PARASITES AND POLLUTION: A STUDY OF SELENIUM UPTAKE IN PARASITE-INFECTED RAINBOW TROUT (*ONCORHYNCHUS MYKISS)*

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ABSTRACT

Given that parasites demonstrate different sensitivity to contaminants and environmental stressors, there is an increasing interest in the use of parasites as biological or ecological indicators of their fish host life conditions as well as bioindicators of heavy metal pollution of aquatic ecosystems. In order to obtain insight into host-parasite relationships in selenium (Se) polluted environments, a study on nematode parasitism of rainbow trout exposed to subchronic dietary selenomethionine (Se-Met) was initiated. Se-Met is the major dietary form of selenium, and exposure to elevated dietary Se has been associated with detrimental effects on fish health and survival.

In the current study, rainbow trout (*Oncorhynchus mykiss*) were experimentally infected with larval stages (L3) of the intestinal nematode *Raphidascaris acus* and subsequently exposed to an environmentally relevant concentraton of dietary Se-Met (4.54 mg/kg wet weight (WW)) for 70 days. The specific research objectives were to determine to what extent *R. acus* and its host accumulate Se from Se-Met fortified diet. In addition, non-specific biomarkers such as condition factor (K), hepatosomatic index (HSI), and gross energy (GE) were calculated to demonstrate the general effects of Se-Met exposure and parasitic infection on trout health. To further investigate joint effects of parasitism and Se-Met exposure on oxidative stress in rainbow trout, levels of superoxide dismutase (SOD) and gluthathione s-transferase (GST) were assessed in the liver and in the head kidney, as these enzymes are involved in the antioxidant response. Fish health variables, muscle Se concentration, and enzyme activity levels were compared between infected trout and uninfected fish exposed to same dietary Se-Met concentration for 56 days, as well as control fish.

Subchronic exposure of uninfected rainbow trout to dietary Se-Met lead to an initial increase in muscle Se concentration reaching a plateau concentration at 5.27 mg Se/kg dry weight (DW), while a continuous increase in muscle Se was observed in infected trout exposed to Se-Met, reaching 7.52 mg Se/kg DW on day 70. Results suggest that continuous Se accumulation by parasitized fish could be due to increased susceptibility of rainbow trout to metal exposure as a result of infection with parasites. Furthermore, depletion in GE stores in infected rainbow trout exposed to Se-Met was observed ($p<0.05$), while no changes in K and HSI were detected between treatments, suggesting that challenging parasitic infection can significantly deplete energy reserves of the fish. In addition, in fish exposed to Se-Met an increase ($p<0.05$) in GST activity was recorded for the initial 7 days of Se exposure, whereas SOD activity was elevated ($p<0.05$) for the duration of the study. In contrast, fish exposed to both parasites and Se-Met showed a decrease ($p<0.05$) in antioxidant enzyme levels when compared to trout exposed to Se-Met alone, suggesting an important role of parasitic infection in suppressing oxidative stress response of the host.

In summary, parasites such as *R. acus* can upregulate absorption of Se in the final host, leading to increased deposition of Se into the muscle tissue. In contrast to its host, *R. acus* did not accumulate large concentrations of Se (1.91 mg Se/ kg DW), and therefore may not be very useful as a biomarker of Se pollution. In addition, parasites negatively influenced expression of general oxidative stress biomarkers (SOD, GST) and therefore should be considered in ecotoxicological studies. To our knowledge, this is the first study to demonstrate effects of combined Se-Met exposure and parasitic infection on rainbow trout health and the oxidative stress response.

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TABLE OF CONTENTS

PERMISSION TO USE ... i
ABSTRACT ... ii
ACKNOWLEDGEMENTS ... iv
TABLE OF CONTENTS ... v
LIST OF TABLES ... viii
LIST OF FIGURES ... ix
LIST OF ABBREVIATIONS ... x
PREFACE ... xii
1.0 GENERAL INTRODUCTION ... 1
1.1 Selenium ... 1
1.1.1 *Properties and sources* ... 1
1.1.2 *Toxicokinetics* ... 2
1.1.3 *Deficiency* ... 4
1.1.4 *Toxicity* ... 5
1.1.5 *Interactions with other metals* ... 9
1.1.6 *Mechanisms of action and resistance* ... 9
1.2 Fish health parameters ... 13
1.2.1 *Fulton's condition factor* ... 13
1.2.2 *Hepatosomatic index* ... 14
1.2.3 *Gross energy* ... 15
1.2.4 *Effects of environmental stressors on general fish health parameters* ... 15
1.3 Oxidative stress ... 17
1.3.1 *Damage from reactive oxidative species* ... 18
1.3.2 *Cellular defenses* ... 19
1.3.3 *Glutathione S-transferase* ... 21
1.3.4 *Superoxide dismutase* ... 23
1.3.5 *Selenium exposure and oxidative stress* ... 24
1.4 Use of parasites as bioindicators of pollution ... 25
1.4.1 *Effects of parasitic infection on fish health* ... 26

1.4.2 *Sub-lethal effects of combined parasitic infection and metal contamination on fish* 30
1.4.3 *Lethal effects of combined parasitic infection and metal contamination on fish* ... 31
1.4.4 *Effects of parasitic infection on oxidative stress response* 32
1.5 Parasitic species of interest: *Raphidascaris acus* 33
1.5.1 *General information on nematodes* 33
1.5.2 *Raphidascaris acus life-cycle* 36
1.5.3 *Pathogenic effects* 39
1.6 Rainbow trout species of interest 40
1.7 Research objectives and hypotheses 41
1.7.1 *Objectives* 42
1.7.2 *Hypotheses* 42
2.0 THE EFFECTS OF SELENIUM EXPOSURE AND PARASITIC INFECTION ON FISH HEALTH INDICATORS AND OXIDATIVE STRESS INDICATORS IN JUVENILE RAINBOW TROUT (*Oncorhynchus mykiss*) 44
2.1 Introduction 44
2.2 Materials and Methods 47
2.2.1 *Chemicals* 47
2.2.2 *Test species* 47
2.2.3 *Experimental protocol* 47
2.2.4 *Selenium analyses* 50
2.2.5 *Fish health parameters* 51
2.2.6 *Measurement of glutathione-S-transferase activity* 51
2.2.7 *Measurement of superoxide dismutase activity* 52
2.2.8 *Protein assay* 52
2.2.9 *Statistical analysis* 53
2.3 Results 53
2.3.1 *Parasite infection* 53
2.3.2 *Selenium analysis* 55
2.3.3 *Fish health parameters* 57
2.3.4 *Effects of selenium and parasite exposure on enzymatic activity* 61
2.4 Discussion 64

2.4.1 *Selenium uptake* 64
2.4.2 *Effects of stressors on fish health* 67
2.4.3 *Oxidative stress response* 68
2.5 Conclusions **71**
3.0 GENERAL DISCUSSION **73**
3.1 Effect of parasitic infection on selenium uptake by rainbow trout **73**
3.2 Selenium accumulation by *Raphidascaris acus* **76**
3.3 Combined effects of *Raphidascaris acus* infection and selenium exposure on fish health **84**
3.4 Effects of combined stressors on oxidative stress response **86**
3.5 Future research **89**
3.6 Overall conclusions **91**
LIST OF REFERENCES **93**

LIST OF TABLES

Table 1.1 Acute selenium (Se) toxicity levels for various fish species and life stages................. 8

Table 1.2 Selected examples of sub-lethal and lethal impacts on fish by parasites in freshwater and marine environments.. 28

Table 2.1 Parasite burden of rainbow trout infected with *Raphidascaris acus* larvae 28 days prior to exposure to dietary selenium. 54

Table 2.2 Morphometric data of rainbow trout exposed to either selenium (Se group) or selenium and parasites (Se+ parasites group). 58

Table 2.3 Fulton's Condition Factor [(weight/length^3)*100] and Hepatosomatic Index [(liver weight)/(body weight)*100] of rainbow trout exposed to either selenium (Se group) or selenium and parasites (Se+ parasites group). 59

Table 3.1 Selected examples of bioaccumulation factors (BF) in parasite species infecting freshwater and marine fish, birds and mammals. 78

LIST OF FIGURES

Figure 1.1 Summary of possible ROS formation by Se-Met in the presence of SOD and other defense mechanisms... 11

Figure 1.2 General morphology of a typical nematode male and female. 35

Figure 1.3 Life-cycle of the parasitic nematode *Raphidascaris acus*. 38

Figure 2.1 Mean Se concentration (mg/kg DW) in the rainbow trout muscle and *Raphidascaris acus* tissues.. 56

Figure 2.2 Mean Gross Energy values of rainbow trout. 60

Figure 2.3 Glutathione S-transferase activity in rainbow trout liver and head kidney over 70 days of exposure to either selenium (Se group) or selenium and parasites (Se+ parasites group). 62

Figure 2.4 Superoxide dismutase activity in rainbow trout liver and head kidney over 56 and 70 days of exposure to selenium (Se group) and selenium and parasites (Se+ parasites group), respectively. 63

LIST OF ABBREVIATIONS

°C	Degrees Celsius
CAT	Catalase
CAR	Carotenoids
Cd	Cadmium
Cu	Copper
Cys	Cysteine
DNA	Deoxyribonucleic acid
DW	Dry weight
EC	Extra-cellular
GE	Gross energy
GPx	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione S-transferase
GSSG	Oxidized glutathione
GSH	Reduced glutathione
H	Hydrogen
$HO^{\bullet}$	Hydroxyl radical
H_2O_2	Hydrogen peroxide
HSI	Hepatosomatic index
K	Condition factor
KB	2-ketobutyrate
LC50	Lethal concentration that kills 50% of the test population
LPO	Lipid peroxidation

MET	Methionase
Met-SeOH	Methylselenol
Mn	Manganese
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NC	No significant change
O_2	Oxygen
$O_2\bullet^-$	Superoxide anion radical
RET	Retinols
ROS	Reactive oxygen species
PP	Protein peroxidation
S	Sulfur
Se	Selenium
SE	Standard error
SK	Saskatchewan
Se-Met	Seleno-methionine
Se-Cys	Seleno-cysteine
SOD	Superoxide dismutase
TOC	Tocopherols
U	Units
USA	United States of America
Zn	Zinc

PREFACE

Chapter 1 is a general introduction into the research topic, while Chapter 2 of this thesis is organized as manuscript for publication in a peer- reviewed scientific journal. Chapter 2 will be submitted to “Environmental Toxicology and Chemistry”. Chapter 3 is a general discussion. Because of this thesis structure some repetition is unavoidable.

1.0 GENERAL INTRODUCTION

1.1 Selenium

1.1.1 *Properties and sources*

Selenium (Se) is a widely distributed metalloid as well as an essential element that belongs to group VIA in the Periodic Table of Elements and has an atomic weight and number of 78.96, and 34 respectively. The greatest selenium concentrations have been found in black shale (600 ppm) and phosphate rock (1-300 ppm) (Haygarth, 1994). Other natural sources include limestone (0.08 ppm), sandstone (0.05 ppm) and igneous rock (0.004-1.5 ppm) (Haygarth, 2004). Selenium is also widely distributed in many soil types and enters ground and surface waters through natural weathering (USEPA, 2004). The concentration of selenium found in most soils and waters ranges between 0.01-2 mg/kg and 0.1-0.4 µg/L, respectively (Mayland, 1994; Maier and Knight, 1994; USEPA, 2004). However, naturally elevated levels of selenium have been reported in soil (>38 mg/kg) and water systems (>10 µg/L) of specific areas (e.g. Elk River Valley, BC, Canada; South Dakota and Colorado, USA; parts of Colombia, Venezuela and Israel) (Combs and Combs, 1986). Additionally, selenium occurs naturally in coal and fuel oil and is emitted in flue gas and in fly ash during combustion (USEPA, 2004). Selenium also occurs in sulfide deposits of copper, lead, mercury, silver and zinc and can be released during the mining and smelting of these ores (USEPA, 2004). Selenium shares a similar electronic configuration and chemical properties with other elements in the group such as sulphur (S), which leads to interactions between these two elements and the substitution of Se and S (Reilly, 2006).

Selenium can exist in four oxidation states or species, each with different chemical, biological and toxicological properties (Maier and Knight, 1994). Naturally occurring oxidation

states of selenium in elemental and combined forms are: selenides (-2), elemental selenium (0), selenites (+4), and selenates (+6). The four valence states are the major aspects of Se chemistry which affect its solubility and movement in nature (Reilly, 2006). Generally, the more soluble and mobile forms of Se (+4 and +6) dominate under aerobic and alkaline conditions such as in natural waters, while Se (-2) and elemental Se (0) are not soluble in water and precipitate into sediments (Muscatello, 2009). Moreover, organic forms of Se like seleno-methionine (Se-Met) and seleno-cysteine (Se-Cys), and volatile forms (e.g. dimethylselenide ($(CH_3)_2Se$), are also present in natural waters (ATSDR, 2003; USEPA, 2004; Simmons and Wallschläger, 2005). Selenium has six stable isotopes with varying degrees of abundance: ^{74}Se (0.87%), ^{76} Se (9.02%), ^{77}Se (7.58%), ^{78}Se (23.52%), ^{80}Se (49.82%), and ^{82}Se (9.19%), and a number of short-lived isotopes (e.g. ^{75}Se) that are commonly used in radiology (Haygarth, 1994).

1.1.2 *Toxicokinetics*

In the aquatic environment selenium is both an essential nutrient and a dietary and waterborne toxicant (Hilton *et al.,* 1982). Due to the narrow margin of safety between essential and toxic levels in tissues along with the propensity of selenium to bioaccumulate in aquatic food webs, it has a considerable biological importance as even a slight increase in environmental selenium can be detrimental (Lemly, 2002a). Selenium has three levels of biological activity: trace concentrations are required for normal growth and development; moderate concentrations can be stored and homeostatic functions maintained; and elevated concentrations can result in toxic effects (Hamilton, 2004).

Industrial and agricultural activity has hastened the release of selenium from geologic sources and made it more available to fish and wildlife in aquatic and terrestrial ecosystems (Hamilton, 2004). Agricultural drain water, sewage sludge, fly ash from coal-fired power plants,

oil refineries, and mining are all sources of selenium contamination of the aquatic environment (Hamilton, 2004). Selenium released into the aquatic environment is actively taken up from the water column by algae, bacteria, and fungi via the same enzymes and processes (e.g. cell membrane permeases) that transport sulfur across cell membranes and incorporate it into various tissues (Bowie *et al.,* 1996; Ogle and Knight, 1996). Organisms require sulfur compounds to synthesize numerous essential biological chemicals, and therefore, have developed uptake mechanisms to transport sulfur compounds from the external media into the organism (Ogle and Knight, 1996). Due to the physical similarity of selenium species to sulfur species, selenium can be inadvertently taken up by the organisms via sulfur uptake mechanisms (Ogle and Knight, 1996).

Algae can take up organic Se 1,000 times more rapidly than inorganic forms (Amweg *et al*., 2003). Bacteria and phytoplankton are generally very tolerant to inorganic Se and convert it into more bioavailable forms such as seleno-methionine resulting in mobilization of Se into the food chain and Se bioaccumulation (Ogle *et al.,* 1988; Bowie *et al.,* 1996). Research has shown that Se can bioaccumulate and biomagnify in the aquatic environment leading to impact on native fish populations (Woock *et al*., 1987; Hamilton and Buhl, 1990; Besser *et al.,* 1993; Lemly, 1997a, 2003; Hamilton, 2004; Holm *et al.,* 2005; Muscatello *et al.,* 2006, 2008).

Uptake of selenium by aquatic biota can occur through water or diet, with dietary exposure being the major source of selenium accumulation in fish (Hamilton, 2004; Sandholm *et al.,* 1973). Selenomethionine is the major dietary source of selenium available to fish, and it has a high bioaccumulation as well as trophic transfer potential compared to other forms of Se (Fan *et al.,* 2002; Besser *et al.,* 1993). Lemly (1997a) indicated that dietary Se requirements for fish are approximately 0.1-0.5 μg/g dry weight (DW); however, concentrations exceeding 3 μg/g DW

can rapidly bioaccumulate in a concentration-dependant manner to levels that can be toxic (Hamilton and Buhl, 1990; Lemly, 1993a, 1997a; reviewed in Janz *et al*., 2010).

There are limited data regarding the mechanisms of Se uptake in fish. Fathead minnows (*Pimephales promelas*) have been reported to accumulate more selenite and Se-Met than selenate, even though selenate was more readily absorbed into the gastrointestinal tract (Kleinow and Brooks, 1986a, b). In mammals (e.g. mice), methionine competes with Se-Met for uptake and is absorbed by passive diffusion in the gastrointestinal tract (Andersen *et al*., 1994; ATSDR, 2003). After uptake, selenium accumulates primarily in the liver and kidney, followed by the gonad and muscle tissues (Kennedy *et al.,* 2000). Toxic effect thresholds have been established at 12 mg/g for liver, 8 mg/g for muscle and 10 mg/g for eggs as described by Lemly (1993a). In salmonids, selenium concentrations within this range have been shown to cause mortality (Hilton *et al.,* 1980; Hunn *et al.,* 1987; Hamilton *et al.,* 1990), haematological alterations (Hodson *et al.,* 1980), reduced smoltification (Hamilton *et al.,* 1986), and reduced growth (Hamilton *et al.,* 1990). Aquatic organisms can excrete Se primarily through urine, although significant amounts of Se are also removed by the gills and bile (Kleinow and Brooks, 1986b; ATSDR, 2003).

1.1.3 *Deficiency*

Selenium was first recognized to have a nutritional role in 1957 (Combs and Combs, 1986). Selenium is important to all living organisms because it is utilized as selenocysteine at the active site of antioxidant enzymes such as gluthathione peroxidase (Reilly, 2006). Selenium was found to have a complementary role to vitamin E in preventing dietary hepatic necrosis and other harmful physiological conditions in mammals and birds (Mayland, 1994). Glutathione peroxidase, superoxide dismutase, and catalase convert free radicals to peroxides, and then to water and oxygen, whereas vitamin E scavenges the free radicals and neutralizes their potential

damaging effects (Mayland, 1994). Thus low Se uptake combined with vitamin E deficiency increases oxidative stress and contributes to the development of oxidative damage (Mayland, 1994). In fish, selenium deficiency leads to decreased glutathione peroxidase activity, decreased liver and plasma Se, and increased pyruvate kinase and glutathione transferase activity (Bell *et al.,* 1986). In farmed salmon, Hitra disease, characterized by muscular and myocardial degeneration, hemorrhages and edema, is associated with Se deficiency in combination with low levels of vitamin E (Watanabe *et al.,* 1997). Other manifestations of deficiency in fish include growth depression, abnormal swimming patterns, and liver and muscle degeneration (Bell *et al.,* 1986; Hilton *et al.,* 1980).

1.1.4 *Toxicity*

With regard to fish, the toxicity of Se varies among the forms or species of Se, fish species, and the life stages of fish (Miller *et al.,* 2007). The most significant biomarker of excess Se in fish is the accumulation of Se in eggs and subsequent larval deformities (Lemly, 1993b, 1997b, c; reviewed in Hamilton, 2004). This is related to parental exposure to Se, maternal deposition of Se (mainly as Se-Met) into eggs, and subsequent exposure of the developing larvae during yolk absorption (Lemly, 1997c; Palace *et al.,* 2004). Selenium-induced teratogenic deformities in fish larvae have been reported in laboratory studies (Goettl and Davies, 1976; Bryson *et al.,* 1984; Klauda, 1986; Woock *et al.,* 1987; Pyron and Beitinger, 1989; Muscatello *et al.,* 2006), experimental stream studies (Schultz and Hermanutz, 1990; Hermanutz *et al.,* 1992; Hermanutz, 1992), artificial crossing experiments (Gillespie and Baumann, 1986), and field investigations (Lemly, 1993b, 1997b, c; Saiki and Ogle, 1995; Hamilton *et al.,* 2001a, b) (reviewed in Hamilton, 2004). Fish deformities include lordosis (concave curvature of the spine), kyphosis (convex spine), scoliosis (lateral curvature of the spine), and head, mouth, gill cover,

and fin deformities, in addition to edema, and brain, heart and eye problems (Hamilton, 2004). Other documented effects of selenosis in fish include skin lesions, swollen gill filament lamellae and decreased white blood cell counts (Lemly, 1993a, 1997c, 2002a; Lohner *et al*., 2001). Liver and kidney necrosis can occur when selenium concentrations in fish exceed 10 μg/g DW (Lemly, 1993a). These pathologies may be lethal as they affect feeding, respiration and may increase predation if they impact swimming ability (Lemly, 2002a).

Chronic Se exposure has also been shown to increase incidence of deformities and to reduce survival in fry (Woock, 1987; Hamilton and Buhl, 1990; Lemly, 1993b, 1997a, 2003; Besser *et al.,* 1993; Holm *et al.,* 2005; Muscatello *et al.,* 2006). Under controlled conditions, high levels of dietary Se decreased the condition factor, damages liver and kidney and eventually led to death (Coughlan and Velte, 1989). Furthermore, concentrations of dietary Se ranging from 9 to 13 μg/g DW have been shown to cause mortality in rainbow trout (Hilton *et al.,* 1980). The form of selenium that is available for uptake also influences its toxicity to the organism (Hamilton, 2004). It has been reported that diets incorporating selenite were not as toxic to fish as diets incorporating selenomethionine (Hamilton, 2004).

The salmonids are very sensitive to selenium contamination, and exhibit toxic effects even when tissue residues are low (Lemly, 2002b). Salmon survival is reduced when whole-body residues exceed 5 μg Se/g (Lemly, 2002a). However, not all species respond to Se in the same manner. Kennedy *et al.* (2000) reported no significant increase in larval deformities in cutthroat trout (*Oncorhynchus clarki*) at egg Se concentrations ranging from 8.7 to 81.3 μg/g DW. In addition, Holm *et al.* (2005) reported no increase in larval deformities in brook trout (*Salvelinus fontinalis*) at egg Se concentrations of 16.9 and 20.0 μg/g DW. However, abundance of larval deformities was elevated in rainbow trout at egg Se concentrations of 22.6 and 29.6 μg/g DW

(Holm *et al.,* 2005). Cold-water fish species including trout (cutthroat, brook, and rainbow), white sucker (*Catostomus commersonii*), and northern pike (*Esox lucius*) can tolerate higher tissue concentrations of Se as compared to warmwater fish, without adverse reproductive effects (reviewed in Chapman, 2007).

The concentrations of Se required to kill 50% of the test population (LC_{50}) for a variety of fish and exposure routes are shown in Table 1.1. Selenium toxicity varies significantly depending on the fish species, form of selenium, and developmental stage of the organisms and many other factors. In general, selenite (+4) is more toxic to fish than selenate (+6) during water exposure, and juvenile fish (especially after gill development) are the most sensitive (Table 1; Buhl and Hamilton, 1991). Selenium concentrations in contaminated aquatic environments are generally less than 10 µg/L, hence the required concentration of waterborne Se necessary to elicit acute responses such as mortality in fish in such environments are unlikely (USEPA, 2004).

Table 1.1 Acute selenium (Se) toxicity levels for various fish species and life stages. Selenite and selenate are expressed as $NaSeO_3$ and $NaSeO_4$ respectively; selenomethionine is expressed as Se-Met.

Organism	Scientific Name	Life Stage	Se Form	Endpoint	Se Concentration (mg/L)	Source
Arctic Grayling	*Thymallus arcticus*	Alevin	Selenate	LC50 (96 hr)	100	Buhl and Hamilton (1991)
		Alevin	Selenite	LC50 (96 hr)	76	Buhl and Hamilton (1991)
		Juvenile	Selenate	LC50 (96 hr)	180	Buhl and Hamilton (1991)
		Juvenile	Selenite	LC50 (96 hr)	34.3	Buhl and Hamilton (1991)
Bluegill	*Lepomis marcrochirus*	Adult	Selenite	LC50	28.5	USEPA (2004)
Brook trout	*Salvelinus fontinalis*	Adult	Selenium dioxide	LC50	10.2	USEPA (2004)
Coho Salmon	*Oncorhynchus kisutch*	Alevin	Selenate	LC50 (96 hr)	379	Buhl and Hamilton (1991)
		Alevin	Selenite	LC50 (96 hr)	80	Buhl and Hamilton (1991)
		Juvenile	Selenate	LC50 (96 hr)	74	Buhl and Hamilton (1991)
		Juvenile	Selenite	LC50 (96 hr)	7.8	Buhl and Hamilton (1991)
Chinook salmon	*Oncorhynchus tshawytscha*	Fry	Se-Met	LC50 (96 hr)	21.60	EPD BC (2001)
Fathead Minnow	*Pimephales promelas*		Selenite	LC50	2.2 - 10.5	Adams (1976)
		Fry	Selenite	LC50 (96 hr)	1.1	Halter *et al.* (1980)
Goldfish	*Carassius auratus*		Selenium dioxide	LC50	26.1	USEPA (1980)
Mosquitofish	*Gambusia affinis*		Selenite	LC50	12.6	USEPA (1980)
Northern pike	*Esox lucius*	Juvenile	Selenite	LC50 (72 hr)	11.1	Klaverkamp *et al.* (1983)
Rainbow trout	*Oncorhynchus mykiss*	Alevin	Selenate	LC50 (96 hr)	47	Buhl and Hamilton (1991)
		Alevin	Selenite	LC50 (96 hr)	118	Buhl and Hamilton (1991)
		Juvenile	Selenate	LC50 (96 hr)	32.3	Buhl and Hamilton (1991)
		Juvenile	Selenite	LC50 (96 hr)	9	Buhl and Hamilton (1991)
		Juvenile	Selenite	LC50 (96 hr)	12.5	Goelttl and Davies (1976)
			Selenite	LC50 (96 hr)	1.8	Hunn *et al.* (1987)
			Selenite	LC50 (96 hr)	7.2	Hodson *et al.* (1980)
			Selenite	LC50 (96 hr)	4.2 - 4.5	Adams (1976)
White sucker	*Catostomus commersonii*	Juvenile	Selenite	LC50 (96 hr)	29	Klaverkamp *et al.* (1983)
Yellow perch	*Perca flavescens*	Juvenile	Selenite	LC50	4.8	Klaverkamp *et al.* (1983)

1.1.5 *Interactions with other metals*

The interactions of selenium with other trace elements can be additive, antagonistic or synergistic (Hamilton, 2004). Selenium toxicity is reduced by antimony, arsenic, bismuth, cadmium, copper, germanium, mercury, silver and tungsten (reviewed in Hamilton, 2004). In addition, arsenic compounds present in diet have been shown to protect against selenite, selenocystine and selenomethionine toxicity (Levander, 1977; reviewed in Hamilton, 2004). Elevated dietary copper reduced the concentration of Se in liver of Atlantic salmon (*Salmo salar*) due to the formation of insoluble Cu-Se complexes in the intestinal lumen or the excretion of Cu-Se complexes from the liver in bile (Lorentsen *et al.,* 1998; Berntssen *et al.,* 1999, 2000; reviewed in Hamilton, 2004). Perhaps one of the most published interactions is that between selenium and mercury (Hamilton, 2004). Series of experiments investigated the ability of selenium to ameliorate the toxic effects on fish inhabiting a mercury contaminated lake (reviewed in Hamilton, 2004). It was furthermore shown that selenium interferes with mercury mobilization through the food web (Hamilton, 2004).

1.1.6 *Mechanisms of action and resistance*

Three major mechanisms have been suggested for Se toxicity: membrane and protein damage from Se-generated reactive oxygen species (ROS), substitution of Se for S during assembly of protein, and inhibition of Se methylation metabolism resulting in hydrogen selenide accumulation (Spallholz *et al.,* 2004; Miller *et al.,* 2007). Reactive oxygen species are by-products of electron transport chains, enzymes and redox cycling and their production may be enhanced by exposure to xenobiotics (Kelly *et al.,* 1998; Winston and Di Giulio, 1991). When ROS become too abundant, this results in the damage to membranes, proteins, and DNA. Reactive oxygen species are removed by cellular defense mechanisms such as glutathione

peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), vitamin E, and carotenoids (Kelly *et al.,* 1998). Details on ROS and oxidative stress are given in chapter 1.3.

The general metabolism of Se involves several reactions to produce hydrogen selenide (H_2Se), a highly toxic selenium compound, which can be methylated for excretion or incorporated into selenoproteins as selenocysteine (Se-Cys) (ATSDR, 2003). Selenocysteine-containing proteins are important as they are largely responsible for the antioxidant properties of selenium (ATSDR, 2003). The primary selenoproteins are glutathione peroxidases, thioredoxin reductases, and iodothyronine 5'-deiodinases (Lobinski *et al.,* 2000; ATSDR, 2003). The activity of these enzymes generally decreases or increases depending on alterations in the concentrations of Se in the body (Lobinski *et al.,* 2000; ATSDR, 2003). Seleno-persulfide (GSSeH) and methylselenol (CH_4Se), two intermediate products of Se metabolism, are significant oxidizing catalysts that can enter a Fenton-type reaction, continuously oxidizing thiols such as reduced glutathione (GSH), while reducing oxygen to produce superoxide radicals (Spallholz and Hoffman, 2002). Since glutathione plays a role as an anti-oxidant and co-factor of anti-oxidant enzymes, its depletion can significantly impact the prevention of oxidative damage to cells and tissues (Hoffman, 2002). Thus, selenium may produce or protect against oxidative stress as it is required for glutathione peroxidase (GPx), but can also lead to generation of ROS (Kelly *et al.,* 1998; Spallholz *et al.,* 2004). In addition, Se -Met can also generate ROS (Palace *et al.,* 2004). It must be first metabolized by methioninase to methylselenol, which then reacts with GSH to produce the ROS (Figure 1.1) (Palace *et al.,* 2004).

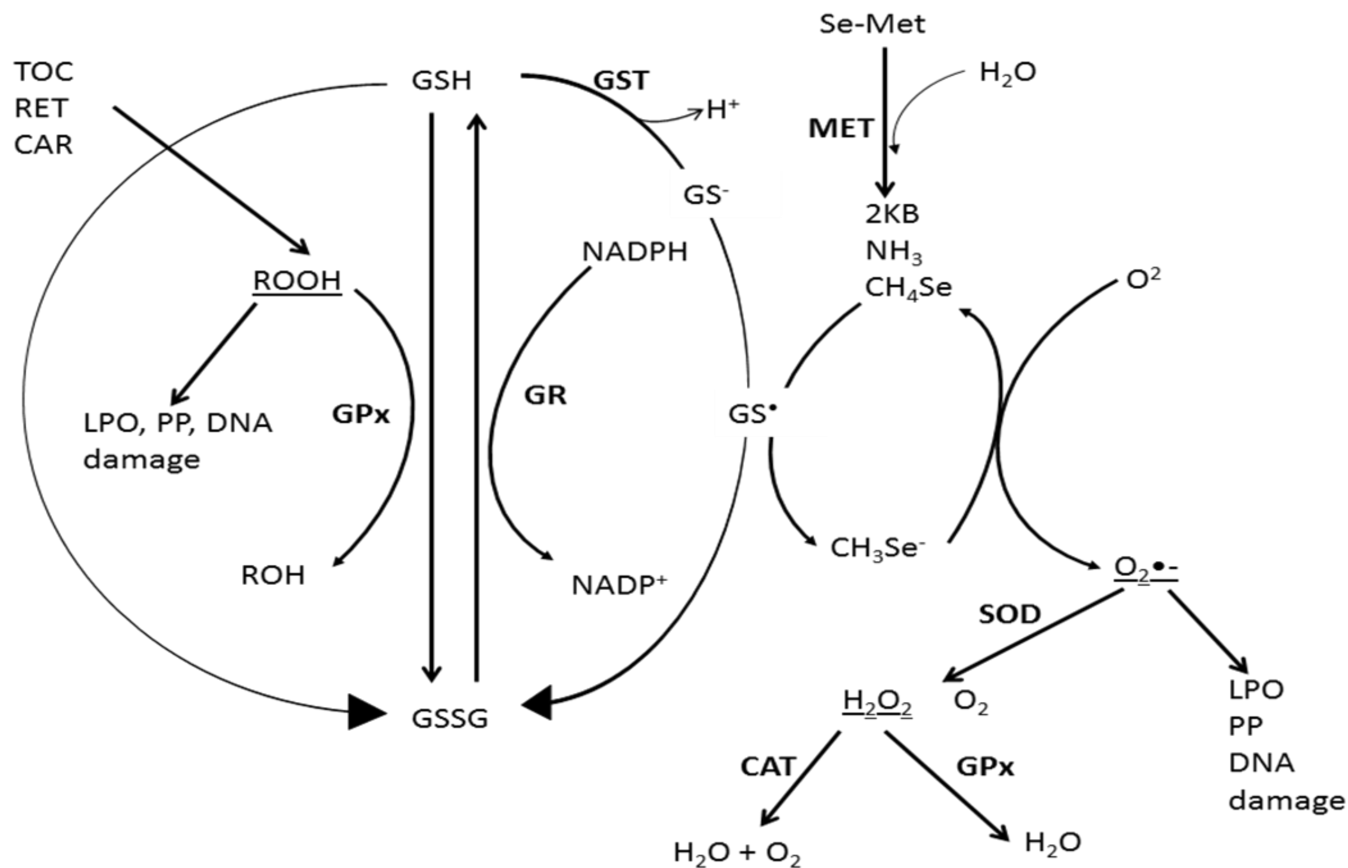


Figure 1.1 Summary of possible reactive oxygen species formation by selenomethionine in the presence of superoxide dismutase and other defense mechanisms. Bold items are enzymes and underlined items indicate reactive species produced. (Modified from Spallholz, 1994; Kelly *et al.,* 1998; Palace *et al.,* 2004.)

Abbreviations: Se- selenium; LPO- lipid peroxidation; PP- protein peroxidation; CAT- catalase; SOD- superoxide dismutase; CAR- carotenoids; TOC- tocopherols; RET- retinols; GR- glutathione reductase; GPx- glutathione peroxidase; KB- 2-ketobutyrate; Se-Met- selenomethionine; MET- methionase; CH_4Se- methylselenol; GSH- reduced glutathione; GSSG- oxidized glutathione.

In fish and animals, when selenium is present in excessive amounts, it is erroneously substituted for sulfur, resulting in the formation of a triselenium linkage (Se-Se-Se) or a selenotrisulfate linkage (S-Se-S); both may prevent the formation of the necessary disulfide chemical bonds (S-S) (Lemly, 2002a). Furthermore, Se can substitute for S in methionine to form the analog, selenomethionine (Spallholz and Hoffman, 2002). Since animals cannot synthesize selenomethionine or distinguish it from methionine, it becomes incorporated into a wide range of Se-containing proteins (Spallholz and Hoffman, 2002). The end result is distorted, dysfunctional enzymes and protein molecules, which impair normal cellular biochemistry (Lemly, 2002a). Although S and Se have similar properties, at biological pH Se is reduced while S is oxidized, altering the disulfide bonds that are necessary for proper protein structure and function (Combs and Combs, 1986). Substitution of Se for S is the mechanism responsible for the damage to hair, feathers and hooves (Spallholz and Hoffman, 2002). In addition, the inhibition of Se methylation process (a detoxification pathway) may lead to more Se available to be substituted for S during protein formation and to create more superoxide radicals (Spallholz and Hoffman, 2002).

The resistance to Se toxicity differs greatly between fish species. Even closely related species show variability in Se toxicity: rainbow trout have higher larval deformity rates than brook trout or cutthroat trout when exposed to increased Se concentrations in the environment (Holm *et al.,* 2005). Moreover, the accumulation potential for Se varies in different fish species. Rainbow trout accumulate higher concentrations of Se in their eggs when compared to brook trout (Holm *et al.,* 2005). Difference in vulnerability of closely related species to Se stress depends primarily on their feeding niche or some adaptive, physiological mechanisms that, in part, make closely related species respond differently (Hamilton, 2004). The physiological

mechanisms potentially responsible for the sensitivity difference include differences in enzyme induction, intestinal availability and differential accumulation rates. For example, Se elimination increased in fathead minnows exposed to increased dietary Se, suggesting that Se may induce proteins and enzymes involved in the elimination process (Kleinow and Brooks, 1986a).

1.2 Fish health parameters

Health can be defined as the condition of an organism with respect to the performance of its vital functions (MedlinePlus Medical Dictionary, 2012). Health is reflective of the body's homeostasis: attempt to maintain a relatively stable internal environment while confronted with changes in the external environment (Health-Biology Encyclopedia, 2012). Failure to do so can result in abnormal function and disease (Health-Biology Encyclopedia, 2012). Condition factor (K) and hepatosomatic index (HSI) are common indicators used in monitoring of fish overall health and bioenergetic status (Busacker *et al.,* 1990). Alterations in condition factor and HSI are well established tertiary stress responses at the whole organism level associated with altered energy distribution (Barton, 2002; Miller *et al.,* 2007). In terms of environmental pollution, depending on fish species and contaminant concentration, condition factor and HSI can be diminished as the fish use their energy for detoxification processes, rather than allocating energy for growth and storage (Laflamme *et al.,* 2000; Cleveland *et al.,* 1993).

1.2.1 *Fulton's condition factor*

During a period when fish have high energy intake, the growth of tissues and the storage of energy in muscle and liver can cause an individual to have a greater than usual weight at a particular length (Bagenal and Tesch, 1978). This excess can be measured by coefficient of condition, or Fulton's condition factor (Bagenal and Tesch, 1978). Condition factor represents

the mass of an individual relative to its body length (assumes isometric growth) and is based on the premise that heavier fish of a given length are in a higher energetic state (Neff and Cargnelli, 2004). In energetic terms, condition can be defined as the amount of energy available to an individual that may be allocated to various life functions including reproduction, foraging and survival (Ricker, 1975). Furthermore, because the condition factor reflects the nutritional state of an individual fish, it can be interpreted as an index of growth rate (Busacker *et al.,* 1990). The value of K can be influenced by a variety of factors, such as age and sex of the fish, stage of maturation, intestinal contents and its fullness, amount of fat reserve, degree of muscular development and season.

Fulton's condition factor has been recently adopted by ecologists studying fish mating systems under the assumption that they reflect an individual's energetic state and overall quality (Neff and Cargnelli, 2004). In addition, condition factors have successfully explained variation in reproductive behavior and success (Nicoletto, 1995; Barber, 2002; Kondoh and Okuda, 2002). Some of the examples of condition factors in naturally occurring populations of fish include trout (1.20-1.80), walleye (0.50-1.90), yellow perch (0.50-2.0), and pike (0.50-1.0) (Schreckenbach, 1996).

1.2.2 *Hepatosomatic index*

In addition to storing energy in muscle tissue, fish can also accumulate energy in the liver during periods of high energy intake (Busacker *et al.,* 1990). Much of this stored energy is in the form of glycogen (Busacker *et al.,* 1990). Therefore, a weight of the liver can be correlated with the nutritional state of the fish and with the growth rate (Busacker *et al.,* 1990). This indirect measure of growth rate is referred to as a hepatosomatic index and is often used in studies of the seasonal and yearly changes in growth of natural populations of fish (Busacker *et al.,* 1990).

Similar to the condition factor, the value of HSI can be influenced by the species of fish, age, activity, and seasonal variation as well as can be influenced by the parasitic infection or exposure to contaminants (Schäperclaus, 1990).

1.2.3 *Gross energy*

Although recently introduced, gross energy (GE) is another method to determine fish health. It has been shown that a strong positive statistical relationship between GE content and percent DW (often at $r^2 = 0.90$) exists in many fish species (Schreckenbach *et al.,* 2001; Wuenschel, 2006). These relationships enable the estimation of energy stores using GE from greater numbers of fish because measurement of percent DW through direct bomb calorimetry is often time consuming (Wuenschel, 2006). Schreckenbach *et al.* (2001) found a significant correlation (gross energy [MJ/kg] = 0.0253 DW [%] $^{1.6783}$) between the dry matter content and gross energy in 17 fish species analyzed. These values correspond closely to the results reported by Hartman and Brandt (1995), which were estimated from literature data and from calorimetric investigations in North American fishes (gross energy [J/g] = 0.04529 dry matter $^{1.507}$).

1.2.4 *Effects of environmental stressors on general fish health parameters*

Various environmental studies have shown diminished condition factor and HSI in fish from polluted environments. For example, yellow perch (*Perca flavescens*) from lakes contaminated with heavy metals had lower HSI than those from clean lakes (Girard *et al.,* 1998; Laflamme *et al.,* 2000). Bluegill (*Lepomis macrochirus*) exposed to waterborne (2.8 mg/l) and dietary (13 mg Se/kg and 25 mg Se/kg) Se for 30 days had significant reductions in condition factor compared to control fish (Cleveland *et al.,* 1993). However, Kelly and Janz (2008) have found no significant difference in condition factor and HSI of northern pike (*Esox lucius*) inhabiting a lake receiving effluent from uranium mill (Key Lake, SK, Canada), versus fish from

a clean reference lake (Unknown Lake, SK, Canada). Similarly, Miller *et al.* (2007) did not observe changes in HSI of juvenile rainbow trout exposed to waterborne selenite from 4 to 30 days. In contrast, juvenile female rainbow trout experimentally exposed to food-borne Se-Met had significantly greater HSI relative to untreated control fish (Wiseman *et al.,* 2011). The condition factor and HSI may be elevated in fish from contaminated systems if the contaminant alters the food web by eliminating competitors or increasing prey abundance (Campbell *et al.,* 2005). It is possible that by inducing teratogenesis in some fish populations, while promoting growth in others at the same concentrations, Se exposure can lead to increased food abundance and availability for survivors.

In addition, parasitic infections have been shown to decrease energy stores of various fish species (Kelly and Janz, 2008; Bakker and Mundwiler, 1999; Neff and Cargnelli, 2004). Kelly and Janz (2008) have reported that intestinal parasite abundance, prevalence and biomass negatively correlate with K and HSI in northern pike. Similarly, in three-spined sticklebacks (*Gasterosteus aculeatus*), parasite infection by *Pomphorhynchus laevis* (Acanthocephala) was negatively correlated with energy reserves and subsequently reproductive success (Bakker and Mundwiler, 1999). Neff and Cargnelli (2004) also found a negative correlation between parasitic density (parasite number divided by host body mass) and condition factor in the bluegill (*L. macrochirus*).

According to Schreckenbach *et al.* (2001), when the gross energy content is below 4 MJ/kg, freshwater fishes are in poor condition, leading to stress-induced energy-deficiency syndrome and resulting in extensive physiological disorders, diseases, and mortality (Spangenberg and Schreckenbach, 1984; Schreckenbach and Spangenberg, 1987; Schreckenbach, 1993). However, it is important to remember that although the formula for

evaluating the gross energy content in freshwater fishes by their dry matter content is a simple and practicable method for the investigation, it also has some limitations. Seasonal, ontogenetic and spatial variations in the energy stores of individual fish and fish populations have to be taken into account (Wuenschel, 2006). However, assessment of the energy status (as measured by GE) in fish should to be considered within ichthyological, ichthyopathological, and ecological investigations in comparison to condition factor and HSI, as latter two might not provide sufficient information about energetic demands posed by natural and anthropogenic stressors (Schreckenbach *et al.,* 2001).

1.3 Oxidative stress

Oxidative stress occurs when there is an imbalance in the generation and removal of reactive oxygen species within an organism resulting in lipid, protein and DNA damage. The condition of oxidative damage can arise through a depletion of antioxidant defences or an increase in ROS production, or both (Halliwell and Gutteridge, 1999). Several cellular processes, such as cellular respiration, lead to the production of ROS; however, exposure to contaminants can also elevate production (Kelly *et al.,* 1998; Palace *et al.,* 2004). The reduction of molecular oxygen (O_2) to water via the electron transport chain during cellular respiration results in the formation of reactive, partially reduced intermediates such as the superoxide anion radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\bullet}$) that may act as prooxidants (Kelly *et al.,* 1998). Other oxygen-derived radicals include peroxyl radical $RO_2^{\bullet}$, hydroperoxyl radical ($HO_2^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) (Livingstone, 2001). Oxidative damage has been implicated in several diseases including Keshan disease (Se deficiency in humans), arthritis, cancer initiation and promotion, malaria, stroke,

multiple sclerosis, and Parkinson's disease (Halliwell, 1987). Moreover, oxidative damage may also be involved in Se toxicity (Palace *et al.,* 2004).

1.3.1 *Damage from reactive oxidative species*

Besides being by-products of cellular respiration (1 to 5% of ROS escape the electron transport chain and damage cellular components), ROS can by produced by several oxidizing enzymes, such as diamine oxidase, cytochrome p450 reductase, glucose oxidase, and nitric oxidase synthase (Kelly *et al.,* 1998). In addition, production of reactive oxygen species may be enhanced by redox cycling of xenobiotics (Kelly *et al.,* 1998). Despite having a potential to cause cellular damage, ROS also have a useful purpose for the organism (Kelly *et al.,* 1998). They may act as cytotoxic agents against pathogenic organisms, can function as neurotransmitters and muscle relaxants, and transcription factors (Bruhwyler *et al.,* 1993; Remacle *et al.,* 1995; Kelly *et al.,* 1998). Once produced, ROS can damage cellular components, lipids, DNA, and tissues. The reactivity and properties of different ROS vary significantly: neither $^{\bullet}O_2^-$ nor H_2O_2 are considered particularly reactive in aqueous solution, while $HO^{\bullet}$ reacts instantly and indiscriminately with virtually all organic molecules (Livingstone, 2001). Superoxide is known to oxidise thiols and inactivate enzymes (e.g. catalase and peroxidases) while $HO^{\bullet}$ radicals lead to the oxidation of lipids and DNA (Kelly *et al.,* 1998).

DNA is particularly susceptible to oxidative damage (Kelly *et al.,* 1998). The primary ROS responsible for DNA damage is $HO^{\bullet}$, as neither H_2O_2 nor peroxyl radicals react directly with DNA (Kelly *et al.,* 1998). The heterogeneity of DNA allows for many susceptible sites for $HO^{\bullet}$ attack, including the nitrogenous bases and the sugar-phosphate backbone (Kelly *et al.,* 1998). This causes formation of intermolecular complexes and protein-DNA crosslinks, as well

as hydroxylation and base fragmentation (Kelly *et al.,* 1998). ROS may also cause protein peroxidation, a covalent modification of the protein (Livingstone, 2001).

ROS can react with lipids in a process called lipid peroxidation (LPO) (Livingstone, 2001). Lipid peroxidation is a chain reaction that occurs when a ROS removes a hydrogen atom from a methylene carbon in a lipid substrate (initiation), resulting in the formation of another ROS that can react with other fatty acids (propagation) (Kelly *et al.,* 1998). Termination of LPO occurs via a coupling of any two ROS to form nonradical products (Kelly *et al.,* 1998). Lipid peroxidation is known to cause structural damage affecting membrane permeability and fluidity (Kelly *et al.,* 1998). LPO has also been shown to decline with increasing Se exposure because Se is a constituent of the antioxidants GPx and thioredoxin reductase, and at lower concentrations protects fish from oxidative damage (Steinbrenner *et al.,* 2006).

Oxygen radicals play a role in generating physical and biochemical lesions in birds exposed to high concentrations of selenium (Spallholz and Hoffman, 2002). Palace *et al.* (2004) reported that the metabolism of selenomethionine in rainbow trout embryos led to ROS generation. An induction of enzymatic antioxidants (SOD and GPx) at low to intermediate doses followed by a sharp decline at the high dose was shown in trout exposed to waterborne sodium selenite (Orun *et al.,* 2005). Meanwhile Miller *et al.* (2007) reported that the concentration of glutathione, one of the most important non-enzymatic antioxidants, decreased in trout exposed to acute concentrations of waterborne sodium selenite.

1.3.2 *Cellular defenses*

There are several mechanisms to remove excess reactive oxygen species and prevent from oxidative damage. The antioxidant systems in aquatic organisms are comprised of

enzymatic processes and scavengers. Enzymatic mechanisms include glutathione peroxidase, catalase, glutathione reductase (GR), and superoxide dismutase.

Glutathione peroxidase is an enzyme responsible for inactivation of H_2O_2 and a large number of organic hydroperoxides with reduced glutathione (Kelly *et al.,* 1998; Orbea *et al.,* 2000). At least two different GPx activities are identified in animal tissue: selenium-dependent GPx reduces both hydrogen peroxide and any other organic hydroperoxides, and selenium-independent GPx which can only reduce the organic hydroperoxides (Aceto *et al.,* 1994). Only the Se-dependent GPx enzyme is present in trout tissues and appears at stage 33 of fry development in rainbow trout (Aceto *et al.,* 1994). Its activity decreases in the kidney, but increases in gills and white muscle as rainbow trout age (Otto and Moon, 1996).

Catalase is a hemoprotein with four atoms of iron per molecule attached to protein and chelated to protoporphyrin IX (Orbea *et al.,* 2000). The primary function of catalase is to split hydrogen peroxide into water and oxygen (Fig. 1.1) by the reaction: $2H_2O_2 \rightarrow 2H_2O+O_2$ (Kelly *et al.,* 1998; Orbea *et al.,* 2000). It is primarily found in peroxisomes, although it is present in the mitochondria and cytosol as well (Kelly *et al.,* 1998). Catalase appears in stage 19-20 of rainbow trout development, but its activity increases during fry development (Aceto *et al.,* 1994). Additionally, it has been shown to decrease in rainbow trout liver as they age, but the activity increases in the gill and red muscle (Otto and Moon, 1996).

Glutathione reductase does not directly remove any ROS, but it reduces oxidized glutathione (GSSG) in the presence of NADPH back to GSH that is required for GPx (Aceto *et al.,* 1994; Kelly *et al.,* 1998). Increased antioxidant enzyme activities to a variety of organic and metal contaminants have been observed in a variety of organisms in laboratory studies, but the

responses are transient and variable for different species, enzymes and single or mixed contaminants (Livingstone, 2001).

Antioxidant scavengers include GSH, vitamin E, vitamin A, vitamin C, and carotenoids. GSH is synthesized in several different cell types and is the most abundant thiol with concentrations up to 10 mM (Kelly *et al.,* 1998). GSH can directly reduce a number of ROS and is oxidized to GSSG in this process (Kelly *et al.,* 1998). GSH also acts as a substrate or co-substrate for many essential enzymatic reactions, such as with GPx (Kelly *et al.,* 1998). Thus, the depletion of GSH during oxidative stress can have a significant impact on the antioxidant balance within a cell (Kelly *et al.,* 1998). Otto and Moon (1996) have shown an increase in GSH activity in the liver, kidney, red muscle, brain, and heart of rainbow trout with age. Vitamin E (tocopherol) is an important lipid soluble antioxidant and scavenges a wide array of ROS (Kelly *et al.,* 1998). The primary antioxidant activity of tocopherols is to stop chain propagation of peroxyl radicals (Kelly *et al.,* 1998). Vitamin A (retinol) combined with peroxyl radicals, quench ROS, or scavenge thiol radicals (Palace *et al.,* 1999). Vitamin C (ascorbic acid), a water soluble antioxidant, is a powerful reducing agent capable of rapidly scavenging a number of ROS (Kelly *et al.,* 1998). In addition, ascorbate reacts with other cellular prooxidants, such as $^1O_2$, hypochlorous acid (HOCI), and thiol radicals (Kelly *et al.,* 1998). Carotenoids (α- and β-carotene) are lipid soluble antioxidants present in lipid membranes serving as provitamin A analogues and singlet oxygen quenchers and peroxyl radical scavengers (Kelly *et al.,* 1998).

1.3.3 *Glutathione S-transferase*

GST activity has been a commonly applied biomarker for assessing different groups of pollutants for several years (Frank *et al.,* 2011). GST is an important family of multifunctional intracellular enzymes that, in addition to the reduction of organic hydroperoxides, catalyse the

conjugation of a large variety of electrophilic alkylating compounds to GSH, thereby protecting cells against potential toxicity (reviewed in Aceto *et al.,* 1994). The GST family plays a physiological role in initiating the detoxication of potential alkylating agents via a -SH group of glutathione, thereby neutralizing their electrophilic sites and rendering the products more water-soluble (Habig *et al.,* 1974). Glutathione conjugates are thought to be metabolized further by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue, to produce the final product, a mercapturic acid (acetylcysteine), which is then excreted (Habig *et al.,* 1974). Since GST enzymes are part of an integrated defence strategy, their effectiveness depends on the combined actions of glutamate cysteine ligase and glutathione synthase to supply GSH as well as the actions of transporters to remove glutathione conjugates from the cell (Hayes *et al.,* 2005).

Three major families of proteins that are widely distributed in nature exhibit GST activity: the cytosolic and mitochondrial GST, which comprise soluble enzymes that are only distantly related, whereas membrane bound microsomal GSTs are a separate GST subfamily belonging to the MAPEG (Membrane Associated Proteins in Eicosanoid and Glutathione metabolism) pathway (Hayes *et al.,* 2005). There are also various isoforms of GST present in fish and mammalian tissues, and so far 14 classes of mammalian GST have been identified (e.g. alpha, mu, pi, sigma, theta, omega, kappa, and zeta GSTs) (George and Buchanan, 1989; Hayes *et al.,* 2005). Although fish GSTs have not been well characterized relative to their mammalian counterparts, all fish species examined to date exhibit tissue GST catalytic activity and express multiple soluble hepatic GST isoforms (Espinosa *et al.,* 2012). Of the various isoforms, the predominant GST in cyprinids, salmonids and gadoids belongs to a pi class GST (Otto and Moon, 1996). In rainbow trout, GST activity appears during stages 19-20 and increases greatly

during fry development reaching the highest concentrations in the liver followed by kidney and heart (Aceto *et al.,* 1994). As rainbow trout age, GST activity decreases in kidney, but the activity increases in the red and white muscle (Otto and Moon, 1996).

1.3.4 *Superoxide dismutase*

Superoxide dismutase acts as an antioxidant by removing of the superoxide anion radical, to form oxygen and hydrogen peroxide (Kelly *et al.,* 1998). There are three different forms of SOD, and all remove free oxygen radicals: manganese SOD (Mn-SOD) is found in the mitochondria, the copper/zinc form (Cu/Zn-SOD) in the cytosol, and the extracellular form (EC-SOD) in the extracellular matrix (Kelly *et al.,* 1998). All forms of SODs are thought to dismutate $^{\bullet}O_2^-$ via a ping-pong reaction: the transition metal prosthetic group is reduced by $^{\bullet}O_2^-$ forming O_2, the metal in the prosthetic group is then immediately re-oxidized by another $^{\bullet}O_2^-$ molecule, resulting in the production of H_2O_2 as shown in Fig.1.1 (Kelly *et al.,* 1998). The cellular activity of SOD is dependent on a variety of environmental factors including changes in pO_2 and exposure to pollutants such as heavy metals and organochlorine chemicals (Palace and Klaverkamp, 1993).

In rainbow trout, SOD activity appears during stages 19-20 and increases greatly during fry development reaching the highest concentrations in the kidneys (Aceto *et al.,* 1994). It decreases in the liver and white muscle of rainbow trout with age, with a corresponding increase in the gill (Otto and Moon, 1996). It has been previously shown that SOD decreases Se-induced ROS production *in vitro* in a chemiluminescent assay (Spallholz *et al.,* 2004) and is induced by low concentrations of sodium selenite (50-100 µM) in rainbow trout hepatocytes (Misra and Niyogi, 2009). Albrecht *et al.* (1994) have demonstrated a decreased SOD activity in livers of rats fed Se-contaminated diet (4.05 mg Se/kg diet). A decrease in SOD activity was recorded in

rainbow trout hepatocytes exposed to 200 μM sodium selenite (Misra and Niyogi, 2009). There is also evidence of an interspecies variation of SOD activity up to four or five fold: Palace and Klaverkamp (1993) reported variations in SOD activity levels in lake charr, white sucker and while pearl dace (*Margariscus margarita*).

1.3.5 *Selenium exposure and oxidative stress*

Selenium prooxidant activity arises from its ability to oxidize thiols (Spallholz, 1994). Glutathione appears to be particularly amenable to form complexes with certain forms of selenium (Palace *et al.,* 2004). In some of its forms, selenium may combine with glutathione to form a selenopersulfide anion that ultimately generates superoxide radicals and leads to damage of cellular components (Spallholz *et al.,* 1998). Related to the complex chemical speciation of Se, not all of its forms are capable of generating superoxide radicals by association with glutathione (Palace *et al.,* 2004). In fact, seleno-methionine, the dominant form of selenium in the eggs of fish and birds was not active in the generation of superoxide in an *in vitro* assay system (Spallholz *et al.,* 2001). However, recently published studies have reported the ability of some cell types to catalyze the metabolism of seleno-methionine to alternative forms that are capable of producing superoxide (Wang *et al.,* 2002; Miki *et al.,* 2001). Moreover, Palace *et al*. (2004) have documented that rainbow trout embryos can metabolize seleno-methionine that accumulates in the eggs, generating superoxide radicals responsible for malformations in the embryo. In addition, selenite can react with GSH to produce ROS and elemental Se (Spallholz, 1994). In adult fish, Se can increase SOD activity as well as the GSH: GSSG balance, as evident in isolated rainbow trout hepatocytes that showed an induction of CAT and SOD activities at 50 and 100 μM of selenite exposure, and an induction of GPx activity at 100 and 200 μM exposure doses (Misra and Niyogi, 2009). Misra and Niyogi (2009) have also demonstrated that selenite

exposure increased intracellular ROS formation and decreased the GSH: GSSG ratio. *In vivo* studies in fish exhibited a 25% reduction in hepatic GSH level during acute exposure to waterborne selenite (Miller *et al.,* 2007).

On the other hand, oxidative stress is also a mechanism associated with selenium deficiency: rainbow trout fed a Se deficient diet demonstrated a decreased GPx activity (Bell *et al.,* 1986). Selenium deficiency in rats also caused a marked inhibition in liver and adrenal GPx activities (Chanoine *et al.,* 2004). The addition of Se to diets of arsenic (As) exposed rats led to restoration of GPx and GST activities back to normal levels (Messaraha *et al.,* 2012). Antioxidant protection by Se in the mammalian cell is mediated by selenoamino acids, either as Se-Cys or Se-Met (ATSDR, 2003). Selenomethionine has GPx-like activity, while the active site of GPx contains selenocysteine residues (ATSDR, 2003). Other selenoproteins (e.g. selenoprotein P and thioredoxin reductase) also have been reported to have antioxidant properties, and can function in the protection against peroxynitrite, by reducing it to nitrite (ATSDR, 2003). Moreover, because of selenium's role in the antioxidant GPx activity, Se also reduces the toxicity of metals in vitamin E-deficient animals (ATSDR, 2003).

1.4 Use of parasites as bioindicators of pollution

In environmental impact studies, certain organisms can provide valuable information about the chemical state of their environment through their ability to concentrate environmental toxicants within their tissues (Sures *et al.,* 2002). Due to the great diversity of parasite species as well as their ability to inhabit a variety of hosts and environments, there is an increasing interest in using parasites as biological or ecological indicators of their host's life conditions (Poulin, 1992; Sures *et al*., 1997a; Sures, 2006; Marcogliese, 2005; Sasal *et al*., 2007; Vidal-Martinez *et*

al., 2009). Parasites are indicative of many biological aspects of their hosts, including diet, migration, recruitment and phylogeny (Williams *et al.,* 1992). As parasites often require vertebrate and invertebrate organisms as their hosts, changes in the structure of a parasite community may reflect differences in the composition of aquatic host species community (Marcogliese and Cone, 1997). Thus, parasites with complex life cycles may provide information about the biological properties of different biotopes within an ecosystem by synthetically recording the presence of intermediate and definitive hosts (Cone *et al.,* 1993; Marcogliese and Cone, 1997). In addition, use of the parasites can complement chemical analysis or traditional biological surveys as indicators of dysfunction at the ecosystem level (Galli *et al.,* 2001). One important advantage of parasite communities as indicators of environmental stress is related to trophic relations: parasites move through the food web and some of them are situated at top of the food chain, integrating the adverse effects of various contaminants (Gelnar *et al.,* 1997). Moreover, parasites may offer advantages over currently used bioindicators as they are more widely spread and have a higher accumulation potential of contaminants as compared to their hosts (Sures *et al.,* 1997a).

1.4.1 *Effects of parasitic infection on fish health*

Parasites have a variety of impacts on their hosts: they impose energetic demands, alter behaviors, affect morphology and appearance, reduce fecundity and growth, and cause mortality (Marcogliese, 2004). In fish, various sub-lethal and lethal pathological effects have been documented, and examples are provided in Table 1.2. Infection of bluegill sunfish (*Lepomis macrochirus*) with *Dactylogyrus* sp., *Ergasilus caeruleus, Proteocephalus* sp., *Spinitectus* sp. and *Leptorhynchoides* sp. lead to a decrease in condition factor (Neff and Cargnelli, 2004). In another study, Thilakaratne *et al.* (2007) demonstrated a negative correlation between condition

factor and parasite numbers in young spottail shiners (*Notropis hudsonius*). In three-spined sticklebacks (*Gasterosteus aculeatus*), infection with cestodes (*Schistocephalus solidus*) have been shown to reduce growth, affect foraging ability, reduce mobility and avoidance behavior, delay maturation, decrease nutrient storage and liver size and increase sensitivity to cadmium (Pennycuick, 1971; Pascoe and Cram, 1977; Barber and Huntingford, 1995). Infection of rainbow trout with *Crepidostomum farionis* (Digenea) lead to decreased hemoglobin concentrations and hematocrit, while infection with *Nanophyetis salmincola* (Digenea) reduced swimming speed and inhibited immune response (Klein *et al.,* 1969; Butler and Milleman, 1971; Jacobson *et al.,* 2003). Fischer and Kelso (1988) showed an increase in bluegill (*Lepomis macrochirus*) parasite-induced mortality as a result of infection with *Allacanthocasmus* sp. (Digenea). The reduced host survival due to the parasitic infection is usually intensity dependent (Minchella and Scott, 1991). Other factors that can affect host mortality associated with parasitic infection include target organ, host size and age, number of invading parasites, as well as time spent in the host.

Table 1.2 Selected examples of sub-lethal and lethal impacts on fish by parasites in freshwater and marine environments. Parasite induced host mortality is expressed as PIHM, and parasite induced vulnerability to predation is expressed as PIVP.

Host Species	Latin Name	Parasite Species	Parasite Group	Impact on the host	Source
Herring	*Clupea harengus*	*Ichthyophonus hoferi*	Fungus	PIHM	Patterson (1996)
		Scolex pleuronectis	Cestode	PIHM, reduced larval feeding	Rosenthal (1967); Heath and Nicoll (1991)
		Lernaeocera sp.	Copepod	PIHM	Rosenthal (1967)
		Hysterothylacium aduncum	Nematode	PIHM	Balbuena *et al*. (2000)
Three-spined stickleback	*Gasterosteus aculeatus*	*Diplostomum gasterostei*	Digenean	PIHM	Pennycuick (1971)
		Schistocephalus solidus	Cestode	Reduced growth, affected foraging ability, diet choice and appearance, reduced mobility, avoidance behaviour, nutrient reserves and liver size, delayed maturation, increased sensitivity to Cd, decreased depth distribution, PIVP,PIHM	Pennycuick (1971); Pascoe and Cram (1977); Barber and Huntingford (1995)
Bluegill sunfish	*Lepomis macrochirus*	*Allacanthochasmus* sp.	Digenean	PIHM	Fisher and Kelso (1988)
		Dactylogyrus sp., *Ergasilus caeruleus, Proteocephalus* sp., *Spinitectus* sp. and *Leptorhynchoides* sp.		Decreased condition factor (K)	Neff and Cargnelli (2004)
		Uvulifer ambloplitis	Digenean	PIHM	Lemly and Esch (1984)
Rainbow trout	*Oncorhynchus mykiss*	*Cryptobia salmositica*	Hemoflaggelate	Reduced respiration and swimming	Kumaraguru *et al*. (1995)

Myxobolus cerebralis	Myxozoan	Deformities, abnormal swimming, lower leukocyte and lymphocyte counts	Densmore *et al*. (2001)
Gyrodactylus sp.	Monogenean	Induced cortisol production	Stoltze and Buchmann (2001)
Crepidostomum farionis	Digenean	Reduced hemoglobin and hematocrit	Klein *et al*. (1969)
		Reduced swimming speed, reduced immune response	Butler and Milleman (1971); Jacobson *et al*. (2003)

1.4.2 *Sub-lethal effects of combined parasitic infection and metal contamination on fish*

There is a growing body of work focusing on combined effects of multiple stressors, including parasites and pathogens, on animal and fish health (Marcogliese and Pietrock, 2011). Most of the research on combined effects of metal exposure and parasitic infection has been done in the laboratory setting, where organisms were exposed to a single species of parasite and a single metal (Marcogliese and Pietrock, 2011). Several researchers, however, have taken a field approach to examine interactions between parasites and abiotic stressors (Marcogliese and Pietrock, 2011). The evidence for impact of parasitic infections on bioaccumulation of contaminants in hosts is equivocal: infected fish have been shown to accumulate both greater (Pascoe and Cram, 1977) and lesser (Sures and Siddall, 1999; Sures *et al.,* 2003) amounts of contaminants than non-parasitized fish. Furthermore, multiple studies have demonstrated that parasitism can increase the susceptibility of fish hosts to a variety of toxicants (Boyce and Yamada 1977; Pascoe and Cram 1997; Moles 1980; Sakanari *et al*., 1984). For example, Guth *et al*. (1977) demonstrated that infections with two trematode species increased the susceptibility of their intermediate host, the mollusk *Lymnaea stagnalis,* to zinc. In the case of combined parasitism and dietary deficiency, the survival of fish infected with *Schistocephalus solidus* (Cestoda) and exposed to cadmium and dietary restriction was correspondingly reduced (Pascoe and Woodworth, 1980). However, the study by McCahon *et al*. (1988) showed that the presence of the larval acanthocephalan *Pomphorhynchus laevis* (at any level of infection) does not modify the effects of sub-lethal Cd toxicity to its intermediate host, the amphipod *Gammarus pulex*, as measured by feeding rate.

Overall, the studies examining the effects of combined stressors (toxicant and parasitic infection) on fish health have demonstrated that toxicological effects depend on host and parasite

species, and type of toxicant. Striped bass *Morone saxatilis* (Walbaum) infected with larval nematodes (*Anisakis* sp.) and exposed to zinc and benzene had lower haematocrit values than fish exposed to single stressors alone (Sakanari *et al.,* 1984). Furthermore, Marcogliese *et al.* (2005) found that levels of lipid peroxidation in the livers of yellow perch *Perca flavescens* (Mitchill) were higher at a polluted location than at a clean location. This effect was more pronounced in fish infected with *Raphidascaris acus* (Nematoda) and *Apophallus brevis* (Trematoda) at the polluted site (Marcogliese *et al.*, 2005). Therefore, to determine if parasites contribute to an enhanced stress response in their hosts under polluted conditions, it is important to use biomarkers that respond to both parasitic infections and natural stressors (Marcogliese and Pietrock, 2011). However, factors such as species, target organ, age, sex, reproductive status, and season, should also be taken into account as well as, since they can affect biomarkers levels (Marcogliese and Pietrock, 2011). For example, sexual differences in lysosomal production and morphology were noted in zebra mussels (*Dreissena polymorpha*) from two sites in northeast France with different concentrations of contamination and exposed to selected parasites (Minguez *et al.,* 2009).

1.4.3 *Lethal effects of combined parasitic infection and metal contamination on fish*

Several experimental studies have demonstrated that the effects of parasitic infection are generally more severe in fishes exposed to contaminants than in those that are not. Moreover, various studies have shown that the survival under polluted condition is significantly reduced in infected organisms compared with non-infected conspecifics (Marcogliese and Pietrock, 2011). Boyce and Yamada (1977) reported an increased in mortality of sockeye salmon *Onchorhynchus nerka* smolts infected with the cestode *Eubothrium salvelini* and exposed to zinc compared to uninfected fish. Cadmium was more toxic to three-spined sticklebacks infected with

plerocercoids of the cestode *Schistocephalus solidus* than to uninfected fish, as mortality rate increased in fish exposed to both stressors compared to either stressor alone (Pascoe and Cram, 1977). In the study of Pascoe and Woodworth (1980), three-spined sticklebacks subjected to three stressors (*S. solidus*, dietary restriction and Cd) jointly and separately experienced significantly higher mortality when exposed to combined stressors as compared to single ones. Gheorgiu *et al.,* (2006) observed higher mortality of guppies *Poecilia reticulata* experimentally exposed to high concentrations of waterborne zinc and the monogenean *Gyrodactylus turnbulli* than those exposed to Zn alone. The snail *Lymnaea stagnalis* infected with the trematode *Schistosoma douthitti* experienced higher mortality when exposed to high concentrations of Zn when compared to uninfected conspecifics (Guth *et al.,* 1977).

1.4.4 *Effects of parasitic infection on oxidative stress response*

Parasites have been shown to induce oxidative stress in various hosts (Dautremepuits *et al*., 2002, 2003; Frank *et al*., 2011). To counter-act tissue and DNA damage, fish have developed various enzymatic and non-enzymatic defensive mechanisms to protect against oxidative stress (Dautremepuits *et al.,* 2009). Thus it is believed that the health of aquatic organism is linked to production of ROS and antioxidant compounds, such as antioxidant enzymes. An increase in antioxidant enzyme (GST, GPx, CAT) levels were recorded in carp (*Cyprinus carpio*) infected with a cestode (*Ptychobotrium* sp.) compared to uninfected fish (Dautremepuits *et al.,* 2002, 2003). Neves *et al*. (2000) reported that shrimp (*Palaemonetes argentinus*) infected with isopod *Probopyrus ringueleti* exhibited lower activity of superoxide dismutase when compared to uninfected individuals. In addition, an increase in tert-butyl hydroperoxide initiated chemiluminescence, as a measure of LPO, was demonstrated in the muscle of fish infected with metacercariae of the trematode *Clinostomum detruncatum* (Belló *et*

al., 2000). Yellow perch from contaminated sites in the St. Lawrence River (Canada) were shown to have higher levels of LPO when infected with nematode *Raphidascaris acus* in comparison to unparasitized fish (Marcogliese *et al.,* 2005).

1.5 Parasitic species of interest: *Raphidascaris acus*

1.5.1 *General information on nematodes*

The nematodes represent a large group of metazoan invertebrate animals that occur from the polar regions to the tropics in all types of the environments, including deserts, high mountain elevations, and great ocean depths (Moravec, 1994; Ruppert and Barnes, 1994). The nematodes show a very wide range of ecological adaptation. Most of them are free-living, whereas others are semiparasitic or parasitic species. Non-parasitic nematodes are benthic animals that live in the interstitial spaces of algal mats, aquatic sediments and soils, while parasitic nematodes attack virtually all groups of plants and animals (Ruppert and Barnes, 1994). The nematodes are parasitic in both marine and freshwater fishes. Their size ranges from one millimeter to a few meters (Ruppert and Barnes, 1994). Both males and females generally lack body coloration, being transparent or with a whitish or yellowish tinted cuticle (Moravec, 1994).

The body of a nematode is elongate, vermiform cylindrical, covered with a well developed cuticle, without cilia, protonephridia, respiratory organs or a blood system (Moravec, 1994). They have four main longitudinal hypodermal chords, a tripartite pharynx, circumenteric nerve ring, copulatory spicules, and one or two tubular gonads opening separately in the female and into the rectum in the male (Moravec, 1994). Most nematodes are dioecious, but hermaphrodites are not uncommon (Ruppert and Barnes, 1994). Males are usually smaller than

females, and the posterior of the male may be curled like a hook or broadened into a fan-shaped copulatory aid, called a bursa (Ruppert and Barnes, 1994; Moravec, 1994).

The nematode cuticle is thick, elastic and tough, and comprised of three layers: thin epicuticle, the median layer and the basal layer. It is sometimes smooth, but usually bears fine transverse grooves (striations), occurring at regular intervals. The epidermis is usually cellular and is responsible for secreting the cuticle, storage of the nutrients, and bears attachment fibers that link the musculature to the cuticle (Moravec, 1994; Ruppert and Barnes, 1994). Most nematodes move forward and backward using sinuous eel-like undulations of the body produced by the alternate contraction of dorsal and ventral longitudinal muscles.

The mouth is located at the centre of the anterior tip and can be surrounded by lips and sensilla of various sorts (Moravec, 1994). The mouth opening leads into the buccal cavity, which is followed by the oesophagus and intestine (Moravec, 1994). Digestive enzymes are produced by the pharangeal glands and the intestinal epithelium (Ruppert and Barnes, 1994). Digestion begins extracellularly, within the intestinal lumen, but is completed. The intestine is also an important site of the yolk synthesis for the developing oocytes (Ruppert and Barnes, 1994). Anatomy of a typical male and female nematode is shown in Figure 1.2.

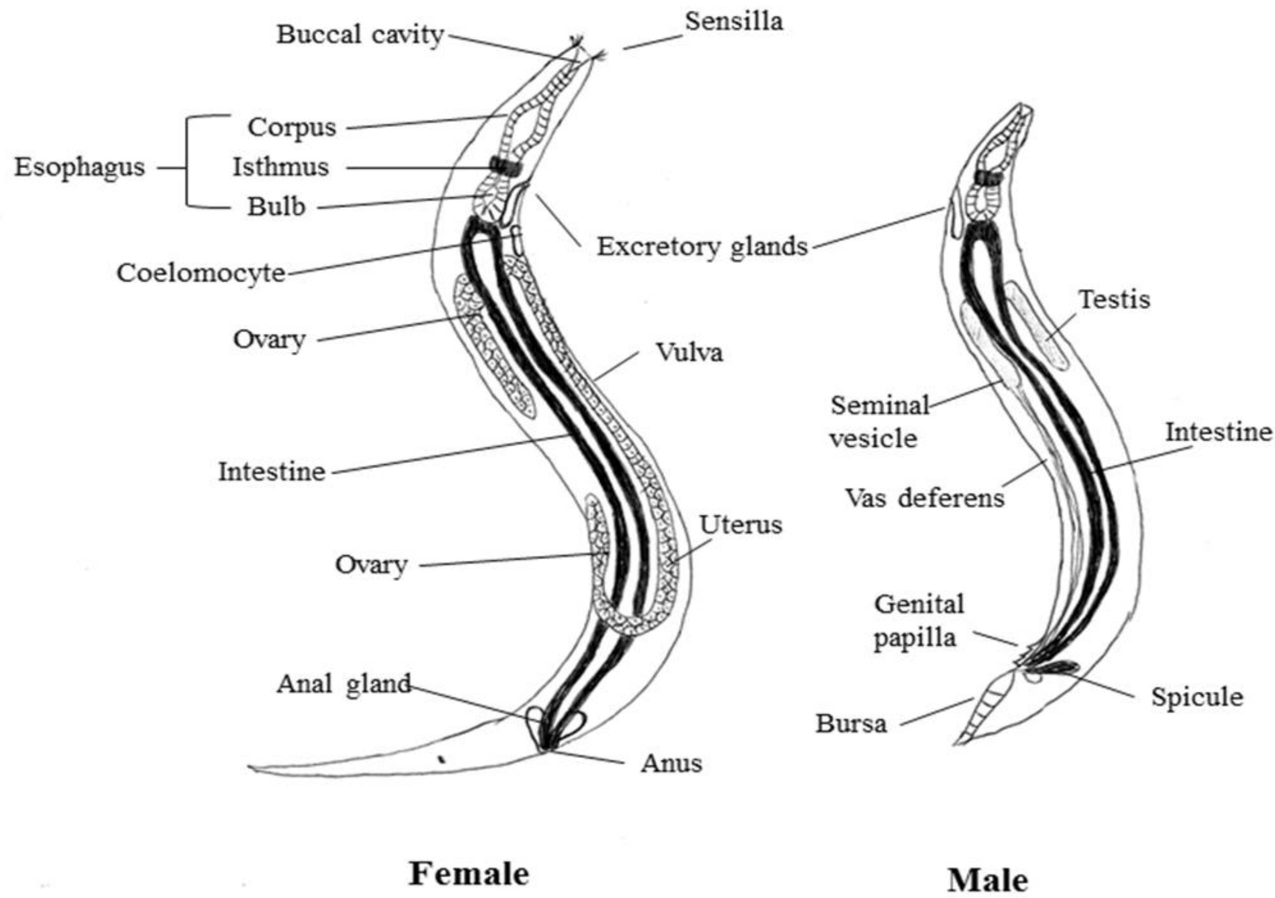


Figure 1.2 General morphology of a typical nematode male and female.

1.5.2 *Raphidascaris acus life-cycle*

Raphidascaris acus (Anisakidae) is widely distributed parasitic nematode that inhabits fresh water invertebrates and fishes (Williams and Jones, 1994). It occurs in both still waters where the principal host is pike, as well as in streams (Moravec, 1994). Its distribution extends up to the sections of the rivers and brooks where the main definitive host, brown trout, occurs. The infection by this parasite is often high in these regions (Moravec, 1994).

The life-cycle of *R. acus* comprises two hosts (Figure 1.3). Adult nematodes live in the intestine of fish where sexual reproduction takes place. So far, members of the families Esocidae, Salmonidae, Anguillidae, Percidae, Lotidae, and Thymallidae have been described to act as the final host (reviewed in Smith, 1984a; Moravec, 1994). The eggs of *R. acus* are think-walled, smooth, and almost spherical (size: 0.066-0.078 x 0.081-0.087 mm). The eggs are laid in the intestine of the fish host from where they are released along with excrements into the aquatic environment where they continue to develop. The eggs are resistant to cold temperatures, and are known to survive even freezing of the surrounding water. The speed of egg development is influenced by the water temperature (Moravec, 1994). Colder environments increase the time it takes for larva to undergo its first molt and to change into the second-stage larva inside the egg shell (Moravec, 1994). Within 7-30 days, these larva hatch from the egg and live for a relatively short time free in the water (this being dependent on water temperature). The further development of the free second-stage larva (size: 0.24-0.38 mm) takes place only when they are consumed (either still inside the egg or liberated from it) by an intermediate host (fish) or a paratenic host (where no parasite development takes place), such as aquatic invertebrates (Smith, 1984; Moravec, 1994). The intermediate host spectrum comprises members of the families

Cyprinidae, Salmonidae, Coregonidae, Esocidae and others. Trout have been found to serve as both definitive host and the main intermediate host (Williams and Jones, 1994).

When eggs are taken up by a fish intermediate host, a larva hatches from the egg, migrates through the intestinal wall and encysts in the host's liver and mesenteries (Smith, 1984b; Valtonen *et al*., 1994). Inside the cyst, the larva (size: 0.74-1.05 mm) develops further for 15-55 days and molts into the infective third-stage larva (Moravec, 1994). Having molted for the second time, larvae grow considerably, reaching a body length of 3-5 mm (Smith, 1984b; Moravec, 1994).

According to Moravec (1994), when an intermediate host is eaten by the final host, *R. acus* larvae undergo two additional molts until they reach adult stage. The first molt occurs as early as 3-4 days post ingestion; the fourth-stage larvae are morphologically similar to the third-stage, differing only by large body size and the development of reproductive organs (Moravec, 1994). However, Smith (1984b) had shown that *Raphidascaris acus* can develop to the fourth stage in the intermediate host as well, and undergo only one molt in the final host. The last molt of the *R. acus* larvae into an adult takes place in the intestinal tract of the final host 21 days post-ingestion (Moravec, 1994). The length of the adult worm ranges from 7.9-9.1 mm (Moravec, 1994). The complete development of *R. acus* in the definitive host from the ingestion of third-stage larva until egg production lasts approximately two months (64 days) at a water temperature of 15°C (Moravec, 1994).

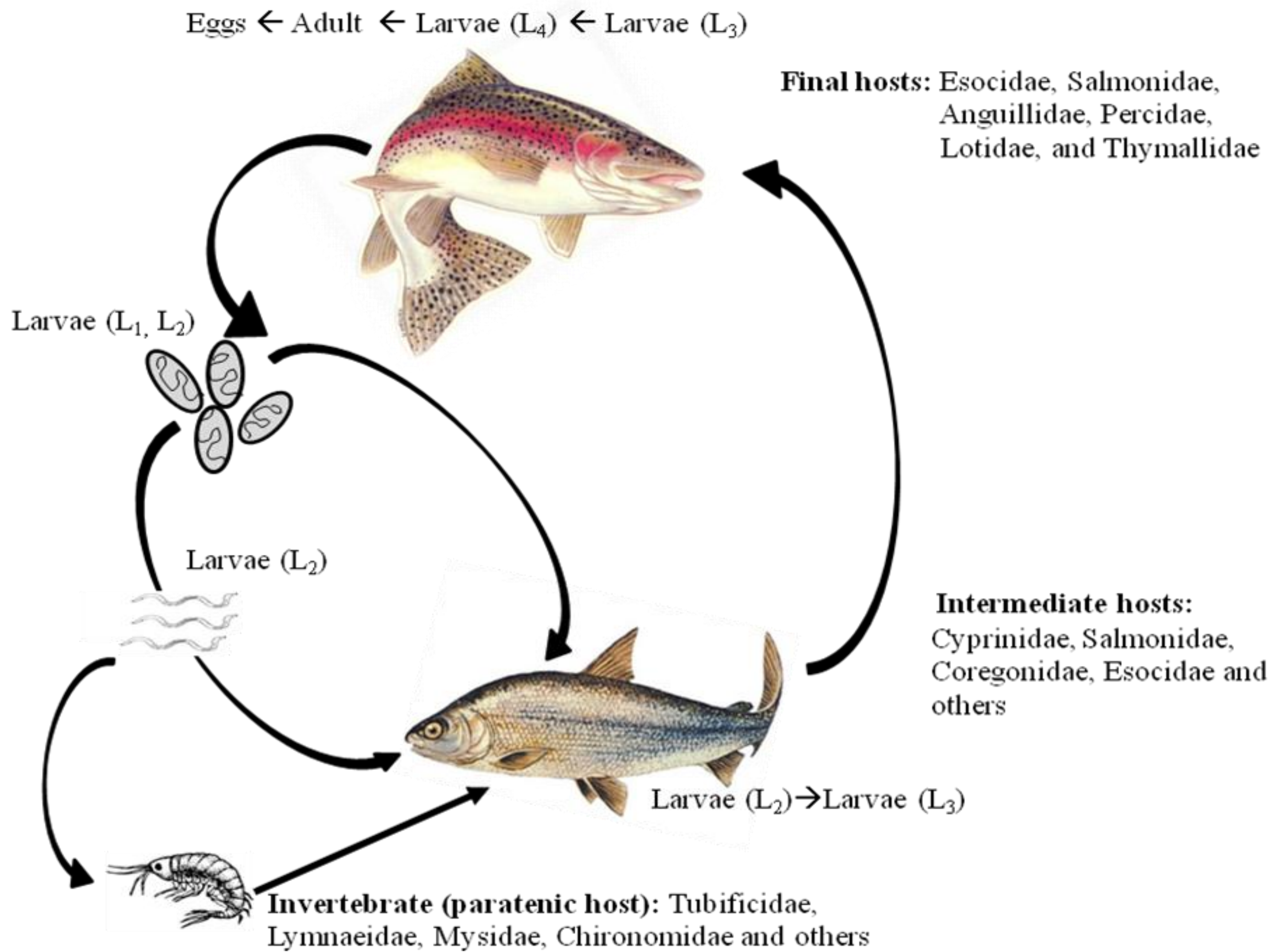


Figure 2.3 Life-cycle of the parasitic nematode *Raphidascaris acus* (modified from Moravec (1994) and Smith (1984b)).

1.5.3 *Pathogenic effects*

The pathogenicity of *R. acus* is not well understood. It appears that adult nematodes do little or no damage to health of the pike, but they may cause mass mortality of trout as was recorded by Carrara and Grimaldi (1960, cited in Moravec, 1994). The larvae of *R. acus* are very pathogenic occurring in various internal organs of the intermediate host fishes with 100% prevalence in some fish and an intensity of more than 1,000 larvae per fish (Moravec, 1994). Larvae of *Raphidascaris acus* often produce numerous nodules surrounding the intestinal wall in trout. They may also cause extensive damage to the hosts' tissues when penetrating through its intestinal wall (Moravec, 1994). This together with gut deformities due to parasitic nodules was observed in rainbow trout by Eiras and Reichenbach-Klinke (1982). Moreover, bream (*Abramis brama*) has been reported to be very susceptible to *R. acus* infections often resulting in considerable mortality of this fish, as observed in some lakes in Russia and Central Asia (reviewed in Moravec, 1994). In streams of Central Europe, loach (*Neomacheilus barbatulus*) exhibited increased mortality rates due to high levels of infection with *R. acus* larvae (reviewed in Moravec, 1994).

The numbers of *R. acus* larvae can accumulate throughout the life of the fish, but dead and degenerating larvae are often present, suggesting that at least some worms are not able to survive as long as their host, or are killed off by the host's immune system (Chubb, 1980). Nonspecific inflammatory responses have been suggested to have a major role in protection against parasitic invasions in fish, resulting in the encapsulation of the parasites (reviewed in Valtonen *et al*., 1994).

1.6 Rainbow trout species of interest

Rainbow trout (*Oncorhynchus mykiss*), a member of the family Salmonidae, are native to North America, extending throughout the eastern North Pacific region, from Mexico through the Aleutian Islands, and to the Kamchatka Peninsula (Thorgaard *et al*., 2002; Fornshell, 2002). Since it is highly regarded as sport and food fish, the species has widely been introduced throughout North America (Wolf and Rumsey, 1985). Successful introductions have also been made in Asia, Africa, Australia, Europe, and South America (Wolf and Rumsey, 1985). The natural habitat of the rainbow trout is a freshwater environment; however, they are versatile and can adapt to a wide array of freshwater and marine environments. This adaptability has been documented for the more than 100 years since rainbow trout were transplanted from its original range (Wolf and Rumsey, 1985). This species can tolerate temperatures from 0° C to 25° C, but the optimum range is 10°-15° C for growth and 1°C-13° C for spawning (Wolf and Rumsey, 1985). Trout are typically generalists and opportunists when it comes to food, preying on a variety of items depending on what is available at any given time (Behnke, 2002). Most fresh water salmonids rely heavily on aquatic invertebrates, such as larvae of aquatic insects; however, as trout become larger, they can prey on smaller forage fish (Behnke, 2002). Rainbow trout usually reach sexual maturity at 2 years of age, and spawn annually (Wolf and Rumsey, 1985). As with other salmonids, their spawning time is regulated by seasonal change in photoperiod (Wolf and Rumsey, 1985).

Although rainbow trout are relatively larger in size than other model fish species, and have fairly long reproductive cycles (2–3 years), they are easy and inexpensive to culture. The widespread cultivation of rainbow trout as a food and sport fish, has led to the development of significant basic biological knowledge about this species. As a result rainbow trout are the most

experimentally tractable of the salmonid fishes and serve as a surrogate for the research on more economically important Atlantic and Pacific salmon (Wolf and Rumsey, 1985). Moreover, trout are more amenable to surgical manipulation than smaller species, and their size allows large amounts of specific tissues and cell types to be isolated for biochemical, immunological, and molecular biological analysis (Thorgaard *et al*., 2002). The closely related species within the *Oncorhynchus*, *Salmo* and *Salvelinus* genera have been so extensively studied that in the past 20 years these species have been associated with over 40,000 reports on their ecology, behaviour, physiology and genetics, with rainbow trout used in half of these studies (Thorgaard *et al*., 2002). A further advantage of using the rainbow trout either as a laboratory animal or as a surrogate for other salmonid species is that a great diversity of strains, greater than for any other salmonid, is available (Wolf and Rumsey, 1985).

1.7 Research objectives and hypotheses

Intestinal parasites, specifically cestodes and acanthocephalans, can accumulate metals to a greater extent than their hosts (Sures *et al*., 1999; Sures, 2001, 2003). However, less is known about accumulation of metals by nematodes, and their role in metal uptake within fish. The focus of this study was to examine *R. acus*- rainbow trout interactions when exposed to environmentally relevant concentrations of dietary selenium. Fish health parameters such as Fulton's condition factor (K), hepatosomatic index (HSI), and gross energy (GE) were calculated and used to determine effects of single and combined stressors on trout health. To investigate joint effects of parasitism and Se pollution on oxidative stress biomarkers in rainbow trout, activities of two enzymes involved in antioxidant response (SOD and GST) were assessed in the liver and in the head kidney, which are major organs of the fish immune system.

1.7.1 *Objectives*

Specific objectives of this thesis were to:

1) Determine to what extent adult *R. acus* will accumulate Se from its host, rainbow trout, experimentally exposed to Se-Met spiked diet.
2) Establish if parasitic infection coupled with Se-Met exposure has an effect on fish health parameters (as determined by Fulton's condition factor, hepatosomatic index and gross energy content).
3) Determine if combined effect of parasitic infection by *R. acus* and Se-Met exposure has an effect on rainbow trout oxidative stress biomarkers (as determined by levels of GST and SOD).

1.7.2 *Hypotheses*

The specific hypotheses of this thesis are:

1) Rainbow trout infected with *R. acus* and exposed to Se-Met will have greater concentration of Se in the muscle than uninfected fish exposed to Se-Met only, as parasitic infection can impact immune response of the host making it more susceptible to contaminants.

<u>Null Hypothesis:</u> No difference in Se mucle concentrations will be detected between parasitized and unparasitized rainbow trout experimentally exposed to dietary Se.

2) Nematodes in fish exposed to dietary Se-Met continuously for 70 days will have greater Se concentrations than their hosts.

<u>Null Hypothesis:</u> There will be no differences in Se concentration between the nematode *R. acus* and its host (rainbow trout) after dietary exposure to Se-Met.

3) The activity of antioxidant enzymes (GST and SOD) will be greater in trout jointly exposed to Se-Met and *R. acus*, than in trout exposed to Se-Met alone, and even greater than enzyme activity in control fish.

<u>Null Hypothesis:</u> There will be no differences in GST and SOD activity levels in parasitized and unparasitized rainbow trout exposed to dietary Se-Met; levels of GST and SOD will not be significantly different between control fish and infected trout exposed to Se-Met.

2.0 THE EFFECTS OF SELENIUM EXPOSURE AND PARASITIC INFECTION ON FISH HEALTH INDICATORS AND OXIDATIVE STRESS INDICATORS IN JUVENILE RAINBOW TROUT (*Oncorhynchus mykiss*)

2.1 Introduction

Selenium is an essential micronutrient required for all vertebrates including fish. Known for its narrow margin of toxicity in tissues along with the propensity to bioaccumulate in aquatic food chains, it has considerable biological importance as even a slight increase in environmental selenium can be detrimental to biota (Lemly, 2002a). Industrial and agricultural activity has hastened the release of selenium from geologic sources and made it more available to fish and wildlife in aquatic and terrestrial ecosystems (Hamilton, 2004). Agricultural drain water, sewage sludge, fly ash from coal-fired power plants, oil refineries, and mining are all anthropogenic sources of selenium contamination of the aquatic environment (Hamilton, 2004). Uptake of selenium by aquatic biota can be through water or diet, dietary exposure being the major source of selenium accumulation in fish (Hamilton, 2004; Sandholm *et al.,* 1973). Under normal physiological conditions, fish require dietary concentrations of 0.1-0.5 µg Se/g dry weight (DW) for maintaining of homeostasis; however, concentrations exceeding 3 µg Se/g DW can produce a rapid concentration-dependant bioaccumulation to concentrations that can be toxic (Hamilton and Buhl, 1990; Lemly, 1993a, 1997a; reviewed in Janz, 2010). In aquatic ecosystems, Se exists in both inorganic (e.g. selenate, selenite) and organic (seleno-amino acids and selenoproteins) forms, with a seleno-amino acid, selenomethionine (Se-Met) being the major dietary source of selenium available to fish (Fan *et al.,* 2002). In freshwater fish, Se exposure has been shown to decrease the condition factor, alter swimming performance, impair reproduction, induce

teratogenic deformities, damage liver and kidney, and increase mortality (Thomas and Janz, 2011; Kennedy *et al.,* 2000; Muscatello *et al.,* 2006; Hamilton, 2004; Holm *et al.,* 2005; Coughlan and Velte, 1989).

Three major mechanisms have been suggested for Se toxicity: membrane and protein damage from Se generated reactive oxygen species (ROS), substitution of Se for S during assembly of protein, and inhibition of Se methylation metabolism resulting in hydrogen selenide accumulation (Spallholz *et al.,* 2004; Miller *et al.,* 2007). Until recently, oxidative damage as a potential mechanism of selenium toxicity in fish has received very little attention. Palace *et al*. (2004) reported that the metabolism of Se-Met in rainbow trout (*Oncorhynchus mykiss*) embryos led to ROS generation, while Orun *et al*. (2005) described an induction of enzymatic antioxidants (superoxide dismutase (SOD) and glutathione peroxidase (GPx)) in trout exposed to waterborne sodium selenite. Furthermore, the concentration of hepatic glutathione (GSH), one of the most important non-enzymatic antioxidants, was reported to decrease in trout exposed to acute concentrations of waterborne sodium selenite (Miller *et al.,* 2007). The induction of catalase (CAT), SOD and GPx activities were observed in primary cultures of rainbow trout hepatocytes exposed to selenite *in vitro* (Misra and Niyogi, 2009). These findings suggest that oxidative stress can be an important factor in selenium toxicity to fish.

While anthropogenic pollution of the environment is an important factor contributing to decline in many animal populations, it is increasingly recognized that organisms are constantly exposed to a variety of stressors which affect animal health. In that context, parasites (and other pathogens) are increasingly a focus of attention as they can interact with anthropogenic stressors to reduce health in a wide spectrum of host and parasite taxa (Marcogliese and Pietrock, 2011). Several experimental studies have shown that the effects of parasitic infection are generally more

detrimental in fishes exposed to contaminants than in those that are not exposed (Boyce and Yamada, 1977; Pascoe and Cram, 1977; Moles, 1980; Gheorgiu *et al.,* 2006). Some of the effects of combined stressors such as contaminant exposure and parasitic infection include lower hematocrit values, increased levels of lipid peroxidation, decreased condition factor, and increased mortality in adults and fry (Sakanari *et al.,* 1984; Marcogliese *et al.,* 2005; Thilakaratne *et al.,* 2007; Boyce and Yamada, 1977; Pascoe and Cram, 1977; Pascoe and Woodworth, 1980). Moreover, digeneans, cestodes, nematodes, and crustaceans which infect fish and invertebrates have been reported to affect antioxidant metabolism and induce oxidative stress (Bello´ *et al*., 2000; Neves *et al*., 2000; Dautremepuits *et al*., 2002, 2003; Marcogliese *et al*., 2005; Frank *et al.,* 2011).

While in the majority of studies, exposure to multiple stressors leads to additive or synergistic effects, there is also evidence that parasites can alleviate detrimental effects of toxicants. Intestinal parasites, specifically cestodes and acanthocephalans, can accumulate pollutants to the greater extent than their host (Sures *et al.,* 1999; Sures, 2001, 2003). Thus, these parasites can play a beneficial role by removing toxicants from the host's body, thereby decreasing the concentrations of contaminants available for uptake. However, little is known about accumulation of metals by nematodes, and their role in metal uptake by fish. In order to obtain insight into host-parasite relationships in polluted environments, a study on nematode parasitism of rainbow trout exposed to subchronic Se was initiated. The specific research objectives were to determine to what extent *Raphidascaris acus*, a parasitic nematode inhabiting the intestine of rainbow trout, and its host accumulate Se from subchronic dietary exposure to an environmentally relevant concentration of Se-Met. Non-specific biomarkers such as condition factor (K), hepatosomatic index (HSI), and gross energy (GE) were calculated to demonstrate the

general effects of Se-Met exposure and parasitic infection on trout health as they are known to be useful to evaluate integrated effects of multiple stressors on individual fish health (Shugart *et al.,* 1992; Pottinger *et al.,* 2002). To further investigate joint effects of parasitism and Se-Met exposure on oxidative stress in rainbow trout, activities of two enzymes involved in antioxidant response (SOD, GST) were assessed in the liver and in the head kidney, which are major organs of the fish immune system.

2.2 Materials and Methods

2.2.1 *Chemicals*

Seleno-L-methionine (purity > 98%) was purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.2.2 *Test species*

Female rainbow trout (*O. mykiss*) approximately 1.5 year of age were randomly selected from an in-house stock reared from eggs obtained from a commercial supplier (Troutlodge, Sumner, WA, USA). Prior to initiation of the study trout were reared in a 1666L flow-through tank receiving dechlorinated Saskatoon City water at approximately 6 °C and maintained under a 12L: 12D photoperiod. Until beginning of Se-Met exposure all fish were fed a commercially available trout feed once daily (Martin Classis Sinking Fish Feed, Martin Mills Inc., Elmira, ON, Canada) at a rate of approximately 2% body weight. Control fish were fed this uncontaminated commercial trout feed throughout the length of the experiment.

2.2.3 *Experimental protocol*

The studies were approved by the University of Saskatchewan's Animal Research Ethics Board (# 20090085), and adhered to the Canadian Council of Animal Care guidelines for

humane animal use. All experiments were conducted in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan's Toxicology Centre.

For the experiment, 232 rainbow trout were randomly assigned to four 719 L tanks supplied continuously with running water at a flow rate of 4 L/min and maintained at approximately 6 °C under 12L:12D photoperiod. The mean weight of trout at initiation of the exposure was 94.6 ± 5.6 g. One of the tanks was randomly designated for control trout and three tanks were randomly designated for Se-Met exposed trout (one Se and two Se+ parasites groups). *Raphidascaris acus* (L3) larvae require approximately 21 days to undergo two moults into an adult stage in its final host, and the complete development of *R. acus* in the definitive host from acquiring infection until egg production takes approximately two months at a water temperature of 15 °C (Moravec, 1994). As a result, 28 days prior to Se-Met exposure, rainbow trout from Se+ parasites groups were fed 1-5 lake whitefish (*Coregonus clupeaformis*) intestine pieces containing cysts with larval (L3) *R. acus* to produce infection levels of 25-30 parasites/fish (Pietrock and Hursky, 2011). This infection method has been verified previously in rainbow trout during a pilot study, obtaining 75% infection rate. Throughout the experiment, trout were fed approximately 1.5% bodyweight of control or Se-Met laced feed 6 days per week, split between 2 daily feedings to ensure complete consumption of the food.

For preparation of food containing Se, a Se stock solution of 1 g/L nominal concentration was prepared by dissolving 250 mg seleno-L-methionine (Sigma-Aldrich) in 100 ml of nanopure water. A 15 ml aliquot of this stock solution was diluted in 375 ml of nanopure water and the resulting solution was then mixed in with 1 kg of crushed trout pellet. The resulting "paste" was processed in a noodle maker to form spaghetti-like pieces which were initially frozen at -80°C and then broken into small pellets, approximately 5 mm^3 in size.

On day 0, 5 fish were sampled randomly from 4 tanks to determine pre-exposure health parameters, muscle Se concentration and antioxidant activity in rainbow trout. Subsequent samples were taken weekly (day 7,14,21,28,35,42,49) from control and the Se group (n=6 fish each) and 8 times (day 7,14,21,28,35,42,49,70) during 70 days from Se+ parasites group (n=16 fish, as trout from two tanks were combined together). On day 56, an additional 5 fish from control and Se groups were sampled. Sampling was performed by carefully netting individual trout and immediately euthanizing them with 150 mg/L MS-222, which induced euthanasia within 2 min. Fish mass (g) and length (cm) were recorded for calculation of condition factor (K); livers were weighed for calculation of the hepatosomatic index (HSI). Head kidney and approximately 0.2 g of liver tissue were excised and washed with a phosphate buffered saline (PBS) solution, pH 7.4, to remove red blood cells and clots. Tissues were homogenized on ice in 1000 µl of 50 mM potassium phosphate, pH 7.0 containing 1mM EDTA and centrifuged at 15,000 g for 15 min at 4°C. Supernatant was then removed and stored at −80°C for further enzyme activity and protein analysis. Muscle tissue (approximately 1.0 g, without skin and bones) was excised from the area posterior to the dorsal fin and frozen at −20°C until later determination of GE levels and Se analysis. Intestines of trout exposed to selenium and parasites were dissected and examined for grossly pathological changes and parasitic infection. Parasites were counted, removed from the fish intestine and rinsed with 0.68% saline solution. Parasite samples were stored in 0.68% saline solution and frozen until further trace element analysis; some *R. acus* samples were transferred into 4% formalin for morphological identification. Nematodes were identified using the key of Moravec (1994).

2.2.4 *Selenium analyses*

To determine the concentration of Se in control and spiked food, samples of each diet were lyophilized and homogenized by use of a mortar and pestle. An aliquot of 100 mg of homogenized food was cold-digested in Teflon vials by use of 5 ml of ultra-pure nitric acid (HNO_3) and 1.5 ml hydrogen peroxide (H_2O_2). Additionally, 30 *R. acus* cysts from intestines of 5 whitefish were dissected out to determine Se concentration of the larval nematodes prior to infection of rainbow trout. For determination of Se concentration in rainbow trout muscle, samples from each fish were thawed and oven dried for 24 h. A 0.10% of total body weight (DW) was calculated for each fish and the corresponding amount was taken from individual samples and ground together for each group/time point. Three 0.1 g subsamples were taken for each group/time point and digested in 6.0 ml of 69 % HNO_3 and 1.6 ml of 30 % H_2O_2. Parasite samples were dried in the oven for 24 h, weighed and digested in 6.0 ml of 69 % HNO_3 and 1.6 ml of 30 % H_2O_2. After digestion, all samples (cysts, fish muscle, and parasites) were processed in the same manner: samples were concentrated to approximately 1 ml on a hot plate (<75 ◦C) and reconstituted in 5 ml of 2% ultra-pure nitric acid. All reconstituted samples were filtered with 0.45 µm filters and stored at 4 °C until analysis. Total concentrations of Se were determined by use of inductively coupled plasma mass spectrometry (ICP-MS X Series^{II}, Thermo Electron Corp.) at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK, Canada). Analysis included monitoring ^{80}Se and application of collision cell technology (with a 93:7 mixture of helium and hydrogen). Recovery of Se was determined using certified reference material (TORT-2, lobster hepatopancreas, NRC, Ottawa, ON, Canada).

2.2.5 *Fish health parameters*

As an indication of general body condition, Fulton's condition factor (K) = [weight/length^3 x 100] as well as hepatosomatic index (HSI) = [(liver weight)/(body weight) x 100] were calculated for control group fish and trout exposed to either Se-Met (Se group) or Se-Met and parasites (Se+ parasites group). Mean gross energy (GE) reserves (MJ/kg) were calculated for each group according to Schreckenbach *et al.* (2001), using the formula GE = [0.0253 * dry matter (%) $^{1.6783}$].

2.2.6 *Measurement of glutathione-S-transferase activity*

Liver samples (approximately 200 mg) and head kidney samples (60-80 mg) were taken from each fish (n=6 control fish, n=6 Se-Met exposed fish, and n= 16 for Se-Met and parasites exposed fish, per time point) and rinsed with 1.5 ml of phosphate buffered saline (PBS) solution (pH 7.4) to remove any red blood cells and clots. Tissues were combined together and homogenized on ice in 1.0 ml of phosphate buffer (50mM KH_2PO_4, pH 7.0 containing 1mM EDTA,) with a sonicator (Sonic Dismembrator 100, Fisher Scientific, USA) and centrifuged at 15,000 g (Sorvall Legend RT$^+$ Centrifuge, Thermo Scientific, Germany) for 15 min at 4 °C to remove nuclei and cell debris. The resulting supernatants were collected for each fish and stored at -80°C until further analysis. For analysis, supernatant samples were pooled for each time point for control fish, Se-Met exposed fish, and fish exposed to Se-Met and parasites. Only supernatants from fish actually showing parasitic infection at the time of sampling were pooled for each time point.

The glutathione-S transferase activity was evaluated using the GST Assay Kit from Sigma-Aldrich (USA) utilizing 1-chloro-2,4 dinitrobenzene (CDNB) as substrate (Habig *et al.,* 1974). Reaction mixture contained Dulbecco's Phosphate Buffered Saline (pH 7.5), 200 mM L-

Glutathione Reduced and 100 mM CDNB. The measurements were determined in a microplate spectrophotometer (Specta Max190, Molecular Devices Corporation, USA) at 25 °C during 6 min as the change in absorbance at 340 nm. One unit of GST was defined as μmol CDNB conjugate formed/min/mg protein using molar extinction coefficient of 5.3 mM^{-1} (path length: 0.552 cm).

2.2.7 *Measurement of superoxide dismutase activity*

The SOD activity was measured in pooled supernatants using a SOD kit (Enzo Life Sciences, USA) where relative SOD activity of the experimental sample was determined from percent inhibition of the rate of formation of WST-1 formazan. The reaction mixture consisted of 10X SOD Buffer, WST-1 Reagent, Xanthine Oxidase, and distilled water. The reaction was initiated by adding 25 μL of 1X Xanthine Solution (one part 10X Xanthine Solution to 9 parts 1X SOD Buffer) to all the wells. Absorbance at 450 nm was measured in a microplate spectrophotometer (Specta Max190, Molecular Devices Coorporation, USA) every minute for 10 minutes at room temperature. The activity of SOD was measured as units/mg protein, where one unit of enzyme activity refers to 50% inhibition of the change in absorbance.

2.2.8 *Protein assay*

Total protein was measured according to Bradford (1976) adapted to a microplate spectrophotometer (Specta Max190, Molecular Devices Coorporation, USA) using the Total Protein Assay Kit (Sigma, MO, USA). Human albumin, 0.3 mg/ml, in saline with 0.1% sodium azide was used as standard and absorbance measured at 595 nm.

2.2.9 *Statistical analysis*

All data are presented as mean ± standard error of mean (S.E.M.). There were 16 fish per sampling event in the Se+ parasites group for health parameter and Se analysis. However, GST and SOD activities were measured only in trout that actually exhibited adult parasites upon dissection (n=1-5 fish per sampling event). Significant differences among the treatment groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test, or by Games Howell post hoc test if homogeneity of variance was not assumed (SPSS, version 18 for Windows). The assumptions of ANOVA (normality of distribution and homogeneity of variances) were verified using the Shapiro-Wilk and Levene's tests, respectively. Data that did not meet the assumptions were log 10 transformed. Significant differences in cases where only two groups were present were analyzed by student's t-test, and homogeneity of variance was tested by Levene's test. Length, weight, condition factor and HSI were analyzed using nonparametric Kruskal- Wallis test followed by Bonferroni-corrected pairwise Mann- Whitney U-test (as the data did not meet normality and homogeneity of variance requirements for ANOVA). A $p \leq 0.05$ was considered significant.

2.3 Results

2.3.1 *Parasite infection*

Overall 128 rainbow trout were fed whitefish intestine fragments each containing several parasite cysts. In total, 23 out of 128 fish from Se+ parasites group were infected with *R. acus,* resulting in overall prevalence of 18% (Table 2.1). Mean intensity (number of parasites/ number of infected fish) was 10.35 parasites/infected fish. Dissections revealed no grossly visible pathological changes to fish intestines.

Table 2.1 Parasite burden of rainbow trout infected with third-stage larvae of *Raphidascaris acus* 28 days prior to exposure to dietary Se-Met.

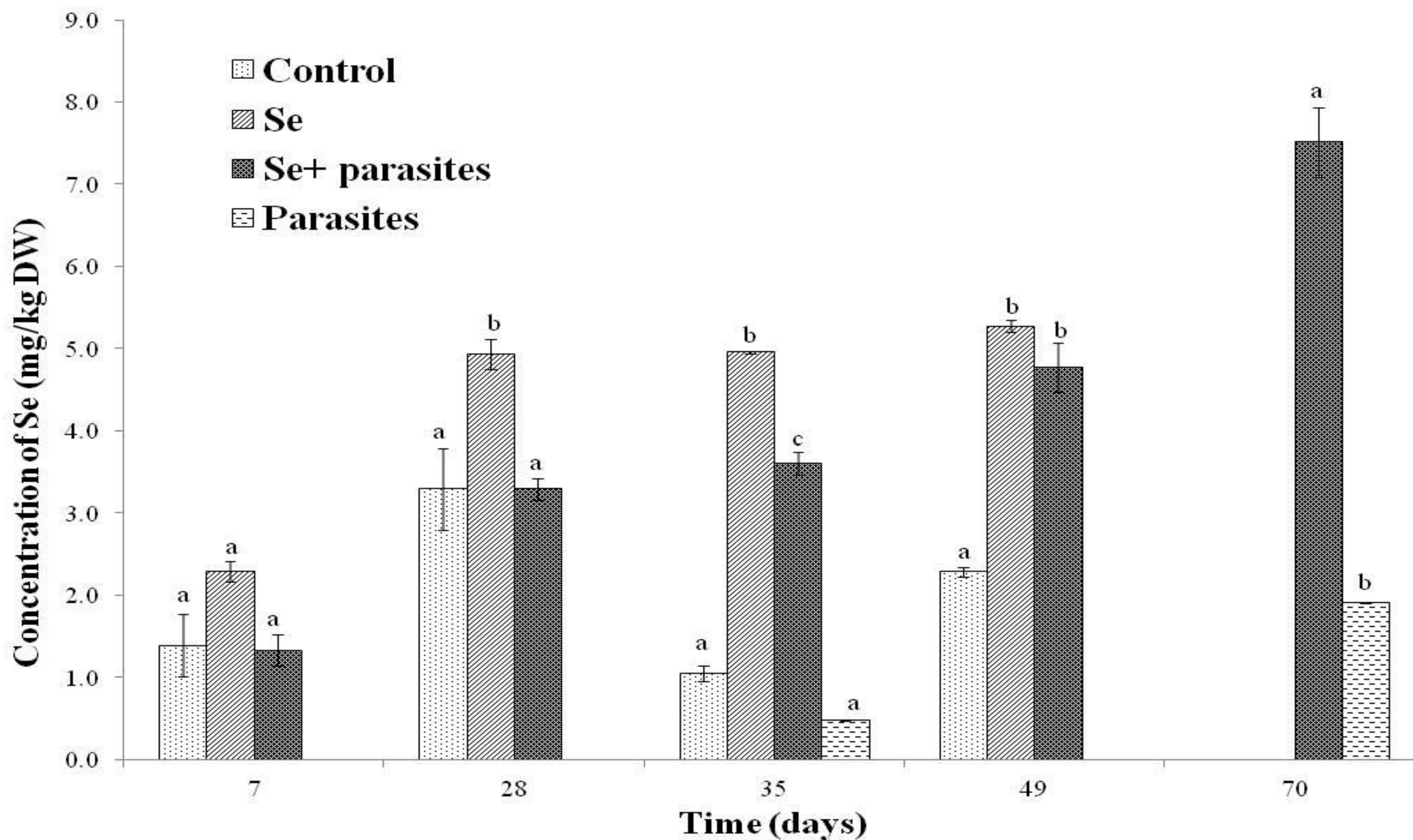
Time (days)*	7	14	21	28	35	42	70
Prevalence (%)	31.25	12.50	6.25	18.75	25.00	18.75	31.25
Mean Intensity	5.80	28.50	1.00	3.33	13.00	4.33	15.20

*post exposure to dietary Se-Met. No parasites were found on day 49.

2.3.2 *Selenium analysis*

The concentration of Se in *R.acus* cysts prior to infection of rainbow trout was 1.263 mg Se/kg DW. Total Se concentration in non-spiked (control) food was 1.10 mg Se/kg wet weight (WW). Measured Se concentrations in the Se spiked food (4.54 mg Se/kg WW) was significantly greater than the control diet ($p < 0.05$). Mean Se concentration (mg Se/kg DW) in the rainbow trout muscle and parasite tissues are presented in Figure 2.1. Concentrations of Se in fish muscle of control fish ranged between 1.05-3.29 mg Se/kg DW. For the first 7 days of the exposure there was no significant difference in Se concentrations between contol, Se and Se+ parasites groups. However, after 28 days, concentrations of Se were significantly lower in control group compared to Se-Met exposed trout for the duration of the experiment ($p <0.05$). Selenium concentration in muscle tissue of fish exposed to Se-Met alone appeared to reach a plateau at around 5 mg Se/kg DW after 28 days of exposure, while a continuous increase in Se concentration with time ($p< 0.05$) was recorded in fish exposed to Se-Met and parasites. Selenium concentration in muscle of infected fish was significantly lower than in Se group between days 28 and 35; however, on day 49, Se concentrations in fish from both groups were similar. Parasite Se concentration was below detection limits during first 7 days of exposure; however, it continued to increase with time (0.025 mg Se/kg DW on day 14, 0.047 mg Se/kg DW on day 35 and 1.91 mg Se/kg DW on day 70, respectively).

Figure 2.1 Mean Se concentration (mg/kg DW) in the rainbow trout muscle and *Raphidascaris acus* tissues. Fish were fed 1.5% bodyweight/day of control or Se-Met spiked food (4.54 mg Se/kg WW) for 56 days (control and Se group) and 70 days (Se+ parasites group), respectively. Data are mean ± S.E.M. of n= 5-6 (control and Se group) and n=16 (Se+ parasites group) samples. Groups with different letters are significantly different within sampling date ($p<0.05$) using one-way ANOVA (Tukey's HSD and Games Howel [day 35] post hoc tests).



2.3.3 *Fish health parameters*

There were no mortalities in either the control group, Se-Met exposed group, or fish exposed to both Se-Met and parasites for the duration of the experiment. No significant difference in weight between groups was found at any time point, and body length of trout exposed to Se-Met was only significantly different from control fish on day 7 and from infected fish on days 49 and 56-70 respectively (Table 2.2). Furthermore, no significant difference was found in condition factor between groups (except day 35), and changes in K over time did not reveal any significant trend (Table 2.3). With the exception of infected trout on day 28, there were no significant differences in hepatosomatic index between the groups at all time points (Table 2.3). Gross energy was significant different between control fish and parasitized trout exposed to Se-Met (Figure 2.2). Trout exposed to both Se-Met and parasites had significantly lower GE values than did control fish. There also appeared to be a non-significant trend showing that fish exposed to parasites and Se-Met have lower GE values than fish exposed to Se-Met alone.

Table 2.2 Morphometric data of rainbow trout exposed to either Se-Met (Se group) or Se-Met and parasites (Se+ parasites group). Data are mean ± S.E.M. of n= 5-6 (control, Se group) and n=16 (Se+ parasites group) respectively. Significance between groups was analyzed using Kruskal-Wallis tests followed by Bonferroni-corrected pairwise Mann-Whitney U-test.

	Time (days)	**0**	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56 - 70**
Weight (g)	Control	94.6 ± 2.4	116.0 ± 12.19	133.75 ± 9.96	143.42 ± 14.71	164.75 ± 18.14	153.08 ± 10.76	168.25 ± 19.38	163.75 ± 9.13	169.00 ± 21.37
	Se	94.6 ± 2.4	107.00 ± 17.75	104.67 ± 7.56	109.00 ± 12.77	141.50 ± 8.51	99.93 ± 25.17	94.08 ± 18.59	113.67 ± 17.87	87.90 ± 16.44
	Se+ parasites	94.6 ± 2.4	145.75 ± 9.70	138.28 ± 9.68	142.25 ± 8.69	162.47 ± 8.91	161.25 ± 8.80	154.16 ± 11.65	183.22 ± 18.29	159.78 ± 13.41
Length (cm)	Control	19.6 ± 0.43	20.60 ± 0.00	22.00 ± 0.52	22.67 ± 1.08	20.67 ± 0.71	23.08 ± 0.54	22.25 ± 0.63	23.08 ± 0.58	23.17 ± 1.39
	Se	19.6 ± 0.43	22.5 ± 0.00 [a]	20.58 ± 0.42	20.67 ± 0.71	22.20 ± 0.48	19.50 ± 1.74	18.92 ± 1.22	20.83 ± 0.75 [b]	19.00 ± 1.10 [b]
	Se+ parasites	19.6 ± 0.43	22.69 ± 0.47	21.94 ± 0.56	22.59 ± 0.78	23.28 ± 0.46	23.25 ± 0.44	22.25 ± 0.54	23.66 ± 0.63	22.84 ± 0.70

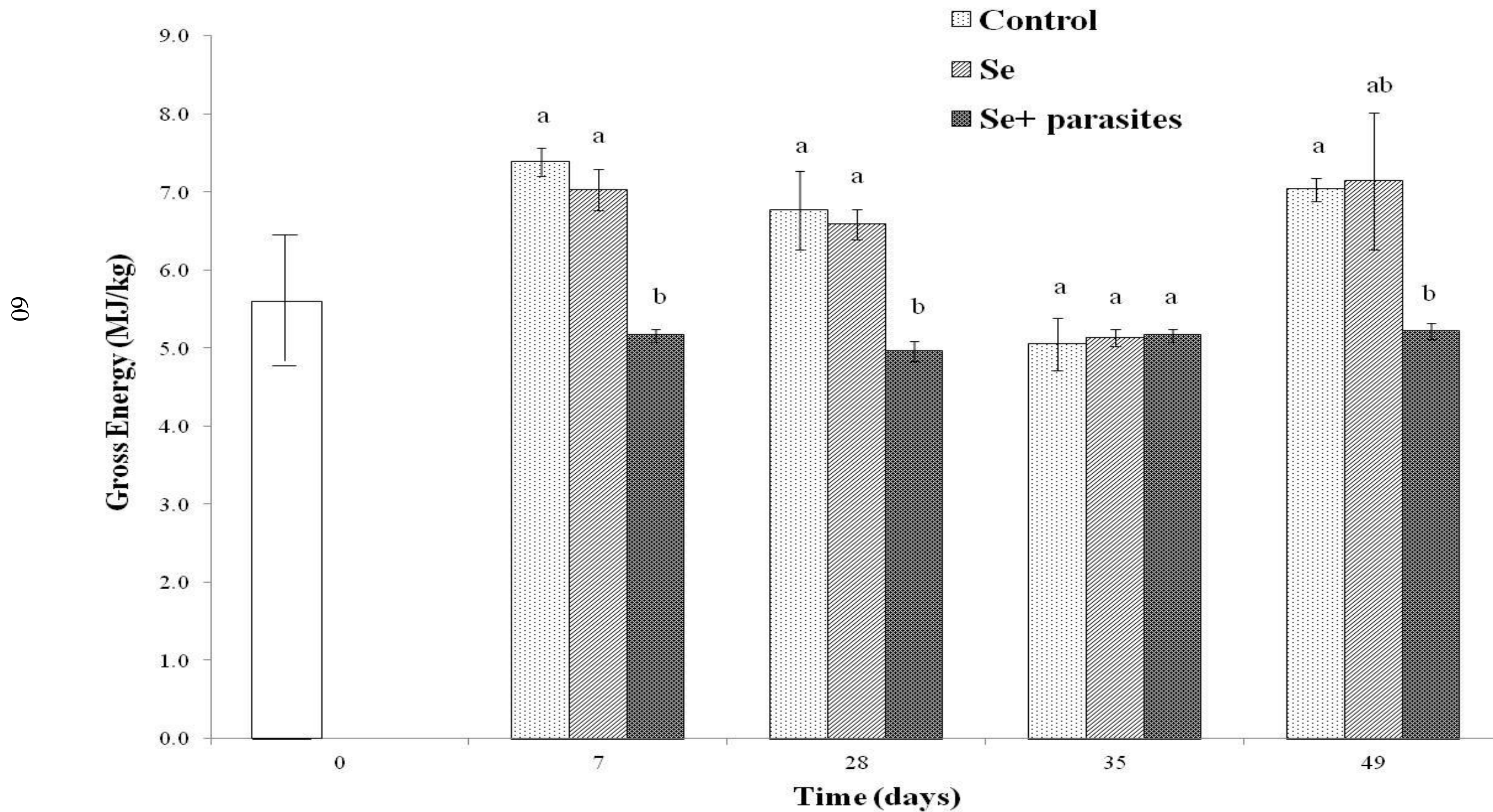
[a] - represents significant difference from control group within sampling date ($p < 0.05$); [b]- represents significant difference from Se+ parasites group within sampling date ($p < 0.05$).

Table 2.3 Fulton's Condition Factor [(weight/length^3)*100] and Hepatosomatic Index [(liver weight)/(body weight)*100] of rainbow trout exposed to either Se-Met (Se group) or Se-Met and parasites (Se+ parasites group). Data are mean ± S.E.M. of n= 5-6 (control, Se group) and n=16 (Se+ parasites group). Significance between groups was analyzed using Kruskal- Wallis tests followed by Bonferroni-corrected pairwise Mann-Whitney U-test.

	Condition factor (K)			Hepatosomatic Index (HSI)		
Time (days)	Control	Se	Se+ parasites	Control	Se	Se+ parasites
0	1.27 ± 0.08	1.27 ± 0.08	1.27 ± 0.08	0.93 ± 0.03	0.93 ± 0.03	0.93 ± 0.03
7	1.02 ± 0.11	1.22 ± 0.20	1.22 ± 0.03	1.23 ± 0.11	1.32 ± 0.09	1.26 ± 0.06
14	1.25 ± 0.03	1.19 ± 0.04	1.29 ± 0.03	1.50 ± 0.10	1.29 ± 0.09	1.65 ± 0.08
21	1.23 ± 0.08	1.20 ± 0.04	1.32 ± 0.15	1.42 ± 0.06	1.70 ± 0.26	1.87 ± 0.11
28	1.44 ± 0.08	1.29 ± 0.05	1.27 ± 0.02	1.28 ± 0.15	1.25 ± 0.10	1.77 ± 0.07[ab]
35	1.24 ± 0.02	1.17 ± 0.06	1.38 ± 0.06[ab]	1.61 ± 0.09	1.73 ± 0.33	1.89 ± 0.14
42	1.53 ± 0.18	1.31 ± 0.07	1.33 ± 0.03	1.51 ± 0.20	1.70 ± 0.09	1.77 ± 0.08
49	1.33 ± 0.05	1.21 ± 0.06	1.22 ± 0.03	1.68 ± 0.12	1.62 ± 0.09	1.43 ± 0.05
56	1.35 ± 0.08	1.22 ± 0.04		1.85 ± 0.22	2.17 ± 0.13	
70			1.29 ± 0.03			1.95 ± 0.17

[a] - represents significant difference from control group within sampling date ($p < 0.05$); [b]- represents significant difference from Se group ($p < 0.05$).

Figure 2.2 Mean Gross Energy values of rainbow trout. Data are mean ± S.E.M. of n= 5-6 (control and Se group) and n=16 (Se+ parasites group), respectively. Groups with different letters are significantly different within sampling date ($p<0.05$) using one-way ANOVA (Games Howell post hoc test). Gross energy (MJ/kg) = 0.0253 * dry matter (%) $^{1.6783}$ was calculated according to Schreckenbach *et al*. (2001). White bar on day 0 represents fish (n=5) that were sampled randomly from 4 tanks to determine pre-exposure gross energy.



2.3.4 *Effects of selenium and parasite exposure on enzymatic activity*

A significant difference between GST activity in control and Se group was observed, although activity fluctuated over time (Figure 2.3) with the highest values noted in control group at time 0 (0.33 mol CDNB/min/mg protein). GST activity of fish exposed to both Se-Met and parasites was generally significantly lower than in the fish exposed to Se-Met alone; a non-significant trend between GST activity in control and Se+ parasites group was found, with fish exposed to both stressors tending to have lower GST activity than control fish. Moreover, a significant induction in SOD activity was recorded in trout exposed to Se-Met over 56 days of exposure (Figure 2.4). In contrast, SOD activity of trout exposed to both stressors (Se-Met and parasites) was not significantly different from control levels (except on days 7 and 21), but always significantly lower than in fish exposed to Se-Met only.

Figure 2.3 Glutathione S-transferase activity in rainbow trout liver and head kidney over 70 days of exposure to either Se-Met (Se group) or Se-Met and parasites (Se+ parasites group). One unit of GST activity is defined as µmol CDNB formed/min/mg protein. Rainbow trout (n=16 per sampling day) were experimentally infected with *R. acus* 28 days prior to beginning of Se-Met exposure. Only fish showing parasite infection on sampling day are shown in this figure (Se+ parasites group). Bars represent a mean of triplicates ± S.E.M. of pooled n= 5-6 fish (control and Se group) and n= 1-5 fish (Se+ parasites group), respectively. Groups with different letters are significantly different within sampling date ($p<0.05$) using one-way ANOVA (Tukey's HSD post hoc test).

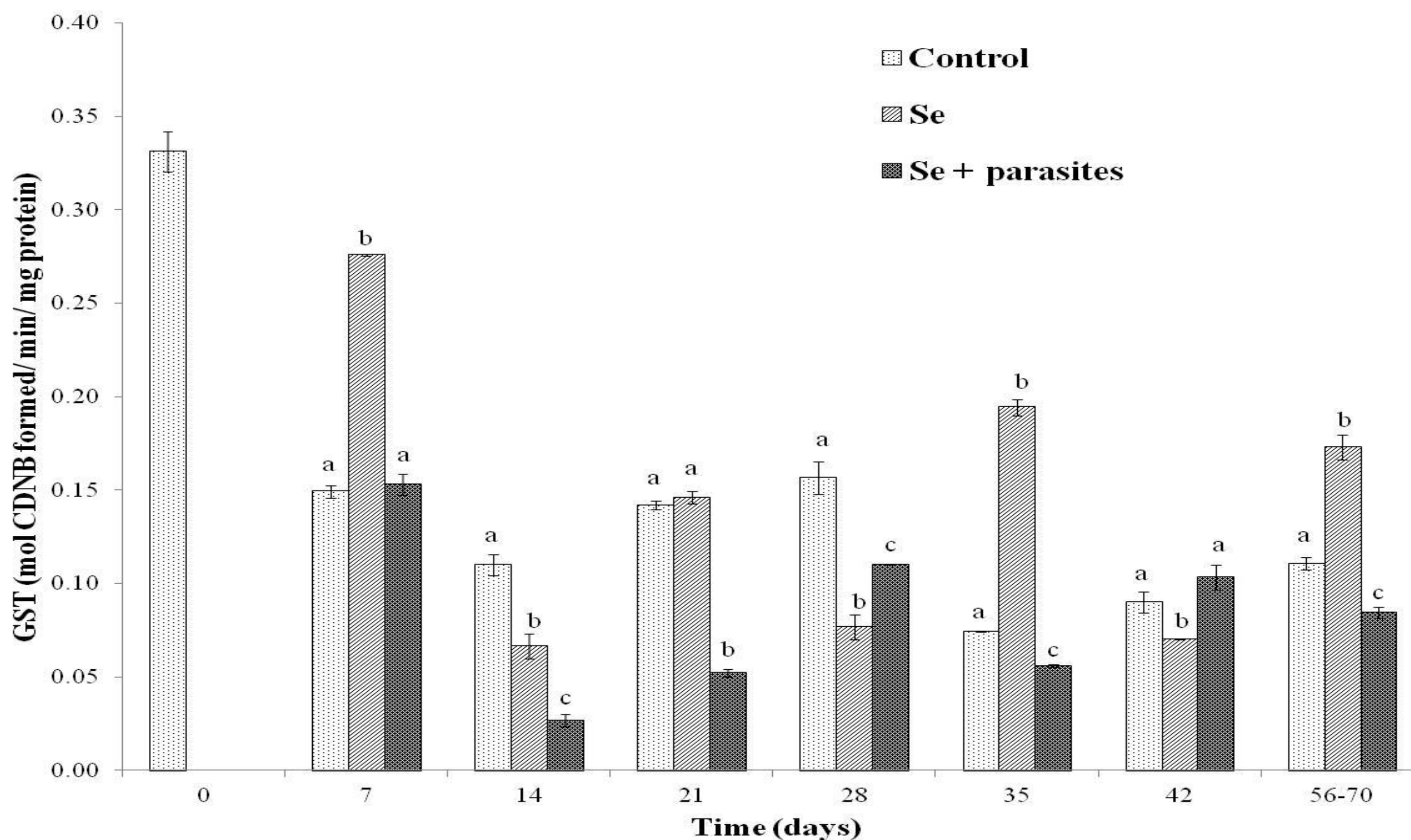
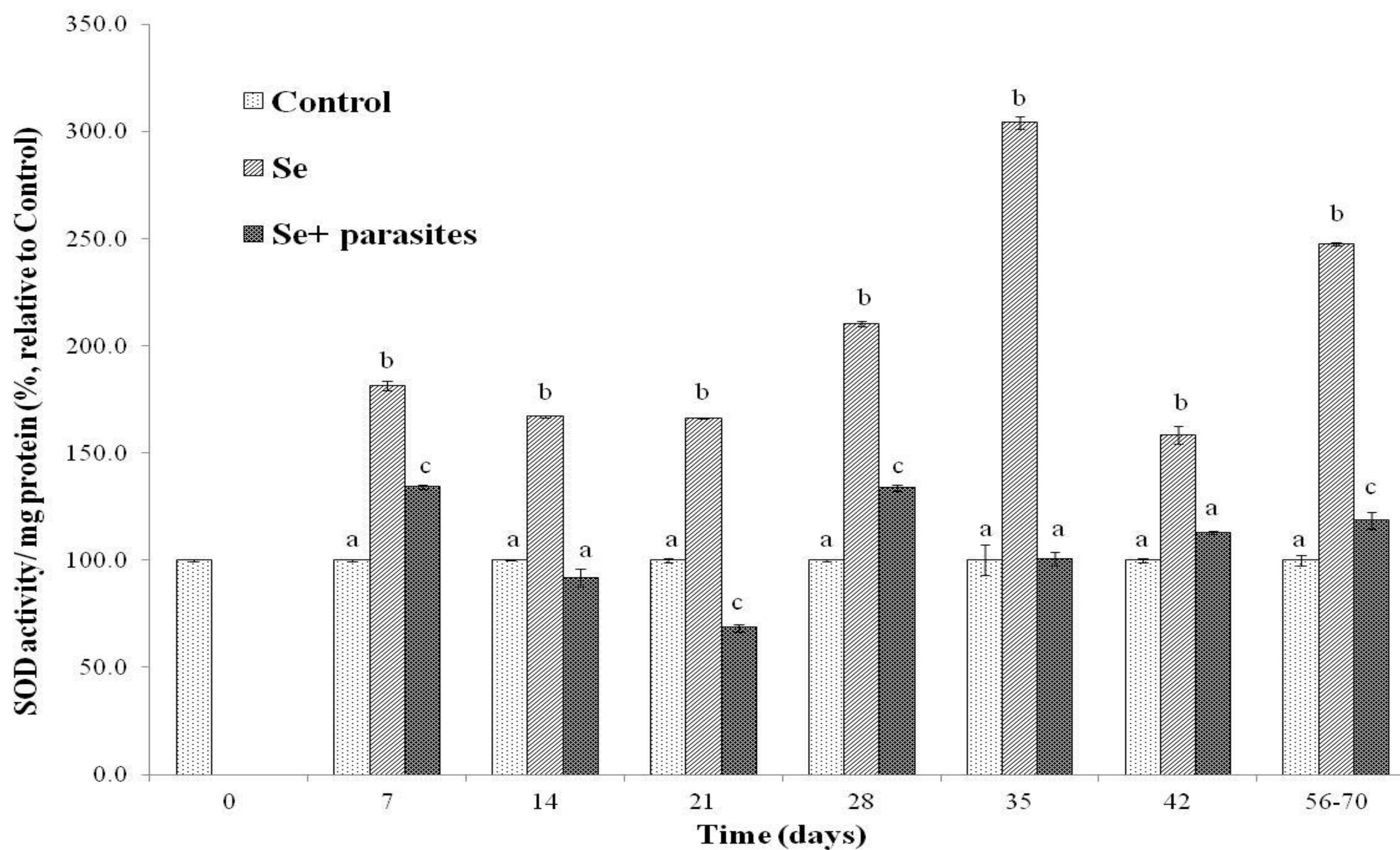


Figure 2.4 Superoxide dismutase activity in rainbow trout liver and head kidney over 56 and 70 days of exposure to either Se-Met (Se group) or Se-Met and parasites (Se+ parasites group), respectively. One unit of enzyme activity refers to 50% inhibition of the changes of absorbance. Rainbow trout (n=16) were experimentally infected with *R.acus* 28 days prior to beginning of Se-Met exposure. Only fish showing parasite infection on sampling day are shown in this figure (Se+ parasites group). Bars represent a mean ± S.E.M. of pooled n= 5-6 fish (control and Se group) and n= 1-5 fish (Se+ parasites group), respectively. Groups with different letters are significantly different within sampling date ($p<0.05$) using one-way ANOVA (Tukey's HSD and Games Howel [day 14] post hoc tests, respectively).



2.4 Discussion

2.4.1 *Selenium uptake*

In the current study rainbow trout were subchronically exposed to environmentally relevant concentrations of Se-Met, and similar concentrations of Se have been reported in prey organisms, such as fish and invertebrates collected from Se affected sites (Lemly, 1997c; Fan *et al.,* 2002; Hamilton, 2004; Muscatello *et al.,* 2006). Since Se is more efficiently assimilated than other metals, accumulation in biota occurs in a concentration-dependent manner from the diet (Baines and Fisher, 2002; Campbell *et al.,* 2005). The muscle tissue concentration of Se in rainbow trout infected with *R. acus* and fed Se-Met spiked diet for 70 days (7.5 mg/kg) was similar to the tissue-based Se criterion to protect aquatic life (7.9 mg/kg DW) proposed by the United States Environmental Protection Agency (USEPA, 2004). Elevated concentrations of whole body and /or tissue Se in adult female fish were reported to cause developmental toxicities and mortalities in offspring (Woock *et al.,* 1987; Muscatello *et al.,* 2006). Skin lesions, inflamed gill filament lamellae, and liver and kidney necrosis were shown to occur when Se concentration in fish exceeded 10 μg/g DW (Lemly, 1993a, 1997c, 2002a; Lohner *et al.,* 2001). Furthermore, concentrations of dietary Se ranging from 9 to 13 μg/g DW have been shown to cause mortality in rainbow trout (Hilton *et al.,* 1980). Salmonids are very sensitive to selenium contamination and exhibit toxic signs even when tissue residues are low (Lemly, 2002b). In the current study, no mortality or macroscopic pathology was noted in rainbow trout exposed to Se-Met alone and fish infected with nematodes and exposed to Se-Met. However, muscle Se concentration in infected trout on day 70 suggests that parasitized fish exposed to Se-Met are at the greater risk for developing chronic toxicity than uninfected fish.

A continuous increase in Se concentration in trout muscle was recorded in fish infected with *R. acus* and exposed to Se-Met, whereas concentrations of Se increased for the first 28 days but then appeared to reach a plateau in uninfected fish exposed to Se-Met alone. The plateau in Se accumulation curve may represent the change from concentration-dependent acute toxicity to subchronic toxicity independent of concentration. Distribution and incorporation of Se into various tissues depends on the presence of selenoproteins, which can become oversaturated with the substrate over time. As a result, a plateau in accumulation was reached and toxicity of Se to the trout was no longer dependant on the concentration of Se-Met in the diet. In contrast, the continuous increase in Se accumulation by infected fish could be due to increased susceptibility of rainbow trout to Se-Met exposure due to infection with parasites. Chronic exposure to contaminants in the presence of parasitic infection can weaken the immune system of the host, as energy resources are spent fighting off the effects of infection rather than dealing with the toxicant (indirect response). This concurs with other studies demonstrating that parasitism can increase the susceptibility of fish hosts to a variety of toxicants, irrespective of whether the fish is a definitive host or an intermediate host (Boyce and Yamada, 1977; Pascoe and Cram, 1977; Sakanari *et al.,* 1984).

To our knowledge this is the first study investigating Se accumulation in fish experimentally infected with nematode parasites. In general, studies have shown that nematodes accumulate low concentrations of pollutants in comparison to their hosts. The concentration of metals (Fe, Cd, Cu, Mn, and Ni) was found to be generally higher in anisakid larvae (Nematoda) than in tissues of European seabass (*Dicentrarchus labrax*) infected with this parasite (Morsy *et al.,* 2012). In contrast, the nematode *Anguillicola crassus*, which parasitizes the swimbladder of the eel (*Anguilla anguilla*), was found to accumulate virtually no lead (Sures *et al*. 1994c). Our

findings demonstrate that although *R. acus* adult nematodes were able to accumulate Se in their tissues, the accumulation capacity was low, as the whole body levels of Se in *R. acus* were significantly lower than corresponding Se concentrations in the host.

However, not all parasite species have similar accumulation capacities. Riggs *et al*. (1987) described elevated Se concentrations in *Bothriocephalus acheilognathi* (Cestoda) in comparison with the tissues of two of its final host species, while Sures *et al*. (1994a, b, c) and Sures and Taraschewski (1995) reported significantly higher Pb and Cd concentrations in adults of three acanthocephalan species when compared with the tissues of their freshwater fish hosts. The contrasting metal-accumulation capacity of nematodes and acanthocephalans and/or cestodes may be reflective of the differences in their modes of nutrition (Sures *et al.,* 1997b). Specifically, nematodes take up nutrients primarily through the mouth and intestine, while their body is enclosed in a cuticle which acts as the first line of defence against pathogens and toxicants (Bush *et al.,* 2002). In contrast, neither cestodes nor acanthocephalans possess a cuticle, but a tegument which absorbs all the nutrients, including metal ions, from the contents of the host's intestine (Bush *et al.,* 2002). Thus it is possible that increased metal accumulation capacity of cestodes and acanthocephalans is related to the lack of an impermeable cuticle to protect from exposure to the toxicants, and an increased area of body surface area that is directly exposed to the contaminants and other ions. Despite the fact that adult *R. acus* bioaccumulate less Se than their hosts, and may therefore not be very suitable as bioindicators of the environmental Se pollution, further investigations are necessary to establish accumulation capacity of molting larvae (L3-L4) as well as adults of this species to other metals/metalloids and/or in other final hosts.

2.4.2 *Effects of stressors on fish health*

In contrast to other studies reporting changes in total body weight and length in response to Se exposure (Bennett and Janz, 2007; Thomas and Janz, 2011; Driedger *et al.,* 2009; Wiseman *et al.,* 2011), we found no significant differences in body weight and length between either parasitized or healthy trout exposed to dietary Se-Met and control fish. This result is similar to the chronic exposure study by Hardy *et al*. (2009) which showed no effect on growth in cutthroat trout fed 11.2 mg/g body weight Se-Met over a 2.5 year period. Furthermore, in the current study the condition factor and HSI values were not altered by Se-Met or parasite exposure. Similarly, Miller *et al*. (2007) observed no change in K and HSI in trout exposed to waterborne selenite for 30 days. Thomas and Janz (2011) reported no change in condition factor in zebrafish exposed to dietary Se-Met for 90 days. Kelly and Janz (2008) also have found no significant difference in condition factor and HSI between northern pike from contaminated (Key Lake, SK, Canada) and clean lakes (Unknown Lake, SK, Canada). Similarly, no difference in K was recorded in the juvenile spottail shiner collected downstream of the Key Lake uranium mill when compared to fish from a reference lake (Goertzen *et al.,* 2012). In contrast, other studies have reported a decrease in condition factor and HSI: yellow perch from lakes contaminated with metals had lower HSI than those from clean lakes (Girard *et al.,* 1998; Laflamme *et al.,* 2000) and bluegill experimentally exposed to waterborne Se had a decreased condition factor compared to control fish (Cleveland *et al.,* 1993). Lower HSI was also recorded in the spottail shiner downstream from uranium mining compared to fish from a clean lake (Goertzen *et al.,* 2012). It might be expected that K and HSI will decrease in response to exposure to a contaminant as the fish use their energy for detoxification purposes, rather than for growth and to build up energy reserves; however, no differences in K and HSI values between control and Se-Met exposed fish

were observed in the current study. This suggests that exposure to Se at the concentration of 4.54 mg/kg WW does not impact these biomarkers of body growth and energy accumulation in liver in rainbow trout, and further correlates with the GE measurement which showed no significant difference between GE stores in control and Se-Met exposed fish. Gross energy levels were used in addition to K and HSI analysis as a measure of tertiary stress response at the whole organism level. We found a difference between control fish and parasitized trout exposed to Se-Met, as trout exposed to combined stressors had significantly lower GE values as compared to control fish. Moreover, a non-significant trend showing that fish exposed to parasites and Se-Met had lower GE values than fish exposed to Se-Met alone was observed. Parasitic infection has been previously shown to decrease energy stores of various fish species (Bakker and Mundwiler, 1999; Neff and Cargnelli, 2004; Kelly and Janz, 2008). Kelly and Janz (2008) have reported intestinal parasite abundance to be negatively correlated with triglycerides in both muscle and liver, and intestinal parasite biomass to be negatively correlated with muscle glycogen levels in northern pike downstream of a uranium mill. Thus depleted energy stores in parasitized rainbow trout exposed to dietary Se-Met (as measured by GE levels) reported in this study, suggest that Se-Met exposure combined with parasitic infection can generate higher energetic demands in the host than Se-Met exposure alone. However, given the experimental design, it is also possible that decreased GE stores in Se+ parasites group were impacted solely by parasitic infection, irrespective of Se-Met exposure.

2.4.3 *Oxidative stress response*

Selenium pro-oxidant activity arises from ability to oxidize thiols, and glutathione appears to be particularly amenable to form complexes with certain forms of selenium (Spallholz, 1994; Palace *et al.,* 2004). GST is an important family of multifunctional intracellular

enzymes and has been commonly used as a biomarker for assessing exposure to different groups of pollutants (Frank *et al.,* 2011). In addition to the reduction of organic hydroperoxides, GST catalyzes the conjugation of GSH thereby protecting cells against potential toxicity (Habig *et al.,* 1974; Aceto *et al.,* 1994). We observed significantly higher level of GST activity in trout exposed to Se-Met when compared to control fish at day 7 post-exposure. However, after 14 days GST activity fluctuated over time: GST activity of Se-Met exposed fish was significantly higher from control levels on day 7, 35 and 56, but it was lower during day 14, 28 and 42. The initial increase of GST activity could be a result of increase in GSH conjugation in response to selenium exposure; however as levels of GSH were depleted with time via oxidation to GSSG, the activity of GST decreased. Interestingly, GST activity in fish exposed to both Se-Met and parasites was generally significantly lower than in the fish exposed to Se-Met alone. Similarly, a study with laboratory bred and laboratory infected three-spined sticklebacks showed a significant reduction of GST activity in parasitized fish when compared to controls (Frank *et al.,* 2011). Reduced hepatic GST activity has also been reported in two other host-parasite systems: infection with either *Dicrocoelium dendriticum* or *Fasciola hepatica* is known to reduce hepatic GST-activity in their respective final hosts (Galtier *et al*., 1983; Skálová *et al*., 2007).

Unlike GST, SOD activity in rainbow trout liver and head kidney was significantly elevated in fish exposed to Se-Met when compared to control fish and parasitized fish for the entire exposure period. The levels of SOD in Se-Met exposed fish increased with time, reaching the highest level on day 35, after which activity began to slowly decrease. The induction of SOD activity in trout fed Se-Met spiked diet could have occurred as a direct response to Se induced superoxide anion production, as primary role of SOD as an antioxidant is to remove the superoxide anion radical to form oxygen and hydrogen peroxide (Kelly *et al.,* 1998). Misra and

Niyogi (2009) and Misra *et al*. (2012) have also reported induction of SOD activity in rainbow trout hepatocytes by sodium selenite exposure *in vitro*. The slow decline in SOD activity observed in our study after 35 days of exposure suggests the change from concentration-dependent acute toxicity of selenium to subchronic toxicity independent of concentration, as Se concentrations in fish muscle remained at around 5 mg/kg DW between days 28-49. Moreover, the decrease in SOD activity after day 35 could be correlated with lower levels of GST activity and concomitant loss of cellular GSH levels. Since the reduction of Se-Met to CH_3Se^-, which produces $O^{\bullet}{}_2^-$, requires two molecules of GSH (Palace *et al.,* 2004), it is possible that GSH depletion might have contributed to the decrease in $O^{\bullet}{}_2^-$ production, and subsequently reducing SOD activity due to substrate limitation. Similar relationship between GSH and SOD activity levels *in vitro* was observed in the study by Misra and Niyogi (2009). In addition, parasitized trout exposed to Se-Met exhibited significantly lower SOD activity than Se group. This difference in enzyme activity persisted throughout the entire exposure period. Similarly, Neves *et al.* (2000) reported that shrimp (*Palaemonetes argentinus*) infected with isopod *Probopyrus ringueleti* exhibited lower activity of SOD when compared to uninfected individuals. As SOD is directly required for dismutation of $O^{\bullet}{}_2^-$, reduction of its activity (as observed in the present study) suggests a decreased capacity of infected trout to prevent cellular damage produced by ROS is associated with Se-Met exposure.

Limited research is available on effects of parasitic infection on fish response to oxidative damage and antioxidant enzyme production. Due to the large number of parasite-host systems, it is difficult to determine if all parasite species inhabiting freshwater fish are capable of producing comparable host responses following Se exposure. Similar to variations in accumulation potential for metals that is species specific and varies among groups of parasites, it is possible

that reduction in GST and SOD activities observed in the current study is also influenced by the specific parasite-host system. For example, Dautremepuits *et al*. (2002, 2003) observed an increase in GST-activity in carp parasitized by the intestinal cestode *Ptychobothrium* sp. when compared with uninfected fish. Furthermore, changes in enzymatic activity could be related to number of species infecting the host, parasite biomass, and organ of infection. Marcogliese *et al*. (2010) reported the number of *Apophallus brevis*, a digenean metacercaria that causes blackspot in the skin and musculature of fish, to be negatively correlated with the activity of the GR in the gills of yellow perch, while the activity of CAT in head kidney was positively correlated with the number of eyeflukes (*Diplostomum* spp.). In addition, a broad range of enzymes and other molecules secreted by helminths is known to subvert the host immune defence in order to maintain a long-term persistence within the host (Dzik, 2006). Examples include helminths secreting SOD and GST enzymes or presenting them on their body surface to ensure parasite survival by neutralizing the toxicants and ROS acting against them (Knox and Jones, 1992; Dzik, 2006). Although the reduction in GST and SOD activity of parasitized trout in our study suggests that the nematode *R. acus* is capable of inhibiting enzyme production, further research is needed to determine the mechanism. Nematodes can produce homologues of cytokines that have been shown to interfere with the host immune cell signaling pathways (reviewed in Dzik, 2006). Therefore, it is possible that *R. acus* can secrete specific anti-enzymatic agents to prevent its host from dealing with ROS as a mechanism of self-preservation.

2.5 Conclusions

In conclusion, we found that subchronic exposure of rainbow trout to an environmentally relevant concentration of dietary Se-Met leads to an initial increase in muscle Se concentrations

reaching a plateau at 5 mg Se/kg DW, while a continuous increase in muscle Se was observed in trout infected with *R. acus* and then exposed to Se-Met. This suggests that continuous Se accumulation by parasitized fish could be due to increased susceptibility of rainbow trout to Se-Met exposure as a result of infection with parasites. One of the possible mechanisms responsible for increased susceptibility to Se is diversion of energy resources from protecting the organism from a contaminant to challenging the parasitic infection. We also observed depletion in gross energy stores in fish infected with *R. acus* and subsequently exposed to Se-Met, suggesting that parasitic infection can deplete energy reserves of the fish exposed to dietary Se-Met. In Se-Met exposed, uninfected fish, an increase in GST activity was recorded for the initial 7 days of Se-Met exposure, whereas the activities of SOD were induced for the duration of the study. Fish exposed to both parasites and Se-Met had lower levels of antioxidant enzyme when compared to trout exposed to Se-Met alone, suggesting an important role of parasitic infection in decreasing oxidative stress response of the host. As we have shown, parasites can negatively influence expression of general oxidative stress biomarkers, and therefore should be taken into account during ecotoxicological studies. To our knowledge, this is the first study to demonstrate effects of combined Se-Met exposure and parasitic infection on rainbow trout energy stores and oxidative stress response.

3.0 GENERAL DISCUSSION

3.1 Effect of parasitic infection on selenium uptake by rainbow trout

Under natural conditions almost every fish is infected with at least one parasite species (Kennedy *et al*., 1986). However, toxicant exposure studies are usually done without considering parasites, despite repeated demonstration of their effects on the physiological homeostasis of their hosts (Sures *et al.,* 2006; Sures, 2008). Infections by parasites have been shown to both increase (Pascoe and Cram, 1977) and decrease (Sures and Siddall, 1999; Sures *et al.,* 2003) contaminant bioaccumulation in fish species. In the current study, a continuous increase in muscle Se concentration over time was recorded in rainbow trout experimentally infected with *R. acus* and exposed to dietary Se-Met. In contrast, the uptake of Se from diet by trout exposed to Se-Met alone followed a sigmoid curve, reaching a plateau in accumulation after 28 days of exposure. Increasing Se accumulation in parasitized fish compared to uninfected conspecifics, observed in the present study, concurs with other research demonstrating that parasitism can increase the susceptibility of fish hosts to a variety of toxicants (Boyce and Yamada, 1977; Pascoe and Cram, 1977; Sakanari *et al.,* 1984). Similarly, in invertebrates, Guth *et al*. (1977) also observed increased susceptibility of the snail intermediate host, *Lymnaea stagnalis*, to zinc as a result of infection with two trematode species.

Continuous Se accumulation with time in parasitized fish exposed to Se-Met suggests that infection with *R. acus* can increase susceptibility of rainbow trout to selenium exposure. However, the exact mechanism by which *R. acus* affects uptake of Se by its host is unclear. One of the possible explanations is a decreased immune response of the host due to parasitic infection. This could indirectly affect Se detoxification mechanisms of trout, as nematodes secrete a variety of enzymes and other molecules to subvert the host's immune defense in order

to maintain a long-term persistence within the host (Dzik, 2006). As fish are infected with gastrointestinal parasites, the cascade of specialized immune responses are initiated by the host, but with time the immune response decreases as parasites release antigens, antioxidant enzymes, and immunosuppressive factors to aid in evading host defenses (Koski and Scott, 2003). Thus it is possible that at the onset of the current study, trout's immune response was up-regulated to fight off the infection; however, over 28 days prior to Se-Met exposure, the parasites established themselves by continuous suppression of host defenses, and continued to suppress host immune system over the duration of the study. Consequently, energetic demands posed by suppression of parasitic infection as well as minimizing pathology decreased energy available for metabolization and detoxification of Se.

Parasites can affect host's absorption of micro- and macronutrients by damaging the lining of the intestine (Koski and Scott, 2003). As parasites require digestion of host tissues for their own nutrition, they rely on proteinases and oxidative damage for tissue penetration and to supply their nutrients (Koski and Scott, 2003). The destruction of the intestinal absorptive surface area by parasites has been shown to diminish absorption of macronutrients; however, the impact of nematodes on absorption of trace elements, and toxicants is not clear. Given the fact that Se-Met is well absorbed by fish (Bell and Cowey, 1989), it is possible that pathological lesions created by nematodes actually increased Se-Met uptake into the blood-stream and consequently into the muscle.

There is however also evidence that some parasite species can decrease contaminant accumulation of their host. For example, periwinkles (*Littorina littorea*) parasitized with digenean parasites had significantly lower concentrations of iron, copper and nickel than uninfected periwinkles (Evans *et al.,* 2001). When exposed to methylmercury in a laboratory

study, grass shrimp (*Palaemonetes pugio*) infected with the isopod *Probopyrus pandalicola*, accumulated lower concentrations of mercury than their unparasitized counterparts (Bergey *et al.,* 2002). Similarly, mummichogs (*Fundulus heteroclitus*) parasitized with the nematode *Eustrongylides* sp. from a mercury contaminated site accumulated lower concentrations of Hg than unparasitized fish (Bergey *et al.,* 2002). With regard to organic contaminants, Heinonen *et al*. (2000) observed slightly lower bioconcentration factors for 2,4,5-Trichlorophenol and benzo-a-pyrene in trematode-infected clams (*Pisidium amnicum*) versus uninfected counterparts. In summary, the observed differences in parasite-modulated effects of contaminants appear to be dependent not only on host and parasite species, but on the toxicant as well. As some parasites have higher accumulation capacity than their hosts, it is possible that decreased concentrations of metals observed in some of the studies are due to detoxification of the host by the parasite. It is suspected that parasites can decrease considerable contaminant loads by accumulating toxicants to greater degree than their hosts and thus reducing host exposure to toxicants.

Further investigations are necessary to determine if Se uptake in rainbow trout would be altered if fish were infected with parasites that have longer developmental time in the final host. Moreover, other parasite species, such as cestodes and acanthocephalans, should be studied as they accumulate greater concentrations of metals than their respective hosts (Table 3.1). Furthermore, in the current study we focused on adult nematodes; however, parasite larvae can have a different effect, and some have been reported to accumulate greater concentrations of metals than their hosts (Pascual and Abollo, 2003; Khaleghzadeh- Ahangar *et al*., 2011; Morsy *et al*., 2012).

3.2 Selenium accumulation by *Raphidascaris acus*

To our knowledge this is the first study investigating Se accumulation by the nematode *R. acus*. Our findings demonstrate that although adult *R. acus* can accumulate Se in their tissues, the accumulation capacity seems to be low, as whole body concentrations of Se in *R. acus* were significantly lower when compared to the muscle Se concentration in its host. It is important to note that low concentrations of Se measured in the larval parasite and their surrounding cysts (1.263 mg Se/kg DW) before rainbow trout infection indicate that there was no potential for significant transfer of Se from the encysted parasite to the final host, *O. mykiss*. It is conceivable that measured Se in parasite cysts was deposited mainly into the cyst itself, rather than into the larval nematode. Moreover, it seems that some of the Se was lost during moulting, as concentrations of Se in adult nematodes on day 14 were lower (0.025 mg Se/kg DW) than Se concentrations measured in the cysts. As the primary role of the cyst is to protect the larva from external environment, it is possible that the walls of the cyst have the ability to accumulate toxicants in order to prevent any accumulation in the parasite. Furthermore, cyst then can act as toxicant burden release mechanism, as it is shed off during infection of the final host. Similarly, electron microscopic X-ray microanalysis of cestode eggs exposed to 1000 and 10,000 μg/L cadmium revealed Cd accumulation on the surface of the egg, with no penetration into the enclosed coracidium (Khalil *et al*., 2009). This suggests that parasite egg shells are not only able to withstand a heavy metal pollutant insult, but are a possible route of metal elimination by the adult worm, as they are shed during moulting and released into the fecal matter of the host (Khalil *et al*., 2009).

Parasite accumulation of metals and other contaminants depends on a variety of factors, including life cycle stage, developmental time in the host, parasite and host species, and tissues

that are infected (Sures and Taraschewski, 1995). Intestinal parasites, such as adult acanthocephalans and cestodes have been reported to be very sensitive bioaccumulators of metals; however not as much is known about accumulation potential of nematodes and their role as indicators of metal pollution in the aquatic environment (Sures *et al.,* 1999; Sures, 2001, 2003). Examples of studies focusing on parasite bioaccumulation of metals and other contaminants in fish, birds, and mammals are summarized in Table 3.1. The evidence on metal accumulation potential of nematodes is equivocal: nematodes of the freshwater and marine fishes have been shown to accumulate both greater (Tenora *et al*., 1999; Pascual and Abollo, 2003; Tenik-Özan and Kir, 2007; Azmat *et al*., 2008; Genc *et al*., 2008; Dural *et al*., 2010; Khaleghzadeh- Ahangar *et al*., 2011) and lesser (Bergey *et al*., 2002; Palikova and Barus, 2003) concentrations of contaminants than their respective hosts, and accumulation potential depends on specific nematode species. For example, the amount of such metals as Fe, Cd, Cu, Mn, and Ni was found to be generally higher in anisakid juveniles (Nematoda) than in tissues of its host, European seabass (*Dicentrarchus labrax*) (Morsy *et al.,* 2012). In contrast, the nematode *Anguillicola crassus*, parasitizing the swimbladder of the European eel, was found to accumulate virtually no Pb (Sures *et al*., 1994c). It appears that parasitic nematodes have bioconcentration factors greater than one for such metals as As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn, but accumulate lesser amounts of Hg than the host. No observation on accumulation of selenium in nematodes has been previously reported.

Table 3.1 Selected examples of bioaccumulation factors (BF) in parasite species infecting freshwater and marine fish, birds and mammals.

Host	Target organ	Parasite	Parasite Taxon	Contaminant	BF	Study type	Source
FISH							
Pike (*Esox lucius*)	Liver	*Raphidascaris acus* (A)	Nematoda	Fe, Zn	>1	FS	Tenik-Özan and Kir (2007)
Cutlass fish (*Trichiurus lepturus*)	Intestine	*Hysterothylacium sp.* (L)	Nematoda	Cd, Pb,	>1	FS	Khaleghzadeh- Ahangar *et al*. (2011)
Squaretail mullet (*Liza vaigiensis*)	Muscle, intestine	*Echinocephalus* sp. *Ascaris* sp.	Nematoda	As, Cd,Fe, Hg, Pb, Zn	>1	FS	Azmat *et al*. (2008)
European eel (*Anguilla anguilla*)	Swim bladder	*Anguillicola crassus* (A)	Nematoda	Cd, Cr, Ni, Pb	>1	FS	Tenora *et al*. (1999)
				Hg, Pb	<1	FS	Palikova and Barus (2003); Sures *et al*. (1994c)
				Fe	> 1	FS	Genc *et al*. (2008)
European seabass (*Dicentrarchus labrax*)	Intestine, muscle	*Anisakid* sp. (L)	Nematoda	Cd, Cu, Fe, Mn, Ni	>1	FS	Morsy *et al*. (2012)
Red sea bream (*Pagellus erythrius*)	Intestine	*Hysterothylacium eduncum*	Nematoda	Cr, Hg, Pb	>1	FS	Dural *et al*. (2010)
Marine killfish (*Fundulus heteroclitus*)	Intestine	*Eustrongylides* sp. (L)	Nematoda	Hg	<1	FS	Bergey *et al*. (2002)
Blue whiting (*Micromesistius poutassou*)	Intestine, muscle, liver	*Anisakis simplex (L)*	Nematoda	Cd, Cu, Pb, Zn	>1	FS	Pascual and Abollo (2003)
Atlantic horse mackerel (*Trachurus trachurus*)	Intestine, muscle, liver			Cd, Cu, Pb, Zn	>1	FS	Pascual and Abollo (2003)

Lesser flying squid (*Todaropsis eblanae)*	Intestine, muscle, digestive gland		Nematoda	Cd, Cu, Pb, Zn	>1	FS	Pascual and Abollo (2003)
European eel (*Anguilla anguilla*)	Intestine	*Proteocephalus macrocephalus* (A)	Cestoda	Cr, Ni,Pb, Zn	>1	FS	Eira *et al*. (2009)
Tench (*Tinca tinca*)	Intestine	*Ligula intestalis* (P)	Cestoda	Cu, Fe, Mn, Zn	>1	FS	Tenik-Özan and Kir (2005)
	Intestine	*Bothriocephalus scorpii*	Cestoda	Cd	>1	FS	Sures *et al*. (1997b)
	Intestine	*Monobothrium wageneri*	Cestoda	Cd, Ph	>1	FS	Sures *et al*. (1997b, c)
Mosquitofish (*Gambusia affinis*) **Fathead minnows** (*Pimephales promela*) **Largemouth yellowfish** (*Labeobarbus kimberleyensis*)	Intestine	*Bothriocephalus acheilognathi* (P, A)	Cestoda	Se, Be, Li, Hg, Mn, Pb, Se, Tl, U	>1	FS	Riggs *et al*. (1987); Retief *et al*. (2006)
Nase (*Chondrostoma nasus*)	Intestine	*Caryophyllaeus laticeps*	Cestoda	Cd, Pb, Zn	>1	FS	Jirsa *et al*. (2008)
Atlantic salmon (*Salmo salar*)	Intestine	*Eubothrium crassum* (A)	Cestoda	PCB	<1	FS	Persson *et al*. (2007)
European perch (*Perca fluviatilis*)	Liver	*Triaenophorua nodulosus (P)*	Cestoda	Cd, Cu, Ni, Zn	>1	FS	Popiolek et al. (2007)
	Intestine	*Acanthocephalus lucii (A)*	Acanthocephala	Cd, Pb	> 1	FS	Sures et al. (1997a); Jankovska et al. (2011); Sures and Taraschewski (1995)
	Intestine	*Acanthocephalus lucii (A)*	Acanthocephala	Al, Ag, Ba, Ca, Cd, Cr, Cu, Ga, Mg, Ni, Sr, Tl, Zn	>1	FS	Sures et al. (1999)

Black rockcod (*Notothrnia coriiceps*)	Intestine	*Pomphorhynchus laevis* (A)	Acanthocephala	Ag, Cd, Cu, Ni, Pb	>1	FS	Sures and Reimann (2003)
Barbel (*Barbus barbus*)		*Pomphorhynchus laevis* (A)	Acanthocephala	Cd, Pb, Zn	>1	FS	Schludermann *et al*. (2003)
Yellow perch (*Perca flavescens*)	Muscle	*Apophallus brevis* (M)	Trematoda	MeHg	<1	FS	Ryman *et al*. (2008)
Bulltrout (*Myoxocephalus scorpius*)	CNS	*Bucephaloides gracilescens* (M)	Trematoda	Lindane		LS	Ruus *et al*. (2001)
BIRDS							
Cormorant (*Phalacrocorax carbo*)		*Contracaecum rudolphii* (A)	Nematoda	Cd	<1	FS	Barus *et al*. (2001)
(*Phalacrocorax auritus*)		*Contracaecum* spp. (A)	Nematoda	Hg	>1	FS	Robintson *et al*. (2010)
MAMMALS							
Red fox (*Vulpes vulpes*)	Intestine	*Toxascaris leonina* (A)	Nematoda	Cr, Cu, Mn, Ni, Pb, Zn	>1	FS	Jankovska *et al*. (2010)
	Intestine	*Mesocestoides* spp. (A)	Cestoda	Cr, Cu, Mn, Ni, Pb, Zn	>1	FS	Jankovska *et al*. (2010)
Wood mouse (*Apodemus sylvaticus*)		*Gallegoide sarfaai (A)*	Cestoda	Pb	>1	FS	Torres et al. (2004)
House mouse (*Mus domesticus*)		*Rodentolepis mictostoma (A)*	Cestoda	Pb	>1	FS	Torres et al. (2011)
Norwegian rat (*Rattus norvegicus*)		*Hymenolepis diminuta (A)*	Cestoda	Pb	>1	LS	Sures et al. (2002)
Sheep (*Rumex acetosella*)	Intestine	*Avitellina lahorea (A)*	Cestoda	Ca, Co, K, Na, Se, Zn	<1	LS	Vijayalakshmi et al. (2003)
Black rat (*Rattus rattus*)		*Moniliformis moniliformis (A)*	Acanthocephala	Cd	>1	FS	Torres et al. (2011)

Striped dolphin *(Stenella coeruleoalba)*	Liver, kidney	*Anisakis simplex* (A)	Nematoda	Cd, Cu, Pb, Zn	>1	FS	Pascual and Abollo (2003)
Long-finned pilot whale (*Globicephala melas)*	Liver, kidney	*Anisakis simplex* (A)	Nematoda	Cd, Cu, Pb, Zn	>1	FS	Pascual and Abollo (2003)

Abbreviations: A- adult; BF- bioconcentration factor (concentration in parasite/ concentration in the host); CNS- central nervous system; FS- field study; L- larva; LS- laboratory study; M-metacercaria; P- plerocercoid.

Furthermore, the majority of the studies focusing on bioaccumulation of toxicants in nematode species have been done in the field, with no control for concentration of pollutants available for uptake as well as length of exposure. Moreover, little or no information is available on the duration of the parasitic infection, as it is possible that longer development within the host increases duration of exposure, and consequently the concentrations of contaminants in parasite tissues. These studies also provide little information on the population of fish sampled in the field; specifically, if the sampled fish are native to the sampling location, or have travelled from areas where exposure concentrations were much higher or lower than at the sampling point. This is especially important in marine environments where fish hosts travel much longer distances, and therefore, can be exposed to variable concentrations of pollutants. Lastly, it is important to remember that in both freshwater and marine environments, parasites are exposed to a number of stressors and contaminants which can act synergistically to increase the susceptibility of a parasite to pollution.

Unlike nematodes, acanthocephalans and cestodes have been proven to be reliable bioindicators of environmental pollution by accumulating much larger concentrations of metals than their hosts. Sures *et al*. (1994a, b, c; 1997a) and Sures and Taraschewski (1995) reported significantly higher concentrations of Pb and Cd, and a number of other elements (Al, Ag, Ba, Ca, Cd, Cr, Cu, Ga, Mg, Ni, Pb, Sr, Tl, and Zn) in several adult acanthocephalan species as compared with the tissues of their freshwater fish hosts (Sures *et al.,* 1999). Schludermann *et al*. (2003) reported a significantly higher bioaccumulation potential for Cd, Pb, and Zn in the acanthocephalan (*Pomphorhynchus laevis*) compared with that in the cyprinid barbel (*Barbus barbus*). A study of an Antarctic host-parasite system revealed that an acanthocephalan (*Aspersentis megarhynchus*) had higher rates of accumulation for Pb, Cd, Ag, Ni and Cu than its

marine host, black rockcod (*Notothenia coriiceps*) (Sures and Reimann, 2003). The study by Riggs *et al.* (1987) described elevated Se concentrations in the cestode *Bothriocephalus acheilognathi* in comparison with the tissues of two of its final host species. Similarly, Retief *et al*. (2006) observed a higher accumulation potential of cestodes (*B. acheilognathi*) for Li, Be, Mn, Se, Hg, Tl, Pb, and U than of its host, largemouth yellowfish (*Labeobarbus kimberleyensis*) in the Vaal Dam, South Africa. Furthermore, concentrations of Hg and Se higher than that of water and sediment were present in the cestodes due to bioaccumulation (Retief *et al.,* 2006). Tapeworms (*Caryophyllaeus laticeps*) infecting intestines of nase (*Chondrostoma nasus*) had significantly higher concentrations of Cd, Pb, and Zn compared to the fish liver (Jirsa *et al.,* 2008).

The contrast in metal-accumulation capacity between nematodes, acanthocephalans, and/or cestodes may be reflective of the presence of cuticle in nematodes that acts as the first line of defense against pathogens and toxicants (Bush *et al.,* 2002). Lack of the cuticle in acanthocephalans and cestodes can lead to increased metal accumulation as there is no protective layer shielding the parasite from contaminants. Moreover, difference in modes of nutrition can play a significant role in metal accumulation of parasite species: while nematodes take up their nutrients primarily through the mouth, the tegument of both acanthocephalans and cestodes is responsible for absorption of all the nutrients (Bush *et al.,* 2002). As a much larger area of the acanthocephalan/cestode body (relative to nematodes) absorbs nutrients and toxicants, greater concentrations of contaminants can be accumulated. It has also been suggested that some intestinal parasites have the ability to interrupt the hepato–intestinal cycling of metals by the host, hence playing an important role as a metal sink within their definitive fish host (Sures and Siddall, 1999). It has been proposed that organometallic complexes formed in the liver of chub in

response to lead exposure pass down the bile duct into the small intestine where they are taken up by the acanthocephalan (*Pomphorhynchus laevis*) concurrently with bile salts, therefore decreasing the amount of lead available for internal uptake by chub (Sures and Siddall, 1999). Cestodes presumably act in a similar manner leading to detoxification of their fish hosts, as demonstrated by their high accumulation potential for metals (Retief *et al.,* 2006). Nothing is known about mechanism of detoxification, if any, by nematodes.

3.3 Combined effects of *Raphidascaris acus* infection and selenium exposure on fish health

Condition factor and hepatosomatic index are tertiary stress responses that have been widely used as indicators of overall fish health and bioenergetic status (Barton, 2002; Busacker *et al.,* 1990). In terms of environmental pollution, depending on e.g. fish species and contaminant concentration, condition factor and HSI can be diminished (Laflamme *et al.,* 2000; Cleveland *et al.,* 1993). Although it might be expected for K and HSI to decrease in response to parasite and Se-Met exposure, in the current study, no difference in K and HSI values between control and Se-Met exposed fish (infected and non- infected) were observed. This suggests that in studies examining combined effects of parasitism and Se-Met exposure on fish, K and HSI may not be sensitive enough as indicators of the energetic costs, as they may not reveal true energetic demands associated with pollution or parasitism. This is further supported by several other studies showing no effect of Se pollution on condition factor and HSI. For example, Miller *et al*. (2007) observed no change in K and HSI in trout exposed to waterborne selenite for 30 days. Similarly, Kelly and Janz (2008) found no significant difference in condition factor and HSI in northern pike from Se contaminated (Key Lake, SK, Canada) and clean lakes (Unknown Lake, SK, Canada), respectively. Moreover, although parasitic infections have been shown to decrease

K and HSI of the various fish species (Kelly and Janz, 2008; Bakker and Mundwiler, 1999; Neff and Cargnelli, 2004), the effect of parasitism on K and HSI can also be dependent on number of parasites present in the host (intensity). Thus it is possible that no effect on K and HSI observed in the present study can be accredited to low parasite loads (maximum number of parasites in the host was 56 *R. acus*) as well as infection of rainbow trout with only one parasite specie. In addition, the effect of combined stressors on GE levels observed in the current study supports the importance of GE measurement in environmental studies, as it has been proven to be a more sensitive indicator of energetic demands posed by parasitism and Se-Met exposure.

We observed no significant difference between GE stores in control and Se-Met exposed fish, suggesting that at the concentration of 4.54 mg/kg WW, dietary Se-Met has no negative impact on energy stores in juvenile rainbow trout. In contrast, GE levels in parasitized fish exposed to dietary Se-Met were significantly reduced in comparison to GE levels of the control fish. Decreased energy available for storage in muscle in parasitized rainbow trout exposed to dietary Se-Met suggest that when combined with trace element exposure, parasitic infection can pose greater energetic demands on the host than metal exposure alone. However, depletion of GE stores in parasitized fish could also be a sole and direct result of parasitic infection, and not at all effected by Se-Met exposure. Decreased GE in infected trout could be attributed to immune mediated response to parasitic infection, as parasites have a considerable impact on defense mechanisms of fish (Williams and Jones, 1994). Since parasites can cause injuries to fish during the infection, the host organism will initiate an inflammatory response aimed at restricting, reducing and ending the damage (Dzik, 2006), consequently using a significant amount of their energy for fighting off and managing parasitic infection, rather than allocating it for storage.

3.4 Effects of combined stressors on oxidative stress response

Until recently, oxidative damage as a potential mechanism of Se toxicity in fish has received little attention. Palace *et al*. (2004) reported that the metabolism of Se-Met in rainbow trout embryos led to ROS generation, while Orun *et al*. (2005) observed an induction of enzymatic antioxidants (SOD and GPx) at low to intermediate doses followed by a sharp decline at the high dose in trout exposed to waterborne sodium selenite. Similarly, Miller *et al*. (2007) recently reported that the concentration of hepatic GSH decreased in trout exposed acutely to waterborne sodium selenite. In the current study, an increase in GST activity levels was observed in trout exposed to dietary Se-Met when compared to control fish during the first 7 days of exposure. The observed increase of GST activity over first 7 days of the study could have resulted from increased GSH conjugation in response to initial Se exposure. However as levels of GSH decreased with time via oxidation to GSSG, the activity of GST decreased as well (days 14, 28 and 42). Furthermore, parasitized rainbow trout exposed to Se-Met had generally lower GST levels than uninfected fish exposed to Se-Met. Research has shown that parasites may lead to reduction in GST levels in the host. For example, in a study with laboratory-bred and infected three-spined sticklebacks, parasitized fish showed a significant reduction of GST activity compared to noninfected fish (Frank *et al.,* 2011). In addition, reduction in hepatic GST activity has also been reported in final hosts of *Dicrocoelium dendriticum* and *Fasciola hepatica* in response to parasitic infection (Galtier *et al*., 1983; Skálová *et al*., 2007).

A significant increase in SOD activity in rainbow trout liver and head kidney was observed in fish exposed to Se-Met when compared to control group for the duration of the exposure. The levels of SOD in Se-Met exposed fish increased with time, reaching the highest level on day 35, followed by a slow decline over the next 20 days. *In vitro* studies have also

shown an induction in SOD activity in rainbow trout hepatocytes resulting from sodium selenite exposure (Misra and Niyogi, 2009; Misra *et al*., 2012). Induction of SOD activity in trout fed Se-Met spiked diet as reported in the current study implied a direct antioxidant response to Se induced superoxide anion production, whereas the slow decline in SOD activity after 35 days of exposure suggests the change from concentration-dependent acute toxicity of Se to subchronic toxicity independent of concentration. Furthermore, the slow decline in SOD activity after day 35 could be correlated with lower levels of GST activity and concomitant loss of cellular GSH level. As the reduction of Se-Met to CH_3Se^- (producing $O^{\bullet}{}_2^-$) requires two molecules of GSH (Palace *et al.,* 2004), GSH depletion might have contributed to the decrease in $O^{\bullet}{}_2^-$ production, and subsequently reduced SOD activity as a result of substrate limitation. A similar relationship between GSH and SOD activity levels was observed in the study by Misra and Niyogi (2009) *in vitro*. Interestingly, significantly lower levels of SOD activity over 70 days of the exposure in trout exposed to *R. acus* and dietary Se-Met when compared to unparasitized fish exposed to Se-Met were observed in the present study. As SOD is directly required for dismutation of $O^{\bullet}{}_2^-$, the observed reduction of its activity suggests decreased capacity of infected trout to prevent cellular damage produced by ROS due to Se exposure. Similarly, the study by Neves *et al*. (2000) reported lower activity of SOD in shrimp (*Palaemonetes argentinus*) infected with isopod *Probopyrus ringueleti* when compared to uninfected individuals.

Limited research is available on the combined effect of parasitic infection and environmental pollution on fish response to oxidative stress and antioxidant enzyme production. Moreover, due to the large number of parasite-host systems, it is difficult to determine if all parasite species inhabiting freshwater fish are capable of producing the same host response to toxicant exposure as it was observed in this study. For example, Dautremepuits *et al*. (2002,

2003) reported an increase in GST activity in carp infected with the intestinal cestode *Ptychobothrium* sp. and exposed to copper when compared with uninfected fish. Furthermore, Marcogliese *et al*. (2005) found that levels of lipid peroxidation in the livers of yellow perch *Perca flavescens* were higher at a polluted location as compared to a clean location, and that this effect was more pronounced in fish infected with *R. acus* and *Apophallus brevis* at the polluted site. Thus it is possible that the observed reduction in GST and SOD activities may be specific to *R. acus* and rainbow trout. In addition, changes in enzymatic activity could be related to parasite intensity and diversity, as well as parasite mass, and organ of infection. Marcogliese *et al*. (2010) reported the number of *Apophallus brevis* to be negatively correlated with the activity of the GR in the gills of yellow perch, while the activity of CAT in the head kidney was positively correlated with the number of eyeflukes (*Diplostomum* spp.).

Furthermore, a broad range of enzymes and other molecules secreted by helminths is known to subvert the host immune defenses in order to maintain a long-term persistence within the host (Dzik, 2006). Studies have reported that gastrointestinal nematodes of both mammals and fish are capable of producing SOD in order to reduce $O^{\bullet}_2{}^-$ radical formation during immune response of their hosts resulting from infection (Knox and Jones, 1992; Dzik, 2006). The protective mucosal response against gastrointestinal nematodes is the development of an inflammatory-type reaction associated with extensive granulocyte infiltration and peripheral blood eosinophilia (Miller, 1984; Secombes and Chappell, 1996). Both activated phagocytes and eosinophils are capable of sustained release of superoxide anions; consequently parasitic nematodes release SODs to protect themselves against oxidative damage to survive within the host (Knox and Jones, 1992; Dzik, 2006). Effective protection of an invading parasite from host-produced ROS depends on levels of scavenger enzymes in the parasite (Dzik, 2006). Studies on

Nippostrongylus brasiliensis infection in rat showed that the increased production of SOD, CAT and GPx is correlated with persistence in the host (Dzik, 2006). In addition, GSTs have been suggested as the biochemical systems that protect cestodes against the host's immune attack (Brophy and Pritchard, 1992). Other examples include helminths secreting GST, SOD and GPx or presenting them on their body surface to ensure survival by neutralizing the toxicants and ROS acting against them (Knox and Jones, 1992; Dzik, 2006). Although the reduction in GST and SOD activity of parasitized trout in our study suggests that the nematode *R. acus* is capable of inhibiting enzyme production or al least diverting resources away from it, at this point it is difficult to determine by which mechanism *R. acus* affects antioxidant enzyme generation by its host. Given that nematodes can produce homologues of cytokines that have been shown to interfere with the host immune cell signaling pathways (reviewed in Dzik, 2006), it is possible that *R. acus* can secrete specific anti-enzymatic agents to prevent its host from dealing with ROS as a mechanism of self-preservation. In addition, *R. acus* could be secreting its own antioxidant enzymes due to Se induced oxidative damage that can interfere with host's antioxidant response. In summary, the antioxidant stress response of infected hosts under polluted conditions is highly complex as both parasites and toxicants may produce ROS and initiate production of it, respectively, and subsequently lead to generation of antioxidant enzymes by the host to prevent oxidative damage (such as Se).

3.5 Future research

Based on the findings of this thesis, there are a few areas of study that can be expanded on, to provide more information on the effect of parasitic infection on fish health, energy stores, and Se accumulation. It would be beneficial to expand experimental design to include a group

exposed only to parasites. This could offer insights on what effects parasitic infection may have on energy stores, fish health parameters, and oxidative stress biomarkers of fish that are not exposed to any contaminants. Specifically, it would enable one to compare GE values between Se, Se+ parasites, and parasites groups to determine if depletion in gross energy stores seen in this study were due to combined effects of parasitic infection and Se-Met exposure or due to parasitic infection alone.

Another possible enhancement to the design could be the addition of a group exposed to dietary Se-Met for 30 days (sub-chronic exposure) which would then be experimentally infected with larval *R. acus* and fed a regular (non-contaminated) food. Parasite samples can be taken every 7 days post-infection to establish if *R. acus* can accumulate Se from its host. As Se is known to be metabolized in the liver and stored in the muscle, it would be useful to establish if there is an indirect transfer of Se from the liver and muscle into the parasite. This would also provide insights into Se accumulation by metabolically active *R. acus* larvae. To further establish Se accumulation capacity of *R.acus* larvae, fish can be infected with *R. acus* (L3) and exposure to Se-Met can begin 1-2 days post-infection. This design allows for parasite samples to be taken 2-3 days after the infection, and for comparison of Se concentration in larvae and adult nematodes.

Given that *R. acus* has a variety of final hosts, one of the possible future research approaches is to test the effects of Se-Met exposure and parasitic infection on another host, for example northern pike. Establishing effects of Se-Met exposure and *R. acus* infection on oxidative stress biomarkers and energy stores in another host will allow for comparison with results obtained in this study. It would also help in determining if current results were specific to the host-parasite system tested, or if *R. acus* can produce similar effects in a different host.

There is a number of antioxidant enzymes involved in oxidative stress response. The focus of this study was on two biomarkers of oxidative stress in fish, GST and SOD. In order to improve our understanding of effects of parasitic infection and Se-Met exposure on oxidative stress response of rainbow trout, levels of glutathione peroxidase, catalase, and glutathione reductase, should also be tested. Moreover, levels of antioxidant enzymes in the parasite tissues can be tested to establish if secretion of such enzymes is a mechanism responsible for decreased oxidative stress response in the host exposed Se-Met.

3.6 Overall conclusions

This is the first study to demonstrate effects of combined Se-Met exposure and parasitic infection on rainbow trout energy stores and oxidative stress responses. We found that subchronic exposure of rainbow trout to an environmentally relevant concentration of dietary Se-Met led to an initial increase in muscle Se concentrations reaching a plateau at 5 mg Se/kg DW, while a continuous increase in muscle Se was observed in trout infected with *R. acus* and exposed to Se-Met. This suggests that continuous Se accumulation by parasitized fish could be due to infection with parasites. We also observed depletion in GE stores in fish infected with *R. acus* and subsequently exposed to Se-Met; whereas no changes in K and HSI were observed between treatments. This finding may indicate that measurement of GE may be a more sensitive indicator of fish condition than determination of K and HSI. In addition, an increase in GST activity was recorded for the initial 7 days of Se-Met exposure, whereas activities of SOD were induced for the duration of the study. Fish exposed to both parasites and Se-Met showed lower levels of antioxidant enzymes when compared to trout exposed to Se-Met alone, suggesting an important role of parasitic infection in inhibiting oxidative stress response of the host. Given that

under natural conditions almost every fish is infected with at least one parasite species (Price, 1980), this will certainly also affect the host's physiological homeostasis and thus might change biomarker levels (Sures, 2008). Parasites can affect expression of biomarkers of pollution, as evident by decreased GST and SOD production by rainbow trout in this study, and therefore should be taken into account during bioaccumulation studies.

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