

Genetic and Neural Modularity Underlie the Evolution of Schooling Behavior in Threespine Sticklebacks

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Summary

Although descriptions of striking diversity in animal behavior are plentiful, little is known about the mechanisms by which behaviors change and evolve between groups. To fully understand behavioral evolution, it will be necessary to identify the genetic mechanisms that mediate behavioral change in a natural context [1–3]. Genetic analysis of behavior can also reveal associations between behavior and morphological or neural phenotypes, providing insight into the proximate mechanisms that control behavior. Relatively few studies to date have successfully identified genes or genomic regions that contribute to behavioral variation among natural populations or species [2], particularly in vertebrates [4–8]. Here, we apply genetic approaches to dissect a complex social behavior that has long fascinated biologists, schooling behavior [9–13]. We performed quantitative trait locus (QTL) analysis of schooling in an F2 intercross between strongly schooling marine and weakly schooling benthic sticklebacks (*Gasterosteus aculeatus*) and found that distinct genetic modules control different aspects of schooling behavior. Two key components of the behavior, tendency to school and body position when schooling, are uncorrelated in hybrids and map to different genomic regions. Our results further point to a genetic link between one behavioral component, schooling position, and variation in the neurosensory lateral line.

Results and Discussion

To dissect the genetic contributions to the evolution of behavior, we focused on schooling, a complex social behavior that is representative of social grouping behaviors seen throughout the animal kingdom. Social grouping provides several key benefits, but it also has associated costs, so the frequency of schooling varies with ecological context [11, 13–15]. We previously developed an assay that utilizes a school of model sticklebacks to reliably elicit divergent schooling behavior between two populations of threespine sticklebacks from distinct habitats. In this assay, lab-raised marine sticklebacks from the Pacific Ocean in Hokkaido, Japan school very strongly, but lab-raised benthic sticklebacks from Paxton Lake in British Columbia, Canada show dramatically reduced schooling behavior [16] (Figures 1A

and 1B). Differences in measures such as time spent with the models and latency to approach the models suggest that marine and benthic sticklebacks differ in their social attraction or tendency to behave socially (Figure 1A). Social attraction is a central feature of two types of collective behavior in fish: shoaling and schooling [13, 17]. Shoals are defined exclusively by social attraction, but for a group to be recognized as a school, individuals must also maintain a coordinated body position with their schoolmates, displaying polarized orientation and synchronized movement [17, 18]. In our assay, marine fish assume a relatively parallel orientation with the models, whereas benthic sticklebacks that follow the school do so with a significantly less parallel orientation (Figure 1B). Because the positions of the models are fixed relative to one another, our assay also permits measurement of the preferred schooling position of the fish among the models, showing that benthic and marine fish assume distinct positions (as assessed by head position) within the model school (Figure 1B).

Thus, the model school assay enables quantification of the two fundamental components of schooling: schooling tendency (comprising time schooling, latency to school, and number of schooling bouts) and schooling position (comprising body orientation and head position). We predicted that because shoals can exist independent of schools [13, 17], these two aspects of schooling behavior would be separable and controlled by distinct genetic modules.

Schooling Behavior Comprises Two Genetically Separable Behavioral Components

We first investigated the modularity of schooling behavior by evaluating phenotypic correlations among 229 benthic-marine F2 hybrids that were tested in the model school assay (Supplemental Experimental Procedures available online). These F2 hybrids expressed a wide range of schooling behaviors (Figure S1) and revealed that the two key features of schooling behavior were indeed genetically separable. Parameters reflecting social attraction or tendency of the fish to school were correlated with one another: time spent schooling was strongly correlated with the latency to join the school and the number of schooling bouts (Figure 1C) (time and latency: Spearman's $\rho = -0.477$, $p < 0.0001$; time and bouts: $\rho = -0.519$, $p < 0.0001$). It is important to note that some of these variables are in part definitionally correlated. For example, latency to school necessarily sets an upper limit on total time spent schooling, although it does not solely dictate the duration of schooling. Variables measuring schooling position were also correlated with one another (Figure 1C): the average body orientation was strongly correlated with the average x and y position of the head relative to the models (orientation and head x position: $\rho = 0.582$, $p < 0.0001$; orientation and head y position: $\rho = 0.683$, $p < 0.0001$). Importantly, measures of schooling position were not strongly correlated with measures of schooling tendency. Most tendency and position variables were not significantly correlated ($p > 0.05$), with the exception of weak correlations between head y position and both latency ($\rho = -0.254$, $p < 0.01$) and time schooling ($\rho = 0.202$, $p < 0.05$). The lack of strong correlation in the

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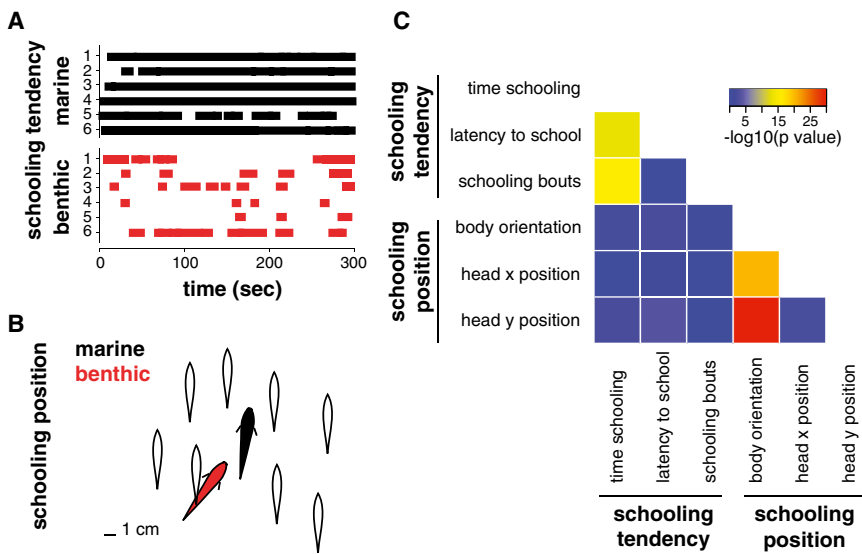


Figure 1. Schooling Tendency and Schooling Position Are Separable Features of Schooling Behavior

(A) Raster plots depicting differences in schooling tendency of typical marine and benthic sticklebacks tested in the model school assay. This assay uses a school of eight model sticklebacks that are positioned and moved to mimic schools of live fish (Supplemental Experimental Procedures). Bars represent schooling episodes (bouts) as a function of time for a random sample of six lab-raised fish from each population [16]. Marine fish have a shorter latency to school and spend more time schooling [16].

(B) Schematic depicting differences in average body orientation and head position of marine and benthic fish when following the model school, based on data from [16]. Open silhouettes represent positions of the model sticklebacks. Marine fish (black silhouette) have a significantly more parallel body orientation than benthic fish (red silhouette). The scale bar represents 1 cm.

(C) Heatmap showing strength of correlations between schooling variables in benthic-marine F2 hybrids. Measures of schooling tendency are not strongly correlated with measures of schooling position in F2 hybrids.

See also Figure S1.

F2 hybrids suggests a separable genetic basis for these behavioral components.

We next asked whether common or disparate genomic regions control schooling tendency versus schooling position. To identify regions of the genome associated with different schooling parameters, we subjected the same benthic-marine F2 individuals to quantitative trait locus (QTL) analysis (Supplemental Experimental Procedures). Fish were genotyped using single-nucleotide polymorphism (SNP) markers that spanned the genome [19, 20]. We identified two significant QTL that were associated with measures of schooling position (Figure 2; Table 1). Both body orientation and head x position mapped to the same region on linkage group 4 (LG4), and body orientation was also linked to a region on LG17 (Figure 2; Table 1). Another QTL on LG20 for y position had a likelihood of odds (LOD) score that reached a genome-wide threshold of $p < 0.1$ and was therefore considered to be a suggestive QTL (Figure 2; Table 1). Measures of schooling tendency did not show any association to these QTL regions (Figure 2; Table 1). We identified a suggestive QTL for latency to school on LG20, but the confidence interval for this QTL was nonoverlapping with the QTL for y position (Figure 2; Table 1). The fact that we detected suggestive QTL for schooling tendency variables reveals that this cross would have sufficient power to identify any potentially overlapping regions of linkage between tendency and position components of schooling behavior. These data provide additional support for the modularity of schooling behavior on a genomic level.

The QTL we detected for schooling position explain a modest amount of the phenotypic variance in these traits. For example, the two QTL for head x position account for just over 20% of the variance (Table 1). This suggests additional undetected QTL and/or an environmental component to this trait. Similarly, the lack of significant QTL for schooling tendency might be due to higher environmental variance and/or a genetic architecture composed of small-effect QTL for these traits. Although QTL of large effect have been detected for several morphological traits in sticklebacks (e.g., [21]), many other morphological and behavioral traits appear

to exhibit a more complex genetic basis (e.g., [5, 20]). Furthermore, our studies are consistent with other behavioral genetic studies: although single genes can have large impacts on behavior [2, 22], many complex behaviors, particularly in vertebrates, are associated with multiple genetic regions of small to modest effect [4, 6, 23, 24].

Genetic Association between Schooling Behavior and Neurosensory Phenotypes

Schooling is a highly precise behavior, requiring sophisticated motor and sensory capabilities. When fish school, they must maintain a characteristic body position and orientation within a highly dynamic group of conspecifics, a task that requires accurately assessing sensory information to quickly respond to changes in the composition of the school. Two sensory systems have previously been implicated in controlling body position when schooling: vision and the lateral line [10, 25, 26]. Vision likely plays a role both in the tendency to school and in influencing body position and orientation when schooling [10, 25, 26]. No work has directly compared the visual ability of the marine and benthic sticklebacks that we study here. However, it is known that there are no differences in relative eye size between these populations [27], and laboratory behavioral studies have revealed that individuals from both populations readily respond to the visual stimulus of conspecifics [5, 28–30]. Despite a lack of evidence for differences in visual capacity between marine and benthic sticklebacks, it remains possible that unexplored differences in visual processing between these populations could contribute to divergent schooling behavior.

The lateral line has been implicated in influencing position and orientation during social grouping in other fish species [25, 26, 31, 32]. The lateral line is a peripheral, mechanoreceptive sensory system consisting of hair cells that respond to water movement and are grouped into structures called neuromasts [33]. Sticklebacks only have superficial neuromasts that are found on the surface of the skin [34]. We have shown striking differences in both the number and arrangement of neuromasts across the bodies of marine and benthic

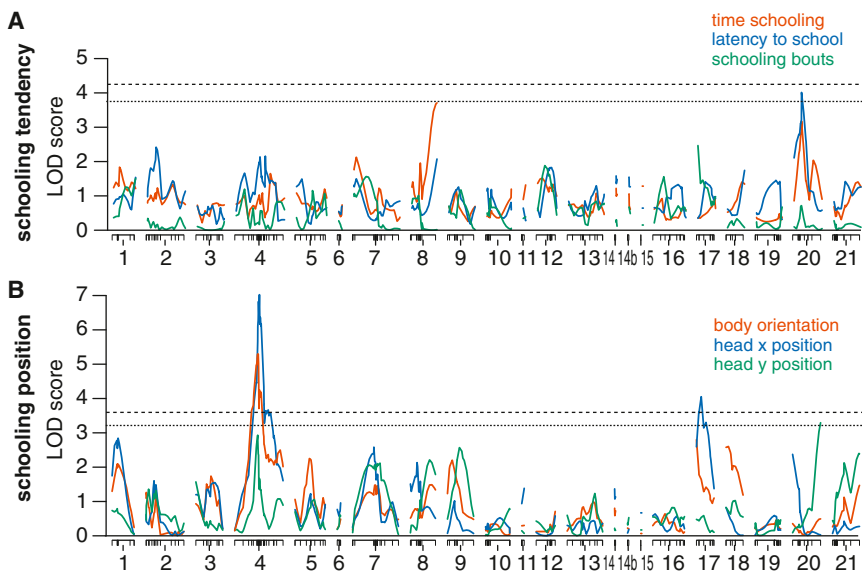


Figure 2. QTL for Schooling Behavior

(A) Graph showing LOD score as a function of linkage group for measures of schooling tendency: time schooling (orange), latency to school (blue), and number of bouts (green).

(B) Graph showing LOD score by linkage group for measures of schooling position: body orientation (orange), head x position (blue), and head y position (green). The dashed line represents genome-wide significance level of $p < 0.05$, and the dotted line represents $p < 0.1$, highlighting suggestive QTL.

See also Table S1.

sticklebacks [20], and we hypothesized that this variation might contribute to their differences in schooling position. In a previous QTL mapping study, we uncovered a modular genetic basis for lateral line anatomy, highlighting multiple genomic regions that control different anatomical portions of the lateral line [20]. This QTL analysis was performed in the same F2 individuals used in the current study, enabling us to ask whether common genomic regions underlie both neurosensory and behavioral variation. Indeed, the schooling position QTL on LG4 also contains QTL for several aspects of lateral line anatomy (Figure 3) [20]. This region is associated with differences in the number of neuromasts in the supratemporal portion of the lateral line [20], and it also contains suggestive QTL for neuromast number in the infraorbital and preopercular lines (LOD = 3.14, $p = 0.095$ and LOD = 3.25, $p = 0.084$, respectively). In addition, this region harbors a major QTL for the pattern of neuromasts in the posterior portion of the main trunk line [20]. Marine alleles at this locus are associated with a paired dorsoventral arrangement of neuromasts, whereas fish with benthic alleles possess a single row of neuromasts [20]. This variant pattern is tightly linked to the presence of bony plates [20], a conspicuous morphological difference between marine and benthic fish that is controlled by the gene *Ectodysplasin (Eda)*, located within this QTL [21]. Body segments that have bony plates contain dorsoventral pairs of neuromasts, whereas neuromasts on unplate segments are found in a single row. Ongoing work in our laboratory is manipulating *Eda* expression through transgenesis to ask whether expression of ectopic plates alters neuromast patterning and/or schooling position. However, there are

several additional candidate genes in this region that have been implicated in lateral line development or in social behavior that are also viable candidates for future functional analysis (Table S1).

In addition to the overlap in neural and behavioral QTL on LG4, we found that the schooling position QTL region on

LG17 also contains a lateral line QTL [20]. In particular, this region has a suggestive QTL (LOD = 3.66, $p = 0.063$) for the number of neuromasts in the supraorbital line, which is located on the dorsal surface of the head (Figure 3). The suggestive QTL that we detected for measures of schooling tendency did not overlap with any lateral line QTL [20], further reinforcing the separate genetic and neural control of these two components of schooling behavior.

The overlap between QTL for lateral line and schooling position phenotypes suggests one of two scenarios. First, these neural and behavioral traits could be mechanistically independent, arising from closely linked genes or from a single gene that has independent pleiotropic effects on both schooling behavior and the lateral line. Alternatively, differences in the lateral line might themselves drive the observed behavioral differences. Because the lateral line has previously been implicated in schooling behavior, we favor the hypothesis that the genetic effects we observe on schooling behavior are likely to act at least in part through the lateral line sensory system. Testing this hypothesis will require targeted ablations of the specific regions of the lateral line that are genetically linked to variation in schooling behavior.

Conclusions

Our work reveals that schooling in sticklebacks is composed of separable behavioral components with distinct genetic architectures. Because shoaling and schooling are distinct behaviors that share the component of social attraction, the neural and genetic mechanisms that control the tendency to school versus the ability to maintain a coordinated body

Table 1. Locations and Effects of Schooling QTL

Trait	LG	cM	Marker	LOD	p Value	PVE	MM	MB	BB
Body orientation (°)	4	48	chrIV:10812344	5.29	$p < 0.001$	10.4	81 ± 1.4	73 ± 1.3	69 ± 1.8
Head x position (cm)	4	51.6	chrIV:13850026	7.02	$p < 0.001$	13.5	4.1 ± 0.5	0.9 ± 0.4	0.3 ± 0.5
	17	10	chrUn:2632376	4.05	$p < 0.03$	7.1	3.9 ± 0.7	1.1 ± 0.3	1.0 ± 0.6
Head y position (cm)	20	58.5	chrXX:232763	3.29	$p = 0.073$	6.6	-2.0 ± 0.4	-0.8 ± 0.2	0.02 ± 0.3
Latency (s)	20	15.5	chrXX:14411783	4.01	$p = 0.084$	6.3	23 ± 6	19 ± 3	48 ± 10

PVE, percent variance explained; MM, homozygous for marine alleles; MB, heterozygous; BB, homozygous for benthic alleles.

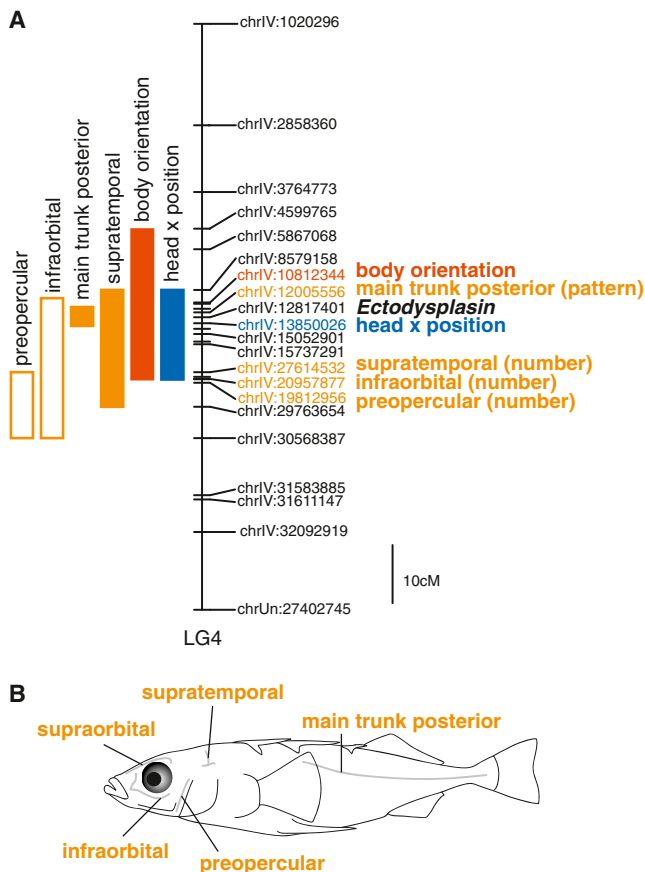


Figure 3. Schooling Position QTL on LG4 Overlap with Lateral Line QTL
(A) Map of LG4 indicating positions of QTL; some markers have been omitted for clarity. Bars on the left side represent 95% confidence intervals for individual QTL. Significant QTL are shown with filled bars and suggestive QTL with open bars. For each trait, the marker at the QTL peak is labeled on the right side. The position of the candidate gene *Ectodysplasin* is indicated on the right side.
(B) Schematic depicting anatomical positions of the lateral line segments that map to LG4 and LG17.

position during schooling are likely to be distinct. Consistent with this prediction, these behavioral features appear to be controlled by separate genetic factors. In addition to genetic modularity, our study reveals putative neural modularity in the control of schooling behavior. Within a single sensory system, the lateral line, some anatomical regions are linked to behavioral variation whereas others are not associated. This finding is consistent with previous work suggesting that different portions of the lateral line may be important for specific behaviors [6, 35].

Our work joins a growing number of studies aimed at dissecting the genetic basis for complex behaviors in natural populations, including those that have used association mapping and/or linkage analysis to identify genomic regions that contribute to behavioral evolution in vertebrates [2, 4–7]. In particular, recent work used a complementary approach to ours to dissect the genetic and sensory basis for differences in schooling behavior in the tetra *Astyanax* [36]. *Astyanax* inhabit both river and cave environments, and those from surface environments school strongly, but cavefish, which are also blind, do not school. Kowalko et al. [36] show that vision plays a major role in the evolution of schooling tendency

in *Astyanax* but also identify vision-independent aspects of divergent schooling. Studies like these—linking genetic, neural, and behavioral variation in natural populations—will continue to provide significant new insights into the proximate mechanisms that underlie behavioral evolution.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, one figure, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2013.07.058>.

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References

- Boake, C.R.B., ed. (1994). Quantitative Genetic Studies of Behavioral Evolution (Chicago: University of Chicago Press).
- Bendesky, A., and Bargmann, C.I. (2011). Genetic contributions to behavioural diversity at the gene-environment interface. *Nat. Rev. Genet.* 12, 809–820.
- Robinson, G.E., Fernald, R.D., and Clayton, D.F. (2008). Genes and social behavior. *Science* 322, 896–900.
- Weber, J.N., Peterson, B.K., and Hoekstra, H.E. (2013). Discrete genetic modules are responsible for complex burrow evolution in *Peromyscus* mice. *Nature* 493, 402–405.
- Kitano, J., Ross, J.A., Mori, S., Kume, M., Jones, F.C., Chan, Y.F., Absher, D.M., Grimwood, J., Schmutz, J., Myers, R.M., et al. (2009). A role for a neo-sex chromosome in stickleback speciation. *Nature* 461, 1079–1083.
- Yoshizawa, M., Yamamoto, Y., O’Quin, K.E., and Jeffery, W.R. (2012). Evolution of an adaptive behavior and its sensory receptors promotes eye regression in blind cavefish. *BMC Biol.* 10, 108.
- Wright, D., Nakamichi, R., Krause, J., and Butlin, R.K. (2006). QTL analysis of behavioral and morphological differentiation between wild and laboratory zebrafish (*Danio rerio*). *Behav. Genet.* 36, 271–284.
- Lim, M.M., Wang, Z., Olazábal, D.E., Ren, X., Terwilliger, E.F., and Young, L.J. (2004). Enhanced partner preference in a promiscuous species by manipulating the expression of a single gene. *Nature* 429, 754–757.
- Pitcher, T.J., and Parrish, J.K. (1993). Functions of shoaling behavior in teleosts. In *Behaviour of Teleost Fishes*, Second Edition, T.J. Pitcher, ed. (New York: Chapman & Hall), pp. 363–439.
- Shaw, E. (1978). Schooling fishes. *Am. Sci.* 66, 166–175.
- Berdahl, A., Torney, C.J., Ioannou, C.C., Faria, J.J., and Couzin, I.D. (2013). Emergent sensing of complex environments by mobile animal groups. *Science* 339, 574–576.
- Herbert-Read, J.E., Perna, A., Mann, R.P., Schaerf, T.M., Sumpter, D.J., and Ward, A.J. (2011). Inferring the rules of interaction of shoaling fish. *Proc. Natl. Acad. Sci. USA* 108, 18726–18731.
- Krause, J., and Ruxton, G.D. (2002). *Living in Groups* (New York: Oxford University Press).
- Hoare, D.J., Couzin, I.D., Godin, J.G.J., and Krause, J. (2004). Context-dependent group size choice in fish. *Anim. Behav.* 67, 155–164.
- Ioannou, C.C., Guttal, V., and Couzin, I.D. (2012). Predatory fish select for coordinated collective motion in virtual prey. *Science* 337, 1212–1215.
- Wark, A.R., Greenwood, A.K., Taylor, E.M., Yoshida, K., and Peichel, C.L. (2011). Heritable differences in schooling behavior among

- threespine stickleback populations revealed by a novel assay. *PLoS ONE* 6, e18316.
17. Pitcher, T.J. (1983). Heuristic definitions of fish shoaling behavior. *Anim. Behav.* 31, 611–613.
 18. Delcourt, J., and Poncin, P. (2012). Shoals and schools: back to the heuristic definitions and quantitative references. *Reviews in Fish Biology and Fisheries* 22, 595–619.
 19. Jones, F.C., Chan, Y.F., Schmutz, J., Grimwood, J., Brady, S.D., Southwick, A.M., Absher, D.M., Myers, R.M., Reimchen, T.E., Deagle, B.E., et al. (2012). A genome-wide SNP genotyping array reveals patterns of global and repeated species-pair divergence in sticklebacks. *Curr. Biol.* 22, 83–90.
 20. Wark, A.R., Mills, M.G., Dang, L.-H., Chan, Y.F., Jones, F.C., Brady, S.D., Absher, D.M., Grimwood, J., Schmutz, J., Myers, R.M., et al. (2012). Genetic architecture of variation in the lateral line sensory system of threespine sticklebacks. *G3 (Bethesda)* 2, 1047–1056.
 21. Colosimo, P.F., Hosemann, K.E., Balabhadra, S., Villarreal, G., Jr., Dickson, M., Grimwood, J., Schmutz, J., Myers, R.M., Schluter, D., and Kingsley, D.M. (2005). Widespread parallel evolution in sticklebacks by repeated fixation of *Ectodysplasin* alleles. *Science* 307, 1928–1933.
 22. Fitzpatrick, M.J., Ben-Shahar, Y., Smid, H.M., Vet, L.E., Robinson, G.E., and Sokolowski, M.B. (2005). Candidate genes for behavioural ecology. *Trends Ecol. Evol.* 20, 96–104.
 23. Mackay, T.F., Stone, E.A., and Ayroles, J.F. (2009). The genetics of quantitative traits: challenges and prospects. *Nat. Rev. Genet.* 10, 565–577.
 24. Flint, J. (2003). Analysis of quantitative trait loci that influence animal behavior. *J. Neurobiol.* 54, 46–77.
 25. Partridge, B.L., and Pitcher, T.J. (1980). The sensory basis of fish schools: relative roles of lateral line and vision. *J. Comp. Physiol.* 135, 315–325.
 26. Pitcher, T. (1979). Sensory information and the organization of behavior in a shoaling cyprinid fish. *Anim. Behav.* 27, 126–149.
 27. Albert, A.Y., Sawaya, S., Vines, T.H., Knecht, A.K., Miller, C.T., Summers, B.R., Balabhadra, S., Kingsley, D.M., and Schluter, D. (2008). The genetics of adaptive shape shift in stickleback: pleiotropy and effect size. *Evolution* 62, 76–85.
 28. Boughman, J.W. (2001). Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* 411, 944–948.
 29. Vamosi, S.M. (2002). Predation sharpens the adaptive peaks: survival trade-offs in sympatric sticklebacks. *Ann. Zool. Fenn.* 39, 237–248.
 30. Wark, A.R., Wark, B.J., Lageson, T.J., and Peichel, C.L. (2011). Novel methods for discriminating behavioral differences between stickleback individuals and populations in a laboratory shoaling assay. *Behav. Ecol. Sociobiol. (Print)* 65, 1147–1157.
 31. Pitcher, T.J., Partridge, B.L., and Wardle, C.S. (1976). A blind fish can school. *Science* 194, 963–965.
 32. Faucher, K., Parmentier, E., Becco, C., Vandewalle, N., and Vandewalle, P. (2010). Fish lateral system is required for accurate control of shoaling behaviour. *Anim. Behav.* 79, 679–687.
 33. Coombs, S., and Van Netten, S. (2005). The hydrodynamics and structural mechanics of the lateral line system. In *Fish Biomechanics, Volume 23*, R.E. Shadwick and G.V. Lauder, eds. (New York: Elsevier), pp. 103–139.
 34. Wark, A.R., and Peichel, C.L. (2010). Lateral line diversity among ecologically divergent threespine stickleback populations. *J. Exp. Biol.* 213, 108–117.
 35. Coombs, S., Janssen, J., and Webb, J.F. (1988). Diversity of lateral line systems: evolutionary and functional considerations. In *Sensory Biology of Aquatic Animals*, J. Atema, R.R. Fay, A.N. Popper, and W.N. Tavolga, eds. (New York: Springer-Verlag), pp. 553–593.
 36. Kowalko, J.E., Rohner, N., Rompani, S.B., Peterson, B.K., Linden, T., Yoshizawa, M., Kay, E.H., Hoekstra, H., Jeffery, W.R., Borowsky, R., et al. (2013). Loss of schooling behavior in cavefish through sight-dependent and sight-independent mechanisms. *Curr. Biol.* Published online September 12, 2013. <http://dx.doi.org/10.1016/j.cub.2013.07.056>.