

## SPECIAL FEATURE: BIOMARKERS IN TROPHIC ECOLOGY

## Polyunsaturated fatty acids in fishes increase with total lipids irrespective of feeding sources and trophic position

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**Abstract.** Trophic transfer and retention of dietary compounds are vital for somatic development, reproduction, and survival of aquatic consumers. In this field study, stable carbon and nitrogen isotopes, and fatty acids (FA) contents in invertebrates and fishes of pre-alpine Lake Lunz, Austria, were used to (1) identify the resource use and trophic level of Arctic charr (*Salvelinus alpinus*), pike (*Esox lucius*), perch (*Perca fluviatilis*), brown trout (*Salmo trutta*), roach (*Rutilus rutilus*), and minnow (*Phoxinus phoxinus*) and (2) examine how polyunsaturated fatty acids (PUFA; i.e., omega-3 and -6 PUFA) are related to total lipid status, littoral–pelagic reliance, and trophic position. Stable isotope data suggest that pike, perch, and minnow derived most of their energy from littoral resources, but minnows differed from pike and perch in their trophic position and PUFA composition. The co-occurrence of cyprinids, percids, and pike segregated these fishes into more lipid-rich (roach, minnow) and lipid-poor (pike, percids) species. Although the relatively lipid-poor pike and percids occupied a higher trophic position than cyprinids, there was a concurrent, total lipid-dependent decline in omega-3 and -6 PUFA in these predatory fishes. Results of this lake food-web study demonstrated that total lipids in fish community, littoral–pelagic reliance, and trophic position explained omega-3 and -6 PUFA in dorsal muscle tissues. Omega-3 and -6 PUFA in these fishes decreased with increasing trophic position, demonstrating that these essential FAs did not biomagnify with increasing trophic level. Finally, this lake food-web study provides evidence of fish community-level relationship between total lipid status and PUFA or stable isotope ratios, whereas the strength of such relationships was less strong at the species level.

**Key words:** cyprinid; fatty acids; percid; salmonid; Special Feature: Biomarkers in Trophic Ecology; stable isotopes; trophic biomarker.

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## INTRODUCTION

Essential dietary nutrients, including omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFA), must be supplied to consumers to

support their somatic growth, reproduction, and survival (Parrish 2009). Thus, understanding trophic dynamics of such essential dietary nutrients in aquatic food webs is an essential component of community ecology. In lakes, food webs

are predominantly fueled by benthic and pelagic primary producers (Vander Zanden and Vadeboncoeur 2002, Vadeboncoeur et al. 2003, Solomon et al. 2011). Benthic periphyton within the littoral photic zone provides a resource for primary consumers, which in turn are predated by a diverse array of benthic macroinvertebrate predators and littoral fishes. In the pelagic component of the food web, phytoplankton are the key resource for zooplankton that are foraged on by planktivorous fishes and ultimately piscivores. Many fish species obtain their dietary energy not only from one particular diet source, but via highly dynamic feeding strategies and the use of multiple feeding grounds (“linked food chains”; Post et al. 2000; e.g., Schindler and Scheuerell 2002, Vander Zanden and Vadeboncoeur 2002, Hayden et al. 2014a). For example, Vander Zanden et al. (2006) investigated lake trophic dynamics in a subalpine lake and showed that fish retrieved their dietary energy from both benthic and pelagic habitats, but demonstrated that fish production was circa five times more supported by benthic than by pelagic resources. Thus, the recognition of different energy pathways to lake fishes has important implication for assessing the availability and retention of dietary energy and its nutritional quality.

The use of stable carbon and nitrogen isotopes (Boecklen et al. 2011) and fatty acid (FA) biomarkers (Brett and Müller-Navarra 1997) has significantly enhanced our understanding of energy flow and trophic dynamics in aquatic systems. For example, benthic periphyton is typically  $^{13}\text{C}$  enriched relative to phytoplankton; thus, pelagic and benthic components of the food web can readily be distinguished by their  $\delta^{13}\text{C}$  stable isotope ratios (Vander Zanden and Rasmussen 1999). Moreover, the FA profile of primary producers also varies between benthic algae and pelagic phytoplankton, in that benthic algae are generally poor in long-chain PUFA compared to pelagic algae and typically richer in n-6 PUFA (Sushchik et al. 2007, Mariash et al. 2011, Taipale et al. 2013).

Lipids and their FA are particularly important for the provision of dietary energy as they support and, in part, enable important physiological processes in fish (Arts and Kohler 2009). In particular, PUFA are required by all consumers as they are integral parts of their cell membranes

and are, at various degrees, imperative for somatic development of benthic invertebrates (Guo et al. 2016), zooplankton (Müller-Navarra et al. 2000), and fish (Murray et al. 2015). Freshwater fish preferentially retain long-chain n-3 PUFA and specifically docosahexaenoic acid (DHA; 22:6n-3; Ahlgren et al. 1994, Kainz et al. 2004). It is thus important to know if and how PUFA contents in fish are related to assimilated diet from pelagic and/or benthic sources. If, for example, preferential feeding on a specific diet source can predict PUFA compounds in fish, it can be inferred that dietary PUFA are conservatively conveyed and retained in fish (i.e., high predictive power of dietary PUFA for fish; Devillettes et al. 1994). Alternatively, if PUFA profiles in lake fish cannot be predicted by their source-specific dietary supply (e.g., benthic vs. pelagic), fish may be able to bioconvert and/or selectively retain PUFA according to their physiological demand. Scrutinizing these two different points of views is still required.

Encouraged by the ability to assess source-specific diet pathways to fish using stomach contents, stable isotopes, and FA, we ask how feeding on benthic and/or pelagic diets affects the trophic transfer and retention of PUFA in fishes in a subalpine lake (Lake Lunz, Austria) that contains pelagic and littoral habitats of approximately equal size. We expect that fish species in Lake Lunz will show variable levels of resource segregation into distinct habitats and utilize either resource in accordance with their functional ecology (Vander Zanden and Vadeboncoeur 2002). This scenario presents an ideal opportunity to assess the effect of benthic vs. pelagic resource use on the PUFA content of fishes. We test two alternative hypotheses: (1) Littoral species such as perch (*P. fluviatilis*) and pike (*Esox lucius*; Amundsen et al. 2003, Hayden et al. 2014b) will exhibit  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  stable isotope ratios and FA profiles typical of littoral consumers, whereas planktivorous species (i.e., Arctic charr, *Salvelinus alpinus*) will be characterized by pelagic isotope and FA values (Heissenberger et al. 2010), and generalist species (e.g., roach *Rutilus rutilus* and minnow *Phoxinus phoxinus*) will display stable isotope (Hayden et al. 2014c) and FA values indicating littoral and pelagic coupling (“resource-determined PUFA partitioning”); or alternatively, (2) irrespective of feeding sources and trophic positions, total

lipid mass fractions (i.e., mg lipids/g dry weight) in muscle tissues of freshwater fishes predict their PUFA mass fractions and thus fishes adjust their PUFA according to their total lipid status (“fish-inherent PUFA adjustment”).

## METHODS

### Field sampling

The study was carried out during spring (May–June) and summer (July–September) 2014 in the oligotrophic (1–5  $\mu\text{g}$  Chl-a/L; 2–8  $\mu\text{g}$  TP/L), subalpine Lake Lunz (47°51' N, 15°03' E, 600 m a.s.l., surface area = 68 ha,  $Z_{\text{max}}$  = 34 m) in Lower Austria. Fish were collected using gill nets, fishing rods (professional fishermen), and authorized electrical fishing. To avoid problems with spawning-related differences in total lipid contents of these fishes (pike, percids, and cyprinids as spring spawners; salmonids as fall spawners), they were collected between both spawning periods. Sampling complied with ethical requirements and principles; professional and licensed fishermen collected fish, which were anesthetized and then killed by cardiac incision following the Federal Act on the Protection of Animals, Austria (<http://www.ris.bka.gv.at>). Total length (1 mm) and mass (1 g) were measured before dorsal white muscle tissue samples were taken. Analysis was restricted to dorsal white muscle tissue in all fishes to limit effects of seasonal changes in FA and lipids (e.g., Murray et al. 2015), because dorsal muscle tissues are generally leaner and contain less storage fats than ventral muscle tissues (Nanton et al. 2007).

Zooplankton were sampled by vertically hauling a zooplankton net (100  $\mu\text{m}$  mesh size, 36 cm diameter) from 25 m depth to the surface. Bulk zooplankton were retained on a 500- $\mu\text{m}$  and smaller organisms subsequently on a 250- $\mu\text{m}$  filter cup, transferred into a falcon tube (50 mL), and put on ice for transport to the laboratory. Benthic invertebrates were sampled using an Ekman sediment grab sampler, collected on a sieve (400  $\mu\text{m}$ ), and sorted (order level) in the laboratory. All samples were kept frozen at cryogenic temperatures (–80°C) until further analysis.

### Laboratory analysis

*Stomach content analysis.*—A subsample of fish was analyzed for stomach contents using a point

method (Swynnerton and Worthington 1940). In species with a defined esophagus and stomach (i.e., perch and pike), stomach fullness was visually estimated using a scale of 0–10, where 0 and 10 indicate an empty and fully extended stomach, respectively. Prey items were identified to the lowest feasible taxonomic level, and the relative volumetric contribution of each prey group was estimated visually. For cyprinid species lacking a defined stomach (i.e., roach, rudd *Scardinius erythrophthalmus*, and minnow), we investigated the first one-third of intestine and the proportions (%) of different prey types were visually estimated.

*Stable isotope analysis.*—All samples were freeze-dried, homogenized to a fine powder, and submitted for carbon and nitrogen stable isotope analysis at the Stable Isotopes in Nature Laboratory, University of New Brunswick, Canada. Samples were combusted and analyzed in a Finnigan Mat Delta Plus continuous-flow, isotope-ratio mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) connected to a Thermoquest NC2500 elemental analyzer (Thermo Fisher Scientific). Isotope ratios are reported relative to international standards, that is, Vienna PeeDee Belemnite carbonate and atmospheric nitrogen for  $^{13}\text{C}$  and  $^{15}\text{N}$ , respectively. Analytical error was calculated as 0.1‰ for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  based on repeated analyses of an in-house standard, bovine liver tissue.

*Fatty acid analysis.*—Lipids were extracted and analyzed from freeze-dried, homogenized samples (ca. 3–10 mg dry weight) using chloroform–methanol (2:1 v/v) as described in detail by Heissenberger et al. (2010). Total lipids were quantified as mass fractions (mg lipids/g dry weight) gravimetrically using duplicate measurements. Known volumes of total lipid extracts were derivatized to fatty acid methyl esters (FAME) using  $\text{H}_2\text{SO}_4$ –methanol (incubated at 50°C for 16 h). Fatty acid methyl esters were dried under  $\text{N}_2$  before being re-dissolved in hexane and run on a gas chromatograph (TRACE GC THERMO coupled to flame ionization detection) with a Supelco™ SP-2560 column (Bellefonte, Pennsylvania, USA) used for separation of FAME (Bellefonte, Pennsylvania, USA). Fatty acid methyl esters were identified by comparison of their retention times with known standards (37-component FAME mix, Supelco 47885-U; bacterial FAs, Supelco 47080-U; and the following individual

FAME standards: stearidonic acid, O5130 SIGMA; and n-3 docosapentaenoic acid, Supelco 47563-U) and quantified with reference to 7-point calibration curves derived from 2.5, 50, 100, 250, 500, 1000, and 2000 ng/ $\mu$ L solutions of the FAME standard for each identified FA. Fatty acid methyl esters were expressed as mass fractions (mg FA/g dry weight) and as individual FA relative proportions (% of total identified FA).

### Data analysis

*Resource partitioning.*—As lipids are depleted in  $\delta^{13}\text{C}$  relative to muscle, the  $\delta^{13}\text{C}$  isotope ratios of all fish were arithmetically transformed prior to further analysis (Kiljunen et al. 2006). The stable isotope analysis in R mixing model (Parnell et al. 2010) was used to determine the relative utilization of pelagic and littoral resources by fishes. The mean ( $\pm$ standard deviation [SD]) pelagic baseline consisted of two samples of Cladocera and four samples of Copepoda ( $>500\ \mu\text{m}$ ), both of which comprised  $>50$  individuals. The mean ( $\pm$ SD) littoral baseline consisted of *Sialis* sp., Trichoptera, Oligochaeta, Chironomidae, *Lymnaea* sp., *Asellus aquaticus*, and Ephemeroptera ( $n = 2\text{--}15$  for all families) collected in littoral habitat ( $<3\ \text{m}$ ). Standard trophic fractionation values for fish muscle tissue ( $\Delta^{13}\text{C} = 1.3\text{‰} \pm 0.3\text{‰}$ ;  $\Delta^{15}\text{N} = 2.9\text{‰} \pm 0.3\text{‰}$ ) were used in the analysis (McCutchan et al. 2003). The trophic level of each individual fish was determined following the model of Post (2002). Mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for zooplankton and littoral benthic invertebrates were used as the pelagic and benthic end members in the model. A nitrogen fractionation value ( $\Delta^{15}\text{N}$ ) of 2.9‰ per trophic level was used in the model (McCutchan et al. 2003).

Differences in FA mass fractions among fishes were analyzed by ANOVA and Tukey's honestly significant difference (HSD) post hoc test. Principal component analysis (PCA) was applied to assess how fish and their invertebrate diet (benthic invertebrates and zooplankton) were classified according to their FA profiles. In an effort to assess how FA in fish of different sizes, but within the same species, were associated with dietary FA, we grouped pike, perch, and roach into small ( $<100\ \text{mm}$ ) and large ( $>100\ \text{mm}$ ) size classes. We examined saturated, monounsaturated, and polyunsaturated FA (arcsin-square-root-transformed relative values) and discriminated among different taxa according

to their FA profiles. We used this multivariate data analysis because we expected differences between groups (taxa) to be greater than those among taxa. The PCA was conducted using SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, IBM Corp., New York, USA).

*Fish-immanent PUFA adjustment vs. dependence on trophic position.*—Linear regression analyses were performed to examine how n-3 and n-6 PUFA were associated with total lipids in fish muscle tissues. This analysis pooled all fishes, independent of their taxa, size, and feeding behavior, to test the predictive power of total lipids for PUFA mass fractions in these fishes. To test for the dependence of trophic position on PUFA mass fractions in fish, regression analysis was applied using  $\delta^{15}\text{N}$  (predictive variable) and n-3 and n-6 PUFA mass fractions (response variables). To determine the relative importance of total lipid and trophic ecology (i.e., benthic–pelagic resource use and trophic level) to PUFA mass fractions in fish, we used generalized linear models to examine the relationship between PUFA (separate models were performed for n-3 and n-6 PUFA), total lipids, and lipid-corrected  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for all fish analyzed. As all fish were sampled from a single system,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were considered representative of resource use and trophic level, respectively, without a requirement to transform values using a mixing model (Post 2002). Models were simplified using stepwise deletion of terms and the best-fitting models were selected according to Akaike information criterion (AIC) score. Models assumed a Gaussian distribution and were performed using the base statistics in R (R Core Team 2015).

## RESULTS

### *Hypothesis 1: resource partitioning*

*Stomach content analysis.*—Stomach content analysis indicated that pike used both fish and invertebrates, whereas perch used both pelagic zooplankton (*Daphnia*) and benthic macroinvertebrates (*A. aquaticus*, Ephemeroptera nymphs, and Trichopteran larvae; Appendix S1). Similar to perch, roach also used zooplankton (*Daphnia*) and benthic macroinvertebrates (Trichoptera larvae, *Valvata* snails). Minnow mainly consumed cladocerans, whereas rudd foraged on macrophytes (Appendix S1).



Table 1. Sample size ( $n$ ), mean ( $\pm$ standard deviation) total length, mass, stable isotope ratios, and trophic level (TL) of the fish species samples from Lake Lunz.

Fish	$n$	Length (mm)	Mass (g)	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	TL	Pelagic	Littoral
Arctic charr	6	–	–	–33.1 (0.3)	4.4 (0.2)	3.3 (0.1)	96 (70–100)	4 (0–29)
Minnow	16	62 (4)	–	–30.3 (0.8)	3.9 (0.6)	2.8 (0.3)	25 (13–36)	74 (63–86)
Perch	18	149 (86)	92 (108)	–28.9 (1.2)	5.4 (0.8)	3.1 (0.2)	1 (0–7)	99 (92–100)
Pike	15	384 (94)	401 (310)	–29.3 (0.8)	5.8 (0.8)	3.3 (0.3)	1 (0–10)	99 (89–100)
Roach	21	126 (97)	75 (127)	–30.8 (2.4)	3.7 (0.9)	2.7 (0.3)	40 (20–54)	60 (45–79)
Brown trout	2	313.0	321 (30)	–27.9 (0.7)	5.6 (0.2)	–	–	–
Rudd	6	323 (86)	530 (402)	–24.3 (1.4)	3.3 (1.3)	–	–	–

Notes: Mode, 95% credibility intervals in parentheses, values for pelagic and littoral resource reliance for each group as defined by the stable isotope analysis in R mixing model are also provided. Trophic-level and resource-use values could not be calculated due to small sample size (brown trout) and inadequate characterization of potential prey resources (rudd).

**Stable isotope analysis.**—Considerable variation in stable isotope ratios was observed between pelagic, littoral, and profundal invertebrate communities (Fig. 1). Littoral benthic invertebrates were enriched in  $^{13}\text{C}$  (mean  $\pm$  SD:  $-31.1\text{‰} \pm 1.6\text{‰}$ ) relative to bulk zooplankton ( $-35.2\text{‰} \pm 0.6\text{‰}$ ), whereas  $^{15}\text{N}$  values were equivalent between both groups (littoral benthic macroinvertebrates:  $1.6\text{‰} \pm 1.3\text{‰}$ ; zooplankton:  $-0.2\text{‰} \pm 1.2\text{‰}$ ). Profundal benthic macroinvertebrates had equivalent  $\delta^{13}\text{C}$  values ( $-35.3\text{‰} \pm 1.0\text{‰}$ ) to zooplankton, but were considerably enriched in  $^{15}\text{N}$

( $4.0\text{‰} \pm 0.2\text{‰}$ ) relative to both other groups. Further variation in the isotope ratios of zooplankton was evident as predatory copepods, mostly cyclopoids (e.g., *Cyclops tatricus*; 250–500  $\mu\text{m}$ ), were enriched in  $^{15}\text{N}$  ( $1.49\text{‰} \pm 0.1\text{‰}$ ) and  $^{13}\text{C}$  ( $-34.4\text{‰} \pm 0.2\text{‰}$ ) relative to larger (>500  $\mu\text{m}$ ) herbivorous cladocerans, in particular *Daphnia longispina* ( $^{15}\text{N}$ :  $-0.7\text{‰} \pm 0.8\text{‰}$ ;  $^{13}\text{C}$ :  $-35.5\text{‰} \pm 0.3\text{‰}$ ).

Trophic-level discrimination was evident within the fish community in Lake Lunz. Sampled pike, Arctic charr, brown trout (*S. trutta*), and perch

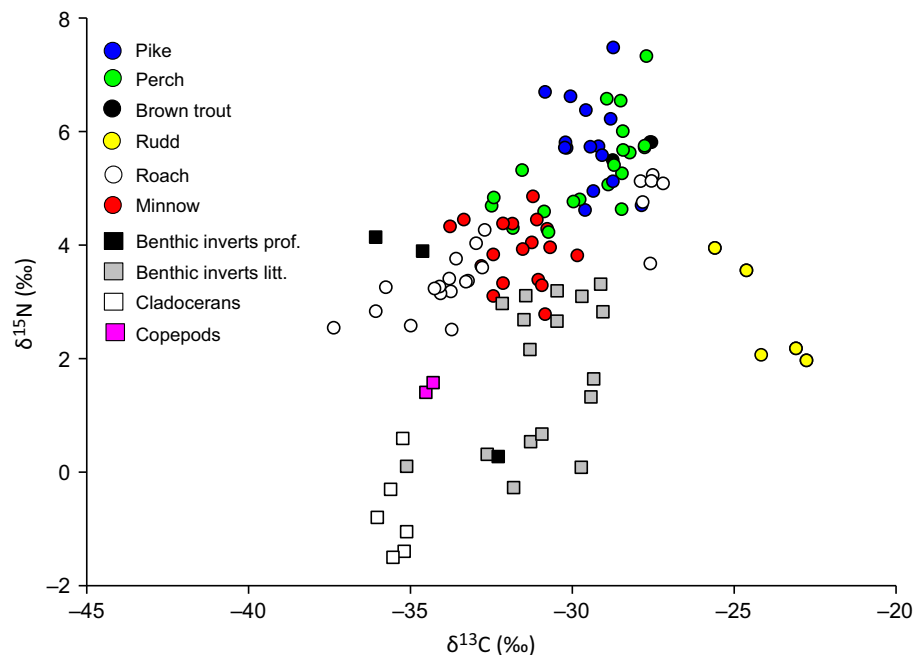


Fig. 1. Stable isotope bi-plot outlining the trophic position of all fish species, zooplankton, and littoral and profundal benthic macroinvertebrates.

Table 2. Total lipids (mg/g dry weight) and relative distribution (%;  $\pm$ standard deviation) of fatty acid groups: SAFA, MUFA, omega-3 (n-3) and omega-6 (n-6) PUFA, and n-3/n-6 PUFA ratios of indicated fish, benthic invertebrates, and zooplankton in Lake Lunz.

Species	Total lipids	SAFA	MUFA	n-3 PUFA	n-6 PUFA	n-3/n-6
Minnow	116.6 $\pm$ 29.5	29.5 $\pm$ 2.6	30.1 $\pm$ 4.1	28.4 $\pm$ 3.7	10.2 $\pm$ 1.0	2.8 $\pm$ 0.4
Perch (in mm)						
<100	77.4 $\pm$ 33.2	29.2 $\pm$ 3.2	23.7 $\pm$ 6.5	35.9 $\pm$ 4.6	10.2 $\pm$ 1.4	3.6 $\pm$ 0.8
>100	38.9 $\pm$ 7.9	32.7 $\pm$ 3.4	18.6 $\pm$ 4.1	38.6 $\pm$ 2.7	9.6 $\pm$ 2.1	4.2 $\pm$ 0.9
Pike (in mm)						
<100	31.2 $\pm$ 5.2	28.7 $\pm$ 4.2	14.2 $\pm$ 3.0	46.7 $\pm$ 4.1	9.9 $\pm$ 0.8	4.8 $\pm$ 0.6
>100	32.1 $\pm$ 5.0	32.1 $\pm$ 4.3	14.5 $\pm$ 2.4	44.2 $\pm$ 3.4	8.5 $\pm$ 1.7	5.4 $\pm$ 1.0
Roach (in mm)						
<100	162.6 $\pm$ 41.7	33.6 $\pm$ 5.3	26.0 $\pm$ 4.0	29.1 $\pm$ 5.6	8.7 $\pm$ 1.8	3.4 $\pm$ 0.5
>100	46.4 $\pm$ 5.3	25.6 $\pm$ 0.9	24.8 $\pm$ 5.6	34.3 $\pm$ 4.6	14.1 $\pm$ 1.1	2.4 $\pm$ 0.3
Brown trout	59.5 $\pm$ 0.5	30.5 $\pm$ 0.1	18.1 $\pm$ 3.1	43.2 $\pm$ 2.1	7.7 $\pm$ 1.0	5.7 $\pm$ 0.5
Rudd	51.3 $\pm$ 23.4	24.6 $\pm$ 2.7	18.2 $\pm$ 4.7	42.1 $\pm$ 3.0	14.3 $\pm$ 0.9	2.9 $\pm$ 0.1
Benth. invertebrates†	88.1 $\pm$ 37.2	24.9 $\pm$ 7.5	30.0 $\pm$ 9.0	18.3 $\pm$ 7.2	24.5 $\pm$ 13.7	0.9 $\pm$ 0.4
Cladocerans	16.3 $\pm$ 2.1	33.3 $\pm$ 0.8	21.3 $\pm$ 1.2	32.4 $\pm$ 1.8	9.7 $\pm$ 0.3	3.3 $\pm$ 0.3
Copepods	19.8 $\pm$ 2.3	32.4 $\pm$ 0.7	17.2 $\pm$ 0.2	36.6 $\pm$ 0.6	10.8 $\pm$ 1.1	3.4 $\pm$ 0.4

Note: SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

† Benthic invertebrates (mostly littoral).

had mean trophic-level values  $>3$  (Table 2), whereas roach and minnow were feeding at a trophic-level values  $<3$ . However, the degree of separation between putative prey and predatory fishes was often less than expected (Fig. 1). Pike and perch predominantly foraged in the littoral food-web compartment, with minimal evidence of pelagic or profundal feeding (Table 1, Fig. 1). Results of the stable isotope mixing model revealed resource segregation among the species (Fig. 2). Arctic charr drew the majority (95% credibility limits of pelagic resource use: 70–100%) of their energy from the pelagic food-web compartment, whereas all the remaining species were to some extent reliant on littoral resources (Fig. 2). Minnow (13–36% pelagic resource reliance) and roach (20–54%) integrated both littoral and pelagic components of the food web, whereas perch (0–7%) and pike (0–10%) fed exclusively from the littoral food-web compartment (Table 1, Fig. 2). The stable isotope ratios of rudd were outside the mixing polygon derived from the basal sources (Fig. 1) and were therefore excluded.

**Fatty acid composition.**—Fatty acid mass fractions in benthic invertebrates, zooplankton, and fishes are listed in Appendix S2. The relative FA composition (%) differed among fish, benthic invertebrates from the littoral (Ephemeroptera, Trichoptera, Hirudinea, *Lymnaea* sp.) and profundal (only a few Oligochaeta, FA not reported),

and zooplankton (*D. longispina*  $>500$   $\mu\text{m}$  and *Cyclops* sp. and *Eudiaptomus* sp.  $<500$   $\mu\text{m}$ ). The first principle component (PC1) explained 33% of the variability in FA (Fig. 3); the proportions of 18-PUFA (i.e., 18:2n-6, 18:3n-3, 18:4n-3) and 20:3n-3, 20:4n-3, and 20:5n-3 were positively correlated, while 20:4n-6 and the 22-PUFA (22:3n-3, 22:4n-6, 22:5n-3, and 22:6n-3) were negatively correlated on PC1. Omega-3 22-PUFA were negatively correlated on PC2, and n-6 20- and 22-PUFA positively (Fig. 3). The 20-PUFA were generally positioned between 18- and 22-PUFA and correlated positively with PC2.

Omega-3 PUFA (%) were two to five times higher than n-6 PUFA in all fish and zooplankton, and only benthic invertebrates had slightly less n-3 than n-6 PUFA (Table 3). Similar to the FA groupings on the PCA, large roach, rudd, and minnow had lower n-3/n-6 PUFA ratios than other fish or zooplankton, and were thus closer to the n-3/n-6 PUFA ratios of benthic invertebrates. Perch, pike, and brown trout had the highest n-3/n-6 PUFA ratios and also grouped together on the PCA plot (Fig. 3).

Proportions (%) of FA in small roach ( $<100$  mm) were associated with FA in pelagic zooplankton and similar to those in minnows, whereas FA in large roach ( $>100$  mm) were closely associated with those in benthic invertebrates (Fig. 3). There was, however, no clear separation

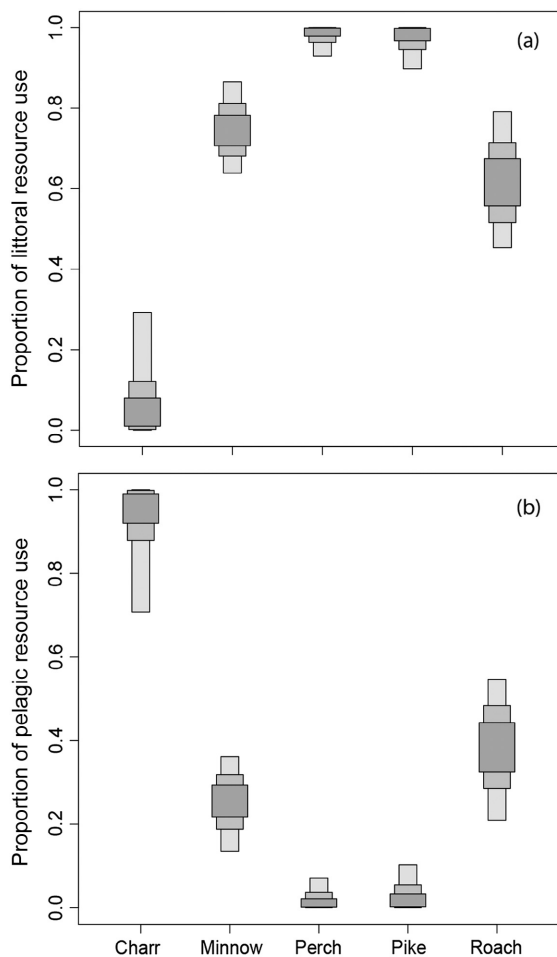


Fig. 2. The littoral (a) and pelagic (b) resource use of fish sampled from Lake Lunz. Gradations denote 50%, 75%, and 95% credibility limits.

in the FA proportions between small and large (>100 mm) pike or perch that were most strongly associated with long-chain (C22) n-3 PUFA. Perch and pike had similarly high n-3/n-6 PUFA ratios (Table 2), whereas smaller roach had higher n-3/n-6 PUFA ratios ( $3.4 \pm 0.5$ ) than larger roach ( $2.4 \pm 0.3$ ).

#### Hypothesis 2: fish-immanent PUFA adjustment

Total lipid mass fractions differed considerably among the different fish taxa: Pike was the leanest fish ( $31.8 \pm 4.9$  mg/g dry weight), followed by large perch and large roach ( $38.9 \pm 7.9$  and  $46.4 \pm 5.3$  mg/g dry weight, respectively), rudd ( $51.3 \pm 23.4$  mg/g dry weight), brown

trout ( $59.6 \pm 0.5$  mg/g dry weight), small perch ( $77.4 \pm 33.2$  mg/g dry weight), minnow ( $116.5 \pm 29.5$  mg/g dry weight), and small roach ( $162.6 \pm 41.7$  mg/g dry weight). By contrasting total lipids and the sum of, respectively, n-3 and n-6 PUFA mass fractions for all fish species, total lipids significantly predicted 85% of the n-3 PUFA and 79% of the n-6 PUFA variation in these fishes (linear regression;  $R^2 = 0.85$ ,  $F_{2,75} = 418$ ,  $P < 0.0001$ , and  $R^2 = 0.79$ ,  $F_{2,75} = 283$ ,  $P < 0.0001$ ; Fig. 4a, b). We performed ln-transformation of these lipid data because of increasing variance with increasing mass fractions of both total lipids and PUFA. Omega-3 PUFA mass fractions differed, however, significantly among these fishes (ANOVA;  $F_{2,32} = 9.4$ ,  $P < 0.001$ ), with pike having the lowest n-3 PUFA mass fractions ( $6.1 \pm 1.1$  mg/g dry weight) of all investigated fish, which were significantly lower (Tukey's HSD;  $P < 0.001$ ) than those of roach ( $20 \pm 10.2$  mg/g dry weight) and minnow ( $17.6 \pm 4.7$  mg/g dry weight).

Total lipids in dorsal muscle tissues predicted saturated fatty acids (SAFA) mass fractions in pike, perch, and trout (linear regression;  $R^2 = 0.94$ ;  $P < 0.0001$ ) and in minnow, roach, and rudd ( $R^2 = 0.97$ ;  $P < 0.0001$ ). However, total lipids in pike, perch, and brown trout provided a higher predictability of monounsaturated fatty acids (MUFA;  $R^2 = 0.93$ ;  $F_{1,43} = 489$ ;  $P < 0.0001$ ) and all PUFA ( $R^2 = 0.97$ ;  $F_{1,43} = 1064$ ;  $P < 0.0001$ ) than in lower trophic-level minnow, roach, and rudd ( $R^2 = 0.74$ ;  $F_{1,33} = 117$ ;  $P < 0.0001$ , and  $R^2 = 0.57$ ;  $F_{1,33} = 55$ ;  $P < 0.0001$ , respectively). Although total lipid mass fractions were significantly correlated with n-3 and n-6 PUFA in fishes at higher trophic positions, total lipids in minnow, roach, and rudd did not significantly predict their DHA (linear regression;  $P = 0.79$ ) or arachidonic acid (ARA;  $P = 0.06$ ) mass fractions.

As hypothesized, n-3 and n-6 PUFA mass fractions decreased exponentially with increasing trophic levels ( $\delta^{15}\text{N}$  signatures) in these fishes ( $R^2 = 0.36$ ,  $F_{1,40} = 22.1$ ,  $P < 0.001$  and  $R^2 = 0.49$ ,  $F_{1,40} = 38.9$ ,  $P < 0.001$ , respectively; Fig. 4e, f). In general, roach (>100 mm) and minnow had higher n-3 and n-6 PUFA mass fractions and lower  $\delta^{15}\text{N}$  signatures than pike, perch, and trout.

Generalized linear models indicated that of the three predictors analyzed (i.e., total lipid,  $\delta^{13}\text{C}$ , and  $\delta^{15}\text{N}$ ), total lipid had the greatest influence on both n-3 and n-6 PUFA (Table 2). Akaike

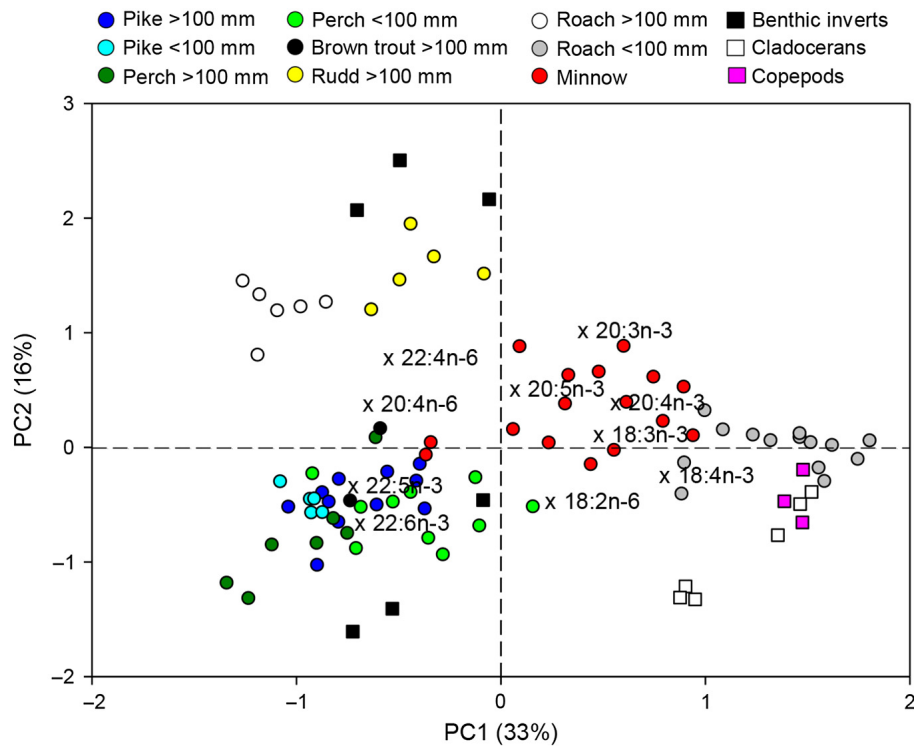


Fig. 3. Principle components analysis of fatty acids for benthic macroinvertebrates, zooplankton, small (<100 mm; pike, perch, roach, and minnow), and large (>100 mm; pike, perch, brown trout, rudd, and roach) fishes in Lake Lunz. Zooplankton, minnow, and small roach are separated from benthic invertebrates and other fishes on PC1. Vectors lengths from origo are shown for polyunsaturated fatty acids.

information criterion scores indicated that the best-fit model in each case included total lipid and one isotope ( $\delta^{13}\text{C}$  for n-3 PUFA and  $\delta^{15}\text{N}$  for n-6 PUFA, respectively). However, the importance

of these isotopes was small and their removal from the models had a minor effect on the AIC score, especially when compared with the removal of total lipid (Table 3).

Table 3. Generalized linear models of the relationship between TL mass fractions (mg/g), littoral–pelagic resource use ( $\delta^{13}\text{C}$ ), trophic level ( $\delta^{15}\text{N}$ ), and omega-3 (n-3 PUFA) and omega-6 (n-6 PUFA) PUFA fractions (mg/g) in fishes sampled from Lake Lunz.

PUFA	Model	TL	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	AIC
n-3	TL + $\delta^{13}\text{C}$	10.38***	-1.72		419.5
	TL	13.88***			420.5
	TL + $\delta^{13}\text{C}$ + $\delta^{15}\text{N}$	8.32***	-1.41	-0.45	421.2
	TL + $\delta^{15}\text{N}$	8.84***		-1.06	421.3
	$\delta^{13}\text{C}$ + $\delta^{15}\text{N}$		-2.46*	-4.73***	469.8
n-6	TL + $\delta^{15}\text{N}$	13.29***		-1.57	171.05
	TL	20.64***			171.57
	TL + $\delta^{13}\text{C}$	16.04***	-1.24		171.99
	TL + $\delta^{13}\text{C}$ + $\delta^{15}\text{N}$	12.7***	-0.69	-1.17	172.54
	$\delta^{13}\text{C}$ + $\delta^{15}\text{N}$		-20.9*	-5.45***	258.05

Notes: AIC, Akaike information criterion; PUFA, polyunsaturated fatty acids; TL, total lipid. Models are arranged in order of increasing AIC score and indicate the effect of stepwise deletion of terms on model score.

\* $P < 0.05$ ; \*\*\* $P < 0.001$ .



## DISCUSSION

Results of this lake food-web study demonstrate that total lipids in fish, independent of their taxa, littoral–pelagic reliance, or trophic level, predicted the n-3 and n-6 PUFA mass fractions in their dorsal muscle tissues and thus confirm our alternative hypothesis that fishes adjust their PUFA according to their total lipid status. In fact, n-3 and n-6 PUFA in these freshwater fishes decrease with increasing trophic position, implying that these essential FA do not accumulate with increasing trophic level. Moreover, in contrast to our first hypothesis, stable isotope-based evidence reveals that pike, perch, and minnow share the littoral as their common feeding ground, but differ in their PUFA composition.

Stable isotope analysis of the Lake Lunz food web indicates that the analyzed fauna can be segregated into two broad ecological units: a pelagic compartment consisting of cladocerans, copepods, small roach, and Arctic charr, and a littoral compartment supporting a greater diversity of primary and secondary consumers. A likely predator–prey relationship was evident in the zooplankton community as the smaller size class, predominantly consisting of predatory cyclo-pods, was enriched in  $^{15}\text{N}$  relative to the larger size class, which was mostly comprised of cladocerans. Assuming a trophic fractionation value of approximately  $\Delta 2.9\text{‰}$  for  $\delta^{15}\text{N}$  (McCutchan et al. 2003), one could infer that small roach prey mostly on cladocerans, whereas Arctic charr,  $^{15}\text{N}$  enriched relative to roach, forage predominantly on copepods. However, isotope ratios of zooplankton in subalpine lakes may exhibit strong seasonal variation (Perga and Gerdeaux 2005), and as muscle tissue of consumers will integrate the isotope ratios over a period of three to six months, further details are required to determine the exact trophic ecology of both fishes (Hayden et al. 2014a).

Stable isotope data suggest that the majority of fish in Lake Lunz are reliant on littoral derived resources. The  $\delta^{13}\text{C}$  values of minnow, perch, pike, and brown trout were analogous with those of littoral benthic invertebrates. This was further evident in the stable isotope mixing models, which ascribed high littoral reliance to all of these species. Roach, a typical generalist (Hayden et al. 2014c), appear to integrate both pelagic

and littoral resources during their ontogeny. Pike, perch, and brown trout occupied the highest trophic positions in the littoral component of the Lake Lunz food web. Due to the isotopic similarity of these three piscivores and their putative prey fishes, resource partitioning at the piscivore level could not be distinguished using the stable isotope mixing models (Fry 2013). However, based on the  $\delta^{13}\text{C}$  values alone, it is evident that this piscivores guild is primarily reliant on littoral resources such as minnow.

Interestingly, piscivorous species such as perch and pike displayed a trophic level only marginally higher than invertebrate consumers such as roach and minnow. It should be noted that both pike and perch had fed on benthic macroinvertebrates and their mean trophic level could be higher, if larger specimens had been captured. This may in part also be a consequence of large  $\delta^{15}\text{N}$  variability in the benthic invertebrate fauna. Similar to the predator–prey relationship evident in zooplankton, the benthic invertebrate community consists of primary and secondary consumers, which are distinguished by their  $\delta^{15}\text{N}$  values. Consequently, predatory invertebrates, for example, some species of *Sialis* sp. or Trichoptera, can have  $\delta^{15}\text{N}$  values within the same range as fish foraging on primary consumers, that is, roach or minnow (Vander Zanden and Rasmussen 1999). In the presented dataset, some *Sialis* sp. and Trichoptera samples along with roach, rudd, and minnow had  $\delta^{15}\text{N}$  values ranging between 2‰ and 3‰. As roach, rudd, perch, and minnow to some extent also forage on predatory invertebrates, this overlap leads to a breakdown of the typical composition of trophic levels within the food web. Thus, the strongest characterization of the Lake Lunz food web available from the stable isotope analysis is into the pelagic and littoral components outlined above with just a coarse overview of trophic levels determined from  $\delta^{15}\text{N}$ .

Similar to the stable isotope results, patterns of FA in fish, pelagic zooplankton (cladocerans and copepods), and benthic invertebrates suggest that particularly small roach feed primarily on pelagic zooplankton, whereas pike, perch, brown trout, rudd, and large roach share a cluster with benthic invertebrates from the littoral component of the food web (i.e., associated with 20- and 22-carbon PUFA). Benthic invertebrate FA were widely

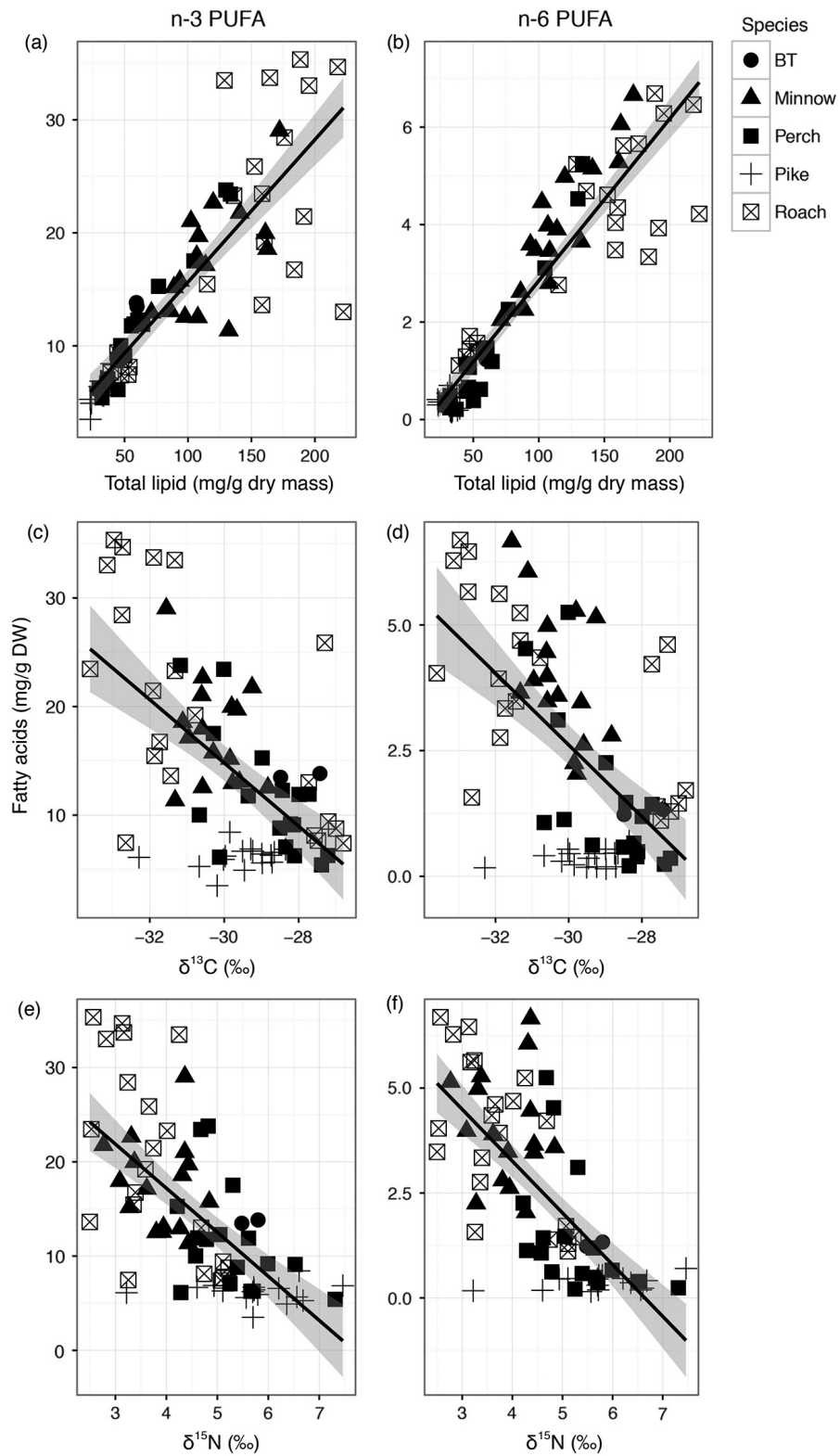


Fig. 4. Scatterplots outlining the relationship between total lipids (a, b),  $\delta^{13}\text{C}$  (c, d), and  $\delta^{15}\text{N}$  (e, f), and

(Fig. 4. Continued)

omega-3 polyunsaturated fatty acids (PUFA; n-3 PUFA) and omega-6 polyunsaturated FAs (n-6 PUFA) in fishes sampled from Lake Lunz. Linear regression (black line) and 95% confidence intervals (gray shading). Generalized linear models describing underlying factors are presented in Table 3. Abbreviation BT means brown trout.

dispersed (PCA), scoring negatively on PC1 and mostly positively on PC2, and being associated with large roach and rudd (more n-6 PUFA) and also with perch, pike, and brown trout (more n-3 PUFA). Combining these FA patterns with n-3/n-6 ratios in large roach, rudd, and minnow shows that they are most closely related to the low n-3/n-6 ratios in the littoral benthic invertebrates. Although it has been suggested that n-3/n-6 ratios in cichlids can track their diet sources (Kuusipalo and Käkekä 2000), the n-3/n-6 ratios in fish of this study provide less clear information. For example, pike, brown trout, and perch contained the highest n-3/n-6 PUFA ratios (3.6–5.7), clearly distinct from the low n-3/n-6 ratios in littoral benthic invertebrates ( $0.9 \pm 0.4$ ), whereas the stable isotope data in contrast indicate a predominant littoral feeding of pike and perch. Suggestively, the predictive power of n-3/n-6 ratios in fish as littoral vs. pelagic diet source indicator gets weaker with increasing trophic level.

Quantification of ontogenetic variation in the resource use of the studied fishes provided further evidence that n-3/n-6 PUFA ratios are better predictors for ontogenetic diet shifts in planktivorous/benthivorous than in carnivorous fish. Fatty acids successfully assessed differences in feeding sources between small and large roach. Much of the resource use of small roach was obtained from pelagic sources as evidenced by the PC1 scores that were associated with FA patterns in zooplankton. Importantly, the FA patterns were distinctly different between small and large roach, the latter being more related to littoral benthic invertebrates. This finding is further supported by lower n-3/n-6 PUFA ratios in larger ( $2.4 \pm 0.3$ ) than in smaller roach ( $3.4 \pm 0.5$ ), the latter being very similar to the ratios observed in zooplankton. Such ontogenetic diet shifts could in contrast not be determined between small and large perch or pike as both fish taxa had similar PCA scores and n-3/n-6 PUFA ratios.

Contrary to a mixed diet contribution from benthic and pelagic sources observed in brown trout

(Vander Zanden et al. 2006) and Arctic charr (this study), perch and pike, but also minnow occupy mostly littoral food-web compartment in the Lake Lunz food web. However, pike and perch had the lowest total lipid mass fractions and their n-3 PUFA were ~3 times and 1.5 times lower than in minnow, and their n-6 PUFA even 11 times and 2.7 times lower, respectively. This clearly shows that sharing the same feeding grounds cannot predict n-3 or n-6 PUFA mass fractions in fish. By contrast, irrespective of fish taxa, feeding grounds, or trophic position, total lipids significantly predicted n-3 and n-6 PUFA in these lake fishes. These results show that lipid and thus PUFA accumulation cannot be equated with increasing trophic position of fish, but that both lipids and PUFA rather are strongly regulated by taxa and their lipid-related metabolism. It must be stressed, however, that not all n-3 or n-6 PUFA in fish are linearly dependent on total lipids; clearly, the highly unsaturated n-3 PUFA DHA and n-6 PUFA ARA were not significantly related to total lipids in lower trophic-level fish such as rudd, roach, or minnow, but significantly predicted by total lipids in predatory fish, such as pike, perch, and trout. This strongly suggests that the retention of DHA and ARA in predatory fish is highly selective and not a mere function of total lipids.

In conclusion, the co-occurrence of cyprinids, percids, and pike in the present lake food web segregates these fishes into more lipid-rich (roach, minnow) and lipid-poor (pike, perch) species. Although the relatively lipid-poor pike and perch occupy a higher trophic position than the cyprinids, there is a concurrent, total lipid-dependent decline in n-3 and n-6 PUFA. Such decline in PUFA with increasing trophic levels of lake fishes demonstrates that n-3 and n-6 PUFA do not biomagnify along the trophic fish cascade, but are a function of total lipids in fishes. Finally, this lake food-web study provides evidence that fishes adjust their PUFA according to their total lipid status, irrespective of their feeding sources and trophic positions.

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