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Article

Loss of Schooling Behavior in Cavefish through Sight-Dependent and Sight-Independent Mechanisms

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Summary

Background: Surface populations of *Astyanax mexicanus*, living in rivers like their common ancestors, school, while several, independently derived cave populations of the same species have lost schooling behavior.

Results: We quantify schooling behavior in individual *A. mexicanus* and identify quantitative trait loci (QTL) for this trait. We find that the evolutionary modulation of schooling has both vision-dependent and -independent components. We also quantify differences in the lateral line and vision between cavefish and surface fish and relate these differences to the evolutionary loss of schooling behavior. We provide evidence that a monoamine neurotransmitter may have played a role in the evolution of schooling behavior.

Conclusions: We find that vision is essential for schooling tendency in *A. mexicanus*, while the lateral line has a small effect on this behavior. Schooling behavior in *A. mexicanus* has evolved both through changes in sensory systems and through changes in genetic loci that likely act downstream of sensory inputs.

Introduction

Most species of fish exhibit schooling behavior during some phase of their life cycle [1]. Schooling benefits fish in a variety of ways, including predator avoidance and foraging [2–5]. However, there are some situations in which schooling behavior is less advantageous. For example, when food is scarce, fish tend to school less [6, 7]. Schooling fish rely on the ability to sense one another. The visual system and the ability to sense water pressure and current through the lateral line have been implicated in schooling behavior [2, 8, 9].

Little is known about how schooling behavior evolves, with the exception of studies in laboratory strains of zebrafish [10]. The Mexican tetra, *Astyanax mexicanus*, provides an excellent opportunity to examine this question. *A. mexicanus* exists in two forms, a sighted surface-dwelling form and a blind cave-dwelling form. Morphological adaptations to life in the caves include an increased number and distribution of taste buds and cranial superficial neuromasts, regressed eyes, and decreased or absent melanin pigmentation [11–13]. Cavefish also have a variety of modified behaviors, including decreases in aggression and in time spent sleeping, a depressed response to alarm substance, an enhanced attraction to vibrations in their environment, modified feeding behaviors, and the absence of schooling [14–19]. While many of these behaviors have been studied to some extent, little is known about their genetic architecture.

Cave and surface forms of *A. mexicanus* are interfertile, allowing for the genetic analysis of cave traits [11]. In particular, quantitative trait locus (QTL) mapping has been used successfully to identify loci underlying the evolution of several morphological traits in these fish [20–25]. Another advantage of studying evolution in *A. mexicanus* is the existence of a number of independently evolved cave populations (reviewed in [26]; Figure S1A available online) with similar morphological characteristics and behaviors, making *A. mexicanus* an ideal system in which to study parallel and convergent evolution (though this is beyond the scope of this paper).

While the surface form of *A. mexicanus* actively aggregates into schools and shoals, the cave forms have reduced this behavior [19, 27, 28]. The apparent absence of macroscopic predators in the caves relieves one selective pressure favoring schooling, suggesting that the loss of schooling behavior could be the result of relaxed selection. Alternatively, the scarcity of food resources in most caves potentially renders clustering of the fish disadvantageous. Thus, the loss of this behavior could be adaptive in the caves. The absence of schooling could also be a secondary consequence of the loss of vision and/or changes in the lateral line system in cavefish or a pleiotropic consequence of other adaptive neurological or morphological changes.

Results

Loss of Schooling Behavior in Cavefish

Schooling and shoaling behaviors occur when individual fish, perceiving and responding to their local environment, interact in the context of larger groups. By following a set of relatively simple rules on the local scale, individuals' behaviors can result in complex group patterns of collective motion (reviewed in [29]). In order to quantify differences in this behavior, we use a relatively simple definition of schooling, the tendency of fish to synchronize their behavior and swim in an oriented manner relative to one another [30]. To quantify schooling behavior, we measured the tendency of fish to follow a model school of plastic fish [31] (Figure 1A). Surface fish follow the model school (Figure 1B and 1D). In contrast,

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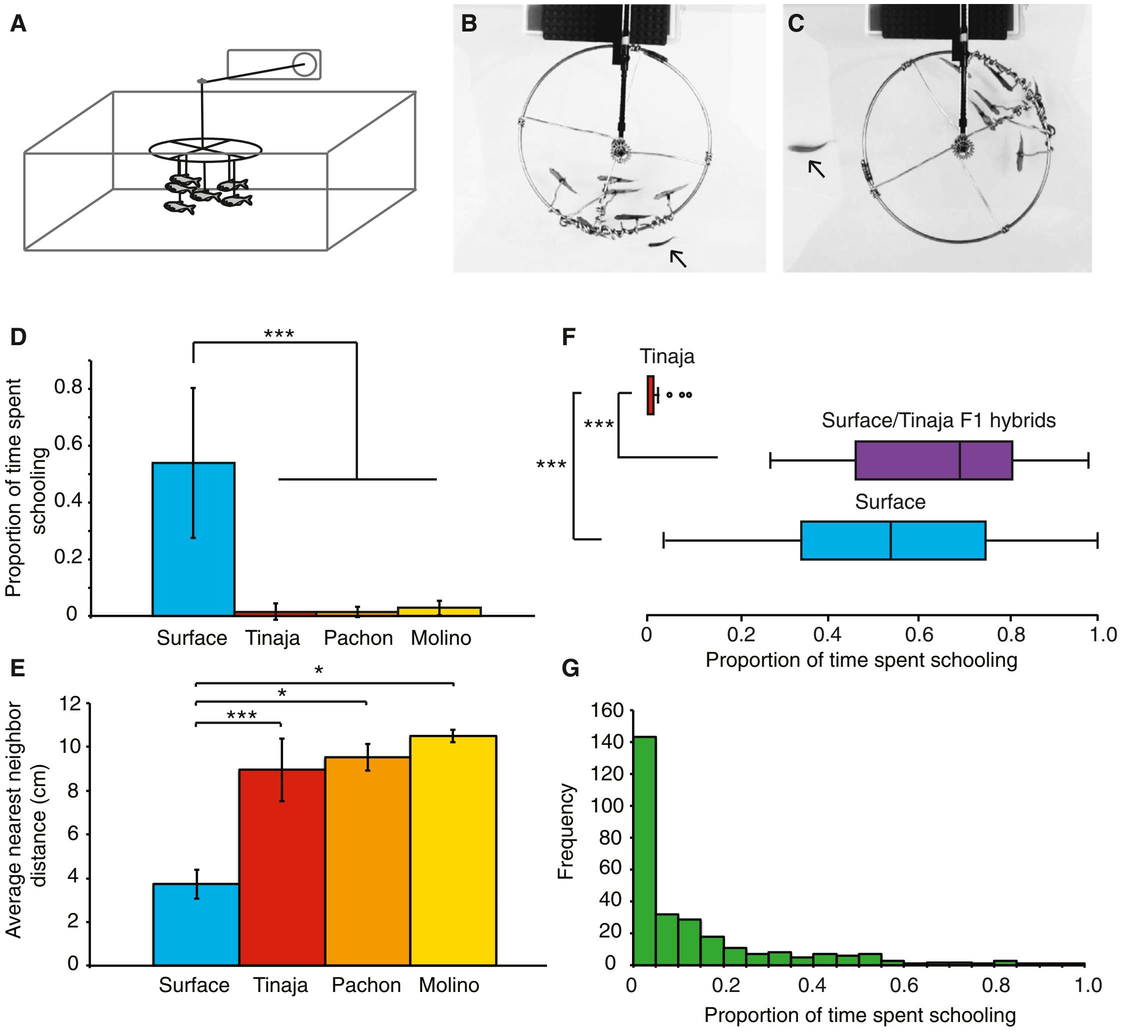

Figure 1. Cavefish Have Lost the Tendency to School

(A) Diagram of the model school behavioral assay.

(B and C) Images from videos of a (B) surface fish and (C) Tinaja cavefish with the model school. Arrows indicate the position of the live fish.

(D) Schooling tendency was quantified as the proportion of time during the trial that each fish spent following the model school. Average time spent following the school was recorded for surface fish (n = 34), and cavefish populations—Tinaja (n = 19), Pachón (n = 10), and Molino (n = 10). Asterisks indicate p values in a Mann-Whitney test.

(E) Shoaling as the average of the nearest neighbor distances (in centimeters) for each fish in a group. Groups of six fish each were measured for surface (nine groups), Tinaja (nine groups), Pachón (three groups), and Molino (three groups) fish. Asterisks indicate p values in a Mann-Whitney test.

(F) Distribution of the proportion of time spent schooling in surface fish (n = 34), surface/Tinaja F1 hybrid fish (n = 12), and Tinaja cavefish (n = 19). Asterisks indicate p values in a Mann-Whitney test.

(G) The distribution of the average proportion of time spent schooling across five trials of 287 F2 fish from a surface/Tinaja F1 hybrid intercross.

All error bars indicate the SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. See also Figure S1 and Table S1.

three independently evolved cave populations (reviewed in [26]) from the Tinaja, Pachón, and Molino caves were significantly different from surface fish and did not display schooling behavior (Kruskal Wallis: $H_4 = 63.6$, $p < 0.001$; Mann-Whitney compared to surface: Tinaja, $U = 3$, $z = -6$, $p < 0.001$; Pachón, $U = 1$, $z = -4.6$, $p < 0.001$; Molino, $U = 4$, $z = -4.6$, $p < 0.001$; surface, n = 34; Tinaja, n = 19; Pachón, n = 9; Molino, n = 10; F1s, n = 12; Figures 1C and 1D).

Shoaling behavior is defined as the tendency of fish to aggregate with other fish of the same species [30] (including schooling). We next measured shoaling for groups of fish, quantifying the average nearest neighbor distance (NND) and the average interindividual distance (IID) (Figures S1B and S1C). Surface fish swam significantly closer together than did fish from any of the cave populations by NND (Kruskal-Wallis: $H_3 = 18.8$, $p < 0.001$; Mann-Whitney test compared to surface: Tinaja, $U < 0.001$, $z = -3.6$, $p < 0.001$; Pachón, $U < 0.001$, $z = -2.5$, $p < 0.05$; Molino, $U < 0.001$, $z = -2.5$, $p < 0.05$; surface, n = 9 groups; Tinaja, n = 9 groups; Pachón, n = 3 groups; Molino, n = 3 groups; Figure 1E) and by IID (Kruskal Wallis: $H_3 = 17.4$, $p < 0.001$; Mann-Whitney compared to surface: Tinaja, $U < 0.001$, $z = -3.6$, $p < 0.001$; Pachón, $U < 0.001$, $z = -2.5$, $p < 0.05$; Molino, $U < 0.001$, $z = -2.5$, $p < 0.05$; Figure S1E). Thus, in multiple, independently evolved natural populations, cavefish have lost the tendency to swim oriented to one another, or school, as well as decreased the tendency to congregate in a group, or shoal.

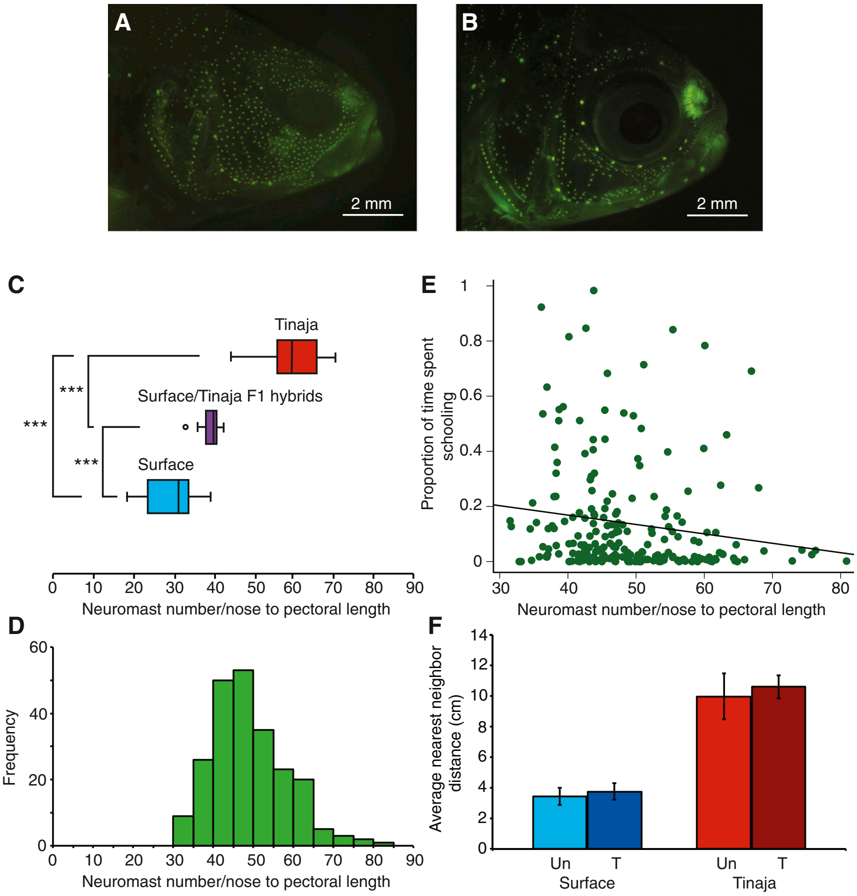

Figure 2. Relationship between Schooling Behavior and the Lateral Line System

(A) Cranial neuromasts in a Tinaja cavefish.

(B) Cranial neuromasts in a surface fish. Neuromasts are visualized using DASPEI.

(C) Distribution of cranial neuromast number corrected for size in surface fish (n = 21), surface/Tinaja F1 hybrid fish (n = 7), and Tinaja fish (n = 21).

(D) Distribution of cranial neuromast number corrected for size in the F2 population (n = 227).

(E) Proportion of the time spent schooling as a function of number of cranial neuromasts corrected for size in the F2 population (n = 214).

(F) Nearest neighbor distances (in centimeters) in groups of surface fish (n = 6) and Tinaja cavefish (n = 6) treated with 0.002% gentamicin (T) or untreated (Un).

Error bars indicate the SD. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S2 and Table S1.

Genetics of Schooling Behavior

Surface fish raised in isolation follow the model school, responding similarly in the assay to group-raised fish ($t_{36} = -0.5$, $p = 0.61$; group raised, n = 34; isolation raised, n = 4; Figure S1D). Thus, this behavior is not learned and is likely to have a genetic basis. To study the inheritance of schooling, we crossed surface fish and Tinaja cavefish to generate F1 hybrid fish. F1 fish follow the model school, similar to surface fish (Mann-Whitney compared to F1: surface, U = 155.5, z = −1.2, p = 1.0; Tinaja, U < 0.001, z = −4.8, p < 0.001; surface, n = 34; Tinaja, n = 19; F1, n = 12), indicating that tendency to school segregates as a dominant trait (Figure 1F).

To probe the genetic architecture of this trait more deeply, we intercrossed F1 fish to generate F2 fish. F2 fish vary widely in their behavior (Figure 1G). These results strongly indicate a polygenic basis for this behavior. Tendency to school in F2 fish differed based on sex (Mann-Whitney: U = 6669, z = −2.1, p < 0.05, n = 252; Figure S1H) and was not correlated with size (Spearman's rho = 0.05, p = 0.37, n = 271; Figure S1I).

An Enhanced Lateral Line in Cavefish Does Not Contribute Significantly to Loss of Schooling Behavior

The lateral line and the visual system have been implicated in schooling behavior in other fish species [2, 8, 9]. Cavefish have enhanced the size and number of superficial cranial neuromasts, the sensory organ of the lateral line system, relative to surface fish [12, 32] (Figures 2A and 2B). It was possible that the larger numbers of cranial neuromasts in cavefish provide a sensory input that repels them from one another, leading to avoidance of conspecifics and a decrease in the tendency to school.

Surface fish indeed have significantly fewer cranial neuromasts than cavefish (one-way ANOVA: $F_{2,46} = 99.2$, $p < 0.001$; surface, n = 21; Tinaja, n = 21; F1, n = 12; Games-Howell surface compared to Tinaja: p < 0.001; Figures 2A–2C). F1 fish have an intermediate number of cranial neuromasts, significantly different from both surface (Games-Howell: p < 0.001) and cavefish (Games-Howell: p < 0.001). The F2 population (n = 227) ranges in number of cranial neuromasts (Figure 2D).

To determine whether the number of cranial neuromasts has an effect on schooling behavior, we compared the number of neuromasts to the proportion of time spent schooling for each fish in the F2 population (Figure 2E). The number of neuromasts in F2 fish accounted for a statistically significant amount of variation in schooling behavior, but the size of this effect was small (Spearman's rho = −0.22, p < 0.001, n = 214). In addition to superficial neuromast number, we also measured superficial neuromast diameter in F2 fish and found no correlation between this measure and the schooling behavior (Spearman's rho = 0.04, p = 0.64, n = 154; Figure S2C). Thus, the increased number and size of neuromasts that evolved in response to the cave environment did not have a large effect on the evolutionary loss of schooling.

Neuromast Ablation Does Not Have a Significant Effect on Aggregation Behaviors

To determine the extent to which the lateral line system is required for schooling and shoaling activity in *Astyanax*, we treated fish with 0.002% gentamicin to ablate neuromast function [17, 33, 34]. Surface fish did not show a significant difference in behavior in the absence of neuromasts, as assessed either by shoaling (NND: $t_{10} = -1.03$, $p = 0.33$; IID: $t_{10} = -1.01$, $p = 0.34$; treated, n = 6 groups; untreated, n = 6 groups) or by schooling (Mann-Whitney: U = 191, z = −0.5, p = 0.63; treated, n = 21; untreated, n = 21; Figures 2F, S2A, and S2B). Treated Tinaja cavefish were not significantly different by NND ($t_{10} = -1.69$, $p = 0.12$; treated, n = 6; untreated, n = 6;

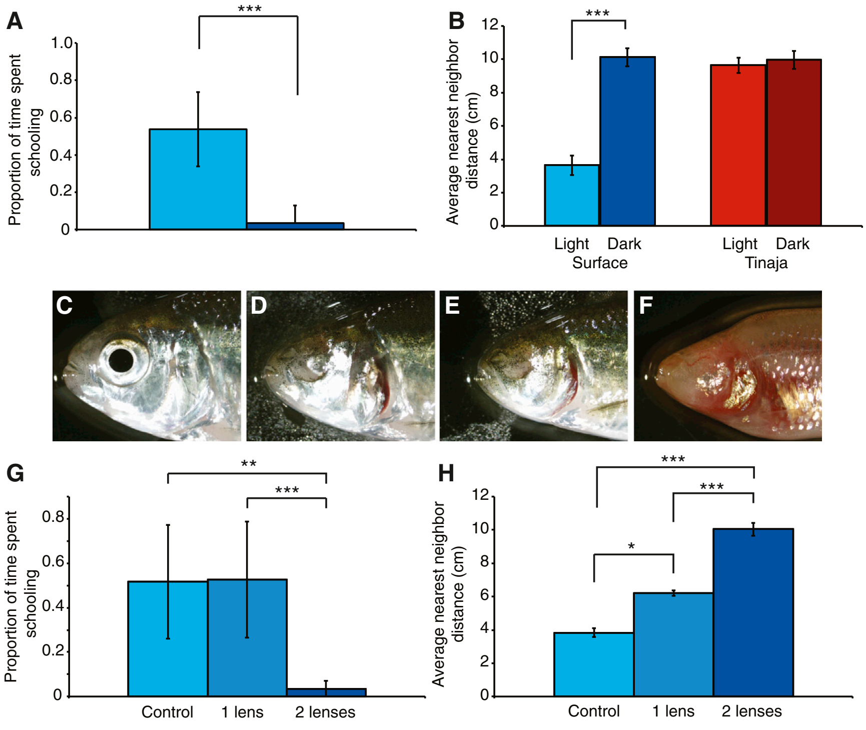

Figure 3. Vision Is Required for Schooling and Shoaling Behavior

(A) Proportion of time spent schooling of surface fish in the light (n = 12) versus the dark (n = 10). Asterisks indicate p value in a Mann-Whitney test.

(B) Shoaling NND measured in groups of six of surface fish (five groups) and cavefish (five groups) in the light and the dark. Asterisks indicate p value in a paired t test.

(C) Eye size in control surface fish.

(D) Partial eye degradation in surface fish with lenses removed.

(E) Complete eye degradation in surface fish with lenses removed.

(F) Eye degradation in cavefish.

(G) Surface fish with zero (n = 7), one (n = 12), or two (n = 8) lenses removed were assayed with the model school. Asterisks indicate p value in a Games-Howell test.

(H) One group each of fish with one lens removed (five trials), fish with two lenses removed (five trials), or control fish (two trials) were assayed for shoaling by NND. Asterisks indicate p value in a Games-Howell test.

Error bars indicate the SD. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S3 and Table S1.

Figure 2F) or IID ($t_{10} = -1.15$, $p = 0.28$; Figure S2B). While not significant, NND and IID in both surface and Tinaja fish were greater in treated fish compared to controls. Therefore, it is unlikely that an enhanced lateral line in cavefish drives the evolution of loss of schooling or shoaling behavior.

Vision Is Essential for Schooling Behavior

Visual function is important for schooling and shoaling behavior in a variety of fish species, either independent of or in conjunction with lateral line function (for example, [8, 35]). It has been previously reported that in *A. mexicanus*, surface fish placed in the dark show a reduction in shoaling [35]. We verified this result in our shoaling assay. Groups of surface fish in the dark swam significantly farther apart compared to the same groups in the light (NND: paired t test, $t_4 = -17.2$, $p < 0.001$, n = 5 groups; IID: paired t test, $t_4 = -15.2$, $p < 0.001$; Figures 3B and S3A). Cavefish were unaffected by the change in lighting conditions (NND: paired t test, $t_4 = -1.2$, $p = 0.31$, n = 5 groups; IID: paired t test, $t_4 = 0.45$, $p = 0.67$; Figures 3B and S3A). Schooling behavior in surface fish was lost in the dark compared to in the light (Mann-Whitney: U = 1, z = −4, $p < 0.001$; light, n = 12; dark, n = 10; Figure 3A).

Loss of schooling in the dark could be due to a learned reliance on vision for schooling behavior. If this were the case, fish that lose vision early in development might school in the absence of sight. Cavefish develop eyes, which undergo apoptosis and degenerate [11, 36, 37]. Cavefish eye degradation can be phenocopied in surface fish [38]. In order to test whether loss of schooling could be rescued if fish lost visual function during development, we removed one, two, or no lenses in surface fish larvae, resulting in a range of adult eye morphology (Figures 3C–3F).

Lens removal had a significant effect on both schooling (one-way ANOVA: $F_{2,24} = 13.9$, $p < 0.001$; control, n = 8; one lens removed, n = 12; two lenses removed, n = 7) and shoaling (NND: Welch ANOVA, F = 253.9, $p < 0.01$; IID: ANOVA, $F_{2,9} = 127.1$, $p < 0.001$; control, n = 2 trials; one lens, n = 5 trials; no lenses, n = 5 trials). Surface fish with both lenses removed schooled significantly differently from control fish and fish with one lens removed (planned-contrast test: $t_{14.1} = -7.8$, $p < 0.001$; Games-Howell test compared to control fish: $p < 0.01$; Games-Howell test compared to fish with one lens removed: $p < 0.001$; Figure 3G) and swam significantly farther away from one another compared to control fish (NND: Games-Howell test, $p < 0.001$; IID: Games-Howell test, $p < 0.001$; Figures 3H and S3B). Surface fish retaining one eye were indistinguishable from control fish in the model school assay (Games-Howell test: $p = 0.996$; Figure 3G). However, these fish were significantly different from both control fish (NND: Games-Howell test, $p < 0.05$; IID: Games-Howell test, $p < 0.001$) and fish with two lenses removed (NND: Games-Howell test, $p < 0.001$; IID: Games-Howell test, $p < 0.001$; Figures 3H and S3B) in the shoaling assay. These results demonstrate that visual function is necessary for schooling and shoaling in surface forms of *A. mexicanus*.

Vision-Dependent and Independent Loss of Schooling Tendency in F2 Fish

Since visual function is required for schooling behavior in *A. mexicanus*, the ancestral fish would have lost the ability to school immediately upon entering the pitch-dark cave environment. Thus, cavefish may have evolved their decreased tendency to school in our assays solely as a consequence of their loss of eyes. Alternatively, loss of schooling behavior may have become fixed in these fish through additional changes, independent of the loss of vision. To distinguish between these possibilities, we assayed visual function in the F2 population.

Visual function in the F2 population can be approximated using the external morphology of the eyes. Both eye ($t_{8.7} = -13.1$, $p < 0.001$; surface, n = 20; F1, n = 5) and pupil ($t_{4.9} = -9.0$, $p < 0.001$) diameters are significantly reduced in F1 fish compared

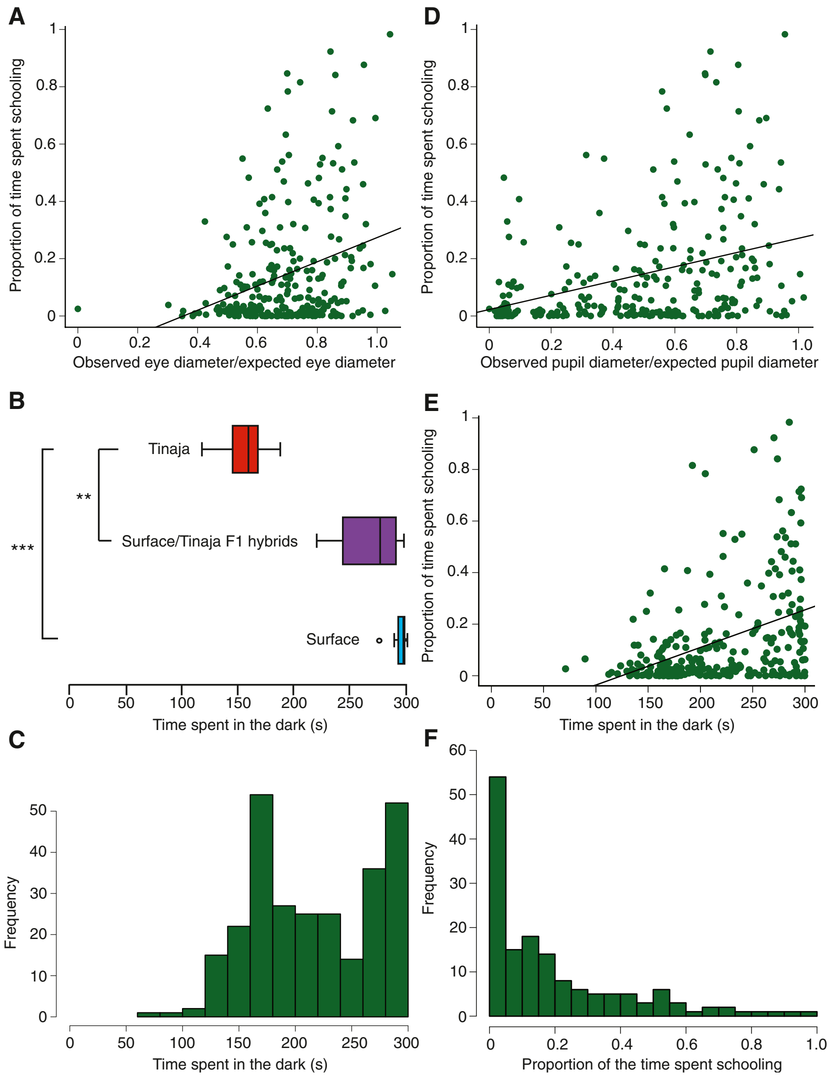

Figure 4. Measures of the Visual System Correlate with Schooling Behavior in F2 Fish

(A) Average proportion of time schooling in the F2 population (n = 270) as a function of eye size.

(B) Surface (n = 9), Tinaja (n = 14), and surface/Tinaja hybrid F1 (n = 4) individuals in an assay for dark preference. Dark preference was quantified as the number of seconds spent in the dark out of a total of 300 s. Asterisks indicate p values in a Mann-Whitney test.

(C) Distribution of average time spent in the dark across three trials for F2 population of fish (n = 275).

(D) Average proportion of the time schooling in the F2 population (n = 270) as a function of pupil size. Both eye and pupil diameters were corrected for the expected size of the eye or pupil of a surface fish of the individual's body length.

(E) Average proportion of the time spent schooling as a function of dark preference in the F2 population (n = 266).

(F) The distribution of the tendency to school in seeing F2 fish, defined as spending an average of 200 s in the dark (n = 151).

$^{**}p < 0.01$, $^{***}p < 0.001$. See also Figure S4 and Table S1.

to surface fish (Figure S4A). Nearly all F2 fish (n = 283) have eyes, although most of them are smaller than surface fish eyes (Figures S4B and S4C). Eye and pupil diameters are highly correlated in the F2 population (Pearson's R = 0.81, p < 0.001, n = 283; Figure S4D). Proportion of time schooling in the F2 population is weakly to moderately positively correlated with both eye (Spearman's rho = 0.27, p < 0.001, n = 270) and pupil (Spearman's rho = 0.35, p < 0.001, n = 270; Figures 4A and 4D) diameters. However, there are individual fish with very large eyes and pupils who still do not school. This suggests that while schooling requires visual function, there may be an independent genetic basis for loss of schooling.

F2 fish with large eyes and pupils may still lack visual function, and fish with smaller eyes may be able to see. Therefore, fish were tested for their ability to sense light. Surface fish display strong negative phototaxis, spending nearly all of their time in the dark. Tinaja cavefish behave significantly differently (Kruskal Wallis: $H_2 = 175.6$, $p < 0.001$; surface, n = 9; Tinaja, n = 14; F1, n = 4; Mann-Whitney: $U < 0.001$, $z = -4$, $p < 0.001$; Figure 4B), showing no preference for either the dark or the light. F1 hybrids display strong negative phototaxis, not significantly different from surface fish (Mann-Whitney: $U = 5$, $z = -2$, $p = 0.15$) and significantly different from Tinaja fish ($U < 0.001$, $z = -3$, $p < 0.01$; Figure 4B). Dark preference in the F2 fish population has a bimodal distribution (n = 275; Figure 4C). Dark preference in the F2 population was moderately positively correlated with both eye diameter (Speaman's rho = 0.36, p < 0.001, n = 265) and pupil diameter (Spearman's rho = 0.47, p < 0.001, n = 265; Figures S4E and S4F).

Dark preference was also moderately positively correlated with schooling in the F2 population (Spearman's rho = 0.42, p < 0.001, n = 266; Figure 4E). A large proportion of nonschooling fish had no dark preference, indicating that many of the F2 fish that displayed no tendency to school have little visual function. However, some F2 individuals that showed a strong dark preference did not show any tendency to school, suggesting that there has been a loss of schooling in cavefish independent of vision.

In order to test for factors that affect schooling behavior independently of vision, we defined a population of F2 fish with visual perception as those fish that spent at least two-thirds of their time in the dark. This cutoff would include F1 and surface fish but exclude Tinaja cavefish. Light-perceiving fish (n = 151) were then analyzed for their propensity to school. Interestingly, within this group of light-perceiving F2 fish, many do not display schooling behavior (Figure 4F).

Groups of light-perceiving and non-light-perceiving F2 fish were tested in the shoaling assay. Light-perceiving, schooling groups of fish swam significantly closer to one another compared to light-perceiving, nonschooling fish groups and non-light-perceiving, nonschooling groups by NND (ANOVA: $F_{2,10} = 7.65$, $p < 0.05$; planned-contrast test: $t_{10} = -3.91$, $p < 0.01$; schooling, n = 6 groups; light-perceiving, nonschooling, n = 4 groups; non-light-perceiving, nonschooling, n = 3 groups; Figure S3C). These groups were

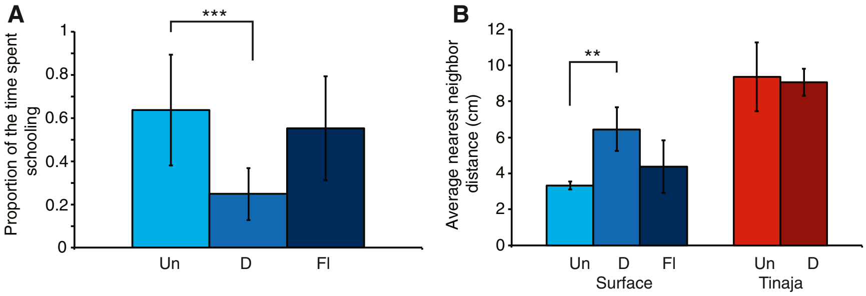

Figure 5. The Effects of Increased Brain Monoamine Levels on Schooling Behavior

(A) Proportion of the time spent schooling in untreated (Un, n = 21), 10 μM deprenyl-treated (D, n = 12), and 14 μM fluoxetine-treated (Fl, n = 21) surface fish. Asterisks indicate p values in a Mann-Whitney test.

(B) Average nearest neighbor distance in groups of untreated (Un, n = 6 groups), 10 μM deprenyl-treated (D, n = 6 groups), and 14 μM fluoxetine-treated (Fl, n = 5 groups) surface fish and untreated (Un, n = 6 groups) and 10 μM deprenyl-treated (D, n = 6 groups) Tinaja cavefish. Asterisks indicate p values in a Games-Howell test. All error bars indicate the SD. $^{**}p < 0.01$, $^{***}p < 0.001$. See also Figure S5 and Table S1.

not significantly different by IID, although they trended in the same direction (one-way ANOVA: $F_{2,10} = 3.5$, $p = 0.07$; Figure S3D). Furthermore, a mixed group of F2s, containing two fish from each of these categories, had a NND of 10.17 and an IID of 22.27. This NND is outside the range of the light-perceiving, schooling F2 fish (Table S1). This confirms that a subset of F2 fish maintain visual function but do not have a tendency to aggregate.

The Roles of Monoamine Neurotransmitters in Schooling Behavior

Recent research has shown that there are differences in levels of monoamine neurotransmitters between cave and surface *Astyanax mexicanus* [39, 40]. In order to determine whether these differences could have an effect on schooling behavior, we treated cave and surface fish with two inhibitors, (R)-(-)-Deprenyl hydrochloride and fluoxetine hydrochloride. Both of these drugs result in an increase in serotonin levels in the brain [40]. However, (R)-(-)-Deprenyl hydrochloride targets monoamine oxidase (MAO), inhibiting the breakdown of multiple monoamines.

The treatments resulted in significant differences in schooling behavior (Kruskal Wallis: $H_2 = 18.4$, $p < 0.001$; untreated, n = 36; Deprenyl, n = 12; fluoxetine, n = 22). (R)-(-)-Deprenyl treatment results in a significant decrease in schooling behavior (Mann-Whitney: $U = 46$, $z = -4$, $p < 0.001$), while fluoxetine does not significantly affect schooling relative to control fish (Mann-Whitney: $U = 313$, $z = -1.3$, $p = 0.38$; Figure 5A). In addition, (R)-(-)-Deprenyl (Welch ANOVA: $H_2 = 18.4$, $p < 0.01$; untreated, n = 6 groups; R-Deprenyl, n = 6 groups; fluoxetine: n = 5 groups; Games-Howell: $p < 0.01$) but not fluoxetine (Games-Howell: $p = 0.35$) results in significantly greater separation between fish in the shoaling assay using NND (Figure 5B) and IDD (Welch ANOVA: $H_2 = 21.6$, $p < 0.01$; R-Deprenyl Games-Howell: $p < 0.01$, Fluoxetine Games-Howell: $p = 0.21$; Figure S5). In contrast, the Tinaja cavefish in the shoaling assay were not affected by treatment with (R)-(-)-Deprenyl hydrochloride (NND: Mann-Whitney, $U = 0.9$, $z = -1.4$, $p = 0.18$; IID: Mann-Whitney, $U = 12$, $z = -0.96$, $p = 0.39$; untreated, n = 6 groups; treated, n = 6 groups; Figures 5B and S5). These data suggest that an increase in brain monoamine levels, but not specifically brain serotonin levels, decreases the tendency to school.

QTL Mapping of Schooling Behavior

Finally, we performed QTL analysis to map the regions of the genome underlying the loss of schooling behavior in the Tinaja cavefish. Using a binary measure of schooling behavior, we mapped a single significant QTL on linkage group 27 that explained 6.4% of the variance (n = 276, $p < 0.05$; Figure 6B and Table 1). Homozygous cave alleles at a marker underlying this QTL result in a decrease in schooling behavior, and a heterozygous genotype result in an intermediate tendency to school (Figure 6E).

In addition to schooling behavior, we mapped a binary measure of dark preference to one significant QTL on linkage group 27 that explains 6.4% of the variance (n = 267, $p < 0.05$; Figure 6A and Table 1). Homozygous cave alleles at a marker underlying this QTL result in less time spent in the dark, while heterozygous genotypes result in an intermediate percentage of time spent in the dark (Figure 6D). This QTL marker mapped to the same location as the schooling QTL.

In order to map the genetic basis of schooling behavior that is independent of visual function, we mapped a binary measure of schooling behavior in light-perceiving fish (defined as described above). This resulted in one significant QTL on linkage group 6 that explains 12% of the variance (n = 143, $p < 0.05$; Figure 6C and Table 1). Somewhat surprisingly, homozygous cave genotypes at a marker underlying this QTL resulted in an increase in schooling behavior, while fish with homozygous surface or heterozygous genotypes schooled a similar amount of time (Figure 6F). This QTL does not fall in the same place as the QTL for dark preference, eye size, or pupil size (Figures 6A and S6A–S6D and Table 1). Thus, this QTL identifies a vision-independent genetic contribution to the evolution of schooling behavior.

Discussion

Here, we determine that while both the visual system and the lateral line affect schooling in surface fish to some extent, it is the loss of sight in cavefish that plays the most significant role in the loss of schooling behavior. In contrast, lateral line enhancement in cavefish plays at most a minor role in schooling behavior loss. Our results suggest that loss of schooling evolved by multiple genetic changes, only some of which are vision dependent.

The Visual System Is Essential in Schooling Behavior in Surface Fish

It has been proposed that while the visual system allows fish to swim closer to one another during schooling, the lateral line provides a repulsive force [8]. We find that only vision, and not lateral line sensation, played a key role in the evolution of this behavior in a cave population of *Astyanax mexicanus*. Surface *Astyanax* do not school and have reduced shoaling in the dark or when they are blinded at 36 hr postfertilization. While there remains a possibility that surface fish choose not to

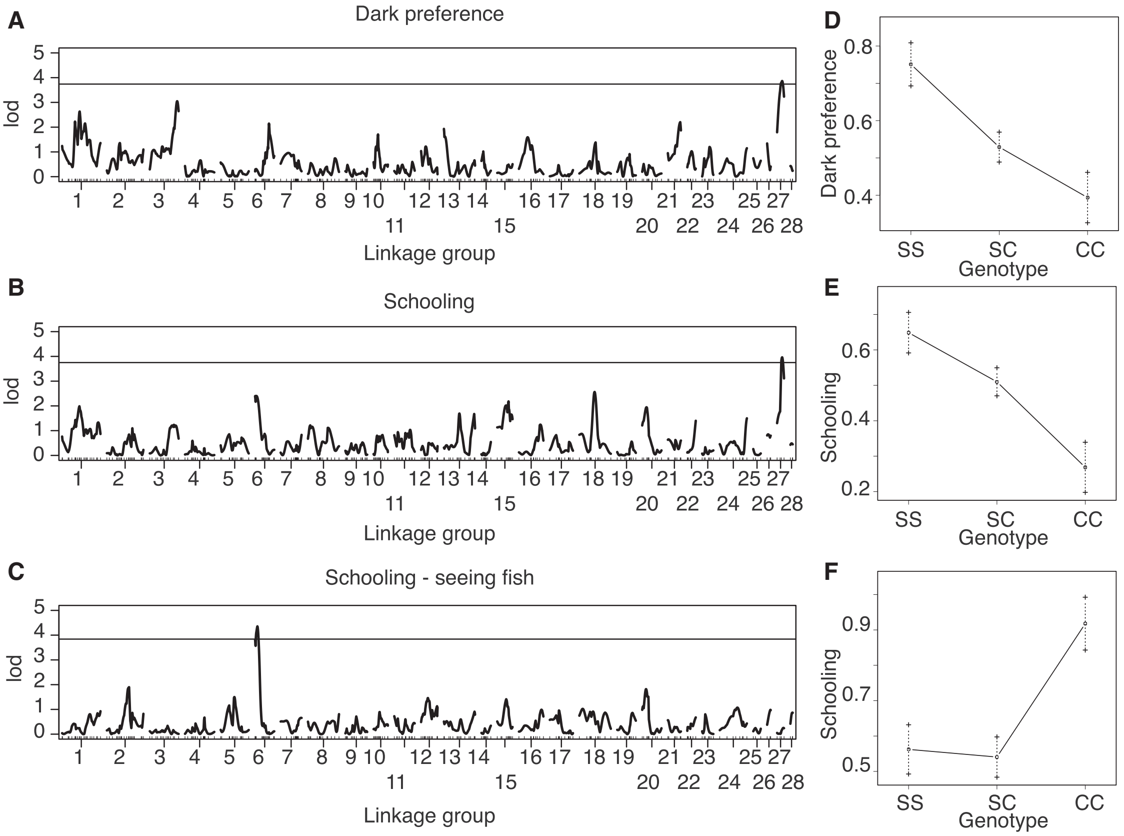

Figure 6. Visual and Nonvisual QTL for Evolutionary Loss of the Tendency to School in Cavefish

(A) QTL for a binary measure of dark preference (n = 267) where fish spending greater than 200 s were scored as preferring the dark. The line indicates a genome-wide significance LOD score for a p value < 0.05.

(B) QTL for a binary measure of the tendency to follow the model school (n = 276). Fish were scored as schooling if they spent more than 5% of their time following the model on average. The line indicates a genome-wide significance LOD score for a p value < 0.05.

(C) QTL for a binary measure of the tendency to follow the model school for the subset of fish that preferred the dark (n = 143).

(D) Effect plot for the QTL for dark preference measured as a binary of time the dark (1, preferring the dark; 0, no dark preference).

(E) Effect plot for the QTL for schooling, measured as proportion of time following the model schooling and then made into a binary trait (1, schooling; 0, nonschooling).

(F) Effect plot for the schooling QTL in light-responsive fish, measured as proportion of time following the model schooling and then made into a binary trait (1, schooling; 0, nonschooling).

Genotypes are for homozygous surface (SS), heterozygous (SC), or homozygous cave (CC) alleles. See also Figure S6 and Table S2.

school without visual cues, vision is likely necessary for both schooling and shoaling behavior.

We also tested the effect of loss of one eye on schooling and shoaling behavior. Interestingly, while fish with one eye could follow the model school, fish with one eye shoaled farther apart from one another. This could result from the importance of two functional eyes in tracking fish swimming in a disorganized manner, while other sensory organs, such as the lateral line, may compensate for the loss of one eye during schooling behavior.

Loss of Vision Has a Large Effect on the Evolutionary Loss of Schooling Behavior, while Enhancement of the Lateral Line Plays a Minor Role

We examined the role of visual function in the evolution of schooling behavior by examining these traits in the F2 population. We found that both morphological and behavioral measures of the visual system were correlated with schooling behavior in the F2 population. Additionally, the QTL for dark preference maps to the same region as a QTL for schooling behavior. This QTL for schooling behavior may explain the proportion of loss of schooling behavior explained by loss of visual function. Alternatively, since the QTL for dark preference does not fall in the same location as QTL for eye or pupil size, it is plausible that the behavioral difference in the dark preference assay mapped to this QTL has to do with a loss of dark preference per se, and not to perception of light. However, it could be related to an eye-size-independent aspect of visual processing still related to light perception, such as retinal degeneration or lens degeneration.

While we found a significant correlation between number of neuromasts and schooling in F2 fish, the correlation was weak, and ablation of neuromasts in surface and cavefish was not sufficient to drive fish to swim closer together or to increase schooling behavior. Therefore, it is unlikely that neuromasts play a large role in the evolution of schooling behavior.

Potential Effects of Increase of Brain Dopamine Levels on Decrease of Schooling

Both dopamine and serotonin have been implicated in Pachón cavefish evolution [39, 40]. There is an increase in the amount of brain serotonin in Pachón cavefish compared

Table 1. Summary of QTL

Trait	LG	cM	LOD	CI	PVE
Schooling	27	20	4.0	16–26 cM	6.4
Dark preference	27	20	3.9	9–27 cM	6.4
Schooling—seeing fish	6	9	4.4	0–14 cM	12
Eye diameter	3	74.1	4.9	65–113 cM	7.9
Pupil diameter	3	74.1	4.6	67–77 cM	7.3

CI, confidence interval; PVE, percent variance explained.

to surface fish [40]. In addition, tyrosinase 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon polypeptide 1 (YWHAE), an enzyme involved in dopamine biosynthesis, is upregulated in Pachón cavefish brains [39]. Both of these pathways are hypothesized to function in cavefish by increasing the amount of time spent foraging relative to surface fish [39, 40]. Therefore, these pathways may have been selected for in cavefish for other behavior purposes, and have a pleiotropic effect on schooling behavior.

We found that potentially increasing levels of multiple monoamines, presumably including both serotonin and dopamine, with (R)-(-)-Deprenyl hydrochloride, decreased both schooling and shoaling tendency in surface fish. Although (R)-(-)-Deprenyl hydrochloride affects brain levels of serotonin in *A. mexicanus* [40], it is unlikely that changing of serotonin levels alone affects schooling behavior given the insignificant effect of fluoxetine. This indicates that another monoamine, not serotonin, plays a role in schooling behavior in *A. mexicanus*. Given the evidence that a molecule involved in the synthesis of dopamine is upregulated in at least one population of cavefish, our results are consistent with an increase in the amount of brain dopamine affecting schooling behavior in cavefish. Thus, brain neurotransmitter levels that have evolved to change adaptive behaviors, such as feeding behavior, may have a secondary, pleiotropic effect on schooling behavior. Testing whether modulation of dopamine specifically affects schooling and shoaling behavior, whether R-deprenyl can induce increased levels of dopamine, and whether cavefish have increased amounts of dopamine compared to surface fish, would be an interesting complement to this work.

Evolution of Schooling Behavior Independent of Loss of Vision

While loss of vision plays an important role in loss of schooling behavior, we also found evidence for a vision-independent loss of schooling behavior. Many F2 fish with a strong response to light still do not follow the model school. This is similar to what was previously seen in shoaling assays in *Astyanax mexicanus* [19]. Interestingly, when the effects of vision are removed by performing QTL analysis on only those fish that are light responsive, a second QTL, which does not fall in the same location as the vision, eye size, or pupil size QTL, emerges. We also performed QTL analysis for neuromast number, and neither of the schooling QTL fall in the same place as the neuromast QTL (data not shown). This suggests that the second QTL for the loss of schooling is vision and lateral line independent. Markers located under this QTL map to zebrafish chromosome 5 (Table S3). Fine-scale mapping, combined with detailed analysis of the genes within this interval, will be necessary to identify the specific genetic changes responsible for the schooling QTL.

Potential Evidence for Relaxed Selection on Schooling in the Cave

Once the ancestors of cavefish entered caves, they would not be able to school due to lack of light, and this could relax selection on schooling behavior. In addition, the ecology of the cave habitat suggests that there would be no counterselection to maintain schooling behavior, in spite of the loss of vision. A likely lack of macroscopic predators in the caves removes one major selective pressure for schooling in the cave environment. One possibility for the evolution of schooling behavior is that once vision was impaired by the lack of light, schooling was no longer under selection, and alterations in genes affecting this behavior would be neutral in consequence. This could be an explanation for identification of a locus where F2 fish with a homozygous cave genotype show an increase in schooling behavior. Since a large percentage of seeing F2 fish still do not school, there are likely to be other loci with cave alleles contributing to loss of schooling behavior. We expect that decreased schooling behavior is caused by many genetic changes and that many of these have effects too small to detect in our current analysis.

Convergence on a Decreased Tendency to School in Multiple Cavefish Populations and Different Fish Species

Here, we demonstrate that multiple, independently evolved cavefish populations have lost the tendency to school. Previous work on *A. mexicanus* also showed a loss of schooling and reduction in shoaling behavior in cave populations [19, 27, 28]. Our work corroborates this previous work. Schooling behavior is also lost in other species of cave populations (reviewed in [28, 41]). The importance of the loss of the visual system for loss of schooling behavior in *A. mexicanus* may be general in cave populations, and it would be interesting to know whether other cavefish species have reduced schooling behavior due to lack of visual cues.

In addition to cavefish, benthic threespine sticklebacks (*Gasterosteus aculeatus*) display reduced schooling behavior [31]. Greenwood et al. explore the genetics of this loss of schooling behavior in marine versus benthic stickleback populations [42]. While cave *Astyanax* and benthic sticklebacks both have a reduced tendency to school, the mechanisms that lead to loss of schooling behavior may be different in these two species. Benthic sticklebacks, which have intact visual systems, still show some tendency to follow a model school, but they position themselves differently within it. In contrast, cave *Astyanax* have lost all tendency to follow a model school. This may be due to differences in habitats and selective pressures. Once they have entered the cave environment, *Astyanax* could no longer school due to loss of visual cues necessary for this behavior. In addition, cavefish do not encounter predators within the cave and have thus lost a selective pressure usually associated with schooling behavior. In contrast, benthic sticklebacks are still confronted with predators but display a shelter seeking behavior rather than a schooling behavior [31].

Interestingly, both cave *Astyanax* and benthic sticklebacks appear to have evolved differences in schooling behavior through modifications of sensory systems. Loss of vision contributes to the evolutionary loss of schooling tendency in cave *Astyanax* and lateral line evolution contributes to the evolution of schooling position in sticklebacks. Thus, convergent reduction of schooling behavior can occur through modulation of different sensory systems and different behavioral components. Together, these studies demonstrate the contribution of sensory system evolution to the evolution of complex behaviors.

Conclusions

In conclusion, we report the results of two behavioral assays for social grouping in one surface and three cave populations of *Astyanax mexicanus*. We show that the loss of schooling behavior in a cave population of *Astyanax* has a genetic basis and is a complex trait, influenced by at least two loci. Vision, but not the lateral line, is important for schooling behavior in surface fish, and vision is not a learned cue for schooling, but instead is required for this behavior. However, vision is not sufficient for schooling behavior. Loss of schooling behavior in cavefish has a genetic basis independent of eye loss. Additionally, we offer evidence that schooling was likely lost due to relaxed selection, as opposed to selection against schooling behavior in cave populations.

Experimental Procedures

Methods are described in the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.07.056.

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