A Behavioral Test Battery to Assess Larval and Adult Zebrafish After Developmental Neurotoxic Exposure

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Abstract

Behavioral batteries are valuable methods which allow outcomes with varying characteristics and neurobiological bases to be assessed and compared in the same animals. This allows investigators to construct a profile of impairments produced by a pharmacological or toxicological challenge, and to propose mechanisms for further study based on those findings. This profile is valuable in the assessment of potentially hazardous substances, including environmental toxicants, drugs of abuse, and other neuropharmacologically active agents. Behavioral tests and batteries have been developed for a number of species, including a relatively recent and growing body of work with the zebrafish, *Danio rerio*. The following chapter discusses the current zebrafish behavioral battery used in our lab, and some of the main factors that drove its development. The principal tests include a motility assay for larval fish (6 days post fertilization, dpf), and a battery intended for adolescent (2–3 months) and adult fish (5+ months), which assay sensorimotor, affective, and cognitive-like functions in these fish. Significant progress has been made in the areas of zebrafish neurobehavioral analysis, although further studies, refinements, and task development efforts will be needed to strengthen this approach in the future.

Keywords

Zebrafish
Neurotoxicology
Behavior
Sensorimotor response
Emotional function
Cognition
Social behavior

1. Introduction

1.1. Rationale for Using Zebrafish in Neurodevelopmental Behavioral Toxicology

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Zebrafish are a useful complementary model for toxicology which carry some of the necessary features provided by higher model species like rodents, while preserving many of the practical and methodological advantages of lower models like invertebrates and in vitro models [1]. As such, they have an important role to play in addressing the backlog of chemicals which need a comprehensive toxicology assessment [2], estimated by the EPA to be in the tens of thousands of chemicals. As vertebrates, zebrafish have substantial genetic and functional homology with mammals including humans, which supports their predictive validity and value in risk assessments and mechanistic studies [3, 4]. Additionally, they have a well-studied genome and can be genetically manipulated for mechanistic analyses of toxicant action and vulnerability, similar to mouse models. Zebrafish have been particularly valued in developmental toxicity studies, where the strengths of this model organism are amplified. As fish embryos are translucent and grow *ex utero*, developmental processes are easily visualized, allowing them to be used for longitudinal studies in early development which cannot be reasonably conducted in mammals. Additionally, their lack of parental dependence and rapid motor development allows neurobehavioral testing to begin within the first day after fertilization and be further conducted at any desired point in their lifespan. As they age, their small size, high housing density, and low material costs allow them to be efficiently maintained for lifespan-length aging studies which are possible, but prohibitively costly, in mammals like rats, mice, and nonhuman primates [5, 6]. This advantage also allows studies to include wider concentration-response ranges and more complex mixtures than could be completed with rodents, based on the strains that numerous animals and treatment groups place on materials and space [7].

It is important to note that as with other lower models, there are limits to the predictive validity of zebrafish relative to mammals. A portion of their genes are not homologous with humans, and some features like sex-determination and reproductive systems are not readily comparable with humans [8, 9]. As ectothermic species with genome duplication (naturally evolved in teleost fish), metabolic regulation in zebrafish does have meaningful differences relative to mammals, and these differences must be considered when interpreting relevant findings. Additionally, the nervous system of fish is considerably less complex than mammals and lacks the structural and functional homology that rodent and primate models can provide. Relevant to toxicant exposures, the route of administration for fish is generally immersion, with chemical transfer across membranes, such as those in the gills, which may have pharmacokinetic differences from traditional mammalian methods such as injection or oral consumption/gavage, and these differences may be exacerbated by any additional differences in metabolism. On the other hand, zebrafish provide a more behaviorally and developmentally relevant view of the vertebrate nervous system than is modeled by invertebrate models and cell or slice cultures. Without a mammalian brain structure, zebrafish can still provide valuable insight into how conserved developmental processes act to build a complete and behaving nervous system, and how networks, cells, neurotransmitters, and ecologically relevant behaviors are affected by toxicant exposures [10, 11].

Along the spectrum of models used in toxicology, zebrafish have a profile of advantages and limitations that allow them to fill a gap between mammals and invertebrate or in vitro models. They have moderate throughput, cost, and complexity, and so may be suited to answering research questions that cannot be adequately addressed using other models. Our perspective on the zebrafish is that it is a complementary model, rather than an alternative or replacement for these other models. For applications to human health, toxicology data from zebrafish may be best used to provide preliminary risk assessments and hypotheses that can drive future mechanism studies and help set priorities for future basic and epidemiological research.

1.2. Zebrafish Husbandry and Exposure Considerations

Zebrafish are relatively simple to maintain in a lab setting, although certain considerations must be made for their housing, breeding, and exposures. Our zebrafish are fed three times per day using a combination of live food (*Artemia Salina,* Brine Shrimp Direct, Ogden, UT, USA, at 9 am and 4 pm) and solid food pellets (Gemma Micro at 12 pm) and maintained in flow-through aquatic racks

(Aquatic Habitats/Pentair AES, Apopka FL, USA; Tecniplast USA, West Chester PA, USA). System water is a mixture of sea salt (Instant Ocean, 0.5 g/L) and buffer (Seachem Neutral Regulator, 0.3 g/L, Seachem Alkaline Regulator, 1 g/L) in deionized water. The flow-through water system connects all tanks to a central reservoir and allows automatic water exchange, temperature control and filtration, as well as community water chemistry testing and maintenance for all tanks on a given system. Importantly, shared water systems share a microbiome of nitrifying bacteria and algae which maintain water quality and combat the buildup of ammonia and nitrites from uneaten food and other waste. These features make flow-through systems superior to similar approaches with standard benchtop tanks, in that they allow a greater degree of control and consistency for housing conditions across all animals in a study. In some cases, we have conducted adult toxicology studies which required adult fish to be housed in benchtop tanks with individual heaters and bubblers for 4–12 weeks [12], and while this can be practically done, precautions must be taken to ensure the health of the fish. Standalone tanks require daily monitoring of the temperature, aerators, and fish health, as well as weekly (at minimum) complete water changes. Sustainable microbiomes cannot be reasonably established in these benchtop tanks, but can be artificially created by supplementing a portion of the tank water either with colonized water from a flow-through system (e.g., 1/3–1/2 of total volume) or with commercial stocks of nitrifying bacteria (e.g., API Quickstart™).

With respect to egg collection, we use a group breeding method to ensure each cohort contains eggs with a high level of genetic homogeneity. Briefly, tanks of mixed-sex breeders (housed at <5 fish per liter) are fed 4 times (rather than the standard 3) and a removable egg collection trap is placed in the bottom of each at ~4 pm. The trap consists of a plastic tray with tapered sides, narrow slits on the side to allow some water circulation, and a latticed lid with square holes large enough to allow eggs to fall through. To encourage the females to lay eggs on this box, artificial vegetation is attached to the lid. When the collection box is placed near the end of the day (4 pm or later), no eggs will be laid or fertilized until the following morning. If they are placed too early, however, some females may lay eggs the same day, resulting in eggs of mixed ages within the same batch.

On the morning of egg collection, the traps are removed and all eggs are washed on a mesh screen to remove debris, rinsed with a 0.01% bleach solution for 60 s to remove microorganisms from outside of the eggs, and then rinsed twice with fresh system water. After this, the eggs are transferred to a petri dish and incubated at 28 ° C (±1°). At 3–4 h post-fertilization (hpf), the embryos are sorted under a

dissecting microscope and selected embryos are randomly divided into glass petri dishes (9 cm diameter × 2 cm depth) at a density of 1 embryo per mL of system water (40 embryos per 40 mL). These petri dishes are then randomly assigned to one of the available treatment conditions for a given study. Our standard embryonic exposure protocol begins with a complete water change of the petri dish at 5 hpf, and then a change of the exposure medium every 24 h until 120 hpf. Most of the compounds we test are lipophilic, so our standard exposure medium is 0.1% dimethyl sulfoxide (DMSO). At each exposure medium change, the embryos are examined for death or dysmorphogenesis, and a log is kept on the type and frequency of any deformities. We note visible deformities including truncated bodies or tails, small or missing eyes, pericardial edema, and lordosis/scoliosis. All embryos with visible deformities are removed prior to the medium change. If a certain concentration leads to a majority of embryos in a dish dying or showing dysmorphogenesis (# dead + # deformed ≥50% of fish), that dish is excluded from behavioral testing. If this pattern of disruption is consistent across replicate plates, the concentration is excluded entirely from subsequent behavioral testing. In our protocol, the primary goal of counting deaths and deformities is to establish the concentration-threshold for lethality and/or anatomical dysmorphology, and to investigate concentration ranges that fall below that threshold. At 120 hpf, all larvae are rinsed twice and housed in fresh system water until the time of larval motility testing (144 h).

With respect to exposures, there are a few important considerations. Our embryos are group-reared in petri dishes, while many other labs individually rear fish in microtiter plates with up to 96 wells [13]. Individually housing the embryos allows for greater tracking and measurement of the development of each fish, as the identity of a group-housed embryo or larva cannot be verified from one time point to another. The inability to track the identity of each embryo is not a major concern for our protocol, which does not take detailed measurements of the embryos and is primarily interested in concentrations below the threshold for overt dysmorphology. Another advantage of the well plate approach is that the fish are housed in the same 96-well apparatus where larval motility is usually tested. This does eliminate the need to rehousing and acclimation prior to testing; however, once the larvae can freely swim, their movements are restricted by the size of the well. Padilla and colleagues [14] did a comparison of the locomotor activity of fish raised in microtiter plates with larger or smaller wells and found that fish raised in 48- or 96-well plates (smaller wells) were substantially less active than those raised in 24-well plates (larger wells). This seems to indicate that raising embryos in restricted spaces may affect their swimming behavior. Group rearing in a petri dish (9 cm diameter)

bypasses this concern and additionally provides the sensory stimulation of living in a shoal of other larvae.

Follow-up neurobehavioral testing can be performed at any age following the end of a drug exposure, although certain considerations may need to be made for housing and testing based on age. Embryonic or larval testing typically takes place during the yolk-sac stage (0–6 days post fertilization (DPF), while the animals do not require feeding. Equivalent testing is sometimes performed within a span of several days (e.g., 10 dpf) following the end of this stage without changes to housing, although water quality parameters will need to be monitored to ensure that uneaten powdered food does not result in potentially hazardous ammonia production. We generally transfer fish from petri dishes to our flow-through water system at 6 dpf, where they stay for the remainder of their lifespan. To support growth, we provide water supplementation to larval to juvenile tanks (up to 30 dpf) as a drip to eliminate any current which the fish would need to swim against, and progressively transition the fish to a higher flow as they grow. Beginning at 30 days of age, these juveniles are transitioned from the powdered baby food to live food (brine shrimp) and from the drip to a typical water flow condition. Follow-up testing generally takes place in adolescence, adulthood, or both. Zebrafish reach sexual maturity around 3 months of age, although in our experience they do not typically reach a normal adult size until closer to 5 months of age. Based on these observations, we test for adolescent effects of an embryonic exposure at 2–3 months of age, and adult effects in 6+ month old fish. In the latter portions of the lifespan, we assess effects in late adulthood at 14–15 months of age, based on our observations that fish stocks show increased rates of spontaneous scoliosis and morbidity when they exceed 18 months of age.

1.3. Behavioral Test Battery

It is important that a behavioral test battery evaluate a variety of functions including assessments of sensorimotor, emotional, social, and cognitive function. As the battery develops, the tests can be refined to improve validity, sensitivity, reproducibility, and efficiency. Validity can be determined in several ways. The environmental and drug challenges that affect behavioral responses in zebrafish should correspond to those that affect behavioral responses in mammalian experimental models and humans. The behavioral response will not be exactly the same across species but rather will be appropriate for the ethology of each species. For example, in the novel tank diving test described in detail below, an anxiolytic response would increase the time spent near the bottom of the tank, while a similar

response in rats would increase the time spent near the sidewalls in an open field or in the wall-bordered arms in an elevated plus maze. Both fit can be interpreted based on a reduction of predatory risk in ways that are specific to their natural environments. Understanding these differences is necessary to ensure the construct validity of each test and the predictive validity between zebrafish and other animal models.

1.3.1. Video Tracking of Behavioral Response

Whenever possible, it is useful to include automated motion tracking in a behavioral battery for zebrafish. A number of companies have developed behavior analysis software which can detect an animal within a video and collect data on its movements, either in real time or using prerecorded testing sessions. These products have a variety of motion detection settings which must be adjusted to optimize animal identification, eliminate the tracking of nonanimal objects, and maintain consistent tracking of the same animal throughout a session. The most common issue with the tracking of zebrafish is a lack of strong color contrast between the fish and the background, which will result in video frames where no subject is being tracked and no data is collected. Our adopted program, EthoVision XT® (Noldus Information Technology, Wageningen, The Netherlands) provides a data filtering option which will include or remove subjects from analysis based on a calculation called “subject not found.” Our image sampling rate is 20 frames per second, and our threshold for inclusion in any dataset is <4% of all frames labeled as “subject not found.” Color contrast can be optimized by using white or backlit backgrounds, improving lighting, and by adjusting the brightness and image quality settings on the camera. Basic nonscientific cameras can be sufficient for this purpose in adult testing, while a specialized setup like the DanioVision™ lightbox (Noldus) or other design with high-resolution video and/or magnification is needed for embryos and larvae. In our protocol, the appropriate camera feed is run directly through the Ethovision software, live scored, and saved in autogenerated video files.

Automated motion tracking products also have a variety of computational options for the data and can report movement data as total or average distances moved, velocity, average distance from an object or zone, and the frequency of crossings between quadrants or zones, among others. All of these outcomes can be scored and calculated by hand, but the automation of this testing will relieve some of the drawbacks to animal behavioral testing, while also protecting the validity of the data.

Automation of both data collection and stimulus delivery has been a major focus in the development of the zebrafish battery for a few reasons. First, this ensures the consistency and quality of each method over the course of each study and between studies using the same battery. Second, it gathers very detailed movement data on the sub-second time scale, third, it improves the throughput of the battery substantially and allows comprehensive data files to be generated, exported, and analyzed, all on the same day that the tests are completed. Finally, it eliminates the need for the experimenter to be within sight of the fish, which may lead to startling or stressing of the fish each time the experimenter moves.

2. Materials and Methods

2.1. Larval Neurobehavioral Testing

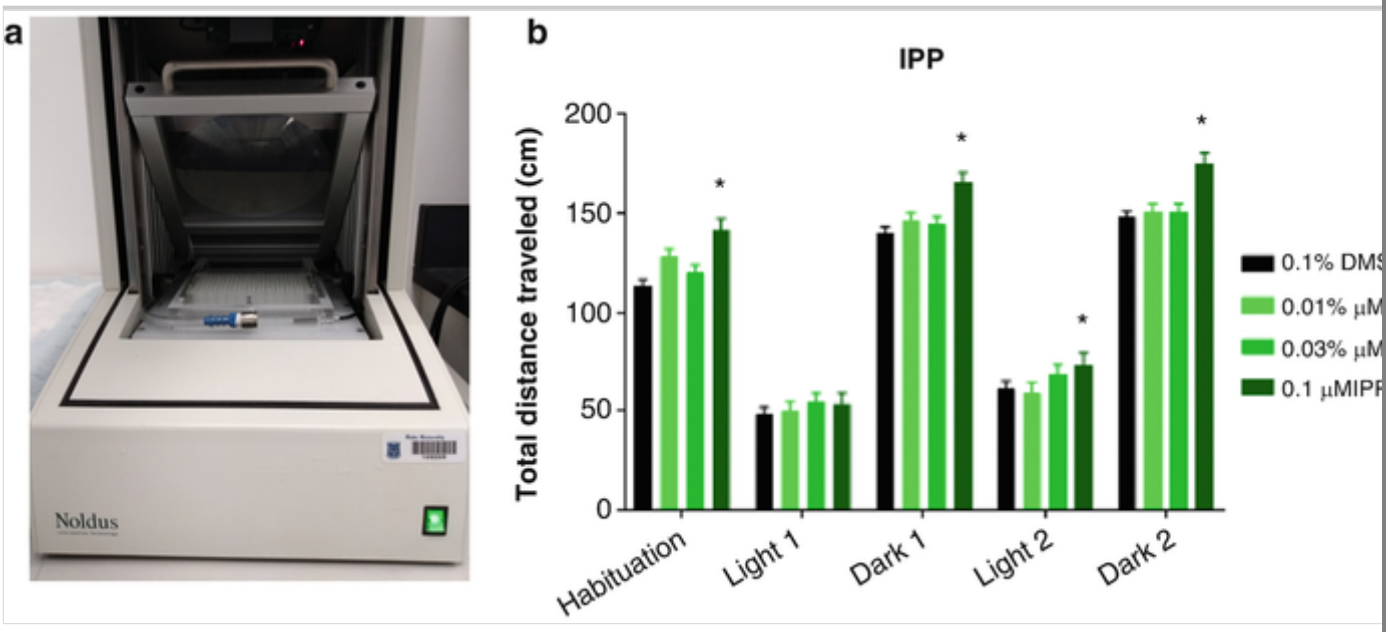
The earliest zebrafish neurobehavioral assays can be conducted during embryonic or larval development. Motor functions develop quickly over the first several days of life, beginning with spontaneous tail flexion in the unhatched embryos around 17–19 hpf. These tail flexions have allowed some groups to observe neurotoxicity-induced locomotor deficits as early as 24–36 hpf [15, 16]. At these early time points, the immature embryos cannot freely swim, but do flex their tails spontaneously at a low rate and a much higher rate in response to a flash of light. This response goes through rapid inhibition and will fail to be elicited by a second flash of light delivered quickly after. As the fish continue to develop, behavior analysis can become more sophisticated and can include swimming and responsivity to other forms of stimulation. For example, Hahn and colleagues have characterized the circuitry underlying a larval startle response and the potential for neurotoxicity within this circuitry [17]. The approach of our lab and others has been to measure motility of free-swimming larvae.

Larval motility is most often tested between 120 hpf and 144 hpf, when the larval swim bladder is inflated and the yolk is still present. In our protocol [18], we perform larval motility testing at 144 hpf, which is 24 h after the end of a standard exposure. Testing is performed in a 96-well plate, fitted with circular 0.5 mL glass well inserts (*see* 96-well plate in DanioVision™ lightbox, Fig. 1a). Since we group-rear our fish in glass petri dishes, the larvae must be acclimated to the 96-well plate prior to testing. On the morning of testing, fish are individually loaded into wells and acclimated for 1 h in a dark incubator. The testing session is 50 min, consisting of 5 alternating periods of dark and lit conditions (*see* representative data, Fig. 1b). The first 10 min are conducted in the dark and treated as an acclimation or

habituation phase. We generally observe elevated levels of locomotor activity during this phase, since this occurs in the dark (0% illumination) and larvae are stimulated under dark conditions. However, the activity in the habituation phase is often noticeably lower than in the later dark phases of testing, likely due to the recent stress of being moved from the incubator to the DanioVision™ lightbox (Noldus). After the 10 min of acclimation, the floor light is turned on (100% illumination, 5000 lux) and left on for 10 min, which decreases locomotor activity for the duration of that lighting phase. Following this, the sequence continues with lights automatically turning off at 20 min, on at 30 min, and off at 40 min. Each lighting change (light-dark, dark-light) is repeated twice in order to detect any interactions between neurotoxic treatments and behavioral adaptations which can occur when stimuli are presented multiple times. The general technique of light and dark motility is widespread in zebrafish toxicology, although a number of variations exist using different numbers of light/dark phases and lengths of time in each phase (e.g., [19, 20, 21]).

Fig. 1

Light/dark larval motility assay, (**a**) DanioVision Apparatus; (**b**) Larval activity data is shown across 5, 10-min time blocks with alternating dark-lit conditions. Embryonic exposure (5–120 hpf) to the flame retardant IPP (0.1 μM) led to locomotor hyperactivity in 4 of the 5 time blocks (mean ± sem, $N = 50+$) [18]



In terms of data analysis, we primarily rely on the distance moved as the measure of activity. This can be further analyzed in multiple ways, including total distance

moved in each min or each lighting phase, average distance per min under lit or dark conditions overall, and the change in activity (either positive or negative) during the transition from dark to light, or light to dark. If there are multiple repetitions of a dark or light phase, as in our protocol, it is statistically necessary to analyze these repetitions as repeated measures factors, prior to any analysis of average-dark and average-light activity score. This is because treatment by replicate interactions could be present (e.g., [18]) and any differences in treatment effects between repetitions must be considered.

2.2. Sensorimotor Response

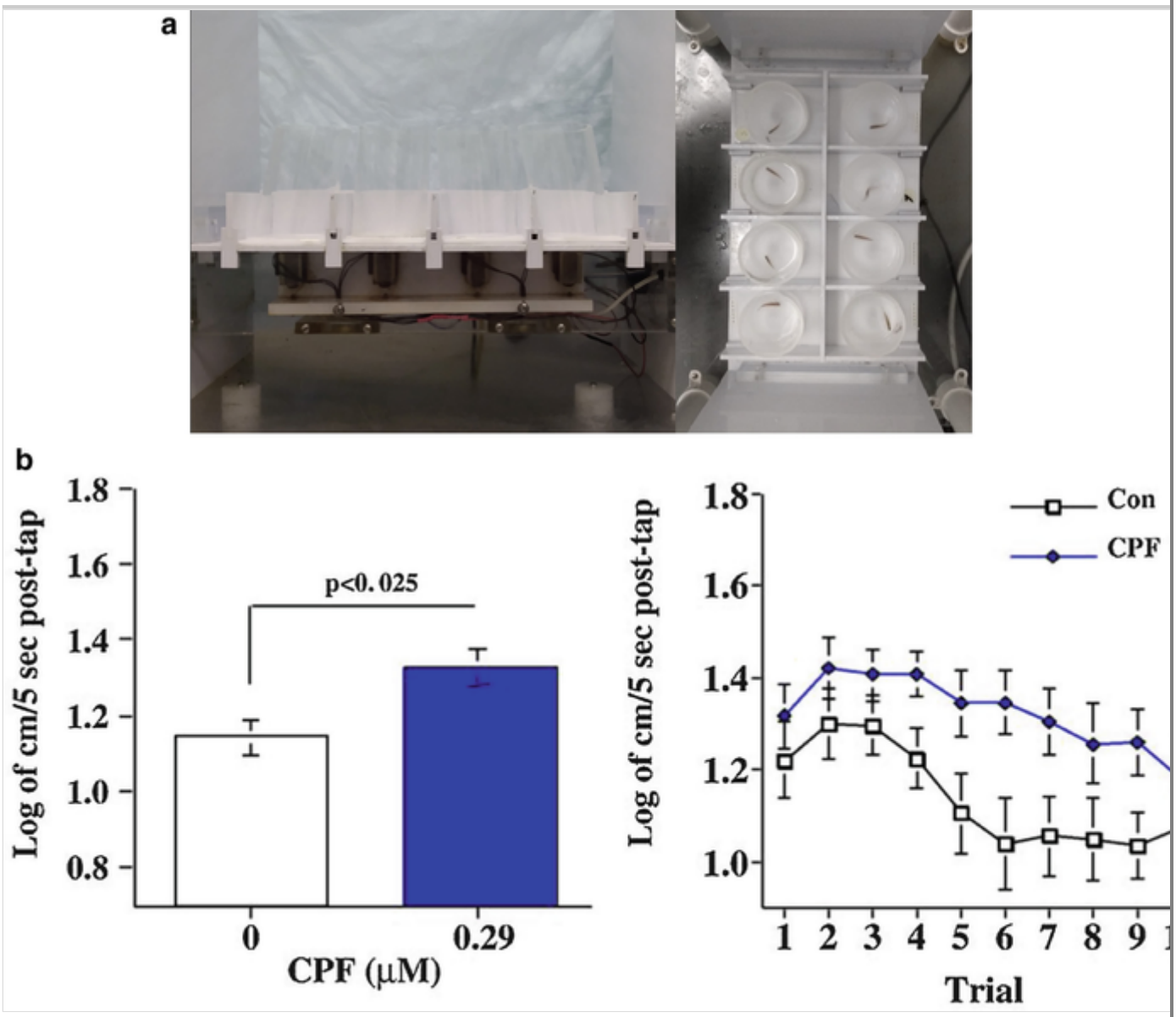
Acoustic startle assays are available for both larval and adult fish, and demonstrate a sensorimotor reflex triggered by a loud noise, or similar vibration through the water. In zebrafish, startle is measured as a brief spike in locomotion following the onset of the sound stimulus [22]. This is in contrast to rodent startle responses, which are characterized by freezing or a stereotyped contraction of the body without locomotion [23]. An adult zebrafish startle can be simply measured by subtracting the baseline levels of locomotion, measured as the distance moved in the 5 s immediately before the stimulus, from the distance moved in the 5 s immediately after the stimulus [24]. This differential represents the amount of activity that can be attributed to the stimulus. As with other forms of reflexive behavior, not all fish will startle every time a stimulus is delivered, and between-group differences will reflect a combination of the rate of the response being elicited and the relative size of the response when it is elicited.

2.2.1. Tap Startle Testing

Our protocol for tap startle was originally developed by Eddins, Cerutti, and colleagues [24], and uses a custom-built tapping apparatus (Fig. 2a). This apparatus consists of eight clear Plexiglas wells (5.5 cm diameter) arranged in a 2 × 4 setup (40 mL system water, depth 2.5 cm), with short white plastic dividers to prevent the fish from seeing one another. The walls of each well are gently angled to provide a clear line of sight to the mounted camera above and eliminate blind spots. The platform for each well is fitted with a centered hole containing a 24-volt DC push solenoid, which strikes the well floor with a metal pin when triggered. Each session of tap startle consists of 10 tap stimuli, presented 1 min apart. The tap sequence begins 30 s after the start of the trial to provide brief acclimation. Tap startles are defined using the distance moved by the fish in the 5 s prior to the tap (pre-tap), the 5 s following the tap (post-tap), and the differential between them (post-tap – pre-tap).

Fig. 2

Tap sensorimotor startle and habituation test, (**a**) Apparatus; (**b**) Post-tap activity (normalized via log-transformation) is reported as an average (left) or for sequential tap stimuli (right). Embryonic chlorpyrifos exposure (0.29 μM, 5–120 hpf) led to elevated post-tap activity and impaired patterns of habituation (mean ± sem, N = 24–40) [24]



We typically analyze tap startle data using raw total distance moved scores (*see* representative data, Fig. 2b), treating the 10 replicate taps as a repeated measures variable (e.g., [12]). Sensorimotor habituation and adaptation are measured as main effects of tap on activity, analyzed separately for pre-tap activity, post-tap activity, and the differential (or tap startle magnitude). Pre-tap activity scores tend to show

an increasing trend across taps, while post-tap and differential scores tend to show a decreasing trend across taps. The distances moved prior to or after the tap can often be skewed, and may require normalization (e.g., log-transformation) for traditional analyses of variance to be performed. A similar statistical method is using a form of linear regression. Linear mixed effects analyses can generate linear models with intercepts and habituation slopes for each subject, and those parameters can then be compared between groups using simplified ANOVAs (as in [18, 25]). This approach does restrict what post hoc analyses can be performed, and so is not suitable for all hypothesis testing.

2.3. Novel Tank Exploration

One of the most common neurobehavioral tests across model species is exploratory behavior in a novel environment. Common variants for rodents include open field and elevated plus maze, which allow measurement of locomotor activity and spatial preferences while exploring the maze [26]. Rodents show a phenomenon called thigmotaxis, which is an innate aversion to open spaces and preference to stay close to walls. This is understood to be a defense mechanism which removes certain angles of attack and minimizes the chances of predation. Zebrafish have a similar innate aversion to the surface and preference for the floor of a novel tank [27]. The magnitude of this aversion, or any attenuation of it by a treatment, is often interpreted as a change in an anxiety-like function. This task has been behaviorally validated as measuring the diving response when the tank is novel but not when it is familiar and pharmacologically validated with attenuation of the diving response after administration of the anxiolytic drugs diazepam and buspirone [28].

Novel tank behavior can be measured in tanks of various shapes and sizes. Using a tank that is both deep and wide allows for diverse swimming patterns, although it does create some technical problems to overcome. Techniques have been developed to map the location of the fish in 3-dimensional space (e.g., [29, 30]), using two cameras to synchronously track the fish as it moves side to side, front to back, and top to bottom within the tank. Two-dimensional analyses are also quite common and can be reasonably accomplished using a narrow tank viewed from the side.

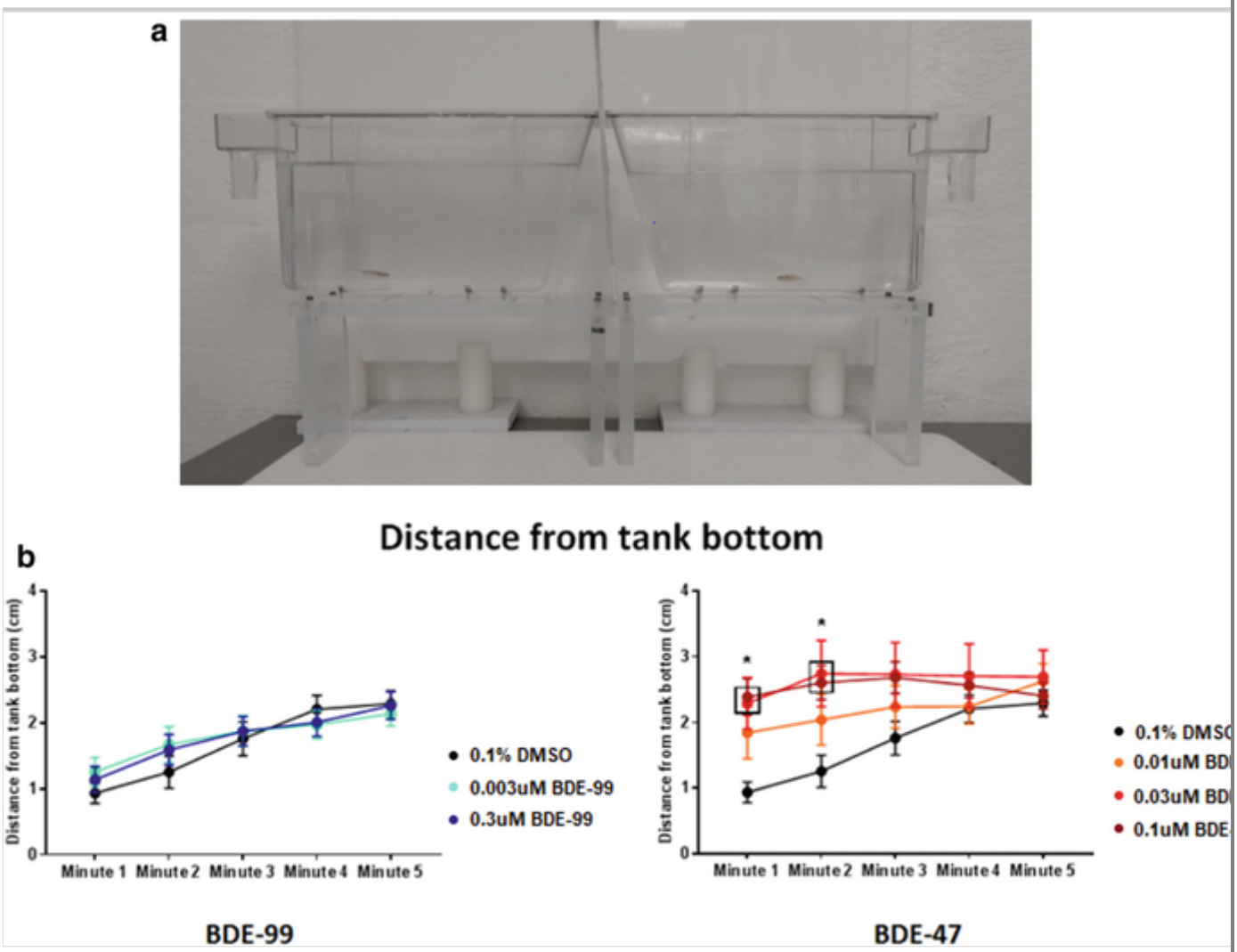
2.3.1. Novel Tank Dive Test

In our protocol (e.g., [18]), adolescent or adult fish are given 5 min to explore a narrow trapezoidal 1.5 L tank (26 cm × 5.5 cm at the top) filled with 10 cm of system water (1.2 L of system water) (*see* test apparatus and tracking pattern, Fig. 3a). In this design, the fish are allowed to dive to the bottom and continue their

stereotypic swimming pattern, which consists of continuous back and forth swimming. Early in the session, the fish will choose to make very few, if any, visits to the surface, but as time passes, they will acclimate to this environment and make progressively more visits to the surface (*see* representative data, Fig. 3b). Similarly, the fish will remain less active early in the session, with some individuals remaining still during the first min(s) of the session, but as time goes on, locomotor activity will tend to increase.

Fig. 3

Novel tank diving test to assess anxiety response vs. risk taking, (**a**) Apparatus; (**b**) Diving responses, represented as distance from the bottom (cm), are shown for two brominated flame retardants (BDE-99, left; BDE-47, right). BDE-47 (0.03–0.1 μM, 5–120 hpf) impaired the diving response during the first two 1-min time blocks of the session (mean ± sem, $N = 21–28$) [25]



The primary measures we gather represent either locomotor activity or anxiety-like floor preference. We generally represent locomotor activity as the distance moved within each min of the session and across the session, although the behavioral software provides other options, including time spent moving and average swim speed. For attraction to the bottom, this bias can be represented in multiple ways as well. In previous iterations of this test (e.g., [28]), we represented attraction to the floor by drawing a zone around the bottom third of the tank and having the software measure how much time the fish spent in that zone. This is a simple and elegant measurement, although the edges of the lower zone are arbitrary and depth may be more accurately judged as a continuous variable. In our current design (e.g., [18]), we place a thin zone around the floor of the tank and have the software generate an average distance from that zone across each min of the session. This distance from the zone measure reliably increases across time. For interpretability purposes, the distance moved and distance from the bottom should be evaluated side by side in order to allow better interpretation of each measure. Treatments which severely suppress locomotion may reduce distance from the bottom as well, if this results in immobile time, where the fish remain still on the bottom of the tank. In other circumstances, these two measures are fairly independent of one another, and it is common to find a treatment effect on one, but not the other.

2.4. Social Behavior

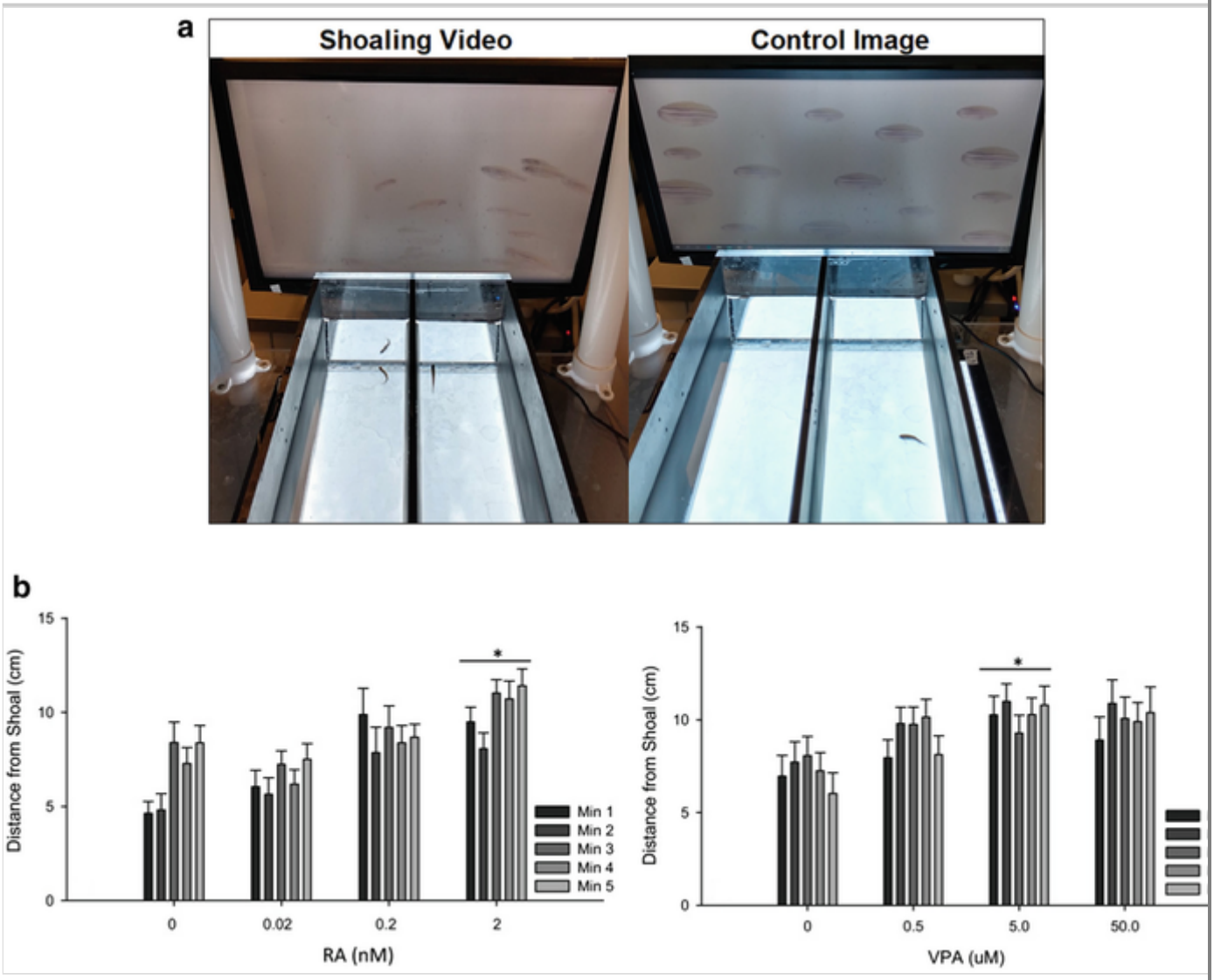
Zebrafish are social animals and regularly swim in a shoal, weaving around other fish in a loose cluster. This attraction to conspecifics can be incorporated in a formal test of social attraction. The best method for measuring this attraction is a matter of some debate. The most naturalistic method for measuring social behavior is by allowing fish to swim in a group, and then assessing how "cohesive" that shoal is based on the attraction of each fish to the others (e.g., [31]). However, this approach provides some computational and statistical challenges. For example, the software required must be able to reliably track and discriminate between each fish, even when their silhouettes merge and separate repeatedly throughout the test. Additionally, the behavior of each fish is not independent from the other fish in its shoal and treatment effects on social cues and social responsiveness may be conflated. An approach that solves this is using untreated fish as the social cues. This can be effectively done by placing live fish into a visible compartment separated by a divider (e.g., [32, 33, 34]), or by playing prerecorded videos of a shoal of zebrafish within view of the test fish (e.g., [18, 35]). In each of these cases, social attraction can be measured using approach behaviors, or relative distance from the shoal when isolated, but in view of the shoal.

2.4.1. Shoaling Assay

Our protocol is adapted from the method developed by Gerlai and coworkers [36] with modifications [18]. Fish are individually housed in narrow 1.5 L tanks for 30 min prior to testing to enhance social attraction. After this deprivation period, the fish are moved to a clear Plexiglas partitioned tank which is separated into lanes (9.25 cm × 28 cm) by black plastic walls, and placed above a light box (Huion Technology, Shenzhen, China) (*see* apparatus and stimuli, Fig. 4a). The light box serves as a backlight and enhances the video tracking of the fish when recorded from above. Each lane is filled with water to a depth of 10 cm (2.75 L of system water). On either end of the tank are 19.5-inch LCD monitors which display either a video of a zebrafish shoal or control shapes, which are unmoving, size-matched ovals patterned after zebrafish stripes. The test session consists of a 2 min baseline with control shapes on both screens, followed by a 5 min period with a shoaling video played on one of the two screens. Social attraction would then be scored by the relative preference for the end of the tank close to the shoal while the video is playing.

Fig. 4

Shoaling Test for assessing social affiliation, (**a**) Apparatus; (**b**) Social approach is shown as the distance from the screen playing the shoaling video, Embryonic exposure (5–120 hpf) to 2 nM valproic acid (left) or to 5 μM vitamin A (right) led to greater distances from the shoaling stimuli (mean ± sem, $N = 30$) [67]



Attraction to the shoal is measured as an average distance from a small zone covering the edge of the tank adjacent to the cue screen (*see* representative data, Fig. 4b). As zebrafish tend to swim back and forth in a stereotyped fashion, they do not spend all of their time adjacent to the screen even when the video is playing. Instead, they make repeated visits of varying durations to the wall of the enclosure, which leads to an overall preference for the shoal-oriented side of the tank. The relative side preference of a fish is analyzed as average distance from the shoal-adjacent wall during the baseline and video phases, as well as using a differential (baseline – video) to represent the change in preference attributable to the video. An additional endpoint is locomotor activity, which we have found to be considerably higher in the backlit partitioned experimental tank than in the novel tank.

2.4.2. Predator Avoidance

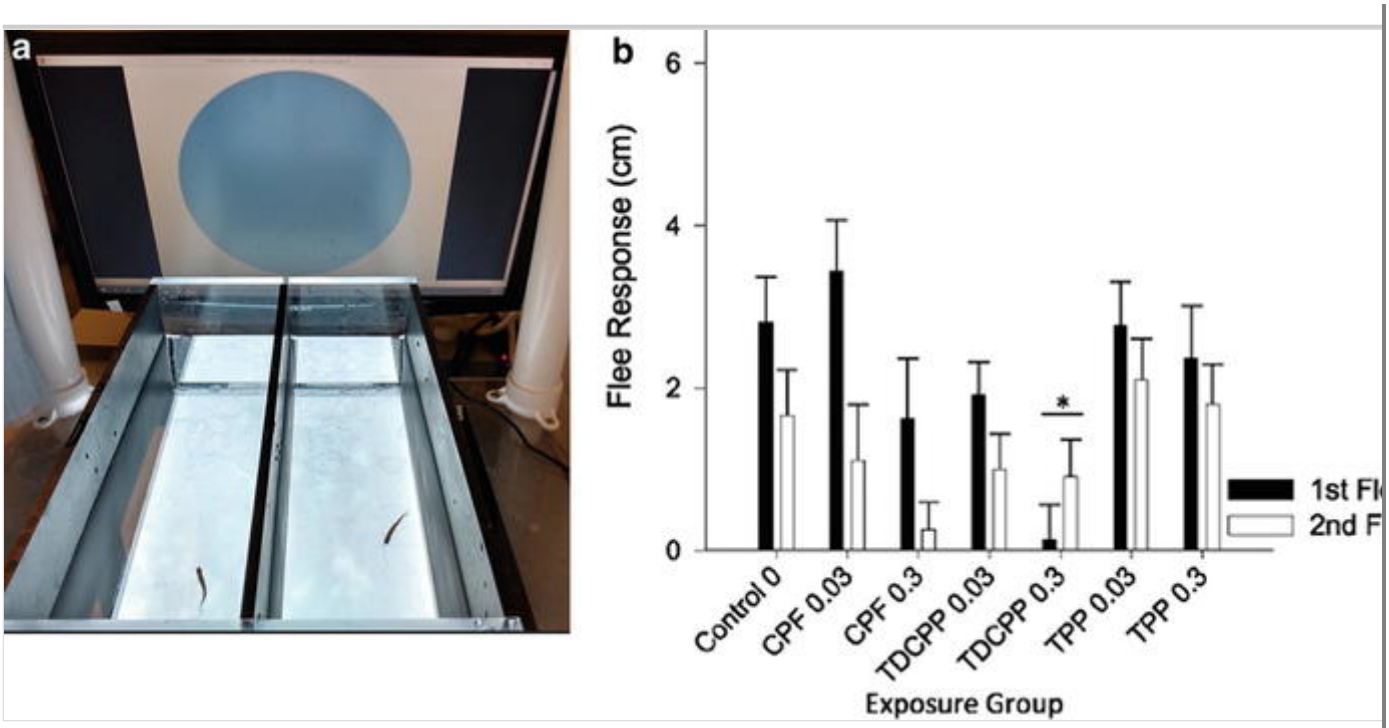
Threat recognition is an important function for zebrafish, as they are prey fish with many natural predators. Vision-based fear-like responses are partly tied to the detection of movement, but zebrafish are also able to discriminate between threatening and non-threatening moving stimuli. For example, zebrafish show fear-like responses to a bird-shaped object moving overhead, videos of Indian leaf fish or needlefish and growing dots, which are 2-dimensional representations of an approaching object [36]. As noted in the previous section, zebrafish can visually identify other zebrafish, and do not show fear-like responses to videos of them. As with other functions, fear-like behaviors can be measured in a variety of ways. Responses to threatening stimuli include an increased preference for the bottom of the tank, erratic movement, jumping or leaping behavior, and freezing or floating [36]. We have further identified spatial aversion to the stimulus (e.g., [37]), which is a directional preference in the opposite direction.

2.4.3. Predator Avoidance Test

Our protocol uses a similar general approach as other fear-response tests [36] with custom stimuli and testing designs [18]. In the current version of this protocol, selected predator stimuli are presented intermittently on one end of the partitioned experimental tank described in the shoaling section above (*see* apparatus and representative stimulus, Fig. 5a). Fish behavior is recorded over a 9-min session containing by four 1-min predator cue presentations corresponding with min 2, 4, 6, and 8 of the session. Each predator cue appears on the same screen (left end of the tank) and a blank white screen is shown during the interstimulus interval, and on the control screen on the opposite end of the tank. Each stimulus presentation consists of a looped video of a growing dot which grows from 1 to 23 cm, to simulate a large object approaching that end of the tank. The intensity of the stimulus can be adjusted by modifying the speed of the dot growth. In our protocol, the first two predator cues are slow moving (4-s duration) while the second two are fast moving (1 s duration). The interstimulus intervals are provided to provide a control or comparison period where no predator is present.

Fig. 5

Predator stimulus escape test for assessing fear response, (**a**) Apparatus; (**b**) Predator avoidance is shown as the magnitude of the fleeing response, or change in position due to the stimulus presentation. Embryonic exposure to 0.3 uM TDCPP (5–120 hpf) led to reduced fleeing responses (mean ± sem, $N = 24–31$) [68]



We primarily measure the fear-like response of fish in this test as an average distance from a small zone covering the edge of the tank adjacent to the cue screen (*see* representative data, Fig. 5b). Zebrafish will tend not to stay against the wall on the opposite side of the tank, but rather continue to swim back and forth in a stereotyped "pacing" manner. As they do this, they will make some approaches toward the cue-adjacent screen. When the cue is present, it can trigger fleeing or an abrupt turn away from the cue when facing it, supporting a shorter pacing pattern that stays further from the screen-adjacent wall. One recent modification of the statistical method has been the treatment of the interstimulus interval. In many cases, but not all, fish will lose their aversion to the predator-paired screen as soon as the cue is removed and explore both sides of the enclosure equally. Based on this, previous iterations of this method have subtracted the baseline, or pre-stimulus distance from the wall from that distance during the cue presentation. This differential would theoretically represent the change in spatial preference due to the cue. However, some recent datasets have shown that the degree to which a fish returns to "no preference" during the interstimulus interval can differ between treatment groups [12]. In some cases, fish maintain an aversion to the cue-paired screen during one or more of the interstimulus intervals, which can be interpreted as a form of fear-based short-term memory. Therefore, the pre-stimulus spatial preference for one cue presentation may be impacted by carryover effects from the previous stimulus. To allow such effects to be detected, we include each cue presentation and interstimulus phase in the repeated measures analysis and consider all potential transitions between cue phases during interpretation.

2.5. Learning and Memory

In non-verbal species, learning is measured as a change in the likelihood of a response due a particular experience, while memory is a situation where current behavior is under the control of cues or events in which took place in the past. Generally speaking, these concepts are related to one another, but they are assessed somewhat differently (e.g., [38, 39]). Measurement of learning, also termed acquisition, requires repeated measurements to establish a change over time or repeated trials, and the evidence for learning is the magnitude of that change (e.g., pretest – posttest). Memory, also termed retention, can be measured on a single occasion, and the evidence for memory is that the rate or likelihood of the response is different than would be expected if no learning had taken place (e.g., observed – expected). A couple examples of learning in zebrafish have already been discussed in this chapter. Specifically, a reduction in the magnitude of the startle response over successive taps shows learning over successive trials, while the tendency to make more trips to the surface of a novel tank over time reflects learning across an extended experience in a novel environment. These are very basic forms of learning reliably shown in zebrafish, but they are perhaps not sufficient for assessing cognition, as they do not have strong face validity for more complex phenomena that are likely to be impaired by toxicant exposures, such as associative conditioning, learning from consequences, and spatial learning or navigation.

To date, a number of learning and/or memory tasks have been developed for zebrafish, largely modeled after rodent paradigms. In these paradigms, zebrafish can be tested for learned preferences for cues or locations (e.g., [40]), navigation of simple mazes (e.g., [41]), learned avoidance (e.g., [42]), and even learning based on interactions with a touchscreen [43]. Overall, however, adoption of these methods has been rather limited. This may be due to the relatively labor-intensive and low throughput of learning tests relative to other tests, and to certain obstacles that limit the adaptation of rodent designs. In terms of measuring complex behavior, zebrafish lack some basic advantages of rodents. The most important differences are that rodents are much more apt to engage with and manipulate objects and can be easily tail marked to allow identification across multiple sessions and multiple days of testing. With few exceptions, zebrafish express their choices and preferences using their location, rather than by actually touching objects, and as mentioned previously, their location in the environment is somewhat relative given their stereotypic back and forth swimming pattern. A related hazard is that zebrafish show freezing behavior when stressed, a phenomenon which can disrupt normal

swimming patterns for up to several min, in our experience. Freezing can make protocols based on repeated handling and testing across trials inefficient and impractical. While a preferable strategy would be to conduct multiple test sessions across multiple days, methods for verifying the identity of a single fish across dates of testing [44] tend to be fairly invasive (e.g., fin clipping, electronic tagging) or cumbersome (e.g., automated color pattern recognition).

In our protocols, we have attempted to develop or adapt learning or memory tests that can provide reliable models of cognitive phenomena, while still remaining practical to run in toxicology studies with large numbers of fish. Below are two paradigms.

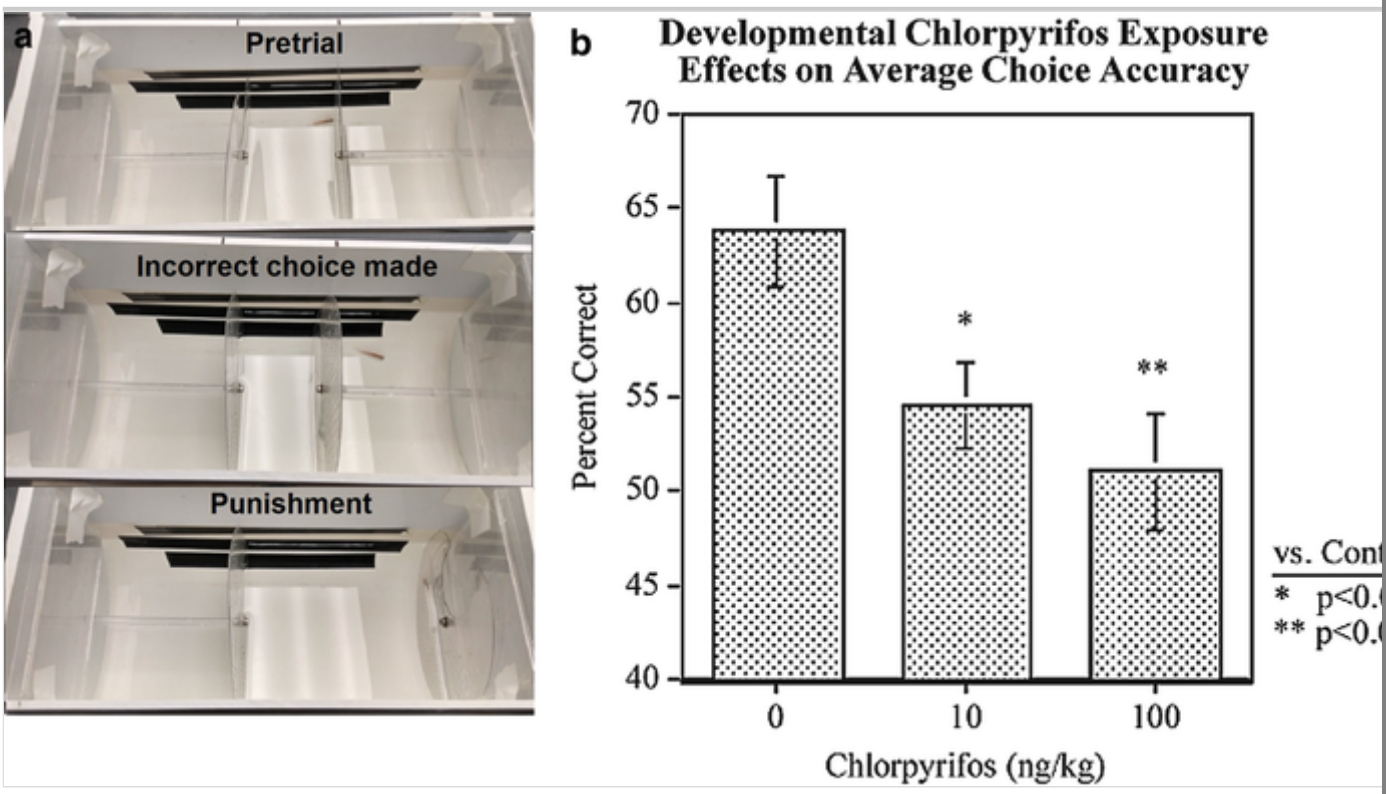
2.5.1. Three-Chamber Task

To measure learning from consequences, we developed a three-chamber test which trains the fish to avoid one chamber and to enter another instead. The 3-chamber task was originally developed with a large 40 L tank in which spatial discrimination, color discrimination, and spatial alternation tasks were developed [45, 46]. Subsequently, it was refined to be conducted in a smaller more efficient apparatus (Fig. 6a) [11]. The apparatus consists of a white PVC cylinder with closed ends (split lengthwise to create a long U-shaped channel) and straight sidewalls attached (overall size – 14.5 cm wide × 28 cm long, × 18 cm high), filled to a depth of 6.5 cm (2.5 L of system water), (*see* apparatus, Fig. 6a). The main channel is subdivided into three compartments by two thin plastic walls which can be moved using handles accessible from the outside of the apparatus. The wall placement is 4 cm from the center of the apparatus, leaving a narrow 8 cm center compartment and two 12 cm end compartments. Moving each wall changes of the size of the adjacent end compartment, which allows the experimenter to either confine a fish in that compartment or expand the swimming space available. In the three-chambered task, changes in the size of the compartment can be triggered as a consequence for entering into a particular end compartment. Zebrafish are relatively claustrophobic and will learn to prefer any compartment where the swimming space is expanded upon entry and avoid any compartment where the swimming space is confined. On the inside of the apparatus, three black lines run down one of the two long walls, to provide cues for spatial orientation.

Fig. 6

Three-chamber test of learning and memory, (**a**) Apparatus; (**b**) Memory is shown as percent choice accuracy, representing the number of trials in which the fish chose the

correct chamber. Embryonic exposure to chlorpyrifos (10–100 ug/l, 5–120 hpf) led to impaired choice accuracy in the three-chamber test (mean ± sem, $N = 12–16$) [69]



To begin the testing sequence, the fish complete a spatial preference test. Each fish is placed into the central chamber of the testing tank with the circular walls positioned so that no doorways are present to allow access to the other two chambers. After 30 s, the dividing walls are rotated so that each contains a circular opening (6 cm diameter). As soon as the fish enters one of the end chambers, the walls are rotated to remove the opening and trap the fish inside. After noting the choice, the fish is returned to the center chamber and the next trial begins. In order to minimize stress, fish are not handled directly when being moved back to the center compartment. Rather, the border wall is turned so that the opening can pass safely over the fish, and carefully slid to force the fish back into the center compartment. Once the fish is in the center again, the wall can be turned to remove the opening and slid until it reaches the starting position. Spatial preference is established when the fish chooses to enter one chamber on three consecutive trials. If no preference is evident after 10 trials, the fish is excluded from further testing. Beginning with the third consecutive entry into a particular chamber, punishment training begins, whereby any entry into the preferred chamber (now referred to as the “incorrect side”) results in the animal being trapped inside and the wall being moved until the chamber is reduced to a width of 1 cm for 60 s. Any entry into the

opposite chamber (now referred to as the “correct side”) results in no consequence and the fish is allowed to remain there for 60 s. Once the fish is returned to the center chamber, the fish remains there for a 30 s intertrial interval, after which the next trial begins. A standard session consists of 7–10 trials, excluding those that established the preferred chamber. Choice accuracy and response latency are recorded manually using a stopwatch and scoring sheet (*see* representative data, Fig. 6b). Learning can be assessed using the raw accuracy scores of each choice (correct = 100% accuracy, incorrect = 0% accuracy) (e.g., [37]) and/or plotting a linear trend to represent their improvement over trials (e.g., [47]).

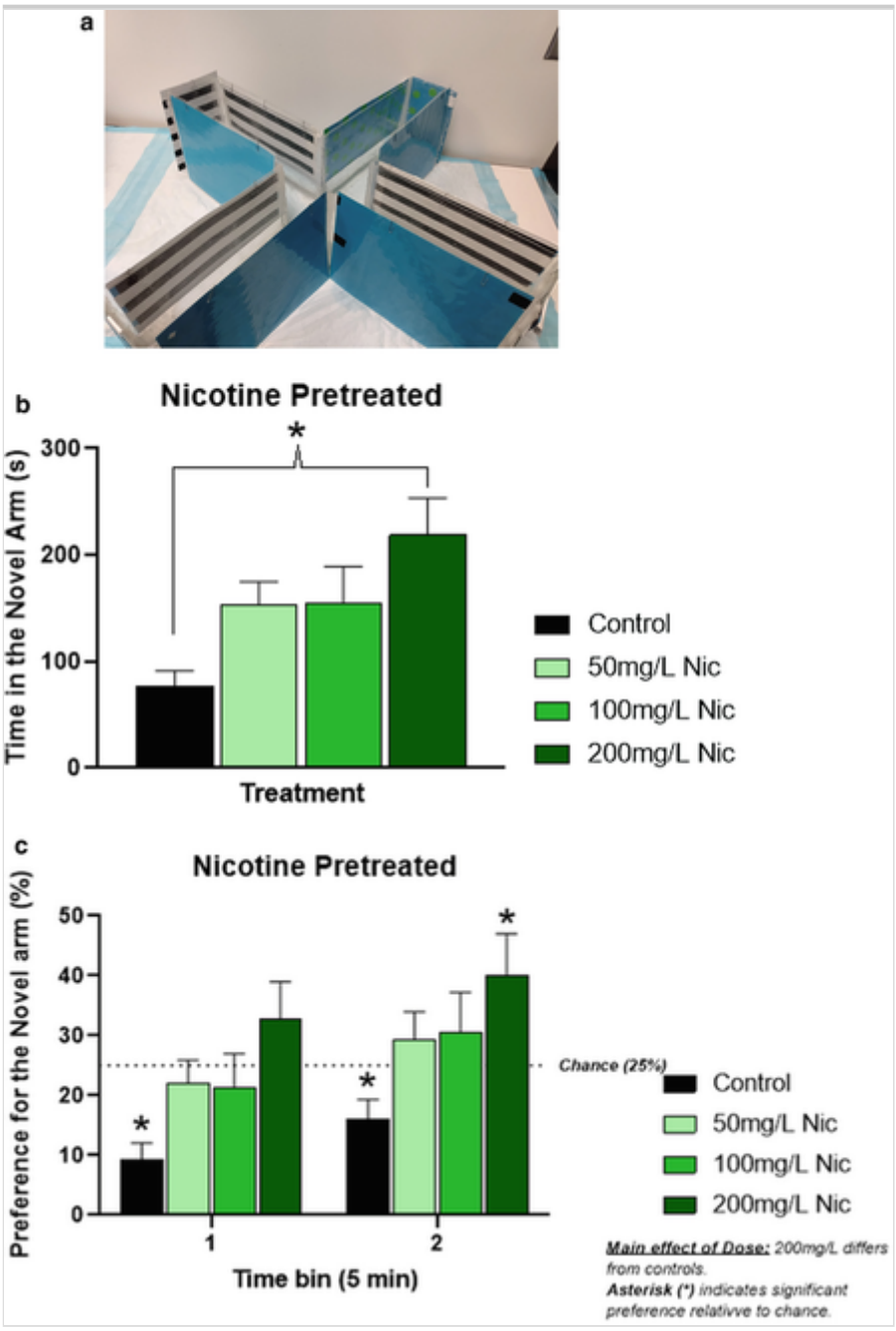
2.5.2. Novel Place Recognition

To measure recognition memory, we recently began using a novel place recognition task [12] adapted from a similar Y-maze design for zebrafish developed by Cognato and colleagues [48] and based loosely on a rodent novel object recognition task we regularly use in rat developmental neurotoxicology studies (e.g., [49]). The apparatus for novel place recognition (NPR) (*see* Fig. 7a) is a clear Plexiglas plus maze with a central hub (10 cm × 10 cm) with four rectangular arms (30 cm x 10 cm) extending outward, filled to a depth of 8 cm (10 L of system water). The clear walls of the arms have replaceable covers which can be used to show distinct visuospatial cues. In our protocol, three of the arms have identical visuospatial cues, consisting of a horizontal black and white stripe pattern, while the fourth arm has different cues, consisting of a blue background with lime green dots. Testing consists of two 10-min sessions. The first is an “AA” session (AA, meaning all stimuli are identical) where the fish can explore the three striped arms for 10 min, while the dotted arm is blocked by an opaque white divider which prevents the fish from exploring or seeing it. After this familiarization session, the fish is placed into a 1.5 L holding tank and remains there for a 2 h intertrial interval. After 2 h, the fish is then placed back into the maze for an “AB” session (where one stimulus does not match the others), with the barrier to the dotted arm removed. This target arm is novel and the fish will react differently to it than to the familiar striped arms. Although Cognato and colleagues [48] demonstrated preference for the novel arm in their Y-maze, our fish demonstrate neophobia, or avoidance of the novel arm. In an unpublished pilot study, we found that the tendency to spend very little time in the novel arm could be attenuated by acute pretreatment with an anxiolytic drug, such as nicotine (Fig. 7b), suggesting that avoidance demonstrates an anxiety-like aversion to the novel arm. At sufficient concentrations, this allowed the fish to show recognition through a preference in the opposite direction (Fig. 7c). Recognition memory for the stripes is demonstrated by an altered amount of total time spent in

the spotted arm in either direction (arm contains 23% of total water volume, so 23% of the time would be the random choice value). As with rodent novel object recognition (Hawkey et al., 2020b), the difference between exploration of the familiar and unfamiliar arms can attenuate over time as the novel cues become more familiar (as in controls in Fig. 7c). Within session-learning can be measured as the change in open arm exploration between the first and second 5-min of the 10-min trial. These time bins can be loaded as a repeated-measures variable in an analysis of variance or used to generate a simple slope which represents the degree of learning across the session.

Fig. 7

Position discrimination in the plus maze, (**a**) Apparatus; (**b**) Memory is shown as time spent in the novel arm of the maze. Acute exposure to the anxiolytic drug nicotine (3 min, immediately prior to the familiarization session) dose-dependently increases novel arm time; (**c**) Memory is shown as preference (% of total time) for the novel arm. Acute exposure to the anxiolytic drug nicotine (3 min, immediately prior to the familiarization session) reverses novel arm preference from avoidance to preference (mean ± sem, $N = 20$) [Unpublished]



With respect to interpretation, it is important to note that the time spent in any one arm is partially conflated with the level of activity, as very low numbers of crossings from arm to arm, likely indicating freezing, do not reflect exploration and artificially inflate the preference for the arm the fish happens to be in while immobile. If sessions are video recorded, then automated motion tracking software can be used to measure the distance moved by the fish, the amount of time spent immobile or freezing, and the frequency or duration of visits to each of the arms. This reduces the labor needed for testing, if not the throughput, and eliminates the need for the experimenter to be in the room with the fish. That being said, plus maze exploration can also be manually scored using a stopwatch to record the time

spent in the novel arm and a score sheet to tally up the number of times the fish crosses from one arm to another.

3. Discussion

3.1. Interpretation of Results

The use of a battery of tests allows greater clarity on a phenotype than one test can accomplish alone. Not only does it provide a broad screen capable of detecting several different neurotoxic impacts, it also improves the interpretation of each significant difference. A heterogeneous result with some tests being positive and other being negative provides important information about the specificity of neurobehavioral toxicity. Most importantly, comparisons of different tests can indicate whether a finding is due to a general deficit which is not the primary aim of a test, such as general motor, perceptual, or motivational ability, or faithfully demonstrates a change in the target function. For example, in a study of the long-term effects of embryonic exposure to two brominated flame retardants [25], we found that low concentrations of both compounds led to reduced locomotor activity over the course of the predator avoidance test. Given that this effect was observed in this test, but not in either the novel tank or the shoaling test, we can conclude that this reduction in activity is a reaction to the test, and does not indicate a general deficit in arousal or motor ability. This distinction could not be made without complementary tasks that would be similarly affected if very basic and generally necessary functions were impacted.

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3.2. Limitations of the Zebrafish Model

One important factor for which zebrafish are likely not a good model are sex differences in neurobehavioral toxicity. Certainly, there are male and female zebrafish, but how they arrive at their sex is not based on an XX vs. XY-like "master switch." Rather, both genetic and environmental factors interact to influence the ratio of males and females within a brood of fish and the development of the juvenile ovary into masculine or feminine gonads (*see* reviews, [50, 51]). Zebrafish do have relatively conserved endocrine systems, including receptors for estrogenic and androgenic signals, and so are also valuable for detecting to endocrine-disrupting chemicals [52]. However, the face validity of zebrafish sex-differences in vulnerability to a toxicant, relative to human health, remains low. Given that zebrafish may still exhibit some behavioral and vulnerability differences between males and females, we load sex as a covariate into our statistics to account

for the degree of variability that is due to this factor, but tend not to include sex differences in the interpretation of data.

Another limitation of the zebrafish model is the complexity of the central nervous system. Zebrafish, like rodents and other vertebrates, have complex brains. The caudal portions of their brains are quite similar in structure to mammals. For example, the general structure and duties of the olfactory system, thalamus, amygdala, and habenula appear to be conserved across fish and mammals [53, 54, 55]. More rostral portions of the zebrafish brain are differently organized. In mammals, the main overlying brain structure is the neocortex, while with zebrafish it is the optic tectum. Likewise, functions carried out in the mammalian hippocampus and basal ganglia are carried out in less conserved networks in the zebrafish pallium [56, 57]. Given the structural differences, a zebrafish brain cannot carry out all necessary functions in the same way as a mammalian brain does, so structure-function relationships, particularly in cognitive functions, should be interpreted carefully. Zebrafish have higher face validity elsewhere, including in the general organization, synaptic structure and principal neurotransmitters in the brain [58, 59], so these endpoints are a preferable focus for mechanistic investigations of zebrafishneurotoxicology.

3.3. Needs for the Future

To date, a variety of behavioral assessments have been developed for young and adult zebrafish. Further development is still needed in order to improve the breadth, efficiency, and informativeness of existing tests and batteries. The keys to improved testing will be the automation of testing, the enhancement of throughput, improving our knowledge of the neuropharmacology of available tests, and a better understanding of how fish behavior and test sensitivity are affected by developmental age.

Of particular concern, a number of learning and cognitive tests are available, but the slow throughput and labor-intensive nature of most of these tests reduces their effectiveness and desirability. A more rapid throughput cognitive test is needed. Our three-chamber test has been sensitive to the neurobehavioral toxicity of varying compounds, including the organophosphate pesticide chlorpyrifos and the stimulant medication methylphenidate [47, 60]. This test can be completed in a single day with a small number of trials. However, few fish can be run per day and testing is quite intensive for the experimenter. The plus maze NPR task requires no manipulation of the fish or stimuli during testing, and these sessions can be recorded and scored using Ethovision software. This minimizes the labor required

for scoring, but again, few fish can be run in a single day. In either case, completing the learning component of the battery for a whole cohort takes as long as the rest of the adult battery, or perhaps longer depending on the number of fish. Improved methods that can be quick and fully automated will be needed in order to best address these functions within an efficient neurobehavioral battery.

An additional concern is our ability to interpret the effect of age on the neurobehavioral effects of toxicant exposures. We typically assess both larval light/dark motility and the adult battery and frequently find discrepancies between the dosimetry and presence of behavioral changes at these two ages (e.g., [18, 25, 61]). Assessments of neurobehavioral toxicity at varying ages across development are needed to better characterize the deficits that are observed, and to explain reportedly discrepant findings. For example, testing at a single time point in early adulthood does not demonstrate whether a finding represents a lifelong deficit or perhaps a temporary deficit due to developmental delays. This is certainly true when considering the radical changes that take place during early life development, but similar issues come up in the study of declines due to aging. With aged fish, multiple testing is needed in order to discriminate between a toxin which exacerbates the processes of aging and one which produces effects that are present early and persist as the animals age (e.g., [12]). Adaptation of tests to novel age ranges is needed, and may have obstacles that will need to be resolved. For example, motion tracking can be problematic with young fish, which are small and light colored or even translucent, so improved test designs and visualization methods are needed. Some standardization can be achieved based on fish size, as testing environments can be made smaller to accommodate for size differences between younger and older fish, although the behavioral relevance of apparatus or stimulus size, water depth, and other parameters will need to be verified as well.

Interpretation will also be aided by the development of an acute pharmacological profile for each behavioral test. Screening each test against drugs with known pharmacological actions is important for two reasons. First, it helps to determine translatable neural mechanisms that underlie the test, and supports the experimenter’s ability to suggest neurochemical mechanisms for follow-up investigations based on behavioral toxicology studies. Second, it offers positive controls which can be used to show the validity and sensitivity of the tests, and to support the validity of findings from the experimental groups. Positive controls are common in molecular and tissue-level analyses, but relatively rare in live animal behavioral studies. This is likely due to the additional cost and space required for additional treatment groups, but these concerns are less relevant for zebrafish

models. Our typical positive control for embryonic exposure studies is the organophosphate insecticide chlorpyrifos.

An additional area of future research concerns studies being conducted across different labs and institutions. Procedures for husbandry, toxicant administration, and testing may all contribute to the behavior of an animal, and sharing and these techniques vary considerably from lab to lab. Likewise, the selection of a particular strain of zebrafish may influence the outcome (e.g [61, 62].). A lesser appreciated aspect that is becoming more recognized is the role of the microbiome (e.g., [63, 64, 65]). Zebrafish have not only an internal microbiome, but also an external biome of microbiota in the tank water. Presently, little is known about the make-up and variability between flow-through water system within a lab or between water systems maintained across labs, institutions, and our field as a whole. Further testing and investigations will be needed to parse out how these issues influence the sensitivity of our fish and our tests to toxicants of interest.

3.4. Summary and Conclusions

Zebrafish offer an outstanding intermediate complementary model that facilitates translation between high throughput in vitro cell-based assays and classic rodent models. Like the cell-based assays, larval zebrafish offer an inexpensive way to assess neurotoxicity of a wide variety of toxicants and mixtures and offer access to the molecular and cellular processes of toxic impact and response. Like classic mammalian models, zebrafish offer an integrated organism [66] with a complex brain and behavior repertoire that is the product of millions of years of evolution. Care must be taken to fashion the behavioral tests around the ethology of the species. For example, the anxiety-like response of zebrafish is to dive to the bottom of the tank rather than the wall hugging seen in rodents. This is just another form of thigmotaxis in the z-dimension that serves the same purpose, to diminish predatory threat. Also, since zebrafish are social species, individual fish are attracted to the sight of a shoal of their conspecifics. This assay likely would not work in a species that was more solitary in habit. Zebrafish show analogous behavioral responses to many toxicant and drug challenges compared with rodents and can provide a critical complementary intermediate model to fill the gap between in vitro and mammalian models.

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