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Evolution of stress reactivity in stickleback

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ABSTRACT

Hypothesis: The stress response involves a series of endocrine cascades that allow an individual to cope with real or perceived stressors to maintain a normal state. Although differences in stress response have been reported between populations facing distinct ecological challenges, whether these responses originate from genetic variation or environmental effects remains unclear. If the variation is genetically based, it could be the result of natural selection.

Question: Does variation in stress response reflect genetic variation?

Organism: Threespine stickleback (*Gasterosteus aculeatus*) originating from two contrasting habitats (a freshwater and a marine population).

Methods: We reared threespine stickleback originating from freshwater and marine populations in a common environment. We exposed them to a 30-minute confinement stress. We measured their ventilation rates during the first and thirtieth minutes of exposure. We also quantified whole-body cortisol concentration in fish from both populations assigned either to an undisturbed control group or to the 30-minute confinement stress.

Results: Marine stickleback exhibited significantly higher stress reactivity as inferred from the ventilation rates. Marine fish also showed higher inter-individual variation in reactivity than freshwater individuals. For all fish there was a strong response in cortisol concentration to the confinement stress. However, the extent of the response differed between the two populations, with freshwater fish showing a more marked change in cortisol concentration.

Conclusion: Genetic variation underlying divergence in stress reactivity is correlated with differences between marine and freshwater habitats.

Keywords: evolution, *Gasterosteus aculeatus*, natural selection, standing genetic variation, stress reactivity, threespine stickleback, ventilation rate.

INTRODUCTION

When an individual faces perturbations such as a storm, an intruder in its territory, or running to catch prey, a stress response is observed (Wingfield and Romero, 2001). This fitness-enhancing response is the main communication channel between the external stressor and the internal disrupted equilibrium that allows an animal to face this stressor. Although such

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stress responses are universal in vertebrates, whether and how an individual responds to a stressor can vary. Variation in stress reactivity has been proposed to be an adaptation stemming from divergent selection pressures in different habitats (Wingfield and Romero, 2001; Williams, 2008; Bell *et al.*, 2010; Dahl *et al.*, 2012). Furthermore, it has been suggested that individuals with high stress reactivity have a higher fitness in certain habitats (e.g. high predation), while individuals exhibiting low stress reactivity have a higher fitness in other conditions (e.g. low predation) (Øverli *et al.*, 2007; Bell *et al.*, 2010; Réale *et al.*, 2010).

An essential first step in determining whether these differences in stress physiology are adaptive is to confirm that between-population differences in stress reactivity are attributable to genetic variation (Williams, 2008). Rearing individuals from natural populations in common environments highlights the effects of genetic variation by minimizing environmental effects, both external and internal (Reid *et al.*, 1998), on the development of the stress physiological regulatory networks. Understanding if genetic differences affect variation in stress reactivity strengthens our knowledge of the ecology and the evolution of species by providing information on potential physiological adaptations that often form an integrated phenotype with, for instance, morphology and behaviour (Williams, 2008; Cohen *et al.*, 2012).

In this study, we wished to determine whether fish originating from isolated populations show genetic variation in their stress reactivity. Ventilation rate, measured immediately when an individual faces a stressor and again after a prolonged period of exposure, has previously been used as an estimate of stress reactivity [e.g. great tits (Carere and van Oers, 2004), salmon (Hawkins *et al.*, 2004)]. Furthermore, respiratory response has been used as a proxy to indicate acute stress in fish (Hawkins *et al.*, 2004; Barreto *et al.*, 2009; Bell *et al.*, 2010). Whole-body cortisol concentration has also been used as an estimate of the reactivity of the stress axis in fish (Yeh *et al.*, 2013). We studied threespine stickleback (*Gasterosteus aculeatus*) from marine and freshwater populations that have evolved separately for 10,000–15,000 years (McPhail, 1994) and that use widely different habitats. These marine (Oyster Lagoon) and freshwater (Hoggan Lake) populations have been shown to diverge in several behaviours (Di Poi *et al.*, 2014), physiological responses to temperature (Morris *et al.*, 2014), and cold tolerance (Barrett *et al.*, 2011). Fish were reared from hatching in a controlled freshwater environment, mimicking the environment marine fish would face when invading a novel freshwater habitat. We hypothesized that the two populations would differ in their stress reactivity when reared in common conditions. We quantified ventilation rate during a confinement stress (after 1 and 30 minutes) and estimated inter-individual variation in stress reactivity within these two populations. We also quantified whole-body cortisol concentrations in a separate group sampled from an undisturbed control or exposed to a 30-minute confinement stress from both populations.

MATERIALS AND METHODS

Populations, common environment rearing, and behaviour

We collected adult threespine stickleback from marine and freshwater populations on the Canadian West Coast of North America. Population genetic studies in threespine sticklebacks from this region have shown extensive gene flow between marine sampling sites as far as 1000 km apart on the West Coast of North America while extensive genetic divergence between marine and freshwater populations has also been documented (Hohenlohe *et al.*, 2010; Jones *et al.*, 2012a).

We collected breeding adults in two sites shaped by fundamentally distinct ecological characteristics. Oyster Lagoon (49°36′48″N, 124°01′47″W) has salinity ranging from 20 ppt in the winter to 32 ppt in the summer, while Hoggan Lake on Gabriola Island (49°09′08″N, 123°00′49″W) is a freshwater body isolated from other populations. Marine sticklebacks in Oyster Lagoon face a wide variety of fish, bird, reptile, and mammal predators (Saimoto, 1993). Hoggan Lake was stocked with rainbow trout (*Oncorhynchus mykiss*) and coastal cutthroat trout (*Oncorhynchus clarkii clarkii*) in 1927 (Ministry of Environment, 2014) and bird predation is likely.

We crossed adults to produce 15 full-sib F_1 families, seven from the freshwater population and eight from the marine population. 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-litre tanks under a 12/12 hour light/dark cycle, at a temperature of 18°C and salinity of 5 ppt. Similar rearing protocols with low salinity and uniform temperature for marine and freshwater juveniles have been used with success by others (Kitano *et al.*, 2010; Jones *et al.*, 2012b; Greenwood *et al.*, 2013). We fed them frozen bloodworms to satiation twice daily. We air-shipped juveniles (8 months old) to the Laboratoire de Recherche en Sciences Aquatiques at Université Laval. We held juveniles in 80-litre tanks (stocking density: 0.11 $g \cdot L^{-1}$) under similar rearing conditions with the exception that all fish were acclimated to 15°C with an 8/16 hour light/dark cycle (winter conditions). We kept juveniles originating from a given environment (marine or freshwater) together during early-life rearing, air transport, and later rearing at Université Laval, such that the information about the family of origin of individuals is not available. We measured body length at time of 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 a genetic sex marker (Peichel *et al.*, 2004).

Sampling was conducted under Department of Fisheries and Ocean Permit #XR 315 2011 and BC Ministry of Environment Permit NA-SU-PE-10-63485. The research adhered 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).

Stress reactivity as measured by ventilation rate

We characterized individual stress reactivity by measuring ventilation rate during a confinement stress that is known to induce a ventilation response in fishes [e.g. cichlids (Barreto *et al.*, 2009), stickleback (Bell *et al.*, 2010)]. In wild-caught stickleback, ventilation rates measured in a non-stress situation did not differ among individuals from 11 freshwater populations, while rates measured during a confinement stress did (Bell *et al.*, 2010). We thus focused on ventilation rates during a confinement stress. Prior to the stress reactivity test, we placed tested fish individually into 45-litre experimental tanks for 5 days under common temperature, salinity, photoperiod, and enrichment (plants and shelter) regimes as in group housing to characterize differences in behavioural phenotypes between the two populations (Di Poi *et al.*, 2014). On the day of the test (day 6 of isolation), we placed individuals in a 150-mL beaker with a low water volume (50 mL) ($n = 36$ per population). We filmed each individual from above using a digital camera (JVC model GZ-MS120). We counted the number of opercular movements in the first 60 seconds of the confinement stress test by watching the recordings at slow speed. Maximum ventilation rates have been shown to occur during the first 30 seconds of post-stress in wild Atlantic salmon juveniles (Hawkins *et al.*, 2004). Individuals were kept in the confinement beaker and covered by light-blocking material for a total of

30 minutes. At the end of this period, we measured the number of opercular movements during one minute to determine if the slope of the recovery approaching the baseline ventilation rate was the same for both populations. As we did not measure baseline ventilation rates in our study, we used baseline ventilation rates measured in wild-caught stickleback from 11 freshwater populations as an estimate, which averaged 72 beats per minute and did not differ between populations (Bell *et al.*, 2010). In addition, two individuals from Hoggan Lake were removed from the study, as samples were mislabelled ($n = 34$).

Whole-body cortisol

Sample preparation

Cortisol was measured from a total of 35 fish sampled from the rearing groups of both populations (different from the fish used to measure ventilation rates). The control treatment (no disturbance) contained 12 freshwater (Hoggan Lake) and 6 marine (Oyster Lagoon) fish, while the stress treatment had 11 freshwater and 6 marine fish. We euthanized individuals with buffered MS-222 (0.4 $mg \cdot mL^{-1}$) and collected the body (brain and interrenal glands removed) to measure whole-body cortisol concentrations. Whole fish were snap-frozen in liquid nitrogen in cryovials and kept at −80°C until cortisol extraction.

Whole-body cortisol was extracted using a modified protocol (Sink *et al.*, 2007; Peterson and Booth, 2010). Whole and frozen stickleback were thawed on ice, dried and weighed. Each carcass was kept in a 50-mL polypropylene tube (Falcon) containing 1 mL of PBS (pH 7.4) and homogenized on ice for 6 minutes with a tissue homogenizer (Tissue tearor homogenizer, Cole-Parmer). An additional 1 mL of PBS was added to the sample to rinse the homogenizer probe. The homogenized samples were vortexed briefly and transferred to a glass test tube (13 × 100 mm, Kimble) to facilitate extraction. The original tube was rinsed with 0.5 mL PBS and added to the larger tube to retrieve any remaining sample (i.e. total volume of PBS = 2.5 mL). Next, 5 mL of ethyl ether was added to each sample and vortexed for 1 minute. The samples were then centrifuged at 3000 rpm for 10 minutes at room temperature and frozen immediately at −20°C for 2 hours to separate the unfrozen upper ether phase (containing cortisol) from the bottom aqueous frozen layer. The upper layer was poured into a new glass test tube (13 × 100 mm) and the ethyl ether was allowed to evaporate under nitrogen for 1–2 hours using a rack evaporator (evap-o-rac, Cole-Parmer). The remaining lipid extract was stored at −20°C until analysis.

Enzyme immunoassay method

Cortisol in the whole-body extracts of individual fish was measured using a commercially available enzyme immunoassay (EIA) kit performed according to the manufacturer's instructions (Cayman Chemical's ACE #00360). Prior to the assay, the whole-body extracts were reconstituted in 50 μL of 95% EtOH, vortexed and placed in an ultrasonic bath (Bransonic) for 10 minutes. Buffer from the EIA kit was then added to each sample (950 μL) to use in the EIA assay. All microplates were read with a microplate reader (Spectramax M2e, Molecular Devices) following the manufacturer's protocol. Whole-body cortisol concentrations are presented as $ng \cdot g^{-1}$.

Cortisol quantification was validated for use with diluted whole-body extracts of three-spine stickleback (1/10 for freshwater fish; 1/25 for marine fish) using the methods of linearity of serial dilution of spiked samples ($R^2 = 0.965$ for freshwater fish; $R^2 = 0.970$ for marine

fish; Spearman correlation test with $P < 0.001$) and parallelism between slopes obtained by serial dilution of samples to the curve created with the kit standards using a linear model (slope comparison: $P > 0.05$ for both populations). The inter-assay and intra-assay coefficients of variation were 13.7% and 9.7%, respectively. The extraction efficiency (percent spiked recovery) of cortisol from whole-body extracts was 107%.

Statistical analyses

All data were analysed in R v.3.2.0 (R Development Core Team, 2013). We estimated the median ventilation rate and variance of ventilation rate for each population at each sampling time point. We tested for normal distributions of the ventilation rate data and for cortisol concentrations using a Shapiro-Wilk normality test and subsequently log-transformed these data sets to achieve a normal distribution. We performed a linear mixed-effect analysis of the relationship between population and ventilation rate during a confinement stress [package nlme (Pinheiro *et al.*, 2015)] on this log-transformed data. The effects on number of opercular movements per minute of population of origin, time of sampling, their interaction, sex, and fish length were assessed using a mixed model that also included fish identity as a random factor, to account for repeated measurements on the same individuals at two time points. We fitted a linear model with log-transformed cortisol data as the response variable using the lm function in R. We included population, treatment (control or facing a confinement stress) and their interaction as the explanatory factors, 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 tests and q-q plots.

RESULTS

Ventilation rate

During the first minute of confinement, the median number of opercular movements was 93 per minute in freshwater stickleback (range 74–124, variance = 159, $n = 34$) and 138 per minute in marine stickleback (range 86–182, variance = 417, $n = 36$), giving a 1.5-fold difference between populations (Fig. 1). Variance in ventilation rates between individuals of a given population during the first minute of confinement was significantly different between the two populations (F-test, $F = 0.3808$, $P = 0.006$). After 30 minutes of confinement stress, a 1.4-fold difference remained, as the median number of opercular movements per minute was 87 in freshwater stickleback (range 64–114, variance = 167, $n = 34$) and 114 in individuals of marine origin (range 52–160, variance = 746, $n = 36$). The linear mixed-effect analysis showed significant effects of population ($P < 0.0001$), time of sampling ($P = 0.03$), and their interaction ($P = 0.03$) on ventilation, while sex and length were non-significant (Table 1).

Cortisol concentration

Although both populations responded physiologically to confinement, the change in cortisol concentration was greater in freshwater than in marine fish (Fig. 2), which was reflected in the significant interaction term between population of origin and treatment ($P = 0.01$).

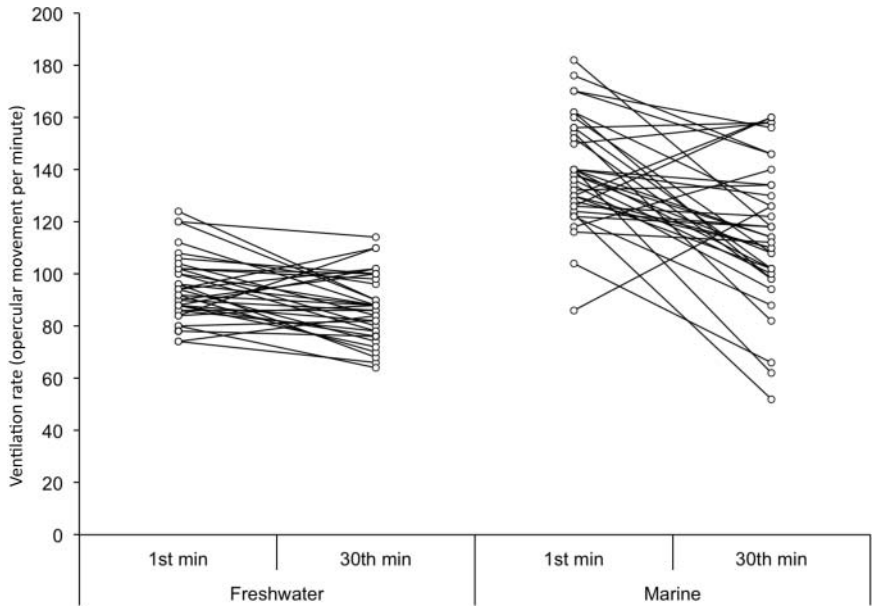

Fig. 1. Variation in ventilation response to a confinement stress in stickleback of freshwater and marine origins. Ventilation rate (number of opercular movements per minute) during the first minute of confinement and after 30 minutes is measured on the same individual (data points linked by a solid line) in laboratory-reared individuals.

Table 1. Results of linear mixed-effect model testing effects on ventilation rate (log number of opercular movements per minute) of population of origin, time of sampling and their interaction, sex, and fish length, with fish identity included as a random factor to account for repeated measurements on the same individuals at two time points

Factor	Value	Standard error	d.f.	*t*-value	*P*-value
Intercept	2.187	0.115	68	18.95	<0.0001
Population	0.202	0.039	66	5.22	<0.0001
Time	−0.037	0.016	68	−2.26	0.027
Sex	−0.013	0.015	66	−0.82	0.416
Length	−0.004	0.002	66	−1.52	0.134
Population × Time	−0.051	0.023	68	−2.22	0.030

Population of origin ($P = 0.03$) and treatment ($P < 0.0001$) had a significant effect on cortisol concentrations, whereas there was no effect of sex or length on cortisol (Table 2).

The complete data set is available at www.evolutionary-ecology.com/data/2964Appendix.txt, which is a tab-delimited text file.

DISCUSSION

In this study, stickleback of freshwater and marine origins differed in their response to a confinement stress when reared in a common controlled freshwater environment. Marine fish showed a higher ventilation response in the first minute of encountering the stressor

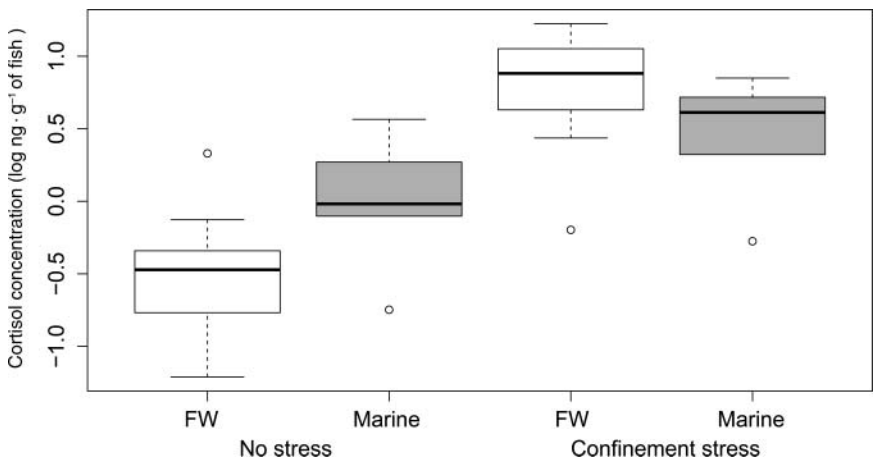

Fig. 2. Freshwater stickleback exhibit a larger cortisol response to 30 minutes of confinement than marine stickleback. Log cortisol concentrations ($ng \cdot g^{-1}$) in laboratory-reared individuals sampled in a baseline treatment (no stress) and in a 30-minute confinement stress. The dark line inside the box represents the median of the data distribution in each population, and the 25th and 75th quartiles are represented by the lower and upper limits of the box respectively. The bottom and top whiskers represent the most extreme data point, which is no more than 1.5 times the inter-quartile range. The small circles represent data outside this limit.

Table 2. Results of a linear model testing the effects on cortisol concentration ($\log ng \cdot g^{-1}$) of population of origin, treatment (control or facing a confinement stress) and their interaction, sex, and fish length

Factor	Sum of squares	d.f.	*F*-value	*P*-value
Intercept	0.22	1	1.283	0.267
Population	0.915	1	5.340	0.028
Treatment	9.238	1	53.910	<0.0001
Sex	0.004	1	0.021	0.886
Length	0.059	1	0.343	0.563
Population × Treatment	1.233	1	7.198	0.012

than freshwater individuals. After 30 minutes, mean ventilation rates of marine fish fell yet still did not match the mean value observed in freshwater fish. There was a significant interaction between population of origin and time of sampling on ventilation rates. Marine fish also showed significantly higher inter-individual variation in ventilation reactivity. On the other hand, the opposite pattern was observed for whole-body cortisol concentrations, with freshwater fish showing a larger mean cortisol response to 30 minutes of confinement than marine fish. Laboratory-reared individuals from both populations experienced the same freshwater environment, suggesting that these differences in physiological responses result from genetic variation in their stress response, a prerequisite for testing hypotheses about the evolutionary forces that are at play in this divergence.

The divergent stress reactivity we observed could result from different natural selection pressures in the populations of origin. Differences in reactivity between populations have

been associated with habitat characteristics, including in stickleback. Wild individuals sampled from freshwater populations facing low predation risk showed lower ventilation rates than freshwater individuals from high predation sites (Bell *et al.*, 2010). However, results on wild-caught individuals include genetic and environmental effects that cannot be dissociated. That fish of freshwater origin showed the lowest acute ventilation response in the present study is consistent with the predicted lower predation pressure in the population of origin compared with the marine stickleback (Di Poi *et al.*, 2014). Fish from the Hoggan Lake freshwater population also show a significantly lower tendency to school than marine individuals from Oyster Lagoon (Di Poi *et al.*, 2014). The isolation and confinement may thus be more stressful to a marine individual than to a freshwater one. On the other hand, the scope of the cortisol response to confinement was greater in freshwater individuals than in marine ones. This result suggests that fish from the two populations seemed to exhibit two different stress coping strategies. The strategy exhibited by the freshwater fish is characterized by a larger hypothalamus–pituitary–interrenal (HPI) axis reactivity (i.e. a combination of a high cortisol response and low ventilation), while the strategy of the marine fish is characterized by a combination of a low cortisol response and high ventilation. In trout, fish selected for a high cortisol response to a handling stress showed a lower increase in plasma epinephrine than trout selected for a low cortisol response (Schjolden *et al.*, 2006). However, our sample size for this part of the study was limited, so caution must be taken when we interpret this trend of the presence of different cortisol responses to stress in these two populations. Nonetheless, our results support the hypothesis that the confinement stress used elicits a clear glucocorticoid stress response in this species. Other physiological traits have been shown to diverge between marine and freshwater stickleback, including regulation of hormonal pathways, maximum oxygen consumption, and freshwater tolerance (Dalziel *et al.*, 2012; Divino *et al.*, 2016; Kitano *et al.*, 2010). A caveat of our study is that we sampled a single population from each environment, which means that the association we find between stress reactivity and habitat differences requires further testing. Furthermore, genetic drift could also be the cause of this difference between populations, and thus direct tests of the presence of selection are required. Finally, these physiological differences may result from epigenetic differences in the form of maternal effects affecting stress reactivity in offspring. More elaborate common garden designs are needed to experimentally elucidate the potential significance of these effects.

Variance in ventilation rates among individuals from a given population was also significantly larger in marine individuals than in freshwater ones in our study, suggesting that large standing genetic variation in the marine population may be a substrate for adaptation (Barrett and Schluter, 2008). All fish were reared at the same salinity from the egg stage. It is possible that the freshwater environment, which the marine individuals would face when invading or becoming trapped in a new inland habitat, affects the stress reactivity of marine individuals differently than that of individuals originating from a freshwater population that has evolved independently for thousands of years. We could take advantage of the large inter-individual variation in stress response in marine individuals to test the hypothesis that natural selection is acting on stress reactivity in the freshwater habitat, as done previously for cold tolerance in stickleback (Barrett *et al.*, 2011). For example, we could expose marine juveniles with a wide range of stress reactivity to *in situ* conditions in a freshwater habitat, and measure fitness in relation to individual stress reactivity. This would allow us to determine whether different ventilation rates affect survival, directly or indirectly, in stickleback. We could also compare the stress reactivity of several

independently evolved pairs of marine and freshwater populations (for example, on the East Coast of North America or in European populations) to potentially uncover convergent evolution, and further test the hypothesis that natural selection acts differently on stress reactivity in these divergent habitats.

Our results suggest a potential functional link between divergent stress reactivity and differences in behaviour previously quantified between these populations. Stickleback originating from the studied freshwater population have been shown to be more aggressive towards a conspecific, more active in their home environment, and to have a lower tendency to school with a group than stickleback originating from the marine population (Di Poi *et al.*, 2014). Stress reactivity measured as ventilation frequency has also been shown to be negatively associated with aggressiveness at the individual level in fish (Barreto *et al.*, 2009). However, wild stickleback from freshwater populations showing lower stress reactivity did not show higher aggressiveness on average (Bell *et al.*, 2010). An association between behavioural variation and basal or post-stress cortisol concentrations has been shown previously in fish using laboratory lines (Øverli *et al.*, 2007; Tudorache *et al.*, 2013) and in wild-caught individuals [aggression (Aubin-Horth *et al.*, 2012), shyness (Fürtbauer *et al.*, 2015)]. The association we observed between divergence in behaviour and in stress reactivity could be the result of a pleiotropic effect of a particular gene or gene product on physiology and behaviour, or of co-selection of stress reactivity type and behaviours (Sih *et al.*, 2004), as found for defence morphology and behaviour in stickleback (Lacasse and Aubin-Horth, 2012; De Winter *et al.*, 2016). Overall, the hormonal and neuronal causes of variation in stress reactivity in threespine stickleback, potentially associated with the adrenergic and glucocorticoid axes (Reid *et al.*, 1998; Wingfield and Romero, 2001), are prime candidates for functional validation in relation to the molecular basis of behavioural variation (Pavey *et al.*, 2012; Aubin-Horth, 2016).

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