A test of the coupling of predator defense morphology and behavior variation in two threespine stickleback populations

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Abstract Among-population differences in morphology and behaviors such as boldness have been shown to co-vary with ecological conditions, including predation regime. However, between- and within-population covariation of predator defense morphology with variation in behaviors relevant to ecology and evolution (boldness, exploration, activity, sociability and aggressiveness, often defined as personality traits when they are consistent across time and contexts) have never been quantified together in a single study in juvenile fish from populations found in contrasting environments. We measured predator defense morphology differences between adults from two freshwater populations of threespine sticklebacks with different ecological conditions. We then quantified five behaviors in juveniles from both populations raised in a common environment. Wild-caught adults showed significant differences in predator defense morphology. One population had significantly lower lateral plate number, shorter dorsal spine, pelvic spine and pelvic girdle. Furthermore, 61% of individuals from that population showed an absence of pelvic spine and girdle. At the population level, we found that differences in defense morphology in adults between the two lakes were coupled with differences in behaviors in juveniles raised in a common environment. Levels of activity, aggressiveness and boldness were higher in juveniles from the population lacking predator defense structures. At the individual level, anti-predator morphology of adult females could not predict their offspring’s behavior, but juvenile coloration predicted individual boldness in a population-specific manner. Our results suggest that ecological conditions, as reflected in adult predator defense morphology, also affect juvenile behavior in threespine sticklebacks, resulting in trait co-specialization, and that there is a genetic or epigenetic component to these behavioral differences [Current Zoology 58 (1): 53–65, 2012].

Keywords Behaviors, Personality, Predator defense morphology, Coloration, Juvenile, Threespine stickleback, Common rearing environment

Phenotypic divergence following colonization of new environments has been found in a myriad of animal and plant species (Schluter, 2000). Several environmental factors have the potential to act as selecting agents in these new environments, resulting in evolutionary changes in phenotype when phenotypic variation is underlined by genetic variation. Phenotypic divergence in a given population is often found for several types of traits simultaneously. Morphological trait divergence is frequently reported as an adaptation to a new environment. These adaptations are often correlated with behavioral and physiological changes and uncovering the relationship that connects these biological levels of organization is thus crucial to understand the process of adaptation in natural populations (Bertossa, 2011).

Threespine stickleback Gasterosteus aculeatus is one of the model species in the study of phenotypic divergence in new ecological conditions. Postglacial stickleback radiations have led to significant phenotypic variations (McKinnon et al., 2002). After the last glacial retreat, 12 000 years ago, marine stickleback underwent repeated episodes of colonization in freshwater (Lauder, 1995), which resulted in evolutionary changes in morphology along with other physiological and behavioral changes (Barrett et al., 2011; Bell et al., 1994; Greenwood et al., 2011; Kitano et al., 2010; Wark et al., 2011). Typically, marine fish have a larger number of lateral bony plates, while freshwater populations show a reduction in the number of these plates (Bell, 2001). These differences are often accompanied by a reduction in the presence and size of other morphological structures, such as pelvic and dorsal spines, as well as of a pelvic girdle (reviewed in (Schluter et al., 2010)). Differences can also be observed between freshwater populations, as the loss of defensive structures can be much higher in some environments than others and differ
in occurrence between regions within the northern hemisphere, e.g., Quebec (Edge et al., 1983), British Columbia (Moodie et al., 1973), Scotland (Campbell, 1979), Spain (Fernandez et al., 2000), Norway (Klepaker et al., 2008)). Inter-population variability in these morphological features is associated with environmental factors such as predation level (Marchinko, 2009; Reimchen, 1994), calcium concentration (Bell et al., 1993) or salinity (Heuts, 1947; Marchinko et al., 2007). Furthermore, it has been shown that differences in predator defense morphology have a genetic basis (Colosimo et al., 2004) and specific genes have been shown to harbor variation associated with these different phenotypes (PitxI- involved in pelvic reduction (Shapiro et al., 2004); EDA - involved in lateral plate change (Colosimo et al., 2005)).

Inter-population variability in behavior has also been uncovered in sticklebacks. When differences in behavior between individuals is consistent across time and contexts, it is defined as a personality trait ((Gosling et al., 1999; Sih et al., 2004). Aggressiveness, sociability, activity, exploration and boldness are among the behaviors that have been found to vary between and within populations in a consistent manner in several species. The average value of a personality trait has been found to vary between populations and to co-vary with ecological differences. In many species, differences in predation pressure are correlated with different mean levels of aggressiveness (Archard et al., 2011; Herczeg et al., 2009a; Riechert et al., 2000), sociability (Herczeg et al., 2009b; Magurran et al., 1991) and boldness (Biro et al., 2004; Brown et al., 2005; Wilson et al., 1993). In sticklebacks, behavior variation in adult individuals, such as aggressiveness and boldness, have been associated with differences in predation levels between populations (Alvarez et al., 2007; Huntingford, 1982). This divergence in behavior may reflect genetic variation or environmental variation between the populations, as environmental variation within the life of an individual may also lead to personality variation. For example, predation pressure applied to a predator-naive population leads to changes in aggressiveness in sticklebacks (Bell et al., 2007). The influence of these two factors must therefore be untangled. In juvenile three-spine stickleback, behavior of an individual varies during development and between populations (Bell et al., 2004). Inter-population variability in aggressiveness in juveniles is associated with variation in predation level between different populations (Dingemanse et al., 2007) and juveniles originating from marine and freshwater sticklebacks populations differ in sociability (Wark et al., 2011).

Divergence in morphology and behaviors between environments with different predation levels may be coupled at the species, population and/or individual level, or may be independent (DeWitt et al., 1999). For example, at the species and population levels, higher average boldness of individual may be found in populations with the strongest anti-predator morphology (Mclean et al., 1989), a correlation obtained through trait compensation (DeWitt et al., 1999). On the other hand, species or populations with the strongest anti-predator morphology can also show the strongest behavioral response to predator pressure, a correlation resulting in trait co-specialization (DeWitt et al., 1999). Similarly, at the individual level, individuals with the strongest predator defense morphology may be the ones that are the boldest within a population or vice-versa (DeWitt et al., 1999).

Sticklebacks vary both in morphology and behaviors within and between populations, allowing testing such coupling between these two types of traits. At the population-level, no covariation was found between anti-predator morphology and risk-taking behavior when juveniles from freshwater benthic populations of sticklebacks with different probability of predation and varying levels of anti-predator morphology were compared (Grand, 2000). Also at the population level, the proportion of juveniles using an open water (shelter less) habitat was significantly lower in a population without pelvic spines than in a population with pelvic spines, when faced with the threat of predation by larger conspecifics (Sillett et al., 2000), suggesting trait compensation. At the individual level, sticklebacks with different lateral plate numbers also showed differences in foraging behavior (Ostbye et al., 2010). However, no study has simultaneously investigated the population-level and individual-level coupling of predator defense morphology with five behaviors (personality traits) of juvenile threespine sticklebacks from populations facing contrasting environments.

Our objective was to quantify predator defense morphology and behavior in two freshwater populations of threespine sticklebacks from neighboring lakes located in Quebec, Canada (Témiscouata and Rond Lakes). We hypothesized that difference in the number of predatory fish species potentially results in a lower probability of piscine predation in Rond Lake than in Témiscouata Lake. Accordingly, sticklebacks from Rond Lake sampled in the 1980s have been shown to have reduced pelvic structures (Edge et al., 1983), supporting the hypothesis of lower piscine predation probability in Rond
Lake. However, no morphological measurements have been made recently in Rond Lake and no other population has been studied in that region. We first measured six morphological traits in adults: standard length, number of plates, spine length (two dorsal spines and pelvic spines) and pelvic girdle length. At the population level, we predicted that defensive morphology would be associated with the probability of predation, with Témiscouata fish showing a higher number of plates and complete spines and Rond lake fish still showing the reduction in defense morphology found 30 years ago by Edge and Coad (1983). We asked whether these morphological differences were coupled with behavioral differences. We measured five behaviors in juveniles reared in a common-environment. Based on the expected predation pressure in the two lakes studied and findings from previous behavioral studies, we predicted that boldness, activity, exploration and aggressiveness would be higher in Rond Lake (Archard et al., 2011; Bakker, 1986; Bell et al., 2004; Brydges et al., 2008), and that sociability would be higher in Témiscouata Lake (Magurran et al., 1991), supporting the hypothesis of trait co-specialization. At the individual level, we tested if female anti-predator morphology could predict her offspring’s behavior and if juvenile coloration was coupled with juvenile behavior.

1 Materials and Methods

1.1 Sampling

The two lakes studied (Témiscouata: 47° 48’ 29” North, 68° 52’ 2” West; Rond: 47° 31’ 0.1194” North, -50° 50’ 5” West) are distant by 3km and globally similar (same ecosystem, same weather, and same latitude) but differ in some characteristics. Rond Lake is characterized by a higher altitude (Rond Lake: 226m, Témiscouata Lake: 149m), is smaller in size (Rond Lake: 1.3 km², Témiscouata Lake: 67 km²) and has a shallower maximum depth (Rond Lake: 25 m, Témiscouata Lake: 74 m). Rond Lake has only one other known species in addition to threespine sticklebacks, *Pimephales promelas*, a non-predatory cyprinid. Brook trout *Salvelinus fontinalis* was also released in the lake in 1976 (SIFA database, Quebec Minister of Natural Resources and Wildlife) although their actual numbers or presence is unknown. In contrast, Témiscouata Lake contains about twenty different species, including common predators of sticklebacks, such as salmonids *Coregonus clupeaformis*, *Salvelinus fontinalis* and perch, *Perca flavescens* (Reimchen, 1994). Bird predation is unknown, but we assume similar predation pressure, as the two lakes are 3 km apart. Insect predation is unknown for both lakes. Adult threespine sticklebacks were collected from both lakes during the reproductive period in June 2010 using minnow traps. Females were then gently squeezed to expel eggs. Seven females from Témiscouata Lake and 5 females from Rond Lake were used. Sperm was collected from sacrificed males by dissecting out the testes. Seven males from Témiscouata Lake and 9 males from Rond Lake were used. Eggs from each container were artificially fertilized with the collected sperm for a total of 9 families from each population (Fig. 1). Fertilized eggs were transported on ice to the “Laboratoire Régional des Sciences Aquatiques” (LARSA) at Laval University, Québec. Fertilized eggs from a given cross were placed in Petri dishes with sterilized 5ppm artificial seawater in an incubator maintained at 18°Celsius with a photoperiod of 12:12 (summer conditions at the original population’s latitude). In each Petri dish, density of eggs varied depending on number of eggs in the clutch. Each day, eggs were checked and dead eggs were removed. No survival difference between the two populations was found. Eggs were washed with 5ppm-sterilized seawater twice daily. When the fry were approximately one cm, we transferred them to 2.8 l rearing tanks (10×20×15 cm (Aquaneering # ZAS550; modular system with central filtration) at a density of approximately ten fish per tank (mean±SE, 10±5), keeping individuals from the same brood together. A total of 25 rearing tanks were used. Temperature and photoperiod were the same as in the incubator. Fry were fed brine shrimp twice daily by hand (10 am and 2 pm). When fry were approximately 2 cm, frozen bloodworms were added to their diet. Fish were subjected to a diet of 4% of the biomass (approximately four bloodworms per individual).

When juveniles reached a size of about 25 mm, behavioral experiments began. The experiment was conducted from November 8 to December 14, 2010, as several series of experiments were performed to reach the final sample size. Fish were thus aged between 138 and 177 days at time of sampling and age was included as a factor in data analyses. Each fish tested (n=56/population) was exposed to five behavioral tests over a 3-day period. In total, there were 7 series of 3 days (8 fish per population tested per series, for a total of 16 fish per series). An average of five fish per family was used, with 9 families tested per population. All tests were done at the same time of day. Fish were fed at the same time and with the same methods as previously described for rearing. On the first day, sociability and
exploration were tested. On the second day, activity was tested. Finally, on the third day, aggressiveness towards a conspecific and boldness were tested. Each behavioral assay was filmed with a digital camera (JVC model GZ-MS120) mounted in front of the tank. The tanks were drained, cleaned and filled with water again between each series.

1.2 Morphology

Adults  Samples of adult threespine sticklebacks were collected from Rond ($n=28$) and Témiscouata Lakes ($n=48$). Adults were used to evaluate morphological variation between populations, as defensive structures only appear completely when fish grow to at least 30 mm (Hagen, 1973) and were thus not quantifiable on common-garden reared juveniles which were 25 mm in length at time of sacrifice (see section above). Furthermore, adult males used for artificial crosses could not be used for morphological analyses, as dissection to obtain gonads destroyed important morphological traits such as the pelvic girdle and pelvic spines. We obtained measurements for females used in artificial crosses. Adult fish were anesthetized in buffered MS222 (40 mg/100ml, Tricaine methanesulphonate; Sigma Aldrich #E10521). The body was placed in 95% ethanol until morphological analysis. Individuals were stained with alizarin red to detect skeletal structures (Peichel et al., 2004) and were digitally photographed (Canon powerShot A720 IS, Fig. 2) along with a size marker used in every picture. Image J (http://rsbweb.nih.gov/ij/) was used for morphological measurements including: standard length, first and second dorsal spine length, pelvic spine length, pelvic girdle length and number of lateral plates recorded from head to tail on the right side (see Fig. 2 in (Marchinko, 2009) for details). Lateral plate morphs were defined as low (less than 10 plates), partial (10-30 plates) or complete (> 30). We used logarithmic transformations (see below) to standardize the length of spines using individual length and mean standard length (58mm) following (Thorpe, 1975).

$$\hat{y}_i = 10^{\frac{y_i - b(\log_{10}x_i - \log_{10}X)}{10}}$$

Where $\hat{y}_i$ is the adjusted character value

$y_i$ is the logarithm of the adjusted character value of the $i^{th}$ specimen

$x_i$ is the body length of the $i^{th}$ specimen

$X$ is the mean of the body lengths of two populations

Juveniles  Fish were randomly selected in different families (13 fish / day of measurement / population) to quantify weight. Mean weight was then used to estimate

| Rond Lake | | | |
|---|---|---|---|
| Female | Male | Juveniles tested |
| FR3 | MR2 | 9 |
| FR3 | MR3 | 5 |
| FR5 | MR5 | 4 |
| FR7 | MR7 | 5 |
| FR7 | MR8 | 10 |
| FR7 | MR9 | 8 |
| FR7 | MR10 | 5 |
| FR8 | MR11 | 5 |
| FR8 | MR12 | 5 |

| Témiscouata Lake | | | |
|---|---|---|---|
| Female | Male | Juveniles tested |
| FT2 | MT2 | 10 |
| FT3 | MT3 | 5 |
| FT4 | MT7 | 5 |
| FT7 | MT8 | 8 |
| FT9 | MT11 | 8 |
| FT10 | MT14 | 5 |
| FT12 | MT15 | 5 |

![Fig. 1](https://example.com/fig1.png) Artificial crosses made for each population

![Fig. 2](https://example.com/fig2.png) Morphological variation between sticklebacks from the Témiscouata Lake (above) and Rond Lake (below) populations

Bone elements were stained with alizarin red. Same scale is used for both individuals.
growth rate (g/day) for each population. Weight was measured three times in each population; at day 57, 85 and 113 post-fertilization. After the behavioral testing period, juveniles were terminally sampled by an overdose of anesthetic and a picture of each fish was taken including a green and gray scale. Dorsal and ventral coloration was then calculated relative to these scales with Image J. Bodies were dissected for further experiments without measurement of predator defense morphology (see above), as these features are only fully developed at a larger size (Hagen, 1973).

1.3 Behavioral assays

Five personality traits have been studied in many species of vertebrates: aggressiveness, sociability, activity, exploration and boldness. Each of these behaviors has been shown to vary between populations and to co-vary with ecological differences, including predation pressure. These five behaviors have been studied both in nature and in the laboratory (Stamps et al., 2010). Repeatability of these behavioral measures was also demonstrated in various species (Reale et al., 2007) including threespine sticklebacks (Aubin-Horth et al., 2011; Dzieweczynski et al., 2011). We thus studied these five behaviors without measuring their repeatability in our populations.

Sociability  Sociability is a measure of the tendency of fish to shoal by joining a group of conspecifics. To measure the level of sociability, we used an assay based on a school of model sticklebacks (Wark et al., 2011). A motorized mobile was constructed on which artificial flexible fish were hung using invisible thread. This mobile reproduced a school of five fish with a similar size (3 cm) and color (olive green) to juvenile sticklebacks. The artificial school was immersed 10 cm under the water surface, 10 cm from the bottom, in an 80 L tank. The focal fish was added in the tank. Following an acclimatization period of 15 minutes, a rotating motor connected to the mobile was turned on to move the fish school in a circle around the tank, during 15 minutes. The focal fish’s behavior in the tank was filmed during these 15 minutes from above the tank. The time-spent swimming within one body length of the group in the same direction was quantified as sociability. After the test, each fish was placed in a new observation tank in which it remained throughout the rest of the behavioral experiment series.

The individual observation tanks had a volume of 45 l (61×25×10 cm) and water temperature and light regime were kept the same as in the group tanks. The individual tanks contained artificial vegetation and a refuge. A single focal fish was placed in this observation tank. During the whole series of behavioral experiments, focal fish were checked each day for signs of stress or disease.

Exploration  Exploration is a measure of an individual’s reaction to a new environment. Immediately after the sociability test, the focal fish was moved into the individual observation tank. The fish was placed in a 7 cm diameter opaque container with an open top, which was then introduced into the tank. Latency to exit the container was quantified as a measure of exploration propensity. Ten minutes after introduction, the container was removed. If the fish was still in the container, it was given the maximum possible latency score. The fish was observed for 5 minutes after removing the container. Time spent swimming in this new environment after leaving the container was used as a measure of activity in an unfamiliar context (see “activity” below) (Bell et al., 2004).

Activity  Activity was measured as the general level of activity in a familiar environment during the second day of test. The movements of each subject were filmed during a 5-minute period. Time spent moving was measured at 10:00 and 14:00. The two time measurements were summed and the combined score was used as a measure of activity. This measure was compared to activity measured in an unfamiliar environment on day 1.

Boldness  Boldness is a measure of the level of risk taken by the individual in an uncertain situation that could affect fitness. During the afternoon feeding session, a simulated predator attack was performed on the focal fish while it was feeding, using a replica of a great blue heron head Ardea Herodias, a natural predator of stickleback (Reimchen, 1994). When the focal fish approached within at least one body length of the food provided by hand of the observer, a heron attack was simulated by striking the water directly over the area where food was distributed. The beak of the great blue heron was plunged about 5 cm below the water surface. The beak was moved in the water for 10 s (Bell, 2005). Behaviors were quantified during 5 minutes following the attack. All fish reacted to the predator attack and could thus be used in the analysis. Latency to resume feeding was used as a measure of boldness.

Aggressiveness  Aggressiveness was measured using the reaction against an intruder. A fish of the same population was placed in a 1L transparent glass container located in the observation tank and the resulting interactions were quantified for a period of 5 minutes.
The glass container was placed in the tank the day before the test to eliminate the effect that could be induced by the introduction of a new object. The intruder and the focal fish were of similar size. The number of bites made by the focal fish against the container where the intruder was introduced was used as a measure of aggressiveness (Huntingford, 1976). At the end of the observation period, the glass containing the intruder was removed from the observation tank. At least eight different fish from each population were used as intruder for each series (16 intruders). In a series, each intruder was used only once. Intruders were re-used for each series.

Fish were terminally anesthetized in buffered MS-222 (40 mg/100ml). Weight and standard length were measured and a picture was taken for morphology measurements (see above). The caudal fin was placed in 95% ethanol to be used for sex determination.

1.4 Sex determination

Genetic sex was determined by genotyping each individual at a specific genetic sex marker for stickleback (Peichel et al., 2004). DNA was extracted from each fin using a salt extraction of genomic DNA (Aljanabi et al., 1997).

All of the procedures were carried out according with national regulation on animal welfare from Canadian Council on Animal Care and local regulation from the CPAUL (Comité de protection des animaux de l’Université Laval, # 2010-066)

1.5 Data Analysis

All statistics were performed in R 2.10.0 (http://www.r-project.org). Normality of behavioral and morphological measures was tested by a Shapiro-Wilk test. For morphology, a student t-test was used to compare means between populations when data was normally distributed or a Mann-Whitney test if data was not normally distributed and could not be transformed. We used a linear mixed model to test for behavioral differences between the two populations. Population, sex and age were included as fixed effects while maternal and paternal identities (and their interaction) were included as random effects. A Ward test was used to measure random effects on juveniles with the same maternal and paternal identity and Pearson correlations to measure correlation. To investigate the influence of morphology on behavior, we used a principal component analyses (PCA) to summarize related morphological traits measured on the females used in the artificial crosses into a single principal component score. We included 6 morphological traits: standard length, number of lateral plates, first and second dorsal spines length, pelvic girdle length and length of pelvic spine. We then used a linear mixed model to determine if juvenile behavior varied according to their mother’s morphology, summarized as a PCA component score. We used the same method to summarize juvenile coloration (green and grey color) in a principal component axis. We then correlated the principal component axis of juvenile coloration with juvenile behaviors.

2 Results

2.1 Morphology

Edge and Coad (1983) showed that the Rond Lake population had 10% of individuals with low lateral plate number (less than 20 plates), as well as 100% of individuals with incompletely developed or absent pelvic structures when sampled in 1981–1982. We show that, 30 years later, 100% of adults sampled in Rond Lake were partially plated (average number of lateral plate = 16, range 11–21) and that in 61% of individuals, pelvic girdles and pelvic spines were absent (Table 1). The low anti-predator morphology is thus still present in this lake. In comparison, adults in Témiscouata Lake had significantly greater mean standard body length and a higher number of lateral plates (average = 24, range 15–29), also corresponding to a partially plated morph. Significantly shorter first and second dorsal spines were found in Rond Lake (Table 1). When pelvic girdle and pelvic spines were present in Rond Lake fish (39% of individuals), they were significantly smaller than those from Témiscouata Lake (Table 1). All differences were P<0.001.

| Population     | Standard length (mm) (Means ± SD) | Plate number (Means ± SD) | First dorsal spine (mm) (Means ± SD) | Second dorsal spine (mm) (Means ± SD) | Pelvic girdle (mm) (Means ± SD) | Pelvic Spine(mm) (Means ± SD) |
|----------------|------------------------------------|---------------------------|--------------------------------------|--------------------------------------|-------------------------------|--------------------------------|
| Rond           | 49.4±3.49                          | 16.04±2.57                | 3.68±0.62                            | 4.50±0.65                            | 11.64±0.76*                   | 4.55±0.64*                     |
| Témiscouata    | 67.05±5.87                         | 24.19±2.28                | 5.93±0.66                            | 7.09±1.00                            | 14.70±1.34                   | 8.57±0.84                      |
| P-value        | <0.001                             | <0.001                    | <0.001                               | <0.001                               | <0.001                        | <0.001                        |

Dorsal and pelvic spines and pelvic girdle sizes are given as the standardized size in a table (calculated following Thorpe, 1975). Asterisk (*) corresponds to value for Rond Lake fish with pelvic structures (n=11) as they are absent for 61% of the individuals in that lake.
Average growth rate was 0.0027 g wet day⁻¹ in Témiscouata Lake and 0.0008 g wet day⁻¹ in Rond Lake between day 57 and 85, while between day 85 and 113 it was -0.001 g wet day⁻¹ in Témiscouata Lake and 0.0018 g wet day⁻¹ in Rond Lake. These average growth rates for the population could not be compared statistically as a single data point was obtained for each population. Individual standard length and weight of juveniles at the time of the behavioral experiment differed between the two populations. In both lakes, the dorsal region was green while the ventral region was grey. However, in Rond Lake, the dorsal and ventral regions were significantly darker than in the Témiscouata population (student t-test: $t=7.71$, $P<0.001$ and $t=10.26$, $P<0.001$ respectively).

### 2.2 Behavior

We observed behavioral divergence between fish from Rond Lake and Témiscouata Lake in juveniles raised in a common environment, (Fig. 3). Fish in Rond Lake were more active and more aggressive than fish in Témiscouata Lake. Aggressiveness measured as the number of bites to an intruder was significantly higher in Rond Lake (mean = 37.75 bites, 95% CI = 8.60) than in Témiscouata lake (mean = 16.94 bites, 95% CI = 5.76) (Linear mixed model: $P<0.001$). All individuals had at least oriented to conspecifics during the test. Activity measured as the time spent swimming in a familiar environment was also higher in Rond Lake (mean = 223.94 sec, 95% CI = 24.33) than in Témiscouata lake (mean = 141.36 sec, 95% CI = 33.48) (Linear mixed model: $P=0.022$). Activity was measured in two contexts: a familiar and a novel environment. We found that the two measurements were significantly correlated (spearman correlation: $r=0.312$, linear mixed model: $P<0.001$). Boldness measured as the latency to resume feeding after a predator attack was twice as high in Témiscouata Lake (mean = 141.36 sec, 95% CI = 33.48) than in Rond Lake (mean = 65.91 sec, 95% CI = 24.33),

**Fig. 3** Behavioral variation between juvenile populations

A. Boldness; latency to resume feeding after a predation attack, in seconds. B. Exploration; latency to exit shelter, in seconds. C. Activity; time in movement in a familiar environment, in seconds. D. Aggressiveness; number of bites. E. Sociability; time spent following the group, in seconds. Bars represent the mean and error bars indicate 95% confidence intervals.
such that Rond fish were bolder, but this difference was not statistically significant after taking parental identity into account (Linear mixed model: $P=0.42$). In this test, all the fish responded to the predator attack by going into hiding in the shelter. Exploration measured as the latency to exit a refuge did not differ between populations (Rond: mean = 343.67 sec, 95% CI = 50.29; Témiscouata: 273.24 sec, 95% CI = 39.88) (Linear mixed model: $P=0.215$). Finally, fish showed low schooling in both lakes, measured as time spent swimming within one body length of the group in the same direction (Rond: mean = 157.42 sec, 95% CI = 47.73; Témiscouata: mean = 143.49 sec, 95% CI = 51.22) which represent an average of 16.72 % of the time spent schooling. Sociability was not significantly different between the two populations (Linear mixed model: $P=1.00$).

Sex and age effects were also found for some behaviors. In both lakes, aggressiveness increased with age (Linear mixed model: $P<0.001$). Males and females in the Témiscouata population were not different in aggressiveness, but females in the Rond population were more aggressive than males (Linear mixed model: $P<0.004$).

Random effects measured on the five behaviors showed a strong maternal effect (Table 2). For each behavioral trait, we observed an effect of female identity on her offspring’s behavior, 4 months after hatching in a controlled environment and without parental care. Sociability was also influenced by the identity of the male used in the artificial crosses. Activity was also affected by a maternal and paternal identity interaction.

### 2.3 Coupling between morphology and behaviors

The six morphological traits measured on the wild adult females used in the artificial crosses could be summarized by a single component axis, PC1, which explained 95% of morphological variations. We confirmed the differences observed between lakes for each morphological trait with a chi-squared test on PC1 (chi-squared test, $P<0.001$). Morphological traits summarized as PC1 values for each female could not predict any of their offspring’s’ behaviors: sociability (linear mixed model including population and the principal component as fixed factors and maternal and paternal identity as random factors: $P=0.121$), aggressiveness (linear mixed model: $P=0.910$), boldness (linear mixed model: $P=0.508$), activity (linear mixed model: $P=0.149$) and exploration (linear mixed model: $P=0.358$).

Juvenile coloration could predict boldness (Linear mixed model: $P<0.001$) and this relationship differed between the two populations. In Témiscouata Lake, relation between juvenile coloration and boldness was negative (Spearman correlation: $r=-0.27$) such that darker individuals were bolder. In Rond Lake, juvenile coloration and boldness were positively correlated (Spearman correlation: $r=0.46$), such that paler individuals were bolder. Other behaviors were not correlated with juvenile coloration: sociability (Linear mixed model: $P=0.557$), aggressiveness (Linear mixed model: $P=0.586$), activity (Linear mixed model: $P=0.286$) and exploration (Linear mixed model: $P=0.903$).

### 3 Discussion

#### 3.1 Population-level coupling of morphological and behavioral traits

We studied two populations that have diverged from a common ancestor after sticklebacks invaded freshwater in the northern hemisphere 12,000 years ago, following the last glacial retreat (Lauder, 1995) to test the presence of coupling of antipredator morphology and behavior. In several species including threespine stickleback and relatives, differences in predation pressure between populations are correlated with different morphology (Marchinko, 2009) and different levels of aggressiveness (Herczeg et al., 2009a; Riechert et al., 2000),

| Random effects on the five behavior traits | Random effects |
|--------------------------------------------|----------------|
| Maternal                                   | Paternal       |
| Aggressiveness                              | 0.13           |
| Boldness                                   | 0.17           |
| Exploration                                | 0.23           |
| Activity                                   | 0.11           |
| Sociability                                | 0.07           |
| Paternal                                   | 0.06           |
| M:P Interaction                             | 0.12           |

Pearson correlation between juveniles with the same maternal identity. Only the significant Pearson correlations between related offsprings are indicated. All correlations have a p-value < 0.001 in the linear mixed model analysis.
sociability (Herczeg et al., 2009b; Magurran et al., 1991) and boldness (Biro et al., 2004; Bleakley et al., 2006; Brown et al., 2005; Wilson et al., 1993). In this study, we found a population-level covariance between adult defense morphology and behaviors of juveniles raised in a common environment. Juvenile morphology also co-varied with juvenile behavior at the population level.

In adults, we found a reduction in the number of lateral plates and an absence of pelvic spines in one of the two populations. This leads us to believe that the environment of the two populations, although very close spatially, contains elements of selection favoring the maintenance of defensive structures in Témiscouata Lake and not in Rond Lake. The loss of armor has been observed repeatedly in several different populations, suggesting adaptive parallel evolution, and several ecological reasons that could explain this repeated loss have been proposed (McKinnon et al., 2002). The predator’s regime (Marchinko, 2009) or the physicochemical environment (Campbell, 1979; Lindsey, 1962; Reimchen, 1980), could explain the difference in morphology observed between two freshwater populations. In accordance with these studies, the piscivorous predation probability in Témiscouata Lake is higher, which is in agreement with the more complete defense morphology found in adults. However, as a salmonid predator may still be present in Rond Lake following introduction in the 1970s, and since insect and bird predation pressure is unknown, we assume that predation may not be totally absent in that lake and the overall effects of these predators is unknown. For example, insect predation has been shown to favor reduced pelvic spine length but not the absence of a pelvic girdle in threespine sticklebacks (Marchinko, 2009). Predation may thus not be the only ecological factor driving the phenotypic divergence in predator defense structure between the two populations. The physicochemical environment also differs between the two lakes, as Rond Lake is located at a higher altitude and the beginning of the summer season (as indicated by ice-cover retreat) is later by approximately one month compared to Témiscouata Lake (Pierre-Emmanuel Chaillon; SÉPAQ; personal communication). This could affect the amount of resources that can be allocated to growth and development of the lateral plates and result in a shorter reproductive season. Furthermore, it has been shown that higher temperature during development may result in a higher lateral plate number for a given genotype in sticklebacks (Lindsey, 1962). This is also in agreement with adult Rond Lake sticklebacks being smaller and showing a loss of defensive structures. Therefore, our correlational study does not allow us to determine the mechanism involved in the morphological differentiation of these two populations. However, as morphological defense structures have a high heritability in sticklebacks (Hagen, 1973; Lindsey, 1962), we can hypothesize that there are one or several selection agents, including but not limited to predation, acting differentially in these two lakes that result in these differences. The nature of these selecting agents and the adaptive nature of these morphological differences will need to be uncovered and confirmed by further testing.

Juveniles raised in a common environment also differed in morphology. Juveniles from Témiscouata Lake were longer and heavier and were paler in dorsal and ventral coloration. This suggests that genetic variation may underlie this coloration divergence. In juveniles, no defensive armor is present in early life and coloration may provide camouflage. Since the two lakes are at different altitudes and have different depths, it is possible that light penetration and sediment bottom coloration differ. Recent studies have shown that light intensity above and below the fish is the first cause of color variation for camouflage (Migaud et al., 2010). Interestingly, Témiscouata Lake has a darker bottom than Rond Lake in spring and summer (pers. obs.), while juveniles from that population have paler dorsal and ventral parts, which is opposite to expectation if that morphological trait serves as camouflage. Further experiments uncovering the functional significance of this genetic variation for coloration are thus needed to determine if survival probability is affected by this morphological trait in the wild (Clarke et al., 2011).

These between-population differences in morphology in adults and juveniles were coupled with differences in juvenile behavior. We found a significant difference in mean activity and aggressiveness levels, with juveniles from the population lacking anti-predator defense morphology (Rond Lake) being more active and more aggressive. We also found that juveniles from Rond Lake were twice as bold, but this large difference was not significant when taking maternal identity into account. These findings are in accordance with predictions based on behavioral studies in other species linking predator pressure and behavior and to the hypothesis of trait co-specialization, but are opposite to prediction based on the model of coupling of morphology and behavior through trait compensation (DeWitt et al., 1999). Indeed, based on this model, one would expect that individuals with a stronger morphological anti-predator protection
would be the boldest, as found for risk-taking during foraging habitat choice in juvenile sticklebacks (Sillett et al., 2000), while we found that they are the least active and aggressive. The expected lower probability of predation in Rond Lake may result in a lower risk of predation for more active individuals than in Témiscouata Lake. Differences in the laboratory in activity could also be the result of a consistent difference in food availability and competition levels between the two lakes, resulting in selection of different activity levels. If for example food is scarce and competition is higher in Rond Lake, a higher activity level could be advantageous, without the cost of higher predation probability. The effect of competition in nature would have to be constant and resulting in selection of a certain type of active individuals to observe these differences in laboratory-reared individuals that have been fed to satiation in a low-density setting since hatching. Our result showing that aggressiveness was lower on average in Témiscouata Lake is in accordance with studies that have shown reduced aggression in adults and juveniles in populations facing higher predation (Archard et al., 2011; Wright et al., 1993), but see (Bell, 2005). It is possible that without predators, the costs of aggressiveness are less significant and individuals have the opportunity to behave aggressively while competing. In Rond Lake, lower food availability, higher competition, lower predation or a combination of these factors may thus have resulted in a divergence of mean activity levels and aggressiveness. If piscine predation probability also acted independently on anti-predator morphology, the observed co-specialization of morphology and behavior could exist (DeWitt et al., 1999; Mikolajewski et al., 2010) rather than observing a trait compensation effect of the level of morphological defense on the behavior expressed (Sillett et al., 2000). Our results suggest that piscine predation has acted on morphology and behavior to create a predator-defense co-specialized phenotype.

3.2 Individual-level coupling of morphological traits and behavioral traits

At the individual level, we showed that anti-predator morphology of adult females did not predict any of the five behaviors measured in their juvenile offspring. Therefore, we did not see a trade-off between morphological and behavioral predator defense strategies, for example more heavily armoured individuals being bolder, more active or more exploratory. It has been shown that lateral plate number varying from 5 (low plated) to 30 (complete) could be associated with variation in feeding behavior in threespine stickleback (Ostbye et al., 2010). The two populations that we studied were in the range of what is considered a “partial” plate morph and among-individual differences in morphology were thus less extensive. Other populations will have to be studied at the individual level to see if this absence of relationship still holds. On the other hand, we found coupling of juvenile coloration patterns and individual boldness level, with a distinct relationship in each lake. In Témiscouata Lake, darker individuals were bolder, while in Rond Lake, the opposite relationship was found, with paler individuals being bolder. Témiscouata Lake provides a darker environment due to its depth and sediment coloration (pers. obs.). Whether these two traits form correlation resulting from trait compensation that is specific to the environment faced by each population, as shown in invertebrates invading new predator-free environments (Mikolajewski et al., 2010), needs further testing.

3.3 Proximate and ultimate mechanisms

The differences in behavior in juveniles were found for individuals that have experienced the same rearing environment. Therefore, while behavior may be modulated by environmental factors (Bell et al., 2007), these differences between populations are most probably due to genetic differences among populations, which could be the result of random genetic drift or natural selection. However, these behavioral differences between populations may also be in part the consequence of epigenetic differences in the form of maternal effects (Giesing et al., 2011; Meaney et al., 2010). We measured significant maternal identity effects on four behaviors measured in juveniles 4 months after hatching and without parental care. It has been shown in fish that stressed females during egg formation have eggs with higher stress hormone levels (cortisol, (Giesing et al., 2011; Mileva et al., 2011) and that eggs exposed to high stress hormone levels result in offspring with a higher sociality tendency (Giesing et al., 2011) or that are more aggressive (Sloman, 2010). We had no information on environmental conditions and life events faced by the wild-caught females used to generate the common-environment reared juveniles in each lake, but if these conditions varied between the two populations and affected the chemical messengers deposited in the eggs used in the artificial crosses, this may be reflected in the behaviors we measured in juveniles, even when reared in a common environment. However, the link between a female’s environment, her hormonal levels, the resulting hormonal levels of her eggs and the behavior of her offspring has not been investigated.
in a systematic fashion for the five behaviors measured in our study.

The proximate and ultimate causes of the covariation between morphology and behavior will need to be investigated. One could hypothesize that those changes in morphology resulting from selection pressure could in turn result in changes in behavior (or vice-versa), that the two are independently affected by the same selection agent, or that these correlated changes result from pleiotropic genetic effects. Further work on the proximate mechanisms resulting in these differences in morphology and behavior would be needed to answer these questions. These two freshwater populations are prime candidates for future mechanistic studies.

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