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Social Status-Related Differences in Motor Activity Between Wild-Type and Mutant Zebrafish

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Abstract. Use of zebrafish as a model organism in biomedical research has led to the generation of many genetically modified mutant lines to investigate various aspects of developmental and cellular processes. However, the broader effects of the underlying mutations on social and motor behavior remain poorly examined. Here, we compared the dynamics of social interactions in the Tüpfel long-fin nacre mutant line, which lacks skin pigmentation, to wild-type zebrafish; and we determined whether status-dependent differences in escape and swimming behavior existed within each strain. We show that despite similarities in aggressive activity, Tüpfel long-fin nacre pairs exhibit unstable social relationships characterized by frequent reversals in social dominance compared to wild-type pairs. The lack of strong dominance relationships in Tüpfel long-fin nacre pairs correlates with weak territoriality and overlapping spatial distribution of dominants and subordinates. Conversely, wild-type dominants displayed strong territoriality that severely limited the movement of subordinates. Additionally, the sensitivity of the startle escape response was significantly higher in wild-type subordinates compared to dominants. However, status-related differences in sensitivity of escape response in Tüpfel long-fin nacre pairs were absent. Finally, we present evidence suggesting that these differences could be a consequence of a disruption of proper visual social signals. We show that in wild-type pairs dominants are more conspicuous, and that in wild-type and Tüpfel long-fin nacre pairings wild-type fish are more likely to dominate Tüpfel long-fin nacres. Our results serve as a cautionary note in research design when morphologically engineered zebrafish for color differences are utilized in the study of social behavior and central nervous system function.

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Abbreviations: M-cells, Mauthner neurons; ROI, region of interest; TLN, Tüpfel long-fin nacre; WT, wild type.

Online enhancements: videos.

Introduction

Recent advancements in genetic approaches propelled zebrafish (*Danio rerio*) to become a popular model organism in biomedical research (Haesemeyer and Schier, 2018). Particularly, the availability of molecular tools to genetically engineer multitudes of mutant lines has improved our understanding of basic biological processes, including developmental, cellular, and neurophysiological principles (Drapeau *et al.*, 2002; Sagasti, 2007; Issa *et al.*, 2012b). However, one particular caveat that has been largely ignored is the fact that the commonly used zebrafish strains (*i.e.*, AB, Tüpfel long-fin [TL], Tübingen [TU], and leopard) differ not only genetically but also morphologically (Singh and Nüsslein-Volhard, 2015). Little attention has been devoted to determine how these genetic and morphological differences impact social behavior. One particular zebrafish strain is the Tüpfel long-fin nacre (TLN), categorized by a complete loss in dark pigmentation and the blue and golden stripes characteristic of wild-type (WT) zebrafish (Fig. 1A, B). The optical clarity of TLN fish is particularly advantageous because it extends the time period during which visualization of developmental processes and functional analysis of brain circuit activity can be examined *in vivo* (Lacoste *et al.*, 2015; Förster *et al.*, 2017). Genetically, the strain is characterized by two homozygous recessive mutations in the connexin 41.8 (*cx41.8*) and microphthalmia-associated transcription factor a (*mitfa*) genes, which are essential in melanophore development, resulting in the TLN strain (Lister *et al.*, 1999). Despite their wide use, the consequences

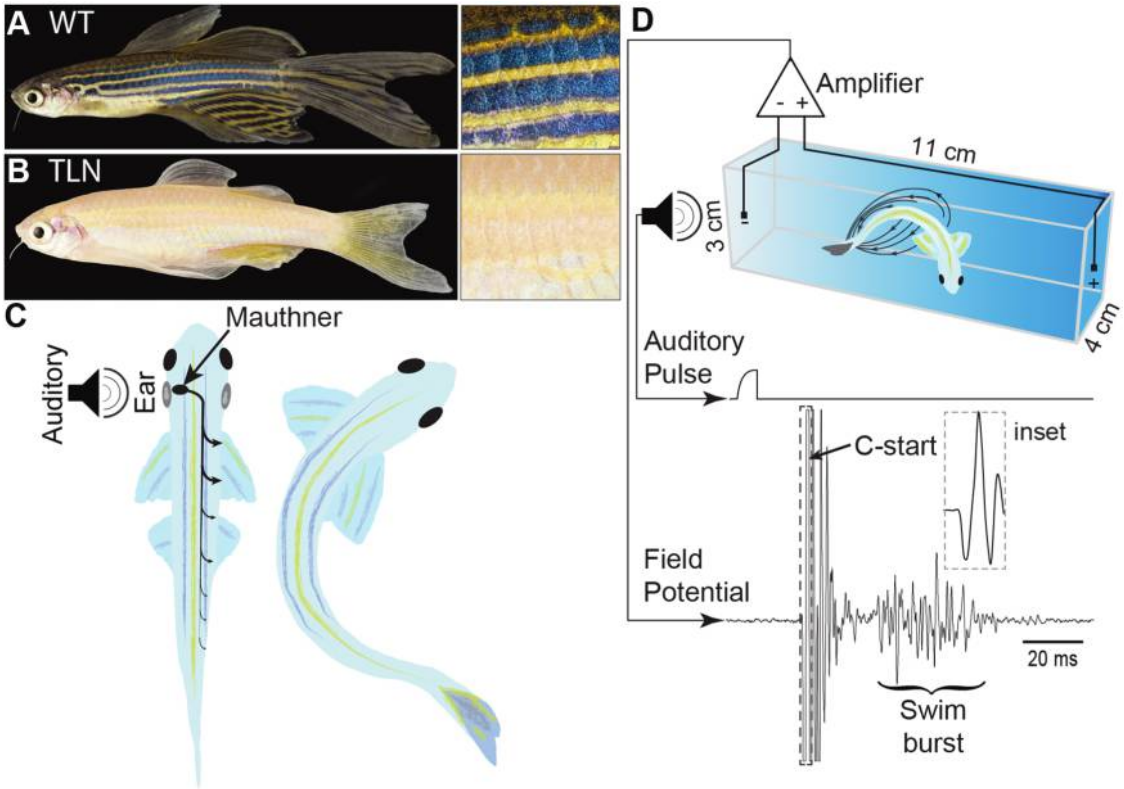

Figure 1. Wild-type (WT) and Tüpfel long-fin nacre (TLN) zebrafish strains are morphologically distinct. (A) WT zebrafish are brightly adorned with blue stripes. (B) TLN zebrafish lack body color pigmentation. Insets in (A) and (B) are high-magnification images taken from the medial part of the animals. (C) Schematic drawing of the C-start escape response mediated by the Mauthner neuron (M-cell). The M-cell receives ipsilateral auditory sensory input from the VIII sensory nerve. Stimulation of the M-cell activates contralateral fast motor neurons that innervate the musculature. (D) The C-start escape can be initiated by a sudden auditory pulse. Rapid contraction of the musculature during C-start escape generates a far-field potential (dashed box indicates C-start) that can be recorded using bipolar bath electrodes placed on either side of the testing chamber. The field potential of the C-start escape is characterized by large amplitude and brief duration (inset). Escapes are typically followed by swims whose field potentials can be detected (Swim burst) and are significantly smaller and longer in duration. Computer-generated auditory pulses and field potentials are digitized and time locked.

of the morphological differences in body coloration and other potential physiological secondary effects mediated by the mutations on social behavior and nervous system function remain unexamined. Recent evidence, however, suggests that the two underlying genetic mutations not only disrupt skin coloration but also cause decreased cortisol levels and a lower habituation rate of the startle response, compared to WT fish (Manuel *et al.*, 2014; van den Bos *et al.*, 2017). These results highlight the importance of characterizing the behavioral phenotype of the TLN mutant line and describing the effects of the mutations on social behavior in adult zebrafish.

WT zebrafish are brightly colored, with longitudinal golden and blue stripes whose color intensity can be regulated during mating and shoaling behaviors through a process called physiological color change (Fujii, 2000; Snekser *et al.*, 2006; Hutter *et al.*, 2012). Physiological color change of melanophores can also be regulated based on the color of the background environment (Logan *et al.*, 2006). Changes in melanophore color intensity are not transient; rather, plasticity in color intensity can be improved by cyclical training, suggesting a learning component and a capacity for long-term adaptation of body coloration to environmental changes (Hatamoto and Shingyoji, 2008). Although these results demonstrate the ability of zebrafish to regulate the intensity of their body color to ethological cues, little information is available on the importance of coloration during dominance hierarchy formation and its effects on zebrafish nervous system function (Pavlidis *et al.*, 2011). This is particularly relevant because visual information is known to be relayed to various brain decision-making nuclei that modulate the excitability of spinal motor circuits involved in mediating startle escape and swim behaviors (Lambert *et al.*, 2012; Mu *et al.*, 2012; Chou *et al.*, 2016; Pantoja *et al.*, 2016). This presents an exciting opportunity to test how changes in visual social cues can alter decision-making pro-

cesses and, consequently, the activation of the escape and swim motor circuits.

The startle escape response and its underlying neural circuit are well characterized behaviorally and physiologically (Eaton *et al.*, 2001; Korn and Faber, 2005; Park *et al.*, 2018). It is centered on a pair of reticulospinal neurons called the Mauthner neurons (M-cells), whose activation is necessary to mediate the C-start escape response that propels the animal away from threats by rapid flexion of the body (Fig. 1C). Visual information modulates the excitability of the M-cells *via* descending dopaminergic input and is contingent on the quality of the visual stimulus. Non-threatening visual stimuli increase the activity of the hypothalamic dopaminergic neurons that lead to suppression in synaptic transmission from the visual center to the escape circuit (Mu *et al.*, 2012). Conversely, threatening visual stimuli inactivate some of these neurons, resulting in dis-inhibition of the visuomotor transformation and escape generation (Yao *et al.*, 2016). In addition, neurons in the medial longitudinal fasciculus, which are crucial for locomotor output, activate during light-evoked swimming, suggesting that they are potentially modulated by visual stimuli as well (Sankrithi and O'Malley, 2010). Taken together, these results strongly suggest that modulation of the escape and swim circuits can be tuned by visual cues to facilitate appropriate behavioral responses. Despite this knowledge, little is known of how the absence of skin pigmentation in TLN zebrafish affects social interactions and the formation of dominance relationships, or how the lack of skin pigmentation influences the activation pattern of escape and swim behaviors.

Here, we conducted a comparative analysis of the social behavior and motor activation pattern of the escape and swim behaviors between WT and TLN zebrafish. We show that TLN fish can form stable social hierarchies, albeit with a higher prevalence of social instability. Second, we show that unlike WT fish, TLN zebrafish do not display social status-dependent place preference or differences in overall swimming activity. Third, we show that unlike WT fish, TLN zebrafish do not exhibit social status-dependent differences in the sensitivity of the C-start escape response. Finally, we illustrate that coloration is socially regulated and that lighter coloration denotes social subordination in WT zebrafish.

Materials and Methods

Animal care and use

Experiments were carried out according to the guidelines and approval of East Carolina University's Institutional Animal Care and Use Committee (Animal Use Protocol D320). Both WT adult AB zebrafish (*Danio rerio* (Hamilton, 1822)) and TLN mutant zebrafish were raised and maintained separately from one another, with no prior social interactions. Each fish strain was raised communally at 28 °C, pH 7.6, and fed pellet and live brine shrimp food twice daily.

Pairings and behavioral observations

Adult males (age 6–12 mo) were initially socially isolated for 1 wk in individual tanks (23 cm × 13 cm × 6 cm). Afterward, animals of equal age and size were randomly paired continuously for 2 wk in a new tank of equal dimensions (WT-WT $n = 6$ pairs, TLN-TLN $n = 16$, and WT-TLN $n = 12$). Social interactions were observed daily for 2 min between 10:00 and 14:00 hours. To assess social rank, we counted the total number of aggressive behaviors. Attacks consisted of bites and chase of an opponent, and submissive behavior consisted primarily of retreats. Social rank was assigned on the basis of the ratio of the total number of attacks divided by the number of retreats. Animals with a higher index were considered dominant in a pair. Unique morphological features allowed the easy identification and tracking of behavior patterns of individual fish (*i.e.*, unique mouth and eye shapes, dorsal fin length, belly size).

Measuring swimming activity

Pairs were filmed daily (early afternoons) for 1 min to monitor changes in swimming activity using a Canon camcorder (MiniDV ZR500, Tokyo, Japan) (WT group-housed $n = 12$ animals, TLN group-housed $n = 6$ animals, WT-WT $n = 7$ pairs, TLN-TLN $n = 12$ pairs). For the group-housed control condition, six males of equal size, age, and strain were selected from communal tanks and grouped together, and their swimming behavior was video recorded and tracked. Videos were digitized, and the movements (distance traveled in centimeters over 1-min periods) of each fish were analyzed using the National Institutes of Health ImageJ software Manual Tracking plug-in (https://imagej.nih.gov/ij/plugins/track/track.html). Instances when animals were interacting with one another resulted in the exclusion of those video frames from analysis. Total tracked distance was normalized by the number of remaining video frames.

Spatial distribution analysis

Using the video recordings as described above, we tracked the movement of each animal in paired (2 animals/tank) and grouped (6 animals/tank) conditions, by using the Manual Tracking plug-in for ImageJ to extract XY coordinates within the housing tank. Videos were down-sampled to three frames per second, and coordinates for dominants and subordinates were loaded into R software (R Core Team, 2016), using a custom script. XY coordinates encompassing periods of social interaction were removed from the analysis. Filled contour plots combining data of all dominant and subordinate animals were produced using two-dimensional kernel density estimations generated by the kde2d function of the MASS package (CRAN repository, Comprehensive R Archive Network, 2018; Venables and Ripley, 2002). The algorithm disperses the mass of the empirical distribution function over a

regular grid of 512 points and uses the fast Fourier transform to combine this approximation with a discretized version of the kernel, followed by linear approximation to evaluate the density at the specified points. Density data were converted into a heat-map probability plot to facilitate illustration of the data set for both social phenotypes. The outcome is an accurate probability predication of the spatial distribution of animals within the experimental arena.

Measurement of field potentials

Experimental setup, measurement of field potentials, and testing of the sensitivity of the C-start escape response were conducted as described previously (Prugh *et al.*, 1982; Issa *et al.*, 2011). In brief, animals were placed individually in a testing chamber (dimensions: 11 cm × 4 cm × 3 cm) filled with reverse-osmosis water with a resistance of ~15 MΩ-cm and a temperature of 25 °C. The fish were allowed to acclimate for 30 min prior to testing (Fig. 1D). The total number of animals tested were as follows: WT group-housed n = 14 animals, TLN group-housed n = 10 animals, WT-WT n = 10 pairs, TLN-TLN n = 12 pairs. M-cell mediated escape electric field potentials were recorded using a pair of conductive electrodes (1-mm bare thickness, 3–5-mm metal exposure) placed on either side of the testing chamber (Fig. 1D). Signals were amplified 1000-fold and were low-pass filtered at 300 Hz and high-pass filtered at 1 kHz, using an AC differential amplifier (model 1700, AM-Systems, Carlsborg, WA). Signals were digitized and then stored (Digidata1550, Axoscope Software, Molecular Devices, Sunnyvale, CA).

Auditory pulses (1 ms in duration) were computer generated using Audacity software (Audacity Team, 2018), and calibration of decibels was performed using a decibel meter (MS6700, Sinometer, Shenzhen, China). The animals' auditory sensitivity and threshold of the escape response were measured by delivering pulses at randomized decibels (dB) at midrange levels (repeated 3–5 times at each level) with a minimum of 2-min intervals between trials. To prevent habituation of the escape response, pulse decibel amplitude was randomized and ranged between 70 and 100 dB re 20 μPa, with 5-dB increments. Trials were averaged, and the data were curved-fitted with a nonlinear regression analysis, using the Boltzmann sigmoidal equation: $Y = Bottom + (Top - Bottom)/(1 + exp((V50 - X)/Slope))$.

Field potential data analysis

The latency of the escape response was measured using the field potential signals recorded by measuring the time between stimulus onset and the beginning of field potential response. The zebrafish escape response is categorized into two main types of escapes. Activation of the M-cell generates a short-latency escape response, with a time onset ranging between 5 and 15 ms (Fig. 1D). Zebrafish also produce long-latency non-M-cell mediated escapes, with a time onset ranging between 15 and 40 ms (Eaton *et al.*, 2001; Kohashi and Oda, 2008). Each type of escape generates a distinct electric signal that permits the reliable identification of escape based on both potential signature and its latency from stimulus onset (Issa *et al.*, 2011). The latency of the electrical signal was defined as the time difference between the onset of the auditory stimulus and the beginning of the large phasic field potential generated during the short-latency M-cell mediated escape response. Data were tabulated into Microsoft Excel and analyzed using Prism (GraphPad Software, San Diego, CA). All values are provided as mean ± SEM unless otherwise stated.

Acquisition and analysis of pigment color intensity

Prior to imaging, the fish (n = 11 pairs) were briefly anesthetized with 0.02% MS-222 diluted in system fish water. The fish were placed onto a petri dish and imaged using an Olympus (Shinjuku, Toyko, Japan) SZX29 stereo microscope equipped with a CCD computer-controlled camera (ToupTek, ToupView software, Zhejiang, China). Images (2048 × 1536) were taken at ambient light while minimizing reflective lighting by using a light diffuser. Multiple images were taken at the same magnification at the anterior and medial parts of the animals. Images were taken of the middle two color bands that extend body length. Once images were taken, the animals were returned to their isolation tanks to recover for 30 min, followed by the onset of pairing for 2 wk.

Using NIH ImageJ, images were converted to grayscale. This process prevented discrimination among the three different types of chromatophores known to contribute to the overall color pattern of zebrafish (Singh and Nüsslein-Volhard, 2015). Nevertheless, this approach allowed the quantification of the overall change in color intensity. For each image, one region of interest (ROI) was selected for each of the colored stripes that extend the full length of the body. Plot profiles of the average grayscale intensity values were generated for each of the selected ROIs. Similar-sized ROIs of background intensity were also collected from regions that lack melanophores (inter-stripe regions), which served as a control of data acquisition and analysis. Grayscale ranged from 0 to 255, where 0 equaled absolute black and 255 equaled absolute white. Data were averaged across all ROIs. The same process of image acquisition and analysis was repeated after two weeks of pairing (day 14). To circumvent potential bias, behavioral observations, image acquisition, and data analysis were conducted blind by three different investigators. One individual was in charge of the behavioral observations. The fish were handed down to a second investigator, who obtained the images. Images were then provided to a third investigator, who analyzed the data. Neither of the investigators who acquired and analyzed the data was aware of the social status of the fish until the data were fully analyzed.

Results

Analysis of social interactions and dominance formation in WT and TLN zebrafish

To examine whether TLN social interactions vary from their WT counterparts, we observed the aggressive interactions of TLN zebrafish pairs for two weeks and compared their behavior to WT pairs. We measured the level of aggression between the two groups by recording the number of aggressive behaviors (attacks) and submissive behaviors (retreats) of each pair (Fig. 2). We found that WT pairs formed stable dominance relationships within two days of pairing. By the third day, dominants (Dom) performed most of the aggressive chasing and biting behavior (Fig. 2A, left panel), while subordinates (Sub) mainly retreated from interactions (Fig. 2A, right panel). The 2-week average number of attacks and retreats differed significantly between the two social phenotypes (2-week avg. no. of attacks: Dom = 5.46 ± 0.59, Sub = 0.72 ± 0.29, Wilcoxon signed rank test, two-tailed, $P = 0.0001$; 2-week avg. no. of retreats: Dom = 0.57 ± 0.25, Sub = 5.19 ± 0.61, Wilcoxon signed rank test, two-tailed, $P = 0.0001$; Dom $n = 6$, Sub $n = 6$). In addition, once dominance was established, it remained stable for the remainder of the observation period (two weeks). TLN pairs displayed similar patterns of aggressive activity, and overall level of aggression did not significantly differ from WT pairs (Fig. 2B1) (avg. no. of attacks for TLN Dom = 5.45 ± 0.28, TLN Sub = 1.58 ± 0.26; $n = 6$ WT pairs, $n = 16$ TLN pairs; avg. no. of attacks of WT Dom *vs.* TLN Dom, Mann Whitney *U* test, two-tailed, $P = 0.8362$; avg. no. of attacks of WT Sub *vs.* TLN Sub, Mann Whitney *U* test, two-tailed, $P = 0.9752$). Although the behavior patterns of TLN pairs appeared similar

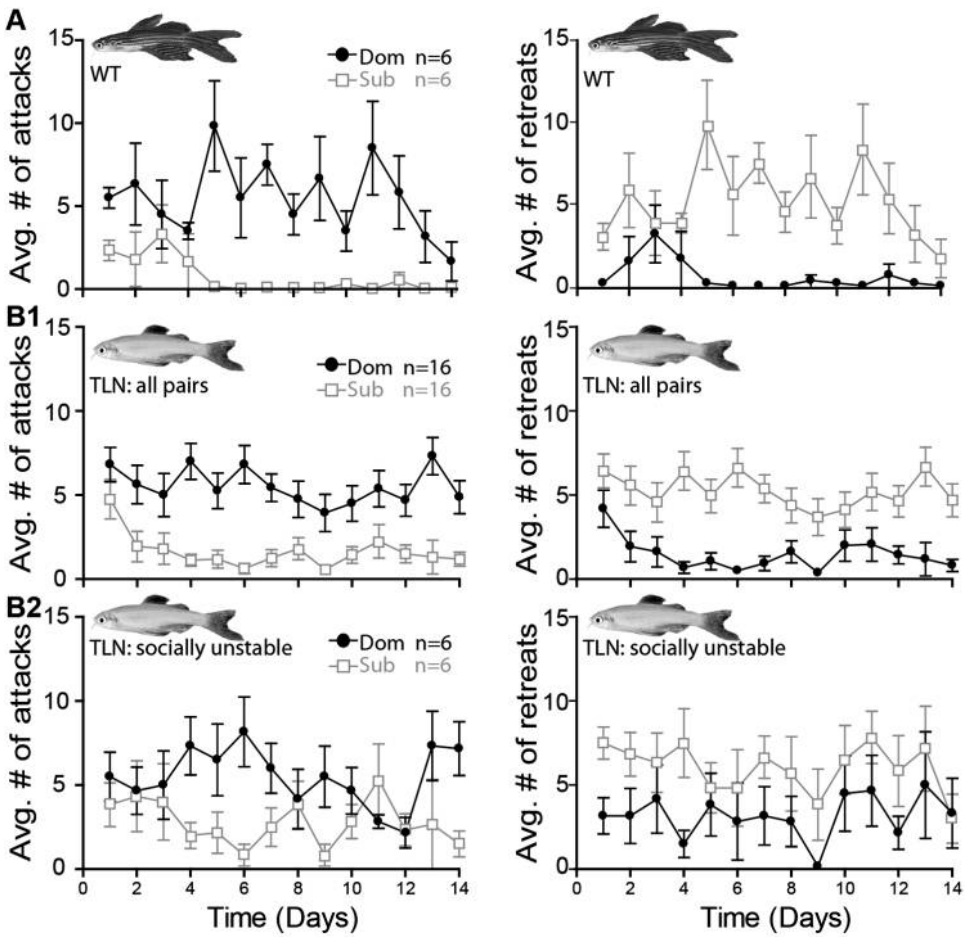

Figure 2. Social dominance relationships in Tüpfel long-fin nacre (TLN) zebrafish are unstable. Both wild-type (WT) and TLN zebrafish engage in agonistic interactions (attacks and retreats). The figure illustrates the dynamics of social agonistic interactions in the two zebrafish strains. (A) Average number of attacks and retreats between WT dominants (Dom, filled circles) and subordinates (Sub, open squares) ($n = 6$ pairs). (B) Average number of attacks and retreats between TLN dominants and subordinates of all TLN pairs regardless of social stability ($n = 16$ pairs). (C) Average number of attacks and retreats between TLN dominants and subordinates of pairs that experienced social instability ($n = 6$ out of 16 TLN pairs).

to those of WT, closer examination showed that more than a third of TLN pairs switched their social status at least once during the pairing period (Fig. 2B2; 6/16 pairs, 37.5%). These frequent reversals in social status were not observed in WT pairs, and reversals were likely a result of an increased frequency of attacks by the TLN subordinates. We compared the aggression level between WT and TLN subordinates and found a significant difference in the average number of attacks over the observation period (WT Sub: 0.72 ± 0.29, TLN Sub = 1.58 ± 0.26, Mann Whitney *U* test, two-tailed, $P = 0.0137$). These results suggest significant behavioral differences in social aggression and stability of dominance relationships in TLN fish compared to their WT counterparts.

Differences in territorial behavior between WT and TLN zebrafish

One of the hallmarks of social dominance is the ability of dominant animals to defend, hold, and have first access to current and future resources within their territory (*i.e.*, food, shelter, or mate). Therefore, we hypothesized that dominant zebrafish would be likely to swim more than subordinates in order to defend and have first access to the daily food supplied. The zebrafish were accustomed since the larval stage to be fed through a specific opening located atop the tank through which food was regularly dispensed (Fig. 3, "food intake"). The fish were often observed to quickly swim to the front of the tank in wait for the food whenever an experimenter approached the tanks, which was indicative of a learned behavior of the daily feeding schedule. Therefore, we examined differences in territorial behavior by recording swimming activity and patterns of spatial distribution in WT zebrafish pairs compared to TLN pairs to determine whether dominants of each strain were capable of defending their territory by keeping subordinates at bay and away from the food source.

First, we measured the average distance swam every day during a 1-min interval for TLN and WT pairs. In addition, we generated density plots of swimming activity to determine the spatial probability distribution of all animals observed over 1 min of filming sampled at 3 frames/s (Fig. 3). We found that as social dominance was established in WT pairs, dominants maintained high levels of swimming activity, while subordinates significantly decreased their swimming behavior compared to dominants and group-housed control fish (Fig. 3A; Kruskal-Wallis test one-way ANOVA, Dunn's multiple comparison test, $P < 0.0001$ [difference in rank sum: group-housed *vs.* Dom = −14.13, group-housed *vs.* Sub = 13.53, Dom *vs.* Sub = 27.67]; group-housed $n = 12$, Dom $n = 7$, Sub $n = 7$). Differences in swimming were apparent in the spatial distribution of the two social phenotypes within the experimental arena. WT dominants exhibited strong territorial displays in which they swam freely throughout the tank, while subordinates were relegated to the back corners (Fig. 3A, right panel; Video 1, available online). Unlike for WT pairs, we found differences in swim frequency and patterns between dominant and subordinate TLN fish to be less pronounced, regardless of the stability of social structure. Statistical analysis of distance traveled for all TLN fish showed a significant difference in the average distance traveled between dominant and subordinate fish but no difference between group-housed and subordinate fish (Fig. 3B1; Kruskal-Wallis test one-way ANOVA, Dunn's multiple comparison test, $P < 0.0001$ [difference in rank sum: group-housed *vs.* Dom = −25.33, group-housed *vs.* Sub = −10.67, Dom *vs.* Sub = 14.67]; group-housed $n = 6$, Dom $n = 7$, Sub $n = 7$). However, among the TLN pairs that displayed social reversals, we did not find significant differences in the average distance traveled (Fig. 3B2; Kruskal-Wallis test one-way ANOVA, Dunn's multiple comparison test, $P < 0.0001$ [difference in rank sum: group-housed *vs.* Dom = −21.51, group-housed *vs.* Sub = −13.27, Dom *vs.* Sub = 8.267]; group-housed $n = 6$, Dom $n = 7$, Sub $n = 7$). Moreover, in both socially stable and unstable TLN pairs, dominants and subordinates did not display territorial behavior, as was observed in WT pairs (Fig. 3B1, B2, right panels; Video 2, available online). These results suggest that the genetic mutations underlying the TLN mutant line affect the dynamics of social interactions, with negative consequences on swimming and territorial behavior.

Differences in C-start escape response between WT and TLN zebrafish

To characterize possible differences in the activation of the C-start escape response in TLN fish, we tested the animals' sensitivity to auditory pulses. To prevent habituation of the escape response, we randomized the intensity of the auditory pulses while maintaining a minimum of 2-min intervals between stimuli (see Methods for details). In WT pairs, subordinate fish displayed heightened sensitivity to auditory stimulation, illustrated by more frequent responses to lower auditory pulses compared to dominants (Fig. 4A). Subordinate fish reached the 50% response threshold at 78.84 dB, dominants at 83.28 dB, and group-housed animals at 84.19 dB. Data were curve-fitted with a nonlinear regression: Boltzmann sigmoidal curve fit; goodness of fit R^2 for group-housed = [0.7765], dominants = [0.7675], subordinates = [0.9096]; group-housed $n = 14$; dominant $n = 10$, subordinate $n = 10$ (one-way ANOVA, $P < 0.001$). This result is consistent with our previous report demonstrating status-dependent differences in the sensitivity of the escape response (Miller *et al.*, 2017). Unlike WT pairs, the probability of the C-start escape response was similar in dominant and subordinate TLN animals (Fig. 4B). Subordinate fish reached the 50% response threshold at 81.18 dB, dominants at 79.1 dB, and group-housed animals at 80.7 dB. Data were curve-fitted with a nonlinear regression: Boltzmann sigmoidal curve fit; goodness of fit R^2 for group-housed = [0.8091], dominants = [0.8434], subordinates = [0.6927]; group-housed $n = 10$; dominant $n = 12$,

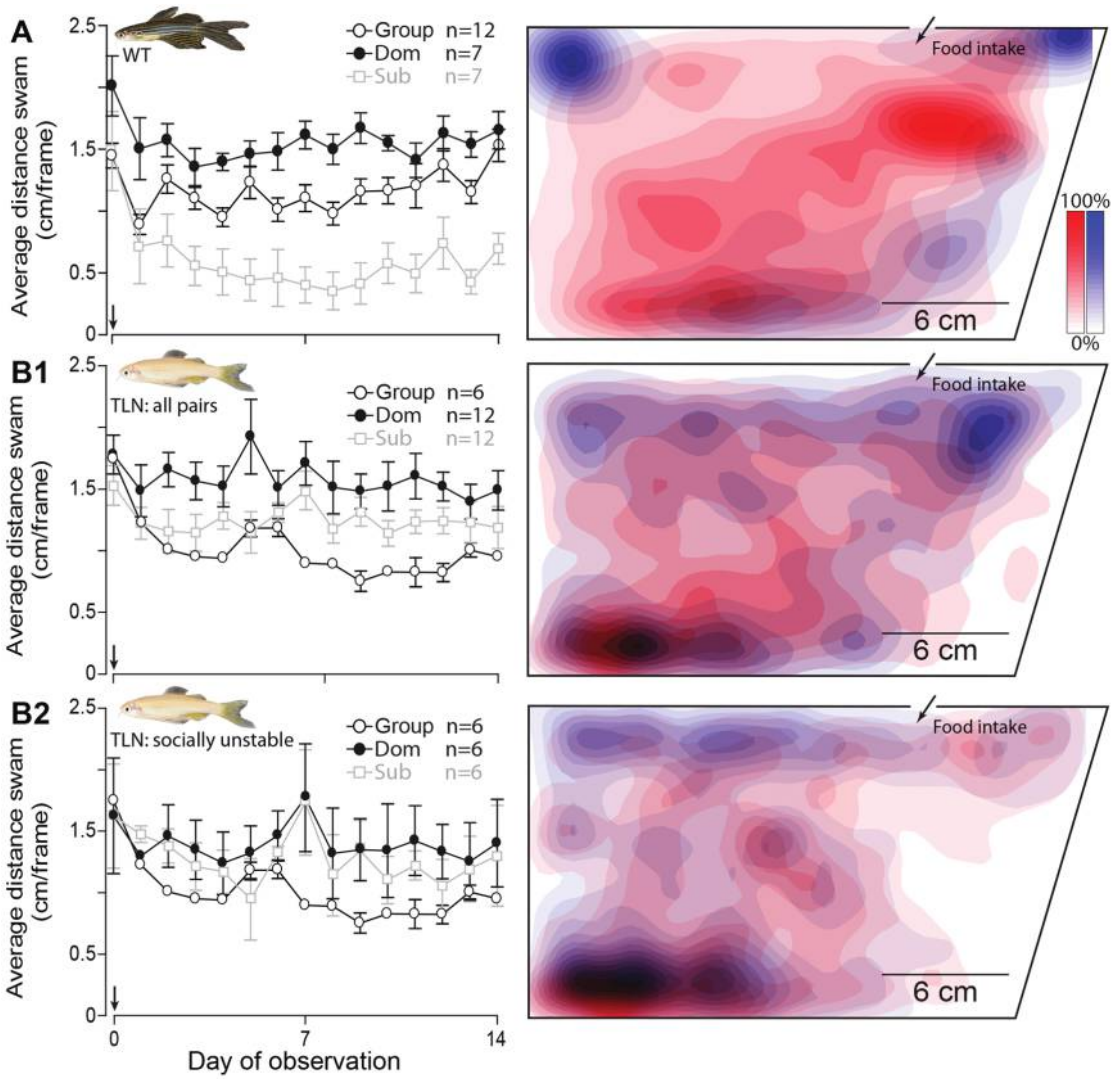

Figure 3. Territoriality and swimming behaviors are disrupted in Tüpfel long-fin nacre (TLN) pairs. Left panels: Average distance traveled over a 1-min period of filming of group-housed (open circles), dominant (Dom, filled circles), and subordinate (Sub, open squares) animals for (A) wild type (WT), (B1) all TLN, and (B2) socially unstable TLN pairs. Right panels: Kernel heat-map estimation plots of swimming activity over a 1-min period of filming for dominants (red) and subordinates (blue) on day 14 post-pairing for each experimental condition. (A) WT condition ($n = 12$ group-housed, $n = 7$ dominants, and $n = 7$ subordinates). (B1) All TLN animals ($n = 6$ group-housed, $n = 12$ dominants, and $n = 12$ subordinates). (B2) Only TLN pairs that experienced dominance reversals ($n = 6$ group-housed, $n = 6$ dominants, and $n = 6$ subordinates). Day 0 marks observations of animals before they were paired (arrow indicates before pairing). "Food intake" arrow in each heat map denotes the location of food dispensed twice daily.

subordinate $n = 12$. Comparison of the sensitivity curves of all three social groups showed that pairwise interactions had no effect on the response sensitivity (Fig. 4B).

Social status regulates intensity of skin pigment in WT zebrafish

The most distinguishing characteristic that sets the TLN fish apart from their WT counterparts is the lack of skin coloration. Therefore, we hypothesized that visual cues may play an important role in regulating social interactions, and disruption of proper visual information in TLN fish may account for social instability and disruption in swimming and escape behavior patterns. If this hypothesis is supported, then it suggests that in WT fish, body coloration is socially regulated and potentially serves as a mechanism to communicate social status. To test this notion, we measured changes in color intensity of WT animals prior to social interactions (day 0) and two weeks later (day 14) (Fig. 5). At first examination, we found that social interactions did not lead to a change in color intensity in

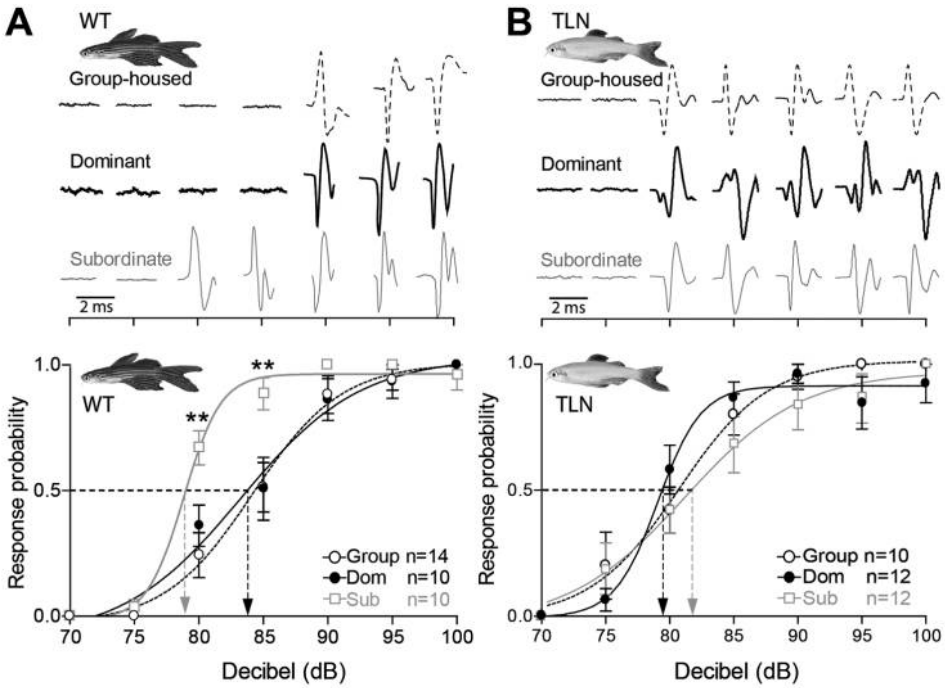

Figure 4. Probability of C-start escape response in Tüpfel long-fin nacre (TLN) is not socially regulated. (A) Individual examples of C-start field potentials from group-housed (open circles), dominant (Dom, filled circles), and subordinate (Sub, open squares) wild-type (WT) animals at increasing decibel intensities (top) and probability of C-start escape response (bottom). Probability of initiating an escape response is significantly higher in subordinates ($n = 10$) compared to dominants ($n = 10$) and group-housed animals ($n = 14$) at the 85–90-dB levels (one-way ANOVA, $^{**}P < 0.001$). A response probability of 0.5 is indicated with the dashed arrow line. (B) Individual examples of C-start field potentials from group-housed, dominant, and subordinate animals at increasing decibel intensities (top) in TLN fish. Probability of C-start escape response in TLN fish (bottom).

dominants but resulted in a decrease in intensity in subordinates (Fig. 5A). Subordinates became lighter in coloration. This was noticeable when comparing the distribution of melanin within the melanophores between day 0 and day 14 (Fig. 5A, subordinate right panel). On day 0, melanin was widely distributed within the cells, preventing the ability to differentiate individual melanophores. However, by day 14, individual melanophores became more distinct, a sign of aggregation of melanin centrally within the cells. This is typically indicative of a decrease in color intensity. To verify this result, we calculated the grayscale intensity profile for each ROI (Fig. 5A, dashed boxes). Intensity plot profiles showed significant differences in intensity patterns between dominants and subordinates after two weeks of interactions (Fig. 5B, C). Dominants became increasingly darker, with a tighter intensity profile distribution, while subordinates became lighter, with widely varied intensity profiles (Fig. 5B). At day 0, color intensities did not differ between the two social groups, nor were there differences in background color intensity (Fig. 5C, Dom $n = 11$, Sub $n = 11$, Wilcoxon signed rank test, two-tailed, $P = 0.0547$). Although statistically not significant, it is important to note that dominants tended to be darker than subordinates prior to pairing, suggesting possible predisposition of color differences that may have affected the outcome of social interactions. Nevertheless, the change in color intensities after two weeks became more pronounced and statistically significant (Fig. 5C, avg. gray intensity day 0: Dom $= 24.73 \pm 4.2$, Sub $= 35.6 \pm 4.4$; avg. gray intensity day 14: Dom $= 18.5 \pm 2.3$, Sub $= 39.6 \pm 6.2$; Wilcoxon signed rank test, two-tailed, $P = 0.0007$). This status-dependent change in intensity was limited to the melanophore-containing stripes and was not observed in the background (Fig. 5D). A closer examination revealed that 8 of 11 dominants increased their color intensity (Wilcoxon signed rank test, two-tailed, $P = 0.0248$), while 5 of 11 subordinates showed a decrease in intensity (Wilcoxon signed rank test, two-tailed, $P = 0.2806$). Moreover, pairwise comparison of the average change in intensity indicated that dominants were darker in intensity than their subordinate counterparts (8/11 pairs). These differences were not intrinsic, because intensities were similar prior to pairing, and significant differences emerged only as a consequence of social interaction.

To validate this result, we paired WT fish with TLN fish. We predicted that if the lack of body coloration served as a cue of social subordination, then WT fish would be more likely than TLN fish to be dominants. Indeed, we found that WT and TLN fish form stable social relationships, in which, on average,

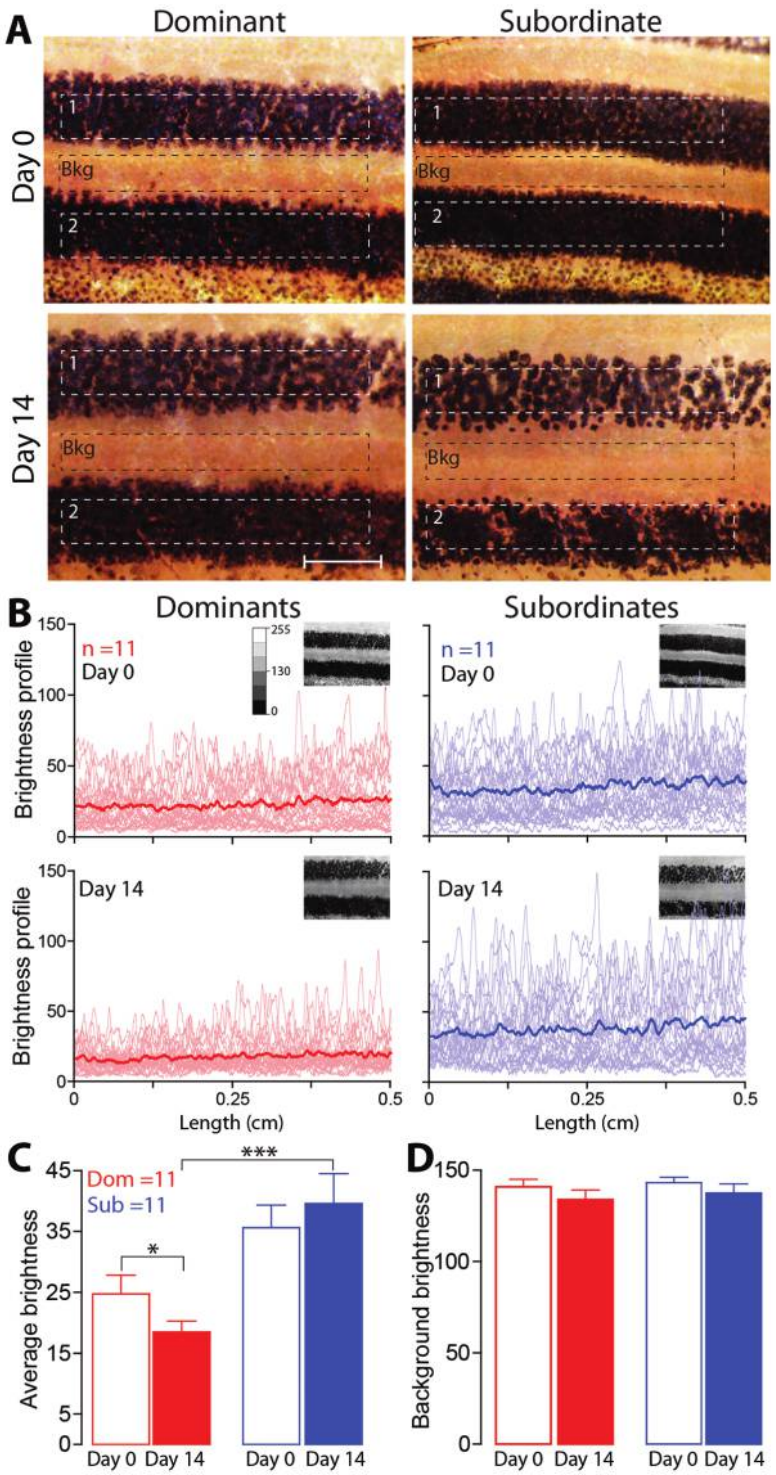

Figure 5. Intensity of body color is socially regulated in wild-type (WT) zebrafish. (A) Representative examples illustrating changes in intensity of body coloration. Illustrations are from a WT dominant and its subordinate counterpart on day 0 and day 14 of pairing. Dashed boxes highlight analyzed regions of interest (ROIs) for color stripes 1 and 2 (scale bar = 125 mm) and background (Bkg) ROIs as controls. (B) Individual plot profiles of the grayscale intensity of all stripes and pairs tested (thin lines) and superimposed averages (thick lines) showing the effect of social status on melanophore intensity. Inset in each panel illustrates representative examples of the grayscale images analyzed for each social condition and time point, along with the grayscale bar (0–255). (C) Average change in color intensity between WT dominant (Dom) and subordinate (Sub) zebrafish on day 0 and day 14 of pairing (Dom $n = 11$, Sub $n = 11$; avg. gray intensity day 0: Dom = 24.73 ± 4.2, Sub = 35.6 ± 4.4; avg. gray intensity day 14: Dom = 18.5 ± 2.3, Sub = 39.6 ± 6.2; Wilcoxon signed rank test, two-tailed, $*P = 0.0248$, $***P = 0.0007$). (D) Average change in background color intensity (Bkg ROIs in A) showing no status-dependent difference or change in background skin color.

WT fish were more likely to dominate their TLN counterparts (WT were dominant in 9 of 12 pairs, and TLN were dominant in 3 of 12 pairs; chi-square = 6, df = 1, $P = 0.0143$, $n = 12$). These dominance relationships tended to be strong and quick to form, evident by the fact that dominance was formed by day 1 (data not shown) and that nearly half of the TLN animals (4/9, 44%) were killed by their WT counterparts during the first week of pairing. This result was concordant with strong territorial displays by WT fish that limited the swimming behavior of TLN fish to only one corner of the arena (Video 3, available online). Taken together, our results suggest that pigment coloration in zebrafish is plastic, socially regulated, and serves as a behavioral mechanism to convey information of social rank.

Discussion

Our results show that the dynamics of social interactions and dominance formation differ significantly between TLN and WT zebrafish. TLN pairs display frequent social reversals and exhibit significant differences in their motor behavior. TLN zebrafish form unstable social relationships and display different escape and swim behavioral patterns compared to WT fish. Although the aggression level and pattern of social interactions were similar to those of WT, social relationships reversed at least once in 37.5% of TLN pairs (Fig. 2). As animals engage in agonistic interactions, they rely on many different types of social information to assess their opponents' strengths (Teles and Oliveira, 2016). Our results show that improper visual cues in TLN fish led to continued competition for dominance, whereas WT fish quickly established stable relationships. Although a significant number of TLN pairs failed to form stable relationships, the remaining 62.5% did, which suggests that other pertinent factors such as body size, pheromones, and inherent motivational differences may compensate for the loss in proper visual cues (Gerlach *et al.*, 2008; Enjin and Suh, 2013; Chou *et al.*, 2016; Teles and Oliveira, 2016).

In many social animals, proper assessment of the strength and aggressive motivation of an opponent determines the outcome of agonistic interactions (Huber *et al.*, 1997; Issa *et al.*, 1999, 2012a; Arnott *et al.*, 2016; Briffa and Lane, 2017). Moreover, with frequent encounters, "winner" and "loser" effects develop and shape the outcome of future interactions (Oliveira *et al.*, 2011). If lighter body coloration serves as a signal of social subordination, as our data suggest, then it is no surprise that TLN pairs showed frequent social instability due to the incongruent social messages that opponents were experiencing: pale coloration as a signal of subordination is inconsistent with displays of aggressive behavior. Conversely, when WT and TLN fish were paired, the exaggerated signal of social subordination (paleness) of the TLN fish was likely to have influenced the aggressive state of their WT counterparts and increased their motivation to fight and win subsequent encounters. The induced "winner" effect in WT fish explains the quick dominance formation between WT-TLN pairs and the higher probability of WT fish to kill their TLN counterparts, a phenomenon that is rarely observed among WT fish.

The frequent social instability in TLN pairs was evident in the lack of differences in swim activity and territorial behavior typical of WT zebrafish pairs (Fig. 3). Within a few days of pairing, WT subordinates were relegated to the corners of the housing tank as a form of shelter and/or avoidance strategy of their dominant counterparts (Fig. 3A; Video 1, available online). However, subordinate TLN fish did not display such behavior, and their swim pattern overlapped extensively with that of dominants (Fig. 3B; Video 2, available online). In addition, while WT subordinates decreased their swimming activity compared to dominants and group-housed fish, TLN subordinates swam as frequently as dominants; this includes TLN subordinates of pairs that formed stable social relationships. This suggests that the individual behavioral components that constitute submissiveness (lack of aggression and swimming activity) can be independently regulated. In the present case, although TLN subordinates reduced their aggressive behavior, they did not reduce their swimming, as was observed in WT subordinates. This suggests that aggression by dominants is necessary to establish a social order and reduce subordinates' aggressive behavior. However, aggression alone is insufficient to change swimming behavior pattern, which may require additional cues to engage specific decision-making circuits responsible for suppressing the swimming circuit.

TLN zebrafish showed no status-dependent difference in the sensitivity of escape response to auditory stimuli (Fig. 4). This was in stark contrast to WT fish, in which the sensitivity of the escape response of subordinates was significantly enhanced compared to dominants and in contrast to previously reported observations showing status-dependent differences in M-cell sensitivity in cichlids (Neumeister *et al.*, 2010; Miller *et al.*, 2017). One possible mechanism that may explain this difference between WT and TLN animals is that the distinct morphological phenotypes between the two strains led to differences in how visual signals were relayed to decision-making circuits, such as the hypothalamic dopaminergic neurons known to modulate the excitability of the M-cell (Mu *et al.*, 2012). An alternative possibility is that the underlying genetic mutations of the TLN strain may have led to presently uncharacterized off-target physiological effects that may account for the behavioral differences observed. If so, then experimental manipulations of skin color in TLN fish will be necessary to determine the exact contribution of the genetic mutations and/or disruption in their social visual communication in regulating social behavior. Currently, these experiments are not possible because simple dying of TLN skin is highly aversive to normal behavior. Alternative genetic approaches will be required to manipulate TLN fish skin color.

It is noteworthy that the sensitivity of the escape response of all TLN fish, regardless of social status, was moderately el-

evated and comparable to that of WT subordinate fish. Although we have no direct evidence, the instability of the social relationship between TLN fish may lead to an increase in stress levels due to the mismatch in social signals. If so, then it is expected that stress hormones can modulate the activation threshold and excitability of spinal motor circuits in a social status-dependent manner. Indeed, in goldfish, cortisol application increases M-cell excitability by increasing M-cell input resistance and the magnitude of M-cell postsynaptic potentials by reducing the strength of local feed-forward inhibition inputs (Bronson and Preuss, 2017). Moreover, exposing zebrafish to unpredictable chronic stress increases their cortisol levels and alters their swimming activity (Manuel *et al.*, 2014). Taken together, this evidence lends credence to our conclusion that proper visual displays are important in social regulation and activation of spinal motor circuits, and disruption of visual information can have profound impact on the dynamics of social interactions and nervous system function.

Status-dependent differences in the intensity of body coloration in WT animals suggest that color plasticity is an adaptive strategy and is evidence that social displays can act as a modulator for behavioral motor output. Without the ability to visually display one's social rank, TLN zebrafish are unable to successfully prioritize adaptive behaviors. In the present case, TLN subordinates continued to swim as often as their dominant counterparts, and TLN pairs displayed prolonged aggressive activity and social instability. This could be due to either a lack of stability of dominance relationships or a disruption of the neuromodulatory signaling pathways that rely on the processing of visual information between members of a social group. The interplay between visual information and neuromodulation of motor circuits is not well understood. However, there is strong evidence showing that visual information activates dopaminergic brain nuclei known to modulate spinal motor circuits such as the escape and swim circuits (Mu *et al.*, 2012). It is unlikely that only one neuromodulator predominates any one neural circuit. Rather, it is probable that excitatory and inhibitory inputs converge and prime the activation of motor circuits in a socially adaptive manner (Petrov *et al.*, 1991; McLean and Fetcho, 2004; Moly and Hatta, 2011; Bacqué-Cazenave *et al.*, 2013; Pantoja *et al.*, 2016). Investigating the social status-dependent changes in these signaling pathways would be a step forward for an improved understanding of the effects of social status on nervous system function.

Our results potentially have broader implications for many zebrafish studies in which genetically engineered lines are used. In this context, utilization of different phenotypic morphs to investigate brain function, particularly studies that examine motivated behavior, should be put in the context of the animals' early development and social environment in order to avoid confounding results. While investigating shoaling behavior of WT and nacre unpigmented zebrafish, Engeszer *et al.* (2004) demonstrated the importance of early life experience on the preference of shoaling behavior at adulthood and determined that shoaling with similar-color morph fish is not innate, but rather learned. Our results expand on their analysis and show the implications of visual displays in regulating motor behavior patterns. However, how disruption of proper visual social cues affects the activation patterns of other brain circuits involved in motivated behavior remains unknown. Therefore, we caution that the life social history should be central in the design and interpretation of results when morphologically engineered zebrafish are used to study the neural bases of behavior.

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