MEASUREMENT OF PLASMA CORTISOL AND HISTOMETRY
OF THE INTERRENAL GLAND OF JUVENILE, PRE-SMOLT
COHO SALMON (ONCORHYNCHUS KISUTCH WALBAUM) DURING
COLD TEMPERATURE ACCLIMATION

by

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of the Requirements for the Degree
of
Master of Science
In the Department of Zoology

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
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ABSTRACT

Juvenile, pre-smolt coho salmon were subjected to a decrease in environmental temperature from 12°C (temperature of acclimation) to 2°C over a period of approximately 72 hours. During this time, plasma cortisol values were estimated by the competitive protein binding (CPB) technique. In addition, an histometric analysis of the interrenal tissues of these fishes was performed as a measure of interrenal activity.

Experimental results indicated that fluctuations in plasma cortisol concentrations occurred within 120 hours of the initiation of temperature alteration. Control levels for plasma cortisol were $2.9 \pm 0.75$ ug cortisol/100 ml plasma (mean ± S.D.). Maximum plasma cortisol concentration, observed at hour 84 after temperature alteration, was $27.0 \pm 2.8$ ug cortisol/100 ml plasma (mean ± S.E.). By hour 96 experimental cortisol values returned to a level just slightly above those of controls and did not change significantly after that time.

Measurements of interrenal nuclear diameters showed a significant increase in interrenal activity 14 days after initiation of exposure to cold. This level of interrenal activity was maintained until the experiment was terminated (20 days exposure to cold).

It is concluded from this study that during acclimation to cold temperature, plasma cortisol values of juvenile,
pre-smolt coho salmon demonstrate an early and rapid increase (within 120 hours exposure to colder temperature) followed by an equally rapid decrease to a level just slightly above that of controls. Furthermore, it is concluded that juvenile, pre-smolt coho salmon treated in this manner show no histologically demonstrable increase in interrenal activity until well after plasma cortisol values have become stabilized at a level slightly above that of controls (14 days exposure to cold).
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section/Procedure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>List of Figures</td>
<td>v</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vi</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vii</td>
</tr>
<tr>
<td>Introduction</td>
<td>viii</td>
</tr>
</tbody>
</table>

**SECTION I - MATERIALS AND METHODS**

- Experimental Animals ......................... 1
- Description of Tanks ......................... 1
  - Holding Tank - Tank A ...................... 2
  - Control Tank - Tank B ...................... 2
  - Experimental Tank - Tank C ............... 2
- Experimental Design
  - Experiment One ............................ 5
  - Experiment Two ........................... 6
- Autopsy Procedures
- Histological Procedures
  - Histological technique .................... 8
  - Karyometry .................................. 8
- Statistical Procedures
  - Cortisol analysis .......................... 9
  - Histological analysis .................... 10

**Quantification of Plasma Cortisol by Competitive Protein Binding (CPB)**

- Introduction .................................. 11
- CPB - Fagerlund modification
  - Materials ................................. 13
  - Method .................................... 14
- Discussion .................................. 18
SECTION II - EXPERIMENTAL RESULTS

Experiment One ........................................ 21
Experiment Two ........................................ 27
Karyometry .............................................. 31

SECTION III - DISCUSSION ............................... 36

SECTION IV - CONCLUSIONS .............................. 48

SECTION V - REFERENCES ................................. 49
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Holding tank</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Control tank</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Experimental tank showing thermo-regulating controls</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>Apparatus for distribution of nitrogen gas</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>Head kidney of Experiment One coho - Day 0 control fish</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>Head kidney of Experiment One coho - Day 0 experimental fish</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Head kidney of Experiment One coho - Day 14 control fish</td>
<td>23</td>
</tr>
<tr>
<td>8</td>
<td>Head kidney of Experiment One coho - Day 14 experimental fish</td>
<td>23</td>
</tr>
<tr>
<td>9</td>
<td>Plasma cortisol concentration of juvenile coho salmon yearlings during cold temperature acclimation - Experiment One</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td>Plasma cortisol concentration of juvenile coho salmon yearlings during cold temperature acclimation - Experiment Two</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>Activity of the interrenal gland of juvenile coho salmon yearlings during cold temperature acclimation</td>
<td>34</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Values for the proportional standard deviation for duplicated non-chromatographed standard determinations.</td>
<td>18</td>
</tr>
<tr>
<td>II</td>
<td>Values for the proportional standard deviation for duplicate plasma sample determinations.</td>
<td>19</td>
</tr>
<tr>
<td>III</td>
<td>Plasma cortisol concentration of juvenile coho salmon yearling during cold temperature acclimation - Experiment One.</td>
<td>24</td>
</tr>
<tr>
<td>IV</td>
<td>Plasma cortisol concentration of juvenile coho salmon yearling during cold temperature acclimation - Experiment Two</td>
<td>28</td>
</tr>
<tr>
<td>V</td>
<td>Activity of the interrenal gland of juvenile coho salmon yearlings during cold temperature acclimation.</td>
<td>32</td>
</tr>
<tr>
<td>VI</td>
<td>Rates of temperature change for Experiment One and Experiment Two.</td>
<td>43</td>
</tr>
</tbody>
</table>
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INTRODUCTION

This introduction includes: a consideration of the supporting evidence for the homology between teleost interrenal tissue and the mammalian adrenal cortex, a discussion of the teleost pituitary-interrenal axis, a review of the general role of this axis in response to diverse environmental conditions, and a consideration of some of the biochemical events associated with adrenocortical stimulation. In addition, in terms of the response of plasma cortisol in the juvenile coho salmon during cold temperature acclimation, there is present a discussion of the events associated with thermal acclimation.

HOMOLOGY

The interrenal gland of teleost fishes is, in most species, a somewhat diffuse tissue lying along the posterior cardinal veins or their branches within the anterior kidney (Overbeeke, 1960; Nandi, 1962). On the basis of its embryology, histology and biochemistry, numerous studies have established that the interrenal gland is homologous to the mammalian adrenal cortex (Pickford and Atz, 1957; Chester Jones and Phillips, 1960; Bern and Nandi, 1964). Valuable evidence favouring this homology involves histochemical (Chavin and Kovacevic, 1961; Chavin, 1966) and fine-structural (Yamamoto and Onozato, 1965; Ogawa, 1967) studies of interrenal tissue and in vitro incubation studies with labelled precursors of steroid synthesis (Phillips and Mulrow, 1959; Nandi and Bern, 1960; Butler, 1965; Arai et al., 1969).
Incubation of teleostean head-kidneys, as well as direct extraction of this kidney region (Fontaine and Leloup-Hatey, 1959), have demonstrated that the portion of the kidney containing interrenal cells is a source of corticosteroids. Adrenocorticosteroids have been clearly demonstrated and identified in the plasma of teleost fishes (Bondy et al., 1957; Chester Jones and Phillips, 1960; Nandi and Bern, 1965; Chester Jones et al., 1970). Cortisol is shown to be a major corticosteroid in the plasma of salmonids (Hane and Robertson, 1959; Schmidt and Idler, 1962; Sandor et al., 1966, 1967; Donaldson et al., 1968; Sandor, 1969) and plasma cortisol levels have been determined as an index of interrenal activity (Fagerlund, 1967; Donaldson and McBride, 1967; Fagerlund et al., 1968; Fagerlund and McBride, 1969).

PITUITARY-INTERRENAL AXIS

A number of recent investigations provide evidence for a pituitary regulation of the interrenal tissue in teleosts. Following hypophysectomy, atrophy of interrenal tissue occurs (Fontaine and Hatey, 1953; Chavin and Kovacevic, 1961; Basu et al., 1965; Overbeeke and Ahsan, 1966; Donaldson and McBride, 1967). Hypertrophy of the interrenal tissue of teleosts subjected to ACTH treatment has been reported (Rasquin, 1951; Fontaine and Hatey, 1953; Chavin, 1956; Krauter, 1958; Basu et al., 1965; Fagerlund et al., 1968). Evidence for the presence of a corticotrophic hormone in the teleost pituitary has been provided by observations of interrenal hypertrophy following implantation of teleost pituitaries into intact or
hypophysectomized Astyanax mexicanus (Rasquin, 1951) and Carassius auratus (Chavin, 1956). Similarly, Overbeeke and Ahsan (1966) demonstrated that injection of pituitary extracts of Pacific salmon (Oncorhynchus kisutch and O. tshawytscha) into hypophysectomized Couesius plumbeus reverses the atrophy of interrenal tissue. Furthermore, a crude extract of pituitary glands from the Pacific salmon, Oncorhynchus keta has been shown to deplete rat adrenal ascorbic acid in a manner similar to that of mammalian ACTH (Rinfret and Hane, 1955).

AXIS AND ENVIRONMENT

It is well established that the pituitary-adrenocortical axis of mammals is stimulated by numerous, diverse environmental conditions referred to as "stressful" (Gorbman and Bern, 1962). For example, many mammalian species show increased mean adrenal weight when subjected to increased population pressure (Gorbman and Bern, 1962; Christian and Davis, 1964). Other studies report a distinct relation between position in a dominance hierarchy and adrenocortical activity (Louch and Higginbotham, 1967). In addition, it has been observed that merely transferring rats from one cage to another results in a significant rise in plasma cortisol levels (Friedman and Ader, 1967). These reports, and others, indicate the sensitive response of the pituitary-adrenocortical axis
of mammals to environmental alterations.

According to W. S. Hoar (1966): "...although the literature is still meagre, it is probable that the pituitary-adrenal system of the lower vertebrates is activated by stress and that differences between the fishes... and mammals are differences in degree rather than kind..." Effects which might be interpreted in terms of the stress concept have been noted among fishes under a variety of conditions. Marked hyperplasia of the interrenal tissue in Pacific salmon accompanying the spawning migration has been reported by Robertson and Wexler (1959). The adrenal tissue of the goldfish is markedly depleted of glucocorticoids by handling (Chavin and Kovacevic, 1961). Circulating glucocorticoids (Leloup-Hatey, 1958) and catecholamines (Nakano and Thomlinson, 1967) are increased after "stress". Both groups of adrenal hormones, especially catecholamines in very low doses, produce hyperglycemia (Falkmer, 1961; Robertson et al., 1963; Mazeaud, 1965; Young and Chavin, 1965). Thus, a pattern of "stress-induced" hyperglycemia-mediated, in part, by the adrenal hormones is present in teleosts. In addition, it has been reported that holding adult rainbow trout in an aquarium with the water level lowered to 2 to 3 cm resulted in a significant increase in plasma cortisol levels (Donaldson and McBride, 1967). Fagerlund (1967), after studying the effects of handling adult sockeye salmon on plasma cortisol levels, concluded
that the pituitary-adrenal system of adult salmon is highly sensitive to various "stressors" and reacts with a manifold increase in the concentration of cortisol in the peripheral plasma.

From the foregoing, several major points are evident. It is clear that the interrenal gland is homologous to the mammalian adrenal cortex. Secondly, it is established that in teleost fishes a pituitary-interrenal axis exists that operates in a manner similar to the same axis in mammals. Finally, it is suggested that the pituitary-interrenal axis of teleost fishes responds in a sensitive way to a variety of "stressors". These relationships frame the theoretical basis of this thesis.

In the current investigation, the fundamental question being asked is: how does the adrenocortical tissue of the juvenile coho salmon respond to the rigors of cold temperature acclimation? This question is experimentally considered in terms of the correlation between the concentration of a specific circulating glucocorticoid, cortisol, and histologically demonstrable alterations in the interrenal cells of fish undergoing cold temperature acclimation. In answering this question, an important qualification is required, namely, a consideration of the conditions inferred in the terms adaptation, acclimation and "stress response".

**STRESS AND ACCLIMATION**

Fishes, with a few exceptions - tuna (Kishiouye, 1923) and
lamnid sharks (Carey and Teal, 1969) - are poikilothermic. Although their body temperature varies, these animals adjust their physiological state in order to compensate for such temperature changes. This process of acclimation involves predominantly behavioural and metabolic adjustments that require one to two weeks (Fry and Hochachka, 1970), depending upon the intensity of the required adjustment.

In the present study, juvenile coho salmon were subjected to a lowering of the environmental temperature from 12°C (temperature of acclimation) to 2°C. This temperature range is within the zone of thermal tolerance established by Brett (1952) and others.

The adjustments of poikilotherms to a temperature change are multiple and exhibit different response times. The rate of temperature variation in these experiments is too rapid to allow for complete acclimation (approximately 1.7°C every 12 hours); however, the process of acclimation is probably initiated during this time period and may involve circulatory and respiratory adjustments, changing levels of circulating hormones, and others.

These initial changes may involve either physiological variations directed towards completion of the process of acclimation or adjustments to the physiological state resulting from a temperature shift away from the initial temperature of acclimation. Such alterations may be of
the type classified in the initial alarm stage as defined by Selye (1950). These will eventually disappear as acclimation to the new temperature is achieved. Thus, in the study reported here, the experimental conditions could evoke two categories of response; those directed towards acclimation and those concerned with maintaining a stable state in an animal in a non-acclimated condition.

Selye (1950) employs the term "stress" to refer to the type of injurious environmental change which, if sustained, will lead to a somewhat standardized collection of responses referred to as the general adaptation syndrome. This syndrome is viewed as being triphasic; that is, as occurring in three definable stages. The first stage, which occurs immediately upon exposure to an injurious stimulus is termed the alarm stage. This is the response by the organism to a sudden exposure to a stimulus for which it is not adapted. If the organism is capable of withstanding this initial phase it then enters the "stage of resistance" which may continue for a long period of time (weeks or months). When the sustained injurious agent finally begins to have its full effect upon the organism, there is an apparent loss of adaptation, and the third stage, the "phase of exhaustion" occurs, which may result in death.

Gorbman and Bern (1962) suggest that the "stress-concept"
proposed by Selye (1950) has been somewhat maligned. In fact, terms such as stress, stressor, alarm reaction and others have achieved legitimacy they do not deserve. This has resulted in a chronic misuse of the term "stress" and its associated synonyms. As a point of clarification, stress, as applied to this investigation, is to be considered in terms of the Selye general adaptation syndrome. Because the thermal treatment of experimental animals in this study is still within their central zone of thermal tolerance as defined by Fry (1947) and others, the presumption is made that the stage of exhaustion as outlined by Selye (1950) will not be achieved. For this reason, any response by these animals to the environmental parameter of temperature is not, in the classical sense, a stress response. If not a stress response then, what is the basis for the change?

From the point of view of the organism, any alteration of an environmental factor which requires it to expend metabolic energy in order to compensate, is stressful. From the observer's point of view, such a response is distinguishable in terms of the final outcome: if the stimulus is such that the animal achieves the stage of exhaustion (a state that usually results in death) then stress is the cause of death; if the animal survives and is able to compensate then the short-term effect is that of adaptation and the long-term effect is that of acclimation.
BIOCHEMICAL VIEW OF ACCLIMATION TO COLD

A great deal has been written concerning the physiological, biochemical and behavioural adjustments to thermal change by teleosts (for major reviews see Precht, 1968; Fry and Hochachka, 1970). Because of the complexity and diversity of the general literature with regard to the topic of temperature acclimation, this introduction is primarily concerned with a discussion of biochemical changes in animals undergoing acclimation to cold temperature.

Adrenocortical hormones are known to influence mammalian intermediary metabolism, stimulating protein catabolism in extrahepatic tissues, particularly skeletal muscle, and promoting gluconeogenesis with a concomitant increase in blood glucose and liver glycogen levels (Long et al., 1940; Cannon et al., 1956; Bellamy and Leonard, 1964). Evidence for the metabolic role of corticosteroids in fishes, although still scarce, is gradually emerging. Except for minor modifications, teleost fishes and mammals share a similar corticosteroid action (Black et al., 1961; Storer, 1967; Chester Jones et al., 1969). In several species of teleosts, an increase in blood glucose or liver glycogen levels follows the administration of ACTH or corticosteroids (Nace, 1955; Falkmer, 1961; Robertson et al., 1963; Kumer et al., 1966; Oguri and Nace, 1966; Butler, 1968). Butler (1968) reported that hypophysectomy or administration of a metabolic inhibitor
of corticosteroid synthesis caused a significant decrease in liver glycogen levels in *Anquilla rostrata*. Storer (1967) found that administration of cortisol to intact goldfish produced a decrease in body weight, an increase in ammonia secretion and an elevation of liver glycogen phosphatase activity. These studies demonstrate that cortisol-type steroids in teleosts, as in mammals, promote gluconeogenesis.

Plasma corticosteroid levels of salmonids are markedly elevated during the spawning migration (Hane and Robertson, 1959; Robertson *et al*., 1961; Schmidt and Idler, 1962), and are accompanied by an obvious hyperplasia and hypertrophy of the interrenal tissue (Robertson and Wexler, 1959). These changes are associated with a substantial catabolism of parietal muscle protein (Idler and Clemens, 1959; Robertson *et al*., 1961), elevation of liver glycogen (Chang and Idler, 1960) and hyperglycemia (Robertson *et al*., 1961). Similar changes are observed when immature rainbow trout are fed cortisol acetate pellets. This evidence suggests that in some way the circulating glucocorticoids mediate the conversion of protein to carbohydrates, at least in fasting fish during their spawning migration. Furthermore, the inference is clear that the carbohydrate so produced may allow the animal to maintain a more normal metabolism during this highly stressful period.
In general, tissues of cold acclimated fish, compared with the tissues of warm acclimated ones, may be described as follows: the rate of glycolysis is increased up to five-fold (Hochachka and Hayes, 1962; Hochachka, 1967); the participation of the pentose shunt may be increased from a negligible contribution to as much as 10% of the total glucose metabolism, (Hochachka and Hayes, 1962; Hochachka, 1967); depending upon the tissue and species, the Krebs cycle may be decreased, unchanged or possibly increased slightly, whereas the electron transfer functions are characteristically increased (Hochachka and Hayes, 1962; Freed, 1969; Caldwell, 1969); lipogenesis is activated, in some cases by only a small factor (Hochachka and Hayes, 1962; Dean, 1969), but in other cases the activations of synthesis of unique fatty acids may be increased by as much as twelve-fold (Knipprath and Mead, 1968); glycogen synthesis rate is increased (Hochachka and Hayes, 1962); the rate of protein synthesis appears to be generally higher, at least in certain species and tissues (Das and Prosser, 1967; Haschemeyer, 1969); an increase in the rate of synthesis and turnover of nucleic acids (especially RNA) (Das, 1967); and, an alteration of the ionic microenvironment (Heiniche and Houston, 1965).

It is evident that in any given tissue not all of the above processes necessarily occur. Most of them probably
take place in liver, in which metabolic organization is unusually complex. In tissue such as brain, gill and muscle, exergonic reactions are coupled to highly specialized work functions; hence metabolic organization may be abbreviated (Fry and Hochachka, 1970).

A mechanistic model attempting to account for changes of the processes outlined above has been proposed (Hochachka, 1967). This model suggests that metabolic adjustments during cold temperature acclimation depends upon an initial induction of a number of new isozymes which are sufficiently sensitive to control so as to be activated or inhibited by preexisting modulators. Although this model has been successful in satisfying a large amount of data, it has been severely criticized (Lardy et al., 1965; Lardy, 1965) as it does not account for two antagonistic events—gluconeogenesis and lipogenesis—occurring simultaneously.

According to the model proposed by Lardy (1965), glucose metabolism is regulated by glucocorticoids. These glucocorticoids initiate two basic events. The first is to release gluconeogenic precursors, such as free amino acids, from the peripheral tissues into the general circulation (an observation consistent with the data reported by Cannon et al., 1956; Bellamy and Leonard, 1964;
Storer, 1967; and others). These gluconeogenic precursors then activate preexisting gluconeogenic enzymes in the liver, thereby yielding an increased product without measurable synthesis of enzymes. Secondly, glucocorticoids induce de novo synthesis of key gluconeogenic enzymes and thus further activate gluconeogenesis.

In summary, it is evident that cold adapting teleost fishes have imposed upon them a metabolic demand that has to be solved in order for them to survive. During the initial stages of adaptation, the primary response systems function over the short-term to maintain a stable internal state. These responses may involve behavioural adjustments (such as seeking a more favourable environment) or physiological changes (such as alterations in respiration, circulation, hormonal levels and so on). During this early period of adaptation the events as outlined by Lardy (1965) are initiated. These should produce an increase in gluconeogenic precursors, a rise in circulating glucocorticoid concentrations, an increase in liver glycogen and others. The net effect of these metabolic adjustments is that over the long-term the organism becomes acclimated to the new conditions and is capable of surviving.

From the foregoing, it is apparent that cortisol and other glucocorticoids play an important role in the regulation of glucose metabolism during cold temperature acclimation.
Hence, the purposes of this investigation are:

(1) to establish if juvenile coho salmon acclimated to $12^\circ\text{C}$ show a significant increase in plasma cortisol concentration while undergoing acclimation to $2^\circ\text{C}$.

(2) to determine by histometric analysis if a relationship exists between interrenal activity and plasma cortisol concentration under the conditions of acclimation just described.
Material and Methods

Experimental Animals

The experimental animal selected for this investigation was the juvenile coho salmon yearling, Oncorhynchus kisutch. This salmonid, considered a primitive teleost, resides in local streams for a period of one year from the time of emergence from the gravel to the seaward migration. The juvenile form was chosen for its abundance in local streams, its sexual immaturity, its ability to tolerate a wide range of temperatures, and its reported hardy characteristics when maintained under laboratory conditions.

Five hundred coho salmon fry were taken from Bertrand Creek (Langley, B.C.) on September 12, 1970 and transferred to the laboratory where they were placed in a holding tank (Tank A). All fry were fed frozen brine shrimp (Artemia, sp.) daily at 0900 hours. The water temperature of tank A was that of the inflow and varied from 12°C on September 12, 1970 to 7°C on January 11, 1971.

Description of Tanks

Holding tank: tank A

The semi-circular fibre-glass holding tank, 111 cm by 51 cm, had a water depth of 35 cm. This 200 litre tank was illuminated by fluorescent lamps that provided an intensity of 150 lux at the water surface. Light intensity measurements for all tanks were made with a model 200 Photovolt photometre. Tank A was covered with several
sheets of transparent plastic. The water supply consisted of continuously aerated, dechlorinated water. The water temperature was that of the inflow.

Control tank: tank B

The rectangular, stainless steel control tank, 61 by 182 cm, had a water depth of 38 cm and a total volume of 480 litres. The flow rate of the continuously aerated, dechlorinated water was approximately 1.5 litres per minute. Tank B was covered by a retangular box whose dimensions matched the perimeter of the tank itself. This top was 18 cm deep and had enclosed within it three equally spaced fluorescent lamps that provided a light intensity at the water surface of 400 lux.

The control tank was heated by a 1000 watt, flexible, nickel-chromium heating unit (Canlab H1960-1) connected in series with a stepdown transformer (Potter and Brumfield-Model KAl1BY) that regulated a mechanical relay (Potter and Brumfield- Model KA9). This apparatus was capable of maintaining a constant temperature of 12 ± 0.5°C.

Experimental tank: tank C

The dimensions of the experimental tank were similar to those of the control tank, tank B. An effort was made to ensure that a minimum of variation existed between the experimental and control tanks. Such variables as water flow
Figure 1:  Holding tank - tank A

Figure 2:  Control tank, tank B, showing thermosensor and heating unit.

Figure 3:  Experimental, Tank C, tank showing electronic thermoregulating controls and heating unit.

Figure 4:  Apparatus for distribution of nitrogen.
rate, light intensity at the water surface, water depth and size of heating assembly were carefully controlled to make tanks B and C as similar as possible.

The temperature control device employed in tank C consisted of a refrigerator component and a heating unit. The refrigerator unit was set in series with a mechanical relay housed in a thermosensor logic box (Versatherm-Model 2156). This logic box controlled a secondary mercury relay (Ebert Electrical Corp.) capable of accepting a surge amperage of 30 amperes. The response time of this temperature control unit; that is, the time between one unit shutting off and the other being activated was set at 30 seconds and provided an accuracy at 2°C of ± 0.1°C. The heating unit was identical to that in tank B.

Experimental Design

Experiment One

At the beginning of Experiment One, 150 fry were taken from the holding tank and 75 fish were each placed in tanks B and C. These fish were then held for three weeks at 12°C and a twelve hour photoperiod. After this period of acclimation, the fish in the experimental tank were subjected to a gradual change in water temperature, until

1 The lowering of the temperature was spread over four days and was effected in three stages: one, from 12°C to 7°C; two, from 7°C to 4°C and three, from 4°C to 2°C.
the present temperature of $2^\circ$C was achieved. Day 0 was defined as the time of initial temperature alteration. Experiment One was terminated on Day 19. From Day 0 to Day 19, fish were sampled in lots of eight every four days. Control fish were sampled within 30 minutes of the sampling of the experimental animals. Sampling was performed at the same time daily (between 1500 and 1600 hours) to reduce possible diurnal effects. The photoperiod for both control and experimental fish was set at twelve hours.

Experiment Two

As for experiment One, Day 0 represented the time of gradual temperature alteration. The rate of temperature decrease was the same as in Experiment One. Experiment Two was terminated on Day 6. From Day 0 to Day 4, fish were sampled at twelve hour intervals. Sampling was performed at 0800 and 2000 hours respectively. From Day 4 to Day 6, fish were sampled every twenty-four hours in identical fashion. The photoperiod was set at twelve hours. No controls were used.

Autopsy Procedures: Experiment One and Two:

To obtain samples, several fish were lightly netted and placed in a large stacking dish filled with water. An individual fish was then removed and "damped-dried". Wet weight was then measured to the nearest tenth of a gram on a Mettler
balance (Model P1200). The fork length was measured to the nearest millimetre. The caudal peduncle was wiped clean with 95% ethanol, dried and severed with a scalpel. Blood was collected from the caudal vessels into a heparinized microhematocrit tube (Fisher Scientific) and centrifuged at 12,000 RPM for 5 minutes in an Adams Microhematocrit Centrifuge (Clay-Adams Model CT 2900) and the hematocrit determined. The centrifuged blood samples were then placed on dry ice for rapid freezing and subsequently stored in a freezer.

It has been shown by Fagerlund (1967) that simply passing a net over adult sockeye salmon produced a rapid and marked increase in plasma cortisol concentration. In a personal communication he suggested that the response time for an elevated plasma cortisol level initiated by netting would be 10 to 15 minutes. For this reason, special sampling procedures were developed to minimize this effect.

When sampling was begun, eight fish were removed from the tank and placed in a large stacking dish filled with water. This procedure reduced the effects of netting to a minimum as it required only a few seconds. In the subsequent handling of the individual animals, two people were involved. Utilizing this system, the time for processing was shortened to a maximum of 15 minutes. Fish plasmas collected in this
manner showed no systematic variation in plasma cortisol concentration when correlated with the order of processing.

Once the blood sample had been taken, the peritoneal cavity was opened and the sex recorded. The head kidney containing the interrenal tissue was then removed with the surrounding muscle mass and fixed in Bouin's fluid. The total handling time from the netting of the fish to fixation of interrenal tissue was no more than 15 minutes. The interrenal tissue was then taken to another laboratory where the histometric analysis was performed with the assistance of Dr. D. J. McLeay.

Histological Procedures

Histological technique:

Following fixation in Bouin's fluid for one week, the head kidneys were dissected from surrounding tissues, dehydrated in ethanol, cleared in benzene and embedded in wax (Paraplast) in the usual manner. Serial transverse sections were cut at 5 microns. These preparations were then post-chromed in 3% potassium dichromate overnight, followed by staining with Mallory Heidenhain's Azan (Humason, 1962).

Karyometry

From each head kidney, five sections were selected for measurement providing an anterior to posterior cross-section
of the distribution of interrenal cells. Under low power magnification, a cluster of interrenal cells was randomly chosen for measurement of 10 nuclei. Diameters of the interrenal cell nuclei were measured directly with an ocular micrometer at 1000X magnification (oil immersion). By changing the focus of the microscope, a series of cross-sections through the preparation is seen. The area of the largest optical cross-section of each cell nucleus was measured. Since coho interrenal cell nuclei are elliptical, mutually perpendicular diameters (longest and shortest) of each nucleus were measured. A computer program was written that computed the average of these two measurements and converted that average to microns. A total of 50 interrenal nuclei from the head kidney of each fish were measured.

 Statistical Procedures
Cortisol Analysis

Means and standard deviations were calculated for each set of cortisol values at a particular treatment time. A Student's "t" test was employed to determine if a significant difference existed between control and experimental values. Analysis of variance was made to establish if variances between means were statistically more significant than
variances within means. Bartlett's test was applied to determine the probability of variances being homogeneous. Scheffe's test for multiple comparisons with unequal sample sizes was used only when variances were homogeneous (Brownlee, 1965). In addition, regression analysis of Experiment One fish lengths, weights, sex and hematocrit against obtained cortisol values was performed. Means were considered significantly different if $p = 0.05$ and very significantly different if $p = 0.01$.

Histological analysis

As described under methods (Histological Karyometry, p. 8) each interrenal nuclear measurement required the estimate of two mutually perpendicular diameters. A computer program was written that: (1) averaged these two measurements and converted them to micron units; (2) calculated the mean and standard deviations for the 10 nuclei measured in each interrenal clump; (3) calculated a grand mean and standard deviations for the 50 nuclei measured in all the interrenal cells counted in an individual fish; and (4) computed (by means of a Student's "t" test), the significance of difference between nuclear diameters of experimental and control animals at the same treatment time. Graphical presentation of histological data utilized grand mean and standard deviations. Means were considered to be
significantly different if $p = 0.05$, and very significantly different if $p = 0.01$. In addition, analysis of variance was calculated for experimental and control treatment. Bartlett's test was applied and if acceptable, a Scheffe's multiple comparison of unequal sample sizes was used.

Quantification of Plasma Cortisol by Competitive Protein Binding

Introduction

Many substances in blood are bound to plasma proteins and in some cases this binding is quite specific and shows a high degree of binding between the ligand and the protein (Berson and Yalow, 1957; Hunter and Greenwood, 1962; Murphy, 1964). Radioimmunoassay of these protein-bound ligands have been developed and include those for the determination of serum insulin (Berson and Yalow, 1957), serum glucagon (Barakat and Ekins, 1961) and cortisol and cortisone (Fagerlund, 1970). Most probably, similar types of ligand-protein binding exists between many other substances and their carrier proteins.

The radioimmunoassay procedures designed to determine the presence of these complexes is currently referred to as competitive protein binding (CPB). In order for this method to be utilized, two basic conditions must be met.
The first is the availability of a ligand-specific protein. The term "ligand-specific" protein refers to a protein that binds only one ligand or a small group of chemically similar ligands. The second major consideration is that a dynamic equilibrium exists between the ligand and its carrier protein. This requirement infers that the interactions of the ligand with its carrier protein follows stoichiometric laws.

Principle of analysis by CPB

If a radioactively labelled form of a substance, \( S^* \), is added to a plasma containing an unlabelled, \( S \), and limited amounts of its specific binding protein, \( P \); and, if a dynamic equilibrium exists between \( S \) and \( P \), then \( S^* \) will distribute itself evenly among the unlabelled \( S \). If the binding affinity between \( S \) and \( P \) is very high, virtually all the \( S^* \) added will be bound until the \( P \) is saturated. At equilibrium,

\[
\frac{SP + S^*P}{\sum (S + S^*)} \quad \text{will equal} \quad \frac{S^* P}{S^*} \quad \text{and} \quad \frac{SP}{S}.
\]

If further \( S \) is added it will also compete for the same binding sites so that the \( S^*P \) will be reduced. From this dynamic interaction, the percentage bound \( S^* \) can be plotted against the total \( S \) by simply increasing or decreasing the amount of \( S \). Such a plot, for a given set of conditions,
produces a standard curve. If, instead of pure, unlabelled S, a sample of plasma from which all the P has been removed and which contains an unknown amount of S is added to the same system, it may be quantified according to the fall in S*P it causes.

Competitive Protein Binding-Fagerlund Modification
(Fagerlund, 1970)

Materials
Thin layer sheets were Eastman silica gel Chromagrams 6060 which were used untreated.

Cortisol-1,2-³H, specific activity 45.0 Ci/mM were supplied by New England Nuclear Corporation, Chicago Illinois, and were used without purification. Purity was checked from time to time in the thin layer chromatography (TLC) system used in this method.

Standards of hydrocortisone (cortisol) (BDH Laboratory Chemicals-0487320) were made up separately into aqueous solutions by dilution of a stock standard containing 40 nanograms of steroid per millilitre.

Chick serum was obtained from the Winley-Morris Company, Montreal, P. Q. The serum was mixed with enough tritiated cortisol to give an activity of 125,000 to 250,000 counts per minute per millilitre and diluted with water to make a 4% stock solution that can be kept for several weeks if refrigerated.
Florisil, 60-100 mesh (Fisher Scientific) was screened by Endecott's test mesh (Fisher Scientific- 80 mesh) to obtain a particle range of 60-80 mesh, which gave a smaller deviation between duplicate determinations than the untreated material (Fagerlund, 1970). The Florisil was dispensed by means of a specially designed measurer made by drilling a Teflon stopcock from a 250 ml separatory funnel and calibrating the hole so drilled. Ten measurements with one such device gave the mean and standard deviation of 41.1 ± 0.43 mg.

All solvents were reagent grade and were distilled slowly through a distillation reflux apparatus. From each gallon distilled a 100 ml forefraction and endfraction was discarded.

Method

Plasma was obtained by thawing the heparinized tubes at room temperature and breaking the tubes just above the level of the compacted red blood cells. The plasma was collected by capillary action by means of a Lang-Levy pipette. The plasma was then placed in a 12 ml, glass-stoppered, conical centrifuge tube (Fisher Scientific) and diluted with 1 ml of distilled water dispensed from a 1 ml volumetric pipette (tolerance ± 0.006 ml). To this was added 8 ml of methylene chloride. Because of the volatility of this reagent, a special delivery syringe was utilized (Luer-Lock- 5 ml syringe; 5 ml metal pipetting holder- Becton, Dickinson and Company, Rutherford, N.J.).
The tubes were then manually shaken for 30 seconds and centrifuged at 2000 RPM in an International Clinical Centrifuge (Model CL) for 2 minutes. The aqueous phase was then removed by aspiration.

Duplicate, 2 ml aliquots, were then removed by means of a specially adapted volumetric pipette (Fisher Scientific-Pipette adaptor 13-682) and evaporated in centrifuge tubes. The evaporation step occurred in a 45°C water bath under nitrogen (Figure 4).

The evaporated extracts were then chromatographed in methylene chloride- methanol- water (150: 9.0: 0.5) (Quesenberry et al., 1965) on TLC sheets cut in half and scored in channels 1 cm in width. The evaporated extracts were transferred to the chromatogram by rinsing four times with two drops of a mixture of methanol and methylene chloride (10% methanol by volume) to points 1 cm apart along a baseline drawn across the longer dimension of the sheet. Cortisol and cortisone markers were applied at the edges and the centre of the chromatogram. Development time is 10 to 15 minutes and produces a solvent front approximately 8 cm from the baseline. The markers were located by ultra-violet light (Ultra-violet Products Inc., San Gabriel, Calif.). The corresponding regions containing the cortisol fraction were then cut into squares, 1 cm by 1 cm, and dropped directly in centrifuge tubes containing 1 ml of distilled water. At this stage, the samples can be stored overnight if refrigerated.
One millilitre of the stock serum solution containing the cortisol binding globulin (CBG) and the tritiated cortisol was added to each tube, giving a final serum concentration of 2%. The tubes were covered with aluminum foil and warmed for 5 minutes in a 45°C water bath to remove any endogenous steroid from the CBG. The tubes were cooled for 2 minutes under a cold water tap and transferred to a cold room (4°C) and allowed three hours to equilibrate to that temperature. All remaining steps were carried out in the 4°C cold room.

After a minimum of two hours cooling, 80 mg of Florisil was added to each tube, which were then stoppered. The tube contents were mixed by simultaneously inverting all tubes. This was accomplished by means of a plywood box padded with foam rubber and pressed against the tube tops. The tube contents were allowed 30 minutes to settle; then 1 ml of the supernatant from each tube was added to 10 ml of Bray's fluid (Bray, 1960) and the radioactivity monitored on a Nuclear Chicago scintillator counter.

For the purposes of determining mean recovery and concentration of unknowns, two kinds of standards are used: a non-chromatographed standard from which the standard curve is made and a chromatographed standard from which mean recovery is determined. For the former, usually three separate standards are sufficient to plot a standard curve. The standards selected
are chosen so as to cover the anticipated range of plasma cortisol concentrations. Each of these standards is determined in the following manner: a volume of standard equal to the volume of unknown used is diluted to 1 ml with distilled water. To this is added 1 ml of the CBG solution and the analysis continues from this point in the same way as for the plasma cortisol determinations. To determine recovery, a volume of standard, equal to the product of the dilution factor and the non-chromatographed standard in concentration, is added to 1 ml of distilled water and subjected to the same procedures as are the unknowns.¹

The amount of cortisol (ug/100 ml plasma) is obtained by determining the counts per minute for the unknown plasma samples and plotting this value on the standard curve. The value is then adjusted as indicated by the mean recovery of the chromatographed standard and multiplied by the dilution factor (in this procedure the dilution factor is 4) to give the number of micrograms per 100 ml of plasma.

¹ The concentration of the chromatographed standard used depends upon the dilution factor for the plasma samples. For example, if the dilution factor is 5, the concentration of the chromatographed standard would be 10 ug/100 ml for estimating the recovery of the 2 ug/100 ml non-chromatographed standard.
Discussion of CPB Technique - Fagerlund Modification

The rationale for the extraction and purification procedures are considered by Fagerlund (1970).

As reported, duplicate samples are prepared for both non-chromatographed standards and chromatographed plasma samples. The values for the proportional standard deviation, S, (Bundy et al., 1957) of duplicate determinations of standards and plasma samples are shown (Tables I and II respectively.

\[
S = \sqrt{\frac{\sum \alpha^2}{2N}} \quad \text{and} \quad \alpha = \frac{(x_1 - x_2)}{x_1 + x_2}
\]

where \(n\) is the number of duplicates and \(x_1\) and \(x_2\) are the results of duplicate determinations.

### TABLE I
VALUES FOR THE PROPORTIONAL STANDARD DEVIATION FOR DUPLICATED NON-CHROMATOGRAPHED STANDARD DETERMINATIONS

<table>
<thead>
<tr>
<th>Concentration of Standard</th>
<th>Standard Deviation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment I</td>
</tr>
<tr>
<td>0</td>
<td>0.046</td>
</tr>
<tr>
<td>1</td>
<td>0.024</td>
</tr>
<tr>
<td>2</td>
<td>0.005</td>
</tr>
<tr>
<td>3</td>
<td>0.062</td>
</tr>
<tr>
<td>4</td>
<td>0.009</td>
</tr>
<tr>
<td>6</td>
<td>0.175</td>
</tr>
<tr>
<td>8</td>
<td>0.012</td>
</tr>
</tbody>
</table>

* Values for the standard deviation, S, were obtained with the formula:

\[
S = \sqrt{\frac{\sum \alpha^2}{2N}} \quad \text{and} \quad \alpha = \frac{(x_1 - x_2)}{x_1 + x_2}
\]
VALUES FOR THE PROPORTIONAL STANDARD DEVIATION FOR DUPLICATE PLASMA SAMPLE DETERMINATIONS

<table>
<thead>
<tr>
<th>Range of Concentrations (ng/100 ml)</th>
<th>0.0-2.5</th>
<th>2.6-5.0</th>
<th>5.1-10.0</th>
<th>10.1-40.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Deviation</td>
<td>0.61</td>
<td>0.37</td>
<td>0.27</td>
<td>0.25</td>
</tr>
<tr>
<td>Number of Duplicates</td>
<td>17</td>
<td>11</td>
<td>15</td>
<td>11</td>
</tr>
</tbody>
</table>

* Standard deviations calculations are based on a combination of Experiment I and Experiment II data.

The proportional standard deviation values reported compare favourably with similar values published by Fagerlund (1970) with respect to non-chromatographed standard calculations (Table I). However, Table II values are considerably higher, especially in the lower concentration ranges. The observation that the variance among duplicate values decreases as the concentration of plasma cortisol increases is supported by the fact that the percentage recovery of chromatographed standards improves as the concentration of that standard is increased. When greater accuracy is required in the low concentration range than is obtained (SD 61% and 37%) it is of advantage to take a larger aliquot of methylene chloride extract for chromatography.
or to use a larger amount of plasma diluted with a proportionately smaller amount of distilled water. This was not done for two reasons: one, the anticipated order of response was within the range of greater accuracy (SD 27% and 25%); and two, the utilization of larger plasma volumes would require the pooling of a greater number of fish plasmas, thus reducing the sensitivity of the experimental design.

The recovery of extracted and chromatographed standards when calculated from seven determinations of triplicate 2 ug/100 ml standards is 85.2 ± 10.5% and shows a mean and standard deviation of 1.89 ± 0.64 ug/100 ml plasma. These values are close to the data reported by Fagerlund (1970), for the recovery of chromatographed standards, of 87.4 ± 10.2%

As mentioned, the solvent system used in this technique was that developed by Quesenberry et al. (1965). This system produced highly reproducible $R_f$ values for cortisol and cortisone. The $R_f$ calculations showed a mean and standard deviation of 0.228 ± 0.022 and 0.415 ± 0.026 for cortisol and cortisone respectively.

In general, the technique of competitive protein binding, under the conditions of this procedure, is quantitatively suspect in the 0 to 5 ug range. However, I am confident that the system used has established an order of relative cortisol values. Because the order of response is high (see Experimental Results, p.30), these relative values take on considerable experimental significance.
Experimental Results

Experiment One

Experiment One was designed to determine if a significant change in plasma cortisol concentration occurs during cold temperature acclimation by juvenile coho salmon. Table III data indicates that such a response took place on Day 4 ($p = 0.05$). The mean and standard deviation for cortisol measurements determined from Day 4 plasmas were $1.8 \pm 1.00$ and $10.2 \pm 3.40$ ug cortisol per 100 ml plasma for control and experimental animals respectively. As shown, (Table III), this was the only measurement during the time course of Experiment One that a significant difference between control and experimental cortisol values was obtained.

Analysis of variances of cortisol values used to determine the mean cortisol value for each treatment time indicated a significant difference ($p \leq 0.05$) between treatment times for both control and experimental groups. Variances within each treatment mean were shown to be non-homogeneous by Bartlett's test. For this reason, the Scheffe's multiple comparison test was not employed. Probability values for $p \leq 0.05$ were considered to be significant and for $p \leq 0.01$ were considered to be very significant.
Figure 5: Head kidney of Day 0 control coho. Clusters of interrenal cells (IC) are distributed irregularly throughout the hematopoietic tissue (HT).
Azan. x 120

Figure 6: Head kidney of Day 0 experimental coho. Comparison with control fish (Figure 5) indicates approximately the same distribution of interrenal cell clusters (IC).
Azan. x 120

Figure 7: Head kidney of Day 14 control coho. Distribution of interrenal clumps is similar to that of Figure 5 and 6.
Azan. x 120

Figure 8: Head kidney of Day 14 experimental coho. Note increase in number of interrenal cell clumps (suggesting hyperplasia) relative to controls
Azan x 120
TABLE III
PLASMA CORTISOL CONCENTRATION OF
JUVENILE COHO SALMON YEARLINGS
DURING COLD TEMPERATURE ACCLIMATION
(EXPERIMENT ONE)

<table>
<thead>
<tr>
<th>Day</th>
<th>Controls</th>
<th>Exp.</th>
<th>Controls</th>
<th>Exp.</th>
<th>t Value</th>
<th>t Prob. Value</th>
<th>Plasma Cortisol Concentration (ug/100 ml Plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Controls Mean ± S.D.</td>
</tr>
<tr>
<td>0</td>
<td>12</td>
<td></td>
<td>12</td>
<td></td>
<td>2(7)</td>
<td>2.33</td>
<td>0.10</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td></td>
<td>2</td>
<td></td>
<td>4(9)</td>
<td>-4.78</td>
<td>0.005**</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td></td>
<td>2</td>
<td></td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>12</td>
<td></td>
<td>2</td>
<td></td>
<td>2(3)</td>
<td>0.99</td>
<td>0.50</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td></td>
<td>2</td>
<td></td>
<td>4(8)</td>
<td>-1.42</td>
<td>0.20</td>
</tr>
</tbody>
</table>

* Numbers in parenthesis represent the number of fish plasmas pooled for each set of determinations.

** Statistically significant at p = 0.01 level.
Changes in plasma cortisol concentrations of juvenile coho salmon yearlings during cold temperature acclimation (Experiment One).

* Plasma cortisol values plotted are mean ± standard deviation.

** Abscissa of plasma cortisol curve is common to that of temperature plot.

*** Day 4 represents time of significant cortisol difference between controls and experimental animals.
FIGURE 9

Plasma Cortisol (µg/100 ml) vs Number of Days

- **Control** (12°C) vs **Experimental** (2°C)

- 0, 1, 2, 3, 4, 8, 14, 20 days

- Data points showing cortisol levels for each condition over time.
Experiment Two

Experiment Two was designed to investigate in a more continuous manner the cortisol response shown by Experiment One. It is evident from the graphical presentation of Experiment Two data (Figure 10) that the fluctuations of plasma cortisol under the conditions of this experiment can be broadly classified into three major orders of response: a minor increase, from hour 24 to hour 36; a major plasma cortisol elevation, from hour 72 to hour 84; and, relatively constant pre-response and post-response values.

The pre-response and post-response levels for plasma cortisol have a mean and standard error of 5.8 ± 1.4 ug cortisol per 100 ml plasma. The calculation of this mean is based upon values for plasma cortisol at hour 0, hour 12, hour 48, hour 96, hour 120 and hour 144. The mean calculated for pre-response and post-response cortisol values was higher than the plasma cortisol measurements from analysis of Experiment One controls (1.7 ± 0.85 ug cortisol per 100 ml plasma).

The region described as a minor response showed a mean and standard error of 11.6 ± 2.2 ug cortisol and the region described as a major increase demonstrated a mean and standard error of 27.0 ± 2.8 ug cortisol per 100 ml plasma.

Figure 10 suggests that a relationship exists between the timing of the increase in plasma cortisol and the changes
TABLE IV
PLASMA CORTISOL CONCENTRATION OF JUVENILE COHO SALMON YEARLINGS DURING COLD TEMPERATURE ACCLIMATION (EXPERIMENT TWO)

<table>
<thead>
<tr>
<th>Hours</th>
<th>Water Temp. (°C)</th>
<th>Number of Cortisol Determinations*</th>
<th>Plasma Cortisol Concentration (µg/100 ml Plasma)</th>
<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>5 (8)</td>
<td></td>
<td>7.2 ± 0.9</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>3 (5)</td>
<td></td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>24</td>
<td>7</td>
<td>2 (4)</td>
<td></td>
<td>11.4 ± 1.6</td>
</tr>
<tr>
<td>36</td>
<td>7</td>
<td>4 (6)</td>
<td></td>
<td>11.6 ± 5.2</td>
</tr>
<tr>
<td>48</td>
<td>4</td>
<td>2 (4)</td>
<td></td>
<td>5.5 ± 1.3</td>
</tr>
<tr>
<td>60</td>
<td>2</td>
<td>3 (6)</td>
<td></td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>72</td>
<td>2</td>
<td>3 (7)</td>
<td></td>
<td>23.8 ± 6.4</td>
</tr>
<tr>
<td>84</td>
<td>2</td>
<td>4 (7)</td>
<td></td>
<td>27.0 ± 2.8</td>
</tr>
<tr>
<td>96</td>
<td>2</td>
<td>4 (8)</td>
<td></td>
<td>6.9 ± 3.1</td>
</tr>
<tr>
<td>120</td>
<td>2</td>
<td>2 (5)</td>
<td></td>
<td>7.2 ± 0.0</td>
</tr>
<tr>
<td>144</td>
<td>2</td>
<td>3 (7)</td>
<td></td>
<td>3.8 ± 3.0</td>
</tr>
</tbody>
</table>

* Numbers in parenthesis represent the number of fish plasmas pooled for each set of determinations.
Changes in plasma cortisol concentration of juvenile coho salmon yearlings during cold temperature acclimation (Experiment Two).

* Values plotted for plasma cortisol are mean ± standard error.

** Abscissa of plasma cortisol curve is common to that of temperature plot.
in water temperature. The minor increase in plasma cortisol, from hour 24 to hour 36, takes place immediately after the environmental water temperature has stabilized at 7°C. Similarly, the major increase in plasma cortisol, from hour 72 to hour 84, occurs immediately after the water temperature reaches 2°C. In addition, Figure 10 indicates that in each instance a significant increase in plasma cortisol occurs, that concentration is maintained for approximately 12 hours and then falls quickly (within 12 hours) to the pre-response level.

Analysis of variances of cortisol values used to determine the mean cortisol measurement for each treatment time indicated a significant difference (p ≤ 0.01) between different treatment times. Variances within each treatment mean were shown to be non-homogeneous by Bartlett's test. Because no controls were kept the Student's t test could not be utilized. Analysis of variances were considered to be significant if p ≤ 0.05 and very significant if p ≤ 0.01.

Karyometry

Measurement of hypertrophy of interrenal nuclei was performed on Experiment One animals only. Table V indicates that control and experimental animals show significantly different values for mean interrenal nuclear diameters only on Day 14 (p = 0.006). Levels of significance were determined by a Student's t test. Probabilities were considered significant if p ≤ 0.05 and very significant
# TABLE V

**ACTIVITY OF THE INTERRENAL GLAND OF THE JUVENILE COHO SALMON (ONCORHYNCHUS KISUTCH) DURING COLD-TEMPERATURE ACCLIMATION**

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of Fish</th>
<th>T Value</th>
<th>T Prob.</th>
<th>Controls Mean ± S.D.**</th>
<th>Experimental Mean ± S.D.**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>0.78</td>
<td>0.484</td>
<td>6.98 ± 0.18</td>
<td>6.87 ± 0.23</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>-0.62</td>
<td>0.587</td>
<td>6.70 ± 0.16</td>
<td>6.83 ± 0.27</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>6.78 ± 0.14</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>-5.75</td>
<td>0.006*</td>
<td>6.86 ± 0.16</td>
<td>7.51 ± 0.16</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>-1.16</td>
<td>0.315</td>
<td>6.97 ± 0.39</td>
<td>7.26 ± 0.31</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>-0.38</td>
<td>0.717</td>
<td>7.07 ± 0.29</td>
<td>7.44 ± 0.22</td>
</tr>
</tbody>
</table>

* Statistically significant at p = 0.01 level

** Mean values for each fish are based on a measurement of ten nuclei from each of at least four interrenal clumps. The grand mean presented above represents the mean value for each group of five fish.
Figure 11

Activity of interrenal gland of juvenile coho salmon yearlings during cold temperature acclimation.

* Interrenal nuclear diameters are presented as mean ± standard deviation.
FIGURE 11

ACTIVITY OF INTERRENNAL GLAND OF JUVENILE COHO SALMON YEARLINGS DURING COLD—TEMPERATURE ACCLIMATION

Control  \( (12^\circ C) \)

Experiment.  \( (2^\circ C) \)
if \( p \leq 0.01 \). Figure II clearly demonstrates the increase in interrenal tissue of Day 14 experimental fish relative to Day 14 and Day 0 controls. Although the extent of hyperplasia was not quantified, examination of Figure 8 suggests that considerable hyperplasia of interrenal nuclei occurred in Day 14 experimental fish.

Analysis of variances of the individual measurements determining the mean for each treatment time indicated a significant difference \((p \leq 0.01)\) between treatment times. Variances within each treatment time were shown to be homogeneous by Bartlett's test. Scheffe's test for multiple comparisons was utilized for control and experimental data analysis. The results of Scheffe's tests indicated that the Day 14 treatment mean was significantly different \((p \leq 0.001)\) from all other experimental treatment means.
DISCUSSION

Straw and Fregly (1967) have reported that rats exposed to cold temperatures over a long period of time demonstrate an increase in adrenocortical hormone concentrations that reached a maximum after 7 days exposure to cold. It is also shown by these authors that adrenal weights, considered an index of adrenal activity, reached a maximum after 14 days in the cold. This kind of response is similar to that obtained in the karyometric analysis in the present investigation, in which a significant increase between control and experimental interrenal nuclear diameters was noted on fish exposed to cold for 14 days, whereas maximum plasma cortisol values were obtained before 6 days of exposure to cold. Hypertrophy of interrenal nuclei is generally considered an index of increased interrenal activity (McLeay, 1970) and others. Straw and Fregly (1967) suggest that in rats such a result is not surprising. The basis for this position is that adrenal size is most probably due to an average daily secretion rate of ACTH over a long period of time rather than a series of sudden spikes in ACTH release. As a result, the lag in the change of adrenal size relative to much earlier increases in plasma adrenocortical hormone levels is understandable. Although
no direct evidence, with respect to salmonids, is available in support of this interpretation, the basic tenet is in agreement with the work of Dear and Guillemin (1960) who demonstrated that hypophysectomized rat adrenals continued to decline in weight for as long as 28 days after hypophysectomy.

The evidence is clear that the pituitary-interrenal axis of salmonids functions by means of a negative feedback system (Donaldson and McBride, 1967). Hypophysectomized adult trout demonstrate a rapid decrease in plasma cortisol within 24 hours of hypophysectomy (Donaldson and McBride, 1967). This is thought to be due to a rapid turnover of endogenous ACTH. Figure 10 of the present investigation indicates a rapid increase and equally rapid decline in plasma cortisol concentration for each region of significant response. These data may be interpretable in terms of the synthesis, release and apparently rapid turnover of endogenous ACTH. In addition, Fagerlund (1969) and others have shown that endogenous cortisol is rapidly converted to cortisone, although the reverse does not appear to be true. By combining these two pieces of information an interpretation of the results shown in Figure 10 as to the rate of change of plasma cortisol levels is possible. Initially, as the animal responds to the metabolic demands of cold acclimation, the plasma cortisol concentration, mediated by adrenocorticotrophin, increase sharply. As the cortisol level rises, the synthesis
and release of ACTH is inhibited, resulting in a decrease in endogenous adrenocorticotrophin and subsequently in a reduction in the interrenal production and release of glucocorticoids. In addition, the conversion of cortisol to cortisone and the normal clearance of these two hormones through kidney action causes a rapid decrease in the levels of these substances. Thus, the rates of fluctuations described by Figure 10 are possible due to negative feedback, hormone interconversion and excretion. This interpretation, however, does not explain the intensity and timing of the changes in cortisol concentrations, but merely suggests why rapid elevations are followed by equally rapid declines.

The data presented in Figures 9 and 10 indicate that cold-acclimating juvenile coho salmon demonstrate a short-term increase in plasma cortisol concentration. The timing of this increase appears to be within 96 hours of the time of temperature alteration. The rise in cortisol subsequent to temperature change is suggested, by this data, to be a relatively rapid rise followed by an equally rapid decline to a level just slightly above that of controls (Figures 9 and 10; Tables III and IV).

Such a response by juvenile coho salmon is complementary to the model proposed by Lardy (1965) and Lardy et al., (1965). According to their thesis, glucocorticoids initiate two basic events during cold acclimation. The first of these
is to release gluconeogenic precursors that activate preexisting gluconeogenic enzymes in the liver, thereby yielding an increase in liver glucose synthesis. In addition, these same glucocorticoids induce \textit{de novo} synthesis of key gluconeogenic enzymes which also results in a net increase in glucose synthesis.

The levels of plasma corticosteroids and their concomitant effects upon teleost fishes is documented, although somewhat sparsely. Responses by teleosts to increase in plasma cortisol have been shown to include obvious interrenal hyperplasia (Robertson and Wexler, 1959), catabolism of parietal muscle protein (Robertson \textit{et al.}, 1961), elevation of liver glycogen (Chang and Idler, 1960) and hyperglycemia (Robertson \textit{et al.}, 1961). These data are also broadly supportive of the model presented by Lardy (1965) of the possible role of glucocorticoids in the regulation of glucose metabolism during cold temperature acclimation. In addition, a number of authors have presented information that suggests that during cold temperature acclimation several of the intermediary metabolic functions undergo major reorganization (see "Introduction to Results" pp. XVIII to XXI). These changes include an increase in glycolysis, an increase in the events associated with the oxidative electron transfer system and an increase in glycogen synthesis. Although
the situation in fact is far more complicated than that reported here, it nonetheless appears that glucose oxidation increases during cold temperature acclimation with a subsequent rise in the availability of high-energy phosphorylated compounds.

If it is acceptable that during cold temperature acclimation, adjustments in glucose metabolism as outlined above actually occur, and that these changes are mediated in part by circulating glucocorticoids, then it logically follows to ask: of what benefit to the organism are these changes?

As far as this author is aware, no precise answer to the question is available. It is, however, reasonable to expect that an organism undergoing acclimation to a new environmental parameter would require some form of metabolic readjustment. Fundamentally, these changes might take the form of energy-requiring isozymic transformations (Hochachka, 1967; Fry and Hochachka, 1970) although supportive evidence is somewhat confused in this regard. Suffice it to say that the experimental evidence collected to date strongly suggests that these changes are energy requiring, although why this is so is not at all clear.

An important qualification in considering the effects of glucocorticoids on the rearrangement of glucose metabolism of salmonids is the fact that the bulk of evidence reported to date concerns adult or migrating adult fish. In the latter instance, conversion of body protein to more
immediately utilizable energy forms has obvious short-term energy advantages. However, in the current investigation, the juvenile coho salmon used were in a post-fingerling to an immediate pre-smolt stage—a stage where a major concern of the animal's metabolism is that of promoting growth. Fagerlund (1971) reported that juvenile coho salmon exposed to changes in ambient water temperatures—from 10°C in September, 1970 to 1°C in January, 1971—showed a relatively high plasma cortisol concentration when compared with post-smolt juveniles. In addition, Fagerlund (1971) demonstrated that the values for plasma cortisol changed little over the period of assay. The tentative conclusions, based on this evidence, is that growth is a more important factor in determining plasma cortisol levels in pre-smolt juvenile coho salmon than is acclimation to a lower water temperature. McLeay (1970) reported different findings. In his studies, it was observed that juvenile pre-smolt coho salmon sampled in winter showed greater interrenal activity than did fingerling coho salmon, sampled from the same population, in the previous summer. From these data, McLeay (1970) suggests that interrenal activity varies inversely with the ambient water temperature although the conclusion that plasma cortisol levels follow the same pattern was only inferred by that author.
In the present investigation, it is noted (Table VI) that the rate of change of water temperature from the temperature of acclimation (12°C) to 2°C is approximately 0.14 and 0.17 °C per hour for Experiments One and Two respectively. The differences in cooling rates were due primarily to variations in the temperature of the inflowing water. These rates of change are considerably more intense than that found in the natural environment. Because of this, it is possible that the results of this investigation more clearly reflect the response of pre-smolt juvenile coho salmon to a change in water temperature than would the designs as presented by McLeay (1970) and Fagerlund (1971), where factors such as predation, photoperiod, availability of food and others would come into effect.

It is important to note, in interpreting the results of Experiments One and Two, that the variances within each treatment mean are of an order that makes it statistically doubtful whether the reported intensities of responses are significant. It would appear that fluctuations in plasma cortisol, as reported for these experiments, should be thought of as an order of response rather than a precisely quantified level of plasma cortisol concentrations. Analysis of variances of treatment means for the karyometric data are of a level that allows for a more confident interpretation in this respect.
TABLE VI

RATES OF TEMPERATURE CHANGE IN
EXPERIMENT ONE AND EXPERIMENT TWO

<table>
<thead>
<tr>
<th>Temperature Interval (°C)</th>
<th>Time Interval (Hours)</th>
<th>Rate of Change * (°C/Hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. I</td>
<td>Exp. II</td>
</tr>
<tr>
<td>12.0-8.0</td>
<td>12.0-7.0</td>
<td>24</td>
</tr>
<tr>
<td>8.0-4.0</td>
<td>7.0-4.0</td>
<td>36</td>
</tr>
<tr>
<td>4.0-2.0</td>
<td>4.0-2.0</td>
<td>12</td>
</tr>
</tbody>
</table>

* The overall rate of change (expressed as °C/Hours) is 0.14 and 0.17 for Experiments I and II respectively.
In the opinion of this investigator, the major reason for the nonhomogeneity of variances within treatment means for Experiment One and Experiment Two is sample size. As outlined in "Material and Methods" (pp 7-8) the sample size used in these experiments was limited to a maximum of 8 animals. This restriction was intended to reduce the possibility of a handling-induced increase in plasma cortisol which was shown in a preliminary test to require 10 to 15 minutes before such a response became significant. Another possible reason for variations within treatment means may be the result of the limitations imposed by the time required between taking sets of samples. Fagerlund (1967) demonstrated that increases in plasma cortisol, resulting from the movement of a net over the heads of adult salmon, require approximately 12 hours to return to pre-netting levels. Because of this, individual variations of true plasma cortisol concentrations may be large for Experiment Two results; that is, differences in the rate of return of cortisol values to normal, among individual animals, may be great. In addition, a very plausible explanation for the divergency of measurements within each treatment mean may well be the technique of cortisol quantification employed (see "Discussion of CPB"; pp18-20). Finally, to test the possibility that variances
within plasma cortisol treatment means were related to individual fish differences as weight, length, sex and hematocrit, a regression analysis of these parameters against plasma cortisol measurements was made. In each case, the slope of the curve so obtained was essentially zero, suggesting that individual variations among fish, with respect to the variables just mentioned, did not bias the reported cortisol values.

Experiment Two differed from Experiment One in several aspects. No controls were used for two basic reasons: one, there was an insufficient number of fish available and two, the results of Experiment One suggested a consistent level of cortisol in control animals throughout the investigation. During the course of the second experiment, no fish weights, lengths and sex were recorded, although hematocrits were consistently taken as an index of fish vitality. Finally, no histological treatment of Experiment Two interrenals was done. This decision was justified by the results of the first experiment in which a clear karyometric picture, both statistically and with regard for similar published works, was obtained. The fact that Experiment One karyometric analysis indicated that hypertrophy of experimental interrenal nuclei relative to controls occurred after 14 days exposure to cold justified not performing this analysis on Experiment Two animals, as the latter experiment was terminated after only 6 days
exposure to cold.

The major sources of error in this study were threefold. The first was the limitation of the sample size to a maximum of 8 fish. The second, related to the restrictions on sample size, was the tremendous individual variations in plasma constituents among different fish exposed to the same treatment. This observation has the support of several other investigators involved in similar research. The third major source of error was the technique of cortisol quantification where standard deviations between duplicate samples of the order of 20% were considered acceptable.

Consistent to the basic question of this thesis is whether an acclimation response and not a stress response was investigated. The arguments in support of the position taken in this study (see "Introduction to Results"; pp XIV-XVII) were predominantly academic and involved a measure of semantic interpretation. Although it would be repetitive to restate the conditions suggesting that the changes investigated were, in fact, responses to acclimation, it is nonetheless admitted by this investigator that criticism of the view taken is justified.

In retrospect, the study reported here has suggested many related avenues for research into the process of acclimation to cold by juvenile coho salmon. Under similar experimental conditions it would be useful to correlate changes in liver glycogen, plasma glucose
and plasma cortisone with that of plasma cortisol. In addition, a histological and histochemical study of the coho pituitary, designed primarily to demonstrate ACTH activity, would also be of value, especially when compared with the biochemical investigations referred to above. Furthermore, a quantification of interrenal hyperplasia, although time consuming and difficult, would clarify the picture. Finally, it should be remembered that the technique of competitive protein binding is rapidly expanding and is extremely useful in dealing with animals having the blood volume restrictions of juvenile salmonids. To this end, investigation into the dynamics of cortisol, cortisone and other related hormones is possible. Similarly, the application of this technique to other important endocrinological factors such as ACTH appears to be a possibility in the very near future. These and other investigations would provide an important contribution in the clarification of the events involved in acclimation to cold temperature by juvenile coho salmon.
CONCLUSIONS

The following conclusions, based on the results of this investigation, are made:

(1) During acclimation to cold temperature, juvenile, pre-smolt coho salmon exhibit increases in plasma cortisol concentration.

(2) Under the experimental conditions imposed in this study, plasma cortisol levels of cold-acclimating juvenile coho salmon achieve control values within 8 days of exposure to cold.

(3) Significant hypertrophy of the interrenal nuclei of juvenile coho salmon occurs by Day 14 of exposure to cold temperature.

(4) Histologically demonstrable increases in interrenal activity lag behind increases in plasma cortisol levels. For the former, a significant increase in interrenal activity occurs by Day 14 of exposure to cold temperature. For the latter, plasma cortisol concentrations approximate those of controls by Day 8.
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