCTION

Populations faced with different ecological challenges diverge in several types of traits, including morphological, physiological, and behavioural traits (Elipot *et al.*, 2013; Corl *et al.*, 2018). As an integrator of form and function (Bertossa, 2011), behaviour can greatly affect fitness in combination with these other types of traits. It is crucial to uncover the proximal physiological and molecular mechanisms of this behavioural divergence in wild populations as a key step towards understanding its evolutionary path (Monaghan, 2014; Aubin-Horth, 2016). Indeed, this knowledge is central to understanding how behavioural divergence arose, and if there are physiological constraints affecting the evolution of that trait alone or in combination with other traits, as a result of pleiotropy or trade-offs (McGlothlin *et al.*, 2007; McGlothlin and Ketterson, 2008; Monaghan, 2014). Studying natural variation in hormonal networks and their evolution is a crucial early step in this endeavour (Swanson and Snell-Rood, 2014; Vitousek *et al.*, 2018; Wingfield, 2018).

Neuropeptides are key candidates in understanding if and how hormonal networks underlying population divergence in behaviour have themselves evolved. Among these neuropeptides are arginine vasopressin (AVP), oxytocin (OT), and corticotropin-releasing hormone (CRH). The fish homologues of these mammalian neuropeptides are vasotocin (AVT), isotocin (IT), and corticotropin-releasing factor (CRF). These neuropeptides have been previously implicated in vertebrate individual behavioural responses to challenges and opportunities, such as expression of social behaviours (aggression, sociality) and stress-response behaviours (locomotor activity, exploration, response to predators), as well as in the physiological stress response (detailed in Fig. 1).

Neuropeptides act through their receptors and thus form molecular networks with them (see Fig. 1). These receptors need also to be studied, since different receptor subtypes may have more specific and localized functional effects than their wide-ranging associated neuropeptides, which makes them more likely to diverge than their ligand (Burns *et al.*, 2014; Swanson and Snell-Rood, 2014; Di Poi *et al.*, 2016a). Based on the demonstrated functional roles of neuropeptides in inter-individual variation in behaviour, we predicted different activity of these neuropeptides and their receptors between populations that diverge in social behaviours and in stress-response behaviours.

Studying molecular networks only in individuals under benign conditions may give an incomplete picture of the divergence in expression between populations. Indeed, levels of these neuropeptides or their receptors could differ constitutively between populations but could also show a difference only in response to a stressor. This type of population

divergence in the response to an environmental challenge has been quantified for hormones in various vertebrates (Partecke *et al.*, 2006; Di Poi *et al.*, 2016b) and is crucial information in our attempt to understand the evolution of hormonal networks (Vitousek *et al.*, 2018). However, there is little information on whether populations that have diverged in behaviours during evolution also diverged in the expression of these neuropeptides and their receptors in the context of a response to an acute stress. Quantifying the *genomic reaction norm*, i.e. the gene expression level in different environmental conditions of a given genotype (Aubin-Horth and Renn, 2009), for two populations that are known to have evolved under different environmental challenges, would give us a more complete picture of the genetic divergence of the molecular networks between these populations (Vitousek *et al.*, 2018). It would thus be necessary to study expression levels of these candidate neuropeptides and of their receptors before and after a stress, in common-environment-reared individuals to isolate the genetic effects on phenotype.

In the present study, we compared laboratory-reared juveniles from two populations of threespine stickleback (*Gasterosteus aculeatus*) found in contrasting ecological conditions. We quantified the genomic reaction norm by measuring brain expression of genes coding for AVT, IT, CRF, and specific receptor subtypes, AVTR1a, ITR, and CRFR1, using quantitative PCR, before and after an acute confinement stress (Fig. 1). We made predictions based on previous studies in other species and known differences in behaviour between Rond and Témiscouata juveniles (see Fig. 1). Individuals from these two populations differ in predator defence morphology and behaviour in the form of trait co-specialization: Lake Témiscouata individuals have more lateral plates, longer pelvic and dorsal spines, and larger pelvic girdles (Lacasse and Aubin-Horth, 2012). When raised in a common environment, Témiscouata fish also show less aggression and are less active than juveniles from Rond Lake (Lacasse and Aubin-Horth, 2012), and exhibit a significant negative relationship between an individual's aggression and its sociability, which Rond Lake fish do not (Lacasse and Aubin-Horth, 2014). We predicted that divergence in aggression and activity would be associated with gene expression differences of AVT, IT, and CRF networks. We predicted that AVT and its AVTR1a receptor would be more highly expressed in more aggressive and more active Rond Lake individuals. We predicted that IT and ITR, as well as CRF and CRFR1, would be more highly expressed in less aggressive and less active Lake Témiscouata individuals. Finally, we predicted that AVT, IT, and CRF expression levels would be raised after a stress (see Fig. 1 for details).

METHODS

Laboratory rearing

We used juveniles reared in a common environment in 2010 who were F1 offspring of wild-caught adults originating from two populations of threespine stickleback (Témiscouata and Rond Lakes) located in Québec, Canada. Detailed crossing, rearing, and behavioural assay procedures and average results for each population are described in Lacasse and Aubin-Horth (2012). In summary, when juveniles reached a size of about 25 mm, an average of five F1-generation fish per family were used in behavioural assays, with nine families tested per population. Each fish was exposed to behavioural tests over a 3-day period before being held for 24 hours in a benign situation and then being terminally sampled.

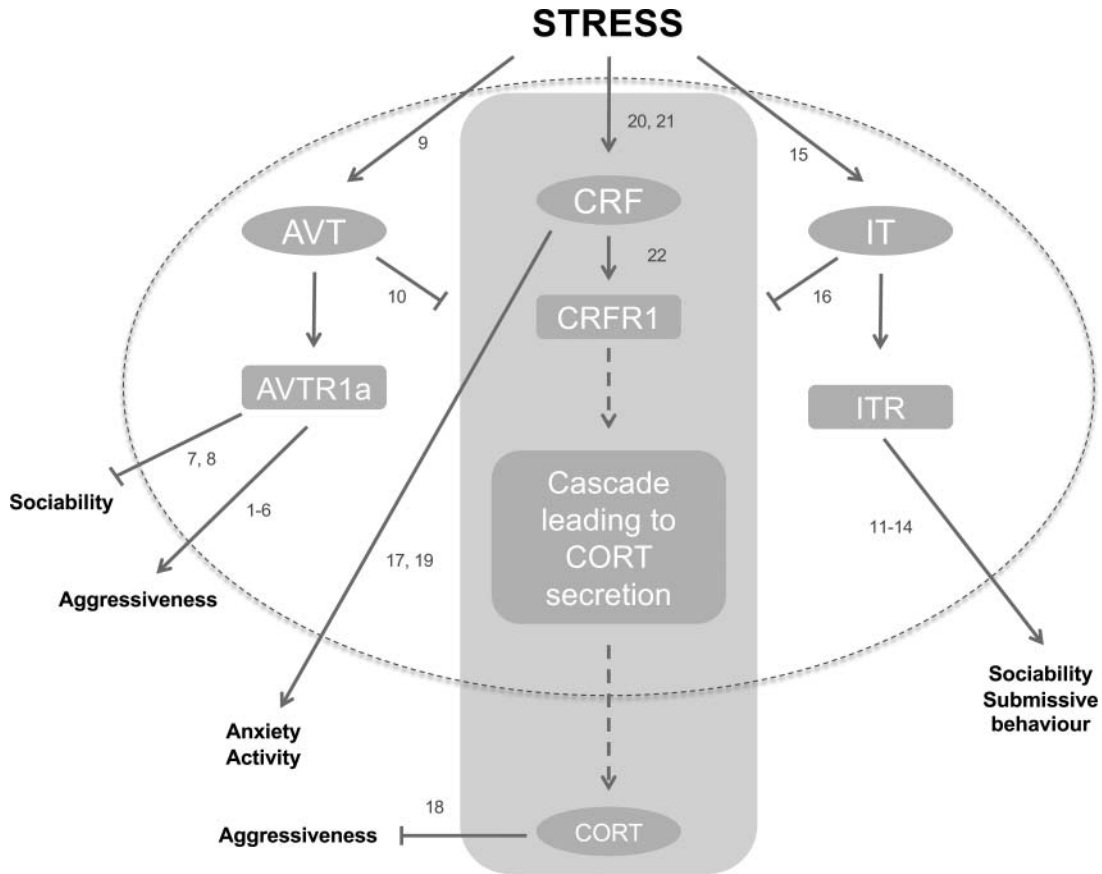

Fig. 1. Candidate neuropeptides and their receptors implicated in social and stress-response behaviours and in the stress response, studied in individuals from two stickleback populations that differ in aggressiveness and activity. Their effects are context-, sex-, and species-specific, such that predictions made here for juvenile stickleback may be opposite to what is found in other studies (Goodson, 2013). Ellipses represent ligands and rectangles receptors. Numbers represent references cited below.

A. The molecular network composed of AVT and its receptors

Aggression. Increasing AVT levels pharmacologically significantly reduces aggression in dominant zebrafish (1) (Filby *et al.*, 2010) and African cichlids (2) (Huffman *et al.*, 2015). Whole-brain gene expression in African cichlids showed higher expression of AVT in dominant and aggressive individuals than in subordinate ones (3, 4, 5) (Aubin-Horth *et al.*, 2007; Renn *et al.*, 2008; Huffman *et al.*, 2015). In individuals ascending in dominance rank in that species, blocking the AVT receptor decreases aggression (6) (Huffman *et al.*, 2015). Increasing AVT increases social preference in zebrafish at intermediate doses (measured as approaching a conspecific), while it reduces it at high doses, and blocking AVTR1 receptors (both V1a and V1b) cancels these effects (7) (Braida *et al.*, 2011). Similarly, increasing AVT levels in goldfish results in lower social approach in individuals with a highly social personality type (8) (Thompson and Walton, 2004).

Activity. A large range of doses does not affect activity in zebrafish (Braida *et al.*, 2011) or goldfish (Thompson and Walton, 2004).

Stress. A confinement stress increases AVT levels in the brain in rainbow trout (9) (Gilchriest *et al.*, 2000). In mammals, AVT dampens the glucocorticoid stress response in the hypothalamus, since blocking the AVP receptor V1 results in a higher hormonal response (10) (Neumann *et al.*, 2000).

B. The molecular network composed of IT and its receptor

Aggression. Increasing IT levels pharmacologically leads to more social approach in individuals that have a low social personality type in goldfish (11) (Thompson and Walton, 2004) and in zebrafish, but in the latter species the effect is reversed at high doses (inverted-U-shaped dose curve) (12) (Braida *et al.*, 2011). Research into ITR expression in whole brain of African cichlids showed higher levels in the more social species compared with its closest less social relative (13) (O'Connor *et al.*, 2015), and pharmacologically increasing IT in that cichlid species increased submissive behaviours (14) (Reddon *et al.*, 2012).

Activity. Manipulating IT using a large range of doses does not affect activity in zebrafish (Braida *et al.*, 2011) or African cichlids (Reddon *et al.*, 2012).

Stress. In mammals, exposure to stress increases OT levels (the mammalian equivalent of IT) in the hypothalamus (15) (Nishioka *et al.*, 1998). Furthermore, increasing OT levels pharmacologically results in a lower behavioural and hormonal stress response (16) (Windle *et al.*, 1997).

C. The molecular network composed of CRF and its receptors

Aggression. CRF administration in fishes decreases aggression while increasing anxiety behaviour, but also increases the probability of winning a fight by lowering latency to attack (17) (Carpenter *et al.*, 2009). CRF increase leads to higher cortisol secretion in rainbow trout, and increasing cortisol levels pharmacologically results in lower aggression in rainbow trout (18) (Øverli *et al.*, 2002).

Activity. Increasing CRF levels by injections results in higher activity in rainbow trout (19) (Carpenter *et al.*, 2007).

Stress. CRF is the first step in the cortisol physiological stress response in fish. For example, CRF has been shown to be more highly expressed in whole brains of rainbow trout exposed to model predator attacks for 7 days compared with control fish (20, 21) (Doyon *et al.*, 2005; Thomson *et al.*, 2012), and raising cerebral CRF through pharmacological manipulations leads to higher circulating cortisol levels in trout (22) (Backström *et al.*, 2011).

In order to quantify the gene expression response to an acute stress and to determine if this response varies between the two populations, a subsample of individuals from each population were submitted to a confinement stress before tissue sampling. This manipulation has been shown to induce a significant response in both the adrenergic stress response (measured using ventilation rates) and the glucocorticoid stress response (measured by cortisol levels) in threespine stickleback (see Di Poi *et al.*, 2016b; Berger and Aubin-Horth, 2018). In this stress treatment, a fish was placed in a beaker filled with 50 mL of water surrounded by opaque material for 30 minutes. Individuals were then put back in their home tank for 3½ hours before being sampled. This 4-hour delay before sampling tissues was chosen to ensure that the stress response was fully triggered and could be detected at the gene expression level, based on a study in rainbow trout that had shown that 4 hours of confinement increased CRF expression significantly in the pre-optic area of the hypothalamus (Doyon *et al.*, 2005). Individuals that were in the control benign treatment were not disturbed before final sampling. We sampled a total of 112 fish, with sample size for each combination of population and stress treatment between 27 and 29 individuals per group.

Tissue sampling

Fish were terminally anaesthetized in buffered MS-222 (tricaine methanesulfonate; Sigma Aldrich #E10521). Individuals were weighed (in grams) and measured using a pair of callipers (standard length, mm). The brain was removed and was frozen in liquid nitrogen and stored at −80°C for later analysis. The whole sampling procedure took less than 2 minutes per fish. Tissue sampling was always carried out between 11.00 and 14.00 hours. All of the procedures were carried out in accordance with national regulations on animal welfare (Canadian Council on Animal Care) and local regulation from the CPAUL (Comité de protection des animaux de l'Université Laval, 2010-066)

Quantitative real-time PCR

Whole-brain levels of mRNA for the six genes coding for the neuropeptides and their receptors were analysed using quantitative real-time polymerase chain reaction (qPCR, see Table 1). We studied the expression of vasotocin, isotocin, and CRF. These neuropeptides have more than one receptor subtype. We chose to study the AVTR1a receptor because it is often implicated in behavioural variation in fish species (Lema, 2010; Kline *et al.*, 2011; Oldfield and Hofmann, 2011; Huffman *et al.*, 2015). While some fish species have two IT receptor subtypes, analysis of the threespine stickleback genome suggests that there is a single ITR, as in most fishes (O'Connor *et al.*, 2015). We studied the CRFR1 receptor in stickleback because studies of the other receptor subtype, CRFR2, have shown no association with behaviour in this species (Aubin-Horth *et al.*, 2012; Di Poi *et al.*, 2016a) and CRFR1 is implicated in the cortisol stress response (Flik *et al.*, 2006). The primers were designed from the stickleback sequence obtained from the Ensembl web site (http://useast.ensembl.org/Gasterosteus_aculeatus/Info/Index). The primers were 18–25 nucleotides in length, with a melting point around 55°C and GC content around 60% (see Table 1 for details for each gene).

Total RNA from individual brain was extracted using the RNeasy Plus Universal Tissue Kit according to the manufacturer's instructions (QIAGEN, #73404). RNA was treated with Dnase Amplification Grade I (Invitrogen). The concentration of each sample was then measured using a Ribogreen fluorescent assay using the manufacturer's standard

Table 1. Primers design for quantitative real-time PCR: list of genes (Gene) studied based on the *G. aculeatus* transcript sequences from Ensembl Genome Browser (Ensembl ID) with forward and reverse primer sequences, and qPCR amplicon size

Gene	Ensembl ID	Forward primer	Reverse primer	Amplicon size (bp)
AVT	ENSGACT00000008867	5′-GAGGAGAACTACCTGCTCAC	5′-ACAGCTCTCTGAGTTACAGC	105
AVTR1a	ENSGACT00000004720 ENSGACT00000000951	5′-CAGGTGATGGGCATGTT	5′-ACGGCTCCACGAAGT	241
IT	ENSGACT00000008706	5′-CGCTGCGAAAGTGCATGTC	5′-GGGGTGAGCAGGTAGTTC	142
ITR	ENSGACT00000001185	5′-TCTCTAACTGTCCCATCG	5′-GTGAACTGCAAACAGGAG	474
CRF	ENSGACT00000003899	5′-CTCTAAAGACTGAAGATTCCTG	5′-ATGGGAAAGAGTTAGTGTCC	249
CRFR1	ENSGACT00000005660	5′-CCATGATCCTCGTCTTAGTG	5′-CTTTCCTGTACTGGATCGTC	111

Note: The primers targeting AVTR1a were designed to amplify products of the two paralogs AVTR1aa and AVTR1ab; the primers for CRF are from Aubin-Horth *et al.* (2012).

Table 2. Average gene expression levels (number of molecules per sample ± S.D., sample size in parentheses) of ligand and receptors of the AVT, IT, and CRF molecular networks for Lake Témiscouata (TEM) and Rond Lake (RD) individuals, in benign (BENIGN) and acute stress (STRESS) treatments, with statistical test results (*P*-value and *df*) for population (POP), stress treatment (STRESS), sex (SEX), and mass (MASS) effects

Gene	RD-BENIGN (mean expression)	TEM-BENIGN (mean expression)	RD-STRESS (mean expression)	TEM-STRESS (mean expression)	POP *P* (*df*)	STRESS *P* (*df*)	MASS *P* (*df*)	SEX *P* (*df*)
AVT	2275 ± 3014 (26)	3659 ± 4847 (26)	2732 ± 3416 (27)	4178 ± 5615 (27)	0.316 (105)	0.798 (105)	0.285 (105)	0.532 (105)
AVTR1a	783 ± 950 (23)	1194 ± 1952 (24)	1169 ± 1 67 (28)	1104 ± 1383 (25)	0.602 (99)	0.735 (99)	0.276 (99)	0.991 (99)
IT	80,339 ± 127,033 (19)	112,046 ± 130,031 (24)	91,882 ± 94,513 (20)	140,987 ± 174,771 (24)	0.366 (81)	0.708 (81)	0.967 (81)	0.895 (81)
ITR	845 ± 1205 (25)	2118 ± 3461 (26)	1863 ± 4799 (25)	4092 ± 6401 (22)	0.006 (92)	0.460 (92)	0.544 (92)	0.278 (92)
CRF	1533 ± 1651 (24)	2147 ± 2731 (27)	1871 ± 2404 (28)	1777 ± 1964 (22)	0.664 (95)	0.684 (95)	0.109 (95)	0.667 (95)
CRFR1	3494 ± 5873 (19)	2880 ± 4222 (20)	2226 ± 3174 (20)	7953 ± 10,683 (22)	0.413 (75)	0.450 (75)	0.561 (75)	0.908 (75)

Note: The interaction between population and stress treatment was not significant for any variable and the results for the model without interaction are presented in all cases.

protocol (Invitrogen, kit R11490) to obtain an accurate RNA amount to reverse-transcribe for each fish. Three hundred nanograms of total RNA were then reverse-transcribed into cDNA using a standard SuperScript protocol (Invitrogen). For each gene, the annealing temperature of primers was optimized by PCR (see Table 1 for details for each gene), while amplification efficiency and specificity of each primer pair was tested by qPCR using a cDNA standard curve (5×10-fold dilutions of pooled samples in duplicates) to quantify expression and a melting curve (50–95°C) following the amplification cycles. The qPCR reaction was performed using a modified protocol for the QuantiTect SYBR Green PCR kit (QIAGEN), prepped in 384 well-plates with an automated liquid handler (Eppendorf), using 7.5 μL of SYBR Green in a total volume of 15 μL and 50 amplification cycles in a Light Cycler 480 instrument (Roche Applied Science). Five nanograms of cDNA were used in each qPCR reaction and each sample was tested in triplicate. All samples were assayed on a single plate for a given gene. A no-template control and a no-primer control were used for each gene. We report gene expression as the number of molecules in a sample calculated using the LRE method (Boyle *et al.*, 2009; Rutledge and Stewart, 2010; Di Poi *et al.*, 2016a). See the Results section for final sample size for each gene, population, and stress condition.

Statistical analysis

We performed all statistical analyses using R software v.3.3.3 (R Development Core Team, 2014). We used the package *lme4* (Bates *et al.*, 2014) to create a linear mixed model including populations, stress treatment, their interaction, mass, and sex as fixed factors and the rearing tank as a random factor. We verified assumptions about homoscedasticity by plotting residuals and fitted data. We tested normality of residuals using a graphical inspection (q-q plot) and a Shapiro-Wilk normality test. As none of the variables were normally distributed, we used the boxcox function from the package MASS to compute and choose the boxcox lambda transformation parameter (Venables and Ripley, 2002). We used the lambda value as an exponent to transform the data. We fitted a linear mixed model using the transformed data to test for significant effects of population of origin, stress treatment, and the interaction of population and stress treatment on expression level of a given gene. We removed the interaction between population and treatment when the interaction was not significant. Then, we tested the effects of population and treatment separately. In those cases, only the *P*-value for that model without interactions are presented (Table 2).

The entire dataset is available in the Appendix (evolutionary-ecology.com/data/3175Appendix.txt).

RESULTS

All candidate genes were found to be expressed at least in some samples (see Fig. 2). We quantified large inter-individual variation in expression levels for all neuropeptides in these common-environment-reared individuals.

AVT pathway

We found no significant effect of population of origin, stress treatment, or their interaction on the expression of AVT (population: $P = 0.32$; stress: $P = 0.80$) or of the AVTR1a receptor (population: $P = 0.60$; stress: $P = 0.74$) (Fig. 2; see Table 2 for full statistics).

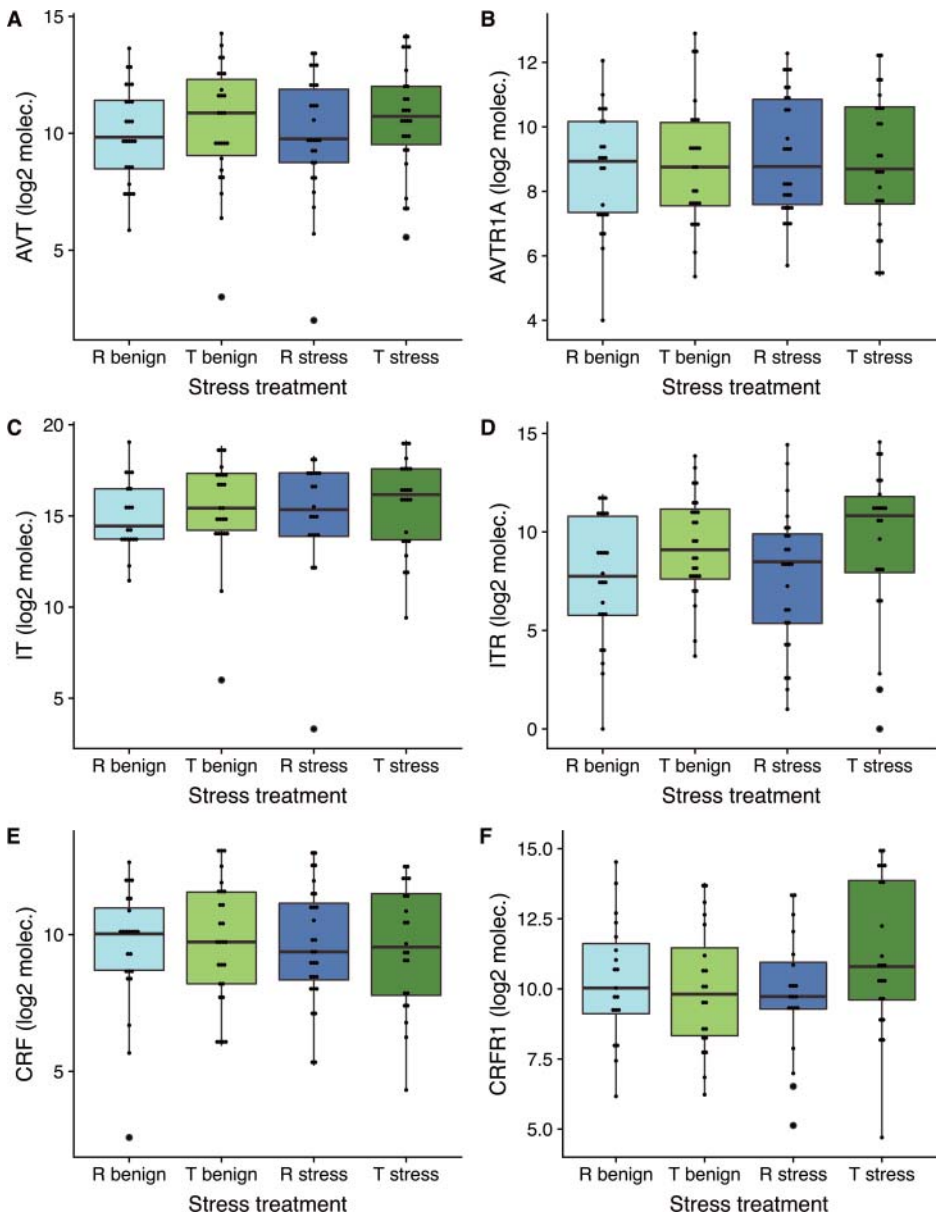

Fig. 2. Candidate gene expression under benign and stressed conditions in individuals from the two populations. Expression levels in number of molecules of AVT and its receptor AVTR1a, IT and its receptor ITR, and CRF and its receptor CRFR1, are presented for control individuals (benign) and acutely stressed ones (stress) for each population, Rond Lake ('R', more aggressive, more active) and Lake Témiscouata ('T', less aggressive, less active). There is a significant difference in expression of the ITR receptor between the two populations in both benign and stress conditions (population: $P = 0.006$; stress: $P = 0.46$). The dark horizontal lines represent the median of the data distribution in each condition.

IT pathway

We found no significant effect of population of origin, stress treatment, or their interaction on the expression of IT (population: $P = 0.37$; stress: $P = 0.71$) (see Fig. 2 and Table 2). We found a significant difference in expression of the ITR receptor between the two populations (population: $P = 0.006$; stress: $P = 0.46$), with Témiscouata fish exhibiting higher expression of that gene, both before and after a stress (Fig. 2 and Table 2).

CRF pathway

We found no significant effect of population of origin, stress treatment, or their interaction on the expression of CRF (population: $P = 0.66$; stress: $P = 0.68$) (see Fig. 2 and Table 2). We observed a tendency for Témiscouata fish to show a stronger expression response for the CRFR1 receptor when under stress (population × stress: $P = 0.13$). However, this interaction was not significant at an alpha of 0.05, so we used the model without interaction, and no significant effect of population or stress was observed (population: $P = 0.41$; stress: $P = 0.45$) (Fig. 2 and Table 2).

DISCUSSION

To understand the nature of the evolutionary divergence in behaviour between populations, one approach is to test if neuropeptides known to be implicated in inter-individual variation in behaviour also diverge between populations (Swanson and Snell-Rood, 2014). Here, we looked at three neuropeptides (AVT, IT, CRF) and specific subtypes of their receptors (AVTR1a, ITR, CRFR1) under a benign and an acute stress condition, in two populations of threespine stickleback that exhibit divergent anti-predator morphology and behaviour. Lake Témiscouata fish have been shown to have larger anti-predator morphological traits (plates and spines) and to be both less aggressive and less active than juveniles from Rond Lake. Contrary to our predictions, we found no significant differences in the expression of AVT, AVTR1a, IT, or CRF between populations, between benign and stress conditions, or in the interaction of these two factors. As predicted, we found that ITR was constitutively more expressed in Lake Témiscouata individuals (in benign and stress conditions). We also found that the CRFR1 receptor showed a trend for a larger response to stress in Lake Témiscouata fish. Our results suggest that the receptors in these neuropeptide networks have evolved in a divergent fashion in these two populations, which could potentially result from natural selection. Our results also suggest that these molecular networks could be functionally implicated in behavioural divergence in juvenile threespine stickleback.

No divergence in activity for most candidate genes

The main result of our study is that most candidate genes did not vary in expression between the two populations. We found no variation between the two populations in AVT and IT, which is contrary to our predictions based on previous studies and on significant divergence in activity and aggression between Lake Témiscouata and Rond Lake individuals (see Fig. 1). This absence of a significant difference between populations could be due to technical reasons (whole-brain sampling, discussed below). It could also reflect species-specific and life-stage-specific functions of AVT and IT in juvenile threespine

stickleback. In zebrafish (*Danio rerio*), a large range of doses of AVT and IT had no effect on activity (Braida *et al.*, 2011), which is concordant with our results. The absence of any association of AVT and IT with aggression in the stickleback was also seen in rainbow trout (*Oncorhynchus mykiss*) selected for a high and low stress response, which resulted in correlated selection of low and high aggression respectively, but not in differences in AVT and AVTR expression levels (Backström *et al.*, 2011). This is also in accordance with results of pharmacological manipulation to raise IT levels that did not change aggression in African cichlids (Reddon *et al.*, 2012). Nonetheless, these results are surprising and further work is required (see below).

We also did not find an expression response to stress for most genes studied, including CRF, which is at the top of the cascade leading to the glucocorticoid stress response, and AVT and IT, which have been found to be elevated after a stress (Nishioka *et al.*, 1998; Gilchriest *et al.*, 2000) (see Fig. 1). We used an appropriate confinement stress treatment known to significantly increase ventilation rates and cortisol levels after 30 minutes in threespine stickleback, including in the Témiscouata population (Di Poi *et al.*, 2016b; Berger and Aubin-Horth, 2018). Our results thus suggest that there is a potential problem with the timing of our final sampling (4 hours after the start of the stress). Studies in other fish species suggest that CRF levels are modulated by the harshness of the experimental stress and its duration (reviewed in Bernier, 2006), and that studying a specific brain area allows the detection of fainter signals than when using whole-brain samples. For example, rainbow trout did not show a significant increase in CRF expression after a 3-hour confinement stress [measured in whole brain (Backström *et al.*, 2011)] but repeated chasing until exhaustion over 4 hours followed by 2 hours of recovery (e.g. sampling done 6 hours after the onset of stress) led to a significant increase in CRF expression when measured in the pre-optic area of the hypothalamus (Doyon *et al.*, 2005). Using 4 hours of confinement in a 1.5-litre box also increased CRF significantly in the same study, but much less than 24 hours of restraint. In carp (*Cyprinus carpio*), a 30-minute confinement stress resulted in no detectable increase in CRF expression 2 or 4½ hours after the start of stress, but a 24-hour restraint stress did [hypothalamus (Huising *et al.*, 2004)]. At 4 hours following our relatively mild confinement stress and using whole brain, we were thus probably in the early phase of the CRF response that is detectable by qPCR. We cannot exclude the possibility that a divergent stress response in the AVT, IT, and CRF pathways might be found between the two populations when focusing on a specific brain area, at a later sampling time, or with a more intense stress treatment.

Isotocin receptor

Divergence in expression levels of IT receptors has been found between closely related African cichlid species that differ in sociality (O'Connor *et al.*, 2015). *Neolamprologus pulcher*, which displays less total aggression, more social motivation to interact with conspecifics, and greater submissiveness than its less social relative, *Telmatochromis temporalis* (Balshine *et al.*, 2017), also has higher ITR1 expression in the brain [whole-brain sampling (O'Connor *et al.*, 2015)]. Our results are in accordance with what has been observed in this species pair, as a higher expression of the ITR receptor in stickleback from Lake Témiscouata was associated with less activity in a familiar environment and lower aggression in this population. Manipulating the IT network has shown that it is positively involved in social approach in goldfish (Thompson and Walton, 2004) and zebrafish (Braida *et al.*, 2011), which may explain some differences in behaviour between the Lake Témiscouata and Rond Lake fish. On the other

hand, manipulating IT does not increase activity in zebrafish (Braida *et al.*, 2011) or African cichlids (Reddon *et al.*, 2012). Therefore, a functional analysis measuring the effects of manipulating isotocin action (both by adding isotocin and by blocking its receptor) on each behaviour of threespine stickleback is required to untangle the implications of our results.

The divergence we uncovered in the expression of ITR illustrates the information that can be obtained by testing different stress conditions and by studying receptors along with ligands (Kitano *et al.*, 2014; Swanson and Snell-Rood, 2014; Wingfield, 2018). Previous studies have shown that receptors of physiological regulatory networks directly implicated in stress reactivity are divergent between a marine population and a freshwater population of threespine stickleback that differ in behaviour (Di Poi *et al.*, 2016a). However, that study quantified expression levels only in acutely stressed fish, such that it was not possible to test if these differences were also found in a control condition. Here we show that the difference in isotocin receptor expression is already present in benign control conditions and is maintained during a stress. This suggests that the higher sensitivity to isotocin in Lake Témiscouata fish is present at all times, which could have effects in a wider range of situations. Studies have shown that studying the receptors of hormones can provide important information on the underlying mechanism of behavioural variation between populations, even if the hormones do not vary themselves [AVP receptors in voles expressing pair bonding (reviewd in Goodson, 2013); androgen receptors in juncos (Burns *et al.*, 2014); receptors of the serotonergic, dopaminergic, adrenergic, and glucocorticoid networks in threespine stickleback (Di Poi *et al.*, 2016a)]. In the present study, the only significant difference between the two populations was in the expression of a receptor. This suggests that the effects of the IT system on behaviour could be different between populations in the brain specifically, while other tissues that express this receptor might not be affected if these other tissues have similar receptor expression levels in both populations. Consequently, a study of spatial localization of receptor gene expression [particularly in the preoptic area (O'Connell and Hofmann, 2011)] would shed further light on the functional meaning of the present results (Goodson, 2013; Kelly and Goodson, 2014).

CRF receptor

We observed a tendency for Témiscouata fish to show higher expression of the CRFR1 receptor after a stress, while individuals from Rond Lake did not show a change in expression levels (population × stress interaction). This result, while only a trend, suggests that there could be genetic variation between the two populations in the CRFR1 genomic reaction norm, i.e. genetic variation for the plastic response to the environment. Overall, previous studies have suggested that higher expression of CRFR1, if it leads to increased CRF network signalling, could result in high anxiety-like behaviour and in individuals being less exploratory and less aggressive, as is found in Lake Témiscouata fish (Fig. 1). The fact that this tendency for more CRFR1 receptors to be expressed in Lake Témiscouata fish appears 3½ hours after exposure to an acute stress suggests that this heightened CRF signalling is specific to a stressful condition. Measuring the CRF response at a later time than 4 hours after the onset of stress could confirm this trend. Furthermore, pharmacological manipulations using antalarmin, an antagonist that specifically affects this CRF receptor subtype (Lastein *et al.*, 2008), could allow testing of whether CRF is implicated in the differences in activity and aggression between the two populations.

Overall, our results suggest that genetic variation in hormonal networks exists between populations that show significant divergence in key social and stress response behaviours

(Swanson and Snell-Rood, 2014; Vitousek *et al.*, 2018, Wingfield, 2018). In the future, the potential functional implication of this association in stickleback will need to be tested using experimental manipulations, in order to make predictions about the role of these physiological networks in behaviour evolution in this species, to test the relationship between these behaviours and fitness in their respective environments, and to determine whether these hormonal networks create physiological constraints on trait evolution (McGlothlin *et al.*, 2007; McGlothlin and Ketterson, 2008; Monaghan, 2014).

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