EFFECTS OF DIETARY AND IN OVO SELENOMETHIONINE EXPOSURE IN ZEBRAFISH (*DANIO RERIO*)

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctorate of Philosophy

In the Toxicology Graduate Program,

University of Saskatchewan

Saskatoon, SK, Canada

By

Jith Kallarakavumkal Thomas

© Copyright Jith Kallarakavumkal Thomas, September, 2014 All Rights Reserved

PERMISSION TO USE STATEMENT

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Chair of the Toxicology Graduate Program

Toxicology Centre

University of Saskatchewan

44 Campus Drive

Saskatoon, Saskatchewan, Canada S7N 5B3

ABSTRACT

Selenium (Se) is an essential trace element to most living organisms, however when compared to other ingested essential trace elements Se has the lowest margin of safety between essential and toxic concentrations. Oviparous vertebrates, especially fishes, are highly susceptible to dietary Se toxicity. Greater incidences of deformities and/or mortalities have been observed in F1 generation larval fishes whose parents were exposed to excess dietary Se in the form of selenomethionine (SeMet), however little information is available on effects of chronic dietary SeMet exposure to adult fish and persistent effects of *in ovo* SeMet exposure to F1 generation fish. This thesis investigated effects of chronic dietary exposure of excess Se in the form of SeMet on swimming performance (U_{crit}), oxygen consumption (MO_2), stored energy (triglycerides and glycogen), and the physiological stress response (cortisol production) in adult zebrafish (*Danio rerio*), as well as immediate (incidence of deformities and mortality) and persistent (e.g. changes in U_{crit}, MO_2, bioenergetics, the physiological stress response and reproduction) effects of *in ovo* exposure to SeMet in F1 generation zebrafish. In addition, the study investigated potential underlying mechanisms of SeMet-induced developmental toxicities in early life stages of zebrafish using embryo microinjection.

Two separate dietary SeMet exposure studies in adult zebrafish and two *in ovo* SeMet maternal transfer studies in F1 generation zebrafish were conducted. The first dietary or *in ovo* exposure study explored effects of excess SeMet exposure on adult zebrafish or the entire life cycle of F1 generation zebrafish. The second study investigated mechanisms of observed SeMet-induced effects on adult or F1 generation zebrafish. In the first feeding study, a significant reduction in U_{crit} and greater accumulation of stored energy were observed in the excess dietary SeMet exposed groups when compared to the Se-sufficient dietary control group.

The second feeding study showed a greater metabolic rate, and impaired aerobic energy metabolism and triglyceride homeostasis in adult fish fed excess dietary SeMet, which was associated with a reduction in swimming performance and accumulation of triglycerides. Embryos collected from adult zebrafish in both dietary SeMet exposure studies were used to investigate effects of *in ovo* SeMet exposure on the entire life cycle of F1 generation fish. The first study showed a greater incidence of mortality, an increasing trend for deformities in F1 generation larval zebrafish, and reduced U_{crit} in F1 generation adult fish exposed to excess SeMet via *in ovo* maternal transfer. However, concentrations of stored energy, cortisol and reproduction were unaltered. The second study found that impaired aerobic performance might have been responsible for the reduction in U_{crit} of F1 generation adult zebrafish exposed to excess SeMet. Since there is a high variability in Se deposition among eggs via natural maternal transfer, SeMet embryo microinjection was adopted to mimic maternal transfer and to investigate potential mechanisms of SeMet-induced developmental toxicities in early life stages of zebrafish. Greater gene expression of oxidant-inducible transcription factors and impairment in gene expression of an enzyme involved in methionine catabolism were observed in early life stages of zebrafish exposed to excess SeMet via *in ovo* microinjection.

The research presented in this thesis suggests that environmentally relevant dietary SeMet exposure can alter physiological responses in adult fishes and reduce survivability of F1generation fishes, which could impact fitness and recruitment of wild fishes inhabiting Se-contaminated aquatic ecosystems. In addition, the study suggests that SeMet-induced developmental toxicities in early life stages of fishes might be related to oxidative stress or impaired methylation, or a combination of these mechanisms.

ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor Dr David Janz for providing me with the opportunity to do a PhD degree in his laboratory. His support, advice, and encouragement were crucial to the completion of this thesis. I would like to thank my PhD committee members Dr Barry Blakley, Dr Som Niyogi, Dr Patrick Krone and Dr Murray Drew for their thoughtful comments, which significantly improved the quality of my research and thesis writing. I would like to thank Dr John Giesy, Dr Paul Jones, Dr Steve Wiseman, Dr Lynn Weber, Dr Markus Hecker, Dr Jorgelina Muscatello, Nicole Sylvain and Bryan Sarauer for sharing their expertise and providing insightful comments about my research. Thanks to my external examiner Dr Dennis Lemly for taking time off to review my thesis and come to my thesis defense.

I would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for funding this research and Toxicology Centre, University of Saskatchewan for a graduate student scholarship.

Completion of this degree wasn't possible without constant support from my family, colleagues and friends. I would like to thank all the past and present toxicology graduate students especially Naveen, Amber, Eric, Sarah, Jordan, Meghan, Anita and Landon for making my student life very exciting and rewarding. Brittany, George, Anna, Lowrance, Lucy, Jerry, Rex, Rebu, Greg, Barb, Jordan, Josh and Kris, thank you for your constant love, support, and encouragement. I would like to thank Adriana, Fiona, and Tina for all the administrative help that you have offered me throughout my degree. Finally, I would like to thank my parents for providing me with an education that has got me this far.

TABLE OF CONTENTS

PERMISSION TO USE STATEMENT i
ABSTRACT ii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS v
LIST OF TABLES xi
LIST OF FIGURES xiii
LIST OF ABBREVIATIONS xix
PREFACE xxii
CHAPTER 1 1
GENERAL INTRODUCTION 1
1.1 Introduction 2
1.2 Properties, sources and uses of selenium 2
1.3 Selenium requirement and toxicity 5
1.4 Selenium in the aquatic environment 7
1.5 Selenomethionine toxicity to freshwater fishes 10
1.6 Mechanisms of selenomethionine toxicity 12
1.7 Swimming physiology of fishes 15
1.8 Use of microinjection techniques in aquatic toxicology 18
1.9 Importance of zebrafish as a test organism 19
1.10 Research objectives and hypotheses 20
CHAPTER 2 22
DIETARY SELENOMETHIONINE EXPOSURE IN ADULT ZEBRAFISH ALTERS SWIMMING PERFORMANCE, ENERGETICS AND THE PHYSIOLOGICAL STRESS RESPONSE 22
Preface 22
2.1 Abstract 23
2.2 Introduction 24
2.3 Materials and methods 27
2.3.1 Test compound 27

2.3.2 Test species ... 27
2.3.3 Diet preparation ... 27
2.3.4 Experimental protocol ... 28
2.3.5 Selenium analysis ... 28
2.3.6 Swimming performance and oxygen consumption ... 29
2.3.7 Image analysis ... 30
2.3.8 Cost of transport ... 30
2.3.9 Tissue preparation ... 31
2.3.10 Triglyceride assay ... 31
2.3.11 Glycogen assay ... 31
2.3.12 Cortisol assay ... 32
2.3.13 Statistical analyses ... 32
2.4 Results ... 32
2.4.1 Selenium analysis ... 32
2.4.2 Fish survival and growth ... 35
2.4.3 Swimming performance ... 37
2.4.4 Oxygen consumption (MO_2) ... 39
2.4.5 Image analysis ... 41
2.4.6 Cost of transport (COT) ... 43
2.4.7 Triglycerides and glycogen ... 45
2.4.8 Cortisol ... 48
2.5 Discussion ... 50
CHAPTER 3 ... 56
EFFECTS OF CHRONIC DIETARY SELENOMETHIONINE EXPOSURE ON REPEAT SWIMMING PERFORMANCE, AEROBIC METABOLISM AND METHIONINE CATABOLISM IN ADULT ZEBRAFISH (*DANIO RERIO*) ... 56
Preface ... 56
3.1 Abstract ... 57
3.2 Introduction ... 59
3.3 Materials and methods ... 63
3.3.1 Test chemical and test species ... 63

3.3.2 Diet preparation and experimental design ... 64
3.3.3 Quantification of selenium ... 65
3.3.4 Oxygen consumption and swim performance ... 66
3.3.5 Determination of standard metabolic rate (SMR), active metabolic rate (AMR), factorial aerobic scope (F-AS) and cost of transport (COT) ... 67
3.3.6 Quantification of triglycerides and glycogen ... 68
3.3.7 Lactate assay ... 68
3.3.8 Real-time polymerase chain reaction (PCR) ... 68
3.3.9 Statistical analyses ... 71
3.4 Results ... 72
3.4.1 Concentrations of selenium ... 72
3.4.2 Survival and growth ... 72
3.4.3 Swim performance and oxygen consumption ... 74
3.4.4 Standard metabolic rate (SMR), active metabolic rate (AMR), factorial aerobic scope (F-AS) and cost of transport (COT) ... 77
3.4.5 Whole-body triglycerides and glycogen ... 80
3.4.6 Whole-body lactate ... 83
3.4.7 Abundances of mRNA of β-hydroxyacyl coenzyme A dehydrogenase (HOAD), citrate synthase (CS), sterol regulatory element binding protein 1 (SREBP 1), protein tyrosine phosphatase 1B (PTP 1B), fatty acid synthase (FAS) and methionine adenosyltransferase 1α (MAT 1A) ... 85
3.5 Discussion ... 88
CHAPTER 4 ... 96
IN OVO EXPOSURE TO SELENOMETHIONINE VIA MATERNAL TRANSFER INCREASES DEVELOPMENTAL TOXICITIES AND IMPAIRS SWIM PERFORMANCE IN F1 GENERATION ZEBRAFISH (*DANIO RERIO*) ... 96
Preface ... 96
4.1 Abstract ... 98
4.2 Introduction ... 100
4.3 Materials and methods ... 103
4.3.1 Test compound ... 103
4.3.2 Diet preparation and experimental design ... 104
4.3.3 Deformity analysis ... 105

4.3.4 Quantification of selenium ... 105
4.3.5 Swim performance ... 106
4.3.6 Image analysis ... 106
4.3.7 Whole-body triglyceride and glycogen assays ... 107
4.3.8 Whole-body cortisol assay ... 107
4.3.9 Statistical analysis ... 108
4.4 Results ... 108
4.4.1 Concentrations of selenium ... 108
4.4.2 Hatchability, mortalities and total deformities ... 111
4.4.3 Swim performance ... 117
4.4.4 Image analysis ... 119
4.4.5 Whole-body glycogen, triglycerides and cortisol ... 121
4.5 Discussion ... 123
4.6 Conclusion ... 131
CHAPTER 5 ... 133
DEVELOPMENTAL AND PERSISTENT TOXICITIES OF MATERNALLY DEPOSITED SELENOMETHIONINE IN ZEBRAFISH (*DANIO RERIO*) ... 133
Preface ... 133
5.1 Abstract ... 134
5.2 Introduction ... 136
5.3 Materials and methods ... 138
5.3.1 Test compound ... 138
5.3.2 Diet preparation and adult exposure ... 139
5.3.3 Deformity analysis ... 140
5.3.4 Quantification of selenium ... 140
5.3.5 Oxygen consumption and swim performance ... 141
5.3.6 Determination of standard metabolic rate (SMR), active metabolic rate (AMR), factorial aerobic scope (F-AS) and cost of transport (COT) ... 142
5.3.7 Statistical analysis ... 142
5.4 Results ... 143
5.4.1 Concentrations of selenium ... 143

5.4.2 Hatchability, mortalities and total deformities 145
5.4.3 Species sensitivity distribution (SSD) and effective concentration (EC10) egg Se versus temperature 149
5.4.4 Swim performance and oxygen consumption 153
5.4.5 Standard metabolic rate (SMR), active metabolic rate (AMR), factorial aerobic scope (F-AS) and cost of transport (COT) 155
5.5 Discussion 157
5.6 Conclusion 162
CHAPTER 6 164
MICROINJECTION OF SELENOMETHIONINE REDUCES HATCHABILITY AND INCREASES MRNA ABUNDANCE OF OXIDANT-RESPONSIVE TRANSCRIPTION FACTOR NF-E2-RELATED FACTOR 2 IN ZEBRAFISH 164
Preface 164
6.1 Abstract 165
6.2 Introduction 167
6.3 Materials and methods 170
6.3.1 Test compound 170
6.3.2 Test animal 170
6.3.3 Microinjection 170
6.3.4 Deformity analysis 172
6.3.5 Quantification of selenium 172
6.3.6 Real-time polymerase chain reaction (PCR) 173
6.3.7 Statistical analysis 175
6.4 Results 175
6.4.1 Concentrations of selenium 175
6.4.2 Hatchability, mortalities and total deformities 178
6.4.3 mRNA abundances of protein tyrosine phosphatase 1b (PTP 1B), methionine adenosyltransferase 1α (MAT 1A), NF-E2-related factor 2A (NRF 2A), NF-E2-related factor 2B (NRF 2B), superoxide dismutase (SOD 1) and glutathione peroxidase 1a (GPX 1A) 181
6.5 Discussion 185
6.6 Conclusion 191
CHAPTER 7 193

GENERAL DISCUSSION 193
7.1 Introduction 194
7.2 Effects of chronic sublethal dietary selenomethionine exposure in adult zebrafish 197
7.3 Effects of maternal selenomethionine exposure through the entire life cycle of F1 generation zebrafish 199
7.4 Effects of embryo microinjection in early life stages of zebrafish 204
7.5 Mechanisms of selenomethionine-induced developmental toxicities in early life stages of zebrafish 206
7.6 Conclusion 209
7.7 Future research 210
REFERENCES 212
APPENDIX 233

LIST OF TABLES

Table 2.1: Total selenium concentrations in food (µg/g dry mass), water (µg/L), and male and female fish (µg/g dry mass). Fish were fed a 5% body weight per day ration with either control or selenomethionine spiked food for 60 days followed by 30-40 days with equal portions (2.5%) of control or selenomethionine spiked food and clean chironomids. Data are mean ± S.E.M. of n = 3-4 samples……………..34

Table 2.2: Mortality, total length, body weight and condition factor in adult zebrafish fed different concentrations of selenomethionine for 90 days. Data are mean ± S.E.M of n = 10-14 fish………………………………………………………………………………………… 36

Table 2.3: Left and right tailbeat amplitudes (average tail stroke distance) and tailbeat frequencies measured at 6.1 cm/s swimming velocity in adult zebrafish fed different concentrations of selenomethionine. Data are mean ± S.E.M. of n = 10-14 fish……………….42

Table 3.1: Gene-specific primer sequences for the quantitative real time PCR used in this study……………………………………………………………………………………..70

Table 3.2: Total selenium concentrations in food and whole fish (µg/g, dry mass), mortality and morphometrics of adult zebrafish fed control or selenomethionine spiked foods for 90 d. Data are mean ± S.E.M of n = 3-5 samples for food and fish selenium analyses and n = 20-21 for morphometrics……………………………………………………………………………...73

Table 4.1: Concentrations of total selenium in eggs, and cumulative mortalities and morphometrics of F1 generation adult zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Cumulative mortalities were calculated from 6-140 days post-fertilization (dpf) and morphometrics were determined on 140 dpf. Data are mean ± S.E.M of n = 3-4 replicates of 60-100 pooled eggs for quantification of selenium, n= 3-4 replicates of 50-75 fish for cumulative fish mortality analysis, and n= 12–14 fish for morphometrics……..110

Table 4.2: Concentrations of whole-body triglycerides, glycogen and cortisol in 140 days post-fertilization F1 generation adult zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Data are mean ± S.E.M of n= 12-14 fish………………………….…..122

Table 4.3: Percent egg viability, embryo hatchability, cumulative mortality and total deformities in progenies (F2 generation) of F1 generation zebrafish exposed to increasing concentrations of selenomethionine via *in ovo* maternal transfer. Data are mean ± S.E.M of n= 3 replicates of 100-150 eggs/larvae………………………………………………………………………….......116

Table 5.1: Concentrations of total selenium in eggs, and cumulative mortalities and morphometrics of F1 generation adult zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Cumulative mortalities were calculated from 6-180 days post fertilization (dpf) and morphometrics were determined on 180 dpf. Data are mean ± S.E.M of n = 3 replicates of 60-100 pooled eggs for quantification of selenium, n= 3 replicates of 40-50 fish for cumulative fish mortality analysis, and n= 9-16 fish for morphometrics……………...144

Table 5.2: Egg selenium concentrations to cause 10% effects (effective concentration [EC] 10) in early life stages of fishes after *in ovo* maternal transfer exposure. Egg selenium EC10s were used to create species sensitivity distribution (SSD), and the EC10s and temperatures were used for correlation analysis……………………..………………………………………………...150

Table 6.1: Gene-specific primer sequences for the quantitative real time PCR used in this study…………………………………………………………………………………………174

Table 6.2: Nominal and measured selenium concentrations in eggs and hatchability of zebrafish exposed to selenomethionine via egg microinjection route. Data are mean ± S.E.M of n = 3-4 replicates of 45-50 pooled eggs for quantification of total selenium, and n= 3-4 replicates of 30-45 embryos for hatchability analysis………………………………………………………...177

Table C4.S1: Percent egg viability and embryo hatchability of adult zebrafish exposed to a selenium sufficient diet (1.3 µg Se/g d.m.), and diet spiked with increasing concentration of selenium (3.7, 9.6 or 26.6 µg Se/g d.m.) for 90d. Data are mean ± S.E.M of n= 4 replicates of 250-600 eggs………………………………………………………………………………236

Table C5.S1: Percent egg viability and embryo hatchability of adult zebrafish exposed to a selenium sufficient diet (1.3 µg Se/g d.m.), and diet spiked with increasing concentration of selenium (3.4, 9.8 or 27.5 µg Se/g d.m.) for 90d. Data are mean ± S.E.M of n= 3-4 replicates of 100-200 eggs…………………………………………………………………………....237

Table C6.S1: Percent hatchability, mortality and total deformities of early life stages zebrafish from a sham or Danieau solution (control group) microinjected treatment groups…………….241

LIST OF FIGURES

Figure 2.1: Critical swimming speed (U_{crit}) in body lengths per second (BL/s) of adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 µg Se/g dry mass). Data are mean ± S.E.M of n = 14 fish. * Significantly different from control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$)……………………………………………38

Figure 2.2: Oxygen consumption (MO_2) in adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 µg Se/g dry mass). Oxygen consumption was measured in n = 12-14 fish from each treatment group swam in a swim-tunnel respirometer at different water velocities. Each point represents mean ± S.E.M………………………………40

Figure 2.3: Cost of transport (COT) as a function of swimming speed in adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 µg Se/g dry mass). Each point represents mean ± S.E.M of n = 12-14 fish……………………………………………44

Figure 2.4: Whole-body triglycerides in adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 µg Se/g dry mass). Data are mean ± S.E.M of n = 7-12 fish. * Significantly different from control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$)………………………………………………………………………………46

Figure 2.5: Whole-body glycogen in adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 µg Se/g dry mass). Data are mean ± S.E.M of n = 9-14 fish. * Significantly different from control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$)………………………………………………………………………………47

Figure 2.6: Whole-body cortisol in adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 µg Se/g dry mass). Data are mean ± S.E.M of n = 9-14 fish. * Significantly different from control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$)………………………………………………………………………………49

Figure 3.1: Critical swimming speed (U_{crit}) in body lengths per second (BL/s) of adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. Solid bars are single exercise critical swimming speed (U_{crit-1}) and open bars are repeat critical swimming speed (U_{crit-2}). Critical swimming speed was significantly altered by the dietary selenium treatment factor ($p = 0.003$) but not the swim challenge factor ($p = 0.717$) in two-way ANOVA. Different lowercase letters denote a significant effect of dietary selenomethionine treatments on U_{crits}. Data are mean ± S.E.M. of n = 12-14 for U_{crit-1} and n = 6-7 for U_{crit-2}…………………………………………………………………………………75

Figure 3.2: Oxygen consumption (MO_2) versus swimming speed in adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. * Significantly different from control group using repeated measures ANOVA followed by Holm–Sidak post hoc tests ($p < 0.05$). Data are mean ± S.E.M. of n = 12-14 fish……………………..76

Figure 3.3: Metabolic capacities (standard metabolic rate [SMR; the minimal maintenance oxygen consumption of unfed fish], active metabolic rate [AMR; the oxygen consumption at the maximum sustainable swimming speed in U_{crit}] and factorial aerobic scope [F-AS; the ratio of AMR to SMR]) of adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. * Significantly different from control group using one-way ANOVA followed by Holm-Sidak post hoc tests (p <0.05). Data are mean ± S.E.M. of n = 12-14 fish……………………………………………………………………..78

Figure 3.4: Cost of transport (COT) as a function of swimming speed in adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. * Significantly different from control group using repeated measures ANOVA followed by Holm-Sidak post hoc tests (p <0.05). Data are mean ± S.E.M. of n = 12-14 fish……………..79

Figure 3.5: Whole-body triglycerides in adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. Solid, striped and crossed bars represent whole-body triglycerides in fish from no swim (fish withheld from swim tests), swim (fish subjected to single U_{crit}) and repeat swim (fish subjected to two U_{crits}) groups, respectively. Both dietary selenium treatment factor and swim status factor had significant effects on concentration of whole-body triglycerides in fish (p <0.001 for dietary selenium treatment factor and p = 0.043 for swim status factor in two-way ANOVA). Different lowercase letters denote a significant effect of dietary selenium treatments on concentration of whole-body triglycerides in adult fish. Post hoc tests did not show any significant differences of swim status factor on whole-body triglycerides in respective dietary selenium treatment groups. Data are mean ± S.E.M. of n = 6-7 fish……………………………………………………………………….81

Figure 3.6: Whole-body glycogen in adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. Solid, striped and crossed bars represent whole-body glycogen in fish from no swim (fish withheld from swim tests), swim (fish subjected to single U_{crit}) and repeat swim (fish subjected to two $_{Ucrits}$) groups, respectively. For whole-body glycogen, p = 0.193 for dietary selenium treatment factor and p <0.001 for swim status factor in two-way ANOVA. Different lowercase letters denote significant differences in concentrations of whole-body glycogen between no swim versus repeat swim groups in respective selenium treatment groups (Holm-Sidak post hoc tests; p <0.05). Data are mean ± S.E.M. of n = 6-7 fish……………………………………………………..….82

Figure 3.7: Whole-body lactate concentrations in adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. Solid, striped and crossed bars represent whole-body lactate in fish from no swim (fish withheld from swim tests), swim (fish subjected to single U_{crit}) and repeat swim (fish subjected to two U_{crits}) groups, respectively. Two-way ANOVA showed significant effects of both dietary selenium treatment factor (p = 0.022) and swim status factor (p <0.001) on whole-body lactate concentrations. Different symbols (@, *) denote significant differences in whole-body lactate between control and fish fed selenomethionine spiked diets, and different lowercase letters denote significant differences in whole-body lactate concentrations between no swim versus repeat swim fish in respective dietary selenium treatment groups (Holm-Sidak post hoc tests; p < 0.05). Data are mean ± S.E.M. of n = 6-7 fish………………………………………………………………84

Figure 3.8: mRNA abundance of (A) sterol regulatory element binding protein 1 (SREBP 1) and (B) β-hydroxyacyl coenzyme A dehydrogenase (HOAD) in liver, and (C) protein tyrosine phosphatase 1B (PTP 1B) and (D) citrate synthase (CS) in muscle of adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. Transcript abundance was determined by quantitative real-time PCR. * Significantly different from control group using one-way ANOVA followed by Holm-Sidak post hoc tests (p <0.05). Data are mean ± S.E.M. of n = 4 liver or muscle samples…………………………………..86

Figure 3.9: mRNA abundance of methionine adenosyltransferase 1α (MAT 1A) in liver of adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. Transcript abundance was determined by quantitative real-time PCR. * Significantly different from control group using one-way ANOVA followed by Holm-Sidak post hoc tests (p <0.05). Data are mean ± S.E.M. of n = 4 liver samples……………………..…...87

Figure 4.1: Percent daily and cumulative mortalities (2-6 days post-fertilization) of embryo or larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. *, Significant difference in mortality compared to control group using Kruskal-Wallis test with Dunn's post-hoc test (whiskers indicate the 5th and the 95th percentile outliers; $p < 0.05$; n = 5-9 replicates of 60-100 embryos)……………………………………………………………113

Figure 4.2: Total morphological abnormalities (sum of skeletal, craniofacial and fin deformities, and edema) in larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. *, Significant difference in total deformities compared to control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$; n = 5-9 replicates of 30-60 embryos)……………………………………………………………………………..114

Figure 4.3: Representative images of normal (A and B) and deformed (C, D, E and F) larval zebrafish taken during deformity analysis. Images A and B show a normal fish, C shows kyphosis and fin deformities, D shows scoliosis, E shows lordosis, and F is a larval fish with multiple deformities and edema…………………………………………………………..115

Figure 4.4: Critical swimming speed (U_{crit}; a measure of swim performance; BL/s = body lengths/second) determined in F1 generation adult zebrafish at 140 days post-fertilization exposed *in ovo* to increasing concentrations of selenium via maternal transfer. *, Significant difference compared to control group using one-way ANOVA followed by Dunnett's test (p < 0.05; n = 12-14 fish)……………………………….……………………………………..118

Figure 4.5: Tailbeat amplitude and tailbeat frequency of F1 generation adult zebrafish at 140 days post-fertilization exposed *in ovo* to increasing concentrations of selenium via maternal transfer. *, Significant difference compared to control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$; n = 6-14 fish)…………………….……………………………120

Figure 5.1: Percent cumulative mortalities (2-6 days post fertilization) of embryo or larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. *,

Significant difference compared to control group using one-way ANOVA followed by the Holm–Sidak post hoc test ($p < 0.05$; n = 3 replicates of 50-200 embryos)……………………147

Figure 5.2: Total morphological abnormalities (sum of skeletal, craniofacial and fin deformities, and edema) in larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. *, Significant difference compared to control group using one-way ANOVA followed by the Holm–Sidak post hoc test ($p < 0.05$; n = 3 replicates of 50-150 embryos)……………………………………………………………………………….148

Figure 5.3: Species sensitivity distribution (SSD) based on egg selenium thresholds (effective concentration [EC] 10s) in early life stages of fishes exposed to excess selenium via maternal transfer. See Table 2 for sources of EC10s obtained to generate the SSD……………………152

Figure 5.4: Swim performance (U_{crit}; 5.4A) and oxygen consumption (MO_2; 5.4B) of F1 generation adult zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Both U_{crit} and MO_2 were determined on 180 days post fertilization in F1 generation adult zebrafish. *, Significant difference compared to control group using one-way ANOVA followed by the Holm–Sidak post hoc test ($p < 0.05$; n = 9-16 fish for Ucrit and n = 7-14 fish for MO_2)……………………………………………………………………………...154

Figure 5.5: Metabolic capacities (standard metabolic rate [SMR; the minimal maintenance oxygen consumption of unfed fish], active metabolic rate [AMR; the oxygen consumption at the maximum sustainable swimming speed in Ucrit] and factorial aerobic scope [F-AS; the ratio of AMR to SMR]; 5.5A) and cost of transport (COT; 5.5B) of F1 generation adult zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Both measurements were carried out on 180 days post fertilization in F1 generation adult zebrafish. *, Significant difference compared to control group using one-way ANOVA followed by the Holm–Sidak post hoc test ($p < 0.05$; n = 7-14 fish for both metabolic capacities and COT)………………………………………………………...……………………156

Figure 6.1: Percent cumulative mortalities (2-6 days post fertilization [dpf]) of early life stages of zebrafish exposed to increasing concentrations of selenium via egg microinjection. *, Significant difference compared to the control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$; n = 3-4 replicates of 30-45 embryos)………………………………..179

Figure 6.2: Total morphological abnormalities (sum of skeletal, craniofacial and fin deformities, and edema) in larval zebrafish exposed to increasing concentrations of selenium via egg microinjection. *, Significant difference compared to the control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$; n = 3-4 replicates of 30-45 embryos)………......180

Figure 6.3: mRNA abundance of protein tyrosine phosphatase 1b (PTP 1B) in zebrafish exposed to either 10 µg Se/g d.m. in the form of selenomethionine or control (Danieau) solution via egg microinjection. Transcript abundance was determined by quantitative real-time PCR at 48, 72 and 96 hours post fertilization (hpf). * Significantly different from the control group using student t-test ($p < 0.05$; n = 3-5 replicates of 20 embryos and/or larval fish)……….….182

Figure 6.4: mRNA abundance of methionine adenosyltransferase 1α (MAT 1A) in zebrafish exposed to either 10 µg Se/g d.m. in the form of selenomethionine or control (Danieau) solution via egg microinjection. Transcript abundance was determined by quantitative real-time PCR at 48, 72 and 96 hours post fertilization (hpf). * Significantly different from the control group using student t-test ($p < 0.05$; n = 3-5 replicates of 20 embryos and/or larval fish)…………...183

Figure 6.5: mRNA abundance of (A) NF-E2-related Factor 2A (NRF 2A) and (B) NF-E2-related Factor 2B (NRF 2B) in zebrafish exposed to either 10 µg Se/g d.m. in the form of selenomethionine or control (Danieau) solution via egg microinjection. Transcript abundance was determined by quantitative real-time PCR at 48, 72 and 96 hours post fertilization (hpf). * Significantly different from the control group using student t-test ($p < 0.05$; n = 3-5 replicates of 20 embryos and/or larval fish)…………………………………………………………………184

Figure C3.S1: mRNA abundance of protein tyrosine phosphatase 1B (PTP 1B), citrate synthase (CS) and fatty acid synthase (FAS) in liver of adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g dry mass) for 90 d. Transcript abundance was determined by quantitative real-time PCR. No significant difference was observed after performing one-way ANOVA. Data are mean ± S.E.M. of n = 4 liver samples…………………………………………………………………………………...234

Figure C3.S2: mRNA abundance of β-hydroxyacyl coenzyme A dehydrogenase (HOAD), sterol regulatory element binding protein 1 (SREBP 1) and fatty acid synthase (FAS) in muscle of adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g dry mass) for 90 d. Transcript abundance was determined by quantitative real-time PCR. No significant difference was observed after performing one-way ANOVA. Data are mean ± S.E.M. of n = 4 muscle samples……………………………………………………....235

Figure C5.S1: Percent cumulative mortalities (2-6 days post fertilization) of embryo or larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Figure shows quadratic relationship between zebrafish egg selenium concentrations and percent larval fish mortality……..…………………………………………………………………....238

Figure C5.S2: Total morphological abnormalities in larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Figure shows quadratic relationship between zebrafish egg selenium concentrations and percent larval fish deformities………...239

Figure C5.S3: Relationship between egg selenium toxicity thresholds (EC 10s) and rearing temperatures (°C) in early life stages of fishes…………………………………....……….240

Figure C6.S1: Total morphological abnormalities in larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Figure shows linear relationship between zebrafish egg selenium concentrations and percent larval fish deformities…………242

Figure C6.S2: Percent cumulative mortalities (2-6 days post fertilization) of embryo or larval zebrafish exposed to increasing concentrations of selenium via *in ovo* microinjection. Figure

shows linear relationship between zebrafish egg selenium concentrations and percent larval fish mortality……………………………………………………………………………………….243

Figure C6.S3: mRNA abundance of (A) superoxide dismutase (SOD 1) and (B) glutathione peroxidase 1a (GPX 1A) in early life stages of zebrafish exposed to either 10 µg Se/g d.m. (nominal concentration) in the form of selenomethionine or control (Danieau) solution via egg microinjection. Transcript abundance was determined by quantitative real-time PCR at 48, 72 and 96 hours post fertilization (hpf). Data are mean ± S.E.M. of n = 3-5 replicates of 20 embryos and/or larval fish……………………………………………………………………………….244

LIST OF ABBREVIATIONS

AHR	Aryl hydrocarbon receptor
AMR	Active metabolic rate
ANOVA	Analysis of variance
BL/s	Body lengths per second
CCME	Canadian Council of Ministers of the Environment
cDNA	Complementary DNA
cm	Centimeters
COPC	Contaminant of potential concern
COT	Cost of transport
CS	Citrate synthase
CV	Coefficient of variation
d	Days
d.m.	Dry mass
EC	Effective concentration
EF 1α	Elongation factor 1α
F-AS	Factorial aerobic scope
FAS	Fatty acid synthase
g	Gram
GPX	Glutathione peroxidase
h	Hours
HDPE	High density polyethylene
HOAD	β-hydroxyacyl coenzyme A dehydrogenase
ICP-MS	Inductively coupled plasma mass spectrometer
J	Joule
KEAP	Kelch-like ECH-associated protein

kg	Kilogram
LD	Lethal dose
LOQ	Limit of quantification
m	Meter
M	Molar
m/s	Meter per second
MAT 1A	Methionine adenosyltransferase 1α
Met	Methionine
mg	Milligram
MNE	Mean normalized expression
MO_2	Oxygen consumption
MS 222	Tricaine methanesulfonate
n	Number of replicate
nm	Nanometers
NRF2	Nuclear factor erythroid 2-related factor 2 /NF-E2-related factor 2
O_2	Oxygen
p	Probability
pg	Picogram
PTP 1B	Protein tyrosine phosphatase 1B
Q-PCR	Quantitative polymerase chain reaction
s	Seconds
S.E.M.	Standard error of the mean
SAM	S-adenosylmethionine
Se	Selenium
SeCys	Selenocysteine
SelO	Selenoprotein O
SelP	Selenoprotein P

SeMet	Selenomethionine
SMR	Standard metabolic rate
SOD	Superoxide dismutase
SREBP 1	Sterol regulatory element binding protein 1
SSD	Species sensitivity distribution
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
Ti	time elapsed at the fatigued velocity
Tii	prescribed time interval
TORT-2	Lobster hepatopancreas
U_{crit}	Critical swimming speed
Ui	Highest velocity maintained by the fish for the entire time interval
Uii	Prescribed increment
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
°C	Degree Celsius
µmol	Micromole
µg/g	Microgram per gram
µg/L	Microgram per liter

PREFACE

This thesis is written and organized as 'manuscript style' format and hence there is some repetition of introduction and material and methods sections throughout this thesis. Chapter 2, 3 and 4 were published in aquatic toxicology (citations listed below), and Chapter 5 and 6 will be submitted for publication in upcoming months. Supplementary data from Chapter 3-6 has been included in the Appendix.

Thomas, J.K., Janz, D.M., 2011. Dietary selenomethionine exposure in adult zebrafish alters swimming performance, energetics and the physiological stress response. Aquatic Toxicology 102, 79-86.

Thomas, J.K., Wiseman, S., Giesy, J.P., Janz, D.M., 2013. Effects of chronic dietary selenomethionine exposure on repeat swimming performance, aerobic metabolism and methionine catabolism in adult zebrafish (*Danio rerio*). Aquatic Toxicology 130-131, 112-122.

Thomas, J.K., Janz, D.M., 2014. In ovo exposure to selenomethionine via maternal transfer increases developmental toxicities and impairs swim performance in F1 generation zebrafish (*Danio rerio*). Aquatic Toxicology 152, 20-29.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Selenium (Se) occurs naturally in the environment. It is the 66th most abundant element in the Earth's crust (Greenwood and Earnshaw, 1997). Selenium was discovered in 1817 by a Swedish chemist, Jöns Jacob Berzelius, and he is also credited with discovering other elements including silicon, thorium, and cerium (Greenwood and Earnshaw, 1997). The name of this element originated from the Greek word for goddess of the moon, *selene* (Weeks, 1956). Berzelius noted that the newly discovered element resembled another element, tellurium, and since tellurium was named after goddess of the Earth (name came from a Latin word, *tellus*), he thought it was appropriate to name the sister element for the Earth's natural satellite, the moon (Weeks, 1956).

1.2 Properties, sources and uses of selenium

Selenium is a metalloid, having properties of both metals and non-metals. It has an atomic number of 34 and atomic mass of 78.96. In the periodic table Se exist in Group 16 (chalcogen group) with other elements including oxygen (O_2), sulfur (S), tellurium (Te), and polonium (Po). Similar chemical properties of Se and other group 16 elements including O_2, S and Te have been widely discussed in literature (Greenwood and Earnshaw, 1997; Mehdi et al., 2013). Selenium has six naturally occurring isotopes with varying degree of abundance, and has many radioactive isotopes (ATSDR, 2003). The most abundant naturally occurring isotope is ^{80}Se (49.61% abundance) followed by ^{78}Se, ^{76}Se, ^{82}Se, ^{77}Se, and ^{74}Se with abundances of 23.7, 9.36, 8.73, 7.63, or 0.87%, respectively (Ralston et al., 2008). With exception of ^{75}Se and ^{79}Se, most radioactive isotopes of Se have a short half-life ranging from seconds to hours (Shaw and Ashworth, 2010). Isotope ^{75}Se has a half-life of 119.8 days and is widely used as a

radiopharmaceutical, in industrial radiography, and as a tracer in environmental studies (Sadek et al., 1983; ATSDR, 2003 ; Kuzelev et al., 2004; Ralston et al., 2008). Isotope ^{79}Se is a by-product of uranium fission reaction and has a half-life of 327,000 years (Jörg et al., 2010). Hence ^{79}Se is the only Se radioactive isotope listed by the Department of Energy (DOE) as a concern in environmental management of sites storing and/or reprocessing of spent uranium fuels (NRC, 2006).

Average concentration of Se in the Earth's crust ranges between 0.05 and 0.09 mg/kg (ATSDR, 2003). Although Se is ubiquitous in soil, its concentration can vary greatly throughout the world depending on the parent rock from which soils are formed (Presser et al., 2004; Maher et al., 2010). The concentration of Se is much higher in cretaceous sedimentary rocks, especially black shale, phosphate rocks and coal, compared to igneous rocks such as basalt, andesite and granite or some of the metamorphic rocks such as quartzite and marble (Presser et al., 2004; Maher et al., 2010). Average soil Se concentrations range from 0.1 to 2 mg/kg, although soil Se concentrations less than 0.1 mg/kg and greater than 100 mg/kg are found in certain regions of the world (Elrashidi et al., 1989). Selenium occurs in one of four oxidation states (-II, 0, IV, VI) and can exist in both inorganic and organic forms in the environment. Inorganic forms of Se are selenide (Se^{2-}), elemental selenium (Se^0), selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), and these forms have varying solubility in water and subsequent bioavailability to plants (Elrashidi et al., 1989; McNeal and Balistrieri, 1989). Selenate is the dominant form of Se present in well-aerated soils whereas selenite is present in soil with moderately oxidizing conditions; these oxyanions are highly soluble and mobile in water, and are bioavailable forms of Se (McNeal and Balistrieri, 1989). Conversely, elemental Se and selenide occur in soil under reducing conditions and these forms are quite insoluble in water and not available forms of Se (McNeal and Balistrieri, 1989).

Organic forms of Se are selenoamino acids (e.g. selenomethionine [SeMet] and selenocysteine [SeCys]) and selenocysteine containing proteins or selenoproteins (e.g. glutathione peroxidases [gpx], selenoprotein P [SelP] selenoprotein O [SelO]) (Gladyshev, 2012; Mehdi et al., 2013).

There are no ores from which Se can be mined as a primary product, however greater concentrations of Se are found in coal deposits, phosphate rocks, crude oil and black shale (Maher et al., 2010; Shaw and Ashworth, 2010). Selenium is produced as a by-product during copper, zinc, lead and nickel ore-refining processes (Butterman and Brown, 2004; Shaw and Ashworth, 2010). It is estimated that greater than 90% of world's Se production comes from copper ores (Butterman and Brown, 2004). Estimated global use of Se is between 2000 and 2800 tons per year, and Japan, Germany, Canada, Russia, and Belgium are the major producers of Se (Butterman and Brown, 2004; Mehdi et al., 2013). Although the Unites States is a major producer of Se, industries in the USA have withheld reporting production of Se since late 1990 (Butterman and Brown, 2004).

Selenium is largely used in glass, ceramic and paint industries as a pigmentation and discoloration agent, in electronic industries as a photovoltaic substance in solar panels and photometers, and as an inner core of semiconductor nanocrystals (Butterman and Brown, 2004; Bouldin et al., 2008; Mehdi et al., 2013). It is used in the rubber industry as an accelerant and vulcanizing agent, and in metallurgy to make alloys to protect machine parts from corrosion (Butterman and Brown, 2004; Mehdi et al., 2013). Selenium is also used in manufacture of pharmaceutical products, especially as a dietary supplement for humans and animals, treatment of dandruff and certain types of skin diseases, and is used in agriculture as a fertilizer and as an insecticide (Mayland, 1994; Varo et al., 1994; Aro et al., 1998; Oldfield, 1999; Janz, 2012).

1.3 Selenium requirement and toxicity

Selenium is known as a 'two-faced' element (Lemly, 1997b; Reilly, 2006). Like moon, Se has two sides, the bright side since it is required for normal physiological function of most living organisms, and the dark side is Se toxicity, which can occur when consumed greater or lower than the recommended daily intake levels (Janz, 2012). One of the distinct features of Se when compared to other ingested essential trace elements is that it has a narrow margin of safety between sufficient and excess supply (Watanabe et al., 1997; Shaw and Ashworth, 2010). Selenium toxicity was first reported by Madison in 1856, and since then several studies observed Se toxicity in livestock and occupational toxicity in humans working in copper mines (Dudley, 1936; James et al., 1989). Selenium was regarded as a naturally occurring toxic element for a century until Schwarz and Foltz (1957) found that dietary Se exposure prevented liver necrosis in rats. Since then several studies reported an essential role of Se in most living organisms (Lobanov et al., 2009; Gladyshev, 2012; Xu et al., 2012). Organisms use trace amount of Se for synthesis of selenocysteine (SeCys) containing proteins or selenoproteins (Gladyshev, 2012). Selenoproteins are identified in all three domains of life, eukaryotes, archaea, and eubacteria (Xu et al., 2012). There are 23-25 known selenoproteins present in mammals including humans whereas fishes possess 30-37 selenoproteins (Lobanov et al., 2009). During evolution, higher plants, yeast and certain insects lost the ability to use Se as an essential trace element and these organisms use a sulfur containing amino acid, cysteine in place of SeCys in proteins (Lobanov et al., 2009). In most living organisms SeCys is co-translationally inserted into nascent polypeptide chain by recoding the UGA codon, and hence SeCys is recognized as the 21st amino acid (Lobanov et al., 2009; Gladyshev, 2012; Xu et al., 2012).

Well characterized selenoproteins in animals are oxidoreductase enzymes such as glutathione peroxidases (GPx), thioredoxin reductases, iodothyronine deiodinases and selenophosphate synthetase-2 (Gladyshev, 2012; Janz, 2012). Although a number of non-enzyme selenoproteins (e.g. selenoprotein P [SelP], selenoprotein M [SelM], selenoprotein N [SelN] selenoprotein W [SelW] are identified in animals, the physiological roles of most of these proteins are not known (Thisse et al., 2003; Gladyshev, 2012). Glutathione peroxidases are major antioxidant enzymes which halt lipid peroxidation by using glutathione (GSH) as a cofactor, thioredoxin reductases are responsible for maintaining intracellular redox state, iodothyronine deiodinases convert thyroxine (T4) to active triiodothyronine (T3), and selenophosphate synthetase 2 is involved in synthesis of selenoproteins. Selenoprotein P is the best characterized non-enzyme selenoprotein and acts as a Se transport protein (Gladyshev, 2012). In mammals, deficiency of dietary Se can affect SeCys synthesis and such an outcome can impair the immune system, thyroid hormone metabolism and spermatogenesis, and severe deficiency of Se can lead to Keshin-Beck disease (a form of heart disease) and Keshan disease (osteoarthropathy) (Rayman, 2000; Beckett and Arthur, 2005). The recommended daily intake of Se is reported to be 75 μg/day for men and 60 μg/day for women (Rayman, 2000). Since Se is recognized as an essential trace element, countries with low soil Se concentrations (e.g. Finland and New Zealand) apply Se as a fertilizer to counteract Se deficiency in diet (Varo et al., 1994; Aro et al., 1998).

Selenium is considered as the second most toxic trace element (second only to mercury) to oviparous vertebrate organisms including amphibians, birds and fishes (Janz et al., 2010; Janz, 2012). Another Se containing amino acid, selenomethionine (SeMet), is reported to be the dominant form of Se present in food (Schrauzer, 2000; Fan et al., 2002). Unlike SeCys, SeMet is

synthesized only by primary producers and certain microorganisms (e.g. yeast and bacteria) (Schrauzer, 2000; Janz, 2012; Kitajima and Chiba, 2013). Since SeMet is structurally similar to the sulfur containing amino acid methionine (Met), SeMet can be nonspecifically inserted to proteins in a concentration dependent fashion (Stadtman, 1974; Sunde, 1984; Behne et al., 1991; Lemly, 1997a; Schrauzer, 2000). Proteins that contain SeMet are not considered selenoproteins. Organisms that live in Se rich environments can bioaccumulate substantial concentrations of Se in the form of SeMet (Schrauzer, 2000; Fan et al., 2002; Hawkesford and Zhao, 2007; Phibbs et al., 2011). Chronic dietary intake of excessive Se is reported to cause alkali disease and blind staggers in livestock, alopecia (loss of hair), nail brittleness, and neurological abnormalities in humans, and teratogenic effects in oviparous (egg laying) organisms (James et al., 1989; Lemly, 1997a,b; Janz et al., 2010; Aldosary et al., 2012).

1.4 Selenium in the aquatic environment

Selenium enters the aquatic environments from both natural and anthropogenic sources primarily as inorganic selenate or selenite, since these oxyanions are highly soluble and mobile in water. Weathering of cretaceous sedimentary rocks, volcanic activities, atmospheric deposition and runoff from seleniferous (Se rich) soils are examples of natural process that contribute Se to the aquatic environment (Nriagu, 1989). Although natural process are involved in mobilization of Se in aquatic environments, anthropogenic activities including mining, coal based power production, oil refining and agriculture on seleniferous soils are the major contributors of Se to the aquatic environments (Lemly, 2004; Maher et al., 2010; Janz, 2012). There are examples of where each of these anthropogenic activities has led to Se contamination of natural aquatic systems. Selenium contamination of the Kesterson Reservoir from agriculture drainage, Belews Lake from a coal-fired power plant cooling drainage, the San Francisco Bay

from oil refining effluent, and the Elk River, British Columbia, Canada from coal mining effluents are well known examples discussed in Se literature (Lemly, 2004; Janz et al., 2010; Janz, 2012). Toxicities are reported in resident fishes and aquatic birds and other terrestrial wildlife feeding from Se contaminated aquatic systems (Lemly, 2004; Ohlendorf, 1989; Beckon and Maurer, 2008; Janz et al., 2010). Hence Se contamination of aquatic environment by anthropogenic activities is identified as an ecotoxicological issue of global concern (Lemly, 2004; Chapman, 2009; Janz et al., 2010, Janz, 2012).

Although the United States Environmental Protection agency (USEPA) and the Canadian Council of Ministers of the Environment (CCME) established ambient water quality criteria of 5 µg Se/L or 1 µg Se/L respectively, for protection of aquatic biota from chronic exposure to Se (USEPA 1987; CCME, 2003), many subsequent studies demonstrated that such guidelines provide limited or no protection against Se toxicity to sensitive oviparous vertebrate organisms (Lemly, 1993b, 2002; Janz et al., 2010; DeForest and Adams, 2011). The reason is that once inorganic forms of Se (selenate or selenite) enter aquatic environments, they are rapidly removed from the water column by primary producers and certain microorganisms and converted to organic forms of Se (SeMet and SeCys) (Fan et al., 2002; Janz et al., 2010). Algae are the major Se accumulators in aquatic environments, and depending on the species of algae and hydrodynamics of an aquatic system, algae can bioaccumulate Se from the water column up to 1,000,000 fold (Janz et al., 2010; Janz, 2012). In addition to accumulating substantial concentrations of Se from the water column, algae and other primary producers are responsible for introducing highly bioavailable organic forms of Se into aquatic food webs (DeForest and Adams, 2011; Janz, 2012). Biomagnification factors ranging from 1.5-6 among plankton, invertebrates and fishes have been observed in Se contaminated aquatic ecosystems (Lemly,

1997b; Muscatello and Janz, 2009). Selenomethionine is the dominant form of Se present in primary producers and consumers inhabiting Se contaminated aquatic ecosystems (Fan et al., 2002; Phibbs et al., 2011). It is reported that greater than 50% of total Se occurs as SeMet in both primary producers and consumers inhabiting aquatic ecosystems contaminated with Se (Fan et al., 2002; Phibbs et al., 2011). Organisms at base of the food webs, especially primary producers and invertebrates, accumulate substantial concentrations of Se (especially SeMet) without any apparent toxicity and acts as vectors transferring Se to sensitive higher tropic level oviparous vertebrate organisms, especially fishes (Janz et al., 2010, Janz, 2012).

Many previous studies demonstrated that although fishes are simultaneously exposed to inorganic Se via water and organic Se via diets, dietary exposure is the dominant route by which Se is toxic to fishes at environmentally relevant concentrations (Lemly, 2002; Fan et al., 2002). Based on this finding, the USEPA (2004) came up with a draft whole-body Se criterion of 7.91 μg/g dry mass (d.m.) for protection of fish populations. Since embryo and/or larval fish deformities and mortality are the most significant toxic effects of organic Se (especially SeMet) exposure in fishes, studies argued that guidelines based on ovary or egg Se rather than whole-body Se provide sufficient protection against Se toxicity to fishes (Lemly, 1993a; Canton et al., 2008; Janz et al., 2010; DeForest and Adams, 2011). Recently, the USEPA proposed a new draft criterion that is undergoing public review while writing this thesis. The major recommendations of the new draft criterion are to implement egg or ovary Se concentration of 15.2 μg/g d.m. in fish, and Se concentrations in whole-body or muscle tissue of fish to 8.1 or 11.8 μg/g d.m., respectively. The new criterion also recommends that Se concentrations should not exceed 4.8 μg/L in lotic (flowing) waters and 1.3 μg/L in lentic (standing) waters more than once in three years on average (USEPA, 2014).

1.5 Selenomethionine toxicity to freshwater fishes

Exposure to waterborne inorganic and/or organic Se at environmentally relevant concentrations is not acutely toxic to freshwater fishes (Janz et al., 2010; DeForest and Adams, 2011). This is because inorganic Se is not efficiently transported across the fish gills, and there are no amino acid transporters identified in the fish gills for the uptake of waterborne organic Se (Campbell et al., 2002; Janz, 2012). In addition, freshwater fishes do not drink water to maintain osmoregulation and hence waterborne Se is less likely to reach the gut for uptake. Concentrations of waterborne sodium selenite or sodium selenate causing 50% mortality (LC50) in juvenile rainbow trout (*Oncorhynchus mykiss*) are 2 to 9.0 mg/L and 32 to 47 mg/L, respectively (Janz, 2012). Similarly, the LC50 of waterborne SeMet in bluegill sunfish (*Lepomis macrochirus*) was reported to be 13 µg/L (Ohlendorf, 2003). It is unlikely to find such high concentrations of waterborne inorganic or organic Se in even the most severely Se-contaminated natural aquatic systems. On the other hand, exposure to environmentally relevant concentrations of organic Se (especially SeMet) in diet is demonstrated to cause toxicities in fishes (Fan et al., 2002; Janz et al., 2010; DeForest and Adams, 2011). Although both inorganic and organic forms of Se can be transported across the gut, dietary exposure of organic Se, especially SeMet, is linked to toxicities in most vertebrate organisms including fishes (Schrauzer, 2000; Fan et al., 2002; Janz et al., 2010).

Embryo-larval and juvenile fishes are highly susceptible to SeMet-induced toxicities when compared to adult fishes (Woock et al., 1987; Teh et al., 2004; Tashjian et al., 2006; Janz et al., 2010). Transfer of organic Se (mainly SeMet) from maternal parent to eggs is the major route of Se exposure to early life stages of fishes (Woock et al., 1987; Lemly, 1997a; Holm et al., 2005; Muscatello et al., 2006; Janz et al., 2010). Throughout this thesis I refer the transfer of Se

from maternal parent to eggs as ‘maternal transfer’. Maternal transfer occurs following exposure of adult female fish to elevated concentrations of dietary Se, resulting in the synthesis and transport of Se-enriched vitellogenin to the ovary, where it is taken up by developing oocytes (Lemly, 1997a; Janz et al., 2010; Janz, 2012). Vitellogenin is the egg yolk precursor protein and serves as the primary food reserve for developing embryo-larval fish (Kunz, 2004). Toxicities in early life stages of F1generation fishes occur when they utilize SeMet-rich yolk protein for development (Janz et al., 2010; Janz, 2012). Developmental toxicities such as deformities and mortality have been observed in early life stages of F1 generation fishes whose parents were exposed to excess dietary SeMet (Woock et al., 1987; Janz et al., 2010) or embryos collected from adult fishes inhabiting Se-contaminated sites (Holm et al., 2005; Muscatello et al., 2006). Although a number of studies have demonstrated Se-induced developmental toxicities in early life stages of F1 generation fishes, to my knowledge no previous studies have evaluated the potential negative effects of subtle Se-induced developmental toxicities and their persistent effects throughout the life cycle of F1 generation fishes.

In addition to SeMet-induced developmental toxicity in larval fishes, chronic dietary exposure of SeMet to juvenile fishes has also been reported to cause various types of toxicities. Chronic dietary SeMet exposure in juvenile Sacramento splittail (*Pogonichthys macrolepidotus*) is demonstrated to cause impaired growth, greater incidence of mortality and deformities, and histopathological changes in the liver (Teh et al., 2004). Dietary SeMet exposure to juvenile white sturgeon (*Acipenser transmontanus*) was reported to cause impaired growth, swimming behaviour and histopathological changes in liver and kidneys (Tashjian et al., 2006). In juvenile rainbow trout, dietary SeMet exposure caused greater accumulation of triglycerides, stimulated gonadal steroidogenesis and attenuation of the cortisol response to an acute stressor (Wiseman et

al., 2011a,b). Although SeMet-induced toxicities in early life stages of F1 generation fishes and juvenile fishes are relatively well understood, little information is available on effects chronic dietary SeMet exposure in adult fishes.

1.6 Mechanisms of selenomethionine toxicity

Several mechanisms are proposed for SeMet-induced developmental toxicities in early life stages of freshwater fishes. One proposed mechanism is altered protein function, which is due to non-specific insertion SeMet in place of Met during protein synthesis (Stadtman, 1974; Sunde, 1984; Martínez et al., 2011). Since both SeMet and Met have similar physico-chemical properties, SeMet can be inserted into proteins in a dose-dependent fashion (Behne et al., 1991; Schrauzer, 2000). There is a disagreement among researchers on whether or not exposure to excess SeMet impairs protein function. Previous studies demonstrated impaired and normal function of proteins after exposure to excess SeMet (Stadtman, 1974; Sunde, 1984; Mechaly et al., 2000; Martínez et al., 2011). In addition, accidental insertion of SeMet is shown to reduce protein stability when compared to wild type Met containing protein (Boles et al., 1991; Kitajima and Chiba, 2013).

The second and more accepted hypothesis for SeMet-induced developmental toxicity is oxidative stress (Palace et al., 2004; Janz et al., 2010). Methylselenol (CH_3SeH), an intermediate metabolite of SeMet, was demonstrated to cause oxidative stress in in vitro studies (Spallholz et al., 2004; Drake, 2006; Kim et al., 2007). Those in vitro studies used a bacterial methionine metabolism enzyme, L-methionine gamma-lyase (EC 4.4.1.11), to demonstrate SeMet-induced oxidative stress. This enzyme has higher substrate specificity for SeMet than methionine (Esaki et al., 1979). L-methionine gamma-lyase activity was recently demonstrated in rainbow trout

(Palace et al., 2004; Misra et al., 2010), but this enzyme is not expressed in mammals. Since CH_3SeH production is observed in mammals, Okuno et al. (2005, 2006) investigated SeMet metabolizing enzymes in mouse liver and found cystathionine gamma-lyase, an enzyme involved in cysteine metabolism, to participate in SeMet metabolism. This enzyme is highly conserved in eukaryotes and its expression or activity is reported in both mammals and fishes (Okuno et al., 2006; Pong et al., 2007; Johnston et al., 2009; Martinović-Weigelt et al., 2011.). It is interesting to note that fishes possess both SeMet metabolism enzymes that are responsible to produce oxidative stress resulting from the SeMet intermediate metabolite, methylselenol.

In many fishes, activities of both superoxide dismutase (SOD) and catalase have been observed during embryonic development (Aceto et al., 1994; Isuev et al., 2008). Under normal physiological conditions, developing embryonic cells are capable of maintaining a balance between activities of antioxidant scavenging enzymes and reactive oxygen species (ROS) generation. However, exposure to an additional pro-oxidant stressor such as SeMet during normal embryo development may alter redox balance, and this could negatively impact cell differentiation that leads to developmental toxicities. It has been reported that even a 15-20% increase in ROS production can alter cellular differentiation of progenitor cells (Smith, et al., 2000; Li et al., 2007).

Oxidative stress is reported to activate many transcription factors in the cell. A widely studied transcription factor in response to oxidative stress is nuclear factor erythroid 2-related factor 2 (NRF 2) (Kobayashi et al., 2002b; Kobayashi et al., 2009; Timme-Laragy et al., 2009; Rubio et al., 2010). This transcription factor is reported to regulate the expression of many antioxidant and detoxifying genes in the cell (Kobayashi et al., 2009; Timme-Laragy et al., 2009). Under normal physiological conditions, NRF 2 is sequestered in the cytoplasm by the

Kelch-like ECH-associated protein 1 (KEAP 1), and oxidative stress modifies KEAP 1 resulting in dissociation of NRF 2 from the NRF 2- KEAP 1 complex (Rubio et al., 2010). Free NRF 2 then translocates to the nucleus and binds to antioxidant response elements (ARE) in the promoter region of various antioxidant genes. Subsequently, increased activities of antioxidant enzymes provide cytoprotection against ROS (Kobayashi et al., 2009; Timme-Laragy et al., 2009). Previous studies have reported increased expression of NRF 2 mRNA in cells exposed to various oxidative stress inducing pollutants (Shinkai et al., 2006; Kobayashi et al., 2009; Timme-Laragy et al., 2009). Co-treatment of a NRF 2 agonist (sulforaphane) with oxidative stress inducing pollutants was reported to increase cell viability when compared to cells treated only with oxidative stress inducing pollutants (Shinkai et al., 2006; Kobayashi et al., 2009; Timme-Laragy et al., 2009). Taken together, NRF 2 plays a crucial role in cytoprotection during in the event of oxidative stress and hence it can be used as a molecular biomarker of oxidative stress.

In addition to protein dysfunction and oxidative stress hypotheses, a recent selenite exposure study reported impaired methylation in early life stages of zebrafish (Ma et al., 2012). This suggests that impaired methylation could possibly be a mechanism of SeMet-induced developmental toxicities in fishes. Methylation plays a pivotal role in cell growth, gene expression, energy homeostasis and biotransformation of chemicals including SeMet (Lu, 2000; Mato et al., 2008; Kobayashi et al., 2002a). S-adenosylmethionine (SAM) is an important donor of methyl groups in multiple methyltransferase reactions and is synthesized from methionine by an enzyme called methionine adenosyltransferase (MAT) (Lu, 2000; Mato et al., 2008). Exposure to excess selenocysteine or selenite is shown to inhibit activity of MAT in mammals (Hoffman, 1977; Hasegawa et al., 1996). However, no fish studies to date have explored potential negative effects of SeMet exposure on methylation and SAM production.

1.7 Swimming physiology of fishes

Swimming is an important survival trait of wild fishes, as it is required for foraging, food acquisition, predator avoidance, schooling and reproduction, and impaired swimming could have organismal, population level and even community level effects (Hammer 1995; Caswell, 1996; Weis et al., 2000, 2001; Scott and Sloman, 2004). It is recognized that in many polluted aquatic ecosystems, most organisms experience sublethal exposure to contaminants, hence studies investigating sublethal toxicities often provide more ecologically relevant information than traditional acute toxicity studies (Little and Finger, 1990; Scott and Sloman, 2004). Swimming performance has been used as an ecologically relevant endpoint for the assessment of sublethal toxicity to fish (Little and Finger, 1990). There are many methods developed to investigate swimming performance of fish, and one of the most commonly used is critical swimming speed (U_{crit}) (Brett, 1964), which challenges fish with incremental changes in velocity until exhaustion. Critical swimming speed is calculated by a formula (Brett, 1964; Plaut, 2001):

$$U_{crit} = Ui + [Uii(Ti/Tii)]$$

Where

Ui = highest velocity maintained by the fish for the entire time interval

Uii = prescribed increment

Ti = time elapsed at the fatigued velocity

Tii = prescribed time interval

In addition to single exercise U_{crit}, studies have also investigated repeat U_{crit}, where fish are subjected to two successive U_{crit} tests (U_{crit-1} and U_{crit-2}) with a short recovery period, normally 40-60 min, between tests (Jain et al., 1998; McKenzie et al., 2007). Repeat swimming performance is commonly used to investigate rates of recovery of fish after exposure to a stressor and is considered a more sensitive indicator of fish health than single exercise U_{crit} (Jain et al., 1998). Tailbeat frequency and tailbeat amplitude are often measured in association with swim performance experiment as these endpoints show direct relationship with swimming speed of fish (Bainbridge, 1958; Herbing, 2002; Tudorache et al., 2010). Tailbeat amplitude measures the maximum tail stroke distance from tip of the tail to midline whereas tailbeat frequency measures complete oscillation of the fish tail and it is represented as number of beats per second (Bainbridge, 1958; Hunter and Zweifel, 1971). Since tailbeat amplitude is a measure of power generated by the tail musculature during swimming (Herbing, 2002; Tudorache et al., 2010), it could be used as an indirect measure on whether SeMet accumulation in muscle proteins alters its function and causes subsequent reduction in swimming. Reduced tailbeat amplitude must be compensated with increasing tailbeat frequency in fish to maintain swimming at a prescribed water velocity (Bainbridge, 1958; Herbing, 2002; Tudorache et al., 2010).

Chemical induced alterations in physiological processes are one of the major causes of impaired fish swimming performance (Scott and Sloman, 2004; Amiard-Triquet, 2009). Commonly studied physiological responses in association with swimming are oxygen uptake, physiological stress responses and energy homeostasis (Scott and Sloman, 2004). These physiological responses are highly interrelated, and alteration in any of these processes can affect others. Oxygen consumption (MO_2) is a measure of metabolic activity of fish, and alteration in MO_2 is an indicator of impaired aerobic performance (Mehrle and Mayer, 1985; MacKinnon and

Farrell, 1992). Exposure to trace elements including Se was reported to alter MO_2 in fishes (Lemly, 1993b; Scott and Sloman, 2004). In teleost fishes, cortisol is the major corticosteroid hormone released from the interrenal cells or head kidney during stress, and cortisol has important roles in energy metabolism and ion homeostasis (Mommsen et al., 1999). Elevated cortisol production by physical or chemical stress can increase MO_2 and may negatively impact fish growth, energy allocation, condition, disease resistance and reproduction (Barton and Iwama, 1991; Mommsen et al., 1999; McGeer et al., 2000; Boeck et al., 2001). Triglycerides and glycogen are the major forms of stored energy in fish (Dange, 1986; Tocher, 2003), and fish utilize these energy stores for swimming, growth, reproduction, and detoxification process. Fishes collected from Se-contaminated sites and fish fed excess dietary SeMet in the laboratory were reported to have a blunted physiological stress response and elevated levels of stored energy (both glycogen and triglycerides) (Bennett and Janz, 2007; Wiseman et al., 2011a,b).

Critical swimming speed is a measure of prolonged aerobic swimming capacity of fish, and triglycerides are used as the primary energy source up to approximately 80% of U_{crit} (Hammer, 1995; Moyes and West, 1995; Plaut, 2001). Alternatively, burst swimming and swimming speed beyond 80% U_{crit} in fish are powered by fast glycolytic white muscle fibers, where the majority of energy comes from anaerobic catabolism of glycogen (Hammer, 1995; Moyes and West, 1995). Fish can maintain burst swimming only for less than 20 sec. This is due to lactate, a by-product of anaerobic energy catabolism, which accumulates in muscle during burst swimming and causes muscle fatigue in fish (Hammer, 1995). Lactate concentration is an indicator of anaerobic metabolic activity and is increased during burst swimming as well as exposure to chemicals (Hammer, 1995; Sancho et al., 1996; Beaumont et al., 2000). Measurement of swimming performance along with other interrelated physiological responses

such as MO_2, stress response, stored energy, production of lactate, and gene expression of enzymes involved in energy metabolism could provide an understanding of sublethal toxicities faced by fishes inhabiting Se contaminated aquatic ecosystem.

1.8 Use of microinjection techniques in aquatic toxicology

Microinjection techniques have been developed to mechanically deliver exogenous compounds into eukaryotic cells (Zhang and Yu 2008a,b). It is extensively used in molecular biology and biotechnology for production of transgenic animals, transfection and introduction of large molecules such as proteins and drugs into a cell (Zhang and Yu 2008a,b). Microinjection was first used in aquatic toxicology to develop an in vivo carcinogenesis assay using rainbow trout embryos (Metcalfe and Sonstegard, 1984). Since then fish embryo microinjection has been extensively used in many developmental and mechanistic toxicity studies, and for production of transgenic fishes (Figueiredo et al., 2007; Colman et al., 2005; Berry et al., 2007). It was demonstrated that chemicals that are likely to deposit in fish eggs by maternal transfer can be effectively studied by microinjection (Walker et al., 1996). Advantages of fish embryo microinjection include, it can be used to investigate toxicity of either parent chemical or its metabolites, microinjection is much quicker than dietborne or waterborne exposure of chemicals and hence many experimental replicates can be generated to get desired statistical power, and since less quantity of chemical is used in microinjection when compared to other routes of exposure, it could be used to study mechanism of highly toxic or expensive chemicals (Walker et al., 1996; Berry et al., 2007). Finally, the most important reason that I have used microinjection to investigate mechanism of SeMet-induced developmental toxicities is that by using microinjection I can deliver uniform doses of SeMet into fish embryos, which cannot be achieved through natural maternal transfer.

1.9 Importance of zebrafish as a test organism

Zebrafish was used as a test organism in my research. Zebrafish has been accepted as a useful vertebrate model for toxicology, pharmacology, developmental biology, immunology and genetic research (Hill et al., 2005). There are well established protocols available to raise zebrafish in a controlled laboratory condition and perform experimental manipulations (Westerfield, 1995; Brand et al., 2002; Gilmour et al., 2002). The whole genome of zebrafish is mapped and hence molecular mechanisms of chemical-induced developmental toxicity can be easily studied (Westerfield, 1995; Brand et al., 2002; Gilmour et al., 2002). Zebrafish has a short maturation time, and one pair of zebrafish can produce up to 200 eggs in a single spawning event, hence zebrafish is an ideal choice for studies investigating transgenerational effect of chemicals (Heiden et al., 2009; Hill et al., 2005). Embryos of zebrafish are non-sticky, clear and transparent which improves successful microinjection of chemicals and is also helpful in conducting morphological and histochemical analysis (Hill et al., 2005; Berry et al., 2007; Timme-Laragy et al., 2012). Zebrafish is an excellent swimmier as it demonstrated by higher U_{crit} values of this fish species when compared to most other fishes studied to date, and has the highest oxygen consumption measured when compared to most small bodied fishes (Palstra et al., 2010). There are commercially available swim tunnel respirometers (e.g. Loligo systems model mini swim tunnel) to study swimming performance and oxygen consumption in zebrafish. All previously mentioned advantages make zebrafish an extremely useful model organism for my research.

1.10 Research objectives and hypotheses

The goals of my research were to investigate direct and transgenerational adverse effects of chronic exposure to excess dietary SeMet in adult zebrafish, and to explore mechanisms of observed SeMet-induced adverse effects in both adult zebrafish and their progeny.

The overall null hypothesis of my research was:

Ho: Chronic exposure to excess dietary SeMet in adult zebrafish does not trigger direct and transgenerational adverse effects.

The specific objectives of this research were:

1. To determine critical swimming speed (U_{crit}), oxygen consumption (MO_2), cost of transport (COT), tailbeat amplitude, tailbeat frequency, and whole-body cortisol, triglyceride and glycogen concentrations in adult zebrafish fed a control (Se-normal) diet or graded concentrations of SeMet-spiked diets.

2. To determine effects of chronic exposure to excess dietary SeMet on repeat swimming performance, oxygen consumption (MO_2), metabolic capacities (standard metabolic rate [SMR], active metabolic rate [AMR], factorial aerobic scope [F-AS] and cost of transport [COT]) and gene expression of energy metabolism and methionine catabolism enzymes in adult zebrafish.

3. To investigate both immediate (incidence of mortality and deformities) and persistent effects (changes in U_{crit}, tailbeat amplitude and frequency, whole-body cortisol, triglyceride and glycogen concentrations and reproduction) of *in ovo* SeMet exposure in F1 generation zebrafish.

4. To describe dose-response relationships between egg Se concentrations and incidences of developmental toxicities in early life stages of F1 generation zebrafish, and to investigate persistent effects of developmental exposure to excess SeMet on swim performance, oxygen consumption (MO_2), metabolic capacities (standard metabolic rate [SMR], active metabolic rate [AMR], factorial aerobic scope [F-AS] and cost of transport [COT]) in F1 generation adult zebrafish.

5. To compare dose-response relationships for SeMet-induced developmental toxicities in early life stages of zebrafish via maternal transfer and embryo microinjection routes, and to investigate oxidative stress and methionine catabolism as potential underlying mechanisms of SeMet-induced developmental toxicities in early life stages of zebrafish via embryo microinjection.

CHAPTER 2

DIETARY SELENOMETHIONINE EXPOSURE IN ADULT ZEBRAFISH ALTERS SWIMMING PERFORMANCE, ENERGETICS AND THE PHYSIOLOGICAL STRESS RESPONSE

Preface

The research described in this chapter was the first dietary SeMet exposure study investigating direct sublethal toxicities in adult fish. The purpose of this study was to understand organismal, physiological and biochemical effects of environmentally relevant dietary SeMet exposure using a toxicological model fish species, zebrafish. I studied swimming performance, tailbeat amplitude, tailbeat frequency, oxygen consumption, concentrations of stored energy (triglycerides and glycogen) and cortisol in adult zebrafish exposed chronically to sublethal concentrations of SeMet spiked diet. Reduced swimming performance and tailbeat amplitude, a trend for greater oxygen consumption, and greater accumulation of stored energy were noticed in adult zebrafish fed excess dietary SeMet. Results of this study suggest that environmentally relevant dietary exposure can cause sublethal toxicities in fishes.

This chapter was published in Aquatic Toxicology 102:79-86, under joint authorship with David M. Janz (University of Saskatchewan). The tables, figures and references cited in this article have been re-formatted here to the thesis style. References cited in this chapter are listed in the reference section of this thesis.

2.1 Abstract

Selenomethionine (SeMet) is the major form of organoselenium present in food. Early life stages of oviparous vertebrate species, especially fish, are highly susceptible to dietary selenium (Se) exposure; however less is known concerning effects in adults. The present study was designed to investigate behavioral and physiological consequences of dietary SeMet exposure to adult zebrafish (*Danio rerio*). Adult fish were fed either control food (1.3 µg Se/g, dry mass or d.m.) or food spiked with varying measured concentrations of Se (3.7, 9.6 and 26.6 µg Se/g, d.m.) in the form of SeMet for 60 d at 5% body weight/ day ration, and an additional 30-40 d with equal ration (2.5%) of control or SeMet spiked foods and clean chironomids. At the end of the exposure period, critical swimming speed (U_{crit}), oxygen consumption (MO_2), cost of transport (COT), tailbeat amplitude, tailbeat frequency, and whole-body cortisol, triglyceride and glycogen levels were determined. Significantly reduced U_{crit} was observed in fish fed 3.7, 9.6 and 26.6 µg Se/g when compared to control fish. Although MO_2 of fish fed > 3µg Se/g was consistently greater than control fish, those values were not statistically significant. There was no difference in COT among different treatment groups. Tailbeat amplitudes of fish fed > 3µg Se/g were lower than control fish, however tailbeat frequencies were not altered. Fish fed 3.7, 9.6 and 26.6 µg Se/g had greater whole-body triglycerides and glycogen levels than control fish. Fish fed the highest concentration of Se (26.6 µg Se/g) had elevated levels of whole-body cortisol compared to control fish. Our results suggest that environmentally relevant dietary SeMet exposure can alter both behavioral and physiological responses in adult fish, and such consequences could threaten fitness of adult fish in Se impacted aquatic ecosystems.

2.2 Introduction

Selenium (Se) is as an essential micronutrient for all vertebrate species, and is known for its narrow margin between essentiality and toxicity in fish (NRC, 2005; Janz et al., 2010). Under normal physiological conditions, fish require dietary concentrations of 0.1 to 0.5 μg Se/g dry mass (d.m.) to maintain their body function and growth, however when concentrations exceed 3 μg/g d.m., Se can be rapidly bioaccumulated in fish tissues to reach toxic concentrations (Lemly, 1997a; NRC, 2005). Selenium can exist in both inorganic forms (e.g. selenite, selenate) and organic forms (e.g. seleno-amino acids, predominantly selenomethionine, and selenoproteins) in aquatic ecosystems. Field-based studies have documented Se bioaccumulation and related toxicities in wild fish (reviewed in Janz et al, 2010). Selenomethionine (SeMet) is the major dietary source of Se available to fish (Fan et al., 2002) and it has high bioaccumulative and trophic transfer properties compared to other forms of Se (Besser et al., 1993; Fan et al., 2002). Mechanisms of SeMet absorption and bioaccumulation are attributed to its structural similarities to the essential amino acid methionine (Met). In mammals both SeMet and Met are mainly absorbed from the intestine via the neutral amino acid transporter, and a similar type of absorption has been reported in fish (Wolffram et al., 1989; Bakke et al., 2010). Inside cells, SeMet is incorporated into the Met insertion site on polypeptides (Schrauzer, 2000), and incorporation of SeMet occurs in a non-specific or random manner (Behne et al., 1991). Thus, excess dietary intake of SeMet results in concentration-dependent bioaccumulation.

Larval and juvenile fish show severe toxicities (e.g. deformities, impaired growth, mortality) when exposed to elevated concentrations of Se. The major exposure pathway of Se to larval and juvenile fish is maternal transfer, which occurs when adult fish are exposed to elevated dietary Se. Although a number of previous studies have reported acute toxic effects resulting

from high Se exposure in adult fish, little information is available on chronic sublethal dietary Se exposure and its consequences in adult fish. Sublethal toxicity studies have many advantages over acute toxicity studies. Usually sublethal endpoints are more sensitive and protective than traditional toxicological endpoints. In natural aquatic ecosystems, most organisms experience sublethal exposure to contaminants, hence studies investigate sublethal endpoints often provide more ecologically relevant information than acute toxicity studies (Little and Finger, 1990; Scott and Sloman, 2004). Swimming behavior is a widely studied endpoint to investigate sublethal exposure of toxicants (Little and Finger, 1990; Cheng and Farrell, 2007). Impaired swimming of fish can alter a number of other behaviors such as foraging, food acquisition, predator avoidance, schooling and reproduction, and such alteration in behaviors could have organismal, population level and even ecosystem level (e.g. increased number of prey species due to loss of predator) consequences (Caswell, 1996; Weis et al., 2000, 2001; Scott and Sloman, 2004). There are many methods developed to investigate swimming capacity of fish, and one of the most commonly used is critical swimming speed (U_{crit}) (Brett et al., 1964). Critical swimming speed is commonly adopted to investigate prolonged and aerobic swimming capacity of fish (Brett et al., 1964; Farrell et al., 1998).

Toxicant induced alterations in physiological processes are one of the major causes of impaired fish swimming behavior (Scott and Sloman, 2004; Amiard-Triquet, 2009). Commonly studied physiological responses are oxygen uptake, physiological stress responses and energy homeostasis (Scott and Sloman, 2004). These physiological responses are highly interrelated, and alteration in either one of the process can affect others. Oxygen consumption (MO_2) is a measure of metabolic activity of fish, and alteration in MO_2 is often related to changes in aerobic performance (Mehrle and Mayer, 1985; MacKinnon and Farrell, 1992). Trace elements

including Se are reported to alter MO_2 in fish (Lemly, 1993b; Scott and Sloman, 2004). In teleost fish, cortisol is the major corticosteroid hormone released from the interrenal cells or head kidney during stress, and cortisol has important roles in energy metabolism and ion homeostasis (Mommsen et al., 1999). Elevated cortisol can increase MO_2 and may negatively impact fish growth, energy allocation, condition, disease resistance and reproduction (Barton and Iwama, 1991; Mommsen et al., 1999; McGeer et al., 2000; Boeck et al., 2001). Normally, circulating (plasma or serum) cortisol is measured in fish as a marker of physiological stress. However, since it is difficult to collect sufficient blood from small fish such as zebrafish to determine circulating cortisol levels, whole-body cortisol has been shown to be a useful surrogate marker for physiological stress (Ramsay et al., 2006; Barcellos et al., 2010). Although previous Se exposure studies showed altered plasma cortisol production in large bodied fish (Mommsen et al., 1999; Miller et al., 2007), to our knowledge no studies have investigated Se-induced stress responses in small bodied fish. Triglycerides and glycogen are the major forms of stored energy in fish (Dange, 1986; Tocher, 2003), and fish utilize these energy stores for locomotion, growth, reproduction, and detoxification process. Toxicant-induced alteration in cortisol production and MO_2 can drain energy stores in fish and such effects may threaten the growth and survivability of fish (Dange, 1986; Lemly, 1993b; McGeer et al., 2000; Miller et al., 2009).

In the present study we used adult zebrafish to investigate chronic sublethal toxicity of dietary SeMet. Zebrafish has been used as a model organism to investigate toxicity of various chemicals and there are many standardized protocols available to raise this fish in controlled laboratory experiments. The objectives of the present study were to investigate the effects of chronic sublethal dietary SeMet exposure on swimming performance, oxygen consumption, physiological stress response and bioenergetic status in adult zebrafish.

2.3 Materials and methods

2.3.1 Test compound

Seleno-L-methionine was purchased from Sigma-Aldrich (Oakville, ON, Canada). Purity of the compound was > 98%.

2.3.2 Test species

Adult zebrafish (approximately 6 months old) were purchased from a local supplier and housed in an environmental chamber with controlled temperature (28.0 ± 1.0 °C) and photoperiod (14 h light and 10 h dark). Mean standard length of fish was 2.8 cm and ranged from 2.6 to 3.1 cm. From 500 purchased zebrafish, 300 were introduced evenly (25 fish/ tank) into twelve 40 L glass aquaria with continuous aeration and filtration, after which treatments were randomly assigned to aquaria, with n=3 replicate aquaria per treatment. Fish were acclimated for 2 weeks to laboratory conditions, and during this time they were fed Nutrafin® basic flake food (Hagen Inc., Montreal, QC, Canada).

2.3.3 Diet preparation

Different nominal concentrations (3, 10 and 30 µg/g d.m.) of Se in the form of SeMet were weighed, dissolved in deionized distilled water, added to flake food, and mixed thoroughly for 10 min. The control diet was made by mixing flake food with an equal volume of water. A freeze dryer (Dura-DryTM MP, FTS Systems, Stone Ridge, NY, USA) was used to remove water from the diets. Freeze dried diets were crushed into flakes and stored at –20 °C in air tight containers. Representative samples of these diets were collected for total Se analysis.

2.3.4 Experimental protocol

Fish were fed twice daily (5% body weight/ day ration) with either control or SeMet spiked foods for 60 d. After 60 d, they were fed equal portions (2.5% body weight/ day) of control or SeMet spiked foods and clean chironomids (Bio-Pure Blood Worms, Hikari Sales Inc., Hayward, CA, USA) for another 30-40 d. Fish were allowed to feed for 1 h, after which excess food was siphoned from the aquarium bottom. During the feeding experiment, 75% of water was renewed from each aquarium every two days. Water samples (n = 1 from each aquaria) for dissolved Se determination were collected 5 h after feeding on day 30 of the feeding experiment. These water samples (n = 3 from each treatment) were filtered using 0.45 μm disposable filters, acidified to pH < 2 using ultra-pure nitric acid, and stored in 25-ml high density polyethylene (HDPE) bottles at 4 °C until Se analysis. After 90 d, n = 4 male and n = 4 female adult fish were collected randomly from each treatment group, euthanized using an overdose of tricaine methanesulfonate (MS 222) (1g/L) and stored at –80°C for total selenium analysis. Swimming behavior, oxygen consumption, bioenergetic status and cortisol levels were measured in remaining adult fish collected from each treatment (n = 10-14).

2.3.5 Selenium analysis

Prior to Se analysis, fish and food samples were lyophilized using a freeze dryer, and homogenized using a motor and pestle. Moisture content of whole zebrafish was 77.0 ± 2.4 %. 100 mg of the homogenized samples were cold digested in Teflon vials using 5 ml of ultra-pure nitric acid and 1.5 ml hydrogen peroxide. After digestion, samples were concentrated on a hot plate (< 75 °C) and reconstituted in 5 ml of 2% ultrapure nitric acid. Reconstituted samples were stored at 4 °C until analysis. Total Se levels in fish, food and water samples were measured

using inductively coupled plasma-mass spectrometry (ICP-MS) in the Toxicology Centre (University of Saskatchewan, Saskatoon, SK, Canada). A method detection limit of 0.02 µg Se/g was determined using method blanks. Selenium recovery was determined using certified reference material (TORT-2, lobster hepatopancreas, NRC, Ottawa, ON, Canada).

2.3.6 Swimming performance and oxygen consumption

Swimming performance and oxygen consumption analyses were carried out using a model mini swim tunnel respirometer equipped with a DAQ-PAC-F1X automated oxygen measurement system (Loligo Systems, Tjele, DK). AutoRespTM 1 software (Loligo Systems) was used to calculate MO_2 consumption during swimming performance trials. Water temperature was maintained at 28.5 ± 1.0 °C during the entire period of experiments using a heated water bath circulator (VWR International, Mississauga, ON, Canada). Critical swimming speed was used to investigate the aerobic swimming capacity of fish (Brett et al., 1964). Briefly, individual adult zebrafish were placed in the swim tunnel respirometer and subjected to stepwise increments in swimming velocity (3.9 cm/s every 5 min) until fish completely fatigued (stopped swimming) (Plaut, 2000). On occasions where fish stopped swimming and rested at the downstream portion of the swim tunnel for the first time, water velocity was reduced for 5-10 seconds to initiate swimming and then the velocity was returned to the previous set point. Water inside the swim tunnel was renewed using a flush pump when the oxygen concentration dropped below 70% saturation. Critical swimming speed was calculated using the equation previously described (Brett et al., 1964). Fish cross sectional area was less than 5% of swim tunnel cross sectional area, hence U_{crit} values were not corrected for solid blocking effect. Critical swimming speed values (cm/s) were corrected for standard body length of each individual fish, and thus U_{crit} values were represented as body lengths per second (BL/s). After the swimming

performance experiments, fish were euthanized using an overdose of MS 222 (1g/L) and stored at –80 °C for subsequent whole-body triglyceride, glycogen and cortisol analyses. Prior to storage, total body length, body weight, and condition factor (body weight/total body length^3) were determined for each fish.

2.3.7 Image analysis

Tailbeat amplitude and tailbeat frequency were measured in SeMet exposed and control fish (Bainbridge, 1958; Hunter and Zweifel, 1971). Tailbeat amplitude measures the maximum tail stroke distance from tip of the tail to midline. A dual-camera high speed video system (Fastec Trouble Shooter, Fastec Imaging, San Diego, CA, USA) was used to record videos during U_{crit} experiments. The camera was mounted on a tripod above the working section of the swim tunnel. Videos were recorded when fish swam in a straight line at a swimming velocity of 6.1 cm/s. Adobe Premiere Element 2.0 (Adobe Systems, San Jose, CA, USA) was used to select three right and three left tailbeat amplitude frames randomly from each video file, and image analysis was conducted on these frames using Image-Pro Express 6.0 (Media Cybernetics Inc., Bethesda, MD, USA). Both right and left tailbeat amplitude values were determined from the three randomly selected frames and those values were averaged to obtain mean left and right tailbeat amplitude values for each fish. Tailbeat frequency measures complete oscillation of the fish tail and it is represented as number of beats per second (Hunter and Zweifel, 1971). Tailbeat frequency was also measured at 6.1 cm/s.

2.3.8 Cost of transport

Cost of transport is used to determine the energetic cost of swimming in fish (Videler, 1993; Claireaux et al., 2006). Cost of transport (J/kg/m) was calculated by multiplying MO_2

values (mg O_2/kg/s) to an oxycaloric value of 14.1 J/mg O_2 and then divided by the corresponding swimming speed (m/s) (Videler, 1993).

2.3.9 Tissue preparation

Whole-body zebrafish samples were thawed on ice and homogenized in 0.2 M sodium citrate buffer (EMD Chemicals Inc., Gibbstown, NJ, USA) using a Tissue Tearor (Fisher Scientific, Houston, TX, USA). Homogenate of each fish was divided into three aliquots and stored at -80 °C until triglyceride, glycogen and cortisol analyses. A SpectaMAX 190 spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA) was used to read absorbances for each of these spectrophotometric assays. For each assay, whole-body homogenate samples were run in triplicate, and mean results with less than 10% coefficient of variation (%CV; standard deviation/mean) were used for data analyses.

2.3.10 Triglyceride assay

The assay for triacylglycerols (triglycerides) was carried out using a kit prepared by Sigma-Aldrich (Oakville, ON, Canada). The kit method was developed by McGowan et al. (1983) and has been previously validated in our laboratory for measuring triglycerides in whole fish homogenates (Weber et al., 2003; Bennett and Janz, 2007; Kelly and Janz, 2008). A glycerol solution was used for the standard curve.

2.3.11 Glycogen assay

A modified version of Gómez- Lechón et al. (1996) was used to measure glycogen levels in whole-body homogenate samples. This method has been previously validated in our lab for whole fish homogenates (Weber et al., 2008). Glycogen assay reagents were purchased from

Sigma-Aldrich (Oakville, ON, Canada). The standard curve was created using purified Type IX bovine liver glycogen.

2.3.12 Cortisol assay

Cortisol was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Oxford Biomedical Research, Oxford, MI, USA). Cortisol in whole-body homogenates from each fish was extracted using diethyl ether. After extraction, ether was evaporated under a gentle stream of nitrogen gas and cortisol was reconstituted in phosphate buffer.

2.3.13 Statistical analyses

All data were tested for normality with the Shapiro-Wilk test and homogeneity of variance with the Levene test using SigmaStat 3.1 (SPSS Inc., Chicago, IL, USA). Data that did not meet the assumptions were log 10 transformed. One-way ANOVA followed by Dunnett's test, when appropriate, was used to test significant differences among treatments. An alpha value ≤ 0.05 was considered significant.

2.4 Results

2.4.1 Selenium analysis

Total Se concentrations measured in food, water and whole-body male and female fish are outlined in Table 2.1. Total Se concentration in non-spiked (control) food was 1.33 µg/g d.m. Measured Se concentrations in the Se spiked foods (3.74, 9.62 and 26.58 µg/g d.m.) were significantly greater than the control diet ($p < 0.05$). Selenium concentrations in water collected from aquaria 5 h after feeding were 0.27, 0.31, 0.63 and 1.18 µg/L in the 1.3, 3.7, 9.6 and 26.6 µg Se/g diet fed tanks, respectively, and were significantly greater than control aquaria in water

collected from aquaria in the two highest dietary treatments ($p < 0.05$; Table 2.1). Whole-body Se concentrations in male and female fish were significantly greater than control fish at the two highest Se spiked dietary treatments ($p < 0.05$; Table 2.1).

Table 2.1: Total selenium concentrations in food (µg/g dry mass), water (µg/L), and male and female fish (µg/g dry mass). Fish were fed a 5% body weight ration with either control or selenomethionine spiked food for 60 days followed by 30-40 days with equal portions (2.5%) of control or SeMet spiked food and clean chironomids. Data are mean ± S.E.M. of n = 3-4 samples.

Nominal diet [Se]	Food	Water	Male fish	Female fish
Control	1.33 ± 0.02	0.27 ± 0.01	1.25 ± 0.38	1.92 ± 1.01
3 µg/g	3.74 ± 0.07*	0.31 ± 0.01	8.19 ± 1.58	6.13 ± 1.01
10 µg/g	9.62 ± 0.35*	0.63 ± 0.04*	11.42 ± 1.38*	13.43 ± 0.96*
30 µg/g	26.58 ± 0.93*	1.18 ± 0.03*	15.28 ± 3.46*	21.93 ± 2.37*

***** Significantly different from the control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$).

2.4.2 Fish survival and growth

Mean mortalities of control, 3.7, 9.6 and 26.6 µg Se/g fed fish after 90 d were 10.7 %, 21.3%, 22.7% and 38.7%, respectively (Table 2.2). Mean mortality was significantly greater for zebrafish fed the 26.6 µg Se/g diet compared to the control group ($p < 0.05$). Approximately 50% of mortalities in each treatment group were observed during the first 30 d of feeding. With the exception of body weight in fish fed 9.6 µg Se/g, fish fed SeMet spiked diets had greater body weight and total length compared to control fish ($p < 0.05$; Table 2.2). Condition factor (body weight/length^3) ranged from 1.13 to 1.24 and was not significantly different among treatment groups (Table 2.2).

Table 2.2: Mortality, total length, body weight and condition factor in adult zebrafish fed different concentrations of selenomethionine for 90 days. Data are mean ± S.E.M of n = 10-14 fish.

Dietary Se (µg/g)	Mortality	Total length (mm)	Weight (g)	Condition factor
1.3	0.11 ± 0.01	37.6 ± 0.4	0.60 ± 0.03	1.13 ± 0.04
3.7	0.21 ± 0.03	40.9 ± 0.7*	0.85 ± 0.05*	1.24 ± 0.04
9.6	0.23 ± 0.07	40.5 ± 1.0*	0.74 ±0.04	1.13 ± 0.06
26.6	0.39 ± 0.06*	41.1 ± 0.7*	0.84 ± 0.06*	1.19 ± 0.05

Condition factor = (body weight/total length^3) x 100,000.

* Significantly different from control group using one-way ANOVA followed by Dunnett's test (p <0.05).

2.4.3 Swimming performance

Significantly reduced swimming performance (U_{crit}) was observed in 3.7, 9.6 and 26.6 µg Se/g fed fish when compared to control fish after 90-100 d of exposure ($p < 0.05$; Figure 2.1). Fish fed 26.6 µg Se/g showed an almost 50% reduction in U_{crit} (4.27 ± 0.79 BL/s) compared to control fish (8.44 ± 0.64 BL/s).

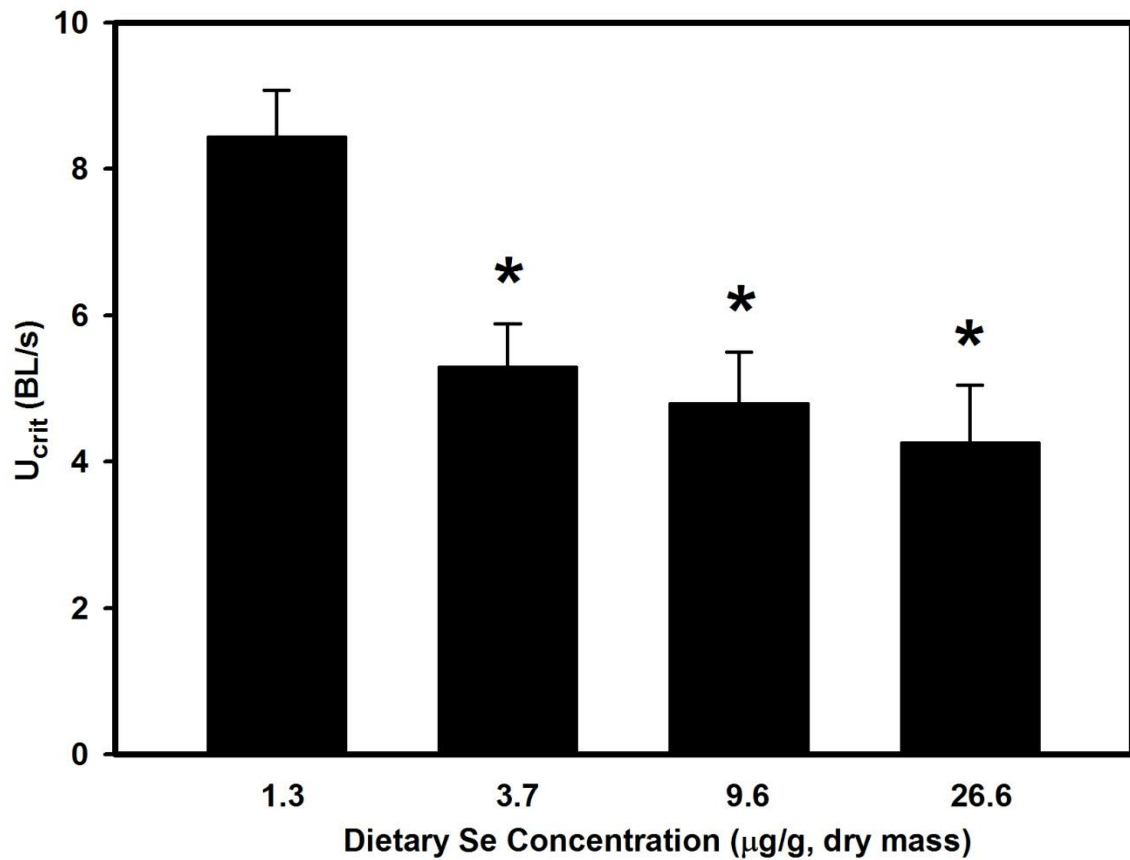

Figure 2.1: Critical swimming speed (U_{crit}) in body lengths per second (BL/s) of adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 μg Se/g dry mass). Data are mean ± S.E.M of n = 14 fish. * Significantly different from control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$).

2.4.4 Oxygen consumption (MO_2)

The relationship between MO_2 and increasing swimming speed is shown in Figure 2.2. Oxygen consumption in all treatment groups was increased with swimming speeds greater than 10 cm/s. Although MO_2 of SeMet fed fish were consistently greater than control fish, there were no statistically significant differences in MO_2 among treatments (Figure 2.2).

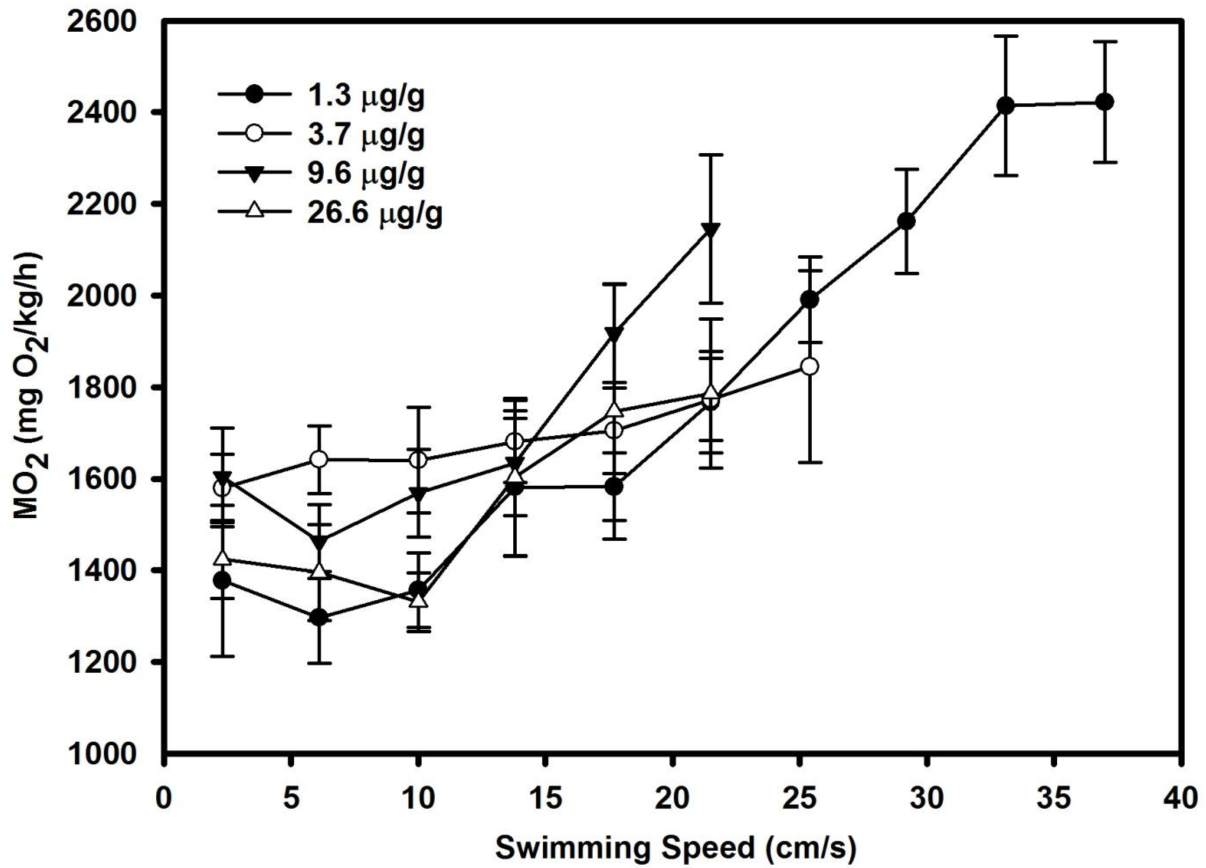

Figure 2.2: Oxygen consumption (MO_2) in adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 µg Se/g dry mass). Oxygen consumption was measured in n = 12-14 fish from each treatment group swam in a swim-tunnel respirometer at different water velocities. Each point represents mean ± S.E.M.

2.4.5 Image analysis

Both tailbeat amplitude and tailbeat frequency were measured at a swimming speed of 6.1 cm/s. Left and right tailbeat amplitudes were significantly lower in fish fed Se spiked diets ($p < 0.05$) with exception of right tailbeat amplitude in fish fed the 26.6 µg Se/g diet (Table 2.3). Although the tailbeat frequency of control fish appeared lower than fish fed Se spiked diets, there were no statistically significant differences in tailbeat frequency among treatments. Mean tailbeat frequencies of control, 3.7, 9.6 and 26.6 µg Se/g fed fish were 7.38 ± 0.30, 8.08 ± 0.31, 8.50 ± 31 and 8.50 ± 0.42 tail beats /s, respectively.

Table 2.3: Left and right tailbeat amplitudes (average tail stroke distance) and tailbeat frequencies measured at 6.1 cm/s swimming velocity in adult zebrafish fed different concentrations of selenomethionine. Data are mean ± S.E.M. of n = 10-14 fish.

Dietary Se (μg/g)	Left tailbeat amplitude (mm)	Right tailbeat amplitude (mm)	Tailbeat frequency (tail beats/s)
1.3	4.13 ± 0.19	3.51 ± 0.15	7.38 ± 0.30
3.7	3.23 ± 0.11*	3.02 ± 0.11*	8.08 ± 0.31
9.6	2.51 ± 0.13*	2.56 ± 0.12*	8.50 ± 0.31
26.6	3.32 ± 0.14*	3.72 ± 0.14	8.50 ± 0.42

* Significantly different from control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$).

2.4.6 Cost of transport (COT)

Cost of transport in adult zebrafish from different treatment groups decreased with increasing swimming speed (Figure 2.3). However, there were no statistically significant differences in COT between control and SeMet fed groups.

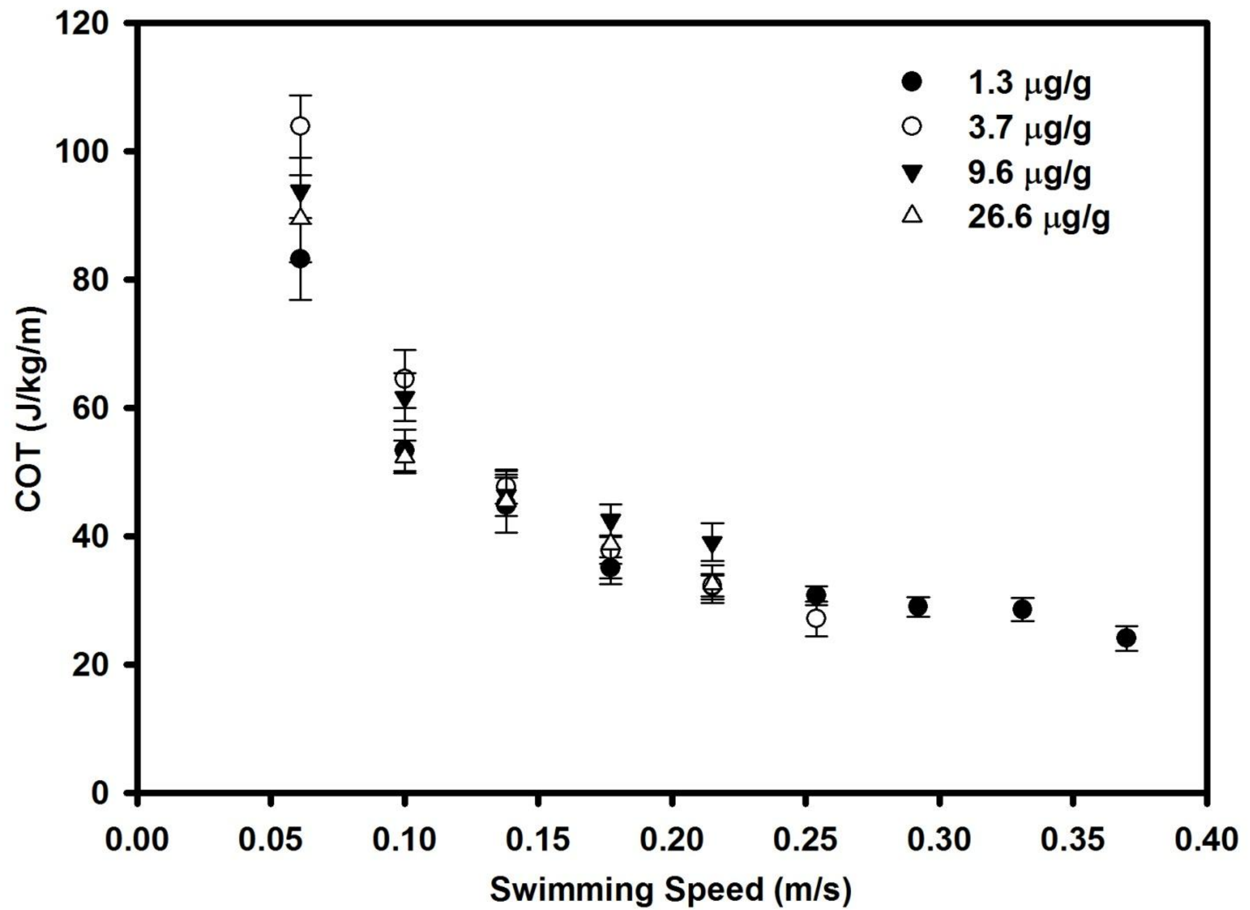

Figure 2.3: Cost of transport (COT) as a function of swimming speed in adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 μg Se/g dry mass). Each point represents mean ± S.E.M of n = 12-14 fish.

2.4.7 Triglycerides and glycogen

Concentrations of whole-body triglycerides in the fish fed 9.6 and 26.6 µg Se/g (47.8 ± 5.1 and 50.0 ± 5.8 mg/g, respectively) were significantly greater ($p < 0.05$) than control fish (32.91 ± 5.43 mg/g; Figure 2.4). Whole-body glycogen concentrations in fish fed 3.7, 9.6 and 26.6 µg Se/g were significantly greater when compared to control fish ($p < 0.05$; Figure 2.5). Glycogen levels in control, 3.7, 9.6 and 26.6 µg Se/g fed fish were 2.6 ± 0.7, 5.5 ± 0.7, 5.6 ± 0.9 and 4.2 ± 0.8 mg/g, respectively.

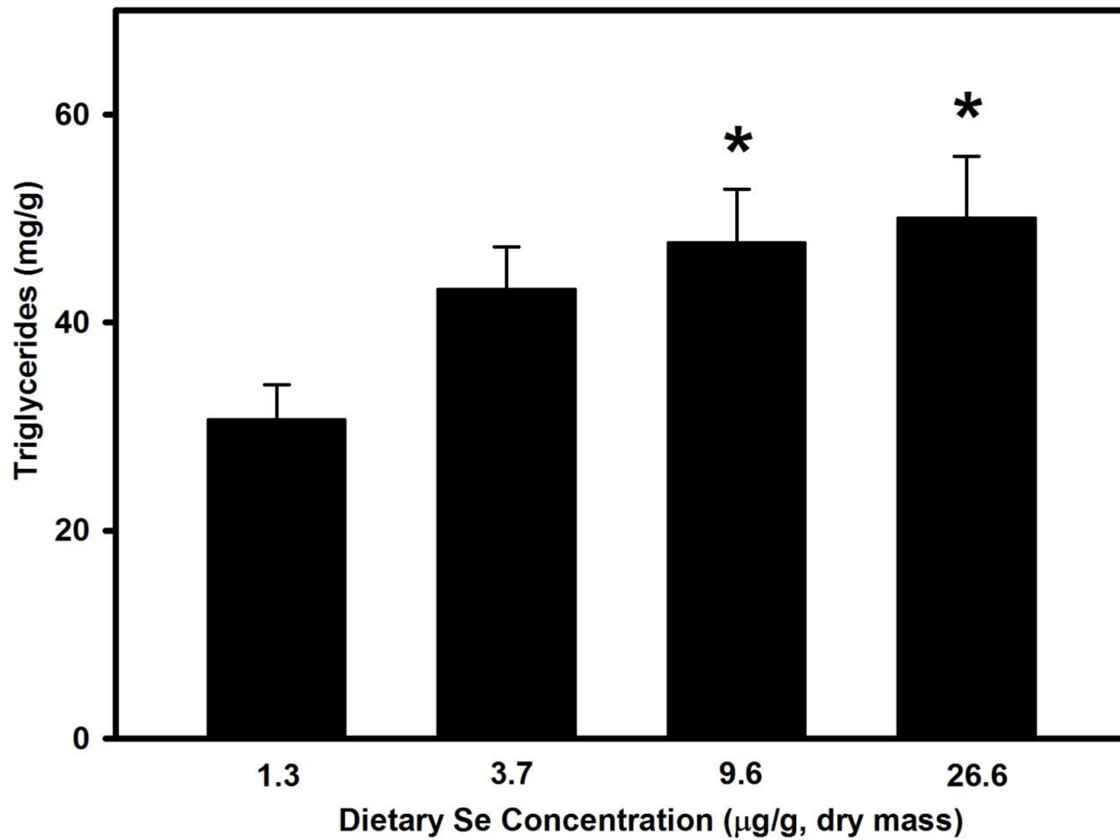

Figure 2.4: Whole-body triglycerides in adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 μg Se/g dry mass). Data are mean ± S.E.M of n = 7-12 fish. * Significantly different from control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$).

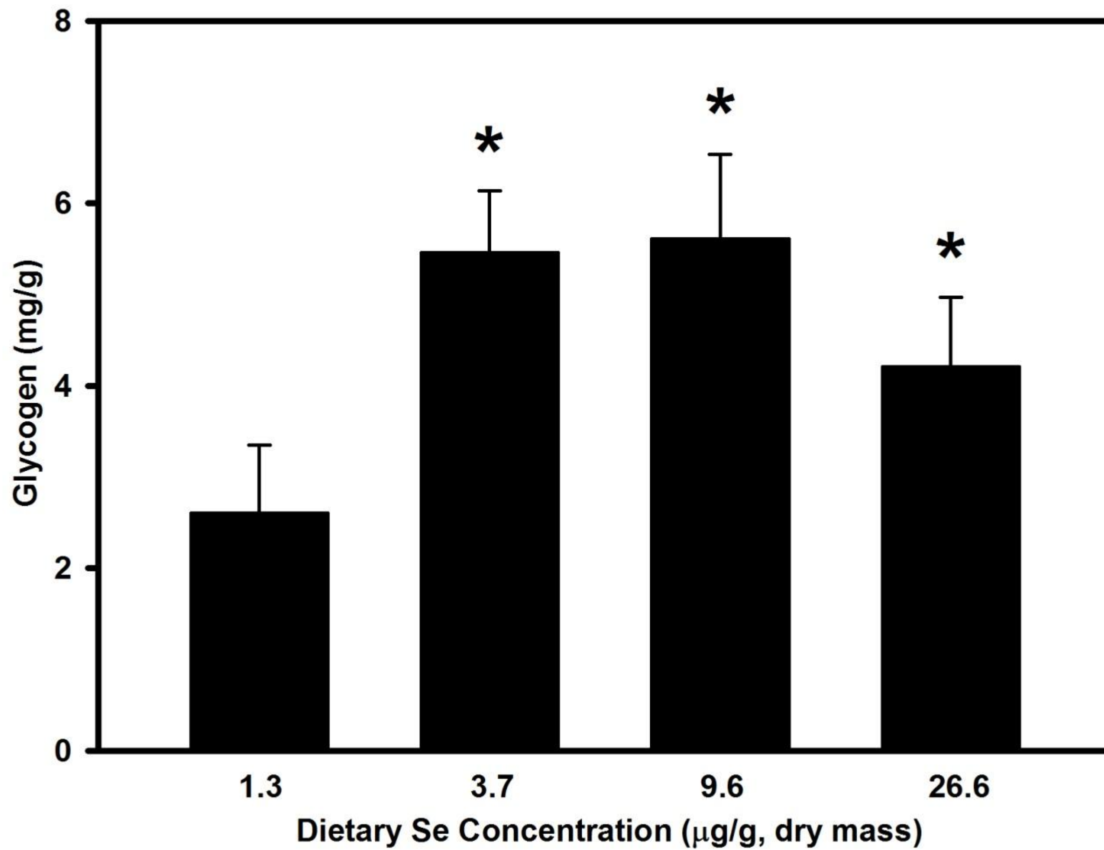

Figure 2.5: Whole-body glycogen in adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 μg Se/g dry mass). Data are mean ± S.E.M of n = 9-14 fish. * Significantly different from control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$).

2.4.8 Cortisol

Whole-body cortisol concentrations in control, 3.7 and 9.6 µg Se/g fed fish were not significantly different (Figure 2.6). Fish fed 26.6 µg Se/g had significantly greater levels of cortisol when compared to control fish ($p < 0.05$). Cortisol levels in the 26.6 µg Se/g and control diet fed fish were 29.9 ± 3.4 and 20.3 ± 2.7 pg/mg, respectively.

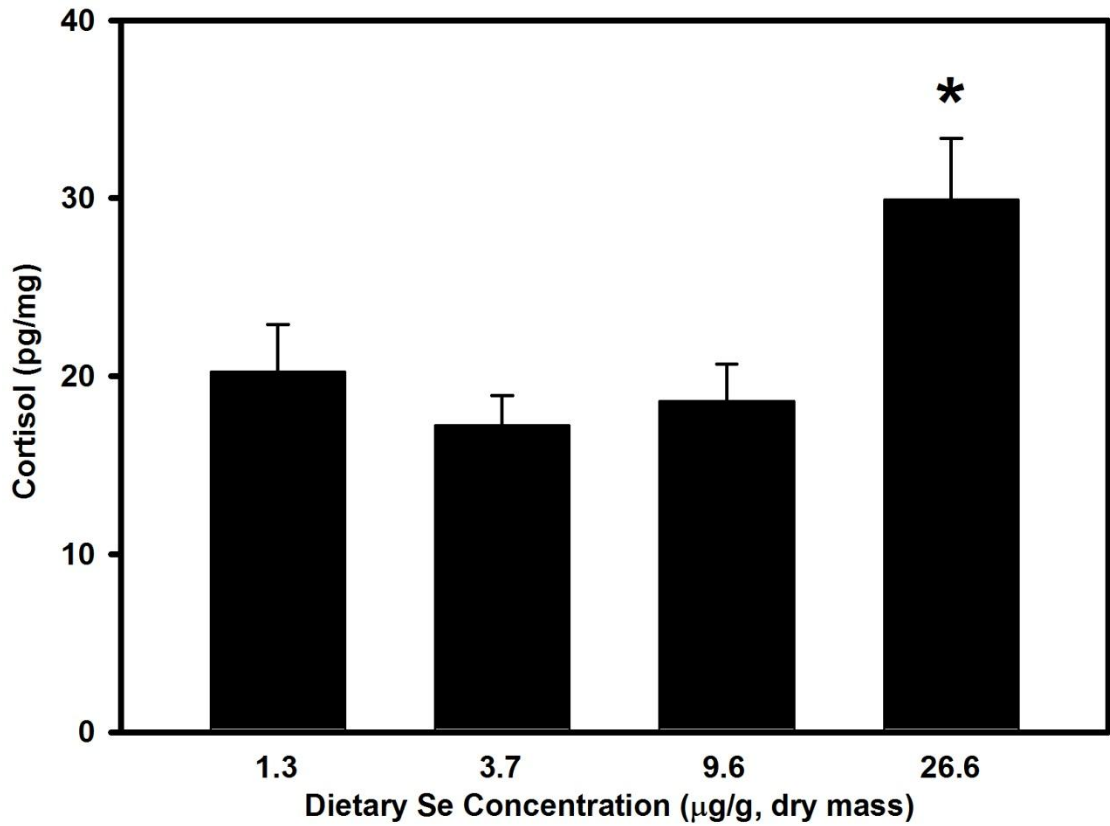

Figure 2.6: Whole-body cortisol in adult zebrafish fed different concentrations of selenomethionine (1.3, 3.7, 9.6, and 26.6 μg Se/g dry mass). Data are mean ± S.E.M of n = 9-14 fish. * Significantly different from control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$).

2.5 Discussion

The results of the present study suggest that chronic sublethal dietary SeMet exposure can alter both behavioral and physiological responses in adult zebrafish. The most important findings of this study were impaired swimming performance (U_{crit}) and elevated energy stores (triglycerides and glycogen) in fish fed greater than 3 µg Se/g in the form of SeMet. The dietary Se concentrations in this study were environmentally relevant, and similar Se concentrations have been reported in both fish and invertebrates collected from Se impacted sites (Lemly, 1997a; Fan et al., 2002; Hamilton, 2004; Muscatello et al., 2006; Muscatello and Janz, 2009). Selenium concentrations in whole-body fish samples were directly proportional to Se concentrations in diet. Similar results were also reported in previous dietary SeMet exposure studies (Teh et al., 2004; Tashjian et al., 2006). Since Se has greater assimilation efficiency than other metals and metalloids, accumulation occurs in a concentration-dependent manner from diet (Baines and Fisher, 2002; Campbell et al., 2005). Elevated levels of whole-body and/ or tissue Se in adult female fish were reported to cause developmental toxicities and mortalities in larval fish (Woock et al., 1987; Muscatello et al., 2006). Fish fed greater than 3 µg Se/g in the present study had greater body weights and lengths than control fish. Increased body weight and total length were reported in juvenile burbot (*Lota lota*) and fathead minnow (*Pimephales promelas*) collected from Se-impacted aquatic systems (Bennett and Janz, 2007; Driedger et al., 2009). Increased body weight in zebrafish fed elevated Se in the present study may have been caused by elevated triglycerides and glycogen levels. However, we were unable to explain the observed difference in total length in zebrafish.

In the present study, after 60 d of dietary SeMet exposure adult zebrafish were fed equal rations (2.5% body weight) of either control or SeMet spiked food and clean chironomids for

another 30-40 d. Clean chironomids were fed to fish to improve egg production because in a companion study we also investigated maternal SeMet transfer and toxicities in F1 generation larval and juvenile fish (article in preparation). At the end of the 90 d exposure period, total Se levels in whole-body fish samples were similar or greater than Se levels in Se spiked food. Whole-body Se concentrations in male and female fish fed the 3.7 µg Se/g diet (6-8 µg/g d.m.) were similar to the tissue-based Se criterion to protect aquatic life (7.91 µg/g d.m.) proposed by the United States Environmental Protection Agency (USEPA, 2004). Whole-body Se concentrations in male and female fish fed the 9.6 and 26.6 µg Se/g diets were approximately 1.5 to 3 times greater than the draft USEPA criteria, and represent environmentally relevant Se body burdens (Hamilton, 2004; Lemly, 1997a). Interestingly, there was significantly greater mortality in adult zebrafish fed the highest SeMet diet (26.6 µg/g) compared to the control group, indicating a direct effect on survival at this dietary exposure.

To our knowledge this is the first study investigating forced swimming capacity of adult fish exposed to sublethal levels of SeMet. We observed reduced U_{crit} in fish fed greater than 3 µg Se/g. Previous studies have investigated swimming behavior of juvenile fish after dietary or aqueous Se exposure. Cleveland et al. (1993) reported abnormal swimming in juvenile bluegill (*Lepomis macrochirus*) exposed to waterborne selenite and selenate. Teh et al. (2004) and Tashjian et al. (2006) reported abnormal swimming behavior (reduced activity, swimming belly up, and confinement to tank bottom) in juvenile sacramento splittail (*Pogonichthys macrolepidotus*) and white sturgeon (*Acipenser transmontanus*), respectively, exposed to dietary SeMet. Both Teh et al. (2004) and Tashjian et al. (2006) observed abnormal swimming activity in fish at much higher dietary Se concentrations (> 20 µg Se/g) than the present study (> 3 µg Se/g). The differences in dietary Se concentrations causing impaired swimming in the present

study and in Teh et al. (2004) and Tashjian et al. (2006) may be due to differences in methods used to study swimming behaviour of fish. The present study investigated forced swimming capacity of fish, whereas Teh et al. (2004) and Tashjian et al. (2006) investigated normal swimming activity of fish.

Although the present study could not explain the mechanism underlying impaired U_{crit} in fish fed diets containing > 3μg Se/g, a number of hypotheses can be proposed for the observed negative effect on swim performance. Selenomethionine substitutes for Met during protein synthesis, and increased accumulation of SeMet in muscle proteins appears to alter normal function of muscle (Ganther, 1974; Stadtman, 1974; Diplock and Hoekstra, 1976; Sunde, 1984). Another hypothesis for impaired swimming performance is oxidative stress, since catabolism of SeMet has been reported to cause oxidative stress in fish (Palace et al., 2004). Oxidative stress can impair muscle function by modifying both muscle structure and release of calcium from sarcoplasmic reticulum (Xia et al., 2004; Musaro` et al., 2010) and such alterations in muscle function could impact swimming behavior of fish. Tailbeat amplitudes of fish fed greater than 3 μg Se/g were lower than control fish when they swam at 6.1 cm/s swimming velocity. This result indirectly supports our muscle dysfunction hypothesis, since reduced tailbeat amplitude explains decreased bend or compression of caudal fin muscle during swimming. Further studies are needed to investigate mechanisms of impaired swimming resulting from SeMet exposure in adult fish.

According to Bainbridge (1958), fish swimming with greater tailbeat amplitudes are likely to travel more distance in a single tailbeat than fish with lower tailbeat amplitude. Tailbeat amplitude of control fish was greater than fish fed elevated dietary Se, suggesting control fish swam more efficiently than higher Se-exposed groups and that this was reflected in lower U_{crit}

values. Lemly (1993b) reported increased oxygen consumption (MO_2) in juvenile bluegill sunfish exposed to both waterborne selenite and dietary SeMet. In the present study we also observed consistently increased MO_2 in adult zebrafish fed greater than 3 μg Se/g, but those values were not significantly different from control values due to large variability in individual fish MO_2. Previous respirometry studies also reported large variability in individual fish MO_2 values (Tang et al., 1994; Reidy et al., 2000; Van Ginneken et al., 2009). Oxygen consumption values in the present study were comparable to previously reported MO_2 values in adult zebrafish (Plaut and Gordon, 1994). Oxygen consumption is an indicator of both metabolic rate and energy requirements of fish (Mehrle and Mayer, 1985; MacKinnon and Farrell, 1992). The present study also calculated the energetic cost of swimming (COT). Similar to previous studies, COT in adult zebrafish was decreased with increasing swimming speed (Sepulveda and Dickson, 2000; Clairreaux et al., 2006). However, energy expenditure to travel a unit distance in adult zebrafish was greater when compared to large bodied fish such as tuna (*Euthynnus affinis*), chub mackerel (*Scomber japonicus*) and sea bass (*Dicentrarchus labrax*) (Sepulveda and Dickson, 2000; Claireaux et al., 2006). This is likely because small bodied fish such as zebrafish expend more oxygen and energy to move a unit mass of their body a given distance than large bodied fish (Cutts et al., 2002; Willmer et al., 2005).

Interestingly, in the present study we noticed elevated levels of stored energy (triglycerides and glycogen) in adult zebrafish fed greater than 3 μg Se/g. Similar results were also observed in fish collected from Se impacted sites (Bennett and Janz, 2007; Kelly and Janz, 2008; Driedger et al., 2009). One of the possible explanations for elevated energy stores in fish fed greater than 3 μg Se/g is impaired energy homeostasis. Impairment of energy homeostasis can occur when a toxicant either alters cortisol production or modifies activities of enzymes

involved in energy metabolism (Gimeno et al., 1995; Mommsen et al., 1999; McGeer et al., 2000; Scott et al., 2002). Several previous studies used whole-body cortisol to assess stress in small fish such as zebrafish (Ramsay et al., 2006; Barcellos et al., 2010). Selenium exposure was reported to alter plasma cortisol levels in rainbow trout (*Oncorhynchus mykiss*) (Miller et al., 2007). Except for greater whole-body cortisol in fish fed 26.6 µg Se/g, fish from other treatment groups had similar levels of cortisol, which may indicate normal head kidney cell function. Elevated cortisol production in 26.6 µg Se/g fed fish may be responsible for the observed reduction in glycogen in the same group. Alterations in activity of enzymes involved in energy metabolism such as phosphofructokinase, lactate dehydrogenase, creatine kinase and citrate synthase have been observed in fish after pollutant exposures (Almeida et al., 2001; Konradt and Braunbeck, 2001). In mammals, dietary SeMet exposure is reported to increase triglyceride synthesis by activating fatty acid synthase (FAS) (Mueller et al., 2008). Another study reported increased activity of FAS and elevated liver triglycerides in Atlantic salmon (*Salmo salar*) exposed to a Met deficient diet (Espe et al., 2010). We hypothesize that if SeMet exposure limits both Met absorption and its incorporation into protein, it could also affect lipid metabolism by affecting FAS activity. To date, no studies have explored mechanisms of Se induced dysregulation of energy homeostasis in fish. Further studies are needed to delineate mechanisms of increased stored energy in adult fish exposed to elevated dietary Se.

In summary, we report that environmentally relevant dietary concentrations of SeMet can impair critical swimming speed and increase energy stores (triglycerides and glycogen) in adult zebrafish. Impaired swimming performance may alter food acquisition, prey avoidance and reproduction, and such alterations could impact fitness and survival of wild fish. Altered energy

homeostasis may be responsible for increased energy stores in fish fed elevated concentrations of dietary SeMet.

CHAPTER 3

EFFECTS OF CHRONIC DIETARY SELENOMETHIONINE EXPOSURE ON REPEAT SWIMMING PERFORMANCE, AEROBIC METABOLISM AND METHIONINE CATABOLISM IN ADULT ZEBRAFISH (*DANIO RERIO*)

Preface

The research described in this chapter was a follow-up study of Chapter 2, which further investigated mechanisms of reduced swimming performance and greater energy accumulation in excess dietary SeMet exposed adult zebrafish. Repeat swimming performance, oxygen consumption, metabolic capacities, stored energy concentrations, and gene expression of energy metabolism and methionine catabolism enzymes were determined to investigate mechanisms by which dietary SeMet exert toxicities in adult zebrafish. The results of this study suggest that SeMet-induced alteration in muscle function and aerobic energy metabolism caused impairment in swimming performance in adult fish, and triglyceride accumulation in excess dietary SeMet fed adult zebrafish is caused by impairment in triglyceride catabolism and /or transport.

This chapter was published in Aquatic Toxicology 130-131:112-122, under joint authorship with Steve Wiseman (University of Saskatchewan), John P. Giesy (University of Saskatchewan), and David M. Janz (University of Saskatchewan). The tables, figures and references cited in this article have been re-formatted here to the thesis style. References cited in this chapter are listed in the reference section of this thesis.

3.1 Abstract

In a previous study we reported impaired swimming performance and greater stored energy in adult zebrafish (*Danio rerio*) after chronic dietary exposure to selenomethionine (SeMet). The goal of the present study was to further investigate effects of chronic exposure to dietary SeMet on repeat swimming performance, oxygen consumption (MO_2), metabolic capacities (standard metabolic rate [SMR], active metabolic rate [AMR], factorial aerobic scope [F-AS] and cost of transport [COT]) and gene expression of energy metabolism and methionine catabolism enzymes in adult zebrafish. Fish were fed SeMet at measured concentrations of 1.3, 3.4, 9.8 or 27.5 µg Se/g dry mass (d.m.) for 90 d. At the end of the exposure period, fish from each treatment group were divided into three subgroups: (a) no swim, (b) swim, and (c) repeat swim. Fish from the no swim group were euthanized immediately at 90 d and whole-body triglycerides, glycogen and lactate, and gene expression of energy metabolism and methionine catabolism enzymes were determined. Individual fish from the swim group were placed in a swim tunnel respirometer and swimming performance was assessed by determining the critical swimming speed (U_{crit}). After both U_{crit} and MO_2 analyses, fish were euthanized and whole-body energy stores and lactate were determined. Similarly, individual fish from the repeat swim group were subjected to two U_{crit} tests (U_{crit-1} and U_{crit-2}) performed with a 60 min recovery period between tests, followed by determination of energy stores and lactate. Impaired swim performance was observed in fish fed SeMet at concentrations greater than 3 µg Se/g in the diet. However, within each dietary Se treatment group, no significant differences between single and repeat U_{crits} were observed. Oxygen consumption, SMR and COT were significantly greater, and F-AS was significantly lesser, in fish fed SeMet. Whole-body triglycerides were proportional to the concentration of SeMet in the diet. While swimming resulted in lesser concentrations of

glycogen in the body, exposure to SeMet in the diet had no significant effect on glycogen content. Exposure to SeMet significantly down-regulated mRNA abundance of protein tyrosine phosphatase 1B (PTP 1B) in muscle, and β-hydroxyacyl coenzyme A dehydrogenase (HOAD), sterol regulatory element binding protein 1 (SREBP 1) and methionine adenosyltransferase 1α (MAT 1A) in liver of adult zebrafish. Overall the results of this study suggest chronic exposure of adult zebrafish to SeMet in the diet can cause both cellular and organismal effects that could affect fitness and survivability of fish.

3.2 Introduction

Among essential trace elements, selenium (Se) is reported to have a narrow margin between beneficial and toxic effects (Janz et al., 2010). For example, fish require dietary Se concentrations of 0.5-1.0 µg Se/g dry mass (d.m.) to maintain normal physiological homeostasis. However, concentrations >3 µg Se/g d.m. in the diet can result in bioaccumulation of Se and cause toxicosis (Lemly, 1997a; Janz et al., 2010). Concentrations of Se in both terrestrial and aquatic environments are increasing due to expansion of mining (coal, uranium, phosphate), power generation (coal-fired power plants) and agriculture (Janz et al., 2010; Janz, 2011). Selenium enters aquatic ecosystems as inorganic selenate and selenite from both point and non-point sources, and these oxyanions are sufficiently soluble to be mobile in water. In aquatic ecosystems, primary producers, such as algae, can convert inorganic forms of Se into organic forms, such as selenomethionine, selenocysteine and selenoproteins. Such organic forms have greater potential to bioaccumulate and be transferred through trophic webs than inorganic Se (Fan et al., 2002). Selenomethionine (SeMet) is the dominant form of selenium (Se) present in food (Fan et al., 2002). Since the molecular structure of SeMet resembles the essential amino acid methionine, absorption and accumulation of SeMet and subsequent incorporation into proteins occurs in a concentration dependent fashion (Behne et al., 1991; Bakke et al., 2010). In aquatic ecosystems contaminated with excess concentrations of Se, 50-70% of total Se occurs in both primary producers and consumers as SeMet (Fan et al., 1998, 2002; Phibbs et al., 2011). Although primary producers and invertebrates accumulate substantial concentrations of SeMet, they are tolerant of SeMet. In contrast, oviparous species, including fish, birds, and amphibians are more susceptible to toxic effects of SeMet (reviewed in Janz et al., 2010; Janz, 2011), and

hence there is a need to investigate mechanisms of toxicity of dietary SeMet to oviparous species.

Exposure of adult fishes to SeMet in the diet is known to cause developmental abnormalities and mortality in F1 larval fish (Lemly, 1997a; Janz et al., 2010). In adult female fish, SeMet is deposited into eggs during vitellogenesis. Subsequent utilization of SeMet in yolk by developing embryos causes abnormalities and mortality of larval fish. Exposure of juvenile fishes to SeMet via the diet has also been reported to attenuate physiological responses to stressors and to modulate endocrine function (Teh et al., 2004; Wiseman et al., 2011a,b). A general notion among aquatic toxicologists is that adult fish are tolerant of chronic exposure to SeMet. Although acute toxicity of Se to adult fishes has been investigated at concentrations that are not environmentally relevant, less information is available on chronic Se toxicity in adult fishes. A previous study demonstrated that dietary exposure of adult zebrafish (*Danio rerio*) to SeMet in the diet resulted in lesser swimming performance, caused accumulation of stored energy and resulted in greater production of cortisol and greater body mass (Thomas and Janz, 2011).

Swimming performance is an important fitness trait in wild fish as it is closely linked to food acquisition, predator avoidance, reproduction, schooling and migration (Hammer, 1995; Plaut, 2001) and has been used as an ecologically relevant endpoint for the assessment of sublethal toxicity to fish (Little and Finger, 1990). The most commonly used test of swimming performance is critical swimming speed (U_{crit}) (Brett, 1964), which challenges fish with incremental changes in velocity until exhaustion. In addition to single exercise U_{crit}, studies have also investigated repeat U_{crit}, where fish are subjected to two successive U_{crit} tests (U_{crit-1} and U_{crit-2}) with a short recovery period, normally 40-60 min, between tests (Jain et al., 1998;

McKenzie et al., 2007). Repeat swimming performance is commonly used to investigate rates of recovery of fish after exposure to a stressor and is considered a more sensitive indicator of fish health than single exercise U_{crit} (Jain et al., 1998). Recovery ratio (U_{crit-2}/ U_{crit-1}) is a relevant measure of the ability of fish to recover from exhaustive exercises (Jain et al., 1998). Stressor exposures have been shown to cause impaired performance in repeat swimming trials and a lesser recovery ratio (Jain et al., 1998; McKenzie et al., 2007).

Oxygen consumption (MO_2) is a measure of metabolic activity of fish and is often measured in combination with U_{crit}. Alteration in MO_2 is an indicator of stress in fish and a number of toxicants including Se are reported to alter MO_2 in fish (Lemly, 1993b; Scott and Sloman, 2004). Swimming respirometry has been used by both fish physiologists and toxicologists to investigate metabolic capacities such as standard metabolic rate (SMR), which is the minimal maintenance metabolic rate of unfed fish, active metabolic rate (AMR), which is the metabolic rate of fish at maximum sustainable velocity in U_{crit} tests, aerobic scope (AS), which is the difference between AMR and SMR, factorial aerobic scope (F-AS), which is the ratio of AMR to SMR, and cost of transport (COT), which is a measure of the energetic cost of swimming (Videler, 1993; Webber et al., 1998; Shingles et al., 2001; Claireaux et al., 2006; McKenzie et al., 2007; Killen et al., 2007). Stressors, including temperature and chemicals have been shown to alter both metabolic expenditure and energetic cost of swimming in fish (Shingles et al., 2001; Claireaux et al., 2006; McKenzie et al., 2007). Triglycerides (triacylglycerols) and glycogen are the major forms of stored energy in fish and are usually used during swimming. In fish, both sustained and prolonged swimming are powered by slow oxidative red muscle fibers and are fuelled primarily by aerobic catabolism of triglycerides (Hammer, 1995; Moyes and West, 1995). Critical swimming speed is a measure of prolonged aerobic swimming capacity of

fish, and triglycerides are used as the primary energy source up to approximately 80% of U_{crit} (Hammer, 1995; Moyes and West, 1995; Plaut, 2001). Alternatively, burst swimming and swimming speed beyond 80% U_{crit} in fish are powered by fast glycolytic white muscle fibers, where the majority of energy comes from anaerobic catabolism of glycogen (Hammer, 1995; Moyes and West, 1995). Lactate, a by-product of anaerobic energy catabolism, accumulates in muscle during burst swimming and serves as a measurable indicator of anaerobic metabolic activity.

Greater accumulation of stored energy was reported in zebrafish and rainbow trout (*Oncorhynchus mykiss*) exposed to augmented dietary SeMet (Thomas and Janz, 2011; Wiseman et al., 2011b) and in native fishes collected from Se-impacted field sites (Kelly and Janz, 2008; Driedger et al., 2009; Goertzen et al., 2012). Stressor exposures have been shown to alter energy metabolism by either impairing MO_2 or altering activity or expression of energy metabolism enzymes (Rajotte and Couture, 2002; McClelland et al., 2006; Goertzen et al., 2011, 2012). Commonly studied enzymes that are associated with aerobic metabolism are citrate synthase (CS) and β-hydroxyacyl coenzyme A dehydrogenase (HOAD). Citrate synthase is a key enzyme involved in the citric acid cycle whereas HOAD is involved in metabolism of triglycerides. Altered expressions or activities of CS and HOAD have been reported to occur after exposure to stressors (Rajotte and Couture 2002; McClelland et al., 2006; Goertzen et al., 2011, 2012). In mammals, exposure to Se via the diet has been shown to result in greater accumulation of triglycerides and cause up-regulation of expression or activities of enzymes, including, protein tyrosine phosphatase 1B (PTP 1B), fatty acid synthase (FAS) and a transcription factor, sterol regulatory element binding protein 1 (SREBP 1), involved in synthesis of fatty acids (Mueller et al., 2008, 2009a,b). In addition, restricted dietary intake of methionine has been reported to

cause greater activity of FAS and accumulation of triglycerides in liver of fish (Espe et al., 2010). Methionine is an important precursor in the synthesis of S-adenosylmethionine (SAM), which is an important donor of methyl groups in multiple methyltransferase reactions. Methionine adenosyltransferase (MAT) is an enzyme responsible for synthesis of SAM. Expression of methionine adenosyltransferase 1α (MAT 1A), a liver-specific MAT gene, can be used as a measure of methionine catabolism, steady-state SAM production and methylation (Lu, 2000; Mato et al., 2002). Exposure to either selenite or selenocystine has been shown to deplete SAM and inactivate MAT in mammalian liver (Hoffman, 1977; Hasegawa et al., 1996). Exposure of mammals to dietary Se has been shown to alter both metabolism of triglycerides and catabolism of methionine, but to date no such studies have been conducted in fishes. Since our previous study showed reduced swimming performance and greater accumulation of stored energy in adult zebrafish exposed to elevated dietary SeMet (Thomas and Janz, 2011), the objectives of present study were to further investigate effects of chronic dietary SeMet exposure on repeat swimming performance, and to elucidate possible mechanisms of SeMet induced impairment of swimming performance and greater accumulation of stored energy in adult zebrafish.

3.3 Materials and methods

3.3.1 Test chemical and test species

Seleno-L-methionine (purity >98%) was purchased from Sigma-Aldrich (Oakville, ON, Canada). Adult zebrafish were purchased from a local supplier and housed in an environmental chamber with controlled temperature (28.0 ± 1.0 °C) and photoperiod (14 h light and 10 h dark). Mean standard length of fish was 2.8 cm and ranged from 2.6 to 3.1 cm. Fish were introduced (25 fish/tank) into twelve 40 L glass aquaria with continuous aeration and filtration, after which

treatments were randomly assigned to aquaria, with n = 3 replicate aquaria per treatment. Fish were acclimated for 3 weeks to laboratory conditions, and during this time they were fed Nutrafin® basic flake food (Hagen Inc., Montreal, QC, Canada).

3.3.2 Diet preparation and experimental design

Nominal concentrations of 3, 10 or 30 µg Se/g d.m. in the form of SeMet were added to Nutrafin flake food as described previously (Thomas and Janz, 2011). The control diet was prepared by adding an equivalent volume of water without SeMet to food. Food was lyophilized in a freeze dryer (Dura-DryTM MP, FTS Systems, Stone Ridge, NY, USA). Freeze dried diets were crushed into flakes and stored at –20 ºC in air tight containers. Representative samples of these diets were collected for determination of total concentrations of Se.

All methods applied in the present study were approved by the University of Saskatchewan's Animal Research Ethics Board (protocol # 20030076), and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Fish were fed twice daily (5% body weight/d ration) with either control or SeMet spiked foods for 90 d. Fish were allowed to feed for 2 h, after which excess food was siphoned from the aquarium bottom. During the feeding experiment, 75% of water was renewed from each aquarium every day. Water samples (n = 1 from each aquaria) for quantification of dissolved Se were collected 5 h after feeding on day 30 of the feeding experiment. These water samples (n = 3 from each treatment) were filtered using 0.45 µm disposable filters, acidified to pH < 2 using ultra-pure nitric acid, and stored in 25-mL high density polyethylene (HDPE) bottles at 4 ºC until Se analysis. After 90 d, fish from each treatment group were divided into three subgroups: (a) no swim, (b) swim, and (c) repeat swim. Fish from the no swim group were euthanized immediately at 90 d and whole-body Se levels (n = 3–5), and whole-body concentrations of triglycerides, glycogen and lactate (n = 6–7)

were determined. In addition, liver and muscle (n = 4) were dissected from adult fish in each treatment group, immediately flash-frozen using liquid nitrogen, and stored at –80 °C until gene expression analyses of energy metabolism and methionine catabolism enzymes. Individual fish from the swim group were placed in a swim tunnel respirometer and swimming performance was determined using the critical swimming speed (U_{crit}) method (Brett, 1964). After both U_{crit} and MO_2 analyses, fish were euthanized and whole-body energy stores and lactate were determined. Similarly, individual fish from the repeat swim group were subjected to two U_{crit} tests (U_{crit-1} and U_{crit-2}) performed with a 60 min recovery period between tests, followed by determination of whole-body energy stores and lactate.

3.3.3 Quantification of selenium

Total concentrations of Se in fish, food and water samples were measured by use of inductively coupled plasma-mass spectrometry (ICP-MS) at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK, Canada). Prior to analysis to determine concentrations of Se, whole-body fish and samples of food were lyophilized and homogenized by use of a mortar and pestle. Moisture content of whole zebrafish was 73.7 ± 1.0 %. Aliquants of 100 mg of homogenized samples were cold digested in Teflon vials by use of 5 mL of ultra-pure nitric acid and 1.5 mL of hydrogen peroxide. After digestion, samples were concentrated on a hot plate (<75 ºC) and reconstituted in 5 mL of 2% ultrapure nitric acid. Reconstituted samples were stored at 4 ºC until quantification. A LOQ of 0.5 µg Se/g was determined using method blanks. Selenium recovery was determined using certified reference material (TORT-2, lobster hepatopancreas, NRC, Ottawa, ON, Canada).

3.3.4 Oxygen consumption and swim performance

Consumption of oxygen and swim performance were conducted in a modified Blazka-type, variable speed, miniature swim tunnel respirometer with a DAQ-M control device and AutoResp™ 1 software (Loligo Systems, Tjele, DK). The system consists of a 170 mL swim tunnel submerged in a 20 L buffer tank supplied with 28.5 ± 0.1 ºC aerated water from a 20 L heated water bath circulator (VWR International, Mississauga, ON, Canada). Measurement of the rate of consumption of oxygen (MO_2) was performed by automated intermittent-flow respirometry in loops of 10 min. Each loop consisted of a 5 min measuring phase followed by a 4 min flushing phase and a 1 min waiting phase. Concentrations of oxygen (O_2) were measured using a fiber optic oxygen dipping probe which was connected to a Fibox 3 minisensor oxygen meter (Precision Sensing GmbH, Regensburg, DE). AutoResp™ 1 software was used to calculate MO_2 and the detailed MO_2 measuring principle is explained elsewhere (Steffensen et al., 1984).

To establish a stabilized minimal rate of metabolism for adult zebrafish after being introduced in the swim tunnel respirometer, fish were acclimated for 1.5–2 h at a minimal water velocity of 0.8 BL/s (body length per second). Fish were not fed 24 h prior to MO_2 and U_{crit} analyses. In the U_{crit} experiment, individual fish were subjected to step-wise increments in swimming velocity (2.7 BL/s every 20 min) until exhaustion. On occasions where fish stopped swimming and rested at the downstream portion of the swim tunnel for the first time, water velocity was reduced for 15 s to initiate swimming and then the velocity was returned to the previous set point. In the repeat U_{crit} experiment, fish were subjected to two successive U_{crit} tests ($U_{crit\text{-}1}$ and $U_{crit\text{-}2}$) with a 60 min recovery period between tests. Critical swimming speed was calculated using the equation previously described (Brett, 1964). Fish cross sectional area was

less than 5% of swim tunnel cross sectional area, hence U_{crit} values were not corrected for solid blocking effect. Critical swimming speed values were corrected for standard body length of each individual fish, and thus U_{crit} values were represented as body lengths per second (BL/s). The recovery ratio of individual fish from the repeat swim test was calculated by dividing the results of the two swim tests (U_{crit-2}/ U_{crit-1}) (Jain et al., 1998). Two MO_2 measurements were determined in each water velocity increment, and the average MO_2 value was used for statistical analysis. After the U_{crit} and MO_2 analyses, fish were euthanized using an overdose of MS 222 (1g/L) and stored at –80 °C for subsequent whole-body triglyceride, glycogen and lactate analyses. Prior to storage, total body length, wet mass, and condition factor were determined for each fish.

3.3.5 Determination of standard metabolic rate (SMR), active metabolic rate (AMR), factorial aerobic scope (F-AS) and cost of transport (COT)

Standard metabolic rate (SMR) was calculated by extrapolating consumption of O_2 back to a water velocity of zero. This was done from a plot of swimming speed (m/s) versus MO_2 (mg O_2/kg/h) and use of nonlinear, curve fitting regression analysis (Webber et al., 1998; Shingles et al., 2001). Active metabolic rate (AMR) is defined as the MO_2 at maximum sustainable speed in the U_{crit} test. The factorial aerobic scope (F-AS) was calculated as AMR/SMR (Webber et al., 1998; Shingles et al., 2001; Killen et al., 2007). Cost of transport (J/kg/m) (COT) was calculated by multiplying MO_2 (mg O_2/kg/s) by an oxycaloric value of 14.1 J/mg O_2 and then dividing by the corresponding swimming speed (m/s) (Videler, 1993).

3.3.6 Quantification of triglycerides and glycogen

Concentrations of triglycerides in whole-body of adult zebrafish samples were measured by use of a kit prepared by Sigma-Aldrich (Oakville, ON, Canada), which follows the McGowan et al. (1983) method. A glycerol solution was used for the standard curve. This method has been previously validated in our laboratory for measuring triglycerides in whole fish homogenates (Kelly and Janz, 2008). Concentrations of glycogen in whole-body adult zebrafish were measured by use of use a modified version of the method of Gómez-Lechón et al. (1996). Glycogen assay reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada). The standard curve was created using purified Type IX bovine liver glycogen. This method has been previously validated in our lab for use in whole fish homogenates (Goertzen et al., 2011, 2012).

3.3.7 Lactate assay

Whole-body concentrations of lactate were measured by use of a commercially available kit according to the manufacturer's instructions (Eton Bioscience Inc., San Diego, CA, USA). The kit method is based on reduction of tetrazolium salt INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) to formazan (a dark red coloured product), and absorbance was measured at 490 nm.

3.3.8 Real-time polymerase chain reaction (PCR)

Expression of mRNA for genes coding for enzymes or transcription factor of interest were quantified by use of quantitative polymerase chain reaction (Q-PCR). Total RNA was extracted from approximately 30 mg of liver using the RNeasy Plus Mini Kit (Qiagen, Mississauga, ON, Canada) and muscle using RNeasy Lipid Tissue Mini Kit (Qiagen) according

to the manufacturer's instructions. Purified total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Integrity of RNA was checked on a 1% denaturing formaldehyde–agarose gel with ethidium bromide and visualized under ultraviolet (UV) light on a VersaDoc 4000MP imaging system (Bio-Rad, Mississauga, ON, Canada). Purified RNA samples were stored at −80 ºC until synthesis of cDNA. A QuantiTect® Reverse Transcription Kit (Qiagen) was used to synthesis cDNA from 1 μg total RNA. The cDNA samples were stored at −20 ºC until further analysis.

Quantitative real-time PCR (Q-PCR) was performed in 96-well PCR plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Gene specific primers were designed against target genes by use of Primer 3 software, and the sequences of primers are shown (Table 3.1). A separate 45 μL PCR mixture consisting of Power SYBR Green master mix (Applied Biosystems), an optimized volume of cDNA, gene specific primers, and nuclease free water was prepared for each cDNA sample and primer pair. A final reaction volume of 20 μL was transferred to each well and reactions were performed in duplicate. The PCR mixture was denatured at 95 ºC for 10 min before the first PCR cycle. The thermal cycle profile was as follows: denature for 10 s at 95 ºC and extension for 1 min at 60 ºC for a total of 40 PCR cycles. Optimal qPCR conditions were established by determining the efficiency of each qPCR assay by with a standard curve of serially diluted cDNA standards. Target gene mRNA abundance was quantified by normalizing to the expression of elongation factor 1α (EF 1α) according to the Mean Normalized Expression (MNE) method of Simon (2003).

Table 3.1: Gene-specific primer sequences for the quantitative real time PCR used in this study

Target	Accession #	Sequence (5'–3')	Annealing temp.
EF 1α	NM_131263.1	F: CTTCAACGCTCAGGTCATCA	60
		R: CGGTCGATCTTCTCCTTGAG	
FAS	XM_682295	F: TGAAGCGGAGGCAGAAA	60
		R: CAAGCAGTGGCGTAAGG	
SREBP 1	NM_001105129.1	F: ACATGGCCCTCAAAATGAAC	60
		R: GTCTGAAGCTGGAGGAGTGG	
PTP 1B	NM130924	F: CTTCACCGAGAGCATCACAA	60
		R: GTTCGTCGGGTTGTTCATTT	
HOAD	NM_001003515.1	F: CTGGTGGTGGAGGCTATTGT	60
		R: ACGTGTTGCTTGCGAATATG	
CS	BC045362	F: ATCCGTTTCCGTGGTTACAG	60
		R: AGACAGCCAACTGACCTGCT	
MAT 1A	NM_199871.1	F: ATGCAGTTCTTGACGCACAC	60
		R: TGGTGTCTCGCACAATCTTC	

3.3.9 Statistical analyses

All data were tested for normality by use of the Shapiro–Wilk test and homogeneity of variance was investigated by use of Levene's test (SigmaStat 3.1, SPSS Inc., Chicago, IL, USA). Data that did not meet the assumptions for parametric statistical procedures were log10 transformed. Non-transformed data are shown in all figures. Significant differences in total Se concentrations in foods, water and whole-body fish as well as total length, wet mass, condition factor, SMR, AMR, F-AS and recovery ratios, and mRNA abundance of energy metabolism and methionine catabolism enzymes of fish fed control or SeMet spiked diets were tested by use of one-way ANOVA followed by the Holm-Sidak post hoc, multiple range test.

Effects of the Se treatment factor (main effect) and swim challenge factor (swim versus repeat swim) on U_{crit} of adult fish, and Se treatment factor and swim status factor (no swim, swim and repeat swim) on whole-body triglyceride, glycogen and lactate were tested by use of two-way ANOVA. There were no interactions between factors for all two-way ANOVAs. When two-way ANOVA showed significant differences, Holm-Sidak post hoc tests were performed to compare differences between control versus SeMet exposed treatment groups, and no swim versus swim or repeat swim groups. At each incremental water velocity tested in U_{crit} analysis, repeated measures ANOVA (RM-ANOVA) followed by Holm-Sidak post-hoc test was used to test for differences in MO_2 and COT of adult fish fed control food or SeMet. Since t-tests did not reveal significant differences between U_{crit} of swim and first U_{crit} (U_{crit-1}) of repeat swim groups within each dietary treatment group, those values were combined for subsequent statistical analysis. A similar approach was followed for both MO_2 and COT data. Data were expressed as mean ± S.E.M. Differences were considered statistically significant at $p \leq 0.05$.

3.4 Results

3.4.1 Concentrations of selenium

The total concentrations of Se in non-spiked (control) and spiked foods (nominal concentrations 3, 10 and 30 µg/g d.m.) were 1.3, 3.4, 9.8 and 27.5 µg Se/g d.m., respectively (Table 3.2). Total concentrations of Se in spiked foods were significantly greater than the control diet ($p < 0.05$). Concentrations of dissolved Se in water from the 1.3, 3.4, 9.8 or 27.5 µg Se/g d.m. fed fish aquaria were 0.3, 0.3, 0.5 and 1.1 µg/L, respectively. Concentrations of dissolved Se in all treatment groups were comparable to the control group with the exception of dissolved Se in aquaria of the greatest SeMet group ($p < 0.05$). Whole-body concentrations of Se in adult zebrafish fed 1.3, 3.4, 9.8 or 27.5 µg Se/g d.m. were 1.6, 4.0, 7.6 and 11.2 µg Se/g d.m., respectively. Although concentrations of Se in adult fish increased with increasing concentrations of SeMet in the diet, only fish fed the greatest concentrations of SeMet had significantly greater whole-body concentrations of Se when compared to the control ($p < 0.05$).

3.4.2 Survival and growth

Mean mortality and condition factor in fish fed greatest concentrations of dietary SeMet were greater than the controls (Table 3.2). Mean mortality in fish fed 1.3, 3.4, 9.8 or 27.5 µg Se/g d.m. was 14.7 %, 20.0 %, 26.7 % and 34.7%, respectively. Mortality was significantly greater for fish fed the 27.5 µg Se/g d.m. than for the controls ($p <0.05$). There were no statistically significant differences in total length or body mass between fish fed SeMet or control diets. However mean condition factors of fish fed the two greatest concentrations of dietary SeMet (1.07 and 1.14) were significantly greater when compared to the control group (0.90) ($p <0.05$).

Table 3.2: Total selenium concentrations in food and whole fish (μg/g, dry mass), mortality and morphometrics of adult zebrafish fed control or selenomethionine spiked foods for 90 d. Data are mean ± S.E.M of n = 3-5 samples for food and fish Se analyses and n = 20-21 for morphometrics.

Dietary Se (μg/g d.m.)	Fish Se (μg/g d.m.)	Mortality (%)	Total length (mm)	Wet mass (g)	Condition factor
1.3 ± 0.01	1.6 ± 0.06	14.7 ± 3.5	38.1 ± 0.3	0.50 ± 0.02	0.90 ± 0.03
3.4 ± 0.09*	4.0 ± 1.00	20.0 ± 2.3	36.7 ± 0.5	0.49 ± 0.03	0.99 ± 0.03
9.8 ± 0.24*	7.6 ± 2.58	26.7 ± 3.5	37.5 ± 0.5	0.56 ±0.03	1.07 ± 0.07*
27.5 ± 1.02*	11.2 ± 1.72*	34.7 ± 3.5*	36.4 ± 0.6	0.55 ± 0.02	1.14 ± 0.04*

Condition factor = (wet mass/total length^3) x 100,000.

* Significantly different from the control group using one-way ANOVA followed by Holm-Sidak post hoc test (p <0.05).

3.4.3 Swim performance and oxygen consumption

Swimming performance was significantly reduced by the dietary Se treatment factor (p = 0.003 for Se treatment factor in two-way ANOVA; Figure 3.1) but not the swim challenge factor (p = 0.717 for swim challenge factor in two-way ANOVA; Figure 3.1). The U_{crit} for adult zebrafish fed diets augmented with SeMet was significantly lesser than that of fish fed the control diet. There were no differences in U_{crit} between swim (U_{crit-1}) and repeat swim (U_{crit-2}) fish within each treatment group. Recovery ratios for adult zebrafish fed 1.3, 3.4, 9.8 or 27.5 µg Se/g d.m. were 0.99 ± 0.05, 1.03 ± 0.03, 0.94 ± 0.05 and 0.98 ± 0.32, respectively. Recovery ratios for fish fed SeMet were not significantly different from that of the control group.

Mean MO_2 of both control and fish fed elevated SeMet were directly proportional to swimming speed (Figure 3.2). Mean routine MO_2, which is MO_2 measured at the least water velocity, of fish fed SeMet were significantly greater than that of the control group ($p < 0.05$). Fish fed elevated SeMet had significantly greater MO_2 than fish fed the control diet at all incremental water velocities tested with the exception of 0.17 and 0.48 m/s ($p < 0.05$; Figure 3.2). Mean fatigue MO_2 of fish fed 1.3, 3.4, 9.8 or 27.5 µg Se/g d.m. were 2572 ± 162.4, 2553 ± 124.1, 2305 ± 106.2 and 2238 ± 161.7 mg O_2/kg/h, respectively. There were no significant differences in fatigue MO_2 among control and fish fed elevated SeMet.

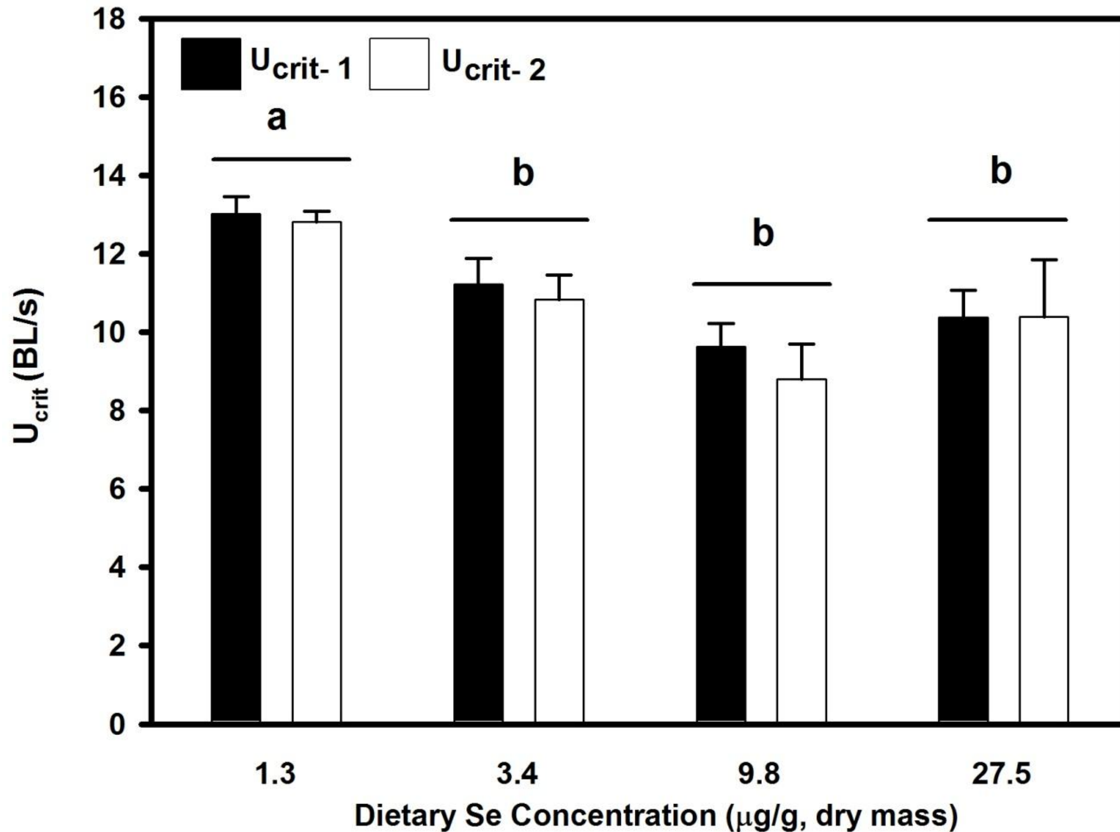

Figure 3.1: Critical swimming speed (U_{crit}) in body lengths per second (BL/s) of adult zebrafish fed control (1.3 μg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 μg Se/g d.m.) for 90 d. Solid bars are single exercise critical swimming speed (U_{crit-1}) and open bars are repeat critical swimming speed (U_{crit-2}). Critical swimming speed was significantly altered by the dietary selenium treatment factor ($p = 0.003$) but not the swim challenge factor ($p = 0.717$) in two-way ANOVA. Different lowercase letters denote a significant effect of dietary selenomethionine treatments on U_{crits}. Data are mean ± S.E.M. of n = 12-14 for U_{crit-1} and n = 6-7 for U_{crit-2}.

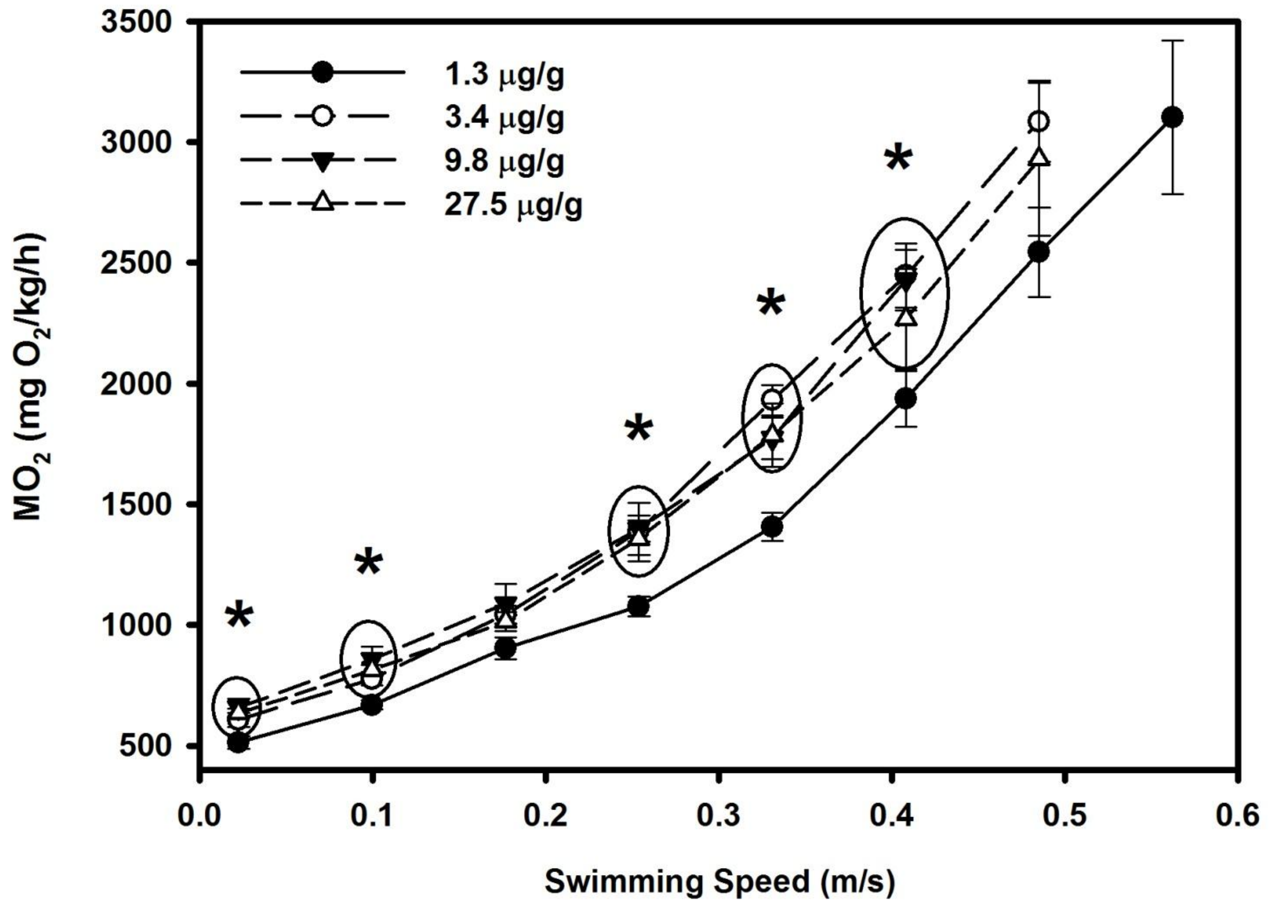

Figure 3.2: Oxygen consumption (MO_2) versus swimming speed in adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. * Significantly different from control group using repeated measures ANOVA followed by Holm–Sidak post hoc tests (p <0.05). Data are mean ± S.E.M. of n = 12-14 fish.

3.4.4 Standard metabolic rate (SMR), active metabolic rate (AMR), factorial aerobic scope (F-AS) and cost of transport (COT)

Elevated dietary SeMet exposure significantly altered metabolic capacities of adult zebrafish (Figure 3.3). Fish fed greater SeMet had significantly greater SMR when compared to fish fed the control diet (p <0.05). Although AMR was lesser in fish fed 9.8 and 27.5 µg Se/g d.m., those values were not significantly different from the control group. Alternatively, F-AS of fish fed 1.3, 3.4, 9.8 or 27.5 µg Se/g d.m were 5.98, 4.82, 4.25 and 3.73, respectively, and F-AS in fish fed the two greatest concentrations of SeMet were significantly lesser than fish fed the control diet ($p < 0.05$; Figure 3.3). Compared to control fish, cost of transport was consistently greater in fish fed diets that had been augmented with SeMet. Cost of transport was significantly greater in fish fed 3.4, 9.8 or 27.5 µg Se/g d.m. compared to control fish at water velocities of 0.09, 0.25, 0.33 or 0.40 m/s (p <0.05; Figure 3.4).

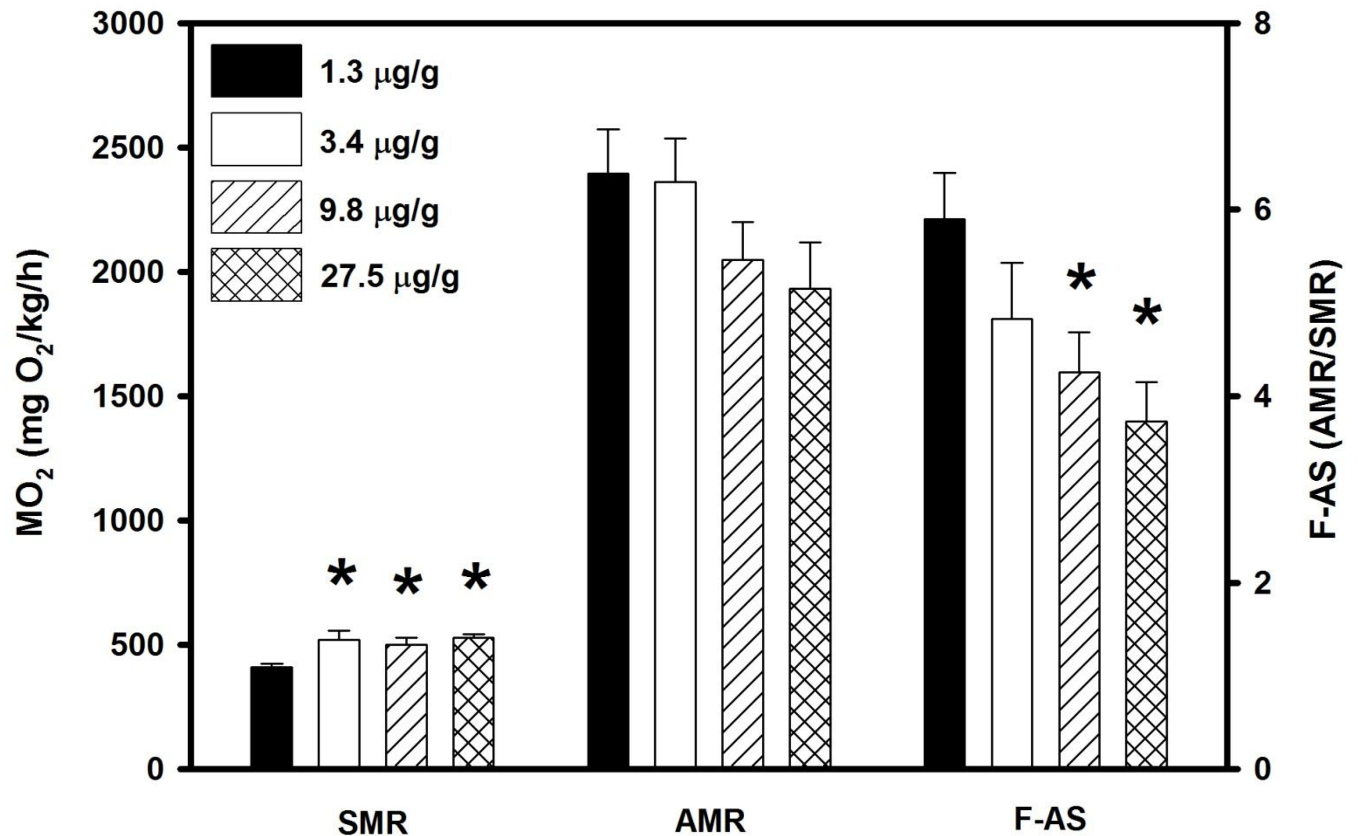

Figure 3.3: Metabolic capacities (standard metabolic rate [SMR; the minimal maintenance oxygen consumption of unfed fish], active metabolic rate [AMR; the oxygen consumption at the maximum sustainable swimming speed in U_{crit}] and factorial aerobic scope [F-AS; the ratio of AMR to SMR]) of adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. * Significantly different from control group using one-way ANOVA followed by Holm-Sidak post hoc tests (p <0.05). Data are mean ± S.E.M. of n = 12-14 fish.

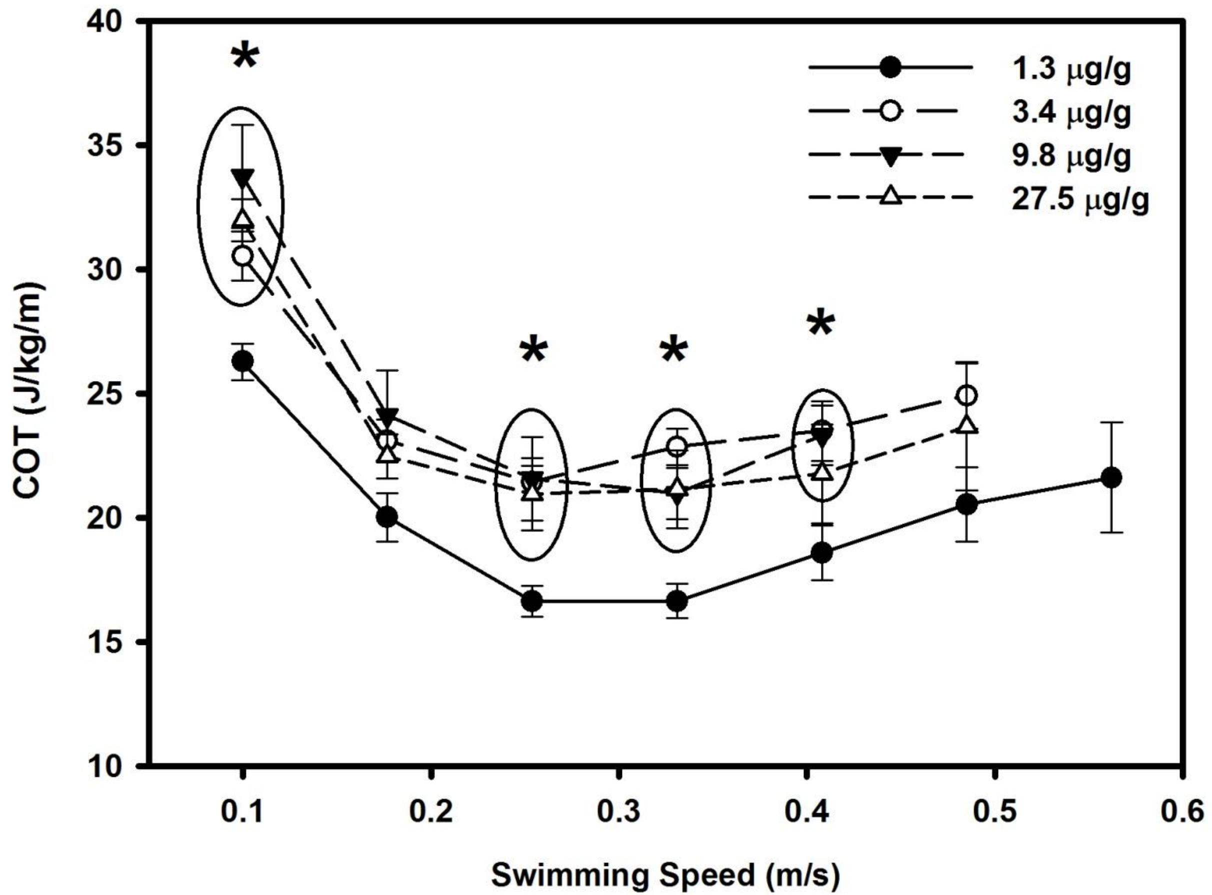

Figure 3.4: Cost of transport (COT) as a function of swimming speed in adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. * Significantly different from control group using repeated measures ANOVA followed by Holm-Sidak post hoc tests (p <0.05). Data are mean ± S.E.M. of n = 12-14 fish.

3.4.5 Whole-body triglycerides and glycogen

Concentrations of triglycerides were significantly altered by both the dietary Se treatment factor and the swim status factor (p <0.001 for Se treatment factor and p = 0.043 for swim status factor in two-way ANOVA; Figure 3.5). Whole-body concentrations of triglycerides were significantly greater in fish fed 9.8 or 27.5 µg Se/g d.m. compared to fish fed the control diet (Figure 3.5). Although two-way ANOVA showed a significant effect of swim status factor on concentrations of whole-body triglycerides in fish, post hoc tests were unable to demonstrate statistically significant differences between no swim versus swim or repeat swim fish within respective dietary Se treatment groups.

The dietary Se treatment factor was not associated with variance of the concentration of glycogen in whole bodies of fish (p = 0.193 for the dietary Se factor in two-way ANOVA; Figure 3.6), but whole-body concentrations of glycogen were significantly associated with the swim status factor (p <0.001 in two-way ANOVA; Figure 3.6). When compared within each concentration of SeMet in the diet, significantly lesser whole-body concentrations of glycogen were observed in repeat swim fish when compared to no swim fish (p <0.05; Figure 3.6).

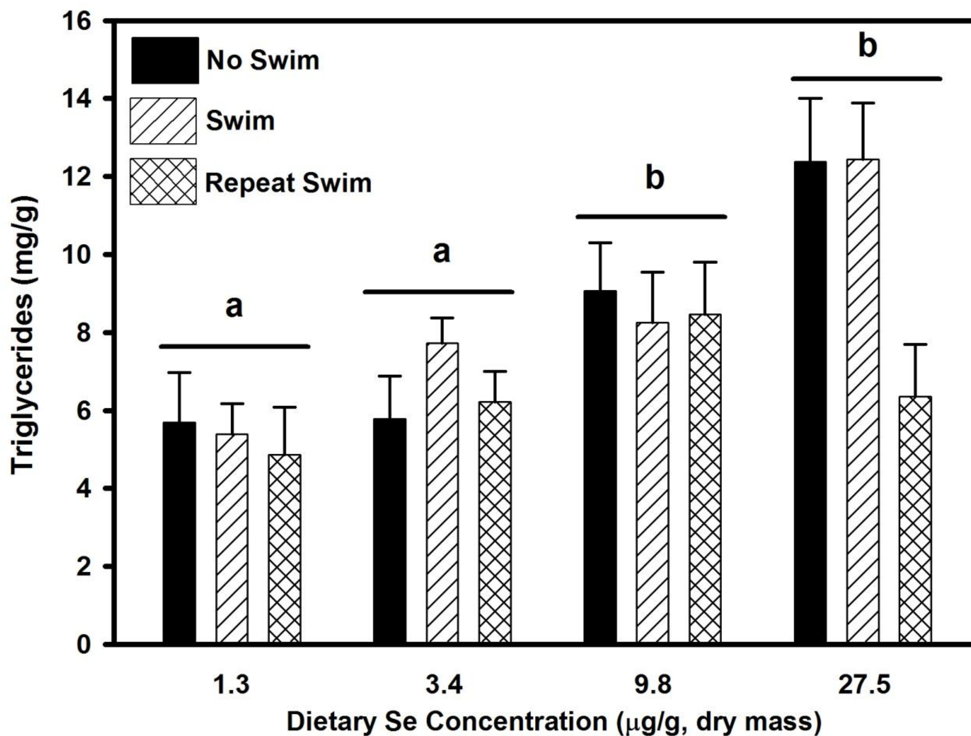

Figure 3.5: Whole-body triglycerides in adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. Solid, striped and crossed bars represent whole-body triglycerides in fish from no swim (fish withheld from swim tests), swim (fish subjected to single U_{crit}) and repeat swim (fish subjected two U_{crits}) groups, respectively. Both dietary selenium treatment factor and swim status factor had significant effects on concentration of whole-body triglycerides in fish (p <0.001 for dietary selenium treatment factor and p = 0.043 for swim status factor in two-way ANOVA). Different lowercase letters denote a significant effect of dietary selenium treatments on concentration of whole-body triglycerides in adult fish. Post hoc tests did not show any significant differences of swim status factor on whole-body triglycerides in respective dietary selenium treatment groups. Data are mean ± S.E.M. of n = 6-7 fish

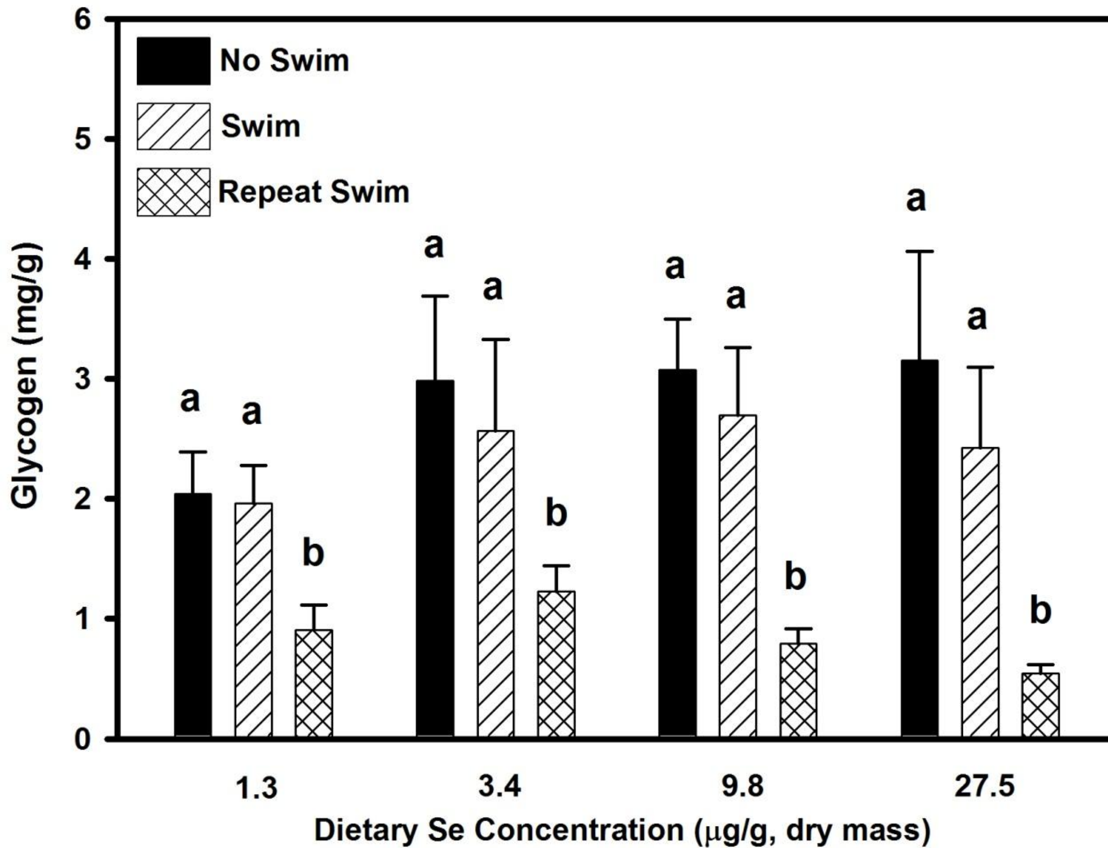

Figure 3.6: Whole-body glycogen in adult zebrafish fed control (1.3 μg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 μg Se/g d.m.) for 90 d. Solid, striped and crossed bars represent whole-body glycogen in fish from no swim (fish withheld from swim tests), swim (fish subjected to single U_{crit}) and repeat swim (fish subjected two U_{crits}) groups, respectively. For whole-body glycogen, $p = 0.193$ for dietary selenium treatment factor and $p < 0.001$ for swim status factor in two-way ANOVA. Different lowercase letters denote significant differences in concentrations of whole-body glycogen between no swim versus repeat swim groups in respective selenium treatment groups (Holm-Sidak post hoc tests; $p < 0.05$). Data are mean ± S.E.M., of n = 6-7 fish.

3.4.6 Whole-body lactate

Whole-body concentrations of lactate in adult zebrafish were significantly altered by both the dietary Se treatment and the swim status factors ($p = 0.022$ for dietary Se treatment factor and $p < 0.001$ for swim status factor in two-way ANOVA; Figure 3.7). Fish fed the greatest concentrations of SeMet showed significantly greater whole-body lactate accumulation when compared to fish fed the control diet ($p < 0.05$; Figure 3.7). Within each concentration of SeMet in the diet, whole-body concentration of lactate was significantly greater in repeat swim fish when compared to no swim fish ($p < 0.05$; Figure 3.7). Mean whole-body concentrations of lactate in repeat swim fish were 36.7, 37.5, 23.8 and 47.2 μmol/g in fish fed 1.3, 3.4, 9.8 or 27.5 μg Se/g d.m., respectively. These values were between 1.5 and 4 times greater than concentrations of lactate in corresponding no swim fish (Figure 3.7).

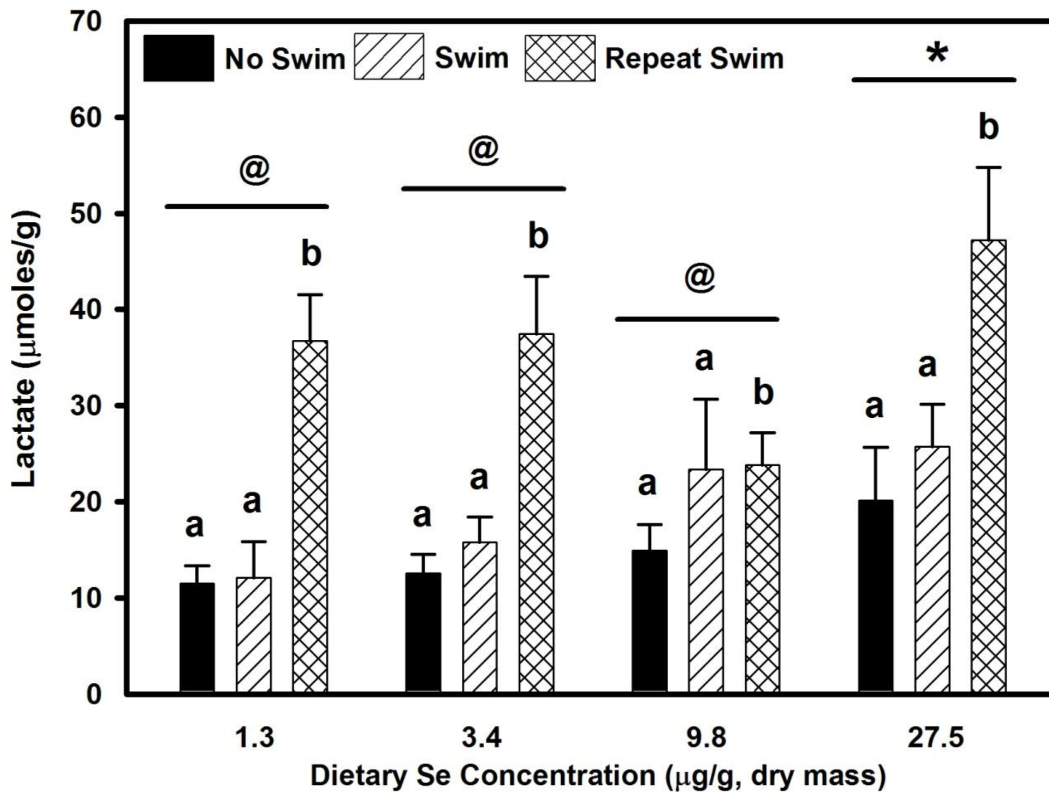

Figure 3.7: Whole-body lactate concentrations in adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g d.m.) for 90 d. Solid, striped and crossed bars represent whole-body lactate in fish from no swim (fish withheld from swim tests), swim (fish subjected to single U_{crit}) and repeat swim (fish subjected two U_{crits}) groups, respectively. Two-way ANOVA showed significant effects of both dietary selenium treatment factor ($p = 0.022$) and swim status factor ($p < 0.001$) on whole-body lactate concentrations. Different symbols (@, *) denote significant differences in whole-body lactate between control and fish fed selenomethionine spiked diets, and different lowercase letters denote significant differences in whole-body lactate concentrations between no swim versus repeat swim fish in respective dietary selenium treatment groups (Holm-Sidak post hoc tests; $p < 0.05$). Data are mean ± S.E.M. of n = 6-7 fish.

3.4.7 Abundances of mRNA of β-hydroxyacyl coenzyme A dehydrogenase (HOAD), citrate synthase (CS), sterol regulatory element binding protein 1 (SREBP 1), protein tyrosine phosphatase 1B (PTP 1B), fatty acid synthase (FAS) and methionine adenosyltransferase 1α (MAT 1A)

Abundances of mRNA of HOAD, CS, SREBP 1, PTP 1B, FAS and MAT 1A in liver and HOAD, CS, SREBP1, PTP 1B and FAS in muscle of adult zebrafish were determined after chronic dietary SeMet exposure. Abundances of mRNA of SREBP 1, HOAD and MAT 1A were significantly down-regulated in liver of fish fed the two greatest concentrations of SeMet (9.8 or 27.5 µg Se/g d.m.) when compared to fish from the control group ($p < 0.05$; Figure 3.8 A,B and 3.9). Significant down-regulation of mRNA abundances of PTP 1B was observed in muscle of fish fed diets augmented with SeMet compared to control fish ($p < 0.05$; Figure 3.8 C). Abundance of mRNA of CS was up-regulated in muscle of fish fed 9.8 µg Se/g d.m. ($p < 0.05$; Figure 3.8 D). There were no statistically significant differences in abundances of mRNA of CS, PTP 1B and FAS in liver and HOAD, SREBP 1 and FAS in muscle of adult zebrafish exposed to SeMet when compared to control fish (see Appendix, Figure C3.S1 and C3.S2).

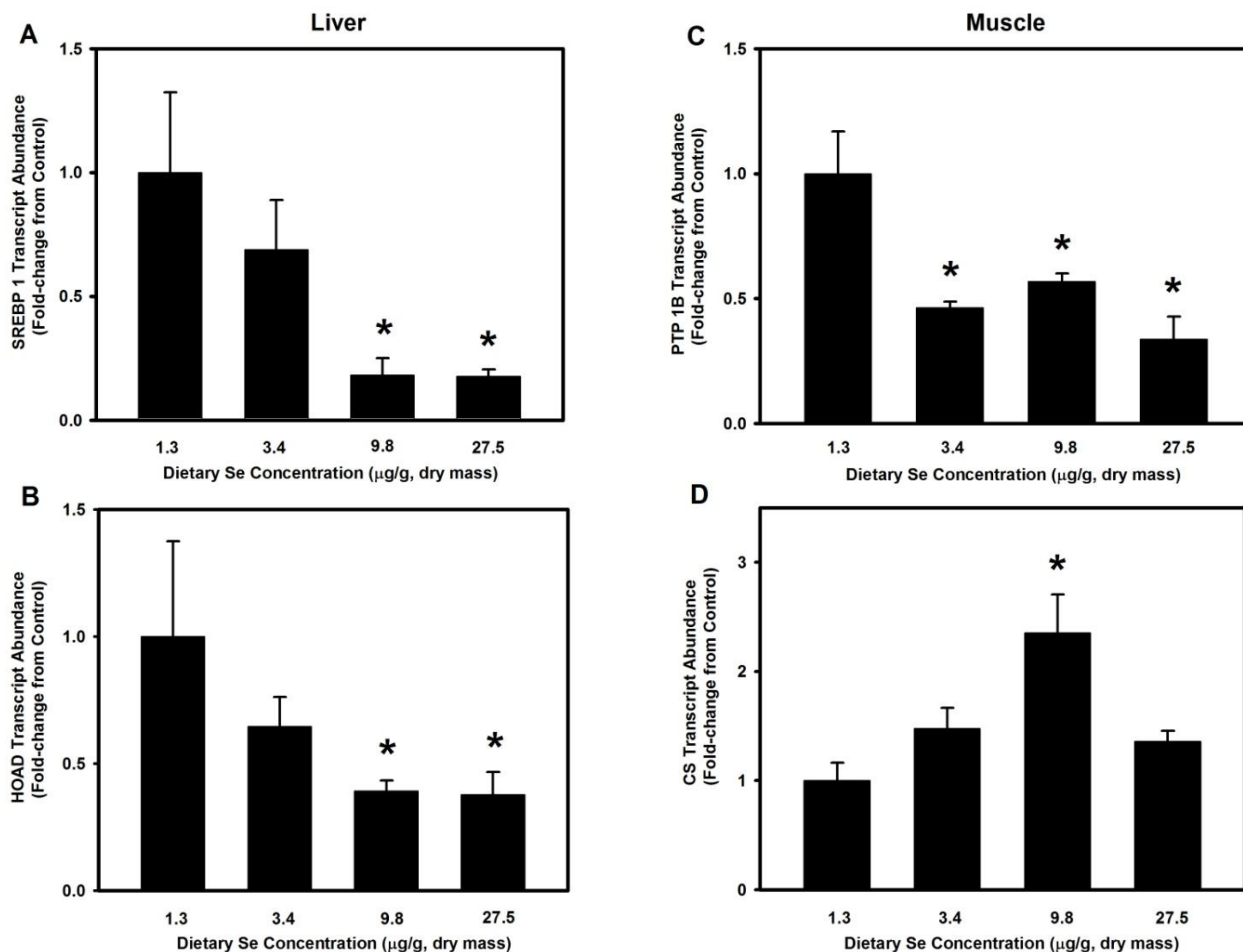

Figure 3.8: mRNA abundance of (A) sterol regulatory element binding protein 1 (SREBP 1) and (B) β-hydroxyacyl coenzyme A dehydrogenase (HOAD) in liver, and (C) protein tyrosine phosphatase 1B (PTP 1B) and (D) citrate synthase (CS) in muscle of adult zebrafish fed control (1.3 μg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 μg Se/g d.m.) for 90 d. Transcript abundance was determined by quantitative real-time PCR. * Significantly different from control group using one-way ANOVA followed by Holm-Sidak post hoc tests (p <0.05). Data are mean ± S.E.M. of n = 4 liver or muscle samples.

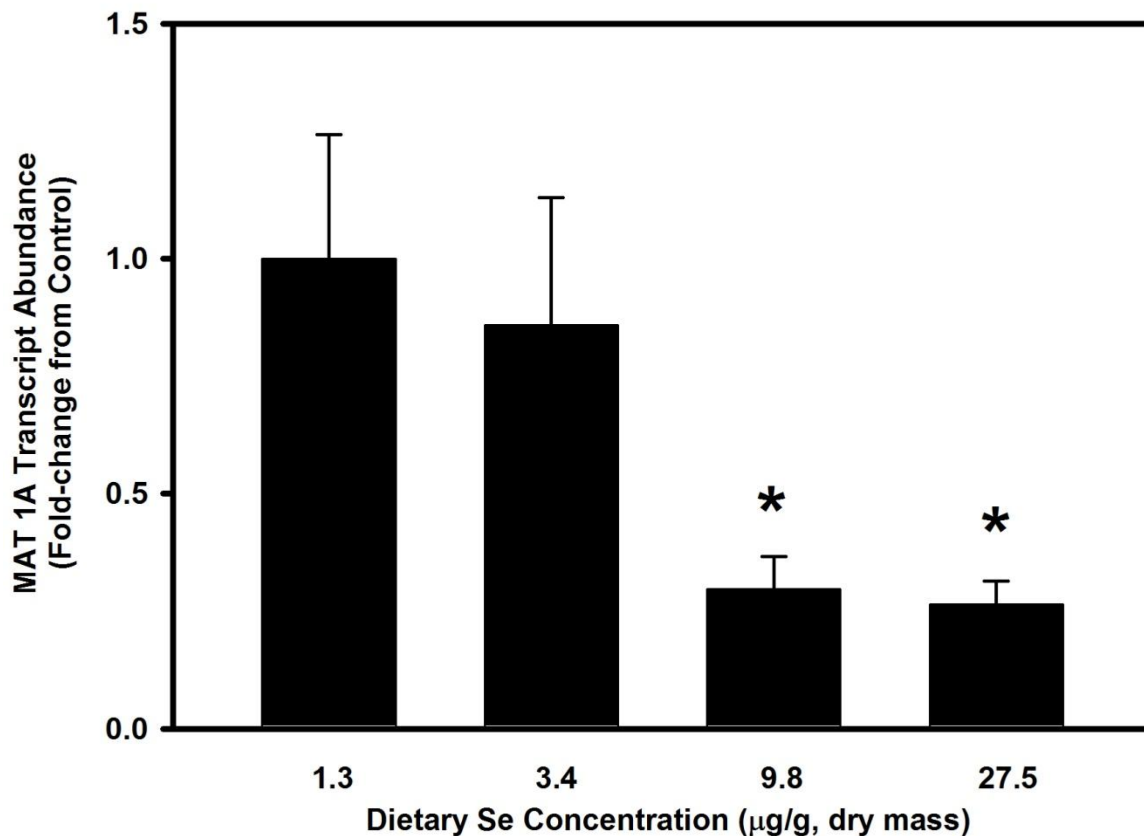

Figure 3.9: mRNA abundance of methionine adenosyltransferase 1α (MAT 1A) in liver of adult zebrafish fed control (1.3 μg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 μg Se/g d.m.) for 90 d. Transcript abundance was determined by quantitative real-time PCR. * Significantly different from control group using one-way ANOVA followed by Holm-Sidak post hoc tests (p <0.05). Data are mean ± S.E.M. of n = 4 liver samples.

3.5 Discussion

The present study is the first to investigate repeat swimming performance, metabolic capacities (SMR, AMR and F-AS) and gene expression of energy metabolism and methionine catabolism enzymes in adult zebrafish after chronic dietary exposure to elevated SeMet. Concentrations of Se used in this study were environmentally relevant and had been previously shown to elicit sublethal effects in adult zebrafish (Thomas and Janz, 2011). Accumulation of SeMet from diets to adult zebrafish occurred in a concentration-dependent manner. Previous studies reported similar accumulation of Se by the Sacramento splittail (*Pogonichthys macrolepidotus*), white sturgeon (*Acipenser transmontanus*), and adult zebrafish exposed to dietary SeMet (Teh et al., 2004; Tashjian et al., 2006; Thomas and Janz, 2011). In addition, since SeMet is the dominant form of Se present in food, the dietary concentrations of SeMet and bioaccumulation of Se in the present study represent values commonly observed in fish inhabiting aquatic ecosystems that are contaminated with Se (Janz et al., 2010).

Greater condition factor in fish fed diets that had been augmented with SeMet in the present study was in contrast to a previous study, where an increase in total length and wet mass but no change in condition factor were observed after exposure to dietary SeMet (Thomas and Janz, 2011). Dietary SeMet has been reported previously to cause changes in the morphology of fishes. Lesser body mass, fork length and condition factor were observed in Sacramento splittail and white sturgeon that had been fed SeMet (Teh et al. 2004; Tashjian et al. 2006), whereas dietary exposure of cutthroat trout (*Oncorhynchus clarki bouvieri*) to SeMet did not affect body mass (Hardy et al., 2010). Significantly lesser body mass and fork length, and greater condition factor were observed in rainbow trout that had been exposed to dietary SeMet (8.47 µg Se/g d.m.) (Wiseman et al. 2011a), whereas in a second study (Wiseman et al., 2011b) greater body

mass and condition factor were observed but there was no statistically significant effect on fork length.

The observation in the present study that, compared to fish fed the control diet, mortality was significantly greater in fish fed the greatest concentration of SeMet (27.5 µg Se/g d.m.) is consistent with the results of previous studies. Greater mortality was observed when zebrafish were fed 26.6 µg Se/g d.m. as SeMet (Thomas and Janz, 2011). A similar result was observed when Sacramento splittail were fed 26.0 and 57.6 µg Se/g d.m. in the form of SeMet for 9 months (Teh et al., 2004) and bluegill (*Lepomis macrochirus*) fed 6.5 and 26 µg Se/g d.m. in the form of SeMet for 60 d (Cleveland et al., 1993).

Since swimming performance integrates many physiological processes, it is commonly used to study whole organismal effects after sublethal exposure to chemicals (Scott and Sloman, 2004). Dietary SeMet exposure had pronounced effect on swimming performance of adult zebrafish. Fish fed greater concentrations of SeMet had reduced swimming performance than the controls. In order to make sure that fish from all treatment groups were able to swim at their maximum swimming capacity before the onset of fatigue, we compared fatigue MO_2 of fish. No significant differences in fatigue MO_2 among fish exposed to control and greater concentrations of SeMet indicated that fish from all treatment groups were able to swim their maximum swimming capacity before the onset of fatigue. Previous studies have reported impaired swimming performance in adult zebrafish exposed to elevated dietary SeMet and aqueous exposure to 2,4-dinitrophenol (Thomas and Janz, 2011; Marit and Weber, 2011). The mean U_{crit} value of zebrafish fed the control diet in the present study (13.5 BL/s) was greater than U_{crit} values reported previously in control fish by Thomas and Janz (2011; 9 BL/s) and Marit and Weber (2011; 5 BL/s). The observed differences in U_{crits} among these studies in adult zebrafish

could be due to differences in acclimation time and U_{crit} protocol adopted in the present study and in those reported by Thomas and Janz (2011) and Marit and Weber (2011). For example, differences in incremental velocity and time interval between velocity increments in U_{crit} tests have been shown to alter swimming performance of fish (reviewed in Hammer, 1995).

Abnormal swimming behaviours were observed in bluegill sunfish exposed to waterborne selenite, and in Sacramento splittail and white sturgeon fed SeMet (Cleveland et al., 1993; Teh et al., 2004; Tashjian et al., 2006). Although fish fed enriched concentrations of SeMet in the present study had significantly impaired U_{crit}, their recovery ratios were not different from fish fed the control diet. The recovery ratio ($U_{crit\text{-}2}$/ $U_{crit\text{-}1}$) is a relevant measure of the ability of fish to recover from exhaustive exercises (Jain et al., 1998). These results suggest that exposure to SeMet only reduces U_{crit} of adult zebrafish and exposure to SeMet is less likely to alter the ability of fish to recover from exercise stress. Similar results were observed in chub (*Leuciscus cephalus*) collected from metal contaminated sites (McKenzie et al., 2007). An explanation for the observed lesser U_{crit} of adult zebrafish exposed to SeMet in the present study is impaired muscle function. Exposure to sublethal concentrations of copper has been shown to reduce U_{crit} in brown trout (*Salmo trutta*) by impairing contraction of muscle fibers (Beaumont et al., 2000). Selenomethionine can substitute for methionine in muscle protein (Stadtman, 1974) and such inappropriate amino acid insertion could possibly cause dysfunction of muscle. Exposure to augmented concentrations of SeMet has been reported to result in lesser amplitude of tailbeat of adult zebrafish (Thomas and Janz, 2011). Since amplitude of tailbeat is functionally related to contraction of musculature of the tail, a lesser amplitude of tailbeat of fish fed SeMet indirectly supports the hypothesis that the cause of this is dysfunction of muscle. In addition, catabolism of SeMet has been reported to induce oxidative stress in cells (Palace et al., 2004) and oxidative

stress has been shown to impair contraction of muscle fibers (Musarò et al., 2010). Taken together, these results suggest that dietary exposure to SeMet has the potential to cause impaired muscle function. However, further studies are needed to test the hypothesis that SeMet directly causes muscle dysfunction in fish.

An alternate explanation for lesser U_{crit} of zebrafish fed SeMet is altered aerobic metabolism. Since U_{crit} is a measure of aerobic swimming capacity of fish, alteration of aerobic metabolism could negatively affect swimming performance. Both MO_2 and activity or expression of key aerobic energy metabolizing enzymes (e.g., CS and HOAD) provide relevant information on aerobic metabolism in fish. Altered aerobic metabolism has been shown to result in impaired swimming in fish collected from metal-contaminated sites and fish exposed to organic and inorganic pollutants (Shingles et al., 2001; Rajotte and Couture, 2002; Pane et al., 2004; Marit and Weber, 2011). In the present study greater values for SMR, exercise MO_2, and COT in fish fed SeMet indicated greater requirements for energy, during both routine activities and forced swimming by those fish to maintain homeostasis, compared to fish fed a control diet. Cray fish (*Procambarus acutus*) and bullfrog (*Rana catesbeiana*) collected from metal-contaminated sites were reported to have higher SMR than reference sites (Rowe et al., 1998, 2001). Elevated SMR may indicate greater energy requirements of organisms to repair toxicant-induced tissue damage and/or eliminate toxicants (Calow, 1991). Both AS and F-AS are commonly calculated from swimming respirometry studies to determine the aerobic capacity of fish (Priede, 1985; Killen et al., 2007). F-AS is the ratio of AMR to SMR and it measures aerobic capacity of fish in a non-mass-specific basis (Killen et al., 2007). Thus, F-AS was used instead of AS to compare aerobic capacity of fish. Significantly lesser values for F-AS in fish fed the two greatest concentrations of dietary SeMet indicates that those fish had a reduced

capacity to support aerobic physiological functions such as swimming. Elevated SMR of fish fed SeMet may also contribute to the observed changes in U_{crit}, FAS and COT.

During aerobic swimming fish utilize triglycerides as their major energy source (Hammer, 1995; Moyes and West, 1995). Although zebrafish fed SeMet had significantly greater whole-body concentrations of triglycerides, they appeared to not utilize this source of energy during swimming as efficiently as control fish. As explained below, down regulation of expression of HOAD and MAT 1A in liver of fish fed elevated SeMet could have altered triglyceride catabolism and transport. During anaerobic metabolism, fish use glycogen as a major source of energy and as a result lactate is accumulated in muscle (Hammer, 1995; Moyes and West, 1995). In large-bodied fishes, lactate in blood plasma is often determined as a measure of anaerobic energy metabolism, whereas in small-bodied fish such as zebrafish, whole-body or muscle lactate concentrations can be determined as a surrogate marker of anaerobic energy metabolism (Sancho et al., 1996; Beaumont et al., 2000; McClelland et al., 2006). Swim performance, temperature and exposure to both organic and inorganic chemicals have been reported to cause greater concentrations of lactate in blood plasma or whole-body of fishes (Sancho et al., 1996; Beaumont et al., 2000; McClelland et al., 2006). In the present study, whole-body concentrations of lactate were proportional to the concentration of SeMet in the diet, where zebrafish fed the greatest concentration of SeMet had significantly greater whole-body concentrations of lactate, compared to control fish. The observation of lesser F-AS, greater accumulation of triglycerides and greater accumulation of lactate in fish fed greater concentrations of SeMet provide further evidence that those fish had an impaired ability to perform aerobic metabolism. Taken together, the lesser U_{crit} of adult zebrafish fed SeMet is most likely related to altered aerobic metabolism.

The observed greater accumulation of whole-body triglycerides in fish fed greater concentrations of dietary SeMet in the present study was agreeable to previous studies. Greater accumulation of triglycerides was reported in zebrafish and rainbow trout exposed to augmented dietary SeMet (Thomas and Janz, 2011; Wiseman et al., 2011b) and in several species of fish collected from Se-impacted field sites (Kelly and Janz, 2008; Driedger et al., 2009). To elucidate potential mechanisms of triglyceride accumulation in fish exposed to elevated SeMet, we investigated gene expression of key enzymes (FAS, PTP 1B and HOAD) and a transcription factor (SREBP 1) involved in triglyceride metabolism. Elevated dietary SeMet exposure resulted in down regulation of mRNA abundance of SREBP 1 and HOAD in liver and PTP 1B in muscle of adult zebrafish. However, there were no differences in mRNA abundance of FAS in both liver and muscle of zebrafish fed elevated dietary SeMet. In contrast, dietary Se exposure in mammals has been shown to increase expression or activities of PTP 1B, FAS and SREBP 1 (Mueller et al., 2008, 2009a,b). It is unclear why the present study observed significant down regulation of SREBP 1 and PTP 1B in fish fed elevated SeMet. However the observed down regulation of HOAD in liver of adult zebrafish exposed to elevated dietary SeMet could potentially result in triglyceride accumulation. β-hydroxyacyl coenzyme A dehydrogenase is a key mitochondrial enzyme involved in β-oxidation of fatty acids. Impaired β-oxidation of fatty acids is shown to increase triglyceride accumulation in both fish and mammals (Fromenty et al., 1990; van den Thillart et al., 2002).

Since elevated SeMet exposure is reported to limit methionine uptake and its insertion into protein, we investigated the role of dietary SeMet exposure in methionine catabolism in adult zebrafish. mRNA expression of the liver-specific MAT gene, methionine adenosyltransferase 1α (MAT 1A), was significantly down regulated in adult zebrafish fed

greater than 9 µg Se/g d.m. in the form of SeMet. To the best of our knowledge, this is first study to report down regulation of MAT 1A in fish after chronic dietary SeMet exposure. Exposure to either selenite or selenocystine has been reported to deplete SAM and inactivate MAT in mammalian liver (Hoffman, 1977; Hasegawa et al., 1996). Reduced cellular methionine concentration and elevated accumulation of s-adenosyl homocysteine, a by-product of methylation, are reported to inhibit expression or activity of liver specific MAT in mammals (Lu, 2000; Mato et al., 2002, 2008). Limited MAT 1A expression is an indicator of both reduced SAM production and impaired methylation (Lu, 2000; Mato et al., 2002). Liver is a major organ responsible for synthesis of triglycerides and methylation plays an important role in transport of triglycerides out of liver (Kerai et al., 1999). Restricted dietary intake of methionine and reduced SAM levels have been shown to increase hepatic triglyceride accumulation in both mammals and fish (Kerai et al., 1999; Rinella et al., 2008; Espe et al., 2010). Based on these results, we postulate that down regulation of both HOAD and MAT 1A in zebrafish liver following elevated dietary SeMet exposure in the present study caused greater accumulation of triglycerides. Both methylation and SAM are vital for regulation of gene expression, transport of triglycerides and biotransformation of toxicants including SeMet (Daniels, 1996; Chiang et al., 1996; Lu, 2000; Kobayashi et al., 2002a). More extensive studies are needed to investigate the role of SeMet exposure in methylation, regulation of gene expression, transport of triglycerides and biotransformation of toxicants in fish.

In summary, environmentally relevant concentrations of SeMet in the diet of adult zebrafish can cause effects at both cellular and organismal levels of organization. Lesser swimming performance of adult fish fed SeMet could alter food acquisition, predator avoidance and migration, and such effects could negatively affect fitness and survivability of fish inhabiting

aquatic ecosystems that are contaminated with Se. Impaired aerobic metabolism in fish exposed to greater concentrations of SeMet in the diet might be responsible for poorer swimming performance. Lesser hepatic β-oxidation of fatty acids and methionine catabolism in fish fed SeMet might have caused triglyceride accumulation in liver. Selenomethionine-induced down-regulation of MAT 1A can impair production of SAM in the liver, and such an effect could possibly reduce cellular methylation reactions. Fish fed the two greatest concentrations of dietary SeMet had better condition factors than fish fed control diet. This result suggests that condition factor of fish is not a good determinant of assessing overall fish fitness after exposure to SeMet.

CHAPTER 4

IN OVO EXPOSURE TO SELENOMETHIONINE VIA MATERNAL TRANSFER INCREASES DEVELOPMENTAL TOXICITIES AND IMPAIRS SWIM PERFORMANCE IN F1 GENERATION ZEBRAFISH (*DANIO RERIO*)

Preface

The research described in this chapter investigated transgenerational effects of dietary SeMet exposure in adult zebrafish. To understand the transgenerational effects of dietary SeMet exposure in adult zebrafish, I collected embryos from a sub-group of adult female zebrafish from the feeding experiment described in Chapter 2 and raised those embryos in clean water. Both immediate and persistent toxicities were determined in F1 generation zebrafish. Egg viability, hatchability, larval fish mortalities and total deformities were determined to investigate immediate effects of maternal transfer of SeMet in F1 generation fish. To study persistent effects of developmental SeMet exposure on the entire life cycle of F1 generation zebrafish, a sub-group of larval zebrafish were raised to adulthood in clean water and fed a Se-normal diet. Swimming performance, tail beat amplitude and tail beat frequency, concentrations of stored energy and cortisol, and reproductive capacity were determined to investigate persistent effect of developmental SeMet exposure in F1 generation adult zebrafish. Maternal exposure of SeMet caused greater incidence of mortality and deformities in early life stages of F1 generation zebrafish, and significantly reduced swimming performance and tailbeat amplitude were observed in F1 generation adult zebrafish exposed to SeMet by maternal transfer. The lack of reproductive impairment in F1 generation adult female zebrafish exposed to SeMet via maternal transfer suggests that developmental exposure to excess Se may not cause transgenerational

effects in fishes. Results of this study suggest that maternal transfer of SeMet exposure can have profound adverse effects in F1 generation fish.

This chapter was published in Aquatic Toxicology 152:20-29, under joint authorship with David M. Janz (University of Saskatchewan). The tables, figures and references cited in this article have been re-formatted here to the thesis style. References cited in this chapter are listed in the reference section of this thesis.

4.1 Abstract

Selenomethionine (SeMet) is the major form of organoselenium present in food. Adult female fish can accumulate greater concentrations of SeMet from food in aquatic ecosystems contaminated with selenium (Se), and maternal transfer to eggs increases the incidence of developmental toxicities and mortality in F1 generation larval fish. The present study was designed to investigate both immediate and persistent adverse effects of graded exposure to SeMet via *in ovo* maternal transfer to F1 generation zebrafish (*Danio rerio*). Adult zebrafish were fed either control food (1.3 μg Se/g, dry mass or d.m.) or food spiked with increasing concentrations of Se (3.7, 9.6 or 26.6 μg Se/g, d.m.) in the form of SeMet for 60 d at 5% body mass/d ration, and an additional 30-40 d with equal rations (2.5%) of control or SeMet-spiked diets and clean chironomids. Concentrations of Se in eggs of adult zebrafish fed 1.3, 3.7, 9.6 or 26.6 μg Se/g d.m. were 2.1, 6.0, 9.6 and 21.9 μg Se/g d.m., respectively. Exposure to SeMet via *in ovo* maternal transfer increased larval zebrafish mortalities in a concentration- and time-dependent fashion. In order to investigate persistent adverse effects of *in ovo* exposure to excess Se, we determined swim performance (U_{crit}), tailbeat amplitude and frequency, energy stores (whole-body triglycerides and glycogen), and a marker of the physiological stress response (whole-body cortisol) of F1 generation zebrafish at 140 days post-fertilization (dpf), and reproductive performance at 180 dpf. Reduced U_{crit} was observed in F1 generation adult zebrafish exposed to ≥ 6.0 μg Se/g d.m. Concentrations of whole-body glycogen in the 6.0 μg Se/g d.m. exposed group were significantly lower than the controls. However, no differences were found in concentrations of whole-body triglycerides or cortisol in adult zebrafish. Mortalities and developmental toxicities in offspring (F2 generation) of F1 generation adult zebrafish exposed to excess Se via *in ovo* maternal transfer were comparable to the controls.

Overall, the results of this study suggest that exposure to greater concentrations of SeMet via *in ovo* maternal transfer can significantly impact the survivability of F1 generation fish, which could impact recruitment of wild fish inhabiting Se-contaminated aquatic ecosystems.

4.2 Introduction

Selenium (Se) is an essential trace element for all animals including fishes, and is an integral component of many enzymes including glutathione peroxidases, thioredoxin reductases, iodothyronine deiodinases, and selenophosphate synthetase-2 (Behne and Kyriakopoulos, 2001). Although Se is required to maintain physiological homeostasis in animals, it has been known to cause toxicities when consumed in excess of recommended nutritional levels. In sensitive oviparous species such as fishes, Se poisoning can occur when dietary concentrations exceed 3 μg Se/g dry mass (d.m.), which is only 7 to 30 times greater than required nutritional levels (Janz et al., 2010). Selenium has emerged as a contaminant of potential concern in many countries including Australia, Canada, New Zealand and the United States due to poisonings reported in fishes and other wildlife (Janz et al., 2010). Wastewater discharges from mining, coal-fired power plants and oil refining activities, and agricultural drainage from seleniferous soils are major contributors of Se into aquatic systems (Lemly, 2002; Janz et al., 2010). Selenium enters aquatic systems primarily as inorganic selenate and/or selenite, where primary producers including algae, plants, and certain bacteria facilitate biomethylation of these inorganic forms into more bioavailable organoselenium forms (Fan et al., 2002). Organisms at the base of food webs can bioaccumulate significant concentrations of Se without any apparent toxicity, and hence can act as vectors transferring Se in to more toxicologically susceptible oviparous vertebrates at higher trophic levels, including fishes, aquatic birds, and amphibians (reviewed in Janz et al., 2010).

In both juvenile and adult fishes, the majority of Se exposure occurs through diets, and selenomethionine (SeMet) is the major form of organoselenium present in food (Fan et al., 2002). In aquatic ecosystems contaminated with Se, approximately 50-70% of total Se occurs in

both primary producers and consumers as SeMet (Fan et al., 2002; Phibbs et al., 2011). Significant body burdens of Se were reported in adult and juvenile fishes collected from Se-impacted sites (Lemly, 2002; Holm et al., 2005; Muscatello et al., 2006; Muscatello and Janz, 2009), and in fishes exposed to dietary SeMet in the laboratory (Thomas and Janz, 2011; Wiseman et al., 2011a,b; Thomas et al., 2013). Although adult fishes can bioaccumulate significant quantities of Se from contaminated diets, they are less sensitive to severe acute Se-induced toxicities than early life stages. However, sublethal toxicities such as reduced swimming performance, greater aerobic metabolism and endocrine disorders have been reported in adult fishes exposed to dietary SeMet (Thomas and Janz, 2011; Wiseman et al., 2011a,b; Thomas et al., 2013) or waterborne inorganic Se (Miller et al., 2007; Massé et al., 2013). Early life stages of fishes are highly susceptible to acute Se toxicity (Lemly, 1997a, 2002; Janz et al., 2010). Fin, cranio-facial and skeletal deformities, and edema have been reported in early life stages of F1 generation fishes whose parents were exposed to elevated dietary SeMet (Lemly, 1997a; reviewed in Janz et al., 2010) or in embryos collected from adult fishes inhabiting Se-contaminated sites (Holm et al., 2005; Muscatello et al., 2006). Transfer of Se (mainly SeMet) from maternal parent to eggs is the major route of Se exposure to early life stages of fishes (Lemly, 1997a; Janz et al., 2010). In the present study we refer the transfer of Se from maternal parent to eggs as 'maternal transfer'. Maternal transfer occurs following exposure of adult female fish to elevated dietary Se concentrations, resulting in the synthesis and transport of Se-enriched vitellogenin to the ovary, where it is taken up by developing oocytes (Lemly, 1997a; Janz et al., 2010; Janz, 2012). Vitellogenin is the egg yolk precursor protein and serves as the primary food reserve for developing embryo-larval fish (Kunz, 2004). Since Se and sulfur (S) have similar chemical characteristics, SeMet can be inserted in place of the S-containing

essential amino acid methionine (Met) during protein synthesis in a non-specific, dose-dependent manner (Behne et al., 1991; Behne and Kyriakopoulos, 2001). Toxicities in early life stages of F1 generation fishes occur when they utilize SeMet-rich yolk protein for development (Lemly, 1997a; Janz et al., 2010).

Although a number of studies have observed Se-induced developmental toxicities in early life stages of F1 generation fishes, to our knowledge no previous studies have evaluated the potential negative effects of subtle Se-induced developmental toxicities and their persistent adverse effects on the entire life cycle of F1 generation fishes. Exposure to endocrine disrupting chemicals such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) or methoxychlor during early stages of development in fishes or mammals has been shown to alter development programming, and such developmental impairment resulted in persistent adverse toxicity in both adults and their offspring (Cantrell et al., 1996; Anway et al., 2005; Heiden et al., 2009). A larval fish with severe Se-induced developmental toxicities might die in the early stages of development (e.g. a severe mouth deformity might kill the fish due to malnutrition), but the fate of larval fish that survive developmental exposure to excess Se is not well understood. The survivability of fishes in their natural environment depends on swim performance, concentrations of stored energy and a functional physiological stress response (Mayer et al., 1992; Adams, 1999; Mommsen et al., 1999; Scott and Sloman, 2004). Swim performance is linked to survival of fish in natural environments, as it determines food acquisition, predator avoidance and migratory capacity of fishes (Hammer, 1995; Plaut, 2001; Scott and Sloman, 2004). Triglycerides (triacylglycerols) and glycogen are the major sources of stored energy in fishes (Hammer, 1995; Moyes and West, 1995; Adams, 1999) and have been shown to be sensitive indicators of physiological and chemical stress (Mayer et al., 1992; Adams, 1999). The physiological stress response in fish

with subtle developmental toxicities might be greater than in normal fish since certain types of subtle deformities could challenge the ability of fish to swim or in general to survive in the natural environment. Cortisol is an important mediator of adaptive responses to environmental and chemical stressors, and studies have shown altered production of cortisol in fishes after exposure to stressors (Mommsen et al., 1999; Miller et al., 2007; Thomas and Janz, 2011; Wiseman et al., 2011b).

The present laboratory study was initiated to investigate potential adverse effects of exposure to elevated concentrations of SeMet via *in ovo* maternal transfer during the entire life cycle of F1 generation zebrafish. We investigated mortalities and developmental toxicities in early life stages of zebrafish after *in ovo* exposure to increasing concentrations of SeMet via maternal transfer, and later, we determined persistent adverse effects of developmental SeMet exposure on swim performance, energetics, the stress response, and reproductive performance of adult zebrafish raised in clean water and fed a normal diet. Zebrafish were used in the present study since they represent an excellent model organism for developmental toxicity studies, standardized protocols are available to raise this fish in controlled laboratory conditions (Westerfield, 1995; Hill et al., 2005), and due to their shorter life cycle, this fish can be successfully used to investigate transgenerational adverse effects of chemicals (Heiden et al., 2009).

4.3 Materials and methods

4.3.1 Test compound

Seleno-L-methionine was purchased from Sigma-Aldrich (Oakville, ON, Canada). Purity of the compound was greater than 98%.

4.3.2 Diet preparation and experimental design

All methods used in the present study were approved by the University of Saskatchewan's Animal Research Ethics Board (protocol # 20030076), and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Adult zebrafish were fed either a control diet (Nutrafin® basic flake food, Hagen Inc., Montreal, QC, Canada) or different nominal concentrations of Se (3, 10 or 30 μg/g [38, 127 or 380 nmol/g]) in the form of SeMet (spiked into flake food) for 60 d, followed by an additional 30-40 d with equal rations (2.5%) of control or SeMet-spiked diets and clean chironomids (Bio-Pure Blood Worms, Hikari Sales Inc., Hayward, CA, USA). The diet preparation and dietary SeMet exposure to adult fish were described previously (Thomas and Janz, 2011). After 90 d of feeding exposure, adult fish from each exposure group were bred 3-4 times and embryos were collected and used to investigate adverse effects of exposure to greater concentrations of SeMet via *in ovo* maternal transfer for the entire life cycle of F1 generation fish. Concentrations of total Se in eggs, percent egg viability, percent hatchability and percent mortality and total deformities were determined in F1 generation larval fish. Percent egg viability was determined as the ratio of number of fertilized eggs to the total number of eggs, and percent hatchability as the ratio of the number of hatched larval fish to the total number of fertilized eggs. Percent mortality was calculated by dividing the number of dead embryos/larval fish by the total number of embryos/larval fish, and then multiplying by 100.

In addition, a sub-group of surviving larval zebrafish from each Se exposure group were reared to 140 days post-fertilization (dpf) to investigate persistent adverse effects of *in ovo* SeMet exposure on swim performance, energy stores and the physiological stress response of adult zebrafish. Remaining F1 generation adult fish were reared to 180 dpf to study the potential

effect of developmental exposure to excess SeMet on reproductive performance. All F1 generation fish were reared in clean (dechlorinated municipal) water and fed the control diet.

4.3.3 Deformity analysis

Total deformity analyses were determined in 6 dpf larval zebrafish. Larval fish used for deformity analyses were euthanized with an overdose of buffered tricaine methanesulfonate (MS-222) (Sigma-Aldrich, Oakville, ON, Canada) and preserved in 10% buffered formalin for 12-13 hours before being transferred to 70% ethanol. All preserved larval zebrafish were examined for malformations in a blind fashion using an Olympus model SZ-CTV dissecting microscope (Olympus, Melville, NY, USA). Each larval fish was examined for skeletal, craniofacial and fin deformities, and edema, and the presence or absence of developmental abnormalities was recorded for each fish. Total percent deformities were calculated by dividing number of malformed larval fish by total number of larval fish, and multiplying by 100.

4.3.4 Quantification of selenium

Concentrations of total Se in pooled egg samples were measured by use of inductively coupled plasma-mass spectrometry (ICP-MS) at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK, Canada). For the quantification of Se we collected 3 replicates of 100 pooled egg samples from adult female zebrafish fed the control diet, and 3-4 replicates of 60 pooled egg samples from adult female fish fed the SeMet-spiked diets. The detailed Se analysis procedure was described previously (Thomas and Janz, 2011; Thomas et al., 2013). A LOQ of 0.5 µg Se/g was determined using method blanks. Concentrations of Se in eggs were measured on wet mass basis, and converted to dry mass based on a moisture content of 93.7 ± 0.1 % determined in a subset of zebrafish eggs.

4.3.5 Swim performance

Swim performance was carried out in a model mini swim tunnel respirometer (Loligo Systems, Tjele, Denmark). Water temperature in the swim tunnel respirometer was maintained at the same water temperature used for fish husbandry (28.5 ± 0.5 °C) by use of a heated water bath circulator (VWR International, Mississauga, ON, Canada). We adopted the critical swimming speed (U_{crit}) method to study aerobic swimming capacity of zebrafish (Brett, 1964). The detailed procedure for this method is discussed elsewhere (Plaut, 2001; Thomas and Janz, 2011). Briefly, fish were forced to swim at different swimming velocities for constant time intervals (5 min) until they fatigued and stopped swimming. During swim performance experiments the oxygen saturation of water in the swim tunnel respirometer was maintained at greater than 85%. Once the experiment was completed, the highest completed swimming velocity and time spent at fatigue velocity were recorded for each fish. Critical swimming speed values were calculated using an equation explained by Plaut (2001). Fish cross sectional area was less than 5% of swim tunnel cross sectional area, hence U_{crit} values were not corrected for the solid blocking effect. Swim performance values (cm/s) were corrected for standard body length of individual fish, and thus U_{crit} values are represented as body lengths per second (BL/s). After the swimming performance experiments, fish were euthanized using an overdose of buffered MS-222 and stored at -80 °C for subsequent determination of whole-body energy stores and cortisol.

4.3.6 Image analysis

Tailbeat amplitude and tailbeat frequency were calculated at maximum sustained swimming velocity (maximum swimming velocity that adult zebrafish swam for an entire 5 min

segment). Tailbeat amplitude measures the maximum tail stroke distance from tip of the tail to midline, whereas tailbeat frequency measures complete oscillation of the fish tail and is represented as number of tailbeats per second (Hunter and Zweifel, 1971). The detailed procedure for image analysis was explained elsewhere (Thomas and Janz, 2011).

4.3.7 Whole-body triglyceride and glycogen assays

Both triglyceride and glycogen assays were carried out using kits purchased from Sigma-Aldrich (Oakville, ON, Canada). Detailed procedures for these assays were explained elsewhere (Thomas and Janz, 2011; Thomas et al., 2013). A glycerol solution was used to create the standard curve for the triglyceride assay, and the standard curve for the glycogen assay was created using purified Type IX bovine liver glycogen. Whole-body homogenate samples were run in triplicate and mean results with less than 10% coefficient of variation were used for concentrations of whole-body triglycerides and glycogen.

4.3.8 Whole-body cortisol assay

Cortisol analysis was carried out using an enzyme linked immunosorbent assay (ELISA) kit (Oxford Biomedical Research, Oxford, MI, USA). Cortisol in whole-body fish homogenate was extracted using diethyl ether (EMD Chemicals Inc., Gibbstown, NJ, USA). After extraction, ether was evaporated and cortisol was reconstituted in phosphate buffer provided by the manufacturer. The assay was run according to the manufacturer’s instructions (Oxford Biomedical Research, Oxford, MI, USA) as described previously (Thomas and Janz, 2011).

4.3.9 Statistical analysis

All data were tested for normality by use of the Shapiro–Wilk test and for homogeneity of variance by use of Levene's test (SigmaStat 3.1, SPSS Inc., Chicago, IL, USA). Data that did not meet the assumptions for parametric statistical procedures were log 10 transformed. One-way ANOVA followed by Dunnett's test was used to test for significant differences among treatment groups from the controls. Data that did not pass the parametric assumptions after transformation were analysed by use of Kruskal-Wallis test followed by Dunn's post-hoc test. Data that passed parametric assumptions were expressed as mean ± S.E.M., and data that did not pass parametric assumptions were expressed as median with 5th and the 95th percentile outliers. Differences were considered statistically significant at $p < 0.05$.

4.4 Results

4.4.1 Concentrations of selenium

Maternal transfer of Se to eggs increased with exposure to increasing concentrations of dietary Se in adult female fish. The concentrations of total Se in diets and in whole-body adult fish samples were reported previously (Thomas and Janz, 2011). Concentrations of total Se in eggs of adult zebrafish fed the 1.3, 3.7, 9.6 or 26.6 µg Se/g d.m. diets were 2.1 ± 0.1, 6.0 ± 0.9, 9.6 ± 0.5, and 21.9 ± 3.8 µg Se/g d.m., respectively (Table 4.1). A significantly greater accumulation of Se was observed in eggs collected from adult female zebrafish exposed to the highest concentration of dietary SeMet (26.6 µg Se/g d.m.) when compared to eggs collected from adult female zebrafish fed the control diet (1.3 µg Se/g d.m.). Although concentrations of Se in eggs collected from adult female zebrafish fed the 6.0 or 9.6 µg Se/g d.m. diets were

greater than the Se concentration in eggs from the control female fish, no statistically significant differences were observed among treatment groups (Table 4.1).

Table 4.1: Concentrations of total selenium in eggs, and cumulative mortalities and morphometrics of F1 generation adult zebrafish exposed to increasing concentrations of Se via *in ovo* maternal transfer. Cumulative mortalities were calculated from 6-140 days post-fertilization (dpf) and morphometrics were determined on 140 dpf. Data are mean ± S.E.M of n = 3-4 replicates of 60-100 pooled eggs for quantification of selenium, n= 3-4 replicates of 50-75 fish for cumulative fish mortality analysis, and n= 12–14 fish for morphometrics

Egg Se (μg/g dry mass)	Cumulative fish mortality (6-140 dpf)	Total length (mm)	Wet mass (g)	Condition factor
2.1 ± 0.1	47.3 ± 6.7	26 ± 0.5	0.167 ± 0.01	0.94 ± 0.02
6.0 ± 0.9	63.3 ± 4.1	24 ± 0.5	0.133 ± 0.01	0.94 ± 0.02
9.6 ± 0.5	58.0 ± 7.2	25 ± 0.8	0.151 ±0.02	0.98 ± 0.03
21.9 ± 3.8*	66.7 ± 9.6	25 ± 0.7	0.154 ± 0.01	1.00 ± 0.03

Condition factor = (wet mass/total length^3) x 100,000.

*, Significantly different from the control group using one-way ANOVA followed by Dunnett's post-hoc test ($p < 0.05$).

4.4.2 Hatchability, mortalities and total deformities

There were no differences observed among exposure groups in percent egg viability or embryo hatchability. Egg viability was 78.0 ± 2.5%, 73.6 ± 2.3%, 76.7 ± 4.2% and 79.1 ± 2.7%, and embryo hatchability was 90.3 ± 3.7%, 89.3 ± 3.5%, 92.7 ± 0.4% and 88.0 ± 2.9% following breeding trials in adult fish fed 1.3, 3.7, 9.6 or 26.6 μg Se/g d.m. diets, respectively (see Appendix, Table C4.S1). However, exposure to excess SeMet via *in ovo* maternal transfer increased larval zebrafish mortalities in a concentration- and time-dependent fashion (Figure 4.1). Significantly greater mortalities were observed in larval fish exposed *in ovo* to 9.6 or 21.9 μg Se/g d.m. on 4, 5 and 6 dpf, and in the 6.0 μg Se/g d.m. exposure group on 6 dpf ($p < 0.05$; Figure 4.1). Cumulative percent mortality from 2 to 6 dpf increased from a mean of 9.1% in the control group to between 55.2 – 68.5% in larvae from fish fed the SeMet-spiked diets ($p < 0.05$; Figure 4.1).

Although the majority of larval zebrafish used in deformity analysis were at 6 dpf, severely deformed larval zebrafish were also euthanized and preserved on 4 and 5 dpf and included in deformity analysis. Total deformities of zebrafish larvae increased from 9.5% in the control group to between 35.2 – 42.8% in larvae from fish fed the SeMet-spiked diets, which was significantly greater than controls in the 21.9 μg Se/g d.m. group ($p < 0.05$; Figure 4.2). The most commonly observed malformations were skeletal curvatures (scoliosis, lordosis and kyphosis) and fin deformities (Figure 4.3). In addition, particularly in the highest exposure group, craniofacial deformities, yolk sac edema, and pericardial edema were also observed (Figure 4.3).

Adult F1 generation zebrafish that survived developmental exposure to SeMet and were raised to 140 dpf on the control diet did not display any significant differences in total length, body mass or condition factor among treatment groups (Table 4.1). Percent egg viability, embryo hatchability, and mortalities and total deformities of offspring (F2 generation) of F1 generation zebrafish were similar among exposure groups (Table 4.3).

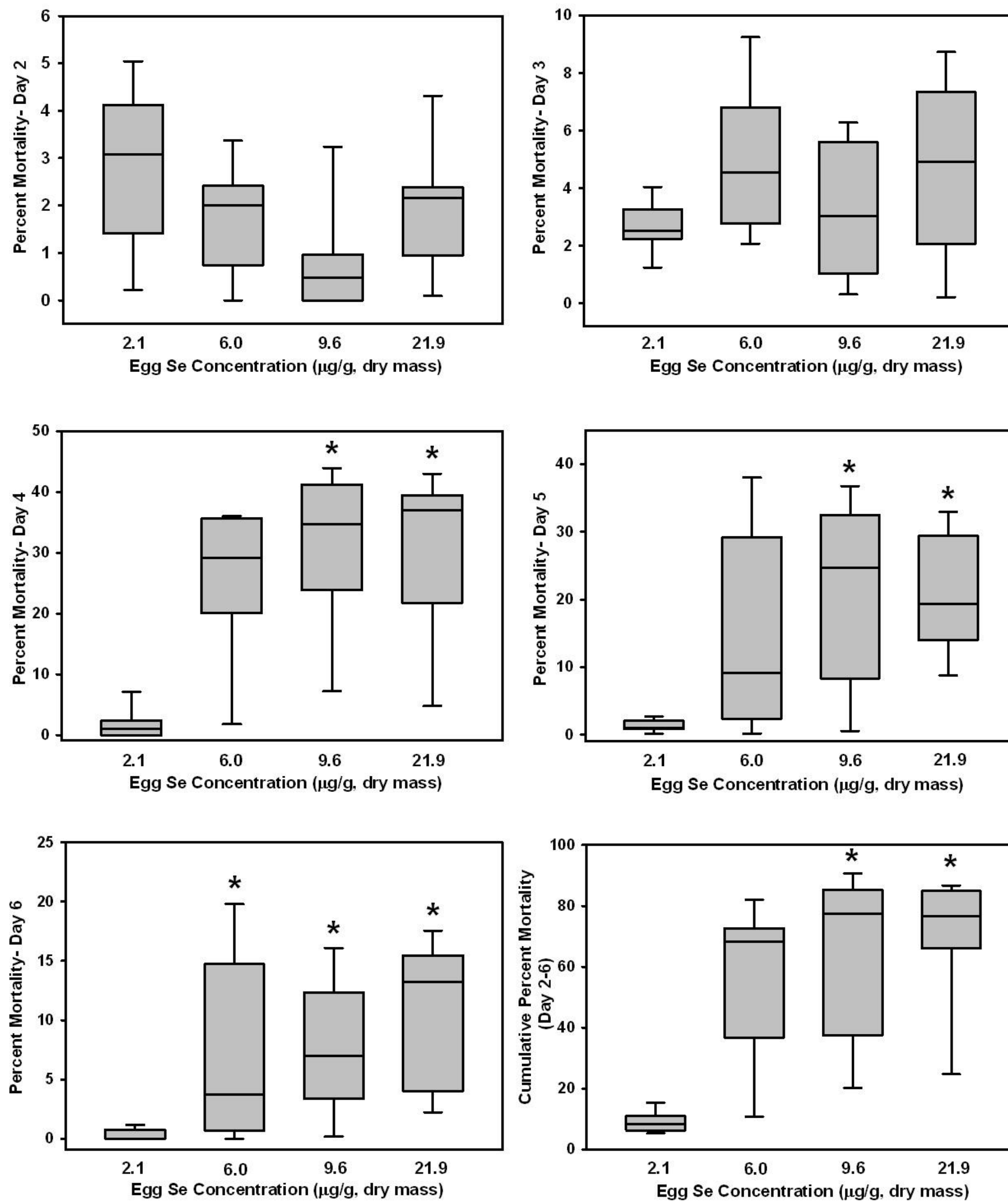

Figure 4.1: Percent daily and cumulative mortalities (2-6 days post-fertilization) of embryo or larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. *, Significant difference in mortality compared to control group using Kruskal-Wallis test with Dunn's post-hoc test (whiskers indicate the 5th and the 95th percentile outliers; $p < 0.05$; n = 5-9 replicates of 60-100 embryos).

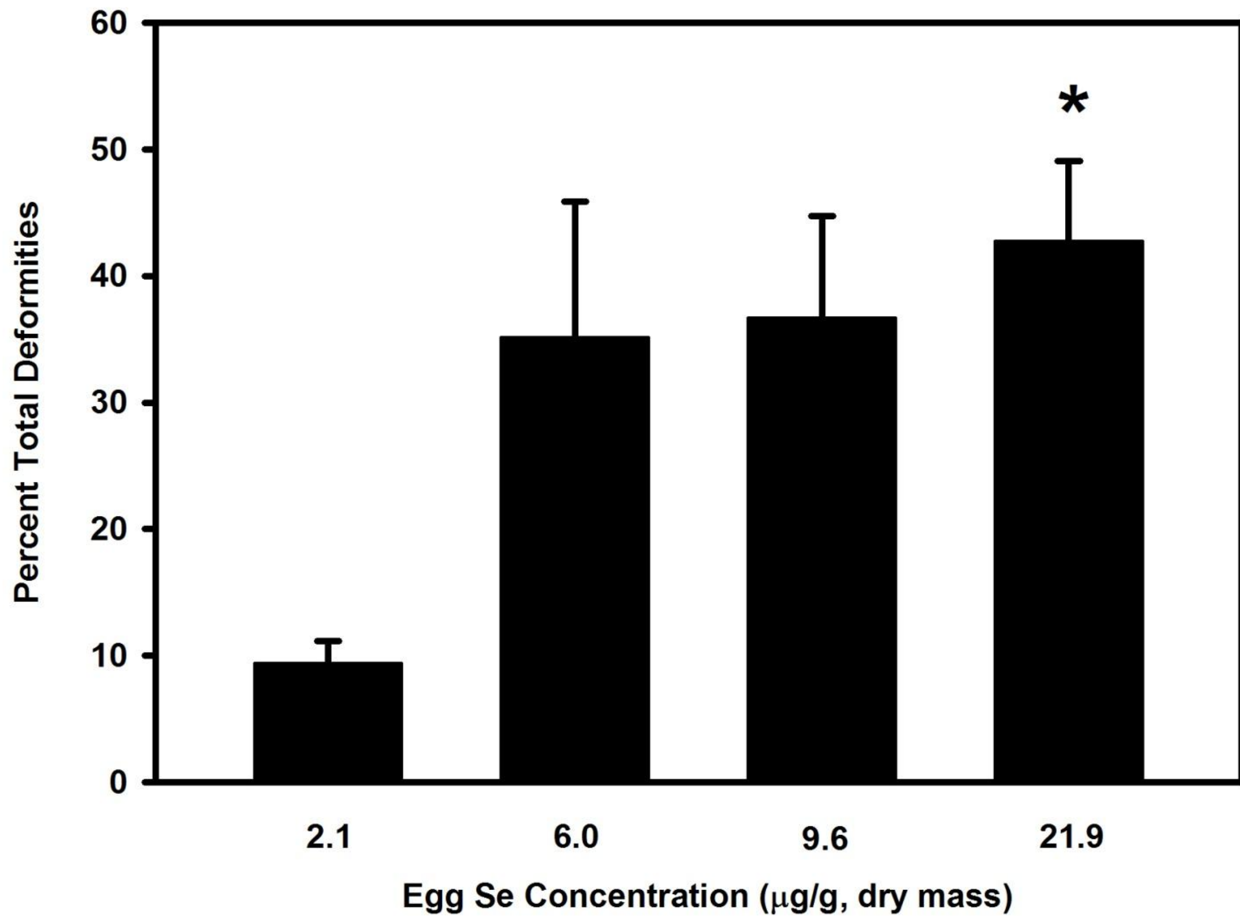

Figure 4.2: Total morphological abnormalities (sum of skeletal, craniofacial and fin deformities, and edema) in larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. *, Significant difference in total deformities compared to control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$; n = 5-9 replicates of 30-60 embryos).

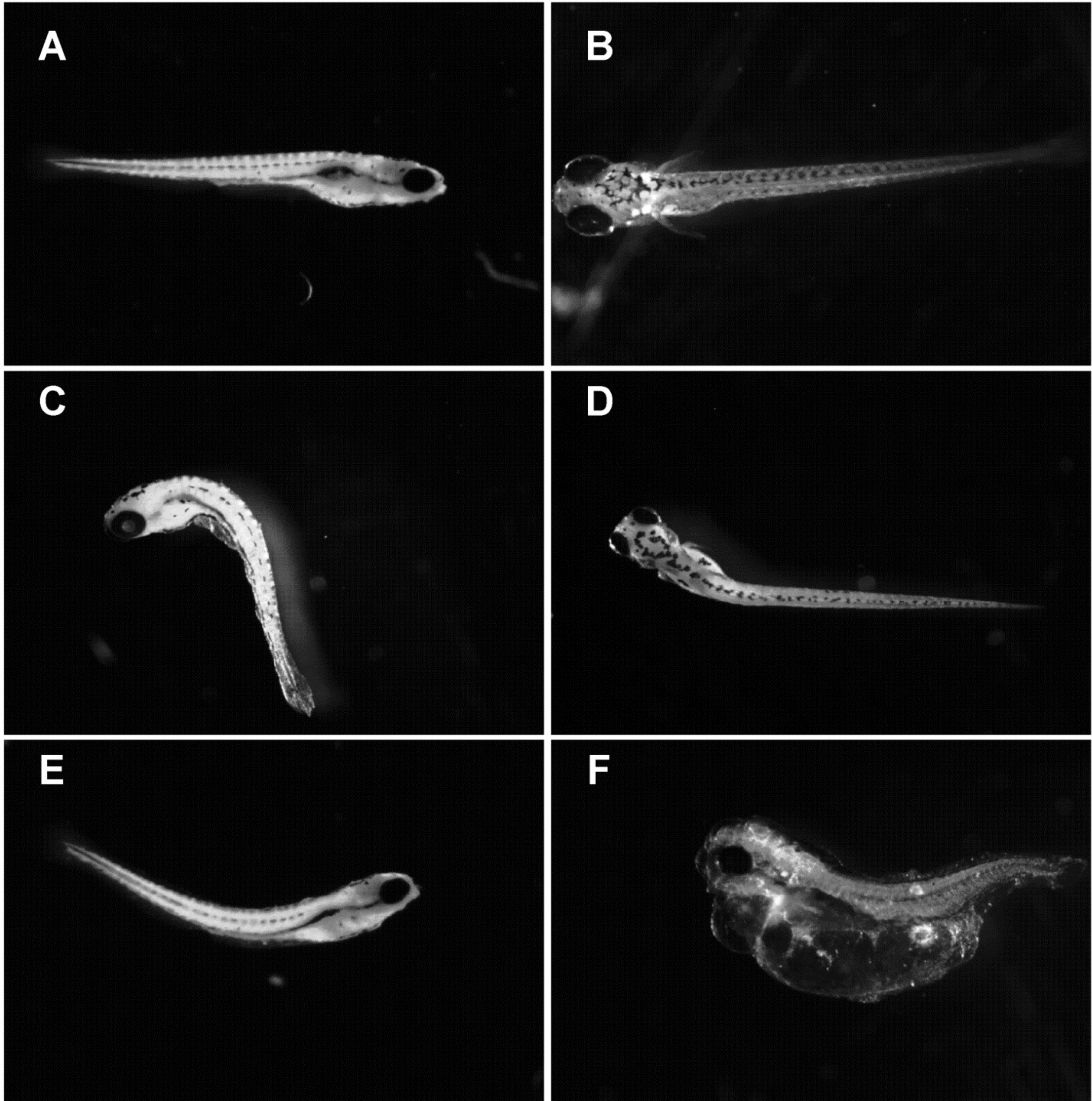

Figure 4.3: Representative images of normal (A and B) and deformed (C, D, E and F) larval zebrafish taken during deformity analysis. Images A and B show a normal fish, C shows kyphosis and fin deformities, D shows scoliosis, E shows lordosis, and F is a larval fish with multiple deformities and edema.

Table 4.3: Percent egg viability, embryo hatchability, cumulative mortality and total deformities in progenies (F2 generation) of F1 generation zebrafish exposed to increasing concentrations of selenomethionine via *in ovo* maternal transfer. Data are mean ± S.E.M of n= 3 replicates of 100-150 eggs/larvae.

F1 Egg Se (µg/g dry mass)	F2 % Egg viability	F2 % Hatchability	F2 % Cumulative mortality (2-6 dpf)	F2 % Total deformities
2.1	82.6 ± 4.0	96.2 ± 2.3	8.8 ± 2.9	9.9 ± 0.9
6.0	78.9 ± 6.8	92.6 ± 2.6	11.7 ± 2.3	8.5 ± 1.6
9.6	79.2 ± 10.6	94.2 ± 2.2	8.2 ± 1.8	7.4 ±1.5
21.9	84.6 ± 3.3	96.0 ± 2.0	7.4 ± 3.0	5.6 ± 1.1

One-way ANOVA did not find any significant differences on egg viability, percent hatchability, cumulative percent mortality and percent total deformities among treatment groups

4.4.3 Swim performance

Developmental exposure to greater concentrations of SeMet via maternal transfer had a significant impact on swim performance (U_{crit}) of F1 generation adult zebrafish (Figure 4.4). Swim performance (U_{crit}) of adult zebrafish exposed *in ovo* to 6.0, 9.6 or 21.9 µg Se/g d.m. were 8.1 ± 0.7, 4.7 ± 0.9, and 5.3 ± 0.7 BL/s, respectively, which were significantly lower ($p < 0.05$) than U_{crit} determined in the control adult fish (12.5 ± 1.1 BL/s). A greater than 50% reduction in U_{crit} was observed in adult zebrafish exposed *in ovo* to the two highest concentrations of Se (Figure 4.4).

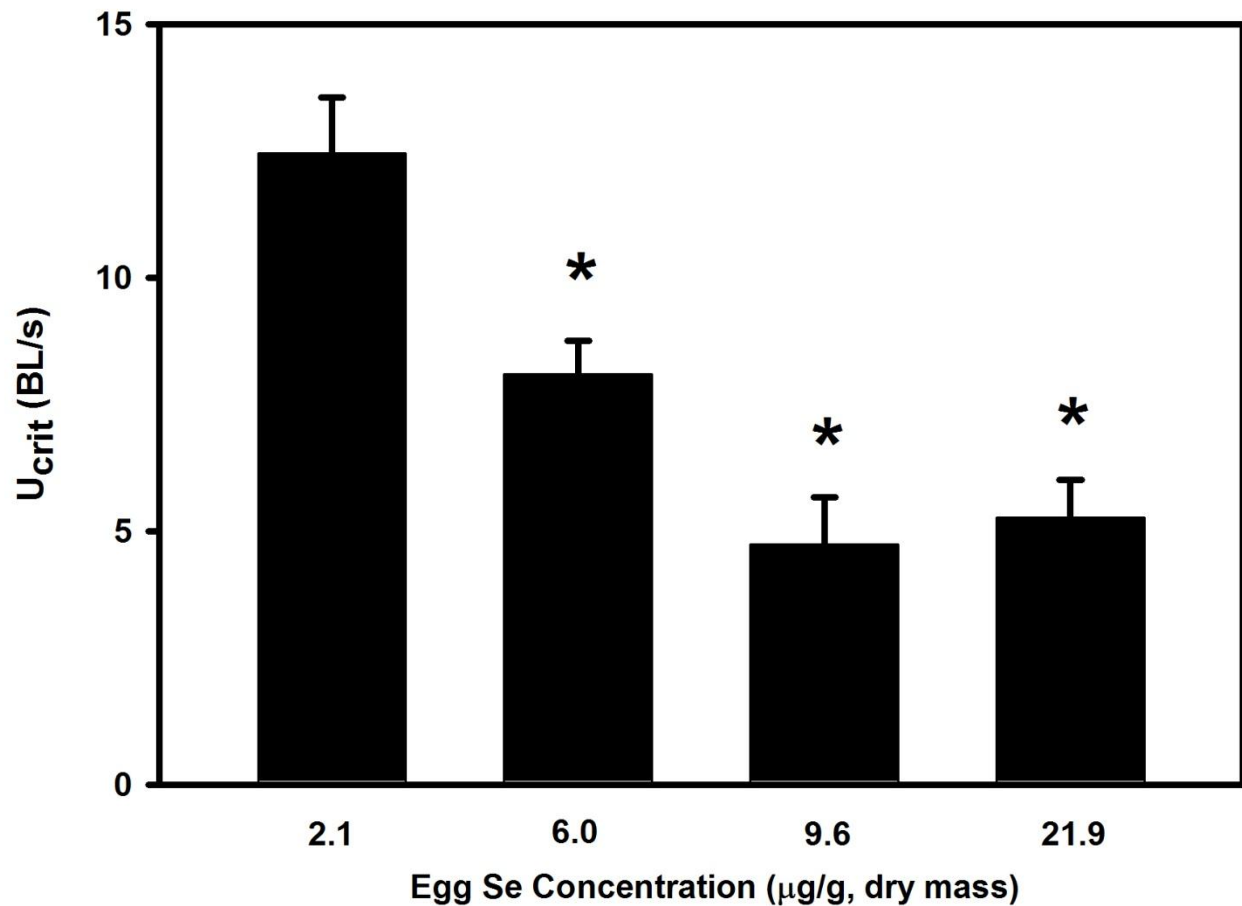

Figure 4.4: Critical swimming speed (U_{crit}; a measure of swim performance; BL/s = body lengths/second) determined in F1 generation adult zebrafish at 140 days post-fertilization exposed *in ovo* to increasing concentrations of selenium via maternal transfer. *, Significant difference compared to control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$; n = 12-14 fish).

4.4.4 Image analysis

Tailbeat amplitude and tailbeat frequency were measured at maximum sustained swimming velocity. Tailbeat amplitude of F1 generation adult fish exposed *in ovo* to ≥ 6.0 µg Se/g d.m. were significantly lower than the controls ($p < 0.05$; Figure 4.5A). However, developmental exposure to excess Se did not alter tailbeat frequency in adult zebrafish (Figure 4.5B).

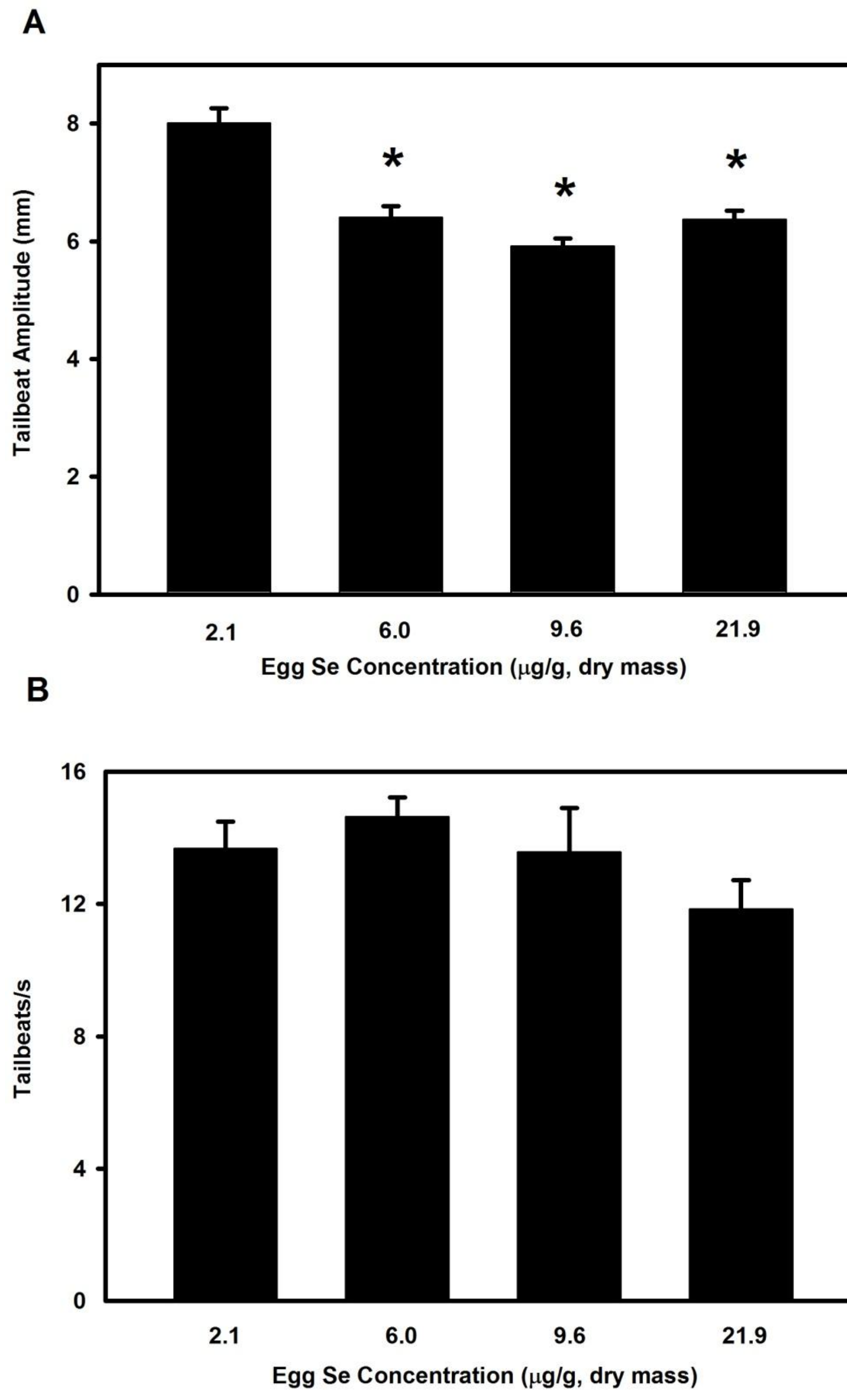

Figure 4.5: Tailbeat amplitude and tailbeat frequency of F1 generation adult zebrafish at 140 days post-fertilization exposed *in ovo* to increasing concentrations of selenium via maternal transfer. *, Significant difference compared to control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$; n = 6-14 fish).

4.4.5 Whole-body glycogen, triglycerides and cortisol

The concentration of whole-body glycogen in adult zebrafish exposed *in ovo* to 6.0 µg Se/g d.m. was significantly lower than the controls ($p < 0.05$; Table 4.2). Concentrations of whole-body glycogen in adult zebrafish from the two higher Se exposure groups were not different from the controls. There were no differences of whole-body triglycerides in adult zebrafish (Table 4.2). Concentrations of whole-body cortisol in adult zebrafish were not significantly different among exposure groups (Table 4.2).

Table 4.2: Concentrations of whole-body triglycerides, glycogen and cortisol in 140 days post-fertilization F1 generation adult zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Data are mean ± S.E.M of n= 12-14 fish.

Egg Se (µg/g dry mass)	Triglycerides (mg/g)	Glycogen (mg/g)	Cortisol (ng/g)
2.1	7.6 ± 1.1	1.8 ± 0.3	4.3 ± 0.7
6.0	8.7 ± 1.2	1.1 ± 0.1*	7.0 ± 1.3
9.6	7.4 ± 1.0	1.9 ±0.2	4.6 ± 0.8
21.9	7.9 ± 0.5	1.7 ± 0.3	5.5 ± 1.1

*, Significantly different from the control group using one-way ANOVA followed by Dunnett's post-hoc test ($p < 0.05$).

4.5 Discussion

In Se-contaminated aquatic ecosystems, diet is the major exposure route of Se to adult fishes, and maternal transfer is the major exposure route of Se to eggs and embryo-larval fishes (Lemly, 1997a, 2002; Janz et al., 2010). To our knowledge this is the first study investigating adverse effects of exposure to increasing concentrations of SeMet via *in ovo* maternal transfer over the entire life cycle of F1 generation fish. The most notable findings of this study were a greater incidence of mortality (especially between 4-6 dpf) and developmental abnormalities in early life stages of larval zebrafish exposed *in ovo* to greater concentrations of Se in the form of SeMet, and reduced swim performance and tailbeat amplitude in F1 generation adult zebrafish that survived acute toxic effects of *in ovo* exposure to excess SeMet. In a previous study (Thomas and Janz, 2011), we investigated sublethal effects of chronic dietary SeMet exposure in adult zebrafish, and in the present study we raised embryos from a sub-group of those adult zebrafish fed control or SeMet-spiked diets. Reported concentrations of Se in the control and SeMet-spiked diets and in adult female zebrafish from our previous study (Thomas and Janz, 2011), and measured concentrations of Se in eggs collected from the adult female zebrafish in the present study were similar to concentrations of Se reported in invertebrates and fishes collected from Se-contaminated sites (Holm et al., 2005; Muscatello et al., 2006; Muscatello and Janz, 2009). This suggests that Se concentrations in diets, adult fish and eggs in the present study are environmentally realistic, and reflect Se exposure scenarios of adult and embryo-larval fishes inhabiting many Se-contaminated aquatic ecosystems.

Eggs collected from adult female zebrafish fed either the control or SeMet-spiked diets did not show any significant changes in egg viability and hatchability. These results were agreeable to laboratory-based Se exposure studies in bluegill sunfish (*Lepomis macrochirus*) and

cutthroat trout (*Oncorhynchus clarki bouvieri*) (Coyle et al., 1993; Hardy et al., 2010) and eggs collected from northern pike (*Esox lucius*), rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*) and westslope cutthroat trout (*Oncorhynchus clarki lewisi*) inhabiting Se-contaminated aquatic ecosystems (Holm et al., 2005; Muscatello et al., 2006; Rudolph et al., 2008). Collectively, results of the present study and most other published studies suggest that exposure to elevated concentrations of Se in adult female fishes is less likely to alter egg viability and hatchability.

Greater incidences of mortality and developmental toxicities were observed in larval zebrafish after *in ovo* exposure to excess Se via maternal transfer. Many laboratory and field based studies observed similar increases in mortality and/or developmental toxicities in F1 generation larval fish whose parents were exposed to dietary and/or waterborne Se (e.g. Coyle et al., 1993; Holm et al., 2005; Muscatello et al., 2006; reviewed in Janz et al., 2010). Both larval fish mortalities and developmental deformities were highly variable in the present study, and such variability might have been caused by differences in deposition of Se to individual eggs, differences in egg quality, and/or differences in the deposition of Se in eggs among breeding trials. High variability in the occurrences of larval fish mortalities and/or developmental deformities have been widely discussed in the fish Se literature (Muscatello et al., 2006; Hardy et al., 2010; Janz et al., 2010), although underlying mechanisms are unclear.

Oviparous vertebrates such as most fishes are highly sensitive to Se toxicity (Lemly, 1997a). An increasing body of literature suggests differences in Se-induced developmental toxicities among fishes. Species-specific differences in deposition of Se from adult female fish to eggs, and potential development of tolerance in fishes inhabiting Se-contaminated aquatic ecosystems are the most common factors hypothesized to cause differences in Se-induced

developmental toxicities among fishes (Holm et al., 2005; Rudolph et al., 2008). In addition, we suggest that other factors such as capital versus income breeding strategies, and concentration-dependent differences in free amino acid deposition (e.g. deposition of free SeMet) in eggs could influence differences in Se-induced developmental toxicities among oviparous fishes. Capital breeders use stored energy for reproduction, whereas income breeders directly depend on energy from food for reproduction (Bonnet et al., 1998; Bunnell et al., 2007). Fishes are thought to be capital breeders, however, a number of recent studies have shown that mixed capital-income breeding strategies exist in many fishes (Bonnet et al., 1998; Bunnell et al., 2007). Since asynchronous spawners lay eggs multiple times in a year, it is possible that those fishes might follow income or a mixed capital-income breeding strategies. Asynchronous spawners such as zebrafish, medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*) lay eggs multiple times per season and hence egg concentrations of Se might differ among spawning events. In fathead minnows, dietary exposure to methyl mercury (MeHg) during oogenesis has been shown to consistently increase concentrations of MeHg in eggs rather than the body burden of MeHg in adult females (Hammerschmidt and Sandheinrich, 2005). In the present study, we collected eggs for Se analysis from the highest Se-exposed group during different spawning events and this might have contributed to the high variability observed in egg Se concentration. Taken together, these results suggest that asynchronous spawners follow an income or a mixed capital-income breeding strategy, and those fishes could accumulate substantial concentrations of Se in eggs if they feed on Se-contaminated dietary items during oogenesis. Synchronous spawners such as Pacific salmon (*Oncorhynchus* spp.) and freshwater eels (*Anguilla rostrata*), and group synchronous spawners such as rainbow trout, are more likely to follow capital breeding strategies, and such fish might be susceptible to chronic dietary exposure of Se rather

than exposure during oogenesis. Collectively, differences in breeding strategies in oviparous fishes could cause differences in Se-induced developmental toxicities, and may be an important consideration underlying species-specific differences in sensitivity.

Another factor that could influence differences in Se-induced developmental toxicities among oviparous fishes is percent free amino acid deposition in eggs. Studies have identified deposition of free amino acids in eggs during oogenesis, and free amino acids serve as an important source of energy during early stages of embryo development (Kamler, 2008). Studies have also observed that concentrations of free amino acids can vary greatly in eggs of fishes (reviewed in Kamler, 2008). Since fish and other vertebrates cannot distinguish between SeMet and Met, we hypothesize that fishes that deposit greater concentrations of free SeMet into eggs could be more susceptible to Se-induced developmental toxicities. Energy production in early life stages of oviparous fishes is well studied. Carbohydrates are the major source of energy used in early stages of embryo development. Once the carbohydrate pool is depleted, the embryo switches to the free amino acid pool for energy requirements. After hatching, both lipid and protein catabolism increases in larval fishes as energy sources (Kamler, 2008). Hatching of zebrafish normally occurs on 3-4 dpf (Westerfield, 1995; Hill et al., 2005). In the present study we observed a greater incidence of larval zebrafish fish mortality from 4-6 dpf, which corresponds to the period when the larval fish are utilizing SeMet-rich yolk proteins for energy requirements prior to swim-up. Since we observed a greater incidence of larval zebrafish mortality from 4-6 dpf, we were interested to see whether exposure to free SeMet during early stages of zebrafish development could significantly increase embryo mortality and/or developmental toxicities. Our ongoing studies have tested this hypothesis by microinjecting free SeMet to early stage zebrafish embryos (between 15 min to 2 h post-fertilization), and

preliminary results indicate greater incidences of zebrafish embryo mortality and reduced hatchability (manuscript in preparation). Based on these findings, we suggest that the degree of free SeMet deposition in eggs of fishes might alter the sensitivity to Se-induced developmental toxicities.

The transition from endogenous to exogenous feeding is a crucial period in oviparous larval fish development, and approximately half of hatched larvae are expected to die during this period due to variation in individual development, availability of food resources, predation, and changes in environmental conditions (e.g. temperature and water quality) (Browman, 1989; Mills et al., 2000; Houde, 2002; Kamler, 2008). Apart from natural causes of larval fish death, exposure to a toxicant before or during this transition period can alter normal fish development and such effects can decrease survival of larval fish (Marit and Weber, 2012). In the present study, larval zebrafish exposed to excess Se via *in ovo* maternal transfer exhibited greater incidences of mortality and developmental toxicities before the onset of exogenous feeding. A number of previous studies where fish were exposed to Se via maternal transfer also reported similar responses (Coyle et al., 1993; Holm et al., 2005; Muscatello et al., 2006; reviewed in Janz et al., 2010). Survival of larval fish has been shown to directly co-relate with recruitment success in fish populations (Mills et al., 2000; Cowan and Shaw, 2002). In contrast, some fish biologists argue that decreased survival of larval fish due to high mortality might be compensated for by higher growth rates and fecundity in adult fish, since mortality of young fish reduces competition for food resources (reviewed in Hodson, 1990; Mills et al., 2000). We argue that the above scenario might be true if a chemical did not accumulate in organisms and transfer through the food web, or if changes in environmental conditions (e.g. changes in water temperature or food resources) persist only for a short period of time. Since Se can

bioaccumulate in organisms and transfer through the food web, fishes inhabiting Se-contaminated ecosystems are continuously exposed to Se. Previous studies from our laboratory and others have observed sublethal toxicities in juvenile and adult fishes exposed chronically to dietary Se (Thomas and Janz, 2011; Wiseman et al., 2011a,b; Thomas et al., 2013). Collectively, these findings suggest that once Se contamination occurs, toxicity may occur in all life stages of fishes, unless fishes are able to avoid a Se-contaminated area and inhabit aquatic ecosystems with less or no Se contamination.

Here we demonstrate for the first time that *in ovo* exposure to elevated concentrations of Se can cause persistent adverse effects in adult zebrafish. Reduced swim performance was observed in F1 generation adult zebrafish exposed to Se via maternal transfer and then reared in clean water and fed a control (Se normal) diet. Similar to the present study, Marit and Weber (2012) reported reduced swim performance in adult zebrafish exposed to TCDD only during early stages of development (2-5 dpf). Greater metabolic rate and cardiovascular deformities in adult zebrafish exposed to TCDD during early stages of development were observed, suggesting that impaired cardiovascular function might have been responsible for reduced swimming in adult zebrafish (Marit and Weber, 2012). Although the toxic mechanism of action of TCDD is different from Se, developmental exposure to both TCDD and Se has been shown to cause oxidative stress. Cantrell et al. (1996) reported that developmental exposure to TCDD in medaka can impair vascular development by a mechanism involving oxidative stress. Developmental exposure to SeMet in rainbow trout and medaka has also been demonstrated to induce oxidative stress (Palace et al., 2004; Lavado et al., 2012). It is possible that SeMet-induced oxidative stress during early life stages of zebrafish development might have caused impairment of cardiovascular development and such impairment could be responsible for the observed

reduction in swim performance of F1 generation adult zebrafish in the present study. Preliminary results from our ongoing respirometry study in adult zebrafish exposed to excess SeMet via maternal transfer observed elevations in both basal and active metabolic rates during swimming performance experiments (manuscript in preparation), again suggesting that developmental exposure to excess Se can alter cardiovascular function in zebrafish. In addition to impaired swimming, we also observed reduced tailbeat amplitude in adult zebrafish exposed to greater concentrations of SeMet via *in ovo* maternal transfer. Although we are unable to explain the mechanism responsible for the reduction of tailbeat amplitude in adult zebrafish in the present study, a number of hypotheses can be put forth for the observed effect. Specifically, the non-specific, dose-dependent insertion of SeMet in place of Met during skeletal muscle development and/or Se-induced oxidative stress might have caused permanent impairment in muscle function in zebrafish. Further studies are needed to investigate mechanisms of reduced tailbeat amplitude and impaired swimming resulting from developmental Se exposure in adult fish.

Both environmental and chemical stressors have been shown to alter stored energy and impair the physiological stress response in fishes (Mommsen et al., 1999; Thomas and Janz, 2011; Wiseman et al., 2011a,b; Thomas et al., 2013). However, in the present study we did not observe any significant changes in whole-body concentrations of stored energy (triglycerides and glycogen) or cortisol. A possible explanation for the observed results in the present study is that F1 generation zebrafish were fed daily and were raised to adulthood in a controlled laboratory environment with minimum stress, favourable conditions that created successful growth and survival of fish.

It is possible that wild fish with subtle developmental toxicities and/or reduced swimming capacity would exhibit reduced survival due to competition for food resources, predation and natural environmental stressors. In the present study, several zebrafish with spinal deformities or a deformed/missing operculum survived to adulthood after exposure to elevated concentrations of Se via *in ovo* maternal transfer. Since adult fish with severe spinal deformities were not able to swim in a straight line in the swim tunnel, those fish were removed from the experiment. Although the deformity analysis observed fin and mouth deformities in 6 dpf larval zebrafish exposed to elevated Se, larval fish with such deformities were not able to survive to adulthood, likely because such deformities would impair the ability to transition to exogenous feeding at swim-up. Although F1 generation zebrafish exposed to increasing concentrations of Se via *in ovo* maternal transfer showed a trend for greater cumulative mortality from 6-140 dpf than the controls, we were not able to detect any significant differences in mortality due to high variability in fish mortality across the treatment groups. In the present study, the observed cumulative mortality from 6-140 dpf in control zebrafish was 47.3%, which is similar to control group mortality reported in other zebrafish rearing studies (Carvalho et al., 2006; Marit and Weber, 2012).

Exposure to endocrine disrupting chemicals such as TCDD or methoxychlor during early stages of development in mammals or fishes have been shown to impair developmental programming by genetic or epigenetic mechanisms, and such developmental impairment caused latent toxicity in both adults and their offspring (Cantrell et al., 1996; Anway et al., 2005; Heiden et al., 2009). Exposure to elevated concentrations of Se during early stages of development can alter developmental programming (Lemly, 2002; Janz et al., 2010; Ma et al., 2012), and a number of studies have reported endocrine disrupting activities of both inorganic and organic

forms of Se in fishes (Miller et al., 2007; Thomas and Janz, 2011; Wiseman et al., 2011a,b). In addition, a recent study in early stages of zebrafish development found impaired genomic DNA methylation resulting from elevated selenite exposure (Ma et al., 2012). Based on these studies, we investigated whether *in ovo* exposure to greater concentrations of Se caused adverse transgenerational effects in zebrafish. Although the present study observed latent toxicity (impaired swimming) in adult zebrafish exposed *in ovo* to greater concentrations of Se, egg viability, embryo hatchability, and mortality and deformities in the offspring of F1 generation adult female zebrafish were not altered. Similar to our finding, Brown et al. (2009) reported an absence of transgenerational effects after developmental exposure to 17α-ethynylestradiol in rainbow trout. The lack of reproductive impairment in F1 generation adult female zebrafish exposed *in ovo* to greater concentrations of Se suggests that developmental exposure to excess Se may not cause transgenerational effects in fishes.

4.6 Conclusion

In conclusion, the results of our study have demonstrated that exposure to environmentally relevant concentrations of Se via *in ovo* maternal transfer increases the incidence of mortality and deformities in larval zebrafish in a concentration-dependent fashion, and such developmental exposure can also cause persistent adverse effects in adult zebrafish (impaired swim performance and tailbeat amplitude). Taken together, both a reduction in juvenile recruitment and persistent alteration in swimming behaviour of fishes exposed to greater concentrations of Se via *in ovo* maternal transfer might significantly alter population dynamics of wild fishes inhabiting Se-contaminated aquatic ecosystems. Reproductive performance of adult zebrafish exposed *in ovo* to elevated Se, and then reared in clean water and fed a control diet was not impaired, suggesting that developmental exposure to excess Se may not cause

transgenerational effects. However, potential transgenerational effects following chronic dietary Se exposure throughout the life cycle of fishes were not investigated in the present study.

CHAPTER 5

DEVELOPMENTAL AND PERSISTENT TOXICITIES OF MATERNALLY DEPOSITED SELENOMETHIONINE IN ZEBRAFISH (*DANIO RERIO*)

Preface

The research described in this chapter was a follow-up study of Chapter 4. The objectives of this study were to develop egg Se toxicity thresholds for mortality and deformities in early life stages of zebrafish after maternal transfer of graded concentrations of SeMet, and to further investigate mechanisms of impaired swimming in F1generation adult zebrafish exposed to excess SeMet via maternal transfer. I collected embryos from a sub-group of adult female zebrafish from the feeding experiment described in Chapter 3 for this study. Egg Se threshold obtained from this study was compared to other published egg Se thresholds in fishes, indicating that early life stages of zebrafish are the most sensitive fish species studied to date. Reduced swimming performance and factorial aerobic scope, and greater oxygen consumption and standard metabolic rates were observed in F1 generation zebrafish exposed to excess SeMet via maternal transfer. Results of this study suggest that impaired cardiovascular performance may have caused reduction in swimming in F1 generation zebrafish exposed to excess SeMet via maternal transfer.

5.1 Abstract

Reproductive toxicities have been identified in both coldwater and warmwater fishes inhabiting aquatic ecosystems contaminated with selenium (Se). Selenomethionine (SeMet) is the dominant organic Se species present in diets and fish tissues in Se contaminated sites. The objectives of present study were to establish egg Se toxicity thresholds for mortality and deformities in early life stages of zebrafish (*Danio rerio*) after exposure to excess SeMet via *in ovo* maternal transfer, and to investigate persistent effects of developmental exposure to excess SeMet on swim performance (U_{crit}), oxygen consumption (MO_2), metabolic capacities (standard metabolic rate [SMR], active metabolic rate [AMR], factorial aerobic scope [F-AS] and cost of transport [COT]) in F1 generation adult zebrafish. Adult zebrafish were fed either control food (1.3 µg Se/g, dry mass or d.m.) or food spiked with increasing concentrations of Se (3.4, 9.8 or 27.5 µg Se/g, d.m.) in the form of SeMet for 90 d at 5% body mass/ d ration. Concentrations of Se in eggs of adult zebrafish fed 1.3, 3.4, 9.8 or 27.5 µg Se/g d.m. were 1.3, 6.8, 12.7 or 34.1 µg Se/g d.m., respectively. Exposure to excess Se via *in ovo* maternal transfer increased mortality and deformities in larval zebrafish in a concentration-dependent fashion, with significantly greater incidences of mortality and deformities observed in larval fish exposed to the two highest concentrations of Se. Concentrations of egg Se to cause a 10, 20 or 50% increase in deformities above controls were 7.0, 11.4 and 29.2 µg Se/g d.m., respectively, and mortalities were 7.5, 13.2 and 39.1 µg Se/g d.m., respectively. Reduced swim performance was observed in F1 generation adult zebrafish exposed to 6.8 and 12.7 µg Se/g d.m. via *in ovo* maternal transfer when compared to the control group. Significantly greater oxygen consumption (MO_2), standard metabolic rate (SMR) and cost of transport (COT) were observed in F1 generation adult fish exposed to 6.8 and 12.7 µg Se/g d.m. via *in ovo* maternal transfer. A species sensitivity distribution (SSD) based on

egg Se toxicity thresholds (effective concentration [EC]10s) suggests that early life stages of zebrafish are the most sensitive fish species studied to date, and thus is a good laboratory model to investigate mechanism of Se-induced toxicities in early life stages of fish.

5.2 Introduction

Although trace concentrations of selenium (Se) are required to maintain physiological homeostasis in all animals, a marginal increase in Se intake can lead to Se accumulation and subsequent toxicity. It has been widely recognized that oviparous species, especially fishes, are highly susceptible to toxic effects of Se (Lemly, 1997a; Janz, 2012). Comparison of the range between essentiality and toxicity of ingested trace elements in freshwater fishes clearly demonstrates that Se is the most toxic essential trace element, with a narrow margin of safety (Lemly, 1997a; Watanabe et al., 1997; Janz et al., 2010). Selenium is classified as a contaminant of potential concern in many countries due to poisonings reported in fishes and other wildlife (Lemly, 2004; Janz et al., 2010). Anthropogenic activities such as mining, coal-fired power plants, oil refining activities, and agriculture on seleniferous soils can significantly augment mobilization of Se into aquatic systems (Janz et al., 2010). Selenium enters aquatic ecosystems mainly as inorganic selenate and selenite, where primary producers and certain bacteria facilitate biomethylation of these inorganic forms into more bioavailable organoselenium forms (Fan et al., 2002). Selenomethionine (SeMet) is identified as the major form of organic Se present in invertebrates and fish tissues collected from Se contaminated aquatic ecosystems, and organic Se has greater bioaccumulative and trophic transfer properties than other forms of Se (Fan et al., 2002; Phibbs et al., 2011). Both laboratory and field studies demonstrated that exposure of excess dietary Se (mainly SeMet) to adult female fishes can cause greater accumulation of Se in their eggs, and such exposure (maternal transfer) can augment Se-induced mortality and deformities in early life stages of F1 generation fishes (Doroshov et al., 1992; Coyle et al., 1993; Holm et al., 2005; Muscatello et al., 2006; Thomas and Janz 2014).

Since the majority of Se exposure in fishes occurs through diet, ambient water quality guidelines or criteria for Se provide limited protection of fish populations against Se toxicity. The United States Environmental Protection agency (USEPA) (2004) proposed a chronic whole-body Se criterion of 7.91 µg/g d.m. for protection of fish populations. Previous studies proposed that egg and/or ovary are the most appropriate tissues for determining Se toxicity thresholds in early life stages of F1 generation fishes after maternal Se exposure (Lemly, 1993a; Canton et al., 2008; Janz et al., 2010). Since species-specific differences in deposition of Se from adult female fish to eggs (Holm et al., 2005; Rudolph et al., 2008) and different breeding strategies (i.e. capital breeding [using stored energy for reproduction] and mixed capital-income breeding [using both stored energy and energy from food for reproduction]) exist in fishes (Bonnet et al., 1998; Bunnell et al., 2007), whole-body Se thresholds might not be as protective as egg Se thresholds for protection of fish populations. The USEPA is revising the current whole-body Se criterion draft to an egg Se based criterion (DeForest et al., 2012). Recently, the USEPA considered using effective concentration (EC) 10s in developing a site-specific Se criterion for the Great Salt Lake (Canton et al., 2008). Lemly (1993a) proposed an egg or ovary Se threshold of 10 µg/g d.m. for protection of fish populations. On the contrary, DeForest et al. (2012) recommended an egg or ovary Se threshold of 20 µg Se/g d.m. for protection of coldwater fish populations. A recent dietary SeMet exposure study (Pilgrim, 2012) reported egg Se thresholds (EC 10) for mortalities in rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*) and cutthroat trout (*Oncorhynchus clarki lewisi*), that were much lower than the egg Se toxicity thresholds (EC10s) used for the same fish species in species sensitivity distribution (SSD) generated by DeForest et al. (2011). Laboratory based dietary SeMet exposure studies suggest that SeMet might be the most toxic form of Se to early life stages of fishes and is the

ideal form of Se to study mechanisms of Se toxicity (Janz et al., 2010; Pilgrim, 2012). In a previous study, Thomas and Janz (2014) reported significantly greater mortality and deformities in early life stages of zebrafish (*Danio rerio*) after exposure to excess SeMet via *in ovo* maternal transfer, and the same study also found reduced swim performance in F1 generation adult zebrafish that survived developmental exposure of excess SeMet. Oxygen consumption (MO_2) is a measure of metabolic activity of fish and is often measured in combination with swim performance experiments (Thomas et al., 2013; Thomas and Janz, 2011). Previous studies have demonstrated that impaired cardiac performance and/or MO_2 can alter swim performance of fishes (Marit and Weber, 2012; Thomas et al., 2013).

The objectives of present study were to establish egg Se toxicity thresholds for mortality and deformities in early life stages of zebrafish after exposure to excess SeMet via *in ovo* maternal transfer, and to investigate persistent effects of developmental exposure to excess SeMet on swim performance (U_{crit}), oxygen consumption (MO_2) and metabolic capacities in F1 generation adult zebrafish. In addition, egg Se EC10s from the present study and other studies were used to generate a SSD to examine the relative sensitivity of zebrafish to Se toxicity.

5.3 Materials and methods

5.3.1 Test compound

Seleno-L-methionine was purchased from Sigma-Aldrich (Oakville, ON, Canada). Purity of the compound was greater than 98%.

5.3.2 Diet preparation and adult exposure

All methods used in the present study were approved by the University of Saskatchewan's Animal Research Ethics Board (protocol # 20030076), and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Adult zebrafish were purchased from a local supplier and housed in an environmental chamber with controlled temperature (28.0 ± 1.0 °C) and photoperiod (14 h light and 10 h dark). Adult fish were acclimated to these laboratory conditions for 3 weeks. During the acclimation period fish were fed Nutrafin® basic flake food (Hagen Inc., Montreal, QC, Canada). Both diet preparation and dietary SeMet exposure to adult fish were described previously (Thomas et al., 2013). Briefly, adult zebrafish were fed twice daily (5% body mass/d ration) with either control or SeMet-spiked diets (nominal concentrations of 3, 10 and 30 μg Se/g d.m.) for 90 d. After 90 d of feeding exposure, sub-groups of adult fish from each dietary treatment group were bred only once to collect eggs for the experiment. Embryos were used to investigate adverse effects of excess SeMet exposure via *in ovo* maternal transfer to F1 generation fish. Concentrations of total Se in eggs, percent egg viability and hatchability, and percent mortality and total deformities in F1 generation larval fish were determined. Percent egg viability was determined as the ratio of number of fertilized eggs to the total number of eggs, and percent hatchability as the ratio of the number of hatched larval fish to the total number of fertilized eggs. Percent mortality was calculated by dividing the number of embryos/larval fish by the total number of live embryos/larval fish, and then multiplying by 100.

In addition, a sub-group of larval zebrafish from each treatment group were reared in clean water (temperature 28.0 ± 1.0 °C) and fed the control diet to 180 days post fertilization

(dpf) to investigate persistent effects of developmental SeMet exposure on U_{crit}, MO_2 and metabolic capacities in F1 generation adult zebrafish.

5.3.3 Deformity analysis

Total deformity analyses were carried out on 6 dpf larval zebrafish. The detailed larval zebrafish preservation protocol for deformity analysis was explained previously (Thomas and Janz, 2014). All preserved larval zebrafish were examined for malformations in a blind fashion using an Olympus model SZ-CTV dissecting microscope (Olympus, Melville, NY, USA). Each larval fish was examined for skeletal, craniofacial and fin deformities, and edema, and the presence or absence of developmental abnormalities was recorded for each fish. Although the majority of larval zebrafish used in deformity analysis were at 6 dpf, severely deformed larval zebrafish were also euthanized and preserved on 4 and 5 dpf and included in deformity analysis. Total percent deformities were calculated by dividing number of malformed larval fish by the total number of larval fish, and multiplying by 100.

5.3.4 Quantification of selenium

Concentrations of total Se in pooled egg samples were measured by use of inductively coupled plasma-mass spectrometry (ICP-MS) at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK, Canada). For the quantification of Se we collected 3 replicates of 100 pooled egg samples from adult female zebrafish fed the control diet and 3 replicates of 60 pooled egg samples from adult female fish fed the SeMet spiked diets. Detailed Se analysis procedure was described previously (Thomas and Janz, 201; Thomas et al., 2013). A limit of quantification (LOQ) of 0.3 μg Se/g was determined using method blanks. Concentrations of Se

in eggs were measured on wet mass basis and converted to dry mass based on a moisture content of 93.7 %.

5.3.5 Oxygen consumption and swim performance

Consumption of oxygen and swim performance were conducted in a modified Blazka-type, variable speed, miniature swim tunnel respirometer with a DAQ-M control device and AutoResp™ 1 software (Loligo Systems, Tjele, DK). The system consists of a 170 mL swim tunnel submerged in a 20 L buffer tank supplied with 28.5 ± 0.1 °C aerated water from a 20 L heated water bath circulator (VWR International, Mississauga, ON, Canada). Concentrations of oxygen (O_2) were measured using a fiber optic oxygen dipping probe which was connected to a Fibox 3 minisensor oxygen meter (Precision Sensing GmbH, Regensburg, DE). AutoResp™ 1 software was used to calculate MO_2. The detailed MO_2 measuring principle is explained elsewhere (Steffensen et al., 1984). We adopted a method called critical swimming speed (U_{crit}) to study aerobic swimming capacity of fish (Plaut, 2001). The detailed procedures for both oxygen consumption and swim performance were discussed elsewhere (Thomas et al., 2013). Fish were not fed 24 h prior to MO_2 and U_{crit} analyses. Fish were acclimated for 1.5–2 h at a minimal water velocity of 0.8 BL/s (body length per second) to achieve a stabilized minimal rate of metabolism after introduced in the swim tunnel respirometer. In the U_{crit} experiment, individual fish were subjected to step-wise increments in swimming velocity (2.9 BL/s every 20 min) until exhaustion. Once the experiment was completed, the highest completed swimming velocity and time spent at fatigue velocity were recorded. Critical swimming speed of F1 generation adult zebrafish was calculated using an equation explained by Plaut (2001). Fish cross sectional area was less than 5% of the swim tunnel cross sectional area, hence U_{crit} values were not corrected for solid blocking effect. Swim performance values (cm/s) were corrected for

standard body length of individual fish, and thus U_{crit} data were represented as body lengths per second (BL/s).

5.3.6 Determination of standard metabolic rate (SMR), active metabolic rate (AMR), factorial aerobic scope (F-AS) and cost of transport (COT)

Standard metabolic rate (SMR) was calculated by extrapolating MO_2 back to a water velocity of zero. This was done from a plot of swimming speed (m/s) versus MO_2 (mg O_2/kg/h) and use of nonlinear, curve fitting regression analysis (Webber et al., 1998; Thomas et al., 2013). Active metabolic rate (AMR) is defined as the MO_2 at maximum sustainable speed in the U_{crit} test. The factorial aerobic scope (F-AS) was calculated as AMR/SMR (Webber et al., 1998; Thomas et al., 2013). Cost of transport (J/kg/m) was calculated by multiplying MO_2 (mg O_2/kg/s) by an oxycaloric value of 14.1 J/mg O_2 and then dividing by the corresponding swimming speed (m/s) (Videler, 1993).

5.3.7 Statistical analysis

All data were tested for normality by use of the Shapiro–Wilk test and homogeneity of variance was investigated by use of Levene's test (SigmaStat 3.1, SPSS Inc., Chicago, IL, USA). Data that did not meet the assumptions for parametric statistical procedures were log 10 transformed. Non-transformed data are shown in all figures. Significant differences in total Se concentrations in egg, egg viability and hatchability, mortality and total deformities of larval fish, U_{crit}, MO_2 and COT at individual water velocities, SMR, AMR, F-AS and morphometrics (total length, wet mass and condition factor) of adult zebrafish from different treatment groups were tested by use of one-way ANOVA followed by the Holm–Sidak post hoc test. Data were expressed as mean ± S.E.M. Differences were considered statistically significant at $p < 0.05$.

Egg Se concentrations to cause 10, 20 and 50 % mortality or deformities in larval zebrafish were calculated by use of TOXSTAT® version 3.5 software (Western Ecosystems Technology [1996], Cheyenne, WY, USA). Abbott's formula was used to adjust mortality or deformities in control groups. Egg Se concentrations to cause 10% effects (EC10s) were taken from the present study and other published studies to generate a species sensitivity distribution (SSD) (Table 2). We represented the most sensitive effect (either mortality or deformities) as EC 10 (to be consistent with other published Se literature). The SSD generator (version V1) from the USEPA was used to create the SSD. If a fish species has more than one published EC10 values, we used the geometric mean of those values to generate the SSD. Correlation analysis was performed between egg Se EC10s (µg Se/g d.m.) versus temperatures (°C) at which different larval fishes were raised in Se exposure studies.

5.4 Results

5.4.1 Concentrations of selenium

The concentrations of total Se in diets and in whole-body adult zebrafish samples were reported in Thomas et al. (2013). Concentrations of Se in eggs of adult female zebrafish were proportional to concentrations of Se in their diets. Concentrations of total Se in eggs of adult zebrafish fed 1.3, 3.4, 9.8 or 27.5 µg Se/g d.m. diets were 1.3 ± 0.2, 6.8 ± 0.4, 12.7 ± 1.5 or 34.1 ± 3.7 µg Se/g d.m., respectively. Significantly greater accumulation of Se was observed in eggs collected from adult female zebrafish fed SeMet spiked diets when compared to eggs collected from adult female zebrafish fed the control diet (1.3 µg Se/g d.m.) ($p < 0.05$; Table 5.1).

Table 5.1: Concentrations of total selenium in eggs, and cumulative mortalities and morphometrics of F1 generation adult zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Cumulative mortalities were calculated from 6-180 days post fertilization (dpf) and morphometrics were determined on 180 dpf. Data are mean ± S.E.M of n = 3 replicates of 60-100 pooled eggs for quantification of selenium, n= 3 replicates of 40-50 fish for cumulative fish mortality analysis, and n= 9-16 fish for morphometrics.

Egg Se (µg/g, dry mass)	Cumulative mortalities (6-180 dpf)	Total length (mm)	Wet mass (g)	Condition factor
1.3 ± 0.2	45.3 ± 5.8	32 ± 0.4	0.269 ± 0.01	0.80 ± 0.04
6.8 ± 0.4*	62.0 ± 11.1	33 ± 0.7	0.303 ± 0.02	0.76 ± 0.04
12.7 ± 1.5*	60.7 ± 9.3	27 ± 1.0*	0.168 ±0.03*	0.86 ± 0.13
34.1 ± 3.7*	80.7 ± 6.4	33 ± 0.7	0.307 ± 0.03	0.79 ± 0.04

Condition factor = (wet mass/total length^3) x 100,000.

*, Significantly different from the control group using one-way ANOVA followed by the Holm–Sidak post hoc test ($p < 0.05$).

5.4.2 Hatchability, mortalities and total deformities

A concentration-dependent increase in mortality and deformities was observed in F1 generation larval zebrafish exposed to excess Se in the form of SeMet via *in ovo* maternal transfer (Figures 5.1 and 5.2). However, excess Se exposure did not alter percent egg viability and hatchability among different treatment groups (see Appendix, Table C5.S1). Significantly greater mortality and total deformities were noticed in larval fish exposed to 12.7 or 34.1 μg Se/g d.m. via *in ovo* maternal transfer when compared to the controls ($p < 0.05$; Figure 5.1and 5.2). Percent mortality of zebrafish larvae increased from 6.0 % in the control group to between 15.2-48.3 % in larval zebrafish exposed to excess Se via *in ovo* maternal transfer, whereas total deformities increased from 4.3 % in the control group to between 10.5- 59.4 % in larvae from excess Se treatment groups. There were significant quadratic relationships between egg Se concentrations and percentage of larval fish mortality ($p = 0.0002$, $r^2 = 0.86$; see Appendix, Figure C5.S1) or percentage larval fish exhibiting total deformities ($p < 0.0001$, $r^2 = 0.94$; see Appendix, Figure C5.S2). Egg Se concentrations to causing 10, 20 or 50% increases in larval zebrafish mortalities compared to the controls were 7.5, 13.2 and 39.1 μg Se/g d.m., respectively and total deformities were 7.0, 11.4 and 29.2 μg Se/g d.m., respectively.

With the exception of the 12.7 μg Se/g d.m. treatment group, adult zebrafish that survived developmental exposure of excess Se via *in ovo* maternal transfer route did not show significant differences in total length, body mass or condition factor (Table 5.1). Significant reductions in body length and body mass were observed in F1 generation adult zebrafish exposed to 12.7 μg Se/g d.m. via maternal transfer ($p < 0.05$; Table 5.1). Although cumulative mortalities of F1 generation zebrafish from 6-180 dpf were greater in excess Se exposed groups than the controls,

no statistically significant differences were observed among treatment groups ($p = 0.09$; Table 5.1).

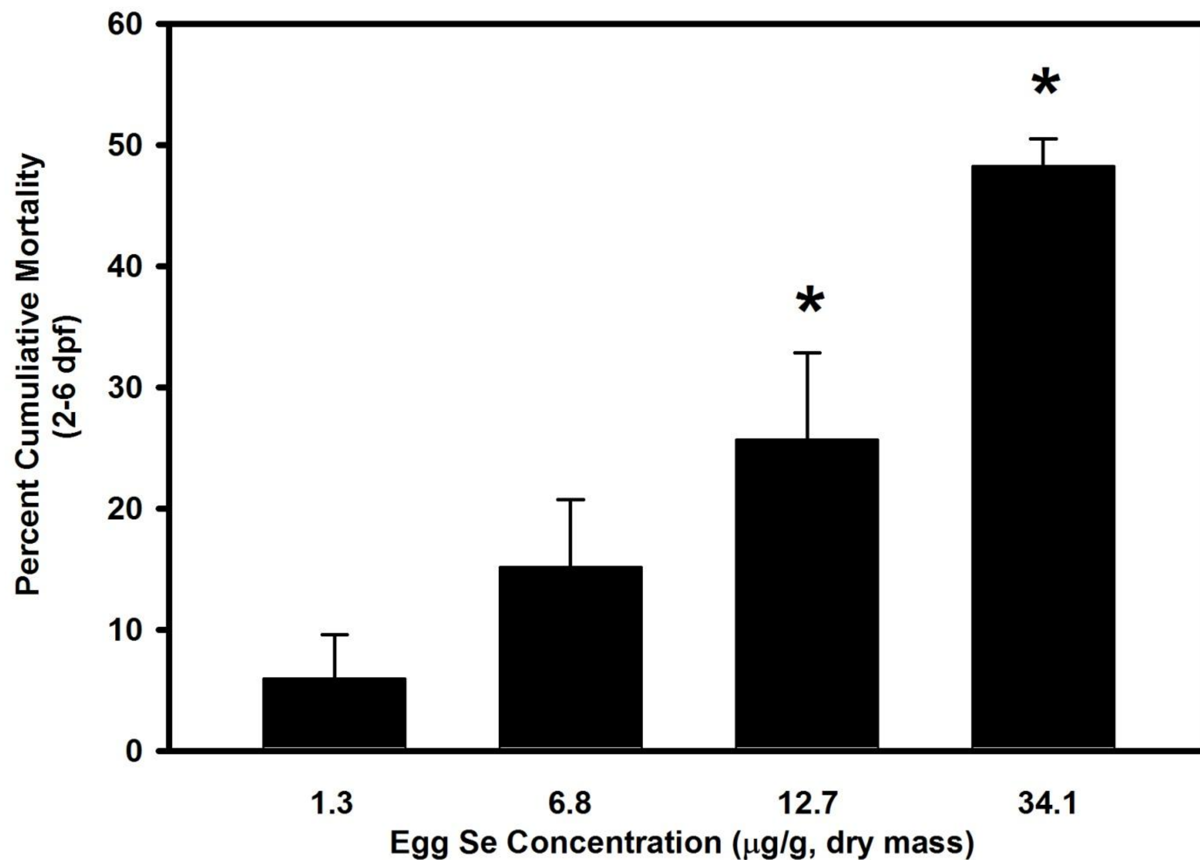

Figure 5.1: Percent cumulative mortalities (2-6 days post fertilization) of embryo or larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. *, Significant difference compared to control group using one-way ANOVA followed by the Holm–Sidak post hoc test ($p < 0.05$; n = 3 replicates of 50-200 embryos).

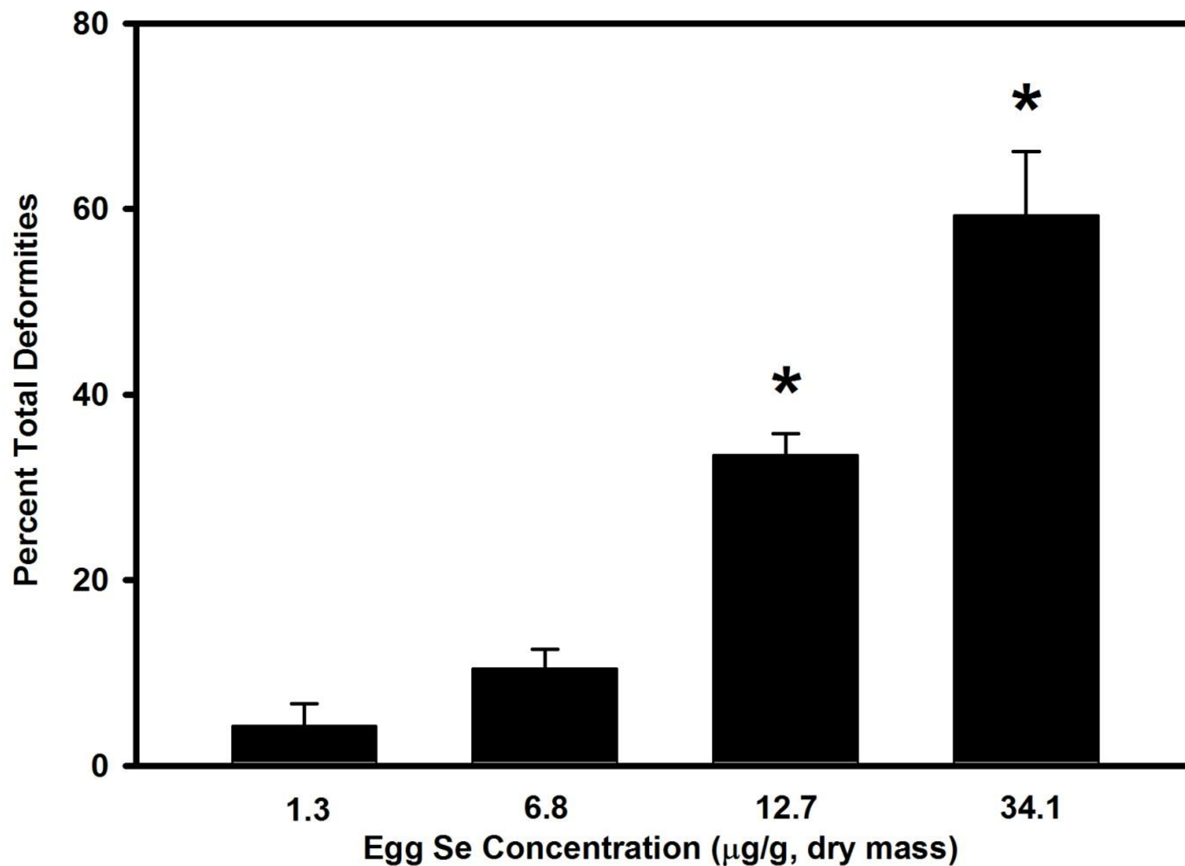

Figure 5.2: Total morphological abnormalities (sum of skeletal, craniofacial and fin deformities, and edema) in larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. *, Significant difference compared to control group using one-way ANOVA followed by the Holm–Sidak post hoc test ($p < 0.05$; n = 3 replicates of 50-150 embryos).

5.4.3 Species sensitivity distribution (SSD) and effective concentration (EC10) egg Se versus temperature

The species sensitivity distribution (SSD) graph indicates that zebrafish is the most sensitive fish species studied to date following *in ovo* exposure to excess Se (Figure 5.3). The SSD plot shows a trend for lower egg Se EC 10 values in the laboratory based Se exposure studies than the field based Se exposure studies (Figure 5.3). Correlation analysis between egg Se toxicity thresholds (EC 10s) in early life stages of fishes versus temperatures (°C) at which larval fishes were reared did not show any association (Table 5.2; see Appendix, Figure C5.S3).

Table 5.2: Egg selenium concentrations causing 10% effects (effective concentration [EC] 10) in early life stages of fishes after *in ovo* maternal transfer exposure. Egg Se EC10s were used to create a species sensitivity distribution (SSD), and the EC10s and temperatures were used for correlation analysis.

Type of adult Exposure	Species	Endpoint	Se (µg/g, d.m.)	Temperature (°C)	Reference
Lab (dietary SeMet)	Zebrafish *(Danio rerio)*	Larval Deformities	7	28	This study
Lab (dietary SeMet)	Brook trout (*Salvelinus fontinalis)*	Larval Mortality	8.9	9.4	Pilgrim, 2012
Lab (dietary SeMet)	Cutthroat trout (*Oncorhynchus clarki lewisi*)	Larval Mortality	11.2	9.7	Pilgrim, 2012
Lab (dietary SeMet)	Rainbow trout (*Oncorhynchus mykiss*)	Larval Mortality	12.4	7.8	Pilgrim, 2012
Lab (dietary SeMet)	White sturgeon (*Acipenser transmontanus*)	Larval Edema and/or Skeletal Deformities	15.3	15.5	Linville, 2006
Lab (dietary SeMet)	Bluegill sunfish (*Lepomis macrochirus*)	Larval Edema	21.2	26	Doroshov et al., 1992
Lab (dietary SeMet and waterborne selenate and selenite)	Bluegill sunfish (*Lepomis macrochirus*)	Larval Mortality	22	28	Coyle et al., 1993; Deforest et al., 2011
Field (dietary and waterborne Se)	Cutthroat trout (*Oncorhynchus clarki lewisi*)	Larval Mortality	17	14.5	Rudolph et al., 2008; Deforest et al., 2011

Field (dietary and waterborne Se)	Cutthroat trout (*Oncorhynchus clarki lewisi*)	Larval Mortality	19	11	Elphick et al., 2009
Field (dietary and waterborne Se)	Brown trout (*Salmo trutta*)	Larval Mortality	19.3	10	Formation Environmental and HabiTech, 2012
Field (dietary and waterborne Se)	Northern pike (*Esox lucius*)	Larval Deformities	20.4	10	Muscatello et al., 2006
Field (dietary and waterborne Se)	Rainbow trout (*Oncorhynchus mykiss*)	Larval Deformities	21.1	8	Holm et al., 2005; Janz et al., 2010
Field (dietary and waterborne Se)	Dolly varden (*Salvelinus malma*)	Larval Deformities	54	5	McDonald et al., 2010

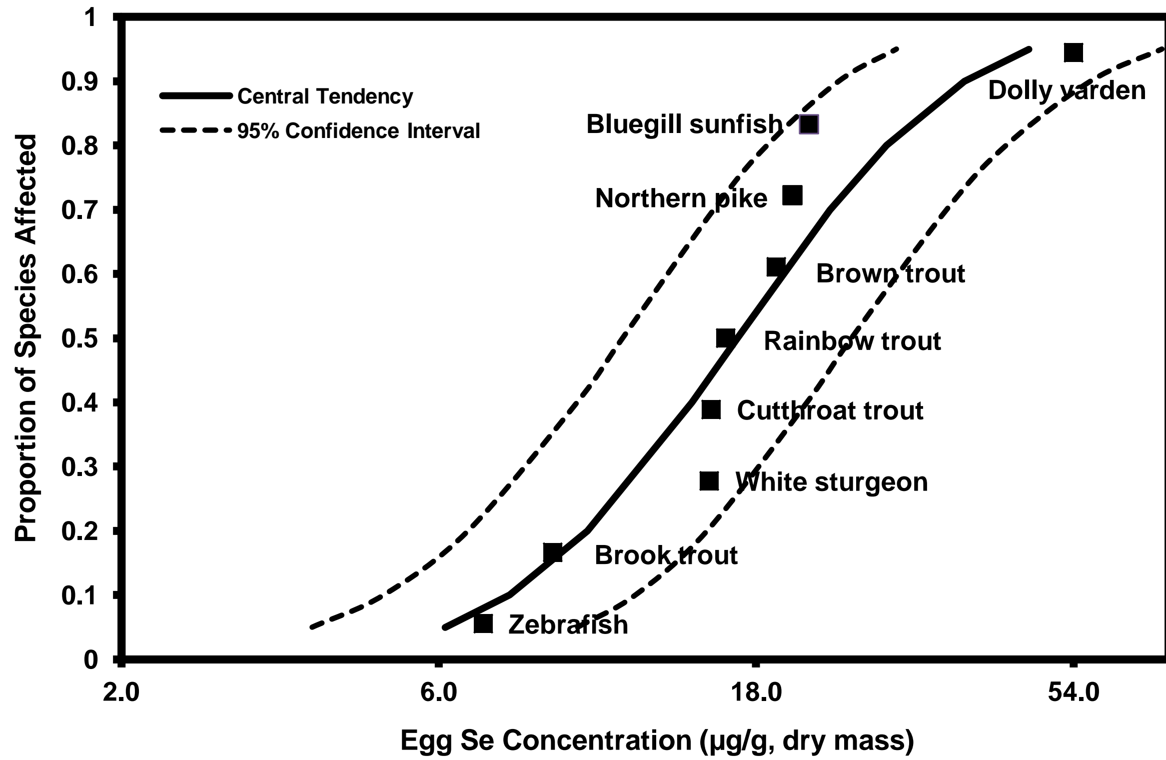

Figure 5.3: Species sensitivity distribution (SSD) based on egg selenium thresholds (effective concentration [EC] 10s) in early life stages of fishes exposed to excess Se via maternal transfer. See Table 2 for sources of EC10s obtained to generate the SSD.

5.4.4 Swim performance and oxygen consumption

Developmental exposure to excess SeMet reduced swim performance (U_{crit}) of F1 generation adult zebrafish. A significant reduction in swim performance (U_{crit}) was observed in adult zebrafish exposed to 6.8 and 12.7 µg Se/g d.m. via *in ovo* maternal transfer when compared to adult fish from the control group ($p < 0.05$; Figure 5.4A). No difference in U_{crit} was observed between adult fish from the highest *in ovo* SeMet exposure group and the control group (Figure 5.4A). Swim performance (U_{crit}) of F1 generation adult zebrafish exposed to 1.3, 6.8, 12.7 or 34.1 µg Se/g d.m. via *in ovo* maternal transfer were 11.6 ± 0.6, 8.8 ± 0.8, 8.7 ± 1.2 and 11.1 ± 0.7 BL/s, respectively.

Rate of oxygen consumption (MO_2) of adult zebrafish increased with increasing swimming speed (Figure 5.4B). Significantly greater MO_2 was observed in adult zebrafish exposed to 6.8 and 12.7 µg Se/g d.m. via *in ovo* maternal transfer at water velocities of 0.02, 0.09 and 0.17 m/s when compared to MO_2 of adult fish from the control group ($p < 0.05$; Figure 5.4B). Significantly higher MO_2 was observed in adult fish from the highest SeMet treatment group at water velocities of 0.09 m/s ($p < 0.05$; Figure 5.4B).

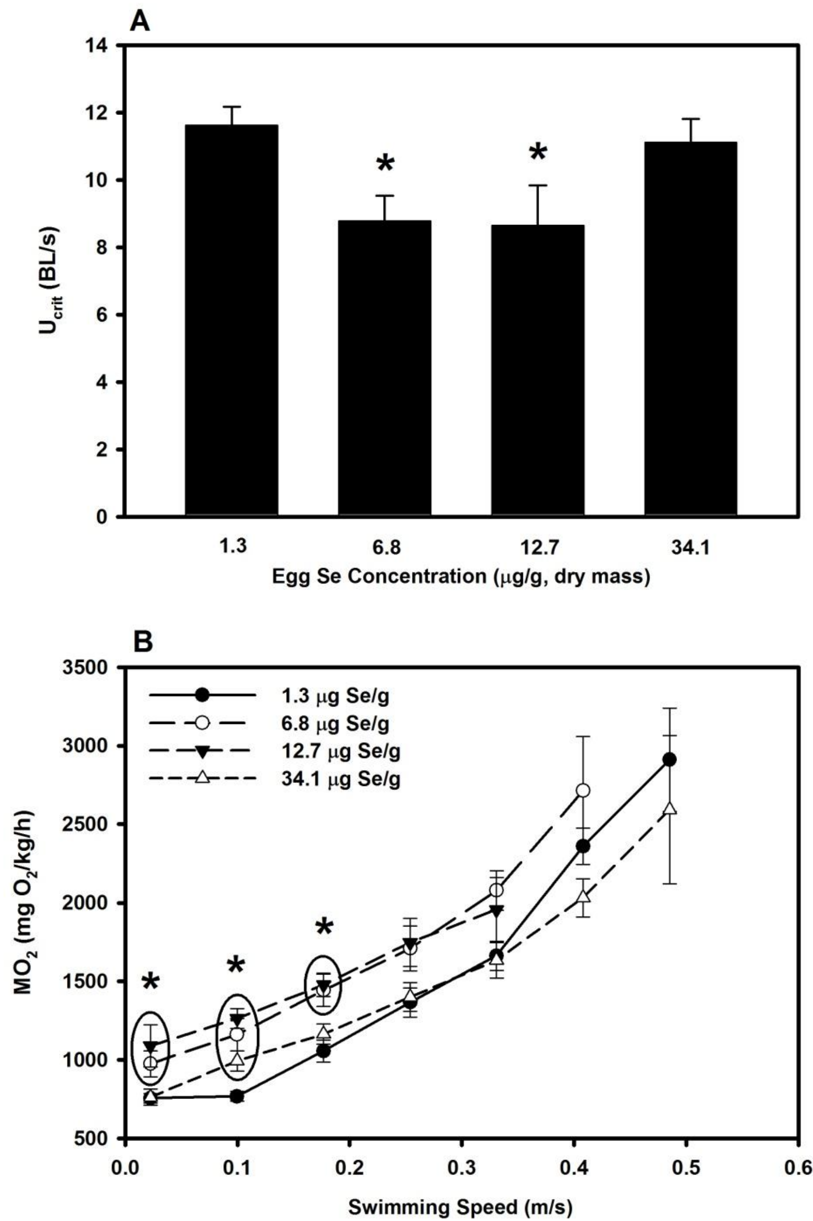

Figure 5.4: Swim performance (U_{crit}; 5.4A) and oxygen consumption (MO_2; 5.4B) of F1 generation adult zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Both U_{crit} and MO_2 were determined on 180 days post fertilization in F1 generation adult zebrafish. *, Significant difference compared to control group using one-way ANOVA followed by the Holm–Sidak post hoc test ($p < 0.05$; n = 9-16 fish for U_{crit} and n = 7-14 fish for MO_2).

5.4.5 Standard metabolic rate (SMR), active metabolic rate (AMR), factorial aerobic scope (F-AS) and cost of transport (COT)

Significantly greater SMR was observed in F1 generation adult fish from 6.8 and 12.7 µg Se/g d.m. treatment groups when compared to adult fish from the control group ($p < 0.05$; Figure 5.5A). No change in AMR was observed among adult fish from the different treatment groups. The F-AS of adult fish exposed to 1.3, 6.8, 12.7 or 34.1 µg Se/g d.m. via *in ovo* maternal transfer were 3.0, 2.6, 1.8 and 2.7, respectively, and the F-AS of fish from the 12.7 µg Se/g d.m. group was significantly lesser than fish from the control group ($p < 0.05$; Figure 5.5A). Cost of transport was consistently decreased with increasing swim speed in adult fish from different treatment groups (Figure 5.5B). At water velocities of 0.02, 0.09 and 0.17 m/s, COT were significantly greater in adult zebrafish exposed to 6.8 and 12.7 µg Se/g d.m. via *in ovo* maternal transfer when compared to adult fish from the control group ($p < 0.05$; Figure 5.5B). With the exception of 0.09 m/s water velocity, COT measured at all other water velocities were similar between adult zebrafish exposed to 34.1 µg Se/g d.m. via *in ovo* maternal transfer and adult fish from the control group (Figure 5.5B). Significantly greater COT was observed in F1 generation adult fish from the highest SeMet exposure group at 0.09 m/s water velocity ($p < 0.05$; Figure 5.5B).

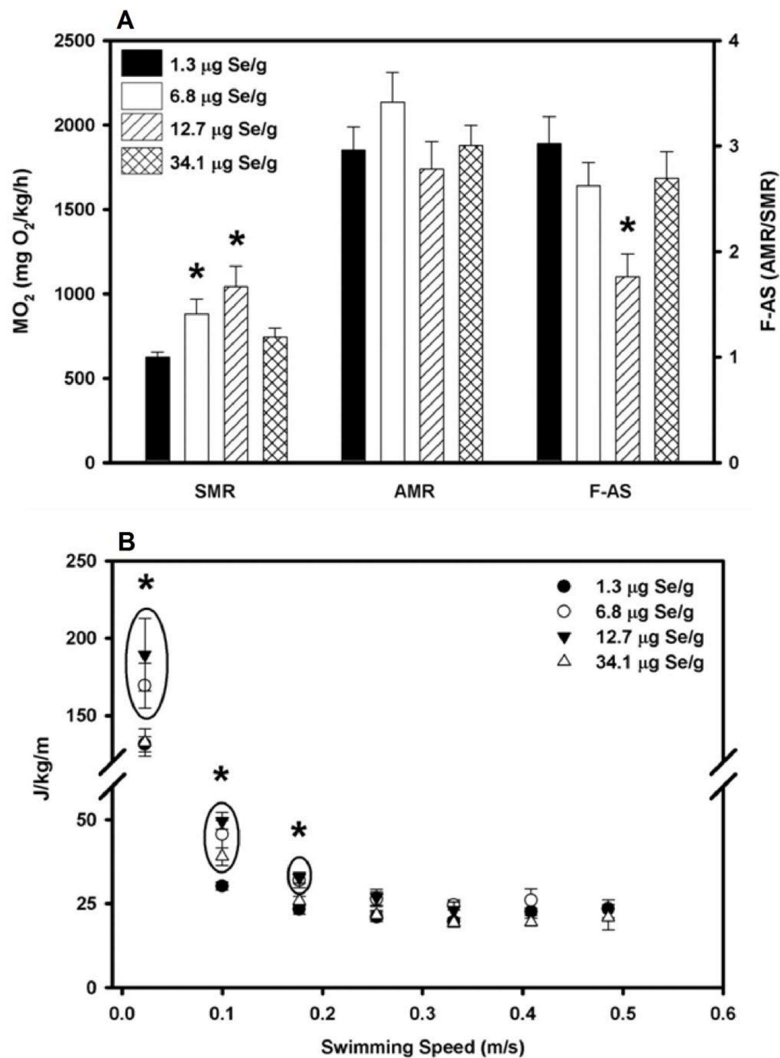

Figure 5.5: Metabolic capacities (standard metabolic rate [SMR; the minimal maintenance oxygen consumption of unfed fish], active metabolic rate [AMR; the oxygen consumption at the maximum sustainable swimming speed in Ucrit] and factorial aerobic scope [F-AS; the ratio of AMR to SMR]; 5.5A) and cost of transport (COT; 5.5B) of F1 generation adult zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Both measurements were carried out on 180 days post fertilization in F1 generation adult zebrafish. *, Significant difference compared to control group using one-way ANOVA followed by the Holm–Sidak post hoc test ($p < 0.05$; n = 7-14 fish for both metabolic capacities and COT).

5.5 Discussion

In aquatic ecosystems contaminated with Se, 50-70% of total Se occurs in diets and fishes as SeMet (Fan et al., 2002; Phibbs et al., 2011). Accumulated Se in adult female fish can transport and deposit in eggs during vitellogenesis and toxicities in early life stages of fishes occur when they utilize Se rich yolk protein for development (Janz et al., 2010). In the present study adult zebrafish were fed dietary SeMet to mimic an environmentally relevant Se exposure (maternal transfer) scenario in F1 generation larval fish, and such exposure was carried out to establish toxicity thresholds for Se-induced mortality and deformities. To our knowledge this is the first study calculating egg Se toxicity thresholds (ECs) for mortality and deformities in early life stages of zebrafish. The most notable findings of this study were a concentration-dependent increase in Se-induced mortalities and deformities in early life stages of zebrafish, and persistent toxicities such as reduced swim performance (U_{crit}), and greater oxygen consumption (MO_2), standard metabolic rate (SMR) and cost of transport (COT) in adult zebrafish that survived developmental exposure of excess Se via *in ovo* maternal transfer. Reported concentrations of Se in zebrafish eggs in the present study were similar to concentrations of Se measured in eggs collected from both coldwater and warmwater adult female fishes inhabiting in Se-contaminated aquatic ecosystems (Lemly, 1997c; Holm et al., 2005; Muscatello et al., 2006; Rudolph et al., 2008; Janz et al., 2010).

Maternal exposure to excess Se did not alter viability and hatchability of zebrafish eggs. These results were agreeable to laboratory based Se exposure studies in zebrafish, bluegill sunfish (*Lepomis macrochirus*) and cutthroat trout (Coyle et al., 1993; Hardy et al., 2010; Thomas and Janz, 2014) and eggs collected from northern pike (*Esox lucius*), rainbow trout, brook trout and westslope cutthroat trout inhabiting Se-contaminated aquatic ecosystems (Holm

et al., 2005; Muscatello et al., 2006; Rudolph et al., 2008). Collectively, results of these studies suggest that maternal exposure to excess Se in adult female fishes do not alter egg viability and hatchability.

Our findings of greater incidences of mortality and deformities in F1 generation larval zebrafish exposed to excess Se via *in ovo* maternal transfer were agreeable to previous laboratory and field based Se exposure studies. Many laboratory and field based studies reported greater incidence of mortality and/or deformities in F1 generation larval fish whose parents were exposed to dietary, or dietary and waterborne, Se (e.g. Coyle et al., 1993; Holm et al., 2005; Muscatello et al., 2006; Thomas and Janz, 2014). Furthermore, regression analysis in the present study revealed significant positive relationships between egg Se concentrations and percent mortality or deformities in larval zebrafish. Similarly, previous Se studies also noticed significant relationships between egg Se concentrations and percent larval fish mortality or deformities in early life stages of fishes (Holm et al., 2005; Muscatello et al., 2006; Janz et al., 2010). In the present study we included severely deformed larval fish from 4 and 5 dpf for deformity analysis. Previous studies have demonstrated that protein catabolism increases in early life stages of fishes immediately after hatching and yolk protein serves as the major source of energy for development of larval fish after hatching (Kamler, 2008). Since larval zebrafish from excess Se treatment groups start utilizing Se rich yolk protein immediately after hatching, it could cause the onset of Se-induced deformities and mortality prior to complete yolk absorption. Thomas and Janz (2014) reported a greater incidence of mortality on 4 and 5 dpf in zebrafish larvae exposed to excess Se via *in ovo* maternal transfer. Hence our decision to include severely deformed larval fish from 4 and 5 dpf for deformity analysis was justifiable.

Comparisons of egg Se EC10s in the present study with other published maternal Se exposure studies (Table 2) suggests that egg Se EC10s differ by a factor of only 3.14 times from the lowest to the highest EC10 in early life stages of fishes (with an exception of EC10 of dolly varden; with dolly varden the factor of difference increases to 7.71). We included egg Se EC10s of both coldwater and warmwater fishes in this calculation, and it suggests that both warmwater and coldwater larval fishes might be equally sensitive to developmental Se toxicities. In addition, we performed correlation analysis between egg Se EC10s reported in both warmwater and coldwater fishes and temperatures at which those fishes reared, and found no association. Collectively, these findings suggest that coldwater fishes are as sensitive as warm water fishes when it comes to developmental Se toxicity. On the contrary, previous studies have argued that coldwater fishes are less sensitive to Se toxicity when compared to warm water fishes (Chapman, 2007; DeForest et al., 2012).

The species sensitivity distribution (SSD) based on egg Se toxicity thresholds (EC10s) in fishes suggests that early life stages of zebrafish are the most sensitive fish species studied to date for Se toxicity, and this fish can be used to investigate mechanism of Se-induced toxicities in early life stages of fishes. It is interesting note that except in bluegill sunfish, egg Se EC 10s obtained from laboratory based dietary Se exposure studies were lower than field based Se exposure studies (see Table 2 and Fig. 3). The reason for this observed difference was that a pure form of SeMet was spiked in diets to feed adult fishes in laboratory Se exposure study, whereas in field studies fishes were exposed to various forms of organic and in organic Se in diets (only 50-70% of total Se in diet is SeMet). Lower egg Se EC10s from laboratory based dietary Se exposure studies suggest that SeMet might be the most toxic form of Se, however further studies are needed to investigate toxicity of different forms of Se to early life stages of

fishes. Both Doroshov et al. (1992) and Coyle et al. (1993) carried out laboratory based dietary SeMet exposures in bluegill sunfish and reported greater egg Se EC10s than field Se exposure studies. Greater egg Se EC10s in bluegill sunfish might be due to not including the most sensitive endpoint (i.e. larval fish mortality) in the Doroshov et al. (1992) study and significantly greater control mortality observed in the Coyle et al. (1993) study.

In the present study egg Se concentrations to cause a 10, 20 or 50% increase in deformities above the controls were 7.0, 11.4 and 29.2 µg Se/g d.m., respectively, and mortalities were 7.5, 13.2 and 39.1 µg Se/g d.m., respectively. In eggs collected from brown trout (*Salmo trutta*) inhabiting Se contaminated aquatic ecosystems, egg Se concentrations to cause 10, 20 or 50% deformities were 19.3, 21.7 and 26.4 µg Se/g d.m., respectively and mortalities were 20.0, 24.5 and 34.7 µg Se/g d.m., respectively (Formation Environmental and HabiTech, 2012). It is interesting to note that although egg EC10s and 20s reported in the present study and Formation Environmental and HabiTech (2012) were different, EC50s reported for larval fish deformities and mortalities from both studies were similar. Collectively, both studies suggest a steep dose-response relationship for Se-induced toxicities in early life stages of fishes, and that a small increase in egg Se accumulation can have profound effect on survival of fishes inhabiting in Se-contaminated aquatic ecosystems. The 10th percentile egg Se concentration from our SSD graph was 7.7 µg Se/g d.m., and if we remove zebrafish from the SSD (since zebrafish not a native fish) 10th percentile egg Se concentration increases to 9.2 µg Se/g d.m. The egg Se threshold obtained in the present study was closer to egg/ovary Se threshold (10 µg Se/g d.m.) proposed by Lemly (1993a), but much lower than the egg/ovary Se threshold (20 µg Se/g d.m.) proposed by DeForest et al. (2011).

In addition to establish egg thresholds for Se toxicity in F1 generation larval zebrafish, we also investigated persistent effects of developmental exposure to excess SeMet via *in ovo* maternal transfer in zebrafish reared to adulthood (180dpf) in clean water and fed a Se nominal diet. Although developmental exposure of excess SeMet caused a trend for greater cumulative mortality from 6-180 dpf in F1 generation fish, those values were not significant. Cumulative mortality observed in control zebrafish in the present study was 45.3 %, which was agreeable to control fish mortalities reported in other zebrafish rearing studies (Marit and Weber, 2012; Thomas and Janz, 2014). Another interesting result of this study was impaired swim performance observed only in F1 generation adult zebrafish exposed to the intermediate concentrations (6.8 and 12.7 µg Se/g d.m.) of Se via maternal transfer, but not in adult zebrafish from the highest Se exposure group (34.1 µg Se/g d.m.). Mean cumulative mortality in the highest SeMet exposure group was greater than 80.0 % and it suggests that fish with developmental deformities might have died prior to 180 dpf, and only embryos exposed to lesser concentrations of Se survived to 180 dpf in that group. Previous studies reported inter or intra species variation in deposition of Se in eggs of adult female fishes exposed to greater concentrations of Se (Holm et al., 2005; Rudolph et al., 2008; Thomas and Janz, 2014) and such variability in egg Se deposition has been shown to alter occurrence of mortality and deformities in early life stages of F1 generation fishes (Holm et al., 2005; Rudolph et al., 2008; Thomas and Janz, 2014).

Developmental exposure to excess selenite has been shown to cause cardiac dysfunction in early life stages of zebrafish (Ma et al., 2012). In the present study we observed cardiac edema in F1 generation larval zebrafish exposed to excess SeMet via *in ovo* maternal transfer. Similarly, developmental exposure of TCDD has been shown to alter cardiovascular

development, which resulted in impairment of swim performance in adult zebrafish (Marit and Weber, 2012). Although mechanism of actions of TCDD and SeMet are different, our recent SeMet microinjection study results indicate that excess SeMet exposure in early life stages of zebrafish can increase mRNA expression of NF-E2-related factor 2 a and b (NRF 2A and NRF 2B), nuclear factors involved in regulation of antioxidant genes and genes involved in phase II biotransformation in vertebrates (Timme-Laragy et al., 2012) (manuscript in preparation). These nuclear factors (NRF 2A and NRF 2B) can also participate in cross-talk with the aryl hydrocarbon receptor (AHR) pathway (a pathway involved in TCDD toxicity) (Timme-Laragy et al., 2012). It suggests a similar underlying downstream mechanism responsible for developmental toxicities such as impaired cardiovascular development in early life stages of fishes exposed to excess SeMet or TCDD. Collectively, developmental exposure of excess SeMet might have altered cardiovascular development in F1 generation zebrafish, which might have caused impairment in swimming of F1 generation adult zebrafish. Greater MO_2 and SMR in F1 generation adult zebrafish exposed to excess SeMet also support our cardiovascular dysfunction hypothesis. Further histological studies are needed to test SeMet- induced impairment in cardiovascular development in zebrafish.

5.6 Conclusion

In conclusion, the present study established egg Se thresholds for mortality and deformities in early life stages of zebrafish. Maternal exposure to excess SeMet increased the incidence of mortality and deformities in early life stages of zebrafish in a concentration-dependent fashion. Developmental exposure to excess Se caused impairments in swim performance (U_{crit}), standard metabolic rate (SMR) and rate of oxygen consumption (MO_2) in F1 generation adult zebrafish. Selenium-induced impairment in cardiovascular development might

be responsible for reduced swim performance of F1 generation adult zebrafish. A species sensitivity distribution (SSD) based on egg Se toxicity thresholds (EC10s) suggests that early life stages of zebrafish are the most sensitive fish species studied to date, and that this species is a good laboratory model to investigate mechanisms of Se-induced toxicities in early life stages of fish.

CHAPTER 6

MICROINJECTION OF SELENOMETHIONINE REDUCES HATCHABILITY AND INCREASES MRNA ABUNDANCE OF OXIDANT-RESPONSIVE TRANSCRIPTION FACTOR NF-E2-RELATED FACTOR 2 IN ZEBRAFISH

Preface

The research described in this chapter investigated mechanisms of SeMet-induced developmental toxicities in early life stages of zebrafish by embryo microinjection. Since there is a high variability in Se deposition in eggs by natural maternal transfer, I used microinjection to deliver uniform doses of SeMet to zebrafish embryos. Embryo microinjection of SeMet in zebrafish caused significant reduction in hatchability and greater incidences of developmental toxicities in early life stages of zebrafish. In addition, embryo microinjection of SeMet significantly up-regulated mRNA abundance of oxidant-responsive transcription factors and down-regulated mRNA abundance of a methionine catabolism enzyme in early life stages of zebrafish. The results suggest that oxidative stress or impaired methylation, or a combination of these mechanisms might be responsible for SeMet-induced developmental toxicities in early life stages of fishes.

6.1 Abstract

Selenomethionine (SeMet) is the dominant organic form of selenium (Se) present in primary producers and consumers inhabiting Se-contaminated aquatic ecosystems. Adult female fishes in Se-contaminated sites transport and deposit Se-rich vitellogenin to their eggs, and such maternal deposition of excess Se can significantly increase mortality and deformities in early life stages of F1 generation fishes. The objectives of the present study were to establish a dose-response relationship for developmental toxicities (mortality and deformities) in zebrafish (*Danio rerio*) after embryo microinjection of Danieau solution (control group) or Danieau solution containing graded concentrations of Se (8, 16 or 32 μg /g dry mass [d.m.] of eggs) in the form of SeMet, and to investigate potential underlying mechanism(s) of Se-induced developmental toxicities in early life stages of zebrafish. Measured egg Se concentrations in the control or excess Se microinjected groups were 1.6 ± 0.1, 11.0 ± 0.6, 18.7 ± 1.0, and 29.3 ± 0.5 μg Se/g d.m., respectively. Significantly reduced embryo hatchability was observed in the 18.7 and 29.3 μg Se/g d.m. microinjected groups when compared to the control group. A dose-dependent increase in occurrences of mortality and total deformities was observed in early life stages of zebrafish exposed to Se via egg microinjection. Egg Se concentrations to cause 10, 20 or 50% mortality were 7.6, 9.6 and 14.9 μg Se/g d.m., respectively, and deformities were 9.0, 13.0 and 26.1 μg Se/g d.m., respectively. The egg Se concentration causing 20% mortality (~10 μg Se/g d.m.) was used to investigate mRNA abundance of protein tyrosine phosphatase 1b (PTP 1B), methionine adenosyltransferase 1α (MAT 1A), NF-E2-related factor 2A and 2B (NRF 2A and NRF 2B), superoxide dismutase (SOD 1) and glutathione peroxidase 1a (GPX 1A) in 48, 72 and 96 hours post fertilization (hpf) zebrafish embryos. Exposure to excess Se via egg microinjection significantly down-regulated mRNA abundance of PTP 1B at 48 hpf whereas

mRNA abundance of MAT 1A was significantly down-regulated at 72 hpf. mRNA abundance of oxidant-responsive transcription factors, NRF 2A and NRF 2B, in 10 µg Se/g d.m. microinjected group were up-regulated at 72 and 96 hpf. Significantly greater up-regulation of NRF 2A mRNA abundance was observed at 96 hpf in the SeMet injected group when compared to the control group. Similarly, significantly greater up-regulation of NRF 2B mRNA abundance was observed at 72 and 96 hpf in the excess Se microinjected group. There were no differences in mRNA abundances of SOD 1 and GPX 1A in early life stages of zebrafish in the excess SeMet exposed group when compared to the controls. Overall, the results of this study suggest that oxidative stress or impaired methylation, or a combination of these mechanisms, might be responsible for Se-induced developmental toxicities in early life stages of fishes.

6.2 Introduction

Selenium (Se) is a well established teratogen to oviparous animals including fishes, aquatic birds and amphibians (Janz et al., 2010; Lockard et al., 2013). As an essential trace element, adequate intake of Se is required to maintain physiological homeostasis in all animals whereas a marginal increase in Se intake can lead to Se poisoning (Watanabe et al., 1997; Janz et al., 2010). Fish Se literature reported a narrow margin of safety for dietary intake, and a steep dose-response relationship for developmental toxicities for this element (Lemly, 1997a, Janz, 2012; Thomas and Janz, 2014; see chapter 5 for details). Anthropogenic activities such as mining, coal-based power production, oil refining, and agriculture can significantly increase mobilization of Se in to aquatic systems (Janz et al., 2010). Bioaccumulation and trophic transfer properties of Se are well established and such properties of Se can cause persistent toxicities to fishes inhabiting Se contaminated sites (Fan et al., 2002; Schmidt et al., 2013). In addition, studies also demonstrated that contamination of aquatic ecosystems with Se can pose a significant threat to terrestrial animals including birds, lizards, snakes and mammals (Ohlendorf, 1989; Beckon and Maurer, 2008; Janz et al., 2010). Since Se is reported to cause adverse effects in wildlife, it is classified as a contaminant of potential concern in many countries (Lemly, 2004; Chapman, 2009).

Selenomethionine (SeMet) is the dominant form of Se present in primary producers and consumers inhabiting aquatic ecosystems contaminated with Se (Fan et al., 2002; Phibbs et al., 2011). It has been reported that approximately 50-70% of total Se occurs in primary producers and consumers as SeMet (Fan et al., 2002; Phibbs et al., 2011; Franz, 2012). During vitellogenesis adult female fishes inhabiting Se contaminated sites transport and deposit Se rich vitellogenin to their eggs and such maternal deposition of excess Se can lead to developmental

toxicities in early life stages of F1 generation fishes (Holm et al., 2005; Muscatello et al., 2006; Janz et al., 2010). Although a number of maternal Se exposure studies reported developmental toxicities in fishes, only a few of these studies attempted to investigate mechanisms of Se-induced developmental toxicities. Since maternal deposition of Se in eggs is highly variable, it is difficult to study mechanisms of Se-induced developmental toxicities in early life stages of fishes (Holm et al., 2005; Thomas and Janz, 2014). Microinjection techniques have been successfully used to deliver uniform doses of teratogenic chemicals, including SeMet, in fish embryos (Walker et al., 1996; Colman et al., 2005; Papoulias et al., 2011). In addition, this technique has been used to investigate expression of genes in early life stages of zebrafish (*Danio rerio*) (Timme-Laragy et al., 2012). Previous studies proposed a number of mechanisms for Se-induced developmental toxicities in early life stages of freshwater fishes. The first proposed mechanism of Se-induced developmental toxicities is altered protein function, which is due to non-specific insertion of SeMet in place of the essential amino acid methionine (Met) during protein synthesis (Stadtman, 1974; Sunde, 1984; Martínez et al., 2011). Since both SeMet and Met have similar physico-chemical properties, SeMet can be inserted into proteins in a dose-dependent fashion (Behne et al., 1991). There is a disagreement among researchers on whether or not exposure to excess SeMet impairs protein function. Previous studies demonstrated impaired or normal function of proteins after exposure to excess SeMet (Stadtman, 1974; Sunde, 1984; Mechaly et al., 2000; Martínez et al., 2011).

The second and more accepted hypothesis for Se-induced developmental toxicity is oxidative stress (Palace et al., 2004; Janz et al., 2010). Catabolism of SeMet is reported to produce methylselenol and selenide anion, and these metabolites were reported to induce oxidative stress (Palace et al., 2004; Janz et al., 2010; Janz, 2012). Developmental exposure to

teratogenic chemicals in fishes has been demonstrated to induce oxidative stress and/or increase mRNA expression of oxidant-responsive transcription factors and enzymes (Timme-Laragy et al., 2009, 2012; Zhao et al., 2013). Nuclear factor erythroid 2-related factor 2 (NRF 2) is an important transcription factor activated during oxidative stress (Timme-Laragy et al., 2012). Zebrafish possess duplicate copies of NRF 2 genes (NRF 2A and 2B) whereas mammals have only one NRF 2 gene (NRF 2A) (Timme-Laragy et al., 2012). In addition to protein dysfunction and oxidative stress hypotheses, a recent selenite exposure study reported impaired methylation in early life stages of zebrafish (Ma et al., 2012). This suggests that impaired methylation could also be a mechanism of Se-induced developmental toxicities in fishes. Methylation plays a pivotal role in cell growth, gene expression and biotransformation of chemicals including SeMet (Lu, 2000; Mato et al., 2008; Kobayashi et al., 2002a). S-Adenosyl methionine (SAM) is produced from methionine by methionine adenosyltransferase (MAT), and SAM is an important substrate for methylation reactions in cells (Lu, 2000; Mato et al., 2008). Methionine adenosyltransferase 1α (MAT 1A) expression or MAT activity is an indicator of cellular methylation status (Lu, 2000; Mato et al., 2008). Exposure to excess Se (SeMet or selenocysteine or selenite) has been reported to impair expression of MAT 1A or inhibit activity of MAT in animals (Hoffman, 1977; Hasegawa et al., 1996; Thomas et al., 2013).

The objectives of present study were to establish a dose-response relationship for mortality and deformities in early life stages of zebrafish (*Danio rerio*) after embryo microinjection of graded doses of Se in the form of SeMet, and to investigate potential underlying mechanism(s) of Se-induced developmental toxicities in early life stages of zebrafish. There are many advantages of using zebrafish for developmental toxicology and embryo manipulation studies. Zebrafish produce clear and non-sticky embryos which aids successful

microinjection of chemicals (Westerfield, 1995; Gilmour et al., 2002; Dooley and Zon, 2000). Since vast information is available on the developmental biology and genome of zebrafish, both developmental and mechanistic toxicological studies can be easily carried out in this fish species (Dooley and Zon, 2000).

6.3 Materials and methods

6.3.1 Test compound

Seleno-L-methionine was purchased from Sigma-Aldrich (Oakville, ON, Canada). Purity of the compound was greater than 98%.

6.3.2 Test animal

All fish housing and experimental procedures adopted in this study were approved by the Animal Research Ethics Board at the University of Saskatchewan (protocol # 20030076), and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Adult zebrafish were purchased from a local supplier and housed in an environmental chamber with controlled temperature (28.0 ± 1.0 °C) and photoperiod (14 h light and 10 h dark). Adult fish were acclimated to laboratory condition for 4 weeks prior to breeding. During the acclimation period fish were fed Nutrafin® basic flake food (Hagen Inc., Montreal, QC, Canada) and clean chironomids (Bio-Pure Blood Worms, Hikari Sales Inc., Hayward, CA, USA).

6.3.3 Microinjection

Zebrafish breeding, maintenance and embryo manipulation were performed as previously described (Westerfield, 1995; Brand et al., 2002; Gilmour et al., 2002). Immediately prior to

microinjection, stock solutions of SeMet were diluted with Danieau solution (Kane and Kishimoto, 2002) to achieve 8, 16 and 32 µg Se/g d.m. in eggs. Phenol red solution was added in injection solutions to monitor injection success. After the acclimation period, adult zebrafish were introduced in breeding tanks overnight (4 male: 8 female ratio). Embryos were collected the following morning and washed with E3 medium (Brand et al., 2002) within 20-30 min after lights came on. Graded concentrations of SeMet or SeMet-free Danieau solution (control group) were injected at a constant volume of 3.14 nL/injection using a Narishige IM-300 microinjector (Narishige Laboratory Instruments Ltd., Tokyo, Japan) into the yolk region of embryos within 2 hours post-fertilization (hpf). A sham injection group was included in the experiment to investigate potential effects of embryo manipulation on hatchability, and incidence of mortalities and deformities in early life stages of zebrafish. After microinjection, embryos were incubated in Petri dishes with daily renewal of the E3 medium. Dead (opaque) eggs removed at 24 hpf from all treatment groups were considered not fertilized and removed from the experiment. Embryos were reared from 48-120 hpf to investigate effects of SeMet injection on embryo hatchability and incidence of mortality and deformities in early life stages of zebrafish. Concentrations of total Se in eggs, percent hatchability, and percent mortality and total deformities in larval fish were determined in all treatment groups. Percent hatchability is the ratio of the number of hatched larval fish to the total number of fertilized eggs. Percent mortality was calculated by dividing the number of dead embryos/larval fish by the total number of live embryos/larval fish, and then multiplying by 100.

In addition, the egg Se concentration to cause 20% mortality (~10 µg Se/g d.m.) was used to investigate mRNA abundance of oxidant-responsive nuclear factors (NRF 2A and NRF 2B)

and enzymes (SOD 1 and GPX 1A), and enzymes involved in methionine catabolism (MAT 1A) and phosphatase activity (PTP 1B) in 48, 72 and 96 hpf zebrafish.

6.3.4 Deformity analysis

Total deformity analysis was carried out on 6 dpf larval zebrafish. The detailed larval zebrafish preservation protocol for deformity analysis was explained previously (Thomas and Janz, 2014). All preserved zebrafish larvae were examined for malformations in a blind fashion using an Olympus model SZ-CTV dissecting microscope (Olympus, Melville, NY, USA). Each larval fish was examined for skeletal, craniofacial and fin deformities, and edema, and the presence or absence of developmental abnormalities was recorded for each fish. Total percent deformities were calculated by dividing number of malformed larval fish by total number of larval fish, and multiplying by 100.

6.3.5 Quantification of selenium

Concentrations of total Se in pooled egg samples were measured by use of inductively coupled plasma-mass spectrometry (ICP-MS) at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK, Canada). From each treatment groups 3-4 replicates of 45-50 pooled egg samples were taken for the quantification of Se. The detailed Se analysis procedure was described previously (Thomas and Janz, 2011; Thomas et al., 2013). A limit of quantification (LOQ) of 0.13 µg Se/g was determined using method blanks. Concentrations of Se in eggs were measured on a wet mass basis, and converted to dry mass based on a moisture content of 92.5 % determined in a subset of zebrafish eggs.

6.3.6 Real-time polymerase chain reaction (PCR)

Expression of mRNA for genes coding for enzymes or transcription factors of interest were quantified by use of quantitative polymerase chain reaction (Q-PCR). Total RNA was extracted from n = 3-5 replicates of 20 embryos and/or larval zebrafish from each treatment group using RNeasy Lipid Tissue Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Purified total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). A QuantiTect® Reverse Transcription Kit (Qiagen) was used to synthesis cDNA from 1 µg total RNA. Detailed procedures of mRNA and cDNA synthesis were explained elsewhere (Thomas et al., 2013)

Quantitative real-time PCR was performed in 96-well PCR plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Gene specific primers were designed against target genes by use of Primer 3 software, and the sequences of primers are shown (Table 6.1). Detailed Q-PCR procedures were explained elsewhere (Thomas et al., 2013). Target gene mRNA abundance was quantified by normalizing to the expression of elongation factor 1α (*ef1α*) according to the Mean Normalized Expression (MNE) method of Simon (2003).

Table 6.1: Gene-specific primer sequences for the quantitative real-time PCR used in this study

Target	Accession #	Sequence (5'–3')	Annealing temp.
EF 1α	NM_131263.1	F: CTTCAACGCTCAGGTCATCA R: CGGTCGATCTTCTCCTTGAG	60
PTP 1B	NM130924	F: CTTCACCGAGAGCATCACAA R: GTTCGTCGGGTTGTTCATTT	60
NRF 2A	NM_182889.1	F: TGGCCCTGAAGAATTTAACG R: TAGGGACAAACCCGAGTCAG	60
NRF2B	HQ661166.1	F: CCTGCCCAACAGACTCTCTC R: CGTCTTTGTCCGACTGTTCA	60
GPX 1A	NM_001007281.2	F: GAAATACGTCCGTCCTGGAA R: CATAAGGGACACAGGGTCGT	60
SOD 1	NM_131294.1	F: AACATGGTTTCCACGTCCAT R: CGGTCACATTACCCAGGTCT	60
MAT 1A	NM_199871.1	F: ATGCAGTTCTTGACGCACAC R: TGGTGTCTCGCACAATCTTC	60

6.3.7 Statistical analysis

All statistical analyses were conducted by use of Sigmaplot 11 (Systat Software Inc., San Jose, CA, USA). Data were tested for normality by use of the Shapiro–Wilk test and for homogeneity of variance by use of Levene's test. Data that did not meet the assumptions for parametric statistical procedures were log 10 transformed. Non-transformed data are shown in all figures. Significant differences in total Se concentrations in eggs, embryo hatchability, and mortality and total deformities of early life stages of zebrafish from the control and the graded SeMet microinjected groups were tested by use of one-way ANOVA followed by Dunnett's test. mRNA abundance of NRF 2A, NRF 2B, SOD 1, GPX 1A, MAT 1A, and PTP 1B in 48, 72 and 96 hpf zebrafish from the control and 10 µg Se/g d.m. injected groups were tested by use of student t-test. Data were expressed as mean ± S.E.M. Differences were considered statistically significant at $p < 0.05$. Egg Se concentrations to cause 10, 20 and 50 % mortality and deformities in early life stages of zebrafish were calculated by use of TOXSTAT® version 3.5 software (Western Ecosystems Technology [1996], Cheyenne., WY, USA). Abbott's formula was used to adjust mortality and deformities in the control groups.

6.4 Results

6.4.1 Concentrations of selenium

Measured and nominal Se concentrations were directly proportional in each treatment group. Measured concentrations of Se in control, 8, 16 or 32 µg Se/g, d.m. microinjected groups were 1.6 ± 0.1, 11.0 ± 0.6, 18.7 ± 1.0, and 29.3 ± 0.5 µg Se/g d.m., respectively. A significantly greater concentration of Se was observed in eggs microinjected with 8, 16 or 32 µg Se/g, d.m.

when compared to eggs microinjected with Se-free Danieau solution (control) ($p < 0.05$; Table 6.2).

Table 6.2: Nominal and measured selenium concentrations in eggs and hatchability of zebrafish exposed to selenomethionine via embryo microinjection. Data are mean ± S.E.M of n = 3-4 replicates of 45-50 pooled eggs for quantification of total selenium, and n= 3-4 replicates of 30-45 embryos for hatchability analysis.

Nominal Se (µg/g, dry mass)	Egg Se (µg/g, dry mass)	Hatchability
Control	1.6 ± 0.1	94.2 ± 0.7
8.0	11.0 ± 0.6*	75.1 ± 4.6
16.0	18.7 ± 1.0*	51.8 ± 7.3*
32.0	29.3 ± 0.5*	19.9 ± 2.7*

*, Significantly different from the control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$).

6.4.2 Hatchability, mortalities and total deformities

Mean embryo hatchability, cumulative percent mortality and total deformities of early life stages of zebrafish from the sham microinjected treatment group were not different from the control group (see Appendix, Table C6.S1). Microinjection of excess Se in zebrafish embryos significantly reduced hatchability ($p < 0.05$; Table 6.2). Percent embryo hatchability was reduced from a mean of 94.2 % in the control group to 75.1-19.9 % in the excess SeMet microinjected groups. Embryo microinjection of excess Se in the form of SeMet increased mortality and deformities of early life stages of zebrafish in a dose-dependent fashion (Figures 6.1 and 6.2). Significantly greater mortality was observed in early life stages of zebrafish from the 11.0, 18.7 and 29.3 µg Se/g d.m. treatment groups when compared to the controls ($p < 0.05$; Figure 6.1). Similarly, significantly greater deformities were observed in early life stages of zebrafish from the two highest Se exposed treatment groups (18.7 and 29.3 µg Se/g d.m.) ($p < 0.05$; Figure 6.2). Cumulative percent mortality increased from a mean of 7.5% in the control group to 32.8-89.8 % in the excess SeMet microinjected groups. Similarly, total deformities increased from a mean of 7.1 % in the control group to 20.2-61.7 % in the excess SeMet exposed groups. Percent total deformities and mortality in early life stages of zebrafish increased with increasing egg Se concentrations ($r^2 = 0.83$ and $p < 0.001$ for total deformities [see Appendix, Figure C6.S1]; $r^2 = 0.95$ and $p < 0.001$ for cumulative mortality [see Appendix, Figure C6.S2]). Egg Se concentrations to cause 10, 20 and 50% mortalities were 7.6, 9.6 and 14.9 µg Se/g d.m., respectively and deformities were 9.0, 13.0 and 26.1 µg Se/g d.m., respectively.

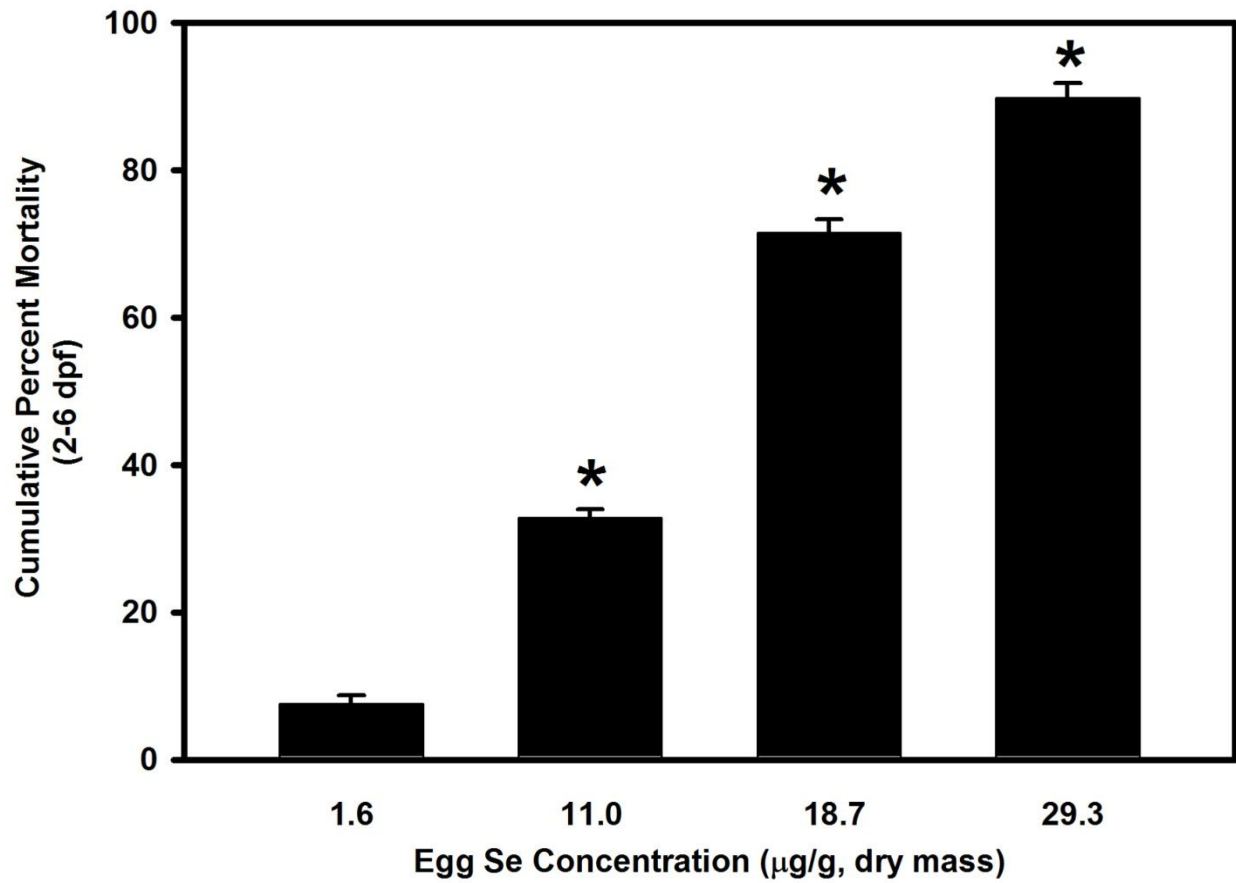

Figure 6.1: Percent cumulative mortalities (2-6 days post fertilization [dpf]) of early life stages of zebrafish exposed to increasing concentrations of selenium via embryo microinjection. *, Significant difference compared to the control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$; n = 3-4 replicates of 30-45 embryos).

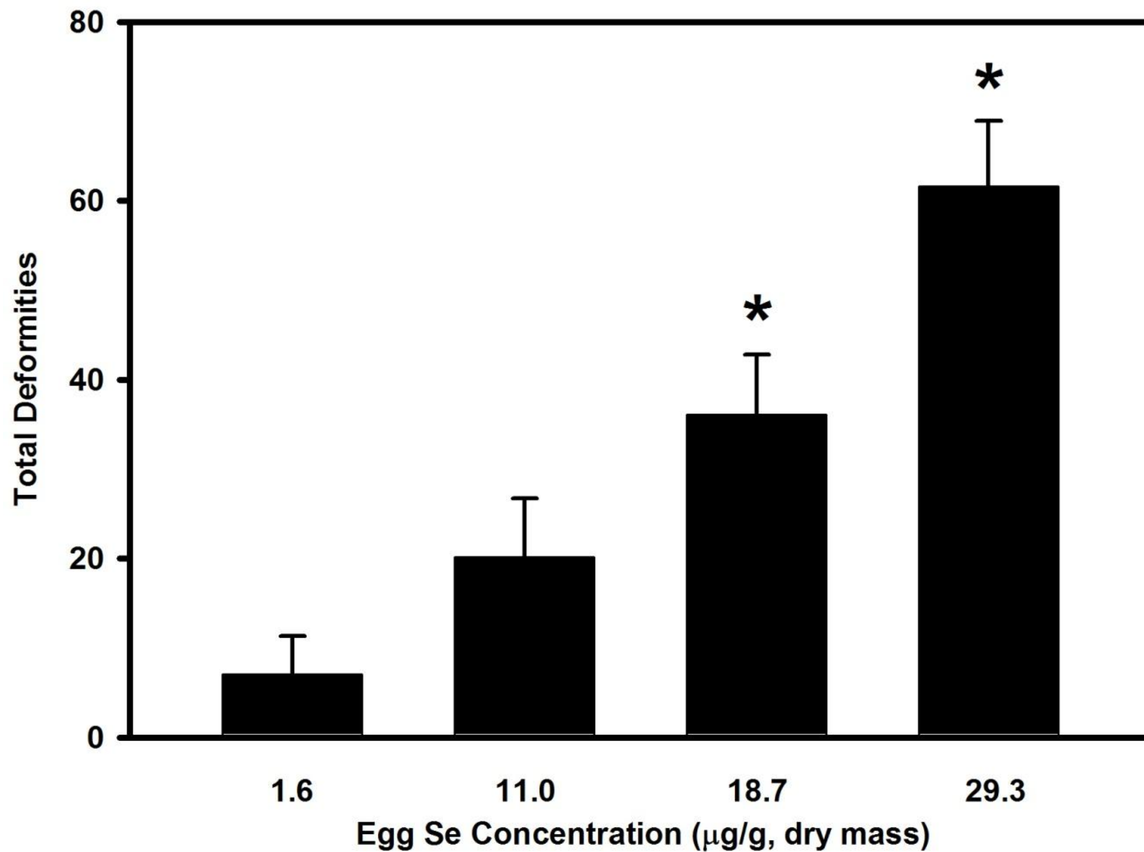

Figure 6.2: Total morphological abnormalities (sum of skeletal, craniofacial and fin deformities, and edema) in larval zebrafish exposed to increasing concentrations of selenium via embryo microinjection. *, Significant difference compared to the control group using one-way ANOVA followed by Dunnett's test ($p < 0.05$; n = 3-4 replicates of 30-45 embryos).

6.4.3 mRNA abundances of protein tyrosine phosphatase 1b (PTP 1B), methionine adenosyltransferase 1α (MAT 1A), NF-E2-related factor 2A (NRF 2A), NF-E2-related factor 2B (NRF 2B), superoxide dismutase (SOD 1) and glutathione peroxidase 1a (GPX 1A)

Abundances of mRNA of PTP 1B, MAT 1A, NRF 2A, NRF 2B, SOD 1and GPX 1A were determined in 48, 72 and 96 hpf zebrafish from the control and the 10 µg Se/g d.m. microinjected group. Abundance of mRNA of PTP 1B was significantly down-regulated at 48 hpf in the excess SeMet exposed group when compared to the controls ($p < 0.05$; Figure 6.3). A trend for down-regulation of abundance of mRNA of MAT 1A was observed in zebrafish from the excess Se exposed group at 48 hpf (Figure 6.4). Similarly, a significant down-regulation of abundance of mRNA of MAT 1A was noticed at 72 hpf in early life stages of zebrafish from the 10 µg Se/g d.m. microinjected group when compared to the controls ($p < 0.05$; Figure 6.4). Abundance of mRNA of NRF 2A and NRF 2B were up-regulated at 72 and 96 hpf in zebrafish from the excess SeMet exposed group when compared to the control group (Figure 6.5A and B). Significantly greater up-regulation of abundance of mRNA of NRF 2A was observed at 96 hpf, whereas abundance of mRNA of NRF 2B was significantly up-regulated at 72 and 96 hpf ($p < 0.05$; Figure 6.5A and B). There were no statistically significant differences in abundances of mRNA of SOD 1 and GPX 1A observed in early life stages of zebrafish from the excess SeMet exposed group and the control group (see Appendix, Table C6.S1).

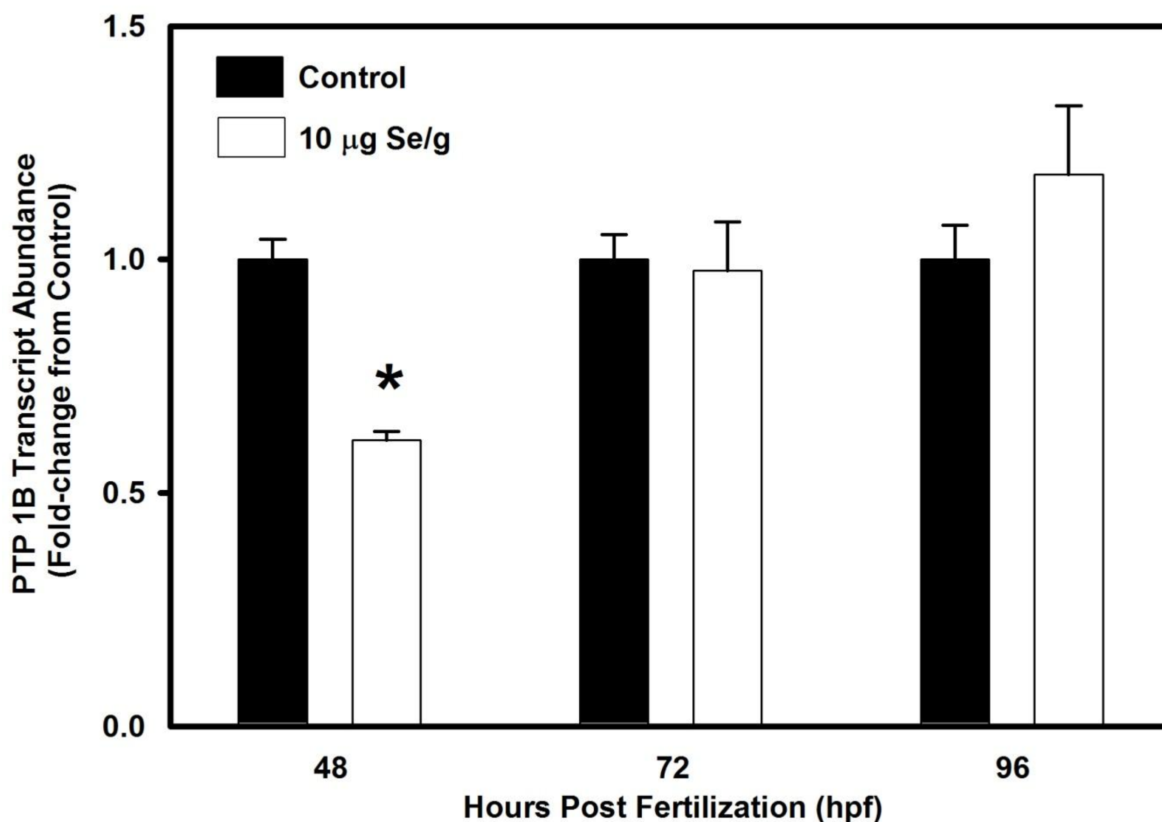

Figure 6.3: mRNA abundance of protein tyrosine phosphatase 1B (PTP1B) in zebrafish exposed to either 10 μg Se/g d.m. in the form of selenomethionine or control (Danieau) solution via embryo microinjection. Transcript abundance was determined by quantitative real-time PCR at 48, 72 and 96 hours post fertilization (hpf). * Significantly different from the control group using student t-test ($p < 0.05$; n = 3-5 replicates of 20 embryos and/or larval fish).

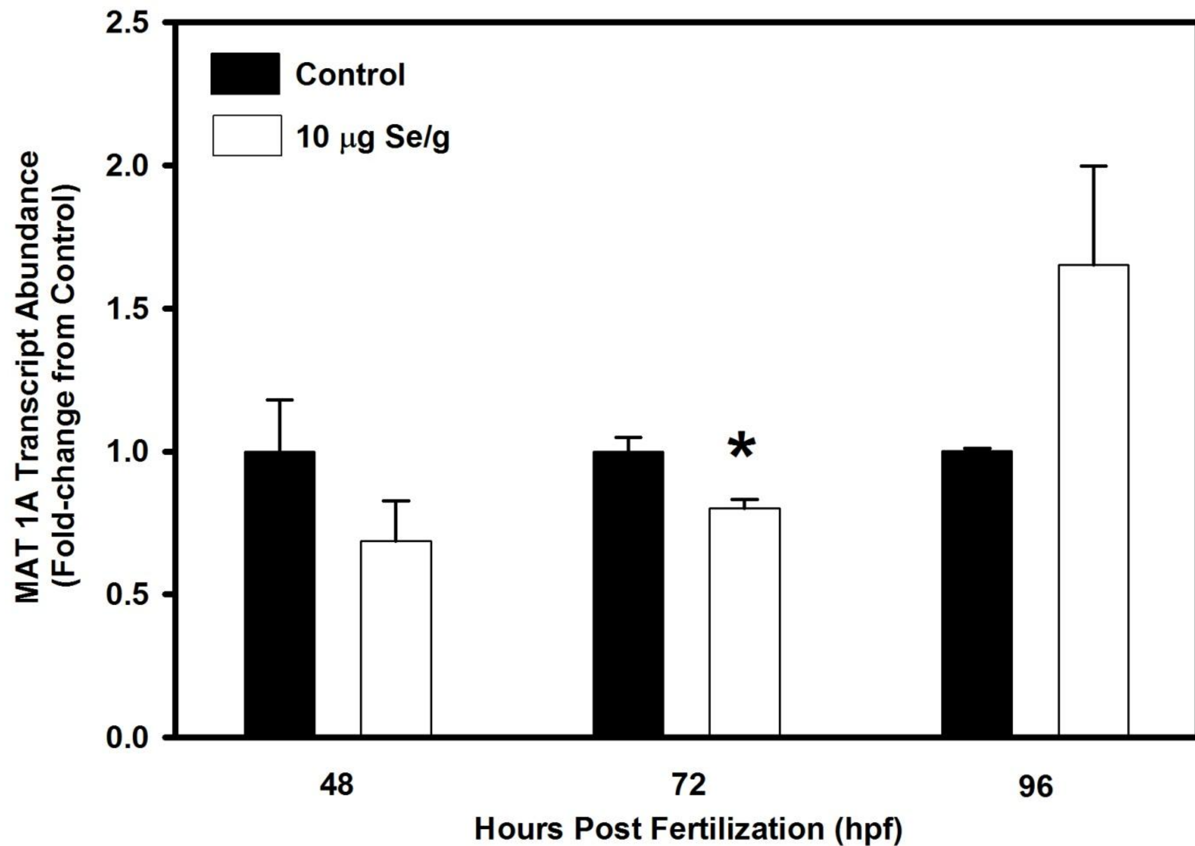

Figure 6.4: mRNA abundance of methionine adenosyltransferase 1α (MAT 1A) in zebrafish exposed to either 10 µg Se/g d.m. in the form of selenomethionine or control (Danieau) solution via embryo microinjection. Transcript abundance was determined by quantitative real-time PCR at 48, 72 and 96 hours post fertilization (hpf). * Significantly different from the control group using student t-test ($p < 0.05$; n = 3-5 replicates of 20 embryos and/or larval fish).

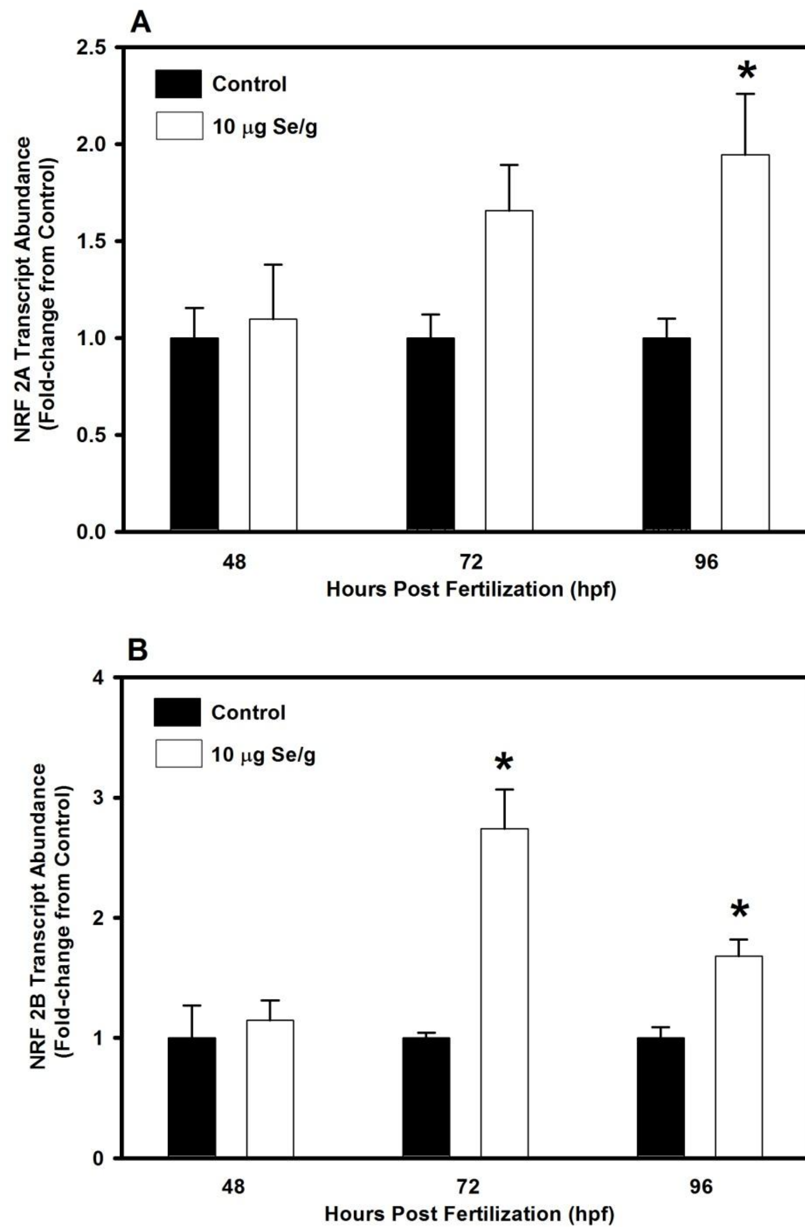

Figure 6.5: mRNA abundance of (A) NF-E2-related Factor 2A (NRF 2A) and (B) NF-E2-related Factor 2B (NRF 2B) in zebrafish exposed to either 10 µg Se/g d.m. in the form of selenomethionine or control (Danieau) solution via embryo microinjection. Transcript abundance was determined by quantitative real-time PCR at 48, 72 and 96 hours post fertilization (hpf). * Significantly different from the control group using student t-test ($p < 0.05$; n = 3-5 replicates of 20 embryos and/or larval fish).

6.5 Discussion

Maternal exposure to excess dietary Se (especially SeMet) has been demonstrated to increase mortality and/or deformities in early life stages of F1 generation fishes (Muscatello et al., 2006; Linville, 2006; Thomas and Janz, 2014; see Chapter 5). The present SeMet embryo microinjection study was conducted with the intention to mimic maternal Se exposure, and was used to explore mechanism(s) of Se-induced toxicities in early life stages of zebrafish. The most notable findings of the present study were a significant reduction in embryo hatchability and a dose-dependent increase in mortality and deformities in early life stages of zebrafish exposed to SeMet via embryo microinjection, and altered mRNA abundances of PTP 1B, MAT 1A, NRF2A and NRF 2B in early life stages of zebrafish exposed to 10 µg Se/g d.m. via egg microinjection. Concentrations of Se used in the study were environmentally relevant and such concentrations have been reported in fish eggs and invertebrates collected from Se-contaminated aquatic ecosystems (Holm et al., 2005; Muscatello et al., 2006; Rudolph et al., 2008; Muscatello and Janz, 2009; Janz et al., 2010).

In the present study, excess SeMet exposure in zebrafish via embryo microinjection caused a significant reduction in hatchability. This result was consistent with embryos of zebrafish and Japanese medaka (*Oryzias latipes*) exposed to waterborne selenite or SeMet in previous studies (Lavado et al., 2012; Ma et al., 2012). However, the majority of maternal Se transfer studies in fishes have reported no significant changes in embryo hatchability (e.g. Holm et al., 2005; Muscatello et al., 2006; Rudolph et al., 2008; Thomas and Janz, 2014; see Chapter 5). Collectively, these results indicate that deposition of the free form of either organic or inorganic Se in eggs causes reduced hatchability in fishes. Previous studies have demonstrated transfer (maternal transfer) of free amino acids from adult female fishes to their eggs (Heming

and Buddington, 1988; Jobling, 1995; Kamler, 2008). Since SeMet is a seleno-amino acid and it has similar physico-chemical properties as the essential amino acid Met, maternal transfer and deposition of free SeMet is possible in fish eggs. Fish literature reported that embryonic fishes use both carbohydrates and free amino acids for energy production, whereas protein-bound amino acids and fats are used for energy production only after hatching (Heming and Buddington, 1988; Jobling, 1995; Kamler, 2008). In addition, studies also suggest that embryonic fishes might preferentially retain free essential amino acids for protein synthesis (Heming and Buddington, 1988; Jobling, 1995). Since SeMet mimics the essential amino acid Met, SeMet might be retained by embryonic fishes for protein synthesis. Thus, regardless of exposure route, embryonic fishes may use accumulated free SeMet for protein synthesis and/or energy production in a dose-dependent fashion. Catabolism of SeMet is reported to cause oxidative stress in developing embryos (Palace et al., 2004), which may negatively affect embryo hatchability. Similarly, accumulation of SeMet in proteins has been reported to alter protein function (Stadtman, 1974; Sunde, 1984; Martínez et al., 2011) and such protein dysfunction could also disrupt embryo development and cause subsequent embryo mortality or reduced hatchability. Collectively, oxidative stress due to catabolism of the free form of SeMet and/or SeMet-induced protein dysfunction might have caused the observed reduction in embryo hatchability in zebrafish.

Dose-dependent increases in mortality and deformities were observed in early life stages of zebrafish after embryo microinjection of SeMet. Greater mortality and/or deformities were also observed after microinjection of excess SeMet in pallid sturgeon (*Scaphirhynchus albus*), shovelnose sturgeon (*Scaphirhynchus platorynchus*) and white sturgeon (*Acipenser transmontanus*) (Linville, 2006; Papoulias et al., 2011). In addition, several field and laboratory

based maternal Se transfer studies also reported similar increases in mortality and/or deformities in F1 generation fishes (Holm et al., 2005; Muscatello et al., 2006; Rudolph et al., 2008; Janz et al., 2010; Thomas and Janz, 2014; see Chapter 5). In the present study, mortality was the most sensitive effect in early life stages of zebrafish rather than deformities. Similar to the present study, previous SeMet microinjection studies in white, pallid and shovelnose sturgeon also demonstrated that embryo-larval mortality is the most sensitive effect (Linville, 2006; Papoulias et al., 2011). Conversely, the majority of laboratory and field based maternal Se transfer studies demonstrated that deformities are the most sensitive effect in early life stages of fishes (Holm et al., 2005; Muscatello et al., 2006; Rudolph et al., 2008; Janz et al., 2010; see Chapter 5). The differences in Se-induced toxicities in early life stages of fishes via maternal transfer and microinjection routes might be due to the relative proportions of free versus protein bound SeMet deposited or introduced to eggs by these routes. In the maternal transfer exposure route, the majority of Se deposition is protein-bound SeMet, and lesser concentrations of free SeMet and other less toxic forms of Se (e.g., selenocysteine, seleno-proteins and inorganic Se) are deposited in eggs. The protein-bound SeMet deposited in embryos via maternal transfer exposure must undergo protein catabolism before being utilized for energy production and/ or protein synthesis. Studies have demonstrated that protein catabolism in early life stages of fishes occur only after hatching (Heming and Buddington, 1988; Jobling, 1995; Kamler, 2008). Hence, it is possible that protein bound SeMet is utilized for energy production and/ or protein synthesis rather slowly than the free form of SeMet. Slow release of SeMet during protein catabolism might delay the onset and progression of Se toxicity in early life stages of fishes and such slow progression of toxicity might induce deformities rather than mortality in early life stages of fishes. In the microinjection exposure route, pure and free form of SeMet is introduced to embryos. Rapid

utilization of the free form of SeMet by developing embryos for energy production and/or protein synthesis could cause quicker onset and progression of Se toxicity and such an event might induce mortality in early life stages of fishes. Collectively, these results suggest that SeMet might be the most toxic form of Se, and the ratio of free to protein-bound SeMet could possibly be used to determine occurrence of deformities or mortality in early life stages of fishes.

Another important finding of this study was the steep dose-response relationship between egg Se and developmental toxicities (mortality and deformities) in early life stages of zebrafish. In the present study, egg Se concentrations to cause 10 and 50% mortality were 7.6 and 14.9 µg Se/g d.m., and deformities were 9.0 and 26.1 µg Se/g d.m., respectively. Microinjection of SeMet in pallid sturgeon and shovelnose sturgeon embryos reported no observed effect concentrations (NOEC) of 7-13 or 9-12 µg Se/g d.m., respectively, and median lethal dose (LD50) of 16.5 or 17.5 µg Se/g d.m., respectively (Papoulias et al., 2011). Taken together, these results suggest that early life stages of zebrafish and sturgeon respond to microinjection of excess SeMet in a similar fashion. A number of maternal Se transfer studies also reported egg Se concentrations to cause 10 and 50 % mortality and/or deformities in early life stages fishes. In zebrafish, egg Se concentrations to cause 10 and 50% mortality were 7.5 and 39.1 µg Se/g d.m., and deformities were 7.0 and 29.2 µg Se/g d.m., respectively (See Chapter 5). Similarly, embryos collected from brown trout (*Salmo trutta*) inhabiting Se-contaminated aquatic ecosystems reported egg Se concentrations causing 10 and 50 % mortality and deformities of 20.0 and 34.7 µg Se/g d.m., and 19.3 and 26.4 µg Se/g d.m., respectively (Formation Environmental and HabiTech, 2012). Egg Se concentrations to cause 10 and 50 % edema and/or skeletal deformities in early life stages of white sturgeon were 15.3 and 29.8 µg Se/g d.m., respectively (Linville, 2006). These studies

further indicate that a steep dose-response relationship exists for Se-induced developmental toxicities in early life stages of both warmwater and coldwater fishes.

The major focus of this study was to investigate mechanisms of Se-induced developmental toxicities in early life stages of fishes. Oxidative stress is one of the hypothesized mechanisms of Se-induced developmental toxicities in early life stages of fishes (Palace et al., 2004; Janz et al., 2010; Janz, 2012). Catabolism of SeMet has been reported to produce highly reactive metabolites such as methylselenol and selenide anion, and these metabolites undergo redox cycling that may result in oxidative stress (Palace et al., 2004; Janz et al., 2010; Janz, 2012). Developmental exposure to teratogenic chemicals in zebrafish has been demonstrated to up-regulate mRNA abundance of oxidant-responsive transcription factors (NRF 2) and/or enzymes (SOD 1 and GPX 1A) (Timme-Laragy et al., 2009, 2012). Duplicate copies NRF 2 genes (NRF 2A and NRF 2B) have been identified in zebrafish, whereas mammals have only one NRF 2 gene (NRF 2A) (Timme-Laragy et al., 2012). Significant up-regulation of mRNA abundance of NRF 2A and NRF 2B in the present study suggest involvement of oxidative stress in Se-induced developmental toxicities in zebrafish. In addition, both NRF 2A and NRF 2B have been demonstrated to participate in cross-talk with the aryl hydrocarbon receptor 2 (AHR 2) signaling pathway (a pathway involved in TCDD toxicity) (Timme-Laragy et al., 2012). Oxidative stress and greater expression of *ahr2* may represent a mechanism of Se-induced developmental toxicities in zebrafish. Since NRF 2B is identified as a negative regulator of expression of several genes during zebrafish embryo development (Timme-Laragy et al., 2009, 2012), it is of interest to investigate whether presence/up-regulation of this particular gene increases susceptibility of zebrafish to developmental Se toxicity. To date, no information is available on whether other teleost fishes possess two copies of the NRF 2 gene, andfurther

studies are needed to determine whether other fishes posses an extra NRF 2 gene and whether or not presence of this gene makes teleost fishes susceptible to developmental Se toxicity. The present study did not find significant up-regulation of transcript abundance of antioxidant responsive genes (SOD 1 and GPX 1A) in early life stages of zebrafish after exposure to excess SeMet via egg microinjection. It is unclear why transcript abundance of oxidant-responsive genes (SOD 1 and GPX 1A) were not up-regulated after developmental exposure to excess Se in zebrafish.

There is a balance between the activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) in cells that plays a pivotal role in cell signaling and animal development (Chiarugi, 2005; Sallee et al., 2006; Challa and Chatti, 2013). Oxidative stress and exposure to chemicals including SeMet have been demonstrated to inhibit mRNA abundance and/or activity of protein tyrosine phosphatase 1B (PTP 1B) both in vitro and in vivo (Chiarugi, 2005; Wang et al., 2010; Rehman et al., 2012; Thomas et al., 2013). Previous studies demonstrated a role of PTP 1B in cell-cell adhesion, angiogenesis, apoptosis and cell migration (Sallee et al., 2006; Nakamura et al., 2008; Burdisso et al., 2013), and all these processes are important for embryo development in fishes and mammals. Hence, we investigated mRNA abundance of PTP 1B in early life stages of zebrafish after developmental exposure to excess SeMet via egg microinjection. Excess SeMet exposure significantly down-regulated mRNA abundance of PTP 1B in zebrafish at 48 hpf. This finding reiterates involvement of oxidative stress in Se-induced developmental toxicities in zebrafish. Maternal exposure of excess SeMet in zebrafish has been shown to cause impairment in cardiovascular performance (measured as altered oxygen consumption) and swim performance (see Chapter 5). The noticed cardiovascular dysfunction and impairment in swim performance in zebrafish in our previous

maternal Se transfer study might be caused by altered cardiovascular development due to Se-induced oxidative stress and subsequent impairment in PTP 1B function.

Exposure to excess Se (SeMet, selenocysteine or selenite) has been demonstrated to inhibit methionine catabolism in animals (Hoffman, 1977; Hasegawa et al., 1996; Thomas et al., 2013). These findings prompted us to investigate mRNA abundance MAT 1A in early life stages of zebrafish after egg microinjection of excess SeMet. Down-regulation of mRNA abundance of MAT 1A was observed at 48 and 72 hpf in early life stages of zebrafish exposed to excess SeMet. Reduced MAT 1A expression is an indicator of both reduced SAM production and impaired methylation (Lu, 2000; Mato et al., 2008). Developmental exposure to selenite in zebrafish has been shown to reduce methylation (Ma et al., 2012). Methylation is vital for cell growth, gene expression and biotransformation of toxicants including SeMet (Daniels, 1996; Chiang et al., 1996; Lu, 2000; Kobayashi et al., 2002a). Reduced methionine catabolism, as indicated by down-regulation of mRNA abundance of MAT 1A in the present study, suggests impaired methylation and/or inhibition SeMet detoxification. Impaired cell growth and gene expression, and inhibition of SeMet detoxification can negatively affect embryo or larval fish development and such effects could also cause developmental toxicities in early life stages of fishes.

6.6 Conclusion

In conclusion, the current study has demonstrated that embryo microinjection of excess SeMet can reduce hatchability and increase developmental toxicities (mortality and deformities) in early life stages of zebrafish in a dose-dependent fashion. In addition, our study suggests that deposition of greater concentrations of the free form of SeMet in eggs might cause mortality

rather than deformities in early life stages of fishes. Developmental exposure of excess SeMet via embryo microinjection up-regulated mRNA transcript abundance of oxidant-responsive transcription factors, NRF 2A and NRF 2B, and suppressed mRNA abundance of PTP 1B and MAT 1A. These results indicate that developmental toxicities in excess SeMet exposed zebrafish could be caused by oxidative stress or impaired methylation, or a combination of these mechanisms. Finally, this study indicates that embryo microinjection techniques can be successfully used to investigate mechanism of toxicity of teratogenic chemicals in early life stages of zebrafish.

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

In Se-contaminated aquatic ecosystems, diet is the major exposure route of Se to adult and juvenile fishes, and maternal transfer is the major exposure route of Se to eggs and embryo-larval fishes (Lemly, 1997a, 2002; Janz et al., 2010). Exposure to dietary organic Se in adult fish is reported to increase the incidence of developmental toxicities in F1 generation larval fish, and similar dietary exposure in juvenile fish is demonstrated to cause greater mortality and deformities, impaired stress response, and histopathological changes in the liver and kidneys (Doroshov et al., 1992; Teh et al., 2004; Tashjian et al., 2006; Wiseman et al., 2011b). However, less is known regarding direct effects of environmentally relevant concentrations of dietary organic Se exposure in adult fishes, and persistent effects of developmental exposure of Se via maternal transfer through the whole life cycle of F1 generation fishes. The overall goal of research described in my thesis was to investigate direct and transgenerational effects of dietary SeMet exposure in zebrafish. The reasons for using SeMet in my research were that it is the dominant organic form of Se found in food organisms and consumers inhabiting aquatic ecosystems contaminated with Se (Fan et al., 2002; Phibbs et al., 2011; Franz, 2012), and that the synthesis, uptake, bioaccumulation, biotransformation, and toxicities of SeMet are relatively well known in animals (Schrauzer, 2000; Muscatello et al., 2006; Muscatello and Janz, 2009; Janz et al., 2010). The results of my research suggest that environmentally relevant concentrations of dietary SeMet exposure in adult zebrafish can alter their swimming performance by interfering with aerobic energy metabolism and muscle function. Maternal transfer of SeMet increased the incidence of developmental toxicities (mortality and deformities) in early life stages of zebrafish, which may have been caused by oxidative stress or impaired methylation, or a combination of these mechanisms. In addition, F1 generation adult fish that survived developmental exposure of

SeMet by maternal transfer demonstrated reduced swimming performance, which was associated with alteration in oxygen consumption and impaired muscle and/or cardiovascular function. The results presented in this thesis rejected the null hypothesis that chronic exposure to excess dietary SeMet in adult zebrafish does not trigger direct and transgenerational adverse effects.

It is important to note that in the present study I used a SeMet spiked artificial diet to investigate effects of SeMet exposure in adult zebrafish and their progenies, and embryo microinjection of free SeMet to investigate mechanisms of SeMet-induced developmental toxicities in early life stages of zebrafish. A common question being asked about my research was the applicability of SeMet spiked diet and SeMet microinjection exposures in investigating toxicity of Se and its mechanisms in fishes. It is well understood that in Se-contaminated aquatic systems, wild fishes accumulate different forms of Se along with SeMet (Phibbs et al., 2011; Franz, 2012). Food organisms inhabiting metal or metalloid contaminated sites are reported to sequester toxic elements in different parts of their body, and such sequestration of toxic elements can significantly alter their bioavailability and toxicity to consumer organisms (Campbell et al., 2002). For example metals or metalloids attached to granules and exoskeleton in food organisms are less bioavailable to consumers, whereas metals or metalloids attached to soft body parts (e.g. metallothionein and other proteins) of food organisms are highly accessible for uptake from the gut of consumers (Campbell et al., 2002). A previous study demonstrated that Se has the highest assimilation efficiency (>90%) compared to other metals and metalloids (Campbell et al., 2002). This suggests that majority of Se is stored as organic Se (especially SeMet) in proteins and other soft body tissues in food organisms and is available for uptake in consumer organisms such as fishes. In addition, aquaculture studies have demonstrated that essential trace elements attached to amino acids are readily taken up from the fish gut when compared to inorganic form of trace

elements (Glover and Hogstrand, 2002). Collectively these studies suggest that the bioavailability of Se is much greater when it is attached to amino acids (e.g. SeMet) or bound to proteins. Resident food organisms and fishes collected from Se contaminated sites reported to accumulate up to 70-80 % of total Se as SeMet (Phibbs et al., 2011; Franz, 2012). Similarly, brine shrimp (*Artemia franciscana*) and brine fly (*Ephydridae sp.*) raised in an artificial Se containing agriculture waste water pond were reported accumulate 82-94% of total Se as SeMet (Schmidt et al., 2013). All these studies clearly suggest that SeMet is the most abundant form of Se present in aquatic systems contaminated with Se. Since SeMet is the most abundant form of Se present in food organisms and assimilation efficiency of protein bound or amino acid attached Se is greater than 90%, my decision to spike SeMet in artificial fish food to study its toxicity was justifiable.

The main reason for using microinjection to investigate mechanisms of SeMet-induced developmental toxicities in early life stages of zebrafish was that by use of microinjection I can deliver uniform doses of SeMet into fish embryos, which cannot be achieved through natural maternal transfer. In addition, studies have demonstrated accumulation of free amino acids from adult female fishes to their eggs, and some researchers argued that embryonic fishes can retain accumulated free essential amino acid for protein synthesis (Heming and Buddington, 1988; Jobling, 1995). Since SeMet mimics the essential amino acid, Met, it was interesting to study whether embryo microinjection of free SeMet affected development of early life stages of zebrafish. Another compelling reason to use microinjection to study SeMet toxicity in early life stages of zebrafish was that it is hypothesized that asynchronous spawners such as zebrafish may follow an income or a mixed capital-income breeding strategy, and fishes following this breeding strategy could accumulate substantial concentrations of Se including free SeMet in eggs if they

feed on Se-contaminated dietary items during oogenesis. It is important to better understand effects of free SeMet accumulation in embryos of fishes following income or a mixed capital-income breeding strategy, and thus microinjection is a very useful technique to investigate such effects.

7.2 Effects of chronic sublethal dietary selenomethionine exposure in adult zebrafish

Relatively few previous studies have investigated direct sublethal physiological effects of sublethal dietary SeMet exposure in adult fish. Hence, I sought to determine effects of sublethal dietary SeMet exposure in adult zebrafish (Chapter 2). Significantly reduced swimming performance and tailbeat amplitudes, and greater accumulation of whole-body stored energy (triglycerides and glycogen) were observed in adult zebrafish fed excess SeMet spiked diets when compared to adult fish fed a control diet. Similarly, previous waterborne or dietary Se exposure in juvenile fishes has been reported to cause abnormal swimming behavior (reduced activity, swimming belly up, and confinement to tank bottom) or greater triglyceride accumulation (Cleveland et al., 1993; Teh et al., 2004; Tashjian et al., 2006; Wiseman et al., 2011b). Since reduced tail beat amplitude can be associated with decreased bend or compression of the caudal fin muscle during swimming, I hypothesized that muscle dysfunction is one of the mechanism by which SeMet causes impaired swimming in adult zebrafish. Muscle dysfunction in adult zebrafish might be caused by non-specific insertion of SeMet in place of Met in muscle proteins, or SeMet-induced oxidative stress. Accidental insertion of SeMet in proteins has been demonstrated to alter their function and stability (Sunde, 1984; Boles et al., 1991; Martínez et al., 2011; Kitajima and Chiba, 2013). Similarly, oxidative stress was reported to impair muscle function by modifying both muscle structure and release of calcium from sarcoplasmic reticulum (Xia et al., 2004; Musaro` et al., 2010). Since a trend for greater oxygen consumption and

greater accumulation of stored energy were observed in adult zebrafish exposed to increasing concentrations of dietary SeMet, I hypothesized that impaired aerobic energy metabolism could also be responsible for impaired swimming in adult zebrafish. To further investigate mechanisms of impaired swimming and stored energy accumulation in adult zebrafish exposed to excess dietary SeMet, a second adult feeding study (Chapter 3) was initiated. Repeat swimming performance, oxygen consumption, metabolic capacities (standard metabolic rate, active metabolic rate, factorial aerobic scope and cost of transport) and gene expression of energy metabolism and methionine catabolism enzymes in adult zebrafish were investigated in this study.

In the second feeding study (Chapter 3), adult fish exposed to graded concentrations of dietary SeMet demonstrated reduced swimming, impaired metabolic capacities, greater oxygen consumption, greater accumulation of whole-body triglycerides and significant down-regulation of gene expression of β-hydroxyacyl coenzyme A dehydrogenase (HOAD) and methionine adenosyltransferase 1 α (MAT 1A) in liver. Greater standard metabolic rate, exercise oxygen consumption and cost of transport, and reduced factorial aerobic scope in adult zebrafish exposed to elevated dietary SeMet suggested impairment in aerobic energy metabolism. Triglycerides are the major form of energy used by resting and moderately active fish and are used in aerobic energy metabolism (Hammer, 1995; Moyes and West, 1995). It was also demonstrated that triglycerides are used as the primary energy source up to approximately 80% of critical swimming speed (U_{crit}; a measure of swimming performance of fish used in this research) (Hammer, 1995; Moyes and West, 1995; Plaut, 2001). β-Hydroxyacyl coenzyme A dehydrogenase (HOAD) is a key mitochondrial enzyme involved in β-oxidation of fatty acids. Impaired β-oxidation of fatty acids was shown to increase triglyceride accumulation in both fish

and mammals (Fromenty et al., 1990; van den Thillart et al., 2002). Methionine adenosyltransferase 1α (MAT 1A) is a liver-specific gene responsible for synthesis of methionine adenosyltransferase (MAT), an enzyme involved in synthesis of S-adenosylmethionine (SAM) (Mato et al., 2008). S-adenosylmethionine (SAM) is an important donor of methyl groups in multiple methyltransferase reactions in the cell (Lu, 2000; Mato et al., 2008). Liver is a major organ responsible for synthesis of triglycerides, and methylation plays an important role in transport of triglycerides out of liver (Kerai et al., 1999). This suggests that elevated dietary SeMet exposure in adult zebrafish has the potential to block triglyceride transport and catabolism, and such effects caused greater accumulation of triglycerides. Exposure to either selenite or selenocystine has been shown to deplete SAM and inactivate MAT in mammalian liver (Hoffman, 1977; Hasegawa et al., 1996). To my knowledge, Chapter 3 in this thesis provided first line of evidence that dietary SeMet exposure can down regulate gene expression of HOAD and MAT 1A in the liver of adult fish, and such impairment caused triglyceride accumulation.

Taken together, the results from Chapter 2 and Chapter 3 suggest that environmentally relevant excess dietary SeMet exposure can impair swimming performance of adult zebrafish by altering both muscle function and aerobic energy metabolism. Triglyceride accumulation in excess dietary SeMet fed adult zebrafish was likely caused by impairment in triglyceride catabolism and /or transport.

7.3 Effects of maternal selenomethionine exposure through the entire life cycle of F1 generation zebrafish

The second main focus of my research was to investigate effects of maternal SeMet exposure throughout the entire life cycle of F1 generation zebrafish. Although a number of

previous studies reported Se-induced developmental toxicities in early life stages of F1 generation fishes, little information is available on the potential negative effects of subtle Se-induced developmental toxicities and their persistent adverse effects on the entire life cycle of F1 generation fishes. Chapter 4 of this thesis investigated effects of maternal SeMet exposure in the whole life cycle of F1 generation zebrafish. Eggs from a sub-group of adult zebrafish from the first feeding study (Chapter 2) were used in this research. Similar to previous maternal Se transfer studies, embryos collected from adult female zebrafish fed either the control or SeMet-spiked diets did not show significant changes in egg viability and hatchability (Coyle et al., 1993; Hardy et al., 2010). Both mortality and deformities in early life stages of zebrafish were greater in excess *in ovo* SeMet exposed groups when compared to the control group. These results were agreeable to previous maternal Se transfer studies (Doroshov et al., 1992; Coyle et al., 1993; Holm et al., 2005). Reduced swimming performance was observed in F1 generation adult zebrafish exposed to SeMet via maternal transfer and then reared in clean water and fed a control (Se normal) diet. This result suggests that developmental exposure to elevated concentrations of SeMet can cause persistent adverse effects in adult zebrafish. Tailbeat amplitude was lower in F1 generation adult zebrafish exposed to elevated SeMet via maternal transfer, suggesting altered muscle function. Similar to the first adult feeding study (Chapter 2), muscle dysfunction appears to be one of the mechanisms of impaired swimming in F1 generation adult zebrafish. It is interesting to note that F1 generation adult zebrafish did not show significant changes in whole-body concentrations of triglycerides or cortisol. Although latent toxicity (impaired swimming) was observed in F1 generation adult zebrafish exposed *in ovo* to greater concentrations of Se, egg viability, embryo hatchability, and mortality and deformities in the offspring of F1 generation adult female zebrafish were not altered. The lack of reproductive

impairment in F1 generation adult female zebrafish exposed *in ovo* to excess Se suggests that developmental exposure to excess Se may not cause transgenerational reproductive effects in fishes. It is possible that wild fish with reduced swimming would exhibit reduced growth, reproduction and survival due to competition for food resources, predation and natural environmental stressors. In this study, F1 generation zebrafish were fed daily and were raised to adulthood in a controlled laboratory environment with minimum stress, favourable conditions that created successful growth, survival and reproduction of fish.

Since I observed immediate and persistent toxicities in F1 generation zebrafish after maternal transfer of elevated SeMet, a second maternal transfer study (Chapter 5) was conducted to develop a dose-response relationship for Se-induced developmental toxicities in early life stages of F1 generation zebrafish, and to further investigate mechanisms of impaired swimming performance in F1 generation adult zebrafish. Eggs collected from a sub-group of adult zebrafish from the second adult feeding experiment (Chapter 3) were used for this study. Similar to the first maternal transfer study (Chapter 4), eggs collected from adult female zebrafish fed either the control or SeMet-spiked diets did not show significant changes in egg viability and hatchability. Greater incidences of mortality and deformities were observed in larval zebrafish exposed to excess SeMet via maternal transfer, which were consistent with other maternal Se exposure studies reported in both coldwater and warmwater fishes (Coyle et al., 1993; Holm et al., 2005; Muscatello et al., 2006). The species sensitivity distribution (SSD) based on egg Se toxicity thresholds (EC10s) in fishes (Figure 5.3) suggests that zebrafish is the most sensitive fish species studied to date for developmental Se toxicity. It is interesting to note that except in bluegill sunfish, egg Se EC10s obtained from laboratory-based dietary Se exposure studies were lower than field-based Se exposure studies (see Table 5.2). This observed difference might be

due to the forms of Se accumulated in fish eggs. Since a pure SeMet spiked diet is commonly used to feed adult female fishes in laboratory-based maternal transfer studies, eggs of these female fishes could accumulate greater concentrations of SeMet and lower levels of other less toxic organic forms of Se. On the other hand, adult female fishes inhabiting Se-contaminated environments are exposed to SeMet and other less toxic forms of Se (e.g. selenoproteins and inorganic Se) from food organisms, and hence deposition of SeMet in their eggs might be relatively lower than laboratory-based dietary SeMet exposure.

In zebrafish, egg Se concentrations to cause 10 and 50% mortality were 7.5 and 39.1 µg Se/g d.m., and deformities were 7.0 and 29.2 µg Se/g d.m., respectively (Chapter 5). Similarly, egg Se concentration to cause 10 and 50% mortality and deformities in brown trout inhabiting Se contaminated aquatic ecosystems were 20.0 and 34.7 µg Se/g d.m., and 19.3 and 26.4 µg Se/g d.m., respectively (Formation Environmental and HabiTech, 2012). Egg Se concentrations to cause 10 and 50 % edema and/or skeletal deformities in early life stages of white sturgeon were 15.3 and 29.8 µg Se/g d.m., respectively (Linville, 2006). These studies indicate that an extremely steep dose-response relationship exists for Se-induced developmental toxicities in early life stages of both warmwater and coldwater fishes. It is interesting to note that the difference between EC10 and EC50 values among these coldwater and warmwater fishes is only 2-4 fold. Based on the SSD described in Chapter 5, the 10th percentile egg Se concentration was 7.7 µg Se/g d.m., and if I remove zebrafish from the SSD (since zebrafish not a native fish species) the 10th percentile egg Se concentration increases to 9.2 µg Se/g d.m. The egg Se threshold obtained in my study was closer to the egg/ovary Se threshold (10 µg Se/g d.m.) proposed by Lemly (1993a), but lower than the new draft egg Se criterion (15.2 µg Se/g d.m.) proposed by the USEPA (USEPA, 2014) and the egg/ovary Se threshold (20 µg Se/g d.m.)

proposed by DeForest et al. (2011). Although previous studies have argued that coldwater fishes are less sensitive to Se toxicity when compared to warmwater fishes (Chapman, 2007; DeForest et al., 2012), my thesis research did not find any evidence to support this argument. Correlation analysis between egg Se EC10s reported in both warmwater and coldwater fishes and temperatures at which those fishes were reared found no association, suggesting both warmwater and coldwater fishes are similarly sensitive to Se-induced developmental toxicities. A similar conclusion (i.e. no difference in sensitivity to Se between coldwater and warmwater fishes) was also made by Muscatello et al. (2006) in northern pike embryos collected from Se impacted lakes in northern Saskatchewan, Canada. Although zebrafish is not a native North American fish species, my study described in Chapter 5 shows similar Se toxicity thresholds (EC10s) for developmental toxicities in zebrafish and North American warmwater and coldwater fishes.

In the SSD graph, I included different families of fishes including Salmonidae (cutthroat trout, rainbow trout, brown trout, brook trout, and dolly varden), Acipenseridae (white sturgeon), Esocidae (northern pike), Centrarchidae (bluegill sunfish), and Cyprinidae (zebrafish). Although zebrafish is not a native North American fish species, zebrafish is a cyprinid and there are 250 species of cyprinids identified in North America. Hence, inclusion of zebrafish in the SSD to represent native cyprinids in North America when developing the new Se draft criterion is a reasonable argument. The new draft Se water quality criteria (USEPA, 2014) is undergoing public review while writing this thesis, and one of the comments was to include zebrafish and medaka Se toxicity data in the final draft of the USEPA Se criterion (Rigby, 2014).

In addition to establishing egg thresholds for Se-induced toxicity in F1 generation larval zebrafish, I also investigated mechanisms of impaired swimming in F1 generation adult zebrafish exposed to SeMet by *in ovo* maternal transfer and reared to adulthood in clean water and fed a

Se-normal diet. Reduced swimming performance, and greater oxygen consumption and standard metabolic rates were observed in F1 generation zebrafish exposed to intermediate concentrations of SeMet via *in ovo* maternal transfer (Chapter 5). It is interesting note that adult zebrafish from the highest *in ovo* SeMet exposed group did not show changes in swimming performance, oxygen consumption and metabolic capacities when compared to adult fish from the control group (Chapter 5). Mortality rate in the highest *in ovo* SeMet exposed group was greater than 80%. Hence, I hypothesized that fish with subtle developmental toxicities might have died prior to the swimming performance experiment, and only embryos exposed to lesser concentrations of Se survived in that group. Previous studies reported inter or intra species variation in deposition of Se in eggs of adult female fishes exposed to greater concentrations of Se (Holm et al., 2005; Rudolph et al., 2008; Thomas and Janz, 2014). Collectively, impaired swimming performance of F1 generation zebrafish exposed to elevated SeMet via maternal transfer might be caused by altered muscle function and impairment in cardiovascular function.

7.4 Effects of embryo microinjection in early life stages of zebrafish

In study 4 and 5, I observed that there was a high variability in egg to egg SeMet deposition occuring by natural maternal transfer exposure, and hence I used microinjection to deliver uniform doses of SeMet into zebrafish embryos to investigate mechanisms of SeMet-induced developmental toxicities. The first step of the microinjection study (Chapter 6) was to develop a dose-response relationship for SeMet-induced developmental toxicities in early life stages of zebrafish. Embryo microinjection of SeMet significantly reduced hatchability of zebrafish. This result was agreeable with previous studies using embryos of zebrafish and medaka exposed to waterborne selenite or SeMet (Lavado et al., 2012; Ma et al., 2012). Collectively these results indicate that deposition of the free form of either organic or inorganic

Se in eggs causes a reduction in hatchability in fishes. Embryonic fishes rely on carbohydrate and free amino acids for development, and at this stage of development fishes cannot process protein-bound amino acids and lipids for energy production (Heming and Buddington, 1988; Jobling, 1995; Kamler, 2008). In addition, studies also suggested that embryonic fishes might preferentially retain free essential amino acids for protein synthesis rather than production of energy for development (Heming and Buddington, 1988; Jobling, 1995). As we already know that SeMet mimics the essential amino acid, Met, SeMet might therefore be retained by embryonic fishes for protein synthesis. Regardless of routes of exposure, embryonic fishes could use accumulated free SeMet for protein synthesis and/or for energy production in a dose-dependent fashion. Catabolism of SeMet is demonstrated to cause oxidative stress in developing embryos (Palace et al., 2004; Chapter 6) and such an event could negatively affect embryo hatchability. Similarly, accumulation of SeMet in proteins has been demonstrated to alter their function and stability (Stadtman, 1974; Sunde, 1984; Lemly, 1993a; Martínez et al., 2011), and such protein dysfunction could also disrupt embryo development, subsequently causing embryo mortality or reduced hatchability. Collectively, oxidative stress due to catabolism of the free form of SeMet and/or SeMet-induced protein dysfunction might have caused the observed reduction in embryo hatchability in zebrafish.

Using microinjection, egg Se concentrations causing 10, 20 or 50% mortality were 7.6, 9.6 and 14.9 µg Se/g d.m., respectively and deformities were 9.0, 13.0 and 26.1 µg Se/g d.m., respectively (Chapter 6). In comparison, the maternal transfer study reported egg Se concentrations causing 10, 20 or 50% mortalities of 7.5, 13.2 and 39.1 µg/g d.m., respectively, and deformities were 7.0, 11.4 and 29.2 µg/g d.m., respectively (Chapter 5). It is important to notice that larval deformities was the most sensitive effect in maternal transfer SeMet exposure

(Chapter 5), whereas mortalities was the most sensitive effect in embryo SeMet microinjection. Since free SeMet was injected into embryos in the microinjection experiment, it was rapidly utilized by developing embryos for energy production and/or protein synthesis. The more rapid onset and progression of free SeMet toxicity may overwhelm homeostatic balance of developing embryonic fishes, and such an event might induce mortality in early life stages of fishes. Conversely, in maternal transfer, protein-bound SeMet is deposited in embryos and must undergo protein catabolism before being utilized for energy production and/ or protein synthesis. As I mentioned earlier, embryonic fishes use only carbohydrates and free amino acid for development whereas proteins and lipids are major sources energy used by fishes after hatching (Heming and Buddington, 1988; Jobling, 1995; Kamler, 2008). This suggests that protein catabolism in fishes occurs only after hatching, and hence protein-bound SeMet is utilized for energy production and/ or protein synthesis more slowly than than the free form of SeMet. I hypothesize that slower release of SeMet during protein catabolism might delay onset and progression of Se toxicity in early life stages of fishes, and such slow progression of toxicity might induce deformities rather than mortality in early life stages of fishes.

7.5 Mechanisms of selenomethionine-induced developmental toxicities in early life stages of zebrafish

A major focus of the microinjection study was to investigate mechanisms of SeMet-induced developmental toxicities in early life stages of zebrafish. Oxidative stress is one of the hypothesized mechanisms of Se-induced developmental toxicities in early life stages of fishes. Catabolism of SeMet has been reported to produce highly reactive metabolites such as methylselenol and selenide anion, and these metabolites have been shown to potentiate oxidative stress (Palace et al., 2004; Janz et al., 2010; Misra et al., 2010). Nuclear factor erythroid 2-

related factor 2 (NRF 2) is an important transcription factor activated during oxidative stress (Kobayashi et al., 2002b, 2009;Timme-Laragy et al., 2009, 2012) and is regarded as a molecular biomarker of oxidative stress. Zebrafish possess duplicate copies NRF 2 genes (NRF 2A and 2B) whereas mammals have only one NRF 2 gene (NRF 2A) (Timme-Laragy et al., 2012). In the present study, significant up-regulation of mRNA abundance of oxidant-responsive transcription factors, NRF 2A and NRF 2B, were observed in early life stages of zebrafish exposed to excess SeMet by embryo microinjection, suggesting that oxidative stress is involved in SeMet-induced developmental toxicities in zebrafish (Chapter 6). In addition, both NRF 2A and NRF 2B have been demonstrated to participate in cross-talk with the aryl hydrocarbon receptor 2 (AHR 2) signaling pathway (a pathway involved in TCDD toxicity) (Timme-Laragy et al., 2012). Oxidative stress and greater expression of AHR 2 might thus be a potential mechanism of SeMet-induced developmental toxicity in zebrafish. Oxidative stress and exposure to chemicals including SeMet have been demonstrated to inhibit mRNA abundance and/or activity of protein tyrosine phosphatase 1B (PTP 1B) (Chiarugi, 2005; Wang et al., 2010; Rehman et al., 2012; Thomas et al., 2013). Roles of PTP 1B in cell-cell adhesion, angiogenesis, apoptosis and cell migration are well documented (Sallee et al., 2006; Nakamura et al., 2008; Burdisso et al., 2013), and all these processes are crucial for embryo development in fishes and mammals. Excess SeMet exposure significantly down-regulated mRNA abundance of PTP 1B in early life stages of zebrafish, and this finding reiterates involvement of oxidative stress in SeMet-induced developmental toxicities in zebrafish.

Methylation is vital for cell growth, gene expression, and biotransformation of toxicants including SeMet (Chiang et al., 1996; Daniels, 1996; Lu, 2000; Kobayashi et al., 2002a). S-Adenosylmethionine (SAM) is an important donor of methyl groups in multiple

methyltransferase reactions in the cell (Daniels, 1996; Lu, 2000). Methionine adenosyltransferase (MAT) is an enzyme involved in synthesis of SAM, and synthesis of this enzyme is controlled by the methionine adenosyltransferase 1α (MAT 1A) gene. Although MAT 1A is generally considered a liver specific gene, its expression was demonstrated throughout larval zebrafish as early as 24 hpf (Lee et al., 2012). Studies have reported that MAT 1A expression is positively associated with SAM concentrations, methionine metabolism, and methylation status in the cell (Daniels, 1996; Lu, 2000; Mato et al., 2008). Down-regulation of mRNA abundance of MAT 1A suggests impaired methionine metabolism, reduced SAM concentrations, and impaired cellular methylation. Impaired SAM production during early life stages of zebrafish development not only alters methylation reactions but also inhibits SeMet detoxification. It is possible that reduced SeMet detoxification due to impaired methylation can also cause developmental toxicity in fish by accidental insertion of SeMet in tissue proteins. Taken together, Chapter 6 of my thesis suggests that both oxidative stress and impaired cellular methylation are involved in SeMet-induced developmental toxicities in early life stages of fishes.

Oxidative stress, impaired PTP 1B function, and methylation were identified as potential mechanisms of SeMet-induced developmental toxicities in early life stages of fishes (Chapter 6). It is important to note that the same mechanisms can also cause impairment in tissues or organ development in fishes that survive *in ovo* exposure to SeMet. In study 4 and 5, I saw persistent toxicities such as impaired swimming, tailbeat amplitude, greater oxygen consumption and impaired metabolic capacities in F1 generation adult zebrafish exposed to SeMet via maternal transfer route. As I mentioned earlier, PTP 1B is essential for cardiovascular and muscle development and hence impairment in PTP 1B function due to SeMet-induced oxidative stress may be associated with adverse effects such as impaired swimming, muscle dysfunction, and

great metabolic rates. Similar effects were observed in F1 generation adult zebrafish exposed to SeMet via maternal transfer in my research (Chapter 4 and 5).

7.6 Conclusion

The goals of my research were to investigate direct and transgenerational adverse effects of chronic exposure to excess dietary SeMet in adult zebrafish, and to explore mechanisms of SeMet-induced adverse effects in both adult zebrafish and their progeny. Chapter 2 and 3 of thesis provide evidence that environmentally relevant concentrations of dietary SeMet exposure in adult zebrafish can alter swimming performance, impair triglyceride metabolism and increase metabolic rate. Impaired aerobic metabolism and muscle dysfunction are potential mechanisms of the observed impairment in swimming performance in adult zebrafish exposed to excess dietary SeMet. Lesser hepatic β-oxidation of fatty acids and methionine catabolism in adult zebrafish fed SeMet might have caused triglyceride accumulation. Chapter 4 and 5 of this thesis investigated transgenerational effects of dietary SeMet exposure in adult zebrafish. Maternal transfer of SeMet caused both immediate and persistent effects in F1 generation zebrafish. Excess SeMet exposure via maternal transfer increased the incidences of mortality and deformities in early life stages of zebrafish in a concentration-dependent fashion. A species sensitivity distribution (SSD) based on egg Se toxicity thresholds (EC10s) suggests that early life stages of zebrafish are the most sensitive fish species studied to date. Developmental exposure to excess SeMet caused reduction in swim performance in F1 generation adult zebrafish. Selenomethionine-induced impairment in cardiovascular function and muscle dysfunction might be responsible for reduced swim performance of F1 generation adult zebrafish. Reproductive performance of F1 generation adult zebrafish exposed *in ovo* to elevated SeMet, and then reared in clean water and fed a Se-normal diet was not impaired, suggesting that developmental

exposure to excess Se may not cause transgenerational effects. Chapter 6 of this thesis provided evidence that developmental toxicities in excess SeMet exposed zebrafish could be caused by oxidative stress or impaired methylation, or a combination of these mechanisms. Finally the research presented in this thesis suggests that environmentally relevant dietary SeMet exposure can alter physiological responses in adult fishes and reduce survivability of F1 generation fishes, which could impact fitness and recruitment of wild fishes inhabiting Se-contaminated aquatic ecosystems.

7.7 Future research

Some novel findings were discovered in my research, but there are a few areas of these studies that could be expanded to provide a more comprehensive understanding of SeMet toxicity in fishes. Areas that require future research attention are listed below.

- In the present study I demonstrated that environmentally relevant dietary SeMet exposure can alter swimming performance in adult fish and their progenies. Future studies should investigate whether non-specific and dose-dependent accumulation of SeMet in muscle proteins or SeMet-induced oxidative stress causes histopathological changes in caudal musculature of fishes.
- In Chapters 4, 5 and 6, I demonstrated that excess SeMet exposure in embryos can cause skeletal deformities is early lifestages of fishes. Future studies should investigate mechanisms of SeMet-induced skeletal malformation in fishes.
- In Chapters 3 and 6, I demonstrated that dietary and embryo SeMet exposure can alter mRNA expression of MAT 1A in zebrafish. Future studies should investigate whether

exposure of SeMet impair mRNA expression of other MAT genes and measure cellular methylation status.

- In Chapter 4, I demonstrated that F1 generation adult zebrafish exposed to SeMet by *in ovo* maternal transfer and reared to adulthood in clean water and fed a Se-normal diet have no reproductive impairment. However, fishes living in Se contaminated aquatic systems are constantly exposed to waterborne and dietary Se. Hence, future studies should investigate whether continuous dietary and aqueous Se exposure alters growth and reproductive ability of F1 generation fishes.
- In Chapter 6, I demonstrated that microinjection is an easy and rapid way to study toxicity of SeMet in early life stages of zebrafish. The same technique can be used to study toxicity of other organic forms of Se to identify other toxic forms of organic Se to early life stages of fishes.
- In Chapter 6, I observed greater mRNA expression of NRF 2 in early life stages of zebrafish after SeMet embryo microinjection. A recent study hypothesized that NRF2 can cross talk with the AHR2 signaling pathway (Timme-Laragy et al., 2012). Hence, future studies should investigate whether developmental exposure to SeMet can activate the AHR2 signaling pathway.
- In my thesis I investigated toxicity of selenomethionine to the entire life cycle of zebrafish. Future studies should investigate and compare toxicity of other organoselenium forms to identify the most toxic organoselenium forms to oviparous organisms.

REFERENCES

Aceto, A., Amicarelli, F., Sacchetta, P., Dragani, B., Bucciarelli, T., Masciocco, L., Miranda, M., Di- Ilo, C., 1994. Developmental aspects of detoxifying enzymes in fish (*Salmo iridaeus*). Free Radical Research 21, 285-294.

Adams, S.M., 1999. Ecological role of lipids in the health and success of fish populations. In: Arts, M.T., Wainman, B.C., (Eds.), Lipids in freshwater ecosystems. Springer, New York, NY, pp.132-160.

Aldosary, B.M., Sutter, M.E., Schwartz, M., Morgan, B.W., 2012. Case series of selenium toxicity from a nutritional supplement. Clinical Toxicology, 50, 57-64.

Almeida, J.A., Novelli, E.L.B., Dal Pai Silva, M., Alves Jůnior, R., 2001. Environmental cadmium exposure and metabolic responses of the Nile tilapia, *Oreochromis niloticus.* Environmental Pollution 114, 169-175.

Amiard-Triquet, C., 2009. Behavioral disturbances: the missing link between sub-organismal and supra-organismal responses to stress? Prospects based on aquatic research. Human and Ecological Risk Assessment 15, 87-110.

Anway, M.D., Cupp, A.S., Uzumcu, M., Skinner, M.K., 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308, 1466-1469.

Aro, A., Alfthan, G., Ekholm, P., Varo, P., 1998. Effect of selenium supplementation of fertilizers on human nutrition and selenium status. In: Frankenberger, W.T., Engberg, R.A., (Eds.), Environmental chemistry of selenium. Marcel Dekker Inc. New York, NY, pp. 81-95.

ATSDR (Agency for Toxic Substances and Disease Registry). 2003. Toxicological Profile for Selenium. U.S. Department of Health and Human Services, Public Health Service, Atlanta, GA, pp.1-418.

Bainbridge, R., 1958. The speed of swimming of fish as related to size and to the frequency and amplitude of the tail beat. Journal of Experimental Biology 35, 109-133.

Baines, S.B., Fisher, N.S., 2002. Assimilation and retention of selenium and other trace elements from crustacean food by juvenile striped bass (*Morone saxatilis*). Limnology and Oceanography 47, 646-655.

Bakke, A.M., Tashjian, D.H., Wang, C.F., Lee, S.H., Bai, S.C., Hung, S.S.O., 2010. Competition between selenomethionine and methionine absorption in the intestinal tract of green sturgeon (*Acipenser medirostris*). Aquatic Toxicology 96, 62-69.

Barcellos, L.J.G., Ritter, F., Kreutz, L.C., Cericato, L., 2010. Can zebrafish *Danio rerio* learn about predation risk? The effect of a previous experience on the cortisol response in subsequent encounters with a predator. Journal of Experimental Biology 76, 1032-1038.

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annual Review of Fish Diseases 1, 3-26.

Beaumont, M.W., Butler, P.J., Taylor, E.W., 2000. Exposure of brown trout, *Salmo trutta*, to a sub-lethal concentration of copper in soft acidic water: effects upon muscle metabolism and membrane potential. Aquatic Toxicology 51, 259-272.

Beckett, G.J., Arthur, J.R., 2005. Selenium and endocrine systems. Journal of Endocrinology 184, 455-465.

Beckon, W.N., Maurer, T.C., 2008. Potential effects of selenium contamination on federally-listed species resulting from delivery of federal water to the San Luis Unit. US Fish and Wildlife Service. Sacramento, CA.

Behne, D., Kyriakopoulos, A., Scheid, S., Gessner, H., 1991. Effects of chemical form and dosage on the incorporation of selenium into tissue proteins in rats. Journal of Nutrition 121, 806- 814.

Behne, D., Kyriakopoulos, A., 2001. Mammalian selenium-containing proteins. Annual Review of Nutrition 21, 453-473.

Bennett, P.M., Janz, D.M., 2007. Bioenergetics and growth of young-of the-year northern pike (*Esox lucius*) and burbot (*Lota lota*) exposed to metal mining effluent. Ecotoxicology and Environmental Safety 68, 1-12.

Berry, J.P., Gantar, M., Gibbs, P.D.L., Schmale, M.C., 2007. The zebrafish (*Danio rerio*) embryos as a model system for identification and characterization of developmental toxins from marine and fresh water microalgae. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology. 145, 61-72.

Besser, J.M., Canfield, T.J., La Point, T.W., 1993. Bioaccumulation of organic and inorganic selenium in a laboratory food chain. Environmental Toxicology and Chemistry 12, 57-72.

Boeck, G.D., Alsop, D., Wood, C., 2001. Cortisol effects on aerobic and anaerobic metabolism, nitrogen excretion, and whole-body composition in juvenile Rainbow Trout. Physiological and Biochemical Zoology 74, 858-868.

Boles, J.O., Cisneros, R.J., Weir, M.S., Odom, J.D., Villafranca, J.E., Dunlap, R.B., 1991. Purification and characterization of selenomethionyl thymidylate synthase from *Escherichia coli*: comparison with the wild-type enzyme. Biochemistry 30, 11073-11080.

Bonnet, X., Bradshaw, D., Shine, R., 1998. Capital versus income breeding: an ectothermic perspective. Oikos 83, 333-342.

Bouldin, J.L., Ingle, T.M., Sengupta, A., Alexander, R., Hannigan, R.E., Buchanan, R.A., 2008. Aqueous toxicity and food chain transfer of quantum dots™ in freshwater algae and *Ceriodaphnia dubia*. Environmental Toxicology and Chemistry, 27, 1958-1963.

Brand, M., Granato, M., Nüsslein-Volhard, C., 2002. Keeping and raising zebrafish. In: Nüsslein-Volhard, C., Dahm, R., (Eds.), Zebrafish. Oxford University Press, Oxford, pp. 7-37.

Brett, J.R., 1964. The respiratory metabolism and swimming performance of young sockeye salmon. Journal of the Fisheries Research Board of Canada 21, 1183-1226.

Browman, H.I., 1989. Embryology, ethology and ecology of ontogenetic critical periods in fish. Brain, Behavior and Evolution 34, 5-12.

Brown, K.H., Schultz, I.R., Nagler, J.J., 2009. Lack of a heritable reproductive defect in the offspring of male rainbow trout exposed to the environmental estrogen 17α-ethynylestradiol. Aquatic Toxicology 91, 71-74.

Bunnell, D.B., Thomas, S.E., Stein, R.A., 2007. Prey resources before spawning influence gonadal investment of female, but not male, white crappie. Journal of Fish Biology 70, 1838-1854.

Burdisso, J.E., González, Á., Arregui, C.O., 2013. PTP1B promotes focal complex maturation, lamellar persistence and directional migration. Journal of cell science, 126, 1820-1831.

Butterman, W.C., Brown, R.D., 2004. Mineral Commodity Profiles, Selenium. US Department of the Interior, US Geological Survey.

Calow, P., 1991. Physiological costs of combating chemical toxicants: Ecological implications. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 100, 3-6.

Campbell, P.G.C., Clearwater, S.J., Brown, P.B., Fisher, N.S., Hogstrand, C., Lopez, R.G., Mayer, L.M., Meyer, J.S., 2005. Digestive physiology, chemistry, and nutrition. In: Meyer, J.S., Adams, W.J., Brix, K.V., Luoma, S.N., Mount, D.R., Stubblefield, W.A., Wood, C.M., (Eds.), Toxicity of dietborne metals to aquatic organisms. SETAC Press, Pensacola, FL, pp. 13-57.

Canton, S.P., Baker, S., Chapman, P.M., DeBruyn, A., DeForest, D.K., Hodaly, A., McDonald, B., Janz, D.M., Muscatello, J.R., 2008. Selenium Tissue Thresholds – Tissue Selection Criteria, Threshold Development Endpoints, and Potential to Predict Population or Community Effects in the Field. Prepared for the North America Metals Council – Selenium Working Group, Washington, D.C. Prepared by GEI Consultants, Golder Associates, Parametrix, Inc., and the University of Saskatchewan, pp.1-178.

Cantrell, S.M., Lutz, L.H., Tillitt, D.E., Hannink, M., 1996. Embryotoxicity of 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD): the embryonic vasculature is a physiological target for TCDD-induced DNA damage and apoptotic cell death in Medaka (*Orizias latipes*). Toxicology and Applied Pharmacology 141, 23-34.

Carvalho, A.P., Araújo, L., Santos, M.M., 2006. Rearing zebrafish (*Danio rerio*) larvae without live food: evaluation of a commercial, a practical and a purified starter diet on larval performance. Aquaculture Research 37, 1107-1111.

Caswell, H., 1996. Demography meets ecotoxicology: untangling the population level effects of toxic substances. In: Newman, MC., Jagoe, C.H., (Eds.), Ecotoxicology a hierarchical treatment. CRC Press, Boca Raton, FL, pp. 255–292.

CCME (Canadian Council of Ministers of the Environment)., 2003. Canadian 'Water Quality Guidelines for Protection of Aquatic Life Guidance for Site–Specific Application of Water Quality Guidelines in Canada and Procedures for Deriving Numerical Water Quality Objectives', Canadian Council of Ministers of the Environment, Winnipeg, MB, pp. 1-146.

Challa, A.K., Chatti, K., 2013. Conservation and early expression of zebrafish tyrosine kinases support the utility of zebrafish as a model for tyrosine kinase biology. Zebrafish 10, 264-274.

Chapman, P.M., 2007. Selenium thresholds for fish from cold freshwaters. Human and Ecological Risk Assessment 13, 20-24.

Chapman, P.M., 2009. Is selenium a global contaminant of potential concern. Integrated environmental assessment and management 5, 353-354.

Cheng, W.W., Farrell, A.P., 2007. Acute and sublethal toxicities of rotenone in juvenile rainbow trout *(Oncorhynchus mykiss)*: swimming performance and oxygen consumption. Archives of Environmental Contamination and Toxicology 52, 388-396.

Chiang, P.K., Gordon, R.K., Tal, J., Zeng, G.C., Doctor, B.P., Pardhasaradhi, K., McCann, P.P., 1996. S-adenosylmethionine and methylation. FASEB Journal 10, 471-480.

Chiarugi, P., 2005. Review PTPs versus PTKs: The redox side of the coin. Free radical research, 39, 353-364.

Claireaux, G., Couturier, C., Groison, A., 2006. Effect of temperature on maximum swimming speed and cost of transport in juvenile European sea bass (*Dicentrarchus labrax*). Journal of Experimental Biology 209, 3420-3428.

Cleveland, L., Little, E.E., Buckler, D.R., Wiedmeyer, R.H., 1993. Toxicity and bioaccumulation of waterborne and dietary selenium in juvenile bluegill (*Lepomis macrochirus*). Aquatic Toxicology 27, 265-280.

Colman, J.R., Twiner, M.J., Hess, P., McMahon, T., Satake, M., Yasumoto, T., Gregory J.D., Ramsdell, J. S., 2005. Teratogenic effects of azaspiracid-1 identified by microinjection of Japanese medaka (*Oryzias latipes*) embryos. Toxicon 45, 881-890.

Cowan, J.H., Shaw, R.F., 2002. Recruitment. In: Fuiman, L.A., Werner, R.G., (Eds.), Fishery science. The unique contributions of early life stages. Blackwell, Oxford, pp. 88-111.

Coyle, J.J., Buckler, D.R., Ingersoll, C.G., Fairchild, J.F., May, T.W., 1993. Effect of dietary selenium on the reproductive success of bluegills (*Lepomis macrochirus*). Environmental Toxicology and Chemistry 12, 551-565.

Cutts, C.J., Metcalfe, N.B., Taylor, A.C., 2002. Juvenile Atlantic salmon (*Salmo salar*) with relatively high standard metabolic rates have small metabolic scopes. Functional Ecology 16, 73-78.

Dange, A.D., 1986. Changes in carbohydrate metabolism in tilapia, *Oreochromis* (*sarotherodon*) *mossambicus*, during short-term exposure to different types of pollutants. Environmental Pollution 41, 165-177.

Daniels, L.A., 1996. Selenium metabolism and bioavailability. Biological Trace Element Research 54, 185-199.

DeForest, D.K., Adams, W.J., 2011. Selenium accumulation and toxicity in freshwater fishes. In: Meador. J.P., (Eds.), Environmental contaminants in biota: interpreting tissue concentrations. CRC Press , Boca Raton, FL, pp.193-229.

DeForest, D.K., Gilron, G., Armstrong, S.A., Robertson, E.L., 2012. Species sensitivity distribution evaluation for selenium in fish eggs: Considerations for development of a Canadian tissue-based guideline. Integrated environmental assessment and management 8, 6-12.

Diplock, A.T., Hoekstra, W.G., 1976. Metabolic aspects of selenium action and toxicity. Critical Reviews in Toxicology 5, 271–329.

Dooley, K., Zon, L.I., 2000. Zebrafish: a model system for the study of human disease. Current opinion in genetics and development 10, 252-256.

Doroshov, S., Eenennaam, J.V., Alexander, C., Hallen, E., Bailey, H., Kroll, K., Restreppo, C., 1992. Development of water quality criteria for resident aquatic species of the San Joaquin River. Davis (CA): University of California–Davis Department of Animal Science. Report to the California State Water Resources Control Board for Contract 7-197- 250-0.

Drake, E.N., 2006. Cancer chemoprevention: selenium as a prooxidant, not an antioxidant. Journal of Medical Hypotheses 67, 318-322.

Driedger, K., Weber, L.P., Rickwood, C.J., Dubé, M.G., Janz, D.M., 2009. Overwinter alterations in energy stores and growth in juvenile fishes inhabiting areas receiving metal mining and municipal wastewater effluents. Environmental Toxicology and Chemistry 28, 296-304.

Dudley, H.C., 1936. Toxicology of selenium II. The urinary excretion of selenium. American Journal of Epidemiology 23, 181-186.

Elphick, J.R., Bailey, H.C., Lo, B.K., Berdusco, J.R., Sword, G., 2009. Effect of selenium on early life-stage development of westslope cutthroat trout. British Columbia Mine Reclamation Symposium. http://hdl.handle.net/2429/24644.

Elrashidi, M.A., Adriano, D.C., Lindsay, W.L., 1989. Solubility, speciation and transformation of selenium in soils. In: Jacobs, L.W., (Eds.), Selenium in agriculture and the environment. American Society of Agronomy and Soil Science Society of America, Madison, WI, pp. 51-69.

Franz, E.D., 2012. Selenium bioaccumulation and speciation in the benthic invertebrate *Chironomus dilutus*: an assessment of exposure pathways and bioavailability. Master of Science dissertation, Toxicology Centre, University of Saskatchewan, SK, pp. 1-159.

Esaki, N., Tanaka, H., Uemura, S., Suzuki, T., Soda, K., 1979. Catalytic action of L-methionine. gamma.-lyase on selenomethionine and selenols. Biochemistry 18, 407-410.

Espe, M., Rathore R.M., Du, Z.Y., Liaset, B., El-Mowafi, A., 2010. Methionine limitation results in increased hepatic FAS activity, higher liver 18:1 to 18:0 fatty acid ratio and hepatic TAG accumulation in Atlantic salmon, *Salmo salar*. Amino Acids 39, 449-460.

Fan, T.W.M., Higashi, R.M., Lane, A.N., 1998. Biotransformations of selenium oxyanion by filamentous cyanophyte-dominated mat cultured from agricultural drainage waters. Environmental Science and Technology 32, 3185-3193.

Fan, T.W.M., Teh, S.J., Hinton, D.E., Higashi, R.M., 2002. Selenium biotransformations into proteinaceous forms by foodweb organisms of selenium-laden drainage waters in California. Aquatic Toxicology 57, 65-84.

Farrell, A.P., Gamperl, A.K., Birtwell, I.K., 1998. Prolonged swimming, recovery and repeat swimming performance of mature sockeye salmon *Oncorhynchus nerka* exposed to moderate hypoxia and pentachlorophenol. Journal of Experimental Biology 201, 2183-2193.

Figueiredo, M.A., Lanes, C.F.C., Almeida, D.V., 2007. Improving the production of transgenic fish germlines: in vivo evaluation of mosaicism in zebrafish (*Danio rerio*) using a green fluorescent protein (GFP) and growth hormone cDNA transgene co-injection strategy. Genetics and Molecular Biology 30, 31-36.

Formation Environmental, HabiTech Inc., 2012. Technical Support Document: Proposed Site-Specific Selenium Criterion, Sage and Crow Creeks, Idaho. Prepared for J.R. Simplot Company, pp.1-256.

Fromenty, B., Fisch, C., Labbe, G., Degott, C., Deschamps, D., Berson, A., Letteron, P., Pessayre, D., 1990. Amiodarone inhibits the mitochondrial β-oxidation of fatty acids and produces microvesicular steatosis of the liver in mice. Journal of Pharmacology and Experimental Therapeutics 255, 1371-1376.

Ganther, H.E., 1974. Biochemistry of selenium. In: Zingaro, R.A., Cooper, W.C., (Eds.), Selenium, Van Nostrand Reinhold, New York, pp. 546–614.

Gilmour, D.T., Jessen, J.R., Lin, S., 2002. Manipulating gene expression in the zebrafish. In: Nüsslein-Volhard, C., Dahm, R., (Eds.), Zebrafish. Oxford University Press, Oxford, pp. 121-143.

Gimeno, L., Ferrando, M.D., Sanchez, S., Gimeno, L.O., Andreu, E., 1995. Pesticide effects on eel metabolism. Ecotoxicology and Environmental Safety 31, 153-157.

Gladyshev, V.N., 2012. Selenoproteins and selenoproteomes. In: Hatfield, D.L., Berry, M.J., Gladyshev, V.N., (Eds.), Selenium. Springer New York, NY, pp. 109-123.

Glover, C.N., Hogstrand, C., 2002. Amino acid modulation of in vivo intestinal zinc absorption in freshwater rainbow trout. Journal of Experimental Biology 205,151-158.

Goertzen, M.M., Driessnack, M.K., Janz, D.M., Weber, L.P., 2011. Swimming performance and energy homeostasis in juvenile laboratory raised fathead minnow (*Pimephales promelas*) exposed to uranium mill effluent. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 154, 420-426.

Goertzen, M.M., Hauck, D.W., Phibbs, J., Weber, L.P., Janz, D.M., 2012. Swim performance and energy homeostasis in spottail shiner (*Notropis hudsonius*) collected downstream of a uranium mill. Ecotoxicology and Environmental Safety 75, 142-150.

Gȯmez-Lechȯn, M.J., Ponsoda, X., Castell, J.V., 1996. A microassay for measuring glycogen in 96-well-cultured cells. Analytical Biochemistry 236, 296-301.

Greenwood, N.N., Earnshaw, A., 1997. Selenium, tellurium and polonium. In: Greenwood, N.N., Earnshaw, A., (Eds.), Chemistry of the elements. The Reed Elsevier, Oxford, OX, pp. 747- 788. pp. 747- 788.

Hamilton, S.J., 2004. Review of selenium toxicity in the aquatic food chain. Science of the Total Environment 326, 1-31.

Hammer, C., 1995. Fatigue and exercise tests with fish. Comparative Biochemistry and Physiology Part A: Physiology 112, 1-20.

Hammerschmidt, C.R., Sandheinrich, M.B., 2005. Maternal diet during oogenesis is the major source of methylmercury in fish embryos. Environmental Science and Technology 39, 3580-3584.

Hardy, R.W., Oram, L.L., Möller, G., 2010. Effects of dietary selenomethionine on cutthroat trout (*Oncorhynchus clarki bouvieri*) growth and reproductive performance over a life cycle. Archives of Environmental Contamination and Toxicology 58, 237-245.

Hasegawa, T., Mihara, M., Nakamuro, K., Sayato, Y., 1996. Mechanisms of selenium methylation and toxicity in mice treated with selenocystine. Archives of Toxicology 71, 31-38.

Hawkesford, M.J., Zhao, F., 2007. Strategies for increasing the selenium content of wheat. Journal of Cereal Science 46, 282-292.

Heiden, T.C.K., Spitsbergen, J., Heideman, W., Peterson, R.E., 2009. Persistent adverse effects on health and reproduction caused by exposure of zebrafish to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin during early development and gonad differentiation. Toxicological Sciences 109, 75-87.

Heming, T.A., Buddington, R.K., 1988. Yolk absorption in embryonic and larval fishes. In: Hoar, W.S., Randall, D.J., (Eds.), Fish Physiology, vol. XI. The Physiology of Developing Fish. Part A. Eggs and Larvae. Academic Press Inc., San Diego, USA, pp. 407–446.

Herbing, I., 2002. Effects of temperature on larval fish swimming performance: the importance of physics to physiology. Journal of Fish Biology 61, 865-876.

Hermanutz, R.O., Allen, K.N., Roush, T.H., Hedtke, S.F., 1992. Effects of elevated selenium concentrations on bluegills (*Lepomis macrochirus*) in outdoor experimental streams. Environmental Toxicology and Chemistry 11, 217-224.

Hill, A.J., Teraoka, H., Heideman, Peterson, R.E., 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicological Sciences 86, 6-19.

Hodson, P.V., 1990. Indicators of ecosystem health at the species level and the example of selenium effects on fish. Environmental Monitoring and Assessment 15, 241-254.

Hoffman, J., 1977. Selenite toxicity, depletion of liver s-adenosylmethionine, and inactivation of methionine adenosyltransferase. Archives of Biochemistry and Biophysics 179, 136-140.

Holm, J., Palace, V., Siwik, P., Sterling, G., Evans, R., Baron, C., Werner, J., Wautier, K., 2005. Developmental effects of bioaccumulated selenium in eggs and larvae of two salmonid species. Environmental Toxicology and Chemistry 249, 2373-2381.

Houde, E.D., 2002. Mortality. In: Fuiman, L.A., Werner, R.G., (Eds.), Fishery science: the unique contributions of early life stages. Blackwell, Oxford, OX, pp. 64-87

Hunter, J.R., Zweifel, J.R., 1971. Swimming speed, tail beat frequency, tail beat amplitude and size in jack mackerel, *Trachurus symmelricus*, and other fishes. Fishery Bulletin 69, 253-266.

Isuev, A.R., Ismailova, S.I., Ozernyuk, N.D., 2008. Comparative study of superoxide dismutase and glutathione peroxidase activity in embryos and skeletal muscles of adult fish. Biological Bulletin 35, 494-498.

Jain, K.E., Birtwell, I.K., Farrell, A.P., 1998. Repeat swimming performance of mature sockeye salmon following a brief recovery period: a proposed measure of fish health and water quality. Canadian Journal of Zoology 76, 1488-1496.

James, L.F., Panter, K.E., Mayland, H.F., Miller, M.R., Baker, D.C., 1989. Selenium poisoning in livestock: a review and progress. In: Jacobs, L.W., (Eds.), Selenium in agriculture and the environment. American Society of Agronomy and Soil Science Society of America, Madison, WI, pp. 123-131.

Janz, D.M., 2012. Selenium. In: Wood, C.W., Farrell, A.P., Brauner, C.J., (Eds.), Fish Physiology Vol 31A, Homeostasis and Toxicology of Essential Metals. Elsevier, San Diego, CA, pp. 327-374.

Janz, D.M., DeForest, D.K., Brooks, M.L., Chapman, P.M., Gilron, G., Hoff, D., Hopkins, W.D., McIntyre, D.O., Mebane, C.A., Palace, V.P., Skorupa, J.P., Wayland, M., 2010. Selenium toxicity to aquatic organisms. In: Chapman, P.M., Adams, W.J., Brooks, M.L., Delos, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P., (Eds.), Ecological Assessment of Selenium in the Aquatic Environment. CRC Press, Boca Raton, FL, pp. 141-231.

Jobling, M., 1995. Development of eggs and larvae. In: Jobling, M., (Eds.), Environmental Biology of Fishes, Fish and Fisheries Series. Chapman and Hall, London, pp. 357-390.

Johnston, I.A., Lee, H., Macqueen, D.J., Paranthaman, K., Kawashima, C., Anwar, A., Kinghorn, J.R., Dalmay, T., 2009. Embryonic temperature affects muscle fibre recruitment in adult zebrafish: genome-wide changes in gene and microRNA expression associated with the transition from hyperplastic to hypertrophic growth phenotypes. Journal of Experimental Biology 212, 1781-1793.

Jörg, G., Bühnemann, R., Hollas, S., Kivel, N., Kossert, K., Van Winckel, S., Gostomski, C.L.V. 2010. Preparation of radiochemically pure ^{79}Se and highly precise determination of its half-life. Applied Radiation and Isotopes 68, 2339-2351.

Kamler, E., 2008. Resource allocation in yolk-feeding fish. Reviews in Fish Biology and Fisheries 18, 143-200.

Kane, D.A., Kishimoto, Y., 2002. Cell labelling and transplantation techniques. In: Nüsslein-Volhard, C., Dahm, R., (Eds.), Zebrafish. Oxford University Press, Oxford, pp. 95-119.

Kelly, J.M., Janz, D.M., 2008. Altered energetics and parasitism in juvenile northern pike (*Esox lucius*) inhabiting metal-mining contaminated lakes. Ecotoxicology and Environmental Safety 70, 357- 369.

Kerai, M.D.J., Waterfield, C.J., Kenyon, S.H., Asker, D.S., Timbrell, J.A., 1999. Reversal of ethanol-induced hepatic steatosis and lipid peroxidation by taurine: a study in rats. Alcohol and Alcoholism 34, 529-541.

Killen, S.S., Costa, I., Brown, J.A., Gamperl, A.K., 2007. Little left in the tank: metabolic scaling in marine teleosts and its implications for aerobic scope. Proceedings of the Royal Society B 274, 431-438.

Kim, A., Oh, J.H., Park, J.M., Chung, A.S., 2007. Methylselenol generated from selenomethionine by methioninase downregulates integrin expression and induces caspase-mediated apoptosis of B16F10 melanoma cells. Journal of Cellular Physiology 212, 386-400.

Kitajima, T., Chiba, Y., 2013. Selenomethionine metabolism and its toxicity in yeast. BioMolecular Concepts 4, 611-616.

Kobayashi, Y., Ogra, Y., Ishiwata, K., Takayama, H., Aimi, N.K., Suzuki, T., 2002a. Selenosugars are key and urinary metabolites for selenium excretion within the required to low-toxic range. Proceedings of National Academy of Sciences of the United States of America 99, 15932-15936.

Kobayashi, M., Itoh, K., Suzuki, T., Osanai, H., Nishikawa, K., Katoh, Y., Takagi, Y., Yamamoto, M., 2002b. Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. Genes to Cells 7, 807-820.

Kobayashi, M., Li, L., Iwamoto, N., Nakajima-Takagi, Y., Kaneko, H., Nakayama, Y., Eguchi, M., Wada, Y., Kumagai, Y., Yamamoto, M., 2009. The antioxidant defense system

Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. Molecular and Cellular Biology 29, 493-502.

Konradt, J., Braunbeck, T., 2001. Alterations of selected metabolic enzymes in fish following long-term exposure to contaminated streams. Journal of Aquatic Ecosystem Stress and Recovery 8, 299- 318.

Kunz, Y.W., 2004. Yolk. In: Kunz, Y.W.,(Eds.), Developmental biology of teleost fishes. Springer, Dordrecht, the Netherlands, pp. 23-40.

Kuzelev, N.R., Zhukovskii, E.A., Khoroshev, V.N., Bogod, V.B., 2004. Contemporary Equipment for Industrial Radiography. Chemical and Petroleum Engineering 40, 248-251.

Lavado, R., Shi, D., Schlenk, D., 2012. Effects of salinity on the toxicity and biotransformation of l-selenomethionine in Japanese medaka (*Oryzias latipes*) embryos: mechanisms of oxidative stress. Aquatic Toxicology 108, 18-22.

Lee, M.S., Bonner, J.R., Bernard, D.J., Sanchez, E.L., Sause, E.T., Prentice, R.R., Burgess, S.M., Brody, L.C., 2012. Disruption of the folate pathway in zebrafish causes developmental defects. BMC developmental biology, 12, 1-11.

Lemly, A.D., 1993a. Guidelines for evaluating selenium data from aquatic monitoring and assessment studies. Environmental Monitoring and Assessment 28, 83-100.

Lemly, D., 1993b. Metabolic stress during winter increases the toxicity of selenium to fish. Aquatic Toxicology 27, 133-158.

Lemly, D., 1997a. A teratogenic deformity index for evaluating impacts of selenium on fish populations. Ecotoxicology and Environmental Safety 37, 259-266.

Lemly, D., 1997b. Environmental implications of excessive selenium: a review. Biomedical and Environmental Sciences 10, 415-435.

Lemly, A.D., 1997c. Ecosystem recovery following selenium contamination in a freshwater reservoir. Ecotoxicology and Environmental Safety 36, 275-281.

Lemly, A.D., 2002. Symptoms and implications of selenium toxicity in fish: the Belews Lake case example. Aquatic Toxicology 57, 39-49.

Lemly, D., 2004. Aquatic selenium pollution is a global environmental safety issue. Ecotoxicology and Environmental Safety 59, 44-56.

Li, Z., Dong, T., Pröschel, C., Noble, M., 2007. Chemically diverse toxicants converge on Fyn and c-Cbl to disrupt precursor cell function. PloS biology 5, e35.

Linville, R.G., 2006. Effects of excess selenium on the health and reproduction of white sturgeon (*Acipenser transmontanus*): implications for San Francisco Bay-Delta. Doctoral dissertation, University of California, Davis, CA, pp. 1-248.

Little, E.E., Finger, S.E., 1990. Swimming behavior as an indicator of sublethal toxicity in fish. Environmental Toxicology and Chemistry 9, 13-19.

Lobanov, A.V., Hatfield, D.L., Gladyshev, V.N., 2009. Eukaryotic selenoproteins and selenoproteomes. Biochimica et Biophysica Acta 1790, 1424-1428.

Lockard, L., Rowe, C.L., Heyes, A., 2013. Dietary selenomethionine exposure induces physical malformations and decreases growth and survival to metamorphosis in an amphibian (*Hyla chrysoscelis*). Archives of environmental contamination and toxicology 64, 504-513.

Lu, S.C., 2000. S-adenosylmethionine. International Journal of Biochemistry and Cell Biology 32, 391-395.

Ma, Y., Wu, M., Li, D., Li, X., Li, P., Zhao, J., Luo, M., Guo, C., Gao, X., Lu, C., Ma, X., 2012. Embryonic developmental toxicity of selenite in zebrafish (*Danio rerio*) and prevention with folic acid. Food and Chemical Toxicology 50, 2854-2863.

MacKinnon, D.L., Farrell, A.P., 1992. The effect of 2-(thiocyanomethylthio) benzothiazole on juvenile coho salmon (*Oncorhynchus kisutch*): sublethal toxicity testing. Environmental Toxicology and Chemistry 11, 1541-1548.

Maher, W., Roach, A., Doblin, M., Fan, T., Foster, S., Garrett, R., Möller, G., Oram, L., Wallschläger, D., 2010. Environmental sources, speciation, and partitioning of selenium. In: Chapman, P.M., Adams, W.J., Brooks, M., Delos, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P., (Eds.), Ecological assessment of selenium in the aquatic environment. CRC Press, Boca Raton, FL, pp. 47-92.

Marit, J.S., Weber, L.P., 2011. Acute exposure to 2,4-dinitrophenol alters zebrafish swimming performance and whole body triglyceride levels. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 154, 14-18.

Marit, J.S., Weber, L.P., 2012. Persistent effects on adult swim performance and energetics in zebrafish developmentally exposed to 2, 3, 7, 8-tetrachlorodibenzo- p-dioxin. Aquatic Toxicology 106, 131-139.

Martínez, J., Lisa, S., Sánchez, R., Kowalczyk, W., Zurita, E., Teixidó, M., Giralt, E., Andreu, D., Avila, J., Gasset, M., 2011. Selenomethionine incorporation into amyloid sequences regulates fibrillogenesis and toxicity. PloS one, 6, e27999.

Martinović-Weigelt, D., Wang, R.L., Villeneuve, D.L., Bencic, D.C., Lazorchak, J., Ankley, G.T., 2011. Gene expression profiling of the androgen receptor antagonists flutamide and vinclozolin in zebrafish (*Danio rerio*) gonads. Aquatic Toxicology 101, 447-458.

Massé, A.J., Thomas, J.K., Janz, D.M., 2013. Reduced swim performance and aerobic capacity in adult zebrafish exposed to waterborne selenite. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 157, 266-271.

Mato, J.M., Corrales, F.J., Lu, S.C., Avila, M.A., 2002. S-adenosylmethionine: a control switch that regulates liver function. FASEB Journal 16, 15-26.

Mato, J.M., Martínez-Chantar, L., Lu, S.C., 2008. Methionine metabolism and liver disease. Annual Review of Nutrition 28, 273-293.

Mayer, F.L., Versteeg, D.J., McKee, M.J., Folmar, L.C., Graney, R.L., McCume, D.C., Rattner, B.A., 1992. Physiological and nonspecific biomarkers. In: Huggett, R.J., (Eds.), Biomarkers: biochemical, physiological and histological markers of anthropogenic stress. Lewis publishers, Boca Raton, FL, pp. 22-26.

Mayland, H.F., 1994. Selenium in plant and animal nutrition. In: Frankenberger Jr, W.T., Benson, S., (Eds.), Selenium in the environment. Marcel Dekker, New York, NY, pp. 29-45.

McClelland, G.B., Craig, P.M., Dhekney, K., Dipardo, S., 2006. Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish (*Danio rerio*). Journal of Physiology 577, 739-751.

McDonald, B.G., DeBruyn, A.M., Elphick, J.R., Davies, M., Bustard, D., Chapman, P.M., 2010. Developmental toxicity of selenium to Dolly Varden char (*Salvelinus malma*). Environmental Toxicology and Chemistry 29, 2800-2805.

McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: iono-regulatory disturbance and metabolic costs. Aquatic Toxicology 50, 231-243.

McGowan, M.W., Artiss, J.D., Strandbergh, D.R., Zak, B., 1983. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. Clinical Chemistry 29, 538 - 542.

McKenzie, D.J., Garofalo, E., Winter, M.J., Ceradini, S., Verweij, F., Day, N., Hayes, R., van der Oost, R., Butler, P.J., Chipman, J.K., Taylor, E.W., 2007. Complex physiological traits as biomarkers of the sub-lethal toxicological effects of pollutant exposure in fishes. Philosophical Transactions of the Royal Society B: Biological Sciences, 362, 2043-2059.

McNeal, J.M., Balistrieri, L.S., 1989. Geochemistry and occurrence of selenium: an overview. In: Jacobs, L.W., (Eds.), Selenium in agriculture and the environment. American Society of Agronomy and Soil Science Society of America, Madison, WI, pp. 1-13.

Mechaly, A., Teplitsky, A., Belakhov, V., Baasov, T., Shoham, G., Shoham, Y., 2000. Overproduction and characterization of seleno-methionine xylanase T-6. Journal of biotechnology, 78, 83-86.

Mehdi, Y., Hornick, J.L., Istasse, L., Dufrasne, I., 2013. Selenium in the environment, metabolism and involvement in body functions. Molecules 18, 3292-3311.

Mehrle, P.M., Mayer, F.L., 1985. Biochemistry/physiology. In: Rand, GM., Petrocelli, S.R., (Eds.), Fundamentals of aquatic toxicology: Methods and application. Hemisphere, New York, NY, pp. 264-282.

Metcalfe, C.D., Sonstegard, R.A., 1984. Microinjection of carcinogens into rainbow trout embryos: an in vivo carcinogenesis assay. Journal of the National Cancer Institute 73, 1125-1132.

Miller, L.L., Wang, F., Palace, V.P., Hontela, A., 2007. Effects of acute and subchronic exposures to waterborne selenite on the physiological stress response and oxidative stress indicators in juvenile rainbow trout. Aquatic Toxicology 83, 263-271.

Miller, L.L., Rasmussen, J.B., Palace, V.P., Hontela, A., 2009. Physiological stress response in white suckers from agricultural drain waters containing pesticides and selenium. Ecotoxicology and Environmental Safety 72, 1249-1256.

Mills, K.H., Chalanchuk, S.M., Allan, D.J., 2000. Recovery of fish populations in Lake 223 from experimental acidification. Canadian Journal of Fisheries and Aquatic Sciences 57, 192-204.

Misra, S., Peak, D., Niyogi, S., 2010. Application of XANES spectroscopy in understanding the metabolism of selenium in isolated rainbow trout hepatocytes: insights into selenium toxicity. Metallomics 2, 710-717.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Reviews in Fish Biology and Fisheries 9, 211-268.

Moyes, C.D., West, T.G., 1995. Exercise metabolism of fish. In: Hochachka, P.W., Mommsen, T.P., (Eds.), Biochemistry and Molecular Biology of Fishes. Elsevier, Amsterdam, The Netherlands, pp. 367-392.

Mueller, A.S., Klomann, S.D., Wolf, N.M., Schneider, S., Schmidt, R., Spielmann, J., Stangl, G., Eder, K., Pallauf, J., 2008. Redox regulation of protein tyrosine phosphatase 1B by manipulation of dietary selenium affects the triglyceride concentration in rat liver. Journal of Nutrition 138, 2328-2336.

Mueller, A.S., Bossea, A.C., Mosta, E., Klomanna, S.D., Schneiderc, S., Pallauf, J., 2009a. Regulation of the insulin antagonistic protein tyrosine phosphatase 1B by dietary Se studied in growing rats. Journal of Nutritional Biochemistry 20, 235-247.

Mueller, A.S., Mueller, K., Wolf, N.M., Pallauf, J., 2009b. Selenium and diabetes: an enigma? Free Radical Research 43, 1029-1059.

Musaro`, A., Fulle, S., Fano`, G., 2010. Oxidative stress and muscle homeostasis. Current Opinion in Clinical Nutrition and Metabolic Care 13, 236-242.

Muscatello, J.R., Bennett, P.M., Himbeault, K.T., Belknap, A.M., Janz, D.M., 2006. Larval deformities associated with selenium accumulation in northern pike (*Esox lucius*) exposed to metal mining effluent. Environmental Science and Technology 40, 6506-6512.

Muscatello, J.R., Janz, D.M., 2009. Selenium accumulation in aquatic biota downstream of a uranium mining and milling operation. Science of the Total Environment 407, 1318-1325.

Nakamura, Y., Patrushev, N., Inomata, H., Mehta, D., Urao, N., Kim, H.W., Razvi, M., Kini, V., Mahadev, K., Goldstein, B.J., McKinney, R., Fukai, T., Ushio-Fukai, M., 2008. Role of protein tyrosine phosphatase 1B in vascular endothelial growth factor signaling and cell–cell adhesions in endothelial cells. Circulation research 102, 1182-1191.

NRC (National Research Council)., 2005. Selenium. In: Mineral tolerance of animals. The National Academic Press, Washington, DC, pp 321-347.

NRC, 2006. Tank waste retrieval, processing, and on-site disposal at three Department of Energy Sites: Final Report. The National Academies Press, Washington, DC, pp 1-78.

Nriagu, J.O., 1989. Global cycling of selenium. In: Inhat, M., (Eds.), Occurrence and distribution of selenium. CRC Press, Boca Raton, FL, pp. 327-339.

Ohlendorf, H.M., 1989. Bioaccumulation and effects of selenium in wildlife. In: Jacobs, L.W., (Eds.), Selenium in agriculture and the environment. American Society of Agronomy and Soil Science Society of America, Madison, WI, pp. 133-177.

Ohlendorf, H.M., 2003. Ecotoxicology of selenium. In: Hoffman, D.J., Rattner, B.A., Burton C.A.J., Cairns, J.J., (Eds.), Handbook of ecotoxicology. CRC Press, Boca Raton, FL, pp. 465-500.

Okuno, T., Motobayashi, S., Ueno, H., Nakamuro, K., 2005. Purification and characterization of mouse hepatic enzyme that converts selenomethionine to methylselenol by its alpha, gamma-elimination. Biological Trace Element Research 106, 77-93.

Okuno, T., Ueno, H., Nakamuro, K., 2006. Cystathionine γ –lyase contributes to selenomethionine detoxification and cytosolic glutathione peroxidase biosynthesis in mouse liver. Biological Trace Element Research 109, 155-171.

Oldfield, J.E., 1999. Selenium world atlas. Selenium-tellurium development association, Grimbergen, Belgium, pp. 1-83.

Palace, V.P., Spallholz, J.E., Holm, J., Wautier, K., Evans, R.E., Baron, C.L., 2004. Metabolism of selenomethionine by rainbow trout (*Oncorhynchus mykiss*) embryos can generate oxidative stress. Ecotoxicology and Environmental Safety 58, 17-21.

Palstra, A.P., Tudorache, C., Rovira, M., Brittijn, S.A., Burgerhout, E., van den Thillart, G.E.E.J.M., Spaink, H.P., Planas, J.V., 2010. Establishing zebrafish as a novel exercise model: swimming economy, swimming-enhanced growth and muscle growth marker gene expression. PloS One 5, e14483.

Pane, E.F., Haque, A., Goss, G.G., Wood, C.M., 2004. The physiological consequences of exposure to chronic, sublethal waterborne nickel in rainbow trout (*Oncorhynchus mykiss*): exercise vs. resting physiology. Journal of Experimental Biology 207, 1249-1261.

Papoulias, D.M., Tillitt, D.E., Annis, M., Nicks, D., Schwarz, M.S., 2011. Laboratory effects of early life Stage exposure to Se on pallid and shovelnose sturgeon. The Society of

Environmental Toxicology and Chemistry (SETAC) North America 32nd Annual Meeting- poster presentation, Boston, MA.

Phibbs, J., Wiramanaden, C.I.E., Hauck, D., Pickering, I.J., Liber, K., Janz, D.M., 2011. Selenium uptake and speciation in wild and caged fish downstream of a metal mining and milling discharge. Ecotoxicology and Environmental Safety 74, 1139-1150.

Pilgrim, N.L., 2012. Multigenerational effects of selenium in rainbow trout, brook trout, and cutthroat trout. Master of Science dissertation, Department of Biological Sciences, University of Lethbridge, AB, pp. 1-134.

Plaut, I., Gordon, M.S., 1994. Swimming metabolism of wild-type and cloned zebrafish *Brachydanio rerio*. Journal of Experimental Biology 194, 209-223.

Plaut, I., 2001. Critical swimming speed: its ecological relevance. Comparative Biochemistry and Physiology Part A: Molecular Integrative Physiology 131, 41-50.

Plaut, I., 2000. Effects of fin size on swimming performance, swimming behaviour and routine activity of zebrafish *Danio rerio.* Journal of Experimental Biology 203, 813-820.

Pong, W.W., Stouracova, R., Frank, N., Kraus, J.P., Eldred, W.M., 2007. Comparative localization of cystathionine-synthase and cystathionine gamma-lyase in retina: differences between amphibians and mammals. Journal of Comparative Neurology 505, 158-165.

Presser, T.S., Piper, D.Z., Bird, K.J., Skorupa, J.P., Hamilton, S.J., Detwiler, S.J., Huebner, M.A., 2004. The phosphoria formation: a model for forecasting global selenium sources to the environment. In: Hein, J.R., (Eds.), Life cycle of the phosphoria formation: from deposition to post-mining environment. Elsevier, New York, NY, pp. 299-319.

Priede, I.G., 1985. Metabolic scope in fishes. In: Tytler, P., Calow, P., (Eds.), Fish Energetics: New Perspectives. Johns Hopkins University Press, Baltimore, MD, pp. 33-64.

Rajotte, J.W., Couture, P., 2002. Effects of environmental metal contamination on the condition, swimming performance, and tissue metabolic capacities of wild yellow perch (*Perca flavescens*). Canadian Journal of Fisheries and Aquatic Sciences 59, 1296-1304.

Ralston, N.V.C., Unrine, J., Wallschläger, D., 2008. Biogeochemistry and analysis of selenium and its species. North American Metals Council - Selenium Working Group, Washington, DC, USA, pp 1-61.

Ramsay, R.M., Feist, G.W., Varga, Z.M., Westerfield, M., Kent, M.L., Schreck, C.B., 2006. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. Aquaculture 258, 565-574.

Rayman, M.P., 2000. The importance of selenium to human health. The Lancet 356, 233-214.

Rehman, K., Chen, Z., Wang, W.W., Wang, Y.W., Sakamoto, A., Zhang, Y.F., Naranmandura, H., Suzuki, N., 2012. Mechanisms underlying the inhibitory effects of arsenic compounds

on protein tyrosine phosphatase (PTP). Toxicology and applied pharmacology 263, 273-280.

Reidy, S.P., Kerr, S.R., Nelson, J.A., 2000. Aerobic and anaerobic swimming performance of individual Atlantic cod. Journal of Experimental Biology 203, 347-357.

Reilly, C., 2006. Introduction. In: Reilly, C., (Eds.), Selenium in food and health. Springer, New York, NY, pp. 1-19.

Rigby, M.C., 2014. Comments on USEPA 2014 draft selenium water quality criteria. pp. 1-5

Rinella, M.E., Elias, M.S., Smolak, R.R., Fu, T., Borensztajn, J., Green, R.M., 2008. Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. Journal of Lipid Research 49, 1068-1076.

Rowe, C.L., Kinney, O.M., Nagle, R.D., Congdon, J.D., 1998. Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. Physiological Zoology 71, 27-35.

Rowe, C.L., Hopkins, W.A., Zehnder, C., Congdon, J.D., 2001. Metabolic costs incurred by crayfish (*Procambarus acutus*) in a trace element-polluted habitat: further evidence of similar responses among diverse taxonomic groups. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 129, 275-283.

Rubio, V., Valverde, M., Rojas, E., 2010. Effects of atmospheric pollutants on the Nrf2 survival pathway. Environmental Science and Pollution Research 17, 369-382.

Rudolph, B.L., Andreller, I., Kennedy, C. J., 2008. Reproductive success, early life stage development, and survival of westslope cutthroat trout (*Oncorhynchus clarki lewisi*) exposed to elevated selenium in an area of active coal mining. Environmental Science and Technology 42, 3109-3114.

Sadek, S.A., Basmadjian, G.P., Hsu, P.M., Rieger, J. A., 1983. New selenium-75 labeled radiopharmaceuticals: selenonium analogs of dopamine. Journal of medicinal chemistry, 26, 947-950.

Sallee, J.L., Wittchen, E.S., Burridge, K., 2006. Regulation of cell adhesion by protein-tyrosine phosphatases II. Cell-cell adhesion. Journal of Biological Chemistry 281, 16189-16192.

Sancho, E., Ferrando, M.D., Andreu, E., 1996. Physiological stress responses of *Anguilla anguilla* to fenitrothion. Journal of Environmental Sciences 31, 87-98.

Schmidt, R., Tantoyotai, P., Fakra, S.C., Marcus, M.A., Yang, S.I., Pickering, I.J., Bañuelos, G.S., Hristova, K.R., Freeman, J.L., 2013. Selenium biotransformations in an engineered aquatic ecosystem for bioremediation of agricultural wastewater via brine shrimp production. Environmental science and technology 47, 5057-5065.

Schrauzer, G.N., 2000. Selenomethionine: a review of its nutritional significance, metabolism and toxicity. Journal of Nutrition 130, 1653-1656.

Schwarz, K., Foltz, C.M., 1957. Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. Journal of the American Chemical Society 79, 3292-3293.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquatic Toxicology 68, 369-392.

Scott, K.D., Fåhræus-Van Ree, G.E., Parrish, C.C., 2002. Sex differences in hepatic lipids of toxaphene-exposed juvenile yellowtail flounder (*Pleuronectes ferrugineus* Storer). Ecotoxicology and Environmental Safety 51, 168-176.

Sepulveda, C., Dickson, K.A., 2000. Maximum sustainable speeds and cost of swimming in juvenile kawakawa tuna (*Euthynnus affinis*) and chub mackerel (*Scomber japonicus*). Journal of Experimental Biology 203, 3089-3101.

Shaw, G., Ashworth, D., 2010. Selenium. In: Atwood, D.A. (Eds.), Radionuclides in the environment. John Wiley and Sons Ltd., Chichester, SXW, pp. 121-130.

Shingles, A., McKenzie, D.J., Taylor, E.W., Moretti, A., Butler, P.J., Ceradini, S., 2001. Effects of sublethal ammonia exposure on swimming performance in rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology 204, 2691-2698.

Shinkai, Y., Sumi, D., Fukami, I., Ishii, T., Kumagai, Y., 2006. Sulforaphane, an activator of Nrf2, suppresses cellular accumulation of arsenic and its cytotoxicity in primary mouse hepatocytes. FEBS Letters 580, 1771-1774.

Simon, P., 2003. Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19, 1439-1440.

Smith, J., Ladi, E., Mayer-Pröschel, M., Noble, M., 2000. Redox state is a central modulator of the balance between self-renewal and differentiation in a dividing glial precursor cell. Proceedings of the National Academy of Sciences 97, 10032-10037.

Spallholz, J.E., Palace, V.P., Reid, T.W., 2004. Methioninase and selenomethionine but not Se-methylselenocysteine generate methylselenol and superoxide in an in vitro chemiluminescent assay: implications for the nutritional carcinostatic activity of selenoaamino acids. Biochemical Pharmacology 67, 547-554.

Stadtman, T.C., 1974. Selenium biochemistry. Science 183, 915-922.

Steffensen, J.F., Johansen, K., Bushnell, P.G., 1984. An automated swimming respirometer. Comparative Biochemistry Physiology Part A: Physiology 79, 437-440.

Sunde, R.A., 1984. The biochemistry of selenoproteins. Journal of the American Chemical Society 61, 1891-1900.

Tang, Y., Nelson, J.A., Reidy, S.P., Kerr, S.R., Boutilier, R.G., 1994. A reappraisal of activity metabolism in Atlantic cod (*Gadus morhua*). Journal of Fish Biology 44, 1-10.

Tashjian, D.H., Teh, S.J., Sogomonyan, A., Hung, S.S.O., 2006. Bioaccumulation and chronic toxicity of dietary L-selenomethionine in juvenile white sturgeon (*Acipenser transmontanus*). Aquatic Toxicology 79, 401-409.

Teh, S.J., Deng, X., Deng, D.F., Teh, F.C., Hung, S.S.O., Fan, T.W.M., Liu, J., Higashi, R.M., 2004. Chronic effects of dietary selenium on juvenile Sacramento splittail (*Pogonichthys macrolepidotus*). Environmental Science and Technology 38, 6085-6093.

Thisse, C., Degrave, A., Kryukov, G.V., Gladyshev, V.N., Obrecht-Pflumio, S., Krol, A., Thisse, B., Lescure, A., 2003: Spatial and temporal expression patterns of selenoprotein genes during embryogenisis in zebrafish. Gene Expression Patterns 3, 525-532.

Thomas, J.K., Janz, D.M., 2011. Dietary selenomethionine exposure in adult zebrafish alters swimming performance, energetics and the physiological stress response. Aquatic Toxicology 102, 79-86.

Thomas, J.K., Wiseman, S., Giesy, J.P., Janz, D.M., 2013. Effects of chronic dietary selenomethionine exposure on repeat swimming performance, aerobic metabolism and methionine catabolism in adult zebrafish (*Danio rerio*). Aquatic Toxicology 130-131, 112-122.

Thomas, J.K., Janz, D.M., 2014. In ovo exposure to selenomethionine via maternal transfer increases developmental toxicities and impairs swim performance in F1 generation zebrafish (*Danio rerio*). Aquatic Toxicology 152, 20-29.

Timme-Laragy, A.R., Van Tiem, L.A., Linney, E.A., Di Giulio, R.T., 2009. Antioxidant responses and NRF2 in synergistic developmental toxicity of PAHs in zebrafish. Toxicological sciences, 109, 217-227.

Timme-Laragy, A.R., Karchner, S.I., Franks, D.G., Jenny, M.J., Harbeitner, R.C., Goldstone, J.V., McArthur, A.G., Hahn, M.E., 2012. Nrf2b, novel zebrafish paralog of oxidant-responsive transcription factor NF-E2-related factor 2 (NRF2). Journal of Biological Chemistry 287, 4609-4627.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11, 107-184.

Tudorache, C., O'Keefe, R.A., Benfey, T.J., 2010. The effect of temperature and ammonia exposure on swimming performance of brook charr (*Salvelinus fontinalis*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 156, 523-528.

USEPA (United States Environmental Protection Agency), 1987. Ambient water quality criteria for selenium, EPA-440/5-87-006, Office of water regulations and standards. Washington, DC, pp. 1-128.

USEPA, 2004. Draft aquatic life water quality criteria for selenium. EPA-822-D-04-001.Office of Water, Washington, DC, pp. 1- 334.

USEPA, 2014. External peer review draft aquatic life ambient water quality criterion for selenium - freshwater 2014. EPA-820-F-14-005, Office of water regulations and standards. Washington, DC, pp. 1-4.

van den Thillart, G., Vianen, G., Zaagsma, J., 2002. Adrenergic regulation of lipid mobilization in fishes; a possible role in hypoxia survival. Fish Physiology Biochemistry 27, 189-204.

Van Ginneken, V., Palstra, A., Leonards, P., Nieveen, M., van den Berg, H., Flik, G., Spanings, T., Niemantsverdriet, P., van den Thillart, G., Murk, A., 2009. PCBs and the energy cost of migration in the European eel (*Anguilla anguilla L.*). Aquatic Toxicology 92, 213-220.

Varo, P., Alfthan, G., Huttunen, J.K., Aro, A., 1994. Nationwide selenium supplementation in Finland- effects on diet, blood and tissue levels, and health. In: Burk, R. F., (Eds.), Selenium in biology and human health. Springer, New York, NY, pp. 198-218.

Videler, J.J., 1993. The costs of swimming. In: Videler, J.J., (Eds.), Fish Swimming. Chapman and Hall, London, The United Kingdom pp. 185-205.

Walker, M.K., Zabel, E.W., Akerman, G., Balk, L., Wright, P., Tillitt, D.E., 1996. Fish egg injection as an alternative exposure route for early life stage toxicity studies: description of two unique methods. In: Ostrander, G.K., (Eds.), Techniques in aquatic toxicology. CRC Press, Boca Raton, FL, pp. 41-72.

Wang, Q., Lu, L., Yuan, C., Pei, K., Liu, Z., Guo, M., Zhu, M., 2010. Potent inhibition of protein tyrosine phosphatase 1B by copper complexes: implications for copper toxicity in biological systems. Chemical Communications 46, 3547-3549.

Watanabe, T., Kiron, V., Satoh, S., 1997. Trace minerals in fish nutrition. Aquaculture 151, 185-207.

Webber, D.M., Boutilier, R.G., Kerr, S.R., 1998. Cardiac output as predictor of metabolic rate in cod *Gadus morhua*. Journal of Experimental Biology 201, 2779-2789.

Weber, L.P., Dubẻ, M.G., Rickwood, C.J., Driedger, K.L., Portt, C., Breton, C., Janz, D.M., 2008. Effects of multiple effluents on resident fish from Junction Creek, Sudbury, Ontario. Ecotoxicology and Environmental Safety 70, 433-455.

Weber, L.P., Higgins, P.S., Carlson, R.I., Janz, D.M., 2003. Development and validation of methods for measuring multiple biochemical indices of condition in juvenile fishes. Journal of Fish Biology 63,637-658.

Weeks, M.E., 1956. Selenium and tellurium. In: Weeks, M.E., Leicester, H.M., (Eds.), Discovery of the element (6th ed.). Journal of Chemical Education, Easton, PA, pp.303-319.

Weis, J.S., Smith, G., Santiago-Bass, C., 2000. Predator/prey interactions: a link between the individual level and both higher and lower level effects of toxicants in aquatic ecosystems. Journal of Aquatic Ecosystem Stress and Recovery 7, 145-153.

Weis, J.S., Smith, G., Zhou, T., Santiago-Bass, C., Weis, P., 2001. Effects of contaminants on behavior: biochemical mechanisms and ecological consequences. Bioscience 51, 209-217.

Westerfield., 1995. The Zebrafish book; a guide for the laboratory use of zebrafish (*Danio rerio*). 3rd Ed. University of Oregon press, Eugene, OR, pp. 1-385.

Willmer, P., Stone, G., Johnston, I., 2005. The problems of size and scale. In: Willmer, P., Stone, G., Johnston, I., (Eds.), Environmental physiology of animals. Blackwell Publishing, Malden, MA, pp. 36-48.

Wiseman, S., Thomas, J.K., Higley, E., Hursky, O., Pietrock, M., Raine, J.C., Giesy, J.P., Janz, D.M., Hecker, M., 2011a. Chronic exposure to dietary selenomethionine increases gonadal steroidogenesis in female rainbow trout. Aquatic Toxicology 105, 218-226.

Wiseman, S., Thomas, J.K., McPhee, L., Hursky, O., Raine, J.C., Pietrock, M., Giesy, J.P., Hecker, M., Janz, D.M., 2011b. Attenuation of the cortisol response to stress in female rainbow trout chronically exposed to dietary selenomethionine. Aquatic Toxicology 105, 643-651.

Wolffram, S., Berger, B., Grenacher, B., Scharrer, E., 1989. Transport of selenoamino acids and their sulfur analogues across the intestinal brush border membrane of pigs. Journal of Nutrition 119, 706-712.

Woock, S.E., Garrett, W.R., Partin, W.E., Bryan, W.T., 1987. Decreased survival and teratogenesis during laboratory selenium exposures to bluegill, *Lepomis macrochirus*. Bulletin of Environmental Contamination and Toxicology 39, 998-1005.

Xia, R., Ganther, H.E., Egge, A., Abramson, J.J., 2004. Selenium compounds modulate the calcium release channel/ryanodine receptor of rabbit skeletal muscle by oxidizing functional thiols. Biochemical Pharmacology 67, 2071-2079.

Xu, X.M., Turanov, A.A., Carlson, B.A., Yoo, M.H., Gladyshev, V.N., Hatfield, D.L., 2012. Selenocysteine biosynthesis and the replacement of selenocysteine with cysteine in the pathway. In: Hatfield, D.L., Berry, M.J., Gladyshev, V.N., (Eds.), Selenium. Springer New York, NY, pp. 23-31.

Zhang, Y., Yu L.C., 2008a. Single-cell microinjection technology in cell biology. Bioessays 30, 606-610.

Zhang, Y., Yu L.C., 2008b. Microinjection as a tool of mechanical delivery. Current Opinion in Biotechnology 19, 506-510.

Zhao, X., Wang, S., Wu, Y., You, H., Lv, L., 2013. Acute ZnO nanoparticles exposure induces developmental toxicity, oxidative stress and DNA damage in embryo-larval zebrafish. Aquatic Toxicology 136, 49-59.

APPENDIX[a]

[a] Supplementary data are included in this chapter. The figure or table number is presented as Cx.Sy format, where 'Cx' indicates Chapter number; 'Sy' indicates figure or table number.

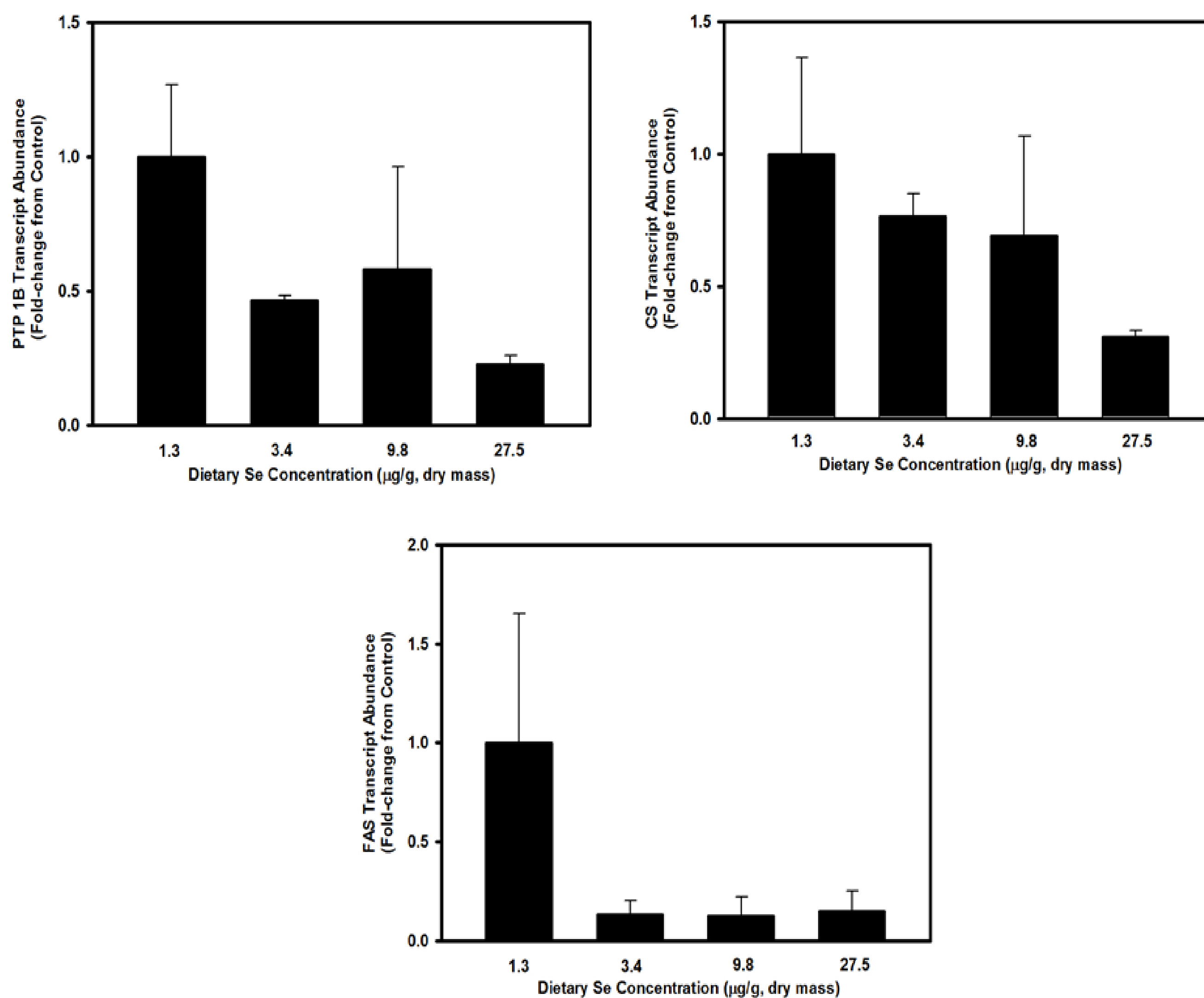

Figure C3.S1: mRNA abundance of protein tyrosine phosphatase 1B (PTP 1B), citrate synthase (CS) and fatty acid synthase (FAS) in liver of adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g dry mass) for 90 d. Transcript abundance was determined by quantitative real-time PCR. No significant difference was observed after performing one-way ANOVA. Data are mean ± S.E.M. of n = 4 liver samples.

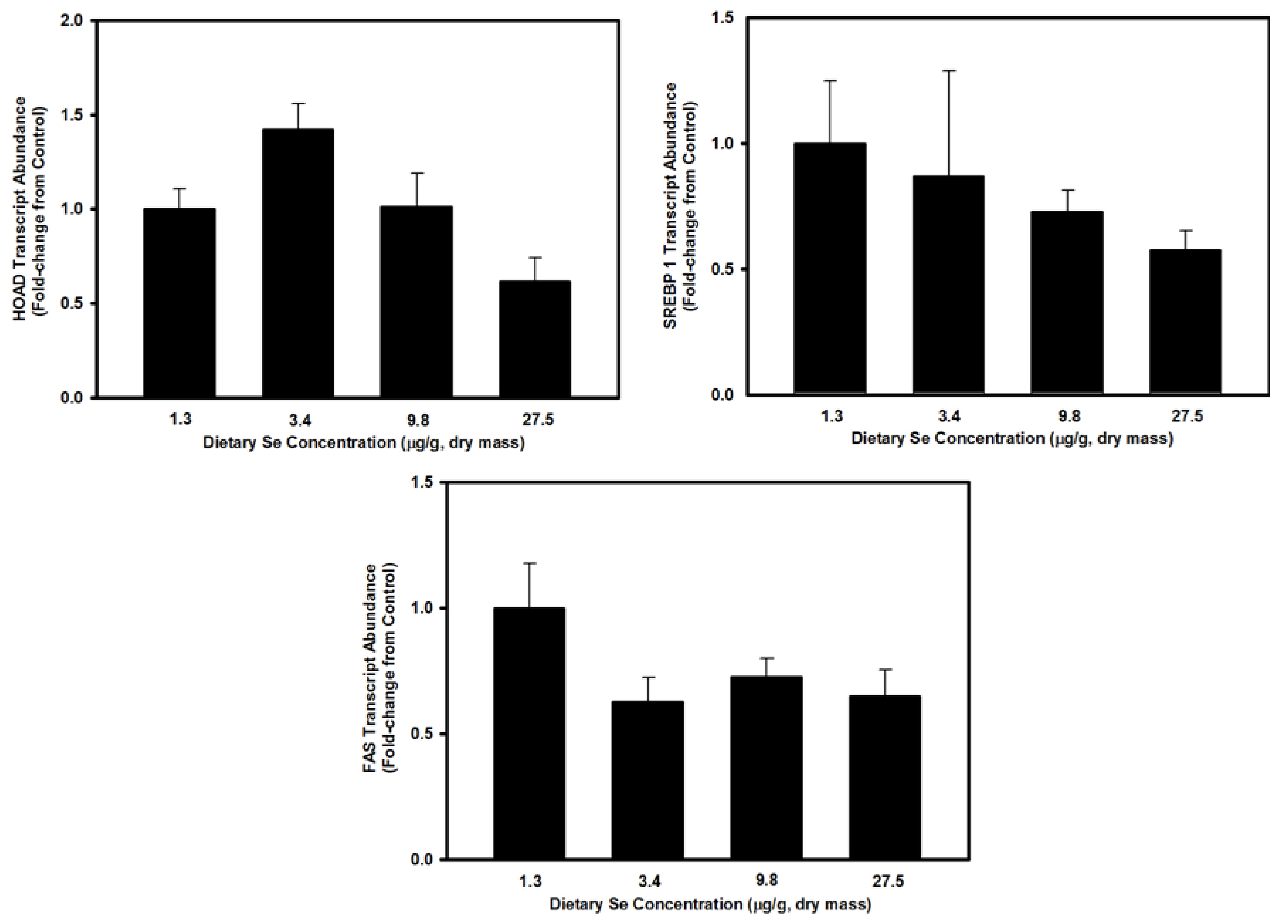

Figure C3.S2: mRNA abundance of β-hydroxyacyl coenzyme A dehydrogenase (HOAD), sterol regulatory element binding protein 1 (SREBP 1) and fatty acid synthase (FAS) in muscle of adult zebrafish fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (3.4, 9.8, and 27.5 µg Se/g dry mass) for 90 d. Transcript abundance was determined by quantitative real-time PCR. No significant difference was observed after performing one-way ANOVA. Data are mean ± S.E.M. of n = 4 muscle samples.

Table C4.S1: Percent egg viability and embryo hatchability of adult zebrafish exposed to a selenium sufficient diet (1.3 µg Se/g d.m.), and diet spiked with increasing concentration of selenium (3.7, 9.6 or 26.6 µg Se/g d.m.) for 90d. Data are mean ± S.E.M of n= 4 replicates of 250-600 eggs.

Dietary Se (µg/g d.m.)	Egg Se (µg/g d.m.)	% Viability	% Hatchability
1.3	2.1	78.0 ± 2.5	90.3 ± 3.7
3.7	6	73.6 ± 2.3	89.3 ± 3.5
9.6	9.6	76.7 ± 4.2	92.7 ± 0.4
26.6	21.9	79.1 ± 2.7	88.0 ± 2.9

Table C5.S1: Percent egg viability and embryo hatchability of adult zebrafish exposed to a selenium sufficient diet (1.3 µg Se/g d.m.), and diet spiked with increasing concentration of selenium (3.4, 9.8 or 27.5 µg Se/g d.m.) for 90d. Data are mean ± S.E.M of n= 3-4 replicates of 100-200 eggs.

Dietary Se (µg/g d.m.)	Egg Se (µg/g d.m.)	% Viability	% Hatchability
1.3	1.3	73.1 ± 3.7	97.7 ± 2.3
3.4	6.8	82.9 ± 5.4	95.6 ± 2.2
9.8	12.7	64.1 ± 2.6	97.9 ± 1.3
27.5	34.1	72.6 ± 8.8	93.0 ± 1.8

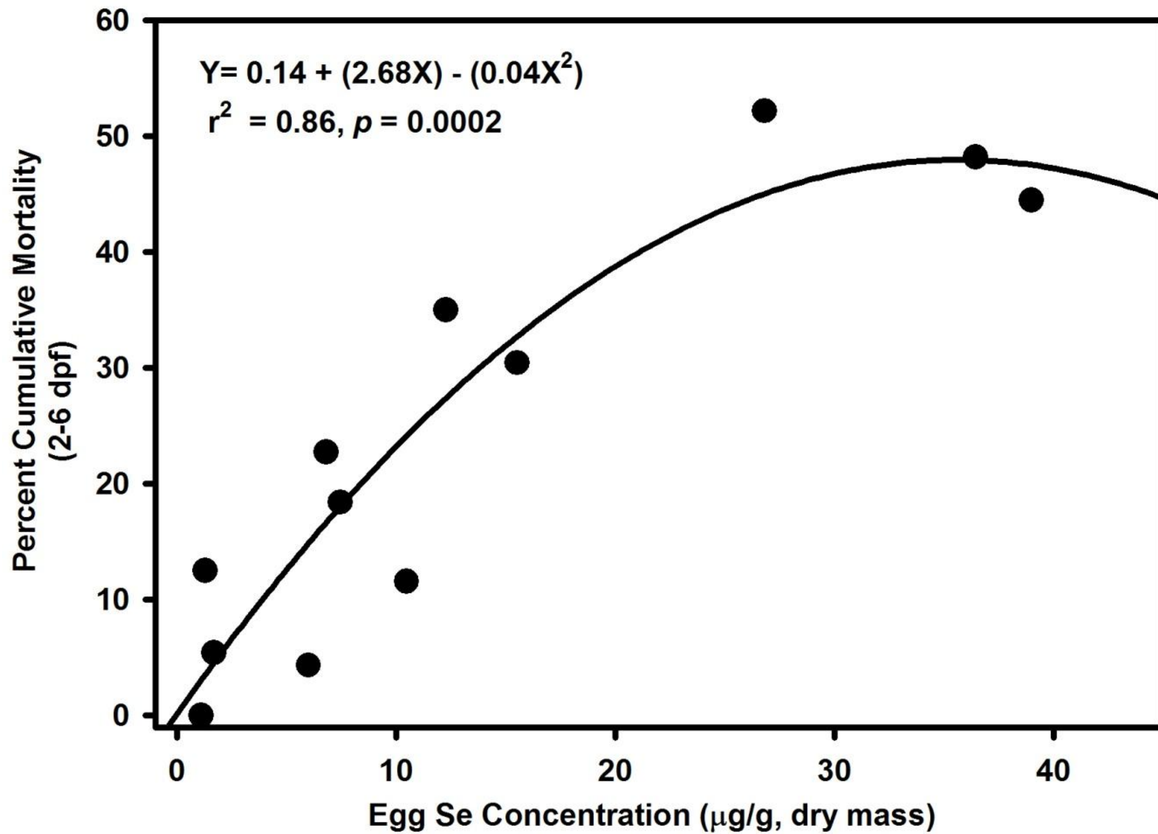

Figure C5.S1: Percent cumulative mortalities (2-6 days post fertilization) of embryo or larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Figure shows quadratic relationship between zebrafish egg selenium concentrations and percent larval fish mortality.

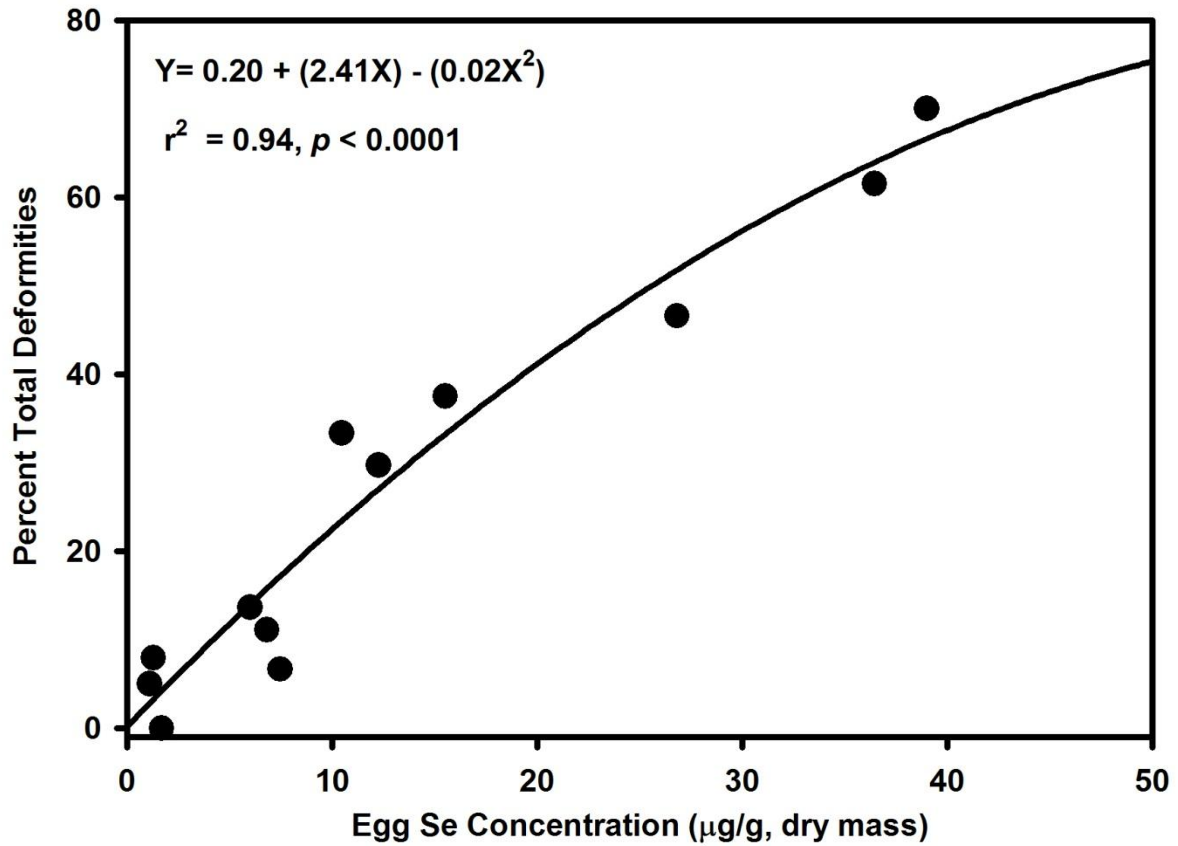

Figure C5.S2: Total morphological abnormalities in larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Figure shows quadratic relationship between zebrafish egg selenium concentrations and percent larval fish deformities.

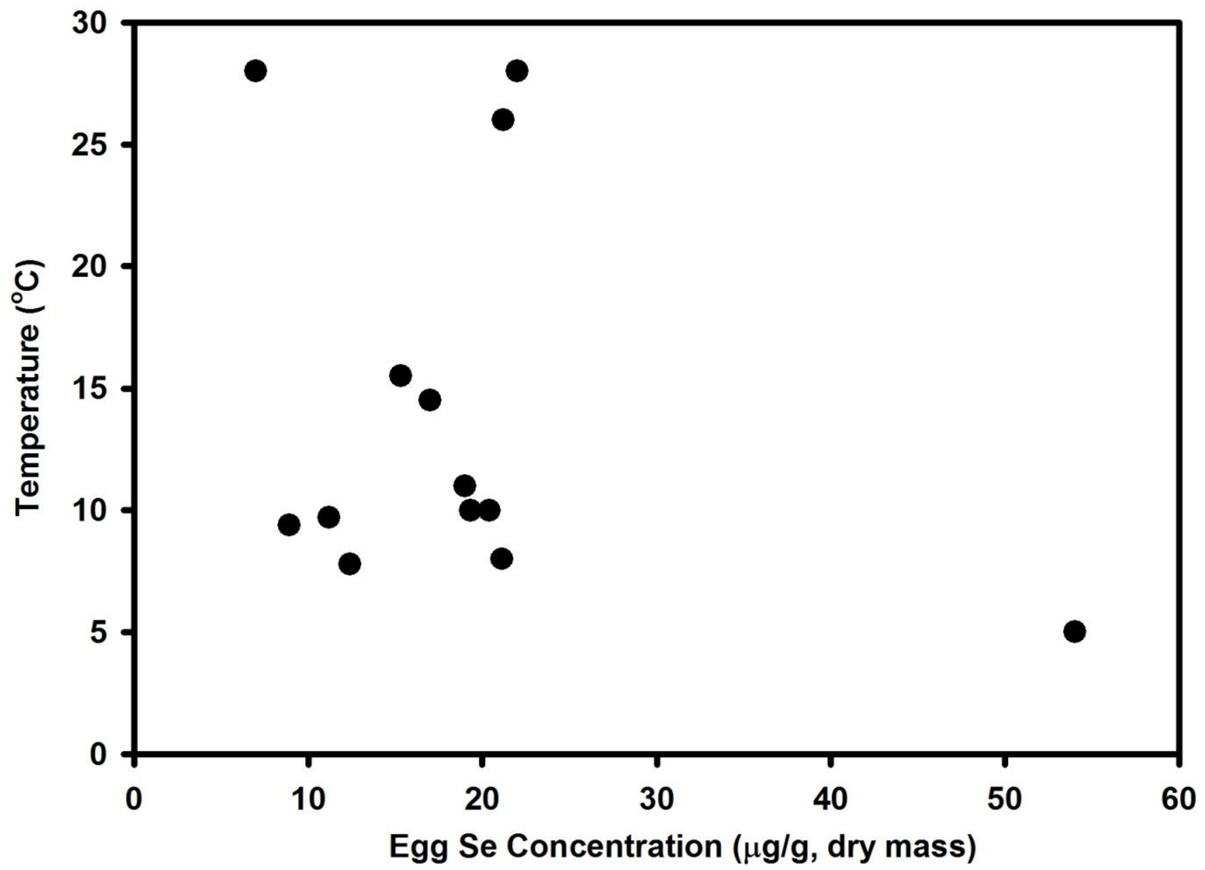

Figure C5.S3: Relationship between egg selenium toxicity thresholds (EC10s) and rearing temperatures (°C) in early life stages of fishes.

Table C6.S1: Percent hatchability, mortality and total deformities of early life stages zebrafish from a sham or Danieau solution (control group) microinjected treatment groups.

Treatment	% Hatchability	% Mortality	% Total Deformities
Sham	95.8 ± 2.5	4.2 ± 2.4	4.7 ± 2.6
Danieau Control	94.2 ± 0.7	7.5 ± 1.2	7.1 ± 4.3

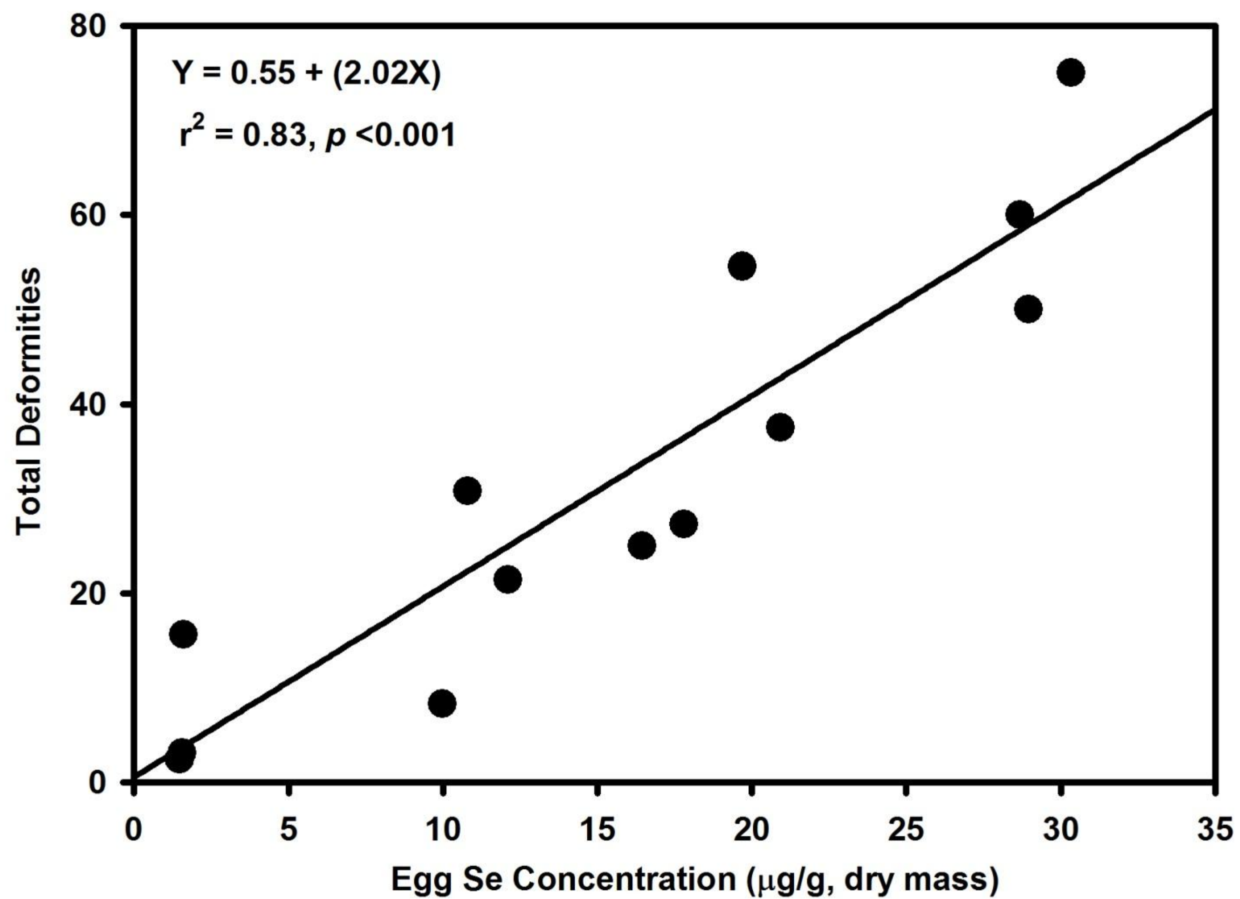

Figure C6.S1: Total morphological abnormalities in larval zebrafish exposed to increasing concentrations of selenium via *in ovo* maternal transfer. Figure shows linear relationship between zebrafish egg selenium concentrations and percent larval fish deformities.

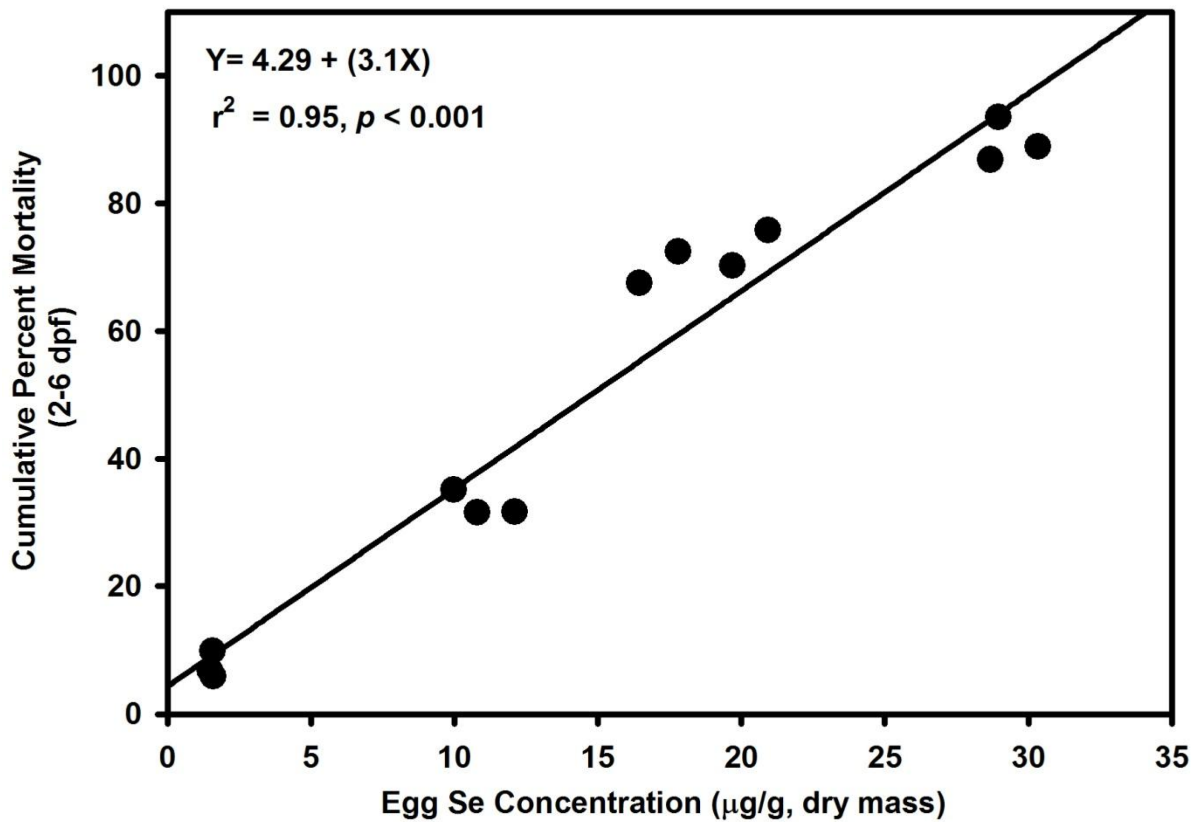

Figure C6.S2: Percent cumulative mortalities (2-6 days post fertilization) of embryo or larval zebrafish exposed to increasing concentrations of selenium via *in ovo* microinjection. Figure shows linear relationship between zebrafish egg selenium concentrations and percent larval fish mortality.

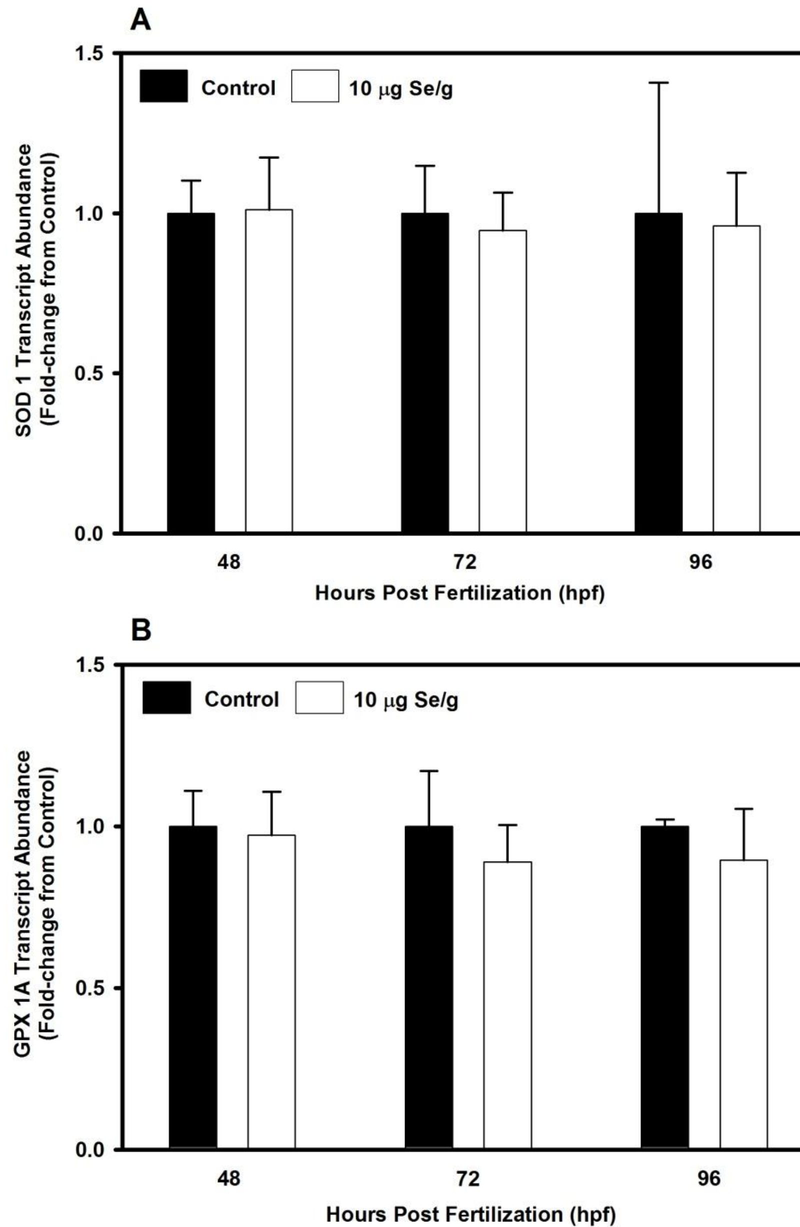

Figure C6.S3: mRNA abundance of (A) superoxide dismutase (SOD 1) and (B) glutathione peroxidase 1a (GPX 1A) in early life stages of zebrafish exposed to either 10 μg Se/g d.m. (nominal concentration) in the form of selenomethionine or control (Danieau) solution via egg microinjection. Transcript abundance was determined by quantitative real-time PCR at 48, 72 and 96 hours post fertilization (hpf). Data are mean ± S.E.M. of n = 3-5 replicates of 20 embryos and/or larval fish.