THE IMPACTS OF AGRICULTURAL CHEMICALS AND TEMPERATURE ON THE PHYSIOLOGICAL STRESS RESPONSE IN FISH

AMIE L. QUINN
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Department of Biological Sciences
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Abstract

Fish are exposed to multiple stressors in their environment. The interactive effects of pesticide exposure and increased temperature on the physiological stress response were investigated in a comparative field study with cold-water (whitefish, *Prosopium williamsoni*) and cool-water (sucker, *Catostomus*) fish from the Oldman River, Alberta, Canada, and in a laboratory study with rainbow trout, *Oncorhynchus mykiss.* Physiogical stress indicators were measured, and exposure to pesticides was estimated using acetylcholinesterase (AChE) inhibition. Species-specific differences in AChE activities and responses of the physiological stress axis were detected in whitefish and suckers, suggesting that whitefish are a more sensitive species to temperature and pesticide stress. *In vivo* Dimethoate exposure inhibited AChE activity in various tissues and disrupted the physiogical stress response. Commercial Dimethoate, *in vitro*, caused a decrease in viability and cortisol secretion while pure grade Dimethoate did not. The results from this study can be used in predictions of fish vulnerability to stress.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
ACTH	adrenocorticotrophic hormone
CB	carbamates
CBG	corticosteroid-binding globulin
ChE	cholinesterase
CRH	corticotrophin releasing hormone
DAG	diacylglycerol
EB	ethylene bisdithicarbamate,
ER	endoplasmic reticulum
Ft. M	Fort MacLeod
HPI	hypothalamo-pituitary-interrenal
LDL	low-density lipoproteins
Mon	Monarch
Na^+/K^+-ATPase	Na^+/K^+-dependent adenosine triphosphatase
OC	organochlorine
OP	organophosphorus insecticides
Pav	Pavan
Per	Pearce Road
PKA	protein kinase A
PKC	protein kinase C
Pop	Popson Park
PY	pyrethroid
StAR protein	Steroidogenic Acute Regulatory protein
Sum	Summerview
TR	triazine

Chapter 1.

Effects of pesticides, temperature and fish species on the physiological stress response – literature review

1.1 Introduction

Fish live in a wide range of habitats all over the world. However, many of their natural habitats are being altered by anthropogenic influences, including physical structures (dams and weirs), agricultural runoff, industrial waste, and urban pollutants. Rivers and lakes are also threatened by global climate change which could lead to changes in water temperature. Fish are exothermic and exposures to warmer water temperatures, particularly in cold-water fish species, can significantly alter their physiological status. Invasion by non-native species is another modification of fish habitat, potentially linked to climate change. Any significant alteration to fish habitat, whether it is physical, chemical or biological, can lead to stress, and prolonged or high intensity exposures to stressors have the potential to impact fitness at individual and population level. Different species of fish may respond to specific stressors differently - while some fish may be resistant to certain types of stress, others may be very sensitive. These species-specific responses can disrupt equilibrium that exists within an aquatic system; however our understanding of the various interactions is limited at present. *This literature review will focus on how the physiological stress response is regulated, how pesticides and temperature affect the stress response, and how different fish species respond to these stressors.*

1.2 Physiological Stress Response

1.2.1 Adrenal Endocrinology - Structure and Function of the Teleostean Head Kidney

a. Anatomy

The physiological stress response is mediated by the adrenal system in all vertebrates; however, the structural organization of the adrenals varies. The adrenal gland of mammals is separated into a medulla composed of chromaffin cells secreting catecholamines (epinephrine and norepinephrine) and a cortex composed of adrenocortical cells secreting steroids, including cortisol. In teleosts, the anterior portion of the kidney, known as the head kidney, is analogous to the mammalian adrenal gland (Norris 1997, Mommsen et al. 1999, Hontela 1997, 2005). The head kidney is not separated into a medulla and cortex but there is close physical association between the chromaffin and adrenocortical (also known as interrenal) cells. The head kidney, which has lost its renal function, consists of interspersed islets of adrenocortical cells that are embedded in a matrix of lymphoid tissue (Hontela 2005).

b. Signalling Pathway Leading to Cortisol Secretion

The secretion of the stress hormones corticosteroids is regulated by the hypothalamo-pituitary-interrenal (HPI) axis in teleost fish, as well as other vertebrates. When an organism is exposed to a stressor, within a few seconds the corticotrophin releasing hormone (CRH) is released from the hypothalamus and stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland (Hontela 2005). The ACTH then stimulates the synthesis and release of corticosteroid hormones, including cortisol, from the adrenals. The biochemical pathways and synthesis of corticosteroids in

teleosts are not as well characterised as those in mammals, but the experiments that have been conducted show a similarity to mammals.

In all vertebrates studied, ACTH targets the adrenocortical cells in the adrenal gland, binds to membrane receptors and stimulates pathways which mobilize cholesterol for production of steroid hormones (Stocco 2000). In mammals, the main pathway involves cAMP and protein kinase A (PKA) while the other involves protein kinase C (PKC) and diacylglycerol (DAG) (Stocco et al. 2005). The importance of ACTH, cAMP, PKA and PKC in the synthesis of cortisol has been also demonstrated in teleost fishes (Patiño et al. 1986, Leblond et al. 2001, Lacroix & Hontela 2001). The cAMP-PKA pathway is activated when ACTH binds to receptors on an adrenocortical membrane and activates G proteins to stimulate adenylate cyclase (Colby & Longhurst 1992, Hontela 1997, Lacroix & Hontela 2001, Stocco et al. 2005). Stimulation of adenylate cyclase increases intracellular levels of cAMP which activates PKA. PKA activation results in the phosphorylation of proteins and transcription factors that are responsible for the activation of the enzyme cholesterol ester hydrolase (Colby & Longhurst 1992, Hontela 1997, Stocco et al. 2005). Cholesterol ester hydrolase releases free cholesterol from cholesterol esters. Cholesterol is supplied to the adrenocortical cells by circulating low-density lipoproteins (LDL) droplets and *de novo* synthesis in the adrenocortical cells (Norris 1997; Stocco 2000). Once cholesterol is present in the outer mitochondrial membrane, it is transported into the inner membrane by the Steroidogenic Acute Regulatory (StAR) protein to be converted into pregnenolone by the side-chain cleavage enzyme, P-450_{scc} (Norris 1997; Geslin & Auperin 2004). Pregnenolone then moves to the smooth endoplasmic reticulum (ER) where it goes through further transformations

(Norris 1997). The conversion of cholesterol into pregnenolone is the rate limiting step in the production of many of the corticosteroids (Colby & Longhurst 1992, Stocco 2000).

c. Cortisol Feedback and Degradation

Secretion of corticosteroids is controlled by a negative feedback loop involving both CRH and ACTH (Colby & Longhurst 1992, Norris 1997, Hontela 1997). When plasma levels of ACTH and corticosteroids are high, they inhibit the release CRH and ACTH, decreasing cortisol synthesis (Colby & Longhurst 1992, Norris 1997, Hontela 1997). The majority of cortisol that is released from the adrenals is bound to a specific plasma protein called corticosteroid-binding globulin (CBG) (Norris 1997, Mommsen et al. 1999, Hontela 2005). Occupied CBG can interact with a target cell membrane which then facilitates dissociation of the glucocorticoid and allows its entrance into the target cell (Norris 1997). The main site for glucocorticoid degradation is in the liver but some degradation may also take place in the kidneys and intestine (Norris 1997, Mommsen et al. 1999).

Glucocorticoids, including cortisol, are metabolized by a series of enzymatic reactions, including transformations by cytochrome P-450 (Norris 1997, Mommsen et al. 1999), increasing water solubility of the steroids and facilitating their excretion via urine, bile or feces.

1.2.2 Physiological Effects of Corticosteroid Hormones

The main corticosteroid in teleosts is cortisol, a steroid that plays a major role in maintaining energy stores and energy mobilization in times of stress (Norris 1997, Hontela 1998, Mommsen et al. 1999, Schreck et al. 2001, Barton 2002). Teleost fish do

not have high levels of mineralocorticoids such as aldosterone, and cortisol has metabolic as well as osmoregulatory effects (Hontela 2005).

a. Metabolic Actions of Cortisol

The main effect of cortisol is energy mobilization by the metabolism of carbohydrates, proteins, amino acids and lipids (Norris 1997). Studies with teleost fish often rely on plasma glucose and liver glycogen stores as indicators of energy metabolism due to stress or exposure to increased cortisol levels. The metabolic actions of cortisol were reviewed by Mommsen et al. (1999). Glucocorticoids are known to increase plasma glucose through degradation and mobilization of proteins, releasing amino acids which are then used through increased activity of gluconeogenic enzymes in hepatic gluconeogenesis. In the liver, cortisol may have some glycogenolytic effects as well; however catecholamines such as epinephrine are mainly responsible for the mobilization of liver glycogen during stress. It has been suggested that during stress, cortisol and insulin interact to not only prevent glycogen breakdown but also increase its synthesis. Thus, as summarized in Mommsen et al. (1999), the overall effect of cortisol in a stressed fish is liver glycogen conservation. Cortisol action on muscle glycogen is not very well understood, but it had been proposed that cortisol may play a role in exercise-induced muscle glycogen mobilization (Milligan 2003). The differences in the stress response and cortisol actions in different species of fish might underlie the variability in responses to cortisol.

b. Osmoregulatory Actions

In freshwater fish cortisol regulates sodium fluxes and stimulates the transfer of sodium across the gill, the mucosa in the gut and the kidney (Norris 1997). The Na^+/K^+-

dependent adenosine triphosphatase (Na^+/K^+-ATPase) and Ca^{2+}-ATPase in the gills, gut and kidney is increased when fish are treated with cortisol (Norris 1997). Cortisol is also known to stimulate the branchial calcium pump and increase calcium absorption by the whole body in freshwater rainbow trout, suggesting that cortisol plays a role in fresh water acclimatization (Mommsen et al. 1999). Cortisol is known to increase during the parr-smolt transformation in salmonids, enhancing the hypo-osmoregulatory ability when anadromous fish move into seawater (Mommsen et al. 1999). Cortisol also facilitates the adaptation of salmonids to seawater by stimulating cellular differentiation of chloride cells and branchial and gill Na^+/K^+-ATPase activity (Mommsen et al. 1999).

c. Reproductive Actions

Cortisol inhibits reproductive functions in both mature and immature fish by affecting production of vitellogenin and oestradiol binding capacity (Mommsen et al. 1999). The stress response increases the amount of cortisol circulating within the body, potentially having a negative effect on the female through decreased gamete quality and progeny fitness (Schreck et al. 2001). Tilapia (*Oreochromis mossambicus*) implanted with cortisol had smaller oocytes, reduced levels of testosterone and 17β-estradiol concentrations (Schreck et al. 2001) and produced smaller larvae (Barton 2002). The effects of these studies clearly show that cortisol has an adverse impact on the reproductive system in many types of fish, and increasingly stressed fish populations may be at a risk of reduced survivorship.

d. Immune Actions

Cortisol production has a negative influence on the immune system in most teleost fish (Barton & Iwama 1991, Hontela 1998, Schreck et al. 2001). The most

extreme example of cortisol's negative impact may be observed in Pacific salmon which all die after spawning. During spawning, Pacific salmon lose control of the pituitary regulatory influences on cortisol secretion by the head kidney and it has been suggested that there is a decrease in the ability to remove cortisol from circulation (Schreck et al. 2001). Cortisol also inhibits the ability of the fish to resist disease or pathogens. High levels of cortisol are known to suppress the ability of fish to form antibodies because the production of leukocytes is depressed (Barton & Iwama 1991, Hontela 1997, Schreck et al. 2001). Studies in brown trout (*Salmo trutta*) have shown that handling stress can cause a significant decrease in leukocytes (Barton & Iwama 1991). When fish are removed from the stressful situation that is causing elevated cortisol levels, the immune system can return to normal (Barton & Iwama 1991, Schreck et al. 2001).

Cortisol also interacts with other hormones, including thyroid hormones, growth hormone and catecholamines (Hontela 1997), particularly during exposures to stressors. The remainder of this review will examine stress with emphasis on cortisol in relation to pesticides, temperature, and species differences. *The effects of multiple stressors (specifically pesticides and temperature) on the physiological stress response and how these stressors affect different species of fish, have not been investigated. These interactions will be the main focus of this thesis.*

1.3 Pesticides

1.3.1 Pesticide Uses and Application

Pesticides are chemicals used to control pests; their primary use is to increase agricultural yields and prevent the spread of vector-borne disease that have implications

to health and the economy (Hayes 1991). Pesticides do not include antibiotics. Pesticides can be classified based on the pest that they are targeting, and include algicides, fungicides, herbicides, insecticides, molluscicides, nematicides, and rodenticides. They are also categorized by their chemical structure; the most commonly used pesticides are organophosphates, carbamates, organochlorines, pyrethroids, triazines, ureas, and aryloxyalkanoic acids (Stenerson 2004).

Pesticides are generally applied during the growing season, but the application times depend on weather, the targeted pest and local development of the pest (Frank & Sirons 1979, Trim & Marcus 1990, Fairchild et al. 1999). Pesticides in Southern Alberta have been detected in the air from April until October (Tuduri et al. 2006b). Waite et al. (1995) found the highest deposition amounts of 2, 4D in mid-June and highest deposition amounts of Triallate in mid-May. Traces of pesticides found outside application areas (e.g. watershed) are usually at the highest concentrations during planting season (Frank & Sirons 1979, Tuduri et al. 2006b). Pesticides are often detected in water due to direct application in water, storm runoff, irrigation tail-water releases, and spray drift or vapour transport (Raupach et al. 2001a, b, Tuduri et al. 2006a, b).

1.3.2 Pesticide Exposure

The main routes of exposure to pesticides are through the integument, ingestion and inhalation, or in fish, uptake through gills (Brooks 1976). Depending on the physical properties of the pesticide, it may enter the blood stream and be carried in solution, bind to proteins, or dissolve in lipid particles (Brooks 1976). Distribution of pesticides and their metabolites depends on their ability to cross cell membranes (Brooks 1976). Many

pesticides biotransform by chemical conversion or enzymatic biotransformation; transformation may increase or decrease their toxicity (Brooks 1976). Original or converted pesticides may need to undergo further transformations into water-soluble conjugates in order to be excreted (Brooks 1976).

Although the use of pesticides has great societal benefits, there are many problems associated with them. Pesticides are biologically active chemicals that pose potential harm to non-target organisms (Hayes 1991). People who are involved with the manufacture or use of pesticides are at high risk of exposure (Marrs 1993). Pesticides can also affect domestic and wild animals that are exposed either directly to the pesticide, its runoff, or by eating contaminated food (Brooks 1976, Hayes 1991). Acute pesticide exposure due to runoff caused reductions in fish populations and large scale fish kills (Trim & Marcus 1990, Fairchild et al. 1999, Fulton et al. 1999). Fairchild et al. (1999) determined that the pesticide Matacil 1.8 was responsible for population reductions of Atlantic salmon in New Brunswick and Newfoundland. Chronic exposures to small amounts of pesticides are known to increase incidence of disease, increase stress, and cause behavioural changes in fish (Austin 1999). *This review will focus on OPs and CBs, and their chemical make-up, and mode of action.*

1.3.3. Organophosphates and Carbamates

a. History and Uses

Organophosphorus insecticides (OPs) were developed in Germany during the 1930s as potential chemical warfare agents (Fulton & Key 2001). The OPs together with carbamates (CBs) (Figure 1.1) have become the most widely used classes of insecticides

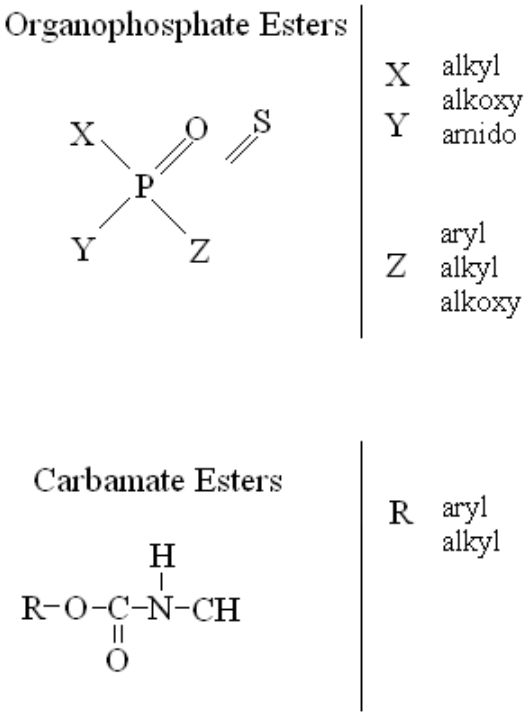

Figure 1.1. The basic structure of organophosphate and carbamate pesticides.

worldwide (Fulton & Key 2001). In 1998, there were 15 types of carbamates and 34 types of OPs sold in Alberta (Byrtus 2000). They are used to control insects on fruits, vegetables, grain crops, and stored seeds, and in the household, to control cockroaches, houseflies, termites, and protect plants of horticultural interest (Fulton & Key 2001). The OPs and CBs have replaced many organochlorine pesticides because they are less persistent in the environment and biomagnification is not a major issue (Tuduri et al. 2006b). A few problems associated with OPs and CBs are their lack of specificity and high water solubility; they are able to enter the aquatic environment where they are toxic to both vertebrates and invertebrates (Tuduri et al. 2006b). Furthermore, under specific conditions, some OPs remain active in soils for up to six months after application, thus increasing the duration they may be harmful to non-target species (Köprücü et al. 2006). OPs have several mechanisms of degradation in natural waters such as oxidation, photodegradation, and hydrolysis, described in a review by Pehkonen & Zhang (2000).

b. ACh and AChE Function in the Nervous System

The OPs and CBs act through the nervous system, inhibiting cholinesterase (ChE) enzymes (Stenerson 2004). Communication between neurons and nerve, muscle or endocrine cells is chemically mediated - an impulse causes the release of the acetylcholine (ACh) from the axon terminal of a presynaptic neuron into the synaptic cleft (Shankland 1976, Stenerson 2004). ACh binds to a receptor on the postsynaptic membrane and the chemical signal is converted into an electrical signal (Shankland 1976). The electrical signal continues until ACh is removed from the receptor on the postsynaptic membrane by acetylcholinesterase (AChE). AChE hydrolyses ACh into acetate and choline, thus stopping the electrical signal to the postsynaptic membrane

(O'Brien 1976, Beauvais et al. 2001). AChE hydrolyzes ACh by forming an enzyme-substrate complex where ACh acetylates AChE and releases choline (O'Brain 1976, Stenerson 2004). AChE is then hydrolyzed and restored (Stenerson 2004); this reaction occurs very fast (O'Brian 1976, Sternerson 2004). There are two main types of cholinesterase enzymes: acetylcholinesterase (true) and butyrylcholinesterase (pseudo). *This review and thesis will focus on acetylcholinesterase.*

c. OP/CB Mode of Action – AChE Inhibition

OPs and CBs inhibit AChE by targeting the serine hydroxyl group on the AChE active site where they bind to and inactivate the enzyme (Grue et al. 1991, Jett et al. 1993, Abbas & Hayton 1997, Stenerson 2004). OPs form a complex with AChE similar to the enzyme-substrate complex made with ACh, but OPs phosphorylate the serine hydroxyl group (O'Brian 1976, Marrs 1993). The reactivation of AChE by dephosphorylation is very slow (considered irreversible) and controls the overall rate of the reaction (O'Brian 1976, Marrs 1993). CBs act differently than OPs by carbamylating the serine hydroxyl group of AChE. The reactivation by decarbamylation step occurs slowly but is not considered irreversible (O'Brian 1976). When AChE is phosphorylated or carbamylated, it is prevented from hydrolyzing ACh which accumulates in the synaptic cleft (Jett et al. 1993, Marrs 1993, Stenerson 2004). The activation of receptors is thus prolonged, leading to an overstimulation of the postsynaptic cholinergic receptors (Jett et al. 1993, Liu & Pope 1996, Jones et al. 1998) and death.

d. Recovery and Adaptations to OP/CB Exposure

Recovery of AChE inhibition by OPs can occur by dephosphorylation or synthesis of new AChE, which are both slow processes (Grue et al. 1991). Recovery of

brain AChE inhibition in mosquitofish (*Gambusia affinis*) exposed to OPs occured between 45 and 60 days post exposure, however, muscle AChE only partially recovered and there was no recovery of liver AChE (Carr et al. 1997). There is evidence that some organisms develop adaptations to AChE inhibition (Beauvais et al. 2001). A study by Abbas and Hayton (1997) reported that rainbow trout can increase the synthesis of AChE by 10%. The downregulation of cholinergic receptors was found to be an adaptive response to overstimulation of the postsynaptic cell (Jett et al. 1993, Jones et al. 1998). Rainbow trout exposed to carbaryl and permethrin decreased the number of cholinergic receptors compared to controls (Jones et al. 1998). Liu and Pope (1996) demonstrated that rats exposed to chlorpyrifos decreased ACh synthesis which reduced the number of ACh molecules present in the synapse, thus lessening the effect of the AChE inhibition. Aliesterases and carboxylesterases in some species of fish are suggested to be a protective mechanism because they bind to the active metabolite of some insecticides, thus preventing OPs and CBs from binding to AChE (Clement 1984, Carr et al. 1997).

e. OP/CB Mechanisms for Toxicity

Many OPs need to be metabolized to an oxon form while carbamates and some OPs inhibit AChE directly, without biotransformation (Grue et al. 1991, Hoffmann & Papendorf 2006). Habig et al. (1988) found that activation of the oxygen analog was required for malathion and parathion to inhibit AChE in channel catfish (*Ictalurus punctatus*). It should be noted, however, that some CB pesticides that act as fungicides (e.g. IPBC and Mancozeb) have been reported to increase AChE rather than inhibit AChE through unknown mechanisms (Jarrard et al. 2004). Areas of high agricultural intensity may use many different kinds of pesticides, some in small amounts. However,

even exposure to trace amounts of different OPs and CBs can cause AChE inhibition because most OPs and CBs have the same mode of action and have additive effects (Grue et al. 1991; Scholz et al. 2006). It has been proposed that in order for an OP or CB to cause toxicity four processes must occur: 1) binding and inhibition of an extensive number of AChE molecules by the OP or CB, causing impairment of ACh degradation, 2) accumulation of ACh in the synaptic cleft of central and/or peripheral neurons, 3) the excessive and continuous stimulation of postsynaptic receptors, and 4) an altered cellular response due to overstimulation of postsynaptic receptors (Pope 1999).

f. Effects of AChE Inhibition in fish

AChE in most vertebrates is present in the nervous tissue, including neuromuscular junctions (O'Brian 1976) and also in some non-nervous tissue such as erythrocytes (Stenerson 2004). The function of AChE in erythrocytes is unknown but it allows for a simple and non lethal way of detecting exposure to OPs and carbamates (Stenerson 2004). OPs and CBs can significantly affect AChE activity in fish, as documented in Table A.1 (Appendix). In some fish such as *Pocilia reticulate,* brain AChE activity can be inhibited by 70% without causing mortality in acute exposures (Frasco & Guilhermino 2002), while in estuarine fish, inhibition > 70% was associated with mortality and sublethal effects on stamina were detected with inhibitions as low as 50% (Fulton & Key 2001). Traces of OPs and CBs have also been found in the tissues of exposed fish. Sapozhnikova et al. (2004) found detectable amounts of OPs in the liver, gonads, gills and muscle of fish in the Salton Sea, California. OPs and CBs are not distributed in tissues equally; Abbas and Hayton (1997) reported that concentrations of paraxon were higher in the liver followed by the brain, heart, and kidney. Moreover,

even though the liver had the highest amount of paraxon in the tissue, the brain was more sensitive to AChE inhibition.

A negative relationship between brain size and AChE activity has been demonstrated in bluegill (*Lepomis macrochirus*) (Beauvais et al. 2002) and golden shiner (*Notemigonus crysoleucas*) but not in rainbow trout (Phillips et al. 2002). Body length and weight had a negative correlation to AChE activity in three-spined stickleback (*Gasterosteus aculeatus*) (Strum et al. 1999) and rainbow trout (Zinkl et al. 1987), whereas Sandahl and Jenkins (2002) did not find a relationship between weight and AChE activity in juvenile steelhead (*Oncorhynchus mykiss*).

1.3.4 Effects of Pesticide in Fish

a. Effects on the Stress Response

Pesticides are toxicants that can activate the stress response in fish, but some can also inhibit cortisol secretion by disrupting the signalling pathways leading to cortisol at specific steps before or after the generation of cAMP (Leblond & Hontela 1999, Leblond et al. 2001, Bisson & Hontela 2002). Table A.2 shows how different pesticides affect the physiological stress response. The understanding of the effects of pesticides on the stress response is limited at present. *This thesis will provide new data on the effects of OP pesticide exposure and how it affects the physiological stress response, under both laboratory and field conditions.*

b. Effects on Behaviour and Development

Sublethal inhibition of AChE activity affects various behavioural endpoints in fish, including swimming capacity and activity (Little & Finger 1990, Beauvais et al.

2001, Table A.3). Changes in behaviour can have consequences to fish by reducing feeding, as well as causing problems avoiding predators, finding mates and migrating (Dutta et al. 1992, Beauvais et al. 2001). OP and CB exposure can affect reproduction and reproductive potential (Bhattacharya 1993, Begum & Vijayaraghavan 1995). Many OPs and CBs cause developmental problems in embryos and juveniles; diazinon caused deformities in larval medaka (*Oryzias latipes*), including growth retardation and formation of edema in yolk sac (Hamm et al. 1998). Chlorpyrifos, another OP, caused malformations (tail abnormalities, ventral swelling, fin damage, haemorrhaging) and morbidity in juvenile guppies, *Poceilla reticulate*, (De Silva & Samayawardhena 2002).

c. Effects on Food Sources

OPs and CBs in water can further affect fish by altering the food webs, including effects on algae and freshwater phytoplankton (DeLorenzo et al. 2001, Ratageri et al. 2006, Sweilum 2006). Dimethoate decreased levels of algal protein and carbohydrates, inhibiting growth and decreasing cell density in *Scenedesmus incrassatulus* at very low concentrations (0.075 mg/L) (DeLorenzo et al. 2001). Dimethoate decreased primary and gross productivity in a water body by 70% after 12 hrs of exposure (Ratageri et al. 2006). Studies in lentic systems exposed to the OP phosphamidon, reported significant reductions in both phytoplankton and zooplankton (Dhar et al. 2004). Pesticides are very useful for protection against pests; however, they can be detrimental to non-target species. As pesticide use continues to increase (Bloomfield et al. 2006), it is important to better understand how pesticides affect non-target species and what the fates of pesticides are in the environment, specifically as the climate changes and water temperatures increase.

1.4 Temperature

1.4.1 Temperature and Projected Effects of Global Climate Change on Aquatic Ecosystems

Human activities such as deforestation, cultivation, irrigation, dam construction and industrial operations can have a negative impact on water quality; moreover, some of these activities lead to an increase in water temperature (Ihnat & Bulkley 1984, Beitinger et al. 2000). Global climate change and specifically an increase in global temperature is also of great concern (De Stasio et al. 1996, Eaton & Scheller 1996, Fang et al. 2004a, b) since with the change in global temperatures an increase in both surface and ground-water temperatures is projected. The Experimental Lakes area in Ontario has already seen a 2°C increase in water temperature in the last twenty years (Morgan et al. 1998). Using modeling and the scenario of doubling of atmospheric carbon dioxide, an increase in mean water temperatures by 1-7°C has been predicted for many lakes and rivers (De Stasio et al. 1996). Studies on the Fraser River, British Columbia, Canada predict water temperature increases of 0.022°C per year (Morrison et al. 2002).

Increases in water temperature are projected to have significant impacts on fish habitats and fish populations, however, the impacts depend on the type of fish (whether it belongs to a cold-, cool- or warm-water fish guild) and the type of aquatic system it inhabits. Fish have rather precise thermal requirements and often confine their distributions to specific water temperatures (Brandt 1993). Body temperatures of most fish are a direct function of water temperature and a combination of environmental, anatomical and physiological factors cause the body temperature of most fish to be

usually only 0.1 to 1°C above ambient water temperatures (Beitinger et al. 2000). Temperature can be viewed as an environmental resource in which fish will compete with each other for favourable temperatures (Beitinger et al. 2000, Beitinger & Magnuson 1975). Field studies have shown that most fish have a total distribution range limited to a 10°C window of their thermal preference and will spend most of their time within 2°C around their thermal preference (Brandt 1993).

The optimal temperature for fish is important because it is the temperature where fish can feed optimally, grow, and complete their life cycle (Brandt 1993, Kroger et al. 1999, Fang et al. 2000a, Myrick & Cech 2004). Fish may be able to live in water temperatures above or below their optimal temperature but are then usually not fit enough to pass their genes on to the next generation (Eaton & Scheller 1996). A review by Beitinger et al. (2000) concluded that the family Salmonidae had the lowest tolerance to high temperatures, as compared to many other fish families.

Temperature can affect not only the physiology but also the habitat of the fish. Changes in stratification within lakes and river pools and decreasing dissolved oxygen will force fish out of their existing habitats and increase competition within and between species for more suitable habitat (Stefan et al. 1993, De Stasio et al. 1996, Fang et al. 2004a, b, Tate & Lancaster 2007). The effects of increasing temperatures and changes in climate patterns are important to fish well being as fish habitat and life histories revolve around water temperatures (De Stasio et al. 1996, Eaton & Scheller 1996, Rahel et al. 1996, Fang et al. 2004a, b).

1.4.2 Projected Effects of Climate Change on Fish

Climate change is predicted to affect both the number of fish kills associated with temperature and increase the number of fish invasions (Trim & Marcus 1990). Fish kills are predicted to increase in both summer and winter: in summer through excessive water temperatures, low dissolved oxygen and increased salinity while in winter only through milder temperatures causing low dissolved oxygen (Trim & Marcus 1990, Fang et al. 2000 a, b, Sapozhnikova et al. 2004). As water temperatures increase, warm water fish may invade lakes and rivers they were previously unable to inhabit due to the low water temperature (Eaton & Scheller 1996, Morgan et al. 1998). These invasions could further exacerbate the effect that global climate change has on a particular ecosystem.

There is the possibility, however, that if raising water temperatures is a slow process, cool- and cold-water fish may be able to acclimatize to warmer waters. Acclimatisation temperature is suggested to be the most important factor influencing temperature tolerance (Cincotta & Stauffer 1984, Beitinger & Bennett 2000, Beitinger et al. 2000). Studies have shown that fish are able to increase their tolerance of high temperatures by more than 10°C when acclimatised to a warm temperature (Cincotta & Stauffer 1984, Ihnat & Bulkley 1984, Beitinger et al. 2000). For example, upper avoidance temperatures in white suckers (*Catostomus commersoni*) changed from 15°C when acclimatised to 6°C, to 30°C when acclimatised at 24°C (Cincotta & Stauffer 1984). Another study in mountain whitefish (*Prosopium williamsoni*) found that fish acclimatised to 15°C preferred warmer temperatures than fish acclimatised to 5°C (Ihnat & Bulkley 1984).

Non-lethal temperature increases can have enormous impacts on fish communities because thermal stress causes fish to divert energy from growth to homeostasis maintenance, including the induction of heat-shock proteins (Iwama et al. 1998, Viant et al. 2003). Both increases and decreases in water temperature can cause reductions in feeding and growth rates (Brett 1971, Bartell et al. 1986, Brandt 1993). Low water temperatures are known to decrease reproduction and fecundity in medaka (Koger et al. 1999). In mountain whitefish, high water temperatures during egg development result in increased egg mortality: 100% mortality at 15°C and 12°C, 97.8 to 100% mortality at 11°C, 34.4 to 95.2% mortality at 10°C, and 6.5 to 9.5% mortality at 6°C (Rajagopal 1979).

Exposure to water temperatures outside the thermal preference of a fish is known to activate the physiological stress response (Table A.4), demonstrated through endpoints such as an increase in cortisol levels (Barton & Peter 1982, Davis & Parker 1990, Davis 2004, Weber & Bosworth 2005, Davis & Peterson 2006), decrease or increase in plasma glucose (Davis & Parker 1990, Davis 2004, Lerman et al. 2004) and decrease in liver glycogen (Viant et al. 2003, Lerman et al. 2004). More information is needed to determine how fish will respond to temperatures changes. This thesis will evaluate how the physiological stress response in fish is affected by temperature. However, it is hypothesised that how a fish responds to temperature depends on the degree of temperature change, acclimatisation temperature, and species of fish.

Temperature also plays an important role in life history characteristics - a study of white suckers reported that fish living in colder subpolar regions took longer to reach maturity and had slower growth rates than fish in warmer temperate regions (Duchesne &

Magnan 1997). Behaviour can be influenced by non-thermal stimuli which can override the preferred temperature response (Beitinger & Magnuson 1975). Juvenile bluegill preferred 31°C over 27° or 34°C but when adult bluegill were present, the juveniles spent the majority of their time outside of the preferred 31°C. Temperature can also affect how a fish will respond to toxicants, including pesticides, in the environment.

1.4.3 Temperature and Pesticides

Global climate change is predicted to have far reaching effects on agriculture. Predicted changes in weather patterns will force changes in farming practices and the use of chemicals associated with agriculture. Canada and the United Kingdom are projected to have milder, wetter winters and hotter drier summers, with an overall change in average temperature and a longer growing season (Smith & Almaraz 2004, Bloomfield et al. 2006). Changes in temperature and growing season affect agricultural production, types of crops that can be grown and the pests associated with the introduction of new crops (Smith & Almaraz 2004, Bloomfield et al. 2006). Changes in crop type and invasion of new pests will shift pesticide use as well (Smith & Almaraz 2004). Climate change is predicted to increase weeds, pests and diseases, which will most likely lead to an increase in pesticide use (Bloomfield et al. 2006).

Changes in temperature are also predicted to change how pesticides act in non-target species such as fish. Temperature is predicted to change pesticide exposures by increasing rates of dispersal and increasing transport distances from source, however, temperature will also increase degradation rates (Bloomfield et al. 2006). The effect of temperature on the toxicity of a pesticide depends on the pesticide itself: some pesticides

are more toxic in warm water while others are more toxic in cold water (Capkin et al. 2006). In fish, the mode of action of OPs and CBs can be temperature-dependent, making the fish more susceptible to OPs and CBs at higher temperatures due to temperature effects on metabolic rate, availability of oxygen in tissues and waste production (Macek et al. 1969). There are conflicting studies on the effect that temperature has on AChE. Studies by Beauvais et al. (2002) in bluegill, Hazel (1969) in mummichog (*Fundulus heteroclitus*), and Phillips et al. (2002) in walleye (*Stizostedion vitreum*) concluded that temperature does not affect AChE, whereas Hogan (1970) in bluegill, Zinkl et al. (1987) in rainbow trout and Hazel (1969) in goldfish (*Carassius auratus*) showed that AChE increases with increasing water temperature. Activity of AChE may not be significantly different if the temperatures are above 10°C and span less than 12°C (Beauvais et al. 2002). A summary of the effects of temperature on fish is presented in Table A.5. *This thesis will investigate the effects of pesticides on the physiological stress response in fish and the interactions with temperature.*

1.5 Species Differences

Different species respond to adverse conditions in different manners. Species-specific differences have been found in regards to toxicity of pathogens (Gordon et al. 2006), AChE inhibition (Davies et al. 1994, Fulton & Key 2001), metal distribution and metallothionein concentration (Linde et al. 1999), stress and the stress response (Lacroix & Hontela 2004, Jentoft et al. 2005), and vulnerability to oxidative stress (Hasspieler et al. 1994). An extensive review was conducted by Vittozzi and De Angelis (1991) comparing the LC_{50} of common test species to a wide range of toxicants. Table A.6

provides a summary of how different species respond to stress, pesticides and temperature. *Very few studies examined species differences in fish that share the same environment; this thesis (Chapter 2) will examine how different species living in the same environment respond to stress, pesticide exposure and temperature.*

1.5.1 Species Differences and Stress

Species differ in their ability to respond to stressors. This ability depends on life history, strain and acclimation temperature (Jentoft et al. 2005). Differences in the stress response have been found in domesticated versus non-domesticated fish (Barton 2000, Jentoft et al. 2005). For example, domesticated rainbow trout was able to habituate to stress while non-domesticated Eurasian perch (*Perca fluviatilis*) were not (Jentoft et al. 2005). When different species are similarly stressed, they may have different capacities to respond to this stress, as indicated by variations in corticosteroid-binding proteins, the metabolic clearance rate of cortisol (Barton 2000) and carbohydrate utilization (Jentoft et al. 2005). When lake trout (*Salvelinus namaycush*) and rainbow trout were exposed to a 30 second handling stress, lake trout had higher levels of plasma cortisol while rainbow trout had higher levels of plasma glucose (Barton 2000). Knowing how fish will respond to stressors is important for aquaculture, and for predicting how fish will respond to environmental challenges, including chemical pollutants and identifying species at risk (Barton 2000).

1.5.2 Species Differences and Pesticides

Pesticides can have different effects on different species (Johnson & Wallace 1987). Sapozhnikova et al. (2004) reported that tilapia exposed in the laboratory had more dimethoate and *pp*-DDE in tissues than covina (*Cynoscion xanthulu*), but covina had more malathion, endrin, and DDD concentrations in tissues as compared to tilapia. Inhibition of AChE activity can be different between species; furthermore the inhibition of different tissues can also be different between species (Carr et al. 1997). One study found that brain tissue in mosquitofish had greater inhibition of AChE activity than largemouth bass (*Micropterus salmoides*), bluegill sunfish and golden shiners but mosquitofish and golden shiners had lower inhibition of muscle AChE activity compared to largemouth bass and bluegill (Carr et al. 1997). Specific activity of AChE can also be different between species; Johnson and Wallace (1987) and Chuiko (2000) found that control levels of AChE activity can be up to 7-fold higher in perch (Percidae) compared to whitefish (Coregonidae). The differences in species reaction to pesticide exposure and AChE inhibition could be due to different levels of aliesterases which have been suggested to provide a protective mechanism to OP exposure (Carr et al. 1997). Species differences to pesticide exposure may be caused by differences in uptake and metabolism of the pesticide (Carr et al. 1997), affinity of the pesticide for AChE (Keizer et al. 1995), amount of enzyme, the rate of AChE phosphorylation (Johnson & Wallace 1987, Intorre et al. 2004) and kinetics of AChE inactivation (Johnson & Wallace 1987).

1.5.3 Species Differences and Temperature

It is well known that different species of fish prefer different water temperatures - fish are even classified according to their thermal preference: cold-water fish have a maximum temperature tolerance under 24.3°C, cool water fish have a maximum temperature tolerance between 26.5 and 29.9°C, and warm water fish have a maximum temperature tolerance over 30°C (Eaton & Scheller 1996). Temperature differences between similar species can be caused by differences in fish size, as metabolic rates decreases when fish are larger (Galbreath et al. 2004). Metabolic rates in polar fish tend to be higher at low temperatures making them better suited for cold water than non-polar fish (van Dijk et al. 1999). High water temperatures cause fish to accumulate anaerobic end products due to an insufficient supply of oxygen to the tissues (van Dijk et al. 1999). This causes a failure in the electron transport chain and will eventually lead to death in some fish, while fish that can handle warm water either clear out the anaerobic end products or have enough oxygen available to the tissues to prevent build-up of anaerobic end products (van Dijk et al. 1999). Temperature is an important consideration in aquaculture - knowing how different species of fish will respond to cold shock, heat shock, and warming of water can influence where aquaculture activities can take place and how a rise in water temperature will affect the fish (Kuo & Hsieh 2006).

1.6 Summary

Functional integrity of the adrenal gland and the head kidney in teleost fish is very important for the well being of the organism. Excessive stress caused by environmental stressors such as temperature and/or toxiciants such as pesticides can have severe consequences to the health and well-being of fish. Furthermore toxicants, such as

pesticides can inhibit normal endocrine function. The adrenal tissue can lose the ability to produces hormones such as cortisol which help fish to properly cope with stressful situations. Excessive stress or an impaired stress response can lead to decreased reproduction, growth, fitness and survivorship. Organisms are often faced with more than one stressor and/or toxicant and it is important to understand how these potentially complex interactions of multiple stressors will affect organisms. The effects of multiple stressors on the physiological stress response are a new area of research and this thesis will examine the stress response in fish exposed to pesticides and warm water temperature. There is very little information regarding how temperature and pesticides affect the stress response and other physiological functions such as acetylcholinesterase activity. Species differences can account for some of the variation in the literature about temperature and pesticide stress but further research is needed. Furthermore, due to the continued use and exposure of organisms to toxicants such as pesticides and to changes in global climate, more research is needed to determine how fish respond to these stressors both in the laboratory and in the field.

The overall objective of this study is to determine, through the use of physiological markers (plasma cortisol, liver glycogen, plasma glucose, condition factor, AChE activity), how temperature and pesticides affect fish. Furthermore, this study will examine how different species of fish that live in the same environment will respond to stressors. The interactive effects of pesticide exposure and temperature on the physiological status will be quantified, and species-specific differences in vulnerability will be assessed, providing new information on the physiological stress response to multiple stressors in fish.

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Chapter 2.

The physiological stress response indicators in whitefish (*Prosopium williamsoni*) and suckers (*Catostomus sp.)* sampled along a gradient of temperature and agricultural chemicals in the Oldman river, Alberta.

2.1 Introduction

Rivers are complex systems where habitats of different species overlap. Species that occupy the same niche may share resources and co-exist, or they may compete with each other. A species better suited for a particular habitat can cause a decline in another species' population. Anthropogenic factors such as physical structures (dams and weirs), water withdrawals or loading of toxicants can cause changes in the aquatic environment (Cormier et al. 2000), potentially stressing and adversely affecting the organisms that live there. Differences in sensitivity to stressors between species can lead to the extirpation of some species and the invasion of others. Species-specific responses of fish have been documented with respect to toxicity (Gordon et al. 2006), acetylcholinesterase (AChE) inhibition (Davies et al. 1994, Fulton & Key 2001), metal distribution and metallothionein concentration (Linde et al. 1999), stress and the stress response (Lacroix & Hontela 2004, Jentoft et al. 2005), and vulnerability to oxidative stress (Hasspieler et al. 1994). Differences in tolerance to heat (Van Dijk et al. 1999) and cold shock (Kuo & Hsieh 2006) have been also reported; fish are classified as cool- or cold-water species according to their thermal preference. Cool- and cold-water fish species often share the same environment, particularly when their movements are restricted by dams and weirs. As climate changes and water temperature increases, temperature sensitive species may be threatened. Cold-water fish species in rivers draining agricultural areas in temperate

zones are particularly vulnerable to pesticides because they are also exposed to high temperatures.

In the agricultural drainages, fish are exposed to short-term spikes of pesticides during spraying and rain events, as well as to chronic exposure in areas where streams and canals drain sprayed areas (Frank & Sirons 1979, Morrison & Wells 1981). Exposure to organophosphate (OP) and carbamate (CB) pesticides lower the activity of the enzyme AChE. Low levels of AChE in fish cause changes in behaviour (Beauvais et al. 2000, Brewer et al. 2001, de Aguiar et al. 2004, Sandahl et al. 2005), metabolism (Samuel & Sastry 1989, de Aguiar et al. 2004), feeding (Pavlov et al. 1992) and reproduction (Bhattacharya 1993). Agricultural chemicals, including pesticides have also been shown to influence the physiological stress response and cortisol secretion in fish (Dorval et al. 2005, Teles et al. 2007; Thangravel et al. 2005). Natural stressors such as increases in temperature can modify the toxicity of pesticides; warmer water temperature increased the toxicity of endosulfan (Capkin et al. 2006) and methicarb (Altinok et al. 2006) to rainbow trout. Moreover, an increase in global temperature may lead to invasions of new and more abundant agricultural pests, thus increasing the amount of pesticides being used (Moore & Folt 1993, Bloomfield et al. 2006).

Fish have precise thermal requirements and will select habitats that are near their optimal temperature (Neill & Magnuson 1974, Dill 1987); if necessary, they will expand their range of temperature in response to food availability (Brett 1971), predators, competition, or social dominance (Neill & Magnuson 1974, Beitinger & Magnuson 1975, Dill 1987). Increasing water temperature is considered a widespread and problematic stressor in salmonids on the West Coast (Sullivan et al. 2000); displacement of cold-

water, northern, high latitude and high altitude species of fish is predicted in many global warming models (De Stasio et al. 1996, Daufresne et al. 2003). Water temperatures outside the thermal preference range, specifically warmer water temperatures, can cause thermal stress which depletes energy reserves (Brett 1971, Viant et al. 2003), decreases growth rates (Brett 1971, Bartell et al. 1986), impairs reproduction (Daufresne et al. 2003) and changes behaviour (Sullivan et al. 2000).

Fish in anthropogenically impacted regions may be exposed to many different concurrent stressors, including loading of agricultural chemicals such as pesticides and fertilizers, increasing water temperatures, and habitat alterations (dams, weirs, and water withdrawals). The physiological stress response is activated when homeostasis is challenged and there are changes to tissue and organ function in an attempt to maintain internal stability (Wendelaar Bonga 1997, Schreck et al. 2001). Physiological indicators of stress such as plasma cortisol and glucose levels, and liver glycogen are often used to determine if fish are stressed and if the hypothalamo-pituitary-interrenal axis functions normally (Hontela 1998). The type and duration of a stressor can have an impact on the stress response: acute exposures to a wide range of chemical pollutants increase plasma cortisol in fish (Hontela 1997) while chronic exposure may disrupt cortisol synthesis. Agricultural chemicals (Dorval et al. 2005) and metals such as Cd, Cu, and Zn (Laflamme et al. 2000, Lacroix & Hontela 2004, Lappivaara & Marttien 2005), can impair the stress response resulting in low levels of cortisol. Pesticides and increasing water temperatures are stressors potentially affecting fish in many regions in North America.

Water temperatures in the Experimental Lake Area of Northern Ontario, Canada have risen by 2°C in the last 20 years (Schindler et al. 1996). Global water temperatures are projected to rise by 1-7°C (depending on type and location of water body) when a doubling of atmospheric CO_2 occurs (Stefan et al. 1993, De Stasio et al. 1996, Eaton & Scheller, 1996). A combination of global climate change and continued runoff of agricultural chemicals may have disastrous effects on fisheries and may decrease the habitat for cold-water and sensitive fish. Yet our understanding of the interactions between temperature and chemical stressors on fish physiological fitness is limited.

The objective of this study is to determine how multiple stressors (pesticides and warm temperature) affect the physiological status and the stress response in cold-water and cool-water fish. Plasma cortisol and glucose, liver glycogen, and condition factor were measured as the physiological stress response endpoints, and plasma AChE activity was used as an indicator of exposure to OP and CB pesticides. *It is hypothesised that species-specific differences to these stressors exist and that at temperature-impacted sites, the cold-water fish species will exhibit an increased stress response compared to cool-water fish.*

2.2 Materials and Methods

2.2.1 Description of the sites and fish species

Fish were sampled at six sites along the Oldman River (Figure 2.1), in southern Alberta, Canada during spring and/or summer 2004 and 2005 (Table 2.1). The Oldman River originates in the Rocky Mountains and flows east, draining an area of intensive grain and livestock production. Runoff from these operations contains agricultural

Figure 2.1. Location of sampling sites in the Oldman River Basin, Southern Alberta. Squares represent site locations, the x is the location of the Oldman Dam and arrows indicate flow direction, with Summerview the most upstream site and Pavan the most downstream site. Figure modified from Oldman Watershed Council (2006)

Table 2.1. Characteristics of sites sampled in the Oldman River, Alberta.

Site	Distance from Oldman dam (km)	Latitude and longitude	Date	Depth* (m)	Velocity* (m/s)	Flow* (m^3/s)
Summerview	7	49°33'N,113°49'W	Summer 2005	0.6	0.4	3.6-6
Fort McLeod	70	49°44'N, 113°13'W	Spring 2005	0.4	0.3	2.0
			Summer 2005	0.3-0.7	0.1-0.6	0.9-5.2
Pearce Road	105	49°48'N, 113°13'W	Spring 2005	0.4-0.7	0-0.5	
			Summer 2005	0.4-0.5	0.1-0.7	0.5-5.5
Popson Park	160	49°44'N, 112°51'W	Spring 2005	0.3-1.1	0.1-0.5	0.5-5.0
			Summer 2005	0.7		
Pavan Park	175	49°38'N, 112°51'W	Spring 2005	0.4-0.6	0.1-0.4	0.6-3.0
			Summer 2005	0.4-0.8	1.4-1.6	9.6-31.5

*Measurements taken at points in the river where fish were seined

chemicals including pesticides that are detected in the water (Table 2.2). Moreover, the river exhibits a west to east temperature gradient, with higher temperatures in the lower reaches (Figure 2.2). Whitefish (*Prosopium williamsoni*) and suckers (*Catostomus* sp.) were sampled for this study because both species are found within the Oldman River and they have different species-specific temperature optima. Whitefish represent a cold-water fish species with a maximum temperature tolerance of 23.1°C (Eaton & Scheller 1996) and a prespawing thermal preference range between 12.8°C to 17.7°C [upper temperature preference (UTP)] (Ihnat & Bulkley 1984). Suckers represent a cool-water species with a maximum temperature tolerance of 27.4°C (Eaton & Scheller 1996) and a thermal preference range of 18.3°C to 24°C (UTP) (Eaton et al. 1995). The UPTs for sucker and whitefish are indicated in Figure 2.2.

2.2.2 Capture and sampling of the fish

Fish were captured by a purse seine (30 x 1.8 m, 0.4 cm mesh) between 10:00 h and 13:30 h each day, at sampling periods indicated in Table 2.1. Fish were transferred from the seine to a floating enclosure (0.68 x 1 x 0.47 m) and were kept in the enclosure until 14:00 h when they were removed in groups of 3 to 5. Fish were anaesthetized in a solution of 0.15 g/L of tricaine methanesulphonate (MS-222) and a blood sample was taken from the caudal vasculature with a 1 or 3 mL heparized syringe. Plasma was separated at 13000 rpm for 5 minutes and frozen in liquid nitrogen. The fish were euthanized by spinal transection, weights and lengths recorded, and the liver was dissected and frozen in liquid nitrogen. Plasma and liver samples were kept frozen at -80°C until analysis.

Table 2.2. Concentrations of chemicals in water detected at sites situated along the Oldman River, Alberta.

	Compound	Summerview	Fort MacLeod	Monarch	Lethbridge	Popson Park	Hwy 36
Herbicide (µg/L)	2,4-D	<0.005(04)[a]			0.005-0.026(04)		0.006-0.082(04)
	Dichlorprop (2,4-DP)	<0.005(04)			<0.005-0.007(04)		<0.005(04)
	MCPA	<0.005(04)			<0.005-0.01(04)*		0.003-0.022(04)
	MCPP (Mecoprop)	<0.005(04)			<0.005(04)		0.004-0.018(04)
	Dicamba (Banvel)	<0.005(04)			<0.005(04)		<0.005-0.006
Insecticide (µg/L)	Dimethoate (Cygon)	<0.05(04)	<0.05(98)	<0.05(98)	<0.05(04)*	<0.05(99)	<0.05(04)*
Other Chemicals† (ng/L)	Cholesterol	74.70(05)				108.15(05)	217.98(05)
	Desmosterol	28.46(05)				36.34(05)	51.58(05)
	Cholestanol	3.61(05)				12.26(05)	36.27(05)
	7-Ketocholesterol	1.65(05)				7.27(05)	9.90(05)
	β-Sitosterol	40.56(05)				48.62(05)	118.74(05)
	Campesterol	9.43(05)				10.12(05)	16.75(05)
	Stigmastanol	2.47(05)				10.64(05)	33.30(05)
	Stigmasterol	19.80(05)				42.01(05)	118.20(05)
	Fucosterol	65.18(05)				111.63(05)	208.14(05)

[a] Year of sampling indicated in bracket; 2004 as (04) and 2005 as (05)

† Samples from Jeffries 2007

Organophospates and Carbamates measured by Alberta Environment were below the detection limit for that pesticide at all sites : Chlorpyrifos-ethyl, Diazinon and Phorate <0.005µg/L, Parathion <0.01 µg/L, Terbufos <0.03 µg/L, Malathion and Triallate <0.05 ug/L, Ethiion <0.1 µg/L, Disulfoton and Gluthion <0.2 µg/L

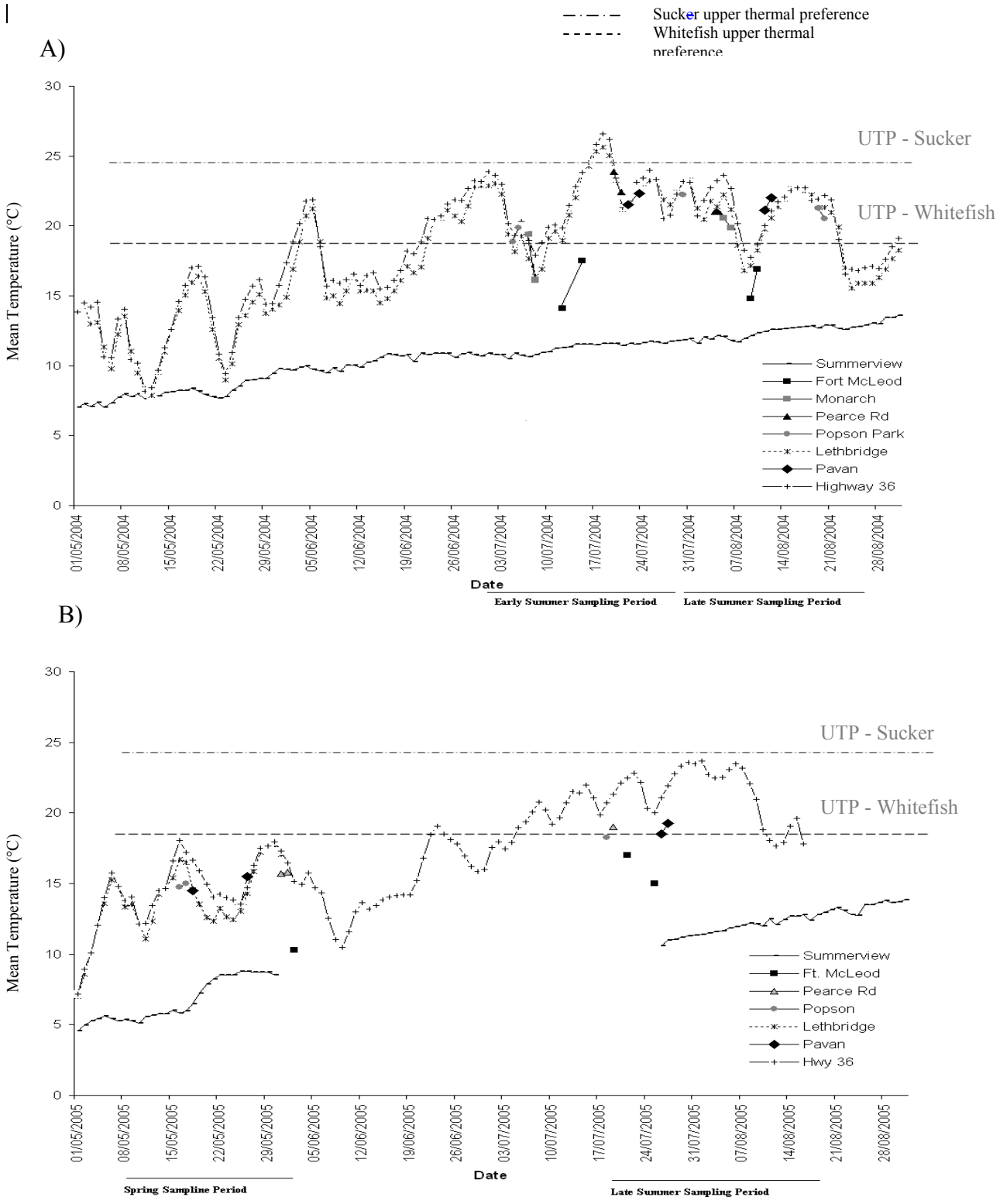


Figure 2.2. Mean water temperatures for each site during sampling periods indicated by black bars below the dates in A) Early Summer and Late Summer 2004 B) Spring and Late summer 2005. Data for Summerview, Lethbridge and Highway 36 received from Alberta Environment. Upper thermal preference (UTP) for sucker and for whitefish are indicated by the lines on the graph for sucker (-.-.-.) and whitefish (-----).

2.2.3 Acetylcholinesterase (AChE)

The AChE activity was measured with an assay modified from Ellman et al. (1961) and Chuiko (2000). Plasma (2 μL) was pipetted into a microplate and 120μL of ISO-OMPA (Sigma Tetraisopropyl pyrophosphoramide, T1505) was added. The samples were incubated at room temperature for 10 minutes and 10 μL of DTNB (Sigma 5,5' Dithiobis [2-nitro-benzoic acid], D8130) and 10 μL of Acethylthiochlorine Iodine (Sigma minimum 98% TLC, A5751) were added, followed by a 10 minute incubation at room temperature. The microplate was read at 405 nm in a microplate reader every 2 minutes for 10 minutes. Concentration of AChE for each sample was measured from the slope; internal standards (Normal Serum Control, TC-TROL[N], Teco Diagnostics) were used to ensure the accuracy of the assay was maintained.

2.2.4 Physiological Stress Response

Concentrations of cortisol in the plasma were determined with diagnostic kits (MP Biomedicals Diagnostics Division 07-221102). Assay characteristics and accuracy were verified, as described previously (Hontela et al. 1995). Plasma glucose was determined with a colorimetric assay using the GOD-PAP reagent (Roche 1929526) at 512nm. Liver glycogen was determined using a method modified from Bleau et al. (1996). Liver samples were digested in KOH (1N) at room temperature; the sample was then precipitated with 1.5N acetic acid and centrifuged at 13000 RPM for 10 minutes. Glycogen from the livers and standards (rabbit glycogen, Sigma, G8876-1G) was hydrolyzed to glucose with aminoglycosidase (Sigma A-7255) in a water bath at 37°C for 30 minutes. Glucose content was measured using the colorimetric assay described above.

2.2.5 Statistical Analysis

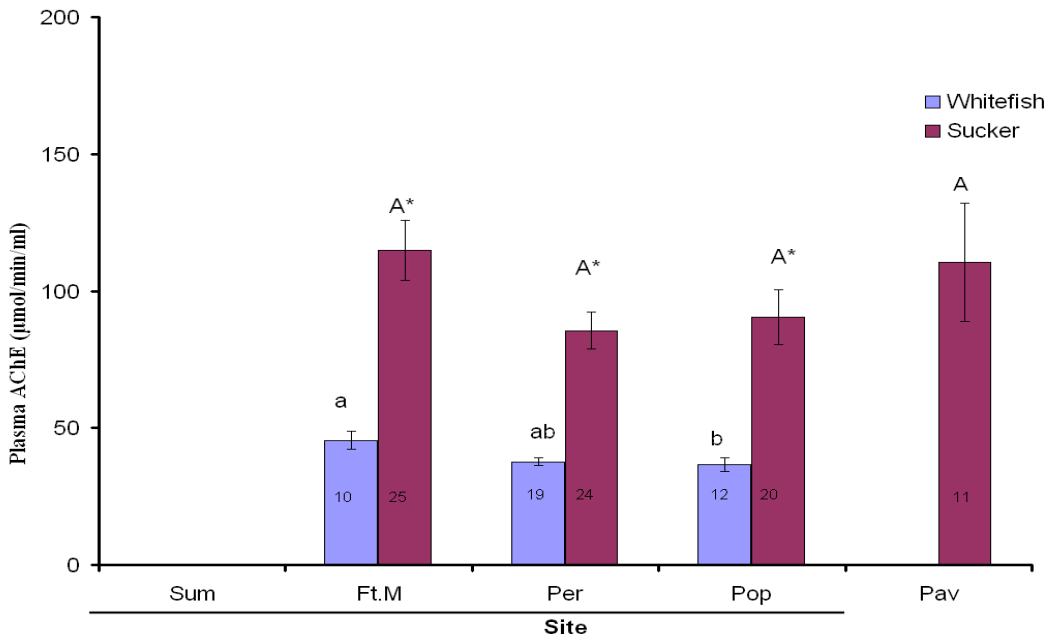
The statistical program JMP IN version 5.1 was used to perform statistical tests at $p<0.05$. One way ANOVA was used for single factor comparisons, a t-test was used to compare species at each site. Significant differences between means were determined using Tukey-Kramer HSD post hoc test and log-normal data were normalized using a log transformation.

2.3 Results

2.3.1 Pesticide Exposure

Water concentrations of herbicides and other chemicals in the Oldman river increased along a west to east transect in 2004 and 2005 (Table 2.2), however several of the OP and CB pesticides that were measured were below the detection limits (0.005-0.1 µg/l depending on the pesticide) (Table 2.2). Plasma AChE activities, used as indicators of exposure to OP and CB pesticides, are presented in Fig. 2.3. At all sites and all sampling times, whitefish had significantly lower plasma AChE activity ($p<0.05$, t-test) compared to suckers (Figure 2.3A & 2.3B). In spring 2005, AChE activities in whitefish were lower at downstream sites while plasma AChE in suckers were not significantly different among sites ($p>0.05$, ANOVA) (Figure 2.3A). Plasma AChE activity in late summer 2005 was higher at downstream sites in both suckers and whitefish (Figure 2.3B).

A) Spring 2005



B) Late Summer 2005

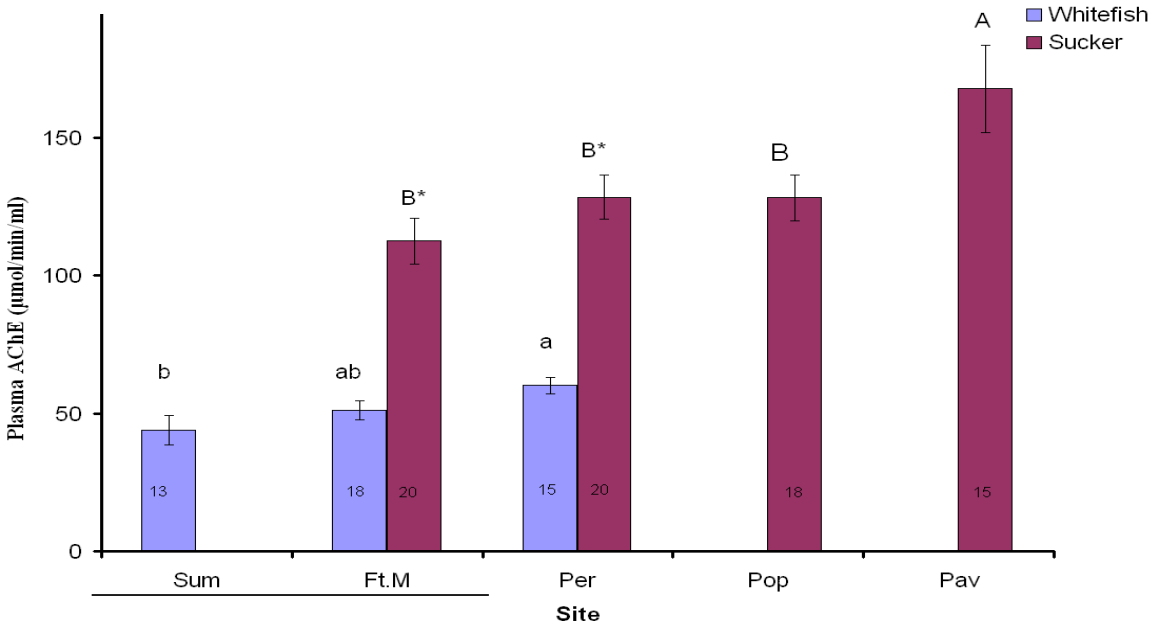


Figure 2.3. Plasma AChE (mean±SE) in whitefish and suckers sampled at sites along the Oldman River in A) spring 2005 B) late summer 2005. Lines under sites represent sites that are within the thermal preference of whitefish. Capital letters represent significant differences among sites for suckers and lower case letters represent significant differences among sites for whitefish (ANOVA and Tukey-Kramer HSD test α=0.05). Asterisk represent significant differences between species at a site (Student's t-test α=0.05). Sites with only one bar indicate that only one species was caught at that site.

2.3.2 Temperature Regimes

Water temperatures fluctuated throughout the sampling period and were always below the maximum temperature tolerance for whitefish and suckers, 23.1°C and 27.4°C respectively (Eaton & Scheller 1996). However water temperatures did exceed the UTP for whitefish (17.7°C) during: early summer 2004 (Figure 2.2A & 2.2B) at Pearce Rd, Monarch (2nd day sampling), Popson, and Pavan (data extrapolated from Alberta Environment), late summer 2004 at Pearce Rd, Monarch, Popson, and Pavan, and in late summer 2005 at Pearce Rd, Popson, and Pavan. In early summer 2004, the water temperature was abnormally high during sampling at Pearce Rd, ranging from 23.0 to 24.8°C. In spring 2005 all sites where whitefish were sampled were within their thermal preference (Figure 2.2B). Whitefish were not found at Popson in 2004, late summer 2005, or at Pavan in any of the sampling periods. Suckers were not present at Summerview in any of the sampling periods.

2.3.4 Physiological Stress Response

Plasma cortisol levels are presented in Figure 2.4. Plasma cortisol levels in whitefish were higher compared to suckers at 7 sampling times (significantly higher at $p<0.05$ at 6 sampling times) out of a total of 11 sampling times. Suckers had significantly higher ($p<0.05$) plasma cortisol compared to whitefish 3 out of 11 sampling times and always at Ft MacLeod. In 2004, there were no significant differences in plasma cortisol for whitefish sampled at different sites but in early summer 2004 whitefish at Pearce Rd have significantly higher plasma cortisol than at all other sites (Figure 2.4A). There was no significant difference ($p>0.05$) in plasma cortisol in suckers in 2004 (Figure

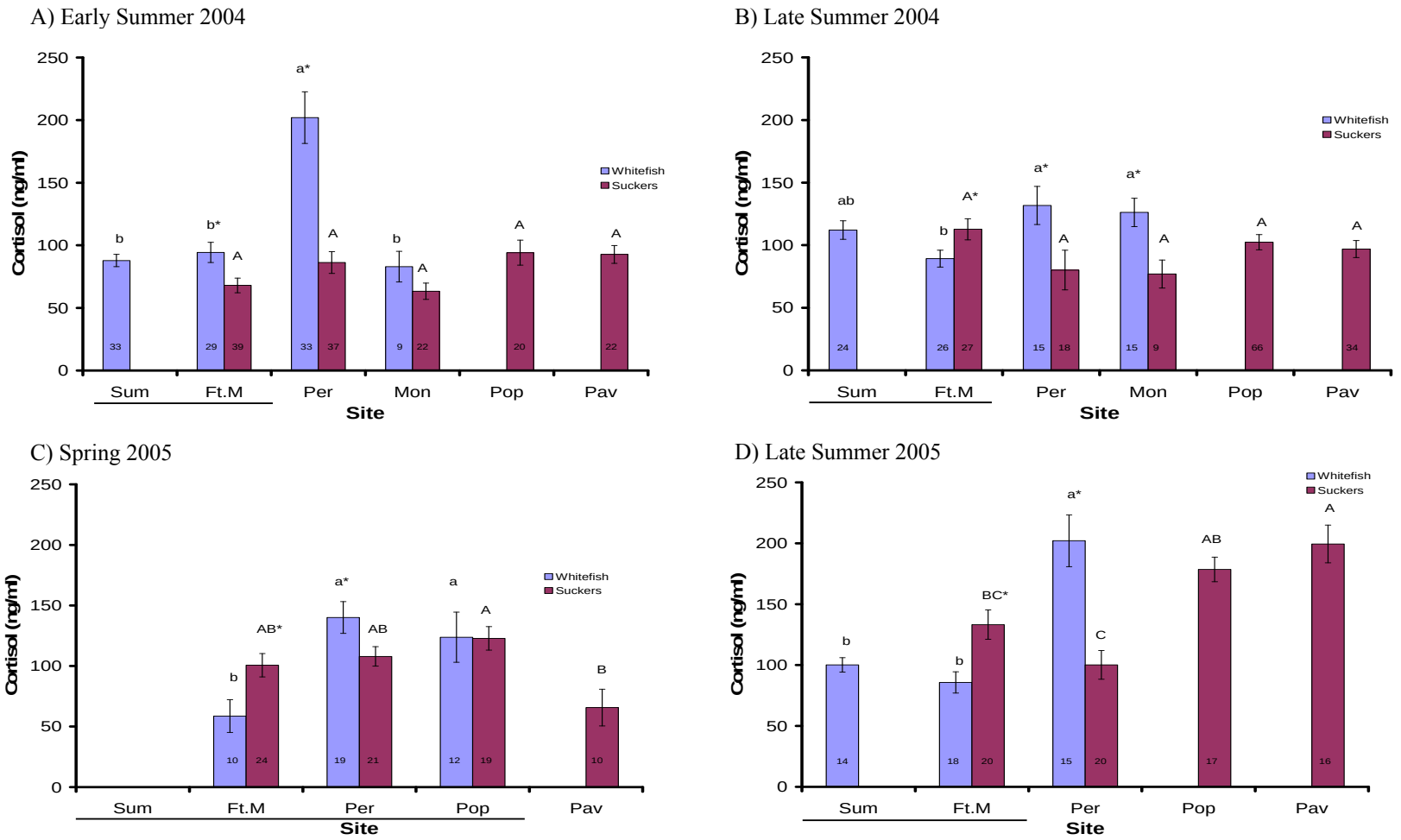


Figure 2.4. Plasma cortisol (mean±SE) in whitefish and suckers sampled at sites along the Oldman River in A) early summer 2004, B) late summer 2004, C) spring 2005, and D) late summer 2005. Numbers in the bars indicate the sample size for each site. Lines under sites represent sites within the thermal preference of whitefish. Capital letters represent significant differences among sites for suckers and lower case letters represent significant differences among sites for whitefish (ANOVA and Tukey-Kramer HSD test α=0.05). Asterisks represent significant differences between species at a site (t-test α=0.05). Sites with only one bar indicate that only one species was present at that site.

2.4B) between sites. In 2005, plasma cortisol of whitefish at downstream sites was higher than upstream (Figure2.4C & 2.4D); plasma cortisol in suckers also increased at downstream sites in late summer 2005 (Figure 2.4D).

The concentrations of liver glycogen (Fig. 2.5), measured as an indicator of energy reserves, were significantly lower ($p<0.05$, t-test) in whitefish compared to suckers at all sites and all sampling times, except at Ft. MacLeod in spring 2005 where the means were not different (Fig. 2.5C). In both fish species sampled in late summer 2004 (Fig. 2.5B), spring 2005 (Fig. 2.5C), and late summer 2005 (Fig. 2.5D), liver glycogen reserves decreased along a west to east gradient. This trend was however not observed in early summer 2004 (Fig. 2.5A).

Plasma glucose levels of whitefish and suckers did not show any site- or species-related trends (Table 2.3). In whitefish there was a decrease in plasma glucose levels at downstream sites in 2005 but suckers did not demonstrate this trend at any sampling period. Condition factor for either whitefish or suckers did not show any observable patterns for any sample period (Table 2.4).

2.4 Discussion

2.4.1 Exposure

The Oldman River flows east from the Rocky Mountains until it joins the Bow River to form the South Saskatchewan River just east of Taber, AB. The temperature of the Oldman River rises as it flows toward the South Saskatchewan River, creating an east to west temperature gradient. The distribution of fish in the Oldman River reflects this temperature gradient, as mainly cold-water species, such as rainbow trout (*Oncorhynchus*

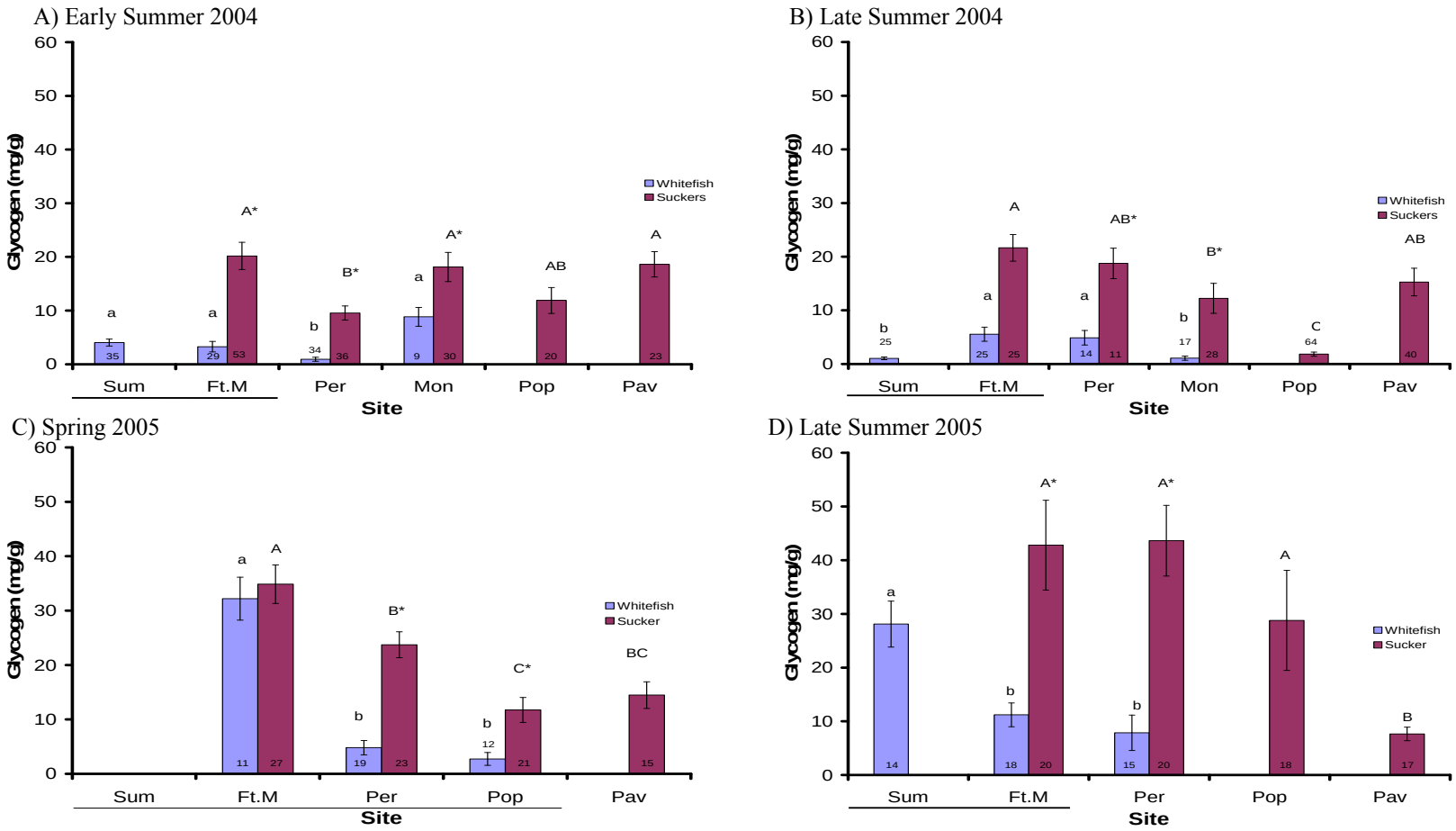


Figure 2.5. Liver glycogen (mean±SE) in whitefish and suckers sampled at sites along the Oldman River in A) early summer 2004, B) late summer 2004, C) spring 2005, and D) late summer 2005. Numbers in the bars indicate the sample size for each site. Lines under sites represent sites within the thermal preference of whitefish. Capital letters represent significant differences among sites for suckers and lower case letters represent significant differences among sites for whitefish (ANOVA and Tukey-Kramer HSD test α=0.05). Asterisk represent significant differences between species at a site (t-test α=0.05). Sites with only one bar indicate that only one species was present at that site.

Table 2.3. Plasma Glucose, mean±SE(n), in whitefish and suckers sampled at sites along the Oldman River in early summer 2004, late summer 2004, spring 2005, late summer 2005.

		Glucose (mg/mL)					
Sampling Period	Species	Sum	Ft.M	Per	Mon	Pop	Pav
E. Summer '04	Whitefish	1.84±0.05 (33) a	1.85±0.06(27) a*	1.55±0.10(34) b	1.93±0.16(9) ab*	-	-
	Sucker	-	1.57±0.11(31) AB	1.64±0.12(33) A	1.40±0.09(23) AB	1.13±0.10(21) B	2.07±0.19(18) A
L. Summer '04	Whitefish	1.63±0.07(24) ab	1.42±0.06(25) b	1.76±0.10(15) a*	1.56±0.08(15) ab	-	-
	Sucker	-	1.98±0.12(22) A*	1.11±0.13(8) BC	1.34±0.14(19) B	1.00±0.06(57) C	1.32±0.07(35) B
Spring '05	Whitefish		1.55±0.08(10) a	1.51±0.09(19) a		1.15±0.11(12) b	-
	Sucker		1.44±0.08(25) A	1.39±0.13(22) A		1.55±0.11(20) A*	1.86±0.26(9) A
L. Summer '05	Whitefish	2.06±0.09(14) a	1.68±0.07(18) b	1.63±0.14(15) b		-	-
	Sucker	-	1.92±0.16(20) A	1.88±0.11(20) A		1.93±0.10(18) A	2.10±0.14(16) A

Lowercase letters represent significant differences within a sampling period for whitefish and capital letters represent significant differences within a sampling period for suckers (ANOVA and Tukey-Kramer HSD test α=0.05). Asterisk represents a significant difference between suckers and whitefish at a site (T-test α=0.05)

- species not caught during sampling period

Table 2.4. Condition factor, mean±SE(n), in whitefish and suckers sampled at sites along the Oldman River in early summer 2004, late summer 2004, spring 2005, late summer 2005.

Sampling periods	Species	Condition Factor					
		Sum	Ft.M	Per	Mon	Pop	Pav
E. Summer '04	Whitefish	1.22±0.01(34) AB	1.07±0.02(31) C	1.30±0.04(33) A	1.09±0.04(9) BC	-	-
L. Summer '04	Whitefish	1.12±0.01(24) AB	0.98±0.02(26) C	1.14±0.02(15) A	1.07±0.01(15) B	-	-
Spring '05	Whitefish		1.11±0.02(11) B	1.19±0.03(18) A		1.09±0.01(12) B	-
L. Summer '05	Whitefish	1.13±0.04(18) A	0.99±0.02(15) B	1.06±0.03(12) AB		-	-
E. Summer '04	Sucker	-	1.18±0.03(57) B	1.16±0.03(37) B	1.06±0.02(30) B	1.27±0.02(21) A	1.14±0.03(23) B
L. Summer '04	Sucker	-	1.04±0.04(25) A	1.13±0.03(11) A	1.36±0.22(28) A	1.11±0.01(66) A	1.12±0.02(42) A
Spring '05	Sucker		1.10±0.01(27) AB	1.15±0.14(22) A		1.07±0.2(20) B	0.99±0.08(14) AB
L. Summer '05	Sucker	-	1.11±0.02(19) A	1.06±0.02(20) A		1.08±0.02(18) A	1.08±0.02(16) A

Letters represent significant differences among sites within a sampling period (ANOVA and Tukey-Kramer HSD test α=0.05). Condition factor = weight*100/length3.

– species not caught during sampling period

mykiss), cutthroat trout (*Oncorhynchus clarki*) and mountain whitefish are present in the upper reaches of the river. In the lower reaches cool-water fish such as suckers (mountain, white, and longnose), mooneye (*Hidon tergisus* Lesueur), goldeye (*Hiodon alosoides*), and northern pike (*Esox lucius* Linnaeus) dominate. In between these extremes, we find both cold- and cool-water fish sharing the same habitat. The pressure of agriculture also increases in an eastward direction, and linked to these activities is an increase in water concentrations of herbicides, such as 2,4-D and MCPP (Mecoprop) and other compounds, such as cholesterol and desmosterol. OPs and CBs were not detected in the Oldman River in the present study; they have a short half-life and are usually only detected in water for a few days after application (Morrison & Wells 1981, Fulton & Key 2001). Furthermore, only a limited number of OPs and CBs were measured by Alberta Environment, due to limited resources. However, the effects, including AChE inhibition, of OPs and CBs can be detected even after a recovery period (Beauvais et al. 2000) and trace amounts of many different OPs and CB can have an affect on organisms because they act through a similar mechanism (Grue et al. 1991, Scholz et al. 2006).

OPs and CBs inhibit AChE, the enzyme that is responsible for the removal of the neurotransmitter acetylcholine, from synaptic clefts and motor endplates (Fulton & Key 2001). Measuring levels of AChE is an easy and inexpensive way of estimating exposure to OPs and CBs. Although there was no detectable gradient of OPs or CBs in the Oldman River, we did observe a species difference in AChE: whitefish always had lower levels of AChE as compared to suckers. The difference in AChE activity between the species could be due to a fundamental physiological difference. Davies et al. (1994)

detected different AChE activities in rainbow trout and four species of Australian fish exposed to pesticides Acephate and Fenitrothion. Van Dolah et al. (1997) reported that Mummichog (*Fundulus heteroclitus*) had lower levels of brain AChE compared to Red Drum (*Sciaenops ocellatus*) when exposed to the same concentrations of Azinphosmethyl. The lower activities of AChE in whitefish could also indicate that whitefish are exposed to higher concentrations of OPs and/or CBs, either through differences in microhabitat increasing exposure to waterborne pesticides, or a difference in pesticide loading through diet. Whitefish feed on dipteran, ephemeropteran, trichopteran and some terrestrial invertebrates (Pontius & Parker 1973, Thompson & Davies 1976, Ellison 1980), while the main food source for suckers is detritus (Saint-Jacques et al. 2000), ostracods, chironomids and amphipods (Johnson & Dropkin 1995).

Along with differences in AchE activities related to fish species, small but significant site-related differences were also observed. The lower AChE activities in whitefish in spring 2005 at easterly sites may be due to an increase in pesticide exposure in early summer, a period of intensive pesticide application in Southern Alberta. Most OPs and CBs have the same mode of action, so while water concentrations of individual pesticides may be at trace amounts and not affect fish, small amounts of many different pesticides acting together may inhibit AChE (Grue et al. 1991). In late summer 2005 (late July to early August), AChE was however lower in western sites for both species. The lower AChE activity at western cooler sites could potentially be due to a temperature effect: Hogen (1970) described a linear relationship between AChE and temperature in bluegills. The relationship between temperature and pesticide exposure on AChE inhibition needs to be explored in greater depth, so that this marker of exposure can be

validated for use in a wide range of fish species and temperature regimes. Furthermore, AChE profile in mountain whitefish and suckers needs to be further characterized. Ours is a first set of data for AChE in whitefish and first seasonal data for sucker. Therefore, at present, it can not be concluded with confidence that the AChE values detected in this study are an indication of inhibition of AChE or if these are normal values.

2.4.2 Physiological Stress Response

The present study also provided a first set of data on plasma cortisol in mountain whitefish. Cortisol is a steroid hormone that plays a major role in maintaining energy stores and energy mobilization at times of stress in fish (Hontela 1998, Mommsen et al. 1999, Schreck et al. 2001, Barton 2002). Temperature is a known stressor and can increase plasma cortisol in fish at water temperatures outside of the thermal preference (Davis 2004). Plasma cortisol levels at sites outside of the whitefish thermal preference range were higher in whitefish than suckers; whitefish sampled at Pearce Rd in early summer 2004 had very high plasma cortisol levels, caused most likely by the high water temperature (23-25°C) on days of sampling. Interestingly, suckers had higher plasma cortisol than whitefish at Ft. MacLeod, the most western and also coldest site where suckers are present in abundance in the Oldman River. Temperature below the optimal temperature range is known in some species to increase plasma cortisol in fish, as temperatures above this range do. Weber & Bosworth (2005) found that channel catfish (*Ictalurus punctatus*) reared at cold temperatures had higher cortisol levels than their controls. However, other studies in striped bass (*Morone saxatilis*) and sunshine bass (*Morone chrysops* X *Morone saxatilis*) have shown that cold water temperature had no effect or lowered plasma cortisol (Davis & Parker 1990, Davis & Peterson 2006).

Several studies have shown, however, that plasma cortisol levels are species-dependent; two species may have different levels of plasma cortisol when exposed to the same stressor (Lacroix & Hontela 2004, Jentoft et al. 2005).

It is difficult to differentiate between the acute and the chronic effects of stressors on plasma cortisol in field studies. Plasma cortisol levels increased in an eastward direction for whitefish in late summer 2004 and during both sampling periods in 2005, while suckers followed the same pattern in 2005. This increase in plasma cortisol follows both the pesticides/chemical gradient and temperature gradient, suggesting that one or both of these factors act as stressors on both whitefish and suckers. However, there is also evidence in the literature to suggest that pesticides impair cortisol secretion in fish chronically exposed to low concentrations (Dorval et al. 2005, Thangavel et al. 2005). Dimethoate, an organophosphate, is known to inhibit steroid hormone production by blocking the steroidogenic acute regulatory (StAR) protein (Walsh et al. 2000). Plasma cortisol levels at all sites for suckers were within the range found for suckers at agriculturally impacted sites and well below the normal values for suckers (Dorval et al. 2005). Thus, our data suggest that the stress response in suckers in the Oldman River is impaired, possibly by agricultural chemicals. Normal plasma cortisol values are not known for mountain whitefish, since this species has not been investigated previously. The cortisol and AChE data suggest that whitefish may be highly vulnerable to the combined stress effects of pesticides and increased temperature. Further studies are needed to determine what the combined effects of pesticides and temperature are in order to determine if cortisol levels in this study were impaired by pesticides.

Liver glycogen is a stored energy source that gets catabolised into a useable energy source, glucose, during times of stress (Wendelaar Bonga 1997). Whitefish had lower levels of liver glycogen compared to suckers at all sites and sampling periods. Our study is a first comparative study to provide data on liver glycogen in these two fish species, thus, as with AChE activities and plasma cortisol, it is difficult to characterize the effects of stressors and the fundamental differences between species. The lower liver glycogen in whitefish could be due to 1) a difference in sensitivity to temperature and/or pesticides, indicating that whitefish show greater signs of stress, or 2) a physiological difference between the two species and a lower capacity for storage of glycogen in the liver of whitefish. Studies that compared liver glycogen in different species have shown that there are species-specific differences in liver glycogen levels (Krogdahl et al. 2004, Tan et al. 2006).

Site differences in liver glycogen reserves were also detected in the present study. Whitefish had lower liver glycogen levels, and very high cortisol levels, at Pearce Rd in early summer 2004, most likely associated with the high temperature during sampling. Liver glycogen reserves in both whitefish and suckers decreased along a west-east gradient, suggesting that the increase of temperature and/or pesticides is recognized as a stressor, leading to a depletion of glycogen reserves. Liver glycogen levels have been shown to decrease when fish are exposed to increased temperature or pesticides; matrinxã (*Brycon cephalus*) exposed to the organophosphate, Folidol 600 (methyl parathion) (de Aguiar et al. 2004) and steelhead trout exposed to increased water temperature both showed a decrease in liver glycogen (Viant et al. 2003).

One of the characteristic responses associated with an increase in plasma cortisol is a rise in plasma glucose, the energy source used to maintain homeostasis (Hontela 1998, Mommsen et al. 1999). Warm water temperature has been found to increase plasma glucose in eelpout (Van Dijk et al. 1999) and other fish. Plasma glucose levels in both whitefish and suckers were similar and were not influenced by site, even at Pearce Rd in summer 2004 where plasma cortisol was very high in whitefish. These results suggest that both fish species were able to maintain their glycaemia, despite other physiological signs of stress (increased plasma cortisol and decreased liver glycogen). Thus, plasma glucose was not a sensitive physiological indicator of stress in fish in our field study.

Condition factor, often used as an indicator of overall health, was not affected in whitefish or suckers sampled in this study. Again, it is at present difficult to conclude whether the condition factors were within the normal range, especially for whitefish, because this species has not been investigated in great detail. One study reported that mountain whitefish at a reference site had a condition factor of 1.14±0.22 while whitefish exposed to bleached kraft mill effluent had a condition factor of 1.20±0.18 and 1.07±0.17 (Swanson et al. 1994). Studies measuring the condition factor in suckers are numerous but condition factor for suckers in reference systems can range from 1.10 to 1.82 while condition factor for suckers in contaminated sites range from 1.23 to 1.72 (Munkittrick & Dixon 1989, Swanson et al. 1994). It is known however that if water temperatures continue to rise, foraging frequency and durations will decrease (Neill & Magnuson 1974), which would lead to a reduction in available energy, and over time would lead to a decreased condition factor.

Global climate change and increased use of pesticides are linked because with increasing air temperatures, new pests and diseases affecting both agriculture and human health are able to survive in areas that were once too cold (Bloomfield et al. 2006). The ability to control these pests usually relies on the use of pesticides (Bloomfield et al. 2006). A study of the impact that global climate change will have on agriculture in Canada and the United Kingdom shows that weeds are likely to evolve rapidly with climate change and that milder winters will increase the survival of some pests and diseases, and allow their establishment in new areas (Smith & Almaraz 2004, Bloomfield et al. 2006). Changes in temperature will also lead to a shift in crop type and with these new crops come their associated pests (Smith & Almaraz 2004, Bloomfield et al. 2006). Some areas will experience an increase in arable land while other areas will need irrigation to adequately supply water to fields, increasing water withdrawals (Bloomfield et al. 2006). Increased water withdrawals will most likely result in further water temperature increases and greater evaporation in rivers used for irrigation (Rosenberg et al. 1999). Furthermore with less water and continued input of pesticides into aquatic systems fish may be exposed to higher concentrations of pesticides.

Global climate change models suggest as water temperatures continue to increase there may also be a shift in the distribution of fish with decreases in cold-water species and the invasion of cool- or warm-water species (De Stasio et al. 1996, Eaton & Scheller 1996). At present, cold-water fish in the Oldman River are protected from the movement of some cool-water fish into the upper reaches of the river due to cold water temperatures and weirs. However, during high waters and/or as a result of improvements to fish ladders, cold-water fish (such as whitefish) might be extirpated from the Oldman River if

water temperatures continue to increase. Daufresne et al. (2003) have been monitoring water temperature increases in the Upper Rhône River in France and they observed a shift in community structure with displacement of cold-water fish and an increase in warm-water fish. Furthermore, studies on zooplankton show that high temperatures and exposure to toxicants lead to smaller bodied organisms which could affect the feeding in fish and lead to changes in fish distribution based on food availability (Moore & Folt 1993).

2.5 Conclusion

In conclusion, species-specific differences in AChE activities and responses of the physiological stress axis were detected in whitefish and suckers, suggesting that whitefish are a more sensitive species to temperature and pesticide stress. Whitefish appear to be at greater risk if water temperatures and pesticides inputs continue to increase. Further monitoring of the Oldman River and a greater understanding of mountain whitefish physiology are needed to determine if whitefish populations are healthy and stable. Studies are also needed to understand the interactions of increased temperature and pesticide exposure on the organisms within the impacted aquatic systems.

2.6 References

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Chapter 3.

Effects of Dimethoate and temperature on acetylcholinesterase activity and cortisol secretion in rainbow trout (*Oncorhynchus mykiss*): *in Vivo* and *in Vitro* exposure

3.1 Introduction

Dimethoate (O,O-dimethyl-S-(N-methylcarbamoylmethyl) phosphorodithioate), an organophosphate (OP) used widely in agriculture for crop protection as a systemic and on-contact anticholinergic pesticide, targets a broad range of insects and mites (WHO 1989). It is used on a variety of fruit, vegetable and field crops, and also to control houseflies indoors (Farag et al. 2006). Dimethoate is a low persistence pesticide which has a degradation half-life of 4-16 days but can last longer depending on the conditions (Wauchope et al. 1992, Athanasopoulos et al. 2000). Alkalinity and temperature influence its degradation, with low temperature and low pH increasing the half-life (Athanasopoulos et al. 2000). Organophosphates such as Dimethoate are highly soluble in water and can leach into nearby water sources and affect aquatic organisms (Tuduri et al. 2006).

Exposure of non-target organisms such as vertebrates to Dimethoate affects behaviour (Dell'Omo et al. 1997, De Mel & Pathiratne 2005, Farag et al. 2006), reproductive function (Begum & Vijayaraghavan 1995, Farag et al. 2006) and muscle glycogen levels (Begum & Vijayaraghavan 1999). Dimethoate also causes a reduction in food consumption and body weight in mammals (Farag et al. 2006), and damage to gill, kidney, and liver tissues in fish (Gill et al. 1988). Exposure to Dimethoate inhibits acetylcholinesterase (AChE) in the brain (Dell'Omo et al. 1997, Frasco & Guilhermino 2002, De Mel & Pathiratne 2005, Farag et al. 2006).

Exposure to stressors, including chemical stressors, such as pesticides, and physical stressors, such as suboptimal water temperature, can activate the physiological stress response, a suite of responses that facilitate maintenance of homeostasis (Wendelaar Bonga 1997, Schreck et al. 2001). Plasma cortisol and glucose levels, or liver glycogen, are physiological indicators used to determine if fish are stressed and if the hypothalamo-pituitary-interrenal axis is functioning normally (Hontela 1998). Acute exposures to pesticides elevate plasma cortisol (Teles et al. 2007) while chronic exposure may actually impair cortisol synthesis in fish species (Dorval et al. 2005, Thangavel et al. 2005). Although the effects of Dimethoate on cortisol synthesis have not been investigated in fish or other vertebrates, Dimethoate has been shown to block the steroidogenic pathway, specifically the StAR protein required for cholesterol uptake by the mitochondrion, in the testis of rats and inhibit synthesis of testosterone (Walsh et al. 2000). Since synthesis of cortisol, another steroid hormone, also relies on StAR, exposure to Dimethoate could block cortisol synthesis in the steroidogenic interrenal tissue in teleosts.

Cortisol synthesis and plasma cortisol levels in fish can be also influenced by temperature. When fish are exposed to temperatures outside their thermal preference range (both above and below); there is an increase in plasma cortisol levels (Davis & Parker 1990, Davis 2004, Davis & Peterson 2006). Suboptimal temperatures can also have negative effects on growth rate and reproduction (Davis & Parker 1990, Martikainen & Rantalainen 1999, Davis 2004, Davis & Peterson 2006). Temperature can not only affect the physiology and behaviour of organisms, but also the fate of chemicals in the environment and their toxicity (Martikainen & Rantalainen 1999). Chemicals that

cause oxygen deficiency or increase metabolic activity tend to be more toxic at higher temperatures (Martikainen & Rantalainen 1999) whereas low temperatures can increase toxicity by increasing exposure duration (Athanasopoulos et al. 2000). The effects of temperature on OP pesticides exposure and the inhibition of AChE vary considerably; some studies (Beauvais et al. 2002, Phillips et al. 2002) reported that temperature does not affect AChE inhibition while others (Hogan 1970, Zinkl et al. 1987) showed a linear relationship between water temperature and AChE inhibition. With the threat of global climate change, it is important to understand the effect that increasing temperatures will have on aquatic organisms and their exposure to chemicals such as pesticides. Pesticide use is expected to increase with increasing global temperature, to control new pests and expanding pest populations. Thus within the climate change scenario, aquatic organisms may be potentially exposed to both increased temperatures and increased pesticide inputs (Bloomfield et al. 2006).

The objectives of this study were to investigate the effects of Dimethoate and temperature on cortisol secretion and the physiological stress response, and AChE activity in rainbow trout (Oncorhynchus mykiss). It is hypothesised that exposure to Dimethoate will inhibit cortisol secretion and AChE activity in all tissues, and higher temperature will increase AChE activity and the toxicity of Dimethoate.

3.2 Materials and Methods

a. Test Species

Immature male and female rainbow trout, *O. mykiss*, obtained from Allison Creek Brood Trout Station (Coleman, Alberta, Canada), were transferred on arrival into a

maintenance tank (600L) for a 2-week acclimation period (12L:12D, 15±1ºC, oxygen saturated water, pH 7.8). Fish were fed commercial trout food (Silver Cup salmon crumbles, Nelson & Sons) once a day.

3.2.1 In Vivo exposure to waterborne Dimethoate (pure grade)

Following the acclimation period, fish (size matched groups of 10) were transferred into 6 experimental tanks (semi-static system, with a water renewal of 20% daily). All the tanks were located in the aquatic room maintained at air temperature of 11ºC; each experimental tank had a submerged heater (Thermal Compact Pre-Set Heater).

a. Experimental treatments

Fish (average body weight 24.7±0.5 g, average body length 12.4±0.1 cm) were acclimatized to the experimental tanks (50 L) for one week, then they were exposed to 0% (control), 0.5%, or 1% LC_{50} (LC_{50} = 7.35 mg/L, Johnson and Finley 1980, WHO 1989) of pure grade Dimethoate (Riedel-de Haën 45449) dissolved in water, and either a cold or a warm water treatment for 21 days. The cold water tanks were maintained at 10±1°C for 21 days, using the air cooling system of the aquatic room. The warm water tanks were maintained at 19±1°C for 10 days using the submerged heaters. On Day 11 all the heaters were removed and for the next 10 days during the daily 20% water renewal, warm water (30±1°C) was added to the warm water treatment tanks at 0830h, to bring the water temperature up to 15±1°C. Water at 11°C was added to the cold water treatment tanks, to insure same level of disturbance in all the tanks. The Dimethoate concentrations were maintained at the experimental levels during water renewal. Water chemistry and temperature during the experiment are presented in Table 3.1 and Fig. 3.1.

Table 3.1. Temperature and water chemistry (mean ± SEM) during Dimethoate (pure grade) and temperature treatments.

Treatment	Temperature Day 1-10 (n=9)	Maximum Temperature Day 11-21 (n=12)	Dimethoate[a] mg/L (n=3)	pH (n=6)	Dissolved Oxygen (n=4)
cold 0% LC_{50}	10.44±0.13	10.17±0.07	<0.1	7.63±0.07	9.98±0.77
warm 0% LC_{50}	20.11±0.18	15.58±0.17	<0.1	7.64±0.09	8.28±0.77
cold 0.5% LC_{50}	9.72±0.12	9.75±0.13	31.6±0.3	7.97±0.06	9.83±0.81
warm 0.5% LC_{50}	18.61±0.27	15.08±0.17	29.6±2.3	7.92±0.09	6.87±0.36
cold 1% LC_{50}	10.89±0.11	11.13±0.07	110.2±28.2	7.80±0.05	7.01±0.41
warm 1% LC_{50}	17.44±0.31	15.13±0.11	78.0±18.9	7.86±0.06	6.25±0.19

[a]Water samples were analyzed for Dimethoate by Alberta Research Council Laboratory (D. Humphries)

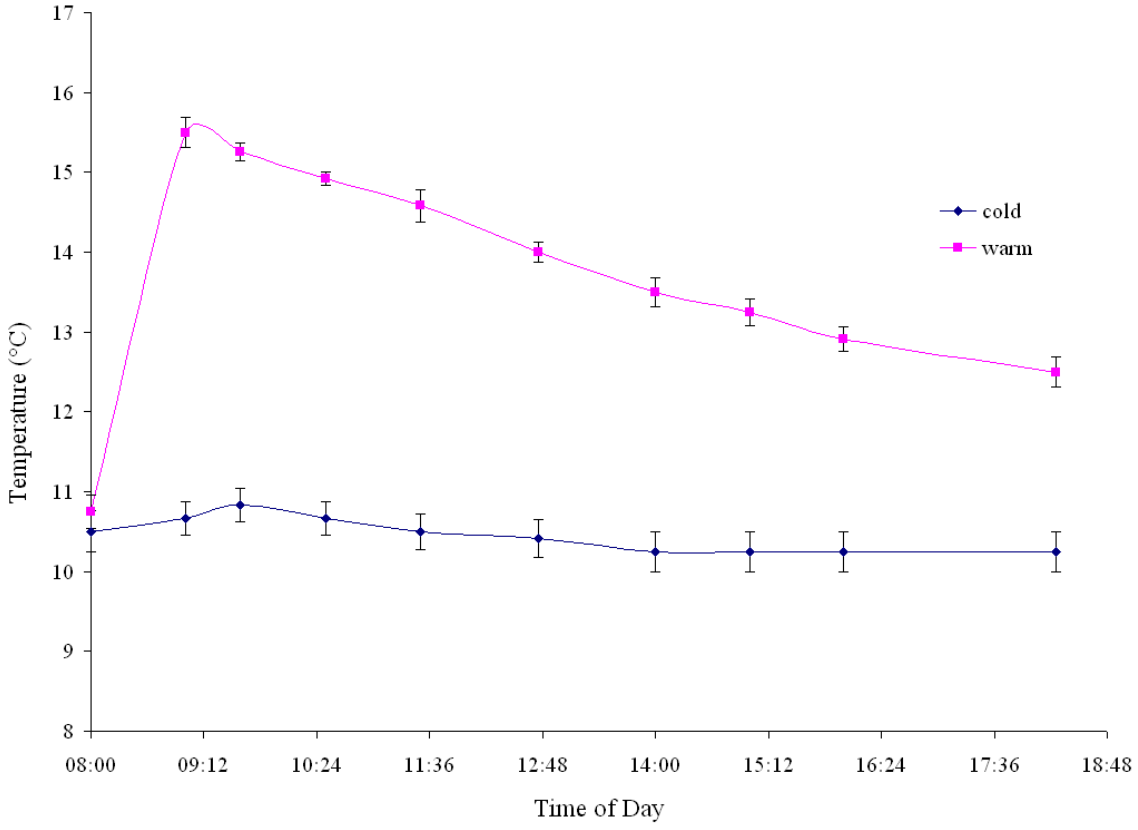


Figure 3.1. Hourly temperature profile for days 11-21 in the experimental treatment tanks.

b. Sampling

Fish were sampled between 0830h and 0930h to minimize the effects of diel hormonal variations. Fish were anaesthetized in a solution of 0.15 g/L of tricaine methanesulphonate (MS-222) and a blood sample was taken from the caudal vasculature with a 1 mL heparized syringe. Plasma was separated at 13000 rpm for 5 minutes and frozen in liquid nitrogen. The fish were euthanized by spinal transection, weights and lengths recorded and, the head kidneys were removed immediately and placed in minimal essential medium (MEM), liver, gills, muscle and brains were dissected and frozen in liquid nitrogen. Plasma and tissue samples were kept frozen at -80°C until analysis.

c. Acetylcholinesterase (AChE)

The AChE activity was measured with an assay modified from Ellman et al. (1961) and Chuiko (2000). Muscle samples were homogenized using Power Max Advanced Homogenizing System (VWR) (1 g of tissue to 3 mL phosphate buffer) and centrifuged at 5°C at 10000 rpm for 10 minutes. Brain samples were homogenized with a Pellet Pestle Motor (Kontes) (1g of tissue to 3 mL phosphate buffer) and centrifuged at 5°C at 13000 rpm for 3 minutes. A 10x dilution was used for both muscle and brain samples. Plasma, brain or muscle homogenate (2 μL) was pipetted into a microplate and 120 μL of ISO-OMPA (AChE only, Sigma Tetraisopropyl pyrophosphoramide, T1505) or 120 μL of Tris Buffer (Total Cholinesterase: AChE and Butyrylchlinesterase (BChE), Tris Buffered Saline Tablets, Sigma T-5030) was added. The samples were incubated at room temperature for 10 minutes and 10 μL of DTNB (Sigma 5,5' Dithiobis (2-nitro-benzoic acid), D8130) and 10 μL Acethylthiochlorine Iodine (Sigma minimum 98% TLC, A5751) were added, followed by a 10 minute incubation period at room

temperature. The microplate was read at 405nm in a microplate reader every 2 minutes for 10 minutes. Concentration of AChE for each sample was measured from the slope; internal standards (Normal Serum Control, TC-TROL(N), Teco Diagnostics) and controls (1U/mL and 2 U/mL of eel acetylcholinesterase, Sigma C3389) were used to ensure the accuracy of the assay was maintained. Protein for muscle and brain samples was measured using the Bradford method (Bradford 1976).

d. Cortisol secretion (ACTH-challenge of head kidneys isolated from Dimethoate-exposed fish)

The head kidney was divided in two, half frozen for later analysis and the remaining portion used for the ACTH-challenge using the microplate method described by Benguira & Hontela (2000). The head kidney was cut into small fragments (1 mm^3) and the coarse homogenate was divided into two portions (one for basal treatment and one for ACTH stimulation), pipetted into a 24 well microplate (1 mL of MEM/well). Following a 2 hrs incubation, the microplate was centrifuged and the supernatant removed. The basal treatment was then incubated in 1mL of MEM while the stimulated treatment was incubated in 1mL of MEM containing 2 IU of porcine adrenocorticotropin hormone (ACTH, Sigma Chemical Co A6303). After 1hr of incubation, the microplate was centrifuged and the supernatant was frozen for later analysis of cortisol by radioimmunoassay.

e. Physiological Stress Response

Concentrations of cortisol in the plasma were determined with diagnostic kits (MP Biomedicals Diagnostics Division 07-221102). Plasma glucose was determined with a spectrophotometer assay (512nm) using the GOD-PAP reagent (Roche 1929526).

Liver glycogen was measured with a method modified from Bleau et al. (1996). Liver samples were digested in KOH (1N) at room temperature; the sample was then precipitated with 1.5 N acetic acid and centrifuged at 13000 rpm for 10 minutes. Glycogen from the livers and standards (rabbit glycogen, Sigma, G8876-1G) was hydrolyzed to glucose with aminoglycosidase (Sigma A-7255) in a water bath at 37°C for 30 minutes. Glucose content was measured using the spectrophotometric assay described above.

f. Gill Na+/K+ ATPase activity

Na^+/K^+ -ATPase activity in the gills was determined using modified methods of Holliday (1985) and Morgan et al. (1998), as described in Levesque et al. (2003). In summary, 0.5g of gill tissue was homogenized in a phosphate buffer using a Pellet Pestle Motor (Kontes). Homogenates were added to 200 µL buffer A (NaCl 167 m*M*, KCl 50 m*M*, imidazole-HCl 33 m*M*, pH 7.2 with NaOH 1 *M*) or buffer B (NaCl 237 m*M*, ouabain 1.67 m*M*, imidazole-HCl 33 m*M*, pH 7.2 with NaOH 1 *M*). The Na^+/K^+ -ATPase activity was obtained by determining the difference in inorganic phosphate production between buffer A and buffer B using a spectrophotometer at 700nm. Protein was measured using the Bradford method (Bradford 1976). Enzyme activity was expressed as micromoles of inorganic phosphate per hour per milligram of protein.

3.2.2. In Vitro exposure of head kidney cells to Dimethoate

a. Preparation of head kidney cell suspensions

The method described by Leblond et al. (2001) was used for the head kidney cell preparations. Fish were anaesthetized with MS-222 (tricaine methanesulphonate), a blood sample was taken from the caudal vein and the fish were perfused through the heart with

a 0.7% saline and heparin solution to remove as much blood as possible. Head kidneys were then dissected out, placed in fresh MEM, cut into small pieces (<1 mm^3) and placed into 2.5 ml MEM with 3.0 mg/mL DNAase (Sigma D8754) and 5.0 mg/mL collagenase/dispase (Boehringer Mannheim: 1-097-113). The cells were incubated at 22ºC for 60 min with gentle agitation and cells were re-suspended every 15 min with a transfer pipette. After the digestion, the cells were agitated with a pipette to further break apart the tissue, then the cells were filtered with a 30 μm mesh cloth and centrifuged at 1000 rpm for 5 min at 15º C. The supernatant was removed, the pellet was re-suspended in MEM and cell density was adjusted to $75x10^6$ cells/mL. The cells were then distributed (75 μL of the cell suspension) in two 96 well microplates, preincubated for 120 min, one plate at 11±1º C and the other at 20±1º C.

b. Exposure to pure grade or commercial Dimethoate

Following the preincubation, the cells were centrifuged at 1000 rpm for 3 min, exposed to either pure grade Dimethoate (0 mg/L, 3 mg/L, 30 mg/L or 300 mg/L) (Riedel-de Haën 45449) or Cygon, the commercial dimethoate (0 mg/L, 3 mg/L, 30 mg/L, 60 mg/L, or 300 mg/L) in Ringer solution (269 mOsm/L), incubated for 60 min at either 11±1ºC or 20±1ºC, washed with 100 μL of Ringer solution, and stimulated with 1 IU/mL ACTH. Supernatant was collected for cortisol analysis.

Viability was determined using the trypan blue solution (0.4%, Sigma, T8154) and a counting chamber (VWR Scientific, 15370). Viability was expressed as a percentage the number of cells that took up the stain (dead) and those that did not (alive).

3.2.3 Statistical Analysis

For the *In Vivo* exposure experiments, a two-way factorial analysis (ANOVA) using JMP IN 5.1, with a post hoc Tukey-Kramer HSD test and a log transformation or Box Cox transformation for non-normal data, was used. Data that could not be transformed were analysed using a non-parametric Wilcoxon test with a Bonferoni correction. Linear regression with inverse prediction using JMP IN 5.1 was used to determine the LC_{50} and EC_{50} for *In Vitro* experiments. A paired t-test was used to determine if the LC_{50} was different than the EC_{50} and the difference between the two temperature treatments.

3.3 Results

3.3.1 In Vivo exposure to Dimethoate

a. AChE activity – a marker of pesticide exposure

The activity of AChE measured in plasma of control fish was lower than muscle and brain AChE (Table 3.2), with the highest activity detected in muscle ($p<0.05$, ANOVA). The activity of AChE decreased in an exposure-dependent pattern in plasma, brain and muscle of rainbow trout exposed to Dimethoate through water, and the greatest inhibition was observed in muscle AChE (Table 3.2). BChE was not detected in any of the tissues (data not shown).

Table 3.2. AChE activity, mean±SE (n), in brain, muscle, and plasma in rainbow trout exposed to Dimethoate.

Temperature treatment	Dimethoate (% LC_{50})	% AChE inhibition compared to controls		
		Brain	Muscle	Plasma
cold	0	0.00±0(10) E	0.00±0 (10) E	0.00±0 (10) E
	0.5	18.89±5.2(10) D	46.55±6.0(10) C	27.65±4.9(10) D
	1	57.22±3.2(9) BC	87.91±2.4(9) A	65.99±2.7(9) B
warm	0	0.00±0 (8) e	0.00±0 (8) e	0.00±0 (8) e
	0.5	2.46±1.5(7) e	44.75±7.6(7) c	25.03±7.4(7) d
	1	42.22±3.5(9) cd	86.12±3.8(9) a	65.57±2.8(9) b

Uppercase letters represent significant differences between brain, muscle, and plasma AChE inhibition for cold water, lowercase letters represent significant differences between brain, muscle, and plasma AChE inhibition in warm water (ANOVA and Tukey-Kramer HSD test α=0.05).

Plasma AChE activity (Figure 3.2A) decreased with increasing exposure to Dimethoate, with significant differences ($p<0.05$) between the control and the group exposed to 1% LC_{50}. The temperature treatments had no effect on plasma AChE activity at any concentration of Dimethoate.

Brain AChE activity was also inhibited with increasing exposure to Dimethoate (Figure 3.2B), again the effect was significant ($p<0.05$) at the 1% LC_{50} of Dimethoate. The warm water treatment decreased brain AChE activity in the control group (0% Dimethoate) but had no effect in the other exposure groups (0.5 and 1% LC_{50}).

Muscle AChE activity decreased significantly ($p<0.05$) with increasing exposure to Dimethoate, in a concentration-dependant pattern, but there was no difference between the different temperature treatments (Figure 3.2C).

b. Physiological Stress Response

Plasma cortisol levels decreased with increasing concentrations of Dimethoate but the temperature treatments did not have an effect on cortisol (Figure 3.3A). Basal and ACTH-stimulated cortisol secretion of head kidneys isolated from fish exposed to Dimethoate in vivo was not different between the exposure groups or temperature treatment (Figure 3.3B). There were however significant differences in the variations between cortisol secretion at the different concentrations of Dimethoate (F-test for unequal variance).

There were no significant differences in plasma glucose or liver glycogen for either temperature treatment or Dimethoate concentrations ($p>0.05$) (Table 3.3). Gill Na^+/K^+ ATPase activity increased with exposure to Dimethoate ($p<0.05$) and warm temperature treatment increased the activity in the control group (Figure 3.4). There were

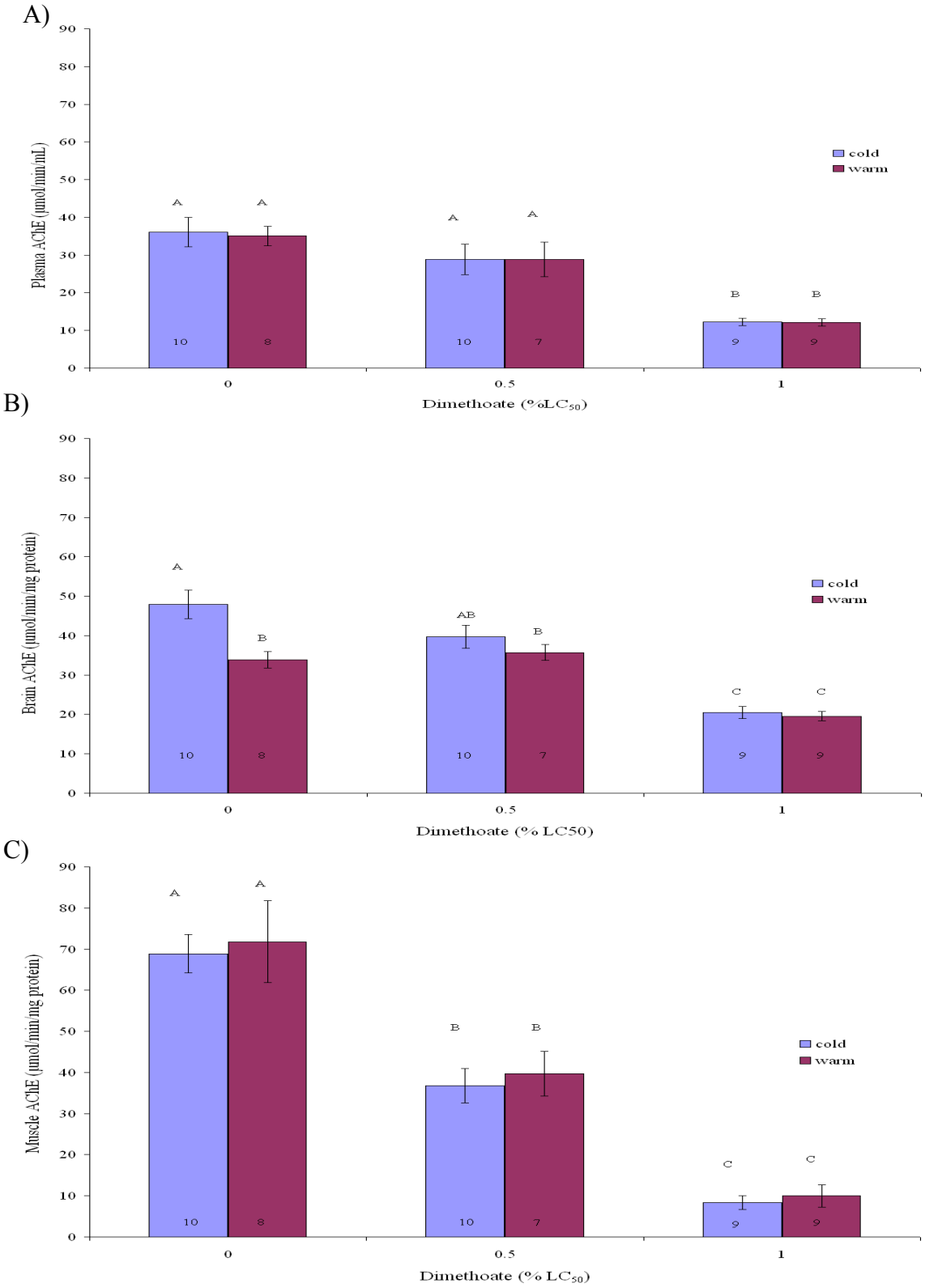


Figure 3.2. AChE activity in tissues of rainbow trout exposed to Dimethoate (pure grade) at two different temperatures. A) Plasma AChE, B) Brain AChE, C) Muscle AChE. Numbers in the bars indicate the number of fish in each treatment. Letters represent significant differences (Two-way ANOVA and Tukey-Kramer HSD test α=0.05).

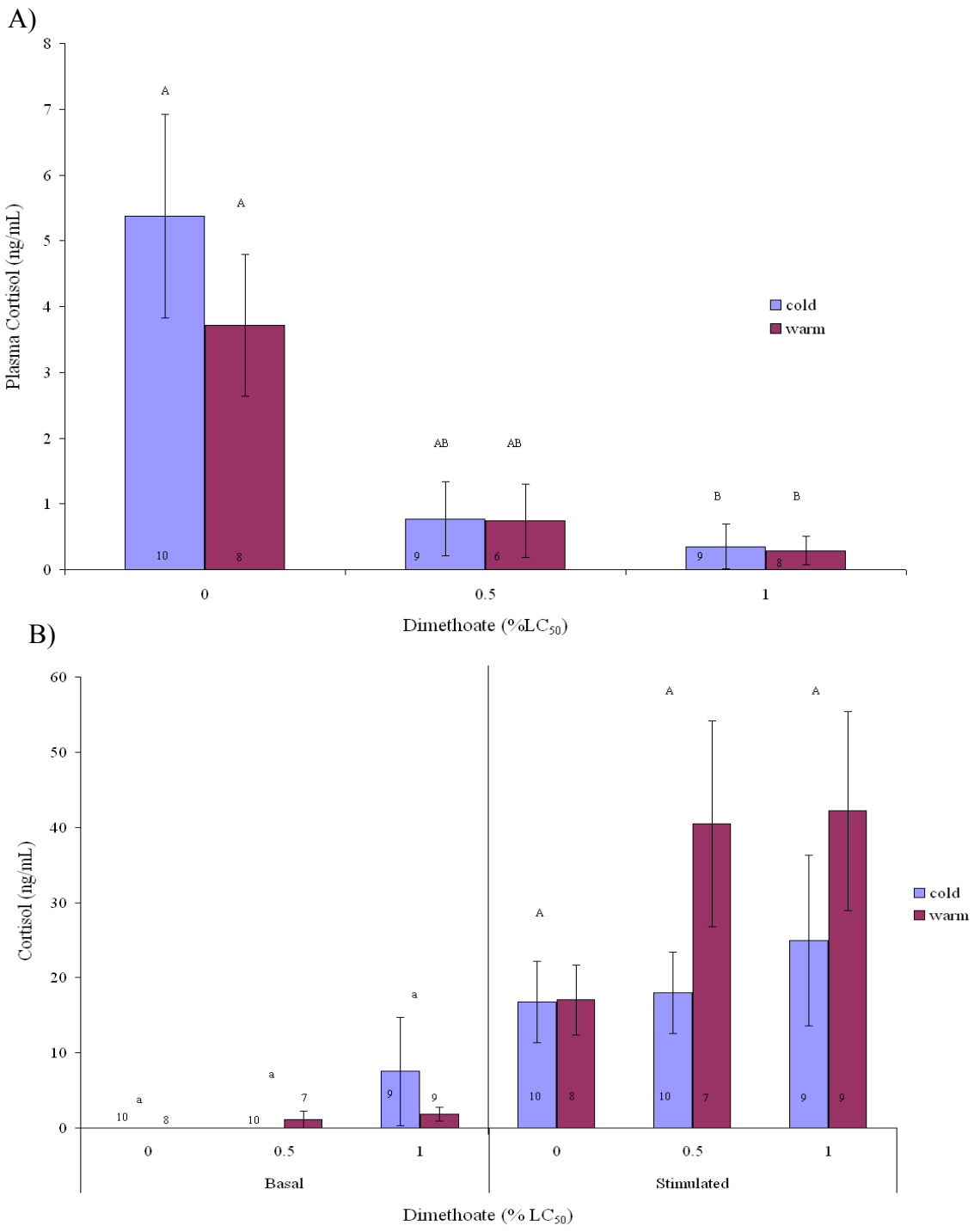


Figure 3.3. A) Plasma cortisol and (B) basal and ACTH-stimulated cortisol secretion (mean±SE) by isolated head kidneys of rainbow trout exposed to Dimethoate (pure grade) at two different temperatures. Numbers in the bars indicate the number of fish in each treatment. A) Upper-case letters represent significant differences (Wilxon non-parametric test with a bonferoni correction and Tukey-Kramer HSD test α=0.05). B) Lower-case letters represent significant difference in basal cortisol while upper-case letters represent significant differences in stimulated cortisol (two-way ANOVA, with Tukey-Kramer HSD test α=0.05).

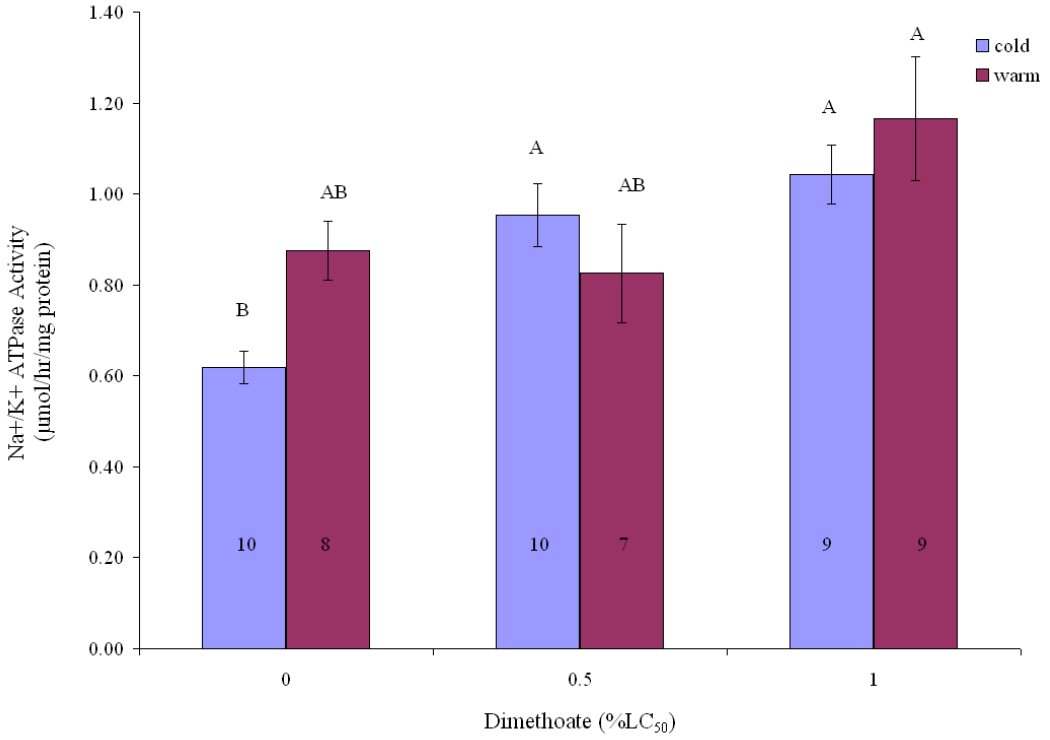


Figure 3.4. Gill Na^+/K^+ ATPase activity (mean±SE) in rainbow trout exposed to Dimethoate (pure grade) at two different temperatures. Numbers in the bars indicate numbers of fish in each treatment. Capital letters represent significant differences (Two-way ANOVA and Tukey-Kramer HSD test α=0.05). Data log transformed for statistical analysis.

Table 3.3. Plasma glucose, liver glycogen, condition factor and liver somatic index, mean ± SE(n), in rainbow trout exposed to Dimethoate (pure grade) at two different temperatures

		Dimethoate Treatment		
	Temperature	0% LC_{50}	0.5% LC_{50}	1% LC_{50}
Plasma Glucose (mg/mL)	Cold Water	0.87±0.04 (10)	0.83±0.07 (10)	0.85±0.11 (9)
	Warm Water	0.87±0.07 (8)	0.80±0.06 (7)	0.84±0.06 (9)
Liver Glycogen (mg/g)	Cold Water	63.73±9.3 (10)	78.12±8.4 (10)	61.59±9.5 (9)
	Warm Water	88.83±11.6 (8)	85.09±10.7 (7)	83.14±14.8 (9)
Condition Factor	Cold Water	1.30±0.19 (10)	1.10±0.03 (10)	1.11±0.01 (9)
	Warm Water	1.12±0.04 (8)	1.13±0.03 (7)	1.13±0.02 (9)
Liver somatic index	Cold Water	1.13±0.09 (10)	1.33±0.07 (10)	1.23±0.06 (9)
	Warm Water	1.48±0.17 (8)	1.42±0.15 (7)	1.31±0.05 (9)

Condition Factor: body weight*100/length^3 , Liver somatic index: (liver weight/body weight)*100 (Two-way ANOVA and Tukey-Kramer HSD test α=0.05). Liver glycogen data log transformed for statistical analysis.

no significant differences for either temperature treatments or for Dimethoate concentrations ($p>0.05$) for condition factor or liver somatic index (LSI) (Table 3.3). Mortality occurred in all the warm water tanks, but was not dependent on Dimethoate concentration (two fish in control and 0.5% LC50 group, one fish in the 1% LC50 group). One fish in the cold water 0.5% LC50) dimethoate group died.

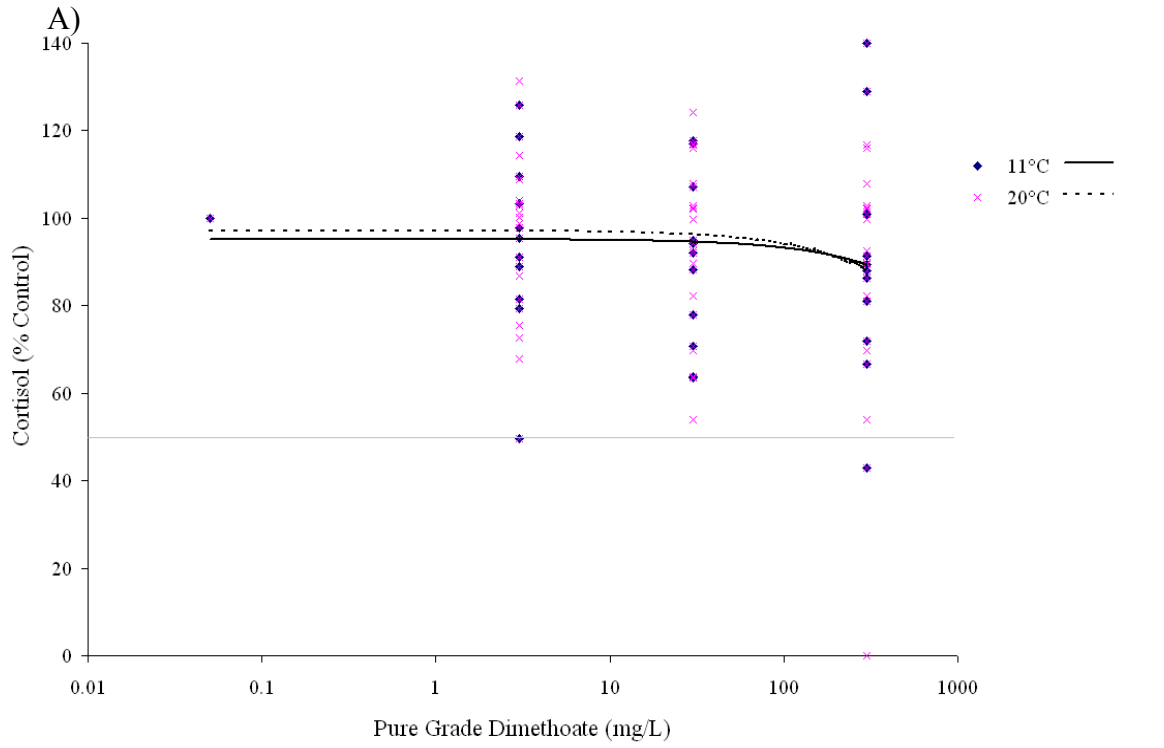
3.3.2 In Vitro exposure of head kidney cells to Dimethoate (pure grade)

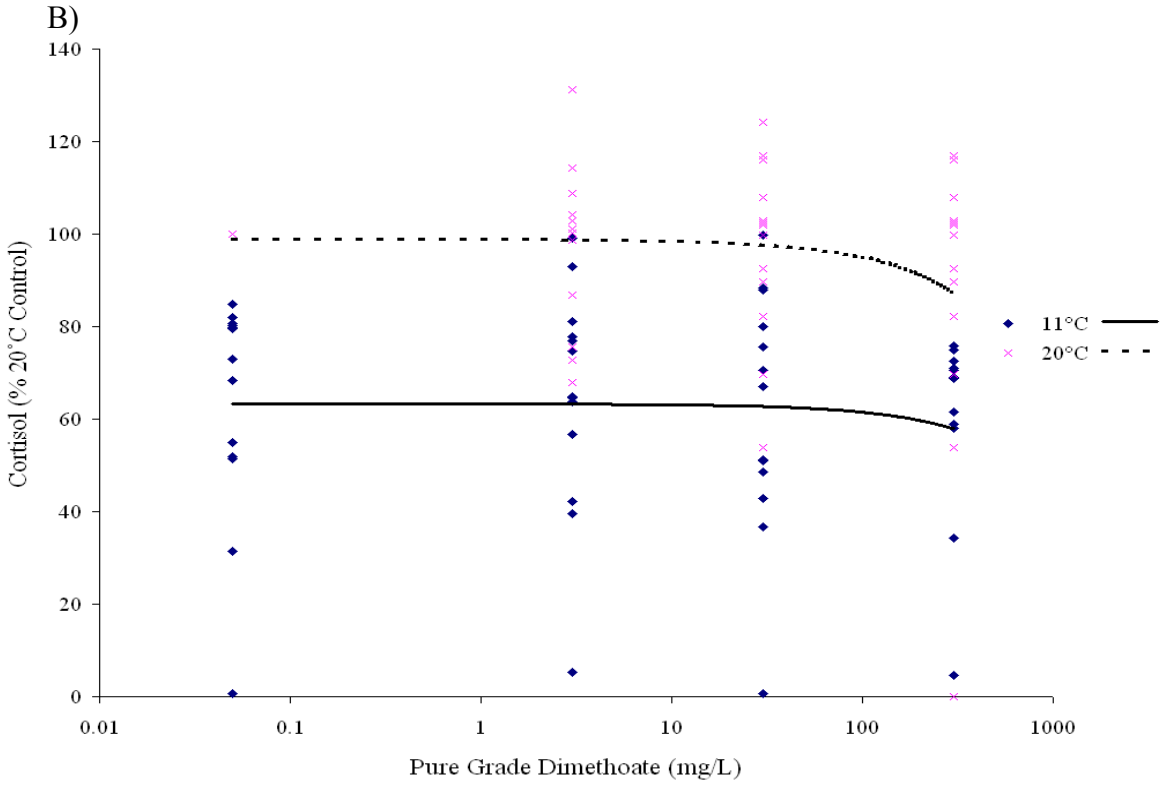
a. Cortisol secretion

Pure grade Dimethoate did not have an effect on ACTH-stimulated cortisol secretion by isolated head kidney cells exposed to the pesticide in vitro at either 11±1°C (R^2=0.04, $P>0.05$) or 20±1°C (R^2=0.33, $P>0.05$) (Figure 3.5A). The EC_{50} (concentration that inhibits 50% of cortisol secretion) for pure Dimethoate could not be determined using inverse prediction because that level of inhibition was not observed. There was a significant difference ($p<0.05$) in cortisol secretion among the two temperatures, with higher cortisol levels detected at 20°C compared to 11°C (Figure 3.5B).

b. Viability

There was no change in viability with increasing concentrations of pure grade Dimethoate at 11°C (R^2=0.04, $P>0.05$, Figure 3.5C) but viability did decrease slightly but significantly (R^2=0.33, $P>0.05$) at 20°C (Figure 3.5C). Again, the LC_{50} (lethal concentration that kills 50% of cells) of pure Dimethoate could no be determined. Temperature did not have an effect on cell viability as there were no differences ($p>0.05$) in viability between 11°C and 20°C.


A)
Cortisol (% Control)
Pure Grade Dimethoate (mg/L)
11°C
20°C
0
20
40
60
80
100
120
140
0.01
0.1
1
10
100
1000


B)
Cortisol (% 20°C Control)
Pure Grade Dimethoate (mg/L)
11°C
20°C
0
20
40
60
80
100
120
140
0.01
0.1
1
10
100
1000

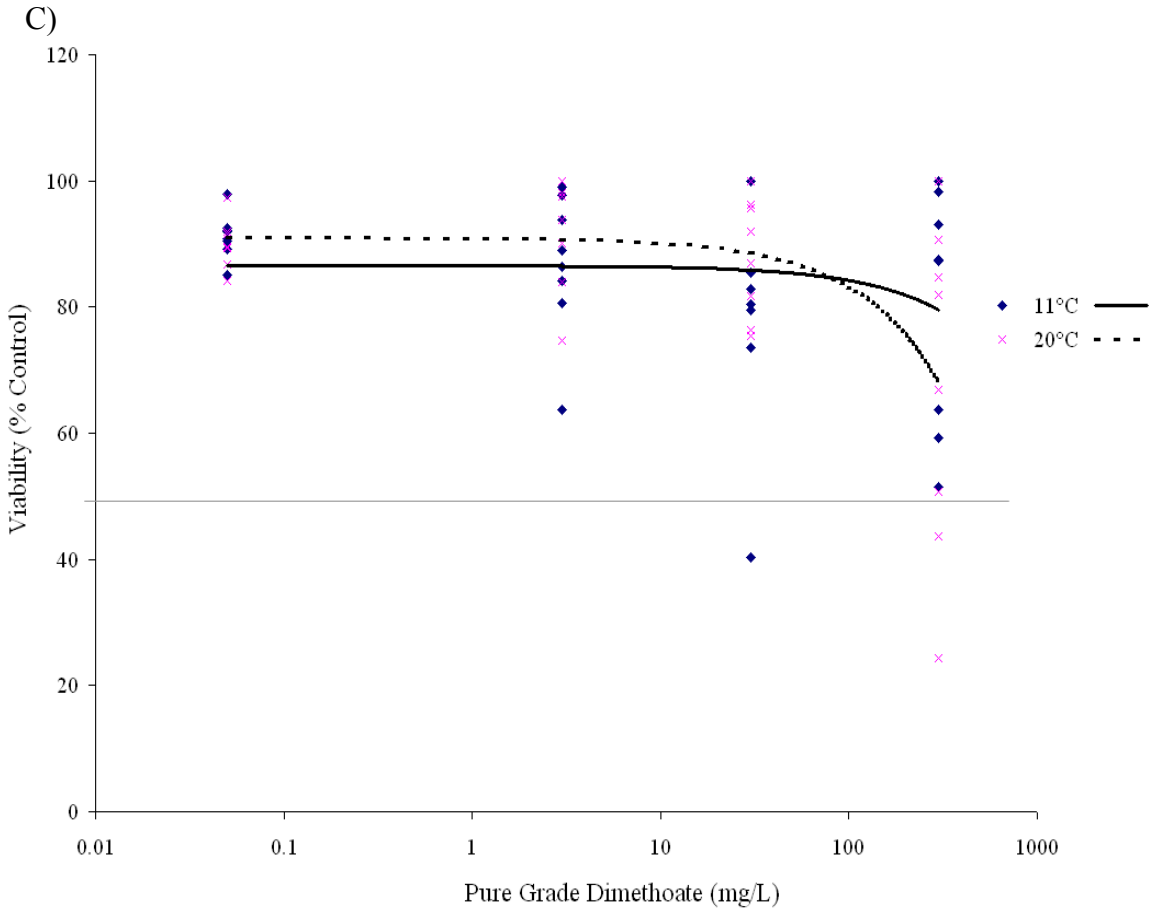


Figure 3.5. Cortisol secretion (ACTH-stimulated) and viability of head kidney cells exposed to Dimethoate (pure grade) in vitro at 11°C and 20°C. A) Cortisol secretion expressed as % Control at either 11°C or 20°C. Linear regression with inverse prediction was used to determine the slope of the line; solid black line is the regression for 11°C [X=(Y+95.17)/0.02], R^2=0.04, dashed line the regression for 20°C [X=(Y+98.87)/0.04], R^2=0.33, n=11 for 11°C and n=13 for 20°C, n indicating number of fish sampled, with individual wells pooled together. The solid grey line represents the predicted LC_{50}. B) Cortisol secretion expressed as % of Control at 20°C. C) Viability (expressed as % of Control at either 11°C or 20°C, The solid line is the regression for 11°C [X=(Y+86.54)/0.02], R^2=0.04, and the dashed line is the regression for 20°C [X=(Y+90.95)/0.08], R^2=0.33. n=8 for all groups.

3.3.3 In Vitro exposure of head kidney cells to commercial dimethoate (Cygon)

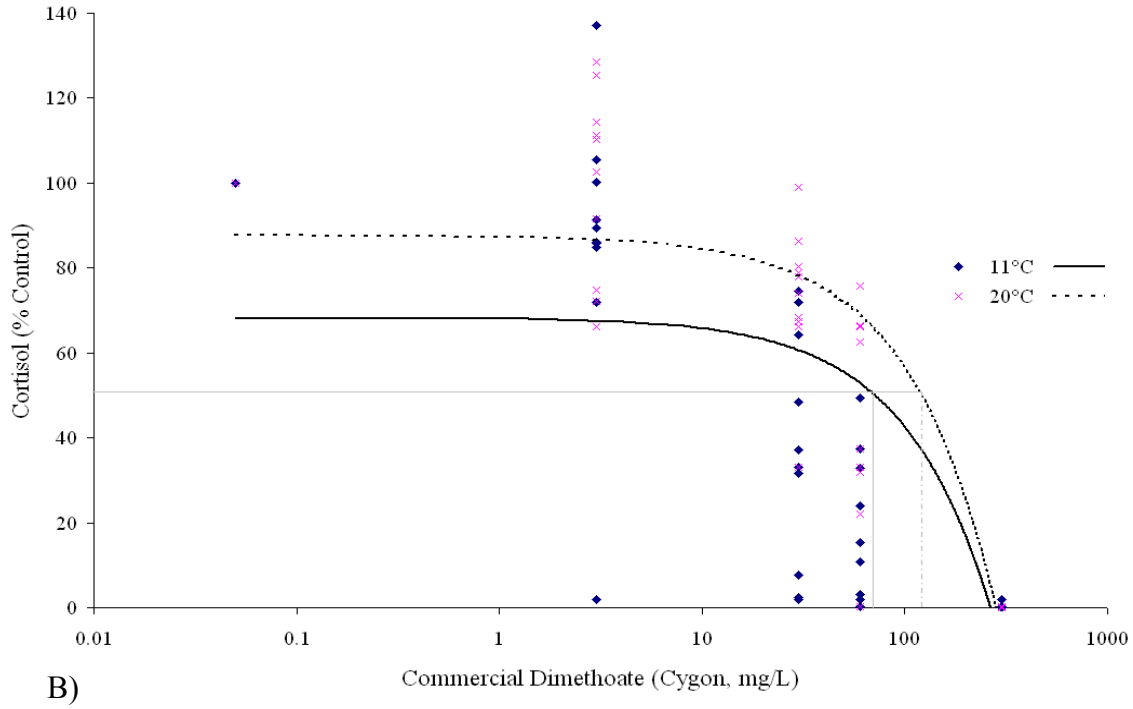
a. Cortisol secretion

Cortisol secretion decreased ($p<0.05$) with increasing concentrations of commercial dimethoate, Cygon, at both 11°C ($R^2=0.45$) and 20°C ($R^2=0.72$) (Figure 3.6A). The EC_{50} at 11°C, determined as 71.45 mg/L Cygon dimethoate (32.18-109.06 mg/L dimethoate 95% confidence limit), was significantly lower than the EC_{50} identified at 20°C as 118.10 mg/L Cygon dimethoate (97.24-141.67 mg/L Cygon dimethoate 95% confidence limit). Temperature had a significant effect ($p<0.05$) on cortisol secretion, with higher cortisol secretion at 20°C than 11°C (Figure 3.6B).

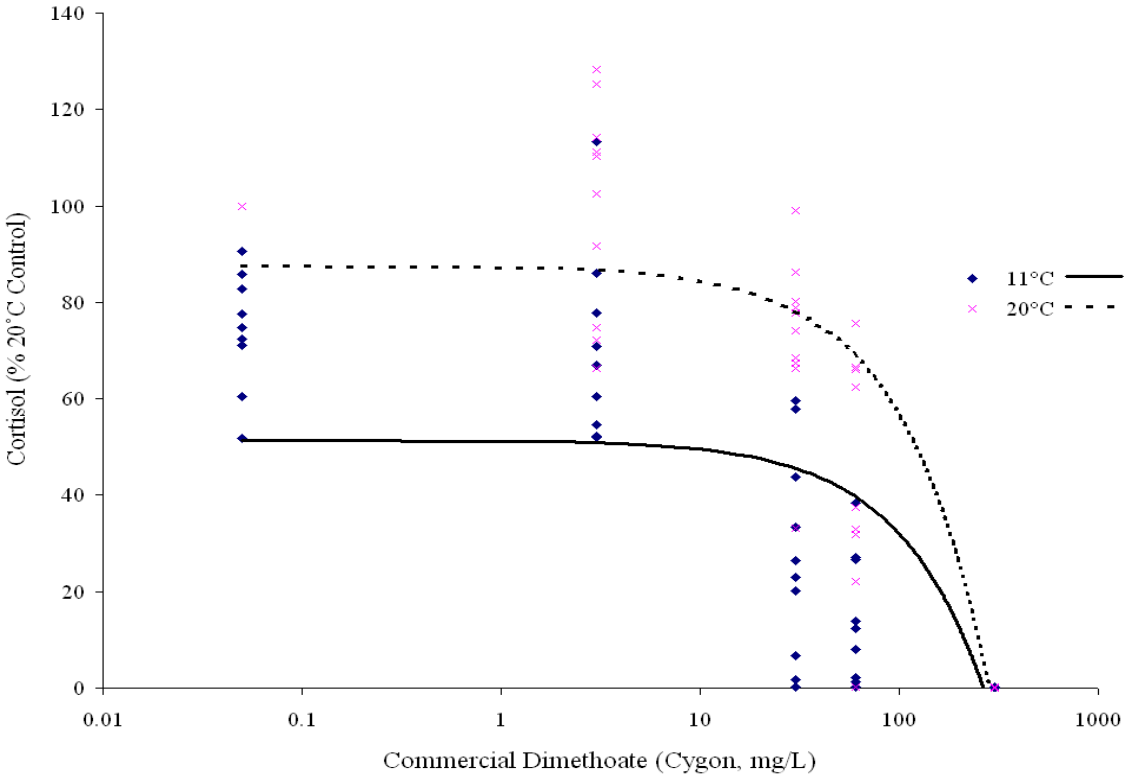
b. Viability

There was a significant decrease ($p<0.05$) in viability with increasing concentrations of Cygon dimethoate at both 11°C and 20°C (Figure 3.6C), however, temperature did not have a significant effect ($p>0.05$) on cell viability. The LC_{50} for 11°C, determined as 101.14mg/L Cygon dimethoate (73.45-131.70mg/L Cygon dimethoate 95% confidence limit), was not significantly different from the LC_{50} at 20°C determined as 93.17 (66.97-120.90 mg/L Cygon dimethoate 95% confidence limit). At 11°C the LC_{50} was significantly greater than the EC_{50} however there was no difference between the EC_{50} and LC_{50} at 20°C.

A)


Cortisol (% Control)
140
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Commercial Dimethoate (Cygon, mg/L)
11°C
20°C

B)


Cortisol (% 20°C Control)
140
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Commercial Dimethoate (Cygon, mg/L)
11°C
20°C

C)

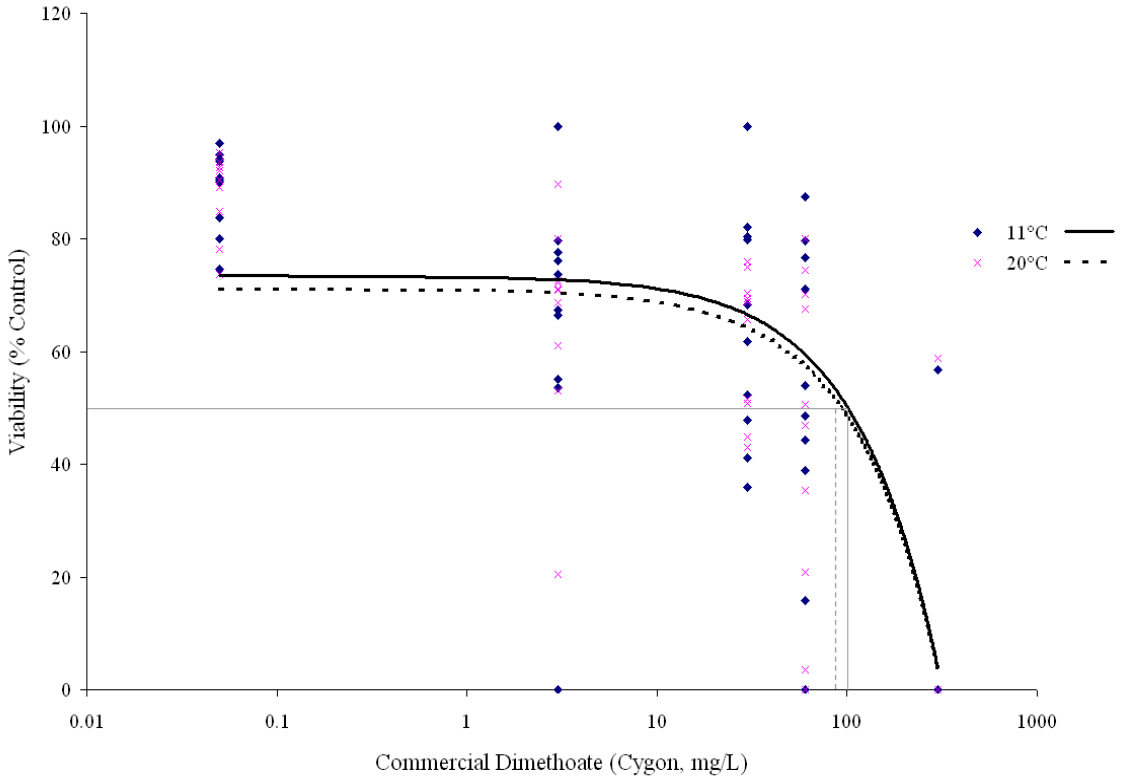


Figure 3.6. Cortisol secretion (ACTH-stimulated) and viability of head kidney cells exposed to Dimethoate (commercial grade) in vitro at 11°C and 20°C. A) Cortisol secretion expressed as % Control at either 11°C or 20°C. Linear regression with inverse prediction used to determine the slope of the line; solid black line is the regression for 11°C [X=(Y+68.40)/0.26], R^2=0.45, the dashed line is the regression for 20°C [X=(Y+86.26)/0.31], R^2=0.72. The grey line represents the EC_{50} for 11°C and 20°C: 71.45 mg/L dimethoate and 118.24 mg/L dimethoate respectively. n=10 for 11°C and 20°C, n indicating number of fish sampled, individual wells were pooled together. B) Cortisol secretion expressed as % of Control at 20°C, C) Viability (expressed as % of Control at either 11°C or 20°C, The black solid line is the regression for 11°C [X=(Y+73.46)/0.23] and the dashed black line is the regression for 20°C [X=(Y+71.14)/0.23]. The grey line represents the LC_{50} for 11°C and 20°C: 101.14mg/L dimethoate 93.17mg/L dimethoate respectively. n=8 for both groups.

3.4 Discussion

3.4.1 AChE activity – a marker of pesticide exposure

The AChE activity was detected in the three tissues tested in rainbow trout, with the activity levels ranked as: muscle AChE > brain AchE > plasma AchE. There was no significant activity of BChE, as previously shown for steelhead trout (*Oncorhynchus mykiss*, Sandahl & Jenkins 2002) and Coho Salmon (*Oncorhynchus kisutch*, Sandahl et al. 2005). Exposure to Dimethoate for 21 days inhibited AChE activity in all three tissues; muscle AChE activity was inhibited the most while brain AChE activity was inhibited the least. Chemical-specific patterns of AChE inhibition may exist: Abbas and Hayton (1997) reported that rainbow trout brain AChE activity was more sensitive than plasma AChE activity to another OP pesticide Paraoxon; however they did not measure muscle AChE. Muscle AChE activity may be more sensitive than brain AChE activity to dimethoate exposure as it takes longer for muscle AChE to recover from pesticide exposures compared to brain (Carr et al. 1997). Our data indicate that inhibition of plasma AChE activity, an endpoint that can be measured with non lethal sampling, is a sensitive marker of exposure to Dimethoate. Muscle AChE was a more sensitive marker, compared to brain AChE, both obtained from lethal sampling. Further research is needed to determine the mechanisms of tissue-specific patterns of AChE inhibition and which type of AChE inhibition causes greatest damage to fish health. In estuarine fish, brain AChE inhibition > 70% was associated with mortality, and sublethal effects on stamina were detected with inhibitions as low as 50% (Fulton & Key 2001). In our study with rainbow trout, inhibition of brain AChE as high as 87.91% was detected in the exposure to Dimethoate at 0.1% LC50, with no dimethoate-exposure dependent effect on condition

factor, liver somatic index or mortality. These results suggest that even though AChE was greatly impacted, fish survived and were still feeding normally. Major differences in tolerance to AChE inhibition seem to exist between fish, and although these differences are important and may determine which species survive pesticide exposures in the field, our understanding of mechanisms mediating these differences remains limited at present.

Temperature was not a factor in AChE inhibition in our study with rainbow trout; however, the warming regime used may not have challenged the fish sufficiently. The effects of temperature on AChE inhibition vary with species and measurement conditions. It has been suggested that to cause a significant change in AChE activity in rainbow trout, water temperature needs to change by at least 12°C, with the lower temperature below 10°C (Beauvais et al. 2002). In our study, the temperature regimes were selected to mimic natural water fluctuations in local rivers (Chapter 2). There was a 10°C difference between warm and cold water treatment groups for the first 10 days, with daily fluctuations of 5-6°C over the cold water group for the next 11 days. The scale and temporal pattern of temperature fluctuations in our study may have been too small to affect AChE activity. Future studies will further investigate the effects of temperature fluctuations on AChE activity in rainbow trout.

3.4.2 Physiological Stress Response

Exposure to Dimethoate decreased plasma cortisol levels in rainbow trout, suggesting that dimethoate disrupted the hypothalamo-pituitary-interrenal pathways leading to production of cortisol. In a mammalian study, Walsh et al. (2000) demonstrated that Dimethoate blocked the steroidogenic pathway in the testis of rats by inhibiting the StAR protein responsible for shuttling cholesterol across the mitochondria

and making it available for steroid synthesis. The decreased plasma cortisol in trout exposed to Dimethoate in the present study is consistent with the effects of Dimethoate in rat testis. However, when the secretory capacity of head kidneys isolated from fish exposed to Dimethoate was tested *in vitro* and the head kidney cells were stimulated with exogenous ACTH, normal cortisol secretion was detected. If the mechanism of cortisol disruption (low plasma cortisol) by Dimethoate involved inhibition of StAR protein, stimulation with ACTH would not cause cortisol secretion as the StAR protein acts downstream from the ACTH step in the steroidogenic pathway. Our *in vitro* results with head kidneys of fish exposed to Dimethoate (pure grade) indicate that the secretory capacity of the interrenal tissue was normal since providing ACTH to the head kidney cells did result in normal secretion of cortisol. To determine whether the low plasma cortisol levels in fish exposed to Dimethoate were caused by disruption of pituitary ACTH, or alterations to cortisol metabolism and half-life, as has been suggested for other pollutants (Vijayan et al. 1997), further investigations would be required. Species differences in mechanisms of Dimethoate endocrine toxicity could be responsible for the difference between the results of Walsh et al. (2000) in rat and our results with fish, however the steroidogenic pathways of different vertebrates are very similar (Vijayan et al. 2005). Differences in chemicals used seem more likely to cause different responses. Walsh et al. (2000) used commercial dimethoate (Cygon) while the current study used pure grade Dimethoate.

To test the hypothesis that differences in Dimethoate formulations, rather than species differences, are responsible for the different effects on steroidogenesis, an *in vitro* approach was used in the present study to investigate the effects of two types of

Dimethoate, pure grade and the commercial form of Dimethoate, Cygon, on cortisol secretion in rainbow trout. The pure grade Dimethoate, same chemical as the one used in the *in vivo* experiment, had no effect on cortisol secretion or cell viability when head kidney cells isolated from uncontaminated trout were exposed *in vitro*. The results confirmed that pure grade Dimethoate was not blocking cortisol secretion or affecting StAR protein. The LC_{50} in vitro (concentration that kills 50% of cells) and the EC_{50} in vitro (concentration that decreases cortisol by 50%) could not be determined. In contrast, in vitro exposure to Cygon, caused a decrease in cortisol secretion and a reduction in cell viability. Commercial grade Dimethoate has the active ingredient of pure grade Dimethoate but also contains proprietary (unspecified) ingredients such as surfactants. Some studies have shown that commercial forms of pesticides, with surfactants added, are more toxic than the pure products (Beusen & Neven 1989), either by crossing the cells membranes faster or by facilitating transformation into toxic metabolites. Many OPs need to be biotransformed into an oxon form by the liver before they can exert their toxic effects (Grue et al. 1991, Hoffman & Papendorf 2006). Pure grade Dimethoate did not have an effect on cell viability or cortisol secretion in vitro because it may require transformation by the liver or it was not able to cross the cell membranes.

The addition of the proprietary ingredients rendered Dimethoate, in the form of Cygon, toxic to the head kidney cells and cortisol production. The EC_{50} , concentration of Cygon at which 50% of cortisol secretion was impaired, at 11°C was lower than the LC_{50}, indicating that the concentration required to disrupt cortisol synthesis was lower than the concentration that killed cells. Thus at 11°C, the secretory processes, possibly function of the StAR protein, were more sensitive to the toxicity of

Cygon than the processes controlling cell viability. Our study also showed that the EC_{50} at 11°C was lower than the EC_{50} at 20°C, providing evidence that the secretory processes were more vulnerable to Cygon at the colder temperature. However at 20°C the EC_{50} was not different from the LC_{50}, suggesting that cortisol secretion was decreased because of mortality of the secretory cells, rather than functional disruption of the secretory processes. Further experimentation is needed to identify the intracellular steps targeted by Dimethoate within the signalling pathway leading to cortisol. In vitro experiments using pure grade or the commercial Dimethoate demonstrated that even though there were no differences in cell viability at 11°C and 20°C, cortisol secretions were consistently higher at 20°C. These results suggest that the vulnerability of cortisol secretion to the pesticide is higher at the colder temperature, even though rainbow trout is a cold water fish species. A previous study by Leblond et al. (2001) reported that cortisol secretion in rainbow trout head kidney cells was lower at 25 and 35°C compared to 5 or 15°C. Further experimentation using a wide range of temperatures is needed to determine the impact that temperature has on cortisol secretion and the stress response.

The physiological stress response is characterized not only by increased plasma cortisol but also by increased plasma glucose and decreased liver glycogen. Dimethoate did not have an effect on plasma glucose or liver glycogen, suggesting that either Dimethoate was not acting as a chemical stressor, or the pathway in the stress response was blocked. These results are consistent with another study where there were no changes in plasma glucose or muscle glycogen in Nile tilapia (*Oreochromis niloticus* L.) exposed to Dimethoate (Sweilum 2006). In our study with rainbow trout, Na^+/K^+ ATPase activity increased with increasing Dimethoate exposure, suggesting that

Dimethoate may alter osmoregulation. Na^+/K^+ ATPase is the enzyme responsible for maintaining transmembrane gradients of Na^+ and K^+, and changes in the enzyme activity provide a measurement of ion-balance disturbances and the capacity of the fish to osmoregulate under chemical stress (Frasco & Guilhermino 2002). Our results are first to report a change in this enzyme in rainbow trout exposed to Dimethoate. A previous study with *Poecilia reticulata* reported no change in Na^+/K^+ ATPase activity in fish exposed to Dimethoate (Frasco & Guilhermino 2002).

Temperature fluctuations used in this study did not have an effect on plasma cortisol and glucose, or liver glycogen. Water temperature outside the thermal preference of a fish has been reported to alter the stress response (Brett 1971, Bartell et al. 1968, Brandt 1993). Warm temperatures increased plasma cortisol (Strange 1980, Davis et al. 1984, Davis & Parker 1990, Davis 2004) and glucose (Carmichael 1984, Van Dijk et al. 1999, Lerman 2004), and decreased liver glycogen (Viant et al. 2003, Lerman 2004). However, a decrease in plasma glucose at warm water temperatures has been also observed (Davis 2004). Carmichael (1984) found that a change of 12°C (from 10 to 22°C) caused an increase in plasma cortisol and glucose, while a change of 6°C (from 10 to 16°C) caused no change. The change in temperature in our study may not have been high enough or sustained long enough to cause a detectable change in the stress responses.

3.5 Conclusions

Exposure to Dimethoate, both *in vivo* and *in vitro*, had significant effects in rainbow trout in the present study. The activity of AChE was inhibited in muscle, plasma, and

brain and while fish did survive a 21 day exposure to 5% LC_{50} of dimethoate, AChE was inhibited as much as 90%. Such inhibition would ultimately, upon longer exposures, cause behavioural effects. Dimethoate disrupted the physiological stress response, causing low plasma cortisol levels in vivo, however further studies are required to determine the exact mechanism of the inhibition. Pure grade Dimethoate, *in vitro*, did not cause a decrease in viability and cortisol secretion while commercial Dimethoate disrupted cortisol secretion and decreased viability. Further toxicity testing of Dimethoate should focus on commercial Dimethoate, the chemical applied in the field. Further investigations are required to determine what causes commercial Dimethoate to be more toxic than pure grade Dimethoate.

3.6 References

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Chapter 4.
General Discussion and Conclusion

4.1 General Overview

The purpose of this project was to investigate the effects of multiple stressors on the physiological status of fish and determine if the physiological stress response was activated or impaired in response to pesticide exposure and increasing water temperature. Furthermore, the responses of different fish species were compared to better understand the processes that make fish vulnerable to environmental stressors. Field sampling of wild fish (whitefish and suckers) and controlled laboratory exposures of rainbow trout (a well validated fish model) were used in this study that provided new data on physiological markers, including plasma cortisol, liver glycogen, plasma glucose, condition factor, Na+/K+ ATPase activity and AChE activity in fish.

4.2 Oldman Study Conclusions (Chapter 2)

The objective of the Oldman study was to determine how multiple stressors (pesticides and temperature) affect the physiological status and stress response in cold-water fish (Mountain Whitefish, *Prosopium williamsoni*) and cool-water fish (Suckers, *Catostomus* sp.). The findings of this study determined that the Oldman River has an west-to-east warm temperature gradient and a gradient for 2,4-D and MCPP (Mecoprop). However, a gradient was not detected for organophosphates (OPs) or carbamates (CBs), possibly because of the limited testing procedures and a short half-life of these pesticides (Morrison & Wells 1981, Fulton & Key 2001). Although we were unable to detect OPs and CBs in the water, site differences and species differences in the activity of AChE, a marker of exposure to OP and CB were detected. Moreover, there was a significant

difference in AChE activity between whitefish and suckers. Whitefish had lower levels of AChE activity, suggesting either there is a physiological difference between the two species or whitefish had a greater exposure to pesticides (possibly through diet). It is difficult to conclude at this time whether the AChE activity is normal for whitefish and suckers since ours are the first data reported for these species in this system. Further studies using whitefish and suckers are needed to determine how pesticide exposure and temperature affect AChE activity but our results suggest that whitefish may be highly vulnerable to pesticides.

Mountain white fish at sites outside of their thermal preference exhibited a higher stress response, as measured by plasma cortisol levels and liver glycogen compared to suckers at the same sites. The increase in plasma cortisol and the decrease in liver glycogen follow both the temperature and pesticide gradient suggesting one or both of these factors act as a stressor for both whitefish and sucker. Again this was the first study of the physiological stress response in whitefish so it is difficult to determine if the values found are within the normal range. Overall this study found species specific differences between whitefish and suckers in regards to AChE activity, plasma cortisol and liver glycogen. The results suggest that whitefish may be more sensitive to water temperature and pesticide exposure and may be more at risk if water temperature and pesticide usage continue to increase. This study suggests that monitoring of whitefish should continue as there is little information regarding their ability to handle stressors. Further information would help determine if whitefish are at risk and whether management strategies are needed to maintain this population.

4.3 Dimethoate Study Conclusions (Chapter 3)

The objective of the Dimethoate study was to investigate the effects of Dimethoate and temperature on the physiological stress response and AChE activity in rainbow trout (*Oncorhynchus mykiss*). This study also looked at the differences between pure grade Dimethoate and commercial dimethoate (Cygon). In the *in vivo* study, rainbow trout were exposed to low amounts (0.5 and 1% LC_{50}) of Dimethoate; these low concentrations decreased AChE activity in a dose dependent manner in the muscle, brain, and plasma. However, temperature did not have an effect on AChE activity. It has been suggested that in order for changes in AChE activity to occur due to temperature, the change in temperature needs to be at least 10°C and the lower temperature below 12°C (Beauvais et al. 2002). This study could be repeated with a lower starting temperature and a greater change in temperature to determine if temperature is a factor is AChE activity.

Plasma cortisol levels decreased with increasing Dimethoate exposure suggesting that Dimethoate disrupts the physiological stress response production of cortisol. However, when the secretory capacity of the isolated head kidney stimulated with adrenocorticotrophic hormone (ACTH) was assessed, cortisol was produced. This suggests that Dimethoate blocks cortisol production within the hypothalamo-pituitary-interrenal axis at a step before ACTH acts. These results are different from other studies that suggest that Dimethoate disrupts cortisol secretion after ACTH, by blocking the StAR protein (Walsh et al. 2000).

To further determine where Dimethoate blocks cortisol secretion, an *in vitro* system was used. First, the head kidney cells of rainbow trout were exposed to pure grade dimethoate (same as the *in vivo* experiment). Pure grade Dimethoate had no effect on cortisol secretion even at the highest concentration. Cygon, a commercial Dimethoate was then used, similar to the original Walsh et al. (2000) experiment. Commercial dimethoate caused a reduction in both cortisol secretion and cell viability. Cortisol secretion decreased more than cell viability indicating that commercial dimethoate blocks cortisol production at lower concentrations of the pesticide than those killing the cells. This study also demonstrated that there are implications to using pure versus commercial grade products in toxicity testing. Commercial products often have proprietary ingredients that can cause cellular damage or allow the toxicant to infiltrate the cell.

Temperature did have an effect on cortisol secretion *in Vitro* both with pure and commercial grade Dimethoate. The higher temperature increased cortisol secretion however temperature did not have an effect on viability. Temperature also did not have an effect on cortisol secretion in the *in Vivo* experiment. Temperature did not affect plasma glucose or liver glycogen, suggesting that temperature does not affect the stress response or that the temperature change was not great enough to cause a change in the stress response.

4.4 Concluding Statements

This study provided novel information regarding the physiological stress response and acetylcholinesterase. This study also addressed important aspects of multiple stressors affecting fish in southern Alberta and Canada as a whole. Climate change is a

very important issue impacting agriculture, industry and government. Changes in climate will be an added stressor to natural and anthropogentic stressors that are already present in aquatic systems. This thesis attempted to discover how temperature would influence pesticide exposure in freshwater fish and it provided new information how these two stresssors affect freshwater fish.

4.5 References

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Appendix

Table A.1. AChE activity in Salmonids and Catostomids exposed to organophosphates (OP), carbamates (CB), or field pesticides

Pesticide	Exposure	Dose	Specific Activity	Species	Tissue	Source
	in water		9.28 AChE (mmol/min/g tissue)	*O. mykiss*[a]	Brain	Davies et al. 1994
	in water		192 (nmol/min/mg protein	*O. mykiss*	Brain	Shao-nan&De-fang 1996
	in Vitro		225 AChE (nmol/min/mg protein)	*O. mykiss*	Brain	Shaonan et al. 2004
Controls	in water	No exposure	16.06 AChE (nmol/g wet tissue)	*O. mykiss*	Brain	Szabó et al. 1991
	in water		4.66 AChE (nmol/g wet tissue)	*O. mykiss*	Gut	
	in water		1.47 AChE (nmol/g wet tissue)	*O. mykiss*	Heart	
	in water		7.08 AChE (mmol/min/g tissue)	*O. mykiss*	Muscle	Davies et al.1994
	in water		3.32 AChE (nmol/g wet tissue)	*O. mykiss*	Muscle	Szabó et al. 1991

[a]Rainbow trout, *Oncorhynchus mykiss*

Pesticide	Exposure	Dose	Effect	Species	Tissue	Source
Aldicarb (CB)	in water	0.5 mg/L	75% AChE inhibition	*O. mykiss*	Brain	Wang et al. 2001
	in water	0.5 mg/L	30% AChE inhibition	*O. mykiss*	Muscle	
Azamethiphos (OP)	in water	0.1 ppm	83% AChE inhibition (60 min exposure)	*O. mykiss*	Brain	Intorre et al. 2004
	in water	0.1 ppm	79 % AChE inhibition (120 min exposure)	*O. mykiss*	Brain	
	in water	0.1 ppm	62% AChE inhibition (240 min exposure)	*O. mykiss*	Brain	
Azinphos methyl (OP)	in water	0.001 mg/L	70% AChE inhibition	*O. mykiss*	Brain	Ferrari et al. 2004
Carbaryl (CB)	in water	188-750 µg/L	14-38% AChE inhibition for 24 hr exposure	*O. mykiss*	Brain	Beauvais et al. 2001
	in water	188-750 µg/L	32-41% AChE inhibition for 96 hr exposure	*O. mykiss*	Brain	
	in Vivo	188-750 µg/L	7-14% AChE inhibition for 48 hr recovery	*O. mykiss*	Brain	
	in Vivo	0.5 mg/L	77% AChE inhibition	*O. mykiss*	Brain	Ferrari et al. 2004
	in Vivo	66 µg/L	50% AChE inhibition	*O. tshawytscha*[b]	Olfactory tissue	Scholz et al. 2006

Pesticide	Exposure	Dose	Effect	Species	Tissue	Source
Carbofuran (CB)	*in Vitro*	14.7 µg/L	32.4% AChE inhibition	*O. kisutch*[c]	Olfactory rosette	Jarrard et al. 2004
	in water	200 µg/L	50% AChE inhibition	*O. tshawytscha*	Olfactory tissue	Scholz et al. 2006
Chlorpyrifos (OP)	in water	0.6 µg/L	23.1% AChE inhibition	*O. kisutch*	Brain	Sandahl et al. 2005
	in water	2.5 µg/L	64% AChE inhibition	*O. kisutch*	Brain	
	in water	0.6 µg/L	12% AChE inhibition	*O. kisutch*	Muscle	
	in water	2.5 µg/L	67% AChE inhibition	*O. kisutch*	Muscle	
	in water	0.625-2.50 µg/L	7-38.4% AChE inhibition	*O. mykiss*	Brain	Sandahl & Jenkins 2002
	in water	1.2 µg/L	8% AChE inhibition	*O. tshawytscha*	Brain	Eder et al. 2004
	in water	7.3 µg/L	15% AChE inhibition	*O. tshawytscha*	Brain	
	in water	7.3 µg/L	85% AChE inhibition compared to solvent control	*O. tshawytscha*	Brain	Wheelock et al. 2005
	in water	7.3 µg/L	8% AChE inhibition	*O. tshawytscha*	Muscle	Eder et al. 2004
	in water	7.3 µg/L	93% AChE inhibition compared to solvent control	*O. tshawytscha*	Muscle	Wheelock et al. 2005

Pesticide	Exposure	Dose	Effect	Species	Tissue	Source
Chlorpyrifos-oxon (OP)	in water	8 µg/L	50% AChE inhibition	*O. tshawytscha*	Olfactory tissue	Scholz et al. 2006
Diazinon (OP)	in water	5 µg/L	50% AChE inhibition	*Catostomus occidentalis*	Brain	Whitehead et al. 2005
	in water	4 µg/L	50% AChE inhibition	*Catostomus occidentalis*	Muscle	
	in water	250-1000 µg/L	16-37% AChE inhibition	*O. mykiss*	Brain	Beauvais et al. 2000
	in water	2.5 µM	50% AChE inhibition	*O. mykiss*	Brain	Keizer et al. 1995
Diazinon-oxon (OP)	in water	525 µg/L	50% AChE inhibition	*O. tshawytscha*	Olfactory tissue	Scholz et al. 2006
Dichlorvos (OP)	in water	1.136 ppm	45-58% AChE inhibition (depending on oxygen levels)	*O. mykiss*	Brain	Høy et al. 1991
Dimethoate (OP)	in water	1300 µg/L	63% AChE inhibition 7 day exposure	*Cyprinus carpio**	Brain	De Mel & Pathiratne 2005
	in water	1301 µg/L	69% AChE inhibition 14 day exposure	*Cyprinus carpio**	Brain	
	in water	1 mg/L	70% AChE inhibition	*Poecilia reticulata**	Head	Frasco & Guihermino 2002
	in water	0.125 mg/L	50% AChE inhibition	*Poecilia reticulata**	Head	

Pesticide	Exposure	Dose	Effect	Species	Tissue	Source
Fenitrothion (OP)	in water	15 to 220 µg/L	1.68- 71.9% AChE inhibition for 10 d exposure	*O. mykiss*	Brain	Davies et al.1994
	in water	15 to 220 µg/L	41.2- 83.9% AChE inhibition for 20 d exposure	*O. mykiss*	Brain	
IPBC (CB)	*in Vitro*	0.0047 µg/L	196% AChE activity of control	*O. kisutch*	Brain	Jarrard et al. 2004
	in Vitro	0.47 µg/L	175% AChE activity of control	*O. kisutch*	Brain	
	in Vitro	47.5 µg/L	186% AChE activity of control	*O. kisutch*	Brain	
Malathion (OP)	in water	20-40 µg/L	19.2-63% AChE inhibition	*O. mykiss*	Brain	Beauvais et al. 2000
Malathion-oxon (OP)	in water	4.1 µg/L	50% AChE inhibition	*O. tshawytscha*	Olfactory tissue	Scholz et al. 2006
Mancozeb (CB)	*in Vitro*	0.0022 µg/L	187% AChE activity of control	*O. kisutch*	Brain	Jarrard et al. 2004
	in Vitro	0.022 µg/L	143% AChE activity of control	*O. kisutch*	Brain	
Methidathion (OP)	in water	1 µg/L	22.2% AChE inhibition	*O. mykiss*	Muscle	Flammarion et al. 1996
	in water	5 µg/L	90.5% AChE inhibition	*O. mykiss*	Muscle	

Pesticide	Exposure	Dose	Effect	Species	Tissue	Source
Paraoxon (OP)	in water	75 ng/ml	58.9% AChE inhibition after 24 hr	*O. mykiss*	Brain	Abbas & Hayton 1997
	in water	76 ng/ml	51.2% AChE inhibition after 24 hr	*O. mykiss*	Heart	Abbas & Hayton 1997
	in water	77 ng/ml	49.6% AChE inhibition after 24 hr	*O. mykiss*	Liver	
Solvent Control (methanol)	in water		11% AChE inhibition	*O. tshawytscha*	Brain	Wheelock et al. 2005
Urban Rivers	field study		no inhibition compared to reference site	*Salmo trutta*	Brain	Payne et al. 1996
	field study		40-50% AChE inhibition compared to reference site	*Salmo trutta*	Muscle	
Yamaska River	field study		39.1-40.8% AChE inhibition compared to reference site	*Catostomus commersoni*	Plasma	Dorval et al. 2005

[b]Chinook salmon - *Oncorhynchus tshawytscha*

[c]Coho salmon - *Oncorhynchus kisutch*

*data on AChE inhibition in salmonids or catostomids exposed to Dimethoate were not available

Table A.2. Physiological indicators of stress in fish exposed to pesticides

Pesticide	Action	Exposure	Dose	Effect	Species	Source
Acephate (OP)	Insecticide	in water	0.2-1.3 mg/L	no change in plasma glucose	*Galaxias maculatus*	Davies et al. 1994
		in water	4.4 mg/L	plasma glucose ↑	*Galaxias maculatus*	
		in water	0.2 mg/L	plasma glucose ↑	*Oncorhynchus mykiss*	
		in water	0.4-4.4 mg/L	no change in plasma glucose	*Oncorhynchus mykiss*	
Atrazine (TR)	Herbicide	*in Vitro*	500 µM	cortisol secretion ↑	*Oncorhynchus mykiss*	Bisson & Hontela 2002
		in Vitro	5 to 0.005 µM	cortisol secretion ↓	*Oncorhynchus mykiss*	
		in water	340 µg/L	plasma glucose ↑	*Oncorhynchus mykiss*	Davies et al. 1994
		in water	1.1-22.7µ/L	plasma cortisol ↑	*Salmo salar*	Waring & Moore 2004
		in water	1-10 µg/L	Na+-K+-ATPase ↓	*Salmo salar*	
Carbofuran (CB)	Insecticide	in water	50 µg/L	plasma glucose ↑ 150%	*Carassius auratus*	Bretaud et al. 2002
		in water	500 µg/L	plasma glucose ↑ 230%	*Carassius auratus*	
		in water	50 µg/L	liver glycogen ↑ 122%	*Clarassius auratus*	
		in water	500 µg/L	liver glycogen ↓ 46%	*Clarassius auratus*	
		in water	7.66 mg/L	liver glycogen ↓	*Clarias botrachus*	Begum 2004
Carbaryl (CB)	Insecticide	in water	4.0-8.0 mg/L	liver glycogen ↓	*Colisa fasciatus*	Singh et al. 2004
		in water	4.0-8.0 mg/L	muscle glycogen ↓	*Colisa fasciatus*	
Pesticide	Action	Exposure	Dose	Effect	Species	Source

Cyanazine (TR)	Herbicide	in water	900-2000 µg/L	plasma glucose ↓	*Oncorhynchus mykiss*	Davies et al. 1994
Cypermethin (PY)	Insecticide	in water	0.49 µg/L	plasma glucose ↓	*Oncorhynchus mykiss*	
Deltamethrin (PY)	Insecticide	in water	0.005 mg/L	liver glycogen ↓	*Clarias gariepinus*	Datta & Kaviraj 2003
		in water	0.005 mg/L	plasma glucose ↑	*Clarias gariepinus*	
Diazinon (OP)	Insecticide	*in Vitro*	500 µM	cortisol secretion ↓	*Oncorhynchus mykiss*	Bisson & Hontela 2002
Dimecron (OP)	Insecticide	in water	0.001 mL/L	serum cortisol ↓	*Saratherodon mossamuicus*	Thangavel et al. 2005
Dimethoate (OP)	Insecticide	in water	21.66 mg/L	muscle glycogen ↓	*Clarias batrachus*	Begum & Vijayaraghavan 1999
		in water	5-20 mg/L	no change in muscle glycogen	*Oreochromis niloticus* L.	Sweilum 2006
		in water	5-20 mg/L	no change in plasma glucose	*Oreochromis niloticus* L.	
		in water	1 mg/L	no change in Na^+/K^+-ATPase	*Poecilia reticulata*	Frasco & Guihermino 2002
Endosulfan (OC)	Insecticide	*in Vitro*	500 µM	cortisol secretion ↓	*Oncorhynchus mykiss*	Bisson & Hontela 2002
		in Vitro	50 µM	cortisol secretion ↓	*Oncorhynchus mykiss*	
		in Vitro	$1x10^{-5}$ M	cortisol secretion ↓	*Oncorhynchus mykiss*	Dorval & Hontela 2003
		in Vitro	$1x10^{-5}$ M	cortisol secretion ↓	*Oncorhynchus mykiss*	Dorval et al. 2003
		in Vitro	$1x10^{-5}$ M	cortisol secretion ↓	*Oncorhynchus mykiss*	Leblond et al. 2001
		in Vitro	$1x10^{-4}$ M	cortisol secretion ↓	*Oncorhynchus mykiss*	

Pesticide	Action	Exposure	Dose	Effect	Species	Source
Fenitrothion (OP)	Insecticide	in water	0.02 ppm	no change in liver glycogen	*Anguilla anguilla*	Sancho et al. 1997
		in water	0.02 ppm	plasma glucose ↑	*Anguilla anguilla*	
		in water	0.04 ppm	liver glycogen ↓	*Anguilla anguilla*	Sancho et al. 1998
		in water	0.04 ppm	plasma glucose ↑	*Anguilla anguilla*	
Folidol 600 (OP)	Insecticide	in water	2 ppm	liver glycogen ↑ 84%	*Brycon cephalus*	de Aguiar et al. 2004
		in water	2 ppm	glucose ↑ 64%	*Brycon cephalus*	
Malathion (OP)	Insecticide	in water	1.0-3.0 mg/L	liver glycogen ↓	*Colisa fasciatus*	Singh et al. 2004
		in water	1.0-3.0 mg/L	muscle glycogen ↓	*Colisa fasciatus*	
		in water	0.5-2.0 mg/L	muscle glycogen ↓	*Oreochromis niloticus L.*	Sweilum 2006
		in water	0.5-2.0 mg/L	plasma glucose ↑	*Oreochromis niloticus L.*	
Mancozeb (EB)	Fungicide	*in Vitro*	50 µM	cortisol secretion ↓	*Oncorhynchus mykiss*	Bisson & Hontela 2002

Pesticide	Action	Exposure	Dose	Effect	Species	Source
Monocrotophos (OP)	Insecticide	in water	10 mg/L	liver glycogen ↓ 2.4%	*Channa punctatus*	Samuel & Sastry 1989
		in water	10 mg/L	muscle glycogen ↓ 35.4%	*Channa punctatus*	
		in water	10 mg/L	plasma glucose ↓ 12.7%	*Channa punctatus*	
o'p DDD (OC)		*in Vitro*	75-200μM	cortisol secretion ↓	*Oncorhynchus mykiss*	Lacroix & Hontela 2003
Yamaska River		Field study		plasma cortisol ↓ compared to reference site	*Catostomus commersoni*	Dorval et la. 2005
		Field study		liver glycogen ↑ compared to reference site	*Catostomus commersoni*	

Pesticide abbrivations: CB - carbamate, EB - ethylene bisdithicarbamate, OC - organochlorine, OP - organophosphate, PY - pyrethroid, TR – triazine

Table A.3. Behavioral changes in fish exposed to organophosphate (OP) and carbamate (CB) pesticides

Pesticide	Dose (exposure period)	Affected Behaviour	Species	Source
Azamethiphos (OP)	0.1 ppm	erratic jumping	*Anguilla anguilla, Dicentrarchus labrax, Oncorhynchus mykiss*	Intorre et al. 2004
Carbaryl (CB)	375 and 750 µg/L	decreased swimming speed	*Oncorhynchus mykiss*	Beauvais et al. 2001
Carbofuran (CB)	5 µg/L (24 hr)	increased burst swimming	*Carassius auratus*	Bretaud et al. 2002
	50 µg/L (24 hr)	increased sheltering and burst swimming	*Carassius auratus*	
	500 µg/L (24 hr)	decreased swimming activity; increased sheltering, burst swimming and grouping	*Carassius auratus*	
	5 µg/L (48 hr)	increased sheltering and burst swimming	*Carassius auratus*	
	50 µg/L (48 hr)	increased sheltering and burst swimming	*Carassius auratus*	
	500 µg/L (24 hr)	decreased swimming activity; increased sheltering, burst swimming and grouping	*Carassius auratus*	
	100 µg/L	decreased attraction and buccal movements; increased sheltering	*Carassius auratus*	Saglio et al. 2003

Pesticide	Dose (exposure period)	Affected Behaviour	Species	Source
Chlorpyrifos (OP)	100 ng/mL	decreased swimming activity	*Danio rerio*	Levin et al. 2004
	0.6 µg/L	decreased swimming rate and total food stikes	*Oncorhynchus kisutch*	Sandahl et al. 2005
	1.2 µg/L	decreased swimming rate, swimming rate during feeding, and total food stikes	*Oncorhynchus kisutch*	
	1.8 µg/L	decreased swimming rate, swimming rate during feeding, and total food stikes	*Oncorhynchus kisutch*	
	2.5 µg/L	decreased swimming rate, swimming rate during feeding, and total food stikes; increased first food strikes	*Oncorhynchus kisutch*	
	0.5 µg/L	abnormal swimming behaviour; signs of paralysis	*Poecilla reticulata*	De Silva & Samayawardhena 2002
	1 µg/L	abnormal swimming behaviour; over 50% paralysis	*Poecilla reticulata*	
	2 µg/L	abnormal swimming behaviour; over 50% paralysis	*Poecilla reticulata*	
Chlorpyrifos-methyl (OP)	1 mg/L	loss of equlibrium; erratic swimming; staying motionless mid-water	*Poecilla reticulata*	Selvi et al. 2005
	2.5 mg/L	staying motionless near surface then falling to aquarium bottom	*Poecilla reticulata*	
	3 mg/L	loss of equlibrium; hanging verticle in water; rapid gill movement; erratic swimming; staying motionless near bottom	*Poecilla reticulata*	

Pesticide	Dose (exposure period)	Affected Behaviour	Species	Source
Chlorpyrifos-oxon (OP)		tremors; flaring of gills; extension of pectoral fins; decreased escapability	*Gambusia affinis*	Carr et al. 1997
Diazinon (OP)	0.08-0.16 mg/L	restlessness; erratic swimming; convulsions; loss of balance; mucous secretion	*Anguilla anguilla*	Ferrando et al. 1991
	45 µg/L	decreased optomotor behaviour; decreased following	*Lepomis macrochirus*	Dutta et al. 1992
	250-1000 µg/L	decreased swimming speeds, turns, and distance, increase in tortuosity	*Oncorhynchus mykiss*	Beauvais et al. 2000
	500-1000 µg/L	decreased distance travelled and swimming speed; fish swam in a more linear path	*Oncorhynchus mykiss*	Brewer et al. 2001
	0.1-1.0 µg/L	no change on basal foraging or swimming activity; increased activity and feeding when exposed to alarm signal	*Oncorhynchus tshawytscha*	Scholz et al. 2000
	10.0 µg/L	no change on basal foraging or swimming activity, increased activity and feeding when exposed to alarm signal, decreased homing success	*Oncorhynchus tshawytscha*	
	2 mg/L	decreased general activity and loss of equilibrium after 72 hr	*Silurus glanis*	Köprücü et al. 2006
	4-64 mg/L	decreased activity; loss of equlibrium; erratic swimming; rapid gill movement; hanging vertically in water; staying motionless on bottom	*Silurus glanis*	
DDVP (OP)	1.87 mg/L	decreased food consumption	*Abramis brama* L.	Pavlov et al. 1992
Folidol 600 (OP)	2 ppm	torpor; reduction in swimming performance; remaining motionless; partial lack of reflexes	*Brycon cephalus*	de Aguiar et al. 2004
Malathion (OP)	20-40 µg/L	decreased swimming speed and distance; increased tortuosity	*Oncorhynchus mykiss*	Beauvais et al. 2000
	20-40 µg/L	positive correlation between AChE and swimming speed, AChE and distance; negative correlation between AChE and turn rate	*Oncorhynchus mykiss*	Brewer et al. 2001
	1 and 10 µg/L	no change in rate of travel, active swimming speed or escape behaviour	*Sciaenops ocellatus*	Alvarez & Fuiman 2006

Pesticide	Dose (exposure period)	Affected Behaviour	Species	Source
Methiocarb (CB)	>5 mg/L	excessive mucus; rapid respiration rate; crowding at water suface then bottom; laterally recumbent	*Oncorhynchus mykiss*	Altinok et al. 2006
Methylparathion (OP)	4.12-5.34 mg/L	restlessness; erratic swimming; convulsions; loss of balance; mucous secretion	*Anguilla anguilla*	Ferrando et al. 1991
Monocrotophos (OP)	20.49 mg/L	dullness; loss of equilibrium; loss of feeding; erratic swimming; decreased distance traveled and swimming speed	*Gambusia affinis*	Kavitha & Venkateswara Rao 2007

Table A.4 Effect of temperature on the stress response in fish

Exposure	Temperature	Effect	Species	Source
acclimatisation temperature	5-35°C	fish acclimated to 5°C and 10°C had ↑ plasma cortisol levels than fish acclimated in warmer water	*Ictalurus punctatus*	Davis et al. 1984
	10-23°C	↑ plasma glucose in fish held at 23°C	*Micropterus salmoides*	Carmichael et al. 1984
Acclimatisation temperature & confinment stress	10°C	slow ↑ in plasma cortisol and glucose	*Ictalurus punctatus*	Strange 1980
	30°C	rapid ↑ in plasma cortisol	*Ictalurus punctatus*	
	20°C	moderate ↑ in plasma cortisol	*Ictalurus punctatus*	
	5-30°C	fish held at 30°C had ↓ plasma glucose than fish at 5°C or 10°C	*Morone chrysops X Morone saxatilis*	Davis 2004
	5-30°C	fish held at 20°C had highest plasma cortisol levels followed by fish at 25°C, 15°C, 10°, 30°C, and 5°C	*Morone chrysops X Morone saxatilis*	
	5-30°C	fish held at 30°C had highest plasma cortisol levels followed by fish at 5°C, 25°C, 21°, 16°C, and 10°C	*Morone saxatilis*	Davis & Parker 1990
	5-30°C	fish held at 5°C had highest plasma glucose levels followed by fish at 16°C, 10°C, 25°, 21°C, and 30°C	*Morone saxatilis*	
	7.5, 12.5 and 21.0°C	plasma cortisol ↑	*Oncorhynchus tshawtscha*	Barton & Schreck 1987
	7.5, 12.5 and 21.0°C	plasma glucose ↑, 21.0°C group had higher plasma glucose than all other groups	*Oncorhynchus tshawtscha*	
	9°C	plasma cortisol ↑	*Salmo clarki clarki*	Strange et al. 1977
	20°C	plasma cortisol ↑ until 70 min where fish failed to maintain increasing cortisol production	*Salmo clarki clarki*	
	5-30°C	fish held at 10°C had the smallest increase of plasma cortisol while fish held at 30°C had the highest increase of plasma cortisol	*Morone saxatilis*	Davis & Parker 1990

Exposure	Temperature	Effect	Species	Source
decrease in water temp	25 to 15°C	plasma glucose ↑	*Chanos chanos, Ctenopharyngodon idella*	Kuo & Hsieh 2006
	25 to 10.5°C	plasma cortisol ↑	*Ictalurus punctatus*	Weber & Bosworth 2005
	10 to 1°C	plasma cortisol ↑ - levels stayed high for at least 48 hr	*Oncorhynchus mykiss*	Barton & Peter 1982
	8 to 1°C	plasma glucose ↑	*Scophthalmus maximus*	Staurnes 2001
increase in water temp	11 to 20°C	liver glycogen ↓	*Oncorhynchus mykiss*	Viant et al. 2003
	0 to 10°C	plasma glucose remains steady until 9°C when it increases rapidly	*Pachycara brachycephalum*	Van Dijk et al. 1999
	20 to 31°C	plasma glucose ↓	*Rhamdia quelen*	Lermen et al. 2004
	20 to 31°C	liver glycogen ↓	*Rhamdia quelen*	
	20 to 15°C	plasma glucose ↑	*Rhamdia quelen*	
	20 to 15°C	liver glycogen ↓	*Rhamdia quelen*	
	3 to 24°C	gradual increase in plasma glucose until 22°C then glucose levels drop	*Zoarces viviparus*	Van Dijk et al. 1999

Exposure	Temperature	Effect	Species	Source
low-water confinement stress	30°C	large ↑ in plasma cortisol	*Morone chrysops X Morone saxatilis*	Davis 2004
	5°C	small ↑ in plasma cortisol	*Morone chrysops X Morone saxatilis*	
temperature change	change of 12°C (10-22°C)	plasma cortisol and glucose ↑	*Micropterus salmoides*	Carmichael et al. 1984
	change of 12°C (10-22°C)	plasma glucose ↑	*Micropterus salmoides*	
	change of 6°C (16-22°C)	no change in plasma cortisol and glucose	*Micropterus salmoides*	
	change of 6°C (16-22°C)	no change in plasma glucose	*Micropterus salmoides*	

Table A.5 Effect of temperature on AChE activity and survival during pesticide exposures

Pesticide	Exposure	Temperature	Effect	Species	Source
Controls	No exposure	5 - 45°C acclimation	Brain AChE activity increased until 35°C then brain AChE activity began to decrease	*Carassius auratus*	Hazel 1969
		temperature of 5°C,15°C or 25°C	fish acclimated to 25°C had highest levels of brain AChE activity followed by 5°C and 15°C	*Carassius auratus*	
		acclimaion temperature of 5°C,15°C or 25°C	no difference in AChE activity	*Fundulus heteroclitus*	
		2 - 27°C	linear ↑ in brain AChE activity	*Lepomis macrochirus*	Hogan 1970
		17.2 - 24.6°C	no change in AChE	*Stizostedion vitreum*	Phillips et al. 2002
		5 - 20°C	linear ↑ in brain AChE activity	*Oncorhynchus mykiss*	Zinkl et al. 1987
		20 - 25°C	slow ↑ in brain AChE activity	*Oncorhynchus mykiss*	
		25 - 35°C	plateau in brain AChE activity	*Oncorhynchus mykiss*	
		>35°C	brain AChE activity ↓	*Oncorhynchus mykiss*	
		20.1 - 30.9°C	no change in AChE activity	*Lepomis macrochirus*	Beauvais et al. 2002

Pesticide	Exposure	Temperature	Effect	Species	Source
Endosulfan (OC)	19.78 µg/L	13.1 - 16°C	↓ survivors from 2.5% to 0% in small fish	*Oncorhynchus mykiss*	Capkin et al. 2006
	19.78 µg/L	13.1 - 16°C	↓ survivors from 42.5% to 0% in medium fish	*Oncorhynchus mykiss*	Capkin et al. 2006
	19.78 µg/L	13.1 - 16°C	↓ survivors from 100% to 77.5% in large fish	*Oncorhynchus mykiss*	Capkin et al. 2006
Methiocarb (CB)	5.43 mg/L	14.9 - 17.4°C	↑ mortality by 8.3% in large fish and 55% in small fish	*Oncorhynchus mykiss*	Altinok et al. 2006

Pesticide abbrivations: CB - carbamate, OC - organochlorine

Table A.6. Species differences in AChE activity and the responses to pesticide exposure and temperature

Exposure	Exposure	Dose	Species Difference	Source
Controls	*in Vitro*		*Coregonus albula* had 7-fold greater brain AChE activity than *Perca fluviatilis*	Chuiko 2000
	in Vitro		*Oncorhynchus mykiss* had 49% less brain AChE than *Pimephales promelas*	Johnson & Wallace 1987
	in water		all species (*Oncorhynchus mykiss, Poecilia reticulata, Brachydanio rerio* and *Cyprinus carpio*) had similar levels of brain AChE activity	Kiezier et al. 1995
	in water		brain AChE activity *Oncorhynchus mykiss* > *E. licius* > *I. nebulosus* > *S. glanis*	Szabó et al. 1991
	in water	no exposure	brain AChE activity *Pseudorasbora parva* > *C.auratus*, *O. mykiss*	Shaonan et al. 2004
	in water		brain AchE activity *Notemigonus crysoleucas* > *M. salmonides,* > *L. macrochirus,* > *G. affins*	Carr et al. 1997
	in water		brain AChE activity *Micropterus salmonides* > *G. affins,* > *L. macrochirus,* > *N. crysoleucas*	
	in water		brain AChE activity *Micropterus salmonides* > *G. affins, N. crysoleucas* and *L. macrochirus*	
Azinphosmethyl (OP)	in water	7.1 and 85.1 µg/L	The LC50 for *Sciaenops ocellatus* and *Fundulus heteroclitus* respectively	Van Dolah et al.1997

Exposure	Exposure	Dose	Species Difference	Source
Azamethiphos	in water	0.1 ppm	brain AChE activity *Anguilla anguilla >D. labrax > O. mykiss*	Intorre et al. 2004
Chlorpyrifos (OP)	Field study	runoff from constuction site	brain AChE activity *Microplerus salmoides > L. macrochirus, > N. crysoleucas, > G. affins*	Carr et al. 1997
Cyclohexyl methylphosphonofluoridate (OP)	*in Vitro*	0.6-2.0 ppb	brain AChE activity *Carassius auratus > P. promelas > Lepomis cyanellus*	Weiss 1958
DDVP (OP)	*in Vitro*	pI_{50} for each species	*Blicca bjoerkna* had 15-fold less brain AChE activity than *Leuciscus idus*	Chuiko 2000
Diazinon (OP)	in water	0.01-100 µM	brain AChE activity *Cyprinus carpio > O. mykiss, > P. reticulata, > B. rerio*	Kiezier et al. 1995
Fenitrothion (OP)	in water	15 to 220 µg/L	*Oncorhynchus mykiss* had greater brain AChE inhibition compared to *Galaxias maculatus*	Davies et al. 1994.
Malaoxon (OP)	*in Vitro*	109-300 nM	*Pimephales promelas* was less sensitive (higher IC50) than *Oncorhynchus mykiss*	Johnson & Wallace 1987
Malathion (OP)	in water	0.25-14.5 ppm	LC_{50} *Pseudorasbora parva < C. auratus, < T. nilotica, < G. affins < O. mykiss*	Shao-nan & De-fang 1996
Malathion - oxidized (OP)	*in Vitro*	0.34-0.81 µmol/l	brain AChE activity *Pseudorasbora parva > C. auratus > O. mykiss*	Shaonan et al. 2004
Paraoxon (OP)	*in Vitro*	2986-20000 nM	*Pimephales promelas* was more sensitive (lower IC_{50}) than *Oncorhynchus mykiss*	Johnson & Wallace 1987
Triazopos - oxidized (OP)	*in Vitro*	0.042-0.16 µmol/l	brain AChE activity *Carassius auratus auratus > P. parva > O. mykiss*	Shaonan et al. 2004

Exposure	Exposure	Dose	Species Difference	Source
Handling stress	in water	30s handling stress	plasma cortisol *Salvelinus namaychush* > *S. fontinalis*, > *S. trutta*, > *O. mykiss*	Barton 2000
	in water	2hr truck transport	plasma cortisol *Salvelinus namaychush* > *S. fontinalis* > *O. mykiss*	
	in water	30s handling stress	plasma glucose *Salmo trutta* > *S. fontinalis*, > *O. myiss*, > *S. namaysush*	
	in water	2hr truck transport	plasma glucose *Oncorhynchus mykiss* > *S. fontinalis* > *S. namaychush*	
	in water	repeatedly held out of water for 60s	Stressed *Perca fluviatilis* showed 34% lower mean body weight and *Oncorhynchus myskiss* showed 22% lower mean body weight	Jentoft et al. 2005
	in water	repeatedly held out of water for 60s	*Perca fluviatilis* showed a reduced cortisol stress response whereas *Oncorhynchus mykiss* seemed to habituate to the stressor	
Cold shock	in water	water temperature reduced from 25 to 15°C	Plasma glucose was 2-fold higher in *Ctenopharyngodon idella* at 15°C than 25°C while plasma gluose was 5-fold higher in *Chanos chanos* at 15°C than 25°C	Kuo & Hsieh 2006
Increased water temperature	in water	temperature increased from 0 to 10°C	*Zoarces viviparus* is more tolerant to heat than *Pachycara brachycephalum*	Van Dijk et al. 1999
	in water	Heating 2°C/day	condition factor *Oncorhynchus mykiss* < *S. fontinalis* < *Salmo trutta*	Galbreath et al. 2004
	in water	Heating 4°C/day	condition factor *Oncorhynchus mykiss* < *S. fontinalis* < *Salmo trutta*	
	in water	Heating 8°C/day	condition factor *Oncorhynchus mykiss* < *S. fontinalis* < *Salmo trutta*	
	in water	Heating 24°C/day	condition factor *Oncorhynchus mykiss* < *S. fontinalis* < *Salmo trutta*	

Exposure	Exposure	Dose	Species Difference	Source
Decreased water temperature	in water	reduced water temperature from 8 to 1°C	Plasma glucose increased by 4-5x in *Scophthalmus maximus* L. but in *Hippoglossus hippoglossus* L. there was no change in plasma glucose	Staurnes 2001
Logan River	Field study	13-18°C	*Salmo trutta* had a higher condition factor than *Oncorhynchus clarkii utah*	McHugh 2005
Logan River	Field study	13-18°C	*Salmo trutta* reduced performance of *Oncorhynchus clarkii utah* in all temperatures but the 18°C	