ENZYME ACTIVITIES IN THE INTESTINAL LAYERS OF TELEOST FISHES: ENDOGENOUS VERSUS EXOGENOUS CONTRIBUTION

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ABSTRACT

Digestive enzymes are essential for the catabolism of macromolecules and are produced endogenously by the animal or exogenously by the gastrointestinal bacteria. This thesis explored the contribution of endogenous versus exogenous trypsin, lipase, and cellulase production across dietary niches by separating the intestines of fed and unfed carnivorous rainbow trout, omnivorous goldfish, and herbivorous central stonerollers into layers where bacteria were present or absent. Trypsin and lipase were both endogenously and exogenously produced in a species-specific manner, whereas cellulase was solely produced by bacteria in all three fishes. As well, feeding generally decreased enzyme activities in most cases. Furthermore, dietary manipulation of goldfish caused the elimination of most trypsin (via endogenous responses) and cellulase (via mostly exogenous responses) activities. Collectively, these findings elucidate the proportional contribution of the microbe host and gastrointestinal bacteria to digestive enzyme production in various fishes with different diets, and provide insight into the digestive functions of environmentally and economically important vertebrates.

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ABBREVIATIONS

ANOVA	Analysis of variance
BAPNA	N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride
CEL	Carboxyl ester lipase
CMC	Carboxymethylcellulose sodium salt
CMCase	Carboxymethyl cellulase
DNS	3,5-dinitrosalicyclic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
F/R primers	Forward/Reverse primers
gDNA	Genomic DNA
MS-222	Tricane methanesulfonate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
rRNA	Ribosomal RNA
SEM	Standard error of mean
TAE	Tris-acetate-EDTA buffer

CHAPTER 1: GENERAL BACKGROUND

1.1. Teleost digestive systems

The teleost digestive system is made up of several organs that work harmoniously together to break down ingested food particles, therefore allowing the animal to use the nutrients effectively for proper growth and development (Gisbert et al. 2018; Figure 1.1). Once food is eaten, mechanical digestion begins in the mouth and pharynx before the bolus continues down into the esophagus. From there, the partially broken-down food enters the stomach (if present), where further mechanical digestion occurs and chemical digestion begins. The chyme (made up of partially digested food, along with secreted enzymes, bacteria, mucus, cells from the upper gastrointestinal tract that have come off during digestion, and water (Bucking and Wood 2006; The Editors of Encyclopaedia Britannica 2014; Zarkasi et al. 2016)) then enters the intestine, where continued chemical digestion and nutrient absorption take place (Grosell et al. 2010). Apart from its role in the digestion of foods, the intestine is multifunctional and is involved in processes relating to the immune system, osmoregulation, and hormone secretion (Buddington et al. 1997; Greenwell et al. 2003; Ringø et al. 2003; Grosell et al. 2010).

1.1.1. Gastrointestinal morphology

Epithelial cells primarily make up the lining of the buccal cavity, the entry to the gastrointestinal tract, where mucous cells and taste buds are also found. Following the buccal cavity is the pharynx, which is also lined with epithelial cells, as well as longitudinal and circular muscle fibres that aid in the physical movement of food into the gastrointestinal tract. The esophagus, subsequent to the pharynx, is short, thick, and tube-like, and is composed of epithelial cells lining the lumen, muscle fibres, and connective tissues (Rogick 1931; Sarbahi 1951). In saltwater fish, the esophagus plays a major role in osmoregulation by desalinating

ingested seawater (Greenwell et al. 2003). The gastrointestinal tract itself can morphologically be described in several ways. First, it can be divided into the two main regions: the intestinal bulb and/or stomach and the intestine proper (Sarbahi 1951; Caceci 1984). The stomach, or intestinal bulb when a stomach is lacking, is for temporary food storage and chemical digestion (Caceci 1984; dos Santos et al. 2015). The intestine proper can be further divided into the anterior, mid, and posterior regions, which can also be referred to as the foregut, midgut, and hindgut, respectively. The anterior intestine is the widest portion, followed by the mid intestine, and finally, narrowing into the posterior region that leads to the anus (Rogick 1931). The main function of the intestine is to continue the digestion of food that started in the previously mentioned structures and to absorb dietary nutrients (Grosell et al. 2010).

Four distinct tissue layers make up the intestine proper, as shown in Figure 1.2A: the serosa, muscularis, submucosa, and mucosa (Rogick 1931; Banan Khojasteh et al. 2009). Each layer has its own purpose and they work together to allow for food digestion and nutrient assimilation to occur. The outermost layer is a protective layer made up of epithelial cells and connective tissue and is known as the serosa (Figure 1.2A). The connective tissue forms the highly vascularized lamina propria, allowing for the transport of oxygen and nutrients (Banan Khojasteh et al. 2009; Nasruddin et al. 2014). The muscularis layer is made up of smooth longitudinal and circular muscle fibres connected through small connective tissues (Rogick 1931; Grosell et al. 2010; Figures 1.2A; 1.2B). It is involved in peristalsis to allow for food to pass down the length of the intestine (Nasruddin et al. 2014). The submucosal layer, made up of vascularized and fibrous connective tissue, connects the muscular and mucosal layers (Rogick 1931; Grosell et al. 2010; Figure 1.2A). Finally, the mucosal layer is an epithelial layer made up of absorptive enterocytes, mucus-producing goblet cells, endocrine cells, and immune cells

(Rogick 1931; Caceci 1984; Minghetti et al. 2017; Figure 1.2A). Numerous enterocytes make up approximately 40% of this layer (Weinberg 1976; Figure 1.2B) while goblet cells, modified mucus-producing epithelial cells, are interspersed with the enterocytes and decrease in abundance as you move posteriorly towards the anus (Rogick 1931; McVay and Kaan 1940; Weinberg 1976). Immune and endocrine cells are also found lining the intestinal lumen, but are found in smaller numbers compared to both enterocytes and goblet cells (Sternini et al. 2008). Intestinal bacteria are also associated with the intestinal mucosa (Pérez et al. 2010; Ringø et al. 2016; Figure 1.2B). The mucosal layer is responsible for the secretion of mucus by goblet cells that provides lubrication to allow for nutrients to be absorbed more readily (Nasruddin et al. 2014), the secretion of digestive enzymes by enterocytes and bacteria (Ray et al. 2012; Gisbert et al. 2018), and the absorption of nutrients by the enterocytes from the chyme (Gauthier and Landis 1972; Figure 1.2B).

Anatomical structure and physiological function are closely related, and in fish, the length and structure of the gastrointestinal tract, specifically the intestine, are dependent on their trophic levels (Buddington et al. 1997; Wagner et al. 2009). In general, algae-eating or herbivorous fish have the longest intestines relative to their body size, while carnivorous fish have the shortest (Kapoor et al. 1975; Kramer and Bryant 1995; Horn et al. 2006), and omnivorous fish species often have intermediate-length intestines (Kramer and Bryant 1995; Horn et al. 2006). This is due to a necessary increase in the intestinal surface area to increase food retention time and nutrient absorption from indigestible and nutrient-poor plant matter (e.g. cellulose), by allowing for both the ingestion of more food in each feeding and an increase in nutrient extraction through increased surface area (Kapoor et al. 1975; German and Horn 2006). As an example, herbivorous central stonerollers *(Campostoma anomalum)* have an esophagus

that leads directly into a long, coiled intestine, as they lack a true stomach (Rogick 1931). Goldfish *(Carassius auratus)*, another minnow species, but an omnivore, has a similar but somewhat shorter intestinal tract (Sarbahi 1951). The digestive tracts of rainbow trout *(Oncorhynchus mykiss)* are comparatively different, as the esophagus leads to a thick, J-shaped stomach (Weinreb and Bilstad 1955). Following the stomach are finger-like projections called pyloric caeca and then a much shorter intestine (Weinreb and Bilstad 1955). Rainbow trout feed on highly digestible and nutrient-dense foods (e.g. protein), and so they have evolved to have shorter intestines and additional structures (stomach and pyloric caeca), which aid in efficient digestion (Kapoor et al. 1975; Buddington et al. 1997). Figure 1.1 shows the anatomy of the gastrointestinal tracts of common carnivorous, omnivorous, and herbivorous fishes.

1.2. Digestive enzymes

The digestive system of an animal works to break down ingested food so that the resulting polymers can be metabolized by the body. Specifically, proteins, fats, and carbohydrates are broken down by the digestive system to produce small peptides and amino acids, fatty acids and glycerol, and simple sugars, respectively (Karasov and Douglas 2013). These nutrients are then absorbed and used in downstream processes to aid in the animal's growth and development (Gisbert et al. 2018). Digestive enzymes found along the gastrointestinal tract are used for efficient catabolism of food into dietary nutrients (Silva et al. 2011; Xia et al. 2018). In fact, the digestive system is rich in enzymes belonging to three separate classes: protease, lipase, and carbohydrase (Bone and Moore 2008). While some of these enzymes can be produced endogenously by vertebrate species (Das and Tripathi 1991; Bairagi et al. 2002; Klomklao et al. 2008; German et al. 2010), the animal may also need to rely on exogenous sources from microbes inhabiting the gut to successfully and efficiently undertake

digestive processes (Lesel et al. 1986; Saha et al. 2006; Kurtovic et al. 2009). Table 1.1 summarizes trypsin, lipase, and cellulase in teleost species, including where these enzymes are secreted, what their release is stimulated by, and where they work within the digestive system.

1.2.1. Trypsin

The breakdown of dietary proteins into smaller peptides and amino acids is referred to as proteolysis. This catabolic process occurs with the help of digestive proteases that cleave peptide bonds holding the peptides together (Grover et al. 2018). Proteins are an important part of the diet because amino acids are required for building and repairing tissues, forming molecules, such as other proteins, enzymes, and hormones, and facilitating chemical reactions in the body (Case et al. 2011). Digestive proteases can either be endopeptidases, where the cleaving of bonds occurs within the polypeptide chain, or exopeptidases, where bonds holding single amino acids at the N- or C-terminus of the polypeptide chain are cleaved (Klomklao 2008). The main class of digestive proteases are endopeptidases known as serine proteases (Hedstrom 2002; Klomklao 2008; Ruiz-Perez and Nataro 2014; Grover et al. 2018). Here, the serine serves as a nucleophile in the active site of the enzyme to attack and break the peptide bond (Hedstrom 2002). Trypsin is a serine protease that hydrolyzes ester and amide bonds on the carboxyl end of lysine and arginine residues of proteins (Hedstrom 2002), breaking them down into small peptides and amino acids. This digestive protease has an important biological role in digestion and the activation of other enzymes (Cao et al. 2000). Trypsin's proenzyme, trypsinogen, is of pancreatic origin and is secreted into and activated in the intestine by enterokinase (Sarbahi 1951; Frossard 2001; Ogiwara and Takahashi 2007), a brush-border enzyme also belonging to the serine protease family (Ogiwara and Takahashi 2007). Once trypsinogen is transformed into trypsin, the activated form of the enzyme can then break down dietary proteins and can even act on other

digestive proenzymes, such as chymotrypsinogen, proelastase, procarboxypeptidases, and some prolipases, to activate them (Frossard 2001; Ogiwara and Takahashi 2007). Studies on mammalian trypsin have shown calcium increases the conversion of trypsinogen into trypsin by stabilizing the enzyme against autolysis (McDonald and Kunitz 1941; Bode and Schwager 1975; Caldwell 1992). Fish are able to synthesize trypsin enzymes that are analogues to mammalian trypsin (Lemieux and Blier 2007; Jesús-de la Cruz et al. 2018), however the effects of calcium on the stability and activity of trypsin from fish is unknown.

1.2.2. Lipase

Lipolysis occurs when lipase hydrolyzes lipid molecules, in the form of fats, oils, or triglycerides, to break them down into free fatty acids and glycerol (Hou and Shimada 2009; Pirahanchi and Sharma 2020). These molecules are then used for energy storage, maintaining cell fluidity, the development of the organism through cell growth, to produce hormones and other lipids, to transport molecules, and for the absorption of fat-soluble vitamins (Olsen and Ringø 1997; Lin and Shiau 2003; de Carvalho and Caramujo 2018). Lipases are a group of enzymes belonging to the esterase class of enzymes (Tocher et al. 2008; Gopalan and Nampoothiri 2016) that work to hydrolyze the ester group from a substrate, releasing an esterified acid (Gopalan and Nampoothiri 2016). Lipase is typically produced in the pancreas and in fish, it has also been found in the pyloric caeca, stomach, and intestine (Tocher 2003; Kurtovic et al. 2009). Mammalian lipases prefer to cleave short chains of fatty acids; however, in fish, digestive lipases tend to cleave long-chain polyunsaturated fatty acids, which are fatty acids with 20 or more carbons (Kurtovic et al. 2009). Pancreatic lipase and carboxyl ester lipase (CEL) are the two types of this enzyme in the digestive system of fish (Kurtovic et al. 2009; Kurtovic et al. 2010). In mammals, there are also calcium-dependent lipases where calcium ions facilitate lipase

activity by minimizing product inhibition (Hwang et al. 2009; Kurtovic et al. 2010). Calcium ions do this by acting as a cofactor to the enzyme and by precipitating fatty acids upon hydrolysis of the substrate (Kurtovic et al. 2010). However, the effects of calcium on lipase activity in teleosts are mostly unknown, as there have been mixed findings (Kurtovic et al. 2009; Kurtovic et al. 2010; Rueda-López et al. 2017). In some instances, teleosts have been found to hydrolyze certain triglyceride molecules (i.e. tributyrin) without calcium, while requiring the ion for the hydrolyzation of other triglyceride molecules (i.e. triolein; Leger et al. 1977). Further, lipases from red sea bream *(Pagrus major)* were determined to be calcium-independent (Iijima et al. 1998).

1.2.3. Cellulase

Cellulose, the most abundant carbohydrate source, is a structural component of plant cell walls and is composed of a homopolymer of glucose residues held together by β-1, 4 glycosidic bonds (Leschine 1995; Saha et al. 2006; Dashtban et al. 2010; Shuangqi et al. 2011). In plant cell walls, it is embedded within other polymers, such as hemicelluloses and proteins (Leschine 1995). Cellulolysis is the process by which cellulose is hydrolyzed into glucose molecules by cellulase enzymes (Shuangqi et al. 2011). The resulting glucose molecules from this reaction are then used in downstream processes that require them, such as energy production and storage through glycolysis and glycogenesis, information processing by the nervous system, and lipogenesis (Polakof et al. 2012). In animals where plant matter makes up a big portion of their diet, the ability to break down cellulose efficiently is essential. There are different kinds of cellulases (e.g. endoglucanases, exoglucanases, and β-glucosidases) that work synergistically to ensure the effective breakdown of cellulose (Dashtban et al. 2010; Merklein et al. 2016). Endoglucanases work by cleaving the internal glycosidic bonds of cellulose, resulting in new

reducing ends (Teter et al. 2014), while exoglucanases cleave the glycosidic bonds that hold cellulose together at the chain ends, producing mostly cellobiose and some glucose molecules (Dashtban et al. 2010). Finally, β-glucosidases are important for breaking the glycosidic bonds of cellobiose to produce glucose (Dashtban et al. 2010; Teter et al. 2014). The action of β-glucosidase is important in preventing end-product inhibition from cellobiose, which is the smallest repeating unit of cellulose, as the animal is unable to use this glucooligomer (Maki et al. 2009; Teter et al. 2014). Carboxymethyl cellulase (CMCase) is a type of endoglucanase that breaks down the β-1, 4 glycosidic bonds in carboxymethyl cellulose, a soluble form of cellulose (Maki et al. 2009). To date, cellulases produced by vertebrates have not been universally discovered. As vertebrates appear to lack the genetic code to produce their own cellulase, these animals must digest cellulose by utilizing cellulases from microorganisms, such as bacteria and fungi (Li et al. 2009). This is the rationale for choosing cellulase as a carbohydrate digestive enzyme for this study compared to another (such as amylase, which is produced by vertebrates), as any activity detected could be contributed to the bacteria within the intestine. This, in turn, created a measurement to compare and contrast with enzymes that could be produced by either the microbe host or the gut bacteria such as trypsin and/or lipase.

1.3. Gastrointestinal microbiota of teleosts

The gastrointestinal tracts of teleosts host a diverse bacterial community that can aid in the breakdown of nutrients by producing additional digestive enzymes, such as trypsin, lipase, and especially cellulase. It has long been known that the intestine of a fish is a non-sterile environment (Trust and Sparrow 1974; Cahill 1990; Ray et al. 2012) that hosts aerobic, obligate, and facultative anaerobic bacterial species (Trust et al. 1979; Ringø et al. 1995; Bairagi et al.

2002; Saha et al. 2006; Izvekova et al. 2007); however, only recently has there been an interest in the role of gut microbes in relation to digestion. Bacteria are found on the mucosal surface of the intestine, closely associated with the intestinal epithelium (Figure 1.2B) and can be classified as either resident or transient bacteria (Cahill 1990; Ringø et al. 2003; Asaduzzaman et al. 2018). The transient communities are free-living and are often associated with the chyme and are found in the lumen of the intestine, whereas the resident communities are the ones on the mucosal surface of the intestine (Ringø et al. 2016; Egerton et al. 2018). The colonization and adhesion of the bacterial community on the epithelial surface of the gastrointestinal tract depends on several things, such as: gastric acidity, bile salts, peristalsis, digestive enzymes, immune response, and the presence of other resident bacteria (Cahill 1990; Ringø et al. 2003). Thus, the microbiota of fish intestines is extremely susceptible to change and this community of microbes is not static, even throughout adulthood. Interestingly, bacteria enter the gastrointestinal tracts of fishes very early on in their development, even prior to active feeding and gut development (Ringø et al. 2003). Fish develop their gut microbiota in the larval stage, and this ecosystem found in their bodies is directly correlated to the microbiota of the egg capsule, the water in which they are being reared in, and the food they are fed (Egerton et al. 2018; Wang et al. 2018).

1.4. Impact of diet

An animal's diet is a result of their ecological niche, anatomy, environment, and food availability. Physiologically, teleost gastrointestinal tracts are designed to ensure efficient food breakdown and nutrient assimilation for sufficient growth. However, a change in their natural diet can alter their intestinal microbiota, assimilation efficacy, enzyme production, and even intestinal morphology (Infante and Cahu 1994; Giri et al. 2000; Wagner et al. 2009; Silva et al. 2011; Michl et al. 2019; Perera et al. 2019). In fact, in fish, digestive enzymes activity levels

often correlate with the diet (Kawai and Ikeda 1972; Fagbenro et al. 2000; German et al. 2010; Ray et al. 2012). The specific influence of the diet on either endogenously produced enzymes or those produced by the bacterial inhabitants of the gastrointestinal tract are currently unknown.

1.5. Rationale

With teleost fishes making up approximately 95% of all extant fish species, it is important to understand the physiology behind their digestive systems. These species are an integral part of the aquatic ecosystem, as they are involved in the nutrient cycles and provide nutrients for other species (Allgeier et al. 2013), and so interest in the gastrointestinal tract and the intestinal microbiota has increased in the recent decades. Moreover, digestive enzymes are important for proper and efficient nutrient assimilation; a key component of the growth and survival of fish. While enzymes and enzyme activities have been studied in depth by many, there is a gap in knowledge about the enzyme activities in the different layers of the intestine in teleosts and how the activity levels compare between various species. As well, little is known about the microbe host (i.e. the fish) versus intestinal bacterial contribution to the overall enzyme activity levels. Therefore, examining digestive enzyme activity levels in the intestines of teleosts will provide insight on where digestive enzymes are produced and what role different enzymes play in the digestive processes of various species. The findings from this study can be used for aquaculture, fisheries management, and the formulation of higher quality foods that improve the digestion abilities of teleosts, which can give them a competitive advantage to other animals and overall help conserve their ecosystems.

1.5.1. Species under study

Studying three fish species that have been acclimated to and kept in very similar holding conditions, with the only change being the differences in their diets, can reveal how enzymatic

activity levels vary in the same intestinal layers of different species. The three fish species used in this study are all fresh- and cold-water fishes, and each have varying diets, therefore making them ideal for comparisons. Central stonerollers *(C. anomalum)* are herbivorous minnows belonging to the Cyprinidae family and are distributed throughout North America (Kraatz 1923; Fowler and Taber 1985; Bisping et al. 2010). They are found in small streams and feed primarily on algae, scraping plant matter off of small surfaces of stones (Kraatz 1923; Evans-White et al. 2001; Burger et al. 2005; Bisping et al. 2010). Central stonerollers are of further value to study because there is limited information available on them, thus any findings can contribute to broadening this knowledge and provide insight on how the digestive functions of herbivores compare to omnivores and carnivores. Goldfish *(C. auratus)* belong to the same family as central stonerollers, Cyprinidae. They maintain an omnivorous diet, feeding on both plant and animal matter (Maier and Tullis 1984). They are excellent model organisms because they are easy to obtain and are easily able to adapt to many changes, including dietary manipulations. Rainbow trout *(O. mykiss)*, found in lakes and rivers, are carnivorous salmonids native to the North American west coast (Daly et al. 2019; National Geographic 2020). In the wild, their diet consists of insects, leeches, and small fishes (National Wildlife Federation 2020). Rainbow trout have been extensively studied, and therefore are useful in the field of comparative physiology.

1.6. Objectives and hypotheses

This study aimed to answer questions about the contribution of endogenous and exogenous digestive enzymes to the overall enzyme activities found in teleosts. First, by dividing the intestines of fish into different layers, including the whole intestine, chyme (if fed), intestinal muscle, epithelial layer (representing the enterocytes) that have bacteria, and epithelial layer with

enterocytes that have been rinsed of any bacteria, I can begin to quantify the contribution of each intestinal structure to digestion. Further, I can quantify the contribution of the microbiota to enzyme activity levels. Then, by altering the diet of these animals, I can observe if enzyme activities can be altered and to what degree the intestinal microbiota may be contributing versus the microbe host.

Specifically, the main focus of this study was to determine whether the aforementioned intestinal layers that have bacteria exhibit higher enzymatic activities than those that are lacking bacteria, therefore suggesting that the gut microbiota plays a greater role in digestive enzyme production compared to the microbe host. This can give insight on the important role that the gastrointestinal microbiota plays in nutrient assimilation and how much the host animal actually relies on their gut microbiota for digestion. My first objective was to determine the microbe host versus bacterial contribution to enzyme activities in the intestinal layers of three teleost species. I hypothesized that trypsin, lipase, and cellulase activities would be higher in the intestinal layers with bacteria present, with cellulase being produced only by bacteria, and trypsin and lipase being produced by both the microbe host and supplemented with production by bacteria residing in the gastrointestinal tract. My second objective was to see if any differences observed were species-specific or due to the varying diets of the three species under study. I predicted that any differences in enzyme activities could be attributed to differences in the diets, as the gastrointestinal microbiota is influenced by diet and feeding habits.

Table 1.1. The site of action and secretory organs of the digestive enzymes (trypsin, lipase, and cellulase) in fish. Adapted from (Sarbahi 1951; German and Bittong 2009; Kurtovic et al. 2009).

Enzyme	Substrate	Substrate Source	Site of Action	Secretory Organs	Stimulated by
Trypsin	Protein	Algae, Detritus, Animals	Lumen of intestine, Chyme	Pancreas, Intestine, Pyloric caeca	Enterokinase
Lipase	Lipid	Algae, Detritus, Animals	Lumen of intestine, Chyme, Stomach, Pyloric caeca	Pancreas, Intestine, Stomach, Pyloric caeca	Potentially calcium*
Cellulase	Cellulose	Algae, Detritus, Wood	Lumen of intestine, Chyme	Unknown in fishes	Presence of cellulose

* Has not been investigated in fishes, but calcium has been found to enhance lipase activity by preventing product inhibition in mammals (Kurtovic et al. 2009).

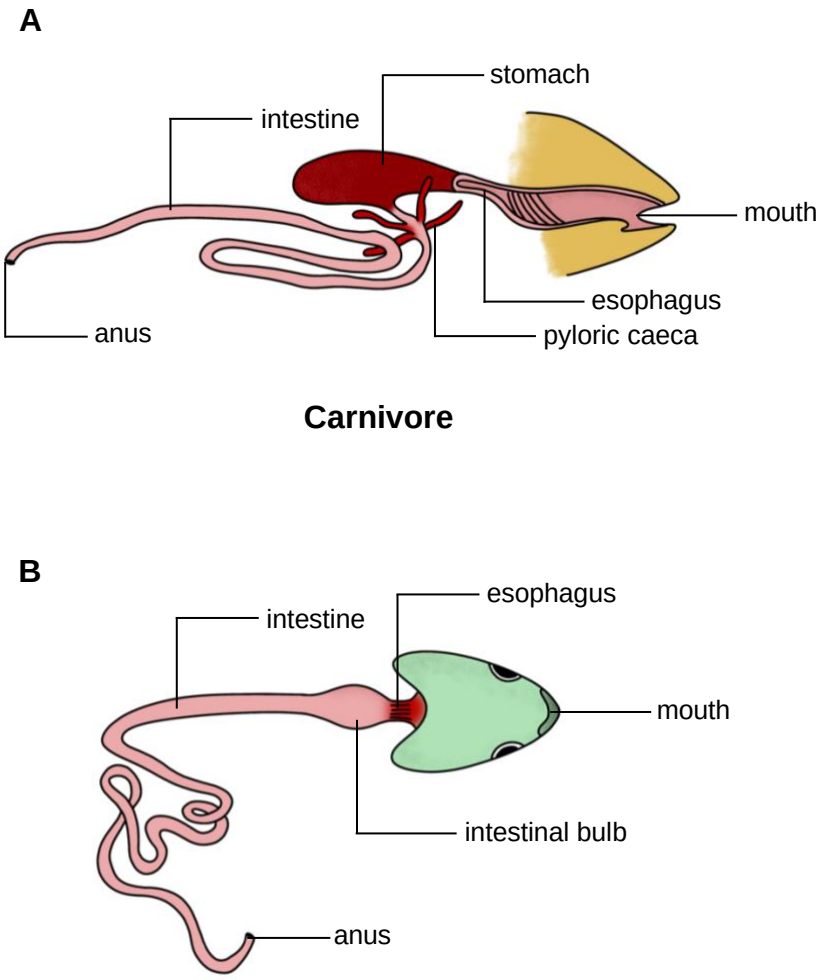

Figure 1.1. The gastrointestinal tracts of a typical **(A)** carnivorous fish with a stomach, pyloric caeca, and short intestine, and **(B)** omnivorous and herbivorous fishes with an intestinal bulb and long intestine. Adapted from (Sarbahi 1951; Palomino 2018).

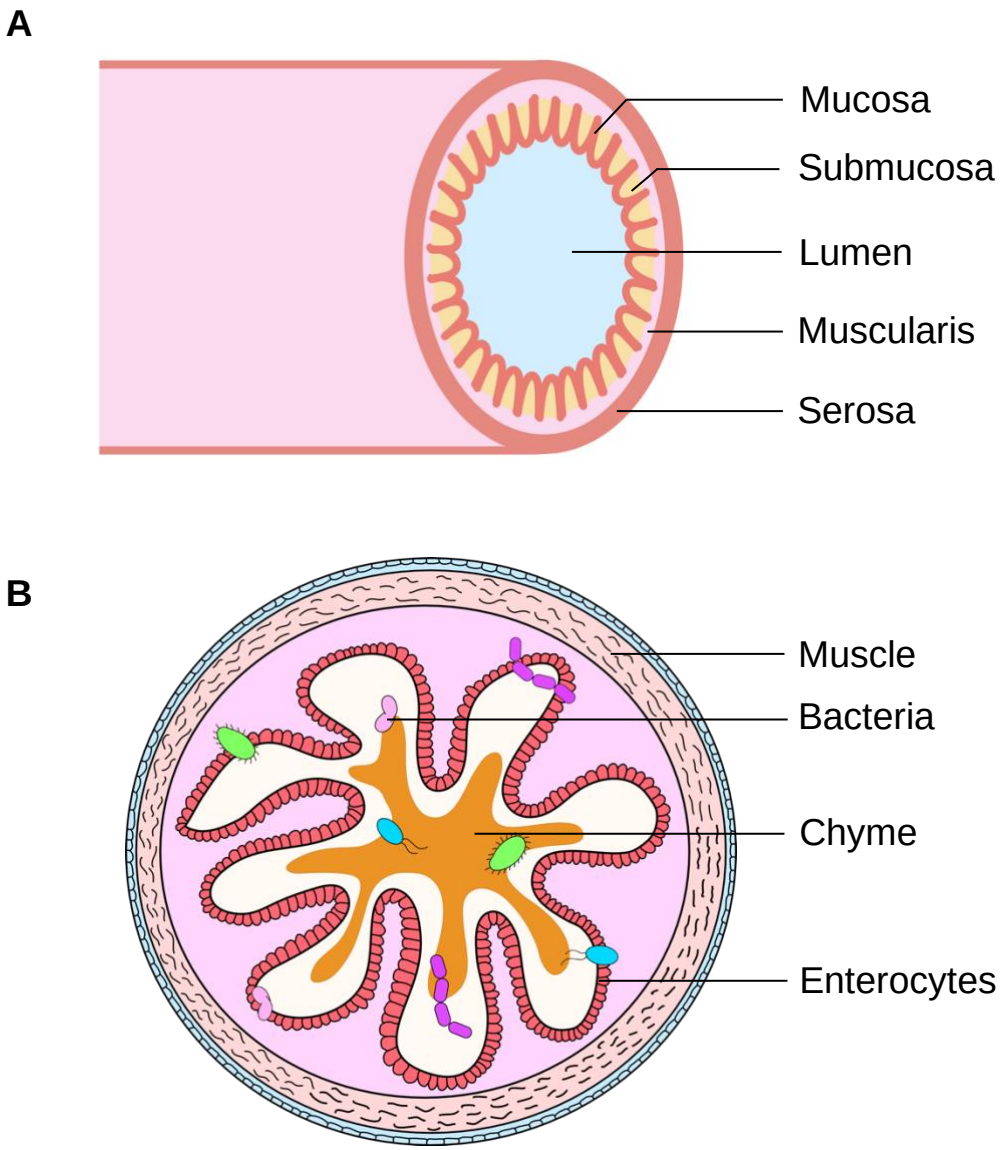

Figure 1.2. Schematic illustration of the intestines of teleosts. **(A)** Layers of the intestine include the serosa, muscularis, submucosa, and mucosa. **(B)** Cross-section of the intestine, showing the intestinal muscle (muscularis layer), enterocytes (in the mucosal layer), chyme (in the lumen), and bacteria (in the mucosal layer and lumen).

CHAPTER 2: TRYPSIN, LIPASE, AND CELLULASE ACTIVITIES IN THE INTESTINAL LAYERS OF THREE TELEOST SPECIES WITH DIFFERENT DIETS

2.1. INTRODUCTION

2.1.1. Endogenous production of digestive enzymes

Digestive enzymes are essential for the breakdown of food into nutrients and eventually energy. Animals have endogenous digestive mechanisms they rely on for this catabolic process (German 2009), and there have been numerous reports of endogenous digestive enzyme production in the gastrointestinal tracts of various teleost species (Das and Tripathi 1991; Bairagi et al. 2002; Klomklao et al. 2008; German et al. 2010). The breakdown of proteins, lipids, and carbohydrates depend on digestive enzymes such as trypsin, lipase, and cellulase (Shuangqi et al. 2011; Grover et al. 2018), respectively. Firstly, it is widely accepted that proteases are produced endogenously by fishes. Multiple studies have examined proteolytic activity in different fish species where native trypsin has been found in the gastrointestinal tract and the surrounding accessory organs (Sarbahi 1951; Sabapathy and Teo 1993; German 2009; German and Bittong 2009; Unajak et al. 2012; Jesús-de la Cruz et al. 2018). Secondly, fishes are also known to produce lipase endogenously. This enzyme was found in larvae before their first exogenous feeding (Caruso et al. 2009), as well as in digestive tissues and organs of several species (Fagbenro et al. 2000; Kurtovic et al. 2009; Xiong et al. 2011). Finally, cellulase likely cannot be endogenously produced, as genes encoding for cellulases have not been found in the genomes of vertebrates (Watanabe and Tokuda 2001). Instead, cellulases found in the digestive tract have been attributed to bacterial sources (Watanabe and Tokuda 2001; Bairagi et al. 2002; Nayak 2010; Sumathi et al. 2011). However, despite this evidence that cellulase is exogenously produced in animals, there have been a few reports of endogenous cellulase production in fishes

(Shcherbina and Kazlawlene 1971 cited in Bairagi et al. 2002; Hlophe et al. 2014). Specifically, some studies have detected cellulase activity in the intestines of fishes where bacteria were not present, therefore suggesting the microbe host was responsible for the production of this enzyme (Prejs and Blaszczyk 1977; Das and Tripathi 1991). The explanation for this contradiction is unclear. Endogenous digestive enzyme production in teleosts is summarized in Table 1.1, including the secretory organs and sites of action.

2.1.2. Exogenous production of digestive enzymes

While animals may be able to produce trypsin and lipase (and possibly cellulase) endogenously, these enzymes may also come from external sources, such as from bacteria (Lesel et al. 1986; Saha et al. 2006; Silva et al. 2011; Xia et al. 2018). In fact, there is a known symbiotic relationship between certain gut bacteria and the human host for producing digestive enzymes (Nicholson et al. 2012). However, little is understood about the host-bacterial interactions in fish (Bairagi et al. 2002; Ray et al. 2012). We do know that there are proteolytic bacteria that are responsible for protease production, which in turn increases the fish's ability to better absorb proteins from their diet (Lesel et al. 1986; Ray et al. 2012; Wu et al. 2012). Studies on the gastrointestinal microbiota of various teleost species have successfully identified proteolytic bacteria in all of the species examined, regardless of diet (Bairagi et al. 2002; Ghosh et al. 2002; Kar and Ghosh 2008). However, these bacteria were found to be more abundant in the gastrointestinal tracts of carnivorous murrel *(Channa punctatus)* than in the herbivorous rohu *(Labeo rohita;* Kar and Ghosh 2008), indicating that there may be a correlation to diet. There are few studies on bacteria that produce lipase in the intestines of fish, but lipolytic bacterial strains have been found in several species with varying diets. Lipase-producing bacteria in the digestive

tracts of teleost species that are herbivorous, omnivorous, or carnivorous have been discovered (Trust et al. 1979; Ringø et al. 1995; Sumathi et al. 2011; Das et al. 2014). One study showed the greatest population density of lipolytic bacteria in the digestive tracts of herbivorous silver carp *(Hypophthalmichthys molitrix;* Bairagi et al. 2002). Further, Ringø (unpublished; cited in Ringø et al. 1995) was able to characterize some aerobic lipolytic bacteria in the carnivorous Arctic charr *(Salvelinus alpinus* L.) intestine. Additionally, anaerobic lipolytic bacteria have also been studied in herbivorous grass carp *(Ctenopharyngodon idella;* Trust et al. 1979*)*. Finally, it is well-accepted that most vertebrates are unable to produce cellulases themselves and therefore, they must rely on the production of these enzymes by microorganisms (Yokoe and Yasumasu 1964; Lesel et al. 1986; Saha et al. 2006). There are known cellulolytic bacteria present in the intestines of herbivorous and omnivorous teleosts, such as Indian major carp *(Catla catla, L. rohita,* and *Cirrhinus mrigala)*, Chinese carp *(H. molitrix* and *C. idella)*, and tilapia *(Oreochromis mossambic;* Bairagi et al. 2002). Interestingly, the authors found no evidence of cellulolytic bacteria in carnivorous species, such as the walking catfish *(Clarias batrachus)* and murrel *(C. punctatus;* Bairagi et al. 2002). Kar and Ghosh (2008) also examined cellulolytic and proteolytic bacterial populations in the gastrointestinal tracts of the herbivorous rohu and carnivorous murrel, and found a higher number of cellulolytic bacteria in rohu, supporting the hypothesis that the microbiota of the gastrointestinal tract correlates to the diet. However, additional studies have shown that the gastrointestinal microbiome of carnivorous species may also contain cellulolytic bacteria, as seen in *Mystus gulio* and other freshwater species (Liu et al. 2016; Asaduzzaman et al. 2018).

2.1.3. Intestinal microbiota

The intestinal microbiota of teleosts is of particular interest to researchers, as the microbes play a crucial role in producing digestive enzymes that help with the breakdown of nutrients (Bairagi et al. 2002). The microbiota of the intestine in fish is established very early on in a fish's development and continuously adapts to changes to their diet, environment, and stress levels (Nayak 2010; Egerton et al. 2018). The microbial composition of the intestine differs between species and is specialized to help break down food particles and assimilate nutrients.

Diet is one major determinant of resident microbial species, and shifts in the microbiota can occur when food sources change and even during periods of starvation (Brown et al. 2012; Ingerslev et al. 2014; Perry et al. 2020). For example, in the Asian seabass *(Lates calcarifer),* bacteria from phylum Bacteroidetes became abundant while bacteria from phylum Proteobacteria were reduced in response to starvation (Xia et al. 2014). In particular, herbivorous and carnivorous species appear to have distinct microbial communities. For instance, Actinobacteria made up more than 10% of the herbivorous grass carp intestinal microbiota (Wu et al. 2012), but only 1% in the carnivorous Asian seabass (Xia et al. 2014). Further, a meta-analysis by Sullam and colleagues (2012) revealed differences in gut bacterial communities of fish in different trophic levels (herbivores: Clostridiales, Bacteroidales, Verrucomicrobiales; omnivores: Rhizobiales, Fusobacteriales, Planctomycetales, Desulfovibrionales; carnivores: Desulfovibrionales, Aeromonadales), further corroborating the effects of diet and microbe host species on intestinal microbial composition. It has been suggested that these differences between the herbivore and carnivore are a result of evolved host-selection differences in response to differing diets (Xia et al. 2014). Indeed, although intestinal bacterial populations are affected by

the external environment, host-specific factors are also at play, as distinct bacterial communities have been found in cohabiting species (Xuemei Li et al. 2012).

2.1.4. Species comparisons

It is clear from the current literature that the gastrointestinal bacterial composition varies across species (e.g. Mondal et al. 2008; Ray et al. 2012; Xia et al. 2014). Further, it is also clear that digestive enzyme activities vary across species (e.g. Sabapathy and Teo 1993; Chan et al. 2004; Ray et al. 2012). The contribution to these differences from bacterial community variation or microbe host phenotype is unclear. Complicating species comparisons is morphology. For example, the intestines of the carnivorous rainbow trout, omnivorous goldfish, and herbivorous central stoneroller are anatomically different due to their varying diets (see Section 1.2.1). This makes comparisons at the whole intestine level complicated by factors such as intestinal size. Although intestinal characteristics may vary across species, the intestinal layers remain the same (Figure 1.2), providing an opportunity for comparison. Further, bacteria inhabit the whole intestine, the chyme of fed individuals, and the enterocytes in the epithelial layer of the intestine (Figure 1.2) but are lacking in the muscle layer. By studying the specific intestinal layers (as opposed to the entire intestine) and bacterial contribution to digestive processes, I can highlight the functional role of intestinal microbial diversity across species.

2.1.5. Hypotheses

I hypothesized that the bacteria inhabiting the intestine of the host fish would contribute digestive enzymes, specifically all of the observed cellulase activity as well as a proportion of the trypsin and lipase activities seen in a species-specific manner depending on diet. First, I

predicted that enzyme activities would be highest in the intestinal layers where bacteria were present. This means that enzyme activity levels would be highest in the whole intestine, chyme (if fed), and epithelial layer (representing the enterocytes) that had bacteria, as there would be endogenous and exogenous enzyme production. Second, I predicted that cellulase activity levels would be absent in the intestinal layers where bacteria were absent, such as the muscle layer and epithelial layer (representing the enterocytes) that were rinsed of any bacteria. Finally, I predicted that in the carnivorous rainbow trout, trypsin activity would be enhanced, in the herbivorous central stoneroller, cellulase and lipase activities would be enhanced, and in the omnivorous goldfish, trypsin, lipase, and cellulase activities would be at an intermediate level. I tested this hypothesis and these predictions by performing trypsin, lipase, and cellulase assays in the aforementioned intestinal layers in the central stoneroller, rainbow trout, and goldfish. I also tested the impact of feeding on these activity rates and compared across the various intestinal layers to observe any trends.

2.2. MATERIALS AND METHODS

2.2.1. Experimental animals, housing, and care

Central stonerollers *(C. anomalum)* were wild-caught from the Grand River in Belwood, Ontario, Canada using minnow traps in the fall of 2018 and 2019. The fish were immediately transported to York University (Toronto, Ontario, Canada) with aeration. Upon arrival at York University, fish were kept in several aerated, flow-through, dechlorinated water tanks (56 L; n=20 per tank), at a temperature of 12 ± 2°C and allowed to acclimate for a minimum of two weeks before experimentation. Central stonerollers were fed to satiation with Omega One™ Super Veggie brown seaweed (OmegaSea LLC; Alaska, U.S.A) once daily. Uneaten food was removed 60 minutes following feeding. For the fed trials, fish were dissected 24 hours following feeding. Fish for the unfed trials were fasted for seven days prior to dissection.

Rainbow trout *(O. mykiss)* were delivered to York University by Humber Springs Trout Club & Hatchery (Orangeville, Ontario, Canada) in fall 2018. As with the central stonerollers, rainbow trout were kept in aerated, flow-through, dechlorinated water tanks (200 L; n=30 per tank) at a temperature of 12 ± 2°C and allowed to acclimate for a minimum of two weeks before experimentation. Fish were fed to satiation with 3.0mm trout food pellets (Corey Aquafeeds; Fredericton, New Brunswick, Canada) every other day. Uneaten food was removed from the tanks 60 minutes following feeding. Fish for the fed trials were dissected 24 hours following feeding, and fish were fasted for seven days before dissection for the unfed trials.

Goldfish *(C. auratus)* were obtained from Big Al's Aquarium Supercentres (Vaughan, Ontario, Canada). The fish were housed in aerated, flow-through, dechlorinated water tanks (56 L; 12 ± 2°C; n=20 per tank) for two weeks before experiments began. Goldfish were fed daily with commercially available goldfish flakes (Cobalt Aquatics; Rock Hill, South Carolina, U.S.A)

to satiation and any remaining food was removed after 60 minutes. As before, for the fed trials, the fish were dissected 24 hours following feeding, and fasted for seven days for the unfed trials.

All experimental procedures have been carried out with approval from the York University Animal Care Committee.

2.2.2. Dissections

Central stonerollers, rainbow trout, and goldfish were all sampled with identical protocols. Individual fish were submerged in a high-dose treatment (2.0g L^{-1}) of buffered tricane methanesulfonate (MS-222; Syndel Canada; Nanaimo, British Columbia, Canada) for euthanization. Subsequently, dissections were performed under sterile conditions. The dissection tools were UV-sterilized for 20 minutes to prevent bacterial contamination from external sources. Further, the dissection surface was sterilized with several washes of 70% ethanol between each fish. Once euthanized, the gastrointestinal tract was carefully removed and the intestine was isolated. The intestine was then separated into the different layers. For the "whole intestine" sample, a small portion of the intestine, immediately posterior to the esophagus in the central stoneroller and goldfish, and immediately posterior to the pyloric caeca in the rainbow trout, was placed in a sterile microcentrifuge tube for genomic DNA (gDNA) extraction. The remaining part of the anterior intestine was separated from the mid- and posterior intestines and was freeze-clamped in aluminum foil for enzyme assays. For fed samples, the chyme was removed from the intestinal lumen by gently pushing it out using UV-sterilized forceps. The chyme was also divided for gDNA extraction and enzyme assays in the same manner as the intestine, and this made up the "chyme" samples. Next, the intestinal muscle and epithelial layer (representing the enterocytes) were mechanically separated. Prior to the mechanical separation of

these two layers, the intestine was rinsed with approximately 1mL of 0.7% NaCl using a sterile syringe and needle for the “saline washed” samples, or with approximately 1mL of 70% sterile ethanol for the “ethanol washed” samples. The intestinal muscle and epithelial layer with enterocytes were mechanically separated by using UV-sterilized forceps and scraping off the epithelial tissue from the intestinal muscle. These were labelled the "muscle" and "epithelial layer (saline or ethanol washed enterocytes)” samples. A portion of both were put into separate sterile microcentrifuge tubes and freeze-clamped in aluminum foils for gDNA extraction and enzyme measurements, respectively. Table 2.1 briefly summarizes the dissected layers of the intestines in fed and unfed treatments. All tissue samples were stored at -80°C until further analysis.

2.2.3. Genomic DNA extraction

Genomic DNA was extracted from all of the samples that were placed in the sterile microcentrifuge tubes during the dissections (Section 2.2.2; Table 2.1) to identify bacterial presence and absence in the various layers and samples. First, gDNA of each dissected sample was extracted under sterile conditions using either the QIAamp DNA Stool Mini Kit (QIAGEN; Toronto, Ontario, Canada) or the QIAamp DNA Microbiome Kit (QIAGEN; Toronto, Ontario, Canada) following the manufacturer’s protocol. These two kits were used interchangeably on random samples, as they have previously been proven to be successful in validating the presence or absence of bacteria in samples by our lab. Forceps, scissors, pipettes, pipette tips, microcentrifuge tubes, and all kit contents were UV-sterilized for 20 minutes before gDNA extractions commenced under the cabinet to prevent external bacterial contamination. Briefly, for the QIAamp DNA Stool Mini Kit, the microbe host cells were lysed with buffer ASL and

heated at 95ºC. InhibitEX tablets were added to remove polymerase chain reaction (PCR) inhibitors, followed by Proteinase K, which works to digest nucleases and proteins to prevent DNA degradation, and buffer AL, which lyses the bacterial cells. Samples were heated at 70ºC to maximize digestion. Ethanol was then added to enhance the binding of DNA to the membrane, while all other proteins and salts were washed out with buffers AW1 and AW2 in a QIAamp UCP Mini Column. Elution buffer AE was used to hydrate and release the extracted gDNA. For the QIAamp DNA Microbiome Kit, the microbe host cells were lysed using buffer AHL. The microbe host DNA was degraded with buffer RDD and Benzonase at 37ºC. Next, Proteinase K was added and the samples were incubated at 56ºC. To lyse bacterial cells, lysis buffer ATL with reagent DX (which reduces foaming during mechanical lysis) were added to samples, which were then transferred into Pathogen Lysis tube L. This tube promotes mechanical digestion of the bacterial cells before the addition of Proteinase K at 56ºC to ensure chemical digestion as well. Buffer APL2 was added to separate the nucleic acids from all other components of the cells. Ethanol and buffers AW1 and AW2 were added as previously described and buffer AVE was used as an elution buffer.

A blank extraction was performed with every set of gDNA extraction to ensure that the extraction process was sterile. In this case, the methods previously described were followed, however, no tissue sample was added. This acted as a contamination control. All extracted gDNA samples were stored at -20ºC until further analysis.

2.2.4. Polymerase chain reaction and gel electrophoresis

Polymerase chain reaction was performed on the extracted gDNA samples using universal primers for the bacterial 16S ribosomal RNA (rRNA) gene sequence. This gene

sequence contains nine hypervariable regions that are separated by nine highly-conserved regions (Chakravorty et al. 2007; Yang et al. 2016). Hypervariable regions are regions in the bacterial genome that are diverse, therefore making it possible to classify bacteria at different taxonomic levels (Chakravorty et al. 2007) whereas the highly-conserved regions are present in the genome of all bacteria. These PCR primers were designed to target the conserved regions that surround the hypervariable regions of interest, which in the current study tested for the presence or absence of all bacterial species (Chakravorty et al. 2007). Table 2.2 shows the primers and respective sequences used in the present study (8F-519R, V1-V3 hypervariable region; 8F-533R, V1-V3 hypervariable region; 338F-785R, V3/V4 hypervariable region; 967F-1177R, V6/V7 hypervariable region; Sigma-Aldrich; St. Louis, Missouri, U.S.A). These primers were specifically selected as they are widely-used in bacterial studies (Chakravorty et al. 2007; Huse et al. 2008; Hamp et al. 2009; Sun et al. 2014; Yang et al. 2016). By using a combination of these primer sets, it allowed me to validate the presence or absence of bacteria within the different intestinal layers with confidence, as it amplified multiple hypervariable regions within the bacterial genome, preventing any efficiency issues to be mistaken as absent bacteria.

Each PCR reaction contained 6.5µL of a ready-to-use solution of *Taq* polymerase, consisting of DreamTaq DNA polymerase, DreamTaq buffer, deoxynucleoside triphosphates (dNTPs), and 4mM $MgCl_2$ (2X DreamTaq PCR Master Mix or 2X DreamTaq Green PCR Master Mix; Thermo Fisher Scientific; Mississauga, Ontario, Canada), 1.25µL each of the forward and reverse primers for each primer set (Table 2.2), 1.75µL of nuclease-free water, and 2µL of the extracted gDNA. A blank PCR reaction where 2µL of nuclease-free water was used in place of a gDNA sample was prepared to ensure the PCR preparation process was sterile. A positive control using 2µL of bacterial gDNA from a previous successful gDNA extraction set

using either the QIAamp DNA Stool Mini Kit or the QIAamp DNA Microbiome Kit was prepared to ensure the PCR reaction was successful. PCR was run using a Mastercycler® gradient thermal cycler (Eppendorf Canada Ltd.; Mississauga, Ontario, Canada) using the steps outlined in Table 2.3.

The PCR products were separated alongside 5µL of GeneRuler DNA ladder mix (0.1µg μL^{-1}; Thermo Fisher Scientific; Mississauga, Ontario, Canada) on a 1.5% agarose (w/v) gel (100V; 30 minutes) using 1X Tris-acetate-EDTA (TAE) buffer. For reactions with 2X DreamTaq PCR Master Mix, 1µL of 6X DNA gel loading dye (Thermo Fisher Scientific; Mississauga, Ontario, Canada) was pipette mixed with 10µL of the PCR product before loading it onto the gel. For reactions with 2X DreamTaq Green PCR Master Mix, this step was skipped, as the master mix already contained a loading dye. The PCR products were imaged using SYBR™ Safe DNA Gel Stain (Invitrogen by Thermo Fisher Scientific; Mississauga, Ontario, Canada) and the imager MiniBIS Pro (DNR Bio-Imaging Systems; Neve Yamin, Israel).

2.2.5. Enzyme assays

The remaining portions of the intestines (Table 2.2) that were freeze-clamped in aluminium foil were used for enzyme activity measurements. Briefly, the samples were homogenized in their respective homogenizing buffers using an ice-cold glass homogenizer and kept on ice. All enzyme assays were performed in BD Falcon™ 96-well microplates (Corning Incorporated; Corning, New York, U.S.A) and enzyme activities were measured using a spectrophotometer (Synergy HT microplate reader; BioTek Instruments; Winooski, Vermont, U.S.A) and the application software Gen5 (version 2.04, BioTek Instruments).

2.2.5.1. Trypsin enzyme assay

Trypsin activity by way of the BAPNA method is measured by the hydrolysis of N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) into N_{α}-Benzoyl-L-arginine and p-nitroaniline (Equation 1). When the peptide bond holding BAPNA together is broken, p-nitroaniline is released, which produces a yellow colour that is read by the spectrophotometer. In the current study, BAPNA was used as the substrate and the amount of p-nitroaniline released from BAPNA was indicative of trypsin activity (Liu et al. 2016).

(1):

$$N\alpha - Benzoyl - L - arginine\ 4 - nitroanilide\ hydrochloride + H_2O \xrightarrow{trypsin}$$
$$N\alpha - Benzoyl - L - arginine + p - nitroaniline$$

Tissues were homogenized in 300µL of 0.05M Tris HCl homogenization buffer at pH 7.5 and centrifuged at 12, 000 x g for 15 minutes at 4°C to pellet insoluble material. 1.95mM BAPNA (Sigma-Aldrich; St. Louis, Missouri, U.S.A) was dissolved in 100mM Tris HCl reaction buffer at pH 7.5 and used as the substrate in this reaction. 10µL of each homogenate was added to a microcentrifuge tube, with either 190µL of the Tris HCl reaction buffer + BAPNA mixture added to the experimental samples, or 190µL of only Tris HCl reaction buffer added to the control samples. The absorbance of each sample was measured at a wavelength of 410nm at 37ºC after 20 minutes and Equation 2A was used to calculate the trypsin specific activity expressed as U mg protein^{-1}. Here, U represents µmol of p-nitroaniline released during the reaction per minute. Equation 2B was used to calculate the trypsin total activity per gram of tissue (U g tissue^{-1}).

(2A):

$$trypsin\ activity = \left(\frac{\left(\frac{slope}{E \times pathlength}\right)_{with\ BAPNA} - \left(\frac{slope}{E \times pathlength}\right)_{control}}{V_{sample} \times C_{protein}} \right)$$

(2B):

$$trypsin\ activity = \left(\frac{\left(\frac{slope}{E \times pathlength}\right)_{with\ BAPNA} - \left(\frac{slope}{E \times pathlength}\right)_{control}}{tissue\ mass} \right)$$

where *E* is the extinction coefficient for BAPNA (9.96), V_{sample} is the sample volume loaded into the microplate well (200µL), $C_{protein}$ is the concentration of protein in the homogenate (mg μL^{-1}), and *tissue mass* is the amount of tissue (g) in the homogenate.

2.2.5.2. Lipase enzyme assay

Lipase assays were performed using the Lipase Activity Assay Kit (Sigma-Aldrich; St. Louis, Missouri, U.S.A) following the manufacturer's protocol with some modifications. Here, lipase activity was determined using a proprietary coupled enzyme reaction, which resulted in a colorimetric (570nm) product proportional to the enzymatic activity. This kit defines one unit of lipase as the amount of enzyme that will generate 1.0µmol of glycerol from triglycerides per minute (Equation 3).

(3):

$$triglyceride + H_2O \xrightarrow{lipase} glycerol + fatty\ acids$$

Modifications included tissues being homogenized in 0.1M phosphate-buffered saline (PBS) buffer at pH 6.8 instead of the lipase assay buffer found in the kit. Briefly, tissues were homogenized in 160µL PBS buffer and centrifuged at 13, 000 x g for 10 minutes at 4ºC to pellet insoluble material. Glycerol standards (0, 2, 4, 6, 8, 10nmol glycerol per well) and reaction mixes (consisting of lipase assay buffer, peroxidase substrate, enzyme mix, and lipase substrate) were prepared. Each well in the microplate was loaded with 2µL of the homogenate, 48µL of lipase buffer, and 100µL of the prepared reaction mix. The absorbance of each sample was measured every five minutes at a wavelength of 570nm for a total of three hours at 37ºC, and a standard curve was produced. The lipase specific activity was calculated using Equation 4A and was expressed as U g protein^{-1}, where U is a measure of nmol of glycerol produced per minute. The lipase total activity was quantified as U g tissue^{-1} using Equation 4B.

(4A):

$$lipase\ activity = \left(\frac{B}{\Delta t \times V_{sample} \times C_{protein}}\right)$$

(4B):

$$lipase\ activity = \left(\frac{B}{\Delta t \times tissue\ mass}\right)$$

where B is the amount of glycerol generated between t_i and t_f in nmol, Δt is ($t_f - t_i$) in minutes, V_{sample} is the sample volume loaded into the microplate well (2µL), $C_{protein}$ is the concentration of protein in the homogenate (mg µL^{-1}), and *tissue mass* is the amount of tissue (g) in the homogenate.

2.2.5.3. Cellulase enzyme assay

Cellulase enzyme assay using the 3,5-dinitrosalicyclic acid (DNS) method is a colorimetric (574nm) assay used to measure the amount of reducing sugars released from the substrate during the reaction. In the present study, carboxymethylcellulose sodium salt (CMC; Sigma-Aldrich; St. Louis, Missouri, U.S.A) was the substrate that released the reducing sugars, measured as glucose (Denison and Koehn 1977). CMC is broken down into glucose and this glucose molecule reacts with DNS to form 3-amino-5-nitrosalicylic acid and gluconic acid (Equation 5). The 3-amino-5-nitrosalicylic acid produces a darker orange-brown colour, which is measured by the spectrophotometer.

(5):

$$(\boldsymbol{i}): carboxymethylcellulose\ sodium\ salt\ (cellulose) \xrightarrow{cellulase} glucose$$

$$(\boldsymbol{ii}): glucose + 3{,}5 - dinitrosalicylic\ acid \longrightarrow$$
$$3 - amino - 5 - nitrosalicylic\ acid + gluconic\ acid$$

Tissues were homogenized in 400µL of 0.1M citrate buffer at pH 5.0 and centrifuged at 12, 000 x g for 20 minutes at 4°C to pellet insoluble material. Microcentrifuge tubes were filled with 125µL of the homogenate and either 125µL of 1% CMC (w/v) was added to experimental samples as a substrate, or 125µL of citrate buffer was added in place of a substrate to the control samples. These samples were briefly vortexed and incubated at 37°C for one hour and the reaction was stopped with the addition of 750µL of DNS reagent to each sample. The samples, along with 250µL of prepared glucose standards (0, 0.125, 0.25, 0.50, 0.75, and 1.00mg glucose

mL^{-1}) also containing 750µL of DNS reagent, were placed in a heating block set at 96°C for 15 minutes until a colour change was observed. 200µL of each sample was loaded onto a microplate. The absorbance of each sample at a wavelength of 574nm at 37ºC was measured and a standard curve was produced. The cellulase specific activity was calculated using Equation 6A and expressed as U mg $protein^{-1}$, where U represents mg of glucose released from cellulose per minute. The total cellulase activity was also quantified as U g $tissue^{-1}$, as seen in Equation 6B. This protocol was adapted from Denison and Koehn (1977) and Liu et al. (2016).

(6A):

$$cellulase\ activity = \frac{(C_{with\ CMC} - C_{without\ CMC})}{V_{sample} \times C_{protein}}$$

(6B):

$$cellulase\ activity = \left(\frac{(C_{with\ CMC} - C_{without\ CMC})}{tissue\ mass}\right)$$

where *C* is the concentration of the sample with and without CMC at 574nm, V_{sample} is the sample volume loaded into the microplate well (200µL), $C_{protein}$ is the concentration of protein in the homogenate (mg μL^{-1}), and *tissue mass* is the amount of tissue (g) in the homogenate.

2.2.6. Bradford protein assay

Bradford protein assays were performed for trypsin, lipase, and cellulase enzyme assays to determine the protein concentration of each homogenized tissue sample using the Bradford assay protocol (Bradford 1976). Microplates were filled with 5µL of homogenates or Quick Start™ bovine serum albumin standard stock solutions (Bio-Rad; Hercules, California, U.S.A) of

concentrations 0.125, 0.25, 0.50, 0.75, and 1.00mg protein mL^{-1}. Each respective homogenization buffer was included for control. 195µL of Quick Start™ Bradford 1X dye reagent (Bio-Rad; Hercules, California, U.S.A) or 250µL of Bradford dye reagent, ready-to-use solution (Alfa Aesar; Thermo Fisher Scientific; Mississauga, Ontario, Canada) was added to each well. Samples with Bradford dye reagent, ready-to-use solution were incubated in the dark for five minutes prior to measurement, as suggested by the manufacturer's protocol. Samples were diluted with their respective homogenization buffers if necessary. The absorbance of each sample at a wavelength of 595nm at 26ºC was measured and a standard curve was produced.

2.2.7. Statistical analyses

Data were transferred into SigmaPlot Software (version 11.0, Systat Software Inc.; San Jose, California, U.S.A) where statistical analyses were conducted, which are described as appropriate in the table and figure captions. If required, data were normalized using simple statistical transformations on SigmaPlot Software (square root, Ln, or Log10 transformations). Grubb's tests were used to identify outliers in normalized data sets using GraphPad Prism (version 8.4.3, GraphPad Software; San Diego, California, U.S.A). To first determine if there was a detectable level of enzyme activity, defined as activity significantly greater than 0.00 U, a one-sample t-test was performed on individual data sets for each intestinal layer of each species for trypsin, lipase, and cellulase enzymes against a set mean value of zero. If the one-sample test failed to detect a difference from zero, the enzyme activity level was considered to be non-detectable or "*n.d.*" and the sample was not included in further statistical testing. When all three species had detectable activity levels, one-way analysis of variance (ANOVA) tests were used to compare enzyme activities between the three different species in each intestinal layer, followed

by a Bonferroni multiple comparison post-hoc test. When conditions of normality and equal variance could not be met, Kruskal-Wallis one-way ANOVA on ranks was performed, followed by a Dunn's post-hoc test. If enzyme activities were undetectable only in one species, comparisons between the two remaining species were conducted using a t-test or Mann-Whitney rank sum test (if conditions of normality or equal variance were not met). Statistical significance was assumed if $p<0.05$. All figures were computed on SigmaPlot Software and data in the tables and figures are expressed as mean values ± standard error of mean (SEM).

Table 2.1. Summary of intestinal tract dissection in unfed and fed central stonerollers, rainbow trout, and goldfish.

Fish	Dissected Intestinal Layer	gDNA Extraction	Enzyme Measurements
Central stoneroller Rainbow trout Goldfish	Whole intestine Chyme* Muscle Enterocytes (saline wash)** Enterocytes (ethanol wash)**	Whole intestine Chyme* Muscle Enterocytes (saline wash)** Enterocytes (ethanol wash)**	Whole intestine Chyme* Muscle Enterocytes (saline wash)** Enterocytes (ethanol wash)**

* Only in fed samples

** Enterocytes here refer to the epithelial layer (which contains enterocytes) that were mechanically separated from the intestinal muscle.

Table 2.2. Primer sequences, annealing temperatures, and the amplified hypervariable region for the primer sets used in this study.

Name	Sequence (5'-3')	Region	Reference	Annealing Temperature (ºC)
8F	AGAGTTTGATCCTGGCTCAG	V1-V3	(Ruff-Roberts et al. 1994; Sun et al. 2014)	*
519R	GWATTACCGCGGCKGCTG			59.6
533R	TTACCGCGGCTGCTGGCAC			63.5
338F	ACTCCTACGGGAGGAGC	V3/V4	(King et al. 2007; Huse et al. 2008)	57
785R	CTACCAGGGTATCTAATCC			
967F	CAACGCGAAGAACCTTACCT	V6/V7	(Hamp et al. 2009)	57
1177R	GACGTCATCCCCACCTTCCT			

F = forward primer; R = reverse primer

* The annealing temperature for 8F is the same as the annealing temperature of the reverse primer used in conjunction with it.

Table 2.3. PCR conditions followed for the primer sets used in the study.

Step	Temperature (°C)	Time	Number of Cycles
Initial denaturation	94	3 minutes	-
Denaturation	94	30 seconds	35
Annealing	*	30 seconds	
Extension	72	1 minute	
Final Extension	72	10 minutes	-
Hold	4	∞	-

* The annealing temperature varies for each primer set (see Table 2.2).

2.3. RESULTS

2.3.1. Bacterial detection in the intestinal layers

Representative sample PCR products from extracted goldfish gDNA samples using universal bacterial rRNA primers that amplified the V1-V3 hypervariable region of the bacterial genome (primer set 8F-519R) are displayed in Figure 2.1. Here, the bands (511bp in size) correspond to the presence of bacteria in the layers of the intestine. Bacteria were detected in the whole intestine, chyme, and saline washed epithelial layer representing the enterocytes. In contrast, bacteria were not detected in the intestinal muscle and the ethanol washed intestinal epithelial layer. Qualitatively, the bands in Lanes 2 (whole intestine) and 3 (chyme) were the brightest, while Lane 5 (saline washed epithelial layer) had a faint band at the expected product size. The presence of bands outside of the expected product size was assumed to be non-specific and due to the large number of PCR cycles. This was repeated and confirmed in all of the tissue samples used for enzyme assays (gel images not displayed). If bacteria were detected in samples presumed to be devoid of bacteria (i.e. ethanol washed samples and muscle), those samples were not used for enzyme analyses.

2.3.2. Trypsin activity levels

2.3.2.1. Intestinal epithelial layers of each species

Detectable levels (i.e. above 0.00 U) of specific and total trypsin activities in extracted intestinal epithelial layers (representing the enterocytes), were found in all samples taken from rainbow trout ($p<0.05$; Figures 2.2A; 2.2B). In the intestinal epithelial layer of unfed rainbow trout, the ethanol wash significantly increased trypsin specific activity (0.445 ± 0.054 U mg protein^{-1}) compared to the saline wash (0.095 ± 0.024 U mg protein^{-1}; $p<0.05$; Figure 2.2A). In

contrast, the wash treatments had no effect on trypsin total activity in unfed samples (5.720 ± 4.225 U g tissue^{-1} in saline wash versus 5.101 ± 0.540 U g tissue^{-1} in ethanol wash; $p>0.05$; Figure 2.2B). In fed rainbow trout, ethanol significantly lowered both trypsin specific (1.023 ± 0.134 U mg protein^{-1}) and total (7.236 ± 0.645 U g tissue^{-1}) activities in the intestinal epithelia ($p<0.05$; Figures 2.2A; 2.2B). Feeding increased trypsin specific activity in the saline washed epithelial layers ($p<0.05$; Figure 2.2A) but had no effect on the total trypsin activity ($p>0.05$; Figure 2.2B). In contrast, feeding appeared to lower both the trypsin specific and total activities in the ethanol washed epithelial layers ($p<0.05$; Figures 2.2A; 2.2B).

In comparison, specific trypsin activities were, again, detected in all samples taken from goldfish ($p<0.05$; Figures 2.3A; 2.3B). Total trypsin activities were likewise detected in almost all samples ($p<0.05$), apart from the saline washed epithelial layer of fed goldfish ($p>0.05$; Figure 2.3B). In unfed goldfish, the ethanol wash treatment did not significantly affect trypsin specific or total activities ($p>0.05$; Figure 2.3A; 2.3B). In contrast to fed rainbow trout where ethanol washes decreased activities (Figure 2.2), washing with ethanol increased both trypsin specific activity in the intestinal epithelial layer of fed goldfish (0.572 ± 0.071 U mg protein^{-1}; $p<0.05$; Figure 2.3A), and total activity above undetectable levels seen with saline washed samples (Figure 2.3B). Finally, feeding lowered the specific activity of saline washed epithelial tissue (0.391 ± 0.002 U mg protein^{-1} versus 1.412 ± 0.158 U mg protein^{-1}; $p<0.05$), unlike rainbow trout, where feeding elevated activity rates (Figure 2.2A). Further, there was no impact of feeding on the ethanol washed samples (Figures 2.3A; 2.3B), whereas in rainbow trout, feeding decreased activity in the ethanol washed epithelia (Figures 2.2A; 2.2B).

Finally, trypsin activity levels in the intestinal epithelial layers of unfed and fed central stonerollers were combined for each wash treatment, as there was no significant impact of

feeding on activity levels ($p>0.05$; Figure 2.4), unlike both rainbow trout and goldfish. Additionally, while there were detectable levels of specific and total activities for both saline and ethanol washed epithelial layers ($p<0.05$), wash treatments did not have a significant effect on trypsin activity levels ($p>0.05$; Figure 2.4), again in contrast to rainbow trout and goldfish.

2.3.2.2. Interspecies comparisons of intestinal epithelial layers

Trypsin activity was further analyzed by comparing across the three fish species. For this comparison, the collapsed data from the central stoneroller (Figure 2.4) was separated into unfed and fed samples. When investigated individually, trypsin total activities were undetectable in the unfed central stonerollers for either of the washes (Table 2.4), while impacts of the washes in the other species were as described above. Overall, unfed goldfish displayed the highest specific activity levels for both saline and ethanol washed epithelial layers (1.412 ± 0.158 U mg protein^{-1} in saline wash and 1.073 ± 0.229 U mg protein^{-1} in ethanol wash; $p<0.05$), compared to the unfed rainbow trout and central stonerollers (Table 2.4). Further, unfed goldfish also had significantly greater total activity than the rainbow trout and the central stoneroller in the saline washed samples ($p<0.05$; Table 2.4). However, this difference between the rainbow trout and goldfish disappeared when examining the total activity levels of the ethanol washed epithelia ($p>0.05$; Table 2.4) due to an increase in variation seen in the goldfish samples.

In contrast to unfed animals, trypsin activities were detectable in both wash treatments of all three species ($p<0.05$), apart from total activity in the saline washed epithelial layer of the goldfish ($p>0.05$; Table 2.5), as described above. Fed rainbow trout had the highest trypsin specific activity in the intestinal epithelial layers rinsed with saline (1.023 ± 0.134 U mg protein^{-1}; $p<0.05$) followed by fed goldfish (0.391 ± 0.002 U mg protein^{-1}) and central stonerollers (0.172 ± 0.027 U mg protein^{-1}; Table 2.5). Total activity was also elevated in rainbow trout

(7.236 ± 0.645 U g tissue^{-1}; $p<0.05$) compared to the central stoneroller (3.499 ± 0.615 U g tissue^{-1}), but was undetectable in the goldfish ($p>0.05$). In contrast to these results, specific and total trypsin activities in ethanol washed epithelial layers were detected in all three species ($p<0.05$), and activity levels were highest in the fed goldfish (0.572 ± 0.071 U mg protein^{-1} and 7.471 ±1.579 U g tissue^{-1}; $p<0.05$), while rainbow trout and central stonerollers showed similar, lower activities ($p>0.05$; Table 2.5).

2.3.2.3. Interspecies comparisons of the whole intestine, chyme, and intestinal muscle

When studying unfed animals, one-sample t-tests detected both trypsin specific and total activities in the whole intestines of all three species ($p<0.05$; Figure 2.5). Significantly higher trypsin specific activity levels were observed in the whole intestines of unfed central stonerollers compared to goldfish ($p<0.05$) but not rainbow trout ($p>0.05$; Figure 2.5A), in contrast to the trends seen in the epithelial layers (both washes) of unfed animals, where goldfish displayed the highest activity rates (Table 2.4). However, trypsin total activity was greatest in the unfed central stonerollers compared to both the goldfish and rainbow trout ($p<0.05$; Figure 2.5B), again, in contrast to the intestinal epithelial layers (Table 2.4).

One-sample t-tests detected activity in the whole intestines and chyme of all fed samples ($p<0.05$; Figures 2.6A; 2.6C; 2.6D), except for trypsin total activity in the whole intestine of central stonerollers due to high variation within this sample ($p>0.05$; Figure 2.6B). In the whole intestine, fed carnivorous rainbow trout had the highest trypsin specific activity (1.514 ± 0.134 U mg protein^{-1}) in contrast to the other two species (0.077 ± 0.008 U mg protein^{-1} in goldfish and 0.347 ± 0.053 U mg protein^{-1} in central stoneroller; Figure 2.6A), which was also in contrast to the trend seen in unfed samples of the whole intestine (Figure 2.5). Likewise, trypsin total activity in the whole intestine of fed rainbow trout was significantly higher than in the

omnivorous goldfish (Figure 2.6B), which once again, was different from unfed samples (Figure 2.5). In the chyme, specific and total activities in the central stonerollers (21.309 ± 3.895 U mg protein^{-1}; 30.927 ± 8.559 U g chyme^{-1}) were greater than in rainbow trout (2.083 ± 0.367 U mg protein^{-1}; 7.184 ± 1.699 U g chyme^{-1}) and goldfish (0.540 ± 0.097 U mg protein^{-1}; 5.273 ± 2.002 U g chyme^{-1}; $p<0.05$; Figures 2.6C; 2.6D). These trends are in contrast to what was observed in the whole intestines (Figures 2.6A; 2.6B) and intestinal epithelial layer (Table 2.5) of fed samples, but similar to the unfed whole intestine (Figure 2.5).

Finally, trypsin specific activities were detected in the intestinal muscle layer of all three species ($p<0.05$), which had data for feeding treatments collapsed due to a lack of differences ($p>0.05$; Figure 2.6E). Here, central stonerollers had significantly greater activity (0.295 ± 0.053 U mg protein^{-1}) than rainbow trout (0.063 ± 0.022 U mg protein^{-1}; $p<0.05$), but not goldfish ($p>0.05$; Figure 2.6E). However, trypsin total activity was undetectable in the central stoneroller ($p>0.05$) and a Mann-Whitney rank sum test revealed no significant differences in total activities in the intestinal muscles of the rainbow trout and goldfish ($p>0.05$; Figure 2.6F).

2.3.3. Lipase activity levels

2.3.3.1. Intestinal epithelial layers of each species

One-sample t-tests detected lipase activity (i.e. above 0.00 U) in all treatments of the intestinal epithelial layers (representing enterocytes) in rainbow trout ($p<0.05$; Figures 2.7A; 2.7B). In the unfed samples, ethanol washes increased both lipase specific and total activities over saline washes ($p<0.05$; Figures 2.7A; 2.7B). Similarly, in fed rainbow trout, the ethanol wash caused a significant increase in lipase specific activity ($p<0.05$; Figure 2.7A); however, this impact was not observed in total activity ($p>0.05$; Figure 2.7B). Further, feeding stimulated

lipase specific activity in the saline washed samples, with fed rainbow trout showing elevated levels (0.833 ± 0.056 U mg protein^{-1}) compared to unfed animals (0.568 ± 0.089 U mg protein^{-1}; $p<0.05$; Figure 2.7A). However, feeding had no impact on specific activities in the ethanol washed tissue ($p>0.05$; Figure 2.7A). In contrast, feeding decreased lipase total activity in both washes of the intestinal epithelial layer ($p<0.05$; Figure 2.7B).

When examining the goldfish, lipase specific and total activities were, again, detected in both wash treatments of unfed goldfish; however, within the fed animals, only the ethanol washed samples showed detectable activity ($p<0.05$; Figure 2.8). In unfed goldfish, the ethanol wash significantly increased lipase specific activity (7.153 ± 0.916 U mg protein^{-1}; Figure 2.8A) compared to the saline wash treatment (2.826 ± 0.527 U mg protein^{-1}), as previously seen in rainbow trout. In contrast, this intensifying effect following the ethanol wash was not seen when measuring lipase total activity in unfed goldfish (Figure 2.8B). Furthermore, unlike in fed rainbow trout, lipase specific and total activities could not be detected in the saline washed epithelial layer of fed goldfish ($p>0.05$; Figure 2.8). However, once again following ethanol washing, lipase activity was enhanced (and now detectable) in the epithelia of fed goldfish (Figure 2.8). Feeding appeared to inhibit lipase specific activity in saline washed epithelial tissue to non-detectable levels, while also causing a decrease in the specific activity of ethanol washed samples ($p<0.05$; Figure 2.8A). Feeding also eliminated total activity in the saline washed intestinal epithelia, but had no impact on activity levels in the ethanol washed tissue (Figure 2.9B), in contrast to rainbow trout (Figure 2.7B).

Unlike rainbow trout and goldfish, statistical testing showed that no lipase specific activity was detectable in the ethanol washed epithelia of unfed central stonerollers ($p>0.05$; Figure 2.9A), but like fed goldfish, fed central stonerollers also had undetectable levels of

specific activity in the saline washed epithelia ($p>0.05$; Figure 2.9A). However, lipase total activities were detectable in all of the feeding and wash treatments of central stonerollers ($p<0.05$; Figure 2.9B). In unfed samples, the ethanol wash completely eliminated lipase specific activity (Figure 2.9A), whereas activity was increased in rainbow trout and goldfish (Figures 2.7A; 2.8A). However, in fed central stonerollers, ethanol enhanced lipase activity above undetectable levels in the saline washed tissue (Figure 2.9A), similar to the goldfish. In the central stoneroller, feeding inhibited specific activity in the saline washed epithelia, whereas it enhanced activity in the ethanol washed layer (Figure 2.9A). Further, wash treatments did not affect lipase total activities in either unfed or fed fish, but feeding caused a significant decrease in activity levels in both wash treatments (Figure 2.9B), which was also seen in the rainbow trout (Figure 2.7B).

2.3.3.2. Interspecies comparisons of intestinal epithelial layers

Lipase specific and total activities were, again, compared across the three species for both unfed (Table 2.6) and fed (Table 2.7) animals. Lipase activities (both specific and total) in the saline washed epithelial layer of unfed central stonerollers were significantly greater than in unfed rainbow trout and goldfish ($p<0.05$; Table 2.6). However, the ethanol wash eliminated lipase specific activity in the unfed central stoneroller, and the goldfish now displayed the greatest activity ($p<0.05$; Table 2.6). Lipase total activity in the ethanol washed epithelial layer was significantly greater in the unfed central stoneroller (175.244 ± 0.292 U g tissue^{-1}; $p<0.05$; Table 2.6) than in the rainbow trout (10.948 ± 0.265 U g tissue^{-1}), while goldfish were indeterminate between the two (47.696 ± 9.741 U g tissue^{-1}).

Following feeding, lipase specific activity was only detectable in the saline washed intestinal epithelial layer of rainbow trout ($p<0.05$), whereas activity was no longer detectable in

the goldfish and central stoneroller ($p>0.05$; Table 2.7), as previously described. In contrast, fed central stonerollers exhibited the greatest total activity in the saline washed epithelia (135.208 ± 23.126 U g tissue^{-1}) compared to the fed rainbow trout (3.235 ± 0.302 U g tissue^{-1}; Table 2.7). These trends are similar compared to the unfed analyses in Table 2.6, however total lipase activities were detectable in the saline washed intestinal epithelia of all three unfed fishes. When washed with ethanol, the intestinal epithelia exhibited detectable levels of lipase activities in all three species ($p<0.05$), unlike in the unfed animals (Table 2.6), with the central stoneroller having the highest activity (9.128 ± 0.300 U mg protein^{-1}; $p<0.05$; Table 2.7). Total activity analyses showed that all three species had significantly different lipase activities compared to each other ($p<0.05$), with rainbow trout having the lowest (2.813 ± 0.300 U g tissue^{-1}) and central stonerollers having the highest (91.597 ± 3.537 U g tissue^{-1}) total activity levels ($p<0.05$; Table 2.7).

2.3.3.3. Interspecies comparisons of the whole intestine, chyme, and intestinal muscle

Lipase specific and total activity levels were detectable in all three species in the whole intestines of unfed animals ($p<0.05$; Figure 2.10). Further, central stonerollers had significantly enhanced lipase specific and total activities ($p<0.05$) in contrast to both the rainbow trout and goldfish, which had comparable activities ($p>0.05$; Figure 2.10). Following feeding, the whole intestine and chyme samples of the three species exhibited detectable levels of lipase specific and total activities for all groups ($p<0.05$; Figures 2.11A; 2.11B; 2.11C; 2.11D), except for specific activity in the whole intestine of goldfish due to the high variation within this sample ($p>0.05$; Figure 2.12A). In the whole intestine, lipase specific and total activities were, once again, significantly enhanced in the fed herbivorous central stonerollers, with a peak activity of 10.985 ± 3.171 U mg protein^{-1} and 121.986 ± 7.088 U g tissue^{-1} ($p<0.05$; Figures 2.11A; 2.11B).

Further, lipase specific activity significantly differed across all three species in the chyme ($p<0.05$; Figure 2.11C), with central stonerollers (44.888 ± 8.268 U mg protein^{-1}) exceeding the rainbow trout (5.227 ± 0.664 U mg protein^{-1}) and goldfish (2.735 ± 0.201 U mg protein^{-1}). Lipase total activity in the chyme was, once again, greatest in the central stoneroller ($p<0.05$), however goldfish and rainbow trout were no longer significantly different ($p>0.05$; Figure 2.11D).

Lastly, lipase specific and total activities were compared in the intestinal muscle of the three species in unfed and fed samples (Figures 2.10C; 2.10D; 2.11E; 2.11F). In the unfed fish, both lipase specific and total activities were detectable in all three species ($p<0.05$; Figures 2.10C; 2.10D). Here, lipase activities were significantly higher in the intestinal muscle of central stonerollers than in the rainbow trout and goldfish ($p<0.05$), following the trends of the unfed whole intestine (Figures 2.10A; 2.10B) and fed whole intestine and chyme samples (Figures 2.11A; 2.11B; 2.11C; 2.11D). Conversely, lipase specific activities were undetectable in the intestinal muscles of all three fed species ($p>0.05$; Figure 2.11E). In contrast, lipase total activity in the intestinal muscle of the fed fish followed the same trend as total activity in the intestinal muscle of unfed fish (Figure 2.10D), chyme (Figure 2.11D), and unfed and fed whole intestine samples (Figures 2.10B; 2.11B), with central stonerollers exhibiting the highest activity (311.670 ± 30.894 U g tissue^{-1}; $p<0.05$; Figure 2.11F) compared to the rainbow trout and goldfish.

2.3.4. Cellulase activity levels

2.3.4.1. Intestinal epithelial layers of each species

Cellulase activities in saline and ethanol washed intestinal epithelial layers (with enterocytes) of rainbow trout are presented in Figure 2.12, where fed and unfed data have been

collapsed because feeding had no significant impact on cellulase activities of either wash treatment ($p>0.05$). Cellulase activities were detected (i.e. above 0.00 U) only in the epithelial layers that were washed with saline ($p<0.05$), whereas the ethanol wash eliminated all detectable activity ($p>0.05$).

When examining goldfish, cellulase specific activity was, as in rainbow trout, only detectable in the saline washed epithelial layer, but this time only in fed samples ($p<0.05$; Figure 2.13A), while all other samples had non-detectable activity levels ($p>0.05$). In the fed goldfish, the ethanol wash eliminated specific activity, again. Furthermore, feeding promoted specific activity in the saline washed epithelia ($p<0.05$), but had no effect on the ethanol washed samples ($p>0.05$). Cellulase total activities were also not detectable for any of the samples in the goldfish, and neither wash nor feeding treatments significantly impacted total activity levels (Figure 2.13B).

Similar to rainbow trout and goldfish, cellulase specific activity was only detectable in the intestinal epithelial layer that was rinsed with saline, in both unfed and fed central stonerollers ($p<0.05$; Figure 2.14A). Here, feeding decreased specific activity in the saline washed epithelia (10.250 ± 0.804 U mg protein^{-1} in unfed versus 0.323 ± 0.093 U mg protein^{-1} in fed; $p<0.05$; Figure 2.14A), unlike the increase observed with goldfish. Similar to the goldfish, cellulase total activities were undetectable for both wash treatments in unfed central stonerollers ($p>0.05$; Figure 2.14B). However, feeding increased total activity to detectable levels in the saline washed epithelial layer alone ($p<0.05$; Figure 2.14B).

2.3.4.2. Interspecies comparisons of intestinal epithelial layers

For interspecies comparisons of cellulase activities, once again, the collapsed data for rainbow trout (Figure 2.12) was separated into unfed (Table 2.8) and fed (Table 2.9) values.

Overall, the unfed herbivorous central stoneroller had significantly higher activity (10.250 ± 0.804 U mg protein^{-1}) in the saline washed epithelial layer than the carnivorous rainbow trout (0.147 ± 0.042 U mg protein^{-1}; $p<0.05$), while the unfed goldfish had undetectable cellulase activity levels ($p>0.05$; Table 2.8). Total activities were undetectable for all species within the saline wash (Table 2.8). Furthermore, ethanol washes eliminated all cellulase specific activities in unfed rainbow trout and central stonerollers, while goldfish activities remained undetectable, along with total activities in all three species ($p>0.05$; Table 2.8).

In contrast to unfed samples, fed goldfish and central stonerollers did not differ in specific activity levels in the saline washed epithelial layer ($p>0.05$), while the rainbow trout had undetectable levels of cellulase specific activity ($p>0.05$; Table 2.9). Cellulase total activities in the fed fish were non-detectable in the rainbow trout or goldfish ($p>0.05$), but were detectable in the central stoneroller ($p<0.05$; Table 2.9). Similar to the unfed samples, ethanol washed intestinal epithelial layers of fed fish failed to produce any detectable cellulase activity in any of the species, for both specific and total measurements ($p>0.05$; Table 2.9).

2.3.4.3. Interspecies comparisons of the whole intestine, chyme, and intestinal muscle

In the unfed whole intestine samples, both cellulase specific and total activities were detectable only in the goldfish ($p<0.05$), but not in the rainbow trout or central stonerollers ($p>0.05$; Figure 2.15). In contrast, detectable levels of cellulase specific and total activities were observed in the whole intestines of all three species when fed ($p<0.05$; Figures 2.16A; 2.16B). Cellulase specific activity was significantly lower in the whole intestine of fed central stonerollers ($p<0.05$), while rainbow trout and goldfish had comparable activity levels ($p>0.05$; Figure 2.16A). In contrast, total activities were comparable in the whole intestines of fed rainbow trout (1.484 ± 0.304 U g tissue^{-1}) and central stonerollers (0.712 ± 0.146 U g tissue^{-1};

$p>0.05$), whereas the goldfish had the greatest cellulase activity (5.835 ± 1.749 U g tissue^{-1}; $p<0.05$; Figure 2.16B). Cellulase specific activity was also detectable in the chyme of all three species (Figure 2.16C); however, total activity was detectable only in the chyme of fed rainbow trout and goldfish (Figure 2.16D). Furthermore, as in the fed whole intestine samples (Figure 2.16B), chyme from goldfish had significantly greater cellulase total activity and also specific activity compared to rainbow trout and central stonerollers ($p<0.05$; Figures 2.16C; 2.16D).

Cellulase activities in the intestinal muscles of each species were collapsed for unfed and fed fish, as feeding treatment had no significant effect on activity levels ($p>0.05$; Figures 2.16E; 2.16F). Detectable levels of specific or total cellulase activities were not observed in any of the species ($p>0.05$; Figures 2.16E; 2.16F).

Table 2.4. Trypsin enzyme activity levels in U mg protein^{-1} (specific activity) and U g tissue^{-1} (total activity) in the intestinal epithelial layers representing the enterocytes (saline wash and ethanol wash) of unfed rainbow trout, goldfish, central stoneroller.

	Epithelial layer (saline wash)		**Epithelial layer (ethanol wash)**	
Species	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**
Rainbow trout	0.095±0.014 **(A#)**	5.720±4.225 **(a#)**	0.445±0.054 **(A#)**	5.101±0.540 **(a#)**
Goldfish	1.412±0.158 **(B#)**	15.177±2.753 **(b#)**	1.073±0.229 **(B#)**	13.840±3.403 **(a#)**
Central stoneroller	0.202±0.038 **(C#)**	4.161±1.206 **(*n.d.*)**	0.213±0.142 **(*n.d.*)**	2.296±1.891 **(*n.d.*)**

Data represent mean ± SEM. Data is taken from Figures 2.2-2.4.

Pound symbol (#) represents significantly detectable levels ($p<0.05$, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity.

Uppercase letters represent significant differences ($p<0.05$, by t-test, Mann-Whitney rank sum test, or Bonferroni test following one-way ANOVA) between specific activity levels within each wash.

Lowercase letters represent significant difference ($p<0.05$, by t-test, Mann-Whitney rank sum test, Bonferroni test following one-way ANOVA, or Dunn's test following Kruskal-Wallis ANOVA on ranks) between total activity levels within each wash.

Table 2.5. Trypsin enzyme activity levels in U mg protein^{-1} (specific activity) and U g tissue^{-1} (total activity) in the intestinal epithelial layers representing the enterocytes (saline wash and ethanol wash) of fed rainbow trout, goldfish, central stoneroller.

	Epithelial layer (saline wash)		**Epithelial layer (ethanol wash)**	
Species	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**
Rainbow trout	1.023±0.134 **(A#)**	7.236±0.645 **(a#)**	0.234±0.032 **(A#)**	2.903±0.517 **(a#)**
Goldfish	0.391±0.002 **(B#)**	3.303±1.450 **(*n.d.*)**	0.572±0.071 **(B#)**	7.471±1.579 **(b#)**
Central stoneroller	0.172±0.027 **(C#)**	3.499±0.615 **(b#)**	0.227±0.023 **(A#)**	3.568±0.326 **(a#)**

Data represent mean ± SEM. Data is taken from Figures 2.2-2.4.

Statistics are as described in Table 2.4.

Table 2.6. Lipase enzyme activity levels in U mg protein^{-1} (specific activity) and U g tissue^{-1} (total activity) in the intestinal epithelial layers representing the enterocytes (saline wash and ethanol wash) of unfed rainbow trout, goldfish, central stoneroller.

	Epithelial layer (saline wash)		**Epithelial layer (ethanol wash)**	
Species	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**
Rainbow trout	0.568±0.089 **(A#)**	8.025±1.135 **(a#)**	1.520±0.290 **(A#)**	10.948±0.265 **(a#)**
Goldfish	2.826±0.527 **(B#)**	29.412±5.007 **(a#)**	7.153±0.916 **(B#)**	47.696±9.741 **(ab#)**
Central stoneroller	19.335±2.743 **(C#)**	225.652±14.119 **(b#)**	19.061±5.826 **(*n.d.*)**	175.244±0.292 **(b#)**

Data represent mean ± SEM. Data is taken from Figures 2.7-2.9.

Statistics are as described in Table 2.4.

Table 2.7. Lipase enzyme activity levels in U mg protein^{-1} (specific activity) and U g tissue^{-1} (total activity) in the intestinal epithelial layers representing the enterocytes (saline wash and ethanol wash) of fed rainbow trout, goldfish, central stoneroller.

	Epithelial layer (saline wash)		**Epithelial layer (ethanol wash)**	
Species	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**
Rainbow trout	0.833±0.056 **(#)**	3.235±0.302 **(a#)**	4.056±1.301 **(A#)**	2.813±0.300 **(a#)**
Goldfish	4.164±1.621 **(*n.d.*)**	38.120±11.815 **(*n.d.*)**	4.383±0.741 **(A#)**	32.242±3.854 **(b#)**
Central stoneroller	12.731±4.369 **(*n.d.*)**	135.208±23.126 **(b#)**	9.128±0.300 **(B#)**	91.597±3.537 **(c#)**

Data represent mean ± SEM. Data is taken from Figures 2.7-2.9.

Statistics are as described in Table 2.4.

Table 2.8. Cellulase enzyme activity levels in U mg protein^{-1} (specific activity) and U g tissue^{-1} (total activity) in the intestinal epithelial layers representing the enterocytes (saline wash and ethanol wash) of unfed rainbow trout, goldfish, central stoneroller.

	Epithelial layer (saline wash)		**Epithelial layer (ethanol wash)**	
Species	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**
Rainbow trout	0.147±0.042 **(A#)**	1.069±0.566 ***(n.d.)***	0.000±0.000 ***(n.d.)***	0.000±0.000 ***(n.d.)***
Goldfish	0.000±0.000 ***(n.d.)***	7.905±7.905 ***(n.d.)***	0.000±0.000 ***(n.d.)***	0.000±0.000 ***(n.d.)***
Central stoneroller	10.251±0.804 **(B#)**	69.195±29.427 ***(n.d.)***	0.031±0.031 ***(n.d.)***	0.333±0.333 ***(n.d.)***

Data represent mean ± SEM. Data is taken from Figures 2.12-2.14.

Statistics are as described in Table 2.4.

Table 2.9. Cellulase enzyme activity levels in U mg protein^{-1} (specific activity) and U g tissue^{-1} (total activity) in the intestinal epithelial layers representing the enterocytes (saline wash and ethanol wash) of fed rainbow trout, goldfish, central stoneroller.

	Epithelial layer (saline wash)		**Epithelial layer (ethanol wash)**	
Species	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**	**Specific activity (U mg protein^{-1})**	**Total activity (U g tissue^{-1})**
Rainbow trout	0.147±0.055 ***(n.d.)***	1.053±0.432 ***(n.d.)***	0.000±0.000 ***(n.d.)***	0.000±0.000 ***(n.d.)***
Goldfish	0.466±0.134 **(A#)**	16.887±7.482 ***(n.d.)***	0.200±0.126 ***(n.d.)***	1.065±0.717 ***(n.d.)***
Central stoneroller	0.323±0.093 **(A#)**	2.381±0.917 **(#)**	0.127±0.127 ***(n.d.)***	1.333±1.333 ***(n.d.)***

Data represent mean ± SEM. Data is taken from Figures 2.12-2.14.

Statistics are as described in Table 2.4.

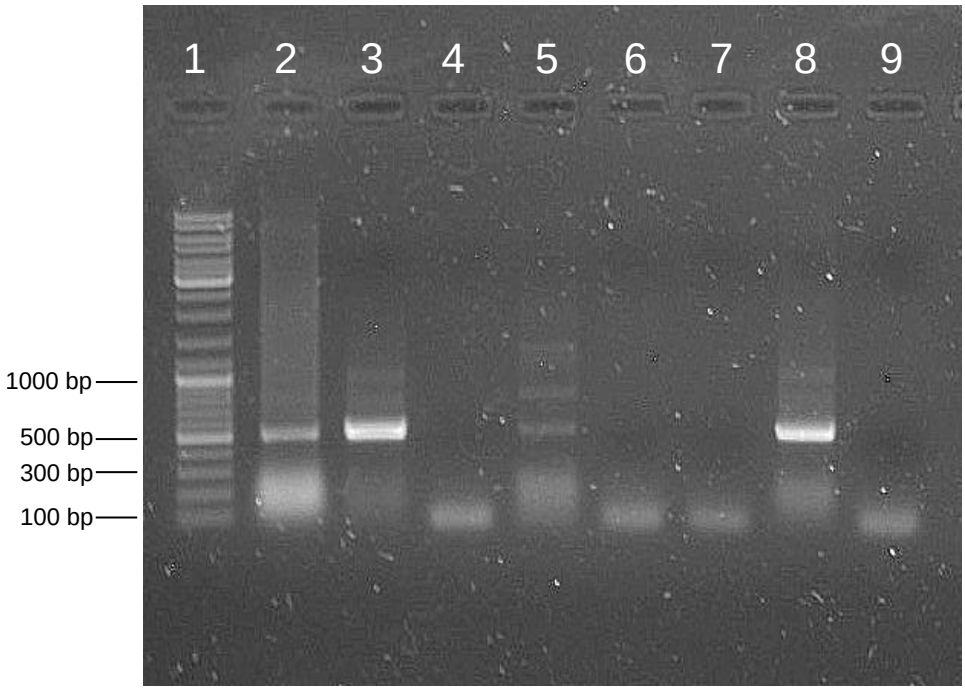

Figure 2.1. Agarose gel electrophoresis (1.5%) of the PCR products in the intestinal layers of fed goldfish using universal bacterial rRNA primers 8F-519R (V1-V3 hypervariable region). Gels were stained with SYBR™ Safe DNA Gel Stain (Invitrogen by Thermo Fisher Scientific) and visualized under UV light. Lane 1: GeneRuler 100bp DNA ladder (Thermo Fisher Scientific); Lane 2: whole intestine; Lane 3: chyme; Lane 4: intestinal muscle; Lane 5: saline washed epithelial layer representing enterocytes; Lane 6: ethanol washed epithelial layer representing enterocytes; Lane 7: gDNA extraction negative control; Lane 8: PCR positive control; Lane 9: PCR negative control. Bands of 511bp in size confirm the presence of bacteria.

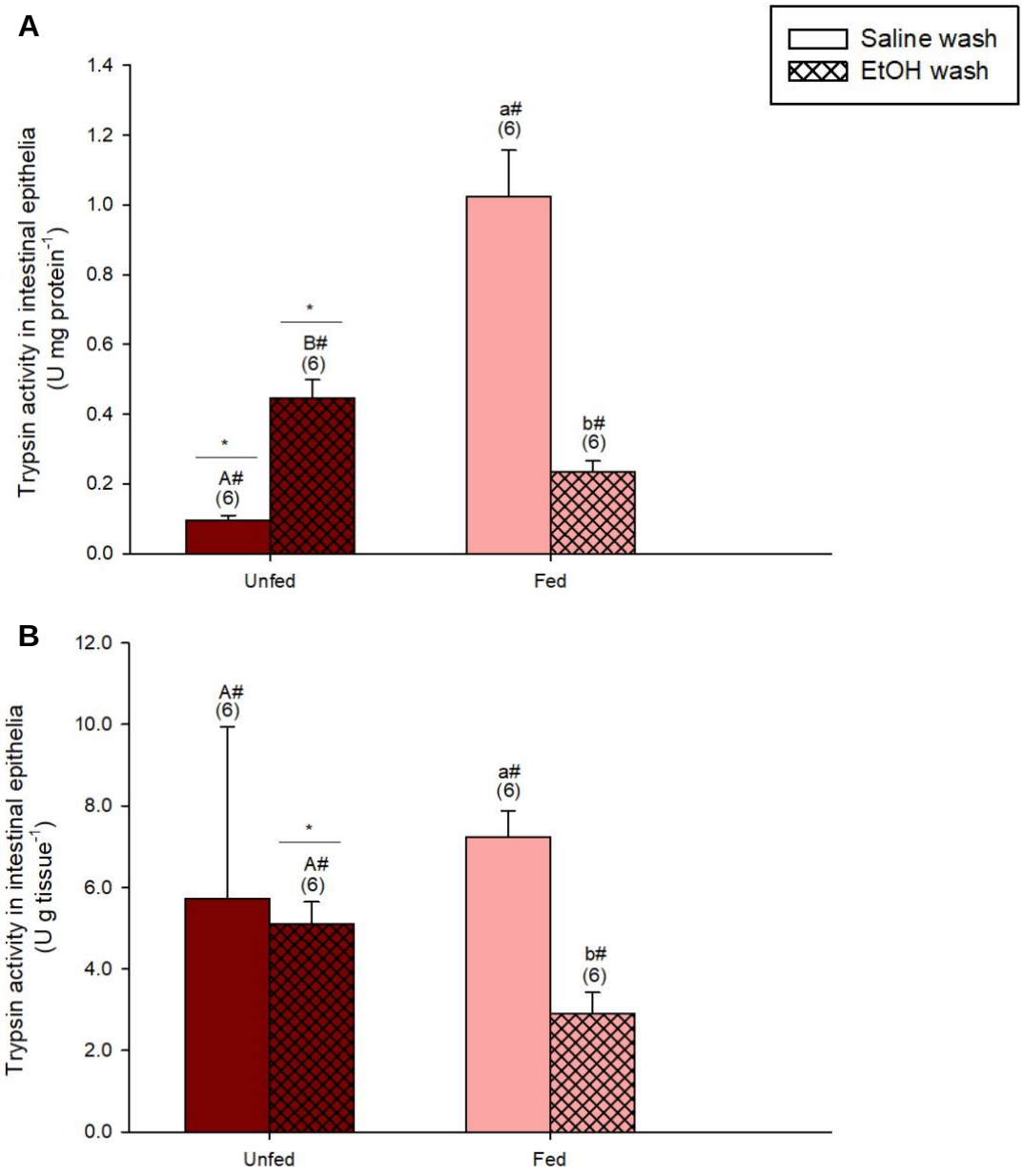

Figure 2.2. Trypsin enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of unfed and fed rainbow trout. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test). Statistically significant differences between saline and ethanol washes within each feeding treatment are denoted by different uppercase (unfed) or lowercase (fed) letters (p<0.05, by t-test or Mann-Whitney rank sum test). An asterisk indicates statistically significant differences between feeding treatments within each wash treatment (p<0.05, by t-test).

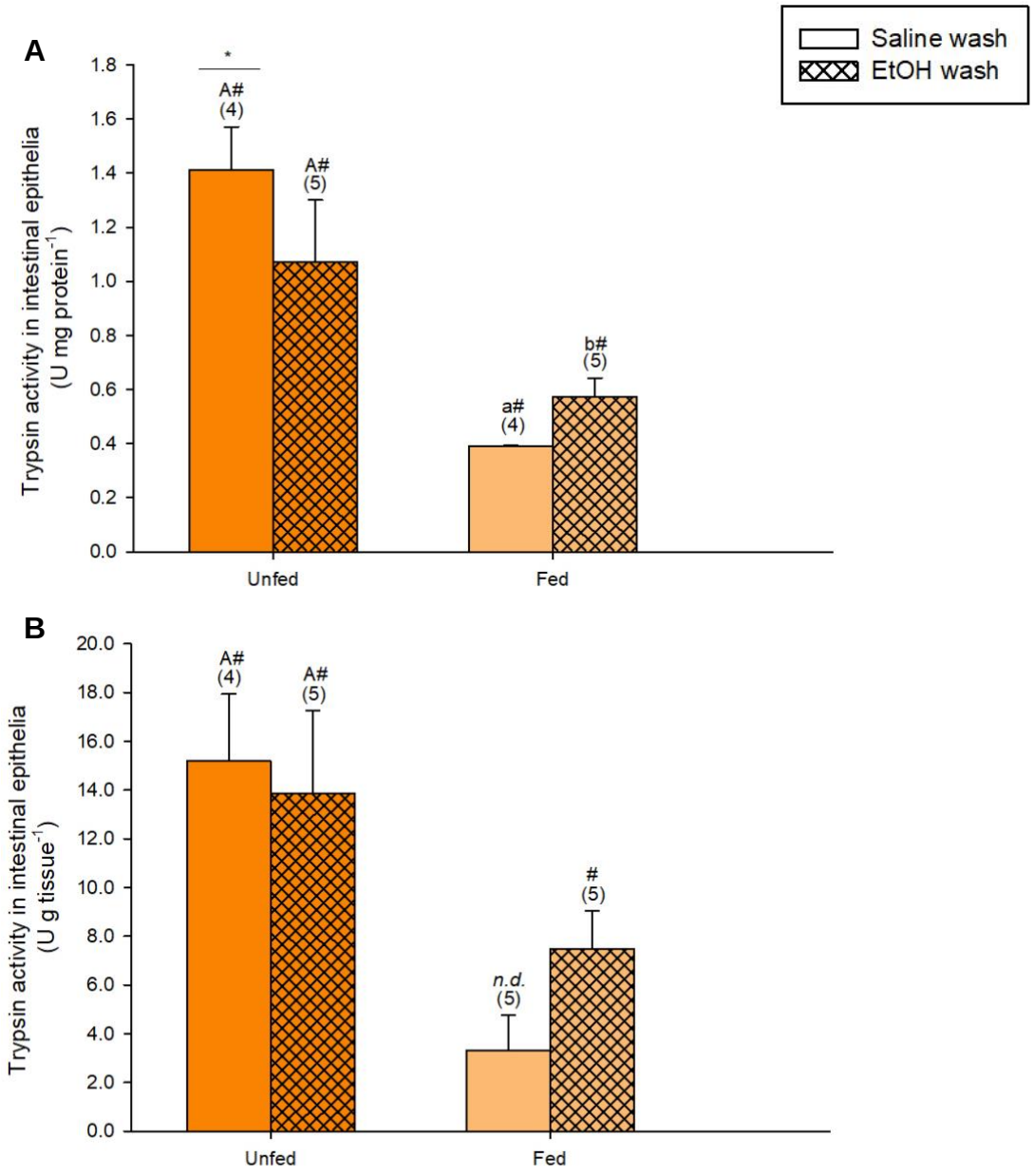

Figure 2.3. Trypsin enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of unfed and fed goldfish. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity. Statistically significant differences between saline and ethanol washes within each feeding treatment are denoted by different uppercase (unfed) or lowercase (fed) letters (p<0.05, by t-test or Mann-Whitney rank sum test). An asterisk indicates statistically significant differences between feeding treatments in the saline wash treatment (p<0.05, by t-test).

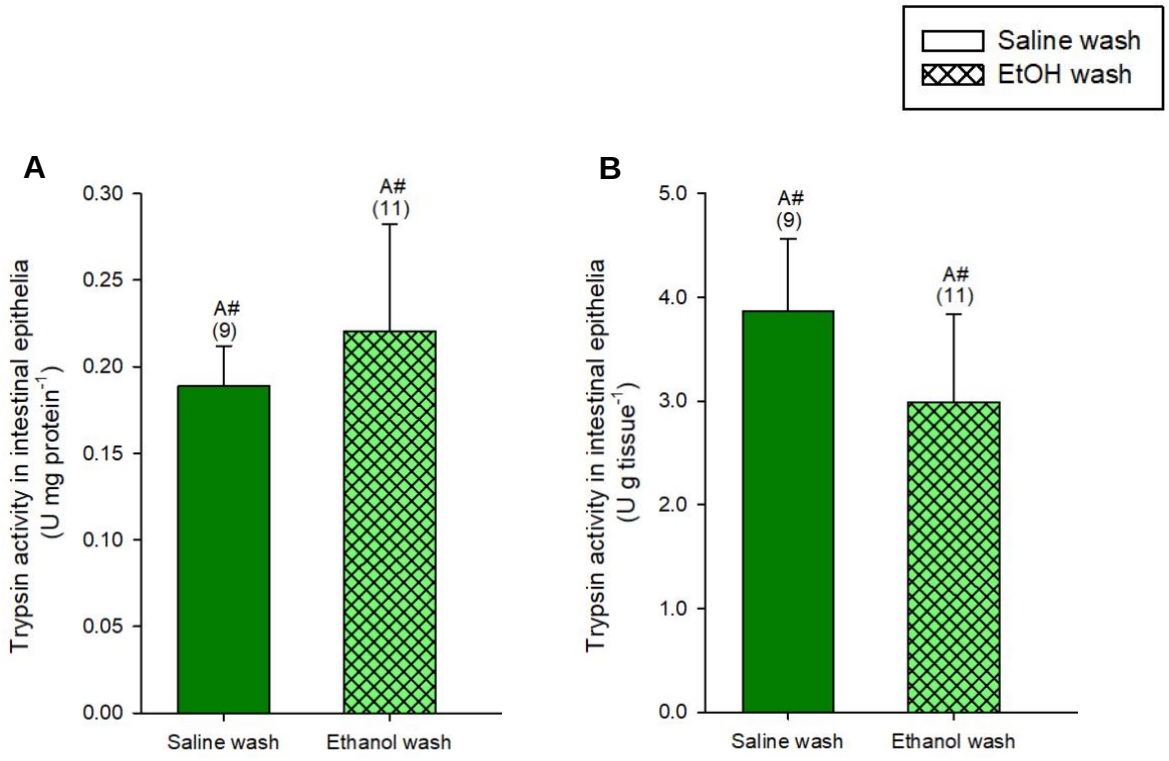

Figure 2.4. Trypsin enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of central stoneroller. Data for feeding treatments were collapsed, as no significant difference was detected (p>0.05, by Mann-Whitney rank sum test). Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test). Bars sharing the same letter between washes are not statistically different (p>0.05, by t-test or Mann-Whitney rank sum test).

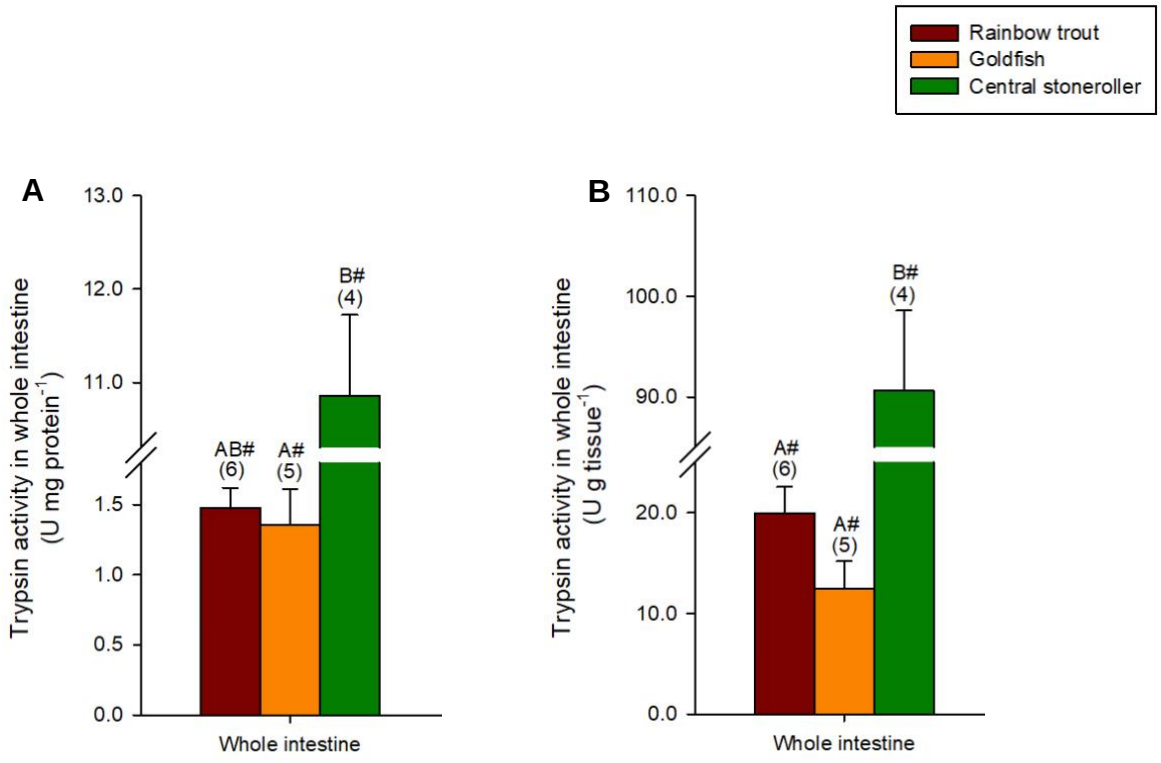

Figure 2.5. Trypsin enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the whole intestine of unfed rainbow trout, goldfish, and central stoneroller. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test). Statistically significant differences between species are denoted by different uppercase letters (p<0.05, by Dunn's test following Kruskal-Wallis one-way ANOVA on ranks or Bonferroni test following one-way ANOVA).

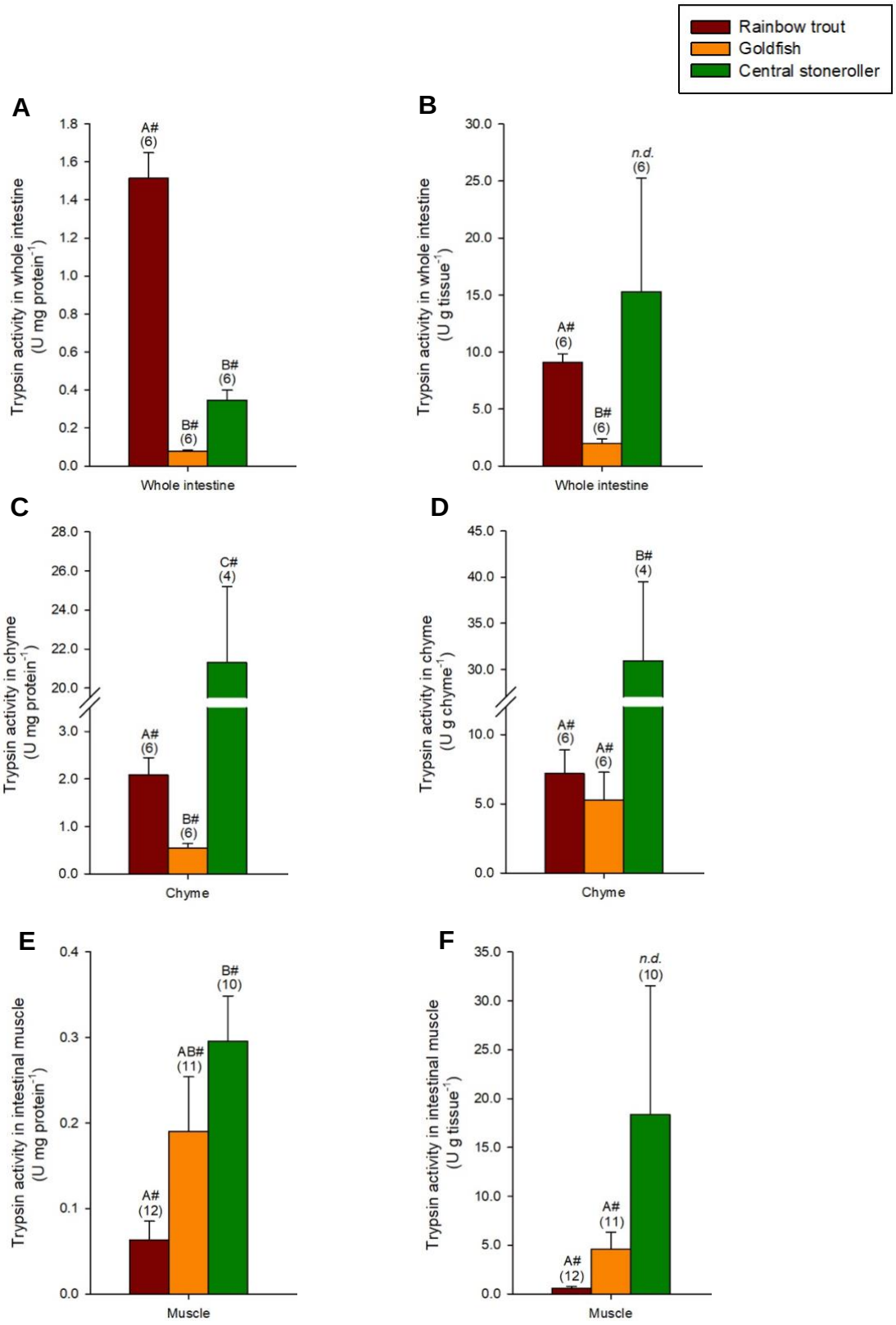
Rainbow trout
Goldfish
Central stoneroller
A
Trypsin activity in whole intestine (U mg protein-1)
A# (6)
B# (6)
B# (6)
Whole intestine
B
Trypsin activity in whole intestine (U g tissue-1)
n.d. (6)
A# (6)
B# (6)
Whole intestine
C
Trypsin activity in chyme (U mg protein-1)
C# (4)
A# (6)
B# (6)
Chyme
D
Trypsin activity in chyme (U g chyme-1)
B# (4)
A# (6)
A# (6)
Chyme
E
Trypsin activity in intestinal muscle (U mg protein-1)
B# (10)
AB# (11)
A# (12)
Muscle
F
Trypsin activity in intestinal muscle (U g tissue-1)
n.d. (10)
A# (11)
A# (12)
Muscle

Figure 2.6. Trypsin enzyme activity levels (U mg protein^{-1}; specific activity, U g tissue^{-1}/U g chyme^{-1}; total activity) in the (A, B) whole intestine, (C, D) chyme, and (E, F) intestinal muscle of fed rainbow trout, goldfish, and central stoneroller. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero ($p<0.05$, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity. Data for feeding treatments in the intestinal muscle were collapsed as no significant difference was detected ($p>0.05$, by t-test or Mann-Whitney rank sum test). Statistically significant differences between species are denoted by different uppercase letters ($p<0.05$, by t-test, Bonferroni test following one-way ANOVA, Mann-Whitney rank sum test, or Dunn's test following Kruskal-Wallis one-way ANOVA on ranks).

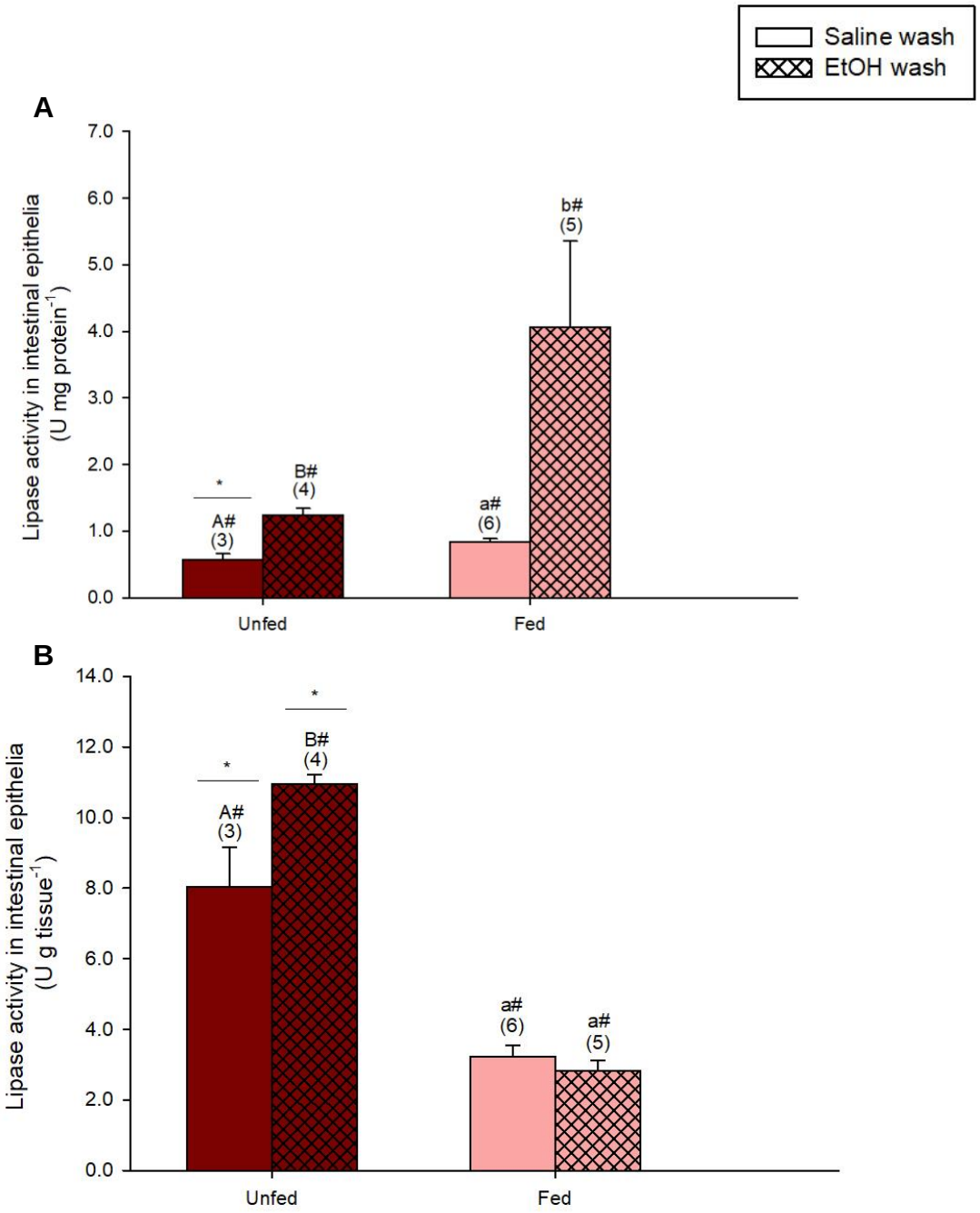

Figure 2.7. Lipase enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of unfed and fed rainbow trout. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test). Statistically significant differences between saline and ethanol washes within each feeding treatment are denoted by different uppercase (unfed) or lowercase (fed) letters (p<0.05, by t-test or Mann-Whitney rank sum test). An asterisk indicates statistically significant differences between feeding treatments within each wash treatment (p<0.05, by t-test).

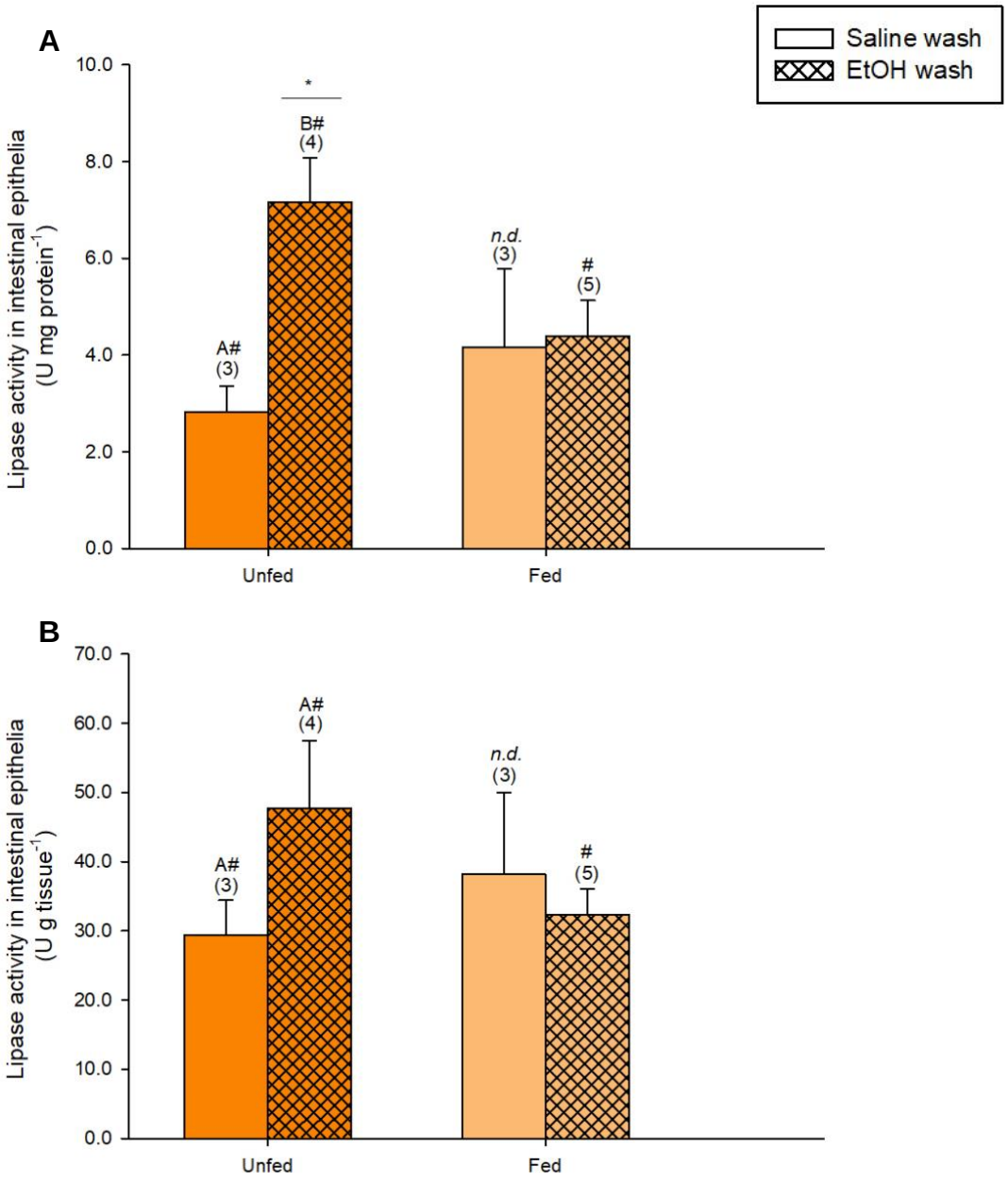

Figure 2.8. Lipase enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of unfed and fed goldfish. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity. Statistically significant differences between saline and ethanol washes within the unfed treatment are denoted by different uppercase letters (p<0.05, by t-test or Mann-Whitney rank sum test). An asterisk indicates statistically significant differences between feeding treatments in the ethanol wash treatment (p<0.05, by t-test).

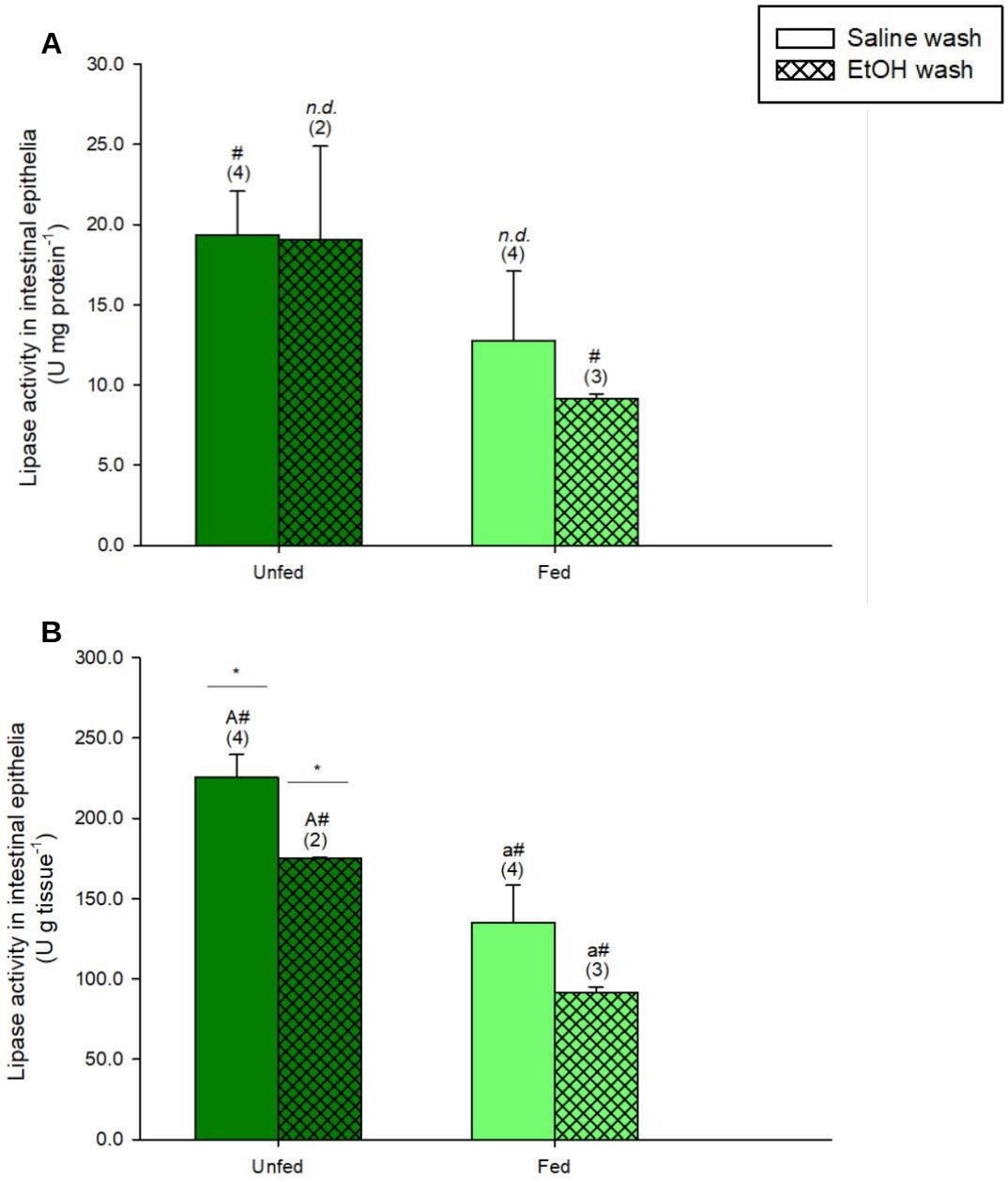

Figure 2.9. Lipase enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of unfed and fed central stoneroller. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity. Bars sharing the same letter in each feeding treatment are not statistically different (p>0.05, by t-test or Mann-Whitney rank sum test). An asterisk indicates statistically significant differences between feeding treatments within each wash treatment (p<0.05, by t-test).

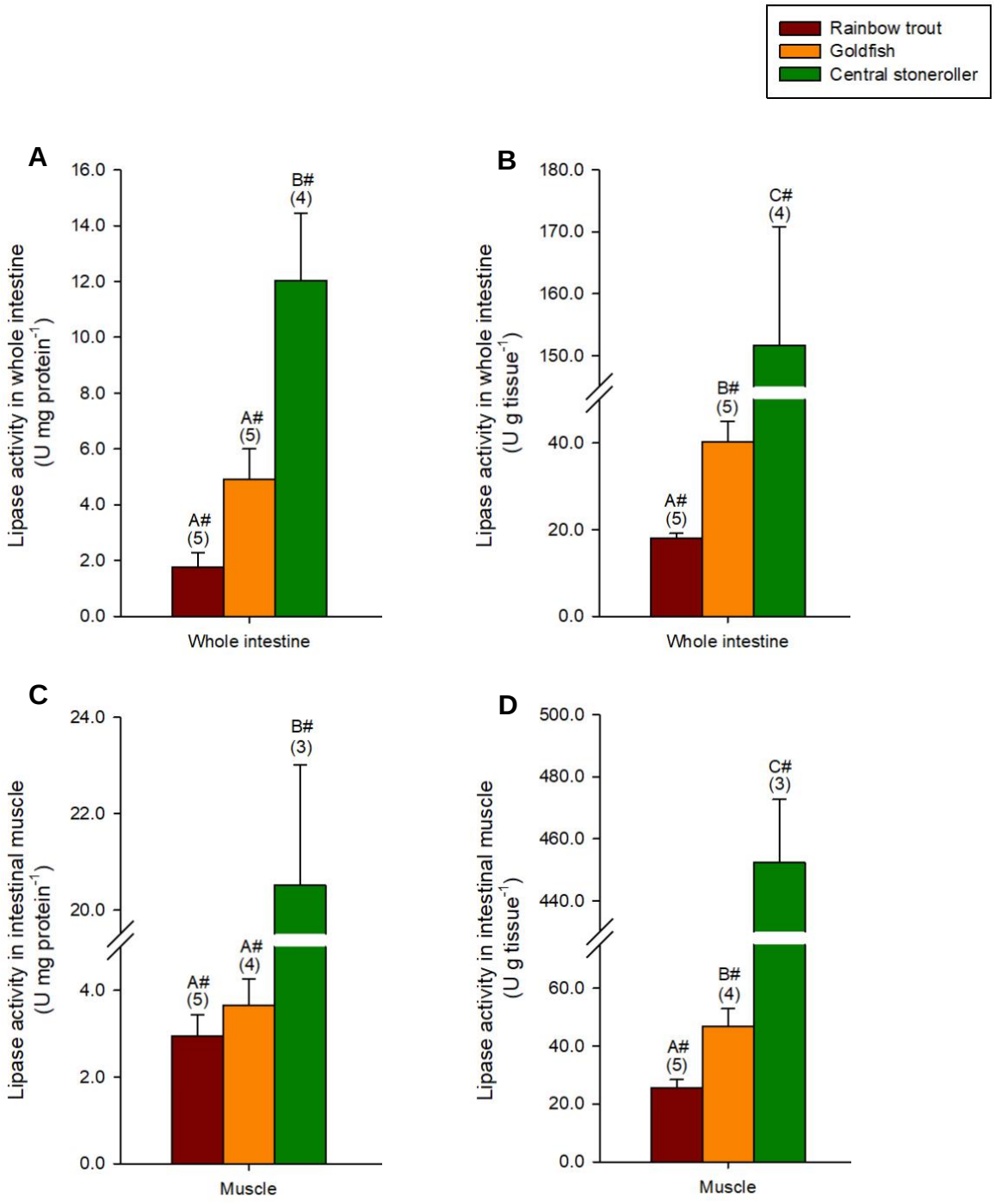

Figure 2.10. Lipase enzyme activity levels (U mg protein^{-1}; specific activity, U g tissue; total activity) in the (A, B) whole intestine and (C, D) intestinal muscle of unfed rainbow trout, goldfish, and central stoneroller. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test). Statistically significant differences between species are denoted by different uppercase letters (p<0.05, by Bonferroni test following one-way ANOVA).

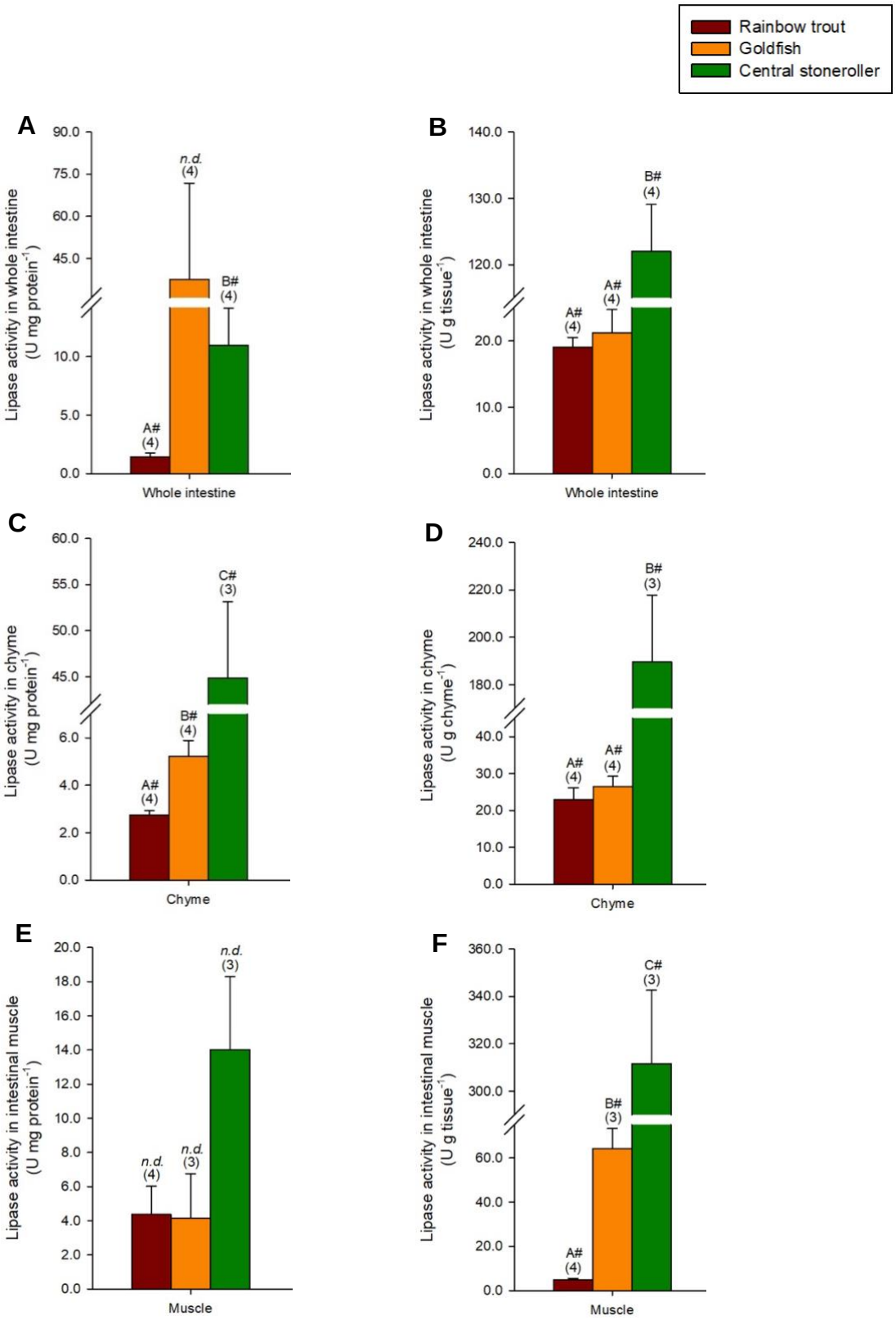
Rainbow trout
Goldfish
Central stoneroller
A
Lipase activity in whole intestine (U mg protein-1)
90.0
75.0
60.0
45.0
10.0
5.0
0.0
n.d. (4)
B# (4)
A# (4)
Whole intestine
B
Lipase activity in whole intestine (U g tissue-1)
140.0
130.0
120.0
20.0
10.0
0.0
B# (4)
A# (4)
A# (4)
Whole intestine
C
Lipase activity in chyme (U mg protein-1)
60.0
55.0
50.0
45.0
6.0
4.0
2.0
0.0
C# (3)
B# (4)
A# (4)
Chyme
D
Lipase activity in chyme (U g chyme-1)
240.0
220.0
200.0
180.0
40.0
30.0
20.0
10.0
0.0
B# (3)
A# (4)
A# (4)
Chyme
E
Lipase activity in intestinal muscle (U mg protein-1)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
n.d. (3)
n.d. (4)
n.d. (3)
Muscle
F
Lipase activity in intestinal muscle (U g tissue-1)
360.0
340.0
320.0
300.0
60.0
40.0
20.0
0.0
C# (3)
B# (3)
A# (4)
Muscle

Figure 2.11. Lipase enzyme activity levels (U mg protein^{-1}; specific activity, U g tissue^{-1}/U g chyme^{-1}; total activity) in the (A, B) whole intestine, (C, D) chyme, and (E, F) intestinal muscle of fed rainbow trout, goldfish, and central stoneroller. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity. Statistically significant differences between species are denoted by different uppercase letters (p<0.05, by t-test or Bonferroni test following one-way ANOVA).

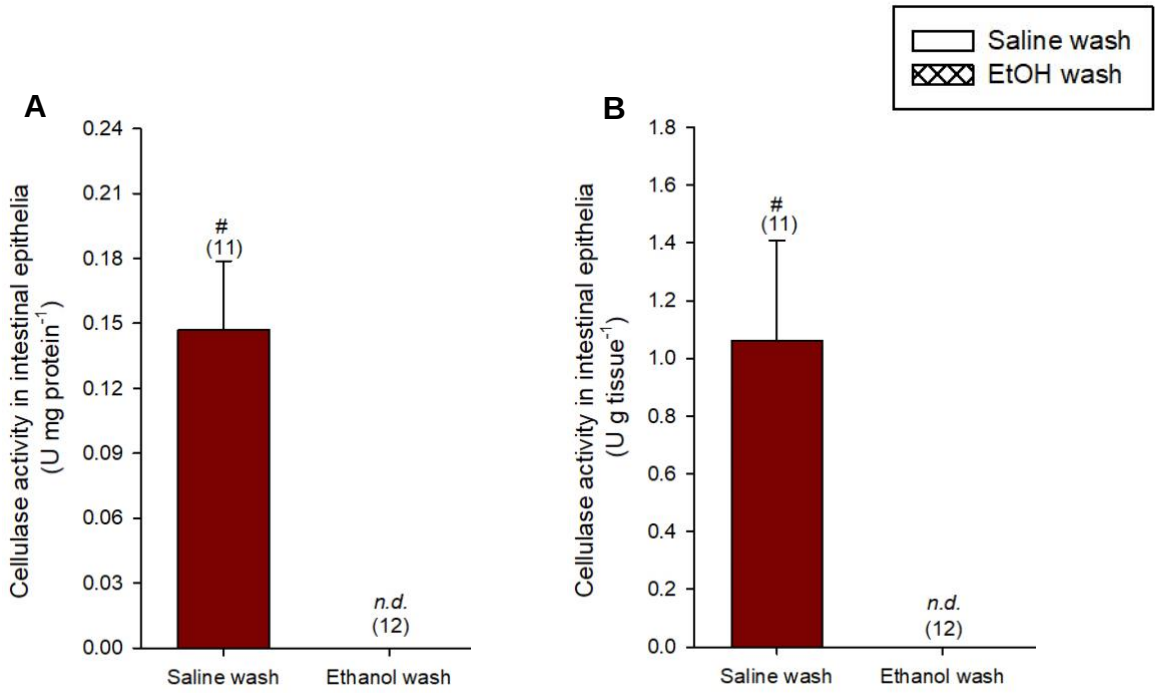

Figure 2.12. Cellulase enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of rainbow trout. Data for feeding treatments were collapsed as no significant difference was detected (p>0.05, by Mann-Whitney rank sum test). Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity.

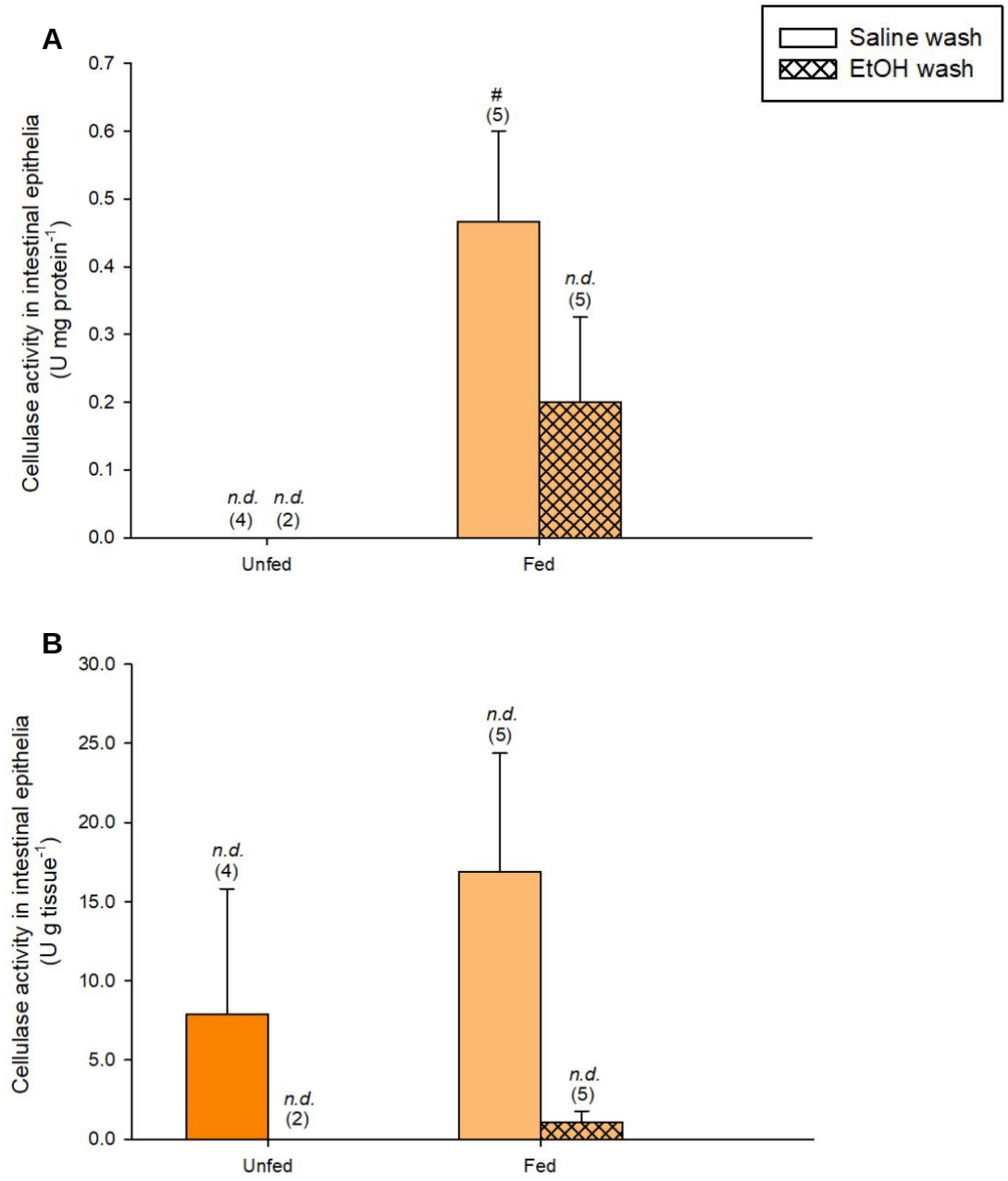

Figure 2.13. Cellulase enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of unfed and fed goldfish. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity.

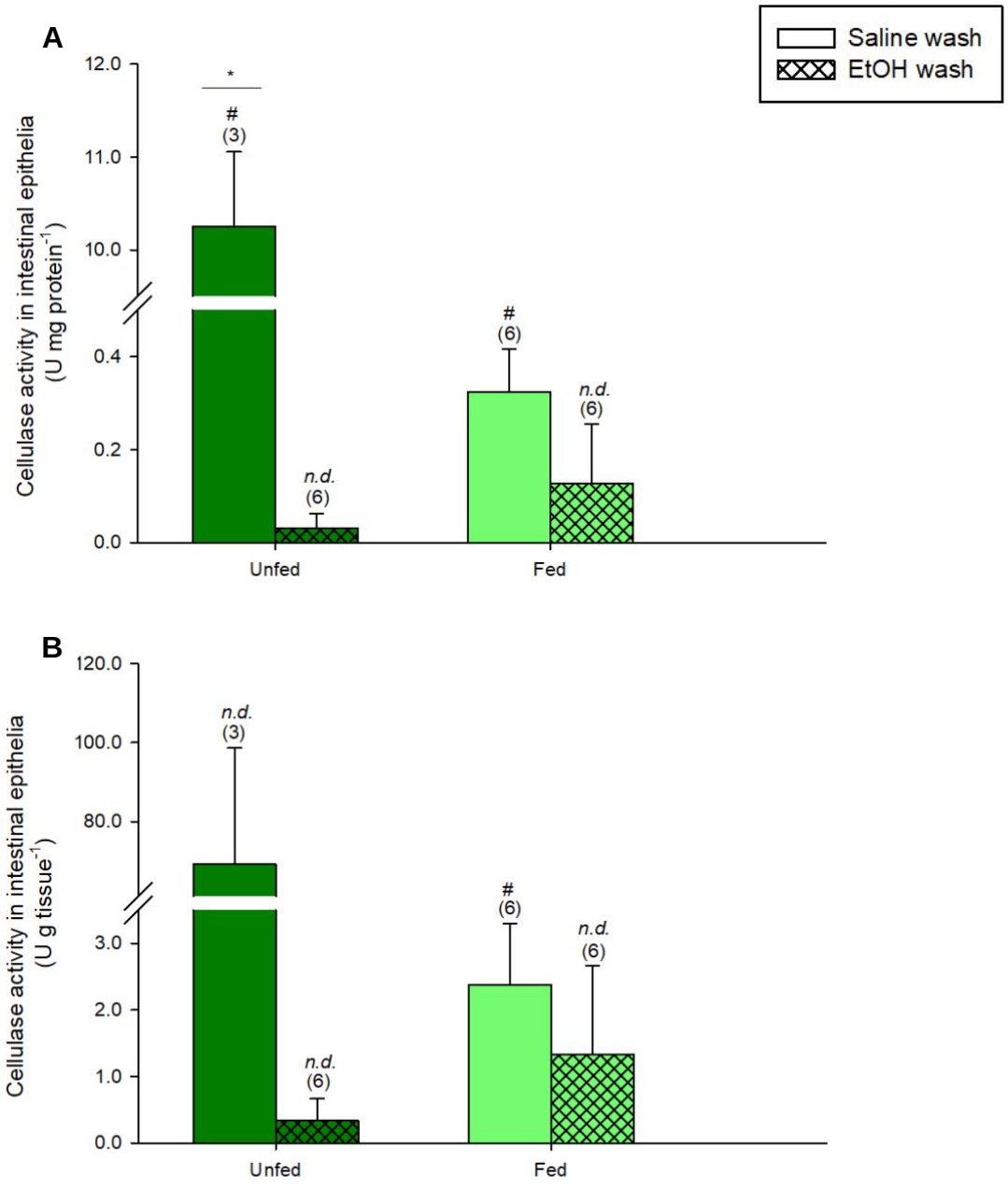

Figure 2.14. Cellulase enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of unfed and fed central stoneroller. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity. An asterisk indicates statistically significant differences between feeding treatments in the saline wash treatment (p<0.05, by t-test).

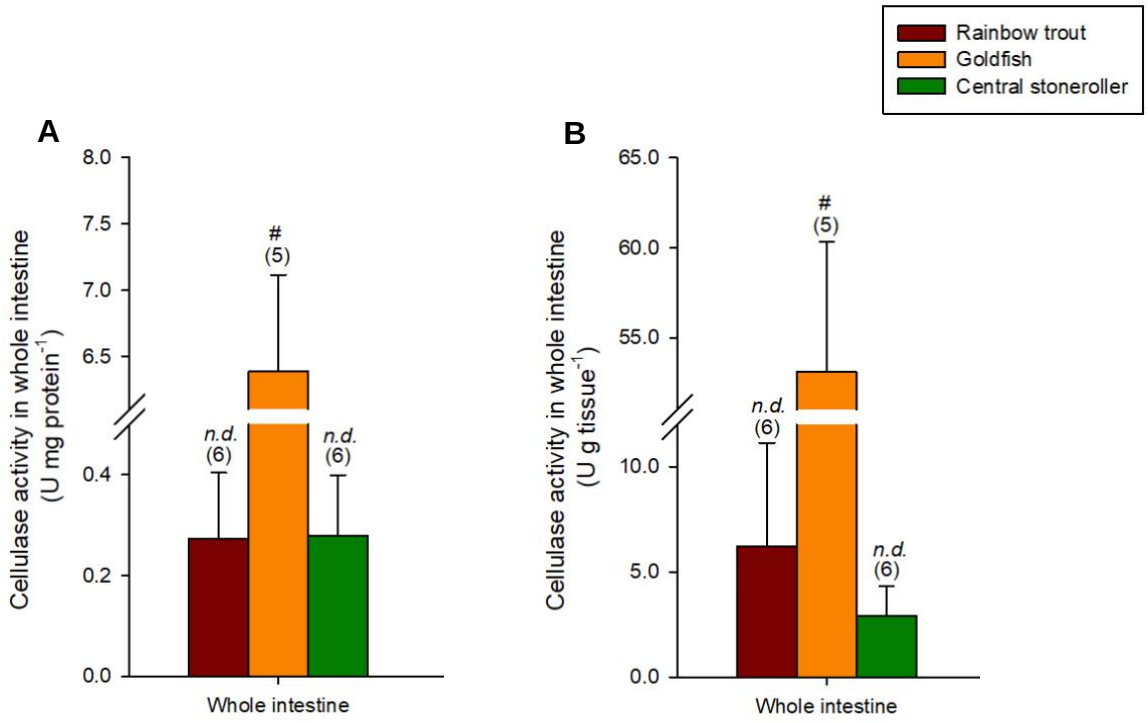

Figure 2.15. Cellulase enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the whole intestine of unfed rainbow trout, goldfish, and central stoneroller. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity.

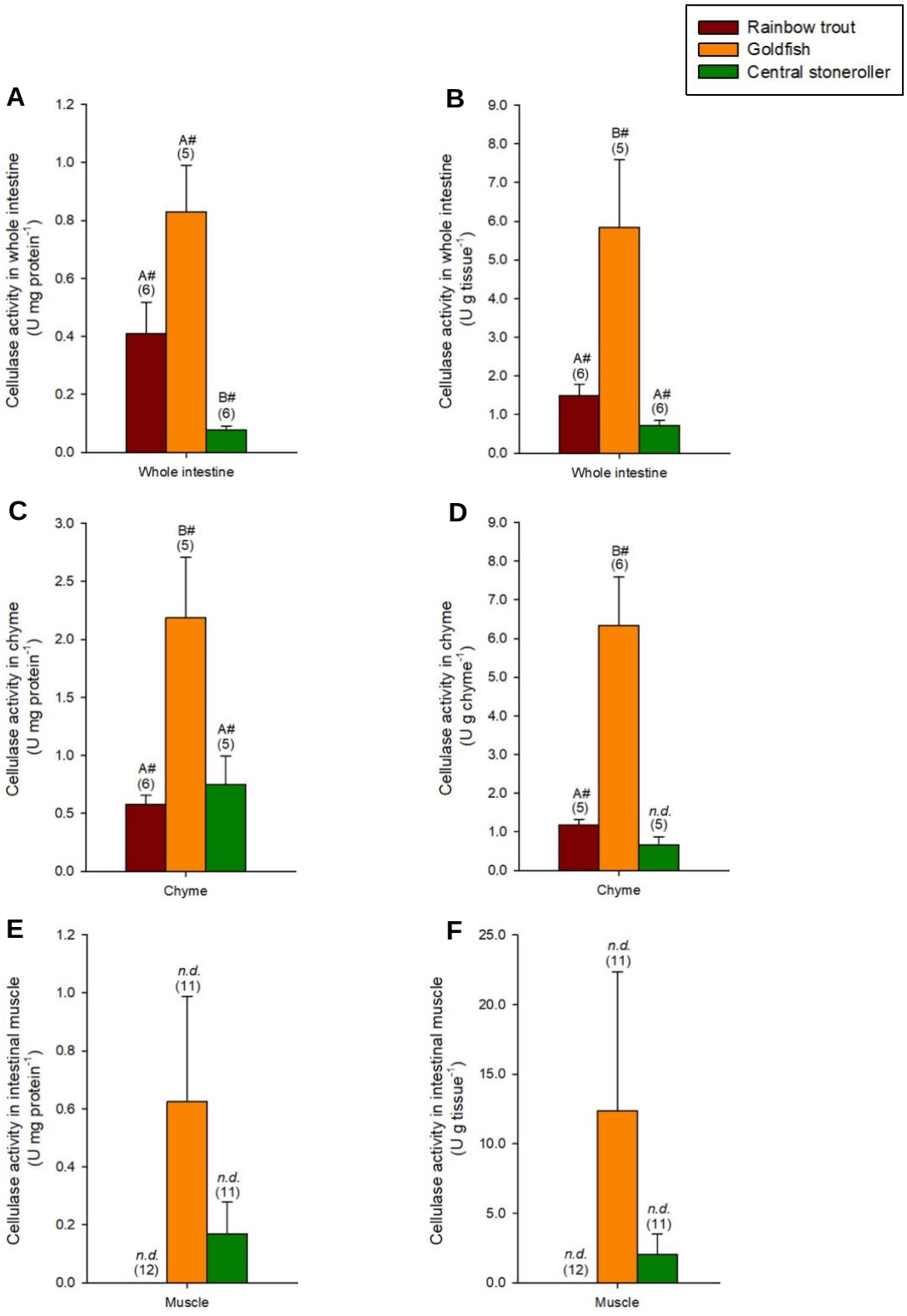
Rainbow trout
Goldfish
Central stoneroller
A
Cellulase activity in whole intestine (U mg protein-1)
A# (6)
A# (5)
B# (6)
Whole intestine
B
Cellulase activity in whole intestine (U g tissue-1)
A# (6)
B# (5)
A# (6)
Whole intestine
C
Cellulase activity in chyme (U mg protein-1)
A# (6)
B# (5)
A# (5)
Chyme
D
Cellulase activity in chyme (U g chyme-1)
A# (5)
B# (6)
n.d. (5)
Chyme
E
Cellulase activity in intestinal muscle (U mg protein-1)
n.d. (12)
n.d. (11)
n.d. (11)
Muscle
F
Cellulase activity in intestinal muscle (U g tissue-1)
n.d. (12)
n.d. (11)
n.d. (11)
Muscle

Figure 2.16. Cellulase enzyme activity levels (U mg protein^{-1}; specific activity, U g tissue^{-1}/U g chyme^{-1}; total activity) in the (A, B) whole intestine, (C, D) chyme, and (E, F) intestinal muscle of fed rainbow trout, goldfish, and central stoneroller. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity. Data for feeding treatments were collapsed as no significant difference was detected (p>0.05, t-test or Mann-Whitney rank sum test). Statistically significant differences between species are denoted by different uppercase letters (p<0.05, by Mann-Whitney rank sum test or Bonferroni test following one-way ANOVA).

2.4. DISCUSSION

2.4.1. Overview

Digestive enzymes can be produced endogenously by the microbe host or exogenously by microorganisms that inhabit the gastrointestinal tract (Das and Tripathi 1991; Bairagi et al. 2002; Kar and Ghosh 2008). However, the proportion that each source of enzyme (microbe host or bacteria) contributes to the overall enzyme activity levels is unclear. Furthermore, it is not known if differences in enzyme activities exhibited by individuals are due to host-specific factors or their diets. I attempted to address the first unknown in this chapter, while partially addressing the second here and further in Chapter 3. Moreover, as a consequence of my experimental design, I was able to explore the impact of feeding on the microbe host and bacterial contributions. Finally, I examined the measurement of enzyme activities, in particular how we quantify activity units and have highlighted flaws and drawbacks.

2.4.2. Removal of bacteria from samples

In the current study, I showed that ethanol effectively removed intestinal tract bacteria, as seen in Figure 2.1, further demonstrating the sterility of my techniques. Thus, I feel I can make conclusions about the attribution of proportions of intestinal enzyme activity to either only the microbe host (when bacteria were removed, i.e. in the ethanol washes), or the microbe host and bacteria together (i.e. in the saline washes, as well as the whole intestines and chyme). Furthermore, based on this ability, my hypothesis that the microbe host and gut bacteria would both produce trypsin and lipase, while only the intestinal microbes would be responsible for cellulase production was mostly supported.

2.4.2.1. Endogenous and exogenous trypsin

My prediction that trypsin activity levels would be significantly higher in the intestinal layers with bacteria (as there would be both endogenous and exogenous enzyme production; German and Bittong 2009) compared to those that were free of bacteria (where only endogenous enzymes would be contributing), was supported for rainbow trout but not central stonerollers or goldfish. Specifically, while microbes had a large effect on trypsin activities in fed rainbow trout, some activity could still be detected in the absence of bacteria (Figure 2.2), which overall suggests trypsin activity in the intestine is due to a microbial enhancement of the fish's own ability to digest proteins (Kar and Ghosh 2008). This has also been suggested in previous studies that indicate the intestinal microbiota enhances enzyme activity, and thus plays an important role in protein breakdown by supplementing the microbe host with an exogenous source of trypsin (Bairagi et al. 2002; Suzer et al. 2008; Liu et al. 2016). Further, it has been proposed that dietary proteins that do not get digested by endogenous trypsin are made available for the gut bacteria to break down (Lyons et al. 2017), which supports the enhancement of endogenous trypsin activity in the rainbow trout following feeding. In contrast, the gastrointestinal microbiota does not appear to have an intensifying effect on trypsin activities in the intestinal epithelial layers of omnivorous goldfish or herbivorous central stonerollers, where all activity appeared to be host-driven (Figures 2.3; 2.4). Proteolytic bacteria have been characterized in the guts of many fishes with varying dietary habits, including omnivorous goldfish (Lesel et al. 1986), walking catfish (Bairagi et al. 2002), and Malaysian mahseer (Asaduzzaman et al. 2018), and herbivorous rohu (Bairagi et al. 2002; Ghosh et al. 2002; Kar and Ghosh 2008) and tilapia (Bairagi et al. 2002). Thus, a lack of trypsin activity by the bacteria in the omnivorous goldfish and herbivorous central stoneroller could suggest that alternative microbial proteases (Sabapathy and Teo 1993;

Kumar et al. 2007; Pujante et al. 2017) may be playing a role in these animals. Further enzyme assays would be required to determine the role of various bacterial proteases other than trypsin in the intestines of fish.

2.4.2.2. Endogenous and exogenous lipase

I hypothesized that lipase would be produced by the host fish as well as by bacteria found in the gastrointestinal tract (Trust et al. 1979; Ringø et al. 1995; Kurtovic et al. 2009; Sumathi et al. 2011; Das et al. 2014), and thus predicted that intestinal layers with bacteria would have enhanced lipase activities. This was observed in fed rainbow trout (Figure 2.7A). Interestingly, bacteria were the only source of lipase in unfed central stonerollers (Figure 2.9A), whereas the microbe host was the only source in fed goldfish (Figure 2.8A). Lipase-producing bacteria have been found in the gastrointestinal tracts of herbivorous (Trust et al. 1979; Bairagi et al. 2002), omnivorous (Sumathi et al. 2011; Das et al. 2014), and carnivorous (Das et al. 2014) fishes, and are thought to aid the fish with lipid digestion by providing an external source of lipase (Sumathi et al. 2011). Dietary lipids provide energy, supply essential fatty acids, and aid in fat-soluble vitamin absorption (e.g. Lin and Shiau 2003; Xiangfei Li et al. 2012). The critical nature of these functions suggests that host organism would have an endogenous supply of lipase to prevent any losses of function with microbial alterations in the gut caused by factors such as environmental alterations (e.g. Sullam et al. 2012; Huyben et al. 2018; Friberg et al. 2019; Yang et al. 2019; Minich et al. 2020; Yukgehnaish et al. 2020). Endogenous lipases have been observed in fish larvae prior to the first feed, and thus bacterial colonization, which supports endogenous and host-specific capabilities of lipase secretion (Fagbenro et al. 2000; Caruso et al. 2009; Kurtovic et al. 2009; Xiong et al. 2011). Both the rainbow trout and goldfish appear to have endogenous supplies; however, the reliance on gut bacteria alone in the central stoneroller is interesting. It is

possible that additional lipases, such as CEL-like enzymes (Kurtovic et al. 2009), are present that were not captured by the lipase assay. Alternative assays to capture the contribution of additional lipase enzymes may reveal endogenous production in the central stoneroller.

An unexpected trend that did not support my prediction was observed in the intestinal epithelial layers of all three fish species. Lipase specific activities were significantly enhanced in the ethanol rinsed epithelia when compared to the saline washed epithelia in unfed and fed rainbow trout (Figure 2.7A) and goldfish (Figure 2.8A), and fed central stonerollers (Figure 2.9A), suggesting an increase in microbe host-driven enzyme production following ethanol washes. This effect was also observed with trypsin specific activity in both the unfed rainbow trout (Figure 2.2A) and fed goldfish (as well as the trypsin total activity levels of fed goldfish; Figures 2.3A; 2.3B). These findings do not support my prediction, and it is unclear why enzyme activities were enhanced in the absence of bacteria. To my knowledge, there are no studies that have examined this phenomenon and so further experimentation is required.

2.4.2.3. Endogenous and exogenous cellulase

I predicted bacteria would be the only source of cellulase in all studied fishes, as it is widely accepted that vertebrates rely on bacterial cellulases to digest cellulose into glucose during nutrient assimilation (Watanabe and Tokuda 2001), and that microorganisms in the gut, such as bacteria, are solely responsible for cellulase production (Saha and Ray 1998). Indeed, in the carnivorous rainbow trout, cellulase specific and total activities were only detected in the epithelial layer that had bacteria (saline wash; Figure 2.12). The observation of cellulase activity in a carnivorous fish is supported by current literature, where cellulase-producing bacteria have been isolated in the gastrointestinal tracts of carnivorous fishes (Stickney and Shumway 1974; Liu et al. 2016). Furthermore, cellulase specific activity was only detected in the presence of

bacteria in the fed goldfish (Figure 2.13) and both unfed and fed central stoneroller (Figure 2.14A). These findings further support my prediction and demonstrate that all three species are reliant on cellulase-producing microbes in their intestines to break down cellulose (Das and Tripathi 1991; Bairagi et al. 2002; Li et al. 2009).

2.4.3. Interspecies comparisons

My prediction that trypsin activity would be highest in the carnivorous rainbow trout because of their high-protein diets and lowest in the central stoneroller, as they feed on low-protein plant matter (Leigh et al. 2018) was not entirely supported, as trypsin activity was not consistently higher in the carnivore. Rainbow trout only displayed the greatest trypsin activity following feeding and in the presence of bacteria in the epithelial layer (Table 2.5) as well as in the whole intestine (Figures 2.6A; 2.6B). This is indicative of an increase in trypsin secretion following feeding in the carnivore to break down the high amounts of protein (Grover et al. 2018) in the food, and appears to be enhanced by bacteria (Liu et al. 2016). Unexpectedly, unfed omnivorous goldfish exhibited greater trypsin activity in the epithelial layer than carnivorous rainbow trout and herbivorous central stonerollers (Table 2.4), as well as in the fed animals in the absence of bacteria (Table 2.5), suggesting that endogenous trypsin secretions were greatest in the omnivore when unfed. Further, central stonerollers exhibited the greatest trypsin activities in the unfed whole intestine and chyme compared to the rainbow trout and goldfish (Figures 2.5; 2.6C; 2.6D). These observations did not support my prediction of trypsin activity positively correlating to protein content in the diet. Higher proteolytic activities in non-carnivorous species when unfed, or in the absence of bacteria, are suggestive of compensatory efforts by the fish to maximize protein absorption (Hofer and Schiemer 1981; Hofer 1982; Lauff and Hofer 1984;

German et al. 2010). Generally, plant proteins are not nearly as nutritious (Hofer and Schiemer 1981; Kumar et al. 2007), yet protein is an essential nutrient for the proper functioning of an animal (Case et al. 2011). In order to compensate for their low-protein diets, herbivores, and even omnivores, have developed mechanisms for maximal protein absorption. These adaptations include higher food consumption, the presence of pharyngeal teeth, and elongated intestines to increase surface area and food retention time (Kapoor et al. 1975; Hofer and Schiemer 1981; Hofer 1982; German et al. 2010) so that they do not waste any proteins ingested. Indeed, herbivorous species have been shown to display enhanced proteolytic activities (Hofer and Schiemer 1981; Hofer 1982; Lauff and Hofer 1984; German et al. 2010) supporting the notion that herbivores attempt to maximize protein absorption from their low protein meals (Kapoor et al. 1975; Hofer and Schiemer 1981; Hofer 1982; German et al. 2010).

I predicted that lipase activity would be highest in the herbivorous central stoneroller because of their dietary habits (German et al. 2004; Caruso et al. 2009; Leigh et al. 2018). Indeed, central stonerollers had the greatest lipase activity in almost all of the samples, regardless of feeding treatment (Table 2.6; 2.7; Figures 2.10; 2.11). Furthermore, lipase activities were created and enhanced when bacteria were washed away in all three fed animals (Tables 2.6; 2.7). Firstly, this is evidence of endogenous mechanisms involved in lipolysis in all three fishes. Endogenous lipases have been previously studied in numerous fish species (Das and Tripathi 1991; Farber et al. 2001; Bairagi et al. 2002; Caruso et al. 2009; Hlophe et al. 2014; Khan et al. 2018), supporting my findings. Secondly, altogether, these findings suggest a significant bacterial contribution to the herbivorous fish, driving unfed total activities above the other species. German et al. (2004) proposed enhanced lipase activities in algae-eating species is a result of maximizing lipid extraction for energy. Further, lipolytic bacteria have been identified

in the guts of fishes, which aid the microbe host in lipid breakdown by producing exogenous lipase (Bairagi et al. 2002; Sumathi et al. 2011). Interestingly, when fed, rainbow trout surpassed goldfish and central stonerollers only in the presence of bacteria (Table 2.7), again suggesting that bacteria are providing additional lipase to rainbow trout following a meal. Though this refuted my prediction of enhanced lipase activity in the herbivore, some studies have shown lipase activity levels are not dependent on the trophic level of the animal (Chakrabarti et al. 1995; Horn et al. 2006). While not fully understood, it has been proposed this may be because all fish use dietary lipids as a source of energy (Chakrabarti et al. 1995), and for fat-soluble vitamin absorption (Lin and Shiau 2003; Xiangfei Li et al. 2012), hence differences would be expected to be negligible.

Finally, I predicted that cellulase activity would be the highest in the herbivorous central stoneroller and lowest in the rainbow trout, again, because of their dietary habits. My prediction was supported in the saline washed epithelia of unfed central stonerollers (Table 2.8). Bacteria further enhanced cellulase total activity in the fed central stonerollers, while the fed carnivore had non-detectable activity levels (Table 2.9). This evidence supports previous studies that have correlated cellulase activities with diet, stating that plant-based diets would result in greater cellulase activity (Prejs and Blaszczyk 1977; Saha and Ray 1998; Liu et al. 2016), and as rainbow trout have little to no cellulose in their food, there was no requirement for cellulolytic bacteria (Divakaran et al. 1999; Bairagi et al. 2002). However, when comparing other tissues, the dominance of the herbivore was replaced with the omnivorous goldfish (Figures 2.15; 2.16). It is unclear why cellulase activities were not higher in the herbivore, but Stickney and Shumway (1974) were also unable to match cellulase activities with feeding habits. To explain the high cellulose breakdown in the omnivore, Stickney (1975 cited in Bairagi et al. 2002) suggested that

omnivorous and carnivorous fishes establish communities of cellulolytic bacteria in their guts from their surroundings, especially from invertebrates. Indeed, studies on tilapia and Chinese grass carp have shown that Gram-positive Bacilli, specifically *Bacillus circulans* and *Bacillus megaterium* are major cellulase producers (Saha et al. 2006). Further, Asaduzzaman et al. (2018) studied enzyme activities in the omnivorous Malaysian mahseer and found that *Alcaligenes* sp. of phylum Proteobacteria are able to produce cellulases that enhance enzymatic reactions. It is possible that differences in the bacterial species identities in the intestines of the three fish species could explain enhanced activity observed in the omnivore (i.e. the goldfish happens to have more established colonies of cellulase producing bacteria for an unknown reason).

Notably, in the intestinal muscle of all three fishes, no cellulase specific or total activities were detected (Figures 2.16E; 2.16F), as expected. These results aid in validating the technique of removing intestinal bacteria with ethanol, as the lack of cellulase activity here suggests that no bacteria were present and further supports the notion that fishes do not possess any endogenous cellulase mechanisms. Further, the intestinal muscle is assumed to be sterile and free of bacteria, as visualized in Figure 2.1, and it is also not known to have digestive enzyme secretory functions. Even so, in the current study, trypsin (Figures 2.6E; 2.6F) and lipase (Figures 2.10E; 2.10F; 2.11F) specific and total activities were detected, and so it can only be assumed that these enzymes were endogenously produced. Beynon and Kay (1978) isolated a proteinase from smooth intestinal muscles of rats, which they classified as a serine protease and characterized as having a trypsin-like nature, but they did not suggest any potential digestive function for the enzyme. Further, lysosomal lipases have been detected in the visceral muscles of rainbow trout (Geromel and Montgomery 1980) and smooth muscles in mammals (Heltianu et al. 2011), and although this has not been expanded to the intestinal muscle, it can possibly explain the findings

of the current study. It is also possible that the mechanical separation of the intestinal epithelial layer and muscle was not completely successful and some residual enterocytes may have been left behind attached to the muscle. The effectiveness of this technique should be further evaluated through histological methods, such as H&E staining.

2.4.4. Effects of feeding on enzyme activity levels and microbe host versus bacterial contributions

I was able to observe the impact of feeding on intestinal digestive enzyme activities as a consequence of the experimental design. Interestingly, increased trypsin activity following feeding in the rainbow trout was likely driven (at least in part) by bacterial secretions (Figure 2.2), suggesting that when fed, rainbow trout rely on trypsin-producing bacteria for efficient proteolysis, even though they able to produce this enzyme endogenously. Indeed, higher proteolytic activity following a meal (Hlophe et al. 2014) has been previously attributed to exogenous sources of proteases (Dabrowski and Glogowski 1977). Carnivorous species have large amounts of protein in their diets for catabolism, especially following a meal, and perhaps microbial contributions are used to maximize the production of nutrients in the intestine (Xiong et al. 2011). Additionally, while feeding had no significant effect on bacteria-free epithelia obtained from goldfish, it appeared to decrease trypsin specific activity in the saline washed epithelia (Figure 2.3A), suggesting that these changes in trypsin activity were also driven by changes in bacteria. Einarsson et al.'s study of protease secretion in Atlantic salmon (1996) showed that trypsin secretion increased during starvation. The authors attributed this to the accumulation proteases in the gut in the absence of substrates, which may be the case with the goldfish. To see the full effects of starvation on trypsin activity, starvation periods would need to

be increased to 21 days, as shown by Rungruangsak-Torrissen and colleagues (2006), who found decreased trypsin specific activities only after this period. Finally, feeding did not affect trypsin activities in the herbivorous central stoneroller in either wash treatment and so the data were collapsed (Figure 2.4), perhaps because they do not heavily rely on amino acids but instead glucose from their meals (Kraatz 1923) for cellular functions.

Following feeding, rainbow trout showed increased lipase specific activity in the intestinal epithelia with bacteria (Figure 2.7A), suggesting feeding, again, stimulated microbe host and bacterial production of lipase (Sumathi et al. 2011; Das et al. 2014) to break down ingested dietary lipids. In contrast, feeding eliminated lipase specific activity in the saline washed epithelial layer of goldfish (Figure 2.8A) and central stonerollers (Figure 2.9A) and in the ethanol rinsed epithelia of goldfish (Figure 2.8A), whereas it promoted lipolysis in the central stonerollers (Figure 2.9A). The reason behind these contradictions is unknown, but this could reflect variation that is not attributable to feeding status. Current literature suggests that being fed or unfed does not greatly affect lipase activity (Divakaran et al. 1999; Xiong et al. 2011). Detectable activities in unfed samples may perhaps be a result of lipase accumulation under fasting conditions by the fishes, as suggested by Einarsson et al. (1996) regarding proteases. Finally, it is unknown why there was a complete shutdown of all lipolytic mechanisms during digestion, including bacterial, as lipolytic bacteria have been identified in the guts of many fishes (Bairagi et al. 2002; Sumathi et al. 2011). Thus, further studies are necessary to better understand the effects of feeding on lipase activity and fat metabolism in the intestine.

Finally, feeding promoted bacterial cellulase specific activity in the intestinal epithelia of goldfish (Figure 2.13), and in the whole intestines of rainbow trout and central stonerollers (Figures 2.16A; 2.16B). This indicates the upregulation of cellulase production post-feeding by

the gastrointestinal microbes to break down dietary cellulose (Lesel et al. 1986). In contrast, feeding diminished specific activity in the intestinal epithelia of central stonerollers and rainbow trout (Figure 2.14A; Table 2.9). The cause of this contradiction is unclear but highlights the importance of specific tissue and activity level comparisons.

2.4.5. Enzyme activity measurements

In the current study, I measured and presented trypsin, lipase, and cellulase enzyme activities both in specific and total activity levels. Specific enzyme activity measures the activity level per milligram of protein (U mg protein^{-1}), while total activity measures the activity level per gram of tissue or chyme (U g tissue^{-1} or U g chyme^{-1}). The trends observed in one activity quantification may be lost in another, as the protein content per gram can differ between the different layers of the intestine in one species and even within one specific intestinal layer between the different species. This difference in protein content can be a result of dietary protein concentrations, gastrointestinal structure, and even the intestinal epithelium morphology. Interestingly, the current available literature on fish digestive enzymes typically has enzyme activity levels presented as specific activity (e.g. McLeese and Don Stevens 1982; Infante and Cahu 1994; Rungruangsak-Torrissen et al. 2006; Suzer et al. 2008; Unajak et al. 2012; Candiotto et al. 2017); however, total activity is also sometimes used (e.g. Lindsay and Harris 1980; Giri et al. 2000; Leigh et al. 2018). Very few authors have presented their results as both units (e.g. Chan et al. 2004). Thus, there is a loss of information in most studies, as well as an issue with comparing between studies that present specific activity and those that present total activity. Both units of measurement are valuable in understanding how digestive enzymes function; however, it is evident from the findings of this study that the same results analyzed in two

different ways do not give the same conclusions. Therefore, it is highly recommended to present the data as both specific and total activity levels to ensure consistency between studies, as well as to provide the most information possible and prevent issues from confounding factors (e.g. intestinal morphology, protein content) between enzymatic studies.

2.4.6. Conclusions

Overall, this study has shown that three different teleosts with varying diets are able to endogenously produce trypsin and lipase but that they also rely on exogenous sources of these digestive enzymes, especially cellulase. Specifically, proteolytic bacteria enhanced trypsin activity in the carnivorous rainbow trout that were fed, but not the omnivorous or herbivorous species. This suggests that the endogenous trypsin mechanisms of goldfish and central stonerollers are adequate for the breakdown of proteins required for cellular processes, whereas rainbow trout require additional trypsin due to their high-protein diets, supporting my prediction. This was further supported by Liu et al.'s (2016) study, where carnivorous fish had enhanced trypsin activity due to their gut microbial composition. Further, my results suggest that the herbivorous central stoneroller may have the highest lipase activity, driven by enhanced bacterial contributions. Additionally, rainbow trout, once again, demonstrated bacterially enhanced digestive enzyme production during feeding, with enhanced lipase activity in the presence of bacteria. However, overall lipase production seems to be related more to endogenous mechanisms rather than the intestinal microbiota. Finally, cellulase was entirely supplied by the intestinal bacteria, creating elevated levels in the unfed herbivore and fed omnivore. Altogether, this signifies the importance of the gastrointestinal microbiota in digestive processes (Silva et al. 2011). Perhaps due to the variable contribution of intestinal bacteria, enzyme activities were not necessarily correlated to dietary habits of the fishes, as initially expected.

CHAPTER 3: THE INFLUENCE OF DIETARY CHANGES ON DIGESTIVE ENZYME ACTIVITIES IN GOLDFISH

3.1. INTRODUCTION

3.1.1. Enzyme activity patterns in fish from different trophic levels

Changes in the diet of an animal can alter digestive enzyme activity levels to allow for the optimal utilization of the enzymes for nutrient extraction. Differences in enzyme activities are seen in fishes from different trophic levels, which often match the makeup of their food (Reimer 1982; Liu et al. 2016). Specifically, trypsin, lipase, and cellulase act on proteins, fats, and carbohydrates, respectively, and enzyme activity levels generally reflect the nutritional substrate amounts in the animal's diet (Reimer 1982). For example, carnivorous species generally demonstrate higher proteolytic activities, while omnivorous and herbivorous species exhibit higher carbohydrase and lipolytic activities (Ugolev and Kuz'mina 1994; German et al. 2004; Caruso et al. 2009; Leigh et al. 2018). Furthermore, enzyme activity levels can be directly manipulated through dietary changes. For example, increasing the amount of dietary plant material fed to carnivorous yellowtail *(Seriola quinqueradiata)* lowered intestinal trypsin activities (Murashita et al. 2019). Similarly, feeding carnivorous walking catfish *(C. batrachus)* increased plant protein significantly lowered protease activity compared to animals fed animal-based proteins (Giri et al. 2000). Further, larval sea bass *(Dicentrarchus labrax)* fed elevated starch levels exhibited increased carbohydrase activities (Infante and Cahu 1994). Information on how diet affects lipase activity levels in teleosts is limited; however, herbivorous *(C. mrigala)* and omnivorous *(Aristichthys nobilis)* fish were found to exhibit overall higher esterase activities than carnivorous fish *(Notopterus notopterus),* and the herbivore had the highest activity in the

intestine (Chakrabarti et al. 1995). Additionally, zebrafish fed high-fiber and low-protein diets had elevated lipase activities (Leigh et al. 2018).

However, not all studies support the correlation of digestive enzyme activities with diet. Hidalgo et al. (1999) suggested proteolytic activity is less dependent on diet than carbohydrase activity. Additionally, changing the nutrient content of zebrafish meals to represent carnivorous, omnivorous, and herbivorous diets created enhanced trypsin, lipase, and carbohydrase activities in the omnivorous group, whereas the carnivorous group had the overall lowest activity levels (Leigh et al. 2018). Further, Chakrabarti and colleagues (1995) found higher protease activity in the intestines of omnivorous fish than carnivorous fish. Several studies also reported lipase activity levels were not affected by the amount of fat in the ingested food (Chesley 1934; Nagase 1964; Das and Tripathi 1991; Murashita et al. 2019). Finally, Stickney and Shumway (1974) also found no evidence supporting this correlation in their study of cellulase activities in 148 fish species, as they observed high, low, and no cellulase activities in species with similar diets. The reason for this contradictory body of literature is unclear; however, an animal's diet is a significant factor that can influence its gastrointestinal microbial community (Nayak 2010; Silva et al. 2011; Michl et al. 2019), and hence the production of exogenous digestive enzymes that aid in digestion. Thus, the variable nature of the gastrointestinal microbiota can potentially explain the enzyme variation observed in the literature.

3.1.2. Intestinal microbial composition of fish from different trophic levels

The gut microbiome is distinct across fishes from different trophic levels (i.e. herbivorous, omnivorous, and carnivorous). While some similarities at the order and family levels may exist within the microbial communities of fishes from different trophic levels (Sullam

et al. 2012; Michl et al. 2017), analyses at the genus level have revealed that there can be a clear difference in which bacteria inhabit the intestines of these fishes (Liu et al. 2016). Interestingly, carnivorous fish have the least phylogenetic diversity in their gut microbiomes, while omnivorous fish have the greatest microbial richness (Givens et al. 2015). This has also been observed in the lab, as the diversity of gut microbiota in fish fed plant-based foods exceed the diversity of fish fed animal-based foods (Liu et al. 2016; Gajardo et al. 2017). Further, omnivores also have large intraspecies variations regarding their gut bacterial composition, whereas herbivorous species are more consistent across individuals (Miyake et al. 2015). This may be because in their natural habitats, omnivores are more flexible in terms of their diet and can feed on a range of foods, while the herbivores and carnivores are more rigid in what they feed on (Givens et al. 2015; Miyake et al. 2015).

Research on the effects of dietary changes on intestinal microbiota have revealed some interesting and opposing findings. Michl et al. (2019) found that the gut microbiome of juvenile brown trout *(Salmo trutta)* was altered after a dietary change. Further, differences in bacterial communities, both at the phylum and order levels, have been found in the gut microbiome of juvenile rainbow trout fed various dietary proteins (Michl et al. 2017). Specifically, dietary plant proteins increased the abundance of bacteria from phylum Proteobacteria and the orders Lactobacillales, Bacillales, and Pseudomonadales, whereas phylum Firmicutes and orders Bacteroidales, Clostridiales, Vibrionales, Fusobacteriales, and Alteromonadales were more abundant in individuals fed animal proteins (Michl et al. 2017). Finally, replacing fish oil with either corn oil or flaxseed oil decreased the abundance of *Aeromonas* bacterial species, which have proteolytic functions, in the carnivorous sablefish (*Anoplopoma fimbria*; Rhodes et al. 2016). In contrast, Sugita and colleagues (1988) failed to see a predictable change in the gut

microbiota of goldfish fed two different diets. Further, a study on the effect of soybean meal-based diet on the intestinal microbiota of rainbow trout found no quantitative differences in lipolytic bacteria in the experimental groups, therefore implying that the new diet caused no microbiological adaptations (Heikkinen et al. 2006). However, although quantitative changes in the intestinal bacteria may be negligible, research has shown that there were changes in the actual species composition, but further studies are required to better understand the impacts of these community-scale changes (Heikkinen et al. 2006; Desai et al. 2012). Overall, these studies all support the idea that the composition of the gut bacterial community may be diet-dependent and can shift to accommodate an animal's feeding habits, possibly to provide functional support.

Unfortunately, studies examining functional correlations between bacteria and enzyme-based digestion capabilities are relatively rare in fish. Cellulose-degrading bacterial species have been found to be more dominant in the guts of herbivorous fishes in contrast to carnivorous fishes, where bacteria producing proteases are more prevalent (Liu et al. 2016). Additionally, the blunt snout bream *(Megalobrama amblycephala)* undergoes dietary transitions during development before it becomes an herbivore at maturity (Wei et al. 2018). Here, cellulase activity was found to be significantly higher once the fish reached the herbivorous-stage compared to fish in the zooplankton- and transition-stages. Individuals in the transition-stage were considered omnivorous and had intermediate levels of cellulase activity and the highest levels of lipase activity (Wei et al. 2018). Bacteria from phylum Actinobacteria, which play an important role in digesting plant matter, such as cellulose (Anandan et al. 2016), were abundant in the guts, specifically in the herbivorous-stage and were less pronounced in the zooplankton-stage groups (Wei et al. 2018). Interestingly the gut microbial communities of herbivorous fishes resemble that of mammalian guts, suggesting that the microbiota serve the same function of gut

fermentation in both groups (Sullam et al. 2012; Talwar et al. 2018). Finally, Talwar and colleagues (2018) and Gajardo et al. (2017) have suggested that similarities of bacterial phyla across species is indicative of gut microbiota that are essential for a variety of host functions, including digestion and immune response. As the gut microbiota can shift in response to dietary changes, alterations in the function of the digestive system can follow (Wu et al. 2012; Rhodes et al. 2016; Gajardo et al. 2017; Michl et al. 2017; Talwar et al. 2018; Michl et al. 2019). Although interest in the gastrointestinal tract microbiota has increased in recent years, there is still a lot that is unknown about how changes in trophic levels by way of dietary manipulation affect the composition of the gastrointestinal tract bacteria. However, due to their role in supplying digestive enzymes, it is possible that variation in bacterial communities could explain some of the variation in the literature, highlighting the importance of researching how diet manipulation affects the intestinal microbiota, along with gut function in fishes.

3.1.3. Hypotheses

By working with one species, the omnivorous goldfish, and feeding them either a carnivorous- or herbivorous-based diet, we can better understand how the intestinal microbiota changes and if these changes correlate with their function of digestive enzyme production. I hypothesized that dietary changes in the goldfish would cause a shift in the composition of the intestinal microbiota, resulting in functional changes in enzyme activity levels. Based on the available literature focusing on the effects of diet on digestive enzyme activity (Nagase 1964; Reimer 1982; Hidalgo et al. 1999; German et al. 2004; Murashita et al. 2019), I predicted that trypsin activity would be highest in the goldfish fed a carnivorous diet, followed by the naturally omnivorous goldfish, and finally in the group fed an herbivorous diet, as the main function of

trypsin is to break down dietary proteins (Cao et al. 2000). Further, I predicted that goldfish fed an herbivorous-based diet would have the highest cellulase activity while the carnivorous-based diet would display the lowest activity rate and omnivore diets would create intermediate levels (German et al. 2004). If the enzyme activity levels differ amongst the three diet treatments, this would suggest that significant differences observed between the three species in Chapter 2 may be due to phenotypic responses to varying diets and not necessarily phylogenetic adaptations.

3.2. MATERIALS AND METHODS

3.2.1. Herbivorous and carnivorous goldfish

All goldfish housing and care were performed as outlined in Section 2.2.1 in Chapter 2. Goldfish were obtained from Big Al's Aquarium Supercentres (Vaughan, Ontario, Canada) and were kept in separate aerated, flow-through, dechlorinated water tanks (56L; 12 ± 2°C; n=20 per tank). Fish were fed once daily with Omega One™ Super Veggie brown seaweed (OmegaSea LLC, Alaska, U.S.A) for the herbivorous-based diet or Hikari® Bio-Pure frozen blood worms (Hikari Sales USA Inc.; Hayward, California, U.S.A) for the carnivorous-based diet for 21 days. Feed composition is outlined in Table 3.1. Uneaten food was removed 60 minutes after feeding. Fish were dissected 24 hours following feeding for the fed trials and there were no unfed trials for this experiment. Once again, the different intestinal layers were separately obtained, as described in Section 2.2.2 and Table 2.1 in Chapter 2.

3.2.2. Genomic DNA extractions, polymerase chain reaction, and gel electrophoresis

The steps outlined in Sections 2.2.3 and 2.2.4 in Chapter 2 were followed to perform gDNA extractions, PCR, and gel electrophoresis in the herbivorous-based diet and carnivorous-based diet goldfish samples.

3.2.3. Enzyme assays

Trypsin and cellulase enzyme assays were performed following the protocols outlined in Section 2.2.5 in Chapter 2. Lipase enzyme assays were not conducted due to time constraints as a result of the COVID-19 pandemic.

3.2.4. Bradford protein assay

Bradford protein assays were performed as outlined in Section 2.2.6 in Chapter 2.

3.2.5. Statistical analyses

Data were transferred into SigmaPlot Software (version 11.0, Systat Software Inc.; San Jose, California, U.S.A) where statistical analyses were conducted and are described as appropriate in the figure captions. If required, data were normalized using a simple square root statistical transformation on SigmaPlot Software. Detection of enzymatic activity was analyzed using one-sample t-tests against a set mean value of zero. Further statistical testing was only carried out on detectable samples (i.e. samples that passed the one-sample t-test). Comparisons between intestinal epithelial layer washes in the omnivorous goldfish were completed through a Mann-Whitney rank sum test. T-tests were used to compare trypsin total activities between the omnivorous goldfish and the goldfish fed a carnivorous-based diet. Statistical significance was assumed if $p<0.05$. All figures were computed on SigmaPlot Software and data in the figures are expressed as mean values ± SEM.

Table 3.1. Nutrient composition (%) of goldfish feed. Goldfish flakes were used for the omnivorous diet, blood worms were used for the carnivorous diet, and brown seaweed was used for the herbivorous diet.

Nutrient	Cobalt Aquatics Goldfish Flakes	Hikari® Bio-Pure Frozen Blood Worms	Omega One™ Super Veggie Brown Seaweed
Crude Protein	Approximately 45%	Approximately 75%	Approximately 23%
Crude Fat	Minimum 10%	Minimum 0.5%	Minimum 3.0%
Crude Fibre	Maximum 5.0%	Maximum 0.9%	Minimum 3.0%

Note: Protein content was previously measured by Bucking et al. (unpublished). Fat and fibre contents are from the manufacturer.

3.3. RESULTS

3.3.1. Bacterial detection in the intestinal layers

Representative sample PCR products from the extracted gDNA of the intestinal layers of goldfish fed an herbivorous diet are shown in Figure 3.1. Bacterial rRNA primers that amplified either the V3/V4 (Figure 3.1A) or V6/V7 (Figure 3.1B) hypervariable regions were used, where bands 447bp and 210bp in size, respectively, correspond to the presence of bacteria. In Figure 3.1A, bacteria were detected in the whole intestine, chyme, intestinal muscle, and saline washed intestinal epithelial layer representing the enterocytes, but not in the ethanol washed epithelial layer. These results validated the effectiveness of the 70% ethanol rinse, as no bacteria were detected in the epithelial layer; however, there is evidence of bacterial contamination in the intestinal muscle. As a result, this particular sample was excluded from enzyme activity measurements. Further gDNA extractions on other intestinal muscle samples and ethanol washed epithelia proved to be free of bacteria (Figure 3.1B). Qualitatively, the whole intestine had the brightest band (Lane 2), while fainter bands were seen in the chyme (Lane 3), intestinal muscle (Lane 4), and saline washed epithelia representing enterocytes (Lane 5; Figure 3.1A). Additional bands outside of the expected product size were presumed to be non-specific and a result of the large number of PCR cycles. The PCR products of the remaining samples of the herbivorous-based diet goldfish and carnivorous-based diet goldfish are not displayed, but the presence or absence of bacteria in the different intestinal layers was validated in the same manner. If bacteria were detected in either the intestinal muscle or ethanol washes, those samples were not tested for enzyme activity.

3.3.2. Enzyme activity levels

Trypsin and cellulase activities were measured in specific activity (U mg protein^{-1}) as well as total activity (U g tissue^{-1} or U g chyme^{-1}), while lipase activities were not studied due to COVID-19 interruptions. Data for goldfish fed an omnivorous diet are from the same data set used in Chapter 2.

3.3.2.1. Trypsin enzyme activity levels

First, trypsin activity levels were determined in the epithelial layers representing enterocytes of goldfish fed either omnivorous, herbivorous, or carnivorous diets (Figure 3.2). One-sample t-tests revealed that trypsin specific activity was detectable (i.e. above 0.00 U) in the intestinal epithelia washed with saline and ethanol only in goldfish fed an omnivorous diet ($p<0.05$; Figure 3.2A). In contrast, activity could not be detected in the epithelial layer (both washes) of goldfish fed the herbivorous and carnivorous diets ($p>0.05$; Figure 3.2A). In the omnivorous diet group, the ethanol wash increased trypsin specific activity in the intestinal epithelial layer (0.572 ± 0.071 U mg protein^{-1}), above the saline washed samples (0.391 ± 0.002 U mg protein^{-1}; $p<0.05$; Figure 3.2A). Saline washed intestinal epithelia had no detectable levels of trypsin total activity in any of the dietary treatments ($p>0.05$; Figure 3.2B). Instead, the ethanol wash in the intestinal epithelial layer enhanced total activity and created detectable levels in the goldfish fed an omnivorous diet ($p<0.05$), but not the herbivorous and carnivorous diet goldfish ($p>0.05$; Figure 3.2B).

Alterations in the diet of a typically omnivorous fish also eliminated trypsin activities in the whole intestine samples as well, where activity was only detectable in the goldfish fed an omnivorous diet ($p<0.05$), but not the herbivorous- and carnivorous-based diets (Figures 3.3A; 3.3B) due to high variation in these samples. Interestingly, in contrast to trypsin specific activity

in the whole intestine and the intestinal epithelial layers, chyme from the goldfish fed the omnivorous diet (0.540 ± 0.097 U mg protein^{-1}) and the carnivorous diet (0.873 ± 0.243 U mg protein^{-1}) both exhibited trypsin activity (p<0.05), but there were no significant differences between the two groups (p>0.05; Figure 3.3C). However, total activity analysis on the chyme samples resembled that of the whole intestine and ethanol washed epithelia, where activity was, again, only detected in the goldfish fed an omnivorous diet (5.273 ± 2.002 U g chyme^{-1}; p<0.05; Figure 3.3D). Herbivorous diet goldfish did not demonstrate any trypsin specific or total activities in any of the samples (p>0.05).

In contrast to the previously discussed intestinal layers, trypsin specific and total activities were non-detectable in the intestinal muscle of the omnivorous diet goldfish (p>0.05; Figures 3.3E; 3.3F). Instead, trypsin activities were detectable only in the muscles of goldfish fed a carnivorous diet (0.277 ± 0.089 U mg protein^{-1} and 2.984 ± 0.970 U g tissue^{-1}; p<0.05; Figures 3.3E; 3.3F). Trypsin activity remained non-detectable in the intestinal muscle of the goldfish fed an herbivorous diet (p>0.05; Figures 3.3E; 3.3F). Hence, both the omnivorous and herbivorous diets resulted in no trypsin activities in the muscle, while the carnivorous diet enhanced activities.

3.3.2.2. Cellulase enzyme activity levels

One-sample t-tests showed that when looking at the intestinal epithelial layers representing enterocytes (Figure 3.4A), cellulase specific activities were only detectable (i.e. above 0.00 U) in the saline washed epithelial layer of the goldfish fed an omnivorous diet (0.466 ± 0.134 U mg protein^{-1}; p>0.05), but the ethanol rinse abolished this activity (p>0.05; Figure 3.4A). In contrast, cellulase specific activity in the saline washed epithelial layer of the goldfish fed a carnivorous-based diet was not detectable (p>0.05), but the ethanol washed epithelia

created detectable levels of activity (0.189 ± 0.045 U mg protein^{-1}; $p<0.05$; Figure 3.4A). The goldfish fed an herbivorous diet failed to show detectable cellulase activity in the epithelia, regardless of wash type ($p>0.05$; Figure 3.4A). Furthermore, cellulase total activities were undetectable in the intestinal epithelial layer, both saline and ethanol wash, of goldfish fed any of the three diets ($p>0.05$; Figure 3.4B).

Overall, changing the diet of a goldfish from omnivorous food to either herbivorous or carnivorous foods, once again, eliminated cellulase enzyme activities completely in the whole intestines and chyme of the animals (Figure 3.5). Specifically, goldfish fed the omnivorous diet was the only group that had detectable cellulase activities ($p<0.05$) in both the whole intestine and the chyme, while cellulase specific and total activities in the goldfish fed herbivorous- and carnivorous-based diets were undetectable ($p>0.05$; Figures 3.5A; 3.5B; 3.5C; 3.5D).

When examining the intestinal muscle layer, cellulase specific activity was detected, for the first time, only in the goldfish fed an herbivorous-based diet (0.3239 ± 0.1259 U mg protein^{-1}; $p<0.05$), but not in the omnivorous or carnivorous diet groups (Figure 3.5E). However, this trend was not seen in total activity analyses, where cellulase activity was undetectable in all three diet treatment groups ($p>0.05$; Figure 3.5F).

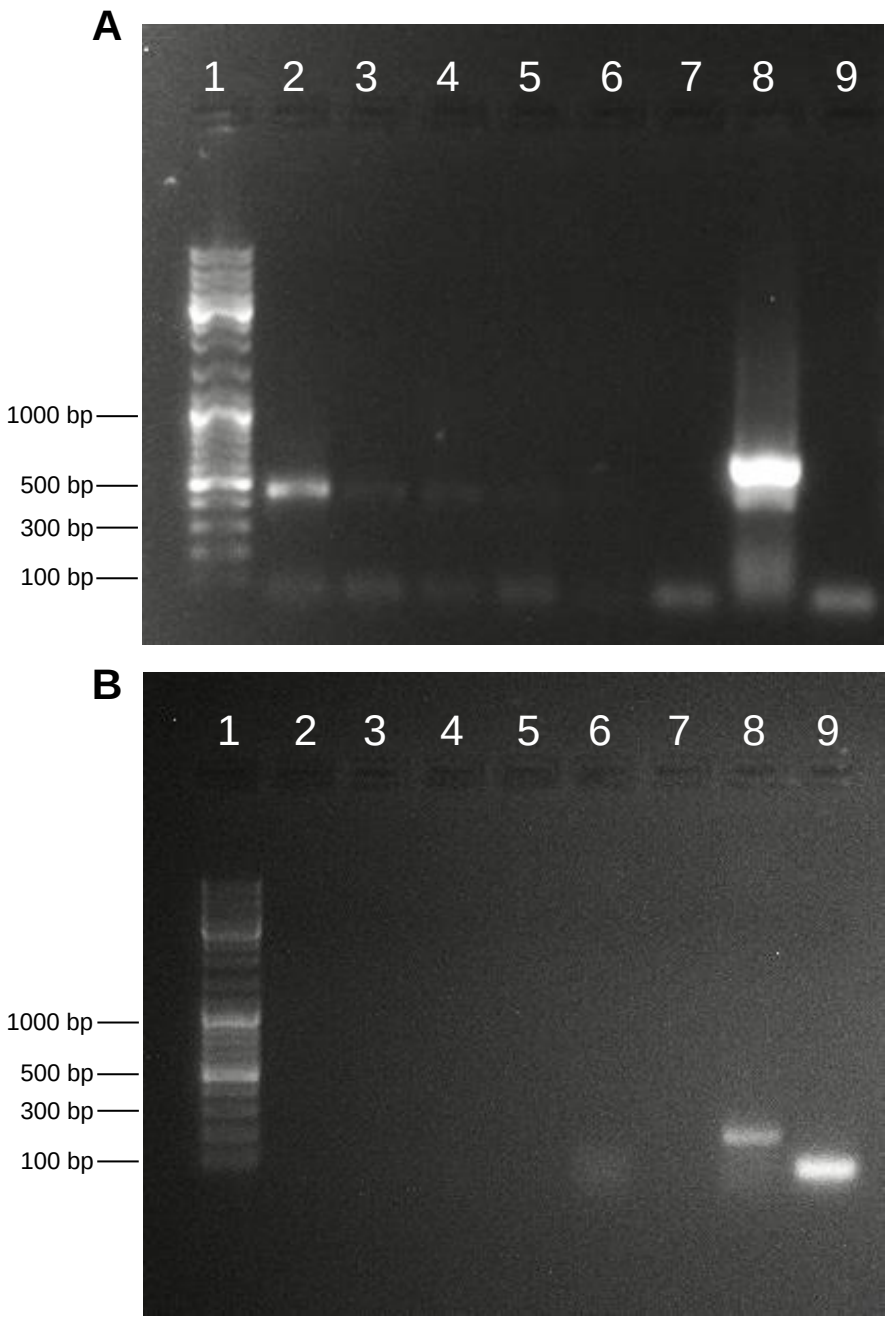

Figure 3.1. Agarose gel electrophoresis (1.5%) of the PCR products in the intestinal layers of goldfish fed an herbivorous diet using universal bacterial rRNA primers. Gels were stained with SYBR™ Safe DNA Gel Stain (Invitrogen by Thermo Fisher Scientific) and visualized under UV light. Lane 1: GeneRuler 100bp DNA ladder (Thermo Fisher Scientific); Lane 7: gDNA extraction negative control; Lane 8: PCR positive control; Lane 9: PCR negative control. **(A)** Primer set 338F-785R (V3/V4 hypervariable region), Lane 2: whole intestine; Lane 3: chyme; Lane 4: intestinal muscle; Lane 5: saline washed epithelial layer representing enterocytes; Lane 6: ethanol washed epithelial layer representing enterocytes. Bands of 447bp in size confirm the presence of bacteria. **(B)** Primer set 967F-1177R (V6/V7 hypervariable region), Lanes 2-4: intestinal muscle; Lanes 5-6: ethanol washed epithelial layer representing enterocytes; Bands of 210bp in size confirm the presence of bacteria.

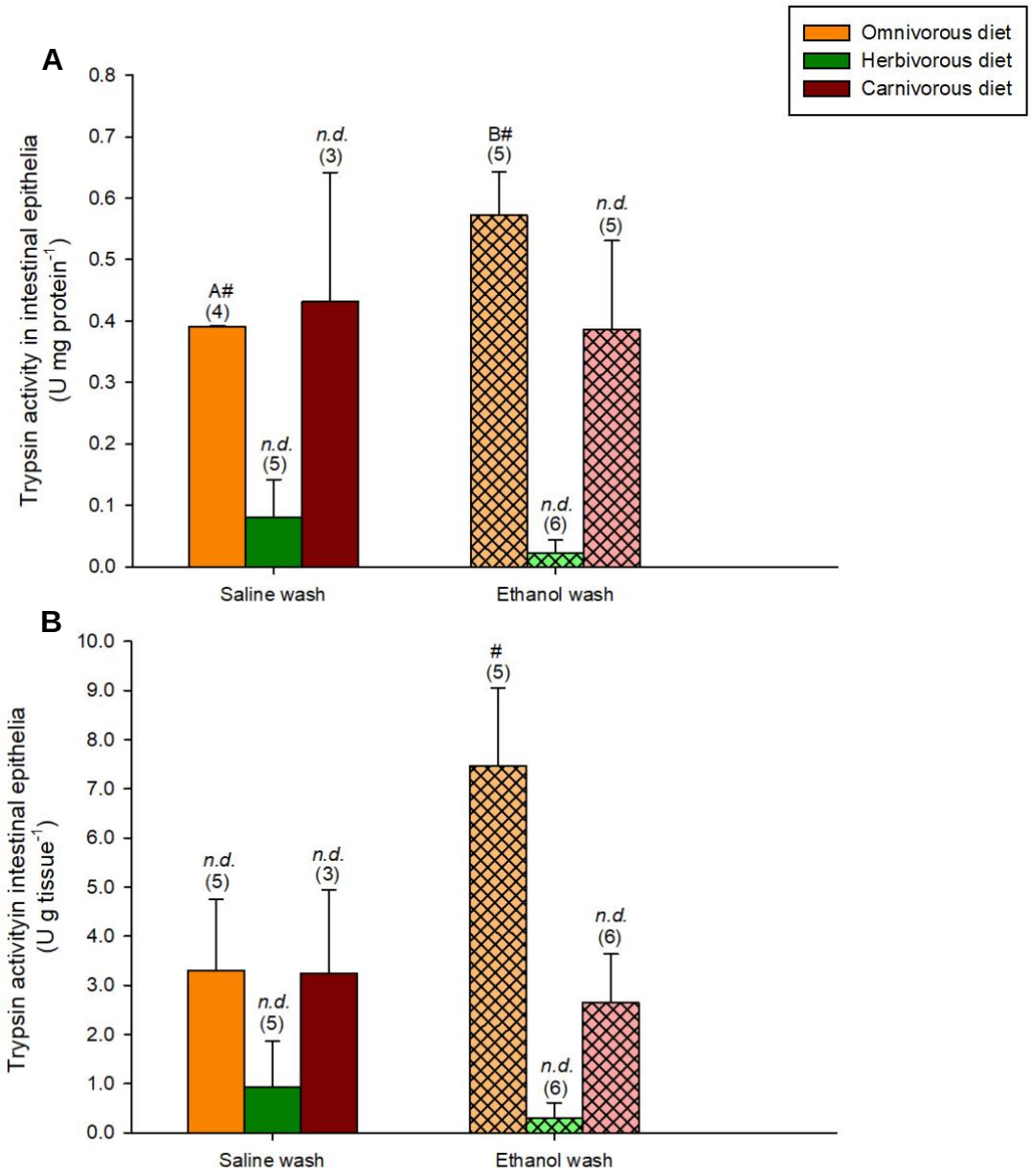

Figure 3.2. Trypsin enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of omnivorous goldfish, herbivorous-based diet goldfish, and carnivorous-based diet goldfish. Each wash treatment of the intestinal epithelial layer has been compared within each diet and the goldfish with the different diets have also been compared within each epithelial layer. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Mean values for the omnivorous goldfish are from the same data set as "Goldfish" in Chapter 2. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity. Statistically significant differences between saline and ethanol washes in the goldfish fed an omnivorous diet are denoted by different uppercase letters (p<0.05, by Mann-Whitney rank sum test).

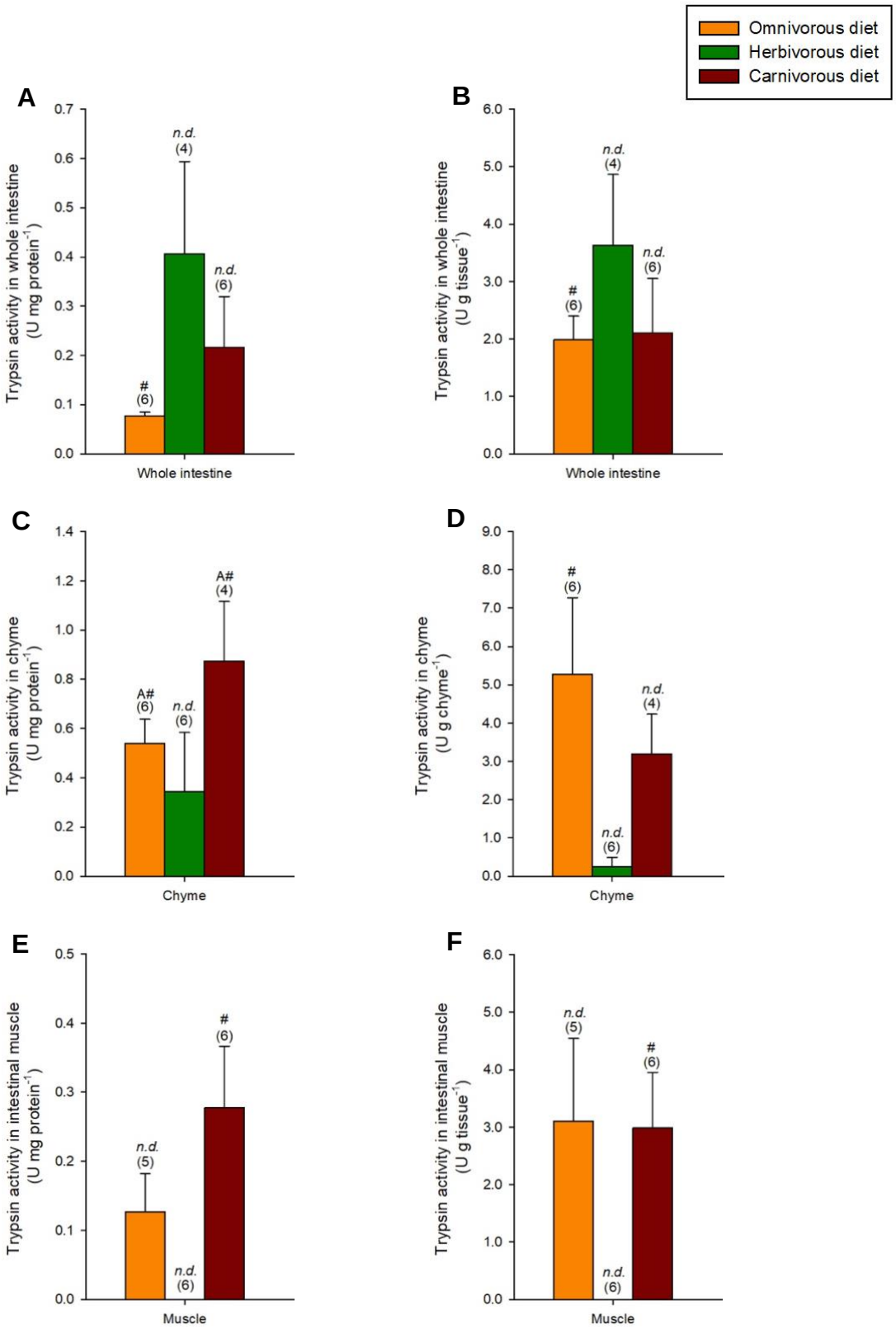
Omnivorous diet
Herbivorous diet
Carnivorous diet
A
Trypsin activity in whole intestine (U mg protein-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
n.d. (4)
n.d. (6)
(6)
Whole intestine
B
Trypsin activity in whole intestine (U g tissue-1)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
n.d. (4)
n.d. (6)
(6)
Whole intestine
C
Trypsin activity in chyme (U mg protein-1)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
A# (4)
A# (6)
n.d. (6)
Chyme
D
Trypsin activity in chyme (U g chyme-1)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
(6)
n.d. (4)
n.d. (6)
Chyme
E
Trypsin activity in intestinal muscle (U mg protein-1)
0.0
0.1
0.2
0.3
0.4
0.5
(6)
n.d. (5)
n.d. (6)
Muscle
F
Trypsin activity in intestinal muscle (U g tissue-1)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
n.d. (5)
(6)
n.d. (6)
Muscle

Figure 3.3. Trypsin enzyme activity levels (U mg protein^{-1}; specific activity, U g tissue^{-1}/U g chyme^{-1}; total activity) in the (A, B) whole intestine, (C, D) chyme, and (E, F) intestinal muscle of omnivorous goldfish, herbivorous-based diet goldfish, and carnivorous-based diet goldfish. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Mean values for the omnivorous goldfish are from the same data set as "Goldfish" in Chapter 2. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity. Bars sharing the same letter in the chyme are not statistically different (p>0.05, by t-test).

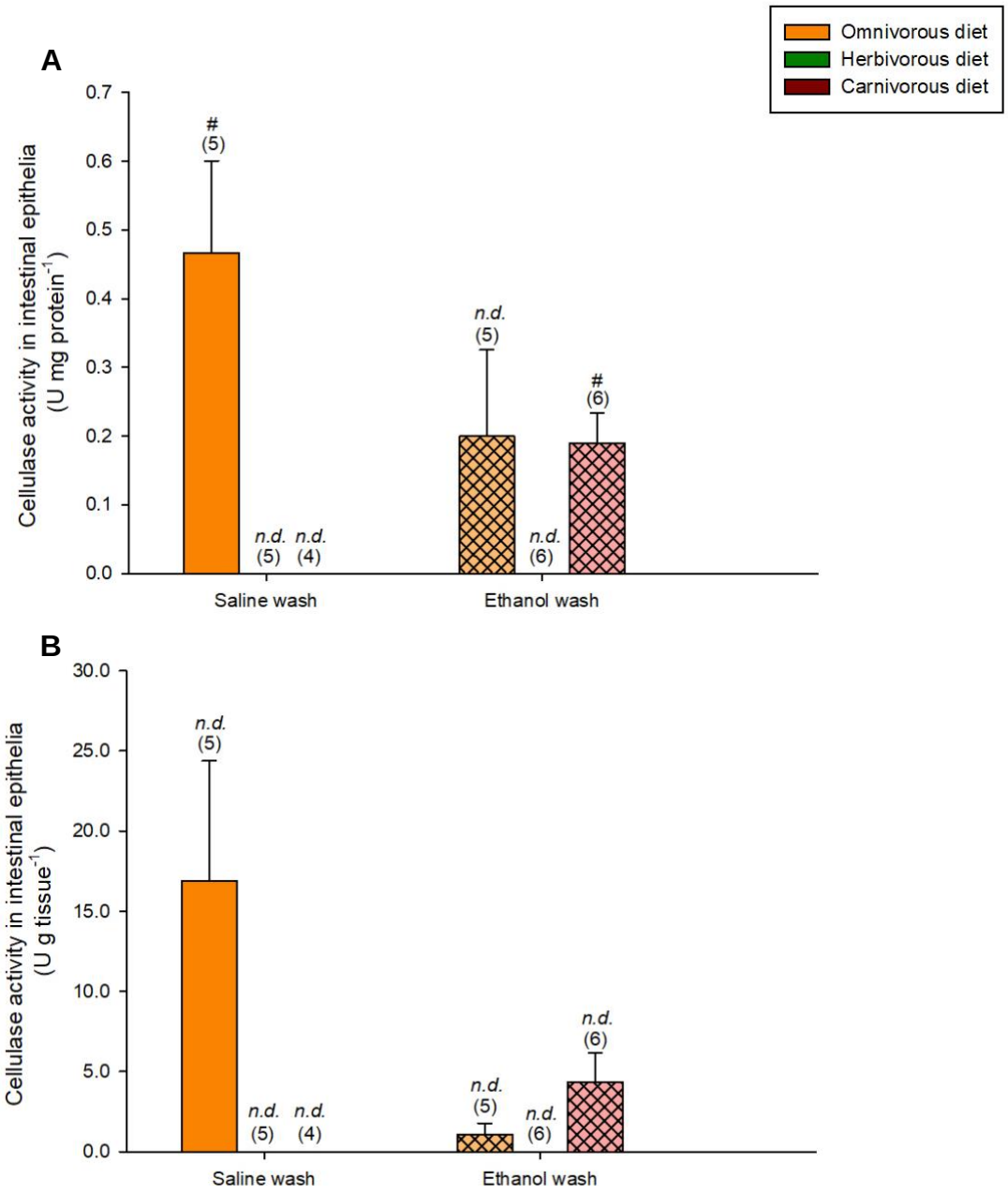

Figure 3.4. Cellulase enzyme activity levels in (A) U mg protein^{-1} (specific activity) and (B) U g tissue^{-1} (total activity) in the intestinal epithelial layers (representing enterocytes; saline wash and ethanol wash) of omnivorous goldfish, herbivorous-based diet goldfish, and carnivorous-based diet goldfish. Each wash treatment of the intestinal epithelial layer has been compared within each diet and the goldfish with the different diets have also been compared within each epithelial layer. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Mean values for the omnivorous goldfish are from the same data set as "Goldfish" in Chapter 2. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity.

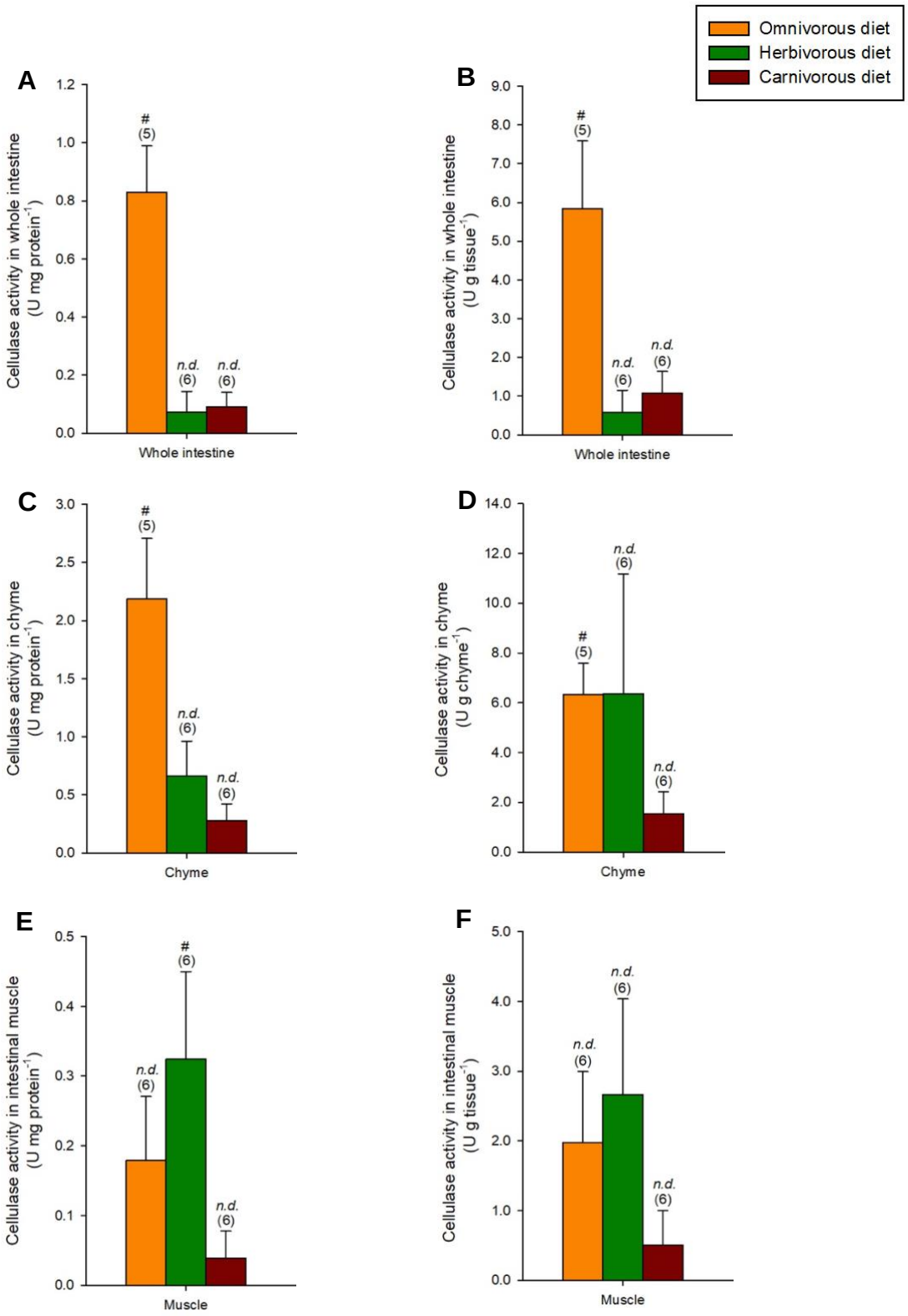
Omnivorous diet
Herbivorous diet
Carnivorous diet
A
Cellulase activity in whole intestine (U mg protein-1)
#
(5)
n.d.
(6)
n.d.
(6)
Whole intestine
B
Cellulase activity in whole intestine (U g tissue-1)
#
(5)
n.d.
(6)
n.d.
(6)
Whole intestine
C
Cellulase activity in chyme (U mg protein-1)
#
(5)
n.d.
(6)
n.d.
(6)
Chyme
D
Cellulase activity in chyme (U g chyme-1)
#
(5)
n.d.
(6)
n.d.
(6)
Chyme
E
Cellulase activity in intestinal muscle (U mg protein-1)
n.d.
(6)
#
(6)
n.d.
(6)
Muscle
F
Cellulase activity in intestinal muscle (U g tissue-1)
n.d.
(6)
n.d.
(6)
n.d.
(6)
Muscle

Figure 3.5. Cellulase enzyme activity levels (U mg protein^{-1}; specific activity, U g tissue^{-1}/U g chyme^{-1}; total activity) in the (A, B) whole intestine, (C, D) chyme, and (E, F) intestinal muscle of omnivorous goldfish, herbivorous-based diet goldfish, and carnivorous-based diet goldfish. Data represent mean ± SEM and the numbers in parentheses above each bar represent the sample size. Mean values for the omnivorous goldfish are from the same data set as "Goldfish" in Chapter 2. Bars with the pound symbol (#) are significantly different from a set mean value of zero (p<0.05, by one-sample t-test), while *n.d.* indicates non-detectable levels of activity.

3.4. DISCUSSION

3.4.1. Overview

Diet is a major determinant of the gastrointestinal microbial composition and the production of gastrointestinal digestive enzymes, but the proportional contribution of endogenous and exogenous enzymes in response to diet acclimation is unknown. In this study, I attempted to correlate trypsin and cellulase enzyme activity levels to diet by acclimating omnivorous goldfish to carnivorous- and herbivorous-based diets. Then, by testing for enzyme activity in the presence and absence of bacteria, I aimed to attribute changes in enzyme activity levels to either the microbe host or the bacteria residing in the gut. As with Chapter 2, I was able to confirm the presence or absence of bacteria in the tissues through gDNA extractions and PCR using universal bacterial primers, as seen in Figure 3.1. Overall, while the diet did indeed alter intestinal enzyme activities, the changes were not reflective of nutritional content in the diets, nor were they definitively attributable to microbe host or bacterial contributions.

3.4.2. Trypsin source and activity levels

Overall, when naturally omnivorous goldfish were fed an herbivorous diet, there was a negative impact on protease activity via elimination of trypsin activity in the intestinal epithelia (Figure 3.2), as well as in the whole intestine and chyme (Figure 3.3). Indeed, I had predicted such a reduction in trypsin activity with the herbivorous diet due to a decrease in the available dietary protein. These results are supported by previous studies that correlate trypsin activity to diet, with herbivorous fish having lower activity (German et al. 2004; Murashita et al. 2019). As trypsin activity in the omnivorous group was attributed solely to endogenous production (Figure 3.2; Chapter 2), the elimination of activity in these fish suggests that this was an endogenous

response to the herbivorous diet, despite prior evidence of the presence of proteolytic bacteria in goldfish (Liu et al. 2016). It is possible that because protein is a major nutrient (Sire and Vernier 1992) for the omnivorous goldfish, they rely on endogenous trypsin mechanisms alone, and the presence of proteolytic bacteria previously observed is unrelated to digestive performance. In fact, naturally omnivorous goldfish have developed adaptations that allow them to break down dietary proteins more efficiently to maximize amino acid absorption required for growth, such as longer intestines and food retention times (Hofer 1982; Hidalgo et al. 1999; German et al. 2010). Further, the lack of microbial trypsin in the current study could also reflect that the same fish species may contain different bacterial communities based on geographic and lab location (Givens et al. 2015; Lyons et al. 2017; Webster et al. 2018). The microbiome of the goldfish used in this study requires characterization to confirm or refute this possible explanation for a lack of microbial trypsin. Regardless, the sole endogenous production of activity in the goldfish (Figure 3.2; Chapter 2) as well as the central stoneroller (Chapter 2) but not the rainbow trout (Chapter 2) suggests that there may be a species-specificity to bacterial control of trypsin activity. Indeed, trypsin can be produced endogenously by the microbe host and exogenously by bacteria residing in the gastrointestinal tract across a variety of fish species (Cao et al. 2000; German and Bittong 2009; Grover et al. 2018; Chapter 2). The determinants of the gut microbial communities, how they are selected and shaped, is currently an area of intense research, however the exact controls are still unknown. They could reflect species-specific host determinants that have evolved to reflect feeding niches.

I also predicted that there would be enhanced trypsin activity with a carnivorous diet, but instead observed a reduction in activity in both the epithelial layers and the whole intestine (Figures 3.2; 3.3A; 3.3B). This is likewise concluded to be an endogenous response, as with the

herbivorous diet. These trends were different from the carnivorous rainbow trout, which displayed elevated trypsin activities in several tissues, which were attributed to both the microbe host and intestinal bacteria (Chapter 2). The reason for this contradiction in results is unclear. It is entirely possible that by altering the diets of the goldfish and examining them after only 21 days was not enough time to observe enhanced changes to trypsin activity levels. Various studies examining the effects of dietary manipulation on physiological processes, including gut microbiota and enzyme activity, acclimated fish to new diets for at least 48 days (Gajardo et al. 2017), approximately two months (Desai et al. 2012; Wang et al. 2016; Michl et al. 2019), or even longer (Gómez-Requeni et al. 2013; Leigh et al. 2018). Furthermore, changes in diet can induce morphological changes in the intestine (Wagner et al. 2009; Leigh et al. 2018) by shortening mucosal foldings (Heikkinen et al. 2006; Wang et al. 2016; Miao et al. 2018), making it more difficult for bacteria to attach to the enterocytes (Ringø and Gatesoupe 1998). Therefore, this can influence the abundance and diversity of the microbial communities within the gut (Miao et al. 2018), which can affect trypsin activity. With more time to adjust to the diets, maybe a change could have been detected, in particular an enhancement through bacterial contributions.

Interestingly, there was no impact on the specific activity in the chyme of goldfish fed a carnivorous diet (Figure 3.3C). Digestive enzymes are particularly active in the chyme in order to catabolize macromolecules for nutrient assimilation. Gajardo et al. (2017) found that the microbial population in the digesta of Atlantic salmon was richer than in the mucosa. The authors concluded that the mucosal microbiota is less dependent on diet, whereas microbiota associated with the digesta are specific to the ingested meal. Furthermore, bacteria that are present within the digesta may not be able to colonize the intestine, which may be why trypsin activity could not be detected in the intestinal epithelial layer of fish fed the non-omnivorous

foods (Figure 3.2; Gajardo et al. 2017). Overall, these studies suggest that the intestinal microbiota was altered as a result of a change in the diet of goldfish, and further studies, such as sequencing of the microbiome, are necessary to understand the extent and impact of this change.

Lastly, trypsin specific and total activities were, once again, detected in the intestinal muscles of goldfish fed a carnivorous diet (Figures 3.3E; 3.3F; Chapter 2), which is interesting because the intestinal muscle is presumed to be free of bacteria (Figure 3.1). This may be suggestive of endogenous trypsin mechanisms playing a role in trypsin production in this group of goldfish (German and Bittong 2009; Bower et al. 2011; Sumathi et al. 2011), but the muscle is not known to have digestive functions. Another possibility is that the method used for the mechanical separation of the enterocytes from the intestinal muscle may have left behind some enterocytes, and so further experimentation, including histological studies (e.g. H&E staining), are required to validate the effectiveness of this technique. However, it is unknown why activity was only observed in the muscle but not in the enterocytes or even the whole intestine. It is possible that trypsin activity in the whole intestine was very weak and it was deemed to be negligible.

3.4.3. Cellulase source and activity levels

Cellulase activity was reliably detectable only in the omnivorous goldfish, specifically in the saline washed epithelia (Figure 3.4A), whole intestine (Figures 3.5A; 3.5B), and chyme (Figure 3.5C; 3.5D), all tissues with bacteria. Further, ethanol eliminated cellulase activity in the omnivorous goldfish (Figure 3.4A). As discussed in Chapter 2, this is highly suggestive of exogenous cellulases from bacteria being active in the goldfish (Stickney and Shumway 1974; Ghosh et al. 2002; Saha et al. 2006; Liu et al. 2016) and further proof that fishes are unable to

endogenously produce cellulase (Saha and Ray 1998; Watanabe and Tokuda 2001; Karasov and Douglas 2013). Cellulase plays as crucial role in allowing the host animal to adequately extract polysaccharides from their diet (Ganguly and Prasad 2012; Karasov and Douglas 2013). As this is particularly important in herbivorous animals, I had predicted that goldfish acclimated to an algae-only diet would exhibit significantly elevated cellulase activities, especially in comparison to goldfish fed a carnivorous-based diet. While the carnivorous diet reduced cellulase activity, as predicted, the herbivorous diet also eliminated cellulase activity (Figures 3.4; 3.5). This is unlike the carnivorous rainbow trout and herbivorous central stonerollers, which demonstrated cellulase activity in the intestinal epithelia with bacteria (Figures 2.12; 2.14; Chapter 2), regardless of their respective dietary cellulose contents. It is unclear why no cellulase activity was observed in the goldfish fed herbivorous diets, as the main compound in their diets was cellulose. Once again, this may be due to the length of the acclimation period (Desai et al. 2012; Wang et al. 2016; Gajardo et al. 2017; Leigh et al. 2018; Michl et al. 2019) or intestinal morphological changes that make it difficult for bacteria to adhere to enterocytes (Ringø and Gatesoupe 1998; Heikkinen et al. 2006; Wang et al. 2016; Miao et al. 2018), as explained in Section 3.4.2.

Interestingly, cellulase specific activity was detected in the ethanol rinsed intestinal epithelial layer of goldfish fed a carnivorous-based diet (Figure 3.4A), as well as in the intestinal muscle of the goldfish fed an herbivorous diet (Figure 3.5E; but this disappeared in total activity analyses (Figure 3.5F)). This was unexpected, as activity in the ethanol washed epithelia and intestinal muscle suggests endogenous cellulase production because bacteria were not present (confirmed through PCR). This is the first instance of cellulase activity in the absence of bacteria in my studies and is refuted by multiple publications which show that vertebrates cannot endogenously produce cellulase themselves (Yokoe and Yasumasu 1964; Lesel et al. 1986; Saha

et al. 2006; Ganguly and Prasad 2012). A possible explanation could lay in contamination of the enzyme assay itself. Contamination may also explain some studies that have seemingly detected a fish's ability to produce endogenous cellulase (German 2009; Hlophe et al. 2014). The prevalence of bacteria in the environment can cause contamination at any stage. Further, a lack of amplifiable DNA does not mean that degraded DNA, and thus bacteria, were not present. However, as a negative control was used in each assay, contamination of the assay itself is not likely. Each reaction would have to be individually contaminated, perhaps by the consumables.

3.4.4. Lipase source and activity levels; enzyme activity measurements

As lipase activities could not be studied, I can only predict the results based on the nutritional composition of the different feeds used in this study (Table 3.1) and the available literature. I would predict that lipase activity would be enhanced in the herbivorous group and lowest in the carnivorous group (Chakrabarti et al. 1995; Leigh et al. 2018). The carnivorous diet has the lowest amount of crude fat (minimum 0.5%), therefore lipase activity would be limited to the substrate concentration and should not exceed those of goldfish fed the omnivorous and herbivorous diets. In contrast, the herbivorous diet has higher fat content, and thus goldfish fed this diet would demonstrate significantly greater lipase activity so that it could use the extracted fatty acids as an additional energy source. However, as my results from Chapter 2 suggest that diet may not shape lipase activity rates and that bacteria may not supply a significant proportion of the activity in goldfish, lipase activities could have remained similar across all samples.

Finally, once again, trends across specific and total enzyme activity levels were not consistent, again highlighting the importance of consistency and standardization in enzymatic

activity studies. This further exemplifies the inconsistencies that can exist because of confounding factors, such as gut morphology and protein content.

3.4.5. Conclusions

Altogether, these findings refuted my predictions that trypsin and cellulase activities would be particularly enhanced in the goldfish fed a carnivorous or herbivorous diet, respectively. It is clear that the gastrointestinal tract physiology of these goldfish was affected by the change in diets, however the effect, a near total elimination of digestive enzyme activity, was unexpected. It may be possible that the carnivorous- and herbivorous-based diets were incompatible with goldfish physiology, which resulted in a much less diverse, active, and effective gut microbiota, along with an inability of the microbe host to endogenously produce trypsin. However, this is unlikely, as the literature states that goldfish can feed on both animal and plant materials with no issues (Maier and Tullis 1984; Bandyopadhyay et al. 2005). Alternatively, the 21-day acclimation period may have been too short of a timeframe for any significant compensations to occur in the composition of the goldfish intestinal microbiota, as well as the microbe host enzyme production levels. Other studies acclimated fish to new diets for over two months before examining the effects on intestinal function (Desai et al. 2012; Wang et al. 2016; Michl et al. 2019), and so it is suggested to extend acclimation periods prior to examining digestive enzyme activities.

CHAPTER 4: CONCLUDING REMARKS AND FUTURE DIRECTIONS

4.1. Concluding remarks

The objective of this thesis was two-fold: first, to determine the contribution of the microbe host and gastrointestinal microbiota to digestive enzyme function, and second, to attribute any significant differences in enzyme activity levels to either host-specific factors or the diet. I concluded that digestive enzymes in teleosts are secreted by both the microbe host and the gut microbiota in a potentially species-specific manner. Specifically, trypsin and lipase were produced endogenously and exogenously (Chapter 2), whereas cellulase was only produced by microbes within the intestinal layers (Table 4.1; Chapter 2). Further, rainbow trout displayed endogenous production of lipase, but relied on bacterial trypsin secretions during digestion, while goldfish appeared to rely predominantly on microbe host production for both enzymes (Table 4.1), regardless of feeding status. Central stonerollers mostly relied on the endogenous trypsin and lipase secretions (Table 4.1). These trends were shown through the separation of the whole intestine into chyme (if fed), epithelial layers representing enterocytes that either contained bacteria or were rinsed of bacteria with ethanol, and the muscle. Interestingly, the effects of feeding were inconsistent across the species, as in some cases, feeding enhanced enzyme activity, whereas activity levels diminished in other cases upon feeding.

To determine if the observed trends were perhaps due to host-specific factors or in response to the differing diets of the rainbow trout, goldfish, and central stonerollers, omnivorous goldfish were acclimated to carnivorous and herbivorous diets. In this case, trypsin and cellulase activities were essentially abolished when goldfish were fed non-omnivorous foods. I concluded that both the intestinal microbiota and the microbe host's physiological response had been altered, as not even endogenous trypsin activity could be detected. However, I was unable to

attribute these changes and the differences between the rainbow trout, goldfish, and central stonerollers to one single factor (i.e. host-specific response or intestinal microbial composition), as further experimentation was required. Overall, it was determined that patterns of digestive enzyme activities were not necessarily associated with the trophic levels of the fishes, as originally predicted. Together, the findings from this study illustrate the effects of host-specific factors and diet on digestive enzymes and further highlight the importance of the gastrointestinal microbiota for proper digestive function. Furthermore, specific and total enzyme activity analyses revealed inconsistencies in current enzyme measurement techniques. Therefore, it was proposed to standardize enzyme activity measurements and analyses, so that accurate comparisons between the available literature could be made. This would also provide valuable information that considers possible confounding factors.

4.2. Perspective

This novel study provides an overview of the amount each layer of the intestine contributes to the specific and total enzyme activities observed in teleosts. These findings can elucidate the relationship between the gastrointestinal microbiota and the host species, which can provide means that allow for better overall fish health. Considering the important role aquatic animals play in fisheries and aquaculture, the findings from this thesis can provide information on how to improve the digestive functions of fishes, as they serve as an important nutrient source in the food chain. Moreover, information on the composition of the gastrointestinal microbiota and enzyme function can be used to formulate higher quality fish feed, including probiotics, to maintain and improve the health and survivability of fishes. Finally, understanding the control of digestive enzymes by the microbe host and bacteria can give us a glimpse at understanding how

and why these relationships evolved in vertebrates. Teleosts are excellent models for studying this, as they are the most diverse group of vertebrates (Ravi and Venkatesh 2008) and their microbiota can easily be manipulated and sampled (e.g. Xia et al. 2014; Zarkasi et al. 2016; Miao et al. 2018; Wei et al. 2018). Despite this knowledge, the extent to which the gastrointestinal microbial composition is dependent on host-specific factors is currently limited. Additionally, fish are often used as environmental indicator models (Burger et al. 2005; Plessl et al. 2017), therefore understanding their digestive physiology can be beneficial for identifying any pollutants or toxins in their environments (Parmar et al. 2016).

4.3. Limitations and future directions

Although this thesis aimed to examine several avenues relating to the intestinal microbiota and digestive enzymes in fish, there are still many things to consider and unanswered questions. The pyloric caeca, presumed to have digestive enzyme function (Buddington and Diamond 1986; Khantaphant and Benjakul 2008), was one part of the intestine where enzyme activities were not examined. Thus, trypsin, lipase, and cellulase activities in the pyloric caeca of rainbow trout should be studied to accurately attribute enzyme function to either the microbe host or gastrointestinal bacteria. Fagbenro et al. (2000) found enhanced digestive enzyme activity in the pyloric caeca of the African bony-tongue fish in contrast to other regions of the gastrointestinal tract. This can further reveal differences between the three species under study, as well as help with understanding the extent to which the goldfish and central stonerollers physiologically compensate, as they lack pyloric caeca.

Additionally, characterizing the intestinal microbiome of the three teleosts used in this study, as well as goldfish fed carnivorous- and herbivorous-based diets through genomic

sequencing can provide valuable information regarding the gut microbial composition. Comparisons between species and diet treatments can then offer explanations as to how the digestive enzyme contributions shift with differences in the intestinal microbiota. Further, experiments from Chapter 3 should be repeated with longer acclimation periods of at least 60 days or more (Desai et al. 2012; Gómez-Requeni et al. 2013; Wang et al. 2016; Leigh et al. 2018; Michl et al. 2019) to observe the full effects of diet composition on intestinal microbial shifts, and thus enzyme activity levels. Further, lipase activities should also be examined to get a better understanding of the lipase source. It would also be interesting to study the enzyme activities in the different layers of the intestines of teleosts at different temperatures. Temperature is known to affect the gastrointestinal tract microbiota (Trust and Sparrow 1974), and so measuring enzyme activities at 12°C, which is reflective of a normal fresh- and cold-water fish's environmental temperature, and again near the thermal optima for each species (Carline and Machung 2001; Chen et al. 2015), can reveal whether temperature has a significant impact on how much the microbe host versus bacteria contribute to the activity levels in each intestinal layer.

A limitation in the current study was that we can only infer but not quantify microbial contribution to enzyme function through the absence of activity. To gain better insight on the contribution of the gastrointestinal bacteria to the enzyme production in these fishes, the bacteria should be isolated and enzyme assays should be completed. To date, attempts at isolating and growing the entire gut microbiome have failed, as current isolation methods do not guarantee the complete removal of intact microbes to then accurately use for enzyme activity measurements. However, an advantage to the current study was that I was able to remove the bacteria without the use of antibiotics. Antibiotics could be used to treat the animals in order to completely rid the

intestines of microbes and further our understanding of endogenous and exogenous enzyme production. As exposing animals to antibiotics may have unwanted side-effects, including reduced growth (e.g. Carlson et al. 2015), higher mortality (e.g. Pindling et al. 2018), and repercussions (e.g. increased antibiotic resistance in waterways), my technique for easily and safely removing the bacteria with ethanol could provide an excellent option. It would be interesting if antibiotic treatments matched my measurements of bacteria removal with ethanol, thus confirming this technique and offering an alternative for future studies.

Table 4.1. General summary of microbe host versus bacterial contribution to digestive enzyme production in rainbow trout, goldfish, and central stoneroller.

Enzyme	Rainbow trout	Goldfish	Central stoneroller
Trypsin	Bacteria	Host*	Host*
Lipase	Host*	Host*	Host*
Cellulase	-	Bacteria	Bacteria

* Host refers to the microbe host (endogenously produced).

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