NEWS AND VIEWS

OPINION

Intestinal microbiota in fishes: what's known and what's not

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High-throughput sequencing approaches have enabled characterizations of the community composition of numerous gut microbial communities, which in turn has enhanced interest in their diversity and functional relationships in different groups of vertebrates. Although fishes represent the greatest taxonomic and ecological diversity of vertebrates, our understanding of their gut microbiota and its functional significance has lagged well behind that of terrestrial vertebrates. In order to highlight emerging issues, we provide an overview of research on fish gut microbiotas and the biology of their hosts. We conclude that microbial community composition must be viewed within an informed context of host ecology and physiology, and that this is of particular importance with respect to research planning and sampling design.

Terrestrial vertebrates maintain diverse assemblages of autochthonous gastrointestinal microbes that play critical roles in nutrition, development of host tissues, facilitation of the immune system and protection from invasive pathogens (Nicholson *et al.* 2012). Although all trophic groups appear to harbour indigenous microbial populations, it is herbivores that have justifiably attracted the most attention due to the role that the gut microbiota plays in digestion and assimilation (Van Soest 1994; Bryant 1997; Mackie 1997). This reflects the importance of microbes in the digestion and assimilation of structural and storage plant components which are resistant to the action of endogenous

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(i.e. host-produced) digestive enzymes (Van Soest 1994; Mackie 1997; Cantarel *et al.* 2012; Hehemann *et al.* 2012). Thus, fermentative digestion facilitated by microbes is a key process in the functioning of terrestrial ecosystems and must be considered when modelling nutrient cycling and carbon flow. Indeed, an understanding of the chemical structure of plant foods and the nature of post ingestive processes has been an essential element in defining herbivory in terrestrial environments and in interpreting the ecological and evolutionary significance of herbivory in ecosystem processes (Van Soest 1994).

High-throughput sequencing approaches have enabled rapid screening of numerous complex samples of gut microbial communities, and this has further enhanced interest in their diversity and functional relationships in different groups of vertebrates (Ley *et al.* 2008a,b; Muegge *et al.* 2011). When linked to phylogenies of major vertebrate groups, novel and exciting insights have emerged into the evolution of the microbiota, their hosts and the emergence of different trophic modes in the history of these host groups (Ley *et al.* 2008a,b; Muegge *et al.* 2011; Zhu *et al.* 2011). The recent interest in exploratory analyses of vertebrate gut communities signals new research initiatives in this area (Lozupone *et al.* 2012; Mueller *et al.* 2012; Relman 2012).

Although fishes represent the greatest taxonomic and ecological diversity of vertebrates, our understanding of their gut microbiota and its functional significance has lagged well behind that of terrestrial vertebrates (Rimmer & Wiebe 1987; Smith *et al.* 1996; Nayak 2010; Ray *et al.* 2012). In fact, the literature on fishes, and especially those associated with coral reefs, lacks a consensus of the nature of marine piscine herbivory and its defining features (Choat & Clements 1998; Clements *et al.* 2009; Comeros-Raynal *et al.* 2012). Unlike the terrestrial literature, in which a focus on postingestive processes has provided the necessary platform for defining herbivory, its functional significance and the role of the microbiota, the study of piscine herbivory has focussed on preingestive processes (e.g. rates of algal removal, feeding behaviour) and its assumed impact on ecosystem function (Clements *et al.* 2009). The failure to adequately define and understand herbivory at a functional level in fishes has resulted in difficulties in attempting to compare aquatic and terrestrial systems under the same functional umbrella (Choat & Clements 1998). We were prompted to attempt to clarify some of these issues by the recent upsurge in interest in fish gut microbial communities (e.g. van Kessel *et al.* 2011; Mouchet *et al.* 2012; Ray *et al.* 2012; Sullam *et al.* 2012; Wong & Rawls 2012; Watts *et al.* 2013; Ye *et al.* 2013). Our intent here is threefold: (i) to provide a brief overview of what is known concerning microbiota–host relationships;

(ii) to highlight factors that may be important in sampling designs for future work, including aspects of the biology of the host fishes; and (iii) to consider our current understanding of the role that fish gut microbiota play in digestion in the context of host phylogeny, ecology and environment.

A historical overview of work on fish gut microbiotas

Early work on these systems largely involved isolated culture experiments. Liston (1957) demonstrated that host-specific pressures in the gut of fishes have a selective effect on hindgut bacteria, and thus fishes harbour an indigenous microbiota (see also Sakata *et al.* 1981). In a seminal paper, Fishelson *et al.* (1985) pointed out that the giant *Epulopiscium* symbionts of tropical herbivorous surgeonfishes were not found outside the host. Fishelson *et al.* (1985) was also the stimulus for subsequent work that examined the role of these microbial communities in digestion (Dabrowski & Portella 2006). Lindsay and Gooday (1985) reported that the digestive tract microbiota of adult cod was specialized and did not reflect allochthonous organisms, and indeed, numerous studies since have revealed that fish gut communities are dominated by autochthonous organisms that have not been detected in the environment. This work was reviewed by Cahill (1990), who concluded that fish harboured complex communities of bacteria. More recent sequence-based approaches show that fish hindgut microbial communities much more closely resemble those of mammals than environmental microbial communities (Fidopiastis *et al.* 2006; Sullam *et al.* 2012), especially in the prevalence of *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (Clements *et al.* 2007; Smriga *et al.* 2010; Sullam *et al.* 2012; Ye *et al.* 2013). These findings indicate that fish, like other vertebrates, harbour specialized gastrointestinal communities.

Many early studies that relied upon simple microscopic characterizations of gastrointestinal microorganisms revealed relationships between host diet and microbiota composition. Fishelson *et al.* (1985) demonstrated that marine herbivorous surgeonfishes harboured a distinctive and diverse gut microbiota, and significant associations between surgeonfish intestinal microbiota composition and host trophic level and taxonomy were identified following comparisons of bacterial morphotypes (Clements *et al.* 1989). Early reviews such as Cahill (1990) and Clements (1997) discussed the ecological and environmental factors shaping gut microbiota composition more generally in fishes, for example diet, phylogenetic affinity of the host, acquisition of symbionts by juveniles, etc. Numerous papers have built on this, demonstrating that many species of herbivorous and omnivorous fishes contain diverse intestinal communities (e.g. Rimmer & Wiebe 1987; Clements *et al.* 1989; Clements 1991, 1997; Martínez-Díaz & Pérez-España 1999; Ray *et al.* 2012) and that herbivorous and detritivorous fish species harbour distinctive microbial populations. Relationships between alimentary tract anatomy and fermentative hindgut communities were discussed in Clements (1997), Clements & Choat (1997), Choat *et al.* (2002) and Mountfort *et al.* (2002).

An ancient pattern of co-evolutionary host–microbiota relationships was demonstrated by phylogenetic analysis of *Epulopiscium* symbionts, in which parallel lineages of both type A1 and type A2 phylotypes are shown to occur in conspecific host surgeonfishes from disparate regions including the Red Sea and the Great Barrier Reef (Flint *et al.* 2005). This suggests that phylogenetic factors such as host physiology and gut anatomy may interact with environmental and ecological factors (e.g. biogeography of host fishes), and thus these must be considered when assessing relationships between microbiota composition and host biology. Accurate information on host diet is also a critical prerequisite for determining patterns between host trophic level and bacterial communities in fishes. For example, of the four host fish species classified as 'herbivores' by Sullam *et al.* (2012) and Wong and Rawls (2012), *Naso tonganus* and *Pomacanthus sexstriatus* are generally considered omnivores (Choat *et al.* 2002; Bellwood *et al.* 2006; Mantyka & Bellwood 2007), while the diet of *Chlorurus sordidus* is dominated by detritus (Choat *et al.* 2002). Host diet is well known to have a strong influence on microbiota composition (Muegge *et al.* 2011; Lozupone *et al.* 2012; Ye *et al.* 2013; David *et al.* 2014), and thus a failure to delineate host diet accurately has the potential to misdirect future studies into the functional significance of fish intestinal communities (see below).

We agree with recent studies that our understanding of the intestinal microbiota of fishes is largely derived from culture-based approaches (e.g. as reviewed by Cahill 1990), which usually reveal only a limited range of microbial diversity (Shiina *et al.* 2006; van Kessel *et al.* 2011; Sullam *et al.* 2012). Nevertheless, recent studies continue to use culture-based approaches to infer microbiota function (e.g. Uchii *et al.* 2006; Mouchet *et al.* 2012), although such work does little to inform our understanding of microbial processes in vivo (Clements 1997; Ray *et al.* 2012). When microorganisms are removed from the gut, and especially when cultured under different environmental conditions, the community composition and function will shift. Published work on Biolog™ plate incubations of fish gut microbiota (Mouchet *et al.* 2012) and plating on selective media incubated in the presence of oxygen (Saha *et al.* 2006), for example, tell more about the metabolism of a few heterotrophic aerobic bacteria than they do about processes occurring in a hindgut ecosystem, as these facultative, metabolically versatile organisms outcompete more specialized and numerically dominant autochthonous anaerobic populations (Preston-Mafham *et al.* 2002; Shiina *et al.* 2006).

Sampling design for fish microbiota studies

Culture-independent approaches such as high-throughput sequencing allow access to the genetic make-up of microbial communities that were once beyond our reach (Pace 2009). We suggest that careful planning of experiments

together with appropriate interpretation of comparative data is essential for advances in understanding the impact of microbial community structure on ecosystem function. First, samples must be processed in a timely manner. Fish gut microbial communities are likely to resemble other microbial communities, changing in both composition and function over time and with perturbation.

Capture and confinement of wild fishes typically leads to a variety of stress responses (Pankhurst & Sharples 1992; Portz *et al.* 2006), and this along with changes in diet and food intake can influence the microbiota in fishes and mammals (Margolis 1953; Uchii *et al.* 2006; Nayak 2010; Dhanasiri *et al.* 2011; Nelson *et al.* 2012; Ye *et al.* 2013). These confounding factors limit the usefulness of analyses using fish collected from the wild but held in captivity before sacrifice to represent the microbiota of wild animals (e.g. Roeselers *et al.* 2011). Surgeonfish lose elements of their microbiota within a couple of days of captivity, even when provided foods collected from the wild (Fishelson *et al.* 1985; Montgomery & Pollak 1988). Intestinal microbiota composition in gizzard shad and silver carp was strongly affected by environmental location and sampling time, respectively (Ye *et al.* 2013). Recent data show that the microbiota composition of cultured rainbow trout is resistant to variations in diet and rearing density, although dietary variation was associated with changes in the relative abundance of *Lactobacillaceae*, *Streptococcus*, *Staphylococcaceae* and *Clostridiales* (Wong *et al.* 2013). The available evidence overall thus suggests that the microbial communities of captive fishes can differ substantially from those of wild populations, although microbiota composition in captive fishes can be resistant to variation in diet and rearing density (Wong *et al.* 2013).

A related issue in molecular characterizations of microbial communities involves the way that gut contents are sampled. The composition and function of microbial communities along the gut differ in different regions and organs (Moran *et al.* 2005; Zhou *et al.* 2009; Ye *et al.* 2013), and processing the contents of entire guts (i.e. from oesophagus to anus) homogenizes symbiotic populations with allochthonous ones. For example, *Cyanobacteria* are a dominant taxon in the gut of grass carp, silver carp and gizzard shad (Wu *et al.* 2012; Ye *et al.* 2013), yet these are highly likely to have been ingested as food (Ye *et al.* 2013). Both van Kessel *et al.* (2011) and Mouchet *et al.* (2012) pooled entire alimentary tract contents and identified aerobic and facultatively anaerobic environmental taxa that were considered part of the resident microbial community. This becomes particularly misleading when these results are used to infer physiological function in the gut. Similar problems arise where data from samples of fish hindgut contents are compared with data derived with sequences from entire intestinal tract contents (e.g. Roeselers *et al.* 2011; Sullam *et al.* 2012). In general, caution should be applied in inferring function on the basis of phylogenetic affiliation alone even when microorganisms are appropriately sampled, as shown by metabolic differences between cultured organisms on different media (e.g. Zinder & Salyers 2001).

Fishes display disparate intestinal morphologies (Stevens & Hume 1995; Choat *et al.* 2002; Clements & Raubenheimer 2006). Some have anatomically delineated alimentary components such as a true stomach or hindgut chamber (Clements & Raubenheimer 2006), while others have no stomach and no morphologically (cf. ultrastructurally) defined sections of the intestine. Despite the lack of defined alimentary sections in many species, there is evidence that distinct populations of microorganisms with different metabolic functions occur in defined regions of the alimentary tract (Montgomery & Pollak 1988; Clements & Choat 1995; Clements 1997; Ye *et al.* 2013). We suggest that a visual assessment of alimentary anatomy, microbial populations (cell density and identification of marker organisms), digesta or the biochemical analyses of enzymatic activities or metabolic by-products could be used to identify distinct regions of the host digestive tract prior to sampling for microbial community composition analyses. This approach would allow for correlative studies between microbial populations and metabolites. Moreover, some assessment of digestion can inform conclusions made about digestive processes (e.g. mechanical, enzymatic).

The role of microbes in digestion

The importance of a robust understanding of host diet and digestion assumes even greater significance when the functional relationships between fish intestinal communities and diet are considered. Sullam *et al.* (2012) suggest that 'herbivorous fish and mammals are united through the process of gut fermentation'. This is an oversimplification, in that herbivory appears to be a more disparate phenomenon in fishes than in mammals, and many herbivorous fishes display low levels of gastrointestinal fermentation (Choat & Clements 1998). It has long been known that many freshwater herbivorous fishes such as grass carp do not rely on microbial cellulolysis, but rather pass large quantities of plant material rapidly through the gut and salvage the soluble sugars and proteins released by the action of pharyngeal teeth (Hickling 1966; Van Dyke & Sutton 1977; Trust *et al.* 1979; Lindsay & Harris 1980; Lesel *et al.* 1986; Gangadhara *et al.* 2004). Despite this, recent studies continue to claim based on either isolation or 16S rRNA gene characterization that the presence of cellulolytic bacteria in cyprinid intestines indicates a significant role for these organisms in the digestion of plant material within the fish intestinal tract (e.g. Li *et al.* 2009; Wu *et al.* 2012). Similar inferences of gastrointestinal cellulolysis based on in vitro culture have been made for so-called wood-eating loricariid catfishes (Nelson *et al.* 1999; Nonogaki *et al.* 2007). Recent studies on the 'wood-eating' catfish *Panaque nigrolineatus* identified 16S rRNA phylotypes related to cellulolytic microorganisms and inferred the presence and expression of *nifH* genes (McDonald *et al.* 2012; Watts *et al.* 2013). These fishes assimilate the biofilm associated with submerged wood. The cellulolytic activity appears to be derived from microbes living on the wood surface that are then ingested as part of the detrital diet

(German 2009; German & Bittong 2009), as indicated by stable isotope signatures consistent with assimilation of carbon and nitrogen from intermediate microbial decomposers in the environment (i.e. biofilms) rather than direct assimilation from wood or endosymbiotic microorganisms (Lujan *et al.* 2011).

Herbivorous marine fishes vary on a continuum from species of damselfishes that guard territories of nutrient-rich filamentous algae, rely on endogenous digestive enzymes and have fast gut throughput times and low levels of intestinal fermentation, to species such as silver drummer that browse tough kelps, and have lengthy gut throughput times and high levels of fermentation in the hindgut (Rimmer & Wiebe 1987; Clements & Choat 1995, 1997; Choat & Clements 1998; Mountfort *et al.* 2002; Choat *et al.* 2004; Crossman *et al.* 2005; Skea *et al.* 2005, 2007; Clements *et al.* 2009). Herbivorous marine fish species with high intestinal short-chain fatty acid (SCFA) concentrations rely on gut microbes to convert unassimilable algal constituents such as mannitol to metabolically useful short-chain fatty acids (Seeto *et al.* 1996; Mountfort *et al.* 2002; White *et al.* 2010), and these fishes display metabolic specializations to hindgut fermentation (Clements *et al.* 1994; Willmott *et al.* 2005). These are the fishes that most resemble foregut- and hindgut-fermenting mammals in terms of symbiont-mediated digestion of plant material. Recent work has also highlighted the role of the human hindgut

microbiota in the digestion of algal carbohydrates (e.g. Hehemann *et al.* 2010, 2012; Cantarel *et al.* 2012). Absorption of SCFA in fishes is largely driven by the osmotic gradient between the intestine and the blood (Titus & Ahearn 1988, 1992), and thus the concentration of these end-products of anaerobic microbial metabolism in the posterior intestine can serve as a rough indicator of the potential importance of microbial digestion. However, intestinal SCFA concentration alone does not necessarily correlate with the metabolic importance of microbial digestion to host fishes, because other factors including fermentation rate and the ratio of fermentative digesta mass to host body mass must be considered (Smith *et al.* 1996; Mountfort *et al.* 2002). Thus, while carnivorous fishes can display relatively high concentrations of SCFA in the intestine (Smith *et al.* 1996), microbial digestion is likely to be less quantitatively important in these species than in herbivorous and omnivorous species.

Interestingly, herbivorous and omnivorous freshwater fishes tend to display shorter gut transit times and thus lower levels of SCFA in the gut (e.g. Smith *et al.* 1996; German *et al.* 2010) than some of their marine counterparts (Fig. 1). This may reflect the differences in herbivorous diets between the two systems, especially in relation to carbohydrate composition (Choat & Clements 1998; Popper *et al.* 2011), but to date no freshwater herbivorous fish species have been identified that appear to rely on hindgut
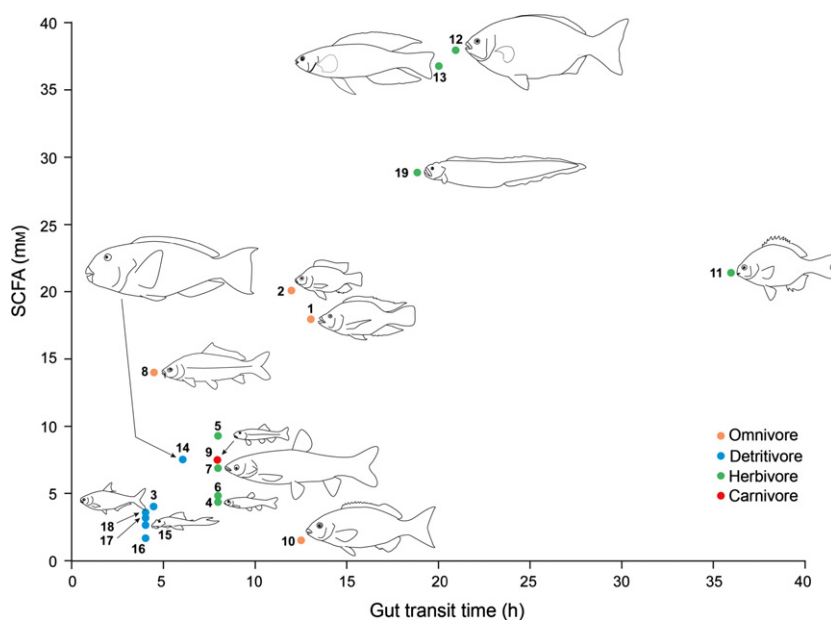
Fig. 1 Intestinal concentration of short-chain fatty acids in marine and freshwater fishes compared to gut transit time. Although gut transit time varies with both temperature and fish size, it is clear that species with high levels of gastrointestinal fermentation are mainly herbivorous marine species. Species line drawings are scaled to relative length of fishes used in each study, and points are colour-coded by diet category. Species identities are as follows: (1) *Oreochromis mossambicus*, (2) *O. niloticus*, (3) *Dorosoma cepedianum*, (4) *Campostoma anomalum*, (5) *C. oligolepis*, (6) *C. pauciradii*, (7) *Ctenopharyngodon idella*, (8) *Cyprinus carpio*, (9) *Nocomis micropogon*, (10) *Girella tricuspidata*, (11) *Hermosilla azurea*, (12) *Kyphosus sydneyanus*, (13) *Odax pullus*, (14) *Chlorurus microrhinos*, (15) *Hypostomus pyrineusi*, (16) *Panaque nocturnus*, (17) *P.* cf. *nigrolineatus*, (18) *Pterygoplichthys disjunctivus*, (19) *Cebidichthys violaceus*. See Table S1 in supporting information for species data and reference sources.

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fermentation to the extent that, for example, marine herbivorous odacines and kyphosids do (Mountfort *et al.* 2002). The highest levels of SCFA in the posterior intestine that we could find reported from 'freshwater' fish species were in two studies on tilapia (*Oreochromis*) species (see Fig. 1). However, it should be noted that one of these studies used wild fish collected from marine habitats (Titus & Ahearn 1988), and the other was based on captive tilapia that were fed cereal grains (Leenhouwers *et al.* 2007). High-starch foods such as cereal grains are well known to increase rates of gastrointestinal fermentation (Van Soest 1994), and indeed, Leenhouwers *et al.* (2007) concluded that the measured SCFA in their fish most likely came from starch fermentation. Smith *et al.* (1996) concluded that fermentation of digesta by the gut microbiota in the freshwater cyprinid *Cyprinus carpio* probably supplied a small but significant proportion of daily energy requirements, but the literature provides little support for gastrointestinal fermentation providing a major component of daily energy requirements in freshwater fishes, and especially for cellulose being a major substrate for gut microbiota in these animals. This is not to downplay the critical importance of gut microbes to nutrition more generally in fishes, as in the recent demonstration that gut microbiota stimulate fatty acid uptake and lipid droplet formation in the intestinal epithelium and liver in zebrafish (Carmody & Turnbaugh 2012; Semova *et al.* 2012).

Hindgut fermentation in at least some marine fishes appears to 'bend the rules' of terrestrial hindgut fermentation (Foley & Cork 1992) in some respects. Differences between fishes and other vertebrates in (i) the distribution and mechanism of protein uptake along the gut and (ii) the characteristics of the gut microbiota suggest that fish hindgut microorganisms may also make significant contributions to host protein metabolism (Clements *et al.* 2009). Uptake of protein from gut symbionts has been demonstrated in freshwater omnivorous tilapia using compound-specific stable isotope analysis of essential amino acids (Newsome *et al.* 2011), and the same phenomenon was recently reported in herbivorous marine turtles (Arthur *et al.* 2014). It is possible that these host–microbiota interactions may be even more quantitatively important in hindgut-fermenting marine herbivorous fishes.

The antiquity of 'fishes' (i.e. nontetrapod vertebrates) and the overlap between their microbiota (viewed at the phylum level) and those of mammals raised the possibility that fishes may have served as the original vertebrate hosts for these gastrointestinal communities (Sullam *et al.* 2012). This implies that these fish symbioses are 'ancient' compared to those with mammals. This may be true in the general sense; however, the radiation of fishes with cranial structures consistent with precise biting and grazing of the substratum, that is, herbivory, is a Cenozoic event (Friedman 2010). The appearance of crown taxa of herbivorous fishes appears to be contemporaneous with mammalian diversification in the Eocene (Bellwood 2003), and many taxa of hindgut-fermenting marine herbivorous fishes are considerably younger. Indeed, while herbivorous odacines

and the early diversification of the aplodactylids trace their evolutionary age to the Miocene (Burridge 2000; Clements *et al.* 2004; Alfaro *et al.* 2009), kyphosids that display specialized alimentary tract anatomy and high levels of intestinal fermentation (Clements & Choat 1997; Mountfort *et al.* 2002) are even younger (S.W. Knudsen and K.D. Clements, in prep.). Herbivory in terrestrial amniotes has a much more ancient pedigree. Mesozoic herbivorous dinosaurs such as sauropods were almost certainly hindgut fermenters (Hummel & Clauss 2011), and herbivorous amniotes that must have relied on intestinal microbiota to digest vascular plant material were well established by the early Permian (Sues & Reisz 1998).

Conclusion

In conclusion, we argue that the field needs to develop through an understanding of the varying role that these communities play in fish, for example the variation in the extent to which the microbiota are involved in digestion, immune responses, and thus contribute to host nutrition and health. The field is developing very rapidly, driven mainly by the ease of generating community profiles of gut microbiota with next-generation sequencing approaches. This progress needs to be matched with advances in our understanding of the sources of intraspecific variation in fish-associated microbial communities. Appropriate testing of hypotheses concerning these sources of variation is critical, and informed development of the field in this respect rests on an understanding of host biology and the ways in which this may influence the gut environment, for example through stress, changes in diet, source location, etc. The correct identification of research priorities for work on the gastrointestinal microbial communities of fishes, and thus informed allocation of research funding in the area, will depend on how well we build on what we know.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Intestinal concentration of short-chain fatty acids in marine and freshwater fishes compared to gut transit time.