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ORIGINAL ARTICLE

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Relaxed risk of predation drives parallel evolution of stickleback behavior

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The occurrence of similar phenotypes in multiple independent populations derived from common ancestral conditions (*viz*. parallel evolution) is a testimony of evolution by natural selection. Parallel evolution implies that populations share a common phenotypic response to a common selection pressure associated with habitat similarity. Examples of parallel evolution at genetic and phenotypic levels are fairly common, but the driving selective agents often remain elusive. Similarly, the role of phenotypic plasticity in facilitating early stages of parallel evolution is unclear. We investigated whether the relaxation of predation pressure associated with the colonization of freshwater ponds by nine-spined sticklebacks (*Pungitius pungitius*) likely explains the divergence in complex behaviors between marine and pond populations, and whether this divergence is parallel. Using laboratory-raised individuals exposed to different levels of perceived predation risk, we calculated vectors of phenotypic divergence for four behavioral traits between habitats and predation risk treatments. We found a significant correlation between the directions of evolutionary divergence and phenotypic plasticity, suggesting that divergence in behavior between habitats is aligned with the response to relaxation of predation pressure. Finally, we show alignment across multiple pairs of populations, and that relaxation of predation pressure has likely driven parallel evolution of behavior in this species.

KEY WORDS: Behavior, parallel evolution, phenotypic plasticity, predation, *Pungitius pungitius*, vector analysis.

Similar environments may impose similar selection pressures on newly colonizing populations, leading to recurrent phenotypes in multiple habitats (Bailey et al. 2015). The evolution of similar phenotypes between independent populations of the same species (hereafter, parallel evolution *sensu* Bolnick et al. 2018; Fang et al. 2021) has long been attributed to natural selection, as only such a deterministic process is expected to result in the occurrence of the same traits in similar environments (Rundle et al. 2000; Schluter et al. 2004). Recent studies of repeated evolution in the wild have greatly advanced our understanding of the population-specific factors influencing the likelihood of parallel evolution (Stern and Lee 2020; Fang et al. 2021, Roberts Kingman et al. 2021) and the genetic underpinnings of similar phenotypic adaptations (Xie et al. 2019; Kemppainen et al. 2021). Yet, the main premise of repeated evolution is that the lineages evolving in parallel should do so in response to a common selection pressure and therefore, identifying the environmental factors driving these responses is central to our understanding of parallel evolution.

Predation is a ubiquitous feature of ecosystems and a driving force of the evolution of species interactions (Abrams 2000). Because of its direct influence on fitness, predation is also a strong selective agent behind the evolution of morphological (Eklöv and Svanbäck 2006), physiological (Rödl et al. 2007), and behavioral traits (Lapiedra et al. 2018) in prey species. Although predation can shape the distribution of phenotypes in prey communities, relaxation of predation pressure—for example, following the colonization of a predator-free habitat—has been suggested to select for traits advantageous in absence of predators (e.g., boldness) and lead to the evolution of novel phenotypes (Bliard et al. 2020). In either case, the presence or absence of predators in the environment is expected to play a central role in adaptive evolution and generate long-term divergence stemming from different levels of predation (Nosil 2004, Nosil and Crespi 2006). Changes in the predation regime of an environment can also induce short-term individual responses through phenotypic plasticity, namely, the expression of different phenotypes from the same genotypes in different environments (West-Eberhard 2003; Benard 2004). For instance, organisms may adjust their behavior when predation risk is high to either increase their probability of survival (Wen and Ueno 2021) or the survival of their offspring (Peluc et al. 2008). Consequently, individual variation in the magnitude and direction of plasticity in a population provides an additional source of phenotypic variation for selection to act on (Abbey-Lee and Dingemanse 2019), and it has been hypothesized that plasticity can sometimes “take the lead” in early stages of adaptive evolution (Scoville and Pfrender 2010; Levis and Pfennig 2016, 2020). Empirical evidence for the role of phenotypic plasticity in repeated evolution of complex traits is still relatively scarce, yet its putative part in paving the way of adaptive evolution holds an important place in the Extended Evolutionary Synthesis (Gilbert et al. 2015; Levis et al. 2018).

Here, we investigated the effects of perceived predation risk on the expression of behavioral traits in two types of locally adapted populations of the nine-spined stickleback (*Pungitius pungitius*). The nine-spined stickleback is a teleost fish distributed across the northern parts of Eurasia and North America. An ecological peculiarity of this species is that it naturally occurs in both marine and freshwater habitats. Marine ancestral populations of *P. pungitius* have colonized multiple freshwater habitats following the last glaciation about 11,000 years ago (Feng et al. 2021) and *P. pungitius* are now found in isolated ponds throughout Northern Europe (Teacher et al. 2011). Although marine populations of *P. pungitius* co-occur with a diverse community of piscine predators, freshwater pond populations have evolved in a virtually predator-free environment where they are often the sole fish species (Herczeg et al. 2010). As a result, it has been hypothesized that pond populations have evolved remarkable phenotypes in response to this relaxation of predation pressure, including gigantism (Herczeg et al. 2009a) and bold aggressive behaviors (Herczeg et al. 2009b). Empirical evidence demonstrated that among-habitat differences in behavior are genetically based and have resulted from divergent selection acting on several behavioral traits (Karhunen et al. 2014). Despite this evidence, whether predation is the likely factor driving behavioral divergence among habitats, and whether such divergence has repeatedly occurred in parallel, is yet to be tested experimentally.

We hypothesized that the relaxation of predation pressure associated with the colonization of predator-free habitats has driven the evolution of behavior in pond populations of *P. pungitius*. Furthermore, we test the complementary hypothesis that the between-habitat divergence in behavior may have resulted from the expression of advantageous plastic phenotypes in response to the relaxation of predation pressure. To this end, we used an experimental test of behavioral response to predation exposure in pond and marine nine-spined sticklebacks, and addressed the following questions: (i) Did behavior evolve in parallel among freshwater *P. pungitius* populations? To answer this question, we verified the expectation that parallel evolution of behavior should be reflected by an alignment between the phenotypic vectors of divergence from an approximation of the marine ancestor, between multiple pond populations. (ii) Is the relaxation of predation pressure likely to be the selective agent underlying the divergence between marine and pond sticklebacks? For this, we tested the theoretical prediction (Lind et al. 2015; Radersma et al. 2020) that the vector of phenotypic plasticity stemming from our predation exposure treatment in an approximation of the marine ancestor should be aligned with the vector of phenotypic divergence between habitats.

Materials and Methods

SAMPLING

Adult *P. pungitius* were sampled during breeding season (May–June 2018) at eight different locations in Finland and Sweden corresponding to four coastal marine and four freshwater pond habitats (Table S1). Pond populations were sampled using minnow traps placed in about 50 cm depth and marine populations were sampled from shallow (about 1 m depth) waters using beach-seine nets. Sampled fish were checked visually to ensure sexual maturity (i.e., black abdomen in males and rounded bellies in gravid females, e.g., McLennan 1996) and subsequently transported to the aquaculture facilities of the University of Helsinki. Wild-caught individuals from each population were housed separately in 1 m^3 plastic aquaria with flow-through freshwater system and fed ad libitum with frozen chironomid larvae twice a day.

COMMON GARDEN EXPERIMENTS

To control for environmental variance and to measure genetically based phenotypic variation among individuals, we set up a common-garden rearing design in the laboratory: for each population, five to 10 full-sib families were produced ($n = 65$; Table S1) by artificial crossing of random pairs of wild-caught individuals. We followed the standard in vitro fertilization techniques and egg husbandry protocols for stickleback crossing (Arnott and Barber 2000) and obtained eggs from gravid females by gently squeezing their abdomens over a petri dish. Males were over-anesthetized using tricaine methanesulfonate (MS-222) to dissect their testes, which were subsequently minced in the petri dish containing the eggs. Eggs and sperm were gently mixed using a plastic pipette to ensure fertilization and kept in freshwater until hatching. Water in the petri dishes was changed twice a day and clutches were visually checked for signs of fungal infections or death, and accordingly removed. At the onset of hatching, each clutch was split once in two replicate 11 × 10 cm plastic boxes. Following yolk resorption, fry was fed ad libitum with live brine shrimp (*Artemia* sp. *nauplii*). All replicated families were transferred to Allentown Zebrafish Rack Systems (hereafter rack; Aquaneering Inc., San Diego, CA, USA). Racks had a closed freshwater circulation system, with multi-level filtering including physical, chemical, biological, and UV filters. All fish were reared in racks under constant temperature and light conditions (15°C; 12:12 LD) for a period of about 1 year (mean age: 316.4 days, SD: 23.8 days) until the start of the behavioral experiment. We ensured that all fish did not show signs of sexual maturity that could affect the expression of behaviors. Before starting the experiments, all families were transferred to holding tanks where they were kept in constant temperature and light conditions (15°C; 12:12 LD) throughout the experimental periods. Replicates of the same family were housed in separate tanks to account for common environment variance.

EXPERIMENTAL SETUP

Two identical experimental aquaria with independent flow-through freshwater systems were built for the experiments (*Methods* in the Supporting Information; Fig. S1). Each aquarium was divided transversely in two sections by a transparent plastic plate separating the behavioral arena and the holding arena. The behavioral arena corresponded to the half of the tank where the focal fish were placed and scored for behaviors, whereas the holding arena corresponded to the half where the predators were introduced (predation treatment) or left empty (control treatment; see below). To investigate the effect of predation risk on stickleback behavior, behavioral tests were conducted in the presence and absence of predators. One of the experimental aquaria was assigned to predation treatment and one to control treatment. In the predation treatment, a pair of wild-caught perch (*Perca fluviatilis*), a natural predator of marine *P. pungitius* (Nelson and Bonsdorff 1990), were placed on the holding arena of the experimental aquarium.

BEHAVIORAL MEASUREMENTS

We measured ecologically relevant behaviors classified into two categories: exploration (an individual's propensity to explore a novel environment), and risk-taking during foraging (an individual's tendency to take risks to obtain food). All behavioral measurements were performed with one fish at a time and fish were starved for 24 h prior to the experiments. Each trial started by transferring the focal fish from the holding tank into the behavioral arena of the experimental tank and running the exploration test followed by the risk-taking test (see also *Methods* in the Supporting Information for details).

The focal fish was caught from its holding tank with a hand net and introduced into an opaque cylinder in the experimental tank (Fig. S1). The fish was left to acclimatize inside the cylinder for 3 min. After this acclimation time, the door of the cylinder was opened allowing the fish to leave the cylinder to explore the experimental tank. Two measurements were recorded: the latency until the head of the fish came out of the cylinder, and the latency until the full body of the fish came out of the cylinder.

Following the exploration test, the cylinder was removed, and the fish was left to acclimatize for 3 min in the behavioral arena. After the acclimation period, chironomid larvae (a familiar food) were pipetted into the open area of the tank in a straight diagonal line from the edge of the refuge to the opposite corner of the tank (see Fig. S1). With this kind of food administration, the more the fish ate, the further it had to move from the refuge, so that the "risk" experienced by the fish (swimming further into the open area and closer to the predator) was proportional to the "reward" (number of food items). Three measurements were recorded: the time spent in the open area (whole body outside the refuge area when viewed from above) in the 5 min following the addition of the first food item; the latency to initiate feeding after the addition of the first food item; and the total number of feeding events measured as the number of successful attacks on the food.

All time variables (latencies) were measured in seconds and each trial was terminated if the fish did not express the behavior after 5 min, so that the maximum value for these measurements was 300 s. At the end of the experiment, a total of 422 individuals were phenotyped across 65 families and eight populations for the four following traits: emergence time, open time (time spent in the open area), feeding (the number of feeding bouts), and risk-taking (the latency to first feeding). Due to the high correlation between the two exploration variables (time-to-head-out and time-to-body-out; Pearson's $\rho = 0.951$ [0.941; 0.959], $P < 0.001$), we used the arithmetic mean of the two variables as a measure of emergence time.

STATISTICAL TESTS OF PHENOTYPIC DIFFERENTIATION

We first investigated behavioral variation between populations, habitats, and the effect of perceived predation using statistical models. Our data consisted of three right-censored (i.e., truncated) time-to-event variables: emergence time, open time, and risk-taking. This type of data is not suitable for classical linear regression approaches (i.e., linear mixed- or generalized linear models; Edelaar et al. 2012) and we thus followed three different statistical frameworks to verify the robustness of our results.

First, we fitted censored regressions using the *censReg* R package (version 0.5-32, Henningsen 2017). We set the right limit for censoring at 300 (the maximum time value in seconds in our experiment). Fixed effects of interests were habitat of origin (marine or pond) and treatment (predation or control) and their interaction. Temporal block of measurements (morning or afternoon, see *Methods* in the Supporting Information) was also set as a fixed effect. To account for the possible effects of body size and age variation in our data, we included age-corrected body size as a covariate, computed from the residuals of a linear regression of body size on age.

Second, we used the Kaplan-Meier survival analysis framework (Crowley and Breslow 1984) and fitted survival curves using the *survival* (version 3.2-11, Therneau 2021) and *survminer* R packages (version 0.4.9, Kassambara et al. 2017). Because the event measured in our time-to-event data is the expression of a behavior rather than actual death or survival as is usually the case in such analyses, the estimated survival curves correspond to the expected proportion of fish having expressed the behavior of interest at a certain time. Original time-to-event variables were binary transformed by assigning a value of zero to all individuals with the maximum value of 300 s and one to all other individuals. This way, each individual assigned with a value of 0 is considered as not having expressed the measured behavior. We used the *Surv* function and input our original measurements (in seconds) as the “time” argument and the binary-transformed data as the “event” into the function. We then estimated survival curves for each habitat (marine or pond) and each treatment (predation or control) using the *survfit* function by including habitat of origin and treatment as categorical variables. We then compared the differences in behavior between habitats and treatments by computing the differences between survival curves using the log-rank test (Kleinbaum and Klein 2012) implemented in the *pairwise_survdiff* function of the *survminer* package and using the Bonferroni *P*-value adjustment method.

Finally, we applied a Box-Cox transformation (Box and Cox 1964) to all right-censored measurements using the *boxcox* function of the *MASS* R package (version 7.3-54, Venables and Ripley 2002) and used the transformed data as response variables in a Linear Mixed Model framework. We used the *lmer* function of the *lme4* R package (version 1.1-27, Bates et al. 2015) and fitted a model with habitat, treatment, and their interaction as fixed effects as well as temporal block and age-corrected body size as covariates. Population of origin, perch pair, and tank identities nested within family term were used as random effects. We tested for the significance of the fixed effects ($P < 0.05$) in all above mixed-effect models (i.e., using the *lme4* package) using Wald chi-square tests using the *Anova* function in the *car* R package (version 3.0-11, Fox et al. 2019).

Count data (i.e., feeding variable) were analyzed with a generalized linear model (GLM) using a negative binomial function with the *glm.nb* function of the *lme4* R package (version 1.1-27, Bates et al. 2015) with habitat of origin, treatment, and their interaction as fixed effects as well as population of origin, perch pair, and holding tank identity as random effects.

Hence, using these modeling approaches, we investigated whether pond and marine fish showed genetically based differences in each behavioral trait (i.e., significant habitat effect), if our predation treatment generated a plastic response in the expression of behaviors (i.e., significant treatment effect) and whether pond and marine fish varied in their plastic response to the two treatments (i.e., significant interaction between habitat and treatment effects). Moreover, a significant interaction between habitat and treatment effects would indicate differences in the reaction norms of pond and marine individuals and suggest an evolution of phenotypic plasticity for these traits (Fraimout et al. 2018).

PHENOTYPIC VECTOR ANALYSIS

We investigated parallelism in behavioral evolution by computing two types of phenotypic vectors: first, we estimated the evolutionary divergence vectors (Δz_{D}) corresponding to the multivariate phenotypic differences between marine and freshwater habitats (Fig. 1a). Specifically, we calculated the vectors of phenotypic change between each pond population from a hypothetical marine ancestral population. The ancestral marine population was estimated as the average multivariate behavioral phenotype from all the marine individuals measured for all behaviors in the presence of predators (Fig. 1c). We used these measurements as representative of a natural marine population experiencing predation pressure. Following the same logic, pond populations in the control treatment (no predation) were used as representative of natural freshwater populations experiencing no piscine predation (Fig. 1). Vectors were calculated as the phenotypic difference between each pond population and the hypothetical ancestral population such that

$$\Delta z_{\mathrm{D}} = \underline{z}_{\mathrm{P}} - \underline{z}_{A}, \tag{1}$$

where $\underline{z}_{\mathrm{P}}$ corresponds to the mean phenotype of a pond population and $\underline{z}_{\mathrm{A}}$ to the mean phenotype in the ancestral marine population. Mean population phenotypes were extracted from separate model coefficients using censored regression for time-to-event data with the *censReg* R package, and a GLM for the feeding count data using the *glm* function of the lme4 R package. In all models, habitat of origin, treatment, and their interaction were set as fixed effects and age-corrected body size was used as a covariate.

Second, we estimated the vectors of phenotypic plasticity (hereafter, plasticity vectors, Δz_{φ}; Fig. 1b) as the phenotypic change induced by predation exposure. We were primarily interested in the plasticity vectors depicting the behavioral changes

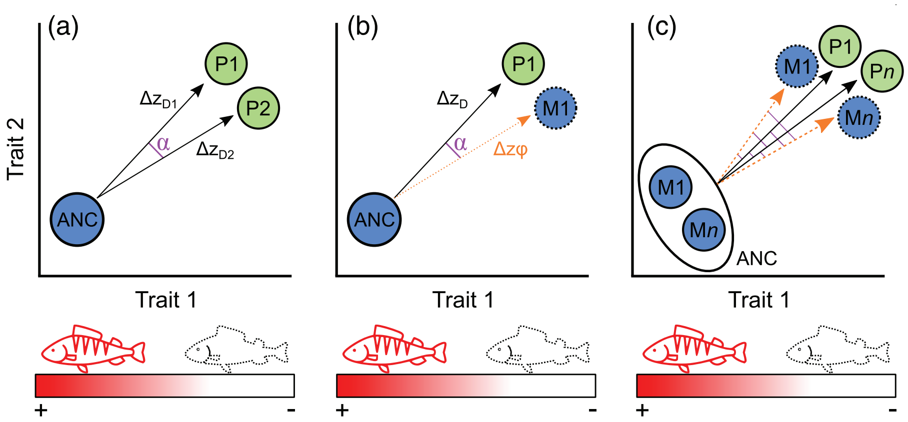

Figure 1. **Graphical representation of the theoretical expectations and empirical test of parallel evolution of divergence and plasticity. (a) Parallel evolution in response to predation relaxation (black-dashed perch outline; –) is expected to result in a small angle (purple solid line, alpha) between the divergence vectors (black solid arrows, Δz_{D}) of two pond populations (green-filled circles; P1, P2) estimated as the divergence in multivariate phenotype from a hypothetical marine ancestor (blue-filled circle; ANC). (b) Alignment between divergence and phenotypic plasticity is inferred from the angle between Δz_{D} and the vector of plasticity (orange dashed arrow, Δz_{ϕ}) calculated as the difference between the marine ancestor and the plastic response to predation release of a marine population (blue-dashed circle, M1). (c) Empirical test of parallel evolution of divergence and plasticity: we estimated the hypothetical marine ancestor (black solid ellipse; ANC) as the average phenotype of replicated extant marine populations (blue-filled circle; M1, Mn) in the predation treatment (red solid perch outline; +) and computed the divergence vectors for each sampled pond population (green-filled circle, P1, Pn) in the control treatment. Plasticity vectors were calculated as the difference in multivariate behavior phenotype between this hypothetical ancestor and each sampled marine population (blue-dashed circle; M1, Mn) measured in the control treatment.**

following the relaxation of predation pressure and thus, equivalent to the colonization of predator-free freshwater habitats by ancestral marine *P. pungitius* populations. To this end, we calculated the plasticity vectors as the phenotypic changes between the hypothetical ancestral population and each marine population measured in the control treatment as

$$\Delta z_{\varphi} = \underline{z}_{\mathrm{M}} - \underline{z}_{\mathrm{A}}, \tag{2}$$

where $\underline{z}_{\mathrm{M}}$ is the mean trait value for the marine population measured in the absence of predators and $\underline{z}_{\mathrm{A}}$ is the same as in equation (1).

Next, we tested for the alignment between all pairs of divergence and plasticity vectors (Fig. 1c). To this end, we calculated the angle α between any two pairs of vectors (Fig. 1c) as

$$\alpha = \cos^{-1}\left(\Delta z_{ANCi}\,\Delta z^{T}_{ANCj}\right), \tag{3}$$

where each vector Δz corresponds to the normalized phenotypic vector of difference between the focal populations i, j, and the estimated marine ancestor *ANC*. Angles were calculated in degrees between all pairwise combinations of divergence and plasticity vectors (Fig. 1c). We assessed the statistical significance of all observed angles by comparing them to the angles calculated from random vectors. To this end, we used the *rnorm* function in R and generated random multivariate vectors similar to our observed vectors (i.e., with four elements each) from a normal distribution. We calculated 10,000 angles between these random vectors and compared this null distribution to our observed angles to assess statistical significance ($P < 0.05$) in our data. This way, we considered significant alignments among pairs of divergence vectors as indicative of parallelism in the evolution of behavior in the ponds (Fig. 1c). Following the same rationale, significant alignments between plasticity and divergence vectors would suggest that the relaxation of predation pressure is likely to be the selective agent underlying the divergence between marine and pond sticklebacks. Finally, significant alignments between plasticity vectors would be suggestive of a shared plastic response to predation exposure among marine populations.

Because we were interested in evaluating the evolution of complex behavior in *P. pungitius*, each phenotypic vector described above was constructed from the multivariate behavioral traits' dataset in each population and treatment. In other words, each vector of divergence or plasticity included the differences in means for all four behavior traits measured, thus providing a multivariate measure of differentiation. To avoid scaling issues due to the differences between count data (i.e., feeding behavior) and time-to-event data, raw measurements were transformed to *z*-scores using the *scale* function in R (version 4.1.1, R Core Team 2021) prior to all phenotypic vector analyses.

We then followed the methodology of De Lisle and Bolnick (2020) to identify the dimensions of parallel change among

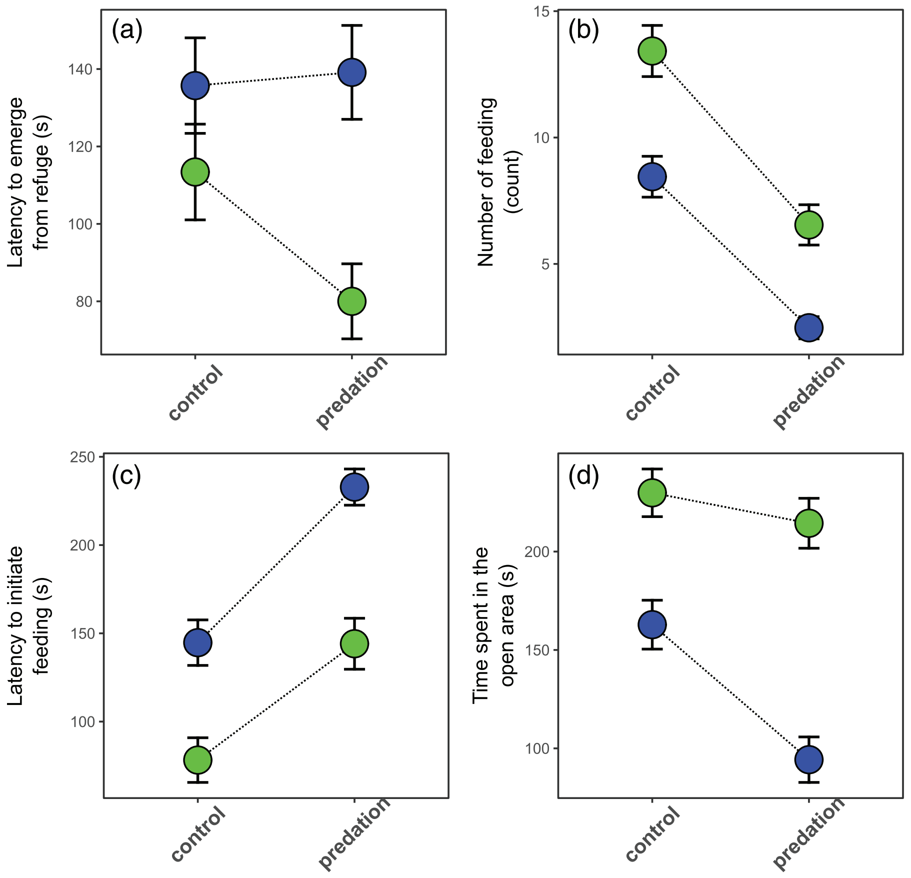

Figure 2. Behavioral variation between habitats and treatments. Mean values (circles) and standard errors (whiskered vertical bars) for the raw behavior measurements are shown for marine (blue circles) and pond (green circles) fish in the control and predation treatments. (a) Emergence time, the latency to emerge from a refuge (in seconds). (b) Feeding, the number of feeding event (count). (c) Risk-taking, the latency to initiate feeding (in seconds). (d) Open time, the time spent in the open area (in seconds). Dashed lines represent the reaction norms for each habitat.

divergence and plasticity vectors by analyzing **C**, the matrix of correlation between replicated pairs of phenotypic vectors. We started this by constructing the matrix **X**, an $m \times n$ matrix (in our case 8 × 4) with m rows containing each a population-specific divergence and/or plasticity vector (i.e., each Δz_{φ} and Δz_{D}) and n columns each containing the difference in behavior from the hypothetical marine ancestor. **C** was calculated as

$$C = XX^{\mathrm{T}}. \tag{4}$$

Eigenanalysis of **C** further allowed us to estimate whether one or more direction in the multivariate space (the eigenvectors) underlined a common parallel direction among our study populations, as well as the extent to which certain populations showed more parallelism among each other (see *Results* section) than others. All analyses were performed in R version 4.1.1 (R Core Team 2021).

Results

PHENOTYPIC DIFFERENTIATION

There was a strong habitat differentiation in all behavior variables and pond sticklebacks were consistently more explorative and took more risks during foraging than marine sticklebacks (Figs. 2a–d, S2, S3; Table S2). Overall, the predation treatment had stronger effects on foraging behaviors than exploration behavior (Figs. 2, S2; Tables S2–S4). Both pond and marine fish reduced the amount of feeding (Fig. 2; Table S2) and took longer time to initiate feeding in the presence of predators (Fig. 2; Tables S2–S4). Emergence time was not significantly affected by the presence of predators (Fig 2; Tables S2–S4) and there was weak statistical support for habitat difference in this trait (Fig. 2; Tables S2–S4). These results were robust across different statistical methods (Tables S2–S4) with the exception of open time: marine individuals were less likely to spend time in the open area in the presence of predators, whereas the predation treatment did

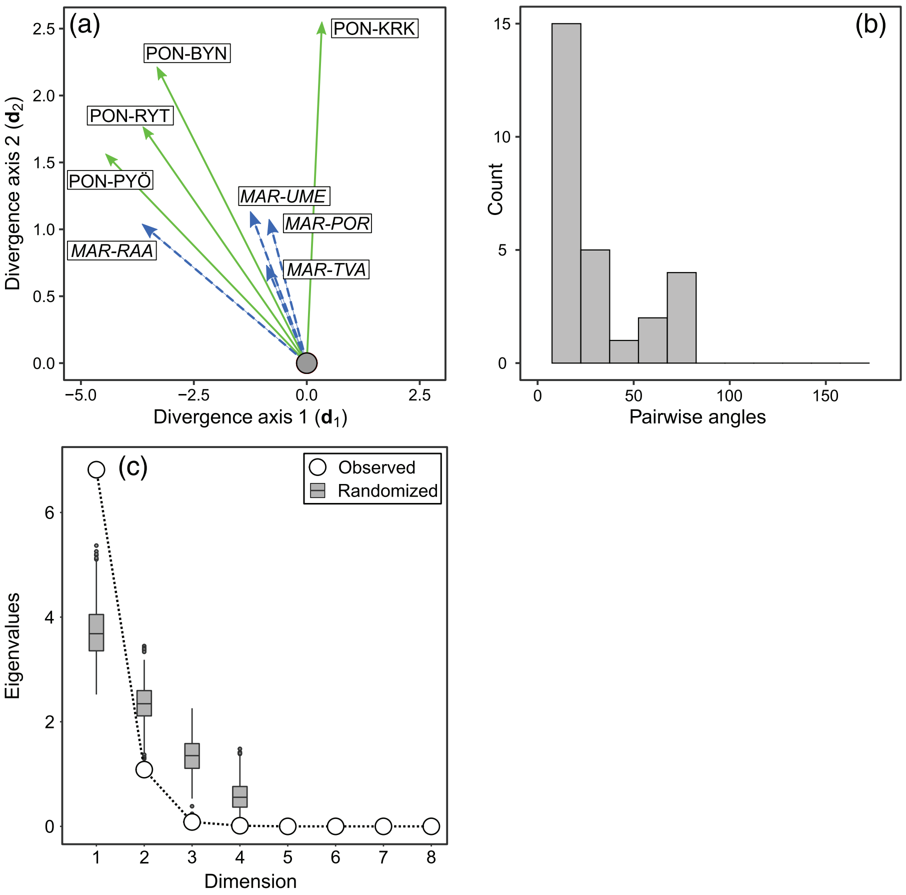

Figure 3. **Results of the phenotypic vector analyses. (a) Graphical representation of the phenotypic vectors. The vectors of divergence (solid green arrows) and plasticity (dashed blue arrows) from the ancestral marine population (gray filled circle) are projected in the multivariate divergence space where d_1 and d_2 represent the first and second main axis of the multivariate divergent covariance matrix. Population codes for pond and marine (italic) are indicated in black text. (b) The distribution of observed vector angles in degree. (c) Results of the multivariate test of parallelism. Eigenvalues from the decomposition of the C matrix calculated from the observed (open circles) and randomized (gray boxplots) data are shown. Boxplots represent the expected (randomized) eigenvalues calculated from sampling a Wishart distribution. Observed eigenvalues greater than expected ones indicate a single significant axis of parallelism among vectors.**

not lead to a significant decrease in open time in pond fish (Fig. 2; Tables S2 and S3) but this result was not reflected by differences in survival curves using the Kaplan-Meier framework (Fig. S2; Table S4). Finally, age-corrected body size had a significant effect on the risk-taking and emergence behaviors with larger fish showing increased latency to emergence and first feeding (Tables S2 and S3).

PHENOTYPIC VECTOR ANALYSES

Phenotypic vector analyses allowed us to investigate three aspects of the evolution of complex behavior in *P. pungitius* (Fig. 3; Table 1): (i) the degree of parallelism between vectors of freshwater adaptation, indicated by the among-ponds comparisons of vectors (Table 1, green cells); (ii) the degree of parallelism between the vectors of phenotypic plasticity, indicated by the among-marine comparisons of vectors (Table 1, blue cells); and (iii) the alignment between the vectors of plasticity and evolutionary divergence, indicative of the effect of predation relaxation on the evolution of behavior from marine to pond habitats (Table 1, red cells). We found that three out of the four pond populations shared a parallel direction of phenotypic divergence from the ancestral marine population, as evidenced by the angles between their divergence vectors, which were found to be more similar than between random vectors (Table 1). The PON-KRK population consistently showed evidence for nonparallelism with the other pond populations (Table 1; Fig. 3). We found that the plastic response to the relaxation of predation pressure was largely shared among marine populations. Two pairs of populations (MAR-POR and MAR-RAA, MAR-UME and MAR-RAA; Table 1) did not show evidence of parallelism between the vectors of phenotypic plasticity. Out of the 16 pairs of plasticity-divergence vectors, 10 showed significant parallelism, as

Table 1. Angles between phenotypic vectors. The angle in degrees between each pairwise vector comparison is shown. Color shading indicates the pairwise comparisons related to the test of parallel evolution among ponds (green), parallel phenotypic plasticity (blue), and the alignment between plasticity and divergence vectors (red, and see *Methods* for rationale). Bold values indicate statistical significance ($P < 0.05$) and italic values nonsignificance for tests of differences between observed and random vectors (see *Methods*).

	PON-PYO	PON-RYT	PON-KRK	PON-BYN	MAR-TVA	MAR-POR	MAR-RAA	MAR-UME
PON-PYO								
PON-RYT	**7.144**							
PON-KRK	*77.850*	*71.676*						
PON-BYN	**15.310**	**11.130**	*63.316*					
MAR-TVA	**23.694**	**20.409**	*56.894*	**11.655**				
MAR-POR	*35.151*	*30.321*	*44.654*	**22.085**	**13.021**			
MAR-RAA	**9.694**	**15.382**	*80.025*	**18.414**	**23.304**	*36.020*		
MAR-UME	**23.438**	**17.105**	*56.137*	**11.006**	**17.207**	**21.079**	*28.883*	

indicated by the low angles between each pair of vectors (Table 1). The six nonsignificant parallel pairs of vectors all included the PON-KRK and MAR-POR populations, indicating that the divergence of the PON-KRK population from the marine ancestor did not follow the global direction of phenotypic plasticity and, conversely, that the plastic response of the MAR-POR population did not align with the divergence vectors of all pond populations (Table 1). Overall, alignments between divergence and plasticity vectors indicate that the direction of behavioral change in the multivariate trait space induced by the relaxation of predation is similar to the direction of change observed in nature between marine and pond habitats.

Finally, we found that the directions of phenotypic changes stemming from the between-habitat divergence and the experimental relaxation of predation treatment were underlined by a single orthogonal dimension or parallelism, as evidenced by the first dimension of the **C** matrix decomposition (Fig. 3) showing greater eigenvalue than expected at random.

Discussion

Our common garden experiment shows that genetically based differences in behavior among pond and marine populations of *P. pungitius* have repeatedly evolved in parallel from marine ancestors. We found that our predation treatment generated a strong plastic response in most behavioral traits in both habitats and that this plastic response was aligned with the direction of evolutionary divergence. Below, we discuss the implications of our results for the study of behavioral evolution in the wild.

The analyses of phenotypic vectors were based on a hypothetical marine ancestral population, corresponding to the average behavioral phenotype of contemporary Baltic Sea populations of *P. pungitius*. The detailed phylogeographic history of the nine-spined sticklebacks in Fennoscandia was recently resolved (Feng et al. 2021) and suggests that the Finnish pond and northernmost Baltic marine populations used in the current study most likely originated from ancestral populations in the White Sea rather than from the Baltic Sea (Teacher et al. 2011; Bruneaux et al. 2013; Feng et al. 2021). Nonetheless, Baltic *P. pungitius* have been shown to be phenotypically similar (particularly regarding behavior) to contemporary populations found in the White Sea (Herczeg et al. 2009a; Karhunen et al. 2014). More importantly, statistical modeling of behavioral phenotypes in relation to genetic coancestry revealed that the behavior of contemporary marine populations of *P. pungitius* (Baltic and White Sea) is akin to the expected ancestral marine behavior (see fig. 3C,D in Karhunen et al. 2014). Our reconstruction of the ancestral population in the current analyses should thus be valid. Furthermore, evolutionary divergence vectors were constructed from the phenotypic differences between marine individuals exposed to predators and pond individuals in absence of predation. Because the results show that pond individuals have retained their plastic response to predation, the pond individuals in our experiments might have expressed exaggeratedly bold behavior in the control treatment that could have affected our inference. However, pond individuals display changes in all behavior variables in the same direction of the phenotypic space (*i.e.*, they are consistently bolder than marine individuals in both treatments), which indicates that the used experimental approach unlikely influenced angles between the plasticity and divergence vectors.

Pairwise comparisons of phenotypic vectors showed that the divergence of one freshwater population (PON-KRK) deviated from that of other pond populations by showing less bold and explorative behavior (Fig. S2). Although we did not record the presence of other fish species at the time of sampling at this location, artificial introduction of potentially predatory trout (*Salmo trutta*) has been reported in this pond (Herczeg et al. 2010) and could explain the observed divergence in behavior of this population. We also note that this population had the lowest sample size of our study and that the estimates may be subjected to some bias. Nevertheless, our multivariate test of parallelism identified a shared direction of phenotypic divergence among all pond populations, providing solid evidence for the parallel evolution of behavior associated with the colonization of freshwater habitat in this species. Moreover, this shared direction of parallelism also indicated that the direction of phenotypic plasticity in marine fish generated by our control treatment (relaxation of predation pressure) is aligned with the direction of evolutionary divergence among habitats.

Phenotypic evolution stemming from ancestral plasticity requires that the plastic response is genetically based and variable between individuals, and that this response would be advantageous in the environment where it is expressed (Ghalambor et al. 2007). Here, we used a common-garden design to ensure the measurement of genetically based differences between individuals and focused on traits known to be heritable in sticklebacks (Bell 2005; Dingemanse et al. 2012; Karhunen et al. 2014). Predation elicited behaviors that could be considered to be advantageous in their corresponding environments and, particularly in the marine (ancestral) individuals. Indeed, in the presence of predators, fish would reduce activity time and foraging rates (thus decreasing their probability of mortality), whereas they increased these behaviors, and consequently their resource intake, in the absence of predators. Selection acting on this new advantageous variation in predator-free habitat would thus promote the evolution of bold behaviors. Nonetheless, Futuyma (2017) argued that "*phenotypic plasticity could be said to truly play a leading role (with genes as followers) if an advantageous phenotype were to be triggered by an environment that really is novel for the species lineage*." In the case of *P. pungitius*—and more generally, in the case of predation—it is difficult to argue that the absence of predators is a truly novel condition to marine ancestors of freshwater-adapted populations. Although our results may not provide direct evidence for the role of plasticity in leading adaptive evolution, our study opens an interesting avenue of research to investigate the fitness effects of predation pressure in *P. pungitius*, and more generally, to consider the role of predation-induced plasticity in the evolution of complex traits.

There were marked behavioral differences between marine and pond sticklebacks and our findings are in agreement with those found in earlier studies (Herczeg et al. 2009a; Herczeg and Välimäki 2011). However, in contrast to earlier studies (*e.g.*, Herczeg et al. 2009a; Herczeg and Välimäki 2011; Laine et al. 2014), all fish in our study were reared in groups. Because nine-spined sticklebacks display social behavior such as schooling (Herczeg et al. 2009c), it is possible that the behaviors measured in our study were affected by this social component. However, such social effects might only accentuate preexisting behavioral differences within groups (Frost et al. 2007) and would not necessarily affect our conclusions. Furthermore, shy and bold behavior could be subjected to maternal effects, as most females in our experiment developed their eggs in the wild while exposed to different levels of predation exposure (Storm and Lima 2010; Roche et al. 2012; McGhee et al. 2012). Although we cannot refute this possibility with the data at hand, maternal effects were found to be low for body size—an important trait involved in the habitat differentiation in *P. pungitius*—(Ab Ghani et al. 2012) and were shown to dissipate throughout growth (Shimada et al. 2011), suggesting that such effects might not be of great concern for our study using adult fish. Overall, our large replicated common-garden design provides robust evidence for the genetic basis of behavioral variation in wild stickleback populations from the two contrasting habitats.

Another important aspect of sociality in the expression of behaviors in *P. pungitius* is intraspecific competition. Indeed, the colonization of predator-free and low-productivity pond habitat is also associated with high levels of intraspecific competition and the evolution of gigantism and bold behaviors in the ponds has also been hypothesized to stem from this increased competition (Herczeg et al. 2009a,b). In such environments, the relaxation of predation pressure and absence of other species sharing similar trophic niche has inevitably led to the need for conspecifics to compete for limited food resources. Hence, it is possible that predation alone would not be sufficient to explain the evolution of bold behaviors and our current experimental setup does not allow to disentangle the effects of predation from the effects of intraspecific competition. However, an important result of our study is that the relaxation of predation pressure directly enhanced the foraging rate—a particularly important life-history trait—in all populations. Therefore, our results suggest that the relaxation of predation pressure would have allowed "quick and heavy" feeders to acquire more resources in predator-free environments, which, in turn, would be favored by the new selection pressure imposed by the pond habitats. Future studies specifically testing for the interaction between predation risks and interspecific competition (*e.g.*, Urban and Richardson 2015) are needed to shed more light on this specific aspect of behavioral evolution in *P. pungitius*.

In conclusion, we have demonstrated that genetically based differences in complex behavior in Fennoscandian nine-spined sticklebacks have repeatedly evolved in similar environments and most likely in response to the same selection pressure. This provides strong evidence that this complex trait has evolved by natural selection in this species (cf. Schluter et al. 2004). We also show that the phenotypically plastic response to the relaxation of predation pressure can align with the direction of evolutionary divergence observed in the wild, suggesting that phenotypic plasticity may have contributed to the early stages of evolution of behavior in freshwater habitats. Overall, our study shows that genetically determined behaviors can evolve through natural selection, and that behavioral traits are well suited to studying local adaptation in general.

AUTHOR CONTRIBUTIONS

AF and JM conceived the study. AF and EP conceived the experimental setup. EP performed all behavioral measurements. AF and EP analyzed the data. AF led the writing of the manuscript with contributions from EP and JM. JM acquired funding.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

Mean population behaviors are available at the Dryad repository: https://doi.org/10.5061/dryad.m63xsj455.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Schematic representation of the experimental aquaria.
Figure. S2. Behavioural variation between populations and treatments.
Figure S3. Kaplan-Meier survival analysis of time-to-event variables.
Table S1. Geographic coordinates of localities, number families and individuals used from each locality.
Table S2. Results of the linear regressions using the original data.
Table S3. Results of the linear mixed model with the Box-Cox transformed time-to-event data.
Table S4. Pairwise comparisons of survival curves.