ASPECTS OF CORTISOL DYNAMICS DURING THE
EARLY ONTOGENY OF THREE SPECIES OF
PACIFIC SALMON (Oncorhynchus sp.)

by
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We accept this thesis as conforming to the required standard

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Abstract

Seven female coho salmon (*Oncorhynchus kisutch*), in the final stages of oogenesis, were exposed to mechanical disturbance (chased for 60 seconds with hand net), twice daily over a two week period. There was no significant difference in mean gonadosomatic index between disturbed (4.63 ± 0.19) and undisturbed (4.76 ± 0.34) females. Mean plasma cortisol level in the disturbed females (227.52 ± 61.51 ng/ml) was higher, though not significantly, than that seen in undisturbed females (140.99 ± 42.70 ng/ml). Mean oocyte cortisol content (22.13 ± 1.32 ng/gm) was significantly higher in the disturbed, than in the undisturbed females (9.90 ± 0.94 ng/gm). It is suggested that the cortisol content of freshly ovulated oocytes in salmonids is reflective of the level of circulating cortisol in the adult female during late oogenesis.

Oocytes from 5 female coho salmon were split into paired groups and one half exposed to water containing exogenous cortisol. The cortisol immersed groups had a mean oocyte cortisol content (232.68 ± 13.93) that was significantly higher than that in the paired untreated groups (37.03 ± 5.43). Mean oocyte cortisol content was monitored from 0 to 56 days post fertilization (dpf). Oocyte cortisol content was seen to decline sharply after fertilization, and there was no significant difference between paired immersed and control groups by 16 dpf. The immersed groups demonstrated no significant difference in terms of mean time to hatch, mean synchrony of hatch, percent mortality, mean yolk sac to body weight ratio at hatch, or mean length and dry weight up to 56
days post fertilization, compared to the paired untreated groups.

Steelhead (*Oncorhynchus mykiss*), embryos subjected to a 60 second emersion every fifteen minutes for two hours, every five days, from -4 to 32 days post hatch (dph), demonstrated a measurable cortisol response beginning at 6 dph. The magnitude of this response increased significantly at each sampling period thereafter with the exception of day 21 post hatch. Coho salmon embryos subjected to a similar treatment every seven days from -5 to 30 dph demonstrated a measurable cortisol response beginning 9 dph, and by 16 dph were demonstrating a cortisol response to emersion that, over an 8 hour period, was similar to that reported for adult and juvenile salmonids to a variety of stimuli. This onset of a measurable cortisol response to an environmental disturbance at 6 and 9 dph for steelhead and coho salmon respectively is an indication of the onset of a functional hypothalamic-pituitary-interrenal (HPI) axis.

Embryonic chinook salmon (*Oncorhynchus tshawytscha*) exposed to a combination of a repeated emersion and exogenous cortisol treatment displayed reduced growth and survival compared to embryos receiving either treatment alone, or neither treatment. The combination of the two treatments was observed to result in a significant increase in whole body cortisol content in chinook embryos beginning at 9 dph. This increase in whole body cortisol content is suggested as a factor in the increased mortality. The reduction in length and weight observed was attributed to physiological responses to disturbance other than the cortisol
response, as embryos treated with exogenous cortisol alone did not display reduced growth, while those receiving the emersion treatment alone did show some significant reduction in weight.
TABLE OF CONTENTS

Abstract ii
Table of Contents v
List of Tables vi
List of Figures vii
Acknowledgement ix

Chapter 1 General Introduction 1

Chapter 2 Transfer of Maternal Cortisol to Maturing Oocytes in Female Coho Salmon (Oncorhynchus kisutch), and its Effects on Subsequent Embryos.
   Introduction 11
   Materials and Methods 14
   Results 20
   Discussion 32

Chapter 3 Ontogeny of the Cortisol Response to an Environmental Disturbance in Steelhead (Oncorhynchus mykiss), and Coho Salmon (O. kisutch), Embryos. 38
   Introduction 39
   Materials and Methods 43
   Results 47
   Discussion 58

Chapter 4 Effects of Cortisol and/or an Environmental Disturbance on Growth and Survival of Chinook Salmon (Oncorhynchus tshawytscha) Embryos. 63
   Introduction 64
   Materials and Methods 66
   Results 69
   Discussion 75

Chapter 5 General Discussion 80

References 85
Table 1. Initial coho salmon (*O. kisutch*) oocyte cortisol levels, percent mortalities, time to 50% hatch, and time from 0 to 100% hatch for control and experimental groups. Experimental groups were immersed for two hours in water containing 600 ug/L cortisol.

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Fig. 1. Competitive binding curves of cortisol standards and extracted oocyte tissue serial dilutions.................24

Fig. 2. Plasma cortisol content of disturbed and undisturbed adult female coho salmon, (O.kisutch)......................25

Fig. 3. Gonadosomatic indices (GSI) of disturbed and undisturbed adult female coho salmon, (O. kisutch)..................26

Fig. 4. Oocyte cortisol content from disturbed and undisturbed adult female coho salmon, (O. kisutch).....................27

Fig. 5. Whole body cortisol content of cortisol immersed and control coho salmon, (O. kisutch) oocytes....................28

Fig. 6. Body length of cortisol immersed and control coho salmon (O. kisutch), embryos 1, 9, and 17 days post hatch......29

Fig. 7. Body weight (dry) of cortisol immersed and control coho salmon (O. kisutch), embryos 1, 9, and 17 days post hatch.................................................................30

Fig. 8. Yolk sac to body weight ratio of cortisol immersed and control coho salmon (O. kisutch), embryos 1 day post hatch.................................................................31

Fig. 9. Whole body cortisol content of steelhead embryos (O. mykiss), exposed to emersion or undisturbed, from -4 to 3 days post hatch.........................................................51

Fig. 10. Whole body cortisol content of coho salmon (O. kisutch) embryos exposed to emersion or undisturbed, over 8 hours, at -5 days post hatch.....................................................52

Fig. 11. Whole body cortisol content of coho salmon (O. kisutch) embryos exposed to emersion or undisturbed, over 8 hours, at 2 days post hatch.....................................................53

Fig. 12. Whole body cortisol content of coho salmon (O. kisutch) embryos exposed to emersion or undisturbed, over 8 hours, at 9 days post hatch.....................................................54

Fig. 13. Whole body cortisol content of coho salmon (O. kisutch) embryos exposed to emersion or undisturbed, over 8 hours, at 16 days post hatch.....................................................55
Fig. 14. Whole body cortisol content of coho salmon (O. kisutch) embryos exposed to emersion or undisturbed, over 8 hours, at 23 days post hatch........................................56

Fig. 15. Whole body cortisol content of coho salmon (O. kisutch) embryos exposed to emersion or undisturbed, over 8 hours, at 30 days post hatch........................................57

Fig. 16. Whole body cortisol content of chinook salmon (O. tshawytscha), embryos either exposed to emersion, exogenous cortisol, given both treatments, or neither treatment (controls)..............................71

Fig. 17. Body length of chinook salmon (O. tshawytscha), embryos either exposed to emersion, exogenous cortisol, given both treatments, or neither treatment (controls).................72

Fig. 18. Body weight of chinook salmon (O. tshawytscha), embryos either exposed to emersion, exogenous cortisol, given both treatments, or neither treatment (controls).................73

Fig. 19. Percent Mortality of chinook salmon (O. tshawytscha), embryos either exposed to emersion, exogenous cortisol, given both treatments, or neither treatment (controls).....74
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CHAPTER 1

GENERAL INTRODUCTION
**General Introduction**

Fish living under natural or artificial conditions will, at almost all points in their life history, be exposed to changes and/or perturbations in their surrounding environment. These can include general environmental shifts in temperature, pH, water quality etc., or can come from direct interaction with predators, conspecifics, or in the case of culture situations, with man. These stimuli can be described as "stressors", and can act to bring about a combination of physiological and behavioural responses in fish often described as "stress".

Stress is an ambiguous term, and one that lacks a clear definition, with its usage seeming to depend largely on the context in which it is being applied. It is sometimes invoked to describe a particular stimulus or an adverse environmental condition, while at other times it is used to describe a response to the same. Selye (1976) described stress in terms of the General Adaptation Syndrome (GAS). In it he proposed three distinct stages through which an animal progresses when exposed to stress. The first phase consists of an immediate physiological response to the stress, and is termed the alarm phase. This is followed by a second, resistance phase, during which the animal attempts to escape or compensate for the adverse conditions which are invoking the stress response. The third and final phase occurs if the animal in unable to escape from or cope with the stress, and experiences exhaustion, and ultimately death.
A general underlying concept in most definitions of stress is proposed by Barton and Iwama (1991), and that is that stress represents a response reaction by fish to a stimulus, and this response may somehow alter the fish’s homeostatic state.

In this thesis I will refrain from using the term stress as much as possible when describing my own work, as this study focused mainly on a single hormonal aspect of the so called stress response. I will instead simply describe the techniques I utilized to try and invoke this particular hormonal response. The terms stress, stress response, and stressor, will still appear when referring to the work of other investigators.

Fish can react to environmental stimuli in a number of ways, both behaviourally and physiologically, and the stress response can be separated into three distinct phases using these criteria. The physiological changes that take place in fish that are exposed to potential or applied stressors includes a neuroendocrine response which has been described as the primary effect, a secondary response, which is described as the physiological consequences of the primary response, and a tertiary response, which encompasses such things as immune response, behaviour, growth and reproductive capabilities (Mazeaud et al., 1977).

The primary stress response is usually characterized by a rapid elevation of circulating levels of catecholamines and corticosteroids. The major corticosteroid found in the plasma of teleosts is cortisol (Donaldson, 1981), and its synthesis and release from the interrenal tissue is the culmination of a hormone
cascade that begins in the brain. An applied or perceived stressor activates nerve impulses to the hypothalamus which in turn releases corticotropin-releasing-factor (CRF). This hormone is carried by nerve fibres to the anterior pituitary gland where it stimulates the corticotrophs of the pars distalis to release the hormone adrenocorticotropin (ACTH) into circulation. It is ACTH which acts at the level of the interrenal to stimulate the synthesis and release of cortisol (Donaldson, 1981; Sumpter et al., 1986).

The interrenal tissue of teleosts is located in the pronephric, or head area of the kidney, in close proximity to the posterior cardinal veins, and is homologous to the adrenal gland found in tetrapods. It is responsible for the synthesis and release of corticosteroids into circulation. This region differs from the tetrapod adrenal in that it does not possess distinct medullary and cortical tissues, and instead possesses two different cell types, the interrenal and chromaffin cells, which are responsible for corticosteroidogenesis and catecholamine production respectively (Bonga, 1993).

Cortisol has been associated with many different effects on the physiology of fish subjected to stressors, including altered blood flow, heart rate, and the mobilization of glucose (hyperglycaemia). Cortisol stimulates the mobilization of the animal's carbohydrate stores, and this initial reaction may be followed by the mobilization of proteins and lipids as further sources of energy in the form of glucose (Freeman and Idler, 1973; Leach and Taylor, 1980; Vijayan et al., 1994). Initially, the
increased circulating levels of cortisol are of a benefit to the fish in that they facilitate its efforts to either avoid, or accommodate, environmental disturbances. However, prolonged exposure to adverse environmental conditions can cause chronic activation of the hypothalamic-pituitary-interrenal (HPI) axis and lead to extended elevation of circulating blood cortisol. This can cause long term mobilization, rather than storage, of glucose, and can ultimately lead to impaired growth and tissue repair, and reduced immune and reproductive capabilities (Barton and Iwama, 1991; Donaldson, 1990).

A decrease in growth has been observed in fish held under adverse environmental conditions, and this reduction has been attributed to the transfer of resources by the fish to catabolic physiological processes associated with the response to these conditions, and away from anabolic processes associated with growth. Brook trout (Salvelinus fontinalis) raised under conditions of low pH displayed reduced growth and elevated plasma cortisol levels (Tam et al., 1988), while brook trout raised under high density also displayed reduced growth, but without elevated plasma cortisol levels (Vijayan et al., 1990). A similar decrease in growth has been reported in fish that were fed exogenous cortisol. One year old rainbow trout (Oncorhynchus mykiss) that were fed cortisol over a ten week period displayed reduced growth, condition factor, and liver glycogen (Barton et al., 1987). Channel catfish (Ictalurus punctatus) fed food containing cortisol for the same period also displayed reduced growth and condition
factor, along with reduced hepatosomatic index (Davis et al., 1985).

Immunocompetence in teleosts has also been demonstrated to be adversely affected by environmental conditions, and has been associated with the accompanying rise in blood cortisol levels (Barton and Iwama, 1991; Snieszko, 1974). The immune system of fish, and other vertebrates, consists of a number of non-specific components such as integument and bacteriolytic enzymes, as well as specific immune functions such as inflammation and antigen recognition and activation of immune effectors (Campbell, 1992). Fish exposed to adverse environmental conditions have demonstrated increased susceptibility to disease, increased mortality rates, as well as reduced amount of lysozyme in various tissues, and reduced numbers of circulating lymphocytes and leucocytes (Barton et al., 1987; Maule et al., 1989; Maule and Schreck, 1990; Mock and Peters, 1990; Pottinger and Pickering, 1992).

Similar indications of reduced immunocompetence have been evoked in fish exposed to exogenous cortisol. Brown trout (Salmo trutta) treated with cortisol, either orally or by implantation, have demonstrated reduced numbers of circulating lymphocytes and increased mortalities due to bacterial and fungal infection, while at the same time demonstrating significantly elevated levels of plasma cortisol (Pickering, 1984; Pickering and Pottinger, 1985; Pickering et al., 1989). Coho salmon (Oncorhyncus kisutch) exposed to emersion from water, or fed food containing cortisol, were seen to display significantly reduced numbers of circulating
lymphocytes, while at the same time having significantly higher lymphocyte numbers in the thymus and anterior kidney, indicating cortisol as a mediating agent in lymphocyte traffic (Maule and Schreck, 1990).

Reproductive ability is another process in fish which has been shown to be adversely effected by exposure to adverse environmental conditions. The substantial physiological investment and reliance on carefully orchestrated endocrine events that characterizes reproductive processes make them prime candidates for disruption due to environmental perturbations. Elevated levels of plasma cortisol have been associated with suppressed plasma androgen levels in mature male brown trout and tilapia (Oreochromis mossambicus) (Foo and Lam, 1993a; Pickering et al., 1987), as well as with reduced 17β-estradiol and testosterone secretion in cultured ovarian follicles of female brown trout (Carragher and Sumpter, 1990; Sumpter et al., 1987). They have also been linked with reduced hepatic estradiol-binding sites in female brown trout (Pottinger and Pickering, 1990) and reduced gonad size, circulating sex steroid and vitellogenin levels, and pituitary gonadotropin content in brown and rainbow trout (Carragher et al., 1989), suggesting that elevated plasma cortisol in response to environmental disturbances can act at the level of the pituitary, the liver and the gonads, and may reduce reproductive ability.

Cortisol, presumably of maternal origin, has been demonstrated to be present in measurable levels in the freshly ovulated oocytes of teleosts (de Jesus et al., 1991; de Jesus and Hirano, 1992;
Hwang et al., 1992; Yeoh et al., 1993), as have sex steroids (Feist et al., 1990; Rothbard et al., 1987) and thyroid hormones (Ayson and Lam, 1993; Leatherland et al., 1989a; Leatherland et al., 1989b). Despite the presence of cortisol in teleost oocytes, its functional significance, if any, has yet to be elucidated.

Endogenous production of cortisol, along with the onset of a functional HPI axis in teleosts, is another area of investigation that has received little attention. In mammals, and specifically rats, the hypothalamic-pituitary-adrenal (HPA) axis has been demonstrated to be sensitive to stimuli in utero, and fetal corticosterone levels respond to hypoxia as early as day 18 of gestation (Ohkawa et al., 1991; Walker et al., 1991). Rats also demonstrate a period of non-responsiveness of the HPA axis that begins shortly after birth (Guillet et al., 1980; Haltmeyer et al., 1966; Sapolsky and Meaney, 1986), and is suggested as being crucial for normal growth and differentiation of tissues and organs, including the central nervous system (Moisan et al., 1992).

Pottinger and Mosuwe (1994) found that rainbow trout embryos demonstrated increased whole body cortisol content in response to mechanical disturbance and confinement beginning at 5 weeks post hatch, while Barry et al., (1995) observed a similar increase in rainbow trout in response to emersion and low water temperature beginning at 2 weeks post hatch. No data is available concerning what effects this early cortisol response to disturbance may have on growth or survival in salmonids.
In summary it can be concluded that exposure of teleosts to adverse environmental conditions and/or disturbances, and the accompanying increase in plasma cortisol that often accompanies this exposure, can have deleterious effects on growth, immunocompetence, and reproductive ability. What is less clear is the role of cortisol in the early ontogeny of teleosts. This investigation was initiated to try and elucidate the relationship between disturbance induced cortisol production in sexually mature female salmonids and the possible transfer of maternal cortisol to their developing oocytes, and what effect varying levels of oocyte cortisol may have on the viability and ontogeny of their subsequent progeny. I also endeavoured to investigate the timing of the onset of endogenously produced cortisol in response to environmental disturbance in embryonic salmonids, as an indicator of the onset of the HPI axis, and what effect this response may have on embryonic growth and survival.
CHAPTER 2

Transfer of Maternal Cortisol to Maturing Oocytes in Female Coho Salmon

(Oncorhynchus kisutch), and its Effects on Subsequent Embryos.
Introduction:

Physiological and behavioural responses to environmental disturbances, such as water borne pollutants and temperature extremes, social interactions, or as a result of aquaculture techniques (handling, sorting etc.), can have deleterious effects on the reproductive capabilities of teleosts (Barton and Iwama, 1991; Billard et al., 1981; Donaldson and Schere, 1983; Donaldson, 1990; Greenberg and Wingfield, 1987). One of the aspects of fish physiology through which environmental perturbations may act to affect reproductive capabilities is via the endocrine response to such environmental disturbances. A nearly universal reaction of teleosts to environmental disturbances is the response of the hypothalamic-pituitary-interrenal axis, a hormone cascade culminating in the release, from the interrenal cells, of the corticosteroid cortisol into the bloodstream (Donaldson, 1981; Sumpter, 1993). The degree to which plasma cortisol levels are elevated in response to an a change in environmental homeostasis is often used as a diagnostic tool to indicate a "stressed" condition among teleosts (Barry et al., 1993; Barton et al., 1986; Davis and Parker, 1983; Davis and Parker, 1986; Flos et al., 1988; Foo and Lam, 1993; Mazur and Iwama, 1993; Pottinger and Pickering, 1992; Sumpter et al., 1986; Thorpe et al., 1987).

Previous work has associated elevated plasma levels of cortisol in salmonids, and other teleosts, with changes in such reproductive parameters as circulating levels of sex steroids, as well as with variable plasma gonadotropin and vitellogenin levels.
It has also been linked with decline in body size, gonadosomatic index, oocyte size, and pituitary gonadotropin content (Carragher et al., 1989; Carragher and Sumpter, 1990; Foo and Lam, 1993; Pickering et al., 1987; Sumpter et al., 1987).

Elevated cortisol levels have also been demonstrated to reduce hepatic estradiol-binding sites in salmonids (Pottinger and Pickering, 1990), and potentiate hepatic vitellogenin mRNA synthesis in tilapia (Ding et al., 1993), suggesting mechanisms by which cortisol levels may impact on oocyte quality. Direct disturbance, in the form of emersion from water, to maturing salmonids has also been demonstrated to negatively effect the quality of gametes in terms of subsequent viability (Campbell et al., 1992). These results indicate a link between the hypothalamic-pituitary-interrenal and hypothalamic-pituitary-gonadal axes, and suggest mechanisms by which external environmental disturbances may act to negatively impact on reproductive capabilities in teleosts.

Another less elucidated aspect of elevated plasma cortisol in response to changes in environmental homeostasis, one that could potentially have a bearing on reproductive success, is the degree and the possible effects of transfer of maternally derived cortisol into developing oocytes. Cortisol has been found to be consistently present in the freshly ovulated oocytes of teleosts (de Jesus et al., 1991; de Jesus and Hirano, 1992; Hwang et al., 1992; Yeoh et al., 1993), as have sex steroid hormones (de Jesus and Hirano, 1992; Feist and Schreck, 1993; Feist et al., 1990;
Rothbard et al., 1987; Yeoh et al., 1993) and thyroid hormones (T3 and T4) (Ayson and Lam, 1993; de Jesus et al., 1991; Leatherland et al., 1989a; Leatherland et al., 1989b).

In anadromous salmonids elevated plasma cortisol levels have been associated with the migration into freshwater that accompanies sexual maturation and reproduction (Krieberg and Blackburn, 1994). Considering the highly variable nature of plasma cortisol levels in response to the presence, or absence, of environmental disturbances; and assuming that oocyte cortisol is of maternal origin, it is not unreasonable to speculate that elevated plasma cortisol in an adult female during the period of oocyte growth and maturation may be reflected in increased oocyte cortisol content.

The purpose of this investigation was to (1), examine the relationship between elevated maternal plasma cortisol levels and subsequent cortisol content of oocytes, along with any effects on ovary weight in relation to body weight, and timing of ovulation (2), to chart the flux of cortisol during ontogeny in oocytes containing natural and experimentally elevated levels of cortisol, and (3), to look for indications of possible effects of elevated oocyte cortisol on a number of developmental parameters.
**Materials and Methods:**

**Applied Disturbance Experiment.**

Sixteen female coho salmon (*Oncorhynchus kisutch*), at the end of their spawning migration, were obtained from the Capilano River Hatchery (North Vancouver, British Columbia) and transported to the Dept. of Fisheries and Oceans research facility at West Vancouver, British Columbia. Preovulatory females were selected by hatchery staff using the degree of hardness of the abdomen as an indication of oocyte maturity, and those selected were deemed to be at least two weeks from ovulation.

Fish were split into two equal groups and held outside in adjacent, identical 4,600 litre circular fiberglass tanks supplied with a combination of flowing Cypress Ck. and dechlorinated city water at a temperature of 4.8 ± 0.6 °C. Fish were left for 48 hours to acclimate to their surroundings before experimental manipulation was initiated.

Fish in the experimental group were exposed to an environmental disturbance consisting of a mechanical agitation twice daily over a period of two weeks. Every day, at randomly chosen times, fish were chased around the tank with a hand net for approximately 60 seconds. Fish in the control group were left undisturbed for the same two week period. A disturbance was not applied to the experimental group on the day of sampling.

At the end of the two week period fish from both groups were netted and placed in a lethal dose of anaesthetic (200 mg/L MS222, buffered 1:1 with sodium bicarbonate). Blood was collected from
each fish via the caudal vasculature and placed on ice for ≤ 30 min. prior to being centrifuged for plasma separation. The ovaries were removed from each fish and ovulation state was determined. The fish were judged to have ovulated if the majority of oocytes in each ovary were detached and free in the body cavity. Ovaries were then weighed and a sample of oocytes was taken from each. The carcass of each fish was also weighed in order to determine gonadosomatic index (GSI). Plasma and oocyte samples were stored at -50°C for later cortisol determination.

Immersion Experiment.

Oocytes from five female coho salmon (Oncorhynchus kisutch) were obtained from the Capilano River Hatchery (North Vancouver, British Columbia) after being selected as having ovulated by hatchery staff. Milt was also collected by staff from three male fish. Oocytes and milt were stored in plastic bags filled with oxygen and placed on ice in a cooler for transportation to the on-site hatchery at the Dept. of Fisheries and Oceans research facility at West Vancouver, British Columbia.

Oocytes from each of the five females were subsequently divided volumetrically into equal control and experimental groups (n≈300 to 700, depending on female). Oocytes were then placed in separate waxed paper containers before milt pooled from the three males was added. Fertilization was initiated by the addition of well water to each paired group simultaneously. This allowed for oocytes from each female to provide both experimental and control specimens.
Control and experimental groups were then placed in two separate compartmentalized trays and placed in 10 litres of well water at 11.7° C to which previously had been added 5 ml of 95% ethanol containing 6 mg of cortisol (Fisher). The concentration of ethanol was 0.05%, and the final cortisol concentration was 600 ug/L. The control group’s water contained 5 ml 95% ethanol alone. Immersion of salmonid alevins in sex steroids utilizing ethanol as a solvent has been shown to be an effective method of manipulating sex differentiation in salmonids (Piferrer and Donaldson, 1992). Trays were left for 2 hours before being placed in the hatchery stack and allowed to flush with running well water (10 liter min⁻¹). The tray containing the immersed group was placed below the control group to avoid cross contamination with cortisol.

Immediately after the 2 hour immersion time a sample of 10 oocytes was removed from each replicate, rinsed for five minutes in running well water to remove any exogenous cortisol, and stored at -50° C for later analysis of cortisol content. Sampling was repeated at 1, 3, and 8 days post fertilization, and every eight days afterwards until 56 days post fertilization.

Three days after fertilization, when there was believed to be no further threat of cross contamination, experimental and control groups were arranged so that all oocytes from each female were in the same tray receiving water at the same temperature (11.2 ± 0.5° C). This was done to standardize the conditions experienced by both control and experimental groups during development.
Developmental parameters such as timing and synchrony of hatch, percent mortality, yolk sac to body weight ratio at hatch, and subsequent dry weight and length of juveniles was monitored at intervals during the extent of the experiment.

**Tissue Extraction.**

Cortisol was extracted from tissue utilizing methods modified from Feist et al., (1990). Frozen oocytes or juveniles were placed singly, or in groups of five, in culture tubes and allowed to sit for approx. 12 hours in 0.1 N NaOH. Groups of 5 eggs were used when cortisol levels in tissue fell below that detectable using a single oocyte. Tissue was then homogenized by hand in a 1.5 ml glass tissue homogenizer (Fisher). The homogenizer was rinsed with distilled water between each sample, and separate homogenizers were utilized for experimental and control groups.

Homogenized tissue was then vortexed for 30 seconds in 10 or 15 ml of diethyl ether (BDH), for single and pooled samples respectively, and the aqueous and organic layers were allowed to separate. The organic layer was decanted off after the aqueous layer was snap frozen by immersion in acetone (BDH) cooled with dry ice. This procedure was repeated for each sample and the organic layers were pooled in a clean culture tube. The ether was then evaporated off by the application of a light stream of compressed air into each tube.

After the tubes had dried, a further 1 ml of ether was used to wash down the sides of the tube and concentrate the extract in the bottom. The ether was again evaporated off before the dried
extract was reconstituted in 100 or 200 ul of phosphate buffered saline (pH 7.4) with 0.1 % gelatin, again for single and pooled samples respectively, and stored at -50° C for later cortisol determination.  

**Cortisol Quantification.**

Cortisol levels in plasma and extracted tissue samples was measured via a commercially available antibody coated tube radioimmunoassay kit (INCstar). The protocol was modified by increasing the aliquot of standards and samples from 10 to 20 ul. This was done to increase the sensitivity of the assay (Sumpter, unpublished data). Validation of the extraction procedure was done via a competitive binding curve utilizing serial dilutions of extracted samples that was seen to lie parallel to the standard curve (Fig. 1).

Extraction efficiency was determined at each extraction interval as specified by the kit manufacturer (INCstar). Efficiency was further validated by the use of [H$^3$]Cortisol. All values are adjusted for extraction efficiency. Extraction efficiency was determined to be 74.4 ± 1.3 % (n=20) for single samples, and 54.3 ± 1.9 % (n=20) for pooled samples (n=5).

**Statistical Analysis.**

Statistical tools utilized were Student’s t-test, ANOVA, Mann-Whitney Rank Sum test, Fisher Exact test and 2 X 2 Chi-square contingency table. The Levene Median and the Kolmogorov-Smirnov tests were used to test for equal variance and normality
respectively. The Student-Newman-Keuls test was utilized to determine significance among means (SigmaStat™, Jandel Scientific Software). Data were $\log_{10}$ transformed to meet requirements of normality and equal variance where appropriate.
Results:

Applied Disturbance Experiment.

One of the undisturbed females demonstrated oocyte cortisol levels (46.84 ± 7.37 ng/gm) that were higher than any other female, control or experimental. This female also demonstrated extremely high plasma cortisol levels (1032.3 ng/ml) when compared to the other females. This plasma cortisol level is indicative of a diseased or moribund fish (Barton and Iwama, 1991; Sumpter et al., 1986), one that was most probably not exhibiting typical cortisol dynamics, and as such was removed from the control group. One of the experimental females died within four hours of transport and was also removed.

Mean plasma cortisol was seen to be higher, though not significantly, in the disturbed group at the time of oocyte sampling compared to the undisturbed group (t-test, p > 0.05) (Fig. 2). Mean gonadosomatic index (GSI) of the disturbed and undisturbed groups was not found to be significantly different at the time of oocyte sampling (t-test, p > 0.05), (Fig. 3). Five of the seven females in the disturbed group were seen to have ovulated, as compared to only one of the seven undisturbed females, though this difference was not seen to be significantly different (Fisher Exact test, p > 0.05).

Mean oocyte cortisol levels in the disturbed adult females was significantly higher than that observed in the undisturbed individuals (Fig. 4), (ANOVA, p < 0.05). Pairwise multiple comparison (Student-Newman-Keuls) revealed that all of the
disturbed females had mean oocyte cortisol levels that were significantly higher than all of the undisturbed females, with the exception of the anomalous control fish.

Immersion Experiment.

The immersion protocol significantly elevated mean oocyte cortisol content in each of the manipulated groups when compared to the untreated group from the same female (t-test, p < 0.0001), and all manipulated groups had mean oocyte cortisol levels that were significantly higher than those in the untreated groups (Table 1) (ANOVA, p < 0.05).

Mean oocyte cortisol content among each of the immersed groups declined over 24 hours (Fig. 5), and were not significantly different from the its paired untreated group by 16 days post immersion (t-test, p > 0.05).

Mortality rates did not differ significantly between paired immersed and untreated groups (Table 1) (Chi square contingency table, p > 0.05). Mean time to 50% hatch, and mean time from beginning to 100% hatch, also did not vary significantly between immersed and untreated groups (Table 1) (Mann-Whitney, p > 0.05).

Body length and dry weight of each paired group was monitored for 24 days post hatch. Fish would have been followed further through development, but a power failure at the facility resulted in significant mortalities taking place among all groups. The experiment was terminated at this point. There was no significant difference seen in mean body length (Fig. 6), or mean weight (Fig.
7) between paired immersed and untreated groups at any of the sampling times (t-test, p > 0.05).

Mean yolk sac to body weight ratio was determined at the first sampling period after hatch for all groups, and no significant difference was seen between paired immersed and untreated groups (Fig. 8) (t-test, p > 0.05).
Table 1. Initial oocyte cortisol levels, percent mortalities, time to 50% hatch, and time from 0 to 100% hatch for control and experimental groups. Experimental groups were immersed for two hours in water containing 600 ug/L cortisol.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CONTROL</th>
<th>EXPERIMENTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Oocyte Cortisol Level</td>
<td>37.03 ± 5.43</td>
<td>232.68 ± 13.93*</td>
</tr>
<tr>
<td>(ng/g wet weight) (n=20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent Mortality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female #1</td>
<td>41.88</td>
<td>42.77</td>
</tr>
<tr>
<td>#2</td>
<td>45.96</td>
<td>40.46</td>
</tr>
<tr>
<td>#3</td>
<td>46.62</td>
<td>43.88</td>
</tr>
<tr>
<td>#4</td>
<td>30.75</td>
<td>30.26</td>
</tr>
<tr>
<td>#5</td>
<td>46.12</td>
<td>46.26</td>
</tr>
<tr>
<td>Time to 50% Hatch (hours) (n=5)</td>
<td>966.9 ± 1.07</td>
<td>962.1 ± 1.82</td>
</tr>
<tr>
<td>Time from 0 to 100% Hatch (hours) (n=5)</td>
<td>80.4 ± 1.82</td>
<td>80.4 ± 2.01</td>
</tr>
</tbody>
</table>

Values represent mean ± SE.
* denotes significant difference between control and immersed groups (p < 0.05).
Figure 1. Competitive binding curves of cortisol standards and serial dilutions of extracted coho salmon (Oncorhynchus kisutch), oocyte tissue (n=10).
Figure 2. Plasma cortisol levels of adult female coho salmon (*Oncorhynchus kisutch*). Experimental fish were disturbed via mechanical agitation (chased with hand net for 60 sec.) twice daily for two weeks. Control fish were left undisturbed. No significant difference was observed (p > 0.05). Bars represent mean ± SE (n=7).
Figure 3. Gonadosomatic indices of adult female coho salmon (*Oncorhynchus kisutch*). Experimental fish were disturbed via mechanical agitation (chased with hand net for 60 sec.) twice daily for two weeks. Control fish were left undisturbed. No significant difference was observed (*p* > 0.05). Bars represent mean ± SE (*n*=7).
Figure 4. Oocyte cortisol content of adult female coho salmon (*Oncorhynchus kisutch*). Experimental fish were disturbed via mechanical agitation (chased with hand net for 60 sec.) twice daily for two weeks. Control fish were left undisturbed. Bars represent mean ± SE (n=7). * indicates experimental groups which differed significantly from all control groups (p < 0.05).
Figure 5. Whole body cortisol content of cortisol immersed and control coho salmon (Oncorhynchus kisutch), oocytes. Immersed oocytes were placed in a solution of 600 ug/L cortisol for 2 hours immediately after fertilization, during the water hardening process. Points indicate mean ± SE (n=20). * indicates immersed groups that differed significantly from controls (p < 0.05).
Figure 6. Body length of coho salmon (*Oncorhynchus kisutch*), embryos from the five paired cortisol immersed and control groups of oocytes 40, 48, and 56 days post fertilization (1, 9, and 17 days post hatch). No significant difference was observed (p > 0.05). Bars represent mean ± SE (n=10).
Figure 7. Body weight (dry) of coho salmon (*Oncorhynchus kisutch*), embryos from the five paired cortisol immersed and control groups of oocytes 40, 48, and 56 days post fertilization (1, 9, and 17 days post hatch). No significant difference was observed (p > 0.05). Bars represent mean ± SE (n=10).
Figure 8. Ratio of yolk sac to body weight (dry) of coho salmon (*Oncorhynchus kisutch*), embryos from the five paired cortisol immersed and control groups of oocytes 40 days post fertilization (1 day post hatch). No significant difference was observed (p > 0.05). Bars represent mean ± SE (n=10).
Discussion:

Mean plasma cortisol levels among the disturbed females was higher than those observed in the undisturbed group (Fig. 2), though this was not seen to be statistically significant. My failure to observe a significant difference between the undisturbed and disturbed females in terms of plasma cortisol may have been due to the combination of a number of factors. The experimental fish were not disturbed on the day of sampling and thus may have had relatively lower plasma cortisol than would have been observed had they been disturbed, or the agitation experienced by the undisturbed fish during netting and application of anesthetic prior to blood sampling may have elevated their plasma cortisol levels to those approaching that of the disturbed fish. It may have also been the case that after 14 days of treatment, the disturbed fish were becoming acclimated to the mechanical agitation protocol and were not responding to the disturbance with as substantial a rise in plasma cortisol.

The proportion of females in the disturbed group that had undergone ovulation over the two week treatment period (5 of 7), appeared to be higher than that observed in the undisturbed group (1 of 7), though this difference was not seen to be statistically significant. However, the p value observed (0.053), was very close to being considered significant (0.05), suggesting that the disturbance protocol may have had some effect on the timing of ovulation. There is some evidence suggesting a role for cortisol in ovulation. Bry (1985) reported that plasma cortisol levels were
low in adult female rainbow trout prior to ovulation, but showed a marked elevation during the immediate post-ovulatory period. It has also been reported that plasma cortisol in adult female rainbow trout attained maximal concentration on the day of ovulation (Bry, 1989). It may be that the female coho in the disturbed group experienced elevated cortisol levels as a result of the applied disturbance, and this elevation in circulating cortisol levels may have influenced the timing of ovulation.

The applied disturbance protocol was seen to be effective in significantly elevating oocyte cortisol levels above those observed in the undisturbed females (Fig. 4). The assumption was made that those females subjected to mechanical agitation would have experienced elevated levels of cortisol in circulation.

It has been speculated that the hormones present in the oocytes and early embryonic stages of teleosts are of maternal origin (Greenblatt et al., 1989; Hwang et al., 1992). Injection of female rabbitfish (Siganus guttatus) with thyroxine (T4) has been demonstrated to increase T4 and triiodothyronine (T3) in maternal circulation, and subsequently in oocytes (Ayson and Lam, 1993). Intraarterial injection of labeled cortisol in Pacific salmon has been demonstrated to result in transfer of cortisol to almost all tissues very shortly after application (Donaldson and Fagerlund, 1972).

The increases in cortisol content I observed in the oocytes of those females which were subjected to mechanical agitation, as compared to those that were not, suggests that increased plasma
cortisol as a result of an environmental disturbance may lead to increased deposition of cortisol in the oocytes, even though I did not observe a significant difference in circulating cortisol levels. It is interesting to note that the female that was excluded from the undisturbed group because of its extremely high plasma cortisol level, also demonstrated the highest oocyte cortisol content among all females.

An applied disturbance during oogenesis in female salmonids has been demonstrated to result in a decrease in the quality of the oocytes in terms of survival to hatch (Campbell et al., 1992). For this reason I chose to artificially elevate oocyte cortisol levels in previously unmanipulated adult females in an attempt to isolate the effect that cortisol may have on subsequent oocyte survival, as well as on the other measured developmental parameters.

All groups of oocytes immersed in cortisol exhibited significantly elevated cortisol levels when compared to controls immediately post immersion (Table 1). Immersion treatment has been demonstrated to be effective in transferring steroid hormones throughout coho salmon oocytes (Piferrer and Donaldson, 1994), and yolk and chorion were not seen to affect accumulation of radioactive steroids in rainbow trout oocytes and embryos (Antila, 1984). In all groups, immersed and control, initial oocyte cortisol levels measured immediately post immersion were higher than those levels observed 24 hours later (Fig. 5).

This pattern of high oocyte hormone levels at fertilization, followed by a rapid decline during early ontogeny has been observed
for cortisol in chum salmon (Oncorhynchus keta) (de Jesus and Hirano, 1992), tilapia (Oreochromis mossambicus) (Hwang et al., 1992) and Japanese flounder (Paralichthys olivaces) (de Jesus et al., 1991), as well as for other steroid hormones in three species of tilapia (O. niloticus, O. mossambicus, and Tilapia hornorum) (Rothbard et al., 1987), rainbow trout (Oncorhynchus mykiss) (Feist and Schreck, 1993; Yeoh et al., 1993), and coho salmon (Feist et al., 1990).

This is not the pattern reported for all hormones detected in teleost oocytes. For example, the thyroid hormones, T4 and T3, have been observed to decrease gradually, or remain relatively constant, in the oocyte and subsequent larvae of rabbitfish (Ayson and Lam, 1993), tilapia (O. mossambicus) (de Jesus et al., 1991) and Pacific salmon (Leatherland et al., 1989a; Leatherland et al., 1989b).

My observation that there was no differences in mortality rates between control and experimental groups suggests that, although an applied disturbance to adult female salmonids undergoing oogenesis has been demonstrated to decrease oocyte quality (Campbell et al., 1992) and to increase oocyte cortisol content (this study), the decline in quality appears to be the result of factors other than cortisol content alone.

The failure to observe any differences in the measured parameters between treated and untreated groups (Table 1), coupled with the observed rapid decline in oocyte cortisol after fertilization (Fig. 5), seems to suggest that maternally derived
Cortisol is cleared from the oocytes early in ontogeny, and does not have a significant impact on later development.

The mechanism underlying the clearance of cortisol from oocytes, whether by metabolic processes or simply by diffusion, is an area for further investigation. Yeoh (1993) has presented evidence supporting cortisol metabolism early in salmonid ontogeny, reporting the presence of cortisol glucuronide in steelhead embryos immediately post fertilization. Yeoh (1993) also observed an increase in the synchrony of hatch of salmonid oocytes immersed in cortisol immediately post fertilization. My experiment did not yield a similar result, and this could be due to a number of factors. Yeoh utilized DMSO (di-methyl-sulfoxide) as a solvent for immersion treatments, and was thus able to raise oocyte cortisol levels higher than I did utilizing 95% ethanol. The increased oocyte cortisol levels may have effected synchrony of hatch, although Yeoh observed an increase in hatch synchrony in oocytes immersed in any hormone (cortisol, thyroxine, testosterone) dissolved in DMSO, and in oocytes immersed in DMSO alone, suggesting that the solvent may have had some effect on the developing embryos.

In conclusion my results indicate that cortisol content in freshly ovulated oocytes of coho salmon, and probably all salmonids, is reflective of the circulating levels of cortisol in the adult female during late oogenesis, and that an increase in ciruclating cortisol levels as a result of an environmental perturbation, if sustained over a long enough period, can result in
elevated oocyte cortisol content. My observation of rapid decline in oocyte cortisol content during early ontogeny, coupled with my failure to observe any differences in the parameters monitored between oocytes with natural, and artificially high, cortisol content suggests that cortisol is not a major factor in early salmonid development.
CHAPTER 3

Ontogeny of the Cortisol Response to an Environmental Disturbance in Steelhead

(Oncorhynchus mykiss), and Coho Salmon

(Oncorhynchus kisutch), Embryos.
Introduction:

The activation of the hypothalamic-pituitary-interrenal (HPI) axis is a nearly universal physiological response of teleosts to environmental disturbances or perturbations. A neuroendocrine response involving corticotropin releasing factor (CRF) that begins at the level of the hypothalamus and results in the release of adrenocorticotropic (ACTH) from the pituitary gland, which precipitates a subsequent release into circulation of the corticosteroid cortisol from the interrenal tissue (Donaldson, 1981; Mazeaud et al., 1977; Sumpter et al., 1986).

This response has been commonly employed as a diagnostic tool to indicate a "stressed" condition in teleosts when circulating titers of cortisol are elevated above baseline levels (Barry et al., 1993; Barton et al., 1986; Davis and Parker, 1983; Davis and Parker, 1986; Flos et al., 1988; Foo and Lam, 1993; Mazur and Iwama, 1993; Pottinger and Pickering, 1992; Sumpter et al., 1986; Thorpe et al., 1987), and has been speculated to have short term adaptive advantages, but may in the long term prove deleterious (Barton and Iwama, 1991).

Most investigations of this response in salmonids have dealt with fish that were either sexually mature, or in the juvenile stage of development (Pickering et al., 1987; Carragher et al., 1989; Pickering et al., 1982; Pottinger and Pickering, 1990; Barton et al., 1980). There is not a great deal of information available concerning the onset of response of the HPI axis in reaction to
environmental perturbations during embryogenesis and early ontogeny.

In birds there is evidence that the pituitary-adrenal axis is established as early as day 14.5 of incubation (Woods et al., 1971), and increased adrenal activity has been associated with hatching in ducks (Tanabe et al., 1983). This has been speculated to be important in adapting the developing animal to a new environment. Embryonic rat adrenals secrete corticosterone in response to a variety of stimuli beginning at the late fetal period (Sapolsky and Meaney, 1986), and the rat fetal hypothalamic-pituitary-adrenal (HPA) axis has been demonstrated to respond to fetal hypoxia as early as day 18 of gestation (Ohkawa et al., 1991). Neonatal rats exhibit basal and stimulated plasma corticosterone levels approaching those of adults at 1-2 days of age (Guillet et al., 1980), and the HPA axis has the functional capacity to respond to a variety of stimuli throughout neonatal life (Walker et al., 1991).

ACTH immunoreactive cells have been observed to first appear in the pituitary of the rainbow trout (Oncorhynchus mykiss), approximately 10 days prior to hatch (Saga, 1993), and in the cloudy dogfish (Scyliorhinus torazame), and tilapia (Oreochromis mossambicus), at the time of, or just after, hatching (Chiba et al., 1992; Fu and Lock, 1990). A similar condition has been reported in the salamander (Hynobius nebulosus) (Oota and Saga, 1991). This suggests a possible role of the HPI axis in hatching,
although corticosteroids did not modulate hatching in the zebrafish (Brachydanio rerio) (Bogsnes and Walther, 1994).

The conversion of steroids to metabolites has been observed in oocytes and early embryos (cleavage to 20 pair-of-somites stage) of rainbow trout (Oncorhynchus mykiss) (Antila, 1984), and the conversion of labelled progesterone to cortisol and cortisone has been demonstrated in rainbow trout oocytes as early as 1 week post fertilization (Pillai et al., 1974). de Jesus and Hirano (1992) have observed changes in whole body cortisol content of chum salmon (Oncorhynchus keta), approximately one week prior to hatch, and similar changes in whole body cortisol content have been reported for coho salmon (Oncorhynchus kisutch), (this thesis, chpt 2). This suggests that embryonic salmonids possess the required enzymatic pathways needed to synthesize cortisol, and that the embryonic interrenal tissue is able to secrete cortisol. It does not indicate at what point this secretion is elicited in response to environmental disturbance.

Salmonids spend the early part of their life history within the gravel redds of their natal streams. This period of time is temperature dependent and includes incubation, hatch, and yolk sac absorption (Neitzel et al., 1985). During this time embryonic salmonids can be exposed to potential environmental disturbances such as temperature extremes, fungal and protozoan infection, variations in water flow, dissolved oxygen concentration, pH, and deposition of sediments and organic matter (Fiss and Carline, 1993; Johnson, 1980; Lisle and Lewis, 1992; Murray and Beacham, 1986;
Neitzel and Becker, 1985; Reiser and White, 1988; Ringler and Hall, 1988; Scrivener, 1988; Scrivener and Brownlee, 1989; Taylor and Bailey, 1979).

It is interesting to speculate whether an integrated HPI response, in terms of cortisol synthesis, to these potential environmental disturbances is advantageous to embryonic salmonids. There is some evidence for movement of salmonid larvae within the gravel redds in response to drying and temperature stimuli (de Leaniz et al., 1993; Mcdonald, Steve, pers comm), and it may be that the energy (glucose) mobilizing properties of cortisol may facilitate this.

The purpose of this investigation was to examine whole body cortisol content in embryonic steelhead and coho salmon for a period immediately prior to, and approximately 30 days post hatch, and to utilize variation in cortisol content in response to an applied disturbance as an indication of a functional HPI axis.
Materials and Methods:

Ontogeny of Cortisol Response to Environmental Disturbance.

Oocytes from a single female, and milt from three male, steelhead trout (*Oncorhynchus mykiss*) were obtained from the Chilliwack River Hatchery (Chilliwack, British Columbia). Gametes were stored on ice in plastic bags filled with oxygen and transported to the Department of Fisheries and Oceans facility at West Vancouver, British Columbia.

Oocytes were fertilized with milt pooled from the three males and were placed in an incubation tray (Heath Techna. Corp.) in the on site hatchery and supplied with flowing well water (10 litres min\(^{-1}\)) at 13.1 ± 1.2° C.

Oocytes were allowed to develop to a point approximately one week prior to hatch, 20 dpf (days post fertilization), as this has been previously reported as the point where an increase in oocyte cortisol has been observed in salmonids (de Jesus and Hirano, 1992; Yeoh, 1993).

A control and experimental set consisting of eight groups of five oocytes each were removed and placed in separate incubation trays. Oocytes were transferred a minimum of four days prior to experimental manipulation, and the procedure was repeated every 5 days, at -4, 1, 6, 11, 16, 21, 26, an 32 days post hatch (dph).

Embryos in the experimental groups were subjected to a repeated environmental disturbance consisting of emersion out of water for 60 seconds, every fifteen minutes over a period of 2 hours. The control group of oocytes or embryos was undisturbed.
Since cortisol quantification required the measurement of whole body cortisol content, as opposed to plasma cortisol, it was felt that repeated application of an environmental disturbance over time was preferable to a single episode. This would allow for the de novo synthesis of cortisol from precursors, and for the accumulation of cortisol in tissue.

At the end of the 2 hour period, after the eighth emersion, pre-hatch embryos in both sets were killed by placement for 15 minutes in a freezer at -50° C. Post-hatch embryos were killed by immersion in an overdose of anaesthetic (200 mg/L MS-222 buffered 1:1 with sodium bicarbonate). Samples were frozen at -50° C for later assessment of whole body cortisol content.

This protocol was repeated every five days from the eyed egg stage, through hatching, up until yolk sac absorption.

**Time Course of Cortisol Response to Environmental Disturbance During Ontogeny.**

Eggs from a single female coho salmon (*Oncorhynchus kistuch*) and milt from three males were obtained from the Capilano River Hatchery (North Vancouver, British Columbia). Gametes were stored on ice in plastic bags filled with oxygen and transported to the Department of Fisheries and Oceans facility at West Vancouver.

Oocytes were fertilized with milt pooled from the three males and placed in an incubation tray (Heath Techna. Corp.) in the on site hatchery and supplied with flowing well water (10 litres min⁻¹) at 10.7 ± 0.3° C.
After the oocytes were allowed to develop to a point approximately one week prior to hatch, 38 dpf, a control and experimental set consisting of eight groups of five embryos each were removed and placed in separate incubation trays. Embryos were transferred a minimum of four days prior to experimental manipulation, and the procedure was repeated every 7 days, instead of 5 as in the previous experiment because increased routine activity by other workers at the facility necessitated that trials be carried out on weekends to ensure minimal disturbance of the control groups.

Oocytes or embryos in the experimental groups were subjected to a repeated environmental disturbance as described above. The control group of oocytes or embryos was undisturbed. Both control and experimental groups were sampled at 1, 2, 4, and 8 hours after the commencement of the trial, and embryos were killed in the manner described above.

Tissue Extraction and Cortisol Quantification.

Embryo tissue was extracted, and cortisol quantification was determined, as described in chapter 2.

Statistical Analysis.

To determine whether there was a significant difference in whole body cortisol content between the experimental and control groups, a students t-test was applied to data for each sampling period (Sigma Stat™, Jandel Scientific).
Whole body cortisol levels within control or experimental groups were analyzed by ANOVA, and pairwise comparisons were done utilizing Student-Neuman-Keuls test.

The Levene Median and the Kolmogorov-Smirnov tests were used to test for equal variance and normality respectively. Data were log_{10} transformed where assumptions of normality and homogeneity of variance were not met.
Results:

Ontogeny of Cortisol Response to Disturbance.

No significant difference in whole body cortisol content was observed in control and experimental steelhead embryos at -4 and 1 dph.

At 6 dph there was a significant increase observed in the whole body cortisol content of those embryos that received the repeated disturbance (t-test, p<0.05) (Fig. 1).

Over the next 5 sampling intervals (11, 16, 21, 26 and 32 dph), the whole body cortisol content of all steelhead embryo groups receiving the repeated disturbance were significantly higher than those observed in the controls (t-test, p<0.05). The difference in whole body cortisol content between the control and experimental groups progressed from a 1.5 fold increase at 6 dph, to a 4.2 fold increase at 32 dph.

The whole body cortisol content in the experimental groups increased as development progressed, from 5.88 ng/g at 6 dph, to 45.57 ng/g at 32 dph, and with the exception of the 21 dph sample, were significantly higher than those levels observed in the previous sample interval (ANOVA, p<0.5). The whole body cortisol levels in the control groups decreased significantly at 1 dph, and increased significantly at 11, 16, and 21 dph (ANOVA, p<0.05). After this there was no significant difference in whole body cortisol content observed between control groups.
Time Course of Cortisol Response to Disturbance During Ontogeny.

There were no significant differences in whole body cortisol content observed between those coho embryos receiving the repeated disturbance, and those that were undisturbed, at -5 and 2 dph during the 8 hour duration of the trial (t-test, p>0.05) (Fig. 2). There were no significant differences between the 0h and any subsequent sampling time in either the control or experimental groups (ANOVA, p>0.05).

At 9 dph there was a significant difference in whole body cortisol content observed between those coho embryos receiving the repeated disturbance and those that did not. There was no significant difference observed at 0h (t-test, p>0.05), but a significant difference was observed at the 1h sampling interval, and at each subsequent sampling interval thereafter (2, 4, and 8h) (t-test, p<0.05) (Fig. 3). There was no significant difference in whole body cortisol content observed between 0h and any other interval within the control group over the duration of the trial (ANOVA, p>0.05).

Whole body cortisol content in the experimental embryos was significantly higher at 1h than at 0h, and remained significantly elevated above the 0h level during the duration of the trial (ANOVA, p<0.05).

At 16 dph there was no significant difference observed in whole body cortisol content between experimental and control groups at 0 and 2h (t-test, p>0.05). Whole body cortisol content was
significantly elevated in the experimental groups at 1, 4, and 8h (t-test, p<0.05) (Fig. 4).

There was no significant difference in whole body cortisol content observed between the 0h and any other sampling interval within the control group over the duration of the trial (ANOVA, p>0.05).

Whole body cortisol content in the experimental embryos was significantly higher at the 1, 2, and 4h sampling intervals, than at the 0h interval (ANOVA, p<0.05), before dropping to a level not significantly different at the 8h interval (ANOVA, p>0.05).

At 23 dph there was no significant difference observed in whole body cortisol content between experimental and control groups at 0 and 8h (t-test, p>0.05). Whole body cortisol content was significantly elevated in the experimental groups at 1, 2, and 4h, (t-test, p<0.05) (Fig. 4).

There was a significant elevation in whole body cortisol content observed between the 0h level and all subsequent levels in both the control and experimental groups (ANOVA, p>0.05).

At 30 dph initial (0h) whole body cortisol content was not significantly different between control and experimental groups (t-test, p>0.05). A significant increase was observed in the experimental groups at the 1, 2, 4, and 8h sampling interval (t-test, p<0.05) (Fig. 5).

There was a significant increase over the 0h level in whole body cortisol content observed in the control embryos at 2h (ANOVA, p<0.05). There was no significant difference observed between the
Oh whole body cortisol content and any of the other sampling intervals in the control group (ANOVA, p>0.05).

Among the experimental embryos there was a significant difference observed in whole body cortisol content between the 0h level and all of the subsequent sampling intervals (ANOVA, p<0.05).
Figure 9. Whole body cortisol content of steelhead salmon (*Oncorhynchus mykiss*), embryos receiving repeated emersion, and left undisturbed, from -4 to 32 days post hatch (dph). Bars represent mean ± SE (n=8). * denotes significant difference between control and experimental groups (p < 0.05). + Denotes significant differences within groups from previous value (p < 0.05).
Figure 10. Whole body cortisol content of coho salmon (*Oncorhynchus kisutch*), embryos over an 8 hour period receiving repeated emersion, and left undisturbed, at -5 days post hatch (dph). No significant difference was observed (p > 0.05). Points represent mean ± SE (n=8).
Figure 11. Whole body cortisol content of coho salmon (*Oncorhynchus kisutch*), embryos, over an 8 hour period, receiving repeated emersion and left undisturbed, at 2 days post hatch (dph). No significant difference was observed (p > 0.05). Points represent mean ± SE (n=8).
Figure 12. Whole body cortisol content of coho salmon (<i>Oncorhynchus kisutch</i>), embryos over an 8 hour period receiving repeated emersion, and left undisturbed, at 9 days post hatch (dph). Points represent mean ± SE (n=8). * denotes significant difference between control and experimental groups (p < 0.05). + denotes significant differences from values at time 0 within groups (p < 0.05).
Figure 13. Whole body cortisol content of coho salmon (*Oncorhynchus kisutch*), embryos over an 8 hour period receiving repeated emersion, and left undisturbed, at 16 days post hatch (dph). Points represent mean ± SE (n=8). * denotes significant difference between control and experimental groups (p < 0.05). + denotes significant differences from values at time 0 within groups (p < 0.05).
Figure 14. Whole body cortisol content of coho salmon (*Oncorhynchus kisutch*), embryos over an 8 hour period receiving repeated emersion, and left undisturbed, at 23 days post hatch (dph). Points represent mean ± SE (n=8). * denotes significant difference between control and experimental groups (p < 0.05). + denotes significant differences from values at time 0 within groups (p < 0.05).
Figure 15. Whole body cortisol content of coho salmon (Oncorhynchus kisutch), embryos over an 8 hour period receiving repeated emersion, and left undisturbed, at 30 days post hatch (dph). Points represent mean ± SE (n=8). * denotes significant difference between control and experimental groups (p < 0.05). + denotes significant differences from values at time 0 within groups (p < 0.05).
Discussion:

Cortisol levels have been observed to increase in salmonid oocytes approximately 1 week prior to hatch (de Jesus and Hirano, 1992; Yeoh, 1993). My results show a significant increase in whole body cortisol content of embryonic coho salmon between 5 days before, and 2 days after, hatch in both control and experimental groups (Figs. 10 and 11).

This would indicate that the interrenal cells in the embryonic coho salmon are capable of synthesizing corticosteroids at this point in development. The fact that I observed no significant differences between undisturbed embryos and those receiving the repeated disturbance at both sampling periods indicates that some components of the HPI axis are immature, or inactive, at this point.

In rats there is a period of nonresponsiveness of the HPA axis that begins shortly after birth (Guillet et al., 1980; Haltmeyer et al., 1966; Sapolsky and Meaney, 1986; Walker et al., 1986) when application of a variety of stimuli fails to elicit an increase in circulating levels of corticosteroids. It has been suggested that this "stress" nonresponsive period (SNRP) is due to enhanced brain and/or pituitary sensitivity to glucocorticoid feedback (Levin et al., 1988; Walker et al., 1986), and is crucial for normal growth and differentiation of a number of tissues and organs, including the central nervous system (Moisan et al., 1992).

The HPA axis prior to this point can be activated by a variety of stimuli. Fetal corticosterone levels are responsive to
environmental disturbance, or application of exogenous ACTH, in mammals prior to birth (Ohkawa et al., 1991; Rose et al., 1982; Walker et al., 1986), and immunoreactive ACTH cells appear in the rat and porcine pituitary as early as days 17 and 40 of gestation respectively (Chatelain et al., 1980; Sasaki et al., 1992).

I observed a significant difference in whole body cortisol content between those steelhead and coho salmon embryos that received a series of disturbances, compared to those that were undisturbed, beginning at 6 and 9 dph respectively (Figs. 9 and 12), but not at -5 and 2 dph for coho (Figs. 10 and 11), or -4 and 1 dph for steelhead (Fig. 9). This indicates the onset of de novo synthesis of cortisol in response to environmental stimuli, and is evidence of a functional HPI axis.

A dissectible pituitary gland mass is first detectable in rainbow trout approximately 15 days post fertilization (Ballard, 1973), but immunoreactive ACTH cells do not appear until approximately 10 days prior to hatch (Saga et al., 1993), which corresponds to the period of corticosteroidogenesis reported in salmonid oocytes (de Jesus and Hirano, 1992; Yeoh, 1993).

Pottinger and Mosuwe (1994), reported a measurable increase in immunoreactive corticosteroids in rainbow trout, in response to mechanical disturbance and confinement, beginning at 5 weeks post hatch. Barry et al., (1995) observed an increase in whole body cortisol content in rainbow trout, in response to suspension out of water and cold temperatures beginning at 2 weeks post hatch.
The variation between these findings and my own could be due simply to the difference in water temperature at which the oocytes and embryos were incubated, especially in the case of Barry et al., (1995) who reported using water at 10°C (Pottinger and Mosuwe, (1995) did not report the temperature of their incubation water), or it may be the result of differences in the application of environmental disturbances. It could be that a repeated disturbance over time, as opposed to a single brief disturbance, allows for greater accumulation of cortisol within tissues, and acts to magnify the differences between control and experimental embryos.

It is difficult to ascertain whether the lack of a measurable cortisol response in the steelhead and coho embryos prior to days 6 and 9 post hatch respectively, is due to a lack of development of the HPI axis, or is homologous to the SNRP reported in rats, and is an area for future investigations.

In adult and juvenile salmonids plasma cortisol levels have been observed to rise significantly after the application of a disturbance (<10 min.), and continue to be elevated if the disturbance is maintained (Sumpter et al., 1986). A single, brief applied disturbance (1 to 5 min. handling) also elicits a rapid elevation in plasma cortisol that is seen to peak within a period of 0.5 to 1 hours, before returning to pre-application levels within 4 to 6 hours (Barry et al., 1993; Barton et al., 1980; Barton et al., 1986; Biron and Benfey, 1994; Pickering et al., 1982).
I observed, beginning at 9 dph in coho salmon, a significant increase in whole body cortisol content in those embryos receiving a series of applied disturbances. At 9 dph this increase was seen to be maintained for the 6 hour period after cessation of the treatment (Fig. 12). This continuation of elevated cortisol levels could be due to a number of factors. It may be that the embryonic HPI axis is still not completely functional, and that cortisol feedback at the level of the hypothalamus and/or pituitary is unresponsive, resulting in continued release of corticotrophin releasing factor (CRF), ACTH, or both. It may also be possible that the metabolic clearance rate of cortisol at this point in development is less efficient, and that cortisol is simply not being cleared from tissues as quickly.

At 16 dph whole body cortisol content in the coho embryos was seen to decline over the 6 hour period after cessation of the treatment, and at 8 hours was not significantly different from pre-treatment levels (Fig. 13). This was also the pattern observed in whole body cortisol content of coho embryos receiving the repeated disturbance at 30 dph (Fig. 15).

At 23 dph the whole body cortisol content of those coho embryos receiving the repeated disturbance was elevated above pre-treatment levels over the duration of the experiment (8 hours). This was also the pattern observed in the undisturbed coho embryos with the exception of the 4 hour interval (Fig 14). This continued elevation in whole body cortisol content observed in the experimental coho embryos, and in the seemingly undisturbed coho
embryos can be explained by the fact that, after the experiment had commenced, another worker at the facility inadvertently entered the hatchery area and disturbed the fish. Even with this disturbance however, the coho embryos in the treatment groups demonstrated significantly higher whole body cortisol contents than those observed in the control groups over the first 4 hours of the experiment. This demonstrates that coho embryos at this stage of development are able to express a graded response to stimuli in terms of cortisol production, an effect observed in juvenile salmonids (Barton et al., 1986).

My results suggest that embryonic steelhead and coho salmon have a functional HPI axis by days 6 and 9 post hatch respectively, and that by day 16 post hatch the cortisol response to an environmental disturbance, over an 8 hour period at least, in coho embryos is similar to that of adults and juveniles.
CHAPTER 4

Effects of Exogenous Cortisol and/or an Environmental Disturbance on Growth and Survival of Chinook Salmon (*Oncorhynchus tshawytscha*) Embryos.
Introduction:

A nearly universal response of teleosts to environmental disturbances or perturbations is the activation of the hypothalamic-pituitary-interrenal (HPI) axis (Sumpter et al., 1993). This neuroendocrine response to environmental disturbances initiates a hormonal cascade that culminates in the release of the corticosteroid, cortisol, from the interrenal cells into circulation (Donaldson, 1981; Bonga, 1993).

The elevation of circulating cortisol above baseline levels in response to varying environmental stimuli has been studied extensively in many varieties of fish (Barry et al., 1993; Davis and Parker, 1986; Foo and Lam, 1993; Lamers et al., 1992; Thorpe et al., 1987), and is often used as a diagnostic tool to indicate the presence of a "stressed" condition in salmonids (Barton et al., 1986; Sumpter et al., 1986; Pottinger and Pickering, 1992; Mazur and Iwama, 1993).

Cortisol has also been demonstrated to cause hyperglycaemia, and appears to stimulate gluconeogenesis and glycolysis from lipid and protein sources in teleosts (Davis et al., 1985; Freeman and Idler, 1973; Leach and Taylor, 1980; Leach and Taylor, 1982; Vijayan et al., 1990; Vijayan et al., 1991), and as such may have an effect on growth and performance (Schreck, 1990). Cortisol has also been observed to modulate lipid metabolism in salmonids (Sheridan, 1986), and to have important effects on immune physiology (Barton and Iwama, 1991). Exposure of salmonids to adverse environmental conditions, and to exogenous cortisol, has
been demonstrated to have deleterious effects on immune function (Pickering and Pottinger, 1985; Pickering and Pottinger, 1988; Maule et al., 1989). This can lead to increased mortality and decreased performance in salmonids under culture conditions, and is of primary concern to fish culturists.

The onset of a functional HPI axis begins early in the ontogeny of salmonids (Pottinger and Mosuwe, 1994; Barry et al., 1995), and has been demonstrated to be partially functional in steelhead (Oncorhynchus mykiss) and coho salmon (Oncorhynchus kisutch), as early as 6 and 9 days post hatch respectively (this thesis, chpt 3).

Since cortisol, and secondary physiological responses to environmental perturbations, have been associated with changes in growth and impairment of immune function in salmonids, it is interesting to speculate whether the repeated activation of this response to environmental stimuli, and the resulting elevation of circulating cortisol, may effect growth and survival during the early ontogeny of salmonids.

The purpose of this investigation was to observe what effects repeated disturbance and/or exogenous cortisol may have on the growth and survival of salmonids immediately prior to hatch, and during early development.
Materials and Methods:

Oocytes from a single adult female, and milt from three adult male, chinook salmon (*Oncorhynchus tshawytscha*) were obtained from the Tenderfoot Ck. Hatchery (Squamish, British Columbia). Oocytes and milt were placed in plastic bags filled with oxygen and placed on ice in a cooler, before being transported to the Department of Fisheries and Oceans research facility in West Vancouver. Oocytes were mixed with milt pooled from the three males before water was added to activate the sperm and initiate fertilization. Oocytes were then placed in a single hatchery tray (Heath Techna Corp.) supplied with flowing well water (10 litres min\(^{-1}\)) at 11.4 ± 0.2° C, in the on site hatchery. Oocytes were allowed to develop to a point approximately 5 days prior to hatch, and were divided into four equal groups, with each group placed in a separate hatchery tray. Each group contained seven replicates of 50 oocytes each.

Beginning approximately 5 days prior to hatch, experimental manipulation was initiated and consisted of two treatments, an applied disturbance consisting of a 60 sec. emersion from water (E), or Cortisol immersion (I), applied to individual trays in a 2 X 2 factorial design. One tray received no E and no I, and acted as a control (C), while the remaining three trays received I and no E, E and no I, or E and I, treatments respectively. Before initiation of treatment, water flow was interrupted to each of the hatchery trays. The E treatment was applied every 15 minutes over a period of two hours. Since cortisol quantification required the measurement of whole body cortisol content, as opposed
to plasma cortisol, it was felt that a series of applications of E over time was preferable to a single episode. This would allow for the de novo synthesis of cortisol from precursors, and for the accumulation of cortisol in tissue. The I treatment consisted of the addition of 6 mg of exogenous cortisol (Fisher) suspended in 5 ml of 95% ethanol to the 10 litre hatchery tray, resulting in a final cortisol concentration of 600 ug/L while keeping the concentration of ethanol at 0.05% (Pifferrer and Donaldson, 1992). The non-immersed groups received 5 ml of 95% ethanol alone.

After two hours water flow was re-established to all trays to allow exogenous cortisol to flush from the I treated embryos. Those trays receiving exogenous cortisol were placed below the non-immersed groups to avoid the possibility of contamination, and the two trays containing the groups receiving the E treatment, and the two that did not, were placed in separate adjacent hatchery stacks. Water flow to the two stacks was carefully adjusted and monitored to insure that each tray received water at the same temperature, and at the same rate of flow during the course of the experiment.

The I and E treatments were applied every 5 days from -1 to 24 days post hatch. At each treatment interval one embryo from each replicate was removed from each group after the two hour treatment period and killed by being placed for 15 minutes in an overdose of anaesthetic (MS222, buffered 1:1 with sodium bicarbonate) at a concentration of 200 mg/L.

Embryos were then weighed and measured before being frozen at -50° C for later assessment of whole body cortisol content.
Mortalities were recorded and removed after each treatment interval.

**Tissue Extraction and Cortisol Quantification.**

Embryo tissue was extracted, and whole body cortisol content was quantified, as described in chapter 2.

**Statistical Analysis.**

Significant differences among, and significant interactions between, treatments in regard to whole body cortisol content, length, and weight at each sampling interval; and cumulative mortalities over the 25 day period, were determined by 2 way analysis of variance (ANOVA) (Sigma Stat™, Jandel Scientific). The Student-Newman-Keuls test was utilized to determine significance among means.

The Levene Median and Kolmogorov-Smirnov tests were used to test for equal variance and normality respectively. Data were log$^{10}$ or square root transformed where assumptions of normality and homogeneity of variance were not met.
Results:

At -1 and 4 dph both groups of embryos receiving the I treatment (with and without E) demonstrated mean whole body cortisol contents that were significantly elevated over those observed in embryos that did not receive I (with and without E) (2 way ANOVA, p < 0.05) (Fig. 16).

At 9, 14, 19, and 24 dph those embryos receiving both the E and I treatments demonstrated mean whole body cortisol contents that were significantly higher than those observed in the three remaining groups (2 way ANOVA, p < 0.05).

During these same sampling intervals those embryos receiving the I, without the E, treatments demonstrated mean whole body cortisol contents that were significantly higher than those observed in the groups receiving the E alone, or neither treatment (C) (2 way ANOVA, p < 0.05).

At 14 and 19 dph those embryos that received the E treatment alone demonstrated mean whole body cortisol contents that were significantly higher than those observed in the C embryos (2 way ANOVA, p < 0.05).

There was a significant interaction between the E and I treatments observed on the mean whole body cortisol content of the embryos at the 9, 14, 19, and 24 dph sampling periods. (2 way ANOVA, p < 0.05).

At 4, 9, 14, 19, and 24 dph those embryos in the E and I treatment group demonstrated a mean length that was significantly lower than that observed in any of the other groups (2 way ANOVA,
p < 0.05) (Fig 17). There was no significant differences observed in the mean length of the remaining three treatment groups at any of the sampling intervals (2 way ANOVA, p > 0.05).

There was a significant interaction between the E and I treatments observed on the mean length of the embryos at the 4, 9, 19 and 24 dph sampling periods. (2 way ANOVA, p < 0.05).

At 14, 19 and 24 dph those embryos in the E and I group demonstrated a mean weight that was significantly lower than that observed in the other three treatment groups (2 way ANOVA, p < 0.05) (Fig 18).

At 19 and 24 dph those embryos in the E group demonstrated a mean body weight that was significantly higher than that observed in the E and I group, and significantly lower than that seen in the I and C groups (2 way ANOVA, p < 0.05).

There was a significant interaction between the E and I treatments observed on the mean weight of the embryos at the 9, 14, and 19 dph sampling periods. (2 way ANOVA, p < 0.05)

There was a significantly higher mean mortality rate among those embryos receiving both the E and I treatments (Fig. 19), and there was a significant interaction between the E and I treatments observed on the mean mortality rate (2 way ANOVA, p < 0.05). There was no significant difference in the mortality rate observed among the other three groups (2 way ANOVA, p < 0.05).
Figure 16. Whole body cortisol content of chinook salmon (*Oncorhynchus tshawytscha*), embryos receiving either repeated emersion (E), exposure to exogenous cortisol (I) (600 µg/L for 2 hrs.), both treatments (E+I), or neither treatment (C). Embryos were exposed every 5 days from 1 day before, to 24 days after, hatch. Points represent mean ± SE (n=7). * denotes a significant difference in whole body cortisol content from the control group at that sampling interval (p < 0.05). + denotes a significant differences in whole body cortisol content from the next highest group (excluding controls) (p < 0.05).
Figure 17. Body length of chinook salmon (Oncorhynchus tshawytscha), embryos receiving either repeated emersion (E), exposure to exogenous Cortisol (I) (600 ug/L for 2 hrs.), both treatments (E+I), or neither treatment (C). Embryos were exposed every 5 days from 1 day before, to 24 days after, hatch. Points represent mean ± SE (n=7). * denotes a significant difference in length from the other three groups at that sampling interval (p < 0.05).
Figure 18. Body weight of chinook salmon (*Oncorhynchus tshawytscha*), embryos receiving either repeated emersion (E), exposure to exogenous cortisol (I) (600 µg/L for 2 hrs.), both treatments (E+I), or neither treatment (C). Embryos were exposed every 5 days from 1 day before, to 24 days after, hatch. Points represent mean ± SE (n=7). * denotes a significant difference in weight from the other three groups at that sampling interval (p < 0.05).
Figure 19. Percent Mortality of chinook salmon (Oncorhynchus tshawytscha), embryos receiving either repeated emersion (E), exposure to exogenous cortisol (I) (600 μg/L for 2 hrs.), both treatments (E+I), or neither treatment (C). Embryos were exposed every 5 days from 1 day before, to 24 days after, hatch. Points represent mean ± SE (n=7). * denotes a significant difference in mortality rate from the other three groups (p < 0.05).
Discussion:

My results indicate that exposure of embryonic salmonids to the combination of an environmental disturbance and exogenous cortisol has significant effects on growth and survival.

It appears that by 9 dph onwards there is an interaction between the E and I treatments that results in increased whole body cortisol content, an increase greater than would be expected by the sum of the two treatments alone. It may be that the combination of the two treatments results in heightened sensitivity of some aspect of the HPI axis and potentiated cortisol synthesis. It may also be possible that some aspect of the embryo's response to the applied disturbance altered its permeability to exogenous cortisol. Catecholamines, which increase in the circulation in response to environmental disturbances, have been associated with increased permeability of tissue to water in salmonids (Mazeaud et al., 1977). The onset of this interaction effect coincided with the onset of the cortisol response to emersion observed in coho salmon (Oncorhynchus kisutch), suggesting that this interaction effect coincides with the onset of a functional HPI axis (this thesis, chpt 3).

The significantly higher mortality rate observed among those embryos receiving both the I and E treatments may be partially attributable to their significantly increased levels of whole body cortisol, as compared to the C and singly treated groups. Environmental disturbances in general, and cortisol in particular, have been demonstrated to suppress the immune response in
salmonids, and as such can make them more vulnerable to infectious agents (for review see Barton and Iwama, 1991; Campbell, 1992). Increased mortality due to disease has been associated with increased interrenal response to environmental disturbance. Atlantic salmon (*Salmo salar*) selected for their high cortisol response, indicated by elevated blood cortisol levels in response to stimuli such as crowding, demonstrated significantly higher mortality rates when challenged by bacterial pathogens than did low response fish (Fevolden et al., 1993). Overwinter mortality rates of S2 atlantic salmon parr (2 year fresh water residency) have also been associated with chronically high plasma cortisol titers and reduced circulating lymphocyte numbers (Pickering and Pottinger, 1988). Resistance to pathogens has been observed to decrease in chinook salmon shortly after handling (Maule et al., 1989), and acute environmental disturbance has also been demonstrated to decrease circulating leukocyte numbers in coho salmon (Maule and Schreck, 1990), as well as circulating numbers of lymphocytes in rainbow trout (*Oncorhynchus mykiss*) (Barton et al., 1987; Pottinger and Pickering, 1992).

Similar immune disfunction has been observed in coho salmon treated with exogenous cortisol, with cortisol appearing to be directly, but not exclusively, involved in changes in leukocyte numbers (Maule and Schreck, 1990). Cortisol administered in food, or via implantation, significantly raised plasma cortisol levels while significantly reducing circulating numbers of lymphocytes, and increased mortality due to bacterial and fungal infection, in

It may have been that the increase in whole body cortisol content of the E and I treated embryos caused them to experience a depression of their immune response, and thus rendered them more susceptible to any infectious agents present. The fact that I observed no increase in mortality among the groups receiving the E or I treatment alone suggests that the significantly higher whole body cortisol content observed among the E and I embryos on days 9 through 30 post hatch was above a level at which deleterious effects were incurred, and that the other groups remained below this threshold level.

The significantly lower mean length of those embryos receiving both the E and I treatments at all sampling intervals, coupled with the significantly lower mean weight in these same embryos observed on the final three sampling dates, suggests a significant effect of these combined treatments on the growth of these embryos. Environmental disturbances have been observed to depress growth in salmonids. Brook trout (*Salvelinus fontinalis*) reared at pH 4.5 demonstrated stunted growth and increased plasma cortisol titers (Tam et al., 1988). Fagerlund et al., (1981) reported that increased fish stocking density was associated with decreased growth and increased interrenal cell nuclear diameter in juvenile coho salmon, while Pickering and Stewart (1984) observed a similar suppression of growth rate in chronically crowded brown trout. They also observed a significant transient increase in plasma
cortisol, though they concluded that suppression of growth rate was not mediated by chronic elevation of corticosteroids. This was also the conclusion reached by Vijayan et al., (1990), who observed decreased growth in brook trout raised at high stocking density, but saw no accompanying elevation in plasma cortisol.

Application of exogenous cortisol however, has been linked to reduced growth rate in some teleosts. One year old rainbow trout fed cortisol for 10 weeks showed a decrease in growth and condition factor (Barton et al., 1987). Davis et al., (1985) observed significantly lower body weight and condition factor among yearling channel catfish (Ictalurus punctatus) fed with food containing cortisol, and suggested this as a possible explanation for the decreased growth of fish under conditions that activate the secretion of endogenous cortisol.

My results suggest that application of exogenous cortisol alone does not affect growth early in ontogeny. I did however, see some reduction in the weight of those embryos subjected to E alone, suggesting that other physiological responses of the embryos, other than the cortisol response, to an environmental disturbance can significantly affect growth. The greatest reduction in both weight and length was observed among those embryos receiving both the E and I treatments. This suggests that there is some interaction between the two factors that acts to negatively impact on growth, and indeed, a statistically significant interaction effect was observed on either embryo weight, length, or both, at each sampling interval. It is difficult to ascertain whether this reduction in
growth is due strictly to elevated whole body cortisol content, or whether some other physiological response(s) to emersion are involved. My observation that the E, and not the I, treatment acted to reduce weight seems to indicate the latter as a more likely determining factor.

I suggest, based on the results of this study, that growth and survival among the chinook embryos as a whole was much more negatively impacted by the combination of the E and I treatments, than by either of these factors in isolation. It may be that the elevated whole body cortisol content experienced by the embryos in the E and I group somehow "overloaded" their capacity to deal physiologically with cortisol, and this resulted in reduced growth and immunocompetence. It is difficult to speculate on the ramifications of exposure of salmonids to the combination of both an environmental disturbance and exogenous cortisol as previous studies done on teleosts usually involved the application of either of these treatments in isolation, and not in conjunction (Davis et al., 1985; Barton et al., 1987). My results suggest that, in terms of growth and survival, chinook salmon embryos appear to be relatively resistant to the effects of emersion or elevated whole body cortisol content, and only begin to exhibit negative impacts when severely treated (treatments combined).
CHAPTER 5

GENERAL DISCUSSION
General Discussion

The results of this study suggest that increased circulating cortisol levels, as a result of an environmental disturbance (mechanical agitation), in adult female coho salmon (*Oncorhynchus kisutch*) during the late stages of oogenesis, can lead to an increase in the cortisol content of their oocytes (Chapter 2). The presence of cortisol has been demonstrated in the freshly ovulated or fertilized oocytes of a number of teleosts, including chum salmon (*Oncorhynchus keta*), coho salmon, rainbow trout (*Oncorhynchus mykiss*), Tilapia (*Oreochromis mossambicus*), and Japanese flounder (*Paralichthys olivaces*) (de Jesus and Hirano, 1992; this thesis, chpt 2; Yeoh et al., 1993; Rothbard et al., 1987; de Jesus et al., 1991). The functional significance of cortisol during the early ontogeny of teleosts is undetermined, although there is some evidence for it effecting hatching rate in salmonids (Yeoh, 1993). The current study investigated the flux of cortisol in developing oocytes, and how parameters such as hatch, growth and survival were affected by different levels of oocyte cortisol (Chapter 2). I saw no significant differences between oocytes with normal, and those with artificially elevated cortisol content, and suggest that the rapid decline in oocyte cortisol observed during very early ontogeny (first 7 days) to relatively low levels (<10 ng/gm) among both control and manipulated oocytes, may preclude the involvement of maternally derived cortisol in later developmental processes.
This study also investigated the onset of endogenous cortisol production, as an indication of a functional hypothalamic-pituitary-interrenal (HPI) axis, in the early ontogeny of steelhead and coho salmon (Chapter 3), and the effects exposure to an environmental disturbance and/or exogenous cortisol may have on growth and survival of chinook salmon (Oncorhynchus tshawytscha) during this same period (Chapter 4).

I was able to demonstrate that embryonic steelhead and coho salmon begin to respond to an environmental disturbance with increased synthesis of endogenous cortisol as early as 6 and 9 days post hatch (dph) respectively. Work by other researchers with rainbow trout has indicated that endogenous production of cortisol, in response to stimuli, begins as early as 5 (Pottinger and Mosuwe, 1994) and 2 (Barry et al., 1995) weeks post hatch. The discrepancy between my observations and theirs may be attributed to a number of factors. An elevation in the water temperature at which my embryos were cultured (13° vs 10° C) may have resulted in their developing at an increased rate, and thus exhibiting responsiveness to disturbance earlier. It is also possible that my application of a repeated disturbance over time, as opposed to a single disturbance, may have allowed for the accumulation of cortisol in tissue, and provided a more sensitive measure of this particular aspect of the "stress response".

The time course of the cortisol response to environmental disturbance in coho salmon was similar to that reported in other studies involving juvenile and adult salmonids (Barry et al., 1993;
Barton et al., 1980; Pickering et al., 1982) by day 16 post hatch. At 9 days post hatch whole body cortisol remained elevated 6 hours after the final emersion application, indicating that either the HPI axis was not fully functional at this time and synthesis of cortisol is still ongoing, or that the clearance of cortisol was not as developed or efficient at this early stage in development.

I was able to demonstrate that embryonic chinook salmon exposed to a repeated environmental disturbance coupled with exposure to exogenous cortisol, during the period when endogenous cortisol production has been observed to begin in salmonids, displayed reduced growth and increased mortality. I also observed a significant interaction effect between these two treatments on whole body cortisol content that coincided with the onset of the cortisol response to environmental disturbance observed in coho salmon (Chapter 3). This resulted in significant increases in whole body cortisol content either by potentiation of endogenous cortisol production, or by increased permeability to exogenous cortisol.

I suggest that this increase in whole body cortisol content was at least in part responsible for the increased mortality observed in the doubly treated group, as cortisol has been demonstrated to reduce immunocompetence in salmonids (Pickering et al., 1989; Pickering and Pottinger, 1985; Maule and Schreck, 1990).

The reduced length and weight observed in those chinook embryos receiving both the emersion and cortisol immersion treatment was most probably due to factors other than elevated
cortisol content. This is supported by the fact that I observed no reduction in weight or length in the chinook embryos receiving exogenous cortisol alone, but did see some reduction in weight in embryos receiving the emersion treatment alone. Reduction in growth has been observed in salmonids raised under adverse conditions (high stocking density) without an accompanying rise in plasma cortisol (Vijayan et al., 1990), and Pickering and Stewart (1984) concluded that growth rate suppression in chronically crowded brown trout was not mediated by chronic elevations of plasma cortisol.

The results of this study suggest that circulating levels of cortisol in adult coho salmon during the period of late oogenesis are reflected in the cortisol content of their oocytes, and that relatively high, or low, oocyte cortisol content during early ontogeny (immediately post fertilization) has little effect on subsequent development or survival.

My results also indicate that the response of the HPI axis to environmental disturbances, as evidenced by de novo synthesis of cortisol, is functional after the first week post hatch in salmonids. Activation of this response alone however, does not adversely effect survival and only minimally decreases growth during early development.
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