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Heritable Natural Variation of Light/dark Preference in an Outbred Zebrafish Population

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Abstract

Anxiety is a fear-like response to stimuli perceived to be threatening. Excessive or uncontrollable anxiety is a debilitating psychiatric disorder which affects many people throughout their lifetime. In unravelling the complex genetic and environmental regulations of anxiety-like phenotypes, models measuring the natural dark avoidance of larval zebrafish have shed light on the individual variation and heritability of this anxiety-related trait. Using the light/dark choice paradigm and selective breeding, this study aims to validate previous findings of variable (VDA) and strong dark aversion (SDA) heritability in AB-WT larval zebrafish using the outbred zebrafish strain EK, which offers more genetic diversity to aid in future molecular mapping efforts. 190 larvae (6 days post fertilization [dpf] and 7 dpf) were tested across four trials and divided into variable (VDA), medium (MDA) and strong (SDA) dark aversion for further in-crosses. VDA and MDA larvae became more explorative with time, whereas SDA larvae rarely left the preferred light zone. The SDA and VDA in-crosses significantly increased the respective phenotypes in the second generation of larvae, whereas VDA×MDA inter-crosses did not. For the second-generation SDA cohort, dark aversion correlated with increased thigmotaxis, which reinforces SDA as an anxiety-like phenotype. Our finding that the dark aversion trait and SDA and VDA phenotypes are heritable in an outbred zebrafish population lays an important foundation for future studies of genetic underpinnings using whole-genome mapping methods. This conserved fear/anxiety-like response in a highly accessible model organism also allows for further pharmacological and behavioral studies to elucidate the etiology of anxiety and the search for novel therapeutics for anxiety disorders.

Keywords

population genetics; behavioral diversity; natural variation; inheritance

2. Introduction:

Anxiety is an evolutionarily conserved stress response to transient states of uncertainty, preparing us for potential dangers (Brosschot, Verkuil & Thayer, 2016; Cryan & Sweeney,

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2011). In anxiety disorders, such as generalized anxiety disorder (GAD), panic disorder and phobias, this natural emotion is dysregulated, giving rise to chronic and excessive fear (American Psychiatric Association, 2013). The high prevalence of pathological anxiety disorders, and their debilitating consequences for affected individuals and society, justify continued efforts in explaining the signaling mechanisms governing this spectrum of behavior (Bandelow & Michaelis, 2015; Baxter, Scott, Vos & Whiteford, 2013). Preclinical animal models play an important part in these efforts, as they can provide initial knowledge regarding the efficacy of therapeutic drugs and how behavior is influenced (Magno, Fontes, Gonçalves & Gouveia, 2015).

One established model for studying anxiety-like behavior is the scototaxis or light/dark assay in zebrafish (*Danio rerio*) (Maximino, Marques de Brito, Dias, Gouveia & Morato, 2010; Steenbergen, Richardson & Champagne, 2011; Stewart et al., 2011). Zebrafish have become an advantageous choice in the biomedical field because their rapid development permits high-throughput screenings of genetic and pharmacological manipulations of embryos, larvae and adults (Bretaud et al., 2007; Gerlai, 2010; Kokel et al., 2010; Macho Sanchez-Simon & Rodriguez 2009; Parmar, Parmar & Brennan, 2010). Their transparency at embryonic and larval stages, together with cost- and space-efficient housing, further warrant the use of zebrafish over conventional rodent models (Lieschke & Currie, 2007). By measuring the time spent in an illuminated versus a dark compartment, the light/dark assay harnesses an innate dark-preference in adult zebrafish (Blaser, Chadwick & McGinnis, 2010; Maximino, de Brito, Colmanetti, et al., 2010; Serra, Medalha & Mattioli, 1999) and a light-preference in larval zebrafish (Steenbergen et al., 2011; Wagle, Nguyen, Lee, Zaitlen & Guo, 2017). These preference behaviors are suggested to be adaptations to avoid predators (Maximino et al., 2007).

The light/dark preference test has been validated as an anxiety measure by exposing zebrafish to anxiolytics, selective serotonin reuptake inhibitors (SSRIs), benzodiazepines and ethanol, all of which attenuate the fear response and increase the time spent in the non-preferred compartment (Chen, Chen, Liu, Zhang & Peng, 2015; Magno et al., 2015; Maximino, da Silva, Gouveia & Herculano, 2011; Steenbergen et al., 2011). In contrast, exposure to anxiogenic substances, like caffeine, reduce exploration and increase the time spent in the preferred compartment (Maximino et al., 2011; Steenbergen et al., 2011). The same effect has been achieved with adverse non-pharmacological stimuli such as heat, cold and UV light (Bai, Liu, Huang, Wagle, & Guo, 2016). Additional validation comes from cortisol assays following these stress responses (Bai et al., 2016; Egan et al., 2009). Given the similarities between the mammalian and zebrafish gluco-corticoid signaling pathway, there is vast potential for the light/dark preference test in studying the basic mechanisms of anxiety (Alsop & Vijayan, 2008).

Wagle et al. (2017) reported a behavioral spectrum of dark aversion in larvae from the laboratory wild-type strain AB, where both variable dark aversion (VDA) and strong dark aversion (SDA) were inherited phenotypes. Using similar methodology as Wagle et al. (2017), this study investigates the dark aversion trait and these behavioral phenotypes in a more genetically diverse outbred strain Ekkwill (EK). Using a new analysis method, we demonstrate how VDA larvae increasingly explore the dark compartment with time, whereas

the SDA larvae remain in the light compartment throughout the behavioral testing. A total of 190 larvae 6 and 7 days post fertilization (6 dpf and 7 dpf) were tested across four trials and an individual mean choice index was calculated (time in dark − time in light)/(time in dark + time in light). Individual larvae with SDA, MDA and VDA phenotypes were selected for further in- and inter-crosses to determine their heritability. Overall, this two-generational study supports the inheritance of anxiety-associated behaviors in larval zebrafish, by demonstrating its heritability in an outbred and genetically more diverse population that is better suited for future genome-wide association studies to reveal the underlying molecular and cellular basis.

3. Materials and methods:

3.1 Animals, housing and crosses:

The larval zebrafish (*Danio rerio*) tested in the experiments were obtained from a mass-cross of the EK-WT strain (n = 72, 50:50 male to female ratio), a wild-type line from Ekkwill Breeders in Florida. Upon arrival, the EK-WT fish had a three-week habituation period in the facility at the University of California, San Francisco, CA. The fish (mean length = 3.55 cm, body weight = 0.87 g) were housed in groups of 5–7 fish per tank (26.5 cm (L) × 8 cm (W) × 17 cm (H), ca 2 L volume) separated by gender. They were fed twice per day with flake food (Tropical Flakes, Aquatic Eco-Systems) and live brine shrimp (Platinum-Grade Argentemia Brine Shrimp, Argent Chemical Laboratories). The fish facility was kept at 28 °C with a 14hr/10hr light/dark cycle. The system water contained 5 g of Instant Ocean Salts (Aquatic Eco-systems) and 3 g sodium bicarbonate per 20 L of reverse-osmosis water (pH 7.6).

On day −1, prior to crossing, the fish were netted into a mating tank containing system water (66 cm (L) × 45 cm (W) × 15 cm (H), water depth = 6 cm). On day 0, embryos from the mass-cross were collected and sorted into 100 mm Petri dishes (50 embryos per dish) with blue egg water (0.12 g of $CaSO_4$, 0.2 g of Instant Ocean Salts, 30 µl of methylene blue in 1 L of H_2O) and kept in a 28 °C incubator. On day 1, 24 hours post fertilization (24 hpf) the embryos were bleached by five-minute immersions in five sterile Petri dishes in the following order: bleach solution (2 µl NaOCl (8.25%) in 50 ml of E3 medium), E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$), bleach solution, E3 medium (twice consecutively). Thereafter, the bleached embryos were transferred to a new sterile Petri dish with 10 µl proteinase (30 mg Proteinase in 1 ml E3 medium) in 50 ml E3 medium to dechorionate embryos. Unhatched embryos had to be manually dechorionated with forceps on day 2. On day 3, the hatched larvae were moved from the 28 °C incubator to a blue surface (VWR underpad Cat no. 82020–845) exposed to a 14hr/10hr light/dark cycle, allowing vision to develop.

At 5 dpf, randomly selected larvae with developed swim bladders from four separate Petri dishes were individualized into 6-well plates containing 7 ml of blue egg water. The wells were labelled in groups of eight: A1-A8, B1-B8… F1-F8 etc., thus each group was split across two 6-well plates.

3.2 Behavioral recording:

Behavioral recording of light/dark choice was carried out as described by Wagle et al. (2017). At 6 dpf, the larvae were habituated on a blue surface for 1 hour in the testing room (27.1°C). The chambers of the light/dark test apparatus (4 cm (L) × 4 cm (W) × 1.5 cm (H)) were constructed out of clear acrylic (McMaster-Carr Supply Company). Opaque black tape and opaque white tape were applied to the outside of the walls, dividing the chamber into one light (4 × 2 cm) and one dark (4 × 2 cm) compartment (Figure 1A). Groups of eight larvae were gently pipetted into individual chambers, containing 10 ml 28 °C blue egg water, and placed on a trans-illuminator (Stratagene light box). The light and dark boundary of the chamber walls was aligned with clear and opaque black (ACRYLITE IR acrylic 11460) acrylic stripes covering the trans-illuminator, thereby only allowing light to pass through the light area (Figure 1B). The setup was enclosed in a dark cabinet and white noise was provided by a fan. A two-camera (Panasonic) setup with infrared filters (ACRYLITE IR acrylic 11460), connected to a PC with Noldus MPEG recorder 2.1, was used for the recordings. Four trials with 8-minute recordings were performed at 6 dpf AM (9 AM to 11 AM), PM (1 PM to 3 PM) and 7 dpf AM, PM. After the four trials, the larvae were fed with paramecium in their individual wells during analysis. This protocol was strictly followed by the same experimenter to ensure a consistent measure of baseline behavior.

After analysis and calculation of the mean Light/Dark Choice Index (CI-LD), (see 3.3), the individual mean CI-LD values of the larvae in a set were arranged in descending order (Microsoft Office 365 ProPlus Excel). For each set, four larvae (8.3%) with the highest CI-LD values (threshold: > −0.3) were categorized as the VDA phenotype, six larvae (12.5%) with the lowest CI-LD values (threshold: −0.9 to −1) were categorized as SDA and 10 larvae (20.8%) in the centre of the spread as MDA (threshold: −0.5 to −0.8). The SDA, VDA and MDA phenotypes of each set were raised as three separate groups under the same conditions to adulthood, giving four complete replicate groups for each phenotype (a total of twelve groups with no more than six fish per tank).

All experiments were conducted in line with the National Institutes of Health's (NIH) principles for the care and use of animals in experimental procedures, as well as in accordance with IACUC regulations.

3.3 Calculations, graphs and statistics:

The behavioral recordings were analyzed by Ethovision XT 13 using the following parameters: duration in the light zone or the dark zone, duration in the inner zone or the outer zone, swim velocity, total distance traveled and latency of zone entry. The Light/Dark Choice Index (CI-LD) was calculated (Microsoft Office 365 ProPlus Excel) using the following formula:

$$\text{CI} - \text{LD} = \frac{(\text{Time in Dark zone} - \text{Time in Light zone})}{(\text{Time in Dark zone} + \text{Time in Light zone})}$$

A value of +1 indicates 100% of the time was spent in the dark zone, i.e. light aversion. Conversely, a value of −1 signifies 100% of the time in the light zone, i.e. dark aversion.

To analyze thigmotaxis in Ethovision, each area was divided into an inner and outer zone. Thigmotaxis Choice Index (CI-Th) was calculated using the following formula:

$$\text{CI} - \text{Th} = \frac{(\text{Time in Inner zone} - \text{Time in Outer zone})}{(\text{Time in Inner zone} + \text{Time in Outer zone})}$$

A value of +1 indicates 100% of the time was spent in the inner zone and −1 signifies 100% of the time in the outer zone.

Pairwise linear correlation coefficient between the two parameters CI-Th (X) and CI-LD (Y) was calculated as follows:

$$\rho(X, Y) = \frac{\text{cov}(X, Y)}{\delta_X \delta_Y}$$

Where $\rho(X, Y)$ is the pairwise correlation coefficient X and Y, $cov(X, Y)$ is the covariance of X and Y and δ_X is the standard deviation of X (Gibbons, 1985).

Graphs were plotted using GraphPad Prism 8.1. The frequency distribution curves were plotted by binning CI-LD data into 0.1 unit-intervals of larvae within that range and then applying the Gaussian distribution curve. One-way ANOVA, Two-way ANOVA, Bonferroni's, Tukey's, Sidak's and Dunnett's Multiple Comparisons Tests were applied. R^2 is the effect size measure and p-values less than 0.05 indicate significance.

4. Results:

4.1 F1 Population:

To determine the heritability of light/dark preferences in the genetically diverse EK-WT population, 192 EK-WT larvae were tested in the light/dark choice assay at 6–7 dpf in four sets of 48 larvae, as described in Materials and methods 3.2 (Figure 1). Data from two larvae were excluded due to failed tracking, leaving data from 190 larvae. The mean CI-LD of each set was: Set 1, −0.61 (SD 0.27); Set 2, −0.58 (SD 0.26); Set 3, −0.57 (SD 0.29); Set 4, −0.57 (SD 0.32) (Figure 2). The mean CI-LD across the four sets was −0.58, with CI-LD values from individual larva ranging from +0.47 to −1. Across the four trials, the larvae displayed variable light/dark preferences (Supplementary Figures 1A-D). Sets 1, 2 and 3 showed a significant difference between trials, whereas the CI-LD of individual larvae in Set 4 was more consistent over the four trials (Supplementary Figures 1E-H). As the distribution curves of the four sets all peak between the negative values −0.63 and −0.78, dark aversion is the most prevalent trait among the tested larvae (Figure 5B, Supplementary Figures 1I-L).

The velocity and the total distance traveled of each larva during the behavioral recordings were also compared (Supplementary Figure 2). The mean velocity and mean total distance traveled were significantly different for Set 2 and Set 3 (Supplementary Figure 2).

4.2 Selected SDA-F1, VDA-F1 and MDA-F1 larvae:

For an equal representation from each of the four sets, 8.3% of larvae were categorized as VDA (CI-LD values > −0.3), 12.5% were categorized as SDA (CI-LD values −0.9 to −1) and 20.8% as MDA (CI-LD values −0.5 to −0.8), within each set. This gave a total of 16 VDA larvae, 24 SDA larvae and 42 MDA larvae selected and raised to adulthood. To measure heritability, the following in- and inter-crosses were performed to produce an F2 population to be tested using the same light/dark assay: SDA×SDA, VDA×VDA and VDA×MDA. The mean CI-LD of each of the F1 phenotype cohorts was: VDA-F1, −0.0003 (SD 0.20, range: +0.471 to −0.252); MDA-F1, −0.634 (SD 0.06, range: −0.506 to −0.780); SDA-F1: −0.956 (SD 0.03, range: −0.912 to −0.998). The SDA phenotype showed little inter-trial variability whereas the VDA phenotype displayed high variability across trials (Figures 3A-B). The time-binned data in Figure 3C illustrates how the mean CI-LD of SDA-F1 larvae remained close to −1 for the duration of the 8-min recording. In contrast, the mean CI-LD of VDA-F1 larvae increased with time and shifted from negative to positive CI-LD values at the four-minute halfway point. The MDA-F1 larvae also showed a gradual increase in mean CI-LD with time, although not to the same extent as the VDA-F1 larvae.

4.3 SDA-F2, VDA-F2 and VDA×MDA-F2 larvae:

The population of F2 larvae was generated through SDA in-crosses, VDA in-crosses and VDA×MDA inter-crosses. Three fish pairs per phenotypic group (SDA, VDA, VDA×MDA), aged 4–5 months, were randomly selected across different F1 sets and crossed pair-wise in smaller tanks (22.5 cm (L) × 11.5 cm (W) × 11 cm (H), water depth = 3 cm)). To maximize survival and prevent pseudoreplication effects, the embryos from crosses with the highest yield out of the three were selected and raised in the same manner as the parental F1 population (Materials and methods 3.1). This set-up was followed for all F2 sets.

The light/dark choice assay was repeated in two sets of 48 larvae for each of the three groups, as described in the Materials and methods 3.2 section. To avoid potential experimenter bias through handling differences, two sets from different phenotypic groups were completed at the same time on the same day. Nine larvae were excluded due to incomplete tracking. There was no significant difference between trials within the sets, except for VDA-F2 Set 1 and VDA×MDA-F2 Set 2 (Supplementary Figures 3I, L). The mean CI-LD of each SDA-F2 set was: Set 1, −0.69 (SD 0.29); Set 2, −0.73 (SD 0.19) (Figure 4A). The overall mean CI-LD of SDA-F2 larvae was −0.71 (SD 0.24, range: +0.007 to −1, n = 96) (Figure 5A). The leftward shift of the distribution curve in Figure 5B, indicates an increase in the SDA phenotype following SDA in-crosses. For each set of VDA-F2 larvae, the mean CI-LD was: Set 1, −0.44 (SD 0.26); Set 2, −0.35 (SD 0.25) (Figure 4A). The overall mean CI-LD of VDA-F2 larvae was −0.40 (SD 0.26, range: +0.35 to −0.93, n = 94) (Figure 5A). In contrast to the SDA-F2 population, the VDA-F2 larvae had a larger inter-trial variation and a rightward shift in the distribution curve compared to the parental F1 population (Figure 5B, Supplementary Figure 3). As with the SDA in-crosses, the VDA in-crosses resulted in a higher prevalence of the VDA phenotype than in the F1 population. The mean CI-LD of the VDA×MDA-F2 sets were: Set 1, −0.61 (SD 0.26); Set 2, −0.59 (SD 0.25) (Figure 4A). The overall mean CI-LD of VDA×MDA-F2 larvae was −0.60 (SD 0.25, range: +0.19 to −0.99, n = 89) (Figure 5A). The VDA×MDA inter-crosses did not

significantly alter the CI-LD (Figure 5A) and exhibited a similar distribution trend to the parental F1 cohort (Figure 5B).

In order to evaluate CI-LD trends across time, data from 10% of the SDA-F2 larvae with the lowest CI-LD values (hereafter denoted SDA-F2^{SDA}) and 10% of the VDA-F2 larvae (VDA-F2^{VDA}) and VDA×MDA-F2 (VDA×MDA-F2^{VDA}) with the highest CI-LD values was time-binned in 30-sec intervals (Figure 4B). The results showed a significant difference between the selected SDA-F2^{SDA} and both the VDA-F2^{VDA} and VDA×MDA-F2^{VDA} larvae (Figure 4B). The constant dark avoidance behavior of the selected SDA-F2^{SDA} larvae (Figure 4B) throughout the recordings was significantly stronger than in the parental SDA-F1 (Figure 3C) (R^2 = 0.816, F (112, 495) = 488.5, ****p<0.0001, Two-way ANOVA; SDA-F2^{SDA} vs. SDA-F1, mean diff. = −0.043, ****p<0.0001, Bonferroni's multiple comparisons test). When the VDA-F2^{VDA} and VDA×MDA-F2^{VDA} larvae (Figure 4B) were compared to the parental VDA-F1 group (Figure 3C), there was no significant difference in CI-LD behavior across time (R^2 = 0.816, F (112, 495) = 488.5, ****p<0.0001, Two-way ANOVA; VDA-F2^{VDA} vs. VDA-F1, mean diff. = 0.050, ns p>0.9999; VDA×MDA-F2^{VDA} vs. VDA-F1, mean diff. = 0.120, ns p = 0.2131, Two-way ANOVA, Bonferroni's multiple comparisons test).

4.4 Heritability:

To further examine the heritability of the dark avoidance trait, the mean CI-LD values of the parental F1, SDA-F2, VDA-F2 and the VDA×MDA-F2 populations were plotted and found to be statistically significantly different (R^2 = 0.1251, F(3, 465) = 22.17, ****p<0.0001, One-way ANOVA) (Figure 5A). The p-value for the parental F1 vs. SDA-F2 was ***p = 0.0007 (Dunnett's multiple comparisons test). For the parental F1 vs. VDA-F2, the p-value was ****p<0.0001 (Dunnett's multiple comparisons test). The mean CI-LD of the VDA×MDA-F2 group was not significantly different from the parental F1 group (ns p = 0.9024, Dunnett's multiple comparisons test). To calculate how heritable the SDA and VDA phenotypes are respectively, the breeder's equation was used:

$$Heritability\, h^2 = \frac{R}{S}$$

Where R = Mean (F2) – Mean parental population (F1), and S = Mean of Specific F1 parents – Mean parental population (F1). Response to Selection (R) is defined as the realized average difference between the parent generation and the next generation. The Selection Differential (S) is defined as the average difference between the parent generation and the selected parents (Falconer & Mackay, 1998).

The mean CI-LD of SDA-F2 was −0.71, giving R = −0.125. The specific SDA-F1 parents had a mean CI-LD of −0.96, giving S = −0.377. Therefore, heritability h^2 for the SDA phenotype was 0.33. For the VDA-F2 group, the mean CI-LD was −0.40, resulting in R = 0.187. The specific VDA-F1 parents had a mean CI-LD of −0.14, resulting in S = 0.446. Heritability h^2 for the VDA phenotype was 0.42. The mean CI-LD of VDA×MDA-F2 larvae was −0.60, therefore R = −0.020. The mean CI-LD of the VDA×MDA-F2 parents was −0.30 and so S = 0.279. These values give a negative h^2 score of −0.07 for the VDA phenotype

following VDA×MDA inter-crosses. Based on the calculated h^2 scores, both the SDA and VDA phenotypes from the F1 in-crosses were inherited by the F2 populations. The VDA phenotype was inherited to a higher degree than the SDA phenotype. Interestingly, the VDA×MDA inter-crosses did not result in a higher prevalence of the VDA phenotype in the F2 offspring.

4.5 Thigmotaxis:

Thigmotaxis, the preference of a chamber's edges over its center, is an established anxiety measure for larval zebrafish (Best & Vijayan, 2018; Colwill & Creton, 2011; Schnörr, Steenbergen, Richardson & Champagne, 2012). Considering the significant difference between thigmotaxis behavior in SDA and VDA larvae observed in Wagle et al. (2017), we proceeded to analyze this parameter using a choice index rather than a percentage. Using Ethovision, inner and outer (10 mm from the edge) zones were applied to the testing chambers. A Thigmotaxis Choice Index (CI-Th) was calculated using the following equation:

$$\text{CI} - \text{Th} = \frac{(\text{Time in Inner zone} - \text{Time in Outer zone})}{(\text{Time in Inner zone} + \text{Time in Outer zone})}$$

Overall, the SDA-F2 group demonstrated a significantly lower mean CI-Th than the VDA-F2 and VDA×MDA-F2 groups (Figures 6A-C, G). Additionally, a positive correlation (ρ = 0.2766) between CI-LD and CI-Th for the SDA-F2 larvae was revealed by a fitting linear regression model and calculating the pairwise linear correlation coefficient (Figure 6D). The stronger the dark avoidance, the closer the larvae swim near the chamber's edges. The line of regression for the VDA-F2 group ($\rho = -0.1661$) suggested a very weak negative correlation (Figure 6E), whereas no correlation was found between CI-LD and CI-Th in VDA×MDA-F2 larvae ($\rho = -0.0750$) (Figure 6F). Finally, the VDA-F2 and VDA×MDA-F2 larvae had a significantly shorter latency of entry into the dark zone than both the parental F1 and the SDA-F2 groups (Figure 6H). On the other hand, the SDA-F2 larvae were significantly slower in entering the dark zone of the chamber than the parental F1, VDA-F2 and VDA×MDA-F2 groups. The increased thigmotaxis in combination with the heightened hesitancy to enter the dark chamber area supports SDA as an inherited anxiety-like phenotype.

5. Discussion:

Using the light/dark paradigm to assess anxiety-like behavior in EK-WT larvae, this study extends previous findings of inherited individual variations in dark avoidance in the AB-WT strain (Wagle et al., 2017) to a more genetically diverse outbred population. The uncovered behavioral spectrum with constant dark aversion at one end, increasing exploration in the middle and indifference at the other end (Figure 3C) is intriguingly similar to the observations of human anxiety-like behavior. A baseline hesitance to approach the unknown is a vital survival mechanism, but with continued exposure to a new environment or situation our apprehension usually tends to diminish (Cryan & Sweeney, 2011). However, as mentioned in the introduction, a persistent fear response can develop into chronic states of anxiety (Brosschot et al., 2016).

The current EK-WT F1 population had a stronger baseline dark avoidance than the laboratory-bred AB strain (Wagle et al., 2017). Consistent with our finding, a previous study of fear and stress-related responses also suggests that AB zebrafish exhibit significantly lower anxiety-like behaviors than wild-derived zebrafish in novel tank and light/dark assays (Wong et al., 2012). A possible explanation for the weaker fear/anxiety-like responses in lab-raised strains is the influence of domestication (Drew et al., 2012). From an evolutionary perspective, domestication involves phenotypic changes based on adaptations to more controlled living conditions (Drew et al., 2012; Larson & Fuller, 2014). In comparison to wild-derived zebrafish, domesticated zebrafish that have been lab-reared for numerous generations tend to feed more frequently and behave more boldly (Drew et al., 2012; Oswald & Robinson, 2008). Consequently, the heightened fear response in zebrafish from the wild makes them more suited for predator avoidance and survival in their natural habitats. Alternatively, the divergence in dark avoidance between AB and EK strains could also be a result of environmental variables during behavioral testing, as the two sets of data were not obtained in side-by-side experiments.

The dark avoidance trait was found to be heritable (Figure 5A). The SDA heritability score of 0.33 after selective breeding in EK was remarkably similar to the 0.39 calculated for SDA inheritance in AB fish (Wagle et al., 2017). A substantially lower heritability was observed for the VDA phenotype in the EK compared to the AB strain (Wagle et al., 2017). The current VDA in-crosses gave a heritability score of 0.42, which is less than half of the 0.89 score for VDA×MDA inter-crosses and VDA×AB-WT back-crosses from Wagle et al. (2017). Furthermore, the current VDA×MDA inter-crosses resulted in a negative heritability score of −0.07 due to the VDA×MDA-F2 larvae having a slightly lower mean CI-LD than the F1 population (ns, Figure 5A). Although no significant difference in CI-LD was detected between the parental F1 and the VDA×MDA-F2, this may be due, at least in part, to lower power to detect a difference. For instance, assuming a h^2 of 0.3 with the sample size used, our power to detect a significant difference at 0.05, adjusted for multiple comparisons, was about 0.92 for the VDA in-cross, about 0.81 for the SDA in-cross and about 0.48 for the VDA×MDA inter-cross.

The positive correlation between CI-LD and CI-Th in SDA-F2 larvae (Figure 6D) combined with their increased latency of entering the dark zone (Figure 6H), strengthens the concept of SDA as having an aggravated anxiety-like characteristic. Curiously, the VDA×MDA-F2 larvae displayed a significant reduction in latency of dark zone entry, even though their mean CI-LD did not significantly differ from the parental F1 larvae (Figure 6H). Moreover, the time-binned data extracted prominent differences between the phenotypic groups (Figure 3C). The SDA-F1 larvae consistently remained in the light zone throughout testing which reflected a stronger innate preference for safe environments than a preference to explore novel environments (Maximino, de Brito, Colmanetti, et al., 2010). This behavior was significantly intensified in the SDA-F2^{SDA} offspring (Figure 4B). MDA-F1 and VDA-F1 larvae initially spent more time in the light zone but gradually increased their visits to the dark zone and thereby appeared to become more explorative with time. Although both the VDA-F2 and VDA×MDA-F2 larvae had negative CI-LD values (Figure 5A), representing a general dark avoidance, the time-binned trends of the VDA-F2^{VDA} and VDA×MDA-F2^{VDA} larvae gave the impression of a more neutral approach to either zone (Figure 4B).

To develop suitable treatments for anxiety disorders, researchers are highly reliant on animal models like zebrafish. Approximately 70% of human genes have zebrafish orthologues (Howe et al., 2013). The zebrafish brain shows considerable homology to mammalian brains, with typical structures including the telencephalon, diencephalon (thalamus, hypothalamus), midbrain, hindbrain, and spinal cord. All major neurotransmitter and neuropeptide systems are found in the larval zebrafish brain (Rinkwitz, Mourrain & Becker, 2011). In the study of motivated behaviour, it is particularly relevant that homologues to the mammalian striatum (Rink & Wullimann, 2001, 2002) and amygdala (Lau, Mathur, Gould & Guo, 2011; Wullimann & Mueller, 2004) have been identified in zebrafish. Anatomical resemblance of zebrafish dorsal pallium to mammalian isocortex have also been uncovered (Mueller, Dong, Berberoglu & Guo, 2011).

Heritable behavioral differences in an outbred zebrafish strain, as demonstrated by this study, can be an ideal candidate for mapping out the genetics underlying natural variations of behavior using genome-wide association studies (GWAS), as they provide a sufficient degree of genetic reshuffling (Parker et al., 2016). By localizing quantitative trait loci (QTL) associated with the phenotypes of interest, GWAS can narrow down regulatory and coding genes implicated in specific behaviors or disease predispositions (Bendesky & Bargmann, 2011; Manolio, Brooks & Collins, 2008; Parker et al., 2016). Using this method, differences in *Drosophila* courtship songs have been attributed to variations of the slowpoke (slo) locus, whereas foraging behavior in honey bees is regulated by the pollen-related QTLs pln1, pln2, pln3, and Amfor (Ding, Berrocal, Morita, Longden & Stern, 2016; Rüppell, Pankiw & Page, 2004). The hippocampal gene *Zmynd11* has been proposed as a regulator of anxiety behavior in mice (Parker et al., 2016). Several human GWAS on anxiety disorders have put forward potential susceptibility markers, but these are often limited by underpowered sample sizes and require further replication (Dunn et al., 2018; Otowa et al., 2016; Strawbridge et al., 2018). Pinpointing the genes involved in SDA and VDA inheritance using GWAS may help in understanding genetic vulnerability to developing anxiety. For this, further research using genome sequencing and statistical genetics is needed.

Much can be gained within anxiety research by selecting zebrafish from the wild and utilizing their naturally preserved sensitivity towards aversive stimuli. Selective breeding of SDA fish as a model of anxiety disorders allows a more focused approach in testing the efficacy of anxiolytics, rather than testing them on whole populations. An interesting future direction is to determine the influence of time on CI-LD. Given enough time to habituate perhaps the SDA fish may venture into dark zone of the chamber. Conversely, additional aversive stimuli such as electric shock may precipitate even stronger pathological anxiety/depression-like phenotypes in SDA as opposed to VDA.

6. Conclusion:

The outbred population of EK larval zebrafish have individual variations of strong (SDA), medium (MDA) and variable (VDA) dark avoidance, where SDA and VDA phenotypes are heritable following in-crosses. The MDA and VDA larvae engage in increasing exploration of the dark area, whereas the SDA larvae remain in the light area. In contrast to AB larval zebrafish, the EK fish have a stronger baseline dark aversion and a lower inheritance of the

SDA and VDA phenotypes. This reflects the conservation of the natural anxiety-like behavioral variation and more varied genetic background of the EK fish. Future use of VDA and SDA strains for molecular genetic, behavioral, and pharmacological studies can provide new insights into the understanding of anxiety-like behaviors and treatment of anxiety disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclosure of interest:

The authors report no conflict of interest. NIH grant number is R01 DA035680.

Biography

Amelia Dahlén is a MSci Neuroscience with Psychology student at the University of Aberdeen, Scotland. During her year at the Guo Lab at the University of California in San Francisco, UCSF, she is working with projects on anxiety- and addiction-like behaviors. Amelia has previous research experience from the Functional Pharmacology group at the Department of Neuroscience, Uppsala University in Sweden.

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Dr. Mahdi Zarei has a background in Applied Mathematics and Software Engineering. In 2015 he obtained his Ph.D. in Information Technology at the Federation University Australia and developed new mathematical models and machine learning algorithms for large-scale brain data analysis. He has continued his research as a computational neuroscientist focusing on the brain's functional connectivity in the Guo Lab at UCSF.

Dr. Su Guo is the Principle Investigator of the Guo Lab at the University of California in San Francisco, where the research focus spans from the molecular genetics of neural development to the understanding of behavior. Dr. Guo has a broad background in molecular biology, genetics, developmental biology and neurobiology as a graduate student at Cornell University and as a postdoctoral fellow at Harvard Medical School and Genentech Inc.

References:

Alsop D, & Vijayan M (2008). Development of the corticosteroid stress axis and receptor expression in zebrafish. American Journal Of Physiology-Regulatory, Integrative And Comparative Physiology, 294(3), R711–R719. doi: 10.1152/ajpregu.00671.2007

American Psychiatric Association, DSM-5 Task Force. (2013). Diagnostic and statistical manual of mental disorders: DSM-5™ (5th ed.). Arlington, VA, US: American Psychiatric Publishing, Inc. 10.1176/appi.books.9780890425596

Bai Y, Liu H, Huang B, Wagle M, & Guo S (2016). Identification of environmental stressors and validation of light preference as a measure of anxiety in larval zebrafish. BMC *Neuroscience*, 17(1). doi: 10.1186/s12868-016-0298-z

Bandelow B, & Michaelis S (2015). Epidemiology of anxiety disorders in the 21st century. Dialogues in clinical neuroscience, 17(3), 327–335. [PubMed: 26487813]

Baxter A, Scott K, Vos T, & Whiteford H (2013). Global prevalence of anxiety disorders: a systematic review and meta-regression. Psychological Medicine, 43(5), 897–910. doi: 10.1017/s003329171200147x [PubMed: 22781489]

Bendesky A, & Bargmann C (2011). Genetic contributions to behavioural diversity at the gene–environment interface. Nature Reviews Genetics, 12(12), 809–820. doi: 10.1038/nrg3065

Best C, & Vijayan M (2018). Cortisol elevation post-hatch affects behavioural performance in zebrafish larvae. General And Comparative Endocrinology, 257, 220–226. doi: 10.1016/j.ygcen.2017.07.009 [PubMed: 28713045]

Blaser R, Chadwick L, & McGinnis G (2010). Behavioral measures of anxiety in zebrafish (Danio rerio). Behavioural Brain Research, 208(1), 56–62. doi: 10.1016/j.bbr.2009.11.009 [PubMed: 19896505]

Bretaud S, Li Q, Lockwood B, Kobayashi K, Lin E, & Guo S (2007). A choice behavior for morphine reveals experience-dependent drug preference and underlying neural substrates in developing larval zebrafish. Neuroscience, 146(3), 1109–1116. doi: 10.1016/j.neuroscience.2006.12.073 [PubMed: 17428610]

Brosschot J, Verkuil B, & Thayer J (2016). The default response to uncertainty and the importance of perceived safety in anxiety and stress: An evolution-theoretical perspective. Journal Of Anxiety Disorders, 41, 22–34. doi: 10.1016/j.janxdis.2016.04.012 [PubMed: 27259803]

Chen F, Chen S, Liu S, Zhang C, & Peng G (2015). Effects of lorazepam and WAY-200070 in larval zebrafish light/dark choice test. Neuropharmacology, 95, 226–233. doi: 10.1016/j.neuropharm.2015.03.022 [PubMed: 25842247]

Colwill R, & Creton R (2011). Imaging escape and avoidance behavior in zebrafish larvae. Reviews In The Neurosciences, 22(1). doi: 10.1515/rns.2011.008

Cryan J, & Sweeney F (2011). The age of anxiety: role of animal models of anxiolytic action in drug discovery. British Journal Of Pharmacology, 164(4), 1129–1161. doi: 10.1111/j.1476-5381.2011.01362.x [PubMed: 21545412]

Ding Y, Berrocal A, Morita T, Longden K, & Stern D (2016). Natural courtship song variation caused by an intronic retroelement in an ion channel gene. Nature, 536(7616), 329–332. doi: 10.1038/nature19093 [PubMed: 27509856]

Drew R, Settles M, Churchill E, Williams S, Balli S, & Robison B (2012). Brain transcriptome variation among behaviorally distinct strains of zebrafish (Danio rerio). BMC Genomics, 13(1), 323. doi: 10.1186/1471-2164-13-323 [PubMed: 22817472]

Dunn E, Sofer T, Gallo L, Gogarten S, Kerr K, & Chen C et al. (2016). Genome-wide association study of generalized anxiety symptoms in the Hispanic Community Health Study/Study of Latinos. American Journal Of Medical Genetics Part B: Neuropsychiatric Genetics, 174(2), 132–143. doi: 10.1002/ajmg.b.32448

Egan R, Bergner C, Hart P, Cachat J, Canavello P, & Elegante M et al. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behavioural Brain Research, 205(1), 38–44. doi: 10.1016/j.bbr.2009.06.022 [PubMed: 19540270]

Falconer DS & Mackay TF (1998). Introduction to quantitative genetics (4th ed.). Essex: Longman. ISBN 978–0-582–24302-6.

Gerlai R (2010). High-Throughput Behavioral Screens: the First Step towards Finding Genes Involved in Vertebrate Brain Function Using Zebrafish. Molecules, 15(4), 2609–2622. doi: 10.3390/molecules15042609 [PubMed: 20428068]

Gibbons J (1985). Nonparametric statistical inference. New York: M. Dekker.

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, ... & McLaren S (2013). The zebrafish reference genome sequence and its relationship to the human genome. Nature, 496(7446), 498. doi: 10.1038/nature12111 [PubMed: 23594743]

Kokel D, Bryan J, Laggner C, White R, Cheung C, & Mateus R et al. (2010). Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nature *Chemical Biology*, 6(3), 231–237. doi: 10.1038/nchembio.307 [PubMed: 20081854]

Larson G, & Fuller D (2014). The Evolution of Animal Domestication. Annual Review Of Ecology, Evolution, And Systematics, 45(1), 115–136. doi: 10.1146/annurev-ecolsys-110512-135813

Lau B, Mathur P, Gould G, & Guo S (2011). Identification of a brain center whose activity discriminates a choice behavior in zebrafish. Proceedings Of The National Academy Of Sciences, 108(6), 2581–2586. doi: 10.1073/pnas.1018275108

Lieschke G, & Currie P (2007). Animal models of human disease: zebrafish swim into view. Nature Reviews Genetics, 8(5), 353–367. doi: 10.1038/nrg2091

Macho Sanchez-Simon F, & Rodriguez R (2009). Expression of the nociceptin receptor during zebrafish development: Influence of morphine and nociceptin. International Journal Of Developmental Neuroscience, 27(4), 315–320. doi: 10.1016/j.ijdevneu.2009.03.008 [PubMed: 19460625]

Magno L, Fontes A, Gonçalves B, & Gouveia A (2015). Pharmacological study of the light/dark preference test in zebrafish (Danio rerio): Waterborne administration. Pharmacology Biochemistry And Behavior, 135, 169–176. doi: 10.1016/j.pbb.2015.05.014

Manolio T, Brooks L, & Collins F (2008). A HapMap harvest of insights into the genetics of common disease. Journal Of Clinical Investigation, 118(5), 1590–1605. doi: 10.1172/jci34772 [PubMed: 18451988]

Maximino C, de Brito T, Colmanetti R, Pontes A, de Castro H, & de Lacerda R et al. (2010). Parametric analyses of anxiety in zebrafish scototaxis. Behavioural Brain Research, 210(1), 1–7. doi: 10.1016/j.bbr.2010.01.031 [PubMed: 20117146]

Maximino C, de Brito TM, de Moraes FD, de Oliveira FVC, Taccolini IB, Pereira PM, . . . Gouveia A Jr. (2007). A comparative analysis of the preference for dark environments in five teleosts. International Journal of Comparative Psychology, 20(4), 351–367. uid: 2009–18155-003

Maximino C, da Silva A, Gouveia A, & Herculano A (2011). Pharmacological analysis of zebrafish (Danio rerio) scototaxis. Progress In Neuro-Psychopharmacology And Biological Psychiatry, 35(2), 624–631. doi: 10.1016/j.pnpbp.2011.01.006 [PubMed: 21237231]

Maximino C, Marques de Brito T, Dias C, Gouveia A, & Morato S (2010). Scototaxis as anxiety-like behavior in fish. Nature Protocols, 5(2), 209–216. doi: 10.1038/nprot.2009.225 [PubMed: 20134420]

Mueller T, Dong Z, Berberoglu M, & Guo S (2011). The dorsal pallium in zebrafish, Danio rerio (Cyprinidae, Teleostei). Brain Research, 1381, 95–105. doi: 10.1016/j.brainres.2010.12.089 [PubMed: 21219890]

Oswald M, & Robison B (2008). Strain-specific alteration of zebrafish feeding behavior in response to aversive stimuli. Canadian Journal Of Zoology, 86(10), 1085–1094. doi: 10.1139/z08-085 [PubMed: 21379405]

Otowa T, Hek K, Lee M, Byrne EM, Mirza SS, Nivard MG, ... & Fanous A (2016). Meta-analysis of genome-wide association studies of anxiety disorders. Molecular psychiatry, 21(10), 1391 10.1038/mp.2015.197 [PubMed: 26754954]

Parker C, Gopalakrishnan S, Carbonetto P, Gonzales N, Leung E, & Park Y et al. (2016). Genome-wide association study of behavioral, physiological and gene expression traits in outbred CFW mice. Nature Genetics, 48(8), 919–926. doi: 10.1038/ng.3609 [PubMed: 27376237]

Parmar A, Parmar M, & Brennan CH (2011) Zebrafish Conditioned Place Preference Models of Drug Reinforcement and Relapse to Drug Seeking In: Kalueff A, Cachat J (eds) Zebrafish

Neurobehavioral Protocols. Neuromethods, vol 51 Humana Press. doi: 10.1007/978-1-60761-953-6_6

Rink E, & Wullimann M (2001). The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). Brain Research, 889(1–2), 316–330. doi: 10.1016/s0006-8993(00)03174-7 [PubMed: 11166725]

Rink E, & Wullimann M (2002). Connections of the ventral telencephalon and tyrosine hydroxylase distribution in the zebrafish brain (Danio rerio) lead to identification of an ascending dopaminergic system in a teleost. Brain Research Bulletin, 57(3–4), 385–387. doi: 10.1016/s0361-9230(01)00696-7 [PubMed: 11922994]

Rinkwitz S, Mourrain P, & Becker T (2011). Zebrafish: An integrative system for neurogenomics and neurosciences. Progress In Neurobiology, 93(2), 231–243. doi: 10.1016/j.pneurobio.2010.11.003 [PubMed: 21130139]

Rüppell O, Pankiw T, & Page R (2004). Pleiotropy, Epistasis and New QTL: The Genetic Architecture of Honey Bee Foraging Behavior. Journal Of Heredity, 95(6), 481–491. doi: 10.1093/jhered/esh072 [PubMed: 15475393]

Schnörr S, Steenbergen P, Richardson M, & Champagne D (2012). Measuring thigmotaxis in larval zebrafish. Behavioural Brain Research, 228(2), 367–374. doi: 10.1016/j.bbr.2011.12.016 [PubMed: 22197677]

Serra E, Medalha C, & Mattioli R (1999). Natural preference of zebrafish (Danio rerio) for a dark environment. Brazilian Journal Of Medical And Biological Research, 32(12), 1551–1553. doi: 10.1590/s0100-879×1999001200016 [PubMed: 10585639]

Steenbergen P, Richardson M, & Champagne D (2011). Patterns of avoidance behaviours in the light/dark preference test in young juvenile zebrafish: A pharmacological study. Behavioural Brain Research, 222(1), 15–25. doi: 10.1016/j.bbr.2011.03.025 [PubMed: 21421013]

Stewart A et al. (2011) Neurophenotyping of Adult Zebrafish Using the Light/Dark Box Paradigm In: Kalueff A, Cachat J (eds) Zebrafish Neurobehavioral Protocols. Neuromethods, vol 51 Humana Press. doi:10.1007/978-1-60761-953-6_13

Strawbridge R, Ward J, Cullen B, Tunbridge E, Hartz S, & Bierut L et al. (2018). Genome-wide analysis of self-reported risk-taking behaviour and cross-disorder genetic correlations in the UK Biobank cohort. Translational Psychiatry, 8(1). doi: 10.1038/s41398-017-0079-1

Wagle M, Nguyen J, Lee S, Zaitlen N, & Guo S (2017). Heritable natural variation of an anxiety-like behavior in larval zebrafish. Journal Of Neurogenetics, 31(3), 138–148. doi: 10.1080/01677063.2017.1343827 [PubMed: 28678579]

Wong R, Perrin F, Oxendine S, Kezios ZD, Sawyer S, Zhou L, Dereje S, & Godwin J (2012). Comparing behavioral responses across multiple assays of stress and anxiety in zebrafish (Danio rerio). Behaviour. 149(10–12). 1205–1240. 10.2307/41720611.

Wullimann M, & Mueller T (2004). Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. The Journal Of Comparative Neurology, 475(2), 143–162. doi: 10.1002/cne.20183 [PubMed: 15211457]

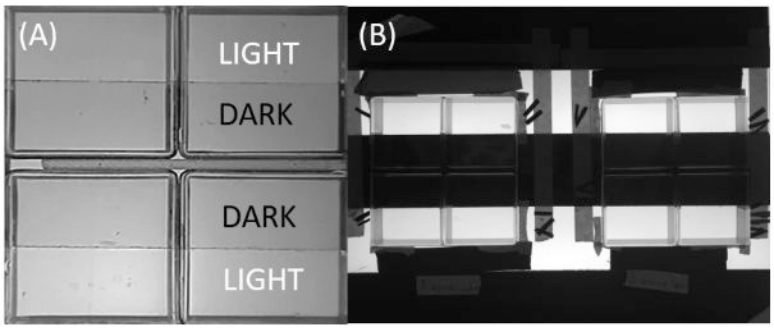

Figure 1.

(A) A snapshot with IR filters of the testing chambers to measure light/dark preference behavior in larval zebrafish. Each behavioral chamber is 4 × 4 cm, divided into 4 × 2 cm light and dark zones. (B) A snapshot of the behavioral setup with the testing chambers positioned on the trans-illuminator.

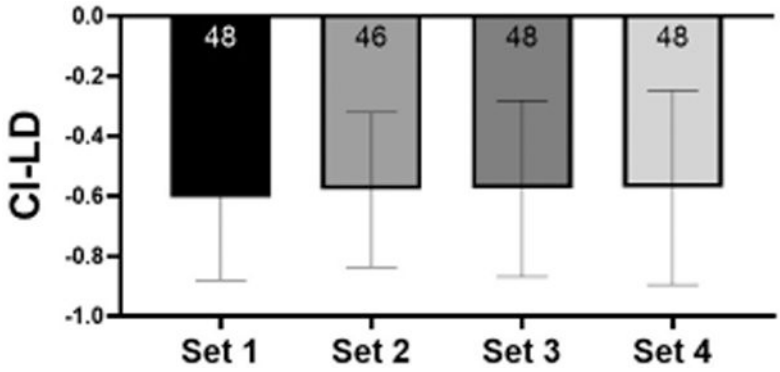

Figure 2.

Mean Light/Dark Choice Index (CI-LD) of four F1 population sets ±SD, with annotation of the n of the sets (R^2 = 0.0022, F(3, 186) = 0.1396, ns p = 0.9362, One-way ANOVA, Bonferroni's multiple comparisons test).

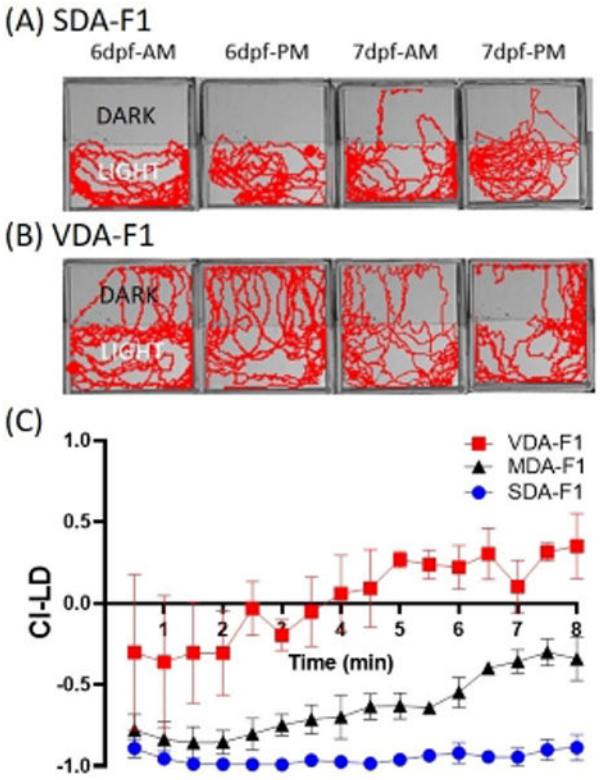

Figure 3.

Representative movement tracks of SDA-F1 (A) and VDA-F1 (B) larvae during 8 min behavioral recordings. (C) Time-binned changes in mean CI-LD across 8 min behavioral recordings for VDA-F1, MDA-F1 and SDA-F1 cohorts ±SD (R^2, = 0.9466, F (56, 135) = 1008.5, ****p<0.0001, Two-way ANOVA; SDA-F1 vs. VDA-F1, mean diff. = −0.976, ****p<0.0001; SDA-F1 vs. MDA-F1, mean diff. = −0.317, ****p<0.0001; VDA-F1 vs. MDA-F1, mean diff. = 0.659, ****p<0.0001, Tukey's multiple comparisons test).

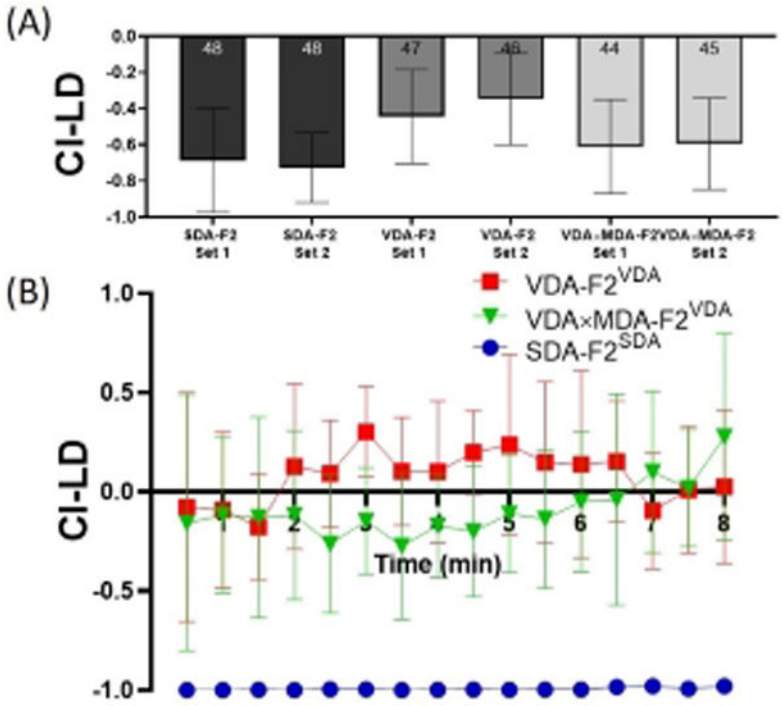

Figure 4.

(A) Comparison of the mean Light/Dark Choice Index (CI-LD) of four trials for each F2 population set ±SD, with annotations of the n of the sets (R^2 = 0.2227, F(5, 272) = 15.58, ****p<0.0001, One-way ANOVA; SDA-F2 Set 1 vs. SDA-F2 Set 2, mean diff. = 0.0411, ns p = 0.8104; VDA-F2 Set 1 vs. VDA-F2 Set 2, mean diff. = −0.0969, ns p = 0.1835; VDA×MDA-F2 Set 1 vs. VDA×MDA-F2 Set 2, mean diff. = −0.0163, ns p = 0.9864, Sidak's multiple comparisons test). (B) Time-binned changes in mean CI-LD across 8 min behavioral recordings for selected SDA-$F2^{SDA}$, VDA-$F2^{VDA}$ and VDA×MDA-$F2^{VDA}$ cohorts ±SD (R^2 = 0.816, F (112, 495) = 488.5, ****p<0.0001, Two-way ANOVA; SDA-$F2^{SDA}$ vs. VDA-$F2^{VDA}$, mean diff. = −1.069, ****p<0.0001; SDA-$F2^{SDA}$ vs. VDA×MDA-$F2^{VDA}$, mean diff. = −0.8991, ****p<0.0001; VDA-$F2^{VDA}$ vs. VDA×MDA-$F2^{VDA}$, mean diff. = 0.1697, **p = 0.0012, Bonferroni's multiple comparisons test).

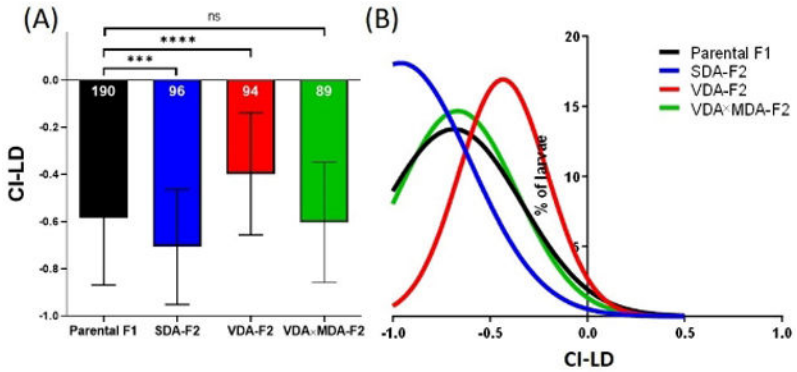

Figure 5.

(A) Mean Light/Dark Choice Index (CI-LD) comparison of parental F1 (black), SDA-F2 (blue), VDA-F2 (red) and VDA×MDA-F2 (green) populations ±SD (R^2 = 0.1251, F(3, 465) = 22.17, ****p <0.0001, One-way ANOVA; Parental F1 vs. SDA-F2, mean diff. = 0.1245, ***p = 0.0007; Parental F1 vs. VDA-F2, mean diff. = −0.1855, ****p<0.0001; Parental F1 vs. VDA×MDA-F2, mean diff. = 0.0201, ns p = 0.9024, Dunnett's multiple comparisons test). (B) Comparison frequency distribution curves of parental F1 (black), SDA-F2 (blue), VDA-F2 (red) and VDA×MDA-F2 (green) populations.

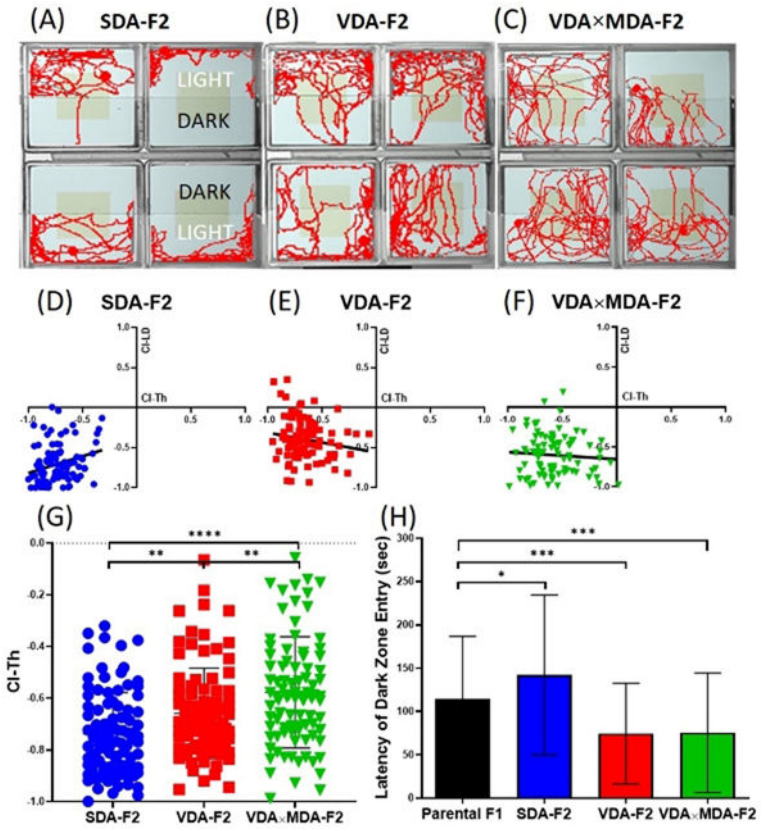

Figure 6.

Representative movement tracks of SDA-F2 larvae (A), VDA-F2 larvae (B) and VDA×MDA-F2 larvae (C) analyzed with thigmotaxis zones. Correlation between Light/Dark Choice Index (CI-LD) and Thigmotaxis Choice Index (CI-Th) in scattered plots for (D) SDA-F2 ($\rho = 0.2766$), (E) VDA-F2 larvae ($\rho = -0.1661$) and (F) VDA×MDA-F2 larvae ($\rho = -0.0750$), with lines of linear regression. (G) Scatter plot comparing individual mean CI-Th of SDA-F2, VDA-F2 and VDA×MDA-F2 larvae ($R^2 = 0.1144$, $F(2, 276) = 17.82$, ****$p<0.0001$, One-way ANOVA; SDA-F2 vs. VDA-F2, mean diff. = −0.0798, **$p = 0.0094$; SDA-F2 vs. VDA×MDA-F2, mean diff. = −0.1612, ****$p<0.0001$; VDA-F2 vs. VDA×MDA-F2, mean diff. = −0.0814, **$p = 0.0091$, Bonferroni's multiple comparisons test). (H) Comparison of latency of entry into dark zone for parental F1, SDA-F2, VDA-F2 and VDA×MDA-F2 populations ±SD ($R^2 = 0.1103$, $F(3, 462) = 19.08$, ****$p<0.0001$, One-way ANOVA; Parental F1 vs. SDA-F2, mean diff. = −27.79, *$p = 0.0176$; Parental F1 vs. VDA-F2, mean diff. = 39.88, ***$p = 0.0001$; Parental F1 vs. VDA×MDA-F2, mean diff. = 39.00, ***$p = 0.0003$, Bonferroni's multiple comparisons test).