© 2019. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2019) 222, jeb209882. doi:10.1242/jeb.209882

RESEARCH ARTICLE

The role of intestinal bacteria in the ammonia detoxification ability of teleost fish

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ABSTRACT

Protein catabolism during digestion generates appreciable levels of ammonia in the gastrointestinal tract (GIT) lumen. Amelioration by the enterocyte, via enzymes such as glutamine synthetase (GS), glutamate dehydrogenase (GDH), and alanine and aspartate aminotransferases (ALT; AST), is found in teleost fish. Conservation of these enzymes across bacterial phyla suggests that the GIT microbiome could also contribute to ammonia detoxification by providing supplemental activity. Hence, the GIT microbiome, enzyme activities and ammonia detoxification were investigated in two fish occupying dissimilar niches: the carnivorous rainbow darter and the algivorous central stoneroller. There was a strong effect of fish species on the activity levels of GS, GDH, AST and ALT, as well as GIT lumen ammonia concentration, and bacterial composition of the GIT microbiome. Furthermore, removal of the intestinal bacteria impacted intestinal activities of GS and ALT in the herbivorous fish but not in the carnivore. The repeatability and robustness of this relationship was tested across field locations and years. Within an individual waterbody, there was no impact of sampling location on any of these factors. However, different waterbodies affected enzyme activities and luminal ammonia concentrations in both fish, while only the central stoneroller intestinal bacteria populations varied. Overall, a relationship between GIT bacteria, enzyme activity and ammonia detoxification was observed in herbivorous fish while the carnivorous fish displayed a correlation between enzyme activity and ammonia detoxification alone that was independent of the GIT microbiome. This could suggest that carnivorous fish are less dependent on non-host mechanisms for ammonia regulation in the GIT.

KEY WORDS: *Etheostoma caeruleum*, *Campostoma anomalum*, Microbiome, Intestinal tract, Ammonia detoxification

INTRODUCTION

The vertebrate gastrointestinal tract (GIT) is an important nitrogen compound-generating organ (Karlsson et al., 2006; Tng et al., 2008), specifically in the form of ammonia released during protein catabolism. Indeed, ammonia spikes in the teleost GIT lumen (as high as 2 mmol l^{-1} during the digestion of a meal) create an appreciable concentration gradient along which ammonia can diffuse into the plasma (Bucking et al., 2013). However, accumulation in the plasma can generate deleterious effects on fish physiology (Arillo et al., 1981; Shingles et al., 2001; Wicks et al., 2002; McKenzie et al., 2003). To mitigate this accrual, ammoniotelic fish excrete up to 85% of this ammonia to the environment through the gills, kidney, skin and GIT (Smith, 1929; Wright et al., 1995; Bucking and Wood, 2012; Bucking et al., 2013). Fish can also rely on the action of enzymes, such as glutamine synthetase (GS), glutamate dehydrogenase (GDH), and aspartate and alanine aminotransferases (AST and ALT), to detoxify the ammonia in the GIT (Tng et al., 2008; Bucking and Wood, 2012; Bucking et al., 2013; Pelster et al., 2014; Turner and Bucking, 2017) and liver (Mommsen et al., 2003; Wilkie, 2002; Ip and Chew, 2010). This beneficially retains the nitrogen for protein synthesis and somatic cell growth (Tng et al., 2008). There is limited evidence that diet trophic level (i.e. herbivore versus carnivore) may dictate reliance on enzymatic ammonia detoxification, with more protein-rich diets generating higher GDH activity levels along with lower GIT lumen ammonia concentrations (Pelster et al., 2014). Interestingly, intestinal enzyme activity levels are also variable within species examined across geographical locations (e.g. rainbow trout, Bucking and Wood, 2012; Rubino et al., 2014). Functional explanations for both between- and within-species variations are not clear. Traditionally, it has been assumed that ammonia detoxification enzymes are created and supplied by the GIT enterocytes. However, they may also be generated by the bacteria that inhabit the GIT. In fact, genes for GS, GDH, ALT and AST are conserved across bacterial and animal kingdoms (Winefield et al., 1995; Andersson and Roger, 2003; Müller et al., 2006; Amon et al., 2010; Harper et al., 2010; Kim et al., 2010; Jing and Zhang, 2011). It is possible that the variation observed across fish species as well as geographic location can be accounted for through variations in the GIT microbiome.

Complex bacteria, archaea and fungal communities constitute the GIT microbiome and by far the most investigated component are the intestinal bacterial communities (IBCs). It is well established that individual fish species have distinct IBCs (Clements et al., 2014). Furthermore, diet is an important factor shaping the IBC of fishes (e.g. Ringø et al., 2006; Clements et al., 2014). Differences in herbivorous and carnivorous GIT microbiomes could reflect differential host reliance on (and/or supplementation by) bacterial ammonia detoxification pathways. Contributions to host digestive processes by the GIT microbiome are well documented, with bacteria supplying a variety of catabolic and anabolic pathways for hosts (Goodrich and Morita, 1977a,b; Lesel et al., 1986; Sugita et al., 1991; Skrodenytė-Arbačiauskienė et al., 2008; Nayak, 2010; Ringø et al., 2012). However, it is unknown whether bacteria specifically support ammonia detoxification in teleosts. Complicating this potential relationship is the impact of the environment on the GIT microbiome, particularly in wild fish populations. The fish GIT is sterile until larvae emerge from the egg (Campbell and Buswell, 1983; Al-Harbi and Uddin, 2004; Dehler et al., 2017), suggesting that fish may form location-specific bacterial communities. Furthermore, external factors not related to

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Received 3 July 2019; Accepted 13 November 2019

Journal of Experimental Biology (2019) 222, jeb209882. doi:10.1242/jeb.209882

host physiology, such as temperature (Clements et al., 2014; Wang et al., 2017), can alter the microbiome composition of the water and the intestine (Wang et al., 2013; Schmidt et al., 2015; Eichmiller 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 or geographic locations). Only a handful of conflicting studies have examined the influence of sampling location on IBCs, with some studies demonstrating a correlation between geographic sampling location and microbiome (Eichmiller et al., 2016; Skrodenytė-Arbačiauskienė et al., 2006; Sullam et al., 2015; Llewellyn et al., 2016; Yang et al., 2018), while others did not (Trust and Sparrow, 1974; Roeselers et al., 2011; Schmidt et al., 2015). It is possible that alterations in the microbiome may impact enzymatic pathways supplied, providing an explanation for experimental variation as seen in Bucking et al. (2013) versus Rubino et al. (2014), as well as observed species differences.

To study the nature of the microbiome–host relationship in GIT nitrogen metabolism, we examined two fish species, the carnivorous rainbow darter (*Etheostoma caeruleum* Storer 1845) and the algivorous central stoneroller (*Campostoma anomalum* Rafinesque 1820), chosen because of their overlapping geographic locations but dissimilar niches. Firstly, we hypothesized that the higher protein content in the carnivore's diet would drive higher intestinal enzyme activity levels in order to detoxify a greater concentration of GIT ammonia (Wilson, 1973; Pelster et al., 2014). We further hypothesized that the dissimilar intestinal enzyme activities and ammonia concentrations would correlate with IBCs (Clements et al., 2014), and that alteration of the IBCs would impact ammonia detoxification enzyme activity. Finally, to measure the strength of this association and to observe in a natural environment, we measured these relationships in several field sampling locations. We predicted that if the bacteria were enhancing host ammonia detoxification, any differences in enzyme activities observed between the sampling locations should also correlate to differences in IBCs. Altogether, this evidence would suggest that the microbiome may ameliorate ammonia loads in the GIT and provide relief for nitrogen detoxification in teleost fish according to dietary trophic level.

MATERIALS AND METHODS

All experiments were carried out according to federal guidelines, collection permits, and approved animal care protocols from York University. All chemical reagents were supplied by Sigma-Aldrich unless otherwise noted.

Fish collection

Fish of mixed sex were collected from the field at three separate points within a single waterbody in late July–early August. The first waterbody examined was the Irvine Creek (Ontario, Canada) where traps were laid at: Wellington Road 16 (Belwood, ON; 43°76′78.74″ N, −80°35′89.88″ W; site 1), Line 2 (Fergus, ON; 43°74′74.97″ N, −80°37′64.64″ W; site 2) and Gerrie Road (Elora, ON; 43°74′03.13″ N, −80°38′98.35″ W; site 3). The second waterbody was the Lutteral Creek where traps were laid at: Line 7 (Belwood, ON; 43°73′41.76″ N, −80°26′20.67″ W; site 4), Line 6 (Rockwood, ON; 43°71′93.43″ N, −80°26′19.75″ W; site 5) and Line 5 (Wellington ON; 43°70′80.51″ N, −80°27′03.03″ W; site 6). At each location, central stonerollers (*Campostoma anomalum*; body mass 5.06±3.03 g, *N*=42; mean±s.e.m.) were collected using minnow traps while rainbow darters (*Etheostoma caeruleum*; body mass 0.56±0.36 g, *N*=42) were caught by hand using dip nets. The temperature (20±0.3°C), oxygen saturation (99.7±0.49%) and pH (7.68±0.024) were similar across all sites according to repeated measures ANOVAs performed on each variable ($P>0.05$). Fish were brought back to York University for dissection in 28-quart (~26 l) Igloo Iceless coolers (Katy, TX, USA), which were filled with creek water obtained from their individual locations, kept at creek temperature (~20°C), and supplied with constant aeration. Rainbow darters and central stonerollers collected from each individual location were transported together; however, fish from different locations were transported separately. Both rainbow darters and central stonerollers from the Irvine Creek alone were sampled at the same locations in the previous year to measure repeatability of variance along the creek from year to year.

Upon arrival at York University, fish were separated into two groups: one for immediate dissection and one for housing and IBC removal. All experiments were conducted with approval from the Animal Care Committee at York University.

Dissection and tissue handling

Fish used for immediate dissection were euthanized in buffered (pH 7.4) tricaine methanesulfonate (MS-222; Sigma-Aldrich, Oakville, ON, Canada) and the entire GITs were removed following an incision along the ventral surface of the fish. Before dissection, all lab surfaces, gloves, and the external surface of the fish were sprayed and wiped with 75% molecular grade ethanol (Sigma-Aldrich) while dissection tools were soaked in 75% ethanol stored in sterile Falcon tubes. Between individual fish dissections, all surfaces were sprayed with ethanol while dissection tools were soaked in a fresh Falcon tube of 75% ethanol. Following removal of the GIT, the lumen was cleared of chyme by gently squeezing with sterile forceps. This chyme was collected for further analysis below. Subsequently, the posterior section of each GIT was placed in sterile bullet tubes and stored for gDNA extraction. The remaining anterior sections were then freeze clamped on dry ice and stored for enzyme assays. All tissues were maintained at −80°C until required.

IBC removal

Fish that were housed at York University were kept in a static, aerated tank containing sterilized (autoclaved) City of Toronto tap water. Fish were housed separately according to species (*N*=20) and creek (*N*=10). However, fish collected from different locations within the waterbodies were kept together. Fish were then further separated into two treatments, antibiotic exposure (*N*=5 for each species and each creek) or sham (*N*=5 for each species and each creek, no antibiotic exposure). For the antibiotic exposure, the fish were exposed to a mixture of antibiotics designed to remove the IBC (75 ppm each of sisomycin, ampicillin, amphomycin and penicillin; Bucking et al., 2013) for a period of 5 days. The water was changed every 12 h, and fresh antibiotics were added. The sham treatment consisted of sterile water changes every 12 h. Following antibiotic exposure (or sham), the animals were dissected as described above. Fish were fed sterile fish feeds. Rainbow darters were fed sterile commercial fish pellets (Zeigler; Pentair, FL, USA), while central stonerollers were fed sterilized algae.

Enzyme assays

Approximately 20 mg of freeze-clamped anterior intestine was homogenized with a glass homogenizer with 200 µl ice-cold homogenization buffer containing 20 mmol l^{-1} HEPES (pH 7.4), 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, per McClelland et al. (2006). Following homogenization, samples were centrifuged (10,000 ***g*** for 5 min) at 4°C in order to pellet cellular debris. The supernatant was placed on ice for enzyme

and protein analyses. All enzyme assays were read at 20°C and were optimized for maximal activity prior to measurement.

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 mmol l^{-1} glutamine, 15 mmol l^{-1} hydroxylamine, 0.4 mmol l^{-1} ADP, 20 mmol l^{-1} $NaAsO_4$, 3 mmol l^{-1} $MnCl_2$ in a 50 mmol l^{-1} 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). Briefly, the assay measured the formation of glutamate, coupled to the oxidation of NADH in the presence of ADP, with 14 mmol l^{-1} 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 mmol l^{-1}), α-ketoglutarate (10.5 mmol l^{-1}; omitted for control), lactate dehydrogenase (10 U ml^{-1}) and alanine (200 mmol l^{-1}). AST running buffer contained NADH (0.2 mmol l^{-1}), α-ketoglutarate (8 mmol l^{-1}; omitted for control), malate dehydrogenase (8 U ml^{-1}) and aspartate (40 mmol l^{-1}).

Enzyme and protein assays were run on 96-well plates and were read using Gen 5 software (v. 4.6) (Thermo Fisher Scientific, Toronto, ON, Canada) on a BioTek microplate reader (BioTek, Wisnooski, VT, USA). Activities for each sample were measured in triplicate and averaged for downstream data analysis. GS activities were calculated as µmol substrate converted min^{-1} g $tissue^{-1}$; all other enzyme activities were calculated as µmol substrate converted min^{-1} mg $protein^{-1}$, using appropriate extinction coefficients for each substrate. Total homogenate protein content was measured using the protocol of Bradford (1976) using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Gut ammonia concentrations

Chyme samples collected from the intestine were centrifuged (7750 ***g*** for 5 min at 4°C), and the supernatant fluid was frozen for later analysis. Ammonia concentrations in chyme supernatant extracts were determined using a Raichem commercial assay (Cliniqa Corporation, San Marcos, CA, USA).

Bacterial gDNA extraction and validation

Extraction of posterior GIT bacterial gDNA was performed using the QIAmp Stool Kit (QIAgen, Toronto, ON, Canada) according to the manufacturer's instructions. The kit reagents, pipettes, pipette tips, tubes and forceps were all UV-irradiated 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 the 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.

Contamination was screened for through polymerase chain reaction (PCR) amplification of 16 s rRNA hypervariable regions V3–V4 and V6–V7. Primers 341f (5′-CCTACGGGNGGCWGCAG-3′) and 785r (5′- GACTACHVGGGTATCTAATCC-3′) were used to amplify the V3–V4 region (e.g. Thijs et al., 2017), while 967f (5′-CAACGCGAAGAACCTTACC-3′) and 1046r (5′- CGACAGCCATGCANCACCT-3′) amplified the V6–V7 region (e.g. Sogin et al., 2006). Both sets of primers were used to ensure detection of any contamination. Each PCR consisted of 35 cycles of 30 s at 94°C, 45 s at the annealing temperature for each respective primer set, and 60 s at 72°C for elongation (DreamTaq; Thermo Fisher Scientific, Waltham, MA, USA). PCR products were verified by electrophoresis on 1.5% agarose gels containing ethidium bromide (Thermo Fisher Scientific). 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. After ensuring no contamination had occurred, as determined by a lack of bands in the negative controls, the extracted DNA was quantified using the Quant-IT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Bacterial quantification

The gDNA extracted from the fish that were housed within York University was used to semi-quantitatively assess the impact of antibiotic (N=5 for each species and each creek) or sham (N=5 for each species and each creek) exposure via qPCR. Isolated chyme gDNA samples were diluted to 40 ng $µl^{-1}$ (NanoDrop ND-1000 Spectrophotometer; Nanodrop Technologies, Wilmington, DE, USA). The PCR reactions [2 µl of mixed universal primers (Pedersen et al., 2013) 804f and 926r (10 µM), 2 µl of LightCycler FastStart DNA Master SYBR Green (Roche), 1.5 µl of DNA template (40 ng $µl^{-1}$) and 14.5 µl of nuclease free water] were carried out in a Roche Light Cycler 96 (Roche). Reaction times and cycling conditions were 95°C for 10 min, 40 cycles of 95°C for 10 s, 58°C for 5 s, and 72°C for 1 min, followed by termination with a melt curve analysis. Obtained Ct values were normalised against the amount of DNA used for the PCR reactions after verification that the primers amplified with an efficiency of about 100% using a standard curve and dilutions of pooled samples.

Bacterial gDNA sequencing

Of the seven fish collected from each location and immediately sampled, only 3 randomly chosen samples were sent for sequencing analysis performed by Génome Québec at McGill University (Montréal, QC, Canada). Génome Québec first created 16S libraries according to manufacturer protocols (Illumina) using the primer pair 341f (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) (e.g. Herlemann et al., 2011) These libraries were created using multiplexing indices and Illumina sequencing adapters and quantified and normalized before sequencing as per manufacturer protocol (Illumina). The libraries were then sequenced using a paired-end format.

Bioinformatics analysis

Data analysis was completed using Quantitative Insights into Microbial Ecology, version 2017.11 (QIIME2) (Caporaso et al., 2010). Data were demultiplexed using the demux plug-in (https://github.com/qiime2/q2-demux) before analysis. 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 cut-off quality score of 30. Sequences were then de-noised to remove sequencing errors and chimeras removed. Phylogenetic trees were created using FastTree2 (Price et al., 2010), and α- and β-diversity analyses were run using default QIIME2 settings. Unweighted and weighted UniFrac analyses (Lozupone et al., 2005, 2007) were carried out 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. Data used in this analysis are available in the SRA repository (BioProject accession number PRJNA549302: www.ncbi.nlm.nih.gov/bioproject/PRJNA549302; SRA number SRP201669: trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP201669).

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Statistics

As bacterial sequence data were non-parametric, comparisons between and within creeks and species were done using PERMANOVA and Kruskal–Wallis tests through QIIME2. Parametric bacterial load quantities, enzyme activity data, and luminal gut ammonia concentrations were analysed with SigmaStat 3.0 (Systat Software Inc., San Jose, CA, USA). The impact of antibiotic exposure on bacterial load quantities and enzyme activities was tested with a paired *t*-test for each species and location. For samples obtained from wild-caught fish, a one-way ANOVA was used to determine whether there was a significant difference between sites within the same creek for each species. Upon determining that differences were not significant within the same creek, enzyme activities and gut ammonia concentrations were averaged for each species within the same creek. The resulting enzyme activities and gut ammonia concentrations were then compared using a two-way ANOVA with fish species and creek location as factors. Since we observed no interaction, we were able to proceed with separate one-way ANOVAs examining each of the factors. $P<0.05$ was considered significant for all statistical tests performed. Interaction effects were not detected for any of the two-way ANOVAs. All values are reported as means±s.e.m. (*N*=individual sample). Statistical power analysis was used to establish appropriate sample sizes.

RESULTS

Enzyme activities and GIT ammonia concentrations

When examining fish caught directly from the wild, there were no significant differences in the enzyme activities or luminal ammonia concentrations found at each site within an individual waterbody. Therefore, for each species the values were averaged along the length of each creek.

Overall, for each enzyme measured, activity was significantly higher for fish sampled from Irvine Creek than it was for those from Lutteral Creek regardless of host identity (GS: $P<0.01$, Fig. 1A; GDH: $P<0.01$, Fig. 1B; ALT: $P<0.05$, Fig. 1C; AST: $P<0.05$; Fig. 1D). Within Irvine creek, GS activities were not different between host species ($P>0.05$, Fig. 1A). In contrast, a host difference was detected in Lutteral Creek where GS activities for the rainbow darters were significantly ($P<0.01$) higher compared with the central stonerollers (Fig. 1A). In contrast, rainbow darters had significantly higher GDH activities than the central stonerollers within Irvine Creek ($P<0.001$) while within Lutteral Creek, there was no significant difference between species ($P>0.05$, Fig. 1B).

As with GDH, ALT activity was significantly higher for rainbow darters sampled from Irvine Creek compared with central stonerollers ($P<0.001$; Fig. 1C). However, as with GS, ALT activity in rainbow darters was also greater than activity in central stonerollers in Lutteral Creek ($P<0.01$; Fig. 1C). AST activities displayed similar differences between species with rainbow darters exhibiting significantly ($P<0.001$) higher activities compared with central stonerollers within both Irvine Creek and Lutteral Creek (Fig. 1D). In general, GIT luminal ammonia concentrations were greater in both fish species from Irvine Creek compared with Lutteral Creek (Table 1), correlating with increased enzyme activities in fish from Irvine Creek (Fig. 1). Furthermore, rainbow darters had significantly higher ammonia concentrations in the lumen compared with central stonerollers (Table 1) correlating with consistently higher ALT and AST activities (Fig. 1C,D).

Microbiome community analysis between host species

Comparing the two host species regardless of stream origin, a similar bacterial species richness, as determined by the observed number of OTUs ($P=0.45$) or the Shannon index ($P=0.39$; Table 2), was observed. However, Simpson's evenness revealed a significant difference between the rainbow darter and the central stoneroller ($P<0.05$; Table 2). Additionally, β-diversity analyses indicated that the host species microbiomes were different using both unweighted (Fig. 2A) and weighted UniFrac analyses (Fig. 2B; Bray–Curtis dissimilarity, $P<0.01$). In particular, the rainbow darter intestine mainly contained bacteria from phyla Proteobacteria (41.11±6.52%, *N*=9), Actinobacteria (17.43±5.10%, *N*=9) and Firmicutes (12.56±3.85%, *N*=9) (Fig. 3A). In contrast, the central stoneroller intestine was dominated by Proteobacteria (70.88±5.98%, *N*=9), followed by

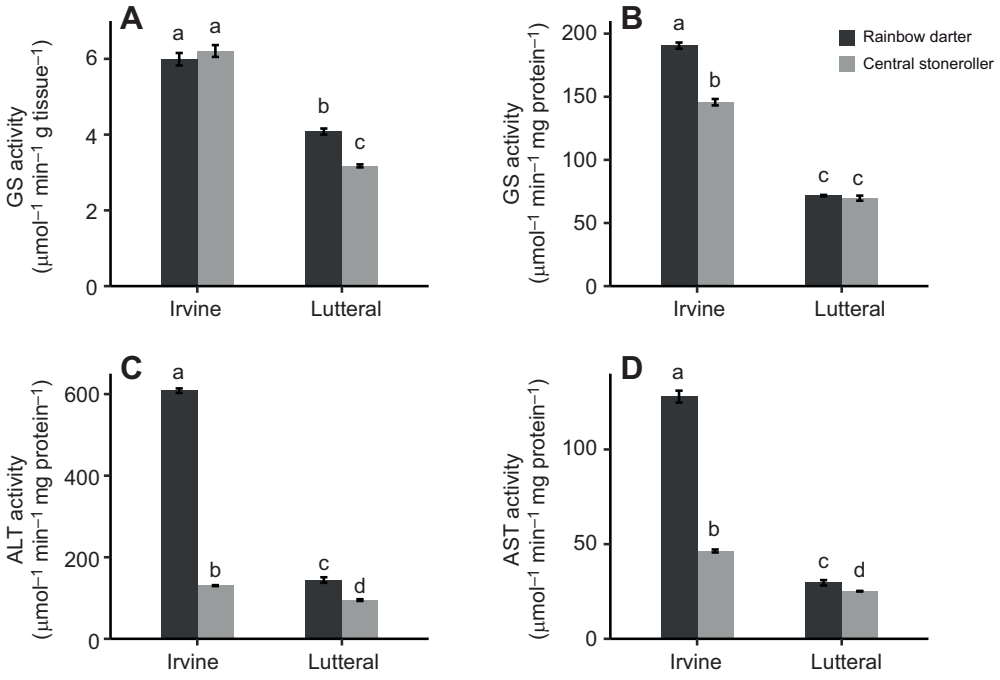

Fig. 1. Intestinal ammonia detoxification enzyme activities of rainbow darters and central stonerollers taken from Irvine Creek or Lutteral Creek. (A) Glutamine synthetase (GS); (B) Glutamate dehydrogenase (GDH); (C) Alanine aminotransferase (ALT); (D) Aspartate aminotransferase (AST). Values are means±s.e.m. *N*=21 rainbow darters and *N*=21 central stonerollers in each location. Bars that share letters within each panel are not significantly different ($P>0.05$). For each enzyme, a two-way ANOVA with creek and species as factors was used to determine statistical differences. There was no interaction between factors ($P>0.05$).

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Table 1. Ammonia concentrations in the gastrointestinal tract lumen of rainbow darters and central stonerollers captured at 6 locations

	Irvine Creek			Lutteral Creek		
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Rainbow darter	1.8±0.4[a]	1.7±0.3[a]	1.9±0.4[a]	1.0±0.2[b]	0.9±0.1[b]	0.9±0.2[b]
Central stoneroller	0.6±0.1[c]	0.5±0.1[c]	0.6±0.1[c]	0.1±0.1[d]	0.1±0.1[d]	0.2±0.1[d]

Lumen ammonia concentration (mmol l^{-1}; mean±s.e.m.) is shown for N=7 fish from each site. Numbers sharing letters indicate no significant difference (determined by a two-way ANOVA with species and site as factors; no interaction effect was detected). Owing to a lack of interaction detected with the two-way ANOVA, each variable was analyzed with a one-way ANOVA.

Fusobacteria (14.68±5.20%, N=9) and Firmicutes (3.23±1.26%, N=9) (Fig. 3B).

Microbiome community analysis of rainbow darters from sampling locations

The microbiome composition of the host species did not change along either creek as shown by similar (P>0.05) α-diversity (Table 2) and β-diversity metrics (unweighted UniFrac, Figs S2A and S3A; weighted UniFrac, Figs S2C and S3C) at each site. This lack of difference along the length of a creek was observed in a previous sampling of Irvine Creek a year earlier (Fig. S4A,B). When comparing between the creeks, both unweighted (Fig. 4A; P>0.50) and weighted UniFrac (Fig. 4B; P>0.30) analyses indicated that bacterial communities were similar regardless of which creek was sampled. Furthermore, there were no differences in the microbiome between creeks according to α-diversity metrics [number of observed OTUs (P>0.40); Shannon Index (P>0.30), Simpson's evenness (P>0.40); Table 2].

Microbiome community analysis of central stonerollers from sampling locations

Comparatively, the microbiome for central stonerollers also did not change along the Irvine Creek in terms of α-diversity (P>0.05; Table 2) or β-diversity metrics (Figs S2B and S3B). This was again supported by previous measurements in the same locations a year earlier (Table S1; Fig. S4C,D). Additionally, the central stoneroller microbiome did not change along the Lutteral creek in terms of α-diversity (Table 2; P>0.05) and β-diversity metrics (Fig. S3B,D). However, according to the unweighted UniFrac analysis (Fig. 4C), bacterial composition was dependent on individual creek location (P=0.018).

Antibiotic treatment of IBC

The impact of antibiotics was evident in the drastic reduction in total bacterial load detected in the intestine (Table 3). Further, exposure to antibiotics significantly reduced ALT activity observed in the central stoneroller obtained from Irvine Creek alone (Table 3). No other enzyme activities were affected in the central stoneroller or rainbow darter regardless of stream origin or treatment.

DISCUSSION

We predicted that rainbow darters and central stonerollers would have dissimilar intestinal nitrogen metabolism levels based on the relative protein content of their diets, signifying a trophic level difference in reliance on enzymatic detoxification of ammonia in the GIT (Pelster et al., 2014). Specifically, we predicted that higher ammonia levels in the carnivorous fish GIT following animal protein digestion would require enhanced ammonia detoxification compared with the herbivorous fish. Indeed, GIT ammonia levels were higher in the carnivore versus the algivore (Table 1) and maximal activities of key ammonia detoxification enzymes in the intestinal tract were correspondingly higher in the rainbow darters compared with the central stonerollers (Fig. 1). Enzyme-catalyzed amino acid deamination during protein catabolism makes the GIT a significant contributor to ammonia 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. This has been observed in the rainbow trout (Rubino et al., 2014), as well as a few wild fish species (Bucking et al., 2013; Pelster et al., 2014). Our findings add additional species to this list and broaden our understanding of this relatively unexplored phenomenon. Higher GS and GDH levels, along with this upward shift in AST and ALT enzyme activities in our study could 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), and may not directly reflect ammonia detoxification. Additionally, lack of correlation between IBC and enzyme activities is not likely to be an artefact of regional differences in bacteria and enzymes between the anterior and posterior intestinal tracts, since there are no obvious morphological barriers that would inhibit movement or exchange of bacteria along the tract. Similar findings were demonstrated in brown trout, wherein mid and posterior intestines had similar microbiome compositions (Al-Hisnawi et al., 2015). Increased aminotransferase activities can indicate enhanced amino acid transamination in order to supply the Krebs cycle with α-keto acids (Prashanth and Neelagund, 2008; Rao, 2006) or to support gluconeogenesis by

Table 2. Alpha diversity indices for rainbow darters and central stonerollers caught at each site

		Irvine Creek			Lutteral Creek			
		Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Mean
Rainbow darter	Observed OTUs	423±99	519±394	373±157	115±55	321±92	187±72	322±71
	Shannon index	5.96±0.82	6.72±1.26	6.90±1.04	6.25±0.14	6.28±1.93	6.34±0.13	6.50±0.34
	Simpson's evenness	0.18±0.05	0.20±0.03	0.23±0.06	0.26±0.06	0.35±0.03	0.21±0.05	0.23±0.02*
Central stoneroller	Observed OTUs	341±182	481±182	294±42	214±60	203±25	291±52	305±43
	Shannon index	5.84±0.53	6.29±0.02	5.66±0.55	5.72±1.15	6.55±0.40	6.08±0.49	6.03±0.22
	Simpson's evenness	0.11±0.03	0.11±0.05	0.09±0.03	0.20±0.09	0.19±0.04	0.16±0.04	0.14±0.02*

N=3 fish for each site. *Significant difference (P≤0.05; one-way ANOVA and Tukey test). The remaining values were not significantly different from one another. All values are means±s.e.m.

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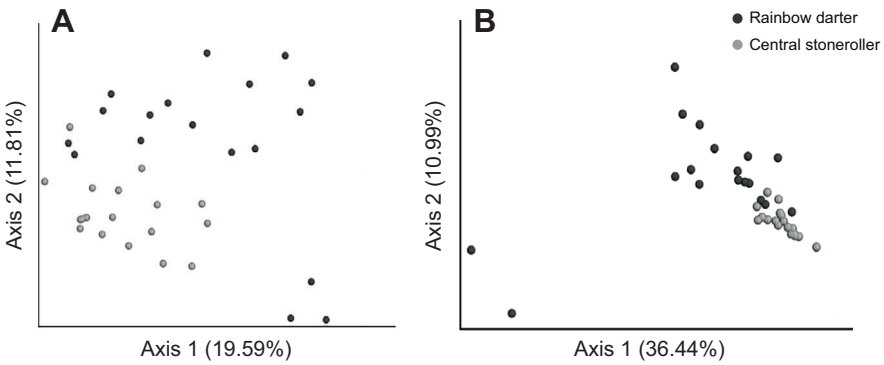

Fig. 2. Effect of host species on microbiome composition in rainbow darters and central stonerollers taken from Irvine and Lutteral creeks. Pairwise community comparisons were determined with (A) unweighted and (B) weighted UniFrac analyses. Each dot represents an individual sample. Significant differences between the species were observed in both the unweighted and weighted UniFrac analyses (P<0.01). N=18 rainbow darters and N=18 central stonerollers.

enhancing the supply of amino acids to the liver (Ramaswamy et al., 1999; Samanta et al., 2014; Prashanth and Neelagund, 2008; Rao, 2006). Although speculative, the water conditions may offer additional insight into the differences observed. Irvine Creek runs through farmland, and thus may contain higher levels of agricultural run-off while Lutteral Creek runs through more forested areas and may be freer from anthropogenic pollution as a result. Higher levels of toxicological stress may be driving higher enzyme activities observed in fish taken from the Irvine Creek (Fig. 1), although a response to toxicants should also reflected in altered microbiomes of both species (Adamovsky et al., 2018). As this was not observed (Table 2, Fig. 4A,B versus C), the enzyme activity differences between creeks may not be driven by such factors. This is an area of possible future investigation.

We proposed that the IBCs may be contributing to the observed differential reliance on enzymatic detoxification (Fig. 1) and that this represents a trophic-level difference found within teleosts. Firstly, the IBCs must then be different between the species and secondly, direct alteration of the IBCs should reflect a change in enzyme activity. Indeed, the rainbow darter and central stoneroller microbiomes were significantly different from one another for both study years (Fig. 2; Fig. S5). Specifically, the rainbow darter intestinal bacterial community was dominated by Proteobacteria, followed by Actinobacteria and Firmicutes (Fig. 3A), in line with previous findings in Perciformes (Sullam et al., 2012; Bolnick et al., 2014). Furthermore, the central stoneroller microbiome was also dominated by Proteobacteria, followed by Fusobacteria and Firmicutes (Fig. 3B), in line with previous work in Cyprinid species (Wu et al., 2013; Li et al., 2014). 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: Wong et al., 2013 versus 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 (Campbell and Buswell, 1983; Martin-Antonio et al., 2007; Ley et al., 2008; de Paula Silva et al., 2011; Sullam et al., 2012). Regardless of which bacteria species may be aiding in ammonia detoxification importantly, when the IBCs in each species were altered through antibiotics, there was a corresponding alteration in the activity of ALT (Table 3); however, this was only observed in the central stoneroller. This suggests that the carnivorous fish is not (or is less) reliant on bacterial inhabitants for ammonia detoxification. This could be due to the higher ammonia content within the gut

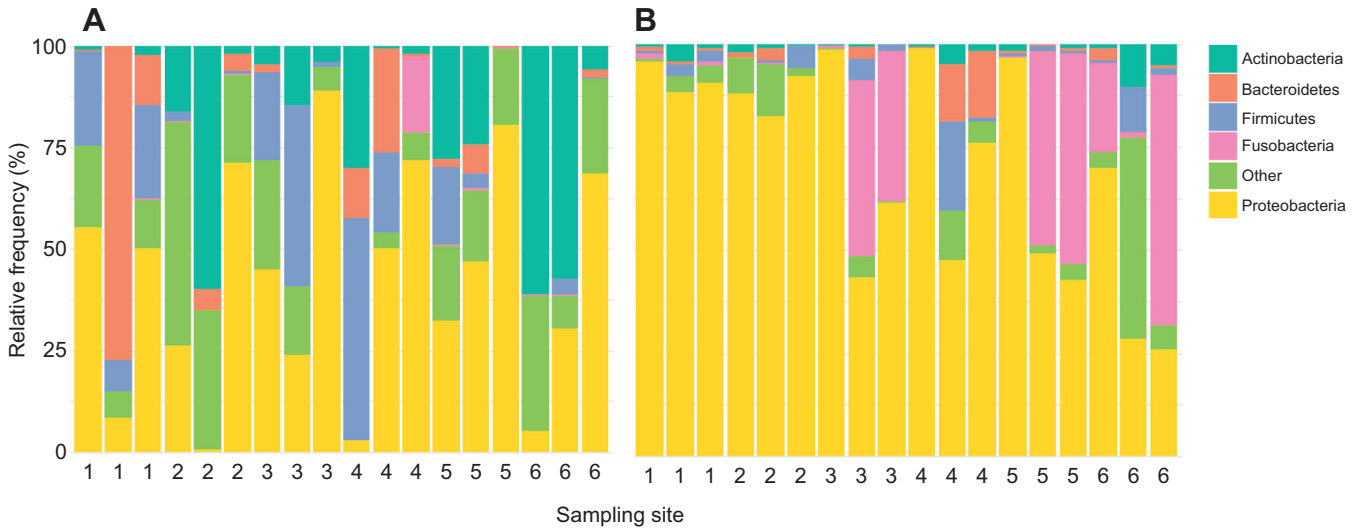

Fig. 3. Relative abundance of intestinal bacterial phyla from rainbow darters and central stonerollers caught at 6 sites. (A) Rainbow darters; (B) central stonerollers. Each bar represents the phyla observed in an individual fish. The sites were as follows: Irvine Creek: site 1 (Belwood, ON), site 2 (Fergus, ON), site 3 (Elora, ON); Lutteral Creek: Site 4 (Belwood ON), site 5 (Rockwood, ON), site 6 (Wellington, ON). The 'Other' bacteria are phyla that represent <10% of the abundance combined into a single category for clarity. In the rainbow darter microbiome, this constitutes approximately 28% of the total diversity, and in the central stoneroller, approximately 12% of the bacterial phyla are counted as 'Other'.

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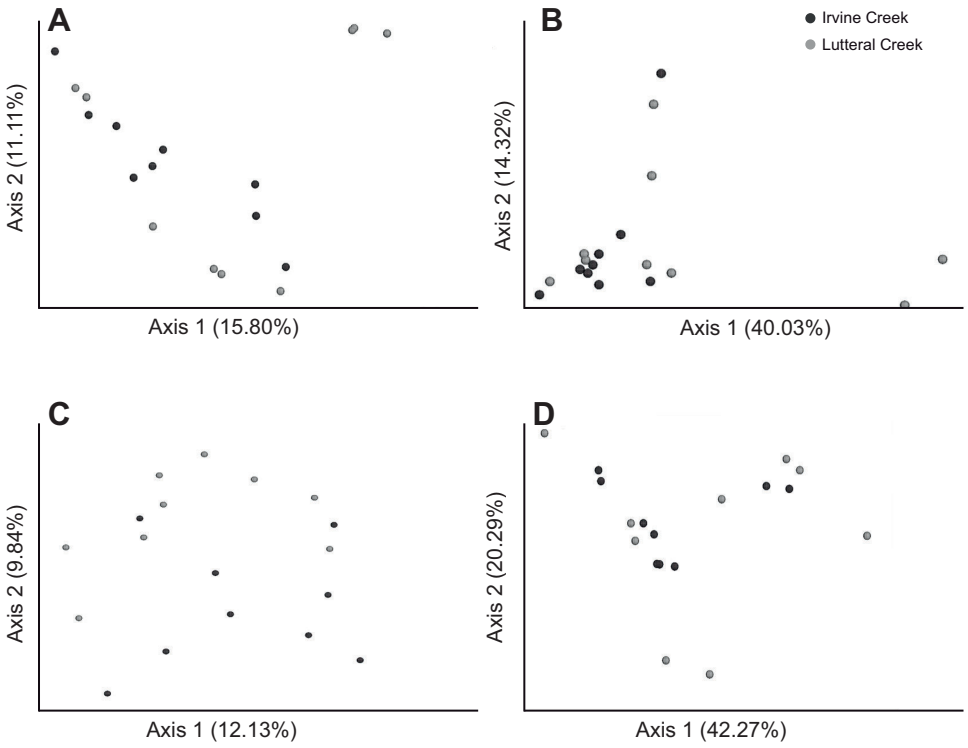

Fig. 4. Effect of sampling location on microbiome composition in fish sampled from Irvine Creek and Lutteral Creek. Each dot represents an individual sample. Pairwise community comparisons of rainbow darters (*N*=18) sampled from the two creeks were determined with (A) unweighted and (B) weighted UniFrac analyses. No significant differences between the creeks were observed in either unweighted (*P*=0.095) or weighted (*P*=0.13) analyses. Central stoneroller (C) unweighted UniFrac analysis determined that the microbiome differed between the two creeks (*P*=0.018; *N*=18), but this was not observed in the (D) weighted UniFrac analysis (*P*=0.524; *N*=18).

(Table 1), which would make reliance on exogenous factors risky. If the bacteria were to change, as can occur with various biotic and abiotic factors (Nayak, 2010; Sullam et al., 2012; Clements et al., 2014; Egerton et al., 2018), the fish could potentially be exposed to lethal ammonia concentrations. However, in contrast, the central stoneroller has ammonia concentrations similar to teleost plasma values (Table 1), suggesting that reliance on bacteria could be feasible as any disruptions would likely not prove toxic. This hypothesis requires further investigation.

In order for this relationship to be meaningful, it should be preserved across locations and be repeatedly detected. As the environment can influence the microbiome composition, we hypothesized that sampling fish from different locations could result in dissimilar microbiome compositions. However, if the fish were depending on the microbiome community for functionality, this alteration in community structure would be reflected in an alteration in provided enzyme pathways. Microbiome composition of both the rainbow darter and central stoneroller did not change along the length of the Irvine Creek (Fig. S4) or Lutteral Creek (Fig. S3), nor did enzyme activities or luminal ammonia concentrations (Fig. 1; Table 1). This was further supported within Irvine Creek during sampling a year earlier (Fig. S2; $P>0.05$). Furthermore, we observed significant qualitative variation in the central stoneroller microbiome between the two creeks (unweighted UniFrac analysis, Fig. 4C) that corresponded to alterations in the enzymatic activity (Fig. 1), supporting a potential role for the IBCs as noted previously. Comparatively, the rainbow darter IBCs did not vary qualitatively or quantitatively between the two creeks (Fig. 4A,B; $P>0.05$) despite variations in enzyme activities (Fig. 1) and ammonia levels (Table 1), further supporting a lack of reliance on bacteria-supplied pathways suggested earlier. Neither host species is migratory (Mundahl and Ingersoll, 1989; Hicks and Servos, 2017), so movement of the fish along the length of the creek can be ruled out as a confounding factor. It is possible that the diet itself is responsible for the static nature of the rainbow darter microbiome and the dynamic nature of that of the central stoneroller. Previous studies have shown a lack of geographic effect on the microbiome in rainbow trout (Trust and Sparrow, 1974) and several Perciform species (Roeselers et al., 2011), which are all carnivorous (e.g. Mundahl and Ingersoll, 1989; Callet et al., 2017; Budge et al., 2012; Wund et al., 2008; Linkowski et al., 1983; Wright et al., 1986), as is the insectivorous rainbow darter (e.g. Turner, 1921; Martin, 1984; Schlosser and Toth, 1984). It could be that the lack of correlation between the rainbow darter microbiome and sampling location is due to the ability of the darter's prey items to relocate outside/along 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 (Fowler and Taber, 1985) have a relatively consistent distribution along the length of a stream (Cortez et al., 2012), but can differ between streams maximizing the effect of sampling creek.

Table 3. Relative impact of antibiotics on intestinal bacterial load and enzyme activities of rainbow darters and central stonerollers

	Irvine Creek					Lutteral Creek				
	Bacterial load	GS activity	GDH activity	AST activity	ALT activity	Bacterial load	GS activity	GDH activity	AST activity	ALT activity
Rainbow darter	0.3±0.1*	1.7±0.5	0.7±0.2	0.9±0.1	1.1±0.2	0.2±0.3*	1.3±0.2	1.1±0.4	0.8±0.3	0.9±0.1
Central stoneroller	0.4±0.2*	0.5±0.3	0.7±0.4	0.6±0.5	0.3±0.1*	0.4±0.1*	0.8±0.3	0.8±0.2	0.9±0.3	0.6±0.4

Values are mean±s.e.m. relative to sham-treated controls. *N*=5; *Significant difference from the sham value determined by an unpaired *t*-test ($P<0.05$).

Conclusion

Our first objective was to assess fish species differences in the ability to detoxify ammonia in the GIT through the production of glutamine (via GS) and glutamate (via GDH, ALT and AST). Our results revealed a species-specific pattern, with carnivorous fish exhibiting higher activities necessitated by higher gut ammonia concentrations. Our second objective was to determine differences in IBCs between host species, which was supported. These differences may reflect differential contributions to ammonia detoxification through enzymatic pathways. If true, differences in enzyme activities detected at sampling locations should also reflect differences in microbiome composition. This was only observed in the central stoneroller, suggesting that enzyme activity differences in the rainbow darter were driven by host enterocyte activity. Finally, disturbing the bacterial composition/load via antibiotic treatment further supported a role for the IBC in the herbivorous fish and ruled out a role in the carnivore. A closer examination of the direct effects of dietary protein on the IBC and intestinal nitrogen metabolism in the central stoneroller is required.

Acknowledgements

The authors would like to acknowledge the reviewers for providing thoughtful feedback.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.A.T., C.B.; Methodology: L.A.T.; Formal analysis: L.A.T., C.B.; Investigation: L.A.T.; Writing - original draft: L.A.T., C.B.; Writing - review & editing: L.A.T., C.B.; Supervision: C.B.; Funding acquisition: C.B.

Funding

C.B. is funded by a Natural Sciences and Engineering Research Council of Canada Discovery Grant (no. 504132), a Canada Foundation for Innovation John R. Evans Leaders Fund Grant and an Ontario Research Foundation grant.

Data availability

Sequence data are available from NCBI BioProject, accession number PRJNA549302 (www.ncbi.nlm.nih.gov/bioproject/PRJNA549302); and the sequence read archive, SRA number SRP201669 (trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP201669)

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.209882.supplemental

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