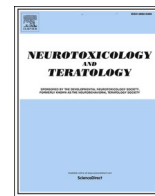




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Neurobehavioral anomalies in zebrafish after sequential exposures to DDT and chlorpyrifos in adulthood: Do multiple exposures interact?



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ABSTRACT

A sequence of different classes of synthetic insecticides have been used over the past 70 years. Over this period, the widely-used organochlorines were eventually replaced by organophosphates, with dichlorodiphenyltrichloroethane (DDT) and chlorpyrifos (CPF) as the principal prototypes. Considerable research has characterized the risks of DDT and CPF individually, but little is known about the toxicology of transitioning from one class of insecticides to another, as has been commonplace for agricultural and pest control workers. This study used adult zebrafish to investigate neurobehavioral toxicity following 5-week chronic exposure to either DDT or CPF, to or their sequential exposure (DDT for 5 weeks followed by CPF for 5 weeks). At the end of the exposure period, a subset of fish were analyzed for brain cholinesterase activity. Behavioral effects were initially assessed one week following the end of the CPF exposure and again at 14 months of age using a behavioral test battery covering sensorimotor responses, anxiety-like functions, predator avoidance and social attraction. Adult insecticide exposures, individually or sequentially, were found to modulate multiple behavioral features, including startle responsivity, social approach, predator avoidance, locomotor activity and novel location recognition and avoidance. Locomotor activity and startle responsivity were each impacted to a greater degree by the sequential exposures than by individual compounds, with the latter being pronounced at the early (1-week post exposure) time point, but not 3–4 months later in aging. Social approach responses were similarly impaired by the sequential exposure as by CPF-alone at the aging time point. Fleeing responses in the predator test showed flee-enhancing effects of both compounds individually versus controls, and no additive impact of the two following sequential exposure. Each compound was also associated with changes in recognition or avoidance patterns in a novel place recognition task in late adulthood, but sequential exposures did not enhance these phenotypes. The potential for chemical x chemical interactions did not appear related to changes in CPF metabolism to the active oxon, as prior DDT exposure did not affect the cholinesterase inhibition resulting from CPF. This study shows that the effects of chronic adult insecticide exposures may be relevant to behavioral health initially and much later in life, and that the effects of sequential exposures may be unpredictable based on their constituent exposures.

1. Introduction

Synthetic insecticides have been widely used in agriculture, vector control and domestic pest management since the mid-20th century. However, their benefits to society have been undermined by frequent and substantial concerns over their toxicity in non-target species, including potential health dangers for humans (Abreu-Villaca and Levin, 2017; Carvalho, 2017). To date, a variety of insecticides have been developed, with varying chemical properties and mechanisms of action

(Abreu-Villaca and Levin, 2017). The use and popularity of specific insecticide classes have been an ever-changing story across time. Some of the earliest and most widely used compounds were organochlorines, such as dichlorodiphenyltrichloroethane (DDT). As time passed, concerns for toxicity in vertebrate species led popular organochlorines to be banned or phased out in favor of alternatives, most notably organophosphate chemicals such as chlorpyrifos (CPF). In recent years, organophosphates have come under similar scrutiny as their predecessors. These concerns led to a partial restriction of compounds like CPF from

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domestic applications in 2001 (Bueschen, 2001; US EPA, 2001), although many scientists continue to push for a full ban on its use (Hertz-Picciotto et al., 2018; Trasande, 2017). Despite the development and diversification of alternative insecticide classes, sales numbers show that organophosphates remain the second largest insecticide sector on the market (Phillips, 2019) behind neonicotinoids and ahead of pyrethroids. Of particular concern for humans, people who apply insecticides are at risk of occupational exposures to these compounds (levels of exposure, see (Crane et al., 2013; Guardino et al., 1996; Thomas et al., 2010)), and may be chronically exposed to multiple classes as new classes of insecticides are phased out and replaced. Considerable research has been done concerning the toxicity of individual insecticides or classes but very little is known about the potential for increased toxicity with sequential exposure.

Traditional insecticides have predominantly targeted human-relevant mechanisms in the nervous system (Coats, 1990; Fukuto, 1990), such as the ability of DDT to block sodium channels and CPF to inhibit the activity of acetylcholinesterase, leaving substantial potential for negative physiological and neurological effects in career agricultural workers. Clinical and epidemiological studies of agricultural workers have identified a variety of potential hazards for mental and brain health in adulthood. In the short term, a history of insecticide exposures is associated with impairments in a variety of cognitive functions (Blanc-Lapierre et al., 2013; Dardiotis et al., 2019; Kamel et al., 2007; Koh et al., 2017; Rothlein et al., 2006; Sullivan et al., 2018), including psychomotor speed and dexterity, mood, attention and short- and long-term memory. Furthermore, those who were chronically exposed to insecticides as adults show adverse outcomes in aging, including greater cognitive decline and risk for Parkinson's disease, Alzheimer's disease and non-Alzheimer's dementia (Aloizou et al., 2020; Hayden et al., 2010; Hernández et al., 2016; Jokanović, 2018; Medehouenou et al., 2019; Mostafalou and Abdollahi, 2018).

Available human data from agricultural workers is concerning, but the short and long-term effects of adult insecticide exposures remain difficult to interpret. Agricultural workers may be exposed to one or more of the many classes of insecticides available, either simultaneously or sequentially, across their careers as the popularity of different insecticide classes rise and fall. Based on the timing of the bans and insecticide introductions, a sizeable group of late-career or retired agricultural workers now experiencing problems in aging would have begun their careers before organochlorine bans in the 1970s and 1980s (Purdue et al., 2007), and then transitioned to organophosphates, thereby experiencing chronic seasonal exposures to both classes in sequence. This scenario is concerning, as the effects of seasonal occupational exposures across a career could be easily misattributed to the toxicity of a single insecticide or class. For example, subjects who report exposure to one class, such as organophosphates (Starks et al., 2012), may systematically differ from one another based on exposures which happened at other points in their careers. More data are needed which clearly define the effects of older and newer generations of insecticides and show the potential for novel consequences when exposures shift from one insecticide to another over time. This will provide greater clarity as to the occupational risks posed by older generations of insecticides and guide the way that differing compounds and classes are treated within epidemiological studies.

To complement existing human data, vertebrate animal models are needed to allow mechanistic and behavioral consequences of different adult insecticide exposures to be examined in controlled studies. One such model is the zebrafish, *Danio rerio*. As vertebrates, zebrafish share a variety of genetic, neural and physiological features with mammals, and can be used to detect changes in neurochemical and pharmacological systems with translational importance to mammalian systems (Goldsmith and Jobin, 2012; Lieschke and Currie, 2007; Sakai et al., 2018), including translatable toxicity from traditional insecticides (Gonçalves et al., 2020). Furthermore, their small size, low cost and dense housing allow zebrafish to be practically maintained for life-span length studies

which are difficult to reasonably conduct with rodent models (Keller and Keller, 2018). Using a zebrafish model, we recently reported the short (1-week post exposure) and long-term effects (14 months of age) of adult exposures to either DDT or CPF (either 2-week or 5-week exposures) (Hawkey et al., 2020). These effects included behavioral effects one-week following the end of exposure, and lingering effects on behavior and cholinergic function at 14-months of age, several months after the end of exposure. In particular, it was observed that some behavioral features were impacted at the one-week follow-up, but not persistently, while others showed impairments only as they reached later adulthood. This work also indicated that organochlorine and organophosphate effects were distinct from one another in zebrafish, further underlining the potential for human populations with different histories of pesticide exposure to experience different long-term consequences on the brain and behavior.

The current study used an adult zebrafish model to expand upon these prior findings by investigating potential differences between the effects of chronic exposures to DDT, CPF, or each in sequence, both in the short term (1-week after the end of the exposure period) after exposure and into late adulthood. Over a 12-week period, four groups of adult zebrafish were chronically exposed to a 0.001% DMSO vehicle, DDT (30 nM) (weeks 2–6), CPF (1uM) (weeks 7–11) or both compounds in sequence. On the last day of the CPF exposure, a subset of fish from each of the four groups (control, DDT-alone, CPF-alone, DDT + CPF) were euthanized and analyzed for brain cholinesterase activity, which is the primary mechanism of toxicity for organophosphate insecticides in insects and vertebrates alike. The remaining fish were then tested on a battery of neurobehavioral tests for locomotor activity, reflexive behaviors, reward, anxiety, fear and learning at two time points, 1-week following the end of the exposure series (or week 13 after the beginning of the experiment) and at 14 months of age. Doses were selected based on the prior study (Hawkey et al., 2020) and pilot work establishing the tolerability of these compounds over the 10-week exposure period. Notably, the CPF concentration was chosen so as to cause significant inhibition of cholinesterase, but at a level well below the 70% threshold for overt toxicity (Clegg and van Gemert, 1999). Across this study, the primary hypotheses concerned: 1) the persistence or attenuation of neurobehavioral effects as time goes on after the end of exposures and 2) the potential for unique or non-additive effects following sequential exposure to organochlorines and organophosphates. These hypotheses were assessed using two parallel analyses, one of which treated each treatment group as a non-overlapping between-subjects variable (hypothesis 1), while the other assessed the overall effects of DDT or CPF exposure (with or without sequential exposure) and any statistical interaction (non-additive) between the two, when viewed in animals with single- or multiple-exposures (hypothesis 2).

2. Materials and methods

2.1. Fish housing and husbandry

The present study was conducted with adult AB* zebrafish, maintained and bred in-house in the Levin Lab at Duke University and all procedures were approved by the Institutional Animal Care and Use Committee at Duke University. Prior to and after the exposure sequence, the zebrafish were housed in mixed-sex 3 L plastic tanks (density ≤ 5 fish/L) on a recirculating water system (Aquatic Habitats, Inc., Apopka, FL, USA). The water chemistry was balanced using sea salt (Instant Ocean, 0.5 parts per thousand) and buffer (Seachem Neutral Regulator, 125 mg/L) in de-ionized water. Standard water quality parameters (temperature, pH, salinity, ammonia, etc) were monitored weekly. Housing areas were maintained on a 14:10 h light:dark cycle and water temperature was maintained at 28 ± 1 °C. While on the flow-through systems, the fish were fed three times daily; morning and afternoon with brine shrimp (*Artemia salina*) hatched in-house over 24 h (eggs from Brine Shrimp Direct, Ogden, UT, USA); and noon feeding with solid

pellet food GEMMA Micro 300 micro-pellets (Skretting USA, Tooele, UT, USA).

During the exposure period, fish were housed in stand-alone 4 L glass tanks (Deep Blue Professional, City of Industry, CA, USA) containing 3.5 L of system water. Each tank was equipped with its own aeration tube and mini-pump, heater and thermometer, and the water was maintained at 26 ± 1 °C. All tanks underwent a complete water change every 7 days, and commercial nitrifying bacteria (API® Quick Start™, 25uL/L) were added prevent the buildup of ammonia and nitrites in the water due to uneaten food and other waste. To further support water quality, the mid-day solid food meal was replaced with an additional brine shrimp feeding during the exposure period.

2.2. Chemicals

Dimethyl Sulfoxide was purchased from Sigma-Aldrich (St. Louis, MO, USA). 4,4'-Dichlorodiphenyltrichloroethane (DDT) and chlorpyrifos was purchased from Chem Service, Inc., (West Chester, PA, USA). Stocks were made by dissolving the insecticides in 100% DMSO. Exposure medium was made by adding stocks to fresh system water at 10 uL DMSO/L (0.001%).

2.3. Exposure of adult zebrafish

Untreated 8–9 month-old breeder fish were sexed and separated into 4 L standalone glass tanks (density, 16 fish per 3.5 L), with equal representation of male and female fish in every tank, as available in each cohort of breeders used. Each tank contained either 12 or 16 fish. Two of the 4 replicate tanks per treatment contained 16 fish, of which 4 were harvested for tissue prior to any behavioral testing (i.e., in the brain cholinesterase analysis). Fish were acclimated to this new housing for one week prior to the first chemical-exposure phase. After one week, each tank was randomly assigned to one of four treatment groups: control, DDT-only, CPF-only, and sequential DDT/CPF. For the next five weeks, all groups exposed to DDT were housed in DDT-exposed system water (30 nM), while all other fish were exposed to vehicle (0.001% DMSO). Following the end of this exposure period, all groups exposed to CPF were housed in CPF-exposed system water (1uM), while all other fish were exposed to vehicle (0.001% DMSO). At the end of this exposure period, a subset of individuals ($n = 8$ per treatment group, with $N = 4$ each coming from 2 replicate tanks) was removed for cholinesterase determinations. All remaining fish were transferred to clean stand-alone tanks and maintained for one week-prior to the first behavioral testing battery. The test batteries, described below, were conducted at two ages. First, we examined behaviors one week after the end of the second exposure period (i.e. six weeks after the end of DDT exposure, one week after the end of CPF exposure). Behaviors were then reexamined at 14 months of age to look for persistence of effects and/or emergence of adverse outcomes with aging. One tank of the sequential DDT/CPF group was excluded from testing due to unrelated health issues (ammonia toxicity), but all groups met a minimum sample size of 30+ fish at the beginning of the behavioral study. The test sequence is shown in Table 1.

2.4. Cholinesterase activity

Zebrafish were briefly anesthetized in ice water, weighed and euthanized by severing of the junction between the skull and spinal cord using scissors. Whole brains were dissected, weighed, wrapped in aluminum foil, flash frozen in liquid N₂ and stored at -80 °C until assayed. Assays were conducted on individual brains. Briefly, brains were homogenized (Polytron) in 0.5% Triton X100, 0.1 M Na₂HPO₄/KH₂PO₄, pH 8.0 and left on ice for 15 min to remove insoluble debris. Homogenates were sedimented at 40,000 xg for 15 min. Aliquots of the supernatant solution were added to final concentrations of 0.5 mM acetylthiocholine iodide and 0.33 mM 5,5'-dithiobis(2-nitrobenzoic

Table 1

Sequence of Testing. Cholinesterase activity analyses were performed immediately after the end of the CPF exposure. Testing at 1-week following the end of the CPF exposure contained 4 tests (ordered from left to right). Testing at 14 months of age contained 5 tests (ordered from left to right).

	ACHE	Novel Tank	Tap Startle	Shoaling	Predator	NPR
Immediately Following CPF Exposure	X					
1-week Post CPF exposure Window		X	X	X	X	
14 Months of age		X	X	X	X	X

Tests are arranged in the order they were performed, from left to right.

acid) in the same buffer without Triton (all reagents from Sigma-Aldrich Chemical Co., St. Louis, MO) to measure cholinesterase activity (Ellman et al., 1961). Assays were incubated at room temperature for 4, 8, 12, 16, and 20 min, and the enzyme activity was assessed from the linear portion of the time course, reading the absorbance at 415 nm. The assay was standardized using mercaptoethanol standards and calculated relative to total protein (Smith et al., 1985).

2.5. Behavioral testing

At each age of testing, a battery of behavioral tests was conducted to evaluate several psychomotor, affective (akin to emotional functions in human subjects), and cognitive functions. One test was conducted each day between 10:00–17:00 h (standard clock), and individuals within each tank were tested across the testing period to control for time of day effects. Fish acclimated to the testing room for 30–60 min prior to each testing session. Tests were conducted and scored using an HD camcorder (VIXIA HFR700; Canon Inc., Tokyo, Japan) and EthoVision XT® automated motion tracking software (Noldus Information Technology, Wageningen, The Netherlands). As data generation required automated tracking of fish within videos, an inclusion criterion of 96% subject tracking (subject found in 96% of collected images) was used to remove individuals from analysis with inconsistent or inaccurate tracking data. All fish completed all tests and the replicated battery consisting of novel tank dive test, tap startle, shoaling, and predator avoidance (excluding novel place recognition, NPR) required 6 consecutive test sessions to complete (shoaling and predator split into 2 days, due to longer trial length). Following the end of the 14 month battery, NPR testing took place, and required approximately 8 sessions (~10 fish tested per day) to complete.

2.6. Startle tap test

Sensorimotor startle and habituation were tested in a custom apparatus, as described in Glazer et al. (Glazer et al., 2018). Briefly, zebrafish were individually placed into eight shallow, clear cylindrical arenas arranged in a 2 × 4 setup. Each arena was equipped with a 24-V DC push solenoid which produced a loud tapping stimulus by driving a pin into the bottom of the arena. The testing session consisted of 10 consecutive taps at 1 min intervals. Startle reactions were measured using locomotor behavior (distance moved in cm) in the 5 s immediately prior to and after each tapping stimulus. The magnitude of the startle response was calculated as the difference between post-tap activity and pre-tap activity. Habituation, a rudimentary form of learning which comes from an attenuation of the reflexive response over repetitions, is observed as a reduction in startle magnitude over successive repetitions of the tapping stimulus. Habituation is associated with an increase in pre-tap activity across successive taps and is validated by a significant (non-zero) negative slope for the linear function of the sequential tap-series.

2.7. Shoaling test

Social attraction was tested using a custom partitioned experimental tank (PET), as described in Glazer et al. (Glazer et al., 2018). The PET sat above a light box (Huion Technology, Shenzhen, China) and was video recorded from above. The two lanes of the tank (9.25 cm × 28 cm) were separated by a black partition. Two 19.5-in. LCD monitors were viewable through either of the narrow ends of the two lanes. A digital video camcorder was placed above the tank. Prior to testing, fish were socially isolated in 1.5 L tanks for 30 min, to facilitate a robust pro-social response. Following this period, each fish was placed into the PET for a 7 min session consisting of a 2-min habituation period followed 5 min in which a video recording of a zebrafish shoal was played on one of the monitors. Social approach was measured as a shift in the distance from the target screen (in cm) during the social video presentation. The magnitude of the approach response was calculated as the difference between the average distance from the screen at baseline (min 1–2) and during the video presentation (min 3–7). Locomotor behavior was measured across the 7, 1-min time bins, as locomotor behavior tends to increase across the session in tasks using the PET, regardless of the cues being presented. This allows changes in locomotion due to the presentation of stimuli to be readily identified (e.g. effects prior to social cue, vs early-, mid- or late-cue presentation).

2.8. Predator avoidance test

Predator avoidance and fleeing were tested using the same custom partitioned experimental tank (PET) used for shoaling, and as described in Glazer et al. (Glazer et al., 2018). For this, fish were individually placed in the PET and recorded for a 9-min testing session. During this session, fish were shown four 1-min presentations of “predator cues”, covering minutes 2, 4, 6, and 8 of the 9 min session. The stimulus consisted of either a blue slow-growing dot (growth from 1 to 23 cm in 4-s) (min 2 and 4) or a red fast-growing dot (growth from 1 to 23 cm in 1-s) (min 6 and 8) appearing repeatedly on one screen. The growing dot is a 2-dimensional representation of a large approaching object. Avoidance was measured as the distance from the screen during the cue presentations or the blank periods between presentations. The magnitude of the approach response was calculated as the difference between a fish’s average distance from the screen in the 1-min prior to each cue and during each cue presentation. Locomotor behavior was measured across the 9, 1-min time bins, as locomotor behavior tends to increase across the session in tasks using the PET, regardless of the cues being presented, and so that changes in locomotion during select cue presentations or removals can be readily identified.

2.9. Novel place recognition

This final test was a novel place recognition task, conducted once at 14 months of age, as described in Hawkey et al. (Hawkey et al., 2020). This test is based on the rodent novel object recognition task, which familiarizes animals with multiple identical stimuli, then presents an array containing both familiar and unfamiliar stimuli which the animal can then investigate. The result of this familiarization is an observable preference between novel and previously presented stimuli. NPR was conducted in a clear Plexiglas plus maze consisting of a central hub (10 × 10 cm) with four, evenly-spaced arms (10 × 30 cm). On the outside of each arm were replaceable covers which could be used to provide visual cues. Testing began with a 10-min session where each fish explored three identical arms of the maze, which were covered with a horizontal black and white striped pattern. A solid white partition blocked entry and viewing of the fourth arm. After this familiarization session, each fish was placed into a separate 1.5 L tank for 2 h. Following this retention period, each fish was returned to the maze with all four arms available for exploration for 10 min (3 arms covered in black and white stripes, 1 arm covered in blue paper with green spots). The general level

of exploration was quantified as the total number of arm entries. Novel arm preference was quantified as the percent of the time that fish spent in the novel arm, relative to the total spent allowed for exploration. This was used to allow the relative approach or avoidance of the novel arm to be quantified for each group. This measure is representative of similar approaches used throughout exploratory behavior studies, which note a characteristic bias towards or away from a given cue. Under control conditions, zebrafish are generally averse to the novel arm, and prefer to remain in the arms present in the pre-test session at above chance levels. However, it must be acknowledged that as a proposed measure of recognition memory, any deviation from chance would be considered evidence for cue retention (either less approach or enhanced approach). Therefore, a discrimination index was also generated, to display the strength of retention, rather than the consistency of the avoidance response, per se. Place discrimination was demonstrated by comparing the percent of the total time spent exploring to novel arm to a chance level of 23%, corresponding with the percentage of the total water volume which was in the novel arm. This was calculated as the absolute value of the difference between chance preference (expected, 23%) and the observed chance level measured in each fish (observed). To assess any within-session learning, the 10-min session was divided into 2, 5 min time blocks.

2.10. Novel tank dive test

Adult zebrafish were tested for exploration in a novel tank as described in Glazer et al. (Glazer et al., 2018). Briefly, fish were video recorded from the side as they explored a narrow trapezoidal 1.5-l tank (Aquatic Habitats) filled with 10 cm of system water. The session length was 5 min. The primary independent measures of interest were total distance traveled (in cm) over each min of testing and the mean distance from the tank floor in cm per min. Distance from the tank floor was included as a measure of “diving”, a stereotypic anxiety-like response which zebrafish show when presented with a novel environment.

2.11. Statistical analysis

All statistical analyses were performed in SPSS v.24 (IBM Corp.). For each set of tests, the general sequence of analysis proceeded as follows. A mixed factorial ANOVA was performed to detect main effects of age (reflecting both developmental age and short vs long latency following exposure), time block (within-session), sex, treatment and cue phase (e.g. social cue present vs absent, slow predator cue vs fast predator cue), as required per test. Housing tank identities were included as covariates. Follow-up tests were performed on interactions at $p < 0.10$ (Snedecor and Cochran, 1967), but a cut-off of $p < 0.05$ (two-tailed) was used as the threshold for significance of all main effect tests and post hoc tests. Post hoc tests with Tukey’s correction for multiple testing were performed on all between-subjects main effects and interactions concerning treatment. Violations of homogeneity of variance were addressed using the Greenhouse-Geisser correction (Greenhouse and Geisser, 1959), which results in degrees of freedom that are not whole numbers.

There were two separate issues addressed in the data analysis. First, we determined whether the effects of DDT, CPF, or the combination, differed from control values and from each other. Second, we wanted to assess whether the effects of the combination were additive (sum of individual effects of DDT and CPF), or non-additive (synergistic, less-than-additive or antagonistic). The first issue required an ANOVA regarding the four treatment groups as one factor (treatment), followed by post-hoc comparisons for intergroup differences. The second issue required that the DDT and CPF treatments be considered as two separate factors, with the interaction term (DDT × CPF) thus testing for additivity: no significant DDT × CPF interaction if the effects of the combination were indistinguishable from additive effects; significant interaction if effects were synergistic, less-than-additive or antagonistic. This approach thus provides an operational framework for determining

if the sequential exposure produced an effect that could not have been anticipated from the effects of the individual exposures. The results of the former ANOVA are presented in graphs. The results of the latter analyses (main effects of DDT or CPF, and interactions involving including both DDT x CPF) are presented in supplemental tables.

3. Results

3.1. Cholinesterase

We assessed brain cholinesterase activity at the end of the exposure period to evaluate the effectiveness of the CPF treatment and to determine whether prior exposure to DDT impacted the CPF effect. There were significant overall treatment effects ($F_{3,28} = 17.3, p < 0.0001$; Fig. 1) that differed among the various groups. By itself, DDT had no significant effect on cholinesterase but CPF alone evoked a significant 25% decrement. Prior exposure to DDT did not alter the ability of CPF to inhibit cholinesterase (significant difference between the combination and control or DDT alone, but not between the combination and CPF alone; no significant DDT x CPF interaction with DDT and CPF treatments regarded as two factors). There were no significant effects of sex or relevant interactions.

3.2. Survival to 14 months of age

In captivity, zebrafish can remain healthy over two years, however a increasing spontaneous attrition is seen as time goes on after 12 months of age (Table 2). During the first round of testing, one week following the end of the CPF exposures, sample sizes were as follows: Control, 46; DDT-alone, 47; CPF-alone, 42; DDT + CPF, 32. Within the second battery, conducted at 14 months of age, sample sizes were as follows: Control, 37; DDT-alone, 35; CPF-alone, 39; DDT + CPF, 23. The attrition rates between this interval were calculated as: Control, 19.6%; DDT-alone, 25.5%; CPF-alone, 7.1%; DDT + CPF, 28.1%.

3.3. Between-group analysis

3.3.1. Tap Startle test

For tap startle behavior (Figs. 1 and 2), the primary measures were locomotor activity immediately pre-tap and post-tap, as well as the difference between pre- and post-tap activities (startle magnitude). For pre-tap activity, there were main effects of tap repetition, $F(7.68, 2013.32) = 6.58, p < 0.05$ (increased over time), as well as interactions of age x tap x sex, $F(7.68, 1924.72) = 2.29, p < 0.05$, and age x sex x treatment, $F(3, 252) = 2.13, p < 0.10$. Stratified by age and sex, a difference between adult, female DDT-alone fish and controls failed to

Table 2

Survival Rates to Aged Testing. Sample sizes are presented at the 1-week post-test period (adult age of testing) and 14-months of age (aged testing). Attrition rates calculated as percent (%) of initial sample size.

Sample Size	DMSO	DDT-Alone	CPF-Alone	DDT/CPF
1-week post CPF exposure window	46	47	42	32
14 months of age	37	35	39	23
% Attrition	19.6	25.5	7.1	28.1

reach significance ($p = 0.08$). No other comparisons reached or approached significance. For post-tap activity, there was a main effect of age, $F(1, 268) = 10.23, p < 0.05$, whereby aged fish exhibited higher post-tap activity scores than adult fish, and interactions of tap x treatment interaction, $F(22.51, 2011.03) = 1.61, p < 0.05$, and age x treatment, $F(3, 286) = 2.60, p < 0.10$. No pairwise comparisons reached significance at individual taps, or overall when stratified by age. For startle magnitude (Stratified by age as per 2-factor analysis, Fig. 2), there was a main effect of tap repetition, $F(7.36, 1920.14) = 4.09, p < 0.05$, as well as interactions of tap x treatment, $F(7.36, 1920.14) = 1.51, p < 0.10$, and treatment x age, $F(3, 261) = 6.19, p < 0.05$. Stratified by age, there was a significant difference between CPF-alone and DDT/CPF fish in adulthood ($p = 0.03$) among adult fish, while a difference in the opposite direction failed to reach significance among aged fish ($p = 0.09$). No other pairwise comparisons reached or approached significance. Fish generally showed habituation, or a reduction in startle magnitude across successive taps, as evidenced by a significant negative slope function ($p < 0.001$).

With the DDT and CPF treatments regarded as two separate factors, an interaction of age x sex x DDT x CPF, $F(1, 262) = 8.95, p < 0.05$, was detected for pre-tap activity, although no pairwise comparisons reached significance when stratified by age and sex. For post-tap activity, a significant age x DDT x CPF interaction was detected, $F(1, 268) = 6.33, p < 0.05$. Stratified by age, no significant treatment effects were observed at either age. For startle magnitude (Fig. 2), interactions were detected for age x DDT x CPF, $F(1, 261) = 16.02, p < 0.05$, and age x sex x DDT x CPF, $F(1, 261) = 3.12, p < 0.10$. The age x DDT x CPF interaction reflected the finding discussed in the prior analysis, that DDT/CPF-exposed fish showed elevated startle responses relative to CPF-alone fish in adulthood. Stratified by age and sex (Table S1), it was found that adult male, but not female, fish with exposure to DDT/CPF differed from both DDT-alone ($p = 0.003$ and CPF-alone ($p < 0.001$) groups, while a similar effect relative to controls failed to reach significance ($p = 0.08$).

3.3.2. Shoaling test

For shoaling behavior, the primary measures were locomotor activity across the seven, 1-min time blocks, and the difference in distance from the screen between the baseline and video-presentation portions of testing (approach score). For locomotor activity (Fig. 3a), there were main effects of time block, $F(3.63, 896.86) = 6.65, p < 0.05$, age, $F(1, 247) = 12.62, p < 0.05$, and treatment, $F(3, 247) = 3.10, p < 0.05$. Locomotor activity increased across the session, and as a function of age ($p < 0.05$). Fish exposed to sequential DDT and CPF were significantly hyperactive relative to those exposed to DDT-alone ($p = 0.002$), while a similar effect relative to controls failed to reach significance ($p = 0.06$). No other comparisons reached significance. For the approach score (Fig. 3b), there was a main effect of sex, $F(1, 247) = 8.93, p < 0.05$, (male < female), and treatment, $F(3, 247) = 5.18, p < 0.05$, as well as an age x treatment interaction, $F(3, 247) = 3.91, p < 0.05$. Among adult fish, differences between the DDT/CPF group and the DDT-alone ($p = 0.09$) and CPF-alone ($p = 0.06$) groups failed to reach significance. Among aged fish, CPF-alone ($p = 0.007$) and DDT/CPF groups ($p = 0.04$) showed reduced approach scores relative to the DDT-alone group. No

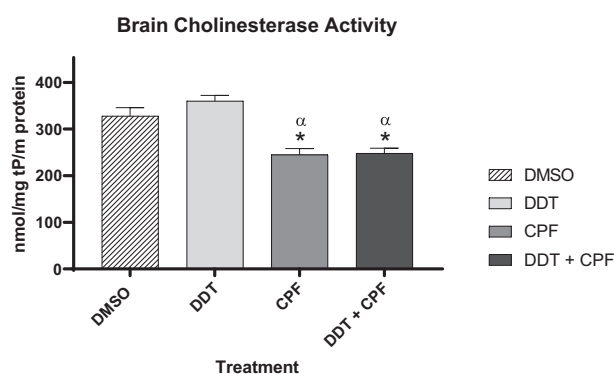


Fig. 1. Brain Cholinesterase Activity. Treatment with CPF led to significantly reduced cholinesterase activity relative to non-CPF-treated groups (mean \pm sem). Asterisk (*) indicates significant difference from controls at the $p < 0.05$ level. Alpha (α) indicates a significant difference from DDT-alone at the $p < 0.05$ level.

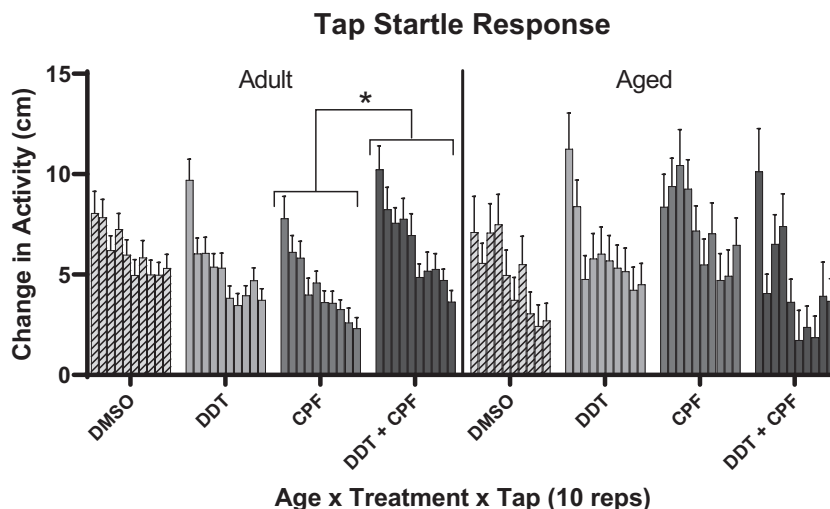


Fig. 2. Tap Startle Test Results: Startle magnitude measured as change in activity (cm) (mean \pm SEM) due to the tap, plotted across the 10 successive taps. Startle magnitude was elevated in the sequentially exposed (DDT/CPF) group relative to the CPF-alone group. Asterisk (*) indicates significant difference at the $p < 0.05$ level.

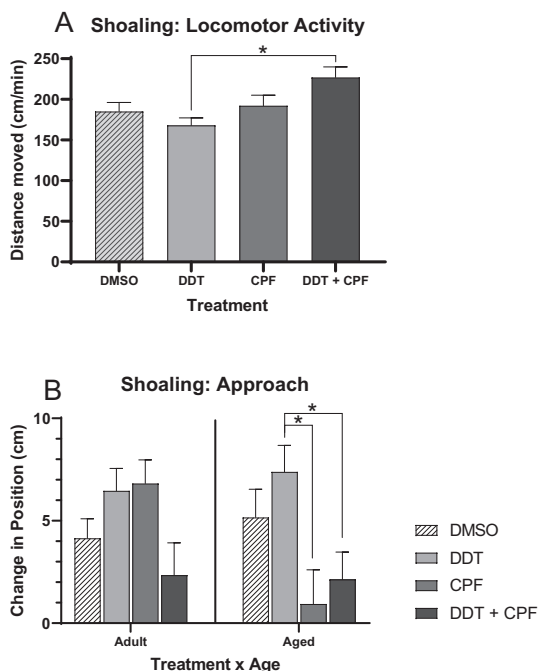


Fig. 3. Shoaling Test Results: (A) Locomotor activity measured as distance moved per minute (cm/min) (mean \pm SEM) averaged across the 7, 1-min time bins. Sequentially exposed fish were hyperactive relative to fish treated with DDT-alone. (B) Social approach measured as change in position within the tank (cm) (mean \pm SEM). Fish exposed to CPF, with and without prior exposure to DDT, showed reduced attraction scores relative to DDT-alone fish at the aged time point. Asterisk (*) indicates significant difference at the $p < 0.05$ level.

other pairwise comparisons reached or approached significance.

Within analysis of treatments as two factors, locomotor activity analyses showed significant interactions of DDT \times CPF, $F(1, 247) = 4.37$, $p < 0.05$, and sex \times age \times DDT \times CPF, $F(1, 247) = 3.22$, $p < 0.10$. When stratified by sex and age (Table S2), it was found that adult males with sequential DDT/CPF exposure were hyperactive relative to males exposed to DDT alone ($p = 0.04$). No effects were found among females, or among aged fish. With respect to the approach response, a main effect was detected for CPF, $F(1, 247) = 9.79$, $p < 0.05$, as was an interaction

of DDT \times CPF, $F(1, 247) = 4.61$, $p < 0.05$. The DDT \times CPF reflected the effect discussed above (Fig. 3b), without additional stratification by age. Overall (Table S3), it was found that DDT/CPF exposed fish showed lower approach responses than DDT-alone exposed fish ($p = 0.003$), while no other pairwise comparisons reached significance. The main effect of CPF-treatment (Table S4) reflected a pattern whereby fish with CPF-exposure (regardless of prior DDT exposure) showed lower social approach scores than non-CPF exposed fish ($p = 0.01$).

3.3.3. Predator avoidance test

For predator avoidance behavior, the primary measures were locomotor activity across the nine, 1-min time blocks, and the difference in the distance from the screen during each cue presentation (predator present vs absent) (fleeing score). For locomotor activity (Fig. 4a), there was a main effect of time block, $F(5.32, 1350.93) = 11.42$, $p < 0.05$, and interactions of time block \times treatment, $F(15.96, 1387.77) = 1.67$, $p < 0.05$, and time block \times sex \times treatment, $F(15.96, 1350.93) = 1.73$, $p < 0.05$. Among males, no pairwise comparisons reached significance. Among females, sequential DDT/CPF fish differed in activity in time block 1 (no cue) compared to DDT-alone ($p = 0.04$), and on time block 5 (post slow cue 2) compared to CPF-alone treated fish ($p = 0.02$). For the fleeing response (Fig. 4b), there were several main effects and interactions observed, including a main effect of cue repetition, $F(1, 254) = 32.83$, $p < 0.05$, as well as interactions of age \times treatment, $F(3, 254) = 3.29$, $p < 0.05$, cue speed \times treatment \times sex, $F(3, 254) = 4.10$, $p < 0.05$, and cue speed \times repetition \times sex \times treatment, $F(3, 254) = 3.42$, $p < 0.05$. Stratified by age (Fig. 4b), post hoc observed that among adults, all insecticide-treated groups showed higher fleeing scores than controls (DDT, $p = 0.01$, CPF, $p = 0.03$, DDT/CPF, $p = 0.01$), but these groups did not differ from one another. No differences were observed among aged fish. When stratified by sex, cue repetition, and cue speed (Fig. 4C), it was observed that all insecticide-treated female groups showed greater fleeing scores than controls due to the initial presentation of the slow cue (DDT, $p = 0.001$, CPF, $p = 0.008$, DDT/CPF, $p < 0.001$), but these groups did not differ from one another. No pairwise comparisons reached significance during the fast-cue fleeing responses, or among male fish.

Within the treatments regarded as two factors, we found an age \times CPF interaction for locomotor activity $F(1, 254) = 3.57$, $p < 0.05$, as well as a significant time block \times DDT \times CPF interaction, $F(5.32, 1350.93) = 2.15$, $p < 0.05$. Follow-up analysis of the time block \times DDT \times CPF interaction parallels the analysis reported above without stratification by sex. Post hocs (Table S5) noted a difference in time block 6 (first fast cue),

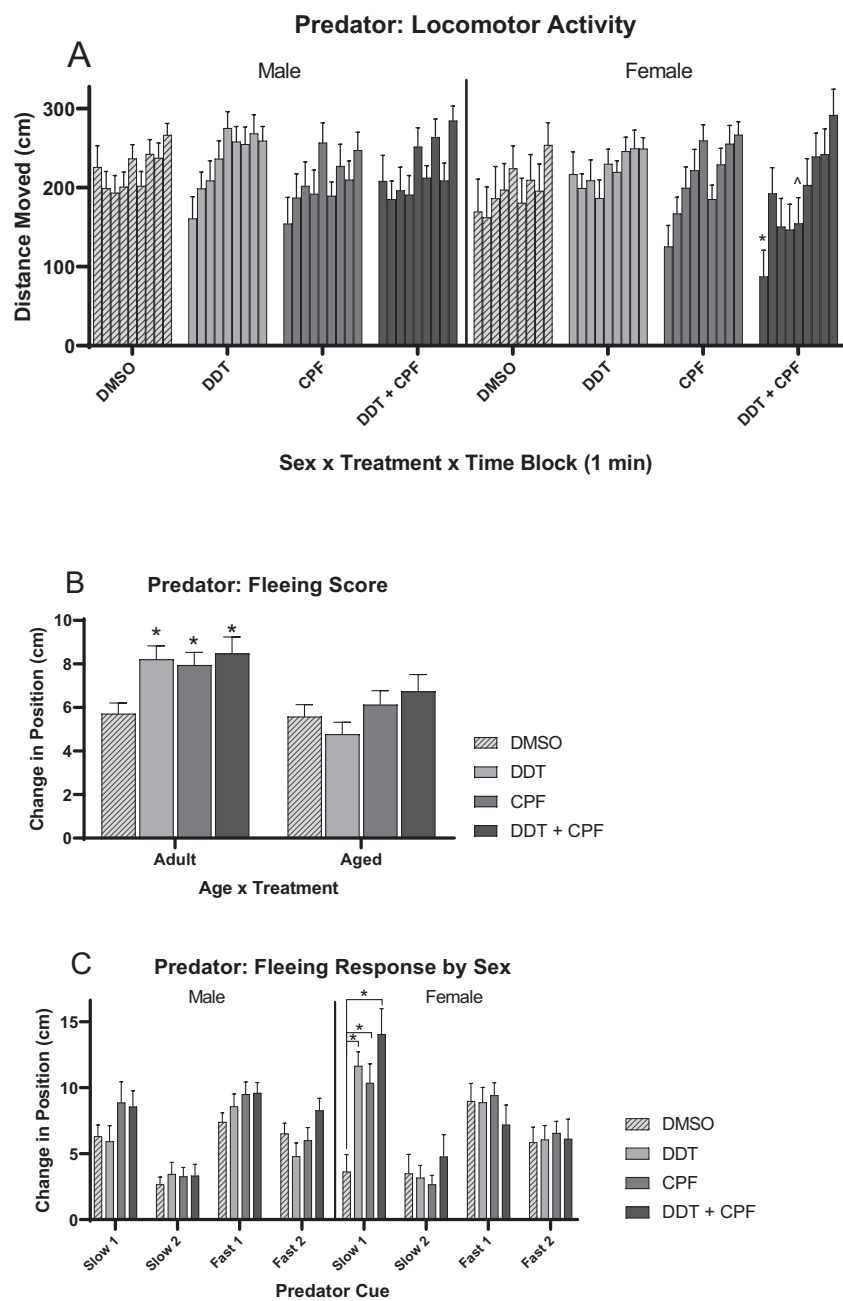


Fig. 4. Predator Avoidance Test Results: (A) Locomotor activity measured as distance moved per minute (cm/min) (mean ± SEM) and plotted across the 9, 1-min time blocks. Females with sequential exposures (DDT/CPF) showed reduced altered locomotor activity at within specific time blocks. Asterisk (*) indicates significant difference from DDT-alone at the $p < 0.05$ level. Carat (^) indicates significant difference from CPF-alone at the $p < 0.05$ level. Slow cues were presented in time blocks 2 and 4, while fast cues were presented in time blocks 6 and 8. (B) Fleeing responses measured as change in position within the tank (cm) (mean ± SEM). Exposure to either insecticide led to increased fleeing response relative to control in adult fish (mean ± SEM). Asterisk (*) indicates significant difference from controls at the $p < 0.05$ level. (C) Fleeing responses plotted by cue presentation and sex. Exposure to either insecticide in females led to an increased fleeing response for the first slow cue relative to controls (mean ± SEM). Asterisk (*) indicates significant difference from controls at the $p < 0.05$ level.

whereby DDT-alone fish showed higher locomotor activity than CPF-alone treated fish ($p = 0.036$).

With respect to the fleeing response, a main effect of CPF was observed, $F(1, 254) = 3.87, p = 0.05$, as were interactions of age x DDT x CPF, $F(1, 254) = 3.87, p < 0.05$, predator cue repetition x DDT x CPF, $F(1, 254) = 4.53, p < 0.05$, sex x cue speed x cue repetition x DDT x CPF, $F(1, 254) = 3.03, p < 0.10$, and sex x age x cue speed x cue repetition x DDT x CPF, $F(1, 254) = 3.03, p < 0.10$. Overall, CPF-treated fish (CPF-alone and DDT + CPF) showed higher fleeing responses than non-CPF-treated fish (Control and DDT-alone) ($p = 0.006$) (Table S6). Post hoc on the highest order interaction (Table S7) showed that adult DDT-alone males showed elevated flee responses relative to controls on the first fast cue presentation ($p = 0.050$), adult DDT-alone and CPF-alone females showed elevated flee responses relative to controls on the first slow presentation ($p = 0.02, p = 0.03$ respectively), and aged DDT/CPF females showed elevated flee responses relative to both CPF-alone fish and controls on the first slow cue presentation ($p = 0.03, p = 0.001$

respectively).

3.3.4. Novel place recognition test

For novel place recognition, the primary measures were the number of arm explorations, the percentage of time spent in the novel arm (preference), and the difference between the observed preference and chance-level preference (discrimination index). A main effect of sex was detected for the frequency of arm explorations, $F(1, 115) = 11.37, p < 0.05$, whereby males made more center crossings than females (mean, male = 41.57 ± 2.38 , female = 30.34 ± 1.88), as was a time block x sex x treatment interaction, $F(3, 115) = 2.63, p < 0.10$, however no post hoc comparisons reached significance when stratified by sex. A main effect of treatment on novel arm preference (Fig. 5a) failed to reach significance, $F(3, 115) = 2.24, p = 0.09$. All groups were significantly averse to novel arm, as verified through an a priori confidence interval calculation (95% confidence interval, not including “chance level” preference, 23%). As fish may technically discriminate the novel from familiar arms

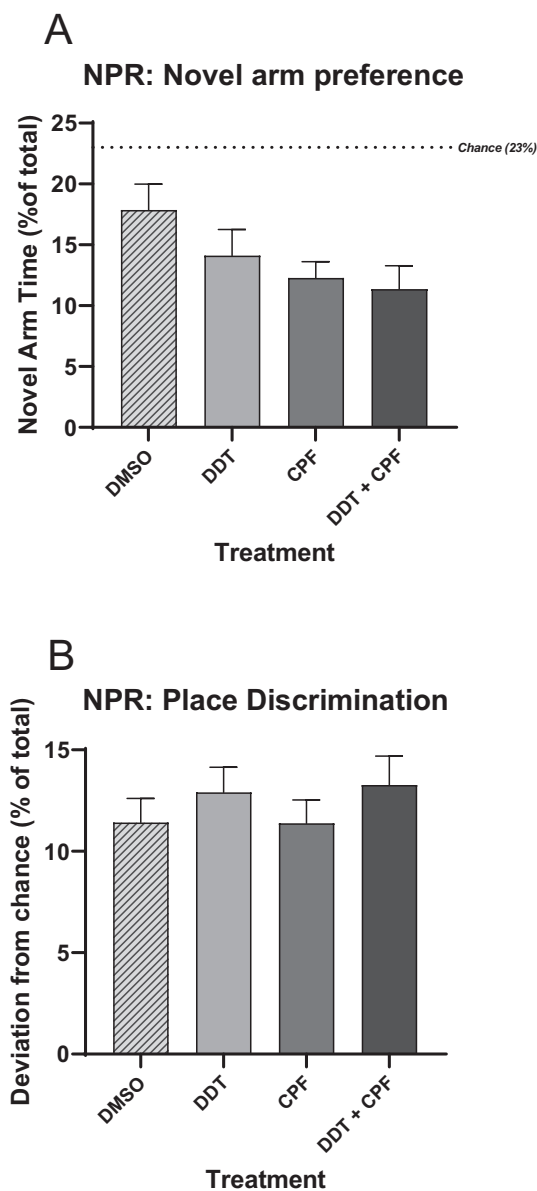


Fig. 5. Novel Place Recognition Task Results: The following results come from 14-month old fish, as NPR testing was not conducted with younger adult fish. (A) Novel arm preference plotted as a percentage of total time spent in the novel arm (% of total) (mean \pm SEM). Aged fish generally showed below chance levels of novel arm preference. (B). Place discrimination, calculated as the deviation from the chance level of 23% ($|\text{observed} - \text{expected}|$)(mean \pm SEM). All groups showed significant (non-chance) levels of novel arm avoidance and place discrimination.

in either direction (i.e. aversion or approach), a secondary analysis was performed using an unbiased discrimination index (Fig. 5g. 5b), calculated as the absolute value of the difference between each preference value (observed) and chance (23%, expected). No main effects of time block, treatment or relevant interactions were observed.

Within the two-factor treatment analysis, a main effect of sex was detected for the frequency of arm explorations, $F(1, 115) = 11.39, p < 0.05$, as was a time block \times sex \times CPF interaction, $F(1, 115) = 6.37, p < 0.05$, although no pairwise comparisons reached significance when stratified by sex and time block. For novel arm preference (Table S8a), there was a main effect of CPF treatment, $F(1, 115) = 4.58, p < 0.05$, whereby CPF-treated fish (CPF-alone or DDT + CPF) showed enhanced aversion (lower preference) to the novel arm relative to non-CPF-treated

fish (Control or DDT-alone) ($p < 0.05$). No other effects or interactions were detected. When adjusted for the direction of preference (discrimination index) (Table S8b), there was a main effect of DDT-treatment, $F(1, 115) = 4.92, p < 0.05$, whereby DDT-treated fish (DDT-alone and DDT + CPF) showed an enhanced discrimination score relative to non-DDT-treated fish (Control and CPF) ($p < 0.05$). CPF and DDT were independently associated with altered novel arm recognition.

3.3.5. Novel tank dive test

For novel tank exploratory behavior, the primary measures were locomotor activity and distance from the bottom of the tank. However, in the current study the control diving data for this test was abnormally low relative to the previous study in this series (Hawkey et al., 2020) and other previous datasets. Therefore the data from this test cannot be interpreted with confidence. It is given in the supplemental data section.

4. Discussion

The current study used an adult zebrafish model to explore the potential for differential toxicological effects following exposure to multiple different classes of insecticides in sequence, specifically when exposures transition from organochlorines to organophosphates over time. With the exception of the novel tank dive test, which was subsequently excluded from interpretation, current patterns and means for controls, DDT-alone and CPF-alone fish were in line with those observed in our prior study (Hawkey et al., 2020), providing support for the doses and time frames selected for testing. Multiple tests in the neuro-behavioral battery presented were sensitive to between group differences, including tap test, shoaling, and predator avoidance. Overall effects of either DDT or CPF, without any indication of DDT \times CPF interactions, were also noted on the novel place recognition (NPR) test. The differences between treatment groups were most prominent in the differences between the individual treatments (DDT-alone or CPF alone) and the sequential exposures (DDT + CPF, sequentially) and were found in multiple cases to be based on the age of testing, interpreted in this case as a combination of developmental age and time since the end of the exposure period. This generally supports the hypothesis that individuals with previous exposure to a given insecticide could systematically differ from one another based on how recent those exposures were and whether those insecticide exposures occurred within a multi-insecticide sequence of exposures.

The second hypothesis tested in this experiment concerned the additivity or non-additivity of the effects which may be produced by exposures to multiple insecticides. This was analyzed through a parallel analysis which included DDT and CPF-exposure as potentially overlapping factors, with a significant DDT \times CPF interaction indicating that the effects described in the traditional analysis were not in line with an additive effect (effect of the combination is either greater or less than expected based on the null hypothesis of additivity). In multiple cases (discussed below) such interactions were observed and used in the interpretation of the primary ANOVA effects. At this point, it is not possible to determine whether these statistical interactions involve processes that are mutually exclusive (i.e. DDT effects only took place during the 5-week DDT exposure) or if there is potential for persistence of DDT itself into the CPF exposure period (i.e. interaction at the level of chemical exposure itself). Certainly, the long biological half-life of DDT would enable the former to take place, and future studies will need to include within-tissue measurements of both compounds after the shift from one insecticide to the other. We can, however, rule out that these interactions involve effects of DDT on the action of CPF on its primary mechanism of toxicity, as we obtained the same degree of cholinesterase inhibition whether CPF was given alone or was preceded by DDT. It is also critical that the degree of cholinesterase inhibition from CPF exposure (25%) was well below the 70% threshold required for acute signs of organophosphate toxicity (Clegg and van Gemert, 1999). Based on this pattern, CPF-exposure in the present study may be interpreted as

representing non-symptomatic organophosphate exposures in humans (Clegg and van Gemert, 1999). Points of potential interaction beyond acetylcholinesterase remain to be investigated and should be included in future work on sequential organochlorine/organophosphate exposures. In any case, our findings of statistically significant interactions emphasize the need for mechanistic studies which corroborate epidemiological observations of unique adverse effects seen in people with sequential occupational exposures to organochlorines and organophosphates (Kamel et al., 2005).

Within the tap startle assay, the present study observed that fish with sequential DDT/CPF exposures exhibited enhanced startle responses relative to fish exposed to CPF alone, and that this pattern was specific to testing which occurred one week following the end of the CPF exposure. The secondary 2-factor analysis indicated that this difference was not consistent with the hypothesis of additivity and appeared to unrelated to any broader pattern related to DDT exposure.

Social shoaling showed multiple elements where fish with sequential DDT/CPF exposure differed from fish exposed to DDT alone, specifically hyperactivity during the test, regardless of the age of testing, and reduced social approach at the aged time of testing. The reduction in social approach relative to DDT alone was also apparent in fish with CPF-alone exposure. With respect to the hyperactivity effect, the secondary 2-factor analysis indicated that the DDT vs DDT/CPF difference was not consistent with the hypothesis of additivity and is therefore not based on any general effect of CPF exposure. With respect to the impaired social approach response in aged fish, the secondary analysis observed a relevant DDT x CPF interaction, but this must be interpreted cautiously. The level of social approach in both CPF-exposed groups approached the floor (zero) for this measure and significantly differed from DDT-alone, so it may be more accurate to interpret this finding as indicating a general effect of CPF-treatment.

Within the predator avoidance test, sequential DDT/CPF exposure to females led to brief hypoactivity at the prior to the first presentation of the predator cue relative to the DDT-alone group, and this difference dissipated once the cue presentations began. Secondary analyses indicated that sex specific activity patterns in this test were consistent with the additivity hypothesis. For the fleeing response, exposures to either of the insecticides, individually and sequentially, were less enhanced fleeing responses relative to controls at the one-week post CPF-exposure time point. Secondary analyses indicated that these effects were non-additive, which indicates that the effects of DDT and CPF on this outcome were redundant, and do not lead to a more severe phenotype. A higher order interaction by sex and predator cue (slow or fast, each presented twice), indicated that the tendency for insecticides to enhance fleeing responses was prominent among females regardless of age, and specifically targeted the very first predator response (slow 1). Again, a corresponding secondary DDT x CPF interaction indicated that these effects were non-additive.

No statistically significant interactions by treatment were noted for the other outcomes of interest. Within the cholinesterase activity analysis, CPF produced an expected reduction in activity, but prior DDT exposure did not alter the pattern. As DDT is a sodium channel blocker which reduces neuronal excitability, it was hypothesized that chronic exposure may lead to adjustments in neurotransmitter functions, including the acetylcholine system which is targeted by organophosphates like CPF. Given that prior DDT exposure did not alter the efficacy of CPF at its main mechanism of action, it is concluded that this is not the case, and that these two compounds exert their toxicity through parallel mechanisms, rather than through a single shared mechanism. This conclusion is partly supported by the effects of these compounds on the fleeing response in the predator avoidance test, where their effects were shown to be redundant, rather than additive. Within the novel place recognition (NPR) task, each insecticide appeared to independently facilitate discrimination, and there was no interaction between the two compounds. Treatment with CPF was associated with greater avoidance of the novel arm, and DDT-exposure was associated with a larger

discrimination response, regardless of the direction of that response.

The present study highlights the importance of vertebrate neurotoxicity testing for insecticides and adds new observations to this important area of research. To date, the non-human literature on insecticide neurotoxicity has largely focused on exposures that occur during early development (Abreu-Villaca and Levin, 2017), when the nervous system is particularly sensitive to interruption by chemical insults. The present data shows that some of the behavioral functions which are sensitive to embryonic neurotoxicity (Crosby et al., 2015; Glazer et al., 2018; Oliveri et al., 2015; Sledge et al., 2011) remain sensitive to similar insults well into adulthood. Further, it complements previous work from our lab and others showing that the effects of neurotoxic exposures depend on the latency of testing and/or the stage of development when testing is performed, as evidenced by discrepancies between larval and adolescent or adult data following an embryonic exposure (Crosby et al., 2015; Glazer et al., 2018) or adult exposures with short- (1 week post test) or long-term testing (several months later, at 14 months of age) (Hawkey et al., 2020).

The present data also provide validation for associations between chronic occupational exposures and short- or long-term psychiatric issues, most notably deviations in mood. A host of human studies of agricultural workers with a history of insecticide exposures or poisonings have reported higher than average incidence of depression, anxiety and/or suicidality (Beard et al., 2014; Harrison and Ross, 2016; Kim et al., 2013; Koh et al., 2017; Serrano-Medina et al., 2019; Weisskopf et al., 2013), particularly for those with exposures to organophosphates. The fish models in the present study similarly show reduced social interest and activity, as well as enhanced fear-like responses, startle reactivity, and novelty avoidance (CPF). Future studies may be able to use these fish to investigate what cell- and molecular-level changes may lead to changes in mood and behavior, and to verify that the apparent similarities in behavioral outcomes result from convergent mechanisms of persistent toxicity.

By contrast, cognitive problems, particularly memory issues in older adults (Dardiotis et al., 2019; Kim et al., 2019), were not well modeled by the current battery. Rather than impairing place discrimination in the NPR task, insecticide exposures tended to enhance reactivity to the novel stimulus, specifically towards aversion for the CPF-exposed fish and generally for DDT-exposed fish. It may be that this familiarization test is less sensitive to such impairment than complex forms of learning and memory which are the focus of human neuropsychological testing, or that the levels of exposure included in this study produce effects which support this place discrimination indirectly (e.g. modulation of anxiety-like avoidance behaviors, rather than memory). Future work with other forms of memory, and at multiple ages, might be necessary for a more comprehensive view of cognitive and neurological decline using a zebrafish model.

With regards to aging more generally, the present study showed some impact of animal age on behavior. Overall, locomotor activity increased as a function of age, as noted previously (Hawkey et al., 2020), as did post-tap activity generally. Within the shoaling test, between-group differences were robust within older adult window of testing, reaching statistical significance at this later time point, while similar differences at the 1-week follow up failed to reach significance. On the other hand, enhancements of startle and fleeing responses were only evident at the 1-week follow-up, and dissipated by the aged point of testing. Each of these findings suggests some potential for symptoms to adjust as time goes on, potentially surpassing threshold for detection or attenuating with time. This lends some evidence to concern over the long-term effects of insecticide exposures, and their potential to interact with processes of aging. However, the interpretation of age in the present study does require some clarification. Recovery time since exposure and repetitions of testing are necessarily confounded with biological age in longitudinal follow-up studies, either in animals or humans (Péres et al., 2012; Rogers et al., 2001), so these adaptations in the response do not necessarily suggest a precise mechanism. Further work is needed to

determine the mechanistic changes which occur after the end of exposure, and parse out the developmental, neuroadaptive, and experience-dependent effects that may lead to differing phenotypes as time goes on.

An additional issue is the sex of the fish. In the predator test and in multiple of the secondary analyses (shown in supplemental tables), sex was a mediator of between-group differences. While this indicates some potential for subpopulations of fish within a treatment to show greater or lesser effects, it would not necessarily be appropriate to interpret these findings as evidence for differential effects in male and female humans with insecticide exposures. Although zebrafish are often used to detect endocrine disrupting toxicants (Segner, 2009), including androgen- and estrogen-based effects, sex determination in teleost fish is a complex process, and one which does not closely mirror mammalian sex differentiation (Liew and Orbán, 2014; Santos et al., 2017). Therefore, similar studies in rodents would likely be needed to make compelling statements about the relevance of sex to insecticide vulnerability in humans.

The compound x compound interactions examined above indicate that although the toxicity of individual insecticides may be informative about their risk on the brain and behavior, additivity may not be a fair assumption when organochlorine and organophosphate exposures have occurred sequentially. Rather, the potential for outcomes which are inconsistent with individual drug effects or redundant with one or both of the multiple exposures must also be considered. More broadly, populations of individuals who report exposures to a single class of insecticides (e.g. organophosphates) may contain multiple subpopulations with varying risk, based on their history of exposure to other classes at other points in their careers. It should be noted that only a single concentration of each compound was tested in this arrangement, and the apparent “sparing” of some behaviors from an interaction may represent ceiling or floor effects, where the selected concentrations of the individual compounds already maximize the adverse outcomes (e.g. shoaling approach scores approaching zero). Future work with lower doses that are barely at the threshold for individual effects of DDT and CPF and a greater number of dose x dose combinations may then reveal a greater spectrum of interactions (Shahid et al., 2019). It should also be noted that in humans, exposures to insecticides may not necessarily be sequential, and the potential for simultaneous mixture effects should also be thoroughly examined in order to fully address the interactions between insecticides. Using inexpensive and quickly developing species like zebrafish, and methods like those using in the current study, we have the opportunity to take a closer look at psychiatric and neurological issues being experienced by present and former agricultural and pest control workers, as well as look into the future at similar problems as they go through the developmental processes of aging.

Declaration of Competing Interest

TAS has received consultant income in the past three years from Gjording Fouser (Boise, ID), Thorsnes Bartolotta McGuire (San Diego, CA), Cracken Law (Dallas, TX), Matthews Law (Houston, TX) and the State of Arizona.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ntt.2021.106985>.

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