The digestive physiology of the Pacific hagfish, *Eptatretus stoutii*

by

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Abstract

Hagfish are considered the oldest living representatives of the vertebrates and as such are of interest for studies in evolutionary biology. Moreover, hagfish occupy a unique trophic niche wherein they are active predators and benthic scavengers of a wide range of invertebrate and vertebrate prey, yet can tolerate nearly a year of fasting. Hagfish are also capable of acquiring nutrients across the integument in a manner similar to that of marine invertebrates, a hallmark of their position between the invertebrate and vertebrate lineages. Despite many interesting modes of feeding, relatively few studies have examined the digestive physiology of the hagfishes.

Feeding and digestion cause many physiological perturbations, which result in elevated metabolic rate. I have characterised the increased metabolic demand (specific dynamic action) and determined that its peak in hagfish corresponds with the peak of acid-base alterations. Specifically, the rate of base efflux reaches a maximum 8 h post-feeding when the intestinal blood supply is significantly more alkaline. The offloading of base to the circulation (alkaline tide) is common of vertebrates that employ an acidic digestion in order to maintain cellular acid-base homeostasis. Likewise, the hagfishes employ a luminal acidification, despite lacking a stomach. I further investigated the cellular mechanisms responsible for hindgut luminal acidification and found that soluble adenylyl cyclase and cAMP are utilised to enhance proton secretion *via* proton-potassium exchangers (HKA), as is found in later-diverging vertebrates. Vacuolar-type ATPase (VHA), which energizes gastric alkalization in some invertebrates, was also present and used for luminal acidification.

In addition to acidic digestion, hagfish also utilize digestive enzymes. I have characterised the distribution and post-feeding activities of six digestive enzymes (α-amylase, maltase, lipase, trypsin, aminopeptidase and alkaline phosphatase) along the hagfish digestive tract. Contrary to previous reports, enzymatic activity is not restricted to the hindgut and is present in the buccal cavity and pharyngocutaneous duct. Furthermore, enzyme activity is differentially regulated post-feeding, indicating the first instance of compartmentalization of gut function in the vertebrates.

Once digested, nutrients are absorbed across the intestinal epithelium using specialized transport proteins. I have identified specific, carrier-mediated transport pathways for glucose, oleic acid, and L-alanyl L-alanine in the hagfish hindgut. Glucose uptake likely occurs *via* sodium-linked glucose transporters (SGLT) and glucose transporters (GLUT) as both sodium-dependent and -independent uptake were observed. Furthermore, specific pharmacological inhibitors of each transporter type effectively reduced glucose acquisition. Oleic acid acquisition was not altered with insulin application, consistent with the behaviour of the intestinal isoforms of mammalian fatty acid transport proteins (FATP). Feeding significantly increased the maximal uptake rate of oleic acid, suggesting an increased apical expression of transporters, possibly as a result of increased surface area. Indeed, post-prandial intestinal remodeling occurred wherein mucosal thickness and microvilli length were significantly increased. Microvilli length rapidly regressed to fasting conditions within 36 h of a feed, suggesting that intestinal regression is utilised to conserve energy in between feeds. Dipeptide (L-alanyl L-alanine) uptake, similarly increased post-feeding and had a sodium-dependent component. This could suggest a role for sodium-proton exchangers (NHE) as has been

identified in mammals. Overall, the mechanisms of uptake and regulation of transporter function appear to be conserved between hagfish and later-diverging vertebrates. Hagfish therefore represent an excellent model for studies of comparative vertebrate digestive physiology as they diverged prior to the genome duplication, have relatively simpler intestinal morphology, and conserved mechanisms of digestion and nutrient acquisition.

Preface

This thesis is an original work by Alyssa Michelle Weinrauch. The research project, of which the thesis herein is a part, received ethics approval from both the University of Alberta Animal Care and Use Committee and the Bamfield Marine Sciences Animal Care Committee. Approval was governed under the following protocols: Project name "Acid-base and ionoregulation in fishes"; University of Alberta, AUP 0001126 (2013-2019); Bamfield Marine Sciences Centre RS-14-13, RS15-31, RS16-19, RS17-03 (2013-2018).

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 (Alyssa M. Weinrauch) performed the majority of the work presented in this original Ph.D. thesis however, owing to collaborative efforts the terms "we" or "our" are used throughout this thesis. Four chapters of this thesis have been previously published. The biographical details for the introduction and data chapters are both listed below and indicated at the beginning of each chapter. The roles of all authors for each chapter along with specific experimental contributions of certain co-authors are briefly described below.

Chapter I:

Weinrauch, A.M., Edwards, S.L., Goss, G.G. 2015. Anatomy of the Pacific hagfish (*Eptatretus stoutii*). In: Edwards, S.L., Goss, G.G. (Eds.), Hagfish Biology. CRC Press, Boca Raton, Fl, pp. 1-40.

This book chapter was conceived and designed by AMW, SLE, and GGG. AMW collected and analysed all tissues and drafted the manuscript. GGG contributed analytical tools. All authors revised and approved the final chapter.

Chapter II:

Weinrauch, A.M., Clifford, A.M., Goss, G.G. 2018. Post-prandial physiology and intestinal morphology of the Pacific hagfish (*Eptatretus stoutii*). J. Comp. Physiol. B. 188:101-112. DOI 10.1007/s00360-017-1118-1.

The study was conceived and designed by AMW and GGG. AMW performed the experiments and AMC assisted in data collection. AMW conducted sample analysis and analysed the data and AMC assisted in data analysis. GGG contributed analytical tools, materials and reagents. AMW drafted the manuscript. All authors revised and approved the manuscript. All experiments performed in Chapter 2 are the results of my individual efforts with the following exception: AMC aided in data collection and analysis.

Chapter III:

Weinrauch, A.M., Kwan, G., Tresguerres, M., Goss, G.G. Cellular mechanisms of ion and acid-base transport following feeding in the Pacific hagfish hindgut.

The study was conceived and designed by AMW and GGG. AMW and GK performed the experiments. AMW conducted sample analysis and analysed the data. GGG and MT contributed analytical tools, materials and reagents. AMW wrote the chapter. All experiments performed in Chapter 3 are the results of my individual efforts with the following exception: GK performed some of the immunohistochemistry and collected images.

Chapter IV:

Weinrauch, A.M., Schaefer, C.M., Goss, G.G. 2019 Activity and post-prandial regulation of digestive enzyme activity along the Pacific hagfish (*Eptatretus stoutii*) alimentary canal. Submitted to PLOSone.

The study was conceived and designed by AMW. AMW performed the experiments and CMS assisted in data collection. AMW analysed the data. GGG contributed analytical tools, materials and reagents. AMW drafted the manuscript. All authors revised and approved the manuscript. All experiments performed in Chapter 4 are the results of my individual efforts with the following exception: CMS assisted in performing the experiments and data collection.

Chapter V:

Weinrauch, A.M., Clifford, A.M., Goss, G.G. 2018. Functional redundancy of glucose acquisition mechanisms in the hindgut of Pacific hagfish (*Eptatretus stoutii).* Comp. Biochem. Physiol. A. 216: 8-13.

The study was conceived and designed by AMW and GGG. AMW performed the experiments, conducted sample analysis and analysed the data. GGG contributed analytical tools, materials and reagents. AMW drafted the manuscript. All authors revised and approved the manuscript. All experiments performed in Chapter 5 are the results of my individual efforts.

Chapter VI:

Weinrauch, A.M., Glover, C.N., Goss, G.G. 2018. Lipid acquisition and tissue storage in hagfish: new insights from an ancient vertebrate. J. Comp. Physiol. B. 189(1): 37-45.

The study was conceived and designed by AMW. AMW performed the experiments, conducted sample analysis and analysed the data. GGG contributed analytical tools, materials and reagents. AMW drafted the manuscript. All authors revised and approved the manuscript. All experiments performed in Chapter 6 are the results of my individual efforts.

Chapter VII:

Weinrauch, A.M., Blewett, T.A., Glover, C.N., Goss, G.G. 2019. Acquisition of alanyl-alanine in an Agnathan: Mechanisms of dipeptide transport across the hindgut of the Pacific hagfish. Submitted to Scientific Reports.

The study was conceived and designed by AMW. AMW performed the experiments, conducted sample analysis and analysed the data. GGG and CNG contributed analytical tools, materials and reagents. AMW drafted the manuscript. All authors revised and approved the manuscript. All experiments performed in Chapter 7 are the results of my individual efforts.

Acknowledgements

First, I'd like to recognize my supervisor, Dr. Greg Goss. I'm very grateful for all of the opportunities you have given me over the years, which have allowed me to grow both as a person and a researcher. Thank you for giving me the independence to make this thesis something that I have been, and continue to be, passionate about. Lastly, thank you for filling out the many workers' compensation forms for the lab record-breaking 17 stitches!

I would also like to express my gratitude to the many people who have contributed to this research and my sanity. Dr. Susan Edwards, my first co-author, I am so grateful we met my first year in Bamfield. You have been a consistent source of encouragement and are the reason I decided to continue on in academia. Thanks to Dr. Martin Tresguerres and Garfield Kwan for welcoming me to Scripps and giving me the opportunity to expand my research toolkit. Dr. Chris Glover for your many discussions, valuable input, and eloquent edits. I am so grateful to have had your support. Dr. Tamzin Blewett, you have changed my life. Thank you for being such a positive female mentor in science and great friend…. When I grow up, I want to be like you. To the most fabulous undergraduate student I could have hoped for – Tina Schaefer. Thank you for showing me the joy that can come from teaching a hard-working and passionate student. Your future is bright and I'm so excited to see what you achieve! Dr. Alexander Clifford thank you for being a passionate teacher who got me involved with the lab in the first place. You've taught me a lot and I appreciate time you took to answer my questions.

As is the case in science, the Goss lab has been ever changing. Throughout my time here I have experienced many wonderful people. Special thanks to: Erik Folkerts - thank you so much for your patient teaching, many questions, and wonderful friendship – I'm so happy we are friends! It's because of you that I understand qPCR and know that we have trout in the basement. Stay perfect! Perrine Delompré, mon petit chef. You've been a fantastic roommate and friend with an amazing humour and the right dose of logic! Thank you for taking jokes, making dinners, and always listening. Carley Wall thanks for the laughs, particularly Bamfield roadtrip 2017, and for being a great movie trivia partner. Dr. Sal Blair, thank you for letting me drop into your office and bother you

about molecular troubleshooting. All. The. Time. I really appreciate it. Dylan Cole you've been a great friend and a lifesaver on the dogfish time course study. Danuta Chamot and Shannon Graham - the superb lab managers who made my life so much easier. Thank you!

Within the department I had a lot of assistance academically, with equipment and reagent loans, and with finances and awards. To my committee members Dr. John Chang and Dr. James Young, thank you for your support. Thank you to Dr. Warren Gallin, Dr. Larry Fliegel, Dr. Colin Brauner, Arlene Oatway, Troy Locke, Cheryl Nargang, Dr. Keith Tierney, Dr. Ted Allison, Tad Plesowicz, Jesse Edgington, Shelley Scott, Dean Wilson, Chesceri Mason, and Dawn Kieller.

Whilst I was banished to Bamfield, I had the opportunity to become a part of the community. Thanks to Janice Cook and John Richards for hagfish collection and Kelley Bartlett (and many assistants) for animal care. Special thanks to Dr. Eric Clelland - research coordinator extraordinaire. You've gone above and beyond your job title and I can't thank you enough. Dr. Gary Anderson, Dr. Chris Wood, Dr. Courtney Deck, Dr. John Morgan, Dr. Dan Baker, and Emily Dong, you've all been fantastic and contributed to troubleshooting, liquid nitrogen rescue, dinners, drinks, and friendship.

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To my friends in the "real world" - Hannah Cook and Jade Waugh. Thanks for providing perspective and great friendship throughout this journey. A very special thanks to my grandparents for welcoming whatever crew I brought to your house en route to/from Bamfield. I appreciate the food, housing, but most of all the time spent with you both. Lastly, thank you to my family (Roselyn, Curtis, Brianna, and Haley) for always responding positively to graphs I sent with no context and providing the light and laughter, wit and humour necessary to make it through the many failures that accompany a PhD. I love you all very much.

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List of symbols, nomenclature and abbreviations

[s]	Substrate concentration
^{14}C	Carbon-14
^{3}H	Tritium
A	Absorbance
AAT	Amino acid transporter
ACS	Aqueous counting scintillant
Ala-ala	Alanyl-alanine
ANOVA	Analysis of variance
Ant HG	Anterior hindgut
AQ	Ammonia quotient
ATP	Adenosine triphosphate
AUP	Animal use protocol
B	Buccal cavity
BAPNA	Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride
BCA	Bicinchoninic acid
BDL	Below detection limit
BMR	Basal metabolic rate
C	Celcius
$C_5H_{14}NOCl$	Choline chloride
CA	Carbonic anhydrase
CaCC	Calcium-activated chloride channels
$CaCl_2$ $2H_2O$	Calcium chloride dihydrate

cAMP	Cyclic adenosine monophosphate
CC	Columnar cells
CFTR	Cystic fibrosis transmembrane conductance regulator
C_i	Initial concentration
Cl^-	Chloride
ClC-2	Chloride channel
cm	Centimeter
CO_2	Carbon dioxide
con β	Control chamber buffering capacity
conH^+final	Final proton concentration in control chamber
conV	Control chamber volume
CPM	Counts per minute
DFO	Department of Fisheries and Oceans
DMSO	Dimethyl sulfoxide
e.g	*exempli gratia*
EDTA	Ethylenediaminetetraacetic acid
exp β	Experimental chamber buffering capacity
exp_{H^+final}	Final total proton concentration in experimental chamber
$exp_{H^+initial}$	Initial total proton concentration in experimental chamber
expV	Experimental chamber volume
FA	Fatty acid
FABP	Fatty acid binding protein
FATP	Fatty acid transport protein

FFA	Free fatty acid
FTR	Fluid transport rate
G	Gauge
g	Gravitational force
GLUT	Glucose transporters
h	Hour
H^+	Proton
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate
HF	Hagfish
HKA	Proton potassium ATPase
i.e	*id est*
IU	International units
J	Joule
J_{amm}	Ammonia excretion rate
J_{H^+Net}	Net proton flux
J_{max}	Maximal transport rate
K^+	Potassium
KCl	Potassium chloride
Kg	Kilogram
KH_2PO_4	Monopotassium phosphate
$KHCO_3$	Potassium bicarbonate
kJ	Kilojoule

K_m	Transporter affinity
kV	Kilovolts
L	Litre
LCFA	Long chain fatty acids
M	Mass
Mf	Final mass
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$ $7H_2O$	Magnesium sulfate heptahydrate
Mi	Initial mass
Mid HG	Mid hindgut
min	Minute
mL	Millilitre
mM	Millimolar
mmol	Millimole
MO_2	Metabolic oxygen consumption
MS 222	Tricaine methanosulfonate
MV	Microvilli
MW	Molecular weight
N	Normal
Na_2PO_4	Disodium phosphate
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide

$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NHE	Sodium proton exchanger
NKA	Sodium potassium ATPase
NKCC	Sodium potassium 2 chloride cotransporter
nM	Nanomolar
nm	Nanometer
NMDG	N-methyl-d-glucamine
nmol	nanomole
O_2	Oxygen
OA	Oleic acid
OMZ	Omeprazole
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline plus Tween 20
PCD	Pharyngocutaneous duct
PCO_2	Partial pressure of carbon dioxide
PE	Polyethylene
PEPT	Peptide transporter
pHi	intracellular pH
PO_2	Partial pressure of oxygen
PO_{2f}	Final partial pressure of oxygen
PO_{2i}	Initial partial pressure of oxygen
Post HG	Posterior hindgut

PVC	Polyvinyl chloride
qPCR	Quantitative polymerase chain reaction
s.e.m.	Standard error of the mean
SA	Surface area
sAC	Soluble adenylyl cyclase
SAct	Specific activity
SCARB3/FAT	Scavenger receptor B class 3/ fatty acid translocase
SDA	Specific dynamic action
sec	Second
SGLT	Sodium glucose-linked transporter
SLC	Solute carrier
t	Time
T_{ammf}	Final total ammonia concentration
T_{ammi}	Initial total ammonia concentration
TCO_2	Total carbon dioxide
TEM	Transmission electron microscopy
tmAC	Transmembrane adenylyl cyclase
Tris-HCl	Trisaminomethane hydrochloride
U	Unit
v	Rate of reaction
V	Volume
v. supraintestinalis	vena supraintestinalis
VHA	Vacuolar-type proton ATPase

V_i	Initial volume
V_{max}	Maximal reaction rate
ZGC	Zymogen granule cell
αO_2	Solubility coefficient of oxygen
β	Buffer capacity
µCi	MicroCurie
µL	Microliter
µm	Micrometer
µM	Micromolar
µmol	Micromole

Chapter I: Introduction

Portions of this chapter have been published in Weinrauch, A.M., Edwards, S.L., Goss, G.G. 2015. Anatomy of the Pacific hagfish (*Eptatretus stoutii*). In: Edwards, S.L., Goss, G.G. (Eds.), Hagfish Biology. CRC Press, Boca Raton, Fl, pp. 1-40.

1.0 Comparative digestive physiology

The digestion and assimilation of nutrients is an essential process in all organisms for energy, growth, reproduction, and maintenance. Ingested food must be broken down into smaller, absorbable constituents (*e.g.* monosaccharides, fatty acids, amino acids) prior to assimilation. Metchnikoff provided the first essential contributions to understanding early metazoan digestion with his pioneering work on phagocytosis in single-celled protists, as well as the identification of an acidic digestion phase in marine invertebrates (Metchnikoff 1893). The earliest eukaryotic protists are predatory phagotrophs (Cavalier-Smith 2013), wherein prey items are internalized *via* invaginations of the cell membrane or the fusion of cytoplasmic vesicles with the outer membrane (Allen 1974). Acidic and enzymatic hydrolysis are carried out within these phagosomes (food vacuoles), using means that are generally conserved across the Metazoa (Kitching 1956; Nilsson 1977; Vonk and Western 1984; Stevens and Hume 1995). In some protsists, the vacuole undergoes alkalization prior to egestion of wastes (Nilsson 1977), much like that observed in later-diverging vertebrates.

As animals became multicellular and more complex, so did their digestive systems. First, certain cells evolved to play a specific role in the digestive process. For example, filter-feeding sponges utilise specialized cells, choanocytes, which create a current for water recycling and simultaneously acquire food particles. The particles are then directly transferred to archaeocytes for intracellular digestion and nutrient storage (Willenz 1980). Extracellular digestion using secreted hydrolytic enzymes then evolved, as did digestive structures opening from a mouth into a blind-ended cavity

(gastrovascular cavity), or terminating in an anus/cloaca (Stevens and Hume 1995). Digestive tracts show considerable variation across animals, and structures (e.g. a stomach) may not be analogous, homologous, or even exist, as the structure and function is dependent upon the ecological niche of a species. For instance, osmoconforming marine invertebrates can acquire nutrients across extra-intestinal structures such as the integument because their internal osmotic milieu is consistent with the surrounding environment (reviewed in Glover et al. 2013). Therefore, an impermeable integument is not necessary and nutrient acquisition across this organ can supplement dietary nutrients. Additionally, morphological differences such as intestine length can be used as an identifier of trophic niche. Indeed, herbivorous animals often have longer guts than omnivorous and carnivorous animals (Kapoor et al. 1975; Karachle and Stergiou 2010). Finally, digestive structures have developed to fill specific roles with many examples across phylogeny including storage organs such as the crop in birds, or the ruminant stomach (Stevens and Hume 1995). There is a clear advancement of digestive systems with the progression along the metazoan lineage however, there is a knowledge gap regarding the transition state between invertebrates and vertebrates. The Agnathans, or jawless fish, occupy the basal position in vertebrate phylogeny and consist of two extant groups, hagfish and lamprey.

Hagfish, of the family Myxinidae, comprise six living genera (*Eptatretus, Myxine, Nemamyxine, Neomyxine, Notomyxine,* and *Paramyxine*), which inhabit all oceans excepting the arctic (Fernholm 1998; Cavalcanti and Gallo 2008; Dayton and Hammerstrom 2018). Exclusively marine, the hagfish are the sole chordate

osmoconformers (Robertson 1963) and represent the transition between invertebrate and vertebrate organisms having diverged from the vertebrate lineage ~500 million years ago (Bardack 1998). Thus, hagfish are useful models for investigations of early vertebrate physiology in an evolutionary context. Hagfish diverged from the vertebrate lineage prior to the lamprey family however, whether hagfish should be included with the lamprey and other vertebrates in a monophyletic group, or branch off as a paraphyletic group, has been a longstanding debate in evolutionary biology (Figure 1.1). Previously, morphological, physiological, and paleontological data supported a paraphyletic relationship (Aldridge and Donoghue 1998; Gess et al. 2006), while molecular data was in support of monophyly (Stock and Whitt 1992; Mallatt and Sullivan 1998; Mallatt and Winchell 2007; Heimberg et al. 2010; Smith et al. 2010). The lack of embryological data contributes to the difficulty in establishing hagfish phylogeny. Hagfish embryos are notoriously difficult to obtain and the majority of embryonic descriptions hail from Bashford Dean in the late 1800's (Dean 1898, 1899). The reproductive life cycle of hagfish is also relatively unknown, with the majority of studies conducted on the Japanese hagfish (*E. burgerii*) owing to an observed, seasonal migration (Nozaki et al. 2000; Ichikawa et al. 2000) and recent successful captive breeding (Ota et al. 2007). More evidence accumulates as time goes on and favours the inclusion of hagfish within the Agnatha as a monophyletic clade (Miyashita et al. 2019).

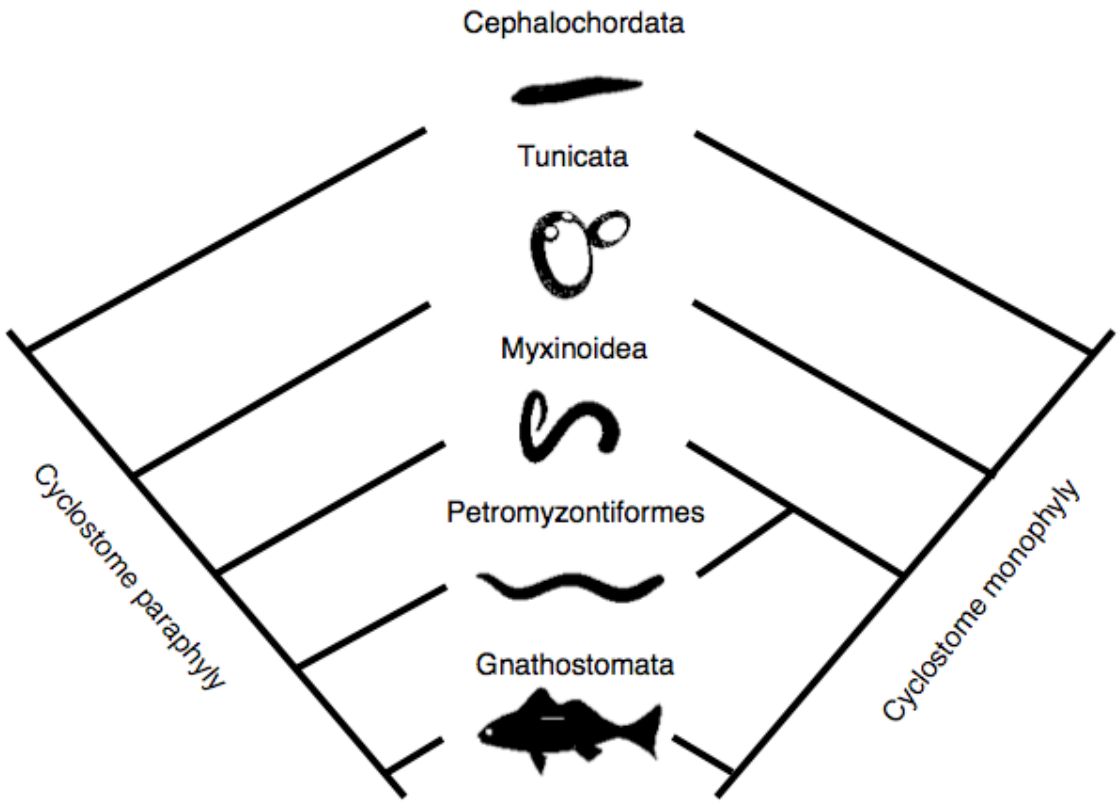

Figure 1.1 Phylogenetic relationships among the major chordate lineages. This depiction includes both the proposed paraphyly and monophyly of the cyclostomes (Myxinoidea, hagfishes; Pteromyzontiformes, lamprey). Tree based upon Heimberg and colleagues (2010).

1.1 Hagfish as a model organism in feeding physiology

Scavenging behaviour has remained a classical descriptor of hagfish since 1758 when Linnaeus classified hagfish as those that "*intrat et devorat pisces*" (*i.e*. enter into and devour fishes). Scavenging is an essential component of nutrient recycling in the marine benthos, yet does not provide sufficient energy for the large biomass of hagfish populations (Knapp et al. 2011). Other feeding modes have been investigated and include active predation, encounter competition, and passive feeding (reviewed in Glover and Bucking 2015), with mode dependent upon environmental factors (Zintzen et al. 2013). The dietary constituents of hagfish are varied and primarily consist of mesopelagic invertebrates (Martini 1998). In addition to invertebrates, the remains of Chondrichthyes, Osteichthyes, Aves, and Mammalia have been found in the hagfish digestive tract (reviewed in Martini 1998) with more recent analysis confirming a generalist diet that extends across multiple trophic levels (McLeod and Wing 2007). Active predation has long been suspected (Strahan 1963; Martini 1998) however, only recently has it been confirmed (Zintzen et al. 2011). Despite the lowest recorded metabolic rate of the vertebrates (Forster 1990), hagfish actively seek out prey items and can utilize slime to incapacitate captured prey (Zintzen et al. 2011). As mentioned above, passive feeding is characteristic of marine invertebrates where the integument is permeable since these organisms osmoconform and do not ionoregulate. Hagfish similarly osmoconform and display this ability to acquire nutrients extra-intestinally across the gill and integument (Glover et al. 2011a, 2015, 2016; Schultz et al. 2014). Given that hagfish are known to enter into carcasses to feed where environmental nutrient load is elevated, passive nutrient acquisition allows for maximal nutrient uptake in an animal that may experience

episodic feeding events as a scavenger (reviewed in Glover and Bucking 2015). This extra-intestinal acquisition strategy again highlights the position that hagfish occupy between the invertebrates and vertebrates.

Accompanying this scavenging lifestyle is the possibility of episodic feeding events. Although hagfish utilize numerous feeding modes as opportunistic feeders, they display an extended tolerance to food deprivation. In fact, there are numerous sources (unfortunately including my own personal observations) demonstrating that hagfish can withstand periods of fasting in captivity for at least 11 months (Foster and Moon 1986; Tamburri and Barry 1999)! Thus, the feeding ecology and tolerance to fasting demonstrate reasons hagfish are of interest for the studying of feeding physiology.

1.2 Morphology of the hagfish alimentary canal

Belonging to the superclass Agnatha, hagfish are lacking jaws and instead have a heart-shaped dental plate housing two rows of bilaterally symmetrical, conical teeth (7-9 per row; Clark and Summers 2007). Continued protraction and retraction of this dental plate rasps away at prey flesh in both juveniles and adults, which is suggestive of similar diets throughout hagfish life history, as well as across species (Dawson 1963; Slavkin et al. 1983; Clark and Summers 2012; Weinrauch et al. 2015). This plate is moved by a large pharyngeal muscle, composed of white muscle only that can exert rapid and powerful force in anaerobic conditions (Dawson 1963; Baldwin et al. 1991). This may be useful when hagfish are immersed within carrion tissues, an environment hypothesized to be anoxic (Bucking et al. 2011b; Clifford et al. 2016).

The alimentary canal of hagfish is a relatively simple, uncoiled tube that is partitioned into a foregut (comprised of the buccal cavity and the pharyngocutaneous duct; PCD) and hindgut (Figure 1.2a). The cellular morphology and proposed functions of each of these sections are vastly different. Unique among the vertebrates, mucus cells are housed strictly within the PCD (Adam 1963; Weinrauch et al. 2015). These cells, along with mature epithelial cells, are derived from undifferentiated cells that rest upon the basement membrane (Weinrauch et al. 2015). The surface of this epithelium is covered with microridges with no apparent pattern and which function to reduce friction and aid in the progression of mucus and food down the tract (Figure 1.2b-d). Irregularly shaped epithelial cells have clear borders with distinct cell-cell junctions and deeper furrows between cells, unlike the typical squamous cell covering of other vertebrate foreguts. Beneath the basement membrane is a *lamina propria*, an area rich in vasculature and adipose tissue. The submucosa contains connective tissue, lymph, and blood vessels, as well as varying amounts of adipose depending on the nutrition state (Adam 1963; Andrew and Hickman 1974) and dispersed lymph vessels that have sometimes been referred to as a diffuse spleen. The submucosa functions to provide turgidity to the intestinal walls, whereas the *muscularis serosa* aids in transport of nutrients by applying peristaltic pressures, with assistance required from body undulation (Andrew and Hickman 1974; Weinrauch et al. 2015; Figure 1.2b,d).

Although hagfish are agastric, a structure resembling the pyloric sphincter is evident at the junction between foregut and hindgut and can be constricted to permit water flow out the posterior-most gill duct, effectively partitioning the foregut from the

hindgut (Adam 1963). The diameter of the hindgut measures 1-2 cm, becoming thinner toward the posterior end and changing colour with nutritional status (Adam 1963). It contains approximately 10 large, permanent zigzag folds, which become somewhat diffuse in the preanal or anal regions (Figure 1.2a inset). The cells of the mucosal region are simple, columnar cells (enterocytes) up to 250 μm in length. They exhibit palpable polarization and a brush border to enhance surface area and aid in passage of food. Interspersed among the columnar cells are zymogen granule cells containing large, acidophilic secretory granules which have been likened to pancreatic enzymes and therefore, all digestion is thought to occur in the hindgut (Adam 1963; Andrew and Hickman 1974; Figure 1.2e-g). They are easily identifiable due to a single nucleolus in the nucleus along with a tapered apex, all of which is contained within a pit reminiscent of the mammalian pancreatic acinar cells (Adam 1963; Andrew and Hickman 1974). Two distinct types of granules appear to reside within the zymogen granule cells, a lightly stained, less-dense granule that is seemingly more basophilic than the alternative, more-dense acidophilic granules. These specialized epithelial cells rest upon a *lamina propria*, containing dense connective tissue and numerous capillaries. At the interface between the basement membrane and the *lamina propria* are distinct nuclei, which are thought to represent lymphatic cell nuclei, a probable necessity at an internal/external junction, given the feeding environment of the hagfish. Similar to lamprey, the mucosal region of hagfish contains more absorptive cells than secretory cells (Appy and Anderson 1971). However, the number of secretory cells appears to increase as you move down the intestine and become more diffuse toward the cloacal region. These cells appear to group and form a secretory cleft or pit, distinct from the surrounding columnar epithelium

(more lightly stained and a reduced brush border), appearing pore-like in scanning electron micrographs. Histochemical and radiotracer flux data suggests that both digestion and acquisition can occur along the entire length of the hindgut (Adam 1963; Glover et al. 2011b; Schultz et al. 2014).

Expansive remodeling of the hindgut may occur following a meal, as is the case in other intermittent feeding organisms. Reptilian intestinal remodeling is well characterised and fluctuates with changing physiological demand (Christel et al. 2007). There are notable increases in both mucosal thickness and microvilli length to enhance surface area and that also likely contribute to the elevated nutrient uptake capacity of these animals post-feed (Secor 2005, 2008; Christel et al. 2007). The extreme regression observed over periods of fasting is postulated to be an adaptive energy-conserving trait, as the energy required for intermittent upregulation is less than that required for constant maintenance (Secor 2005).

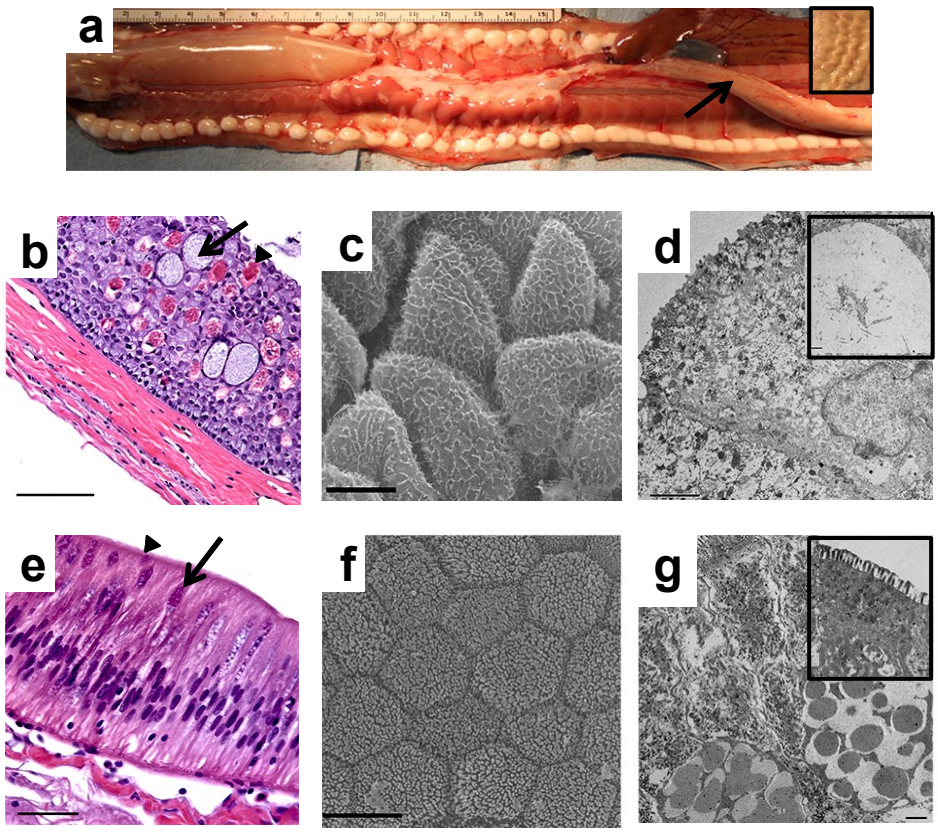

Figure 1.2 The morphology of the hagfish alimentary canal. (a) The straight intestinal tube of the Pacific hagfish partitioned into the anterior pharyngocutaneous duct and the posterior hindgut (arrow). Inset depicts the large, permanent zigzag folds found on hindgut mucosal surface. (b) The pharyngocutaneous duct is comprised of small (arrow head) and large (arrow) mucus cells, alongside undifferentiated cells. Scale bar = 100 µm. (c) The mucosal surface is covered with microridges to aid in food passage. Scale bar = 5 µm. (d) Transmission electron micrograph depicting the microridges on the small mucus cell. Scale bar = 2 µm. Inset demonstrates the disperse nature of the large mucus cell. Scale bar = 2 µm. (e) The hindgut contains columnar cells with a brush border for absorption (arrow head) and digestive granules within zymogen granule cells (arrow). Scale bar = 100 µm. (f) Scanning electron micrograph depicting the brush border microvilli. Scale bar = 5 µm. (g) Transmission electron micrograph of the zymogen granule cells. Scale bar = 2 µm. Inset highlights the fine brush border. Scale bar = 0.5 µm.

1.3 Physiological changes as a result of feeding

Feeding yields a range of notable effects on homeostatic physiology. The elevated metabolism/energy use associated with feeding is termed specific dynamic action (SDA) and is the summation of ingestion, digestion, absorption, and nutrient assimilation (McCue 2006; Secor 2009). Typically, indirect calorimetry is used in comparative studies, with metabolic oxygen consumption rate ($\dot{M}O_2$), and/or carbon dioxide production a proxy for energy use (Secor 2009). The energy requirements of an animal can be determined by the method of nutrient acquisition, which in turn will set the metabolic rate of the animal. The basal metabolic rate (BMR) is defined as the metabolic rate of a resting, post-absorptive animal not experiencing stress (Secor 2009). Following feeding, a number of SDA-related parameters can be defined. The factorial scope of peak can be calculated by dividing the peak postprandial metabolic rate by that of the BMR. A number of factors, such as meal content or meal size, have been shown to alter the peak, and the time to peak for many animals. Large, proteinaceous diets will elicit a larger SDA peak than a carbohydrate- or fat-rich meal across vertebrate phyla, as it will require more energy input to digest (Secor 2009). The length of time required for the metabolic rate to return to that of the BMR, is classified as the SDA duration and is often longer in binge-feeding animals looking to maximize nutrient extraction from infrequent meals (Secor 2009).

While these components of SDA can be calculated, the specific factors contributing to energy use, which can range from morphological remodeling (Chapter II) to enzyme secretion (Chapter III), acid secretion (Chapter IV), and/or nutrient absorption

(Chapters V, VI, VII), cannot be delineated. To my knowledge, a comprehensive examination of each of these contributions to hagfish digestion was lacking from the literature prior to this thesis.

1.3a Mechanisms of digestion: Digestive enzymes

Chemical digestion of food is a primary contributor to the digestive process through the hydrolysis of carbohydrates, proteins, and lipids into smaller compounds suitable for absorption. Derived from pancreatic or salivary glands, zymogen granules are storage organelles of the exocrine pancreas that house the inactive forms of digestive enzymes for each type of macronutrient (*i.e.* carbohydrates, fats, proteins). In many cases, the application of gastric HCl is necessary to convert the inactive zymogens into active enzymes (Bakke et al. 2010), as well as to denature the proteins themselves (see below section 1.3b). As mentioned above, digestive enzymes are known to exist in the hagfish hindgut as determined using histochemistry (Adam 1963). However, aside from some preliminary protease activity quantification (Nilsson and Fänge 1970), there is a paucity of reports concerning hagfish digestive capacity.

Digestive enzyme activity has been localized to the enterocytes themselves across a number of invertebrate and vertebrate species (Gibson and Dixon 1969; Wilson and Castro 2010). These include epithelial-derived enzymes such as alkaline phosphatase, disaccharidases, leucineaminopeptidase, and di-/tri-peptidases, alongside enzymes such as lipase, esterase, α-amylase, and carboxypeptidase, which are apically expressed along the microvilli (Kuz'mina and Gelman 1997). Carbohydrases are important in fish species in order to break down glycogen stores in prey. Carbohydrates are known to be the

preferred fuel source in hagfishes (Sidell et al. 1984) and α-amylase activity has been localized to the hagfish hindgut using histochemistry (Adam 1963), yet to my knowledge no reports on carbohydrase biochemical activity existed prior to this thesis. α-amylase is responsible for the hydrolysis of α(1-4) glucoside-linkages, which yields maltose and other branched oligosaccharides (Bakke et al. 2010). Maltase can then be utilized to break down maltose into the easily absorbed glucose using glucose transporters (GLUT) or sodium-linked glucose transporters (SGLT; see below in section 1.4a and Chapter V). Each of these carbohydrases plays a crucial role in carbohydrate digestion.

During extended periods of fasting, lipids are an essential energy source for hagfish (Emdin 1982; Foster and Moon 1986). Lipases are essential for the digestion and assimilation of lipids and require emulsifying agents like bile acids for effective digestion (Tocher 2003). There are numerous sources and varying activities of lipases along the digestive tract with effective regulation of activity depending upon diet (Bakke et al. 2010). The acquisition of lipids can occur through both passive diffusion and active processes regulated by specific carrier proteins (see below in section 1.4b and Chapter VI).

Finally, proteolytic enzymes are essential for protein digestion and the amount and activities of these enzymes is likewise dependent upon an organism's natural diet (Bakke et al. 2010). Large proteins can be hydrolyzed into smaller peptides (di- and tri-peptides) or further still into their smallest subunit, amino acids. While a number of proteases are released into the digestive tract, the final hydrolysis occurs at the brush

border surface or within the enterocytes themselves (Bakke et al. 2010). Amino acid acquisition in the hagfish hindgut had been previously examined (Glover et al. 2011b) and therefore, this thesis examines the acquisition strategies of dipeptides (see section 1.4c and Chapter VII).

1.3b Mechanisms of digestion: Acidic digestion

In addition to enzymatic hydrolysis, a well-characterized phenomenon of digestion is acid secretion to the lumen of the gastrointestinal tract, which is often accompanied by an equimolar secretion of bicarbonate (HCO_3^-) to the circulation (termed the alkaline tide; Hersey and Sachs 1995; Niv and Fraser 2002). The H^+ and HCO_3^- are derived from the hydration of carbon dioxide (CO_2) produced as a result of cellular respiration, a reaction catalyzed by carbonic anhydrase (CA; see below).

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \qquad (1)$$

Gastric acid is secreted *via* the gastric proton/potassium adenosine triphosphate (H^+-K^+-ATPase; HKA). Numerous secretagogues (for example, neurotransmitters such as acetylcholine, peptide hormones such as gastrin, or organic compounds such as histamine) lead to increased intracellular cyclic adenosine monophosphate (cAMP). This, in turn, stimulates H^+ secretion from the apical membrane in an electroneutral exchange for K^+, and forms concentrated HCl in the lumen using Cl^- effluxed from chloride channels (Gleeson 1992; Smolka et al. 1994; Hersey and Sachs 1995). Later-diverging vertebrates have distinct acid- and pepsinogen-secreting cells whereas earlier-diverging vertebrates, such as fish, have oxynticopeptic cells with dual function (Bomgren et al.

1998). Despite differing cellular morphology, the transporters responsible for gastric acidification appear to be conserved across vertebrate taxa. HKA has been localized to Chondrichthyan tissues and coincidentally this group represents the earliest phylogenetic appearance of gastric acidification in the vertebrates (Smolka et al. 1994). Typically, organisms are continually acid-secreting and this is likely true of hagfish with a fasting hindgut pH of ~ 5.5-6.0 however, as hagfish lack intestinal carbonic anhydrase, researchers postulated that should the acid-secretion mechanism exist, the mechanism could differ in hagfish (Nilsson and Fänge 1970).

Cellular acid secretion leads to alterations in intracellular acid-base homeostasis and consequently, the cell basolaterally extrudes HCO_3^- in an equimolar amount to rectify the intracellular environment (Niv and Fraser 2002). This "alkaline tide" has been identified in many vertebrate taxa (Wolosin and Forte 1984; Paradiso et al. 1989; Niv and Fraser 2002; Andrade et al. 2004; Wood et al. 2005a, 2007), but is not universally present. For instance, two marine teleosts, the Gulf toadfish and European flounder, do not display this phenomenon, despite exhibiting a marked gastric acidification post-feeding (Taylor and Grosell 2006; Taylor et al. 2007). It has been hypothesized that the intestine can efficiently excrete the alkaline equivalents and thereby nullify the alkaline tide in these species. Alternatively, an "acidic tide" has been characterised in the agastric killifish, which uses alkaline digestion (Wood et al. 2010). In those animals that experience a tide, the resultant alkalosis (or acidosis) of the circulation is excreted across the respiratory surface. In air-breathing vertebrates ventilation is restricted to permit elevation of blood partial pressure of carbon dioxide (P_{CO2}), followed by HCO_3^- secretion

in the small intestine, which coincidentally aids in activation of digestive enzymes (Wang et al. 2001). In water-breathing organisms however, O_2 is the limiting factor and changes in P_{CO2} are not achievable *via* altered ventilation (Gilmour 2001). Marine teleosts utilize the intestine for osmoregulation by secreting HCO_3^- and thus, acid-base homeostasis can be restored through the secretion of base equivalents into the intestine, as mentioned briefly above (Grosell et al. 2005; Taylor and Grosell 2006). In the osmoconforming (yet ionoregulating) group, the Chondrichthyans, base is excreted directly to the environment *via* v-type H^+ ATPase (VHA) at the gills (Tresguerres et al. 2007b). VHA has been previously localized to the hagfish gill (Tresguerres et al. 2007a) and therefore represents a potential means by which acid-base homeostasis can be maintained following feeding, should acidification/alkalization be utilized for digestion in hagfish.

1.4 Intestinal nutrient acquisition

The acquisition of each nutrient is regulated *via* specific transport proteins, which are mechanistically conserved across vertebrate phylogeny (Tocher 2010). The ubiquitous sodium-potassium ATPase (Na^+-K^+-ATPase; NKA) provides the energetics for the majority of transporters. This electrogenic transporter establishes an inwardly directed sodium gradient in terms of both concentration and charge by excreting sodium for potassium at a ratio of 3:2 (Skou and Esmann 1992).

The discrimination of regulated transport from passive uptake is a key component of the kinetic characterisation of transport proteins. The addition of increasing substrate concentrations will lead to saturable uptake if there are a finite number of transport

proteins, whereas passive uptake will increase in a linear fashion. This relationship was founded by Michaelis and Menten (1913) and is mathematically defined as follows:

$$v = \frac{v_{max}[S]}{k_m + [S]} \qquad (2)$$

where, v is the rate of reaction, v_{max} is the maximal reaction rate, [S] is the substrate concentration, and K_m represents the substrate concentration at which half-maximal reaction rate is achieved (a proxy for transporter affinity). With the parameters derived from this equation (*i.e.* v_{max} and K_m) comparative analysis can be conducted across substrates, tissues and/or organisms. As fish and other poikilotherms are often functioning at lower temperatures, the nutrient absorption process is slower and therefore, the transporters themselves tend to have a higher affinity for each substrate (Bakke et al. 2010).

In addition to saturable uptake, regulation of transporter function or reaction rate can be utilized to establish the presence of carrier-mediated transport. For instance, feeding is known to induce significant increases in maximal nutrient absorption in certain reptilians (Secor 2005). Additionally, apical expression of nutrient transporters changes in response to post-prandial cues (*e.g.* insulin), enabling enhanced capacity for absorption (Stahl et al. 2002). Regulation of uptake suggests that specific mechanisms are responsible for nutrient acquisition.

1.4a Glucose acquisition

Glucose is a ubiquitous energy source that is commonly stored as the branched polysaccharide, glycogen (Bell et al. 1990). Glycogen stores are the first energy reserve to become depleted at the on-set of fasting in hagfish (Foster and Moon 1986) and a 90% reduction in liver and muscle glycogen content is observed alongside decreased blood glucose and insulin content after fasting (Emdin 1982). Despite low concentrations of free glucose in marine environments, carbohydrates are the preferred metabolite of the hagfish cardiac system (Sidell et al. 1984). In addition, hagfish maintain glucose homeostasis using well-known hormones such as insulin, which has a highly conserved structure with that of mammals (Cutfield et al. 1979). Indeed, hagfish demonstrate the first occurrence in phylogeny of somatostatin- and insulin-containing islet cells (Falkmer and Winbladh 1964). The canonical effects of insulin persist (*i.e.* lowering of blood glucose) with a greater potency observed in Eptatred species, than Myxinids (Falkmer and Matty 1966; Thomas et al. 1973; Inui and Gorbman 1978). Yet, glucagon appears to be lacking in these animals (Falkmer and Matty 1966; Ostberg et al. 1976; Vigna 1979) and so somatostatin is instead hypothesized to perform the role of insulin regulation (Stewart et al. 1978).

Dietary glucose can be actively acquired *via* sodium-linked glucose transporters (SGLT; SLC5A family) or using equilibrative movement in a passive manner *via* glucose transporters (GLUT; SLC2A family; Figure 1.3). There are 14 members of the GLUT family distributed across nearly every tissue that operate based upon concentration gradients and thus are routinely expressed in the basolateral membrane to facilitate

glucose efflux from the cells to the circulation (Raja et al. 2012; Mueckler and Thorens 2013). However, at high glucose concentrations, GLUT2 isoforms can be recruited to the apical membrane for heightened acquisition in a rapid and reversible manner (Hediger and Rhoads 1994; Kellett and Helliwell 2000; Uldry and Thorens 2004; Raja et al. 2012). Similarly, GLUT4 isoforms can be recruited to the apical surface following hormonal stimuli in certain tissues such as muscle or adipose (Uldry and Thorens 2004).

SGLT is an active transporter that utilizes the electrochemical charge established by NKA (as described above). Crane (1960) was the first to propose that uphill glucose transport is coupled to a sodium gradient. Since this time, 12 members of the SLC5 family have been identified in mammals and the SLC5 family is now one of the most well studied SLC families (Wright et al. 2011). The high-affinity, low-capacity SGLT1 is often intestinally expressed and therefore responsible for dietary glucose uptake (Hediger and Rhoads 1994; Wright et al. 2011). At a cellular level, the regulation of SGLT movement is dependent upon phosphorylation state (Subramanian et al. 2008) with an intracellular vesicle pool available for rapid membrane insertion (Kipp et al. 2003). Following feeding, SGLT expression is increased in the apical membrane and permits heightened glucose acquisition (Williams and Sharp 2002).

Pharmacological inhibitors have been essential in the elucidation of mechanisms of nutrient acquisition. Phloretin is an effective GLUT inhibitor and can also inhibit SGLT, although to a lesser degree (Kellett and Helliwell 2000; Raja et al. 2012). Cytochalasin B however, is the most commonly used inhibitor in studies of facilitated

glucose diffusion. It is membrane permeant and binds to GLUT transporters at the intracellular face and can also diminish SGLT function at high concentrations (Estensen and Plagemann 1972).Phlorizin is chiefly used as the potent inhibitor of SGLT because it binds to the same exofacial loops as that of glucose, although with a 1000-fold greater affinity (Ehrenkranz et al. 2005). At heightened concentrations, phlorizin can also inhibit GLUT (Ehrenkranz et al. 2005). This inhibitor has been effectively used in Myxine species previously. Flöge and colleagues (1984) demonstrated the presence of a sodium-dependent, phlorizin-inhibitable glucose acquisition mechanism in the archinephric duct (hagfish kidney equivalent). However, the mechanisms of dietary glucose acquisition were never before investigated in hagfish.

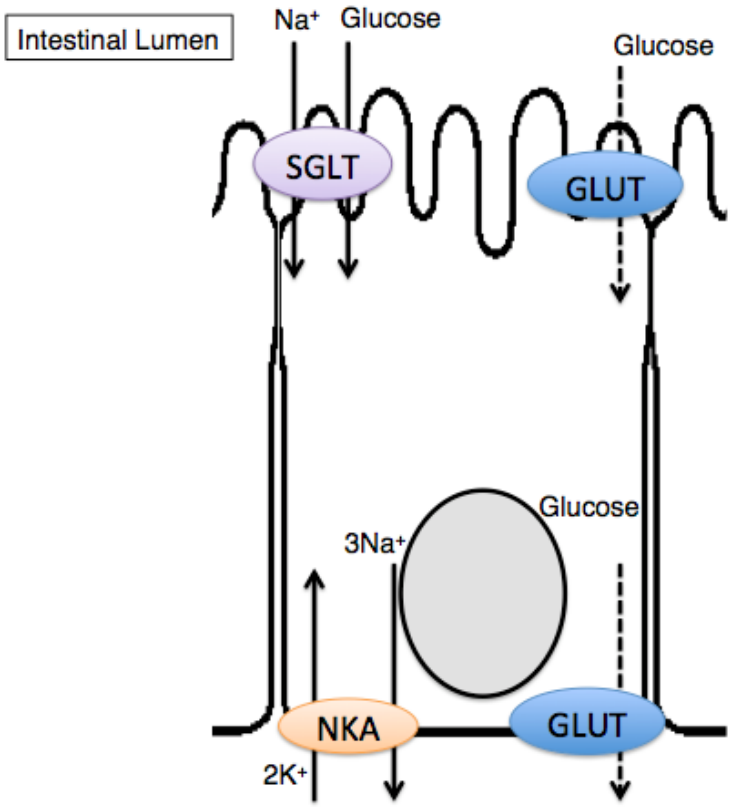

Figure 1.3 Schematic representation of enterocyte glucose acquisition mechanisms. Sodium glucose-linked transporters (SGLT) operate based upon the sodium electrochemical gradient that is established by the sodium-potassium ATPase (NKA). Glucose transporters (GLUT) are facilitated transporters (dotted lines) that can be apically expressed following a meal when the luminal concentration of glucose is elevated.

1.4b Oleic acid acquisition

Lipids are organic compounds that are insoluble in water, yet are an essential energy reserve in the marine hagfish during fasting (Foster and Moon 1986). Dietary lipids are hydrolysed by numerous lipases along the digestive tract into constituents such as fatty acids (Chapus et al. 1988). Oleic acid (18:1 cis-9) is a monounsaturated long chain fatty acid (13-21 carbons) and the most common fatty acid in nature (Alasalvar et al. 2002). Therefore, it represents an excellent model fatty acid for the understanding mechanisms of dietary fatty acid acquisition.

For many years, it was believed that fatty acids were acquired *via* a passive "flip-flop" mechanism owing to their lipophilic/amphipathic nature (Schwenk et al. 2010). However, the inability to regulate transmembrane movement of fatty acids would likely not be sufficient to meet metabolic demands and leaves the cell subjected to fluctuating fatty acid concentrations, which can not be controlled. Thus, a carrier-mediated mechanism is highly desirable for the cells and over the past few decades, such a system has been identified (reviewed in Stahl 2004).

Fatty acid transport proteins (FATP; SLC27 family) are largely evolutionarily conserved, existing in yeast (*e.g. Saccharomyces cerevisiae*; DiRusso et al. 2013), invertebrates (*e.g. Caenorhabditis elegans*; Hirsch et al. 1998), and vertebrates (*e.g. Homo sapiens*; Hirsch et al. 1998). As mammalian FATP variants were the first to be identified, the majority of studies have focused on mouse. The intestinal isoforms are FATP2 and FATP4, which are localized to the apical brush border to permit dietary

uptake (Stahl 2004). The expression of this transporter is regulated by nutrients, hormones and cytokines, however the action appears to be tissue-dependent. For example, insulin application can alter FATP1 adipocyte expression, but has no effect on FATP4 abundance in the small intestine (Stahl et al. 2002). In cooperation with FATP, it has been put forward that fatty acid translocase or scavenger receptor B3 (SCARB3/FAT) can increase LCFA uptake. The proposed model suggests that SCARB3/FAT will acquire the LCFA and pass it to FATP for internalization of the nutrient (Stahl 2004; Figure 1.4). Upon internalization, the LCFA is esterified by enzymes such as acyl-coA synthetase to prevent efflux and then shuttled around *via* fatty acid binding proteins (FABP; Stahl 2004; Schwenk et al. 2010). However, some researchers dispute this claim and state that FATP are enzymes (like acyl-coA synthetase), responsible for the metabolic trapping of fatty acids intracellularly (Glatz and Luiken 2017). Although cloning of fatty acid transporters has proven difficult, the targeted deletion of SCARB3/FAT in mouse models shows a definitive reduction in fatty acid uptake in specific tissues (Coburn et al. 2000), and provides further evidence for regulated means of fatty acid acquisition. Although fatty acid fate is not well understood in fish, the mechanisms are presumed to occur as in mammals (Tocher 2003).

The regulation of these carrier-mediated proteins has been most rigorously tested in response to insulin. Analogous to GLUT transport, SCARB3/FAT is mobilized from an endosomal compartment following insulin application in the heart and muscle (Luiken et al. 2002; Jain et al. 2009). Furthermore, the timeframe of translocation is similar

between GLUT and SCARB3/FAT (van Oort et al. 2008), suggesting that the action of one hormone can induce broad impacts in terms of nutrient acquisition.

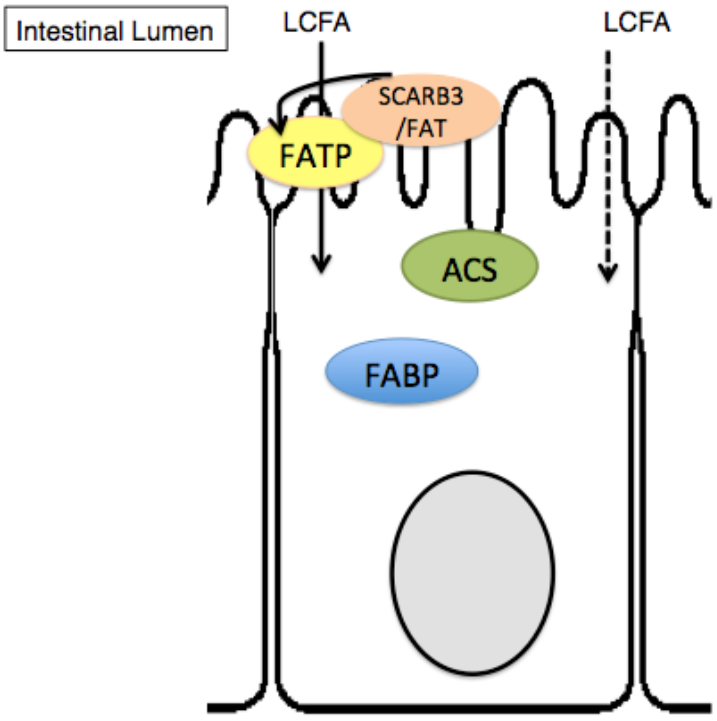

Figure 1.4 Schematic representation of proposed enterocyte long chain fatty acid uptake. Long chain fatty acids (LCFA) may bind to fatty acid transport protein (FATP) for internalization or first be acquired by scavenger receptor B class 3/ fatty acid translocase (SCARB3/FAT) and subsequently passed to FATP. Inside the cell an acyl coA synthetase (ACS) will esterify the LCFA, effectively trapping it within the cell. Fatty acid binding proteins (FABP) shuttle LCFA to specific destinations within the cell. Conversely, LCFA may passively enter the cell (dotted line). Figure based upon Stahl et al. 2004.

1.4c Dipeptide acquisition

Dietary proteins constitute a large portion of a carnivorous diet. These proteins are broken down by an array of proteases and peptidases derived in many places of the gastrointestinal tract and in fact, a number of proteases have been identified in the Atlantic hagfish intestine (Nilsson and Fänge 1970). The proteases produce a variety of peptides, as well as free amino acids. Amino acid acquisition has long been established in a variety of organisms, including the hagfish. In fact, amino acids (l-alanine and glycine) were the first nutrients examined in the hagfish hindgut in terms of uptake (Glover et al. 2011b). Glover and colleagues (2011a,b) characterised carrier-mediated pathways in the gills, skin, and hindgut of the Pacific hagfish. The intestinal carriers conformed to Michaelis-Menten kinetics, displayed sodium-dependence, and were inhibited by putative competitors, which allowed the authors to identify potential amino acid transporter families that have been characterised in mammals and may be present in hagfish.

Although amino acids are an essential dietary nitrogen source, the majority of dietary amino nitrogen is acquired in the form of di- and tri-peptides (Daniel 2004). The peptide transporter family (SLC15A) is promiscuous and able to acquire 400 di-peptides and 8000 tri-peptides, yet is specific for peptides alone (Adibi 2003; Daniel 2004). Despite numerous studies identifying a sodium-linked contribution to peptide uptake (Cheeseman and Devlin 1985), further analysis has led to the hypothesis that peptide transporters work in conjunction with sodium proton exchangers (NHE), or VHA (Cheeseman and Devlin 1985; Con et al. 2017). Furthermore, flux studies have revealed that there is a pronounced increase in uptake when the extracellular pH is decreased

(Daniel 2004), suggesting that these transporters function using an inwardly directed H^+ gradient (Ganapathy and Leibach 1985). Upon internalization, the peptides are then hydrolysed into individual amino acids and transported across the basolateral surface *via* as-of-yet unidentified transporters (Matsumoto et al. 1994; Figure 1.5).

As observed with other carrier-mediated transporters, peptide transporters are modulated by hormones such as insulin, which function to increase transport activity (Daniel 2004). Furthermore, the presence of dietary nutrients can also elicit increases in transporter activity (Shiraga et al. 1999), which could be essential for the hagfish that undergo extended fasting periods.

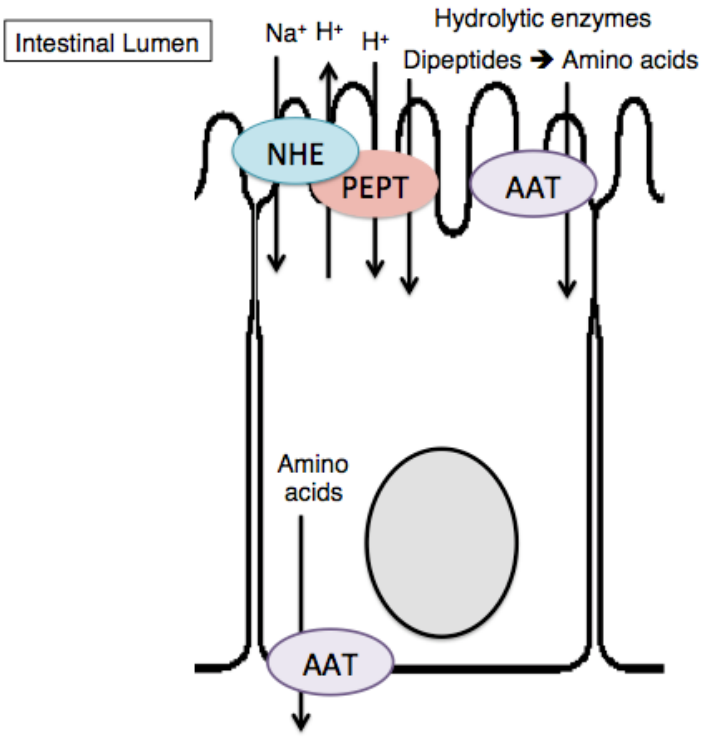

Figure 1.5 Schematic representation of mechanisms of enterocyte dipeptide acquisition. Dipeptides can be acquired *via* the peptide transporter (PEPT), which uses a proton motive force established by sodium proton exchangers (NHE). Dipeptides may be hydrolysed into individual amino acids both in the intestinal lumen and within the cell itself. The amino acids can then be transported by amino acid transporters (AAT) that are able to discriminate between amino acid groups.

1.5 Perspectives and objectives of the thesis

Hagfish are integral members of the benthic community, responsible for nutrient recycling and habitat turnover. With prey items ranging across trophic levels, as well as commercial fisheries becoming more prevalent, an understanding of hagfish digestive function and capacity is a necessary and missing component of the scientific literature. Furthermore, as the earliest extant representatives of the first vertebrates, hagfish are useful models for the understanding of early vertebrate physiology and its evolution.

This thesis addresses hagfish post-prandial intestinal physiology using multiple levels of biological organization, thereby enhancing our understanding of hagfish post-prandial physiology from multiple perspectives. Chapter II examines a selection of whole organismal responses to feeding in the Pacific hagfish. The goal of this study was to characterise standard metrics of feeding including SDA, nitrogenous waste production and acid/base balance. Morphometric analysis of the intestine post-feeding was also analysed given the extreme regression of intestinal tissue/function observed in other intermittent feeders. To assess the enzymatic digestive capacity, Chapter III examined tissue enzyme activity along the alimentary canal and in response to feeding, while Chapter IV examined potential cellular mechanisms of luminal hindgut acidification. This is the first definitive demonstration of acidic digestion in the agastric hagfishes and now the first phylogenetic appearance of acidic digestion in the vertebrate lineage. The nutrient uptake capacity of the hagfish hindgut was examined in Chapters V, VI, and VII, where kinetic profiles of glucose, oleic acid, and alanyl-alanine uptake were established, respectively. In addition, I examined some potential regulators of transporter activity and

expression. This collection of complementary research is summarized in Chapter VIII where I address the major findings and future avenues of research to better understand hagfish digestive physiology.

Chapter II: Post-prandial physiology of the Pacific hagfish (*Eptatretus stoutii*)

A version of this chapter has been published.

Weinrauch, A.M., Clifford, A.M., Goss, G.G. (2018). Post-prandial physiology and intestinal morphology of the Pacific hagfish (*Eptatretus stoutii*). *Journal of Comparative Physiology B. 188*:101-112. Reproduced with permission of the co-authors of the manuscript.

Introduction

Hagfish are the oldest extant representative of the craniates having diverged from the vertebrate lineage ~550 million years ago (Falkmer and Matty 1966; Thomas et al. 1973; Inui and Gorbman 1977) and have thus been used as an evolutionary model across a range of disciplines including morphology (Oisi et al. 2015; Miyashita and Diogo 2016), immunology (Pancer et al. 2005; Li et al. 2013), endocrinology (Evans and Harrie 2001; Clifford et al. 2017a) and acid-base and ion homeostasis (Evans 1984). The hagfish are a useful model for nutrient acquisition given their unique feeding lifestyle and mode of feeding wherein they scavenge both living (Zintzen et al. 2011) and dead/decaying carrion (Martini 1998) and can withstand food deprivation for extended periods (at least 11 months in captivity; Foster and Moon 1986).

As scavengers, hagfish have broad dietary constituents ranging from polychaetes to cetaceans (Martini 1998); the latter prey option sometimes involving burrowing into the decaying carcasses. The hagfish then become enveloped in a nutrient-rich broth (Glover et al. 2011a; Clifford et al. 2015, 2017b) and researchers have examined the physiological effects of such an environment, postulated to be hypercapnic (Baker et al. 2015), hypoxic/anoxic (Bucking et al. 2011b; Gillis et al. 2015; Clifford et al. 2016), rich in dissolved organic matter (Glover et al. 2011a; Bucking et al. 2011b; Schultz et al. 2014) and ammonia (Clifford et al. 2015, 2017b). Of the aforementioned studies, the nutrient transport capabilities were examined *ex vivo* (Glover et al. 2011a; Bucking et al. 2011b; Schultz et al. 2014) demonstrating a capacity to acquire nutrients across multiple

epithelia (gills, skin, intestine); however, there is little knowledge of the physiological effects of feeding on intact hagfish.

The post-prandial increase in energy expenditure over resting metabolic rate is commonly referred to as specific dynamic action (SDA) and is the result of metabolic processes involved in digestion, assimilation and absorption (for comprehensive reviews see: McCue 2006; Secor 2009). In aquatic organisms, the measured proxy for SDA is oxygen consumption (M_{O_2}), which is elevated following a feeding bout and displays a distinct pattern with a peak (termed factorial metabolic scope; measured as the peak divided by the resting M_{O_2}) and defined duration (Secor 2009). The peak and duration of SDA vary with meal composition (proportion of carbohydrate, fat, protein), meal toughness, and meal size, with large, protein-rich meals eliciting the greatest factorial metabolic scope (McCue 2006).

The post-prandial changes in nitrogen metabolism have been characterized in a number of teleost and elasmobranch species (Wood et al. 2005a, 2007; Bucking and Wood 2008). Protein deamination of a meal leads to increased production of nitrogenous waste, most of which is excreted passively as ammonia across the gills in the majority of teleost fishes (reviewed by: Wood 2001; Bucking 2016). A recent study examined the post-prandial nitrogen catabolism and urea-N excretion in Pacific hagfish following natural feeding in baited traps (Wilkie et al. 2017). Both ammonia-N and urea-N excretion rates were significantly elevated following feeding and returned to control levels by 24-72 h. Given that hagfish were permitted to feed at any point over a 12 h

period, we sought to quantify ammonia excretion (J_{Amm}) rates in this study for a comparative analysis of elevated post-prandial J_{Amm}.

Meal ingestion has been demonstrated to alter acid-base homeostasis across many vertebrate taxa (Coulson et al. 1950; Secor and Diamond 1995; Wood et al. 2005a; Taylor et al. 2007) primarily because of the 'alkaline tide phenomenon'. To maintain cellular acid-base homeostasis, alkalization of the blood *via* HCO_3^- (alkaline tide) occurs in response to gastric parietal cell secretion of HCl; a process that remains conserved from elasmobranchs to mammals (Hersey and Sachs 1995; Niv and Fraser 2002; Wood et al. 2005a). The hagfishes, however, are agastric and instead have a straight intestinal tube that is partitioned into pharyngocutaneous duct (foregut) and hindgut at a cardiac region where cross-striated muscle fibers cross the intestine (Adam 1963). Distinct differences occur between these regions with the foregut uniquely housing all mucus cells while the hindgut is comprised of columnar cells with a distinct brush border and zymogen granule cells (Adam 1963; Weinrauch et al. 2015). Digestive enzymes (lipases, amylases and proteases) have been verified in the Atlantic hagfish hindgut and are hypothesized to drive digestion (Adam 1963), although the presence of a further acidic component of digestion has never before been examined. The post-prandial acid-base response in another agastric species, the killifish (*Fundulus heteroclitus*), has previously been examined and no alkaline tide was observed, likely because of the absence of an acid-secreting stomach (Wood et al. 2010).

Given the absence of an alkaline tide in some teleosts (European flounder, Gulf Toadfish; Taylor and Grosell 2006; Taylor et al. 2007) and the presence in others (Rainbow trout; Bucking et al. 2009) including in an earlier diverging chondrichthyan (Spiny dogfish; Wood et al. 2005a), we sought to quantify the acid-base status of the Pacific hagfish both internally and with the environment using net proton flux measurements. Furthermore, we quantified the bile pH in an attempt to determine whether the bile acids could account for a luminal acidification of the hindgut should a subsequent plasma alkalization not occur.

Hagfish are known to be opportunistic and intermittent feeders (Lesser et al. 1997). Other organisms that feed infrequently have diminished gastrointestinal function and regress their metabolically demanding tissues (such as the intestine) during periods of fasting to reduce energy expenditure (Secor 2005; Lignot et al. 2005; Christel et al. 2007; Secor et al. 2012). In fact, mucosal oxygen consumption rates are significantly lower in the fasted intestines of infrequently feeding snakes in comparison to frequently feeding snakes (Secor et al. 2012), and estivating anurans demonstrate a doubling of intestinal mass accompanied with a significant increase (6-10-fold) in intestinal nutrient uptake capacity post-feeding (Secor 2005). It is speculated that this cycling of up- and down-regulation of the intestine is less costly than would be the maintenance of such tissue in frequently fasting organisms (Secor et al. 2012). This gastrointestinal plasticity would aid in energy conservation when hagfish must survive months between meals, yet maximize uptake during feeding.

The goal of the present study was to characterize aspects of post-prandial physiology and intestinal morphology of the Pacific hagfish. We aimed to address the hypotheses that feeding would increase M_{O_2}, and J_{Amm} as typically observed in animals. Alterations to acid-base status were not expected to occur because of the absence of an acid-secreting stomach and the proposed enzymatic nature of hagfish digestion. We hypothesized that Pacific hagfish would display distinct increases in mucosal thickness and microvillar length in the fed state as observed in other intermittent feeders. This increase would regress in the fasted state as a means to preserve energetic stores for critical tissue function during extended periods of food deprivation.

Materials and Methods

Animal Care and Husbandry

Pacific hagfish (*Eptatretus stoutii;* N = 70; mean mass 77 ± 6 g, range 29 – 206 g) were collected using baited traps in Trevor Channel, Bamfield, B.C. Canada and immediately transported to Bamfield Marine Sciences Centre (2015, 2016). Twenty-five animals were housed in darkened 80 m^3 tanks that received continuously flowing seawater and had open-ended PVC tubes for added shelter as previously described (Clifford et al. 2014). Animals were fasted for 4-5 weeks prior to experimentation. Of note, *E. stoutii* collected in 2015 readily ate hake from Aug-Oct but only infrequently ate squid from Nov-June. For experimental consistency, only animals that consumed hake were used in experiments and thus, a second batch of hagfish were collected in 2016 and fasted 4-5 weeks prior to feeding with hake. All experiments were conducted with the approval of the University of Alberta Nos. AUP0001126 (2014-2016) and Bamfield Marine Sciences Centre Nos. RS-14-13 (2014), RS-15-31 (2015) and with DFO Collection Permit Nos. XR-192-2014, XR-310-2015, XR-202-2016.

Chemicals

All chemicals for electron microscopy were obtained from Electron Microscopy Sciences (Hatfield, PA, USA) while all other chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA), unless otherwise noted.

General Experimental Procedure

A single, intact North Pacific hake (*Merluccius productus*; Ucluelet Harbour Seafoods Ltd.) was placed in the bottom of the tank and *E. stoutii* were permitted to feed

to apparent satiation (~10 min; characterized by the *E. stoutii* swimming away from the carcass after an active feeding bout and into the PVC housing). Notably, *E. stoutii* bore a single hole through the ventral surface of the fish and the soft inner organs (liver and stomach) were consumed (Fig. 2.1). To account for possible diurnal variations in oxygen consumption, feeding always commenced at midnight when fish were actively swimming. Unfortunately, accurate assessment of food mass consumed was not possible due to the fact that the hagfish did not eat when separated into individual chambers and evacuated their gut contents when anaesthetized. Immediately following a single feeding (or fasting; control), unanaesthetized *E. stoutii* were gently moved from the tank and placed into individual 1350 mL chambers in a sea table receiving continuously flowing seawater for temperature control (12 ± 1°C). *E. stoutii* were then allowed to acclimate for a 2 h period as preliminary experiments demonstrated variable M_{O_2} values for the first 2 h measurement period. *E. stoutii* handling protocols, including wearing nitrile gloves and gently cupping the coiled hagfish, were employed to minimize slime production and any trials that contained slime were not included in analysis. Importantly, given that *E. stoutii* did not reliably feed, control measurements were unable to be paired with post-prandial measurements from the same individual. Rather, control measurements were obtained from non-fed animals that were thereafter returned to the main population for feeding experiments. Time series experiments were conducted for 4 h intervals over the ensuing 36 h for M_{O_2}, net proton flux ($J_{Net}^{H^+}$), J_{Amm} (collected simultaneously for each subject), acid-base status and morphology (see below).

Measurement of metabolic oxygen consumption

Following 2 h of acclimation, oxygen content (P_{O2}; Torr) was measured *via* sensor spots and optical fiber cables connected to Witrox 4 (Loligo systems, Tjele, Denmark) at the beginning and end of each 2 h measurement period, conducted every 4 h over a 36 h time course. The oxygen content was never less than 80 Torr and *E. stoutii* M_{O_2} remains stable until oxygen tensions of ~ 15 Torr (Drazen et al. 2011). Chambers were well mixed prior to measurement using a magnetic stir bar. Following each measurement period, the chambers were flushed with flowing seawater until the beginning of the next measurement period to remove metabolic wastes and replenish oxygen content. At experiment completion, *E. stoutii* (N = 70) were anaesthetized (4 g L^{-1} MS-222 neutralized with 1.2 g L^{-1} NaOH), blotted dry and weighed.

M_{O_2} (μmol kg^{-1} h^{-1}) was calculated as previously described (Clifford et al. 2016) as:

$$M_{O_2} = \frac{[P_{O_2 i} - P_{O_2 f}] \times \alpha_{O_2} \times V}{M \times t} \qquad (1)$$

where, $P_{O_2 i}$ (Torr) is the initial PO_2 of the chamber and, $P_{O_2 f}$ (Torr) is the final PO_2 of the chamber, α_{O_2} is the solubility coefficient of oxygen in seawater at 12°C (1.7747 μmol O_2 L^{-1} $Torr^{-1}$) as determined by (Boutilier et al. 1984), *V* is the volume of the chamber less the volume of the animal (L), *M* is the animal wet mass (kg) and *t* is the time (h). With the resulting M_{O_2} values the mean factorial scope was calculated as peak M_{O_2} divided by the resting $M_{O_2}$and SDA was determined from the total increase in oxygen uptake above resting levels and standardized to kJ using the conversion factor of 1 μmol O_2 = 0.45 J (Secor 2009).

Net ammonia fluxes

For each 2 h flux period, initial and final water samples (1 mL) were collected in 4 h intervals for 36 h from each individual chamber (N= 70). Water ammonia concentration was determined using the salicylate hypochlorite colourimetric assay described by Verdouw and colleagues (1978). J_{Amm} (μmol kg^{-1} h^{-1}) was determined using the following equation:

$$J_{\mathrm{Amm}} = \frac{[T_{\mathrm{Ammf}} - T_{\mathrm{Ammi}}] \times V}{M \times t} \qquad (2)$$

where, T_{Ammf} and T_{Ammi} are the final and initial ammonia concentrations (μmol L^{-1}) in each water sample, *V* is the volume (L) of the given chamber less the fish volume, *M* is the animal mass (kg) and *t* is the duration of the flux (h). Ammonia quotient (AQ) was calculated as ammonia excreted (μmol kg^{-1} h^{-1}) per amount of oxygen consumed (μmol kg^{-1} h^{-1}).

Net Proton Fluxes

E. stoutii (N=70) $J_{\mathrm{Net}}^{\mathrm{H}^+}$ was determined using techniques previously described by (Clifford et al. 2014). Briefly, initial and final water samples (30 mL) were collected using a disposable 25 mL pipette from each chamber. The time course persisted over 36 h with 2 h fluxes conducted every 4 h. The samples were then placed in a 10°C water bath and bubbled with 100% CO_2. While bubbling, the pH was measured with a thermojacketed Orion ROSS Micro pH electrode for at least 6 min (no drift in pH units for 1 min). To determine buffering capacity, the pH change following the addition of known amounts of 0.1011 N HCl standard or 0.1000 N NaOH standard were determined while samples were stirred and aerated with 100% CO_2. Buffering capacity was

calculated as previously described (see Clifford et al. 2014for details). The $J_{\mathrm{Net}}^{\mathrm{H}^+}$ was determined using the following equation:

$$J_{\mathrm{Net}}^{\mathrm{H}^+} = \left[\left[\frac{\left(expH_{final}^{+} - expH_{initial}^{+}\right)}{exp\beta} \times expV\right]\right] - \left[\left[\frac{\left(conH_{final}^{+} - conH_{initial}^{+}\right)}{con\beta} \times conV\right]\right] \times \frac{1}{M} \times \frac{1}{t} \qquad (3)$$

where, $J_{\mathrm{Net}}^{\mathrm{H}^+}$ is the net proton flux, $expH_{final}^{+}$ and $expH_{initial}^{+}$ are the total proton concentrations in the experimental chambers, $conH_{final}^{+}$ and $conH_{initial}^{+}$ are the total proton concentrations in the control chambers, $expV$ and $conV$ are the volume in the experimental and control chambers subtracting fish weight, $exp\beta$ and $con\beta$ are the measured buffering capacities of the experimental and control water, *M* is the animal mass (kg) and *t* is the flux time (h). Negative flux values denote base excretion.

Blood and Bile Sampling

Fasted and post-fed (8 h and 36 h) *E. stoutii* (N = 21) were euthanized (4 g L^{-1} MS-222 buffered with 1.2 g L^{-1} NaOH) and a blood sample was collected *via* caudal puncture as described previously (Tresguerres et al. 2007a; Clifford et al. 2014). Single sampling was necessary as this method of blood sampling prompted incontinence. Briefly, *E. stoutii* were held vertically to pool blood in the caudal sinus and a heparinized 23-G needle was used to extract a 200 µL blood sample from the sinus. Given that the caudal sinus contains a mixture of arterial and venous blood that could diminish acid-base changes, we also collected a blood sample from the *vena supraintestinalis* to observe acid-base changes directly at the intestinal level. The *vena supraintestinalis* runs on the ventral side of the hindgut and acts as the principle efferent hepatic vein running

from the posterior liver lobe (Johansen 1963). Additionally, a 23-G needle was used to pierce and drain the gall bladder of biliary fluids. In all collected samples, pH was measured immediately using an Orion ROSS Micro pH electrode (Fisher Scientific, Massachusetts, USA) thermojacketed at 10°C. Blood was then centrifuged (14, 000 × *g* for 30 sec) and plasma total carbon dioxide (T_{CO_2}) was subsequently measured using a Corning 965 Carbon Dioxide Analyzer (Ciba Corning Diagnostic, Halstead, Essex, UK). Volumes of blood drawn from the *vena supraintestinalis* were too low to be analyzed for T_{CO_2}. Blood [HCO_3^-] was calculated by inserting the measured pH and T_{CO_2} values into the Henderson-Hasselbalch equation (Boutilier et al. 1984).

Morphological assessment

Sample processing for microscopy occurred as previously conducted (Weinrauch et al. 2015). To reduce variation stemming from body mass differences, *E. stoutii* of similar sizes were selected for this experimental series, as this was the only variable not standardized by weight in the calculations (N = 9; mean mass = 43 ± 4 g; no significant size differences among animals used; $F_{2,6}$ =0.64; p = 0.56). Briefly, tissues were collected from 3 fish in each treatment (fasted, 8 h, 36 h post-fed) at 2 points along the intestine, anterior hindgut immediately posterior to the liver (first 1/3 of the hindgut) and posterior hindgut (last 1/3 of the hindgut). Light microscopy samples were fixed in a 4% paraformaldehyde solution at 4°C overnight followed by two washes in ice-cold 70% ethanol. Tissues were trimmed and embedded in paraffin following an ethanol dehydration series prior to sectioning (5 microns). Post-staining with haematoxylin and eosin was conducted prior to visualization on a Zeiss Scope.A1 microscope with image capture using an Optronic camera. Samples collected for TEM were cut in 2 mm^3

sections and fixed in a 4% paraformaldehyde, 2.5% glutaraldehyde, 0.05 mmol L^{-1} sodium cacodylate buffer for 3 h at 4°C. Following ethanol dehydration, samples were then rehydrated to 0.1 mol L^{-1} PBS, rinsed with PBS and osmicated in 1% osmium tetroxide for 1 h before full dehydration to 100% ethanol and immersion in a Spurr resin:ethanol (1:1) mixture for three hours. Overnight curing in pure Spurr resin occurred prior to sectioning (80 –100 nm) with a diamond knife on a Reichert-Jung ultramicrotome Ultracut E and post-staining with 4% uranyl acetate and Reynold's lead citrate in CO_2-free conditions (Reynolds 1963). Images were captured on a Philips FEI transmission electron microscope (Model: Margagni 268) operating at 80 kV and a Gatan Orius CCD camera. All images were transferred to Adobe Photoshop CS6 for resizing, sharpening and color correction only. For each *E. stoutii*, 2 representative images were chosen (anterior and posterior hindgut) and 10 randomly selected measurements were made of mucosal thickness (from the basement membrane to the top of the columnar cells) and microvilli length with ImageJ software (National Institute of Health). The mean of the 10 measurements for each treatment were used in the analysis (3 values per treatment; 1 per animal).

Statistical Analysis

Data are presented as mean ± standard error of the mean (s.e.m). Normality was tested using the D'Agostino and Pearson omnibus normality test. Datasets that were normally distributed were analyzed with a one-way analysis of variance (ANOVA) and further tested for homogeneity of variance using the Brown-Forsythe test. Differences between treatments and control were determined using Dunnett's multiple comparisons *post-hoc* test. Datasets that failed the assumptions for ANOVA were analyzed with a non-

parametric Kruskal-Wallis ANOVA followed by Dunn's multiple comparison *post-hoc* test to distinguish differences from controls. The fiducial limit was set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, USA).

Results

Post-prandial patterns of M_{O_2}, J_{Amm}, and $J_{Net}^{H^+}$

Control basal M_{O_2} of fasted *E. stoutii* averaged 642 ± 51 µmol kg^{-1} h^{-1} and increased significantly to 1869 ± 272 µmol kg^{-1} h^{-1} 8 h following feeding ($F_{7,60}$ = 2.63; p = 0.003; Fig. 2.2a), resulting in a factorial metabolic scope of peak M_{O_2} of 2.92 and an SDA of 0.552 kJ. Metabolic rates returned to basal levels by 12 h post-feeding and remained unchanged from basal values ($p \geq 0.160$), 12-36 h post-feeding. Baseline J_{Amm} averaged 17.3 ± 6.4 µmol kg^{-1} h^{-1} in control fasted *E. stoutii* (Fig. 2.2b). Rates of J_{Amm} increased 16-fold post-prandially to 278 ± 101 µmol kg^{-1} h^{-1} at the final 36 h measurement period (p = 0.022). Basal excretion rates of net base equivalents (denoted as negative $J_{Net}^{H^+}$) averaged 267 ± 373 µequiv kg^{-1} h^{-1} (Fig. 2.2c). Net base equivalent excretion rates did not differ significantly from control excretion rates at any time point in the 36 h time course (p> 0.999).

Post-prandial blood and bile acid-base status

Control, fasted *E. stoutii* had blood pH values of 7.89 ± 0.03 (Fig. 2.3a). Blood collected from the caudal sinus 8 h following feeding was not significantly different from control values (pH = 7.97 ± 0.02; $F_{3,18}$ = 4.12; p = 0.41); however, a significant alkalosis (8.15 ± 0.05; p = 0.01) was found in samples collected 8 h post-prandially from the *vena supraintestinalis.* Plasma pH values remained unchanged from control levels (7.91 ± 0.05; p = 0.99) by 36 h. No changes in caudal blood T_{CO_2} values were observed among fasted (6.27 ± 0.76 mmol CO_2 L^{-1}), 8 h (6.73 ± 0.35 mmol CO_2 L^{-1}) or 36 h post-fed *E. stoutii* (6.36 ± 0.61 mmol CO_2 L^{-1}; $F_{2,15}$ = 0.14, p = 0.87; Fig. 2.3b).

Plasma [HCO_3^-] concentrations did not vary among control (6.18 ±0.74 mmol L^{-1}), 8 h (6.27 ± 0.44 mmol L^{-1}) or 36 h (6.22 ±0.48 mmol L^{-1}) post-fed *E. stoutii* ($F_{2,17}$ = 0.007, p = 0.993; Fig. 2.3c). Bile pH in fasted *E. stoutii* averaged 7.17 ± 0.05 (Fig. 2.3d) and while no significant differences were observed throughout the time series, there was a trend of increasing bile acidity 8 h post-feeding (pH = 6.83 ± 0.19; $F_{2,16}$= 1.79, p = 0.20; Fig. 2.3d).

Intestinal Morphology

The hagfish hindgut lacks mucus cells and instead displays prominent zymogen granule cells (ZGC) amongst the brush-border topped columnar cells (CC; Fig. 2.4a-c). Mucosal thickness was significantly elevated 8 h following feeding (116 ± 2 µm; p < 0.0001) when compared to control (97.4 ± 1.8 µm). Mucosal thickness remained significantly elevated in comparison to control in 36 h post-fed *E. stoutii* (111 ± 2 µm; $F_{2,6}$ =7.96; p < 0.0001; Fig. 2.4d). Mucosal thickness did not vary along the length of the tract of any fed or fasted *E. stoutii* investigated (data not shown). The lengths of the microvilli (MV) comprising the enterocyte brush border were significantly elevated 8 h following feeding (1.2 ± 0.02 µm; $F_{2,6}$ = 0.644; p < 0.0001) and returned to fasting lengths (0.5 ± 0.01 µm) by 36 h post-feeding (0.6 ± 0.01 µm; p= 0.61; Fig. 2.5a-d).

Figures

Figure 2.1 Hagfish enter a carcass to consume the soft inner organs. A single hake (*Merluccius merluccius*) was placed into a tank with multiple hagfishes at midnight. The hagfish immediately bore a hole (note in image above) into the flesh of the fish and consumed the soft inner organs such as the liver.

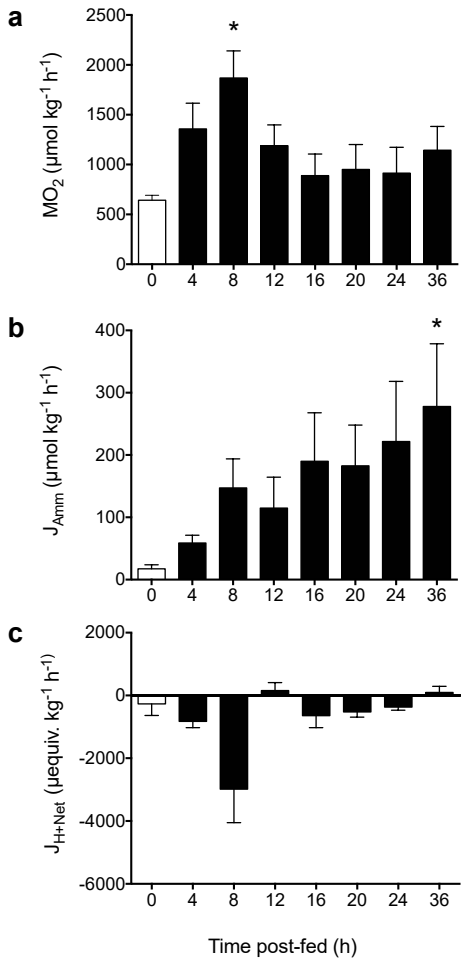

Figure 2.2 Time course of M_{O_2}, J_{Amm}, and $J_{Net}^{H^+}$, following feeding in the Pacific hagfish. Pacific hagfish were fasted (white bars) for one week or permitted to feed *ad libitum* (black bars) on Pacific hake (*Merluccius productus*). The animals were then transferred to individual, static containers for measurement of metabolic oxygen consumption (M_{O_2}; **a**), net ammonia excretion (J_{Amm}; **b**) and net proton flux ($J_{Net}^{H^+}$; **c**) in 2 h flux periods. Water was refreshed in between measurement periods to prevent anoxia and the build-up of nitrogenous waste products. Data are presented as means ± s.e.m (n = 6 –9). Negative values denote base equivalent excretion (**c**). Asterisk (*) denotes statistical difference from control ($p < 0.05$; ANOVA; **a** or Kruskal Wallis; **b,c**).

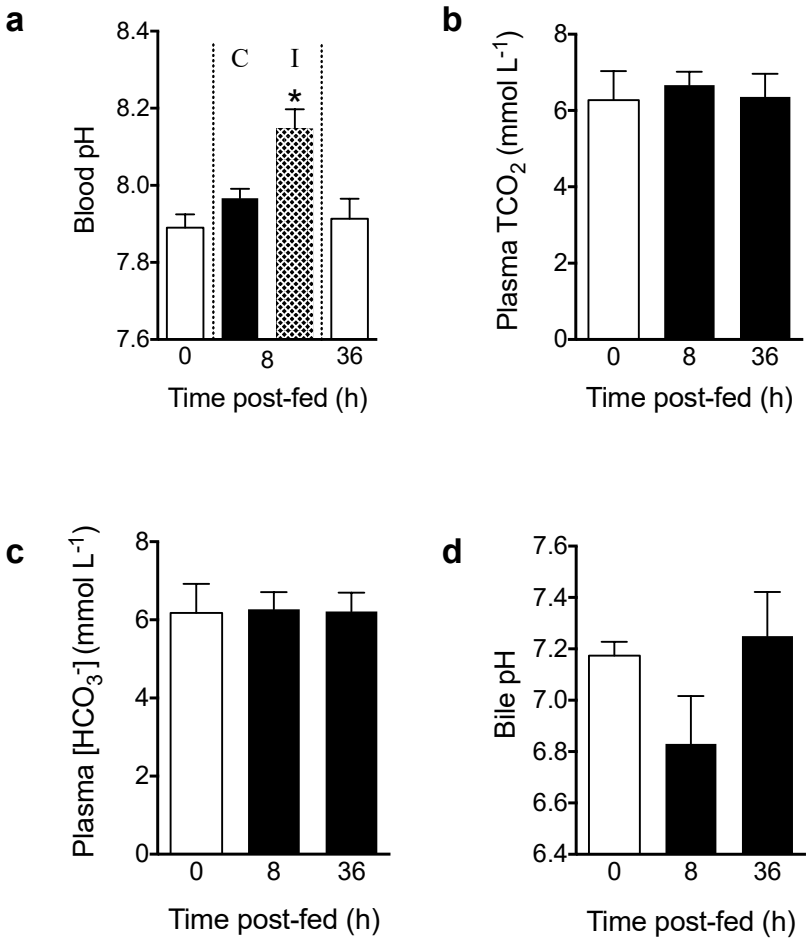

Figure 2.3 Feeding does not affect whole animal plasma acid/base status, but causes an alkalization of the *vena supraintestinalis.* Hagfish were fasted (white bars, time = '0') for one week or fed hake (*Merluccius productus*) *ad libitum*. Caudal blood (C) samples and bile samples were collected 8 h and 36 h post-feeding (black bars) or from the *vena supraintestinalis* (I; dotted bar) 8 h post-feeding. Blood pH (**A**), plasma total CO_2 (T_{CO_2}) concentration (**B**), plasma bicarbonate concentration [HCO_3^-] (**C**), and bile pH (**D**) were determined at each time point. Data are presented as means + s.e.m. (n = 4 – 9). *Asterisk* (*) denotes a statistical difference from control ($p < 0.05$; one-way ANOVA).

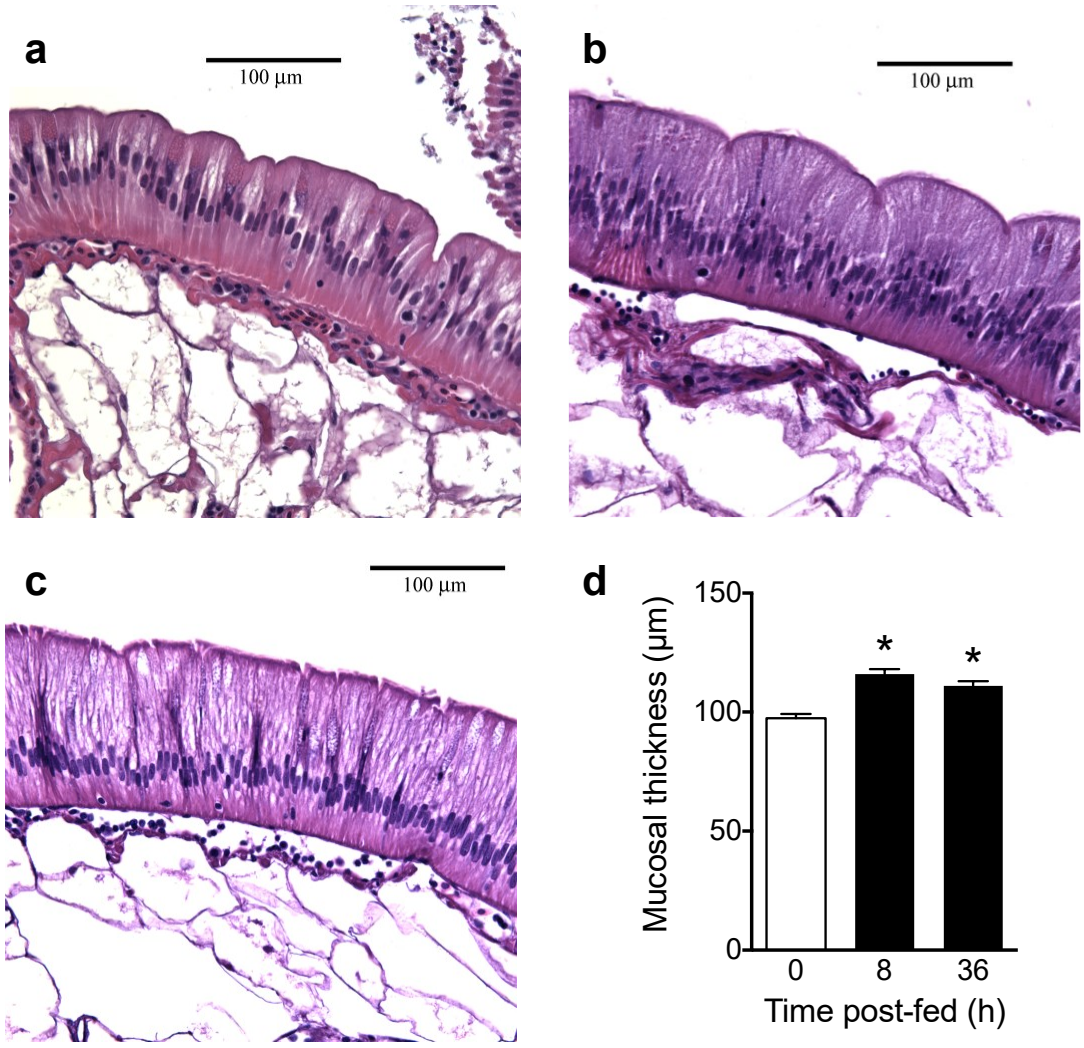

Figure 2.4 Feeding causes a significant increase in mucosal thickness that persists until at least 36 h post-feeding. Light micrographs depict the mucosal thickness of the hindgut in one-week fasted (**a**), or 8 h (**b**) or 36 h (**c**) post-fed hagfish. The average mucosal thickness was calculated from three fish in each treatment group (**d**). Data are presented as means + s.e.m. (n = 3). Asterisk (*) denotes statistical difference from control ($p < 0.05$; one-way ANOVA).

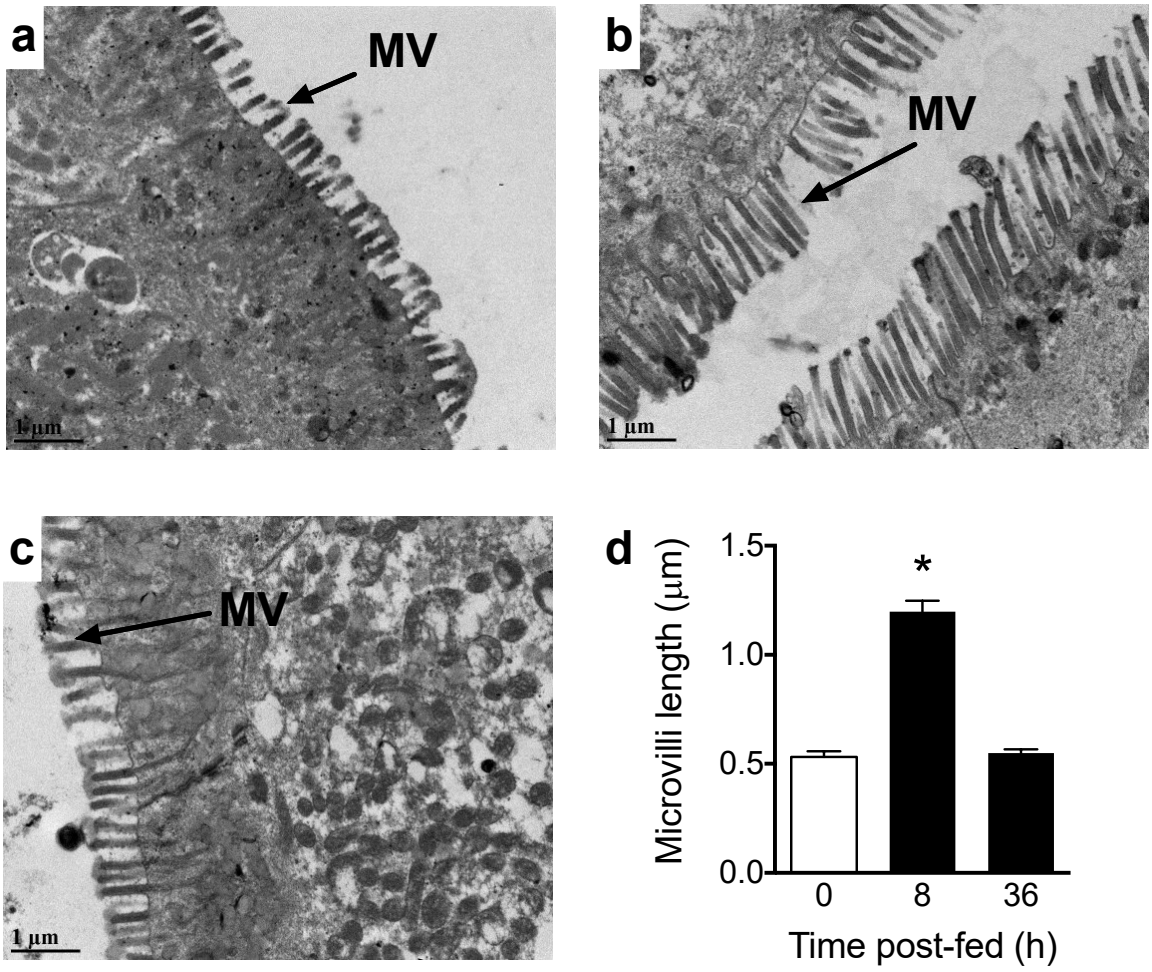

Figure 2.5 Feeding causes a significant and transient increase in microvilli length. Transmission electron micrographs depict the microvilli (MV) length in one-week fasted (**a**), or 8 h (**b**) or 36 h (**c**) post-fed hagfish. The average MV length was calculated from three fish in each treatment group (**d**). Data are presented as means + s.e.m. ($n = 3$). Asterisk (*) denotes statistical difference from control ($p < 0.05$; one-way ANOVA).

Discussion

Post-prandial M_{O_2} and N-excretion

We have identified evidence for intestinal remodeling that coincides with the peak of SDA 8 h post-feeding. While we did not observe evidence for an alkaline tide in the total circulation, a localized alkalization did occur in the *vena supraintestinalis*, the major portal vein leading from the intestinal circulation, thus further study of the potential for acid excretion into the intestinal lumen following feeding is required (see Chapter IV). The mean factorial scope of peak postprandial M_{O_2} of *E. stoutii* is 2.92 and falls within the typical 2-3-fold increase found in other marine organisms (Robertson et al. 2002). Indeed, despite differing diets of fish, squid and beef heart respectively, the Swellshark (*Cephalosyllium ventriosum*), Small-spotted catshark (*Scyliorhinus canicula*) and West African lungfish *(Protopterus annectens*) all fall within this range with mean factorial scope values of 2.30 (Ferry-Graham and Gibb 2009), 2.99 (Sims and Davies 1994) and 2.30 (Smith 1935, p. 193), respectively. This pattern continues throughout the teleost lineage; for example, the Atlantic cod (*Gadus morhua*) and largemouth bass (*Micropterus salmoides*) have factorial scope values of 2.25 (Jordan and Steffensen 2007) and 2.76 (Glass 1968) respectively when fed fish diets. Thus, hagfish appear to conform to the majority of fishes, which is not surprising given both their phylogenetic relationship and shared aquatic habitat with fishes. This peak M_{O_2} value was obtained within 8 h of feeding after which M_{O_2} values returned to control/fasting values. There is a great deal of variability in time required to reach peak M_{O_2} following prandial events across both invertebrates and vertebrates, which is altered depending on the meal composition and

size (for extensive review see: McCue 2006; Secor 2009). For instance, within the teleosts, time to peak M_{O_2} can span from 3 h in cichlids fed a formulated pellet diet (Somanath et al. 2000), to 48 h in sculpin fed a shrimp/squid diet (Johnston and Battram 1993). The elevated M_{O_2} of *E. stoutii* SDA returned to control values within 4 h after the observed peak (12 h post-feeding) and parallels that recorded for the Swellshark (12 h), which also is thought to feed intermittently and like hagfish, reportedly remains sedentary throughout the day with active bouts through the night (Ferry-Graham and Gibb 2009). As carnivores, *E. stoutii* will likely not experience great variation in meal protein composition and the amount of food consumed, as well as the degree of toughness (i.e. soft flesh or tougher muscle) will be the largest contributors to variation in SDA profile. The conclusions drawn in this study in regards to hagfish SDA are limited, given that we were unable to quantify the amount of food eaten by each hagfish. Attempts to measure the hagfish weight pre- and post-meal were made; however, given the vigorous swimming at the onset of anaesthesia the gut contents were always evacuated and dispersed through the water. Upon dissection, a liquid paste was observed along the intestinal lining of fed hagfish but the low intestinal volumes and the propensity for hagfish to become incontinent during anesthesia made collection of this liquid impossible to any repeatable degree.

As expected, the ammonia excretion rates significantly increased following feeding with the final reading obtained at 36 h demonstrating a near 16-fold increase from fasting rates. Some variation observed in this study likely stems from the differing proportions of protein ingestion between animals. The basal AQ (rates of ammonia

excreted to oxygen consumed) was 0.008. At peak M_{O_2}, the AQ increased to 0.073 and was the greatest 16 h post-feeding (0.251). The maximal post-fed AQ in hagfish (0.251) is similar to Sockeye salmon (0.240; Kutty 1978), as would be expected due to the bolus protein load that requires deamination during digestion. Of note, we did not quantify a nitrogen quotient (encompassing all nitrogenous wastes) because previous reports demonstrate that the detoxification of ammonia to urea does not occur in hagfish (Clifford et al. 2015).

A complete profile of post-prandial nitrogenous waste handling has recently been published (Wilkie et al. 2017) and demonstrates a higher J_{Amm} (674 μmol kg^{-1} h^{-1}) for the first hour following feeding before declining to rates reported in this study. We suggest these discrepancies may exist owing to different presentation of food as Wilkie et al. (2017) presented hake in baited traps which were confined spaces and likely had high, localized environmental ammonia in addition to metabolically derived ammonia.

Acid-base regulation

The alkaline tide has been documented in a limited number of fish and elasmobranch species (Wood et al. 2005a, 2007; Bucking and Wood 2008; Cooper and Wilson 2008), and does not appear to be present in other examined marine teleosts such as the European flounder (Taylor et al. 2007) and Gulf toadfish (Taylor and Grosell 2009). In marine teleosts, the intestine plays a major role in acid-base balance and osmoregulation *via* HCO_3^- excretion; a mandatory component of osmoregulation (Taylor and Grosell 2006). Hagfish however, are osmoconformers (Robertson 1954; Bellamy and

Jones 1961; Currie and Edwards 2010) and thus any acid-base patterns observed here would be purely digestive, rather than osmoregulatory in nature.

We did not observe an alkaline tide, as there were no significant increases in base excretion rates to the water, nor any changes in plasma [HCO_3^-], T_{CO_2} or pH. However, a significant alkalization of the blood drawn from the intestinal portal vein (8 h post-fed) was observed and does suggest that localized acid-base changes occurred in the intestinal tissue. The acid-base changes relating to the alkaline tide in most vertebrates occur because of gastric K^+-stimulated H^+ATPases (HKA) in conjunction with Cl^-/HCO_3^- exchangers to produce a localized luminal acidity (Niv and Fraser 2002). While at least one HKA has been identified in an elasmobranch (Smolka et al. 1994), the identification of these transporters in hagfish hindgut have yet to be confirmed (see Chapter IV). Mammalian enterocytes have been shown to create pH microdomains, likely through Na^+/H^+ exchanger (NHE) working in conjunction with H^+-linked solute co-transporters or even Na^+-linked co-transporters (Thwaites et al. 1999; Turner and Black 2001). The futile cycling of H^+ at the apical membrane creates a local acidification, which permits the maintenance of pH_i as well as the driving gradient for H^+-linked solute transport (Thwaites et al. 1999). One hypothesis is that protons are used to drive these processes in the hagfish intestine, thereby explaining the local alkalization of the *vena supraintestinalis*. We did attempt to measure the luminal pH throughout this experiment, however the volumes collected were too low to be measured in a repeatable fashion. Clearly, an in-depth examination of transporters known to be involved in the alkaline tide phenomenon of other vertebrates, paired with measurements of luminal pH/H^+ excretion

capacity are necessary to provide further evidence to support or reject the presence of both an acidic component to digestion and/or, an alkaline tide in *E. stoutii.*

The degree to which acid-base homeostasis is altered varies between taxa, with some of the most extreme alkalizations occurring in opportunistically feeding reptiles which display a 2.5 fold increase in plasma [HCO_3^-] (Coulson et al. 1950; Secor and Diamond 1995; Wang et al. 2001). We were unable to detect a significant alkalization (or coincident alteration to T_{CO_2} and [HCO_3^-]) in the blood drawn from the caudal sinus. This could be due to either pooling of both arterial and venous blood in the caudal sinus which minimizes turnover and maximizes mixing, the large blood volume in hagfish (*c.a*. 14% body weight; Forster et al. 2001), or the result of the relatively high buffering capacity of hagfish blood (Tresguerres et al. 2007a; Parks et al. 2007; Clifford et al. 2014; Baker et al. 2015). Additionally, the bile pH was slightly acidic (6.83; Fig. 2.3c) 8 h after feeding and it is possible that some of the base produced (as observed here in the *v. supraintestinalis*) is used to neutralize this acidic chyme, as has been suggested to occur in alligators (*A. mississipiensis*; Coulson and Hernandez 1983). Each of these would contribute to reducing the ability to detect pH changes in the caudal sample. Similar to our observed results, there was variation among individuals of *A. mississipiensis*, with some individuals appearing to be alkalotic and others acidotic during periods of fasting (Coulson and Hernandez 1983). An alternative explanation for the lack of blood pH elevation is that hagfish are able to more rapidly excrete HCO_3^- at the gills/skin than the rate at which HCO_3^- accumulates within the bloodstream, as hagfish possess a high capacity for HCO_3^- excretion (Clifford et al. 2014). A similar phenomenon has been observed in the Pacific dogfish (Wood et al. 2005a) where post-prandial VHA

translocation occurs in the gill and provides a mechanism for enhanced post-prandial HCO_3^-excretion *via* carbonic anhydrase (CA)-mediated mechanisms (Tresguerres et al. 2007b). Hagfish are able to use gill VHA and alter expression during 24 h of repeated bicarbonate infusions (Tresguerres et al. 2007a) suggesting a capacity for up-regulation in the face of post-prandial HCO_3^- elevation. However, given that there were no associated net base secretions (Figure 2.2c) to the water post-prandially, this mechanism does not appear to occur in *E. stoutii* following feeding.

Intestinal Morphology

In the present study, we demonstrate significant and rapid morphological alteration of the hagfish hindgut epithelia following feeding. Increases in both mucosal thickness and microvilli length were observed and provide a beneficial increase in absorptive surface area. The energy required to induce this change must be substantially less than that which is required for long-term maintenance of a greater surface area/absorptive potential (Secor et al. 2012), lending merit to this method of proliferation and regression following feeding in organisms that may go months between meals. It has been found that snakes feeding once every 2 weeks utilize moderate regulation of the gut, whereas those that feed once every 4 weeks or longer undergo more drastic changes (Secor et al. 2012). Furthermore, the metabolic activity of the stomach and intestine increases following a feeding event (Secor et al. 2012), which demonstrates site-specific post-prandial regulation and decreased fasting metabolism.

Hagfish have been documented to persist up to 11 months without feeding (Foster and Moon 1986) and thus, the wide changes in hindgut morphology are consistent with

snakes feeding every 4 weeks or more. In fact, the fasted state of intestinal morphology may be further regressed over extended time periods (11 months). Additionally, the regression of this metabolically active organ may contribute to the low resting M_{O_2} of hagfish, the lowest recorded of the vertebrates (Munz and Morris 1965; Forster 1990; Clifford et al. 2016), and aid in their ability to withstand extended periods of food deprivation. This parallels data recorded for snakes where resting metabolic rates are ~ 50% lower in infrequently feeding species when compared to frequently feeding species (Ott and Secor, 2007).

Mucosal thickness is often further characterized by the changes present in morphology of the enterocytes themselves. Generally, fasted epithelia are tightly packed, pseudostratified columnar cells that transition into a more stratified layer post-feeding (Lignot et al. 2005; Day et al. 2014). Given the relatively thin columnar cells of *E. stoutii*, along with ill-defined cellular borders, we did not measure metrics associated with enterocytes (width, height, volume). However, the enterocytes still appear pseudostratifed 8 h post-feeding, and may be classified as in a transition phase by 36 h.

The ~58% decrease in hindgut microvilli length in fasted compared to fed *E. stoutii* is similar to values reported in certain aestivating anurans such as the African bullfrog (*P. adspersus;* 40% decrease) or the eastern spadefoot toad (*S. holbrooki*; 45% decrease; Secor and Lignot 2010). Given that the increase in length drastically increases the surface area upon feeding, the potential capacity for nutrient transport is likely similarly increased. Future studies should be undertaken in *E. stoutii* to determine

whether nutrient transport capacity is altered post-feeding (see Chapters VI and VII). Alterations in nutrient transport capacity have been measured in a number of teleost species and these variations are both species- and nutrient-dependent. For instance, Day et al. (2014) found no changes in post-feeding transport capacity for L-leucine, L-proline or D-glucose in grass carp (*Ctenopharyngodon idella*), but an increased uptake capacity for all nutrients tested in the channel catfish (*Ictalurus punctatus*). Given that these fish do not undergo extensive periods of fasting like *E. stoutii*, we hypothesize that uptake mechanisms will be upregulated particularly for glucose (as carbohydrates are a preferred metabolite; Sidell et al. 1984; Foster and Moon 1986), as well as lipids (which appear to provide the long-term energy over fasting; Emdin 1982) that are essential to acquire in fasted hagfish. Furthermore, because *E. stoutii* are able to acquire amino acids (glycine and L-alanine) *via* multiple epithelia (intestine, gill, skin; Glover et al. 2011a,b), it may be plausible that these mechanisms of transport may be unchanged (given the multiple pathways) or indeed, upregulated at each of the tissues.

Conclusions

We demonstrate a robust SDA in Pacific hagfish and while there is no evidence for an alkaline tide, the significant alkalization of the *v. supraintestinalis* suggests that further research is required to definitively characterize changes in localized acid-base status. Additionally, we report extensive remodeling of the hindgut, in terms of both mucosal thickening and lengthening of microvilli, which significantly increase the surface area for absorption in this intermittent feeder. This study illuminates many additional questions relating to hagfish post-prandial physiology and future studies will elucidate the nature of evolution of digestive processes in this early diverging craniate.

Chapter III: Activity and post-prandial regulation of digestive enzyme activity along the Pacific hagfish (*Eptatretus stoutii*) alimentary canal

A version of this chapter has been submitted for publication to PLOSone.

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Introduction

Digestion is essential for the catabolism and hydrolysis of ingested macronutrients into smaller molecules suitable for transport. It is carried out using mechanical, chemical and enzymatic methods with digestive enzymes released from multiple locations along the alimentary canal. There are a multitude of digestive enzymes for each type of macronutrient, with specifications for substrate and optimal reaction conditions (*e.g.* acidic *vs*. alkaline), which correspond to their location in the digestive tract and can be derived from the stomach, exocrine pancreas, or the intestinal mucosa itself (reviewed in Bakke et al. 2010). The capacity for an organism to digest certain foods predominantly depends upon the presence of appropriate enzymes (Smith 1980). The complement of digestive enzymes found in bony fishes are consistent with what is found in other vertebrates (Hidalgo et al. 1999), however few reports exist focusing on hagfish.

The hagfishes are useful models for studies of evolutionary comparison as they are the oldest extant representatives of the first vertebrates (Bardack 1998). They have a wide range of prey items consisting of both living and dead invertebrate and vertebrate species (Martini 1998; Zintzen et al. 2011) and the consumption of decaying organisms provides the hagfish with a vital ecological role in the nutrient cycling and substrate turnover of the marine benthos (Knapp et al. 2011). The hagfish alimentary canal has been morphologically characterized with various microscopy techniques in both the Atlantic (*Myxine glutinosa*) and Pacific (*Eptatretus stoutii*) species (Adam 1963; Weinrauch et al. 2015). With a structure unique to chordates, the mucus cells are contained solely within the foregut whereas the hindgut consists of a monolayered

epithelium of both absorptive columnar cells and zymogen granule cells containing digestive enzymes (Adam 1963). Parallels have been drawn between the pancreatic tissue and the dispersed zymogen granule cells in the hagfish hindgut (Andrew and Hickman 1974) and enzymatic activity in the hindgut has been examined, albeit solely on the Atlantic species, *M. glutinosa*. Adam (1963) histochemically demonstrated the presence of amylase, lipase, and leucinaminopeptidase in all regions of the hindgut and postulated that feeding could induce changes in enzyme activity. In addition, Nilsson and Fänge (1970) characterized the activities of trypsin, chymotrypsin, carboxypeptidase A, leucineaminopeptidase and a catheptic-type protease. The question yet remains as to whether Pacific hagfish: (1) possess a full complement of digestive enzymes, (2) have differential enzyme distribution along the alimentary canal, and (3) modulate enzyme activity post-feeding. To address these queries we investigated the enzymatic activity for each class of macronutrient. α-amylase and maltase activity were assessed to determine the potential for carbohydrate digestion, as polysaccharides such as glycogen are obtained from the liver tissue of prey. α-amylase hydrolyses large polysaccharides, into smaller molecules of maltose and glucose, whereas maltase yields glucose from maltose and is an excellent metric to assess complex carbohydrate digestion (Caviedes-Vidal et al. 2000). The potential for lipid digestion was quantified by measure of lipase activity as it converts dietary lipids, such as fats and triglycerides, into transportable monoglycerides and fatty acids (Svendsen 2000). Finally, protein digestion capacity was measured by trypsin, aminopeptidase and alkaline phosphatase activity. Trypsin is a digestive protease produced in the pancreas, while aminopeptidase is derived from the small intestine and cleaves individual amino acids from proteins (Taylor 1993; Krogdahl and Sundby 1999).

Alkaline phosphatase operates at an alkaline pH and has numerous physiological roles including mediation of inflammation, nutrient absorption, and maintenance of intestinal pH (Lallès 2014). We hypothesized that the varied diet of hagfish, from the glycogen- and lipid-rich liver tissue to proteinaceous muscle tissue (Martini 1998), would necessitate a full complement of digestive enzymes that would have elevated activity in the hindgut compared to the anterior alimentary tract (buccal cavity (B) and pharyngocutaneous duct (PCD)) owing to the presence of zymogen granule cells. Additionally, we expected reduced rates of tissue enzyme activity following feeding, as the digestive contents of the zymogen granule cells would have been released at the onset of feeding.

Materials and Methods

Animal care and husbandry

Twenty-four Pacific hagfish (*Eptatretus stoutii*; 65.3 ± 3.5 g; mean ± standard error of the mean (s.e.m)) were collected using baited traps in Trevor Channel, Bamfield, B.C., Canada and immediately transferred to ~5000 L tanks holding tanks with continuously flowing seawater at Bamfield Marine Sciences Station. The hagfish were then shipped to the University of Alberta and housed in a recirculating artificial salt water system (Instant Ocean SeaSalt; Spectrum Brands, Blacksburg, VA, USA) at 12 ± 2°C. Fasted animals were starved for at least one month prior to experimentation, whereas fed animals were fed squid to satiation 2 h prior to experimentation. All experiments were conducted with the approval of the University of Alberta Animal Care (No. AUP0001126 (2017)) and under DFO collection permit No. XR-136-2017.

Tissue preparation

Hagfish were euthanized by an overdose of MS-222 (5 g L^{-1}; Syndel Laboratories Ldt., Nanaimo, B.C., Canada) in artificial seawater. The animals were dissected along the mid-ventral line and the digestive tract was excised and gently flushed with 0.5 M NaCl to clear contents. The digestive tract was then divided into 5 equal portions as follows: buccal cavity (B), pharyngocutaneous duct (PCD), anterior hindgut (Ant HG), mid hindgut (Mid HG), and posterior hindgut (Post HG); see Fig. 3.1). The tissues were immediately placed in homogenization buffer (50 mM imidazole, 2 mM EDTA; pH 7), homogenized on ice (Polytron PT 1200 E; Kinematica AG, Lucerne, Switzerland) and stored in aliquots at -80°C until biochemical analysis. Of note, luminal contents were also

collected and analysed. However, the amount of activity detected was orders of magnitude below that detected in the tissue. For this reason, no further analysis of luminal content activity was conducted or reported herein.

Enzymatic assays

All assays of digestive enzymatic activity were carried out as previously described and listed below. The tissue activity of each enzyme was measured in each portion of the digestive tract and compared to a substrate only blank to account for endogenous product in that solution. Samples were read on a microplate spectrophotometer (Spectromax 190, Molecular Devices, Sunnyvale, CA, USA) using clear, flat-bottom 96-well microplates. Unless otherwise noted, all chemical compounds, reagents and enzymes were supplied by Millipore-Sigma (St. Louis, MO, USA).

α-amylase activity

α-amylase activity was measured according to the Somogyi-Nelson method (Nelson 1944; Somogyi 1952), with modifications (Shao and Lin 2018). Briefly, solution 1 (53.2 mM sodium potassium tartrate, 283 mM sodium carbonate, 238 mM sodium bicarbonate, 1.27 M sodium sulfate) and solution 2 (80 mM copper sulphate pentahydrate, 1.27 M sodium sulfate) were prepared and stored separately in brown glassware. Solution 1 and 2 were mixed (4:1) to create a working reagent immediately prior to analysis. Homogenate (60 µL), working reagent (60 µL) and substrate (60 µL of 1% starch boiled in 0.8 M sodium citrate buffer; pH 7) were combined. Following cooling, Nelson reagent (60 µL of 0.28 M ammonium molybdate, 0.38 M sodium arsenate dibasic, 21 mL concentrated sulphuric acid – stored at 37°C for 24 h in brown

glassware) was added and the reaction proceeded for 15 min before termination with 1 µL of 1 M HCl. Samples were centrifuged (2 min @ 14, 000 *x* g), plated, read at A600 nm and compared to a glucose standard curve. α-amylase activity was expressed as nmol glucose liberated min^{-1} mg $protein^{-1}$.

Maltase activity

Maltase activity was determined as previously (Dahlqvist 1968). The homogenate (50 µL) was combined with substrate (50 µL of 62.5 mM maltose) and incubated for 1 min. The reaction was terminated with 1 µL of 1 M HCl and the samples were centrifuged (2 min @ 14, 000 *x* g). Following an incubation period of 5 min, glucose content was determined by combining supernatant (10 µL) with glucose cocktail (200 µL of 0.22g $MgCl_2$, 0.05g NAD, 0.05g ATP, 2.4µ/mL glucose-6-phosphate dehydrogenase in 50mL triethanolamine hydrochloride; pH 7.5). The difference at A340 nm before and 15 min after the addition of hexokinase (5U/ sample) was recorded and compared to a glucose standard curve. Maltase activity was expressed as nmol glucose liberated min^{-1} mg $protein^{-1}$.

Lipase activity

Lipase activity was measured using modified methods (Iijima et al. 1998). The homogenate (6 µL) was incubated for 15 minutes in 86 µL of solvent (5.2 mM deoxycholic acid in 250 mM tris-HCl; pH 7.5) and 2.5 µL of 10 mM ethanol. The substrate (5.5 µL of 20 mM *p*-nitrophenyl myristate dissolved in ethanol) was added to the homogenate mixture and incubated for 15 min before centrifugation (2 min @ 6100 *x*

g). Samples were read at A405 nm and compared to a *p*-nitrophenol standard curve. Lipase activity is expressed as µmol *p*-nitrophenol liberated min^{-1} mg $protein^{-1}$.

Trypsin activity

Trypsin activity was measured as previously (Preiser et al. 1975) wherein the homogenate (100 µL) was activated by enterokinase (4 U mL^{-1} in 40 mM succinate buffer; pH 5.6) for 15 min. The substrate (350 µL of 2mM Na-benzoyl-l-arginine-*p*-nitroanilide hydrochloride (BAPNA) dissolved in DMSO (0.1%) in 100 mM tris-HCl; pH 8) was added to the activated homogenate (50 µL) and incubated for 1 h at 15˚C. The reaction was terminated with 100 µL of 30% acetic acid and the samples centrifuged (2 min @ 14,000 *x* g) and read at A550 nm against a *p*-nitroaniline standard curve. Activity was expressed as nmol *p*-nitroaniline liberated min^{-1} mg $protein^{-1}$.

Aminopeptidase activity

Aminopeptidase activity was measured as previously (Roncari and Zuber 1970). Homogenate (30 µL) was combined with substrate (80 µL of 2.04 mM l-alanine-*p*-nitroanilide HCl in 200 mM sodium phosphate buffer; pH 7) and incubated for 15 min. Following centrifugation (30 s @ 14,000 *x* g) the samples were plated, read at A410 nm, and compared to a *p*-nitroaniline standard curve. Final activity was expressed as nmol *p*-nitroaniline produced min^{-1} mg $protein^{-1}$.

Alkaline phosphatase activity

Measurement of alkaline phosphatase activity was conducted as previously (Walter and Schütt 1974). Homogenate (25 µL) was combined with substrate (55 µL of 20 mM *p*-nitrophenyl phosphate dissolved in 100 mM ammonium bicarbonate buffer

containing 1 mM $MgCl_2$; pH 7.8) and incubated for 15 min. Following centrifugation (30 s @ 14,000 *x* g), the samples were plated, read at A405 nm, and compared to a *p*-nitrophenol standard curve. Aminopeptidase activity was expressed as µmol *p*-nitrophenol liberated min^{-1} mg $protein^{-1}$.

Protein assays

All protein assays were conducted using commercial kits (bicinchoninic assay (BCA) or Bradford's reagent for amylase assays) according to the manufacturer's instructions (Thermo Fisher Scientific; Waltham, MA, USA).

Statistical analysis

Datasets were first analysed using a Kruskal-Wallis 1-way analysis of variance (ANOVA) on ranks to discern if differences occurred between the anterior digestive tract (B and PCD) and the posterior digestive tract (anterior, mid and posterior hindgut). If a significant difference was found between these sections, the datasets was analysed independently using a 2-way ANOVA in each section (effect of feeding and effect of location). The sole exception was for lipase activity. Lipase activities in the anterior section were not of equal variation and so an ANOVA could not be utilized. In this case, we analyzed the B and PCD separately using the Mann-Whitney Rank Sum Test. If no difference was detected between the anterior and posterior sections, a 2-way ANOVA was run on the entire tract length. Significance was accepted at $\alpha = 0.05$ for all tests. In the instances where significant differences were detected, all pairwise multiple comparisons were made using a Bonferroni t-test post hoc analysis. All statistical analyses were conducted in SigmaPlot ver. 14 (Systat software Inc, integration, San Jose,

CA, USA). Datasets were graphed using Prism 6 (GraphPad Software INC., La Jolla, CA, USA).

Results

For each of α-amylase, lipase, trypsin, and alkaline phosphatase, the anterior and posterior sections differed substantially (see Table 3.1; $H_1 = 49.4$, $P < 0.001$; $H_1 = 9.36$, $P < 0.002$; $H_1 = 58.4$, $P < 0.001$; $H_1 = 68.4$, $P < 0.001$). Therefore, the effect of feeding and location was examined independently in each section for these enzymes *via* a 2-way ANOVA or Mann-Whitney Rank Sum Test (see methods). Notably, the anterior region (B and PCD) was not analyzed for α-amylase, trypsin, or aminopeptidase owing to the activities being below detectable limits. All other enzymes were analyzed using a 2-way ANOVA taking all sections into consideration.

α-amylase activity was either minimal (fed fish) or below detectable limits (fasted fish) in the anterior portion of the digestive tract (B and PCD); however, the activity was significantly higher in all tested regions of the hindgut in both feeding states. Feeding significantly lowered the hindgut activity of α-amylase (Fig. 3.2 & Table 3.2; 37.4%, $F_{1,40} = 56.038$, $P < 0.001$). No significant differences were observed in maltase activity, either in regard to location or feeding status (Fig. 3.3 & Table 3.2; feeding: 71.7%, $F_{1,62} = 2.826$ $P = 0.099$; location: $F_{4,62} = 0.926$ $P = 0.456$).

Lipase activity was detected at all points along the alimentary canal and was significantly elevated in the anterior segments in comparison to the posterior segments (mid- and post-HG; Fig. 3.4 & Table 3.2; $H_1 = 4.11$, $P = 0.043$). There was no effect of either feeding or location in the posterior location (feeding: 73.1%, $F_{1,50} = 1.172$, $P = 0.285$; location: $F_{2,50} = 0.642$, $P = 0.531$). Differences between feeding state were found for both the B and PCD ($P < 0.001$).

Trypsin activity was not detected in the anterior alimentary canal in either feeding state (Fig. 3.5). The hindgut had detectable activity although there were no significant effects of feeding status or location within the hindgut (Table 3.2; feeding: 57.1%, $F_{1,34}$ = 2.19, P = 0.149; location: $F_{2,34}$ = 0.675, P = 0.517). Aminopeptidase activity was most prominently expressed in the PCD however, no effects of feeding were observed at any location (Fig. 3.6 & Table 3.2; H_4 = 13.2, P = 0.010). Finally, alkaline phosphatase activity was not discernable in the anterior regions of the tract (B & PCD) however, significant decreases in hindgut enzyme activity occurred following a feeding event (Fig. 3.7 & Table 3.2; 17.5%, $F_{1,45}$ = 6.643, P = 0.014).

Figures

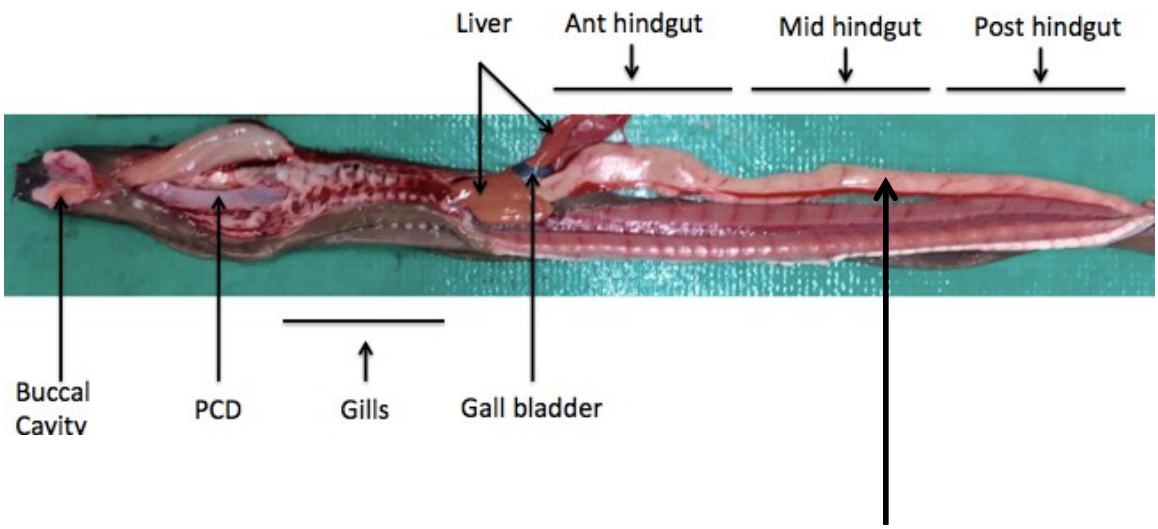

Fig. 3.1 Regions of interest along the hagfish alimentary canal. Following euthanasia, hagfish were dissected along the dorsal midline and the inner organs were exposed. The buccal cavity and pharyngocutaneous duct (PCD) were each collected and considered the foregut, while the hindgut was divided into three equal segments: anterior (Ant), Mid, and posterior (Post) hindgut.

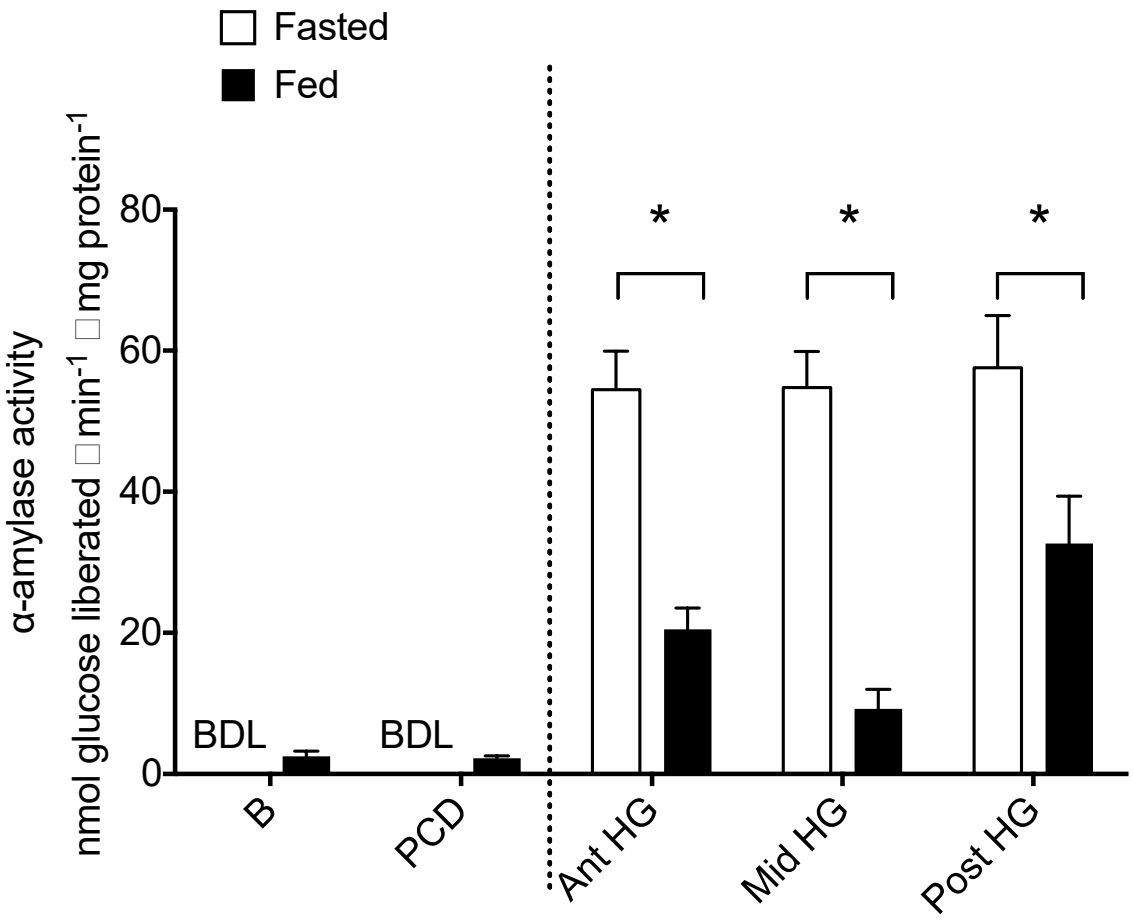

Figure 3.2 Changes in α- amylase activity along the length of the Pacific hagfish alimentary canal in fed and starved hagfish. Activity (nmol glucose liberated min^{-1} mg $protein^{-1}$) was measured in both fasted (white bars) and fed (black bars) hagfish in five locations along the alimentary canal (B – buccal cavity, PCD – pharyngocutaneous duct, Ant HG – anterior hindgut, Mid-HG – mid-hindgut, Post-HG – posterior hindgut). Bars represent means + s.e.m. of 5-8 hagfish. A Kruskal-Wallis determined that there were significant differences between the anterior and posterior segments of the tract, thus a 2-way ANOVA to determine effect of feeding and location was conducted in the hindgut only (right of the dotted line). Asterisks (*) denote significant differences with significance accepted at $\alpha = 0.05$.

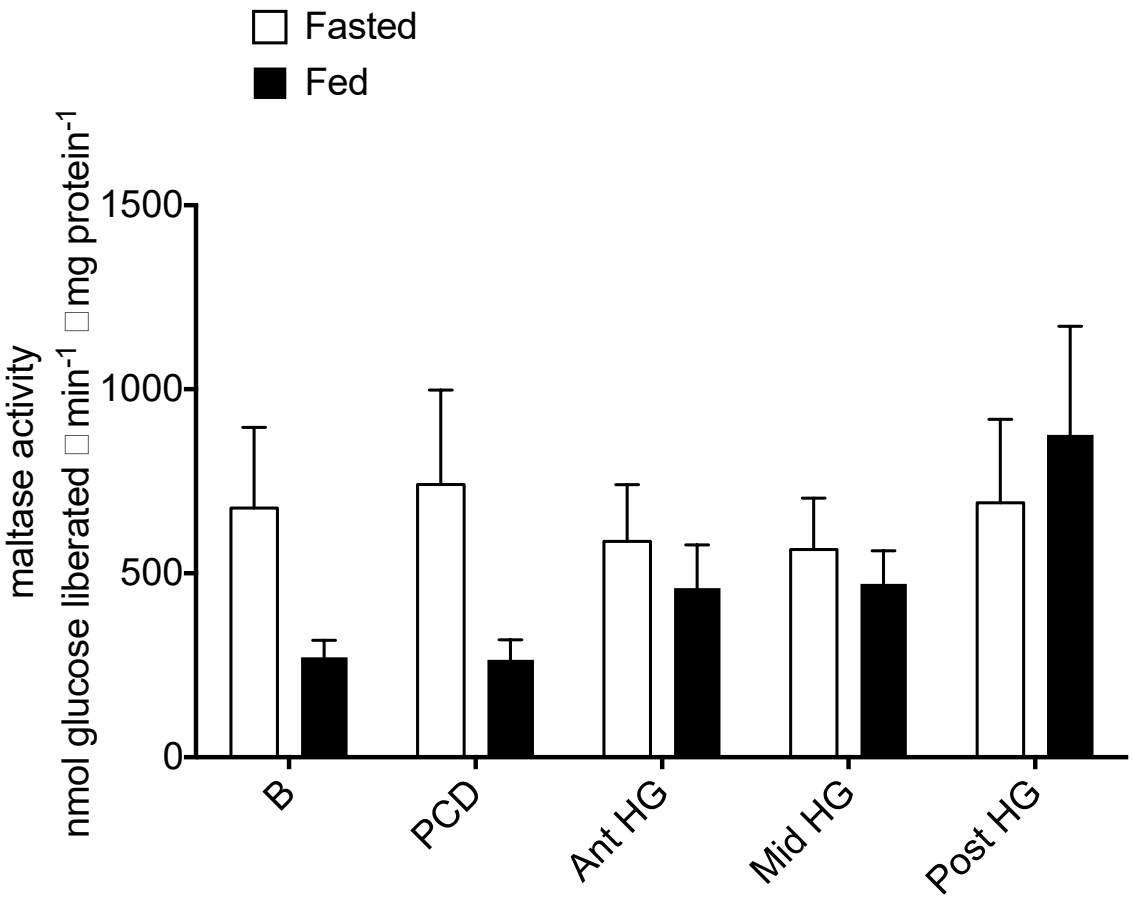

Figure 3.3 Maltase activity does not change with feeding or location in the Pacific hagfish alimentary canal. Activity (nmol glucose liberated min^{-1} mg $protein^{-1}$) was measured in both fasted (white bars) and fed (black bars) hagfish in five locations down the alimentary canal (B – buccal cavity, PCD – pharyngocutaneous duct, Ant HG – anterior hindgut, Mid-HG – mid-hindgut, Post-HG – posterior hindgut). Bars represent means + s.e.m. of 4-7 preparations. A 2-way ANOVA of the entire tract determined there were no significant effects of feeding or location ($\alpha = 0.05$).

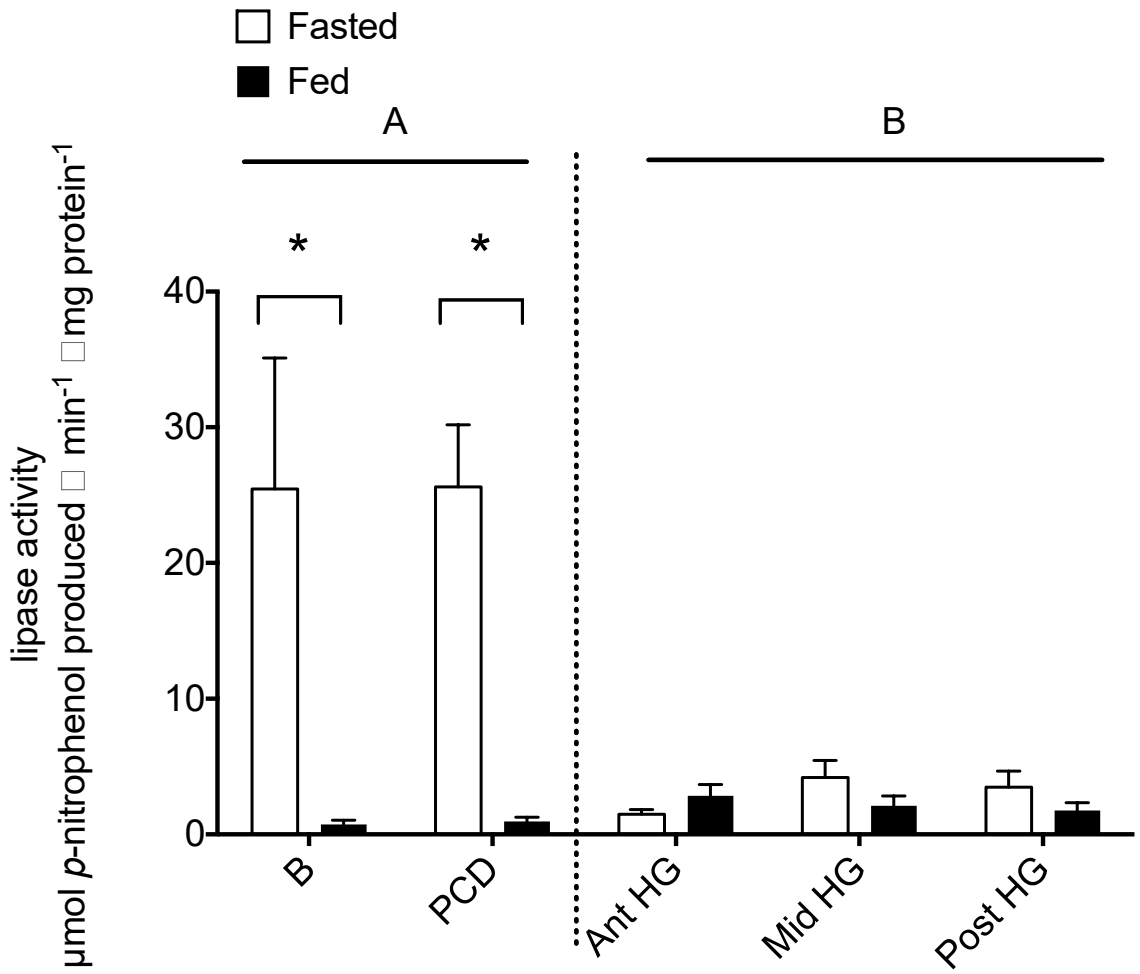

Figure 3.4 Lipase activity is dependent upon location within the alimentary canal and significantly decreases post-feeding in the anterior segment. Activity (μmol *p*-nitrophenol min^{-1} mg $protein^{-1}$) was measured in both fasted (white bars) and fed (black bars) hagfish in five locations down the alimentary canal (B – buccal cavity, PCD – pharyngocutaneous duct, Ant HG – anterior hindgut, Mid-HG – mid-hindgut, Post-HG – posterior hindgut). Bars represent means + s.e.m. of 5-11 hagfish. Letters denote significant differences between locations as determined by a Kruskal-Wallis ($\alpha = 0.05$), whereas * denote a difference in feeding state as determined using a Mann Whitney Rank Sum Test ($\alpha = 0.05$).

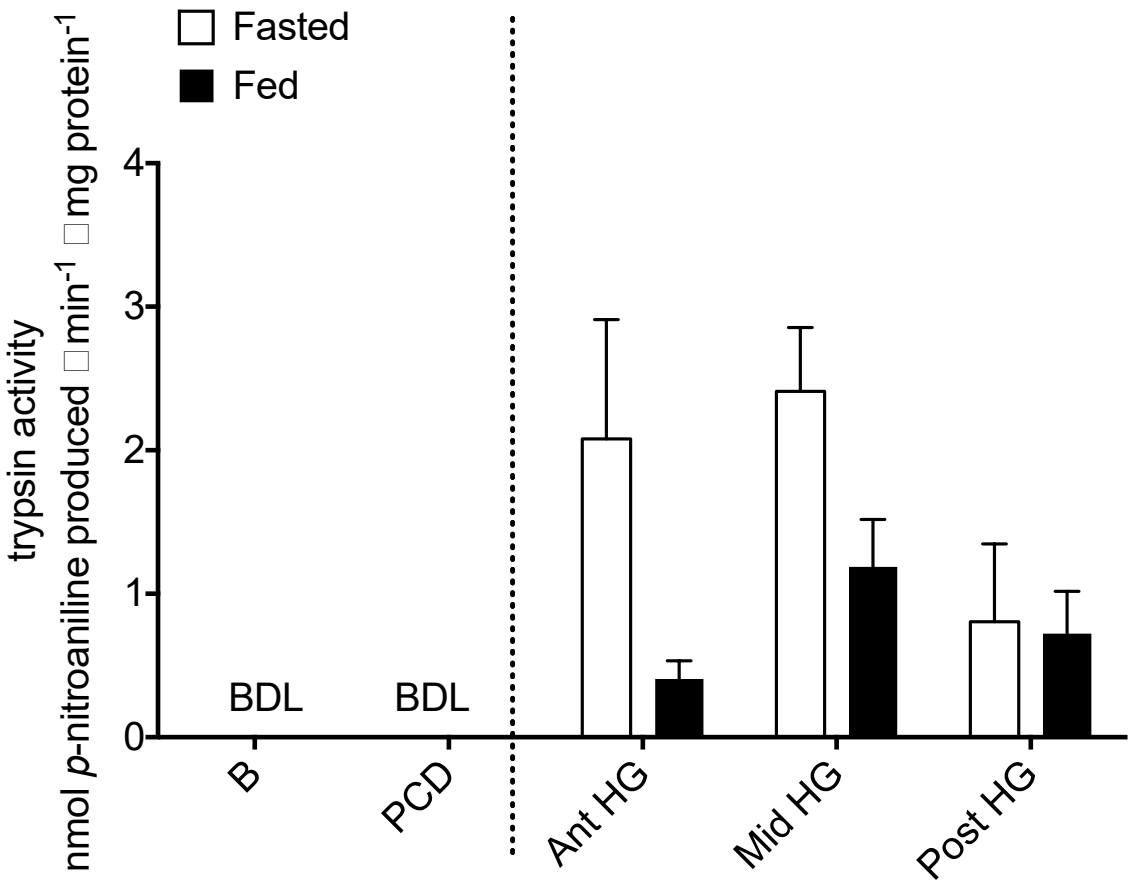

Figure 3.5 Trypsin activity along the entirety of the Pacific hagfish hindgut does not change with feeding status. Activity (nmol p-nitroaniline produced min^{-1} mg $protein^{-1}$) was measured in both fasted (white bars) and fed (black bars) hagfish in five locations down the alimentary canal (B – buccal cavity, PCD – pharyngocutaneous duct, Ant HG – anterior hindgut, Mid-HG – mid-hindgut, Post-HG – posterior hindgut). Bars represent means + s.e.m. of 3-8 hagfish. BDL = below the detectable limits of the assay. A Kruskal-Wallis determined that there were significant differences between the anterior and posterior segments of the tract, thus a 2-way ANOVA to determine effect of feeding and location was conducted in the hindgut only (right of the dotted line). Significance was accepted at $\alpha = 0.05$.

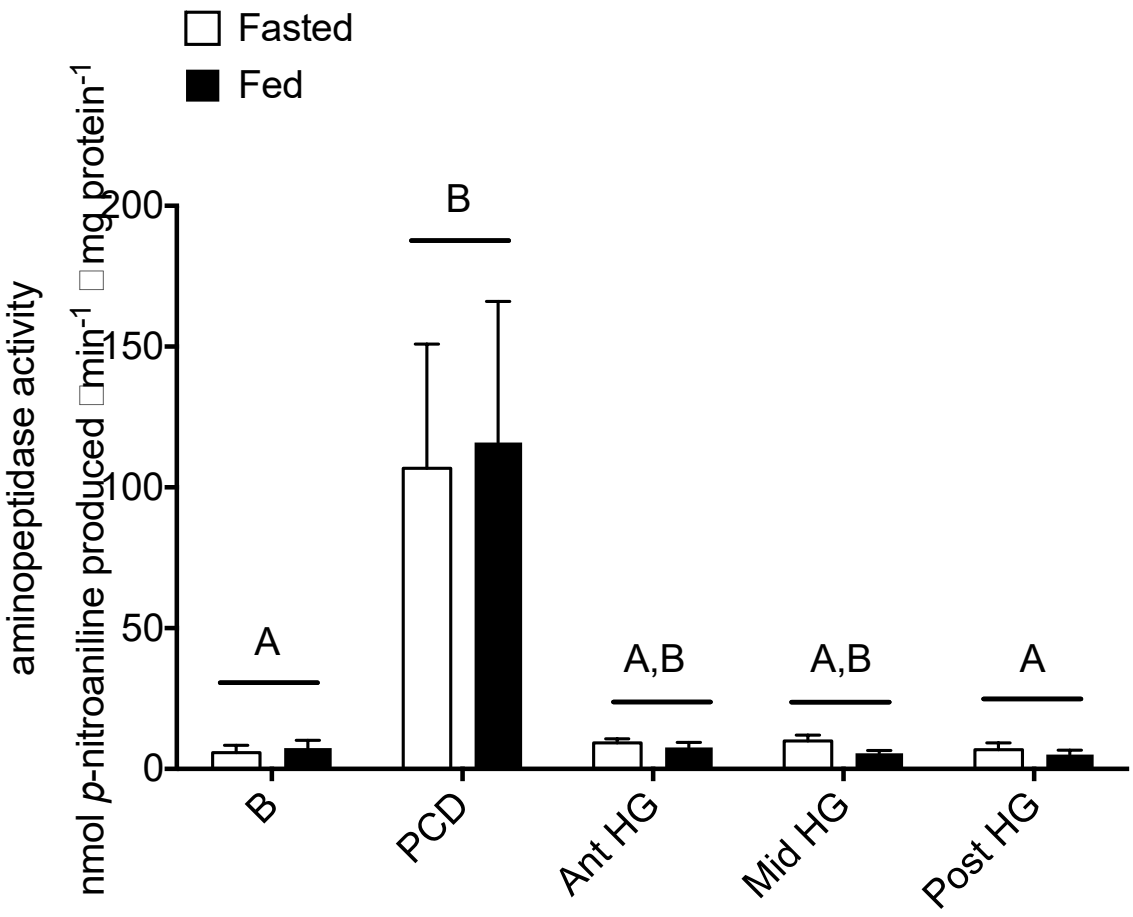

Figure 3.6 The activity of aminopeptidase varies with location along the Pacific hagfish alimentary canal. Activity (nmol *p*-nitroaniline produced min^{-1} mg $protein^{-1}$) was measured in both fasted (white bars) and fed (black bars) hagfish in five locations down the alimentary canal (B – buccal cavity, PCD – pharyngocutaneous duct, Ant HG – anterior hindgut, Mid-HG – mid-hindgut, Post-HG – posterior hindgut). Bars represent means + s.e.m. of 5-12 hagfish. A Kruskal-Wallis determined that there were significant differences in activity between different locations, with differences indicated by different letters. Significance was accepted at $\alpha = 0.05$.

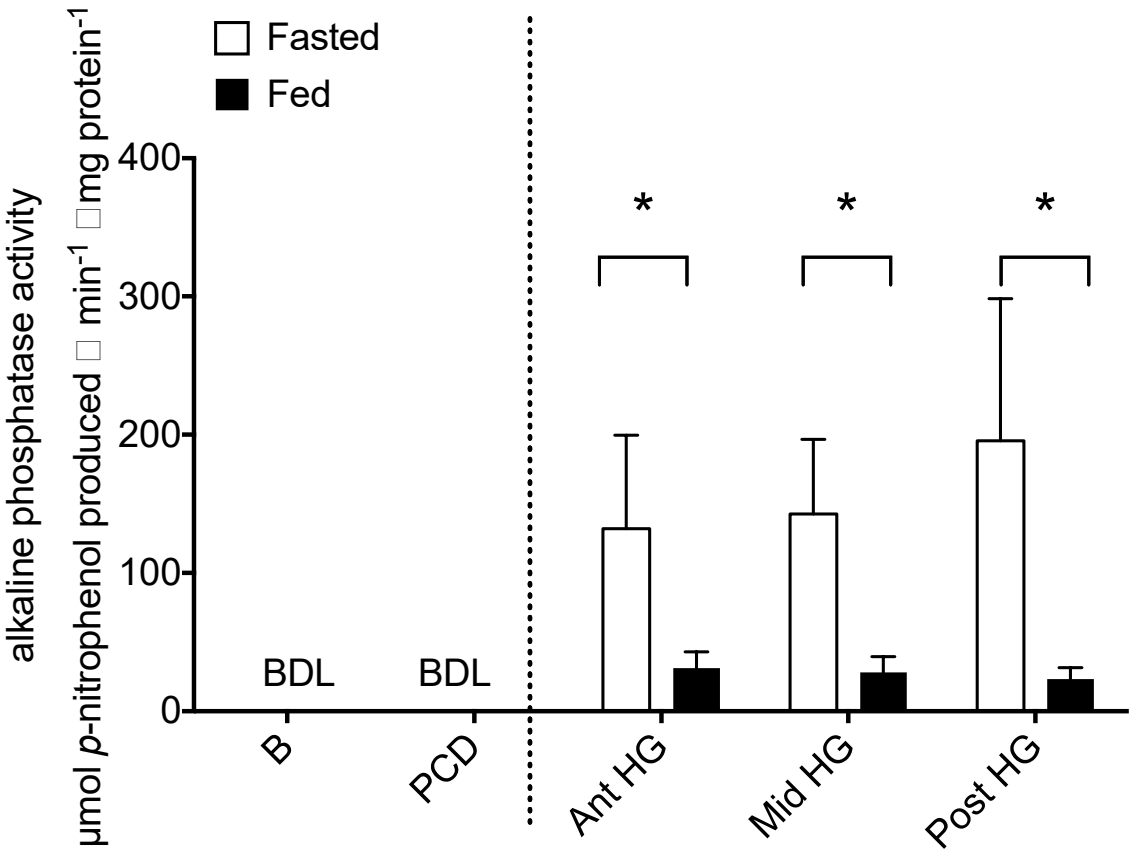

Figure 3.7 Feeding alters alkaline phosphatase activity within the entire hagfish hindgut. Activity (μmol *p*-nitrophenol produced min^{-1} mg $protein^{-1}$) was measured in both fasted (white bars) and fed (black bars) hagfish in five locations down the alimentary canal (B – buccal cavity, PCD – pharyngocutaneous duct, Ant HG – anterior hindgut, Mid-HG – mid-hindgut, Post-HG – posterior hindgut). Bars represent means + s.e.m. of 6-12 preparations. No activity was detected in the anterior portion of the alimentary canal (BDL = below detectable limits). Therefore, a 2-way ANOVA was conducted for the hindgut regions alone (right of the dotted line) with significance accepted at $\alpha = 0.05$. Asterisks (*) denote significant differences between feeding states.

Tables

Table 3.1 Summary of statistics for Kruskal-Wallis comparisons between the anterior and posterior segments of the hagfish alimentary canal

Enzyme	Statistical Summary
Amylase	$H_1 = 49.4$ $P < 0.001$
Maltase	$H_1 = 3.109$ $P = 0.078$
Lipase	$H_1 = 4.11$ $P = 0.043$
Trypsin	$H_1 = 58.407$ $P < 0.001$
Aminopeptidase	$H_1 = 1.398$ $P = 0.237$
Alk. Phosphatase	$H_1 = 68.411$ $P < 0.001$

Table 3.2 Summary of statistics for 2-way comparisons along the length of the hagfish alimentary canal and with differing feeding states

Enzyme	Location	Feeding	Interaction
Amylase	$F_{2,40} = 2.621$ $P = 0.087$	$F_{1,40} = 56.0$ $P < 0.001$	$F_{2,40} = 1.568$ $P = 0.222$
Maltase	$F_{4,62} = 0.926$ $P = 0.099$	$F_{1,62} = 2.826$ $P = 0.099$	$F_{4,62} = 1.092$ $P = 0.370$
Trypsin	$F_{2,34} = 0.675$ $P = 0.517$	$F_{1,34} = 2.190$ $P = 0.149$	$F_{2,34} = 2.314$ $P = 0.116$
Lipase – post.	$F_{2,50} = 0.642$ $P = 0.531$	$F_{1,50} = 1.172$ $P = 0.285$	$F_{2,50} = 2.202$ $P = 0.122$
Aminopeptidase	$F_{4,79} = 10.791$ $P < 0.001$	$F_{1,79} = 0.0036$ $P = 0.953$	$F_{4,79} = 0.0379$ $P = 0.997$
Alk. Phosphatase	$F_{2,45} = 0.108$ $P = 0.898$	$F_{2,45} = 6.643$ $P = 0.014$	$F_{2,45} = 0.171$ $P = 0.843$

Discussion

Overall, *E. stoutii* have digestive enzymes that catabolize each of the major macronutrient classes (carbohydrates, fats, proteins). Much like stomachless teleosts, the lack of a stomach does not appear to impact digestive flexibility or capacity (Horn et al. 2006; Day et al. 2011). Each enzyme has a unique distribution along the alimentary canal and, for the most part, it seems that the majority of digestive activity takes place within the hindgut as previously suggested (Adam 1963); however, some digestive activity (maltase, lipase and aminopeptidase) was noted in the anterior regions (B and PCD). The typical vertebrate observation of decreasing enzyme activity in the posterior-most segments (Cox and Secor 2008; German et al. 2010) was not evident along the hindgut of hagfish for any studied enzyme. Furthermore, there were differential effects of feeding for certain enzymes. The differential distribution and retention of some enzymatic function post-feeding, supports the previous hypothesis that hagfish must maximize digestive function in their relatively straight and simple alimentary canal (Glover and Bucking 2015). Owing to the difficulties in making quantitative comparisons with other species because of differences in methodology, reported units of activity, ontogenic stages, and the amount and composition of the diet (German et al. 2004), the digestive enzymes in this study were not directly compared to other calculated activities.

Digestive enzyme activity

It has long been known that carbohydrates are the preferred metabolic fuel of the hagfish (Emdin 1982; Sidell et al. 1984). Hagfish preferentially feed upon the glycogen-rich liver when presented with a whole carcass in captivity (Weinrauch et al. 2017). The

capability to digest polysaccharides has been investigated previously with α-amylase histochemically detected (Adam 1963) and maltase activity expressed (Weinrauch et al. 2018a; Chapter V) uniformly across the hindgut. This study has confirmed that α-amylase is essentially restricted to the hindgut regions of the hagfish digestive tract. It is likely contained within the digestive zymogen granule cells, as our results demonstrate a significant decrease in activity post-feeding (Fig. 3.2), which corresponds to the release of granules at the commencement of digestion, much like that of vertebrate salivary glands. Indeed, the zymogen granule cells have been likened to both salivary and pancreatic glands of vertebrates (Adam 1963; Andrew and Hickman 1974). Given that α-amylase is a primary protein that is stored in a bound zymogen granule prior to export from vertebrate salivary glands and pancreatic secretions (Cope and Williams 1973; Uys and Hecht 1987), it is not surprising to find α-amylase activity in the hindgut containing a similar structure. Interestingly, α-amylase activity has also previously been detected around the slime glands and in the skin (Adam 1963). It is known that hagfish, akin to some marine invertebrates, acquire nutrients such as amino acids, inorganic phosphate, and other trace metals across the skin (Glover et al. 2011a, 2015, 2016; Schultz et al. 2014). Perhaps additional nutrients, such as glucose, can likewise be acquired cutaneously. An alternative suggestion is that the slime possesses digestive activity thereby providing a means of external digestion while the prey is encased in a slime cocoon (Weinrauch et al. 2018a). Each of these hypotheses require further examination. Finally, previous characterization of hagfish α-amylase suggests a relatively elevated optimum pH of 8-9 (Adam 1963), despite typical optima in the range of pH 6-8 (Nagase 1964). This is puzzling as there are indications that hagfish acidify the hindgut lumen

upon feeding (Nilsson and Fänge 1970) and the zymogen granule cells react positively to acidophilic stains (Adam 1963; Weinrauch et al. 2015). However, α-amylase activity is known to differ depending upon food item (Aiso and Tamura 1998). Therefore, since the previously recorded optimal pH of hagfish α-amylase was determined *in vitro*, it is possible that an *in vivo* study may yield different results, particularly if in fact the lumen does become relatively acidic (Chapter IV).

The recorded maltase activity was comparable to previously calculated rates in fasted *E. stoutii* (~ 200-1000 nmol min^{-1} mg $protein^{-1}$; Weinrauch et al. 2018a; Chapter V). Similarly, the rate of activity was unchanging along the length of the alimentary canal, regardless of the nutritional state of the hagfish (Fig. 3.3). This is comparable to the unchanging rates of amylase activity that reflect the natural diets of some teleosts (Buddington and Diamond 1987; German et al. 2004). The immutable rates of maltase activity would ensure a continual ability to digest this favoured macronutrient of hagfish (Sidell et al. 1984) and maximize uptake along the entire tract (Weinrauch et al. 2018a) when polysaccharides become available. Similar reports exist for some fish species and are suggested to be effective means to maximize nutrient assimilation along a relatively short tract with a rapid transit rate (Day et al. 2011). The differential expression and regulation of these polysaccharide-digesting enzymes is in accordance with their distinct substrate requirements. The zymogen release of α-amylase will cleave the larger polysaccharides into short and linear branched chains, with the final and pivotal transformation into the transported glucose conducted by maltase. Vertebrate maltase is known to be a membrane-bound enzyme (Sim et al. 2008), and considering the continual expression in this study, membrane-bound maltase is a possibility for hagfish as well.

Future studies should utilize imaging techniques (such as immunocytochemistry) to determine the specific localization of each of these enzymes. For this study, I mined both the published *E. burgerii* genome (GenBank assembly accession: GCA_900186335.2) and an Illumina transcriptome of *E. stoutii* to discern whether there was sequence conservation with commercially available antibodies for each of these digestive enzymes. Unfortunately, the degree of conservation was not satisfactory (*i.e.* < 40% conservation). The development of hagfish-specific antibodies would be a useful endeavour to confirm our hypotheses of digestive enzyme location.

Lipids are an invaluable energetic source for hagfish, particularly during extended periods of starvation, which can persist at least 11 months in captivity (Emdin 1982; Foster and Moon 1986). Recent evidence has suggested that hagfish have a regulated and specific mechanism by which lipids can be acquired in the hindgut (Weinrauch et al. 2018b; Chapter VI) and lipases are essential for the digestion and assimilation of dietary lipids. There were significantly elevated lipase activities in the anterior tract (B and PCD) in comparison to all regions of the hindgut (Fig. 3.4). Prominent lipase expression has been observed in the anterior digestive tracts of a number of teleost species (Al-Hussaini and Kholy 1953; Nagase 1964). We saw a significant reduction in anterior lipase activity following a feeding event, suggestive of enzyme release. Lipase activity can be influenced by a multitude of factors including dietary components (Bogevik et al. 2009; German et al. 2010) as well as the endogenous source of the lipase. For example, some lipases require bile salt for activation (Lie et al. 1987). Hagfish have a large gall bladder yet any biliary secretions would be missed in this study, as lipase was not detected in the luminal contents of fed fish, wherein the gall bladder secretions would have existed.

Furthermore, the variability in lipase substrate specificity (Svendsen 2000), likely means there are additional lipases with activities that were not recorded with our single substrate assay.

Protease activity has been previously demonstrated in the Atlantic hagfish (*M. glutinosa*; Nilsson and Fänge 1970) and a broad spectrum of proteases is unsurprising given the carnivorous feeding habits of hagfish. While a different suite of proteolytic enzymes was examined, we too observed an unchanging activity along the length of the hindgut (Fig. 3.5-3.7). Trypsin activity was only detected in the hindgut regions and did not appear to differ with nutritional status (Fig. 3.5). Trypsin is restricted to the duodenum of vertebrates (Louvard et al. 1973), but the lack of differentiation along the hagfish hindgut may once again result from the absence of compartmentalization of function in favour of maximizing nutrient uptake. Of note, trypsin is generally stored in the inactive form of trypsinogen before activation by enterokinase in vertebrates (Ogiwara and Takahashi 2007). In this study, we utilized a recombinant enterokinase despite uncertainty of whether: a) hagfish have this hormone, b) the heterologous hormone would be effective, or c) if hagfish have an inactive trypsinogen form. Irrespective of the outcomes of these queries, we were able to effectively detect trypsin activity in the hagfish.

The activity of aminopeptidase was significantly elevated in the PCD region (Fig. 3.6). Aminopeptidase is a primary brush border enzyme that is anchored in the plasma membrane of the vertebrate small intestine (Huërou-Luron 2002), which perhaps accounts for the unchanging activities found with feeding state. Furthermore, aminopeptidases are also involved in numerous functions including the initiation of a

peptide anti-inflammatory response. Peptidases involved in such a response have been localized to the mammalian nasal passage (Ohkubo et al. 1998), which is similar morphologically to the PCD in hagfish. Moreover, the other examined proteases were restricted to the hindgut region perhaps indicating that initial digestion could occur *via* aminopeptidase in the anterior tract.

Finally, we investigated was alkaline phosphatase, which was also restricted to the hindgut, and may be utilized to demarcate between functional units of the intestine (Wang et al. 2017b). Feeding resulted in a significant reduction of tissue alkaline phosphatase activity (Fig. 3.7). This enzyme plays many roles in the intestine including pH regulation, fat acquisition, anti-inflammatory responses, as well as potential regulation of the gut microbiome (reviewed in Lallès 2014). The digestive contribution of alkaline phosphatase is not firmly established (Buddington and Doroshov 1986). However since we observe a significant decrease in activity post-feeding, it is likely that our measurements relate to feeding in some way. Whether this is for digestion of the incoming meal or perhaps a more indirect role, such as gut mucosal defence (Fawley and Gourlay 2016), remains uncertain. Such mucosal defences may be of particular import for hagfish when they feast upon dead and decaying matter.

Given that the pH optima for these proteases fall in the alkaline range, we must again consider the post-prandial acidification of the hagfish lumen (Nilsson and Fänge 1970; Weinrauch et al. 2017). We characterized each enzyme using a single pH value and the possibility exists that higher activities could result if the pH was altered. Nilsson and Fänge (1970) demonstrated a biphasic response of protease activity to changing pH. A strong proteolytic activity was observed at each of pH 4 and pH 9. If the animals do have

a luminal acidification, does it persist along the entire length of the digestive tract? Is there a transition from acidic to alkaline in a time-dependent manner? These, among other questions, should be investigated in order to have a holistic understanding of hagfish post-prandial physiology.

Environmental influences on digestive enzyme activity

The current viewpoint of digestive enzyme physiology suggests that activity correlates well with feeding ecology (Fernández et al. 2001). The suite of enzymes reflects the opportunistic feeding habits of hagfish and their ability to utilize a wide range of nutrients efficiently. Since most digestive enzymes can accept multiple substrates, the relative contributions of each type of enzyme (carbohydrase, lipase, protease) cannot be conclusively determined from this study. However, maltase appears to be of significant importance to hagfish as its rate of activity was an order of magnitude above the other examined enzymes. Throughout this study we utilized a single food source (squid). It is very likely that enzyme activity will vary with diet however we predict that the trends would remain constant with our observations. For example, those enzymes with decreasing activity post-feeding are likely derived from the zymogen granule cells and thus, should continue to demonstrate reduced post-prandial activity irrespective of diet composition. The mechanism by which zymogen granules are released remains unknown. Despite vagal innervation, electrical stimulation did not yield any changes to gastric fluid production (Nilsson and Fänge 1970), and it is possible that mechanical/stretch stimulus or a hormonal cue is responsible for granule release. This remains to be studied.

Diet composition can impact enzyme affinity and regulation, but can also induce changes to the gut microfauna. Bacteria are often associated with an organism's digestive system and contribute to the success of nearly all studied animals. Digestive activity increases in regions of the digestive tract where the microbes are most densely populated (Skea et al. 2007; German and Bittong 2009) and contribute to the overall digestion within an animal. Yet, the microflora itself is often a relatively unconsidered source of enzymatic activity. The gut microbiome of the hagfish has yet to be studied and will only serve to increase our knowledge of these scavengers. As mentioned above, a mucosal defense strategy within the hagfish digestive tract is likely important owing to their feeding behaviours. The alkaline phosphatase we detected along the hindgut may simultaneously inactivate bacterial pathogens, while recruiting commensal bacteria (Fawley and Gourlay 2016). The hagfish gut microbiome constituents must either tolerate periodicity of feeding events or there will be a general turnover of the community depending on duration of fast or dietary composition.

Differences in digestive enzyme activity have also been attributed to circadian rhythms. For instance, in a sea cucumber species, rates of α-amylase and pepsin activity were elevated during times when this species is most active (Sun et al. 2015). We conducted our trials at the same time of day to ensure we would avoid differences induced by such rhythms. Hagfish are nocturnal and it is possible that our results underestimate maximal enzyme activity. However, we hypothesize that those enzymes that exist as zymogen granules will not change with time, as it is a stimulus-induced release rather than a membrane-bound protein with the possibility for up-regulation or altered affinity. We believe this is supported by the fact that hagfish can persist for many

months without feeding and it would be a futile effort to continually alter enzyme activity and/or production. *Eptatretus burgerii* have a demonstrable seasonal migration (Ichikawa et al. 2000) and may therefore, display a more regulated rhythmicity of digestive enzyme activity.

Conclusions

This experiment has quantified an array of digestive enzyme activities in the Pacific hagfish, comparable to their varied diet and metabolic requirements. Contrary to previous reports, digestive activity is observed along the entire length of the digestive tract however, the majority of enzymes function within the hindgut region of the alimentary canal. The variable expression of these enzymes along the tract may be the first indications of compartmentalization of gut function. Although there is an obvious difference between the anterior and posterior tract in terms of cellular morphology, this is the first time that a physiological function other than lubrication is shown in the anterior portions. Functional differentiation along the hindgut is unlikely as there were no observed differences in activity along the length of the hindgut. As previously hypothesized, this likely permits a maximization of digestive function and nutrient assimilation across a relatively short digestive tract (Glover and Bucking 2015).

Chapter IV: Cellular mechanisms of ion and acid-base transport following feeding in the Pacific hagfish hindgut

Introduction

Hagfish diverged from the vertebrate lineage ~300-500 million years ago (Bardack 1998) and alongside the lamprey, are the sole remaining Agnathans. With a typical feeding lifestyle as both a predator and opportunistic scavenger (Martini 1998; Zintzen 2014), hagfish are useful for comparison across the vertebrate lineage, unlike the specialized lamprey that transition from filter-feeding juveniles to parasitic adults (Rovainen 1996). Contrary to most vertebrates however, hagfish are agastric and previous reports have therefore suggested that digestion is accomplished using digestive enzymes along the straight, uncoiled hindgut (Adam 1963). Nevertheless, a recent report identified a significant post-prandial alkalization of the intestinal venous blood supply (Weinrauch et al. 2017; Chapter II) and digestive fluids have a recorded pH of 5.5-5.8 (Nilsson and Fänge 1970). Thus, although a whole-animal increase in base equivalent concentration (alkaline tide phenomenon) was not observed, there are several possibilities presented by the authors for mitigation of significant acid-base changes. First, the pooling of blood in the hagfish circulatory system may mitigate any measurable differences in acid-base homeostasis, as could the high blood buffering capacity of hagfish (Weinrauch et al. 2017; Chapter II). Alternatively, hagfish may be able to rapidly excrete HCO_3^- across the gills as is observed in Chondrichthyan species (Wood et al. 2005a; Tresguerres et al. 2007b). Thus, it is possible still that hagfish may utilize an acidic digestion, albeit likely *via* alternative mechanisms to those described in other vertebrates since the intestine apparently lacks CA (Nilsson and Fänge 1970; Esbaugh et al. 2009). If acid-forming mechanisms do indeed persist in hagfish, the cells responsible are likely akin to the oxynticopeptic cells found in elasmobranchs (Rebolledo and Vial 1979), which

secrete both digestive enzymes and acid (rather than the more specialized parietal cells of mammals), owing to the homogenous morphology of the hagfish hindgut (Adam 1963; Weinrauch et al. 2015).

Gastric acid secretion is a well-studied component of vertebrate digestion. The acid is derived from the hydration of metabolic CO_2, a reaction that is catalyzed by carbonic anhydrase (CA) and results in the production of both protons (H^+) and bicarbonate (HCO_3^-). The H^+ are secreted across the apical surface of the mammalian parietal cells primarily *via* the gastric H^+/K^+ATPase (HKA) and the cellular pH_i is maintained by extrusion of HCO_3^- across the basolateral surface into the blood supply (the alkaline tide phenomenon; reviewed in Niv and Fraser 2002). Efficient efflux of intracellular HCO_3^- is necessary to maintain whole-animal acid/base homeostasis. Prior to its excretion, the HCO_3^- may first initiate an adenylyl cyclase signal transduction cascade and elicit post-prandial changes in ion transporter abundance and/or activity, as described herein.

Adenylyl cyclases are one of the foremost effectors of signal transduction pathways (Tresguerres et al. 2011). Within this classification are the transmembrane associated adenylyl cyclases (tmAC) and the cytoplasmic soluble adenylyl cyclase (sAC), however only sAC is sensitive to HCO_3^-. The bicarbonate anion allosterically modifies sAC, resulting in an increased reaction rate (V_{max}), yet unchanged affinity (K_m; Tresguerres et al. 2014; Levin and Buck 2015). Thus, sAC has been termed an acid/base sensor. This is because changes in CO_2 or H^+ can be near-instantaneously translated into

readable HCO_3^- in the presence of CA (Chang et al. 2017). The activation of sAC with increased pH leads to an increase of intracellular cyclic adenosine monophosphate (cAMP), which initiates downstream mechanisms integral to gastric acid secretion, including the translocation of important H^+ transporters such as H^+K^+ATPase (HKA) and vacuolar-type H^+-ATPase (VHA; Malinowska et al. 1988; Vucic et al. 2003; Pastor-Soler et al. 2003; Breton and Brown 2007; Levin and Buck 2015).

While gastric acidification and the accompanying alkaline tide have been well researched in mammalian models for a few decades (Wolosin and Forte 1984; Paradiso et al. 1989; Niv and Fraser 2002), recent work has examined these phenomena in teleost fishes. An alkaline tide has been identified in some species (e.g. Rainbow trout, Bucking and Wood 2008; Cooper and Wilson 2008), yet is absent in others (e.g. Gulf toadfish, Taylor and Grosell 2006; European flounder, Taylor et al. 2007). Interestingly, the agastric killifish displays an alkaline digestion and subsequent 'acidic tide' following a feeding event (Wood et al. 2010). To date, the earliest appearance in vertebrate phylogeny of a paired acidic digestion and alkaline tide appears within the Chondrichthyans (Smolka et al. 1994; Wood et al. 2005b, 2007). Furthermore, some of the cellular mechanisms responsible for gastric acidification in mammals have also been identified in Chondrichthyan species (e.g. HKA; Smolka et al. 1994), thus suggesting some degree of conservation of function. The alkaline tide is subsequently offloaded as base equivalents to the surrounding water (Wood et al. 2007), as opposed to the typical respiratory acidosis observed in air-breathing vertebrates (Wang et al. 2001), or the intestinal secretion of calcium carbonate precipitates in marine teleosts used for

osmoregulation (Taylor et al. 2007). Although this is currently the earliest vertebrate phylogenetic appearance of such processes, the oldest extant representative of the earliest vertebrates, the hagfish, remains unstudied.

With this in mind, the current study sought to identify post-prandial changes in ion and fluid transport to better understand hagfish intestinal function prior to and during digestion. Additionally, we sought to elucidate whether digestion occurs using a luminal acidification of the hindgut. Should this acidification occur, we sought to identify the mechanisms at play both in terms of signal transduction pathways (i.e. sAC and cAMP), and the apical transporters responsible for H^+ secretion. We first examined the cellular mechanisms using the pharmacological stimulant, forskolin. Forskolin is an activator of adenylyl cyclases that leads to increases in cAMP concentration (Alasbahi and Melzig 2012) and is known to promote gastric acid secretion (Hersey and Sachs 1995). We hypothesized that the application of forskolin would stimulate acid secretion in an unfed fish. To further elucidate the role of adenylyl cyclases, we applied the sAC-specific inhibitor KH7 (Tresguerres et al. 2011) following feeding, with the assumption that the post-prandial acidification would be prevented if sAC is involved. Finally, we applied the inhibitors omeprazole or bafilomycin post-prandially to determine whether HKA and VHA are involved in luminal acidification, respectively. In combination with these *in vitro* results, we conducted immunocytochemistry to further confirm the cellular mechanisms involved in hagfish hindgut acidification.

Materials and Methods

Animal care and husbandry

Pacific hagfish (*Eptatretus stoutii*; $N = 49$; 85.9 ± 5.1 g; mean ± s.e.m.) were collected using baited traps in Trevor Channel, Bamfield, B.C., Canada and immediately transferred to ~5000 L tanks holding tanks with continuously flowing seawater at Bamfield Marine Sciences Station. All experiments were conducted with the approval of the Canadian Council of Animal Care under protocols for the University of Alberta Animal Care (No.AUP0001126 (2017)), Bamfield Marine Sciences Animal Care (No.RS17-03) and with a collection permit from the Department of Fisheries and Oceans (No.XR-136-2017). Animals were fasted at least one week prior to experimentation and were fed squid to satiation where indicated. Fed animals were sacrificed 8 h following a feeding bout as previous research indicates this is the time where post-prandial alkalization of the portal vein occurs (Weinrauch et al. 2017; Chapter II).

Chemicals and Reagents

Unless otherwise noted, all chemical compounds, reagents and enzymes were supplied by Millipore-Sigma (St. Louis, MO, USA).

General Experimental Procedure

Fed and fasted hagfish were euthanized with neutralized (NaOH) 4 g L^{-1} MS-222 (Syndel Laboratories, Nanaimo, BC, Canada) and dissected bilaterally on the ventral side. The hindgut was removed in its entirety and blotted dry prior to recording wet

weight. Small sections of the hindgut were dissected away and fixed for immunohistochemistry (see below).

Net Proton Fluxes

Hindguts were tied into sacs as previously described (Glover et al. 2011b). Briefly, hindguts were sutured at either end with a piece of flared PE-50 tubing in one end to permit sample loading and retrieval. The sacs were filled with a low buffering capacity hagfish saline (in mM: NaCl 450, KCl 8, $CaCl_2$ $2H_2O$ 5, $MgSO_4$ $7H_2O$ 4, $MgCl_2$ 11, $NaHCO_3$ 1, glucose 1) and placed in aerating solution of the same composition. Net proton flux (J_{H^+}) into the intestinal lumen was calculated using a double end-point method (Hills 1973; Wilson et al. 2002). Briefly, a sample (4 mL) was collected from the intestinal lumen and the pH was measured. Then, 0.02 N HCl was added to each sample with continuous pH measurement using a thermojacketed pH electrode (Fisher Scientific accumet glass body combination electrode) until a stable reading was obtained (drift < 0.002 pH units 10 s^{-1} for 1 min). NaOH (0.02 N) was then added in a similar fashion until the pH surpassed that of the original sample and stabilized (as above). The titration data (μequivalents H^+ required for change in pH unit) was plotted and the slope of a linear regression analysis was determined to calculate buffer capacity (β; μmol/pH). The net proton secretion (J_{H^+}) was calculated as follows:

$$J_{H^+} = \frac{\Delta pH \times \beta}{SA \times t} \qquad (1)$$

where, Δ pH is the difference between the lowest and highest recorded pH, β is the buffer capacity of the solution (μmol/pH), SA is the surface area of the gut sac (cm^2) and t is the flux time (h).

The *vena supraintestinalis* was cannulated using PE-20 and forskolin (10 μM) was perfused through at a rate of 4 mL h^{-1} for 15 min (Bucking et al. 2011b). The cannula was removed and the sac was then filled with fresh mucosal saline, transferred to a bath of aerating saline, and a 2 h flux was conducted to determine J_H^+ (as above). All subsequent drugs were paired with a 0.1% DMSO control. Following an initial 1 h control flux period with saline containing DMSO, the mucosal saline was refreshed and contained omeprazole (50 μM), KH7 (10 μM) or bafilomycin (10 μM) dissolved in DMSO. A subsequent 1 h flux was conducted and J_H^+ was calculated (as above).

Ion absorption and fluid transport rates

Ion concentrations were determined using atomic absorption spectrophotometry (iCE 3000 Series, AA Spectrophotometer; Thermofisher Scientific, MA, USA) with the appropriate matrix modifier or, in the case of chloride, using a digital chloridometer as per the manufacturer's specifications (Labconco, MO, USA). Ion absorption rates (μmol cm^{-2} h^{-1}) were calculated as follows:

$$Ion\ absorption\ rate\ = \frac{(V_i \times C_i) - (V_f \times C_f)}{SA \times t} \qquad (2)$$

where, V_i and V_f represent the initial and final volumes in the intestinal sac (mL), C_i and C_f represent the initial and final measured concentration of the respective ion (μmol mL^{-1}), SA is the surface area of the intestinal sac (cm^2), and t is the time of the flux (h). Fluid transport rates (FTR; μL cm^{-2} h^{-1}) were calculated as follows:

$$Fluid\ transport\ rate\ (FTR) = \frac{(M_i - M_f)}{SA \times t} \qquad (3)$$

where, M_i and M_f are the final and initial masses of the full intestinal sac (µL) representing the changing volume within the sac over time, *SA* is the surface area of the intestinal sac (cm^2) and *t* is the duration of the flux (h).

Immunocytochemistry

Hindgut segments were fixed in 4% paraformaldehyde, 0.05 mM sodium cacodylate (pH 7.4; Electron Microscopy Sciences, Hatfield, PA, USA) overnight at 4°C and then serially dehydrated in a graded ethanol series. Following paraffin embedding, samples were sectioned in 7 µm sections and placed on Superfrost plus microscope slides (Thermofisher Scientific, Ottawa, ON, Canada). Slides were then deparaffinized by incubating for 10 min in each of the following solutions: SafeClear (x3), 100% ethanol, 95% ethanol, 70% ethanol, and PBS-T (1X PBS and 0.2% Tween 20). Slides were incubated in 95°C citrate unmasking buffer (10 mM citric acid, Tween 20, pH 6) 5 X 3 minutes for antigen retrieval, followed by incubation in sodium borohydrate (1mg/mL in PBS; 5 X 5 min) to reduce autofluorescence. After incubation in blocking buffer (PBS-T, 2% normal goat serum, 0.02% keyhole limpet hemocyanin, pH 7.7) for 1 h, slides were incubated overnight in primary antibody (1:125; NKA, anti-dogfish sAC (Roa and Tresguerres 2016), HKA, or 1:250; VHA). Antibodies were obtained from the Tresguerres lab. Slides were washed 3 times in PBS-T and incubated in the appropriate secondary antibody (1:500 goat-anti rabbit or goat-anti mouse) for 1 h, before a 10 min incubation in Hoescht 33342 nuclear stain (1:1000 in blocking buffer; Invitrogen, Grand Island, NY). Finally, the slides were washed 3 times in PBS-T (5 min) and mounted with FluoroGel (Electron Microscopy Sciences, Hatfield, PA, USA) for visualization on a Zeiss AxioObserver ZA. Zeiss Axiovision software and Adobe Photoshop were used to

adjust contrast and brightness only. Pre-absorption controls showed no visible immunoreactivity for any antibody. Peptide absorption controls (300-fold excess) were conducted for sAC and VHA and did not show any visible immunoreactivity.

Statistical Analysis

Datasets are presented as means ± s.e.m. Normal datasets, as determined *via* a Shapiro-Wilk test, were further analysed using a paired, or un-paired t-test where appropriate, with significance set as $p < 0.05$ (see figure captions). The exception was sodium absorption rate post-feeding, which was not normal and therefore statistical analyses was conducted using a Mann Whitney U test. Analyses and graphical outputs were completed using Prism 6 (GraphPad, La Jolla, CA, USA) or SigmaPlot v 11.0 (Systat Software Inc., San Jose, CA, USA).

Results

Effect of feeding on intestinal acid and ion secretion rates

Rates of fluid transport were significantly elevated following feeding (Fig. 4.1a), which coincided with an increase in the overall weight of the intestine (Fig. 4.1b). Chloride absorption rates were unchanged, however there was a trend towards chloride absorption in a fasted state and chloride secretion in a fed state (Fig. 4.1c). All measured cations (Na^+, K^+, Mg^{2+}), excepting calcium (Ca^{2+}), had increased rates of absorption following feeding (Fig. 4.1d-g). Feeding induced a significant increase in intestinal proton secretion rate (J_{H^+}; Fig. 4.2a).

Effect of pharmacological agents on intestinal acid and ion secretion rates

There was no effect of DMSO on acid secretion rates in either fed or fasted states (Fig. 4.3a). The application of forskolin to fasting fish led to increased proton secretion rates by 87.2% (Fig. 4.3b), while the application of KH7 to fed fish suppressed acid secretion by 57.8% (Fig. 4.3c). The addition of omeprazole following feeding led to a reduction in the amount of proton efflux by 53.2% (Fig. 4.4a), as did the VHA inhibitor, bafilomycin by 58.6% (Fig. 4.4b).

N^+K^+ATPase, H^+K^+ATPase, v-type H^+ATPase, and soluble adenylyl cyclase localization

As expected, Na^+/K^+-A TPase was localized to the bottom third of both the enterocytes and the zymogen granule cells in both fed and fasted fish (Fig. 4.5a,b). sAC appeared to have a fairly ubiquitous expression being found in the enterocyte cytoplasm (primarily basolateral expression), within the zymogen granule cells, as well as showing a

punctate appearance along the brush border membrane in both feeding states that is seemingly enhanced in the apical cytoplasm of fed animals (Fig. 4.6a,b). HKA was localized to the apical brush border in animals of both feeding states, and additionally appeared within the zymogen granule cells (Fig. 4.7a,b). VHA was observed along the apical brush border in both fed and fasted individuals. There was no indication of VHA within the zymogen granule cells (Fig. 4.8a,b). Sections incubated in no primary antibody, as well as those with a peptide absorption control (where available) showed no staining (Fig. 4.9a,b).

Figures

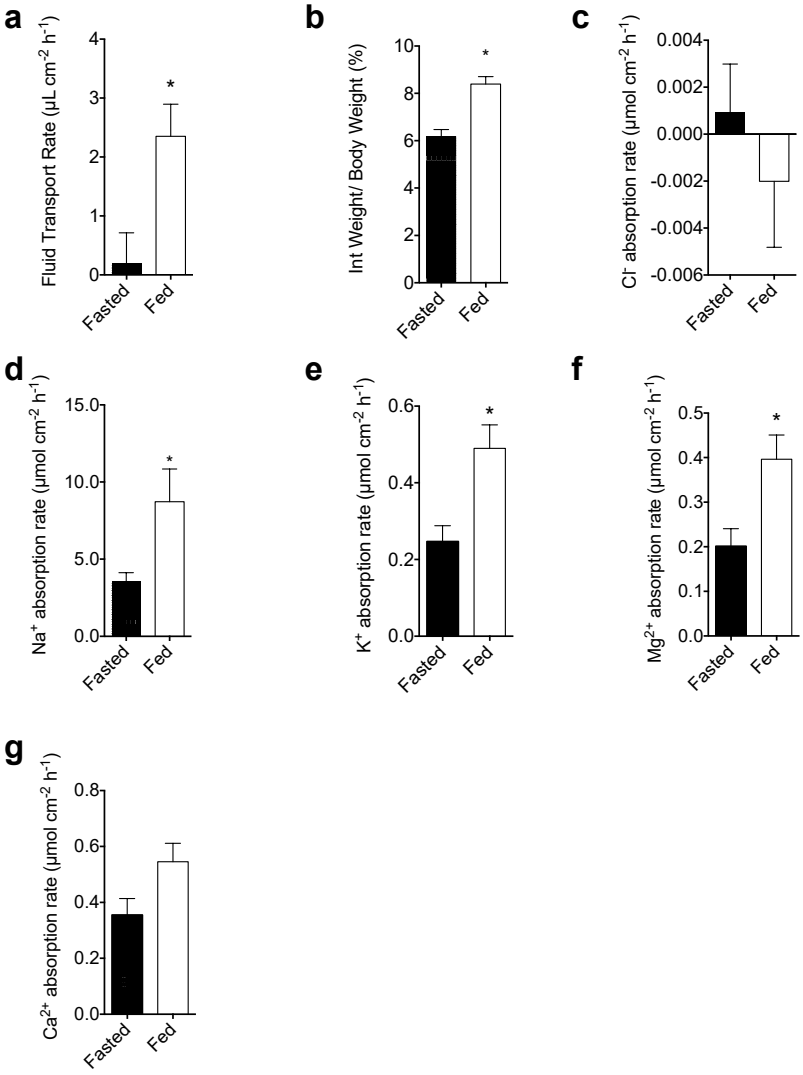

Figure 4.1 Fluid and ion transport rates of the Pacific hagfish hindgut in fed and fasted conditions. The change in intestinal fluid transport rate (**a**), intestinal weight (**b**) and ion absorption rates for chloride (Cl^-; **c**), sodium (Na^+; **d**), potassium (K^+; **e**), magnesium (Mg^{2+}; **f**) and calcium (Ca^{2+}; **g**) in fasted (black bars) and fed (white bars) hagfish. Positive values indicate net ion absorption, whereas negative values indicate net ion secretion. Data is presented as means ± s.e.m. ($N = 6$). An asterisk (*) denotes a significant difference when compared using an unpaired t-test ($p < 0.05$).

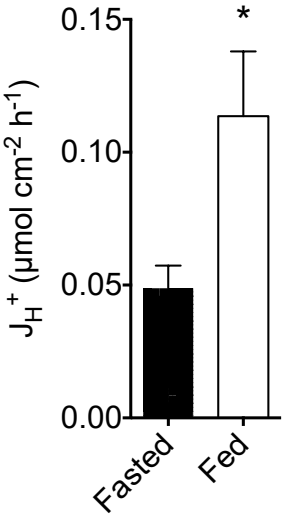

Figure 4.2 Proton secretion rate increases into the hindgut lumen following feeding. Rate of proton secretion (J_H^+) into the hindgut lumen of fasted (black bars) or fed (white bars) Pacific hagfish (*Eptatretus stoutii*). Values are presented as means + s.e.m. ($N = 6$). An asterisk (*) denotes a significant difference when compared using an unpaired t-test ($p < 0.05$).

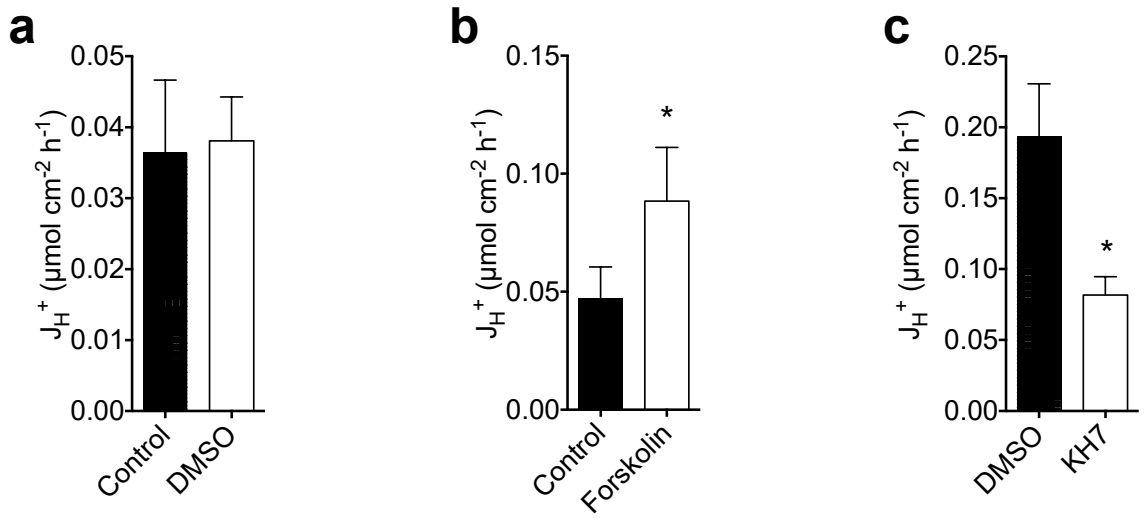

Figure 4.3 Proton secretion rate in hagfish hindgut following application of DMSO, forskolin, or KH7. Rates of proton secretion (J_H^+) into the hagfish hindgut following application of the DMSO control (0.1%; **a**), or the DMSO control followed by: the cAMP stimulator, forskolin (10 µM; **b**), or the sAC inhibitor, KH7 (10 µM; **c**). Of note, **a** and **b** were conducted on fasted animals with forskolin utilised to elicit a 'fed' response whereas **c** represents flux calculated on a fed animal to demonstrate the effect of inhibiting sAC. Datasets are presented as means + s.e.m. (N = 5–9). An asterisk (*) denotes a significant difference when compared using a paired t-test ($p < 0.05$).

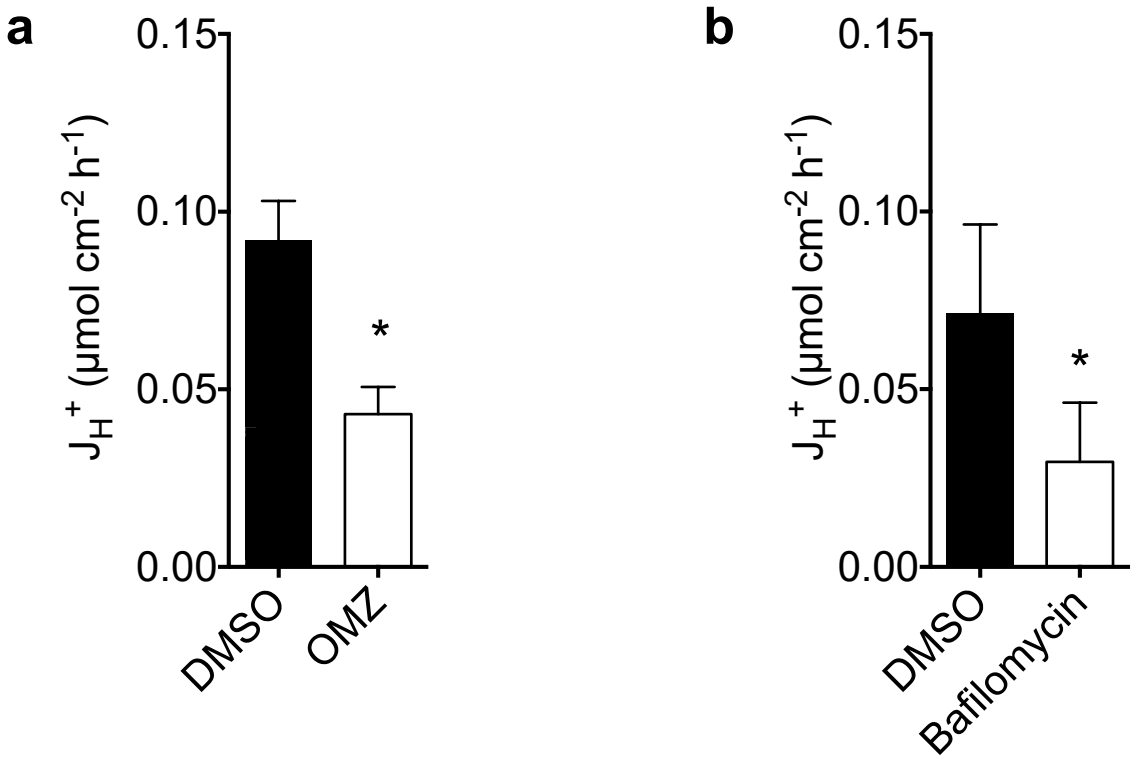

Figure 4.4 Proton secretion rate into the hagfish hindgut decreases following omeprazole or bafilomycin application. Rates of proton secretion (J_H^+) into the hagfish hindgut following application of: the HKA inhibitor, omeprazole (50 µM, OMZ; **a**) to fed fish, or the VHA inhibitor, bafilomycin (10 µM; **b**) to fed fish. Datasets are presented as means + s.e.m. (N = 4–8). An asterisk (*) denotes a significant difference when compared using a paired t-test ($p < 0.05$).

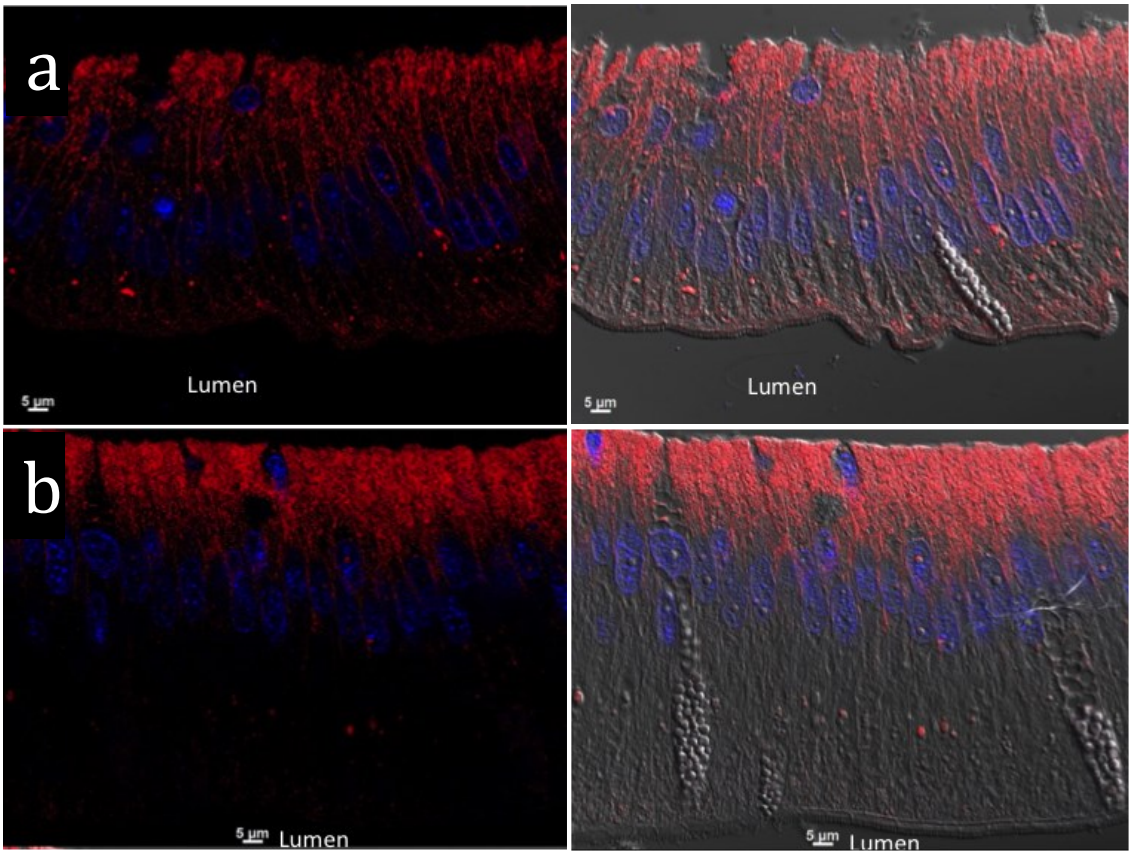

Figure 4.5 Representative micrographs of NKA immunolocalization in fed and fasted hagfish hindgut. Sections (7 µm) of fasted (a) or fed (b) hagfish hindgut immunolabeled for Na^+K^+ATPase (NKA; red) and Hoescht staining for nuclei (blue). Images are presented with the bright field overlay to the immediate right.

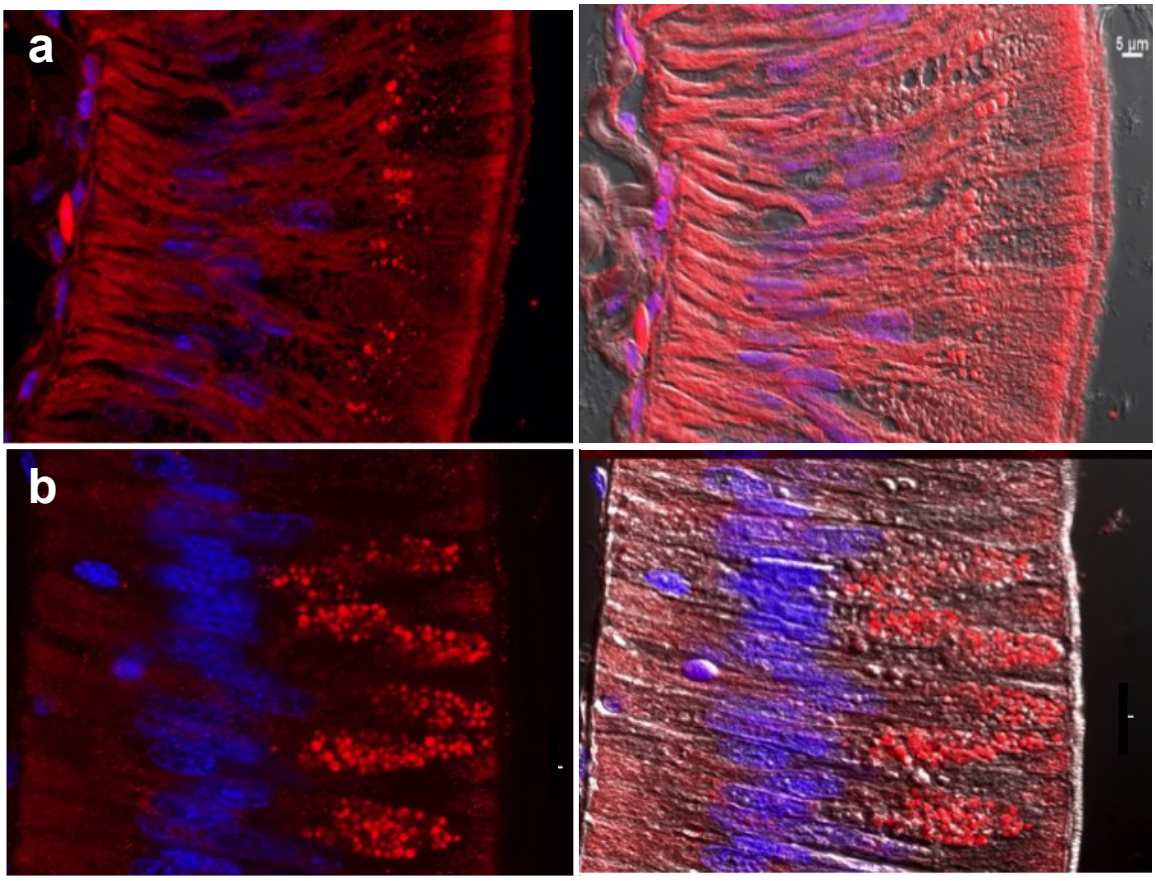

Figure 4.6 Representative micrographs of sAC immunolocalization in fed and fasted hagfish hindgut. Sections (7 µm) of fasted (A) or fed (B) hagfish hindgut immunolabeled for soluble adenylyl cyclase (sAC; red) and Hoescht staining for nuclei (blue). Images are presented with the bright field overlay to the immediate right.

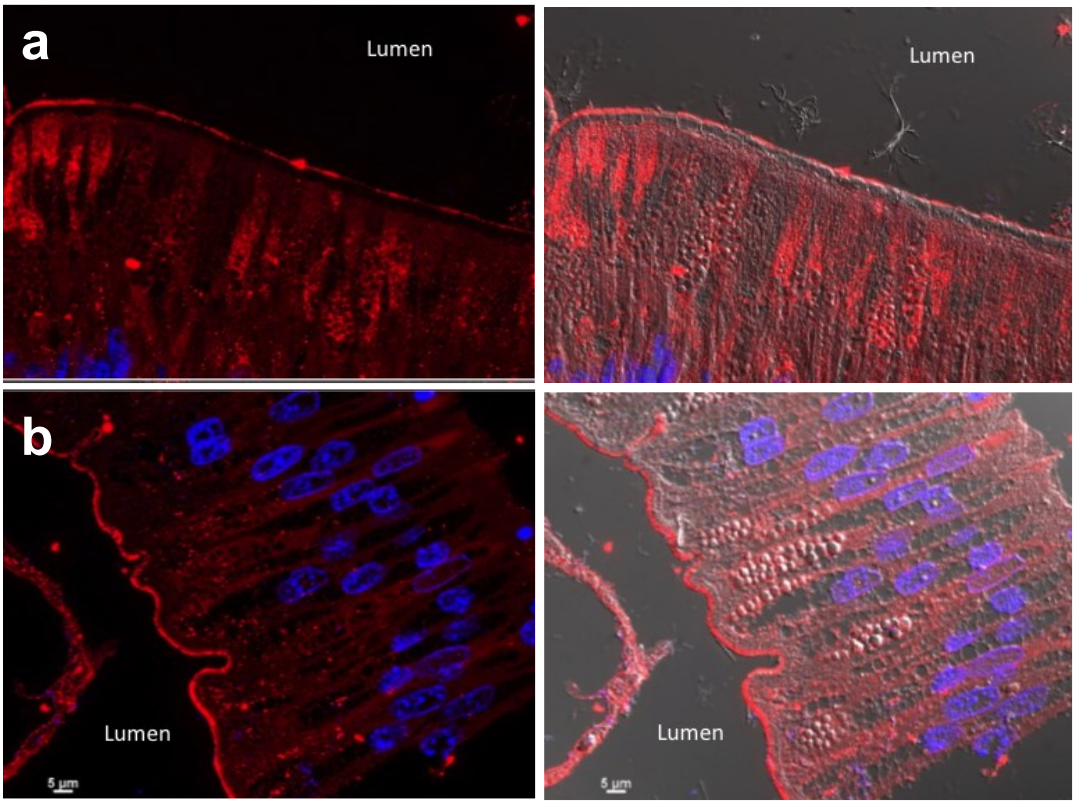

Figure 4.7 Representative micrographs of HKA immunolocalization in fed and fasted hagfish hindgut. Sections (7 µm) of fasted (A) or fed (B) hagfish hindgut immunolabeled for H^+K^+ATPase (HKA; red) and Hoescht staining for nuclei (blue). Images are presented with the bright field overlay to the immediate right.

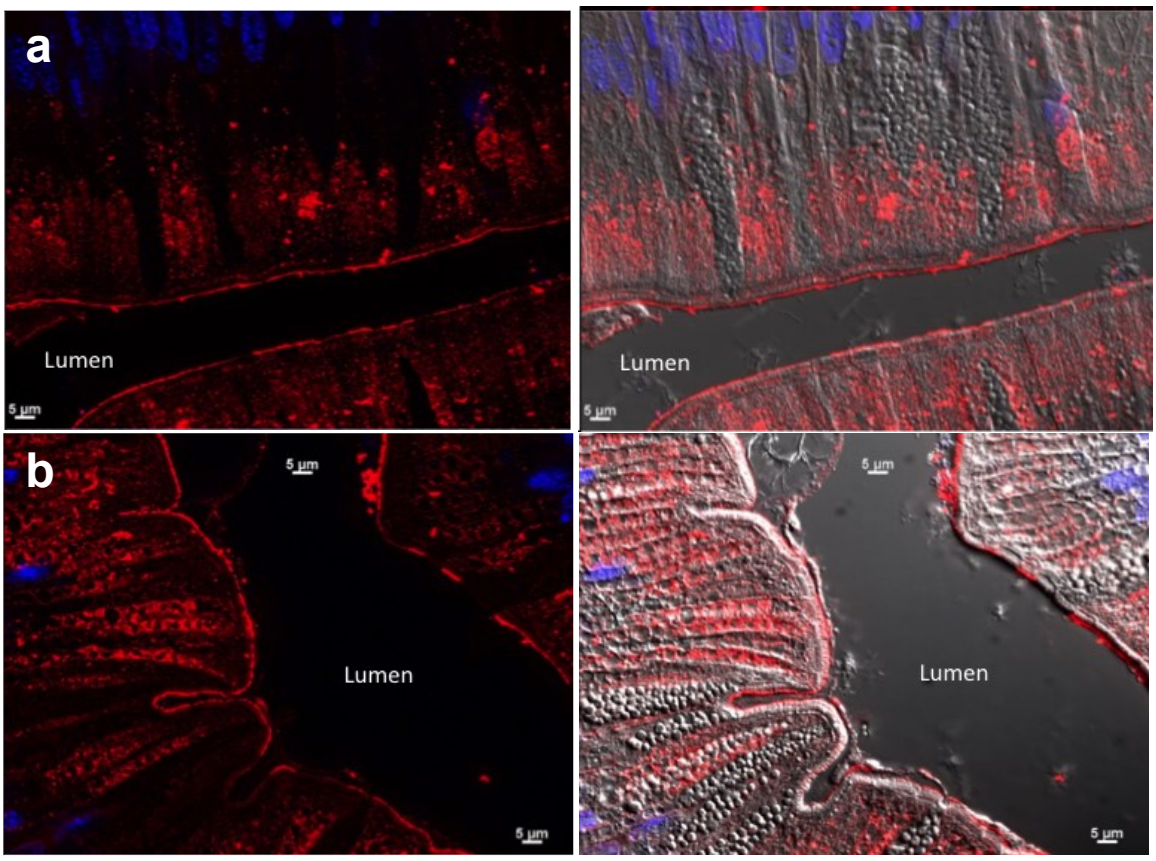

Figure 4.8 Representative micrographs of VHA immunolocalization in fed and fasted hagfish hindgut. Sections (7 µm) of fasted (A) or fed (B) hagfish hindgut immunolabeled for v-type H^+ ATPase (VHA; red) and Hoescht staining for nuclei (blue). Images are presented with the bright field overlay to the immediate right.

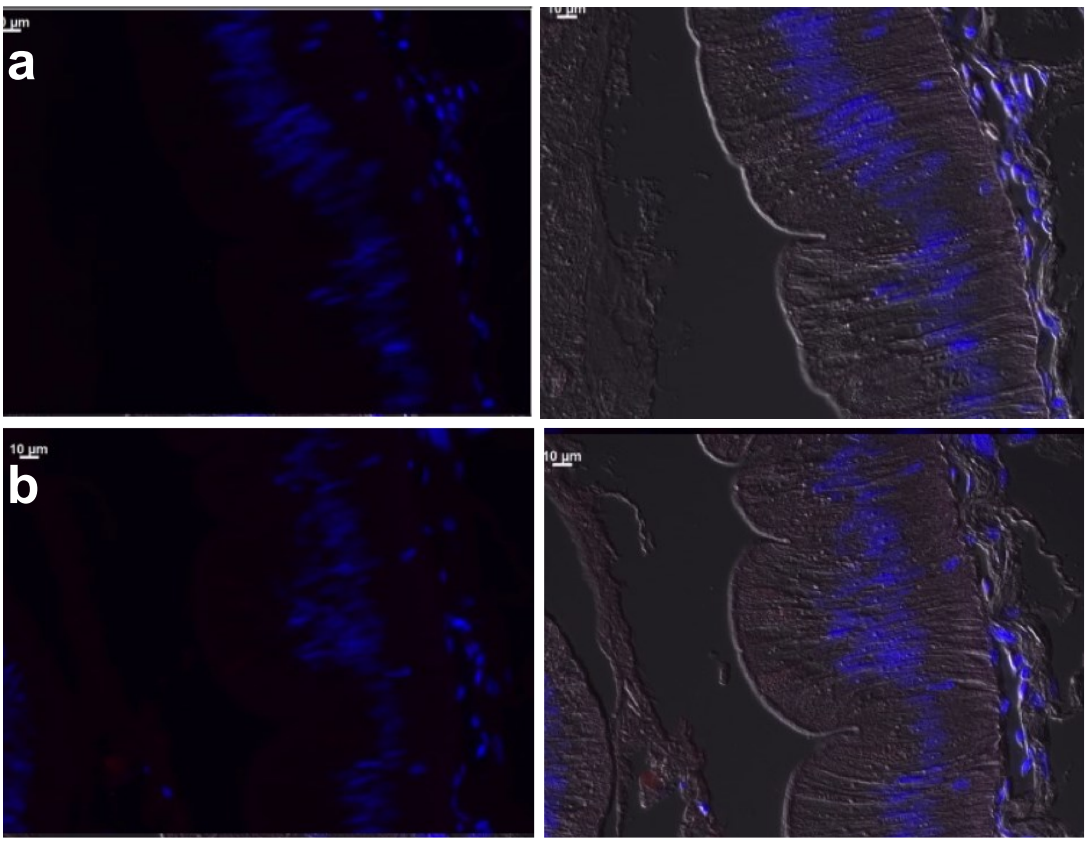

Figure 4.9 Representative micrographs of primary absorption controls and peptide absorption controls in fed and fasted hagfish hindgut. Sections (7 µm) of hagfish hindgut primary absorption control for VHA (A) or sAC peptide absorption control (B) with Hoescht nuclear staining (blue). No fluorescence was detected. Images are presented with the bright field overlay to the immediate right.

Discussion

Fluid and ion transport

The transport activity of the hagfish hindgut is significantly increased following feeding. In a quiescent state, the hindgut experiences a near-zero fluid transport rate however, upon feeding the amount of fluid transport increases over 10-fold (Fig. 4.1a). A recent study has confirmed that hagfish do not drink when fasting, yet are highly permeable to water flux (Glover et al. 2017). Thus, the act of imbibing water alongside a food source will bring the water into the hindgut where it can be readily fluxed into the enterocytes. Conversely, during fasting periods the constrictor cardiae (a structure analogous to the pyloric sphincter in other vertebrates; Adam 1963) will shunt water flow out the posterior-most gill duct, thereby limiting water uptake. The high luminal solute concentration can also enhance passive influx (Fig. 4.1b), as predicted for dogfish (Wood et al. 2009). Further potential sources of increased intestinal weight include nutrient acquisition, changes in morphology, or enhanced intestinal blood flow. Lipid influx specifically, contributes to reptilian post-prandial weight increases (Starck and Beese 2001) and lipid uptake is enhanced post-feeding in hagfish (Weinrauch et al. 2018b; Chapter VI). Increased mucosal thickness and microvilli length have likewise been characterized at this time point previously (Weinrauch et al. 2017; Chapter II). Finally, changes in intestinal blood volume were not accounted for in these wet weight measurements, yet there are noticeable increases in intestinal blood flow following feeding in hagfish (personal observation; Weinrauch et al. 2017) although the magnitude of difference has never before been calculated.

Alongside changes in fluid transport rate, were significant increases in sodium, potassium, and magnesium absorption rates (Fig. 4.1d-f). Chloride and calcium absorption remain unchanged with feeding status (Fig. 4.1c,g). The rate of sodium absorption far exceeds that of all other measured ions, which is unsurprising as it contributes nearly half of the osmotic composition of seawater. Additionally, sodium is known to provide the electrogenic force for the absorption of many nutrients. A phlorizin-inhibitable and sodium-dependent glucose transporter (likely SGLT) has been characterised in the Pacific hagfish hindgut (Weinrauch et al. 2018a; Chapter V) and this study confirms the presence of the ubiquitous NKA on the basolateral surface of the enterocytes (Fig. 4.5a,b) to establish the sodium electrochemical gradient. Thus alongside nutrients, the rate of sodium uptake will increase. Active transport *via* HKA could account for increased potassium absorption, correlating with the increased post-prandial proton efflux (Fig. 4.2a) and its inhibition following omeprazole treatment (Fig. 4.4a). The apical, absorptive isoform of $Na^+ K^+ 2Cl^-$ (NKCC) could also contribute by simultaneously absorbing sodium and potassium (as observed), in addition to chloride. Net chloride transport hovers around zero in this study, yet appears to alternate from net absorption to net secretion following feeding, albeit at very minute rates (~0.002 μmol cm^{-2} h^{-1}; Fig. 4.1c). There are a multitude of putative chloride channels (*e.g*. cystic fibrosis conductance regulator (CFTR), calcium-activated chloride channel (CaCC), or the chloride channel type-2 (ClC-2)), which could contribute to net chloride secretion, however these channels remain unstudied in hagfish hindgut thus far. A post-prandial net chloride secretion supports the hypothesis that hagfish are utilizing similar mechanisms of digestive acidification (HCl formation; see below). Seawater acclimated trout similarly

secrete chloride following feeding from the oesophagus/stomach and switch to chloride absorption over time, whereas the intestine of these fish routinely absorbs chloride (Bucking et al. 2011a). The hindgut must play the dual role of stomach and intestine in the hagfish, and therefore future studies should examine chloride flux for extended periods following feeding.

Magnesium and calcium are regulated within the hagfish plasma at concentrations lower than that of the surrounding environment (Morris 1965). Interestingly, only magnesium absorption was increased with feeding while calcium uptake was not significantly altered (Fig. 4.1f,g). Magnesium is an essential co-factor for many enzymes (Schweigel and Martens 2000) and is of importance for ATP-dependent transporters (Bijvelds et al. 1998), many of which we identify in this study (e.g. NKA, HKA, VHA). Furthermore, magnesium is an allosteric modifier of haemoglobin and can increase hypoxia tolerance (Terwilliger 1998; Terwilliger and Brown 1993; Watanabe et al. 2011). When feeding on decaying carrion, hagfish likely experience hypoxia (Bucking et al. 2011b; Clifford et al. 2016), and the maintenance of magnesium content could therefore be of high priority. The mechanisms of dietary magnesium acquisition are not yet well understood in fish (Bijvelds et al. 1998), but passive paracellular movement (Kayne and Lee 1993) and specific channels/transporters have been suggested (Karbach and Feldmeier 1991), each of which have never before been examined in hagfish. Calcium, similarly, is an essential element integral for many physiological processes. However, dietary-sourced calcium may not be a requirement for some aquatic teleosts whereby branchial absorption can meet calcium demand (Hossain and Furuichi 1999). Since

hagfish maintain calcium concentrations below seawater and dietary calcium may only be acquired intermittently (Tamburri and Barry 1999), branchial calcium-handling mechanisms likely exist. The intestine may then have a reduced role in calcium regulation, explaining the unchanging uptake rates observed across feeding states. A comprehensive examination of the ion transporting machinery present in the hagfish hindgut is required to better understand the role of the intestine in maintaining hagfish magnesium and calcium homeostasis.

Intracellular signals leading to luminal acidification

In between meals, the majority of vertebrates secrete protons at a basal rate and significantly increase proton secretion after feeding (Papastamatiou and Lowe 2004). The fasting hindgut environment of the hagfish is slightly acidic (pH 5.5-6; Nilsson and Fänge 1970; Glover et al. 2011b) and this study confirms that hagfish routinely secrete protons from their hindgut. The rate of proton secretion doubles following a feeding; identifying that acidification is utilized for digestion in this earliest representative of the vertebrates (Fig. 4.2). Increased proton secretion is often accompanied by changes in cell morphology, metabolic activity, fluid secretion, and ion transport (Malinowska et al. 1988), as observed and discussed in the previous section.

The cellular mechanisms responsible for proton secretion are well characterised in vertebrates (see introduction) and we sought to elucidate whether a conserved mechanism is present in the hagfish. Although forskolin leads to unphysiologically elevated stimulation of the cAMP pathway (reviewed in Tresguerres et al. 2011), it was useful to ascertain whether cAMP plays a role in luminal acidification. The post-fed condition was

mirrored in fasted fish treated with forskolin, suggesting a cAMP-mediated pathway is involved in luminal acidification (Fig. 4.3b). Numerous stimuli can increase intracellular cAMP concentrations, one of which is the bicarbonate-sensitive sAC (see chapter introduction). As forskolin does not stimulate sAC (Chang et al. 2017), we utilized the sAC-specific inhibitor KH7 which resulted in a significantly reduced proton flux following feeding (Fig. 4.3c). Therefore, we suggest that the activation of sAC will ultimately lead to proton secretion *via* the cAMP pathway. sAC was localized throughout the enterocytes, within the zymogen granule cells, and in clusters on the brush border membrane (Fig. 4.6a,b). Metabolically generated HCO_3^- could stimulate sAC activation in any/each of these locations, as could extracellular sources of HCO_3^-. Hagfish do not have intestinal CA (Nilsson and Fänge 1970; Esbaugh et al. 2009) and it has been postulated that digestive acid must be derived in a unique fashion, relative to other vertebrates (Nilsson and Fänge 1970). However, the low metabolic rate of hagfishes (Munz and Morris 1965; Forster 1990) may assuage the need for rapid hydration of CO_2 by CA, much like the proposition made by Peters and colleagues (2000) regarding the low blood CO_2 transport capacity of hagfish. Moreover, rapid HCO_3^- offloading may not be necessary given that hagfish can withstand alkaline loads (elevated pH) that would otherwise be fatal (Tresguerres et al. 2007a; Baker et al. 2015; Clifford et al. 2017c). Consequently, the rapid conversion of HCO_3^- by CA may not be required in these animals.

Finally, cAMP has been identified as a stimulus for secretagogue-sensitive zymogen release and sAC appears to modulate enzyme activation (Kolodecik et al.

2012). The localization of sAC throughout the zymogen cells (Fig. 6a,b) indicates a cellular source to retain digestive granule activity in the tissue, which could be particularly important for long-term digestion or anticipation of future meals. Additionally, the acinar cells in the mammalian pancreatic duct are an important HCO_3^- source for the neutralization of stomach contents in the small intestine (Steward et al. 2005). It is possible that the hagfish zymogen granule cells may have a similar role in neutralization/ alkalization (beyond the 8 h time point investigated in this study). As HCO_3^- is created with cellular respiration, sAC is activated and downstream increases in cAMP could lead to zymogen release (Kolodecik et al. 2012). The expansive distribution of sAC within the enterocytes could permit 'early' and 'late' zymogen release, as mediated by changing HCO_3^- content, and requires further investigation.

Apical enterocyte transporters involved in luminal acidification

HKA establishes the largest differential ion gradient in vertebrates and is the primary apical transporter responsible for gastric proton secretion (Hersey and Sachs 1995). Despite being agastric, hagfish displayed significantly reduced post-prandial proton secretion following omeprazole application (Fig. 4.4a). There are both gastric and non-gastric isoforms of HKA and the gastric variant is expressed in mammalian pancreatic cells (Wang et al. 2015). Similarly, HKA appears in the pancreatic-like zymogen granule cells of the hagfish, in addition to the apical cytoplasm and brush border of the enterocytes (Fig. 4.7a,b). We speculate that there is potential for increased abundance of HKA in the apical membrane post-feeding. Therefore, we are currently conducting Western blots to quantify the amount of protein in each nutritive state. Increased membrane expression of HKA would correlate with the observed increase in

proton secretion. The localization of HKA within the zymogen cells may be useful for the activation of zymogens (Bakke et al. 2010). It has been previously determined that some hagfish proteases have optimal activity at pH 4 (Nilsson and Fänge 1970), and HKA could provide a proton source in the granule cells. Further examinations of the means of HKA activation are required.

VHA is a known transporter of the pancreatic ducts and increases membrane expression in a cAMP-dependent fashion (Wang et al. 2015). VHA is also responsible for luminal acidification of marine teleost intestines, albeit for osmoregulatory purposes (Guffey et al. 2011), and responds to a HCO_3^- load (Niv and Fraser 2002) as is generated following feeding (Weinrauch et al. 2017). Bafilomycin successfully reduced the proton secretion rate (Fig. 4.4b), which strongly supports the hypothesis of VHA contribution to luminal acidification. VHA was expressed in the apical cytoplasm of the enterocytes and had distinct brush border localization in both fasted and fed animals (Fig. 4.8a,b). It was conspicuously absent from the zymogen granule cells. VHA is known to translocate to the basolateral surface of dogfish gill cells to facilitate bicarbonate offloading in a post-fed state (Tresguerres et al. 2007b). We propose that the same machinery operates in the post-fed hagfish hindgut, however the transporters are trafficked to opposing membranes (i.e. VHA to apical). This would enable basolateral HCO_3^- secretion, leading to the observed alkalization of the intestinal blood supply (Weinrauch et al. 2017; Chapter II), and apical proton secretion as observed herein.

Conclusions

We conclude that the Pacific hagfish hindgut alters transporter activity following feeding. There were notable increases in fluid transport rate, as well as the sodium, potassium, and magnesium absorption rates. The cellular mechanisms responsible for each of these ions, as well as chloride and calcium, require further examination in this species. Furthermore, we have established that hagfish utilize luminal acidification as a means of digestion. Subcellularly, sAC (likely influenced by HCO_3^-) can elicit changes in cAMP concentrations that ultimately lead to proton secretion into the luminal space. Despite lacking intestinal CA, it appears that protons are contributed using the evolutionarily conserved HKA, likely owing to their incredibly low metabolism. In addition to HKA, VHA was localized to the hindgut and contributed to luminal acidification. We propose that the mechanisms outlined in the post-prandial dogfish gill function here in the post-prandial hagfish hindgut to promote luminal acidification. Finally, each of sAC and HKA were widely distributed across both cell types of the hagfish hindgut, including the zymogen granule cells. Within these cells, we predict that sAC and HKA can enable zymogen activation, demonstrating both enzymatic and acidic digestive processes in this oldest extant craniate.

Chapter V: Mechanisms of Glucose Acquisition in the Hagfish Hindgut

A version of this chapter has been published.

Weinrauch, A.M., Clifford, A.M., Goss, G.G. (2018) Functional redundancy of glucose acquisition mechanisms in the hindgut of Pacific hagfish (*Eptatretus stoutii*). *Comparative Physiology and Biochemistry Part A. 216*:8-13. Reproduced with permission of the co-authors of the manuscript.

Introduction

The hagfish diverged from the vertebrate phylogeny ~500 million years ago (Bardack 1998) and so are representatives of the evolution of early vertebrates. Hagfish are thus of great interest as evolutionary models and are of particular use in the study of nutrient transport system evolution owing to their unique feeding environment. Occupying a distinct trophic niche, hagfish consume both living (Zintzen et al. 2011) and dead prey items, ranging from polychaetes to cetaceans, and may become fully immersed within a carcass (Martini, 1998). Interestingly, it has been noted that hagfish preferentially consume the glycogen-rich liver (Weinrauch et al., 2017), which conforms with the favoured use of carbohydrates for fuel in particular tissues (Sidell et al. 1984). Further evidence for the importance of glucose has been demonstrated in multiple hagfish species. Glucose loading induces immediate hyperglycaemia lasting two days in *Myxine glutinosa* (Falkmer and Matty, 1966), while a one-month starvation period in *Eptatretus stoutii* incites decreased basal levels of glucose and insulin (Emdin, 1982). Additionally, the archinephric duct demonstrates glucose resorption as only 12-18% of an injected glucose load is recovered in the urine (Falkmer and Matty, 1966). Hormonal glucose regulation is also demonstrable in hagfish, albeit with variable results between species. Large quantities of exogenous insulin (1000-3000 IU/kg) were required to incite hypoglycaemia 2-3 days post-injection in *Myxine glutinosa* (Falkmer and Matty, 1966), while *Eptatretus stoutii* had sensitivities akin to mammals, wherein 0.5 IU/kg of bovine insulin produced a pronounced hypoglycaemia within 12-24 hours that was rectified in the span of 5 days (Inui and Gorbman 1977). Hormonal regulation of glucose suggests

that it is an important molecule for hagfish and glucose uptake will therefore be regulated by transport systems.

Glucose is an energetically important molecule that is a primary energy source for eukaryotes (Bell et al., 1990). The plasma membrane is impermeable to large polar molecules (Bell et al., 1990), and so the uptake of glucose necessitates membrane-associated proteins such as the sodium-dependent and phlorizin-sensitive sodium glucose-linked transporters (SGLT; SLC5A family), as well as cytochalasin-B and phloretin-sensitive glucose transporters (GLUT; SLC2 family). SGLT acquires glucose against a concentration gradient due to the electrochemical gradient of sodium established by the ubiquitous basolateral sodium-potassium ATPase (NKA), while GLUT functions to move glucose down its concentration gradient and is perpetually expressed on the basolateral membrane, but may be inserted apically for enhanced uptake. Such transporters have been identified in hagfish tissues with sodium-dependent, phlorizin sensitive (SGLT) glucose uptake identified in *M. glutinosa* archinephric duct (Flöge et al., 1984) and cytochalasin b sensitive (GLUT) glucose uptake observed and characterized in *E. stoutii* erythrocytes (Ingermann et al. 1984; Young et al. 1994). The objective of this study was to characterize the mechanisms of glucose acquisition in the hindgut of the Pacific hagfish (*E. stoutii*) owing to its clear importance as an energy source for this organism.

The agastric hagfish have distinct morphological differences along the intestinal tract, with mucus cells restricted to the foregut and digestive/absorptive cells along the

length of the hindgut (Adam, 1963; Weinrauch et al., 2015; Introduction). As such, previous histochemical and histological studies suggest that digestive activity (including amylases, lipases, and proteolytic enzymes) is restricted to, and are present consistently along the hindgut (Adam, 1963). This study sought to examine whether digestive enzymes for maltose (presumably maltase) were present along the length of the intestine as a means to digest complex carbohydrates into the simpler molecule glucose, over an extended surface area. Using *in vitro* gut fluxes, we sought to characterize the kinetics, sodium-dependence and effect of pharmacological inhibitors (phloretin, phlorizin and cytochalasin b) on intestinal glucose acquisition.

Materials and Methods

Animal care and husbandry

Pacific hagfish (*Eptatretus stoutii*) were collected from the Trevor channel, Vancouver Island, B.C, Canada using bottom-dwelling traps (2014-2017). Fish were immediately transported to the Bamfield Marine Sciences Centre and housed in aerated, darkened 20 m^3 tanks with flow-through water. Hagfish were starved at least one week prior to experimentation. All animals were used under the licenses of the Department of Fisheries and Oceans Canada (collection permits XR-192-2014; XR-310-2015; XR-202-2016; XR-136-2017) and approved by the Bamfield Marine Science Centre (RS-14-13 (2014), RS-15-31 (2015), RS16-19 (2016), RS17-03 (2017)), and University of Alberta Animal Care (No. AUP00001126; 2014-2017). Unless noted, all chemical compounds, reagents and enzymes were supplied by Sigma-Aldrich Chemical Company (St. Louis, MO).

Solutions

Glucose-free hagfish saline (HF saline) was used for all serosal fluid. It contained: NaCl, 490 mM; KCl 8.0 mM; $CaCl_2$ $2H_2O$ 5.0 mM; $MgSO_4$ $7H_2O$ 3.0 mM; $MgCl_2$ $6H_2O$ 9.0 mM; Na_2PO_4 2.06 mM; $NaHCO_3$ 8.0 mM; HEPES 20 mM; pH 7.6. To test for sodium-dependent glucose uptake, modifications were made to reduce the sodium concentration in the HF saline, replacing it instead with choline chloride as per previous sodium-free studies: [$C_5H_{14}NOCl$ 480 mM; $CaCl_2$ $2H_2O$ 5.0 mM; $MgSO_4$ $7H_2O$ 3.0 mM; $MgCl_2$ $6H_2O$ 9.0 mM; KH_2PO_4 2.06 mM; $KHCO_3$ 8.0 mM; HEPES 20 mM; pH 7.6]; Schultz et al. 2014). Matched mucosal salines containing all above mentioned chemicals

were used for either sodium-containing or sodium-free experiments and osmotically balanced with mannitol with measurement using a Vapro vapor pressure osmometer (Model 5520; Wescor, Logan, UT). The mucosal saline also contained 0-10 mM glucose for concentration dependent experiments and 5 mM glucose in all other solutions (Bucking et al. 2011b). An atomic absorption spectrophotometer (Thermo Scientific model iCE 3300) was utilized to ensure solutions had reduced sodium concentrations (<1 mM) prior to experimentation.

Excised intestine was rinsed and soaked in sodium-free, aerating hagfish saline for 30 min prior to the preparation of individual gut sacs in order to remove free endogenous sodium. Serosal fluid was again measured using the atomic absorption spectrophotometer following experimentation and no significant changes in sodium concentration were detected. Radiolabelled D-[1,^{14}C] glucose (Perkin Elmer, Boston, MA, USA) was added at a specific activity of 0.05 μCi/mL to the mucosal saline. Glucose uptake was measured with and without the presence of pharmacological inhibitors. Phloretin, a weak inhibitor of SGLT and GLUT, was used at concentrations of 0, 10, 50, 100, 500, and 1000 μM. Phlorizin, a selective inhibitor of SGLT, was used at concentrations of 0, 0.1, 1, 10, 100, and 1000 μM. Cytochalasin-B, an inhibitor of GLUT, was utilized at a concentration of 200 μM. Each inhibitor was dissolved in DMSO and added to solutions at a final concentration of 0.1%. A 0.1% DMSO control was used for each inhibitor experiment.

Intestinal fluxing protocols

Hagfish (N=56; 81.4 ± 4.1 g; mean ± s.e.m.) were euthanized using 4 g L^{-1} MS-

222 (Syndel Laboratories Ltd., Vancouver, B.C, Canada) neutralized with 1.2 g L^{-1} NaOH. Weight measurements were obtained prior to dissection where the intestine was removed in two pieces; a region of the pharyngocutaneous duct anterior to the first gill pouches, and the hindgut from the biliary duct to the cloaca, which was then further divided into three pieces. Additional tissue was flash frozen and stored at -80°C until enzymatic analysis (see below). Mesentery and gonads (if present) were removed and the intestinal tube was flushed multiple times with glucose-free hagfish saline. Gut sacs were prepared as reported previously (Glover et al. 2011b; Schultz et al. 2014). Briefly, each intestinal piece had one end tied off with suture thread and a flared piece of polyethylene (PE)-90 tubing sutured on the other side for sample insertion/removal. The sacs were filled with glucose-containing mucosal hagfish Ringer and placed in aerating serosal Ringer solution for the flux period. A 200 µL sample was removed at both the beginning and end of the flux period and the sacs weighed to determine flux volume. Two 50 µL subsamples were taken and combined with ACS (aqueous counting scintillant; Amersham Bioscience, Baie d'Urfe, Quebec, Canada) and radioactivity measured on a Beckman Coulter LS6500. The remaining sample was stored at -80 °C until further analysis of glucose content within a 2 week period. Preliminary studies demonstrated no glucose in the serosal fluid following a flux period (data not shown). Following a flux period, the sacs were cut open and spread on graph paper where surface area was determined using Image J Software (National Institute of Health). Glucose concentration was determined using the following procedure. Briefly, 20 µL of sample was added to 200 µL of glucose cocktail (50 mL Triethylamine-hydrochloride pH 7.53; 0.22 g $MgCl_2$; 0.050 g ATP; 0.050 g NAD; 2.4 U/mL glucose-6-phosphate dehydrogenase) and

absorbance (A340 nm) was recorded using a spectramax 190 (Molecular Devices, Sunnyvale, CA, USA). A final A340 measurement was conducted after 15 min of incubation following the additional of hexokinase (5U/sample) and the difference between initial and final absorbance represents the concentration of glucose when corrected to µmol/L using a standard curve (Bergmeyer 1983). The disappearance of glucose from the mucosal saline was then calculated using the following equation:

$$J_{glucose}\ (nmol\ cm^{-2}h^{-1}) = \frac{\Delta CPM/SAct}{SA/t} \qquad (I)$$

where **Δ**CPM represents the decrease of 1-^{14}C-D-glucose radioactivity inside the gut sac, SAct is the specific activity (CPM nmol^{-1}), SA is the surface area (cm^2) and t is the flux time (h). Of note, the data for Figs. 5.1, 5.5a, and 5.5b were collected in 2014 and Figs. 5.3, 5.4 and 5,5c were collected in 2017 (see discussion).

Presence of maltase in the hagfish intestine

Maltase presence was measured indirectly by homogenizing different intestinal segments in a homogenization buffer (PBS 23 mM, Tris-HCl 5 mM, EDTA 1 mM, pH 7.0) modified from Cox and Secor (2008). The homogenate (50 µL) was added in a 1:1 (*v*:*v*) ratio to maltose (62.5 mM prepared in distilled water) and was allowed to incubate for 15 seconds (time determined during preliminary experiments). Following this incubation, 1 µL of 1N HCl was added to inhibit enzymatic activity (preliminary experiments demonstrate no effect of acidification on the glucose measurement) and glucose concentration was measured using the aforementioned assay, while protein content was calculated using the Bicinchoninic Assay (BCA) as per the manufacturer's

instructions (Thermo Fisher Scientific; Waltham, MA, USA). Glucose produced/ mg protein of intestine added was calculated as follows:

$$Maltase\ Presence\ = \frac{[glucose_{sample}]-[glucose_{maltose}]}{mg\ protein\ added} \qquad (2)$$

with the assumption that any glucose produced in addition to the background concentrations measured in the maltose were the result of hagfish intestinal maltase.

Statistical Analysis

The assumption of homogeneity of variance was not met when analyzing enzymatic activity along intestinal length, thus differences in enzyme activity along the intestinal length were determined using a non-parametric Kruskal-Wallis test with Dunn's multiple comparison *post-hoc* test. All remaining data were determined to be normal and of equal variance and are presented as means + s.e.m. Glucose uptake along the intestinal length was analyzed using a one-way ANOVA with Tukey's *post-hoc* test where differences did occur. Similarly, the effects of ranging concentrations of pharmacological inhibitor were analyzed using a one-way ANOVA with Dunnett's *post-hoc* test where appropriate. The effect of cytochalasin-b was analyzed using a paired one-tailed t-test while sodium-dependence was determined using an unpaired t-test. Concentration-dependence was first assessed by a linear regression ($y = 13.71x + 0$). This equation was utilized to calculate the amount of glucose uptake that was attributed to diffusive pathways and subtracted from the total value for kinetic analysis at glucose concentrations ≤ 2 mM. All data were analyzed in GraphPad Prism6 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Maltose digestibility

The combination of hagfish intestinal samples with maltose resulted in the production of glucose in all segments tested (Fig. 5.1). The negative control (NaCl with no maltose present) produced significantly less glucose in all areas of intestine tested

Concentration-dependent glucose uptake

Initial experiments determined that there was no radioactivity above background observed in the serosal fluid following a flux period. Given the metabolic transformation of glucose upon entry into a cell (into glucose-6-phosphate *via* hexokinase), we chose to measure disappearance of radioactivity from the mucosal solution (in nmol cm^{-2} h^{-1}). Rates of glucose uptake did not differ along the length of the digestive tract, including the foregut (Fig. 5.2). However, given the distinct morphological differences between the foregut and hindgut, we opted to utilize the entirety of the hindgut as replicates in all subsequent experiments. Hindgut intestinal segments were systematically rotated within experiments to further ensure no effect due to region analyzed.

1-^{14}C-$_D$-glucose was acquired from the mucosal solution in a concentration-dependent manner. We observed both a linear/diffusive component ($\leq$ 2 mM; Fig. 5.3a) and a saturable/active component (that was obtained by subtraction of the associated diffusive flux from the measured value; Fig. 5.3b). The apparent affinity constant (K_m) for saturable component of glucose uptake in the hagfish hindgut was 0.37 mM, while the estimated maximal velocity of transport (J_{max}) was calculated as 8.48 nmol cm^{-2} h^{-1}.

Transporter characterisation

To examine potential sodium dependence of glucose uptake, we reduced sodium content in both mucosal and serosal solutions and measured glucose uptake. Rates of glucose uptake remained unaltered in low sodium conditions at 5 mM glucose concentrations (Fig. 5.4).

The rate of glucose transport was not significantly altered with the addition of phloretin at all tested concentrations (0-1 mM; Fig. 5.5a). However, phlorizin did cause a significant depression in glucose transport rates at all concentrations tested (>0.0001 mM; Fig. 5.5b). Similarly, application of 200 μM cytochalasin-b resulted in a significant reduction in glucose transport when compared to control 0.1% DMSO (Fig. 5.5c).

Figures

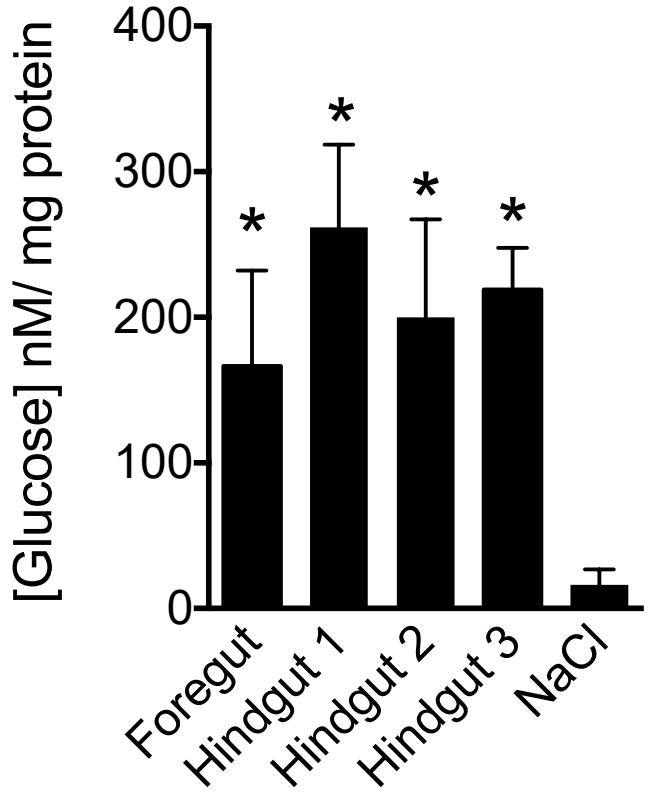

Figure 5.1 Maltase activity along the length of the hagfish digestive tract. Intestinal segments were incubated in either maltose or NaCl (negative control). The production of glucose from each substrate was calculated per mg protein of intestinal tissue. Data are presented as means + s.e.m. ($n = 6$). Bar with an asterisk (*) are statistically different from the negative control (NaCl; $p < 0.05$) as determined *via* a Kruskal-Wallis non-parametric test with a Dunn's *post-hoc* test to determine where differences occurred.

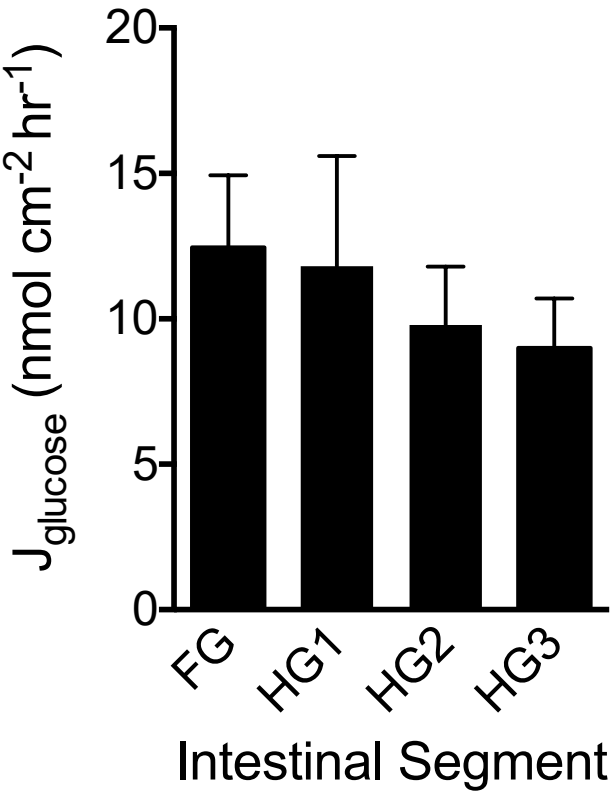

Figure 5.2 Rates of ^{14}C- D-glucose acquisition are consistent along the length of the hagfish digestive tract. Segments were removed from each section along the intestinal tract and $J_{glucose}$ (nmol/cm^2/h) was quantified. Data are presented as means + s.e.m. (*n* = 7–9). No statistical differences were observed as calculated using a one-way ANOVA.

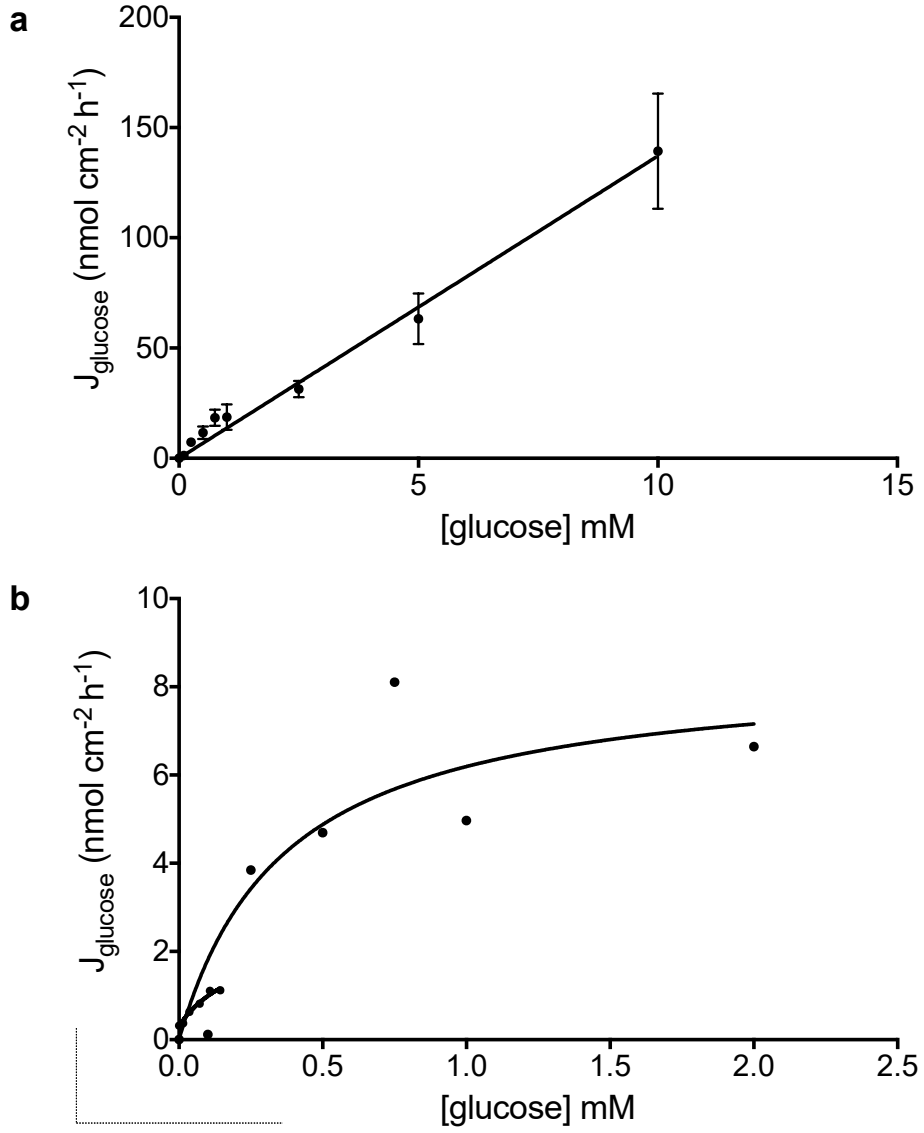

Figure 5.3 Concentration-dependent uptake of 1-^{14}C D-glucose (nmol/cm^2/h) across the hindgut of the Pacific hagfish. Uptake rates in the *in vitro* preparations increased with increasing concentration (**a**). A second uptake pathway that conformed to Michaelis-Menten kinetics was observed at lower concentrations (**b**; values obtained by subtraction of associated diffusive flux observed in **a**). Plotted points are means ± s.e.m. (n = 5–11). Kinetic analysis was conducted using Prism6 software (GraphPad Prism 6).

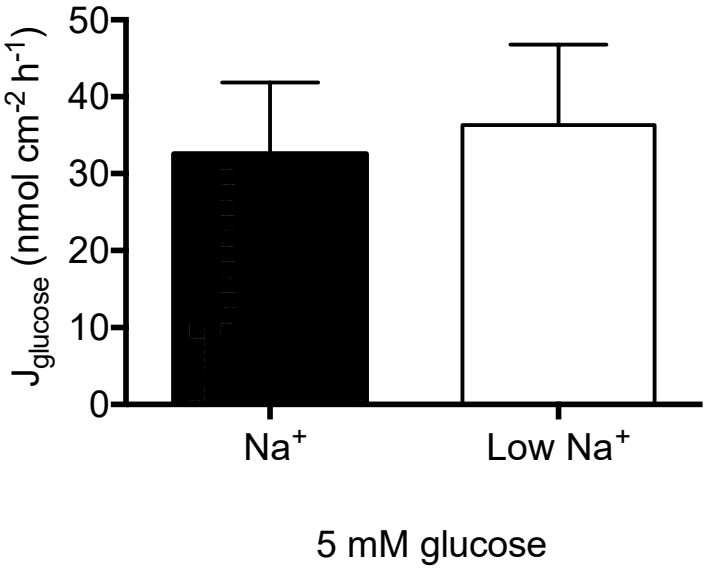

Figure 5.4 1-^{14}C D-glucose uptake in the hagfish hindgut was not sodium dependent. Glucose flux at 5 mM glucose was determined both in sodium-containing (black bar) and sodium-limiting (white bar) conditions. Data are presented as means + s.e.m. (*n* = 6–8). No statistical differences were detected as determined with an unpaired Students t-test.

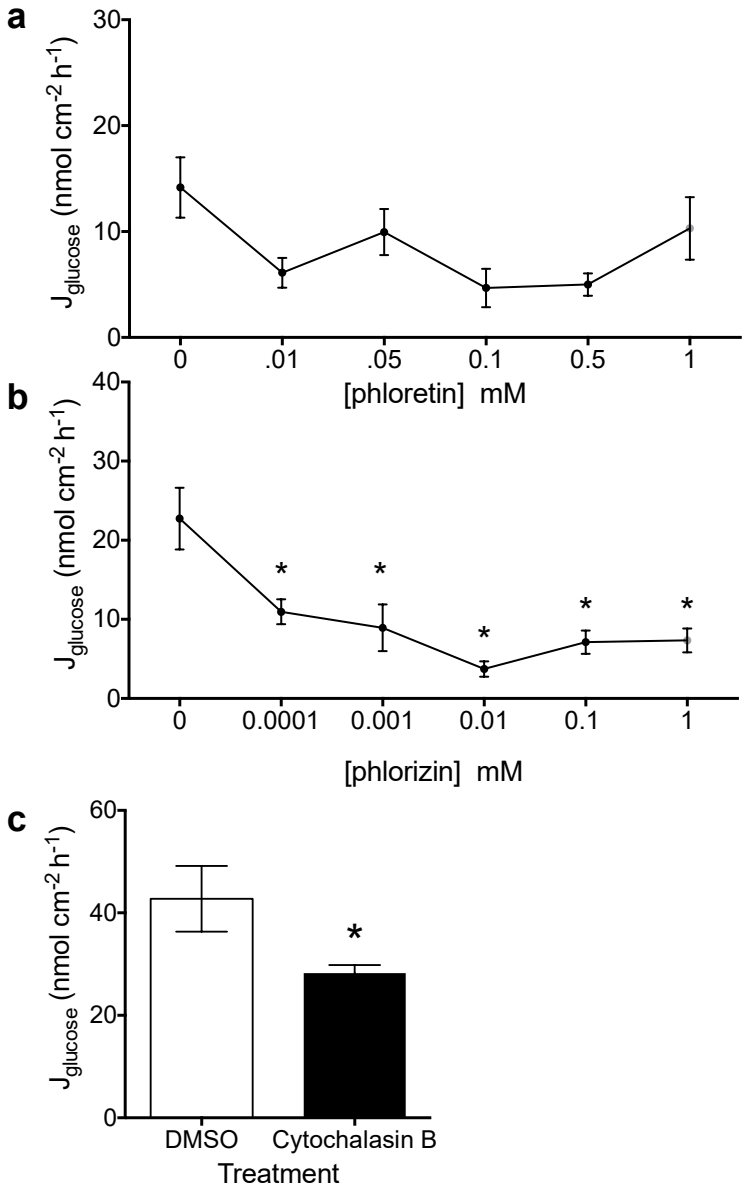

Figure 5.5 Effects of potential pharmacological inhibitors of glucose transport on 1-^{14}C D-glucose flux in the hagfish hindgut. Phloretin (0-1 mM; **A**), phlorizin (0-1mM; **B**) and cytochalasin-b (200 μM; **C**) were applied to the mucosal saline and effect on glucose transport was assessed. Data is presented as mean ± s.e.m. (n = 3 –7). An asterisk (*) indicates significant difference from control (0 mM pharmacological agent or DMSO) as determined using a one-way ANOVA with Dunnett's multiple comparisons *post-hoc* test where differences were apparent (**A, B**), or a paired one-tailed t-test (**C**).

Discussion

The preferred use of carbohydrates has been well documented in a number of hagfish species (Sidell et al. 1984; Foster and Moon 1986; Gillis et al. 2015). This investigation has demonstrated the ability of Pacific hagfish (*Eptatretus stoutii)* to digest the disaccharide maltose into its simpler subunits of glucose. Furthermore, we demonstrate the presence of multiple glucose uptake pathways in the hindgut of this primitive vertebrate.

Disaccharide Digestibility

Following the addition of maltose, the presence of glucose was detected in each section of homogenized intestine at levels significantly elevated compared to control (NaCl added rather than maltose). Interestingly, this was also apparent in the foregut region; a region previously thought to provide lubrication only (Adam, 1963). However, given the lack of folding of the intestine and therefore a reduced surface area, commencement of digestion in this pharyngeal cavity can only enhance the hagfish's ability to extract nutrients from a scavenged meal. We did also observe a consistent production of glucose from maltose along the length of the hindgut, suggesting that enzymes capable of breaking down disaccharides are present throughout the intestine, and supports both histochemical and biochemical evidence of amylase in *M. glutinosa* (Adam, 1963). Adam (1963) postulated that amylase is necessary for glycogen digestion, which would be required to digest the preferred liver tissue (Weinrauch et al., 2017). Interestingly, amylase activity was also detected in the slime glands as well as the skin, albeit to a lesser extent than that of the intestine (Adam 1963). Given the role that the

skin and gills play in nutrient acquisition, trace metal uptake (Glover et al. 2011b, 2015, 2016; Schultz et al. 2014), and acid-base and ammonia homeostasis (Clifford et al. 2014, 2015a,b, 2017) as a likely means to enhance surface area for transport when burrowing in prey tissue, it is possible that these digestive enzymes are prevalent on external surfaces to aid in the digestion of macromolecules *ex vivo* and further increase their availability for transport. Predatory behavior of certain hagfish species may also include suffocating prey by encasing them in slime within the hagfish burrow (Zintzen et al., 2011). It is possible then that the slime itself may begin digesting the prey prior to hagfish consumption and this requires further investigation. While some ion uptake is negatively impacted by the presence of slime (zinc and nickel), other neutrally charged molecules ($_{L}$-alanine) were unaffected by its presence (Glover et al., 2015). Given that glucose is likewise in a neutral state, it is possible that it would be allowed to pass through the slime, although its size (MW: 180 Daltons) may prove limiting for diffusion. Future research should examine the potential for slime digestibility as well as to quantify whether the skin and gills are capable of acquiring (and/or digesting) di- or mono-saccharides.

Mechanisms of intestinal glucose acquisition

Surprisingly, the foregut demonstrated a similar capacity to acquire glucose as the hindgut (Fig. 5.2). The lack of a brush border in the foregut had previously deterred researchers from believing it held a capacity to acquire nutrients (Adam, 1963). However, the abundance of mucus may indicate glucose adsorption to the tissue rather than effective transport. Unfortunately, the delicate nature and small size of the foregut prevented further characterization of the transporters present by the methods employed in

this study. Future studies should focus on localizing glucose transport mechanisms in hagfish *via* immunostaining, *in situ* hybridization or qPCR.

A consistent rate of uptake along the length of the hindgut is in accordance with that found for other nutrients including phosphate and amino acids (Glover et al. 2011b; Schultz et al. 2014) and demonstrates maximal use of surface area in the intestinal tube. We found that transport of glucose was biphasic (Fig. 5.3) and likely the result of multiple transport pathways. The reported K_m of SGLT varies widely between both vertebrate species and specific sugar but range anywhere from 0.012 -23 mM (Hirayama et al. 1996; Kellett and Helliwell 2000; Au et al. 2002). These differences may be due to isoform variation, tissue specificity, different techniques or substrates (Hirayama et al., 1996), or in our intermittent feeder, depend upon time since last feeding or even the season (see below). Our observed glucose K_m of 0.37 mM suggests that hagfish have a relatively high affinity glucose transport in the intestine; however, post-prandial luminal glucose concentrations may well exceed the capacity of this transporter, much like that observed in a number of other vertebrates (Au et al. 2002; Driedzic et al. 2013). As such, it is likely that the hagfish system mirrors that of the mammalian system and employs the use of a facilitated GLUT following a meal. Whether hagfish have a sustained presence of GLUT in the apical membrane or if it is recruited to the membrane post-feeding remains to be determined and warrants further investigation. Notably, we did observe variability in the uptake rates between years, which has been observed in terms of glucose production rates in hagfish (Foster and Moon, 1986) and may be the result of differing nutritional states between those years, or the effect of season (Evans 1984).

Explicitly, the data collected in 2014 occurred during winter months after a longer period of starvation (Figs. 5.2, 5.5a and 5.5b) and show significantly lower transport rates than those collected in 2017 during summer months and a brief period of starvation (~1-2 weeks; Figs. 5.3, 5.4 and 5.5c). The transport rates are comparable within a year, with the exception of a noted statistical difference between the mid- and posterior hindgut, and that of the 0 μM phlorizin (Figs. 5.2 and 5.5b). The phlorizin data was collected a few months after the length assessment and did include a feeding period approximately 1 month before its collection. It appears then, that the regulation of glucose transport mechanisms varies incredibly in the hagfish following feeding and perhaps includes a further regression during the winter months. It is reported that hagfish intestinal tissue begins to regress as soon as 36 h following a meal likely due to the high metabolic demand of maintaining intestinal tissue in an infrequently feeding animal (Weinrauch et al. 2017). Such regression is accompanied by reduced rates of nutrient transport in Burmese python (Cox and Secor, 2008) as we likely observed here. Additionally, it has been suggested that in nature hagfish will experience food limitation in winter months (Hardisty 1979; Foster and Moon 1986). Perhaps then, the hagfish collected in 2014 winter had already begun a seasonal regression, accounting for the depressed uptake rates we observed.

The fact that we did not observe a sodium-dependent component may be explained by the mere presence of GLUT in the same tissue as SGLT. It is known that the primary post-prandial mechanism of uptake is *via* GLUT at a rate 2-3 fold greater than SGLT in the mammalian small intestine (Kellet and Helliwell, 2000). Therefore, if the

driving mechanism for glucose acquisition *via* SGLT was reduced, glucose could still be routinely acquired via GLUT.

Pharmacological results further suggest that both GLUT and SGLT are present within the hagfish hindgut. Phlorizin caused a significant reduction in glucose uptake rates at concentrations as low as 100 nM (Fig. 5.5b). It is well established that phlorizin is not an effective inhibitor of any of the 12 identified mammalian GLUTs (Bell et al., 1990) and is classified as a SGLT-specific inhibitor (reviewed by Ehrenkranz et al., 2005). Mechanistic characterization is required to ensure that this is true of the hagfish SGLT as well. Cytochalasin b, a known GLUT-specific inhibitor (Estensen and Plagemann, 1972), significantly reduced glucose uptake at 200 μM (Fig. 5.5c) and provides additional support for GLUT in the hagfish hindgut. Finally, we observed no effect of phloretin at the tested concentrations. It is a weaker inhibitor of SGLT than phlorizin (Chan and Lotspeich 1962), which may explain the lack of effect and also contribute to the variability observed. Another possibility is that the concentration of glucose used during inhibitor fluxes (5 mM) was still able to outcompete the inhibitor at these concentrations because the glucose has a higher affinity for the transporter. This response was observed in the red blood cells of a few fish species where inhibitors had no effect at 5 mM glucose, but were able to significantly reduce uptake at 0.5 mM glucose (Driedzic et al., 2013).

Evolutionary perspectives

Glucose transport proteins are conserved from bacteria to eukaryotes in terms of sequence and function (Coady et al. 1990; Baldwin 1992). Mechanistic characterization

of a Pacific hagfish erythrocyte GLUT demonstrates close functional and structural similarity to mammals not seen in other vertebrates, or even the other extant agnathan, the lamprey (Young et al., 1994). Such similarity between hagfish and mammals continues with our observation of both SGLT and GLUT in the hagfish intestine and suggests that this strategy evolved early to maximize the acquisition of a major metabolic energy source. Additionally, the appearance of islet cells containing insulin and somatostatin (Falkmer and Windbladh, 1964) emerges with this earliest-diverging vertebrate (Emdin, 1982); thus, the role of both this organ and its associated hormones on glucose acquisition and homeostasic mechanisms represent interesting avenues for future study, particularly as this may be the earliest appearance of such mechanisms. Indeed, insulin is known to regulate hagfish blood glucose concentration much like higher vertebrates (Falkmer and Matty, 1966; Inui and Gorbman, 1977), so perhaps other physiological roles of insulin, such as the post-prandial insertion of GLUT into apical membranes, are also evident. This would support our finding that both GLUT and SGLT are present in the hindgut, with GLUT likely present for instances of elevated glucose concentrations. Answering such questions will elucidate the earliest roles of insulin and the subsequent mechanisms of glucose acquisition and maintenance. That the primitive hagfish appear to conform to the mammalian strategy makes them a useful model for the early evolution of vertebrate glucose uptake mechanisms, particularly because these transporters are not ubiquitously expressed throughout vertebrate phylogeny (*e.g.* certain chondrichthyan species apparently do not express intestinal SGLT; Scharlau et al. 2004). Future molecular characterization and expression profiles of glucose transport mechanisms in a variety of lower vertebrates species will provide a more thorough

understanding of the origin, regulation and evolution of vertebrate transport systems and homeostatic mechanisms.

Chapter VI: Mechanisms of Oleic Acid Acquisition in the Hagfish Hindgut

A version of this chapter as been published.

Weinrauch, A.M., Glover, C.N., Goss, G.G. (2019) Lipid acquisition and tissue storage in hagfish: new insights from an ancient vertebrate *Journal of Comparative Physiology B*. 189(1): 37-45. Reproduced with permission of the co-authors of the manuscript.

Introduction

Owing to their basal position in vertebrate phylogeny, hagfish are models for studying the origin and evolution of vertebrate traits, including nutrient transport (Bardack 1998). However, while recent work has elucidated novel pathways of amino acid uptake in hagfish (Glover et al. 2011a), to date nothing is known regarding their transport of lipids. Lipids are of particular importance to hagfish owing to their vital role during extended periods of fasting (at least 11 months; Foster and Moon, 1986), wherein energy use transitions from glycolytic to lipolytic in nature and lipid energy reserves are mobilized (Emdin 1982). These energy reserves are evident as tissue lipid droplets across multiple organs (Mallatt and Paulsen 1986; Baldwin et al. 1991), with ~13% of the upper intestinal wet weight quantified as lipid (Spencer et al. 1966). Lipids have additional importance beyond their roles as an energy substrate, contributing to cellular structure and physiology as membrane-forming entities, cellular signaling moieties, and as transcriptional regulators, and thus must be routinely and effectively acquired.

In mammalian systems it is well known that lipids are ingested and hydrolyzed by digestive enzymes in the lumen of the anterior intestine to produce free fatty acids (FFA) and monoacylglycerols, which, when combined with bile salt, form micelles that are then absorbed (Olsen and Ringø 1997; Hirsch et al. 1998). Importantly, once in the intracellular compartment, these lipids are re-esterified into inert triglycerides before cycling on to various fates (Johnston and Borström 1964; Robinson and Mead 1973) in a process that appears conserved between mammals and teleost fishes (Sheridan 1988). Until recently, dietary lipids were thought to be acquired *via* passive diffusion, as

characterized by linear uptake rates and minimal substrate competition (Schoeller et al. 1995). However, the low aqueous solubility of fatty acids (FA), along with the need to regulate intracellular lipid stores, mandates regulation of lipid transport into cells and between tissues (Glatz and Luiken 2017). Supporting this, a number of evolutionarily-conserved transport proteins have since been identified as regulators of FA uptake. These include fatty acid transport proteins (FATP), acyl-coA synthetases (ACS), fatty acid translocase (FAT or scavenger receptor class B member 3), and fatty acid binding proteins (Hirsch et al. 1998; Nassir et al. 2007; Kazantzis and Stahl 2012; Glatz and Luiken 2017), each of which regulates the movement of the relatively insoluble FA across and between cells.

Given the reliance of hagfish on lipids for survival, as well as their basal position in vertebrate evolution, this study sought to: (1) examine the presence of lipid droplets across multiple tissues to assess their capacity for energy storage to support long-term fasting and, (2) characterize the mechanisms of intestinal oleic acid (OA; 18:1 cis-9) acquisition in the Pacific hagfish (*Eptatretus stoutii*) using *in vitro* gut sac preparations. OA was selected as a model long-chain fatty acid because it is a naturally-occurring lipid found in animal tissues, and has been identified as a primary monoenoic fatty acid in fish (Alasalvar et al. 2002). Further evidence for carrier-mediated transport was obtained by assessing the active regulation of OA acquisition following feeding. Remodeling of the hindgut to increase surface area while feeding is common amongst binge-eating animals including hagfishes (Weinrauch et al. 2017; Chapter II). Thus, we hypothesized that a greater density of carrier-mediated transporters could be apically expressed due to the

greater surface area and feeding should elevate OA uptake rates much like the pattern of FA uptake observed in fed anurans (Secor 2005).

Finally, insulin sensitivity has been demonstrated for specific isoforms of both FATP and SCARB3/FAT in multiple animal models (Luiken et al. 2002; Stahl et al. 2002). Insulin is also a known regulator of lipid metabolism in both fish and mammalian models, although the outcomes of these experiments are inconsistent and include studies that demonstrate lipolytic, lipogenic and/or no effect of this hormone (Harmon and Sheridan 1992; Navarro et al. 2006; Zhuo et al. 2015). Therefore, we sought to determine whether insulin-dependent fatty acid acquisition occurs in this insulin-sensitive ancient craniate (Inui and Gorbman 1977).

Materials and Methods

Animal care and husbandry

Pacific hagfish (*Eptatretus stoutii*; 48.8 ± 5.8 g; mean ± s.e.m.) were collected (Fisheries and Oceans Canada Collection Permit No. XR-202-2016) from Barkley Sound (Vancouver Island, Canada) using baited traps, before being transported to the University of Alberta and housed in a recirculating artificial salt water system (Instant Ocean SeaSalt; Spectrum Brands, Blacksburg, VA, USA) at 12 ± 2°C. Animals were permitted to acclimate to this system for three months, fed weekly prior to experimentation, and were fasted for one week unless otherwise stated. All experiments were conducted with the approval of the animal care committees of the University of Alberta (No. AUP0001126) and Bamfield Marine Sciences Centre (No. RS16-19).

In vitro intestinal uptake assay

Hagfish (N = 15) were euthanized in NaOH-buffered MS-222 (4 g L^{-1}; Syndel Laboratories Ltd., Nanaimo, Canada), and the hindgut (intestinal segment from immediately posterior to the bile duct to immediately anterior to the cloaca) was extracted and placed in aerating hagfish Ringer (in mM: NaCl 490; KCl 8; $CaCl_2$ $2H_2O$ 5; $MgSO_4$ $7H_2O$ 3; $MgCl_2$ $6H_2O$ 9; NaH_2PO_4 2; $NaHCO_3$ 8; HEPES 20; glucose 5; pH 7.6; all chemicals from Sigma-Aldrich Chemical Company, St Louis, USA). To effectively dissolve the lipid, deoxycholic acid was added to all mucosal solutions at a concentration of 2 mM. Gut sacs were tied as previously described (Glover et al. 2011b). Briefly, the intestinal tube was divided into equal segments that were secured at one end with suture. A sampling port made from a piece of flared PE-50 tubing was placed in the opposing

end, secured with suture and the sac was then filled with hagfish Ringer containing OA (5-500 μM) spiked with 2.5 μCi mL^{-1} of ^{3}H-OA (PerkinElmer, Boston, USA). The sac was then placed in OA-free aerating hagfish Ringer (termed serosal fluid). Mucosal and serosal samples were collected at the start and end of each 2 h flux period, combined with ScintiVerse ™ BD Cocktail scintillant (Fisher Scientific, Ottawa, Canada) and measured on a Beckman Coulter LS6500. Given that there was no statistical difference detected in OA uptake rate along the length of the intestine (mean uptake 1662 ± 232 pmol cm^{-2} h^{-1}; $p = 0.64$), the hindgut was divided into six equal segments. Each segment was assigned a treatment (e.g. concentration) that was altered with every hagfish to ensure an unbiased representation and even distribution of each section of the hindgut (anterior, medial and poster) across data points. Preliminary tests detected no radioactivity above background in the serosal fluid following a 2 h flux. Disappearance of OA from the mucosal solution was calculated using the following equation:

$$J_{oleic\ acid}\ (pmol\ cm^{-2}h^{-1}) = \frac{\Delta CPM/SAct}{SA/t} \qquad (1)$$

where **Δ**CPM represents the decrease of ^{3}H-OA radioactivity inside the gut sac, SAct is the specific activity (CPM $pmol^{-1}$), SA is the surface area (cm^{2}; calculated by spreading dissected gut sacs on graph paper and imaging with ImageJ; National Institute of Health). and t is the flux time (h).

Effect of feeding or insulin injection

Hagfish (N = 11) were fed freshly thawed squid until satiated. Eight hours following feeding, the intestine was flushed three times with 0.5 M NaCl and OA uptake was calculated as described above. This time point was chosen in accordance with peak

values for other previously measured post-prandial metrics (Weinrauch et al. 2017; Chapter II). To investigate the effects of insulin on intestinal OA transport and blood glucose concentration, hagfish (N = 10) were anaesthetized (1 g L^{-1} MS-222) and a 500 µL blood sample was drawn from the caudal sinus, and immediately spun at 14, 000 *x g*, as previously described (Tresguerres et al. 2007a). The resulting plasma was stored at -80°C until further analysis of glucose content. Bovine insulin (0.5 IU/kg) suspended in 0.16% sodium acetate and 3.5% sodium chloride was then injected intramuscularly as previous (Inui and Gorbman 1977). Control (sham) hagfish were injected with the sodium acetate/sodium chloride mixture alone. Animals were placed in 1500 mL containers with continuously flowing seawater. Twenty-four hours post-injection, a second blood sample was obtained and processed as above, before the determination of OA uptake in excised hindgut as detailed above. Plasma glucose concentration was measured in the collected blood samples according to previous studies (Weinrauch et al. 2018a; Chapter III,V). Briefly, 20 µL of sample was combined with 200 µL of glucose cocktail (50 mL triethylamine hydrochloride; 0.22 g $MgCl_2$; 0.050 g ATP; 0,050 g NAD; 2.4 U mL^{-1} glucose-6-phosphate dehydrogenase; pH 7.53). After a 5-min incubation at room temperature, absorbance at 340 nm was recorded on a Spectramax 190 (Molecular Devices, Sunnyvale, CA, USA) and 5 U per sample of hexokinase dissolved in triethylamine hydrochloride (pH 7.53) was added. Samples were incubated for 15 min after which a final reading (A340) was obtained. Glucose concentration was calculated as the change in absorbance between the initial and final readings and converted to µM using a glucose standard curve.

Transmission electron microscopy

Tissue samples (2 mm^3; myotomal red muscle, second gill pouch on the left, liver, gall bladder, foregut, hindgut, archinephric duct, ventricle muscle of the systemic heart) were collected from fasted (1 week) hagfish (N = 6) and processed as described previously (Weinrauch et al. 2015). Images were captured using a Philips FEI Transmission electron microscope (Morgagni 268) operating at 80 kV, and a Gatan Orius CCD camera.

Statistical analysis

Concentration dependence of intestinal OA uptake was assessed using sigmoidal curve-fitting on SigmaPlot 11 with SigmaStat integration 3.5 (Systat software Inc., San Jose, CA, USA):

$$y = \frac{a}{1+ e^{-(x-\frac{x_0}{b})}} \qquad (2)$$

where *a* represents the maximal rate of uptake (i.e. equivalent to a Michaelis-Menten J_{max}; (Glover et al. 2011a), *b* represents the slope at the transition point, and x_o represents the substrate concentration at the transition point (i.e. equivalent to a Michaelis-Menten K_m). OA uptake rates obtained from fed/fasted and sham-/insulin-injected hagfish passed tests of equal variance and normality. As such, unpaired t-tests with equal variance were used on all data, apart from plasma glucose concentrations, which were analyzed with a paired t-test with equal variance in Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). All data have been expressed as means ± s.e.m. and significance for all tests was accepted at $p < 0.05$.

Results

Lipid droplet tissue distribution

Multiple lipid deposits were observed in all tissues examined (red muscle, gill, liver, gall bladder, foregut, hindgut, archinephric duct, ventricle muscle; Fig. 6.1a-h). Deposits were evident as electron-dense spheres within specific cells. The largest droplets were observed in liver tissue (up to 4.2 μm in diameter), gall bladder (up to 2.5 μm in diameter) and archinephric duct (up to 2.8 μm in diameter). The droplet diameter recorded in all other tissues ranged from 0.5 μm (ventricle muscle, foregut) to ~1.1 μm (red muscle, hindgut, gill).

Concentration-dependent OA uptake

^{3}H-OA was absorbed across the hindgut of the Pacific hagfish in a concentration-dependent manner, such that increasing substrate concentration led to increasing uptake, up until 200 μM where further increases in OA led to only minimal increases in transport (Fig. 6.2). The data best fit a sigmoidal distribution ($R^2 = 0.84$), rather than conforming to hyperbolic Michaelis-Menten kinetics ($R^2 = 0.71$). From the sigmoidal fit, the uptake range of 1311 ± 97 pmol cm^{-2} h^{-1} (*a*; J_{max}-like value), slope at the transition point of 22 ± 5 (*b*), and affinity constant of 55 ± 7 μM (OA concentration at the point of transition; x_0; K_m-like value) were determined.

Effect of feeding or insulin on OA uptake

Feeding induced significant increases in OA flux when compared to fasting rates at both 75 μM (2.2 fold increase; $p = 0.0086$; Fig. 6.3a) and 150 μM (5.3 fold increase; *p*

= 0.0036; Fig. 6.3b). Application of bovine insulin did not induce an effect on the OA transport rates at either 75 μM (Fig. 6.4a; p =0.66) or 150 μM OA (Fig. 6.4b; p = 0.28). However, the hormone did induce a significant lowering of plasma glucose concentrations 24 h post-injection when compared to controls (Fig. 6.4c; p = 0.01).

Figures

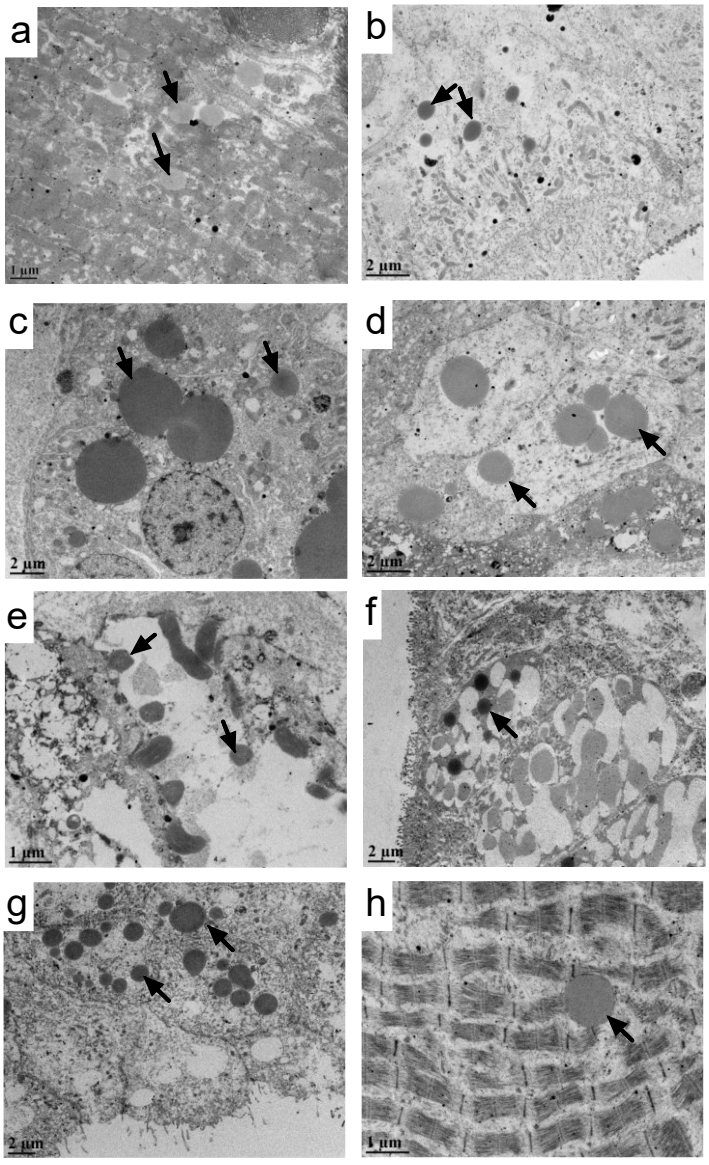

Figure 6.1 Representative TEM micrographs demonstrating the presence of lipid droplets (arrows) within Pacific hagfish tissues. Ventricular muscle (a), gill (b), liver (c), gall bladder (d), foregut (e), hindgut (f), archinephric duct (g), myotomal red muscle (h).

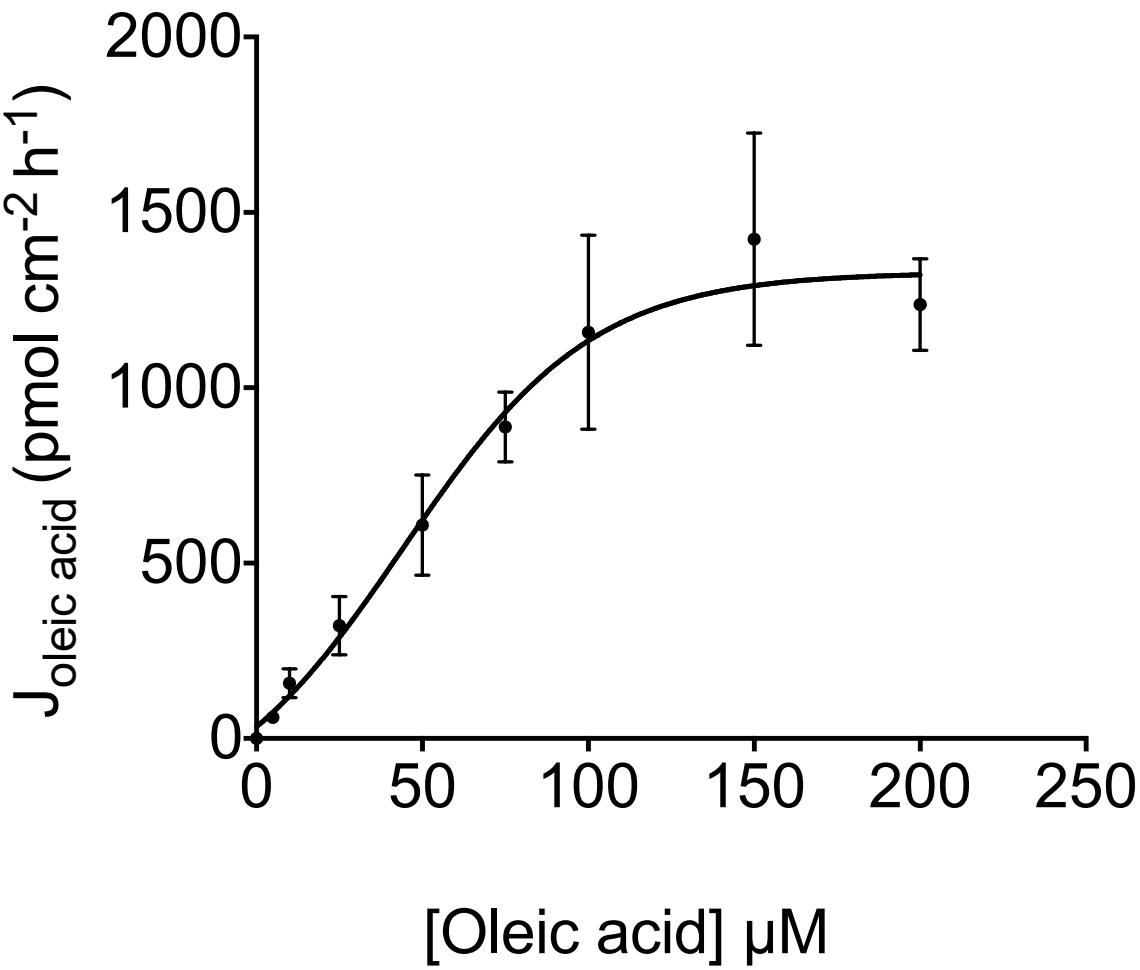

Figure 6.2 Concentration-dependent uptake of ^{3}H-oleic acid into the hindgut of fasted Pacific hagfish (*Eptatretus stoutii*). Equal segments of hagfish hindgut were tied into sacs and muscoal disappearance of ^{3}H-oleic acid was quantified and normalized across surface area. Plotted points represent means ± s.e.m of 5-6 preparations. Curves and calculated apparent K_m and J_{max} were modeled on raw data using SigmaPlot (11.0).

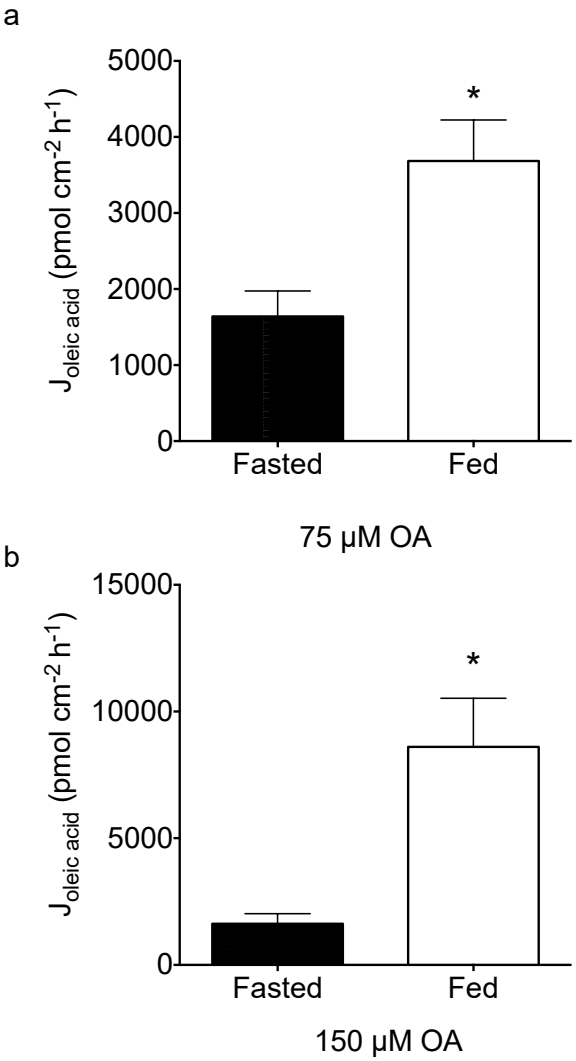

Figure 6.3 Feeding induces significant increases in oleic acid acquisition in comparison to fasted animals. Oleic acid (OC) acquisition was measured at both 75 µM (a) and 150 µM (b). Bars represent the means + s.e.m of 5-6 hagfish. Data were analyzed with unpaired t-tests of equal variance in GraphPad Prism 6.0. * denotes significant difference within a concentration treatment with $p < 0.05$

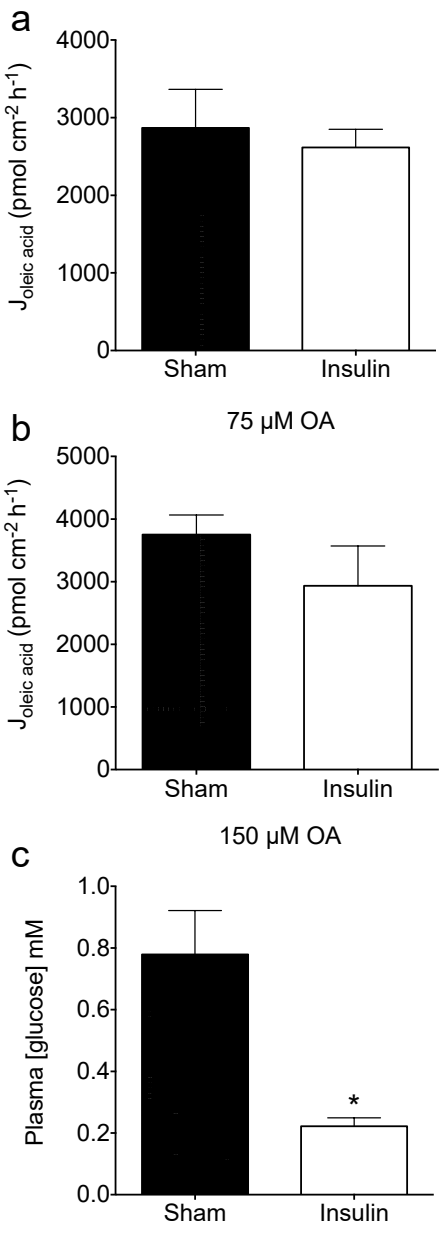

Figure 6.4 Bovine insulin does not affect hindgut oleic acid acquisition but does reduce plasma glucose concentration. The effect of sham (black bars) or insulin (white bars; 0.5 IU/kg) injection on oleic acid (OA) uptake at 75 µM (a) and 150 µM (b), or on plasma glucose concentrations (c). Bars represent means + s.e.m of 5 hagfish. Data were analyzed with unpaired t-tests with equal variation (a/b) or a paired t-test (c) in GraphPad Prism 6.0. * denotes significant difference with $p < 0.05$.

Discussion

This study is the first to kinetically characterize facilitated OA uptake in any fish intestine and provides confirmation of an evolutionarily conserved process for FA uptake in an agnathan. The sigmoidal nature of concentration-dependent uptake suggests that multiple transport pathways are utilized in the hagfish intestine, which could conceivably consist of FATP and SCARB3/FAT, alongside other molecules such as ACS and FABP (Hirsch et al. 1998; Kazantzis and Stahl 2012). A significant increase in OA uptake rate was observed after feeding, but it is unclear whether this was due to a decrease in lipolytic enzyme activity in fasted animals, or an increase in the apical expression of transport proteins. We did not observe an effect of insulin on OA uptake but this may be the result of a constitutively expressed transporter, similar to that observed in mammals, or lack of potency of the heterologous hormone.

Tissue lipid droplets

Hagfish are a unique model within which to examine lipid acquisition. The extensive periods of starvation (11 months; Foster and Moon, 1986) that they may undergo as scavengers necessitates a long-term energy store, and a transition from carbohydrate-based fuels, which are the preferred energy substrate, to fat-based fuels (Emdin 1982). Similar lipid mobilization has been observed in a number of teleost species including rainbow trout, albeit over much shorter fasting durations (Jezierska et al. 1982). Consistent with the concept that lipid is an energy store in hagfish, intracellular lipid deposits were always observed throughout studied tissues, including in intestine, liver, gall bladder, gill, ventricle muscle and archinephric duct (Fig. 1a-h), with no

apparent adipose deposits grossly visible in these tissues. These findings are consistent with previous studies that have focused on single tissues. For example, a detailed histochemical characterization of Atlantic hagfish striated muscle fibres also documented significant lipid stores (1-10 μm in diameter) in red and intermediate fibres, albeit to a lesser extent in the latter fibre type (Mellgren and Mathisen 1966). Red muscle lipid droplets are typical across vertebrate lineages as they provide energy to the numerous mitochondria and support aerobic muscle movement (Lin et al. 1974; Walther and Farese Jr 2012). Fish store fat in the red muscle to withstand long periods of fasting (Kiessling and Kiessling 1993) or for extra energy during migration (Doucett et al. 1999). The hagfish may similarly draw upon these lipid stores during extensive fasting periods or during burst activity such as when actively predating (Zintzen et al. 2011).

Our finding of lipid droplets within each gill cell type, is a feature not common to other fishes, but has been previously observed in hagfish (Mallatt and Paulsen 1986). Mallatt and Paulsen (1986) suggested that the lipid stores in pavement, intermediate and basal cells are utilized in the generation of the secreted lipo-mucus, but that the largest store, found in the pillar cells, may in fact be an energy store. It is well known that liver has a high capacity to store fat and accordingly the hagfish liver contained the largest lipid droplets that we measured, with diameters 2-3 μm in range. Liver lipid stores are often dynamic, being used and deposited continuously (Walther and Farese Jr 2012), a process that would give rise to the varying droplet sizes we observed (Fig. 1c).

Similar to the pattern seen in the liver, lipid droplets of varying size were observed along the entirety of the hagfish intestine (Fig. 1e,f). This corresponds to observations in the mammalian small intestine where differences in lipid droplet size were attributed to the dynamic nature of the intestine and the corresponding variation in lipid profile that changes with dietary fat levels (Zhu et al. 2009). Furthermore, a lack of droplet variation is in keeping with the equivalent uptake rates of lipid we observed independent of gut segment assayed. Spencer (1966) also reported the presence of lipid-(triglyceride) filled vacuoles in the luminal portion of hagfish intestine, similar to those observed in the present study. Surprisingly, these droplets were still present after ~1 week of starvation in the current study. In mammals, triglycerides are absorbed intestinally and form lipid droplets until they are shuttled from the cell as a component of chylomicrons minutes to hours following a meal (Walther and Farese Jr 2012). As regression of hagfish intestinal tissue occurs within 36 h of a meal (Weinrauch et al. 2017; Chapter II), we expected lipid stores to be exported to tissues that maintain functional capacity during starvation, yet one week after a meal intestinal lipid droplets remained. Whether these enterocyte stores persist over extended periods of food deprivation in hagfish is unknown and warrants further investigation. Hagfish therefore exhibit a phenotype of absorption, with local storage in gut tissues, seemingly at the expense of post-absorptive lipid distribution to other body tissues. This description is very similar to a human disorder, congenital β-lipoprotein deficiency, which can result in a retention of intestinal lipid content, even following fasting (Dobbins 1966; Spencer et al. 1966). Consequently, hagfish may be a model organism for the study of this disease.

The archinephric duct had a particular abundance of lipid droplets (Fig. 1g) and these may be akin to those found in the adrenal cortex of mammals. Lipid stores in the adrenal cortex of mammal are believed to be involved in steroid synthesis and we hypothesize that a similar function may occur in the hagfish. In hagfish, prominent catecholamine stores occur in cardiac tissue (Perry et al. 1993), despite synthesis typically occurring in the adrenal tissues. While this is a hypothesis that remains to be confirmed in hagfish (Clifford et al. 2017a), the large lipid deposits associated with the archinephric duct would provide a ready source of steroid hormone precursors. Finally, we noted lipid droplets in the ventricle muscle of the hagfish (Fig. 1a). Although this is a pathogenic state in many mammalian models, the hagfish heart is known to utilize glycogen along with a secondary fuel source during anoxia (Gillis et al. 2015). Gillis et al. 2015 hypothesized that the breakdown of triglycerides into glycerol may be that secondary fuel source and the deposits we observed provide support for this idea.

Concentration-dependent kinetics of oleic acid transport

Intestinal uptake of OA in hagfish was saturable and modulated by feeding. These are two important criteria for a regulated transport pathway and suggest that OA transport is mediated by specific transporters rather than solely by diffusion, although a diffusive component was evident at higher substrate concentrations (500 μM; data not shown). Similar profiles of FA uptake which include a carrier-mediated component at lower concentrations and a diffusive component at higher substrate concentrations persist in soft-bodied marine invertebrates and tapeworms (Arme and Read 1968; Testerman 1972), as well as in isolated enterocytes of rainbow trout (Oxley et al. 2005), mammalian

small intestine sacs (Molina et al. 1990), stable cell lines (Schaffer and Lodish 1994) and adipocytes expressing mammalian FATP (Stahl et al. 2002).

The sigmoidal rather than hyperbolic saturation we observed for OA uptake is likely a result of multiple transport pathways with overlapping affinities (Brauchi et al. 2005), or from cooperativity between subunits or substrates for transport. Given the likelihood of multiple transport proteins (SCARB3/FAT, FATP) and esterification enzymes (ACS), coupled with the tight regulation of lipid circulation (FABP), a more complex, sigmoidal fit for our data is not unexpected. Alternatively, modifications to the substrate (*e.g.* esterification) or the manner in which FA are retrieved by the transporter (whether passed from FABP or SCARB3/FAT; Stahl et al. 1999) could result in allosteric modification of a single transporter, which would provide a sigmoidal kinetic curve. With each of these potential contributors to FA uptake yet to be fully characterized even in mammalian models, the exact nature of FA transport and its complexities remains unexplored.

Although there are difficulties in comparing kinetic parameters across studies using different methodologies and tissues of interest, our calculated kinetic parameters (apparent K_m of 55 μM) are comparable to those previously reported. For example, the K_m of OA in Atlantic salmon hepatocytes is 46 μM (Zhou et al. 2010). However, in a polychaete whole body study, a K_m of of 1.2 μM was observed (Testerman 1972). The lower affinity of OA uptake hagfish gut may be explained by the high concentrations of

FA in the hagfish gut lumen, relative to the low seawater lipid concentrations that would bathe the integument of marine invertebrates.

Consideration of the cellular metabolism of OA and its potential effect on OA acquisition is also required. Although FA are esterified upon reaching the intracellular compartment, and so should maintain an inwardly-driven concentration gradient, Zhou et al. (2010) suggest that uptake saturation may be reached as intracellular metabolism is a rate-limiting step. However, this is unlikely to explain the saturable kinetics seen in the current study, as at the highest tested concentration (500 μM), the presence of a diffusive component increased total uptake, implying that saturation was not the result of intracellular metabolic constraints.

Regulation of oleic acid transport

We did not see a difference in OA uptake rates along the length of the hindgut. This is in contrast to reports from multiple teleost species where differential rates of uptake occur along the digestive tract. Indeed, turbot demonstrate a greater lipolytic capacity and decreased total lipid in a food bolus, paired with increased FFA at the posterior intestine (Koven et al. 1994), whereas the majority of lipolysis and absorption occurs in the anterior intestine of cod (Lie et al. 1987). The lack of regional specialization in hagfish intestinal uptake is, however, consistent with the lack of differentiation in morphology along the length of the hagfish hindgut. The entirety of the hindgut in hagfish is comprised of zymogen granule cells interspersed amongst columnar cells that are topped with a brush border (Adam 1963; Weinrauch et al. 2015). This simple, straight hindgut is relatively lacking in surface area and so the entire length of the hindgut is

likely involved in nutrient acquisition. This would be of benefit to the animals because the infrequency of meals would prioritize the need to maximize nutritive uptake capacity (Glover and Bucking 2015).

Another mechanism that facilitates nutrient acquisition is the upregulation of transport following feeding. Previous studies have shown that surface area of the hagfish gut is increased after a feeding event by the lengthening of the brush border microvilli and overall thickening of the mucosa (Weinrauch et al. 2017; Chapter II). This morphological remodeling, in combination with recruitment of more FA transporters to the apical membranes, is likely able to account for the significant increase in post-prandial OA uptake (Fig. 6.3a,b). In the current study, we detected significant effects of feeding at two tested substrate concentrations, one representing saturation (150 μM), and thus indicative of an effect on the number of available transporters, and one concentration representative of the exponential phase of uptake (75 μM), suggestive of an alteration in transporter affinity or isoform. Alternatively, lipolytic enzyme activity may be increased after feeding, yielding elevated uptake rates, as has been observed in teleosts (Overnell 1973). However, it is unlikely enzymatic activity alone could account for the drastic increases in lipid uptake.

It is also worth noting that the metabolic substrate an organism utilizes is altered depending upon which fuels are available. Hagfish demonstrate a preference for carbohydrates when provided with both carbohydrate and lipid sources (Sidell et al. 1984) and trout myotubes similarly decrease OA metabolism when the more

energetically favourable glucose is available (Sánchez-Gurmaches et al. 2010). Perhaps then, the glucose within the hagfish saline (5 mM), or within the meal itself, had an effect on OA metabolism. It is therefore possible that our reported increased uptake rates for OA underestimate the potential for OA uptake in fed hagfish.

Insulin is a major regulator of teleost lipid homeostasis as it reduces circulating free FA (Polakof et al. 2010), and increases both lipase activity (Albalat et al. 2006) and lipid deposition (Navarro et al. 2006). In hagfish, insulin induces a similar increase in lipid deposition; however, circulating FFA are not altered (Emdin 1982). In the present study, bovine insulin did not induce an effect of OA uptake in hagfish (Fig. 6.4a,b), but did reduce plasma glucose levels (Fig. 6.4c). One explanation for the absence of effect of insulin on hagfish FA uptake is that a heterologous hormone was used. Although hagfish insulin is relatively similar in structure to mammalian insulin, even small changes may manifest differences in terms of receptor activation (Cutfield et al. 1979). Nonetheless, Kleveland et al. (2006) utilized bovine insulin (3.3 μM) in an Atlantic salmon model and demonstrated an increase in SCARB3/FAT expression in a hepatocyte culture, while porcine insulin (1 μM) was sufficient to increase FA uptake in Atlantic salmon cultured myotubes (Sánchez-Gurmaches et al. 2011). Their responses were evoked using insulin concentrations that are greater than those considered physiologically relevant, yet they suggested higher concentrations might be necessary for activation to compensate for structural differences between the insulin molecules that could impact activity. Perhaps doses well beyond those that are physiologically relevant would have elicited a significant increase of OA uptake in the present study, although the reduction in plasma

glucose argues against this. It is more likely that the insulin-insensitivity may relate to the phylogenetic position of hagfish. Having diverged from the vertebrate lineage prior to a vertebrate-gnathostome transition gene duplication (Escriva et al. 2002), fewer isoforms of FA transporters may be present, and in fact, the basal state may be one of insulin insensitivity. Interestingly, mammalian intestinal isoforms of FATP are also insulin insensitive (Stahl et al. 2002). Perhaps this isoform is the more ancient isoform and is constitutively expressed to routinely acquire FA.

Conclusions

Our study supports hagfish as a useful model for FA uptake owing to: (a) their evolutionary position, (b) their extended tolerance to food deprivation and subsequent use of lipids as an energy source, and (c) the presence of active and regulated uptake of FA. In addition, our kinetic analysis demonstrates that hagfish gut transport of lipids is of a similar affinity to that demonstrated in vertebrate models. As hagfish can survive several months without feeding they must maximize nutrient uptake when feeding is possible. Our demonstration of elevated post-feeding FA acquisition supports this. While there are a multitude of post-prandial signals that could elicit this effect, we have determined that insulin is not the responsible hormone. Hormone-containing islet cells first appear with the hagfishes (Falkmer and Winbladh 1964) and while cyclostomes have a wide range of hormonal peptides that resemble those of higher vertebrates (Van Noorden 1990), they do not have a full hormonal complement (Conlon and Falkmer 1989). Thus, hagfish could be a useful tool to examine early actions of hormones and their regulation of FA acquisition. Finally, given that hagfish have an extensive supply of lipid droplets across tissues, future examination into the regulated use of these stores could provide insight

into the incredible tolerance to food deprivation that the hagfish demonstrate and could also be applicable to the understanding of human diseases of lipid regulation dysfunction.

Chapter VII: Mechanisms of Dipeptide Acquisition in the Hagfish Hindgut

A version of this chapter has been submitted for publication.

Weinrauch, A.M., Blewett, T.A., Glover, C.N., Goss, G.G. (201X) Acquisition of alanyl-alanine in an Agnathan: Characteristics of dipeptide transport across the hindgut of the Pacific hagfish *Scientific Reports* Reproduced with permission of the co-authors of the manuscript.

Introduction

Hagfish are a living representative of the invertebrate to vertebrate transition, owing to their basal position in vertebrate phylogeny (Bardack 1998). Indeed, hagfish are capable of acquiring nutrients across multiple epithelia including the skin and gills, much like soft-bodied marine invertebrates (Glover et al. 2011a). Although these animals are agastric and have a primitive digestive system consisting of a straight, undifferentiated hindgut (Adam 1963; Weinrauch et al. 2015), nutrient transport mechanisms appear to be highly conserved relative to those of later-diverging vertebrates (Young et al. 1994; Glover et al. 2011b; Schultz et al. 2014; Glover et al. 2016; Weinrauch et al. 2018a,b; Chapter V, VI). These animals are therefore ideally suited for studies aiming to understand the evolution of early vertebrate nutrient transport systems.

The carnivorous hagfish have a varied, protein-rich diet, owing to their behaviour as both an active benthic predator and opportunistic scavenger of vertebrate and invertebrate species (Martini 1998; Zintzen et al. 2011). Upon ingestion, proteins are catabolised by an array of proteases, with trypsin, carboxypeptidase A, and leucineaminopeptidase activities all having been quantified in the hagfish hindgut (Nilsson and Fänge 1970). Amino acids are the ultimate products of protein catabolism and are essential nutrients with critical roles as energy substrates, and as the building blocks of nitrogen-containing compounds including hormones, neurotransmitters and proteins. Whilst amino acids can be directly acquired across the gut lumen, *via* specific and regulated mechanisms previously characterised in hagfish (Glover et al. 2011b),

~80% of dietary amino acids are instead absorbed as di- and tri-peptides (Leibach and Ganapathy 1996), which have not been examined in this species.

Intestinal dipeptide acquisition is chiefly mediated by proton-driven transporters located on the enterocyte brush border (Adibi 2003). In effect, the ubiquitous sodium-potassium ATPase (NKA) works in concert with the sodium proton exchanger (NHE) and creates a cellular gradient favouring efflux of protons from the cell, which is coupled to the influx of peptides into the cell (Mackenzie et al. 1996; Thwaites et al. 2002). Once within the enterocyte, the peptides can be further hydrolyzed by cytoplasmic peptidases into free amino acids and transported across the basolateral membrane using various amino acid transporters, or the dipeptide may be transported intact into the circulation *via* an, as of yet, unidentified peptide transporter (Hu et al. 2008; Boudry et al. 2010; Berthelsen et al. 2013). Since the majority of dietary amino nitrogen is acquired in this fashion, the characterisation of dipeptide transport in the hagfish will contribute significantly to an understanding of their nutrition.

Thus, the objective of the present study was to utilise a craniate, with the potential for insight into the evolution of nutrient transport systems, to model mechanisms of early vertebrate intestinal peptide uptake. Consequently the model dipeptide, L-alanyl L-alanine (L-ala- ala), was used to examine absorption across the hindgut of the Pacific hagfish (*Eptatretus stoutii*) using standard *in vitro* intestinal transport assays. Concentration-dependent transport kinetics of the substrate were examined to confirm the presence of saturable transport, indicative of a specific transporter or transport pathway. To ensure

that the transport measured was that of the dipeptide and not of hydrolyzed monomers appearing in the mucosal medium (Glover et al. 2011b), we conducted dipeptide transport assays in the presence of high alanine (concentration 10-fold that of $_{L}$-ala-ala). If apical transport of the dipeptide $_{L}$-ala-ala occurred *via* a specific peptide transporter, then no effect of mucosal alanine addition would have been expected. Furthermore, we sought to determine whether hagfish peptide transport was dependent upon sodium and/or proton concentrations (thereby implicating a role for NHE) as discussed above. Finally, we established whether hagfish experience a postprandial up-regulation of transport rates following a meal, indicative of transporter regulation and evident in other binge-feeding animals (Secor 2005).

Materials and Methods

Animal care and husbandry

Pacific hagfish (*Eptatretus stoutii*; N = 33, 78 ± 6 g; mean ± standard error of the mean (s.e.m.)) were collected using baited traps in July and August 2017 from Barkley Sound, Bamfield, BC, Canada under license from Department of Fisheries and Oceans Canada (permit number XR-136-2017). Upon capture, hagfish were immediately transferred to Bamfield Marine Sciences Centre and placed in 500-L tanks receiving flow-through seawater (12 ± 2°C). Hagfish were fasted one week prior to experimentation. The exception was feeding studies, where hagfish were fed squid until satiation. As previous experiments have demonstrated significant physiological effects 8 h following feeding(Weinrauch et al. 2017), we opted to use intestinal sacs from euthanized fed fish at this time point for all feeding experiments. All procedures were approved by the Canadian Council on Animal Care Committee under protocols for each of the University of Alberta Animal Care (AUP00001126) and Bamfield Marine Sciences Animal Care and Use Committee (RS17-03).

Solutions

Hagfish Ringer (in mM: NaCl 490, KCl 8, $CaCl_2$ $2H_2O$ 5, $MgSO_4$ $7H_2O$ 3, $MgCl_2$ $6H_2O$ 9, NaH_2PO_4 2, $NaHCO_3$ 8, HEPES 20, glucose 5; pH 7.8) was utilised for all serosal salines. The mucosal saline was of the same composition but contained $_{L}$-ala-ala (0-10,000 μM) and 0.05 μCi mL^{-1} ^{3}H-$_{L}$-ala-ala (American Radiolabelled Chemicals, St. Louis, MO, USA). To examine the effect of pH on dipeptide uptake (5000 μM $_{L}$-ala-ala), the mucosal pH was adjusted from 5.8 - 8.8 with minute amounts of concentrated HCl or

KOH. To examine the effect of reduced sodium on dipeptide uptake (5000 μM L-ala-ala), both mucosal and serosal salines were sodium-substituted as follows (in mM: N-methyl D-glucamine (NMDG) 488, KCl 8, $CaCl_2$ $2H_2O$ 5, $MgSO_4$ $7H_2O$ 3, $MgCl_2$ $6H_2O$ 9, KH_2PO_4 2.06, $KHCO_3$ 8, HEPES 20, glucose 5, and adjusted to pH 7.8). To ensure that the dipeptide was not being hydrolyzed and that the measurement of transport was not that of individual amino acids (alanine), an experiment was conducted where a high L-alanine concentration (50 μM) was added to a mucosal L-ala-ala concentration of 5 μM. Prior to all experimentation, each solution was osmotically balanced with mannitol on a Vapro vapor pressure osmometer (model 5520; Wescor, Logan, UT, USA). All chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) unless otherwise specified.

Radioisotope transport assays

Hagfish were euthanized with neutralized 4 g L^{-1} MS-222 (Syndel Laboratories, Nanaimo, BC, Canada) and dissected on the ventral surface to expose the intestine. The hindgut (from the bile duct to the cloaca) was excised, rinsed with hagfish Ringer and placed into temperature-controlled, aerating hagfish Ringer. The hindgut was separated into 2 – 4 cm sections and tied into sacs as described previously (Glover et al. 2011b). Of note, uptake in the pharyngeocutaneous duct was not conducted owing to its small, fragile nature and the conglomeration of mucus cells, as opposed to brush border 'absorptive' cells (Weinrauch et al. 2015). Briefly, one end of the intestinal segment was tied off with suture silk and a flared piece of PE-90 tubing was inserted into the opposing end and subsequently secured with suture. Mucosal hagfish Ringer containing the radiolabeled substrate was added to the sac by way of the flared PE-90 until the sac was

turgid. An initial sample was drawn from both the mucosal and serosal compartments and the sac was weighed. The sac was then placed in aerating hagfish Ringer with temperature maintained at 12 ± 2°C using flow-through seawater. Following a 2-h flux period, the sac was blotted dry and re-weighed, and final mucosal and serosal samples were obtained. Each sample was combined with 4 mL of Optiphase liquid scintillate (Perkin Elmer, Boston, MA, USA) and kept in the dark for at least 2-h before counting on a Beckman Coulter scintillation counter (LS6500; Beckman Coulter Inc., Miami, FL, USA). The sac was then dissected lengthwise and spread on graph paper with surface area determined using ImageJ Software (National Institute of Health). The flux of dipeptide was calculated as follows:

$$J_{ala-ala} = \frac{\Delta CPM/SAct}{SA/t} \qquad (1)$$

where ΔCPM represents the change in radioactive counts per minute measured as either mucosal disappearance or serosal appearance (see below), SAct is the specific activity (amount of ionizing radiation per minute per total quantity of the molecule; CPM $nmol^{-1}$), SA is the surface area (cm^2) and t represents time of flux (h). In order to examine the two steps of absorption (*i.e.*, movement of dipeptide from gut lumen into tissue, and then movement of substrate from gut tissue into the serosa), uptake was determined both on the basis of mucosal disappearance, and on the basis of serosal appearance. To ascertain whether there were regional differences in dipeptide transport along the length of the hindgut, the source of each sac (i.e. anterior or posterior hindgut) was recorded. Statistical analysis revealed that there were no differences in transport rate along the length of the hindgut (Fig. 7.1). Consequently, different hindgut sections from an individual hagfish were methodically rotated across concentrations/treatments, such that

an equal distribution of each section of the hindgut (anterior and posterior) was tested for each experimental treatment. No treatment was tested more than once from a single hagfish to avoid pseudoreplication.

Statistical analysis

The Shapiro Wilk normality test was used to verify normality of all datasets (presented as means + s.e.m.). Concentration-dependent kinetics of dipeptide uptake were determined using SigmaPlot (ver. 13; with Systat software Inc, integration, San Jose, CA, USA). Datasets were fit using both sigmoidal distribution kinetics ($Eq1.\ y = \frac{a}{1+ e^{(\frac{-(x-x_0)}{b})}}$; $R^2 = 0.9163$) and Michaelis-Menten kinetics ($Eq2.\ y = \frac{v_{max} \times X}{K_m+X}$; $R^2 = 0.9159$) with sigmoidal analysis resulting in the better fit. Tests of statistical significance were performed using GraphPad Prism 6 (La Jolla, CA, USA) with an unpaired t-test with equal variance (for analysis of anterior *vs*. posterior uptake or fed *vs*. fasted datasets where gut sacs were not pair-matched), a paired t test (for analysis of high alanine and low sodium treatments where pair-matched analysis was conducted within each animal) or a one-way ANOVA (testing the effect of mucosal pH), where appropriate. Differences were considered significantly different at $p < 0.05$.

Results

Kinetics of Dipeptide Uptake

There were no statistical differences in dipeptide uptake rates along the length of the hagfish hindgut (i.e. more anterior sections of hindgut displayed statistically similar uptake rates for a given treatment as more posterior sections of hindgut; Fig. 7.1; p = 0.76). All subsequent analyses were thus conducted independently of gut sac source along the length of the hindgut. Mucosal disappearance of dipeptide was concentration-dependent and contained both a diffusive component (Fig. 7.2a), as well as a saturable component. The diffusive component was prevalent only at substrate concentrations in excess of 5000 μM. For the determination of saturable kinetics, only substrate concentrations equal to or less than this threshold were used. The data best fit a sigmoidal distribution (R^2 = 0.9163), rather than hyperbolic Michaelis-Menten kinetics (R^2 = 0.9159). Using the sigmoidal equation (*Eq1.* Fig 7.2b), the calculated maximal uptake (*a*; equivalent to Michaelis-Menten J_{max}) was calculated as 70 ± 3 nmol cm^{-2} h^{-1}. The concentration at the slope of the transition point (x_0; *Eq1.,* equivalent to Michaelis-Menten K_m) was calculated as 1072 ± 81 μM. Calculation of uptake kinetics based on serosal appearance yielded a single saturable curve with Michaelis-Menten kinetics yielding a maximal uptake (J_{max}) of 70 ± 14 nmol cm^{-2} h^{-1} and a substrate affinity (K_m) of 2696 ± 1573 μM (Fig. 7.2c).

Effect of changing pH and sodium concentration on dipeptide uptake

The presence of a high concentration of mucosal alanine (10X the concentration of ala-ala) did not significantly alter dipeptide transport (Fig. 7.3; p = 0.37). The

dipeptide transport rate also remained unaffected by a change in pH (Fig. 7.4; $p = 0.59$). However, the concentration of sodium in the mucosal solution did alter rates of transport (Fig. 7.5) with low sodium eliciting a decrease in transport rates in comparison to control rates (mucosal disappearance = 41%; $p = 0.01$).

Effect of feeding on dipeptide uptake

A significant 78% increase in the rate of dipeptide uptake was noted in intestinal sacs obtained from hagfish 8 h after feeding (Fig. 7.6a; $p = 0.04$). However, this effect was only observed when transport was calculated in terms of mucosal disappearance. There was no difference in rates of $_{L}$-ala-ala transport when determined on the basis of serosal appearance between fed and fasted animals (Fig. 7.6b; $p = 0.12$).

Figures

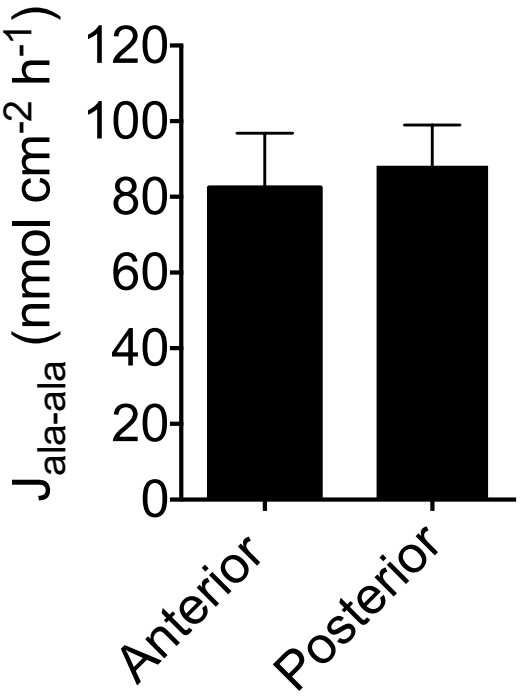

Figure 7.1 Rate of $_{L}$-ala-ala uptake are equal in the anterior and posterior portions of the hagfish hindgut. Rates of $_{L}$-ala-ala uptake ($J_{ala-ala}$; nmol cm^{-2} h^{-1}) in anterior and posterior Pacific hagfish hindgut at 5000 μM. Bars represent means + s.e.m of 5-6 animals. Analysed using an unpaired t-test at an α-level of 0.05.

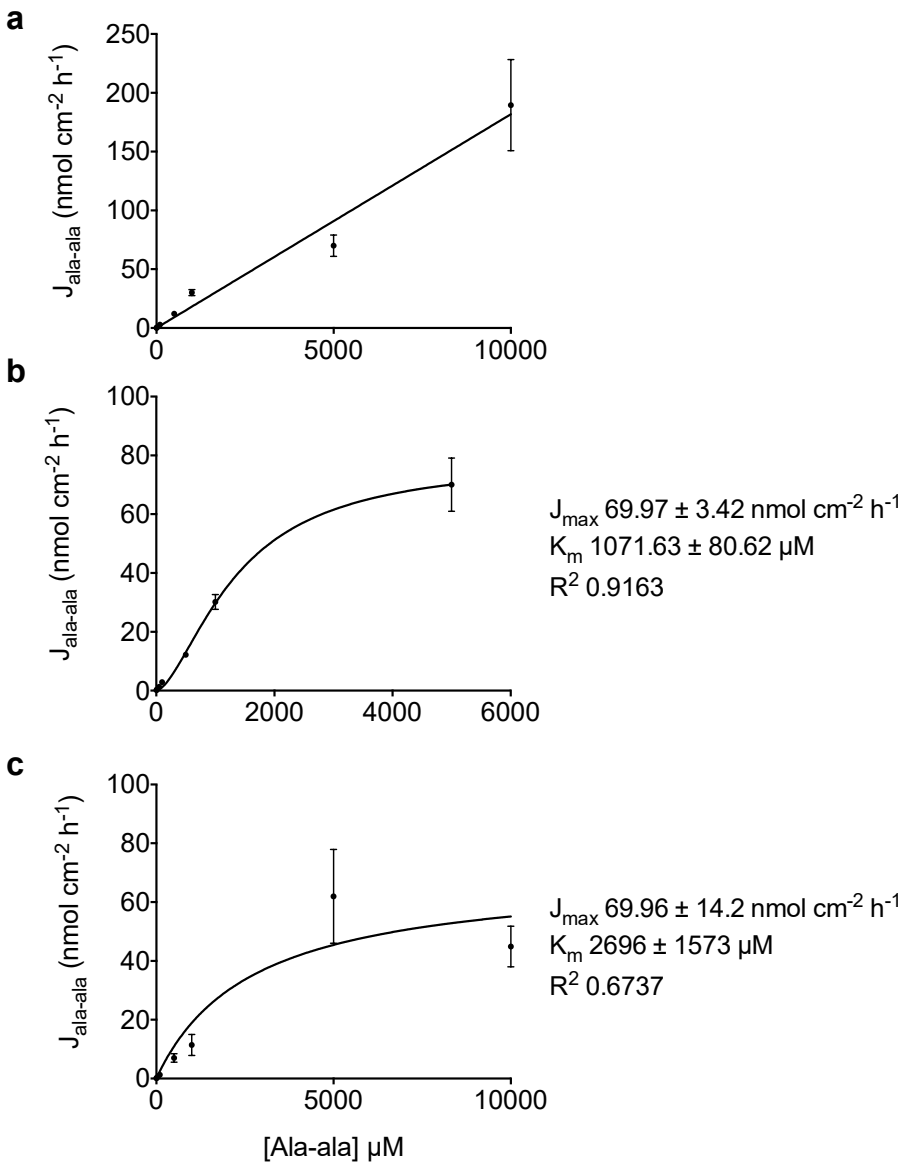

Figure 7.2 Dipeptides are acquired linearly at elevated concentrations but demonstrate saturable uptake at lower concentrations. Concentration dependent uptake of L-ala-ala (nmol cm^{-2} h^{-1}) across Pacific hagfish hindgut. (**a**) Linear transport of L-ala-ala at elevated concentrations (0-10,000 µM) as determined by mucosal disappearance. (**b**) Sigmoidal kinetic uptake of L-ala-ala from 0-5000 µM as determined by mucosal disappearance (best fit R^2 = 0.9163; see methods). (**c**) Saturable transport of ala-ala from 0-10000 µM as determined by serosal appearance. Data points represent means ± SEM of 4-6 animals. Curves were fitted using SigmaPlot (ver. 13; Systat).

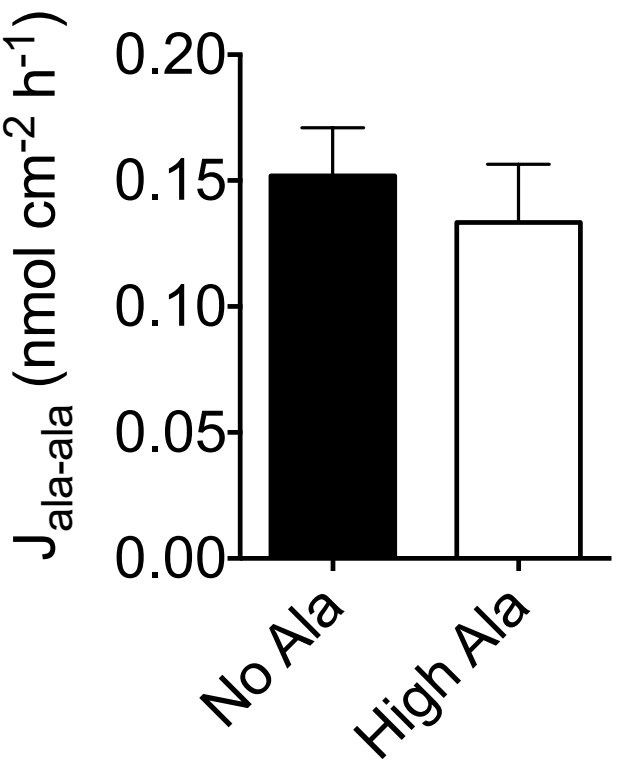

Figure 7.3 Addition of elevated alanine does not change L-ala-ala transport rate indicating dipeptides are acquired intact and not as hydrolysed amino acids. L-ala-ala transport rate (nmol cm^{-2} h^{-1}; control; black bar) at 5 μM L-ala-ala during putative competition substrate of 50 μM alanine (white bar). Bars represent means + s.e.m of 5 animals. Analysed using an unpaired t-test at an α-level of 0.05.

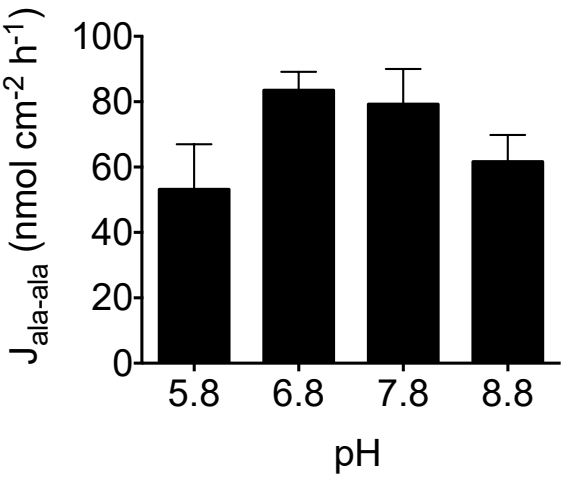

Figure 7.4 L-ala-ala transport rate is unchanging with variable mucosal pH. L-ala-ala transport rate (nmol cm^{-2} h^{-1}) in response to changing pH at 5000 μM. Bars represent means + s.e.m of 7 hagfish. Analysed using a one-way ANOVA with a Tukey's multiple comparisons post hoc analysis and an α-level of 0.05.

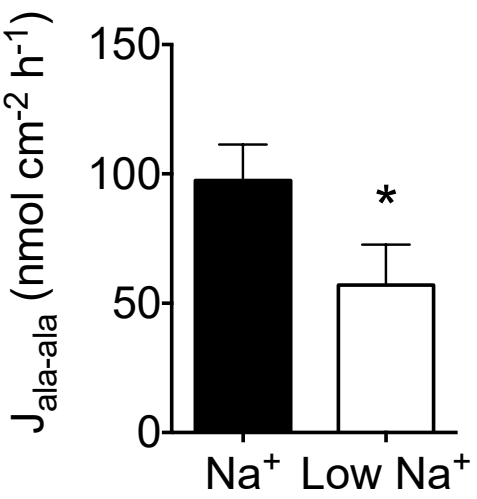

Figure 7.5 L-ala-ala transport rate is significantly decreased in a low sodium environment. L-ala-ala transport rate (nmol cm^{-2} h^{-1}) in response to reduced sodium conditions at 5000 μM ala-ala. Bars represent means + s.e.m of 5 hagfish. * indicates significant difference as determined by a paired t-test with an α-level of 0.05.

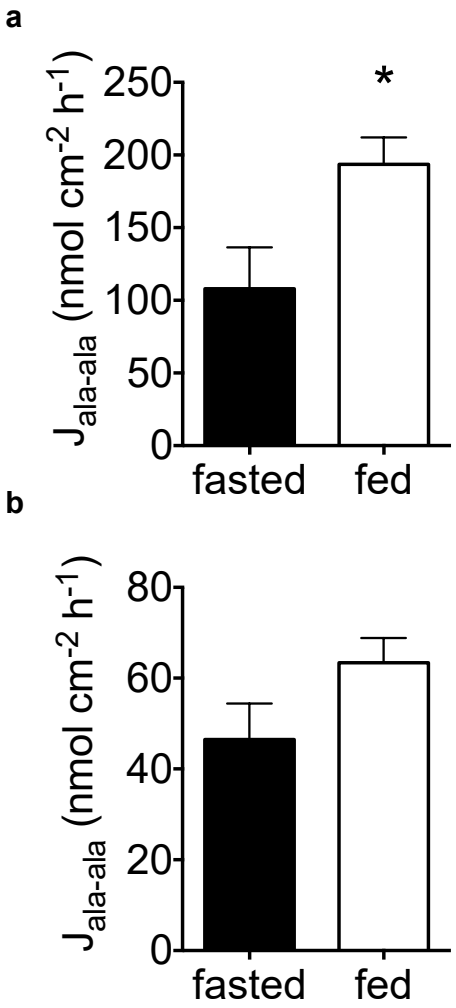

Figure 7.6 Mucosal L-ala-ala transport is significantly increased following feeding, while serosal transport rates are unchanged. Alterations to L-ala-ala transport rate (nmol cm^{-2} h^{-1}) in response to feeding at 5000 μM ala-ala. Intestines were extracted from fasted (black bars) or 8-10 h post-fed (white bars) hagfish and the mucosal disappearance (**a**) or serosal appearance (**b**) of L-ala-ala was calculated. Bars represent means + s.e.m of 5 animals. * indicates significant difference as determined using an unpaired t-test and an α-level of 0.05.

Discussion

In the present study, we show that the model dipeptide $_{L}$-ala-ala was transported *via* a specific pathway across the Pacific hagfish hindgut. Transport was saturable, specific, and regulated in response to feeding, all characteristics that indicate a dedicated dipeptide acquisition mechanism.

Kinetic characterisation of dipeptide acquisition

The rate of $_{L}$-ala-ala uptake was unaltered along the length of the hindgut (Fig. 7.1). This feature is characteristic of hagfish intestinal nutrient uptake (Glover et al. 2011b; Schultz et al. 2014; Weinrauch et al. 2018a,b; Chapter V, VI), and could conceivably be the result of both feeding behaviour and intestinal morphology. Hagfish obtain food episodically through both active predation (Zintzen et al. 2011) and opportunistic scavenging (Martini 1998). The variable timespan between meals, compounded with the relatively simple digestive tract, highlights the necessity for maximal nutrient absorption when a meal becomes available. Specifically, the tract begins with the pharyngocutaneous duct; a relatively thin structure comprised of mucus cells, epithelial cells, acidophilic secretory granules and undifferentiated cells (Adam 1963; Weinrauch et al. 2015). The cellular composition indicates a role for lubrication to aid in the progression of the food bolus down the tract. The straight, uncoiled hindgut is quite distinct with zig-zag folds that are retained during intestinal distention. Digestion is accomplished with zymogen granules, released from distinct cells of the hindgut and absorption occurs across the apical brush border of the columnar cells (Adam 1963; Weinrauch et al. 2015, 2018a,b). The morphological data suggests hagfish have a hindgut

lacking regional functional specialization, which would permit maximal nutrient uptake following a binge. Namely, the whole tract can serve in food storage, digestion, and assimilation, which is important in a relatively short organ that may only periodically receive meals. A generalized hindgut function may also aid the animal in pre-emptively preparing for future meals (Glover and Bucking 2015). However, there are data confirming the presence of proteases, and specifically dipeptidases, along the hindgut (Nilsson and Fänge 1970), leading to speculation that the proportion of dipeptides to amino acids would thus decrease along the length of the tract, and indicating a need for a low affinity, high-capacity transporter in the anterior hindgut and a high-affinity, low-capacity transporter in the posterior region. This very arrangement has been described in Atlantic salmon where the distal intestine apparently absorbs dipeptides at concentrations >25 mM in an unsaturable fashion (Bakke-McKellep et al. 2000). However, hagfish are unique wherein they can binge on a meal so that undigested food passes through the anus while they continue to feed (Baldwin et al. 1991). In this case, the concentration of dipeptides may not vary along the tract length, as it does in other fishes. A comprehensive characterisation of the activity and distribution of hagfish intestinal dipeptidases (Nilsson and Fänge 1970), in addition to luminal dipeptide measurements, would greatly enhance our understanding of the post-fed hagfish intestine and its capacity for nutrient digestion and assimilation.

The transport kinetics of $_{L}$-ala-ala were first determined in terms of mucosal disappearance. A sigmoidal relationship was the best fit ($R^2 = 0.9163$), however a similar fit was obtained when modeled using Michaelis-Menten kinetics ($R^2 = 0.9159$). The

sigmoidal uptake pattern is characteristic of other nutrient transport processes in hagfish (Glover et al. 2011b), and has been explained by the presence of allosteric modulators (e.g. sodium, or protons) impacting transporter function. Alternatively, the sigmoidal curve could reflect the effects of mucus binding, wherein at low dipeptide concentrations, the mucus layer covering the gut binds to, and effectively limits the bioavailability of, the substrate. However, additional dipeptide saturates binding, and more substrate becomes available to the transporter for absorption. This leads to the distinct sigmoidal pattern of uptake observed in the current work, and in studies of *in vivo* zinc transport in rainbow trout where uptake was also based on mucosal disappearance (Glover and Hogstrand 2002). It is also possible that the sigmoidal distribution of dipeptide transport may arise from the presence of multiple transporters (Weinrauch et al. 2018a; Chapter V). Given that the presence of digestive enzymes in hagfish intestine has been long established (Adam 1963; Nilsson and Fänge 1970), it is likely that some hydrolysis of the dipeptide occurred, resulting in some uptake *via* amino acid transporters. Yet, our 10-fold excess addition of alanine to the transport assays suggests this likely does not contribute substantially to overall uptake (Fig. 7.3). Uptake occurring *via* an alanine transporter (i.e. through hydrolysis of the dipeptide) should elicit a marked decline in uptake rates as a result of substrate dilution, much like the pattern observed in Atlantic salmon (Bakke-McKellep et al. 2000). However, the presence of even small amounts of labeled alanine could contribute to complex kinetic parameters and result in a sigmoidal curve having a better fit than a hyperbolic curve. Two transporters with slightly different properties could contribute to a sigmoidal relationship, although hagfish arose prior to the gene

duplication (Escriva et al. 2002), and ongoing molecular characterization is needed to clarify the exact nature of the observed concentration-dependent kinetics.

Irrespective of the specific nature of the phenomenon leading to sigmoidal kinetics, it is clear from the current study that dipeptide transport is achieved by a specific transport pathway. Our calculated K_m (1.07 mM) is distinct from that calculated for alanine in this species of hagfish (7 mM; Glover et al. 2011b), further supporting our observation that we are measuring dipeptide uptake, rather than that of the hydrolysed alanine. Additionally, this K_m corresponds with those reported for dipeptides in numerous studies in fish. These range from ~0.1-10 mM as calculated *via* numerous techniques including brush border membrane vesicle uptake, everted sleeves and vector expression systems (reviewed in: Verri et al. 2010; Wang et al. 2017a). A similar range in dipeptide K_m values has also been reported in mammalian systems (Ganapathy and Leibach 1985; Abe and Ohmama 1987; Fei et al. 1994). Given the wide range of affinities and the multitude of techniques employed, it is difficult to infer the similarities and/or differences between hagfish and other organisms. This relatively wide range could be the result of many factors including differences in dietary composition, or digestive enzymes, as well as transporter expression, abundance and cooperativity. Furthermore, the specific dipeptide varies with each study and the binding characteristics of each dipeptide may differ even with the same transporter. This is particularly likely if an oligopeptide transporter is present as they have promiscuous binding sites that accept ~400 dipeptides and 8000 tripeptides (Adibi 2003; Daniel et al. 2006; Ahn et al. 2013).

While we did observe a saturable uptake component, there was evidence for a high-capacity, low affinity transporter at elevated concentrations (10 mM; Fig. 7.2a). Akin to some salmonids, this may be indicative of an abundance of low affinity transporters that were unsaturated at these concentrations (Ferraris and Ahearn 1984; Bakke-McKellep et al. 2000). Since hagfish and salmonids are both carnivorous, and concentrations of 25 mM did not yield saturable uptake in the Atlantic salmon, it is entirely possible that a second transport system persists in hagfish too. Alternatively, paracellular movement may contribute to uptake at elevated concentrations.

In addition to characterizing the kinetics of mucosal disappearance, we also examined serosal appearance. Generally, mucosal loss will more strongly reflect apical transport phenomena, whereas serosal appearance will reflect intracellular and basolateral characteristics. While patterns were generally similar, indicating that apical transport processes are controlling uptake and absorption, there were subtle differences. For example, serosal appearance of radiolabel was a better fit for a hyperbolic Michaelis-Menten relationship. The hyperbolic nature of our serosal appearance data suggests that basolateral transfer of the radiolabel may be occurring *via* amino acid transporters, which have previously been shown to conform to Michaelis-Menten kinetics in hagfish gut (Glover et al. 2011b). Furthermore, while maximal uptake rates were essentially the same between mucosal disappearance and serosal appearance calculations (~70 nmol/cm^2/h; Fig. 7.2b,c), the affinity for basolateral transport was nearly doubled. This is to be expected, as the cellular compartment will have hydrolyzed the dipeptides into amino

acids, doubling the available substrate, thereby decreasing the need for an elevated affinity.

Effect of changing mucosal pH and sodium concentration on dipeptide uptake rates

Peptide transport is known to be a proton-dependent process in mammals (Thwaites et al. 2002; Adibi 2003). Interestingly, there was no significant change in L-ala-ala uptake rate with altered mucosal pH (Fig. 7.4). This is distinct from studies in both *C. elegans* and mammals, which describe pH-dependence of dipeptide uptake, and an optimum uptake occurring from pH 5.5-6.0 (Fujisawa et al. 2006; Benner et al. 2011; Spanier 2014). Conversely, zebrafish have an elevated rate of transport at more alkaline pH (8.5). This likely stems from the fact that zebrafish are stomachless and their digestive tract lumen is generally more alkaline under normal physiological conditions (>pH 7.5; Verri et al. 2003). Albeit agastric, previous reports suggest the intestinal pH of fasting hagfish to be generally lower than most aquatic vertebrates at ~5.5-5.8 (Glover et al. 2011b; Glover and Bucking 2015), and there is an indication that post-prandial luminal acidification occurs (Nilsson and Fänge 1970). Thus, the microclimate of the lumen may provide the necessary protons to continue effective dipeptide uptake at each pH investigated in this study. In fact, if hagfish secrete additional acid upon feeding, the environment may be very similar to that described in mammals where an acidic microclimate is created amongst the brush border microvilli (and is thus very difficult to accurately measure; Daniel et al. 1985; Thwaites et al. 1999). On the other hand, there are multiple animals that have not displayed pH-dependence of dipeptide uptake. For instance, the European sea bass (Sangaletti et al. 2009), Antarctic icefish (Rizzello et al. 2013), and seawater-acclimated killifish (Bucking and Schulte 2012), do not present

evidence for a pH-dependent dipeptide acquisition strategy. Isolated preparations of specific transporter candidates (e.g. isoforms of the SLC15 family) are necessary to determine the exact role of protons in hagfish dipeptide acquisition. Con et al. (2017) suggest that there is a potential role for v-type H^+ ATPase (VHA) in tilapia peptide absorption (Con et al. 2017). Since protons not only provide the driving force for peptide uptake but also can enhance the affinity of the transporter (Verri et al. 2003), VHA could create an optimal pH for peptide absorption (Con et al. 2017). While the proton transporters present in the hagfish have yet to be identified, this is a viable option for post-prandial proton extrusion (Nilsson and Fänge 1970), particularly because VHA has been localized to certain hagfish tissues (Tresguerres et al. 2006) and VHA contributes to gastric alkalization of some studied marine invertebrates (Stumpp et al. 2015). Given their phylogenetic position, it is possible that the hagfish may have this feature of VHA-driven luminal acidification. Additionally, hindgut VHA immunolocalization is presented in Chapter III of this thesis. Another feasible option is the sodium proton exchanger (NHE), which is the more widely discussed proton transporter in peptide uptake, likely owing to the longstanding link between peptide uptake and sodium concentration. Regardless of the proton-transporting entities found in hagfish intestine, we were unable to detect an effect of changing pH on peptide transport.

Previous studies have demonstrated a clear link between sodium and peptide uptake in the guts of vertebrates (Cheeseman and Devlin 1985; Ganapathy and Leibach 1985; Daniel 2004). Consistent with these observations, hagfish intestinal L-ala-ala transport was significantly inhibited under reduced sodium conditions (Fig. 7.5). In our study a

42% reduction in dipeptide transport was observed, similar to that noted for other dipeptides in mammalian systems, where inhibition in the range of ~35-65% has been observed in low sodium experiments (Ganapathy and Leibach 1985). The apparent sodium contribution to transport has been attributed to interplay between NHE and peptide transporters (e.g. PepT), yet the exact mechanism by which this occurs is unknown (Ganapathy and Leibach 1985; Thwaites et al. 2002; Verri et al. 2016). Pharmacological inhibition of NHE (for example with amiloride) would help to delineate the role of NHE in hagfish dipeptide acquisition, however the highly saline nature of both seawater and hagfish plasma precludes such an experiment owing to the reduced solubility of amiloride at elevated salinities. Sodium is also important for amino acid uptake in hagfish (Glover et al. 2011b), however we do not attribute the reduction in transport to amino acid transporters owing to the aforementioned results of the high alanine control, and the distinct affinities between amino acid and dipeptide transporters. Additionally, the rate of dipeptide transport at 5000 μM (~100 nmol/cm^2/h) far exceeds that of alanine at this concentration (~25 nmol/cm^2/h), which is archetypical of intestinal dipeptide and amino acid uptake rates (Boge et al. 1981; Reshkin and Ahearn 1991). Therefore, the reduced transport observed in conjunction with reduced sodium levels (Fig. 7.6) does imply a role for sodium in dipeptide acquisition. Much like the studies conducted in other organisms, we cannot yet determine the exact role for sodium.

Effect of feeding on dipeptide transport

Hagfish demonstrate an increase in dipeptide transport following a meal, similar to other vertebrates (Fig. 7.6; reviewed in Verri et al. 2016), and suggestive of a specific and regulated transport pathway. The magnitude of this response (J_{max} fasted rate: ~100

nmol/cm^2/h; fed rate: ~ 200 nmol/cm^2/h) is similar to that reported in other studies (2-fold; Adibi 2003). Furthermore, the uptake rate was only significantly impacted at the apical surface (mucosal disappearance; Fig. 7.6a). Additionally, the transport rates were at least double that of the basolateral surface (serosal appearance; Fig. 7.6b), which suggests distinct mechanisms for peptide uptake at each surface with only the apical surface impacted by feeding at this time point. The increase in transport after a meal has a clear benefit to the animal, allowing them to maximize nutrient acquisition from a meal. This is particularly important in species such as hagfish, which can go long periods without feeding (Tamburri and Barry 1999). We have previously identified substantial morphological alterations in the hagfish hindgut post-feeding, leading to an increased surface area (Weinrauch et al. 2017; Chapter II). Similar changes occur in other binge-feeding vertebrates and correspond to increased nutrient uptake rates (Secor 2005). The mechanism by which transport is increased cannot be discerned from the current study, but it could involve an increased apical expression of transporters. Alternatively, an increase in transport could be a result of an increased affinity of existing transporters. These two hypotheses could be tested by conducting a full kinetic characterization of uptake in both fed and fasted animals, an analysis that was beyond the scope of the current work. Feeding also induces a host of physiological alterations that could be involved in the altered dipeptide uptake rates. For instance, the act of feeding itself will alter the luminal contents in terms of ion composition and pH. The nature by which hagfish gorge themselves could also induce intestinal stretch receptors, leading to a cascade of cellular effects, ultimately affecting peptide uptake. For example, mechanical stretch is known to alter cellular cascades in mammals, leading to a change in intestinal

physiology and the expression of hormone precursors (Shi et al. 2011). Furthermore, the aforementioned peptidases may have altered activities depending upon nutritional status (Day et al. 2011), which could alter substrate composition within and along the digestive tract.

Conclusions

Collectively, the data presented herein predicates an apparent conservation of intestinal dipeptide acquisition strategies in hagfish relative to other vertebrates that diverged millions of years later. We observed a saturable transport mechanism that effectively absorbed L-ala-ala along the hagfish hindgut. While changing pH did not alter uptake rates, hagfish dipeptide uptake was affected by reduced sodium concentrations. Further regulation of this system was observed as feeding yielded significant increases in uptake. The barrage of physiological changes induced by feeding leaves many open-ended questions that require study in the future. Molecular characterisation and confirmation of the transport proteins involved in dipeptide uptake would be of additional value and would elevate our understanding of the evolution of early vertebrate nutrient transport systems.

Chapter VIII: Conclusions and Perspectives

My doctoral thesis examined the digestive physiology of the earliest extant craniate, the Pacific hagfish. The experiments herein have focused on addressing: whole animal physiological effects of feeding (Chapter II), profiles of selected digestive enzymes (Chapter III), cellular mechanisms of acidic digestion (Chapter IV), and the characterisation of nutrient acquisition mechanisms using representative macronutrients (Chapters V, VI, VIII). This chapter serves as a general summary where I highlight the major findings and connections between chapters, as well as describe avenues for future research.

8.1 Whole animal effects of feeding

The SDA peak occurred 8 h after natural feeding, which corresponded with the peak of base efflux and intestinal blood alkalization, as well as increases in mucosal thickness and microvilli length. The magnitude and duration of SDA measured here is quite different from that of a more recent study in the New Zealand hagfish (*Eptatretus cirrhatus*; Glover et al. 2019). Pacific hagfish have a greater magnitude (2.9- *vs.* 1.9-fold increase) and shortened duration (8 h *vs.* 48 h) of SDA, which may be the result of their smaller size and higher basal metabolic rate. Comparisons are difficult to make however, because I could not measure the meal size or content since the hagfish were not often willing to feed or easy to manipulate (see section 8.4 below). Changes to intestinal architecture likely contribute to the elevated metabolism, as would the increased rates of transport for both nutrients (Chapters VI and VII), and ions (Chapter IV), similar to that described for pythons (reviewed in Secor 2008).

The localized intestinal alkalization spurred the interest for Chapter IV where I identified an acidic digestion facilitated by conserved cellular pathways and transporters. The confirmation of luminal acidification, along with the localized alkalization and increasing trend of base efflux, suggests that hagfish have an alkaline tide, the magnitude of which is likely mitigated by a large blood volume with high buffering capacity and mixing of arterial and venous blood. Base efflux is likely accomplished using plasma-accessible gill CA (Esbaugh et al. 2009) and VHA translocation (Tresguerres et al. 2007a), as has been described in dogfish (Tresguerres et al. 2007b). Given that hagfish are "champions of CO_2 tolerance" and can rapidly offload plasma HCO_3^- using CA-mediated mechanisms (Clifford et al. 2017c), VHA upregulation may not be required, as the animals are equipped to deal with much larger base loads. Clearly, future examination is required to understand the mechanisms of post-prandial base efflux. Ideally, CA inhibitors (e.g. acetazolamide or the membrane-impermeant C18) would be injected before feeding to elucidate the CA contribution. In my experience this is a logistical nightmare, as hagfish will not feed when stressed (see section 8.4).

8.2 Enzymatic digestion

Hagfish are capable of digesting nutrients along the entirety of the hindgut. In this thesis I have characterised the distribution and activity of six digestive enzymes with substrates representing each of the macronutrients. Previously, enzymes were believed to be restricted to the hindgut (Adam 1963), however the results of Chapter III indicate that they preside throughout the foregut as well. Feeding results in decreased tissue activity

for only some enzymes, suggesting differential distribution across cell types and likely unique cues (e.g. mechanical, neural, chemical) for their activation/release. This differential expression and response to feeding presents the first evidence in vertebrate phylogeny for compartmentalization of gut function.

Chapter III is a preliminary foray into understanding hagfish digestive enzymes. The kinetic properties (J_{max} and K_m) of each enzyme could be investigated with varying substrates or conditions. Of particular interest to me is the effect of pH on enzyme activity. In Chapter IV I demonstrate that hagfish undergo a marked post-prandial acidification. Although a hagfish catheptic enzyme works optimally at pH 4, most of these studied enzymes are typically of pancreatic origin and operate at alkaline conditions (Nilsson and Fänge 1970). Does this indicate that the cellular mechanisms utilized for acid secretion could stimulate the release of some enzymes and not others? Transporters capable of supplying protons (HKA) are housed within the zymogen granule cells (Chapter IV). Perhaps stimulants of HKA, such as histamine, can contribute to zymogen granule release and simultaneously activate HKA to create an acidic environment. Once acidic digestion is complete, the pancreatic-like zymogen granules may act akin to vertebrate pancreatic cells to secrete HCO_3^- and promote a secondary alkaline digestive phase. Experiments that extend past the 8 h time frame are necessary to determine whether a luminal alkalization occurs and if so, what cellular signals and machinery are responsible.

8.3 Mechanisms of nutrient acquisition

With Chapters V, VI, and VII, I have identified regulated means of nutrient acquisition in the hagfish hindgut. A distinct, carrier-mediated transport process was identified for each of glucose, oleic acid, and L-alanyl L-alanine (Table 8.1). Dipeptides had the lowest affinity which could be the result of high protein concentrations in the diet, or a promiscuous transporter (Daniel 2004), each of which reduces the need for high affinity transporters. The relatively higher affinities for glucose and oleic acid transport hint toward their energetic importance for these animals.

In my experiments, I utilised pharmacological agents and altered mucosal environments (e.g. low sodium or altered pH) to elucidate the mechanisms of nutrient uptake. Overall, acquisition strategies appear to be conserved between hagfish and later-diverging vertebrates. Feeding elicits many physiological changes, which lead to increased nutrient uptake capacity. The delineation of which specific signals regulate nutrient transporter function is an important future direction, particularly in regards to hormones (see below section 8.6e).

Of obvious importance, is the molecular determination of the transport proteins involved. Only recently has the genome of a related hagfish species (*Eptatretus burgerii*) become available, which should provide a means to identify homologous sequences in *E. stoutii*. This will facilitate the molecular identification and phylogenetic relationships of various transporters and allow the application of multiple techniques (e.g. qPCR, Western

blotting, immunocytochemistry) to quantify transporter abundance and localization after various treatments (e.g. feeding, hormone or drug application).

Table 8.1 Summary of transporter kinetic parameters in the Pacific hagfish hindgut.

Nutrient	J_{max}	K_m
Glucose	8.5 nmol/cm^2/h	0.37 mM
Oleic acid	1.3 nmol/cm^2/h	0.06 mM
L-alanyl L-alanine	70 nmol/cm^2/h	1.1 mM

8.4 Methodological considerations

The study of hagfish presents ample challenges, particularly in regards to feeding. Hagfish are notoriously picky eaters. Over the course of a year, the animals did not feed despite numerous dietary options. Appetite/satiation cues in hagfish are unknown and feeding studies would greatly benefit from their elucidation (see section 8.6e). Hagfish tend to feed in groups more readily than alone and any added stressors (e.g. transferred that same day, injected, or anaesthetized), makes the animals too agitated to eat. As unreliable eaters, the measured content or size of the meal was unable to be quantified. This means that inter- and intra-species comparisons regarding SDA cannot be conducted. I did gavage a series of hagfish but emesis of the meal was common once the hagfish revived.

When they do feed, it aligns with their nocturnal activities and therefore, the feedings commenced at midnight throughout this thesis. This limits the variation across studies herein, but does not provide much information on any diurnal cycle. Sea cucumbers, for example, have a distinct circadian rhythm for digestive enzyme activity (Sun et al. 2015), which would be unaccounted for in this thesis. Indeed, seasonal effects might also contribute to different nutrient handling strategies. For instance, the data collected across years in Chapter V suggests that there is variability in glucose acquisition depending on nutritive state and season. A long-term study examining nutrient handling over the course of a year would contribute greatly to our understanding of hagfish digestive physiology.

The whole body measurements were not repeated measures because hagfish do not have tight control of their anal musculature. Both the act of anaesthetization and/or the method of obtaining a caudal blood sample cause incontinence. Therefore, the observed variability might be the result of individual variation and contribute to the lack of significant base efflux. I did attempt to cannulate the hagfish in order to collect repeated samples for analysis of blood parameters. However, the blood pressure of hagfish is incredibly low and almost non-existent in the intestine of a fasting hagfish. Without sufficient pressure, an adequate blood sample could not be obtained due to immediate collapse of the vessel when drawing blood.

Many chapters in this thesis utilised gut sac techniques to measure nutrient or proton flux and thus the pitfalls of this technique should be discussed. First, because the

experiments are conducted *ex vivo* the intestine is removed from both the neural innervation as well as the blood supply. The lack of a blood supply could augment the transport characteristics if the nutrient is increasing within the intestine, rather than being exported to the circulation. Additionally, the regulation of gut function is a complex process and hormonal influence would not be accounted for in these experiments where circulation was removed.

Hagfish are demersal creatures and so are subjected to increased pressure in their natural habitat. This work was conducted entirely within a laboratory where the effects of pressure were not considered. However, hagfish metabolic rate does not differ when measured at depth and in the laboratory (Drazen et al. 2011), indicating that pressure does not affect metabolic rate. However, deep-sea animals have adaptations such as altered membrane fluidity, or structural protein changes to withstand heightened pressure (Somero 1992), which could be examined in hagfish in the future.

8.5 Environmental relevance

The environmental concentrations of nutrients that hagfish encounter are difficult to determine owing to a wide range of prey. The studied concentrations (glucose 0-10 mM; oleic acid 0-200 μm; alanyl-alanine 0-10 mM) vary widely to account for the diversity that hagfish would naturally encounter. Given that the affinities (Table 8.1) fall within the tested concentrations, the natural range has likely been captured in these studies. Digestive function varies with meal size and content (Nagase 1964), which are unaccounted for in this thesis using single feedings. The repeated feeding and varied diet

of the hagfish could lead to changes in whole animal physiology (length and peak of SDA, N-excretion), digestive enzyme activity/expression, nutrient availability and therefore, transporter activity.

Notably, within this thesis I examined the uptake of each nutrient individually. A combination of nutrients will likely alter uptake capacity through their interaction, and by altering enterocyte metabolism. As addressed in Chapter IV, the presence of the favoured glucose in the hagfish saline alongside the oleic acid (or alanyl-alanine), may underestimate the transport capacity for oleic acid (or alanyl-alanine) because glucose would be preferentially acquired.

8.6 Future directions

While my thesis provides novel information regarding hagfish digestive strategies and mechanisms of nutrient assimilation, there is still a substantial body of research that needs to be completed to contribute to our understanding of hagfish digestive physiology. Presented below are some key areas that future studies should address.

8.6a What role does extended fasting have on digestive capacity?

My thesis contributes a substantial amount regarding hagfish feeding, yet the extreme lengths of fasting are not considered. Particularly interesting is the widespread distribution of lipid droplets across tissues (Chapter VI). Does each organ amass stored lipids in order to tolerate extended fasting? Future examination of stored lipid content and lipid use during fasting could highlight which tissues are most metabolically demanding.

As discussed in Chapter VI, the retention of droplets within the enterocytes is reminiscent of the human disorder, congenital β-lipoprotein deficiency, wherein lipids are acquired normally but not distributed efficiently (Spencer et al. 1966). The mechanisms of enterocyte lipid retention could be examined in hagfish to understand the cellular components of use for therapeutic agents.

I demonstrate that intestinal function is significantly reduced during fasting in hagfish. Does this mean that circulating hormone activity is similarly reduced? What are the cellular mechanisms of rapid microvilli lengthening/shortening? How does the microbiome function during these extended periods of fasting? Although the majority of hagfish physiology studies are conducted in fasted animals for ease of replication and comparison, they do not focus on fasting as a nutritive state, which should be a priority in the future.

8.6b How do hagfish sense nutrients and prey?

The degenerate sensatory capabilities of the hagfishes are apparent as soon as one watches a hagfish feed in captivity. Multiple accounts indicate that the hagfish can swim within centimetres of a prey item, yet miss it entirely (Greene 1925; Holmes et al. 2011; personal observation). How can this be? Particularly as hagfish inhabit a benthic environment likely with transient prey (Glover and Bucking 2015), thus suggesting sensory cues are requisite for survival. Investigations have focused on visual, auditory, olfactory and chemosensory roles, with a greater dependence seemingly placed upon the latter two systems. The chemosensory organs in hagfish (Schreiner organs) are widely distributed along the body surface, as well as within the nasal canal. The neural

requirement for these organs is immense with 10% of medullary volume dedicated to Schreiner organ operation (Ronan 1988). While these organs are obviously of great import in terms of hagfish sensory capabilities, there is no data on potential stimuli, the sensitivity of these organs, or a definitive biological role whether physiologically or behaviourally.

8.6c Does the intestine play a role in calcium and magnesium homeostasis?

Calcium and magnesium are conspicuously regulated at levels below that of seawater (Morris 1965) and in Chapter IV I demonstrate that magnesium uptake increases with feeding while calcium uptake is maintained. The specific channels or transporters responsible for calcium and magnesium uptake have not been examined in hagfish thus far in any tissue. Like other aquatic animals, dietary sources may not be necessary since other tissues such as the gill, or skin in the case of hagfish, can likewise acquire these cations (Bijvelds et al. 1998). Intestinal calcium is important in marine teleosts for osmoregulation (Taylor and Grosell 2006). This is unlikely to be important in the osmoconforming hagfish, however the machinery may be present.

8.6d Do the kinetics of intestinal uptake vary with environmental conditions?

Hagfish have long been known as scavengers that enter their prey (Linneaus 1758), which presents the opportunity for heightened environmental nutrient load. Since hagfish are known to have extra-intestinal nutrient uptake mechanisms in place (Glover et al. 2011, 2015, 2016; Schultz et al. 2014), multiple sources could alter plasma nutrient load. Future studies could examine whether increasing plasma nutrient load reduces the

hindgut uptake capacity as a result of decreased inwardly-directed concentration gradients.

When feeding within decaying carrion, hagfish must endure a pernicious environment. A previous study has examined the effects of hypoxia and elevated ammonia concentration on hagfish nutrient transport capabilities, determining that transport is maintained or elevated (Bucking et al. 2011b). Another prominent stress hagfish face while feeding is hypercapnia (Clifford et al. 2017c), which deserves future examination, as does a combination of these potential feeding stressors.

8.6e What hormones are involved in hagfish digestive physiology?

As mentioned throughout, the first phylogenetic appearance of somatostatin- and insulin-containing islet cells occurs with the hagfishes (Falkmer and Winbladh 1964). In addition to maintenance of plasma glucose homeostasis, insulin is a known regulator of nutrient acquisition. We did not see a change in oleic acid uptake with insulin application, however this is a feature similar to that observed in the mammalian intestine (Stahl et al. 2002). Whether insulin can alter fatty acid acquisition in other tissues (similar to mammals) remains to be determined. The role of insulin on glucose acquisition also deserves future attention.

Regulation of gut function is accomplished with a suite of hormones, some of which have been investigated in hagfish. However, the typical role of a hormone may not correspond to its role in the hagfish, solely because hagfish lack both a stomach and a discrete pancreas. Additionally, the role observed in the hagfish may be an earlier action

of the hormone. For example, cholecystokinin (CCK) is responsible for gall bladder contraction and pancreatic enzyme release in mammals. In hagfish, differential enzyme secretion was observed (Vigna and Gorbman 1979) and could be responsible for selective enzyme activation as discussed above (section 8.2). Furthermore, CCK may also enact the role of gastrin (stomach hormone that stimulates gastric acid release; Ostberg et al. 1976) as the amphibian/reptilian gastrin appears to have evolved from a CCK-like molecule (Van Noorden 1990), and could provoke the proton secretion observed in Chapter IV. Finally, the vasodilator, substance P, is found in hagfish intestine and elicits vasoactive function (Reinecke 1987). Its release post-feeding may account for the drastic changes in blood flow that I observed, and which requires further study. Other hormones, such as gastrin-releasing peptide and vasoactive intestinal peptide, are known to influence many varied factors (vasodilation, bicarbonate secretion, gastric acid secretion and gut motility) in other long-term fasting vertebrates (e.g. pythons; Secor et al. 2001), but have yet to be investigated in hagfish.

Hagfish represent an interesting model for feeding/satiation cues namely because of the extended tolerance to food deprivation (11 months; Foster and Moon 1986). Much like the python model (reviewed in Secor et al. 2001), hagfish rapidly respond to a meal and have large changes in intestinal morphology (Chapter II) and nutrient acquisition (Chapters VI and VII). CCK is a known mediator of satiation (Secor et al. 2001) and, as mentioned above, is found in hagfish. The relative simplicity of the hagfish intestinal system, along with a reduced hormonal complement, designates hagfish as a model

organism for the understanding of early digestive hormones and their integrative regulation of digestive physiology.

8.7 Summary

Overall, my thesis demonstrates that hagfish utilize both zymogens and acid for digestion along the alimentary canal. The differential enzyme distribution and expression post-feeding is an early indication of compartmentalization of gut function. Although hagfish lack a stomach, the hindgut is capable of producing a significant acidification using cellular mechanisms that are conserved across the vertebrate lineage. Thus, I conclude that the localized alkalization of the intestinal blood supply is the manifestation of a hagfish alkaline tide. Whether the typical alkalization that is observed in vertebrate intestines persists in the hagfish remains to be seen. Finally, I have demonstrated that hagfish have specific carrier-mediated transport mechanisms for glucose, oleic acid, and alanyl-alanine. The means of regulation and transport characteristics suggest that nutrient acquisition strategies are conserved between hagfish and later-diverging vertebrates.

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