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LETTER

Individuals' diet diversity influences gut microbial diversity in two freshwater fish (threespine stickleback and Eurasian perch)

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Abstract

Vertebrates' diets profoundly influence the composition of symbiotic gut microbial communities. Studies documenting diet-microbiota associations typically focus on univariate or categorical diet variables. However, in nature individuals often consume diverse combinations of foods. If diet components act independently, each providing distinct microbial colonists or nutrients, we expect a positive relationship between diet diversity and microbial diversity. We tested this prediction within each of two fish species (stickleback and perch), in which individuals vary in their propensity to eat littoral or pelagic invertebrates or mixtures of both prey. Unexpectedly, in most cases individuals with more generalised diets had less diverse microbiota than dietary specialists, in both natural and laboratory populations. This negative association between diet diversity and microbial diversity was small but significant, and most apparent after accounting for complex interactions between sex, size and diet. Our results suggest that multiple diet components can interact non-additively to influence gut microbial diversity.

Keywords

Diet mixing, *Gasterosteus aculeatus*, generalist, individual specialisation, microbiota, *Perca fluviatilis*, perch, stable isotopes, threespine stickleback.

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INTRODUCTION

Vertebrate digestive systems contain diverse and abundant microbial communities (Ley *et al*. 2008), which provide valuable services to the host including nutrition, and immune and developmental regulation (Lathrop *et al*. 2011). Atypical gut microbiota can disrupt these services, causing immunological and metabolic disorders (Turnbaugh *et al*. 2008; Turnbaugh & Gordon 2009; Sanz *et al*. 2011; Koeth *et al*. 2013). Consequently, there is great interest in identifying genetic and environmental factors that regulate microbiota composition and diversity (Benson *et al*. 2010; Spor *et al*. 2011). Environmental factors are particularly interesting, as they offer potentially simple mechanisms for treating dysbiosis (De Filippo *et al*. 2010; Haiser & Turnbaugh 2012), and improving animal health and productivity (Merrifield *et al*. 2010).

Host diet is among the most important environmental factors influencing gut microbiota composition (Turnbaugh *et al*. 2008; Muegge *et al*. 2011; Wu *et al*. 2011; Sullam *et al*. 2012). Unfortunately, most diet-microbiota studies focus on simple diet effects such as discrete diet treatments like high fat vs. low fat diets (Parks *et al*. 2013), or linear univariate measures such as caloric intake. In contrast, in nature most individuals consume a mixture of foods, rather than specialising on single items. Furthermore, within a given population, some individuals may specialise more than others (Bolnick *et al*. 2003; Araújo *et al*. 2011). That is, individuals can differ not just in which foods they consume, but also food diversity. At present, it remains unclear whether combinatorial mixing of foods affects the gut microbiota. Here, we evaluate whether host diet diversity affects gut microbial diversity.

Symbiotic microbiota present a perfect example of a metacommunity (Leibold *et al*. 2004): host individuals are transient habitat patches colonised and inhabited by microbial communities, whose composition depends on colonisation processes and filtering of colonists by local environmental conditions (Costello *et al*. 2012). Diet-associated microbes represent a source of potential colonists (Adlerberth & Wold 2009;

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Costello *et al.* 2012). We therefore expect generalists, which consume more diverse foods, to be exposed to and carry more diverse microbes. Microbe composition also depends on nutrients in the gut (Laparra & Sanz 2010), so a mixed diet might increase microbial diversity by providing more diverse nutrients. Alternatively, diet could indirectly alter microbiota by changing host physiology (Hooper *et al.* 2012; Nicholson *et al.* 2012), foraging success or body condition (Bolnick & Lau 2008), or parasite exposure and immune status (Walk *et al.* 2010). These indirect effects could plausibly increase or decrease microbial diversity. Thus, while it seems intuitive that dietary generalists should harbour more diverse gut microbiota, the opposite relationship is plausible.

To test whether diet diversity affects gut microbial diversity, we sampled two wild populations of fish that exhibit among-individual diet differences (Svanbäck & Persson 2004; Svanbäck *et al.* 2008; Bolnick & Paull 2009; Matthews *et al.* 2010). We show that within each species dietary generalists have lower microbial diversity, although this effect is sex- and size-dependent in perch. Laboratory manipulations of stickleback diet also found lower microbial diversity in mixed-diet fish.

MATERIALS AND METHODS

Study systems

Fish inhabiting temperate lakes typically consume two general invertebrate prey categories: large substrate-dwelling ('littoral') insect larvae, and small open-water ('pelagic') zooplankton, predominantly crustaceans. Note that 'benthic' and 'limnetic' are sometimes used in place of littoral or pelagic, especially in reference to ecologically divergent species of stickleback (Schluter & McPhail 1992). Within any given population, individual fish fall along a continuum ranging from littoral to pelagic specialists (Bolnick *et al.* 2003), with many intermediate generalists consuming mixtures of both resources. Thus, within populations individuals differ not just in what food they consume, but also in prey diversity. We use this natural among-individual variation to test whether diet diversity affects gut microbial diversity, in two populations of temperate fish (threespine stickleback, and Eurasian perch), each previously known to exhibit individual specialisation (Bolnick 2004; Svanbäck *et al.* 2008).

Sample collection

In June 2008 we sampled 398 stickleback (*Gasterosteus aculeatus*) from Cedar Lake on Vancouver Island, Canada (50°12′09″ N, 125°33′58″ W). This lake contains a single panmictic and morphologically unimodal population (not subdivided into discrete ecomorphs), with typical levels of phenotypic, dietary and genetic diversity for lakes in the region. We caught fish using unbaited minnow traps set along ≈200 m of shoreline, between 0.5 and 3 m deep. In July 2009 perch were sampled from Lake Erken, Sweden (59°51′26″ N, 18°35′52″ E), (Svanbäck *et al.* 2008). We captured 255 perch in 1 day, using survey-link gill nets set along the shoreline and offshore (<100 m apart).

Experimental diet manipulations

Using stickleback, we tested whether diet alters microbial composition, and whether diversity alters gut microbiota. We mixed 9-month old fish from each of 50 families derived from wild-caught parents, and distributed the mixture among 100-L aquaria subjected to the three diet treatments: littoral, pelagic or mixed diet (frozen chironomid larvae, *Daphnia*, or an equal mixture by mass respectively). Aquaria were on a shared recirculating water supply to maintain identical ambient microbiota. After 1 month, we sequenced the microbiota as described below, to contrast microbial diversity of mixed vs. pure diets. Microbiota was also characterised for *Daphnia* and chironomids and water from each aquarium (two replicate samples each), to test whether fish have microbiota characteristic of their respective foods or water.

Isotopic measures of diet variation within species

Carbon and nitrogen stable isotope ratios are widely used to study feeding ecology in wild populations (Post 2002; Fry 2006; Araujo *et al.* 2007; Newsome *et al.* 2007; Boecklen *et al.* 2011). Using isotopes from mussels and snails as baselines (pelagic and littoral primary consumers respectively), we used standard formulas to calculate α, the proportion littoral carbon in an individual fish's diet and *tpos*, the trophic position (Post 2002; Matthews *et al.* 2010). Previous studies of diet variation within stickleback and perch populations have confirmed that $\delta^{13}C$ and $\delta^{15}N$ are correlated with individual variation in foraging microhabitat use, stomach contents and trophic morphology (Snowberg & Bolnick 2008; Bolnick & Paull 2009; Quevedo *et al.* 2009; Matthews *et al.* 2010; Bolnick & Araújo 2011). We used caudal peduncle muscle to measure $\delta^{13}C$ and $\delta^{15}N$ at the University of California at Davis stable isotope facility.

Quantifying variation in gut microbiota

We sequenced the gut microbiota from 183 perch and 187 stickleback, subsampled from the larger isotope sample. Lacking information on microbial substructure within the intestine, we extracted DNA from entire stickleback intestines, and a 100 mg medial section of perch intestines, using the Powersoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) using sterile dissection techniques. DNA from laboratory prey was extracted in a similar manner. Microbial DNA from aquarium water samples was extracted using the MO BIO UltraClean Water DNA Isolation Kit. We amplified the V4 hypervariable region of the 16S rRNA gene (positions 515–806, based on *E. coli* numbering) using PCR conditions of the Earth Microbiome Project standard PCR protocol (Caporaso *et al.* 2011, 2012), individually barcoding samples using primers and barcodes described in (Bates *et al.* 2011; Bergmann *et al.* 2011). Negative controls (no sample added) were included in both the DNA extraction and 16S PCR amplification stages to test for contamination; these PCRs yielded negligible DNA concentrations during Picogreen quantitation (within measurement error of 0 ng μL^{-1}; insufficient for pooling for amplicon sequencing), indicating contamination was

not a detectable problem. Thus, any contaminant of our samples would yield comparatively few sequence reads and generally be lost during rarefaction. Amplicon pools were sequenced on an IlluminaHiSeq 2000 sequencer at the University of Colorado as described by (Caporaso *et al.* 2012) yielding 100 bp paired end reads. Captive population samples were sequenced on an Illumina MiSeq (250 bp paired-end reads).

Data analysis was performed using the open-source software system Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.* 2010; Kuczynski *et al.* 2012), following quality filtering (Bokulich *et al.* 2012). Sequences were demultiplexed and quality filtered using default QIIME parameters. OTUs were picked using a closed-reference OTU picking protocol against the Greengenes database 12_10 release, http://greengenes.secondgenome.com/ (De Santiz *et al.* 2006) pre-filtered at 97% identity, discarding reads with less than 97% similarity to any reference sequence. We retained between 22 and 60% of quality-filtered reads; discarded sequences were primarily host mitochondrial 16S sequences, spiked-in phiX, and presumptive sequencing errors that had no BLAST match. Closed-reference OTU picking discarded approximately 25% of the sequences retained by open-reference OTU picking, but yielded higher quality taxonomic assignments for calculating phylogenetically weighted alpha or beta diversity metrics. Open-reference OTU picking yields diversity measures that are about twice as large as, but highly correlated with, closed-reference OTU diversity (e.g. in stickleback $r = 0.951$). Consequently, relationships between diet and microbial diversity reported below are supported whether we use open- or closed-reference OTU picking, to minimise repetition we focus solely on the latter. Taxonomic assignments for OTUs were based on the Greengenes reference sequence defining that OTU, and the Greengenes tree was used for computing phylogenetic diversity metrics.

Individual hosts' microbial alpha diversity was calculated as the phylogenetic diversity [PD; (Lozupone & Knight 2008)] rarefied to 10 000 sequences per sample, removing the few samples with insufficient read depths. In general, any measure of community diversity is sensitive to sampling effort (for microbiota or any ecological community). To be sure that our results are not an artefact of choosing a particular rarefaction depth, we recalculated PD at various levels of rarefaction from 1000 through 10 000 sequences, and reran our analyses. PD is highly correlated ($r > 0.98$) across an order of magnitude variation in rarefaction depth, and is unrelated to original sequencing depth, so we feel confident our measures of diversity are biologically informative. We emphasise that 16S sequencing can underestimate diversity among microbes with highly similar 16S, and provides information on relative abundance but not actual cell density (see Lozupone & Knight 2008 for further discussion). We also calculated phylogenetically naïve diversity metrics including species richness, Pielou's evenness, and Shannon diversity metrics from OTU tables rarefied to 10 000 reads.

Data analysis – wild fish

To evaluate whether diet (α and *tpos*) affects the microbiota, we used general linear models (GLMs) with quasibinomial link functions to test whether the relative abundance of each common OTU (> 0.01% of total sequence reads) depends on diet within each host population. More thorough analyses of among-individual microbial variation (beta diversity and taxon composition) are reported elsewhere, as here we focus on microbial alpha diversity.

To test whether diet has nonlinear effects on microbial diversity, we ran bivariate quadratic regressions of PD as a function of isotope signatures, α and *tpos*, separately for perch and for stickleback, testing whether PD depends on α, *tpos*, α^2, $tpos^2$, $\alpha \times tpos$, $\alpha^2 \times tpos^2$, sex, size (standard length) and interactions between sex, size and diet, with complex models first reduced using AIC model selection criteria. These analyses were also applied to phylogenetically naïve diversity metrics. It is important to note that we are measuring diet diversity not in terms of the number of prey species consumed, but in terms of how evenly an individual uses littoral vs. pelagic prey. Because littoral prey are predominantly insect larvae, whereas pelagic zooplankton are predominantly crustaceans, littoral/pelagic generalists use a more diverse combination of prey at a deep taxonomic level (different ratios of Subphyla). We anticipate that the diversity of closely related prey species (e.g. various cladocera) would have a comparatively modest effect on microbial diversity.

Changes in microbial diversity must coincide with altered taxonomic composition. We repeated our quasibinomial GLM analyses of individual taxa, this time testing for quadratic relationships between taxon relative abundance and diet (using the first PC axis of isotope variation to characterise diet), to identify microbes that are more or less common in intermediate-diet fish. We focused on the relative abundance of higher taxonomic groups (Classes) which are more likely to drive wholesale changes in microbial phylogenetic diversity, but we also examined other taxonomic levels to ensure our results were not dependent on one taxonomic rank.

Data analysis – laboratory diet manipulation

MANOVAS of leading weighted and unweighted PCoAs tested whether microbiota composition differed between lab diet treatments. We used an ANOVA to test for experimental diet effects on PD, including sex and sex × diet effects in lab-reared stickleback. To account for the ordinal relationship between diet treatments, we used quadratic regression to test whether PD depends on proportion littoral prey (100, 50, and 0% for chironomid-fed, mixed-diet, and *Daphnia*-fed fish).

We tested whether microbial differences between food sources can explain the observed diet effects on the microbiota. First, we generated unweighted PCoA axis scores from UniFrac distances among all fish, food and water samples. For each of the top 21 PCoA axes (explaining 50% of the cumulative microbial beta diversity), we used *t*-tests to evaluate whether each axis differed between food sources, or guts of stickleback fed the different food sources. If diet effects arise primarily via ingestion of different food-associated microbes, PCoAs that are larger (or smaller) in *Daphnia* should also be larger (smaller) in *Daphnia*-fed fish, so we expect a positive correlation between prey- and diet-effect sizes (t statistics).

RESULTS

Diet variation within populations

Stable isotopes confirmed that individuals ranged from littoral specialists to pelagic specialists, with many intermediate generalists (Fig. 1). In stickleback, individuals consumed anywhere from 12 to 100% littoral carbon, with a mean $\alpha = 0.58$ (SD = 0.17), and spanned nearly a full trophic position (*tpos* = 3.14–3.89). On average, the perch population used less littoral carbon than stickleback (mean $\alpha = 0.19$, ranging from 0.04 up to 0.67). Perch are at about the same trophic level as stickleback (mean *tpos* = 3.52) but are more variable, ranging over a full trophic level (2.79–3.95). As is typical of lacustrine fish, α and *tpos* are negatively correlated, forming a principal component axis (> 70% of variance in each population) that distinguishes between littoral and pelagic specialists and recapitulates isotopic differences between benthic and limnetic species pairs (Matthews *et al.* 2010). In Cedar Lake stickleback, diet variation among individuals exceeds differences between incipient sympatric species pairs of stickleback (Fig. 1).

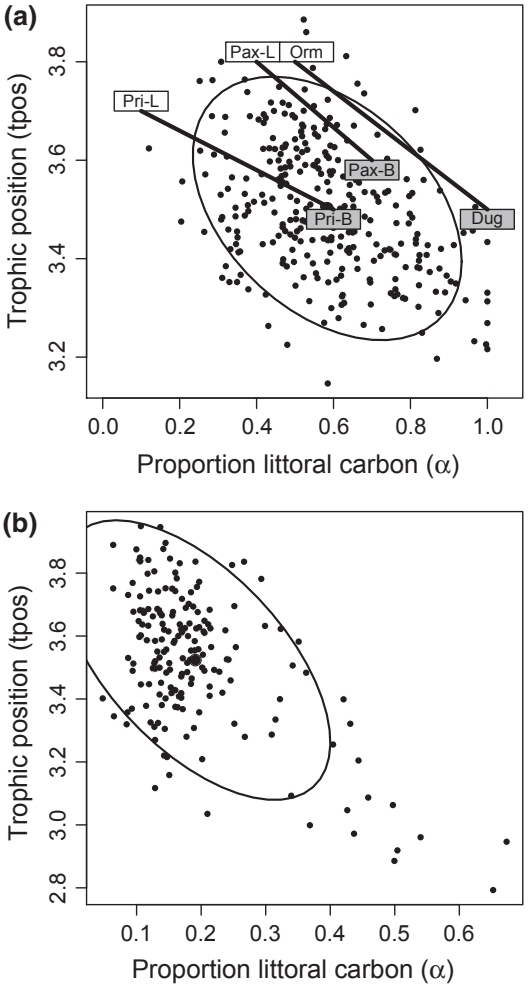

Figure 1 Isotopic evidence of diet variation within each fish population. (a) Among-individual variation in the proportion littoral carbon and trophic position in stickleback from Cedar Lake, calculated from stable isotopes (Post 2002). A 95% density ellipse indicates the major axis of covariation between α and *tpos*. For comparison, we plot the centroid values of α and *tpos* for three pairs of benthic (grey rectangles) and limnetic populations (white), showing that the major axis of diet within the Cedar Lake stickleback population is parallel to the classic benthic-limnetic diet axis in other stickleback populations (Priest Lake: Pri-B and Pri-L; Paxton Lake: Pax-B and Pax-L; and a parapatric benthic and limnetic population pair from Dugout and Ormond Lakes: Dug and Orm). Here, we use littoral/pelagic rather than benthic/limnetic, to avoid confusion with these distinct species pairs. (b) Among-individual variation in the proportion littoral carbon and trophic position in Eurasian Perch from Lake Erken.

Diet effects on the gut microbiota composition

Diet affects overall microbial composition, and the abundance of particular microbial taxa, in both wild-caught fish, and in lab stickleback. Quasibinomial general linear models (GLMs) indicated that the relative abundances of many common OTUs are correlated with host diet (α and *tpos*) within each host species (Table S1). Of 530 common OTUs in stickleback (> 0.01% relative abundance), α and *tpos* affected the relative abundance of 31 and 34 OTUs, respectively ($P < 0.05$), significantly more than a 5% false positive rate (Fisher's exact test $P < 0.001$). Over half of these OTUs are significant after FDR corrections ($q < 0.05$). Perch exhibit stronger support for diet effects, with significant α and *tpos* effects in, respectively, 50 and 70 of 512 common OTUs (9.8 and 13.4% of OTUs, more common than 5% false positive rate, $P > 0.0001$). After FDR corrections for multiple comparisons, 3.3 and 9.8% of OTUs still exhibit significant associations with α and *tpos*. Laboratory feeding experiments confirmed that diet alters the gut microbiota (Fig. S1), and the gut microbiota of mixed-diet fish is not simply a mixture of pure diet microbiota.

Effects of mixed diets on gut microbial diversity in wild fish

Within-host microbial community diversity varied among individuals by an order of magnitude. In stickleback, phylogenetically weighted diversity (PD) ranged from 6.1 to 32.1. Omitting OTUs found in a single host individual, OTU richness (rarefied to 10 000 reads) ranged from 42 to 594 OTUs per host (median of 211). Perch PD ranged from 4.5 to 37.5 with 31 to 633 OTUs per host (median of 163). For all individuals alpha diversity was substantially less than aggregate microbial diversity in the population as a whole (e.g. 5404 and 5149 OTUs found in the stickleback and perch samples, respectively, using only OTUs found in at least two individuals). Individual PD was less than null expectation for PD if individuals randomly sampled OTUs from a pooled microbial metacommunity (Fig. S2). Thus, individuals harbour a non-random and phylogenetically clustered subset of available microbes.

Contrary to the intuitive expectation that generalists should have higher gut microbial diversity than specialists, in stickleback there was a positive quadratic relationship between PD and location along the littoral-pelagic diet axis. This U-shaped relationship implies that individuals with intermediate diets, who consume a roughly equal mixture of littoral and pelagic prey, have less diverse microbiota than littoral or pelagic specialists (Fig. 2a). This quadratic effect was significant in a model with only linear and squared diet effects (α and *tpos*).

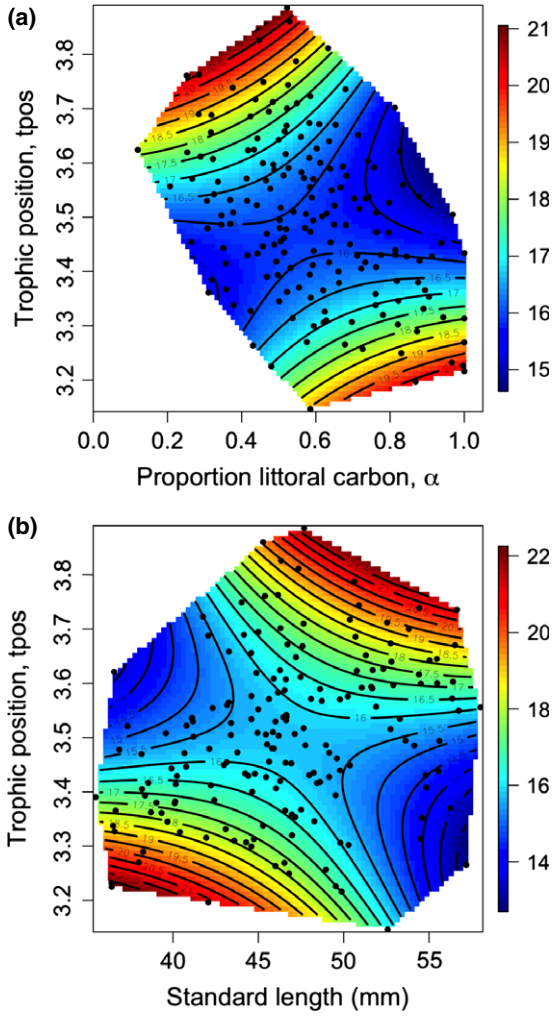

Figure 2 Gut microbial phylogenetic diversity (PD) is minimised for individual stickleback with intermediate diets. (a) Microbial phylodiversity of stickleback as a quadratic function of both proportion littoral carbon and trophic position. Contour lines and colour indicate PD, points indicate individual fish. The heat colour indicates estimated values of phylogenetic diversity (PD) from a quadratic regression, ranging from low (blue) to high (red). Pelagic and littoral specialists are in the top left and bottom right respectively (see Fig 1a). (b) The same relationship as in (a) but focusing on the interaction between body size and the quadratic effect of trophic position, which is strongest for individuals with intermediate size. The range of PD values differs among panels because in (a) & (b) the coloured surface indicates the values predicted from a quadratic regression, rather than the raw PD values.

There is also a significant interaction between quadratic terms ($\alpha^2 \times tpos^2$ $P = 0.024$) which reflects a transverse valley of low PD running diagonally across diet space, separating peaks of higher PD associated with typical littoral (high α and low *tpos*) from pelagic (low α high tpos) diets. The quadratic effect of diet on PD is recapitulated at any level of rarefaction between 1000 and 10 000 reads per individual [PD scores are highly correlated ($r > 0.98$) across these different rarefaction levels], and is not correlated with original sequencing depth, allowing us to rule out the possibility that our finding is an artefact of choosing a particular rarefaction level. Quadratic diet effects were seen at comparable frequencies in higher taxonomic ranks as well.

When we add sex and size (standard length) and their interactions with diet to the model (Table S2) we retain the quadratic diet effects but also find that (1) males have higher PD than females, (2) PD increases with size more strongly in males than in females and (3) there is an interaction between diet diversity and size (length × $tpos^2$, $P = 0.00067$). This interaction reflects a stronger quadratic effect in intermediate-sized stickleback, whereas PD declines (increases) linearly with *tpos* in the smallest (largest) individuals.

Microbial phylogenetic diversity can be low if a few microbe taxa dominate the community. Indeed, PD is higher when microbial taxa are more evenly represented ($r = 0.43$ between PD and Pielou's evenness index, $P < 0.001$), although PD is still more strongly correlated with OTU richness ($r = 0.786$, $P < 0.001$, richness and evenness are uncorrelated: $r = 0.049$, $P = 0.513$). Consistent with these correlations, when examining these phylogenetically naïve diversity measures we also found positive quadratic effects of diet. Species richness, Pielou's evenness and inverse Simpson's diversity are all lowest for individuals with intermediate diets ($tpos^2$ effect $P = 0.009$, 0.046 and 0.037 respectively). Unlike PD, these effects are only detected in models that include interactions between $tpos^2$ and sex and size. Note that for all of the above results, effect sizes are small, with typical $r^2 < 0.1$, due to substantial among-individual microbiota differences even for fish with similar diets. Another diet diversity metric (*G*, see Fig. S3) also reveals a negative relationship between diet and microbial diversity.

Unlike in stickleback, effects of perch diet on microbial phylodiversity (PD) were only detectable when including effects of sex and size (without these, all $P > 0.8$). A model with sex and length and all interactions is significant ($P = 0.021$), but a simpler model (Tabe S3) is preferred, judging by both AIC and LRT ($P = 0.0109$). We find female perch PD is lowest for individuals with intermediate *tpos*, consistent with our findings in stickleback, whereas male perch show the opposite trend, leading to a sex × $tpos^2$ interaction ($P = 0.0197$, Fig. 3). In both sexes, their respective quadratic $tpos^2$ effect on microbial diversity (Fig. 3b, $P = 0.0083$) gets weaker in larger individuals (length × $tpos^2$ $P < 0.0001$), which tend to have higher microbial diversity in general ($P = 0.0001$), particularly in females (sex × size $P = 0.0469$). Females have higher PD than males (Fig. 3; $P = 0.016$, but not significant in models with sex alone). In a model that accounts for these sex- and size-dependent trends, we find a net interaction $\alpha^2 \times tpos^2$ ($P = 0.0106$) that recapitulates our finding from stickleback that individuals with intermediate diets have lower PD. Phylogenetically naïve measures of species richness and evenness, recapitulate the effects described for PD, and so are not presented here in detail.

Focusing on the 64 microbial Classes found in at least five fish, we found many clades that were most or least common in intermediate-diet fish. Using quasibinomial GLMs, 19.1% of Classes in stickleback (19.6% in perch) exhibited significant quadratic effects ($P < 0.05$) of diet, roughly evenly split between positive and negative effects. For example, in both host species Gammaproteobacteria were most abundant in

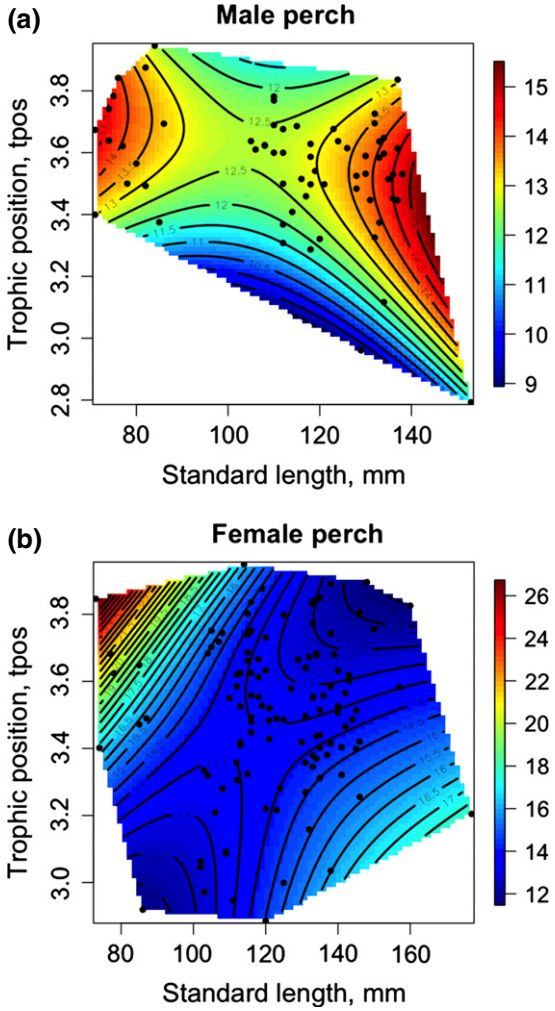

Figure 3 Quadratic fit of phylodiversity (PD) as a function of trophic position and body size in (a) male and (b) female perch. Isoclines and colour indicate PD values (scale to right). Points represent individual observations. Note the different scales for PD for each sex.

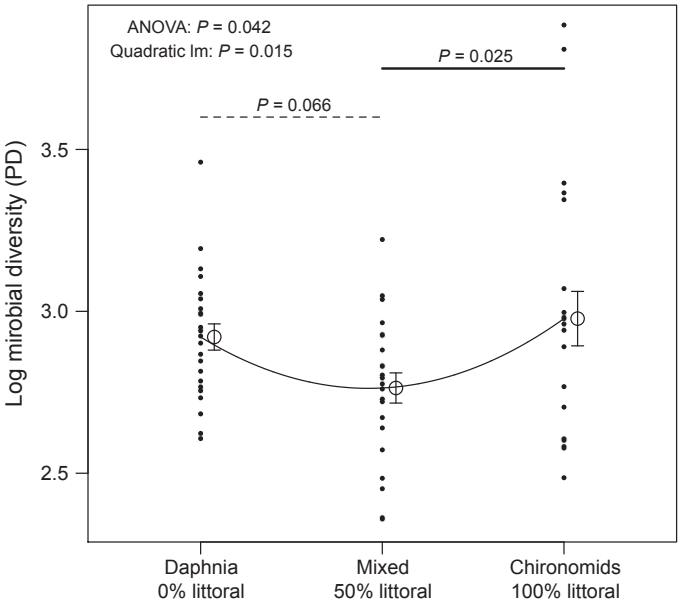

Figure 4 Effect of diet on microbial phylodiversity (PD) in laboratory-reared stickleback. Solid points represent the microbial diversity of individual fish fed either a pure *Daphnia* diet, mixed diet or pure chironomid diet. Open circles represent treatment means with standard error bars. The curve fit through these means is the estimated quadratic linear model (LM) estimated relationship between PD and diet (% littoral prey consumed); the significant positive quadratic coefficient supports the observation from wild-caught fish that mixed-diet (generalist) fish have less diverse microbiota. Dashed and solid lines at the top of the panel indicate non-significant and significant *post hoc* Tukey tests between treatment groups, with corresponding *P*-values. Sex and sex × diet effects were not significant in the ANOVA or quadratic regression and so were dropped from analyses and are not presented.

intermediate-diet fish (Fig. S4). In stickleback, Gammaproteobacteria are typically less than 20% of the microbiota but in generalist individuals can comprise over 90%, with corresponding reductions of common clades like Bacilli (or Clostridia in perch). Because 16S sequencing estimates relative but not absolute abundances we cannot determine whether the increased relative abundance of Gammaproteobacteria in generalist fish entails exclusion of other microbes, or is in addition to the normal community.

Effects of mixed diets on gut microbial diversity in captive stickleback

Microbial diversity (PD) differed significantly among lab-reared stickleback fed either a littoral, pelagic or mixed diet (ANOVA; $F_{2,62} = 3.31$; $P = 0.043$). Species richness shows the same pattern ($P = 0.034$). Sex and sex × diet interactions have no discernable effect on either PD or richness. The effect of diet is driven by a significant difference in microbial diversity between mixed-diet vs. single-diet fish. Mixed-diet stickleback had significantly lower microbial diversity (PD) than chironomid-fed fish, and marginally significantly lower diversity than Daphnia-fed fish (Fig. 4; Tukey test corrected pairwise t-tests $P = 0.025$ and 0.066 respectively). *Daphnia* and chironomid-fed fish were not significantly different ($P = 0.35$). Combining the two single-diet treatments, single-diet fish had higher gut microbial diversity than mixed-diet fish in general ($t = -2.74$, $P = 0.008$). Quadratic regression confirms that PD has a significant U-shaped relationship with the proportion of littoral prey, diversity being lowest for intermediate-diet fish (Fig. 4; linear coefficient $t = -2.23$, $P = 0.029$, quadratic coefficient $t = 2.49$, $P = 0.015$), corroborating our results from wild-caught stickleback. Species richness and evenness yield similar results.

To identify the microbe taxa underlying the non-additive effect of mixed diets, we used quadratic quasibinomial GLMs to identify OTUs whose relative abundance is nonlinearly related to diet. We found 46 out of 263 OTUs had significant nonlinear (quadratic) responses to diet, significantly more than 5% false positive expectations, $P < 0.001$). A majority of these OTUs exhibited positive quadratic effects (Fig. S5, Table S4), being disproportionately rare in mixed-diet fish, consistent with lower diversity in those fish.

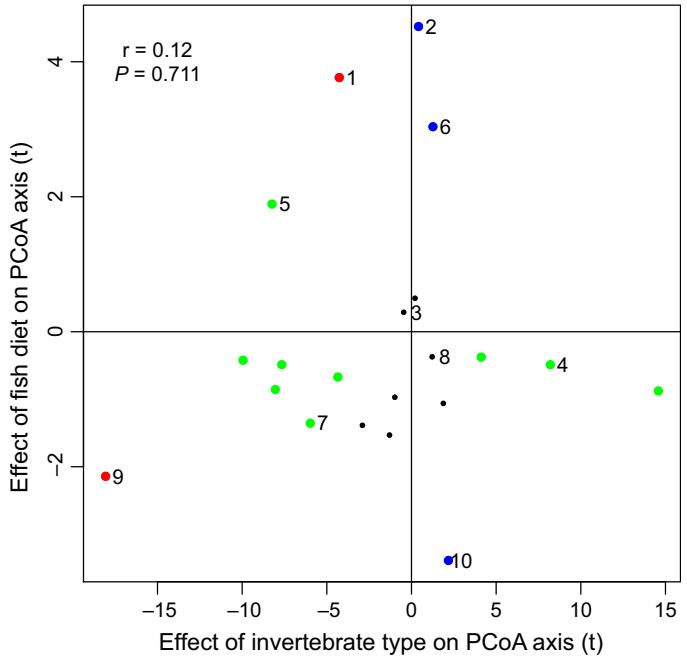

Figure. 5 Differences between prey microbial communities do not explain diet effects on stickleback microbiota. We used t-statistics to measure the effect size and direction of prey type (*Daphnia* or chironomids) on each unweighted PCoA axis. Positive values indicate larger PCoA values in *Daphnia* than chironomids. Similarly, t-statistics measure effect size of fish diet on each PCoA axis. A positive correlation between prey and diet effect sizes would suggest that ingested prey-associated microbes underlie diet effects. We plot diet vs. prey effect sizes for the top 21 PCoA axes (50% cumulative variation), labelling PCoA axes 1–10. Blue points differ significantly between prey, green points between diet treatments and red points differ both between prey and diets ($P < 0.05$). Small points are axes that do not differ between prey or diets. We do not observe the expected positive correlation for any subset of PCoA axes, weighted or unweighted.

Differential exposure to microbes does not explain diet effects

Comparing the microbiota obtained from lab-reared stickleback, their food (chironomids or *Daphnia*), and their aquarium water, we found no evidence that differential exposure to microbes explains the observed diet effects. Prey samples differed significantly in microbial composition along 12 of 21 leading PCoA axes, whereas stickleback fed different foods differed significantly along 7 PCoA axes. However, there is no tendency for PCoA axes enriched in *Daphnia* (or chironomids) to be enriched in *Daphia*-fed (chironomid-fed) stickleback (Fig. 5). Only one PCoA axis (9) shows significant differences, in the same direction, for both prey and stickleback. There is also no tendency for fish to carry more microbial taxa unique to their food source (Fig. S6). Indeed, the trend is in the opposite direction but not significant in either diet treatment (*Daphnia*-fed fish carry slightly more chironomid OTUs, $\chi^2 = 1.99$, $P = 0.156$; chironomid-fed fish carry slightly more *Daphnia* OTUs, $\chi^2 = 1.42$, $P = 0.231$). Finally, stickleback gut microbial diversity (mean PD = 21.4, 17.9, and 17.2 for chironomid-fed, *Daphnia*-fed and mixed-diet fish respectively) was consistently lower than any of the food items (PD = 46.0 and 35.7 for chironomids and *Daphnia* respectively) or the aquarium water (PD = 36.18), all $P < 0.0001$.

DISCUSSION

It is now well established that host diet affects the taxonomic composition of vertebrates' gut microbiota (Muegge *et al.* 2011; Wu *et al.* 2011). In contrast, little is known about how mixtures of different foods jointly influence the gut microbial community. Given that most vertebrates consume diverse mixtures of foods, this represents a major gap in our understanding of the assembly and regulation of animals' symbiotic microbial communities. The intuitive expectation is that individuals consuming a more varied diet would carry a more diverse microbiota, either because they are exposed to more microbial taxa, or because their gut lumen contains a more diverse nutritional environment. Instead, we found a weak but statistically significant tendency for individuals with mixed diets to carry less diverse microbes, within each of two natural populations of hosts (stickleback and perch) and in laboratory diet manipulations of captive stickleback. This quadratic relationship between dietary and microbial diversity was independent of sex in stickleback, whereas in perch the quadratic effect was sex- and size-dependent. Female perch exhibited lower diversity for dietary generalists, as in stickleback, whereas the opposite trend held in perch. Nonetheless, the fact that we observe lower PD in intermediate diet animals (controlling for sex and size effects) in each of two evolutionary divergent host species, and across field and lab environments, suggests a potentially general theme. While we cannot extrapolate beyond the north temperate fish studied here, our findings do show, for the first time, the potential for combinatorial mixtures of host foods to non-additively affect gut microbial communities.

Our results shed new light on a major theme at the intersection of community ecology and human health: how are symbiotic microbial communities assembled? Metacommunity theory describes situations in which transient habitat patches (e.g. individual vertebrates) appear and disappear and are colonised by species (microbial OTUs) from the surrounding matrix of other such patches (Leibold *et al.* 2004). Community diversity within patches depends both on the diversity of colonising species, and how patch characteristics (e.g. host genotype, diet) determine colonists' persistence. Clearly, hosts that consume more food types will be exposed to more microbial taxa. For example, *Daphnia* and chironomids share only about 44% of their microbial OTUs, so individuals consuming both foods will be exposed to 39% more OTUs (Fig. S6). However, fish gut microbiota do not disproportionately resemble the microbes associated with consumed foods, nor do generalists carry more diverse microbes or mixtures of prey-associated foods. We conclude that the effect of diet on gut microbiota is not mediated primarily by microbial colonisation during feeding, and fish gut microbiota are not a subset of the microbes of their prey and water. Instead, we posit that host physiology or gut nutrition must underlie diet effects on microbial composition. This inference is supported by the phylogenetic clustering of individual gut microbial communities (Fig. S2), relative to null models of random community assembly. Such phylogenetic clustering is considered a

hallmark of habitat filtering of colonists in community phylogenetic studies (Webb *et al*. 2002).

Why might mixed diets alter the host gut environment in a manner that reduces microbial diversity? We enumerate a few hypotheses for future testing. First, generalists might have more nutritionally diverse gut environments that can sustain a few competitively dominant microbes at high abundance, which would otherwise be rare or unable to persist in specialists. Supporting this hypothesis, generalist stickleback exhibited a substantial increase in Gammaproteobacteria, which may be a competitive dominant in a mixed nutritional environment. As a result, in dietary generalists microbial relative abundances are significantly less even (more skewed). Low evenness in turn can explain the lower microbial phylogenetic diversity documented here. Consequently, the simultaneous pattern of low evenness and low PD in generalist stickleback suggests that proliferation of a few microbes changes PD via reduced evenness.

Second, each food might contain chemicals that inhibit certain microbes; if generalists consume more such inhibitors, fewer microbial taxa could persist. This explanation is also consistent with our results, because loss of microbe taxa should result in lower OTU richness in generalists, which we do observe (indeed, richness is much more strongly correlated with PD than was microbial evenness). However, because we measure relative rather than absolute abundances, we cannot tell whether uneven abundances occur because of the proliferation of one or a few clades (e.g. Gammaproteobacteria), or the extirpation of other clades. Either way, both hypotheses entail direct effects of diet on microbes, although indirect effects via host physiology are also possible. For example, mixed diets might affect host nutrition, with ancillary effects on host immune defence or tolerance.

A second open question is whether effects of diet mixing on microbial diversity have any broader consequences, particularly given the modest effect sizes found here. Our analyses of host body condition (Fig. S7) suggest that microbial diversity may indeed have fitness consequences for hosts. For example, in male perch, dietary specialists had lower microbial diversity which in turn coincided with higher host condition (relative mass). These partial correlations suggest that microbiota may be a mechanism by which diet diversity affects a common proxy for host fitness. This result is not general: lab stickleback showed the opposite trend (a positive correlation between PD and condition), and wild male stickleback exhibited no effect of PD on condition. Despite these inconsistencies, it is clear that microbial diversity is sometimes associated with variation in host condition. This also holds in humans, where low microbial diversity has been associated with diseases including obesity (Turnbaugh & Gordon 2009). Emerging treatments for these diseases include dietary changes, such as elemental diets, that appear to act, in part, by altering the gut microbiota composition and diversity (Langille *et al*. 2013).

It remains to be determined whether diet mixing has non-additive effects on microbial diversity in other host species. Our hope is that the findings presented here stimulate additional investigations, in diverse organisms, into whether mixing diet items has non-additive effects on microbial composition. It would be particularly valuable to determine whether food sources interactively affect the human gut microbiota. If so, then therapeutic diet changes intended to treat dysbiosis might need to consider not just what foods are consumed, but also account for combinatorial effects foods on microbial community dynamics.

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AUTHORSHIP

The study was conceived and designed by DIB, RS and RK. Samples were collected and lab work done by LKS, PH and CLL. Bioinformatic analyses were done by JGC, statistical analyses and writing by DIB in consultation with all co-authors.

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