# The Role of Carboxylesterase in Organophosphorous Pesticide Toxicity at the Olfactory Epithelium of Coho Salmon (*Oncorhynchus kisutch*)

by

**Andrew Jeyin Wan**
B.Sc. (Agr) University of British Columbia, 1999

PROJECT SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF ENVIRONMENTAL TOXICOLOGY
in the
Department of Biological Sciences


SIMON FRASER UNIVERSITY
April, 2004

APPROVAL

Name: Andrew Jeyin Wan

Degree: Master of Environmental Toxicology

Title of Project:

The role of carboxylesterase in organophosphorous pesticide toxicity at the olfactory epithelium of Coho salmon (*Oncorhynchus kisutch)*

Examining Committee:

Chair: Dr. I. Novales Flamarique

Dr. C.J. Kennedy, Associate Professor/Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. R.A. Nicholson, Associate Professor
Department of Biological Sciences, S.F.U.

Dr. F.C.P. Law, Professor
Department of Biological Sciences, S.F.U.
Public Examiner

April 13, 2004

Date Approved

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The role of carboxylesterase in organophosphorous pesticide toxicity at the olfactory epithelium of Coho salmon (*Oncorhynchus kisutch)*

**Author:**

(signature)

Andrew Jeyin Wan

(name)

April 15, 2004

(date)

# ABSTRACT

Salmon rely on olfactory cues for the detection of prey, synchronization of sexual maturation, migration, and predation. Reliance on olfactory cues makes them particularly vulnerable to olfactory toxicants. Organophosphosphorous (OP) pesticides are known to inhibit olfaction and consequently, olfactory-mediated behaviours.

Carboxyesterase enzymes (CEs) hydrolyze many compounds including OPs. Because the primary olfactory tissue, the olfactory epithelium, is directly exposed to aquatic olfactory toxicants, CEs may be important in the biotransformation of OPs and may play a role in modulating their olfactory toxicity. Therefore, objectives of this study were to determine if CE: 1) is present in the salmon olfactory epithelium, 2) activity in the salmon olfactory epithelium can be modulated through *in vivo* exposure of fish to xenobiotics, and 3) activity modulation in the olfactory epithelium alters the olfactory toxicity of an OP.

CE was histochemically localized to the olfactory sensory epithelium. Distribution of CE in olfactory tissues was similar to that of mammals. No activity was present in the non-sensory epithelium of the primary or secondary lamellae. It was possible, for the first time, to measure the activity of carboxylesterase in the telost olfactory epithelium. Induction and inhibition of carboxylesterase activity in the olfactory epithelia was 174 and 40% of control CE activity respectively after *in vivo* exposure of fish to pyridine and triphenylphosphate. On average, liver CE activity (99.3 nmol/min/mg protein) was 390% higher than rosette CE activity (25.5 nmol/min/mg protein). To determine if altered CE

activity inhibited olfaction, coho salmon were first pre-exposed to pyridine or triphenylphosphate, then exposed to diazinon. Fish were finally exposed to an olfactory-based alarm substance, which is known to induce a stress response in fish. Plasma cortisol concentration, an indicator of stress in organisms, was similar in all exposure and control groups.

Results from this study demonstrate that an interaction between xenobiotics and olfactory epithelium CE does take place. Whether or not this interaction is relevant in the scope of salmon survival is still unclear as results in the behavioural portion of this study were inconclusive. Further studies that focus on how xenobiotic-carboxylesterase-olfactory toxicant interactions affect salmon behaviour are necessary.

# ACKNOWLEDGMENTS

I would like to first and foremost acknowledge my gratitude to Dr. Chris Kennedy of Simon Fraser University for accepting me as his graduate student, thus providing me with the opportunity to learn about environmental toxicology. My gratitude extends also to the members of my committee Dr. Russell Nicholson and Dr. Francis Law for their valuable insight and encouragement to pursue graduate studies. A very deep and profound appreciation is extended to Dr. Hugh Jarrard. His amazing insight and knowledge, patience, direction, encouragement and understanding were greatly appreciated throughout the course of this project. I would also like to thank the managers and employees at the Capilano Fish Hatchery in North Vancouver for providing me with the juvenile Coho Salmon used in the experiments.

A special note of appreciation is extended to my parents Dr. Michael and Mary Wan, as well as my siblings Esther Wan and Ethel Wan-Sharp for supporting me. I however, reserve my deepest gratitude and appreciation to Angela Yeasting for always being there for me and encouraging me through it all.

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## General Introduction

*Overview*

The purpose of this project was to explore the potential role of carboxylesterases (CEs) in the sublethal effects of the organophosphorous (OP) pesticide, diazinon, particularly on the behavioural capacity of the salmon olfactory system. Because of the reliance of salmon on olfactory cues for a variety of behaviours such as the detection of prey, the synchronization of sexual maturation between sexes, migration into natal streams etc., it is important to determine the effects of xenobiotics on salmon olfactory ability. CE is a hydrolytic enzyme common to many organisms and has the ability to hydrolyze a wide variety of structurally diverse compounds including OPs. There is a substantial amount of literature available regarding the biotransformation of xenobiotics by CEs, however, very few studies have examined the relevance of this enzyme with respect to the detoxification of olfactory toxicants. The complexity of enzyme-xenobiotic interactions and their sublethal consequences is an area of rapid growth in toxicology.

*Importance of salmon to British Columbia and challenges for survival*

Salmon are important to humans and the environment in that they provide opportunities for sport and commercial uses, and have sustained aboriginal peoples for centuries. For example, 80, 000 metric tons of salmon were harvested in BC with a landed value of nearly $170 million (BC Ministry of the

Environment, Land and Parks, 1993). Clearly, salmon harvesting from commercial fisheries is an important contributor to the economy. From an environmental standpoint, the survival of other animal species depends on the survival of salmon. Mature salmon do far more than produce another generation; arriving in autumn to spawn, they provide sustenance to many animals hence allowing them to prepare for and survive the harshness of winter. Top predatory species such as bears, eagles, mink, river otters, seals and sea lions are particularly dependant on salmon as a food source (Washington Department of Fish and Wildlife, 1999).

Salmon, unfortunately, are facing threats to their survival from many different sources, most of which are anthropogenic in origin. Salmon have been impacted by widespread devastation of old growth forests, overgrazing, excessive water diversions, dams, and chemical pollution (EPA, 2003).

Although trend data in BC for representative salmon stocks between 1976 and 1990 indicate that 76% of BC salmon stocks are considered stable or increasing, worrisome is the fact that such stocks are increasingly reliant on artificial salmon enhancement efforts such as hatcheries (BC Ministry of Environment, Lands and Parks, 1993). This implies that the majority of wild stocks may either be stable or declining.

Excess nutrients such as nitrogen and potassium originating from sewage treatment plants, stormwater runoff from lawns and agricultural lands, faulty or leaking septic systems, sediment in runoff, animal wastes, atmospheric deposition and groundwater discharges may contribute to fish diseases,

increases in algae and low dissolved oxygen concentrations which have been known to cause fish kills (EPA, 2003).

The destruction of habitat by human activities also plays a considerable role in the survival of fish (Solomon et al., 1993). Humans have reclaimed habitat for many purposes but especially relevant to fish, aquatic habitats have been converted to terrestrial habitats (land reclamation) where water bodies are drained and built upon. Habitat destruction reduces an organism's biological range and ability to survive. Destruction of fish habitat from stream blockage, dams and other impairments poses risks to salmon populations (BC Ministry of the Environment, Land and Parks, 1993). For example, a survey of selected forest cut blocks on Vancouver Island found that logging has had some adverse effect on 34% of the 53 surveyed streams. These impacts were found in streams with the highest potential to support salmon species (Tripp et al., 1992).

Overexploitation of fish stocks occurs when commercial, sport or native fisheries catch an excessive number of individuals such that the ability of the species to maintain its population levels is compromised. This issue is especially relevant in Eastern Canada where overfishing has been blamed for the extremely low population of turbot (Myers et al., 1996; Nakken, 1998). Overexploitation of salmon in other jurisdictions such as the Baltic Sea however, has reduced local stocks below acceptable levels, with increased risk of genetic deterioration and considerable economic losses (Sers, 1996).

Finally, toxic substances including metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), heavy metals, and

pesticides can play considerable roles in threatening the survival of fish (Servizi, 1989).

*Impacts of pollutants and pesticides on salmon*

Tributaries that flow into BC rivers, are the birthplaces of several economically important species of salmon. Unfortunately, the environmental concerns described previously have compromised the ability of salmon to survive in recent years. The Fraser River flows though the Greater Vancouver Regional District (a heavily populated area), and the Lower Fraser Valley where much of the province's agricultural community resides. These two factors weigh heavily on the water quality of the Fraser River as effluents enter via ditches, waterway storm-drains, industrial discharges and runoff from lawns, streets and farmlands; sewage treatment plants municipal sewage and surface runoff, and agriculture discharge (BC Ministry of Environment, Lands and Parks, 1993). These effluents can contain toxic substances including metals, PAHs, PCBs, and frequently, pesticides. For example, the organophosphorus pesticides fensulfothion, parathion, diazinon and dimethoate are frequently detected in farm ditch water at concentrations between 0.04 and 1.08 µg/L (Wan et al., 1994).

The problem of pesticide exposure to salmonids and other fish is not endemic to BC. Exposure can occur in any stream or river near industrial or agricultural activity. The US Geological survey detected 49 different pesticides in the surface waters of California's Central Valley (Dubrovsky et al., 1998), 50 in Oregon's Willamette Basin (Wentz et al., 1998) and 23 in urban streams in the Puget Sound Basin (Wentz et al., 1998). OPs and carbamates frequently

contaminate western rivers; they accounted for 12 of 19 pesticides detected in the surface waters of the California Central Valley (Dubrovsky et al., 1998).

Discharge into local rivers and streams typically occur via storm water runoff and ground water discharge with rain events producing pesticide pulses in rivers and streams (Domagalski, 1996). Such pulses are well defined, lasting anywhere from a few days to weeks (Kuivila & Foe, 1995). For example, in-stream concentrations of diazinon have been measured as high as 36.8 µg/L in the San Joaquin river system (Menconi & Cox, 1994), however, they are commonly less than 10 µg/L. Although a typical pulse of diazinon lasting for a few days at 10 µg/L is not likely to kill salmon outright, sublethal effects may be evident in concentrations as low as 1 µg/L (Moore & Waring, 1996).

*Overview of pesticides and their uses*

The term pesticide is all encompassing, as defined by federal legislation, to include any material used to control, prevent, destroy, repel, attract or reduce pest organisms (Pest Control Products Act, 2002). Insecticides, herbicides, fungicides, rodenticides, insect growth regulators, plant defoliants, and surface, as well as swimming pool disinfectants, are all considered pesticides. Pesticides are available in a multitude of mixtures and formulations depending on their particular use. Much of the success in controlling pests and associated diseases has been attributed to the use of chemical pesticides.

There are several important benefits in using pesticides. The prevention of malarial pandemics, stemming from the use of DDT, and other insect-transmitted diseases has been attributed to the use of pesticides (Isman, 1997). Pesticides

help lower the cost of food production significantly while at the same time, increasing food supplies, as over half of the world's potential human food supply is lost to pests before or after harvest (Isman, 1997). Without pesticides, these losses would be more extreme. Pesticides are more effective than alternative methods and are the only viable option at the economic threshold, that is, the pest density at which management intervention, typically through the application of chemical pesticides, must be taken to prevent the pest from causing a measurable economic impact on a farmer's income (Gullan & Cranston, 1996). Below this threshold, crop losses will have a minimal impact on income. As such, pesticides have the ability to control most pests quickly and at a reasonable cost. In some countries, a greater and more dependable production of food has eliminated famine and thus pesticides contribute as much to health as to the economy. Arguably, the health risks of pesticides are insignificant compared with their benefits (Ames & Gold, 1997). It has been estimated that 99.99% of the pesticides we consume in our food are natural chemicals produced by plants (Ames & Gold, 1997).

There are also costs associated with pesticide use (Isman, 1997). Pesticides may be a temporary solution because insects have the ability to develop genetic resistance against them. It is possible that insects, especially those that breed very rapidly, can become resistant to pesticides through natural selection within a short period of time (McKenzie, 1996). The use of broad-spectrum insecticides can also eliminate not only the pest, but also the pest predators, leading to secondary pest outbreaks. The "Pesticide Treadmill" is a situation where farmer's costs escalate for a pesticide-based pest control

program that often becomes less effective as genetic resistance develops and applications of stronger pesticides are required (McKinney & Schoch, 2004). Finally, depending on the formulation and chemistry, pesticide use may threaten countless non-target aquatic and terrestrial organisms through drift, granule consumption, and groundwater contamination. The contamination of ground water is especially problematic as drinking water supplies and animal species residing in streams to which ground water flows will be exposed to pesticides and their transformation products (Rand, 1995).

*Uses, production and environmental levels of pesticide actives*

A 1999 pesticide survey, conducted as part of the Georgia Basin Ecosystem Initiative (GBEI) by ENKON Environmental Limited (2001) for the British Columbia Ministry of Environment, Lands and Parks and Environment Canada, included a survey of pest control services in the Lower Mainland that were licensed to apply pesticides for the purposes of landscaping and agriculture. Landscape services in the Lower Mainland used 91 different active ingredients, of which 10 accounted for 87% of the pesticides applied. Lower Mainland agriculture services applied 86,565 kg of 101 different active ingredients during 1999 with 66% of the pesticides applied accounted for by two fumigants based on the active ingredients, metam-sodium and methyl bromide. One of the more frequently used active ingredients was diazinon (7%). Based on this survey, the organophosphorous pesticide diazinon has the potential of being an environmentally relevant pollutant to fish including salmonids.

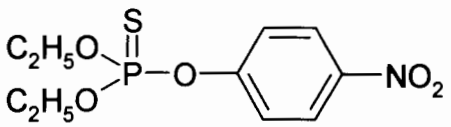
*Organophosphorous pesticides*

Organophosphorous (OPs) pesticides are a group of chemical compounds used throughout the world. In Canada, OPs are registered as an insecticide in agriculture, horticulture, greenhouses, turf and domestic uses (Pest Management Regulatory Agency, 2004). They are also used as additives in plastics and petroleum products (Klaasen, 1996). As pesticides, OPs are heavily used in BC (see previous section). With exceptions, OPs are less persistent than organochlorine pesticides and generally do not accumulate in body fat, however, they tend to be acutely toxic to many organisms (Tomlin, 1995). The structures of several commonly used OP's are shown in Figure 1.1.

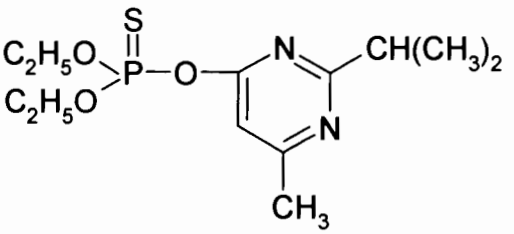
The technical-grade active ingredient diazinon (Figure 1.1), an OP insecticide, is registered in Canada (Pest Control Product - PCP #22584) and is available in a wide range of formulations including dusts, granules, seed dressings, wettable powders, and emulsifiable concentrates (PMRA, 2004). It is a contact poison and has moderate residual activity. Registered uses of diazinon include commercial terrestrial fruit and vegetable sprays, as well as seed treatments (PMRA, 2004). The use of diazinon is intended for the control of a very wide variety of flies, home and garden pests, turf insects and commercial agricultural insect pests (PMRA, 2004).

*Acute toxicity and mechanisms of OPs*

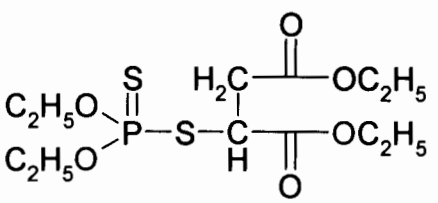
Insecticides such as parathion, azinphos-methyl and diazinon are bioactivated to highly toxic oxon forms by the cytochrome P450 systems in



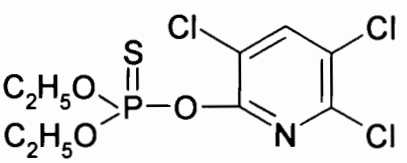
Parathion



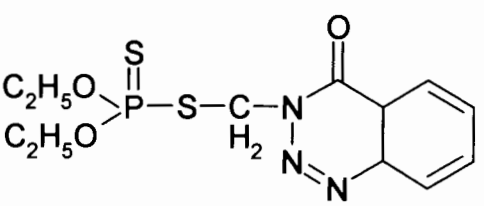
Diazinon



Malathion



Chlorpyrifos



Azinphosmethyl

Figure 1.1. Chemical structures of commonly used organophosphorous pesticides registered in Canada

endotherms and ecotherms (Woods, 1999). The oxons are potent inhibitors of acetylcholinesterase (AChE), an enzyme responsible for hydrolyzing acetylcholine, a highly conserved neurotransmitter in many animal species (Woods, 1999). This inhibition results in excessive agonism of synaptic acetylcholine receptors in the autonomic and central nervous systems, because of the inactivation (phosphorylation) of this enzyme (Gallow & Lawryk, 1991). AChE has a serine-OH amino acid at its esteratic site, a characteristic it shares with some CEs and proteases. The multiplicity of binding sites (those containing a serine-OH amino acid) gives rise to a wide range of possible sites and modes of actions besides AChE inhibition. In fact, OPs phosphorylate a number of other enzymes, inhibiting their action. Some of these include butyl CEs (pseudo cholinesterase), lipase, phosphatase, trypsin, dehydrogenase, glycogenphosphorylase, neurotoxic esterase (NTE), and sulfydryl enzymes (Gallo & Lawryk, 1991).

The electrophilic phosphorus atom in OPs, which has a partial positive charge, undergoes nucleophilic attack by the serine-hydroxy groups of some enzymes resulting in enzyme phosphorylation. It is thought that a partial negative charge on the oxygen atom of the serine-hydroxy group (conferred upon it by an adjacent histidine side chain) nucleophilically attacks the partial positive charge on the phosphorus atom (Eto, 1974). The reactivation of the phosphorylated enzyme is very slow and may take days to weeks. Ageing is a process where the OP is itself dealkylated while having phosphorylated the enzyme. This process strengthens the bond between the OP and the serine site making it very stable – essentially an irreversible complex.

The result of AChE inhibition is the accumulation of acetylcholine at receptors in the brain and spinal cord, at neuromuscular junctions, at ganglia of the autonomic nervous system and at parasympathetic (muscarinic) nerve endings (Gallow & Lawryk, 1991). The resulting excess cholinergic drive and continued high level of receptor activation leads to the characteristic symptoms and signs of acute OP toxicity. In humans, symptoms include salivation, lacrimation, urination, defecation, gastroenteritis, and emesis (Woods, 1999). In insects, overexcitation of the nerve endings leads to paralysis and eventually death (BC Environment, 1995). OP exposure does not always result in AChE inhibition. Moribund fish exposed to 20 ug/L of parathion showed 50% AChE inhibition, whereas those exposed to 750 ug/L showed only 25% AChE inhibition (Murty, 1986). In fact, there have been confirmed cases of human deaths from OP exposure without significant depression of AChE levels (Gallow & Lawryk, 1991).

The $LD_{50}$'s of diazinon to terrestrial and aquatic species are shown in Tables 1.1 and 1.2. Clearly, diazinon's toxicity extends to many species. In general, aquatic invertebrates are the most acutely susceptible to diazinon, followed by fish, birds, and mammals.

*Sublethal toxicity of OPs*

Chronic and sublethal responses may be longer term and more difficult to quantify than acute effects. OP-induced-delayed-neuropathy is a very well studied sublethal consequence of OP exposure (OPIDN). OPs, besides being known as acute inhibitors of AChE activity, may also have a chronic component

in their ability to cause in birds and mammals, which is the primary sublethal effect caused by OP exposure. OPIDN is different from the acute effectsy caused by most AChE-inhibiting OPs, in that OPIDN causes a substantial inhibition of a secondary target, neuropathy target esterase (NTE) (Johnson, 1982, 1990). Inhibition thought to cause a pattern of axonal and terminal degeneration in the central and peripheral nervous systems (Cavanagh, 1964; Cavanagh & Patangia, 1965; Tanaka & Bursian, 1989; Tanaka et al., 1990). Degeneration of the distal ends of longer axons of some distal and spinal nerves as a result of OP exposure is followed by myelin breakdown, Schwann cell proliferation and macrophage accumulation (Steenland et al., 1994).

The mechanism is believed to be associated with the phosphorylation and ageing of a NTE, within neurons by processes analogous to those described for acetylcholinesterase (Ames et al., 1995; Stephens et al., 1995).

In fish tissue, OPs have been known to cause a number of histological changes. Such changes include detachment, necrosis, hypoplasia and haemorrhage of primary gill lamellae (Cengiz & Unlu, 2003; Edith, 2003), swelling, bile stagnation, necrosis, atrophy and vacuolization of the liver (Arnold et al., 1996; de Lara Rodrigues, 2001; Edith, 2003).

Other sublethal effects in fish include a decline in liver protein content (Begum & Vijayaraghavan, 1995; Singh, 1998), changes in muscle glycogen, lactate, lactate dehydrogenase and glycogen phosphorylase (Begum, 1999), and general follicular degeneration in ovaries of malathion-exposed catfish (Dutta et al., 1994). Reduced weight gain, growth rate and food conversion efficiency (Davies et al., 1994; Pathiratne, 1999; Ramani et al., 2002), lowered

carbohydrate metabolism (Begum and Vijayaraghavan, 1995), decrease in swimming performance (Heath et al., 1993; Beauvais et al., 2000; Islam et al., 2001), lowered oxygen consumption (Pathiratne & Ranasinghe, 1992), and aggressive behaviour (Islam et al., 2001) have also been observed. The studies above indicate that OPs cause several whole-body changes in the physiology of fish and other animals.

*Salmonid olfaction*

The importance of the olfactory system to salmonids cannot be overstated. Living in a medium that can limit visual information, the aquatic environment readily exposes the olfactory organ to a variety of chemicals that relay critical information about the environment to the fish (Stacey et al., 2003). Salmonids, including coho salmon, depend on their sense of smell for a variety of purposes including feeding, defense, schooling, migration and reproduction (Kleerekoper, 1969).

The ability of salmonids to navigate to their home streams is renowned. During smoltification, the process by which salmon prepare for their sea run migration, juveniles imprint on the unique chemical composition of the water at their natal spawning ground (the home-stream olfactory bouquet or "HSOB") (Carruth, 2002). The four major aquatic odourants constituting HSOB that fish recognize in their natal streams are: amino acids, bile salts, steroid hormones, and prostaglandins (Hara, 1993 a,b). The olfactory system is able to discriminate between very low concentrations of these odours; particulary amino acids (down to $10^{-6}$ to $10^{-7}$ M; Hara, 1992a, b), and bile salts ($10^{-8}$ to $10^{-10}$; Doving et al., 1980;

| Aquatic Organisms | | | |
|---|---|---|---|
| **Species** | **Duration (h)** | **Average $LC_{50}$ (mg/L) (range in other studies)** | **Reference** |
| Snail *(Gillia altilis)* | 96 | 11 | Robertson & Mazzella (1989) |
| Cladoceran (*Daphnia pulex*) | 48 | 0.001 (0.0006-0.0011) | Mayer & Ellersieck (1986) |
| Shrimp *(Hyallela azteca)* | 96 | 0.004-0.006 | Collyard et al. (1994) |
| Insect larva (*Pteronarcys californica*) | 96 | 0.025 (0.020-0.030) | Mayer & Ellersieck (1986) |
| Blugill sunfish (*Lepomis macrocharis* | 96 | 0.17 (0.012-0.22) | Mayer & Ellersieck (1986) |
| Rainbow trout (*Oncorynchus mykiss*) | 96 | 0.09 | Mayer & Ellersieck (1986) |
| Cutthroat trout (*Salmo clarkii*) | 96 | 2.76 (2.28-3.33) | Mayer & Ellersieck (1986) |
| Walking catfish (*Clarias batrachus*) | 96 | 2.41 | Tripathi (1992) |
| Eel (*Anguilla anguilla*) | 96 | 0.08 (0.06-0.10) | Ferrando et al. (1991) |
| Channel catfish (*Channa punctatus*) | 96 | 3.1 | Sastry & Mallik (1982a) |
| Goby (*Gobius*) spp. | 96 | 0.25 (0.23-0.28) | Ebere & Akintonwa (1992) |
| Lake trout (*Salvelinus namaycush*) | 96 | 0.6 (0.4-0.9) | Mayer & Ellersieck (1986) |

| Terrestrial organisms | | | |
|---|---|---|---|
| **Species** | **Route of Administration** | **$LD_{50}$ (mg/kg)** | **Reference** |
| Mallard duck (*Anas platyrhynchus*) | oral single dose, 24h | 4.3 | Sachsse & Ullmann (1975b) |
| Bobwhite quail (*Colinus virginianus*) | oral single dose, 24h | 85 | Fletcher & Pedersen (1988b) |
| Rat | oral single dose, 24h | 300 | Piccirillo (1978) |
| Rat | oral single dose, 24h | 422 | Bathe & Gfeller (1980) |

| | | | |
|---|---|---|---|
| Rat | oral<br>single dose,<br>24h | 1012 | Schoch & Gfeller (1985) |
| Guinea pig | oral<br>single dose,<br>24h | 320 | Gasser (1953) |
| Rabbit | oral<br>single dose,<br>24h | 130 | Gasser (1953) |

Table 1.1. Acute toxicity of diazinon to several aquatic and terrestrial species.

Hara, 1992a, b). Evidence suggest that combinations of odourants in natal streams are responsible for providing a "fingerprint" on which salmon may imprint (Hasler & Scholtz, 1983; Hara, 1992a, b).

During the first few months in the life of a salmon, not only does olfaction play a role in stream imprinting, it is also important for the ability of salmon to feed in streams and in the ocean. The olfactory sensitivity of salmon can best be demonstrated by the preference of salmon fry for daphnids of high nutritional value based on the amount of free amino acids (FAA's) leaking from the daphnids (Holm & Walther, 1988). This ability gives the salmon fry a nutritional profile of their prey before predation ensues. This ability is advantageous to the salmon fry to economize its efforts at prey capture as well as prey digestion (Holm & Walther, 1988).

Salmonids, along with other fish, avoid predators primarily through visual stimulation, and stimulation of their lateral-line sensing system. There is evidence however, that fish also use chemical alarm signaling systems (Brown & Smith, 1997, 1998). In fact, chemical alarm signaling systems can be divided into two categories based on whether the signals are emitted before (disturbance signals) or after the predator captures the prey (damage-released signals) (Chivers & Smith, 1998). Disturbance signals simply indicate that the sender was attacked, maimed or captured by the predator. The substance that constitutes the damage-released signal is called alarm substance (Smith, 1992; Scott et al., 2003). Sufficient skin damage from predatory attack causes the release of alarm substance from specialized epidermal cells in the fish skin (Renberg & Schreck, 1986). Hypoxanthine-3-N-oxide, pyridine-N-oxide, aspartic acid, arginine, L-

alanine, L-methionine, L-proline, and thaurocholic acid present in the epithelial club cells of fish skin are thought to be the principle constituents of alarm-substance (Brown et al., 2001; Karlsen et al., 2001; Brown et al., 2002; Brown et al., 2003).

Another critical aspect of salmon olfaction is its role during sexual maturation. It is known that prostaglandins (PGFs) are important in the process of ovulation in fish (Goetz et al., 1989). $PGF_{2a}$ has been identified as a potent odourant that has a role in synchronizing spawning physiology and behaviour between the two sexes (Moore & Scott, 1992; Sorenson, 1992). $PGF_{2a}$ has a priming effect on plasma steroid and gonadotropin levels in mature male salmon parr (Moore & Waring, 1996).

Noting the overall dependence of salmonids on their olfactory sense for the above-mentioned functions, this same system has the potential to make them particularly vulnerable to xenobiotics such as OPs.

*Structure and function of olfaction in fish*

Various approaches based on a broad range of anatomical, physiological, biochemical, and especially molecular studies have been used over the past three decades to increase the understanding of olfactory mechanisms in fish. It has recently become clear that the various approaches used are converging on a basic set of principles of functional organization that begin with the encoding of molecular information at the level of sensory transduction in olfactory receptor cells (ORCs) and can be followed through several stages of neural circuits to the level of higher-order processing (Hildebrand & Shepherd, 1997). It is interesting

to note the fact that many of these principles have emerged from studies of both invertebrates and vertebrates. Despite morphological differences between phyla, a common set of neural mechanisms appears to have evolved across phyla for detecting and discriminating among olfactory stimuli (Dethier,1990; Ache, 1991; Smith & Getz 1994). In the most basic of levels, the characteristic olfactory organs of vertebrates (sensory epithelia within a nasal chamber) and invertebrates (appendages such as antennae) are comprised of complex structures adapted to collect odor molecules and guide them to the ORCs, the structures which play major roles in setting the sensitivity of the olfactory system (Kaissling, 1990).

The olfactory system of fish is situated such that during swimming, water flows through the anterior afferent nostril and exits through the posterior efferent nostril. The nasal bridge, septum or flap that separates the afferent and efferent nostrils serves to deflect water, along with odourants, into the olfactory pit where water is forced up and over the surfaces of the lamellae. Water then exits the efferent nostril.

The basic structure of the teleost olfactory system is similar to other vertebrates (Byrd & Brunjes, 1995). The olfactory system of vertebrates is composed of a centre for chemoreception, the olfactory epithelium. In fish, the olfactory epithelium is organized into a semi-circular rosette. The olfactory rosette itself contains transverse primary lamellae, which radiate to the wall of the olfactory pit (Pfeiffer, 1963; Yamamoto, 1982) (Figure 1.2). These lamellae greatly increase the available surface area in contact with water-borne odourants. The rosette contains all the olfactory receptors.

Microscopy performed by Moran et al. (1992) (Figure 1.2) show that a single lamella from a brown trout olfactory rosette is itself a primary fold, and that this primary fold contains secondary folds. It is known that non-sensory epithelium is comprised mainly of ciliated epithelial cells (Moran et al., 1992), and large numbers of goblet cells (Saucier et al., 1999). Whereas non-sensory epithelium is located on the ridges of both primary and secondary lamellae, sensory epithelium is located in the troughs of these very same lamellae (Moran et al., 1992). Cell types associated with the sensory epithelium include ciliated epithelial cells, ciliated sensory neurons, ciliated olfactory receptor cells, microvillar olfactory receptor cells, basal cells and sustentacular cells (Figure 1.4).

Goblet cells located in the non-sensory epithelium region are responsible for mucous production, and are home to high activities of biotransformational enzymes, especially CYP1A1 (Monod et al., 1994; Saucier et al., 1999) compared to other cells in the non-sensory epithelium. Interestingly, a body of evidence further indicates that biotransformational enzymes located in the olfactory epithelium are involved in the actual process of olfaction as oxidation and glucuronidation of a number of odourants inhibited their ability to stimulate receptors (Lazard et al., 1991). Therefore, goblet cells in the olfactory epithelium of salmonids may play a multifunctional role in mucous production; production of xenobiotic biotransforming enzymes for the purpose of inhibiting olfaction, and perhaps the production of xenobiotic biotransforming enzymes for the purpose of detoxification.

As with most vertebrates, signals from the olfactory rosette travel along the

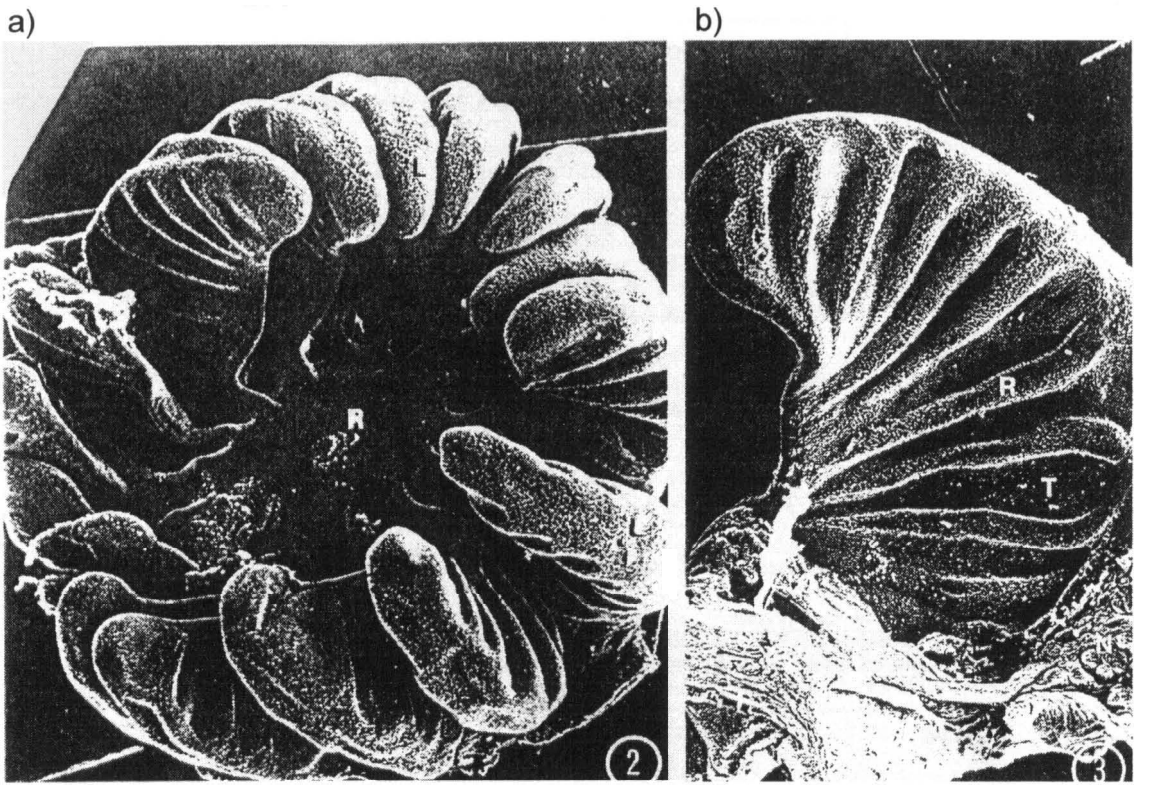



Figure 1.2. a) SEM of an olfactory rosette taken from the nasal cavity of a wild brown trout. Sixteen lamellae (L), which bear the olfactory epithelium, project from the midline raphe (R). (x50); b) SEM of a single lamella from a brown trout olfactory rosette shows that its surface is thrown into secondary folds. The ridges (R) are covered by non-sensory, "indifferent" epithelium; the troughs (T) between the ridges are lined with olfactory epithelium. From *Moran et al., 1992.*

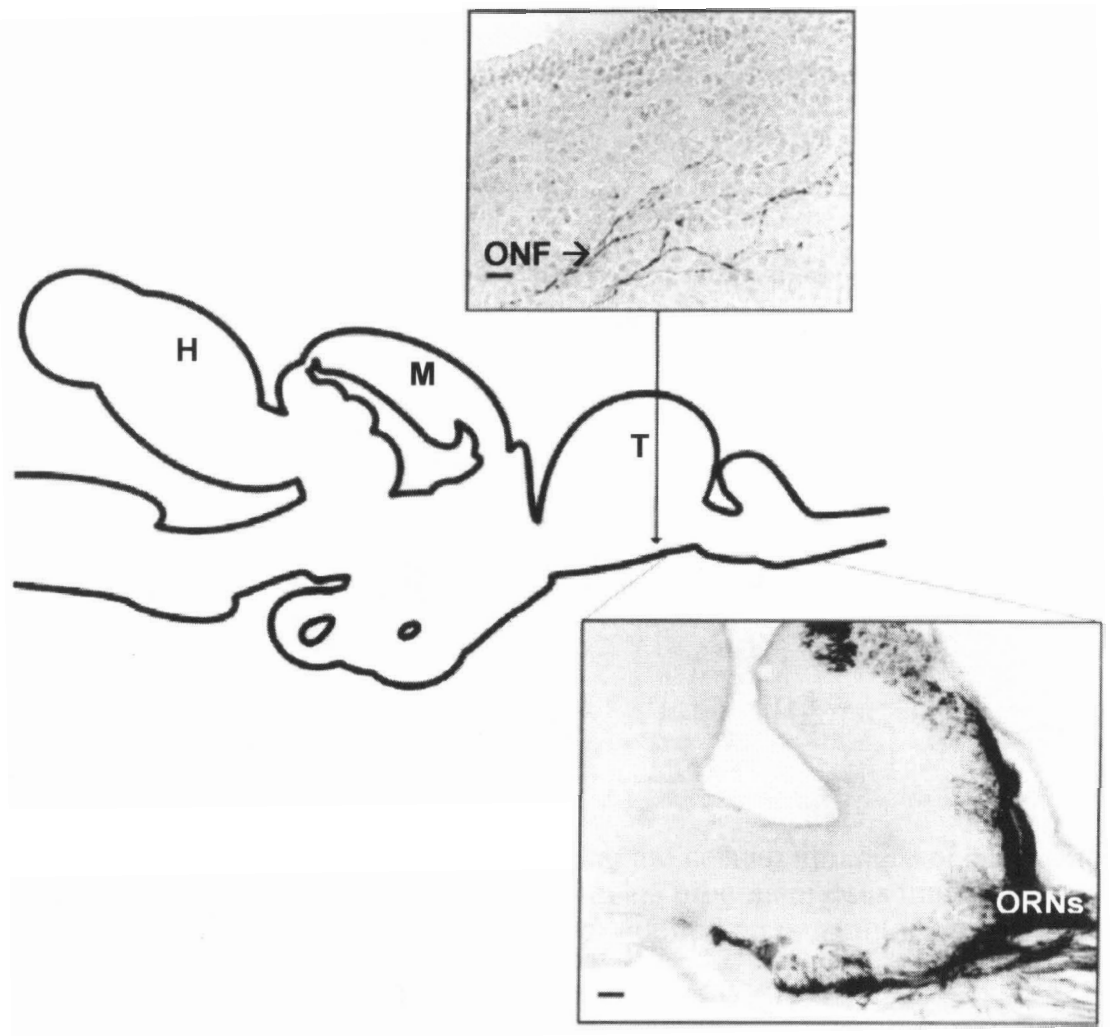



Figure 1.3. A parasagittal section of the olfactory bulb in rainbow trout (*Oncorhynchus mykiss*). The olfactory receptor neurons (ORN) in this diagram were labelled by the application of 5% biotinylated dextran-amine to the olfactory epithelium. The primary olfactory neuron fibres (ONF) are shown in more detail in the micrograph above. Scale bar is 25 µm in the box above and 100 µm in the box below. (T) Telencephalon, (M) Midbrain, and (H) Hindbrain. From *Laberge & Hara, 2001*.

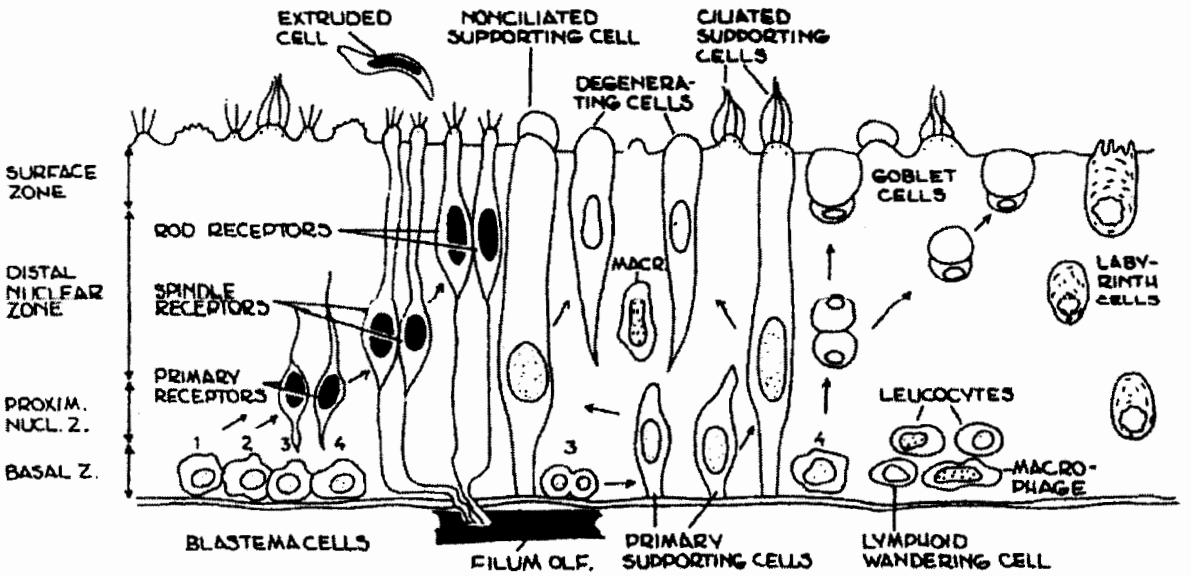



Figure 1.4. A schematic diagram depicting the cellular structure of the salmon olfactory epithelium. The basal zone contains progenitor cells that migrate and differentiate into either goblet, receptor, or supporting (sustentacular) cells. From *Bertmar, 1971*.

axons of the olfactory nerve and into the olfactory bulb (Figure 1.3). The olfactory bulb of salmon has the same cell types and functions as in other animals in that the axons of the olfactory nerve synapse with mitral cells in the glomerular layer of this organ (Byrd &m Brunjes, 1995). In addition to being a simple junction between neurons, the glomeruli appear to enhance or reduce sensory information en route to progressively higher levels of processing in the olfactory bulb (Duchamp et al., 1990). From the synapses within the glomerular layer, mitral cells then transmit signals to the telencephalon where they undergo higher levels of processing. The telencephalon is a rudimentary organ that is homologous to the cerebrum in higher animals (Laberge & Hara, 2001).

*Effects of OPs on olfaction*

OPs have been shown to cause a variety of effects on olfaction and consequently, olfactory-mediated behaviours. Only a few studies of this nature exist, and have been performed mainly on mice, rats and fish. The following sections review most, if not all, of these studies which date back to the early 1990s.

Studies in rats and mice suggest that mechanisms governing OP-mediated olfactory inhibition may be caused by AChE inhibition. For example, a dose-dependent inhibition of olfactory reactivity was demonstrated in rats receiving subcutaneous injections of 50, 250 and 500 µg/kg of diisopropylfluorophosphate (DFP) (Prendergast et al., 1997). In the lowest dose group of this study, olfaction did not deteriorate across 10 days of observation. In the 250 µg/kg dose, olfactory inhibition was observed only for the first 6 days

following treatment after which olfaction recovered. Olfaction did not recover in the 500 µg/kg group. The effects of OP AChE inhibitors can also be histological. In another study examining the enzymological alterations in the olfactory bulbs of mice fed 1 and 1.5 mg/kg of the OP phorate, AChE activity in the higher dose group of 1.5 mg/kg was absent in the neurons and fibres of the external plexiform, mitral, internal plexiform, granule cell, and olfactory tract layers (Vandana & Zzaman, 1997). These layers are found in the olfactory bulbs of mice and other vertebrate animals.

The glomerular layer, a possible target site of AChE-inhibitors, normally stains heavily for AChE activity and is rich in cholinergic terminals (Gerebtzoff, 1959). The synapses in the glomerular layer are both adrenergic and cholinergic in nature (Shanthaveerappa, 1965). Despite the ubiquitousness of AChE in brain, olfactory bulbs and other components of the olfactory system, the role AChE plays in signal transmission to the brain remains unknown. The studies above show that diazinon inhibits olfaction in rats and mice (Prendergast et al.,1997; Vandana & Zzaman, 1997). However, the fact that diazinon is an AChE inhibitor, and that this enzyme is well represented within the components of the olfactory system, a possible mode of action seems apparent with respect to olfactory inhibition. No studies however, are available that can confirm this link. Furthermore, no studies can confirm that diazinon inhibits olfaction via inhibition of signal transmission in this system.

In their 1996 paper, Moore & Waring (1996) examined two aspects of the salmon olfactory system that relate to the possible sub-lethal effect of diazinon. First, an electrophysiological study was undertaken to determine the response of

male Atlantic salmon to prostaglandin $PGF_{2a}$ after exposure to various concentrations of diazinon. Second, serum levels of the reproductive steroids 17, 20-ß-dihydroxy-4-pregnen-3-one, testosterone, and gonadotropin II were examined in male diazinon-exposed fish prior to exposure to ovulated female salmon urine. $PGF_{2a}$ is a potent odourant that has a role in synchronizing spawning physiology and behaviour between the two sexes (Moore & Scott, 1992; Sorenson, 1992). A priming effect on plasma steroid and gonadotropin levels by $PGF_{2a}$ was found in mature male salmon parr (Moore & Waring, 1996). Responses to $PGF_{2a}$ were reduced significantly at diazinon concentrations as low as 1ug/L. Furthermore, upon examination of their blood serum, levels of the sex steroids of the male salmon were greatly depressed. In conclusion, the inhibition of olfaction in male fish prevented the detection of $PGF_{2a}$ which subsequently resulted in no increase in male sex steroids. The authors were unable to ascertain the mechanism by which diazinon exposure affects olfactory ability.

The studies above indicate that OPs, especially diazinon, can be potent olfactory inhibitors. Because of this inhibition and the reliance of fish on olfaction for the reasons stated above, it is worthwhile to further explore the effects and implications of diazinon with respect to olfaction.

*Role of metabolism in toxicity*

Considering the exposure of the salmonid olfactory system to xenobiotics and that the olfactory tissues contain biotransformation enzymes, it stands to reason that these enzyme systems may play a role in limiting or enhancing the impact of toxicants.

All organisms are exposed constantly and unavoidably to xenobiotics. Lipophilicity, which enables these substances to be absorbed, can also be major problem for their elimination (Rand, 1995). In many cases, organisms including fish have devised biotransformation mechanisms which allow excretion of these lipophilic compounds. Therefore, the elimination of xenobiotics from an organism is often determined by their conversion to more water-soluble metabolites by enzymes in the liver and other tissues, which facilitate their elimination.

In most organisms, the biotransformation of xenobiotics is a two-step process that involves oxidation, hydrolysis, or reduction reactions and subsequent conjugation with polar constituents through glucuronidation, sulfation, acetylation, methylation, glutathion conjugation, or amino acid conjugation (Klaasen et al., 1996). By undergoing biotransformation, considerable changes in the biological activity of xenobiotics may occur. In most cases, a chemical is detoxified, however, the toxicity of a chemical can increase if biotransformation of a protoxin results in an active, toxic form of a compound. This effect is apparent in the transformation of OPs where oxidation results in the more toxic oxon form.

*Role of biotransformation enzymes in diazinon toxicity*

Diazinon shows selective toxicity to organisms. Mentioned previously, aquatic invertebrates in general are the most acutely susceptable to diazinon toxicity, followed by fish, birds and mammals. For example the oral acute toxicity ($LD_{50}$) of diazinon to rats and birds is 450, and 4.5 mg/kg respectively, with a resulting selectivity ratio of 100 (Walker, 2001). The $LC_{50}$'s of diazinon to fish such as guppy, trout, zebrafish and carp are much lower at 0.7, 1.28, 7, and 14

mg/L (Keizer et al., 1995). $LC_{50}$ ratios between species can range from a factor of 10 to 300 (Keizer et al., 1991; Vittozzi et al., 1991). Such selectivity is well documented, not only with OPs, but several other classes of pesticides as well (Table 1.2). Metabolic differences between animal classes account for the selective action of OP pesticides (Keizer et al., 1991).

*Role of CE*

CEs represent a multigene family that hydrolyze an exceptional range of structurally diverse compounds such as drugs, pesticides, environmental chemicals, food additives, and endogenous substances containing carboxylic ester, amide, thioester, phosphoric acid ester, and acid anhydride functional groups (Satoh 1987; Klaasen, 1996; Satoh & Hosokawa, 1998).

Because of the wide array of substrates that can be hydrolysed via CEs, both inactivation and activation of xenobiotics may occur. In humans, a significant number of drugs and exogenous compounds are substrates of CEs, including dipivefrinhydrochloride, carbonates, cocaine, salicylates, capsaicin, palmitoyl-coenzyme A, haloperidol, imidapril, pyrrolizidine, alkaloids, and steroids (Klaasen, 1996).

Besides humans, many other species require CE activity to metabolize xenobiotics. An increase in CE activity and a corresponding decrease in the oxidation of an applied insecticide, such as diazinon, are thought to be the principle means by which pesticide resistance to OPs develops in insect pests (McKenzie, 1996). For example, overproduction of an otherwise unaltered CE has been recorded in the green peach aphid, *Myzus persicae* (Devonshire &

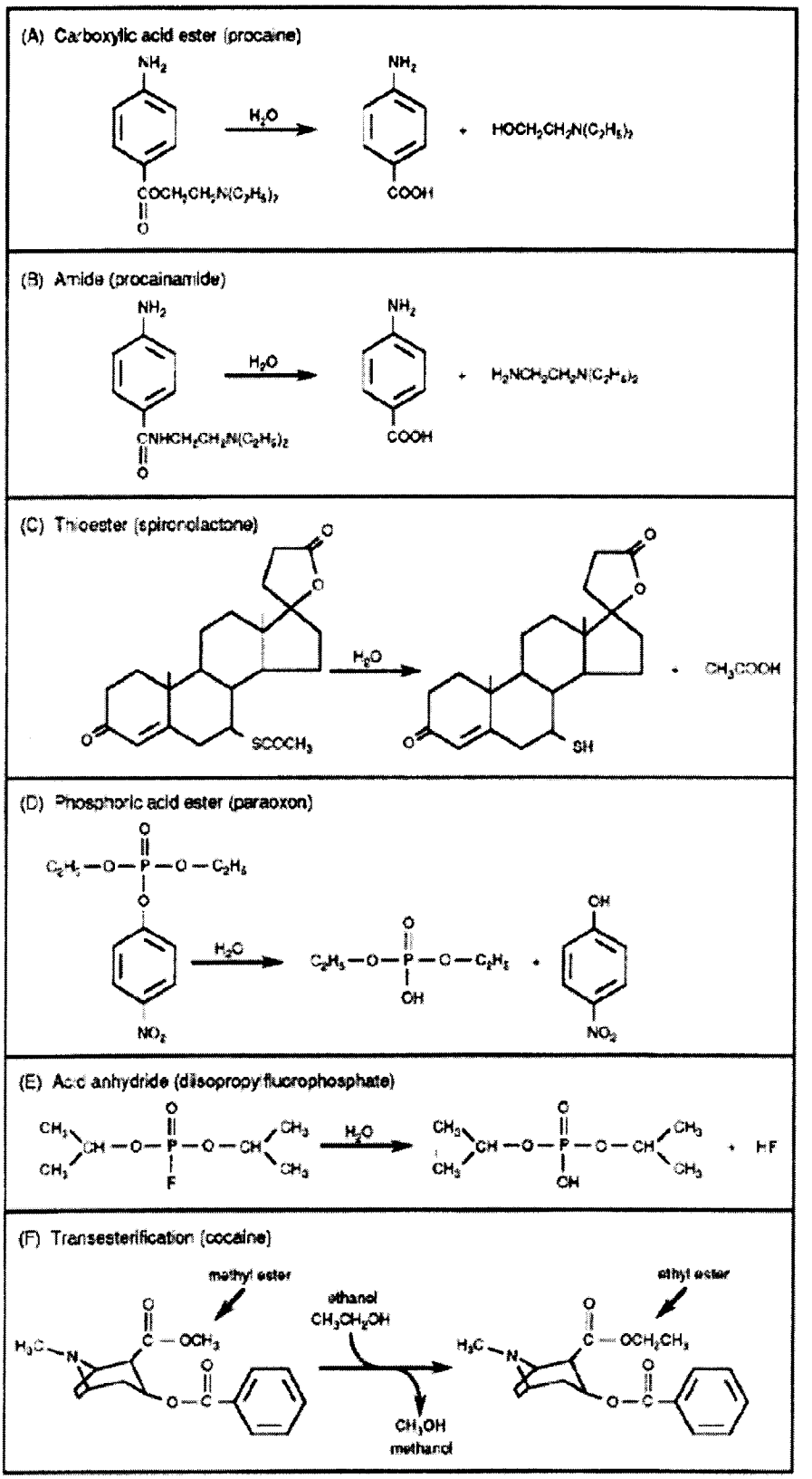



Figure 1.5. Substrates of CE's include carboxylic esters, thioesters, amides, phosphoric acid esters, acid anhydrides and transesters. From Klaasen, 1996.

| Compound | Acute Oral LD50 (mg/kg) | | | Topical/Dermal LD50 (mg/kg) | | |
|---|---|---|---|---|---|---|
| | Rat | Birds | SR | Rat | Insects | SR |
| **Organophosphorus insecticides** | | | | | Housefly | |
| Dimethoate | 250 | 26(4) | 9.9 | 925 | 0.2 | $4.6x10^3$ |
| Fenitrothion | 462 | 332(4) | 1.4 | >3000 | 5.7 | 526 |
| Dichlorvos | 27 | 9.6(2) | 2.8 | 488 | 0.8 | 610 |
| Diazinon | 450 | 4.5(4) | 100 | 850 | 1.9 | 447 |
| Malathion | 1650 | 685(3) | 2.4 | >4000 | 17.4 | >230 |
| Pirimiphosmethyl | 1400 | 162(3) | 8.6 | | | |
| Pirimophosethyl | 138 | 6.5(2) | 21 | | | |
| **Organochlorine insecticides** | | | | | Housefly | |
| DDT | 400 | 923(3) | 0.43 | 2500 | 14.0 | 179 |
| Dieldrin | 40 | 91(7) | 0.44 | 75 | 1.0 | 75 |
| γ HCH | 200 | 118[1] | 1.7 | 750 | 3 | 250 |
| **Carbamate Insecticides** | | | | | | |
| Carbaryl | 500 | 990(5) | 0.5 | >4000 | >500 | |
| Baygon (Propoxur) | 135 | 26(6) | 5.2 | >2400 | 25 | >96 |
| Carbofuran | 6 | 2.4(4) | 2.5 | | 7 | |
| Aldicarb | 6 | 3.6(4) | 1.6 | 1 | 6 | 0.16 |
| Zectran | 39 | 12(6) | 3.2 | | | |
| **Pyrethroids** | | | | | Bee | |
| Permethrin | 500 | >13000(4) | <0.04 | >2500 | 0.017 | $>1.47x10^5$ |
| Cypermethrin | 250 | >10000 | <0.025 | >4800 | 0.11 | $>4.4x10^4$ |
| Fenvalerate | 451 | >4000(3) | <0.11 | 4000 | 0.21 | $2.4x10^4$ |
| Deltamethrin | 129 | 4000[2] | <0.03 | >800 | 0.035 | $2.3x10^4$ |

For birds an average value of $LD_{50}$ is usually given. A number in brackets ( ) indicates the number of different species used to obtain average.
Where ranges of $LD_{50}$s are given in the original source, an average has been calculated to simplify presentation.

Selectivity Ratio (SR) = $\frac{LD_{50}\ rat}{LD_{50}\ other\ species}$

[1] Value for Pheasant
[2] Value for Duck

Table 1.2. Selective toxicity of some pesticides. From *Walker, 1994.*

Sawicki, 1979; Devonshire & Field, 1991) and in *Culex* mosquitoes (Mouches et al., 1986) as a result of gene amplification in each species, leading to OP resistance. CE activity also mitigates the toxic effects of OPs in mammals (Atterberry et al., 1997). Endogenous CE provided significant protection to rats against the *in vivo* toxicity of soman, sarin, tabun, and paraoxon (Maxwell et al. 1992). The importance of this enzyme system is demonstrated by its wide distribution in the tissues of many organisms including vertebrates, insects (McKenzie, 1996), plants (Hill et al., 1978), and mycobacteria. In addition, the system's high degree of genetic conservation (Satoh, 1987) in many species points to its importance with respect to xenobiotic metabolism.

The difference in the rate of OP biotransformation by CE vs. P450 may be responsible for their differential toxicity to various animal groups. Mentioned previously, many OPs including diazinon require the oxidation of the parent compound to the toxic "oxon" form. CE, on the other hand, hydrolyzes the ester bond joining the phosphorus to the leaving group, which is in the case of diazinon, is a heterocyclic group. CE activity, being generally high in mammals, lower in birds and absent in insects, may help to explain why OPs are highly acutely toxic to some organisms and relatively less acutely toxic to others (Yang et al., 1971; Brealy et al., 1980; Lasker et al., 1982; Mackness et al., 1987; Walker et al., 1987). High expression of CEs in insects is known to render them resistant to OP-insecticides including diazinon (McKenzie, 1996).

The gene products of the CEs are localized in the smooth endoplasmic reticulum (SER) and cytosol in a wide variety of organs and tissues in mammals (Satoh & Hosokawa, 1998). Although activity is greatest within the centrilobular

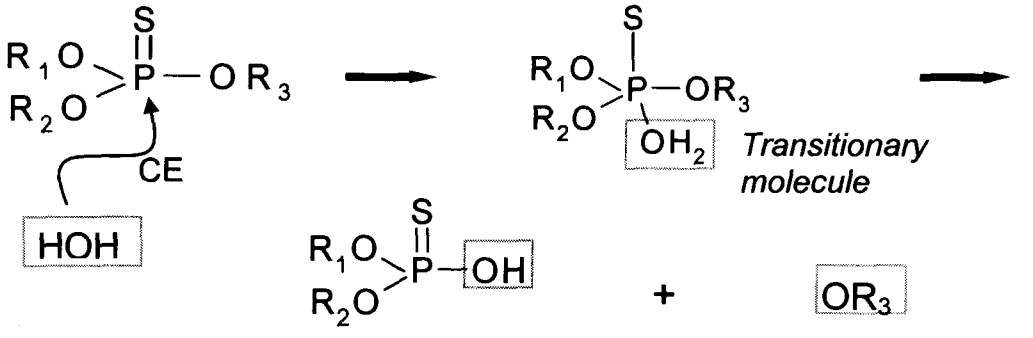



Figure 1.6. Diagramatic representation of the hydrolysis of phosphorothioate organophosphorus pesticides. Red box indicates the fate of water as a hydrolysing substance within the reaction.

region of the liver (Klaasen, 1996), CE activity is also present within the proximal tubules of the kidney (Tsujita et al., 1988; Tsujita, & Okuda, 1993), small intestine epithelial cells of rats (McCracken et al., 1993), lung (Gaustad, R. et al., 1991), skin (Nomeir et al., 1989; McCracken et al., 1993), heart (Dean et al., 1995), muscle (Nambu et al., 1987), and blood (Grubic et al., 1988; McCracken et al., 1993; Van Lith et al., 1993). The testis, nasal and respiratory tissues (Bogdanffy et al., 1987), adipose tissues (Tsujita & Okuda, 1992), leukocytes (Laohapand et al., 1985), and central nervous system of the rat (Dean & Zhang, 1995) also contain cells that exhibit a high degree of CE expression.

CE is widely distributed throughout the tissues of fish as there are considerable concentrations of CE within the gill, intestine, heart, liver, serum, and serum yolk sac (Li & Fan, 1997; Barron et al., 1999). CE activities in juvenile rainbow trout, channel catfish, fathead minnows, and bluegill sunfish whole fish homogenates were found to be similar to each other (Li & Fan, 1996, 1997; Barron et al., 1999) indicating that CE is expressed in roughly similar levels in different fish species.

*Classification*

Although a systematic nomenclature system for classifying CEs remains to be established, a broad classification scheme developed by Aldridge (1953) is still widely used. This scheme allows for the classification of enzymes based on a practical, toxicological point of view. As such, there are three classes of CE: 1) A-esterases – those that hydrolyze OPs, 2) B-esterases – those that are inhibited by OPs, and 3) C-esterases – those that do not interact with OPs (Hodgson &

Levi, 1994; Klaasen, 1994).

Despite such a scheme, much confusion appears to exist with respect to isozyme classification. Because of the broad substrate specificity of this enzyme system, classification based on the reactions they catalyze appears to be difficult at best. The term "CE" is misleading because enzymes possessing this activity can also function as amidases, phosphatases and/or oxonases. A reference was made to B-class CEs that "protect against the toxicity of OP and organophosphorothioate insecticides in both mammals and fish" (Maxwell et al., 1992). In other studies no distinction is made between A, B and C-CE (Asperen, 1962; Devonshire et al., 1992; Moores et al., 1996; Li & Fan, 1997). Furthermore, elevated CE activities are suggested to be responsible for the resistance to many carbamate, OP, and pyrethroid insecticides (Goh et al., 1995; Zhao et al., 1996; Harold & Ottea, 2000). Although a CE classification scheme exists, such scheme remains to be widely used.

*CE, diazinon and olfactory toxicity*

The study of xenobiotics and their effects on organisms commonly revolves around single compounds simply due to logistics (Hodgson & Levi, 1994). Evaluating the toxicity of single compounds is usually not as complicated as evaluating binary, tertiary or quaternary mixes as one does not have to evaluate the effects of the chemicals on the receptor plus the interactions of chemicals. Intuitively however, organisms are exposed to, and affected by a multitude of xenobiotics simultaneously *via* different modes of entry, modes of action, and metabolic pathways. From a toxicological point of view, we have only

begun to examine the intricacies of toxicity-related interaction between different xenobiotics. In addition to serving as substrates for a number of enzymes, xenobiotics may also be inhibitors or inducers of other enzymes (Hodgson & Levi, 1994). Using the detoxification of diazinon as an example, it would seem likely that exposure to xenobiotics may induce or inhibit CE activity such that the rate of diazinon detoxification increases, decreases, or ceases altogether.

Since CEs are an enzyme class responsible for the transformation of diazinon, and since diazinon is a known olfactory toxicant, this would seem to suggest a possible link between this enzyme system and olfactory toxicity. Modulation of CE activity may potentiate or antagonize the sublethal (and acute) effects of diazinon. The following discusses the implications of CE modulation with enzyme inducers and inhibitors, and their effects with respect to xenobiotic toxicity.

*Research objectives*

The possibility exists that the modulation of CE activity in olfactory tissues may affect the olfactory toxicity of diazinon. Currently, few studies have examined the role that CEs play in protecting the salmon olfactory system from OP pesticides. In addition, no studies have been undertaken to examine the ability of xenobiotics to modify the activity of olfactory CEs. Therefore, the specific goals of this experiment were to demonstrate if:

1) CE is present in the salmon olfactory epithelium,
2) CE activity in the salmon olfactory epithelium can be inhibited or induced, and

3) modulation of CE activity in the olfactory epithelium affects diazinon olfactory toxicity.

# CHAPTER 2

## Histochemical Localization of Carboxylesterase in the Olfactory Epithelium

### Introduction

As mentioned earlier in this thesis, biotransformational enzymes located in the olfactory tissue of animals break down airborne or waterborne compounds that elicit olfactory responses, and additionally may play a role in the biotransformation of olfactory toxicants. Considering the possible dual purpose of such enzymes, it is important to briefly discuss the types of enzyme in olfactory tissues and their possible roles.

*Olfactory enzyme systems in mammals*

Xenobiotic metabolizing enzymes in the rat nasal cavity clearly show a substantial biotransformation capacity (Dahl & Hadley, 1991). Enzymes contributing to this capacity include various forms of cytochrome P450 (Bond, 1983; Hadley & Dahl, 1983; Baron et al., 1986; Foster et al., 1986; Baron et al., 1988), aromatase and reductases (Lupo et al., 1986), benzo(a)pyrene hyroxylase (Barron et al., 1986), FAD containing monooxygenase (McNulty et al., 1983), aldehyde dehydrogenase (Casanova-Schmitz, et al., 1984; Bogdanffy et al., 1986), formaldehyde dehydrogenase (Keller et al., 1990), CE (Bogdanffy et al., 1987), carbonic anhydrase (Brown, et al., 1984), UDP-glucuronyl transferase (Lazard et al., 1992), glutathione-S-transferase (Baron et al., 1986; Baron et al.,

1988) and rhodanase (Lewis et al., 1991). Of the various tissues within the nasal cavities of rats and mice, the olfactory epithelium in general contains relatively high activities of xenobiotic detoxifying enzymes (Dahl & Hadley, 1991). More specifically, sustentacular cells (Figure 1.5), and cells comprising the Bowman's glands and ducts were identified as containing the greatest activities of these enzymes through a combination of histochemical and immunohistochemical methods (Bogdanffy et al., 1987; Olsen et al., 1987; Randall et al., 1987; Dahl & Hadley, 1991; Banger et al., 1994; Brittebo, 1997). Functionally, high activities of xenobiotic biotransformation enzymes in the Bowman's glands and sustentacular layer of nasal tissue may be important in detoxifying olfactory toxicants in addition to playing an active role in olfaction. Considering that most odourants that are foreign to a living organism are by definition, xenobiotics, enzymes present in the olfactory epithelium can serve to prevent excessive stimulation of olfactory receptor cells through the biotransformation of odourants, and potentially biotransform xenobiotic substances that exhibit toxicity to the olfactory epithelium (Merriam-Webster, 2004).

*Olfactory enzyme systems in fish*

Very few studies have examined the xenobiotic transformational capacity of the fish olfactory organ. In one study, the cellular and subcellular distributions, and enzymatic capabilities of P4501A1 were characterized in goblet and ciliated nonsensory cells of the olfactory rosette in rainbow trout after exposure to β-naphthoflavone, (Saucier et al., 1999). In another study, biotransformational

enzymes including P4501A1, *p*-nitrophenol hydroxylase and glutathione *S*-transferase were present and expressed at similar levels in the olfactory organ, at levels found in liver (Monod et al., 1994).

As mentioned earlier, the basic structure of the salmon olfactory system is similar to other vertebrates however, there are several subtle differences (Byrd & Brunjes, 1995). For example, Bowman's glands, which contain xenobiotic biotransformational enzymes in rats, mice, and other terrestrial animals, are absent in the fish olfactory rosette (Saucier et al., 1999). Activity appears to be localized in the mucous-producing goblet cells instead (Saucier et al., 1999). This suggests a possible functional convergence between Bowman's glands in terrestrial animals and goblet cells in fish. No studies have histochemically determined if CE exists in the salmonid olfactory epithelium despite the widespread presence of this enzyme in many other tissues and organs in fish.

The objective of this experiment was to determine if CE is present in the salmonid OE, and if so, to determine the cell type(s) that contain CE activity. The underlying premise of this experiment is that tissue sections containing CE should be receptive to various substrates and stains that will make the enzymes visible upon examination using optical microscopy. The localization of CE in the olfactory organs of fish will address fundamental questions regarding its possible roles in olfaction and olfactory toxicity, and will begin to lay the groundwork for future studies of this enzyme system in the olfactory organs of fish.

**Materials and Methods**

*Fish*

Coho salmon (19 months-old; 15 ± 2 cm, 17-35 g) used in this experiment were obtained from the Capilano Hatchery (Department of Fisheries and Oceans, North Vancouver, BC). The animals were maintained in locally dechlorinated and aerated water, acclimated to a temperature of 15°C in outdoor 1500 L fiberglass tanks, and fed synthetic BioDiet feed (BioProducts Inc., OR) daily *ad libitum* for at least 7 days prior to an experiment.

*Tissue preparation*

Fish were anesthetized with MS-222 at a concentration of 0.2 g/L (buffered with 0.2 g/L $NaHCO_3$) and then perfused as described below, for 10 minutes with a solution of 0.1 M phosphate buffer (pH 7.6). The purpose of perfusion was to eliminate any serum CE in tissues. Fish were dissected to expose the heart and a blunted surgical syringe inserted and clamped into the conus arteriousus through which buffer was pumped by a peristaltic pump (flow rate = 1 mL/min) for 15 minutes. The inferior vena cava was severed to allow for the drainage of blood. Fish were pinned onto a styrofoam tray containing ice during phosphate buffer perfusion. Olfactory rosettes from each fish were then dissected out under a stereo microscope and cryoprotected in a 30% sucrose solution for 2 hours on ice.

Prior to freezing the rosette tissue for sectioning, a spot of embedding medium (Fisher, NH) was placed on a metal cryostat-mounting disc. This spot was immersed into liquid nitrogen until the embedding medium had a thick syrupy consistency. The dissected rosette was placed directly on the embedding

medium and re-positioned into a dorso-ventral orientation. Additional OCT was then applied to cover the inserted tissue sample. Finally, the whole mounting disc containing the positioned tissue sample was immersed into liquid $N_2$-chilled iso-pentane.

*Sectioning*

Sectioning was performed on a cryostat (Leica model #2800E Jung Frigocut, Heidelburg, GER). Frozen tissue samples were allowed to equilibrate with the temperature of the cryostat mounting disc which was set at -25°C. Samples were cut into 10 μm sections and thaw-mounted on silanized glass slides. Slides containing the cut sections were then stored at -20°C until histochemical analysis was performed.

*Histochemical Localization*

Several kits are available that measure CE, including those that are based on α-naphthylacetate and α-naphthylbutyrate as substrates (Sigma-Aldrich, MO). In both assays, enzymatic hydrolysis of ester linkages liberates free naphthol compounds. These couple with a diazonium salt, forming highly coloured deposits at sites of enzyme activity.

Sections were brought to room temperature and air-dried to ensure that they adhered to the slides. An α-naphthyl butyrate kit (Sigma® #181-B) was used to stain for CE activity. Sodium nitrite solution (1.5 mL of a 0.1 M solution) was added to 1.5 mL of pararosaniline solution (40 g/L in 2 M HCl), mixed via

inversion and allowed to stand for 5 minutes. This was then added to 40 mL of a 0.1 M phosphate buffer solution (pH 7.6). An α-naphthyl butyrate solution (5 mL of a 2.4 g/L in methanol solution) was added to this previous mixture. Slides containing the cut tissue samples were placed into this solution and incubated for 1 hour at 37°C. Following incubation, slides were rinsed with a steady and gentle stream of deionized water and immediately cover-slipped in pure glycerol. To prevent the formation of air-pockets within the cover-slipped slides, the tissues were not allowed to dry between rinsing and coverslipping.

To demonstrate that reaction formation was CE-specific, some sections were pre-incubated for 10 minutes in the presence of 200 μM bis-nitrophenylphosphate (BNPP, an inhibitor of all non-specific esterases) in 0.1 M phosphate buffer (Bogdanffy et al., 1987). These sections were then co-incubated in the complete reaction mixture containing the inhibitor.

## Results

*General morphology*

The general morphology of the coho olfactory rosette is similar to other fish (Pfeiffer, 1963; Moran et al., 1992; Byrd & Brunjes, 1995). The olfactory epithelium is arranged in primary and secondary lamellae, which are contained within an olfactory rosette (Figures 1.2, 2.1). The olfactory epithelium was easily discernable from the lamina propria, the underlying supporting connective tissue which normally contains nerves and blood vessels. Further examination of the morphology at the microscopic level was not possible because the tissues were not fixed. The use of unfixed tissue was necessary in this study

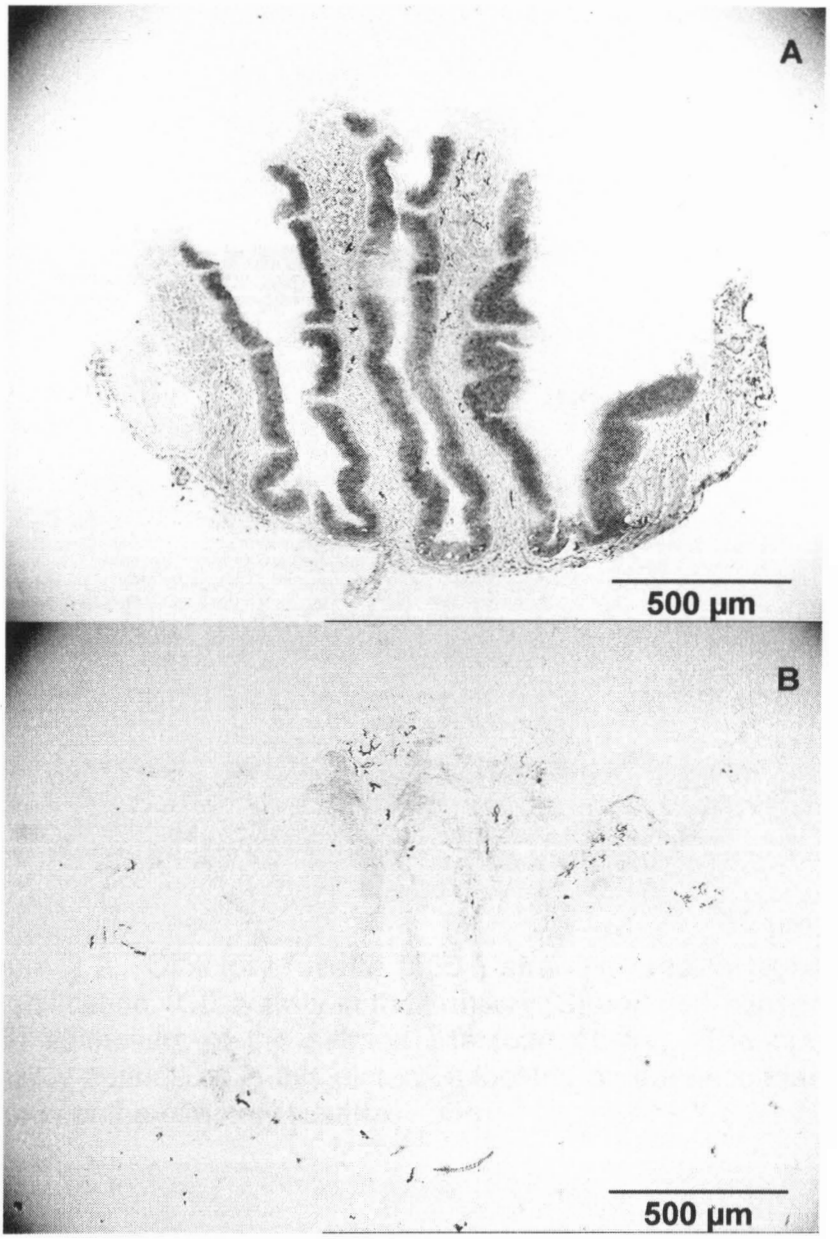



Figure 2.1. Comparison of CE activity in dorso-ventral sections (a) of the coho salmon olfactory lamellae at 40x magnification. CE activity was present in all tissues at varying degrees of intensity. Control sections (b) were CE inhibited via pre-incubation with *bis*-nitrophenyl phosphate. Lack of staining in (b) demonstrates the proliferation of CE in olfactory rosette tissues. Box in (A) is enlarged in Figure 2.2.

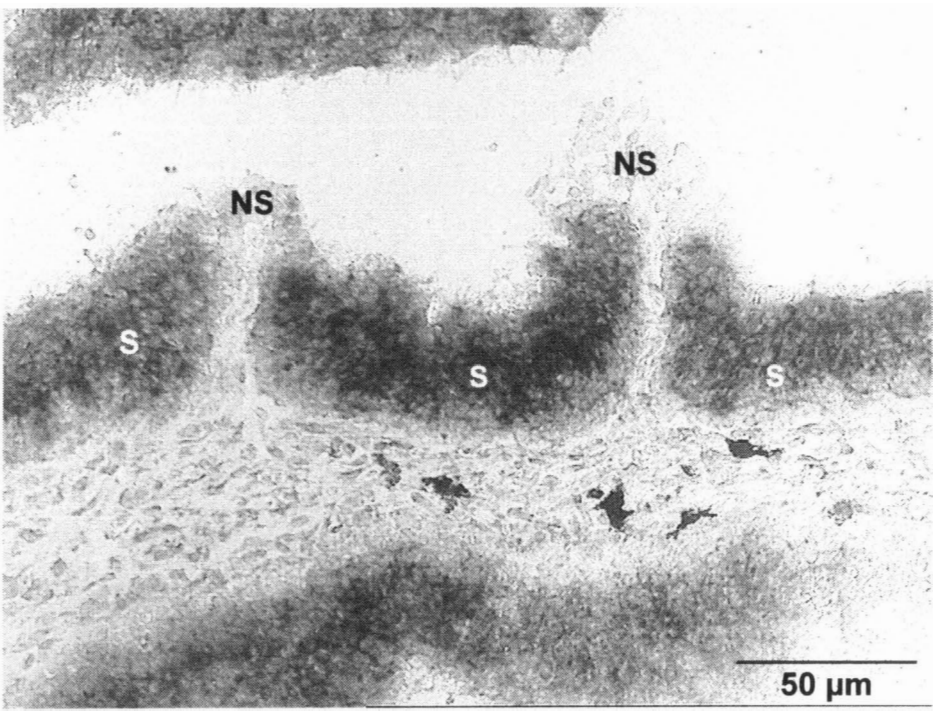



Figure 2.2. Olfactory rosette (400x) showing area in higher magnification. CE activity in the sensory (S) and non-sensory (NS) epithelium of the salmon olfactory rosette. The non-sensory epithelium in this picture is located on the secondary lamella of the olfactory rosette.

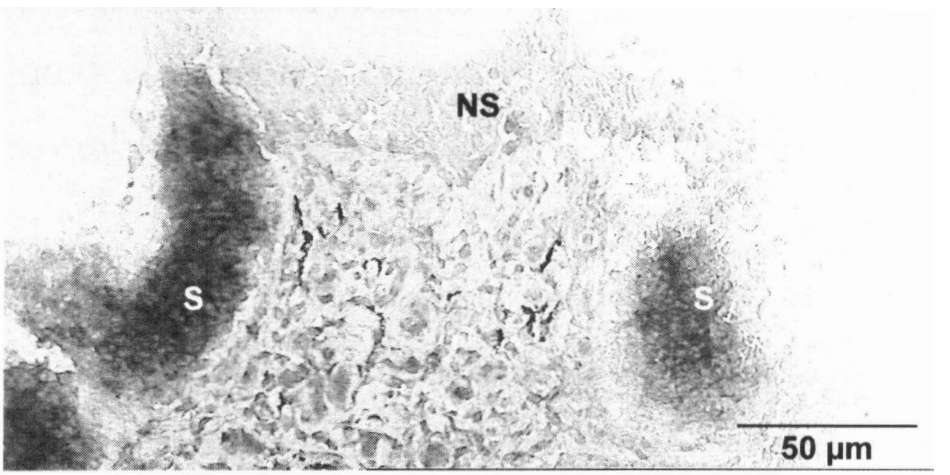



Figure 2.3. Tip of a primary lamella (400x). Areas exhibiting α-naphthyl-butyrate staining denotes CE activity. Sensory (S) and non-sensory (NS) epithelium of the salmon olfactory rosette are denoted.

to preserve CE activity was found to be necessary in a previous trial with fixed tissue.

*Localization of CE activity*

CE activity manifested itself as areas of dark reddish brown precipitate (Figures 2.1a, 2.2, 2.3). Generally, staining was prominent throughout the sensory epithelium. Staining varied from little to no staining on the tips of the lamellae, to moderate in the supporting connective tissue and finally moderate to heavy throughout the sensory epithelium.

BNPP was used as a CE inhibitor to demonstrate the specificity of the histochemical substrate for the CE enzyme. Figure 2.1(b) shows complete inhibition of CE as opposed to Figure 2.1a, where CE activity was easily localized in the sensory epithelium. Figures 2.2 and 2.3 respectively show close-up views of the secondary lamella and primary lamella tip. Non-sensory epithelium (NS) located in these areas did not demonstrate CE activity. Minor staining of the connective tissues of the rosette demonstrated some degree of CE activity (Figures 2.1, 2.2 and 2.3).

**Discussion**

Routine histological examination of tissues involves a series of steps; fixation dehydration, embedding and subsequent sectioning. Arguably, the most important step in the histological process is fixation since its purpose is to preserve the general structure of the tissue at the molecular level, and to prevent microbial, thermal and oxidative degradation of the dissected tissue. Routine

fixation involves the chemical

cross-linking of proteins (to prevent enzyme action and digestion) and the removal of water to further denature the proteins of the cell. A common means of preparing tissues for histochemical analysis is to fix them in formalin solutions of varying concentrations, followed by embedding in wax, prior to sectioning. The method just described has been in use for many decades and has changed little since the inception of histological tissue examination. A large variety of fixatives are now available, but no single substance or known combination of substances has the ability to preserve and allow the demonstration of every tissue component (Leong, 1994). It is for this reason that some fixatives have only special and limited applications, and in other instances, a mixture of two or more reagents is necessary to employ the special properties of each. The selection of an appropriate fixative is based on considerations such as the structures and entities to be demonstrated and the effects of short-term and long-term storage.

Histological examination of the salmon olfactory rosette posed a number of challenges such as: 1) the juxtaposition of delicate rosette tissue with much harder cartilaginous tissue and 2) the ability to produce visually acceptable tissue sections without compromising CE activity. Previous attempts to isolate relevant tissues involved sectioning whole fish heads containing both cartilaginous and olfactory tissue. The difficulty in obtaining clean rosette sections from whole fish head tissues prompted the dissection of the olfactory rosette from the fish head prior to mounting and sectioning.

Previous studies with rat nasal tissues involved the embedding of tissues in a glycolmethacrylate monomer (Bogdanffy et al., 1986; Bogdanffy et al., 1987).

It is likely that this method was used because rat tissue samples contained bone and cartilage, and required a more robust supporting medium. Attempts at the use of glycolmethacrylate-based embedding medium in the current study indicated that CE was sensitive to the glycolmethacrylate embedding process as no activity was present in these tissues using this method.

CE activity was absent in previous trials utilizing tissue fixed with a variety of fixing agents such as paraformaldehyde, gluteraldehyde, ethanol, and acetone-based fixatives. This result was contrary to the published literature were CE activity was successfully localized in rat nasal tissue following fixation in a 10% neutral buffered solution (Robinson et al., 2002). It is possible that olfactory tissue structures containing CE activity in salmon olfactory rosettes differ sufficiently from similar structures in rat olfactory epithelium. This difference may manifest itself by an increased susceptibility of the fish CE to the crosslinking action of tissue fixatives compared to rat tissue, the end result being inhibition of enzyme activity. More likely however, is the possibility of a much lower activity of CE activity in salmon compared to rats. Subsequently, any effects of fixative on activity would be noticeable, in contrast to rats where only a slight deficit in CE activity would occur. These suppositions will be further evaluated in Chapter 3 of this thesis. Because of the lack of CE activity in fixed tissue, it was decided that further histology should proceed using unfixed tissue. Much higher CE activity was subsequently demonstrated in unfixed olfactory rosette tissue compared to formalin-fixed tissue.

Sectioning proved to be another challenge as conventional media (eg. glycol methacrylate) normally used to support tissue was insufficient for unfixed

tissue. Tissue sections were incompletely infiltrated and hardened by the glycol-methacrylate infiltration process. The use of unfixed tissues in this case may have prevented proper hardening from taking place, as it is known that dehydration to absolute alcohol is required, especially for dense, bloody, or fatty tissue specimens. Furthermore, stained tissue sections derived from this technique did not show any CE activity, despite previous histochemistry performed with glycol methacrylate (Bodganffy et al., 1987). There was no attempt at paraffin embedding and sectioning as it is well known that excessive heat denatures many enzymes.

Because of the difficulties discussed above, it was decided that a cryofreezing technique would yield the best results. Cyrofreezing has the distinct advantage of being able to process unfixed tissue, and maintain the integrity and activity of enzyme systems (Donovan & Preston, 1994). There are however, disadvantages to this technique, the main one being that some cell-membrane damage will invariably occur due to ice-crystal formation unless cryoprotected in a 30% sucrose solution. Despite this disadvantage, sections showed enough detail such that gross localization of CE activity could be determined. With a refined technique, it may be possible to localize activity at the cellular level.

Areas of tissue containing heavy staining corresponded to the sensory epithelium, indicating that these cell types do indeed possess CE. Ridges of both primary and secondary lamellae, which contain non-sensory epithelium, did not contain CE activity. It was not possible to differentiate the individual cell types that demonstrate CE activity, because the tissues were not fixed. As mentioned, the use of unfixed tissue was necessary in this study to preserve CE activity.

The absence of CE in the non-sensory epithelium seems counterintuitive to the fact that the goblet cells located in this region are responsible for mucous production, and are home to high activities of other biotransformational enzymes, especially CYP1A1 (Monod et al., 1994; Saucier et al., 1999). It has been suggested that goblet cells may not just be responsible for mucous production, but may also secrete elements into the surrounding mucous that are involved in the biotransformation of xenobiotic compounds (Saucier et al., 1999) including CE. Findings from this study however, show clearly that no CE activity is present in these areas. Nevertheless, the lack of staining in at least the ciliated epithelial cells is not surprising since these cells, which possess motile cilia, are thought to be responsible for the creation of water currents along the olfactory organ (Doving & Thommeson, 1977; Yamamoto & Ueda, 1977; and Breucker, 1979). Ciliated epithelial cells in the non-sensory epithelium were found in this study not to contain CE, therefore it may be inferred that activity is localized either to ciliated olfactory receptor cells, microvillar olfactory receptor cells, or sustentacular cells of the sensory epithelium.

The uniform distribution of CE in the sensory epithelium in the current study (Figures 2.1, 2.2 and 2.3) is similar to the distribution of CE in rat nasal epithelium (Bogdanffy et al., 1997). No attempt at localization of CE was made at the cellular level of this study (Bogdanffy et al., 1987). Excessive thickness of the sections (10 μm) used in the current study and others (Bogdanffy et al., 1987) is the factor most likely preventing localization of cellular CE. An immunohistochemical study of CE in rat tissue however, localized CE to the sustentacular cell cytoplasm, cells comprising the Bowman's gland and very

small amounts in the basal cells (Olson et al., 1993). More specifically, CE is known to be localized within the endoplasmic reticulum of terrestrial vertebrates (Potter et al., 1998). Some structural homology exists between the olfactory epithelium of coho salmon and rats as both species share similar cell types including sustentacular cells, basal cells and olfactory receptor neurons. The presence of biotransformational enzymes in goblet cells in fish and Bowman's glands in rats may further suggest functional homology. Given similar cell types in salmonid and rat olfactory epithelium, with exception of goblet cells and Bowman's glands respectively, it is reasonable to suggest that sustentacular cells may be an important contributor of CE in the salmonid olfactory epithelium. An immunohistochemical study would confirm this.

Olfactory epithelial sustentacular cells are thought to support the survival and function of olfactory receptor neurons in vertebrates (Pixley et al., 1997). No studies are available to identify the specific roles of these cell types. Considering the close proximity of sustentacular cells with the olfactory receptor neurons, localization of CE in these cells and the importance of CE in the biotransformation of exogenous compounds, sustentacular cells may play an active role in the biotransformation of odourants and possibly olfactory toxicants in the olfactory epithelium of both fish and rats.

# CHAPTER 3

## Quantitative Analysis of CE Activity in the Olfactory Rosette

### Introduction

Organisms can be exposed to, and affected by, a multitude of xenobiotics simultaneously. From a toxicological point of view, examinations of interactions between different xenobiotics in a single organism have been few.

Xenobiotics can be inhibitors or inducers of enzymes in addition to serving as substrates (Hodgson & Levi, 1994). This has special implications with respect to pesticide toxicity since the exposure of organisms to xenobiotics can inhibit or induce biotransformational enzymes theoretically alter toxicity.

In regard to CE specifically, inhibition and induction may modify the toxicity of some pesticides. Several studies have found that CE inhibitors and OPs can act synergistically (Maxwell et al., 1992; Li & Fan, 1997; Atterberry et al., 1997). No studies however, have been undertaken to determine if a link exists between the induction of CE activity and subsequent reductions in the acute toxicity of pesticides. However, several studies have shown that the clearance rate of some chemicals can increase as a result of CE induction (Barron et al., 1989; Barron et al., 1990).

In order to address the potential link between CE activity and pesticide toxicity, a suitable method is required which will enable the determination of enzyme activity. The broad substrate specificity of CEs suggests many possible substrate-enzyme combinations that could be used for the purpose of CE activity

determination (Dary et al., 1990; Klaasen, 1996). This non-specificity has provided many possible avenues for the development of an assay that is both easy and inexpensive to perform. A colorimetric assay has been developed that exploits the ability of CEs to hydrolyze aryl-esters (Gomori, 1953). This method measures the formation of a chromophoric product between a diazonium ion dye (Fast Garnet GBC) and an aryl-ester ($\alpha$-naphthylacetate).

This assay has been used successfully by researchers for the determination of CE activity in the tissues of rats (Sonne et al., 1991), humans (Slatter et al., 1997), birds (Siddiqui & Walker, 1996), as well as fish (Li & Fan, 1997). An extensive use of this assay has been in studies on insecticide resistance (Dary et al., 1990; Devonshire & Field, 1991; Devonshire et al., 1992; Harold & Ottea, 2000). There have been only a few studies that have examined CE activity in fish. Table 3.1 lists the activity of CE in various fish tissues. Although the CE activity of several tissue types as well as whole body homogenates have been obtained, a direct comparison of activity between studies may be inappropriate since subtle differences in methodology may affect enzyme rate measurements. What can be concluded from these studies, however, is that CE activity is expressed in many tissue types and at all life stages in fish. Currently, no studies have examined CE activity in the olfactory epithelium. Results from the histological section of this study indicated that general CE enzymes are present throughout the olfactory epithelium. This objective of this study was to determine the activities of CE enzymes located in the salmon olfactory epithelium and in the

| Tissue type | Method of Determination | CE activity (nmols/min/mg protein) | Reference |
|---|---|---|---|
| Whole-body (fathead minnows) | p-nitrophenyl-acetate spectrophotometry (405nm) | 30-50 | Denton et al., 2003 |
| Whole-body (juvenile fathead minnows) | p-nitrophenyl-acetate spectrophotometry (405nm) | 80 | Barron et al., 1999 |
| Whole-body (juvenile bluegill sunfish) | | 65 | |
| Whole-body (juvenile fathead minnows) | | 80 | |
| Whole-body (juvenile channel catfish) | | 83 | |
| Liver CE from hepatic *microsomes (nile* talapia adult) | p-nitrophenyl-acetate spectrophotometry *(405 nm)* | 500 | Pathiratne & George, 1998 |
| Malathion CE from hepatic microsomes | Talcot, (1979) using malathion as a substrate | 4.5 | |
| Liver (rainbow trout) | α-naphthylacetate spectrophotometry (550nm) | 142.2 | Li & Fan, 1997 |
| Intestine (rainbow trout) | | 153.9 | |
| Heart (rainbow trout) | | 122.9 | |
| Muscle (rainbow trout) | | 8.3 | |
| | | | |
| Whole body (mosquito homogenates) | α-naphthylacetate spectrophotometry (550nm) | 28 | Dary et al., 1990 |
| Liver (rat) | p-nitrophenyl-acetate spectophotometry (405 nm) | 1930 | Hosokawa et al., 1990 |
| Liver (rat) | p-nitrophenyl-acetate spectophotometry (405 nm) | 2500 | Morgan et al., 1994 |

Table 3.1. Activities of CE in tissues of various animals. Activities were determined colourimetrically using various substrates as indicated.

liver using α-naphthylacetate as a substrate.

## Materials and Methods

### *Fish*

Coho salmon (*Oncorhynchus kisutch,* 19-months old) used in this experiment were maintained in a same manner as in Chapter 1.

### *Chemicals*

Chemicals used in this experiment were as follows: triphenylphosphate (TPP) (98%), pyridine (99%), α-naphthylacetate (99%), α-naphthol (99%), 1,5-bis-(4-allyldimethylammonium-phenyl)-pentan-3-one dibromide (BW284C51), triton X-100 (0.5%), fast garnet GBC, bovine serum albumin, and coomassie protein assay reagent (Pierce Biotechnology Inc., IL). Unless otherwise stated, all stock reagents and chemicals were purchased from Sigma.

### *Induction and inhibition*

Prior to the assessment of CE activity, fish were exposed to a previously reported inhibitor or inducer of CE (Li & Fan, 1996, 1997). Triphenylphosphate (TPP) has been used as an *in vivo* inhibitor of CE in various fish species at a static concentration of 1 mg/L (Li & Fan, 1996, 1997). In other studies, various derivatives of pyridine have been used to induce CE activity in the rat olfactory system (Hotchkiss et al., 1993; Maxwell et al., 1994). The exposure route for

TPP in fish was via the water, while rats were exposed to pyridine vapour. No studies have yet examined the induction of CE in fish. As such, the present experiment relied on an arbitrary 1 mg/mL concentration of pyridine to induce CE activity in fish. The 1 mg/L concentration was used alongside that of the 1 mg/L concentration of the TPP CE inhibitor.

Prior to exposure of fish to either inducer or inhibitor, fish were transferred to 20 L tanks (n = 5 fish/tank) and acclimated to 14±1$^{O}$C with aeration for 24 hours. TPP or pyridine was added to exposure tanks hence to achieve final concentrations of 1 mg/L TPP and 1 mg/L pyridine. Exposure durations were 12 hours. Acetone alone was used in control tanks as carrier control.

*Tissue Preparation*

The fish were anesthetized and perfused in the same manner as in Chapter 1. The olfactory rosettes of each fish (left and right) were dissected out of the fish by excising the surrounding cartilage of the olfactory rosettes under a stereo dissecting microscope and combined. Both rosettes and livers were dissected out and immediately homogenized in 200 μL or 2 mL of 0.1 M phosphate buffer (pH = 7.6) containing 0.1% Triton X-100, respectively, using a Teflon homogenizer. Aliquots (140 μL) of liver and rosette homogenates were transferred to centrifuge tubes and diluted with 1.26 mL of 0.1 M phosphate buffer (pH 6.5) containing 0.1% Triton X-100 and centrifuged for 15 sec in a microcentrifuge (16000 g). The supernatant was then transferred into a microcentrifuge tube. This fraction was used for protein determination (Bradford

et al., 1976), and subsequent CE determination (Dary et al., 1990). The complete process was conducted at 4°C.

*Determination of α-naphthyl CE activity*

The microplate method employed by Dary et al., (1990) was used for the determination of CE activity in olfactory rosettes and livers. The substrate-containing solution used for the microplate assay was prepared immediately before use by mixing 7 mL distilled water, 2.5 mL of 0.1 M phosphate buffer (pH = 6.5) containing 0.5% Triton X-100, 0.5 mL of 60 mM α-naphthylacetate in ethanol, and 0.02 mL of 2 mM 1,5-bis-(4-allyldimethylammonium-phenyl)-pentan-3-one dibromide (a general cholinesterase inhibitor) in deionized water. Similar to the histology study, a substrate, α-naphthylacetate, undergoes hydrolysis by CEs present in the homogenate to form the product α-naphthol. This product then couples with a diazonium salt, in this case, fast-red GBC, forming highly coloured deposits that can be detected by a spectrophotometer (Appendix C).

Assays were performed in 96-well, curved bottom microtitre plates (Fisher Scientific, NH). Blanks contained 50 µL of 0.1 M phosphate buffer (pH 6.5) containing 0.1% Triton X-100. Prior to starting the assay, 100 µL of substrate-containing solution was added to all wells. The assay reaction was initiated by adding 50 µL of either rosette or liver homogenate sample to each well at the appropriate time. Reactions were allowed to continue for 10, 20, 30, 40, 50 and 60 minutes. At the end of each incubation period, 100 µL of 0.8 mg/mL Fast Red

GBC was added to all wells and the plate incubated at 20°C for a further 5 minutes. Following this incubation, the plate was read at 550 nm in a microplate reader (Molecular Devices SpectraMax 340, Sunnyvale, CA).

The amounts of α-naphthol produced were calculated from a standard α-naphthol curve (Appendix A) using absorbance values. α-Naphthyl standards used for the CE activity assay contained 12.5, 25, 50, 100, 200 and 400 μmols/L α-naphthyl. A five-minute incubation time was chosen because this was well within the linear region for α-naphthol generation (Appendix B). Although separate standard curves were prepared with each separate experiment, the curves did not differ significantly from each other (data not shown).

*Determination of protein content*

The protein content of the liver and olfactory rosette homogenate fractions was determined (Bradford 1976). Dye reagent (diluted 1:4 with deionized water) was combined with 10 μL of homogenate fraction in each microplate well and incubated for 5 minutes at room temperature. The plate was read at 595 nm on a microplate reader. Absorbance values were compared to a bovine serum albumin standard curve to calculate the amount of protein produced. Protein standards used for protein determination contained 0.02, 0.04, 0.08, 0.16, 0.32 mg/mL of bovine serum albumin in 0.1 M phosphate buffer pH 6.5 and 0.1% Triton X-100.

*Data analysis and statistics*

The rate of α-naphthol produced per minute was calculated from the slope of the α-naphthol concentration vs. time curve. Production rate was normalized to protein content. CE activity is expressed as nmoles of α-naphthol produced per minute per mg of protein (nmol/min/mg protein). See appendix A for activities for individual fish.

A paired t-test using Microsoft Excel 2000 two-sample t-test assuming equal variances (Bluman, 1995) was used to compare the CE activities from each rosette and liver TPP and pyridine groups, and their respective rosette and liver controls. Results are expessed as means ± standard error. Since the fish used for all groups originated from the same hatchery cohort and that acclimation conditions were the identical in all exposure and control groups, the expected variance in each exposure group was assumed to be equal.

**Results**

*Symptomology*

Fish showed some signs of sublethal toxicity during exposure to the inhibitor TPP at 1 mg/L. Symptoms included slight lethargy, congestion, degeneration and haemorrage of the smaller blood vessels. Similar symptoms were reported by in a study investigating a water-extractable toxic compound in vinyl upholstery fabric, which was identified as TPP (Ahrens et al., 1978). No effects were observed with the fish exposed to the CE inducer pyridine.

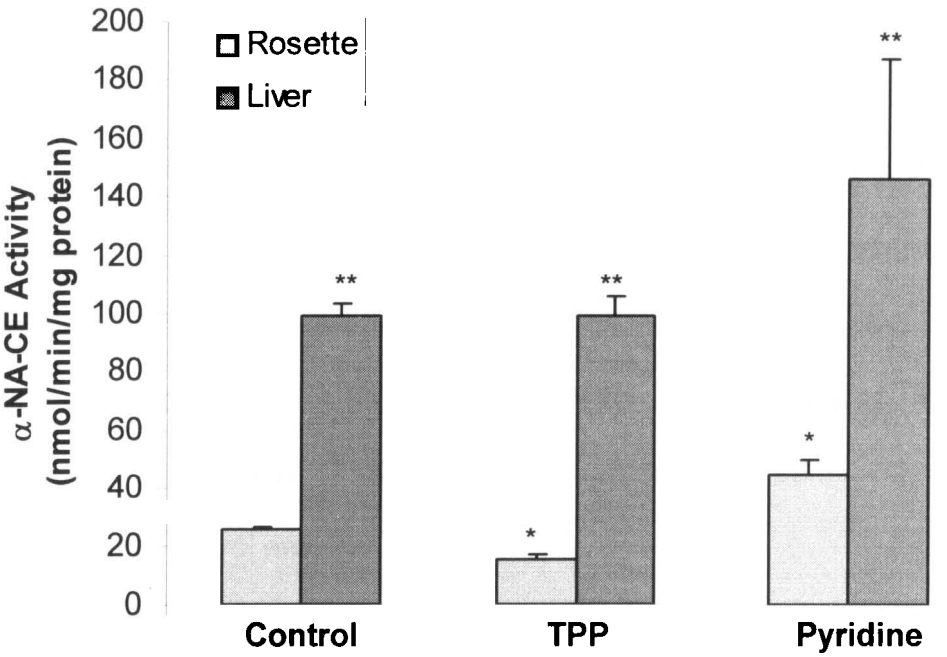



Figure 3.1. Olfactory rosette and liver homogenate α-naphthyl-acetate CE activity (nmols/min/mg protein) in control, TPP, and pyridine exposed groups. Values are means ± SE. (n = 5 fish). * denotes a significant difference from controls at $P < 0.05$. ** denotes a significant difference between liver and olfactory rosette homogenates at $P < 0.05$.

*CE activity in tissues*

The mean control olfactory rosette tissue CE activity is shown in Figure 3 and was 25.5 ± 0.7 nmol/min/mg protein (95% confidence interval = 23.3 - 27.7). Exposure of fish to 1 mg/L TPP resulted in a statistically significant decrease ($P < 0.05$) in olfactory CE activity compared to control animals (Figure 3.1). CE activity of fish in the TPP group was lowered at 15.3 ± 1.7 nmol/min/mg protein (95% confidence interval = 9.95 - 20.6) representing 60.0% of the control group activity. Exposure of the fish to 1 mg/L of pyridine resulted in a statistically significant increase ($P < 0.05$) in CE activity. CE activity was 44.4 ± 5.41 nmol/min/mig protein (95% confidence interval = 29.4 - 59.4) representing 174% of the control activity.

Mean CE activity in control liver was 99.3 ± 4.3 nmol/min/mg protein (95% C.I. = 85.6 to 113 nmol/min/mg protein) (Figure 3.1). CE activity in the TPP and pyridine groups was not significantly different from controls. The CE activity of the control, TPP and pyridine groups for liver (Figure 3.1) were significantly greater ($P < 0.05$) than respective olfactory rosette TPP and pyridine homogenate groups exposed to either pyridine or TPP. CE activity in control rosette tissue was approximately 27.3% of control liver CE.

**Discussion**

A microplate assay technique using α-naphthylacetate as a substrate was used in this study to quantify CE in both Coho salmon liver and olfactory tissue. A particular advantage of this method compared to others is the fact that only small amounts of tissue are required for a reliable measurement. In a study of

esterase activity in insects, only 10 to 100 mg of tissue was required for accurate assessment of esterase activity in houseflies, (Van Asperen, 1962). In the present study, the microplate assay, originally used for determining esterase activity in humans and insects, was adopted for use in fish tissues. As such, determining CE activity in small organs such as the olfactory rosette of salmon was possible.

Results from this study indicate that CE activity in the olfactory rosette was significantly lower than activity in liver. Although direct comparisons cannot be made to CE activities in other studies, CE activity of liver in the present study were similar to activities found in rainbow trout liver, intestine and heart, and higher than those found in whole-body homogenates of several other fish species (Table 3.1). CE activity in rat liver was higher than that of the liver activity in the current study. On average, rat liver CE activity was 4x higher than fish liver CE activity (Barron et al., 1999).

One likely reason that may contribute to the observable difference between fish and mammalian CE activity is body temperature. At 25°C, rat liver CE activity is 1.8 mmols/min/mg protein and increases to 3.3 mmols/min/mg protein at 40°C (Barron et al., 1997). Activity of rainbow trout liver CE is 400 nmols/min/mg protein at 0°C and also increases to 1.2 mmols/min/mg protein at 25°C (Barron et al., 1997). Many enzymes have an optimum temperature at which they operate (Solomon et al., 1993). At 25°C, fish and mammalian CE activity seem similar.

Differences of CE activities between liver and olfactory rosette homogenates can be explained by the distribution of CEs in both tissues.

Results from Chapter 2 demonstrated that CE distribution is limited to the sensory epithelium of the olfactory rosette lamellae. CEs are thought to play a role primarily in the biotransformation of odourants (Lazard et al., 1991). On the other hand, it is well known that xenobiotic-detoxifying enzymes including CEs are well distributed within liver tissues, where they biotransform both endogenous and exogenous compounds (Klaasen et al., 1996; Pathiratne & George, 1998). Therefore, one can expect that activity in the liver would be higher than that in the olfactory rosette.

The use of TPP as a CE inhibitor is well documented (Li & Fan, 1997; Oppenoorth & Welling, 1976). In the present study, the inhibitory effects of TPP can be seen clearly (Figure 3.1) where the mean activity of CE in olfactory rosette homogenates was significantly lower (60.0%) of controls. No significant inhibition in liver CE activity was found, results which are contrary to that of a previous study (Li & Fan, 1997) in which liver CE activity of goldfish and topmouth gudgeon exposed to 1 mg/L TPP for 12 hours decreased to 50.5 and 62.1% of controls, respectively. Species sensitivity may account for this difference as the uptake, elimination and subsequent target-site concentration of TPP may be different in coho salmon than other fish.

An enzyme such as CE can be inhibited via competitive, non-competitive, uncompetitive and irreversible inhibition. The mechanism, by which CE inhibition takes place following exposure to TPP is not known. TPP is considered to be a member of the OP family of compounds. Exposure to OP pesticides such as diazinon, parathion, soman and tri-orthocresyl-phosphate (TOCP) can cause neurotoxicity by preventing the hydrolysis of acetylcholine by

acetylcholinesterase. Irreversible inhibition of acetylcholinesterases involves covalent phosphorylation of their esteratic serine site. However, acetylcholinesterase may not be the only target of OP pesticides. In rat plasma, CE was found to function as a scavenger which removes OP-anticholinesterases before they reach their target acetylcholinesterases (Yang & Dettbarn, 1998). This suggests that covalent phosphorylation of the serine esteratic site in both CE and butylesterase enzymes play a pivotal role in the development of tolerance in OP-resistant organisms (Yang & Dettbarn, 1998). If phosphorlyation of CEs does occur as a result of TPP exposure, then irreversible inhibition may indeed be the mechanism by which inhibition occurs. Therefore, acetylcholinesterase inhibition may proceed after available CE enzymes are inhibited (Yang & Dettbarn, 1998).

It has been demonstrated however, that CE exhibited no ageing after inhibition by any OP pesticides tested (Maxwell & Brecht 2001). In their study, OP-inhibited CE exhibited spontaneous reactivation with reactivation rates that decreased as the structural size of the OP increased. In light of this information, CE inhibition by TPP may be reversible, and may act via other means. Moreover, TPP, although clearly an OP, does not appear to share many of the classic traits that other members of this family share, primarily, covalently phosphorylating the serine esteratic site of the target enzymes.

The exposure of salmon to 1mg/L pyridine led to a significant increase (174% over controls) in olfactory rosette tissue CE activity. In contrast, there was no statistically significant induction of liver CE activity from exposure of fish to 1 mg/L of pyridine. No previous experiments on fish have been performed that

have examined the inducibility of CE in various tissues, therefore a comparison cannot be made with other fish species.

Chemical induction is dependent on many factors. Further examination of the results obtained in this study and others lead to the conclusion that the inducibility of CE is highly dependent on several factors including time, inducer concentrations, and possibly species sensitivity.

First, the expression of an enzyme requires transcription and translation of DNA. Although there are no published studies that have documented the time required for CE expression after induction, one may obtain general idea from studies on Cyp1A1. In one study, Cyp1A induction in *Fundulus heteroclitus* was found to be dependent on temperature (Kloepper & Stegeman, 1992). An increase in CYP1A1 mRNA content precedes a gradual increase in CYP1A1 EROD activity, and takes place in a matter of days. In rats, four days of pyridine inhalation exposure was required for measurable increases in nasal CE activity (Nikula et al., 1995). These observations may help explain why there was no significant increase in liver CE activity. Subjects were exposed to pyridine for only a 12-hour period and immediately sacrificed for the determination of CE activity. A longer period of time may be necessary for CE induction in the liver. In this present study, the 12-hour induction period was chosen because a parallel examination of CE induction and inhibition was desired.

The proximity of olfactory rosette CE to the inducer agent may be a factor which explains the inducibility of CE in this tissue vs. liver. Olfactory tissues, by nature are continually bathed in the external environment. Pyridine may have been able to diffuse readily into the olfactory epithelium hence eliciting an

induction response. Somatic concentrations of pyridine conversely, may have not reached a concentration sufficient to induce hepatic CE, possibly due to the time required for pyridine to reach a concentration in somatic tissues that causes hepatic CE induction.

The concentration of CE inducing agents can alter the induction of CEs in a dose-dependent manner (Yang et al., 2001). A dose-dependent decrease in mRNA levels was found in conjunction with an increase in CE levels and activity (Yang et al., 2001). Furthermore, a study examining the effects pyridine vapour exposure on nasal CE induction in rats indicated insignificant induction at 5 mg/L and significant induction at 444 mg/L (Nikula et al., 1985). These studies suggest that the concentration of pyridine used in the present study may not have been high enough to illicit CE induction in the liver.

Species sensitivity may also play a critical role in the inducibility of CE. It is well known that a wide range of drugs and other xenobiotics are capable of altering the expression of CEs. However, induction has now been shown to vary depending on the species in question (Morgan et al., 1994a; Satoh & Hosokawa, 1998; Zhu et al., 2000). Depending on the chemical, some have similar effects across species while other compounds show profound species differences. For example, phenobarbital causes the induction of CEs in both rodents and humans. A different chemical, dexamethasone, suppresses CE expression in rats, while causing induction in humans (Zhu et al., 2000). It is not known if species selectivity plays a role in CE induction by pyridine. Pyridine is known to induce CE in the rat olfactory epithelium and from the current study, coho olfactory rosette CE.

Considering these factors, it is likely that the exposure duration and inducer concentration were sufficient for induction of CE in the olfactory rosette tissue, but were insufficient for induction of liver CE. Further studies that vary the exposure dose and duration should provide a clearer picture of CE inducibility by pyridine.

From the standpoint environmental relevance in the current study, induction of CEs via in vivo exposure or exposure from the natural environment may protect the animal from the effects of an olfactory toxicant. It has also been shown that ester hydrolysis significantly reduced the accumulation of several hydrophobic esters in rainbow trout (Barron et al., 1989; Barron et al, 1990). Metabolism of di-2-ethylhexyl phthalate (DEHP), a widely used commercial plasticizer, by CE present in the gill of rainbow trout lowered the amount of unhydrolyzed DEHP entering and hence accumulating within the fish (Barron et al., 1989). In rats, mice and hamsters, DEHP is also to induce hepatic microsomal CE isozymes (Hosokawa et al., 1994; Sato & Hosokawa, 1998).

Animals exposed to CE inhibitors generally demonstrate increased mortality and sensitivity to xenobiotics; the exception being protoxins. The inhibitory effects of xenobiotics to CE activity have been studied extensively in a variety of animals including rats and fish. Rats treated with a specific CE inhibitor [2-(O-cresyl)-4H-1,3,2-benzodioxaphosphorin-2-oxide (CBDP)] demonstrated between a two to ten-fold decrease in their respective $LD_{50}$'s to the OP phorate (Maxwell et al., 1992). In another study, age-related changes in CE seemed to contribute to the age-dependent toxicity of the acetylcholinesterase inhibiting insecticide chlorpyrifos in rats (Atterberry et al., 1997). With respect to fish, TPP

increased the toxicity of malathion in five species of fish while piperonyl butoxide, a P450 inhibitor, did not (Li & Fan, 1996). When rainbow trout were exposed to the selective CE active-site inhibitor BNPP (Chapter 2), inhibition of CE activity caused a 7-fold increase in the bioconcentration of triclopyr butoxyethyl ester and an eightfold increase for di-2-ethylhexyl phthalate (Karara & Hayton, 1988; Barron, 1989).

Inhibition, with respect to the action of TPP, is especially relevant from environmental point of view. Continued phosphorylation of free CEs, as a result of chronic exposure to CE inhibitors, would reduce the amount of free CE, hence resulting in a cumulative effect. By inhibiting CE in this manner, there would be fewer free CEs remaining to perform its critical functions, foremost being the biotransformation of relevant exogenous xenobiotics. Such a mechanism may have profound implications from the point of view of chronic inhibitor exposure, as a permanent inhibition of free and microsomal CE may render the organism defenseless to many xenobiotics that are detoxified by CE.

With respect to salmon olfaction, a decrease in the detoxification of certain compounds that target the olfactory system may lead to loss of function. Whether induction or inhibition of CE plays a role in potentiating or mitigating the olfactory-inhibiting effects of OPs remains to be established. The next chapter investigates the potential role of CE inhibition and induction on diazinon olfactory toxicity.

# CHAPTER 4

## The Role of Carboxylesterase Activity in Modulating the Effect of OP Toxicity in the Olfactory System

### Introduction

Histological evidence obtained in Chapter 2 shows that CEs are well distributed within the olfactory sensory epithelium of Coho salmon. In addition, it was possible to inhibit and induce the activity of CE in the olfactory epithelium through exposure to TPP and pyridine, respectively (Chapter 3). The environmental relevance of CE inhibition or induction however, is unknown with respect to the ability of salmonids to detect odourants following exposure to the olfactory toxicant diazinon.

*Diazinon as an olfactory toxicant*

The mechanism by which diazinon inhibits the olfactory ability of salmon is unclear. It was suggested that diazinon acts upon the general cell function, sensitivity, or transduction mechanisms of the olfactory receptor cells (Moore and Waring, 1996)

Mentioned previously, diazinon is an olfactory toxicant to fish at concentrations as low as 1 µg/L. This is especially important considering the fact that in-stream concentrations of diazinon have been detected as high as 36.8 µg/L in the San Joaquin river system following rain events (Menconi & Cox, 1994). In farm ditches of the Lower Fraser Valley of British Columbia, diazinon concentrations were much lower, between 0.05 and 0.12 µg/L (Wan et al., 1994).

However, no indication was given if these measurements were taken following precipitation.

*Possible mitigatory action of CEs*

The hydrolysis of many exogenous compounds including pesticides by CEs is well-known (Satoh, 1998). It has been established that the biotransformation of diazinon in many vertebrates is accomplished primarily through CE activity (Yang et al., 1971; Brealy et al., 1980; Lasker et al., 1982; Mackness et al., 1987; Walker et al., 1987). In light of the established effects of diazinon on salmon olfaction, and the ability of CE's to hydrolyse diazinon, there is the distinct possibility in that up or down-regulation of this enzyme system may either potentiate or mitigate toxic effects.

*Assessing the impact of xenobiotics on olfactory performance*

Olfaction in fish is an integrated response and may function as a sensitive measure of the sublethal effects of a chemical (Rand, 1995). Various endpoints in have been used to determine the olfactory toxicity of pesticides. For example, serum 17,20β-dihydroxy-4-pregnen-3-one, testosterone, and gonadotrophin II, can be measured in male salmon pre-exposed to diazinon to determine olfactory performance in response to ovulating female salmon urine (Moore & Waring, 1996, 1998; Waring & Moore, 1997; Moore & Lower, 2001). The feeding and swimming activities of fish exposed to olfactory toxicants and subsequent alarm pheromone exposure may also be used (Scholtz et al., 2000). Other behaviours

suitable for use as endpoints in olfactory toxicity include swimming patterns (eg. preference/avoidance patterns [Saglio et al., 2000]).

*The stress response in fish*

The stress response in fish, mediated by the hypothalamo-sympathetic-chromaffin cell (HSC) and hypothalmo-pituitary interrenal cell (HPI) axes, is comprised of a coordinated set of behavioural and physiological responses thought to be compensatory and adaptive, enabling the animals to overcome a threat (Wendelaar, 1997). The HSC axis mediates the release of catecholamines into the circulation, which increases cardiac output, blood flow to muscle and gills, respiration and mobilization of energy reserves. The HPI-axis mediates the release of cortisol into the circulation and is highly correlated with the stress response in fish (Wendelaar, 1997). As a result of endocrine stimulation the increase in general body metabolism, heightened optical acuity, and mobilization of energy reserves together may provide the fish with additional energy for emergency situations requiring a "fight or flight" response (Reubush & Heath, 1996),. The stress response can thus be triggered by detection of alarm substance by fish (Scott et al., 2003).

*Stress as an assessment endpoint*

Stimulation of the HSC-axis, and HPI-axis can occur by stimulation of the olfactory system through exposure to alarm substance (Wendelaar, 1997). The stress response therefore, may be used as an assessment endpoint for the determination of olfactory performance. The correlation between stress and

stimulation of the olfactory organ by alarm substances suggests that plasma cortisol concentration, which increases during stress, may be used as a measurement endpoint to determine indirectly the ability of fish to smell (Ackerman et al., 2000; Scott et al., 2003).

This experiment was designed test the idea that the biotransformation of diazinon by CEs is a key factor in determining the ability of diazinon-exposed salmonids to detect alarm-based pheromones. Decreasing visual acuity, by increasing turbidity of the water, has been found to increase the reliance of fish on olfaction (Huber & Rylander, 1992; Van Staaden *et al.*, 1995; Hartman & Abrahams, 2000). Consequently, an increased reliance on olfaction from increased turbidity may enhance the reliability of plasma cortisol levels in the determination of olfactory performance. Therefore, a method was developed, to test how the inhibition and induction of CE affects the ability of coho salmon to detoxify diazinon and modulate its effects on the salmon olfactory system. By inhibiting CE activity, very little metabolism of diazinon should occur which should then result in pesticide-induced anosmia with no stress responses (elevated cortisol) to alarm-scent exposure.

## Methods and Materials

*Fish*

Coho salmon (20 weeks-old, 3-5 g) were obtained from the Capilano Hatchery (North Vancouver, BC) and maintained as described previously in Chapters 2 and 3.

For this particular experiment, fish were placed individually, in respective 20 L aerated glass tanks. These tanks were of a flow-through design with an average flow rate of 0.6 L/min maintained at 14±1$^{O}$C and a photoperiod of 16:8 day/night. Tanks were covered with a combination of plastic mesh and black plastic to provide shelter and isolate them visually

*Chemicals*

TPP (1 mg/L) and pyridine (1 mg/L) were successfully used as an inhibitor and inducer of olfactory rosette CE respectively, as the experiments described in the previous chapter. As in the previous chapter, both chemicals were prepared for use in a stock solution of 1mg/mL with acetone. A cortisol ELISA kit (Neogen Corporation, product #402710, KY) was used for the determination of plasma cortisol concentration.

*Stress water (Alarm substance)*

Stress water which contains alarm pheromone can be obtained from fish in a stressed situation (Toa, 2003). Water was prepared by transferring 35, 90-week old salmon smolts into a 150 L plexiglass tank containing locally dechlorinated water. Fish were repeatedly chased with a broom-stick for 5 minutes. This water (containing alarm pheromone) was then ready to use for the experiment as is.

*Exposures*

In order to determine the effects of diazinon exposure in the presence of CE inhibitors and inducers, fish were divided into eight treatment groups: 1) Turbidity + Acetone (carrier) control (TAC), 2) Turbidity + Stress Water control (TSC), 3) Diazinon + Turbidity control (DTC), 4) Diazinon + Turbidity + Stress Water control (DTSC), 5) TPP + Diazinon + Turbidity control (TDTC), 6) TPP + Diazinon + Turbidity + Stress Water (TDTS), 7) Pyridine + Diazinon + Turbidity control (PDTC), and 8) Pyridine + Diazinon+ Turbidity Stress Water (PDTS). TPP and pyridine concentrations were 1 mg/L in tanks. Exposure to TPP or pyridine was 12 h prior to exposure to diazinon. Acetone-carrier alone was used in the carrier, and stress control tanks (TAC and TSC tanks). Fish were acclimated in tanks for 7 days prior to exposure.

Following the 12-hour exposure period, diazinon was added to the DTC, DTSC, TDTC, TDTS, PDTC and PDTS exposure tanks to achieve a final concentration of 10 µg/L. Exposure was continued for an additional 2.5 hours together with the inhibitor or inducer. The concentration and exposure time used was based on a study by Moore and Waring (1996). In their study, perfusion of the olfactory epithelium for 30 mins with 1ug/L diazinon was sufficient to decrease the olfactory response to PGF2a by 70%.

To reduce the visual clarity of the water (to heighten reliance on the olfactory system for sensory inputs), a bentonite clay slurry was added to all experimental tanks after the 2.5 hour diazinon exposure period. This slurry was prepared by adding 10 g of dry bentonite clay directly to each 20 liter exposure tank. The fish were then allowed to swim in the clouded experimental water for 1 hour together with the inducer or inhibitor, and diazinon.

When fish had been exposed to the appropriate chemicals or combinations, they were exposed to stress water by first siphoning off 50% (9 L) of the experimental tank volume and replacing it with the stress water preparation via an intake pipe. This procedure was repeated again such that the majority of water that contained the xenobiotics and diazinon was replaced with the stress water. Fish were exposed to the stress water for a period of 1 hour. Each exposure tank contained solution-introduction tubing as well as exit tubing. This allowed for the introduction of solutions and draining of water to prevent exposure of the fish to additional stress.

*Blood sampling*

Immediately following the 1-hour exposure of fish to the stress water, fish were removed, stunned by a blow to the head, and blood extracted from the caudal vasculature by heparanized microcentrifuge tubes and spun in a haemofuge for 5 min. Following centrifugation, the plasma was removedand stored at –80$^{o}$C freezer until analysis.

*Determination of plasma cortisol concentration*

Plasma cortisol concentration was determined using an ELISA kit (Neogen Corporation, MI). The plasma was first diluted 200x with diluted extraction buffer. Fifty μL of each diluted plasma sample was then pipetted into respective microplate wells together with 50 μL of diluted enzyme conjugate. Samples were assayed in triplicate. Contents in the microplate wells were mixed by gently

shaking on a tabletop. The plate was then covered with plastic film, and incubated at room temperature for 1 hour. Following incubation, the microplate contents were disposed of and the plate then tapped out on paper towels to eliminate any remaining solution in the wells. Each well was then washed with 0.3 mL of the diluted wash buffer and disposed of again. This washing procedure was repeated twice. Colour-developing substrate (150 µL) was added to each well immediately following washing and incubated for 30 min at room temperature. Following incubation, the plate was read at 650 nm on a plate reader (Molecular Devices SpectraMax 340 Nanometer plate reader, Sunnyvale, CA). Absorbances were compared to a cortisol standard curve. Cortisol standards used for this assay contained 0, 0.04, 0.1, 0.2, 0.4, 1, 2, and 10 ng/mL of cortisol in EIA buffer. Blanks for both assay standards consisted of substrate only.

*Data analysis*

Following determination of plasma cortisol concentration a one-way ANOVA, using Microsoft Excel 2000 ANOVA single factor (Bluman, 1995), was used to compare the results from each experimental group with the turbid-acetone control. All activity units were expressed as ng of cortisol per mL of plasma.

## Results

*Fish symptomology*

As described in Chapter 3, exposure of fish exposed to 1 mg/L TPP showed some signs of sublethal toxicity. Symptoms included slight lethargy, congestion, degeneration and haemorrage of the smaller blood vessels. Neither fish exposed to pyridine (1 mg/L) nor diazinon (10 µg/L) showed signs of sublethal toxicity.

*Plasma cortisol concentration*

The mean plasma cortisol concentrations in fish in each treatment group are shown in Figure 4.1. There were no statistically significant differences in plasma cortisol concentrations between the experimental groups and the turbid-acetone control. Furthermore, concentrations of plasma cortisol did not follow the expected pattern of distribution.

**Discussion**

The use of plasma cortisol concentrations to determine, by indirect means, the effect of *in vivo* pesticide exposure on salmon olfaction has been successfully performed on juvenile rainbow trout in a previous study (Scott et al., 2003). In that study, exposed to 2ug Cd/L for 7 days showed no antipredatory behavioural responses to alarm substance (Scott et al., 2003). This observation was characterised by a short-term elevation in plasma cortisol in response to alarm substance under control conditions, and an inhibition in plasma cortisol elevation in cadmium-exposed fish.

In the current study, no discernable effects were observed between any treatment groups, and the turbid-acetone control (TAC). It was expected that

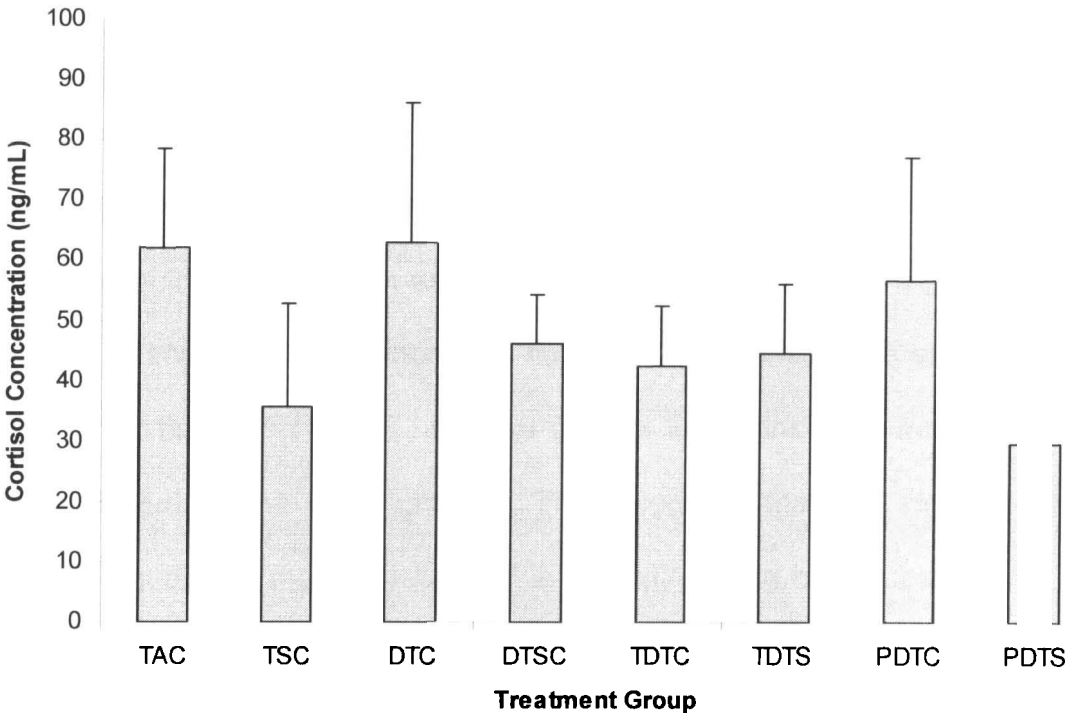



Figure 4.1. Plasma cortisol concentration in various treatment groups: No statistically differences were detected between the treatment groups, and the turbid-acetone control (TAC). Acetone carrier control cortisol concentrations were elevated compared to published control cortisol values. Groups are as follows: 1) Turbidity + Acetone (carrier) control (TAC), 2) Turbidity + Stress Water control (TSC), 3) Diazinon + Turbidity control (DTC), 4) Diazinon + Turbidity + Stress Water control (DTSC), 5) Triphenylphosphate + Diazinon + Turbidity control (TDTC), 6) Triphenylphosphate + Diazinon + Turbidity + Stress Water (TDTS), 7) Pyridine + Diazinon + Turbidity control (PDTC), and 8) Pyridine + Diazinon+ Turbidity Stress Water (PDTS).

plasma concentrations in the TAC would be low, and turbid-alarm-scented controls (TSC) would have been higher. This is because the unscented water would not have elicited an antipredatory responses while scented water would have increased stress and plasma cortisol levels.

Based on the result of Scott et al. (2003) plasma cortisol concentrations of the the turbid-diazinon (DTC) and turbid-diazinon-stress water (DTSC) control groups, as well as the TPP-diazinon-turbid-alarm-scented (TDTG) and its control (TDTC), should all have been as low as the TAC group. Such a plasma profile would have implied that diazinon was causing an anosmic state and preventing detection of the alarm scent. Plasma cortisol levels instead, were high in all groups, including the TAC group. This suggests that an element in the experimental design may be responsible for maintaining high stress levels in all fish. Plasma cortisol concentrations were comparable to other studies in stressed fish (Pottinger et al., 1999; Pottinger et al., 2002; Scott et al., 2003).

Increases in plasma cortisol concentrations, as a result of stress, follows similar patterns whether it is caused by chemical contaminants, or physical stress originating from confinement or handling (Barton & Iwama, 1991). Blood cortisol levels typically increase within minutes of stress and while still imposed, these levels may remain elevated or may begin to decrease after a period of time depending on the type and intensity of the stress (Barton & Iwama, 1991; Folmar, 1993). In salmonids, such cortisol levels rarely exceed 200-300 ng/mL even after exposure to severe stressors (Barton & Iwama, 1991). In a study by Ackerman et al. (2000), plasma cortisol levels of cutthroat trout peak at 115 ng/mL one-hour

after exposure to a 45 second handling stress. Control plasma cortisol levels were 0 and 4 ng/mL respectively in the above two studies.

It was difficult to draw conclusions with respect to this study because plasma cortisol levels were elevated in all groups compared to control values in the published literature (Ackerman et al., 2000; Scott et al., 2003). This observation would suggest that all groups had experienced a high degree of stress. In all groups stress could have been caused by several factors. First, the process of emptying and filling the tanks during exposure to the stress-water may itself have been a stressor. Second, another likely cause of an increased level of stress may have taken place during the final capture and sampling of the animals. The most accurate measurement of stress should theoretically take place when plasma samples are obtained without stressing the fish. This was not entirely the case however, as it was difficult to capture the fish in the turbid test water. In several instances, it took a few minutes of searching by net in order to find the fish. Again, plasma cortisol levels are can rise rapidly within 45 seconds after exposure to an acute stressor (Barton & Iwama, 1991; Wendelaar, 1997) which provides a limited window from which to obtain the plasma sample. The excessive time lag from fish capture to plasma sampling most likely resulted in an increase in plasma cortisol levels. Thirdly, the loss of visual acuity as a result of the introduction of turbidity in all exposure groups may in itself have caused a stress response. Clearly, several elements in this particular experiment, especially those pertaining to stress induction and blood extraction, may need to be redesigned in order to reduce ambient stress levels in the fish. Future experiments may also focus on the use of individual pheromone compounds that

may be added directly to the exposure tanks. This would avoid the excessive filling and emptying of tanks as required in this study.

In summary, no significant differences in plasma cortisol levels were detected between control and fish exposed to alarm substance or chemicals. This was most likely to high ambient stress caused by the experimental design of this study.

# Chapter Five: Summary and Conclusions

A number of studies have shown that CE activity is responsible for mitigating the toxicities of OPs such as diazinon. Because of the importance of CE activity with respect to xenobiotics biotransformation, the susceptibility of the olfactory system to this pesticide, and its widespread use of in agriculture, the overall aim of this study was to determine the significance of CE activity to the olfactory toxicity of diazinon in Coho salmon. The major findings of this project were:

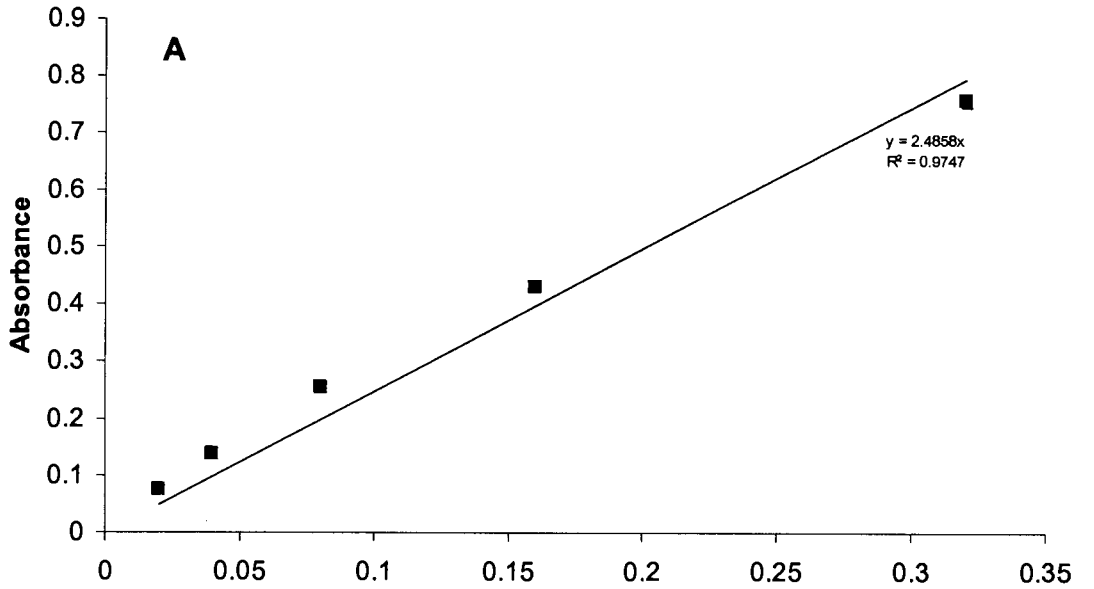
1) The present study is the first to report carboxylesterase activity in the teleost olfactory epithelium.
2) Carboxylesterase activity was histochemically localized to the olfactory sensory epithelium of the olfactory rosette. No activity was present in the non-sensory epithelium of the primary and secondary lamellae.
3) The activity of carboxylesterase in the olfactory rosettes of Coho was 25.5 nmol/min/mg protein and can be significantly induced and inhibited by pyridine and TPP respectively.
4) Differences in plasma cortisol levels between control and treatment groups after *in vivo* induction and inhibition of carboxylesterase activity and subsequent exposure to the olfactory toxicant diazinon and alarm substance were inconclusive. Results indicated that experimental conditions were responsible for the increased ambient plasma cortisol

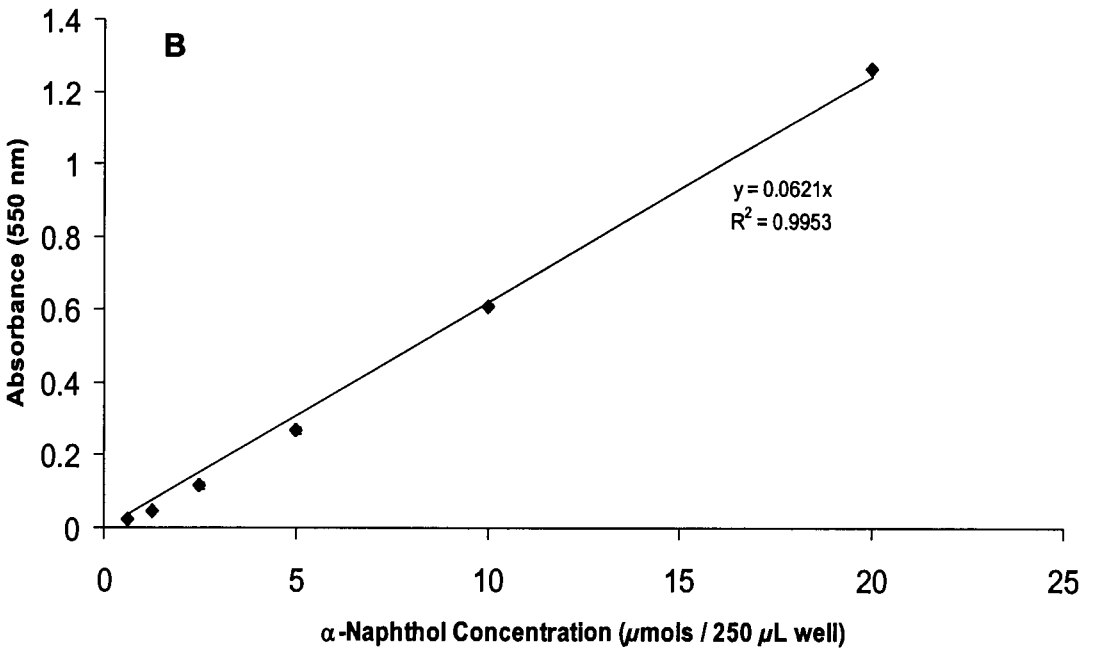
levels. This prevented an accurate determination of stress levels between treatment groups.

The presence of CE in the olfactory epithelium is a feature that is generally conserved in almost all vertebrates and is now confirmed in teleosts. As pointed out earlier, one of the functions of biotransformation in the olfactory epithelium is to prevent the excessive stimulation of olfactory receptors odourants through the metabolism of odourants. It was suggested in this study that biotransformation may also serve to detoxify olfactory toxicants. The induction and inhibition of CEs, as demonstrated in this study, shows that an interaction between xenobiotics and olfactory epithelium CE does indeed take place. Whether or not this interaction is relevant in the scope of salmon survival is unclear as the results in the behavioural portion of this study were inconclusive. Further studies that focus on how xenobiotic-CE-olfactory toxicant interactions affect salmon behaviour are necessary.

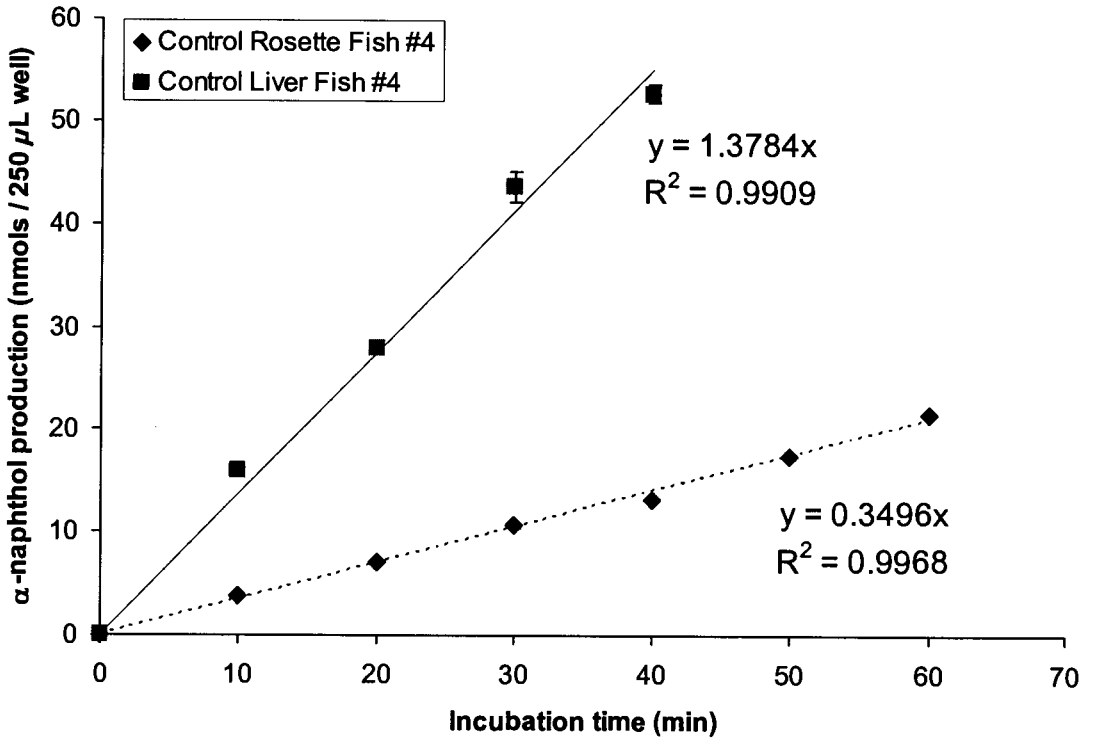
# Appendix

**Appendix A:** **Standard curves for bovine serum albumin (A) and α-naphthol (B) used in the determination of protein content and α-naphthol production**



**Appendix B:** **Control α-NA-CE curves for liver and olfactory rosette homogenate obtained from from one fish Liver (♦), Rosette (■)**



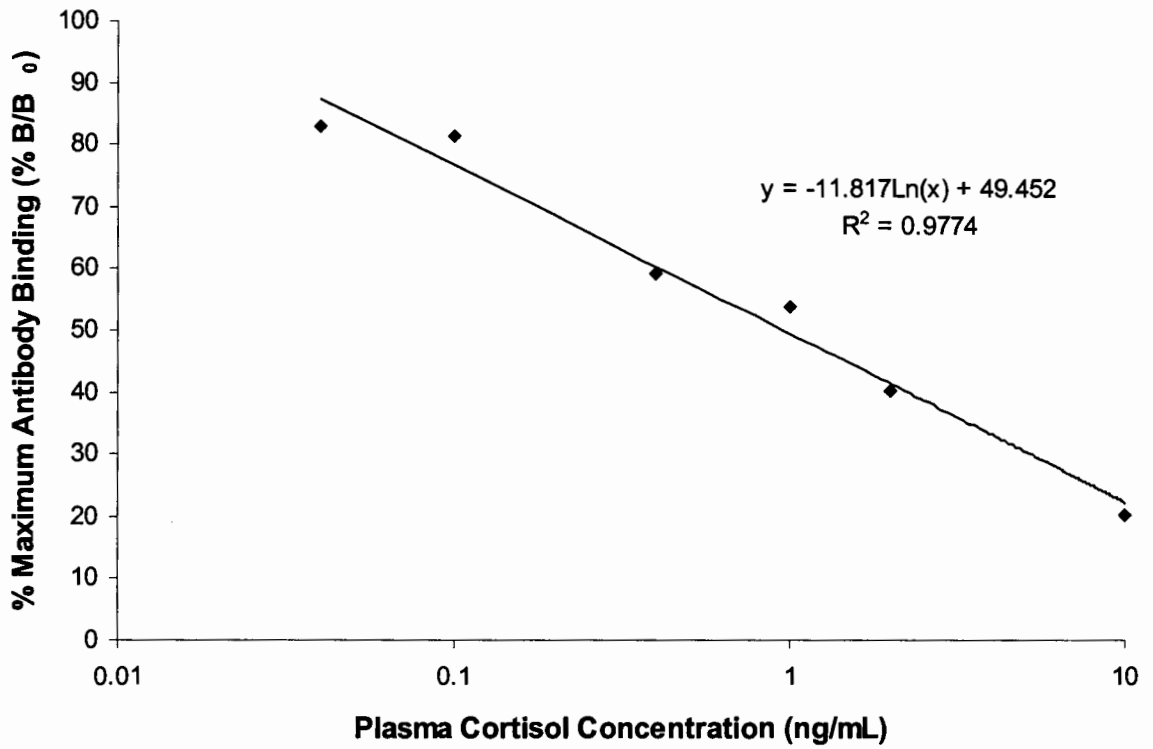
## APPENDIX C: Principle of CE activity determination and localization

1) $\alpha$ – naphthylacetate (or butyrate) $\xrightarrow{\text{CE}}$ $\alpha$ – naphthol

2) $\alpha$ – naphthol + diazonium salt (pararosaniline or fast red GBC) $\rightarrow$ highly coloured deposits

(activity determined by measuring deposit concentration via spectrophotometry)

**APPENDIX D:** **Plasma cortisol standard curve. $\%B/B_0$ is the percentage absorbance of the plasma cortisol sample absorbance (B) to the blank absorbance ($B_0$).**



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