Plasma cholinesterase activity as a biomarker for quantifying exposure of green sturgeon (*Acipenser medirostris*) to carbaryl following applications to control burrowing shrimp in Washington State

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Abstract

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Willapa Bay, located in Southwestern Washington State (USA), is one of the rare intertidal locations where large-scale pesticide applications occur. Since the 1960s, carbaryl has been applied to mudflats in Willapa Bay to control populations of burrowing shrimp (*Neotrypaea californiensis* and *Upogebia pugettensis)* that decrease oyster (*Crassostrea gigas*) productivity. Green sturgeon (*Acipenser medirostris*) are present in Willapa Bay, and population declines (Southern Distinct Population Segment) have resulted in their listing under the Endangered Species Act (ESA). White sturgeon (*Acipenser transmontanus*) are also found in Willapa Bay, are closely related to green sturgeon, but are not ESA listed. To determine potential impacts of carbaryl on cholinesterases in sturgeon brain and plasma, seawater-acclimated and laboratory maintained white sturgeon (average wt = 1.1 kg) were exposed to each of six concentrations of carbaryl (0, 30, 100, 300, 1,000 and 3,000 µg L^{-1}, n = 5 per treatment) for 6 h and brain acetylcholinesterase (AChE) and plasma butyrylcholinesterase (BChE) activities were measured.

Enzyme recovery was measured in additional cohorts (n = 4 sturgeon per time point, average wt = 1.8 kg) exposed to 1,000 µg L^{-1} carbaryl for 6 h with brain AChE and plasma BChE activity measured at 0, 24, 48 and 72 h post-exposure. Brain AChE activity was statistically ($p \leq 0.05$) reduced at all concentrations except 30 µg L^{-1}. Compared to controls, brain AChE was inhibited 15% in sturgeon exposed to 30 µg L^{-1}, and 27%, 32%, 35% and 42% respectively at the higher concentrations. In the enzyme recovery trial, brain AChE was inhibited ($p \leq 0.05$) after the 6-h exposure to carbaryl, followed by recovery over 72 h. Compared to controls, brain AChE was inhibited 36%, 23%, 28% and 13% at 0, 24, 48 and 72 h recovery post-exposure. Plasma BChE activities in the dose-response and recovery fish were not statistically different from controls, with activities indicative of elevation of the enzyme as opposed to inhibition. Another cohort of sturgeon (n = 16, average wt = 0.5 kg) was exposed to 0 or 3,000 µg L^{-1} carbaryl for 6 h to clarify the results. Compared to controls, brain AChE was inhibited 29% in treatment sturgeon while plasma BChE was elevated by 30%, with both differences in enzyme activity statistically significant ($p \leq 0.05$). Plasma samples were also collected from white sturgeon in the Columbia River and green sturgeon in Willapa Bay prior to application of carbaryl in 2012, and from green sturgeon in Willapa Bay 4-5 d after the last application. On average, white sturgeon plasma BChE activity (0.326 units/ml plasma) was statistically greater than that of green sturgeon (0.151 units/ml plasma; $p \leq 0.05$). Plasma BChE activity from green sturgeon collected post-carbaryl application was statistically lower (37%) than that pre-application, indicative of carbaryl exposure. IC_{50}s were calculated for captive white sturgeon brain AChE (8.7 µM) and plasma BChE (15.8 µM) and suggest brain AChE is slightly more sensitive than plasma BChE. Comparable values for plasma BChE from wild white (20.3 µM) and green (18.5 µM) sturgeon were similar and suggest white sturgeon are a good surrogate for green sturgeon. At the lowest carbaryl concentration to which white sturgeon were exposed in the laboratory (30 µg L^{-1}), both brain AChE and plasma BChE activities were inhibited. At the higher doses (≥ 100 µg L^{-1}), brain AChE activity was further inhibited, while plasma BChE activity was elevated. This suggests a concentration threshold (e.g., < 100 µg L^{-1}) above which white sturgeon livers appear to release stored BChE into the plasma and/or increase BChE synthesis (induction) and secretion. The laboratory results suggest that no significant brain AChE inhibition would be expected at lower concentrations, indicative of little risk to sturgeon. If, however, the release of BChE from white sturgeon liver is masking plasma BChE inhibition, it is possible that the magnitude of brain AChE and plasma BChE inhibition mirror each other, and plasma BChE inhibition in Willapa Bay green sturgeon is indicative of similar brain AChE inhibition, which would indicate greater risk to the fish. Plasma BChE activity appears to be a conservative biomarker of exposure of sturgeon to carbaryl when inhibition is detected, as in the case of green sturgeon in Willapa Bay following carbaryl applications to control burrowing shrimp. Although it is unlikely that the level of BChE inhibition detected in wild green sturgeon is life-threatening, the relationship between plasma BChE inhibition and brain AChE activity is unclear. Further studies are needed to better understand the magnitude and effects of carbamate exposure on threatened and non-threatened sturgeon in Willapa Bay, and other Western US surface waters, including green sturgeon spawning grounds.

Introduction

As the economic and nutritional importance of aquaculture rises, so too do the concerns about effects of practices of the aquaculture industry on the surrounding environment and native species. To date, aquaculture is a fast-growing animal food producing sector, with just under half of all fish consumed globally originating from aquaculture (Waite et al. 2014). By 2050, the world population is projected to reach 9.6 billion (United Nations 2012), and if global per capita fish consumption is to rise without putting further pressure on already stressed wild fish stocks, aquaculture production would need to more than double (Waite et al. 2014). This expansion may be constrained by availability of land, water, feed and energy – limited resources that have significant environmental impacts (Waite et al. 2014). Moreover, the use of pesticides in aquaculture is of growing concern (Bergqvist and Gunnarsson 2013). Just as pests present a problem to terrestrial agriculture, they also damage and decrease productivity of aquaculture crops. A common way to address these pests in terrestrial agriculture is to treat the affected area with pesticides. This practice introduces chemicals into the environment that may potentially impact non-target species. When applying pesticides to an aquatic environment, it is easier for the chemical to spread to areas other than those intended for treatment compared to terrestrial ecosystems. In intertidal areas, dispersion of chemicals may be facilitated by currents, wind, tidal fluctuations, and movement of contaminated target and non-target species. Because of the difficulty in controlling the pesticide impact zone, pesticides are rarely applied to intertidal habitats in the USA (Labenia et al. 2007).

Willapa Bay, in southwestern Washington, is one of the few locations where pesticides are directly applied on a large-scale in an intertidal setting (Fig. 1). Willapa Bay is the second largest estuary on the Pacific Coast, covering 418 km^2 of water surface, and is considered one of

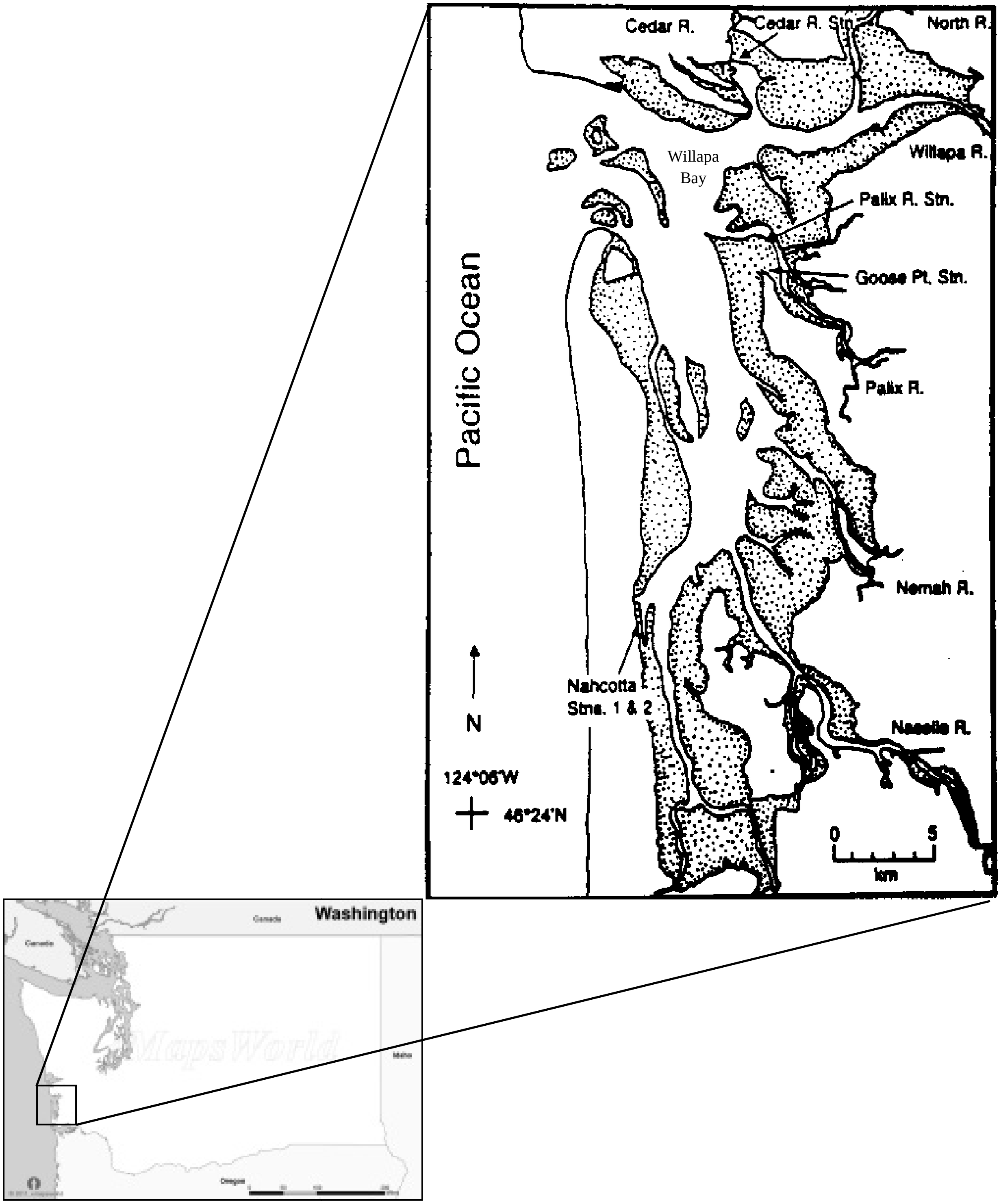

Figure 1**:** Willapa Bay, WA. Dotted regions indicate intertidal habitats (Feldman et al. 2000, emapsworld.com).

the most pristine estuaries in the United States (US Fish and Wildlife Service 2012). Located between the Columbia River to the south, Grays Harbor to the north and Long Beach Peninsula to the west, over 85 percent of this shallow estuary never reaches a depth greater than 7 m. Tides are semidiurnal, with fluctuations of up to 4 m. At low tide, over half of the surface area is exposed. The tidal flats drain into intertidal creeks that subsequently drain into deeper subtidal channels (Feldman et al. 2000). Eelgrass beds (native: *Zostera marina* and exotic: *Zostera japonica*) are common in Willapa Bay, providing food and shelter for many benthic invertebrates, fishes, and waterfowl (Wyllie-Echeverria et al. 1994). Of particular interest are the bare mudflats, which can be dominated by burrowing shrimp.

In some areas, burrow densities can reach up to 600 holes m^{-2} (Armstrong et al. 1992). These shrimp pose a threat to the economically important Pacific oyster (*Crassostrea gigas*) aquaculture industry in the Bay by destabilizing the sediments onto which the young oysters are seeded. When the shrimp burrow, they create extensive subterranean tunnels up to 90 cm in depth (Swinbanks and Luternauer 1987). As a result, sediment compaction decreases to the point that young oysters seeded on the tide flat sink into the unconsolidated mud, where they suffocate, thus threatening the productivity of the commercial oyster operations (Feldman et al. 2000). In order to control indigenous populations of burrowing ghost shrimp (*Neotrypaea californiensis*) and mud shrimp (*Upogebia pugettensis*), the carbamate pesticide carbaryl (1-napthyl n-methyl carbamate) has been applied since 1963 by the Willapa - Grays Harbor Oyster Growers Association (WGHOGA).

Overall, Washington State produces approximately 25% of the country's oysters (Dumbauld and Harlan 2009), and over 60% of the State's production comes from Willapa Bay and Grays Harbor (Hoines 1996). Originating as a capture fishery for the native Olympia oyster

(*Ostrea lurida*) in the mid to late 1800s, the stock quickly became overexploited by the early 1900s. In 1928, the Pacific oyster was introduced from Japan, and the oyster industry once again thrived. With better regulatory laws, including restocking mudflats with shucked oyster shell crucial for larval settlement, Washington State is now home to a successful large-scale oyster farming industry (Dumbauld et al. 2011). As of 2012, the value of Washington's oyster industry was estimated at $37 million (National Oceanic and Atmospheric Administration 2012). Thus, the economic benefits provided by this industry are the main impetus for mitigating detrimental effects caused by burrowing shrimp.

The seasonal application of carbaryl to the tide flats remains controversial, highlighted by Labenia et al. (2007) who suggested that the carbamate may have detrimental effects on non-target species, particularly cutthroat trout (*Oncorhynchus clarki*). Many of the studies examining non-target effects of carbaryl have focused on salmonids (Beauvais et al. 2001, Major et al. 2005, Labenia et al. 2007, Tierney et al. 2007, Grue et al. 2009, Troiano et al. 2013) in part due to their abundance and economic importance, as well as the fact that 29 out of the 52 designated Evolutionary Significant Units for salmonids on the West Coast are listed under the Endangered Species Act (ESA). With few exceptions (Major et al. 2005, Grue et al. 2009, Troiano et al. 2013), existing studies were conducted in the laboratory using exposures to the pesticide that may not be representative of what non-target fishes encounter in nature.

In 2001, WGHOGA entered into a non-binding legal agreement with the Washington Toxics Coalition to work towards an Integrated Pest Management strategy focused on finding an alternative to carbaryl for the control of burrowing shrimp, to gradually reduce the amount of carbaryl applied, and ideally phase out the chemical completely by 2012 (Dumbauld et al. 2011). Currently, the WGHOGA has received a label from the US Environmental Protection Agency

(EPA) for the use of the neonicotinoid insecticide, imidacloprid (N-[1-[(6-chloropyridyn-3-yl)methyl]-4,5-dyhydroimidazol-2-yl]nitramide) to control burrowing shrimp in Willapa Bay and Grays Harbor, but is awaiting a National Pollutant Discharge Elimination System (NPDES) Permit from the State. Until the permit is granted, the growers continue to use carbaryl under an extension to the existing NPDES permit for shrimp control.

In addition to salmonids, both white (*Acipenser transmontanus*) and green (*Acipenser medirostris*) sturgeon are present in Willapa Bay. In 2006, green sturgeon were listed as near-threatened according to the International Union for Conservation of Nature (IUCN) Red List (St. Pierre and Campbell 2006) and the ESA by the National Marine Fisheries Service (Department of Commerce 2009). Green sturgeon are large, anadromous fish with long lifespans, delayed maturation, high fecundity, and far-ranging movements, and they are the most marine-oriented sturgeon species. Juveniles spend 1-4 years in freshwater (FW) prior to migrating to the ocean, where sexual maturity is not reached until 13-20 years of age, after which fish return to natal rivers to spawn every 2-5 years. Their range extends along the West Coast of North America from Mexico to Alaska, yet the only known spawning locations for these fish are in Oregon and California rivers (Adams et al. 2007). Spawning habitat loss is thought to be the main factor contributing to population declines. Other factors possibly contributing to population loss and the subsequent ESA listing of green sturgeon include reduced growth and survival as a result of increased water temperatures, entrainment by water diversions, exposure to contaminants, competition from exotic species, and loss as bycatch in commercial fisheries (Adams et al. 2007, Israel et al. 2009). It is unclear why the spawning grounds are disappearing, but there is concern that anthropogenic forces may be playing a role (St. Pierre and Campbell 2006).

During winter and spring months, green sturgeon live in the coastal sub-littoral zone in depths less than 100 m (Erickson and Hightower 2007). In the summer and early fall, however, they aggregate in coastal estuaries, particularly in the Columbia River estuary, Willapa Bay, and Grays Harbor (Moser and Lindley 2007). Based on spawning patterns, green sturgeon are grouped into two distinct population segments (DPSs). The Northern DPS includes fish that spawn in the Klamath and Rogue rivers, and the Southern DPS encompasses fish that spawn in the Sacramento River (Israel et al. 2004). Mixed stock analysis indicates that ca. 80 percent of green sturgeon found in the Columbia River and Willapa Bay belong to the Southern DPS, which is ESA listed (Israel et al. 2009). In the Columbia River, white sturgeon greatly outnumber green sturgeon, and constitute one of the healthiest sturgeon populations, while in Willapa Bay, green sturgeon are more abundant (Dumbauld et al. 2008).

In 2009, critical habitat was established for the Southern DPS, extending throughout the freshwater, estuarine, coastal, and marine areas regularly occupied by green sturgeon, including all marine waters up to 110 m depth from Monterey Bay to the US-Canada border. Within this critical habitat, primary constituent elements were established to ensure normal behavior, growth, and survival of the southern DPS through maintenance of abundant food resources and suitable water and sediment quality. Willapa Bay and Grays Harbor fall within these designated critical habitats (Department of Commerce 2009).

The oyster beds in Willapa Bay have been aerially treated with carbaryl on a 3-year rotation in the summer, primarily during July low tides (Sevin® 80 WSP or Sevin® 4F, 8 lb active ingredient [ai] ac^{-1}[8.96 kg ai ha^{-1}], ca. 400 ac annually). This is, in part, due to the fact that shrimp activity and subsequent bioturbation of the sediment increases in spring and summer as a result of increasing temperatures, salinities, and availability of food (Feldman et al. 2000).

These months also coincide with the period during which green sturgeon reside in Willapa Bay. Following the seasonal application of carbaryl to the tide flats in Willapa Bay, concentrations of carbaryl in the water on the beds and in the surrounding channels decrease rapidly, and reach levels below detection within a few days (Troiano et al. 2013). While this would indicate that carbaryl exposure in the surface water is likely minimal, sturgeon may be exposed through their foraging for benthic invertebrates including burrowing shrimp on the tide flats. Although stomach content analyses have been inconclusive, it is thought that sturgeon feed on both species of burrowing shrimp found in Willapa Bay (Dumbauld et al. 2008). This is supported by fishers who have reported shrimp in the stomachs of some sturgeon, particularly those caught as incidental bycatch in commercial salmon fisheries (Dumbauld et al. 2008). Feeding is thought to occur when the fish move onto the beds during high tide (Moser and Lindley 2007). Specially adapted sensory organs called Ampullae of Lorenzini allow sturgeon to locate prey - in this case, burrowing shrimp - by sensing electrical signals given off by the organisms (Zhang et al. 2012). Once the shrimp are located, the sturgeon excavate and suck the detected shrimp out of their burrows, including whole sediment (Johnson et al. 1997), leaving “feeding pits” (Fig. 2) that are

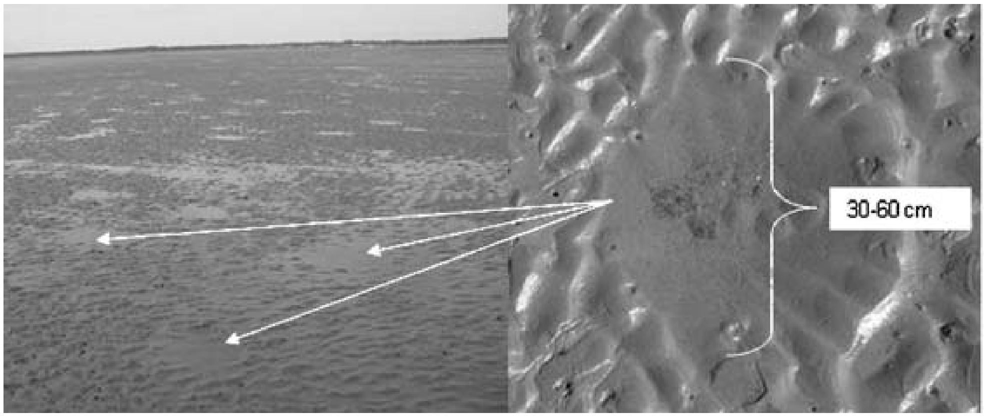

Figure 2: Multiple feeding pits (left) and an individual feeding pit (right) on an exposed tide flat in Willapa Bay, WA. (Dumbauld et al. 2008).

visible on tide flats as evidence of their predation. Concentrations of carbaryl within the sediment post-application are greater and persist longer than those within the water column (Felsot and Ruppert 2002). The amount of chemical within whole sediments and shrimp, in addition to the amount of sediment and shrimp a sturgeon consumes on a contaminated bed may play a large role in whether or not the sturgeon are being exposed to appreciable amounts of carbaryl. Some argue that declines in sturgeon and other shrimp predator populations may be part of the reason shrimp populations began to rise in the 1950s, creating the pest problem as it exists today (Dumbauld et al. 2008).

The primary concern with the effect of carbaryl on non-target species is based on its mode of action. Carbamates, as a class, bind to and inhibit cholinesterases (ChEs) in tissues throughout the body. These include acetylcholinesterase (AChE) in nerves and muscles, and AChE and butyrylcholinesterase (BChE) in plasma. AChE is essential for hydrolyzing acetylcholine, an important neurotransmitter at neuronal synapses. Organisms exposed to enough carbaryl are not able to break down acetylcholine, leading to tetany and muscle and respiratory paralysis. As a carbamate, carbaryl exhibits the property of spontaneous reactivation where, with time, the bond between carbaryl and the enzyme may be spontaneously hydrolyzed, decreasing the level of enzyme inhibition. In addition, synthesis of new enzyme is a continuous process, maintaining tissue and plasma enzyme activity. In the field, carbaryl degradation occurs via hydrolysis, biodegradation, and photolysis, with a half-life of a few days or less (Roberts and Hutson 1999, Felsot and Ruppert 2002). *In vivo*, the main metabolic pathways are hydroxylation, hydrolysis and expoxidation by esterases and cytochrome P-450 enzymes to form metabolites, mainly 1-naphthol (and its conjugates), which are eliminated via excretion (Statham et al. 1975). Other metabolites include 4-hydroxycarbaryl and 5-hydroxycarbaryl (Roberts and

Hutson 1999). In some monitoring studies, 1-naphthol has been used as a surrogate for carbaryl (Moser et al. 2013). Studies in the field have indicated that salmonids exposed to carbaryl in Willapa Bay may experience reduced brain AChE activity, but not at the level that would overtly affect behavior (≤ 30% inhibition; Zinkl et al. 1991). In light of these concerns and the potential for continuing litigation to restrict the use of carbaryl in Willapa Bay, it is important to understand how carbaryl affects associated non-target species.

Activity of ChE in blood and other tissues in animals (e.g., brain and muscle) has been widely used as a tool for diagnosing organophosphate (OP) or carbamate exposure in animals (Fairbrother et al. 1991). The use of plasma or serum ChE as a biomarker is a valuable technique for inferring exposure without necessitating lethal sampling of the organism under assessment (Hooper et al. 1989). In addition to non-lethal sample collection, plasma ChEs are inhibited before brain ChEs, so exposure can be detected sooner and serve as an indication of recent exposure (Gard and Hooper 1993). Recently, plasma AChE and BChE activities were characterized in nine species of native Australian birds in order to document baseline activity levels to aid in future monitoring for exposure to ChE-inhibiting pesticides that are applied throughout the country to control agricultural pests (Fildes et al. 2009). Plasma ChE activities were determined in migratory shorebirds during migration between North America and South America in order to compare samples from individuals captured in areas with no known sources of OP or carbamate exposure to those from areas where agrochemical applications occur. The unexposed samples served as a baseline to determine whether individuals in agricultural areas were recently being exposed to ChE inhibitors (Strum et al. 2010). The importance of assessing the normal range of fish blood plasma chemistry, including ChE activity, was addressed in a study with brook trout (*Salvelinus fontinalis*) reared in aquaculture conditions (Kopp et al. 2013).

Plasma BChE activity was measured in juvenile red-bellied pacu (*Piaractus brachypomus)* exposed to fenthion, and the results showed that BChE activity was a good biomarker for environmental monitoring of waterborne exposure to the OP (Borbón and Mantilla 2012). In order to successfully use ChE as a biomonitoring tool, it is crucial to determine the type of ChE that predominates in the tissue being analyzed, as well as to optimize the assay conditions for the given species and tissue (Habig and Di Giulio 1991).

The goal of this study was to determine the utility of using plasma ChE as a biomarker for exposure of white and green sturgeon to carbaryl following application of the pesticide to control burrowing shrimp in Willapa Bay and Grays Harbor, WA. While this study aims to provide answers to questions related specifically to sturgeon in Willapa Bay and Grays Harbor, Washington, the biomarker developed would have application for monitoring the exposure of both species of sturgeon to ChE-inhibiting pesticides in the freshwater and estuarine habitats within their entire ranges. OP and carbamate pesticides are found in western US surface waters including the Sacramento River and San Joaquin-Tulare basins (Laetz et al. 2009), which are known spawning grounds for green sturgeon. Ultimately, this monitoring tool will inform assessments of the risk this use of the pesticide poses to green sturgeon and population recovery efforts.

A surrogate species, the white sturgeon, was used for the laboratory portion of this study due to the ESA listed status of green sturgeon. Since the 1973 ESA prohibits “take” of listed species, the use of surrogate species has become commonplace, especially in toxicological studies investigating effects of pesticides on endangered species (Banks et al. 2010). While using surrogates can lead to difficulties in extrapolation, attention to both physiological similarities as well as life history traits can lead to defensible results when estimating from surrogates to threatened species. For example, rainbow trout (*Oncorhynchus mykiss*) were shown to be good surrogates for the threatened bull trout (*Salvelinus confluentus)* when determining exposure to rangeland herbicides (Fairchild et al. 2008). White and green sturgeon are closely related (Lee et al. 2011), and both are found in Willapa Bay. To determine the potential impact of carbaryl exposure on activity of ChEs in green and white sturgeon, the main objectives of this study were as follows:

(1) Develop a dose-response relationship for brain AChE inhibition by carbaryl in seawater-acclimated white sturgeon.
(2) Develop a dose-response relationship for plasma ChE inhibition by carbaryl to correspond with brain AChE levels determined in objective (1).
(3) Determine recovery curves for the activity of both enzymes following inhibition.
(4) Develop carbaryl sensitivity profiles for brain AChE in white sturgeon and plasma ChE in white and green sturgeon *in vitro.*
(5) Compare the sensitivities of plasma ChE to carbaryl between the two species.
(6) Apply the plasma ChE assay to determine the extent to which green sturgeon are exposed to carbaryl in Willapa Bay.

Materials and Methods

1. Controlled Exposure Studies

Two cohorts of juvenile hatchery-reared FW white sturgeon were utilized in this study. The Spokane Indian Tribe provided the first cohort of sturgeon. These fish were raised at the Washington Department of Fish and Wildlife (WDFW) Columbia Basin Hatchery in Moses Lake, WA as part of the Lake Roosevelt White Sturgeon Recovery Project (Spokane Tribe of Indians 2014). The second cohort was obtained from the Yakima Nation Fisheries. These fish were raised at the Marion Drain Hatchery in Toppenish, WA as part of the Yakima Sturgeon Management Project (Yakima Nation Fisheries 2014).

First cohort - 2010

Young-of-the-year white sturgeon were transported in FW with supplemental aeration from the Columbia Basin Hatchery to the University of Washington (UW), where they were maintained for 1 year to ensure sufficient size and maturity for seawater (SW) acclimation prior to exposure to carbaryl. Temperature (14.0 ± 1.0°C), dissolved oxygen (D.O.; 10.0 ± 1.0 mg L^{-1}) and pH (7.1-7.4) were monitored daily from the flowing de-chlorinated City of Seattle water in the circular tanks in which the fish were held. Fish were fed dependent on body weight. The average mass of the fish was calculated each month, and BioBrood, 4.0 mm (Bio-Oregon) was fed to the fish once daily. The feeding rate decreased from 1.95 to 0.95% of body weight as fish grew over approximately 6 months, at which time, fish were fed BioBrood 6.0 mm once daily at a rate of 0.95% body weight.

SW acclimation. Once the fish reached 2 years of age (Allen and Cech 2007), they were transported with supplemental aeration to the United States Geological Survey (USGS) Western Fisheries Science Center, Marrowstone Marine Station (MMS) in Nordland, WA. Transportation occurred in de-chlorinated City of Seattle water at 5.3 parts per thousand (ppt) salinity (Instant Ocean, Blacksburg, VA), 14.4°C, and 100% saturation. SW acclimation occurred gradually in a large (2.7 m diameter, 6,300 L) circular tank with an adjustable mixture of flowing sand-filtered SW (salinity = 30 ppt) and flowing de-chlorinated City of Port Townsend FW. Initially, fish were held for 2 d at an isosmotic concentration of 8 ppt, after which the FW and SW flows were adjusted such that the salt concentration in the tank was increased by 5 ppt every 2 d, followed by a final 2 ppt increase to full SW, for a total of 12 d (8, 13, 18, 23, 28 and 30 ppt). The fish were then held at 30 ppt in the SW tank, covered with opaque black plastic, with supplemental aeration for approximately 3 months prior to carbaryl exposure. Temperature (10.0 ± 1.0°C), D.O. (9.02 – 10.15 mg $L^{-1)}$, and salinity (30.0 ± 1.0 ppt) were monitored daily; pH was not measured. BioBrood 6.00 mm was delivered once daily at a rate of 0.95% body weight – based on monthly average body mass calculations.

Exposure scenario. Sturgeon were exposed to either 30, 100, 300, 1,000 or 3,000 µg L^{-1} carbaryl (Chipco Sevin® 80 Wettable Soluble Powder [WSP], 80% ai, 20% inert ingredients; Aventis Environmental Science, Montvale, NJ) for 6 h. Concentrations selected included the maximum reported in the middle of the water column above treated beds (application rate = 8.96 kg ai carbaryl ha^{-1}) at the first high tide (first inundation, 30 µg L^{-1}; Grue et al. 2009); the maximum water concentration tested by Labenia et al. (2007) in their study with cutthroat trout (1,000 µg L^{-1}), and the estimated maximum concentrations (3,050 µg kg^{-1} wet weight) based on the measured maximum concentration in sediment to a depth of 25 cm (4,360 µg kg^{-1} dry weight; Felsot and Ruppert 2002) immediately post-application assuming a moisture content of 30% characteristic of Willapa Bay sediments (Grue and Grassley 2013). The 6-h exposure represented the time period sturgeon would be most likely to be in the water column above the beds during the first high tide and when water depth would be adequate and their exposure to the pesticide would be greatest (3 h before and 3 h after the high). Concentrations in the water column and sediment decrease rapidly (1-2 orders of magnitude) within the first 24 h post-application (Felsot and Ruppert 2002, Troiano et al. 2013). AChE activity in brain tissue of SW adapted juvenile Chinook (O*ncorhynchus tshawytscha*) was inhibited 34% following a 6 h exposure to 30 µg L^{-1} and 70% at 300 µg L^{-1} (C.E. Grue, unpublished data) without mortality. Labenia et al. (2007) reported that brain AChE activity was inhibited by 20% in cutthroat trout following exposure to 32 µg L^{-1} for 6 h and 75% at 1,000 µg L^{-1} also without mortality. These studies suggest that the exposures selected for the present study should reflect those sturgeon may receive following carbaryl applications in Willapa Bay and that detectable plasma and brain ChE inhibition would be expected, at least at the higher concentrations (1,000 and 3,000 µg L^{-1}).

Dose-response test. All fish in the holding tank were weighed prior to the beginning of testing to determine the weight range of the cohort. Fish weight ranges were determined to minimize variation within treatment groups, but minimize loading (g L^{-1} fish) per treatment tank. Fish were removed one at a time from the holding tank and weighed. If they fit within the desired weight range (1.1 ± 0.2 kg), they were randomly assigned to a treatment tank for the duration of the 6-h exposure (average length: 65 ± 5 cm). All tanks were covered with opaque black plastic for the duration of the experiment. Given the relatively short time of exposure, and the large size of the fish, an adjusted loading of 2.67 L treated water per g fish weight was used for the 6-h duration of the test to comply with standard 96-h acute static exposure guidelines (ASTM 2002). Temperature, DO, pH and salinity were measured at the beginning and end of each exposure period. Temperature was held at 12.0 ± 1.0°C, D.O. at 10.0 ± 1.0 mg L^{-1}, pH at 6.9-7.2, and salinity at 30.0 ± 1.0 ppt. At the end of the exposure period, fish (n = 5) were removed from the tanks for sample collection.

Recovery test. Fish were again weighed to determine whether they fit within the predetermined weight range (1.7 ± 0.2 kg). If they did, they were randomly assigned to a treatment tank where they were exposed to 1,000 µg L^{-1} carbaryl for 6 h (average length: 73 ± 3 cm). After 6 h, fish (n = 4) from one tank were removed and plasma was collected to represent 0 h recovery. The remaining fish were transferred into tanks with clean flowing SW for either a 24, 48 or 72 h recovery period prior to sample collection. All tanks were covered with opaque black plastic for the duration of the experiment. Temperature, DO, pH and salinity were measured at the beginning and end of each exposure and recovery period. Temperature was held at 12.0 ± 1.0°C, D.O. at 10.0 ± 1.0 mg L^{-1}, pH at 6.8-7.1, and salinity at 30.0 ± 1.0 ppt.

Plasma collection and storage. Fish were removed from exposure tanks one at a time and stunned by a blow to the head. Length (0.5 cm) and weight (g) were recorded. First gill arches were severed, and blood was extracted from the exposed severed artery by pre-heparinized 5-ml syringes, and then transferred into 15-ml conical tubes. Plasma was isolated by centrifuging the conical tubes (1,900 x *g*, 10 min) and transferred into 1.5-ml microcentrifuge tubes and stored at -80°C. The remaining bodies were held at -20°C until an entire tank was processed, then placed in closed coolers on dry ice for transfer back to UW where they were stored at -40°C until brains were excised immediately prior to analysis.

Second cohort – 2014

SW acclimation. Two-year-old white sturgeon were transported directly from the Marion Drain Hatchery to the USGS Western Fisheries Science Center, MMS in de-chlorinated City of Seattle water at 8 ppt salinity (Instant Ocean; Blacksburg, VA), 12.0°C, and 11.03 mg L^{-1} D.O. Upon arrival, fish were placed in a large (2.4 m diameter, 3,560 L) circular tank with an adjustable mixture of flowing sand-filtered SW (salinity = 30 ppt) and flowing de-chlorinated

City of Port Townsend FW. Sturgeon were held at 8 ppt for 2 d, after which the FW and SW flows were adjusted such that the salt concentration in the tank was increased by 8 ppt every 2 d, until full SW, for a total of 8 d (8, 16, 24, 30 ppt). The fish were then held at 30 ppt in the SW tank with supplemental aeration for ca. 1 d prior to being placed in treatment tanks. Temperature averaged 10.8°C during the acclimation. The fish were fed once per day at a rate of 0.77% body weight, in keeping with the feeding plan used at the hatchery.

Exposure scenario. The purpose of the second exposure trial (2014) was to verify the lack of inhibition of plasma BChE measured in the fish from the first trial. Because enzyme activity varied significantly among individual fish, treatments (carbaryl concentrations) were reduced to two (control [0] or 3,000 µg L^{-1}, the maximum in the previous test), and sample size was increased. There were a total of 16 fish per treatment and four treatment tanks, giving two control and two treatment tanks (1.7 m diameter, 1,425 L), each with eight fish. In addition, each fish served as its own control; i.e., blood was collected 5 d pre-exposure and immediately following the 6-h exposure.

Exposure tests. All fish obtained were approximately 0.5 kg and 45 cm with eight fish assigned to each of four tanks so as not to exceed an adjusted loading rate of 5.7 L treated water per g fish weight (ASTM 2002). Fish were randomly removed one at a time from the holding tank and 1 ml blood was collected. Blood was drawn from the caudal vein using a 21G needle attached to a 3cc syringe, then immediately expunged (without the needle attached) into a 4-ml heparinized vacuum reservoir (BD Vacutainer; Becton Dickinson, Franklin Lakes, NJ), which was inverted then held on wet ice until centrifugation. Prior to centrifugation, blood was pipetted into 1.5-ml microcentrifuge tubes, plasma was isolated by centrifugation at 4,500 x *g* for 7 min, after which the plasma was transferred into 1.5-ml microcentrifuge tubes and stored at -80°C.

Following the blood draw, each fish had a fin clipped for future identification prior to being placed in one of the four treatment tanks. To be able to accurately identify each of the eight fish per tank, fin clips were as follows: dorsal, left pectoral, right pectoral, left pelvic, right pelvic, anal, caudal, and no fin clip. Fish were then held in their respective treatment tanks (flowing SW with supplemental aeration) for 5 d prior to carbaryl exposure. The fish were not fed for 24 h following the initial blood draw, fed for the next 2 d, and then fasted for the 2 d prior to dosing.

To begin the 6-h exposure tests, water flow was turned off in each tank in a randomly selected order, with the start time for each tank staggered by 2 h. The control fish were held static in clean SW for 6 h while the treated fish were exposed to static 3,000 µg L^{-1} carbaryl for the same amount of time. All tanks had supplemental aeration and were covered with opaque black plastic. Water samples were collected at the beginning and end of the test from the treatment tanks, and sent to Pacific Agricultural Laboratory (PAL, Portland, OR) for quantification of carbaryl. Temperature, DO, pH and salinity were measured at the beginning and end of each exposure period. Temperature was held at 11.0 ± 1.0°C, D.O. at 10.0 ± 1.0 mg L^{-1}, pH at 6.8-7.1, and salinity at 30.0 ± 1.0 ppt. At the end of the 6-h exposure, fish were removed from each tank one at a time, stunned by a blow to the head, identified by fin clip, and length (cm) measured. Blood (5-6 ml) was drawn from the caudal vein using a 21G needle attached to a 3 cc syringe and immediately expunged (without the needle attached) into a 4-ml heparinized vacuum reservoir (BD Vacutainer), which was gently inverted, then held on wet ice until centrifugation. Gill arches were then severed to drain the remaining blood, and fish were placed on wet ice until the entire tank was processed. Plasma was then isolated in the same way as it was during the pre-exposure sampling, and each fish was removed from ice, weighed (g) and livers were extracted with gall bladders intact, weighed (mg), placed into 50-ml conical tubes, and stored at -80°C. Fish bodies were then placed in closed coolers on dry ice for transport to UW where they were stored at -40°C.

2. Sturgeon Plasma Pre- and Post-carbaryl Application

Blood samples from white and green sturgeon were collected in 2012 in collaboration with the WDFW as part of their annual sturgeon monitoring program under guidelines set by the National Marine Fisheries Service (National Marine Fisheries Service 2010). Fish were captured on a chartered commercial fishing vessel using long-line gill nets at multiple locations in proximity to carbaryl test beds (pre-application) during high tide. Green sturgeon (minimum n = 30) were captured prior to application (Nahcotta, 1 July) and 4-5 d post-application (Nahcotta, 25-26 July) of carbaryl in Willapa Bay, and white sturgeon (n = 30) were captured in the lower Columbia River on 1-2 August, where exposure to carbaryl is unlikely. Due to the lower abundance of white sturgeon found in Willapa Bay, sufficient white sturgeon plasma samples could not be collected following carbaryl application as part of the WDFW monitoring. Following capture, 5-10 ml of blood (depending on fish size) was drawn from the caudal vein by needle attached to a 5- or 10-ml heparinized vacuum reservoir (BD Vacutainer). The blood was placed on ice in a closed cooler for 2-4 h until sampling was complete. Upon returning to shore, plasma was isolated (3,000 x *g*, 10 min) and 2-ml aliquots from each fish were stored in 2-ml microcentrifuge tubes on dry ice in a closed cooler until they were transferred to the UW and stored at -80C.

3. ChE Assays

Brain ChE analyses

Brain AChE assays were performed using methods described by Ellman et al. (1961) as modified by Zinkl et al. (1991) over 2 d with fresh reagents. All fish from the recovery test were analyzed on the first day, and all fish from the dose-response test were analyzed on the second. On both days, one randomly selected fish from each treatment was analyzed in a rotation so that fish from each treatment group were represented throughout the day. Sturgeon bodies were removed from the freezer and placed at room temperature to allow the heads to thaw sufficiently to remove the brains while still frozen. Whole brains were weighed (mg) and homogenized in 15x Tris buffer with 1% Triton X-100 (Sigma Life Science, St Louis, MO). Further homogenate dilutions were selected to provide a linear enzyme activity response for 3 min with absorbance determined every sec. DTNB (2.8 ml of 0.00025 M 5,5-dithio[bis-2-nitrobenzoic acid]; Aldrich Chemistry, St Louis, MO) in 0.1 M Tris buffer (pH 8.0) was dispensed into a 4.5-ml, two-sided spectrophotometric cuvette (K-06343-10; Cole-Parmer, Vernon Hills, IL). Brain homogenate (0.1 ml) was then added. The addition of 0.1 ml of a 0.156 M solution of acetylthiocholine iodide (AThChI; Sigma Life Science, St Louis, MO) initiated the reaction. All reagents were kept on wet ice except for DTNB that was chilled in a water bath to 14ºC. Absorbance was determined in duplicate samples of each homogenate using a spectrophotometer (model 1200; Cole-Parmer Instrument, Vernon Hills, IL) with a water jacket (15ºC) at 405 nm for 180 sec. Duplicate background (DTNB + AThChI) samples were also run each day and daily averages were subtracted from the corresponding sample absorbance values. Brain AChE activity was expressed as percent that of controls.

Plasma ChE analyses

Plasma ChE activities were measured using the method of Ellman et al. (1961) as modified by Gard and Hooper (1993) for use on a 96-well plate reader (Sunrise Basic Absorbance Reader; Tecan, Grodic, Austria) used in conjunction with a computer equipped with Magellan™ Data Analysis Software (Tecan Trading AG, Männedorf, Switzerland). The spectrophotometer was set in a kinetic mode and measured absorption at 405 nm for 3 min, with readings taken at approximately 7 sec intervals with 1 sec of shaking between each cycle. All samples were run in triplicate at 16-18°C with a final volume of 250 µl/well. Optimal enzyme dilution and substrate (AThChI) concentration were determined on non-study samples prior to analysis. Non-study samples were pre-incubated for 5 min with a specific BChE inhibitor, tetraisopropyl pyrophosoramide (iso-OMPA; Sigma Life Science, St. Louis, MO) at final concentrations (FC) in the assay ranging from 10^{-10} to 10^{-3} M to differentiate AChE from BChE. Iso-OMPA inhibited nearly all plasma ChE activity in a linear fashion, indicating BChE as the only ChE active in sturgeon plasma (Appendix A). Total ChE was measured in all plasma samples and reported as BChE.

Plasma from MMS and wild sturgeon (Willapa Bay green and Columbia River white) was diluted 16-fold and 8-fold, respectively, with 0.05 M Tris buffer (pH 7.4; Sigma Life Science, St. Louis, MO) to ensure linearity of substrate hydrolysis with time. All reagents were kept on wet ice except for the buffer (pH 8.0), which was kept at room temperature. Assay component FCs and reagent volumes were as follows: 0.05M Trizma buffer (pH 8.0), 170 µl; DTNB, 20µl, 3.23×10^{-4} M; 16x diluted plasma enzyme source, 30 µl; and AThChI, 30 µl, 1×10^{-3} M. In background blank wells, buffer (pH 8.0) replaced enzyme volume. All of the plasma samples from a given group were analyzed on a single day with the same mix of reagents. The groups were the dose-response and recovery from the first cohort of white sturgeon, the dose-response from the second cohort of white sturgeon, the clean white and green sturgeon plasma from Willapa Bay and the Columbia River, and the post-carbaryl application green sturgeon samples (with corresponding controls).

Sensitivity profile curves were created by spiking clean, unexposed plasma with pre-determined concentrations of carbaryl. An initial acetone stock was mixed by dissolving 30 mg of technical grade carbaryl (Pestanal®, analytical standard; Fluka Analytical, St. Louis, MO) in 2.0 ml of acetone to produce a 0.075 M acetone stock solution. This solution was capped to prevent evaporation and stirred until all carbaryl was dissolved. Immediately prior to use in the assay, 0.147 ml acetone stock was pipetted (using a Hamilton syringe) into 9.853 ml buffer (pH 7.4) to yield a 1.1×10^{-3} M lab stock buffer. This stock was then serially diluted by adding 0.1 ml stock (or previous dilution) to 0.9 ml buffer (pH 7.4). All dilutions used ice cold buffer. Assay FCs ranged from 1×10^{-4} M to 1×10^{-15} M. Three replicates were run for each group of samples: Willapa Bay green sturgeon, Columbia River white sturgeon, MMS 2014 white sturgeon plasma and brains. Serial dilutions of carbaryl were mixed fresh before each replicate was run. ChE activities were converted from optical density units min^{-1} to µmol AThChI hydrolyzed/min (or "units") per ml plasma or g brain using the extinction coefficient, 13,600 $\text{cm}^{-1}\text{M}^{-1}$.

4. Analytical Chemistry

Water samples from treated tanks were collected in 200-ml amber glass bottles for carbaryl analyses immediately after the initiation of dosing and/or at the end of the 6-h exposure and analyzed by PAL. Samples were stored on wet ice and shipped with cold packs overnight to PAL. Upon arrival at PAL, 160 µL of 1.0 N HCl were added to stabilize and preserve the samples prior to analysis. Neutral extractions (3535A) were performed within 14 d of receipt/acidification. In 2010, carbaryl was quantified using EPA Method 8321B, direct injection LC-MS analysis (single quadrupole), with a limit of quantification (LOQ) of 10 µg L^{-1}. Samples in 2014 were analyzed using the same method but with direct injection LC-MS/MS (triple quadrupole) with an LOQ of 1.0 µg L^{-1}. Mean percent recovery in 2010 was 110% (range = 97-123%, n = 2 blank spikes) and 101% (101%, n = 2 blank spikes) in 2014. Concentrations reported were not corrected for percent recovery.

5. Data Analyses

IBM SPSS Statistics 21.0 software was used to identify statistical differences among brain and plasma samples from fish from different carbaryl exposures or recovery times. Brain and plasma ChE activity from both cohorts of hatchery white sturgeon were analyzed using a one-way ANOVA, and when necessary, a Student-Newman-Keuls (SNK) post-hoc test was applied to determine where significant differences existed. SigmaPlot (Version 12.5, Systat Software Inc) graphing software was used for smoothing sensitivity profile curves and calculating IC_{50} values. The sigmoidal inhibition curves were fitted using a four parameter sigmoid dose-response with hillslope equation:

$$y = \mathrm{min} + \frac{\mathrm{max} - \mathrm{min}}{1 + 10^{(\mathrm{logEC50} - \mathrm{x})\mathrm{Hillslope}}}$$

IC_{50} values were calculated by interpolating the equation using the fitting parameters.

Results

MMS Acute Toxicity Tests

Measured vs. nominal concentrations

Immediately after dosing each tank with carbaryl, water samples were collected to verify nominal concentrations. Measured carbaryl concentrations exceeded nominal (3-30%) in 2010, and were very close to nominal (-3 to +7%) in 2014 (Table 1).

Brain ChE activity

Brain AChE activity was measured in each individual white sturgeon as part of the acute toxicity test conducted at MMS in 2010, and activities were averaged within each treatment. The dose-response test indicated decreased AChE activity with increasing concentration of carbaryl. Differences among treatments were statistically significant ($p < 0.001$; ANOVA). All concentrations other than the lowest 30 µg L^{-1} (85% AChE activity) were statistically different from controls, and AChE activity was lower ($p \leq 0.05$) at the maximum, 3,000 µg L^{-1} treatment group (58% activity), as compared to the other concentrations (SNK post-hoc test; Fig. 3). The enzyme activity recovery test indicated AChE recovery to 87% of control values by 72 h post-exposure. An ANOVA ($p = 0.013$) and SNK post-hoc test differentiated the 0 h (64%) and 48 h (72%) groups from the control (100%), 24 h (77%), and 72 h (87%) groups (Fig. 4).

Brain AChE activity was also measured in control and treated fish in the 2014 MMS toxicity tests. The treated fish demonstrated lower AChE activity than the controls, similar to that seen in the 2010 study. There were no statistical differences in AChE activity between the two control tanks or between the two treatment tanks, but activities in both control tanks were statistically higher than in both treatment tanks ($p < 0.001$; ANOVA and SNK post-hoc test).

Activities from both tanks in a treatment were pooled, and brain AChE activity was inhibited 29% in treated fish as compared to controls (Fig. 5).

Table 1. Nominal and measured concentrations (µg L^{-1}) of carbaryl in SW during acute toxicity tests with 2-year-old SW-acclimated white sturgeon. Percent recoveries were 97-123%.

Year	Test	Nominal	Measured		LOQ
			0 h	6 h	
2010	Recovery	1,000	1,300; 1,300		50
	Dose-response	30		31	10
		300		340	10
		3,000		3,800	100
2014	Dose-response	3,000	2,900; 2,900	3,200; 3,000	50

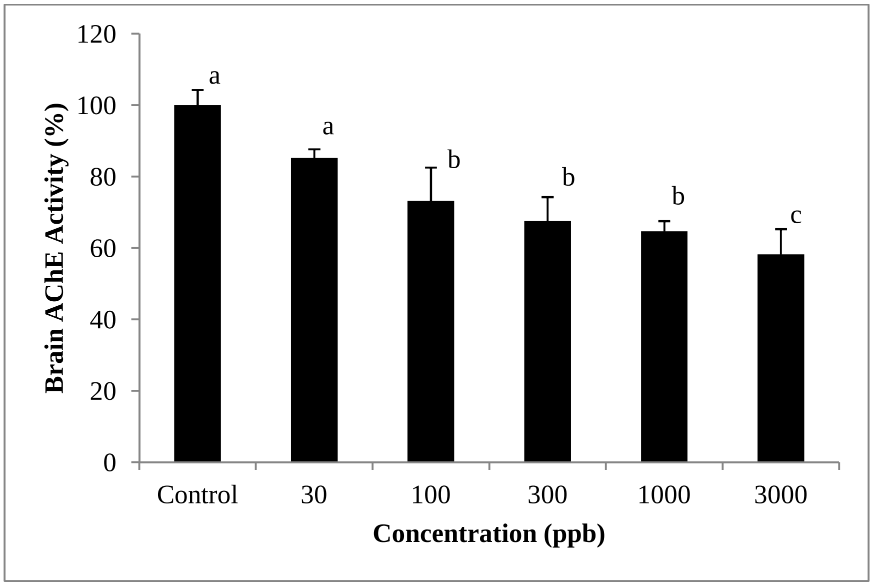

Figure 3: Brain AChE activity in 2-year-old SW-acclimated white sturgeon from the 2010 cohort following a 6-h exposure to carbaryl (30-3,000 µg L^{-1}, clean SW for controls). Data are average ± SE, n = 5 per dose. Percent activity based on control (100% = 2.1 units/g brain tissue). Groups that do not share the same superscript varied statistically from one another ($p \leq 0.05$).

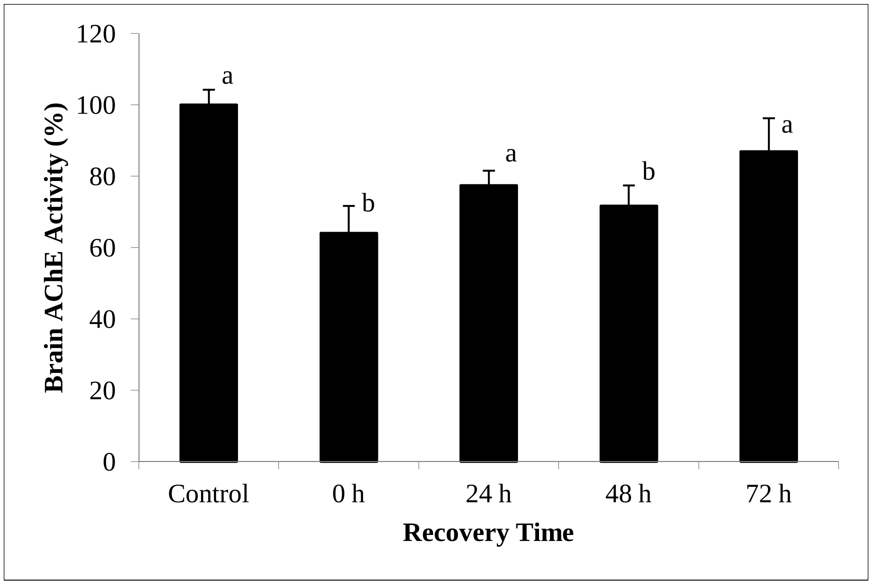

Figure 4: Brain AChE activity in 2-year-old SW acclimated white sturgeon from the 2010 cohort following a 6-h exposure to 1,000 µg L^{-1} carbaryl followed by a recovery period (0-72 h) in clean SW. Data are average ± SE, n = 4 per recovery time. Percent activity based on control (100% = 2.5 units/g brain tissue). Groups that do not share the same superscript varied statistically from one another ($p \leq 0.05$).

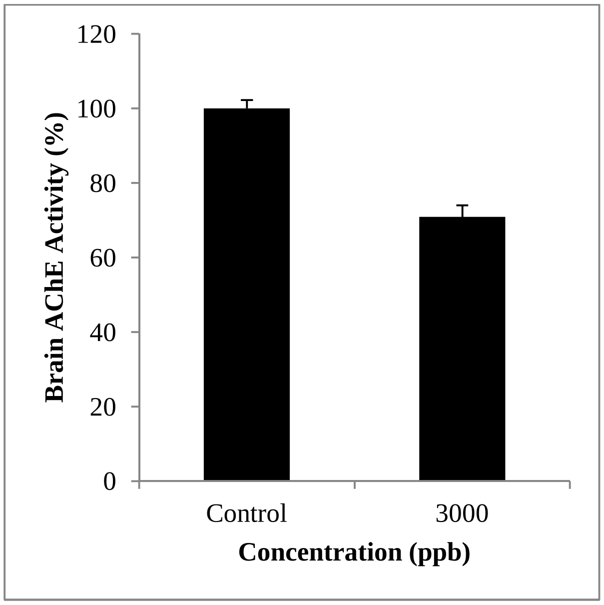

Figure 5: Brain AChE activity in 2-year-old SW-acclimated white sturgeon from the 2014 cohort following a 6-h exposure to clean SW or 3,000 µg L^{-1} carbaryl. Data are average ± SE, n = 16 per treatment. Percent activity based on control (100% = 4.9 units/g brain tissue). Difference was statistically significant ($p \leq 0.05$).

Plasma ChE activity

Plasma BChE activity was measured in the same fish used for the brain AChE analyses. Prior to measuring test sample ChE activity, white sturgeon plasma was characterized and optimized for the ChE assay (Appendix A). The optimal enzyme dilution was determined to be 16x, iso-OMPA indicated total plasma ChE was BChE, and the optimal substrate concentration was found to be $1x10^{-3}$ M (FC). Again, individual activities were averaged for each treatment. Unlike the results of the brain analyses, the plasma demonstrated an increasing trend in BChE activity associated with exposure to increasing concentrations ($\mu g\ L^{-1}$) of carbaryl (control = 100%, 30 = 84%, 100 = 99%, 300 = 120%, 1,000 = 134%, 3,000 = 82%). Differences among treatments in the 2010 samples were analyzed using a one-way ANOVA, which yielded no statistical differences ($p = 0.363$) among treatment groups (Fig. 6). The recovery test showed an initial increase in activity peaking at 24 h, followed by a decrease to below control activity levels at 72 h (control = 100%, 0 h = 111%, 24 h = 134%, 48 h = 103%, 72 h = 78%). Differences among recovery times were not statistically significant ($p = 0.517$; ANOVA, Fig. 7). The plasma BChE activities of individual fish varied greatly.

To verify the trends seen in the 2010 MMS plasma BChE studies, plasma BChE activity was measured in the 2014 MMS toxicity test. In 2014, plasma was drawn 5 d prior to exposure, and again following the 6-h test, so each fish could serve as its own control. Again, despite the decrease in brain AChE activity with exposure to 3,000 $\mu g\ L^{-1}$ carbaryl (29% inhibition), plasma BChE activity increased in exposed fish, as seen in 2010. At the end of the 6-h experiment, average plasma BChE activity in both control tanks was lower than pre-test activities, and average plasma BChE activity in both treatment tanks was higher (Fig. 8). There were no statistical differences between the two control tanks or between the two treatment tanks, but activities in both control tanks were statistically lower than in both treatment tanks (p = 0.002; ANOVA and SNK post-hoc test). Post-test activities from both tanks in a treatment were pooled, and plasma BChE activity in exposed fish was 30% higher than in control fish (Fig 9). The reproducibility of the results is supported by the fact that the activity from the 2010 control fish was not significantly different from the activity of the 2014 control fish (p = 0.205), but the increased sample size (from 5 to 16) greatly reduced the variance (Fig. 10).

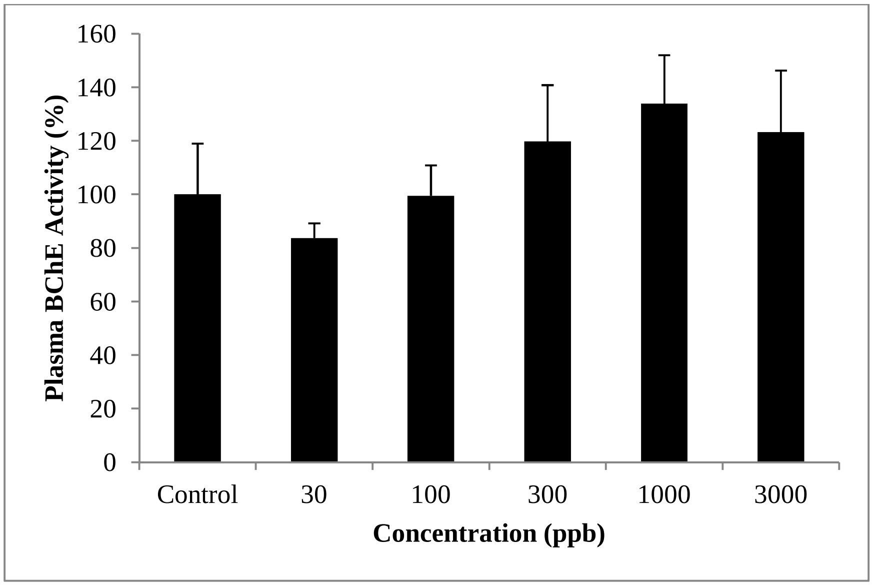

Figure 6: Plasma BChE activity in 2-year-old SW-acclimated white sturgeon from the 2010 cohort following a 6-h exposure to carbaryl (30-3,000 µg L^{-1}, control = clean SW). Data are average ± SE, n = 5 per dose. Percent activity based on control (100% = 0.343 units/ml plasma). Differences were not statistically significant ($p > 0.05$).

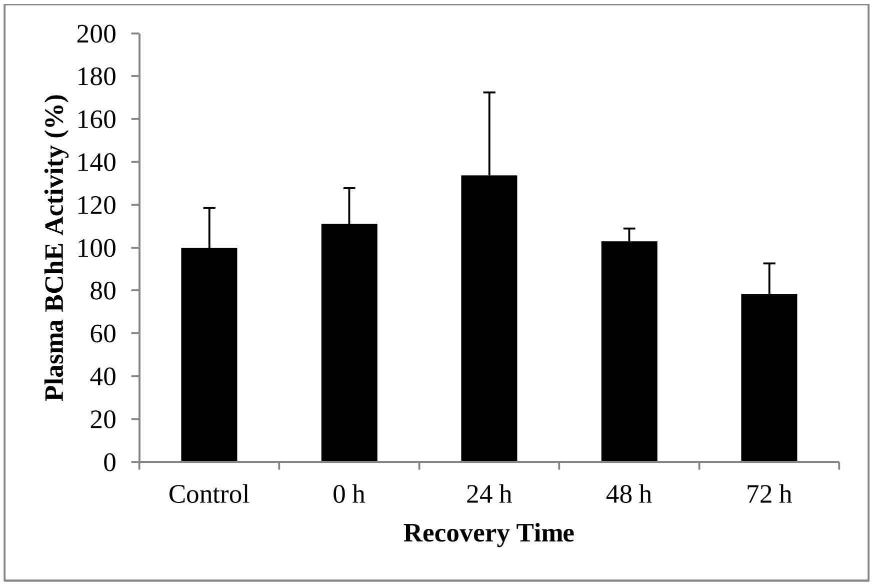

Figure 7: Plasma BChE activity in 2-year-old SW-acclimated white sturgeon from the 2010 cohort following a 6-h exposure to 1,000 µg L^{-1} carbaryl followed by a recovery period in clean SW. Data are average ± SE, n = 4 per recovery time. Percent activity based on control (100% = 0.379 units/ml plasma). Differences were not statistically significant ($p > 0.05$).

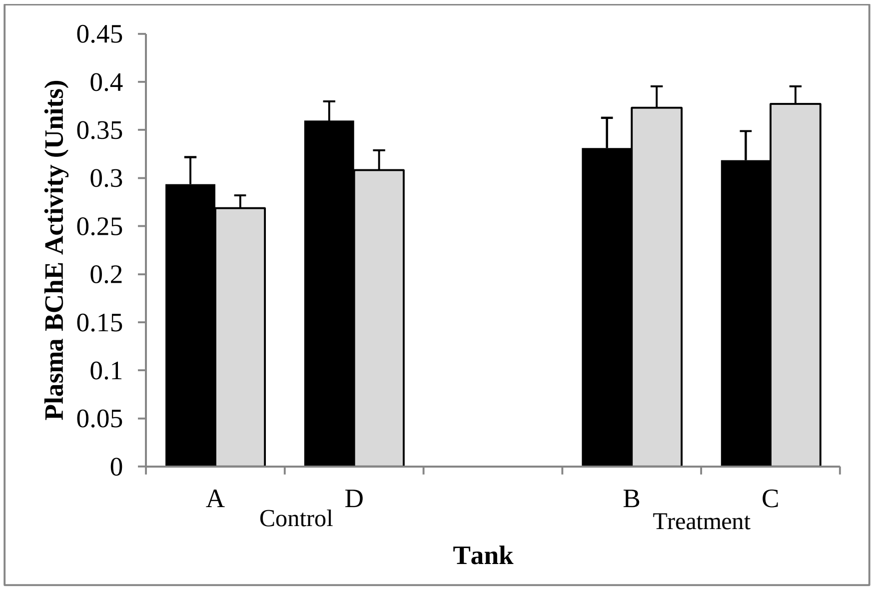

Figure 8: Plasma BChE activity in 2-year-old SW-acclimated white sturgeon from the 2014 cohort 5 d before (black) and immediately after a 6-h exposure (grey) to clean SW (control) or 3,000 µg L^{-1} carbaryl (treatment). Data are average ± SE, n = 8 per tank. Activity is separated by tank.

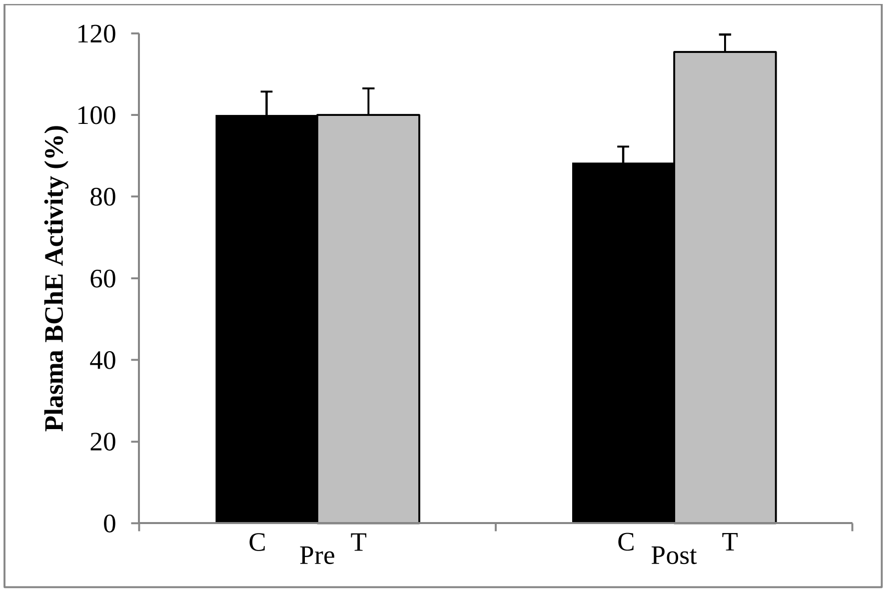

Figure 9: Plasma BChE activity in 2-year-old SW-acclimated white sturgeon from the 2014 cohort 5 d before and immediately after a 6-h exposure to clean SW (black) or 3,000 µg L^{-1} carbaryl (grey). Data are average ± SE, n = 16 per treatment. Percent activity based on pre-exposure activity (100% = 0.325 units/ml plasma). Activity was statistically greater ($p \leq 0.05$) following exposure.

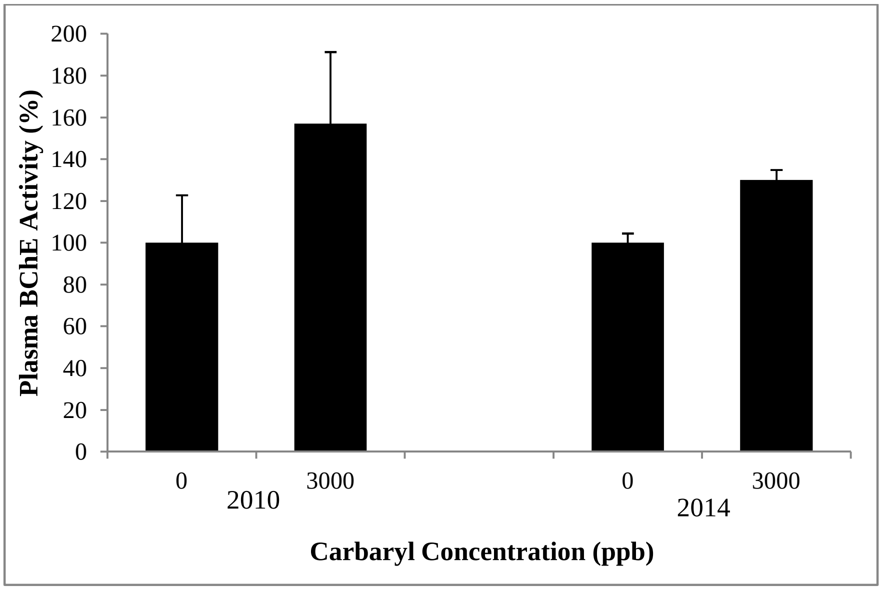

Figure 10: Comparison of plasma BChE activity in 2-year-old SW-acclimated white sturgeon following a 6-h exposure to clean SW or 3,000 µg L^{-1} carbaryl in 2010 and 2014. In 2010, n = 5, whereas in 2014, n = 16 per treatment. Data are average ± SE. Percent activity based on control (100% = 0.288 units/ml plasma).

Sensitivity Profiles

Plasma and brain homogenates from MMS white sturgeon in 2014, and plasma from green (Willapa Bay) and white (Columbia River) sturgeon collected pre-carbaryl application in 2012 were incubated with serial dilutions of technical grade carbaryl (FC 10^{-4} to 10^{-10}) in order to develop a sensitivity profile for each tissue and species. IC_{50}s were calculated from three replicates (average ± SE), each of which was run with independent inhibitor preparations. Average IC_{50} values calculated from Willapa Bay green sturgeon plasma (18.5 ± 2.0 µM) were slightly lower than Columbia River white sturgeon plasma (20.3 ± 5.9 µM; Fig. 11), but the difference was not statistically significant ($p = 0.79$). Average IC_{50} values indicated that MMS white sturgeon brain AChE (8.7 ± 0.6 µM) was more sensitive than plasma BChE (15.8 ± 1.8 µM; Fig. 12), and the difference was statistically significant ($p = 0.03$). The average IC_{50} s for Willapa Bay and MMS white sturgeon were not statistically different ($p = 0.44$).

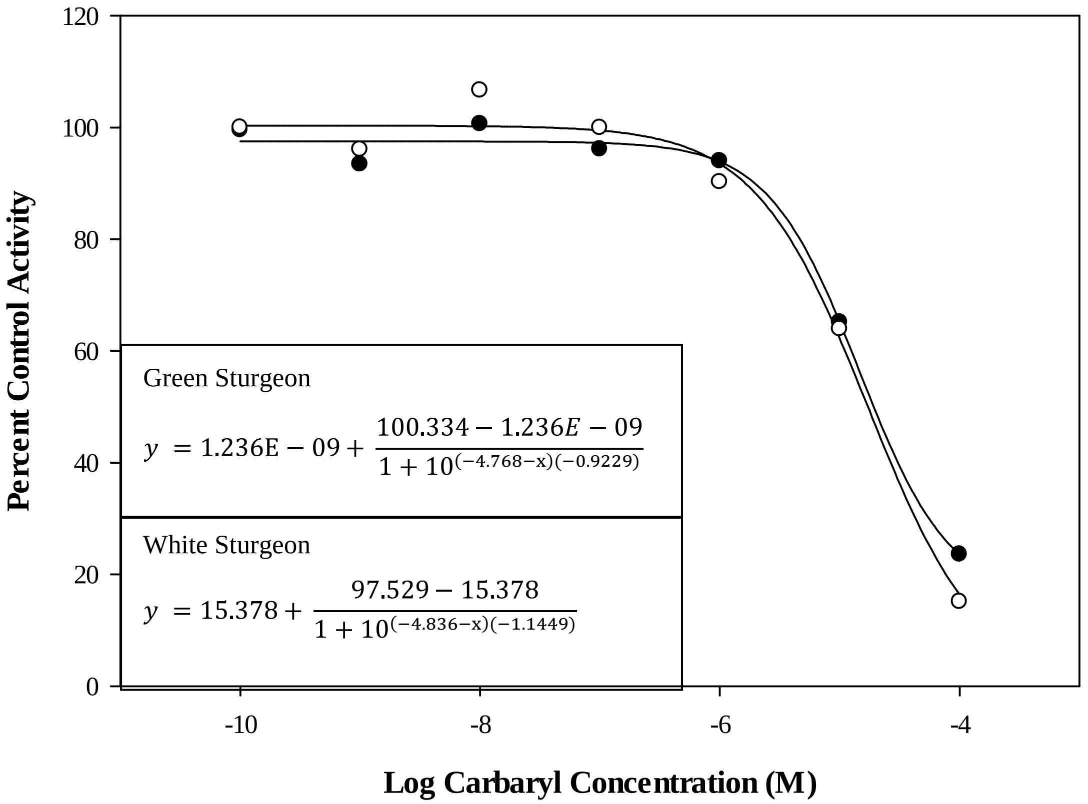

Figure 11: Relationship between representative *in vitro* sigmoidal dose-response curves for green and white sturgeon plasma BChE. Percent activity was calculated based on total green or white sturgeon plasma BChE activity measured with no carbaryl incubation. Solid circles = white sturgeon, open circles = green sturgeon. Demonstrated plots are representative of 3 independent determinations per species.

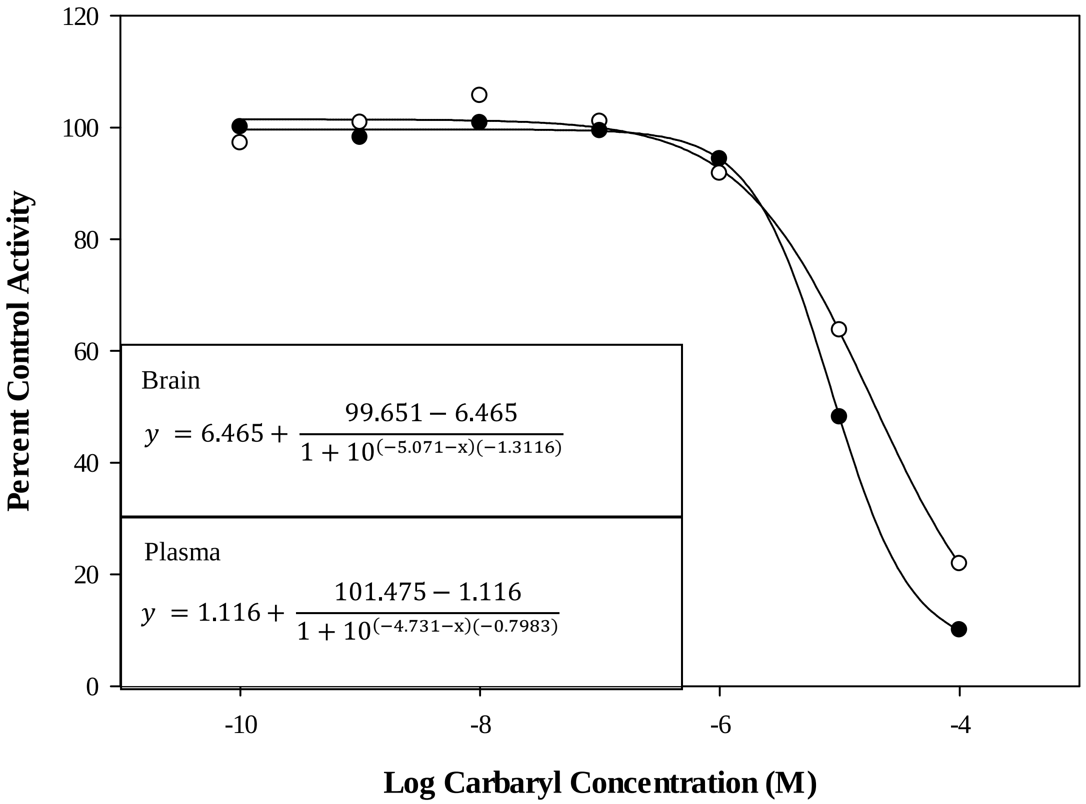

Figure 12: Relationship between *in vitro* sigmoidal dose-response curves representative for brain AChE and plasma BChE. Percent activity was calculated based on total brain AChE or plasma BChE activity measured with no carbaryl incubation. Solid circles = brain, open circles = plasma. Demonstrated plots are representative of 3 independent determinations per tissue.

Sturgeon Plasma Pre- and Post-carbaryl Application

Plasma BChE activity was measured in samples collected from wild green sturgeon (n = 30, length = 138.5 ± 2.7 cm) in Willapa Bay in 2012 prior to that year's application of carbaryl. In addition, wild white sturgeon (n = 30, length = 116.0 ± 4.6 cm) were collected from the lower Columbia River. There was no apparent correlation between plasma BChE activity and fish length in either species (Fig. 13), but average activity of white sturgeon was significantly higher than that of green sturgeon ($t_{58,\,0.05} = -7.717$, $p < 0.001$). Average plasma BChE activity in control white sturgeon did not differ statistically across the various groups of fish analyzed, regardless of age or wild vs. captive origin (Table 2). In addition, plasma was collected from green sturgeon in Willapa Bay 4-5 d post-application of carbaryl and compared to a second cohort of pre-spray green sturgeon plasma (n = 30). There was no apparent correlation between green sturgeon plasma BChE activity and fish length pre- or post-application (Fig. 14). On average, activity in the samples collected post-application (length = 128.9 ± 3.4 cm) was 37% lower than that of the control samples (length = 137.5 ± 3.2 cm) collected in Willapa Bay pre-spray ($t_{58,\,0.05} = -4.442$, $p < 0.001$; Fig. 15). Only one post-application fish had plasma BChE activity greater than the average pre-application activity, with 4 other fish having activities within -20% of average control activity (benchmark inhibition; Labenia et al. 2007). The remainder of the fish (n = 25) had activities lower than the -20% benchmark for exposure (Fig. 16). All but six fish fell within -2 SD of the average pre-application activity (95% confidence interval = ca. 50% inhibition), so using this benchmark, only 20% of the fish sampled would be considered exposed (Fig. 16).

Table 2: Average plasma BChE activity (units/ml plasma) for all white sturgeon analyzed. There were no statistical differences among average activities (independent t-test, $p \leq 0.05$)

Location	Year	Time	Sample Size	Activity ±SE
MMS	2010	Post-test	5	0.343 ± 0.065
Willapa Bay	2012	Pre-carbaryl	30	0.326 ± 0.021
MMS	2014	Pre-test	32	0.326 ± 0.014
MMS	2014	Post-test	16	0.289 ± 0.013

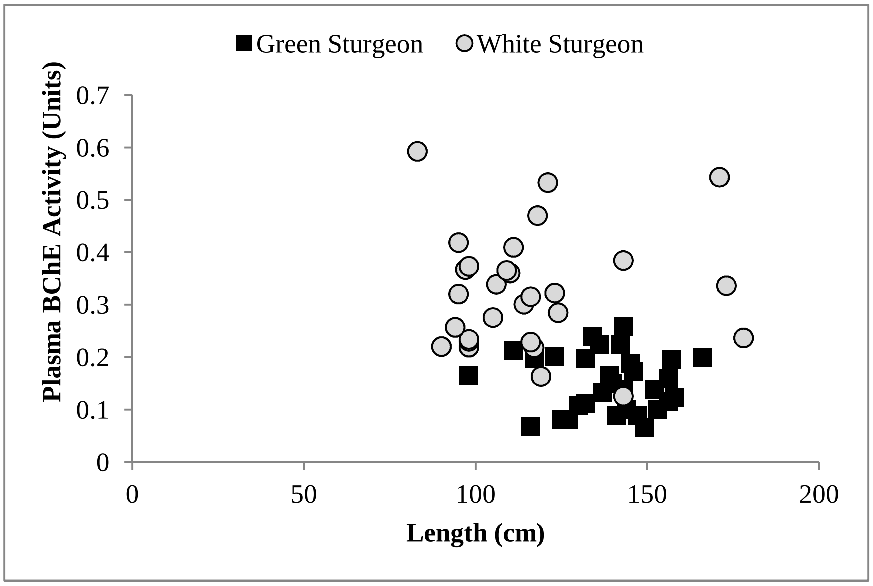

Figure 13: Plasma BChE activity in wild caught white and green sturgeon (n = 30 per species) prior to carbaryl application in Willapa Bay, 2012. Individual activities are plotted against fish length. Squares represent green sturgeon, circles represent white sturgeon. Average activity in white sturgeon was statistically higher ($p \leq 0.05$) than that in green sturgeon.

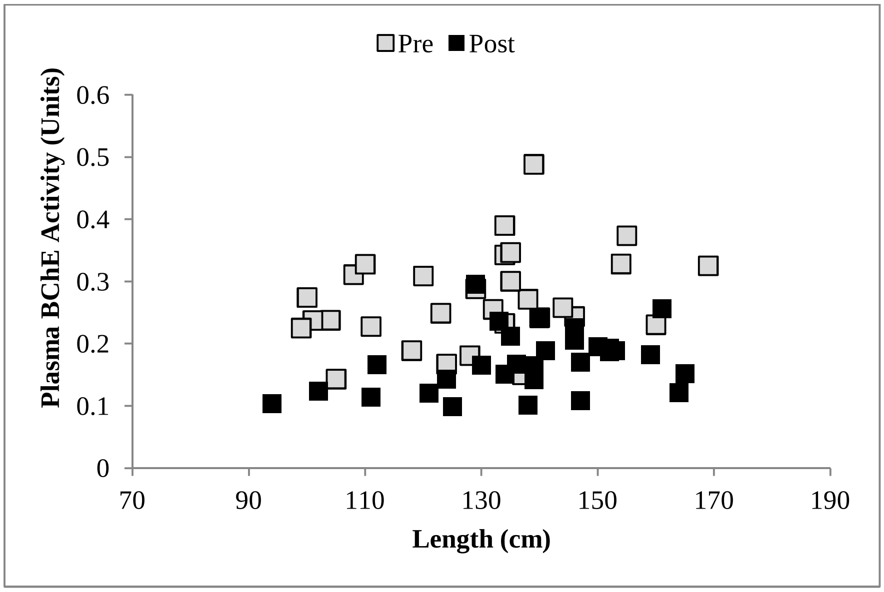

Figure 14: Plasma BChE activity in wild caught green sturgeon (n = 30) prior to, and 4-5 d post-carbaryl application (n = 30) in Willapa Bay, 2012. Individual activities are plotted against fish length. Grey squares represent pre-carbaryl samples, and black squares represent post-carbaryl samples. Average BChE activity in post-carbaryl plasma samples was statistically lower than that in pre-carbaryl plasma samples ($p \leq 0.05$).

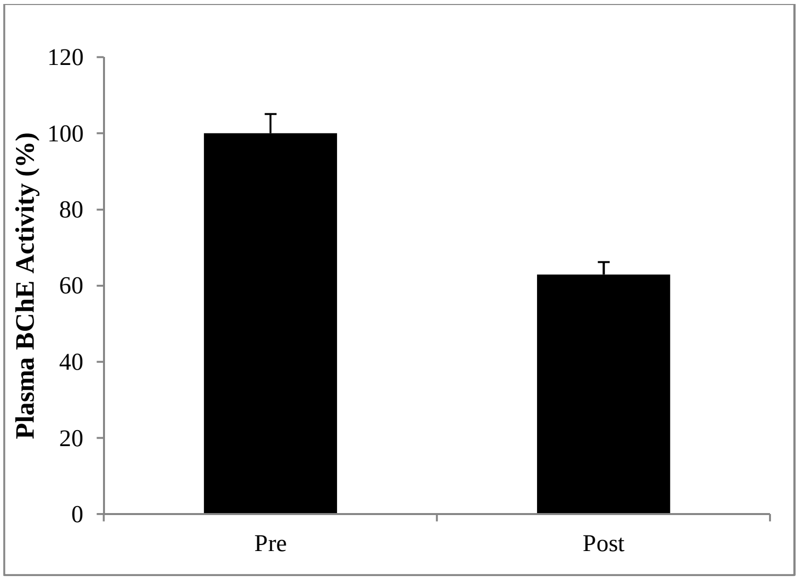

Figure 15: Plasma BChE activity in wild green sturgeon (n = 30) prior to, and 4-5 d post-carbaryl application (n = 30) in Willapa Bay, 2012. Data are average ± SE. Percent activity based on pre-exposure activity (100% = 0.272 units/ml plasma). Average activity in post-carbaryl plasma was statistically lower than that in pre-carbaryl plasma ($p \leq 0.05$).

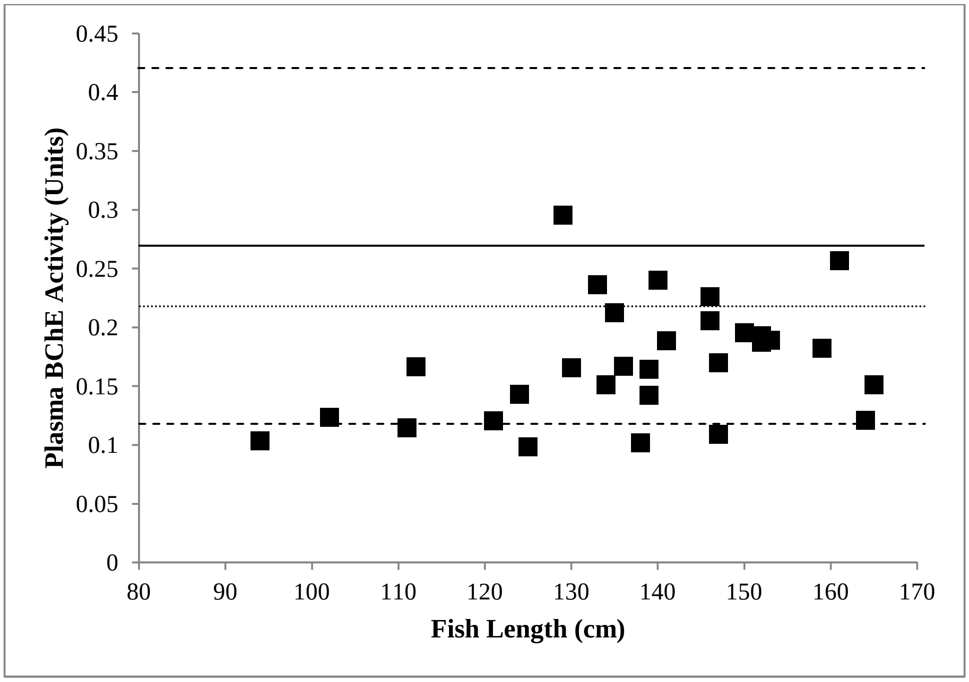

Figure 16: Plasma BChE activity in wild caught green sturgeon (n = 30) 4-5 d post-carbaryl application in Willapa Bay, 2012. Activities are plotted against individual fish length. Solid line represents average activity of green sturgeon pre-carbaryl application, dotted line represents benchmark -20% of control activity (Labenia et al. 2007), dashed lines represent ±2 SD of average control activity (95% confidence interval).

Discussion

The effect of carbaryl exposure on ChE activity in white and green sturgeon was investigated with the goal of evaluating the use of plasma ChE as a biomarker for exposure in the ESA-listed green sturgeon. Acute laboratory toxicity tests with white sturgeon as a surrogate indicated that plasma BChE inhibition was not correlated with brain AChE inhibition, complicating the interpretation of the biological significance of the results and questioning the role of BChE as a biomarker for exposure. In both 2010 and 2014, brain AChE behaved as predicted, with decreased AChE activity corresponding to increased carbaryl concentration (Zinkl et al. 1991), whereas in both years, plasma BChE activity was elevated at concentrations $\geq$100 µg L^{-1}. In 2014, brain AChE inhibition in fish exposed to 3,000 µg L^{-1} was lower than in fish exposed to the same concentration in 2010 (29% and 42%, respectively), but measured water concentrations were higher in 2010 than 2014 (3,800 and 2,900 µg L^{-1}, respectively), and likely contributed to the difference between years. Additionally, the measured variance in activity decreased in 2014 due to an increase in sample size from 5 to 16. Brain AChE activity in sturgeon exposed to carbaryl at concentrations $\geq$ 100 µg L^{-1} was statistically lower than control activity in both years, confirming that carbaryl was transported to the brain. The recovery test showed inhibition immediately following a 6-h exposure to 1,000 µg L^{-1} carbaryl, followed by an increase in activity over 72 h once fish were transferred to clean SW. By 72 h post-exposure, brain AChE activity was not statistically different from control activity. The activity at 24 h was higher than the activity at 48 h post-exposure, and the reason for this is unclear, but could be due to the small sample size (n = 4). In similar recovery studies, brain AChE activity in cutthroat trout exposed to 500 µg L^{-1} carbaryl for 6 h recovered to pre-exposure levels after 42 h (Labenia et al. 2007) and brain AChE activity in rainbow trout exposed to 1,000 µg L^{-1} carbaryl for 24 h returned to normal between 48 and 72 h (Zinkl et al. 1991).

Since carbaryl is carried to the brain via the blood, a similar dose-response in ChE activity in plasma was expected. Channel catfish fingerlings (*Ictalurus punctatus)* exposed to the carbamate, aldicarb, demonstrated significant decreases in both brain and plasma AChE activity following a 48-h exposure to 0.1 mg L^{-1} of the carbamate (Perkins and Schlenk, 2000). Similarly, brain and plasma AChE activity showed a negative trend with increasing concentration in African sharptooth catfish (*Clarias gariepinus*) exposed to the OP pesticide, chlorfenvinphos (0.0003 – 0.06 μM) or carbaryl (0.0005 – 0.05 μM) for 24 h, although only reductions in plasma AChE activity at chlorfenvinphos concentrations greater than 0.03 µM were statistically significant (Mdegela et al. 2010). The observed increase in white sturgeon plasma BChE activity was therefore unexpected in light of the numerous studies where plasma ChE inhibition has been used as an indicator of exposure to ChE inhibitors in a variety of species, irrespective of whether the inhibited enzyme was BChE or AChE (e.g., Grue et al. 2002, Fildes et al. 2009, Strum et al. 2010, Borbón and Mantilla 2012).

Also unexpected was the result of the characterization of sturgeon plasma. In most fish, plasma ChE is composed primarily (or completely) of AChE (Chuiko 2000), whereas the ChE in the plasma of both white and green sturgeon appeared to be entirely BChE. Plasma BChE is present in various quantities in birds, mammals, and reptiles, but is much less common in fish. Presence of BChE in fish plasma was first documented in 1979 in blue bream (*Abramis ballerus*) and roach (R*utilus rutilus*), and has since been documented in various quantities in other fish from the family Cyprinidae (Chuiko, 2000), the piaussu (*Leporinus macrocephalus*; Salles et al. 2006), red-bellied pacu (Borbón and Mantilla 2012), and grass carp (*Ctenopharyngodon idella*;

Pucher et al. 2014). Of greater relevance, Kruse and Scarnecchia (2002) reported a plasma composition of 92.4% BChE and 7.6% AChE in Kootenai River white sturgeon; values similar to those observed in the present study. Following increased iso-OMPA incubation, plasma ChE activity was reduced linearly to nearly zero, and as a result, total sturgeon plasma ChE is reported as BChE. With sturgeon being amongst the most primitive Actinopterygii, it is possible that the transition from plasma BChE to AChE occurred later in the evolutionary tree, although the presence of plasma BChE in more advanced species (e.g., various cyprinids and characids) would indicate otherwise. Although the results of the characterization are interesting, carbamate pesticides inhibit both AChE and BChE and therefore the characterization does not explain the elevated plasma BChE activity in the sturgeon exposed to carbaryl.

A potential explanation for the elevated BChE activity in 2010 was the processing of the plasma samples. In some cases, the centrifugation protocol (1,900 x *g*, 10 min) produced a cloudy upper plasma layer, with an undefined buffy coat layer, and a defined bottom red blood cell layer. The plasma, therefore, may have contained some fraction of leukocytes and/or platelets due to incomplete isolation. Some of the samples were very cloudy despite fasting of the fish for ca. 72 h prior to testing that should have prevented retention of fat. Some samples had a noticeably pink tinge that may have indicated the presence of hemoglobin from lysed red blood cells. Plasma BChE activities were plotted for each individual fish and plasma color. The "pink" samples were randomly distributed throughout the enzyme activity levels, suggesting the colored samples did not skew the results. Small sample size also proved to be a problem. The large variance associated with a sample size of five fish only allowed detection of ≥ 53% reduction in enzyme activity ($p \leq 0.05$, one-tailed t-test). These concerns together with the unexpected increase in ChE activity led to the more powerful study design in 2014 in which 16 fish were exposed to 3,000 µg L^{-1} carbaryl. Blood was spun such that the plasma layer was clear (4,500 x *g*, 7 min). Again, BChE activity in the carbaryl exposed fish exceeded controls, indicating the elevated activity observed in the initial test was reproducible.

Based on the scope of this study, it is hard to determine why plasma BChE activity was elevated following a 6-h exposure to carbaryl, especially in view of the fact that brain AChE activity was significantly inhibited (42% at 3,000 µg L^{-1} in 2010, 29% in 2014). It is possible that plasma BChE activity would be inhibited if fish were exposed to carbaryl for a longer period of time at the higher concentrations tested, but such exposure is unlikely in the field. In contrast to chronic laboratory exposures at high carbaryl concentrations, the wild green sturgeon plasma results suggest that acute or chronic, low-level exposure may lead to plasma BChE inhibition up to a certain threshold, beyond which, enzyme activity is elevated. It is also possible that sturgeon plasma BChE is inhibited very quickly, within a matter of hours (< 6), before release of BChE is stimulated.

Unlike AChE, which is found at neuromuscular junctions and on red blood cell membranes, BChE is a liver enzyme. The main physiological function of BChE is still largely unknown, although many roles have been suggested. In vertebrates, BChE gene expression appears to play an important role in neurogenesis, and in humans, BChE activity in the brain increases with age, and increased brain BChE activity is associated with a variety of neurodegenerative disorders (Mack and Robitzki 2000). Other proposed functions include lipoprotein metabolism, cellular adhesion and neurogenesis, and scavenging of toxic molecules (Patocka et al. 2004) before the chemicals reach the brain (Grue et al. 2002). BChE has been shown to protect against naturally occurring esterase inhibitors such as cocaine and eserine (Mack and Robitzki 2000), and also binds to, and thus, protects against certain nerve agents, including OPs, preventing the inhibition of AChE and blocking of cholinergic synapses (Grue et al. 2002, Patocka et al. 2004, Ghazala et al. 2014). It is also possible that carbaryl exposure may lead to injury to or stimulation of liver tissues, which is rapidly followed by elevated BChE levels either by release of stored BChE, or production of new enzyme (*de novo* synthesis, induction).

There are no studies linking an increase in plasma BChE to carbaryl-induced hepatotoxicity, but there are studies linking liver toxicity to carbaryl exposure in various species of fish. In one study, rainbow trout were exposed to carbaryl at 1.4 and 2.6 mg L^{-1} for 96 h (96-h LC_{50} = 1.39 mg L^{-1}), after which livers were removed (along with controls). No histopathological lesions were seen in control fish. Intercellular edema, cytoplasmic vacuolation indicative of necrosis in hepatocytes, and pyknotic nuclei were seen in livers from all treated fish, with severity increasing with higher concentration (Boran et al. 2010). While livers can degrade toxic compounds, their regulatory mechanisms can be overwhelmed by elevated concentrations, leading to structural damage (Boran et al. 2010). In another study, Nile tilapia (*Oreochromis niloticus*) were exposed to 0.25 or 0.5 mg L^{-1} carbaryl for 21 d (96-h LC_{50} = 1.5 mg L^{-1}; Matos et al. 2007). In contrast with the study of Boran et al. (2010), limited histological alterations were observed, with the exception of increased hepatocellular basophilia, and a few necrotic foci at day 7. There was also an increase in vacuolization due to lipid accumulation, indicating glycogen depletion, which is a common, non-specific liver response to toxicants (Matos et al. 2007, Venkateshwarlu et al. 2011). In addition to liver histology, various enzymes related to oxidative stress have been used as indicators of hepatotoxicity. Exposure to contaminants may lead to oxidative damage of biological systems via an increase in intracellular reactive oxygen species (ROS). These ROS can be detoxified by enzymes in an enzyme defense system, including superoxide dismutase (SOD) and catalase (CAT). Over the first 14 d of the study with Nile tilapia, SOD, CAT, glutathione reductase (GR) and glutathione S-transferase (GST) levels were lower than controls. While there was no mortality, and no visible disease observed in the fish throughout the test, decreased enzyme levels indicated altered hepatic function (Matos et al. 2007). A similar study exposed rainbow trout to 1 and 3 mg L^{-1} carbaryl for 24, 48 and 96 h, and measured liver carboxylesterase (CaE), glutathione (GSH), CAT, GST, and cytochrome P450-1A (CYP1A) levels. Significant reductions were seen in CaE and GSH levels, while GST, CAT, and CYP1A showed initial increases in activity (0-48 h) followed by significant decreases after 48 h. The early induction followed by a decrease may be indicative of delayed oxidative stress (Ferrari et al. 2007). These studies indicate carbaryl exposure can affect fish livers in various ways depending on the concentration and the species, and to a certain extent, the liver can play a role in mitigating damage to the remainder of the organism.

The liver has also been directly linked to elevation in plasma BChE activity in dogs exposed to carbon tetrachloride (CCl_4) at concentrations that would not cause extra-hepatic damage (Brauer and Root 1947). The authors of this study conclude that CCl_4 poisoning results in an increase in plasma ChE activity of up to 50% above normal which is due to an increase in circulating plasma ChE, and not a failure of destruction or excretion of the enzyme. The study also experimentally linked much of the increased plasma ChE activity to a transfer of the enzyme from the liver into the blood stream. These results indicate the existence of a mobile store of preformed plasma ChE in the liver of the dog, which can rapidly replace at least 25% of the circulating plasma ChE activity, with the remainder of the elevation in enzyme activity due to increased synthesis in the liver (Brauer and Root 1947). A similar study exposed rats to CCl_4 and showed a substantial increase in plasma ChE activity within the first 8 h, followed by a decline to basal activity levels within 24 h (Bhatnagar 1970). These studies support the theory that the liver could play a role in releasing BChE into the plasma of exposed white sturgeon, leading to elevated plasma BChE levels observed.

The results from the sensitivity profiles for plasma BChE and brain AChE from hatchery white sturgeon indicate the IC_{50} for plasma is approximately twice as high as the IC_{50} for brain. While this difference was statistically significant ($p = 0.03$), its biological relevance is unclear as significant differences in ChE susceptibility are generally measured in differences across orders of magnitude (Mortensen et al. 1998). Calculated brain AChE IC_{50}s for carbaryl in the literature for various fish species span at least 4 orders of magnitude, ranging from 0.003 μM (African sharptooth catfish; Mdegela et al. 2010) to 33.8 μM (tambaqui, *Colossoma macropomum*; Assis et al. 2012), and the IC_{50} for white sturgeon (8.73 μM) falls within this range. When plasma BChE is more sensitive than brain AChE, the plasma enzyme can act as a buffer, absorbing carbaryl at lower doses and decreasing its movement into the brain at concentrations affecting AChE, with the level of protection dependent on the magnitude of the difference in sensitivity. As this appears not to be the case in sturgeon, plasma BChE inhibition in wild sturgeon may actually be indicative of brain AChE inhibition levels in these species.

There was no significant difference ($p > 0.05$) between the IC_{50} values calculated for MMS white sturgeon plasma as compared to the Columbia River white sturgeon plasma, suggesting plasma BChE activity and sensitivity does not vary based on age or size between 2-year-old and adult white sturgeon. In addition, there was no significant difference ($p > 0.05$) in the IC_{50} values for green and white sturgeon plasma from Willapa Bay and the Columbia River, respectively. Results suggest white sturgeon are a good surrogate for greens despite the fact that the elevation of plasma BChE seen in the laboratory complicates extrapolation from the laboratory tests to the field. Statistically significant inhibition was seen in plasma BChE collected from green sturgeon following carbaryl application in Willapa Bay in 2012. Given the elevated plasma BChE activity in the laboratory dose-response tests with white sturgeon, enzyme inhibition in wild green sturgeon was unexpected.

Threshold for elevated plasma BChE. Based on the results from the laboratory toxicity tests, measurable elevation in the post-carbaryl plasma BChE activity would have suggested exposure of green sturgeon in Willapa Bay to concentrations at least as high as in the acute toxicity test for at least 6 h. Had there been no difference in plasma BChE activity pre- and post-application, one could have concluded little or no exposure to carbaryl. Inhibition, however, could indicate exposure below a concentration threshold, above which, plasma BChE activity is elevated. In the laboratory white sturgeon, both plasma BChE and brain AChE activities were slightly, but not statistically inhibited at 30 µg L^{-1}, while at 100 µg L^{-1} and higher, plasma BChE activity was elevated, and brain activity was reduced. A concentration threshold could therefore fall between 30 and 100 µg L^{-1}, and at concentrations below this threshold, plasma BChE inhibition would be measureable, as seen in the wild green sturgeon plasma samples. Given that clean white and green sturgeon plasma spiked directly with carbaryl showed BChE inhibition (*in vitro*), elevated levels of plasma BChE activity must be due to a physiological process *in vivo* that occurs above the aforementioned concentration threshold. The inhibition seen in wild green sturgeon plasma BChE would therefore be attributed to acute or more chronic exposures below this threshold.

More chronic, lower exposures to carbaryl in Willapa Bay are possible based on the timing of the applications, and the environmental fate of carbaryl in Willapa Bay. Carbaryl has been applied in Willapa Bay twice each summer in July, ca. 2 weeks apart, and the control green sturgeon samples were collected before the first application in 2012. The post-application green sturgeon plasma was collected 4-5 d after the second application. The maximum carbaryl concentration previously reported in the mid-water column (ca. 1 m below the surface) above treated beds at the first high tide ca. 6 h following application was 30 µg L^{-1} (Grue et al. 2009), which was the minimum concentration in the dose-response test. In most cases, concentrations in the water column above treated beds fall below 1 µg L^{-1} within 30 h (Troiano et al. 2013). Concentrations in adjacent channels that drain treated beds are generally greater than those above the beds and persist longer, but concentrations are still low (maximum < 7 µg L^{-1}, Troiano et al. 2013). Therefore, sturgeon in Willapa Bay are likely not exposed to water concentrations greater than 30 µg L^{-1} for greater than 6 h. More chronic exposure to carbaryl is probably the result of their feeding behavior. Average concentrations of carbaryl in burrowing shrimp were 8,700 and 13,800 µg kg^{-1} immediately after application of 7.5 and 10 lbs ai ac^{-1}, respectively, and decreased by an order of magnitude within 24-96 h (Washington Department of Fisheries and Washington Department of Ecology 1992; wet or dry weight not specified). Comparable residues in annelid worms were 57.0 to 75.7 mg kg^{-1} immediately after application at the two rates (Washington Department of Fisheries and Washington Department of Ecology 1992; wet or dry weight not specified). The current application rate (8 lbs ai ac $^{-1}$) is between the rates used in these studies. When extracting prey from within the substrate, sturgeon also ingest whole sediment (sediment + pore water). Analysis of the stomach contents of Atlantic sturgeon (*Acipenser oxyrinchus*) indicated that 26-75% by weight was sand and organic debris ingested during feeding (Johnson et al. 1997). Carbaryl concentrations in whole sediment are greater and persist longer than those in the water column. Maximum concentrations within the top 25 cm averaged 2,773 µg kg^{-1} (dry weight) immediately after application, with an average concentration of 72 and 6 µg kg^{-1} (dry weight) 1 and 14 d post-application (Felsot and Ruppert 2002).

Low risk scenario. One way of interpreting the laboratory and field results leads to a conclusion of low risk to wild green sturgeon from carbaryl applications in Willapa Bay. Even at the high concentrations utilized in the laboratory toxicity tests, no overt effects were observed, suggesting that white sturgeon can survive acute exposure to carbaryl in water of at least two orders of magnitude greater than the maximum reported in the water column above treated beds in Willapa Bay. Whereas plasma BChE was elevated at these high concentrations in the laboratory, brain AChE activity was inhibited. At the lowest concentration (30 µg L^{-1}), both brain AChE and plasma BChE were inhibited (15% and 17% respectively), above which, plasma BChE activity was elevated, and brain AChE activity was reduced. Since plasma BChE from wild green sturgeon was on average inhibited by 37%, exposure could be attributed to concentrations less than 30 µg L^{-1}. Based on the trend from the brain dose-response test, no significant brain AChE reduction would be expected at concentrations lower than 30 µg L^{-1}. Given the relationship between brain AChE inhibition and physiological and behavioral effects in anti-ChE exposed organisms (for review, see Grue et al. 2002), the presence of plasma BChE inhibition without brain AChE inhibition would suggest that carbaryl applications pose little hazard to green sturgeon in Willapa Bay.

Higher risk scenario. Alternatively, if the plasma BChE inhibition detected in wild green sturgeon (37%) in Willapa Bay were associated with comparable or higher levels of brain AChE inhibition, it would indicate greater risk to these fish. It is likely that the elevation of plasma BChE observed in the laboratory white sturgeon was due to a release of BChE from the liver, and the release of BChE may have masked carbaryl-induced plasma BChE inhibition. The release of hepatic BChE is likely due to hepatotoxicity caused by exposure to carbaryl or the primary metabolite, 1-naphthol, which is also toxic to fish (see discussion below).

Carbaryl is broken down into 1-naphthol by hydrolysis, biodegradation, and photolysis. Subsequently, 1-naphthol is primarily degraded by photolysis and biodegradation. In sterile SW with artificial sunlight, carbaryl and 1-naphthol are rapidly degraded with half-lives of 5 h and less than 2 h, respectively (Armbrust and Crosby 1991) which, in conjunction with dilution by tidal inundations, results in low persistence in the water column in Willapa Bay. Carbaryl is hydrolyzed according to pseudo-first-order kinetics in sterile SW in the dark with a half-life of 24 h (pH = 7.9; Armbrust and Crosby 1991). Under the same conditions, 1-naphthol was not degraded over the course of 3 d. In raw SW in the dark, both carbaryl and 1-naphthol were degraded to undetectable levels within 96 h (Armbrust and Crosby 1991). In unfiltered SW in the dark, carbaryl degradation occurred via hydrolysis and biodegradation, but 1-naphthol degradation was due to biodegradation, as it was shown not to undergo significant hydrolysis (Armbrust and Crosby 1991). The MMS laboratory tests were conducted with raw SW in a dark environment (no sunlight, pH = 6.8-7.2), and would therefore closely resemble the conditions reported by Armbrust and Crosby (1991). While the lack of sunlight and flowing water in the laboratory tests may have decreased the rate of 1-naphthol breakdown, carbaryl concentrations were measured at the time of dosing, and at the end of the 6-h exposure, and no carbaryl was lost over this time period (Table 1). If fish were metabolizing carbaryl, however, and excreting it into the tank water, there is the possibility that laboratory fish would be exposed to higher concentrations of 1-naphthol than in the field, potentially contributing to hepatotoxicity. Rainbow trout exposed to 250 $\mu g\ L^{-1}$ carbaryl for 24 h showed biliary and possible urinary excretion of metabolites (Statham et al. 1975) that could lead to 1-napthol in test water.

If sturgeon in the laboratory were exposed to higher concentrations of 1-naphthol than they would experience in the wild, the relative toxicities of carbaryl and 1-naphthol to fish become relevant. Four species of FW fish were exposed to carbaryl (Sevin®) or 1-naphthol for 96-h and the calculated LC_{50} values for all species indicated that 1-naphthol was 1.5 (*Catla catla*) *to* 13.9 (*Mystus cavasius*) times more toxic than carbaryl (Tilak et al. 1981). Similarly, 1-naphthol was four times more toxic to *Cirrhinus mrigala* than carbaryl (Sevin® 85% WP) based on 96-h LC_{50} values (Rao et al. 1984). In addition, 24-h EC_{50} values indicate that 1-naphthol was 1.6, 2.1, and 3 times more toxic than carbaryl (Sevin®) to *Cymatogaster aggregata, Gasterosteus aculeatus*, and *Parophrys vetulus*, respectively (Stewart et al. 1967). While these studies do not discuss the mechanism of 1-naphthol toxicity¸ they consistently indicate that 1-naphthol is more toxic than carbaryl to the species of fish tested, and the same may be true for white and green sturgeon.

In the 2010 MMS toxicity test, plasma BChE activity was inhibited by 17% at 30 $\mu g\ L^{-1}$, although this reduction was not statistically significant. At higher concentrations, plasma BChE activity was equal to or higher than controls, indicating release of BChE from the liver. The duration and magnitude of the elevated BChE in the plasma would depend on the extent of release of stored enzyme and the synthesis of new enzyme in the liver. Secretion of BChE into plasma from the liver has been shown to be a transient phenomenon (<8 h in rats; Bhatnagar 1970), and therefore may not be expected to counteract BChE inhibition or hepatotoxicity indefinitely, as would be the case in more chronic exposure scenarios, such as what may occur in Willapa Bay.

In addition, there is evidence that white sturgeon plasma BChE and brain AChE inhibition may parallel each other fairly closely. The IC_{50} values calculated for the two different tissues only varied by a factor of two, which is unlikely to be a biologically relevant difference. Furthermore, the ChE inhibition seen at 30 µg L^{-1} was similar in both brain (15%) and plasma (17%) samples from MMS in 2010. While inhibition was not statistically significant at the low concentration in either tissue, there was a dose-response, inhibition was very similar, and in conjunction with the similar IC_{50} values, the results suggest that brain AChE and plasma BChE have similar sensitivities to carbaryl in white sturgeon. It follows that the wild green sturgeon sampled might be experiencing brain AChE inhibition similar to that in the plasma. The degree to which release of BChE from the livers of the sampled fish may have masked carbaryl-induced BChE inhibition is not known, so the amount of inhibition measured likely represents a conservative estimate. If brain AChE inhibition mirrors the 37% decrease in plasma BChE activity, green sturgeon in Willapa Bay may be at greater risk due to exposure to carbaryl. While a decrease in brain ChE activity of 37% has not been associated with overt effects in fish, more subtle behavioral effects have been recorded in fish with brain ChE inhibition of this magnitude (Zinkl et al. 1991).

The severity of effects associated with brain AChE inhibition in fish appears to be directly correlated with the magnitude of enzyme inhibition, with inhibition > 70% associated with overt behavioral effects, such as impaired locomotor function (for review, see Fulton and Key 2001, Grue et al. 2002, Labenia et al. 2007). A similar relationship exists for birds and mammals and is likely related to the physiological mechanisms responsible for the biphasic recovery of enzyme activity in these taxa (i.e., a threshold of approximately 40-60% brain AChE inhibition; Grue et al. 2002). Other studies have documented effects in fish with levels of enzyme inhibition < 70% (reviewed by Zinkl et al. 1991). For example, swimming stamina was decreased by 23-44% in three species of salmonids with ca. 50% brain AChE inhibition (Post and Leasure 1974). Sandahl et al. (2005) reported a decrease in spontaneous swimming rate (27%) in juvenile coho salmon (*Oncorhynchus kisutch*) with brain AChE inhibition of 23%, whereas feeding, swimming rate, time to first feeding strike, and total feeding strikes were not impaired until enzyme inhibition reached 51%. More recently, Tierney et al. (2007) reported impaired swimming performance in juvenile coho salmon after a threshold of approximately 32 or 50% brain AChE inhibition was reached depending on the type of performance test used. Whether or not comparable effects occur in wild green sturgeon following carbaryl applications is not known.

Another factor that may affect the toxicity of carbaryl as well as the extrapolation of the results of the present study to other parts of the range of green sturgeon is the salinity of the water in which they are exposed (Hooper et al. 2013). Willapa Bay is a SW estuary, and as such, salinity varies depending primarily on FW inputs and tidal flushing (15% of the volume of Willapa Bay is exchanged during each daily tidal cycle; for review, see Banas et al. 2004). In the summer when carbaryl is applied, salinity is greatest along the length of the estuary (26-31.5 ppt in July) and is associated with low river flows coupled with coastal upwelling (Banas et al. 2004). In comparison, salinity within the toxicity tests at MMS was 30 ± 1 ppt. The extreme regime of SW intrusion within Willapa Bay and other estuaries in the Northwest appears to be different from other coastal estuaries in North America and more closely resembles the north coast of Europe and the British Isles (Banas et al. 2004).

The toxicity of ChE-inhibitors to anadromous fish may be increased when the fish are exposed in SW. Studies with coho salmon have shown increased toxicity to the OP, phorate, with increasing salinity (Lavado et al. 2011). Similar results were reported for rainbow trout exposed to the carbamate, aldicarb; elevated salinity increased muscle AChE inhibition and toxicity (Wang et al. 2001). One explanation for the salinity-related increase in toxicity appears to be related to the differential expression of flavin-containing mono-oxygenases (FMOs), which are involved in osmoregulation and metabolism of xenobiotics. To support this, Wang et al. (2001) exposed hybrid striped bass (*Morone saxatilis* x *chrysops*), which express FMOs that are nonresponsive to salinity induction, to aldicarb. Neither muscle nor brain AChE activity nor toxicity was affected by increases in salinity (Wang et al. 2001). Whether sturgeon have FMOs that are responsive to salinity or not may affect the extent to which salinity affects sensitivity to carbaryl and other carbamates throughout their home range. As this is yet unknown and salinities may vary, it is hard to extrapolate risk to green sturgeon outside of Willapa Bay.

Results from the present study indicate green sturgeon are exposed to carbaryl following application of the insecticide to control burrowing shrimp in Willapa Bay. On average, BChE was inhibited 37% compared to controls. The distribution of enzyme activities in sturgeon collected after the final carbaryl treatment in Willapa Bay in 2012 compared to the control average indicated that all but one was below the control average. The significance of these results is increased when one considers: (1) the low sampling fraction (1.5%, 30 sturgeon in a population estimated to be ca. 2,000 in July; Olaf Langness, personal communication); (2) telemetry studies that indicate green sturgeon move extensively throughout Willapa Bay, but are concentrated near areas with the highest burrowing shrimp densities (and therefore carbaryl applications; Mary Moser and Brett Dumbauld, personal communication); and (3) the small proportion of Willapa Bay's intertidal mudflats that are sprayed with carbaryl each year in July (410 acres in 2012 = 1% of the intertidal mudflat or 4.5% of the cultivated oyster ground).

Unfortunately, the results from the acute toxicity tests conducted with SW-acclimated white sturgeon suggest one can interpret the observed plasma BChE inhibition in wild green sturgeon in two ways, leading to opposite conclusions about the risk carbaryl applications in Willapa Bay pose to this ESA-listed species. In one scenario (low risk), the observed plasma BChE inhibition is not associated with the comparable brain AChE inhibition, as BChE activity was elevated in white sturgeon exposed to ≥ 100 carbaryl, not inhibited. In the second scenario (higher risk), release of BChE from the liver is masking BChE inhibition that is associated with comparable brain AChE inhibition, in this case, 37%, as well as potential behavioral effects. As a result, one should consider the level of carbaryl exposure in green sturgeon in Willapa Bay to be a conservative estimate.

To better understand the effects of carbaryl on ChE activity in sturgeon, the risks associated with exposure, and the role of BChE as a biomarker for exposure, additional studies are needed. A dose-response test with SW-acclimated white sturgeon using the same concentrations (30-3,000 µg L^{-1}) could be conducted, with fish sampled at frequent intervals within the 6-h exposure to determine whether BChE inhibition is occurring rapidly (before the plasma enzyme is released from the liver), as well as the onset of AChE inhibition. Water samples could also be collected at the various time points and analyzed for both carbaryl and 1-naphthol in order to better understand the production and accumulation of the metabolite over the course of the 6-h exposure. A comparable dose-response test using much lower concentrations would be helpful in identifying the concentration threshold for hepatotoxicity and the release of BChE. In addition to brain and plasma samples, analysis of liver tissue for BChE activity would confirm the source of the elevated plasma BChE activity when fish are exposed to high concentrations of carbaryl. If possible, the use of water exchanges (static-renewal or flow-through) instead of a 6-h static exposure would reduce the potential for the accumulation of 1-naphthol in test tanks as it is unlikely that such exposure would occur in the field.

At present, plasma BChE activity appears to be a conservative biomarker for exposure of sturgeon to carbaryl when inhibition is detected, as in the case of green sturgeon in Willapa Bay following carbaryl applications to control burrowing shrimp. Although it is unlikely that the level of BChE inhibition detected is life-threatening, as sturgeon exposed to 3,000 $\mu g\ L^{-1}$ carbaryl for 6 h did not exhibit overt effects, but brain AChE was inhibited 42%, the relationship between plasma BChE inhibition and brain AChE activity is unclear. Further studies are needed to better understand the magnitude and effects of carbamate exposure on threatened and non-threatened sturgeon in Willapa Bay, and other western US surface waters, including green sturgeon spawning grounds.

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Appendix A

Characterization of white and green sturgeon plasma ChEs, including determination of appropriate plasma dilution factor and substrate and iso-OMPA concentrations, was conducted on plasma samples from non-study individuals. Concurrent analysis of both horse serum and sturgeon plasma allowed comparison with ChEs of known properties. The optimal plasma dilution was selected from the linear range of the enzyme dilution curve, and was 32-fold for horse serum, and 16-fold for MMS white sturgeon plasma. Iso-OMPA inhibition of BChE was determined after 5 min incubation over a range of iso-OMPA concentrations from 10^{-10} to10^{-3} M. The optimal concentration selected was that at which all BChE was inhibited, but where AChE activity remained constant. In horse serum, this plateau occurred from 10^{-4} to 10^{-3} M (Fig. A-1), so an iso-OMPA concentration of 10^{-3} M was selected for use. In white sturgeon plasma, ChE activity decreased throughout the range of concentrations used without reaching a noticeable plateau (Fig. A-2). Activities at 10^{-3} M were routinely 2-6% of total control activity. Sturgeon plasma was incubated with additional higher concentrations of Iso-OMPA to see if a plateau was visible (Fig. A-3). There still was no obvious plateau, and activity at 10^{-3} M was low, approaching zero. Sturgeon plasma was then incubated for 30 min (rather than the normal 5 min) at the higher concentrations (Fig. A-4), and plasma activity reached zero linearly with no plateau, indicating an absence of AChE and that BChE was the form of ChE in sturgeon plasma. Moving forward, plasma ChE was measured and reported as BChE.

In some of the iso-OMPA runs, there was a gradual decrease in activity (ca. 10%) from 10^{-10} M to 10^{-6} M. Iso-OMPA inhibition of plasma CaE activity was measured in an attempt to explain the decrease, but there was no decrease in CaE activity from 10^{-10} to 10^{-6} M to help explain the ChE decrease (Fig. A-5). Although CaE did not explain this trend, the gradual decrease in ChE activity was not seen in all iso-OMPA titration runs, and would not change the conclusion of total ChE being reported as BChE, so no further attempts to explain the trend were pursued.

Substrate affinity was determined by measuring AChE and BChE activities over a range of AThChI concentrations from 10^{-6} to 10^{-2} M, with peak activity in a bell-shaped AChE curve being the appropriate assay substrate concentration (Krupka 1963). In horse serum, this was found to be 10^{-3} M, but in white sturgeon plasma, without the presence of measurable AChE, there was no peak AChE activity. A substrate concentration of 10^{-3} M consistent with standardized ChE analysis in other fish species (Zinkl et al. 1991) was selected for use in the white sturgeon assays.

Cholinesterase inhibition by carbamates may be influenced by temperature, so to avoid reactivation, reagents and enzyme sources were kept on ice until the assays were performed. To verify that no reactivation was occurring within the time it took to analyze a plate, carbaryl-exposed samples were left to incubate for 5 (average time from substrate addition to end of analysis), 10, 20 and 30 min and then analyzed. There was no noticeable increase in inhibition, or increase in activity over the course of the 30 min, indicating there would be no reactivation occurring in the time it took to analyze a plate.

White sturgeon brain homogenate was incubated for 10 min with a range of Iso-OMPA concentrations (10^{-3} M to 10^{-10} M), to determine ChE composition of the tissue. No inhibition was seen, verifying brain ChE to be AChE (Fig. A-6).

A brief characterization was performed with control plasma samples collected from wild sturgeon from Willapa Bay (green) and the Columbia River (white). The only difference between assay parameters for the wild sturgeon and MMS plasma was the optimal enzyme dilution, which was 8-fold instead of 16-fold, respectively. Total plasma ChE was reported as BChE in wild white and green sturgeon, and a substrate concentration of 10^{-3} M was utilized.

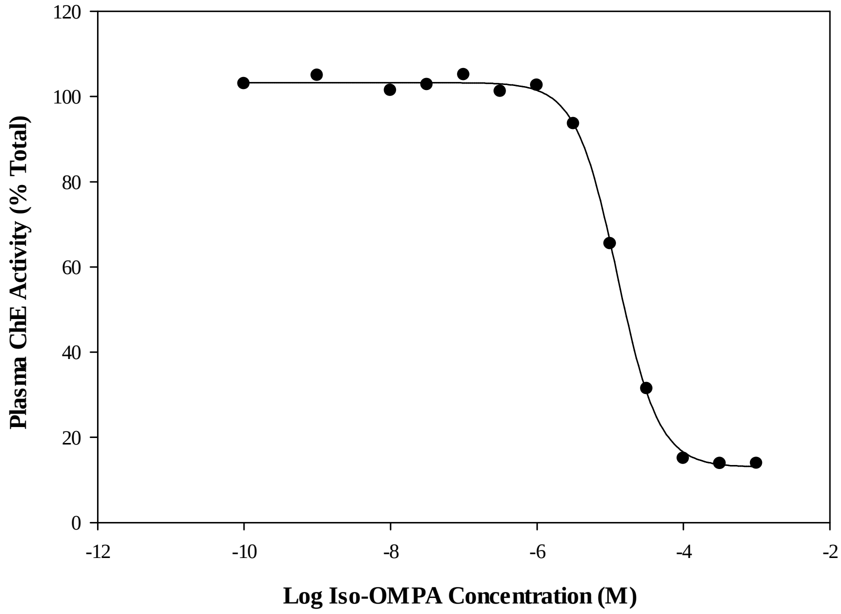

Figure A-1: Iso-OMPA titration in horse serum ChE. IC_{50} = 12.5 µM.

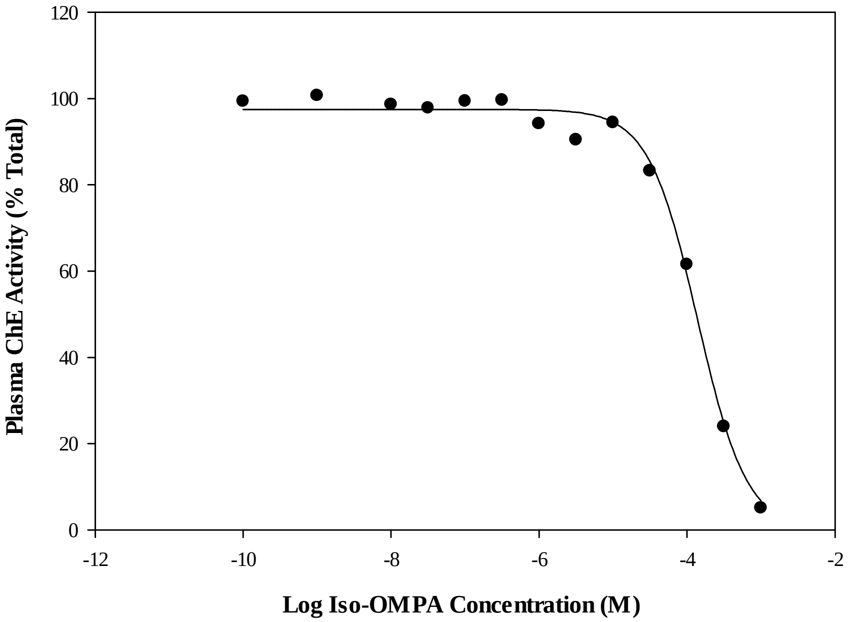

Figure A-2: Iso-OMPA titration in white sturgeon plasma ChE. Average IC_{50} = 143 ± 29 µM.

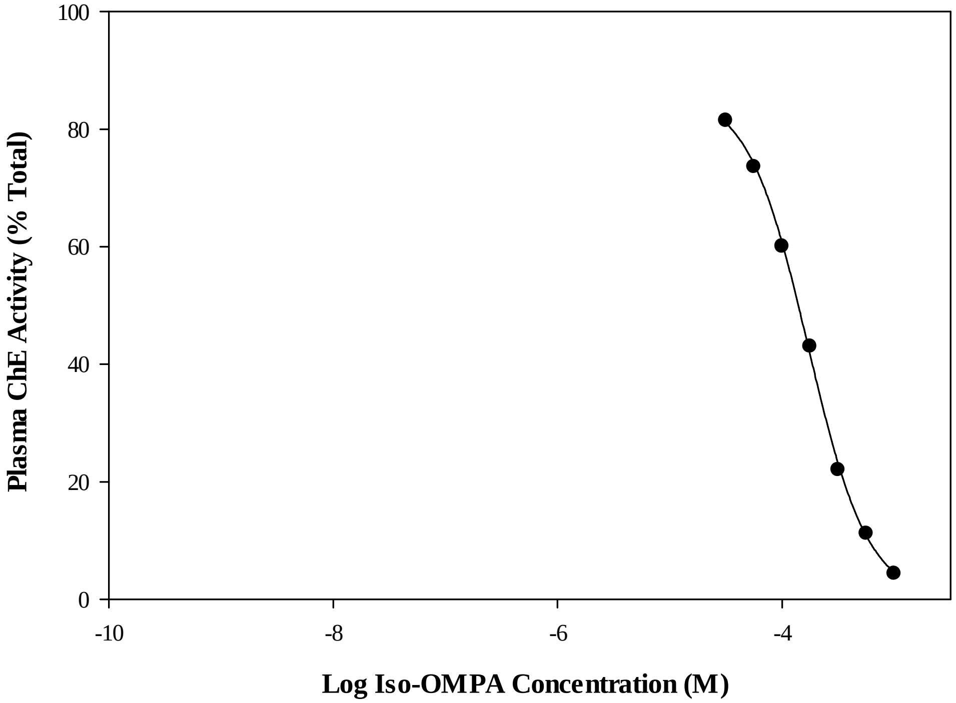

Figure A-3: Iso-OMPA titration in white sturgeon plasma ChE with increased high concentration incubations

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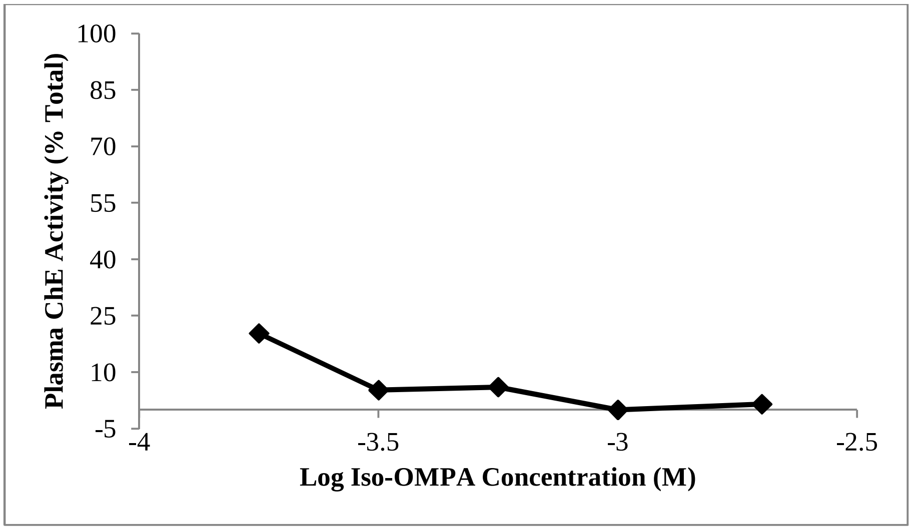

Figure A-4: High-concentration iso-OMPA titration with 30 min incubation in white sturgeon plasma ChE.

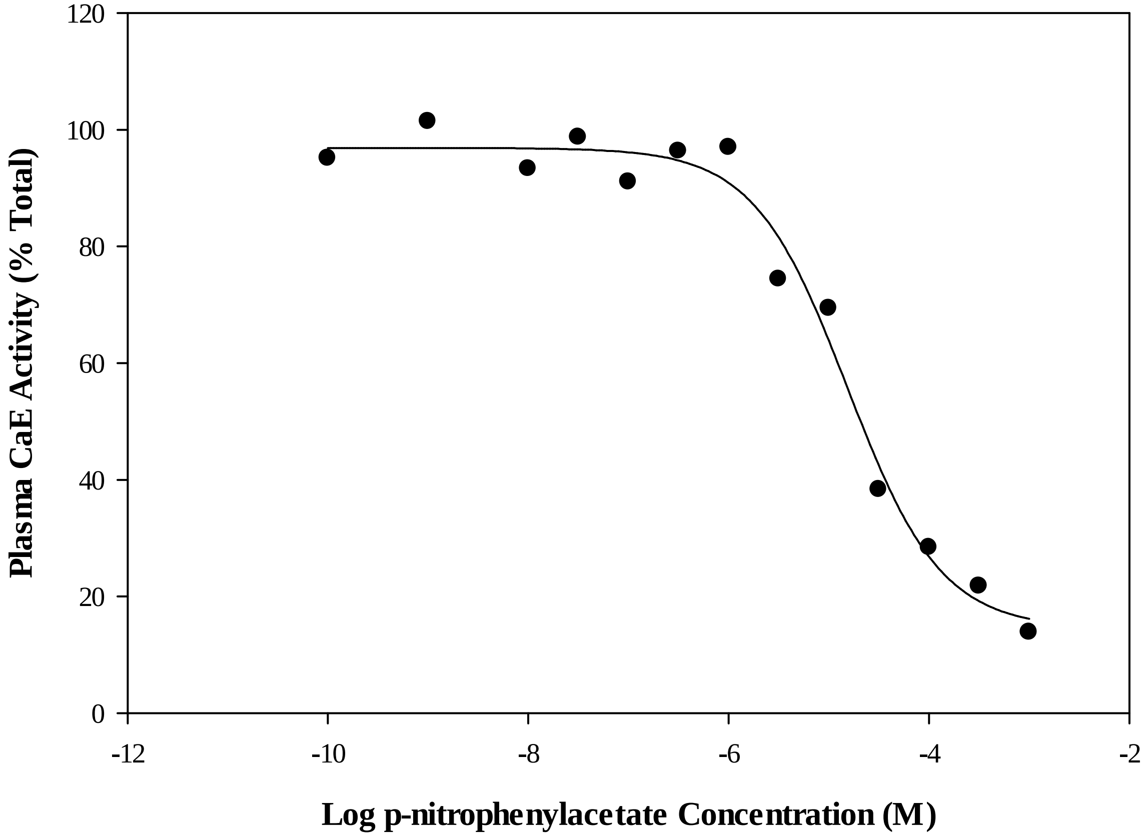

Figure A-5: Iso-OMPA titration in white sturgeon plasma CaE

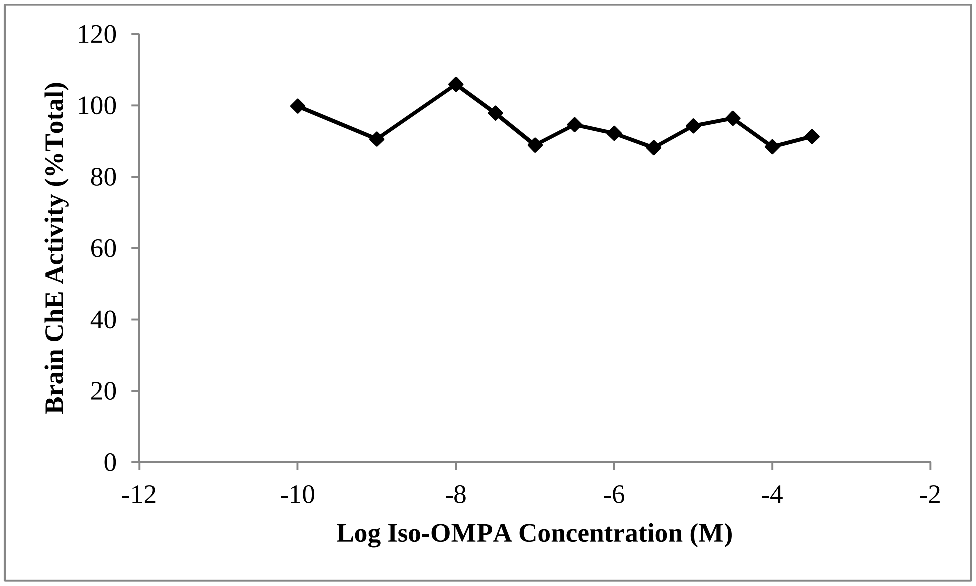

Figure A-6: Iso-OMPA titration in white sturgeon brain ChE. No presence of BChE indicated.