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Neurobehavioral Impairments Caused by Developmental Imidacloprid Exposure in Zebrafish

Emily B. Crosby[1], **Jordan M. Bailey**[2], **Anthony N. Oliveri**[3], and **Edward D. Levin**[2,3]

[1]Department of Biology, Duke University, Durham, NC, USA, 27710

[2]Department of Psychiatry and Behavioral Sciences, Duke University School of Medicine, Durham, NC, USA, 27710

[3]Department of Pharmacology and Cancer Biology, Duke University School of Medicine, Durham, NC, USA, 27710

Abstract

BACKGROUND—Neonicotinoid insecticides are becoming more widely applied as organophosphate (OP) insecticides are decreasing in use. Because of their relative specificity to insect nicotinic receptors, they are thought to have reduced risk of neurotoxicity in vertebrates. However, there is scant published literature concerning the neurobehavioral effects of developmental exposure of vertebrates to neonicotinoids.

METHODS—Using zebrafish, we investigated the neurobehavioral effects of developmental exposure to imidacloprid, a prototypic neonicotinoid pesticide. Nicotine was also administered for comparison. Zebrafish were exposed via immersion in aqueous solutions containing 45 μM or 60 μM of imidacloprid or nicotine (or vehicle control) from 4 h to 5 d post fertilization. The functional effects of developmental exposure to both imidacloprid and nicotine were assessed in larvae using an activity assay and during adolescence and adulthood using a battery of neurobehavioral assays, including assessment of sensorimotor response and habituation in a tactile startle test, novel tank swimming, and shoaling behavior.

RESULTS—In larvae, developmental imidacloprid exposure at both doses significantly decreased swimming activity. The 5D strain of zebrafish were more sensitive to both nicotine and imidacloprid than the AB* strain. In adolescent and adult fish, developmental exposure to imidacloprid significantly decreased novel tank exploration and increased sensorimotor response to startle stimuli. While nicotine did not affect novel tank swimming, it increased sensorimotor response to startle stimuli at the low dose. No effects of either compound were found on shoaling behavior or habituation to a startling stimulus.

Communicating author: Edward D. Levin, Ph.D., Department of Psychiatry and Behavioral Sciences, Box 104790, Duke University Medical Center, Durham, NC 27710, USA, edlevin@duke.edu, Phone: 1-919-681-6273; Fax: 1-919-681-3416.

DISCUSSION—Early developmental exposure to imidacloprid has both early-life and persisting effects on neurobehavioral function in zebrafish. Its developmental neurotoxicity should be further investigated.

Keywords

Imidacloprid; Neonicotinoid; Nicotine; Neurodevelopment; Zebrafish; Behavior

1. Introduction

Compounds that disrupt normal cholinergic signaling such as the nicotinic cholinergic agonist nicotine are widely classified as neurotoxicants (Chao & Casida, 1997; Yamamoto & Casida, 1999). Nicotine has detrimental effects on behavioral function in many species(Eddins et al., 2010; Eddins et al., 2009; Elliott et al., 2004; Levin & Chen, 2004), particularly with exposure during neurodevelopment when cholinergic systems play a morphogenic role. Despite this, concern over the developmental neurotoxicity associated with organophosphate (OP) pesticides (a once widely-used pesticide) use has led to the development of a new class of pesticides: the neonicotinoids, which share structural similarities to nicotine. The neonicotinoids are thought to have reduced toxicity compared to OP pesticides due to their presumed selectivity for insect over vertebrate nicotinic cholinergic receptors. An effective and widely used neonicotinoid pesticide is imidacloprid (1-(6-chloro-3-pyridylmethyl)-*N*-nitro-imidazolidin-2-ylideneamine) (Sheets, 2012).

Neonicotinoids are widely used and quite effective for control of sucking-insects on crops and for flea control on cats and dogs (Schenker et al., 2003). Like nicotine, the efficacy of neonicotinoids as pesticides (and therefore as neurotoxicants) comes from their ability to act as agonists at nicotinic acetylcholine receptors, an action they have in insects and mammals alike (Tomizawa & Casida, 2000; Tomizawa et al., 1995; Sheets, 2002). However, neonicotinoids are thought to selectively bind to insect nicotinic receptors with less action at vertebrate nicotinic receptors (Tomizawa & Casida, 2003; 2005). Thus, imidacloprid and other neonicotinoids are thought to have lowered toxicity profiles for mammals, birds, and fish, making them a popular alternative to organophosphates in commercial agriculture.

The effects of neonicotinoids on neurobehavioral development in vertebrates have not been well characterized, and assumptions regarding their safety have been made in the absence of thorough investigation. While some reports have failed to find overt morphological teratogenic effects on embryogenesis following imidacloprid exposures up to 50 μM (Scheil & Kohler, 2009), the potential lasting behavioral effects of these exposures are largely unknown. Due to the structural and functional similarities between imidacloprid and nicotine (Kimura-Kuroda et al., 2012), and in fact the organophosphate pesticides (Fig. 1), it is possible that developmental imidacloprid exposure could similarly affect neurodevelopment and influence behavior later in life. Thus, we sought to clarify both the short and long-term effects of imidacloprid on neurodevelopment by investigating the effects of developmental exposure on behavior immediately after exposure and again during adolescence and adulthood.

In this study, we utilized zebrafish (*Danio rerio*) as a model organism for determining the effects of imidacloprid on neurobehavioral function. Zebrafish are a useful preclinical vertebrate model due to their capacities for complex behavior and higher throughput data collection than mammalian models (Levin & Cerutti, 2008). The small size and relatively easy maintenance of zebrafish also offers economic and logistic benefits over mammalian models. Zebrafish spawn within approximately 30 min of morning light, hatch in 2-3 days post fertilization (dpf), develop complex behavior within the first week, and reach sexual maturity in 2-3 months. Thus, the effects of exposure to a compound can easily be observed over the entire lifespan of the organism (e.g., Levin et al., 2006). Additionally, their ability to absorb compounds through the water facilitates pharmacological and toxicological studies, particularly for water soluble chemicals. Due to the popularity of zebrafish, well-established protocols exist for a number of different behavioral tests, allowing us to assess elements of sensorimotor plasticity, emotional function, social behavior, and cognition (Levin & Cerutti, 2008; Bailey et al., 2013). The goal of the present study was to investigate behavioral endpoints across the lifespan, with larval, adolescent and adult testing times. We used two zebrafish strains (AB* and 5D) for larval assessments. For adolescent and adult assessments we used the type of zebrafish most frequently described in the literature, the AB* strain.

2. Methods

2.1: Design

All zebrafish, in each testing age group (i.e. larval, adolescent and adult), were treated with imidacloprid, nicotine or vehicle control for 0-5 dpf (with daily renewal of solutions). Larval activity levels, assessed at 6 dpf, quantified swimming behavior as well as reactivity to bright and dark environments (Ahmad et al., 2012). A separate cohort was dosed and raised identically and tested at 1.5 months of age to examine neurobehavioral function (sensorimotor response and habituation, social behavior, and novel tank swimming) during adolescence. A third cohort was dosed and raised identically and tested at 3 months of age, at sexual maturity, to examine sensorimotor response and habituation, social behavior, novel tank swimming, and predator avoidance during adulthood. Nicotine exposures were included to permit comparisons between two nicotinic cholinergic receptor acting compounds: nicotine (which has been widely studied) and imidacloprid (of primary interest here).

2.2: Subjects

The procedures were approved by the Duke University Institutional Review Committee for the use of animal subjects. Zebrafish (*Danio rerio*) from two wild-type strains, AB* and 5D, were maintained in a colony room at approximately 28.5°C on a 14:10-h light/dark cycle. Tanks used de-ionized H_2O, sea salt (Instant Ocean, 9.0 g/5 gal H_2O), and neutral regulator (Seachem, 2.5 g/5 gal H_2O) to maintain a pH of 7, and were kept with continual aeration and filtration. Fish were fed twice daily with lab-grown brine shrimp (45 g salt, 5 g eggs/2L deionized H_2O) and ground flake fish food (TetraMin Tropical Flakes). All behavioral testing of larvae was completed between 4:00 PM and 6:00 PM during the light phase. Due to the time-course of the adolescent and adult behavioral tests, this testing took place

throughout the day (9:00 AM to 6:00 PM), with subjects from each exposure group counterbalanced among testing times to eliminate any potential confounding effects of time of day on behavior.

All subjects used in the study were bred in the lab from AB* and 5D progenitors. Zebrafish embryos were collected at the beginning of the 14-h light cycle on the morning following the pairing of same strain adult breeders. Embryos were inspected before use, and those unfertilized or showing obvious malformations were excluded. All subjects used in the larval activity analyses and adolescent/adult tests were without overt physical malformation at the beginning of exposures. Several adolescent or adult zebrafish across all exposure groups exhibited a mild mini-fin mutant phenotype (Connors et al., 1999), which was only apparent as the fish increased in size). This is a genetic malformation of the tail that can alter swimming; consequently, all mini-fin subjects were excluded from behavioral testing. As this occurred across all groups, mini-fin exclusions did not disproportionately affect any group, and there was no difference in N among any of the exposure groups or control at adolescent or adult time points (p's>0.05).

2.3: Chemical exposures

Animals destined for larval, adolescent or adult testing were dosed separately but identically: approximately 2 h post fertilization (hpf), eggs were inspected under a microscope, and placed in glass Petri dishes at a density of 35-eggs/50 mL aqueous solution and maintained in an incubator (28°C, 14 h light/10 h dark cycle) for 5 d. Solutions were renewed daily (including control) using normal aquarium water and nicotine hydrogen tartrate salt (Sigma Aldrich, St. Louis, MO, USA) or imidacloprid (Sigma Aldrich, St. Louis, MO, USA). The five doses included a control (home aquarium water), low dose nicotine (45 μM nicotine solution), high dose nicotine (60 μM nicotine solution), low dose imidacloprid (45 μM) and high dose imidacloprid (60 μM), each renewed daily from 0-5 dpf. This dose range was selected based on prior (unpublished) pilot, dose-ranging studies.

On 5 dpf all larvae were transferred to un-dosed aquarium water. On 5 dpf all embryos (N=35 per exposure group) destined for larval testing were distributed pseudo-randomly across 96-well plates containing 45 μL un-dosed aquarium water for behavioral testing 24 h later, on 6 dpf. In this way, each exposure group was represented within a 96-well plate. On 5 dpf all embryos destined for adolescent or adult testing were transferred to un-dosed aquarium water and placed on the adult colony aquaria rack for rearing.

2.4: Behavioral assessment

i. Larval motility assay—In order to quantify developmental effects of exposure in larval zebrafish, we utilized a behavioral test for assessing larval swimming activity (i.e. distance traveled) and the capacity to adapt to changing environmental stimuli (i.e. alternating periods of light and dark) (see Willemsen & Van der Linde, 2010). Larval zebrafish are typically more active under low light conditions than they are under relatively brighter conditions (Ahmad et al., 2012); therefore, this arrangement allows us to quantify behavior in two distinct environments. DanioVision™ hardware equipped with an infrared camera combined with EthoVision XT® tracking software (Noldus, Wageningen, The Netherlands)

permitted the tracking of individual zebrafish larvae during alternating periods of white light ("100% illumination", 5,000 lux) and dark ("0% illumination", <1 lux). An initial acclimation dark period of 10 min was followed by 2 phases of a 10 min light/10 min dark period, and larval motion was tracked 30 times/sec over the course of the 50 min trial. Video data were analyzed and total distance traveled for each individual larva during each min of each 10 min phases was calculated.

ii. Adolescent Assessment

Startle response and habituation: Sensorimotor function and habituation to a startle stimulus were determined using the tap-elicited startle reflex (see Eddins et al., 2010). Control zebrafish (and most other animals), will habituate to a repeated stimulus (exhibiting a reduced response following stimulus onset). The habituation curve that is generated from this trial is thought to provide information about neuroplasticity and adaptation to an environmental stimulus. On the day of testing, zebrafish were brought from the colony room to the adjacent testing room in their home tanks. In the testing room, they were netted and placed individually into test arenas and left for a 5-minute acclimation before testing. Introduction to the test arena produced 1 to 2 min of rapid swimming, followed by a stable pattern of swimming after acclimation.

The tap-elicited startle response experimental apparatus has been widely used by our group and the methods used here are identical to those published elsewhere (see Eddins et al., 2010). Briefly, 8 fish can be tested simultaneously in a 2 × 4 array of swim arenas. Arenas were plastic containers 55 mm in diameter and filled with 30 mL of aquarium water. Each container was clear with horizontal bottoms and slightly angled sides to enable complete visibility to the camera fixed overhead. Opaque screens separated the arenas, isolating subjects from each other to eliminate shoaling behavior. Below each arena was a centrally located push solenoid that created a sudden physical tap to the test environment when activated.

For video acquisition, a digital-video camera was centrally positioned above the arena display, 75 cm above the water level, and fluorescent ceiling lights provided light for video recording. The video output from the camera was imported into a computer running EthoVision™ tracking software (Noldus, Wageningen, The Netherlands). Each fish was located six times/s. Timing of experimental events was done with the tracking software that sent logic pulses at scheduled times to a second computer via a parallel port connection. Event timing by the control computer was done with the multimedia hardware clock. Motor startle responses were assessed for 10 trials with 1 min intervals between trials to determine the initial startle response and habituation with repeated stimulus presentation. The dependent measures for tap-elicited swimming were total distance traveled for the 5 s preceding and 5 s following each of the 10 stimulus deliveries.

Novel Tank Exploration: Exploration of an unfamiliar environment was assessed using a novel tank exploration arrangement (see Bencan & Levin, 2008). When placed in an unfamiliar tank, zebrafish, like many other prey fish, avoid the more exposed upper regions of the tank and dive to the tank floor, thereby demonstrating a behavior that is often used as

an index of anxiety (Bencan & Levin, 2008; Cachat et al., 2010). However, as they acclimate to a tank devoid of aversive stimuli, zebrafish gradually rise and begin to explore the tank (Cachat et al., 2010). Because of this phenomenon, anxiolytic or anxiogenic effects of drugs can be observed with an automated assessment of diving response that utilizes video tracking of a zebrafish in a novel tank. For each min of a five-min trial, the distance from the tank floor and the total distance traveled were measured to assess tank exploration.

Shoaling Behavior: To assess group affiliation, a shoal location reversal task was utilized to quantify shoaling behavior. Typically, zebrafish have strong shoaling tendencies and will rapidly approach a group of conspecifics. To simulate the presence of a shoal, we developed an automated version of a previously described shoaling protocol (see Miller et al., 2013). The testing apparatus consisted of a 46 cm clear tank positioned beneath a video camera and between two monitors (Fig. 2). Each monitor was programmed to show 1 min video clips of a group of 10-15 shoaling zebrafish, filmed earlier in the lab. Before trials, subjects were placed individually in tanks surrounded by opaque dividers for 1 h isolation periods. Once deprived of social interaction for 1 h, they were placed in the test arena. Trials began with a 1 min baseline period, after which one of the screens showed the shoaling fish for 1 min. After that the shoal location was reversed, now appearing on the opposite monitor. This reversal process took place a total of 3 times in the 5 min trial, for a total of two left presentations and two right presentations (starting location was counter-balanced between subjects). EthoVision XT® software calculated swim speed, location and total distance traveled. Social affiliation was measured by calculating the distance of the fish from the video shoal (i.e. distance from the wall next to the video of shoaling fish).

iii. Adult assessment—Each of the behavioral assays described above for adolescent testing (startle habituation, novel tank exploration, shoaling) was used to evaluate neurobehavioral function during adulthood in a separate cohort of AB zebrafish. Additionally, a predator escape task was added to the test battery.

Predator Escape and Avoidance: To capture fear and escape behavior in zebrafish, an image of a blue dot, which increased in size from 1.3 cm in diameter to 30.5 cm in diameter within 5 s was displayed on a computer monitor located on one side of a rectangular 1.5-L tank. Presumably this simulated the appearance of a rapidly approaching figure. A single fish occupied a test tank and following a 1 min acclimation, the video alternated between a 1 min “predator on” condition in which the stimulus was presented 12 times and a 1 min “predator off” condition in which the screen was blank. These conditions alternated twice, and combined with the initial 1 min baseline, constituted a 5-min trial (Fig 3). Control zebrafish will typically flee rapidly from the presentation of this stimulus and allocate most of their swimming to the farthest end of the tank from the predator, however, when the stimulus is off control fish will typically explore the tank space approaching the tank wall associated with the predator. Therefore, by utilizing the on/off design here, we are able to capture both predator escape (when the stimulus is on) and predator avoidance (when the stimulus is off).

2.5 Statistical Analysis

All data were analyzed with SYSTAT 13 (Systat Software, San Jose, CA, USA) and SuperAnova (SAS Institute, Cary, NC, USA) using a repeated measures analysis of variance (ANOVA), with dependent variables appropriate to each behavioral test, and Dunnett's post hoc to identify differences of each exposed group from controls. All statistical analyses utilized an alpha level of 0.05 (two-tailed) as a threshold to determine significance.

Results

3.1 Larval activity

There was a significant main effect of treatment on larval activity ($F(4,334)=5.85$, $p<.001$) and a significant interaction between illumination condition and treatment ($F(4,334)=2.98$, $p<0.025$). In the light condition, the effect of treatment was not significant, but in the dark, there was a significant effect of treatment ($F(4,334)=7.45$, $p<0.0005$). Both doses of imidacloprid significantly and robustly decreased activity ($p<0.0005$) in the dark condition. The 45 μM ($p<0.05$) and 60 μM ($p<0.005$) nicotine doses also significantly reduced activity in the dark (Fig. 4). Additionally, a significant difference was found between activity of zebrafish strains ($F(1,334)=10.90$, $p<0.0005$), with 5D zebrafish being less active than the AB* strain.

3.2 Adolescent Neurobehavioral Test Battery

Sensorimotor response and habituation—The distance traveled 5 s before and 5 s after the stimulus delivery were measured, which provided assessments of general/baseline swimming speed and swimming in response to the stimulus delivery. There was a significant linear function such that distance traveled (after stimulus delivery) decreased across trials on the 5 s after stimulus delivery, indicating that the zebrafish habituated to the repeated stimulus presentations ($F(1,220)=33.18$, $p<0.001$). However, there was an overall effect of treatment on distance traveled after each startle ($F(4,220)=5.02$, $p<0.005$). This was due to a hyperactive response to startle stimuli in 45 μM ($p<0.05$) and 60 μM imidacloprid dose groups ($p<0.01$), as well as in the 45 μM nicotine dose group ($p<0.0005$) (Fig. 5). The treatment effects were selective to the startle response. No significant treatment-induced differences or trial effect in distance traveled occurred during the 5 s before each stimulus delivery.

Novel tank swimming—Distance from the tank floor and total distance traveled by min was measured. There was no significant main effect of treatment on distance from the tank floor ($p>0.06$), but there was an interaction between treatment and time ($F(16,236)=1.81$, $p<0.05$), driven by behavior during the 4^{th} ($p<0.05$) and 5^{th} ($p<0.01$) mins of the trial. Zebrafish treated with imidacloprid spent more time during the 4^{th} and 5^{th} mins near the tank floor (Fig. 6). Importantly, this effect occurred in the absence of any effect of treatment on total distance traveled during the session.

Shoaling—Two dependent measures were assessed: distance to shoal and total distance traveled, providing orthogonal measures of overall swimming speed and reaction to the shoal. There was a significant main effect of time (i.e. shoal location) on distance to shoal

(F(1,60)=322.91, p<0.0005). No significant effects of nicotine or imidacloprid on shoaling behavior were found. For total distance traveled, there was a significant difference between exposure groups (F(4,60)=2.55, p<0.05), with nicotine-exposed (45 μM) fish swimming significantly (p<0.05) farther than controls. There was also a significant effect of time (F(1,60)=5.62, p<0.025).

3.3 Adult Neurobehavioral Test Battery

Sensorimotor response and habituation—Similar to the adolescent cohort, no effect of treatment or trial number was found on distance travelled during the 5 s preceding each stimulus delivery. However, while trending towards significance, there was no significant effect of dose on distance traveled during the 5 s following the tap stimuli, unlike the effect in adolescents. There was a significant effect of trial number (i.e. stimulus presentation number) (F(4,620)=22.64, p<0.001) (see Fig. 8), indicating habituation to the repeated stimulus delivery.

Novel tank swimming—In adults, a significant effect of treatment was detected when distance to the tank floor was analyzed over the course of the 5 min trial (F(4,85)=2.98, p<0.025) with both nicotine doses and the higher imidacloprid dose causing the fish to remain significantly (p<0.05) closer to the tank floor compared to control fish. A main effect of time (F(4,340)=9.96, p<0.0005) was also detected on distance to the tank floor. Interestingly, however, there was no effect on total distance traveled among the treatment conditions, indicating that tank location preference was not driven by changes in motoric function. There was a main effect of time on total distance traveled, as with distance from the floor (F(4,340)=23.89, p<0.001) (Fig. 9).

Shoaling—Similar to the adolescent cohort, there was no effect of treatment on social affiliation in the adult cohort. A significant effect of time (i.e. shoal location, as location changes as a function of minute) on both distance to shoal (F(3,216)=3.88, p<0.05) and total distance traveled (F(3,216)=6.12, p<0.05) was found (Fig. 10).

Predator Avoidance—Three dependent measures were analyzed for each condition, predator present (“ON”) and predator absent (“OFF”): distance to the predator (horizontal location preference), distance to the tank floor (vertical location preference) and total distance traveled (overall swim speed). A significant effect of distance to the floor emerged both in the presence of the predator stimulus (F(4,58)=3.49, p<0.025) and during the intervals between stimulus presentations (F(4,58)=3.76, p<0.01), in which all treated groups remained closer to the tank floor than the control animals (Fig. 11, panel B). Not surprisingly, there was also a main effect of predator condition (ON vs. OFF) on distance from the predator wall (F(1,58)=142.51, p<0.001) (Fig. 11, panel A). There was no effect of treatment on total distance traveled.

Discussion

The behavioral effects of early life exposure to the neonicotinoid insecticide imidacloprid are not well characterized in vertebrates, despite its widespread use in agriculture and structural similarity to compounds widely regarded as neurotoxic (e.g. nicotine,

chlorpryifos). To capture the immediate effects of early life exposure to imidacloprid, the swimming activity of 6 dpf zebrafish was quantified under two conditions of illumination (dark and light). Diverse and subtle behavioral endpoints are more reliably quantified in older zebrafish; as such a test battery was employed to characterize the long-term effects of early life exposures to imidacloprid and nicotine. This battery included procedures thought to measure cognitive (learning, anxiety/fear and sociability) and sensorimotor function in zebrafish. We hypothesized that early life exposure during critical developmental periods to a neonicotinoid insecticide, which shares some functional similarities with nicotine and OP pesticides, would be behaviorally toxic, at doses too low to cause overt morphological toxicity.

Indeed, early developmental exposure to imidacloprid altered behavior in zebrafish both immediately after exposure (24 hr later) and following months (1.5 or 3 mo) living in a clean environment. Imidacloprid, like nicotine, significantly reduced swimming activity of larval zebrafish during the dark phase of the larval swimming assay. The nicotine effect here resembles that reported in previous studies, which showed depressed larval activity 6 dpf, albeit at lower doses (for a review, see Klee et al., 2011). Within the context of a stress response, decreased swimming in the dark would draw less attention to a zebrafish larva increase its chance of survival. Thus, a compound that heightens anxiety could cause this kind of “cautious” behavior. Similarly, when tested later in life, imidacloprid fish engaged in more bottom-dwelling when placed in a novel environment and were hyperactive in response to startling stimuli compared to their counterparts who did not experience imidacloprid for the first five days of life. The finding of decreased larval activity combined with the effects seen during adolescence and adulthood supports our hypothesis that developmental exposure to imidacloprid is associated with alterations in behavior, which might align with the neurobehavioral profile of toxicity associated with OP exposure.

Interestingly, zebrafish strain modulated the effect of these exposures. The 5D strain of zebrafish swam significantly less than the AB* strain of zebrafish when exposed to both nicotine and imidacloprid (Fig. 4). Because no behavioral difference was found between AB* and 5D control fish, our findings suggest that 5D zebrafish might be more sensitive to the effects of these exposures; a novel finding which could have implications on strain choice for future studies. Previous studies have found differences in susceptibility between other strains, but these particular strains have not yet been widely compared (e.g. Dlugos & Rabin, 2003). However, in an effort to facilitate comparisons with other studies and reconcile space and logistical constraints, adult testing commenced with the widely used AB* strain only. Because only AB* fish were raised for adolescent and adult testing here, future studies should follow up with both AB* fish and 5D fish to determine whether the strain effects found here at 6 dpf persist into adolescence and adulthood.

Generally, the effects of these chemicals manifested very early (i.e. 6 dpf) and persisted through adolescence and into adulthood. The adolescent and adult studies detected many of the same responses on the neurobehavioral test battery following early developmental exposure to imidacloprid or nicotine. Where there are exceptions, as with the dive test, the effect of exposure approached statistical significance ($p=0.051$). Imidacloprid and 45 μM nicotine both caused hyperactivity in response to a startle stimulus, which is consistent with

previous studies demonstrating that nicotine at low doses (15 and 25 μM) causes hyperactivity in response to a startling stimulus (Eddins et al., 2010; also see Parker & Connaughton, 2007). This heightened response is generally attributed to an increase in anxiety, which is consistent with our findings of imidacloprid's larval effects. However, 60 μM nicotine did not have any effects on distance traveled following the stimulus delivery. This could possibly be explained by the inverted U-shaped curve nature of nicotine – although, as only two doses were used here, there is not a sufficient range to make definitive comparisons to studies reporting the U-shaped curve associated with nicotine. Additionally, as expected, there was no significant effect of treatment on activity in the 5 s preceding the tap ($p>0.05$), meaning that the activity differences after the tap were due to varying responses to the startle stimulus and not generalized hyperactivity or hypoactivity of particular groups.

Imidacloprid also impacted novel tank exploration, causing adult (i.e. via significant main effect) and adolescent (i.e. via significant interaction) zebrafish to remain near the tank floor for longer than control fish. As this type of diving behavior is often characterized as a defense or protective mechanism, it is often indicative of exposure to otherwise anxiogenic compounds and this diving behavior is well-supported as a model for anxiety in zebrafish, as it is reliably and predictably increased or decreased by compounds known to be anxiogenic or anxiolytic (respectively) in rodents and humans (Egan et al., 2008; Levin et al., 2007; Maximo et al., 2010). The fact that this effect occurred in the absence of any effect on total distance traveled during the session is important, as it defines this difference as a deficit in adapting to a new environment rather than handicapped swimming ability. Thus, it appears that developmental exposure to imidacloprid has long-term anxiogenic effects in zebrafish. However, some inconsistent effects of nicotine did emerge between adolescent and adult testing. For instance, no significant effects of nicotine were found on novel tank exploration when tested during adolescence, although one appeared during adult testing. This might indicate that the time course for some of nicotine's effects on behavior might be domain-specific, or otherwise under the control of age-related environmental stimuli (in this case, adult fish have a longer cumulative history of inhabiting a tank than the adolescent fish).

These findings may be understood in the wider context of other pesticides that interrupt acetylcholine signaling, notably the organophosphate (OP) insecticides. OP compounds inhibit the enzyme responsible for catabolizing acetylcholine in the synapse, acetylcholinesterase, leading to increased levels of acetylcholine signaling, paralleling the agonistic effects of nicotine and imidacloprid at acetylcholine receptors. Developmental exposure to OP pesticides has long been associated with adverse neurobehavioral outcomes - in zebrafish alone, they can lead to hyperactivity in larval fish (Levin et al., 2004), and adult fish exposed as embryos display decreased startle habituation and increased activity in a novel environment (Sledge et al., 2011) and decreased accuracy in a spatial discrimination test (Sledge et al., 2011, Levin et al., 2003). Although many of these findings are abnormal in the opposite direction from those seen in this study with nicotine and imidacloprid, it is clear that the same behavioral domains are being altered by each class of compounds. While it may be hypothesized that the OPs exert their effects through increased signaling via nicotinic acetylcholine receptors, it should be noted that OP pesticides have been shown to

produce neurobehavioral effects at levels too low to significantly reduce acetylcholinesterase activity (see Aldridge et al., 2005) and that alterations to monoamine systems in rats (Aldridge et al., 2005) and zebrafish (Eddins et al., 2010) have also been implicated, which may explain the diverging results between the two pesticide classes.

Thus far, mechanistic research into imidacloprid neurotoxicity is limited. Imidacloprid, as well as other neonicotinoids, do indeed seem to interact with mammalian nicotinic acetylcholine receptors as nicotine does (Kimura-Kuroda et al., 2012), and developmental exposure in rats produces abnormal histopathology in the motor cortex and hippocampus (Abou-Donia et al., 2008). At this point, the best insights concerning downstream mechanisms of imidacloprid and neonicotinoid neurobehavioral teratology might come from what we already know about nicotine's effects on the developing brain (see Slotkin, 2004 for a review).

One caveat in the study is the photodegrading quality of aqueous solutions of nicotine (half-life = 3 d) and imidacloprid (half life approximately 45 m - 3 h, depending on water conditions) (Fuentes et al., 2015; Warnhoff & Schneider, 1999). Because illumination is part of a normal diurnal light cycle, which is required for zebrafish to develop normally, protecting the solution (and therefore larvae) from light was not desired. However, one measure that minimized this photodegradation effect was dosing fish late in the afternoon so as to maximize dark exposure time. In addition, Petri dishes were emptied and refilled with freshly prepared solution every 24 h to counter evaporation in the incubator and degradation of the compounds. This was repeated for days 0-4 after fertilization. Moreover, the photodegredation of the compounds would have only decreased the toxicity of the exposures. If anything, the neurotoxic risk posed by imidacloprid and nicotine would be higher absent the photodegradation.

Although past experiments have demonstrated a significant decrease in shoaling tendencies in zebrafish with acute exposure to nicotine at doses of 4 mg/L and 8 mg/L, (approximately 9 and 20 μM, respectively) this trend was not seen as a long-term effect of developmental exposure to nicotine here (Nishimura et al., 1994). The data shown here indicate a significant difference across dose groups for swimming distance in shoaling tests for the adolescents, suggesting hyperactivity in nicotine-exposed groups, especially at the 45 μM dose. Because this difference in total swimming distance contradicts the results in the novel tank diving test, it is possible that other factors are contributing to this effect. It is possible that nicotine-exposed fish are exhibiting hyperactivity in response to a shoal, but do not exhibit hyperactivity in the absence of such stimuli. Although neonicotinoids were designed to be safer versions of nicotine-like compounds, imidacloprid was associated with several behavioral effects that were not altered by nicotine. In fact, further investigation into the mechanism by which imidacloprid affects development would be an interesting follow-up to this experiment. However, a possible explanation for lessened effects from nicotine could be photodegradation. As previously described, the solutions used here were changed daily, but it is still possible that the two drugs degraded quickly or at unequal rates and thus were not present in the concentrations as anticipated.

In conclusion, we found that developmental imidacloprid exposure causes neurobehavioral defects in zebrafish that persist into adolescence and adulthood. This risk, from a drug designed to have fewer implications for vertebrate health, creates some concerns for human health, as imidacloprid is widely used in the United States. Although imidacloprid does break down over time in the presence of sunlight, decreasing its risk in surface bodies of water, it certainly has the potential to leak into groundwater, where it can survive for extended periods and thereby pose a threat to human health. Additionally, the pharmacological effects of its degradation intermediate, imidacloprid-urea, are unknown (Liu et al., 2006). Although much of Europe has already banned imidacloprid as a pesticide due to its effects on bees (see Whitehorn et al., 2012), our findings suggest that imidacloprid may pose a threat to more than just insects and should be further investigated. Future studies conducting additional behavioral tests, extending the research to mammals, and finding the dose thresholds of imidacloprid that pose neurotoxic risks to developing vertebrates will provide valuable information to the field of environmental toxicology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Highlights

- Embryonic exposure of zebrafish to imidacloprid significantly decreased larval swimming activity.
- Embryonic exposure of zebrafish to imidacloprid had significant long term effects decreasing novel tank exploration.
- Embryonic exposure of zebrafish to imidacloprid had significant long term effects increasing startle response.
- Embryonic imidacloprid has early-life and persisting neurobehavioral effects in zebrafish.

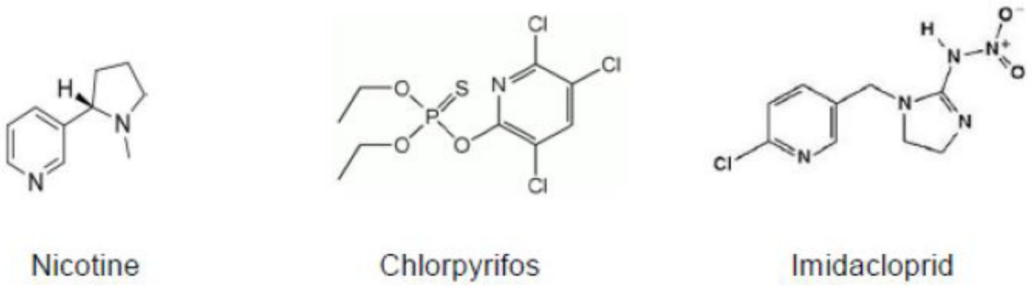

Figure 1. Molecular structures of nicotine, chlorpyrifos, and imidacloprid

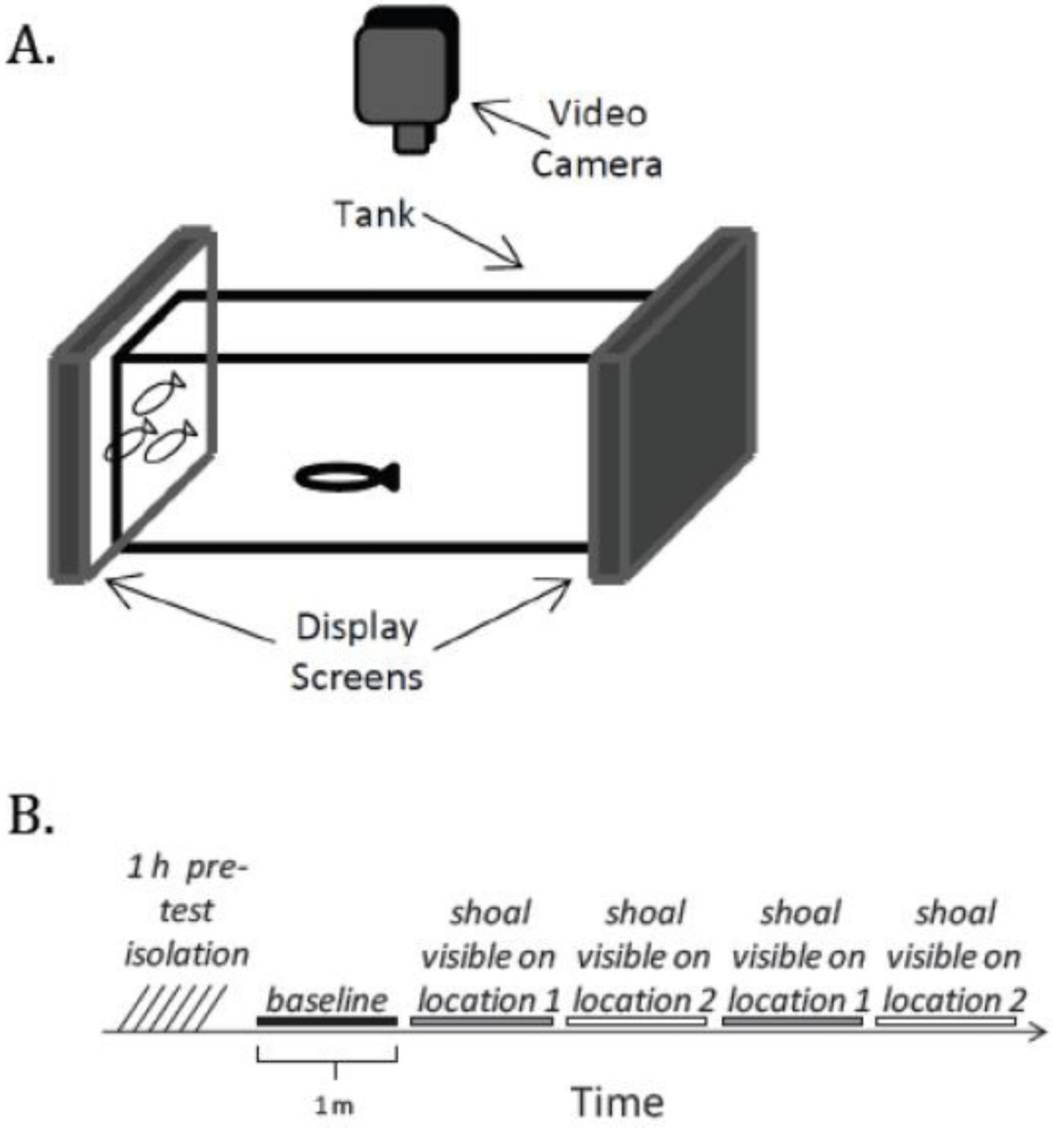

Figure 2. Diagram of apparatus used to measure social affiliation

Panel A: Fish were isolated for 1 h prior to testing then gently submerged into the center of the test tank. After a 1 min baseline period, a pre-recorded video image of 15 conspecifics engaging in shoaling behavior was displayed on either the left or right screen for 1 min after which the video was immediately displayed on the opposite screen for 1 min. The display location alternated three times, which resulted in two presentations on the left and two on the right. Starting location was counter-balanced within each treatment group. Distance from the active shoal was measured in cm via overhead recording and simultaneous tracking. Panel B: Timeline of experimental events.

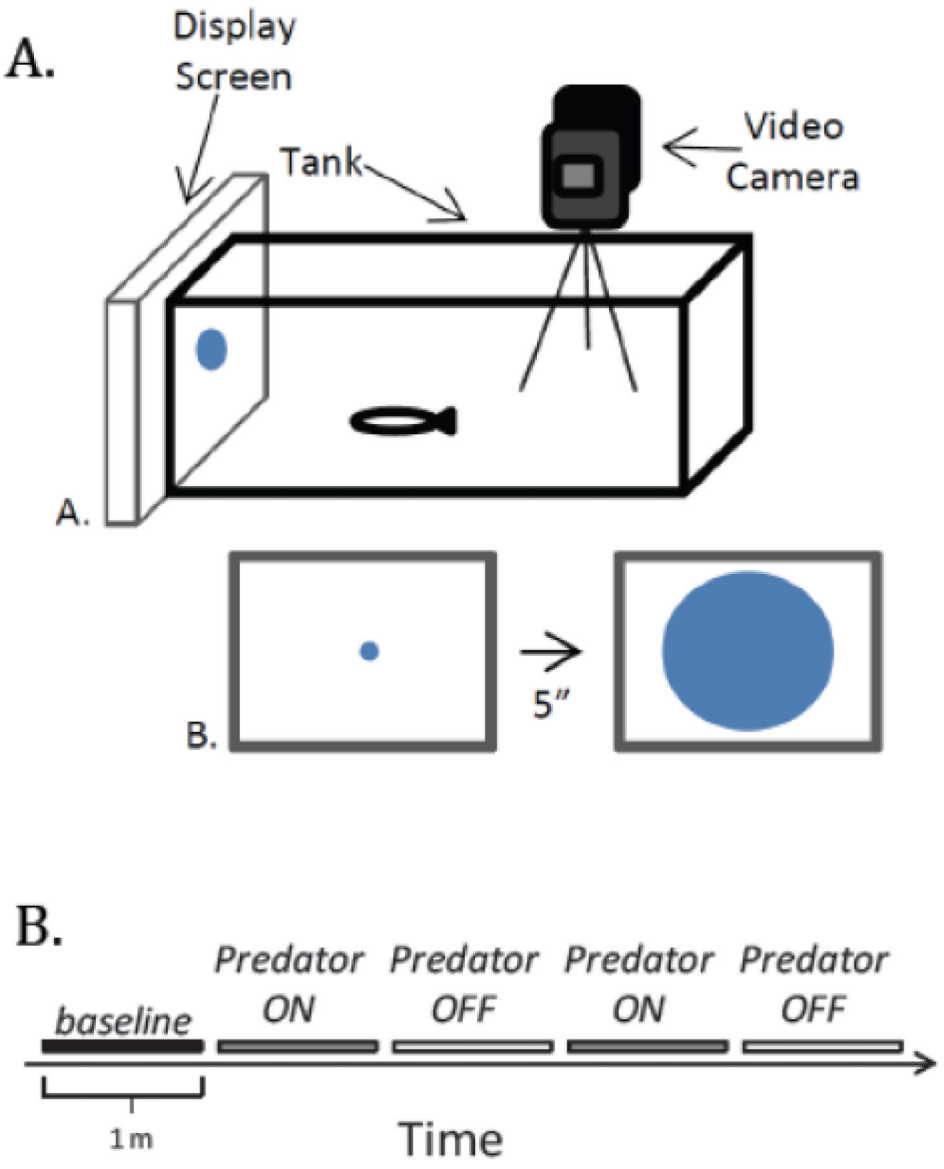

Figure 3. Diagram of apparatus used to measure predator escape and avoidance

Panel A: Individual fish were gently submerged into the center of the test tank. After a 1 min baseline period, an animated image of circle saw displayed in the center of a monitor located at one end of the rectangular tank. The circle grew in diameter from an initial .5 in to 10 in within 5 sec, after reaching maximum diameter the circle was reset to the starting size and began increasing again. The circle grew and was reset a total of 12 times within 1 min; this is labeled the “predator ON” phase. Then, the screen was blank for 1 min (“predator OFF”), followed by the onset of the predator stimulus for 1 min. The screen alternated between 1 min phases of predator OFF and predator ON for a total of 5 min, displaying the predator stimulus twice. Panel B: Timeline of experimental events.

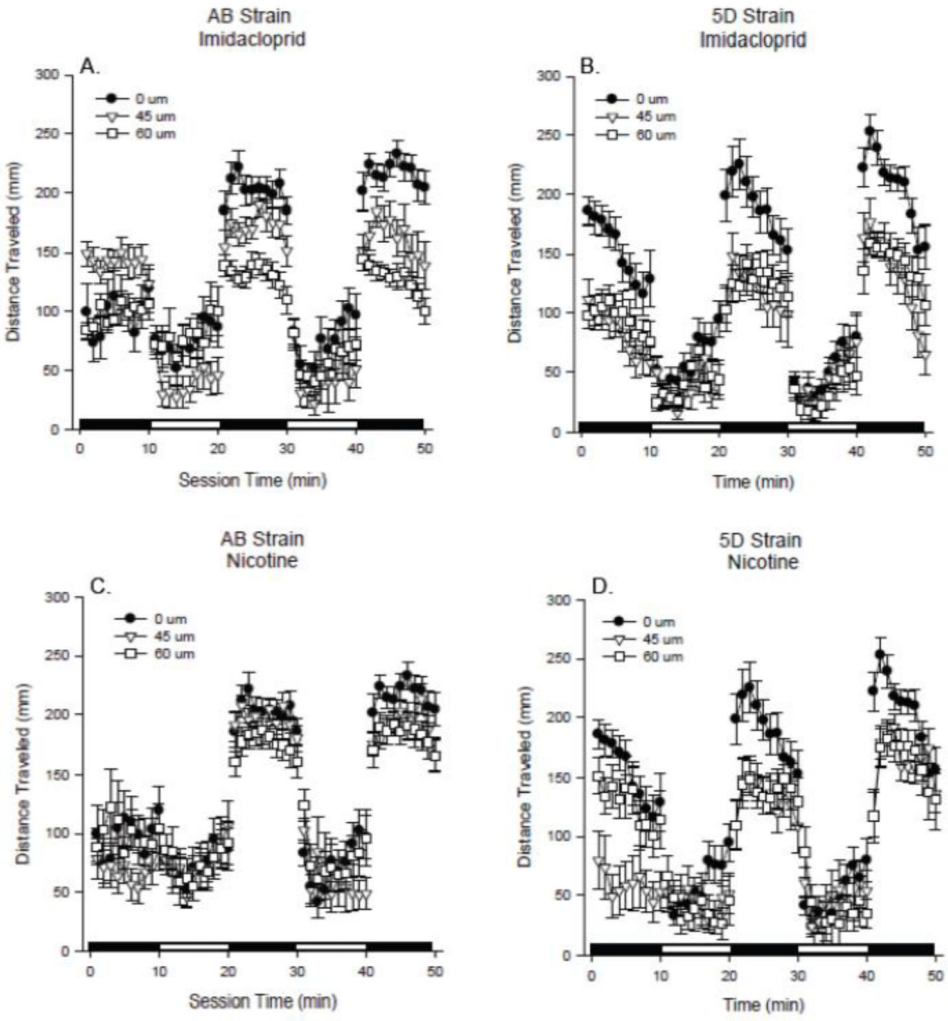

Figure 4. Larval sensorimotor behavior

Distance traveled (mm) is plotted as a function of time for each of the three exposure groups (0, 45, 60 μM) for the AB strain exposed to imidacloprid (Panel A), 5D strain exposed to imidacloprid (Panel B), AB strain exposed to nicotine (Panel C) and 5D strain exposed to nicotine (Panel D). Breaks in the plots correspond to alternating illumination conditions, which are illustrated via shading above the x-axis. Error bars represent SEM. N=35 per exposure condition.

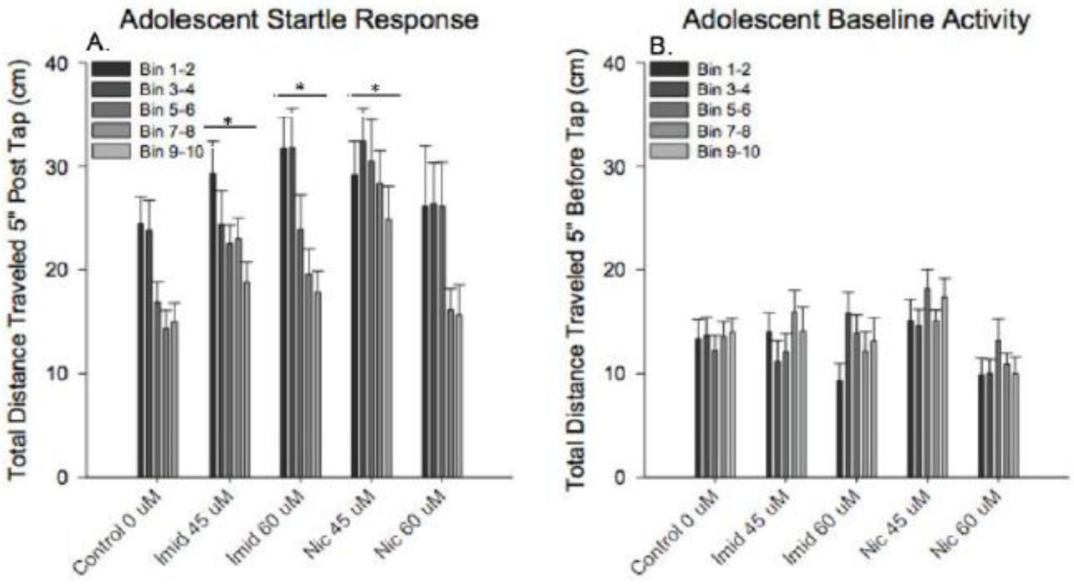

Figure 5. Adolescent zebrafish, sensorimotor habituation

Mean distance traveled in the 5 s following the delivery of the tap stimulus (Panel A). Mean distance traveled in the 5 s preceding the delivery of the tap stimulus (Panel B). Each bar represents the mean distance traveled across a bin of two tap deliveries. Error bars represent SEM, "*" indicates significant post-hoc (significantly different from control). N=15-18 per exposure condition.

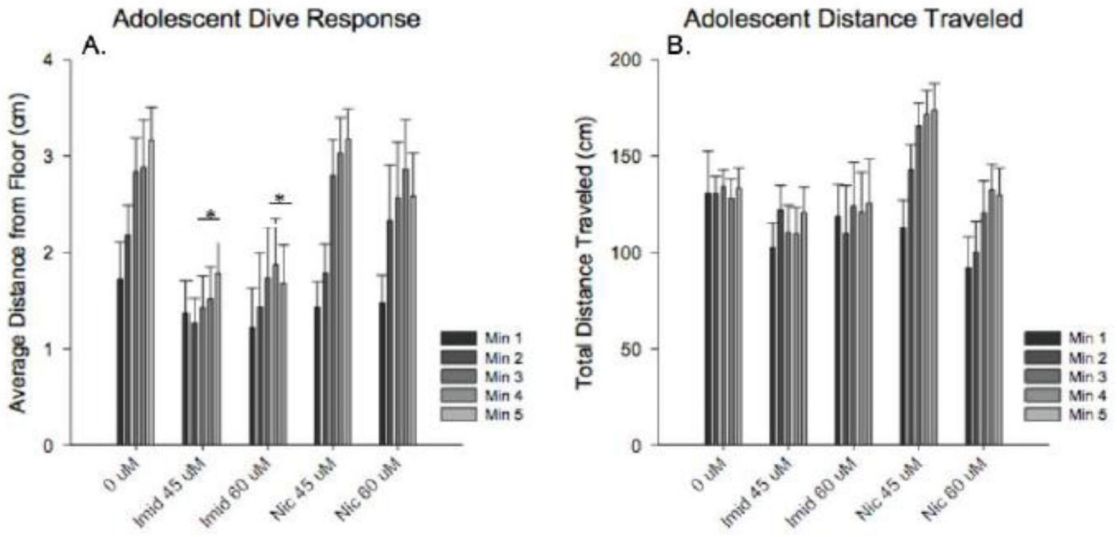

Figure 6. Adolescent zebrafish, novel tank exploration

Distance from the tank floor for each minute of a five-minute novel tank exploration trial (Panel A). Total distance traveled for each minute of a five-minute novel tank exploration trial (Panel B). Each bar represents the mean distance for one minute (min 1-5); data are organized by exposure group. Error bars represent SEM, "*" indicates significant post-hoc (significantly different from control). N=15-18 per exposure condition.

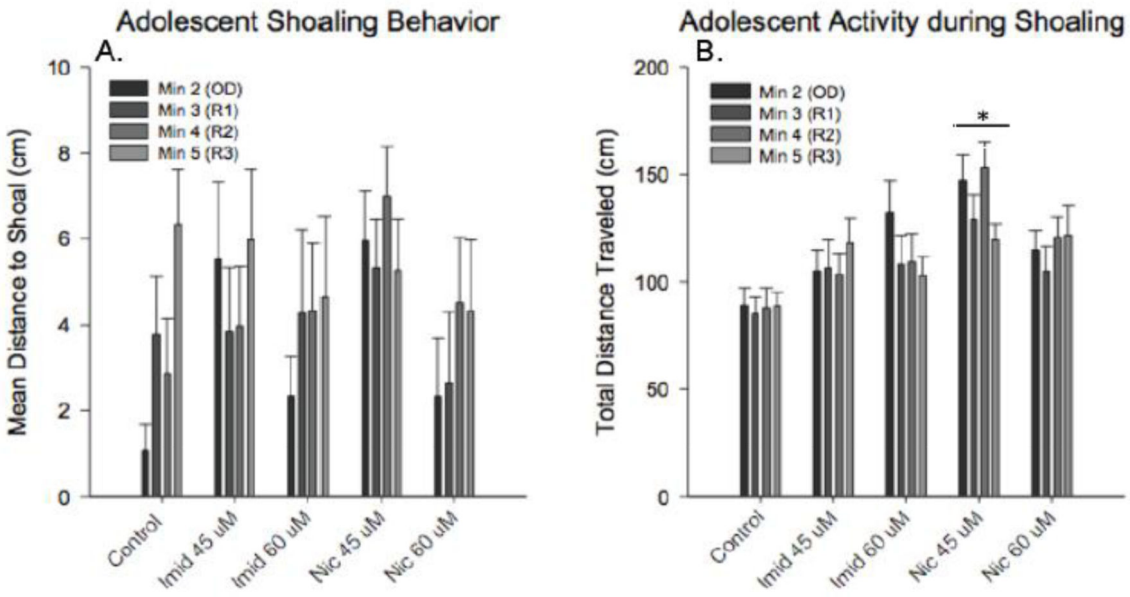

Figure 7. Adolescent zebrafish, social affiliation

Distance to the tank side that is actively displaying the video recording of conspecifics for each minute of the active trial (baseline, min 1, is not shown as it is not possible to calculate distance from an active shoal during that time) is plotted by exposure group (Panel A). Total distance traveled for each minute of the active trial is plotted by exposure group (Panel B). "OD" refers to the original discrimination (side left or right), "R1" refer to reversal #1, "R2" to reversal #2 and "R3" to reversal #3. Error bars represent SEM, "*" indicates significant post-hoc (significantly different from control). N=15-18 per exposure condition.

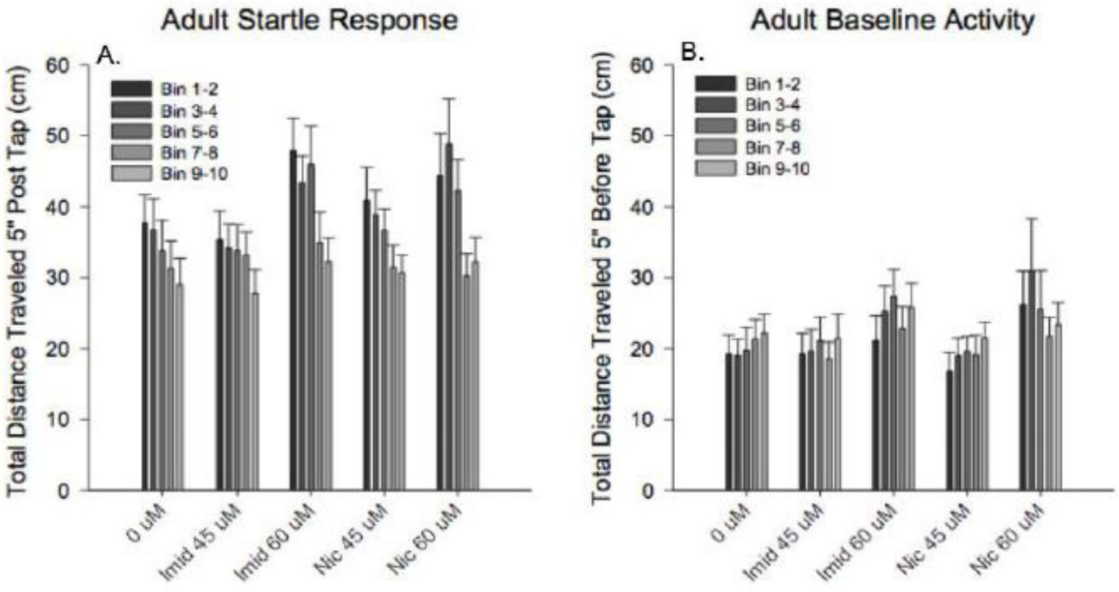

Figure 8. Adult zebrafish, sensorimotor habituation

Data are plotted exactly as those in Figure 5 for the adolescent cohort. N=30-34 per exposure condition.

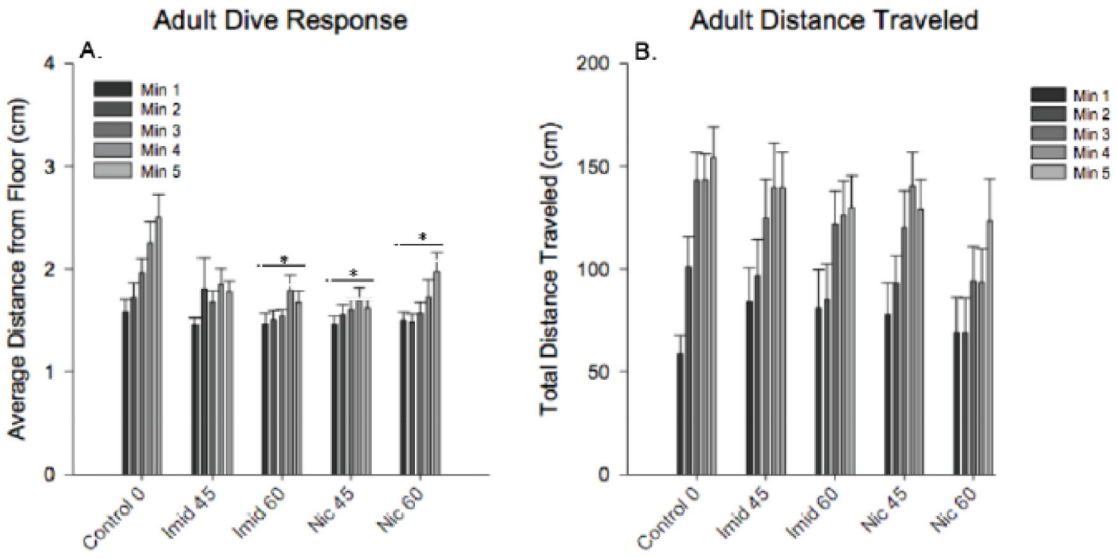

Figure 9. Adult zebrafish, novel tank exploration

Data are plotted exactly as those in Figure 6 for the adolescent cohort. N=30-34 per exposure condition.

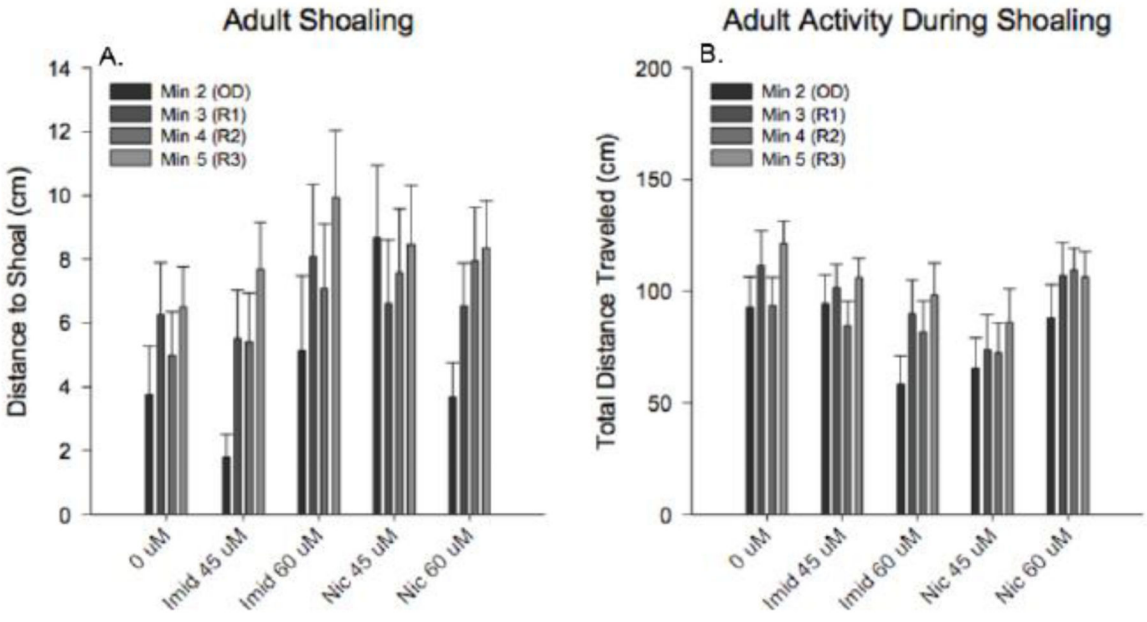

Figure 10. Adult zebrafish, social affiliation

Data are plotted exactly as those in Figure 7 for the adolescent cohort. N=30-34 per exposure condition.

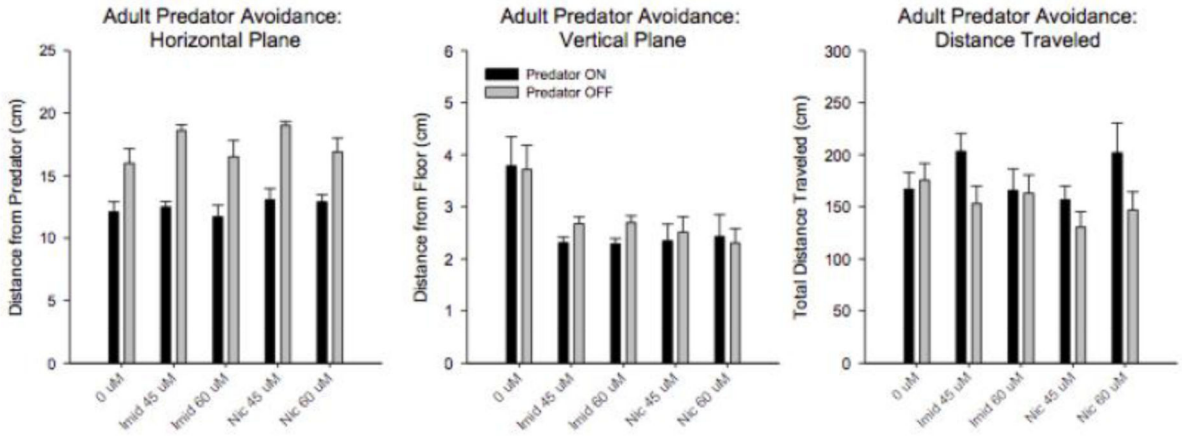

Figure 11. Adult zebrafish, predator escape and avoidance

Mean distance from the tank wall displaying the predator stimulus is plotted for each exposure group in the presence (filled bars) and absence (open bars) of the predator stimulus (Panel A). Similarly, distance from the tank floor is plotted (Panel B) and total distance traveled (Panel C). Filled bars correspond to the "escape" component of this task and the open bars correspond to the "avoidance" component. Error bars represent SEM, "*" indicates significant post-hoc (significantly different from control). N=30-34 per exposure condition.