The stress steroid system of the Pacific hagfish, *Eptatretus stoutii*

by

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Abstract

The Pacific hagfish, *Eptatretus stoutii*, is a basal, jawless marine fish considered key to understanding the evolution of vertebrates as is their sister group, the lampreys. Cyclostomes like hagfishes and lampreys differ from gnathostomes in their stress response system including the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis. For hagfishes, little is known about the identity of their primary corticosteroid(s), steroidogenic enzyme(s), and steroidogenic tissue(s).

Through bioinformatic analyses, including ancestral character reconstruction (ACR), cytochrome p450 (CYPs) and hydroxysteroid dehydrogenase (HSDs) enzymes involved in the stress steroid synthesis pathway were examined as traits in fishes to predict the primary corticosteroid pathway of hagfishes. Hagfishes were predicted to possess genes for 3β-HSD, CYP11a1, CYP17, and CYP21. The presence of these implies that hagfishes could synthesize 11-deoxycorticosterone (DOC) and 11-deoxycortisol (11-DOC). Previous studies, however, did not detect DOC nor 11-DOC in hagfish plasma.

Physiological experiments were also performed on Pacific hagfish. These experiments involved injecting hagfish with steroid implants containing an upstream steroid (pregnenolone or progesterone) early in the steroid synthesis pathway to induce mineralocorticoid and glucocorticoid responses. Fish were also exposed to either an acid (HCl) injection or an exercise stressor (30 minutes of handling) to induce a stress response. Hagfish plasma analyzed via targeted liquid chromatography tandem mass spectroscopy (LC-MS/MS) detected pregnenolone and progesterone in the low concentration range (< 3 ng/mL) in stressed fish without steroid injection. The detection of these two steroid products present in the steroidogenesis pathway implies the presence of two key steroidogenic enzymes, CYP11a1 and

3β-HSD, in hagfish. No cortisol was detected by LC-MS/MS thereby providing further support that cortisol is not a corticosteroid found in hagfish and that CYP11b, the enzyme that assists in the synthesis of cortisol, is absent or not expressed by hagfish. It should be noted that steroid standards for DOC and 11-DOC were not present in this analysis, and therefore, we could not determine whether these steroids were present or absent from Pacific hagfish.

Preliminary work to perform real-time quantitative polymerase chain reaction (RT-qPCR) resulted in four reference genes suitable for mapping gene expression in the gonad, the gut (fore-, mid-, and hindgut regions), and the kidney (upper and lower regions). These primers can be used to continue the investigation of the expression of steroidogenic enzymes of hagfish tissues.

In response to the exercise stressor, plasma glucose concentrations in hagfish increased. Significant increases in plasma glucose levels in exercised fish without steroid injection ranged from 0.8 – 9.5 mM on average over the time course of the experiment (48 h). While exercised pregnenolone and progesterone-injected fish had plasma glucose levels ranging from 0.4 – 5.5 mM and 0.6 – 6.4 mM over 48 h, respectively. The injection of either pregnenolone or progesterone resulted in a decline of the gluconeogenic response in hagfish compared to exercise-stressed fish without any steroid injection, suggesting that these steroids have a suppressive effect on glucose release.

In response to the acid stressor, all fish experienced metabolic acidosis (mean pH 6.4 – 6.8, bicarbonate ion concentration ranging 2.12 – 5 mmol/L) within the first 2 h post treatment. Morphometric analysis of hagfish gills at 48 h post-acid stressor demonstrated a decrease in the thickness of the lamellar epithelium compared to control fish. Changes in lamellar microplicae due to the acid stressor were also observable via scanning electron microscopy. Fish treated

with the pregnenolone injection and acid had elongated microplicae compared to control fish microplicae that were shorter and rounder in appearance.

The findings of this research demonstrate the advancement of methods and understanding of the stress steroid system of the Pacific hagfish. Hagfish do not possess cortisol as their predominant corticosteroid thereby demonstrating a novel stress steroid profile that is distinct from the rest of the gnathostome lineage.

Preface

This thesis is an original work by Christiana R. Frost. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Animal Care and Use Committee and the Bamfield Marine Sciences Animal Care Committee. Approval was granted under the following protocols: Project name "Acid-base and ionoregulation in fishes"; University of Alberta, Animal Use Protocol (AUP) 00001126_AME6 (2019 – 2021); Bamfield Marine Sciences Centre (BMSC) RS-20-14 and RS-21-04 (2020 – 2021).

Some of the research conducted for this thesis forms a part of international research collaborations led by Professor G. G. Goss of the University of Alberta. I (Christiana R. Frost) performed the majority of the work presented in this original MSc. thesis. However, owing to collaborative efforts, the terms "we" and "our" may be used in this thesis. Chapter II was submitted and is under consideration for publication in the Journal of General and Comparative Endocrinology (GCE). The biographical details of this chapter are listed below and indicated at the beginning of the chapter. The roles of all authors for this chapter along with specific contributions are described below. Other contributions are also presented below.

Chapter II:

Frost, C.R. and Goss G.G. 2021. Absence of some cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes results in novel stress steroid profiles in hagfishes. Journal of General and Comparative Endocrinology. Submitted 47 pages on Dec. 6, 2021. Manuscript ID: GCE-D-21-00379.

This work was conceived and designed by GGG and CRF. CRF performed the research and conducted analysis. CF and GGG analyzed the data and together they wrote, drafted, and revised the manuscript.

Chapter III:

The liquid chromatography tandem mass spectroscopy (LC-MS/MS) work and analysis were done in collaboration with The Metabolomics Centre for Innovation (TMIC) and Dr. David Wishart's lab at the University of Alberta. Study coordination was performed by Rupa Mandal. Sample preparation was performed by Edison (Ying Wei) Dong and Mathew Johnson. Analysis was performed by and sample preparation and LC-MS/MS protocol were obtained from Jiamin (Tammy) Zheng.

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Table of Contents

List of Tables

List of Figures

List of symbols, nomenclature, and abbreviations

[ ]	concentration
11-DOC	11-deoxycortisol
11β-HSD	11-beta-hydroxysteroid dehydrogenase
17β-HSD	17-beta-hydroxysteroid dehydrogenase
^{125}I	iodine-125
18S	18S ribosomal RNA
3β-HSD	3-beta-hydroxysteroid dehydrogenase
ACE	ancestral character estimation
ACR	ancestral character reconstruction
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
AR	androgen receptor
ATPase	adenosine 5'-triphosphatase
AU	approximately unbiased
Au/Pd	gold/palladium
AUP	animal use protocol
B	brain
BIC	Bayesian Information Criterion
BLAST	basic local alignment search tool
BMSC	Bamfield Marine Sciences Centre
BP	bootstrap proportion
C	Celsius

CAD	collision gas
cDNA	complementary DNA
CE	collision energy
cm	centimetres
Cnot ctd	CCR4-NOT Transcription Complex Subunit 1
CO_2	carbon dioxide
COX1	cytochrome c oxidase subunit 1
CR	corticosteroid receptor
CRF	corticotropin-releasing factor
C_t	threshold cycle
CUR	curtain gas
CXP	cell exit potential
CYP	cytochrome p450
CYP11A1	cytochrome P450 Family 11 Subfamily A Member 1
CYP11B	cytochrome P450 Family 11 Subfamily B
CYP17	cytochrome P450 Family 17
CYP19	cytochrome P450 Family 19
CYP21	cytochrome P450 Family 21
DFO	Department of Fisheries and Oceans
DHEA	dehydroepiandrosterone
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOC	deoxycorticosterone

DP	declustering potential
DPX	dibutylphthalate polystyrene xylene
EDTA	ethylenediaminetetraacetic acid
Ef1a	translation elongation factor 1 A
EP	entrance potential
ER	estrogen receptor
EtOH	ethanol
F81	Felsenstein 1981
FG	foregut
g	gram
G	gauge
GAS1	gas 1
GAS2	gas 2
GC	guanine-cytosine
gDNA	genomic DNA
Gi	gill
Go	gonad
GR	glucocorticoid receptor
h	hours
H&E	hematoxylin and eosin
H+	hydrogen ion
HCl	hydrochloric acid
HCO_3^-	bicarbonate ion

HG	hindgut
HMDS	hexamethyldisilazane
HPA/I	hypothalamic-pituitary-adrenal/interrenal
HPLC	high-performance liquid chromatography
HSD	hydroxysteroid dehydrogenase
ISTD	internal standard
KH	Kishino-Hasegawa
KX	thousand times
L	litre
LC-MS/MS	liquid chromatography tandem mass spectroscopy
LK	lower kidney
m	metre
M	moles per litre
MBSU	Molecular Biology Services Unit
MCR	melanocortin receptor
MG	midgut
min	minutes
ML	maximum likelihood
mL	millilitre
mM	millimolar
mmol/L	millimoles per litre
MPPA	marginal posterior probabilities approximation
MR	mineralocorticoid receptor

mRNA	messenger RNA
MS	mass spectroscopy
MTBE	methyl tert-butyl ether
MYA	million years ago
N	normal
N_2	nitrogen
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NBB	non-bicarbonate buffer
NCBI	National Center for Biotechnology Information
NEB	New England BioLabs
ng	nanogram
NH_3	ammonia
NK	sodium potassium
PBS	phosphate-buffered saline
PCO_2	partial pressure of carbon dioxide
PCR	polymerase chain reaction
pK_{app}	apparent pK
PLL	phylogenetic likelihood library
PmCR	*Petromyzon marinus* CR
PMSF	phenylmethylsulfonyl fluoride
PR	progesterone receptor
PVC	polyvinyl chloride

QC	quality control
Rhcg	Rhesus blood group c glycoprotein
RIA	radioimmunoassay
RIN	RNA integrity number
RNA	ribonucleic acid
Rpl22	ribosomal protein L22
rpm	rotations per minute
RT-qPCR	real-time quantitative polymerase chain reaction
s	seconds
SD	standard deviation
SE	standard error of the mean
SEM	scanning electron microscopy
SH	Shimodaira-Hasegawa
SI	Supplementary Information
StAR	steroidogenic acute regulatory protein
t	time
TCO_2	total carbon dioxide
TMIC	The Metabolomics Centre for Innovation
TMS	tricaine methanesulfonate
UFBoot	ultrafast bootstrap
UK	upper kidney
UPLC	ultra performance liquid chromatography tandem mass spectrometry
v	version

V	volts
VLR	variable lymphocyte receptor
WGD	whole-genome duplication
μ	micro
μL	microlitre
μm	micrometre
μM	micromolar
αCO_2	solubility coefficient of carbon dioxide
β-Actin	beta actin

Chapter I – Introduction

1.1 Introduction to hagfish

Hagfishes are marine organisms considered key to understanding basal vertebrate evolution, specifically the divergence of cyclostomes and gnathostomes (Ota and Kuratani 2007; Shimeld and Donoghue 2012). Unlike the jawed fishes, hagfishes belong to the jawless fishes, Cyclostomata, a group that also includes the lampreys, with the agnathans considered basal to the gnathostome lineage, the jawed fishes. There are approximately 87 species of hagfishes, while only approximately 39 for lampreys (Fernholm et al. 2013; Froese and Pauly 2021). The specific evolutionary relationships between hagfish and lamprey within the Cyclostomes has long been debated since the 1970s when a paraphyletic relationship was advocated based on morphological evidence (Fig. 1.1; Delarbre et al. 2002). However, with the advent of molecular tools, data accrued supporting a monophyletic relationship with hagfishes and lampreys as sister groups in the phylogenetic tree of life (Delarbre et al. 2002; Heimberg et al. 2010; Shimeld and Donoghue 2012). Within the jawless fishes, one may expect hagfishes and lampreys to share many similarities, but there remain many traits that are unique to each group. For example, hagfishes and lampreys have differences in both genome organization and by the differing chromosome size, number, and guanine-cytosine (GC) -content (Delarbre et al. 2002; Kuraku and Kuratani 2006). More recent research including a fossil hagfish species, *Tethymyxine tapirostrum*, and morphological data demonstrates further support for a monophyly of hagfishes and lampreys (Miyashita et al. 2019). Miyashita et al. (2019) also support that hagfishes are organisms with specialized morphology rather than primitive features. The unique traits of these organisms can also be challenging to ascertain whether they are a result of homology (sharing with a common ancestor) or homoplasy (gained or lost independently in evolutionary lineages) (Smith et al. 2010; Shimeld and Donoghue 2012). This

makes hagfishes and lampreys even more interesting to study since they are the only extant members of jawless fishes. Therefore, they are excellent models to compare specific characteristics of basal vertebrates which will lead to a better understanding of early vertebrate evolution (Shimeld and Donoghue 2012). However, one consideration that should be stated is that the extant representatives of each group of current hagfishes and lampreys should not be seen as direct representations of ancestral vertebrates (Shimeld and Donoghue 2012). Rather, they should be viewed as organisms with their own independent evolution in the past approximate 500 million years after divergence from the rest of the vertebrate lineage.

1.2 Divergence in steroid profiles between cyclostomes and gnathostomes

One key trait of incongruence between cyclostomes and gnathostomes is that the steroidal profiles of the stress hormones in the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis are quite different (Nozaki et al. 2007). In most vertebrate animals, when an organism experiences a stressor, the HPA/I axis is activated by stimulating the secretion of specific corticosteroids (primarily cortisol in most vertebrates) from glands that allow the organism to respond appropriately to the stressor (Fig. 1.2; Schreck and Tort 2016). The pathways for production and steroids present are both well understood and well conserved in teleost fishes and other vertebrates (Fig. 1.3).

Hormones are classified based on their chemical structure (i.e., peptide/protein, biogenic amine, steroid, and eicosanoid), and their mechanism of action (i.e., binding to a receptor on the plasma membrane or an intracellular receptor). Steroid hormones are cholesterol derivatives commonly producing either sex steroids (usually in the gonads) or stress steroids (usually produced in the adrenal cortex of the kidney in mammals or in other tissues in other vertebrates lacking distinct adrenal glands). Synthesis of these hormones occurs on demand via enzymatic

modification of cholesterol in the smooth endoplasmic reticulum and mitochondria. Corticosteroids bind to corticosteroid receptors (CRs) that are typically produced when corticotropin-releasing factor (CRF) is released from the hypothalamus. CRF then activates the anterior pituitary to release adrenocorticotropic hormone (ACTH) which promotes the production and release of corticosteroids from peripheral steroid-producing tissues. ACTH binds to melanocortin receptors (MCR) in these steroid-producing tissues and effectuates a response in the cytosol. In hagfishes, ACTH-like cells have been reported in the adenohypopsis region via immunohistochemistry (Nozaki et al. 2005; Nozaki et al. 2007). While MCRs were absent from the genomes of some invertebrates, MCR-related genes were found in vertebrate genomes of hagfish, lamprey, and other fishes (Dores 2013). MCR orthologs usually found in higher vertebrates include MC1R, MC2R, MC3R, MC4R, and MC5R, and these receptors can be activated by ACTH or melanocyte-stimulating hormone (MSH; (Dores 2013; Dores et al. 2016; Bouyoucos et al. 2021; Takahashi 2021). The MC2R ortholog of teleost and tetrapods, however, is selective for ACTH such that it cannot be activated by MSH.

When the MCR is bound, this triggers a cascade of events inside the cytosol. Cholesterol accumulates outside the mitochondrial membrane and indirectly interacts with Steroidogenic Acute Regulatory protein (StAR), a transport protein that regulates its movement into the mitochondria. A cholesterol-protein binding complex forms and interacts with a transporter complex. Then, StAR interacts with the transporter complex to allow cholesterol movement into the inner mitochondrial membrane (Rone et al. 2012). Now, cholesterol can undergo conversion to pregnenolone via CYP11A1. Pregnenolone exits the mitochondria and is transported into the smooth endoplasmic reticulum where it is further modified by additional

steroidogenic enzymes to become the terminal corticosteroid for that animal/species (Metz et al. 2006; Cerdá-Reverter et al. 2011).

Steroids are hydrophobic and therefore have the ability to simply diffuse across lipid membranes (i.e., plasma, nuclear) since they are hydrophobic and with the help of carrier proteins (Chen and Farese 1999; Wilkenfeld et al. 2018). The modes of action of corticosteroids and gonadal steroids are similar in that they may occur via genomic or non-genomic mechanisms (Wierman 2007; Wilkenfeld et al. 2018). The hormone (ligand) enters the cell from the extracellular fluid into the cytoplasm of the cell. The ligand can then bind to an intracellular receptor in the cell's cytosol or continue through the nuclear membrane to bind to a receptor inside the cell's nucleus. Once bound, a conformational change occurs, and the receptor binds to DNA to induce a change in gene expression. These hormones cannot be stored and therefore must be synthesized on demand. They can also be excreted and either travel short distances for paracrine activation or bind to carrier proteins and travel longer distances in circulation to target tissues (Chen and Farese 1999). The effects of these hormones may be faster or slower depending on the distance it must travel before binding and initiating a response. There are two main types of steroid hormones: stress steroids (glucocorticoids, for example, cortisol and mineralocorticoids like aldosterone); and sex steroids (e.g., progesterone, testosterone, estrogen). This thesis focuses entirely on the synthesis of glucocorticoids (e.g., cortisol and its precursors) and will not examine the sex steroids.

In most teleost fishes, the activities of steroidogenic enzymes to synthesize corticosteroids are located in the interrenal tissue of the kidney. Once synthesized, the corticosteroids are released into the bloodstream and travel to their target tissues where they induce specific effects (the second phase of the stress response; Schreck and Tort 2016). In

most vertebrates, the terminal steroid is cortisol which exerts its effects through interaction with specific corticosteroid (CR), mineralocorticoid (MR) or glucocorticoid (GR) receptors (Fig. 1.4). However, in hagfishes and lampreys, cortisol is not used as the circulating stress steroid (Fig. 1.5) and this unique feature is the subject of this thesis.

The lack of cortisol as a stress steroid in lampreys and hagfishes has been postulated to their feeding lifestyle when one feeds parasitically on live animals (lampreys) while the other (hagfishes) feeds on recently deceased vertebrates, respectively (Yalden 1985; Clark and Uyeno 2019). Although it has been observed in some species of hagfish that they hunt their prey rather than solely feed on dead vertebrates (Zintzen et al. 2011). Each of these live or dead vertebrates would have high levels of circulating cortisol in the blood. Hence a loss of responsiveness to cortisol by cyclostomes may be advantageous since the feeding is then not associated with a stress response. However, these speculations are not enough to explain the lack of evidence for cortisol in cyclostomes. Lampreys have been shown to produce 11-deoxycortisol (11-DOC) as a terminal steroid although these findings have been contested (see section 1.3 below; Close et al. 2010). For hagfishes, Weisbart et al. (1980) suggested cortisol, corticosterone, deoxycorticosterone (DOC) and 11-DOC were present in plasma. However, these findings are not supported by more current research by Clifford et al. (2017) who found that neither cortisol, corticosterone, deoxycorticosterone (DOC) or 11-DOC were present in plasma after a variety of stressors after induction of synthesis following injection of implants containing upstream steroids (see details below; Weisbart et al. 1980; Clifford et al. 2017). While there are demonstrated glucocorticoid-like and mineralocorticoid-like responses in hagfishes to various stressors, the corticosteroid(s) that facilitate these responses remain undescribed (Bouyoucos et al. 2021).

1.3 Receptor profiles and activity

In vertebrates, including fishes, corticosteroid synthesis occurs by highly conserved enzymes belonging to two families: the cytochrome P450 (CYPs) and hydroxysteroid dehydrogenase (HSDs). These steroids bind to nuclear corticosteroid receptors (CRs) in target tissues. In most vertebrates, the CR receptor sub-types include glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), or alternatively sex steroid receptors (androgen (ARs), estrogen (ERs) and progesterone receptors (PRs). The multiplicity of CR receptors present in higher vertebrates is the result of multiple gene-duplication events that occurred during vertebrate evolution (Bridgham et al. 2006; Baker et al. 2007; Bury and Sturm 2007). Cyclostomes, which split from the vertebrate lineage about 500 MYA, prior to the first whole genome duplication event, do not possess the same breadth of receptors as do the gnathostomes (Shimeld and Donoghue 2012; Miyashita et al. 2019). While cyclostomes have homologous ER and PR sequences (termed SR in Cephalochordates), cyclostomes are thought to only possess/express a single CR homologous to both the GR and MR in other gnathostomes. These have not been found in more basal lineages like in the Urochordates and Cephalochordates.

The natural ligand for the lamprey and hagfish CRs has been a subject of keen interest (Baker et al. 2013; Baker and Katsu 2020). To elucidate the lamprey CR ligand, Close et al. (2010) collected plasma from stress-treated lamprey and used a combination of radioimmunoassays (RIAs), high-pressure liquid chromatography (HPLC) and mass spectrometry (MS) to suggest that DOC and 11-DOC were the probable CR agonists (Close et al. 2010). Following injection of either CRF or sea lamprey pituitary extracts, they saw an increase in the concentration of 11-DOC and a dose-dependent increase in plasma 11-DOC levels. They also used 11-DOC implants in sea lamprey and found increased concentrations of

11-DOC and DOC and a reduction in the concentrations of circulating sex steroids. To demonstrate that DOC and 11-DOC were the natural ligands for the lamprey CR, they also performed a competitive binding assay where 11-DOC had the highest affinity for the CR, at concentrations similar to what might be found in plasma (Close et al. 2010).

Although the study by Close et al. (2010) revealed some interesting findings, there were some caveats to be considered in their study. Thornton and Carroll (2011) argued that ancestral states cannot be determined from a single descendant and that the *Petromyzon marinus* CR (PmCR), and the GR and MR of extant vertebrate species are all the same evolutionary distance from the ancestral CR. Additionally, it was unconfirmed that the PmCR is only activated by endogenous 11-DOC as there may be other molecules capable of binding to the PmCR. The binding demonstrated in the Close et al. (2010) study may be due to other molecules present, and that binding, rather than activation, was measured. Phylogenetic analyses support that the ancestral CR has broad promiscuity for different ligands and that specificity for ligands is a derived trait (Baker et al. 2013). Therefore, it cannot be claimed that 11-DOC is the sole activator of the ancestral CR.

Since this publication, other studies have been conducted that support 11-DOC as a corticosteroid in lamprey (Rai et al. 2015; Shaughnessy and McCormick 2021). The most recent study done by Shaughnessy and McCormick (2021) subjected sea lamprey (*Petromyzon marinus*) to a physical stressor. By monitoring plasma glucose concentrations and performing mRNA expression work, they found that changes in plasma glucose levels are attributable to the actions of 11-DOC, and that there was mRNA expression of *star* (the gene coding for StAR) only in the lamprey kidney, respectively. Thus, they provide further support that 11-DOC is the

predominant corticosteroid in lamprey and that the kidney may be the main site of steroidogenesis.

Hagfishes have a single CR rather than both a GR and MR (Bridgham et al. 2006). It is known that hagfish elicit both glucocorticoid and mineralocorticoid responses, corresponding to an elevation of plasma glucose and active sulfate removal, respectively (Bury et al. 2015; Clifford et al. 2017; Baker 2019). Interestingly, the hagfish CR is activatable by multiple corticoids, but only at elevated pharmacological doses *in vitro* (Bury et al. 2015; Clifford et al. 2017). There has been initial, albeit unsuccessful, research to discover the natural ligand for the hagfish CR conducted by Clifford et al. (2017). In that study, steroid precursor injections of cortisol, corticosterone, 11-DOC, and DOC were used, hagfish were then subjected to a series of stressors (e.g., sulfate injection, exercise), and plasma samples were collected over several days. To validate the steroid injection implant, hagfishes were dissected and visually inspected for implant location, and an RIA was used to measure the plasma cortisol levels. The plasma cortisol levels were used as a proxy for the other steroid injections. From the results of the study, it was demonstrated that hagfishes are not able to synthesize cortisol, corticosterone, DOC nor 11-DOC (Clifford et al. 2017).

1.4 Synthesis of steroids: enzymes profiles

Steroidogenic enzymes, in addition to steroidogenic ligands, are another area where lampreys and hagfishes appear to differ. Steroid hormone synthesis begins with cholesterol and, via the use of enzymes, other steroid products are synthesized (Fig. 1.6). Since 11-DOC is recognized as the primary corticosteroid in lampreys, they must express all the enzymes required (CYP11A1, CYP17, CYP21, and 3β-HSD) to synthesize the steroids between cholesterol and 11-DOC.

Despite the vast amount of research on steroid synthesis in vertebrates, we are unaware of any studies on hagfishes examining the enzymes required for stress steroid synthesis. It was suggested by Clifford et al. (2017) that hagfishes express cyp11a1 and 3β-hsd based on transcriptome analysis. Similarly, a study of sex steroids in hagfish plasma demonstrates the presence of estradiol, testosterone, and progesterone which could suggest that the enzymes CYP17 and 3β-HSD are present in hagfish tissue (Nishiyama et al. 2013). However, the expression of other CYPs and HSDs normally involved in the conversion of steroids in hagfish is unknown (Bury et al. 2015; Clifford et al. 2017). It is plausible that the absence of specific functional enzymes involved in the steroid synthesis pathway terminates the synthesis of downstream steroids prematurely. This could occur through either loss of function through mutation, loss of expression through mutation of regulatory elements, loss of the gene itself through chromosomal re-arrangement or major deletion events, or perhaps these enzymes were not present in the evolution of the hagfish lineage. It is possible that hagfishes, without the ability to synthesis a full complement of steroids, employ the use of upstream precursor molecules such as pregnenolone or progesterone as CR agonists. Detection of the steroid(s) responsible for the glucocorticoid and mineralocorticoid responses in hagfishes forms a central component of my thesis.

1.5 Thesis objectives

To understand the stress response of an organism, the identity of primary corticosteroid(s), the identity of steroidogenic enzymes, and the predominant site(s) of steroidogenesis are important to know. Since the current research on these aspects of the stress system in hagfish is not entirely established, the long-term goal of my research is to identify the primary stress steroid(s), steroidogenic enzymes, and steroidogenic tissue(s) involved in the

stress response of Pacific hagfish, *Eptatretus stoutii*. My findings can be used to further the understanding of the stress system of this particular species and potentially to the hagfish lineage.

1.5.1 Objective # 1: Bioinformatic examination of the enzymes in the stress steroid synthesis pathway

The first objective of my thesis research is to examine the enzymes responsible for stress steroid synthesis. I hypothesized that hagfishes have the enzymes Cyp11a1, Cyp17, and 3β-HSD in the stress steroid synthesis pathway. If hagfishes possess these enzymes, then they would be capable of synthesizing up to the steroid progesterone because these enzymes aid in the conversion of cholesterol to pregnenolone and pregnenolone to progesterone. I do not expect hagfishes to possess the enzyme CYP21 because this enzyme would be needed for the synthesis of DOC and 11-DOC, corticosteroids which currently have not been identified in Pacific hagfish (Clifford et al. 2017). It is possible though that hagfishes could have remnants of the gene for CYP21 in their genome, but it may be non-functional. To accomplish this objective, phylogenetic analyses were performed to understand the evolution of these steroidogenic enzymes. This analysis was conducted to predict the presence/absence of these enzymes in hagfishes and secondarily, to predict the terminal corticosteroid in hagfish tissues. It should also be noted that the presence/absence of DOC and 11-DOC have a limited ability to reliably predict the presence/absence of CYP and HSD genes.

1.5.2 Objective # 2: Identify the primary stress steroid(s) of the Pacific hagfish

The second objective is to determine the physiological stress steroid present in hagfishes. I hypothesized that hagfishes use a less-derived upstream steroid like pregnenolone

or progesterone to regulate their stress response. If hagfishes use a corticosteroid like pregnenolone or progesterone, then these should be detectable in hagfish plasma. It is not expected that hagfish use downstream corticosteroids like DOC or 11-DOC based on previous research by Clifford et al. (2017). To accomplish this objective, Pacific hagfish were subjected to a couple of stressors (acid injection or exercise) and received implantation of either pregnenolone or progesterone as a steroid delivery system. Through subsequent analyses including liquid chromatography tandem mass spectrometry (LC-MS/MS) of the plasma and real-time quantitative polymerase chain reaction (RT-qPCR) of the tissues of hagfish, I examined the plasma for the presence of steroid products and the required enzymes, respectively to identify both the terminal steroid and the site(s) of production (see Fig. 1.7).

1.6 Figures

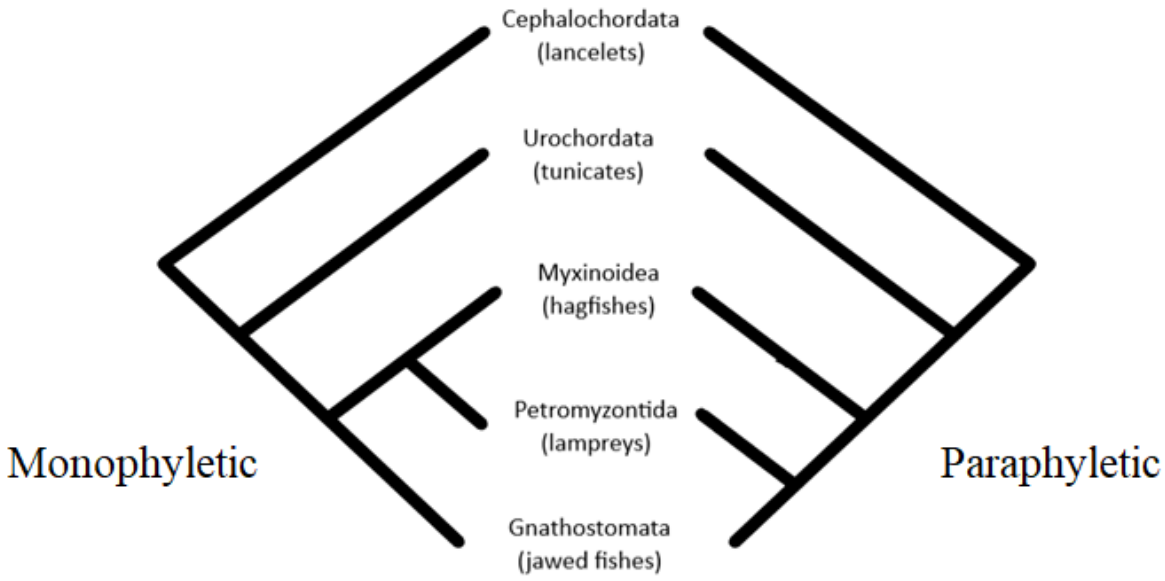


Figure 1.1 The monophyletic versus paraphyletic relationship between hagfishes and lampreys in vertebrate evolution. Morphological evidence supports a paraphyletic relationship between hagfishes and lampreys while molecular evidence supports a monophyletic relationship (Heimberg et al. 2010; Shimeld and Donoghue 2012).

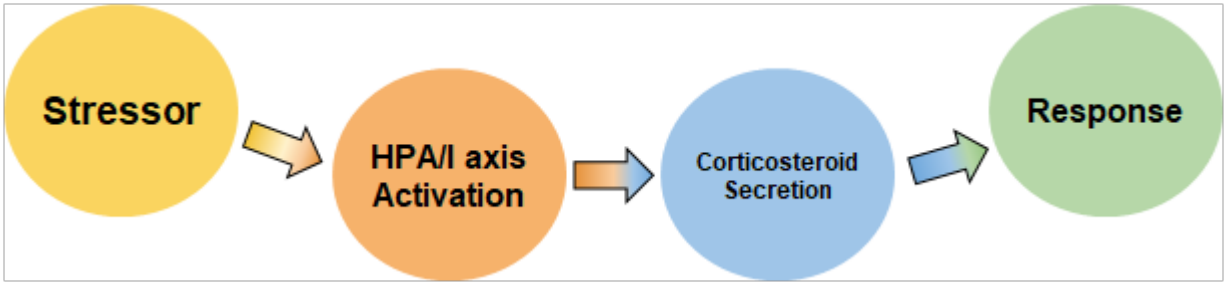


Figure 1.2 The general stress response pathway. For most vertebrates experiencing a stressor, the chain of events is as follows: 1) presence of a stressor (physical, chemical, or perceived stressor); 2) Activation of the hypothalamic-pituitary-adrenal/interrenal (HPA/I); 3) Secretion of corticosteroids (these are steroids involved in mitigating the stress response); and 4) A response (the second phase of the stress response).

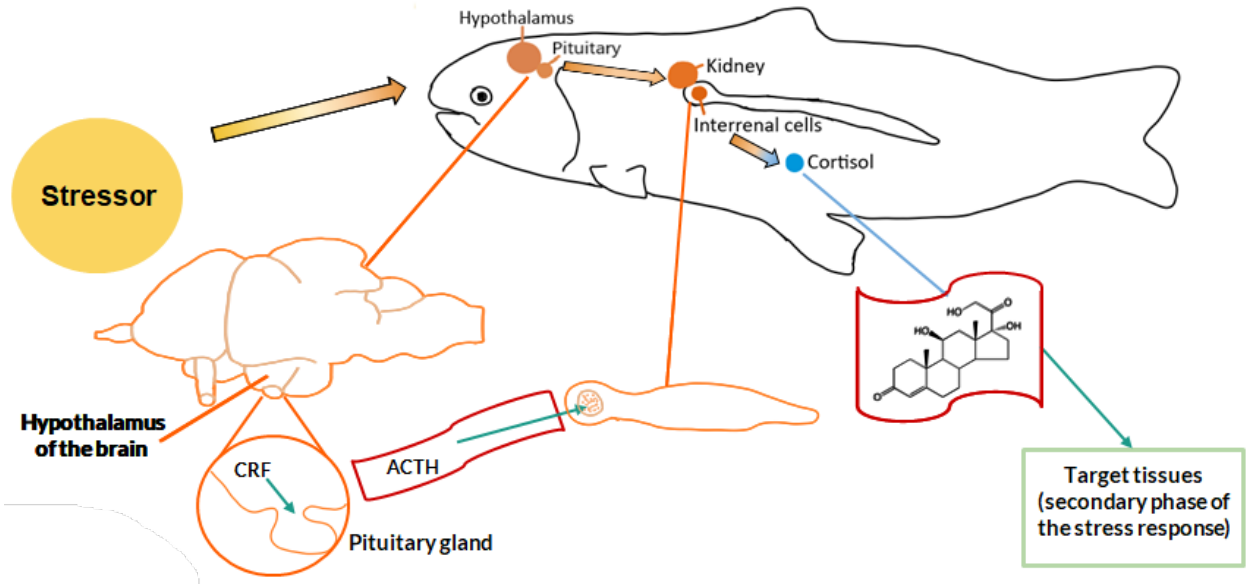


Figure 1.3 The stress system of most teleost fishes. This steroid production pathway is well-conserved in teleost fishes and other vertebrates. In most fishes, this is the process that occurs where a stressor is perceived by the hypothalamus region of the brain. CRF is released into the pituitary gland which stimulates the secretion of ACTH into the bloodstream. ACTH goes to the target organ, the kidney, where it binds to a melanocortin receptor (MCR) in the interrenal cells. Binding stimulates corticosteroid production and the release of cortisol into the bloodstream. Cortisol goes to target tissues and binds to a nuclear corticosteroid receptor (CR) and initiates the second phase of the stress response.

Figure 1.4 Steroid receptors and ligands present in the stress steroid system of fishes. A basic phylogeny of vertebrates based on the vertebrate phylogenies described in the literature (Shimeld and Donoghue 2012; Green and Bronner 2014; Baker et al. 2015; Baker 2019). Corticosteroid (CR), glucocorticoid (GR), and mineralocorticoid (MR) receptors are identified with corresponding ligands for each group of vertebrates. Unidentified ligand labelled by a "?".

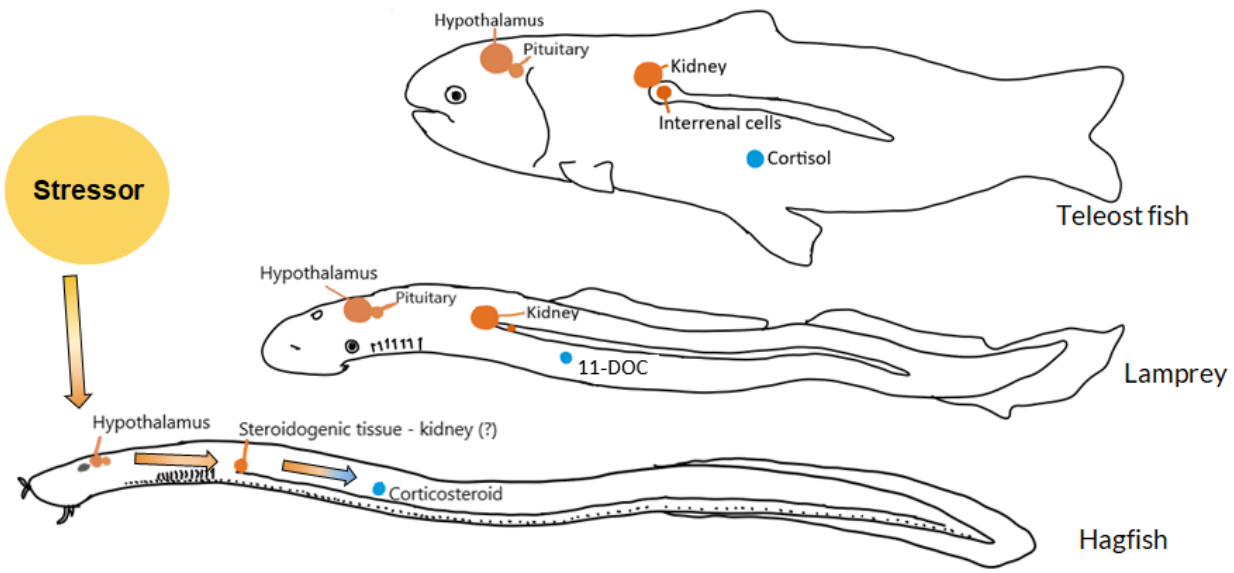


Figure 1.5 Comparison of the stress system of teleost fish, lamprey, and hagfish. The steroid production pathway in lampreys and hagfishes is thought to be similar to that of other vertebrates as seen in teleost fish. In lamprey, current research shows that they employ 11-deoxycortisol as their predominant corticosteroid and that steroidogenesis may occur in the kidney (Shaughnessy and McCormick 2021). In hagfishes, however, the site(s) of steroidogenesis and the primary corticosteroid have yet to be confirmed.

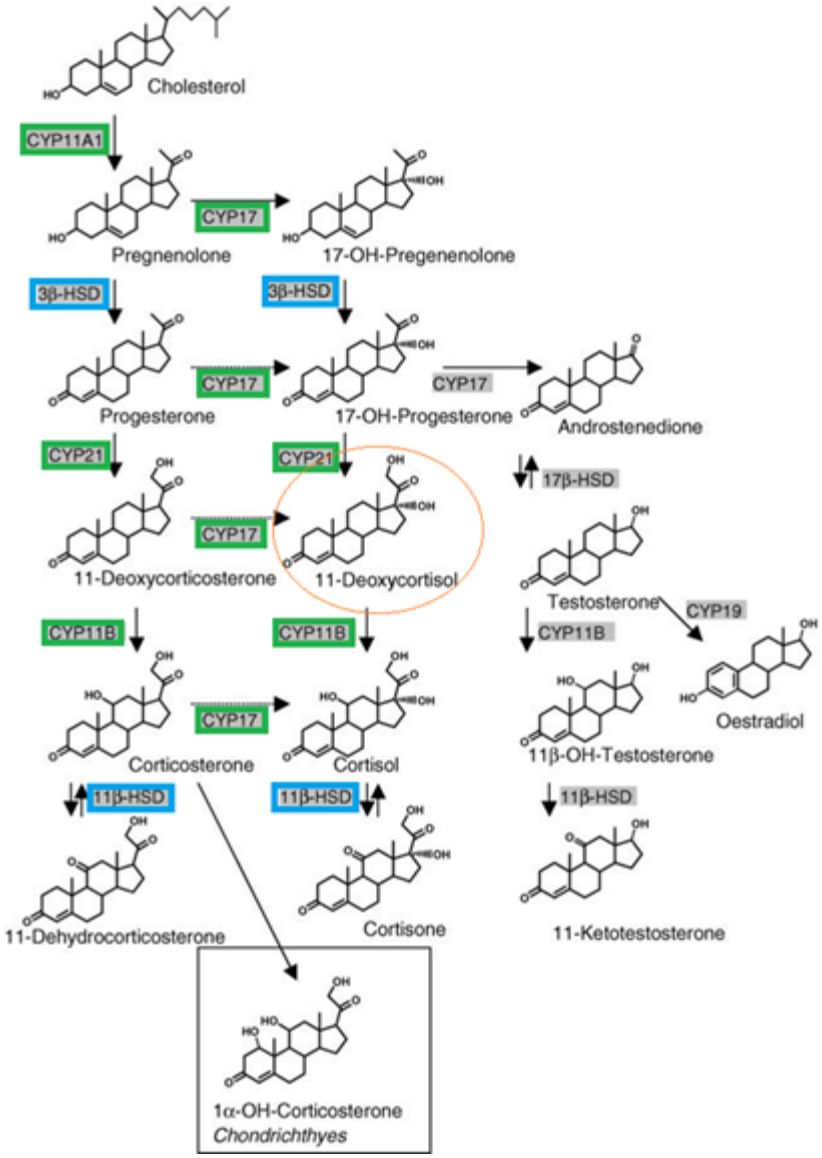


Figure 1.6 Steroid synthesis pathway in fishes. CYPs and HSDs of interest are highlighted in green and blue boxes, respectively. The primary corticosteroid of lamprey, 11-deoxycortisol (11-DOC), is circled in orange. This image is adapted from the steroid synthesis pathway by Bury and Sturm (2007).

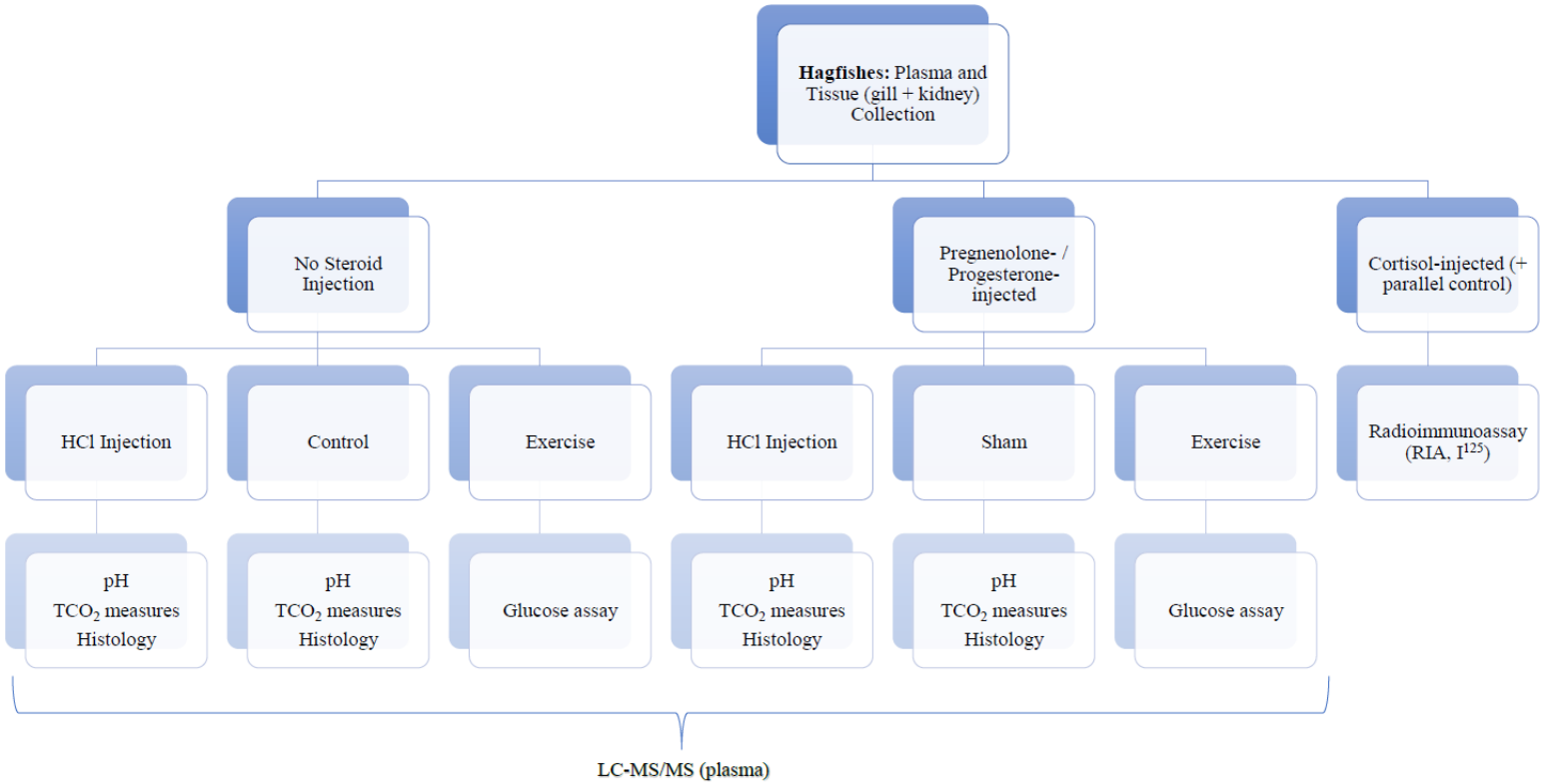

Hagfishes: Plasma and Tissue (gill + kidney) Collection
No Steroid Injection
Pregnenolone- / Progesterone-injected
Cortisol-injected (+ parallel control)
HCl Injection
Control
Exercise
HCl Injection
Sham
Exercise
Radioimmunoassay (RIA, I^{125})
pH
TCO_2 measures
Histology
pH
TCO_2 measures
Histology
Glucose assay
pH
TCO_2 measures
Histology
pH
TCO_2 measures
Histology
Glucose assay
LC-MS/MS (plasma)

Figure 1.7 Experiment workflow with treatments, control and experimental groups, and corresponding assay(s).

Chapter II - Absence of some cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes results in distinct stress steroid profiles in hagfishes

A version of this chapter has been submitted for publication.

Frost, C.R. and Goss G.G. 2021. Absence of some cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes results in novel stress steroid profiles in hagfishes. Journal of General and Comparative Endocrinology. Submitted 47 pages on Dec. 6, 2021. Manuscript ID: GCE-D-21-00379.

This work was conceived and designed by GGG and CRF. CRF performed the research and conducted analysis. CF and GGG analyzed the data and together they wrote, drafted, and revised the manuscript.

Abstract

When an organism experiences a stressor and its equilibrium is disrupted, a stress response begins. During the stress response of vertebrates, corticosteroids are synthesized from cholesterol by steroidogenic enzyme catalysts belonging to two main families: the cytochrome p450s (CYPs) and hydroxysteroid dehydrogenases (HSDs). The action of these steroidogenic enzymes allows the genesis of the terminal active corticosteroids 11-deoxycortisol (11-DOC), 1α-hydroxycorticosterone (1α-OH-B), or cortisol in different fish species. However, for Cyclostomes like hagfishes, the terminal corticosteroid is still undefined. In this study, we examined the CYPs and HSDs as traits in fishes to elucidate the primary corticosteroid synthesis pathways of the hagfishes. We used published cytochrome c oxidase I (COXI) amino acid sequences to construct a phylogeny of fishes and then mapped the CYPs and HSDs as morphological traits onto the tree to predict the ancestral character states through ancestral character reconstruction (ACR). There is a clear phylogenetic signal for CYP (i.e., CYP11a1, 17, 21, and 11b) and HSD (i.e., 11-βHSD and 3β-HSD) derivatives of interest throughout the more derived fishes. Using trait-based ACR, we also found that hagfishes possess genes for 3β-HSD, CYP11a1, CYP17, and CYP21. Importantly, the presence of CYP21 implies that hagfish can synthesize 11-deoxycorticosterone (DOC) and 11-DOC. In contrast, previous research demonstrated that despite hagfish having CYP21, neither DOC nor 11-DOC could be detected in hagfish. This discrepancy between the presence of steroidogenic enzymes and products brings into question the expression and/or function of CYP21 in hagfishes.

2.1 Introduction

In the event of a stressor, organisms have a complex but highly conserved stress response that empowers them to respond to that stressor. The hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis activates and initiates the secretion of catecholamines and corticosteroid hormones from various glands that play key roles in this stress response (Selye 1936; Schreck and Tort 2016; Bouyoucos et al. 2021). Corticosteroids fall into two primary classes based on the physiological responses elicited and interaction with glucocorticoid (GR) or mineralocorticoid (MR) receptors (Faught et al. 2016; Timmermans et al. 2019). The primary corticosteroid synthesized varies between different groups of organisms wherein teleosts generally use cortisol as their primary corticosteroid while chondrichthyans (i.e., sharks and rays) use 1α-hydroxycorticosterone (1α-OH-B; Anderson 2012; Bouyoucos et al. 2021) and lampreys arguably use 11-deoxycortisol (11-DOC; Close et al. 2010), although this has been questioned (Thornton and Carroll 2011). Most recent studies support the finding that lampreys use 11-DOC as their predominant corticosteroid (Rai et al. 2015; Shaughnessy and McCormick 2021). The aforementioned studies demonstrate that fishes use a diversity of corticosteroids to manage the stress response (Schreck and Tort 2016). In all vertebrates, these steroids are synthesized from cholesterol via the use of enzymes belonging to two families, the cytochrome P450s (CYPs) and the hydroxysteroid dehydrogenases (HSDs) (Fig. 2.1).

Cyclostomes are a group of jawless fishes that include the lampreys and hagfishes (Janvier 2008; Ota 2018). These fishes are considered primitive vertebrates, and extant members of these families are representatives of a monophyletic lineage of basal vertebrates (Kuraku et al. 1999; Delarbre et al. 2002; Heimberg et al. 2010; Miyashita et al. 2019) that diverged from the common ancestor ~ 520 MYA. Close et al. (2010) described 11-DOC as the

terminal steroid in lampreys and postulated that the absence of cortisol was due to the parasitic lifestyle of the adult form and the need to avoid stress hormones during feeding. For hagfishes, neither the steroid profile nor the terminal steroid has been definitively established. While there are earlier descriptions of cortisol being present in hagfish plasma (Freeman and Idler 1971), these have been invalidated in more recent studies (Clifford et al. 2017). Studies conducted by Clifford et al. (2017) attempted to identify the corticosteroid profile in hagfish by implant injection with the putative upstream precursors corticosterone, DOC, and 11-DOC and monitoring gill ATPase activity and changes in plasma glucose levels as indicators of mineralocorticoid and glucocorticoid activity, respectively. Despite clear mineralocorticoid and glucocorticoid activities in response to multiple stressors, there was no detectable cortisol nor 11-DOC found in hagfish plasma. The authors suggested that the lack of these terminal steroids could be due to the absence of expression of key CYP or HSD enzymes required for terminal corticosteroid synthesis (Bury et al. 2015; Clifford et al. 2017), but this has not yet been explored.

Hagfishes are known to undergo significant genomic rearrangement and deletion of up to 21% of the genome in somatic cells during development. However, this deletion does not happen in germline cells (Kubota et al. 2001). Therefore, the absence of activity in corticosteroid-producing tissues (which have not yet been identified) could be due to either selective loss of the genes responsible, dysregulation of expression, or mutation rendering the genes non-functional. Notably, the sex steroids estradiol, testosterone, and progesterone are detectable in plasma of female and male brown hagfish, *Paramyxine atami*, suggesting the enzymes needed to synthesize sex steroids from cholesterol (CYP17, 3β-HSD) are present in hagfish tissues (Nishiyama et al. 2013). Consequently, the enzymes involved in sex steroid

biosynthesis should also be present since there is an overlap with CYP17, and 3β-HSD is also used to produce stress hormones. With the recent publication of the inshore hagfish (*Eptatretus burgeri*) genome, we can now examine the possible presence/absence of enzymes required to synthesize stress steroids (in RIKEN Kobe PL, 2020). Using a phylogenetics-based approach, our goal in the present study was to investigate the presence and absence of steroidogenic enzymes in fishes to elucidate the reasons for the noted unique steroidal profiles found in hagfishes.

2.2 Materials and methods

See Figure 2.2 for an overview of the workflow highlighting the methods used.

2.2.1 Taxonomic sampling

We included 99 species of fish for the scope of this study, including at least two species per genus and representatives from each major class (e.g., Myxini, Petromyzontida, Chondrichthyes, Elasmobranchii) or superclass (e.g., Actinopterygii, Sarcopterygii). A total of seven taxa were included as outgroups. Since the ingroups are all fishes and consist of taxa within subphylum Vertebrata, we selected taxa external to vertebrates, including subphyla Urochordata (Tunicata) and Cephalochordata. Cytochrome P450 enzymes, for example, have not all been described in these groups or they are absent from these groups (Goldstone et al. 2016). Four Urochordata taxa within genera *Ascidia* and *Acidiella* and three Cephalochordata taxa within the genus *Branchiostoma* were selected as suitable outgroups for building a phylogeny of various fish species. Therefore, with 99 ingroup taxa and seven outgroup taxa, a total of 106 taxa were used in this study.

2.2.2 Molecular sampling

We obtained amino acid sequences for cytochrome c oxidase subunit 1 (COX1) for each of the 106 taxa from previously published sequence data available on the National Center for Biotechnology Information (NCBI) website database (https://www.ncbi.nlm.nih.gov/). COX1 is a commonly used mitochondrial gene used in phylogenetics and for identifying organisms (i.e., DNA barcoding) (Hebert et al. 2003; Ward and Holmes 2007). Sequences for this gene are readily available online for many taxa. Of the 99 ingroup taxa, 25 had full COX1 protein sequences (~ 500 amino acids) published, while 74 had partial COX1 protein sequences. For the outgroup taxa, three had full COX1 sequences (515 amino acids) and four had partial COX1 sequences (minimum 212 amino acids). In the fishes identified, the shortest COX1 sequence had 107 amino acids, whereas the next shortest sequences were all 180 amino acids minimum.

2.2.3 Morphological sampling

We gathered protein information on CYP and HSD enzyme family members from published data on the NCBI database (https://www.ncbi.nlm.nih.gov/). The enzymes of interest include CYP11a1, CYP17, CYP21, CYP11b, 3β-HSD, and 11β-HSD. Given that the same enzymes have multiple nomenclatures, we completed multiple searches using different terms to ensure all possible sequences in the database were found. Search terms included “cyp450”, “cytochrome p450”, “cytochrome 450”, “hsd”, “3 beta hsd”, “hydroxysteroid dehydrogenase”, “3 beta hydroxysteroid dehydrogenase”, “corticosteroid”, “11 beta”, and “steroid”. These were used in conjunction with genus and species names to return the optimal quantity and quality of results. Sequences without annotation were not used. A morphology matrix was assembled using sequences for each enzyme listed above for each selected taxon. Sequences for each taxon were assigned a value of 0, 1, or ?, indicative of protein data on the database and/or in the

literature being “absent”, “present”, or “missing”, respectively. For example, the taxon *Oreochromis aureus*, superclass Actinopterygii, has logged data for a “CYP general/unspecified”, CYP17, CYP21, CYP11b, and a “CYP other”. This taxon does not have a reported CYP11a1 sequence. Even though sequence data for the CYP11a1 enzyme for this fish was absent from the database, we know that CYP11a1 is involved at the beginning of the stress steroid synthesis pathway converting cholesterol to pregnenolone. Therefore, this taxon is considered to have a CYP11a1 sequence and was scored a value of 1 for “present”. A trait is “absent” if the taxa does not have it while “missing” describes data that we do not have for which we do not know if the trait is “present” or “absent”.

2.2.4 Alignment of sequences

Protein sequences were downloaded in FASTA format and compiled into a NEXUS file. COX1 protein sequences were aligned using the online Multiple Alignment using Fast Fourier Transform (MAFFT v 7) with default settings (https://mafft.cbrc.jp/alignment/server/; Kuraku et al. 2013; Katoh et al. 2019). The resulting aligned sequences were visualized using Mesquite v 3.61 (http://www.mesquiteproject.org/; Maddison and Maddison 2009; The Mesquite Project Team 2021).

2.2.5 Partitions and model selection

To determine the model of evolution for the COX1 protein partition, we performed ModelFinder in IQ-TREE v 2.0.6 using command line to select the best-fit model of evolution for this partition (http://www.iqtree.org/; Nguyen et al. 2015; Kalyaanamoorthy et al. 2017). The model of evolution allows estimates to be made about the data, including the rate of

substitution and rate heterogeneity. This accounted for sites within a sequence that evolve at differing rates.

2.2.6 Maximum likelihood (ML)

We next performed an ML analysis of the COX1 protein partition using the model found in Section 2.2.5 and including the seven outgroups as previously defined (IQ-TREE v 2.0.6; Nguyen et al. 2015). Default settings used include 1) initial parsimony tree: phylogenetic likelihood library (PLL) and 2) the candidate tree set starting tree: default setting (100 parsimony trees + BIONJ tree). Ten thousand bootstrap replicates were run using IQ-TREE's Ultrafast Bootstrap (UFBoot) (Hoang et al. 2018). This first analysis aimed to construct a phylogeny of fishes using the COX1 protein partition according to ML criteria and determine the probability of the data set fitting a generated tree topology. The tree was initially visualized in FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and edited using the Interactive Tree of Life (iTOL) v 6.1.1 (Letunic and Bork 2021).

2.2.7 Topology testing

Following the construction of a set of unconstrained COX1 trees using ML, constraints were added to arrange taxa by superclass and class to better reflect the current understanding of the phylogeny of fishes. To execute this, we took the COX1 ML consensus tree and grouped species of the same genus and those of the same superclass and class together. Following each change to the original unconstrained tree, we visualized the tree in FigTree v 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) to check for readability of the altered code and better resolution of the desired constraints. Since there is keen interest in the relationship between hagfishes and lampreys in vertebrate evolution (Ota and Kuratani 2007; Heimberg et al. 2010),

we constructed two constrained trees: one representative of monophyly between these groups; and the other representative of paraphyly between these groups. Thus, we tested three trees with different resolutions.

The topology of the unconstrained tree versus the constrained trees was tested using the bootstrap proportion (BP) test, the Kishino-Hasegawa (KH) test (Kishino and Hasegawa 1989), the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 1999), and the approximately unbiased (AU) test (Shimodaira 2002) via IQ-TREE v 2.0.6 within a single command (Nguyen et al. 2015). The settings were set at default with a recommended minimum number of 1000 RELL resamplings.

2.2.8 Ancestral character reconstruction (ACR)

The constrained COX1 ML tree representative of monophyly between hagfishes and lampreys was selected for this next set of analyses. This tree more closely represents our current understanding of the relationships between taxa. Tests for phylogenetic signal were run using R v 4.0.2 (https://www.r-project.org/) and R Studio v 1.3.1093 (https://rstudio.com) using packages adephylo, phylobase, and phytools. Both data partitions were used for this portion of the study with COX1 protein data used for the phylogeny and the CYP and HSD morphological data used to perform trait mapping onto the phylogeny. Morphological and protein data were used to see similarities and differences between taxa for testing the phylogenetic signal. We tested each CYP and HSD for phylogenetic signal using the Abouheif-Moran test with a default of 999 permutations. We then reconstructed the ancestral states for each CYP and HSD by character mapping and conducted ancestral character estimation (ACE). ACR was performed in PastML v 1.9.33 (https://pastml.pasteur.fr/; Ishikawa et al. 2019). PastML allows for ancestral states to be inferred using a rooted phylogenetic tree and a set of criteria which is then

visualized. The rooted constrained COX1 ML phylogenetic tree was uploaded to the PastML website in Newick format. A separate .csv file containing the tip states for the characters whose ancestral states were to be reconstructed with PastML was also uploaded. The recommended marginal posterior probabilities approximation (MPPA) coupled with the Felsenstein 1981 (F81) character evolution model was chosen. With MPPA, for each node, a subset of likely states is selected, minimizing the prediction error as indicated by the Brier score. Brier scores range from 0 to 1 providing a measure of the accuracy of probabilistic predictions (Ishikawa et al. 2019). The lower the score for a set of predictions or the closer the score is to 0, the better the predictions are. This scoring method is appropriate when the outcomes are categorical (i.e., true or false) or binary, as is the case with the morphology matrix. As noted on the PastML website, it is important to remember that states not retained by MPPA may still be significant despite being less probable.

2.3 Results

2.3.1 Maximum likelihood unconstrained and constrained trees

From the aligned COX1 amino acid sequences, an unconstrained tree using ML criterion was first constructed (Fig. 2.3). While taxa belonging to superclasses Actinopterygii and Sarcopterygii, in addition to the major classes, Myxinoidea, Petromyzontida, and Chondrichthyes grouped within this phylogeny, the Actinopterygians (ray-finned fishes) interspersed with lampreys (Petromyzontida), and sharks and rays (Chondrichthyes). The data of the COX1 protein partition have low bootstrap support for numerous nodes in the topology and do not fit the current understanding for the phylogeny of fishes. The Cyclostomes include the Myxinoidea and Petromyzontida and are understood to be sister groups. The constrained

COX1 tree allowed for better resolution of the superclasses and major classes of fishes (Fig. 2.4), with Petromyzontida now farther up the phylogeny and in a monophyletic resolution with its Cyclostome neighbour, the hagfishes.

2.3.2 Topology testing

Topology tests for the unconstrained ML COX1 tree and the two constrained trees (monophyletic and paraphyletic for Cyclostomes) demonstrate that the topology of the constrained trees is not significantly better than that of the unconstrained tree (Table 2.1). Nevertheless, the constrained monophyletic tree represented our current understanding of phylogenetic relationships better and thus, was chosen for ACR.

2.3.3 Ancestral character reconstruction (ACR) analyses

The results obtained from the Abouheif-Moran test for phylogenetic signal provide differing histograms for each character (Fig. 2.5). For the character CYP11a1 (Fig. 2.5 A), the observed position of the C mean falls on the edge of the expected sampling distribution. This reveals that CYP11a1 displays statistically significant autocorrelation and phylogenetic signal (Münkemüller et al. 2012). The histograms of the other three characters, CYP17, CYP21, and CYP11b (Fig. 2.5 B – D), also have the C mean falling outside the distribution, demonstrating phylogenetic signal. The same results are observed in Fig. 2.6 A, B) for the HSD characters. Phylogenetic signal occurs when more closely related species are more analogous to one another than when compared to their distant relatives (Arnaudo et al. 2019). The opposite of this is observable when there is a greater degree of similarity across distantly related species for a character, when there is a greater degree of dissimilarity for a trait across closely related

species, or when a trait varies randomly in a phylogeny. This is demonstrative of weaker phylogenetic signal.

The results of each character were mapped onto the constrained tree for ancestral character estimation (ACE) using PastML and are shown in Figures 2.7 – 2.12. A summary of results for hagfishes is presented in Figure 2.13. When ACR is performed using PastML, the program visualizes both a compressed and a full tree. The compressed tree shows a condensed representation of ancestral scenarios that essentially summarizes the results of the analysis without having to look at the entire tree. This is useful for immediately seeing the main findings while having the minor details concealed. Conversely, the full tree shows the entire tree with all details viewable with all relationships and nodes. Each node is represented as a shape, usually a circle, that may only show the tip state (present or absent) represented by different colours or it may be divided into two coloured halves with the left half identifying the state of the trait (i.e., present or absent), and the right half representing the group of taxa it belongs to (e.g., Urochordates, Cephalochordates, etc.). A number in the node indicates the number of taxa sharing the state for a given trait. If a unique state for a character cannot be selected, the node is left uncoloured.

The trait CYP11a1 is present in all taxa in this analysis (Fig. 2.7). This is demonstrated in Fig. 2.7 A by the left half of each node sharing the same dark green colour and in the most compressed tree version in Fig. 2.7 B by a single node in the same dark green colour with "Present" and the total number of taxa in this analysis. Differences among taxa tip states are noticeable for CYP17, indicating that most taxa have this protein, but four of the seven taxa in the outgroup do not possess CYP17 (Fig. 2.8). All taxa in the outgroup (non-vertebrates) do not possess the CYP21 enzyme sequences, but all the vertebrate taxa possess CYP21 sequences

(Fig. 2.9). Interestingly, CYP11b, which is required for final cortisol synthesis, is absent in both all outgroup taxa and also absent in the Cyclostomes (hagfish and lampreys) but is present in all the rest of the vertebrate taxa included in our analysis (Fig 2.10).

The enzyme 3β-HSD is present in all taxa included in our analysis, including both outgroup taxa and all vertebrate taxa (Fig. 2.11). However, for 11β-HSD, the results are not as clear. In the outgroups, 11β-HSD appear to be present in the three cephalochordate taxa while absent the four urochordate taxa. In the vertebrates, the lampreys appear to lack 11β-HSD, and the rest of the vertebrates (except the hagfish) have 11β-HSD. However, it should be noted that for hagfish, our prediction method was unable to determine or estimate the character state for the hagfishes (Myxinoidea) and is demonstrated by the white node and the half dark green, half orange node (Fig 2.12). This means we cannot conclude whether hagfish possess have 11β-HSD or not.

2.4 Discussion

The results of this study suggest that all the vertebrate taxa studied, including the hagfishes and lampreys, are predicted to possess each of the enzymes CYP11a1, CYP17, CYP21 and 3β-HSD. However, the Cyclostomes (hagfishes and lamprey) do not appear to possess CYP11b. Interestingly, 11β-HSD appears to be absent in lampreys but present in the more-derived vertebrates. For hagfishes, bioinformatic analysis of 11β-HSD could not determine if 11β-HSD is either present or absent. These findings imply that hagfish and lamprey do not have complete steroidal synthesis pathways for stress steroids. These findings are congruent with a previous study by Close et al. (2010), who demonstrated that sea lamprey can synthesize 11-DOC but cannot synthesize further derivatives downstream of 11-DOC including cortisol and corticosterone. Similarly, for hagfishes, Clifford et al. (2017) also

demonstrated that hagfish do not synthesize cortisol, DOC, or corticosterone. Therefore, given the absence of CYP11b in both hagfishes and lampreys, the synthesis of the steroids cannot proceed past 11-DOC (Fig. 2.1).

ACR analyses demonstrate consensus amongst taxa for a trait as is evident in our results for CYP11a1 and 3β-HSD, where steroidogenic enzymes at the beginning steps of the steroid synthesis pathway of vertebrates are conserved. This is not surprising since some steroidogenic enzymes like CYP11a1, CYP17, and CYP19 along with gonadal steroids have even been reported in some Cephalochordates like amphioxus (Baker 2007). From our ACR analysis, hagfishes have both CYP11a1, CYP 17, and 3β-HSD, which are all required for synthesizing 17-OH-pregnenolone from pregnenolone and 17-OH-progesterone from progesterone. While Close et al. (2010) and Shaughnessy and McCormick (2021) found that sea lampreys have circulating 11-DOC in their plasma, Clifford et al. (2017) were unable to detect circulating 11-DOC in plasma from hagfish experiencing a variety of stressors. To generate 11-DOC from either progesterone or 17-OH progesterone requires the enzyme CYP21. The absence of circulating 11-DOC in hagfishes when our analysis demonstrates the presence of the enzyme implies that either the CYP21 gene is not expressed appropriately or that mutation has rendered the gene non-functional in hagfishes. Alternatively, it is possible hagfishes synthesize other corticosteroids from pregnenolone or progesterone but not 11-DOC or cortisol. From a quick search of the genes for these enzymes on the NCBI, there does not appear to be gene synteny for these genes (Table 2.2). Resolution of circulating corticosteroids by liquid chromatography tandem mass spectrometry (LC-MS/MS) or other advanced analytical techniques is required to resolve the unique steroidal profile of the hagfishes.

It is challenging to find annotated protein and gene sequences for hagfishes through online databases. This makes phylogenetic analyses difficult as there is a lack of annotated CYP and HSD sequences available in the more ancient vertebrate taxa. Abouheif-Moran tests for phylogenetic signal for each CYP and HSD enzyme in the stress steroid synthesis pathway were designed to demonstrate if taxa belonging to the same group have the same trait or not. We found that there is not very much variation within groups of taxa. This is not surprising given the central role of these enzymes in the vertebrate stress response. Using a phylogenetic signal analysis, variation amongst taxa for a particular trait can be examined and is useful for detecting larger trends in data. However, phylogenetic signal tests alone cannot determine which taxa may have or not have a particular enzyme. It is also important to acknowledge that Abouheif's C mean and Moran's I are not based on evolutionary models and are not robust enough for quantitative interpretation (Münkemüller et al. 2012).

Many fish species included in this study did not have catalogued CYP and HSD sequences in the publicly available databases. For species belonging to Actinopterygii, we assumed that these fishes have the necessary CYPs and HSDs for cortisol synthesis given that many papers have demonstrated cortisol in these Actinopterygians, implying the presence of each required CYP and HSD derivative (Mommsen et al. 1999; Bury and Sturm 2007; Alsop and Vijayan 2008). A future direction could involve the use of the Basic Local Alignment Search Tool (BLAST) to find more sequences.

We used Bayesian Information Criterion (BIC) for the ML analyses. BIC chooses the best model that best fits the data for any given tree. The resulting topology provides the best representation of the tree and estimates of branch lengths. The branch lengths are assigned and generated based on substitution rates if sites evolve independently (Makarenkov et al. 2006).

One caveat is that the rate of substitution of amino acid residues can be estimated, but it does not account for the structure of the protein (i.e., secondary structure like motifs) or the function of the resultant protein. There could also be difficulty modelling genes in fishes due to whole-genome duplication (WGD) events in teleosts (Ohno 1970; Glasauer and Neuhauss 2014). In our analysis, we used the mitochondrial enzyme COX1 to build the phylogenetic relationships between our taxa. However, if there were higher mutation rates for some taxa for this gene (COX1) compared to other taxa, this would explain why the unconstrained ML tree did not show taxa thought to be closely related congregating together. Therefore, we constrained the ML tree for use in ACR by imposing constraints that mirrored consensus relationships between taxa (Near et al. 2013; Glasauer and Neuhauss 2014; Betancur-R et al. 2017; Hughes et al. 2018; Betancur-R et al. 2013). Our main goal was to examine the relationships between superclasses and major classes for the stress steroid synthesis enzymes. There was no attempt to further resolve the deeper nodes within the tree due to the inherent issues with using a single locus to construct a species tree. While future studies could include adding multiple markers/multiple loci (e.g., cytochrome b, cytochrome c oxidase II) to improve results with the unconstrained tree, this would likely not alter the relationships at the class and superclass level, which was the focus of this study.

2.5 Conclusions

Our study identified the steroidogenic enzyme sequences for CYP11a1, CYP17, CYP21, and 3β-HSD enzymes in both hagfishes and lampreys (Cyclostomes). However, CYP11b and 11β-HSD appear absent in both Cyclostome lineages, which can account for the absence of cortisol and cortisone, respectively, in the Cyclostomes. In addition, while experimental evidence suggests that 11-DOC is absent in hagfishes but present in lamprey, our analysis

indicates that both hagfishes and lampreys possess the enzyme CYP21 required to convert upstream steroids to 11-DOC (Close et al. 2010; Clifford et al. 2017). This discrepancy raises questions regarding the expression or function of CYP21 in hagfishes compared to lamprey, and this requires more empirical laboratory research in combination with further bioinformatic analyses.

This study highlights the ability to use phylogenetic analyses to detect phylogenetic signals and patterns of ancestral character states (e.g., CYPs and HSD steroidogenic enzymes). In fishes, the resolution of unique steroidal profiles in hagfishes remains a key question for our understanding of vertebrate evolution.

2.6 Tables

Table 2.1 Topology testing results of unconstrained versus the constrained ML trees. Constrained Tree B was designated with monophyly between hagfishes and lampreys, while Constrained Tree C was designated with a paraphyly. DeltaL represents the logL difference from the maximal logL in the set of trees and bp-RELL represents the bootstrap proportion using the RELL method. Four types of comparison tests were run via IQ-TREE and are present with their p-values below. The minus (-) sign denotes significant exclusion while the plus (+) sign denotes 95% confidence sets.

Tree	logL	deltaL	bp-RELL	p-KH	p-SH	p-ELW	p-AU
Unconstrained Tree A	-5499.62	0	0.976 +	0.972 +	1 +	0.975 +	0.975 +
Constrained Tree B	-5554.76	55.13	0.0015 -	0.0132 -	0.0132 -	0.00179 -	0.00444 -
Constrained Tree C	-5543.08	43.45	0.0225 -	0.0282 -	0.0282 -	0.0228 -	0.0388 -

Table 2.2 Chromosome location of genes for enzymes of interest. Enzymes of interest include CYP11a1, CYP17, CYP21, CYP11b, 3β-HSD, and 11β-HSD. The loci of each gene were obtained from the NCBI gene database.

Gene name on NCBI	Gene ID	Species	Common name	Loci (chromosome #)	Chromosome ID
cholesterol side-chain cleavage enzyme, mitochondrial	116968174	*Amblyraja radiata*	Thorny skate	44	NC_045999.1
steroid 21-hydroxylase	118231182	*Anguilla anguilla*	European eel	1	NC_049201.1
steroid 21-hydroxylase-like	113116301	*Carassius auratus*	Goldfish	16	NC_039258.1
cytochrome P450 11B, mitochondrial-like	113059430	*Carassius auratus*	Goldfish	41	NC_039283.1
cyp11a1	80374	*Danio rerio*	Zebrafish	25	NC_007136.7
cyp17a1	399692	*Danio rerio*	Zebrafish	13	NC_007124.7
CYP11A1	1583	*Homo sapiens*	Human	15	NC_000015.10
CYP17A1	1586	*Homo sapiens*	Human	10	NC_000010.11
CYP21A2	1589	*Homo sapiens*	Human	6	NC_000006.12
CYP11B1	1584	*Homo sapiens*	Human	8	NC_000008.11
CYP11B2	1585	*Homo sapiens*	Human	8	NC_000008.11
HSD3B1	3283	*Homo sapiens*	Human	1	NC_000001.11
HSD11B1	3290	*Homo sapiens*	Human	1	NC_000001.11
HSD11B2	3291	*Homo sapiens*	Human	16	NC_000016.10
hsd3b1	100304475	*Ictalurus punctatus*	Channel catfish	6	NC_030421.1
Cyp11a1	13070	*Mus musculus*	House mouse	9	NC_000075.7
Cyp17a1	13074	*Mus musculus*	House mouse	19	NC_000085.7
Cyp21a1	13079	*Mus musculus*	House mouse	17	NC_000083.7

Cyp11b1	110115	*Mus musculus*	House mouse	15	NC_000081.7
Cyp11b2	13072	*Mus musculus*	House mouse	15	NC_000081.7
Hsd11b1	15483	*Mus musculus*	House mouse	1	NC_000067.7
cholesterol side-chain cleavage enzyme, mitochondrial-like	110501505	*Oncorhynchus mykiss*	Rainbow trout	2	NC_048566.1
cyp17a1	100137017	*Oncorhynchus mykiss*	Rainbow trout	1	NC_048565.1
cyp11b	100135891	*Oncorhynchus mykiss*	Rainbow trout	3	NC_048567.1
cyp17	100125816	*Oryzias latipes*	Japanese medaka	15	NC_019873.2
cholesterol side-chain cleavage enzyme, mitochondrial	121896942	*Thunnus maccoyii*	Southern bluefin tuna	5	NC_056537.1

2.7 Figures

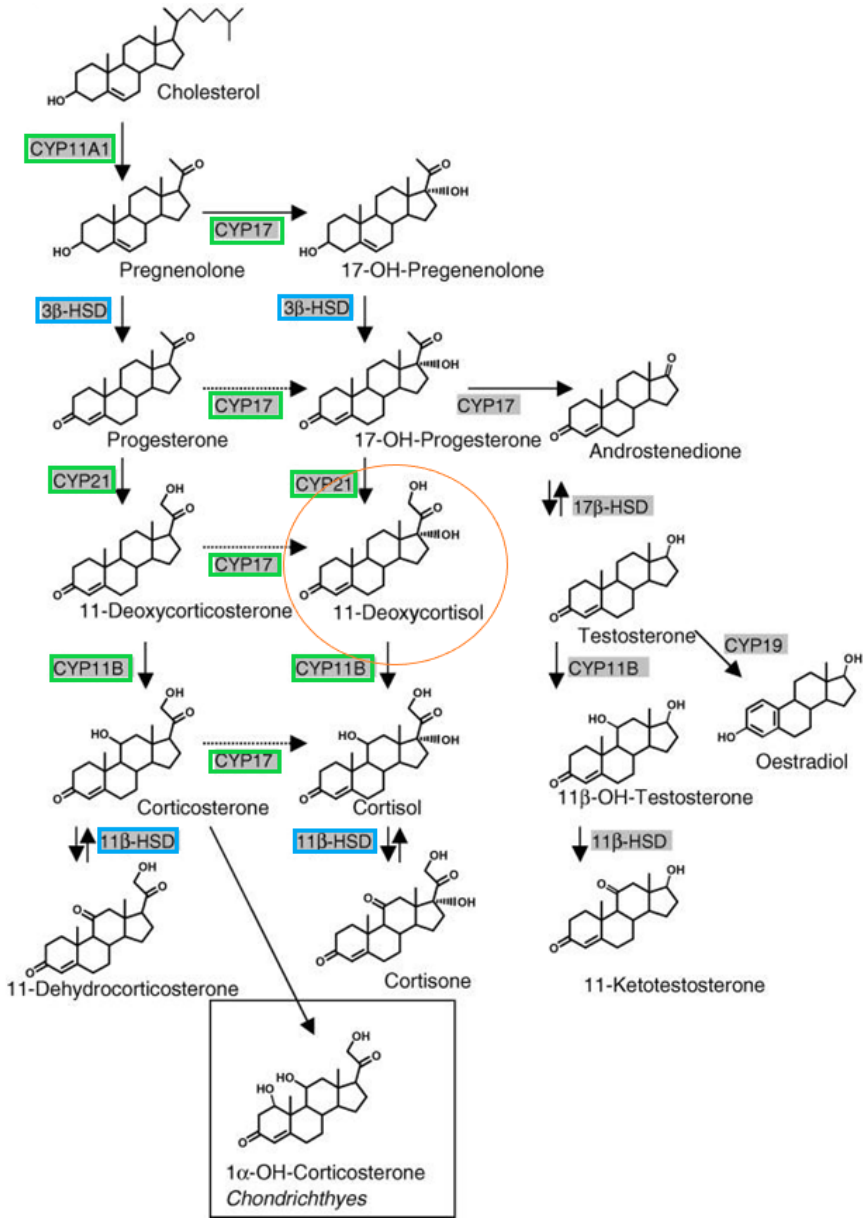


Figure 2.1 Steroid synthesis pathway in fishes. CYPs and HSDs of interest are highlighted in green and blue boxes, respectively. The primary corticosteroid of lamprey, 11-deoxycortisol (11-DOC), is circled in orange. This image is adapted from the steroid synthesis pathway by Bury and Sturm (2007).

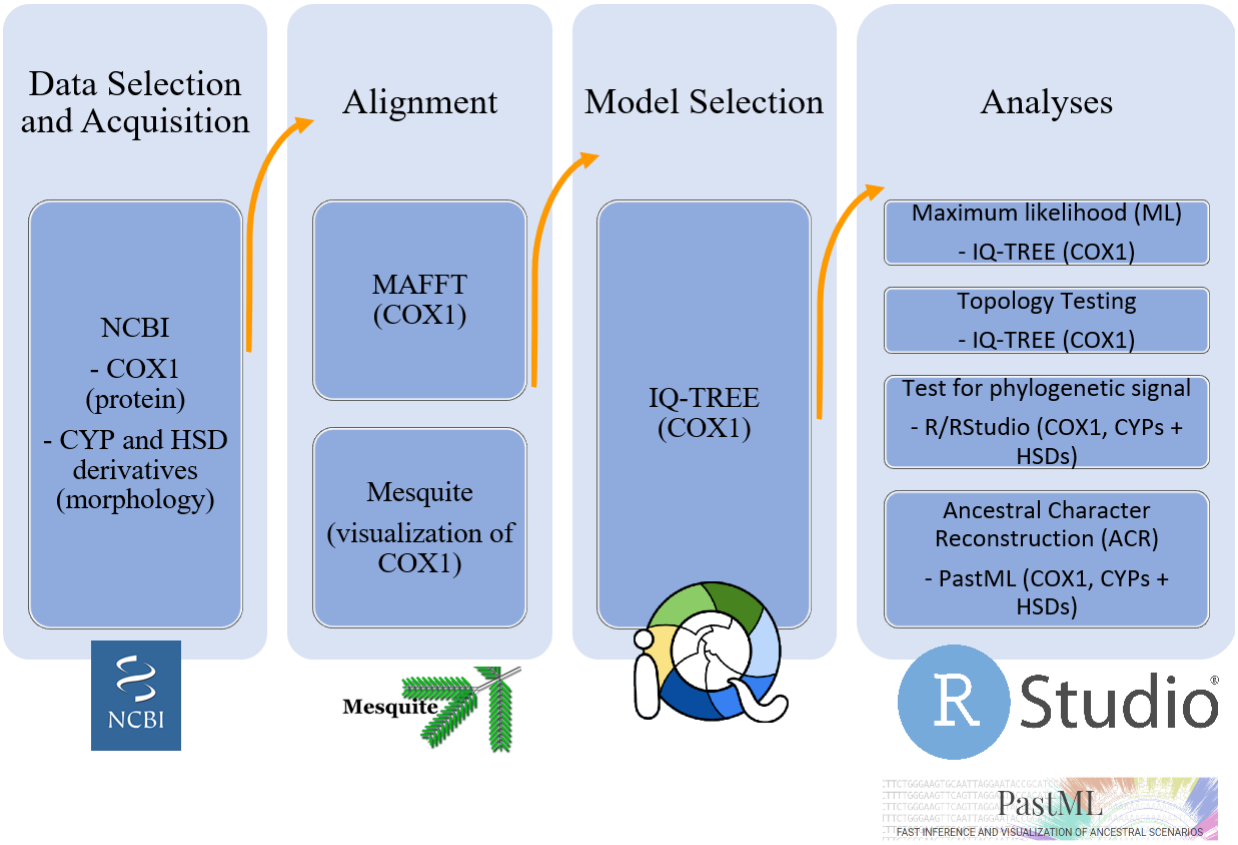


Figure 2.2 Workflow summary of methods.

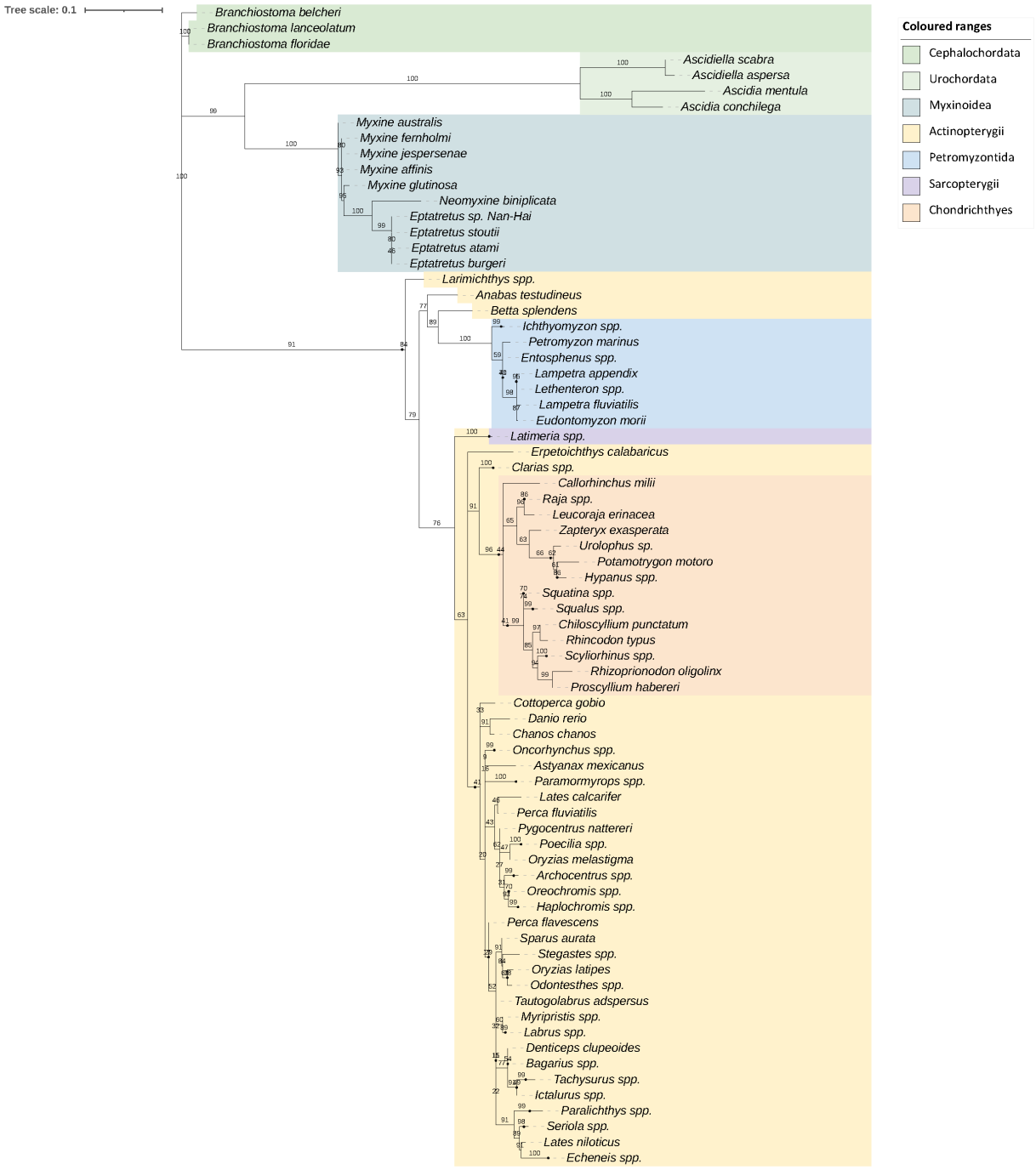

Tree scale: 0.1
Branchiostoma belcheri
Branchiostoma lanceolatum
Branchiostoma floridae
Ascidiella scabra
Ascidiella aspersa
Ascidia mentula
Ascidia conchilega
Myxine australis
Myxine fernholmi
Myxine jespersenae
Myxine affinis
Myxine glutinosa
Neomyxine biniplicata
Eptatretus sp. Nan-Hai
Eptatretus stoutii
Eptatretus atami
Eptatretus burgeri
Larimichthys spp.
Anabas testudineus
Betta splendens
Ichthyomyzon spp.
Petromyzon marinus
Entosphenus spp.
Lampetra appendix
Lethenteron spp.
Lampetra fluviatilis
Eudontomyzon morii
Latimeria spp.
Erpetoichthys calabaricus
Clarias spp.
Callorhinchus milii
Raja spp.
Leucoraja erinacea
Zapteryx exasperata
Urolophus sp.
Potamotrygon motoro
Hypanus spp.
Squatina spp.
Squalus spp.
Chiloscyllium punctatum
Rhincodon typus
Scyliorhinus spp.
Rhizoprionodon oligolinx
Proscyllium habereri
Cottoperca gobio
Danio rerio
Chanos chanos
Oncorhynchus spp.
Astyanax mexicanus
Paramormyrops spp.
Lates calcarifer
Perca fluviatilis
Pygocentrus nattereri
Poecilia spp.
Oryzias melastigma
Archocentrus spp.
Oreochromis spp.
Haplochromis spp.
Perca flavescens
Sparus aurata
Stegastes spp.
Oryzias latipes
Odontesthes spp.
Tautogolabrus adspersus
Myripristis spp.
Labrus spp.
Denticeps clupeoides
Bagarius spp.
Tachysurus spp.
Ictalurus spp.
Paralichthys spp.
Seriola spp.
Lates niloticus
Echeneis spp.
Coloured ranges
Cephalochordata
Urochordata
Myxinoidea
Actinopterygii
Petromyzontida
Sarcopterygii
Chondrichthyes

Figure 2.3 Maximum likelihood unconstrained consensus tree. Tree was created using the mtZOA+R4 model for cytochrome c oxidase 1 (COX1) amino acid sequences of 99 fish species and seven outgroup taxa. The best-fit model of evolution was chosen according to Bayesian Information Criterion (BIC) in IQ-TREE v 2.0.6. The analysis was run using default settings with 1000 bootstrap replicates using UFBoot. Fish taxa are highlighted in blues, yellow, orange and purple, and outgroup taxa are highlighted in greens. Bootstrap supports are present, and the scale bar represents branch lengths.


Tree scale: 0.1
Coloured ranges
Cephalochordata
Urchordata
Myxinoidea
Petromyzontida
Chondrichthyes
Sarcopterygii
Actinopterygii
Branchiostoma belcheri
Branchiostoma lanceolatum
Branchiostoma floridae
Ascidiella scabra
Ascidiella aspersa
Ascidia mentula
Ascidia conchilega
Myxine australis
Myxine fernholmi
Myxine jespersenae
Myxine affinis
Myxine glutinosa
Neomyxine biniplicata
Eptatretus sp. Nan-Hai
Eptatretus stoutii
Eptatretus burgeri
Eptatretus atami
Ichthyomyzon gagei
Ichthyomyzon fossor
Ichthyomyzon unicuspis
Petromyzon marinus
Entosphenus lethophagus
Entosphenus tridentatus
Lampetra fluviatilis
Eudontomyzon morii
Lethenteron camtschaticum
Lampetra appendix
Lethenteron reissneri
Callorhinchus milii
Leucoraja erinacea
Raja polystigma
Raja miraletus
Zapteryx exasperata
Urolophus halleri
Urolophus cruciatus
Potamotrygon motoro
Hypanus sabinus
Hypanus americanus
Squalus blainville
Squalus acanthias
Squatina africana
Squatina occulta
Squatina guggenheim
Chiloscyllium punctatum
Rhincodon typus
Scyliorhinus torazame
Scyliorhinus canicula
Rhizoprionodon oligolinx
Proscyllium habereri
Latimeria menadoensis
Latimeria chalumnae
Larimichthys crocea
Larimichthys polyactis
Anabas testudineus
Betta splendens
Erpetoichthys calabaricus
Clarias fuscus
Clarias batrachus
Cottoperca gobio
Danio rerio
Chanos chanos
Oncorhynchus tshawytscha
Oncorhynchus mykiss
Astyanax mexicanus
Paramormyrops kingsleyae
Paramormyrops gabonensis
Lates calcarifer
Perca fluviatilis
Pygocentrus nattereri
Oryzias melastigma
Poecilia latipinna
Poecilia formosa
Archocentrus multispinosus
Archocentrus centrarchus
Haplochromis desfontanii
Haplochromis burtoni
Oreochromis niloticus
Oreochromis aureus
Perca flavescens
Sparus aurata
Stegastes nigricans
Stegastes partitus
Odontesthes bonariensis
Oryzias latipes
Odontesthes hatcheri
Tautogolabrus adspersus
Myripristis berndti
Myripristis murdjan
Labrus mixtus
Labrus bergylta
Denticeps clupeoides
Bagarius yarrelli
Bagarius bagarius
Bagarius suchus
Ictalurus punctatus
Ictalurus furcatus
Tachysurus vachellii
Tachysurus fulvidraco
Paralichthys olivaceus
Paralichthys lethostigma
Lates niloticus
Echeneis neucratoides
Echeneis naucrates
Seriola dumerili
Seriola lalandi dorsalis
Seriola quinqueradiata

Figure 2.4 Maximum likelihood constrained consensus tree with branch lengths. Tree was created using the mtZOA+R4 model for cytochrome c oxidase 1 (COX1) amino acid sequences of 99 fish species and seven outgroup taxa. The best-fit model was chosen according to BIC in IQ-TREE v 2.0.6 and the analysis was run using default settings with 1000 bootstrap replicates using UFBoot. The tree was constrained to resemble the current phylogeny by grouping taxa of the same superclass or class together. Fish taxa are highlighted in blues, yellow, orange and purple, and outgroup taxa are highlighted in greens. The scale bar represents branch lengths.

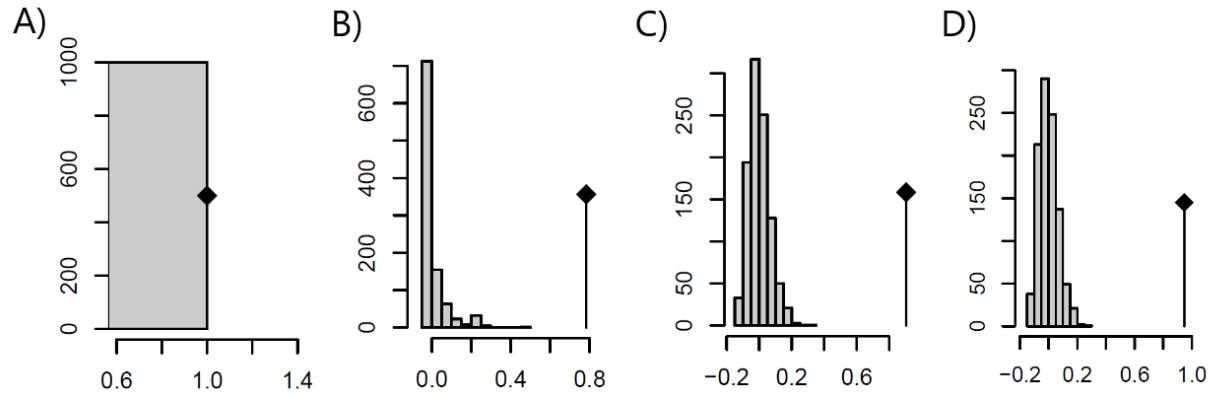


Figure 2.5 Phylogenetic signal identified in CYP derivatives according to the Abouheif-Moran test for phylogenetic signal. CYP derivatives A) CYP11a1, B) CYP17, C) CYP21, and D) CYP11b. The distribution represents the hypothesis developed by randomizations along the tips of the phylogeny. The observed position of the C mean is represented by the black diamond on a stick. A significantly different C mean position relative to the expected sampling distribution means that there is strong autocorrelation or phylogenetic signal.

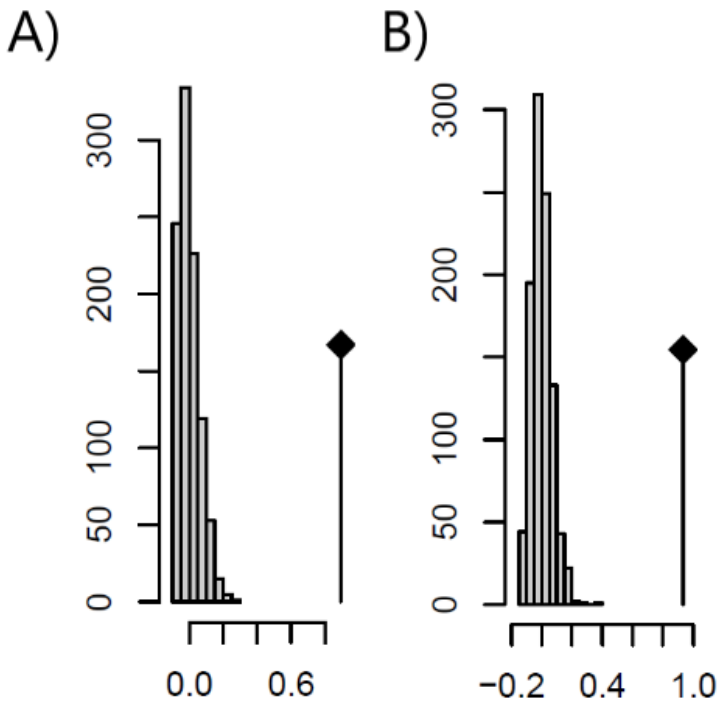


Figure 2.6 Phylogenetic signal identified in HSD derivatives according to the Abouheif-Moran test for phylogenetic signal. HSD derivatives A) 11β-HSD, and B) 3β-HSD. The distribution represents the hypothesis developed by randomizations along the tips of the phylogeny. The observed position of the C mean is represented by the black diamond on the stick.

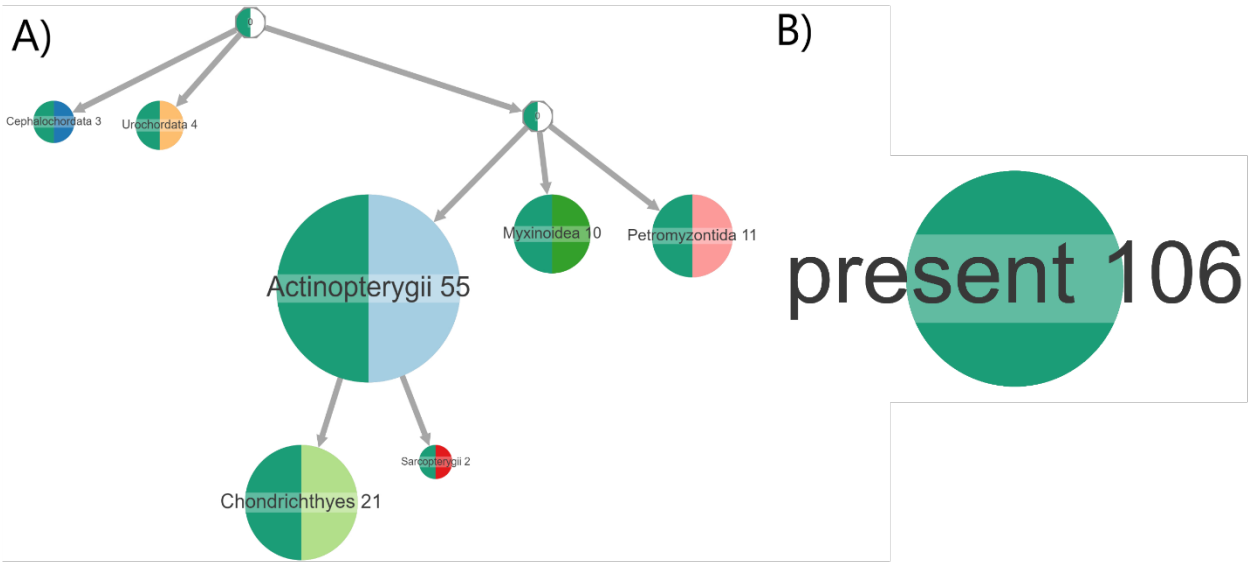


Figure 2.7 PastML results for the constrained COX1 ML tree using MPPA with F81 for the character CYP11a1. The A) expanded tree with group and tip states and B) most compressed tree with tip states only. Dark green indicates that the character CYP11a1 is present for the taxa identified at each node.

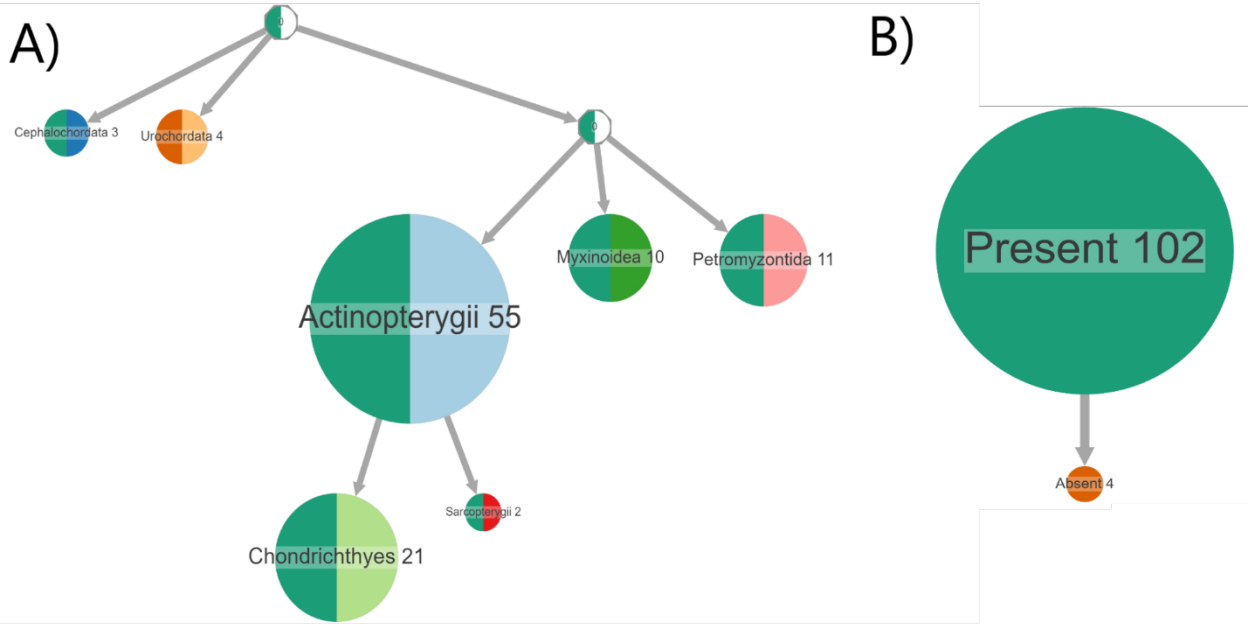


Figure 2.8 PastML results for the constrained COX1 ML tree using MPPA with F81 for the character CYP17. The A) expanded tree with group and tip states and B) most compressed tree with tip states only. Dark green indicates that the character CYP17 is present while orange indicates the absence of this trait.

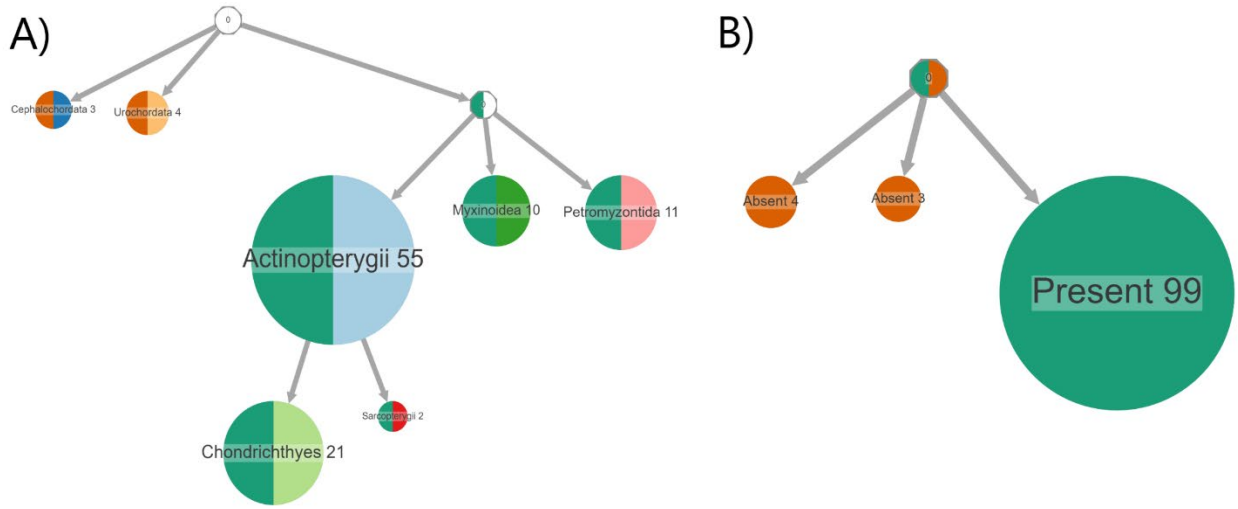
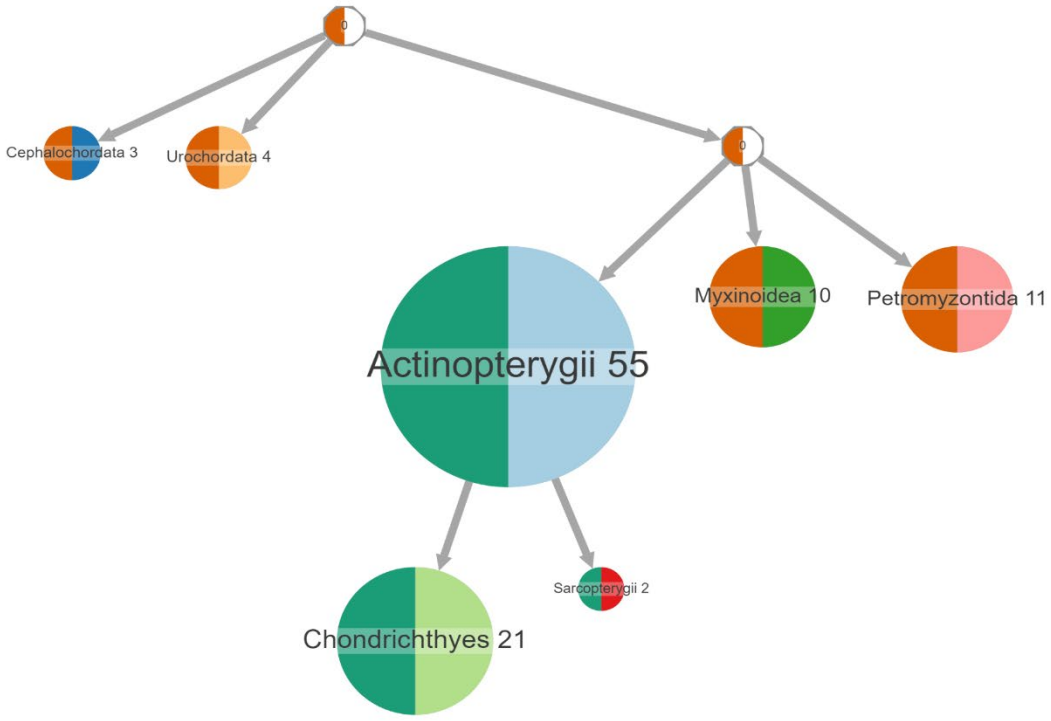


Figure 2.9 PastML results for the constrained COX1 ML tree using MPPA with F81 for the character CYP21. The A) expanded tree with group and tip states and B) most compressed tree with tip states only. Dark green indicates that the character CYP21 is present while orange indicates the absence of this trait.

A)


0
Cephalochordata 3
Urochordata 4
0
Myxinoidea 10
Petromyzontida 11
Actinopterygii 55
Sarcopterygii 2
Chondrichthyes 21

B)

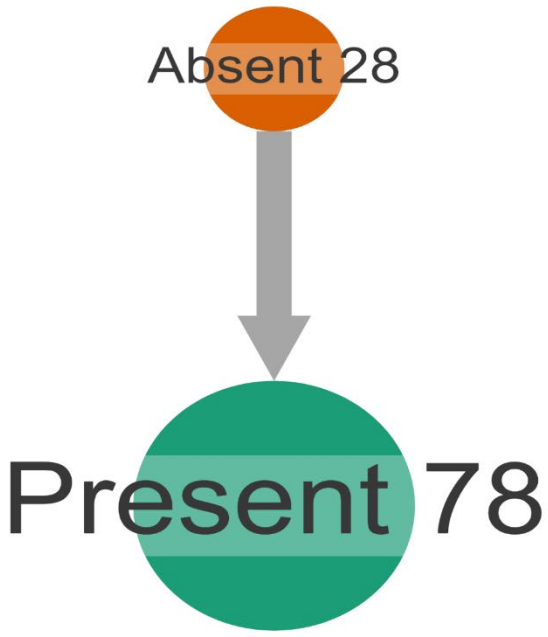

Absent 28
Present 78

Figure 2.10 PastML results for the constrained COX1 ML tree using MPPA with F81 for the character CYP11b. The A) expanded tree with group and tip states and B) most compressed tree with tip states only. Dark green indicates that the character CYP11b is present while orange indicates the absence of this trait.

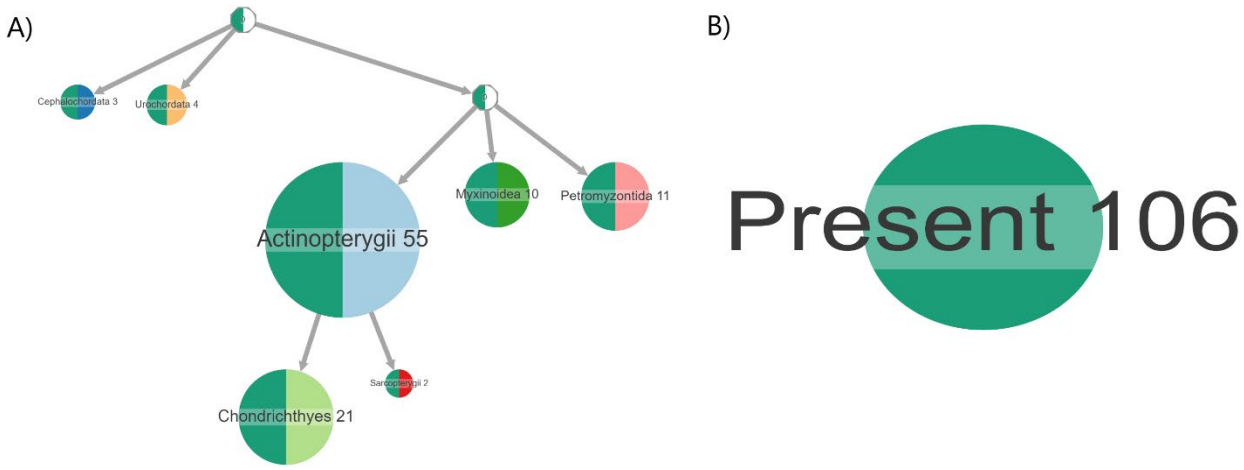


Figure 2.11 PastML results for the constrained COX1 ML tree using MPPA with F81 for the character 3β-HSD. The A) expanded tree with group and tip states and B) most compressed tree with tip states only. Dark green indicates that the character 3β-HSD is present for the taxa identified at each node.

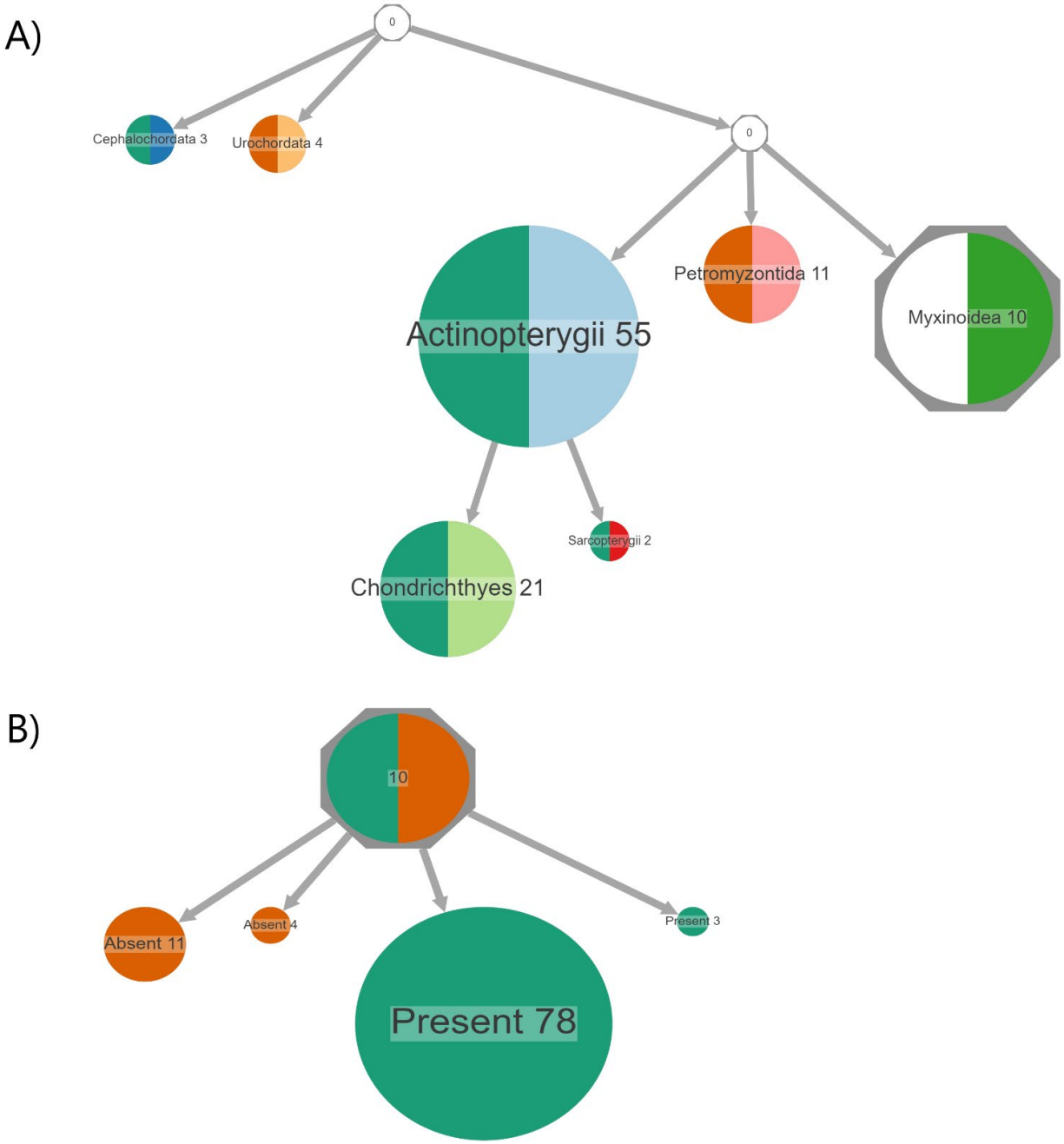
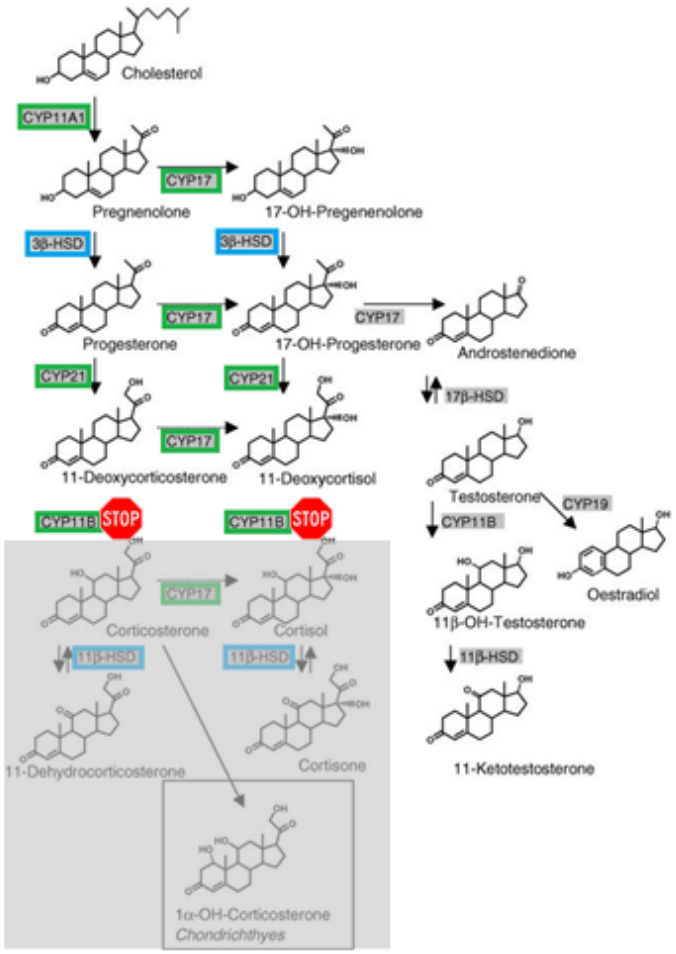

A)
0
Cephalochordata 3
Urochordata 4
0
Actinopterygii 55
Petromyzontida 11
Myxinoidea 10
Sarcopterygii 2
Chondrichthyes 21
B)
10
Absent 11
Absent 4
Present 78
Present 3

Figure 2.12 PastML results for the constrained COX1 ML tree using MPPA with F81 for the character 11β-HSD. The A) expanded tree with group and tip states and B) most compressed tree with tip states only. Dark green indicates that the character 11β-HSD is present, orange indicates the absence of this trait, and white indicates that a unique state could not be chosen for this particular character.

Present

CYP11a1
CYP17
CYP21
3β-HSD



Absent

CYP11b

Undetermined

11β-HSD

Figure 2.13 Ancestral character reconstruction suggests that steroid synthesis cannot proceed past DOC nor 11-DOC in hagfishes. Summary of bioinformatic analyses shows traits predicted to be present, absent, and undetermined in hagfishes. Steroid synthesis pathway image adapted from Bury and Sturm 2007.

Chapter III - Primary stress steroid identification and physiological effects in Pacific hagfish (*Eptatretus stoutii*)

The liquid chromatography tandem mass spectroscopy (LC-MS/MS) work and analysis were done in collaboration with The Metabolomics Centre for Innovation (TMIC) and Dr. David Wishart's lab at the University of Alberta. Study coordination was performed by Rupa Mandal. Sample preparation was performed by Edison (Ying Wei) Dong and Mathew Johnson. Analysis was performed by and sample preparation and LC-MS/MS protocol were obtained from Jiamin (Tammy) Zheng.

3.1 Introduction

Hagfish are unique vertebrates that belong to the group of jawless fish also known as the Cyclostomes (Shimeld and Donoghue 2012). These fish are recognizably unique because of their abilities, in particular, to produce copious amounts of slime and to go for months without eating. Hagfish possess paired slime glands along the length of their body consisting of gland mucous cells and gland thread cells (Fudge et al. 2015). When a hagfish faces a stressful situation, such as a potential predatory attack, the muscles in proximity to the glands contract, releasing the sequestered mature gland mucous and thread cells through the slime gland pores. These cells then release their contents, mucin vesicles and a coiled slime thread (skein), that when in contact with seawater begin to swell. The resulting slime sticks to the predator's gills which usually ceases any other attacks from the predator. Hagfish feeding habits are also an interesting characteristic of this vertebrate. Previous work found that these organisms can go up to 11 months without feeding (Foster and Moon 1986). These fish feed off of decaying carcasses found on the seafloor (Martini 1998) and their skin allows them to absorb salt, water, and amino acids (Glover and Weinrauch 2019). Inside these carcasses, they can be exposed to several stressors such as hypercapnia (elevated water CO_2) which is known to reduce blood pH (acidosis), a physiological stressor by which hagfish are known to recover from rapidly (Parks et al. 2007; Baker et al. 2015). Interestingly, although hagfish skin is quite permeable to water, it was demonstrated that these fish do not drink (Glover et al. 2017a). Rather, their skin allows for great permeability, reducing the movement of fluids between their body and the environment, thus contributing to them being osmoconformers (Glover et al. 2017a,b).

Although a better understanding of the defense and feeding strategies of hagfish has grown in the past few years, a complete understanding of the stress system of the Pacific

hagfish, a basal vertebrate, remains elusive. Presently, there is no evidence identifying the primary corticosteroids(s), steroidogenic enzymes, or the site(s) of steroidogenesis in these fish. Previous studies that aimed to elucidate the primary stress steroid(s) of this unique Cyclostome confirmed that Pacific hagfish possess both mineralocorticoid and glucocorticoid responses, through measurements of gill ATPase activity and plasma glucose levels, respectively (Clifford et al. 2017). The authors injected fish with steroid implants containing probable upstream precursors of corticosteroids, corticosterone, DOC, or 11-DOC, with the goal of upregulating the stress response and monitored physiological responses under multiple stressors. While changes in responses to stressors occurred, they were unable to detect the steroids cortisol or 11-DOC in hagfish plasma, and therefore, the identity of the primary hagfish stress steroid(s) remains unknown (Clifford et al. 2017). However, it is known that hagfishes and their sister group, the lampreys, do not produce cortisol like other vertebrates, and this sets them apart from the rest of the vertebrate lineage (Clifford et al. 2017; Bouyoucos et al. 2021). Current evidence suggests that the lamprey has 11-deoxycortisol (11-DOC) as one of its corticosteroids (Close et al. 2010; Rai et al. 2015; Shaughnessy and McCormick 2021). Given that these two species diverged as a monophyletic lineage prior to the true vertebrates, it might be expected that they might share many similarities. However, with regards to the stress steroids, these two species may present more differences than similarities because 11-DOC was not found in hagfish plasma (Clifford et al. 2017).

The goal of this study is to identify the primary corticosteroid(s) produced by the Pacific hagfish (*Eptatretus stoutii*) as well as the identification of the site(s) of steroidogenesis, by determining the responses of hagfish to various stressors. To accomplish this, a series of physiological stress experiments were performed, and tissue and plasma diverse samples were

collected for analysis of physiological changes, as a continuation of the previous work done by Clifford et al. (2017). Steroid implants of upstream corticosteroid precursors were chosen as a method to both provide upstream steroids for synthesis of the terminal corticosteroid and induce mineralocorticoid and glucocorticoid responses. Since the downstream products of the stress system pathway, DOC and 11-DOC, were not found in Pacific hagfish, pregnenolone and progesterone were used as upstream steroids. Pregnenolone is the first product synthesized from cholesterol in this pathway with help from the enzyme CYP11a1, and progesterone is the steroid synthesized from pregnenolone with the use of the enzyme 3β-HSD. I hypothesized that if one of these steroids induces an upregulation of the stress response in hagfish (i.e., by an increase in plasma glucose concentration), and is detectable in hagfish plasma then this could be a possible candidate corticosteroid involved in the hagfish stress response.

3.2 Materials and methods

3.2.1 Animal capture and holding

Pacific hagfish (*Eptatretus stoutii*) were captured and collected in the summers of 2020 and 2021 from Barkley Sound near Bamfield, British Columbia (BC), Canada (Table 3.1). Barrel and bucket traps baited with rotting Pacific hake (*Merluccius productus*) were lowered and left overnight. The following day, the traps were raised, hagfish sorted by size, and placed in large buckets with seawater until transport to the laboratory. Fish smaller than 45 g were returned to the ocean. Hagfish were transferred into large, 500-L tanks with running seawater (12 ± 2°C) at the Bamfield Marine Sciences Centre (BMSC) in Bamfield, BC. The fish were left to acclimate for two weeks to acclimate to establish a baseline metabolism and fasted prior to experimentation. Polyvinyl chloride (PVC) pipes were placed in the tanks for habitat

enrichment. Fish were slid from the PVC tubes into a 10-L bucket for transportation to the lab. Fish were then separated into individual experimental chambers, darkened with black electrical tape, and monitored twice daily for water flow, temperature, airflow, and animal health. All experimental chambers were placed inside a wet table with constant water flow for temperature maintenance (12°C). All fish were collected and maintained under the Department of Fisheries and Oceans (DFO) Canada collection permits XR 214 2020 and XR 157 2021 and the University of Alberta Animal Use Protocol (AUP) 00001126_AME6 and BMSC AUP RS-20-14 and RS-21-04.

3.2.2 Reagents

Reagents were supplied by Sigma-Aldrich Canada Co. (Oakville, ON, CA). Reagents and kits for molecular assays were supplied by Thermo Fisher Scientific (Waltham, MA, USA). Steroids cortisol (4-pregnen-11β, 17, 21-triol-3, 20-dione), pregnenolone (5-pregnen-3β-ol-20-one), and progesterone (4-pregnen-3, 20-dione) were obtained from Steraloids, Inc. Newport, RI, USA (www.steraloids.com). Coconut oil was purchased from a local health food store in Edmonton, AB, CA. Tricaine methanesulfonate (TMS) was supplied by Syndel laboratories in Nanaimo, BC, Canada (www.syndel.com).

3.2.3 Experiments

Experiments were performed in two separate field seasons. The initial set of experiments were conducted to examine the physiological response of Pacific hagfish to two stressors (acid injection and exercise) and to see if the injection of an upstream steroid would affect their response to the stressors. The goal was to induce a stress response in the fish such that we may be able to report physiological changes and detect the presence of corticosteroids in

the fish. The injection of upstream steroids was designed to make more of a necessary upstream steroid available to the fish to use in their stress response and for us to record possible amplification of the stress response. The second set of experiments were conducted to again examine the physiological response of hagfish to a stressor (with and without upstream steroid injection) and to collect a series of tissues for molecular analysis. The goal of this set of experiments was to use molecular tools to locate the site of steroidogenesis in hagfish. These experiments are described in detail below.

3.2.3.1 Steroidal profiles in hagfish during environmental stressors

To determine the corticosteroid profiles of hagfish, a series of environmental stressors were used to initiate a stress response:

1) Acid (HCl) injection: To lower the blood pH, fish were injected in the caudal sinus with a dose of ~ 6000 μequiv/g (12 μL/g hagfish) of a 250 mM NaCl/250 mM HCl solution (Parks et al. 2007).
2) Exercise: Fish were exercised by 30 minutes of handling stress. This exhausted the fish and resulted in the release of large volumes of slime.

Each of the above stressors was induced with a parallel control group of animals not undergoing any stress treatment. Therefore, there were three treatment groups (one control group and two experimental groups).

The goal of this experiment was to examine the time course of an environmental stressor on hagfish plasma and tissues that may be involved in steroidogenesis. For each experiment, fish were placed into individual 0.5-L darkened glass containers. Each container had two holes drilled into the lid that allowed for the supply of running seawater and air, and constant running seawater on the exterior of the container. Fish were held overnight in these containers to

acclimate. At the start of the experiment, 2.5 mL of 200 g/L TMS solution buffered with 10 g $NaHCO_3$ was added to the 0.5-L container, allowing for rapid anaesthesia (< 2 min.) and blood sampling of the animal. Anaesthesia was confirmed by unresponsiveness to a pinch of the fish tail using forceps. Following this, a 0.4 mL blood sample was taken from the caudal sinus using a heparinized 1-mL syringe and a 20 G needle. After the blood sampling, the animal was returned to its container filled with fresh seawater to recover, usually within 2 min. Serial blood samples were taken at 0 (pretreatment), and 2, 4, 8, 24, and 48 hours post treatment (post stressor treatment, either acid injection or exercise) according to the above protocol. Blood pH was measured immediately using a micro pH electrode (ROSS Orion) and a pH meter (Fisher Scientific Accumet Research, AR15) at a controlled water temperature of 12°C using a water bath (Fisher Scientific isotemp 3016D). Then, whole blood was centrifuged allowing plasma separation from red blood cells. Total carbon dioxide (TCO_2) was measured in fresh plasma for control and acid-stressed groups using a TCO_2 analyzer (Corning Limited 965, Halstead Essex, England). The remaining plasma was flash-frozen in liquid N_2 and stored at – 80°C for later analysis by liquid chromatography tandem mass spectroscopy (LC-MS/MS) in collaboration with The Metabolomics Innovation Centre (TMIC) at the University of Alberta. LC-MS/MS provides the mass and structural information of the compounds within each plasma sample and serves to identify any steroid products circulating in the fish plasma.

When the experiments were complete, fish were euthanized using 5 g/L TMS buffered with 10 g sodium bicarbonate ($NaHCO_3$). Gills were dissected and samples were fixed in a glass vial with 2.5 mL of 25 % glutaraldehyde for later histological examination while kidney samples were each put into a 2.0 mL Eppendorf tube with 0.75 mL of RNA later for RNA isolation.

In the second field season, the environmental stressor used was the same acid (HCl) injection. Blood samples and parameters (pH and TCO_2) were taken at t = 0 h and following treatment (t = 6 h), just prior to euthanasia as described above.

3.2.3.2 Steroids for the control of mineralocorticoid responses in hagfish

The purpose of this experiment was to manipulate the corticosteroid profiles of hagfish using coconut oil implants infused with the upstream steroids pregnenolone and progesterone. We hypothesize that an appropriate steroid injection treatment would accelerate the production of the terminal steroids and affect terminal steroid-dependent ionoregulatory processes. The goal was to compare the time course and examine the responses of the steroids to determine mineralocorticoid and glucocorticoid responses in hagfish. For example, the return of blood acid-base parameters to control levels when compared to sham-injected fish after 48h.

Fish were grouped into two main groups: those without any upstream steroid injection (no steroid), and those with an injection (steroid or sham, vehicle only). Fish with a steroid injection received either pregnenolone or progesterone while sham-injected fish only had an injection of coconut oil. Coconut oil was melted at 27°C and the appropriate amount of steroid was added to yield a final plasma steroid concentration of 200 ng/L or an equivalent volume of coconut oil was added for the sham group.. The coconut oil was drawn into a 1 mL Hamilton syringe with a 20 G needle. The syringe and needle were kept warm on a hotplate to maintain fluidity of the injection. Fish were anesthetized as previously described and the injections were administered into the peritoneal cavity of the fish four days prior to subjection to a stressor (see above). No inflammation around the injection site was visible in any of the fish. The coconut oil hardens inside this region of the fish and gradually releases the steroid at a relatively stable

rate over the course of seven days (Clifford et al. 2017). Correct placement of the implant was confirmed following euthanasia of the fish.

Fish were divided into three cohorts, each with a different treatment as described in the first experiment, with 22 - 24 fish per treatment group (total 66 - 72 fish). One cohort had no steroid injection. The second and third cohorts were injected with pregnenolone or progesterone, respectively. Parallel control groups for each of the steroid injected fish cohorts were fish injected with sham.

In the second field season, the experiment began with no steroid, sham, or steroid-infused pregnenolone injection as described above. Placement of the implant was verified after euthanasia. Four days after the implant was injected, some hagfish were subjected to the acid (HCl) injection treatment as done before (Section 3.1.3.1). Fish were divided into three cohorts: no steroid injection (control); pregnenolone injection; and pregnenolone + acid injection. Each of these cohorts had two groups of fish at different timepoints, t = 0 and 6 h. Fish at t = 0 h were prior to subjection of the treatment whereas fish at t = 6 h were six hours posttreatment. There were 12 fish per cohort with six fish per timepoint plus one fish for testing the methods (total 37 fish).

3.2.4 Steroid implant validation

To validate the method and doses of the steroidal implants, two separate groups of fish were also injected with either sham or cortisol (total 12 fish) and serial blood samples were taken over the course of seven days (Clifford et al. 2017). Cortisol, a steroid known to have no effect in hagfish, was used to measure the steroid dose in a smaller number of fish (n = 6) with the sham (n = 6) as a parallel control group. A commercially available cortisol radioimmunoassay (RIA) kit was used to measure the cortisol levels of fish (ImmuChem™

Coated Tube Cortisol ^{125}I RIA Kit, MP Biomedicals, LLC, Diagnostics Division, 13 Mountain View Avenue, Orangeburg, NY, 10962). It is assumed that the concentration of cortisol would approximate the concentrations of upstream steroids, pregnenolone or progesterone, in fish with the steroid implant.

3.2.5 Exercise stressor validation

To validate the exercise stressor, a glucose assay was used on plasma from exercised fish along with parallel controls. When fish are stressed by the 30 minutes of handling, there should be an increased secretion of glucose into circulation as a response to this stressor.

3.2.6 Acid-base measurements and acid injection validation

Acid-base measurements (pH and total CO_2 as described above) were taken for each treatment group (no steroid, pregnenolone injection, and progesterone injection) for control and acid-injected fish. These measurements serve to validate the method of injection and to confirm that fish experienced a metabolic acidosis. pH and TCO_2 measures were analysed for each treatment group. The solubility coefficient of carbon dioxide (αCO2) and the apparent pK of CO_2 were determined (Giacomin et al. 2018) based on the equations presented for 12°C (Heisler 1984). Using the results of these two equations, the PCO_2 of plasma was determined using the measured TCO_2 and pH in a modified Henderson-Hasselbalch equation (Giacomin et al. 2019):

$$PCO_2 = TCO_2/[\alpha CO_2 \times (1 + \text{antilog}(pH - pK_{app}))]$$

TCO_2 (mmol/L) was determined and used along with the pH to calculate the concentration of HCO_3^- (mmol/L). The concentration of HCO_3^- was calculated using the equation (Giacomin et al. 2019):

$$[HCO_3^-] = TCO_2 - (\alpha CO_2 \times PCO_2)$$

The average blood pH, PCO_2 and concentration of HCO_3^- of control pretreatment fish (t = 0) were calculated and then used to determine the formula of the non-bicarbonate buffer (NBB) line (Wells et al. 1986). The averages of these values for each of the other treatment groups and time points were also calculated. This data was plotted into pH/HCO_3^- plots, also known as Davenport diagrams, using GraphPad Prism v 9.3.0.463.

3.2.7 Gill tissue collection

Gill samples were collected from all three treatments and stressors while at the BMSC. At the end of the time course of the experiments, fish were euthanized, and gills were collected for later histological analysis using light microscopy. Whole gill pouches were collected from each hagfish and placed in a glass vial with 2.5 mL of a 25 % glutaraldehyde and a 150 mM sodium cacodylate trihydrate solution, pH 7.35 solution on ice and then held at 4°C overnight. The next day, tissues were rinsed twice in 70 % ethanol (EtOH) and kept in 70 % EtOH at 4°C until processing.

At the end of the experiments in the second field season, fish were terminated, and gill samples were also collected from all fish by fine scale dissection using a dissecting microscope. Gill pouches were cut for conventional scanning electron microscopy (SEM) according to the diagram in the Appendix (SI 1.). Each section was each placed in a glass vial with 1 mL of a 2.5 % glutaraldehyde, 2 % paraformaldehyde, 0.1 M phosphate buffer fixative solution at 4°C until processing.

Hematoxylin and Eosin (H & E) staining

Three gills from each treatment group (no steroid injection, pregnenolone injection and progesterone injection) for the acid stressor and parallel controls were processed for histology.

Gills were dehydrated in a series of EtOH and toluene washes overnight using a tissue processor (Leica TP1020 Tissue Processor, Leica Biosystems, IL, USA) at the Microscopy Facility at the University of Alberta, Edmonton, AB. The following day, tissues were embedded in paraffin wax and sectioned to 5 and 7 μm using a microtome (Leica RM2125 RTS, Leica Biosystems, IL, USA), placed on microscope slides, and left overnight to dry in an incubator at 37°C. Next, paraffin was removed with a series of washes involving toluene, EtOH, and distilled water. The tissues were then stained with hematoxylin, rinsed with water followed by 70% EtOH, and then stained with eosin. Finally, tissues were rinsed with EtOH and toluene and a coverslip was applied with DPX (dibutylphthalate polystyrene xylene) mounting medium to each slide. The slides dried overnight in an incubator at 37°C.

Morphometrics

The H & E processed gill sections (n = 3) of control and acid-stressed fish (t = 48 h) were visualized and photographed five times at random using a brightfield microscope (Zeiss Axio Scope A1, Carl Zeiss AG, TO, CA) with a mounted camera. A grid made up of 2.5 x 2.5 cm squares was created and overlaid atop each image in Adobe Photoshop (v 23.0.2). The thickness of the lamellar epithelium was measured using ImageJ (v 1.53e) and converted from width in pixels to μm. At the intersections of the grid where there is tissue present, a measurement of the lamellar epithelial width was taken. Where there was no overlay between the intersections of the grid and this structure, no measurement was taken. See Appendix for an example of the grid atop a microscopy image (SI 2.). Lamellar epithelial width measurements were averaged, and the standard error of the mean (SE) were calculated (Fig. 3.7). A representative photo of main structures and from each control group and experimental group for each treatment is included below (Fig. 3.5 and 3.6).

Scanning Electron Microscopy (SEM)

Three gill sections from each treatment group (no steroid injection, pregnenolone injection, pregnenolone + acid injection) and at two timepoints (0, 6 h) were processed for conventional SEM. Gills were dehydrated in a series of EtOH washes followed by hexamethyldisilazane (HMDS) washes in a fume hood. Samples were air dried overnight. The next day, SEM stubs were coated with a thin layer of nail polish prior to mounting each sample. Because some gill samples were larger, the application of nail polish was done to improve contact of the tissue with the stub for better conductivity. Samples were sputter coated with gold/palladium (Au/Pd) and examined with a scanning electron microscope (Zeiss Evo 10, Carl Zeiss AG, TO, CA). A representative photo from each group taken at 10 and 15 KX is included below in section 3.5.

3.2.8 Targeted liquid chromatography tandem mass spectroscopy (LC-MS/MS) in collaboration with The Metabolomics Innovation Centre (TMIC) at the University of Alberta

Sample preparation

Hagfish plasma samples were thawed on ice in the dark before use. A sample volume of 100 µL (PBS as blank sample, calibration standards, QC standards and plasma samples) mixed with 20 µL of ISTD mixture solution were pipetted into Eppendorf tubes. A volume of 100 µL of PBS buffer was then added to each tube and vortexed for 30 s. A volume of 1000 µL of methyl tert-butyl ether (MTBE) was then added to each tube for extraction. The samples were shaken at 1000 rpm for 15 min. After that, samples were centrifuged at 13,000 rpm at 4°C for 15 min, and 750 µL of supernatants were transferred into high-performance liquid chromatography (HPLC) vials and dried under nitrogen purge until completely dry. To the

dried tubes, 100 µL of derivatization solution (1.5 M Hydroxylamine in HPLC grade water) was added, followed by shaking at 150 rpm for 15 min. All the tubes were then incubated at 60°C for 1 hour and then 20 µL was injected into an UHPLC-equipped QTrap 4000 mass spectrometer for LC-MS/MS analysis.

LC-MS/MS method

An Agilent 1260 series UHPLC system (Agilent, Palo Alto, CA) was used for LC-MS/MS analysis with an AB Sciex QTrap 4000 mass spectrometer (Sciex Canada, Concord, ON). The controlling software for the LC-MS system was Analyst 1.5.2. For the HPLC work, solvent A was 0.1% formic acid in water; and solvent B was 0.1% formic acid in methanol. The gradient profile for the UHPLC solvent run was set as follows: t = 0 min, 10% B; t = 1.50 min, 10% B; t = 2.50 min, 55% B; t = 7.50 min, 95% B; t = 8.50 min, 95% B; t = 8.60 min, 10% B; and t = 12.0 min, 10% B. The flow rate was 0.5 mL/min and the sample injection volume was 20 µL. The mass spectrometer was set to a positive electrospray ionization mode with multiple reaction monitoring (MRM). The IonSpray voltage was set at 5500 V and the temperature at 550°C. The curtain gas (CUR), ion source gas 1 (GAS1), ion source gas 2 (GAS2) and collision gas (CAD) were set at 40, 60, 60 and medium, respectively. The entrance potential (EP) was set at 10 V. Likewise, the declustering potential (DP), collision energy (CE), collision cell exit potential (CXP), MRM Q1 and Q3 were set individually for each analyte and ISTD.

3.2.9 Additional tissue collection for RT-qPCR

Tissue collection and RNA isolation

Tissue samples including brain, gill, intestine (foregut, midgut, and hindgut), kidney, and gonad were collected and placed into individual 2.0 mL screw cap Eppendorf tubes each with 1 mL of TRIzol reagent on ice. Previous research in our lab has demonstrated that to get optimal quality of RNA extraction of hagfish tissue, extraction must be performed on freshly isolated tissues without freezing. A whole gill pouch was removed from each fish, placed in a Petri dish with RNA later and then cut finely with a scalpel under a dissecting microscope. A small piece of each gill was placed into a tube with TRIzol reagent, as noted above, to facilitate rapid entry of the reagent into the tissue. Hagfish intestine was removed from the body and placed on RNase-zapped aluminum foil-covered ice packs where it was cut open and laid flat. The tissue was rinsed with an ice-cold 2 mM PMSF, 500 mM NaCl and 10 mM EDTA solution to inhibit serine proteases of the gut. The intestine was divided into three pieces: fore-; mid-; and hindgut. After cutting the gut, each piece was briefly immersed into a 50 mL Falcon tube with 10 mL of TRIzol reagent. This slightly stiffened the mucosal lining of the intestine. Then, the internal lining of each section was scraped using an RNase-zapped microscope slide. The collected tissue from each section was placed into separate tubes with TRIzol reagent. Hagfish kidney was collected from the upper and lower regions of the organ and cut into smaller pieces to ease homogenization.

Tissues were homogenized using a hand-held homogenizer (Polytron PT 12000 E). The homogenizer tip was rinsed between each tissue with a series of soap and water, ELGA water, and 70% EtOH to prevent contamination between tissues. After homogenization, RNA was

isolated from each tissue sample using a TRIzol extraction protocol from our lab. Samples were aliquoted into new tubes (30 µL/tube) and stored at – 80°C until use.

Genomic DNA (gDNA) removal

RNA quantity and quality were tested using a nanodrop (ND-1000 Spectrophotometer, Thermo Fisher Scientific) for each tissue: Brain (B); Gill (Gi); Foregut (FG); Midgut (MG); Hindgut (HG); Upper Kidney (UK); Lower Kidney (LK); and Gonad (Go). Of the eight different tissue sections collected, six (FG, MG, HG, UK, LK, and Go) were chosen for further processing. These tissues were selected because they are hypothesized to be possible steroidogenic tissues based on the steroidogenic tissues of other fish (i.e., teleosts – kidney, lamprey – kidney). If the concentration of the sample was high (greater than 2000 ng RNA/µL), the sample was diluted by a factor of 10. gDNA was removed using the DNase I, RNase-free, kit (Thermo Fisher Scientific) according to manufacturer instructions, including a phenol-chloroform purification (Richter 2019). Samples were stored at – 80°C until processing. Quantity and quality were measured using a nanodrop spectrophotometer (ND-1000) and six randomly selected RNA samples were tested for RNA integrity on a 0.8 % RNase-free agarose gel.

Complementary DNA (cDNA) synthesis

RNA samples with concentrations > 100 ng μL^{-1} were diluted to a common concentration for each tissue prior to cDNA synthesis. Concentrations (ng μL^{-1}) of each RNA sample per tissue were Go (50), FG (85), MG (50), HG (62), UK (40), and LK (27). A first-strand cDNA synthesis kit was used for this procedure (Superscript III Reverse Transcriptase, invitrogen, Thermo Fisher Scientific). Additional reagents supplied by Invitrogen (Thermo

Fisher Scientific) were necessary for this protocol: 50 µM Oligo(dT)$_{20}$ Primer; 10 mM dNTP mix; and RNaseOUT Recombinant Ribonuclease Inhibitor. cDNA samples were stored at –20°C until further use.

Primer design

Primers for six genes of interest (*cyp11a1*; *cyp17*; *cyp21*; *cyp11b*; *3βhsd*; and *11βhsd*) involved in the steroidogenesis pathway were designed. We developed primers using publicly available sequences from closely related species (NCBI; SI 4), a publicly available genome for *Eptatretus burgeri* (EMBL-EBI; Yamaguchi et al. 2020), and a newly constructed Pacific hagfish transcriptome. To obtain *E. burgeri* transcripts, hagfish gut was collected as described above, samples were diluted to < 500 ng/µL, and tested using an Agilent 2100 Bioanalyzer (Agilent Technologies). Anterior gut samples with an RNA integrity number (RIN) higher than 9 (on a scale of 1 – 10) were sent to Genome Québec for processing. Forward and reverse sequences for the twenty individual fish (unpublished) were uploaded to OmicsBox (v 2.0.36; BioBam Bioinformatics 2019 Mar 3) where default parameters were used to generate transcripts. This resulted in sequences, which were imported to Geneious Prime v. 2021.2.2 (www.geneious.com), along with a publicly available genome (Yamaguchi et al. 2020) and specific sequences from closely related species from the GenBank Public Database (Altschul et al. 1997). Primer3 software (Koressaar and Remm 2007; Untergasser et al. 2012) was used to generate primers. Primers were obtained from Integrated DNA Technologies (IDT; www.idtdna.com/pages).

Primer testing

gDNA from hagfish kidney was isolated using a commercial extraction kit (DNeasy Blood & Tissue Kit, QIAGEN) and run on a nanodrop to determine concentration. A polymerase chain reaction (PCR) was used to amplify 200 ng of gDNA using dNTPs (10 mM of each dATP, dCTP, dGTP and dTTP; Thermo Fisher Scientific), *Taq* DNA Polymerase, 0.2 µM primers, 1X *Taq* Buffer from New England BioLabs (NEB), according to manufacturer instructions for the *Taq* polymerase (NEB).

gDNA from select samples were visualized on a 1.5 % agarose gel with GelRed Nucleic Acid Stain (Biotium) using a transilluminator (AlphaImager 2200, Alpha Innotech). Only reactions that produced single bands at the approximate predicted molecular weight (SI 4) were used for sequencing and RT-qPCR analysis. See Appendix for gel results (SI 3).

Sanger Sequencing

PCR products were purified using the QIAquick PCR purification kit (QIAGEN) according to manufacturer instructions, and then quantified using a nanodrop spectrophotometer (ND-1000). PCR products (75 ng) were sequenced on a 3730 Genetic Analyzer with either 0.25 µM forward or 0.25 µM reverse primers. Sequences were imported into Geneious (v. 2021.2.2 (www.geneious.com), forward and reverse sequences aligned (if possible) to each other and the resulting consensus sequences were aligned using custom databases within Geneious against the *E. burgeri* genome and transcriptome, in addition to the basic local alignment search tool (BLAST) from NCBI (SI 4).

Real Time quantitative Polymerase Chain Reaction (RT-qPCR)

Four reference genes were found suitable for testing on RT-qPCR: Rpl22 (ribosomal protein L22), β-Actin, Cnot ctd (CCR4-NOT Transcription Complex Subunit 1), and Ef1a (translation elongation factor 1 A). The prepared cDNA was processed and examined via RT-qPCR for expression of reference genes in hagfish tissues.

An aliquot of each cDNA sample was combined and five 1:5 dilutions were generated. These dilutions were used to generate a standard curve for RT-qPCR for each primer pair. Each cDNA sample was serially diluted 1:5 twice prior to RT-qPCR.

RT-qPCR was performed in triplicate 15 µL reactions using 1x SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad Laboratories, Mississauga, CA), 0.3 µM forward and reverse primers and 10 ng cDNA. The assay was run using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Mississauga, CA) with the following program: 95°C (20 s), 40 cycles of 95°C (15 s), and 60°C (30 s). Melt curve analysis was performed using the 7500 Software v 2.3 (Life Technologies Corporation) to test reaction specificity and demonstrated a single PCR product. LinRegPCR (v 2013:0; Ruijter et al. 2009) was used to determine threshold cycle (C_t) values and amplification efficiencies for each sample and gene. C_t values were also examined in qbase+ v 3.2 (201807021216). Replicates with efficiencies > 10 % from the group median and those that had no plateau were removed from downstream analysis.

3.2.10 Statistical analyses

All data, unless otherwise noted, are presented as means ± standard error of the mean (SE) and calculated using GraphPad Prism v 9.3.0.463 for Windows (GraphPad Software, San Diego, USA, www.graphpad.com). Differences between treatment groups were tested using a

two-way ANOVA. A Šidák's multiple comparisons test ($p < 0.05$) was used to test for differences across treatment groups at a specific timepoint, and a Dunnett's multiple comparisons test ($p < 0.05$) was used to test for differences within each treatment group across different timepoints. A two-tailed, unpaired t-test was used to compare two independent, unrelated groups, assuming that the variance between the groups is equal, to determine if there was a significant difference between the two groups. RT-qPCR data is presented as means with ± standard deviation (SD).

3.3 Results

3.3.1 Elevation of plasma cortisol concentrations post-injection

The results of the RIA demonstrate that fish 24 hours after injection with cortisol had elevated cortisol concentrations that were gradually depleted from circulation over the next six days (Fig. 3.1). Fish injected with a sham implant did not have any cortisol present in their blood, as is expected since this steroid is not known to circulate in hagfishes (Clifford et al. 2017).

3.3.2 Increase of plasma glucose levels following the exercise stressor

Plasma glucose levels of fish without steroid pretreatment are presented in Figure 3.2 A. Control and exercise-stressed fish both experienced an increase in plasma glucose levels over 48 h as a result of the sampling treatment. At 2, 4, and 8 h, plasma glucose concentrations were significantly (*) higher in exercised fish when compared to time-matched control fish demonstrating a 14-, 8- and 3-fold increase, respectively. Additionally, the plasma glucose concentrations of control fish at 8, 24, and 48 h were significantly increased compared to the

time 0 h (control) sample while plasma glucose concentrations of exercised fish were significantly higher at every time point compared to the time 0 h control.

In control fish and fish pre-injected 4 days earlier with a sham treatment (Fig. 3.2 B), plasma glucose levels were also increased in both treatments because of the repetitive sampling protocol (represented as either + or x) similar to the control and exercised fish seen in Fig. 3.2 A. However, the plasma glucose levels of fish without the exercise stressor (Fig. 3.2 B) were not as elevated as those that were subjected to the exercise stressor.

All exercise, and steroid-injected fish (with either pregnenolone or progesterone) experienced an increase in plasma glucose concentrations over the time course of the experiment (Fig. 3.2 C). At 2, 4, 8, and 48 h, plasma glucose concentrations were significantly (*) higher (2 – 2.5-fold increases) in exercised fish without any steroid injection compared to time-matched pregnenolone-injected + exercise fish. Exercise-stressed fish without any steroid treatment had significantly higher (+) levels of plasma glucose at all timepoints compared to at pretreatment (0 h). Pregnenolone-treated fish also had significantly elevated (x) plasma glucose levels at all timepoints compared to 0 h, prior to the exercise stressor. Progesterone-treated fish had significantly higher (•) plasma glucose levels only at 2, 8, and 48 h compared to pretreatment (0 h). Interestingly, plasma glucose concentrations of exercised fish without any steroid treatment were greater than both groups of fish with steroid treatment at 24 and 48 h post treatment.

3.3.3 Fish experience metabolic acidosis post-acid (HCl) stressor

Control fish blood was slightly alkaline throughout the experiment (Fig. 3.3 A), with the average blood pH being 7.76. All fish had HCO_3^- concentrations higher than that of the NBB. The P_{CO2} lies between 2.5 and 10 Torr. Fish subjected to the acid stressor but without the

steroid treatment (Fig. 3.3 B) experienced metabolic acidosis within the first two hours post treatment where the average blood pH of the fish was around 6.4 and the HCO_3^- concentration was below 5 mmol/L. In the hours following treatment, the blood pH of the fish gradually rose to an average of 7.52, becoming less acidic, and the HCO_3^- concentration slowly reached an average of 5.66 mmol/L at 48 h post treatment. Interestingly, the blood pH of the fish is lower at 48 h compared to 24 h post treatment (Fig. 3.3 B).

The fish subjected to the acid stressor and treated with either the pregnenolone injection (Fig. 3.3 C) or the progesterone injection (Fig. 3.3 D) also experienced metabolic acidosis post treatment. Pregnenolone-treated fish (Fig. 3.3 C) had average blood pH levels of 6.46 at 2 h post stressor that rose to an average of 7.35 at 48 h. This group of fish also had mean HCO_3^- concentrations of 2.12 and 5.66 mmol/L at 2 and 48 h post treatment, respectively. Progesterone-treated fish (Fig. 3.3 D) displayed similar mean blood pH levels of 6.83 at 2 h and 7.31 at 48 h post treatment with mean HCO_3^- concentrations of 2.35 (2 h) and 4.31 mmol/L (48 h), too. Over the course of 48 h, the fish gradually balance their blood pH and bicarbonate concentration.

Fish subjected to the acid stressor with a pregnenolone injection in field season #2 also had significantly different blood pH levels following stress (6 h) compared to pretreatment (0 h; Fig. 3.4). Control and pregnenolone-injected fish without the acid injection, however, did not experience a significant change in blood pH.

3.3.4 Changes in lamellar epithelium in acid-stressed fish

The structure of the hagfish gill is unique amongst the fishes and Figure 3.5 is provided to demonstrate the internal structures of the hagfish gill pouch. A sagittal H and E-stained section from a control fish is included to identify the filament (F), lamellae (L), epithelium (E),

cartilage (C), and lamellar epithelium (*). The filament is a large, thick-walled structure containing cartilaginous tissue (C) stained pink at its centre while the filament and lamellar epithelia (*) are stained purple. Lamellae have cartilaginous tissue and are the primary site of gas transfer and ion regulation.

In control fish, the lamellae are contiguous and have clean, distinguishable edges (Fig. 3.6 A). Acid-stressed fish (48 h; Fig. 3.6 B) have lamellae epithelia that do not appear to show any significant alterations when compared to control fish. However, the morphometric analysis demonstrates that acid treatment alone results in a thinning of the lamellar epithelia by 1.21 % (Fig. 3.7).

3.3.5 Changes in microplicae between control and acid-treated fish via SEM

The surface profiles of the main structures of hagfish gill pouches including lamellae, cell-to-cell junctions, mucous cells, and microplicae are shown by SEM (Fig. 3.8). Under control conditions, the lamellae show significant infoldings. Cell-cell junctions (CJ) are not readily apparent in control animals but appear as shallow indentations to the trained eye (Fig 3.8 A). In control animals, the surface microplicae are generally short and rounded in form as seen in the higher magnification image (Fig 3.8 B).

When hagfish are pre-treated with pregnenolone, there is a distinct increase in roughness of the gill epithelia with an increase in folding of the epithelia noted for treated fish (Fig. 3.8 C, E). Moreover, the microplicae present on the surface tend to be more elongated compared to control fish epithelia (Fig. 3.8 D, F). When pregnenolone-treated fish are exposed to an acid stressor, the microplicae change in appearance to become even further elongated with many cells showing flattened and more prominent microplicae when compared to the control group

(Fig. 3.8 G – H). Finally, in pregnenolone-treated and acid-stressed fish, the cell-cell junctions between epithelial cells become easily distinguishable (Fig. 3.8 G).

3.3.6 Steroid products identified via targeted LC-MS/MS

A sample chromatogram identifying the main peaks of steroids present in hagfish plasma are presented in Fig. 3.9. Preliminary results from the targeted LC-MS/MS analysis of hagfish plasma from all eight fish without steroid pretreatment after either acid stress (n = 4) or exercise stress (n = 4) demonstrated low concentrations (< 3.0 ng/mL) of pregnenolone, progesterone, and dehydroepiandrosterone (DHEA) (Fig. 3.10). There was one of the exercise-stressed fish that had detectable 17-hydroxyprogesterone and estrone in their plasma while the other three did not have these steroids in sufficient quantities for detection. For all other steroids, no steroid peaks corresponding to the known steroid standards used were detected. There was no detectable peak for cortisol at a retention time of 2.2 min. All other small peaks noted were considered below detection limits and did not run at elution times to allow identification. It should be noted that two of the four fish subjected to the acid injection stressor had progesterone detected in their plasma while all four fish subjected to the exercise stressor had progesterone detection. All fish had pregnenolone and DHEA detected in their plasma.

3.3.7 Primers developed for reference genes for RT-qPCR

Considerable effort was expended to validate a method to both extract RNA from hagfish tissues that are of high enough quality for molecular investigation and to design and validate reference genes for RT-qPCR since these methods have not been reported used in Pacific hagfish previously. While there was good stability of gene expression for the reference genes within each tissue, there was considerable variability between tissues as seen in Fig 3.11.

Results of the Standard (2 ng/µL) of the reference genes suggest variability in the expression of the four reference genes for hagfish in all tissues tested with gonad showing lowest level of each reference gene expression compared to other tissues which showed consistent but higher expression of each other reference genes. The lowest expression levels for all tissues were found for Cnot ctd and Rpl22. Unfortunately, the primers designed for each of the steroidogenic enzymes of interest (Cyp11a1, Cyp17, Cyp21, Cyp11b, 3β-HSD, and 11β-HSD) were unable to be validated so those results cannot be verified at this point.

3.4 Discussion

The results of the analyses completed for this study confirm that hagfish have a novel stress steroid profile in that they do not possess cortisol as the primary corticosteroid. Of the plasma analyzed from Pacific hagfish, only significant concentrations of pregnenolone, progesterone, and DHEA were detected via LC-MS/MS. To my knowledge, this is the first study using LC-MS/MS for the detection of corticosteroids in hagfish plasma although there have been other studies performed using UPLC-MS/MS for the detection of bile acids in sea lamprey (*Petromyzon marinus*) plasma and tissues (Wang et al. 2015), and thyroid hormones (Bussy et al. 2017).

The absence of cortisol detection in hagfish plasma is consistent with previous findings that this downstream steroid is not known to circulate in this fish (Clifford et al. 2017). Moreover, in lampreys, the other primary cyclostome lineage, cortisol is also thought to be absent (Close et al. 2010). This gives further credulity to the idea that the cyclostome lineage is distinct from the gnathostomes. While the steroids found in the plasma samples from stressed hagfish are in the low range (< 3 ng/mL), they are similar when compared to some other fish species. For example, a study examining fish steroid levels in plasma and bile of a flounder

species (*Platichthys flesus*) had mature flounders with pregnenolone and progesterone levels of < 0.4 ng/mL and ranging from 1.4 - 4.4 ng/mL, respectively (Budzinski et al. 2006). The same study also reported 17-OH-pregnenolone at 1.8 - 9.5 ng/mL and 17-OH-progesterone at 0.5 - 1.3 ng/mL in unstressed flounder. In my study, since pregnenolone and progesterone were found in the hagfish plasma, this would imply the presence of two functional steroidogenic enzymes, CYP11a1 and 3β-HSD, in Pacific hagfish. These two enzymes are necessary for the synthesis of pregnenolone from cholesterol and progesterone from pregnenolone. The absence of cortisol would imply that CYP11b is either absent or not expressed in hagfish and this concurs with my trait-based analysis conducted in Chapter II.

Initial work developing protocols for RT-qPCR demonstrates that there are now four validated reference genes appropriate for use in analyzing gene expression in the following tissues: gonad, the fore-, mid-, and hindgut regions of the gut, and in the upper and lower kidney. It was challenging to find verifiable primers for use in hagfish as there are only a handful of studies with RT-qPCR primers for hagfish. Some previous studies have published primers for the variable lymphocyte receptor (VLR) in the Pacific hagfish (*E. stoutii*; (Li et al. 2013), 18S (18S rRNA; Li et al. 2013), or the Rhcg (Rhesus blood group c glycoprotein) in the Atlantic hagfish (*Myxine glutinosa*; Edwards et al. 2015). Although a primer for 18S was designed in my study, it could not be validated and therefore could not be used. However, the work done to prepare the four other primers for the reference genes (Rpl22, β-Actin, Cnot ctd, and Ef1a) can now be applied to further RT-qPCR work involving cDNA from these same hagfish tissues as used in my study to examine for the presence and expression patterns of steroidogenic enzymes or any other mRNA expression. Unfortunately, while I did design primers, I was unable to complete a validation (sequencing of products to confirm specificity)

for each of the enzymes involved in steroidogenesis before the end of my studies. This remains to be completed.

This is the first study, to my knowledge, that performed morphometric analysis of the gills of Pacific hagfish subjected to various treatments (no steroid, or steroid injection; no stressor or acid stressor, or exercise stressor). Previous work done on hagfish gills did demonstrate the expression of specific transport proteins (H+ ATPase, NK ATPase) in the gill lamellar membrane following acid infusion (Parks et al. 2007) or base infusion by immunohistochemistry (Tresguerres et al. 2007). However, no morphometric analysis was conducted. My study demonstrated that lamellar thickness appears to be reduced following an acid stressor. Changes in the thickness of the lamellar epithelium have been described in numerous species under a variety of treatments and are thought to indicate changes in physiological function. For example, the gills of silver catfish (*Rhamdia quelen*) exposed to increasing pH levels coupled with increased levels of ammonia (NH_3) showed gill lamellae hyperplasia followed by fusion and necrosis in some gill samples (Miron et al. 2008). Another study with *Sparus aurata* (gilt-head bream) found hypertrophy of the lamellar epithelium, fusion of lamellae, and necrosis across gills of these fish post exposure to Okadaic acid as released in the environment by some dinoflagellate species (Souid et al. 2018). Although these effects seen in the literature are not identical to those seen in this study, there are recurring morphological changes in response to acidosis that alter the width of the lamellar epithelium.

The changes in gill morphometrics following acid injection in Pacific hagfish were also observable as changes of the external surface topography of the lamellae as viewed by SEM. The use of SEM to observe these responses to a physiological stressor in the gills of Pacific hagfish is also a first to my knowledge. Changes in surface morphology of gill epithelia

as viewed via SEM across other fish species (*Anguilla rostata*, *Ictalurus nebulosus*, and *Oncorhynchus mykiss*) have also been observed (Perry et al. 1992). This study found that the overall appearance of gill epithelia differs substantially between the fish species tested and that the density of chloride cells was elevated in each species following cortisol treatment when compared to control fish. The magnitude of change in the density of chloride cells also varied across each fish species. Moreover, previous work on gill epithelia in response to acid and base environmental stressors in rainbow trout (*Oncorhynchus mykiss*) also demonstrated alterations in gill surface morphology via SEM (Goss et al. 1994). These changes in the increase of the chloride cell fractional surface area in rainbow trout were correlated to changes in gill transport function. Another study that exposed mangrove killifish (*Kryptolebias marmoratus*) to hypercapnic conditions found a decrease in the lamellar surface area of the gills (Ong et al. 2007). In my study, elongation of the lamellar microplicae is a change seen in pregnenolone-injected fish treated with the acid stressor. A similar observation has been made in a previous work examining the morphology of the gills of *Ictalurus nebulosis* (brown bullhead catfish) when subjected to a different acidotic stressor, hypercapnia (Goss et al. 1992). The gills of the brown bullhead catfish displayed a similar increase in the density and length of pavement cell microvilli at 48 h.

Plasma glucose levels in hagfish across all treatments were low when compared to other fish species. Plasma glucose concentrations of control Pacific hagfish pretreatment (0 h) averaged < 1 mM while average literature values under control conditions are much higher for other species including rainbow trout: 3 – 10 mM (Bucking and Wood 2005; Polakof et al. 2010; Short and Driedzic 2018), Atlantic cod: ~5 mM (Short and Driedzic 2018), and black bullhead catfish: 3 mM (Legate et al. 2001). The reasons for the low resting glucose are not

understood at this point although data obtained in our lab suggests that lipids are the primary energy source for hagfish while carbohydrates playing a minor role under control conditions (Weinrauch et al. 2019). One possible reason for the low plasma glucose levels is that hagfish were fasted for > 2 weeks prior to experimentation. Moreover, hagfish have a very slow metabolism (Drazen et al. 2011; Weinrauch et al. 2019) thus likely have a low demand for metabolic fuel. The increases in plasma glucose levels following handling stress are consistent with previous work done by Clifford et al. (2017) where hagfish were subjected to the same exercise stressor and showed a similar increased plasma glucose concentration.

One new interesting finding of this research is that the repetitive anaesthetic and blood sampling treatment causes a substantial increase in glucose secretion in control hagfish over the 48 h. This finding is similar to that found in zebrafish where plasma glucose was elevated following multiple anaesthesia treatments (Eames et al. 2010). Interestingly, the prior steroid treatment in both pregnenolone or progesterone-injected fish in my study caused reduced gluconeogenic responses in their plasma when compared to exercise-stressed fish without steroid treatment. This suggested that pregnenolone or progesterone may have a suppressive effect on glucose release from stores. A previous study that involved administering a single injection of progesterone in rats found an increase in plasma glucose levels from 30 – 60 min and at 5 h post treatment, but a return to control levels at around 2 h (Mei-Po and Yang 1970). Despite having a steroid injection of progesterone in this study with rats, there was not a suppression of glucose production as seen in my study because glucose levels continued to rise 2 h post treatment.

One of the caveats with respect to my finding of novel stress steroid profiles in hagfish is that I was unable to examine the possibility/presence of DOC and 11-DOC in hagfish plasma.

Unfortunately, I did not have the steroid standards available for targeted LC-MS/MS analysis for DOC or 11-DOC. These steroids are downstream products of progesterone and 11-OH-progesterone, respectively, each synthesized by the enzyme CYP21. Although these downstream steroid products (DOC or 11-DOC) were not previously found by Clifford et al. (2017) using an RIA in hagfish plasma, it would have been beneficial to confirm the absence of these products by LC-MS/MS. In addition, previous studies suggested that lamprey, the other main cyclostome lineage, has 11-DOC but not cortisol in the plasma and that lamprey use 11-DOC as a corticosteroid (Close et al. 2010; Rai et al. 2015; Shaughnessy and McCormick 2021). By including DOC or 11-DOC steroid standards in a future analysis, this would provide further evidence to support whether CYP21 is a functional steroidogenic enzyme in hagfish or not. In addition, given that my trait-based analysis of CYP21 in the bioinformatic analyses of Chapter II produced mixed results, it is essential to investigate the potential for DOC or 11-DOC to be present (or absent) in hagfish plasma. Further improvements to the results should include increased sample size of the LC-MS/MS results as well as examinations of plasma samples from various treatment groups (control, pregnenolone- and progesterone-injected) with and without a stressor (acid or exercise). These are currently being processed by TMIC, but because of delays due to both COVID-19 restrictions and instrument malfunctions, analysis has not yet been completed.

This research demonstrated progress in methods and understanding the stress system of the Pacific hagfish. There was significant progress made in molecular analyses that can be continued in future research done in our lab. Physiological experiments with an acid stressor demonstrated changes in hagfish gill structure via light microscopy and SEM and exercise stressor experiments demonstrated gluconeogenic responses in hagfish. Although not all stress

steroids have been examined via LC-MS/MS, there is the opportunity to investigate the presence of additional stress steroids in future work in this field.

3.5 Tables

Table 3.1 Hagfish mass (g) data of fish collected in Barkley Sound near Bamfield, BC, Canada and kept for experiment use over two field seasons.

Season	# fish	Average mass (g)	Median mass (g)	Smallest fish (g)	Largest fish (g)
Summer 2020	80	98.7	97.1	48.4	222.0
Summer 2021	37	122.5	120.2	72.0	190.4

3.6 Figures

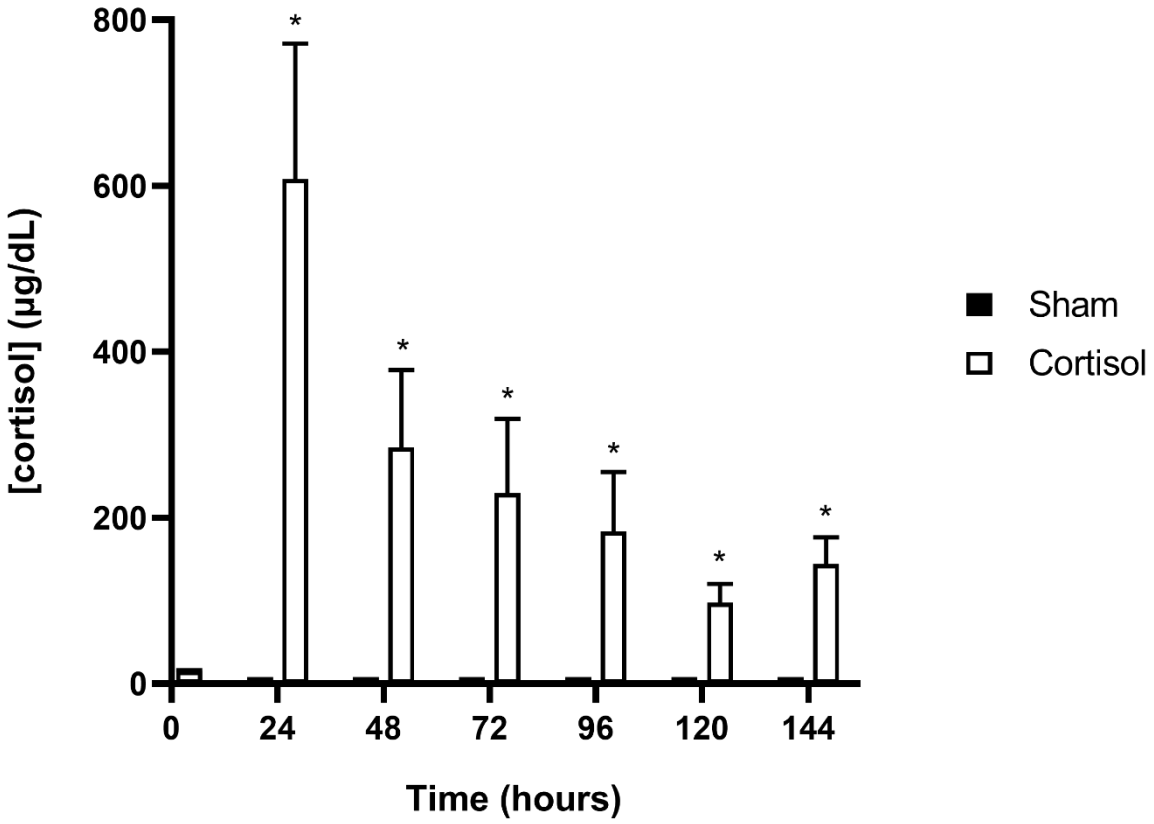
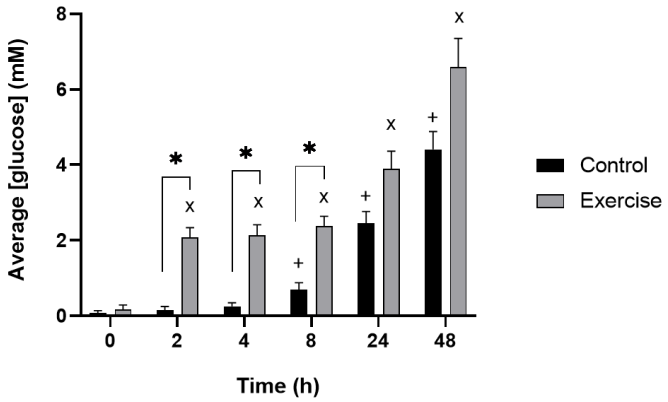
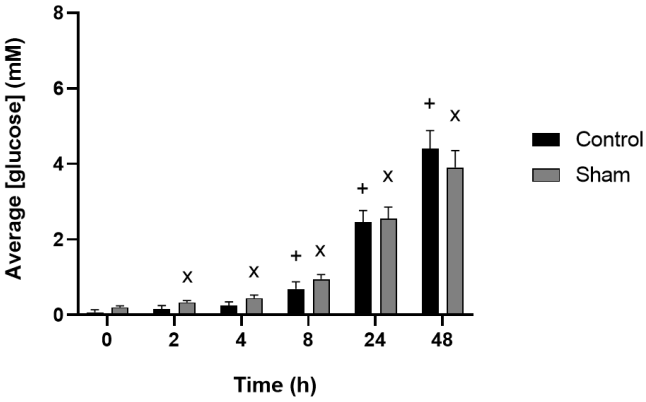


Figure 3.1 Cortisol RIA concentrations. Average Cortisol and Sham data for hagfish injected with cortisol (n = 6) or sham (n = 6) over seven days. Concentrations of cortisol are significantly different (*) from sham-treated fish. Bars indicate ± SE.

A)


Average [glucose] (mM)
Time (h)
0
2
4
8
24
48
Control
Exercise

B)


Average [glucose] (mM)
Time (h)
0
2
4
8
24
48
Control
Sham

C)

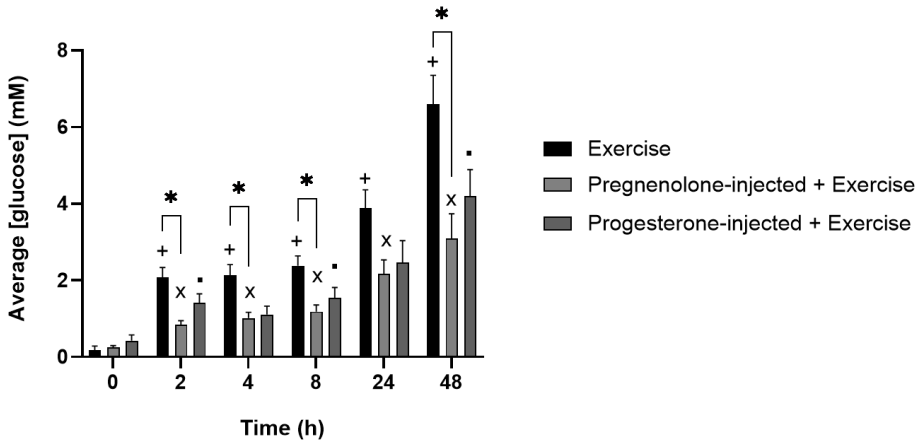

Average [glucose] (mM)
Time (h)
0
2
4
8
24
48
Exercise
Pregnenolone-injected + Exercise
Progesterone-injected + Exercise

Figure 3.2 Hagfish plasma glucose concentrations in A) Control vs. Exercise, B) Control vs. Sham, and C) Exercise vs. Pregnenolone-injected + Exercise vs. Progesterone-injected + Exercise over the time course of the experiment. A two-way ANOVA was performed using Šidák's multiple comparisons test ($p < 0.05$) and Dunnett's multiple comparisons test ($p < 0.05$) to test for significant difference across treatment groups (*; effect of treatment) and within treatment groups (+, x, and • ; effect of time) compared to the corresponding treatment at 0h, respectively.

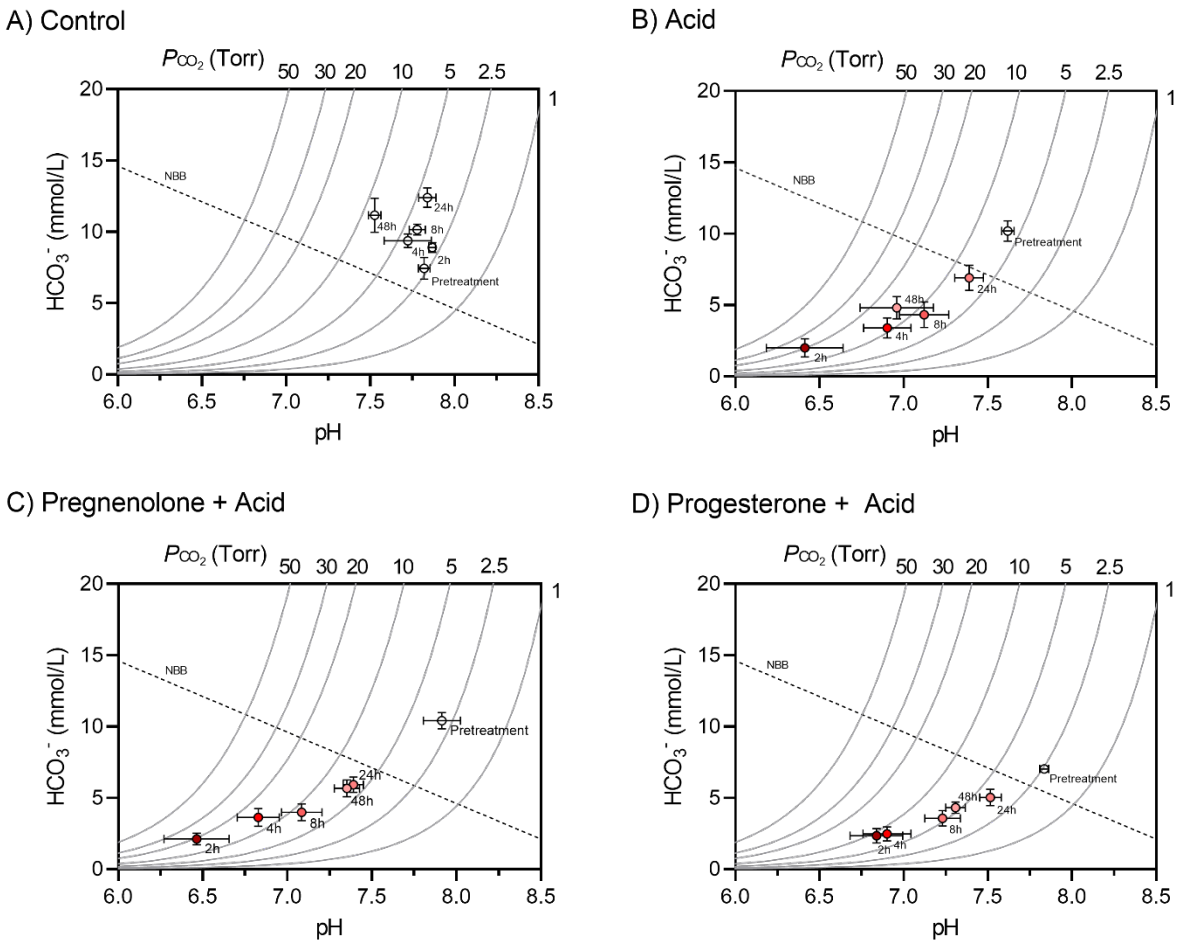


Figure 3.3 Hagfish blood acid-base measurements for fish with and without steroid injection. Davenport diagrams of A) Control, B) Acid-stressed, C) Pregnenolone + Acid-stressed, and D) Progesterone + Acid-stressed fish showing blood pH, bicarbonate (HCO_3^-), the partial pressure of carbon dioxide (P_{CO2}) in grey isopleths along the course of the experiment (t = 0, 2, 4, 8, 24, 48 h), and the non-bicarbonate buffer (NBB) line. Data are means ± SE (n = 8 except for control where n = 7).

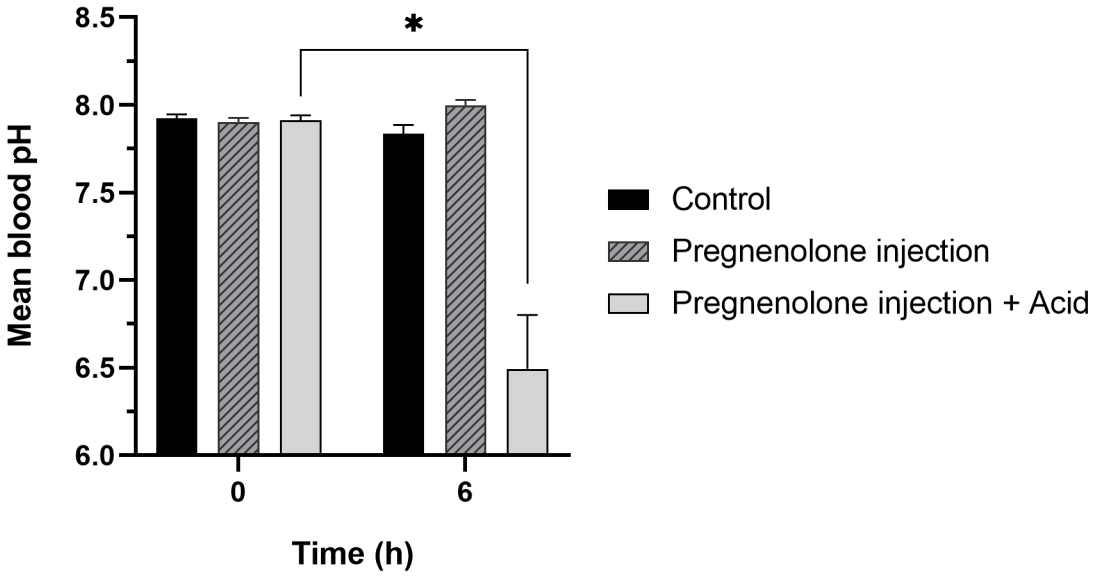


Figure 3.4 Hagfish blood pH measurements for fish without steroid injection and fish with pregnenolone injection and acid (HCl) injection from field season #2. A two-way ANOVA was performed using Šidák's multiple comparisons test ($p < 0.05$) to test for significance (*) across treatment groups. Data are means ± SE (n = 6).

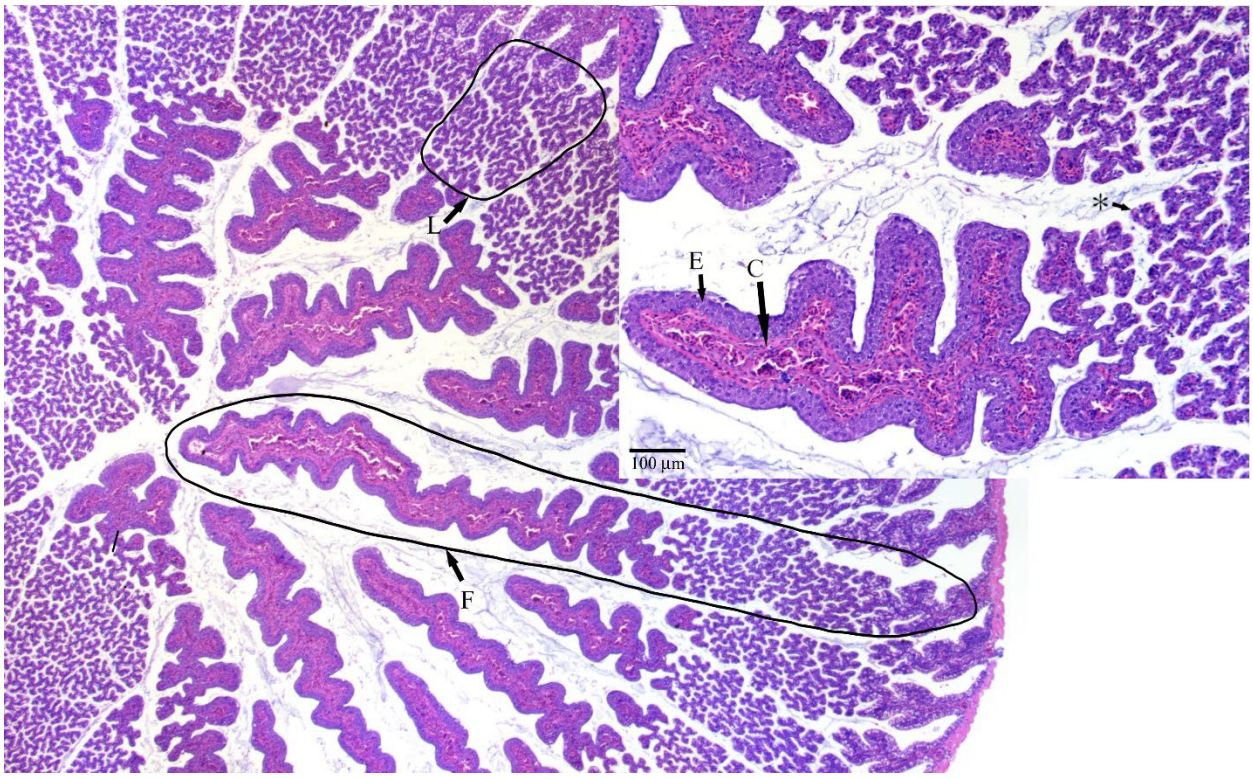
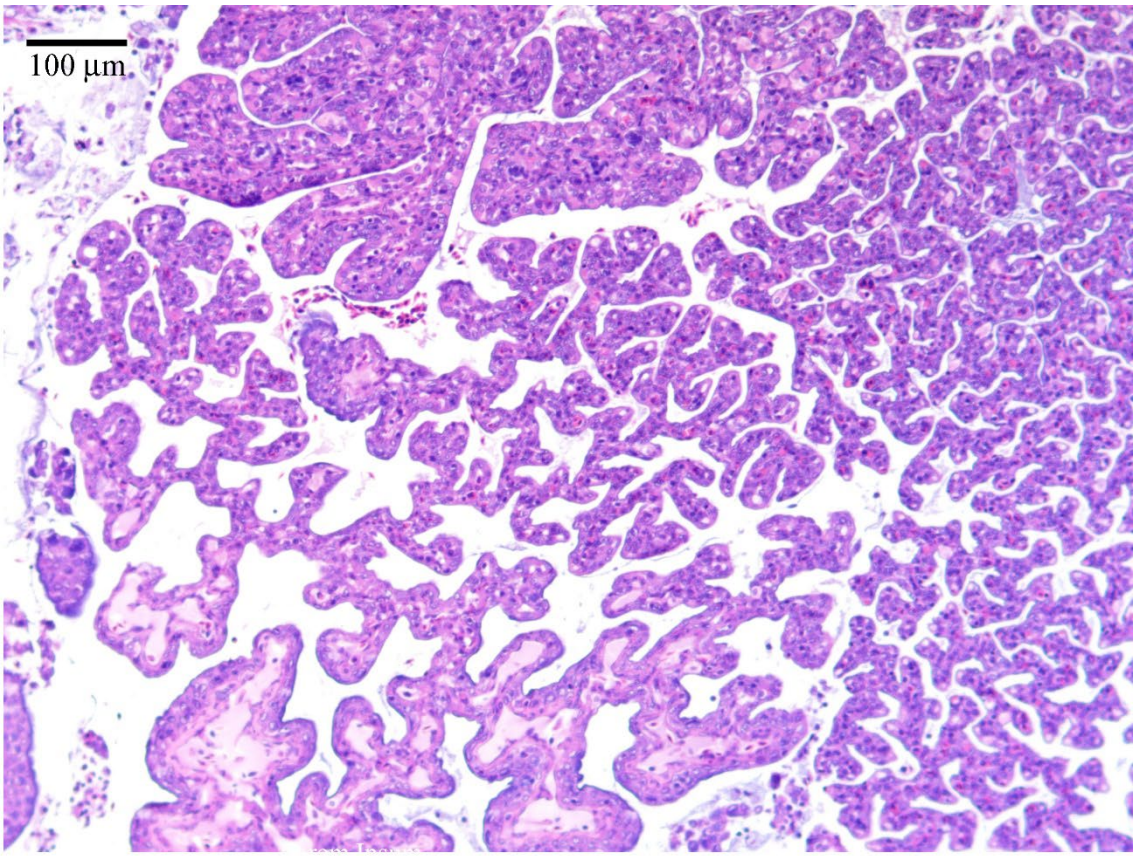


Figure 3.5 Sagittal section of the hagfish gill with H&E staining at 2.5X with inset taken at 10X. Right inset structures identified include filament (F), lamellae (L), epithelia (E), cartilage (C), and lamellar epithelium (*) in black. Scale bar is 100 µm.

A)



B)

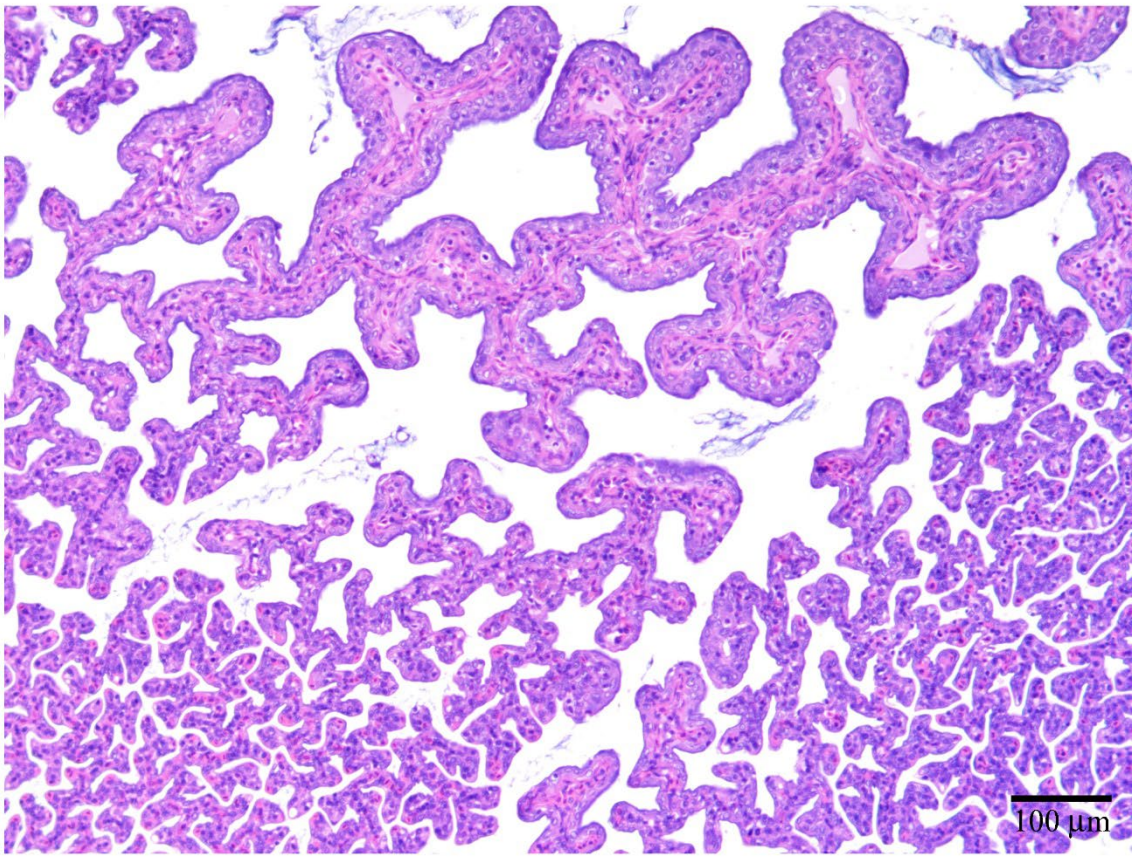


Figure 3.6 Representative images of hagfish gill filaments and lamellae from A) Control fish and B) Acid-stressed fish (t = 48 h) stained with H&E. Images taken at 10X and scale bar is 100 μm.

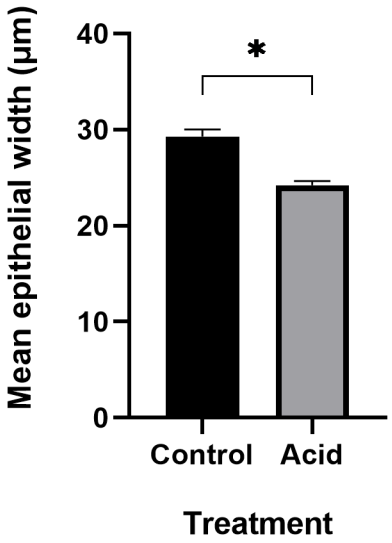


Figure 3.7 Mean epithelial width (µm) of hagfish gill lamellae for control and acid-stressed fish. Three gills per treatment group were processed with a total of five images per gill taken (total = 15 images/treatment group). A two-tailed, unpaired t-test was used to compare control and acid-stressed groups for significance (*). Bars are means ± SE across all measurements taken for each treatment group of gills.

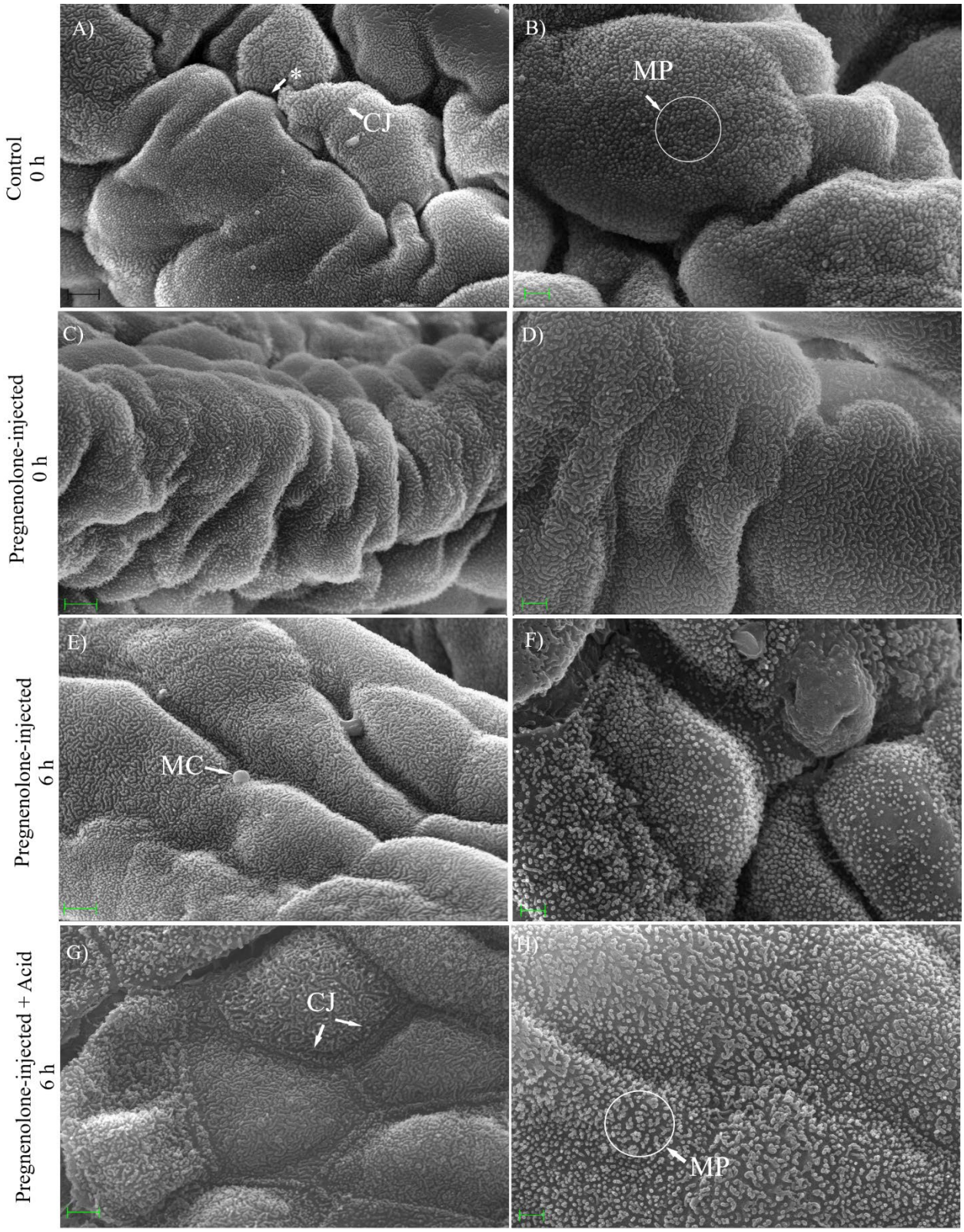

Control
0 h
A)
*
CJ
B)
MP
Pregnenolone-injected
0 h
C)
D)
Pregnenolone-injected
6 h
E)
MC
F)
Pregnenolone-injected + Acid
6 h
G)
CJ
H)
MP

Figure 3.8 Representative scanning electron microscopy (SEM) images of the external surface of hagfish gill lamellae from various treatments at t = 0 and 6 h. Fish without steroid injection (control) and fish with pregnenolone injection for both control and acid-stressed fish are present. Images on the left-hand side and right-hand side were taken at 10 KX (scale bar = 2 μm) and 15 KX (scale bar = 1 μm), respectively. Lamellae folds (*), microplicae (MP), cell-to-cell junctions (CJ), and mucous cells (MC) are labelled in white.

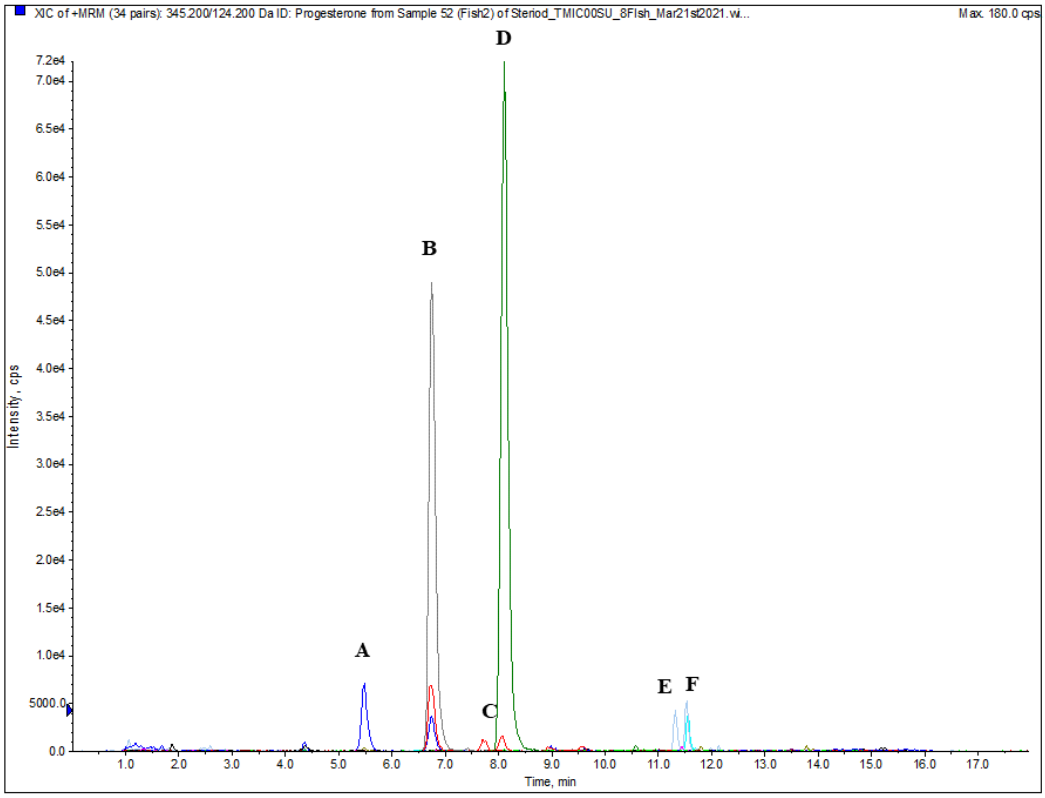


Figure 3.9 Representative chromatogram from targeted LC-MS/MS analysis of hagfish plasma from a fish without steroid injection. Main peak regions identified are A) Estrone, B) Dehydroepiandrosterone, C) 17-Hydroxyprogesterone, D) d3-Testosterone, and E) Progesterone, and F) Pregnenolone.

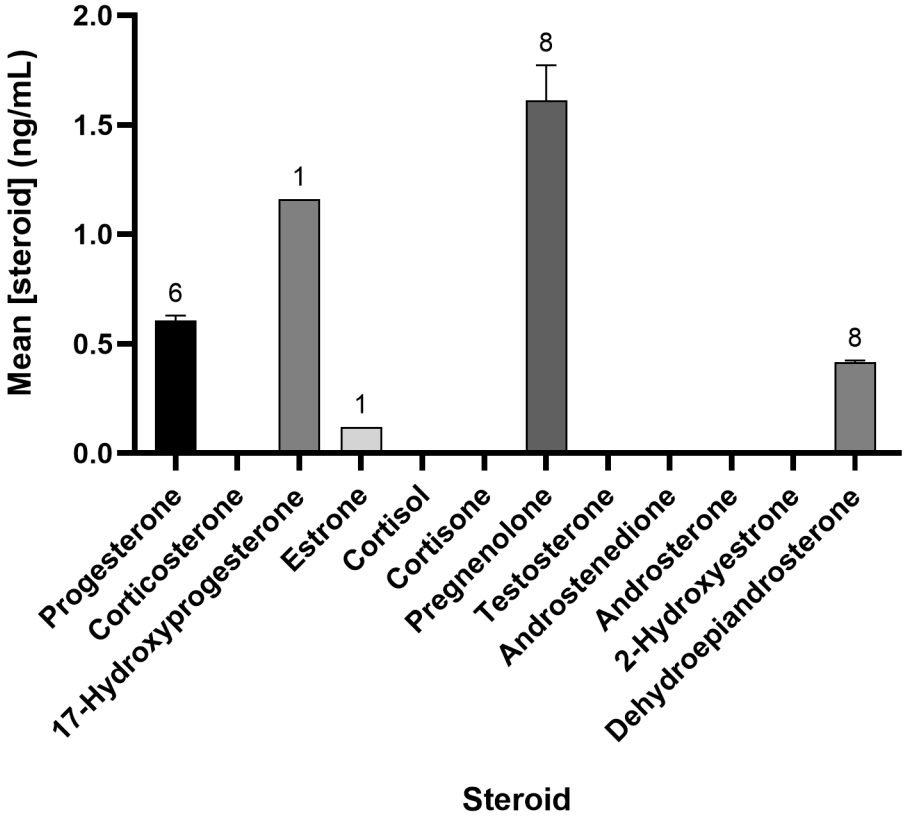


Figure 3.10 Targeted LC-MS/MS results of plasma from fish without steroid injection. Thirteen steroid standards were used in this targeted analysis of Acid- (n = 4) and Exercise-stressed (n = 4) fish. Numbers above bars indicate the number of fish with the corresponding steroid detected. Mean steroid concentrations (ng/mL) were calculated and bars ± SE are presented.

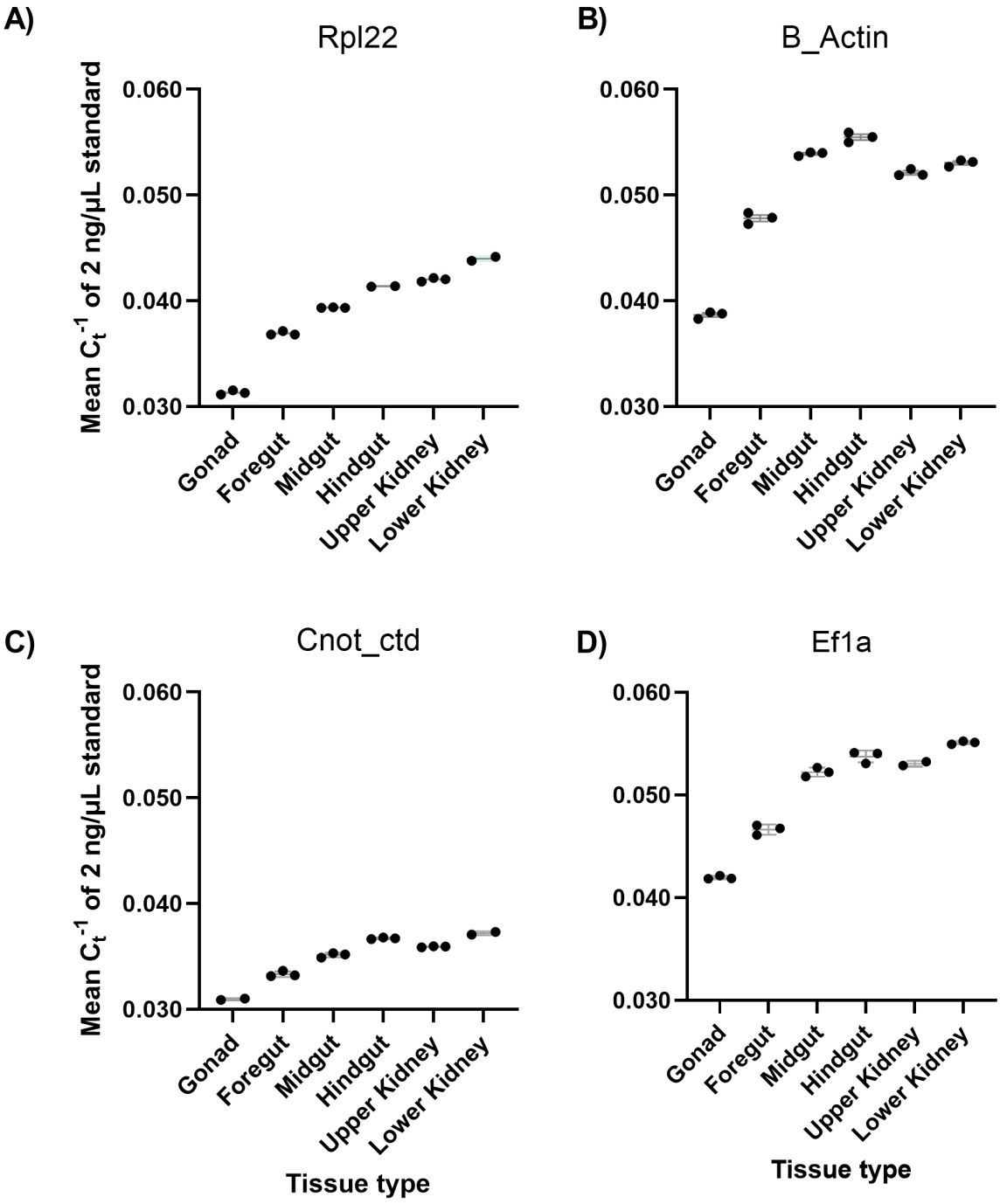

A)
Rpl22
Mean C_t^{-1} of 2 ng/µL standard
0.060
0.050
0.040
0.030
Gonad
Foregut
Midgut
Hindgut
Upper Kidney
Lower Kidney
B)
B_Actin
C)
Cnot_ctd
D)
Ef1a
Tissue type

Figure 3.11 Standard curves for reference (housekeeping) genes across six tissues from hagfish for RT-qPCR. The mean of the inverse threshold cycle (C_t^{-1}) with standard deviation (SD) are shown for each tissue type where there is expression of the gene. Each dot in black represents one replicate (n ranging from 2 – 3) of a pooling of cDNA from the corresponding tissue type for each fish. Lines for tissue type are representative of the mean and ± SE.

Chapter IV – General Conclusions

Summary

Hagfishes are compelling organisms to study because of their life history and physiology. My thesis research encompassed key questions about their stress physiology, including the identity of their primary stress steroid(s), the steroidogenic enzymes, and the site(s) of steroidogenesis. In the first set of physiological experiments where hagfish were subjected to an acid stressor, there was an expected decrease of blood pH and bicarbonate ion concentration within the first two hours following injection, resulting in the fish entering metabolic acidosis. This acidosis occurred in both fish with, and without, either steroid pretreatment, and all groups experienced a similar subsequent gradual recovery. In the second set of physiological experiments, hagfish were stressed for 30 min by repeated handling, resulting in significant production and secretion of glucose into circulation. However, the injected steroids, pregnenolone and progesterone, effectuated a suppression of this anticipated response. Histologically, there were distinct changes to the width of the lamellar epithelium between control and steroid injected fish with acid injection. I documented a thinning of the lamellar epithelium in steroid-injected fish as a result of the acid injection. Investigation of gill structures using SEM revealed topographical changes in the cells of the gill lamellae between control versus pregnenolone-injected fish and those treated with acid. The microplicae of pregnenolone-injected acid-treated fish were found to be elongated whereas those microplicae in control fish were found to be more compact and rounded in appearance.

Since I was able to confirm that the fish experienced stress, the next portion of my work was to examine their plasma and cDNA from various tissues for the presence of corticosteroids and gene expression of genes coding for steroidogenic enzymes, respectively. The preliminary results of the LC-MS/MS analyses showed stressed hagfish without steroid injection with low

concentrations of steroids (e.g., pregnenolone, progesterone, DHEA) in their plasma. At this point, it cannot be concluded what steroid(s) may be key in the stress response of these fish. Based on the sequencing results for each primer pair, only my reference genes could be validated. These reference genes had expression in all tissue types at varying degrees. Unfortunately, without confirmed sequencing products for target genes of interest (i.e., Cyp11a1, Cyp17, Cyp21, and 3β-HSD), implications about the steroidogenic enzymes present in hagfish cannot be made at this time.

Future directions

This research substantially furthers the understanding of the stress steroid system of the Pacific hagfish, *Eptatretus stoutii*. Both acid injection and exhaustive exercise are appropriate physiological stressors to induce physiological responses in the organism. Importantly, my research demonstrates that the steroidal profile of Pacific hagfish is substantially different from the rest of the gnathostomes.

My work in this area would greatly benefit from more LC-MS/MS and RT-qPCR analyses. Unfortunately, the LC-MS/MS results at present do not describe the steroid plasma composition of control (unstressed fish without steroid injection), pregnenolone or progesterone-injected fish. While these experiments have been performed and the plasma collected, the analysis remains to be completed. When the analysis of these samples is complete, we will be able to validate the plasma pregnenolone and progesterone levels in each respective hagfish treatment group both immediately before acid or exercise stress and the time-dependent changes as a result of the treatment. The resulting plasma pregnenolone and progesterone levels could be compared to those of the cortisol-injected fish and be used to determine the rate that these steroids diminish from circulation over the post-stressor period.

Completing these analyses would permit a more comprehensive examination of steroids present or absent from hagfish circulation. The inclusion of other steroid standards in my analysis will strengthen the argument whether DOC and/or 11-DOC are possible terminal corticosteroids, or not.

To examine the differential expression of genes coding for steroidogenic enzymes across tissues, there must be working primers generating anticipated products that match the gene(s) of interest. Without these validated reference genes, there will be no confidence in the results generated. Currently, the preliminary results can only provide a snapshot of what the expression levels across hagfish tissues may look like. Executing these other RT-qPCR runs will allow for the expression pattern to be mapped across both tissues and differing treatments (i.e., control versus pregnenolone-injected fish versus pregnenolone and acid injected fish). If sequencing products for target steroidal enzyme genes can be generated and validated, it will be possible to identify changes in the levels of expression after fish experience a stressor and identify steroidogenic tissues as well.

Overall, my thesis research continues to support the contention that hagfish are the most basal vertebrates in evolution. A full understanding of the Pacific hagfish stress system remains an enigma, but I have added some new information as to their primary stress steroid(s), steroidogenic enzymes, and steroidogenic tissues.

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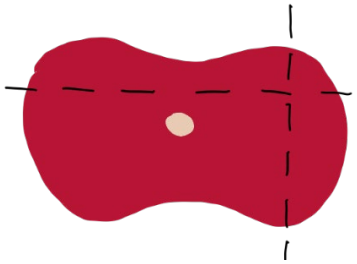
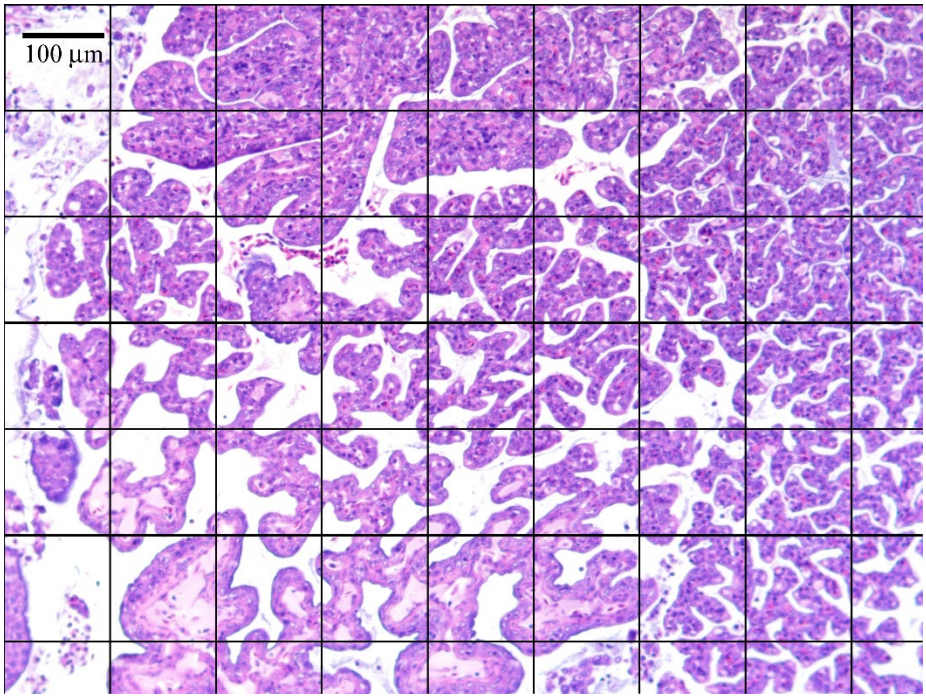
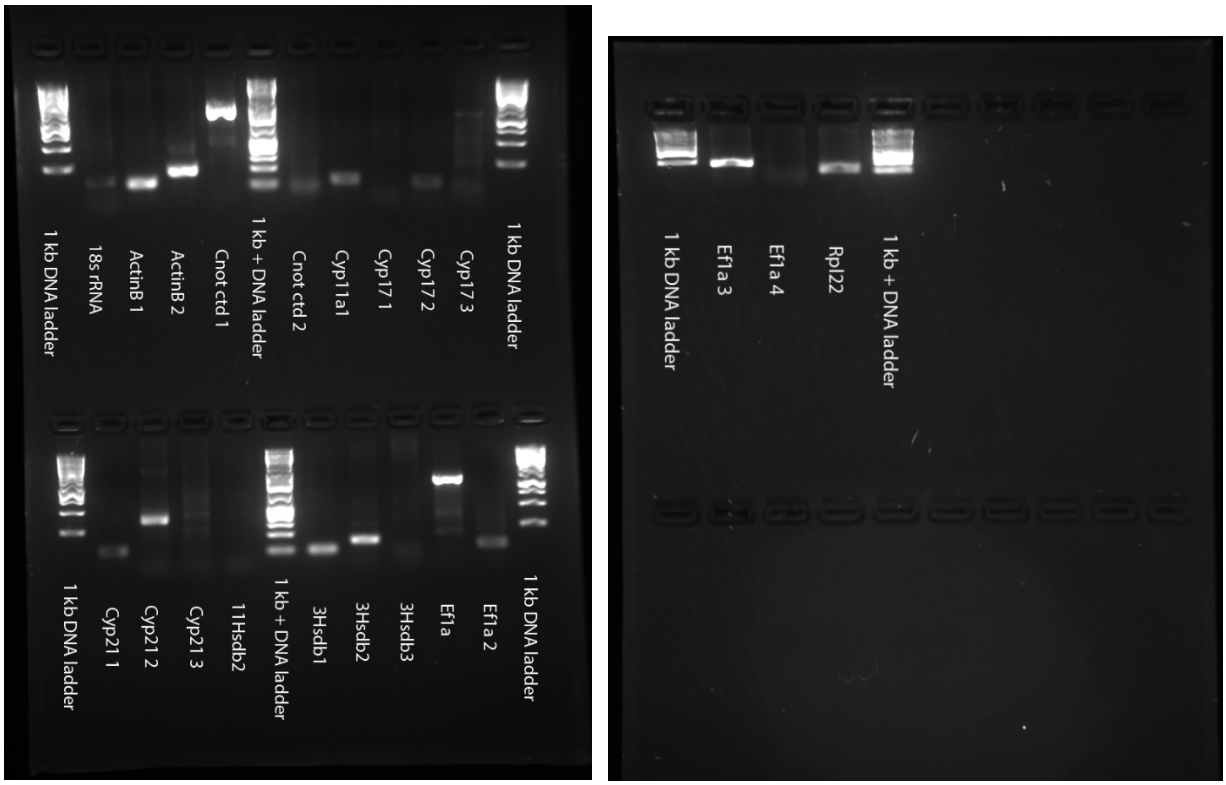
Appendix



SI 1. Diagram of hagfish gill specimen collection for SEM. Cuts are represented by the dotted lines.



SI 2. A 2.5 x 2.5 cm grid was created and overlaid on each image from H&E-stained gill sections. See methods for details.



SI 3. PCR product results of agarose gel electrophoresis for DNA separation. A total of 21 probable primer pairs for reference and target genes were run.

Gene	F Seq.	R Seq.	Designed from	Intron spanning	Product size	Gel size	F align	R align	Consensus	BLASTn	BLAST vs. genome	Validated
Rpl22	CCTTCGAGATTGGCTGCGTGT	TTCCTCCTTCCTCTTCCTGGT	*E. stoutii* Transcriptome hit	N	31	100	Y	N	60 bp	Rpl22	N	Y
ActinB_1	TGTCATCTTCTCCCGGTTGGC	GGCACCACACCTTCTACAACG	*E. atami* (AB546742.1) & transcriptome alignment	U	104	106	Y	Y	Y	*E. atami* mRNA for beta actin, partial cds	N/A	Y
Cnot_ctd_1	GCAACAACCACCAAGAGC	AGGCTCCACGATACGGTCC	*P. marinus* (XM_032960811.1) & transcriptome alignment	Y	123	>5000	N	N	N	80 bp of 77-R (exon and intron) matches to *D. aesculapii* genome	-	36bp from 77-R-Cnot-1 may match up with the second exon, BLAST hit to *P. tetrazona* Cnot1
Efa1_3	CCATCCCGCCCAACCGATAAG	GACCACCACCAGGCTTCAA	*E. stoutii* Transcriptome hit	Y	123	250	Y	Y	Y	*N. hemipeltis* elongation factor-1 alpha gene, exons 4 through 8 and partial cds	Yes, blast 300bp *A. australis* elongation factor 1 (eef1) gene, partial cds	Y

Gene	F Seq.	R Seq.	Designed from	Intron spanning	Product size	Gel size	F align	R align	Consensus	BLASTn	BLAST vs. genome	Validated
Cyp11a1	GGTCCTTGTAACGGTGCTGCT	TCACTTTCTGCAATTCGTCGCC	*E. burgeri* genome (hit to *E. atami* Cyp11a AB985739)	U	98	106	N	Y (6 of 7)	N	N/A	N/A	Only 6-7 bp. Further sequencing needed.
Cyp17_2	ACGAGGCTGCTTGGGTGAA	GCGAGAGGGTGAAGGTCTGTA	*E. burgeri* genome	N	83	75	N	Y (5 of 6)	N	N/A	N/A	Only 6bp, only 81-R. The Reverse primer overhangs the exon by 8bp, so may not work well for gDNA.
Cyp21_1	TTGCCTCTTCCGTGACTTCCG	GGCTTCAAGACGGCACCCAT	*E. burgeri* genome	N	87	100 - 120	Y	Y (5 of 6)	Y	No hits	100% (including R primer); when copying 200bp total around hit, blast hit to *C. undulatus* steroid 21-hydroxylase (LOC121513960), mRNA	
Hsd3b	GGGAGCCCGTGTCTTTGTGTT	GCTTCTGCCTTGCTGCGACTA	Transcriptome hit	U	141	138	Y	Y	Y	No hits	Expand out to ~300bp, Pantherophis guttatus 3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase type 1-like (LOC117661842), mRNA	Y

SI 4. Primers result summary.