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*Published in:*  
 Behavioral Ecology

*DOI:*  
[10.1093/beheco/arab121](https://doi.org/10.1093/beheco/arab121)

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*Document Version*  
 Publisher's PDF, also known as Version of record

*Publication date:*  
 2022

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Ramesh, A., Groothuis, T. G. G., Weissing, F. J., & Nicolaus, M. (2022). Habitat fragmentation induces rapid divergence of migratory and isolated sticklebacks. *Behavioral Ecology*, 33(1), 167-177. <https://doi.org/10.1093/beheco/arab121>

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Original Article

# Habitat fragmentation induces rapid divergence of migratory and isolated sticklebacks

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Received 6 April 2021; revised 7 September 2021; editorial decision 27 September 2021; accepted 30 September 2021; Advance Access publication 23 November 2021.

The adaptive capacity of many organisms is seriously challenged by human-imposed environmental change, which currently happens at unprecedented rates and magnitudes. For migratory fish, habitat fragmentation is a major challenge that can compromise their survival and reproduction. Therefore, it is important to study if fish populations can adapt to such modifications of their habitat. Here, we study whether originally anadromous three-spined stickleback populations (*Gasterosteus aculeatus*; “migrants”) changed in behavior and morphology in response to human-induced isolation. We made use of a natural field-experiment, where the construction of pumping stations and sluices in the 1970s unintendedly created replicates of land-locked stickleback populations (“resident”) in the Netherlands. For two years, we systematically tested populations of residents and migrants for differences in morphology and behavioral traits (activity, aggressiveness, exploration, boldness, and shoaling) in lab-based assays. We detected differences between migrant and resident populations in virtually all phenotypic traits studied: compared with the ancestral migrants, residents were smaller in size, had fewer and smaller plates and were significantly more active, aggressive, exploratory and bolder, and shoaled less. Despite large ecological differences between 2018 and 2019, results were largely consistent across the two years. Our study shows that human-induced environmental change has led to the rapid and consistent morphological and behavioral divergence of stickleback populations in about 50 generations. Such changes may be adaptive but this remains to be tested.

**Key words:** animal personality, anthropogenic changes, behavioral syndrome, *Gasterosteus aculeatus*, migration.

## INTRODUCTION

Humans induce unprecedented fast changes in many habitats, thereby imposing new selective pressures to animal populations. Animals thus need to implement quick adaptive responses to these changes to maintain their ability to survive and reproduce. One of the first responses to these challenges is often behavioral as behavior directly mediates how individuals interact with their environment. Consequently, it is expected that individuals will respond to human-induced changes through behavioral modifications as a first step, which then may pave way for other morphological

and/or physiological adaptations (Sih et al. 2011; Tuomainen and Candolin 2011; Wong and Candolin 2015).

Animal personalities are behavioral traits that are consistent across time or contexts and are often correlated to form “behavioral syndromes” (Stamps and Groothuis 2010). Animal personalities presumably have significant consequences for the speed and the outcome of adaptation processes to changing environments (Bolnick et al. 2011; Dall et al. 2012; Sih et al. 2012; Wolf and Weissing 2012). For example, personality variation may slow-down or speed-up rate of microevolution depending on whether personality structure retards adaptive evolution (Dochtermann and Dingemans 2013) or provides “pre-adapted” phenotypes, which drive faster adaptation in multiple dimensions (Wagner and Altenberg 1996; Barrett and Schluter 2008; Wolf and Weissing

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2012; Van Gestel and Weissing 2018). Furthermore, existence of personalities and mechanisms maintaining such intraspecific variation within populations can have an immense effect on the adaptive potential of these populations in response to environmental change (Réale et al. 2007; Bolnick et al. 2011; Dall et al. 2012; Wolf and Weissing 2012; Moran et al. 2016).

Human-driven changes have disproportionately affected freshwater species, which have suffered the largest declines of 84% on average (WWF living planet report 2020). One of the greatest threats is habitat fragmentation that decreases habitat size and functional connectivity between habitats (LeGrand et al. 2017). Migratory fish species, in particular, rely on moving between sea and freshwater or between other habitats to reach spawning and nursery habitats (Fullerton et al. 2010). Hence, blocking access to these habitats can compromise the reproduction and survival of such migratory species (Lucas and Baras 2001). The important questions that connect the fields of animal personality, conservation, ecology, and evolution are whether and how migratory fish can adapt to the sudden isolation. Our study system in the north of the Netherlands is well-suited to address such questions: In the last 50 years, man-made barriers (such as pumping stations and sluices) have been extensively built in rivers to maintain water levels below sea-level, with the consequence that it has blocked some of the side arms of main river channels. This created an unintended natural field experiment, wherein several populations of anadromous three-spined sticklebacks (*Gasterosteus aculeatus*) (“migrants”) have become land-locked (“residents”) in some of these replicate side-arms of the river. Over contemporary time-scales, we expect resident populations of sticklebacks to have experienced very different selection pressures by completing their life-cycle entirely in freshwater as opposed to their ancestral migrants, that spend a significant part of juvenile growth at the sea, during winter. We used this opportunity to study whether resident populations exhibit consistent phenotypic differences (morphology and personality) compared with ancestral anadromous sticklebacks, as a result of this recent human-driven change.

Three-spined sticklebacks have become a model system for studying rapid phenotypic divergence because populations generally harbor high standing phenotypic variation (e.g. Jones et al. 2012), which enables them to adapt to a multitude of environments and

through various proximate mechanisms (genetic, hormones, developmental plasticity, parental effects) (see review for freshwater colonization in Table 1). Likewise, other examples are phenotypic differences that have been repeatedly among populations with and without exposure to predation (Bell and Sih 2007; Dingemanse et al. 2007, 2009; Stein et al. 2018; Dingemanse et al. 2020). Yet, little is known about population phenotypic divergence (including behavior and morphology) following habitat fragmentation over shorter time-scales. To fill this knowledge gap, we sampled resident and migrant stickleback populations more than two years and quantified differences in morphology and in behavioral traits involved in movements and anti-predator strategies: activity, aggressiveness, exploration, boldness, and sociability (Seghers and Magurran 1994; Wolf et al. 2008, 2011; Cote et al. 2010, 2013; Chapman et al. 2011; Trompf and Brown 2014; Sommer-Trembo et al. 2017). In this species, these behaviors are moderately repeatable (e.g. for the classical behavioral assays in Dingemanse et al. 2007, repeatability ranges between 0.339 and 0.552,) and can be phenotypically integrated (Bell and Stamps 2004; Bell 2005; Bell and Sih 2007; Dingemanse et al. 2007, 2020; Kim and Velando 2015). Our field system provides a good opportunity to answer whether ~50 years of isolation have been sufficient to induce morphological and behavioral differences between resident and migrant populations. We thus studied populations of residents and migrants in our system more than two study years. Based on the synthesized literature on freshwater adaptation in this species (Table 1), we expect that individuals in resident populations should exhibit smaller body size with less armature as well as decreased levels of activity, exploration, boldness, and shoaling compared with the ancestral migratory population.

## METHODS

### Study populations and data collection

Our study sites were located along two main rivers, Terunterzijldiep and Westerwoldse Aa originating from the Ems Dollard estuary in the province of Groningen, the Netherlands. We caught incoming migrants at the two sea locks (“TER” [53°18'7.24", 7°2'17.11"] and “NSTZ” [53°13'54.49", 7°12'30.99"]), whereas resident sticklebacks were caught in two adjacent land-locked polders (“LL-A”

**Table 1**

**Overview of freshwater adaptations from marine and migratory three-spined sticklebacks. Sticklebacks can adapt to freshwater via a multitude of proximate mechanisms. This table provides a non-exhaustive overview of these mechanisms that may be at play in our migrant-resident study system**

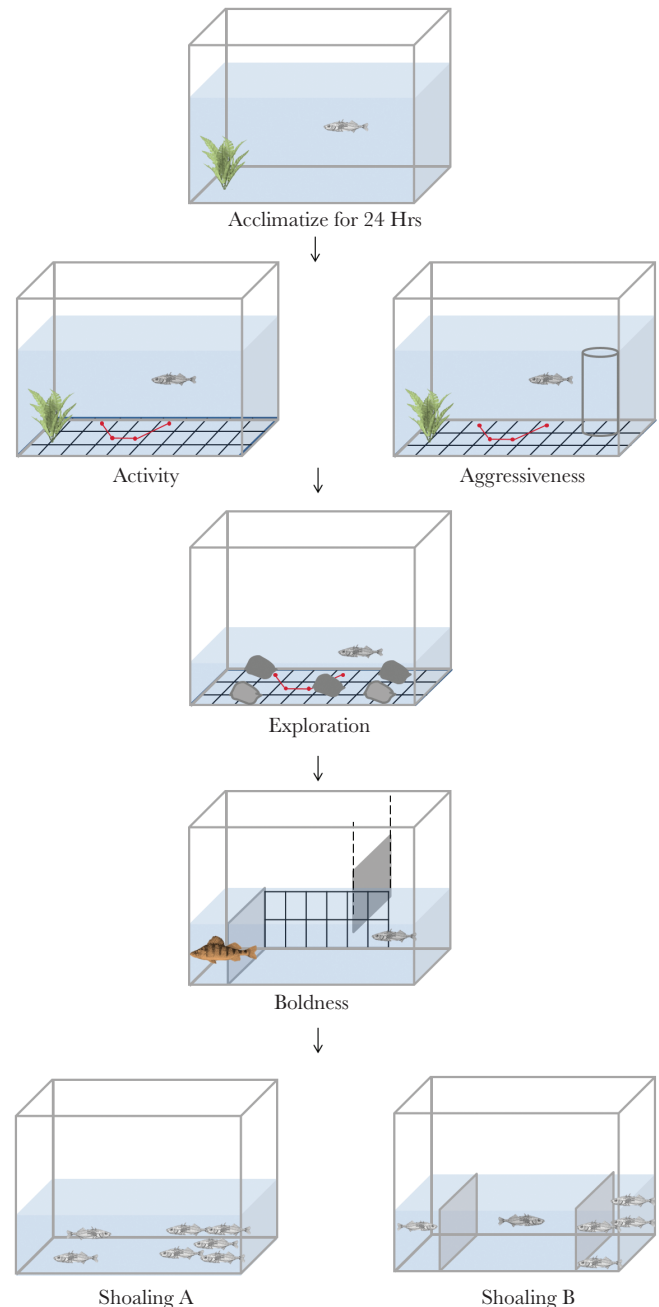
Trait	Change following freshwater adaptation	Mechanism	Reference
Life history	Younger and smaller at maturity, Lower growth rates, association between growth rate and plate morphology in freshwater (low-plated morphs grow faster in freshwater compared with high-plated morphs)	Genetic and/or developmental plasticity	(Snyder 1991; Marchinko and Schluter 2007; Robinson 2013)
Morphology	Reduction in number and size of lateral plates, reduction in size of dorsal spines, diminution or absence of pelvic spines	Genetic and/or developmental plasticity	(Bell et al. 1993; Colosimo 2005)
Physiology	Lower thyroid levels, lower metabolic rates, osmoregulation, tolerance to freshwater	Genetic, Developmental plasticity, Transgenerational plasticity, Parental effects	(Lam and Hoar 1967; Kitano et al. 2010, 2012a; b; Kitano and Lema 2013; Kusakabe et al. 2017)
Swimming ability / Buoyancy	Lower swimming endurance, interaction between plates and size of swim-bladder (reduce tissue density / lateral plates or increase size of swim-bladder)	Genetic	(Tudorache et al. 2007; Dalziel et al. 2012)
Behavior	Decrease in schooling, shoaling, anti-predator behavior toward freshwater predators, parental care	Genetic, Developmental plasticity, Transgenerational plasticity, Parental effects	(Wark et al. 2011a; Di-Poi et al. 2014; Stein and Bell 2014 2019; McGhee et al. 2015)

[53°17′56.14″, 7°2′1.28″] and “LL-B” [53°17′16.52″, 7°2′26.46″]) (Supplementary Information 1a). The land-locked populations are a part of a network of isolated freshwater ditches from the side arms of the main river, with depths less than three meters and width up to 8 meters with ample vegetation. LL-A is blocked from the main river by a historic sluice that is not functional (Supplementary Information 1b) and LL-B is separated by a pumping station (Supplementary Information 1c). To prevent sampling biases, we used lift-, hand- and fyke-nets in resident populations and lift netting for incoming migrants directly at the fish passages at the sea locks, ensuring that fish of all behavioral types were caught. Apart from the spatial separation, which aided us in assigning migrant and resident status to fish, we also repeatedly sampled either in and outside the land-locked polders as well as in and outside of the migratory season (with electrofishing) in order to confirm our assignment of “residents”. We consistently found resident fish all year round while migrants were absent outside the breeding season.

All individuals were transported to the laboratory within 2 h of capture in aerated bags. After acclimatization to the laboratory conditions (temperature and 1% salinity water) for 1 h, we took the following morphological measurements of all individuals: total length (the length from the tip of the snout to the end of the tail), standard length (the length from the tip of the snout to the base of the tail), body mass, category of lateral plating (fully-plated, partially-plated and low-plated forms) (Bell and Foster 1994), and clipped fins and/or spine of individuals for unique individual identification. We could not count the plates accurately in live specimens and used a rule of thumb that follows this description: low-plated (10 or fewer plates), partially-plated (11–20 plates), or fully-plated (21–30 plates). When we applied this classification to fish where the plates could be counted accurately (e.g. dead specimens), it turned out to be quite reliable. Sometimes, partially-plated resident fish were classified as completely-plated (or vice versa), but we are confident that our classification is largely correct. We used *standard length* as proxy for size in all analyses because this measure is highly correlated with the two other measures namely, total length and body mass and was less error prone than total length (Supplementary Information 3).

Thereafter, we placed each fish in an individual “home tank” (30 × 16 × 18 cm [L × W × H]) that was visually isolated from others and enriched with one artificial plant. Fish were fed frozen blood worms and brine shrimps (3F Frozen Fish Food bv.), ad libitum. On the following day, the fish were allowed to acclimatize to the new environment and laboratory conditions (day 0). From day 1 to day 4, fish were subjected to a range of behavioral tests (Figure 1). On day 6 or 7 fish were released in the wild at their site of origin or kept in the lab for further breeding experiments. The laboratory conditions were set to mimic the natural conditions in terms of air temperature (range 5 to 20 °C, depending on season) and photoperiod (range 10:14 L:D to 16:8 L:D, cycled with natural levels). Fish were monitored every day and individuals that showed signs of injury or sickness were removed immediately or before the behavioral assay ( $n = 65$ ).

Data collection occurred between March and May in the years 2018 and 2019. These years were drastically different in terms of the weather conditions of the summer and winter of the previous years (see Supplementary Information 2). Compared with winter of 2017, the winter of 2018 was particularly cold with frozen ditches and main canals until March and the following summer was in contrast very warm and dry leading to small ditches partly drying up (Maximum daily temperature (2017 vs. 2018): 29.9 °C vs. 35.7 °C; mean annual precipitation (2017 vs. 2018): 25.9 cm vs. 17.88 cm;



**Figure 1**

**Behavioral assays.** The flow chart represents the order in which assays were performed along with illustration of different behavioral assays and the placement of grids used for extracting different parameters.

data from Royal Netherlands Meteorological Institute). In 2018, a total of 251 fish were caught (189 migrants and 62 residents) and in 2019, 74 fish were caught (38 migrants and 36 residents). It is noted that in 2019, we were successful in capturing migrants from only one population (“NSTZ”). Our sample size was determined by the number of fish we could successfully catch, while ensuring that batches were caught at different time intervals to avoid confounding effects of season.

Wild animals were sampled using a fishing permit from *Rijksdienst voor Ondernemend Nederland* (The Netherlands) and an angling permit from the Hengelsportfederatie Groningen-Drenthe. Housing and



testing of behaviors were in adherence to the project permit from the *Centrale Commissie Dierproeven* (The Netherlands) under license number *AVD1050020174084*.

## Behavioral assays

Five behaviors were scored for both migrants and residents: general activity in home tank, aggression towards a conspecific from the same location, exploration of a novel environment, boldness in a predator inspection trial, and shoaling tendency (Figure 1). Activity, aggression, and exploration were live-scored by five observers whereas the boldness tests and shoaling assays were filmed and subsequently scored using the software BORIS v.6.2.4. (Friard and Gamba 2016). Details of each assay are given below. The tests were performed during the light period (usually between 9 am and 6 pm). The sequence of fish to be tested was drawn at random. It was not possible to be blind to the status of fish, as migrants and residents exhibited large size differences.

Several variables were measures for behavioral traits (except shoaling) and morphology. We used one of these variables as a proxy of the behavior of interest, as it is easier to interpret. We thus performed principal component analyses (PCA) including all measured variables per behavior under study and used the first principal component PC1 (explaining most of the variance) as a proxy for the activity and exploration (Supplementary Information 3) and we chose other variables or a composite of it based on biological reasons for aggression and boldness, as mentioned below. The PCA-based results (not reported), did not differ from the results on the single variables.

## Activity

The general activity level of each individual was recorded in their home tank using a grid at the bottom of the tank (Figure 1). Each individual was observed for a period of 60 s and its position in a 10 × 6 square grid space was recorded every 5 s. With the recorded position the following values were calculated: unique squares visited, number of square changes, and total distance travelled (adapted from Dingemanse et al. 2007). In the analyses reported in the main text, we used *number of square changes* as a proxy for “activity”.

## Aggression

Immediately after the activity test, we introduced an empty transparent glass in one corner of the home tank, in order to acclimatize the focal fish to the new object (120 s). Subsequently, the empty glass was replaced with a similar one containing a smaller conspecific from the same population (“intruder”). During the following 120 s, we scored the position of the focal individual and its response towards the intruder (bites, spine-up display) every 10 s (Figure 1). The mean and minimum distance to the intruder and the total number of bites were then calculated (adapted from Bell and Stamps 2004). Spine-up threat display was hard to notice for residents because of their smaller spines and subsequently dropped from observations. We re-used intruders for a maximum of five different trials and controlled for intruder identity in the later analyses. To disentangle aggression from sociability, we used the *total number of bites* as a proxy for “aggression”, rather than the time spent near the intruder.

## Exploration

For studying exploration in a novel environment, the focal fish was placed into an opaque acclimatization compartment (4 × 6 cm)

within a tank of size equal to the home tank, a water level of 5 cm, and with a 10 × 6 square grid at the bottom. The tank included five stones that extend to the top of the water surface to block the view and force the fish to swim around them to gather information about the environment (Figure 1). After an acclimatization period of 120 s, the compartment was gently removed, releasing the fish into the arena and the subject started the exploration test, lasting for 300 s. During this period, the position of the focal fish was recorded every 5 s. With the recorded position the following values were calculated: unique squares visited, number of square changes, and total distance travelled (adapted from Dingemanse et al. 2007). In the analyses reported in the main text, we used *number of square changes* as a proxy for “exploration”.

## Boldness

In the boldness tests, we measured the responses of the focal fish toward a model of a predator, European perch (*Perca fluviatilis*), with jointed soft body that moves realistically when displaced remotely using a thread (Kozak and Boughman 2012). European perches naturally occur in our field sites and are considered one of the primary predators of sticklebacks (Hoogland et al. 1956). The focal fish was moved from its home-tank into a bigger, novel tank (60 × 30 × 30 cm) with three compartments, filled with 10 cm of water. Of the three compartments, the predator model was presented in the left compartment while the focal fish was released from the right compartment. The space between the “fish” compartment and “predator” compartment was divided into 8 equally spaced grids with one fish-distance (6 cm) between the subsequent grids (Figure 1). The focal fish was first placed into the fish compartment of the tank. The barrier at the fish compartment was removed while the opaque barrier at the predator compartment was retained. Subsequently, the focal fish could explore the novel tank for a period of 120 s without the predator being visible. After that period, the focal fish was gently pushed back into the fish compartment and the barrier was replaced. Meanwhile, the opaque barrier to the predator model was removed and replaced by a transparent barrier. After this was done, the barrier of the fish compartment was removed again to allow the focal fish to view the predator. The boldness trial of 300 s was recorded with a camera. In the subsequent video-scoring, the latency to exit the fish compartment, the number of inspection bouts (i.e. directed swimming towards the predator crossing at least one square and ending when the fish swam back into the opposite direction), the total duration of inspection bouts, the number of predator visits (i.e. visiting the last grid next to the predator compartment, <6 cm), the total duration spent near the predator compartment, and the minimum distance to the predator compartment were recorded. If a fish did not exit, its latency to exit amounted to the maximum or 300 s and all other values were recorded as NA (adapted from Wilson and Godin 2009). At least half of the water was replaced after testing 10 fish in the arena.

We used the *number of inspection bouts* towards the predator (number of inspection bouts performed in the first minute after the focal fish entered the arena) as a proxy for “boldness”. This measure is preferable to *latency to exit*, as it is less related to activity (an early exit may reflect higher activity level) and to *time spent near predator*, as it takes into account the total time the fish spent in the test arena.

## Shoaling – A

In 2018, individual shoaling tendency was scored in a group of ten fish. Fish that were captured on the same day and within the

same population were placed into a larger tank (60 × 30 × 30 cm) filled with 10 cm of water where they could interact freely with each other (Figure 1). After 120 s, all shoaling fish and then all non-shoaling fish were caught, identified, and shoal composition noted. Fish were considered to shoal if they associated with another fish within one-fish distance (<6 cm) at the end of the test. The procedure was repeated three times to calculate a shoaling score or ratio (1.0 is when individual was found to be associated with the shoal in all three trials, adapted from Wark et al. 2011).

## Shoaling – B

The shoaling assay conducted in 2018 adopted a setting where individuals were able to interact with one another. However, this captured very little among-individuals differences. Hence, we re-adjusted this test in 2019 by assaying individual shoaling in a large tank divided into three compartments: a central testing arena where the focal fish was released and two end compartments containing the stimulus shoal ( $n = 5$  unfamiliar conspecifics) and two distracter fish ( $n = 2$  unfamiliar conspecifics) (Figure 1; adapted from Wark et al. 2011). The stimulus shoal and distracter fish comprised of migrants if the focal fish was migrant and residents otherwise. The stimulus shoal and distracter fish compartments were switched in sides to prevent a place or side bias and the fish were replaced with new stimulus shoal and distracter fish after five trials. At the start of the test, the focal fish was allowed to acclimatize for 120 s in the central arena without viewing the ends compartments that were covered with opaque barriers. The focal fish was returned to its home-tank momentarily and the opaque barriers were replaced with transparent barriers. The focal fish was then reintroduced to the center of the focal arena to record its shoaling behavior for 300 s (shoaling time, spending one fish-distance (<6 cm) from the shoal). The water was partially replaced after testing 10 fish in the arena.

## Statistical analyses

To test whether resident and migrant fish differ in the proportions of the three common lateral plate morphs, we used a Chi-squared test for each year separately. We then analyzed variation in standard length and all the behavioral traits measured (activity, aggression, exploration, standardized scores for predator inspection, and shoaling) in Linear Mixed Models (LMMs) with Gaussian errors. *Status* (resident vs. migrant, with migrant being the reference category), *year* (2018 vs. 2019, 2019 being the reference category), and *status* × *year* interactions were included as fixed effects. Date and time of testing did not have a significant effect and were thus removed from the models. In all models, *observer ID* and the combination of *population ID* × *year* (four populations in 2018 and 3 populations in 2019, giving 7 levels, as population is the unit of replication for our inferences) were fitted as random effects. We did not detect any sex differences in any of the behaviors (Supplementary Information 4), and thus decided to pool data from both sexes. All GLMMs were constructed in R v. 3.6.1, R Core Team (2019) using the lmer function of the “lme4” package (Bates et al. 2015). The statistical significance of fixed effects was assessed based on the 95% confidence interval (CI): an effect was considered significant when its 95% CI did not include zero. The sample sizes slightly varied between tests due to missing data and are reported with the outcome of each statistical test.

To establish the existence and structure of behavioral syndromes in migrants and residents, we ran multivariate mixed models that

estimate covariances and correlations among all traits. However, due to lack of model convergence, covariances could not be estimated this way. Other advocated methods (e.g. Structural equation modelling [SEM]; Dingemanse et al. 2010) could not be applied due to limited sample sizes. Hence, we estimated syndromes based on pairwise Spearman correlation with sequential Benjamini-Hochberg correction for multiple testing (Benjamini and Hochberg 1995). Data were zero-inflated in some behaviors (Aggression in residents; Activity and Aggression in migrants). We discarded these behaviors from the correlational analyses to prevent spuriously high correlation coefficients. Correlation analyses focus on pairwise relationships between traits, thus ignoring higher-order effects (Dingemanse et al. 2010). To overcome this, we also compared the results of a PCA approach to summarize the structure of relationship between all the behaviors within categories of migrants and residents between the years, which did not yield qualitatively different results (not shown).

## RESULTS

### Morphological differentiation

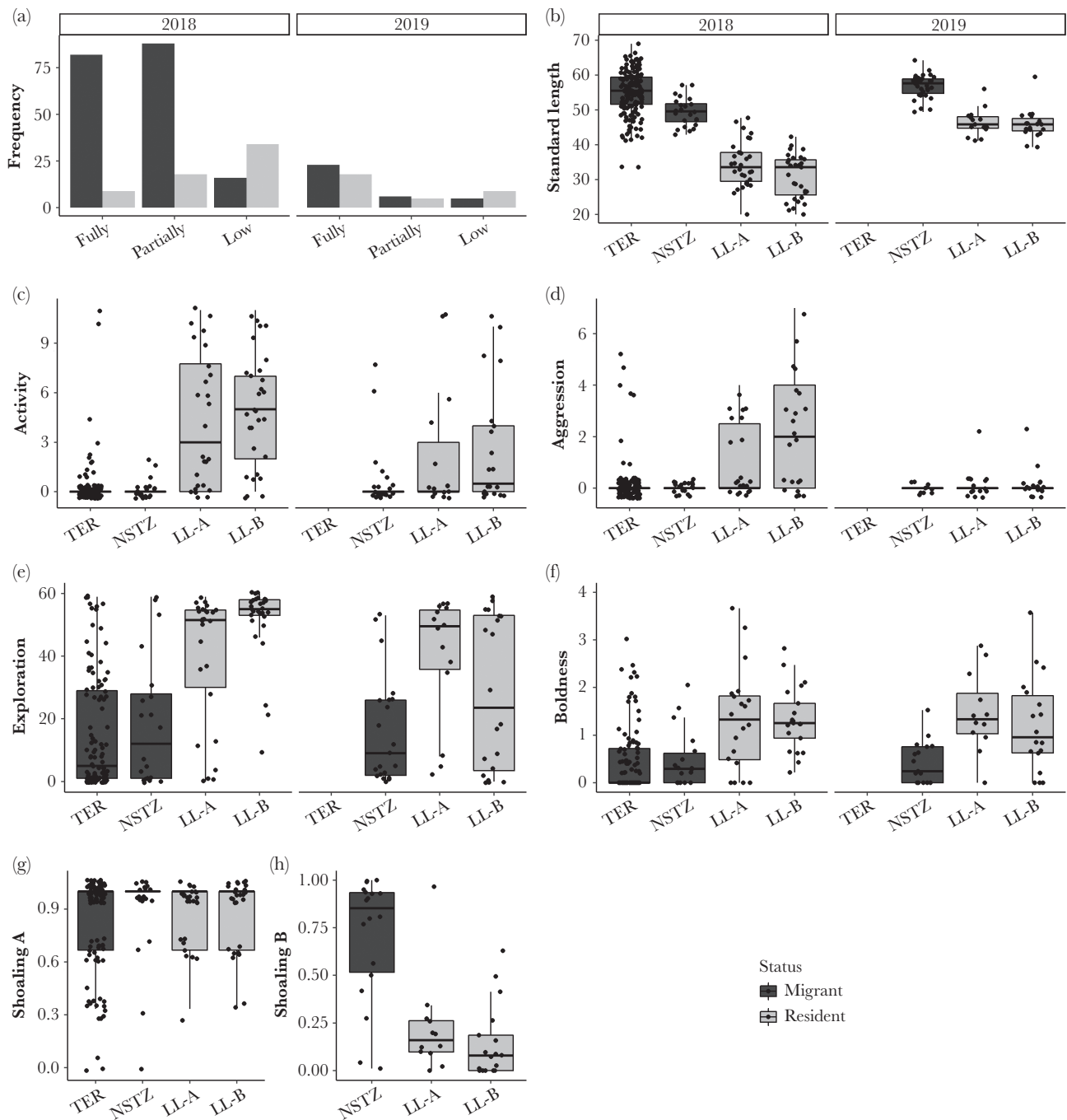
Residents had more low-plated forms compared with migrants in 2018, but not in 2019 (Figure 2a), although the difference between migrants and residents seem to display a similar pattern in both years. This is confirmed by chi-square test on the relative proportions of lateral plate morphs between residents and migrants (Proportions of fully, partial and low plated morphs in 2018 = 0.44, 0.47, 0.09 in migrants and 0.15, 0.30, 0.56 in residents respectively;  $\chi^2$  (df = 2,  $N = 247$ ) = 64.536,  $P < 0.01$  and in 2019 = 0.68, 0.18, 0.15 in migrants and 0.56, 0.16, 0.28 in residents respectively;  $\chi^2$  (df = 2,  $N = 66$ ) = 1.785,  $P = 0.410$ ). Residents were significantly smaller than migrants in both years (Table 2; Figure 2b). All fish were also larger in 2019 compared with 2018 (Table 2; Figure 2b).

### Behavioral differentiation

In both years, residents were significantly more active (87.5% of the migrants did not exhibit movements at all in their home-tanks), more exploratory and bolder compared with migrants (Table 2, Figure 2c, e, f, respectively). Compared with previous studies in sticklebacks (Huntingford 1976; Bell 2005; Dingemanse et al. 2007), we found only a marginal proportion of aggressive individuals outside of the breeding period. In 2018, residents were significantly more aggressive than migrants and in 2019, this pattern disappeared (significant *status* × *year* in Table 2; Figure 2d). The shoaling A assay performed in 2018 did not reveal differences between residents and migrants. However, the shoaling B assay performed in 2019 showed that residents shoaled much less than migrants (Table 2; Figure 2h).

### Behavioral syndromes

Behaviors were not correlated and there was little evidence for the existence of syndromes in both populations: only 2 of the 32 pair-wise correlations were significant after correcting for multiple testing and the correlation structure was not stable across years in either group (Figure 3). In 2018, the only significant result was the positive correlation between exploration and predator inspection in migrants (Figure 3, Supplementary Information 5;  $\rho = 0.29$ , corrected  $P = 0.009$ ). In 2019, the only significant result was the positive correlation between activity and exploration in residents (Supplementary Information 5;  $\rho = 0.68$ , corrected  $P = 0.002$ ). Most of the other correlations were far from significant.



**Figure 2**

**Individual size and behaviors (median  $\pm$  quartiles) of populations of residents and migrants more than two years.** (a) Lateral plate morph distribution ( $N_{2018} = 247$ ,  $N_{2019} = 66$ ). (b) Standard length ( $N_{2018} = 249$ ,  $N_{2019} = 72$ ). (c) Activity – number of square changes ( $N_{2018} = 203$ ,  $N_{2019} = 56$ ). (d) Aggression – number of bites to intruder ( $N_{2018} = 187$ ,  $N_{2019} = 44$ ). (e) Exploration – number of square changes ( $N_{2018} = 183$ ,  $N_{2019} = 54$ ). (f) Boldness – number of inspection bouts / minute ( $N_{2018} = 164$ ,  $N_{2019} = 48$ ). (g) Shoaling A – only 2018, fraction of trials spent with shoal ( $N_{2018} = 180$ ). (h) Shoaling B – only 2019, fraction of time spent near stimulus shoal ( $N_{2019} = 46$ ).

## DISCUSSION

This study investigated if resident populations of sticklebacks, which are cut off from the sea due to human water management measures in the 1970s, exhibit consistent morphological and behavioral differences compared with their ancestral migrant counterparts. Our results reveal that  $\sim 50$  generations of isolation were sufficient to induce substantial morphological and behavioral differences.

## Phenotypic divergence between derived residents and ancestral migrants

We found clear phenotypic differentiation between migrants and residents in almost all traits studied in both years. In line with previous literature on morphological adaptations of sticklebacks to freshwater that occurred over the last glaciation event ( $\sim 12\,000$  years), we found that residents were about half the size

**Table 2.**

Summary of linear mixed models on behavior and morphology. The table gives the summary of the LMMs on the selected variable for each behavior and morphology. Estimates of fixed effects ( $\beta$ ) are given with their 95% confidence intervals (CI) computed by bootstrapping method and variance components ( $\sigma^2$ ) are given with their standard deviation (SD). Significant fixed effects are denoted in bold. Sample size ( $N$ ) represents number of individuals. For shoaling assays, the analyses were done separately for each year as they were different procedures and analyzed using linear models.

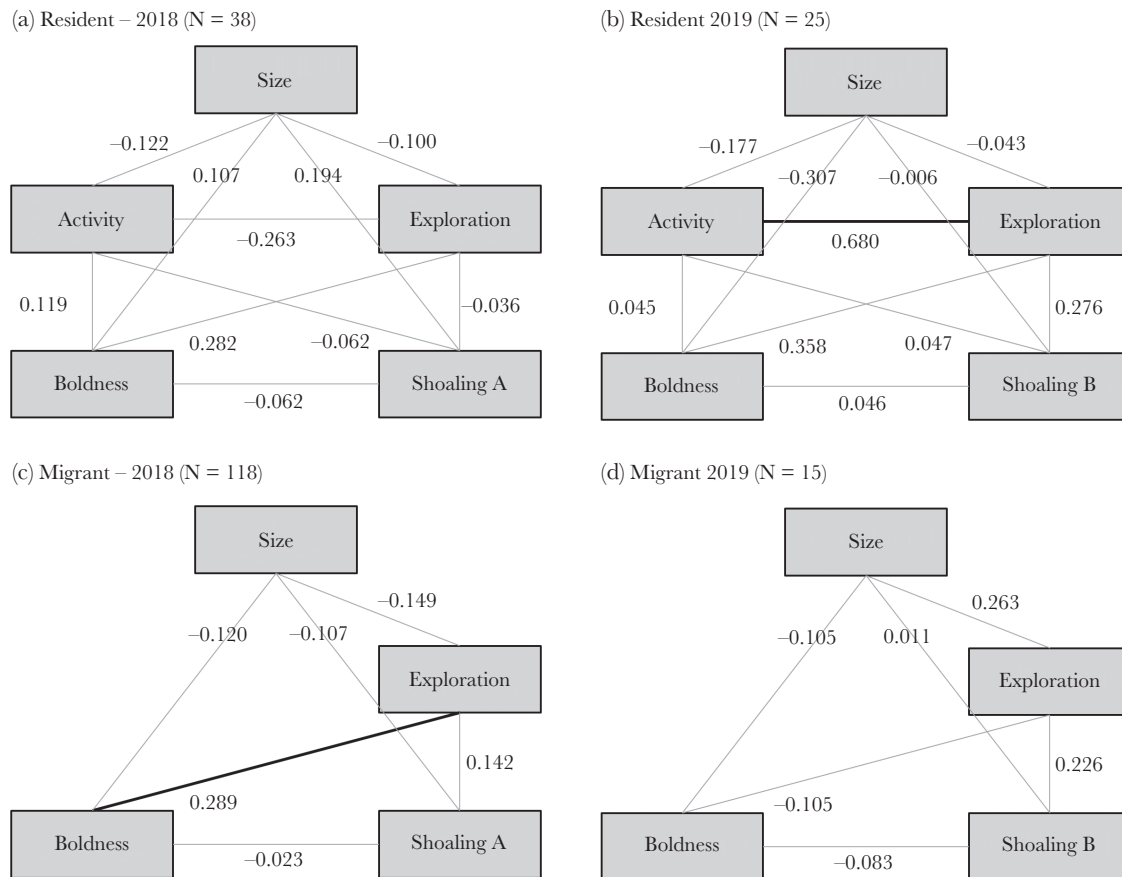
	Size ( $N = 317$ )	Activity ( $N = 258$ )	Aggression ( $N = 233$ )	Exploration ( $N = 239$ )	Boldness ( $N = 214$ )	Shoaling A ( $N = 181$ )	Shoaling B ( $N = 47$ )
Fixed effects	$\beta$ (95% CI)	$\beta$ (95% CI)	$\beta$ (95% CI)	$\beta$ (95% CI)	$\beta$ (95% CI)	$\beta$ (95% CI)	$\beta$ (95% CI)
Intercept	52.43 (48.55, 56.05)	0.36 (-0.40, 1.11)	0.18 (-1.26, 1.56)	19.28 (7.63, 30.47)	0.75 (0.08, 1.41)	0.85 (0.80, 0.89)	0.71 (0.58, 0.83)
Status (resident)	-19.81 (-25.11, -14.51)	4.34 (3.40, 5.35)	3.09 (1.04, 5.24)	29.01 (16.43, 41.32)	0.72 (0.33, 1.06)	0.04 (-0.04, 0.12)	-0.53 (-0.69, -0.37)
Year (2019)	4.39 (-2.41, 11.19)	0.61 (-0.53, 1.72)	2.55 (-0.33, 5.56)	2.47 (-16.14, 18.48)	-0.17 (-0.80, 0.48)		
Status resident x Year 2019	9.11 (0.43, 17.69)	-3.03 (-4.80, -1.15)	-5.37 (-9.01, -1.50)	-17.18 (-37.13, 2.27)	0.37 (-0.51, 1.23)		
Random effects	$\sigma^2$ (SD)	$\sigma^2$ (SD)	$\sigma^2$ (SD)	$\sigma^2$ (SD)	$\sigma^2$ (SD)		
Observer ID		0.17 (0.41)	0.00 (0.00)	44.72 (6.69)	0.35 (0.59)		
Population - year	6.74 (2.60)	0.00 (0.00)	0.81 (0.90)	25.00 (5.00)	0.00 (0.00)		
Residuals	32.78 (5.73)	6.11 (2.47)	10.14 (3.18)	346.81 (18.62)	1.19 (0.92)		

of migrants and were characterized mostly by low-plated forms. Although the resident fish from 2019 comprised of more fully plated forms than the residents in 2018, they showed reduction in plate width (personal obs.) in contrast to the robust armature spanning the width of the body, in fully- or partially-plated migrants. Lateral plate polymorphisms take many forms in sticklebacks, from variation in the number of plates (Reimchen et al. 2013; Eriksson et al. 2021) to variation in thickness and width of plates (Leinonen et al. 2012; Wiig et al. 2016), depending on the ecological conditions. In our case, we find reduction in lateral plate coverage via reduction in the width of plates, similar to that observed in populations with limited allelic variation for low-plated morph (Leinonen et al. 2012).

The morphological difference between populations is most easily explained by the necessity for flexibility to maneuver through vegetation in residents as compared with the demanding robustness and swimming abilities for migrants (Tudorache et al. 2007; Dalziel and Schulte 2012; Dalziel et al. 2012) and decreased resource availability in freshwater during growth (Snyder 1991; Wund et al. 2012). Furthermore, the reduction in number and size of lateral plates are also known to occur in response to different predator regimes present in the freshwater system (with fewer piscivorous predators and mainly dominated by invertebrate predators like dragonfly naiads) through selection on *eda* gene underlying lateral plate polymorphism (Colosimo et al. 2005; Marchinko and Schluter 2007; Leinonen et al. 2011). These observations therefore suggest that the reduced size and the reduced armament of our resident fish likely follow the same pattern of adaptive evolution seen during freshwater colonization of marine sticklebacks over the last glaciation event.

As for individual behavioral scores, we found that residents were more active, aggressive (in the year 2018), exploratory, bolder and showed lower shoaling tendencies than migrants (in the year 2019). The majority of our findings with wild-caught sticklebacks are in line with the only other study that compared similar behaviors in populations of residents and migrants in lab-bred F1 sticklebacks (Di-Poi et al. 2014). In this study, the authors found that residents were more active, more aggressive, and shoal less than migrants. Functional explanations for the behavioral differences can be given, but they include quite some speculation. Compared with the sea, land-locked ditches in our study sites are characterized by small and shallow streams, enriched with vegetation, low mean annual productivity (Gross et al. 1988), lower density of piscivorous fish yet with the presence of invertebrate predators (Reimchen 1980; Marchinko 2009) and birds. Hence for residents selection may favor higher levels of aggression and exploration that facilitate the discovery, acquisition, and monopolization of limited resources (Budaev 1997; Brown et al. 2005; Huizinga et al. 2009; Herczeg et al. 2013; Greenwood et al. 2016; Moran et al. 2017). Such “risk-prone” behaviors may then be traded-off against shoaling, explaining why residents shoaled less compared with migrants (Ward et al. 2004). Differences in shoaling tendencies may also stem from the fact that migratory lifestyle involves group schooling during migration and presumably high shoaling tendencies in the sea due to “openness” of habitats. In migrants, lowered activity level could further be an indication that freezing is an adaptive response to higher perceived predation when not protected by a shoal (Huntingford and Wright 1993). Furthermore, the robust armature and larger spines, characteristic of migrants, are known to impede them in escape behavior,





**Figure 3**

**Syndrome structure of migrants and residents in two years.** Significant correlations after sequential Benjamini-Hochberg correction are represented with bold black lines. The numerical values represent pairwise Spearman correlation coefficients ( $\rho$ ).

thus potentially favoring freezing behavior (Andraso and Barron 1995). In addition, reduced aggressive interactions could be due to the highly shoaling lifestyle of migrants as these two behaviors were shown to be incompatible in sticklebacks (Lacasse and Aubin-Horth 2014). Despite the substantial differences in ecological conditions across the two study years, the differences in morphology and behavior between migrants and residents were relatively consistent, suggesting that the observed population differences are related to the different life styles of migrants and residents, rather than due to stochastic annual changes.

In our system, we know with reasonable certainty that the resident populations have been isolated from the migratory ancestors about fifty generations ago due to barriers. However, some divergence may already have taken place before the isolation event. For example, local stickleback populations that are located further away from the sea may exhibit partial migration (McKinnon et al. 2004), that is, a polymorphism where only part of the population migrates to the sea, while the rest of the population overwinters on site. If our “resident” populations originated from such partially migrating populations, the adaptations to a resident lifestyle may, to a certain extent, already have been in place. Additionally, the barriers to migration, especially the pumping station at LL-B (Supplementary Information 1c) pose as impenetrable barriers only to adult sticklebacks. Juveniles and fry may potentially cross over, especially to the open river with the direction of water flow and consequently making it possible to have reduced and biased gene flow from resident to ancestral migrant population. There exist many other

possibilities for gene flow, for example transportation of eggs/fry via birds. In any case, it is even more surprising that that the stark behavioral differences in wild-caught fish from these resident and migrant populations exist, despite the potential of reduced gene flow to hamper local adaptation (Raeymaekers et al. 2014). Further population genetic studies are needed to uncover exact population structure and divergence in our system.

### Rates of phenotypic change

Is the rate of phenotypic differentiation observed in our study comparable to other documented instances of rapid evolution? The literature on population differentiation in response to anthropogenic changes (Hendry et al. 2008), specifically in sticklebacks, acts as a useful yardstick (even though most of this literature only considers morphological traits). Quantifying rates of change in “haldanes” (Haldane 1949; Gingerich 1993) yields a measure that allows comparisons across populations and study systems. One way of calculating this is by quantifying absolute change in trait standard deviations per generation. In our study, we found that rate of change in size was  $-0.007$  haldanes; in other words, the size of resident fish decreased by 0.007 standard deviations per generation. The rates of change in our behavioral measures were 0.01 haldanes for activity, 0.001 haldanes for exploration, 0.014 haldanes for boldness, and  $-0.149$  haldanes for shoaling behavior. Overall, these values are quite comparable to the evolutionary rates reported for diverse traits in sticklebacks (Bell and Aguirre 2013) and other organisms (Hendry et al. 2008). In contrast, the

rate of change in size was much smaller than the rate reported in another anadromous-freshwater system of three-spined sticklebacks (0.234 haldanes for females and 0.365 haldanes for males, Baker et al. 2011). It is possible that the values reported above are underestimates of the rates of change that occurred in the initial phase after isolation: we averaged the rates of change linearly more than 50 generations, while a major part of differentiation typically occurs in the first few generations of isolation. Indeed more recent studies on sticklebacks isolated from marine to freshwater habitats have found evidence for evolution on contemporary timescales of decades to even seasons (Lescak et al. 2015; Hosoki et al. 2019; Garcia-Elfring et al. 2021).

### Population differences in syndromes

A previous study with twelve freshwater stickleback populations reported a positive correlation between boldness and aggression toward a conspecific in five out of the six populations where predators were present (Dingemanse et al. 2007, 2009). There were also tight correlations among other behaviors including activity, exploration, aggressiveness, and boldness in predator-sympatric populations (correlation coefficients range from 0.03 to 0.74). These tight behavioral correlations are thought to result from predation that enhances habitat heterogeneity by creating risky and non-risky areas and thus favors alternative behavioral strategies (e.g. Bell and Sih 2007; Dingemanse et al. 2007; Dhellemmes et al. 2020). Surprisingly, (but in line with an earlier study on freshwater and marine sticklebacks, Di-Poi et al. 2014), none of our stickleback populations, including migrants that should be exposed to higher predation pressure, exhibited stable syndromes across years and only few correlations between traits were detected. Boldness-Exploration was one of the stronger correlations in migrants ( $\rho = 0.289$ ), but still was weaker compared with previous studies ( $\rho = 0.667$ , Dingemanse et al. 2007). Activity-Exploration syndrome in residents was observed in the second study year ( $\rho = 0.680$ ), which was comparable to those reported from predator-sympatric populations ( $\rho = 0.754$ , Dingemanse et al. 2007). This lack of syndromes could be because the behaviors selected are not under correlated selection or that we lack the power to detect syndromes. Alternatively, in our system, predation risk and change in life-history may not systematically select for phenotypic trait integration (Sommer-Trembo et al. 2017).

### CONCLUSIONS

We have shown that behavior and morphology diverged in sticklebacks after human disturbance, blocking migration over about 50 generations. The observed phenotypic differences between migrants and residents clearly show that barriers to migration have thus major consequences for the phenotype and potentially life-histories and population dynamics of sticklebacks as correlated life-history characteristics (growth rate, size at maturity, number, and size of eggs) are also known to change on adaptation to freshwater in sticklebacks. Nevertheless, at least some populations can cope to a drastic loss of migration opportunity as they seem to thrive in land locked conditions. Next step would be to test whether the observed divergence is adaptive and to identify how it came about. One way to delineate the relative roles of genetic inheritance, non-genetic inheritance, developmental plasticity, and phenotypic plasticity is through common-garden experiments combined with cross-fostering experiments and through experiments where juveniles are exposed to different

selective regimes in semi-natural mesocosms. This would give us insight into role of personality in adaptation to novel environmental conditions.

### SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

### ACKNOWLEDGEMENTS

We thank Louis de Vries, Colleen Illing, Diederik Blaauw, Lisette Borchers for helping with data collection, the animal care takers and Suma Murthy for helping with animal care and lab work. We thank Peter Paul Schollema, at the Water Authorities Hunze en Aa's and Jeroen Huisman at van Hall Larenstein, university of applied sciences for help with acquiring samples of sticklebacks. We thank Jakob Gismann for commenting on the manuscript. We also thank two anonymous reviewers for their useful comments, which greatly improved the manuscript.

### FUNDING

This work is supported by PhD fellowship of the Adaptive Life programme of the University of Groningen to AR. FJW acknowledges funding from European Research Council (ERC Advanced Grant No. 789240). This work was also supported by grants from the Waddenfonds - Ruim Baan voor Vissen 2 (01755849/WF-2019/200914) and the Dr. J.L. Dobberke Foundation (KNAWWF/3391/1911).

Conflict of interest: The authors declare no competing interests.

Data availability: Analyses reported.

Handling editor: Ulrika Candolin

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