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Effects of Chlorpyrifos on Cholinesterase and Serine Lipase Activities and Lipid Metabolism in Brains of Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT

Chlorpyrifos is an organophosphorus insecticide that elicits acute toxicity through inhibition of acetylcholinesterase (AChE), leading to acetylcholine accumulation and prolonged stimulation of cholinergic receptors throughout the central and peripheral nervous systems. Previous studies have indicated that neurodevelopment may also be impaired through alternative pathways, including reduction of cyclic adenosine monophosphate (cAMP)-catalyzed downstream events. The upstream initiating events that underlie noncholinergic neurological actions of chlorpyrifos and other organophosphorus compounds remain unclear. To investigate the potential role of fatty acid signaling disruption as a mechanism of toxicity, lipid metabolism and fatty acid profiles were examined to identify alterations that may play a critical role in upstream signaling in the central nervous system (CNS). Juvenile rainbow trout were treated for 7 days with nominal chlorpyrifos concentrations previously reported to diminish olfactory responses (10, 20, and 40 μg/l). Although lethality was noted higher in doses, measured chlorpyrifos concentrations of 1.38 μg/l (nominal concentration 10 μg/l) significantly reduced the activity of AChE and two serine lipases, monoacylglycerol lipase, and fatty acid amide hydrolase in the brain. Reductions in lysophosphatidylethanolamines (16:0, 18:0, 18:1, and 22:6) derived from the phosphatidylethanolamines and free fatty acids (palmitic acid 16:0, linolenic acid 18:3, eicosadienoic acid 20:2, arachidonic acid 20:4, and docosahexaenoic acid 22:6) were also noted, suggesting that chlorpyrifos inhibited the metabolism of select phospholipid signaling precursors at sublethal concentrations. These results indicate that in addition to AChE inhibition, environmentally relevant chlorpyrifos exposure alters serine lipase activity and lipid metabolites in the trout brain, which may compromise neuronal signaling and impact neurobehavioral responses in aquatic animals.

Key words: pesticide; neurotoxicology; organophosphate; lipidomics.

Chlorpyrifos (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate, CPF) is an organophosphate insecticide that is widely used for agriculture purposes in the United States and around the world. Its residential use has been restricted in the United States since 2000, primarily due to developmental neurotoxicity studies in rodents and epidemiological studies in

humans suggesting significant risk to the developing brain (Campbell *et al.*, 1997; Qiao *et al.*, 2003; Rauh *et al.*, 2006). Numerous studies have shown that CPF is bioactivated by cytochrome P450 monooxygenases to its corresponding oxon, a potent irreversible inhibitor of acetylcholinesterase (AChE) (Amitai *et al.*, 1998), responsible for the breakdown of the neurotransmitter acetylcholine. Reduced AChE following CPF exposure leads to hyperactivity at cholinergic synapses in neurons and neuromuscular junctions (Sandahl *et al.*, 2005; Sultatos, 1994). High exposures in mammals can cause acute lethality due to respiratory failure from both central and peripheral actions (Eddleston *et al.*, 2006). The cause of death in fish is less certain.

Measurable concentrations of CPF have been detected in many waterways along the western coast of the United States (Sargeant *et al.*, 2013; Zhang *et al.*, 2012), generally at concentrations well below those necessary to induce acute lethality in fish. Therefore, recent attention has focused on the effects of chronic, lower-level CPF exposures. The behavioral effects of AChE inhibition following sublethal CPF exposure have been well-documented in several fish species, with effects on swim performance, feeding, predatory cue detection, and overall activity (Levin *et al.*, 2004; Rice *et al.*, 1997; Sandahl *et al.*, 2005; Tierney *et al.*, 2007). Loss of predatory cue detection has been associated with reduced predator avoidance and increased mortality risk (McIntyre *et al.*, 2008, 2012).

Recent evidence has suggested other sublethal impacts of CPF in the developing brain that are unrelated to AChE inhibition. Specifically, altered signal transduction in the olfactory bulb may be related to declines in odor-evoked behaviors in CPF-exposed fish. Olfactory function in zebrafish is disrupted by CPF through inhibition of G-protein-coupled receptor signaling and odorant binding, leading to reduced olfactory responsiveness (Tilton *et al.*, 2011). Genes involved in second messenger signaling, neurotransmitter signaling, and calcium signaling were consistently downregulated in salmon with reduced odor avoidance behaviors (Wang *et al.*, 2016), and the magnitude of overevoked field potentials was inhibited in electrophysiological recordings in response to CPF (Sandahl *et al.*, 2004). Previous studies in our laboratory using rainbow trout have indicated that reduced physiological and behavioral responses following CPF exposure correspond with transcriptomic changes in the brain for genes involved in neuronal signal transduction and are associated with diminished cAMP and calcium signaling (Maryoung *et al.*, 2014).

The initiating events for transcriptomic changes associated with CPF exposure are unclear. However, recent developments in lipidomic methodologies have indicated that novel free fatty acids (FFAs) derived from lipids either from plasma or from organelle membranes can have significant impacts on transcriptional regulation (Jump, 2004). Free fatty acids bind to nuclear receptors in a manner similar to hydrophobic hormones (Göttlicher *et al.*, 1992) and can directly affect gene transcription through peroxisome proliferator activated receptors (PPARs) and NF-κB (Bocos *et al.*, 1995). Each of these signaling pathways can alter expression of genes important in neuronal signaling and cognitive function (Bright *et al.*, 2008). Indirect effects of FFA on transcription can occur via effects on protein kinase C (PKC) signal transduction and changes in membrane lipid composition, altering G-protein-coupled receptors (Jump, 2004). Protein abundance and mRNA turnover rate can also be affected by FFAs (Duplus *et al.*, 2000; Jump, 2004). The summation of FFA-induced changes can alter cellular growth, differentiation, signaling, and metabolism (Diascro *et al.*, 1998).

Alterations in FFA and other lipids in the brain following CPF exposure may occur through serine lipase inhibition. FFA content is regulated by a number of serine hydrolases that cleave ester bonds anchoring fatty acids to the glycerol backbone. Previous studies have shown that organophosphate oxons, such as CPF-oxon, can bind to and inhibit serine hydrolases in a similar manner as AChE (Deutsch *et al.*, 1997; Quistad *et al.*, 2006). Inhibition of hydrolases/lipases by CPF could alter the population and distribution of FFAs as well as other lipid-derived molecules in the brain, initiating FFA-induced transcriptional changes. Monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) are two serine lipases highly sensitive to many organophosphate oxons, and inhibition of FAAH or MAGL in mice drastically alters the brain lipidome, an effect that varies greatly by brain region (Leishman *et al.*, 2016).

In order to test the hypothesis that CPF inhibits serine hydrolases in rainbow trout brain that are important in regulating lipid signaling at concentrations previously reported to impair neurological function, juvenile rainbow trout were exposed to CPF for 7 days. Following treatment, the brains were evaluated for activities of the cholinesterases (AChE and its "sister" enzyme butyrylcholinesterase, BChE), activities of the serine lipases MAGL and FAAH, and changes in lipid profiles. *Ex vivo* inhibition of AChE and BChE by chlorpyrifos oxon was also compared. Alterations in lipidomic content and serine lipases induced by CPF were examined in the context of their potential impacts on neuronal signaling pathways in the developing brain.

MATERIALS AND METHODS

Exposures. After acclimation for 1 month, juvenile rainbow trout (parr stage, mean ± SEM, mass= 54.0 ± 4.4 g, length= 17.4 ± 1.5 cm, $n = 33$) from Jess Ranch Hatchery (Apple Valley, California) were placed in 20-l glass aquaria (1 fish per aquaria) for 7 days in 12°C water with a 14:10 light dark cycle. Aquaria were placed on an exposure table with a recirculating chiller to maintain 12°C water temperature. Chlorpyrifos (99.5% purity, Chem Services, West Chester, Pennsylvania) was dissolved in 100% ethanol, with stock solutions kept in the dark at −20°C. Exposure solutions were prepared by diluting a working stock solution with dechlorinated water, with a final vehicle concentration <0.01% ethanol within all treatment groups and vehicle control. Exposure groups included a solvent control (0.01% ethanol) and nominal CPF treatments of 10, 20, and 40 μg/l for mortality range finding, with an *n* of 8 animals per treatment group. Fifty percent water changes were performed daily and replaced with fresh water. A stock CPF solution was then spiked into each exposure aquarium to maintain initial nominal concentrations of CPF. Trout were fed Skretting trout food every 48 h prior to re-exposure and allowed 2 h to consume the food. Each animal was fed 3% of its body weight and the remaining food was siphoned out during the daily water change. After 7 days, brains from remaining live fish from sublethal concentrations were removed for biochemical and lipidomic measurements. Brain removals were conducted by first euthanizing an animal in tricaine methane sulfonate (MS-222, Argent Chemical Laboratories, Redmond, Washington), followed by cervical dislocation on the bench top. Tissues were immediately flash frozen in liquid nitrogen following extraction. Concentrations of CPF and duration of exposure were based on previous studies showing impairment of electrophysiological olfactory function and behavior (Maryoung *et al.*, 2014; Sandahl *et al.*, 2004).

Experimental protocols were approved by the University of California Riverside Institutional Animal Care and Use Committee (A-20130010). Sex was not determined for the animals.

Water chemistry analysis. Water samples were collected throughout the duration of the exposure at the same time each morning (8:00), prior to the daily water change, and stored at 4°C in 1-l amber glass bottles. Samples were processed within 1 week of sampling. Extractions were performed in accordance with Environmental Protection Agency (EPA) method 3510C. Briefly, 1 l of sample was mixed with 50 ml methylene chloride (Thermo Fisher Scientific) in a separatory funnel and shaken for 2.5 min. Following phase separation, the lower methylene chloride layer was drained and filtered through a layer of burned anhydrous sodium sulfate into a round-bottom flask. This extraction was repeated 2 more times, for a total of 3 times, and all extractions were combined. The sample was dried down on a Rotevaporator to approximately 1 ml and the flask was rinsed with hexane, then further dried under nitrogen. The sample was reconstituted in 1 ml hexane and stored at −20°C until analysis. Samples were run on an Agilent 6890N/5973B GC/MSD with a 30 m × 0.25 m DB-5MS column with the following parameters: Initial temperature of 80°C for 1 min, followed by a temperature increase to 200°C at a rate of 25°C/min, increased to 300°C at a rate of 5°C/min and held at 300°C for 9 min. Helium was used as the carrier gas under a flow rate of 1.2 ml/min. A 6-point standard curve was used to determine sample concentrations with serial dilutions of an internal standard.

PCB 209 was used as an internal standard and added to each sample prior to extraction. Although PCB 209 is a very hydrophobic compound with a log Kow of around 8, it has commonly been used as a surrogate standard in studies of pyrethroid insecticides and other hydrophobic pesticide products.

Cholinesterase and serine lipase assays. Brain tissues were homogenized 1:10 (w/v) in phosphate buffered saline (100 mM phosphate containing 1.54 M NaCl, pH 7.4) for measurements of AChE, BChE, MAGL, and FAAH activity. AChE, BChE, and MAGL were measured using a photometric assay (Anderson *et al.*, 2018) based on the method of Ellman *et al.* (1961). A cocktail containing substrate (1 mM acetylthiocholine, 1 mM butyrylthiocholine, or 0.1 mM 2-arachidonoyl thioglycerol) and 0.1 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) in Tris-E (10 mM Tris-HCl containing 1 mM EDTA, pH 7.2) was added to start the reaction. Change in absorbance at 412 nm was measured for 5 min at 25°C using a Spectramax M2 microplate reader (Molecular Devices; Sunnyvale, California). Enzyme activity was estimated based on the rate of appearance of the reaction product, 2-nitro-5-thiobenzoate ($\varepsilon = 14,150\ M^{-1}cm^{-1}$). FAAH activity was measured in a plate reader-based fluorometric assay (Huang *et al.*, 2007). Tissue homogenates were added into a plate with 125 mM sodium phosphate buffer containing 1% glycerol and 0.1% Triton-X-100, pH 8.0. The reaction was started by the addition of 0.5 mM N-(6-methoxypyridin-3-yl) octanamide (OMP) (dissolved in 1:1 DMSO/EtOH) and incubated for 30 min at 25°C. Following incubation, the fluorescence intensity of the product (5-amino-2-methoxypyridine [AMP]) was measured (excitation: 320 nm, emission: 396 nm). A contemporaneous standard curve of AMP was used to convert relative fluorescence units (RFU) to nmol of product. OMP was a generous gift from Dr Bruce Hammock, UC Davis. All enzyme activities were normalized to protein concentration using the method described by Bradford (1976) and bovine serum albumin as the standard.

Ex vivo AChE and BChE inhibition assays. *Ex vivo* sensitivity of AChE and BChE to inhibition by chlorpyrifos oxon was evaluated similarly to Johnson and Russell (1975). Frozen brain tissue was homogenized 1:30 (w/v) in 50 mM potassium phosphate buffer, pH 7.0 (KPi). Equal volumes of homogenate and either vehicle (KPi containing 1% ethanol; KPi/EtOH) or one of a range of concentrations of CPF-oxon (0.6 nM–1 μM in KPi/EtOH) were mixed and preincubated for 20 min at room temperature. Following preincubation, the reaction was started by addition of [^{3}H]acetylcholine or [^{3}H]butyrylcholine (1 mM final concentration). [^{3}H]acetylcholine iodide (specific activity 65.5 mCi/mmol) and [^{3}H]butyrylcholine iodide (specific activity 95 mCi/mmol) were purchased from Perkin Elmer (Waltham, Massachusetts). Reactions proceeded at room temperature for 10 min (AChE) or 360 min (BChE) and were terminated by addition of an acidified stop solution, followed by extensive vortexing. A hydrophobic scintillation cocktail was added and product ([^{3}H]acetic acid) was separated by liquid-liquid exchange and then counted in a scintillation counter (Perkin Elmer Tri-Carb 2810 TR; 61% efficiency). Preliminary assays were conducted to determine the appropriate tissue concentration and incubation time to capture a linear rate of substrate hydrolysis. Data from three independent samples were normalized based on vehicle controls (100% activity) and fit using a variable slope nonlinear regression model to estimate half maximal inhibitory concentration (IC_{50}) and 95% confidence intervals (Graphpad Prism version 6.07; La Jolla, California).

Lipid extraction and analysis. Samples were homogenized in phosphate buffer saline (10:1, w/v) using a glass homogenizer. An aliquot of 50 μl of each sample was added into a glass vial followed by addition of deuterated internal standards (SPLASH mix, Avanti, Alabama). Lipid extraction was accomplished by the Bligh and Dyer (1959) method using water:methanol:chloroform (2:1:1.5 v/v/v). The lower layer chloroform was collected, concentrated to dryness, and reconstituted into 100 μl MeOH:toluene (9:1 v/v) prior to analysis.

Separation and quantification of lipid classes was performed on an AB Sciex Qtrap 6500 LC-MS/MS using a hydrophilic interaction liquid chromatography (HILIC) column. LC conditions were operated at a flow rate of 0.5 ml/min and a gradient consisting of acetonitrile:water (95:5 v/v) (A) and acetonitrile:water (50:50 v/v) (B) with 10 mM ammonium acetate. Scheduled multiple reaction monitoring (sMRM) was used for identification of lipid classes (eg, phospholipids, sphingolipids, glycerols, and FFAs).

RESULTS

Mortality and Water Chemistry Analysis

Nominal CPF concentrations of 10, 20, and 40 μg/l were used for mortality range finding for sublethal CPF concentrations in rainbow trout. Seventy five percent mortality was observed in both 20 and 40 μg/l CPF treatments, with no acute mortality observed in control or 10 μg/l CPF exposures. Therefore, all subsequent chemical and biochemical analyses were performed at a nominal CPF concentration of 10 μg/l.

Measured CPF concentration in the solvent controls was below the detection limit at all sampling timepoints. Measured CPF concentration from the 10 μg/l nominal

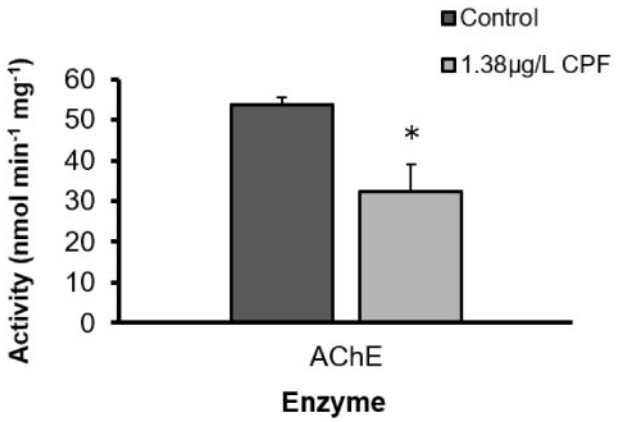

Figure 1. Acetylcholinesterase inhibition following chlorpyrifos (CPF) exposure. Acetylcholinesterase activity in brain from rainbow trout exposed to 1.38 µg/l CPF for 7 days. (*$p \leq .05$, unpaired *t* test, two-tailed, $n = 4$ per treatment group).

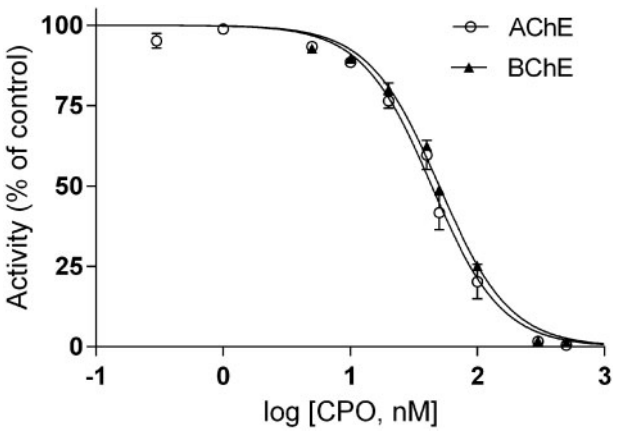

Figure 2. *Ex vivo* inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) by chlorpyrifos (CPF)-oxon. Brain tissue from untreated juvenile rainbow trout was used to assess the sensitivity of AChE and BChE to ex vivo inhibition by CPF-oxon. The data represent the mean ± SEM ($n = 3$ replicates per treatment group).

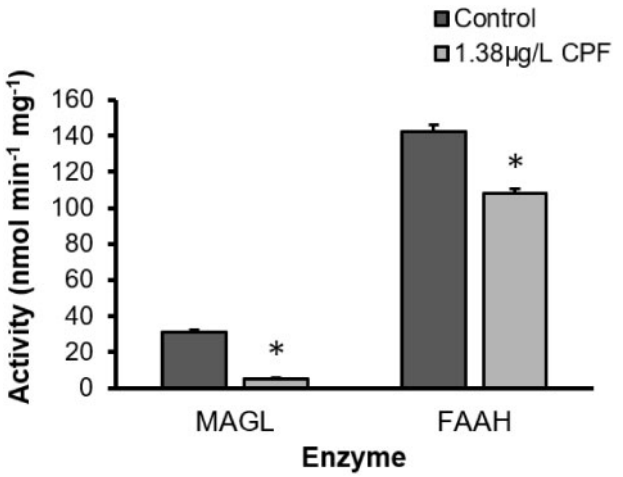

Figure 3. Inhibition of monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) by chlorpyrifos (CPF). Inhibition of MAGL and FAAH activity in rainbow trout brain tissues following whole-animal exposure to CPF for 7 days. Values represent mean ± SEM (*$p \leq .05$, unpaired *t* test, two-tailed, n = 3 per treatment).

concentration, taken over the duration of the exposure, were 1.38 ± 2.58 µg/l. Recovery values, calculated from a PCB 209 standard, were 104% ± 16.9%. The 10 µg/l nominal CPF concentration will be presented as the measured concentration throughout.

Biochemical Changes

Brain AChE was significantly inhibited in animals exposed to 1.38 µg/l CPF (40% inhibition, Figure 1). The basal activity of BChE was significantly lower than AChE activity in trout brain and was not inhibited *in vivo* by CPF (control BChE activity: 0.646 nmol $min^{-1} mg^{-1}$ ± 0.04; 1.38 µg/l CPF activity: 0.694 nmol $min^{-1} mg^{-1}$ ± 0.03). To further test the sensitivity of AChE and BChE to CPF-oxon, *ex vivo* inhibition of each enzyme was determined (Figure 2). IC_{50} values (25°C, 20 min) and 95% confidence intervals were calculated. In contrast to the *in vivo* findings, AChE and BChE appeared relatively similar in sensitivity to CPF-oxon *ex vivo* (AChE, IC_{50} = 43.7 nM [39.72–48.13]; BChE, IC_{50} = 50.1 nM [47.24–53.07]).

Activity of two serine lipases, MAGL and FAAH, were also assessed at the sublethal dose of 1.38 µg/l CPF. MAGL activity was significantly decreased (84% inhibition) by CPF at 1.38 µg/l, and FAAH activity was also significantly reduced following exposure (24% inhibition; Figure 3).

Lipidomics

Lipids were separated by HILIC chromatography based on their retention times. Targeted analysis of lipids was performed using 1300 sMRMs in both positive and negative mode and lipids were graphed based on lipid categories. No significant differences were observed between controls and trout exposed to CPF (1.38 µg/l) for sphingomyelins, phosphatidylcholines, or lysophosphatidylcholines. However, reductions were noted for lysophosphatidylethanolamines (LPEs), triacylglycerides (TAG), FFAs, and phosphatidylethanolamines (PE).

Free fatty acids showed the greatest reduction in CPF-treated animals (Table 1). Reductions were observed in a number of lipids including 16:0, 18:3, 20:2, 20:4, and 22:6. Significant reductions were also noted for 4 out of the 5 LPEs measured, including the 16:0, 18:0, 18:1, and 22:6 compounds (Figure 4A). This was accompanied by a trend toward an increase in the parent PE (P16:0/18:1), suggesting a reciprocal parent/daughter relationship (Figure 4B). Significant decreases were observed for 2 other PEs, PE (18:1/20:5) and PE (P18:0/16:1). A significant decrease was also noted for 2 TAGs (TAG52:4-FA18:2 and TAG52:3-FA18:1) in the brains of CPF-treated animals (Figure 5). Notably, TAG52:3-FA18:1 was highly concentrated in control brains (5.85 µM) but was absent in the brain of CPF-treated animals.

DISCUSSION

In order to evaluate the molecular initiating event for impaired signal transduction observed in previous sublethal studies with CPF in fish (Maryoung *et al.*, 2014; Wang *et al.*, 2016), a lipidomic screen and evaluation of serine hydrolase enzymes important in the cleavage of FFAs and other signaling lipids from glycerol backbones was carried out. Lipidomic evaluations were conducted at the sublethal concentration of 1.38 µg/l CPF, where AChE, MAGL, and FAAH activities were significantly reduced, without inducing acute lethality. Concentrations exceeding 3 µg/l have been measured in the San Jaoquin region in

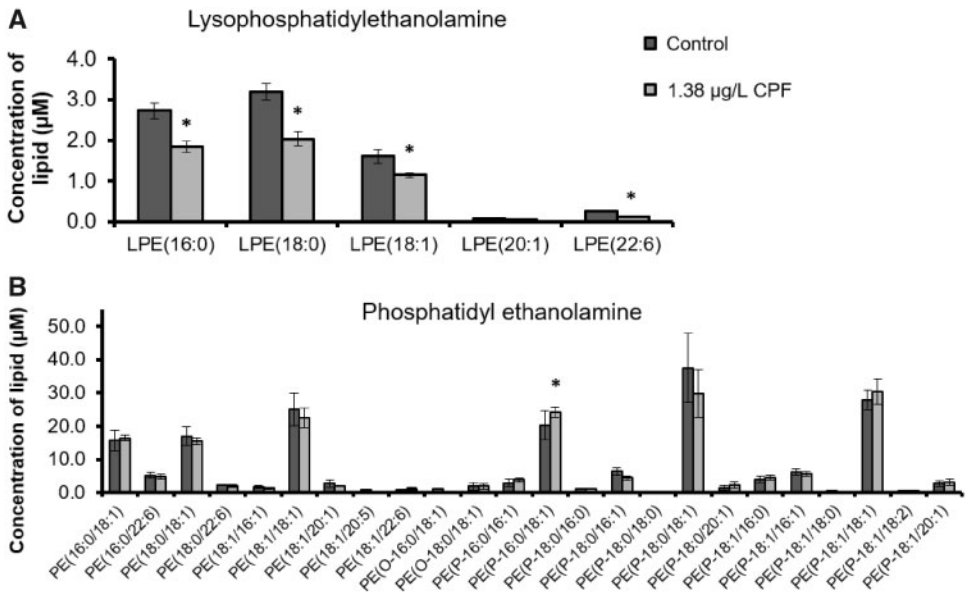

Figure 4. Lysophosphatidylethanolamine (LPE) and phosphatidylethanolamine (PE) concentrations following exposure. Concentrations of (A) LPEs and (B) PE in the brain of control and chlorpyrifos (CPF)-treated trout (1.38 µg/l for 7 days). Values represent the mean ± SE (controls, $n = 6$; 1.38 µg/l CPF, $n = 4$, $^*p \leq .05$, unpaired *t* test, two-tailed).

Table 1. Free Fatty Acid Concentrations

Free Fatty Acid	Control Concentration (µM)	1.38 µg/l CPF Concentration (µM)	% Change
14:0	0.24 ± 0.09	0.16 ± 0.02	↓ 33
14:1	0.18 ± 0.05	0.14 ± 0.02	↓ 22
*16:0	3.81 ± 0.61	0.66 ± 0.14	↓ 83
16:1	0.51 ± 0.18	0.29 ± 0.18	↓ 43
16:2	0.36 ± 0.09	na	na
18:0	1.89 ± 0.80	0.87 ± 0.19	↓ 54
18:1	2.07 ± 1.08	0.77 ± 1.08	↓ 63
18:2	0.37 ± 0.12	na	na
*18:3	0.27 ± 0.07	0.12 ± 0.05	↓ 56
20:0	1.57 ± 0.23	1.18 ± 0.21	↓ 25
20:1	0.61 ± 0.23	0.42 ± 0.07	↓ 31
*20:2	0.28 ± 0.06	0.12 ± 0.03	↓ 57
20:3	0.41 ± 0.13	0.21 ± 0.06	↓ 49
*20:4	5.23 ± 1.87	1.89 ± 0.59	↓ 64
20:5	12.77 ± 7.64	6.03 ± 1.90	↓ 53
22:4	0.36 ± 0.10	0.17 ± 0.00	↓ 53
22:5	3.17 ± 1.07	1.41 ± 0.42	↓ 56
*22:6	35.46 ± 11.11	15.75 ± 4.50	↓ 56
24:0	0.30 ± 0.10	0.20 ± 1.04	↓ 33
24:1	1.08 ± 0.50	0.83 ± 0.19	↓ 23

Free fatty acid concentrations from brain tissues following CPF exposure for 7 days. Values represent the mean ± SE. Na indicates that the concentration was below the detection limit (controls, $n = 6$; 1.38 µg/l CPF, $n = 4$, $^* p \leq .05$, unpaired *t* test, two tailed).

California, thus the CPF concentration used in this study represents an environmentally relevant scenario.

Measured CPF concentrations of 1.38 µg/l resulted in 40% inhibition of AChE in rainbow trout brains compared with controls. Inhibition of AChE by CPF has been observed with concentrations as low as 0.4 µg/l for 96 h in the brain of coho salmon (Sandahl *et al.*, 2005), and concentrations less than one part per billion (approximately 1 µg/l) in steelhead (Sandahl and Jenkins, 2002), thus it was unsurprising that AChE was significantly inhibited in rainbow trout in this study. Although both AChE and BChE appeared similar in sensitivity to CPF-oxon *ex vivo*, BChE activity levels in the brain were near the limit of detection, indicating that BChE may not be present in the rainbow trout brain. This corroborates other studies showing that AChE is the predominant cholinesterase source in the brain in rainbow trout and other *Salmonidae* species (Sandahl *et al.*, 2005; Sandahl and Jenkins, 2002; Sturm *et al.*, 2007) and suggests that cholinesterase-induced alterations in the brain of CPF-exposed rainbow trout are likely due exclusively to AChE inhibition.

Reduced behavioral responses to olfactory cues following CPF exposure suggest CPF alters neuronal signaling in the olfactory bulb. However, cholinergic synapses have not been identified in olfactory rosettes (Scholz *et al.*, 2006), and reduced reception to predatory cues occurs in the absence of AChE inhibition, suggesting a non-AChE mechanism of action. Other studies have indicated inhibition of olfactory receptor neuron signaling pathways, alterations in downstream cAMP-catalyzed events, and altered Ca^{2+} regulation following CPF, each independent of neuromuscular impairment and AChE inhibition (Maryoung *et al.*, 2014; Wang *et al.*, 2016).

An alternative pathway may be alterations in lipid-derived signaling molecules. In addition to AChE, 1.38 µg/l CPF inhibited both MAGL and FAAH in the brain of treated animals. MAGL and FAAH are membrane-bound serine hydrolases that hydrolyze 2-arachidononylglycerol (2-AG) and 2-arachidonoylethanolamine (AEA), respectively, the two endogenous ligands of cannabinoid receptors in the brain. Repeated low-dose CPF exposure also inhibits MAGL and FAAH in the forebrain of juvenile rats, leading to elevated 2-AG and AEA levels that could disrupt proper endocannabinoid (eCB) signaling that is crucial to proper brain development in juveniles (Anavi-Goffer and Mulder, 2009; Carr *et al.*, 2011). Moreover, FAAH inhibition and increased AEA levels occur at low CPF concentrations that do not elicit cholinesterase inhibition (Carr *et al.*, 2014). We also observed greater inhibition of MAGL (84%) than AChE (40%) in rainbow trout. Although other enzymes may also play a role in AEA and 2-AG hydrolysis (Blankman *et al.*, 2007), organophosphate inhibition of MAGL

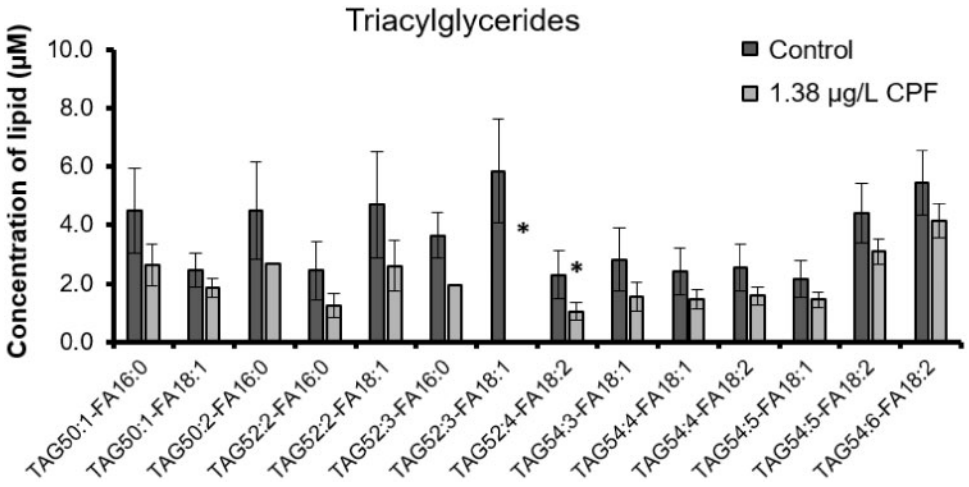

Figure 5. Triacylglyceride (TAG) concentrations following chlorpyrifos (CPF) exposure. The sum total of all TAGs was significantly decreased in CPF-exposed animals. Values represent the mean ± SE (controls, $n = 6$; 1.38 µg/l CPF, $n = 4$, $^*p \leq .05$, unpaired *t* test, two tailed).

and FAAH results in elevations in >1000% elevation in 2-AG and AEA and cannabinoid behavioral effects in mice (Nomura *et al.*, 2008). Thus, although the analytical methods used in this study were not suitable for measuring tissue eCBs, FAAH and MAGL inhibition noted here suggests that elevated concentrations of AEA and 2-AG may occur in the brain of CPF-exposed trout, with potential alterations in eCB signaling and related signaling pathways.

Altered eCB signaling via serine lipase inhibition in the olfactory bulb could be involved in diminished olfactory behavioral responses following CPF exposure (Maryoung *et al.*, 2014). Overactivation of eCB signaling suppresses excitatory transmission in the nervous system via reduced glutamate release (Pouille and Schoppa, 2018), and cannabinoid receptors are highly expressed in excitatory corticofugal feedback (CFF) neurons in the olfactory bulb that make connections onto glutamatergic neurons involved in odor-evoked output and odor-driven behaviors (Boyd *et al.*, 2015; Martin *et al.*, 2004; Otazu *et al.*, 2015). Thus, overactivation of eCB signaling and suppression of neurotransmission between CFF and glutamatergic neurons could play a direct role in diminished olfactory behavioral responses and inhibition of olfactory receptor neuron signaling pathways in CPF-exposed fish (Maryoung *et al.*, 2014; Wang *et al.*, 2016). Cannabinoid receptor 1 (CB1) agonist suppression of transmission is greater in juvenile rats than adults, suggesting juvenile trout may also be more susceptible than older trout (Kang-Park *et al.*, 2007). Furthermore, the G-protein-coupled cannabinoid receptors are primarily of the Gi and Go classes, which rapidly decrease cAMP levels by inhibition of adenylyl cyclase activity (Howlett *et al.*, 2004). Overactivation of eCB signaling would support transcriptional evidence of diminished cAMP-catalyzed events observed previously and could play a role in altered olfactory signal transduction in juvenile rainbow trout (Maryoung *et al.*, 2015).

CPF exposure at concentrations eliciting serine lipase inhibition led to significant reductions in the concentration of specific lipids in the brain, most notably in the overall reduction of FFAs. The reduction in FFA may be related to the observed inhibition of MAGL. Inhibition of MAGL in PC3 and DU145 cell lines leads to accumulation of MAGs and subsequent reductions in their corresponding FFAs, including C20:4 and 16:0 that were altered in this study (Nomura *et al.*, 2011). This is consistent with previous observations of FAAH and MAGL inhibition altering brain lipid content in mice (Leishman *et al.*, 2016). Docosahexaenoic acid (DHA; FFA 22:6 n-3) comprises 40% of the poly unsaturated fat in the human brain (Singh, 2005) and was also the most abundant FFA in the brain of rainbow trout. Docosahexaenoic acid showed significant decreases in the brain of CPF-treated animals, which has also been implicated in altered eCB signaling through CB1 (Pan *et al.*, 2011; Watanabe *et al.*, 2003). However, another FFA with a trend toward decreased concentration, eicosapentaenoic acid (EPA, FFA 20: 5), causes the opposite results, with decreased expression on CB1 following treatment. This suggests a complex relationship between FFA changes and altered eCB signaling in the brain. Further studies are needed to determine how the summation of FFA changes alter eCB signaling in the olfactory bulb, and how these may be related to altered behavioral responses in trout.

Deficiencies in DHA have been implicated in other cognitive declines through effects on ion channel activity, neurotransmission, and importantly, deficiencies in the acquisition of an olfactory learning set (Catalan *et al.*, 2002; Lukiw *et al.*, 2005). In membrane phospholipids it can affect membrane receptor activities and cell signaling (Akbar *et al.*, 2005). Similar findings have also been reported for other FFAs showing significant declines in this study. Palmitic acid (FFA 16:0) deficiency alters serotonin receptor densities in the frontal cortex in rats, again suggesting alterations in learning (Delion *et al.*, 1994). Palmitic acid deficiency also leads to declines in visual acuity in infant rhesus monkeys (Neuringer *et al.*, 1984). Arachidonic acid (FFA 20:4) can activate PKCα, inducing mitogenic signaling via activation of Fos, Jun, and AP-1 transcription factors. Other FFAs with reduced concentrations, eicosadienoic acid (FFA 20:2) and linolenic acid (FFA 18:3), were very minor components of the lipidome, and little is known about their importance in the trout brain.

Changes in FFA in the brain can directly alter gene expression through transcription factors, particularly through PPARs, PPARα and PPARγ, part of the steroid hormone receptor subfamily (O'Sullivan, 2016). All PPAR subtypes bind C_{16}-C_{20} fatty acids (Xu *et al.*, 1999), of which four showed deceased concentrations in CPF animals. PPARs form heterodimers with retinoid X receptor and bind to their constituent response elements in the DNA, leading to changes in transcription of genes involved in cell differentiation, fatty acid metabolism, and neuronal function (Menendez-Gutierrez *et al.*, 2012; Schoonjans *et al.*, 1995). PPARγ mRNA is expressed at high levels in the olfactory bulb of adult

mice (Liu, Huang, *et al.*, 2015) and plays an important role in development and lipid metabolism (Peters *et al.*, 2000). Activation of PPARα may also have neuroprotective effects and reduce permeability of the blood-brain barrier (Hind *et al.*, 2015; Zhou *et al.*, 2012). Inhibition of FAAH observed may further affect PPAR signaling. FAAH in the brain hydrolyzes palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), 2 other PPAR agonists (Leishman *et al.*, 2016). Inhibition of FAAH led to increased PEA and OEA in rat brain (Liu, Parsons, *et al.*, 2015), which could further alter PPAR signaling if these metabolites are altered in trout following CPF exposure. Other studies have reported that CPF alters expression of transcription factors (AP1 and Sp-1) in the developing brain of neonatal rats, indicating that CPF interferes with brain development through transcription factors involved in cell replication and differentiation (Crumpton *et al.*, 2000). Although there is no reported evidence of FFA effects on AP1 or Sp-1, these results suggest CPF-induced transcription factor changes occur and could alter associated signaling in the brain.

Activation of PPARα is a major regulator of both intra- and extra-cellular lipid metabolism by increasing the size and number of peroxisomes, responsible for cholesterol and lipid metabolism (Michalik *et al.*, 2006), and activation of lipoprotein lipase gene expression, important players in triglyceride metabolism. Triacylglycerides are synthesized from 3 fatty acid molecules bonded to the C1-C3 positions in a glycerol "backbone" and are the primary form of fat energy storage in adipose tissue. This may in part explain our observation of an overall decrease in triglycerides. Reduced FFAs could mean less material to form TAGs, combined with increased PPAR activity and metabolism of present TAGs. Triacylglyceride can be used as a fuel source for the brain by the metabolism of the glycerol backbone into glucose. There are little data about the effects of TAGs on brain activity, thus it is difficult to predict the impacts of reduced TAG concentrations in the brain due to CPF.

Although ethanolamides were not measured in the current study, significant differences were observed with LPEs at nearly every carbon length. Lysophosphatidylethanolamine can be generated from PE, a component of the cell membrane, via a phospholipase A-type reaction. In typically low concentrations in human serum, LPE has been shown to activate MAPK in fungi and initiate neuron differentiation in PC12 cells (Nishina *et al.*, 2006). However, it is unclear if altered LPE and PE could affect neuronal processes *in vivo*.

To our knowledge, this is the first study to identify serine lipase inhibition and lipidomic changes as effects of CPF exposure on the developing brain of fish. Non-AChE mediated alterations in transcriptional regulation have previously been implicated in neuronal deficits and reduced prey behavior. The inhibitory effects of CPF on serine lipases and downstream lipidomic profiles indicate potential upstream pathways, such as eCB and PPAR signaling, that could initiate transcriptomic changes that ultimately lead to behavioral deficits. Moreover, MAGL inhibition was more severe than AChE inhibition at low CPF concentration. These data have important implications for sublethal effects of CPF on juvenile fish that could affect survival in the wild.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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