Mechanisms of Social Behavior in the Anti-Social Blind Cavefish (*Astyanax mexicanus*)

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Abstract

The evolution of social behavior in *Astyanax mexicanus*, which exists as a sighted, surface-dwelling morph and a blind, cave-dwelling morph, provides a model for understanding how environmental pressures shape social behaviors. We compared the shoaling behavior of blind and surface *A. mexicanus* to that of zebrafish (*Danio rerio*), and examined the effects of nutritional state and the neuropeptides isotocin (IT) and arginine vasotocin (AVT) on their social behavior. Blind cavefish not only fail to form shoals, but actively avoid conspecifics, with hunger further diminishing their social cohesion. Administration of low doses of AVT and an IT antagonist partially restored social behavior in blind cavefish, reducing distances between individuals, whereas surface fish exhibited minimal or opposite responses to these hormonal manipulations. Our findings suggest that the loss of shoaling behavior in blind cavefish is not a consequence of visual impairment alone, as they remain capable of detecting and responding to others. Instead, this behavior likely reflects an adaptive response to their resource-poor, predator-free cave environment, where shoaling may be disadvantageous. The differing responses to nonapeptides between the morphs indicate that blind cavefish may have lost the motivation to shoal rather than the ability, highlighting how ecological pressures can shape social behavior.

Keywords: shoaling, Astyanax mexicanus, social behavior, cavefish, neuropeptides

Introduction

Social behaviors vary widely across species, often shaped by diverse environmental pressures. Among vertebrates, fish exhibit an extensive range of social behaviors, from predominantly solitary species (1) to those that engage in cooperative breeding (2), establish dominance hierarchies (3), or display reciprocal altruism (4). Most social fish species engage in collective movement, also called shoaling (5-8). Shoaling offers significant fitness benefits, including predator avoidance, as it enables quicker predator detection and creates predator confusion when faced with multiple identical targets (9-16). Shoaling also enhances foraging efficiency by enabling fish to locate and exploit larger food patches and increasing feeding efficiency by reducing the need for individual vigilance (15,17-23). However, aggregation can also have costs, such as increased competition for limited resources (24,25) or an elevated risk of attracting predators to large groups (12,13,26).

The balance between the benefits and costs of shoaling raises intriguing questions about the evolution of this behavior and how environmental conditions shape it (27-28). For instance, Trinidadian guppies (*Poecilia reticulata*) derived from populations originating in low-predator environments display reduced social cohesion compared to descendants of fish from high-predation areas (29-35). Desert pupfish (*Cyprinodon macularius*), which evolved in predator-free environments, appear to have lost defensive behaviors such as responses to alarm pheromones (36).

Collective behaviors may also respond to variations in current environmental conditions. Factors like pollution, waterway obstruction, and noise modify group cohesion (37-40). Zebrafish (*Danio rerio*) exposed to simulated predator risks form denser shoals (5). Gulf menhaden (*Brevoortia patronus*) form tighter, more synchronized groups in physically complex environments (41), while zebrafish exhibit the opposite pattern (42). Hungry zebrafish prefer to shoal with well-fed conspecifics, potentially to increase their chances of finding food while minimizing competition (43). When food is present, zebrafish also increase their distance from neighbors (5). Food deprivation reduces shoaling tendencies across species; food-deprived banded killifish (*Fundulus diaphanus*), walleye pollock (*Theragra chalcogramma*) and three-spined sticklebacks (*Gasterosteus aculeatus*) display a reduced tendency to maintain tight shoals and display less cohesion with conspecifics (44-46). European sea bass (*Dicentrarchus labrax*) exhibit fewer interactions with others when food-deprived (47).

The Mexican tetra (*Astyanax mexicanus*) is a valuable model for studying the evolution of social behavior in fish. Approximately 150 to 200 thousand years ago, some surface-dwelling populations of this species migrated into pitch-black caves, where they evolved a blind cave-dwelling form (48-49). Cave-adapted populations are now found in up to 34 different caves (50), likely originating from two independent colonization events (49). While the repeated evolution of similar cave-adapted traits across these populations suggests convergent evolution (51-54),

evidence of gene flow between cave populations indicates that some adaptations may have spread through genetic exchange (48). Blind *A. mexicanus* populations underwent a host of morphological, physiological, and behavioral adaptations (55-64), believed to have been driven not only by the absence of light but also by the lack of predators and extreme scarcity of food in their cave habitats. Blind *A. mexicanus* feed on low-nutrition organic matter that occasionally drifts into the caves, such as detritus, algae, fungi, bat guano, and the remains of other cave-dwelling organisms (51,65-68). Many populations of blind *A. mexicanus* are characterized by their relentless pursuit of food and have been suggested to be insatiable (55,58).

In addition to losing their eyes and pigmentation, cave-dwelling blind *A. mexicanus* have an enhanced lateral line system, a sensory adaptation crucial for survival in the dark (52,69-71). The lateral line, an array of pressure-sensitive cells along the sides of fish, acts as a sensory organ that detects the velocity of water flow generated by the fish's own movements or by external currents (72-74). Blind *A. mexicanus* possess a higher density of these cells, improving their sensitivity to water movements, aiding in the detection of food and obstacles (75-82). They also generate a flow as they swim and can detect distortions in the flow caused by nearby objects (83-85).

Although born with eyes, blind *A. mexicanus* undergo lens apoptosis early in development, a process driven by increased expression of the *sonic hedgehog* (*Shh*) gene (86-88). Elevated *Shh*, alongside Fibroblast Growth Factor (*Fgf*) signaling, contributes to many of the adaptations seen in blind *A. mexicanus*, such as larger jaws, an increased number of taste buds, and expanded forebrain regions, including the olfactory system and hypothalamus, which result in a higher number of neurons associated with feeding behavior (89-92). Consequently, blind *A. mexicanus* sleep less, spend more time foraging, and have heightened appetites—adaptations that enhance their ability to maximize food intake and forage more efficiently in the dark (55,57-58,68,88,93).

Alongside these physiological adaptations, blind *A. mexicanus* have undergone several behavioral changes (70), most notably their complete loss of shoaling behaviors (51,58,62,65,67,94-98). Although this has often been attributed to their loss of vision (51,62,94), it is essential to consider the broader ecological context: in addition to scarce food, blind cavefish have no predators (51,65). Therefore, the adaptive benefits of shoaling, such as predator avoidance and collective foraging, are greatly reduced in their environments. Here, we propose that the loss of shoaling in blind *A. mexicanus* results more from a decrease in their motivation to shoal than an inability to aggregate.

While shoaling remains a vital survival mechanism for surface-dwelling *A. mexicanus*, solitary foraging might be a more effective strategy in habitats with high competition for limited resources (99-100). Swimming independently might also improve lateral line function by enhancing the signal-to-noise ratio, aiding navigation and foraging in complete darkness (58,79).

Blind *A. mexicanus* also exhibit a reduced response to alarm substances, further suggesting that the absence of predators has relaxed the selective pressures maintaining shoaling and defensive behaviors in their surface-dwelling relatives (62,101). Even hybrids of surface and cave morphs exhibit reduced shoaling, despite retaining functional vision, indicating that inherited predispositions from blind *A. mexicanus*, rather than vision loss alone, contribute to this behavioral shift (62).

Alongside the loss of shoaling, blind *A. mexicanus* show a significant reduction in aggressive behaviors, such as a complete loss of territoriality and hierarchical dominance. In contrast, surface *A. mexicanus* exhibit hierarchical dominance and high levels of aggression (58,67,94-96,102). As with shoaling, this loss of agonistic behaviors in blind *A. mexicanus* was initially attributed to a loss of vision (94-95,103). However, subsequent studies have demonstrated that surface-dwelling sighted *A. mexicanus* remain aggressive even in complete darkness (58), and hybrid populations also exhibit reduced aggression (104). This behavioral shift likely stems from changes in food-seeking behavior and alterations to the serotonergic system (58,96,102).

Hormones likely play a crucial role in shaping social behaviors in blind *A. mexicanus*, as in many fish species (105-106). Adaptations in hypothalamic brain regions not only enhance their foraging abilities but also contribute to changes in their neuroendocrine system by reducing arginine vasopressin (AVT)-producing neurons (92). AVT, known for its role in stress regulation (107), may contribute to the distinct behaviors of blind *A. mexicanus*, such as their lower baseline cortisol levels, compared to surface *A. mexicanus* (108). As AVT also influences social behavior, these neuroendocrine changes may help explain blind *A. mexicanus*'s reduced shoaling. Examining nonapeptides like AVT could thus offer valuable insights into the mechanisms underlying social behavior—or its absence—in this species.

Neuropeptides such as AVT and isotocin (IT)—the fish analogs of the mammalian arginine vasopressin (AVP) and oxytocin (OT)—play essential roles in regulating social behaviors across vertebrate taxa (109-110). In goldfish (*Carassius auratus*), IT decreases proximity to conspecifics, while AVT promotes shoaling (111). In guppies, IT increases social proximity, while AVT decreases grouping (112; but see 113). In zebrafish, AVT reduces social interactions and aggression (114-115), but it increases aggression in species such as bluehead wrasse (*Thalassoma bifasciatum*; 116-117), brown ghost knifefish (*Apteronotus leptorhynchus*; 118), and beaugregory damselfish (*Stegastes leucostictus*; 119), highlighting species-specific differences in neuropeptide effects. Peripherally administered nonapeptides have been shown to cross the fish blood-brain barrier (114,116,119-122), which is likely more permeable to neuropeptides than its mammalian counterpart (123-124).

Cichlids also show varied responses: in male *Neolamprologus pulcher*, IT reduces grouping but increases sensitivity to social stimuli, while an IT-receptor antagonist promotes

grouping (120-121). *N. pulcher* also has higher expression of IT-related genes than the non-social cichlid *Telmatochromis temporalis* (125). In African cichlids (*Astatotilapia burtoni*), AVT helps maintain social hierarchies, with dominant males showing higher AVT expression than subordinates (126-127). Guppies exposed to predation risk show increased AVT expression without a corresponding change in IT expression, suggesting that predator presence may specifically enhance AVT's role in social regulation (128). In mosquitofish (*Gambusia affinis*), IT modulates social behaviors in context-specific ways, such as reducing interactions with males while maintaining associations with conspecific females under conditions of male harassment (129). This supports the social salience hypothesis that OT/IT increases the salience of social stimuli, thus adapting social behavior to environmental context (129-131).

In mammals, OT enhances positive social interactions, increasing proximity in lions (132), partner-seeking behavior in marmosets (133), and caregiver attention in infant macaques (134). In vampire bats, OT increases food donation size and allogrooming (135), in naked mole-rats, it boosts huddling and proximity to familiar conspecifics (136), and in meerkats, it increases cooperative behaviors, such as pup-feeding, while reducing aggression (137). In humans, OT is linked to trust, empathy, and social connectedness (138-140). In contrast, AVP is more often linked to aggression, territoriality, and defense. AVP increases aggression in male rodents (141) and AVP analogs help maintain social bonds and manage dominance hierarchies in birds, similar to their roles in fish (142).

To investigate whether the loss of shoaling in blind *A. mexicanus* represents an adaptive strategy rather than a physiological constraint, we examined the shoaling tendencies of surface-dwelling and cave-dwelling *A. mexicanus* morphs alongside zebrafish—a well-studied shoaling species used as a control. Zebrafish have been extensively used in research examining collective behavior (5) and the effects of AVT and IT on social behavior (114-115). We also compared these groups to a theoretical shoaling-null model that assumed they were swimming randomly, ignoring one another. We hypothesized that blind *A. mexicanus* would exhibit reduced shoaling compared to sighted *A. mexicanus* and zebrafish, reflecting an adaptive response to their resource-scarce, predator-free cave environments. Next, we manipulated the nutritional state of blind *A. mexicanus* to examine the effects of hunger on shoaling. Fish were observed in three states: fasted (24 hours after feeding), post-absorptive (3-5 hours after feeding), and fed (10 min after feeding). We hypothesized that hunger would reduce social cohesion while feeding would enhance it, as it does in other species (44-47). Finally, we administered varying doses of IT and AVT and their antagonists to both *A. mexicanus* morphs. We hypothesized that hormonal responses would differ between morphs, reflecting their contrasting reliance on social interactions and differences in their neuroendocrine regulation. Together, these experiments aim to reveal how adaptations to extreme ecological conditions, like the complete absence of light, contributed to the loss of shoaling in cave-dwelling populations, advancing our understanding of the mechanisms underlying social behaviors across species.

Methods

Ethics statement

All experimental procedures were approved by the Wilfrid Laurier Animal Care Committee (AUP R22007) and followed all Canadian Council on Animal Care regulations.

Subjects and Housing

Subjects were 35 wild-type zebrafish (*Danio rerio*) bred in-house, 180 Pachón cavefish (*Astyanax mexicanus*) acquired from a local supplier (Tropical Fish Room, Brantford, ON), and 120 surface-dwelling *Astyanax mexicanus*, also bred in-house. In Experiment 1, we tested 35 zebrafish, 35 blind *A. mexicanus*, and 30 surface *A. mexicanus* on shoaling. Experiment 2 involved 55 blind *A. mexicanus* reused from Experiment 4 to investigate the effects of hunger on shoaling. Experiment 3 tested 90 blind *A. mexicanus* and 90 surface fish to evaluate the impact of hormone administration. Experiment 4 involved 55 blind *A. mexicanus* and focused on the effects of increased dosages of certain hormones. With the exception of Experiment 2, each fish was only tested once. Fish in Experiment 2 had completed experiment 4 at least one month earlier.

Zebrafish were housed in 10-liter tanks in a high-density rack system (Pentair), with no more than 10 fish per tank, while blind and surface-dwelling *A. mexicanus* were housed in groups of 5 to 20 in 10-gallon tanks (50 x 25 x 30 cm). All tanks were maintained at 23 ± 1°C with a 12 h:12 h light-dark cycle (lights on at 7:00 am). Water quality parameters were monitored daily. All fish were fed dried brine shrimp daily *ad lib*, except during Experiment 2, where we manipulated nutritional state.

Experimental Setup

Each experiment was conducted in a featureless, white circular tank (60 cm diameter; Figure S1), filled with 10 cm of water at 23 ± 1°C. The water in the experimental tank was changed daily, with temperature and salinity matched to those of the housing tanks. Behavioral trials were recorded using a video camera (Canon Vixia HF R700) mounted directly above the tank.

Procedure

In all experiments, groups of fish (N = 5) were first gently netted from their home tanks into a bucket filled with home-tank water. Fish were then either transferred directly to the testing arena (Experiments 1 and 2) or given injections before testing (Experiments 3 and 4; see below). Fish were released into the center of the experimental tank and recorded swimming freely for 10 minutes (Videos S1 and S2). At the end of each trial, fish were returned to their home tanks.

In Experiment 1, seven groups of zebrafish, seven groups of blind *A. mexicanus*, and six groups of surface fish were tested to assess baseline shoaling. In Experiment 2, eleven groups of blind *A. mexicanus* were randomly divided into two conditions. Six groups were food-deprived for 24 hours before testing (fasted condition), and five groups were fed *ad lib* 10 minutes before the testing (fed condition). To minimize the number of fish used, data from the unmanipulated blind *A. mexicanus* in Experiment 1, which were fed *ad lib* approximately 3 to 5 hours before testing, were used as the post-absorption condition.

In Experiment 3, we tested the effects of nonapeptides on shoaling in both blind and sighted *A. mexicanus*. Subjects were randomly selected from their home tanks and assigned to one of five treatment groups immediately prior to testing, receiving an injection of either isotocin (IT; Carbetocin acetate, *MilliporeSigma Canada*), AVT ([Arg^8]-Vasotocin, *VWR International*), an IT antagonist (L-368,899 hydrochloride, *Cayman Chemical*), an AVT antagonist (Manning Compound, *VWR International*), or saline (0.9% physiological saline). We note that some of these compounds may have weak non-specific effects on other systems.

Before injection, each fish was weighed by being placed into a beaker of water of known weight, and injection volumes were calculated individually. Fish were positioned upside-down in a slit within a damp sponge for injection. Drugs were administered via intraperitoneal injection using a 10 μL, 26-gauge syringe (Hamilton 701N). The entire procedure took approximately 30 seconds per fish. Each group of five fish was injected sequentially and then transferred to a recovery tank for 10 minutes before testing, ensuring enough time for the neuropeptides to enter the brain (113-114). All drugs were administered at 10 μg/g body weight, with injection volumes between 2 and 8 μL. For blind *A. mexicanus*, five groups received saline, three received IT (denoted IT+), four received AVT (AVT+), three received the IT antagonist (IT-), and three received the AVT antagonist (AVT-). For surface fish, three groups received saline, four received IT+, three received AVT+, four received IT-, and four received AVT-.

In Experiment 4, we tested the effects of higher hormone dosages in blind *A. mexicanus* only. Fish were randomly selected from their home tanks and assigned to one of four treatment groups immediately prior to testing, receiving either 20 μg/g or 40 μg/g doses of AVT+ or IT- following the same procedures as Experiment 3. Three groups received 20 μg/g of AVT, three received 40 μg/g of AVT, and five received 40 μg/g of IT-. Data from the saline-injected groups in Experiment 3 were used as a control. Our dosages were informed by previous research on nonapeptide administration in small fish species (114-116,122), as no previous studies have manipulated IT and AVT in *A. mexicanus*.

Analysis

Fish movements were tracked from videos using an automated tracker (*IDTracker*; 143), which provided the location of each fish in each frame. Data were then processed in R (144) to extract standard metrics of collective movement: the inter-individual distance (IID; the mean

distance between an individual and all others, averaged across all fish), nearest-neighbor distance (NND; the distance between each fish and its nearest neighbor, averaged over all individuals), and polarization (the degree to which individuals are oriented in the same direction). These metrics are widely recognized as robust indicators of group cohesion and alignment (5,145). All distance measurements were converted from pixels to centimeters using a scaling factor derived from the arena's known diameter of 60 cm.

To assess whether fish were actively shoaling or ignoring each other, we created a null model in R to simulate random movement. We generated 10,000 iterations of random point distributions (five points per iteration) within the experimental arena, calculating IID, NND, and polarization values for each configuration. These randomly generated values served as a baseline for comparison with the observed experimental data.

All statistical analyses were performed in *Mathematica* (v.12.0, *Wolfram Technologies*). Analyses of variance (ANOVA) were used to evaluate significant differences between species, experimental conditions, and hormonal treatments. One-way ANOVAs were applied to assess differences in IID, NND, and polarization between treatment groups or species. Two-way ANOVAs were used to examine interactions between morphs/species and treatment conditions (hormonal treatments, nutritional state). When the ANOVA indicated significant effects, post-hoc tests were conducted, and a Bonferroni correction was applied to all tests. Comparisons of a group to the theoretical null model were conducted using T-tests. We additionally report the power of each test, using η^2 for ANOVAs and Cohen's D for T-tests.

As we tracked our fish in every frame of the video (30 fps), the values of all our measures are not independent across frames, violating the assumptions of our analysis methods. To address this, before conducting any analyses, we down-sampled our data to one frame from each minute of each trial (i.e., we took 10 evenly spaced frames from the data for each trial), reducing the autocorrelation in the dataset.

All data are available on our OSF repository, at https://osf.io/9jky4/?view_only=c8e10cd8b43c4e58b3d84d0de6f0d876.

Results

Experiment 1

We found significant differences between all species or morphs on all measures of shoaling (Figure 1 A-C. Means ± SD in Table S1, post-hoc tests in Table S2. IID: $F(2,197) = 1216.4$, $p < 0.00001$, $\eta^2 = 0.93$; NND: $F(2,197) = 927.7$, $p < 0.00001$, $\eta^2 = 0.90$; Polarization: $F(2,197) = 233.1$, $p < 0.00001$, $\eta^2 = 0.70$), suggesting that blind *A. mexicanus* shoals are less polarized and looser than those of either zebrafish or sighted *A. mexicanus*, and sighted *A. mexicanus* form tighter and more polarized shoals than zebrafish. We additionally compared the

experimental data to our null distribution to test whether blind *A. mexicanus* were ignoring each other (means ± SD in Table S1). Both zebrafish and sighted *A. mexicanus* shoaled more tightly than predicted by the model (NND: zebrafish, $T(52.7) = 5.13$, $p < 0.00001$, Cohen's $D = 1.11$; sighted *A. mexicanus*, $T(50.9) = 6.90$, $p < 0.00001$, $D = 1.44$. IID: zebrafish, $T(62.1) = 6.97$, $p < 0.00001$, $D = 1.45$; sighted *A. mexicanus*, $T(54.4) = 10.75$, $p < 0.00001$, $D = 2.23$) but blind *A. mexicanus* maintained *greater* distances between individuals than predicted by the model (NND: $T(53.8) = -3.8$, $p = 0.0004$, $D = 0.82$. IID: $T(58.1) = -8.9$, $p < 0.00001$, $D = 1.88$), suggesting that they do not merely ignore but actively avoid each other. Zebrafish and sighted *A. mexicanus* groups were more polarized than the model (zebrafish: $T(67.3) = -8.44$, $p < 0.00001$, $D = 1.72$; sighted *A. mexicanus*: $T(71.5) = -10.0$, $p < 0.00001$, $D = 2.02$) but there was no difference in polarization between the model and blind *A. mexicanus* ($T(56.8) = 0.26$, $p = 0.80$, $D = 0.05$).

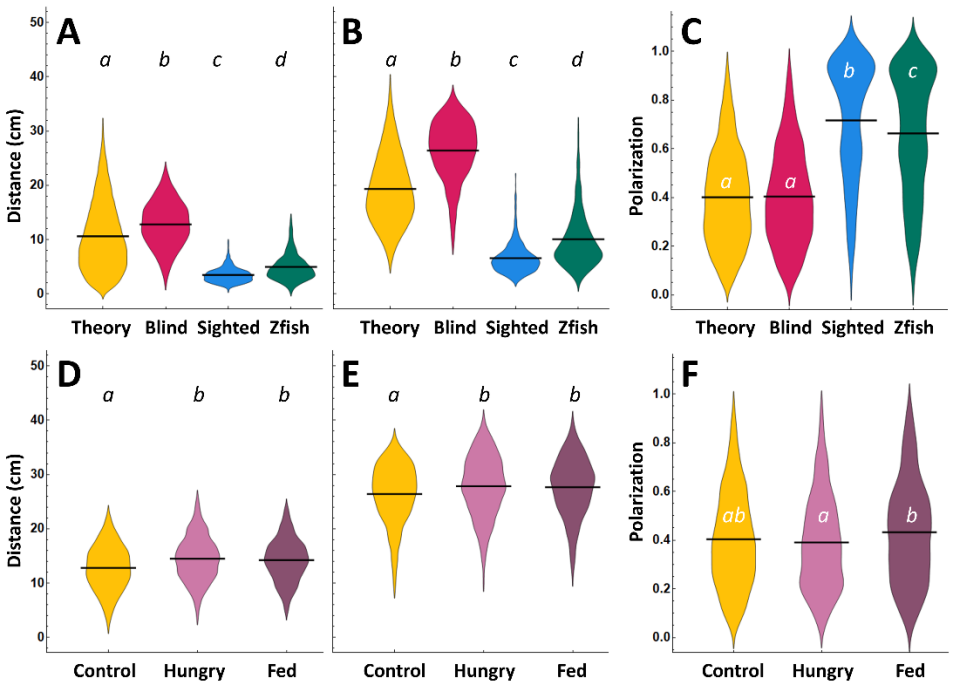

Figure 1. Results of Experiments 1 and 2. Distribution plots of nearest neighbor distances (NND; A, D), inter-individual distances (IID; B, E), and polarization (C, F) for the different species/morphs in Experiment 1 (A-C) and the different feeding conditions in Experiment 2 (D-F). Horizontal black lines indicate the mean of each distribution. Italic letters above or inside each distribution indicate statistical similarity.

Distributions of polarization in both zebrafish and sighted *A. mexicanus* (Figure 1C) displayed a characteristic bimodality, indicating the presence of two kinds of shoaling (sometimes referred to as schooling and shoaling; 146), but no such distinction was evident in the polarizations of blind *A. mexicanus*.

Experiment 2

Hunger levels had a significant impact on shoaling behavior in blind cavefish (Figure 1 D-F). Both hungry and recently fed fish shoaled less tightly than control fish (means ± SD in Table S1; post-hocs in Table S3. IID: $F(2,177) = 10.34$, $p = 0.00006$, $\eta^2 = 0.10$; NND: $F(2,177) =$

29.57, $p < 0.00001$, $\eta^2 = 0.25$). Polarization also differed significantly across conditions ($F(2,177) = 7.05$, $p = 0.001$, $\eta^2 = 0.07$), with fed fish showing higher polarization than hungry fish (Table S3).

Experiment 3

We found a significant main effect of morph and an interaction between morph and drug treatment but no main effect of drug treatment on both IID (Figure 2. Means ± SD in Table S1; post-hocs in Tables S4-S5. Morph: $F(1, 350) = 6712$, $p < 0.00001$, $\eta^2 = 0.94$; drug: $F(4, 350) = 2.78$, $p = 0.03$, $\eta^2 = 0.002$; morph x drug: $F(4, 350) = 16.79$, $p < 0.00001$, $\eta^2 = 0.01$) and NND (morph: $F(1,350) = 6547$, $p < 0.00001$, $\eta^2 = 0.94$; drug: $F(4,350) = 1.60$, $p = 0.17$, $\eta^2 = 0.001$; morph x drug: $F(4,350) = 11.71$, $p < 0.00001$, $\eta^2 = 0.007$). These results suggest that AVT modulated the dynamics of shoaling in both *A. mexicanus* morphs, but in different directions. Blind *A. mexicanus* given AVT+ swam closer together while sighted *A. mexicanus* moved further apart. Reducing IT levels also moved blind *A. mexicanus* closer together. On polarization, we found main effects of both morph and drug, as well as an interaction (morph: $F(1,350) = 650$, $p < 0.00001$, $\eta^2 = 0.59$; drug: $F(4,350) = 15.0$, $p < 0.00001$, $\eta^2 = 0.05$; morph x drug: $F(4,350) = 10.04$, $p < 0.00001$, $\eta^2 = 0.04$), suggesting that IT+ greatly reduced polarization in sighted *A. mexicanus* and AVT- increased polarization in blind *A. mexicanus* shoals.

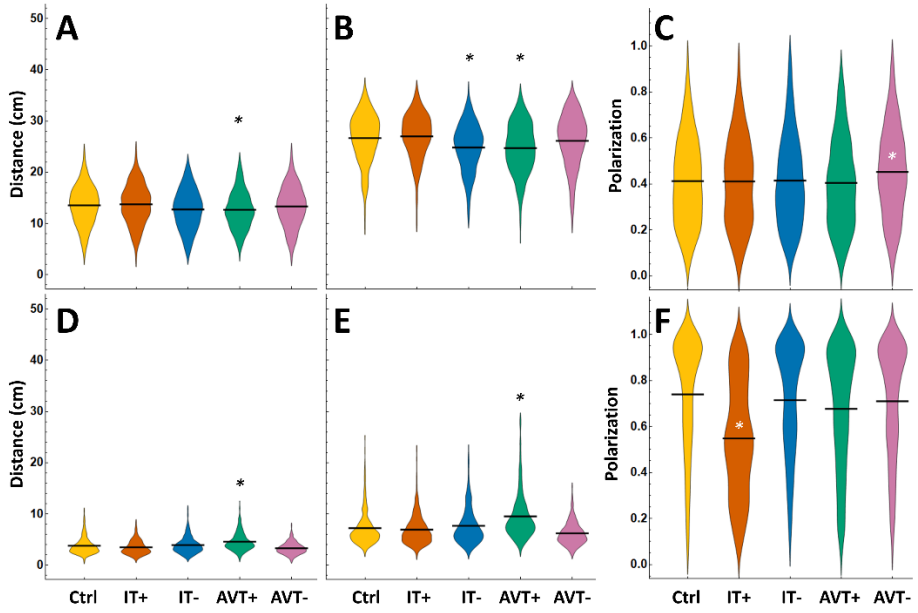

Figure 2. Results of Experiment 3. Distribution plots of nearest neighbor distances (NND; A, D), inter-individual distances (IID; B, E), and polarization (C, F) for the blind (A-C) and sighted *A. mexicanus* (D-F) in Experiment 3. Ctrl = control, IT+ = isotocin, IT- = isotocin antagonist, AVT+ = vasotocin, AVT- = vasotocin antagonist. Horizontal black lines indicate the mean of each distribution. Asterisks above or inside each distribution indicate significant differences ($p < 0.01$) from the control condition.

Experiment 4

Since we found, in Experiment 3, that increasing AVT in blind *A. mexicanus* decreased the distances between them, we next examined whether how effect varied with dosage. Administering higher AVT+ doses (at 20 and 40 µg/g) in blind *A. mexicanus* resulted in significantly looser shoals compared to the control and 10 µg/g doses (Figure 3. Means ± SD in

Table S1; post-hocs in Table S6. IID: $F(3,146) = 15.49$, $p < 0.00001$, $\eta^2 = 0.24$; NND: $F(3,146) = 15.19$, $p < 0.00001$, $\eta^2 = 0.24$), that were also less polarized ($F(3,146) = 4.28$, $p = 0.006$, $\eta^2 = 0.08$). In other words, higher doses of AVT had the opposite effect on shoaling distances as the low dose administered in Experiment 3. We also administered a higher dose of IT-, as it similarly decreased distances between blind *A. mexicanus* in Experiment 3. Increased doses of IT- (40 µg/g) led to significantly lower IID compared to the control group ($F(2,127) = 13.36$, $p < 0.00001$, $\eta^2 = 0.17$), but not NND ($F(2,127) = 3.94$, $p = 0.022$, $\eta^2 = 0.06$), and had no effect on polarization ($F(2,127) = 1.49$, $p = 0.23$, $\eta^2 = 0.02$). In other words, in contrast to AVT, higher IT- doses had the same effect as lower doses.

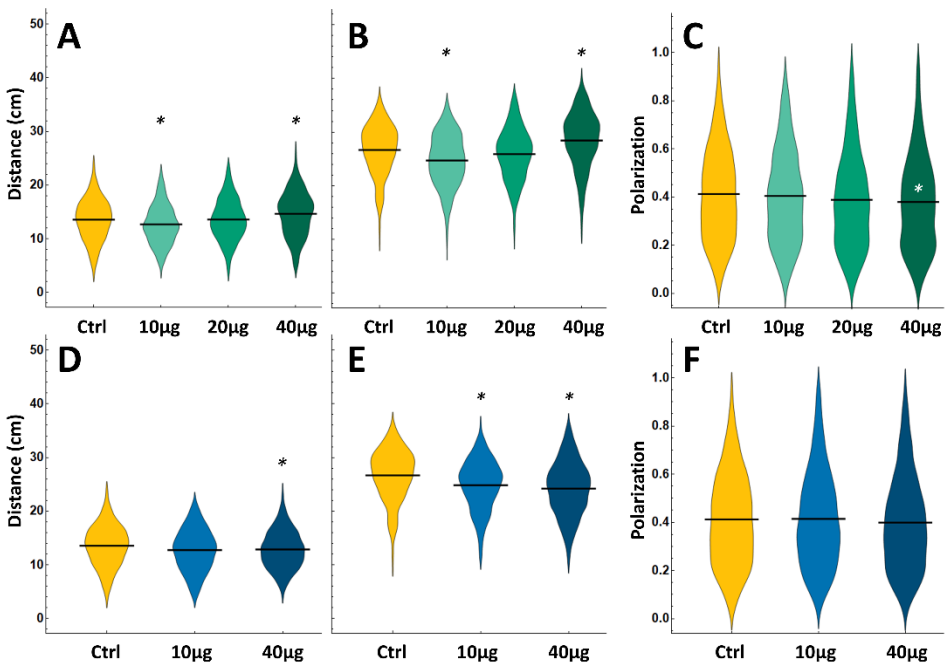

Figure 3. Results of Experiment 4. Distribution plots of nearest neighbor distances (NND; A, D), inter-individual distances (IID; B, E), and polarization (C, F) for increasing concentrations of vasotocin (A-C) and an isotocin antagonist (D-F) in Experiment 4. Horizontal black lines indicate the mean of each distribution. Asterisks above or inside each distribution indicate significant differences ($p < 0.01$) from the control condition.

Discussion

We compared the shoaling of blind *Astyanax mexicanus* with their surface-dwelling conspecifics and zebrafish, examining how nutritional state and neurohormonal modulation influence these behaviors. We found, unsurprisingly, that both sighted *A. mexicanus* and zebrafish formed cohesive shoals, maintaining smaller distances than would be expected by chance (Figure 1 A-B; Video S2), and displayed coordinated movement, as evidenced by high polarization values and bimodal distributions characteristic of shoaling behavior (Figure 1C; 148). Sighted *A. mexicanus* formed tighter and more polarized shoals than zebrafish, possibly a response to more challenging ecological conditions. Blind *A. mexicanus*, in contrast, neither coordinated their movements (their polarization was identical to what would be expected by chance) nor formed shoals. Notably, they maintained significantly *greater* distances between

individuals than would be expected if they were simply ignoring each other, suggesting active avoidance rather than social indifference (Video S1).

We found that both food deprivation and feeding increased distances between blind *A. mexicanus* (Figure 1 D-E), and groups of recently fed fish were more polarized than fasted groups (Figure 1F). Blind *A. mexicanus* also moved closer together when we increased AVT or blocked the action of IT (Figure 2 A-B), though the former effect was reversed at higher concentrations of AVT (Figure 3 A-B). Interestingly, AVT had the opposite effect on sighted *A. mexicanus*, moving them further apart (Figure 2 D-E). Increasing IT caused a drastic reduction in the polarization of sighted *A. mexicanus* groups, and the polarization of blind *A. mexicanus* groups varied inversely with AVT: decreasing AVT increased polarization (Figure 2C), while high doses of AVT reduced it (Figure 3C).

Our findings indicate that altering nutritional state or manipulating levels of IT and AVT in blind *A. mexicanus* affects group cohesion. Although the specific directions and magnitudes of these effects were not always as predicted (see below), the mere fact that hunger and hormonal changes impact shoal density suggests that blind *A. mexicanus*'s lack of shoaling stems more from a reduced motivation to aggregate than from an inability to detect conspecifics. This conclusion is further strengthened by our finding that groups of unmanipulated blind *A. mexicanus* do not simply ignore one another but actively avoid each other, directing their movements based on the positions of others but being repulsed from rather than attracted to them. Together, these results provide valuable insights into the evolutionary pressures that shape social behaviors.

While blind *A. mexicanus* retain the ability to perceive their surroundings through their lateral line system, our results suggest that their motivation to engage in social behaviors has been lost since they split from the sighted morph. This behavioral shift likely reflects adaptation to cave environments where aggregation is disadvantageous due to resource scarcity and the absence of predators, as shoaling could increase risks of kleptoparasitism and competition (99). Similar shifts away from sociality have been observed in other species experiencing changes in predation pressures, including guppies (29). Solitary swimming may also allow the lateral line system to detect subtle water movements with greater precision by reducing interference from conspecifics, enhancing navigation (58,79,83,148). While blind *A. mexicanus* have often been described as 'asocial' (147), the term 'anti-social' may better capture their active avoidance of conspecifics.

Our results also reveal that nutritional state modulates social behavior in blind *A. mexicanus*, which are constantly food-oriented (55,58). Contrary to our initial expectations, both fasted and fed groups maintained greater distances from each other than the control group (Figure 1 D-E), suggesting that both hunger and feeding may shift behavioral priorities toward

foraging or digestion. This likely reflects an energy-conserving adaptation, advantageous in environments where food resources are both scarce and unpredictable (51,65-68).

The social behaviors we observed in surface-dwelling *A. mexicanus* under increased AVT are consistent with findings in other social fish species, where AVT administration tended to promote social distancing or even aggression rather than cohesion (107,111-112,114,126). These findings align with AVT's known role in enhancing territoriality and defensive behaviors. We found that increasing AVT increased cohesion in groups of blind *A. mexicanus*, but decreased it in sighted *A. mexicanus*. This, together with the reduced density of AVT-producing cells in the blind *A. mexicanus* brain (92), provides strong evidence that this molecule and its receptors have been the targets of strong selection since the two morphs diverged. However, we also found that higher AVT doses reversed the effect in blind *A. mexicanus*, causing reduced cohesion. This pattern of results suggests that neuropeptide dose-response relationships in this species may be U-shaped. Exploring these dose-response dynamics in detail remains an interesting direction for future research. Higher AVT doses may have been necessary to elicit a comparable response to that seen in sighted *A. mexicanus* with lower doses, suggesting reduced sensitivity to AVT in the blind morph, possibly due to variations in the density of AVT-producing neurons. In either case, as AVT is closely involved in social cognition in a wide range of species, changes in the AVT system may largely be responsible for having reversed how blind *A. mexicanus* react to conspecifics, replacing attraction with repulsion.

The role of IT in modulating social behavior also differed between the two morphs. In blind *A. mexicanus*, blocking IT receptors decreased distances between individuals, partially restoring shoaling-like behavior. This suggests that IT typically functions to suppress social cohesion in blind *A. mexicanus* (Figure 2 A-B). No such effect was observed in sighted fish; however, increasing IT decreased their polarization. These findings contrast with research on other fish species, such as guppies and goldfish, as well as studies on OT in mammals, where this nonapeptide often enhances social bonding, facilitating group cohesion (133-140).

However, our results from blind *A. mexicanus* align with those seen in male *N. pulcher*, where IT administration inhibits grouping behavior, while IT antagonists increase group cohesion (120). These cichlids have higher levels of IT and lower levels of AVT than closely related less social species (125,149). Although their social behaviors contrast (blind *A. mexicanus* are solitary while *N. pulcher* are social), their neurohormonal responses to IT manipulation, as well as their baseline hormone levels, reveal notable similarities. This suggests that, despite divergent ecological demands, certain neuroendocrine systems may share evolutionary pathways, shaping distinct social strategies through similar mechanisms.

One possible explanation for the IT antagonist's increasing proximity in blind *A. mexicanus* is the social salience hypothesis, which suggests that OT/IT increases the salience of social stimuli (129-131). In blind *A. mexicanus*, where shoaling may be maladaptive, social

stimuli may be perceived as aversive, leading them to actively avoid conspecifics. Blocking IT, however, may reduce their sensitivity to these aversive social signals, leading to an increase in proximity, as we observed in our IT-antagonized blind *A. mexicanus* groups.

Conclusion

Neuropeptides such as AVP/AVT and OT/IT play a complex role in shaping social behaviors across taxa. Our findings reveal that the solitary nature of blind *A. mexicanus* is not due to an inability to detect conspecifics but reflects a decreased motivation to maintain social proximity. This highlights how blind *A. mexicanus* have adapted their social behavior to their cave environments, where predators are absent and competition for scarce resources favors solitary foraging (51). The contrasting responses to AVT in blind and surface *A. mexicanus* suggest that changes in the AVT system may have played a major role in restructuring social behavior since these morphs diverged. AVT enhances social cohesion in blind *A. mexicanus*, whereas it promotes social distancing in the sighted morph, as it does in other social fish species (107,126). Future work could profitably address the neural and molecular mechanisms by which these systems appear to have quickly diverged in the two morphs (e.g., 92).

Previous QTL studies have mapped loci associated with schooling in *A. mexicanus*, but have not identified AVT- or IT-related genes as part of this species' cave-specific adaptations (62). In other fish species, AVT or AVT-receptor knockouts show modified social behaviors such as aggression, courtship and mating (150). Investigating whether AVT- and IT-related genes are linked to schooling in *A. mexicanus* could clarify whether selection has directly acted on these genes during cave adaptation.

Overall, these findings not only shed light on the evolution of social behavior in cavefish but have broader implications for understanding how environmental pressures shape sociality. Our ability to modulate social behaviors through hormone administration suggests that similar mechanisms might drive the evolution of new social dynamics in other species experiencing drastic ecological changes. For instance, intense fishing pressures can select against shoaling in marine populations, leading to social shifts that mirror those seen in blind *A. mexicanus* (151). Further exploration of these neurohormonal systems will improve our ability to predict behavioral responses to shifting ecosystems, including those impacted by human activity, ultimately aiding conservation efforts and the management of species under pressure.

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Supplementary Information

Table S1. Means of all measures in all experiments. The table gives the mean measure (IID or NND, in cm; Polarization in unitless values) for each condition in each experiment. The table is divided by experiment (1-4). AM = *A. mexicanus*.

Experiment 1 (comparison of zebrafish, sighted AM, blind AM, and theoretical null model):

	Theory null	Blind AM	Sighted AM	Zebrafish
NND (cm)	10.61 ± 6.40	12.80 ± 9.39	3.48 ± 2.45	4.96 ± 4.69
IID (cm)	19.34 ± 6.53	26.42 ± 7.62	6.57 ± 3.31	10.06 ± 6.41
Polarization	0.40 ± 0.20	0.40 ± 0.20	0.72 ± 0.24	0.66 ± 0.25

Experiment 2 (comparison of fed and hungry blind AM [compared in the text to control data from Experiment 1]):

	Hungry	Fed
NND (cm)	14.50 ± 8.79	14.24 ± 8.81
IID (cm)	27.85 ± 7.84	27.66 ± 7.80
Polarization	0.39 ± 0.20	0.43 ± 0.21

Experiment 3 (comparison of drug effects on blind and sighted AM):

Blind AM	Saline	IT+	IT-	AVT+	AVT-
NND (cm)	13.56 ± 3.92	13.75 ± 3.95	12.75 ± 3.94	12.67 ± 3.70	13.34 ± 4.03
IID (cm)	26.67 ± 4.92	27.01 ± 4.53	24.84 ± 4.79	24.72 ± 4.97	26.14 ± 5.24
Polarization	0.41 ± 0.19	0.41 ± 0.19	0.41 ± 0.20	0.40 ± 0.20	0.45 ± 0.20
Sighted AM					
NND (cm)	3.79 ± 1.73	3.50 ± 1.53	3.94 ± 1.69	4.59 ± 1.78	3.31 ± 1.19
IID (cm)	7.24 ± 3.50	6.95 ± 2.98	7.69 ± 3.71	9.53 ± 4.50	6.23 ± 2.44
Polarization	0.74 ± 0.25	0.55 ± 0.25	0.71 ± 0.24	0.68 ± 0.26	0.71 ± 0.25

[continued on next page]

Experiment 4 (comparison of higher doses of AVT+ and IT- on schooling in blind AM [compared in the text to saline and 10 μg/g data from Experiment 3]):

	AVT+ @ 20 μg/g	AVT+ @ 40 μg/g	IT- @ 40 μg/g
NND (cm)	13.59 ± 7.79	14.64 ± 9.05	12.87 ± 7.57
IID (cm)	25.91 ± 6.96	28.45 ± 7.66	24.21 ± 7.00
Polarization	0.39 ± 0.20	0.38 ± 0.20	0.40 ± 0.20

Table S2: ANOVA Post-hoc test results for Experiment 1. Post-hoc tests were run using the Bonferroni correction. Each cell gives the T statistic for the test along with a p-value and the value of Cohen's D. Each sub-table gives results for a different measure (IID, NND, or Polarization). AM = *A. mexicanus*.

IID	Blind AM	Sighted AM
Sighted AM	T(124.1) = -53.1, p < 0.00001, D = 9.11	
Zebrafish	T(138) = -35.2, p < 0.00001, D = 5.96	T(114.7) = 8.35, p < 0.00001, D =1.42
NND		
Sighted AM	T(113.4) = -41.7, p < 0.00001, D = 7.06	
Zebrafish	T(138) = -31.0, p < 0.00001, D = 5.24	T(120.4) = 7.26, p < 0.00001, D = 1.24
Polarization		
Sighted AM	T(96.0) = -20.4, p < 0.00001, D = 3.70	
Zebrafish	T(118.5) = -18.4, p < 0.00001, D = 3.10	T(128) = -3.00, p = 0.004, D = 0.53

Table S3: ANOVA Post-hoc test results for Experiment 2. Post-hoc tests were run using the Bonferroni correction. Control data are taken from Experiment 1. Each cell gives the T statistic for the test along with a p-value and the value of Cohen's D. Each sub-table gives results for a different measure (IID, NND, or Polarization). AM = *A. mexicanus*.

IID	Hungry	Fed
Control	T(128) = -4.06, p = 0.00008, D = 0.71	T(118) = -3.57, p = 0.0005. D = 0.66
Fed	T(108) = -0.41, p = 0.69, D = 0.08	
NND		
Control	T(128) = -6.92, p < 0.00001, D = 1.22	T(118) = -5.76, p < 0.00001, D = 1.07
Fed	T(108) = -0.95, p = 0.34, D = 0.18	
Polarization		
Control	T(128) = 1.45, p = 0.15, D = 0.25	T(118) = -2.35, p = 0.02, D = 0.43
Fed	T(108) = -4.05, p = 0.0001, D = 0.77	

Table S4: ANOVA Post-hoc test results for Experiment 3, for comparisons between strains (blind and sighted). Post-hoc tests were run using the Bonferroni correction. Each cell gives the T statistic for the test along with a p-value and the value of Cohen's D. Each row gives results for a different measure (IID, NND, or Polarization [POL]). AM = *A. mexicanus*.

	Saline	IT+	IT-	AVT+	AVT-
IID	T(78) = 42.0, p < 0.00001, D = 9.71	T(68) = 47.47, P < 0.00001, D = 11.46	T(68) = 29.24, P < 0.00001, D = 7.06	T(68) = 23.83, P < 0.00001, D = 5.75	T(45.4) = 44.59, P < 0.00001, D = 11.48
NND	T(78) = 42.3, P < 0.00001, D = 9.77	T(68) = 42.00, P < 0.00001, D = 10.12	T(42.2) = 23.76, p < 0.00001, D = 6.20	T(67.3) = 34.77, P < 0.00001, D = 7.95	T(38.5) = 36.54, P < 0.00001, D = 9.69
POL	T(34.2) = 13.34, P < 0.00001, D = 3.72	T(61.3) = 6.52, P < 0.00001, D = 1.45	T(51.6) = 13.57, P < 0.00001, D = 2.93	T(34.7) = 11.18, P < 0.00001, D = 3.02	T(63.7) = 11.18, P < 0.00001, D = 2.50

Table S5: ANOVA Post-hoc test results for Experiment 3, for comparisons between saline and the drug treatment conditions. Post-hoc tests were run using the Bonferroni correction. Each cell gives the T statistic for the test along with a p-value and the value of Cohen's D. Each row gives results for a different measure (IID, NND, or Polarization). The top part of the table gives results for blind AM, the bottom for sighted AM. Each drug condition is being compared to the saline condition. AM = *A. mexicanus*.

Blind	IT+	IT-	AVT+	AVT-
IID	T(78) = -0.75, p = 0.45, D = 0.17	T(78) = 3.70, p = 0.0004, D = 0.85	T(88) = 4.35, p = 0.00004, D = 0.92	T(78) = 1.09, p = 0.28, D = 0.25
NND	T(78) = -0.80, P = 0.42, D = 0.19	T(40.3) = 2.17, P = 0.04, D = 0.57	T(88) = 3.79, P = 0.0003, D = 0.80	T(78) = 0.72, P = 0.47, D = 0.17
Polarization	T(78) = 0.16, P = 0.87, D = 0.04	T(78) = -0.07, P = 0.94, D = 0.02	T(88) = 0.85, P = 0.40, D = 0.18	T(46.7) = -2.72, P = 0.009, D = 0.68
Sighted				
IID	T(68) = 0.64, p = 0.52, D = 0.15	T68) = -0.79, p = 0.43, D = 0.19	T(58) = -3.37, p = 0.001, D = 0.87	T(68) = 2.41, p = 0.02, D = 0.58
NND	T(61.9) = 1.23, P = 0.22, D = 0.30	T(68) = -0.67, P = 0.50, D = 0.16	T(58) = -3.64, P = 0.0006, D = 0.94	T(68) = 2.46, P = 0.02, D = 0.59
Polarization	T(68) = 6.51, P < 0.00001, D = 1.57	T(68) = 0.76, P = 0.45, D = 0.18	T(58) = 1.81, P = 0.07, D = 0.47	T(68) = 0.90, P = 0.37, D = 0.22

Table S6. ANOVA Post-hoc test results for Experiment 4, comparing each drug concentration to saline (saline results taken from Experiment 3). Post-hoc tests were run using the Bonferroni correction. Each cell gives the T statistic for the test along with a p-value and the value of Cohen's D. Each row gives results for a different measure (IID, NND, or Polarization). AM = *A. mexicanus*.

	AVT+ @ 20 μg/g	AVT+ @ 40 μg/g	IT- @ 40 μg/g
IID	T(78) = 1.26, P = 0.21, D = 0.29	T(45.8) = -3.12, P = 0.003, D = 0.79	T(86) = 5.06, P < 0.00001, D = 1.01
NND	T(78) = -0.36, P = 0.72, D = 0.08	T(78) = -4.10, P = 0.0001, D = 0.95	T(87.3) = 2.62, P = 0.01, D = 0.52
Polarization	T(78) = 2.47, P = 0.02, D = 0.57	T(78) = 3.03, P = 0.003, D = 0.70	T(98) = 1.53, P = 0.13, D = 0.31

Figure S1. Frame of video from a trial of blind *A. mexicanus* in Experiment 1. The tank was 60 cm in diameter.