PERSISTENT BEHAVIORAL EFFECTS FOLLOWING EARLY LIFE EXPOSURE TO RETINOIC ACID OR VALPROIC ACID IN ZEBRAFISH

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INTRODUCTION

ABSTRACT

Background. During pregnancy, women are administered a variety of medications and supplements. Some of these drugs have been hypothesized to induce behavioral and/or morphological abnormalities in the developing fetus; these abnormalities may persist into adulthood, permanently impairing cognitive domains or anatomical features in the fetus. In this study, we used zebrafish embryos as a model system to study how prenatal exposure to certain doses of retinoic acid and valproic acid may be associated with persisting behavioral abnormalities. *Methods*. During development, zebrafish were exposed to either retinoic or valproic acid. From 0-5 dpf (days post-fertilization), larvae were reared in aqueous solutions containing retinoic acid (0, 0.02, 0.2 or 2 nM) or valproic acid (0, 0.5, 5.0 or 50 uM). Fish were screened in early life for physical malformation; those exhibiting no critical physical malformations were subjected to behavioral testing. One cohort of zebrafish was assessed during early life (6-dpf) using a locomotor activity screen; another cohort was reared to adulthood and assessed using a neurobehavioral test battery (startle habituation, novel tank exploration, shoaling, and predator escape/avoidance). *Results*: No significant increase in physical malformation was observed in the experimental group as compared to the control group. Both retinoic acid and valproic acid exposure during development disrupted larval activity and induced behavioral changes that persisted into later life, manifesting most significantly as decreased social affiliation. *Conclusions:* Social behavior and some aspects of motor function were altered in fish exposed to retinoic or valproic acid early in life.

BIOCHEMISTRY OF TARGET DRUGS RETINOIC ACID AND VALPROIC ACID

The scientific basis for this study hinges on the biochemical basis of the drugs tested.

Retinoic acid (RA) is a derivative of vitamin A, commonly found in prenatal vitamins. The developmental role of RA involves regulating gene expression, as well as controlling the differentiation, proliferation, and migration of stem cells (Rhinn and Dolle´, 2012). Accuracy and efficiency of RA signaling pathways are therefore critical components of mammalian development; interferences in this process can disrupt development severely and, potentially, irreversibly. This has been demonstrated by studies linking increases in the dosage of vitamin A administered during pregnancy, regardless of whether the increase arises from dietary (ex. Animal livers) or pharmaceutical (ex. Preformed vitamin A) sources, with the disruption of embryonic RA signaling (Lammer et al., 1985; Koch et al., 1983; Hathcock et al., 1990; Coberly et al., 1996; Hayman and Dalziel, 2012; Duerbeck and Dowling, 2012). Retinoic acid-acting drugs (a.k.a. 'RARs', or 'retinoic acid receptor-acting drugs') are another class of drugs that may demonstrate these detrimental effects; it is worth noting that the other target drug in this study, valproic acid, is an RAR.

CLINICAL SIGNIFICANCE

Consequences of elevated doses of vitamin A and related RAR-acting drugs include severe CNS morphological abnormalities and organ damage. Neural tube defects such as spina bifida provide examples of the severity of structural defects. Other serious effects of exposure to elevated doses of

vitamin A during development include newborn lethality, growth stunting, and behavioral dysfunction (Shaw et al., 1997; Steele et al., 1983; Shenefelt, 1972). Among the impairments to behavioral and cognitive domains is autism spectrum disorder (e.g. Rodier et al., 1996).

As stated, valproic acid (VPA) is related to RA as an RAR-acting compound. The clinical applications of VPA include its use as a prominent anti-epileptic medication; thus, in the context of our study, it is most relevant as a drug administered to epileptic women during pregnancy. However, VPA has also been shown to be associated with developmental impairments in studies performed on both mice (Kawanishi et al., 2003; Faiella et al., 2000) and, more recently, humans (Tomson et al, 2011). VPA exerts its molecular regulation as a histone deacetylase (HDAC) inhibitor, a role which has been posited to facilitate the regulatory effects of RA on gene expression (Li et al., 2014; Boudadi et al., 2013). One of the most significant families of genes that is targeted by VPA is the Hox family of genes; importantly, Hox genes control fundamental aspects of embryonic development, including determination of the body plan, segmentation, and determination of the rostro-caudal axis (Menegola et al., 2006).

SELECTING THE ZEBRAFISH MODEL

Zebrafish have highly conserved neural architecture, allowing the results from a study involving zebrafish to be considered with respect to other organisms such as humans. The popularity of the zebrafish as a model organism also spiked following the launch of a project to sequence the zebrafish genome, initiated by the Wellcome Trust Sanger Institute in 2001. Using data from this project, subsequent genomic comparative studies estimated that 70% of human genes had a zebrafish orthologue (Howe et al, 2013), further validating the relevance of this model. Extensive characterization of the zebrafish genome has in fact enabled them to serve as a modern replacement for rodents, which were previously among the most common model organisms used in teratological studies.

Additional benefits of the zebrafish include ability to engage in complex social behavior while accommodating high throughput data collection (Gerlai et al., 2000; Kimura-Kuroda et al., 2012). Additionally, zebrafish spawn overnight, hatch in 2–3 days post fertilization (dpf), develop complex behavior within the first week of life, and reach sexual maturity in 2–3 months, allowing for studies to proceed efficiently and facilitating the collection of a large volume of data. A final advantage afforded by this model is that embryos are translucent, allowing for the observation and identification of morphological defects from the earliest stages of development.

PURPOSE AND SIGNIFICANCE OF STUDY

A deeper understanding of the alterations of retinoid signaling by RA and VPA have important implications for maternal nutrition, prescription, and prenatal care. This, in turn, may help elucidate the effect of RARs in downstream processes, such as dopaminergic or serotonergic pathways, which determine behavior. Disrupted RAR signaling may further offer insight into such neurobehavioral disabilities as autism spectrum disorder and disruptive mood dysregulation disorder.

The present study thus aims to characterize, in zebrafish, how early life disruptions in RA signaling impact later-life behavior. The premise for this study is that, by observing how zebrafish react in a

variety of social simulations, and by noting the differences in behavior between exposure and control groups, the extent to which early-life chemical exposure may be linked to later-life behavioral abnormality may be deduced. Simulations were designed to imitate real-life stimuli that inspire responses of anxiety, fear, habituation, and social affiliation in zebrafish. These studies can form the foundation for future work to elucidate the mechanism by which RA singling disruptions induce lasting impairments, as well as how clinicians may continue to use these important drugs in a way that effectively treats the mother while preserving the health of the developing fetus.

METHODS

Zebrafish embryos were immersed in various solutions of toxins. Two cohorts of zebrafish were designated; one group was tested at a larval stage, and the other was reared into adulthood and tested at an adult stage. This allowed us to test both the acute effects of toxin exposure and the persisting effects.

The toxicant concentrations were as follows:

RA: 0.02, 0.2 or 2-nM (solvent: DMSO)

VPA: 0.5, 5.0 or 50-mM (solvent: aquarium water)

A follow-up study was also conducted by Jordan M. Bailey and Anthony N. Oliveri to more precisely define the hazardous dosage within the bracket tested in the initial stage. The solutions used for the follow-up stage were as follows:

RA: 0.02, 0.1, 0.2, 0.33, 1, 2 and 3.3 nM

VPA: 15 and 30 mM

Eggs were inspected under a dissecting microscope at 2- hours post fertilization (hpf), and those unfertilized or exhibiting physical malformations were excluded. This precluded the possibility of baseline impairments interfering in behavioral testing during adult. Larvae were randomly distributed among all aqueous exposure conditions. All behavioral testing of larvae was completed between the hours of 3:00 PM and 5:00 PM to ensure temporal consistency. Exposure was conducted at a density of 60 eggs/ 40-mL aqueous solution (Easton and Goulson, 2013), which was done in duplicate twice (for a total of four dishes per exposure group).

A total of 60 zebrafish larvae per VPA exposure group and 70 zebrafish larvae per RA exposure group were used for the larval motility assay. For the adult assays, 30 zebrafish per VPA exposure group and 30 zebrafish per RA exposure group were used.

All fish care and experimental procedures were approved by the Duke University Institutional Animal Care & Use Committee.

DESCRIPTION OF BEHAVIORAL ASSESSMENTS

1) Larval Motility Assay[1]
   Zebrafish are known to exhibit general hypoactivity in the presence of light. This effect was hypothesized to be exaggerated in the group of larvae subjected to higher doses of RA and VPA. Additionally, the higher exposure groups were expected to demonstrate a less dramatic response to changes in illumination and more activity in conditions of darkness, consistent with their normal physiological response.

   Following a 10 min acclimation period, we implemented 2 cycles of 10 min light/10 min dark for a 50 min trial. Larval motion was tracked 30 times/s using EthoVision XT® software (Noldus, Wageningen, The Netherlands).

2) Startle/Habituation
   Zebrafish habituate to an ongoing physical stimulus, though this response was expected to be blunted in higher exposure groups.

   Fish were tested in groups of eight, each sequestered in an individual tank located directly above a push solenoid. When activated, the solenoids delivered a tap to each tank. Each trial consisted of 10 repetitions of a tap, separated by 1 min- intervals, for a total trial duration of 10 mins.

3) Novel Environment Exploration
   When placed in an unfamiliar environment, zebrafish initially exhibit anxiety. In a tank environment, this manifests in a behavioral tendency to linger toward the bottom of the tank (Bencan and Levin, 2008; Levin and Cerutti, 2008). As the fish become more familiar with their new environment, they begin to explore. This increases both their distance from the bottom of the tank and the total distance they travel. It was expected that anxiety and exploratory behavior would be altered in high exposure groups.

   For this test, zebrafish were placed in an unfamiliar tank. The total distance traveled by each subject, as well as its distance from the bottom of the tank, were tracked over the course of a 5 min- trial.

4) Shoaling (Social Affiliation)
   In response to the presence of conspecifics, zebrafish are known to 'shoal', a behavior indicating association between the subject and its other fish. This response was expected to be diminished in higher exposure groups.

   We placed two monitors on two opposite ends of a tank. After a 1 min acclimation period, we simulated the presence of conspecifics by playing a video of zebrafish swimming. After 1 min, the video switched to the monitor on the opposite end of the tank. This reversal occurred three

[1] The larval motility assay was the only test conducted at the larval stage. The other tests were performed on adult fish that had been exposed to toxins as larvae. These other tests comprise the NBTB, or 'neurobehavioral test battery'. All tracking during the NBTB was performed with EthoVision XT®.

times, allowing for two presentations on one side of the tank and two on the other. The total trial time was 5 min. The shoal location reversal model was developed by Gerlai and colleagues (Saverino and Gerlai, 2008; Fernandes et al., 2015).

5) Predator Escape and Avoidance
   Zebrafish tend to flee from approaching predators, or from any stimulus reminiscent of an approaching predator. When there is no longer a predatory threat, zebrafish resume exploration of their environment. In conducting this test, we placed zebrafish in a tank adjacent to a monitor. To simulate the approach of a predator, we played a video of a rapidly expanding blue dot (diametric increase from 1.3 cm to 30.5 cm over the course of 5 s) on the monitor.

   We alternated between 2 conditions: 'predator on' and 'predator off'. In the 'predator on' phase, the blue dot expanded 12 times in the duration of 1 minute. In the 'predator off' phase, no stimulus was presented. 'Predator on' and 'predator off' were administered to evaluate behaviors of predator escape and avoidance, respectively. After a 1 min- acclimation period, 4 alternations of the conditions ('on'-'off'-'on'-'off') were presented, for a total trial time of 5 mins.

STATISTICAL ANALYSIS

Log transformations were performed on raw data if funneling was observed or if distributions were skewed. All statistical analyses were performed using Superanova/Statview (SAS; Cary, NC, USA) and all graphs were made using SigmaPlot (SYSTAT Software Inc., Richmond, CA). The Type 1 error rate (α) was set at 0.05. A repeated-measures analysis of variance (RMANOVA) was performed for each dependent variable of interest. In the RMANOVA, dose served as between-subject factors; tap number, time block within session, or stimulus condition served as within subject factors. When appropriate, e.g. where the adjustment to the degrees of freedom was less than 0.8, Greenhouse–Geiser adjustments to the degrees of freedom were used to account for lack of sphericity in the dataset. Dunnett's comparisons (two-tailed) were used to test for differences between the dose groups and control.

Statistical analyses were led by Jordan M. Bailey and Anthony Oliveri. They performed transformations and adjustments when necessary. I assisted them in running the statistical tests.

RESULTS

1) Survival rate upon exposure to RA and VPA
   As mentioned, eggs that were inviable or that exhibited obvious physical malformations were excluded immediately and not reared to adulthood. The survival rate of larvae in our control group, when reared from embryo to adult, was ~60%. This rate was similar to that achieved by previous studies performed by the Levin Lab, as well as by other groups (Bourrachot et al., 2014). After 10-dpf, no further deaths or malformations were observed (100% of embryos that reached the 10-dpf stage were successfully reared to adulthood; this survival rate was also consistent with other studies). Larvae from higher exposure groups did not exhibit a lower rate

of survival or morphologically-appropriate development than larvae from lower exposure groups (p's>0.05), indicating that neither RA nor VPA played a significant role in impairing morphological development. Displayed in Figure 1 are survival plots for both RA and VPA, which show the proportion of larvae that survived and that exhibited no obvious physical malformations after monitoring through 6-dpf.

2) Larval Activity

The highest exposure dose of RA (0.2 nM) was associated with a significant increase in swimming activity ($p<0.05$) relative to the control group (See Figure 2A). Doses of VPA were linked with a significant differential effect ($p<0.0005$) during the light and dark phases, indicating that among fish exposed to VPA, variations in swimming activity varied significantly between the two phases. Though none of the doses were associated with significant changes in activity relative to control groups during the light phase, the highest dose of VPA (50 uM) was associated with a significant reduction in activity ($p<0.01$) during the dark phase, relative to the control group (See Figure 2B). It is important to note that the control group was not immobile during this trial. The control group in fact swam at a near-constant rate throughout the trial, as opposed to all other groups, which all showed a dramatic reduction in activity during the light phases.

The follow-up study yielded a significant ($p<0.0005$) main effect of treatment on activity (See Figure 2C). Comparing the treatment groups to controls via Dunnett's tests showed that the 0.2 nM RA exposure group (the highest dose of RA) was associated with significant ($p<0.05$) overall hyperactivity. Though the higher and lower doses of VPA were not found to be associated with significant changes in activity, the 30 uM dose was associated with significant ($p<0.01$) overall hypoactivity. Because there was a significant ($p<0.0005$) interaction of exposure x light-dark condition in this follow-up study (indicating that at least two of the groups were significantly different, but not providing information as to which specific groups differed), further analyses were performed to determine the main simple main effects of exposure (i.e. the effect of each individual exposure group) in conditions of both illumination and darkness. In these additional analyses, it was found that the 15 uM VPA exposure was associated with significant ($p<0.05$) hyperactivity relative to the light condition, while the 30 uM VPA exposure was associated with significant ($p<0.01$) hypoactivity during the dark condition.

3) Startle/Habituation

Neither RA nor VPA exposure was found to be associated with a significantly different response to the physical tap stimulus. However, the typical habituation response ($p<0.0005$) was observed in both exposure groups as the trial progressed (see Figures 3A and 3B).

4) Novel Tank Exploration

The RA doses were not associated with a significant change either in total distance traveled or in distance from the tank bottom. Main effects of time, in 1 minute-bins, were observed on both total distance traveled and distance from tank bottom ($p<0.05$); this indicated that, as the trial progressed, the total distance traveled and the distance from the tank bottom changed. In fact,

values of both dependent variables increased, consistent with the fact that all fish in the trial exhibited increased exploration following the typical dive response (see Figures 4A and 4B).

Likewise, none of the VPA doses were associated with significant changes in distance traveled or distance from the tank bottom. Significant main effects were again observed of time on both total distance traveled ($p<0.0005$) and distance from the tank bottom ($p<0.01$), with both measures increasing—again, this was indicative of exploration following the typical dive response (see Figures 4C and 4D). A significant dose x time interaction on total distance traveled was also observed ($p<0.05$); as interpreted in conjunction with our main effect, this simply provides further indication that over time, within each dose, significant changes were observed on total distance traveled.

5) Shoaling (Social Affiliation)
   Exposure to the 2 nM dose of RA (the highest dose) was associated with a significant increase ($p<0.05$) in distance from the video of conspecifics, as compared to the control group (see Figures 5A and 5B). Notably, this result is consistent with diminished sociability. The 5 uM VPA dose was also associated with a significant increase ($p<0.05$) in distance from the video of conspecifics (see Figures 5C and 5D). With respect to both RA and VPA, there were significant main effects ($p<0.05$) of time within session, as the fish tended to affiliate with the shoaling image less strongly as the trial progressed. Neither RA nor VPA doses were found to be associated significantly with total distance traveled.

6) Predator Escape and Avoidance
   Neither RA nor VPA exposure was associated with a significant change in the distance from the predator stimulus, indicating that exposure to neither chemical was found to have a significant association with predator escape or avoidance. A main effect of condition ('predator on', 'predator off', acclimation) on distance from predator stimulus was observed among fish treated with RA and those treated with VPA ($p<0.001$), simply indicating that all fish did display the expected behavior of fleeing from the stimulus upon presentation (see Figures 6A and 6B).

DISCUSSION

The analysis and writing comprising the following discussion is the work of Jordan Bailey, Anthony Oliveri, Ed Levin, and myself. The initial idea for this project was developed by Ed Levin, who also performed some of the previous studies that lay the groundwork for this study's central premise. Experiments were designed and developed by Ed Levin, Jordan Bailey, Anthony Oliveri, myself, and Roy AJ Brooks. Analysis of the literature and previous studies was done by Ed Levin, Jordan Bailey, and Anthony Oliveri.

In this study, we administered two chemicals involved in retinoid signaling to developing zebrafish. The premise was that exposure to these chemicals would disrupt critical signaling pathways, which would in turn induce impairments that would last into adulthood and interfere with normal behavior. The doses we used were determined by previous studies, as recorded in the relevant literature (Wang et al., 2014;

Aluru et al., 2013; Farooq et al., 2012); additionally, we selected a dose range encompassing doses high enough to disrupt retinoid signaling, but not high enough to potentiate overt physical malformation or lethality. The clinical relevance of our study stemmed from the hypothesis that neonatal exposure to certain doses of RA and VPA could affect developing fetuses in such a way as to impair aspects of their behavior through to adulthood.

The data from our study indicates that early life retinoid disruption by means of exposure to VPA and RA does, in fact, disrupt several domains of behavior, including social affiliation, exploratory behavior and anxiety response, and light-sensitive activity (during a larval stage). The results of exposure to RA and VPA manifested most prominently in larval activity response to illumination and, in adulthood, social affiliation.

Excess VPA exposure was also found to be associated with hyperactivity during the novel tank exploration test, a result indicative of diminished anxiety. Excess RA exposure did not yield a significant result in this test, though RA-exposed fish did actually exhibit a trend toward hypoactivity.

Both RA and VPA significantly diminished social affiliation during the shoal trial. It is important to note that the results during this trial were most likely due solely to the effects of chemical exposure as opposed to any sort of impairment in visual or motor ability—this was evidenced by the fact that, during the predator avoidance trial, the same fish fled from the visual predator stimulus, indicating functional visual ability in sensing the stimulus and functional motor ability in escaping.

We can posit an interpretation for the behavior observed in the trial—namely, that exposure to RA and VPA might reduce sensitivity to reinforcement. This would make sense in the context of the social affiliation trial, in which the appetitive stimulus presented elicited only a weak response from subjects. This would also make sense in the context of the predator escape and the startle/habituation trials, in which no significant results were observed. These trials presented an explicitly aversive stimulus, and responses to appetitive and aversive stimuli have been linked to divergent neural networks (Samad et al., 1997). It would then be reasonable to posit that a chemical that diminishes the response to an appetitive stimulus would have a similar effect on the response to an aversive stimulus.

During the larval assay, the effects of VPA were more pronounced than those of RA—thus, VPA appears to more significantly impair motor ability. Similarly, adult fish that had been subjected to VPA exhibited more motor impairments on adult tests. The larval motility assay in fact produced a U-shaped curve with regards to RA exposure dose and activity, in which those fish having been exposed to a moderate dose of RA, but not the highest or the lowest dose, exhibited the most hyperactivity. This result was replicated in the follow-up study. The purpose of the larval assay, however, is simply to serve as a predictor of behavioral capacities that are tested during adulthood. It is important that results of the larval test be analyzed in conjunction with those of the adult tests, as the adult tests have been shown to be more sensitive in characterizing complex behavior (Eddins et al., 2010; Levin et al., 2014; Yen et al., 2011).

We can make an important deduction about the effect of RA and VPA on Hox genes based on the observation that no significant morphological abnormalities were observed after exposure to these chemicals. The lack of significant morphological abnormality must mean that Hox genes were not critically affected by RA or VPA, debunking a hypothesis that was formerly posited, which indicated that RA and VPA exerted their effects via a mechanism convergent upon certain cellular signaling pathways.

This explanation would also account for the sometimes contrasting behaviors following RA and VPA exposure—for example, in the novel tank exploration test, the tendency toward hyperactivity versus hypoactivity, respectively.

In part, the utility of this study arises from its ability to address a domain of neurological capacity relatively unexplored, specifically with respect to retinoid signaling: emotional and psychosocial responses. Certain aspects of these responses have been explained to some degree, however; for example, a previous study used behaviors from certain brainstem nuclei projection patterns to show that RA and similar chemicals could be associated with autism spectrum disorder (ASD) (Rodier et al., 1996). Additionally, VPA, when administered at mid-gestation, has been shown to affect dopaminergic and serotonergic pathways (Chen et al., 2007). Lower doses of VPA have also been associated with ASD (Ornoy, 2009). In a study examining a strong but relatively poorly understood emotional response, it was hypothesized that connections exist between excess vitamin A and suicidal behavior, as determined from patients using such drugs as isotretinoin (e.g. Marqueling and Zane, 2007). Others studies have shown that regions of the brain with high densities of RA receptors are engaged under conditions of stress and depression (Bremmer and McCaffery, 2008). The data we present here complements these studies, taking a whole-organismal level, downstream approach to demonstrate that exposure to RARs does influence aspects of behavior associated with emotional domains, including anxiety and sociability. Future studies may aim to further elucidate the mechanism of action and the genetic targets of retinoid signalers.

FIGURES

All figures and captions courtesy of Jordan M. Bailey and Anthony Oliveri. They generated these figures and wrote the captions.

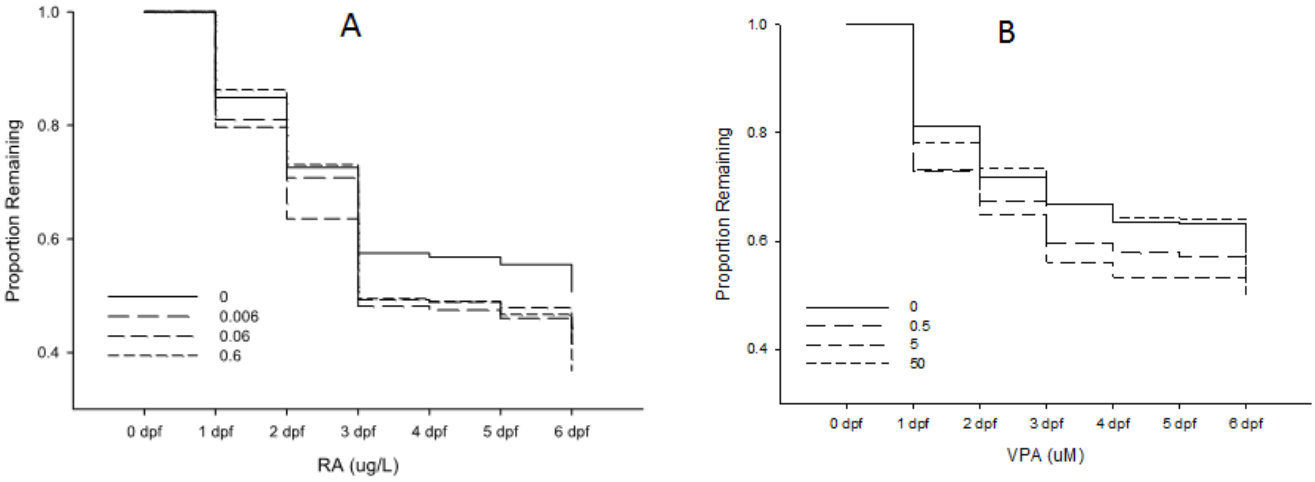

Figure 1. The proportion of zebrafish larvae remaining after unfertilized eggs, embryos with arrested development and dead or malformed zebrafish were removed from the RA (0-5 dpf) treated group (Panel A) and from the VPA (0-5 dpf) treated group (Panel B).

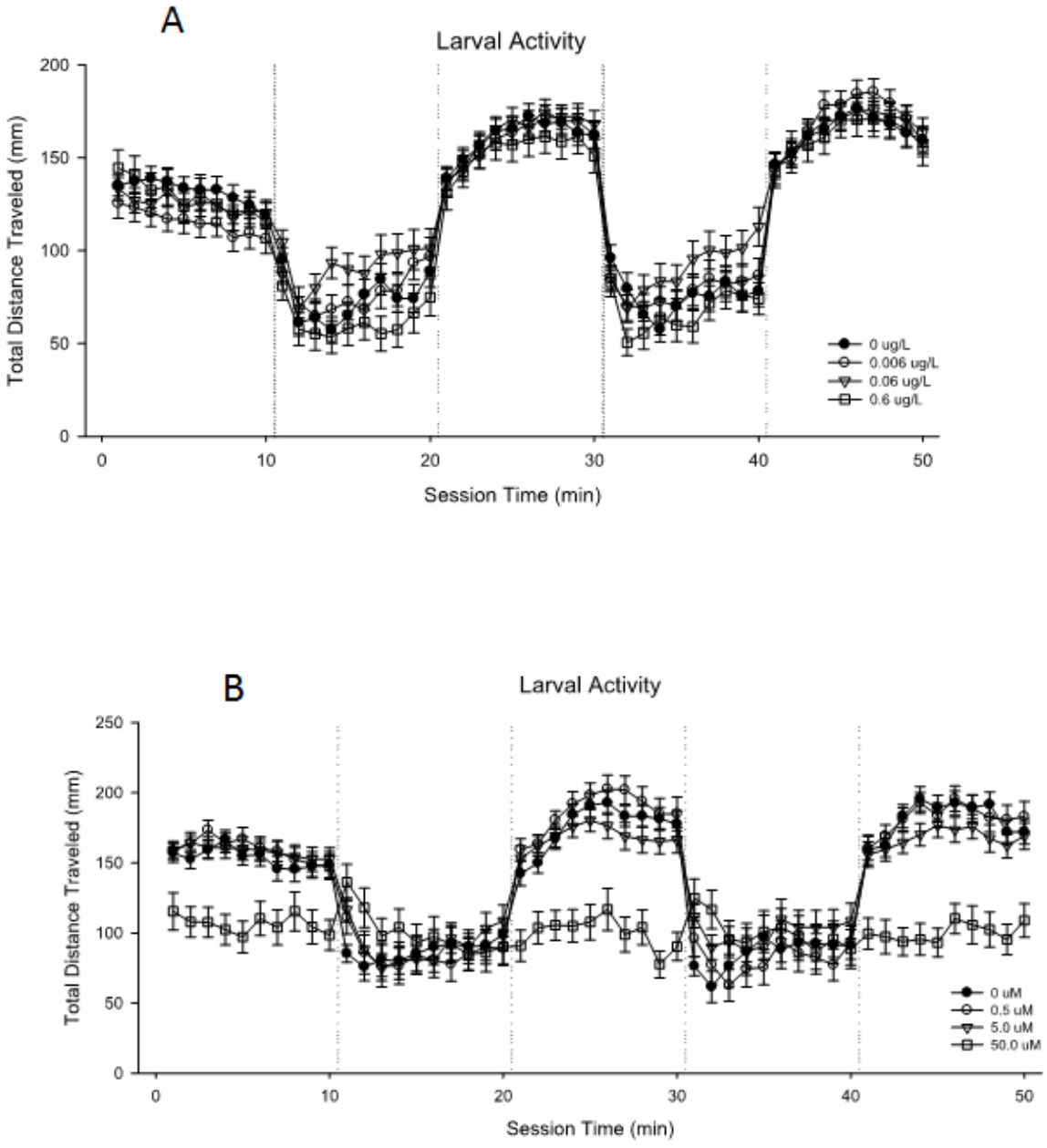

Figure 2. Larval activity data are plotted as a function of time, across 10-min condition blocks, for each group of RA treated fish (Panel A) and VPA treated fish (Panel B). Error bars represent SEM.

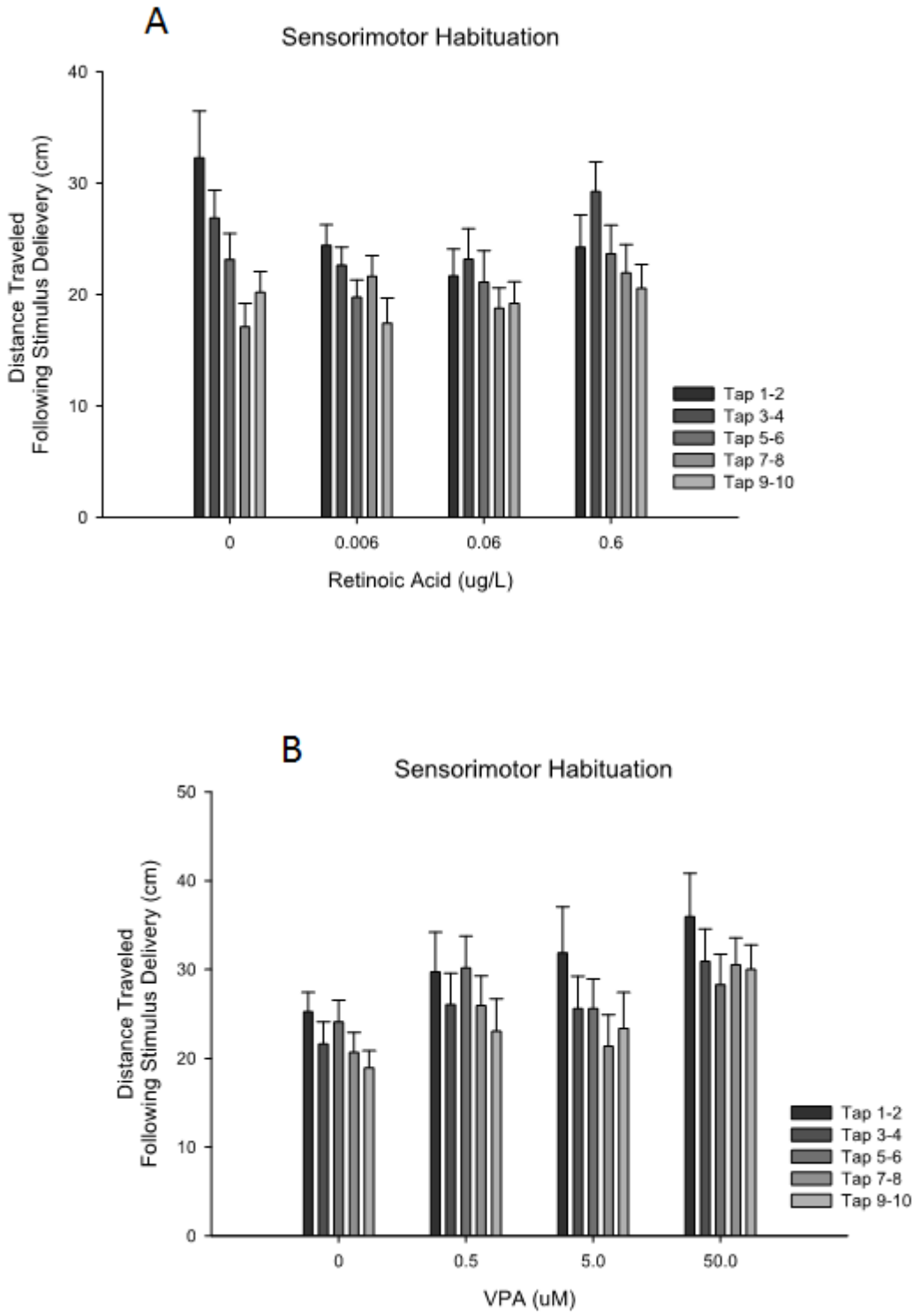

Figure 3. Distance traveled following the tap stimulus presentation is plotted for each RA (Panel A) and VPA (Panel B) dose condition (grouped bars). Each bar represents the mean of two tap presentations. Error bars represent SEM.

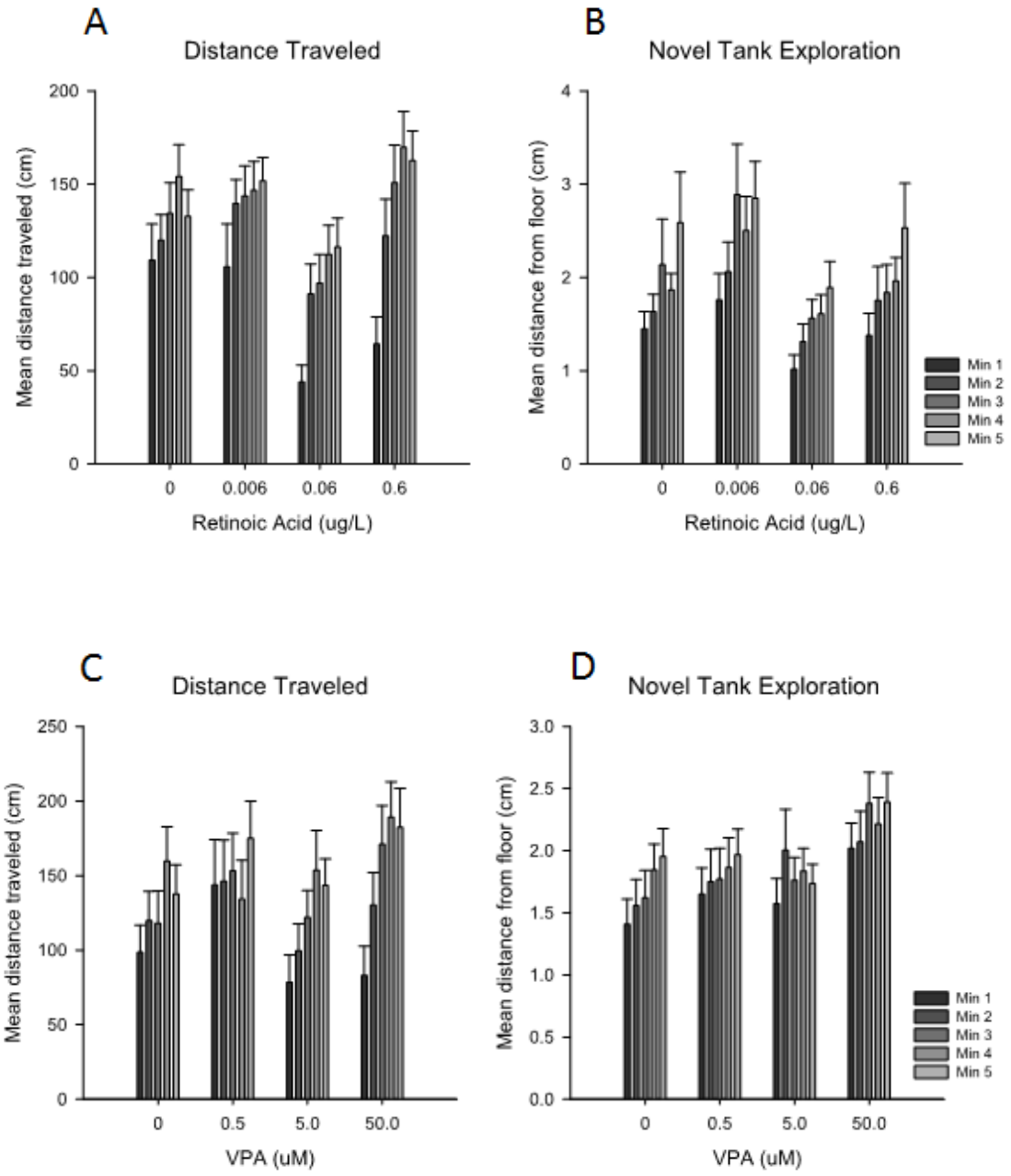

Figure4. Mean distance traveled for each min of the trial is plotted by RA dose (Panel A) and VPA dose (Panel C) condition (grouped bars). Mean distance from the tank floor (a measure of tank location) is plotted by RA dose (Panel B) and VPA dose (Panel D) condition (grouped bars). Error bars represent SEM.

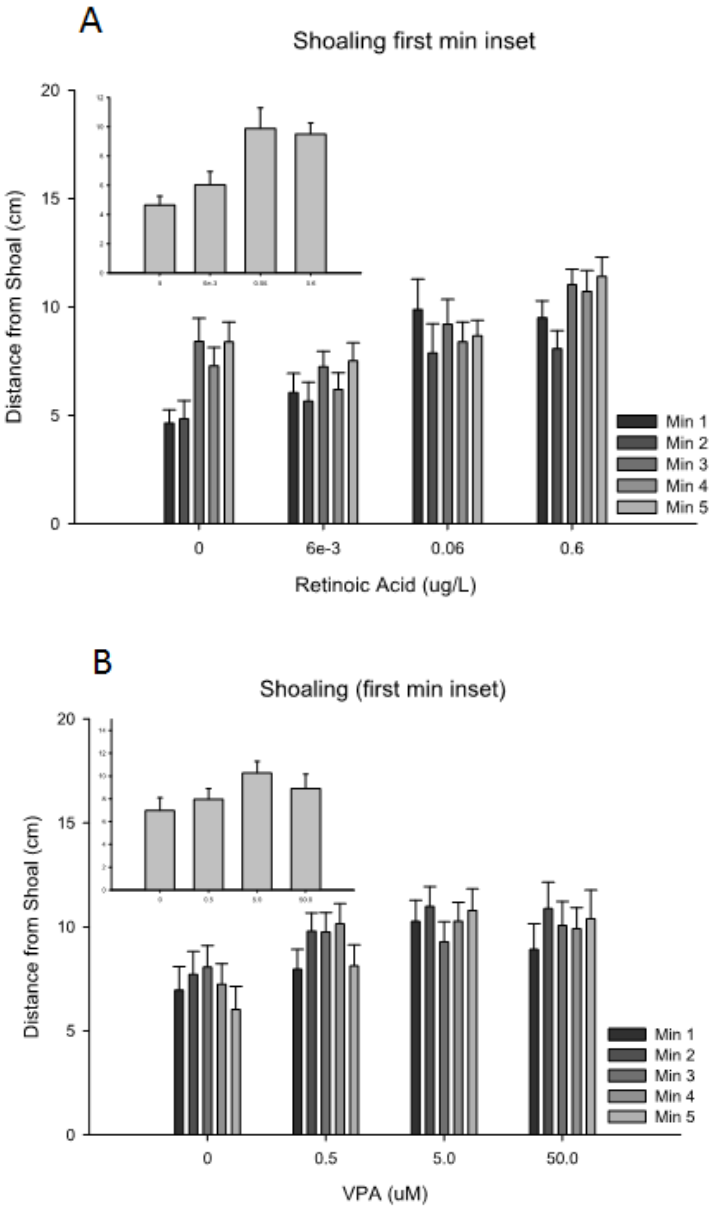

Figure 5. Mean distance from the shoal (cm) for each min of the trial is plotted by RA dose (Panel A) and VPA dose (Panel C) condition (grouped bars), inset graphs plot the mean distance from the shoal (cm) for first one minute of the session, which highlights the initial difference among groups for RA (Panel B) and VPA (Panel D) conditions. Error bars represent SEM. An "*" denotes $p<0.05$, difference from control (Dunnett's post-hoc).

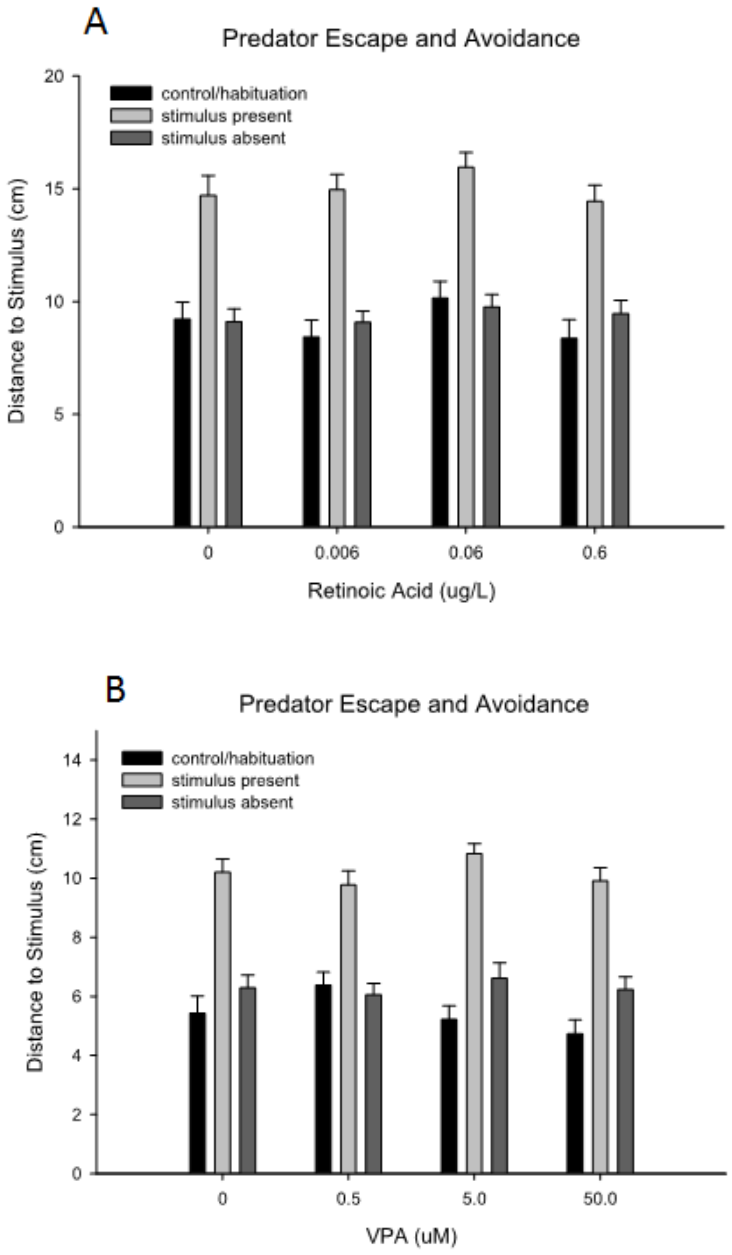

Figure 6. Mean distance to predator stimulus is plotted by RA dose (Panel A) and VPA dose (Panel B) condition (grouped bars). Error bars represent SEM.

CREDITS

All credits listed in their respective sections. This thesis is based on a study published in 2015 (Bailey, Oliveri, Karbhari, et al, 2015).

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