Receptors rather than signals change in expression in four physiological regulatory networks during evolutionary divergence in threespine stickleback

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Abstract

The molecular mechanisms underlying behavioural evolution following colonization of novel environments are largely unknown. Molecules that interact to control equilibrium within an organism form physiological regulatory networks. It is essential to determine whether particular components of physiological regulatory networks evolve or if the network as a whole is affected in populations diverging in behavioural responses, as this may affect the nature, amplitude and number of impacted traits. We studied the regulation of four physiological regulatory networks in freshwater and marine populations of threespine stickleback raised in a common environment, which were previously characterized as showing evolutionary divergence in behaviour and stress reactivity. We measured nineteen components of these networks (ligands and receptors) using mRNA and monoamine levels in the brain, pituitary and interrenal gland, as well as hormone levels. Freshwater fish showed higher expression in the brain of adrenergic (adrb2a), serotonergic (htr2a) and dopaminergic (DRD2) receptors, but lower expression of the htr2b receptor. Freshwater fish also showed higher expression of the mc2r receptor of the glucocorticoid axis in the interrenals. Collectively, our results suggest that the inheritance of the regulation of these networks may be implicated in the evolution of behaviour and stress reactivity in association with population divergence. Our results also suggest that evolutionary change in freshwater threespine stickleback may be more associated with the expression of specific receptors rather than with global changes of all the measured constituents of the physiological regulatory networks.

Keywords: behaviour, evolution, gene expression, hormones, monoamines, physiological regulatory networks

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Introduction

Many striking examples of behavioural divergence between populations have been demonstrated, but our knowledge of the molecular mechanisms underlying behavioural evolution following colonization of novel environments is limited (Aubin-Horth 2016). Molecules do not act alone but rather form networks of interacting components. These physiological regulatory networks (PRNs) are defined to comprise ‘molecules and their regulatory relationships that maintain and adjust homeostasis and facilitate performance at the whole-organism level’ [as defined in Cohen et al. (2012)]. It is essential to determine whether particular components of physiological regulatory networks evolve or if the whole network is changed in populations diverging in behavioural responses, as this may affect the nature, amplitude and...
number of impacted traits (Kitano et al. 2014). For example, changes in a ligand that can bind several receptors or act in more than one PRN [integrators (Cohen et al. 2012)] could have wider ranging effects on phenotypes than changes in a receptor subtype that binds to a single ligand (Taniguchi et al. 2006; Adkins-Regan 2008).

The adrenergic, serotonergic, dopaminergic and glucocorticoid physiological regulatory networks have been associated with modulation of behavioural responses in association with opportunities and challenges faced by an individual ([O’Connell & Hofmann 2011], Fig. 1). On an evolutionary scale, we know much less about how the components of these four PRNs are regulated in natural populations of a species that have evolved different behavioural responses (Elipot et al. 2013).

We studied the regulation of these four physiological regulatory networks in freshwater and marine populations of threespine stickleback (*Gasterosteus aculeatus*). After the last glacial retreat in the Northern Hemisphere, marine sticklebacks invaded freshwater habitats that contrast in ecological conditions from the ones faced by the marine ancestor and evolved independently (Hohenlohe et al. 2010; Jones et al. 2012a). In addition to morphological (Bell & Foster 1994; Miller et al. 2014; Ellis et al. 2015) and physiological changes (Kitano et al. 2010; McCairns & Bernatchez 2010; Dalziel et al. 2012; Morris et al. 2014; Di Poi et al. 2016), this colonization is also associated with divergence in behaviour (Bakker & Feuth-De Bruin 1988; Ishikawa & Mori 2000; Peeke & Morgan 2000; Messler et al. 2007; Greenwood et al. 2013; Di Poi et al. 2014). We used individuals from marine and freshwater stickleback populations that were previously characterized as diverging in sociability, aggressiveness and activity (Di Poi et al. 2014), as well as in stress reactivity (Di Poi et al. 2016), when reared in a common environment. We quantified divergence in the regulation of components of the adrenergic, serotonergic, dopaminergic and glucocorticoid networks. We measured mRNA, monoamine and metabolite levels in the brain, pituitary and interrenal gland, and hormonal levels, for a total of 19 molecular and physiological traits that reflect the activity of these PRNs (Fig. 1).

### Material and methods

#### Populations and common environment

We collected adult threespine sticklebacks from marine and freshwater populations in two breeding sites presenting different ecological characteristics (Saimoto 1993; Rogers et al. 2012). Population genetics study in threespine sticklebacks have shown extensive gene flow between marine sampling sites as far as 1000 km apart on the west coast of North America and genetic divergence of freshwater populations, both compared to marine samples and to each other (Hohenlohe et al. 2010). The first site, Oyster Lagoon, is located on the eastern side of the strait of Georgia, British Columbia, Canada (49°36'48"N 124°01'47"W), and has a salinity range between 20 ppt in winter and 32 ppt in summer. Hoggan Lake on Gabriola Island, British Columbia, Canada (49°09'08"N 123°50'00"W), is a freshwater body. The lake is not connected to the ocean, and this population has been isolated from marine ancestors for approximately 10 000 years. Previous studies showed that common environment-reared fish originating from these

![Fig. 1](https://example.com/fig1.png) The components of the physiological regulatory networks studied. (a) Adrenergic, (b) serotonergic, (c) dopaminergic and (d) glucocorticoid networks. Ligands are represented by ovals, monoamine metabolites by italic font and receptors by rectangles. A full line borders components that we quantified in the present study, while components not measured are bordered by a dashed line. Components of the PRN significantly more expressed in freshwater individuals are shown as dark-filled shapes, while components significantly more expressed in marine individuals are shown as clear shapes.
populations differed in behaviours, stress reactivity, transcriptomic response to rearing temperature and cold tolerance (Barrett et al. 2011; Di Poi et al. 2014, 2016; Morris et al. 2014).

We crossed adults to produce pure lines of F1 freshwater and marine individuals resulting in 14 full-sib families, seven from each population, that were reared in common environments. We kept juveniles in a common environment in the Life and Science Animal Research Centre at the University of Calgary. We kept them in 110-L tanks under a 12-h L:12-h D cycle, with temperature at 18 °C and salinity of 5 ppt. Similar rearing protocols using low salinity and uniform temperature for marine and freshwater juveniles have been used successfully by other groups (Kitano et al. 2010; Jones et al. 2012b; Greenwood et al. 2013). This freshwater rearing mimics what the marine individuals would face when invading a new inland freshwater habitat. We fed them frozen bloodworms to satiation twice daily. We air-shipped juveniles (8-month old) to the Laboratoire de Recherche en Sciences Aquatiques at Université Laval. We held juveniles in 80-L tanks (stocking density: 0.11 g/L) under similar rearing conditions with the exception that all fish were acclimated to 15 °C with a 8-h L:16-h D photoperiod (winter conditions). We kept juveniles originating from a given environment (marine or freshwater) together during air transport and later rearing, such that the information about the family of origin of individuals is not available. We measured body length at time of tissue sampling, which averaged 46.4 ± 0.6 mm for fish from Hoggan Lake and 42.5 ± 0.5 mm for fish from Oyster Lagoon. We determined sex using the IDH genetic sex marker (Peichel et al. 2004).

Regulation of the physiological regulatory networks

We aimed to measure 19 components in four PRNs. Several central signalling molecules (ligand) in these networks cannot be quantified using transcriptomics (notably monoamines: noradrenaline, serotonin, dopamine; and steroid hormones: cortisol, Fig. 1). Furthermore, dynamic responses to a challenge at the cellular level implicate both changes in mRNA levels through gene transcription and changes in protein levels through translation and degradation (Jovanovic et al. 2015). Likewise, changes in monoamine levels can result from an increase in their release or a decrease in their turnover into a metabolite. The metabolite: monoamine ratio representing the turnover rate may thus be more informative than absolute values (Bell et al. 2007). Therefore, measurements of gene expression, monoamine and hormonal levels must be combined to obtain an integrated view of these networks.

Five days prior to sampling, we placed tested fish individually in 45-L experimental tanks at the same temperature, salinity and light cycles as in group housing, with plants and shelter as enrichment, in order to measure behaviours (Di Poi et al. 2014). On the day of sampling, we placed individuals in a 150-mL beaker with a low water volume (50 mL) (n = 36 per population) to test stress reactivity (Di Poi et al. 2016). Individuals stayed in the confinement beaker, covered on the outside by light-blocking material, for a total of 30 min. Fish facing this confinement stress show significantly higher cortisol levels than undisturbed individuals [see Di Poi et al. (2016)]. Immediately after the confinement stress, we euthanized individuals with buffered MS-222 (0.4 mg/mL). We collected the brain and the pituitary in two different ways depending on downstream measurements (transcriptomics and brain monoamine, see below). This means that a fish used to measure gene expression levels in the brain cannot have monoamine measurements in the same organ, since this tissue can only be used for one protocol or the other. We also collected the interrenal glands for transcriptomics, the teleost homologue of the adrenal cortex of mammals. Finally, we collected the body, minus the selected organs, to measure whole-body cortisol levels. All gene expression, monoamine and cortisol level measurements were thus done on individuals that had faced a confinement stress [for complete data sets on behaviour and ventilation rates during a confinement stress in the same individuals used here, see Di Poi et al. (2014, 2016)]. For transcriptomic assays, the whole brain (n = 17 freshwater and n = 19 marine), the pituitary (n = 12–13 freshwater and n = 16–18 marine, depending on the gene) and interrenal glands (n = 33 freshwater and n = 34 marine) were stored separately in RNA later (Life Technologies) in 0.5-mL tubes and kept at −20 °C until RNA extraction. For brain monoamine assays, we snap-froze the whole brain (without the pituitary) (n = 17 freshwater and n = 17 marine) in liquid nitrogen in separate 0.5-mL microcryovials and kept them at −80 °C until protein extraction. For cortisol measurement, whole fish were snap-froze (n = 39 freshwater and n = 39 marine) in liquid nitrogen in cryovials and kept at −80 °C until cortisol extraction.

Gene expression

RNA extraction. We extracted total RNA in the brain using a standard Trizol reagent protocol (Life technologies) and in the pituitary using a RNEasy Plus microkit (Qiagen) and stored it at −80 °C. After spectrophotometer quantification of concentration and 260/280 ratios (Nanodrop, Thermo Scientifics), we treated RNA with DNase Amplification Grade I (Invitrogen). We
quantified RNA content of the DNase-treated sample using a Ribogreen quantification (Invitrogen). We reverse-transcribed 200 ng of whole-brain RNA in duplicate (total of 400 ng), 25–125 ng of RNA from the pituitary (one replicate) and between 29 and 200 ng for the interrenals (one replicate) of each fish, using Superscript II, oligo-dT (500 ng/μL) and random hexamer (100 ng/μL) primers and RNAselOut (Invitrogen). To confirm RT success, we used a small amount of cDNA from each sample in a PCR using 18S primers and visualized using an electrophoretic gel. All samples had successfully amplified (data not shown). We pooled the cDNA replicates from each whole-brain sample and standardized each cDNA sample to a concentration of 1 ng/μL.

Quantitative real-time PCR. We measured 12 components of the candidate physiological regulatory networks using qRT-PCR (Fig. 1). In whole brain, we quantified expression of the beta 2-adrenergic receptor (adrb2a) in the adrenergic network, three serotonin receptor subtypes (htr1a, htr2a and htr2b) in the serotonergic network and the dopamine receptor D2 (DR2) in the dopaminergic network. We also quantified the expression of the corticotropin-releasing factor receptor 2 (CRHR2), the glucocorticoid receptor 1 and 2 (GR1, GR2), and the mineralocorticoid receptor (MR) in the glucocorticoid network. In the pituitary, we measured the two proopiomelanocortin paralogs found in sticklebacks, pomca and pomcb, also part of the glucocorticoid network. In the interrenal gland, we measured the mc2r receptor, a component of the glucocorticoid network. We used previously published primers (Aubin-Horth et al. 2012) or designed them using cdna sequence from the Ensemble Genome Browser for Gasterosteus aculeatus [Ensembl ID, forward and reverse primer sequence, amplicon length and thermocycling temperature in Celsius in Table S1 (Supporting information)]. We verified PCR efficiency, the absence of primer dimers and the specificity of amplification for each primer pair using a qRT-PCR experiment and melting curves (50–90 °C) in a RealPlex2 instrument (Eppendorf) using a cdna standard curve consisting of 5 × 10-fold dilutions (of pooled samples) in duplicates. We calculated the efficiency using the formula \(E = 10^{(−1/\text{slope})} − 1\) (Pfaffl 2001) with the slope calculated from the relationship between the log cDNA quantity of a sample and its quantification cycle. We measured gene expression in experimental samples by qRT-PCR with a scaled-down version of the manufacturer’s protocol: 5 μL of cDNA, 7.5 μL of SYBR Green PCR Master Mix (Qiagen), 1.5 μL of nucelease-free water (Ambion) and 1 μL of primer pairs in a total volume of 15 μL, in a 384-well plate using an EPmotion 5075 pipetting robot (Eppendorf, Hamburg, Germany). Amplifications were carried out in a LightCycler 480 (Roche) (Boyle et al. 2009; Rutledge & Stewart 2010). We assayed all samples for a given gene on a single plate, such that interplate variation is not an issue. We report gene expression as the number of molecules in a sample calculated using the LRE method (Boyle et al. 2009; Rutledge & Stewart 2010).

Brain monoamines

Protein extraction. We extracted proteins from stickleback brains following a published protocol (Bell et al. 2007). We homogenized the whole brain (the pituitary was not included) in 100 μL of ice-cold 1% perchloric acid (PCA) containing 40 ng/mL DHBA as internal control on ice. We placed the sample in an ultrasonic bath for 10 min, centrifuged it at 15 800 g for 10 min at 4 °C and kept the supernatant at −80 °C for HPLC analysis. We kept the pellet separately at −80 °C to quantify the total protein content of the sample using a standard Bradford assay (Bio-Rad). We also extracted a negative control sample containing no brain tissue.

HPLC methods. We analysed extracts for norepinephrine (NE), serotonin (5HT), dopamine (DA) and their metabolites (MHPG, 5-HIAA, DOPAC, respectively), as well as their turnover rate by high-pressure liquid chromatography [HPLC, modified from Bell et al. (2007)]. We used a HPLC system model 1200 (Agilent) coupled with an electrochemical detector model 400 (EG&E Princeton Applied Research) equipped with a thin-layer flow cell (BASI) 0.002″ (51 μm) membrane spacer. We set the electrode potential at +0.6 V using 3-mm glassy carbon working cell with an Ag/AgCl reference. Briefly, we injected a 40 μL aliquot of extract into the reverse phase column (Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 5 μm, Agilent) at 25 °C. The mobile phase consisted of 14 g citric acid, 8.6 g sodium acetate, 110 mg 1-octanesulfonic acid, 150 mg EDTA disodium salt and 120 mL of methanol in 1 L of deionized water (Sigma-Aldrich, St. Louis MO) at a flow rate of 1.2 mL/min. We calibrated the instrument (2.5–100 ng/mL) for each component. We prepared solutions in 1% perchloric acid. We corrected results for dihydroxybenzylamine recovery, which was added as an internal control during the protein extraction step, as described in a previously published protocol (Bell et al. 2007). We measured concentrations of monoamines and of their metabolites in ng/mL. We corrected these values using the total protein content of the sample (mg/mL) and thus express the monoamine results as ng/mg. We calculated the turnover rate using the ratio of concentration of metabolites on the concentration of the monoamine.
A high ratio is indicative of elevated catabolic activity and has been interpreted as a high monoamine demand in the brain (Elipot et al. 2014; Loveland et al. 2014).

Cortisol levels

We extracted whole-body cortisol using a previously published protocol (Di Poi et al. 2016). Briefly, whole sticklebacks (after removal of brain, pituitary and interrenals) were homogenized in PBS (pH 7.4) using a hand-held homogenizer (Tissue Tearor, Cole-Parmer). After successive additions, the final volume of PBS was 2.5 mL. 5 mL of ethyl ether was added to all samples which were centrifuged at RT at 1480 g for 10 min and then frozen immediately at −20 °C for 2 h. The unfrozen upper phase (ether layer containing cortisol) was separated from the frozen layer (aqueous phase) at the bottom. The ethyl ether was evaporated under nitrogen for at least 1 h (2 h maximum). The remaining lipid extract was stored at −20 °C until analysis.

We used commercial enzyme immunoassay kits (Cayman Chemical’s ACE #00360) to quantify cortisol in the whole-body extracts of individual threespine stickleback reconstituted in EtOH 95%. Cortisol quantification was validated for use with diluted whole-body extracts (1/10 for freshwater fish; 1/25 for marine fish) using the methods of linearity and parallelism [full details on EIA validation in Di Poi et al. (2016)]. The interassay and intra-assay coefficients of variation were 13.7% and 9.7%, respectively. The estimated extraction efficiency of cortisol from whole body (following the cold spike protocol from Cayman chemical, which is based on comparing two aliquots of a sample, one spiked with pure cortisol and the other unspiked) was 107.0%. Results are expressed as ng/g of fish.

Statistical analyses

We performed all statistical analyses using R software version 3.0.2 (R Core Team 2013). We assessed normal distribution of data and homogeneity of variances using Shapiro–Wilk and Bartlett tests, respectively. Most variables (except cortisol levels) were not normally distributed even after transformation attempts, so we used a linear model for each variable using untransformed data, with P-values obtained by permutation tests [lmPerm package, Permutation tests for linear models in R (Wheeler 2010)]. We used these models to test for differences between the two populations. These models included population, sex and length of the fish as fixed effects. Cortisol levels were log-transformed prior to analysis to achieve normal distribution. We fitted a linear model with log-transformed cortisol data as the response variable using the lm function in R. We included population as the explanatory factor, as well as sex and fish length. We used the ‘ANOVA’ function from the Car package to test the null hypothesis of equal group means with the ANOVA table using a type III test. We verified assumptions about the residuals of the model using Shapiro-Wilk test and q-q plot. Only population effects are reported in the results section when there were no effects of sex or fish length. The entire data set is available as Table S2 (Supporting information).

Ethics statement

Wild animals were sampled under the Department of Fisheries and Ocean Permit #XR 315 2011 and the BC Ministry of Environment NA-SU-PE-10-63485 permit. The research adheres to the ASAB/ABS Guidelines for the Use of Animals in Research and was approved by the Comité de Protection des Animaux de l’Université Laval (permit #2010012-1).

Results

The twelve candidate genes from the adrenergic, serotonergic, dopaminergic and glucocorticoid physiological regulatory networks were expressed at detectable levels in sticklebacks (see mean expression levels for each population and complete statistical results in Table 1). All six candidate monoamines and metabolites were also detected in our samples (see mean concentrations for each population and complete statistical results in Table 2). Cortisol was at detectable levels in all samples.

Adrenergic network

We found higher expression of the adrb2a receptor in freshwater fish when compared to marine sticklebacks (P = 0.038, Fig. 2a, Table 1). No differences were observed between populations in NE monoamine levels, MHPG metabolite levels and NE turnover rate (Table 2). We found an effect of sex on NE levels (P = 0.04) and NE turnover rate (P < 0.0001). NE turnover rates were higher in males (mean = 0.30 ± 0.01, n = 13) than in females (mean = 0.23 ± 0.01, n = 21, Fig. 3b).

Serotonergic network

We found higher expression of the htr2a receptor (population: P = 0.0098) and lower expression of the htr2b receptor (population: P = 0.034) in the brain of freshwater fish (Fig. 2b and c, Table 1), but no differences
Values in bold are statistically significant.

**Table 1** Average gene expression levels (in number of molecules per sample ± SE, sample size in parenthesis) of ligand (L) and receptor (R) components of the adrenergic (A), serotonergic (S), dopaminergic (D) and glucocorticoid (G) physiological regulatory networks (PRN) for individuals of freshwater and marine origin, and statistical results for population, sex and length effects

<table>
<thead>
<tr>
<th>PRN and function</th>
<th>Gene</th>
<th>Freshwater mean expression</th>
<th>Marine mean expression</th>
<th>Pop. (P-value)</th>
<th>Sex (P-value)</th>
<th>Length (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-A</td>
<td>adrb2a</td>
<td>435 ± 38 (17)</td>
<td>286 ± 33 (19)</td>
<td>0.04</td>
<td>0.65</td>
<td>0.94</td>
</tr>
<tr>
<td>R-S</td>
<td>htr1a</td>
<td>1980 ± 204 (17)</td>
<td>1917 ± 149 (19)</td>
<td>0.94</td>
<td>0.50</td>
<td>0.96</td>
</tr>
<tr>
<td>R-S</td>
<td>htr2a</td>
<td>6004 ± 1026 (17)</td>
<td>4224 ± 543 (19)</td>
<td>0.01</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>R-S</td>
<td>htr2b</td>
<td>1981 ± 495 (17)</td>
<td>3845 ± 584 (19)</td>
<td>0.03</td>
<td>0.35</td>
<td>1.0</td>
</tr>
<tr>
<td>R-D</td>
<td>DRD2</td>
<td>3894 ± 352 (17)</td>
<td>2802 ± 239 (19)</td>
<td>0.02</td>
<td>0.34</td>
<td>0.96</td>
</tr>
<tr>
<td>R-G</td>
<td>CRHR2</td>
<td>2163 ± 272 (17)</td>
<td>2106 ± 296 (19)</td>
<td>0.86</td>
<td>0.02</td>
<td>0.68</td>
</tr>
<tr>
<td>L-G</td>
<td>pomca</td>
<td>4619 ± 1075 (12)</td>
<td>3028 ± 724 (16)</td>
<td>0.34</td>
<td>0.13</td>
<td>0.43</td>
</tr>
<tr>
<td>L-G</td>
<td>pomcb</td>
<td>10 376 ± 3846 (13)</td>
<td>13 874 ± 4063 (18)</td>
<td>0.52</td>
<td>0.47</td>
<td>0.96</td>
</tr>
<tr>
<td>R-G</td>
<td>Mc2r</td>
<td>3953 ± 402 (33)</td>
<td>2735 ± 363 (34)</td>
<td>0.01</td>
<td>0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>R-G</td>
<td>GR1</td>
<td>16 327 ± 1841 (17)</td>
<td>15 748 ± 1684 (19)</td>
<td>1.0</td>
<td>0.66</td>
<td>0.92</td>
</tr>
<tr>
<td>R-G</td>
<td>GR2</td>
<td>15 484 ± 2210 (17)</td>
<td>12 556 ± 1102 (19)</td>
<td>0.58</td>
<td>0.86</td>
<td>0.74</td>
</tr>
<tr>
<td>R-G</td>
<td>MR</td>
<td>15 848 ± 2117 (17)</td>
<td>16 683 ± 2705 (19)</td>
<td>0.78</td>
<td>0.77</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Values in bold are statistically significant.

**Table 2** Average levels of brain monoamines (ligands, L) and metabolites (in ng/mg), and turnover ratio (ratio of metabolite to monoamine) of components of the adrenergic (A), serotonergic (S) and dopaminergic (D) physiological regulatory networks (PRN) for individuals of freshwater and marine origin, and statistical results for population, sex and length effects. All sample sizes = 17 per group

<table>
<thead>
<tr>
<th>PRN and function</th>
<th>Measurement</th>
<th>Freshwater</th>
<th>Marine</th>
<th>Population</th>
<th>Sex</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-A</td>
<td>NE</td>
<td>3.16 ± 0.25</td>
<td>3.52 ± 0.29</td>
<td>0.82</td>
<td>0.04</td>
<td>0.57</td>
</tr>
<tr>
<td>A</td>
<td>MHPG</td>
<td>0.81 ± 0.06</td>
<td>0.86 ± 0.06</td>
<td>0.64</td>
<td>0.82</td>
<td>0.80</td>
</tr>
<tr>
<td>A</td>
<td>NE turnover</td>
<td>0.26 ± 0.01</td>
<td>0.26 ± 0.02</td>
<td>0.84</td>
<td>&lt;0.0001</td>
<td>0.19</td>
</tr>
<tr>
<td>L-S</td>
<td>5HT</td>
<td>1.16 ± 0.1</td>
<td>0.96 ± 0.07</td>
<td>0.08</td>
<td>0.98</td>
<td>0.29</td>
</tr>
<tr>
<td>S</td>
<td>5-HIAA</td>
<td>0.27 ± 0.02</td>
<td>0.27 ± 0.03</td>
<td>0.37</td>
<td>0.01</td>
<td>0.40</td>
</tr>
<tr>
<td>S</td>
<td>5HT turnover</td>
<td>0.24 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>0.16</td>
<td>&lt;0.0001</td>
<td>0.62</td>
</tr>
<tr>
<td>L-D</td>
<td>DA</td>
<td>0.37 ± 0.03</td>
<td>0.39 ± 0.03</td>
<td>0.98</td>
<td>0.23</td>
<td>0.71</td>
</tr>
<tr>
<td>D</td>
<td>DOPAC</td>
<td>0.01 ± 0.0</td>
<td>0.01 ± 0.0</td>
<td>0.84</td>
<td>0.02</td>
<td>0.23</td>
</tr>
<tr>
<td>D</td>
<td>DA turnover</td>
<td>0.04 ± 0.0</td>
<td>0.04 ± 0.0</td>
<td>0.84</td>
<td>0.02</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Values in bold are statistically significant.

between populations in htr1a receptor expression (Table 1), 5HT monoamine levels, 5-HIAA metabolite levels or 5HT turnover rate (Table 2). An effect of sex was observed on 5-HIAA levels (P = 0.01) and 5HT turnover rate (P < 0.0001). 5HT turnover rates were higher in females (mean = 0.29 ± 0.01, n = 21) than in males (mean = 0.21 ± 0.01, n = 13, Fig. 3c).

**Dopaminergic network**

We found higher expression of the DRD2 receptor in freshwater fish (P = 0.024, Fig. 2d, Table 1), but no difference between populations in DA monoamine levels, DOPAC metabolite levels or DA turnover rate (Table 2). We found an effect of sex on DOPAC levels (P = 0.02) and DA turnover rate (P = 0.02). DA turnover rates were higher in females (mean = 0.04 ± 0.0, n = 21) than in males (mean = 0.03 ± 0.0, n = 13, Fig. 3d).

**Glucocorticoid network**

The expression of the mc2r receptor was significantly higher in the interrenal glands of freshwater sticklebacks than in marine individuals (P = 0.011, Fig. 2e, Table 1). None of the measured components of the glucocorticoid network in the brain (CRHR2, GR1, GR2, MR) or the pituitary (pomca, pomcb) differed in expression between marine and freshwater sticklebacks, although mRNA could be measured (Table 1). Mean cortisol levels did not differ between marine (mean = 6.14 ng/g, SE = 0.71, n = 36) and freshwater (mean = 7.94 ng/g, SE = 1.62, n = 34) fish (population: P = 0.18; sex: P = 0.83; length: P = 0.06). We found an effect of sex on brain expression of CRHR2 (P = 0.02). Expression levels were higher in males (mean number of molecules = 2703 ± 363, n = 16) than in females (mean = 1678 ± 156, n = 20, Fig. 3a).
Discussion

An integrated view of physiological regulatory networks

Studying several components of a PRN allowed us to draw an integrated portrait of the evolution of network regulation and to show that measuring changes in receptors is as crucial as quantifying levels of ligand (Adkins-Regan 2008). At the evolutionary scale, house sparrow populations at a colonization front show divergent expression levels [MR/GR ratio (Liebl & Martin 2013)], and dark-eyed junco males from different subspecies that differ in ornaments show divergence in androgen receptor expression (Bergeon Burns et al. 2014). It has been shown that rainbow trout strains artificially selected to differ in their stress responsiveness also diverge in the expression of hormone receptors in the brain [MR (Johansen et al. 2011); CRHR2 (Backstrom et al. 2011)], that surface and cavefish populations of Astyanax diverge in the expression of several receptors in the brain (Strickler & Soares 2011) and that vole species with divergent affiliative behaviour show differences in spatial pattern of expression of the AVPRIA receptor (Young et al. 1997). We can predict that the change we observe in abundance of certain receptors in a physiological regulatory network where several receptor subtypes are present will have more specific effects than if changes would be found in levels of signalling molecules that could interact with several receptors (Adkins-Regan 2008). Indeed, a given ligand, such as cortisol or serotonin, could potentially bind to all the receptor subtypes present in a tissue, which will have potentially broadscale effects (McGlothlin & Ketterson 2008). In contrast, each receptor potentially has a particular and narrower cellular effect. The differential regulation of specific receptors rather than of a ligand thus allows for fine manipulation of phenotypes associated...
with a regulatory axis rather than large-scale pleiotropic effects (McGlothlin & Ketterson 2008; Cohen et al. 2012). Our results thus support the model that to study the phenotypic output of hormonal systems and how they evolve, quantifying hormonal titres is essential but it must be done in conjunction with measurements of receptors and of their downstream effects.

**Functional implications of the differential regulation of PRNs**

As we assayed several components in each PRN, we can make predictions about the potential total outcome of a given pathway, in each population. In the brain serotonergic network, we found that 5HT levels and the turnover rate of this monoamine did not differ between populations, while the expression levels of two of the three receptors did, in opposite direction (htr2a expression was higher in freshwater fish and htr2b expression was higher in marine fish). This suggests that the specific functions and signalling associated with these receptor subtypes could be affected in opposite directions in freshwater and marine individuals. Among the various receptor subtypes that mediate 5HT function, the htr2a receptors have been shown to directly affect reproductive behaviour when manipulated in fish (Smith & Combs 2008), and higher mRNA levels are found in the telencephalon of subordinate males compared to dominant ones (Loveland et al. 2014). The htr2b receptor is expressed in several organs in puffer fish (*Tetraodon fluviatilis*), including in the heart and brain (De Lucchini et al. 2001), although its function in fish brain remains unclear. Finally, we quantified expression of the htr1a receptor, which has been linked to aggressiveness in fish (Clotfelter et al. 2007; Filby et al. 2010), as well as to the modulation of anxiety (Connors et al. 2014). However, we found that this receptor was not differentially expressed between the two stickleback populations in the condition tested. In the dopaminergic physiological regulatory network, we found that the DRD2 receptor was more expressed in the brain of freshwater individuals. We could thus predict that this would result in higher signalling, which would be transmitted at the phenotypic level. The dopaminergic receptors have been associated with aggressiveness in many vertebrates, including in cavefish (Strickler & Soares 2011) and zebrafish (Filby et al. 2010). Our finding of higher expression of the DRD2 receptor in the brain of freshwater sticklebacks is consistent with their previously reported higher aggressiveness towards conspecific, lower sociability and higher activity than in individuals of marine origin (Di Poi et al. 2014).

We found that the adrenergic and glucocorticoid physiological regulatory networks, which are directly implicated in stress reactivity, were divergent between the marine and freshwater populations. The higher expression of the receptor subtype and not of the ligand associated with the adrenergic pathway suggests that
the total outcome would be specific. We found that freshwater sticklebacks have higher expression of beta-2 adrenergic receptors (adrβ2a) in the brain. These receptors are expressed in a variety of tissues in fish, including the brain [Pimephales promelas (Giltrow et al. 2011)], but their role in the central nervous system is less studied. Our results uncover a potential new association between this receptor and stress physiology, and suggest that the functional implication of their higher expression in more aggressive, more active, less social and less reactive fish (Di Poi et al. 2014, 2016) should be further studied. In the glucocorticoid network, we found higher expression of the mc2r receptor in the interrenal glands of freshwater fish. More receptors should result in a stronger signal for the interrenal glands to produce cortisol, the glucocorticoid hormone involved in the slower stress response. From our result, we would predict that freshwater stickleback would have a higher glucocorticoid stress response than marine ones. Although we found no significant difference in the mean poststress cortisol levels between the two populations in the present study, individuals from this same freshwater population have been previously shown to have a larger change in cortisol level from baseline to poststress level than individuals from the marine population (Di Poi et al. 2016). Linking measurements in the same individual of receptor levels and amplitude of cortisol level changes from baseline to levels following a stress would be necessary to establish a functional link.

In vertebrates, brain levels of monoamines are correlated with the manifestation of aggressive behaviour (Clotfelter et al. 2007; Filby et al. 2010). The absence of significant difference in noradrenaline, serotonin and dopamine brain levels that we quantified in the two divergent stickleback populations even though aggressiveness differences have been shown for these populations (Di Poi et al. 2014) contrasts with findings in other fish systems in which the evolution of neurochemical profiles was studied. For example, evolutionary divergence in aggressive response to a conspecific by blind cavefish and their surface-dwelling close relatives has been shown to be due to changes in the serotonergic network (Strickler & Soares 2011; Elipot et al. 2013), including changes in rates of degradation of monoamines (Elipot et al. 2014). Behavioural data suggest that aggressiveness and sociability in sticklebacks and blind cavefish have evolved between natural populations (Strickler & Soares 2011; Elipot et al. 2013), but comparatively to cave fish, it seems to be expressed in sticklebacks through alternative changes in the PRNs that have equivalent phenotypic effects [alternative physiological structures, as defined in Cohen et al. (2012)]. Furthermore, as with all experiments that focus on gene expression to infer protein levels in cells and that do not directly measure signalling activity of a PRN, we do not assess other levels of biological complexity that could affect the signalling outcome of the studied pathways, such as post-translational modifications or receptor turnover. We also could not make predictions based on specific spatial expression patterns, as we used whole-brain gene expression, which would have permitted us to connect our transcriptomic results more directly to specific neural circuits underlying physiological output. While we took the additional step of also measuring ligand levels by studying monoamines and circulating hormones, we could not address the potential effects of differential affinity of these ligands to different receptor subtypes, which could play a role in the measured physiological output of a PRN. Therefore, the absence of changes in certain components does not mean that these PRNs as a whole are not divergent between these populations, but only that the levels of biological complexity that we assessed were not.

The mechanisms of evolution

The population differences in gene expression in four physiological regulatory networks we measured in common environment-reared juveniles could be the result of nonmutually exclusive processes: natural selection and genetic drift. Molecular network regulation has been shown to diverge in new habitats with strongly different selection pressures, such as in the threespine stickleback (thyroid hormone network [Kitano et al. 2010], osmoregulation-associated genes [McCairns & Bernatchez 2010], thermal response [Morris et al. 2014]) and the blind cavefish (serotonin network [Elipot et al. 2013]). In the present study, if this divergence is the result of natural selection, one would predict a fitness advantage of having differentially active PRNs in the freshwater habitat and the same pattern should be found repeatedly in independent freshwater-marine population pairs. On the other hand, if genetic drift is the main force acting to result in the observed divergence, we should not observe convergent changes in PRN regulation in comparable freshwater habitats. To test this, one could study candidate behaviours from Di Poi et al. (2014) and gene expression uncovered in the present study in several other marine and freshwater population pairs. Our study is thus the first essential step in such a larger endeavour. Furthermore, analysing the phenotypic output of these PRNs will further connect these variations with fitness-relevant traits, which can also play a role in uncovering evolutionary forces at play.
As all fish were reared since the egg stage in freshwater, our experiment mimics the development of an individual of marine origin that has newly invaded a freshwater habitat. A different developmental response of the PRNs in marine individuals versus freshwater ones could result in the observed divergence in receptor expression. This genetic variation for the response to the environment (genotype-by-environment interactions) has been proposed to have major effects on the speed and direction of evolution in novel environments (West-Eberhard 2003; Morris et al. 2014). Quantifying PRNs of individuals reared in the opposite environment (freshwater fish reared in a marine environment) would allow defining the developmental reaction norms of these PRNs and to further address this question.

Overall, our results provide novel information on the natural variation in the molecular machinery in four interrelated physiological regulatory networks, which is crucial to understand evolution of hormone-mediated phenotypes (Williams 2008).

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N.A.H. and C.D.P. conceived the study, C.D.P. and N.A.H. performed tissue sampling, C.D.P. and N.A.H. performed laboratory protocols, D.B. performed the H.P.L.C. analysis, N.A.H. performed the statistical analyses, N.A.H. wrote the manuscript, S.C.R. and M.A. provided material, reagents and technical support, and all authors revised and approved the final text.

**Data accessibility**

The complete data set is available in tab-delimited text format as Table S2 (Supporting information).

**Supporting information**

Additional supporting information may be found in the online version of this article.

**Table S1.** cDNA sequence accession number from the *Ensembl Genome Browser*, forward and reverse primer sequence, ampli-con length in base pair and thermocycling temperature in Celsius.

**Table S2.** Dataset in tab-delimited text format.