

2009

The roles of organic and inorganic zinc and selenium sources in the nutrition and promotion of health in rainbow trout (*Oncorhynchus mykiss*)

Rider, Sebastien A. B.

<http://hdl.handle.net/10026.1/2712>

<http://dx.doi.org/10.24382/3674>

University of Plymouth

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.

**The roles of organic and inorganic zinc and selenium
sources in the nutrition and promotion of health in rainbow
trout (*Oncorhynchus mykiss*)**

by

Sebastien A. B. Rider

A thesis submitted to the University of Plymouth

in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

(February 2009)

Department of Biological Sciences

Faculty of Science

In partnership with Alltech Inc. Biotechnology

The roles of organic and inorganic zinc and selenium sources in the nutrition and promotion of health in rainbow trout (*Oncorhynchus mykiss*)

Sebastien A. B. Rider

ABSTRACT

Three nutritional feeding trials were undertaken to investigate the nutritional role of zinc (Zn) and selenium (Se) in the promotion of health in rainbow trout (*Oncorhynchus mykiss* Walbaum). Organic and inorganic sources of both elements were assessed by determining bioavailability, effects on growth, feed utilisation, immuno-competence, oxidative status, fillet quality and interactions with other trace elements. A focus was made on the use of practical diets throughout the study.

The first experiment assessed the bioavailability of residual Se and Zn from a white fishmeal based practical diet during a 10-week trial in comparison to diets containing either supplemental Se-yeast and Zn proteinate (Zn-pr) or sodium selenite (Na_2SeO_3) and Zn sulphate (ZnSO_4). Se-yeast and Zn-pr were hypothesised to be more digestible and more effective in raising Se and Zn status than inorganic sources. Apparent digestibility of residual Zn and Se was 21.9% and 54.2% respectively. No conclusive difference in overall Zn bioavailability was observed between treatments. Se-yeast significantly increased Se digestibility to 63.7%, Se levels in all tissues, and the activities of hepatic thioredoxin reductase (Trx-R) and glutathione peroxidase (GSH-Px). Selenite supplementation only resulted in increased GSH-Px activity. Se-yeast was found to be a highly bioavailable Se source, effectively raising Se status.

The second experiment determined the efficacy and effect on health of residual Zn in comparison to 125, 312 and 781 mg kg⁻¹ supplemented Zn-pr or Zn sulphate in a practical diet. It was hypothesised that Zn-pr would be more efficacious in the promotion of health than Zn sulphate. The retention of both Zn sulphate and Zn-pr followed the same exponential decay ($R^2 = 0.978$) with increasing dietary Zn loading. This was accompanied with a lack of effect of 12-weeks Zn supplementation on standard growth rate (SGR), feed conversion ratio (FCR), Zn enzyme activity (Cu/Zn-superoxide dismutase and alkaline phosphatase), oxidative status (hepatic and pyloric caeca malondialdehyde (MDA), total plasma antioxidant capacity (TAC) and oxidative DNA damage), immuno-competence (superoxide generation, lysozyme and leukocyte counts), hematocrit, nuclear abnormalities (micronuclei, and notched and blebbed nuclei), and fillet quality (drip loss and astaxanthin). Both dietary Zn-pr ($R^2 = 0.573$) and Zn sulphate ($R^2 = 0.453$) interacted with hepatic manganese (Mn) but not with iron (Fe) or copper (Cu). No significant

differences were observed between sources and a strict homeostatic regulation of dietary Zn in rainbow trout was observed.

Preliminary investigations were carried out to establish the effects of seven days chronic husbandry related stressors on immuno-competence and oxidative status. It was hypothesised that chronic husbandry related stressors would result in oxidative stress and impaired immuno-competence. Alternating between netting and confinement stressors resulted in oxidative stress as determined by decreased TAC, increased oxidative DNA damage and modulated superoxide generation by leukocytes in the whole blood.

The final experiments investigated the efficacy and effects on health of residual Se in comparison to 2, 4, and 8 mg kg⁻¹ supplemental sodium selenite or Se-yeast in a commercial trout grower diet. It was hypothesised that supplemental Se would confer benefits to the health of both stressed and un-stressed fish, and Se-yeast would be more efficacious than selenite in its effects. Following a 10-week trial, the implication of seven days husbandry related stressors were also evaluated. Se retention was reduced in fish fed selenite up to 4 mg kg⁻¹ in comparison to fish fed the basal diet; in contrast Se-yeast increased Se retention. SGR, FCR, hepatic GSH-Px and Trx-R, plasma TAC, hepatic MDA, respiratory burst, lysozyme, nuclear abnormalities, and hematocrit were not affected by the supplemented dietary Se. However, in pre-stress fish fillet quality (drip loss) may be improved by Se-yeast supplementation; this was not observed with selenite. Stress decreased whole body Se in all treatments and increased GSH-Px to the greatest extent in Se supplemented diets suggestive of an increased Se utilisation during stress. Se had no protective effects on immuno-competence and oxidative status post stress. On the contrary, 8 mg kg⁻¹ Se from selenite increased hepatic MDA and resulted in the lowest GSH-Px increases amongst supplemented fish, which may be indicative of the sub-lethal effects of Se in fish fed a high level of inorganic Se; this was not observed with Se-yeast. Selenite ($R^2 = 0.6611$), but not Se-yeast ($R^2 = 0.073$), significantly interacted with whole body Cu. No interactions were found between dietary Se and Fe or Mn. Levels of whole body Fe, Mn, Cu and Zn were not affected by husbandry related stressors.

The study concludes that Se-yeast supplemented in fishmeal based diets is more bioavailable and efficacious in the maintenance of health than selenite. The efficacy of Zn-pr was not distinguished from that of Zn sulphate in the supplementation of practical diets. No difference was observed between Zn-pr and Zn sulphate in terms of bioavailability or efficacy. This may be due to the tight homeostatic regulation of dietary Zn above marginal levels. Se utilisation may increase during chronic husbandry related stress. Due to the lack of any measurable toxic effects and increased retention, Se-yeast may be more suitable for the delivery of Se in fish exposed to husbandry related stress, which was shown to result in oxidative stress. Higher quality practical diets may meet requirements for Se in un-stressed fish, but in conditions of stress, maximal GSH-Px and Se status may only be achieved by diets containing supplemental Se.

All experimental work involving animals was carried out in accordance with the 1986 Animals Scientific Procedures Act under Home Office project license # 30/2135 and personal license # 30/7809

LIST OF CONTENTS

Copyright statement	1
Title page	2
Abstract	3
Home Office Licence	5
List of Contents	6
List of Tables	15
List of Figures	17
List of Plates	18
Author's Declaration	19
Conferences attended and work presented	20
Acknowledgements	21
List of Abbreviations	23

Contents

CHAPTER 1.

SELENIUM AND ZINC NUTRITION: ORGANIC AND INORGANIC SOURCES, AND ROLES IN FISH HEALTH AND NUTRITION.....	25
1.1. Trace elements in fish	25
1.2. Zinc chemistry and biochemistry	30
1.2.1. The chemistry of zinc as a micronutrient.....	30
1.2.2. The biochemistry and biological basis of zinc.....	30
1.3. Zinc metabolism and regulation in fish	33
1.3.1. Zinc absorption	33
1.3.2. Zinc excretion	34
1.3.3. Zinc regulation and storage.....	35

1.4. Zinc and its role in health in mammals and fish	36
1.4.1. Zinc requirements and deficiency in salmonids.....	36
1.4.2. Zinc and the maintenance of oxidative status.....	39
1.4.3. Zinc and immunity.....	40
1.5. Organic and inorganic zinc	42
1.5.1. Overview of organic and inorganic zinc sources.....	42
1.5.2. Organic and inorganic zinc sources in fish nutrition	44
1.6. Factors affecting zinc utilisation in farmed fish	46
1.6.1. The disparity in zinc status between farmed and wild fish.....	46
1.6.2. Effect of diet upon zinc utilisation.....	47
1.6.3. Interactions between zinc and other trace elements.....	47
1.7. Selenium chemistry and biochemistry	48
1.7.1. Selenium compounds and selenium biochemistry.....	48
1.7.2. Selenium containing proteins and their function	50
1.8. Selenium metabolism and regulation in fish.....	52
1.8.1. Selenium absorption	52
1.8.2. Selenium excretion	53
1.8.3. Selenium regulation and storage.....	54
1.9. Selenium and its role in health in mammals and fish	55
1.9.1. Selenium requirements and deficiency in salmonids.....	55
1.9.2. Selenium and the maintenance of oxidative status	57
1.9.3. Selenium and immunity.....	59
1.9.4. Toxicity of dietary selenium.....	61

1.10. Dietary selenium in fish feeds.....	61
1.11. Factors affecting selenium utilisation in farmed fish.....	62
1.11.1. The disparity in selenium status between farmed and wild fish	62
1.11.2. Dietary selenium digestibility in fish feeds	63
1.11.3. Organic and inorganic selenium sources in fish nutrition.....	64
1.11.4. Interactions between selenium and other trace elements	65
1.12. Conclusions and study objectives	65

CHAPTER 2.

GENERAL ANALYTICAL METHODS.....	68
2.1. Overview.....	68
2.2. Rearing facilities and maintenance of water quality.....	68
2.3. Experimental fish.....	71
2.4. Feed and weighing.....	71
2.5. Growth and feed performance	71
2.6. Proximate analysis of diets and carcass	72
2.6.1. Moisture.....	72
2.6.2. Ash.....	72
2.6.3. Lipid.....	72
2.6.4. Crude protein	73
2.6.5. Gross energy	74
2.7. Trace element analysis.....	74
2.7.1. Sample preparation and digestion.....	74

2.7.2. Trace element analysis of digests	75
2.8. Trace element digestibility and retention.....	76
2.9. Mineral premixes	77
2.10. Determination of selenium and zinc dependent enzymes.....	77
2.10.1. Sample protein	78
2.10.2. Assay of glutathione peroxidase	78
2.10.3. Assay of thioredoxin reductase.....	79
2.10.4. Assay of carboxypeptidase B.....	80
2.10.5. Assay of alkaline phosphatase	81
2.10.6. Assay of copper/zinc superoxide dismutase	81
2.11. Markers of oxidative status.....	82
2.11.1. Malondialdehyde	82
2.11.2. Total antioxidant capacity.....	83
2.11.3. Single cell gel electrophoresis (SCGE) or 'Comet' assay	84
2.12. Hematological parameters and assay of nuclear abnormalities	86
2.12.1. Hematocrit	86
2.12.2. Blood Smears.....	86
2.12.3. Total leukocyte counts	86
2.12.4. Micronucleus and nuclear abnormalities	87
2.13. Immune parameters.....	87
2.13.1. Turbidimetric assay for lysozyme activity.....	87
2.13.2. Determination of respiratory burst.....	88
2.14. Drip loss of fillets.....	88

2.15. Astaxanthin extraction and determination of fillets and feed	89
2.16. Statistical analysis	90

CHAPTER 3.

THE BIOAVAILABILITY OF CO-SUPPLEMENTED ORGANIC AND INORGANIC ZINC AND SELENIUM IN A WHITE FISHMEAL BASED DIET. 91

3.1. Introduction.....	91
3.2. Methods.....	94
3.2.1. Experimental fish.....	94
3.2.2. Experimental design and diets.	94
3.2.3. Analytical procedures	95
3.2.4. Statistical analysis.....	97
3.3. Results.....	98
3.3.1. Growth rates and feed performance	98
3.3.2. Selenium and zinc digestibility.....	99
3.3.3. Tissue and whole body zinc.....	99
3.3.4. Tissue and whole body selenium	100
3.3.5. Selenium and zinc dependent enzyme activity	102
3.4. Discussion	104
3.4.1. Improved Se-yeast digestibility in fishmeal based diets.....	104
3.4.2. Improved deposition of selenium from Se-yeast across tissues.....	104
3.4.3. Increased activity of selenium dependent enzymes by Se-yeast.....	105
3.4.4. A lack of difference in zinc digestibility between zinc sources.....	106
3.4.5. Increased tissue zinc by dietary zinc supplementation	107

3.4.6. Lack of response by zinc dependent enzymes	109
3.4.7. Diet formulation and zinc and selenium interactions.....	110
3.4.8. Conclusions.....	111

CHAPTER 4.

THE EFFECT OF NUTRITIONAL, SUPRA-NUTRITIONAL, AND HIGH ORGANIC AND INORGANIC ZINC SUPPLEMENTATION IN A PRACTICAL DIET ON ZINC STATUS AND HEALTH.....

112

4.1. Introduction.....	112
------------------------	-----

4.2. Methods.....	116
-------------------	-----

4.2.1. Experimental fish.....	116
-------------------------------	-----

4.2.2. Diets and experimental regime	116
--	-----

4.2.3. Analytical procedures	119
------------------------------------	-----

4.2.4. Statistical analysis.....	120
----------------------------------	-----

4.3. Results.....	121
-------------------	-----

4.3.1. Growth rates and feed performance.....	121
---	-----

4.3.2. Response of tissue and whole body zinc to supplemental zinc.....	122
---	-----

4.3.3. Response of zinc enzymes to zinc supplementation	126
---	-----

4.3.4. Effect of zinc supplementation on markers of oxidative status	126
--	-----

4.3.5. Effect of zinc supplementation on immuno-competence.....	128
---	-----

4.3.6. Response of haematological parameters to zinc supplementation.....	129
---	-----

4.3.7. Effect of supplemental zinc on fillet quality.....	130
---	-----

4.3.8. Interactions between dietary zinc and other hepatic trace elements	131
---	-----

4.4. Discussion.....	133
----------------------	-----

4.4.1. Homeostatic regulation of supplemented dietary zinc	133
4.4.2. Response of growth and zinc enzymes to zinc supplementation	135
4.4.3. Zinc supplementation and oxidative status	138
4.4.4. Zinc supplementation and immuno-competence	140
4.4.5. Effect of dietary zinc on haematological parameters.....	142
4.4.6. Zinc supplementation and fillet quality	143
4.4.7. Zinc supplementation and interactions with other essential elements	144
4.4.8. Zinc supplementation and toxicity.....	145
4.4.9. Conclusions.....	145

CHAPTER 5.

PRELIMINARY INVESTIGATIONS INTO THE EFFECT OF CHRONIC HUSBANDRY RELATED STRESSORS ON OXIDATIVE STATUS AND IMMUNOLOGICAL FUNCTION	147
--	------------

5.1. Introduction.....	147
5.2. Methods.....	152
5.2.1. Fish maintenance and experimental conditions	152
5.2.2. Sample analysis.....	152
5.2.3. Statistical analysis.....	153
5.3. Results.....	154
5.3.1. Chronic husbandry related stressors and the stress response	154
5.3.2. Chronic husbandry related stressors and health responses	155
5.4. Discussion	159
5.4.1. The effect of chronic husbandry stressors on hematocrit and cortisol.....	159

5.4.2. The modulation of plasma TAC by chronic husbandry stressors	160
5.4.3. The effect of chronic husbandry stressors on oxidative DNA damage.....	161
5.4.4. The effect of chronic husbandry stressors on respiratory burst	162
5.4.5. Conclusions.....	163

CHAPTER 6.

SUPRA-NUTRITIONAL DIETARY INTAKE OF SELENITE AND SELENIUM YEAST IN NORMAL AND STRESSED RAINBOW TROUT: IMPLICATIONS ON SELENIUM STATUS AND HEALTH RESPONSES.....

165

6.1. Introduction.....	165
------------------------	-----

6.2. Methods.....	170
-------------------	-----

6.2.1. Experimental fish.....	170
-------------------------------	-----

6.2.2. Diets and experimental regime	170
--	-----

6.2.3. Analytical procedures	171
------------------------------------	-----

6.2.4. Statistical analysis.....	172
----------------------------------	-----

6.3. Results.....	173
-------------------	-----

6.3.1. Growth rate and feed performance	173
---	-----

6.3.2. Response of body selenium to supplemented selenium and stress	174
--	-----

6.3.3. Response of selenium enzymes to dietary selenium and stress	176
--	-----

6.3.4. Effect of selenium and stress on markers of oxidative status	178
---	-----

6.3.5. Effect of selenium and stress on hematocrit, immune parameters and cytogenetic damage	180
--	-----

6.3.6. Effect of dietary selenium and stress on fillet drip loss	182
--	-----

6.3.7. Effect of dietary selenium and stress on other trace elements	186
--	-----

6.4. Discussion.....	187
----------------------	-----

6.4.1. Retention of selenite and Se-yeast	187
6.4.2. Selenium status and stress; differences between selenite and Se-yeast	188
6.4.3. Effects of selenium and physical stress on oxidative status.....	189
6.4.4. Selenium and health; implications of physical stress.....	191
6.4.5. Effect of selenium and physical stress on fillet quality	193
6.4.6. Effect of selenium and physical stress on other trace elements.....	194
6.4.7. Conclusions.....	194
CHAPTER 7.	
GENERAL DISCUSSION	196
7.1. Overview of findings	196
7.1.1. The bioavailability of organic and inorganic zinc and selenium	196
7.1.2. Organic and inorganic zinc supplementation and health	198
7.1.3. Husbandry stress; effects on oxidative status and immuno-competence	200
7.1.4. Selenium supplementation, health, and implications of husbandry stress	200
7.2. Further Research	204
7.2.1. Evaluation of selenium and zinc nutrition	204
7.2.2. Selenium and zinc utilisation; disease, stress and life cycle	206
7.2.3. Selenium and zinc utilisation; diet and species.....	208
7.3. Conclusions.....	208
8. APPENDIX.....	210
8.1. Analysis of zinc and selenium in selected feed ingredients.....	210
8.2. Zinc and selenium analysis of selected commercial trout grower diets.....	211

8.3. Validation of thioredoxin reductase assay	212
8.4. Methods of cryo-preservation of trout erythrocytes for the comet assay	213
8.4.1. Introduction.....	213
8.4.2. Methods	213
8.4.3. Results.....	214
8.4.4. Discussion.....	214
9. REFERENCES	215

LIST OF TABLES

Table 1.1. Trace elements important for mammals, birds and fish.....	27
Table 1.2. Typical trace element requirement ranges for cultivated fish ^{1,2}	29
Table 1.3. Zinc involvement in the organism.	37
Table 1.4. Zn deficiency consequences in fish and mammals.	38
Table 3.1. Basal diet formulation.....	95
Table 3.2. Composition of experimental diets ¹ and growth performance and feed utilisation of trout given diets with basal, organic or inorganic supplemental selenium and zinc.....	96
Table 3.3. Mean tissue and whole-body Zn ¹ of trout given a basal fishmeal diet, and diets with supplemented organic or inorganic zinc and selenium sources.	100
Table 3.4. Mean tissue and whole-body Se ¹ of trout given a basal fishmeal diet, and diets with supplemented organic or inorganic zinc and selenium sources.	101
Table 3.5. Mean Zn dependent enzyme activity and activity/tissue Zn ratios of trout given a basal fishmeal diet, and diets with supplemented organic or inorganic zinc and selenium sources.....	102
Table 3.6. Mean Se dependent enzyme activity and activity/tissue Se ratios of trout given a basal fishmeal diet, and diets with supplemented organic or inorganic zinc and selenium sources.....	103
Table 4.1. Basal diet formulation.....	117

Table 4.2. Proximate and trace element analysis of experimental diets ¹	118
Table 4.3. Mean growth performance, feed utilisation and mortality of rainbow trout given various levels of supplemental Zn sulphate or Zn-pr for 12 weeks.	121
Table 4.4. Mean net Zn retention and tissue Zn of rainbow trout given various levels of supplemented Zn sulphate and Zn-pr for 12 weeks.	123
Table 4.5. Mean activity of plasma ALP and SOD in rainbow trout given various levels of supplemented Zn sulphate and Zn-pr for 12 weeks.....	125
Table 4.6. Mean TAC, MDA and percentage tail DNA of rainbow trout given various levels of supplemented Zn sulphate and Zn-pr for 12 weeks.	127
Table 4.7. Mean immune parameters, respiratory burst, lysozyme activity and leukocyte counts, of rainbow trout given various levels of supplemented Zn sulphate and Zn-pr for 12 weeks.	128
Table 4.8. Mean hematocrit and nuclear abnormalities of rainbow trout given various levels of supplemented Zn sulphate and Zn-pr for 12 weeks.	129
Table 4.9. Mean fillet quality, astaxanthin and water retention, of rainbow trout given various levels of supplemental Zn sulphate or Zn-pr for 12 weeks.	130
Table 6.1. Mean growth performance, feed utilisation and net Se retention of rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks.	173
Table 6.2. Mean whole body Se of rainbow trout given various levels supplemental Se pre and post 7 days stress. .	174
Table 6.3. Mean activity of hepatic glutathione peroxidase and thioredoxin reductase of rainbow trout given various levels supplemental Se pre and post 7 days stress.	177
Table 6.4. Mean hepatic malondialdehyde and total antioxidant capacity of rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks pre and post 7 days stress.....	179
Table 6.5. Mean hematocrit, serum lysozyme and peak respiratory burst of rainbow trout given various levels of organic or inorganic Se for 10 weeks pre and post 7 days stress.....	181
Table 6.6. Mean nuclear abnormalities and leukocyte frequency of rainbow trout given various levels of organic or inorganic supplemental Se.....	182

Table 6.7. Mean fillet drip loss of rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks pre and post 7 days stress.....	183
Table 6.8. Mean whole body copper and zinc in rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks pre and post 7 days stress.....	184
Table 6.9. Mean whole body manganese and iron in rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks pre and post 7 days stress.....	185
Table 8.1. Mean zinc and selenium levels in feed ingredients.....	210
Table 8.2. Mean zinc and selenium of commercial trout grower diets.....	211
Table 8.3. Assay of Trx-R activity in fish tissues.....	212

LIST OF FIGURES

Figure 1.1. Hypothetical relationship between dietary nutrient level and optimal health.....	28
Figure 1.2. The dismutation of superoxide by superoxide dismutase. The spontaneous dismutation of superoxide to hydrogen peroxide and oxygen is accelerated by superoxide dismutase.....	40
Figure 1.3. Metabolic pathway of dietary Se in humans.....	50
Figure 1.4. Reduction of hydrogen peroxide by glutathione peroxidase.....	51
Figure 3.1. Mean apparent digestibility of basal and supplemented zinc and selenium sources from a fishmeal based diet.....	98
Figure 4.1. Percentage Zn retention in rainbow trout supplemented increasing levels of Zn-pr and Zn sulphate.....	122
Figure 4.2. Response of whole body Zn to 12 weeks dietary Zn-pr and Zn sulphate supplementation.....	124
Figure 4.3. Response of hepatic manganese to various levels of dietary Zn sulphate and Zn-pr supplemented for 12 weeks in rainbow trout.....	131
Figure 4.4. Response of hepatic iron to various levels of dietary Zn sulphate and Zn-pr supplemented for 12 weeks in rainbow trout.....	132
Figure 4.5. Response of hepatic copper to various levels of dietary Zn sulphate and Zn-pr supplemented for 12 weeks in rainbow trout.....	132

Figure 5.1. 95 % confidence interval bars of mean percentage hematocrit of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts.	154
Figure 5.2. 95 % confidence interval bars of mean plasma cortisol concentration (ng ml ⁻¹) of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts.	155
Figure 5.3. 95 % confidence interval bars of mean plasma TAC of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts.	156
Figure 5.4. 95 % confidence interval bars of mean percentage tail DNA of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts.	156
Figure 5.5. 95 % confidence interval bars of mean micronuclei frequency per 2000 cells of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts.	157
Figure 5.6. PMA activation of NADPH oxidase of trout leukocytes in diluted whole blood of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts.	158
Figure 6.1. Response of whole body Se concentration to 10 weeks organic and inorganic dietary Se supplementation and 7 days handling stress in rainbow trout.	175
Figure 6.2. Response of whole body Cu concentration to 10 weeks organic and inorganic dietary Se supplementation both pre and post 7 days handling stress in rainbow trout.	186
Figure 8.1. Effect of storage methods on DNA stability of rainbow trout erythrocytes.	214

LIST OF PLATES

Plate 2.1. Annotated collage of recirculation system 'F' located at the University of Plymouth.	69
Plate 2.2. Comet images from individual trout erythrocytes showing various degrees of DNA damage.	86
Plate 2.3. Nuclear abnormalities in rainbow trout erythrocytes.	87

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was jointly funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and Alltech Biotechnology through a CASE studentship.

Word count: 49,762

Signed.....

Date.....30-9-09.....

Conferences attended and work presented

Alltech's 22nd International Feed Symposium. April 23rd – 26th 2006. Lexington, USA

XII International Symposium on Fish Nutrition and Feeding (XII ISFNF 2006). May 28th – June 1st 2006. Biarritz, France.

Alltech's 3rd European Aquaculture Meeting. November 29th 2006. Dunboyne, Ireland

7th International Comet Assay Workshop. 24th – 27th June 2007 Coleraine, Northern Ireland.

Poster: Reduced genetic stability, antioxidant status and leukocyte activation in rainbow trout subjected to chronic husbandry related stressors. In: Abstracts from the 17th International Comet Assay Workshop held at the University of Ulster, Coleraine, Northern Ireland. *Mutagenesis* 2007. 22(6) e1-e22.

Alltech Selenium Roundtable Meeting. 6th July 2007. Stamford, UK.

European Aquaculture Society Aquaculture Europe Conference. 24th – 27th October 2007, Istanbul Turkey.

Poster: Reduced genetic stability, antioxidant status and leukocyte activation in rainbow trout subjected to chronic husbandry related stressors.

Alltech's 4th European Aquaculture Meeting. November 7th 2007. Dunboyne, Ireland

Presentation: Dietary zinc and selenium bioavailability in practical rainbow trout diets: the developments and application of techniques.

Alltech's 24th International Animal Health and Nutrition Symposium. April 20th – 23rd 2008. Lexington, USA

Presentation: The bioavailability of Selplex[®] and selenite in practical diets.

Publications

Davies, S., Rider, S., Sweetman, J., 2008. The nature of selenium and its role in fish nutrition. *International Aquafeed* 11 (4), 28-33.

Rider, S., Sweetman, J., 2008. Aquaculture turns eye on organic selenium. *Feed Mix* 16 (1), 13-15.

Rider, S.A., Davies, S.J., Jha, A.N., Clough, R., Sweetman, J.W., 2009. Bioavailability of co-supplemented organic and inorganic zinc and selenium sources in a white fishmeal based rainbow trout (*Oncorhynchus mykiss*) diet. *Journal of Animal Physiology and Animal Nutrition*. *In press*.

Rider, S.A., Davies, S.J., Jha, A.N., Fisher, A., Knight, J. Sweetman, J.W., 2009. Supra-nutritional dietary intake of selenite and selenium yeast in normal and stressed rainbow trout (*Oncorhynchus mykiss*): Implications on selenium status and health responses. *Aquaculture*, 295 (3-4), 282-291.

Davies, S., Rider S., Lundebye, A., 2009. Selenium and zinc nutrition of farmed fish - new perspectives in feed formulation to optimise health and production. Society for Experimental Biology, Book chapter. *In press*.

Acknowledgements

I would like to express my sincere gratitude to all the following people who have helped me during the completion of this project.

Prof. Simon Davies, my director of studies for his guidance, help, patience, eternal optimism and many ideas – “it will all come together”!

Dr Awadhesh Jha, my second supervisor, for his guidance, his thorough input into the preparation of posters and publications and advice on genetic assays.

Prof. Peter Surai, my industrial supervisor at Alltech Ireland Ltd., for his guidance in the initial stages of the programme.

John Sweetman, my contact with Alltech Ireland Ltd. throughout the study, for his encouragement given prior to presentations, securing extra funding for selenium analysis, his enthusiasm for my work and his encouragement regarding presentations and publications.

The Staff at Alltech Inc., for being most approachable and helpful with technical issues, organising flights etc for meetings, supplying copies of relevant books and publications and much inspiration.

The BBSRC and Alltech Ireland Ltd. for funding the project.

My friends and colleagues within the university department, James Reeves, Matt Owen, Daniel Merrifield, Arkadios Dimitroglou, Martin Canty, Siddiq El-Ajnaf, Jerome Laporte, Mark Rawling, and Daniel Leeming for being there to help on long sampling days, their many ideas, the endless banter, and being there in times of both celebration and commiseration.

Dr Robert Clough and Dr Andrew Fisher for their greatly appreciated help with trace element analysis.

Robert Serwata and Benjamin Eynon, the aquarium technical staff for help with fish husbandry.

Liz Folland, Peter Russel, Roger Haslam, Andy Atfield, Paul Waines, Lynne Cooper, Nikki Blatchford, and Ricki Preston, technical staff who would often go above and beyond their call of duty.

Dr Jan Knight, the director of Knight Scientific Ltd., for supplying assay kits and her technical advice. In addition, Kenneth Armstrong, James Reeves, and Xiaoli Gong, post and present staff at Knight Scientific Ltd. for their help and advice with assays.

To my father, mother and two brothers for their endless support and everything they have given me over the last 29 years.

To my partner Gemma Stevens for being her wonderful self and help during long sampling days.

To all my friends, particularly those who shared my passion for an adventure of the non-academic kind!

To the great outdoors for always being there!

Dedication

I would like to dedicate this thesis to Bill Grant, a close family friend who sadly passed away in December 2008. Your words of wisdom, great humour and many acts of kindness will be greatly missed.

Abbreviation List

ALP	Alkaline phosphatase
ANOVA	Analysis of variance
BKD	Bacterial kidney disease
CPB	Carboxypeptidase B
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DPX	Dibutyl phthalate xylene
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
FCR	Feed conversion ratio
Flame AA	Flame atomic absorption spectrometry
fMLP	<i>N</i> -formylmethionylleucylphenylalanine
FPG	Formamidopyrimidine glycosylase
GI	Gastro-intestinal
GSH	Glutathione
GSH-Px	Glutathione peroxidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICP-MS	Inductively coupled plasma mass spectrometry
IHN	Infectious haematopoietic necrosis
IPN	Infectious pancreatic necrosis
ISA	Infectious salmon anaemia
IUPAC	International Union of Pure and Applied Chemistry
LMA	Low melting point agarose
MDA	Malondialdehyde
MN	Micronucleus
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MS-222	Tricane methanesulfonate
MT	Metallothionein

NADPH	Nicotinamide adenine dinucleotide phosphate
NMA	Normal melting point agarose
OES	Optical emission spectrometry
PBS	Phosphate buffered saline
PMA	Phorbol myristate acetate
RNA	Ribonucleic acid
PUFA	Polyunsaturated fatty acid (s)
Redox	Reduction-oxidation
ROS	Reactive oxygen species
RS	Recirculation system (s)
SAV	Salmonid alphavirus
SCGE	Single cell gel electrophoresis
SD	Standard deviation
SEM	Standard error of the mean
Se-Cys	Selenocysteine
Se-Met	Selenomethionine
Se-yeast	Selenium yeast
SGR	Standard growth rate
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TGA	Thymine, adenine and guanine
Trx	Thioredoxin
Trx-R	Thioredoxin reductase
VEA	Vitamin-E analogue
VHS	Viral haemorrhagic septicaemia
ZIP	Zrt- and Irt-like proteins
ZnT	Zinc transporter
Zn-AA	Zinc chelated or complexed amino acids
Zn-Lys	Zinc lysine
Zn-Met	Zinc methionine
Zn-pr	Zn-proteinate (s)

CHAPTER 1.

SELENIUM AND ZINC NUTRITION: ORGANIC AND INORGANIC SOURCES, AND ROLES IN FISH HEALTH AND NUTRITION

1.1. Trace elements in fish

Due to the expansion in the culture of aquatic organisms and the ever increasing diversity of species cultivated, there has been a mounting quantity of research in aquaculture, particularly involving disease management, production methods and nutrition. Over the past two decades, nutritional research into the macro nutrients has been relatively significant in comparison to that of the micro nutrients. Typically cultivated fish rely on one feed source; consequently it is essential that diets satisfy requirements for all nutrients. As with humans and other mammals, trace elements are required for the functioning of essential life processes including metabolism, growth, and reproduction. These micro nutrients are involved in vital processes including skeletal formation, the maintenance of colloidal systems, regulation of acid-base equilibrium and the function of biologically active compounds including hormones and enzymes (Watanabe et al., 1997). Despite the importance of trace elements in fish nutrition and health, knowledge of their biological roles and requirements in aquacultural environments is fragmentary and limited.

Currently 29 of the 90 naturally occurring elements are known to be essential for animal life. The majority of living matter consists of carbon (C), hydrogen (H), nitrogen (N), oxygen (O), phosphorus (P) and sulphur (S), and these are required in grams per kilogram (g kg^{-1}). Also required in gram amounts, are the macro elements, calcium (Ca), magnesium (Mg), sodium (Na), potassium (K) and chlorine (Cl). The remaining elements are the so called trace

elements, which are present in much lower amounts with dietary requirements being in milligrams per kilogram (mg kg^{-1}). Trace element metabolism differs from that of most other nutrients in that they are neither produced nor consumed. As organic mineral sources are metabolised, such as is the case for organic selenium (Se) sources, this applies to the actual element rather than any associated compounds and molecules. There are sixteen trace elements considered to be essential in mammals and fish (Lall, 2002). Frieden (1984) gives the following description of an essential element:

“An element is considered essential when a deficient intake produces an impairment of function when restoration of physiological levels of the element prevents or relieves the deficiency. The organism can neither grow nor complete its life cycle without the element in question. The element should have a direct influence on the organism and be involved in the metabolism. The effect of an essential element cannot be wholly replaced by any other element.”

Currently the essentiality of only eight of micro nutrients has been verified in fish, and requirements and deficiency signs of the remaining eight have yet to be established (Table 1.1.).

Table 1.1.

Trace elements important for mammals, birds and fish.

Zinc	Molybdenum ¹
Iron	Vanadium ¹
Copper	Fluorine ¹
Manganese	Lithium ¹
Selenium	Nickel ¹
Iodine	Silicon ¹
Chromium	Arsenic ¹
Cobalt	Lead ¹

¹ limited information on fish (Watanabe et al., 1997).

The physiological importance of trace elements is such that deficiencies, i.e. levels below requirements, can cause biochemical, structural and functional pathologies, the severity of which will depend on factors including, duration of deficiency, environmental conditions and age. To compensate for dietary fluctuations, homeostatic mechanisms operate within organisms that maintain element levels to that required for normal metabolic activities. Should excess amounts be ingested and assimilated by either waterborne or dietary uptake, essential elements may become toxic. The thresholds between normal dietary requirements and toxicity will depend on the element of concern and may be narrow (Fig. 1.1.).

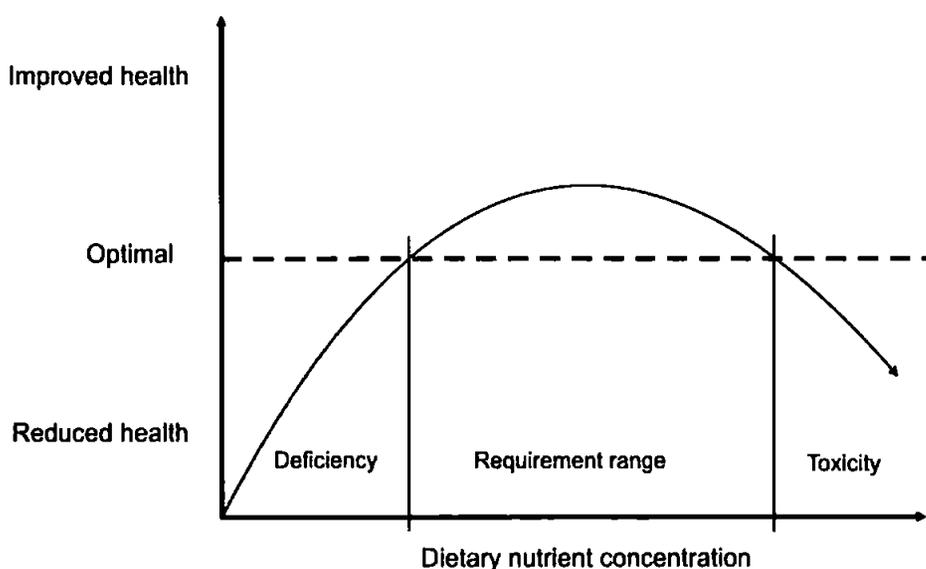


Figure 1.1. Hypothetical relationship between dietary nutrient level and optimal health.

Trace element requirements have largely been determined for rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*) for seven of the trace elements (Watanabe et al., 1997) (Table 1.2.). However, a better understanding is required of the nutritional and physiological role of trace elements in the health of cultivated fish. Mineral requirements of farmed fish may be influenced to differential degrees by the use of practical diets, fish physiology, environmental conditions and fish husbandry practices.

Table 1.2.

Typical trace element requirement ranges for cultivated fish^{1,2}.

Trace element	Requirement ¹
Iron	30-170
Copper	1-5
Manganese	2-20
Zinc	15-40
Cobalt	0.05-1.0
Selenium	0.15-0.5
Iodine	1-4

¹ Expressed as mg kg⁻¹ diet (NRC, 1993).

² Rainbow trout, channel catfish and common carp

Practical diets by nature of their ingredients and formulation vary considerably in composition and quality as opposed to semi-purified and purified experimental diets. Commercial diets may be manufactured from a range of fishmeals derived from a variety of fish species, notably pelagic fish species such as blue whiting (*Micromesistius poutassou*), capelin (*Mallotus villosus*) and herring (*Clupea harengus*) or a variety of fish by-products. Due to the increasing demand for an increasingly scarce resource, the replacement of fishmeals by inclusion of various plant proteins, such as soybean meal and wheat middlings, is becoming increasingly common place. Consequently, research into trace mineral nutrition needs to consider fish cultivation practices, diets, species, life cycle, and cultivation environments. There are many cultivated fish species where requirements for these elements have not been determined, particularly for marine species such as sea bass (*Dicentrarchus*

labrax) and sea bream (*Sparus aurata*) where the rearing seawater causes complications due to the significant quantities of waterborne minerals present (Watanabe, 1988).

1.2. Zinc chemistry and biochemistry

1.2.1. The chemistry of zinc as a micronutrient

Zinc, atomic number 30 and molecular weight 65.3, belongs to the heavy metals in group IIB and is particularly suited to a role in biological processes due to a combination of factors. Zinc has two electrons in its outer shell, and as the Zn^{2+} ion does not change oxidation state in physiological conditions it does not participate in redox reactions (Reilly, 2004). As a result of its stable valency, Zn (and its salts), unlike copper (Cu) and iron (Fe), has the advantage that it does not promote free radical damage in biological systems (Shankar and Prasad, 1998). As Zn is a small ion, it has a highly concentrated charge and strong electrostatic binding capabilities. Its high affinity for electrons makes Zn a strong Lewis acid that can form bonds with bases by accepting their electrons; this makes Zn ideal for use as a metal cofactor in reactions requiring a redox stable ion (McCall, 2000). Cadmium (Cd), which also belongs to the group IIB elements, has chemical similarities to Zn and is known to substitute for Zn in several enzymes. However, Cd is a much rarer element, and thus less readily available to biological systems (Reilly, 2004).

1.2.2. The biochemistry and biological basis of zinc

Zinc is ubiquitous in cellular metabolism and its biological role is most often associated with proteins, where it may be either involved in the active site of an enzyme or provide structural stability to the tertiary protein structure. Zn is an essential component of the catalytic site of around 300 enzymes in at least one enzyme of all six IUPAC enzyme classes. Zn can have

three different functional roles in enzymes: catalytic, coactive and structural (Vallee and Falchuk, 1993).

Catalytic role

In its catalytic role the Zn ion is directly involved in the catalytic site of the metalloenzyme. Carboxypeptidase A (EC 3.4.12.2) and B (EC 3.4.17.2) are examples, which as with all such enzymes are inactive without the Zn ion (Folk et al., 1960). One Zn ion is typically coordinated by four ligands; three amino acids and a water molecule. The water molecule is also involved in catalytic activity, which is either ionised or polarised to form hydroxide ions at neutral pH. Alternatively, the water molecule may be displaced in Lewis acid catalysis (McCall, 2000).

Coactive role

In coactive multi-metal enzymes, Zn will function with another Zn ion or other metal ion co-catalytically; one ion participating directly in bond making/breaking and the other increasing the catalytic activity of the site. Examples of co-catalytic enzymes are; alkaline phosphatase (ALP) (EC 3.1.3.1), which has two Zn and one magnesium (Mg) ion and leucine aminopeptidase, which has two Zn ions. Cu/Zn superoxide dismutase (Cu/Zn-SOD) (EC 1.15.1.1) requires Zn for its synthesis and although not required in directly in its catalytic activity, its Zn ion provides structural stability to the apoenzyme; Cu is present as a cofactor (Vallee and Falchuk, 1993). Zn metalloenzymes differ in their affinity for Zn; those with high affinity being more common. However, affinity can change with pH, for example α -mannosidase has a high affinity for Zn at pH 7, but above this activity is lost. Histidine is the most

common amino acid to act as a ligand in Zn metalloenzyme catalytic sites, followed by glutamic and aspartic acids (Chesters, 1997).

Structural role

Zinc also plays a structural role in enzymes by stabilizing the protein structure through cross-linking remote regions within and between polypeptides to modify tertiary protein structure and function. With its high affinity for electrons Zn^{2+} can interact with several amino acid side chains, especially those containing sulphur and nitrogen atoms, such as cysteine and histidine (Reilly, 2004). Although cysteine rarely acts as a ligand in the active site of metalloenzymes, it is common where Zn provides structural stability, such as in alcohol dehydrogenase, in which Zn is coordinated tetrahedrally by four cysteine residues. Structural Zn atoms are also found in, aspartate transcarbamylase and protein kinase C. Alcohol dehydrogenase is unique in that it is the only Zn enzyme known to require Zn for both catalytic and structural purposes (Vallee and Falchuk, 1993).

In addition to Zn requiring enzymes, Zn is found in two classes of non-enzymatic Zn proteins; the metallothioneins and gene regulatory proteins. Composed of 62 amino acids, 20 of which are cysteine residues, metallothionein (MT) contains $5-7 \text{ g mol}^{-1}$ Zn, but can also bind to other heavy metals including, Cu and Cd. Zn MT contains two distinct Zn thiolate clusters, and has a stronger affinity and holds higher Zn concentrations than most other Zn proteins (Reilly, 2004). Consequently, Zn cannot move freely from its binding sites in MT to lower affinity proteins without the assistance of effectors that enhance its release and transfer (Maret, 2000). The function of MT is not fully understood as yet. It has been proposed to be involved in the storage of Zn, to detoxify heavy metals, stabilise membranes, regulate Cu and Zn metabolism, supply Zn to newly synthesized apoenzymes, scavenge free radicals and

regulate gene expression (Vallee and Falchuk, 1993, Kang, 1999, Powell, 2000; Wood, 2000; Maret, 2000).

25 % of cellular Zn in the rat liver is found in the nucleus and around 1 % of all mammalian genes encode for transcriptional activators; previously classed as Zn finger proteins. These Zn containing nucleoproteins link to the double helix of DNA and are involved in its repair (both base excision and nucleotide excision repair), replication and transcription (Dreosti, 2001). The linking of Zn fingers to corresponding sites on DNA initiates the transcription processes. These transcription proteins, which contain three or more such 'Zn fingers', have a series of polypeptide loops and are configured by Zn ligated to two anti-parallel beta sheets (Chesters, 1997). Zn also provides structural integrity to a second series of DNA transcription proteins originally also classed as 'Zn finger proteins' (Chesters, 1997). The Zn binding sites of these transcription proteins, now so called 'Zn twists', are distinctly different from Zn fingers and contain two Zn atoms per molecule, each ligated by four cysteine residues (Vallee et al., 1991).

1.3. Zinc metabolism and regulation in fish

1.3.1. Zinc absorption

Unlike mammals, fish have two routes of Zn absorption – the GI tract and in addition the gills (Bury et al., 2003). In common with mammals, in rainbow trout Zn absorption occurs in the anterior gastro-intestinal (GI) tract via an unknown saturable active transport mechanism most probably involving Zn transporter proteins (Glover and Hogstrand, 2002a; Bury et al., 2003) and a putative transport via Ca^+ channels. At high dietary Zn levels a passive uptake is dominant. However at environmentally relevant intestinal Zn levels ($50 \mu\text{mol l}^{-1}$) the passive component is unlikely to be of much significance (Bury et al., 2003). The affinity of the

intestinal Zn uptake component in rainbow trout is the highest reported in any organism, with that of the gill being ten-fold higher. Intestinal Zn uptake is described as low affinity and high capacity in comparison to the gill, which is high affinity but low capacity (Glover et al., 2003). Brachial Zn uptake is 240-410 nmol kg⁻¹ h⁻¹; in comparison the gut has a maximal uptake rate of 933 nmol kg⁻¹ h⁻¹ (Bury et al., 2003). Intestinal uptake is consequently of much greater importance to body Zn status (Spry et al., 1988; Glover and Hogstrand, 2002a). Typical freshwater Zn levels are low (<10 µg l⁻¹), consequently freshwater Zn does not contribute significantly to whole body Zn; only high waterborne Zn may contribute sufficient Zn to avoid severe deficiency (Spry et al., 1988).

1.3.2. Zinc excretion

Mammals regulate Zn by adjusting dietary absorption and endogenous excretion, which has contributed to the difficulty in measuring Zn status (King et al., 2000; Salguero et al., 2000). The same is true in fish (Clearwater et al., 2002), where excretion of endogenous Zn via excretions of bile and intestinal sloughing are thought to be the main mechanism of Zn homeostasis (Hardy et al., 1987; Bury et al., 2003). Indeed in trout Zn retention decreases with increasing dietary Zn sulphate (ZnSO₄) (Spry et al., 1988; Apines et al., 2001; Glover and Hogstrand 2002a). The regulation of dietary Zn means that dietary levels of Zn sulphate as high as 1700 mg kg⁻¹ Zn are relatively non-toxic (Wekell et al., 1983). As Zn is actively transported from the circulatory portal to the gills, a brachial excretion as well as faecal excretion of dietary Zn is also thought to occur (Hardy et al., 1987; Maage and Julshamn, 1993). The integument and its associated mucus may also play a role in Zn excretion, as high levels of Zn are found in this tissue (Spry et al., 1988), but this has yet to be evaluated. Only 0.1-1 % dietary Zn is lost via the urine, thus although Zn is found at relatively high levels in the kidney, it does not imply an excretory role for this organ (Hardy et al., 1987).

1.3.3. Zinc regulation and storage

The uptake, distribution, intracellular metabolism and excretion of Zn all contribute to its homeostasis. In trout and other fish, of all metabolic organs, Zn accumulates most rapidly and to the highest level in the intestine (Ogino and Yang, 1978; Hardy et al. 1987; Maage and Julshamn, 1993; Sun and Jeng, 1998). As well as a Zn regulatory organ the intestine is also regarded as a Zn storage organ, as Zn can be mobilised from this tissue during periods of low dietary Zn (Maage and Julshamn, 1993). The intestinal mucus is thought to play a role in Zn regulation by facilitating absorption by holding Zn close to the apical surface during deficiency, and obstructing Zn absorption during exposure to high dietary Zn by increased mucus production (Glover et al., 2003).

The bone tissues, including the vertebrae, effectively accumulate Zn sulphate. As it is proposed that fish may mobilise Zn from the vertebrae during deficiency (4-5 weeks) (Maage and Julshamn, 1993; Do Carmo E Sa et al., 2004), the bone is considered a Zn storage tissue in fish. The assimilation of Zn by the bone is expected to contribute significantly to whole body Zn with increasing dietary Zn (Hardy and Shearer, 1985; Wekell et al., 1986; Maage and Julshamn, 1993; Lorentzen and Maage, 1999); in humans 29 % of body Zn is in the bone (Rink and Gabriel, 2000). In carp, Zn increases in the skeletal tissues before the muscle (Jeng and Sun, 1980). In fish, most Zn is associated with a slowly exchanging pool; at most only 40 % may be involved in a second rapidly exchanging pool (Hogstrand & Wood, 1996).

In contrast to mammals where Zn is stored in the liver as metallothioneins (Salguerio et al., 2000), the consensus with fish is that the liver plays a metabolic rather than a storage role. Hepatic Zn does not change in response to dietary Zn, except at levels higher than 440 mg kg⁻¹ (Ogino and Yang, 1978; Wekell et al., 1986; Overnell et al., 1988; Maage and Julshamn, 1993). Although containing 57 % of body Zn in humans (Rink and Gabriel, 2000), Zn in the

muscle is not modulated significantly by dietary Zn (Wekell et al., 1986, Overnell et al., 1988) in trout or turbot (*Psetta maxima*). The lack of a significant response of the liver to dietary Zn in fish is consistent with the fact that considerable changes in dietary Zn result in little or no change in the transcription of hepatic Zn transporter genes (Feeney et al., 2005). As the ionic charge of Zn makes it water soluble it cannot freely pass through cell membranes, consequently Zn transporter proteins are crucial in regulating Zn concentrations in the cytosol and lumen of organelles. ZIP and ZnT are both families of Zn transporters found in humans (Liuzzi and Cousins, 2004) and their orthologues have also been identified in zebra fish (*Danio rerio*) (Feeney et al., 2005). Zn transporters are also important in the delivery of Zn to enzymes such as ALP (Suzuki et al., 2005).

In rainbow trout 99 % of total plasma Zn is bound to proteins (Bettger, et al., 1987). In mammals 60-70 % of protein bound plasma Zn is loosely bound to albumin and tightly bound to α -2-globulin, and plasma is not regarded to play a role in Zn storage; it is only a Zn carrier containing 0.1 % of total body Zn (Salguerio et al., 2000). This is confirmed in fish, as above deficiency levels plasma Zn does not change over a wide range of either waterborne or dietary Zn (Spry et al., 1988).

1.4. Zinc and its role in health in mammals and fish

1.4.1. Zinc requirements and deficiency in salmonids

Zinc, after Fe, is the second most abundant trace element in the human body (McCall et al., 2000). The normal human body Fe and Zn content is 4-5 g and 1.4-2.3 g, respectively (Eastwood, 2003; Stefanido et al., 2006). In contrast, Zn is the most abundant trace mineral in fish (Schwartz, 1995) with whole body Zn and Fe levels in salmonids being $\sim 30 \text{ mg kg}^{-1}$ and $\sim 8 \text{ mg kg}^{-1}$, respectively (Andersen et al., 1997). As a consequence of its role in a large number of enzymatic proteins, Zn is vital for a wide range of functions essential for the

maintenance of optimal health (Fracker et al., 1986; Vallee and Falchuk, 1993; Berg and Si, 1996; Shankar and Prasad; 1998; Rink and Gabriel, 2000; Salgueiro et al. 2000; Powell 2000; McCall et al., 2000; Grahn et al., 2001; Dreosti, 2001; Stefanidou et al., 2006) (Table 1.3.)

Table 1.3.

Zinc involvement in the organism.

Immune function
Antioxidant status
Enzymatic cofactor
Vitamin A metabolism
Insulin storage and release
Energetic metabolism
Protein synthesis
Stabilization of macromolecules
Regulation of DNA transcription
Cellular division
Eye function

Adapted from: Salgueiro et al. (2000).

The minimum Zn requirement for rainbow trout, determined from the minimum level of dietary Zn required for maximal growth in fish fed a purified egg white diet, is 15-30 mg kg⁻¹ (Ogino and Yang, 1978; NRC, 1993). Severe Zn deficiency in rainbow trout (1-2 mg kg⁻¹ dietary Zn), causes anorexia nervosa, low growth rate, high mortality rate (45 %), cataracts (50 % of fish), and fin erosion (86 % of fish); at 5 mg kg⁻¹, except for an impairment in growth, these aforementioned pathologies do not occur (Table 1.4.) (Ogino and Yang, 1978; Hidalgo et al., 2002).

Table 1.4.

Zn deficiency consequences in fish and mammals.

¹ Growth retardation (Ogino and Yang, 1978).	² DNA damage
¹ Decreased feed efficiency (Watanabe et al., 1980)	² Skin changes
¹ Cataractas (Satoh et al., 1987a)	² Mental lethargy
¹ Fin erosion (Ogino and Yang, 1978)	² Hyperammonaemia
¹ Increased mortality (Ogino and Yang, 1978)	² Taste abnormalities
¹ Short body dwarfism (Satoh et al., 1987a)	² Reproductive complications
¹ Anorexia (Ogino and Yang, 1978)	² Delayed wound healing
¹ Oxidative stress (Hidalgo et al., 2002).	² Recurrent infections
¹ Impaired immunity (Paripatananont and Lovell, 1995)	² Increased abortion risk

Adapted from Salgueiro et al. (2000).

¹ References pertaining to pathologies also found in fish.

² Consequences of Zn deficiency ascertained in mammals but equivalent pathologies yet to be described in fish.

Severely Zn deficient rainbow trout also suffer short body dwarfism, a result of the arrested longitudinal growth of the vertebrae (Satoh et al., 1987a) and decreased feed efficiency (Watanabe et al., 1980; Hidalgo et al., 2002). Reduced feed utilisation is associated with a reduced protein and carbohydrate digestibility; possibly a result of the reduced activity of the Zn dependent digestive enzyme carboxypeptidase (Ogino and Yang, 1978). Many Zn deficiency symptoms are revisable through dietary supplementation, as repletion of Zn improves growth rate and feed efficiency (Hidalgo et al., 2002). Zn deficiency also affects the status of other minerals. Fe and Cu in the liver, whole body, vertebrae and intestine are increased during Zn deficiency (1-5 mg kg⁻¹ Zn) (Ogino and Yang, 1978). During Zn

deficiency, tissue levels of Zn decrease in the vertebrae, intestine, eye and whole body but hepatic Zn is conserved (Ogino and Yang, 1978).

1.4.2. Zinc and the maintenance of oxidative status

Zinc plays an important role in the maintenance of oxidative status (the balance between the production and removal by antioxidants of free radicals) with deficiencies known to cause oxidative stress in mammals (Yousef et al., 2002). Typically the Zn ion does not act directly as an antioxidant but rather exerts its effects indirectly (Powell, 2000). One of the primary cellular antioxidant defence enzymes present in the cytosol is Cu/Zn-SOD, which dismutates $O_2^{\cdot-}$ radicals to H_2O_2 and $2H^+$ (Halliwell and Gutteridge, 1999) (Figure 1.2.). Zn MT has antioxidant properties in humans (Kang, 1999) and acts as a redox active protein due to the association of Zn with cysteine sulphur ligands, which can be oxidised and reduced with concomitant release and binding of the otherwise redox inert Zn (Maret, 2000). Antagonism by the Zn ion may offer site specific radical protection to biological molecules by the displacement of Cu and Fe, which gives protection against Fenton type reactions with H_2O_2 that otherwise oxidise biological molecules through the formation of hydroxyl radicals (OH^{\cdot}) (Powell, 2000). Zn ions may also modulate the redox potential of sulphur by protecting sulphhydryl/thiol groups in functional proteins from oxidation by either or; direct binding to sulphhydryl groups, binding to another nearby protein causing hindrance to oxidation, or causing conformational change by binding to another site on the protein (Powell, 2000; Klotz et al., 2003). In mammals, Zn deficiency impairs the absorption of lipid soluble vitamins, namely vitamins E and A, due to defective formation of chylomicrons (lipoproteins that transport dietary lipids) in the enterocyte, which may indirectly contribute to oxidative stress (Salgueiro et al., 2000).

Zn is also important in the protection of DNA. Zn deficient rats develop an increased frequency of single strand DNA breaks (Castro et al., 1992), which may either be due to increased oxidative damage due to the loss of Cu/Zn-SOD or Zn MT, or the loss of activity of Zn containing DNA-repair enzyme fapy glycosylase, which repairs oxidised guanine (Ames, 2001). Zn MT is effective in reducing radiation induced DNA damage demonstrating its role in the protection of DNA from oxidative damage (Cai and Cherian, 2003). Few studies have assessed the role of Zn in the maintenance of oxidative status in fish. In rainbow trout, dietary Zn below requirements results in the production of malondialdehyde (MDA), a lipid peroxidation product (Kucukaby et al., 2006). In rats, Zn supplementation protects against ethanol induced lipid peroxidation in the liver and kidney (Al-Damegh, 2007). Zn deficiency is also associated with a decrease in two of the three hepatic Cu/Zn-SOD isoenzymes found in trout. A possible compensation by catalase, most likely due to an increased production of hydroxyl radicals may ameliorate the effects of reduced Cu/Zn SOD during Zn deficiency (Hidalgo et al., 2002).



Figure 1.2. The dismutation of superoxide by superoxide dismutase. The spontaneous dismutation of superoxide to hydrogen peroxide and oxygen is accelerated by superoxide dismutase (SOD).

1.4.3. Zinc and immunity

In mammals, Zn deficiency causes reduced disease resistance, poor wound healing (lymphocytopenia) and recurrent infections due to impaired immunity (Fraker et al., 1985; Shankar and Prasad, 1998). Zn is required in non-specific immunity and is involved in polymorphonuclear leukocyte function, natural killer cell activity and complement activity. In specific immunity, Zn modulates numbers and functions of T and B lymphocytes and macrophages (Vallee and Falchuk, 1993; Shankar and Prasad, 1998). Despite its importance,

the effects of mineral deficiency on piscine immunity have not been well determined (Blazer, 1992). Zn has several roles in both the specific and non-specific immune system, the former of which is not as well developed in fish in comparison to mammals. In mammals, Zn deficiency damages epidermal cells and reduces wound healing (Fraker et al., 1985), which may be particularly significant in fish due to the role of the integument as a first line of defence against waterborne pathogens (Shephard, 1994).

There is little consistency in the literature with regard to the effect of Zn on immune function in fish. In catfish, mortality of non-immunised catfish post *Aeromonas hydrophila* challenge is lowest in Zn deficient fish, possibly due to a reduced ability of pathogens to produce virulence factors and replicate in Zn deficient conditions (Scarpa and Gatlin, 1992). In mammals the onset of disease can also decrease Zn status, as inflammations and acute infections can increase Zn excretions and Zn may be redistributed to the liver decreasing the Zn serum pool (Rink and Gabriel, 2000). This has been reported in vaccinated rainbow trout and may be attributed to an innate defence mechanism against bacterial pathogens that require Zn (Simko et al., 1999). Zn deficiency has been associated with nodular gill disease and fungal infestation in rainbow trout (Spry et al, 1998). Supplemental Zn has no effect on both bacterial kidney disease (BKD) in sockeye salmon (*Oncorhynchus nerka*) (Bell et al., 1984) and *Edwardsiella ictaluri* challenge in catfish (Lim et al., 1996). This is however contrary to a study finding enhanced disease resistance to *E. ictaluri* in catfish (Paripatananont and Lovell 1995b). Dietary Zn increases percentage neutrophils and chemotaxic responses to *E. ictaluri* (Lim et al., 1996), but has no effect on neutrophil levels in catfish post *A. hydrophila* challenge (Scarpa and Gatlin., 1992). Dietary Zn has no effect on antibody titres post *A. hydrophila* challenge (Scarpa and Gatlin, 1992) but raises titres post *E. ictaluri* challenge in catfish (Paripatananont and Lovell, 1995b). Supplemented Zn does

not affect percentage lymphocytes, thrombocytes or macrophages and decreases phagocytic activity of macrophages in catfish (Lim et al., 1996).

1.5. Organic and inorganic zinc

1.5.1. Overview of organic and inorganic zinc sources

Of importance to Zn nutrition are the types of Zn sources that may be ingested by fish and indeed used as supplements. Inorganic Zn salts include zinc oxide (ZnO) and Zn sulphate, both of which are principally used in the fortification of feeds as well as in mammalian and piscine mineral research. Although Zn may be present in inorganic forms, particularly in plant food stuffs (Reilly, 2004), in living organisms and feedstuffs of animal origin, very little Zn is free in solution, but is rather organically chelated or complexed by a variety of biological or synthetic compounds collectively known as ligands (Vallee and Falchuk, 1993). In a Zn complex, such as metallothionein and alcohol dehydrogenase, only one of the ligand atoms donates its free electron pair to the Zn ion. In a chelate, two or more atoms of the ligand donate electron pairs to the Zn cation, such as occurs in the active site of the carboxypeptidase enzymes (Swinkels et al., 1994). Several organic sources of Zn have been used for the supplementation of Zn in fish research and include; mixtures of amino acids chelated or complexed with Zn (Zn-AA), Zn methionine (Zn-Met), Zn lysine (Zn-Lys), Zn gluconate, zinc acetate ($\text{Zn}(\text{O}_2\text{CCH}_3)_2$), Zn picolinate, Zn propionate, and Zn proteinates (Zn-pr) (Chesters, 1997 in mammals; Maage et al., 2001 in Atlantic salmon (*Salmo salar*); Apines et al., 2001; Kucukbay et al., 2006 in rainbow trout). Zn-pr are defined as products resulting from the chelation of a soluble salt with amino acids and (or) hydrolysed protein. The term Zn-pr is a loose definition for any product containing Zn chelated or complexed compounds and thus may not produce reproducible results from usage to usage (Ashmead, 1992).

In the GI tract, some organically bound Zn compounds are restricted in their ability to enter into chemical reactions with other dietary compounds. This can dramatically change the chemical and physical characteristics of organic Zn over the 'free' ionic form characteristic of inorganic Zn salts. The formation of chelates and complexes is a reversible process, and a continuous exchange of ligands occurs in intraluminal and intracellular conditions depending on the given conditions. The strength of a Zn – ligand complex/chelate varies between different Zn compounds. In addition the conversion of inorganic Zn to organic Zn compounds during the manufacturing process of Zn-pr varies depending on product quality. Consequently, the type of ligating compounds present and the proportion of chelated Zn to inorganic in Zn-pr will determine the chemical nature of any manufactured Zn chelates/complexes (Cao et al., 2000).

The presence and type of organic ligands in an organic Zn source will greatly influence the efficacy of an organic Zn compound as a nutrient. Whilst chelates and complexes can be used to enhance metal delivery, to be nutritionally effective the chelate must be stable enough to be absorbed intact but also available enough for degradation at sites of metabolic requirement. In the highly acidic environment of the stomach (pH ~2.6-3.0) Zn solubility is increased and consequently the ion may dissociate from some chelates or complexes thus giving them the same properties as inorganic Zn once in the anterior intestine. In the intestine solubility is lost and ions may bind to anions or ligands forming insoluble complexes rendering the Zn ions unavailable for absorption (Cao et al., 2000). Should the chelate remain intact, organic Zn sources may be more digestible than inorganic Zn salts due to protection of the Zn ion from the formation of such insoluble complexes in the gut lumen (O'Dell, 1984; Wedekind et al., 1992). Subsequently chelated Zn may be then transported into the mucosal cells via the uptake mechanisms for amino acids and dipeptides (Ashmead, 1992). In animal nutrition there is a trade off between having an organic Zn source that is so strongly chelated

that it does not dissociate at the site of utilization, and a weakly chelated Zn chelate that dissociates in the acidic environment of the stomach. Consequently, it is of importance to the study of Zn nutrition that chemical variations between Zn sources are measured by differences in animal health and performance rather than merely Zn status alone (Cao et al., 2000) as such parameters will require the effective utilisation of Zn from uptake to incorporation into active metabolic processes.

1.5.2. Organic and inorganic zinc sources in fish nutrition

It is evident that one of the most important factors in Zn bioavailability (availability of a nutrient to the site of its metabolism) is dietary Zn digestibility (the uptake of a nutrient from the GI tract into the circulatory system). Despite the potentially increased efficacy of organic some Zn sources (Ashmead, 1992; Wedekind et al., 1992) few studies have compared the digestibility (uptake from the GI tract) of organic and inorganic Zn sources in fish. The mean concentration range for Zn in commercial salmonid feeds in 2004, 2005 and 2006 were 148 mg kg⁻¹ (range: 96-191 mg kg⁻¹, *n*=40), 122 mg kg⁻¹ (range: 31-254 mg kg⁻¹, *n*=23) and 141 mg kg⁻¹ (range: 68-241 mg kg⁻¹, *n*=49), respectively (Maage et al., 2007) and represents both residual and supplemented Zn. The maximum limit for Zn in animal feed is 200 mg kg⁻¹ (88 % dry matter, regulation (EC) 1831/2003). This limit was reduced in 2003 (commission regulation (EC) 1334/2003) from 250 mg kg⁻¹ based on Zn requirements in farm animals and environmental concerns regarding Zn in excreta.

The bioavailability of different inorganic Zn compounds in a white fishmeal-based diet varies considerably. Based on growth, at 20 mg Zn kg⁻¹ the bioavailability of inorganic dietary Zn compounds decreases in the following order in rainbow trout; Zn sulphate, Zn nitrate, Zn chloride (Sato et al., 1987a). In fish, organic forms of Zn can be more bioavailable than inorganic Zn. However, this depends on the species, diet, and the parameter measured. In

rainbow trout, based on increased ALP, Zn/Cu-SOD expression and Zn retention, Zn-AA is more bioavailable than Zn sulphate, Zn-Met or Zn embedded glass (Apines et al., 2001; Apines-Amar, 2004). Supplementing 40 mg kg⁻¹ Zn to a practical diet, Apines et al. (2003b) found that Zn-AA and Zn sulphate were not significantly different when assessing growth, feed conversion, and plasma or liver Zn. However, activity of alkaline phosphatase (ALP) was significantly higher, and bone deposition of Zn marginally higher for Zn-AA. In catfish, Zn-Met is more effective in preventing deficiency symptoms (Paripatananont and Lovell, 1995a) and maintaining host defence against *E. ictaluri* (Paripatananont and Lovell, 1995b). Also, in catfish Zn-pr is more digestible than Zn sulphate in both purified and soybean meal diets (Paripatananont and Lovell, 1997).

Some studies have however observed little or no difference between Zn sources. The bioavailabilities of Zn sulphate, Zn-Met and glass embedded Zn are not significantly different in rainbow trout (Apines et al., 2001). In catfish, growth, feed conversion, and bone Zn were the same for both Zn sulphate and Zn-Met using dietary Zn levels between 50 and 140 mg kg⁻¹. This study also showed that despite claims from the manufacturer, in practical diets 45 mg kg⁻¹ supplemental dietary Zn from Zn-pr did not increase bone Zn to the same extent as 200 mg kg⁻¹ Zn sulphate (Li and Robinson, 1996). In rainbow trout, Zn-pr has an increased bioavailability over Zn sulphate in diets containing low to medium levels of calcium and phosphate; however, the bioavailability of both Zn-pr and Zn sulphate is comparable in diets containing high levels of calcium and phosphate (Hardy and Shearer, 1985). In Atlantic salmon, Zn gluconate and Zn sulphate supplemented to a practical diet at 50 and 180 mg kg⁻¹ were equally efficient at raising Zn status (Maage et al., 2001). Also, in catfish immune responses and resistance to *E. ictaluri* were not improved by Zn-Met or Zn sulphate (Lim et al., 1996). This may have been a result of using a purified diet where due to a lack of anti-nutritional factors the benefits of organic Zn may not be realized. Based on bone

mineralization, Zn bioavailability was significantly lower for fish given Zn-AA than Zn oxide or Zn sulphate in Tilapia (Do Carmo et Sa, 2005), which suggests there may be differences in Zn utilisation between species.

1.6. Factors affecting zinc utilisation in farmed fish

1.6.1. The disparity in zinc status between farmed and wild fish

Measured Zn levels are higher in wild than cultivated salmonids. Hepatic Zn was found to be 33 mg kg⁻¹ (wet wt.) in wild Atlantic salmon but 26 mg kg⁻¹ (wet wt.) in farmed equivalents (Peppe et al., 1985). In Coho salmon (*Oncorhynchus kisutch*) smolts, carcass Zn in wild fish was 16.8 mg kg⁻¹ (wet wt.) and 11.5 mg kg⁻¹ (wet wt.) in hatchery-reared fish (Felton et al., 1994). The Zn content of wild fish is significantly more variable than that of farmed fish, presumably due to the uniformity of commercial feeds and the feeding schedule of farmed fish (Felton et al., 1994). The decreased Zn content of farmed fish may be due to inadequate levels in feed, growth rates, low digestibility from commercial diets and (or) stress factors, including disease. Studies in mammals show that Zn is drawn to the liver and spleen in response to cytokines released during stress and infection (Thompson, 1991). Indeed corticosteroids are known to decrease plasma Zn by 30-40 % in mammals and this is associated with increased hepatic uptake (Vallee and Falchuk, 1993). Interestingly, Zn retention is decreased in heat stressed quail (Sahin and Kucuck, 2003). Despite the modulation of Zn metabolism by stress in mammals, no studies have assessed Zn physiology in relation to stress in fish; many stressors are associated with intensive aquaculture (Pickering, 1992).

1.6.2. Effect of diet upon zinc utilisation

Several studies using fishmeal have shown that the Zn requirement is higher in practical fishmeal diets than purified diets and this is particularly prevalent in diets based on white fishmeal (Ketola, 1979; Satoh et al., 1987a,b). The reduced bioavailability of Zn in fishmeal-based diets, particularly white fishmeal diets, is due to increased levels of hydroxyapatite, mainly in the form of tri-calcium phosphate from skeletal material (Satoh et al., 1987a,b). Although un-supplemented fishmeal based diets typically contain around Zn 65 mg kg⁻¹, due to reduced Zn bioavailability this can be insufficient to sustain whole body Zn in Atlantic salmon (*Salmo salar*) (Maage and Julsham, 1993; Lorentzen and Maage, 1999). In rainbow trout, supplementation of 40 mg kg⁻¹ inorganic Zn to white fishmeal-based diets was required to obtain normal growth (Satoh et al., 1987a). Plant-based feeds have a lower Zn content than those containing fishmeal (NRC, 1993) and contain phytates, which also reduce the availability of Zn to fish (Satoh, 1987a; Storebakken et al., 2000). The current trend of fishmeal replacement by plant products in salmonid feeds will therefore almost certainly necessitate Zn supplementation. In rainbow trout, a soybean-based diet required 150 mg Zn kg⁻¹ to obtain optimal growth (Satoh, 1987a). Cataracts occur in salmonids fed un-supplemented commercial and white fishmeal diets containing 60 and 65 mg kg⁻¹ Zn, respectively (Ketola, 1979; Watanabe et al., 1980). These studies demonstrate the need for Zn supplementation in fishmeal based diets, particularly those containing white fish meal and increased levels of plant material.

1.6.3. Interactions between zinc and other trace elements

Mineral interactions can occur both pre and post absorption from the GI tract. Minerals may compete for ligands, some of which may form insoluble complexes that cannot be absorbed. A second interaction is for active transport carriers which convey cations from the lumen to

the cytoplasm of intestinal cells; for example, both Cu and Zn share the same carrier protein in the cell membrane. A third type of interaction is the reduction in capacity of cells to either produce metal binding proteins due to interferences by non-essential metals or the activation or inactivation of metal enzymes, such as the prevention of Zn incorporation into the Zn requiring enzyme carboxypeptidase by cobalt (Ashmead 1992).

There is a significant antagonistic relationship between Zn and Fe in fish. In rainbow trout, whole body Fe increases as whole body Zn decreases from 40 to ~ 4 mg kg⁻¹ dietary Zn. Above 40 and up to 170 mg kg⁻¹ Zn, whole body Zn increases at the expense of whole body Fe (Wekell et al, 1986). Dietary inorganic Zn of 500-1000 mg kg⁻¹ reduces hepatic Cu in rainbow trout (Knox et al., 1984). In a study with catfish no such effects were found supplementing Zn up to 200 mg kg⁻¹ (Gatlin et al., 1989), suggesting that the antagonistic effect of Zn on Cu only occurs at high levels of dietary Zn, with only a considerable dietary excess causing an imbalance in mineral distribution (Knox et al., 1984). Zn is reported to have no interactive effects with aluminium (Al), cobalt (Co), potassium (K), phosphorus (P) or strontium (Sr) (Wekell et al., 1986). No interactive effects between Zn and Se occur in salmonids (Julshamn, et al., 1990).

1.7. Selenium chemistry and biochemistry

1.7.1. Selenium compounds and selenium biochemistry

Selenium, atomic number 34 and atomic weight 78.9, belongs to group IV along with sulphur and oxygen. Se can exist in a reduced form as Se²⁻ (selenide) or oxidised as Se⁴⁺ (SeO₃²⁻, selenite) or Se⁶⁺ (SeO₄²⁻, selenate). Similar outer valence shell electronic configurations and atomic sizes make Se and sulphur biochemically alike. At present Se supplementation is not common place in fish feeds and any Se fortification is largely with sodium selenite (Na₂SeO₃). In feedstuffs of animal and plant origin Se is predominantly organically bound,

but in lesser amounts some inorganic forms of Se may also be present (Daniels, 1996). Se is organically bound in biological materials by the substitution of sulphur by Se in the amino acids cysteine and methionine to form selenocysteine (Se-Cys) and selenomethionine (Se-Met), respectively. In addition to individual Se amino acids, organic Se sources include Se-yeasts, which contain predominantly Se-Met (Schrauzer, 2006) but also a multitude of up to 15 known lesser organic Se compounds, including varying amounts of inorganic Se depending on the quality of manufactured yeast (Hymer and Caruso, 2000). Some of the other Se compounds present in Se-yeasts include Se-Cys, Se-methyl-selenocysteine, γ -glutamyl-Se-methyl-selenocysteine and Se-adenosyl-selenohomocysteine, some of which are proposed to be responsible for the anti-cancer properties of Se (Rayman et al., 2008).

Se is of particular biochemical significance as it is a better redox catalyst at physiological pH than sulphur (Brigelius-Flohe, 1999). Se is present as Se-Cys in the active site of at least 25 identified selenoproteins in humans (Kryukov et al, 2003), many of which are involved in redox reactions and form antioxidant enzymes (Birringer et al., 2002). Unlike Se-Met which can not be synthesised, Se-Cys is synthesised via selenide by higher organisms from selenate, selenite or Se-Met (Fig. 1.3.). Se-Cys is incorporated into active selenoproteins via its own TGA codon, and is thus often referred to as the 21st amino acid. Se is the only trace element to be encoded by the genetic code in such a manner (Rayman, 2000). Se can also be non-specifically incorporated into plant and animal proteins via Se-Met, as methionine transfer RNA does not discriminate between Se-Met and its sulphur analogue (Schrauzer, 2003) (Figure 1.3.). Unless non-specifically incorporated, selenoproteins typically contain one Se-Cys residue per polypeptide, an exception being mammalian selenoprotein P, which has 10 Se-Cys residues (Behne and Kyriakopoulos, 2001).

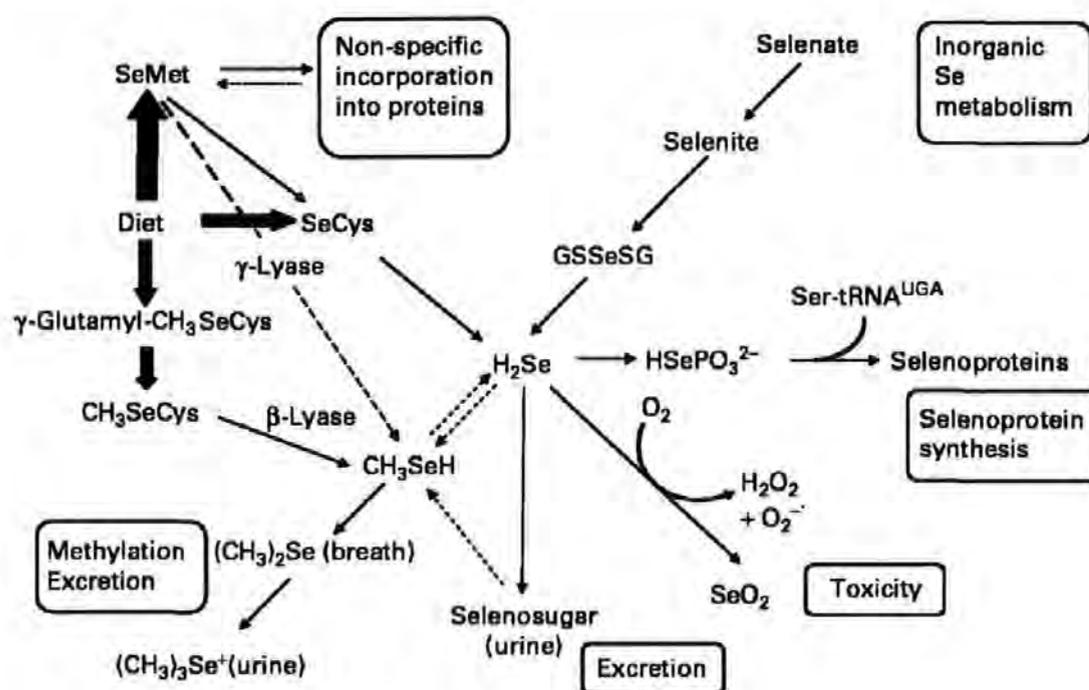


Figure 1.3. Metabolic pathway of dietary Se in humans (Rayman et al., 2008). Se-Met, selenomethionine; Se-Cys, selenocysteine; GSSeSG, selenodiglutathione; γ -glutamyl-CH₃SeCys, γ -glutamyl-Se-methyl-selenocysteine; H₂Se, hydrogen selenide; HSePO₃²⁻, selenophosphate; CH₃SeCys, Se-methyl-selenocysteine; CH₃SeH, methyl selenol; (CH₃)₂Se, dimethyl selenide; SeO₂, selenium dioxide; (CH₃)₃Se⁺, trimethyl selenonium ion.

1.7.2. Selenium containing proteins and their function

25 selenoproteins have been identified in mammals, but 30 homologues have been identified in zebra fish (Kryukov and Gladyshev, 2000). One of the best studied selenoproteins is the enzyme glutathione peroxidase (GSH-Px) (EC 1.11.1.9), which catalyses the reduction of toxic peroxides (Arthur, 2000) (Figure 1.4.). To date there are seven known GSH-Px isoenzymes in mammals and fish, which principally contain Se as Se-Cys; two contain Cys at the active site as opposed to Se-Cys (Toppo et al., 2008). The GSH-Px enzymes are key antioxidant enzymes involved in the primary cellular antioxidant defence along with catalase and SOD (Halliwell and Gutteridge, 1999; Arteel and Sies, 2001) and are named on the basis

of their location and latterly in their order of discovery. Cystolic or classical (cGSH-Px), gastrointestinal (GI-GSH-Px) and plasma (pGSH-Px) are homotetrameric in form and all reduce hydrogen peroxide and alkyl hydroperoxides. Phospholipid hydroperoxide (PHGSH-Px) is unique in that it is a monomer with a hydrophobic surface and is associated with the plasma membrane where it can reduce lipid hydroperoxides in addition to hydrogen peroxide (Brigelius-Flohe, 1999). PHGSH-Px plays a key role in the protection of biological membranes, which is of particular importance in fish due to their high levels of peroxidation-prone polyunsaturated fatty acids (PUFA) (Winston and Di Giulio, 1991). In addition exist two non-selenocysteine GSH-Pxs where the Se-Cys is replaced by cysteine; phospholipid GSH-Px (NPGPx) and a GSH-Px produced in the epididymal fluids. Lastly is a GSH-Px solely found in the epithelium of the olfactory system (Margis et al., 2008), which may be of particular importance to fish, which have an intensive ability to sense minute odours.

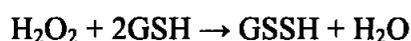


Figure 1.4. Reduction of hydrogen peroxide by glutathione peroxidase. GSH-Px removes H_2O_2 by coupling its reduction to H_2O with the oxidation of reduced glutathione (GSH).

Se is also present in thioredoxin reductase (Trx-R) (EC 1.8.1.9), which provides reducing equivalents for many redox dependent systems (Figure 1.5.), including DNA synthesis and antioxidant defence, including some of the GSH-Px reactions (Ganther, 1999; Arner and Holmgren, 2000).

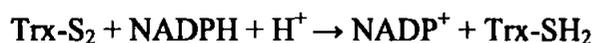


Figure 1.5. The redox reaction of Trx-R and reduced nicotinamide adenine dinucleotide phosphate (NADPH) catalysed by thioredoxin reductase. Trx-R reduces oxidised thioredoxin (Trx-S_2) to reduced thioredoxin (Trx-SH_2).

Iodothyronine deiodinases are also selenoenzymes and are involved in thyroid hormone metabolism catalysing the production of active thyroid hormone (T3) from thyroxine (T4) (Berry and Larsen, 1992). Thought to primarily function as a Se transport protein in mammals, selenoprotein P is present in 60-70 % of total plasma Se. This glycoprotein is also thought to function as an antioxidant and has been shown to have metal binding properties (Saito, et al., 1999; Saito and Takhashi, 2002; Burk and Hill, 2005). Selenoprotein W is typically found in the muscle and may be involved in antioxidant defence. Selenophosphate synthetase (SPS) is an enzyme involved in Se-Cys biosynthesis (Fig. 1.3.). There are also selenoproteins R, N, T, and the 15 and 56 kDa selenoproteins. Selenoprotein J is a recently discovered selenoprotein, which interestingly is unique to fish (Castellano et al., 2005). The number of selenoproteins being discovered is ever increasing, but currently their functions are unknown or at best poorly understood (Bhene and Kyriakopoulos, 2001; Papp et al., 2007).

1.8. Selenium metabolism and regulation in fish

1.8.1. Selenium absorption

Knowledge of Se metabolism in fish is fragmentary and has been dominated by studies involving selenite on rainbow trout. Fish have an additional Se absorption pathway to mammals due to the efficient uptake of inorganic waterborne Se by the gills, which is a function of water Se concentration (Hodson et al., 1980). However, waterborne Se does not contribute significantly to total Se, as it is typically present in very low concentrations ($<0.1 - 0.4 \mu\text{g l}^{-1}$) and drinking of water by trout is low ($0.1 \text{ ml kg}^{-1} \text{ h}^{-1}$) (Hodson et al., 1980; Hilton et al., 1980; Hodson and Hilton, 1983). The uptake and metabolism of Se varies according to the form ingested. Inorganic selenite is rapidly absorbed in the anterior intestine by passive diffusion (Daniels, 1996). Inorganic selenate is absorbed by co-transport with sodium ions,

which unlike selenite is competitively inhibited by sulphate due to its shared absorption route (Jacques, 2001). Although not ascertained in fish, in mammals, intestinal absorption of selenate is more efficient than for selenite; apparent absorption for selenate and selenite is 95 % rather than 62 %, respectively (Daniels, 1996). Se-Met is absorbed in the small intestine via the Na⁺ dependent neutral amino acid transport system, which is competitively inhibited by methionine (Schrauzer, 2003). Apparent absorption of Se-Met in mammals is 95-98 % (Daniels, 1996) and is more efficient than for selenite (Humaloja and Mykkanen, 1986 in chicks); this is also reported in fish (Bell and Cowey, 1989). Se-Cys is thought to be transported by a similar means to Se-Met by the carrier mechanism for basic amino acids (Jacques, 2001). Although Se containing proteins are catabolised in the GI tract, Se in Se-Cys and Se-Met is strongly bound by covalent bonds, consequently Se is not expected to dissociate from Se-Cys or Se-Met in the acidic environment of the stomach and organic Se will remain intact and uptake will be as an amino acid. The metabolism of Se in rainbow trout is dependent on its route of uptake, as dietary Se is transported to the liver by the circulatory portal, whereas waterborne Se is transported to all organs except the liver (Hodson and Hilton, 1983). Dietary absorption of selenite by the small intestine is efficient but decreases with increasing dietary Se (Hilton et al., 1982). This may be due to either reduced uptake or increased elimination of excess Se at high dietary levels (Hilton et al., 1982).

1.8.2. Selenium excretion

The excretion pathway of Se in fish depends on the route of intake. Excretion of Se following aquatic exposure is directly proportional to waterborne Se concentration, suggesting excretion is by passive diffusion (Hodson and Hilton, 1983). Dietary Se is thought to be excreted by the urinary system involving the kidney, the respiratory system via the gills, and a fixed proportion by faecal excretion; either via secretions of bile or intestinal release into

the caecum (Hilton et al., 1980). Faecal Se is not affected by dietary Se level but is influenced by injected Se, which suggests that faecal Se arises from Se released into the intestinal tract rather than unabsorbed Se (Hilton et al., 1982). Unlike the passive excretion of waterborne Se, the excretion of dietary Se increases disproportionately with Se loading suggesting it involves an 'active' energy requiring metabolic process (Hodson and Hilton, 1983). Increased liver concentrations with high dietary and waterborne Se exposures may suggest a limit in the methylation of Se, which is necessary for excretion (Fig 1.3.) (Hilton et al., 1982). Like mammals, Se homeostasis in fish is regulated by excretion rather than absorption (Hilton et al., 1980).

1.8.3. Selenium regulation and storage

The highest concentrations of Se in fish are found in the liver, gill, kidney and intestinal tract, with low Se present in the blood. The plasma is regarded as a Se transport medium rather than a storage organ (Hilton et al., 1982). Although not ascertained in fish, in humans Se is distributed in the plasma between GSH-Px (15 %), Selenoprotein P (52-70 %) and albumin (9-17 %) (Ducros et al., 2000). Se-Met is incorporated into non-active selenoproteins of the skeletal muscle (Bell and Cowey, 1989; Wang and Lovell, 1997; Schrauzer, 2000). As Se-Met can not be synthesised, selenite is not non-specifically incorporated to any appreciable extent in the skeletal muscle of Atlantic salmon (Bell and Cowey, 1989 and Lorentzen et al., 1994) but increases in muscle Se have been reported in catfish (Galtin and Wilson, 1984) suggesting possible differences in Se metabolism between fish species. Similarities in Se metabolism between mammals and fish suggest that two Se pools are also present in fish (Lorentzen et al., 1994). One Se pool is rapidly exchanging and involves the synthesis and metabolism of all forms of Se and principally occurs in the liver. This pool cannot exchange with the slowly exchanging pool, which comprises of Se-Met containing proteins in the

skeletal tissues. The Se-Met pool can however contribute to the rapidly exchanging pool (Daniels, 1996). Rainbow trout, like mammals, have the highest Se concentration in the kidney; however at dietary levels above $0.35 \text{ mg dietary Se kg}^{-1}$ the liver has the highest levels. Consequently, unlike mammals, the liver in fish may play a role in Se storage as well as a metabolic and excretory function (Hilton et al., 1980; Hilton et al., 1982).

1.9. Selenium and its role in health in mammals and fish

1.9.1. Selenium requirements and deficiency in salmonids

The dietary selenium requirement of Se has been determined for rainbow trout based on the minimum dietary selenite level required for maximal plasma GSH-Px activity in a semi-purified diet as 0.35 mg kg^{-1} (Hilton et al., 1980). However, other functional roles of Se may require additional Se than for maximal GSH-Px activity; such as optimal immunity, genetic stability or the activity of other selenoproteins such as Trx-R, the iodothyronine deiodinases and selenoprotein P. Se requirements have been based on sodium selenite and have not been determined for organic Se sources in salmonids, which may be more suitable and efficacious Se sources. Based on growth, the requirement for selenite in catfish was found to be 0.28 mg kg^{-1} , whereas the requirement for Se-Met and Se-yeast were 0.09 and 0.11 mg kg^{-1} , respectively (Wang and Lovell, 1997). The measurement of requirements should include a suite of biochemical markers involving several active Se proteins and should include functional markers such as, effects on genetic stability, antioxidant status and immunological competence. The Se requirement of salmonids may be variable, and may decrease with age (Hilton et al., 1980) and be dependent on previous dietary intake (Lorentzen et al., 1994).

Severe Se deficiency in salmonids (below 0.025 mg kg^{-1} dietary Se), even with adequate vitamin E, causes histopathological lesions, including damage to the axon sheathes of the nerve cord, losses in integrity of the endoplasmic reticulum and mitochondria, increased

erythrocyte fragility, and decreased hepatic vitamin E. Increased ataxia (abnormal swimming behaviour), decreased packed cell volume, and increased plasma pyruvate kinase (an indicator of muscle damage) are also associated with severe Se deficiency (Bell et al., 1986; Bell et al., 1987). Depressed growth and increased mortality is only found in salmonids and catfish below dietary Se levels of 0.06 - 0.017 mg kg⁻¹ (Gatlin and Wilson, 1984; Bell et al., 1987; Wang et al., 1997).

In salmonids, depressed plasma and liver GSH-Px levels are associated with Se deficiency (Poston et al., 1976, Bell et al., 1985 and Bell et al., 1986). Dietary levels of ≤ 0.35 mg kg⁻¹ result in decreased hepatic GSH-Px activity in rainbow trout; above 0.35 mg kg⁻¹ a GSH-Px plateau occurs (Hilton et al., 1980; Bell et al., 1985). However, inexplicably increases in hepatic GSH-Px up to 11.1 mg kg⁻¹ have since been recorded in Coho salmon (Felton et al., 1996). Depressed GSH-Px activity is independent of vitamin E levels in salmonids and no compensatory Se-independent glutathione (GSH) transferase peroxidase activity occurs during deficiency; in mammals GSH can exhibit peroxidase activity during deficiency to compensate for decreased GSH-Px (Bell et al., 1985).

In trout, severe Se deficiency (0.017-0.025 mg kg⁻¹ Se) decreases Se in the blood, liver and kidney in a manner reflecting dietary Se (Bell et al., 1985; Bell et al., 1986; Bell et al., 1987). Cerebral Se levels also decrease as a result of severe Se deficiency, but to a lesser extent than plasma or hepatic Se indicating a possible conservation of Se in this organ (Bell et al., 1987). Haemorrhagic syndrome (Hitra disease) was postulated to be caused by a Vitamin E and Se deficiency (Folstad and Heyeraas, 1985; Poppe et al., 1986), but prophylaxis with these nutrients had no effect. Hitra disease is now known to be caused by the bacterium *Vibrio salmonicida* (Salte et al., 1988). It has been found that as with mammals, Vitamin E and Se act synergistically in salmonids and other fish species. Se deficiencies often manifest to a

lesser extent when adequate vitamin E is supplied, showing a 'sparing' effect of vitamin E during Se deficiency (Poston et al., 1976; Bell et al., 1985; Bell et al., 1987). Mild Se deficiency does not result in histopathological lesions or degeneration of the liver and muscle as found in severe Se deficiency. However, if accompanied with a vitamin E deficiency exudative diatheses will occur (Poston et al., 1976; Hilton et al., 1980; Bell et al., 1985). Supplementation of both vitamin E and Se is required in a purified diet to prevent muscular dystrophy in juvenile salmon (Poston et al., 1976). Waterborne Se is not thought to significantly affect tissue Se content in salmonids (Bell et al., 1985), but may be sufficiently abundant in freshwaters to prevent severe Se deficiency (Hodson and Hilton, 1983). Due to the level of Se in fishmeal, severe Se deficiency is unlikely in fish cultivation, however low Se status may occur and could lead to increased disease susceptibility and sub-optimal health in fish.

1.9.2. Selenium and the maintenance of oxidative status

A large quantity of selenoproteins act as cellular antioxidant enzymes, consequently Se plays an important role in the control of oxidants produced during normal metabolism, stress, exhaustive exercise, and those that arise from exogenous origins. It is well documented that oxidative stress is detrimental to animal health by the loss of cell function due to the peroxidation of membranes and inhibition of damaged enzymes (Hermes-Lima et al., 1995; El-Bayoumay, 2001). Se exhibits a protective effect against lipid peroxidation due to the reduction of hydroperoxides in the cell compartment and membranes by cGSH-Px and membrane bound PHGSH-Px, respectively (Ursini and Bindoli, 1987). Lipid peroxidation measured by the presence of malondialdehyde (MDA) is reduced by supplemented Se and vitamin E in smoke exposed mice (Fiskin et al., 2005). Similarly, supplemented sodium selenite has been shown to protect rat tissues against spontaneous and *tert*-butyl

hydroperoxide (t-BHP) induced lipid peroxidation in rat kidney and liver (Leibovitz et al., 1989). In Se deficient children, MDA products were higher than controls, and selenite supplementation reduced MDA products to control levels (Wilkie et al., 1992). However, Se does not always decrease lipid peroxidation as found in calves given supplemented sodium selenite (Walsh et al., 1993). In mammals, Se has been found to have a sparing effect on vitamin E with regard to lipid peroxidation, again highlighting the synergy between these two nutrients (Ursini and Bindoli, 1987). This is also found in fish; in salmonids, low values for microsomal lipid peroxidation were only found if both vitamin E and Se were supplemented in conjunction (Bell et al., 1985). However, in catfish selenite supplemented up to 0.2 mg kg^{-1} had no effect on pro-oxidant induced lysis of erythrocytes (Wise et al., 1993). This may be due to the low levels of Se used, the use of selenite, or differences in techniques used. There is a lack of studies on the *in vivo* effects of both organic and inorganic Se supplementation on lipid peroxidation in fish.

Organic Se supplementation has been shown to reduce single strand breaks as measured by the alkaline comet assay in mammalian prostate tissue and peripheral lymphocytes. In addition, apoptosis is increased by organic dietary Se, which may eliminate cells with extensive DNA damage (Waters et al., 2003). The effects of dietary Se on genetic stability depend on the source. *In vitro* studies on mammalian cell lines show that increases of selenite induce DNA lesions measured by 8-hydroxydeoxyguanosine adducts. Conversely, Se-Met does not produce DNA lesions (Stewart et al., 1999). The protective effect of organic Se on DNA is supported by the fact that Se deficiency in mice is associated with oxidative stress and decreased genetic stability (Rao et al., 2001). However, in another study selenite, but not Se-Met, reduced DNA adduct formation induced by 3,2'-dimethyl-4-aminobiphenyl in the rat colon (Davis et al., 1999). Se has also been shown to prevent DNA methylation, micronuclei induction, and chromosomal aberrations in mammals (El-Bayoumay, 2001). It is essential to

determine which dietary Se compounds and levels provide optimal protection against genetic damage and may be indicative of nutritional requirements (Fenech, 2002). No studies have yet assessed the effects of dietary Se on DNA stability or genotoxicity in fish.

Despite its role as an antioxidant and its protective effects on DNA, Se can also contribute to oxidative stress and DNA damage, via production of the selenide anion (Se^{2-}) by reaction of selenite with glutathione; this does not occur with Se-Met (Stewart et al., 1999) and highlights the importance of source in Se supplementation. Indeed, Se-Met is thought to act as a cellular antioxidant, as it can reduce peroxynitrites and hydrogen peroxide to produce methionine selenoxide, which can easily be reduced by glutathione back into Se-Met; this does not occur with methionine (Schrauzer, 2003). In lambs supplemented Se-yeast or selenite a lower total plasma antioxidant status was recorded in animals given Se-yeast. This may be a result of non-specifically incorporated Se-Met re-circulating and releasing a continuous supply of selenide within the plasma. The constant supply of selenide means albumin is constantly bound with selenide, thus reducing its antioxidant activity; depending on the measurement, a significant portion of total plasma antioxidant activity is provided by albumin (Boldizarova et al., 2005). Considering both the antioxidant and pro-oxidant properties of Se, very few studies have assessed the ability of different Se sources to raise or indeed decrease antioxidant status in fish.

1.9.3. Selenium and immunity

Se significantly affects all components of the immune system, i.e. non-specific, humoral, and cell mediated (Kiremidjian-Schumacher and Stotzky, 1987; Turner and Finch, 1991; McKenzie et al., 1998; Beck, 1999; Arthur, 2003), but little is known on the role of Se in piscine immunity (Lall, 2000). Se has three major functions in the immune system. Se reduces organic and inorganic peroxides formed by free radical chain reactions, it is involved

in the lipoxygenase and cyclooxygenase pathways of the arachidonic cascade, which produce hydroperoxides that lead to the synthesis of leukotrienes, thromboxanes, prostaglandins and lipoxins and Se modulates superoxide and H_2O_2 produced during the respiratory burst (Spallholz et al., 1990).

In mammals, Se supplementation has been shown to, increase chemotaxis and the random migration of phagocytic cells, modulate phagocytosis, and increase the effectiveness of phagocytic burst due to an increased production of reactive oxygen species (Kiremidjian-Schumacher and Stotzky, 1987). Increases of dietary selenium up to 0.4 mg kg^{-1} decrease mortality from *E. ictaluri* in catfish and potency was increased for Se-Met and Se-yeast over selenite (Wang et al., 1997). However, when challenged with *E. tarda*, no changes in mortality were found in tilapia supplemented $0.2 - 0.5 \text{ mg kg}^{-1}$ Se as selenite (Kim et al., 2003). In Chinook salmon (*Oncorhynchus tshawytscha*), prevalence of *Renibacterium salmoninarum* (bacterial kidney disease) was not affected by selenite and vitamin E supplementation (Thorarinsson et al., 1994). This shows that the effects of Se on immunity in fish may depend on the pathogen, the species and the level and source of supplemented Se.

In catfish, increased dietary Se increases intracellular macrophage superoxide anion production and only organic Se increases macrophage chemotaxis with *Escherichia coli* antigen (Wise et al., 1993). As these increases were found mainly below the Se requirement, they may be due to a factor of Se deficiency, rather than an increase above the norm. Complement activity and lysozyme activity have not been assessed in fish with regards to dietary Se, and it has not been ascertained whether or not phagocytic activity is affected by Se in fish. In mammals, supplemented Se can increase the potency of vaccinations by producing increases in antibody titres (Spallholz et al., 1990); however, a lack of effect was found in cows (Nemec et al., 1990). In catfish, antibody titres were increased after exposure

to *E. ictaluri*, with an increased potency for organic sources of Se (Wang et al., 1997). The effects of dietary Se on fish immunity is in need of further study, with a particular focus on organic Se sources. It would be particularly pertinent to assess the role of Se in fish mucus; an important primary defence mechanism in fish (Shephard, 1994).

1.9.4. Toxicity of dietary selenium

The toxicity of dietary selenium is a particular concern as there is a narrow threshold between nutritional and toxic dietary Se levels. Dietary Se becomes toxic at only 10 times that of normal dietary levels (1-2 mg kg⁻¹); dietary Se supplemented as sodium selenite is toxic at 13 mg kg⁻¹ in salmonids, causing elevated mortality, reduced feeding, slower growth, high feed/gain ratios and liver paleness (Hilton et al., 1982). Hilton and Hodson (1983), supplementing salmonids with similar levels of selenite (11.4 mg kg⁻¹) also reported reduced growth and higher feed/gain ratios, but not at 6.6 mg kg⁻¹. Up to 13.1 mg kg⁻¹ dietary Se has no effect on blood Fe, hematocrit or erythrocyte counts (Hilton et al., 1980). Dietary selenite supplemented above 0.35 mg kg⁻¹ results in Se being stored in the liver, presumably for methylation for detoxification and excretion (Hilton et al., 1982). In Chinook salmon, Se-Met does not exhibit signs of toxicity up to 18 mg kg⁻¹ (Hamilton et al., 1990). A disparity between the toxicity of organic and inorganic Se is also found in terrestrial species, including poultry (Todorovic et al., 2004). Increases of organic dietary selenium in fish feeds may, as in mammals, prove be less toxic than increases of sodium selenite; the most widely used Se source in fish dietary trace element research.

1.10. Dietary selenium in fish feeds

Although not well defined, Se in animal products is largely present as selenoproteins containing Se-Cys and Se-Met (Sunde, 1997; Jacques, 2001; Rayman et al., 2008). Se in Cod (*Gadus morhua*) muscle is predominantly organic containing at least 50 % protein bound Se-

Met (Huerta et al., 2004) and 12 % selenite (Crews et al., 1996). Se levels in fishmeal are high in comparison to plant products, which will result in lower Se levels in fish feeds where fishmeals are partially replaced with plant products (NRC, 1993). The mean Se levels found in commercial salmonid feed in 2004, 2005 and 2006 were 1.3 mg kg^{-1} (range: $0.7\text{-}4.1 \text{ mg kg}^{-1}$, $n=40$), 1.3 mg kg^{-1} (range: $0.18\text{-}3.8 \text{ mg kg}^{-1}$, $n=23$) and 1.2 mg kg^{-1} (range: $0.7\text{-}3.5 \text{ mg kg}^{-1}$, $n=49$) respectively (Maage et al. 2007) and reflects naturally present residual Se. Within the European Union, Se (selenite, selenate, Se-Met and Se-yeast) may not be supplemented in fish feeds that contain above 0.5 mg kg^{-1} Se (88 % dry matter, regulations (EC) 1831/2003, 1750/2006 634/2007). Fishmeals contribute a significant proportion of the Se (Lunde, 1973) in aquafeeds, thus the bioavailability of Se is of crucial importance to cultivated fish.

1.11. Factors affecting selenium utilisation in farmed fish

1.11.1. The disparity in selenium status between farmed and wild fish

Comparing Se levels in the tissues and feeds of wild and farmed fish may be a useful gauge of normal Se status. Se levels in fishmeal are often above requirements and EC limits; despite this Se levels in tissues of farmed fish are considerably lower than that of wild fish. Hepatic Se is five to seven times higher in wild Atlantic salmon than farmed equivalents (Poppe et al., 1985; Julshamn et al., 1990). Felton et al. (1990) also found lower carcass Se in hatchery reared than wild pacific salmon smolts from the same progeny. Wild salmon fillets are also reported to have greater tissue Se than farmed counterparts (Lorentzen et al., 1994). The lower Se content in cultivated fish may be due to; differences in growth rates and feeding regimes (Maage et al., 1991); its depletion by increased utilisation during stress associated with culture conditions; and (or) a reduced level of Se in commercial fish diets than present in the prey of wild fish. The wild crustacean prey of salmonids can contain up to $3\text{-}4.7 \text{ mg kg}^{-1}$ Se (Hodson and Hilton, 1983; Julshamn et al., 1990). Carcass Se is depleted in Chinook

salmon exposed to transportation stress (Halver et al., 2004) and higher dietary Se levels were found to provide greater protection against chemical and physical stresses (Felton et al., 1989). Felton et al. (1996) found that a dietary Se of 8.6 mg kg^{-1} (supplemented as selenite) was required to achieve a whole body Se level similar to wild Coho salmon smolts. Although fish feeds contain Se above requirements, supplementation of dietary Se, namely organic sources, may result in Se levels comparable to wild fish and may ensure Se requirements are met in all conditions including periods of stress.

1.11.2. Dietary selenium digestibility in fish feeds

The utilisation of dietary Se is primarily dependent on its effective uptake. The absorption of Se may be affected by the compounds of Se present in different fish feeds and interactions with other nutrients, e.g. the antagonist effects of copper (Lorentzen et al., 1998). In broiler chicks, the bioavailability of supplemented selenite assessed on the ability to prevent exudative diatheses, was 86 % for corn, 60 % for soybean meal and only 25-16 % for herring and menhaden fishmeals (Cantor, 1975). Overall diet digestibility is not expected to be responsible for low Se bioavailability in fishmeal diets, as animal derived feedstuffs are generally more digestible than plant derived ingredients. Similarly, a low digestibility of Se from fishmeal is reported in Atlantic salmon. Se digestibility in a fishmeal based diet is lower (47 %) in comparison to Se added to a purified diet as selenite (63 %), Se-Cys (52 %) and Se-Met (91 %) (Bell and Cowey, 1989). In catfish, selenite has a lower digestibility than organic Se-proteinate, and this is not affected by the diet used; in this case soybean (selenite 51 %; Se-proteinate 90 %) and egg white based diets (selenite 66 % and Se-proteinate 91 %) (Paripatananont and Lovell, 1997). This shows that both the form of Se and the dietary constituents are important factors in Se digestibility and highlights the need to determine Se digestibility and requirements in practical diets. The cause of low Se bioavailability in

fishmeal and fish products is still unknown. Se from Se-Met enriched fillets is reported to be highly bioavailable (Ornsrud and Lorentzen, 2002), suggesting the low availability of Se from fishmeal is due to tissues other than fish muscle in practical diets. It has been proposed that the low availability of Se from seafood products may be due to an interaction with any mercury present (Hg), as Se and Hg have a high affinity for one another; this has yet to be verified (Rayman et al., 2008). A high proportion of Se in fillets is covalently bound as Se-Met (Huerta et al., 2004) and consequently is not expected to interact with Hg.

1.11.3. Organic and inorganic selenium sources in fish nutrition

Ingested Se is ultimately required in important metabolic processes. However, Se deposited in the tissues may not necessarily be available for utilisation in active proteins. Se-Met effectively increases apparent Se status as it is non-specifically incorporated into proteins, but it must be catabolised into an inorganic precursor before it can be incorporated into catalytic Se enzymes as Se-Cys (Rayman, 2000). Organic Se, especially Se-Met, is reported to be more bioavailable than inorganic Se in both mammals and fish. Comparisons of the bioavailabilities of different Se sources in fish has largely been assessed in salmonids and catfish by determining apparent net Se digestibility, GSH-Px activity, tissue Se, growth, and responses to bacterial challenge (Wang et al., 1997; Bell et al., 1989; Lorentzen et al., 1994; Cotter et al., 2008). Organic Se sources are often found to be more bioavailable, but studies in fish come to different conclusions, and this may largely be an artefact of the methods used and parameters measured between different studies. If GSH-Px activity is measured, organic Se is not always found to be any more bioavailable than selenite. In salmonids the dietary source of Se affects GSH-Px/plasma Se ratios, which are lower for Se-Met and Se from fishmeal diets than selenite or Se-Cys (Bell and Cowey, 1989). However, rather than being interpreted as an increased bioavailability of selenite this is due to an increased plasma Se by

Se-Met; GSH-Px activity was similar between treatments due to the normal plateau in its activity above 0.35 mg kg^{-1} dietary Se. In fish, Se bioavailability studies at sub-optimal levels seem to show differences in growth and GSH-Px activity, but not at higher levels. Se-Met may affect metabolic pathways other than that of GSH-Px and may be why the outcome of bioavailability studies depend on the parameters measured. In broiler chicks, Se-Met is four times more efficacious in the prevention of pancreatic atrophy than selenite or Se-Cys, but the latter are more effective at preventing pancreatic diathesis (Cantor et al., 1975).

1.11.4. Interactions between selenium and other trace elements

Dietary Se has been found to have no effect on Zn, Cu or Fe, as plasma levels of these nutrients between low (0.6 mg kg^{-1}) or high (2.6 mg kg^{-1}) dietary Se do not change (Julshamn et al., 1990). However, high supplemental dietary Cu was found to reduce hepatic Se in salmon (Lorentzen et al., 1998). Hilton and Hodson (1983) however, found a synergistic relationship between liver Cu and Se in salmon; increasing dietary Se from 0.6 to 11.4 mg kg^{-1} increased hepatic Cu. The interaction between dietary Se and Cu, and effects of Se source needs to be clarified in salmonids.

1.12. Conclusions and study objectives

Zinc and Se have pivotal roles to play in the nutrition and health of farmed fish. Both elements have key functions in immunity, the maintenance of oxidative status, and in optimal growth and feed utilisation. Research of both Se and Zn in fish has largely been limited to the assessment of the status and nutritional physiology of these elements, with few studies pertaining to the functions and requirements of these nutrients for optimal health. In addition, established requirements have only been determined with inorganic sources, namely sodium selenite and Zn sulphate. However, there is an increasing move towards the use of organic sources in mammalian research, particularly with regards to Se. Organic trace element

sources are chemically more akin to naturally occurring trace element compounds, are often reputed to be more bioavailable and typically exhibit larger thresholds between toxicity and essentiality. Despite the advantages emerging in terrestrial animal research, the understanding and use of organic minerals in fish nutrition is very limited, particularly with regard to health. Increasingly it is realized that both Se and Zn may be pivotal in the maintenance of optimal health during the onset of physiological stress, which is particularly prevalent in the intensive cultivation of fish, as fish are often subjected to various environmental and husbandry related stressors.

This study was designed to establish the suitability of organic Se and Zn as effective dietary supplements for cultivated fish. There are a limited number of organic Se and Zn producers worldwide and the quality of manufactured products varies. As the efficacies of organic minerals are wholly dependent on the presence of key biological compounds, this project assessed those produced by a world leader whose products have undergone the scrutiny of through scientific research. The metabolism and utilisation of organic minerals are increasingly realised to be fundamentally different to traditionally used inorganic supplements. As the mechanisms of trace element utilisation are poorly understood, this study made a thorough assessment of the benefits of organic versus inorganic sources by the use of general health markers relevant to the nutritional physiology of the trace elements in question. Although actual requirements were not determined, the project assessed how current fish cultivation practices, which involve the use of practical diets and exposure to numerous stressors, affect Se and Zn utilisation. The findings ascertained the utilisation of novel organic minerals and verified use their use as additives in diets for cultivated salmonids. The research is expected to be of relevance to the legislation surrounding the use of trace element supplements and the formulation of commercial fish diets. Although trout were used as a model species, findings of the study are relevant to all cultivated fish species

and build on the understanding of Se and Zn nutrition in all animal life. In view of the current limitations in the understanding of Zn and Se nutrition in fish the project has several key aims:

1. Determine the bioavailability of organic Se-yeast and Zn-proteinates in comparison to traditionally used inorganic sources (sodium selenite and Zn sulphate) in diets relevant to current salmonid cultivation practices by the assessment of element digestibility, tissue levels and specific enzyme activities.
2. Compare the efficacy of Zn-proteinates in comparison to Zn sulphate for optimal health and Zn status through the assessment of oxidative status, immuno-competence, interactions with other trace elements, and growth and feed utilisation.
3. Undertake preliminary investigations into the effect of chronic husbandry stressors on markers of immuno-competence and oxidative status.
4. Contrast the beneficial and detrimental effects of supra-nutritional Se-yeast and selenite supplementation on fish health and selenium status using commercial salmonid diets. In addition, the implications of chronic husbandry stressors on Se utilisation will be determined. Se status, oxidative status, immuno-competence, interactions with other trace elements, growth, and feed utilisation will all be evaluated.

CHAPTER 2.

GENERAL ANALYTICAL METHODS

2.1. Overview

The following analytical procedures were fundamental to the experimental analyses undertaken within the present study. Other methods unique to particular trials (including diet formulation) are described in the relevant experimental chapters. Unless otherwise stated, all materials, chemicals and reagents were sourced from Fisher Scientific Ltd. (Loughborough, Leicestershire, UK) and Sigma-Aldrich Ltd. (Poole, Dorset, UK). All experimental work involving fish was carried out under the Home Office project licence # 30/2135 and personal license # 30/7809.

2.2. Rearing facilities and maintenance of water quality

All experimental trials were conducted within a freshwater recirculation system (RS), number 'F', at the University of Plymouth's Aquaculture and Fish Nutrition Research Aquarium (see plate 2.1.). In order to effectively function and provide a suitable environment for the rearing of fish, water quality parameters in a RS must be effectively managed to remain within optimal ranges for the rearing of trout.



Plate 2.1. Annotated collage of recirculation system 'F' located at the University of Plymouth. Outgoing (A) and incoming (B) water to and from carbon filter and chiller (not shown) respectively. Sump and filtered water intake for experimental tanks (C). Drum filter (D). Waste water pipe (E) with arrow showing flow from experimental tanks to drum filter (complete pipe not visible under experimental tanks). Arrows show the direction of incoming water flow from pipes marked A and B to experimental tanks, and the flow of sump water from the drum filter through the biological filter (not visible) to the water intake (C).

The facility used was a closed freshwater RS with a total volume of ~ 6000 l. Twenty experimental 130 l fibreglass tanks each received water at a rate of ~ 800 l h^{-1} . An automated 12 h dark and light regime was maintained throughout all experimental trials. The system received a constant 22 l h^{-1} flow of municipal water to maintain losses from evaporation and alleviate any potential build up of nitrates and trace elements. An activated carbon filter (Commandomatic TCF, Waterco Ltd., Sittingbourne, Kent, UK) removed chlorine and

organic compounds from the incoming water. As a by product of the breakdown of proteins, fish excrete ammonia gas (NH_3) via the gills, which is highly toxic to fish. Ammonium ions (NH_4^+) are relatively non-toxic to fish and are also always present in amounts relative to water pH. Ammonia is removed from re-circulated water by a submerged biological filter of nitrifying bacteria. The biological oxidation of ammonia to relatively harmless nitrate (NO_3^-) is a two stage process; ammonia is firstly oxidised to less toxic nitrite (NO_2^-) by the *Nitrosomonas* species, which is subsequently converted to nitrate by the *Nitrobacter* species. Nitrogenous compounds were monitored on a weekly basis using a Hach Lange DR 2800 and cuvettes for ammonia (Lange LCK 304), nitrite (Lange LCK 341) and nitrate (Lange 340) (Hach Lange Ltd., Salford, UK). The following levels of nitrogenous compounds were considered acceptable; ammonia (un-ionized) $< 0.1 \text{ mg l}^{-1}$, nitrite $< 1.0 \text{ mg l}^{-1}$ and nitrate $< 50 \text{ mg l}^{-1}$. If required, nitrogenous compounds were controlled by a partial water exchange.

Particulate material arising from faecal material, undigested feed and sloughed bacteria from the bio-filter was constantly mechanically removed by a rotating drum screen filter (Aquasonic DF100, Aquasonic Ltd, Wauchop, Australia). The saturation of dissolved oxygen needs to be maintained above 60 % for both fish and bio-filter (Masser et al., 1999) and was maintained at $> 80 \%$ by a side supply of compressed air (compressor: Rietschle, UK) delivered via air-stones and a perforated pipe to each tank and sump water respectively. Temperature was maintained at $15.1 \pm 1 \text{ }^\circ\text{C}$ with a thermostatically controlled chiller (Optipac R407C, PSA, Saint Barthelemy, D'Anjou, France). Typically there is a natural decrease of pH in a RS, as nitrification both consumes OH^- ions and produces acids, and carbon dioxide produced by fish forms carbonic acid (H_2CO_3) in the aqueous environment. The pH of recirculating water was maintained between 6.5-8 by use of the alkaline buffer sodium bicarbonate (NaHCO_3) as required. Oxygen saturation, pH, and temperature were monitored daily with an electronic meter (Hach HQ4d).

2.3. Experimental fish

Juvenile female rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) obtained from Hatchlands Fisheries (Greysheet Lane, Rattery, South Brent, Devon, UK) were used for all experimental trials. Fish were transported directly from the fishery to the aquarium facility in a 1000 l tank supplied with pure oxygen (BOC, UK); transport time was ~30 min. Fish were gradually acclimated to the temperature of the aquarium facility over a period of one hour. All fish were checked on arrival, monitored daily and fed *ad libitum* a commercial trout diet until grading (under anaesthetic). A period of at least three weeks was allowed until fish were randomly allocated into experimental tanks prior to experimental trials. Any anaesthetisation of fish was carried out ethically within Home Office procedures with tricane methanesulfonate (MS-222) (Pharmaq Ltd. Fordingbridge, Hampshire, UK) at a dose rate of 0.06 g l⁻¹ and buffered with sodium bicarbonate.

2.4. Feed and weighing

All fish in each experimental tank were weighed at $t = 0$ and fed relative to % biomass per day in two equal rations twice daily at ~09:00 and ~18:00 hours. Throughout trials fish were re-weighed every two weeks and within this period feed input adjusted daily based on a predicted feed conversion ratio (FCR) of 1. Feed input was adjusted accordingly in the event of any mortality.

2.5. Growth and feed performance

Growth performance and feed utilisation was assessed by specific growth rate (SGR) and FCR. Calculations were as follows:

$$SGR = ((\ln \text{ final wt. (g)} - \ln \text{ initial wt. (g)}) / (\text{Days fed})) \times 100$$

$$FCR = \text{feed intake (g)} / \text{live weight gain (g)}$$

2.6. Proximate analysis of diets and carcass

Diets and carcass (for moisture only) were subjected to analysis for the determination of moisture, ash, lipid, and gross energy. All diets were ground by use of a house hold blender and analysed on a wet weight basis. Analysis was conducted in duplicate according to protocols of the AOAC (2007) as described in the following sub sections.

2.6.1. Moisture

Diets (in duplicate) and carcasses (whole with peritoneal cavity opened and viscera exposed) were weighed and dried at 105 °C with a fan assisted oven (Genlab Ltd., UK) until a constant weight was achieved. Percentage moisture was determined by:

$$\text{Moisture (\%)} = ((\text{wet wt. (g)} - \text{dry wt. (g)}) / (\text{wet wt. (g)})) \times 100$$

2.6.2. Ash

Ash (total mineral or inorganic content) content was determined in duplicate by adding a known sample weight (~500 mg) to a pre-weighed ceramic crucible. The crucibles and samples were then incinerated in a muffle furnace (Carbolite, Sheffield, UK) at 550 °C for 12 hours. After cooling in a dehumidification chamber, percentage ash was determined from the sample residue by:

$$\text{Ash (\%)} = ((\text{Sample residue (g)} - \text{crucible wt. (g)}) / (\text{initial sample wt. (g)})) \times 100$$

2.6.3. Lipid

Lipid content was determined in duplicate using the Soxhlet extraction method. Diets were weighed (~3 g) and placed into a cellulose thimble lightly plugged with cotton wool and inserted into the condensers (raised into the 'rinsing' position) of a SoxTec™ extraction system (Tecator Systems, Högnäs, Sweden; model 1043 and service unit 1046). Pre-weighed

cups containing 40 ml of petroleum ether were clamped into the condensers and extraction levers moved to the 'boiling' position for 30 min, after which extraction levers were set to the 'rinsing' position for 45 min. The cups containing extracted lipid were then transferred to a fume cupboard, cooled for 30 min and weighed. Total lipid content was determined as:

$$\text{Total lipid (\%)} = ((\text{cup (incl. lipid) wt. (g)} - \text{cup wt. (g)}) / (\text{initial sample wt. (g)})) \times 100$$

2.6.4. Crude protein

Determination of total crude protein in diets and feed ingredients was achieved in duplicate by the Kjeldhal method, which measures protein from the total nitrogen content of samples. Total nitrogen is multiplied by a factor of 6.26 (5.72 for proteins of plant origin) to calculate apparent protein content. In brief, ~100 mg of sample was weighed directly into a micro Kjeldahl tube along with one catalyst tablet (3g K₂SO₄, 105 mg CuSO₄ and 105 mg TiO₂; BDH Ltd., Poole, UK) and 10 ml concentrated sulphuric acid (H₂SO₄) (Sp. Gr. 1.84, BDH Ltd. Poole, UK). Digestion was performed with a Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) with the following schedule; 100 °C for 30 min, 225 °C for 45 min (1 h if samples had particularly high lipid content) and 380 °C for 1 h. Once digestion was complete and following a cooling period, the samples were distilled using a Vodapest 40 automatic distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany), the distillate was neutralised with concentrated H₂SO₄ and from the titration value crude protein determined using:

$$\text{Crude protein (\%)} = (((ST - BT) \times 0.10 \times 14 \times 6.25) / SW) \times 100$$

2.6.5. Gross energy

Gross energy was determined in duplicate in MJ kg⁻¹ with a Parr Adiabatic Bomb Calorimeter model 1356 (Parr Instrument Company, IL, USA). Briefly, ground feed was compressed into a 1 ± 0.5 g pellet and weighed. The pellet was then loaded into a nickel crucible with a 10 cm length of fuse wire, which was formed into a 'U' shape to touch the pellet. The 2 kg water used by the instrument to determine released heat energy was exactly weighed to 2 kg prior to loading the bomb. The crucible was then loaded and sample weight keyed into the calorimeter for determination of MJ gross energy per kg as calculated by the instrument.

2.7. Trace element analysis

2.7.1. Sample preparation and digestion

All trace element analysis was carried out in triplicate nitric acid digested samples unless otherwise stated. For the analysis of individual tissues, samples were freeze dried (Super Modulyo freeze-drier; Girovac, UK) and percentage moisture determined from initial wet weight and final dry weight. For the analysis of diets and whole body, samples were dried and percentage moisture determined (see: 2.6.1. *Moisture*). Dietary ingredients were analysed by wet weight. All dried samples were homogenised using a household blender prior to digestion. For the analysis of all elements except Se ~100 mg sample was weighed directly into each micro Kjeheldahl tube and digested in 7 ml nitric acid (70 % ANALAR grade). As the Se levels of some tissues and diets are close to the limits of detection, for Se the amount of sample digested was increased to a maximum of ~450 mg in 7 ml nitric acid (except for faecal material). Samples were digested using a Gerhardt Kjeldatherm 40 tube digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) using the following schedule; 60 °C for 1 h, 100 °C for 1 h, 120 °C for 30 min, 135 °C for 2 h (an additional hour was given

for samples containing a high lipid content). After a cooling period of 30 min, 2 ml 30 % (w/w) hydrogen peroxide was added and samples further digested at 135 °C for 1 h. Samples were then transferred into pre weighed 50 ml polypropylene vials (25 ml for Se) and diluted to ~ 50 ml (~25 ml for Se analysis) with ultra pure Milli-Q water (Millipore Corp., MA, USA) and total weight determined. To ensure 100 % recovery of all elements the certified reference materials, DORM-2 (dogfish muscle) or TORT-2 (lobster hepatopancreas) (National Research Council, Canada), were included in all analyses confirming 100 % recovery for both elements, ± 10 % for Se and 5 % for Zn. Duplicate blanks comprising of only nitric acid and hydrogen peroxide were also analysed in each digestion.

2.7.2. Trace element analysis of digests

Unless otherwise stated, Zn was analysed by Flame Atomic Absorption (Flame AA) spectrometry (Varian SpectrAA 50B, Varian Inc. CA, USA) using a 213.9 nm wavelength. Cu, Fe, Mn, Yttrium (Y) and Zn were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian 725-ES OES spectrometer, Varian Inc. CA, USA) using the following wavelengths for each element (nm); Cu 324.75, Ir 224.268, Fe 238.204, Mn 257.610, Zn 213.857 and Y 360.074. For samples containing Y, a 1 ppm internal iridium (Ir) standard was added to all sample digests; for samples not containing Y a 1 ppm internal Y standard was used. Unless otherwise stated, Se (isotope 82) was analysed by high resolution inductively coupled plasma mass spectrometry (ICP-MS) (VG Axiom sector field ICP-MS, VG Elemental, Winsford, UK.) using a 10 ppb indium standard (isotope 115) and four external standard additions. Se⁸² was chosen to avoid the major argon dimer (Ar₂) interferences associated with the other Se isotopes (Featherstone et al., 2004). Polyatomic interferences and a poor signal response of Se (a result of its high first ionisation potential) make Se analysis by ICP-MS difficult. Se isotopes are subject to varying degrees of spectral

interference by polyatomic ions, notably those involving argon (Sieniawska et al., 1999). In consideration of the matrix effect inherent in the analysis of Se in biological sample digests, standard additions (2.5, 5, 10 and 15 ppb Se) were used. Concentrations of each element in digests were determined in parts per million (ppm) (also mg kg^{-1}) against an external standard calibration and concentrations in original samples calculated using the following calculation:

$$\text{Sample conc. (ppm)} = ((\text{digest wt. (g)} / (\text{sample wt. (g)})) \times \text{digest conc. (ppm)})$$

2.8. Trace element digestibility and retention

For the determination of net apparent trace element digestibility, the inert marker yttrium oxide was added to diets at 1 g kg^{-1} yttrium oxide as recommended by Ward et al. (2005). Under anaesthetic, faecal material was stripped from every fish in each experimental tank by hand. Pressure was gently applied on the hind portion of the peritoneal cavity. Faecal material was pooled for each replicate tank, dried at $105 \text{ }^\circ\text{C}$ for 24 h, and homogenised prior to trace element analysis. Percentage apparent digestibility was determined from the faecal and dietary concentrations of the element in concern relative to reciprocal concentrations of Y using the following equation (Paripatananont and Lovell, 1997):

Net apparent digestibility (%)

$$= 100 \times (1 - ([^Y \text{ diet}] \times [^{\text{Element}} \text{ faeces}] / ([^Y \text{ faeces}] \times [^{\text{Element}} \text{ diet}]))$$

Trace element retention was determined from dietary and whole body element concentration relative to feed and element intake concentration by the following equation:

Element retention (%)

$$= ((\text{final body wt.} \times [\text{final body element}] - \text{initial body wt.} \times [\text{initial body element}]) /$$

$$(\text{Feed consumed} \times [\text{dietary element}])) \times 100$$

2.9. Mineral premixes

Unless otherwise stated, both organic and inorganic minerals were added via premixes based on cornstarch. In Zn and Se variable premixes inorganic Zn (zinc sulphate heptahydrate, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) and Se (sodium selenite, Na_2SeO_3) (both sourced from Sigma, Poole, Dorset, UK) were added 'wet' by being dissolved in a sufficient quantity of lukewarm water. The mineral solutions were then added to a quantity of cornstarch (less than the final required weight), thoroughly mixed, and dried at 40 °C in a fan assisted oven for 18 h (Labmark-VSL, Boro Labs Ltd, Aldermaston, UK). The dried cornstarch and mineral mixture was then re-powdered by use of a household blender (taking steps to ensure minimal losses), sieved, any lumps powdered, and then transferred to a polythene 'zip tie' bag. Cornstarch was added to the required final weight before the bag was air inflated and the mixture thoroughly mixed by shaking. This method was also used in the preparation of a Zn and Se free premix, which supplied the following inorganic minerals in mg kg^{-1} to each diet: Fe, 34 ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$); Cu, 5 ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$); I, 6 (KI), Mn, 15 ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$) as similarly used by Anderson et al. (1997) and Lorentzen and Maage (1999). All inorganic minerals were sourced from Sigma, Poole, Dorset, UK. In Zn and Se variable premixes organic Se (Selplex[®]) and Zn (Zn-Bioplex[®]) sources (sourced from Alltech Ltd, Stamford, Lincolnshire, UK) were added dry at the expense of cornstarch to the required concentration and thoroughly mixed in a 'zip tie' bag.

2.10. Determination of selenium and zinc dependent enzymes

Activities of all Se and Zn dependent enzymes were assayed in duplicate and normalised to sample protein concentration or volume plasma in the case of plasma alkaline phosphatase (ALP).

2.10.1. Sample protein

Sample protein was determined by the spectrophotometric bicinchoninic acid (BCA) method as described by Smith et al. (1985) (Kit: Sigma; BCA1-1KT). The assay measures protein by use of a copper/protein complex that forms relative to the amount of protein present. Prior to analysis tissue homogenates were diluted 1:10 with distilled water. Briefly, 1 part reagent A (copper (II) sulphate) was mixed with 50 parts reagent B (BCA solution). In a clear 96 well microplate, 25 μl of sample was added to 200 μl reagent mixture. The plate was then incubated for 30 min at 37 °C (Heraeus, Kendo Laboratory Products, Hanau, Germany) before being immediately read at 550 nm (Optimax Tuneable Microplate Reader, Molecular Devices, CA, USA). Sample protein was determined in mg ml^{-1} against a standard curve of bovine serum albumin (BSA).

$$\text{Sample protein (mg ml}^{-1}\text{)} = ((A_{550} \text{ change over 30 min}) \times (\text{slope of absorbance vs. mg protein}) - \text{intercept of absorbance vs. mg protein}) \times \text{dilution factor}$$

2.10.2. Assay of glutathione peroxidase

Hepatic glutathione peroxidase (GSH-Px) was measured according to Bell et al. (1986). Briefly, liver samples were homogenised 1 in 9 volumes 100 mM Tris-HCl buffer (pH 7.4) with, 2.5 mM EDTA, 0.01 % Triton-X100 and 2.5 mM sodium azide. A 100 μl homogenate was added to the reaction mixture, which contained 0.96 mM reduced glutathione (GSH), 0.96 units ml^{-1} glutathione reductase and 0.19 mM NADPH. The rate of non-GSH-Px NADPH oxidation in samples was determined at 340 nm (V-530 UV/VIS spectrometer, Jasco, Easton, MD, USA) for five min and subtracted from the rate of GSP-Px NADPH oxidation by the addition of 0.25 mM H_2O_2 . To account for the spontaneous non-enzymatic reaction between GSH and H_2O_2 at pH 7.6 a blank with extraction buffer instead of sample was included and subtracted from total activity. The typical rate of this blank was 18.2-21.7

nmol NADPH oxidised min^{-1} . The family of glutathione-S-transferases can also have GSH-Px activity, but only with organic hydroperoxides and not hydrogen peroxide (Arthur, 2000). Although fish do not appreciably exhibit non-Se dependent GSH activity (Bell et al., 1986), Se dependent GSH-Px activity was measured with H_2O_2 unless otherwise stated. GSH-Px activity was calculated as nmol NADPH oxidised min^{-1} mg protein^{-1} .

$$\text{GSH-Px activity (nmol NADPH oxidised min}^{-1} \text{ mg protein}^{-1}) =$$

$$(V \times (\text{net } A_{340} \text{ sample change} - A_{340} \text{ blank change}) / (e \times d \times v)) / \text{mg protein}$$

Where: V = total assay volume (1.35 ml) in sample assay; e = extinction coefficient of substrate (NADPH, $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ at 340 nm); d = light path of cuvette; v = volume of sample or blank

2.10.3. Assay of thioredoxin reductase

The assay of hepatic thioredoxin reductase (Trx-R) developed by Hill et al. (1997) for determination of Trx-R in rat liver was modified for measurement in fish. The concentration of oxidised Trx was increased from $5 \mu\text{M}$ to $16 \mu\text{M}$, incubation time was increased from 10 min to 45 min and temperature was decreased from 37°C to 25°C . A positive Trx-R control was included in each assay. Liver samples were homogenised 1 in 9 volumes and dialyzed in 0.01 M phosphate buffer saline (PBS) (2.7 mM KCl and 0.137 M NaCl) containing 2 mM EDTA (pH 7.4). Dialysis was performed over 26 h to remove endogenous GSH using 12-14000 Daltons Visking tubing. The assay mixture contained 0.104 units oxidised thioredoxin, 0.38 mg insulin and 0.12 mg NADPH in $160 \mu\text{l}$ of 1 M HEPES with 10 mM EDTA (pH 7.6). The reaction was started by adding $20 \mu\text{l}$ sample and incubated for 45 min at 25°C . $750 \mu\text{l}$ of 0.4 mg ml^{-1} DTNB in 6 M guanidine hydrochloride was added to stop the insulin reaction and the absorbance was read at 412 nm. Reciprocal assays were run with

thioredoxin replaced by water to determine the activity of non Trx-R dependent reduction, which was subtracted from the Trx-R dependent reaction and activity given in A_{412} units $\times 1000 / \text{min mg protein}^{-1}$ (Holmgren and Bjornstedt, 1995). As there are two redox centres in Trx-R, insulin is used as a substrate so only activity from the Se-Cys residue is measured (Ganther, 1999).

$$\text{Trx-R activity } (A_{412} \cdot 1000 \text{ min}^{-1} \text{ mg protein}^{-1}) =$$

$$\text{net non-Trx-R } A_{412} - \text{net Trx-R } A_{412} / 45 \text{ min} \times \text{mg protein}$$

2.10.4. Assay of carboxypeptidase B

Carboxypeptidase B (CPB) was measured in the whole pyloric caeca according to the method of Ramseyer et al. (1999). Whole pyloric caeca samples were homogenised 1 in 9 volumes PBS buffer. A 25 μl sample was added to 2.975 ml assay cocktail containing 25 mM Tris-HCl (pH 7.65) with 100 mM NaCl and 1.0 mM Hippuryl-L-Arginine. Reaction rate at 25 °C was followed over 5 min at 254 nm (V-530 UV/VIS spectrometer, Jasco, Easton, MD, USA). Units CPB were calculated using a molar extinction coefficient of hippuric acid of 0.36 (Folk et al., 1960).

$$\text{Units/ml enzyme} =$$

$$(A_{254} \text{ nm/min sample} - A_{254} \text{ nm/min blank} \times 3 \times \text{df}) / (0.36 \times 0.1)$$

Where:

3 = total volume in assay in ml; df = dilution factor; 0.36 = Millimolar extinction coefficient of hippuric acid at 254 nm; 0.1 = volume (ml) of enzyme used.

Values were normalised to sample protein by:

$$\text{Units mg protein}^{-1} = \text{units/ml enzyme} / \text{mg protein/ml enzyme}.$$

One unit will hydrolyse 1.0 mole of hippuryl-L-arginine per minute at pH 7.65 at 25 °C.

2.10.5. Assay of alkaline phosphatase

Alkaline Phosphatase (ALP) was determined on intestinal tissue homogenates and plasma using a method based on that of Walter and Schutt (1974). Tissues were homogenised 1 in 9 volumes PBS buffer. 50 μ l of homogenate was added to 2 ml 97.5 mM diethanolamine substrate solution (pH 9.8) with 0.5 mM MgCl and 1.21 mM *p*-nitrophenylphosphate (*pnpp*) and incubated at 25 °C for 30 min. The reaction was terminated with 10 ml 0.05 M NaOH and absorbance read at 405 nm (Jenway 6300 spectrophotometer, Patterson Scientific, Luton, UK.). For each sample the background activity of a blank (sample added after the 10 ml 0.05 M NaOH) was subtracted. For tissue ALP activity was normalised to sample protein. For plasma ALP units ALP l⁻¹ were calculated (see below) as by Walter and Schutt (1974).

$$\text{Units ALP per litre} = (\text{Absorbance} \times \text{total reaction vol.} \times 1000\text{ml}) /$$

$$(\text{Reaction time} \times \text{extinction coefficient} \times \text{sample vol.})$$

The extinction coefficient of 4-nitrophenol in alkaline solution is 18.5 $\mu\text{M}^{-1} \text{cm}^{-1}$ at 405 nm.

2.10.6. Assay of copper/zinc superoxide dismutase

Hepatic Cu/Zn superoxide dismutase (SOD) was determined in duplicate by use of a Fluka SOD determination kit (Kit 19169, Sigma, Poole, UK) based on 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1). Liver samples were homogenised in 1 in 9 in 0.25 M sucrose buffer adjusted to pH 7.4 (0.25 M sucrose, 10 mM Tris-BASE, 1 mM EDTA) and further diluted 1 in 20 with buffer. Homogenates were then centrifuged at 10,000 g for 15 min at 4 °C. The assay was performed

according to the manufacturer's instructions. Briefly, 20 μ l of sample was added to each plate well. 200 μ l WST working solution and 20 μ l of enzyme working solution were added and the plate mixed thoroughly. The plate was then incubated at 37 °C (Heraeus, Kendo Laboratory Products, Hanau, Germany) for 20 min and the read at 450 nm with a microplate reader (Optimax Tuneable Microplate Reader, Molecular Devices, CA, USA). SOD activity (% inhibition rate) was determined by the following calculation:

SOD activity =

$$(((A \text{ blank } 1 - A \text{ blank } 2) - A \text{ sample}) / (A \text{ blank } 1 - A \text{ blank } 2)) \times 100$$

Where; 'A blank 1' is the absorbance of a blank where sample is substituted for distilled water; 'A blank 2' is the absorbance of a blank which includes enzyme dilution buffer instead of enzyme working solution. 'A sample' is the absorbance of the sample.

Units Cu/Zn SOD were determined from a standard curve of units SOD vs. % inhibition and normalized to sample protein (mg).

Units Cu/Zn SOD mg protein⁻¹ =

$$((\% \text{ inhibition} \times \text{slope of } \% \text{ inhibition vs. units SOD}) - \text{intercept of } \% \text{ inhibition vs. units SOD}) / \text{mg sample protein}$$

2.11. Markers of oxidative status

2.11.1. Malondialdehyde

Malondialdehyde (MDA) is a product of lipid peroxidation (Esterbauer et al., 1991) and was determined in duplicate colorimetrically as described by Gerard-Monnier et al. (1998) with modifications. The use of 1-methyl-2-phenylindole to detect MDA was used in the present study as it is more specific for MDA than the thiobarbituric acid reactive substances

(TBARS) assay, which is affected by interferences from side reactions of thiobarbituric acid (TBA) and endogenous compounds at high temperature and pH (Gerard-Monnier et al., 1998). Briefly, liver samples were homogenised in 4 volumes (pyloric caeca 9 volumes) 20 mM Tris-chloride assay buffer (pH 7.4). Samples were added (200 μ l) to a 650 μ l solution of 10.3 mM 1-methyl-2-phenylindole and 5 mM 2, 6-di *tert*-butyl-4-methylphenol (BHT) in a mixture of 3 parts acetonitrile to 1 part methanol. The reaction was started by the addition of 150 μ l 37 % HCl. After incubation at 45 °C for 40 min the mixture was cooled to room temp, centrifuged at 15,000 g for 10 min, and absorbance read at 586 nm (Jenway 6300 spectrophotometer, Patterson Scientific, Luton, UK.). MDA was determined from A_{586} (A_{586} no sample blank subtracted) sample values in μ mol MDA per g tissue from a standard curve of 1,1,3,3-tetramethoxypropane.

$$\mu\text{mol MDA g tissue}^{-1} =$$

$$(\text{A}_{586} \text{ of sample} \times \text{slope of absorbance vs. } \mu\text{mol MDA}) - \text{intercept of absorbance vs. } \mu\text{mol MDA}) \times \text{dilution factor}$$

2.11.2. Total antioxidant capacity

Total antioxidant capacity (TAC) is a measure of all known and unknown antioxidants (Nourooz-Zadeh et al., 2006) and was measured in duplicate in EDTA plasma using the novel Analysis By Emitted Light (ABEL[®]) antioxidant test kit (41M2) with Pholasin[®] and peroxyntirite (ONOO⁻) (Knight Scientific Ltd, Plymouth, UK); the first application of this TAC assay kit in fish (Knight, 2008, personal communication). Pholasin[®], a chemiluminescent probe, is a unique photoprotein from the rock boring bioluminescent mollusc *Pholas dactylus*, which emits light upon reaction with ROS (Knight et al., 2002). The assay determines TAC as the peroxyntirite scavenging capacity of plasma against a water soluble vitamin-E analogue (VEA) equivalent. Peroxyntirite is formed virtually

instantaneously in the assay after injection from a reaction between superoxide and nitric oxide released from 3-morpholino-sydnonimine (SIN-1). On reaction with peroxyntirite, Pholasin[®] quickly emits light and reaches a peak of intensity. The presence of antioxidants in the sample will compete with Pholasin[®] for peroxyntirite and delay the time at which the peak occurs. TAC is determined from the time to the peak of emitted light measured against VEA equivalent standards. In brief, 5 μ l plasma samples were manually plated in a white 96 well microplate in duplicate. Once in the plate reader, 95 μ l assay buffer, 50 μ l Pholasin[®] and 50 μ l SIN-1 were injected automatically at time zero to all wells. Assays were performed at 30° C using a BMG Lumistar Galaxy Luminometer. A 'no cell' plasma control was also included in all assays. TAC was determined in VEA equivalents (μ mol l⁻¹)

TAC (μ mol VEA) =

(time to peak of light intensity \times slope of time to peak light intensity vs. μ mol VEA) -
(intercept of time to peak light intensity vs. μ mol VEA)

2.11.3. Single cell gel electrophoresis (SCGE) or 'Comet' assay

Single strand breaks and oxidised purines were detected in erythrocytes using the SCGE or 'Comet' assay (Singh et al. 1988) modified with a formamidopyrimidine DNA glycosylase (FPG) enzyme digestion, which detects major purine oxidation product 8-oxoguanine and other altered purines by catalysing the conversion of oxidised purines into DNA strand breaks (Dusinska and Collins, 1996) as used by Aniagu et al. (2006) in chub (*Leuciscus cephalus*) erythrocytes. Unless otherwise stated, whole blood samples were diluted into 900 μ l freezing medium for cryopreservation at -80 °C (see 8.3.). The freezing medium contained; 20 % (v/v) bovine serum albumin (Invotrogen, Gibco, Paisley, UK), 40 % (v/v) RPMI-1640 medium (Sigma, Poole, UK) and 20 % DMSO (v/v). In brief, frosted microscope slides were coated with 1.5 % normal melting agarose (NMA) and left to air dry. Whole blood was diluted 1 in

5000 with CaCl_2 and MgCl_2 free DPBS physiological saline solution (Gibco, Invitrogen, Paisley, UK) to give a cell count of approximately 2.4×10^5 cells ml^{-1} . Cells were then resuspended in 0.75 % low melting agarose (LMA). In duplicate, 170 μl of the cell/agarose suspension was then placed on to each slide as two 85 μl spots, covered with 22 mm^2 cover slips and left to set at 4 °C for one hour. The cover slips were then removed from both gels and slides and immersed in lysis solution (2.5 mM NaCl, 100 mM Na_2EDTA , 10 mM tris-BASE, 10 % DMSO, 1 % Triton X-100, 1 % *n*-lauroyl-sarcosinate and adjusted with NaOH to pH 10) for one hour. Slides were then removed and washed in enzyme reaction buffer twice for 5 min (4 mM HEPES, 0.01 M KCl, 0.05 mM Na_2EDTA and 0.02 g ml^{-1} BSA at pH 8). 50 μl FPG ($1.5 \mu\text{g ml}^{-1}$) was added to each duplicate gel, covered with cover slips and incubated at 37 °C for 45 min (Heraeus, Kendo Laboratory Products, Hanau, Germany). Enzyme reaction buffer controls were prepared using the same procedure. Slides were then placed in a horizontal electrophoresis chamber and DNA left to unwind for 10 min (unless otherwise stated) in electrophoresis buffer to a level <1 mm above gels (0.3 M NaOH and 1 mM EDTA). Unless otherwise stated, after unwinding electrophoresis was performed for 20 min using a current of 300 mA and 25 V (Apelex ST 1006T electrophoresis unit). After electrophoresis slides were washed in neutralisation buffer (0.4 M Tris-chloride pH adjusted to 7.5 with HCl) for 10 min followed by distilled water for 10 min. To facilitate scoring by ensuring all DNA was on the same focal plane, slides then left to air dry for 24 h before staining with 80 μl 2 $\mu\text{g ml}^{-1}$ ethidium bromide solution and covering with 24/50 mm cover slips. Stained slides were scored immediately by use of a fluorescence microscope (Leica DMR) and Komet 5.0 image analysis software (Kinetic Imaging Ltd., Liverpool, UK). Percentage tail DNA, a common and robust measurable parameter of DNA damage (see plate 2.2.), was measured in 50 nucleoids per gel (100 per slide) (Kumaravel & Jha, 2006).

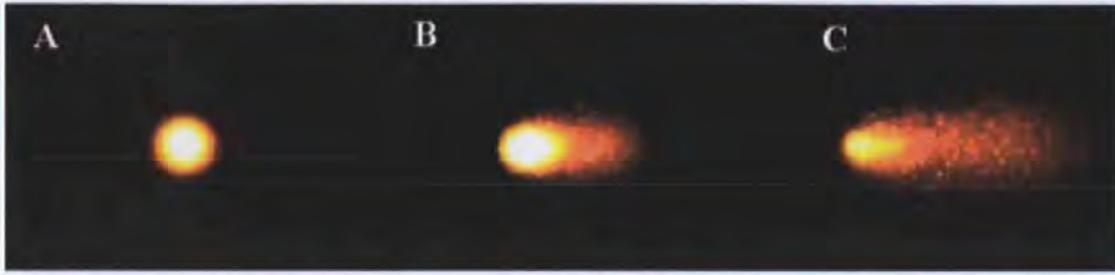


Plate 2.2. Comet images from individual trout erythrocytes showing various degrees of DNA damage. A: Normal undamaged DNA. B and C: Increasing comet tail of DNA super coils indicates increasing DNA strand breaks.

2.12. Hematological parameters and assay of nuclear abnormalities

2.12.1. Hematocrit

Hematocrit (packed cell volume) of whole blood was determined in duplicate as described by Klontz (1997). Whole blood was collected into 300 μ l heparinised hematocrit tubes and subsequently separated using a Centurion hematocrit centrifuge at 10,500 g for 5 min. Hematocrit was determined as the total percentage packed cell volume using a Hawksley hematocrit reader.

2.12.2. Blood Smears

Blood smears were prepared with 5 μ l heparinised whole blood. Smears were fixed in methanol for three min, stained in 6 % Giemsa stain solution (Giemsa/buffer, BDH, Poole, UK) for seven minutes and washed twice for one min in distilled water. Slides were air-dried and mounted with cover slips with the aid of DPX before analysis.

2.12.3. Total leukocyte counts

Total leukocytes counts (lymphocytes, thrombocytes, granulocytes) were scored 'blind' per 1000 cells on blood smears (see 2.12.2. *Blood smears*) at 400x magnification.

2.12.4. Micronucleus and nuclear abnormalities

The frequency of nuclear abnormalities including micronuclei, blebbed nuclei, and notched nuclei as described by Carrasco et al. (1990) and Del Barga et al. (2006) (see plate 2.3.) were determined 'blind' in 1000 cells per blood smear (see 2.12.2. *Blood smears*) under 1000x magnification. To be considered as micronuclei, cells were non-refractory, the same colour as the cell nucleus, round or ovoid shaped and with a diameter of 1/3-1/20 of the size of the main nucleus. Two clear nuclei (without any 'nuclear bridge' as in blebbed nuclei, see plate 2.3., number 4) of similar size were considered binucleate.

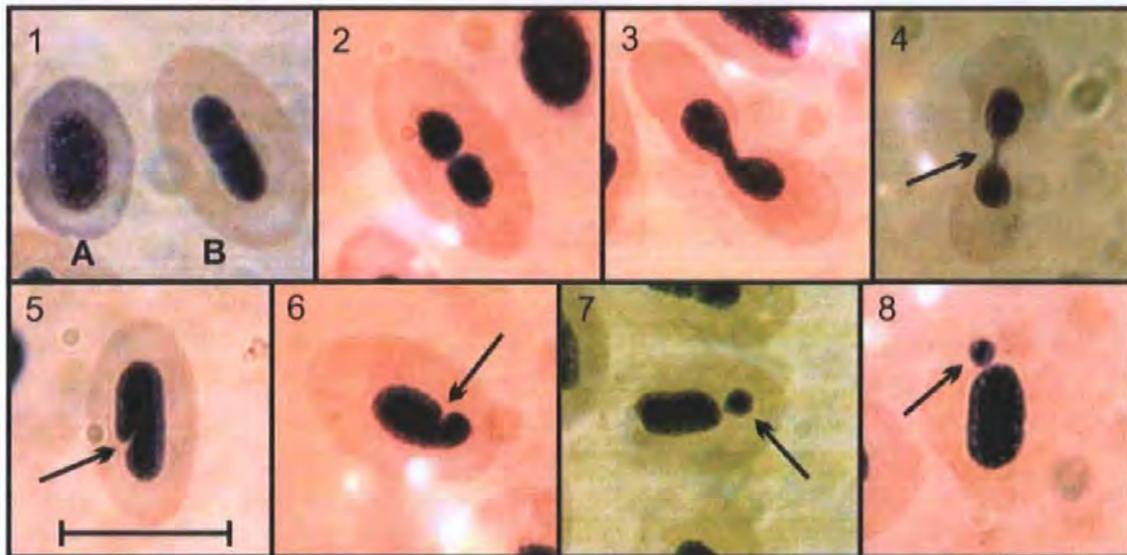


Plate 2.3. Nuclear abnormalities in rainbow trout erythrocytes. Scale bar = 10 μ m. 1 = Normal erythrocytes; A immature and B mature; 2 = Bi-nucleated erythrocyte; 3 and 4 = Blebbed; 5 and 6 = Notched; 7 and 8 = Micronuclei.

2.13. Immune parameters

2.13.1. Turbidimetric assay for lysozyme activity

Lysozyme activity was measured in duplicate in serum or plasma using the turbidimetric assay in a 96 well microplate according to Ellis (1990). Briefly, 15 μ l serum/plasma was

added to 285 μl 0.05 M sodium phosphate buffer (Na_2HPO_4) (pH 6.2) with 0.2 mg ml^{-1} lyophilised *Micrococcus lysodeikticus*. After 30 s absorbance was followed at 530 nm (Optimax Tuneable Microplate Reader, Molecular Devices, CA, USA) for 4 min at 25 °C and units lysozyme calculated. 1 unit of lysozyme activity is the amount of enzyme causing a decrease in absorbance of 0.001 min^{-1} .

2.13.2. Determination of respiratory burst

The production of extracellular superoxide anion ($\text{O}_2^{\cdot-}$) was determined in duplicate in diluted whole blood using an ABEL[®] cell activation test kit (04M) with Pholasin[®] and Adjuvant-K[™] (Knight Scientific Ltd., Plymouth, UK). This study is the first application of this assay technique in fish (Knight, 2008, personal communication). Leukocytes in whole blood were tested with both fMLP and PMA. No response was observed with fMLP thus PMA was subsequently used. Decreasing the assay temperature from 37 °C to 22 °C increased the response of fish leukocytes. The length of the assay was increased from that used for human blood due to an increased time to initial peak. Briefly, fresh EDTA treated whole blood was diluted 1/100 with dilution buffer and 20 μl added to 90 μl assay buffer, 50 μl Pholasin[®] and 20 μl Adjuvant-K[™] (enhances luminescence of Pholasin[®]). Leucocytes were activated by injecting 20 μl phorbol myristate acetate (PMA) providing a final concentration of 0.5 $\mu\text{g PMA ml}^{-1}$ (final blood dilution 1/1000). The assay was run in a 96 well white microplate at room temperature (22 °C) and emitted light read every 27 s for 105 min using a luminometer (Lumistar Galaxy, BMG, Offenburg, Germany). The integral of relative emitted light units was determined for each sample.

2.14. Drip loss of fillets

The drip loss of fillets was determined by the following method. Fillets were weighed, placed flat on an absorbent pad in an airtight zip bag and frozen at -40 °C until analysis. Drip loss

was quantified in thawed fillets kept in the dark at 4 °C for 48 hours. Fillets were re-weighed and percentage moisture loss determined using the following equation:

$$\text{Moisture loss (\%)} = ((\text{initial fillet wt.} - \text{final fillet wt.}) / (\text{initial fillet wt.})) \times 100$$

2.15. Astaxanthin extraction and determination of fillets and feed

Astaxanthin content was determined in whole fillets in duplicate by solvent extraction followed by spectrophotometric determination. Both feeds and whole fillets (with integument removed) were homogenised prior to analysis. Approximately 0.2 g and 4.0 g feed and fillet respectively were weighed directly into 50 ml centrifuge tubes and 6 ml distilled water and 12 ml HPLC grade methanol added. Samples were homogenised for 1 min, shaken vigorously and left in the dark at room temp for 15 min. 12 ml HPLC grade dichloromethane was then added, shaken vigorously and left for a further 15 min. Samples were then centrifuged at 3000 rpm for 10 min, the supernatant decanted, and 2 ml of the dichloromethane and carotenoid containing hyperphase recovered and transferred to a 4 ml darkened glass vial. The dichloromethane was evaporated over a period of ~ 2 hours under a constant stream of nitrogen. The remaining carotenoids were resuspended in 1 ml *n*-hexane. The solution was then transferred to a 1.7 ml centrifuge tube and centrifuged at 13,000 rpm and the absorbance read at 470 nm (Jenway 6300 spectrophotometer, Patterson Scientific, Luton, UK.). Five standard additions were added to both a random feed and fillet sample. Additions of 0.1, 0.2, 0.3, and 0.4 mg ml⁻¹ pure astaxanthin were prepared from a stock solution of 10 mg ml⁻¹ and ensured a 100 % recovery ± 5 %. Values of recovered astaxanthin were as expected given the supplementation level in prepared feeds. Astaxanthin (mg kg⁻¹) was determined using an E_{1%} of astaxanthin in *n*-hexane of 2100 at 470 nm (Britton, 1995) by:

$$\text{Astaxanthin} = (((A_{470} \times E_{1\%} \text{ of solvent}) \times \text{Dilution}) / (\text{Sample wt.})) \times 100$$

Dilution = 6 (extracted in 12 ml dichloromethane and 2 ml astaxanthin/dichloromethane solution resuspended in 1 ml *n*-hexane).

2.16. Statistical analysis

All statistical analysis was carried out using SPSS for Windows version 15. Unless otherwise stated all means are reported a standard error of the mean (SEM) or pooled SEM. Prior to analysis all data was tested for homogeneity of variance (Levene test) and normality of distribution (Kolmogorov-Smirnov test). Unless otherwise indicated, differences across treatments were either modelled by one-way ANOVA or a two-way nested ANOVA. Where appropriate, a *post hoc* Fisher's least significant difference (LSD) test was performed to make pair wise comparisons between individual treatments. Data violating the assumptions of parametric tests after log transformation were tested with the equivalent non-parametric Kruskal-Wallis and Mann-Whitney *U* tests. Any relationships in dose responses were modelled by linear, logarithmic or exponential decay regression analysis. Exponential decay regression analysis was modelled using Sigma Plot version 10. All percentage data was arcsine transformed prior to analysis. Unless otherwise stated, differences were considered significant at a value of $p \leq 0.05$.

CHAPTER 3.

THE BIOAVAILABILITY OF CO-SUPPLEMENTED ORGANIC AND INORGANIC ZINC AND SELENIUM IN A WHITE FISHMEAL BASED DIET.

Hypotheses:

- i) Organic Se and Zn will be more digestible than inorganic sources in a fishmeal based diet.**
- ii) Se and Zn supplementation will increase tissue levels of these elements above that of fish fed a basal fishmeal diet; organic sources resulting in the greatest increases.**
- iii) Se and Zn supplementation will raise specific Se and Zn dependent enzyme activity and organic sources will result in greater levels of enzyme activity.**

3.1. Introduction

Dietary Zn and Se are both essential elements for metabolic processes and may be supplemented to meet dietary requirements for fish. In rainbow trout, minimum requirements for Zn (15-30 mg kg⁻¹ Ogino and Yang, 1978) and Se (0.35 mg kg⁻¹ Hilton et al., 1980) have been determined in experiments with semi-purified diets containing inorganic minerals. Fishmeal based diets often contain levels of Se and Zn well above requirements, but the bioavailability of both elements from such diets is lower than that from semi-purified diets (Bell and Cowey, 1989; Lorentzen and Maage, 1999), consequently requirements may be higher in practical diets. Consequently, under intensive aquaculture conditions trace element supplementation, particularly with Zn, is often necessary to ensure requirements are met. Due to the higher mineral levels in practical diets, EU maximum permitted dietary levels of Se

(0.5 mg kg⁻¹) and Zn (200 mg kg⁻¹) may not leave much scope for supplementation. It is therefore essential that supplemented Se and Zn sources are highly bioavailable. Currently, any fortification of commercial and experimental fish diets is largely with inorganic sources, namely Zn sulphate, Zn oxide, and sodium selenite.

Organically chelated minerals are becoming increasingly favoured trace element supplements over inorganic sources due to their increased bioavailability. Organic Zn sources are those in which the Zn ion is chelated or complexed by organic tri or di peptides (Zn-proteinates) or single amino acids (Ashmead, 1992). Organic Se is covalently bound in selenoproteins by the substitution of sulphur in the amino acids cysteine and methionine forming 'seleno' analogues Se-Cys and Se-Met (Allan et al., 1999). These organically bound minerals, as opposed to inorganic mineral salts, are referred to as 'organic' hereon. Natural feed ingredients largely contain minerals bound to proteins and amino acids; consequently the use of organic minerals is regarded as a more natural method of trace element supplementation (Tucker and Taylor-Pickard, 2005).

The chelation of trace elements by organic compounds may protect metal ions against anti-nutritional factors present in practical diets. The dietary ingredients of commercial catfish and salmonid diets contain calcium, phosphorus, and phytic acid, which can form insoluble and indigestible Zn compounds in the gut (Gatlin and Wilson, 1984; Hardy and Shearer, 1985; Richardson et al., 1985; Satoh et al., 1987a; Satoh et al., 1989; Storebakken et al., 2000). Chelated/complexed Zn sources are increasingly thought to be more bioavailable than inorganic Zn sources due to the protection of the Zn ion from the formation of insoluble complexes in the digestive tract, thus facilitating absorption from the intestinal lumen (Ashmead, 1992). This has been reported with Zn-AA in trout (Apines et al., 2001) and Zn-pr in catfish (Paripatananont and Lovell, 1997). The organic Zn compounds present, and thus the degree of chelation of Zn-pr may vary significantly between manufactures, despite this

few studies exist on the bioavailability of Zn-pr in salmonid fish. An increased digestibility of supplemented Zn would be advantageous for environmental reasons, as the 87 % increase of Zn in sediments under salmon farm cages is a consequence of dietary Zn excretion (Dean et al., 2007).

Fishmeal diets often contain elevated levels of Se resulting from the high levels present in fishmeal; 2 mg kg⁻¹ in brown LT fishmeal, 1.8 mg kg⁻¹ in white fishmeal (see 8.1.), and between 1.36 and 2.15 mg kg⁻¹ for other various fishmeals (NRC, 1993). However, it has been reported by Bell and Cowey (1989) that Se in fishmeal is poorly digestible compared to Se-Cys, Se-Met or selenite supplemented to purified diets. Unlike Zn, Se is not cationic and consequently should not bind to ligands present in practical diets. Se availability from fishmeal is thought to be dependent on the digestibility of the Se compounds present within the diet and their subsequent metabolism (Wang and Lovell, 1997).

The aim of this study was to determine the bioavailability of Se-yeast, containing predominantly Se-Met, and Zn-pr in a white fishmeal based diet in comparison to inorganic sodium selenite and Zn sulphate in rainbow trout. SGR and FCR were determined for each treatment. Apparent mineral digestibility was used as a measure of net mineral uptake from the gut. Tissue and whole body levels, which are mandatory as a measure of element uptake in trace element studies (Cowey, 1992), were used to show the uptake of Se and Zn once digested. The specific activities of selected enzymes dependent on Se and Zn were determined as a measure of the uptake of these elements into metabolically active proteins. Se enzymes GSH-Px and Trx-R were used to assess Se status; the latter being previously unreported in fish with respect to dietary Se intake. Zn uptake was measured in metabolic processes by the enzymes activities of CPB and ALP.

3.2. Methods

3.2.1. *Experimental fish*

300 rainbow trout mean weight 31.9 ± 2.1 g (\pm SD) were acquired and acclimated (see 2.3.) for a period of 3 weeks and fed *ad libitum* using a standard commercial trout diet (15-45 Grower Feed XS, Aller Aqua, Christiansfeld, Denmark). Post acclimation, fish were stocked into one of nine 130 l tanks at a density of 25 fish per tank, and each tank was randomly assigned to receive a dietary treatment; each consisting of three replicate tanks. Water quality was monitored daily (see 2.2.). Total mortality during the trial was <1 %.

3.2.2. *Experimental design and diets.*

A feeding trial was initiated with three dietary treatments based on a common basal fishmeal diet (Table 1); a control (un-supplemented) diet containing only residual Zn and Se; a diet containing supplemental organic Se and Zn, and a diet containing supplemental inorganic Se and Zn. Dietary ingredients were analysed (see 8.1.) and the basal diet subsequently formulated (see Table 3.1.) to contain relatively low levels of Se and Zn with a high level of anti-nutritional factors. Inorganic Zn and Se were supplemented using Zn sulphate and sodium selenite, respectively. Organic Se and Zn were supplemented using Selplex[®] (Se-yeast) and Bioplex Zn[®] (Zn-protein) (Alltech inc., Kentucky, USA) respectively (see 2.9.), respectively. Fish were fed the experimental diet at 2.2 % biomass per day and maintained for a ten week period (see 2.2. and 2.4.) and SGR and FCR determined (see 2.5.).

Table 3.1.
Basal diet formulation.

Ingredient	% of diet
White fishmeal ¹	64
Marine fish oil ²	15
Wheat middlings (4-05-205) ³	19.82
Vitamin premix ⁴	0.5
Se/Zn variable premix ⁵	0.5
Se/Zn free premix ⁶	0.18

¹ Provimi 66 white fishmeal, Provimi Ltd, Thirsk, North Yorkshire, UK.

² Seven Seas Ltd, Hull, UK.

³ Elliots farm, Venn Ottery, Ottery St. Mary, Devon, UK.

⁴ Skretting, Preston, Lancashire, UK.

⁵ 5 % variable Se and Zn premix with yttrium oxide (Y_2O_3) to provide 1 mg kg^{-1} Y_2O_3 . Inorganic Se (Na_2SeO_3) and Zn ($ZnSO_4 \cdot 7H_2O$) Sigma, Poole, UK. Organic Se yeast (Selplex[®]) and Zn-Proteinat (Bioplex Zn[®]), Alltech inc., Kentucky, USA.

⁶ Se and Zn free premix (supplementing the following in mg kg^{-1} ; Fe 34 ($FeSO_4 \cdot 7H_2O$), Cu 5 ($CuSO_4 \cdot 5H_2O$), I 6 (KI), Mn 15 ($MnSO_4 \cdot H_2O$).

3.2.3. Analytical procedures

Energy, protein, lipid, ash and moisture were determined in each diet (see 2.7. and 2.6.). Trace element analysis was performed on all diets and tissues (see 2.7.). Se analysis was performed on 'in house' sample digests by LGC, Teddington, UK, by ICP-MS with standard additions. For proximate and elemental composition of diets refer to Table 3.2. Although supplemented diets were designed to be equimolar, the Zn-pr diets contained a significantly ($p < 0.001$) higher (by approximately one third) concentration of Zn.

Table 3.2.

Composition of experimental diets¹ and growth performance and feed utilisation of trout given diets with basal, organic or inorganic supplemental selenium and zinc.

Diet	Basal	Organic Se & Zn	Inorganic Se & Zn	⁵ Dietary effect p=
Moisture ²	4.0 ± 0.2	4.2 ± 0.2	4.6 ± 0.3	
Protein ²	43.6 ± 0.2	42.9 ± 0.4	42.9 ± 0.1	
Lipid ²	19.8 ± 0.5	21.3 ± 0.2	20.1 ± 0.2	
Ash ²	11.9 ± 0.5	12.3 ± 0.1	11.9 ± 0.3	
Energy ³	20.6 ± 0.0	20.9 ± 0.0	20.7 ± 0.1	
Iron ⁴	182 ± 0	178 ± 3	173 ± 0	
Copper ⁴	41.1 ± 0.1	42.1 ± 0.8	44.2 ± 1.2	
Manganese ⁴	75.6 ± 1.0	73.0 ± 2.0	75.7 ± 4.0	
Zinc ⁴	157 ± 3	306 ± 3	246 ± 1	
Selenium ⁴	1.2 ± 0.0	1.9 ± 0.0	1.8 ± 0.0	
SGR ⁶	2.07 ± 0.03	1.87 ± 0.08	1.95 ± 0.07	0.163
FCR ⁶	1.06 ± 0.02	1.15 ± 0.05	1.12 ± 0.04	0.300

¹ Proximate analysis of experimental diets on as fed wet weight basis (± SD).

² Value in %

³ Value in MJ kg⁻¹

⁴ Value in mg kg⁻¹

⁵ Statistical difference between dietary treatments (*n*=3).

⁶ SGR, standard growth rate; FCR, feed conversion ratio.

Fish were sampled for all analysis at the end of the 10-week trial period (see 2.3.), which by nature of the feeding schedule included one day of feed withdrawal prior to sampling. All sampled fish were euthanized by cerebral destruction under anaesthetic. Three random fish per tank (9 per treatment) were sampled and whole blood recovered by a caudal vein puncture using heparinised syringes for the determination of Se, Zn and plasma ALP (see 2.10.5.). The liver, pyloric caeca and remaining intestinal tissue was excised for tissue

enzyme analysis. The pyloric caeca was processed whole (fat deposits removed) for the analysis of CPB (see 2.10.4.) and the remaining intestinal tissue was also sampled, with any remaining digesta removed and cleaned with 0.7 % NaCl for the analysis of ALP (see 2.10.5.). The liver was sampled whole for the analysis of GSH-Px (see 2.10.2) and Trx-R (see 2.10.3.). GSH-Px was assayed with 1.23 mM cumene hydroperoxide. All sampled tissues were stored at -80 °C until analysis. A further three fish per tank (9 per treatment) were sampled for trace element analysis (see 2.7.) and the following tissues sampled: liver (whole); kidney (whole); all gills (gill arches removed); pyloric caeca (whole); mid and hind intestinal tissue (digesta removed and cleaned with 0.7 % NaCl); spleen (whole); muscle and corresponding integument (2 cm³ sections beneath both sides of the dorsal fin); and the caudal fin (whole). The remaining carcass was heated at 100 °C for 20 min such that the whole vertebrae could be removed and sampled from surrounding soft tissues. All tissues were freeze dried and percentage moisture determined from initial and final weights. Dried tissues were pooled per treatment and homogenised. Four fish per tank (12 per treatment) were also sampled for the analysis of whole body Se and Zn (see 2.7.) For the analysis of apparent digestibility faecal material was recovered from all fish on two separate occasions in week 8 and pooled for each replicate tank (see 2.8.).

3.2.4. Statistical analysis

All means are reported with SEM and statistical analysis was performed on SPSS version 15. Data was tested for homogeneity of variance (Levene and Kolmogorov-Smirnov tests), and modelled by one-way ANOVA. Percentage digestibility data was arcsine transformed prior to statistical analysis. Any significant differences among treatment means given by the ANOVA model were evaluated by a *post hoc* Fisher's LSD test. Significance was set at $p \leq 0.05$ for all statistical tests except Se dependent enzyme activities, which were accepted as significant at $p \leq 0.1$.

3.3. Results

3.3.1. Growth rates and feed performance

After ten weeks all fish almost quadrupled in weight to a mean (\pm SEM) of 125 ± 19 g. Growth rates and feed conversion were within expected ranges for trout under production conditions. Throughout the ten-week period there was no significant difference between treatments for SGR and FCR (Table 3.2.). Despite being designed to be equimolar, Zn was significantly greater in the Zn-pr than the Zn sulphate supplemented diet ($p < 0.001$).

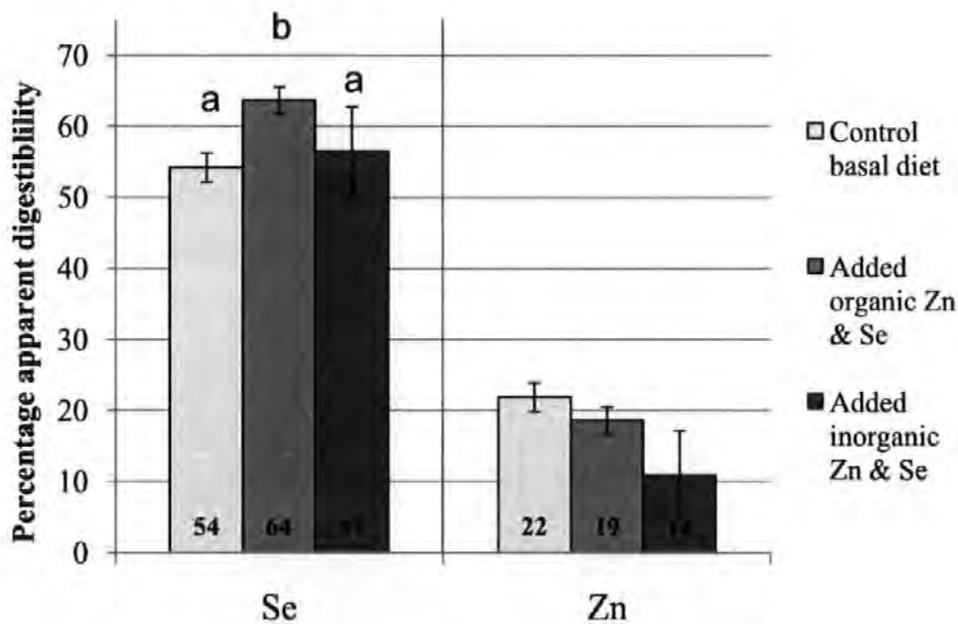


Figure 3.1. Mean apparent digestibility of basal and supplemented zinc and selenium sources from a fishmeal based diet. Bar graph of mean (\pm SEM.) percentage apparent digestibility of dietary Se and Zn. Different lower case letters indicate significant difference between treatments ($p \leq 0.05$).

3.3.2. Selenium and zinc digestibility

Digestibility of Se from the Se-yeast fortified diet was significantly higher than Se from either the control or selenite supplemented diets. Zn digestibility was similar across all treatments. Mean (\pm SEM) apparent digestibility (Fig. 3.1.) of residual Zn and Se in the basal diet was 22 ± 2 % and 54 ± 1 %, respectively. Apparent Zn digestibility (Fig 3.1.) was highest from the basal diet followed by the Zn-pr and Zn sulphate supplemented diets; the latter having the lowest digestibility. However, trends in Zn digestibility were not significantly different between diets (p 0.089). Digestibility of Se in the control and selenite supplemented diets, were not significantly different (p 0.340).

3.3.3. Tissue and whole body zinc

Significant differences in whole body Zn were observed between treatments. Both Zn sources increased whole body Zn with Zn sulphate supplementation resulting in a greater increase than Zn-pr (Table 3.3.). In those tissues which responded to supplemented Zn, increases were greater for Zn sulphate; the only exception being the liver (Table 3.3.). Despite the higher Zn level in Zn-pr than the Zn sulphate diet, Zn in the muscle, vertebrae and plasma was only significantly increased by Zn sulphate. A similar trend was found in the gill tissue, where both sources of supplemented Zn resulted in increased levels of the element, but by a significantly greater extent from Zn sulphate than Zn-pr. Neither Zn sulphate nor Zn-pr significantly increased Zn in the whole blood, integument or caudal fin (Table 3.3.). Zn sulphate supplementation did not significantly increase kidney Zn above that of the basal diet (p 0.186), and Zn-pr supplementation resulted in a slight reduction of kidney Zn. Only Zn-pr resulted in a significant increase of hepatic Zn above that of fish fed the basal diet. The higher Zn levels in the Zn-pr diet were not reflected in the pyloric caeca, only Zn sulphate significantly increased Zn in this tissue. A similar trend was found in the posterior intestinal

tissue, where both sources increased intestinal Zn, but with Zn sulphate resulting in a significantly greater increase.

Table 3.3.

Mean tissue and whole-body Zn¹ of trout given a basal fishmeal diet, and diets with supplemented organic or inorganic zinc and selenium sources.

Diet	Basal	Zn -proteinate	Zn-sulphate	² Dietary effect <i>p</i> =
Whole body	28.8 ± 0.5 ^a	31.2 ± 0.4 ^b	32.7 ± 0.2 ^c	<0.001*
Gill	105 ± 1 ^a	121. ± 1 ^b	129 ± 1 ^c	<0.001*
Plasma	10.6 ± 0.2 ^a	11.8 ± 0.6 ^a	13.7 ± 0.5 ^b	0.003*
Vertebrae	86 ± 5 ^a	80 ± 2 ^a	160 ± 8 ^b	<0.001*
Integument	42 ± 2	47 ± 2	47 ± 1	0.147
Whole blood	11.0 ± 0.5	11.0 ± 0.3	11.9 ± 0.4	0.283
Muscle	4.8 ± 0.0 ^a	5.1 ± 0.1 ^{ab}	5.2 ± 0.1 ^b	0.023*
Caudal fin	48 ± 2	51 ± 1	54 ± 4	0.338
Liver	17.1 ± 0.2 ^a	19.0 ± 0.3 ^b	18.4 ± 0.6 ^a	0.008*
Kidney	21.5 ± 0.3 ^a	20.0 ± 0.3 ^b	22.0 ± 0.3 ^a	0.002*
Pyloric caeca (including digesta)	186 ± 1 ^a	217 ± 9 ^a	258 ± 8 ^b	0.005*
Posterior intestinal tissue	307 ± 13 ^a	392 ± 14 ^b	405 ± 3 ^{bc}	0.016*

¹ Mean values in mg kg⁻¹ (wet weight) ± SEM.

² Means in the same row not sharing a common superscript are significantly different (*p* ≤ 0.05); * highlights significant difference between treatments. All tissues analyzed *n*=3.

3.3.4. Tissue and whole body selenium

Se-yeast supplementation significantly increased whole-body Se but selenite supplementation had no significant effect in comparison to the whole body level of fish fed the basal diet (*p* 0.54) (Table 3.4.). The response of individual tissues to Se supplementation differed between fish fed selenite and Se-yeast (Table 3.4.). There was a significantly lower Se content in the pyloric caeca of the Se-yeast than selenite supplemented trout. In all remaining tissues except the liver, fish given Se-yeast had significantly higher tissue Se levels than counterparts fed

the basal and selenite supplemented diets. Both Se-yeast and selenite supplementation significantly increased Se in the kidney, plasma, integument and whole blood, but increases of Se was significantly lower in tissues of fish given selenite than Se-yeast. Hepatic Se was significantly elevated to a similar extent by both Se-yeast and selenite supplementation. Selenite did not significantly increase muscular Se above that of fish fed the basal diet.

Table 3.4.

Mean tissue and whole-body Se¹ of trout given a basal fishmeal diet, and diets with supplemented organic or inorganic zinc and selenium sources.

Diet	Basal	Se-yeast	Selenite	² Dietary effect <i>p</i> =
Whole body	0.24 ± 0.01	0.036 ± 0.01	0.26 ± 0.01	<0.001*
Muscle	0.21 ± 0.01 ^a	0.37 ± 0.01 ^b	0.23 ± 0.01 ^a	<0.001*
Integument	0.40 ± 0.00 ^a	0.61 ± 0.01 ^b	0.46 ± 0.00 ^c	<0.001*
Gill	0.40 ± 0.00 ^a	0.51 ± 0.00 ^b	0.43 ± 0.00 ^c	<0.001*
Whole blood	0.45 ± 0.00 ^a	0.58 ± 0.01 ^b	0.52 ± 0.00 ^c	<0.001*
Plasma	0.14 ± 0.00 ^a	0.19 ± 0.00 ^b	0.17 ± 0.00 ^c	<0.001*
Spleen (<i>n</i> =1) ³	0.77	1.01	0.80	
Kidney	0.90 ± 0.02 ^a	1.07 ± 0.02 ^b	0.99 ± 0.01 ^c	0.001*
Liver	2.04 ± 0.04 ^a	3.65 ± 0.07 ^b	3.75 ± 0.05 ^b	0.001*
Pyloric caeca (including digesta)	0.73 ± 0.01 ^a	0.92 ± 0.01 ^b	1.17 ± 0.01 ^c	<0.001*
Posterior intestinal tissue (excluding digesta)	0.36 ± 0.00 ^a	0.38 ± 0.01 ^{ab}	0.34 ± 0.00 ^{ac}	0.043*

¹ Mean values in mg kg⁻¹ (wet weight) ± SEM.

² Means in the same row not sharing a common superscript are significantly different (*p* ≤ 0.05); * highlights significant difference between treatments. All tissues analyzed *n*=3 unless stated.

³ Statistical comparisons could not be made due to lack of replication.

Table 3.5.

Mean (\pm SEM) Zn dependent enzyme activity and activity/tissue Zn ratios of trout given a basal fishmeal diet, and diets with supplemented organic or inorganic zinc and selenium sources.

Diet	Basal	Zn-protein	Zn-sulphate	⁵ Dietary effect $p=$
Plasma ALP ¹ ($n=6$)	139 \pm 12	157 \pm 8	122 \pm 12	0.128
Intestinal ALP ²	0.14 \pm 0.02	0.16 \pm 0.04	0.18 \pm 0.03	0.512
P.C. CPB ²	1.1 \pm 0.1	1.1 \pm 0.1	1.1 \pm 0.1	0.875
Plasma ALP/Zn ratio ^{1,3} ($n=6$)		13.2 \pm 0.8 ^a	8.8 \pm 0.6 ^b	0.012*
Intestinal ALP/Zn ratio ^{2,4}		0.41 \pm 0.6	0.44 \pm 0.4	0.648
P.C. CPB/Zn ratio ^{2,4}		4.9 \pm 0.5	4.1 \pm 0.4	0.223

¹ Units ALP litre plasma⁻¹.

² Units ALP mg protein⁻¹.

³ Tissue Zn in mg kg⁻¹ (wet weight).

⁴ Tissue Zn in g kg⁻¹ (wet weight).

⁵ Means in the same row not sharing a common superscript are significantly different ($p \leq 0.05$); * highlights significant difference between treatments. All tissues $n=9$ unless stated.

3.3.5. Selenium and zinc dependent enzyme activity

Activities of intestinal and plasma ALP, and pyloric caeca CPB were not affected by either forms of Zn supplementation. Zn enzyme activity/tissue Zn ratios were also not significantly different for intestinal ALP or CPB. However, an increased plasma ALP ratio in Zn-pr over Zn sulphate supplemented fish was observed (Table 3.5.). Selenium dependent Trx-R activity significantly increased as a result of Se-yeast supplementation but not selenite supplementation. GSH-Px activity increased in response to both sources of supplemented Se with no difference between sources. Both GSH-Px and Trx-R/tissue Se ratios were significantly greater for Se-yeast than selenite (Table 3.6.).

Table 3.6.

Mean (\pm SEM) Se dependent enzyme activity and activity/tissue Se ratios of trout given a basal fishmeal diet, and diets with supplemented organic or inorganic zinc and selenium sources.

Diet	Basal	Se-yeast	Selenite	⁴ Dietary effect $p=$
Hepatic Trx-R ¹	0.77 \pm 0.09 ^a	1.15 \pm 0.15 ^b	0.83 \pm 0.09 ^{ab}	0.048*
Hepatic GSH-Px ²	68.3 \pm 3 ^a	79.0 \pm 1.6 ^b	77.8 \pm 4.3 ^b	0.059*
Hepatic Trx-R /Se ratio ^{1,3}		0.32 \pm 0.04 ^a	0.22 \pm 0.02 ^b	0.056*
Hepatic GSH-Px/Se ratio ^{2,3}		21.6 \pm 0.4 ^a	20.7 \pm 1.2 ^b	0.065*

¹ A₄₁₂·1000 min⁻¹ mg protein⁻¹

² nmol NADPH oxidised min⁻¹ mg protein⁻¹

³ Tissue Se in mg kg⁻¹ (wet weight).

⁴ Means in the same row not sharing a common superscript are significantly different ($p \leq 0.1$); * highlights significant difference between treatments. All tissues $n=9$.

3.4. Discussion

3.4.1. Improved Se-yeast digestibility in fishmeal based diets

Digestibility of endogenous Se was relatively low and was slightly improved by Se-yeast supplementation. The digestibility of residual Se from the basal diet was similar to that found in Atlantic salmon fed a fishmeal based diet (46 %) (Bell and Cowey, 1989). In the present study net Se digestibility increased as a result of the supplementation of Se-yeast. This did not occur with selenite and shows that the digestibility of organic Se within a practical is better than that of selenite. This is expected to be due to the fact that being covalently bonded, Se in Se-yeast is protected from any interactions with anti-nutritional factors in the GI tract. Both Se sources accumulated in the pyloric caeca, with the highest levels resulting from selenite supplementation. Given the lower Se level in the whole blood of selenite fish, this is proposed to be due to the reduced absorption of inorganic Se in comparison to Se-yeast. Conversely, the lower pyloric caeca Se level of the Se-yeast group is expected to be due to increased Se uptake, which corresponds with the increased plasma Se and digestibility. Bell and Cowey (1989) reported that in Atlantic salmon Se-Met has a 92 % digestibility in a semi purified diet. Despite supplementing an additional 62 % Se-yeast, which contains predominantly Se-Met, only a 16 % improvement in digestibility was achieved. This suggests that Se absorption from fishmeal, especially for selenite, may be affected by anti-nutritional dietary factors that are yet to be identified. Mineral interactions such as the antagonistic effects of Cu may be one of the contributing factors in reduced Se digestibility from fishmeals, as proposed by Lorentzen et al. (1998).

3.4.2. Improved deposition of selenium from Se-yeast across tissues

Tissue and whole body Se analysis demonstrated that Se-yeast is more bioavailable than selenite and suggests that both sources follow a different metabolic pathway. Whole body Se

is a good measure of Se status (Hilton et al., 1980; Lin and Shiau, 2005) and was significantly greater for Se-yeast than selenite supplementation. This has also been reported in Atlantic salmon (Lorentzen et al., 1994) and catfish (Wang and Lovell, 1997). The elevation of Se from selenite in the liver, but not the muscle and whole body as occurred with Se-yeast, is consistent with differential metabolism of Se-yeast and selenite. Selenite will follow the regular pathway for metabolism of Se but Se-yeast with a high Se-Met content will also follow pathways for methionine (Burk, 1976). Se-yeast supplementation of trout resulted in elevated Se in all tissues and is expected to be principally due to the non-specific incorporation of Se-Met into proteins of the soft tissues (Schrauzer, 2000). Likewise, Lorentzen et al. (1994) showed that the non-specific incorporation of selenite does not occur to any significant extent in salmon and this is verified in the present study for trout. As fillet Se is highly bioavailable (Ornsrud and Lorentzen, 2002), the Se enrichment of fillets by Se-yeast may be of potential as a fortified feed to European consumers, who are generally thought to be Se deficient (Rayman, 2000; Cotter et al. 2008).

3.4.3. Increased activity of selenium dependent enzymes by Se-yeast

Both GSH-Px and Trx-R are dependent on Se for their activity (Allan et al., 1999). As GSH-Px responds well to marginal Se status, it is routinely assessed in relation to Se intake in piscine as well as mammalian studies. However, this Se dependent enzyme only responds to dietary Se at marginal levels (Bell et al., 1986; Bell et al., 1987). At higher dietary Se, a plateau in GSH-Px activity occurs, which is commonly used to establish dietary Se requirements (Hilton et al., 1980). Despite being above the established requirement (0.35 mg kg⁻¹) this study reports an increase in GSH-Px in a low quality practical diet. Both studies by Hilton et al. (1980) and Bell and Cowey (1989) used semi-purified diets where nutritional factors may have less effect on trace element digestibility. Although not significant, the

lowest GSH-Px values obtained by Bell and Cowey (1989) were from fish given a white fishmeal diet as opposed to a Se supplemented semi-purified diet. In the present study, and in hybrid striped bass, GSH-Px activity was not affected by dietary Se source (Cotter et al., 2008). GSH-Px activity may be affected by *in vivo* physiological conditions. Se in GSH-Px is required to be in a reduced rather than oxidised form such that the enzyme may function. Substrate peroxides oxidise Se within the active site of GSH-Px from selenol (E-SeH) to selenic acid (E-Se-OH) or seleninic acid (E-SeO₂H), which decreases the activity of the enzyme (Ganther and Kraus, 1984). The level of reducing agents such as GSH and ascorbic acid may therefore affect *in vivo* GSH-Px activity (Nakano et al., 1992). Requirements based solely on one Se dependent enzyme are questionable; the assessment of a battery of Se enzymes is preferable. In cell lines and live animal studies, a hierarchy of selenoprotein expression exists during Se deprivation and repletion (Papp et al., 2007). In mammals, the activities of Trx-R and selenoprotein P have been found to be optimised at higher levels than GSH-Px (Burk and Hill, 2005; Berggren et al., 1999). This has been reported to be due to an increased incorporation of Se rather than increased protein synthesis (Berggren et al., 1999). The determination of Trx-R activity was therefore particularly relevant to this study, as the practical diet used was expected to contain dietary Se above the minimum required for maximal GSH-Px activity. The increase in Trx-R activity by Se-yeast in the present study shows that the requirements of Se enzymes for Se may differ and that Se source is an important factor.

3.4.4. A lack of difference in zinc digestibility between zinc sources.

Digestibility of residual Zn in the basal diet was low and no difference in Zn digestibility was found between supplemented sources. White fishmeal, with larger concentrations of calcium and phosphorus in comparison to higher quality brown fishmeals (NRC, 1993), and phytic

acid from plant material, can significantly reduce Zn digestibility due to the formation of insoluble complexes with the Zn cation (Richardson et al., 1985; Satoh et al., 1987a&b and 1993; McClain and Gatlin 1988; Ramseyer et al., 1999; Storebakken et al., 2000). As a consequence, white fishmeal diets often require Zn supplementation (Satoh et al., 1987b). Previous studies, which have used significantly lower levels of dietary Zn than the present study, have reported a significantly higher digestibility of chelated Zn sources than Zn sulphate (Paripatananont and Lovell, 1997; Apines et al., 2001). The lack of significant difference between the digestibility of Zn-pr and Zn sulphate in the present study may have been affected by 'diminished returns' as Zn retention decreases with increasing dietary Zn (Spry et al., 1988). Both Zn sources accumulated in the pyloric caeca and posterior intestine, but the highest levels were found in the Zn sulphate group, despite a higher dietary Zn in the Zn-pr diet. The decreased Zn in the pyloric caeca and intestine of Zn-pr supplemented trout is expected to be due to a decreased accumulation and subsequent uptake in these tissues of Zn-pr than Zn sulphate. This would be consistent with the increased retention of Zn from Zn sulphate in all tissues including the gill and plasma in Zn sulphate supplemented fish. The levels of Zn in the intestine and gills, which are key tissues in the excretion of dietary Zn (Hardy et al. 1987; Maage and Julshamn, 1993), were lower for Zn-pr. However static Zn levels may not necessarily reflect Zn turnover in individual tissues and cannot be depended on as a sole indication of bioavailability. The similar digestibility between sources may be due to the dissociation of the Zn ion from its complex in Zn-pr in the acidic environment of the salmonid stomach.

3.4.5. Increased tissue zinc by dietary zinc supplementation

Zn in the whole body and majority of tissues increased to a greater extent by Zn sulphate than Zn-pr supplementation indicating a higher retention for inorganic Zn in diets above Zn

requirements. In salmonids, whole body Zn correlates well with Zn status (Wekell et al., 1986). Despite the higher Zn in the Zn-pr group, Zn sulphate supplemented trout had a marginally greater whole body Zn indicating a slightly higher retention of Zn sulphate. Increases in whole body Zn by Zn supplementation were much lower than found in previous salmonid studies using lower levels of dietary Zn (Apines et al., 2001, Maage et al., 2001). As the basal diet contained levels of dietary Zn above established requirements, this is likely to be a result of the regulation of dietary Zn (Clearwater et al., 2002). Gatlin and Wilson (1984) reported a plateau in bone Zn concentration in catfish above 150 mg kg^{-1} using Zn-oxide. This is consistent with the increased Zn in the gills (particularly the Zn sulphate group) of supplemented fish. Zn-pr has been found to effectively raise whole body Zn, but its increased bioavailability decreases markedly in diets containing high levels of calcium phosphate such as those used in the present study (Hardy and Shearer, 1985). Zn sulphate supplementation significantly elevated plasma and vertebrae Zn, but this was not found for Zn-pr, again showing a higher retention of Zn sulphate. A slight increase in liver Zn was found in the Zn-pr supplemented fish but may merely reflect the higher Zn in this diet than the Zn sulphate diet. Owing to its metabolic rather than storage role, Zn turns over rapidly in the liver (Vallee and Falchuk, 1993), and consequently a measurement of Zn turnover or expression of Zn transporter proteins may be a better determinant of Zn status than static Zn levels. The results of Zn in the vertebrae were similar to those of Do Carmo e Sa et al. (2005) in Nile tilapia fed practical diets, who found reduced bone deposition of Zn from Zn-AA than Zn sulphate. The different response in tissue Zn, particularly in bone tissue, suggests that organic and inorganic Zn sources may be metabolised differently post absorption.

3.4.6. Lack of response by zinc dependent enzymes

Zn supplementation did not affect the activity of Zn dependent enzymes, which confirms that Zn requirements were met by the basal diet. The activity of both CPB and ALP are dependent on the presence of Zn at their active site (Folk et al., 1960; Coleman, 1992). Zn deficiency also influences the activity of ALP by destabilisation of the enzyme (Reinhold and Kfoury, 1969). Using 61 % plant based diets in rainbow trout, Ramseyer et al. (1999) reported that both CPB and intestinal; ALP increased between dietary Zn levels of 51 and 133 mg kg⁻¹. In the present study basal Zn levels were higher (157 mg kg⁻¹) and Zn enzyme activities remained the same across all treatments. Using a cod muscle and corn meal based diet, plasma ALP plateaus between 40 and 80 mg kg⁻¹ in Atlantic salmon (Maage and Julshamn, 1993), which also supports the notion that the basal diet met Zn requirements. Below 100 mg kg⁻¹ dietary Zn, Zn-AA increases plasma ALP activity to a greater extent than Zn sulphate supplemented diets (Apines et al., 2001 and 2003; Apines-Amar et al., 2004). Although Zn sulphate raised plasma Zn to a greater extent, the plasma ALP/plasma Zn ratios show that more Zn from Zn-pr is incorporated into ALP than Zn sulphate. This suggests that Zn from Zn-pr may be more available to plasma ALP than Zn sulphate; an assessment of Zn dependent enzyme/tissue Zn ratios in other tissues would reveal if this is common to all other tissues. Although Zn sulphate increased tissue Zn, it does not imply that inorganic Zn ultimately more available for Zn requiring metabolic processes. Amino acids, such as histidine that chelate Zn ions, may facilitate the passage of Zn to Zn transporters (Glover et al., 2003); Zn transporters (ZnT7 and ZnT5) are involved in the loading of Zn into ALP (Suzuki et al., 2005).

3.4.7. Diet formulation and zinc and selenium interactions

The nutritional value of a trace element depends both on dietary levels and bioavailability, which can be affected by both physiological and dietary factors. Dietary factors that influence trace element bioavailability in practical diets include the chemical forms of elements in the diet, the formation of insoluble complexes by interactions with dietary components, and antagonistic effects of other elements (O'Dell, 1984). Such that the effects of dietary anti-nutritional factors on Se and Zn sources could be maximised, the basal diet of the present study was formulated with white fishmeal and wheat middlings to contain relatively high levels of potential Zn inhibitors and a low Se and Zn content. As mineral supplementation in fishmeal diets may often require both Se and Zn, they were co-supplemented and the bioavailability of both evaluated using markers specific to each element. Previously House and Welch (1989) showed that in rats high levels of either Zn or Se can have antagonistic effects on one another. In Atlantic salmon, dietary Se has no significant effect on hepatic Zn between 0.66 and 2.6 mg Se kg⁻¹ (Julshamn et al., 1990). As both Se and Zn were above requirements in the present study, any such interactions are not expected to be significant. Although a preliminary assessment of Se and Zn levels in several ingredients was made, both Se and Zn levels were higher than anticipated in the final diet. This is expected to have occurred due to the intrinsic variability of these elements between different batches of ingredients. Although comparisons have been made between Zn-pr products from different manufactures, as other Zn-pr products may differ in their biological properties, their nutritional value may differ (Cao et al., 2000). It has been reported in rainbow trout that the chemical nature of a Zn chelates are more influential on Zn absorption than simply chelation itself (Glover and Hogstrand, 2002a).

3.4.8. Conclusions

In summary, this study has shown that the bioavailability of organic and inorganic Se sources differ in a white fishmeal diet. Zn digestibility and Zn dependent enzymes were not affected by either forms of supplemented Zn suggesting a similar bioavailability. Zn sulphate accumulated to a greater extent than Zn-pr in tissues showing a higher retention when Zn is above requirements. However, based on an increased plasma ALP/plasma Zn ratio, Zn from Zn-pr may be more available for Zn requiring metabolic processes. Se levels in the basal diet may not have met requirements suggesting that diet quality may affect Se bioavailability. In all factors assessed, Se bioavailability was greater for organic Se-yeast than selenite. Organic Se-yeast proved to be highly efficacious, increasing digestibility of overall dietary Se, efficiently raising whole body and tissue Se status, as well as increasing Trx-R and GSH-Px activity. This was not the case with selenite, which in the present study did not prove to be as highly bioavailable.

CHAPTER 4.**THE EFFECT OF NUTRITIONAL, SUPRA-NUTRITIONAL, AND HIGH ORGANIC AND INORGANIC ZINC SUPPLEMENTATION IN A PRACTICAL DIET ON ZINC STATUS AND HEALTH****Hypotheses:**

- i) Zn supplementation will increase Zn status in a fishmeal based diet; Zn-pr will be equivalent to Zn sulphate.**
- ii) Zn supplementation of a fishmeal diet will confer benefits to growth, oxidative status, immuno-competence, DNA stability, haematological parameters and fillet quality; Zn-pr will be as efficacious as Zn sulphate.**
- iii) Both dietary Zn sources will interact with other trace elements.**
- iv) Supra-nutritional and high dietary Zn supplementation will not be toxic to rainbow trout.**

4.1. Introduction

Zn is an essential element for fish (Watanabe et al., 1997) and as farmed fish derive the majority of their nutrients from a single diet, it is essential for optimal health that practical diets are formulated with the correct levels of this micronutrient. Zn is a ubiquitous trace element and is present as a co-factor of over 300 enzymes involved in many metabolic processes including DNA synthesis, growth, membrane stability, bone formation and wound healing (Salgueiro et al., 2000; Stefanidou et al., 2006). However, excess supplementation of

Zn may result in sub-optimal health, interactions with other nutrients, and unnecessary contamination of the environment in open cultivation systems. As was observed in the previous experiment, due to the natural levels of Zn in fishmeal and plant ingredients some practical diets contain this mineral above dietary requirements. However, due to dietary anti-nutritional factors present in practical diets including phytate (Richardson et al., 1985; McClain and Gatlin, 1988, in tilapia) and tri-calcium phosphate (Satoh et al., 1987a; Satoh, et al., 1993), Zn supplementation of some practical diets with lower basal levels of Zn can be necessary (Watanabe et al., 1980; Lorentzen and Maage, 1999). Whole body and hepatic Zn in farmed salmonids is lower than wild equivalents (Poppe et al., 1985; Magge et al., 1991; Felton et al., 1994). This disparity may be due to differences in dietary intake, growth rates and (or) life histories. Due to an increase in the pH of the stomach, the antagonistic effect of tri-calcium phosphate on Zn uptake from the GI tract is greatest in fish with a stomach, such as salmonids, than stomachless fish such as carp (Watanabe et al., 1988).

Much research on Zn nutrition in fish has focused on effects of dietary anti-nutritional factors on uptake and classical measurements of Zn status including tissue levels, growth and Zn dependent enzymes; relatively few studies have assessed the role or compared the toxicity of organic and inorganic dietary Zn sources on health. In mammals, Zn is known to play a key role in growth (Rossi et al., 2000), the immune system (Shankar and Prasad, 1998; Rink and Gabriel, 2000), in antioxidant functions (Powell, 2000), DNA replication and transcription (Dreosti, 2001), and in the ocular system (Grahn et al., 2001). In fish Zn deficiency is known to cause oxidative stress (Hidalgo et al., 2002; Kucukbay et al., 2006), cataracts (Ketola, 1979), and decreased disease resistance (Paripatananont and Lovell, 1995b). Zn deficiency has also been reported to increase DNA instability in rats (Castro et al., 1992), but this has not been ascertained in fish. Despite the importance of the innate immune response to fish, no studies have assessed the role of dietary Zn in respiratory burst or lysozyme to date. As well

as being essential, Zn can be toxic to mammals (Barceloux, 1999). Dietary Zn is reported to be relatively non-toxic to fish (Hogstrand and Wood, 1996, Clearwater et al., 2002), yet few studies have assessed the sub-lethal toxicity of organic dietary Zn in fish. Levels of Zn can be particularly high in some commercial diets, with up to 1162 mg kg⁻¹ reported in a larval diet (Tacon and De Silva, 1983).

In catfish and rainbow trout chelated Zn, Mn and Cu are all more bioavailable than the most highly available inorganic form, Zn sulphate (Sato et al., 1987a; Paripatananont and Lovell, 1997; Apines et al., 2001, 2003a,b; Apines-Amar, et al., 2004). The plasma ALP / plasma Zn ratios of the previous experiment (Chapter 3.) suggested that although Zn-pr was not absorbed to the same extent in the plasma it may be more available for metabolic processes post absorption. Amino acids may act as effective transfer molecules and may facilitate the transfer of Zn to Zn transporters for incorporation into enzymes (Apines et al., 2001). Although the previous study showed that digestibility and tissue deposition of organic and inorganic Zn were similar, further clarification is required to the availability of organic and inorganic Zn to their ultimate site of physiological requirement. Chelated Zn sources be metabolised differently post uptake from the GI tract and within cell compartments, as a result differences in toxicity may be observed.

The objectives of the present study were to determine the effects of dietary organic and inorganic Zn supplementation on the health of rainbow trout fed a practical diet. So that a thorough assessment of the potential benefits and sub-lethal toxic effects of dietary Zn could be determined, nutritional (125 mg kg⁻¹), supra-nutritional (312 mg kg⁻¹) and high (781 mg kg⁻¹) Zn supplementation of both Zn sulphate and Zn-pr was assessed. Seven diets comprising of six supplemented diets (three incremental levels of either Zn sulphate or Zn-pr) and a control basal practical diet were fed to rainbow trout for a period of 12-weeks. The effects of dietary Zn on, growth, Zn-status, immuno-competence, oxidative status, DNA

stability, haematological parameters, interactions with other essential trace elements and fillet quality were determined.

4.2. Methods

4.2.1. Experimental fish

400 rainbow trout were acquired and acclimated according to methods outlined in section 2.3. During the acclimation period fish were fed *ad libitum* a commercial trout grower diet (EWOS Micro 20P, Bathgate, West Lothian, UK).

4.2.2. Diets and experimental regime

Seven experimental diets differing in Zn content were based on a common fishmeal based experimental diet, specifically formulated to contain a low residual Zn content (Table 4.1.). Zn was supplied via a Zn variable premix added at 1 % (see 2.9.). Experimental diets were prepared at Dragon Feeds Ltd., Endeavour Close Industrial Estate, Port Talbot, UK. The treatments comprised of a basal un-supplemented diet and six diets formulated to contain three 2.5 x Zn increments; (125, 312, 781 mg kg⁻¹) of either Zn sulphate or Zn-pr giving a total of seven dietary treatments.

25 fish per tank, mean weight 25 ± 1 g (\pm SD) were randomly distributed amongst 14 experimental tanks. Each tank was allocated a dietary treatment, each consisting of two replicate tanks ($n=2$). Duplicate tanks were chosen due to constraints on the number of available experimental tanks; at least triplicate tanks would have otherwise been preferable. Fish were fed the experimental diet at 2 % biomass per day, maintained for a 12-week period (see 2.2. and 2.4.) and SGR and FCR determined (see 2.5.). Each diet and six initial fish (trace element analysis only) were subjected to proximate and trace element analysis (see 2.6., 2.7. and 2.15.). The proximate and trace element composition of each experimental diet is outlined in table 4.2. Despite attempts to make Zn sulphate and Zn-pr diets equimolar at each supplementation concentration, ANOVA and *post hoc* Fishers LSD show that these

reciprocal diets differed significantly in their Zn content ($p < 0.001$); the Zn-pr diets contained approximately one third additional Zn. This was subsequently found to be due to the supply of a 15 % Zn-pr which was labelled and supplied as containing 10 % Zn. This is also the cause of the Zn differences between supplemented diets in the first experiment (Chapter 3.).

Table 4.1.
Basal diet formulation.

Ingredient	g kg ⁻¹ diet
LT fishmeal ¹	640
Marine fish oil ²	150
Maize Gluten Meal ³	193.8
Vitamin premix ⁴	5
Zn variable premix ⁵	5
Zn free premix ⁶	5
Barox Plus ⁷	0.2
Astaxanthin ⁸	1

¹ Scottish LT fishmeal (blue whiting; capelin; herring): United fish products Ltd., Greenwell place, Aberdeen, UK.

² Seven Seas Ltd, Hull, UK.

³ Elliots farm, Venn Ottery, Ottery St. Mary, Devon, UK.

⁴ Skretting, Preston, Lancashire, UK.

⁵ Variable Zn premix supplementing 125, 312 or 718 mg kg⁻¹ ZnSO₄.7H₂O or Zn-Bioplex[®].

⁶ Zn free supplementing the following in mg kg⁻¹; Fe 34 (FeSO₄.7H₂O), Cu 5 (CuSO₄.5H₂O), I 6 (KI), Mn 15 (MnSO₄.H₂O).

⁷ Antioxidant from Kemin UK Ltd, Brigg, Lincolnshire, UK.

⁸ Astaxanthin: CAROPHYLL[®] Pink, DSM nutritional product (formerly F. Hoffmann-La Roche), Basel, Switzerland.

Table 4.2.

Proximate and trace element analysis (\pm SD) of experimental diets¹.

Added Zn ²	Basal	Zn-sulphate			Zn-proteinate		
	0	125	312	781	125	312	781
Moisture ³	5.5 \pm 0.2 ⁵	5.5 \pm 0.2	5.4 \pm 0.3	5.3 \pm 0.2	5.6 \pm 0.3	5.3 \pm 0.2	5.4 \pm 0.1
Protein ³	47.5 \pm 0.1	47.9 \pm 0.6	47.9 \pm 0.6	48.6 \pm 0.9	47.1 \pm 0.1	47.1 \pm 1.7	46.9 \pm 0.1
Lipid ³	20.1 \pm 0.1	20.3 \pm 0.1	20.5 \pm 0.1	20.6 \pm 0.6	20.2 \pm 0.1	20.6 \pm 0.1	20.1 \pm 0.1
Ash ³	6.1 \pm 0.4	6.1 \pm 0.9	6.3 \pm 0.3	6.0 \pm 0.9	6.2 \pm 0.8	6.3 \pm 0.6	6.2 \pm 0.4
Energy ⁴	23.2 \pm 0.4	23.2 \pm 0.0	23.3 \pm 0.3	22.9 \pm 0.1	22.9 \pm 0.6	23.4 \pm 0.0	23.0 \pm 0.1
Astaxanthin ²	103 \pm 1	103 \pm 3	102 \pm 6	100 \pm 3	97 \pm 4	101 \pm 6	105 \pm 4
Zinc²	67.4 \pm 0.7	149 \pm 1.6	404 \pm 3.2	935 \pm 8.8	238 \pm 1.6	544 \pm 6.8	1459 \pm 50
Copper ²	11.9 \pm 1.2	12.3 \pm 1.0	12.5 \pm 1.7	11.9 \pm 0.7	12.1 \pm 1.7	12.0 \pm 0.4	12.0 \pm 0.7
Manganese ²	37.2 \pm 2.9	38.5 \pm 2.4	39.3 \pm 1.9	38.9 \pm 0.9	39.3 \pm 1.8	37.5 \pm 0.3	39.9 \pm 1.0
Iron ²	233 \pm 19	235 \pm 16	230 \pm 12	231 \pm 11	230 \pm 16	223 \pm 6	226 \pm 9

¹ As fed (wet weight) basis.² Value in mg kg⁻¹.³ Value in %.⁴ Value in MJ kg⁻¹.

4.2.3. Analytical procedures

At the end of the 12-week period (by nature of the feeding regime this included one day feed withdrawal) fish were ethically anaesthetised (see 2.3.) and euthanized by cerebral destruction. Blood was sampled from six fish per tank and transferred to 300 μ l heparinised tubes for the immediate analysis of hematocrit (see 2.12.1.) and the preparation of blood smears (see 2.12.2.) for determination of leukocyte counts (see 2.12.3.) and nuclear abnormalities (see 2.12.4.). Whole blood was also aliquoted into vials containing freezing medium and stored at -80 °C for the analysis of DNA damage using the 'comet' assay (see 2.11.3.); to achieve cell DNA that could be effectively scored, electrophoresis and unwinding times were both reduced to 5 min. Serum was recovered from remaining heparinised blood and stored at -80 °C until determination of serum ALP (see 2.10. and 1.10.2.) and lysozyme (see 2.13.1.). 300 μ l was also aliquoted into EDTA treated tubes for the immediate determination of respiratory burst (blood kept at room temp ~20 °C) (see 2.13.2.). Plasma was retained from the remaining EDTA treated blood and stored at -80 °C until analysis of TAC (see 2.11.2.). The remaining fish carcasses were frozen at -40 °C for determination of percentage moisture (see 2.6.) and pooled (per tank) for whole body Zn analysis (see 2.7.). Blood was sampled from six further fish and pooled by tank for the determination of Zn (see 2.7.). Fish were then dissected for portions of the livers and pyloric caece (washed in 0.7 % NaCl), which were kept on ice until homogenisation in buffers corresponding to the assays of MDA (see 2.11.1.) and SOD (see 2.10.6.); homogenates were stored at -80 °C until analysis. The caudal fin was also removed and along with remaining liver tissue (both tissues pooled per tank) stored at -40 °C until trace element analysis (see 2.7.). Both fillets were prepared whole with the integument removed and stored at -40 °C until determination of fillet drip loss (see 2.14.) and astaxanthin (see 2.15.). Zn retention was determined according to method 2.8. Actual

supplemented Zn was determined by subtracting total Zn from the measured Zn content of the basal diet.

4.2.4. Statistical analysis

All statistical analyses were carried out according to methods outlined in section 2.16. SGR, FCR, whole body Zn, percentage mortality, and tissue element levels were all subjected to one-way ANOVA. Zn retention was modelled by a 3 phase exponential decay using Sigma plot version 10. All other data was analysed with a nested ANOVA; unless indicated no significant differences were observed between replicate tanks.

4.3. Results

4.3.1. Growth rates and feed performance

Fish more than quadrupled in weight from a mean (\pm SD) of 25 ± 1 g to 109 ± 9 g within the 12-week period. During the feeding trial mortality was high with a mean of 25 ± 10 % across treatments. Fish receiving the highest levels of dietary Zn sulphate and Zn-pr had the greatest percentage mortality; however percentage mortality was not significantly different between treatments (Table 4.3.).

Table 4.3.

Mean growth performance, feed utilisation and mortality of rainbow trout given various levels of supplemental Zn sulphate or Zn-pr for 12 weeks.

Zn ¹	SGR ²	FCR ³	Mortality ⁴
67	1.78	1.07	16
149 sulphate	1.91	0.93	20
404 sulphate	1.63	1.18	24
935 sulphate	1.50	1.20	38
238 proteinate	1.67	1.15	22
544 proteinate	1.74	1.07	22
1459 proteinate	1.56	1.21	30
<i>Pooled SEM</i>	<i>0.01</i>	<i>0.01</i>	<i>1.2</i>
⁵ <i>Dietary effect p=</i>	<i>0.062</i>	<i>0.125</i>	<i>0.280</i>

¹ mg kg⁻¹ Zn (wet weight) and supplemented source.

² SGR; specific growth rate ($n=2$).

³ FCR; feed conversion ratio ($n=2$).

⁴ Percentage mortality ($n=2$).

⁵ p = significance of dietary effects across treatments.

The best growth and feed performance was observed in the 82 mg kg⁻¹ Zn sulphate supplemented fish (projected supplementation level 125 mg kg⁻¹) and a trend towards decreased growth in any fish receiving >150 mg kg⁻¹ total Zn was observed. However, trends in growth performance and feed utilisation were not significantly different between treatments over the 12-week period (Table 4.3.).

4.3.2. Response of tissue and whole body zinc to supplemental zinc

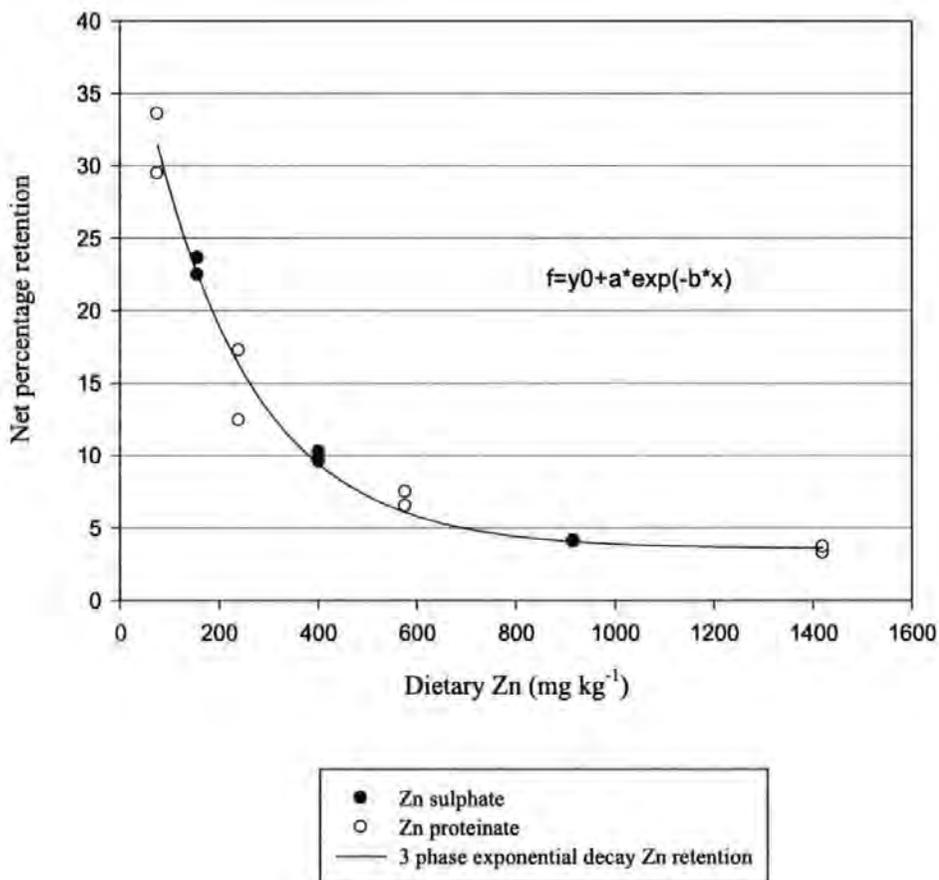


Figure 4.1. Percentage Zn retention in rainbow trout supplemented increasing levels of Zn-pr and Zn sulphate. Scatter graph showing percentage apparent net Zn retention ($n=2$) of rainbow trout given graded levels of either Zn-pr or Zn sulphate for 12 weeks. Percentage Zn retention of both Zn sources follow the same 3 parameter exponential decay (R^2 0.978 $p < 0.001$).

Zinc retention significantly decreased with increasing dietary Zn (Figure 4.1.) from 32 % in control fish to between 3.5 and 4.1 % in fish given the highest levels of dietary Zn-pr and Zn sulphate, respectively (Table 4.3.). The pattern of decreased Zn retention followed the same response for both Zn sources.

Table 4.4.

Mean net Zn retention and tissue Zn of rainbow trout given various levels of supplemented Zn sulphate and Zn-pr for 12 weeks.

Added Zn ¹	Zn retention ²	Caudal fin Zn ³	Whole blood Zn ⁴	Hepatic Zn ³
67	31.5 ^a	32.3 ^a	21.2 ^{ac}	25.4 ^a
149 sulphate	23.0 ^b	46.6 ^b	21.0 ^a	26.5 ^b
404 sulphate	9.9 ^{cf}	39.7 ^c	18.3 ^b	29.7 ^c
935 sulphate	4.1 ^{dg}	40.9 ^{cd}	20.2 ^{ab}	29.7 ^c
238 proteinate	14.8 ^c	41.8 ^d	19.6 ^{ab}	27.6 ^{bc}
544 proteinate	7.0 ^f	42.1 ^d	21.4 ^{ac}	25.1 ^c
1459 proteinate	3.5 ^e	48.3 ^e	23.5 ^c	31.2 ^d
<i>Pooled SEM</i>	<i>0.3</i>	<i>2.3</i>	<i>1.5</i>	<i>1.3</i>
⁵ <i>Dietary effect p=</i>	<i><0.001*</i>	<i><0.001*</i>	<i>0.002*</i>	<i><0.001*</i>

¹ mg kg⁻¹ Zn (wet weight) and supplemented source.

² percentage net retention based on wet body weight (*n*=2).

³ mg kg⁻¹ wet weight (*n*=2).

⁴ mg l⁻¹ whole blood (*n*=2).

⁵ *p*= significance of dietary effects across treatments (*n*=2). Different lower case superscript letters indicate significant (*p* ≤ 0.05) difference between dietary Zn treatments); * highlights significant difference.

Caudal fin Zn was significantly elevated in fish given Zn supplemented diets in comparison to those receiving the basal diet. In Zn sulphate supplemented fish, caudal fin Zn was significantly greater in those fish supplemented 82 mg kg⁻¹ Zn; increasing Zn

supplementation above this level (337 and 886 mg kg⁻¹) significantly decreased caudal fin Zn. This response was reversed in Zn-pr supplemented groups, with the 1392 mg kg⁻¹ Zn supplemented fish having significantly greater levels of Zn than those given 171 and 477 mg kg⁻¹ Zn-pr (Table 4.4.).

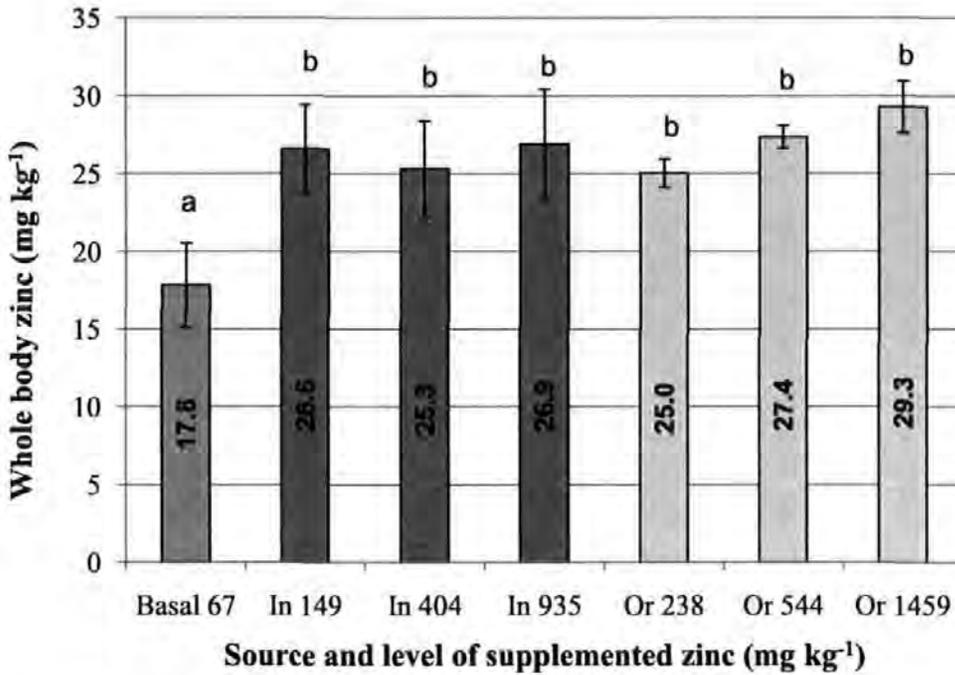


Figure 4.2. Response of whole body Zn to 12 weeks dietary Zn-pr and Zn sulphate supplementation. Bar graph with SEM bars ($n=2$) showing mean response of whole body Zn (mg kg⁻¹ wet weight) to various levels of supplemented Zn sulphate (In) and Zn-pr (Or) Zn (mg kg⁻¹). There is a significant difference in whole body Zn across treatments (ANOVA, $p < 0.01$). Significant differences ($p \leq 0.05$) between pair-wise comparisons of treatments are indicated by different lower case letters.

Whole blood Zn was only significantly increased above basal levels in fish given 1392 mg kg⁻¹ Zn-pr. Fish supplemented 337 mg kg⁻¹ Zn sulphate had a significantly lower whole blood Zn than control fish and those supplemented 82 and 886 mg kg⁻¹ Zn sulphate (Table 4.4.).

Dietary Zn had a significant effect on hepatic Zn. Zn supplementation at all levels and sources significantly increased hepatic Zn above that of fish fed the basal diet. Maximum hepatic Zn in inorganic groups was attained by 337 mg kg⁻¹ Zn sulphate supplementation. Of all Zn supplemented fish, 1392 mg kg⁻¹ Zn-pr supplementation resulted in the greatest elevation of hepatic Zn (Table 4.4.).

Whole body Zn significantly increased in all Zn supplemented fish over that of fish fed the basal diet. However, whole body Zn was not significantly affected by the level of supplemented Zn sulphate or Zn-pr (Figure 4.2.).

Table 4.5.

Mean activity of plasma ALP and SOD in rainbow trout given various levels of supplemented Zn sulphate and Zn-pr for 12 weeks.

Added Zn ¹	ALP ²	Cu/Zn-SOD ³
67	188	0.57
149 sulphate	200	0.56
404 sulphate	164	0.58
935 sulphate	177	0.59
238 proteinate	149	0.55
544 proteinate	171	0.61
1459 proteinate	168	0.51
<i>Pooled SEM</i>	5.5	0.16
⁴ <i>Dietary effect p=</i>	0.805	0.936

¹ mg kg⁻¹ Zn (wet weight) and supplemented source.

² Units l plasma⁻¹ (*n*=2).

³ Units SOD mg protein⁻¹ (*n*=2).

⁴ *p*= significance of dietary effects across treatments.

4.3.3. Response of zinc enzymes to zinc supplementation

Neither plasma alkaline phosphatase (ALP) nor hepatic Cu/Zn superoxide dismutase (Cu/Zn-SOD) responded to Zn supplementation (Table 4.5.).

4.3.4. Effect of zinc supplementation on markers of oxidative status

Total antioxidant capacity (TAC) was not significantly affected by either Zn-pr or Zn sulphate supplementation. There was a trend towards a decreased TAC with 886 mg kg⁻¹ Zn sulphate and an increased TAC in all Zn-pr supplemented diets but these differences were not significant (Table 4.6.).

Lipid peroxides derived from PUFA decompose to form a complex series of compounds including carbonyl compounds, of which MDA is the most abundant (Esterbauer et al., 1991). The lipid peroxidation product malondialdehyde (MDA) was not significantly modulated by Zn supplementation in either the pyloric caeca or the liver. MDA was more concentrated in the pyloric caeca than the liver (Table 4.6.).

Single strand DNA breaks as measured by the standard comet assay and purine base oxidation as determined by the FPG modified assay were not affected by Zn supplementation. There was a trend towards an increased percentage tail DNA in the FPG modified assay in all treatments but this was not significant (Table 4.6.).

Table 4.6.

Mean TAC, MDA and percentage tail DNA of rainbow trout given various levels of supplemented Zn sulphate and Zn-pr for 12 weeks.

Added Zn ¹	TAC ²	Pyloric caeca MDA ³	Hepatic MDA ³	Tail DNA buffer ⁴	Tail DNA FPG ⁵	⁷ Effect of FPG p=
67	144	109	41.8	21.5	23.2	0.664
149 sulphate	14	83	35.2	19.7	23.7	0.370
404 sulphate	143	91	52.0	20.6	27.2	0.159
935 sulphate	135	119	47.4	23.6	27.1	0.304
238 proteinate	151	117	4.1	24.8	26.8	0.625
544 proteinate	147	102	40.3	22.1	30.7	0.067
1459 proteinate	152	106	45.5	20.5	21.9	0.681
<i>Pooled SEM</i>	3	5	1.0	0.7	0.5	
⁶ Dietary effect P=	0.906†	0.332†	0.206	0.461	0.326†	

¹ mg kg⁻¹ Zn (wet weight) and supplemented source.

² Vitamin E equivalents (μmol l⁻¹) (n=2).

³ Malondialdehyde (MDA) in μM g tissue⁻¹ (n=2).

⁴ Percentage tail DNA determined by standard assay (n=2).

⁵ Percentage tail DNA determined by FPG modified assay (n=2).

⁶ p= significance of dietary effects across treatments; † indicates significant difference between replicate tanks (p ≤ 0.05).

⁷ Denotes significance between mean percentage tail DNA in normal and FPG modified comet assay.

4.3.5. Effect of zinc supplementation on immuno-competence

Markers of immuno-competence were not affected by Zn supplementation. The generation of superoxide during the respiratory burst of leukocytes within the blood showed high inter-individual variation. Although the highest levels of superoxide in whole blood were observed in fish supplemented 337 mg kg⁻¹ Zn sulphate and 171 mg kg⁻¹ Zn-pr, differences among treatments were not significant. In addition, both lysozyme and total leukocyte counts were not modulated by the dietary Zn treatments (Table 4.7.).

Table 4.7.

Mean immune parameters, respiratory burst, lysozyme activity and leukocyte counts, of rainbow trout given various levels of supplemented Zn sulphate and Zn-pr for 12 weeks.

Added Zn ¹	Respiratory burst ²	Lysozyme ³	Leukocyte counts ⁴
67	4.0	691	67
149 sulphate	4.2	596	68
404 sulphate	5.8	606	70
935 sulphate	5.3	779	54
238 proteinate	5.7	678	58
544 proteinate	4.9	587	60
1459 proteinate	4.8	544	67
<i>Pooled SEM</i>	<i>1.5</i>	<i>23</i>	<i>2</i>
⁵ <i>Dietary effect p=</i>	<i>0.685</i>	<i>0.136†</i>	<i>0.517</i>

¹ mg kg⁻¹ Zn (wet weight) and supplemented source.

² Integral relative light units x 10⁴ (n=2).

³ Units lysozyme ml⁻¹ (n=2).

⁴ Total leukocytes per 1000 cells (n=2).

⁵ p= significance of dietary effects across treatments. † indicates significant difference between replicate tanks (p ≤ 0.05).

4.3.6. Response of haematological parameters to zinc supplementation

There was a trend towards a slightly decreased percentage hematocrit in fish supplemented 886 mg kg⁻¹ Zn sulphate and 477 and 1392 mg kg⁻¹ Zn-pr in comparison to all other treatments, but these differences were not significant. There was a trend towards an increased frequency of micronuclei in fish fed the un-supplemented diet, and a higher number of blebbed nuclei in fish given the highest levels of both Zn-pr and Zn sulphate. However these marginal differences were not significant (Table 4.8.).

Table 4.8.

Mean hematocrit and nuclear abnormalities of rainbow trout given various levels of supplemented Zn sulphate and Zn-pr for 12 weeks.

Added Zn ¹	Hematocrit ²	Micronucleus ³	Notched ³	Blebbed ³
67	32.9	1.3	8.9	1.6
149 sulphate	34.3	0.8	9.2	2.0
404 sulphate	34.7	1.0	7.4	1.5
935 sulphate	29.5	0.6	5.4	3.9
238 proteinate	36.4	1.1	6.1	1.5
544 proteinate	30.5	0.8	9.4	2.8
1459 proteinate	31.4	0.8	6.5	3.4
<i>Pooled SEM</i>	0.2	0.1	0.3	0.3
⁴ Dietary effect <i>p</i> =	0.287	0.475	0.538	0.155†

¹ mg kg⁻¹ Zn (wet weight) and supplemented source.

² percentage hematocrit (*n*=2).

³ nuclear abnormalities per 1000 erythrocytes (*n*=2).

⁴ *p*= significance of dietary effects across treatments; † indicates significant difference between replicate tanks (*p* ≤ 0.05).

4.3.7. Effect of supplemental zinc on fillet quality

There was a trend towards a reduced deposition of the carotenoid astaxanthin in fish given higher levels of Zn sulphate (337 and 886 mg kg⁻¹) in comparison to other treatments, however this was not significant. Fillet water loss during 48 hours at 4 °C was not significantly affected by dietary Zn supplementation (Table 4.9.)

Table 4.9.

Mean fillet quality, astaxanthin and water retention, of rainbow trout given various levels of supplemental Zn sulphate or Zn-pr for 12 weeks.

Added Zn ¹	Astaxanthin ²	Water loss ³
67	3.1	8.4
149 sulphate	2.9	6.2
404 sulphate	2.5	8.4
935 sulphate	2.5	8.5
238 proteinate	2.8	8.6
544 proteinate	3.1	7.0
1459 proteinate	2.8	7.7
<i>Pooled SEM</i>	<i>0.1</i>	<i>0.2</i>
⁴ <i>Dietary effect p=</i>	<i>0.055</i>	<i>0.108</i>

¹ mg kg⁻¹ Zn (wet weight) and supplemented source.

² mg kg⁻¹ wet weight (*n*=2).

³ Percentage water loss over 48 hours at 4 °C (*n*=2).

⁴ *p*= significance of dietary effects across treatments.

4.3.8. Interactions between dietary zinc and other hepatic trace elements

Both sources of dietary Zn significantly interacted with hepatic Mn (Figure 4.3.). No significant interaction was observed between dietary Zn sulphate or dietary Zn-pr and hepatic Fe (Figure 4.4.). A trend towards a slight decrease in hepatic Cu was observed with increasing dietary Zn but this was not significant for either Zn sulphate or Zn-pr (Figure 4.5.).

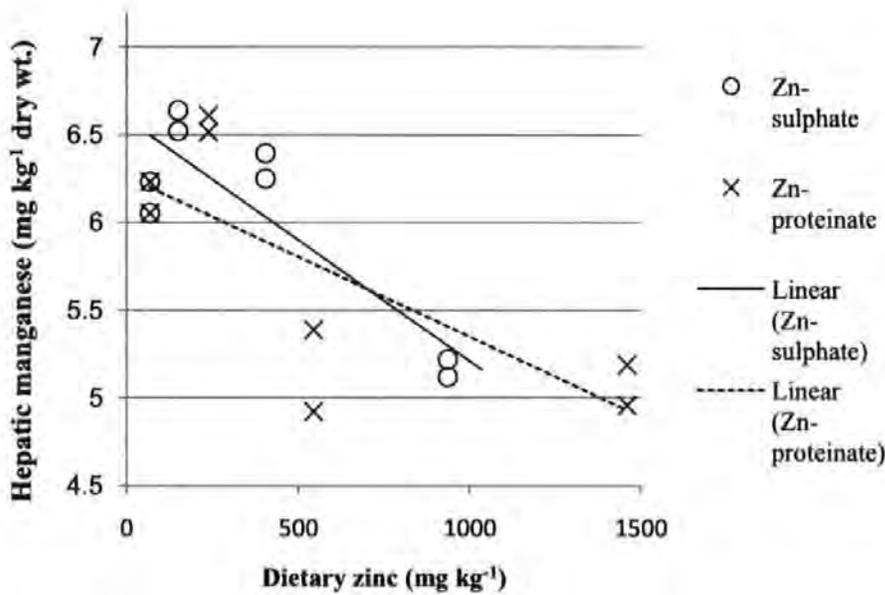


Figure 4.3. Response of hepatic manganese to various levels of dietary Zn sulphate and Zn-pr supplemented for 12 weeks in rainbow trout. Linear regression analysis ($n=2$) shows a decreasing hepatic Mn concentration in response to increasing Zn sulphate (R^2 0.453, p 0.001) and Zn-pr (R^2 0.573, p <0.001) supplementation.

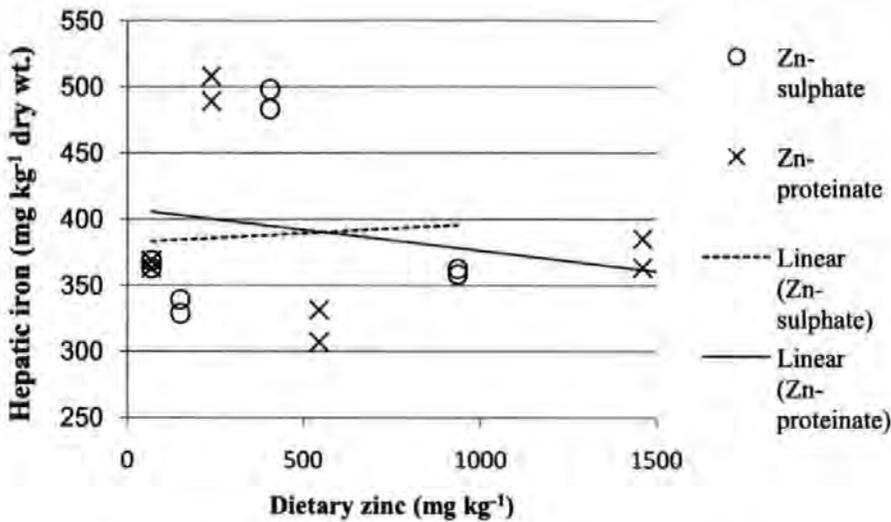


Figure 4.4. Response of hepatic iron to various levels of dietary Zn sulphate and Zn-pr supplemented for 12 weeks in rainbow trout. Linear regression analysis ($n=2$) shows no significant response of hepatic Fe in response to increasing Zn sulphate (R^2 0.173, p 0.097) or Zn-pr (R^2 0.037, p 0.443) supplementation.

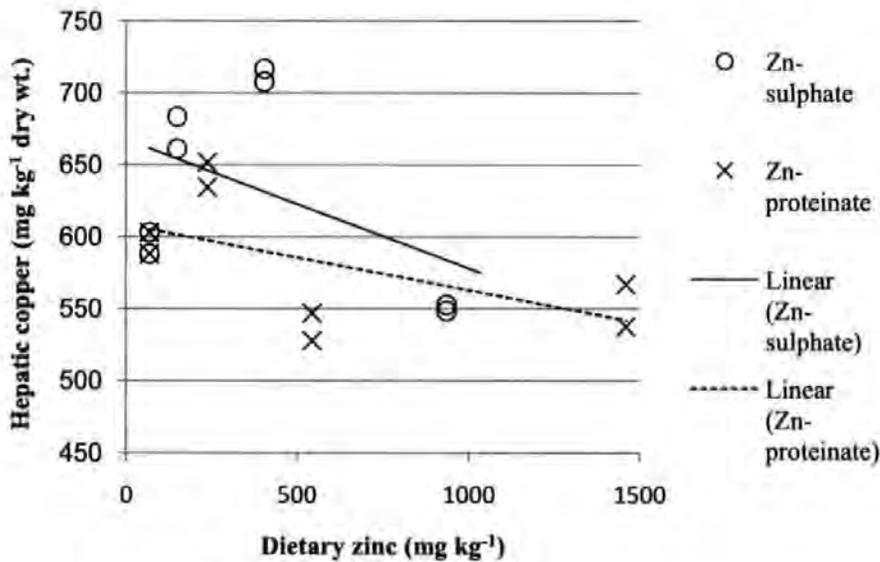


Figure 4.5. Response of hepatic copper to various levels of dietary Zn sulphate and Zn-pr supplemented for 12 weeks in rainbow trout. Linear regression analysis ($n=2$) shows a trend towards a decreased hepatic Cu in response to Zn supplementation but this is not significant for either Zn sulphate (R^2 0.013, p 0.650) or Zn-pr (R^2 0.188, p 0.063).

4.4. Discussion

4.4.1. Homeostatic regulation of supplemented dietary zinc

The trends in whole body retention of the present study suggest that the uptake of both Zn sulphate and Zn-pr are similar. Previous studies in rainbow trout have shown organic Zn sources (Zn-AA) are retained more effectively than Zn sulphate (Apines et al., 2001). Chelated Zn may assist the movement of Zn to the cell and for storage as amino acids have a unique ability of to act as Zn transfer molecules. In rainbow trout L-cysteine increases post-intestinal Zn transfer into the plasma either by drawing Zn into the plasma or acting as a Zn chelate (Glover and Hogstrand, 2002b) and histidine may facilitate Zn transport across the brush border membrane by either acting as a donor molecule to intestinal Zn transporters or forming a transportable Zn-histidine complex (Glover et al., 2003). Although the increased utilisation of some amino acid chelates and complexes has been observed in terms of uptake and enzyme activity in trout (Apines et al., 2001), the data of the present study show that there is little difference between the utilisation of Zn-pr and Zn sulphate. Zn complexes and chelates may remain intact in the acidic stomach environment and be ultimately be absorbed as amino acids or peptides. However, in the acidic stomach environment of the GI tract Zn ions may dissociate from complexes and chelates, particularly in fish species with a stomach including salmonids. As pH is increases in the anterior intestine, from the highly acidic environment of the stomach any disassociated Zn ions will undergo the same fate as inorganic Zn, including the formation of insoluble complexes that may not be absorbed.

The decreased retention of dietary Zn with increasing dietary Zn loading has been previously reported in rainbow trout, (Wekell et al., 1983) and this study further reveals that the response of Zn retention to supplemented dietary Zn is comparable between Zn sulphate and Zn-pr. Whole body Zn values were typical ($\sim 30 \text{ mg kg}^{-1}$) of that found in Atlantic salmon (Maage et

al., 2001) and rainbow trout (Kjoss et al., 2006), but did not reflect the 20 fold supplementation of Zn. This has similarly been reported in rainbow trout and turbot supplemented up to 1000 mg kg⁻¹ Zn from Zn sulphate, Zn-pr, Zn-methionine and Zn-oxide in practical diets containing 91 to 102 mg Zn kg⁻¹ (Kjoss et al., 2006; Overnell et al., 1988). A similar plateau of Zn accumulation in the liver (and muscle) has also been reported in Nile tilapia (Do Carmo et Sa et al., 2005). Of the tissues assessed all have been shown to respond to dietary Zn. However, this study confirms that whole body (Shearer, 1984; Maage and Julshamn, 1993; Schwarz, 1995), caudal fin, and whole blood (Wekell et al., 1983) Zn levels are only responsive within marginal dietary Zn levels.

The response of tissues and whole body Zn to increased dietary Zn in the present study agrees with the strict homeostatic regulation of both dietary Zn-pr and Zn sulphate in salmonids. The effective maintenance of Zn homeostasis is also documented in humans (King et al., 2000). Interestingly in carp, which have a different gastrointestinal physiology with regard to stomach pH than salmonids, whole body Zn increases linearly up to 1974 mg kg⁻¹, which highlights differences in Zn utilisation between fish some species (Jeng and Sun, 1981; Sun and Jeng, 1998). The distribution, intracellular metabolism and excretion mechanisms of Zn are still relatively poorly understood (Hogstrand and Wood, 1996). However, the findings of the present study are consistent with a known maintenance of internal (Spry et al., 1988) and cellular (Bury et al., 2003) Zn over a wide range of waterborne and dietary Zn in rainbow trout.

The GI tract plays an important role in the regulation of dietary Zn, which appears to be effective up to 10 times (>1000 mg kg⁻¹) the normal level of dietary Zn. Zn uptake via the intestinal tract in rainbow trout is now known to only involve a saturable low affinity high capacity active carrier mediated mechanism, which may its self regulate sub-epithelial Zn

accumulation (Glover and Hogstrand, 2002a). In humans, expression of Zn transporters in the gut is down regulated with increasing dietary Zn (Cragg et al., 2005). Due to its polyanionic nature, mucus has a large metal binding capacity at neutral pH (Handy et al., 1989) and is also postulated to play a role in Zn regulation. High dietary Zn may result in an intestinal muco-secretory response, which regulates Zn by preventing Zn ions reaching the uptake surface. The regulatory role of mucus is also thought to occur via shedding of mucus containing Zn, thus allowing surplus Zn to be excreted (Glover and Hogstrand, 2002a); this also occurs with potential contaminants at other uptake surfaces in fish (Shephard, 1994). In contrast, mucus may also facilitate Zn absorption during deficiency by accumulating Zn close to the intestinal epithelium where a pH gradient acts to release it (Glover and Hogstrand, 2002a).

The sequestration of Zn into less active skeletal tissues including the scales and vertebrae are also postulated to contribute to dietary Zn homeostasis (Clearwater et al., 2002). In Atlantic salmon, sequestered Zn in the skeletal tissue and the GI tract may be mobilised during deficiency (Maage and Julshamn, 1993), however this was not found in the vertebrae of salmon by Vangen and Hemre (2003). In rats the skeletal tissues are regarded a Zn 'sink' rather than a store, as Zn deficiency does not promote Zn removal from the bone (Murray and Messer, 1981). Zn transporter (family of ZIP and ZnT proteins) genes also play an important role in teleost Zn homeostasis, being up or down regulated depending on Zn levels; the pattern of gene regulation varies considerably amongst tissues (Feeney et al., 2005).

4.4.2. Response of growth and zinc enzymes to zinc supplementation

Dietary Zn supplementation had no appreciable effect on SGR, or FCR in the present study and confirms that growth is not affected by supplemental Zn in fish fed practical diets containing >50-60 mg Zn kg⁻¹ (Sato et al., 1987a,b). As growth does not respond above

marginal levels of dietary Zn, growth is not deemed an appropriate index of Zn bioavailability in studies utilising practical diets. Growth has frequently been used to assess Zn status in fish as it is reduced during Zn deficiency (Gatlin and Wilson, 1984; Ogino and Yang, 1978; Watanabe et al., 1980, Hidalgo et al., 2002). Growth and feed utilisation may be affected through the involvement of Zn in DNA transcription factors such as DNA polymerase, which require Zn (Dreosti, 2001) and by a reduced protein utilisation through the decreased activity of Zn dependent pancreatic carboxypeptidases in the GI tract (Ramseyer, 1999). In rainbow trout supplemented Zn, an increased expression of DNA polymerase is concurrent with increased growth, which may result from an enhanced rate of protein synthesis (Apines et al., 2003a; Apines-Amar et al., 2004).

The lack of effect of supplemented Zn on growth and ALP activity strongly suggests that the basal diet of the present study ($67.4 \text{ mg Zn kg}^{-1}$) met established dietary Zn requirements (Ogino and Yang, 1978). The lack of effect of supplemental Zn on growth in fish fed practical diets has also been reported by Lorentzen and Maage (1999) with Zn sulphate and Wekell et al., (1983) with both Zn sulphate and Zn-pr. This suggests that despite the presence of anti-nutritional factors in practical diets, some such diets contain sufficient residual Zn to meet the requirements of trout.

ALP is a suitable indicator of Zn status (Swinkels et al., 1994) as it decreases during Zn deficiency (Reinhold and Kfoury, 1969). ALP is synthesised in the bone, liver, and gut and is subsequently released into the circulatory portal (Kaplan, 1972), which makes it particularly relevant to the measurement of Zn status given the key roles these organs play in Zn regulation. However, as found in the present study, plasma ALP activity stabilises in Zn replete catfish (Gatlin and Wilson, 1983), tilapia (Do Carmo e Sa et al., 2004) salmon (Maage

and Julshamn, 1993) and rainbow trout (Ramseyer et al., 1999). This is expected to have contributed to the lack of any difference observed between treatments in the present study.

No alterations in Cu/Zn-SOD were found in the present study. In rainbow trout, Zn deficiency decreases Cu/Zn-SOD isoenzymes II and III, whilst isoenzyme I remains conserved. The decrease of these Cu/Zn-SOD isoenzymes is not due to a repression of encoding genes but either due to the lack of Zn being inserted during post-trans-locational modification or loss of the metal and a subsequent loss in activity (Hidalgo et al., 2002). Hepatic Cu/Zn-SOD activity correlates well to dietary Cu but it appears that this is not the case above marginal dietary Zn for catfish (Gatlin et al., 1989) or rainbow trout. In rainbow trout, Cu/Zn-SOD correlates with dietary Zn but only in diets also containing high (495 mg kg⁻¹) Cu. This is expected to be due to the antagonistic effect of dietary Cu on dietary Zn (Knox et al, 1984), however no such interaction was observed in the present study. The lack of effect of Zn on Cu/Zn-SOD consequently suggests dietary Zn levels were above those considered marginal. Synthesis of Cu/Zn-SOD includes the addition of a Zn ion during post-trans-locational modification. Zn plays a secondary structural role in the Cu/Zn-SOD and the Cu ion functions in electron transfer. Unlike the Zn ion, the Cu ion can not be replaced without loss of activity by any other ion (Fridovich, 1986). High Zn supplementation has been observed to decrease Cu/Zn-SOD in rats (Labbe and Fischer, 1984), but this did not occur in the present study, which is consistent with the efficient homeostatic Zn regulation observed. Interestingly, due to its capacity to bind relatively large amounts of Cu and Zn, Cu/Zn-SOD is thought to play a role in cellular Zn homeostasis in mammals (Wei et al., 2001). Cu/Zn-SOD mRNA expression is higher in amino acid chelated Cu, Zn and Mn trout than equivalent sulphate supplemented fish (Apines-Amar et al., 2004), which suggests some organic mineral sources may be more available for incorporation into SOD, however this may only be observed in fish given marginal levels of these elements.

This study shows that the stabilisation of ALP activity occurs before the stabilisation of tissue Zn, consequently comparisons between Zn sources need to be assessed below the saturation level of ALP activity and possibly Cu/Zn-SOD activity. Indeed it appears that the majority of studies that have revealed any differences between organic and inorganic Zn sources using ALP activity have been those utilising purified diets with deficient to marginal Zn levels. The homeostasis of Zn and its implication in the comparison of Zn sources has also been documented in mammals (Swinkels et al., 1994). As tissue enzyme activity and tissue Zn respond best to deficient dietary Zn levels, future studies evaluating differences between organic and inorganic Zn should include a range of treatments within marginal Zn.

4.4.3. Zinc supplementation and oxidative status.

An assessment of dietary Zn on health was made as increased tissue Zn levels may not necessarily reflect Zn available for crucial metabolic processes. TAC, lipid peroxidation and DNA instability are all measures of oxidative status that have been shown to be modulated by Zn. Unlike Se, Zn is not directly required for the activity of any antioxidant enzymes; SOD activity can still occur without the presence of the Zn ion (Fridovich, 1986). Nevertheless, Zn acts as an antioxidant indirectly via; the induction of metallothioneins, the protection of enzyme sulphhydryl groups, and antagonism of redox active transition metals, particularly Fe and Cu (Powell et al., 2000). In addition, metallothioneins have been shown to scavenge superoxide radicals produced by xanthine/xanthine oxidase reactions and hydroxyl radicals (Kang, 1999). Consequently Zn deficiency results in increased oxidative injury induced by oxidative stress in mammals (Powell, 2000; Sefanidou et al., 2006) and fish (Hidalgo, et al., 2002). In rainbow trout, Zn deficiency induces hepatic and intestinal lipid peroxidation (Hidalgo et al., 2002). Despite the role of Zn in the maintenance of optimal oxidative status,

these parameters were not affected by supplemented dietary Zn in the present study, which is consistent with the fact that dietary Zn in the basal diet was not marginal.

The protective effect of Zn against lipid peroxidation is well demonstrated by studies in rats, which show that Zn deficiency increases MDA (Yousef et al., 2003). Zn supplementation reduces MDA production induced by ethanol and chlorpyrifos (organophosphorus insecticide) exposure (Goel et al., 2005; Al-Damegh, 2007). In rainbow trout fed a fishmeal diet (47.6 mg kg^{-1}) supplemented with 30 and 60 mg kg^{-1} Zn from Zn-picolinate, MDA in the serum, liver and whole body is reduced (Kucukbay et al., 2006). The increased MDA in the GI tissue over that of hepatic tissue found in the present study is postulated to be a result of a higher proportion of PUFA in the GI tract tissues.

As many degenerative diseases are in part caused by damage to DNA, optimal requirements of minerals should be defined as those preventing damage to both nuclear and DNA damage (Fenech, 2002). The FPG modified comet assay has been successfully used in fish erythrocytes in the previous study and that of other authors for the assessment of oxidative stress resulting from genotoxic compounds (Akcha et al., 2003) and exhaustive exercise (Aniagu et al., 2006). Chromosome breaks may be caused by increased oxidative damage, due to an excess of free radicals, or loss of activity of fapy glycosylase, which is involved in the repair of oxidised guanine (Ames, 2001). In rats, Zn deficiency results in increased single DNA strand breaks (Castro et al. 1992). Tail DNA values obtained from frozen erythrocytes in the present study were higher than previously found using fresh cells, consequently unwinding and electrophoresis times were decreased. However, it is suspected that due to excessively high background levels and significant *in vitro* sensitivity to assay conditions, *in vivo* effects of DNA damage were not discernable. Six weeks freezing significantly increased the sensitivity of DNA in the present study but previously 18 hours freezing did not

significantly increase DNA damage (see 8.3.). This suggests that DNA stability is decreased during long term storage using the current cryo-preservation protocol, and is thus unsatisfactory for the cryo-preservation of trout erythrocytes. Similar complications have also been reported using turbot tissues and erythrocytes (Belpaeme, et al., 1998). This effect may be augmented in fish erythrocytes over other cell types including hepatocytes due to an increased number of alkali liable sites. Some cell types are associated with a high control response in the comet assay (Mitchelmore and Chipman, 1998) with DNA packaging and background alkali-liable sites postulated to be the cause rather than endogenous single strand breaks (Singh et al., 1989). Storage protocols need to be established for the cryo-preservation of erythrocytes for analysis by the comet assay. Although a proven marker of oxidative DNA damage, without the ability to either store or a large number of samples or develop a high throughput assay, the use of this assay will be limited in nutritional feeding trials. The effect of Se (and Zn) supplementation on the stability of DNA in both stressed and un-stressed fish may be a sensitive marker of the requirements of these nutrients in fish as well as mammals (Fenech, 2002).

TAC is a measure of the total number of water and lipid soluble antioxidant compounds including a 70 % contribution by uric acid (in humans) (Nourooz-Zadeh et al., 2006). Zn is not directly involved in any antioxidant enzymes (Powell, 2000) and no evidence of oxidative stress was observed, which may explain the lack of effect of dietary Zn on TAC in the present study.

4.4.4. Zinc supplementation and immuno-competence

No effect was observed by dietary Zn or source on parameters of immuno-competence in the present study. Despite its requirement by both the innate and adaptive immune system (Fracker et al., 1986) few studies have assessed the role of Zn in piscine immunity (Blazer,

1992). Supplementing dietary Zn above requirements for growth did not affect resistance to bacterial kidney disease (Bell et al., 1984). Deficient catfish and sockeye salmon (*Oncorhynchus nerka*) showed decreased mortality after inoculation of *A. hydrophilia* (Scarpa et al., 1992) and *Renibacterium salmonarium* (Bell et al., 1984), respectively and is expected to be due to a decreased replication of pathogens and reduced formation of virulence factors due to a depleted plasma Zn; as similarly occurs with Fe. A multivalent bacterin vaccination (*Aeromonas salmonicida*, *Vibrio anguillarum*, *V. ordalii* and *V. salmonicida*) in rainbow trout results in hypozicaemia, which is thought to be an innate defence against bacterial pathogens requiring Zn for their metabolism and replication (Simko et al., 1999).

Other studies do show a requirement for Zn in the piscine immune system. Catfish fed semi-purified diets required Zn for resistance against *E. ictaluri* (Paripatananont and Lovell, 1995b). Paripatananont and Lovell (1995a) also reported a greater potency of Zn-Met than Zn sulphate in restoring disease resistance and growth. In rainbow trout cytotoxicity by natural killer-like activity of leukocytes is depressed by Zn deficiency and restored in Zn replete fish (Inoue et al., 1998). Leukocyte counts in Nile tilapia have been shown to increase linearly with increasing dietary Zn up to 100 mg kg⁻¹ (Hidalgo et al., 2002), which is not surprising due to the essentiality of Zn in the immune system; a highly proliferating cell system (Shankar and Prasad, 1998; Rink and Gabriel, 2000). Lysozyme is an important innate defence enzyme (Saurabh and Sahoo, 2008) and was not affected by Zn in the present study. However, its activity in Zn deficient fish has yet to be determined. In rainbow trout exposed to 10 and 50 µg l⁻¹ waterborne ZnCl₂ for 30 days lysozyme increases (Sanchez-Dardon et al., 1999). However in the aforementioned study Zn deficiency cannot be discounted as a reason for increased lysozyme activity as dietary Zn was not disclosed and the effect of Zn deficiency on lysozyme is unknown. The only studies pertaining to superoxide anion generation by macrophages is by 21-30 days waterborne Zn exposure where additional Zn

had no effect; this was associated with no increase in gill Zn (Dethloff et al., 1999; Sanchez-Dardon et al., 1999). The present study shows no beneficial or immuno-repressive effects of dietary Zn supplementation, which again may be explained by the strict homeostatic regulation of supplemented Zn in the present study.

4.4.5. Effect of dietary zinc on haematological parameters

Zinc deficiency is known to reduce hematocrit in Nile tilapia (Do Carmo e Sa et al., 2004) and rainbow trout (Spry et al., 1988). In rainbow trout, chelated Mn, Cu, and Zn increased hematocrit to a greater extent than sulphate minerals and is postulated to be due to the role of Zn containing transcription factors in erythrocyte development or hematopoiesis (Apines-Amar et al., 2004). However, decreased hematocrit has been reported in catfish given 200 mg kg⁻¹ dietary Zn sulphate (Gatlin et al., 1989). As no deficiency was observed in the present study a lack of effect on hematocrit is expected. In contrast to Gatlin et al. (1989) in catfish, high Zn supplementation did not result in a markedly reduced hematocrit; a decrease was observed in fish given 886 mg kg⁻¹ Zn from Zn sulphate, but this was not significant.

Despite the important role of Zn in DNA replication (Dreosti, 2001), few studies have assessed micronuclei frequency in relation to dietary Zn. The effect of dietary Zn and micronuclei (the loss of genetic material during mitosis) induction has not been studied in fish. In rats, Zn deficiency does not induce micronuclei (Morgan et al., 1995), but high levels of intraperitoneally administered Zn have been shown to increase micronuclei frequency (Piao, et al., 2003). Similarly, this has been observed in rainbow trout exposed to mixtures of waterborne Zn and Cu, and may be due to effects of Zn on the sulphhydryl groups of tubulin, which is involved in chromosome segregation (Bagdonas and Vosyliene, 2006). No effect of Zn on micronuclei frequency was observed in the present study and may be explained by the tighter regulation of dietary Zn than waterborne Zn in trout (Clearwater et al., 2002). Blebbed

nuclei (nucleoplasmic bridges) are proposed to form when the centromeres of dicentric chromatids are pulled to opposite poles of the cell at anaphase and have been validated as biomarkers of DNA damage in humans (Fenech and Crott, 2002). Blebbed nuclei and notched nuclei have been used in addition to micronuclei for the assessment of cytotoxic compounds in fish (Cavas and Ergene-Gozukara, 2003; Souza and Fontanetti, 2006; Guilherme et al., 2008). The trend towards increased blebbed nuclei in the erythrocytes of fish given the highest dietary Zn warrants further investigation with regard to the possible effect of excess Zn on cytokinesis.

4.4.6. Zinc supplementation and fillet quality

Nutrition is known to have several effects on fillet quality including colour, appearance, odour, taste, shelf life and texture (Lie, 2001). Astaxanthin, the major carotenoid in salmonids, can account for a significant cost of feed ingredients and is poorly utilized due to poor intestinal absorption and fillet retention. Its absorption can be marginally increased with additional dietary α -tocopheryl acetate (Bjerkeng et al., 1999; Storebakken and No, 1992). Astaxanthin, like antioxidant vitamins, is prone to lipid peroxidation (Bustos et al., 2003) and consequently may be protected both pre and post absorption by a diet rich in antioxidants. The present study found no such evidence with regard to Zn.

Zn has a key role in the stabilisation of biological molecules and deficiency is known to lead to the destabilisation of membranes and increased lipid peroxidation (Stefanidou et al., 2006). Muscle lipid peroxidation is known to occur in Zn deficient rainbow trout (Hidalgo et al., 2002), which may affect the post mortem quality of fish fillets. Muscle tissue contains 70-75 % water, of which 90 % is bound to proteins, some of which is lost post mortem due to structural and biochemical changes that affect the water holding ability of muscle proteins (Honikel, 1987). In the present study no effect was found with Zn and fillet water holding

capacity. Similar to the findings of the present study, in cattle there is no effect of dietary Zn level or Zn source on Zn status, muscle Zn or drip loss (Kessler et al., 2003).

4.4.7. Zinc supplementation and interactions with other essential elements

Supplementing dietary elements in isolation can significantly modulate the status of other trace elements via nutrient interactions. Minerals with similar chemical properties may vie for transport proteins or other uptake mechanisms in addition to competing for organic ligands, which may facilitate or hamper absorption (Sandstrom, 2001). Negative interactions between dietary Zn and whole body Fe (Wekell et al., 1986), and dietary Zn and hepatic Cu have been previously reported in fish (Knox et al., 1984). However, other studies have observed no significant interactions between dietary Zn and Fe or Cu in Atlantic salmon (Maage and Julshamn, 1993), rainbow trout (Kucukbay et al., 2006) or catfish (Gatlin et al., 1989). Dietary Zn does not interact with Co, K or P (Wekell et al., 1986). Some of these 'so called' interactions may be a recovery from Zn deficiency rather than a negative effect of dietary Zn. Studying interactions at marginal levels should be treated with caution as such animals are not physiologically normal, and may be the cause of so called interactions. For instance, Zn deficiency is known to lead to mucosal dystrophy, which may affect the uptake of other nutrients (Sandstrom, 2001). In the present study, in which fish were not Zn deficient only an interaction between dietary Zn and hepatic Mn was observed. No Zn/Mn interactions in the whole body were reported by Kucukbay et al. (2006) using Zn-picolinate, which may be due to a difference in the behaviour of this organic Zn source in the GI tract. However, due to the compartmentalization of metal burdens, whole body metal levels may not accurately reflect trace element interactions (Glover and Hogstrand, 2003).

4.4.8. Zinc supplementation and toxicity

The regulation of dietary Zn is expected to be a key factor in the lack of toxicity of either supplemented organic or inorganic Zn observed in the present study. Zn toxicity occurs via two principal mechanisms; mortality may result from either hypoxia caused by gross morphological damage to the gills or impairment of Ca uptake causing hypocalcemia, which may become terminal (Hogstrand and Wood, 1996). Wekell et al. (1983) only observed 2 % mortality in fish given 1700 mg kg⁻¹ Zn. The lack of a significant difference in mortality between treatments indicates that the high mortality observed in the present study was not directly related to dietary Zn. However, as there was a trend towards elevated mortality in fish given high Zn, it may have exacerbated it. Supplementing up to 1000 mg kg⁻¹ Zn sulphate and Zn-pr do not result in any signs of toxicity after 15 days in rainbow trout (Kjoss et al., 2006). The cause of high mortality in the present study was not identified. Post-mortem examination revealed some haemorrhaging of the GI tract in some fish, which may have been a result of irritation by the diet or an unidentified pathogen. Recovered moribund and deceased fish were largely bigger fish, which may indicate a dietary factor, as larger fish are expected to consume greater amounts of feed; however this was not confirmed and the presence of a disease can not be rule out.

4.4.9. Conclusions

The study found a lack of any significant benefit or toxicity on Zn status and measured health parameters by either supplemented organic or inorganic Zn. This may be attributed to the homeostatic regulation of both Zn sulphate and Zn-pr. At the dietary Zn levels used, the lack of difference between organic and inorganic Zn sources is consistent with similar previous studies assessing the efficacy of inorganic and chelated Zn above marginal levels (Li and Robinson, 1996; Lim, et al., 1996; Magge et al., 2001; Kjoss, 2006). In the present study Zn

supplementation of a practical diet did not optimise Zn dependent enzymes and health markers. As trout effectively regulate both organic and inorganic dietary Zn, additions to a practical diet between $\sim 150 \text{ mg kg}^{-1}$ and above the E.U. limit of 200 mg kg^{-1} are superfluous. The study provided no evidence that organic Zn is any more or less efficacious than Zn sulphate. However, any difference in efficacy between sources may have been masked by 'diminished returns' due to the homeostatic regulation of supplemented Zn. Future studies should evaluate the bioavailability of different Zn sources in diets containing marginal levels of Zn (Swinkels et al., 1994). The thorough assessment of the efficacy of different Zn sources should involve the determination of optimal, rather than minimal, requirements and should consist of a battery of relevant indicators.

CHAPTER 5.

PRELIMINARY INVESTIGATIONS INTO THE EFFECT OF CHRONIC HUSBANDRY RELATED STRESSORS ON OXIDATIVE STATUS AND IMMUNOLOGICAL FUNCTION

Hypothesis: Chronic husbandry related stressors will induce a primary stress response and will impair oxidative status and immuno-competence.

5.1. Introduction

The intensive culture of finfish is typically associated with various stressors including those arising from husbandry, such as overcrowding and physical disturbance. Due to the nature of intensive fish production, reared fish can be subjected to various husbandry stressors, which result in the manifestation of a physiological 'stress response' (Pickering, 1992). The stress response evolved to enable fish to cope with hostile environments. However, in aquacultural conditions where no escape is possible, the stress response may no longer be beneficial, but can in fact become damaging to health. The effects of stress on fish can, depending on severity, result in impaired growth, feeding, reproduction and increased susceptibility to disease and mortality (Wendelaar-Bogna, 1997). A full understanding of the impact of husbandry related stressors on cultured fish is consequently of considerable relevance to the aquacultural industry.

The physiological events involved in the piscine stress response have received much attention. Under stress, fish switch from an anabolic to catabolic state. This primary stress

response involves the release of many hormones, notably catecholamines and glucocorticoids (e.g. cortisol), changes of which are frequently used as indicators of the primary stress response and its severity (Wendelaar-Bogna, 1997). The stress response is also associated with secondary effects. Modulations in blood glucose and hematocrit are often studied and used to determine the level of secondary stress responses (Barton & Iwama, 1991). However, the evaluation of secondary stress responses, in particular those arising from chronic rather than acute stressors, are less well determined, in particular effects on antioxidant status, DNA stability and respiratory burst. These health parameters, the responses of which may be interrelated, are directly related to fish health and survival and have not been studied in-depth in relation to stress.

Normal metabolism produces ROS. Excess ROS, i.e. those not used for cell signalling etc, are scavenged by antioxidants, which convert them into non-toxic metabolites. This prevents oxidative damage to cellular macromolecules such as DNA, lipids and proteins. It is essential this balance is maintained to avoid the onset of oxidative stress (Halliwell & Gutteridge, 1999). During physiological stress, energy is reallocated to intensify activities such as locomotion and respiration (Wendelaar-Bogna, 1997). As a result of the altered metabolism that may be associated with stress, a concurrent increase in the generation of ROS and DNA damage is expected, as occurs with humans (Hartman et al., 1994) and rats (Leeuwenburgh & Heinecke, 2001; Bejma & Ji, 1999, Packer et al., 2008, Sachdev and Davies., 2008) subjected to exercise that is exhaustive in nature. This is not generally associated with moderate levels of exercise as in this scenario antioxidants are up regulated (Packer et al., 2008, Sachdev and Davies., 2008). As with mammals, in fish increased exhaustive exercise causes oxidative stress and associated DNA damage (Aniagu et al., 2006). During oxidative stress, increased levels of ROS cause DNA strand breaks and oxidation of nitrogenous bases, i.e. purines and pyrimidines. The FPG modified alkaline Comet assay measures the level of oxidised purines

by FPG conversion to single strand breaks in addition to pre-existing induced single strand breaks (Collins, 2004; Dusinska & Collins, 1996). The FPG modified Comet assay therefore allows the indirect determination of the production of 8-hydroxyguanine (8-OH-G); the most widely measured product of purine oxidation by ROS during oxidative stress (Valavanidis et al., 2006; Martinez et al., 2003). The repeated exposure to chronic physical stressors is expected to result in oxidative DNA damage due to associated exercise that may be exhaustive in nature.

The effect of environmental stressors including temperature, season and oxygen saturation on antioxidant levels in fish have been determined (Martinez-Alvarez et al., 2005) but knowledge into the effect of husbandry related stress on TAC has not been assessed. TAC considers the cumulative effect of all antioxidants present in plasma and is a useful tool in the evaluation of the redox status of plasma (Ghiselli et al., 2000). Oxidative stress may occur in physically stressed fish due to an increased production of ROS, which are associated with increased metabolic rates (Sachdev et al., 2008). In humans and rats, exhaustive exercise is known to modulate the production of antioxidants, generally up regulating antioxidants in trained individuals and decreasing them in un-trained individuals performing a single bout of exhaustive exercise (Gomez-Cabrera, et al., 2008). Continual physical activity increases reactive oxygen species, which act as signalling molecules for an up regulation of antioxidants, which enhances TAC in rabbits (Alipour et al., 2006) and trained humans (Sachdev and Davies, 2008). However, should increased metabolic activity associated with physical stress be exhaustive, a decrease in TAC is expected as antioxidant defences are overwhelmed. Decreased TAC may also occur as a result of nutritional stress. Chronic cortisol administration is known to reduce appetite and feed conversion in fish (Gregory and Wood, 1999). As key antioxidants including ascorbic acid and tocopherol are dietary nutrients and major contributors to TAC, nutritional stress associated with physical stress

may decrease plasma TAC. A Study in cattle concluded that transportation stressors may result in nutritional stress, which reduces TAC resulting in oxidative stress as measured by increased lipid peroxidation (Chirase et al., 2004). TAC in stressed fish may be modulated by the response of antioxidants to altered metabolic rate or nutritional stress associated with decreased feeding.

The damaging effect of physical stressors on the cellular immune system has been relatively well studied with regards to acute stressors, but to a much lesser extent concerning the application of chronic stressors. The innate immune response involves both cytotoxic and phagocytic cells, which provide a fast and effective response against pathogenic attack (Roberts, 1989). The respiratory burst, so called due to the large amount of oxygen consumed in the process, is a key part of the piscine immune system. The cytotoxic respiratory burst in teleosts, as in mammals, involves an initial release of microbiocidal superoxide ($O_2^{\cdot-}$) produced by NADPH oxidase, which is then dismutated to hydrogen peroxide, either spontaneously or catalysed by the enzyme superoxide dismutase (SOD) (Chanock et al., 1994). Detectable after the initial release of superoxide and catalysed by myeloperoxidase (MPO) is the later release of hypohalites, chloroamines, and singlet oxygen by the MPO reaction during degranulation. This chain of events can be initiated by either adherence of particles to the cell membrane or soluble activating agents within the cell such as phorbol-myristate-acetate (PMA) (Chung & Secombes, 1988). Previous studies have shown that components of the respiratory burst are modulated by acute physical stressors, being either stimulated (Pulsford et al., 1994) or suppressed (Vazzana et al., 2002; Angelidis et al., 1987).

Given the backdrop of the above information, and to assess the designed stress protocol for further experiments, the main objective of this preliminary study was to determine if chronic physical handling stressors result in oxidative stress and impaired immunity in rainbow trout. This was achieved by adopting an integrated approach measuring a range of stress and health

markers to evaluate the stress response and the effect of chronic physical stressors on health parameters relevant to Se and Zn nutrition. Cortisol levels and hematocrit values were measured to differentiate between stressed and unstressed fish. The health parameters measured included, TAC, the induction of DNA strand breaks using Comet assay, induction of micronuclei as a cytogenetic parameter to determine chromosomal damage and loss (i.e. clastogenic and aneugenic effects), and the respiratory burst of leukocytes.

5.2. Methods

5.2.1. Fish maintenance and experimental conditions

Fifty rainbow trout mean weight 105 ± 16 g (\pm SD) were obtained, introduced and acclimated for four weeks to the experimental rearing facility (see 2.3. and 2.2.). Three days prior to the study fish were separated into two 130 l tanks (25 fish per tank) comprising of one 'control' and one 'stressed' tank ($n = 1$). Replicate fish would have been preferable but only 50 fish were available and trout could not be further divided, as below 20-30 fish per tank trout become aggressive and form hierarchies. Throughout the experiment, fish were fed twice daily a commercial diet (15-45 Grower Feed XS, Aller Aqua, Christiansfeld, Denmark) at 1.8 % body weight per day. Water quality and fish were routinely monitored (see 2.2.). In order to lessen the chance that fish may desensitize to the physical stressors, two types were used alternately as similarly preformed by Barton et al. (1987) who used three different stressors (disturbance, netting and confinement). Husbandry related stressors were applied alternating daily between either handling or confinement. Fish were confined once daily for 45 min in a similar manner to (Contreras-Sanchez et al., 1998) by being subjected to a water level to just below the tip of the dorsal fins by use of a shortened stand pipe. Handling stress involved dip netting and aerial exposure for 30 s twice daily. The stressors started and finished with the netting stressor. The netting stressor was only applied once just prior to sampling on the 7th day.

5.2.2. Sample analysis

Prior to sampling, all fish were anaesthetised with MS-222. On the 7th day whole blood was sampled from the caudal vein using a needle and syringe from 12 random individual fish and kept at room temperature (22 ± 1 °C) in EDTA treated tubes until immediate analysis of percentage hematocrit (2.12.1.) and respiratory burst (within ~2 hours) in eight fish per tank

(see 2.13.2.). Plasma was recovered and stored at $-80\text{ }^{\circ}\text{C}$ until analysis of TAC in 10 fish per tank (see 2.11.2.).

Blood samples were taken from a further five fish per tank and stored on ice in heparin treated tubes until analysed by the Comet assay (2.11.3.). Blood smears were prepared (see 2.12.2.) for the determination of micronuclei frequency, and plasma recovered and stored at $-80\text{ }^{\circ}\text{C}$ until analysis of cortisol.

The MN assay for the detection of cytogenetic damage was performed as described by Del Barga et al. (2006) with slight modifications. Blood smears were prepared in duplicate from six fish per tank (see 2.12.2.) To determine MN frequency 1000 cells per slide were scored under $1000\times$ magnification, giving a total of 2000 cells per fish as recommended by Udrouiu (2006) (see 2.12.4.). Plasma cortisol was measured in duplicate in 10 fish per tank using the same method as King et al. (2005) in a 96 well ELISA cortisol kit (DRG Diagnostics, Germany). Serial dilutions (1/2, 1/4 and 1/8) were made of a random 70 ng ml^{-1} sample to test linearity giving percentage recovery of 103 %, 51 % and 44 %, respectively, showing linearity between a 1/1 and 1/2 dilution. The plate was read at 450 nm using an Optimax microplate reader with kinetic software SOFTmax Pro[®] (Molecular Devices, USA). Cortisol was calculated in ng ml^{-1} against cortisol standards using a four-parameter line of best fit ($R^2 = 0.999$)

5.2.3. Statistical analysis

As this was a preliminary study and tanks were not replicated, statistical analysis was not performed; individual fish in a tank are not independent of one another. 95% confidence interval bars allowed for a visualisation of the preliminary data collected.

5.3. Results

5.3.1. Chronic husbandry related stressors and the stress response

No mortality occurred as a result of the applied stressors throughout the study. Plasma cortisol and hematocrit levels were measured to confirm the presence of a primary physiological stress response. Despite being subjected to chronic stressors for seven days, there was no significant effect on either stress marker. However, feeding was markedly reduced in fish subjected to the chronic stressors; although all feed was eaten, the time to feed and feeding response was reduced. It was observed that during the netting stress increased mucus was produced and this response continued throughout the experimental period. A slight decrease in percentage hematocrit was observed as a result of stress (Fig. 5.1.). Mean cortisol only increased slightly in stressed fish (Fig. 5.2.).

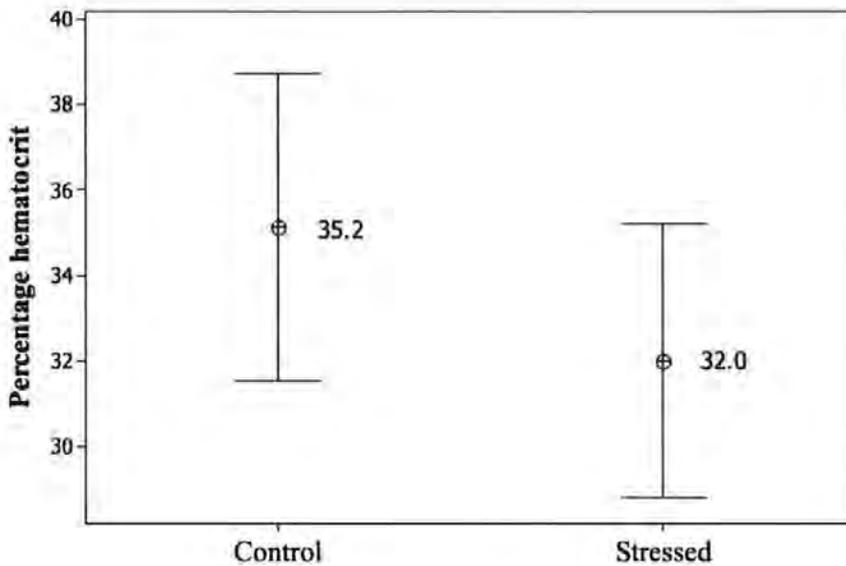


Figure 5.1. 95 % confidence interval bars of mean percentage hematocrit of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts.

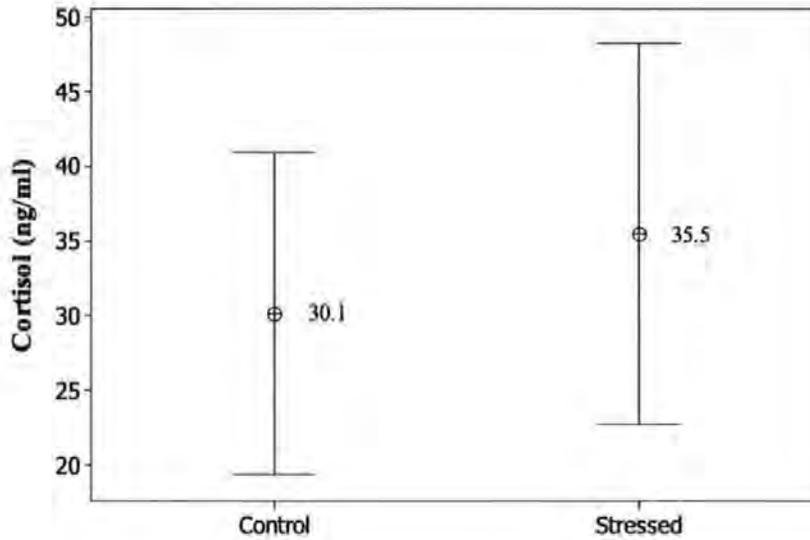


Figure 5.2. 95 % confidence interval bars of mean plasma cortisol concentration (ng ml^{-1}) of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts.

5.3.2. Chronic husbandry related stressors and health responses

With the exception of MN frequency and the stress markers, the measured health parameters responded to the applied chronic physical stressors. TAC, measured by the peroxynitrite scavenging ability of plasma, was lower in stressed fish (Fig. 5.3.). Chronic physical stressors increased DNA instability as measured by the modified Comet assay (Fig. 5.4.). Single strand breaks, measured with the standard alkaline Comet, increased but not to the same extent as that measured by the FPG modified assay. Purine base oxidation, measured by the FPG modified assay was markedly increased in fish subjected to the physical stressors. Cytogenetic damage, measured by the induction of micronuclei, was not affected by the seven days husbandry related stressors (Fig. 5.5.).

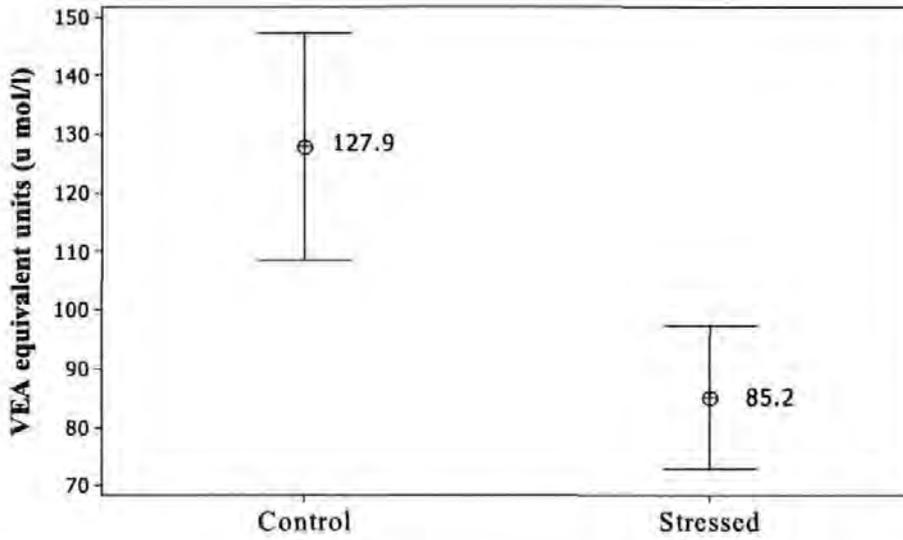


Figure 5.3. 95 % confidence interval bars of mean plasma TAC ($\mu\text{mol l}^{-1}$ VEA units) of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts.

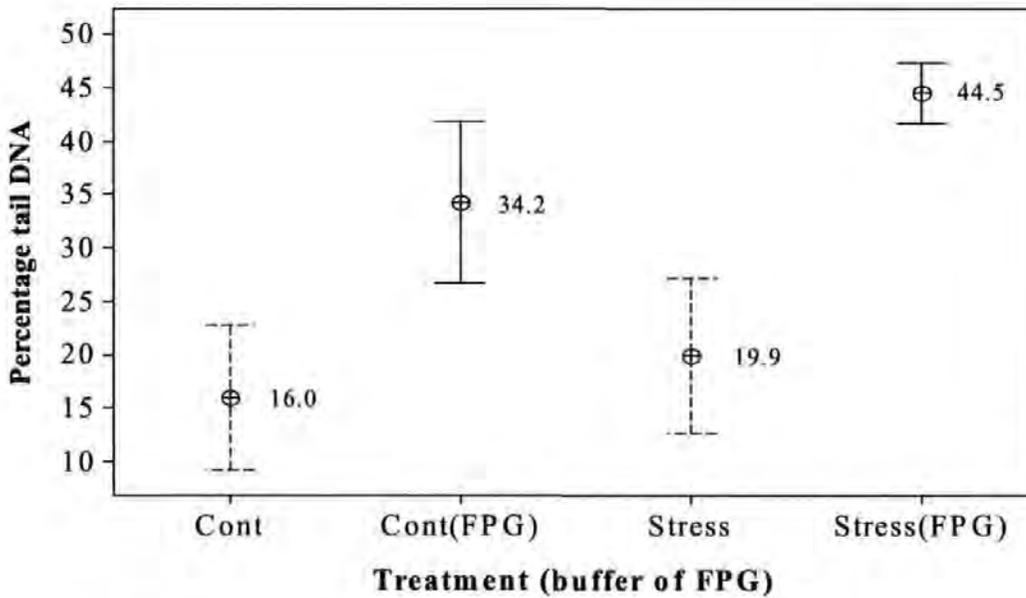


Figure 5.4. 95 % confidence interval bars of mean percentage tail DNA of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts. Percentage tail DNA for single strand breaks (dashed bars) and FPG sensitive sites (solid bars).

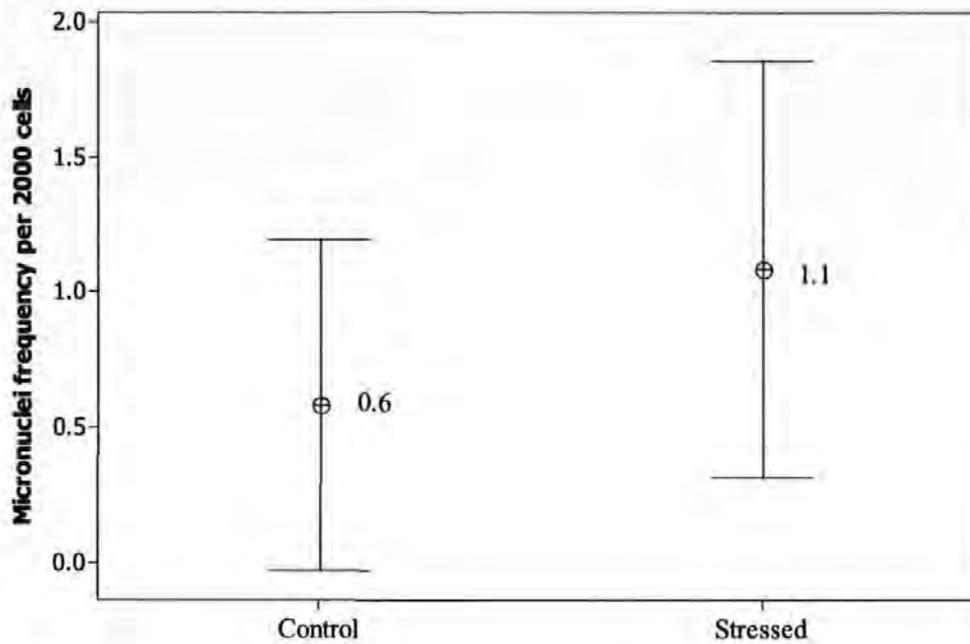


Figure 5.5. 95 % confidence interval bars of mean micronuclei (MN) frequency per 2000 cells of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts.

There was a trend towards suppression of the activity of PMA activated leukocytes after ~50 min as a result of seven days handling stress. The first peak of activity at 11.5 min was not dissimilar between stressed and un-stressed fish (Fig. 5.6.). This indicates that physical stressors may modulate the later stages of the respiratory burst but not the first peak of activity.

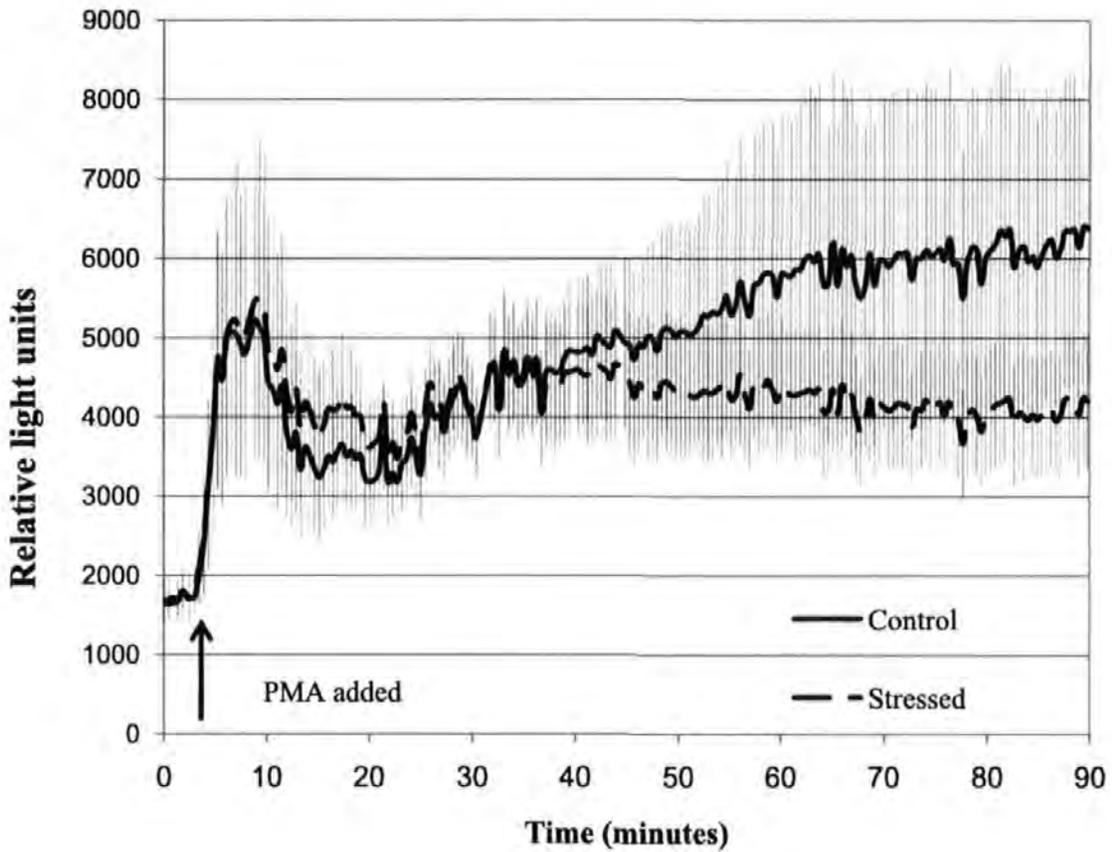


Figure 5.6. PMA activation of NADPH oxidase of trout leukocytes in diluted whole blood of rainbow trout subjected to seven days chronic handling stressors in comparison to un-stressed counterparts. Mean relative units \pm 95 % confidence interval bars of control and stressed fish. PMA activation at time 3 min. Integral (over 90 min) leukocyte activity was slightly higher in un-stressed control fish ($6.55 \pm 2.83 \times 10^5$) than those that were subjected to the chronic stressors ($5.05 \pm 2.05 \times 10^5$).

5.4. Discussion

5.4.1. *The effect of chronic husbandry stressors on hematocrit and cortisol*

The results of this study show that exposure to seven days husbandry related stressors may impair the health of rainbow trout, causing a decrease in DNA stability and antioxidant status, and a modulated respiratory burst of the innate immune system. Due to the measured impact of the applied physical stressors on health, the apparent lack of a response in the measured stress markers may be a result of the stress markers measured and the nature of the response to chronic stress. The response of hematocrit to acute and physical stress varies amongst the literature. Circulating stress hormones during stress are thought to affect hematocrit by increased release of erythrocytes via contraction of the spleen, increased erythrocyte fragility, or ageing leading to increased erythrophagocytosis (Pulsford et al., 1994). Studies applying similar stressors found no effects on hematocrit after two weeks physical stress (Barton et al., 1987) or acute confinement stress (Vazzana et al., 2002). A lack of a cortisol response to chronic physical stressors has also been reported by Barton et al. (1987). The normal increase in cortisol associated with acute physical stressors (Sadler et al., 2000) is believed to require a novel stimulus, otherwise habituation occurs. This may have been the cause of the lack of a plasma cortisol response in the present study. Although no studies have used the present methods for plasma cortisol analysis in rainbow trout, cortisol levels measured by the same method in un-handled black sea bass *Centropristis striata* (King et al., 2005) were consistent with those of the present study (25 ng ml⁻¹). However, in acutely stressed fish, cortisol increased to >50 ng ml⁻¹; significantly higher levels than found in the present study. Basal cortisol levels depend on assay specificity, husbandry conditions and capture procedures, and in salmonids are reported to be in the region of <5 – 50 ng ml⁻¹ (Wendelaar-Bogna, 1997). In salmonids increases up ~75 ng ml⁻¹ as a result of a single 2.5 h bout of confinement stress have been reported in salmonids (Sadler et al., 2000). This supports the notion that stressed

fish may have adapted to the chronic stressors, despite alternating between two types. Cortisol can return to basal values despite the continued presence of stress and numbers of stress receptors are known to decrease before any significant reductions in cortisol occur in trout (Pickering 1992). The findings of the present study therefore suggest that fish may desensitise to chronic physical stressors by adapting and compensating to stress (Barton et al., 1987), therefore other markers of the primary stress response may need to be used to measure the cumulative effects of stress.

5.4.2. The modulation of plasma TAC by chronic husbandry stressors

In the present study, chronic physical stressors caused a significant reduction in TAC, as determined by the peroxynitrite scavenging capacity of plasma using Pholasin[®]. Pholasin[®] is known to be sensitive to alterations in plasma TAC (Knight et al., 2002) and this is the first time this unique photoprotein has been used in the determination of TAC and respiratory burst in fish. Although not previously reported in fish, reduced TAC arising from transportation stress has been measured in cattle by the chemiluminescence of isoluminol (Chirase et al., 2004). The mechanism by which TAC is reduced by physical stress in the present study is unknown. The decreased TAC may have been a result of an increased metabolic activity associated with the increased physical activity associated with netting and aerial exposure and (or) nutritional stress. Decreased TAC post acute exercise occurs in untrained rats (Ficicilar et al., 2003). The decreased TAC indicates that the stress protocol used was similar to exercise that is exhaustive, as antioxidant defences were not up regulated, as otherwise occurs with animals subjected moderate exercise (Gomez-Cabrera et al., 2008). The only study of this type in chub found no significant effect on the antioxidants SOD and reduced GSH in the gill, liver and blood after acute exhaustive physical activity (Aniagu et al., 2006). TAC is theoretically a measure of the cumulative effect of all known and unknown

antioxidants, however it may not be the case in practice (Prior and Cao, 1999; Ghiselli et al., 2000). In fish (Carp), uric acid is a major antioxidant but its concentrations are 6-8 times lower than in humans. Ascorbic acid, followed by tocopherol, are reported to be the major plasma antioxidants in fish and could contribute significantly to TAC in fish (Xue et al., 1998). A reduced feeding and feed conversion associated with stress (Gregory and Wood, 1999) may have resulted in nutritional stress in the present study. A lack of dietary nutrients present in the plasma, including ascorbic acid and tocopherol, may result in an inability of stressed fish to increase TAC. The contributions of ascorbic acid, tocopherol and uric acid to TAC in fish, and its relation to stress may provide clues as to the mechanism by which physical stress reduces TAC in fish.

5.4.3. The effect of chronic husbandry stressors on oxidative DNA damage

The FPG modified Comet assay has been used successfully in fish erythrocytes to measure DNA damage as a result of exposure to genotoxic compounds (Akcha, et al., 2003) and as a marker of oxidative stress resulting from exhaustive physical activity (Aniagu et al., 2006). This is the only study to our knowledge which shows that physiological stress can cause an increase in FPG-sensitive sites using the FPG modified Comet assay. As stressed fish had both increased DNA damage and reduced TAC, this suggests that stressed fish were in a state of oxidative stress. The data also show a relatively high percentage tail DNA in the controls indicating a high sensitivity of erythrocyte DNA, which is thought to be due to the high pH in the electrophoresis conditions used. A high level of alkali liable sites is characteristic of human sperm DNA, and chicken erythrocyte DNA. The DNA of these cell types is highly condensed and result in a high level of background DNA breaks, which is not found in pH neutral conditions. The mechanism for high single strand breaks in highly condensed DNA is not yet known (Singh et al., 1989). Fish erythrocytes are also reported to have a high level of

alkali labile sites due to a highly condensed chromatin (Frenzilli et al., 2004). Although stress caused oxidative DNA damage, it did not cause significant cytogenetic damage as evaluated by MN induction. Cytogenetic damage may therefore not be significantly affected by physical stressors under the present experimental conditions. It is possible that the duration of applied stressors was insufficient to cause significant elevations in MN frequency, which is a cell-cycle dependent phenomenon. The onset of oxidative stress is cumulative, so considering peaks in micronucleated erythrocytes only occur one to five days after exposure (Udroiu, 2006) seven days exposure to physical stressors may have been insufficient for increased micronuclei formation. The reduced sensitivity to genotoxic compounds of the MN compared to the Comet assay has been previously reported in fish erythrocytes using *in vivo* exposure to Ethyl methanesulphonate (EMS) (Belpaeme et al., 1995).

5.4.4. The effect of chronic husbandry stressors on respiratory burst

This study showed a trend towards a depression in the superoxide generation of trout leukocytes in the whole blood measured using Pholasin[®]. The respiratory burst has been measured in fish leukocytes indirectly using the nitroblue tetrazolium (NBT) assay to detect O_2^- , the horseradish peroxidase (HPO) dependent conversion of phenol red to detect H_2O_2 , and the chemiluminescence assay to detect singlet oxygen (Blazer, 1991). The use of these aforementioned assays allows for quantification of only parts of the respiratory burst. Pholasin[®] provides an advantage over other methods as it allows the kinetics of released radicals to be followed simultaneously after activation with PMA (Arnhold, 2004). In this study the initial peak in respiratory burst activity, expected to be due to extracellular O_2^- release, is not dissimilar between stressed and un-stressed fish. However, after 50 min, during what is postulated to be the later degranulation phase, there is a trend towards a reduction in the response of leukocytes in stressed fish. A previous study on dab (*Limanda limanda*),

using a one off acute physical stressor, showed increased respiratory burst activity of kidney leukocytes post PMA activation (measured by HPO) in physically stressed fish (Pulsford et al., 1994). Ortuno et al. (2001) however, found no effect of acute physical stressors on hydrogen peroxide production by the respiratory burst of kidney leukocytes, which is consistent with findings of this study as they measured the initial respiratory burst during the first 30 min rather than 90 min assessed in the present study. Using luminol, another chemiluminescent probe that detects MPO activity, Vazzana et al. (2002) and Angelidis et al. (1987) both found a reduction in the activity of head kidney leukocytes following the application of an acute handling stressor. By using Pholasin[®] this study has shown that only the latter phase of the respiratory burst may be modulated by physical stressors. Angelidis et al. (1987) proposed that reduced respiratory burst activity is due to corticosteroids causing a 'stiffening' of membranes, which affects lysosomal exocytosis and consequently reduces degranulation. However, this cannot be confirmed in the present study due to a lack of a significant cortisol response. That said a higher cortisol level would have been expected before any habituation occurred during the experimental period. Alternatively, a change in the proportions of different leukocytes due to stress may also have affected the kinetics of the respiratory burst in the present study.

5.4.5. Conclusions

In conclusion, the study demonstrates that seven days chronic handling and confinement stress in rainbow trout may impair oxidative status and modulate immuno-competence. Antioxidant status is reduced as a result of stress. The increased nitrogenous base oxidation of erythrocyte DNA as measured using the FPG modified Comet assay confirmed the onset of oxidative stress. The induction of cytogenetic damage as measured by the micronucleus assay, a cell-cycle dependent phenomenon, was not significantly different from the un-

stressed group. The effects of oxidative stress may considerably increase the susceptibility of fish to disease and mortality in practical aquacultural situations.

CHAPTER 6.

SUPRA-NUTRITIONAL DIETARY INTAKE OF SELENITE AND SELENIUM YEAST IN NORMAL AND STRESSED RAINBOW TROUT: IMPLICATIONS ON SELENIUM STATUS AND HEALTH RESPONSES.

Hypotheses:

- i) Supplemental Se will raise Se status in un-stressed fish fed a fishmeal based diet and Se-yeast will be more effective than selenite.**
- ii) Supplemented Se will confer benefits to growth, oxidative status, haematological parameters, immuno-competence, frequency of nuclear abnormalities and fillet quality in both stressed and un-stressed fish with Se-yeast being more effective than selenite.**
- iii) Se utilisation will increase in stressed fish and Se-yeast will be more effective in the maintenance of Se status than selenite.**
- iv) Neither Se-yeast nor selenite supplementation up to 8 mg kg⁻¹ will be detrimental to fish health.**

6.1. Introduction

Selenium, with its pivotal role against oxidative cellular injury is an essential trace element for fish as well as mammals (Watanabe, 1997), and may confer a protective role against pathologies associated with oxidative stress in fish following exposure to physical stress. Se

is present in a multiplicity of functional selenoproteins as the Se amino acid selenocysteine (Se-Cys) (Beckett and Arthur, 2005); to date 18 have been identified in fish (Kryukov and Gladyshev, 2000). Often referred to as the 21st amino acid, Se is the only trace element to be genetically encoded for via a Se-Cys insertion codon (Hatfield and Gladyshev, 2002). In higher organisms, Se plays a critical role in the maintenance of oxidative and immune status, and is thus essential for the preservation of optimal health (Brown and Arthur, 2001; Rayman, 2000). In salmonids, Se deficiencies lead to ataxia, muscular dystrophy, lipid peroxidation and decreased GSH-Px activity (Poston, 1976; Hodson and Hilton, 1983; Bell et al., 1985; Bell et al, 1986; Bell et al, 1987). The principal function of Se is in the protection of biological compounds, namely DNA, proteins and lipids, against attack from oxidants and peroxides produced during normal metabolism. The maintenance of cellular oxidative status by Se is principally via GSH-Px and Trx-R; the most abundant Se containing proteins in mammals (Tujebajeva et al., 2000). Fish, particularly salmonids, may have a relatively high requirement for the antioxidant properties of Se to protect their elevated levels of PUFA, which are prone to lipid peroxidation (Winston and Di Giulio, 1991). GSH-Px has been shown to both protect and repair trout red blood cell membranes subjected to oxidative haemolysis (Falcioni, et al., 1987). Se is also required for the efficient functioning of many components of the immune system (Kiremidjian-Schumacher and Stotzky, 1987; Arthur et al., 2003); this is of particular importance in intensive fish culture, which can suffer significant losses from disease outbreak.

In farmed fish, requirements for Se may be elevated due to the low availability of Se from fishmeal based diets and the effect of various physical and environmental stressors. Dietary Se requirements of rainbow trout are 0.35 mg kg⁻¹ (Hilton et al., 1980); however despite containing Se above determined requirements (0.53-1.23 mg kg⁻¹ Se, see 8.2.), commercial trout diets may not meet the requirements of intensively raised fish. Cultivated salmonids

have been found to have a markedly decreased tissue Se than wild counterparts (Poppe et al., 1985; Felton et al., 1990; Julshamn et al., 1990) and the cause is yet unknown. This discrepancy may be due to combination of factors including, differences in growth rates and feeding regimes (Maage et al., 1991), decreased dietary Se / Se digestibility in cultivated fish diets, and (or) Se losses resulting from stresses associated with intensive fish cultivation. Se digestibility is significantly lower from diets containing fishmeal (46-54 %), than organic and inorganic Se sources supplemented to semi-purified diets (Bell and Cowey, 1989; Rider et al., 2009). Physical stress intensifies activities such as locomotion and respiration (Pickering, 1998), which when exhaustive is associated with a concurrent increase in ROS (Leeuwenburgh and Heinecke, 2001; Bejma and Ji, 1999). The increased DNA damage and reduced TAC found in the previous experiment in fish subjected to stress (Chapter 5.) demonstrates that physical stress in fish increases oxidative stress. Consequently, Se utilization may increase during stress due to an increased demand for antioxidant enzymes, including GSH-Px and Trx-R. In Chinook salmon, transportation stress results in increased GSH-Px activity and a loss of carcass Se (Halver et al., 2004). Also, Coho salmon required 7.5 mg kg^{-1} supplemental Se from selenite to attain maximum survival during seawater adaptation and eviscerated carcass Se levels comparable to wild counterparts (Felton et al., 1996). Se or vitamin E supplementation is also required for maximum survival during seawater adaptation in Chinook salmon (Thorarinsson et al., 1994). Physical stress is well known to have a detrimental effect on the non-specific immune response (Wendelaar-Bonga, 1997) with knock on effects on aquaculture. As Se utilisation may increase during physical stress, it is crucial that adequate Se reserves are available for the maintenance of a healthy immune system as well as for antioxidant defence.

Should Se supplementation be required to maintain Se reserves in all conditions, Se-yeast may be more efficacious and less toxic to salmonids than selenite. Much Se research in fish

to date has focused on inorganic Se; namely sodium selenite. In feedstuffs Se is naturally present organically bound in selenoproteins (Surai, 2006). Containing predominantly Se-Met and several other lesser selenocompounds (Schrauzer, 2006), Se-yeasts can provide Se similar in nature to naturally occurring Se. Se-Met is non-catalytic, does not participate in redox reactions and may even act as an antioxidant; consequently it is less toxic than selenite (Schrauzer, 2003). Conversely, selenite can exhibit pro-oxidant properties in biological systems (Spallholz, 1997; Stewart et al., 1999), thus thresholds between toxicity and nutritional levels are narrower for inorganic than organic Se sources. This is of particular importance, as levels of selenite that may be beneficial during stress are close to those of toxicity (Felton et al., 1996). In rainbow trout, selenite is chronically toxic at $>10 \text{ mg kg}^{-1}$ (Hodson and Hilton, 1983), and although not determined in rainbow trout, in Chinook salmon Se-Met does not exhibit signs of toxicity up to 18 mg kg^{-1} (Hamilton et al., 1990). The disparity between the toxicity of organic and inorganic Se is also found in terrestrial species, including poultry (Todorovic et al., 2004). In fish, organic Se sources have been found to have greater potency in terms of bioavailability and effects on health than inorganic Se, but this can depend on the parameter measured. Unlike selenite, supplemented dietary Se rich in Se-Met is effectively incorporated into muscular tissues of fish (Lorentzen et al., 1994; Wang and Lovell, 1997; Cotter et al., 2008, Rider et al., 2009). In addition, organic Se is more effective in raising GSH-Px activity in carp and catfish (Wang et al., 1997; Jovanovic et al., 1997); however this has not been observed in Atlantic salmon (Bell et al., 1989), rainbow trout (Rider et al., 2009) or hybrid striped bass (Cotter et al., 2008,). Studies in catfish show that sub optimal dietary Se decreases disease resistance, and organic Se is more potent than selenite in restoring immune defences (Wang et al., 1997). Relatively little is known about the effect of trace elements, including Se, in the piscine immune system (Lall, 2000). Due to the possibility of interactions between trace elements when supplementing a single element

(Sandstrom, 2001) an assessment of other essential elements should be considered. As organic and inorganic Se sources use different uptake mechanisms, interactive effects may differ between sources; this has not yet been ascertained in fish.

Given the backdrop of the above information the aim of this study was to determine if a commercial trout diet required Se supplementation and if supra-supplementation of sodium selenite or Se-yeast conferred benefits to the health of physically stressed rainbow trout. Graded supra-nutritional (levels above those that are typically regarded as nutritionally beneficial) levels of Se were chosen to include concentrations that have been previously found to be beneficial during stress (Felton et al, 1996). A suite of markers were used to assess the effects of supplemented Se and the implications of physical stress on; Se levels and utilisation, growth, oxidative status, immuno-competence, haematological parameters and levels of other essential elements.

6.2. Methods

6.2.1. *Experimental fish*

420 rainbow trout mean weight 26 ± 3 g (\pm SD) were acquired and acclimated according to methods outlined in section 2.3. During the acclimation period fish were fed *ad libitum* a commercial trout grower diet (EWOS Micro 20P, Bathgate, West Lothian, UK).

6.2.2. *Diets and experimental regime*

Post acclimation fish were randomly stocked into one of fourteen 130 l tanks at a density of 30 fish per tank. Each tank was randomly assigned to receive a dietary treatment, each consisting of two replicate tanks ($n=2$). Fish were fed the experimental diet at 2% biomass per day and maintained for a 10-week period according to methods 2.2. and 2.4. SGR and FCR were determined according to method 2.5. Fish were fed one of even experimental dietary treatments based on a common commercial trout grower diet (Elite 45, Skretting, Preston, UK) supplemented with various levels of selenite or Se-yeast for ten-weeks. The diet was ground down and re-pelleted with supplemented Se via prepared premixes (see 2.9.). One treatment comprised of an un-supplemented basal diet, and in light of the study by Felton et al. (1996) three diets were supplemented with 2, 4, and 8 mg kg⁻¹ Se from Se-yeast and three reciprocal diets supplemented with sodium selenite. The basal diet was subjected to proximate analysis (see 2.6.) and contained (\pm SD); 43.1 \pm 0.3 % protein, 18.6 \pm 0.1 % lipid, 7.1 \pm 0.1 % ash, 6.2 \pm 0.2 % moisture, and provided 21.7 \pm 0.1 MJ kg⁻¹ energy. The measured Se concentrations in each diet were (mg kg⁻¹); basal (0.73); inorganic 2 (2.3), 4 (3.9), 8 (7.1); organic 2 (2.4), 4 (4.1), 8 (7.4) (see 6.3.). Both Se sources were supplemented via a Se variable premix and included at 1 % (see 2.9.). Six initial fish were pooled and prepared for trace element analysis (see 2.7.1. and 6.3.). Following the 10-week feeding trial, fish remained on the experimental regime and after two days (pre-stress sampling) were subjected

to seven days husbandry related stressors. Husbandry related stressors were applied alternating daily between either handling or confinement. Fish were confined once daily for 45 min in a similar manner to (Contreras-Sanchez et al., 1998) by being subjected to a water level to just below the tip of the dorsal fins by use of a shortened stand pipe. Handling stress involved dip netting and aerial exposure for 30 sec twice daily. The stress phase started and finished with the netting stressor.

6.2.3. Analytical procedures

Fish were sampled for all analysis at the end of the 10-week feed trial period, which within the regime included one days feed withdrawal. All sampled fish were euthanized with MS-222 followed by cerebral destruction. Blood samples were obtained using a 2 ml syringe without anticoagulant via a caudal puncture. Blood was sampled from six fish per tank and used immediately for the determination of hematocrit using heparinised hematocrit tubes (see 2.12.1.). Serum was collected from the remaining blood and stored at -80 °C until analysis for the determination of lysozyme (see 2.13.1.). Livers were excised and separate portions immediately homogenised (livers pooled into two random groups from each tank of three individuals for Trx-R and GSH-Px) in cold buffers (4 °C) corresponding to the assays of MDA (see 2.11.1.), GSH-Px (see 2.10.2.) and Trx-R (see 2.10.3.) and samples stored at -80 °C until analysis. Blood samples were taken from six further fish per tank and immediately aliquoted into 300 µl EDTA tubes for the immediate analysis of extracellular respiratory burst (see 2.13.2.) and preparation of blood smears (see 2.12.2.) for leukocyte counts (see 2.12.3.) and nuclear abnormalities (see 2.12.4.). Plasma was retained from remaining blood and stored at -80 °C until analysis for the determination of TAC (2.11.2.). These fish were then frozen whole at -20 °C for determination of whole body trace elements. Whole body samples were pooled per tank and homogenised for trace element analysis (see 2.7.) Se in all

dietary and whole body samples was determined by ICP-MS with standard additions by Ultra Traces Analyses Aquitaine, Pau, France. Cu, Fe, Mn and Zn were determined in the same digests by ICP-OES at the University of Plymouth. This sampling procedure was repeated (except nuclear abnormalities and leukocyte counts) at the end of the seven day stress phase. The last application of netting stress was immediately prior to sampling.

6.2.4. Statistical analysis

All statistical analyses were carried out according to methods outlined in section 2.16. One-way ANOVA was performed on SGR, FCR, Se retention, and whole body element data. All other data was modelled with a nested ANOVA; unless indicated no significant differences were observed between replicate tanks.

6.3. Results

6.3.1. Growth rate and feed performance

No mortality occurred during the entire feeding trial or period of stress. Growth rates were within normal ranges for rainbow trout under production conditions and supplemental Se had no effect on SGR or FCR across treatments (Table 6.1.). During the 10-week period fish more than quadrupled in weight (\pm SD) from a mean of 26.3 ± 1.2 g to 117 ± 5.6 g.

Table 6.1.

Mean growth performance, feed utilisation and net Se retention of rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks.

Selenium ¹	Growth performance and feed utilisation		Se retention ⁴
	SGR ²	FCR ²	
0.7	2.28	0.85	44.6 ^{ac}
2.3 Selenite	2.27	0.85	26.9 ^b
3.9 Selenite	2.31	0.83	29.0 ^b
7.1 Selenite	2.26	0.86	40.5 ^a
2.4 Se-yeast	2.21	0.89	46.1 ^c
4.1 Se-yeast	2.32	0.83	54.8 ^d
7.4 Se-yeast	2.37	0.82	51.5 ^{dc}
<i>Pooled SEM</i>	<i>0.01</i>	<i>0.00</i>	<i>0.5</i>
³ Dietary effect <i>p</i> =	0.252	0.290	<0.001*

¹ mg kg⁻¹ Se and supplemented source.

² SGR, specific growth rate; FCR, feed conversion ratio.

³ *p*= significance of dietary Se intake and source (*n*=2); * highlights significant difference. Different lower case superscript letters indicate significant (*p* ≤ 0.05) difference between dietary Se treatments.

⁴ Percentage net Se retention.

Table 6.2.

Mean whole body Se of rainbow trout given various levels supplemental Se pre and post 7 days stress.

Selenium ¹	Pre stress	Post stress	Mean change	⁴ Stress effect <i>p</i> =
Whole body Se accumulation ²				
0.7	0.9 ^a	0.6 ^a	-0.3	0.083
2.3 Selenite	1.6 ^b	1.3 ^{ab}	-0.3	0.032*
3.9 Selenite	2.8 ^c	2.1 ^{cd}	-0.7	0.058
7.1 Selenite	6.2 ^d	4.0 ^c	-2.2	0.040*
2.4 Se-yeast	2.8 ^{cc}	2.4 ^{fd}	-0.4	0.054
4.1 Se-yeast	4.8 ^f	3.8 ^{eg}	-1.1	0.042*
7.4 Se-yeast	8.3 ^g	8.2 ^h	-0.6	0.282
Pooled SEM	0.0	0.0		
³ Dietary effect <i>p</i> =	<0.001*	<0.001*		

¹ mg kg⁻¹ Se and supplemented source.

² Whole body Se in mg kg⁻¹ dry wt.

³ *p*= significance of effect of dietary Se intake and source (*n*=2); * highlights significant difference. Different lower case superscript letters indicate significant (*p* ≤ 0.05) difference between dietary Se treatments.

⁴ *p*= significance of stress induced changes within individual dietary Se treatments (*n*=2); * highlights significant difference pre and post-stress.

6.3.2. Response of body selenium to supplemented selenium and stress

The retention of Se from selenite was significantly lower than that of Se-yeast in all groups. Retention of 2.3 and 3.9 mg kg⁻¹ inorganic Se and retention of 4.1 and 7.4 mg kg⁻¹ organic Se was significantly lower and higher respectively than residual Se from the basal diet (Table 6.1.). Regression analysis showed that selenite increased whole body Se exponentially, which reflects the particularly high Se retention in fish given 7.1 mg kg⁻¹ inorganic Se relative to the lower selenite supplemented groups (Fig. 6.1.). Regression analysis illustrates a linear response of whole body Se to increased dietary Se-yeast (Fig. 6.1.). Whole body Se in the Se-yeast supplemented trout was significantly higher than those supplemented with selenite.

There was a trend towards decreased whole body Se after the application of stressors in all groups; this loss was significant in the 2.3 and 7.1 mg kg⁻¹ selenite and the 4.1 mg kg⁻¹ Se-yeast supplemented fish. Despite the increased retention in fish given 7.1 mg kg⁻¹ Se from selenite compared to the lower inorganic Se groups, fish within this treatment proportionally lost the greatest amount of whole body Se when subjected to the husbandry related stressors (Table 6.2.).

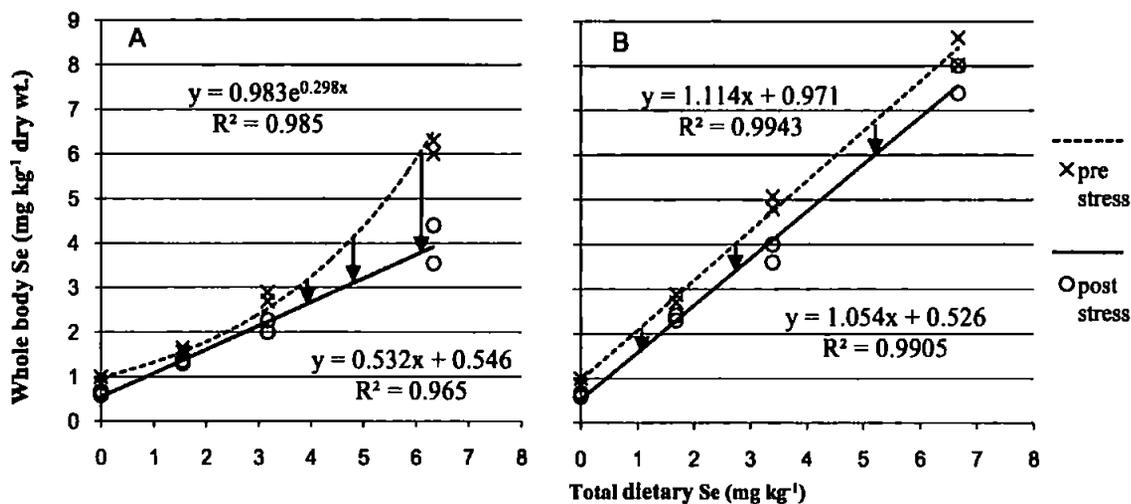


Figure 6.1. Response of whole body Se concentration to 10 weeks organic and inorganic dietary Se supplementation and 7 days handling stress in rainbow trout. Whole body Se accumulation (n=2) of rainbow trout pre and post-stress fed a commercial fishmeal diet supplemented with various levels of Se from sodium selenite (A) and Se-yeast (B). Whole body Se accumulation from selenite follows an exponential dose response pre-stress (regression $p < 0.001$) and a linear dose response post-stress (regression $p < 0.001$). Whole body Se accumulation from Se-yeast follows a linear dose response pre-stress (regression $p < 0.001$) and post-stress (regression $p < 0.001$).

6.3.3. Response of selenium enzymes to dietary selenium and stress

Neither organic nor inorganic Se supplementation had an effect on hepatic GSH-Px or Trx-R activity pre stress. Handling stress resulted in a trend towards increased GSH-Px in all groups, but only significantly in fish fed the diet supplemented 7.4 mg kg⁻¹ Se-yeast. The lowest increases in GSH-Px were in fish given 8 mg kg⁻¹ inorganic Se and the un-supplemented basal diet (Table 6.3.). For both Se sources there was a trend towards elevated Trx-R up to ~4 mg kg⁻¹ both pre-stress and post-stress, however this was not significant. An insignificant trend towards decreased Trx-R activity was observed in all treatments post-stress (Table 6.3.).

Table 6.3.

Mean activity of hepatic glutathione peroxidase and thioredoxin reductase of rainbow trout given various levels supplemental Se pre and post 7 days stress.

Selenium ¹	Pre stress	Post stress	Mean change	³ Stress effect <i>p</i> =
Hepatic glutathione peroxidase ⁴				
0.7	10.8	14.3	+3.5	0.152
2.3 Selenite	11.5	24.7	+13.2	0.066
3.9 Selenite	9.9	18.2	+8.3	0.212
7.1 Selenite	9.2	10.7	+1.5	0.449
2.4 Se-yeast	9.3	18.2	+8.9	0.228
4.1 Se-yeast	10.0	21.3	+11.3	0.139
7.4 Se-yeast	8.7	22.5	+13.8	0.031*
<i>Pooled SEM</i>	0.3	0.8		
² Dietary effect <i>p</i> =	0.805	0.235		
Hepatic thioredoxin reductase ⁵				
0.7	0.63	0.55	-0.08	0.624
2.3 Selenite	0.78	0.60	-0.18	0.445
3.9 Selenite	0.99	0.80	-0.19	0.147
7.1 Selenite	0.96	0.64	-0.32	0.123
2.4 Se-yeast	0.72	0.57	-0.15	0.123
4.1 Se-yeast	1.17	0.67	-0.50	0.228
7.4 Se-yeast	0.99	0.77	-0.22	0.436
<i>Pooled SEM</i>	0.03	0.02		
² Dietary effect <i>p</i> =	0.848	0.194		

¹ mg kg⁻¹ Se and supplemented source.

² *p*= significance of effect of dietary Se intake and source (*n*=2); * highlights significant difference. Different lower case superscript letters indicate significant (*p* ≤ 0.05) difference between dietary Se treatments.

³ *p*= significance of stress induced changes within individual dietary Se treatments (*n*=2); * highlights significant difference pre and post-stress.

⁴ nmol NADPH oxidised min mg⁻¹ protein⁻¹

⁵ A₄₁₂.1000 min mg⁻¹ protein⁻¹.

6.3.4. Effect of selenium and stress on markers of oxidative status

Supplemented Se had no effect on hepatic MDA or serum TAC pre-stress (Table 6.3.). Hepatic MDA increased as a result of handling stress, but this was only significant in four of the Se supplemented groups. The level of hepatic MDA was significantly increased post-stress in fish given 7.1 mg kg^{-1} Se from selenite (Table 6.3.). Serum TAC increased significantly post-stress in all groups (Table 6.3.).

Table 6.4.

Mean hepatic malondialdehyde and total antioxidant capacity of rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks pre and post 7 days stress.

Selenium ¹	Pre stress	Post stress	Mean change	⁴ Stress effect <i>p</i> =
Malondialdehyde²				
0.7	15.2	18.6 ^a	+ 3.4	0.073
2.3 Selenite	13.9	25.4 ^a	+ 11.5	0.005*
3.9 Selenite	18.9	24.4 ^a	+ 5.5	0.156
7.1 Selenite	20.1	38.6 ^b	+ 18.5	0.010*
2.4 Se-yeast	13.5	20.7 ^a	+ 7.2	0.003*
4.1 Se-yeast	19.1	21.6 ^a	+2.5	0.226
7.4 Se-yeast	13.1	21.0 ^a	+7.9	<0.001*
<i>Pooled SEM</i>	0.5	1.2		
³ Dietary effect <i>p</i> =	0.142	0.012*		
Total antioxidant capacity⁵				
0.7	160	219	+59	0.002*
2.3 Selenite	144	236	+91	<0.001*
3.9 Selenite	130	198	+68	<0.001*
7.1 Selenite	148	199	+51	0.001*
2.4 Se-yeast	137	223	+87	<0.001*
4.1 Se-yeast	138	201	+63	0.001*
7.4 Se-yeast	156	211	+55	0.013*
<i>Pooled SEM</i>	1	1		
³ Dietary effect <i>p</i> =	0.231	0.101		

¹ mg kg⁻¹ Se and supplemented source.

² μmol g liver⁻¹.

³ *p*= significance of effect of dietary Se intake and source (*n*=2); * highlights significant difference. Different lower case superscript letters indicate significant (*p* ≤ 0.05) difference between dietary Se treatments.

⁴ *p*= significance of stress induced changes within individual dietary Se treatments (*n*=2); * highlights significant difference pre and post stress.

⁵ Vitamin E equivalents (μmol l⁻¹).

6.3.5. Effect of selenium and stress on hematocrit, immune parameters and cytogenetic damage

Supplemental Se had no significant effect on percentage hematocrit. However, pre-stress fish given 7.1 mg kg^{-1} Se from selenite exhibited a trend towards a decreased hematocrit in comparison to all other treatments; post-stress hematocrit in this group significantly increased to a level comparable to all other treatments (Table 6.5.). Lysozyme activity was not affected by dietary Se. Stress raised lysozyme activity in all dietary treatments; this was to a significant level in five of the seven treatments (Table 6.5.). A high amount of inter-individual variation was observed in peak respiratory burst activity. Although differences were found post stress these cannot be attributed to dietary Se or stress, as significant differences were observed between replicate tanks (Table 6.5.). Pre-stress, leukocyte frequency was significantly lower in fish given 7.4 mg kg^{-1} Se-yeast than control fish (Table 6.6.). Se supplementation had no significant cytotoxic effect on nuclear abnormalities. The greatest frequencies of micronuclei and blebbed micronuclei coincided with the 7.1 mg kg^{-1} Se selenite supplemented trout and the highest numbers of notched nuclei were observed in fish fed the basal diet followed by the fish given 7.1 mg kg^{-1} selenite; frequencies were not however significantly different between treatments (Table 6.6.).

Table 6.5.

Mean hematocrit, serum lysozyme and peak respiratory burst of rainbow trout given various levels of organic or inorganic Se for 10 weeks pre and post 7 days stress.

Selenium ¹	Pre stress	Post stress	Mean change	⁴ Stress effect <i>p</i> =
Hematocrit²				
0.7	33.5	33.3	+0.2	0.388
2.3 Selenite	33.5	32.9	-0.6	0.794
3.9 Selenite	33.3	30.3	-3.0	0.359
7.1 Selenite	29.8	33.9	+4.1	0.005*
2.4 Se-yeast	31.7	30.9	-0.8	0.720
4.1 Se-yeast	33.8	32.4	-1.4	0.468
7.4 Se-yeast	31.8	28.8	-3.0	0.302
<i>Pooled SEM</i>	0.1	0.1		
³ Dietary effect <i>p</i> =	0.186	0.162		
Lysozyme activity⁵				
0.7	749	1104	+355	0.008*
2.3 Selenite	938	1283	+345	0.027*
3.9 Selenite	931	1136	+205	0.265
7.1 Selenite	710	1181	+471	0.001*
2.4 Se-yeast	896	1207	+311	0.045*
4.1 Se-yeast	878	1101	+223	0.214
7.4 Se-yeast	817	1333	+516	0.006*
<i>Pooled SEM</i>	20	24		
³ Dietary effect <i>p</i> =	0.729	0.362		
Respiratory burst⁶				
0.7	2.5	2.8 ^{ab}	+0.3	0.140†
2.3 Selenite	2.0	5.2 ^b	+3.2	0.074†
3.9 Selenite	1.7	2.2 ^{ab}	+0.5	0.293
7.1 Selenite	2.5	1.4 ^{ab}	-1.1	0.257
2.4 Se-yeast	2.0	2.1 ^{ab}	+0.1	0.341†
4.1 Se-yeast	1.5	1.2 ^a	-0.3	0.596
7.4 Se-yeast	3.8	2.6 ^{ab}	-1.2	0.432†
<i>Pooled SEM</i>	1.6	1.7		
³ Dietary effect <i>p</i> =	0.326†	0.018*†		

¹ mg kg⁻¹ Se and supplemented source.

² Percent.

³ *p*= significance of effect of dietary Se intake and source (*n*=2); * highlights significant difference. † indicates significant variability between replicate tanks (*p* ≤ 0.05). Different lower case superscript letters indicate significant (*p* ≤ 0.05) difference between Se treatments.

⁴ *p*= significance of stress induced changes within individual dietary Se treatments (*n*=2). † indicates significant variability between replicate tanks (*p* ≤ 0.05).

⁵ Units lysozyme ml⁻¹.

⁶ Relative light units (x 10³).

Table 6.6.

Mean nuclear abnormalities and leukocyte frequency of rainbow trout given various levels of organic or inorganic supplemental Se.

Selenium ¹	Micronuclei ² (‰)	Blebbled ² (‰)	Notched ² (‰)	Leukocytes ³ (‰)
0.7	0.5	4.3	3.7	72 ^{ab}
2.3 Selenite	0.8	1.2	2.1	59 ^b
3.9 Selenite	0.4	2.9	2.6	70 ^{ab}
7.1 Selenite	1.4	5.3	3.6	74 ^{ab}
2.4 Se-yeast	0.6	2.6	2.5	82 ^a
4.1 Se-yeast	0.3	2.9	1.3	65 ^{ab}
7.4 Se-yeast	0.4	3.8	2.7	40 ^c
<i>Pooled SEM</i>	<i>0.1</i>	<i>0.2</i>	<i>0.2</i>	<i>2</i>
⁴ <i>Dietary effect p =</i>	<i>0.063</i>	<i>0.137</i>	<i>0.431</i>	<i>0.018*</i>

¹ mg kg⁻¹ Se and supplemented source.

² Nuclear abnormalities per 1000 cells.

³ Total leukocyte counts per 1000 cells.

⁴ *p* = significance of effect of dietary Se intake and source (*n*=2); * highlights significant difference. Different lower case superscript letters indicate significant (*p* ≤ 0.05) difference between dietary Se treatments.

6.3.6. Effect of dietary selenium and stress on fillet drip loss

Pre stress the post mortem percentage drip loss of fillets was significantly affected by both the level and source of dietary Se; however there was also a significant variability between duplicate tanks. Pre-stress, the highest moisture loss over the 48 hour period was observed in fish fed the un-supplemented basal diet. A trend towards decreased drip loss was observed in fish given 3.9 mg kg⁻¹ Se selenite supplementation, but selenite did not significantly decrease moisture loss at any supplementation level. Se-yeast supplementation decreased water loss in comparison to the basal diet, with the 2.4 and 7.4 mg kg⁻¹ Se yeast supplemented fish displaying a significantly lower post mortem drip loss than fillets from the control fish. There

was no significant difference in drip loss of fillets of post stress fish. Stress decreased percentage loss in each treatment to a significant extent except that of the 3.9 selenite, and 4.1 and 7.4 mg kg⁻¹ Se-yeast supplemented groups (Table 6.7.); however there was significant variability observed between duplicate tanks.

Table 6.7.

Mean fillet drip loss of rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks pre and post 7 days stress.

Selenium ¹	Pre stress	Post stress	Mean change	⁴ Stress effect <i>p</i> =
Drip loss ²				
0.7	14.6 ^a	8.1	-6.5	< 0.001*†
2.3 Selenite	12.7 ^{ba}	8.0	-4.7	<0.001*
3.9 Selenite	10.3 ^{ba}	8.2	-1.8	0.066†
7.1 Selenite	13.2 ^{ba}	8.5	-4.7	0.022*
2.4 Se-yeast	10.0 ^b	8.2	-1.8	0.111†
4.1 Se-yeast	10.3 ^{ba}	8.9	-1.4	0.024*†
7.4 Se-yeast	9.3 ^b	8.8	-0.5	0.588†
<i>Pooled SEM</i>	0.3	0.1		
³ Dietary effect <i>p</i> =	0.003*†	0.924†		

¹ mg kg⁻¹ Se and supplemented source.

² Percentage drip loss of rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks pre and post 7 days handling stress.

³ *p*= significance of effect of dietary Se intake and source (*n*=2); * highlights significant difference, † indicates significant difference between replicate tanks (*p* ≤ 0.05). Different lower case superscript letters indicate significant (*p* < 0.05) difference between dietary Se treatments.

⁴ *p*= significance of stress induced changes within individual dietary Se treatments (*n*=2); * highlights significant difference pre and post stress, † indicates significant difference between replicate tanks (*p* ≤ 0.05).

Table 6.8.

Mean whole body copper and zinc in rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks pre and post 7 days stress.

Selenium ¹	Dietary concentration ²	Pre stress ³	Post stress ³	⁵ Stress effect p=
<i>Copper</i>				
0.7	12.7	3.2 ^a	3.2 ^{bc}	0.914
2.3 Selenite	12.6	3.3 ^a	3.3 ^{bc}	0.954
3.9 Selenite	13.5	3.9 ^a	4.5 ^a	0.150
7.1 Selenite	12.9	6.7 ^b	7.7 ^b	0.457
2.4 Se-yeast	12.9	3.0 ^a	2.9 ^c	0.792
4.1 Se-yeast	12.9	2.8 ^a	3.5 ^{bc}	0.070
7.4 Se-yeast	13.2	2.9 ^a	3.0 ^{bc}	0.633
<i>Pooled SEM</i>		0.1	0.1	
⁴ Dietary effect p=		0.008*	0.011*	
<i>Zinc</i>				
0.7	134	50.3	56.7	0.402
2.3 Selenite	133	58.0	53.2	0.523
3.9 Selenite	135	53.5	58.4	0.133
7.1 Selenite	136	57.3	51.6	0.194
2.4 Se-yeast	135	51.2	46.4	0.291
4.1 Se-yeast	135	56.7	51.6	0.157
7.4 Se-yeast	136	49.9	50.6	0.896
<i>Pooled SEM</i>		0.5	0.5	
⁴ Dietary effect p=		0.054	0.427	

¹ mg kg⁻¹ Se and supplemented source.

² mg kg⁻¹ wet weight.

³ mg kg⁻¹ dry weight.

⁴ p= significance of effect of dietary Se intake and source (n=2); * highlights significant difference. Different lower case superscript letters indicate significant (p ≤ 0.05) difference between dietary Se treatments.

⁵ p= significance of stress induced changes within individual dietary treatments (n=2).

Table 6.9.

Mean whole body manganese and iron in rainbow trout given various levels of organic or inorganic supplemental Se for 10 weeks pre and post 7 days stress.

Selenium ¹	Dietary concentration ²	Pre stress ³	Post stress ³	⁵ Stress effect <i>p</i> =
<i>Manganese</i>				
0.7	28.6	1.83	2.74	0.207
2.3 Selenite	28.5	2.40	2.23	0.775
3.9 Selenite	28.9	1.89	2.45	0.176
7.1 Selenite	29.4	2.77	2.73	0.924
2.4 Se-yeast	29.1	2.06	1.60	0.164
4.1 Se-yeast	28.8	2.65	2.02	0.063
7.4 Se-yeast	29.8	2.25	2.01	0.672
<i>Pooled SEM</i>		0.08	0.11	
⁴ Dietary effect <i>p</i> =		0.330	0.190	
<i>Iron</i>				
0.7	165.6	22.2	24.4	0.362
2.3 Selenite	165.9	24.0	21.2	0.254
3.9 Selenite	168.2	21.4	24.3	0.060
7.1 Selenite	165.4	21.3	21.4	0.971
2.4 Se-yeast	163.8	20.3	19.4	0.674
4.1 Se-yeast	163.1	19.2	23.3	0.068
7.4 Se-yeast	177.0	19.8	22.2	0.430
<i>Pooled SEM</i>		0.9	1.2	
⁴ Dietary effect <i>p</i> =		0.319	0.374	

¹ mg kg⁻¹ Se and supplemented source.

² mg kg⁻¹ wet weight.

³ mg kg⁻¹ dry weight.

⁴ *p*= significance of effect of dietary Se intake and source (*n*=2). Different lower case superscript letters indicate significant (*p* ≤ 0.05) difference between dietary Se treatments.

⁵ *p*= significance of stress induced changes within individual dietary treatments (*n*=2).

6.3.7. Effect of dietary selenium and stress on other trace elements

Handling and confinement stress and had no significant effect on whole body levels of Cu, Fe, Mn or Zn (Table 6.8. and 6.9.). Both pre-stress and post-stress an interaction between selenite and whole body Cu was observed; no such interaction was found with Se-yeast (Fig 6.2.). Levels of whole body Fe, Mn and Zn were unaffected by dietary Se (Table 6.8. and 6.9.).

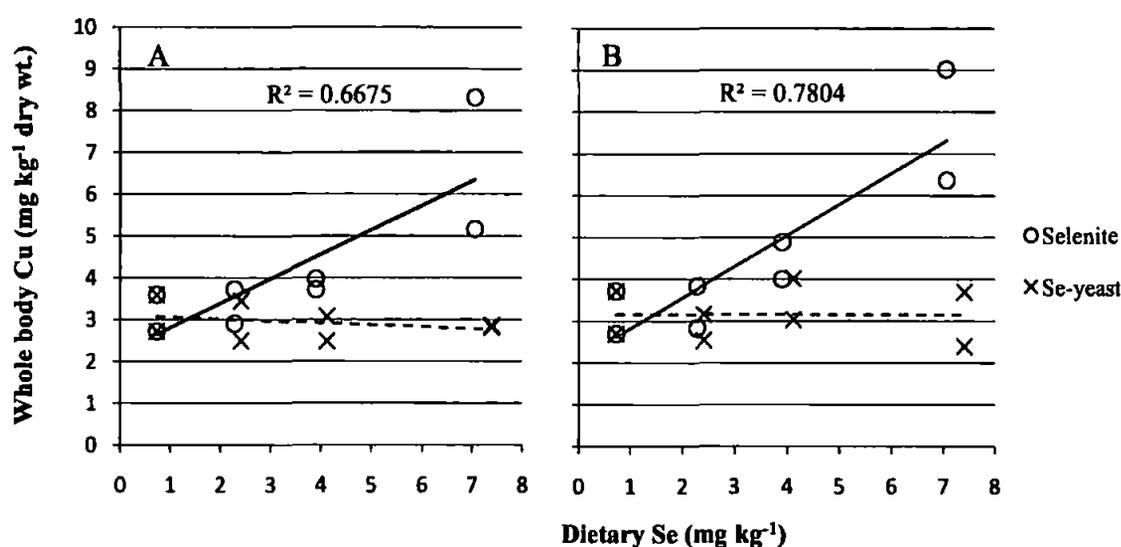


Figure 6.2. Response of whole body Cu concentration to 10 weeks organic and inorganic dietary Se supplementation both pre and post 7 days handling stress in rainbow trout. Whole body Cu ($n=2$) of rainbow trout given a commercial fishmeal diet supplemented with various levels of Se from Se-yeast or selenite pre stress (A) and post stress (B). Pre stress increasing selenite supplementation elevates whole body Cu in a linear dose response (regression $p < 0.001$); Se-yeast supplementation has no effect on whole body Cu (regression $R^2 0.073$, $p 0.310$). The same trend is found post stress with selenite and Cu (regression $p < 0.001$) and Se-yeast and Cu (regression $R^2 < 0.001$, $p 0.673$).

6.4. Discussion

6.4.1. Retention of selenite and Se-yeast

Net whole body Se retention and accumulation was significantly greater for Se-yeast than selenite in trout. Similarly in hybrid striped sea bass, Se-Met is 3.3 fold more bioavailable than selenite (Jaramillo et al., 2009). The increased Se retention of Se-yeast in the whole body may be explained by the lower digestibility of selenite compared to organic Se in fishmeal and the effective non-specific incorporation of Se-Met into proteins of skeletal tissues (Schrauzer, 2003), which is known to occur in salmonids (Bell et al., 1989; Rider et al., 2009). In fish and other higher organisms, absorption of selenite occurs in the anterior intestine by passive diffusion (Daniels, 1996), but uptake of Se-Met via the Na⁺ dependent neutral amino acid transport system is more efficient and as shown in the present study with fish, not affected by dietary Se concentration (Humaloja and Mykkanen, 1986; Schrauzer, 2003). Homeostasis of Se is not regulated during uptake but via excretion. Se can only be excreted once methylated and the rate at which this occurs may limit Se excretion in salmonids (Hilton et al., 1982). A limited Se excretion rate may explain the disproportional retention of selenite when supplemented at 7.1 mg kg⁻¹. The increased accumulation of Se in Se-yeast supplemented fish may confer a benefit to Se deficient consumers as a fortified functional food (Cotter et al., 2008; Rayman, 2004); Se-Met enriched fillets provide a highly bioavailable form of Se to rats (Ornsrud and Lorentzen, 2002). The lower Se accumulation in selenite supplemented fish compared to those fed the basal diet may be due to the increased uptake of inorganic Se compounds at the expense of the residual organic Se; inorganic Se can not be non-specifically incorporated in salmonids (Lorentzen et al., 1994).

6.4.2. Selenium status and stress; differences between selenite and Se-yeast

Background Se from the practical diet appeared to maintain Se status pre-stress, but subsequent husbandry related stressors increased Se utilisation and dietary supplementation may be necessary to meet elevated Se requirements during periods of stress. Handling and confinement are two of the most common physical stressors encountered by intensively farmed fish, and have been routinely used by biologists for research into the effects of stress (Pickering, 1998). In un-stressed fish, supra-supplementation of Se appeared to be superfluous. The lack of a response by both GSH-Px and Trx-R from either supplemented Se-yeast or Selenite indicates that Se requirements were met by the basal diet. This is consistent with previous findings in other salmonids fed practical diets containing 1-2 mg kg⁻¹ Se (Lorentzen et al., 1994). Although Trx-R has not been used to determine requirements in fish, it has been found to correlate well to Se status in mammals (Hill et al., 1997); even to supra-nutritional levels in rats (Berggren et al., 1999). In the present study, a trend towards increased Trx-R activity by both Se sources was observed up to ~4 mg kg⁻¹ Se but this was not significant. Whole body Se decreased and a trend towards increased GSH-Px was observed as a result of chronic handling and confinement stress, which is indicative of an increased requirement for Se, as found in stressed Chinook salmon (Halver et al., 2004). The different pattern of Se loss in fish supplemented selenite suggests inorganic Se is mobilised from different reserves than in Se-yeast supplemented fish. Two pools of Se are thought to exist, one rapidly exchangeable metabolic pool involving the liver where all forms of Se, including inorganic Se, are metabolised and synthesis of selenoproteins occurs, and a second Se pool comprising of Se-Met proteins in skeletal as well as the visceral tissues. The exchangeable pool does not contribute to the Se-Met pool but the Se-Met pool can contribute to the exchangeable pool (Daniels, 1996). The pattern of Se loss found in the stressed fish of the present study is consistent with the notion of two such Se pools. The lower loss of Se

from Se-yeast than selenite supplemented fish at $>7 \text{ mg kg}^{-1}$ Se suggests that organic Se may be mobilised from the slower exchanging Se-Met pool and selenite is only stored within the rapidly exchanging pool involving the liver. Previous studies principally show that selenite accumulates in the liver and not skeletal tissues, whereas Se-yeasts can raise both hepatic and skeletal Se reserves (Rider et al., 2009) (Chapter 3.). The release of Se-Met from the skeletal muscle may involve the catabolism of proteins that occurs during chronic stress (Pickering, 1998). GSH-Px levels revealed that Se requirements may increase as a result of combined handling and confinement stress and un-supplemented diets may not meet increased Se requirements. There was a trend toward increased GSH-Px over the basal diet in all treatments but increases were only significant in trout receiving 8 mg kg^{-1} Se-yeast, showing Se-yeast to be more effective in raising GSH-Px during stress. The trend toward increased activity in the 2.3 mg kg^{-1} selenite diet observed indicates that both organic and inorganic Se can be incorporated into GSH-Px. The trend towards decreased GSH-Px activity with increasing selenite supplementation may be indicative of a toxic effect of high selenite supplementation, which was not observed with Se-yeast. An increased GSH-Px by stress has also been reported in transported Chinook salmon and is postulated to be due to an increased phospholipid and cytosolic GSH-Px activity to provide protection against peroxidation of PUFA in cell membranes and other biological molecules (Halver et al., 2004).

6.4.3. Effects of selenium and physical stress on oxidative status

Oxidative status was unaffected by Se supplementation pre-stress, but physical stressors increased both TAC and lipid peroxidation regardless of Se level. The adequate Se status of pre-stressed fish in the present study is consistent with a lack of effect of supplemental Se on oxidative status, as measured by serum TAC and hepatic MDA. Deficiencies of Se are known to cause tissue peroxidation due to a reduced oxidative capacity resulting from a decreased

GSH-Px activity (Bell et al., 1987; Gatlin et al., 1986). Husbandry related stressors increased both MDA and TAC, and are indicative of oxidative stress; which confirm the findings of the previous experiment involving physical stress. However, no beneficial effects of Se were observed on these markers. In mammals, the increased metabolic rate concurrent with exhaustive exercise raises the production of free radicals, and as a consequence antioxidant defences are up regulated (Ji, 1999; Leeuwenburgh and Heinecke, 2001). In chub, increased physical activity causes oxidative stress as verified by an increased oxidative DNA damage (Aniagu et al., 2006). The increased TAC in stressed fish in the present study suggests that confinement and handling stressors, which involve increased physical activity, increase free radical production. Increased TAC as a result of chronic exercise has also been reported in rabbits (Alipour et al., 2006). The lack of effect of Se on TAC, which is a measure of the combined activity of all known and unknown antioxidants, can be explained by the fact that ascorbic acid, tocopherol and uric acid are major contributors of plasma antioxidant status in fish (Xue et al., 1998). MDA, a product of lipid peroxidation, is frequently measured as a marker of oxidative stress (Dotan et al., 2004; Esterbauer et al., 1991) and increased as a result of physical stress in the present study. Se is involved in the primary cellular antioxidant defence as cystolic and phospholipid GSH-Px along with antioxidant enzymes SOD and catalase (Halliwell and Gutteridge, 1999). The importance of Se in the maintenance of cellular oxidative status during increased metabolic activity is demonstrated by Reddy, et al. (1998), who showed that free radical production is greater in exercised rats with a Se and vitamin E deficiency than those that are replete. MDA was not reduced by Se supplementation in the present study, but was in fact increased by the highest level of selenite supplementation. A lack of protective effect by selenite on exercise induced lipid peroxidation had also been reported in rats (Brady et al., 1979). These findings are surprising due to protective role of phospholipid GSH-Px against hydroperoxide induced lipid

peroxidation (Brigelius-Flohe, 1999). Increased lipid peroxidation in high selenite supplemented fish may be due to the mobilisation of stored hepatic selenite (or selenide) during stress resulting in selenite exhibiting its pro-oxidant properties (Sphaloz 1994). This shows a sub-lethal toxic effect of selenite at 7.1 mg kg^{-1} Se. No evidence of pro-oxidant related toxicity was observed in high Se-yeast supplemented fish. Organic Se is organically bound to proteins and is consequently expected to be released slowly through protein catabolism.

6.4.4. Selenium and health; implications of physical stress

Se supplementation had no beneficial effect on growth performance, immuno-competence or any other health parameter both pre and post-stress. The lack of effect of supplemental Se on immune function and health is consistent with an adequate Se status of un-stressed fish fed the basal diet. Growth is only decreased in severe Se deficiencies (0.07 mg kg^{-1}) or at toxic levels (Hilton et al., 1980); the Se levels used in this study were therefore neither overtly toxic nor deficient confirming the reduced toxicity of dietary Se in fish than mammals. The extra cellular secretion of superoxide post activation with PMA, as measured by Pholasin[®] displayed a high level of inter-individual variation and consequently differences could not be conclusively attributed to either stress or Se supplementation. Post PMA activation, extracellular superoxide (O_2^-) can be followed due to chemiluminescence by Pholasin[®] on reaction with the superoxide anion (Arnhold, 2004). A lack of effect of Se on extracellular superoxide production has been reported in catfish (Wise et al., 1993). The lack of effect of organic Se on lysozyme has also been reported by Cotter et al. (2008) in hybrid striped bass. In contrast to their study, no increase by selenite supplementation was observed. Nuclear abnormalities are frequently used in the assessment of cyto-genotoxicity (Costa and Costa, 2007). Se compounds can be either anti-genotoxic or genotoxic in mammals (Itoh and

Shimada, 1996). Inorganic Se has been shown to induce micronuclei in fish erythrocytes (Al-Sabti and Metcalfe, 1995). In the present study there was a trend towards increased nuclear abnormalities in erythrocytes of fish supplemented with 7.1 mg kg^{-1} selenite; however this was not significant. The especially low leukocyte counts for 7.4 mg kg^{-1} Se-yeast supplemented fish may be due to excess organic dietary Se. Waterborne Se contamination has been shown to increase leukocyte counts in sunfish (*Lepomis cyanellus*) (Lemly, 2002), but this was not observed by inorganic Se in the present study. In terrestrial species a decreased proliferation of peripheral leukocytes occurs in response to excess dietary selenite (Rampal et al., 2008). The decrease of leukocytes by 7.4 mg kg^{-1} Se-yeast but not selenite may be a result of increased organic Se accumulation in the head kidney tissue (Rider et al., 2009). This shows that the effective accumulation of organic Se may be detrimental in some tissues showing a sub-lethal toxic effect of organic Se. The trend towards decreased hematocrit in the 7.1 mg kg^{-1} selenite supplemented fish may be another sub-lethal toxic effect of inorganic Se, as reported in sunfish exposed to high levels of waterborne Se (Lemly, 2002). Selenite is reported to increase eryptosis (suicidal death of erythrocytes) due to cell membrane scrambling; this effect did not occur with selenate (Sopjani, et al., 2008). The return of hematocrit to normal values in stressed fish given 7.1 mg kg^{-1} Se from selenite may be due to the release of erythrocytes from the spleen (Randall and Perry, 1992) as a result of stimulation by catecholamines released during severe stress responses (Perry and Bernier, 1999). Of the aforementioned health and immune parameters, only lysozyme was conclusively affected by the physical stressors used. An increased lysozyme activity in rainbow trout has also been associated with handling stress in previous studies (Saurabh and Sahoo, 2008). Differences in PMA induced extracellular superoxide production between stressed and un-stressed fish could not be conclusively attributed to the applied stressors as a significant level of variability occurred between replicate tanks. Ortuno et al. (2001) found no

effect of acute crowding stress on the production of superoxide production by head kidney leukocytes. As previously reported in rats (Rao et al., 2001), the assessment of DNA stability by use of the Comet assay may be a sensitive marker of both the protective and damaging effects of dietary Se in fish. However, storage protocols are required such that the assay may be used in feeding trials involving large numbers of samples.

6.4.5. Effect of selenium and physical stress on fillet quality

Post mortem fillet water holding capacity was significantly improved by all levels of Se-yeast supplementation but only by 3.9 mg kg⁻¹ Se from selenite. As significant variability occurred between replicate tanks, these findings should be interpreted with caution. In salmonids, only organic Se sources are effectively deposited in muscle tissue due to the non-specific incorporation of Se-Met (Rider et al., 2009). Increased Se in the fillet may increase GSH-Px activity. GSH-Px increases in activity during storage at -50 °C, which may protect the post mortem muscle from oxidative deterioration during storage; modified GSH-Px activity may be due to alterations in enzyme configuration (Nakano et al., 1992). Oxidation of lipids is a major quality issue in fish fillets (Lie, 2001) and further research may reveal the potentially protective role of supplemental organic Se and its relationship with GSH-Px activity on fillet quality. The decrease in drip loss due to stress was not expected. Handling stress and exercise is known to lead to a disruption of normal metabolism in the white muscle of fish. After death, glycogen continues to be converted to lactic acid in the muscle. However in stressed fish glycogen is depleted and lactate levels are reduced which results in a higher muscle pH. This causes modulations in muscle pH, which can increase drip loss (Pottinger, 2001).

6.4.6. Effect of selenium and physical stress on other trace elements

Inorganic Se exerted a positive interaction with whole body Cu but no such interaction was observed with Se-yeast. In salmonids positive interactions between dietary selenite and Cu have been previously reported (Hilton and Hodson, 1983; Julshamn et al., 1990; Lorentzen et al., 1998). The interaction between selenite and Cu may occur either within the GI tract where the low pH may result in the formation of insoluble compounds or post absorption. Post absorption, Cu is released into the plasma and transported within albumin to the liver for incorporation and subsequent distribution via the plasma within ceruloplasmin (Clearwater et al., 2002). One mechanism causing the selenite/Cu interaction may be competition between Se and Cu for albumin binding sites. The higher Cu in the 8 mg kg⁻¹ supplemented fish may have also contributed to the increased lipid peroxidation in fish of this treatment as high levels of Cu can result in oxidative stress (Ozcelik et al., 2003). The lack of interaction between Se and Fe or Zn has also been reported by Julshamn et al. (1990). Interestingly this study also shows that unlike Se, whole body Fe, Mn, Zn and Cu are not decreased by handling stress and therefore reserves of these minerals may be maintained during physical stress. However the assessments of whole body trace elements levels do not consider the compartmentalisation of elements between various tissues. Plasma Zn has been shown decrease in response to corticosteroids (Vallee and Falchuk, 1993) and in sea trout (*Salmo trutta*) Zn decreases in upstream migrating fish possibly due to gonad development and (or) stress (O'Grady, 1981). The assessment of Zn in individual tissues during stress may shed light on the utilisation of Zn and other trace elements during stress.

6.4.7. Conclusions

In conclusion, this study has shown that without the presence of any stressors, practical diets may contain sufficient residual Se to meet requirements. However, physical stress results in

an increased Se utilisation and Se supplementation may therefore be necessary. European legislation on feed additives limits Se supplementation in diets containing $> 0.5 \text{ mg kg Se}^{-1}$, which is of particular concern, as commercial diets often contain Se above this threshold. The pivotal role of Se on immunity and fish health is of particular importance during adverse conditions such as exposure to husbandry related stress and seawater adaptation (Thorarinsson et al., 1994). The lack of a capability to supplement Se may consequently be detrimental to the welfare of intensively farmed fish, which are frequently exposed to physical and environmental stressors, as well as disease. Due to the increased requirement of Se during stress events, further studies are required to optimise the dietary levels of Se, particularly organic Se sources, beneficial to fish exposed to adverse conditions including the onset of disease. Despite containing high levels of Se, fish fed practical diets supplemented with up to $\sim 4 \text{ mg kg}^{-1}$ Se do not exhibit any signs of Se toxicity, which is consistent with the fact that the prey of wild salmonids, notably crustaceans, can contain $> 3 \text{ mg kg}^{-1}$ Se (Julshamn, 1990). The study also highlights that Se source is crucial, as organic Se exhibits several advantages over selenite including; increased retention in the slowly exchanging Se pool; decreased whole body loss during stress; no capacity to induce lipid peroxidation; increased capacity to raise GSH-Px during stress; no definitive interactions with other trace elements, and the potential to produce a Se enriched food for the consumer.

CHAPTER 7.

GENERAL DISCUSSION

7.1. Overview of findings

The objective of the present study was to assess the role of Zn and Se in the maintenance and promotion of the health of cultivated rainbow trout. Due to the increased realization of the potential advantages of organic trace elements the study involved a particular focus on the comparison of organic and inorganic Zn and Se sources. Such that the study may be relevant to conditions typical of intensive aquaculture the study made use of practical diets and exposure to stress rather than classical requirement experiments involving semi-purified diets and favourable experimental rearing conditions. The results of the present study indicate that assessments of mineral requirements in experimental situations involving semi-purified diet differs considerably to trace element utilisation in intensively farmed fish fed practical diets and cultured in marginal environments. To date the majority of research on trace minerals in fish nutrition has focused on inorganic sources. In common with mammalian research, this study provides evidence that for some trace elements organic sources may be more bioavailable and efficacious than traditionally used inorganic sources and should be a focus of further research.

7.1.1. The bioavailability of organic and inorganic zinc and selenium

Of primary importance in the function of a trace element is its bioavailability; the uptake from the diet and subsequent incorporation into metabolic processes (O'Dell, 1984). Much research has focused on the availability of Se and Zn from purified and semi-purified diets,

yet it is well established that due to the presence of various anti-nutritional factors, the bioavailability of Se (Bell et al., 1989), and Zn (Sato et al 1987a,b; Sato et al, 1993) to salmonids is reduced in practical diets. The findings of the first feeding trial (Chapter 3.) indicate that organic Se is more bioavailable than selenite, but no conclusive differences were observed between the digestibility and bioavailability of Zn-pr in comparison to Zn-sulphate.

The digestibility of Zn in the white fishmeal based diet of the first trial (Chapter 3.) was low and no significant differences were observed between Zn sources. There is still no conclusive evidence of increased organic Zn bioavailability in salmonids fed practical diets. The only studies to report increased bioavailability or organic Zn over Zn-sulphate in practical diets is by Apines et al. (2003b) and Apines et al. (2001) using Zn-AA. Other studies with salmonids, including the present, report no significant differences in the availability of Zn-sulphate in comparison to Zn-gluconate (Maage et al., 2001), Zn-Met or Zn-pr (Bioplex[®]) (Kjoss et al., 2006). An explanation for the similar bioavailabilities of both Zn sources may be the dissociation of the Zn ion from some chelates or complexes due to the high pH within the salmonid stomach. The strength of a chelate or complex is crucial to its uptake from the GI tract and subsequent metabolism. Consequently the bioavailability of organic Zn sources may vary significantly between different compounds. Due to variations in the physiological conditions of the GI tract, the behaviour of organic Zn sources is expected to vary between species.

In the first experiment the use of practical diets was successful and revealed some novel findings with regard to the comparison of Se sources. The significantly higher digestibility of Se-yeast than selenite from a practical diet provides evidence that organic Se may be less susceptible to the effects of anti-nutritional factors in fishmeal based diets. As found in chicks (Cantor et al., 1975) and Atlantic salmon (Bell et al., 1989) the relatively low availability of

Se from fishmeal in comparison to other feedstuffs may be effectively overcome with Se-yeast. Consequently, organic Se appears to be a more suitable supplement than selenite for supplementation of practical fish diets.

As typically practical diets often contain residual Se at twice the level of requirements (see 8.2), the determination of additional Se enzymes, in this case Trx-R, which can respond to supra-nutritional Se levels, was particularly relevant. In the first feeding trial (Chapter 3.), Trx-R activity increased as a result of Se-yeast but not by selenite supplementation and GSH-Px responded to both sources. As an increase in GSH-Px activity occurred at higher dietary Se levels than previously reported in trout, this suggests Se requirements may be higher than in salmonids fed practical diets than those fed semi-purified experimental diets.

7.1.2. Organic and inorganic zinc supplementation and health

The role of chelated and inorganic Zn sources in health processes was assessed in the second feeding trial (chapter 4). Zn sulphate and Zn-pr were supplemented to a practical diet and markers specific to Zn utilisation and related health responses were assessed. Although nutritional levels (125 mg kg^{-1}) of both Zn-sulphate and Zn-pr raised whole body and tissue Zn, Zn supplementation did not improve any of the measured health parameters. The essentiality of Zn for optimal growth (Ogino and Yang, 1978), oxidative status (Hidalgo et al., 2002; Kucukbay et al., 2006) and immune function (Paripatananont and Lovell, 1995a,b) has been established in salmonids. The findings are most likely explained by the observation of a significant negative correlation between dietary Zn and Zn retention in the present study. Although the regulation of Zn sulphate was previously observed in some studies (Clearwater et al., 2002), the present study shows that Zn-pr is also under the same strict homeostatic control in rainbow trout. Recently, it has been reported that dietary Zn up to 4800 mg kg^{-1} is

effectively regulated in trout, with the carcass accumulating 84-90 of dietary Zn (the carcass makes up to 80 % of body mass) (Sappal et al., 2009).

The efficient homeostatic control of dietary Zn explains the lack of either beneficial or toxic effects of supplemental Zn found in the present study and possibly also the lack of effect of dietary Zn in the first trial. This highlights the complications associated with the use of practical diets in the comparison of dietary Zn sources. In the study by Apines et al. (2003b) and Apines et al. (2001), supplemented Zn was 40 mg kg^{-1} and the total Zn content only totalled a maximum of $\sim 72\text{-}100 \text{ mg kg}^{-1}$. However, in the present study, and that of Maage et al. (2001) and Kjoss et al. (2006), minimum dietary Zn was $>120 \text{ mg kg}^{-1}$. Although the use of practical diets is relevant with regards to the presence of anti-nutritional factors, the comparison of Zn sources in practical diets is unsatisfactory due to complications resulting from the high levels of residual Zn. The efficient regulation of dietary Zn may also have contributed to the lack of differences observed between sources in studies involving catfish and turbot (Overnell et al., 1988; Paripatananont and Lovell, 1995a,b; Li and Robinson, 1996; Lim et al., 1996). Given the confirmation of the efficient regulation of dietary Zn, this study supports the reduction of the EU limit from 250 to 200 mg kg^{-1} in 2003.

Although the diet of the second trial was specifically formulated to contain low Zn, the residual Zn was still higher than desirable. The formulation of diets using muscle meal (Maage and Julshamn, 1993; Andersen et al., 1997) and an increased substitution by plant based feedstuffs rather than fishmeal may achieve lower basal Zn levels and ameliorate difficulties encounter with high levels of residual Zn. However, due to the lack of phosphate containing skeletal material, muscle meal is expected contain less anti-nutritional factors than typically formulated practical diets. Due to the high mortality experienced in the second trial it was not possible to assess the roles of Zn during exposure to stress.

7.1.3. Husbandry stress; effects on oxidative status and immuno-competence

The effect of both chronic and acute stressors on the physiology of cultivated fish has received much attention due to the implications of stress on disease resistance, health and production (Pickering, 1992). However, relatively few studies have assessed the effect of chronic husbandry related stressors on oxidative status, which is of particular relevance to Se and Zn intake due to their roles in anti-oxidative processes. The preliminary experiments on stress (Chapter 5.) suggest that seven days chronic confinement and netting stress results in oxidative stress in rainbow trout. Husbandry related stressors increased oxidative DNA damage and modulated plasma TAC, which may be either a result of the increased metabolic activity associated with these physical stressors, particularly netting stress, or possibly the onset of nutritional stress due to reduced feeding and feed utilisation. Similarly, oxidative stress has been reported in chub subjected to exhaustive exercise (Aniagu et al., 2006). The chronic physical stressors did not increase plasma cortisol and may be due to a desensitisation of this stress hormone in chronic stress. This suggests that impaired oxidative status may be a suitable marker of the cumulative effects of chronic physical stress. Due to the roles of Zn and Se in immuno-competence and oxidative status, the findings of this study prompted further evaluation of the nutritional role of Se in the maintenance of these health parameters during exposure to husbandry related stressors. There is currently little research on the relationship between oxidative stress, nutritional stress and mineral utilisation. This is of particular concern as fish compromised by stress or disease at best typically have a significantly reduced feed intake.

7.1.4. Selenium supplementation, health, and implications of husbandry stress

Following the findings of the low bioavailability of Se from a fishmeal based diet, and the possibility to increase Se status by Se-yeast supplementation (Chapter 3.), the role of Se

supplementation in a commercial diet on various relevant markers of health was assessed. In addition, the role of Se utilisation during chronic physical stress was evaluated. Se utilisation has been reported to increase as a result of stress, during which it may play a pivotal role in the maintenance of oxidative status (Halver et al., 2004) and immune function. The preliminary experiments on stress (Chapter 5.) established that the chronic husbandry stress protocol used resulted in the onset of oxidative stress. Due to its nutritional role, supplemented Se was expected to minimise the effects of husbandry related stress on oxidative status. The increased retention of Se-yeast over selenite confirmed findings of the first experiment. However, in un-stressed fish the adequate level of residual Se in the basal diet was concurrent with a lack of any appreciable effects on immuno-competence and oxidative status by supplementation of either Se-yeast or selenite up to 8 mg kg^{-1} . The lack of effect of selenite or Se-yeast on hepatic Trx-R and GSH-Px activity indicated that requirements may have been met by the basal diet (0.73 mg kg^{-1}), which is consistent with previous studies in Atlantic salmon using practical diets (Lorentzen et al., 1994). The increased Se status by Se-yeast supplementation in the first trials (Chapter 3.) may have been due to the use of a lower quality white fishmeal diet than the commercial diet used in the Se/stress trial (Chapter 6.). This may suggest that as with Zn, diet quality may affect Se uptake and needs further evaluation.

However, although maximal hepatic Se enzyme activity was achieved, organic Se supplementation may be necessary for optimal post-mortem fillet quality (drip loss) as it was improved by Se-yeast supplementation. Post mortem fillet quality may consequently require higher levels of Se than for maximal hepatic enzyme activity. The benefit only seen with Se-yeast is consistent with the fact that Se-yeast and not selenite effectively accumulates in the fillet (Chapter 3.). The increased Se in the fillet is proposed to reduce drip loss due to the protective effects of an increase in fillet GSH-Px activity. However, this has yet to be tested

in salmonids; the presence of this enzyme in the fillet may reduce post mortem oxidative deterioration (Nakano et al., 1992).

Although the interaction between selenite and Cu has been previously reported in salmonids (Hilton and Hodson, 1983; Julshamn et al., 1990), this is the first study in fish to report the lack of interaction between Cu and organic Se. The increased digestibility of Se and the lack of interaction with Cu in fishmeal based diets is an indication that Se-yeast may be less prone to potential anti-nutritional factors in practical diets.

The present study found that during periods of physical stress, Se utilisation may increase and requirements may not be met by commercial diet in some adverse cultivation conditions. Seven days chronic husbandry stress decreased whole body Se and increased GSH-Px activity. Importantly, the lowest increase in GSH-Px activity was observed in fish fed the basal unsupplemented diet. This indicates that supplementation of practical diets may be necessary to maintain optimal GSH-Px activity and Se status during stress. An increased requirement for Se during physical stress will have particular implications in the production of farmed fish, which can often involve significant handling, such as in the case of brood stock handling, vaccinations, fish transfer and grading. No studies have assessed the roles of Se in milt or egg quality and hatchability; Se is known to be essential for sperm quality (Surai, 2006). Findings of increased Se utilisation during stress are of particular concern with regard to fish harvest and fillet quality. Prior to harvest fish are normally either transported in the confines of a well boat or crowded, which given the findings of the present study is expected to decrease fillet Se and reduce any protective effects on the post mortem fillet. Se supplementation may therefore be of particular benefit for a period prior to the harvest of fish.

The effects of stress also revealed differences in the metabolism and properties of Se-yeast and selenite. The large loss of whole body Se in the 8 mg kg⁻¹ selenite supplemented fish suggests that hepatic Se from selenite may be more mobile than that from Se-yeast, and at high dietary Se levels the mobility of Se was a disadvantage rather than a benefit. The tissue analysis undertaken in the first experiment (Chapter 3.) suggests that selenite only contributes to the short term storage pool involving the liver. The rapid loss of Se in the liver during stress may be due to the mobilisation selenide that has accumulated due to a limit in the methylation of excess Se. Selenite appears to largely accumulate in the liver, and although it may be utilised within the liver for GSH-Px, it may also be implicated Se toxicity during stress. Although Se-yeast contributes to the long term Se pool, Se-yeast still had the ability to increase GSH-Px indicating it is also available to the short term Se pool involving selenide, as also reported in mammals (Daniels, 1996).

This study has provided evidence that the EU 0.5 mg kg⁻¹ limit on Se in practical diets may not be warranted with regards to intensively cultivated salmonids. This limit fails to consider the bioavailability of Se from fishmeal and the increased Se utilisation in stressed animals. Se requirements were also largely influenced by research based on selenite, which is increasingly regarded as a toxic form of Se. In addition, without the ability to supplement organic Se there is no potential to produce Se enriched fillets. Such a functional food could offer a valuable and convenient source of organic dietary Se that may confer protection against certain cancers in Se-deficient populations, such as those in parts of Europe and New Zealand (Rayman, 2004 and 2008). The established EU Se limit in feeds is the same as that given for terrestrial organisms. However, this study has demonstrated that the nutritional physiology of Se is fundamentally different in fish in which Se utilisation may be greater than in terrestrial organisms.

7.2. Further Research

7.2.1. Evaluation of selenium and zinc nutrition

This study has shown that Se-yeast is not only a suitable trace element supplement, but can be more effective than traditionally used inorganic compounds. Despite the fact that organic trace elements are potentially less toxic and more akin to naturally occurring biologically bound trace elements, the study of the metabolism and requirements of Se and Zn in fish have primarily focused on selenite and Zn-sulphate. It is becoming evident that from uptake mechanisms to incorporation into metabolic processes, the metabolism of some inorganic trace element compounds are different to those that are organically bound. As this study demonstrates, some organic minerals clearly have a place in the supplementation of future salmonid diets. It is therefore desirable that the metabolism and utilisation of the several manufactured organic Se and Zn sources are established and understood. Indeed the metabolism of inorganic elements is still poorly understood and requires further investigation.

The nutritional assessment of both Zn and Se are difficult and few markers of mineral status are satisfactory. A thorough assessment of requirements needs to involve a battery of both direct measures of trace element metabolism and other indirect relevant functional markers as attempted in the present study. The metabolism of Zn appears to have a rapid turnover within the organism and cell. Recent studies in trout have shown that 40 % of Zn in the gill is present in the nuclei-cellular debris (again reflects the essentiality of Zn in nucleic acid synthesis) and 81-90 % of brachial Zn is metabolically active (that present in the nuclei, mitochondria, microsomes-lysosomes and heat denatureable proteins), and Zn exposure does not induce shifts between Zn in the active metabolic pool and the metabolically detoxified pool (heat stable proteins and NaOH-resistant granules). This suggests that sequestration and chelation are not major mechanisms of cellular Zn homeostasis (Sappal et al., 2009). It also

highlights the limitations of using total Zn in fish tissues as a measure of Zn uptake and metabolism and suggests that Zn storage in tissues such as the vertebrae may be of little significance in terms of available Zn. Further knowledge in the metabolism of Zn may lead to the development of more accurate markers of Zn status. Typically levels of a limited number of Se and Zn dependent enzymes are determined and only in the liver or plasma. This study shows that the availability organic and inorganic trace elements sources can differ between tissues. The assessment of a multitude of Se and Zn requiring proteins across a range of tissues would make for a more comprehensive assessment of Zn utilisation in an array of biological processes throughout the organism. Messenger RNA expression of Zn transporter proteins in different tissues, or indeed other proteins responsive to Zn metabolism, may be used in the assessment of different Zn sources and Zn status (Wood, 2000). As Zn is metabolised rapidly, particularly in tissues such as the liver, a measure of Zn turnover, such as by the use of isotopic Zn compounds, may be more reflective of Zn status than static levels (Thompson, 1991).

The response of selenoprotein mRNA expression or activity of selenoproteins other than GSH-Px such as Trx-R and selenoprotein P needs to be evaluated in both normal and stressed fish. In mammals, the activities of Trx-R and selenoprotein P have been found to be optimised at higher levels than GSH-Px (Burk and Hill, 2005; Berggren et al., 1999). In cell lines and animal studies a hierarchy of selenoprotein expression exists during Se deprivation and repletion (Papp et al., 2007); little is known of the distribution of Se amongst Se requiring proteins at different levels of Se status and stress. A comprehensive measure of Se bioavailability determining the presence of individual selenoproteins in various key tissues will soon be made possible as novel speciation analysis becomes more reliable. Due to the emerging role of individual selenoproteins, the future assessment of Se status will go beyond that of just total Se.

Nano particle trace elements may be suitable alternative trace element sources. New research has shown that in crucian carp (*Carassius auratus*) nano-particle Se accumulates to a greater extent in the muscle than Se-Met, and is as effective as Se-Met at raising muscle GSH-Px (Zhou et al., 2009). However, a thorough assessment of the metabolism and toxicity, of nano particle compounds, is required to establish the potential nutritional role of these artificial compounds.

7.2.2. Selenium and zinc utilisation; disease, stress and life cycle

This study has only assessed a very limited number of stressors. Further studies are required to assess the role and utilisation of Se and Zn during exposure to various diseases, environmental stressors, such as oxygen saturation, temperature, and pH, and other husbandry related stressors including stocking density and seawater adaptation. Recently, supplementation of a practical diet containing 0.8 mg kg⁻¹ Se with 15-0.30 mg kg⁻¹ of both selenite and Se-met have been found to decrease muscle MDA and increase muscle GSH-Px in trout exposed to 12 weeks crowding stress (Kucukbay et al., 2009). As the duration of stress was longer than that used in the present study, this suggests that the duration of stress may be an important factor in the effects of Se in the maintenance of oxidative status. The study also found that Se-Met was more effective in its protective effect than selenite, which is consistent with the findings of increased organic Se bioavailability in the present study.

Se and Zn may be of particular importance in reducing susceptibility to stress and disease and should be a focus of future research. Se deficient organisms are known to not only be more susceptible to viral infection, but it is during Se deficiency that avirulent viruses may change into those that are virulent (Beck, 1999). Consequently, the effect of Se status on significant viral fish diseases such as infectious salmon anaemia (ISA), salmonids alphavirus (SAV), infectious pancreatic necrosis (IPN), viral haemorrhagic septicaemia (VHS) and infectious

haematopoietic necrosis virus (IHNV) should be evaluated. The gut microflora plays an important role in disease resistance and health in both humans (Guarner and Malagelada, 2003) and fish (Gomez and Balcazar, 2008). In pigs, it has been reported that Zn deficiency may alter the gut microflora (Lalles et al., 2007). The effects of Zn and Se status on the gut microflora and its subsequent consequences on health and disease resistance have not been assessed in fish to date. Mucus in the GI tract and integument is an important pathogen barrier in fish, yet little is known of the role of trace elements in this 'first line of defence'. Zn is known to play a key role in wound healing (Salgueiro et al., 2000) but this has not been evaluated in fish, which would be of particular relevance due to the lesions resulting from grading and lice damage.

Although this study has shown that levels of whole body Zn, Mn, Fe and Cu do not alter as a result of physical stress, due to the compartmentalisation of trace elements, an assessment of trace minerals during stress in individual tissues needs to be made. Zn is known to be mobilised from the liver in response to corticosteroids (Vallee and Falchuk, 1993). In birds, stress (heat) has been found to reduce Zn digestibility (Sahin and Kucuk, 2003); the effect of stress on mineral digestibility has yet to be determined in fish. The assessment of Zn in the plasma and the bone will reveal if skeletal tissues are indeed Zn important reserves during stress or deficiency as often claimed.

The present study has focused on a certain size class of fish. However, currently little is known of the roles and requirements of Zn and Se in reproduction and within different life cycle stages. It has been shown that Zn tissue levels and requirements may vary with age and life cycle in sea bream (Carpene, et al., 1999). As reported in mammals and birds, Se (Surai, 2006) and Zn (Vallee and Falchuk) are expected to play key roles in fish reproduction, including the development of reproductive tissue, the hatchability of eggs, the development

of larvae, and the prevention of deformities. The role of Se and Zn in these different life stages have received little attention in fish to date.

7.2.3. Selenium and zinc utilisation; diet and species

As there is a need to reduce the reliance on fishmeal in the cultivation of many fish species, the availability of Zn and Se from diets containing a spectrum of alternative protein sources needs to be established. As physiologies of the GI tract there are significantly different amongst fish species, an assessment of the requirements of different Se and Zn sources in different species is required. Supplementation levels may then be tailored for individual species, diets and cultivation conditions. Requirements have not been determined to the following cultivated species; arctic char (*Salvelinus alpinus*), barramundi (*Lates calcarifer*), carp, cobia (*Rachycentron canadum*), halibut, milk fish (*Chanos chanos*), cod, sea bream, sea bass, tuna (*Tunnus sp.*), tench (*Tinca tinca*), turbot (*Hippoglossus hippoglossus*) and tilapia. The role of these elements in cultivated crustaceans is also required. The assessment of Se and Zn utilisation in saltwater species has been limited to date due to difficulties in the assessment of trace element nutrition in salt water fish. However, knowledge in the requirements and behaviour of trace elements in fish residing in seawater is lacking. Due to the very different physiological processes that occur in seawater fish, requirements may differ significantly to their freshwater counterparts.

7.3. Conclusions

The previous focus of inorganic Zn and Se sources in research, and the supplementation of practical diets have led to a lack of understanding and appreciation of the advantages of organic trace elements in fish nutrition and health. This study has revealed not only the suitability but also the advantages of organic trace element supplementation, namely Se, in the formulation of practical diets. As organic minerals are often found to be increasingly

efficacious and less toxic, future research in the Se and Zn nutrition of cultivated fish (and terrestrial animals) should turn its focus on these more natural forms of trace elements.

The advantages of organic minerals are repeatedly claimed by the numerous manufacturers, which appears to be a contributing factor to the scepticism surrounding their use in research and diet formulation. However, it is at the expense of fish health, fish production, and the understanding of trace element nutrition should the potential of organic mineral not be realised. The exact chemical nature of the organic compounds present in manufactured products is of paramount importance with regard to the efficacy of organic minerals. Despite this, there is great variation in the quality of man-made organic mineral products between manufacturers. It is recommended that manufactures claims are not relied upon (as found by Li and Robinson. (1996) with Zn) and rigorous independent scientific testing establishes the true value of individual organic mineral products. The concurrent use of both organic and inorganic sources may also have its place and should be investigated, particularly with Zn. With advances in speciation analysis, particularly for Se, the role of individual specific organic compounds will be an exciting area of future research. Evidence is emerging that the anti-cancer properties of Se are due to the presence of specific organic Se compounds i.e., γ -glutamyl-Se-methyl-selenocysteine rather than simply total Se (Rayman, 2008). By understanding the roles of particular Se and Zn compounds we may come to have a more complete understanding of the roles and advantages of organic minerals both to individual species and within the food chain.

8. APPENDIX

8.1. Analysis of zinc and selenium in selected feed ingredients

Such that a low Zn and Se diet could be formulated, Se and Zn levels in key ingredients were analysed. For methods of analysis (see 2.7.).

Table 8.1.

Mean (\pm SD) zinc and selenium levels in feed ingredients (mg kg^{-1}).

Ingredient	International feed number	Selenium	Zinc
LT fishmeal ¹	5-02-000	2.04 \pm 0.08	96.0 \pm 10.2
White fishmeal ²	5-02-025	1.79 \pm 0.03	106.6 \pm 12.2
Wheat middlings ³	4-05-205	0.08 \pm 0.01	77.3 \pm 6.9
Soybean meal ³	5-04-612	0.05 \pm 0.00	62.4 \pm 10.7

¹ Provimi 66 white fishmeal, Lichfield, Staffordshire, Provimi Ltd, UK.

² Scottish LT fishmeal; United fish products Ltd., Greenwell place, Aberdeen, UK.

³ Elliots Farm, Venn Ottery, Ottery St. Mary, Devon, UK.

8.2. Zinc and selenium analysis of selected commercial trout grower diets

An audit of Se and Zn levels in a selection of trout grower diets was performed. Digests were prepared and Se was analysed by LGC, Teddington, UK and Zn analysed by ICP-OES (see 2.7)

Table 8.2.

Mean (\pm SD) zinc and selenium of commercial trout grower diets (mg kg^{-1}).

Manufacturer	Feed	Selenium	Zinc
Skretting ¹	Standard Expanded 40	0.53 \pm 0.00	133 \pm 2
Aller Aqua ²	15-45 XS	0.61 \pm 0.00	183 \pm 14
Dana Feed ³	Trout Dan-Ex 2844	0.92 \pm 0.02	110 \pm 6
Biomar ⁴	Ecolife 66	0.88 \pm 0.01	292 \pm 4
EWOS ⁵	EWOS sigma 50	1.23 \pm 0.00	160 \pm 4

¹ Shay Lane, Longridge, Preston, Lancashire, UK.

² Aller Aqua, Allervej, Christiansfeld, Denmark.

³ Dana Feed, Haven, Horsens, Denmark.

⁴ Biomar Ltd., North Shore road, Grangemouth Docks, Grangemouth, UK.

⁵ Westfield, Nr Bathgate, West Lothian, UK.

8.3. Validation of thioredoxin reductase assay.

Trx-R assay was limited by amount of Trx so concentration was increased from 0.016 to 0.104 units Trx.

The assay was verified by use of a 0.5 unit per ml positive control. Various tissue were analysed for TRx-R activity.

Table 8.3.

Assay of Trx-R activity in fish tissues

Tissue	Rep.	Trx +/-	A ₄₁₂	A change	A412*1000/min	Mean	%CV
Liver 20ul	a	+	0.726				
Liver 20ul	a	-	0.526	0.199	4.4		
Liver 20ul	b	+	0.731				
Liver 20ul	b	-	0.532	0.198	4.4	4.4	0.4
Liver 40ul	a	+	1.215				
Liver 40ul	a	-	0.853	0.361	8.0		
Liver 40ul	b	+	1.225				
Liver 40ul	b	-	0.833	0.392	8.7	8.4	5.6
Spleen	a	+	1.813				
Spleen	a	-	1.737	0.076	1.7		
Spleen	b	+	1.816				
Spleen	b	-	1.742	0.074	1.6	1.7	2.0
Plasma	a	+	0.396				
Plasma	a	-	0.389	0.006	0.1		
Plasma	b	+	0.385				
Plasma	b	-	0.377	0.008	0.2	0.2	19.4
+ive cont.	a	+	0.912				
+ive cont.	a	-	0.002	0.909	20.2		
+ive cont.	a	+	0.989				
+ive cont.	a	-	0.001	0.987	22.0	21.1	5.8

The data indicates that the assay can determine Trx-R activity in fish tissues. The liver had the highest levels followed by the spleen. Little activity was recorded in the plasma. The liver was chosen for determining tissue Trx-R.

8.4. Methods of cryo-preservation of trout erythrocytes for the comet assay

8.4.1. Introduction

An effective method for the preservation of blood samples is required for use of the comet assay in feeding trials involving the simultaneous sampling of many individual fish. Previous studies in turbot (*Psetta maximus*) reveal that cryo-preservation of kidney, gill and blood cells leads to increased DNA strand breaks, the former being least affected by cryo-preservation (Belpaeme et al., 1998). The aim of this study was to determine the effect of tissue cryo-preservation on DNA stability using the standard and FPG modified comet assay using trout erythrocytes and assess the suitability of mediums containing the permeating agents dimethyl sulfoxide (DMSO) and Glycerol.

8.4.2. Methods

Blood samples were retrieved from three fish via a caudal puncture by use of a heparinised syringe. Individual whole blood (100µl) samples were diluted into 900µl of either freezing medium for cryo-preservation or DPBS solution (-CaCl₂ and MgCl₂, Gibco, Invitrogen, Paisley, UK) for refrigeration. Freezing mediums contained; 20% bovine serum albumin (Invitrogen, Gibco, Paisley, UK) and 40% RPMI-1640 medium (Sigma, Poole, UK) and either 20% DMSO or 20% Glycerol. The dilution medium contained 100% RPMI.

Those samples in freezing medium were frozen at -80 °C for 18 hours and reciprocal samples in RPMI refrigerated at 4 °C for 18 hours.

All samples were the assayed simultaneously with the Comet assay (see 2.11.3.).

8.4.3. Results

Cryo-preservation increased DNA strand breaks over that of the control in both the standard Comet assay and FPG modified assay, but this increase was not significant (ANOVA buffer; p 0.684; FPG p 0.739) Of the two tested freezing mediums, the medium based on DMSO was slightly more effective at maintaining genetic stability (Fig. 8.1.).

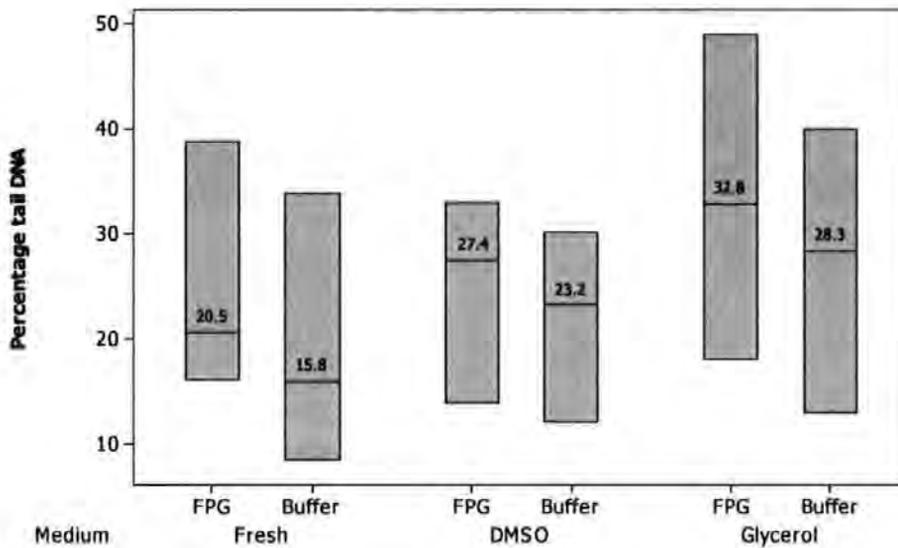


Figure 8.1. Effect of storage methods on DNA stability of rainbow trout erythrocytes. Boxplots showing mean percentage tail DNA of rainbow trout erythrocytes subjected to different storage methods; stored at 4 °C in buffer or cryo-preserved at -80 °C in glycerol or DMSO based mediums; both standard and modified FPG assays presented.

8.4.4. Discussion

As found by Belpaeme et al. (1998) in turbot tissues frozen for seven days, freezing reduces DNA stability in rainbow trout erythrocytes as measured by the Comet assay. However, by use of a freezing medium this can be minimised. Freezing medium containing DMSO is preferred as it was the most effective cryo-preservation technique. This is expected as DMSO can reduce oxidative DNA damage.

9. REFERENCES

- Akcha, F., Vincent Hubert, F., Pfol-Leszkowicz, A., 2003. Potential value of the comet assay and DNA adduct measurement in dab (*Limanda limanda*) for assessment of in situ exposure to genotoxic compounds. *Mutation Research* 534, 21-32.
- Al-Damegh, M.A., 2007. The protective role of zinc sulphate on ethanol-induced liver and kidney damages in rats. *Saudi Journal of Biological Sciences* 14 (2), 283-292.
- Alipour, M., Mohammadi, M., Zarghami, N., Ahmadiasl, N., 2006. Influence of chronic exercise on red cell antioxidant defence, plasma malondialdehyde and total antioxidant capacity in hypercholesterolemic rabbits. *Journal of Sports Science and Medicine* 5, 682-691.
- Allan, C.B., Lacourciere, G.M., Stadtman, T.C., 1999. Responsiveness of selenoproteins to dietary selenium. *Annual Review of Nutrition* 19, 1-16.
- Al-Sabti, K., Metcalfe, C.D., 1995. Fish micronuclei for assessing genotoxicity in water. *Mutation Research* 343, 121-135.
- Ames, B.N., 2001. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. *Mutation Research* 475, 7-20.
- Andersen, F., Lorentzen, R., Waagbo, R., Maage, A., 1997. Bioavailability and interactions with other micronutrients of three dietary iron sources in Atlantic salmon, *Salmo salar*, smolts. *Aquaculture Nutrition* 3, 239-246.
- Angelidis, P., Baudin-Laurencin, F., Youinou P., 1987. Stress in Rainbow trout, *Salmo gairdneri*: effect upon phagocyte chemiluminescence, circulating leukocytes and susceptibility to *Aeromonas salmonicida*. *Journal of Fish Biology* 31, 113-122.

Aniagu, S.O., Day, N., Chipman, K.J., Taylor, E.W., Butler, P.J., Winter, M.J., 2006. Does exhaustive exercise result in associated DNA damage in Chub (*Leuciscus cephalus*)? *Environmental and Molecular Mutagenesis* 47, 616-623.

AOAC, 2007. *Official Methods of Analysis of the Association of Analytical Chemists*. 18th Edn., AOAC, Maryland, USA.

Apines, M.J., Satoh, S., Kiron, V., Watanabe, T., Aoki, T., 2003a. Availability of supplemental amino acid-chelated trace elements in diets containing tricalcium phosphate and phytate to rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 225, 431-444.

Apines, M.J., Satoh, S., Kiron, V., Watanabe, T., Fujita, S., 2003b. Bioavailability and tissue retention of amino acid-chelated trace elements in rainbow trout *Oncorhynchus mykiss*, *Fisheries Science* 69, 722-730.

Apines, M.J., Satoh, S., Kiron, V., Watanabe, T., Nasu, N. and Fujita, S., 2001. Bioavailability of amino acids chelated and glass embedded zinc to rainbow trout, *Oncorhynchus mykiss*, fingerlings. *Aquaculture Nutrition* 7, 221-228.

Apines-Amar, M.J.S, Satoh, S., Ciapang, C.M.A., Kiron, V., Watanabe, T., Aoki, T., 2004. Amino acid-chelate: a better source of Zn, Mn and Cu for rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 240, 345-358.

Arner, E.S.J., Holmgren, A., 2000. Physiological functions of thioredoxin and thioredoxin reductase. *European Journal of Biochemistry* 267, 6102-6109.

Arnhold, J., 2004. Free radicals – friends or foes? Properties, functions, and secretion of human myeloperoxidase. *Biochemistry (Moscow)* 69 (1), 4-9.

- Arteel, G.E., Sies, H., 2001. The biochemistry of selenium and the glutathione system. *Environmental Toxicology and Pharmacology* 10, 153-158.
- Arthur, J.R., 2000. The Glutathione peroxidases. *Cellular and Molecular Life Sciences*, 57, 1825-1835.
- Arthur, J.R., McKenzie, R.C., Beckett, G.J., 2003. Selenium in the immune system. *Journal of Nutrition* 133, 1457-1459.
- Ashmead, H.D., 1992: *The roles of amino acid chelates in animal nutrition*. Noyes Publications, New Jersey, USA.
- B'Hymer, C., Caruso, J.A., 2000. Evaluation of yeast based selenium food supplements using high performance liquid chromatography and inductively coupled plasma mass spectrometry. *Journal of Analytical Spectrometry* 15, 1531-1539.
- Bagdonas, E., Vosyliene, M.Z., 2006. A study of toxicity and genotoxicity of copper, zinc and their mixture to rainbow trout (*Oncorhynchus mykiss*). *Biologija* 1. 8-13.
- Barceloux, D.G., 1999. Zinc. *Clinical Toxicology* 37 (2), 279-292.
- Barton B.A., Schreck, C.B., Barton L.D., 1987. Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. *Diseases of Aquatic Organisms* 2, 173-185.
- Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effect of corticosteroids. *Annual Reviews of Fish Diseases* 1, 3-26.
- Beck, M.A., 1999. Selenium and host defence towards viruses. *Proceedings of the Nutrition Society* 58, 707-711.

- Beckett, G.J., Arthur, J.R., 2005. Selenium and endocrine systems. *Journal of Endocrinology* 184, 455-465.
- Behne, D., Kyriakopoulos, A., 2001. Mammalian selenium-containing proteins. *Annual Review of Nutrition*, 21, 453-473.
- Bejma, J., Ji, L.L., 1999. Ageing and acute exercise enhance free radical generation in rat skeletal muscle. *Journal of Applied Physiology* 87, 465-470.
- Bell J.G., Pirie B.J.S., Ardon J.W., Cowey C.B., 1986. Some effects of selenium deficiency on glutathione peroxidase (EC 1.11.1.9) activity and tissue pathology in rainbow trout (*Salmo gairdneri*). *British Journal of Nutrition*. 55, 305-311.
- Bell, G.J., Cowey, C.B., 1989. Digestibility and bioavailability of dietary selenium from fishmeal, selenite, selenomethionine and selenocysteine in Atlantic salmon (*Salmo salar*). *Aquaculture* 81, 61-68.
- Bell, G.R., Higgs, D.A., Traxler, G.S., 1984. The effects of dietary ascorbate, zinc and manganese on the development of experimentally induced bacterial kidney disease in sockeye salmon (*Oncorhynchus nerka*). *Aquaculture* 36, 293-311.
- Bell, J.G., Cowey, C.B., Adron, J.W., Pirie, J.S., 1987. Some effects of selenium deficiency on enzyme activities and indices of tissue peroxidation in salmon parr (*Salmo salar*). *Aquaculture* 65, 43-54.
- Bell, J.G., Cowey, C.B., Adron, J.W., Shanks, A.M., 1985. Some effects of vitamin E and selenium deprivation on tissue enzyme levels and indices of tissue peroxidation in rainbow trout (*Salmo gairdneri*). *British Journal of Nutrition* 53, 149-157.

- Belpaeme, K., Cooreman, K., Kirsch-Volders, M., 1998. Development and validation of the in vivo alkaline comet assay for detecting genomic damage in marine flat fish. *Mutation Research* 415, 167-184.
- Belpaeme, K., Delbeke K., Zhu L., Kirsch-Volders, M., 1995. Cytogenetic studies of PCB77 on brown trout (*Salmo trutta fario*) using the micronucleus test and the alkaline comet assay. *Mutagenesis* 11, 485-492.
- Berg, J.M., Si, Y., 1996. The galvanisation of biology: A growing appreciation for the roles of zinc. *Science* 271, 1081-1085.
- Berggren, M.M., Mangin J.F., Gasdaska, J.R., Powis, G., 1999. Effect of selenium on rat thioredoxin reductase activity. *Biochemical Pharmacology* 57, 187-193.
- Berry, M.J., Larsen, P.R., 1992. The role of selenium in thyroid hormone action. *Endocrine Reviews* 13(2), 207-219.
- Bettger, W.J., Spry, D.J., Cocknell, K.A., Cho, Y., Cho, C., Hilton, J.W., 1987. The distribution of zinc and copper in plasma, erythrocytes and erythrocyte membranes of rainbow trout (*Salmo gairdneri*). *Comparative Biochemistry and Physiology* 87C (2), 445-451.
- Birringer, M., Pilawa, S., Flohe, L., 2002. Trends in selenium biochemistry. *Natural Product Reports* 19, 693-718.
- Bjerkeng, B., Hamre, B., Hatlen, B., Wathne, E., 1999. Astaxanthin deposition in fillets of Atlantic salmon *Salmo salar* L. given two dietary levels of astaxanthin in combination with three levels of α -tocopheryl acetate. *Aquaculture Research* 30, 637-646.

- Blazer, V.S., 1991. Piscine macrophage function and nutritional influences: A review. *Journal of Aquatic Animal Health* 3 (2), 77-86.
- Boldizarova, K., Gresakova, L., Faix, S., Mellen, M., Leng, L., 2005. Antioxidant status of lambs fed on diets supplemented with selenite or Se-yeast. *Journal of Animal and Feed Sciences* 14, 245-253.
- Brady, P.S., Brady, L.J., Ullrey, D.E., 1979. Selenium, vitamin E and the response to swimming stress in the rat. *Journal of Nutrition* 109, 1103-1109.
- Brigelius-Flohe, R., 1999. Tissue specific functions of individual glutathione peroxidase. *Free Radical Biology and Medicine* 27 (9/10), 951-965.
- Britton G., 1995. UV/Visible spectroscopy. In *Carotenoids*, Vol. IB: Spectroscopy (Eds. Britton G., Liaaen-Jensen S. and Pfander H.), pp 13-62.
- Brown, K.M., Arthur, J.R., 2001. Selenium, selenoproteins and human health: a review. *Public Health and Nutrition* 4 (2B) 593-599.
- Burk, R.F., 1976: Selenium in man. In: A.S. Prasad (Ed.), *Trace Elements in Human Health and Disease*. Academic Press, London, 105-134.
- Burk, R.F., Hill, K.E., 2005. Selenoprotein P: An extracellular protein with unique physical characteristics and a role in selenium homeostasis. *Annual Review of Nutrition* 25, 215-235.
- Bury, N.R., Walker, P.A., Glover, C.N., 2003. Nutritive metal uptake in teleost fish. *Journal of Experimental Biology* 206, 11-23.
- Bustos, R., Romo, L., Yanez, K., Diaz, G., Romo, C., 2003. Oxidative stability of carotenoid and polyunsaturated fatty acids in microparticulate diets containing krill oil for nutrition of marine fish larvae. *Journal of Food Engineering* 56, 289-293.

- Cai, L., Cherian, G.M., 2003. Zinc-metallothionein protects from DNA damage induced by radiation better than glutathione and copper- or cadmium-metallothionein. *Toxicology Letters* 136, 193-198.
- Cantor, A.H., Scott, M.L., Noguchi, T., 1975. Biological availability of selenium in feedstuffs and selenium compounds for prevention of exudative diathesis in chicks. *Journal of Nutrition* 105, 96-105.
- Cao, J., Henry, P.R., Guo, R., Holwerda, R.A., Toth, J.P., Littell, R.C., Miles, R.D., Ammerman, C.B., 2000. Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. *Journal of Animal Science* 78, 2039-2054.
- Carpene, E., Serra, R., Manera, M., Isani, G., 1999. Seasonal changes of zinc, copper, and iron in gilthead sea bream (*Sparus aurata*) fed fortified diets. *Biological Trace Element Research* 69, 121-139.
- Carrasco, K.R., Tilbury, K.L., Myers, M.S., 1990. Assessment of the piscine micronuclei test as an in situ biological indicator of chemical contaminant effects. *Canadian Journal of Fisheries and Aquatic Science*. 47, 2123-2136.
- Castellano, S., Lobanov, A.V., Chapple, C., Novoselov, S.V., Albrecht, M., Hua, D., Lescure, A., Lengauer, T., Krol, A., Gladyshev, V.N., Guigo, R., 2005. Diversity and functional plasticity of eukaryotic selenoproteins: Identification and characterisation of the SelJ family. *Proceedings of the National Academy of Science* 102, 16188-16193.
- Castro, E.C., Kaspin, L.C., Chen, S., Nolker, S.G., 1992. Zinc deficiency increases the frequency of single strand DNA breaks in the rat liver. *Nutrition Research* 12, 721-736.

- Cavas, T., Ergene-Gozukara, S., 2003. Micronuclei, nuclear lesions and interphase silver-stained nucleolar organizer regions (AgNORs) as cyto-genotoxicity indicators in *Oreochromis niloticus* exposed to textile mill effluent. *Mutation Research* 538, 81-91.
- Chanock, S.J., El Benna J., Smith R.M., Babior B.M., 1994. The respiratory burst oxidase. *The Journal of Biological Chemistry* 269 (40), 24519-24522.
- Chesters, J.K., 1997. Zinc. In: *Handbook of Nutritionally Essential Mineral Elements*. Marcel Dekker, Inc. New York. pp 185-230.
- Chirase, N.K., Greene, W.L., Prudy, C.W., Loan, R.W., Auvermann, B.W., Parker, D.B., Walborg, E.F., Stevenson, D.E., Xu Y., Klaunig J.E., 2004. Effect of transport stress on respiratory disease, serum antioxidant status, and serum concentration of lipid peroxidation biomarkers in beef cattle. *American Journal of Veterinary Research* 65 (6), 860-864.
- Chung S., Secombes C.J., 1988. Analysis of events occurring within teleost macrophages during the respiratory burst. *Comparative Biology and Physiology* 89B (3), 539-544.
- Clearwater, S.J., Farag, A.M., Meyer, J.S., 2002. Bioavailability and toxicity of dietborne copper and zinc to fish. *Comparative Biochemistry and Physiology* 132C, 269-313.
- Coleman, J.E., 1992. Structure and mechanism of alkaline phosphates. *Annual Review of Biophysics and Biomolecular Structure* 21, 441-483.
- Collins, A.R., 2004. The comet assay for DNA damage and repair. *Molecular Biotechnology* 26, 246-261

Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. *Official Journal of the European Union* L133, 1-65.

Commission Regulation (EC) No 634/2007 of 7 June 2007 concerning the authorisation of selenomethionine produced by *Saccharomyces cerevisiae* NCYC R397 as a feed additive. *Official Journal of the European Union* L146, 14-16.

Commission Regulation (EC) No 1750/2006 of 27 November 2006 concerning the authorisation of selenomethionine as a feed additive. *Official Journal of the European Union* L330, 9-11.

Contreras-Sanchez W.M., Schreck C.B., Fitzpatrick M.S., Pereira C.B., 1998. Effects of stress on the reproductive performance of rainbow trout (*Oncorhynchus mykiss*). *Biology of Reproduction* 58, 439-447.

Costa, P.M., Costa, M.H., 2007. Genotoxicity assessment in fish peripheral blood: a method for a more efficient analysis of micronuclei. *Journal of Fish Biology* 71 (supplement A), 148-151.

Cotter, P.A., Craig, S.R., Mclean, E., 2008. Hyperaccumulation of selenium in hybrid striped bass: a functional food for aquaculture? *Aquaculture Nutrition* 14, 215-222.

Cowey, C.B., 1992. Nutrition: estimating requirements of rainbow trout. *Aquaculture* 100, 177-189.

Cragg, R.A., Phillips, S.R., Piper, J.M., Varma, J.S., Campbell, F.C., Mathers, J.C., Ford, D., 2005. Homeostatic regulation of zinc transporters in the human small intestine by dietary zinc supplementation. *Gut* 54, 469-478.

- Crews, H.M., Clarke, P.A., Lewis, J.D., Owen, L.M., Strutt, P.R., 1996. Investigation of selenium speciation in *in vitro* gastrointestinal extracts of cooked cod by high-performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray mass spectrometry. *Journal of Analytical Atomic Spectrometry* 11, 1177-1182.
- Daniels, L.A., 1996. Selenium metabolism and bioavailability. *Biological Trace Element Research* 54,185-199.
- Davis, C.D., Feng, Y., Hein, D.W. and Finley, J.W., 1999. The chemical form of selenium influences 3,2'-dimethyl-4-aminobiphenyl DNA adduct formation in the rat colon. *Journal of Nutrition* 129, 63-69.
- Dean, R.J., Shimmield, M., Black, K.D., 2007. Copper, zinc and cadmium in marine cage fish farm sediments: an extensive survey. *Environmental Pollution* 145, 84-95.
- Del Barga, I., Frenzilli, G., Scarcelli, V., Nigro, M., Malmvarn, A., Asplund, L., Forlin, L., Sturve, J., 2006. Effects of algal extracts (*Polysiphonia fucoides*) on rainbow trout (*Oncorhynchus mykiss*): A biomarker approach. *Marine Environmental Research* 62, S283-S286.
- Dethloff, G.M., Schlenk, D., Hamm, J.T., Bailey, H.C., 1999. Alterations in physiological parameters of rainbow trout (*Oncorhynchus mykiss*) with exposure to copper and copper/zinc mixtures. *Ecotoxicology and Environmental Safety* 42, 253-264.
- Do Carmo e Sa, M.V., Pezzato, L.E., Barros, M.M., De Magalhaes Padilha, P., 2005. Relative bioavailability of zinc in supplemental inorganic and organic sources for Nile tilapia *Oreochromis niloticus* fingerlings *Aquaculture Nutrition* 11, 273-281.

- Do Carmo E Sa, M.V., Pezzato, L.E., Lima, M.M., De Magalhaes Padilha, P., 2004. Optimum zinc supplementation level in Nile tilapia *Oreochromis niloticus* juvenile diets. *Aquaculture* 238, 385-401.
- Dotan, Y., Lichtenberg, D., Pinchuk, I., 2004. Lipid peroxidation cannot be used as a universal criterion of oxidative stress. *Progress in Lipid Research* 43, 200-227.
- Dreosti, I.E., 2001. Zinc and the gene. *Mutation Research* 475, 161-167.
- Ducros, V., Ferry, M., Faure, P., Belin, N., Renversez, J., Ruffieux, D., Favier, A., 2000. Distribution of selenium in plasma of French women: relation to age and selenium status. *Clinical Chemistry* 46 (5), 731-733.
- Dusinska M., Collins A., 1996. Detection of oxidised purines and UV-induced photoproducts in DNA of single cells, by inclusion of lesion-specific enzymes in the comet assay. *Alternatives to Laboratory Animals, Thirteenth Scandinavian Cell Toxicology Congress* 24, 405-411.
- Eastwood, M., 2003. Principals of Human Nutrition. Blackwell Publishing, Oxford, UK. pp 311-353.
- El-Bayoumy, K., 2001. The protective role of selenium on genetic damage and on cancer. *Mutation Research*, 475, 123-139.
- Ellis, A.E., 1990. Lysozyme Assays, in: Stolen, J.S., Fletcher, T.C., Anderson, D.P., Robrson, B.S., van Muiswinkel, W.B. (Eds), *Techniques in Fish Immunology-1* (1st ed). SOS Publications, NJ, USA, pp.101-103.

- Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine*. 11 (1), 81-128.
- Falcioni, G., Cincola, G., Brunori, M., 1987. Glutathione peroxidase and oxidative hemolysis in trout red blood cells. *Journal of the Federation of Biochemical Sciences* 221(2), 335-358.
- Featherstone, A.M., Townsend, A.T., Jacobson, G.A., Peterson, G.M., 2004. Comparison of methods for the determination of total selenium in plasma by magnetic sector inductively coupled mass spectrometry. *Analytica Chimica Acta*, 512, 319-327.
- Feeney, G.P., Zheng, D., Kille, P., Hogstrand, C., 2005. The phylogeny of teleost ZIP and ZnT zinc transporters and their tissue specific expression and response to zinc in zebrafish. *Biochimica et Biophysica Acta* 1732, 88-95.
- Felton, S.P., Smith, L.S., Ji, W., Halver J.E., 1989. Implications of selenium involvement during chemical and physical stress in salmonids. *Proceedings of the Society for Experimental Biology and Medicine* 190, 303.
- Felton, S.P., Ji, W., Mathews, S.B., 1990. Selenium concentrations in coho salmon outmigrant smolts and returning adults: a comparison of wild versus hatchery-reared fish. *Diseases of Aquatic Organisms*, 9, 157-161.
- Felton, S.P., Grace, R., Landolt, M., 1994. Significantly higher levels of zinc and copper found in wild compared to hatchery-reared coho salmon smolts *Oncorhynchus kisutch*. *Diseases of Aquatic Organisms* 18, 233-236.

- Felton, S.P., Landolt, M.L., Grace, R., 1996. Effects of selenium dietary enhancement on hatchery-reared coho salmon, *Oncorhynchus kisutch* (Walbaum), when compared to wild coho: hepatic enzymes and seawater adaptation evaluated. *Aquaculture Research* 27, 135-142.
- Fenech, M., 2002. Micronutrients and genomic stability: a paradigm for recommended dietary allowances (RDAs). *Food and Chemical Toxicology* 40, 1113-1117.
- Fenech, M., Crott, J.W., 2002. Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes – evidence for breakage-fusion bridge cycles in the cytokinesis-block micronucleus assay. *Mutation Research* 504, 131-136.
- Ficiclar, H., Zergeroglu, A.M., Tekin, D., Ersoz, G., 2003. The effects of acute exercise on plasma antioxidant status and platelet response. *Thrombosis Research* 111, 267-271.
- Fiskin, K., Ozkan, A., Ayhan, A., 2005. Protective effect of selenium and vitamin E on lipid peroxidation in smoke-exposed male mice. *Turkish Journal of Biochemistry* 30(3), 232-235.
- Fjolstad, M., Heyeraas, A.L., 1985. Muscular and myocardial degeneration in cultured Atlantic salmon, *Salmo salar* L., suffering from 'Hitra disease'. *Journal of Fish Diseases* 8, 367-372.
- Folk, J.E., Piez K.A., Carrol, W.R., Gladner, J.A., 1960. Carboxypeptidase B. IV. Purification and characterization of the porcine enzyme. *Journal of Biological Chemistry* 235 (8), 2272-2277.
- Fraker, P.J., Gershwin, E.M., Good, R.A., Prasad, A., 1986. Interrelationships between zinc and immune function. *Federation Proceedings* 45, 1474-1479.

- Freiden, E., 1984. In: *Biochemistry of the Essential Ultra Trace Elements*. (Frieden, E., Ed.) Plenum, New York. p 1.
- Frenzilli, G., Scarcelli, V., Del Barga, I., Nigro, M., Forlin L., Bolognesi, C., Sturve J., 2004. DNA damage in eelpout (*Zoarces viviparus*) from Goteborg harbour. *Mutation Research* 552, 187-195.
- Fridovich, I., 1986. Superoxide dismutases. *Methods in Enzymology* 58, 61-97.
- Gabrielsen, B.O., Opstvedt, J., 1980. Availability of selenium in fishmeal in comparison with soybean meal, corn gluten meal and selenomethionine relative to selenium in sodium selenite for restoring glutathione peroxidase activity in selenium-depleted chicks. *Journal of Nutrition* 110, 1096-1100.
- Ganther, H.E., 1999. Selenium metabolism, selenoproteins and mechanisms of cancer prevention: complexities with thioredoxin reductase. *Carcinogenesis*, 20 (9), 1657-1666.
- Ganther, H.E., Kraus, R.J., 1984. Oxidation states of glutathione peroxidases. *Methods in Enzymology* 107, 593-602.
- Gatlin, D.M., Phillips, H.F., Torrans, E.L., 1989. Effects of various levels of dietary copper and zinc on channel catfish. *Aquaculture* 76, 127-134.
- Gatlin, D.M., Poe, W.E., Wilson, R.P., 1986. Effects of singular and combined dietary deficiencies of selenium and vitamin E on fingerling catfish (*Ictalurus punctatus*). *Journal of Nutrition* 116, 1061-1067.
- Gatlin, D.M., Wilson, R.P., 1983. Dietary zinc requirement of fingerling catfish. *Journal of Nutrition* 113, 630-635.

- Gatlin, D.M., Wilson, R.P., 1984a. Zinc supplementation of practical channel catfish diets. *Aquaculture* 41, 31-36.
- Gatlin, D.M., Wilson, R.P., 1984b. Dietary selenium requirement of fingerling channel catfish. *Journal of Nutrition*, 114, 627-633.
- Gerard-Monnier, D., Erdelmeier, I., Regnard, N.M., Yadan, J., Chaudiere, J., 1998. Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation. *Chemical Research and Toxicology* 11, 1176-1183.
- Ghiselli, A., Serafini, M., Natella, F., Scaccini, C., 2000. Total antioxidant capacity as a tool to assess redox status: Critical view and experimental data. *Free Radical Biology and Medicine* 29 (11), 1106-1114.
- Glover, C.N., Bury, N.R., Hogstrand, C., 2003. Zinc uptake across the apical membrane of freshwater trout intestine is mediated by high affinity, low affinity, and histadine-facilitated pathways. *Biochimica et Biophysica Acta* 1614, 211-219.
- Glover, C.N., Hogstrand, C., 2002a. *In vivo* characterisation of intestinal zinc uptake in freshwater rainbow trout. *Journal of Experimental Biology* 205, 141-150.
- Glover, C.N., Hogstrand, C., 2002b. Amino acid modulation of *in vivo* intestinal zinc in freshwater rainbow trout. *The Journal of Experimental Biology* 205, 151-158.
- Glover, C.N., Hogstrand, C., 2003. Effects of dissolved metals and other hydrominerals on *in vivo* intestinal uptake in fresh water rainbow trout. *Aquatic Toxicology* 62, 281-293.

- Goel, A., Dani, V., Dhawan, D.K., 2005. Protective effects of zinc on lipid peroxidation, antioxidant enzymes and hepatic histoarchitecture in chlorpyrifos-induced toxicity. *Chemico-Biological Interactions* 156, 131-140.
- Gomez, G.D., Balcazar, J.L., 2008. A review on the interactions between gut microflora and innate immunity of fish. *Immunology and Medical Microbiology* 52, 145-154.
- Gomez-Cabrera, M., Domenech, E., Vina, J., 2008. Moderate exercise is an antioxidant: upregulation of antioxidant genes by training. *Free Radical Biology and Medicine* 44, 126-131.
- Grahn, B.H., Paterson, P.G, Gottschall-Pass, K.T., Zhang Z., 2001. Zinc and the eye. *Journal of the American College of Nutrition* 20 (2), 106-118.
- Gregory, R.T., Wood, C.T., 1999. The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. *Physiological and Biochemical Zoology* 73 (3), 286-295.
- Guarner, F., Malagelada, J.R., 2003. Gut flora in health and disease. *The Lancet* 360, 512-519.
- Guilherme, S., Valega, M., Pereira, M.E., Santos, M.A., Pacheco, M., 2008. Erythrocytic nuclear abnormalities in wild caged fish (*Liza aurata*) along an environmental mercury contamination gradient. *Ecotoxicology and Environmental Safety* 70, 411-421.
- Halliwell, B., Gutteridge J.M.C., 1999. *Free Radicals in Biology and Medicine* (3rd ed.). Oxford University Press, Oxford, pp. 105-245

- Halver, J.E., Felton, S.M., Zbanyszek, R., 2004. Carcass selenium loss as an indicator of stress in barrage transported Chinook salmon (*Oncorhynchus tshawytscha*), *Aquaculture Research* 35, 1099-1103.
- Hamilton, S.J., Buhl, K.J., Faerber, N.L., 1990. Toxicity of organic selenium in the diet to Chinook salmon. *Environmental Toxicology and Chemistry* 9, 347-358.
- Handy, R.D., Eddy, F.B., Romain, F.B., 1989. *In vitro* evidence for the ionregulatory role of rainbow trout mucus in acid, acid/aluminium and zinc toxicity. *Journal of Fish Biology* 35, 737-747.
- Hardy, R.W., Shearer, K.D., 1985. Effect of dietary calcium phosphate and zinc supplementation on whole body zinc concentration of rainbow trout (*Salmo gairdneri*). *Canadian Journal of Animal Science* 42, 181-184.
- Hardy, R.W., Sullivan, C.V., Koziol, A.M., 1987. Absorption, body distribution, and excretion of dietary zinc by rainbow trout (*Salmo gairdneri*). *Fish Physiology and Biochemistry* 3 (3), 133-143.
- Hartman, A., Plappert, U., Raddatz, K., Grunert-Fuchs, M., Speit, G., 1994. Does physical activity induce DNA damage? *Mutagenesis* 9, 269-272.
- Hatfield, D.L., Gladyshev, V.N., 2002. How selenium has altered our understanding of the genetic code. *Molecular and Cellular Biology* 22 (1), 3565-3576.
- Hermes-Lima, M., Willmore, W.G., Storey, K.B., 1995. Quantification of lipid peroxidation in tissue extracts based on Fe(III)xylenol orange complex formation. *Free Radical Biology and Medicine* 19(3), 271-280.

- Hidalgo, C.M., Exposito, A., Palma, J.M., Higuera, M., 2002. Oxidative stress generated by dietary Zn-deficiency: studies in rainbow trout (*Oncorhynchus mykiss*). *The International Journal of Biochemistry and Cell Biology* 34, 183-193.
- Hill, K.E., McCollum, G.W., Boeglin, M.E., Burk R.F., 1997. Thioredoxin reductase activity is decreased by selenium deficiency. *Biochemical and Biophysical Research Communications* 234, 293-295.
- Hilton, J.W., Hodson, P.V., 1983. Effect of increased dietary carbohydrate on selenium metabolism and toxicity in rainbow trout (*Salmo gairdneri*). *Journal of Nutrition* 113, 1241-1248.
- Hilton, J.W., Hodson, P.V., Slinger, S.J., 1980. The requirement and toxicity of selenium in Rainbow trout (*Salmo gairdneri*). *Journal of Nutrition* 110, 2527-2535.
- Hilton, J.W., Hodson, P.V., Slinger, S.J., 1982. Absorption, distribution, half-life and possible routes of elimination of dietary selenium in juvenile rainbow trout (*Salmo gairdneri*). *Comparative Biochemistry and Physiology* 71C, 49-55.
- Hodson, P.V., Hilton, J.W., 1983. The nutritional requirements and toxicity to fish of dietary and waterborne selenium. *Ecological Bulletin* 35, 335-340.
- Hodson, P.V., Spry, D.J., Blunt, B.R., 1980. Effects on rainbow trout (*Salmo gairdneri*) of chronic exposure to waterborne selenium. *Canadian Journal of Fisheries and Aquaculture Science* 37, 233-240.
- Hogstrand, C. and Wood, C.M., 1996. The physiology and toxicity of zinc. In: *Toxicology of Aquatic Pollution: Physiological, Cellular and Molecular Approaches*. Cambridge University Press. pp 61-84.

- Holmgren, A., Bjornstedt, M., 1995. Thioredoxin and thioredoxin reductase. *Methods in Enzymology* 252, 199-208.
- Honkiel, K.O., 1987. Critical evaluation of methods detecting water holding capacity in meat. In: *Accelerated Processing of Meat*. Romita, A., Valin, C., Taylor, A.A. (Eds). Elsevier Applied Science, London, UK. pp 225-239.
- House, W.A., Welch, R.M., 1989. Bioavailability of and interactions between zinc and selenium in rats fed wheat grain intrinsically labelled with ^{65}Zn and ^{75}Se . *Journal of Nutrition* 119, 916-921.
- Huerta, V.D., Sanchez, M.L.F., Sanz-Medel, A., 2004. Quantitative selenium speciation in cod muscle by isotope dilution ICP-MS with a reaction cell: comparison of different reported extraction procedures. *Journal of Analytical Atomic Spectrometry* 19, 644-648.
- Humaloja, T., Mykkanen, H.M., 1986. Intestinal absorption of ^{76}Se -labelled sodium selenite and selenomethionine in chicks: effect of time, segment, selenium concentration and method of measurement. *Journal of Nutrition* 166, 142-148.
- Inoue, M., Satoh, S., Maita, M., Kiron, V., Okamoto, N., 1998. Recovery from derangement of natural killer-like activity of leukocytes due to Zn or Mn deficiency in rainbow trout, *Oncorhynchus mykiss* (Walbaum), by the oral administration of these elements. *Journal of Fish Diseases* 21, 233-236.
- Itoh, S., Shimada, H., 1996. Micronucleus induction by selenium, and suppression by metallothionein inducer. *Mutation Research* 367, 233-236.
- Jacques, K.A., 2001. Selenium metabolism in animals: the relationship between dietary selenium form and physiological response. In: *Biotechnology in the Feed and Food Industries*. Nottingham University Press. pp 319-348.

- Jaramillo, F., Peng, L., Gatlin, D.M., 2009. Selenium nutrition of hybrid striped bass (*Morone chrysops* X *M. saxatilis*) bioavailability, toxicity and interaction with vitamin E. *Aquaculture nutrition* 14, 160-165.
- Jeng, S.S., Sun, L.T., 1981. Effects of dietary zinc levels on zinc concentrations of tissues of common carp. *Journal of Nutrition* 111, 134-140.
- Ji, L.L., 1999. Antioxidants and Oxidative Stress in Exercise. *Proceedings of the Society for Experimental Biology and Medicine* 222, 283-292.
- Jovanovic, A., Grubor-Lajsic, G., Djukic, N., Gardinovacki, G., Matic, A., Spasic, M., 1997. The effect of selenium on antioxidant system in erythrocytes and liver of the carp (*Cyprinus carpio* L.). *Critical Reviews in Food Science* 37 (5), 443-448.
- Julshamn, K., Sandnes, K., Lie, O., Waagbo, R., 1990. Effects of dietary selenium supplementation on growth, blood chemistry and trace element levels in serum and liver of adult Atlantic salmon (*Salmo salar*). *Fiskeridirektoratets Skrifter. Serie Ernaering III* (2), 47-58.
- Kang, J.Y., 1999. The antioxidant function of metallothionein in the heart. *Proceedings of the Society for Experimental Biology and Medicine* 222, 263-273.
- Kaplan, M.M., 1972. Alkaline Phosphatase. *Gastroenterology* 62 (3), 452-68.
- Kessler, J., Morel, I., Dufey, P.A., Gutzwiller, A., Starn, A., Geyer, H., 2003. Effect of organic zinc sources on performance, zinc status and carcass, meat and claw quality in fattening bulls. *Livestock Production Science* 81, 161-171.
- Ketola, G.H., 1979. Influence of dietary zinc on cataracts in rainbow trout (*Salmo gairdner*), *Journal of Nutrition* 109, 965-969.

- Kim, K., Wang, X., Choi, S. Park, G. Koo, J., Bai, S.C., 2003. No synergistic effects by the dietary supplementation of ascorbic acid, α -tocopheryl acetate and selenium on the growth performance and challenge test of *Edwardsiella* in fingerling Nile tilapia, *Oreochromis niloticus* L. *Aquaculture Research* 34, 1053-1058.
- King, J.C., Shames, D.M., Woodhouse, L.R., 2000. Zinc homeostasis in humans. *Journal of Nutrition* 130, 1360S-1366S.
- King, W.V., Hooper, B., Hillsgrove, S., Benton, C., Berlinsky, D. L., 2005. The use of clove oil, metomidate, tricane methanesulphonate and 2-phenoxyethanol for inducing anaesthesia and their effect on the cortisol stress response in black sea bass (*Centropristis striata* L.). *Aquaculture Research* 36, 1442-1449.
- Kiremidjian-Schumacher, L., Stotzky, G., 1987. Selenium and immune responses. *Environmental Research* 42, 277-303.
- Kjoss, V.A., Wood, C.M., McDonald, G.D., 2006. Effects of different ligands on the bioaccumulation and subsequent depuration of dietary Cu and Zn in juvenile rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Fisheries and Aquatic Science* 63, 412-422.
- Klontz, G.W., 1997. Fish Haematology, in: Stolen, J.S., Fletcher, T.C., Rowley, A.F., Zelikoff, J.T., Kaattari, S.L., Smith, S.A. (Eds), *Techniques in Fish Immunology-3* (2nd ed), SOS Publications, NJ, USA.
- Klotz, L., Kroncke, D., Buchczyk, D.P., Sies, H., 2003. Role of copper, zinc, selenium, and tellurium in the cellular defence against oxidative and nitrosative stress. *Journal of Nutrition* 133, 1448S-1451S.

- Knight, J., Ganderton, M., Hothersall J., Zitouni K., Nourooz-Zadeh J., 2002. The ABEL[®] peroxy-nitrite antioxidant test with Pholasin[®] measures the antioxidant capacity of plasma to protect against peroxy radical attack. *Luminescence* 17, 95-96.
- Knox, D. Cowey, C.B., Ardon, J.W., 1984. Effects of dietary zinc uptake upon copper metabolism in rainbow trout (*Salmo gairdneri*). *Aquaculture* 40, 199-207.
- Kryukov, G.V., Castellano, S., Novoselov, S.V., Lobanov, A.V., Zehtab, O., Guigo, R., Gladyshev, V.N., 2003. Characterisation of mammalian selenoproteomes. *Science* 300, 1439-1443.
- Kryukov, G.V., Gladyshev, V.N., 2000. Selenium metabolism in zebrafish: multiplicity of selenoprotein genes and expression of a protein containing 17 selenocysteine residues. *Genes to Cells* 5, 1049-1060.
- Kucukbay, F.Z., Yazlak, H., Karaca, I., Sahin, N., Tuzcu, M.N., Cakmak, M.N., Sahin, K., 2009. The effects of dietary organic and inorganic selenium in rainbow trout (*Oncorhynchus mykiss*) under crowding conditions. *Aquaculture Nutrition*. In press.
- Kucukbay, F.Z., Yazlak, H., Sahin, N., Tuzcu, M., Cakmak, M.N., Gurdogan, F., Juturu, V., Sahin, K., 2006. Zinc picolinate supplementation decreases oxidative stress in rainbow trout. *Aquaculture* 257, 465-469.
- Kumaravel, T.S., Jha, A.N., 2006. Reliable measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutation Research* 605, 7-16.
- Labbe, M.R., Fischer, P.W.F., 1984. The effects of high dietary zinc and copper deficiency on the activity of copper-requiring metalloenzymes in the growing rat. *Journal of Nutrition* 114, 813-822.

- Lall, S., 2000. Nutrition and health of fish, in: Cruz-Sua' rez, L., Ricque-Marie, D., Tapia-Salazar, M., Olvera-Novoa, M., Civera-Cerecedo, R., (Eds.), *Avances en Nutricio'n Acu'cola V. Memorias del V Simposium Internacional de Nutricio'n Acu'cola*, 19–22 November 2000, Me'rida, Yucata'n, Mexico, pp. 13–23.
- Lalles, J.P., Favier, C., Jondreville, C., 2007. A diet moderately deficient in zinc induces limited intestinal alterations in weaned pigs. *Livestock Science* 108, 153-155.
- Leeuwenburgh C., Heinecke J.W., 2001. Oxidative stress and antioxidants in exercise. *Current Medicinal Chemistry* 8, 829-838.
- Leibovitz, B. Hu, M., Tappel, A.L., 1990. Dietary supplements of vitamin E, β -carotene, coenzyme Q₁₀ and selenium protect tissues against lipid peroxidation in rat tissue slices. *Journal of Nutrition* 120, 97-104.
- Lemly, D.A., 2002. Symptoms and implications of selenium toxicity in fish: the Belews Lake case example. *Aquatic Toxicology* 57, 39-49.
- Li, M.H., Robinson, E.H., 1996. Comparison of chelated zinc and zinc sulphate as zinc sources for growth and bone mineralization of channel catfish (*Ictalurus punctatus*) fed practical diets. *Aquaculture* 146, 237-243.
- Lie, O., 2001. Flesh quality-the role of nutrition. *Aquaculture Research* 32 (1), 341S-348S.
- Lim, C., Klesius, P.H., Duncan, P.L., 1996. Immune response and resistance of channel catfish to *Edwardsiella ictaluri* challenge when fed various dietary levels of zinc methionine and zinc sulfate. *Journal of Aquatic Animal Health* 8, 302-307.
- Lin, Y., Shiau, S., 2005. Dietary requirements of juvenile grouper *Epinephelus malabaricus*. *Aquaculture* 250, 356-363.

- Liuzzi, J.P., Cousins, R.J., 2004. Mammalian zinc transporters. *Annual Reviews of Nutrition* 24, 151-172.
- Lorentzen, M., Maage, A., 1999. Trace element status of juvenile Atlantic salmon *Salmo salar* L. fed a fish-meal based diet with or without supplementation of zinc, iron, manganese and copper from first feeding. *Aquaculture Nutrition* 5, 163-171.
- Lorentzen, M., Maage, A., Julshamn, K., 1998. Supplementing copper to a fish meal based diet fed to Atlantic salmon parr affects liver copper and selenium concentrations. *Aquaculture Nutrition* 4, 67-72.
- Lorentzen, M., Maage, A., Julshamn, K., 1994. Effects of dietary selenite or selenomethionine on tissue selenium levels of Atlantic salmon (*Salmo salar*). *Aquaculture* 121, 359-367.
- Lunde, G., 1973. Trace metal contents of fishmeal and of the lipid phase extracted from fish meal. *Journal of Food Science and Agriculture* 24, 413-419.
- Maage, A., Julshamn, K., 1993. Assessment of zinc status in juvenile Atlantic salmon (*Salmo salar*) by measurement of whole body tissue levels of zinc. *Aquaculture* 117, 179-191.
- Maage, A., Julshamn, K., Ulgenes, Y., 1991. A comparison of tissue levels of four essential trace elements in wild and farmed Atlantic salmon (*Salmo salar*). *Fiskeridirektoratets Skrifter. Serie Ernaering* IV (2), 111-116.
- Maage, A., Julshamn, K., 1993. Assessment of zinc status in juvenile Atlantic salmon (*Salmo salar*) by measurement of whole body tissue levels of zinc. *Aquaculture* 117, 179-191.
- Maage, A., Julshamn, K., Berge, E., 2001. Zinc gluconate and zinc sulphate as dietary sources for Atlantic salmon. *Aquaculture Nutrition* 7, 183-187.

- Maage, A., Julshamn, K., Hemre, G., Lunestad, B.T., 2007. Overavakningsprogram for forvarer til fisk og andre akvatiske dyr: Årsrapport, 2006; NIFES Nasjonalt Institutt for ernærings og sjømatforskning, Bergen, Norway, 53pp
- Maret, W., 2000. The function between zinc metallothionein: a link between cellular zinc and redox state. *Journal of Nutrition* 130, 1455S-1458S.
- Margis, R., Dunand, C., Teixeira, F.K., Margis-Pinheiro, M., 2008. Glutathione peroxidase family – an evolutionary overview. *The FBES Journal* 275, 3959-3970.
- Martinez, G.R., Loureiro, A.P., Marques, S.A., Miyamoto, S., Yamaguchi, L.F., Onuki, J., Almeida, E.A., Garcia, C.C., Barbosa, L.F., Medeiros, M.H., Di Mascio, P., 2003. Oxidative and alkylating damage in DNA. *Mutation Research* 544, 115-127.
- Martinez-Alvarez, R.M., Morales, A.E., Sanz, A., 2005. Antioxidant defences in fish: Biotic and abiotic factors. *Reviews in Fish Biology and Fisheries* 15, 75-88.
- Masser, M.P., Rakocy, J., Losordo T.M., 1999. Recirculating aquaculture tank production systems. Management of recirculating systems. In: *Southern Regional Aquaculture Center* Publication no: 452 Stoneville, MS, USA, 12 pp.
- McCall, K.A., Huang, C. and Fierke, C.A., 2000. Function and mechanism of zinc metalloenzymes. *Journal of Nutrition* 130, 1437-1446.
- McClain, R.W., Gatlin, D.M., 1988. Dietary zinc requirement of *Oreochromis aureus* and effects of dietary calcium and phytate on zinc bioavailability. *Journal of the World Aquaculture Society* 19 (3), 103-108.
- McKenzie, R.C., Rafferty, T.S. and Beckett, G.J., 1998. Selenium: an essential element for immune function. *Trends in Immunology Today* 19(8), 342-345.

- Mitchelmore, C.L., Chipman, J.K., 1998. DNA strand breaks in aquatic organisms and the potential value of the comet assay in environmental monitoring. *Mutation Research* 399, 135-147.
- Morgan, P.N., Wher, C.M., MacGregor, J.T., Woodhouse, L.R., King, J.C., 1995. Zinc deficiency, erythrocyte production, and chromosomal damage in pregnant rats and their foetuses. *Journal of Nutritional Biochemistry* 6, 263-268.
- Murray, E.J. and Messer, H.H., 1981. Turnover of bone zinc during normal and accelerated bone loss in rats. *Journal of Nutrition* 111, 1641-1647.
- Nakano, T., Sato, M., Takeuchi, M., 1992. Glutathione peroxidase of fish. *Journal of Food Science* 57 (5), 1116 – 1119.
- National Research Council, 1993. *Nutrient Requirements of Fish*. National Academy Press, Washington D.C.
- Nourooz-Zadeh, J., Ziegler, D., Shor, D., Betteridge, J.D., Knight, J., Hothersall, J., 2006. The use of Pholasin[®] as a probe for the determination of plasma total antioxidant capacity. *Clinical Biochemistry* 39, 55-61.
- O'Dell, B.L., 1984. Bioavailability of trace elements. *Nutrition Reviews* 42 (9), 301-308.
- O'Grady, K.T., 1981. The resorption of zinc from scales of sea trout (*Salmo trutta*) during the upstream spawning migration. *Freshwater Biology* 11, 561-565.
- Ogino, C., Yang, G., 1978. Requirement of rainbow trout for dietary zinc. *Bulletin of the Japanese Society of Fisheries Science* 44 (9), 1015-1018.

- Ornsrud R., Lorentzen, M., 2002. Bioavailability of selenium from raw or cured selenomethionine-enriched fillets of Atlantic salmon (*Salmo salar*) assessed in selenium-deficient rats. *British Journal of Nutrition* 87, 13-20.
- Orunto, J., Esteban, M.A., Meseguer, J., 2001. Effects of short-term crowding stress on the gilthead sea bream (*Sparus aurata* L.) innate immune response. *Fish and Shellfish Immunology* 11, 187-197.
- Overnell, J., Fletcher, T.C., McIntosh, R., 1988. The apparent lack of effect of supplementary dietary zinc on zinc metabolism and metallothionein concentrations in the turbot, *Scophthalmus maximus* (Linnaeus). *Journal of Fish Biology* 33, 563-570.
- Ozcelik, D., Ozaras, R., Gurel, Z., Uzun, H., Aydin, S., 2003. Copper-mediated oxidative stress in rat liver. *Biological Trace Element Research* 96, 209-214.
- Packer, L., Cadenas, E., Davies, K.J., 2008. Free radicals and exercise: an introduction. *Free Radical Biology and Medicine* 44, 123-125.
- Papp, L.V., Lu, J., Holmgren, A., Khanna, K.K., 2007. From selenium to selenoproteins: synthesis, identity, and their role in human health. *Antioxidants and Redox Signalling* 9(7), 775-806.
- Paripatananont, T., Lovell, R.T., 1995a. Chelated zinc reduces the dietary zinc requirement of channel catfish, *Ictalurus punctatus*. *Aquaculture* 133, 73-82.
- Paripatananont, T., Lovell, R.T., 1995b. Responses of channel catfish fed organic and inorganic sources of zinc to *Edwardsiella ictaluri* challenge. *Journal of Aquatic Animal Health* 7, 147-154.

- Paripatananont, T., Lovell, R.T., 1997. Comparative net absorption of chelated and inorganic trace minerals in channel catfish *Ictalurus punctatus* diets. *Journal of the World Aquaculture Society* 28 (1), 62-67.
- Perry, S.F., Bernier, N.J., 1999. The acute humoral adrenergic stress response in fish: facts and fiction. *Aquaculture* 177, 285-295.
- Piao, F., Yokoyama, K., Ma, N., Yamauchi, T., 2003. Subacute toxic effects of zinc on various tissues and organs of rats. *Toxicology Letters* 145, 28-35.
- Pickering, A.D., 1992. Rainbow trout husbandry: management of the stress response. *Aquaculture* 100, 125-139.
- Pickering, A.D., 1998. Stress responses of farmed fish, in: Black, K.D., Pickering, A.D. (Eds.), *Biology of Farmed Fish* (1st ed.). Blackwell, Oxford, UK, Pp. 222-255.
- Poppe, T., Hastien, T., Frosli, A., Norheim, G., Heum, M., 1985. Vitamin E, selenium, copper and zinc in Atlantic salmon (*Salmo salar*): comparative studies of wild and farmed fish. *Bulletin of the European Association of Fish Pathologists* 5 (2), 28.
- Poston, H.A., Combs, Jr, G.F., Leibovitz, L., 1976. Vitamin E and selenium interrelations in the diet of Atlantic salmon (*Salmo salar*): gross, histological and biochemical deficiency signs. *Journal of Nutrition* 106, 892-904.
- Pottinger, 2001. Effects of husbandry stress on flesh quality indicators in fish. In: Kestin, S.C., Warriss, P.D. (Eds) *Farmed Fish Quality*. Blackwell Science, Oxford, UK. Pp 145-160.
- Powell, S.R., 2000. The antioxidant properties of zinc. *The Journal of Nutrition* 130, 1447S-1454S.

- Prior, R.L., Cao, G., 1999. In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radical Biology and Medicine* 27(11/12), 1173-1181.
- Pulsford, A.L., Lemaire-Gony, S., Tomlinson, M., Collingwood, N., Glynn, P.J., 1994. Effects of acute stress on the immune system of the dab *Limanda limanda*. *Comparative Biochemistry and Physiology* 109C (2), 129-139.
- Rampal, S., Kumar, R., Randhawa, C.S., Sood, N., 2008. Maturation arrest of neutrophils – a possible reason for the leucopenia in sodium selenite induced sub-chronic selenosis in cow calves. *Environmental and Toxicological Pharmacology* 25, 39-42.
- Ramseyer, L., Garling, Jr. D., Hill, G., Link, J., 1999. Effect of dietary zinc supplementation and phytase pre-treatment of soybean meal or corn gluten meal on growth, zinc status and zinc-related metabolism in rainbow trout, *Oncorhynchus mykiss*. *Fish Physiology and Biochemistry* 20, 251-261.
- Randall, D.J., Perry, S.F., 1992. Catecholamines, in: Hoar, W.S., Randall, D.J., Farrell, A.P. (Eds), *Fish Physiology - The Cardiovascular System*, Vol XIIB. Academic Press, New York, pp 225-300.
- Rao, L., Puschner, B., Prolla, T.A., 2001. Gene expression profiling of low selenium status in the mouse intestine: transcriptional activation of genes linked to DNA damage, cell cycle control and oxidative stress. *Journal of Nutrition* 131, 3175-3181.
- Rayman, M.P., Infante, H.G., Saegent, M., 2008. Food-chain selenium and human health: spotlight on speciation. *British Journal of Nutrition* 100, 238-253.
- Rayman, M.P., 2000. The importance of selenium to human health. *The Lancet* 356, 233-241.

- Rayman, M.P., 2004. The use of high-selenium yeast to raise selenium status: how does it measure up? *British Journal of Nutrition* 92, 557-573.
- Reddy, K.V., Kumar, T.C., Prasad, M., Reddanna, P., 1998. Pulmonary lipid peroxidation and defences during exhaustive physical exercise: the role of Vitamin E and selenium. *Nutrition* 14 (5), 448-451.
- Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. *Official Journal of the European Union* L268, 29-43.
- Reilly, C., 2004. Zinc. In: *The Nutritional Trace Metals*. Blackwell Publishing, Oxford. Pp 82-117.
- Reinhold, J.G., Kfoury, G.A., 1969. Zinc-dependent enzymes in zinc depleted rats; intestinal alkaline phosphatase. *The American Journal of Clinical Nutrition* 22 (9), 1250-1263.
- Richardson, N.L., Higgs, D.A., Beames, R.A., McBride, J.R., 1985. Influence of dietary calcium, phosphorous, zinc and sodium phytate level on cataract incidence, growth and histopathology in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). *Journal of Nutrition* 115, 553-567.
- Rider, S.A., Davies, S.J., Jha, A.N., Clough, R., Sweetman, J.W., 2009. Bioavailability of co-supplemented organic and inorganic zinc and selenium sources in a white fishmeal based rainbow trout (*Oncorhynchus mykiss*) diet. *Journal of Animal Physiology and Animal Nutrition*. In press.
- Rink, L., Gabriel, P., 2000. Zinc and the immune system. *Proceedings of the Nutrition Society* 59, 541-552.

- Roberts R.J., 1989. The Immunology of Teleosts. In: *Fish Pathology*, 2nd edition, Bailliere Tindal. 492pp
- Rossi, L., Migliaccio, S., Corsi, A., Marzia, M., Bianco, P., Tetti, A., Gambelli, L., Cianfarani, S., Paoletti, F., Branca, F., 2000. Reduced growth and skeletal changes in zinc-deficient growing rats are due to impaired growth plate activity and inanition. *Journal of Nutrition* 131, 1142-1146.
- Sachdev, S., Davies, K.J., 2008. Production, detection, and adaptive responses to free radicals in exercise. *Free Radical Biology and Medicine* 44, 215-223.
- Sadler, J., Wells, R.M.G., Pankhurst, P.M., Pankhurst, N.W., 2000. Blood oxygen transport, rheology and haematological responses to confinement stress in diploid and triploid Atlantic salmon, *Salmo salar*. *Aquaculture* 184, 249-361.
- Sahin, K., Kucuk, O., 2003. Zinc supplementation alleviates heat stress in laying Japanese quail. *Journal of Nutrition* 133, 2808-2811.
- Saito, Y., Hayashi, T., Tanaka, A., Watanabe, Y., Suzuki, M., Saito, E., Takahashi, K., 1999. Selenoprotein P in human plasma as an extracellular phospholipid hydroperoxide glutathione peroxidase. *The Journal of Biological Chemistry* 274 (5), 2866-2871.
- Saito, Y., Takahashi, K., 2002. Characterisation of selenoprotein P as a supply protein. *European Journal of Biochemistry* 269, 5746-5751.
- Salgueiro, M.J., Zubillaga, B.M., Lysionek, A., Sarabia, M.I., Caro, R., Paoli, T. Hager, A., Weill, R., Bioch, J.B., 2000. Zinc as an essential micronutrient: a review. *Nutrition Research* 20 (5), 737-755.

- Salte, R., Asgard, T., Liestol, K., 1988. Vitamin E and selenium prophylaxis against 'Hitra disease' in farmed Atlantic salmon – a survival study. *Aquaculture* 75, 45-55.
- Sanchez-Dardon, J., Voccia, I., Hontela, A., Chilmonczyk, S., Dunier, M., Boermans, H., Blakley, B., Fournier, M., 1999. Immunomodulation by heavy metals tested individually or in mixtures in rainbow trout (*Oncorhynchus mykiss*) exposed in vivo. *Environmental Toxicology and Chemistry* 18, 1492-1497.
- Sandstrom, B., 2001. Micronutrient interactions: effects on absorption and bioavailability. *British Journal of Nutrition* 85 (2), 181S-185S.
- Sappal, R., Burka, J., Dawson, S., Kamunde, C., 2009. Bioaccumulation and sub cellular partitioning of zinc in rainbowtrout (*Oncorhynchus mykiss*): Cross-talk between waterborne and dietary uptake. *Aquatic Toxicology*, 91, 281-290.
- Satoh, S., Izume, K., Takeuchi, T., Watanabe, T., 1987a. Availability to rainbow trout of zinc contained in various types of fish meals. *Bulletin of the Japanese Society of Fisheries Science* 53 (10), 1861-1866.
- Satoh, S., Poe, W., Wilson, R. P., 1989. Effect of supplemental phytate and/or tricalcium phosphate on weight gain feed efficiency and zinc content in vertebrae of channel catfish. *Aquaculture* 80, 155-161.
- Satoh, S., Porn-Ngam, N., Takeuchi, T., Watanabe, T., 1993. Effect of various types of phosphates on zinc availability to rainbow trout. *Bulletin of the Japanese Society of Fisheries Science* 59 (8), 1395-1400.
- Satoh, S., Takeuchi, T., Watanabe, T., 1987b. Availability to rainbow trout of zinc in white fish meal and of various zinc compounds. *Bulletin of the Japanese Society of Fisheries Science* 53 (4), 595-599.

- Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defence molecule of fish innate immune system. *Aquaculture Research* 39, 223-239.
- Scarpa, J., Gatlin, D.M., 1992. Effects of zinc and calcium on select immune functions of channel catfish. *Journal of Aquatic Animal Health* 4, 24-31.
- Schrauzer, G.N., 2000. Selenomethionine: A review of its nutritional significance, metabolism and toxicity. *Journal of Nutrition* 130, 1653-1656.
- Schrauzer, G.N., 2003. The nutritional significance, metabolism and toxicology of selenomethionine. *Advances in Food and Nutrition Research* 47, 73-112.
- Schrauzer, G.N., 2006. Selenium yeast: Composition, quality, analysis, and safety. *Pure and Applied Chemistry* 78, 105-109.
- Schwarz, F.J., 1995. Determination of mineral requirements of fish. *Journal of Applied Ichthyology* 11, 164-174.
- Sefanidou, M., Maravelias, C., Dona, A., Spiliopoulou, C., 2006. Zinc: a multipurpose trace element. *Archives of Toxicology* 80, 1-9.
- Shankar, A.H., Prasad, A.S., 1998. Zinc and immune function: the biological basis of altered resistance to infection. *American Journal of Clinical Nutrition* 68, 447S-463S.
- Shearer, K.D., 1984. Changes in elemental composition of hatchery-reared rainbow trout, *Salmo gairdneri*, associated with growth and reproduction. *Canadian Journal of Aquatic Science* 41, 1592-1601.
- Shephard, K.L., 1994. Functions for fish mucus. *Reviews in fish Biology and Fisheries* 4, 401-429.

- Spallholz, J.E., Boylan, M. and Larsen, H.S., 1990. Advances in understanding selenium's role in the immune system. *Annals of the New York Academy of Sciences* 587, 123-139.
- Spallholz, J.E., 1997. Free radical generation by selenium compounds and their pro-oxidant toxicity. *Biomedical and Environmental Science* 10 (2-3), 260-270.
- Spry, D.J., Hodson, P.V., Wood, C.M., 1988. Relative contributions of dietary and waterborne zinc in the rainbow trout, *Salmo gairdneri*. *Canadian Journal of Aquaculture Science* 45, 32-41.
- Stefanidou, M., Maravelias, C., Dona, A., Spiliopoulou, C., 2006. Zinc: a multipurpose trace element. *Archives of Toxicology* 80, 1-9.
- Stewart, M.J., Spallholz, J.E., Neldner, K.H., Pence, B.C., 1999. Selenium compounds have disparate abilities to impose oxidative stress and induce apoptosis. *Free Radical Biology and Medicine* 26(1-2), 42-48.
- Storebakken, T. and No, K.H., 1992. Pigmentation of rainbow trout. *Aquaculture* 100, 209-229.
- Storebakken, T., Shearer, K.D., Roem A.J., 2000. Growth, uptake and retention of nitrogen and phosphorous, and absorption of other minerals in Atlantic salmon *Salmo salar* fed diets with fish meal and soy-protein concentrate as the main sources of protein. *Aquaculture Nutrition* 6, 103-108.
- Sun, L.T.; Jeng, S., 1998. Comparative zinc concentrations in tissues of common carp and other aquatic organisms. *Zoological Studies* 37 (3), 184-190.
- Sunde, R.A., 1997. Selenium. In: *Handbook of Nutritionally Essential Mineral Elements*. Marcel Dekker, Inc. New York. pp 493-556.

- Sieniawska, C.E., Mensikov, R., Delves, T., 1999. Determination of total selenium in serum, whole blood and erythrocytes by ICP-MS. *Journal of Analytical Spectrometry*, 14, 109-112.
- Singh N.P., McCoy M.T., Tice, R.R., Schneider E.L., 1988. A simple technique for determination of low levels DNA damage in individual cells. *Experimental Cell Research* 175, 184-191.
- Singh, N.P. Danner, D.B., Tice, R.R., McCoy, M.T., Collins, G.D., Schneider, E.L., 1989. Abundant alkali-sensitive sites in DNA of human and mouse sperm. *Environmental Cell Research* 184, 461-470.
- Simko,E., El-Mowafi, A., Bettger, W.J., Ostland, V.E., Ferguson, H.W., Hayes, M.A., 1999. Alterations in iron, zinc and major plasma proteins of rainbow trout, *Oncorhynchus mykiss* (Walbaum), and brook trout, *Salvelinus fontinalis* (Mitchill), with sterile peritonitis induced by oil-adjuvanated multivalent bacterium vaccination. *Journal of Fish Diseases* 22, 81-90.
- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* 150, 76-85.
- Sopjani, M., Foller, M., Gulbins, E., Lang, F., 2008. Suicidal death of erythrocytes due to selenium compounds. *Cellular Physiology and Biochemistry* 22, 387-394.
- Souza, T., Fontanetti, C.S., 2006. Micronucleus test and observation of nuclear alterations in erythrocytes of Nile tilapia exposed to waters affected by refinery effluent. *Mutation Research* 605, 87-93.
- Spallholz, J.E., 1994. On the nature of selenium toxicity and carcinostatic activity. *Free Radical Biology and Medicine* 17(1), 45-64.

- Spallholz, J.E., Boylan, M. and Larsen, H.S., 1990. Advances in understanding selenium's role in the immune system. *Annals of the New York Academy of Sciences* 587, 123-139.
- Sphallholz, J.E., 1997. Free radical generation by selenium compounds and their pro-oxidant toxicity. *Biomedical and Environmental Science* 10 (2-3), 260-270.
- Spry, D.J., Hodson, P.V., Wood, C.M., 1988. Relative contributions of dietary and waterbourne zinc in the rainbow trout, *Salmo gairdneri*. *Canadian Journal of Aquaculture Science* 45, 32-41.
- Stefanidou, M., Maravelias, C., Dona, A., Spiliopoulou, C., 2006. Zinc: a multipurpose trace element. *Archives of Toxicology* 80, 1-9.
- Stewart, M.J., Spallholz, J.E., Neldner, K.H., Pence, B.C., 1999. Selenium compounds have disparate abilities to impose oxidative stress and induce apoptosis. *Free Radical Biology and Medicine* 26(1-2), 42-48.
- Storebakken, T. and No, K.H., 1992. Pigmentation of rainbow trout. *Aquaculture* 100, 209-229.
- Storebakken, T., Shearer, K.D., Roem A.J., 2000. Growth, uptake and retention of nitrogen and phosphorous, and absorption of other minerals in Atlantic salmon *Salmo salar* fed diets with fish meal and soy-protein concentrate as the main sources of protein. *Aquaculture Nutrition* 6, 103-108.
- Sun, L.T.; Jeng, S., 1998. Comparative zinc concentrations in tissues of common carp and other aquatic organisms. *Zoological Studies* 37 (3), 184-190.
- Sunde, R.A., 1997. Selenium. In: *Handbook of Nutritionally Essential Mineral Elements*. Marcel Dekker, Inc. New York. pp 493-556.

- Surai, P.F. 2006. Selenium in Nutrition and Health. Nottingham University Press, Nottingham, UK, 974 pp.
- Suzuki, T., Ishihara, K., Migaki, H., Matsuura, W., Kohda, A., Okamura, K., Nagao, M., Yamaguchi, Y., Kambe, T., 2005. Zinc transporters, ZnT5 and ZnT7, are required for the activation of alkaline phosphatase, zinc requiring enzymes that are glycosylphosphatidylinositol-anchored to the cytoplasmic membrane. *Journal of Biological Chemistry* 280, 637-643.
- Swinkels, J.W.G.M., Kornegay, E.T., Verstegen, M.W.A., 1994. Biology of zinc and biological value of dietary organic zinc complexes and chelates. *Nutrition Research Reviews* 7, 129-149.
- Tacon, A.G.J., De Silva, S.S., 1983. Mineral composition of some commercial fish feeds available in Europe. *Aquaculture*, 31, 11-20.
- Thompson, R.P.H., 1991. Assessment of zinc status. *Proceedings of the Nutrition Society* 50, 19-28.
- Thorarinnsson, R. Landolt, M.L., Elliott, D.G., Pascho, R.J., Hardy, R.W., 1994. Effect of dietary vitamin E and selenium on growth, survival and the prevalence of *Renibacterium salmoninarum* infection in Chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture* 121, 343-358.
- Todorovic, M., Jovanovic, M., Jokic, Z., Hristov, S., Davidovic, V., 2004. Alterations in liver and kidneys of chickens fed with high levels of sodium selenite or selenized yeast. *Acta Veterinaria-Beograd* 54 (2-3), 191-200.
- Toppo, S., Vanin, S., Bosello, V., Tossato, S.C.E., 2008. Evolutionary and structural insights into the multifaceted glutathione peroxidase (Gpx) superfamily. *Antioxidants and Redox Signalling* 10 (9), 1501-1513.

- Tucker L.A., Taylor-Pickard J. A., 2005. *Re-Defining Mineral Nutrition*. Nottingham University Press, UK. 295pp
- Tujebajeva, R.M., Ransom, D.G., Harney, J.W., Berry, M.J., 2000. Expression and characterisation of nonmammalian selenoprotein P in the zebrafish *Danio rerio*. *Genes to Cells* 5, 897-903.
- Turner, R.J., Finch, J.M., 1991. Selenium and immune response. *Proceedings of the Nutrition Society* 50, 275-285.
- Ubels, J.L., Edelhauser, H.F., 1987. Effects of corneal epithelial abrasion on corneal transparency, aqueous humor composition and lens of fish. *The Progressive Fish Culturist* 49, 210-224.
- Udroiu, I., 2006. The micronucleus test in piscine erythrocytes. *Aquatic Toxicology* 79, 201-204.
- Ursini, F. and Bindoli, A., 1987. The role of selenium peroxidases in the protection against oxidative damage of membranes. *Chemistry and Physics of Lipids* 44, 255-276.
- Valavanidis, A., Valhogianni, T. Dassenakis, M., Scoullou, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety* 64, 178-189.
- Vallee, B.L., Coleman, J.E., Auld, D.S., 1991. Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Proceedings of the National Academy of Sciences* 88, 999-1003.
- Vallee, B.L., Falchuk K.H., 1993. The biochemical basis of zinc physiology. *Physiology Reviews* 73 (1), 79-118.

- Vangen, B., Hemre, G., 2003. Dietary carbohydrate, iron and zinc interactions in Atlantic salmon (*Salmo salar*). *Aquaculture* 219, 597-611.
- Vazzana, M., Cammarata, M., Cooper, E.L., Parrinello, N., 2002. Confinement stress in sea bass (*Dicentrarchus labrax*) depresses peritoneal leukocyte cytotoxicity. *Aquaculture* 210, 231-243.
- Walsh, D.M., Kennedy, G.D., Goodall, E.A., Kennedy, S., 1993. Antioxidant enzyme activity in the muscles of calves depleted of vitamin E or selenium or both. *British Journal of Nutrition* 70, 621-630.
- Walter, K., Schutt, C., 1974. Phosphatases in: *Methods of Enzymatic Analysis* (2nd Ed. Vol. 2), Academic Press, NY. pp 860-864.
- Wang, C., Lovell, R.T., 1997. Organic selenium sources, selenomethionine and selenoyeast, have higher bioavailability than an inorganic selenium source, sodium selenite, in diets for channel catfish (*Ictalurus punctatus*). *Aquaculture* 152, 223-234.
- Wang, C., Lovell, R.T., Klesius, P.H., 1997. Response to *Edwardsiella ictaluri* challenge by channel catfish fed organic and inorganic sources of selenium. *Journal of Aquatic Animal Health* 9, 172-179.
- Ward, A.D., Carter, C.G., Townsend, A.T., 2005. The use of yttrium oxide and the effect of faecal collection timing for determining the apparent digestibility of minerals and trace elements in Atlantic salmon (*Salmo salar*, L.) feeds. *Aquaculture Nutrition* 11, 49-59.
- Watanabe, T, Satoh, S., Takeuchi, T., 1988. Availability of minerals in fish meal to fish. *Asian Fisheries Science* 1, 175-195.

- Watanabe, T., Takeuchi, T., Ogino, C., 1980. Effect on rainbow trout and chum salmon of deletion of trace elements from fish meal diet. *Bulletin of the Japanese Society of Scientific Fisheries* 46 (12), 1521-1525.
- Watanabe, T., Kiron, V., Satoh, S., 1997. Trace minerals in fish nutrition. *Aquaculture* 151, 185-207.
- Waters, D.J., Shen, S., Cooley, D.M., Bostwick, D.G., Qian, J., Combs, Jr, G.F., Glickman, L.T., Oteham, C., Schlittler, D., Morris, S.J., 2003. Effects of dietary selenium supplementation on DNA damage and apoptosis in canine prostate. *Journal of the National Cancer Institute*, 95(3), 237-241.
- Wedekind, K.J., Hortin, A.E., Baker, D.H., 1992. Methodology for assessing zinc bioavailability: efficacy estimates for zinc-methionine, zinc sulfate and zinc oxide. *Journal of Animal Science* 70, 178-187.
- Wei, J.J., Srinivasan, C., Han, H., Valentine, J.S., Gralla, E.B., 2001. Evidence for a novel role of copper-zinc superoxide dismutase in zinc metabolism. *Journal of Biological Chemistry* 276 (48), 44798-44803.
- Wekell, J.C., Shearer, K.D., Gauglitz, E.J., 1986. Zinc supplementation of trout diets: tissue indicators of body zinc status. *Progressive Fish Culturist* 48, 205-212.
- Wekell, J.C., Shearer, K.D., Houle, C.R., 1983. High zinc supplementation of rainbow trout diets. *The Progressive Fish Culturist* 45 (3), 144-147.
- Wendelaar-Bogna, S.E., 1997. The stress response in fish. *Physiological Reviews* 77 (3), 591-625.

- Wilkie, B.C., Vidailhet, M., Favier, A., Guillemin, C., Ducros, V., Arnaud, J., Richard, M.J., 1992. Selenium, glutathione peroxidase (GSH-Px) and lipid peroxidation products before and after selenium supplementation. *Clinica Chimica Acta* 207, 137-142.
- Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology* 19, 137-161.
- Wise, D.J., Tomasso, J.R., Gatlin, D.M., Bai, S.C., Blazer, V.S., 1993. Effects of dietary selenium and vitamin E on red bloods cell peroxidation, glutathione peroxidase activity, and macrophage superoxide anion production in channel catfish. *Journal of Aquatic Animal Health* 5, 177-182.
- Wood, R.J., 2000. Assessment of marginal zinc status. *Journal of Nutrition* 130, 1350-1354.
- Xue, C., Yu, G., Hirata, T., Sakaguchi, M., Terao, J., 1998. Antioxidative activity of carp blood plasma on lipid peroxidation. *Bioscience, Biotechnology and Biochemistry* 62 (2), 201-205.
- Yousef, M.I., El-Hendy, H.A, El-Demerdash, F.M., Elagamy, E.I., 2002. Dietary zinc deficiency induced-changes in the activity of enzymes and the levels if free radicals, lipids, and protein electrophoretic behaviour in growing rats. *Toxicology* 175, 223-234.
- Zhou, X., Wang, Y., Gu, Q., Li, W., 2009. Effects of dietary selenium sources (selenium nanoparticle and selenomethionine) on growth performance, muscle composition and glutathione peroxidase enzyme activity of crucian carp (*Carassius auratus gibelio*). *Aquaculture* 291, 78-81.

Copyright Statement

This copy of the thesis has been supplied on the condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the author's prior consent.