Assessing the effects of temperature, feeding status, and the microbiome on the maximal activities of several enzymes important to energy production and ammonia detoxification in the intestines of teleost fish

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Abstract

This thesis examined the effects of temperature, feeding status, and the microbiome on the maximal activities of several enzymes important to energy production and ammonia (NH_3) detoxification in the intestines of three species of teleosts: the goldfish, rainbow darter and central stoneroller. In warm-acclimated goldfish, intestinal and branchial tissues were more responsive to feeding, while renal tissues were not affected by digestion or thermal acclimation. This was reflected in the maximal enzyme activities of Na^+, K^+-ATPase (NKA), citrate synthase, pyruvate kinase, and glutamine synthetase (GS). Increased NKA activity following a meal suggested an increase in ATP demand, and amplified GS activity likely assists in enhanced NH_3-detoxification processes in the intestinal tract. There was a strong effect of host species on intestinal tract bacteria composition and on enzyme activities in the rainbow darter and central stoneroller, and sampling location was a significant determinant of the central stoneroller intestinal microbiome.

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Chapter 1: Introduction

The importance of fish

As an industry, fisheries and aquaculture were responsible for over 4.7 billion Canadian dollars (CAD) in gross output in the Canadian economy in 2017 (Canada 2017), and these industries employed over 76,000 Canadians (Canada 2017). Recreational anglers spent over 8 billion CAD in Canadian provinces and territories in 2010 on fishing trips and related items (Barrow et al. 2012). Fish are among the taxonomically and ecologically diverse of the vertebrates (Clements et al. 2014), and they occupy an important place in the food chain for human and animal alike. This value cannot be quantified in dollars.

The importance of fish digestive physiology

Understanding the piscine digestive system is important to both aquaculture and wild fish stocks. In particular, examining the role of digestive enzymes in nutrient absorption and assimilation has practical applications in the aquaculture industry, such as the improvement of commercial fish feed (Hidalgo et al. 1999; Furné et al. 2005; Krogdahl et al. 2015). Establishing baselines for digestive enzyme function in the absence of stressors in both wild and farmed fish can be of use for understanding how the application stressors, such as temperature, can alter the capacity for these enzymes to assist with nutrient catabolism and assimilation.

The digestive process

The digestive process requires the breakdown, or catabolism, of ingested food, in order to access and absorb the nutrients contained within. These functions are largely mediated by enzymes. For

example, nutrient transport into the cells of the intestinal tract (enterocytes) relies on electrochemical gradients, which can be created by enzymes such as Na^+, K^+-ATPase (NKA) (Poulsen et al. 2010). Once inside the enterocytes, those nutrients are converted into energy by other enzymatic pathways, such as glycolysis and the Krebs cycle. While beneficial, however, the digestive process can produce deleterious by-products, such as ammonia (NH_3). This occurs when proteins are broken down into their amino acid components. Fortunately, this NH_3 can be detoxified through the action of enzymes, such as glutamine synthetase (GS) and glutamate dehydrogenase (GDH).

Digestive enzymes

Na^+, K^+, ATPase (NKA)

This enzyme is an important ion exchanger situated on the basolateral membrane of the cell (Randall and Tsui 2002; Bucking et al. 2013; Wright et al. 2016). It acts by pumping three sodium (Na^+) ions out of the cytosol in exchange for two potassium (K^+) ions from the extracellular space, consuming one adenosine triphosphate (ATP) molecule as energy in the process (Poulsen et al. 2010). As mentioned above, NKA is critical to the digestive process, as the ionic gradient it creates facilitates Na^+-dependent secondary transport, enabling nutrients to enter the enterocytes, and it acidifies the stomach lumen, creating the optimal pH for other digestive enzymes to act (Poulsen et al. 2010). Teleost fish are ammoniotelic, meaning that aside from being able to detoxify digestion-produced NH_3, they can also excrete it directly through the gills in order to eliminate it (Bucking et al. 2013). The ammonium ion (NH_4^+) can replace K^+ in NKA, which allows NH_4^+ to enter gill cells for eventual excretion into the water (Randall and Tsui 2002).

Citrate synthase (CS)

CS is a critical enzyme in the regulation of aerobic energy production (Hochachka et al. 1970; Mommsen 1984), as it catalyses the initial step of the Krebs cycle (Cooper and Plum 1987) by converting acetyl coenzyme A (acetyl-CoA) and oxaloacetate into citrate and coenzyme A (Wiegand and Remington 1986). The rate at which CS catalyses this step is a reflection of the flux capacity of the cycle as a whole, and by extension, the ability of the organism to make energy efficiently (Schnurr et al. 2014).

Pyruvate kinase (PK)

As CS activity is a reflection of the organism's ability to generate energy aerobically, so the activity of PK reflects the ability of the organism to generate energy anaerobically (Mommsen et al. 1980; Mommsen 1984; Baumgarner et al. 2012; Schnurr et al. 2014). It catalyses the final step of glycolysis by converting phosphoenolpyruvate (PEP) to pyruvate and ATP (Ainsworth and MacFarlane 1973), acting as a rate-limiting enzyme in this process (Childress and Somero 1979; van den Thillart and Smit 1984; Bickler and Buck 2007; Baumgarner et al. 2012). PK levels can also be indicative of the organism's ability to use dietary carbohydrate as an energy source (Meton et al. 1999).

Glutamine synthetase (GS)

As mentioned above, the breakdown of proteins into amino acids results in the production of toxic NH_3 in the digestive tract. An increase in NH_3 levels can trigger cell death in neurons, initially affecting parts of the central nervous system locally, culminating in damage to the brain, and

eventually death (Svoboda et al. 2007). Another toxic effect of NH_3 is that it is alkaline, and when it accumulates in the cell, this results in a dramatic increase in pH. Cellular enzymes involved in protein catabolism optimally catalyze their reactions at an acidic pH, but these processes are inhibited at more alkaline pH levels (Campbell 1991). GS detoxifies this NH_3 that is generated during protein catabolism (Bucking and Wood 2012; Rubino et al. 2014) by incorporating NH_3 into a glutamate molecule to create glutamine (Webb and Brown 1976; Cooper and Plum 1987; Randall and Tsui 2002). Glutamine is significantly less toxic than ammonia (Bucking and Wood 2012), and is one of two major endpoints in ammonia detoxification.

Glutamate dehydrogenase (GDH)

GDH is another ammonia-detoxifying enzyme, incorporating ammonium (NH_4^+) into α-ketoglutarate to produce glutamate (Randall and Tsui 2002), the other major endpoint of ammonia detoxification. High levels of GDH activity in the liver can be indicators of elevated energy production through amino acid catabolism, or through the aerobic metabolism of carbon from the glutamate molecule (Mommsen et al. 1980). This may be a sign of the stress response, as has been observed in rainbow trout (Morales et al. 1990), as more fuel is required to sustain the increased metabolism that accompanies stress.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Transaminases are also key enzymes involved in ammonia detoxification. They also mediate the metabolism of amino acids, catalysing their conversion into an α-keto acid (pyruvate in the case of ALT, Jing and Zhang 2011; oxaloacetate in the case of AST, Cooper and Plum 1987), and a glutamate molecule (Cooper and Plum 1987) from a precursor α-ketoglutarate and an NH_3

molecule, making them important ammonia detoxifiers. Elevated activities of ALT and AST have been used as stress biomarkers in several fish species (see Chapter 2).

The contribution of intestinal tract bacteria to the digestive process

Enzymes that are measured in the GIT can be attributed to two sources; the host and the bacteria that populate the GIT. (Goodrich and Morita 1977b; Goodrich and Morita 1977a; Lesel et al. 1986; Sugita et al. 1991; Skrodenyte et al. 2008; Nayak 2010; Tapia-Paniagua et al. 2010; Mouchet et al. 2012; Ringø et al. 2012; Clements et al. 2014; Pelster et al. 2014; Ye et al. 2014). This is particularly important if the host does not produce these enzymes endogenously. In this case bacteria-produced, or exogenous, enzymes can allow the host to break down food items it would not otherwise be able to digest, such as chitin (Goodrich and Morita 1977a; Goodrich and Morita 1977b; Skrodenyte et al. 2008; Nayak 2010; Tapia-Paniagua et al. 2010; Ringø et al. 2012; Clements et al. 2014) and cellulose (Lesel et al. 1986; Nayak 2010; Mouchet et al. 2012; Pelster et al. 2014; Ye et al. 2014), making these energy sources available to the host. Intestinal bacteria have also been demonstrated to synthesize vitamin B_{12} in fish, such that the host does not require a dietary source of that vitamin (Sugita et al. 1991). This process is entirely mediated by exogenous bacterial enzymes. However, bacteria can also contribute to endogenously produced enzymes such as GS and GDH (ref). Such contributions may create commensal or mutualistic relationships between the bacteria and the host and may act to shape the bacterial communities.

The effect of temperature on fish and bacteria

Fish are ectotherms, meaning that they do not generate their own body heat, and as such, their biological functions, which are largely enzyme-driven, are temperature-dependent. Climate change, especially warming temperatures, has already triggered disruptions in seasonal events, and

has altered food availability and diversity within many ecosystems (Pörtner and Farrell 2008). Given that fish are ectothermic, increases in water temperature triggers increases in the metabolic rates of organisms within marine and freshwater ecosystems (Acuña et al. 2008). The survival of species under increasing thermal stress is dependent on their ability to acclimate to new temperature regimes (Schnurr et al. 2014). The microbiome is also sensitive to perturbations in the surrounding environment (Llewellyn et al. 2014), including temperature (Niemi and Taipalinen 1982).

Objectives

There were two main objectives of this thesis. The first was to determine the effect of temperature changes on the maximal activities of enzymes involved in nutrient assimilation, energy production and ammonia detoxification in the goldfish, *Carassius auratus* (Chapter 2). The second was to better understand how the environment influences the intestinal tract microbiome, and in turn, how the microbiome might assist carnivorous rainbow darter (*Etheostoma caeruleum*) and herbivorous central stoneroller (*Campostoma* anomalum) hosts in detoxifying intestinal ammonia generated during digestion (Chapter 3). Enzyme activities were measured according to established methods. Impacts of the environment on the microbiome were assessed using key ecological measures of species diversity, such as α-diversity (the bacterial species diversity within the fish host) and β-diversity (a comparison of the α-diversities *between* fish hosts).

Chapter 2: The interactive effect of digesting a meal and thermal acclimation on maximal enzyme activities in the gill, kidney, and intestine of goldfish (*Carassius auratus*)

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Abstract

Surrounding environmental temperatures affect many aspects of ectotherm physiology. Generally, organisms can compensate at one or more biological levels, or allow temperature to dictate processes such as enzyme activities through kinetic effects on reaction rates. As digestion also alters physiological processes such as enzyme activities, this study determined the interacting effect of thermal acclimation (8 and 20°C) and digesting a single meal on maximal enzyme activities in three tissues of the goldfish (*Carassius auratus*). Acclimation to elevated temperatures decreased branchial Na^+, K^+, ATPase (NKA) activity. In contrast, acclimation to elevated temperatures had no effect on citrate synthase (CS) or pyruvate kinase (PK) activity in any tissue, nor were renal NKA or glutamine synthetase (GS) activities impacted. Warm water-acclimation exaggerated the positive impact of digestion on intestinal and branchial NKA activities and intestinal GS activity only, but digestion had no effect in the kidney. CS and PK did not display intestinal zonation; however, there was a distinct increase towards the distal intestine in NKA and GS activities. Zonation of NKA was more prominent in warm-acclimated animals, while acclimation temperature did not affect intestinal heterogeneity of GS. Finally, the impact of tissue protein content on enzyme activity was discussed. I conclude that the intestine and gill of warm-acclimated goldfish exhibited an augmented capacity for increasing several enzyme activities in response to digestion while the kidney was unaffected by thermal acclimation or digesting a single meal. However, this amplified capacity was ameliorated by alterations in tissue protein content. Amplified increases in NKA activity may ultimately have implications for ATP demand in these tissues, while increased GS activity may beneficially increase ammonia-detoxifying capacity in the intestine.

Introduction

Generally aquatic ectotherms rely on the external environment to set their internal temperature. Accordingly, thermal conditions alter the physiology of teleost fish through direct and indirect impacts of temperature and exert influence on fish biology across multiple biological levels. Thermal conditions can directly affect enzyme activity (such as Na^+, K^+, ATPase (NKA) activity) through kinetic effects on catalytic rates to which Precht (1958) proposed several potential responses. First, overcompensation or positive adaptation may occur resulting in higher enzyme rates at colder temperatures compared to warmer temperatures. Second, perfect compensation results in the maintenance of functional rates regardless of thermal conditions. Third, partial compensation and fourth, no compensation, both result in a decrease in functional rates at colder temperatures to varying degrees. Finally, inverse compensation (or negative adaptation) decreases enzyme rates below those due to lower temperature effects alone. Compensation of several enzyme activities in gill, liver, kidney, and intestinal tissues have been recorded in numerous fish species (reviewed by Hochachka and Somero 2002). These compensatory responses likely counteract the impact of temperature on enzyme catalytic rates and hence improve cellular function at lower temperatures (e.g. Guderley 2004; Kinsey et al. 2011).

Feeding, and conversely fasting, also alter tissue enzyme activity, especially in the intestine (e.g. Bucking and Wood 2012; Hayes and Volkoff 2014). The vast majority of studies examining the fish intestine have focused primarily on brush-border membrane enzymes (BBMEs) that are associated with catabolizing consumed nutrients, such as disaccharidase, and alkaline phosphatase, and pancreatic enzymes, such as lipases (e.g. Krogdahl and Bakke-McKellep 2005; Furné et al. 2008; German et al. 2010; Ahmad et al. 2014; Hayes and Volkoff 2014). Nutrient assimilation and growth are supported by these final stages of digestion, and lower acclimation temperatures tend

to create lowered digestibility and efficiency (e.g. Bernreuther et al. 2013; Ahmad et al. 2014; Wu et al. 2015), possibly through effects on BBME and lipase activities (Kuz'mina and Gelman 1997). In contrast, examination of enzymes involved with key metabolic processes of the enterocytes themselves are rare (e.g. citrate synthase (CS) and pyruvate kinase (PK); Bélanger et al. 2002; Mommsen et al. 2003), despite evidence that temperature acclimation may alter cytosolic and mitochondrial enzymes involved with nutrient metabolism, including gluconeogenesis, lipogenesis, and protein synthesis (Peres and Oliva-Teles 1999; Van Ham et al. 2003). Additionally, these studies examined the long-term impacts of dietary manipulation on enzyme activity, not the immediate, postprandial impact of the process of digestion itself (Bucking and Wood 2012; Rubino et al. 2014). Finally, while previous studies have revealed postprandial integrated responses of the intestine, kidney, and gill (Bucking and Wood 2006; Bucking and Wood 2007; Bucking et al. 2010), integrated enzyme responses have not been examined.

Here I observed the impact of digesting a single meal on several key metabolic enzyme activities across tissues (intestine, kidney, and gill) in two seasonal temperature treatments, one to approximate winter and one to approximate summer in the temperate zone. Enzymes examined play either important roles in animal homeostasis and metabolism (NKA, CS, PK) or are detoxifying enzymes in the gastrointestinal tract hypothesized to ameliorate ammonia toxicity during digestion [glutamine synthetase (GS); Bucking and Wood 2012; Rubino et al. 2014]. Our hypothesis was that a lower acclimation temperature would result in higher enzyme activity levels to compensate for reduced enzyme catalytic capacity in each tissue. Postprandial enzyme activities in ectotherms are altered by thermal acclimation (Ahmad et al. 2014; Bolinger and Rodnick 2014), as are metabolism (Gillooly et al. 2001), foraging efforts and frequency, and food consumption (e.g. Buentello et al. 2000; Biro et al. 2007; Ahmad et al. 2014; Wu et al. 2015). Hence, I further

predicted that digesting a meal would increase enzyme activity disproportionately in warm-acclimated animals due to their higher metabolic demands.

Materials and Methods

Animal care

Goldfish (*Carassius auratus*; body weight 10.1 ± 0.9 g), of mixed sex were purchased from a commercial supplier (Big Al's; Toronto, Ontario, Canada) and held in 50L flow through tanks supplied with dechlorinated City of Toronto tap water and constant aeration. Animals were acclimated to laboratory water temperature (7.2 ± 0.1 °C; monitored daily) and were exposed to a light:dark cycle of 12 h:12 h. Animals were fed daily to satiation with commercial pellets (Wardley's Goldfish food, Hartz; Seaucus, NJ, USA), 3 h following the start of the light cycle and uneaten food was removed 1 h following feeding. The pellets contained crude protein (minimum 35%), fat (minimum 4%), fibre (maximum 3%) and 9% moisture. All experiments were conducted with approval from the Animal Care Committee at York University.

Temperature acclimation

Animals (~180 total) were divided equally into two thermal acclimation treatments to approximate temperate zone winter temperatures (T = 8 °C) and summer temperatures (T = 20 °C). Several tanks (N ~ 30) for each temperature treatment were established to avoid pseudo replication and during animal collection they were randomly sampled from different tanks. Water temperatures were maintained using flow-through water and submersion heaters, and water temperature was monitored and recorded daily. Water ammonia, nitrate, and nitrite levels were monitored in each temperature treatment for the duration of the experiment. Photoperiod and feeding were maintained as described above. Animals were acclimated to temperature treatments for 4 weeks in accordance with previous literature (e.g. Hazel 1972; Cossins 1977).

Sampling protocol and tissue collection

Following acclimation to the two temperature treatments, animals (N = 5) were removed and sacrificed [through immersion in MS-222 (Tricane mesylate; Western Chemical Inc., Ferndale, WA, USA)] for tissue collection immediately before the scheduled feeding (referred to as unfed animals). Immediately following sampling of the unfed fish, the acclimated animals were fed to satiation and sacrificed at 3, 6, 9, 12, and 24 h post-meal consumption (N = 5 per time point; referred to as fed animals). Animals were then subjected to fasting, and sacrificed after 3 days (N = 5) and 7 days (N = 5) of food deprivation. To obtain tissues, following sacrifice, the animals were dissected, and the intestinal tract was removed, weighed, and then freeze-clamped in liquid nitrogen. The intestinal tracts from fed fish were cleaned of any chyme (by squeezing the length of the intestinal tract with forceps) before weighing. Animals that had not fed were excluded from analysis. This sampling protocol was used to determine an optimal post-prandial time point for further sampling in the subsequent experiments.

Following determination of an optimal post-prandial time point, a second group of temperature acclimated animals were again sampled immediately before the scheduled feeding as before (N = 10; unfed) and then at a single time point following feeding (N = 10; 6 h post-feeding). The animals were sacrificed, dissected, and multiple tissues (gill, distal kidney, and intestinal tract) were removed, weighed, and then freeze-clamped in liquid nitrogen as before. Chyme was removed from the intestine (fed treatment only) as previously described.

To determine zonation of enzyme activity along the intestinal tract, a third group of temperature acclimated animals were sampled (N = 16) from each temperature-acclimation treatment 6 h following scheduled feeding. For this collection, the entire intestinal tract was removed, cleared of

chyme as before, and separated into seven equal divisions based on the length of the intestine from esophagus to rectum (referred to as esophagus, anterior intestine, ant/mid intestine, mid intestine, mid/post intestine, post intestine, and rectum). Due to the small size of the individual divisions, samples were pooled from two individuals to create a single sample. These pooled divisions were then freeze-clamped in liquid nitrogen as before.

Finally, ration consumption was determined in both temperature treatments by feeding animals to satiation as before and sampling immediately following cessation of feeding ($N = 5$ for each temperature acclimation). The body mass of the fish was determined and then the chyme was removed from the intestine, weighed, dried overnight at 65 °C and weighed again. Ration size was then determined as dried chyme mass % of fish body mass. These preliminary experiments revealed that cold-acclimated animals consumed ~0.6% body mass ration (0.59 ± 0.18; $N = 5$), while warm-acclimated animals consumed a ~2.0% body mass ration (2.01 ± 0.39; $N = 5$). To determine the effect of ration size on enzyme activity rates, a group of warm-acclimated animals were fed various ration sizes (0.5, 1, 1.5, 2%) and the intestine sampled as before at 6 h post-feeding ($N = 5$ for each ration size).

All tissues were transferred to −80 °C for storage until enzyme analysis.

Enzyme analysis

Obtained tissues were ground into a fine powder under liquid nitrogen and stored at −80 °C before analysis. Subsamples of this powder were weighed and then added to a homogenization buffer (see below for specific enzymes) and homogenized on ice using a modified motorized pestle in 1.5-ml

conical centrifuge tubes. Samples were then centrifuged (10,000 × *g* for 5 min) at 4 °C to pellet cellular debris. The remaining supernatant was used for subsequent enzyme and protein analyses.

Before running each enzyme assay described below, random subsamples of frozen tissues were pooled and homogenized. These homogenates were then used to test and verify that the assays were functioning (at maximal activity) through serial dilutions of homogenate, comparisons with known values from other species, as well as increasing the concentration of substrate to ensure no effect on maximal activity was observed. During measurement, each enzyme was measured separately, and was not suspended in homogenization buffer for >30 min before analysis, with the exception of CS, which was subjected to three freeze–thaw cycles before measurement.

NKA (EC 3.6.3.9) activity was measured in all tissues after homogenization of preweighed powdered tissue in 100 µl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and 25 µl of ice-cold SEID buffer (SIE buffer, 12 mM sodium deoxycholate, pH 7.3). NKA activity was subsequently determined using a microassay method (McCormick 1993; Bucking et al. 2013) with 1-mmol l^{-1} ouabain. Due to a lack of tissue, the NKA activity assay was run on the intestinal zonation samples of fed animals only.

For GS (EC 6.3.1.2) activity measurements, pre-weighed powdered tissue was added to four volumes of extract buffer (50 mM HEPES, pH 7.5, 50 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, and 50% glycerol). The homogenate was centrifuged for 5 min at 10,000 × *g* to obtain a supernatant for testing. Briefly, GS was measured by the glutamyl transferase assay as previously described (Bucking et al. 2013). The reaction mixture contained glutamine (6 mM), hydroxylamine (15 mM), ADP (0.4 mM), KH_2AsO_4 (20 mM), $MnCl_2$ (3 mM) and HEPES (50 mM; pH 6.7).

For all other enzymes, pre-weighed powdered tissue was added to four volumes of extract buffer [20 mM HEPES, pH 7.5, 1.0 mM EDTA, 0.1% Triton-X, 1 mM DTT (excluded for CS)] before enzyme analysis (assay methods according to Mommsen et al. 1980; Mommsen 1984). The homogenate was centrifuged for 5 min at 10,000 × *g* to obtain a supernatant for testing as before. CS (EC 2.3.3.1) was assayed in Tris–HCl buffer (75 mM pH 8.0) containing DTNB (5,5′-dithio-bis (2-nitrobenzoic acid); 0.1 mM) and oxaloacetate (OXA; 0.5 mM) and monitored at 412 nm. To measure PK activity (EC 2.7.1.40), the formation of ATP was coupled to the oxidation of NADH by the reaction of lactate dehydrogenase recorded spectrophotometrically at 340 nm. The reaction buffer was composed of imidazole (50 mM), KCl (100 mM), $MgSO_4$(10 mM), PEP (2.5 mM; omitted for control), ADP (5 mM), NADH (0.15 mM) and 3 U/mL lactate dehydrogenase, and its pH was adjusted with KOH to 7.5. Due to a lack of tissue, the GS, CS, and PK activity assays were run on the intestinal zonation samples of fed animals only.

Bradford protein assays were run on all homogenates to determine the total protein content (Biorad, Hercules, CA, USA). NKA activity is expressed as μmol ADP mg^{-1} protein h^{-1}. All other enzymes are expressed as U g^{-1} protein, where U = μmol substrate converted min^{-1} (using appropriate extinction coefficients for each substrate). To determine the effect of tissue protein content enzyme rates were also quantified as either μmol ADP mg^{-1} tissue h^{-1} or U g^{-1} tissue for NKA and all other enzymes, respectively.

All enzyme and Bradford protein assays were run in 96-well format on a BioTek microplate reader using Gen 5 software 4.6 (Fisher Scientific Company, Toronto, ON, Canada). All assays were carried out at 26°C to standardize between water acclimation treatments, and in accordance with previous literature (Webb and Brown 1976; Shankar and Anderson 1985; Hardewig et al. 2000;

McClelland et al. 2006; Bremer and Moyes 2011; Gao and Moyes 2016). All chemicals were obtained from Sigma Aldrich (MilliporeSigma; CA, USA). The activity of each enzyme was determined in triplicate for each replicate, and the average activities of these replicates was used for further data analysis.

Statistics

All data were first examined for normality and heterogeneity of variance. Comparisons of enzyme activity between temperature-acclimation treatments and feeding regimes was tested with a two-way ANOVA with acclimation temperature and feeding status (fed or unfed) or time post-meal as the main factors. If no significant interaction effect was found, the individual main factors were examined using multiple comparisons (Tukey's post hoc test). Zonation of enzymes within the intestine was tested with a one-way repeated measures ANOVA followed by a Tukey's post hoc test. A *t* test was used to compare between identical zones at different acclimation temperatures. The effect of ration size on enzyme activities was determined with a one-way ANOVA followed by a Tukey's post hoc test. A $p < 0.05$ was considered significant. Statistics were carried out using SigmaStat 3.0 (Systat Software Inc., San Jose, CA, USA).

Results

Tissue mass

The relative total mass (mg tissue mass g^{-1} fish mass) and the protein content (mg protein g^{-1} tissue mass) of all tissues were not significantly altered during any time point post-meal or during fasting (data not shown). Additionally, the two-way ANOVA failed to show a significant interaction between the examined factors of feeding status and temperature treatment. Hence, values for fed and unfed animals were combined for each temperature treatment (Table 1). The intestine showed a significant impact of acclimation temperature, with relative total intestinal mass increasing at the higher acclimation temperature ($p < 0.05$; Table 1). The relative total mass of the gill and the kidney were unaffected (Table 1). Temperature acclimation did not have a detectable significant impact on protein content of the intestine, kidney, or gill (Table 1), although the decrease in protein content of the intestine in warm-acclimated fish was close to significance ($p = 0.062$).

Time course of enzyme activity

Following the ingestion of a single meal, intestinal NKA activity increased in both thermal acclimation treatments (Fig. 1). There was no detectable significant interaction of treatments (feeding and temperature; $p = 1.00$), nor was there a detectable effect of acclimation temperature on activity ($p = 0.658$). There was a significant impact of time on intestinal NKA activity ($p < 0.001$) which appeared to peak between 6 and 9 h post-meal ingestion, as these time points were significantly elevated over unfed time points in both temperature acclimation treatments. There was no significant difference in NKA activity over 3 (72 h) and 7 days (168 h) post-feeding

compared to immediately preceding feeding (Fig. 1), suggesting values were not influenced by meal expectation.

Maximal enzyme activities

No significant effect of temperature treatment was detected in NKA activity in the intestine (Fig. 2a, $p > 0.05$), nor was there an interaction with feeding status ($p > 0.05$), hence, unfed NKA activity averaged approximately 2.68 ± 0.29 μmol ADP mg protein^{-1} h^{-1} across both temperature treatments. There was a significant effect of feeding ($p < 0.01$) and NKA activity in fed animals increased to 4.17 ± 0.31 μmol ADP mg protein^{-1} h^{-1} across acclimation temperatures ($N = 20$; Fig. 2a). Thus, in the intestine, digestion of a single meal resulted in a significant elevation of NKA activity at both temperature treatments, with a larger increase (2.1- vs. 1.3-fold) for the warm-acclimated fish (Fig. 2a). In comparison, the kidney also showed no significant effects of temperature treatment on NKA activity in unfed or fed animals. In addition, there was no effect of feeding status, nor was there an interaction effect detected between treatments. Hence, NKA activity averaged 7.41 ± 0.54 μmol ADP mg protein^{-1} h^{-1} ($N = 40$) across all treatments (Fig. 2b). Branchial NKA activity in unfed cold-acclimated controls was 3.01 ± 0.35 μmol ADP mg protein^{-1} h^{-1}, and the activity decreased significantly ~1.7-fold to an activity of $1.78 \pm$ μmol ADP mg protein^{-1} h^{-1} in unfed warm-acclimated animals ($p < 0.001$; Fig. 2c). Digestion of a single meal also resulted in a significant 1.8-fold increase in branchial NKA activity in the warm-acclimated animals while cold-acclimated animals exhibited a 1.3-fold increase during digestion (Fig. 2c). There was no significant interaction effect detected by the two-way ANOVA ($p = 0.64$).

When maximal NKA activities were examined based on tissue mass (Fig. 2d–f), impacts of feeding observed were similar to those seen when based on protein concentration (Fig. 2a–c), with activity significantly increasing in the fed group for both the intestine and gill while the kidney was not

affected. However, due to the slightly lower intestinal protein content in warm-acclimated animals (Table 1), a novel effect of temperature was observed with warm-acclimated animals displaying significantly lower NKA rates while the magnitude of increase following feeding was decreased from 2.1- to 1.7-fold (Fig. 2d). Due to the slightly higher gill protein content in warm-acclimated animals (Table 1), the impact of temperature was eliminated in the gill (Fig. 2f).

Acclimation to cold water temperatures did not affect CS activity in the intestine, kidney, or gill tissue ($p > 0.05$; Fig. 3a–c). Intestinal CS activity averaged 16.3 ± 2.2 U g^{-1} protein ($N = 40$; Fig. 3a), while renal CS rates averaged 13.9 ± 2.1 U g^{-1} protein ($N = 40$; Fig. 3b) and branchial rates averaged 16.4 ± 2.2 U g^{-1} protein ($N = 40$; Fig. 3c) across thermal treatments. Examining CS activity based on tissue mass slightly, but non-significantly, decreased intestinal CS activity in the warm-acclimated animals (Fig. 3d) while there was a slight but non-significant increase in gill CS activity (Fig. 3f) resulting in a similar lack of significant effect of either temperature or feeding.

Similarly to CS, but in contrast to NKA, there was no effect of acclimation temperature on PK activities in all tissues of unfed animals examined ($p > 0.05$; Fig. 4a–c), suggesting perfect compensation in PK activity with altered thermal conditions. No effect of acclimation temperature was also observed when PK activities were quantified based on tissue weights ($p > 0.05$; Fig. 4d–f). Digestion of a single meal did not affect PK activity in the kidney or the gill ($p > 0.05$; Fig. 4b, c) regardless of quantification method (Fig. 4e, f). However, similar to NKA activities, there was a significant effect of digestion on PK activity in the intestine ($p < 0.01$). PK activity in this tissue increased 1.5-fold (from 84.3 ± 13.6 to 128.8 ± 13.9 U g−1 protein) during the digestion of a meal in warm-acclimated animals, but only 1.2-fold in cold-acclimated animals (Fig. 4a). This increase was similar between the treatments (1.35-fold in cold-acclimated animals, 1.40-fold in warm-acclimated animals) when PK rates were quantified based on tissue mass ($p < 0.05$; Fig. 4d).

There was once again no significant effect of acclimation temperature on the intestine ($p > 0.05$), with GS activity averaging 6.91 ± 1.32 U g−1 protein across treatments ($N = 20$). However, similarly to NKA and PK, digesting a single meal resulted in a significant ~2-fold elevation of intestinal GS activity in the warm-acclimated fed fish, along with a significant ~1.3-fold activity increase in cold-acclimated animals ($p < 0.01$; Fig. 5a). Feeding significantly increased intestinal GS activity (U g−1 tissue), however, the increase in the warm-acclimated animals was blunted (1.6-fold increase) compared to the cold-acclimated animals (1.4-fold increase; Fig. 5b). The kidney again showed no significant effects of temperature treatment or feeding status on GS activity (average rate of 4.14 ± 0.37 U g−1 protein across treatments, $p > 0.05$; $N = 40$; Fig. 5b) a trend that was maintained regardless of quantification method (Fig. 5e).

When examining the branchial GS values (U g−1 protein), there was a significant effect of temperature ($p = 0.045$), a significant effect of feeding status ($p = 0.012$), as well as a significant interaction effect ($p = 0.032$). As such it is difficult to ascribe changes in individual treatments to either of the main factors (Fig. 5c). The branchial GS rates (U g−1 tissue) exhibited the same trends, despite a slight increase in activity observed in warm-acclimated animals (Fig. 5f). Hence, branchial GS activity is determined by both feeding and acclimation temperature as well as a combination of the two regardless of quantification method.

Zonation and impact of ration size

Zonation of NKA activity along the intestine of fed goldfish showed a proximal–distal gradient with increasing values found towards the posterior intestine compared to the esophagus at both acclimation temperatures (Fig. 6). However, the magnitude of change was greater in the fed, warm-acclimated animals, showing a 2.9-fold increase between the minimal and maximal activities observed in warm-acclimated animals, compared to a 1.6-fold increase in cold-

acclimated animals (Fig. 6), however this effect was again blunted (2.0-fold increase) when enzyme rates were determined per gram tissue (data not shown). Additionally, values in the rectum fell from the previous section and were not significantly different from the esophagus in both temperature treatments (Fig. 6). Decreased acclimation temperature significantly increased NKA activity in the nearly all portions of the intestine, except the mid and posterior intestine (Fig. 6). GS activity also increased towards the distal potions of the intestine of fed goldfish, however activity in the rectum was maintained in contrast to NKA activity (Fig. 7). Additionally, unlike NKA activity, there was no impact of acclimation temperature on GS activity in any section of the intestine of fed animals (Fig. 7). There was no evidence of zonation for CS or PK along the goldfish intestine in either temperature treatment following digestion (data not shown). Due to a lack of tissue, unfed animals were not tested. Maximal intestinal enzyme activities were maintained regardless of ration size for all enzymes examined (Table 2).

Figures and Tables

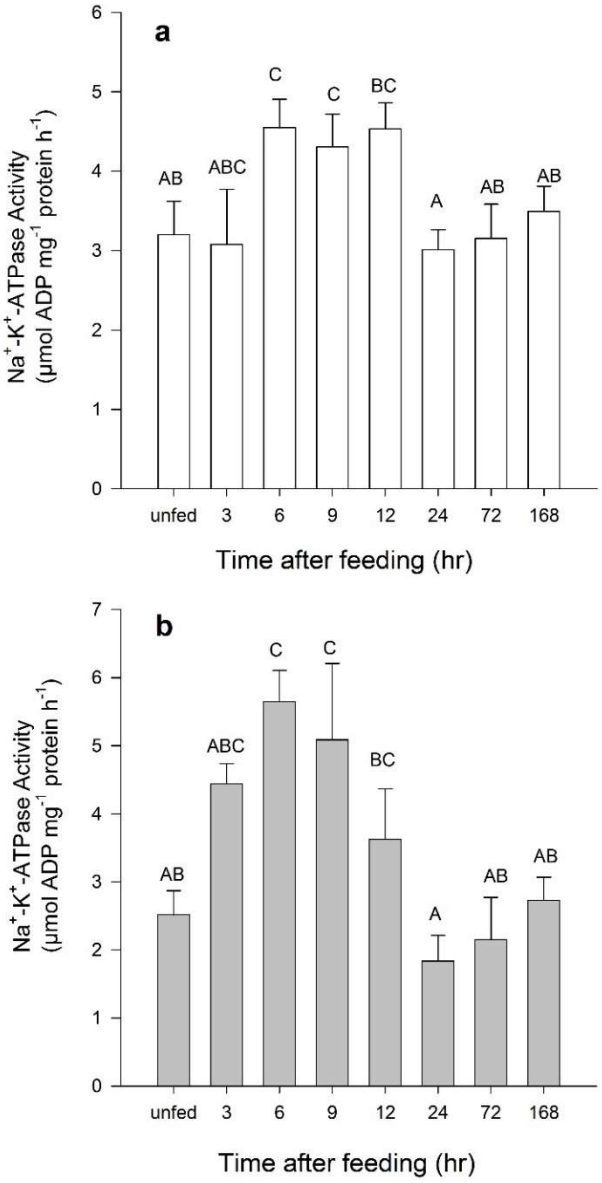

Fig. 1 Time course of whole intestinal Na^+, K^+, ATPase (NKA) activity (µmol ADP mg^{-1} protein h^{-1}) immediately preceding a meal (unfed) and following the ingestion of a single meal (3 h post-ingestion to 168 h (7 days) post-ingestion). a White bars are fish acclimated to 8 °C. b Gray bars are fish acclimated to 20 °C. Values are means ± SEM. Bars that share letters are not significantly different ($p < 0.001$ time point after feeding; $p = 0.851$ acclimation temperature; $p = 0.899$ interaction between time point and acclimation temperature). N = 5 in each treatment.

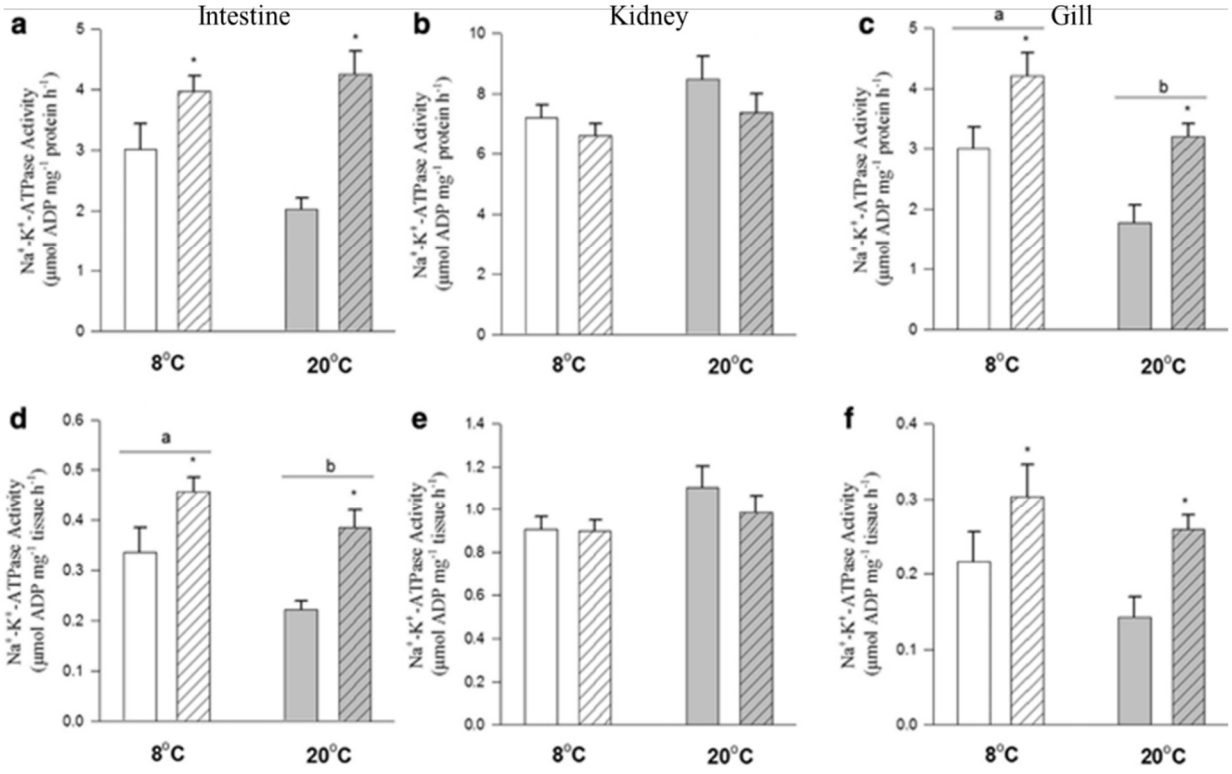

Fig. 2 Na^+, K^+, ATPase (NKA) activity (µmol ADP mg^{-1} protein h^{-1}) in response to acclimation temperature and feeding status in the a) Intestine, b) Kidney, and c) Gill, and expressed as µmol ADP mg^{-1} tissue h^{-1} in d) Intestine, e) Kidney, and f) Gill. White bars are fish acclimated to 8°C. Gray bars are fish acclimated to 20°C. Solid Bars are unfed animals, hatched bars are from fed animals (6 hrs post-ingestion). Values are means + SEM. Within each panel, a * indicates a significant effect of feeding, lines and letters indicate a significant effect of acclimation temperature. Statistical testing did not reveal a significant interaction between parameters for any tissues. ($p < 0.05$). N=10 per treatment.

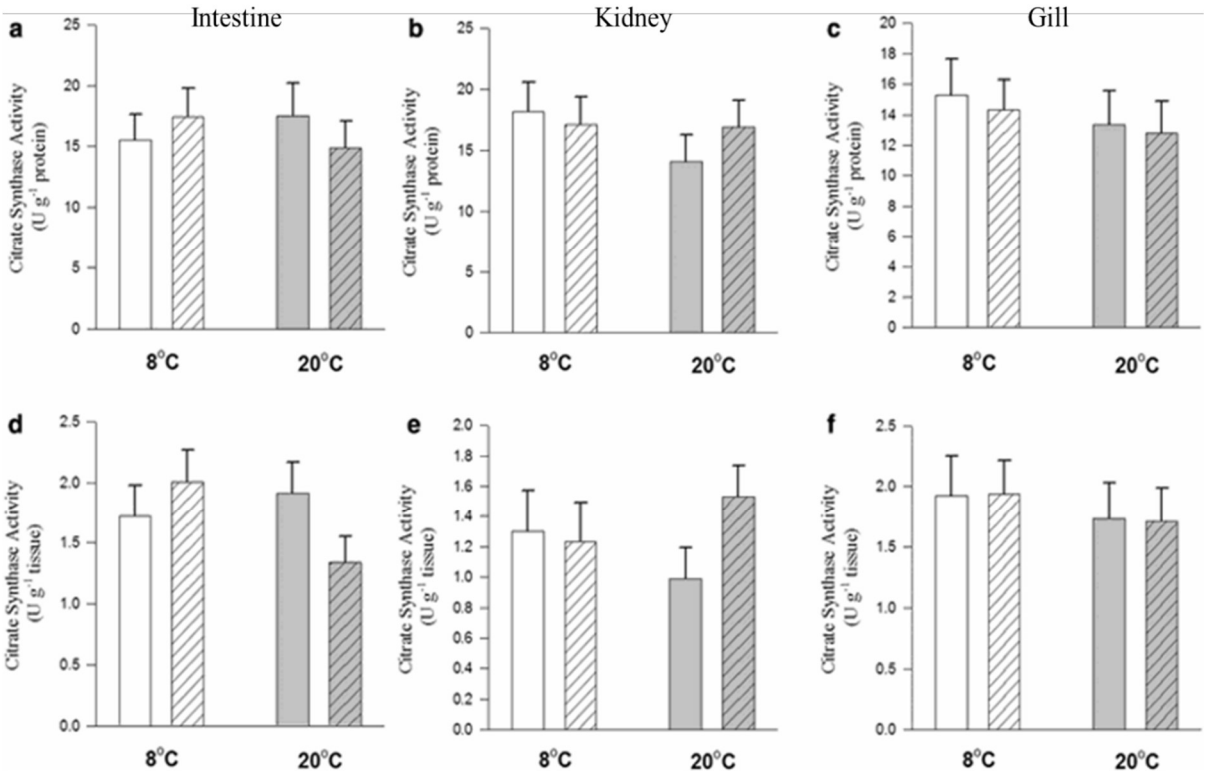

Fig. 3 Citrate Synthase (CS) activity (U g^{-1} protein) in response to acclimation temperature and feeding status in the a) Intestine, b) Kidney, and c) Gill, and expressed as U g^{-1} tissue in d) Intestine, e) Kidney, and f) Gill. White bars are fish acclimated to 8°C. Gray bars are fish acclimated to 20°C. Solid Bars are unfed animals, hatched bars are from fed animals (6 hrs post-ingestion). Values are means + SEM. Within each panel, lines and letters indicate a significant effect of acclimation temperature. Statistical testing did not reveal a significant interaction between parameters or effect of feeding for any tissues. ($p < 0.05$). N=10 per treatment.

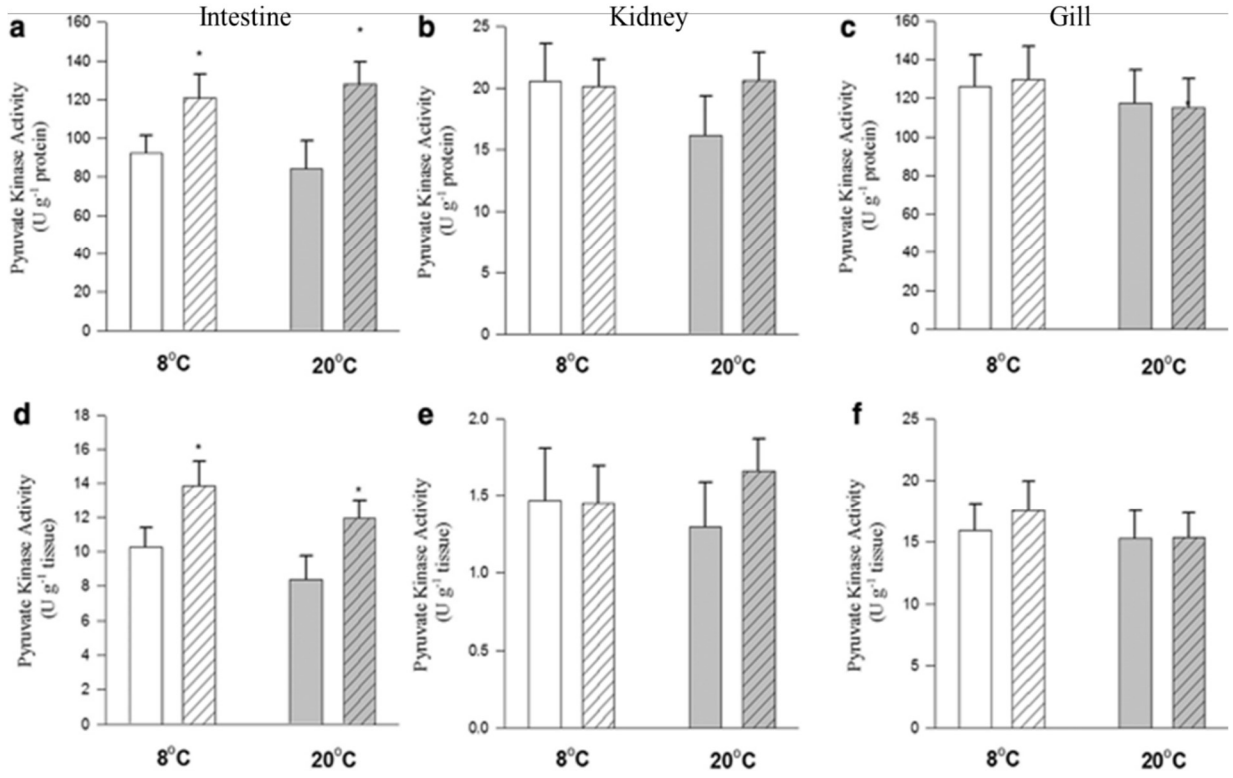

Fig. 4 Pyruvate Kinase (PK) activity (U g^{-1} protein) in response to acclimation temperature and feeding status in the a) Intestine, b) Kidney, and c) Gill, and expressed as U g^{-1} tissue in d) Intestine, e) Kidney, and f) Gill. White bars are fish acclimated to 8°C. Gray bars are fish acclimated to 20°C. Solid Bars are unfed animals, hatched bars are from fed animals (6 hrs post-ingestion). Values are means + SEM. Within each panel, a * indicates a significant effect of feeding. Statistical testing did not reveal a significant interaction between parameters or effect of acclimation temperature for any tissues. ($p < 0.05$). N=10 per treatment.

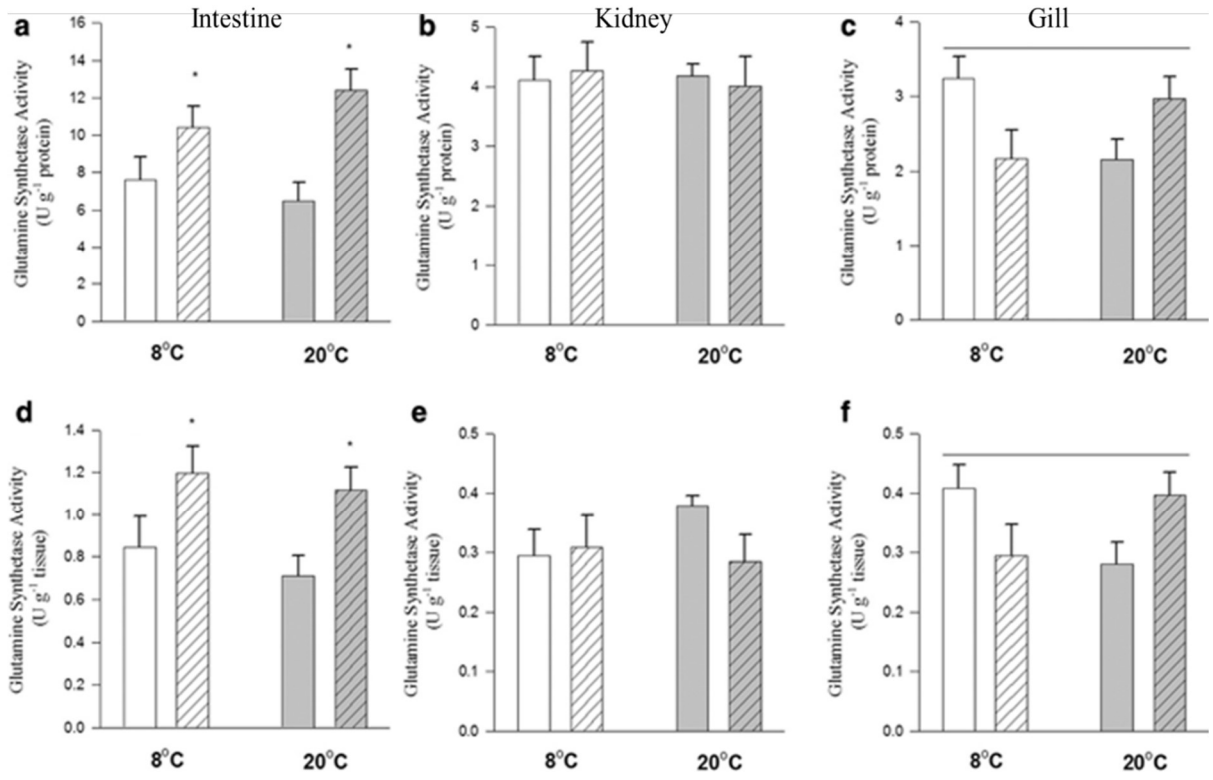

Fig. 5 Glutamine Synthetase (GS) activity (U g^{-1} protein) in response to acclimation temperature and feeding status in the a) Intestine, b) Kidney, and c) Gill and expressed as U g^{-1} tissue in d) Intestine, e) Kidney, and f) Gill. White bars are fish acclimated to 8°C. Gray bars are fish acclimated to 20°C. Solid Bars are unfed animals, hatched bars are from fed animals (6 hrs post-ingestion). Values are means + SEM. Within each panel, a * indicates a significant effect of feeding. Statistical testing did not reveal a significant interaction between parameters, or an effect of thermal acclimation for either the intestine or kidney. However, a significant interaction effect was detected in the gill (shown by line). ($p < 0.05$). N=10 per treatment.

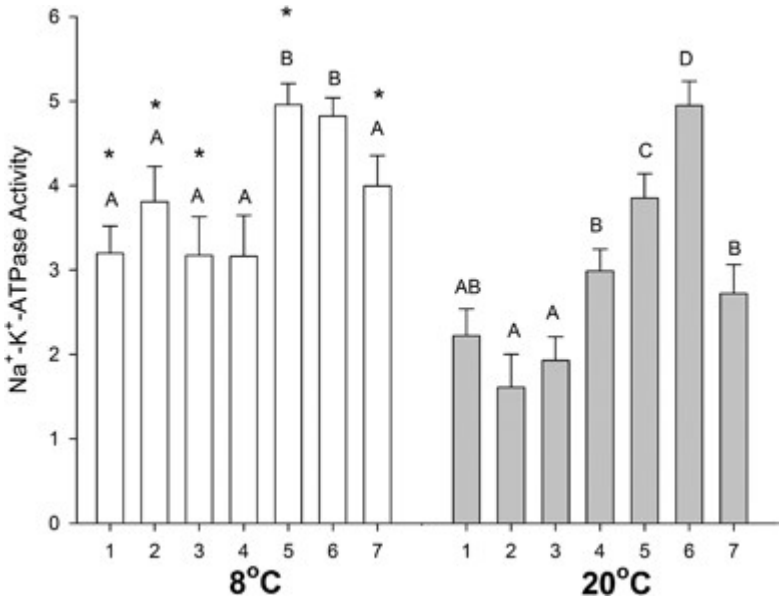

Fig. 6 Zonation of Na^+, K^+, ATPase (NKA) activity (μmol ADP mg^{-1} protein h^{-1}) in response to acclimation temperature in 7 sections of the intestine from fed animals (6 hrs post-ingestion). 1) Esophagus, 2) Anterior intestine, 3) Anterior/Mid intestine, 4) Mid intestine, 5) Mid/Posterior intestine, 6) Posterior intestine, and 7) Rectum. Within each temperature treatment, bars that share letters are not significantly different according to a repeated-measures ANOVA. * indicate a significant difference from similar section at 20°C according to a *t*-test. Values are means + SEM. N=4 per treatment.

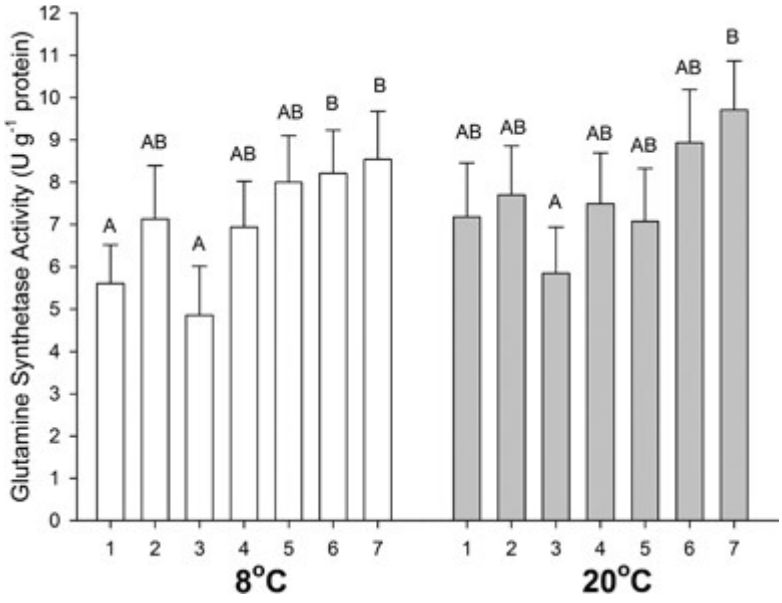

Fig. 7 Zonation of Glutamine Synthetase (GS) activity (U g^{-1} protein) in response to acclimation temperature in 7 sections of the intestine from fed animals (6 hrs post-ingestion). 1) Esophagus, 2) Anterior intestine, 3) Anterior/Mid intestine, 4) Mid intestine, 5) Mid/Posterior intestine, 6) Posterior intestine, and 7) Rectum. Within each temperature treatment, bars that share letters are not significantly different according to a repeated-measures ANOVA. There were no significant differences between temperature treatments according to a *t*-test. Values are means + SEM. N = 8

Table 1 Effect of temperature on the relative tissue mass (mg tissue mass g^{-1} fish mass) and tissue protein content (mg protein g^{-1} tissue mass) for the intestine, gill and kidney of goldfish. Values were combined across fed and unfed animals for each acclimation temperature treatment.

Temperature	Relative intestine mass	Intestine protein content	Relative gill mass	Gill protein content	Relative kidney mass	Kidney protein content
8°C	0.95 ± 0.06	114.96 ± 9.28	0.39 ± 0.05	74.49 ± 5.63	0.04 ± 0.01	128.98 ± 10.38
20°C	1.34 ± 0.04*	100.32 ± 5.46	0.45 ± 0.06	81.81 ± 4.18	0.04 ± 0.01	131.13 ± 9.45

*Significant difference between temperatures
Values are means ± SEM. N = 20.

Table 2 Effect of ration size on maximal intestinal enzyme activities

Enzyme	Ration level (% of total body mass)			
	0.5	1	1.5	2
NKA	3.78 ± 0.69	3.84 ± 0.69	4.29 ± 0.40	3.80 ± 0.64
CS	13.993 ± 2.43	14.42 ± 1.89	14.14 ± 1.33	15.435 ± 1.04
PK	121.12 ± 12.63	129.22 ± 12.57	127.25 ± 11.41	138.0 ± 12.46
GS	12.38 ± 1.44	12.36 ± 2.87	11.01 ± 1.14	12.68 ±2.03

Values are means ± SEM. N = 5
NKA activities are expressed as μmol ADP mg^{-1} protein h^{-1}
No significant differences were detected between ration sizes (one way ANOVA)
CS, PK, GS activities are expressed as U g^{-1} protein

Discussion

Our results showed that following a single meal, the gill and intestine exhibited an increased capacity for digestion-related increases in several enzyme activities in warm-acclimated animals, however, this augmented response was reduced, although not eliminated, when enzyme activities were calculated based on tissue mass and not protein content. Regardless, the increase in activity was not driven by a larger consumed ration, as activity rates were unaffected by meal size. Additionally, unlike the gill and intestine, the kidney showed almost no effect of thermal acclimation or digesting a single meal, revealing that the tissue undergoes perfect compensation to temperature treatments and may be primed to accommodate any prandial demands successfully.

While increased intestinal protein degradation (Houlihan et al. 1988) and decreased fractional rate of protein synthesis (McMillan and Houlihan 1989) occur in well-nourished fish during early phases of fasting, the interval between consuming a single meal did not alter the protein content of tissues (Table 1) in the current study. However, while the protein content of the warm-acclimated fish intestine was not significantly lower than that of the cold-acclimated animals (Table 1; $p = 0.062$), when enzyme activity was quantified per gram tissue mass, there was an amelioration of the augmented response to feeding observed for both NKA and GS (Figs. 2a, 5a) driven by this slight reduction in protein content. As warm-water acclimation increased total relative intestinal mass (Table 1), this suggests an increase in intestinal tissue water content, as previously observed (Kitchin and Morris 1971; Groot et al. 1983) could be driving the relative decrease in the fed, warm-acclimated animals (compared to rates quantified per gram of protein). Intestinal regulation of enzyme activity has been previously attributed to tissue level control of total amount of enzyme expressed through organ-mass alterations rather than genetic or proteomic

level regulation (Krogdahl and Bakke-McKellep 2005; German et al. 2010; Baumgarner et al. 2013; Hayes and Volkoff 2014).

Covalent regulation, such as phosphorylation, is another means by which enzyme activities are modulated (e.g. Petersen et al. 1987; McDonough and Farley 1993; Poulsen et al. 2010) and while not investigated in the current study, may offer a possible explanation for the difference (or lack thereof) in enzyme activities observed here. Several enzymes may also be regulated on a genetic level in response to feeding status (Baumgarner et al. 2012, 2013; Hayes and Volkoff 2014), at least in response to short- and long-term fasting (Bucking and Wood 2012). Importantly, the current study examined the maximal enzyme activity at an assay temperature that was higher than either acclimation temperatures. Thus, differences in activity must be interpreted cautiously and may not be representative of activities in vivo, but instead represent the maximal capacity at a set temperature. As well, the entire intestinal tissue was assayed, tissue that includes enterocytes but also smooth muscle cells. Hence, alterations in enzyme activity cannot be attributed solely to absorptive intestinal cells. Finally, it is also noteworthy that levels of enzyme activities were assessed only following feeding at a single time point. While it reflected the peak activity time for intestinal NKA (Fig. 1), it is possible that other enzymes may be regulated on a different time scale, both within the intestine and among the other tissues. Indeed, a lack of substrate availability to the kidney may be reflected in the lack of enzymatic responses observed, however as branchial responses were noted this indicates that substrates were available for some extra-intestinal tissues. Any compensatory response will likely be slower at reduced acclimation temperatures; however, intestinal NKA activity would suggest that this may not be true for all enzymes (Fig. 1).

NKA

The current study supports previous observations (Smith and Ellory 1971; Paxton and Umminger 1983) of compensation of NKA activity in the kidney of cold-acclimated goldfish (Fig. 2b). Additionally, previous studies suggest increased branchial sodium absorption in cold-acclimated animals (Maetz 1972) is supported by overcompensation in NKA activity at colder acclimation temperatures (Jurss et al. 1987) as is observed in the current study (Fig. 2c). Despite this compensation and overcompensation respectively, cold-acclimation of goldfish reduced sodium plasma levels (Catlett and Millich 1970; Mackay 1974), indicating that this compensatory mechanism was not sufficient to maintain ion homeostasis. Consuming a meal further increased branchial NKA activity (Fig. 2c) suggesting the incapability to maintain sodium balance may not be due to an inability to further increase NKA activities; however, this ability may be dependent on nutrition status. In contrast to playing a role in ion regulation, an upregulation in NKA activity in the unfed intestine (Fig. 2a; Smith and Ellory 1971) of cold-acclimated goldfish may represent an upregulation in resting nutrient absorption capacity. Indeed, rates of valine absorption in the goldfish intestine increase in cold-acclimated animals (Kitchin and Morris 1971), which if occurring through Na^+-linked amino acid transporters such as B^0 system (Broer 2008), would rely on Na^+-dependent symport and hence Na^+ gradients established by the NKA.

The intestinal tract and its associated organs can account for up to 40% of an animal's metabolic rate (Cant et al. 1996), hence any changes that reduce energetic costs, perhaps through reduced NKA activity (e.g. Webb and Brown 1980), may be important for survival. These morphological (Montgomery and Pollak 1988; Hall and Bellwood 1995; German et al. 2010; Zeng et al. 2012), and enzymatic (Krogdahl and Bakke-McKellep 2005; Furné et al. 2008; Zeng et al. 2012)

changes occur in as little as 2 days following the cessation of feeding (Krogdahl and Bakke-McKellep 2005), resulting in a decrease in both oxygen consumption and metabolism (e.g. Yang and Somero 1993; Zeng et al. 2012), and therefore, energetic savings. In the short-term interval between meals, downregulating the activity of the NKA (Figs. 1, 2a, c; Jurss et al. 1987; Xu et al. 2016), not only in the intestine but also gill, may reflect adaptive reductions in energetic costs.

Metabolic enzymes

Several examples of both cytosolic and mitochondrial metabolic enzymes were investigated in this study. Overall, CS activity appears insensitive to the effects of digesting a single meal or thermal acclimation in the current study (Fig. 3a–c). Fasting had no impact on CS activity in the intestine of the Nile tilapia, with the exception of a single proximal portion (Mommsen, Osachoff, et al. 2003). Bélanger et al. (2002) also showed that CS activity in the intestine of the Atlantic cod was decreased after >1 month of fasting, but not over short term intervals between meals. Finally, the calculated Q10 of CS across eight species of teleosts is ~1.60, indicating that temperature acclimation may not influence this mitochondrial enzyme (Driedzic 1992; Schaarschmidt et al. 1999).

In the current study, PK was not responsive to temperature treatment in any tissue; however, there was a significant response to digesting a meal (Fig. 4). Hepatic PK was likewise not altered by acclimation to environmental temperature in the sea bream or sea bass (Enes et al. 2006, 2008). In contrast, muscle PK activities responded in a positive manner to colder rearing temperatures in larval zebrafish (Schnurr et al. 2014), suggesting the potential of tissue- and/or species-specific regulation. Goldfish have a low glycolytic capacity compared to other fish (van den Thillart and Smit 1984), but compensate with a high glycogen content. Decreasing

acclimation temperatures induced a rise in glycogen content and rate of glycogen synthesis in the closely related crucian carp (Varis et al. 2016), the authors concluding that lowering water temperature is the main trigger in preparation for winter anoxia in this species. Compensation of PK in the cold-water acclimated animals (Fig. 4) may reflect this process.

Glutamine synthetase

It has been suggested that enterocyte GS is involved in detoxifying the large ammonia load created in the intestine during digestion (Bucking and Wood 2012; Rubino et al. 2014); correspondingly, intestinal GS activity increased during the digestion of a single meal (Fig. 5a, d; Bucking and Wood 2012). Interestingly, ammonia excretion rates positively correlate with increasing environmental temperatures in fish species (e.g. Maetz 1972; Luo and Xie 2009; Frisk et al. 2013; Bucking 2017) reflecting higher rates of deamination of amino acids to fuel increased metabolic rate (e.g. Luo and Xie 2009; Frisk et al. 2013). Excretion of the increased ammonia load at the gill and kidney would suggest that brachial and renal GS activities may also increase with acclimation temperature, to avoid cellular toxicity during excretion. However, renal GS activities were not affected by thermal acclimation or digestion (Fig. 5b) and branchial GS activities displayed an interactive effect of both thermal conditions and feeding status (Fig. 5c). Finally, transcellular branchial ammonia transport is driven in part by the NKA in fish gills (reviewed by Wright and Wood 2009). Hence, sodium gradients in the gill are important for ammonia excretion rates and the increased branchial NKA activity (Fig. 2c) may not only relate to ion transport directly, but also to increased ammonia excretion during digestion (Bucking and Wood 2010).

Zonation and impact of ration size

GS and NKA displayed a proximal–distal increase in activity (rainbow trout; Almansa et al. 2001; Bucking and Wood 2012; Sullam et al. 2015), however, only the NKA activity was affected by temperature treatment, exaggerating the zonation (Figs. 6, 7). Previous studies have correlated GS zonation with increasing luminal ammonia concentrations during the digestion of a meal (Rubino et al. 2014) and scanning electron microscopy (SEM) has revealed morphological differences along the goldfish intestinal tract (Caceci 1984), which correlate to the differences in enzymatic activities along its length. How temperature acclimation affects zonation of nutrient absorption and digestion and if they may be predicted by changes in NKA and GS zonation (Figs. 6, 7), remains to be investigated. Mommsen et al. (2003) established the local heterogeneity of several enzyme activities in the intestine of teleost fish, and that large species differences exist, confirming earlier studies examining BBMEs (Harpaz et al. 2005a, b). In particular, CS showed distinct zonation along the intestine of the trout (with highest levels at the proximal portions), but this appears to be species-specific (Mommsen et al. 2003; Bélanger et al. 2002) as it was lacking in the goldfish. In the current study, PK also showed a lack of heterogeneity along the goldfish intestine, despite such being observed in other species (Mommsen et al. 2003).

The augmented increase in enzyme activity levels of warm-acclimated fed animals (Figs. 2a, c, 4a, 5a) were likely not in response to an elevated ration consumption (Table 2). This possibly reflects a lack of graduated response of these enzyme activities in response to meal size, and instead represents a standard response of consuming a meal at these ration sizes. This has been previously observed for other aspects of digestion such as feed efficiency, protein efficiency

ratio, and retention of protein and digestibility of nutrients for fish fed 1–2.5% body mass rations, however, these parameters were altered when feeding levels were increased above 3% body mass ration (e.g. Abbas and Siddiqui 2009).

It was hypothesized that higher enzyme activity levels would serve as a compensatory mechanism for reduced enzyme catalytic capacity in cold-acclimated animals, and that those fish that were warm-acclimated would demonstrate an increase in enzyme activity disproportionately large to those fish that were cold-acclimated. Our present findings confirm these hypotheses, although there are enzyme- and tissue-specific responses to thermal conditions and digestion in the goldfish. Additionally, there are impacts of tissue protein content (and hence possible tissue water content) that influence the magnitude of the observed responses. Ration size of the meal consumed was likely not responsible for the observed impacts of temperature or feeding.

Acknowledgements

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Chapter 3: Host trophic level and diet are influential in shaping the intestinal microbiome and ammonia detoxification ability of two native Ontario fish

Abstract

An unexplored area of bacteria-host interaction is whether bacteria aid in host detoxification of nitrogenous waste in the intestinal tract. Digestive enzymes such as glutamine synthetase, glutamate dehydrogenase, and alanine and aspartate aminotransferases are key for ammonia detoxification and are conserved across many animal and bacterial phyla. This suggests that bacteria could aid in ammonia detoxification by providing additional biochemical pathways. However, bacterial communities are altered by both abiotic and biotic factors and correlations with ammonia detoxification may be obscured by geographic location among other factors. Therefore, rainbow darters (N=30) and central stonerollers (N=30) were sampled from six sites within the Grand River watershed. The intestinal bacteria were inventoried using Illumina Mi-Seq while intestinal tracts were simultaneously assayed for activities of ammonia detoxification enzymes. There was a strong effect of host species on the composition of the intestinal tract bacteria as well as on the enzyme activities, suggesting a relationship between host physiology and bacterial community. Within a creek, the intestinal tract microbiome did not change with sampling location and neither did the enzyme activity, suggesting that this relationship is robust within a creek. However, central stoneroller intestinal microbiome populations varied between the creeks, while those of the rainbow darter did not. Furthermore, enzyme activities were higher in samples of both species taken from Irvine Creek but these differences could not be attributed to intestinal microbiome composition, nor to other environmental factors measured. Sampling location is a predictor of intestinal microbiome composition in the central stoneroller, but more study is needed to examine possible mechanisms of selection pressures within that microbiome, and of possible impacts of agriculture pollution as drivers of increased enzyme activities in the intestinal tracts of the rainbow darter and the central stoneroller.

Introduction

The gastrointestinal tract (GIT) is an important ammonia-generating organ (Karlsson et al. 2006; Tng et al. 2008). In rainbow trout, the hepatic portal vein, which brings blood from the intestinal tract to the liver, contains high post-prandial ammonia (NH_3) concentrations, indicating that the intestinal tract is a significant source of digestive ammonia to the liver (Karlsson et al. 2006). Indeed, whole animal nitrogen excretion has been shown to increase 6-fold in the plainfin midshipman following a meal (Bucking et al. 2013). Local spikes in GIT ammonia concentration can be as high as 2 mM, and with plasma NH_3 concentrations of 100-200 μM, there is an appreciable concentration gradient along which there is potential for the ammonia to diffuse into the tissues (Bucking et al. 2013). Elevated plasma ammonia has been linked to impaired swimming performance (Shingles et al. 2001; Wicks et al. 2002; McKenzie et al. 2003), a spike in the stress hormone cortisol (Wicks and Randall 2002), disruptions to cellular energy metabolism (Wicks et al. 2002) and brain hypoxia (Arillo et al. 1981). To prevent these deleterious outcomes, teleost fish excrete large amounts of ammonia to the surrounding environment, as much as 85% through the gill, and to a lesser extent, the kidney, skin and GIT (Smith 1929; Wright et al. 1995; Bucking and Wood 2012; Bucking et al. 2013). Teleost fish can also rely on biochemical enzyme reactions to detoxify ammonia instead of excreting it. Indeed, the action of glutamate-forming enzymes, such as glutamate dehydrogenase (GDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), or the glutamine-forming glutamine synthetase (GS) can detoxify the ammonia in the intestinal tract (Tng et al. 2008; Bucking and Wood 2012; Bucking et al. 2013; Turner and Bucking 2017), as well as other tissues such as the liver (Wilson 1973; Wilkie 2002; Mommsen, Busby, et al. 2003; Ip and Chew 2010). By relying on these biochemical pathways to

detoxify ammonia, fish are able to retain the nitrogen for protein synthesis and somatic cell growth (Tng et al. 2008).

Host-supplied enzymes are not the only possible source of ammonia detoxification in the GIT. Bacterial GS, GDH, ALT and AST, are conserved across bacterial phyla (Feldman and Gunsalus 1950; Fottrell and Mooney 1969; Müller et al. 2006; Amon et al. 2010; Harper et al. 2010; Kim et al. 2010; Jing and Zhang 2011; Rubino et al. 2014), which makes it possible that these symbionts may be important contributors to the host's NH_3-detoxification processes. It is unknown at this time whether symbiotic bacteria can help fish detoxify the NH_3 produced in the intestinal tract during digestion. However, bacteria are known to supply catabolic and anabolic pathways for hosts (e.g. chitin [Goodrich and Morita 1977a; Goodrich and Morita 1977b; Skrodenyte et al. 2008; Nayak 2010; Tapia-Paniagua et al. 2010; Ringo et al. 2012; Clements et al. 2014] and cellulose catabolism [Lesel et al. 1986; Nayak 2010; Mouchet et al. 2012; Pelster et al. 2014; Ye et al. 2014] and vitamin B_{12} synthesis [Sugita et al. 1991]), making this a distinct possibility. If the bacteria are enhancing important detoxification pathways, correlations between bacterial community members and ammonia detoxification activity levels are expected.

Complicating the bacteria-host relationship are variations in microbiome communities that are driven by external factors not related to host physiology. In fact, the composition of the intestinal tract bacterial population is not static and can be influenced by abiotic factors in the environment. For example, temperature fluctuations on diurnal, seasonal and spatial scales can dictate the temperature of the intestinal tract of the ectotherm host, and that of the bacteria living within. Bacterial biochemistry is largely governed by temperature, and each bacterial phylum has a temperature optimum. Changes to GIT temperature allow certain bacterial phyla to flourish at the expense of other phyla in the tract (Gajardo et al. 2016; Wang et al. 2017). Water quality can also

affect symbiotic bacteria, as environmental pollution can weaken immune function (Nayak 2010; Buchtíková et al. 2011; Mouchet et al. 2012; Ringø et al. 2012; McFall-Ngai et al. 2013), allowing pathogenic bacteria to take hold in the GIT (Hansen and Olafsen 1999). High levels of inorganic nitrogen and phosphorous in the water stimulate algal growth, which in turn provide substrate on which bacteria may feed and develop (Allan and Castillo 2007; Dehler et al. 2017). This can affect the microbiome composition of the water, which can impact vertebrate hosts by making certain types of bacteria more readily available for intestinal colonization than others. Finally, the diet can also influence the GIT bacterial composition. The carbohydrate-rich diet of an herbivorous fish favours cellulase-producing bacteria by providing ample substrate on which they can flourish (Tinh et al. 2008; Nayak 2010; Bolnick et al. 2014). The high-protein diet of carnivorous fish correlates with a greater proportion of bacteria that produce proteases that enhance protein catabolism during digestion (Kar and Ghosh 2008; Das et al. 2014; Liu et al. 2016).

Altogether, this suggests that bacterial community structures may vary across environments, both on a fine-scale (i.e. along an individual water body) and on a large-scale (i.e. between watersheds). Only a handful of studies have examined the influence of environment on wild species by sampling the same species from different locations (e.g. Trust and Sparrow 1974; Skrodenyte-Arbaciauskiene et al. 2006; Roeselers et al. 2011; Ye et al. 2014; Schmidt et al. 2015; Sullam et al. 2015; Eichmiller et al. 2016; Llewellyn et al. 2016) despite the established impact of the environment on the GIT microbiome. These studies present conflicting evidence for the effect of sampling location on GIT bacterial community composition, with some studies finding no correlation between geographic sampling location and microbiome (Trust and Sparrow 1974; Roeselers et al. 2011; Schmidt et al. 2015), while others found that sampling location was a determinant of microbiome composition (Skrodenyte-Arbaciauskiene et al. 2006; Sullam et al.

2015; Eichmiller et al. 2016). If the intestinal bacterial community assists the host with ammonia detoxification, this relationship should persist despite environmental differences observed between sampling locations. If the correlation disappears between sampling locations, the bacteria may not be essential for detoxification and/or redundancy is present in the communities.

This study has several aims. Firstly, the correlation between diet and ammonia detoxification in two species was studied. I hypothesized that the higher protein diet should drive higher GS, GDH, ALT and AST activities in the intestinal tract of the insectivorous Perciform, the rainbow darter (*Etheostoma caeruleum*) as compared to those of the herbivorous Cyprinid, the central stoneroller (*Campostoma anomalum*) in line with findings from previous literature (Wilson 1973; Pelster et al. 2014). Furthermore, given the influence of diet on the composition of the intestinal tract community, I hypothesize that the rainbow darter and herbivorous central stoneroller will have distinct intestinal microbiota with community members that correlate with enzyme activity. Lastly, if the bacteria are enhancing host NH_3 detoxification, any differences in enzyme activities observed between the sampling locations should correlate to differences in intestinal bacterial composition between samples taken from the different locations. I hypothesize that fish sampled from distinct locations will have different intestinal tract bacterial composition and functional bacterial genomes from one another that correlate with different enzyme activities.

Materials and Methods

All experiments were carried out according to federal guidelines and approved animal care protocols. Each step described within this section is summarized in a flowchart (Fig. 1).

Sample Collection

In August 2016, the following sites from Irvine Creek were sampled. Wellington Road 16, Belwood Ontario (ON) (43° 76'78.74" N -80° 35'89.88" W), Line 2, Fergus ON (43° 74'74.97" N -80° 37'64.64" W) and Gerrie Road, Elora ON (43° 74'03.13" N -80° 38'98.35" W) (Fig. 3). In August 2017, these sites were re-sampled, and three additional sites were chosen along the Lutteral Creek: Line 7, Belwood ON (43° 73'41.76" N -80° 26'20.67" W), Line 6, Rockwood ON (43° 71'93.43" N -80° 26'19.75" W), and Line 5, Wellington ON (43° 70'80.51" N -80° 27'03.03" W) (Fig. 3). The Irvine Creek sampling sites were surrounded by lands used for agriculture (mixed crops and livestock), while the Lutteral Creek sites were predominantly surrounded by tree cover.

Approximately 7 fish were sampled from each location. Rainbow darters (Etheostoma caeruleum; body mass 0.56 ± 0.36 g) were caught by hand, using dip nets, and central stonerollers (Campostoma anomalum, body mass 5.06 ± 3.03g) were caught using minnow traps baited with commercially available dog food. Fish were brought back to York University in 28-quart (~26L) Igloo Iceless coolers (Katy, Texas, USA) where they were kept at creek temperature and supplied with constant aeration.

Dissection and tissue handling

Upon arrival at York University, the fish were sacrificed in buffered tricaine methanesulfonate (MS-222; Sigma, Oakville, ON, CA) and the intestinal tracts were removed under sterile conditions. Sterile dissection conditions were established by UV-irradiating dissection tools and molecular-grade bullet tubes. The lab bench, gloves and external surface of the fish were sprayed

with 75% ethanol (Sigma, Oakville, ON, CA). Between dissections, the surfaces were wiped and sprayed with the ethanol. Intestines were cleared of any chyme, and the anterior sections were freeze clamped on dry ice and stored for enzyme assays. Posterior sections were stored in the sterile bullet tubes and stored for extraction. All tissues were maintained at -80°C until required.

Enzyme Assays

Approximately 20 mg of freeze-clamped anterior intestine from August 2017 samples was homogenized with a glass homogenizer with 200 µl ice-cold homogenization buffer containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 0.1% TritonX-100, per McClelland et al. (2006). Following homogenization, samples were centrifuged (10 000 x g for 5 minutes) at 4°C in order to pellet cellular debris. The supernatant was placed on ice for enzyme and protein analyses. This step was repeated just before each enzyme assay took place. All enzyme assays were read at 25°C, and were optimized for maximal activity prior to measurement. All reagents were obtained from Sigma-Aldrich unless otherwise noted.

Glutamine synthetase (GS; EC 6.3.1.2) was measured using a glutamyl transferase assay (Bucking et al. 2013) with a running buffer containing 6 mM glutamine, 15 mM hydroxylamine, 0.4 mM ADP, 20 mM $NaAsO_4$, 3 mM $MnCl_2$ in a 50 mM HEPES buffer (pH 6.7), and 50 µl homogenate. The reaction was terminated using a ferric chloride stopping buffer.

Glutamate dehydrogenase (GDH; EC 1.4.1.3) activity was measured using previously published methods (Pelster et al. 2014; Rubino et al. 2014). Briefly, the assay measured the formation of glutamate, coupled to the oxidation of NADH in the presence of ADP, with 14 mM α-ketoglutarate as the substrate (omitted for control).

Alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) activity assays were carried out according to the protocol of Pelster et al. (2014). ALT running buffer contained NADH (0.2 mM), α-ketoglutarate (10.5 mM; omitted for control), lactate dehydrogenase (10 U/ml), and alanine (200 mM). AST running buffer contained NADH (0.2 mM), α-ketoglutarate (8 mM; omitted for control), malate dehydrogenase (8 U/ml), and aspartate (40 mM). See Appendix A for all enzyme reaction equations.

Total homogenate protein content was measured using the protocol of Bradford (1976) using the Biorad Protein Assay Kit (Biorad, Hercules, CA, USA).

Enzyme and protein assays were run on 96-well plates and were read using Gen 5 software (v. 4.6) (Fisher Scientific, Toronto, ON, CA) on a BioTek microplate reader (BioTek, Wisnooski, VT, USA). Activities were measured in triplicate and averaged for downstream data analysis. GS activities are expressed as μmol substrate converted min^{-1} g $tissue^{-1}$; all other enzyme activities are expressed as μmol substrate converted min^{-1} mg $protein^{-1}$, using appropriate extinction coefficients for each substrate.

Bacterial gDNA extraction, validation and sequencing

Extraction of anterior intestinal bacterial gDNA extractions were performed in a biosafety cabinet using the QIAmp Stool Kit (QIAgen, Toronto, ON, CA) according to manufacturer's instructions. The cabinet, kit reagents, pipettes, pipette tips, tubes and forceps were all UV-irradiated for 20 minutes before extractions were completed, to ensure sterile extraction conditions. During the cell lysing step, samples were heated to 95°C in order to ensure extraction of gram-positive bacterial DNA, per manufacturer's instructions. A blank extraction containing all kit reagents, except for

tissue, was used as a contamination control to ensure that no lab bacteria contaminated the extraction.

Universal primers amplifying prokaryotic 16s rRNA hypervariable regions V3-V4 and V6-V7 (Table 1) were used for PCR of the extracted DNA, using DreamTaq (Thermo Fisher Scientific, Waltham, MA, USA). Hypervariable regions are parts of the bacterial genome which are unique to each bacterial species, but are bound on either side by highly conserved sections of the genome (Chakravorty et al. 2007). The conserved regions enable the use of universal primers to amplify the more variable regions, serving the dual purpose of verifying whether bacteria are present, and allowing for sequencing of the bacteria in order to determine its taxonomic identity (Chakravorty et al. 2007). A known sample of bacterial gDNA was used as a positive control while blank reagent samples were used as negative controls to ensure no lab bacterial contamination during the extraction process. The final validation step was quantification of samples using the Quant-IT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. gDNA concentrations of at least 10 ng μl^{-1} were required for the sequencing step. Samples were forwarded to Génome Québec at McGill University (Montréal, QC, CA) for Mi-Seq sequencing.

Bioinformatics analysis

Data analysis was completed using Quantitative Insights into Microbial Ecology, version 2017.11 (QIIME2) (Caporaso et al. 2010) and the following steps are summarized in Fig. 3. The bacterial gDNA was sequenced in a single reaction, with each sample assigned a unique barcode. This process increases sequencing efficiency, since it reduces the amount of time required to complete the sequencing, and keeps costs low (Parameswaran et al. 2007). Following sequencing, each paired-end read has a barcode and quality data attached to it. This metadata must be separated, or

demultiplexed using the demux plug-in (https://github.com/qiime2/q2-demux), before analysis can begin. The paired-end sequences were then joined with vsearch (Rognes et al. 2016), and the lowest-quality reads were filtered (Bokulich et al. 2013), with a cutoff quality score of 30 (Sullam et al. 2015). Sequences were then denoised to remove sequencing errors and the lower quality reads were trimmed to 212 base pairs. This ensures the integrity of the data (Amir et al. 2017). Phylogenetic trees were created using FastTree2 (Price et al. 2010), and α- and β-diversity analyses to establish species diversity within each sampling location, and between sampling locations, respectively (Whittaker 1960; Whittaker 1972), were run using default QIIME2 settings. Richness, which is an α-diversity metric which takes into account the total number of species present within a sample area (Heip et al. 1998), was measured using the Chao1 index. Evenness is an assessment of how well distributed the species are within a given community (Heip et al. 1998), while the Shannon index measures diversity by accounting for both species richness and evenness (Peet 1975; Jost 2006). Unweighted (Lozupone and Knight 2005) and weighted UniFrac analyses (Lozupone et al. 2007) to compare differences in species' presence or absence (unweighted) and species abundance (weighted) between sites were carried out, again using default QIIME2 settings. Operational taxonomic units (OTUs) were picked using the GreenGenes database 13_8 release (http://greengenes.secondgenome.com) (DeSantis et al. 2006) at a 97% sequence identity in line with previously published work (Bolnick et al. 2014a; Givens et al. 2015; Sullam et al. 2015; Eichmiller et al. 2016).

Prediction of functional genomic profiles

A key component of biodiversity that is relevant to the study of the intestinal microbiome is that of functional diversity. Functional diversity takes into account the role of various organisms within a given ecosystem (Petchey and Gaston 2006). Understanding the functional diversity of the

collective genome, or metagenome, of the microbiome is integral to understanding the importance of symbiotic bacteria to the host (Wang et al. 2017). In this study, the functional genome of the microbiome was determined using a Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) pipeline (Langille et al. 2013) running in Microbiome Helper (Comeau et al. 2017). PICRUSt sorts the bacterial metagenome by gene family using published databases in order establish the functional composition of the microbiome (Langille et al. 2013). In order to do this, OTUs must first be picked against the GreenGenes v. 13_5 database at 97% similarity, to generate the .biom tables required for PICRUSt analysis. The nearest sequenced taxon index (NSTI) is then calculated, which is an estimate of the phylogenetic relatedness of the intestinal bacterial sequences to those found within the genome database (Gajardo et al. 2016; Bestion et al. 2017). OTU tables were then corrected and the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologue (KO) were predicted. The KEGG database characterizes the higher order functions of both prokaryotic and eukaryotic genes (Kanehisa and Goto 2000), allowing for analysis of the metagenomic function of the intestinal microbiome. Statistical Analysis of Metagenomic Profiles (STAMP) (Parks et al. 2014) software was then used to visualize and assess the functional genomic differences between samples.

Statistics

Most of the bacterial abundance data were non-parametric, so comparisons between and within creeks and species were done using PERMANOVA and Kruskal-Wallis tests through QIIME2.

Enzyme activity data were analysed with SigmaStat 3.0 (Systat Software Inc., San Jose, CA). One-way ANOVAs were used to determine whether there was a significant difference between sites *within* the same creek. Upon determining that differences were not significant within the same creek, enzyme activities within the same creek were averaged, and were compared against each

other, again using a one-way. Finally, enzyme activities of central stonerollers were compared to those of rainbow darters both within and between creeks using a two-way ANOVA (to identify differences between the two fish species both within and between the creeks of interest. $p < 0.05$ was considered significant for all statistical tests performed. Enzyme data is presented at mean ± S.E.M (N=individual sample).

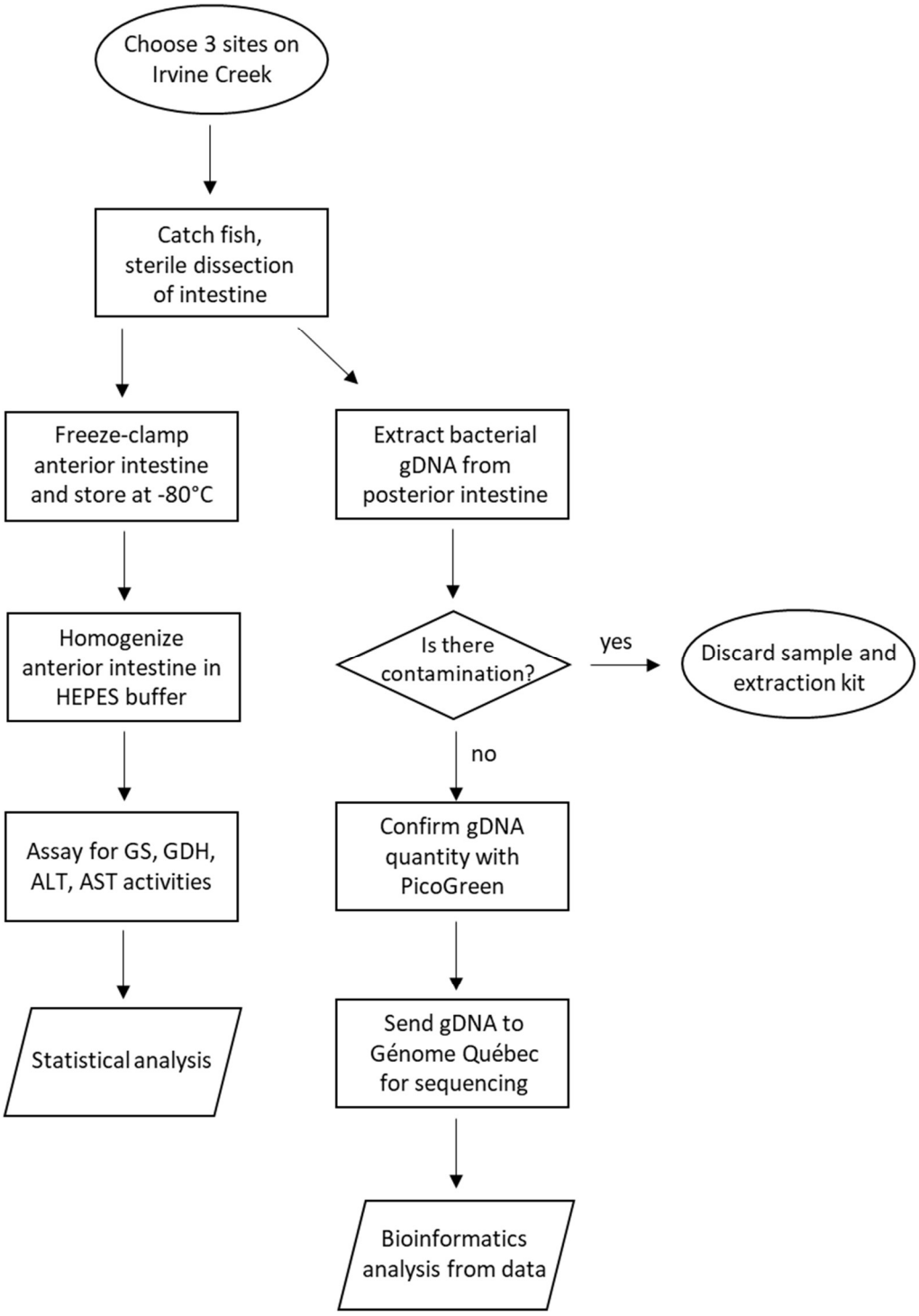

Fig. 1 Flow chart depicting methodology used for this project. Ovals represent start- or end-points in the methodology. Rectangles indicate a process, while diamonds indicate a decision-making point. Parallelograms indicate a data collection point.

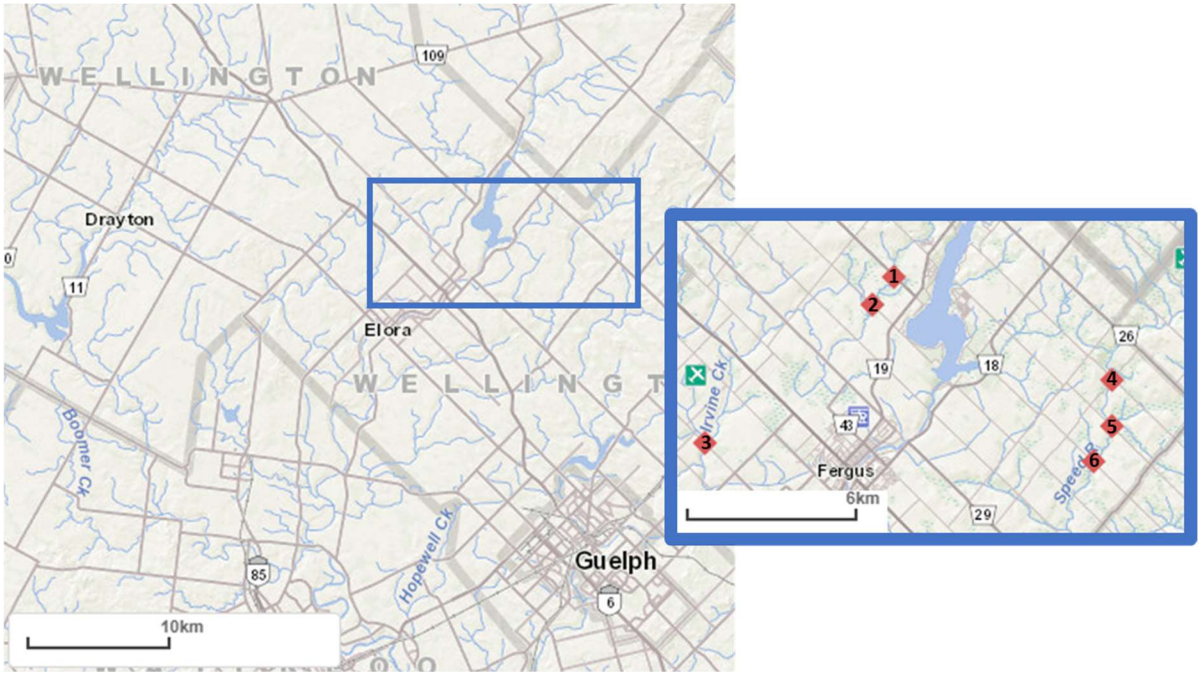

Fig. 2 Sampling locations within the Grand River watershed. August 2016 sites are (1) Wellington Road 16, Belwood ON (43° 76’78.74” N -80° 35’89.88” W), (2) Line 2, Fergus ON (43° 74’74.97” N -80° 37’64.64” W) and (3) Gerrie Road, Elora ON (43° 74’03.13” N -80° 38’98.35” W). In August 2017, sites 1, 2 and 3 were re-sampled, and three additional sites were chosen along the Lutteral Creek: (4) Line 7, Belwood ON (43° 73’41.76” N -80° 26’20.67” W), (5) Line 6, Rockwood ON (43° 71’93.43” N -80° 26’19.75” W), and (6) Line 5, Wellington ON (43° 70’80.51” N -80° 27’03.03” W).

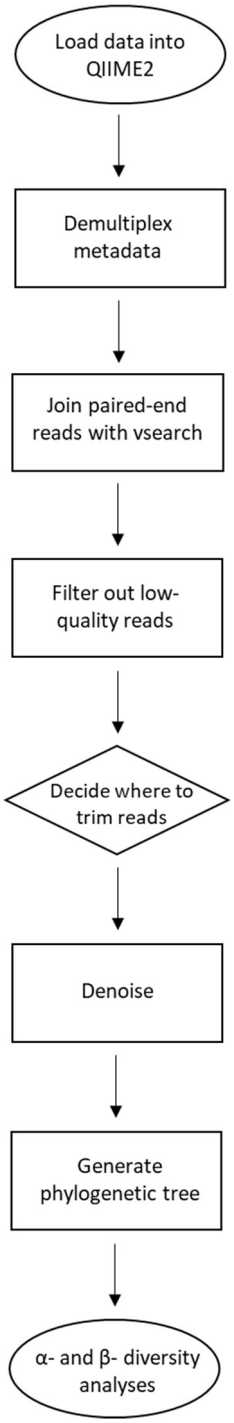

Fig. 3 Flow chart depicting bioinformatics workflow. Ovals represent start- or end-points in the methodology. Rectangles indicate a process, while diamonds indicate a decision-making point.

Table 1 Primers used for PCR amplification of extracted intestinal bacterial genomic DNA from rainbow darters and central stonerollers. Primers used were taken from Chakravorty et al. (2007). Positions of genes within the genome are included in brackets, and are based on Escherichia coli numbering (Bolnick et al. 2014b).

Primer		
Name	**Sequence**	**Amplified hypervariable region**
V3F	5' CCAGACTCCTACGGGAGGCAG 3' (334-354)	V3 (334-537)
V3R	5' CGTATTACCGCGGCTGCTG 3' (519-537)	
V6F	5' TCGATGCAACGCGAAGAA 3' (961-78)	V6 (986-1043)
V6R	5' ACATTTCACAACACGAGCTGACGA 3' (1062-85)	

Results

There were no significant differences in the enzyme activities along Irvine creek for all enzymes measured within each species. Therefore, the values for each species were combined and averaged along the creek for comparison below.

Glutamine synthetase (GS)

Glutamine synthetase (GS) activity was significantly higher for fish sampled from Irvine Creek than it was for those from the Lutteral Creek for both species ($p < 0.05$; Fig. 4) but was not different between species within the Irvine Creek ($p > 0.05$). Activities for the rainbow darters of the Lutteral Creek (4.08 ± 0.08 μmol min^{-1} g $tissue^{-1}$; $N = 5$) were significantly higher than they were for the central stonerollers (3.17 ± 0.04 μmol min^{-1} g $tissue^{-1}$; $N = 7$) within that creek ($p > 0.05$).

Glutamate dehydrogenase (GDH)

GDH activity was significantly higher for fish sampled from Irvine Creek than for the Lutteral Creek ($p < 0.05$). Within Irvine Creek, the rainbow darters had significantly higher enzyme activities ($190.483 \pm$ μmol min^{-1} g $protein^{-1}$; $N = 7$) than the central stonerollers (145.68 ± 0.609 μmol min^{-1} g $protein^{-1}$; $N = 11$) within the same creek (Fig. 5, $p < 0.001$). Within the Lutteral Creek, there was no significant difference between species (69.67 ± 0.609 μmol min^{-1} g $protein^{-1}$; $p > 0.05$).

Alanine aminotransferase (ALT)

Alanine aminotransferase (ALT) activity was significantly higher for rainbow darters sampled from both the Irvine Creek (608.42 ± 1.59 μmol min^{-1} g $protein^{-1}$; N = 12) and the Lutteral Creek (144.16 ± 1.96 μmol min^{-1} g $protein^{-1}$; N = 12) than it was for the central stonerollers ($p < 0.001$; Fig. 6; N = 21). Within the Irvine Creek, ALT activities were significantly lower for the central stonerollers than the rainbow darters ($p < 0.001$), and this pattern was repeated in the Lutteral Creek ($p < 0.001$).

Aspartate aminotransferase (AST)

Aspartate aminotransferase (AST) activities followed a similar pattern as those for ALT, wherein overall enzyme activities for samples of both species from the Irvine Creek (rainbow darters, N = 12; central stonerollers, N = 10) were higher than those of the Lutteral Creek ($p < 0.05$; Fig. 7; rainbow darters, N = 13, central stonerollers, N = 10). Within the Irvine Creek, AST activities were significantly higher for the rainbow darters (127.90 ± 3.16 μmol min^{-1} g $protein^{-1}$).

gDNA extraction and validation

Bacterial gDNA was not contaminated by any lab bacteria before being sent for sequencing, as determined by a lack of bands in the extracted sample and negative control lanes, and bands of expected size found within the positive control lanes as visualized on 1.5% agar gel (see Appendix B).

Microbiome community analysis between species

August 2016 rainbow darters and central stonerollers did not differ in terms of the number of observed OTUs, with 188 and 192 OTUs respectively ($p = 0.07$). α-diversity analyses within the samples did not uncover differences between the two host species according to bacteria species richness (chao1; $p = 0.183$), evenness ($p = 0.67$) and the Shannon ($p = 0.924$) index. In terms of β-diversity, the host species had different microbiome compositions from one another according both unweighted (Fig. 8a) and weighted UniFrac analyses (Fig. 8b; $p=0.01$). This means that the rainbow darters and central stonerollers were dissimilar to one another in terms of β-diversity in both bacterial species identity and in abundance. Specifically, the 2016 *E. caeruleum* intestines were dominated by Proteobacteria (45.38%), followed by Bacteroidetes (15.44%) and Firmicutes (12.30%) (Fig. 12a). *C. anomalum* posterior intestine microbiota were comprised primarily of Proteobacteria (48.90%), followed by Fusobacteria (20.18%) and Tenericutes (9.80%) (Fig. 15a).

There were 100 OTUs observed in the August 2017 rainbow darter intestines, while there were 120 within the central stoneroller; these differences were not significant ($p = 0.1945$). Species diversity (Shannon's index) and richness (chao1) were not significantly different ($p > 0.05$) between the species, nor was the species evenness ($p=0.194$) as in 2016. From a β-diversity standpoint, the species were again different from one another according both unweighted (Fig. 8a) and weighted UniFrac analyses (Fig. 8b) based on Bray-Curtis dissimilarity ($p=0.001$). The 2017 rainbow darter intestine mainly contained bacteria from phyla Proteobacteria (36.27%), Actinobacteria (16.25%) and Firmicutes (12.91%) (Fig. 12b). The 2017 central stoneroller intestine was dominated by Proteobacteria (69.62%), followed by Fusobacteria (8.86%) and Firmicutes (5.33%) (Fig. 15b).

Microbiome community analysis between rainbow darters caught from the two creeks

The microbiome of rainbow darters sampled in August 2016 remained unchanged along the creek gradient in terms of all α-diversity metrics: richness ($p = 1.00$), evenness ($p > 0.77$), Shannon ($p = 1.00$) and observed species ($p = 1.00$). β-diversity did not differ between sites according to both the unweighted (Fig. 10a; $p > 0.1$) and weighted (Fig. 10b; $p > 0.48$) UniFrac analyses. Additionally, there was no significant difference in the microbiome composition of rainbow darters caught from different sites in August 2017 within a single creek by any α- or β-diversity metric ($p > 0.10$) as in 2016. Interestingly, there were no differences in the microbiome between creeks ($p=0.401$) in terms of the number of observed OTUs, nor any α-diversity metric (Shannon, richness, evenness; $p > 0.40$). Both unweighted (Fig. 11a; $p = 0.095$) and weighted (Fig. 11b; $p = 0.13$) UniFrac analyses revealed no differences between the creeks in terms of presence-absence, nor abundance data, respectively. Overall, this suggests that the GIT bacterial communities within the rainbow darters does not differ along a single creek, nor does it differ between creeks despite difference in enzyme activities.

Microbiome community analysis between central stonerollers caught from the two creeks

The August 2016 central stoneroller microbiome did not change along the stream gradient in terms of diversity (Shannon; $p > 0.66$), species evenness (Pielou; $p > 0.833$), nor species richness (chao1; $p = 1.00$). In terms of β-diversity, the unweighted (Fig. 13a; $p > 0.2$) and weighted UniFrac analyses (Fig. 13b; $p > 0.05$) revealed no change to the microbiome composition along the creek gradient ($p > 0.06$). As with the rainbow darters, the microbiome communities of samples taken

from within the same creek were not significantly different from one another by any α- or β-diversity metric ($p > 0.1$) in 2017 either. As with rainbow darters, there were no α-diversity differences between the creeks (Shannon, Faith's, Evenness; $p > 0.1$), nor were there differences according to the weighted UniFrac analysis (Fig. 14b; $p > 0.50$). However, unlike the darters the samples taken from either creek were significantly different from one another, as measured by the unweighted UniFrac analysis (Fig. 14a; $p = 0.018$). This suggests that the bacterial species composition is different between the two creeks in terms of presence-absence of bacterial species.

Functional genomics analysis

The nearest sequenced taxon index (NSTI) values are summarized in Table 2. The bulk of the predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for all samples from both species, from all creeks and all years sampled were metabolism, genetic information processing and environmental information processing. Metabolic pathways were dominated by genes encoding for carbohydrate, amino acid and energy metabolism. Genetic information processing pathways were concentrated on replication and repair and translation, and environmental information processing pathways were heavily weighted in genes for membrane transport. A comparison of the 2016 and 2017 rainbow darters to one another (Table 4) and the 2016 and 2017 central stonerollers to one another (Table 5) revealed no differences in KEGG pathways ($p > 0.05$). The 2017 rainbow darters taken from both creeks shared similar KEGG pathways ($p > 0.05$;Table 6) as did the 2017 central stonerollers taken from both creeks ($p > 0.05$; Table 7). A comparison of the 2017 rainbow darters and central stonerollers demonstrated that the two species had different KEGG pathways to one another ($p < 0.05$; Fig. 16).

Figures

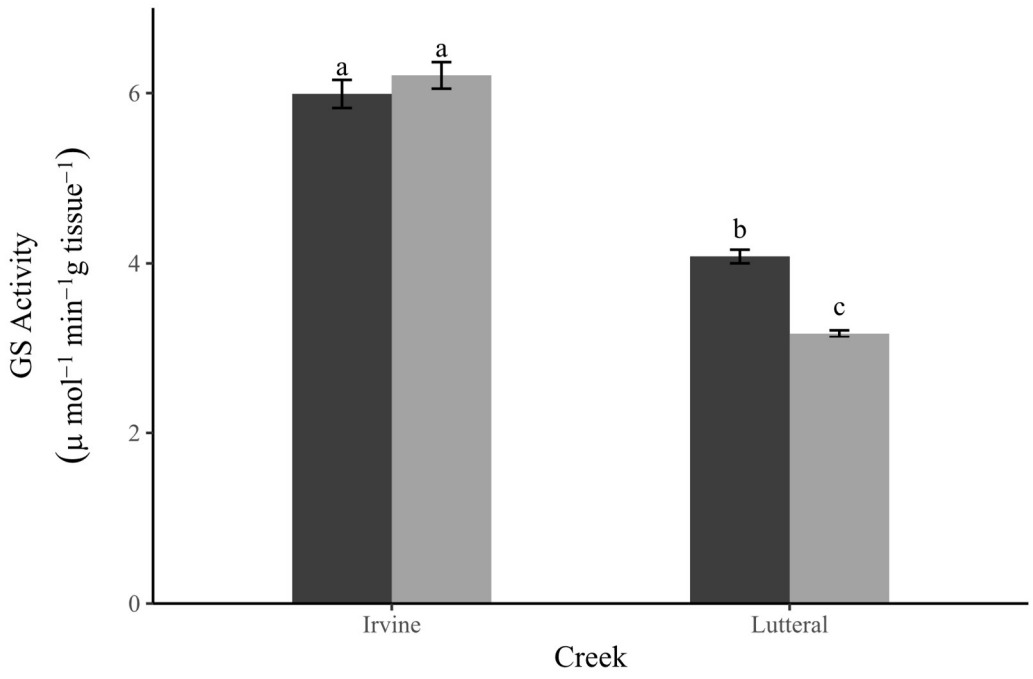

Fig. 4 Mean glutamine synthetase (GS) activity (μmol min^{-1} g tissue^{-1}) in the anterior intestinal tracts of wild-caught rainbow darters (N=9) and central stonerollers (N=14). Dark grey bars are rainbow darters, and light grey bars are central stonerollers. Bars on the left of the graph represent samples taken from the Irvine Creek, and those on the right are samples taken from the Lutteral Creek. Values are means ± SEM, and a two-way ANOVA was used to establish the effect of species and creek on enzyme activities. Bars that share letters are not significantly different, while bars that share letters are significantly different from one another. GS activities within the Irvine Creek are the same for both species ($p > 0.05$), but in the Lutteral Creek, rainbow darter GS activities are higher ($p < 0.05$). Mean differences between the two creeks are higher in the Irvine Creek ($p < 0.05$).

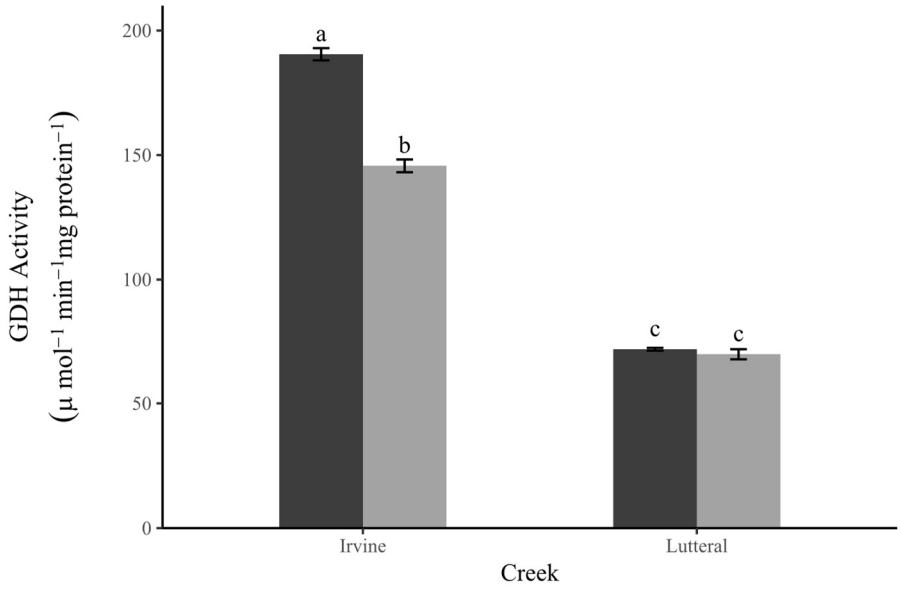

Fig. 5 Mean glutamate dehydrogenase (GDH) activity (μmol min^{-1} g $protein^{-1}$) in the anterior intestinal tract*s* of wild-caught rainbow darters (N = 14) and central stonerollers (N=22). Dark grey bars represent rainbow darters, and light grey bars represent central stonerollers. Bars on the left of the graph represent samples taken from the Irvine Creek, and those on the right are samples taken from Lutteral Creek. Values are means ± SEM, and a two-way ANOVA was used to establish the effect of species and creek on enzyme activities. Bars that share letters are not significantly different. GDH activities between the species within Irvine Creek are significantly different ($p < 0.001$) and are significantly higher than those of the Lutteral Creek ($p < 0.001$). Within the Lutteral Creek, GDH activities between the species are the same ($p > 0.05$).

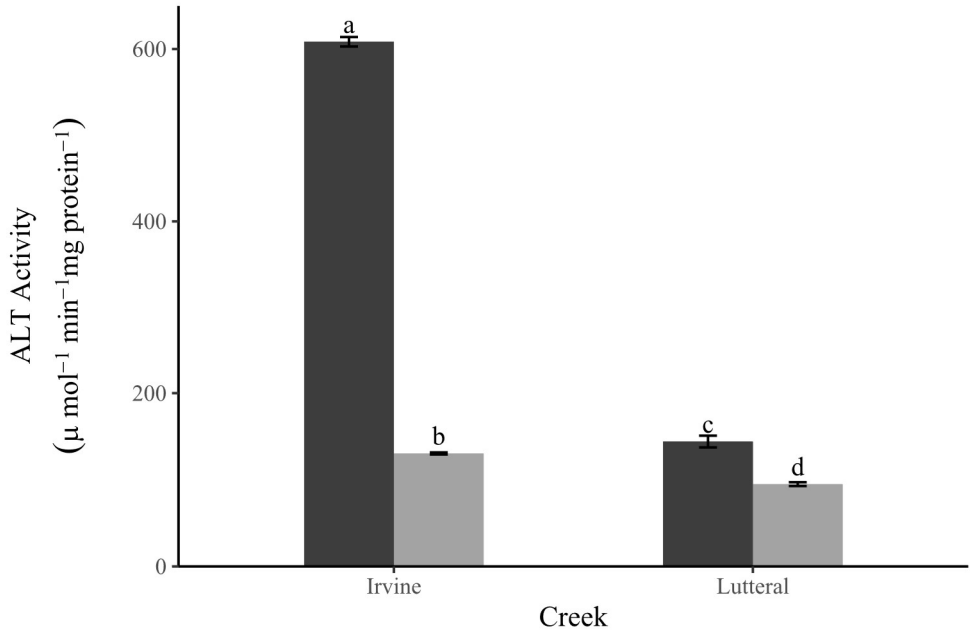

Fig. 6 Mean alanine aminotransferase (ALT) activity (μmol min^{-1} g $protein^{-1}$) in the anterior intestinal tracts of wild-caught rainbow darters (N=24) and central stonerollers (N=21*).* Rainbow darters are indicated in dark grey, and central stone rollers are indicated by the light grey bars. Bars on the left of the graph represent samples taken from the Irvine Creek, and those on the right are samples taken from Lutteral Creek. Values are means ± SEM, and a two-way ANOVA was used to establish the effect of species and creek on enzyme activities. Bars sharing the same letters are significantly different. ALT activities are higher for the rainbow darters than they are for the central stonerollers in both creeks ($p < 0.01$), and the central stonerollers from Irvine Creek have higher ALT activities than those from Lutteral Creek ($p < 0.05$). Overall ALT activity levels in Irvine Creek are higher ($p < 0.001$) than they are in the Lutteral Creek.

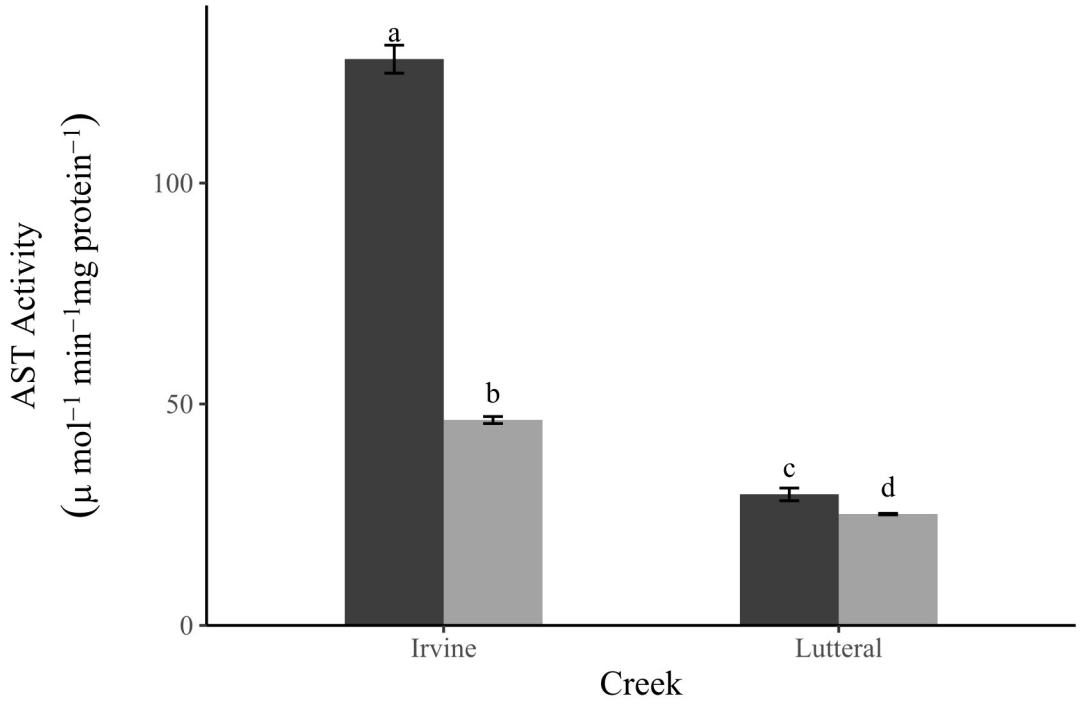

Fig. 7 Mean aspartate aminotransferase (AST) activity (μmol min^{-1} g $protein^{-1}$) in the anterior intestinal tracts of wild-caught rainbow darters (N=25) and central stonerollers (N=20*).* Dark grey bars are rainbow darters, and light grey bars are central stonerollers. Bars on the left of the graph represent samples taken from the Irvine Creek, and those on the right are samples taken from Lutteral Creek. Values are means ± SEM, and a two-way ANOVA was used to establish the effect of species and creek on enzyme activities. Bars that share letters are not significantly different from one another. AST activities are significantly higher for rainbow darters in the Irvine Creek ($p \leq 0.001$) and in the Lutteral Creek ($p < 0.05$) than they are for the central stonerollers. Overall AST activities are higher in the Irvine Creek than in the Lutteral Creek ($p < 0.05$).

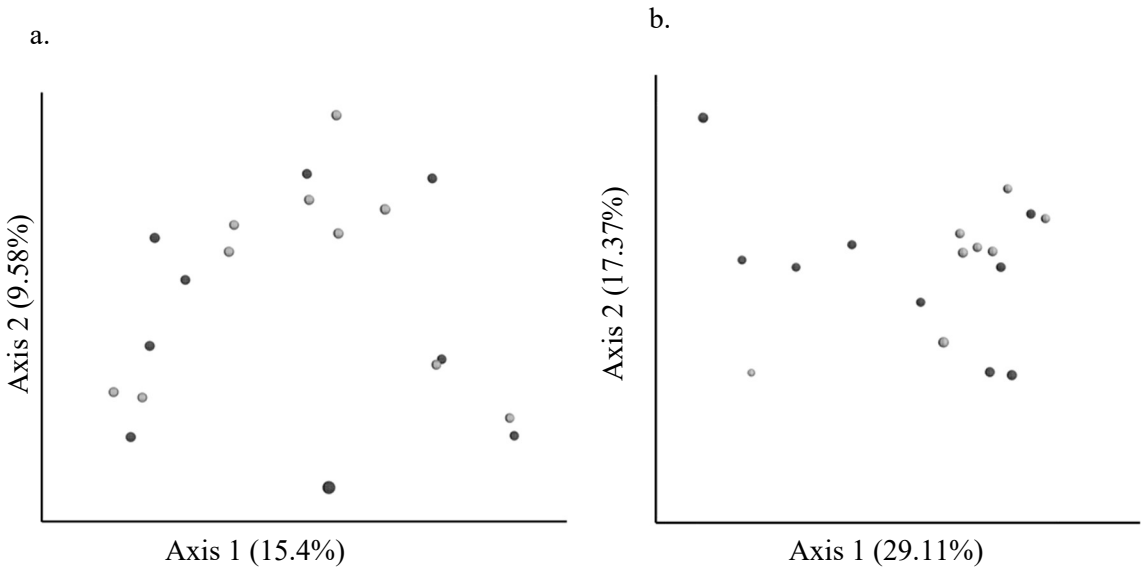

Fig. 8 Effect of host species on microbiome composition in rainbow darters (N= 9) and central stonerollers (N=10) taken from Irvine creek in August 2016. Sequences are based on V3-V4 hypervariable regions of intestinal bacterial 16s rRNA. Pairwise community comparisons were determined with (a) unweighted and (b) weighted UniFrac analyses. Each dot represents a sample, with rainbow darters and central stonerollers indicated by dark and light grey, respectively. Significant differences between the species were observed in both the unweighted and weighted UniFrac analyses (p=0.01).

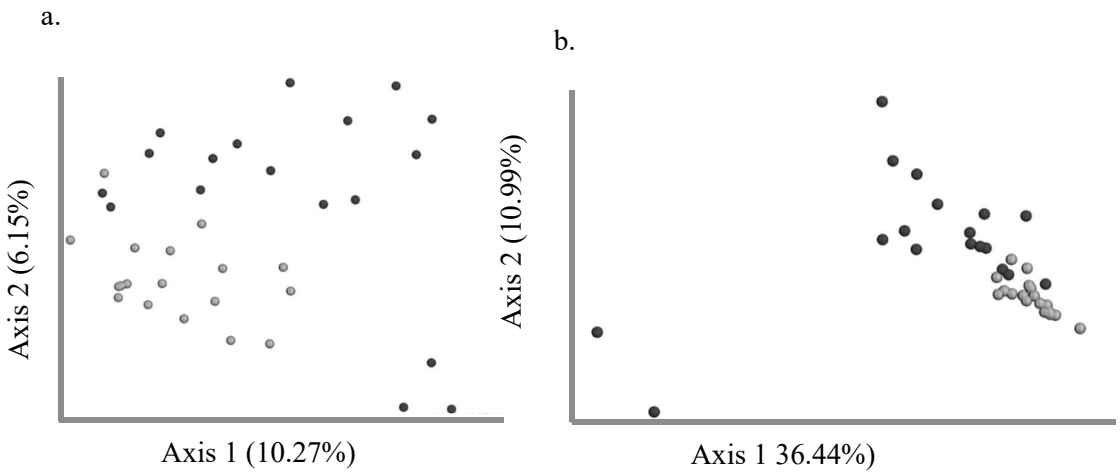

Fig. 9 Effect of host species on microbiome composition in rainbow darters (N = 18) and central stonerollers (N = 18) taken from Irvine and Lutteral creeks in August 2017. Sequences are based on V3-V4 hypervariable regions of intestinal bacterial 16s rRNA. Pairwise community comparisons were determined with (a) unweighted and (b) weighted UniFrac analyses. Each dot represents a sample, with rainbow darters and central stonerollers indicated by dark and light grey, respectively. Significant differences between the species were observed in both the unweighted and weighted UniFrac analyses (p=0.001).

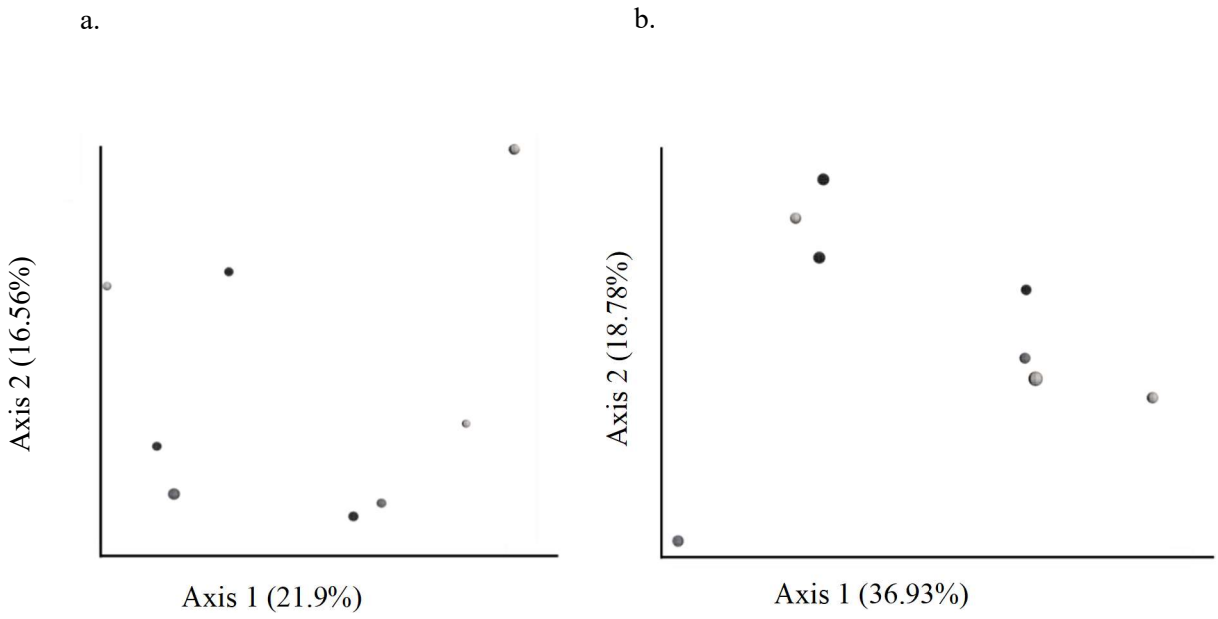

Fig. 10 Effect of sampling location on microbiome composition in rainbow darters (N= 9) taken from Irvine creek in August 2016. Sequences are based on V3-V4 hypervariable regions of intestinal bacterial 16s rRNA. Pairwise community comparisons were determined with (a) unweighted and (b) weighted UniFrac analyses. Each dot represents a sample. Samples taken from Wellington Road 16 are in black, Line 2 samples are in dark grey, and Gerrie Road samples are light grey. No differences between the sites were observed in either the (a) unweighted ($p > 0.110$), nor the (b) weighted ($p > 0.48$) UniFrac analyses.

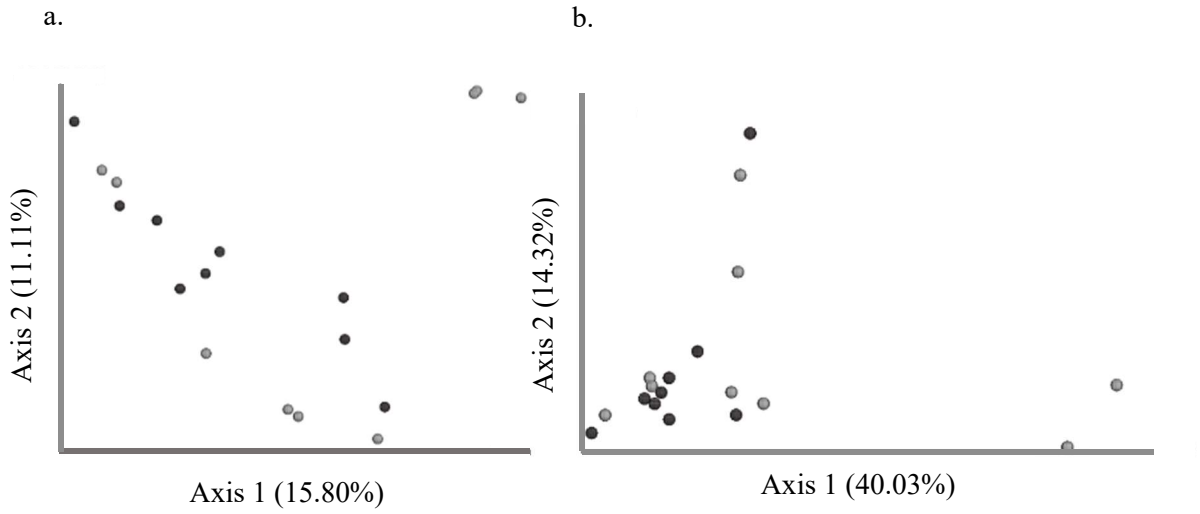

Fig. 11 Effect of sampling location on microbiome composition in rainbow darters (N= 18) taken from Irvine and Lutteral creeks in August 2017. Sequences are based on V3-V4 hypervariable regions of intestinal bacterial 16s rRNA. Pairwise community comparisons were determined with (a) unweighted and (b) weighted UniFrac analyses. Each dot represents a sample, with Irvine Creek samples coloured in dark grey, and Lutteral Creek samples in light grey. No significant differences between the creeks were observed in neither unweighted (p=0.095) nor weighted (p=0.13) UniFrac analyses.

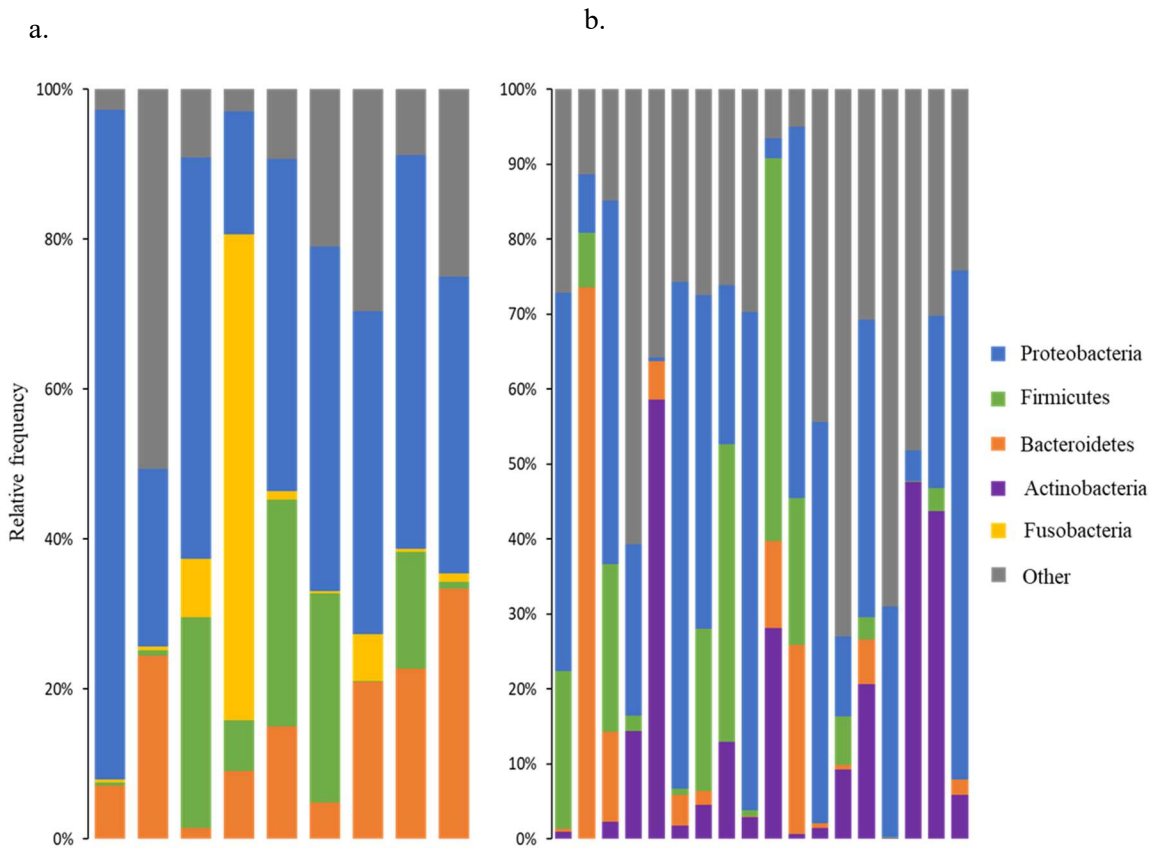

Fig. 12 Rainbow darter intestinal tract bacterial composition from (a) August 2016 and (b) August 2017 samples by relative abundance of each bacterial phylum in the posterior intestinal tract. Each bar represents a sample. Proteobacteria dominates most 2016 rainbow darter intestinal tracts sampled with a relative abundance of 45.38%. Bacteroidetes (15.44%) and Firmicutes (12.30%) are the next most abundant phyla. The 2017 intestinal tracts are dominated by Proteobacteria with a relative abundance of 36.27%. Actinobacteria (16.25%) and Firmicutes (12.91%) are the next most abundant phyla. The "other" bacteria shown were all phyla that represented < 1% of the abundance combined into a single category to provide more clarity.

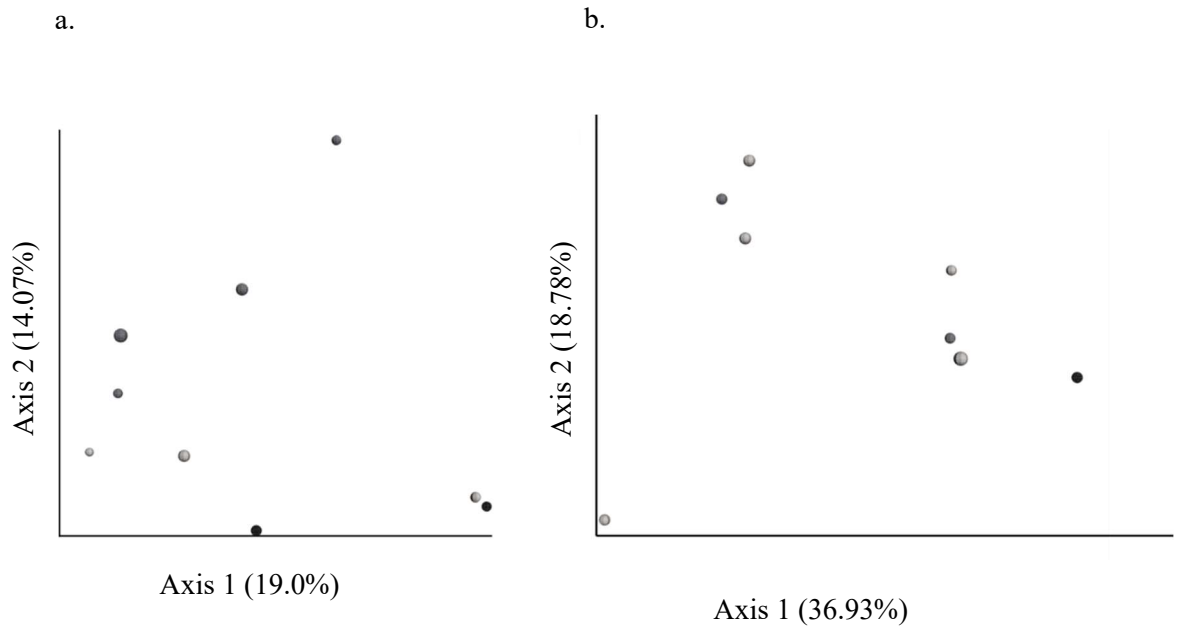

Fig. 13 Effect of sampling location on microbiome composition in central stonerollers (N = 9) taken from Irvine creek in August 2016. Sequences are based on V3-V4 hypervariable regions of intestinal bacterial 16s rRNA. Pairwise community comparisons were determined with (a) unweighted and (b) weighted UniFrac analyses. Each dot represents a sample. Samples taken from Wellington Road 16 are in black, Line 2 samples are in dark grey, and Gerrie Road samples are light grey. No differences between the sites were observed in the (a) unweighted ($p > 0.211$). In the (b) weighted UniFrac analysis, samples from the Wellington Road 16 and Line 2 sites are significantly different ($p < 0.025$), while samples from Wellington Road 16 and Gerrie Road, and samples from Line 2 and Gerrie Road are not different from one another ($p > 0.230$).

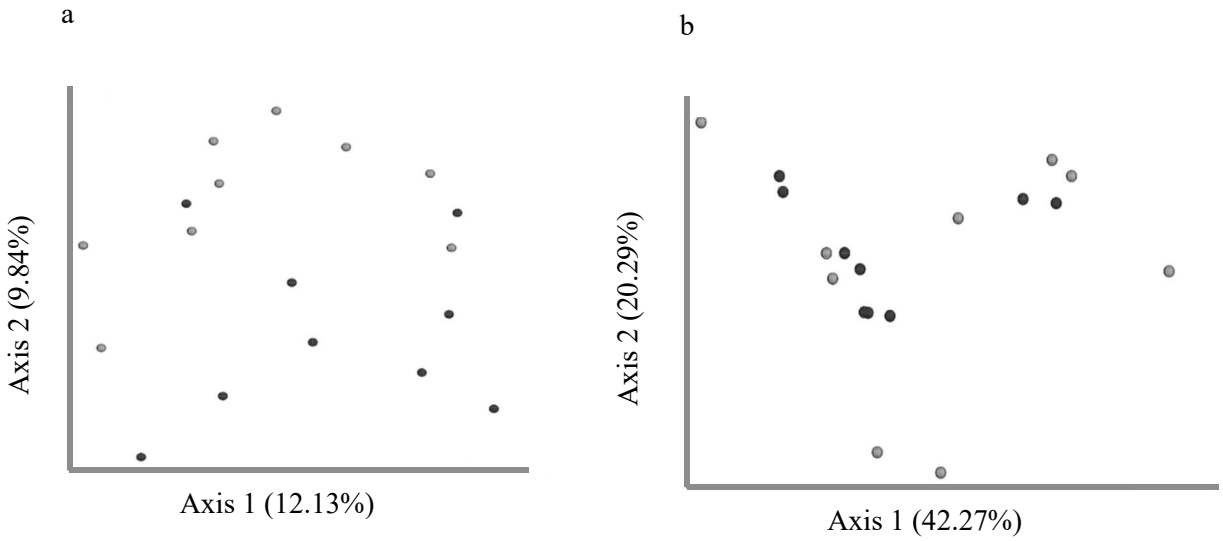

Fig. 14 Effect of sampling location on microbiome composition in central stonerollers (N= 18) taken from Irvine Creek and Lutteral Creek in August 2017. Sequences are based on V3-V4 hypervariable regions of intestinal bacterial 16s rRNA. Pairwise community comparisons were determined with (a) unweighted and (b) weighted UniFrac analyses. Each dot represents a sample, with Irvine Creek samples coloured in dark grey, and Lutteral Creek samples in light grey. Significant differences between the creeks were observed in the unweighted analysis (p=0.018), but not in the weighted (p=0.524) UniFrac analysis.

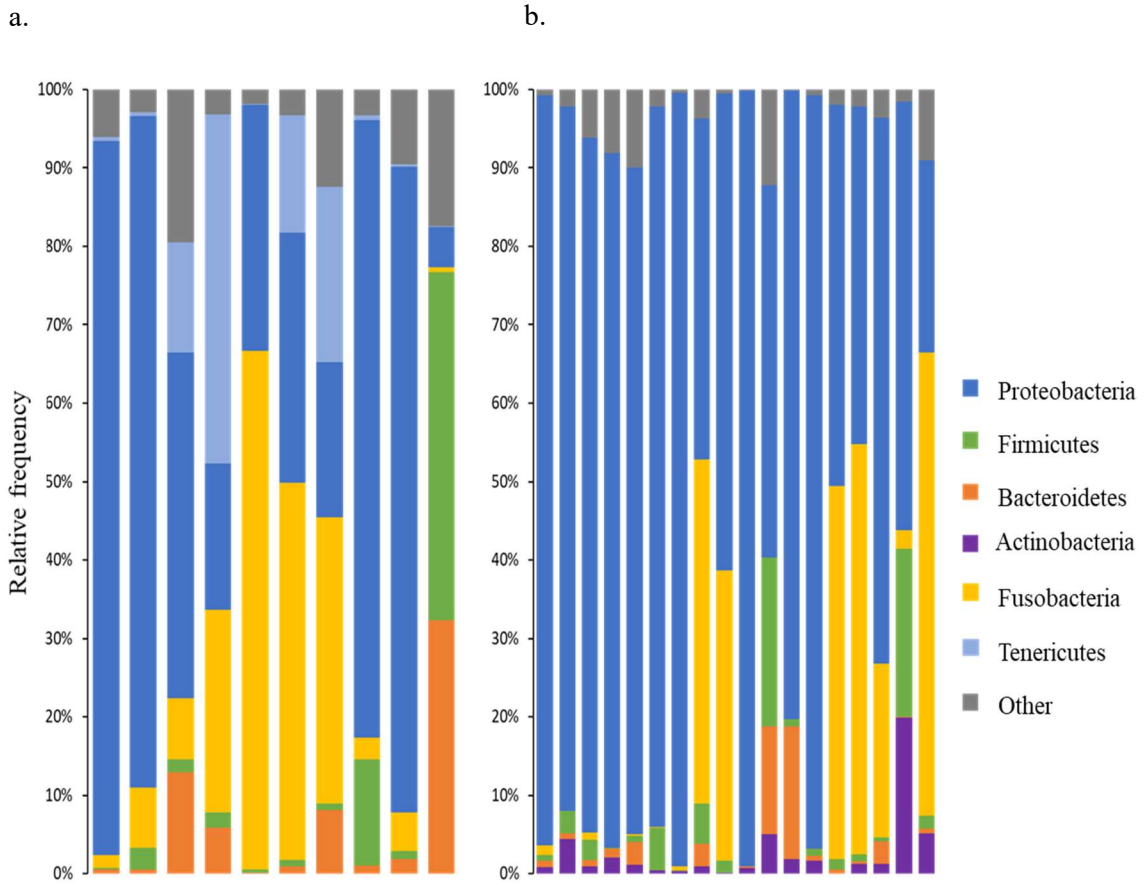

Fig. 15 Central stoneroller intestinal tract bacterial composition from (a) August 2016 and (b) August 2017 samples by relative abundance of each bacterial phylum in the posterior intestinal tract. Each bar represents a sample. Proteobacteria dominates most 2016 stoneroller intestinal tracts sampled with a relative abundance of 48.9%. Fusobacteria (20.18%) and Tenericutes (9.80%) are the next most abundant phyla. In the 2017 samples, Proteobacteria dominated the tract with a relative abundance of 70.36%, followed by Fusobacteria (14.34%) and Firmicutes (3.61%). The "other" bacteria shown were all phyla that represented < 1% of the abundance combined into a single category to provide more clarity.

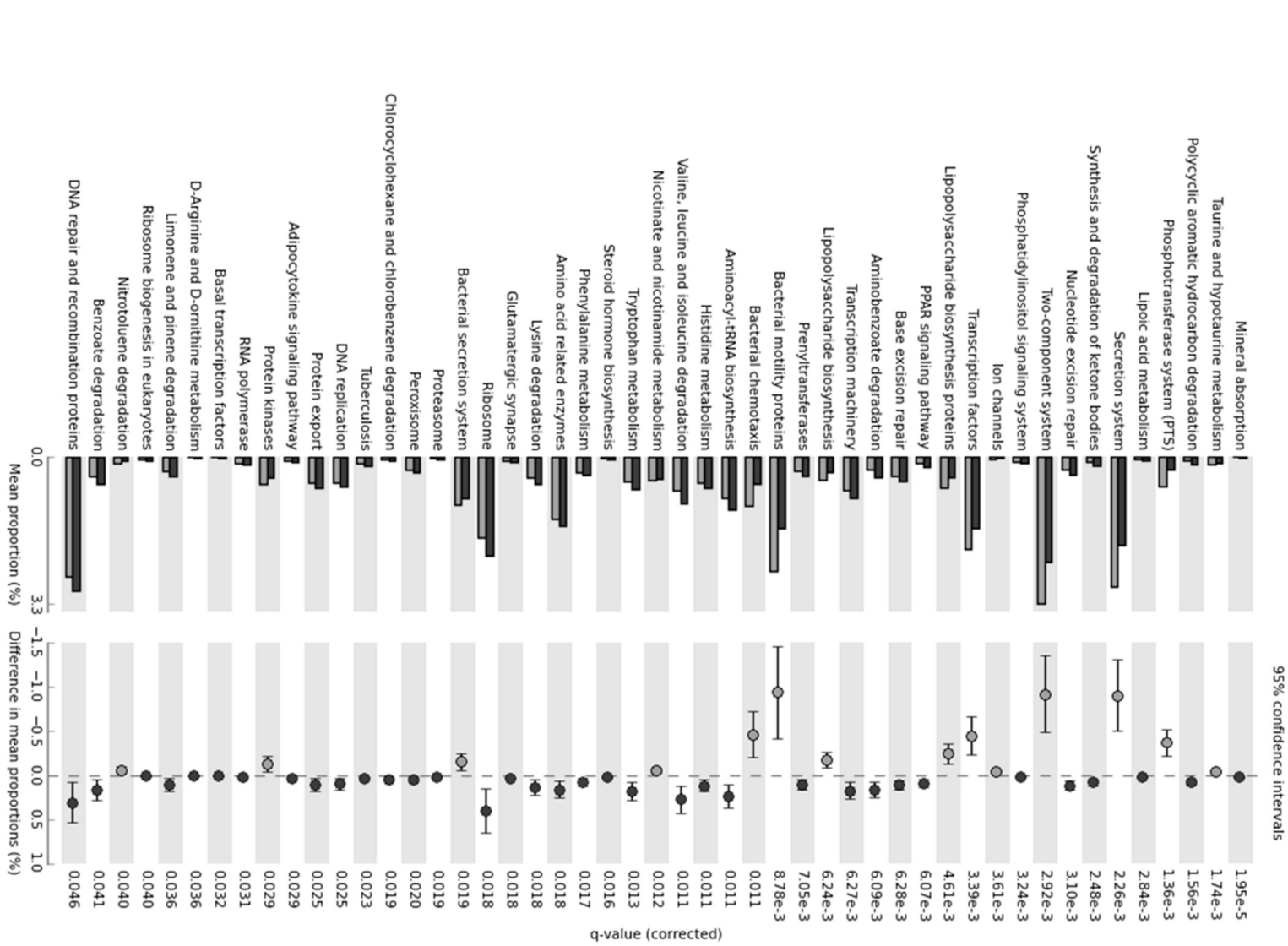

Fig. 16 Predicted functional genomic Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of rainbow darters (N = 9; dark grey) and central stonerollers (N = 9; light grey) sampled from the Irvine and Lutteral Creeks in August 2017. Only pathways significantly different between samples are shown.

Tables

Table 2 Nearest sequenced taxon index (NSTI) values for the rainbow darters (E. caeruleum; N = 9) and the central stonerollers (C. anomalum; N = 10) sampled from Irvine Creek in August 2016, and from Irvine and Lutteral Creeks in August 2017. Closed-reference OTUs were selected at 97% similarity using the GreenGenes 13_5 database.

	NSTI value	
Sampling year	*E. caeruleum*	*C. anomalum*
2016	0.095 ± 0.033	0.078 ± 0.033
2017	0.095 ± 0.037	0.082 ± 0.034

Table 3 Mean relative frequency (%) of KEGG pathways of the rainbow darters (E. caeruleum; N = 9) and central stonerollers (C. anomalum; N = 10) sampled from Irvine Creek in August 2016. Although the microbiota of the two fish species differed from one another, the functional genomes of the bacteria were not different between the rainbow darters and central stonerollers ($p > 0.05$).

	Mean relative frequency (%)	
Pathway	*E. caeruleum*	*C. anomalum*
Metabolism	46.57	46.58
Carbohydrate metabolism	9.35	9.61
Amino acid metabolism	9.32	9.24
Energy metabolism	5.54	5.89
Genetic information processing	16.22	16.33
Replication and repair	7.10	7.12
Translation	4.12	4.34
Environmental information processing	14.73	15.00
Membrane transport	11.96	12.14

Table 4 Mean relative frequency (%) of KEGG pathways of the rainbow darters sampled from Irvine Creek in August 2016 (N = 9) compared to those sampled in 2017 (N = 9). Although the microbiome was different between the two years, the functional genomes of the bacteria did not differ substantially ($p > 0.05$).

	Mean relative frequency (%)	
Pathway	2016	2017
Metabolism	55.73	58.43
Amino acid metabolism	11.05	12.32
Carbohydrate metabolism	11.61	11.35
Energy metabolism	6.58	7.28
Genetic information processing	19.24	20.19
Replication and repair	8.43	8.95
Translation	4.89	5.66
Environmental information processing	17.53	15.27
Membrane transport	14.19	12.81

Table 5 Mean relative frequency (%) of KEGG pathways of the central stonerollers sampled from Irvine Creek in August 2016 (N = 10) compared to those sampled in 2017 (N = 9). Although the microbiome was different between the two years, the functional genomes of the bacteria did not differ substantially ($p > 0.05$).

	Mean relative frequency (%)	
Pathway	2016	2017
Metabolism	49.82	47.71
Amino acid metabolism	9.46	9.52
Carbohydrate metabolism	10.13	9.18
Energy metabolism	6.86	6.33
Genetic information processing	18.86	17.99
Replication and repair	7.12	6.65
Translation	4.34	4.00
Environmental information processing	15.04	16.46
Membrane transport	12.14	13.09

Table 6 Mean relative frequency (%) of KEGG pathways of the rainbow darters sampled from Irvine (N = 9) and Lutteral Creeks (N = 9) in August 2017. Neither the microbiome composition, nor the functional genome of the bacteria differed substantially in samples taken from either creek ($p > 0.05$).

	Mean relative frequency (%)	
Pathway	Irvine Creek	Lutteral Creek
Metabolism	49.82	47.41
Amino acid metabolism	10.65	10.60
Carbohydrate metabolism	9.78	9.73
Metabolism of cofactors and vitamins	4.58	4.37
Genetic information processing	17.06	16.39
Replication and repair	7.67	7.39
Translation	4.73	4.38
Environmental information processing	12.73	14.40
Membrane transport	11.05	12.32

Table 7 Mean relative frequency (%) of KEGG pathways of the central stonerollers sampled from Irvine (N = 9) and Lutteral Creeks (N = 9) in August 2017. Although the microbiome differed between the two creeks, the functional genome of the bacteria did not differ substantially ($p > 0.05$).

	Mean relative frequency (%)	
Pathway	Irvine Creek	Lutteral Creek
Metabolism	44.97	46.48
Amino acid metabolism	9.35	9.50
Carbohydrate metabolism	9.08	9.91
Energy metabolism	5.08	5.18
Environmental information processing	16.46	16.39
Membrane transport	13.09	13.53
Genetic information processing	15.52	15.96
Replication and repair	6.65	6.87
Translation	4.00	4.28

Discussion

The objectives of this study were three-fold. The first was to assess host species differences in the ability to detoxify NH_3 in the GIT through the production of glutamine (via GS) and glutamate (via GDH, ALT and AST). The second objective was to determine differences in GIT microbiome composition between host species and to quantify any correlations with the ability of the host to detoxify NH_3 through enzymatic pathways. The last objective was to assess the correlation between the GIT microbiome composition, NH_3 detoxification abilities, and sampling location to observe the impact of the environment on this relationship. I examined these relationships by sampling two fish species from several locations within two creeks running through two distinct environments within the same watershed. I then analysed the intestinal bacterial community (IBC) using culture-independent methods alongside the enzyme activities of GS, GDH, ALT and AST in the anterior intestinal tracts of the insectivorous rainbow darter, and the herbivorous central stoneroller. It was hypothesized that the two fish species should have different microbiome compositions and enzyme activities reflecting their dissimilar diets. I further hypothesized that rainbow darters and central stonerollers sampled from various locations along different creeks should have differing intestinal tract bacteria (IB) composition. Finally, I hypothesized that if the symbiotic bacteria were enhancing host ammonia detoxification processes, then differential abilities of hosts to detoxify NH_3 via enzymatic pathways should correlate to differences in the qualitative and quantitative composition, as well as the functional genome of the IBC.

Enzyme activities of glutamine synthetase, glutamate dehydrogenase, and alanine and aspartate aminotransferases

I predicted that the two fish species would have different GIT ammonia detoxification activity levels based on the relative protein content of their diets; specifically, that the protein-rich diet of the rainbow darter would produce higher levels of ammonia via protein catabolism requiring enhanced NH_3 detoxification compared to the herbivorous central stoneroller (Pelster et al. 2014). Indeed, maximal activities of key NH_3 detoxification enzymes in the intestinal tract were significantly higher in the rainbow darters than in the central stonerollers (e.g. Fig. 6; Fig. 7). Enzyme-catalyzed amino acid deamination during protein catabolism makes the GIT a significant contributor to NH_3 production during digestion (Karlsson et al. 2006; Tng et al. 2008; Rubino et al. 2014), necessitating increased detoxification enzyme activities in order to remediate potential toxic local effects of ammonia in the intestinal tract and the tissues at large. As has previously been demonstrated, carnivores can have higher activities of GS, GDH, ALT and AST than do herbivores (Pelster et al. 2014).

Increased GS and GDH levels, along with this upward shift in AST and ALT enzyme activities in our study may also reflect a restructuring of protein metabolism in order to account for the increased energy demands that accompany toxicological stress (Sreedevi et al. 1992; Ramaswamy et al. 1999; Samanta et al. 2014). Aminotransferases act as intermediaries between protein and carbohydrate metabolism (Ramaswamy et al. 1999), and increased activity levels of these enzymes can indicate either enhanced amino acid transamination in order to supply the Krebs cycle with α-keto acids (Rao 2006; Prashanth and Neelagund 2008) or to support processes of gluconeogenesis

by enhancing the supply of amino acids to the liver (Ramaswamy et al. 1999; Rao 2006; Prashanth and Neelagund 2008; Samanta et al. 2014). The correlations that I are observing may reflect this alteration in metabolic pathways and not directly reflect ammonia detoxification. In order to determine the exact pathway that is being augmented, additional experiments that measure ammonia concentration in the GIT are needed.

Microbiome composition of rainbow darters and central stonerollers

Based on previous work demonstrating the effects of species and diet on the composition of the microbiome (e.g. Desai et al. 2012; Sullam et al. 2012; Ghanbari et al. 2015), I hypothesized that the rainbow darters and central stonerollers would have different microbiome compositions from one another. Generally, the GIT of carnivores is dominated by bacteria that produce proteases, while there is a high proportion of cellulase-producing bacteria within the herbivore digestive tract (Liu et al. 2016). The dominance of Proteobacteria in the rainbow darter and central stoneroller (Fig. 12**;** Fig. 15) intestinal tracts for the 2016 and 2017 samples is well-documented across both Perciformes (Goldschmidt-Clermont et al. 2008; Sullam et al. 2012; Bolnick et al. 2014b) and Cyprinids (Wu et al. 2013; Li et al. 2014). The microbiome of the Asian sea bass is similar to that of the rainbow darter in this study (Fig. 12), with a dominance of Proteobacteria (48.8%) followed by Firmicutes (15.3%) (Xia et al. 2014). The gibel (Wu et al. 2013) and bighead (Li et al. 2014) carp had similar microbiome composition to the central stoneroller in this study (Fig. 15), being dominated by Proteobacteria, Fusobacteria and Firmicutes. At present, there does not appear to be a consensus in the literature as to which bacterial phyla are dominant in the carnivorous versus the herbivorous piscine intestinal microbiome, with conflicting reports on the effects of diet manipulation, even between members of a given species (e.g. rainbow trout; Desai et al. 2012;

Wong et al. 2013; Michl et al. 2017). It is generally accepted, however, that microbiomes of carnivores and herbivores of most vertebrate species are different from one another, indicating a broad influence of diet on microbiome composition (Ley et al. 2008; Sullam et al. 2012), although this largely appears to be at the Order or Genus level, rather than at the phylum level (e.g. Campbell and Buswell 1983; Martin-Antonio et al. 2007; de Paula Silva et al. 2011).

Given the contribution of these bacteria to many metabolic functions (Langille et al. 2013), I further predicted that these differences in microbiome composition would correlate to differences in the functional genomes of the IBC. The composition of the rainbow darter and central stoneroller microbiomes were significantly different from one another in both 2016 (Fig. 8) and 2017 (Fig. 9), in line with expectations. Interestingly, however, the function of the microbiome is preserved across species (Table 3) and sampling years (Table 4; Table 5), and between sampling creeks (Table 6; Table 7), despite differences in the bacterial phyla present. This suggests that there is redundancy in the metabolic pathways supplied by the IBC and that multiple bacterial species may be providing identical functions. This may further explain why there are so many conflicting studies about the influence of diet on IBC composition in different species (e.g. rainbow trout; Desai et al. 2012; Wong et al. 2013; Michl et al. 2017). The preservation of microbiome function despite a difference in microbiome composition observed in this study has been also observed previously (Mouchet et al. 2012; Ferrer et al. 2013; Staley et al. 2014). Many metabolic genes are conserved across bacterial phyla (Staley et al. 2014; Ish-Am et al. 2015), so it is possible that despite phylogenetic differences in OTU identity that the genome function would be preserved. Functional genomic studies have their limitations; in this study, NSTI values were above the ideal threshold of 0.03 (Eichmiller et al. 2016), and the accuracy of PICRUSt analyses are limited to the accuracy of the database used for closed-reference OTU picking (Bowman and Ducklow 2015).

Therefore, the functional genomics data collected from this study require additional *ex silico* experimental confirmation (Mukherjee et al. 2017). The exception to these functional similarities is revealed in a comparison of the rainbow darters and central stonerollers sampled in 2017 (Fig. 16), wherein bacterial functional genomic differences the in bacteria mirrored phylogenetic differences within the IBC of the two host species. The reason for this is unclear but may reflect the greater proportion of Proteobacteria in the 2017 central stonerollers (69.62%;Fig. 15b) as compared to the 36.27% found in the rainbow darters of that year (Fig. 12b), compared to 2016, when the two host species had similar relative abundances of that phylum (Fig. 12a; Fig. 15a). This difference is likely driving the differential functional genomes between the two host species.

Regardless of sampling year or location, however, differences in the host species were correlated with differences in microbiome compositions, which is suggestive of an interaction between host and microbiome, leaving room for the possibility that the host may be selecting for a given set of bacterial functions, rather than selecting for the bacteria themselves. Studies investigating the microbiome composition and function are still rare (Xing et al. 2013; Lyons et al. 2017), but perhaps future studies will elucidate selection mechanisms in this context.

Influence of sampling location on rainbow darter and central stoneroller intestinal tract bacterial composition

Since the biotic and abiotic forces within a given environment can influence the microbiome composition, I hypothesized that sampling fish from different locations within a creek and between creeks would result in different microbiome compositions. Rainbow darter (Fig. 10*;* $p > 0.05$) and central stoneroller (Fig. 13; $p > 0.05$) microbiome compositions did not change along the Irvine Creek gradient in the August 2016 samples. Therefore, in August 2017, I resampled these sites

and compared them to sites from a second stream (the Lutteral Creek). As in 2016, the 2017 IBC of the rainbow darter did not vary substantially, qualitatively (Fig. 11a; $p > 0.05$) or quantitatively (Fig. 11b; $p > 0.05$) between sites within the same creek, nor between the two creeks (Fig. 11a, b; $p > 0.05$). There was qualitative variation in the central stoneroller microbiome between the two creeks (unweighted UniFrac analysis; Fig. 14a). However, once weighted abundances were accounted for, these differences were obscured (Fig. 14b). The overwhelming abundance of Proteobacteria, Fusobacteria, Tenericutes and Firmicutes in the *C. anomalum* bacterial community masks more subtle variation between the two creeks. Several studies support the findings that the bacterial community does not change on a geographic basis, as was observed in the rainbow darter. For example, wild-caught and hatchery-raised rainbow trout caught from several lakes were found to share IBCs, regardless of sampling location (Trust and Sparrow 1974). Furthermore, intestinal bacterial communities were found to correlate with the phylogenetic profile of the host, rather than geographic location, in Perciformes sampled from two distinct environments (Roeselers et al. 2011). The IBC of threespine stickleback correlated with habitat type (i.e. river, lake, estuary), rather than geographic location (Schmidt et al. 2015).

Neither host species migrates (Mundahl and Ingersoll 1989; Hicks and Servos 2017), so movement of the fish along the creek gradient can be ruled out as a confounding factor. It is likely that the *diet* of the host is responsible for the static nature of the rainbow darter microbiome and the dynamic nature of that of the central stoneroller. The rainbow trout, threespine stickleback and various Perciformes discussed above are all carnivorous (Linkowski et al. 1983; Wright et al. 1986; Choat et al. 2002; Detrich et al. 2005; Wund et al. 2008; Budge et al. 2012; Callet et al. 2017), as is the rainbow darter, which feeds on mayflies, chironomids and midges (Turner 1921; Adamson and Wissing 1977; Wynes and Wissing 1982; Martin 1984; Schlosser and Toth 1984). It appears

that the lack of correlation between the rainbow darter microbiome and sampling location is due to the capacity for the darter's prey items to relocate outside of the stream from which the fish were sampled, thus weakening the effect of sampling location on microbiome composition. In contrast, the algae and diatoms of the central stoneroller diet (Kraatz 1923; Fowler and Taber 1985; Mundahl and Ingersoll 1989) have a relatively consistent distribution along a stream gradient (Cortez et al. 2012), but can differ between streams, minimizing the effect of diet on microbiome composition and maximizing the effect of sampling creek. A similar effect of the confluence of diet and lifestyle was observed during the sea lamprey transition from larval to parasitic adult stages. The sea lamprey microbiome transitions from one of great phylogenetic diversity during the stationary larval stage to a predominance of bacteria from genus *Aeromonas* once the lamprey entered the adult stage, wherein the lamprey could feed on a variety of prey items from different geographic locations (Tetlock et al. 2012).

The current study demonstrates that broad assumptions about factors shaping the fish microbiome, such as environment and diet may be oversimplified at present. Whether the environment *per se* influences the microbiome appears to be diet-dependent, so it may be more accurate to say that the primary determinant of microbiome structure is the diet, which in and of itself is derived from the environment. The ability of mosquitoes to lay their eggs in any body of water within a given geographic range masks the effect of location on the rainbow darter microbiome, while the inability of algae to leave the stream in which it grows amplifies the effect of location on the microbiome of the central stoneroller.

Conclusion

Ammonia detoxification enzyme activities were generally higher in the carnivorous rainbow darter than in the herbivorous central stoneroller in this study, in line with expectations. I also predicted that sampling location would influence the microbiome of these host species, but this was only true of the central stoneroller. It is possible that this is due to differences in the diet, insofar as the rainbow darter prey items can move out of the creek, minimizing the effect of sampling location on the microbiome in the darter host. This avenue requires further study. Furthermore, differences in the microbiome composition between the two host species did not reflect bacterial functional genomic differences in the ability to detoxify ammonia, which warrants further investigation. This is the first work to address the role of intestinal tract bacteria in the detoxification of nitrogenous waste and is one of only a few studies that measure alanine and aspartate aminotransferases in the intestinal tract of teleosts. The long-term effect on the host of a continuously high rate of glutamine and glutamate production has yet to be determined, and no study that I are aware of has provided insight into the ability of the IBC to assist in host ammonia detoxification. At present, a great deal about host-bacterial interaction in teleosts in general is still unknown, perhaps owing to the vast phylogenetic diversity among fish hosts in comparison to that of other animals (Rimmer and Wiebe 1987; Smith et al. 1996; Nayak 2010; Ray et al. 2012; Clements et al. 2014). A closer examination of the effects of diet in terms of food abundance and diversity, as well as the interactive effects of temperature and diet on the microbiome would be informative.

Chapter 4: Overall significance, conclusions, and future directions

This thesis had two main objectives, which were to assess the effects of temperature, feeding status (Chapter 2) and the microbiome (Chapter 3) on the maximal activities of several enzymes important to energy production and ammonia (NH_3) detoxification in the intestines of teleost fish. To evaluate the effects of temperature and feeding on digestive enzyme activities, I acclimated goldfish to two temperatures (8 and 20°C) and two feeding regimes (fed and unfed) and measured the activities of Na^+, K^+-ATPase (NKA), citrate synthase (CS), pyruvate kinase (PK) and glutamine synthetase (GS) in the kidney, intestine and gill. Temperature is influential on the metabolism of ectotherms, such as fish. Increased temperatures generally increase enzyme activities (e.g. Hazel 1972; van den Thillart and Smit 1984; Jurss et al. 1987; Guderley 2004; McClelland et al. 2006; Couto et al. 2008; Ahmad et al. 2014), although in chapter 2, I observed that the level of the response varies with each enzyme and tissue (Fig. 2-5). It is also possible that temperature can influence the bacteria living within the intestinal tract of the fish host, although due to time constraints, I was not able to measure these effects in the goldfish.

I did, however, assess the effects of sampling location on the composition of the microbiome in the rainbow darter and central stoneroller (Chapter 3). I also examined the contribution of that microbiome to enzyme-mediated NH_3 detoxification, by collecting those fish from two different creeks, on the premise that if the environment shapes the microbiome, fish from different locations should have different gastrointestinal tract (GIT) bacteria. Any differences in bacterial composition in the GIT would correlate to differences in the ability of the host to detoxify post-prandially generated NH_3 Through the activities of GS, as well as glutamate dehydrogenase (GDH), and alanine (ALT) and aspartate aminotransferases (AST).

Overall, I found that the NH_3-detoxification enzyme activities were generally higher in the rainbow darter (Chapter 3, Fig. 4-7). This was in line with findings from previous work that demonstrated a similar pattern in carnivores and herbivores, owing to the higher protein in the carnivore diet (Pelster et al. 2014). I also found that the rainbow darter microbiome did not change between creeks (Chapter 3, Fig. 10-11); however, the enzyme activities were higher in samples from the Irvine Creek than those from the Lutteral Creek. Therefore, I could not attribute the differences in the enzyme activities to changes in the rainbow darter microbiome. In contrast, the stoneroller microbiome was observed to change with sampling location, although an analysis of the functional genome of the bacteria did not correlate with differential NH_3-detoxification abilities between creeks. The lack of correlation between the microbiome and the enzyme activities of these fish suggested that additional factors may be shaping the host physiology. The intestinal tract of the ectotherm is typically similar in temperature to the surrounding water, and this can have impacts on the bacterial community of the GIT, both by increasing the metabolism of the bacteria (Niemi and Taipalinen 1982; Corkrey et al. 2012; Zarkasi et al. 2014), and by changing the composition of the bacterial community (Sugita et al. 1989; Ghanbari et al. 2015; Gajardo et al. 2016; Bestion et al. 2017; Wang et al. 2017). Indeed, I observed microbiome differences between the 2016 and 2017 rainbow darters and central stonerollers in Chapter 3, which correlate with temperature differences between the summers of those two years. Spring and summer of 2017 were colder and had more precipitation than spring and summer 2016 (Government of Canada 2018). Since bacteria-produced digestive enzymes have been shown to assist the host in a number of metabolic functions (reviewed by Clements et al. 2014), shifts in the composition of the microbiome can potentially affect the ability of the fish host to digest and assimilate food.

There are several other confounding factors that deserve consideration. As demonstrated in Chapter 2, the ingestion of a meal, or lack thereof, can also affect the activities of digestive enzymes in goldfish, and many other fish species (e.g. Bélanger et al. 2002; Mommsen et al. 2003; German et al. 2010; Zeng et al. 2012; Zaldúa and Naya 2014). While it was not possible to control for these factors in the wild-caught fish in Chapter 3, and it is not likely that this factor is responsible for enzyme differences between the two creeks, the effect of feeding or fasting cannot be ignored as an influencing factor in enzyme activity levels. Due to difficulty in obtaining wild caught samples, I was unable to directly measure the effects of temperature and feeding status on the enzyme activities in the rainbow darters and central stonerollers. I instead measured these metrics in the goldfish to better appreciate the effects of temperature acclimation on enzyme activities, but repeating this experiment with the wild-caught fish from Chapter 3 would be useful. Another factor that may account for the enzyme activity differences between the two creeks is that of pollution. The sampling sites along the Irvine Creek were surrounded by farmland, and previous work has demonstrated that sublethal exposure to the heavy metals, inorganic nitrogen compounds, herbicides and pesticides that enter waterways through agricultural runoff can trigger upward shifts in ammonia detoxification enzymes in several fish species (Natarajan 1985; Asztalos et al. 1988; Asztalos et al. 1990; Sreedevi et al. 1992; Bálint et al. 1995; Oruç and Üner 1999; Ramaswamy et al. 1999; De Smet and Blust 2001; Rao 2006; Prashanth and Neelagund 2008; Kumar et al. 2012; Samanta et al. 2014). Although this phenomenon was not measured in the current study, it remains a possible explanation for the differential enzyme activities between the two creeks. Follow-up studies assessing water quality from a toxicological perspective would appreciably shed light on the enzyme results. An extension of the enzyme assays to assess the activity of acetylcholinesterase

(AChE) in the brain of fish caught from these creeks would be helpful, as AChE is a valuable tool for assessing pesticide stress in teleosts (Das et al. 2004).

Future Directions

As with any study involving wild-caught samples, it is impossible to completely control for the effects of sex, age, feeding status, or other random abiotic or environmental factors, such as pollution, water flow rates and precipitation. Follow-up studies wherein fish are housed in the lab for a period to control for these factors would be warranted to further explore the effects of the environment on the intestinal bacterial community. It would also be helpful to sample the same species from rivers in a completely different watershed to further examine the effects of geographic distance on the GIT microbiome.

Furthermore, examining the role of temperature and feeding status on the microbiome and enzyme activities in the goldfish would enhance our understanding of how these variables interact to influence the digestive process not only in the intestinal tract, but in the gill and kidney as well.

Gathering data on the impact of temperature on ectotherms, such as fish, and the bacterial communities within them, has important applications to both commercial and recreational fisheries. Identifying temperature optima can assist aquaculture facilities with finding the ideal rearing conditions for fish stocks (Alcorn et al. 2002; Van Ham et al. 2003; Couto et al. 2008; Bernreuther et al. 2013; Ahmad et al. 2014), and understanding the role of the GIT microbiome in digestion can enhance the development of probiotic supplements in aquaculture to improve fish nutritional status and to strengthen the immune systems of intensively raised fish stocks (Thune et al. 1993; Sun et al. 2010; Sun et al. 2013; Piccolo et al. 2014; Li et al. 2015). In terms of wild fish, understanding the relationship of temperature and microbiome to the activities of GIT enzymes

can provide us with a baseline by which I can determine the effects of climate change on different fish species (Somero 2011). Climate change is predicted to be responsible for the extinction of a number of animal species in this century (Ikeda et al. 2017), and as such, animals whose thermal optima is more broad stand a better chance of survival (Pörtner and Farrell 2008). Data from studies like these can be used to better understand the impacts of climate change on fishes (Ikeda et al. 2017).

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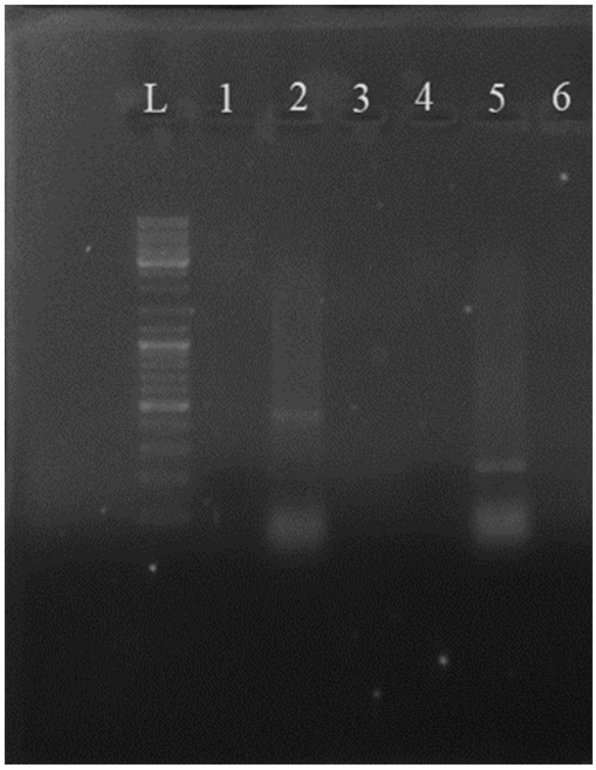
Appendix A: gDNA validation results

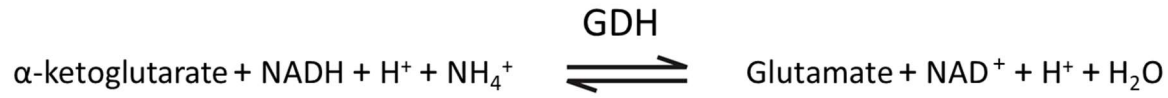


PCR gel of the extraction validation. Lanes are as follows: L) ladder, 1) extraction blank, 2) gDNA control, 3) PCR blank, 4) extraction blank, 5) gDNA control, 6) PCR blank. Lanes 1-3 were prepared with the V3/V4 primer set and lanes 4-6 were prepared with the V6-V7 primer set.

Appendix B: Ammonia detoxification enzyme equations

Glutamine synthetase (GS)

Glutamate dehydrogenase (GDH)



Alanine aminotransferase (ALT)

Aspartate aminotransferase (AST)

Appendix C: Abbreviations used in the thesis

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)

5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB)

Acetylcholinesterase (AChE)

Acetyl coenzyme A (acetyl-CoA)

Adenosine triphosphate (ATP)

Alanine aminotransferase (ALT)

Ammonia (NH_3)

Ammonium (NH_4^+)

Aspartate aminotransferase (AST)

Brush-border membrane enzyme (BBME)

Canadian dollars (CAD)

Citrate synthase (CS)

Dithiothreitol (DTT)

Ethylenediaminetetraacetic acid (EDTA)

Gastrointestinal tract (GIT)

Glutamine synthetase (GS)

Glutamate dehydrogenase (GDH)

Intestinal bacterial community (IBC)

Intestinal bacteria (IB)

Intestinal tract (IT)

KEGG Orthology (KO)

Kyoto Encyclopedia of Genes and Genomes (KEGG)

Na^+, K^+, ATPase (NKA)

Nearest sequenced taxon index (NSTI)

Nicotinamide adenine dinucleotide (NADH)

Operational taxonomic units (OTUs)

Oxaloacetate (OXA)

Phosphoenolpyruvate (PEP)

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt)

Phosphoenolpyruvate

Pyruvate kinase (PK)

Polymerase chain reaction (PCR)

Quantitative insights into microbial ecology (QIIME)

Operational Taxonomic Units (OTUs)

Scanning electron microscopy (SEM)

Statistical Analysis of Metagenomic Profiles (STAMP)

Sucrose, EDTA, imidazole (sodium deoxycholate) buffer (SEI(D))

Tricaine methanesulfonate/mesylate (MS-222