Identification of a Putative Corticosteroid in Pacific Lamprey
(Entosphenus tridentatus)

by

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Abstract

The mechanisms and interplay of multiple axes as well as the physiological components of the stress response have been studied in many vertebrate groups; however, the intricacies and chemical messengers involved in the stress response in lamprey have not been fully resolved. 11-Deoxycortisol is a functional corticosteroid in sea lamprey, but the identity of the functional corticosteroid used by other lamprey species and the evolution of the stress response in lamprey lineage remains unknown. Identification of the functional corticosteroid in a greater number of lamprey species will help elucidate the evolution of the stress response in lamprey. I have demonstrated that 11-deoxycortisol, a steroid precursor to cortisol, may be a functional corticosteroid in Pacific lamprey. The putative hormone was identified in Pacific lamprey plasma by employing an array of methods such as radioimmunoassay (RIA), high performance liquid chromatography (HPLC) and mass spectrometry analysis. I have demonstrated that plasma levels of 11-deoxycortisol significantly increased in Pacific lamprey 0.5 and 1 hr after stress exposure and that sea lamprey corticotropin releasing hormone (CRH) injections increased circulating levels of 11-deoxycortisol, suggesting that 11-deoxycortisol secretion is under control of the hypothalamic neuropeptide CRH. However, plasma 11-deoxycortisol concentrations did not increase in response to sea lamprey adrenocorticotropic hormone (ACTH) injections. The effect of artificially elevated 11-deoxycortisol on the expression of glucocorticoid receptor and progesterone receptor mRNA was examined in liver, gill, muscle, heart and testis using RT-qPCR. 11-Deoxycortisol was chronically elevated using coconut oil implants with two different doses of 11-deoxycortisol (0.5 and 1.0 µg per g of body weight). After 7 days of elevated plasma 11-deoxycortisol concentrations there was
no significant difference in the expression of either receptor in the tissues examined.
Overall, the results of this thesis demonstrate 11-deoxycortisol is a putative corticosteroid in Pacific lamprey; however, despite the evidence for hypothalamic control of 11-deoxycortisol release, the results do not conclusively support Pacific lamprey having a hypothalamic pituitary interrenal (HPI) axis.
Preface


The section on “Liquid chromatography-mass spectrometry” and “Identification of 11-deoxycortisol by UHPLC/MS/MS MRM” were conducted and drafted by S.-S. Yun.

The corticotropin-releasing hormone injections and blood collection in the section “CRH dose response” were conducted by Brent Roberts.
# Table of Contents

Abstract ......................................................................................................................... ii

Preface .......................................................................................................................... iv

List of Tables .................................................................................................................. vii

List of Figures ................................................................................................................ viii

List of Abbreviations .................................................................................................... x

Acknowledgements ....................................................................................................... xii

Dedication ...................................................................................................................... xiii

Chapter 1: General Introduction .................................................................................. 1

1.1 Introduction to Steroids & Steroid Receptors .......................................................... 1

1.2 Stress Response & Stress Axes ................................................................................. 2

1.3 Steroid Receptors & Gene Expression ..................................................................... 6

1.4 Steroid and Steroid Receptor Evolution ................................................................... 7

1.5 Pacific Lamprey as Model Species .......................................................................... 10

1.6 Lamprey CR and Corticosteroid Secretagogues: ...................................................... 12

1.7 Thesis Objectives and Hypotheses ......................................................................... 14

Chapter 2: Identification of a Putative Corticosteroid in Pacific Lamprey ............... 17

2.1 Introduction ............................................................................................................. 17

2.2 Materials and Methods ......................................................................................... 18

2.2.1 Materials ........................................................................................................... 18

2.2.2 Experimental Animals ..................................................................................... 18

2.2.3 Blood collection ................................................................................................. 19

2.2.4 Radioimmunoassay .......................................................................................... 19

2.2.5 Screen of HPLC Fractions from Pacific Lamprey Plasma .................................. 20

2.2.7 Acute Stress Time Series Experiment ............................................................... 22

2.2.8 CRH Injection Experiment .............................................................................. 23

2.2.9 ACTH Injection Experiment .......................................................................... 23

2.2.10 Liquid Chromatography-Mass Spectrometry .................................................... 24

2.2.11 Implant Injections – Experimental Design and Sampling Procedure .............. 24

2.2.12 Real Time PCR ............................................................................................... 25

2.2.13 Statistical Analyses ......................................................................................... 28

2.3 Results ................................................................................................................... 28
2.3.1 Steroid extraction, HPLC fractionation and RIA screening for corticosteroids ........................................... 28
2.3.2 Pre- and Post- Acute Stress and changes in concentrations of 11-deoxycortisol ........................................ 29
2.3.3 Identification of 11-deoxycortisol ............................................................................................................. 29
2.3.4 Acute Stress Time Series ......................................................................................................................... 29
2.3.5 CRH Injection Experiment ....................................................................................................................... 30
2.3.6 ACTH Injection Experiment ..................................................................................................................... 30
2.3.7 11-Deoxycortisol Implant ....................................................................................................................... 31
2.3.8 Effects of 11-deoxycortisol implant on PR and CR expression ................................................................. 31
2.4 Discussion .................................................................................................................................................... 32
  2.4.1 Identification of 11-deoxycortisol in Pacific lamprey plasma ................................................................. 32
  2.4.2 Acute Stress Time Series ....................................................................................................................... 34
  2.4.3 CRH Injections ......................................................................................................................................... 35
  2.4.4 ACTH Injections ....................................................................................................................................... 36
  2.4.5 Effect of Chronically Elevated 11-deoxycortisol Concentration on Expression of CR and PR mRNA Transcript .................................................................................................................... 37

Chapter 3: Conclusions ........................................................................................................................................ 50
  3.1 Thesis Summary ........................................................................................................................................... 50
  3.2 Thesis Objectives and Hypotheses ....................................................................................................... 51
  3.4 Implications ............................................................................................................................................... 52
  3.5 Research Limitations & Future Directions ............................................................................................... 52

References .......................................................................................................................................................... 55
List of Tables

Table 1: Gene-specific primer sequences were used for reverse transcriptase PCR and real-time PCR. Forward (F) and reverse (R) primers are listed for each gene. CR, corticosteroid receptor; PR, progesterone receptor; 18S, 18S ribosomal RNA.
List of Figures

**Figure 1:** HPLC/RIA identification: Concentration of (A) corticosterone, 11-deoxycorticosterone, (B) cortisol and 11-deoxycortisol (ng/fraction) from adult Pacific lamprey plasma following HPLC fractionation. Arrows show the elution point of standard (A) corticosterone, 11-deoxycorticosterone, (B) cortisol and 11-deoxycortisol..........................41

**Figure 2:** HPLC/RIA on pre and post stressed Pacific lamprey plasma. Concentrations of (A) 11-deoxycorticosterone (ng/fraction) and (B) 11-deoxycortisol in fractions 41-70 for pre- and post- acute stressed individuals (n=4). Results are mean ± SEM and asterisks indicate a significant difference (P<0.001) with a two-way ANOVA followed by Bonferroni post-tests.................................................................42

**Figure 3:** Identification of 11-deoxycortisol by LC/MS analysis. Multiple reaction monitoring (MRM) was performed with transition m/z 347 > 109 using UHPLC-MS (positive mode) over 8 min. Note that 11-deoxycortisol was detected at 4.75 min in both standard (A) and natural (B) samples........................................................................................................43

**Figure 4:** Acute Stress Experiment: Concentration of 11-deoxycortisol in plasma of female Pacific lamprey (n= 7-10) at different time points after acute stress by dewatering. Results are mean ± SEM. Asterisks indicate a significant (**P < 0.01; ***P < 0.001) difference with one-way ANOVA followed by Dunnett’s comparison to controls.................................................................44

**Figure 5:** CRH injections: Concentration of 11-deoxycortisol in plasma of adult Pacific lampreys after injection of sea lamprey CRH (females n=6-13; males n=1-3). Results are mean ± SEM. Asterisks indicate a significant (*P < 0.05; ***P < 0.001) difference with one-way ANOVA followed by Dunnett’s comparisons to controls.................................................................45

**Figure 6:** ACTH injections: Concentrations of 11-deoxycortisol in plasma of adult Pacific lamprey (females n=5; males n=5) with injections of either saline or a mixture of 4 sea lamprey ACTH peptides (ACTH(1-60), ACTH(1-60P), ACTH(1-59), ACTH(1-59P)) at levels of 100 µg/kg of each peptide. Each group (saline and ACTH injected). Results are mean ± SEM. Significant differences between groups were determined using a t-test.................................................................46
**Figure 7:** Effects of 11-deoxycortisol implants on concentration of plasma 11-deoxycortisol in Pacific lamprey (n=8). Results are mean ± SEM.

**Figure 8:** Effect of 11-deoxycortisol implants on corticosteroid receptor (CR) mRNA abundance in adult Pacific lamprey in different tissues, 7 days post implantation of either coconut oil alone or coconut oil and 11-deoxycortisol at doses of 0.5 or 1.0 µg 11-deoxycortisol per g or non-injected controls. CR mRNA from muscle, liver, heart, gill and testis was quantified using real-time PCR and normalized to 18S rRNA. Values represent the means ± SEM (n=7-8; except testis which had n=4-7).

**Figure 9:** Effect of 11-deoxycortisol implants on progesterone receptor (PR) mRNA abundance in adult Pacific lamprey in different tissues, 7 days post implantation of either coconut oil alone or coconut oil and 11-deoxycortisol at doses of 0.5 or 1.0 µg 11-deoxycortisol per g or non-injected controls. PR mRNA from muscle, liver, heart, gill and testis was quantified using real-time PCR and normalized to 18S rRNA expression. Values represent the means ± SEM (n=4-7; except testis which had n=1-5).
List of Abbreviations

ACTH  adrenocorticotropic hormone
ANOVA  analysis of variance
AR  androgen receptor
CR  corticosteroid receptor
CRH  corticotropin-releasing hormone
DBD  DNA binding domain
DPM  disintegrations per minute
ERα  estrogen receptor-alpha
ERβ  estrogen receptor-beta
GC  glucocorticoid
GnRH  gonadotropin releasing hormone
GR  glucocorticoid receptor
GTH  gonadotropin
hGR  human glucocorticoid receptor
HPA  hypothalamic pituitary adrenal
HPI  hypothalamic pituitary interrenal
HPLC  high performance liquid chromatography
HSP  heat shock protein
HRE  hormone response element
LBD  ligand binding domain
MC  mineralocorticoid
MR  mineralcorticoid receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>MSH</td>
<td>melanocyte stimulating hormone</td>
</tr>
<tr>
<td>MYA</td>
<td>million years ago</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PD</td>
<td>pars distalis</td>
</tr>
<tr>
<td>PI</td>
<td>pars intermedia</td>
</tr>
<tr>
<td>POC</td>
<td>proopiocortin</td>
</tr>
<tr>
<td>POM</td>
<td>proopiomelanotropin</td>
</tr>
<tr>
<td>POMC</td>
<td>proopiomelanocortin</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>rtGR</td>
<td>rainbow trout glucocorticoid receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SR</td>
<td>steroid receptor</td>
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To my mom
Chapter 1: General Introduction

1.1 Introduction to Steroids & Steroid Receptors

Hormones are chemical messengers that are synthesized in one cell type, released into the blood in micromolar concentrations (Bolander, 2004) and induce a transcriptional or non-transcriptional response in target cells, thus providing a mechanism for cell-cell communication between cells located in different parts of the body. Steroid hormones (hereafter referred to as steroids and not to be confused with non-hormonal steroids which are not discussed in this thesis) play central roles in many aspects of an organism's life including gametogenesis, gestation, growth, development, metabolism and regulation of hydromineral balance (Evans, 1988; Baker, 1997; Whitfield et al., 1999).

Steroids are a sub-class of hydrophobic hormones synthesized from cholesterol; the major groups of steroids in vertebrates are the estrogens, androgens, progestins, glucocorticoids (GCs), and mineralocorticoids (MCs). Each of the aforementioned steroid hormone classes has their respective receptor classes: the alpha and beta estrogen receptors (ERα and ERβ, respectively), androgen receptors (ARs), progesterone receptors (PRs), glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). In vertebrate species where both the respective ligands and receptors are present, each pair can elicit specific physiological changes in target cells, furthermore, these specific changes can be generalized into a group of related functions for each pair: the estrogens are involved in gonadal growth and sexual development, the androgens in spermatogenesis and development of secondary sex characteristics, the progestins in oocyte maturation, spermiation, myogenesis and sexual development, the GCs in regulation of glucose
metabolism and the stress response, and finally the MCs in hydromineral balance (Evans, 1988; Baker, 1997; Whitfield et al., 1999). Despite these specific pairings, there is evidence for modern vertebrate steroid receptors (SRs) having promiscuity for multiple ligands and it has been hypothesized that ancestral SRs had lower specificity for steroids compared to modern day SRs (Baker, 2002). Studies using a cloned human MR have reported that the MR has high affinity for GCs (cortisol and corticosterone), MC (aldosterone) and a progestin (progesterone) (Myles and Funder 1996; Funder, 1997). Using ancestral gene reconstruction and a reporter assay, it was shown that the ancestral corticosteroid receptor (CR) from which the modern day GR and MR arose, had lower steroid specificity than modern day GR because the reconstructed ancestral CR was able to bind aldosterone and this aldosterone sensitivity was later lost in the GR during evolution (Bridgham et al., 2006). With their involvement and regulation of the aforementioned physiological processes, the evolution of the SR family, SR specificity and steroid action has been of great interest with regards to their role in providing early vertebrates with competitive advantages over organisms lacking some or all of these SRs (Baker, 1997, 2003).

1.2 Stress Response & Stress Axes

The ability to perceive and respond to stressful situations is considered an adaptive response (Wendelaar Bonga, 1997; Barton, 2002) and the stress response is found across all vertebrate groups (Tort and Teles, 2011). Stress can be defined as the condition under which a stimulus, referred to as a stressor, disrupts or threatens the body's homeostatic balance (Wendelaar Bonga, 1997). The stressor can be physical, chemical (e.g. exposure to contaminants), pathological, fluctuations in abiotic factors (e.g. extreme temperature, hypoxia or salinity changes), nutritional limitations or predation. The stress response acts
to re-establish the deviation from normal resting state to allow the organism to cope with or overcome the stressor by increasing the amount of available energy within the body and restore homeostasis.

Immediately following stressor recognition, the central nervous system activates two axes: the sympathetic-chromaffin axis and the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis. Within seconds of activating the sympathetic nervous system, the sympathetic neural fibers release neurotransmitters directly onto the cholinergic receptors of the chromaffin cells (located in adrenals in mammals or as chromaffin tissue in the anterior region of the kidney in teleosts) and trigger the release of stored catecholamines into the blood (Barton, 2002). The catecholamine release triggers physiological changes associated the fight or flight response: increased heart rate, increased respiration, glycogenolysis to increase glucose mobilization and blood diversion towards skeletal muscles and away from the digestive and reproductive tracts (Wendelaar Bonga, 1997; Randall and Perry, 1992).

The interrenal cells of the head kidney in fish are homologous to the adrenals in the higher vertebrates (Butler, 1973; Wendelaar Bonga, 1997), and for this reason the HPA axis is termed the hypothalamic-pituitary-interrenal (HPI) axis in fish; this axis is highly conserved among vertebrates (Denver, 2009). Activation of the HPA/I triggers the release of a neuropeptide, corticotropin releasing hormone (CRH), from the hypothalamic paraventricular nucleus or preoptic nucleus, in mammals and fish respectively (Tort and Teles, 2011). CRH binds to corticotrophin-releasing hormone receptors on corticotrophs located in the pars distalis of the pituitary, and triggers the release of adrenocorticotropic hormone (ACTH). ACTH enters the circulation and binds to melanocortin receptors on the
adrenal gland in tetrapods and the interrenal tissue in fish to stimulate synthesis and release of GCs (Wendelaar Bonga, 1997; Tort and Teles, 2011).

The active GC is taxon dependent and includes cortisol in most mammals and ray finned fish (Idler and Truscott, 1972; Mommsen et al., 1999; Barton, 2002), corticosterone in most amphibians, birds, and reptiles (Tyrell and Cree, 1998; Moore and Jessop, 2003), and possibly 1α-hydroxycorticosterone in elasmobranchs (Idler and Truscott, 1969; Anderson, 2012). Recently, a putative corticosteroid in a modern representative of the most primitive vertebrates, sea lamprey (*Petromyzon marinus*), was shown to be 11-deoxycortisol, which is a direct precursor molecule to cortisol in the steroidogenic pathway (Close et al., 2010).

The stress response is mediated by GCs acting on GRs, causing changes in the expression of stress responsive genes and ultimately affecting the metabolism of carbohydrates, proteins and lipids. Despite the growing body of work looking at the molecular mechanisms of GCs and the number of stress responsive genes they regulate in fish, the literature regarding the molecular mechanisms of stress physiology in fish not completely resolved (Prunet et al., 2008). There is, however, a plethora of information on the molecular mechanisms of GCs in response to stress in mammals compared to fish. In mammals, GCs generally increase gluconeogenesis and glycogenesis (Charmandari et al., 2005). The increase in gluconeogenesis is attributed to increased phosphoenolpyruvate carboxykinase (PEPCK) expression and destabilization of mRNAs of other hepatic proteins to increase the hepatic precursor supply (Mommsen et al., 1999); the increase in glycogenesis is attributed to increased activity of glycogen phosphatase which increases activation of glycogen synthase (Mommsen et al., 1999). Exposure to increased GCs in fish
can cause plasma glucose and liver glycogen to increase, decrease or remain unchanged and there is high interspecies and intraspecies variability on these measures, thus questioning the usefulness of these measures as indicators of metabolic status (Mommsen et al., 1999). The general consensus is that GCs will increase the rate of gluconeogenesis despite the fact that in some cases there is no change or a decrease in plasma glucose (Mommsen et al., 1999). Cortisol has been shown to increase the activity of key gluconeogenic enzymes such as PEPCK, glucose-6-phosphate, and 1,6-bisphosphatase in mammals and teleostean fish (Mommsen et al., 1999). Furthermore, acute stress in rainbow trout caused up regulation of 40 genes involved in energy metabolism, immune function and protein degradative pathways in the liver one hour post stressor exposure (Wiseman et al., 2007).

Activation of the stress response for increased duration or frequency can become maladaptive to the organism (Barton, 2002). At high levels, GCs have been shown to have suppressive effects on the immune system, growth and reproduction (Mommsen et al., 1999). GCs have been shown to reduce immunocompentence in fish by reducing lymphocyte levels and antibody-production capacity thus potentially increasing susceptibility to pathogens (Barton and Iwama, 1991; Wendelaar Bonga, 1997). GCs have also been shown to down modulate proinflammatory transcription factors and therefore suppress their activity in the immune response (Herrlich and Gottlicher, 2002; Hayashi et al., 2004). Stress also reduces the amount of available energy for growth due to the catabolic actions of corticosteroids (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Stress in fish is known to reduce concentrations of gonadal steroids which regulate sexual maturation, and is associated with higher ovarian atresia rates, reduced egg size in females and lower sperm
counts in males, which can lead to direct influences on reproductive success (Barton and Iwama, 1991; Iwama et al., 1997).

1.3 Steroid Receptors & Gene Expression

Like other nuclear receptors, SRs have 4-5 domains: an amino terminal A/B region involved in transcriptional activation, the C region containing a DNA-binding domain (DBD), a hinge D region, an E region with a ligand binding domain (LBD) and some contain an F region (Markov et al., 2010). The DBD is highly conserved among the SRs (Bury and Sturm, 2007), has two zinc fingers which are important in stabilizing the 3D structure, and is involved in binding to the HRE in the promoter region of the gene. The D region is responsible for nuclear localization of the ligand-receptor complex. The E region has four major roles: steroid binding, transcriptional activation, dimerization and HSP binding. SR ligand specificity lies in the LBD within the E region. The F region is the region closest to the carboxyl terminal and is sometimes completely absent.

SRs are referred to as ligand-activated transcriptional factors (Baker, 1997; Kumar and Thompson, 1999; Bolander, 2004) because of their ability to alter gene expression once bound to their appropriate ligand. In their unliganded state, SRs form a protein complex with heat shock proteins (HSPs), HSP-organizing proteins and immunophilins in the cytoplasm (Kumar et al., 2006; Bury and Sturm, 2007). The HSPs act as molecular chaperones to stabilize the unliganded inactive receptor and maintain high affinity for the ligand (Funder, 1997; Stolte et al., 2006; Kumar et al., 2006). In their liganded state, the SR-HSP complex dissociates, and the SR-ligand complex will transverse into the nucleus.

Activated SRs form homodimers with other activated SRs in the nucleus and then bind to specific hormone response elements (HREs) within the promoter region of
responsive genes (Bury and Sturm, 2007; Guerriero, 2009). A given steroid-SR complex can control the expression of a number of genes within a cell. Binding of the steroid-SR complex to the HRE recruits co-activators and RNA polymerase II to the DNA and causes induction or suppression of gene transcription (Beato et al., 1995). Genes contain many response elements and a specific hormone-receptor pair can have cognate HREs present in many different promoter regions of genes. Therefore, one hormone-receptor pair can affect many genes but these genes can also be simultaneously regulated by other transcription factors and other stimuli. A given steroid will be able to regulate gene expression in a cell if the appropriate SR is present, thus allowing for tissue specificity. Additional SR regulation is due to the presence of different SR isoforms in some species, these different isoforms allow for different transactivational and transrepressional sensitivities. For example, rainbow trout have 2 GRs (rtGR1 and rtGR2) that have different transrepressional sensitivities, rtGR2 is able to suppress the activity of transcription factors involved in the immune response at lower concentration compared to rtGR1 (Bury and Sturm, 2007).

Steroids typically act via classical steroid action where the hormone diffuses into the cell and binds to a cytosolic receptor, allowing the receptor-ligand complex to enter the nucleus and regulate gene transcription. However some steroids are known to act via non-classical action where the steroid binds to membrane bound G-protein coupled receptors, and then activates signal transduction pathways to ultimately bring about a rapid change without changing protein expression (Cato et al., 2002).

1.4 Steroid and Steroid Receptor Evolution

SRs, along with retinoic acid receptor, thyroid hormone receptor, prostaglandin receptors and others, belong to the nuclear receptor superfamily (Escriva et al., 1997;
Bertrand et al., 2004), which act as ligand-activated transcriptional factors (Bolander, 2004). It is hypothesized that serial duplications of SRs led to the evolution of the six vertebrate SRs. “New” receptors appear after a gene duplication event of an extant receptor gives rise to a nascent receptor that can more readily accept mutations since it is no longer under the constraint of maintaining its original function. Mutations to the nascent receptor’s LBD can lead to changes in specificity for a different ligand and therefore lead to the creation of a new receptor-ligand partnership. The appearance of “new” steroids can be due to a new enzyme or mutations in an existing enzyme in the steroidogenic pathway. The appearance of multiple SRs in early vertebrate evolution may have provided early vertebrates with an advantage to survive global catastrophes and compete with other organisms (Baker, 1997).

Phylogenetic analysis indicates that two successive duplications occurred in the early evolution of SRs (Baker, 1997), one before and one after the divergence of lamprey and gnathostomes (Thornton, 2001), and these duplications are thought to correspond to the genome size duplications hypothesized to have occurred in the early evolution of vertebrates (Baker, 1997). Among the SRs, the ER is the closest to the ancestral SR (Baker, 1997, 2001a, 2002b; Thornton, 2001) and Thornton (2001) provides evidence for the ER being the ancestral receptor of the adrenal and sex SR clade of nuclear receptors.

Phylogenetic analysis of the LBD of SRs indicates that the AR, PR, MR and GR form a distinct clade and evolved from an ancestral SR by two successive gene duplications (Baker, 1997; 2001a). The AR and PR cluster on one branch and the MR and GR cluster on another, thus indicating the AR and PR are decedents of a common ancestor and the MR and GR are decedents of a common ancestor (Baker, 1997). Thornton (2001) provides evidence for the
second ancestral SR being a 3-keto SR that bound 21-carbon steroids with the likely ligand being progesterone.

Three SR orthologs have been identified in sea lamprey: the lamprey ER, lamprey PR and lamprey CR (Thornton, 2001). These SRs have sequence similarities to gnathostome receptors; the lamprey PR is most similar to the gnathostome PR, the lamprey ER is most similar to the gnathostome ERα and ERβ, the lamprey CR is similar to both the gnathostome GR and MR (Thornton, 2001). It is hypothesized that a large scale genome duplication event in the gnathostome lineage is responsible for creating the six SR orthologs present in the sarcopterygians: the ERα, ERβ, PR, AR, GR and MR (Baker, 1997; Thornton, 2001).

Skates and sharks have two distinct orthologs for mammalian GR and MR (Baker et al., 2013). Skate MR is transcriptionally activated by corticosterone, cortisol, 11-deoxycorticosterone, and aldosterone and skate GR has low affinity for human corticosteroids and 1α-hydroxycorticosterone (Baker et al., 2013). Phylogenetic analyses estimate that the duplication event leading to GR and MR occurred >450 MYA (Bridgham et al., 2006). Studies of elasmobranchs support 1α-hydroxycorticosterone as the functional corticosteroid (Idler and Truscott 1969; Anderson 2012). Teleosts have two distinct GRs present (GR1 and GR2) and a MR (Bury et al, 2003; Prunet et al., 2006). The GR1 has splice variants, GR1A and GR1B, with GR1A having a nine amino acid insert in the DBD (Bury et al., 2003; Stolte et al., 2006). It is hypothesized that a gene duplication event happened in the teleost lineage to produce these two GRs and this is consistent with known duplicated genes in teleosts (Stolte et al., 2006). Rainbow trout GRs have shown different transactivational activities (Bury et al., 2003) which leads to interesting questions
regarding the potential differences in physiological functions of the GRs. Cortisol is the functional GC and MC in teleosts and acts through the MR and GRs (Mommsen et al., 1999). Lobe finned fish have a GR and MR and are the ‘earliest’ vertebrates known to synthesize aldosterone (Baker et al., 2013). Tetrapods have two corticosteroid receptors which have divergent functions and ligands, the MR whose ligand is aldosterone and the GR whose ligand is either corticosterone or cortisol depending on the group (Bury and Sturm, 2007). The MR has similar affinity for cortisol and aldosterone and cortisol is found at much higher concentrations than aldosterone. Mammalian MR is co-expressed with 11-βHSD2, which converts cortisol into the inactive GC cortisone, in the distal kidney tubule which ensures the MR is an aldosterone specific receptor that regulates the MC response (Funder, 1997; Prunet et al., 2006; Baker et al., 2007).

1.5 Pacific Lamprey as Model Species

Lamprey are modern representatives of the oldest lineage of vertebrates, the agnathans, which diverged from the vertebrate lineage about 500 MYA (Forey and Janvier, 1993). The phylogenetic position of lamprey makes them an ideal species to elucidate the evolution of complex physiological processes present in modern vertebrates today. The advent of SR signaling and the responses mediated by these receptors is thought to have had an important role in the early evolution of vertebrates as well as their survival (Baker, 1997). Resolving the mechanism by which steroids and SR partnerships have evolved has been of great interest for the past several decades and a better understanding of the steroid signaling and steroid-SR partnership of lamprey is critical to gain a better understanding of the steroid signaling mechanisms.
Lamprey belong to the order Petromyzontiformes which is made up of three families: Petromyzontidae, in the Northern Hemisphere, and Geotriidae and Mordaciidae, in the Southern Hemisphere. Pacific lamprey (*Entosphenus tridentatus*), belong to the family Petromyzontidae and are primarily found along the Pacific coast of North America and Asia. Pacific lamprey are anadromous and migrate from the sea to freshwater to spawn semelparously. Sea lamprey belong to the family Petromyzontidae and are found in the Great Lakes, the western Mediterranean Sea and along the shores of Europe and North America in the northern Atlantic Ocean. Close et al., (2010) found that 11-deoxycortisol acts as a functional corticosteroid that has both MC and GC activities in sea lamprey. Both human CRH and sea lamprey CRH act as 11-deoxycortisol secretagogues in sea lamprey (Close et al., 2010; Roberts et al., 2014), and pituitary extract injections cause an increase in sea lamprey plasma 11-deoxycortisol levels (Close et al., 2010). These results support the presence of a functional HPI axis in sea lamprey and the identification of a corticosteroid and presence of a functional HPI axis in Pacific lamprey will help elucidate the evolution of the stress axis in the lamprey lineage.

Pacific lamprey populations are declining (Close et al., 2002). Dams, poor water quality, predation, and prey decline have contributed to the decline of Pacific lamprey populations (Renaud, 1997). Furthermore, these aforementioned environmental factors have the potential of activating the stress response, and chronic stressors have shown to have maladaptive consequences to organisms (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Rainbow trout experiencing daily stress show reduced growth, liver glycogen, and circulating lymphocytes levels and increased resting plasma glucose (Barton et al., 1987). Acute stress has the ability to cause a shift in the body's energy stores and chronic stress...
exposure can severely reduce the amount of energy available to go towards reproduction and immunity and could potentially affect a species on the individual and population level. Identification of the functional corticosteroid used by Pacific lamprey will provide a better understanding of the stress response in these fish and can be used by fisheries management as an indicator of stress in these fish along the West coast of North America (Close et al., 2009; Ward et al., 2012).

1.6 Lamprey CR and Corticosteroid Secretagogues:

Lamprey and hagfish have a single CR (Bridgham et al., 2006). 11-Deoxycortisol has been shown to have MC and GC activity with high specificity for the sea lamprey CR (Close et al., 2010). However, transactivation studies of sea lamprey CR-LBD suggests that sea lamprey CR is a promiscuous receptor binding to cortisol, corticosterone, 11-deoxycorticosterone, 11-deoxycorticosterone and aldosterone (Bridgham et al., 2006). Close et al., (2010) proposed that the contradictory results may be due to the lack of the entire receptor, lack of lamprey specific chaperones, or assay conditions used in the transactivation studies carried out by Bridgham et al., (2006) since binding assays on gill cytosol indicates high specificity for 11-deoxycortisol by the native lamprey CR (Close et al., 2010).

Baker et al., (2011) analyzed the LBD of a crystallized lamprey CR and found that 11-deoxycortisol and 11-deoxycorticosterone have similar contacts to the lamprey CR LBD but 11-deoxycortisol makes an extra contact with Leu-220 and Met-299. Crystallized lamprey CR shows Van der Waals contact between Cys-227 in helix 3 and Met-264 in helix 5 which are absent in human MR and human GR. Mutant human MR and mutant human GR with Van der Waals contact between helix 3 and helix 5 have enhanced responses to
progesterone and GCs, respectively (Baker et al., 2011). It is therefore proposed, that the helix 3-helix 5 interaction was present in the CR and lost during the evolution of the MR and GR and was important in the evolution of corticosteroid specificity in vertebrate MR and GR.

CRH and ACTH are considered to be the most important corticosteroid secretagogues in many vertebrates (Wendelaar Bonga, 1999). The CRH gene is highly conserved among vertebrates (Lovejoy and Balment, 1999; Chang and Hsu, 2004; Lovejoy and Jahan, 2006) and the function of CRH has also been conserved. Sea lamprey CRH peptide sequence is very similar to a wide range of other known CRH peptide sequences (Roberts et al., 2014). Sea lamprey increase plasma 11-deoxycortisol concentration in response to human CRH injections (Close et al., 2014) and sea lamprey CRH injections (Roberts et al., 2014) providing evidence for functional similarity between sea lamprey CRH and human CRH. Similarities between lamprey CRH and gnathostome CRH may be indicative of early establishment of CRH function in the vertebrate lineage.

In contrast to the conserved state of the CRH gene among vertebrates, there are key differences with regards to lamprey ACTH and gnathostome ACTH in length and precursors. In the gnathostomes ACTH is encoded as a part of a larger precursor molecule called POMC, which encodes ACTH, melanophore-stimulating hormones (MSHs), and β-endorphin and is expressed in both the pars distalis (PD) and pars intermedia (PI) (Heinig, 1995). In contrast, lamprey possess two types of POMCs that are expressed in different parts of the pituitary: proopiocortin (POC) which is expressed in the PD and proopiomelanotropin (POM) which is expressed in the PI (Heinig et al., 1995; Takahashi et al., 1995b). In lamprey, the POC-producing cells of the PD encode ACTH and β-endorphins
and the POM-producing cells of the PI encode MSHs and β-endorphins which are different from those expressed in the PD (Heinig et al., 1995; Takahashi et al., 1995b); therefore, the POC-producing cells of the PD and the POM-producing cells of the PI in lamprey are functionally similar to the POMC producing cells in gnathostome PD and PI respectively (Takahashi et al., 2001). Another key difference between lamprey ACTH and gnathostome ACTH is that lamprey ACTH is made up of 60 residues (ACTH₁₋₆₀) with further post-translational modification to produce ACTH₁₋₅₉ with either the presence or absence of phosphorylation at Ser35 (Takahashi et al., 2006c), whereas gnathostome ACTH is composed of 39-40 residues (Takahashi and Kawauchi, 2006). Despite these marked differences between lamprey ACTH and gnathostome ACTH, there is evidence for ACTH-like activity in the pituitary of least brook lamprey (Eastman and Portanova, 1982) and European river lamprey (Baker and Buckingham, 1983) using a rat adrenal bioassay.

1.7 Thesis Objectives and Hypotheses

Close et al., (2010) identified 11-deoxycortisol as a functional corticosteroid in sea lamprey, but the identification of the functional corticosteroid used by other lamprey species and the evolution of the stress axis in the lamprey lineage remains unknown. Identification of the functional corticosteroid in a greater number of lamprey species will help elucidate the evolution of the stress axis in lamprey. The general objective of this thesis is to identify a putative corticosteroid in Pacific lamprey to help elucidate the evolution of the stress axis in lamprey. This thesis consists of three main objectives:

**Objective 1: Identify corticosteroids in Pacific lamprey plasma**

Tasks to achieve objective 1: a) Fractionate Pacific lamprey plasma with HPLC and screen fractions with RIA; b) confirm identity of corticosteroids with mass spectrometry.
Adult sea lamprey have both 11-deoxycortisol and 11-deoxycorticosterone present in their plasma (Close et al., 2010). I predicted that Pacific lamprey plasma will also have 11-deoxycortisol and 11-deoxycorticosterone.

**Objective 2: Determine if Pacific lamprey possess a functional HPI axis.**

Tasks to achieve objective 2: Use RIA to evaluate if putative corticosteroid concentrations increase in response to a) acute stress b) sea lamprey CRH injections c) sea lamprey ACTH injections

Like the higher vertebrates, fish increase plasma corticosteroids concentrations in response to acute stressors and concentrations return to basal levels within several hours (Barton, 2002). I hypothesized that the stress response in Pacific lamprey would be similar to that in gnathostomes. Therefore, I predicted that the putative corticosteroid that I identified in Objective 1 would increase in response to acute stress 1-2 hrs after stressor exposure and return to basal levels within several hours and increase in response to sea lamprey CRH and sea lamprey ACTH injections in Pacific lamprey.

**Objective 3: Determine if elevated concentration of the putative corticosteroid has an effect on receptor expression in Pacific lamprey.**

Tasks to achieve objective 3: Measure CR and PR mRNA transcript levels in muscle, liver, gill, heart and testis using RT-qPCR after exposure to 7 days of elevated corticosteroid levels.

Studies on elevated GC levels and chronic stress in teleosts have shown changes in the concentration and binding affinity of GRs as well as changes in expression of the GR mRNA transcript levels (Shrimpton and Randall, 1994; Vijayan et al., 2003; Sathiyaa and Vijayan, 2003; Teles et al., 2013). I hypothesized that this response would be conserved
between lamprey and teleosts, and therefore I predicted that CR mRNA transcript levels, but not PR mRNA transcript levels, would decrease in the tissues examined after 7 days of exposure to elevated corticosteroid levels.
Chapter 2: Identification of a Putative Corticosteroid in Pacific Lamprey

2.1 Introduction

The HPA/I axis is a feature found across the vertebrate lineage and is activated in response to stressors. The HPA/I axis begins with the release of CRH from the hypothalamus which stimulates the release of ACTH from the PD which enters the bloodstream and stimulates the release of a GC (the active glucocorticoid is taxa specific) from the adrenals or interrenals, in mammals and fish, respectively. The GC will induce or suppress gene expression in target cells and ultimately cause an increase in available energy to cope with the stressor.

The phylogenetic position of lamprey makes them an ideal species to study the HPI axis because they are extant members of the earliest vertebrate group, the agnathans, which diverged from the rest of the vertebrate lineage about 500 MYA (Kumar and Hedges, 1998). The evolution of the stress axis in lamprey remains poorly understood. Close et al., (2010) identified 11-deoxycortisol as a functional corticosteroid in sea lamprey, but the identification of the functional corticosteroid used by other lamprey species remains unknown. Identification of the functional corticosteroid in a greater number of lamprey species will help elucidate the evolution of the stress axis in lamprey. Therefore, this study aims to identify a functional corticosteroid used by Pacific lamprey. The specific objectives of this thesis are to: 1) Identify corticosteroids in plasma of Pacific lamprey 2) Determine if Pacific lamprey possess a functional HPI axis 3) Determine if elevated concentration of the putative corticosteroid has an effect on receptor expression in Pacific lamprey.
2.2 Materials and Methods

2.2.1 Materials

11-Deoxycortisol antibody was purchased from American Research Products (Waltham, MA, USA), antibodies for 11-deoxycorticosterone and corticosterone were purchased from Novus Biologicals (Littleton, CO, USA) and cortisol antibody was purchased from Millipore (Billerica, MA, USA). Standard 11-deoxycortisol, 11-deoxycorticosterone, cortisol and corticosterone were purchased from Sigma (Sigma Aldrich., St. Louis, MO, USA). 11-Deoxycortisol, 11-deoxycorticosterone, cortisol and corticosterone radioligands were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). The putative sea lamprey CRH was custom synthesized by New England Peptide (Gardner, MA, USA) based on the deduced amino acid sequence identified from the Sea lamprey genome database. ACTH was custom synthesized by BachemInc (Torrance, California, USA). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Ottawa, Canada), formic acid (puriss. p.a. for mass spectroscopy) from Fluka (Steinheim, Germany), and ultra pure water was prepared in our laboratory using a Milli-Q Synthesis system (Millipore). All other chemicals were purchased from Sigma Aldrich.

Coconut oil was purchased from Omega Nutrition (Vancouver, Canada).

Tricainemethanesulfonate MS-222 was purchased from Argent Chemical Laboratories, Inc. (Redmond, WA, USA).

2.2.2 Experimental Animals

Adult Pacific lampreys were collected in August 2012 from Stamp River, Port Alberni, B.C. with the use of dip nets in vertical slot fish ladders and in August 2013 from
Skeena River, Morristown, B.C. with the use of dip nets. Fish were transported to the University of British Columbia and held in insulated tanks kept 7-10°C with flow through dechlorinated tap water. Fish were allowed to acclimate for at least 2 weeks prior to any experiments. All fish were maintained in accordance with the Canadian Council on Animal Care and research experiments performed were approved by the UBC Animal Care Committee (A11-0245).

2.2.3 Blood collection

Pacific lamprey were anesthetized using buffered tricainemethanesulfonate (MS-222) and then blood samples (1-2 mL) were taken from the caudal vein into heparinised vacutainer tubes. Blood was spun at 1,000 x g at 4°C for 12 min, plasma was transferred into 1.5 mL tubes and stored at -80°C until use.

2.2.4 Radioimmunoassay

All plasma samples were subjected to ether extraction using a protocol outlined in Brenner et al., (1973) before assayed with radioimmunoassay (RIA). Briefly, twenty µL of plasma sample was ether extracted in duplicate. Two hundred µL of diethyl ether was added to each tube and tubes were vortexed for 30 sec. The tubes were centrifuged (1,000 x g for 2 min at 4°C) to allow the phases to be separated. The bottoms of the tubes were dipped into a bucket containing dry ice and methanol to freeze the aqueous layer, and then the organic phase was transferred to a clean test tube. This process was carried out for a total of three times to extract the steroids from the plasma. The organic phase was dried under a stream of N₂ gas. The dried tubes received 20 µL of RIA buffer and then were used for RIA.
RIA was performed as in Scott et al., (1980). Briefly, RIA was conducted in glass culture tubes (10 mm x 75 mm, Fisher Scientific) in duplicate. Assay buffer was made of 50 mM sodium phosphate, pH 7.4, 0.2% BSA, 137 mM NaCl, 0.40 mM EDTA, and 0.77 mM sodium azide. Nine standard tubes were made ranging from 1.95-500 pg of standard steroid (11-deoxycortisol, 11-deoxycorticosterone, cortisol or corticosterone) in 100 μl assay buffer. Blank, total and maximum tubes received 100 μl of assay buffer. Sample tubes received 20 μl of plasma and 80 μl assay buffer. Radiolabel-buffer solution was made such that 100 μl of this solution would contain 5000 disintegrations per minute (DPM). 100 μl of this solution was added to blank tubes. Antibody was added to the label-buffer solution such that 50% of the radiolabel was bound to the antibody in the absence of any standard steroid. All tubes (except blank tubes) received 100 μl of this antibody-label-buffer solution. Tubes were incubated overnight at 4°C. The next day tubes were placed on ice and 500 μl of ice cold charcoal solution (50 mM sodium phosphate, pH 7.4, 0.1% gelatin, 1.0% dextran-coated charcoal) was added to all tubes (except total tubes which received 500 μl assay buffer). After 15 minutes, the tubes were centrifuged at 1,000 x g, 4°C for 12 minutes, decanted into 7 mL scintillation vials (Fisher Scientific), and mixed with 5 mL scintillation cocktail (RPI Corp.). DPM were counted with an LS-6500 scintillation counter (Beckman Coulter, Mississauga, Ontario, CAD) using a 5 min counting program.

**2.2.5 Screen of HPLC Fractions from Pacific Lamprey Plasma**

Five Pacific lampreys were netted, anesthetized and blood samples were immediately taken. After centrifugation of the blood, 5 mL of plasma was pooled together and filtered using a 0.45 μm filter (Millipore, Billerica, MA, USA). The filtered plasma was passed through an activated Sep-Pak (Waters Associates Inc., Milford, MA) and eluted with
10 mL of methanol. The methanol extract was then dried down using a CentriVap Concentrator (Labconco, Kansas City, MO, USA) and Benchtop Freeze Dryer (VirTis, Gardiner, NY, USA). The dried sample was re-suspended in 700 µl of solvent A (0.01% formic acid in deionized water) and 300 µl of solvent B (70% acetonitrile and 0.01% formic acid in deionized water) and then passed through a 45 µm PVDF filter (Fisher Scientific). The sample was centrifuged at 12,000 x g for 10 min and then loaded onto a C18 reverse-phase HPLC column (Alltima, 4.6 mm x 250 mm, Alltech, Dearfield, IL, USA) fitted with a guard module. Oven temperature was kept at 40°C. Solvents A and B were used to create the column gradient and the development pattern was as follows: 0-10 min: 28% B; 10-60 min: 28-100% B; 60-90 min 100% B. Total running time was 90 min (flow rate: 0.5 mL/min) and fractions were collected every 1 min between 21-80 min into vials. Fractions 21-80 were dried down in the CentriVap Concentrator and Benchtop Freeze Dryer and then re-suspended with 50 µL of 100% ethanol and 450 µL of RIA buffer and vortexed. Fractions 21-80 were then screened using RIA (described above) to determine the concentration of 11-deoxycortisol, 11-deoxycorticosterone, cortisol and corticosterone. Position of standard 11-deoxycortisol, 11-deoxycorticosterone, cortisol and corticosterone was determined by loading 6 µg of synthetic steroids (dissolved in 700 µL solvent A and 300 µL solvent B) onto the C18 reverse phase HPLC column, in separate runs, and the eluate was monitored for UV absorption with a photodiode array detector (Analytical Technologies Group, Gorton, CT, USA).
2.2.6 Pre- and Post- Acute Stress Experiment & Confirmation of Changes in 11-deoxycorticosterone and 11-deoxycortisol

Four Pacific lampreys were allowed to acclimate for one week before the experiment. Following acclimation, Pacific lampreys were netted, blood samples were taken (pre-stress sample), and then the Pacific lamprey were placed into a dry bucket for 5 minutes while the bucket was lightly shaken by hand. After the stressor, the fish were returned to the holding tanks. The fish were netted once again, one hour after the stressor and blood samples were taken for the second time (post-stress sample). Plasma was frozen at -80° C until use. Steroid extraction and HPLC fractionation of the individual plasma samples was performed as described above, with slight modification in elution volume of 5 mL methanol. Fractions 41-70 were collected, dried down and screened with RIA for 11-deoxycorticosterone and 11-deoxycortisol.

2.2.7 Acute Stress Time Series Experiment

Female Pacific lampreys (n=7-10) were allowed to acclimate in their respective tanks for one week. Following the acclimation, the fish were netted and placed into a dry bucket for 5 minutes while the bucket was lightly shaken by hand and then placed back into the tanks. Fish were netted once again, placed into a bucket containing anesthetic and blood samples were immediately taken at different time intervals: 0.5, 1, 2, and 4 hr post stress. Control groups were left undisturbed and blood samples were immediately taken after being netted out of the tank. Plasma was stored at -80° C until use. Plasma levels of 11-deoxycortisol were determined using ether extraction and RIA (as described above).
2.2.8 CRH Injection Experiment

Plasma samples from a CRH injection experiment carried out by Brent Roberts (Masters’ dissertation, 2012) using saline control injections and CRH injections were analyzed for concentrations of 11-deoxycortisol. Briefly, following a one week acclimation, fish were netted and injected intraperitoneally with either 0.9% saline as a control (female n= 6-13; male n=1-3), or a specific dose of sea lamprey CRH peptide (0.1, 50, 100 μg/kg body weight dissolved in 0.9% saline solution). Fish were placed in a recovery bucket and then returned to holding tanks after injections and blood samples were taken 1 hr after injections. Plasma samples were stored at -80°C until use. Concentration of plasma 11-deoxycortisol in these plasma samples was determined using ether extraction and RIA (as described above).

2.2.9 ACTH Injection Experiment

Following one week acclimation, Pacific lamprey were netted and placed into a bucket containing water and MS-222. Once anesthetized, the lamprey were intraperitoneally injected with either 0.9% saline (n=10; 5 male and 5 female) or a mixture of 4 ACTH peptides (n=10; 5 male and 5 female). Four ACTH peptides (ACTH (1-60), ACTH (1-60P), ACTH (1-59), ACTH (1-59P)) were mixed together in saline and injected at a dose of 100 μg of each peptide per kg body weight dissolved in 0.9% saline. Fish were placed back into holding tanks after the injections and blood samples (as described above) were taken 1 hour after injections. Plasma samples were stored at -80°C until use to determine plasma levels of 11-deoxycortisol using RIA on ether extracted plasma (as described above).
2.2.10 Liquid Chromatography-Mass Spectrometry

The HPLC fraction with 11-deoxycortisol immunoreactivity (fraction 48) and synthetic 11-deoxycortisol were further analyzed by using ultra high performance liquid chromatography-tandem mass spectrometer (UHPLC/MS/MS) in separate runs to confirm the identity of 11-deoxycortisol in fraction 48.

2.2.11 Implant Injections – Experimental Design and Sampling Procedure

Coconut oil was melted at 40°C and 11-deoxycortisol dry steroid was added and vials were then vortexed until steroids were completely dissolved. 11-Deoxycortisol was dissolved at concentrations of 0.1 and 0.2 mg 11-deoxycortisol/1.0 mL coconut oil to achieve 0.5 and 1.0 µg/g body weight dose levels respectively. The coconut oil vials remained on a heater until use. Fish were divided into 4 groups (n=8): control (no injection), sham injected (coconut oil injection), 0.5 µg/g 11-deoxycortisol implant and 1.0 µg/g 11-deoxycortisol implant and allowed to acclimate in their respective tanks for one week before the experiment. Pacific lampreys were netted, placed in a bucket containing a buffered solution of MS-222 (0.06 g/l) and NaHCO3 (0.15 g/l), weighed and injected with either 11-deoxycortisol dissolved in coconut oil or coconut oil alone via ip injection with a 500 µL Hamilton syringe with an implant volume of 0.5 mL/100 g body weight (control fish were weighed but not injected). Fish were immediately placed into recovery buckets post injection (or post weighing for the control fish) and then returned to holding tanks. Fish were sacrificed by placing them in a bucket with a kill dose of buffered anesthetic 7 days post implantation. Blood was collected from anesthetized Pacific lamprey via caudal vein using heparinised vacutainer tubes and spun at 1,000 x g at 4°C for 12 min. Plasma was
stored at -80°C until use. After euthanasia by decapitation, tissues were collected, snap frozen with liquid nitrogen and stored in the -80°C until use.

2.2.12 Real Time PCR

Total RNA Extraction and Quantification

Muscle, liver, heart, gill and testis samples collected from the 11-deoxycortisol implant study were stored at -80°C and used for RT-qPCR. All 32 samples for a tissue type were handled on the same day to minimize differences between different days (i.e. gill samples were extracted on same day, muscle samples were extracted on the following day). Roughly 100 mg of tissue was suspended in an eppendorf tube containing 700 µL of TRIzol and allowed to thaw on ice. Ten 1 mm zirconium oxide beads were added to the tubes and homogenized in a beat homogenizer for 3 min. Gill tissue was not sufficiently homogenized after 3 min and had to be homogenized one more time (3 min) after being cut into smaller pieces with scissors. Tubes were allowed to sit for 5 min at room temperature, and after adding 200 µL of chloroform tubes were shaken by hand for 30 sec. Following a 3 min incubation at room temperature, the tubes were centrifuged for 15 min at 12, 000 x g at 4°C. The top layer (roughly 400 µL) was pipetted into new tubes to which 500 µL of isopropanol was added, the tubes were inverted 5-10 times, incubated for 10 min at room temperature and centrifuged for 10 min at 12, 000 x g at 4°C. The isopropanol phase was decanted and 1 mL of 75% ethanol was added to the tubes. The tubes were lightly vortexed to allow the pellet to be suspended in the ethanol, and centrifuged for 5 min at 7,500 x g at 4°C. The ethanol phase was removed and tubes were incubated at 37°C for 5 min to allow all the ethanol to evaporate. Culture water (60-100 µL) was added to the tubes, and the
tubes were placed in a water bath at 55°C for 5-10 min to allow the pellet to dissolve. The tubes were then stored at -80°C until use. The RNA extracts were thawed on ice and RNA integrity of the samples was examined by electrophoresis using a 1% agarose gel at 140 V with 1 μL of RNA. RNA was quantified spectrophotometrically in triplicates by measuring absorbance at 260 nm and the DNA/RNA ratio was calculated by the OD260/280 ratio; a ratio between 1.8-2.0 indicates the sample is composed of RNA.

**DNase Treatment and cDNA Synthesis**

Samples were subjected to DNase treatment using a DNA-free kit (Ambion, Austin, TX) by following the manufacturer’s instructions using 2 μg of RNA. The DNase treated RNA was then used for first strand cDNA synthesis using the high capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. Briefly, 10 μL of master mix (volume/reaction are as follows: 2.0 μL buffer, 0.8 μL dNTP, 2.0 μL random primers, 1.0 μL inhibitor, 1.0 μL Taq (Ambion) and 3.2 μL culture water) was added to strip tubes for each of the cDNA reactions along with 1 μL of DNase treated RNA. The strip tubes were placed in the Peltier Thermal Cycler (PTC-200, MJ research) and thermal settings were as follows: 40 min at 25°C, 2 hr at 37°C, and 5 min at 85°C. cDNA samples were stored at 4°C.

**Reverse Transcriptase PCR**

Primers for Sea lamprey CR were designed using Primer3. Primers for Sea lamprey PR and 18S were designed using Primer Express 3.0. PCR was performed using 1 μL of Pacific lamprey liver cDNA with 2.5 μL 10x buffer, 0.4 mM dNTPs, 1.25 units of Taq Polymerase, 2 μM MgCl₂, 0.4 μM forward and reverse primer, for a total reaction volume of
25 μL. The tubes were placed in the thermal cycler and the PCR cycling conditions were as follows: 3 min at 94°C, and then 40 cycles of 30 sec at 94°C, 30 sec at the primers annealing temperature (55°C, 58°C, and 59°C for sea lamprey CR, PR and 18S, respectively), 2 min at 72°C. PCR products were analyzed by electrophoresis using a 1% agarose gel at 140 V. A single band of the appropriate size appeared for the three different genes, thus the remainder of the PCR products were purified using GeneJET PCR Purification Kit (Thermo Scientific, Canada) and sent for sequencing to Integrated DNA Technologies.

**Real Time PCR**

Primers based on Pacific lamprey CR, PR and 18S sequences were designed using Primer Express 3.0 and synthesized by Integrated DNA Technologies. Separate standard curves were made for each tissue type; 1 μL from each cDNA sample in a particular tissue type was mixed and a dilution series (1:5) was performed to generate a standard curve. The plate was kept on ice while loading. Twenty μL of the real time PCR master mix (10 μL SYBR Green and 0.2 μM of forward primer and reverse primer) was loaded onto a 96 well plate along with either 1 μL of Pacific lamprey cDNA sample, standard curve dilution, culture water to serve as a no enzyme control, or RNA to serve as a no reverse transcriptase control, in duplicates. PCR on Pacific lamprey 18S was carried out with 1 μL of a 1/500 dilution of cDNA samples. The plate was sealed with adhesive tape before being spun at 100 x g for 1 min and the real time PCR was carried out on the plate using the ABI PRISM 7000 sequence detection system (Applied Biosystems). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min, then a dissociation of 95°C for 15 sec, 60°C for 20 sec and 95°C for 15 sec.
2.2.13 Statistical Analyses

Data are expressed as mean ± SEM, and statistical significance is assumed at P<0.05. Statistical analyses were performed using Prism 5.00 (GraphPad Software Inc, California, USA). Two-way ANOVA followed by a Bonferroni Post test was performed on the pre- and post-acute stress experiments to evaluate differences between the means for each of the fractions in the two groups. For the ACTH injection an unpaired t-test and F-test was used to compare significant differences in the variances and means between the groups. One way ANOVA followed by Dunnett’s comparisons was used to assess significant differences between means for the CRH injection experiment and acute stress time series experiment. PR and CR expression was normalized with expression of the 18s gene. For the CR and 18s, replicates with a standard deviation of less than 0.2 Ct were accepted. Large differences in the final amount of amplified material between replicates can be produced if samples have an initially low transcript level (Peccoud and Jacob, 1996) and therefore replicate values with a standard deviation of less than 0.35 Ct were accepted for the PR transcript.

2.3 Results

2.3.1 Steroid extraction, HPLC fractionation and RIA screening for corticosteroids

The highest immunoreactive peak was at fraction 48 with the 11-deoxycortisol antibody (Fig 1B). The cortisol and 11-deoxycorticosterone antibodies also showed immunoreactivity with fraction 48. Standard 11-deoxycortisol elutes at fraction 48 under the same HPLC conditions, corresponding to the highest immunoreactive peak from the HPLC fractions. Two small immunoreactive peaks were also present; one at fraction 46 with the corticosterone antibody and the other at fraction 57 with the 11-deoxycorticosterone antibody (Fig 1A). Authentic standard corticosterone and 11-
deoxycorticosterone elute at fractions 46 and 57, respectively. Immunoreactive cortisol did not appear to be above background levels. Authentic standard cortisol elutes at fraction 39.

2.3.2 Pre- and Post- Acute Stress and changes in concentrations of 11-deoxycortisol

There was no significant difference in concentration of 11-deoxycorticosterone between pre- and post- acute stress plasma HPLC fractions (Fig 2A). A significant increase (P<0.001) in plasma 11-deoxycortisol concentration was seen 1 hour after acute stress exposure in comparison to the pre-stress concentration. The concentration of 11-deoxycortisol pre-stress was 0.17 ± 0.03 ng/mL (mean ± SEM) and increased to 1.13 ± 0.07 ng/mL in the same individuals post-stress. No other fractions showed significantly different immunoreactivities between the pre- and post- stress groups.

2.3.3 Identification of 11-deoxycortisol

Multiple reaction monitoring (MRM) experiments using m/z 347.0 → 109.2 transition on fraction 48 and synthetic 11-deoxycortisol confirmed the presence of 11-deoxycortisol, as evidenced in the peak at 4.75 min (Fig. 3). The peak at 4.75 min produced from fraction 48 coincided with that from the synthetic standard, confirming the identity of 11-deoxycortisol.

2.3.4 Acute Stress Time Series

Acute stress induced by 5 min of dewatering caused circulating concentrations of 11-deoxycortisol to significantly (P<0.001) increase by 0.5 and 1 hr (P<0.001; P<0.01, respectively). Basal levels of 11-deoxycortisol were 0.59 ± 0.057 ng/mL (mean ± SEM) and significantly increased to 1.15 ± 0.11 ng/mL and 0.91 ± 0.06 ng/mL by 0.5 and 1 hr, respectively (Fig. 4). Plasma 11-deoxycortisol concentrations returned to basal
concentrations by 2 hr after the acute stress (0.62 ± 0.09 ng/mL) and remained at basal levels at 4 hr (0.57 ± 0.04 ng/mL).

2.3.5 CRH Injection Experiment

Pacific lamprey injected with sea lamprey CRH showed significantly increased plasma concentrations of 11-deoxycortisol in comparison to saline injected controls (female n= 6-13/treatment; male n=1-3/treatment) (Fig. 6). Saline injected Pacific lamprey had plasma 11-deoxycortisol concentrations of 0.60 ± 0.09 ng/mL (mean ± SEM) for females and 0.55 ± 0.15 ng/mL for males and 0.1 μg/kg CRH dose induced a statistically significant (P<0.05) increase in females with plasma 11-deoxycortisol rising to 3.39 ± 0.60 ng/mL and an increase, although not significant, in males to 2.07 ± 0.47 ng/mL. Plasma 11-deoxycortisol levels were significantly increased in the 50 μg/kg of females and males to 2.91 ± 0.29 ng/mL and 3.27 ± 0.39 ng/mL, respectively and a significant increase in females injected with the 100 μg/kg doses to 3.53 ± 0.29 ng/mL. Due to low sample size, statistical analyses on males in the 100 μg/kg treatment group could not be performed.

2.3.6 ACTH Injection Experiment

Pacific lamprey injected with the 4 ACTH peptides used did not have increased plasma concentrations of 11-deoxycortisol (Fig. 6) compared to the saline injected control group. Saline injected Pacific lamprey had plasma 11-deoxycortisol concentrations of 2.48 ± 0.44 ng/mL and 2.90 ± 0.71 ng/mL for females and males respectively whereas Pacific lamprey injected with 100 μg/kg of an ACTH peptide mixture had plasma concentrations of 2.32 ± 0.69 ng/mL and 2.36 ± 0.64 ng/mL for females and males, respectively.
2.3.7 11-Deoxycortisol Implant

Plasma 11-deoxycortisol levels 7 days post implantation of only coconut oil in the sham group were not significantly different than levels in the non-injected control group, 2.38 ± 0.45 ng/mL and 2.29 ± 0.54 ng/mL, respectively (Fig 7). Plasma 11-deoxycortisol levels in the 0.5 µg/kg treatment group (4.43 ± 1.15 ng/mL) were not significantly higher than levels in the control group. There was a significant increase (P<0.0001) in plasma 11-deoxycortisol levels in the 1.0 µg/kg treatment group which had 9.46 ± 1.16 ng/mL, in comparison to the control groups with 2.29 ± 0.54 ng/mL.

2.3.8 Effects of 11-deoxycortisol implant on PR and CR expression

There was no significant difference in expression of the CR or PR gene in the muscle, liver, heart, gill or testis for the two treatment groups with the 11-deoxycortisol implant of 0.5 and 1.0 µg/g in comparison to the control group. The CR transcript was more highly expressed compared to the PR transcript in all tissues analyzed with exceptions at the 1.0 µg/g treatment group for the gills, the CR transcript was 0.82 ± 0.08.
2.4 Discussion

Results from the experiments conducted indicate that 11-deoxycortisol may function as a corticosteroid in Pacific lamprey. First, 11-deoxycortisol was identified in the plasma of Pacific lamprey using HPLC fractionation, RIA and mass spectrometry analyses. Second, plasma 11-deoxycortisol levels increased after exposure to acute stress in a time-dependent manner. Third, injections of the hypothalamic neuropeptide, CRH, caused an increase in plasma 11-deoxycortisol levels, thus there is support for hypothalamic control over 11-deoxycortisol secretion in Pacific lamprey. Plasma 11-deoxycortisol levels did not change after injection of a mixture of sea lamprey ACTH. Despite 11-deoxycortisol levels not responding to sea lamprey ACTH injections, the possibility of Pacific lamprey possessing an axis similar to the vertebrate HPA/I axis is plausible if Pacific lamprey possess a pituitary 11-deoxycortisol secretagogue which has yet to be identified. 11-Deoxycortisol implants did not change expression of CR or PR mRNA expression in muscle, liver, heart, gills or testis. Possible reasons for why elevated 11-deoxycortisol levels did not change CR or PR mRNA expression in the tissues tested will be discussed below. Close et al. (2010) showed that 11-deoxycortisol is present and functions as a corticosteroid in sea lamprey. The results presented in this thesis provide support for 11-deoxycortisol being a functional corticosteroid in Pacific lamprey.

2.4.1 Identification of 11-deoxycortisol in Pacific lamprey plasma

11-Deoxycortisol was isolated and identified from adult Pacific lamprey plasma by using HPLC, RIA and mass spectrometry. Pooled Pacific lamprey plasma was fractionated using HPLC and the collected fractions were screened with an RIA developed for 11-deoxycortisol and other corticosteroids. Fraction 48 was the only fraction out of the 60
fractions screened that was immunoreactive with the 11-deoxycortisol antibody, this fraction was analyzed using mass spectrometry and the steroid was identified as 11-deoxycortisol. The cortisol and 11-deoxycorticosterone RIAs also showed some immunoreactivity with fraction 48, which is likely due to cross reactivity. The combination of the aforementioned methods provides direct evidence for the presence of 11-deoxycortisol in the circulation of Pacific lamprey.

Plasma 11-deoxycortisol concentrations, and not plasma 11-deoxycorticosterone concentrations, increased after stress exposure. This is evidenced by measuring concentrations of both the steroids in HPLC fractionated pre- and post- stress Pacific lamprey plasma. Fraction 48 in the post- stress group was the only fraction which had significantly higher concentrations of 11-deoxycortisol compared to the pre- stress group in the RIA developed for 11-deoxycortisol. This eliminates 11-deoxycorticosterone as a candidate corticosteroid since the fraction that standard 11-deoxycorticosterone elutes was not significantly higher in the post-stress group compared to the pre-stress group. This supports the hypothesis that 11-deoxycortisol is the functional corticosteroid used by Pacific lamprey.

In the steroidogenic pathway, 11-deoxycortisol is the direct precursor to cortisol, requiring the CYP11B1 enzyme to attach a hydroxyl group at the C-11 position of 11-deoxycortisol (Payne and Hales, 2004). Close et al. (2010) have shown that cortisol is absent in sea lamprey plasma and they were also unable to find any CYP11B1 orthologs in the sea lamprey genome database. Most teleosts use cortisol as their stress hormone (Wendelaar Bonga, 1997); the absence of a CYP11B1 gene in the sea lamprey genome would prevent the conversion of 11-deoxycortisol into cortisol, thus permitting 11-
deoxycortisol to act as the stress hormone in lamprey. The presence of 11-deoxycortisol and absence of cortisol in the plasma of Pacific lamprey are in line with the fact that the CYP11B1 gene is absent in lamprey genome. If the absence of CYP11B1 gene is the ancestral state of all vertebrates, then the arrival of CYP11B1 sometime after the divergence between agnathans and gnathostomes would allow the production of cortisol or corticosterone and its later functional use. However, a phylogenetic analysis on a greater number of lamprey species looking for the presence of CYP11B1 would be required to help elucidate the ancestral vertebrate state.

2.4.2 Acute Stress Time Series

Acute stress is known to elicit corticosteroid release among vertebrates; however, different species have different latency periods between stressor recognition and significant increases in corticosteroid levels (Barton, 2002). Five min handling stress and confinement in brown trout caused significant increases in cortisol levels and highest plasma levels were detected at 60 min (Sumpter et al., 1985). Cortisol concentrations significantly increased in sparid red porgy after 8 min of net handling and were highest at 2 hr following stress exposure (Rotllant and Tort, 1997). Net handling and confinement of gilthead sea bream caused a significant increase in cortisol concentrations by 1 hr (Rotllant et al., 2001). Close et al. (2010) found similar results that sea lamprey significantly increased 11-deoxycortisol plasma concentrations 1 hr after dewatering stress and that concentrations of 11-deoxycortisol were back to basal levels 24 hr post stress. Although the rate of corticosteroid release during HPI axis activation varies, it is commonly accepted that the rise in corticosteroid concentrations generally occurs within minutes rather than hours in teleosts (Pankhurst, 2011). Exposure to acute stress caused a significant increase of
plasma 11-deoxycortisol in Pacific lamprey 0.5 and 1 hr post stress with a return to basal levels by 2 hr. This reflects the same trend seen in most fish where the highest elevation in plasma is seen 0.5-1 hr post stress (Barton and Iwama, 1991; Barton, 2002).

2.4.3 CRH Injections

The CRH gene is highly conserved among vertebrates (Lovejoy and Balment, 1999; Chang and Hsu, 2004; Lovejoy and Jahan, 2006) and Roberts et al., (2014) have shown that the sea lamprey CRH peptide and other known CRH peptide sequences have sequence and functional similarity. Human CRH and sea lamprey CRH have high sequence similarity in addition to functional similarity, as shown by Close et al. (2010) where human CRH injections caused a significant increase in plasma 11-deoxycortisol concentrations in sea lamprey. The CRH peptide derived from sea lamprey genome database was custom synthesized and injected to determine the effect of CRH on plasma 11-deoxycortisol levels. The observed sequence similarity of the CRH peptide of sea lamprey and other higher vertebrates suggests that CRH gene has been conserved and may play a similar role in these species. Although Pacific lamprey-CRH was not used, it is expected that the sea lamprey-CRH would act similarly to endogenous CRH in Pacific lamprey due to the highly conserved state of the CRH peptide among vertebrates. I demonstrated that plasma 11-deoxycortisol concentrations increased in Pacific lamprey in response to sea lamprey-CRH injections. The elevation of 11-deoxycortisol concentration in response to sea lamprey CRH injections provides support for a hypothalamic neuropeptide regulating corticosteroid release in Pacific lamprey which is similar to what is seen in other vertebrate taxa where corticosteroid release is regulated at the hypothalamus by CRH.
2.4.4 ACTH Injections

Sea lamprey ACTH was first isolated and characterized by Takahashi et al (1995a) and sequence analysis of purified sea lamprey pituitary showed the presence of four different ACTH peptides which differed in either length or phosphorylation state. ACTH\(_{(1-59)}\) and ACTH\(_{(1-60)}\) are unphosphorylated and ACTH\(_{(1-59P)}\) and ACTH\(_{(1-60P)}\) are thought to be modified by a phosphorylation at Ser35 (Takahashi et al., 2005). Differences between lamprey ACTH and gnathostome ACTH were also noted. First, gnathostome ACTH is typically 39-41 residues long whereas lamprey ACTH is 59-60 residues long. Second, the POMC precursor is expressed in the PD and PI in gnathostomes with post translational processing producing: ACTH, MSHs, and β-endorphins. In contrast to lamprey which express the POC gene in the PD and the POM gene in the PI, with POC encoding ACTH and β-endorphins and POM encoding MSHs and different β-endorphins (Heinig et al., 1995; Takahashi et al., 1995b). In the present study, an injection of all four peptides based on the sea lamprey ACTH forms into Pacific lamprey did not significantly increase plasma concentrations of 11-deoxycortisol. Similar results were seen in sea lamprey where a 100 µg/kg injection of the same four ACTH peptides did not cause a significant increase in plasma 11-deoxycortisol (Roberts et al., 2014). Potential reasons for why ACTH injections do not elicit an increase in concentrations of plasma 11-deoxycortisol were provided by Roberts et al., (2014): 1) the ACTH forms used may not be the functional adrenocorticotropic hormone; 2) the synthesized ACTH molecules may have not formed the correct structural conformation; 3) the ACTH molecules may have degraded rapidly upon injection; or 4) potentially, 11-deoxycortisol may not be the functional corticosteroid and thus does not respond to ACTH. There is evidence for hormones other than ACTH
acting as cortisol secretagogues in teleost fish. Cortisol secretion in rainbow trout is stimulated by urotensins I and II and angiotensin II has been shown to act synergistically with ACTH to stimulate cortisol secretion (Mommsen et al., 1999). α-MSH levels become elevated post stress in tilapia and show corticotrophic activity (Lamers et al., 1992). Salmonid gonadotropins (GTHs) have also been shown to increase cortisol levels in coho salmon (Schreck et al., 1989). Roberts et al., (2014) showed that sea lamprey injected with lamprey gonadotropin releasing hormone (GnRH) I, GnRH III or arginine vasotocin caused an increase in plasma 11-deoxycortisol. Although ACTH injections on their own did not change in plasma 11-deoxycortisol in Pacific lamprey, it is possible that one of the aforementioned hormones acts as the functional corticotropic hormone, or is co-released with ACTH to induce corticotropic activity in these fish, this an obvious area for further studies to identify 11-deoxycortisol secretagogues.

2.4.5 Effect of Chronically Elevated 11-deoxycortisol Concentration on Expression of CR and PR mRNA Transcript

Changes in CR mRNA have been shown to be associated with chronic stress (Shrimpton and Randall, 1994; Vijayan et al., 2003). Elevated 11-deoxycortisol levels were used to mimic physiological chronic stress to determine the effects of stress on CR and PR mRNA abundance in different tissues. Although no changes in muscle, liver, heart, gill or testis CR or PR mRNA abundance were present, there are several possible reasons for these results: 1) 11-Deoxycortisol may have an effect on CR and/or PR protein abundance and/or affinity; 2) The experimental approach used may not have been able to induce a true chronic stress response due to low implant doses of 11-deoxycortisol or the duration of the experiment may have been too short or the sample size may not have been large enough to
see a significant change; 3) Developmental stage of Pacific lamprey may have an effect on the responsiveness of receptors to stress.

Since changes in mRNA transcript levels are not always correlated with changes in protein content, the total amount of CR and/or PR protein levels may have changed in response to the 11-deoxycortisol implants even though changes in mRNA transcript levels were not seen. Trout hepatocytes exposed to physiological dose of cortisol significantly increased GR mRNA content but significantly decreased GR protein content (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003), the decrease in GR protein content is thought to drive the increase in GR mRNA abundance since a proteasomal inhibitor that prevented GR protein degradation eliminated the cortisol-mediated upregulation of GR mRNA. A GR antagonist was shown to decrease protein expression and increase GR mRNA levels in rainbow trout liver (Alderman et al., 2012). 11-Deoxycortisol may have caused changes in CR and/or PR affinity without changing levels of mRNA transcript. Dexamethasone administration and cortisol implants in rainbow trout caused a significant decrease in GR concentration and affinity (Pottinger, 1990; Lee et al., 1992). Cortisol implants in juvenile coho salmon caused a decrease in CR concentration and affinity for cortisol 10 days after implantation (Shrimpton and Randall, 1994).

It has also been noted that corticosteroids can change the expression of receptors in a time series and organ specific manner (Teles et al., 2012). Studies have also indicated that GCs may affect GR mRNA expression in a time series and tissue specific manner, Tort et al., (2012) used a cortisol implant for 7 and 14 days in gilthead sea bream and found an increase in GR mRNA in heart and head kidney 7 and 14 days post implantation, an increase in liver and muscle on day 14 but not on day 7, and an increase in gills on day 7
but a return to control levels by day 14. Although no change in CR or PR mRNA was seen 7 days post implantation of 11-deoxycortisol in Pacific lamprey, changes in CR or PR mRNA changes could require more than 7 days to see significant differences in the tissues analyzed.

Life stage is another factor which can change the responsiveness of receptors to their ligands. The number and affinity of CRs also change seasonally during the parr-smolt transformation (Shrimpton et al., 1994) and the responsiveness of gill tissue to cortisol was seen to vary with developmental stage in salmonid species (McCormick et al., 1991). The cortisol peak associated with smolting will differ depending on prior exposure to stress or cortisol as cortisol causes a reduction in CR protein in gills and therefore could retard the ability of stressed fish to smolt (Shrimpton and Randall, 1994).

In conclusion, by identifying 11-deoxycortisol in the plasma using several different methods as well as looking at the biological effects of induced stress and sea lamprey CRH injections on circulating plasma 11-deoxycortisol levels, I have demonstrated that 11-deoxycortisol is a putative corticosteroid hormone in Pacific lamprey. This is the second species of lamprey in which 11-deoxycortisol has been identified and shown to increase plasma levels in response to stress. This work along with future studies on lamprey corticosteroid signaling can contribute to the understanding of the evolution of corticosteroid signaling in the lamprey lineage.
Figure 1: HPLC/RIA identification: Concentration of (A) corticosterone, 11-deoxycorticosterone, (B) cortisol and 11-deoxycortisol (ng/fraction) from adult Pacific lamprey plasma following HPLC fractionation. Arrows show the elution point of standard (A) corticosterone, 11-deoxycorticosterone, (B) cortisol and 11-deoxycortisol.
Figure 2: HPLC/RIA on pre and post stressed Pacific lamprey plasma. Concentrations of (A) 11-deoxycorticosterone (ng/fraction) and (B) 11-deoxycortisol in fractions 41-70 for pre- and post- acute stressed individuals (n=4). Results are mean ± SEM and asterisks indicate a significant difference (P<0.001) with a two-way ANOVA followed by Bonferroni post-tests.
Figure 3: Identification of 11-deoxycortisol by LC/MS analysis. Multiple reaction monitoring (MRM) was performed with transition \( m/z \) 347 > 109 using UHPLC-MS (positive mode) over 8 min. Note that 11-deoxycortisol was detected at 4.75 min in both standard (A) and natural (B) samples.
Figure 4: Acute Stress Experiment: Concentration of 11-deoxycortisol in plasma of female Pacific lamprey (n= 7-10) at different time points after acute stress by dewatering. Results are mean ± SEM. Asterisks indicate a significant (***P < 0.01; ****P < 0.001) difference with one-way ANOVA followed by Dunnett’s comparison to controls.
Figure 5: CRH injections: Concentration of 11-deoxycortisol in plasma of adult Pacific lampreys after injection of lamprey CRH (females n=6-13; males n=1-3). Results are mean ± SEM. Asterisks indicate a significant (*P < 0.05; ***P < 0.001) difference with one-way ANOVA followed by Dunnett’s comparisons to controls.
Figure 6: ACTH injections: Concentrations of 11-deoxycortisol in plasma of adult Pacific lamprey (females n=5; males n=5) with injections of either saline or a mixture of 4 ACTH peptides (ACTH (1-60), ACTH (1-60P), ACTH (1-59), ACTH (1-59P)) at levels of 100 µg/kg of each peptide. Each group (saline and ACTH injected). Results are mean ± SEM. Significant differences between groups were determined using a t-test.
**Figure 7**: Effects of 11-deoxycortisol implants on concentration of plasma 11-deoxycortisol in Pacific lamprey (n=8). Results are mean ± SEM.
Figure 8: Effect of 11-deoxycortisol implants on corticosteroid receptor (CR) mRNA abundance in adult Pacific lamprey in different tissues, 7 days post implantation of either coconut oil alone or coconut oil and 11-deoxycortisol at doses of 0.5 or 1.0 µg 11-deoxycortisol per g or non-injected controls. CR mRNA from muscle, liver, heart, gill and testis was quantified using real-time PCR and normalized to 18S rRNA. Values represent the means ± SEM (n=7-8; except testis which had n=4-7).
Figure 9: Effect of 11-deoxycortisol implants on progesterone receptor (PR) mRNA abundance in adult Pacific lamprey in different tissues, 7 days post implantation of either coconut oil alone or coconut oil and 11-deoxycortisol at doses of 0.5 or 1.0 µg 11-deoxycortisol per g or non-injected controls. PR mRNA from muscle, liver, heart, gill and testis was quantified using real-time PCR and normalized to 18S rRNA expression. Values represent the means ± SEM (n=4-7; except testis which had n=1-5).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>Sea lamprey CR</td>
<td>F: TCT GCC ACA TCC TTT CGT C</td>
</tr>
<tr>
<td></td>
<td>R: TGT CAG GAA ACT CCA CAC TCC</td>
</tr>
<tr>
<td>Sea lamprey PR</td>
<td>F: ACG TTT CGG GGC TCT CCT A</td>
</tr>
<tr>
<td></td>
<td>R: ACT GCA CGA AGG TGT GGA AG</td>
</tr>
<tr>
<td>Sea lamprey 18S</td>
<td>F: ACC AAA ACC AAT CCG GGC T</td>
</tr>
<tr>
<td></td>
<td>R: CCC GCC AGT CCC TCT TAA TC</td>
</tr>
<tr>
<td>Pacific lamprey CR</td>
<td>F: AGT CCC GCA CAG TCC TCA GT</td>
</tr>
<tr>
<td></td>
<td>R: GCA GCC CGA AGC TTC ATC</td>
</tr>
<tr>
<td>Pacific lamprey PR</td>
<td>F: ATG GGC CTT ATG GCC TTT G</td>
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<td></td>
<td>R: CCC GTT GGC GAG CTT GTA</td>
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<tr>
<td>Pacific lamprey 18S</td>
<td>F: ACG CCT GAA TAG TGC AGC TGG GAA T</td>
</tr>
<tr>
<td></td>
<td>R: AAT CAT GGC CTC CGT TCC GAA AAC C</td>
</tr>
</tbody>
</table>

**Table 1:** Gene-specific primer sequences were used for reverse transcriptase PCR and real-time PCR. Forward (F) and reverse (R) primers are listed for each gene. CR, corticosteroid receptor; PR, progesterone receptor; 18S, 18S ribosomal RNA.
Chapter 3: Conclusions

3.1 Thesis Summary

The research presented in my thesis provides evidence for 11-deoxycortisol acting as putative corticosteroid in Pacific lamprey, however, the results do not conclusively support or reject Pacific lamprey having an HPI axis which is present in other vertebrate taxa. However, it is clear that the hypothalamic neuropeptide, CRH, does have a role in regulating 11-deoxycortisol release which is similar to what is seen in other vertebrate taxa. 11-Deoxycortisol was found in the plasma of adult Pacific lamprey. 11-Deoxycortisol, and not 11-deoxycorticosterone, was found to increase in concentration one hour after acute stress, thus providing support for 11-deoxycortisol and not 11-deoxycorticosterone being the active corticosteroid which is involved in the stress response.

Pacific lamprey increased plasma 11-deoxycortisol concentrations one hour after being injected with sea lamprey CRH. However, an injection of a mixture of four sea lamprey ACTHs (ACTH_{1-60}, ACTH_{1-60P}, ACTH_{1-59}, ACTH_{1-59P}) did not cause a significant change in plasma 11-deoxycortisol concentration in Pacific lamprey one hour post injection. These results do not provide evidence for the presence of an HPI axis, however more research needs can elucidate whether this is true. These results are similar to those seen in sea lamprey where sea lamprey responded to sea lamprey CRH injections, but not sea lamprey ACTH injections, with an increase in plasma 11-deoxycortisol (Roberts et al., 2014). Further research is required to elucidate whether lamprey possess a pituitary peptide which acts as a corticosteroid secretagogue. If lamprey ACTH does not have
corticotropic activity, it is possible that ACTH gained its corticotropic activity sometime after agnathans split from the vertebrate lineage.

Acute stress caused by dewatering caused plasma 11-deoxycortisol concentration to significantly increase by 0.5 and 1 hr and concentrations returned to basal concentrations by 2 hr. This is similar to other vertebrates species where a significant increase in corticosteroid concentrations is seen 1 hr post stress (Barton, 2002).

Elevated plasma 11-deoxycortisol for 7 days did not cause levels of CR or PR mRNA transcript to change in muscle, gill, liver, heart or testis.

3.2 Thesis Objectives and Hypotheses

Objective 1: Identify corticosteroids in plasma of Pacific lamprey

11-Deoxycortisol was found in the plasma of adult Pacific lamprey plasma.

Objective 2: Determine if Pacific lamprey possess a functional HPI axis.

CRH injections, but not ACTH injections, caused plasma 11-deoxycortisol concentrations to increase. Acute stress by dewatering caused plasma 11-deoxycortisol concentration to increase by 0.5 and 1 hr and returned to basal levels by 2 hr.

Objective 3: Determine if elevated concentration of the putative corticosteroid has an effect on receptor expression in Pacific lamprey.

Seven days of elevated 11-deoxycortisol concentrations did not cause CR or PR mRNA transcript levels to change in any of the tissues examined.
3.4 Implications

Pacific lamprey populations have declined tremendously in abundance and distribution in the past few decades and this species’ existence is threatened due to factors such as habitat loss, damming and stream degradation. A widely used clinical indicator of stress is to measure circulating plasma levels of corticosteroids in animals. The identification of 11-deoxycortisol as a corticosteroid in Pacific lamprey can be used as a clinical indicator of stress and a means to monitor stress in Pacific lamprey.

Pacific lamprey are modern representatives of the most basal vertebrate group, the agnathans, and identification of the functional corticosteroids in a greater number of lamprey species is vital to elucidating the identity of corticosteroids in early vertebrates and the evolution of the stress response in the vertebrate lineage.

3.5 Research Limitations & Future Directions

The present study serves as a starting point in understanding the role of 11-deoxycortisol in the stress response of Pacific lamprey. Pacific lamprey CRH and Pacific lamprey ACTH were not used in the experiments due to lack of genomic database for Pacific lamprey. Only one male was used in the CRH 100 µg/kg treatment group, having a larger number of males in this treatment group could help elucidate the presence of a dose response relationship between CRH injection and rise in plasma 11-deoxycortisol.

Future work should use Pacific lamprey-CRH to better understand the effect on plasma 11-deoxycortisol and ACTH, and then determine whether there is a dose-response relationship between CRH and plasma 11-deoxycortisol levels.
Future research should focus on the presence and identification of a pituitary corticotropin in Pacific lamprey. The presence of a corticotropin in the pituitary can be accomplished by injecting lamprey with pituitary extracts and determining if there is a rise in plasma 11-deoxycortisol concentration. If 11-deoxycortisol levels become elevated in response to pituitary extract injections, then the next step would be to isolate the 11-deoxycortisol secretagogue. Isolation of the pituitary peptide which is responsible for increased 11-deoxycortisol synthesis can be done several ways. The pituitary extract can be fractionated and then each fraction can be tested for its 11-deoxycortisol secretagogue activity. One way would be to inject groups of lamprey with one of the fractions and see which group has elevated plasma 11-deoxycortisol concentrations. Another way would be to incubate each of the fractions with Pacific lamprey interrenal tissue (with appropriate tissue medium) and a radioactive 11-deoxycortisol precursor (e.g. pregnenolone or progesterone) and measure which fraction(s) caused the highest yield of radioactive 11-deoxycortisol in the tissue medium. The next step would be to identify the 11-deoxycortisol secretagogue in the fraction by mass spectrometry or another method. Further studies would still be necessary to support the corticotrophin activity of the identified pituitary peptide.

Despite elevated levels of 11-deoxycortisol not affecting CR or PR mRNA abundance, the effects of elevated 11-deoxycortisol on CR and PR protein abundance and binding affinity should be examined since changes in mRNA transcript levels are not indicative of changes occurring at the protein level. Changes in receptor concentration or binding affinity will change tissue responsiveness to circulating steroids. Elevated 11-deoxycortisol levels may cause significant differences at the protein level despite the absence of a
significant difference in mRNA levels, and thus may still effect the tissue’s responsiveness to 11-deoxycortisol. The changes in expression of corticosteroid-responsive genes (e.g. PEPCK, alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase) in response to elevated 11-deoxycortisol levels should be examined to determine if 11-deoxycortisol has a role in the stress response process and/or CR signaling. Changes in plasma sex steroid concentrations during long term exposure of elevated 11-deoxycortisol should also be examined to see if 11-deoxycortisol suppresses sex steroidogenesis.
References


