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Trophic pathways of omega-3 polyunsaturated fatty acids in stream food webs

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Abstract

In series of five studies, the ecological significance of ***stream algae*** in small and shaded rivers was investigated, with the focus on the dietary contribution of algal-derived ***polyunsaturated fatty acids (PUFAs)*** to secondary production in stream ecosystems. The research addressed the question of whether stream consumers (e.g., benthic invertebrates and fish) primarily rely more on autochthonous (benthic algae, epilithon) or allochthonous (fresh or submerged leaf litter) sources for essential nutrients, while also considering internal processes, such as the enzymatic bioconversion of precursor PUFAs to long-chain polyunsaturated fatty acids (LC-PUFAs), which are particularly important for the development of neural tissues in vertebrates (e.g., brain and eyes). This question challenges long-standing ecological paradigms (e.g., River Continuum Concept, RCC), which traditionally emphasized the importance of terrestrial inputs to ***low-productivity headwaters*** leading to a systematic underestimation of their ecosystem function and, consequently, their ecological significance.

All five studies traced the source and fate of essential fatty acids in river food webs by using field investigations, experimental analyses, and/or advanced compound-specific stable isotope analysis (CSIA) and revealed that stream algae, rather than terrestrial inputs, are the primary source of LC-PUFAs for invertebrates and higher trophic levels (e.g., fish). These findings have significant implications for nutrient cycling and energy transfer within river ecosystems, highlighting the crucial role of stream algae in providing ***eicosapentaenoic acid*** (20:5n-3, EPA) to higher trophic levels.

This research revised current ecological models of ***riverine productivity*** and underscored the need to preserve freshwater ecosystems and their algal resources, particularly given the detrimental impact of climate change on the nutritional quality at the trophic base of sensitive river habitats.

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Introduction

In the following five trophic ecology studies, we delved into the ecological significance of stream algae in small and shaded rivers. The goal was to enhance the understanding of dietary contribution of algae to secondary production in streams and their role in providing essential algal-derived nutrients for aquatic consumers and their neural tissues.

Trophic research provide a profound understanding of ecosystem functioning and powerful frameworks on ecosystem-level processes such as nutrient cycling and energy fluxes (Odum, 1968), leading to better insights into community structures (Elton, 1927; Lindeman, 1942) and ecosystem connectivity by cross-boundary resource-use (Baruch et al., 2021; Baxter et al., 2005; Polis & Strong, 1996) shaping pathways of dietary *energy* and *organic matter* within and across ecosystems. While such trophic linkages are common to all natural systems, they have been extensively researched in cases in which habitats significantly differ in *productivity*, such as aquatic versus terrestrial ecosystems (Huxel et al., 2002; Polis & Strong, 1996; Polis & Hurd, 1996). Consequently, this ecological interface has undergone extensive investigations. Food sources produced within the system (in this case: rivers) are termed *'autochthonous'* while those from other are labelled *'allochthonous'* (e.g. riparian vegetation). However, the latter can contribute significantly to secondary production in low-productivity systems.

Productivity in freshwater ecosystems, especially that of forest rivers, has often been considered insignificant compared to marine and terrestrial ecosystems. This misconception partly arises from direct comparisons of standing crops (terrestrial inputs > riverine biofilms) or *net ecosystem production* (NEP,Fisher & Likens, 1973) without considering turnover rates or nutritional quality (Brett et al., 2017; Elser et al., 2000; Mayer & Likens, 1987). So far, a large body of empiric studies on riverine metabolism solidified the conception about the distinct *heterotrophy* of temperate rivers (average annual gross primary productivity, GPP: 331 g C m^{-2} $years^{-1}$, ER: −591 g C m^{-2} $year^{-1}$, NEP: −260 g C m^{-2} $year^{-1}$; Battin et al., 2023; Cummins, 1974). Consequently, many ecologists have mistakenly concluded that terrestrial inputs must be crucial for the growth and somatic reproduction of stream consumers (Brett et al., 2017).

The paradigm that headwater systems are heterotrophic and that their consumers heavily rely on terrestrial inputs, became further backed by Vannote's *River Continuum Concept* (RCC, Vannote et al., 1980). This conceptual framework linked predictive, longitudinal changes in environmental factors (*e.g.,* light availability, nutrients, stream size) along rivers to productivity, and consequently, to food availability. These gradual changes in environmental factors and food supply provoke shifts in the taxonomy and functionality of macroinvertebrate communities (Minshall et al., 1985). *Vannote et al.* (1980) integrated the ratio of gross primary productivity (P) to community respiration (R) as a proxy for measuring riverine productivity (Cummins, 1974; Fisher & Likens, 1973; Odum, 1956). Their hypothesis suggested that small and shaded headwaters tend to be more heterotrophic (P/R < 1) than larger rivers (stream order > 3) and thus, headwater consumers are more reliant on extra-carbon from terrestrial inputs (*e.g.,* leaf litter) as they are short on in-stream food sources. However, the metabolic state of a river alone is a weak indicator of the dietary importance of terrestrial subsidies for higher trophic levels (Brett et al., 2017; Thorp & Delong, 2002), a contradiction later termed the "*Heterotrophy Paradoxon*". Moreover, analysis of the mere *gut content* (Bogatov et al., 2024; Cummins et al., 1966; Mecom & Cummins, 1964) done in the past and even to date, fails to draw conclusions about diet uptake to consumer tissues. Consequently, the RCC overestimates *carbon quantity* and neglects the importance of its

dietary quality retained in stream consumers. That conflicts with the current state of knowledge regarding the importance of autochthonous carbon (riverine and algal) as a high-quality food source for secondary production of stream consumers (Brett et al., 2017; Guo et al., 2016; Hayden et al., 2016; Rosi-Marshall & Wallace, 2002). Consequently, the significance of rivers for ecosystem functionality was underestimated systematically for decades. Inconsistencies and contradictions in both historical and contemporary research findings (Bogatov et al., 2024; Thorp & Bowes, 2017) have fuelled a persistent debate about *'allochthony versus autochthony in streams'* and have led to multiple extensions of the RCC, introducing additional factors and complexities (Doretto et al., 2020; Humphries et al., 2014; Winemiller et al., 2010).

Although Vannote's RCC made a remarkable contribution to changing views (*e.g.,* "passive" riverine pipe) on significant carbon pathways and compartments (*e.g.,* landlocked water bodies) which had been overlooked for decades in the global carbon budget , it received significant criticism (Barmuta & Lake, 1982; Doretto et al., 2020; Humphries et al., 2014; Thorp & Delong, 1994) and was particularly challenged by the shift from *ingestion* to *assimilation* food webs. This change was facilitated by the integration of modern methods such as the inclusion of *elemental* compositions (Andersen & Hessen, 1991; Mattson, 1980; Redfield, 1958), e.g. *ecological stoichiometry* (Elser et al., 1996; Reiners, 1986), *markers* and *tracers* (radio- and stable isotopes, Cabana & Rasmussen, 1994; Fry, 1988). However, with further progress in analytical techniques (Bartle & Myers, 2002; Snow, 2006), food web methodologies could evolve beyond the quantification of *elements* to *bulk* macromolecules (e.g., lipids, proteins), and ultimately to individual molecular units (*e.g.,* fatty or amino acids) at relatively low cost and with decreasing effort. This progress of ongoing improvements in automation and analytic biochemistry, coupled with increasing analytical sensitivity and declining costs, has been crucial in furthering trophic ecology. As this trend continues, research on smaller scales (e.g., individual metabolomics), enabled by *compound-* or *position-specific stable isotope analysis* (CSIA and PSIA), becomes increasingly common and emerges as new state-of-the-art techniques in trophic research (Burian et al., 2020; Pickett et al., 2024; Whiteman et al., 2019).

CSIA allows researchers to trace *isotopic signatures* (isotopic fingerprint) of specific compounds to reveal pathways through which organisms acquire, process, and allocate these. The established disparity in food quality between terrestrial and aquatic diets (*e.g.,* stream algae) is largely defined by the abundance of long-chain polyunsaturated fatty acids (LC-PUFAs), notably eicosapentaenoic acid (EPA). EPA is essential for the survival of both, vertebrate and invertebrate organisms, serving as a fundamental component for their physiological processes and overall survival. This essential compound is absent in non-genetically modified plants (Napier, 2006; Twining et al., 2019) and thus, riparian leaf litter entering aquatic habitats, a focal point in the RCC, is initially devoid of vital EPA. However, the complexity arises from the conditioned leaf litter (plant material overgrown by aquatic biofilms) serving as the 'grey zone' within the RCC, blurring differences between terrestrial and aquatic food sources (Dunstan et al., 1993; Feckler et al., 2024; Taipale et al., 2013). In addition to the allo- or autochthonous sources, some organisms possess the remarkable ability to biosynthesize LC-PUFA from alpha-linolenic acid (ALA) by converting it to EPA and subsequently to docosahexaenoic acid (DHA, Monroig et al., 2022; Ribes-Navarro et al., 2023; Twining et al., 2018). Moreover, ALA, the precursor to EPA, can derive from both, terrestrial and aquatic sources. This adds another layer of complexity to the pathways through which vital fatty acids are acquired. Consequently, EPA in the tissues of aquatic consumers may originate from various *trophic pathways*, including *de novo synthesis* and dietary intake with subsequent *trophic retention* from stream algae as well as conditioned leaf litter.

As mentioned, EPA acts as a precursor for the internal synthesis of DHA - the predominant fatty acid found in vertebrate neural tissues (*e.g.,* brain and eyes). Consequently, DHA is pivotal for *neurogenesis*, cognitive function, and visual acuity, unveiling a novel dimension in understanding how diet can affect consumer's survival (Vagner et al., 2024). Naturally, organisms are inclined to prioritize dietary LC-PUFA (EPA as well as DHA) over endogenous synthesis to minimize metabolic costs (Závorka et al., 2023). The elucidation of EPA's sources and pathways challenges conventional ecological paradigms such as the RCC. By revealing the complex dynamics of nutrient flow and energy transfer within aquatic ecosystems, researchers gain a more nuanced understanding of trophic interactions and ecosystem processes. Such knowledge can enhance our ability to predict and manage ecosystem responses to environmental change, ultimately contributing to the conservation and sustainable management of critically threatened freshwater ecosystems. Freshwaters, especially alpine and mountainous rivers, are particularly sensitive to climate change and face declining nutritional quality at the base of food webs, which detrimentally affect consumer health in freshwaters and adjacent habitats (Calderini et al., 2023; Colombo et al., 2020; Kim et al., 2024; Yan et al., 2024).

In a series of five peer-reviewed research publications (see list of contents), the overall objective was to provide empirical evidence for the significance of stream algae as the principal LC-PUFA source for higher trophic levels (benthic invertebrates, fish, and their neural tissues), exploring both direct and indirect dietary pathways of their acquisition in riverine ecosystems. Benthic invertebrates and fish are crucial for ecosystem functioning, acting as essential links between aquatic and terrestrial habitats. Through trophic transfer as well as insect emergence, algal components can support secondary production even in adjacent terrestrial habitats (Baxter et al., 2005; Gladyshev et al., 2013; Mathieu-Resuge et al., 2022). By comprehensive field investigations, experimental analyses, and the inclusion of CSIA, this research sought to uncover LC-PUFA sources and complex interactions between stream algae, benthic invertebrates, and fish and their neural tissues, shedding light on the mechanisms driving ecosystem productivity. Further, these studies provide field and experimental evidence that autochthonous rather that allochthonous fatty acids are selectively retained in stream consumers with fish neural tissues particularly rich in LC-PUFA.

In the research article "*Preferential retention of algal carbon in benthic invertebrates: Stable isotope and fatty acid evidence from an outdoor flume experiment*" (Kühmayer et al., 2020) it was revealed that, contrary to predictions of the RCC, stream macroinvertebrates - the shredder *Gammarus* and the grazer *Ecdyonurus* - primarily rely on algal carbon and PUFA rather than on allochthonous sources. This tracer-based feeding experiment showed that while *Gammarus* feeds on leaf litter, it assimilates PUFA from attached biofilms, and *Ecdyonurus* uses leaf litter primarily as a substrate for algal growth, with both taxa preferentially derive their carbon and PUFA from algae—a pattern likely applicable to other stream macroinvertebrates.

Building on this, the journal article "*Basal resources of river food webs largely affect the fatty acid composition of freshwater fish*" (Guo et al., 2022) demonstrated that epilithon quality significantly impacts fish LC-PUFA levels. Reduced epilithon quality lowers EPA and DHA in *Salmo trutta* and *Cottus gobio*, adversely affecting fish health and potentially harming freshwater ecosystems. The study recommends that future fish management plans should consider and protect nutritional quality of epilithon.

Expanding further, the primary study "*Longitudinal variation in the nutritional quality of basal food sources and its effect on invertebrates and fish in subalpine rivers*" (Guo et al., 2021) highlighted the role of FA in stream food webs, particularly the trophic transfer of LC-PUFA from the base of food webs to carnivorous fish. This study demonstrated that stream consumers obtain their LC-PUFAs from epilithon, regardless of their feeding modes or variations in leaf litter inputs and riparian shading. However, the diet quality of epilithon varies longitudinally and affects consumer nutrition. The study proposes a high-quality, epilithon-dominated energy pathway from upstream to downstream.

The subsequent large-scale field study "*Polyunsaturated fatty acids in fish tissues more closely resemble algal than terrestrial diet sources*" (Ebm et al., 2021) confirmed that algae, not terrestrial sources, provide essential PUFA for headwater consumers. Algal PUFA, especially the diatom-derived EPA, are first retained by benthic invertebrates and then by fish. Freshwater fish then might utilize EPA for bioconversion to DHA which is critical to neural organs, particularly brain and eyes. However, it remains unclear whether neural DHA is sourced from the liver or produced locally in brain cells.

Finally, the follow-up study "*Compound-specific stable isotopes resolve sources and fate of polyunsaturated fatty acids in biota of headwater streams*" (Ebm et al., 2023) found that in oligotrophic, temperate, and DHA-limited aquatic systems, winter consumers preferentially retain EFA from epilithon or conditioned leaves over terrestrial PUFA (fresh leaves), with no evidence for internal transformation from ALA to EPA. Among LC-PUFA, only fish livers produced DHA endogenously in substantial amounts, with weak evidence for additional *de novo* LC-PUFA synthesis in fish brains or eyes under natural conditions.

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SPECIAL ISSUE

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Preferential retention of algal carbon in benthic invertebrates: Stable isotope and fatty acid evidence from an outdoor flume experiment

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Abstract

1. According to the River Continuum Concept, headwater streams are richer in allochthonous (e.g. terrestrial leaves) than autochthonous (e.g. algae) sources of organic matter for consumers. However, compared to algae, leaf litter is of lower food quality, particularly ω-3 polyunsaturated fatty acids (n-3 PUFA), and would constrain the somatic growth, maintenance, and reproduction of stream invertebrates. It may be thus assumed that shredders, such as *Gammarus*, receive lower quality diets than grazers, e.g. *Ecdyonurus*, that typically feed on algae.
2. The objective of this study was to assess the provision of dietary PUFA from leaf litter and algae to the shredder *Gammarus* and the grazer *Ecdyonurus*. Three different diets (algae, terrestrial leaves, and an algae–leaf litter mix) were supplied to these macroinvertebrates in a flume experiment for 2 weeks. To differentiate how diet sources were retained in these consumers, algae were isotopically labelled with ^{13}C.
3. Both consumers became enriched with ^{13}C in all treatments, demonstrating that both assimilated algae. For *Gammarus*, n-3 PUFA increased, whereas n-6 PUFA stayed constant. By contrast, the n-3 PUFA content of *Ecdyonurus* decreased as a consequence of declining algal supply.
4. Results from compound-specific stable isotope analysis provided evidence that the long-chain n-3 PUFA eicosapentaenoic acid (EPA) in both consumers was more enriched in ^{13}C than the short-chain n-3 PUFA α-linolenic acid, suggesting that EPA was taken up directly from algae and not from heterotrophic biofilms on leaf litter. Both consumers depended on algae as their carbon and EPA source and retained their EPA from high-quality algae.

KEYWORDS

compound-specific stable isotopes, food quality, food webs, headwaters, River Continuum Concept

1 | INTRODUCTION

In their seminal work, Vannote, Minshall, Cummins, Sedell, and Cushing (1980) introduced the River Continuum Concept (RCC), suggesting that riparian vegetation in forested headwater streams limits autotrophic production by shading and supplies considerable amounts of allochthonous organic matter (OM). It has often been assumed that benthic invertebrates in headwater streams are dependent on this allochthonous OM as dietary energy, an assumption that has been recently questioned (Lau, Leung, & Dudgeon, 2008b; Rasmussen, 2010; Torres-Ruiz, Wehr, & Perrone, 2007). There is still uncertainty as to whether terrestrial leaves can provide sufficient dietary energy and resources to support consumer fitness and answering this question requires detailed information on the dietary quality of autochthonous and allochthonous OM for invertebrate consumers in streams.

Food sources differ in their nutritional quality in stream ecosystems (Brett et al., 2017; Guo et al., 2018; Lau, Leung, & Dudgeon, 2008a). For aquatic consumers, terrestrial leaves are considered as a low-quality resource (Allan & Castillo, 2007) because they contain a high content of recalcitrant OM, such as cellulose, lignin, and hemicelluloses (Meyers & Ishiwatari, 1993), and short-chain polyunsaturated fatty acids (PUFA), e.g. linoleic acid (LIN; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3; Torres-Ruiz et al., 2007). They are also deficient in high-quality dietary nutrients, such as long-chain PUFA, in particular eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3; Lau, Leung, & Dudgeon, 2009). After leaves enter stream channels, fungi and bacteria colonising leaves can increase their nutritional quality in terms of lower C:N ratios and increased protein content (Allan & Castillo, 2007). However, fungi and bacteria also lack long-chain PUFA, including EPA and DHA, that support somatic growth and reproduction of invertebrates (Guo, Kainz, Sheldon, & Bunn, 2016a; Lau et al., 2009). Studies on terrestrial and marine fungi reported that 16:0, 18:0, 18:1n-9, and LIN are the most common fatty acids (FA) in fungi (Cooney, Doolittle, Grahl-Nielsen, Haaland, & Kirk, 1993; Stahl & Klug, 1996). Bacteria contain 15:0, 17:0 and their branched derivatives, as well as vaccenic acid (18:1n7; Desvilettes, Bourdier, Amblard, & Barth, 1997), but generally lack ω-3 PUFA.

In contrast, algae are of higher nutritional quality with more easily accessible carbon, in particular the long-chain PUFA: arachidonic acid (ARA, 20:4n6), EPA, and DHA; it is therefore expected that aquatic consumers will derive high-quality carbon and essential biomolecules from algae (Brett et al., 2017). Algal PUFA, especially the long-chain n-3 PUFA EPA and DHA, enhance the somatic growth and reproduction of aquatic consumers, yet algal taxa differ markedly in their FA composition and thus in their nutritional quality. For example, diatoms contain EPA and DHA (Brett & Müller-Navarra, 1997), whereas cyanobacteria lack these PUFA as well as nutritionally important sterols (Martin-Creuzburg, Von Elert, & Hoffmann, 2008). However, most aquatic consumers have a limited ability to convert the short-chain PUFA LIN or ALA to long-chain PUFA (Brett & Müller-Navarra, 1997). The physiological mechanism for such conversion is still poorly studied for most invertebrates (Guo, Bunn, Brett, & Kainz, 2017; Torres-Ruiz et al., 2007).

The two long-chain PUFA most often shown as essential for stream macroinvertebrates are EPA and ARA, whereas DHA is generally lacking (Guo et al., 2018). Eicosapentaenoic acid plays a critical role for the reproduction and emergence of insects (Stanley-Samuelson, 1994) and ARA for somatic growth and reproduction (Ahlgren, Vrede, & Goedkoop, 2009 for chironomids and ephemerids). Although DHA is not retained in most stream macroinvertebrates, high DHA contents generally found in stream fish (Heissenberger, Watzke, & Kainz, 2010) require bioconversion from dietary precursors, such as EPA, to DHA in their hepatocytes (Murray, Hager, Tocher, & Kainz, 2014). For macroinvertebrates, the consumption of algae that contain EPA leads to a higher growth rate and reproduction in shredders (Guo, Kainz, Valdez, Sheldon, & Bunn, 2016c) and grazers (Guo et al., 2016a). Algal biofilms growing on leaf surface can increase the nutritional quality for consumers (Findlay, 2010; Guo et al., 2018) and support their somatic growth (Manning et al., 2015). Such biofilms can be considered as *peanut butter on crackers* (Cummins, 1974), emphasising that only part of the ingested diet is of high nutritional value.

Fatty acids are also used as biomarkers of dietary bacterial, algal, and terrestrial sources in aquatic organisms (Jardine et al., 2015; Kainz & Mazumder, 2005). As such, the FA composition of diets and consumers can be used to assess how similar the FA retained in consumers are to dietary FA. Bulk stable isotopes (Perga, Kainz, Matthews, & Mazumder, 2006) and compound-specific stable isotopes (Taipale, Kainz, & Brett, 2011) used in combination with FA can yield more detailed information about trophic transfer for OM sources. Although there is increasing evidence that algae FA are more highly retained in aquatic consumers than terrestrial FA (Brett, Kainz, Taipale, & Hari, 2009; Guo, Kainz, Sheldon, & Bunn, 2016b; Torres-Ruiz et al., 2007), it remains to be elucidated if aquatic macroinvertebrates can convert dietary FA from either algae or terrestrial sources to build their somatic biomass. For example, if headwater consumers mainly feed on terrestrial leaves, as suggested by the RCC, does carbon from allochthonous sources make an important contribution to the tissues of headwater stream invertebrates?

Based on such uncertainties, we designed an outdoor flume experiment to assess the provision of dietary PUFA from leaf litter (allochthonous source) and algae (autochthonous source) to stream macroinvertebrates using an isotopic tracer. Algae were labelled with ^{13}C to differentiate the dietary uptake of these two sources in consumers. By artificially manipulating the abundance of the heavy carbon isotope (^{13}C), differences in uptake by different producers (e.g. fast uptake by microalgae, but slow uptake by terrestrial leaves due to difference in tissue turnover rates) were thought to result in differential labelling of the producers, thereby allowing to track specific food sources when the consumers are analysed at predetermined times after enrichment. We tested the hypotheses that: (1) invertebrates from different feeding guilds obtain most of their

carbon from algae and not from leaf litter; and (2) long-chain PUFA in both consumers are derived directly from algae and not converted from precursors.

2 | METHODS

2.1 | Flume experiment

This study was performed in an outdoor experiment, consisting of 18 flumes, for 14 days (11–25 July 2017) at WasserCluster Lunz (Austria). Two feeding guilds were used representing a common shredder (*Gammarus* sp., an amphipod) and a common grazer (*Ecdyonurus* sp., a heptageniid mayfly), and all fed with three different diets: (1) naturally growing stream algae (treatment A); (2) leaf litter (treatment L); or (3) mixed diet, i.e. algae and leaf litter (treatment M), with six replicated flumes for each diet. The flumes were composed of acrylic plastic (300 cm long, 5 cm width, and 10 cm high) and their upper end was closed, whereas the lower end was open to allow stream water to freely flow out. The flumes were covered with a light transparent sheet to avoid rainwater or leaves directly entering the flumes. The flume water was supplied from a nearby stream (Lunzer Seebach) and pumped into each of the flumes at a constant supply rate (0.1 L/s), creating an average velocity of 0.1 m/s. In each flume, two stainless steel nets (5.5 × 11 cm) were installed; one 50 cm downstream of the flume inflow and the other 100 cm before the outflow to prevent invertebrates from escaping due to drift.

The experimental stretch of the flumes between the nets was covered with unglazed ceramic tiles (5 × 5 × 0.5 cm) to allow biofilm development. Twenty-seven days before the start of the experiment, the flumes were supplied with unfiltered stream water and algae from the stream water formed biofilms on the tiles; the leaf litter treatment was not covered with tiles over the first 27 days to limit algae growth. No algal growth was observed at the channel walls.

Leaf litter from beech (*Fagus sylvatica*; a common tree species around the study area) was collected from nearby streams and added to the flumes 1 day before the start of the experiment to treatments L and M. Leaf litter was conditioned for 1 week in the dark to minimise photosynthetic biofilm growth, but some periphytic taxa may have survived on leaf litter without light. Six stones from the adjacent stream were added, and regularly brushed from algae, to each flume to better simulate stream conditions and offer hiding places for invertebrates.

The macroinvertebrates *Gammarus* and *Ecdyonurus* were collected 1 day before the start of the experiment from a nearby stream (Lunzer Seebach). The genus *Gammarus* is represented by the single species *Gammarus fossarum* (Koch, 1835) in these streams. Several species of *Ecdyonurus* are present within the studied headwater stream, including *Ecdyonurus dispar* (Curtis, 1834), *Ecdyonurus helveticus* (Eaton, 1885), *Ecdyonurus picteti* (Meyer-Dür, 1864), and *Ecdyonurus venosus* (Fabricius, 1775) (J. Waringer, personal communication). The *Gammarus* and *Ecdyonurus* were separated into different flumes to avoid food competition and 20 individuals added to each, resulting in three replicates per treatment per taxon.

2.2 | Flume labelling

To distinguish dietary carbon from leaf litter and algae in the invertebrates, all treatments were enriched in ^{13}C by labelling the flumes with ^{13}C enriched $NaHCO_3$. In experiment L, algae were carefully brushed from leaf litter every second day to minimise dietary contribution of ^{13}C-labelled periphytic lipids. Previous studies showed that the addition of ^{13}C enriched $NaHCO_3$ did not label plant stems, so leaf litter carbon would isotopically retain its natural background abundance (Maddi, Carman, Fry, & Wissel, 2006). This isotopic tracer was first added 12 days before the start of the experiment and then continuously supplied every second day until completion. For this tracer addition, the water flow was blocked off for 1 hr (always between 10 and 11 a.m., during which time the photosynthetic productivity was assumed to be high), but water temperature did not increase considerably. Ten millilitres of 0.74 g/L ^{13}C-labelled $NaHCO_3$ solution was added to each of the A and M treatment flumes to double the ^{13}C content from 0.029 to 0.057 mM. This increased the delta ($\delta^{13}C$) values of the water to approximately +980‰. However, because the labelling was only 1 hr every second day and algae also fixed carbon during the remainder of the day (additional 15 hr in July), the $\delta^{13}C$ value of the algae was expected to be around +20‰. Nevertheless, photosynthetic efficiency naturally differs during daytime, weather conditions and physiological variability of the algae. Thus, the realised $\delta^{13}C$ values were expected to differ somewhat from the expected +20‰ target. Data loggers (HOBO™) were used to log light and temperature every hour during the experiment.

2.3 | Sample collection

At the beginning (T1) and the end of the experiment (T2), algae samples for FA and carbon content measurements were collected from each flume. Additionally, three random samples were taken after 1 week (T1.5). From each flume, one randomly chosen tile covered with algae was scraped and immediately cooled and frozen at −20°C. For the determination of FA and carbon of leaf litter, three replicate leaf samples from each flume were taken at the start and at the end of the feeding experiment, respectively. Leaf materials were stored at −20°C. At the end of the experiment, all living invertebrates were collected from the flumes, frozen at −80°C and subsequently freeze-dried (Virtis™ Genesis Freeze Dryer) for 2 days and then ground and homogenised using a pestle and mortar.

2.4 | Algal chlorophyll-a

Chlorophyll-*a* (Chl-*a*) was measured for algal biomass assessment on the tiles and on leaf litter prior to and after the experiment. From

each flume, biofilm from randomly picked tiles or leaves was scraped, filtered on a Whatman GF/F (25 mm diameter) filter. The Chl-*a* was then extracted using acetone (90%) and subsequently measured on a fluorescence spectrophotometer (Hitachi F-7000).

2.5 | Algal community composition

Randomly chosen tiles from the flumes were scraped to determine the autotrophic community composition. Samples were diluted using MQ-water (15 ml) and fixed with Lugol solution (1:50 dilution). The autotrophic community composition was determined and counted using an inverted microscope (Nikon™ Eclipse TS 100; objective 20×).

2.6 | Invertebrate somatic growth assessment

The initial dry weight of each consumer that was put into the flumes was estimated by a regression equation between body dry weight and body length, and/or wet weight. For *Gammarus*, the initial wet and dry weights of 38 individuals were measured, and the following regression equation was established; i.e. dry weight (y) = 0.1313 times body length (x) + 1.151, r^2 = 0.81. The regression equation between *Ecdyonurus* body length (head to end of abdomen in mm) and dry weight (mg) was established by measuring 44 individuals, i.e. dry weight (y) = 2.6104 times body length (x) − 16.808, r^2 = 0.88.

For the 20 individuals that were placed in each flume, initial wet weight or body length was measured and a mean for the 20 individuals was calculated. Based on these data, we calculated the starting mean dry weight per individual for each flume (3.18 mg for *Gammarus*, 6.05 mg for *Ecdyonurus*).

After 14 days, all living invertebrates (67% *Gammarus* and 76% *Ecdyonurus* survived) from each flume were collected and freeze-dried, weighed, and divided by the number of remaining invertebrates to determine the mean dry weight per individual at the end of the experiment. The difference between the start and end point was the increase in dry weight over 2 weeks. The difference divided by the initial dry weight was the somatic growth per mg initial weight (Crenier et al., 2017). Somatic growth was expressed as gain or loss of weight (in %) relative to the initial weight.

2.7 | Fatty acid analysis

All samples were freeze-dried (Virtis Genesis Freeze Dryer) and homogenised before lipid and FA analysis. Lipids from algae (*c*. 20 mg) and leaf litter (*c*. 150 mg) were extracted and methylated according to the methods reported elsewhere (Guo et al., 2016c). We used nonadecanoic acid (19:0) as an internal standard. In brief, fatty acid methyl esters (FAME) were analysed using a gas chromatograph (THERMO Trace; FID 260°C, Carrier gas: He: 1 ml/min, Detector gases: H_2: 40 ml/min, N_2: 45 ml/min, air: 450 ml/min, temperature ramp: 140°C (5 min) − 4°C/min–240°C (20 min) = 50 min) equipped with a temperature-programmable injector and an autosampler. FAME were separated by a Supelco™ SP-2560 column (100 m, 0.25 mm i.d., 0.2 µm film thickness), identified by comparison of their retention times with known standards (37-component FAME Mix, Supelco 47885-U; Bacterial Acid Methyl Ester Mix, Supelco 47080-U) and quantified with reference to seven-point calibration curves based on known standard concentrations. The retention of FA in consumers is defined as the ability of organisms to regulate ingested FA detected in their tissues (Kainz, Arts, & Mazumder, 2004).

2.8 | Bulk stable isotope analysis

Freeze-dried leaf litter (600 ± 30 µg) and algae or invertebrate samples (300 ± 15 µg) were weighed in tin capsules (duplicates) for subsequent carbon determination, and their stable isotopes ($\delta^{13}C$) values were quantified using an A Flash HT Plus CNSOH elemental analyser interfaced with a Conflo IV device (Thermo, Bremen, Germany) to a continuous flow stable isotope ratio mass spectrometer (Delta V Advantage IRMS). Our reference gas was CO_2 (messergroup.com). The mass spectrometer was calibrated to the at-air international standards using CH7 and USGS24 for carbon. All stable isotope values were reported in the δ notation where $\delta^{13}C = ([R_{sample}/R_{standard}] - 1) \times 1{,}000$, and R is ^{13}C:^{12}C.

2.9 | Compound-specific stable isotope analyses

Compound-specific stable isotope analyses were used to assess the origin and isotopic composition of different FA molecules. Fatty acids were separated using a gas chromatograph (Trace 1310 Thermo™) with helium as the carrier gas. The GC was coupled to an Isolink 2 where the separated FA where combusted at 1,000°C. The generated CO_2 was transported with the carrier gas (helium) to the Conflo IV where each sample was diluted with the carrier gas helium and connected with the reference CO_2 gas. Finally, all CO_2 molecules were analysed in the IRMS (Delta V Advantage).

2.10 | Data analysis

All statistical analyses were performed using R (R Studio 0.99.902). The significant level for all tests was set at 0.05. A Student *t* test or Mann–Whitney *U* test was used to compare paired samples, depending on the distribution of the data. An ANOVA or Kruskal–Wallis *H*-test was applied to compare multiple samples. Principal components analysis was conducted to characterise the differences in FA compositions between algae, leaf litter, and macroinvertebrates, using the *vegan* package in R on arcsin-transformed FA values (%). The level of statistical significance for all tests was set at $p < 0.05$.

3 | RESULTS

3.1 | Algal biomass

Algal biomass, assessed by its Chl-*a* content, was significantly greater on tiles than on leaf litter ($p < 0.001$, *t* test). The Chl-*a* content on the tiles decreased, but not significantly, over time from 7.7 to 4.3 µg Chl-*a*/cm^2 ($p = 0.057$, *t* test) because of an uncontrollable influx of organic particles from the stream water entered the flumes. The Chl-*a* content on the leaf litter also increased but not significantly throughout the experiment, from 0.04 to 0.07 µg Chl-*a*/cm^2 ($p = 0.059$, *t* test).

3.2 | Algal community composition

The algal density decreased significantly during the experiment from *c*. 3.6 × 10^5 to *c*. 2.1 × 10^5 cells/cm ($p = 0.023$, *t* test). Diatoms (mostly composed of the genera *Achnanthidium*, *Diatoma*, and *Cymbella*) were the most abundant group (>90% of identified cells), followed by chlorophytes (mostly *Chlamydomonas* and *Pediastrum*; <10%) and cyanobacteria (<1%). Although the relative abundance of diatoms increased, the absolute abundance of diatoms and chlorophytes decreased significantly ($p < 0.05$, *t* test).

3.3 | Invertebrate somatic growth

No significant somatic growth for both consumers was observed in any of the three treatments after the feeding experiment. For *Gammarus*, the overall body mass increase was 28.3% in treatment A, 21.2% in treatment L, and 21.8% in treatment M, but was not significant among the three diet treatments ($p = 0.695$, ANOVA). Somatic growth in *Ecdyonurus* also did not differ significantly among treatments ($p = 0.442$, ANOVA), but in contrast was −15.4% in treatment L, −13.6% in treatment A and −2.85 in treatment M.

3.4 | Fatty acid variation

Fatty acid patterns in diets and consumers (*Gammarus* and *Ecdyonurus*) showed clear separation from each other (Figure 1). Algae and leaf litter were associated with long-chain, saturated FA (22:0 and 24:0) and had very low variation in their FA content before and after the experiment. The FA composition of *Gammarus* differed from *Ecdyonurus* mainly by LIN and DHA, whereas *Ecdyonurus* differed mainly by EPA, ALA, and typical diatom FA, such as 16:1n-7 (palmitoleic acid).

Algae had on average 7× higher n-3 PUFA, 5× higher n-6 PUFA, and 2× higher total lipid contents than leaf litter (i.e. 4.4 versus 0.6 mg n-3 PUFA g/dry weight; 1.4 versus 0.2 mg n-6 PUFA g/dry weight; and 36 versus 18 mg total lipids g/dry weight, respectively). However, the algal FA content changed significantly over

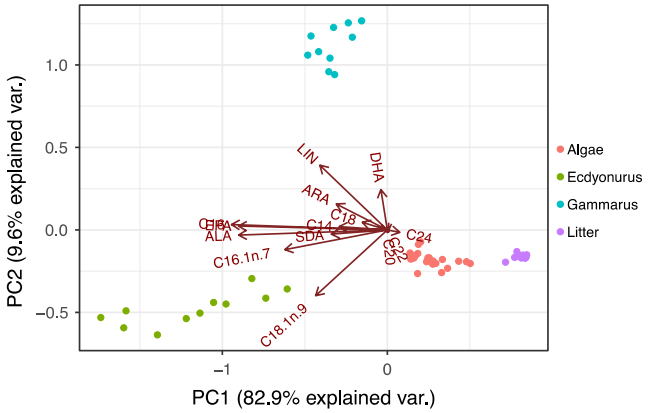

FIGURE 1 Principal components analysis of arcsin-transformed fatty acids (%) of algae, leaf litter, and *Ecdyonurus* and *Gammarus* before and after the feeding experiment. See text for fatty acid abbreviations; the vectors of 16:0 and eicosapentaenoic acid overlap

time. LIN and EPA were higher at T1.5 ($p < 0.01$, ANOVA; *H*-test: $p = 0.023$, respectively) compared to T1 and T2. ALA, DHA, and n-6 PUFA decreased after T1.5 ($p < 0.001$, ANOVA; $p < 0.001$, *H*-test; $p < 0.001$, ANOVA; respectively). Omega-3 and total PUFA increased from T1 to T1.5 ($p < 0.001$, ANOVA), but decreased from T1.5 to T2 ($p < 0.01$, ANOVA). Arachidonic acid did not change significantly, but slightly increased at T1.5. Total lipids decreased significantly from T1 to T2 ($p = 0.017$, H-test). In contrast, the FA content in leaf litter hardly changed over time, whereas EPA and the n-3/n-6 ratio differed significantly during the experiment (*U* tests: $p < 0.01$).

The total lipid and FA content in *Gammarus* and *Ecdyonurus* differed among the three treatments (Figure 2). In *Gammarus*, total lipids were highest in treatment *M* (91 ± 3 mg/g dry weight), followed by treatment L (87 ± 4 mg/g dry weight) and treatment A (79 ± 2 mg/g dry weight). *Gammarus* contained lower n-6 PUFA (LIN and ARA) in treatment A compared to those at the beginning of the experiment (Figure 2a). Omega-6 PUFA were higher in *Gammarus* that fed on litter and mixed diets relative to treatment A. The essential n-3 PUFA, ALA, was higher in *Gammarus* in all diet treatments relative to *Gammarus* from the stream. Similarly, EPA was significantly higher in *Gammarus* feeding on algae, litter, and the mixed diet compared to *Gammarus* from the stream. Moreover, of all PUFA, the EPA content was most highly retained in *Gammarus*, whereas only very little DHA was detected. The n-3/n-6 ratio was lowest in *Gammarus* from the stream (1.1 ± 2.5) and highest in treatment A (2 ± 0.1).

In *Ecdyonurus*, however, total lipids and the FA contents did not differ significantly among different treatments, although total lipids, ω-6 (LIN and ARA) and ω-3 PUFA (ALA, EPA, and DHA) decreased during the experiment relative to lipids and FA in *Ecdyonurus* at the beginning of the experiment (Figure 2b). In contrast to *Gammarus*, the n-3/n-6 ratios in *Ecdyonurus* were much higher and ranged between treatment *M* (6.0 ± 0.1) and treatment L (6.6 ± 0.13).

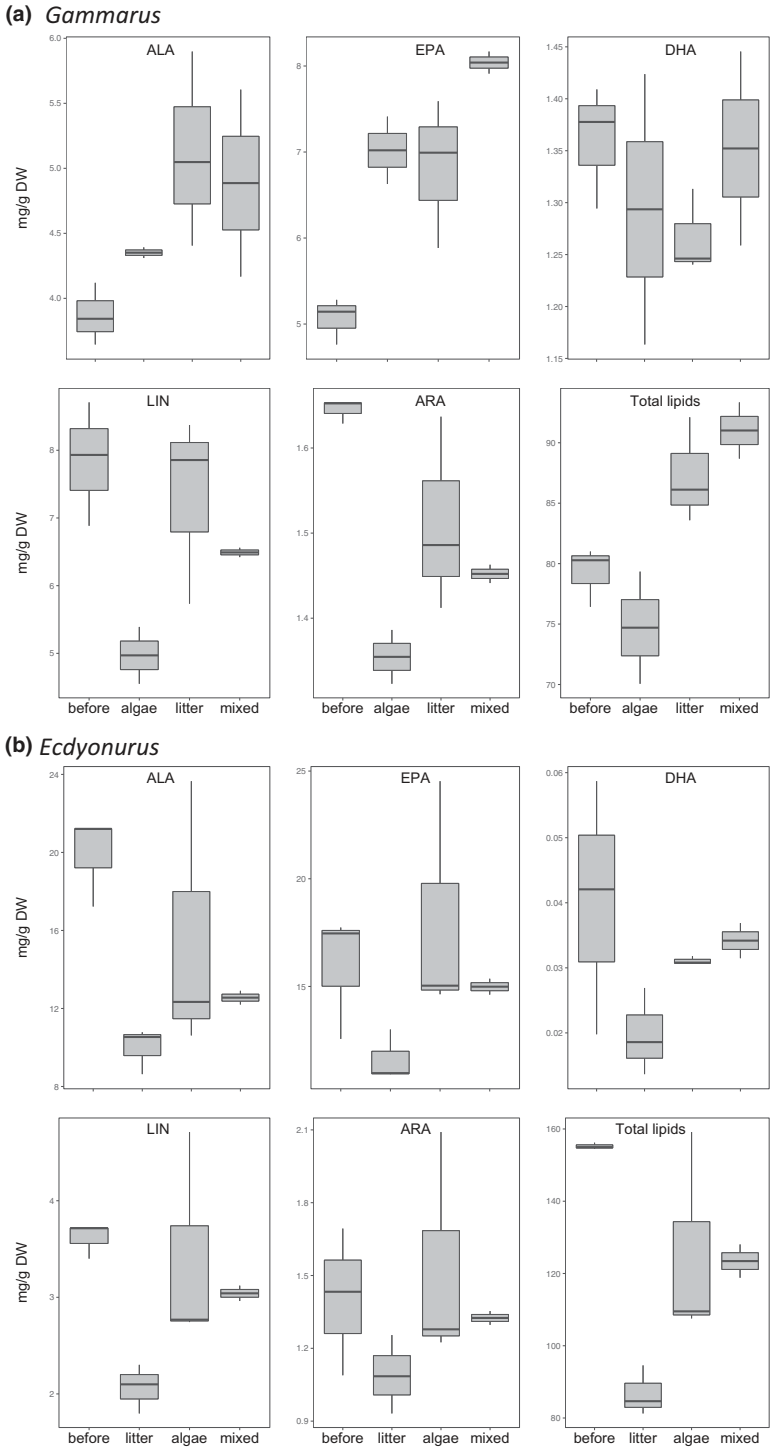

FIGURE 2 Total lipids and fatty acids in (a) *Gammarus* and (b) *Ecdyonurus* before the feeding experiment and after feeding on algae, leaf litter, or, mixed diet. ALA: alpha-linolenic acid (18:3n-3); EPA: eicosapentaenoic acid (20:5n-3); DHA: docosahexaenoic acid (22:6n-3); LIN: linoleic acid (18:2n-6); ARA: arachidonic acid (20:4n-6).

3.5 | Bulk stable carbon isotopes

Algal $\delta^{13}C$ values varied over the duration of the experiment from −21.0‰ to +34.0‰, whereas the $\delta^{13}C$ values of the litter material remained relatively constant (−30.2 ± 0.6‰, mean ± *SD*). The $\delta^{13}C$ values of *Gammarus* were enriched in all treatments compared to the start of the experiment (Figure 3). *Gammarus* in the treatments A (−21.2 ± 1.2‰) and *M* (−21.5 ± 0.9‰) were significantly enriched in ^{13}C compared to *Gammarus* from the nearby stream (starting conditions, −26.3 ± 0.1‰) or treatment L (−24.4 ± 0.8‰; $p < 0.001$, ANOVA; Figure 3a). The $\delta^{13}C$ values of *Ecdyonurus* were enriched, but not significantly different from the start of the experiment

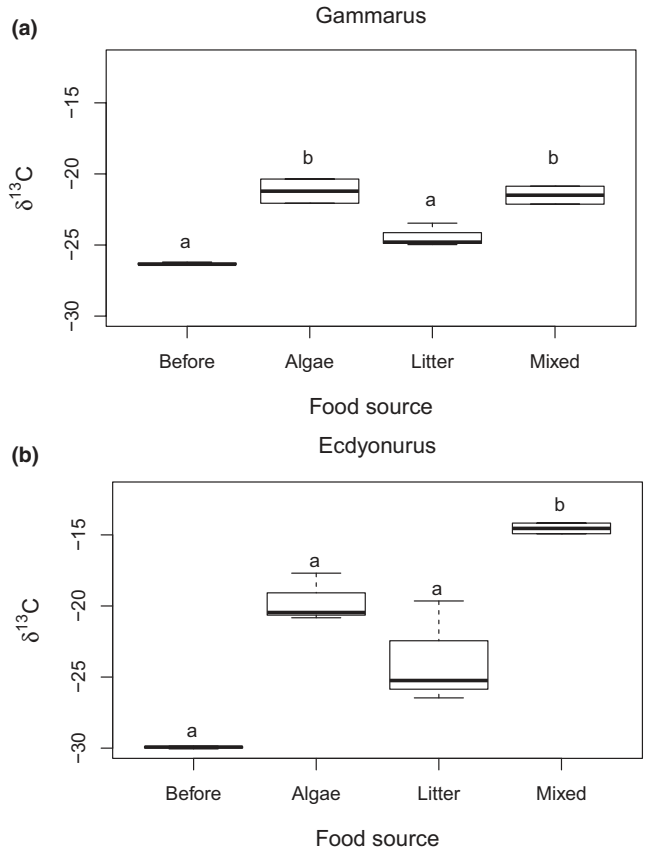

FIGURE 3 Delta ^{13}C values in (a) *Gammarus* and (b) *Ecdyonurus* before the feeding experiment and after feeding on algae, leaf litter, or, mixed diet

(−29.9 ± 0.1‰), except in treatment *M* (−14.5 ± 0.5‰; $p = 0.035$, *H*-test). *Ecdyonurus* in treatment L were isotopically lighter (−23.8 ± 3.6‰) than in the labelled treatment A (−19.7 ± 1.7‰; Figure 3b).

3.6 | Compound-specific stable isotope analyses

Three PUFA (LIN, ALA, and EPA) in algae were isotopically enriched (resulting in positive $\delta^{13}C$ values) during the experiment and could thus be distinguished from the more negative $\delta^{13}C$ values in the leaf litter (Table 1). Although periphyton was removed from leaf litter (see above), the PUFA on leaf litter were isotopically enriched, but not as strongly as the PUFA from treatment A, except for EPA.

In both consumers, LIN, ALA, and EPA were enriched in ^{13}C in each treatment. At the end of the experiment, $\delta^{13}C_{EPA}$ values in *Ecdyonurus* and *Gammarus* were more enriched relative to the start of the experiment (Δ = 16.5 and 8.9‰, respectively). Compared to $\delta^{13}C_{EPA}$, $\delta^{13}C_{ALA}$ (Δ = 4.6 and 8.8‰, respectively), $\delta^{13}C_{LIN}$ values (Δ = 10.7 and 2.3‰, respectively) were less enriched in both consumers. For *Ecdyonurus*, $\delta^{13}C_{ALA}$ values were more depleted compared to $\delta^{13}C_{LIN}$, whereas the $\delta^{13}C_{EPA}$ values were more enriched compared to $\delta^{13}C_{LIN}$ and $\delta^{13}C_{ALA}$.

For *Gammarus*, $\delta^{13}C_{ALA}$ did not differ from $\delta^{13}C_{LIN}$ in treatments L and M, but in treatment A the $\delta^{13}C_{ALA}$ values were enriched compared to $\delta^{13}C_{LIN}$. The $\delta^{13}C_{EPA}$ values were highly enriched in all treatments. Finally, consistent with patterns in bulk $\delta^{13}C$ values of these consumers (Figure 3), changes in $\delta^{13}C_{LIN}$, $\delta^{13}C_{ALA}$, and $\delta^{13}C_{EPA}$ values of *Ecdyonurus* and *Gammarus* were highest in treatments A and M, and lowest in treatment L.

4 | DISCUSSION

In contrast to the assumption of the RCC that upstream consumers depend more on terrestrial than algae-derived diet, our flume experiment suggests that the shredder *Gammarus* and the grazer *Ecdyonurus* retained a greater proportion of carbon from algae. Our results also demonstrate that both consumers used algae as their main dietary carbon and PUFA source, which is consistent with recent experimental and field studies (Brett et al., 2009; Guo et al., 2018; Lau et al., 2009). Even when fed on leaf litter, both consumers retained more algal carbon, indicating that algae growing on leaves were their preferential dietary resource.

The enriched $\delta^{13}C$ values in the shredder *Gammarus* and the grazer *Ecdyonurus*, in particular in treatment L, indicate the importance of biofilms attached to leaf litter for stream consumers (Cummins, 1974; Findlay, 2010). Since algal biofilm on leaf litter can account for up to 35% of primary production of streams (Naiman, 1983), algae on leaf litter are a potential food source for invertebrates (Allan & Castillo, 2007; Manning et al., 2015). If invertebrates were exclusively feeding on the biofilm of the leaf litter, the retained carbon would be far more enriched compared to the leaf carbon. Indeed, the $\delta^{13}C$ values of *Gammarus* and *Ecdyonurus* were very similar when feeding on algae or on the mixed diet, suggesting that both consumers were retaining mainly algal carbon. Similarly, in treatment L, the equally enriched $\delta^{13}C$ values of *Gammarus* and *Ecdyonurus* suggest that when stream invertebrates are provided with both algae and litter, they preferentially retain algal carbon.

The Chl-*a* content in the flumes declined over the course of the experiment, as did the grazer biomass. Even low algal biomass can be an important subsidy for stream consumers (Thornton, 1974), although the remaining algal biomass in the present study growing on leaf litter was probably too low to improve the somatic growth of *Ecdyonurus*. Especially before emergence, this grazer needs lots of dietary energy, including lipids and their FA, which consequently leads to a decrease in PUFA content during metamorphosis (Hanson, Cummins, Cargill, & Lowry, 1985). *Ecdyonurus* do not feed after emergence, thus these grazers need to obtain all of their dietary energy for adult life and reproduction prior to emergence. High PUFA demand is also inferred by comparing the initial PUFA contents of *Ecdyonurus* and *Gammarus*. The decreasing PUFA and lipid content in *Ecdyonurus* suggests that algal biomass was too low to provide adequate food in our experiment. In addition, high grazing activity reduces resource biomass, but may increase algae population growth, which can compensate for the algae consumption by

TABLE 1 Compound-specific stable isotope values (mean $\delta^{13}C$ values; ‰) of linoleic (LIN), α-linolenic (ALA), and eicosapentaenoic acids (EPA) in algae and leaf litter before and after the experiment, and in the grazer *Ecdyonurus* and the shredder *Gammarus* before and after feeding on algae (A), leaf litter (L), or the mixed diet (M; algae + leaf litter); Δ = difference in $\delta^{13}C$ values (‰) of LIN, ALA, and EPA between before and after the experiment

	LIN			ALA			EPA		
	Before	After	Δ	Before	After	Δ	Before	After	Δ
Algae	5.7	6.8	1.1	10.27	6.25	−4.0	−9.36	14.73	24.1
Leaf litter	−37.4	−28.8	8.6	−39.2	−34.7	4.5	−40.3	8.5	48.8
Ecdyonurus A	−36.0	−24.4	11.6	−40.6	−36.7	3.9	−34.9	−18.4	16.5
Ecdyonurus L	−36.0	−27.2	8.8	−40.6	−36.7	3.9	−34.9	−25.5	9.4
Ecdyonurus M	−36.0	−24.2	11.8	−40.6	−34.7	5.9	−34.9	−12.1	22.8
Gammarus A	−33.4	−30.4	3.0	−37.7	−23.5	14.2	−36.1	−25.7	10.4
Gammarus L	−33.4	−33.1	0.3	−37.7	−33.7	4.0	−36.1	−31.1	5.0
Gammarus M	−33.4	−29.8	3.6	−37.7	−29.6	8.2	−36.1	−24.7	11.4

consumers (Lamberti & Resh, 1983). However, in our experiment, the algal biomass decrease was not due to feeding pressure, but probably due to the accumulation of sediment particles (field observation). Therefore, it seems that settling sediments reduced algae production, which then limited the somatic growth of consumers. Similar to other emerging insects, mayflies require a substantial amount of stored energy for metamorphosis, egg production, and mating swarms (Winkelmann & Koop, 2007), emphasising the importance of high-quality food for *Ecdyonurus* to successfully conduct its post-larval life stage.

Relative to the initial PUFA composition in *Gammarus*, n-6 PUFA (LIN and ARA) decreased or remained similar, whereas the n-3 PUFA EPA and ALA increased, indicating selective retention of algal n-3 PUFA (Guo et al., 2016b) even during decreasing algae supply, as was the case in treatment A. The concurrent decrease in algal supply and n-6 PUFA in *Gammarus* suggests n-6 to be less important than n-3 PUFA for *Gammarus*. Furthermore, the n-3 PUFA EPA and ALA in *Gammarus* were higher in all the treatments compared to *Gammarus* from the stream. The large contribution of diatoms to the algal community probably accounted for the high n-3 PUFA content in the consumers since diatoms are rich in EPA (Taipale et al., 2013). The low DHA in *Gammarus* can be attributed to generally low dietary DHA availability, as seen in other studies (Guo et al., 2016b), and/or the ability of *Gammarus* to bioconvert DHA, at low rates, from dietary precursors. However, the long-chain n-3 PUFA, in particular EPA and DHA, are not provided by terrestrial leaves (Mills, Mcarthur, Wolfe, Aho, & Rader, 2001), but probably by some algal sources. In general, these results suggest that algae were the most important PUFA provider for *Gammarus*.

Compound-specific stable isotopes provide evidence that both consumers acquired their FA mostly from algae. Fatty acids had generally 5–15‰ more depleted $\delta^{13}C$ values than bulk carbon due to isotopic fractionation during lipid synthesis (Gladyshev, Sushchik, Kalachova, & Makhutova, 2012). Relative to these initially depleted values representing natural background, the n-3 PUFA in consumers were ^{13}C-enriched. The strongest ^{13}C-enrichments occurred in EPA derived from the biofilm. Consumers selected and/or preferentially retained high-quality resources that already contained this essential PUFA. Consistent with changes in bulk $\delta^{13}C$ of these consumers, whereby *Ecdyonurus* appear to turn over algal carbon twice as fast as *Gammarus*, EPA turnover in *Ecdyonurus* appeared to be *c.* 2× faster than in *Gammarus* as indicated by their difference in algal $\delta^{13}C_{EPA}$ values between the beginning and the end of this experiment. The isotopically enriched EPA in both consumers from treatment L suggests dietary access of ^{13}C-labelled algae, although periphyton was regularly removed from leaf litter, and indicates that traces of labelled algae-derived EPA were available and retained in these consumers.

We attribute the high variation in algae $\delta^{13}C$ values to several factors. First, there is a naturally large natural variation in $\delta^{13}C$ among different algal taxa (Vuorio, Meili, & Sarvala, 2006) and the various enrichments observed probably reflect different patches of actively photosynthesising algae. Second, different physiological processes among algae or incorporation efficiency of the labelled CO_2 may have caused the observed high variability in the $\delta^{13}C$ values. Finally, the different supply of particles in the flume may have *diluted* algae biomass and induced different $\delta^{13}C$ values. However, even with differing $\delta^{13}C$ values in algae, our data indicate that the benthic consumers were all enriched in algal carbon.

In conclusion, in contrast to the predictions of the RCC, our study suggests that stream macroinvertebrates, both the shredder *Gammarus* and the grazer *Ecdyonurus*, strongly rely on algae as their main carbon source for somatic tissues, and very little on allochthonous leaf litter. Algae provide more readily available carbon with nutritionally important PUFA to both *Gammarus* and *Ecdyonurus*. However, *Gammarus*, which fed preferentially on leaf litter, nonetheless assimilated FA from attached biofilms; thus, leaf litter itself does not supply PUFA to *Gammarus*, as shown by the discrepancy in FA between leaf litter and consumers. We note that *Gammarus* and other shredders as trophic conveyors of dietary energy, both in terms of quantity and nutritional quality, to higher consumers strongly depend on dietary algal supply. *Ecdyonurus*, referred to as typical grazer, may not be limited to grazing on algae and other

easily available diet from rocks, but also from leaf litter. Thus, litter is contributing minimally directly as food source, but serves as an important substrate for algae production (Cummins, 1974; Findlay, 2010; Guo et al., 2016c). Finally, we conclude that both consumers preferentially obtained their carbon and PUFA from algae. Although only two taxa were investigated in this study, it is possible that other grazers and shredders may similarly select and retain their dietary carbon and PUFA from algae.

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DATA AVAILABILITY STATEMENT

Presented data are original data generated for this study. Upon request, data will be made available.

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RESEARCH ARTICLE

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Longitudinal variation in the nutritional quality of basal food sources and its effect on invertebrates and fish in subalpine rivers

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Abstract

1. There is growing recognition of the importance of food quality over quantity for aquatic consumers. In streams and rivers, most previous studies considered this primarily in terms of the quality of terrestrial leaf litter and importance of microbial conditioning. However, many recent studies suggest that algae are a more nutritional food source for riverine consumers than leaf litter. To date, few studies have quantified longitudinal shifts in the nutritional quality of basal food resources in river ecosystems and how these may affect consumers.
2. We conducted a field investigation in a subalpine river ecosystem in Austria to investigate longitudinal variations in diet quality of basal food sources (submerged leaves and periphyton) and diet source dependence of stream consumers (invertebrate grazers, shredders, filterers and predators, and fish). Fatty acid (FA) profiles of basal food sources and their consumers were measured.
3. Our results indicate systematic differences between the FA profiles of terrestrial leaves and aquatic biota, that is periphyton, invertebrates and fish. Submerged leaves contained very low proportions of long-chain polyunsaturated fatty acids (LC-PUFAs), which were conversely rich in aquatic biota. While the FA composition of submerged leaves remained similar among sites, the LC-PUFAs of periphyton increased longitudinally, which was associated with increasing nutrients from upstream to downstream.
4. Longitudinal variations in periphyton LC-PUFAs were reflected in the LC-PUFAs of invertebrate grazers and shredders, and further tracked by invertebrate predators and fish. However, brown trout *Salmo trutta* contained a large proportion of docosahexaenoic acid (DHA, 22:6ω3), a LC-PUFA almost entirely missing in basal sources and invertebrates. The fish accumulated eicosapentaenoic acid (EPA, 20:5ω3) from invertebrate prey and may use this FA to synthesize DHA.
5. Our results provide a nutritional perspective for river food web studies, emphasizing the importance of algal resources to consumer somatic growth and the need to account for the longitudinal shifts in the quality of these basal resources.

KEYWORDS
brown trout, diatoms, fatty acids, food quality, food webs

1 | INTRODUCTION

The importance of food quality over food quantity for aquatic consumers has been increasingly recognized in recent years (Brett et al., 2017; McInerney et al., 2020). Food quality is central for consumer growth, reproduction and survival, in all food webs (Simpson & Raubenheimer, 2012). In rivers and streams, previous studies framed under the River Continuum Concept (RCC; Vannote et al., 1980) hypothesized that terrestrial food sources were a major dietary carbon source supporting aquatic food webs (Junk et al., 1989; Wolff et al., 2013). Such conclusions were largely based on gut content analyses and the feeding behaviour of benthic invertebrates. However, with the application of biochemical tracers, such as stable isotopes, fatty acids (FA) and amino acids, it has become evident that algae are a more nutritionally important food source for riverine consumers (Bunn et al., 2003; Lau et al., 2009; Thorp & Bowes, 2017). While Vannote et al. (1980) predicted increased dependence on algae in the wider mid-river reaches, few studies have quantified longitudinal shifts in the nutritional quality of basal resources and how these basal resources may affect consumers.

The nutritional quality of resources is generally determined by specific biochemical compounds that are essential for animal fitness but cannot be synthesized by animals or can only be synthesized at very low rates. Long-chain polyunsaturated fatty acids (LC-PUFAs) are one of these groups of compounds, in particular the omega-3 (ω3) LC-PUFA eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3), and the omega-6 (ω6) LC-PUFA arachidonic acid (ARA, 20:4ω6), which are essential to support maintenance, growth and reproduction of aquatic consumers (Arts et al., 2001; Gladyshev et al., 2018; Sargent et al., 1999; Tocher, 2003). Aquatic consumers must obtain these molecules directly from their diets due to their limited ability to synthesize LC-PUFAs. Diets low in LC-PUFAs can constrain somatic growth and reproduction and affect survival at the individual level, and may result in reduced secondary production and food chain efficiency at the ecosystem level (Muir et al., 2014; Müller-Navarra et al., 2004; Twining et al., 2016).

In river and stream ecosystems, terrestrial leaves and algae have contrasting LC-PUFA profiles. Periphyton growing on large woody debris and rock surfaces, especially diatoms that are rich in LC-PUFAs, provide high-quality resources for consumers, whereas terrestrial leaves are of low-quality due to the lack of LC-PUFAs, high C:N ratios and presence of other compounds (Guo et al., 2016; Torres-Ruiz et al., 2007). Fungal and bacterial colonization on submerged leaves can increase the nutritional quality of leaves, for example lower C:N ratios and increased protein content (Tant et al., 2015), but remain low-quality food compared with algae, because fungi and bacteria generally lack LC-PUFAs (Desvilettes et al., 1997; Stahl & Klug, 1996). Despite their low biomass, algae growing on the surfaces of submerged leaves have been reported to be an important source of dietary LC-PUFAs for leaf shredders (Guo et al., 2018; Kühmayer et al., 2020). The preferential assimilation of high-quality periphyton over low-quality leaves has been found for primary consumers, even in headwaters where terrestrial inputs dominate (Kühmayer et al., 2020). Primary consumers feeding on high-quality periphyton in turn provide a source of high-quality prey for secondary consumers, including fish and humans (Brett et al., 2017; Ebm et al., 2021; Guo et al., 2017).

However, periphyton LC-PUFA profiles are very sensitive to variations in environmental conditions (Cashman et al., 2013; Guo et al., 2015; Hill et al., 2011), especially light, nutrients and temperature, which change longitudinally in river ecosystems. For example, spatial differences in riparian canopy cover may regulate algae LC-PUFA synthesis. High canopy cover (low-light intensity) generally increases the relative content of EPA and ARA in periphyton, and reduces the relative availability of the short-chain PUFAs alpha-linolenic acid (ALA, 18:3ω3) and linoleic acid (LIN, 18:2ω6; Guo et al., 2015; Hill et al., 2011). Periphyton LC-PUFAs also change in response to variations in nutrient concentrations. Moderate nutrient inputs lead to increases in LC-PUFAs (Hill et al., 2011), but high nutrient supply, in particular phosphorus, favours cyanobacteria (Müller-Navarra et al., 2004), which have no LC-PUFAs. Furthermore, low temperatures can lead to increases in periphyton EPA and DHA, as well as decreases in ALA and LIN (Guschina & Harwood, 2006). Despite these apparent broad patterns, it is largely unknown how periphyton PUFA profiles are affected by longitudinal gradients of the above environmental factors in river ecosystems, and how this variation in periphyton food quality might affect stream consumers.

While the RCC proposes increased dependence on algae in the wider mid-reaches of rivers, recent studies suggest that consumers most likely derive their LC-PUFAs primarily from high-quality periphyton regardless of stream location (Guo et al., 2018; McInerney et al., 2020). The diet source dependence of stream consumers has been extensively studied, with most studies focusing on shifts in macroinvertebrate functional feeding groups (FFG), that is shredders, grazers, filterers and predators, and fish (Greathouse & Pringle, 2006; Hayden et al., 2016; Rasmussen, 2010; Rosi-Marshall et al., 2016). Since most freshwater macroinvertebrates have a very low ability to synthesize LC-PUFAs, they must obtain LC-PUFAs directly from the diet. Given that periphyton are the exclusive source of LC-PUFAs (Guo, Kainz, Sheldon, et al., 2016; Torres-Ruiz et al., 2007), invertebrate primary consumers should have a FA composition that largely reflects the FA composition of periphyton (Guo, Bunn, et al., 2021; Torres-Ruiz et al., 2010) and this periphyton-derived LC-PUFAs can be consequently transferred from primary consumers to predators (Guo et al., 2018). It is thus likely that the FA profiles of macroinvertebrates should reflect and/or track the spatial patterns of periphyton diet quality in river ecosystems.

Fish FA profiles are largely affected by dietary LC-PUFAs (Tocher, 2010). In a feeding experiment, the incorporation of dietary LC-PUFAs into the eyes, brains, livers and white muscle of turbot was determined by the percentage of these FAs in their diet (Estevez et al., 1999). In river ecosystems, the LC-PUFA profiles in fish tissues were found to more closely resemble invertebrate and algal than terrestrial sources (Ebm et al., 2021). The FA profiles in a range of riverine fish species have been demonstrated to significantly reflect the concurrent differences in periphyton FAs from upstream to downstream, for example sauger and white bass in the Ohio River (Dayhuff & Wells, 2005), channel catfish in the Kaskaskia River (Young et al., 2016), bluegill across Illinois River reaches (Rude et al., 2016) and Australian lungfish in the Brisbane River (Tao et al., 2020). However, it is not clear to what extent the LC-PUFA composition of fish will reflect dietary supply or how PUFAs are innately regulated within fish.

We conducted a field study in a subalpine catchment in Austria to examine longitudinal variations in diet quality of basal food sources (i.e. submerged leaves and periphyton) and the dietary dependence of stream consumers (i.e. invertebrate grazers, shredders, filterers and predators, and fish) on them. We tested the hypotheses that: (a) the quality of basal resources, especially periphyton, will change in response to longitudinal variations in light, temperature and nutrients, (b) the FA profiles of macroinvertebrate FFG will reflect that of their primary dietary source and (c) the FA profiles of fish will also reflect that of their local prey.

2 | MATERIALS AND METHODS

2.1 | Site selection

This study was carried out in the subalpine River Ybbs catchment, Austria (47°45′N, 15°12′E). The area has a temperate climate with evenly distributed precipitation over the year. The primary land use in the catchment is forestry, with alpine meadows and agriculture constituting only a small area in the lower catchment. All study streams were selected in the upper catchment, *Weiße Ois*, which had little or no human disturbances in the study reaches or their upstream catchments. The substrata of study streams were cobbles, and the underlying geology was primarily limestone. Nine study streams were chosen (Figure 1A), with consideration of different levels of riparian canopy cover (shade vs. open), altitude (associated with temperature) and nutrient concentrations [dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP)], including two headwater streams with shaded canopy, two headwater streams with open canopy, two midstream sites with open canopy and three downstream sites with open canopy.

2.2 | Field collection

Basal food sources, that is submerged leaves and periphyton, and their consumers, that is macroinvertebrates and fish were collected from nine streams in October 2016 (Figure 1). All samples were collected along a 20-m reach from each stream. Submerged leaves were picked from water by hand. Periphyton and macroinvertebrates were sampled using a 1.5 m × 1.5 m quadrat. Three replicated samples of periphyton were collected from three quadrats, respectively, and each periphyton sample was collected from five different cobbles by scraping them with brushes. Macroinvertebrates clinging to those cobbles within each quadrat were washed into a white tray, identified to genus and assigned to FFG (Cummins & Klug, 1979). Macroinvertebrate grazers in the study streams included *Ecdyonurus* sp. and *Baetis* sp.; shredders were *Nemoura* sp., *Leuctra* sp., *Allogamus* sp. and *Potamophylax* sp.; the only filterer collected was *Hydropsyche* sp.; predators included *Rhyacophila* sp., *Plectrocnemia* sp., *Perla* sp., *Perlodes* sp. and *Isoperla* sp. A separate invertebrate sample was preserved for further taxonomic identification. Fish samples were collected by electrofishing, anesthetized and killed in situ according to the Federal Act on the Protection of Animals, Austria (http://www.ris.bka.gv.at). Total body length (mm) of each collected fish was measured.

All samples were placed in ziplock plastic bags, stored on ice and kept in the dark in a portable freezer in the field. The dorsal muscle tissue of fish samples was extracted immediately for FA analyses when back in laboratory. All FA samples were placed in a −80°C freezer until further processing, and the separate invertebrate sample was preserved in 75% ethanol for invertebrate identification.

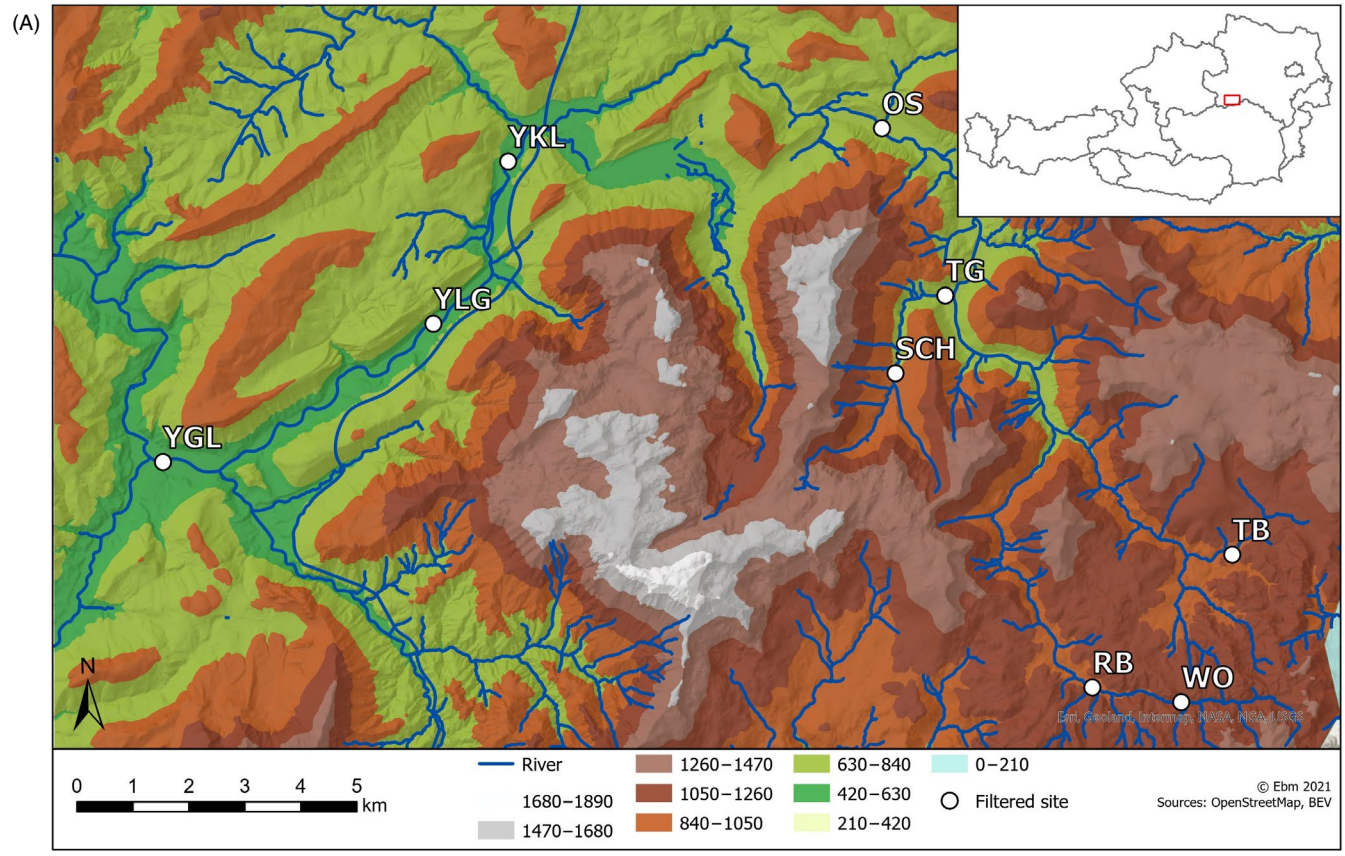
At each site, triplicate water samples were collected to determine the concentrations of DIN, including nitrate (NO_3–N), nitrite (NO_2–N) and ammonium nitrogen (NH_4–N), and SRP. Nutrient samples were stored in a portable dark cooler in the field and filtered through 0.7-μm glass microfiber filters (GF/F; WhatmanTM, GE Healthcare) in the laboratory.

Altitude was measured by an outdoor app 'Runtastic Altimeter GPS' (Runtastic GmbH, Linz) and, when necessary, verified with cartographical data. Stream temperature and pH were measured using an HQD portable measuring meter (HQ30D – Multi/1 Channel, HACH LANGE). Current velocity was measured using a mechanical flow meter (Hydro-Bios, Kiel). Riparian canopy cover was estimated in situ, and followed the methods described in the study by Guo et al. (2018).

2.3 | Laboratory analysis

Three fish species were collected in the study catchment, including brown trout *Salmo trutta*, rainbow trout *Oncorhynchus mykiss* and European bullhead *Cottus gobio*. However, only juvenile brown trout

FIGURE 1 Nine study sites in the River Ybbs catchment, Austria. (A) Sampling map. Sites were selected based on the gradients of riparian canopy cover (*shade vs. open*), altitude (associated with temperature) and nutrient concentrations from upstream to downstream, including two headwater streams with shaded canopy (TB and SCH), two headwater streams with open canopy (RB and WO), two midstream sites with open canopy (TG and OS) and three downstream sites with open canopy (YGL, YLG and YLK). (B) Biplot of principal component analysis (PCA) summarizing the variations in environmental characteristics of nine study sites


(A)
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TG
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0 1 2 3 4 5 km
River
1680–1890
1470–1680
1260–1470
1050–1260
840–1050
630–840
420–630
210–420
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Filtered site
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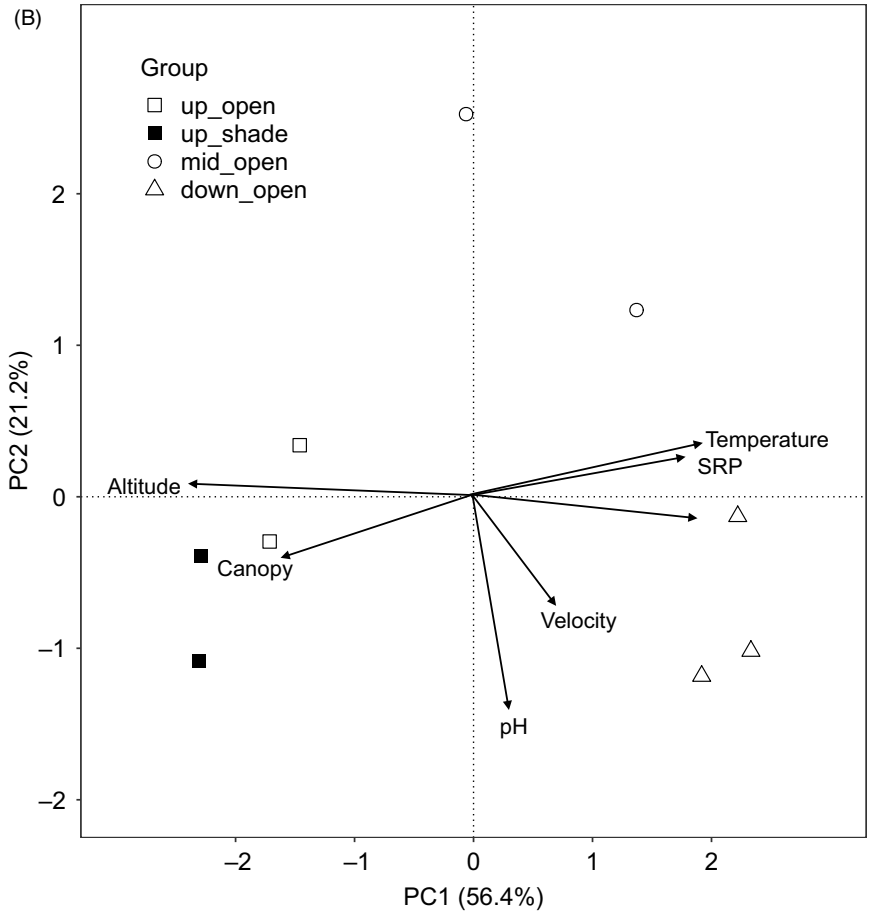
Sources: OpenStreetMap, BEV


(B)
Group
up_open
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Velocity
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PC2 (21.2%)
PC1 (56.4%)
2
1
0
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−2
−2
−1
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2

were abundant and occurred in most sampling sites, and therefore this species was used for FA analyses. Their body lengths ranged from 55 to 218 mm.

All FA samples of fish, macroinvertebrates, periphyton and leaves were freeze-dried (Virtis Genesis Freeze Dryer). After freeze-drying, each sample was homogenized with a glass rod and/ or a food processor. A dry mass of each invertebrate sample (5–7 mg), each fish sample (5–7 mg), each periphyton sample (10 mg) and each leaf sample (50 mg) were used for lipid extraction and FA methylation according to the methods described in the study by Guo et al. (2016). Fatty acid methyl esters (FAME) were analysed using a gas chromatograph (Thermo Trace) equipped with a temperature-programmable injector and an autosampler. FAME were separated by a Supelco^{TM} SP-2560 column (100 m, 25 mm i.d., 0.2-μm film thickness), identified by comparison of their retention times with known standards (37-component FAME mix, Supelco 47885-U; Bacterial Acid Methyl Ester Mix, Supelco 47080-U) and quantified with reference to 7-point calibration curves based on known standard concentrations. FA compositions were expressed as percentage values relative to total FA (FA%).

Dissolved nutrients (DIN and SRP) were analysed by a continuous flow analyser (Alliance instrument GmbH, 5020; Table 1).

2.4 | Data analysis

Environmental characteristics of nine stream sites were analysed using principal component analysis (PCA). Monte Carlo randomization with 1,000 permutations was applied to test the significance of the resulting eigenvalues from PCA. All environmental factors were standardized for the analysis to minimize the effects of differences in measurement units and variance and render the data dimensionless (Zhang et al., 2009).

Differences in overall FA profiles between consumers and their basal food sources were visualized and estimated by non-metric multidimensional scaling (NMDS). NMDS was based on Bray–Curtis dissimilarity matrix, and the stress value <0.2 was accepted (Clarke, 1993). The dissimilarity in overall FA profiles between consumers and their basal food sources was further assessed by permutational multivariate analysis of variance (PERMANOVA), with taxa (e.g. periphyton, leaves, invertebrates and fish) as the fixed factor and sites as the blocking term.

To visualize the longitudinal variations in diet quality, the FA composition of basal food sources (i.e. submerged leaves and periphytons), invertebrate primary (grazers, shredders and filterers) and secondary (predators) consumers and fish *Salmo trutta* from headwater streams to downstream sites were plotted. The differences among sites were estimated by one-way ANOVA, followed by Tukey's HSD multiple comparisons. Due to the very low proportions of DHA, EPA and ARA in submerged leaves, and DHA and ARA in periphyton, the differences of these FAs among sites were not examined.

The trophic transfer of LC-PUFAs to fish from basal food sources was examined by piecewise structural equation models (SEM). Specifically, a priori directed acyclic graph was constructed according to the trophic links from basal food sources (submerged leaves and periphyton) to fish (Guo et al., 2018; Jardine et al., 2015). The graph was then translated into a set of linear equations, each of which was fitted using restricted maximum likelihood (Shipley, 2009). Model fit was evaluated using the directed separation test, based on the Fisher's *C* statistic and a chi-square distribution significance test (Shipley, 2009). The test of directed separation assumes that all variables are conditionally independent, which implies no missing relationships among unconnected variables (Shipley, 2000). The *p*-value for the chi-square test >0.05 indicates support for the set of the conditional independence (Shipley, 2009). Path coefficients were then calculated for each component model. Invertebrate filterers were not included in the analysis because of their small sample size.

Piecewise SEM was undertaken for ω3 LC-PUFAs, the sum of DHA and EPA, for three reasons: (a) Results of ANOVA and NMDS showed that EPA was high in invertebrates (~14.6%), and DHA was high in fish (~24.1%); (b) EPA is critical for invertebrate growth and reproduction (Stanley-Samuelson, 1994), and DHA is key for fish neural development and vision (Tocher, 2003); and (c) it is also likely that freshwater salmonids obtain most of their DHA via elongating and desaturating EPA (Barry & Trushenski, 2020). Therefore, the sum of EPA and DHA was used to construct the models.

The relative importance of dietary FAs, fish body size and spatial environmental factors for the overall FA composition of *Salmo trutta* was partitioned and quantified by partial redundancy analysis (partial RDA). The fish FA data were used as the response variable, whereas the three sets of data, that is dietary FAs, fish body size and spatial environmental factors were explanatory variables. Dietary FAs included the overall FA profiles of invertebrate shredders, grazers, filterers and predators in this study, and fish body size was the body length. Spatial environmental factors included altitude, riparian canopy, DIN, SRP, velocity, pH and temperature. Partial RDA was performed based on a series of combinations of different explanatory variables with or without covariables to partition the influence of explanatory variables.

All statistical analyses were conducted in the statistical software R version 4.0.0 (R Core Team, 2020), using the extension package VEGAN for PCA, NMDS, PERMANOVA and partial RDA (Oksanen et al., 2013), and ggm for SEM (Marchetti et al., 2020). All FA percentage data were arcsine-square-root-transformed for normal distribution approximation before analysis (Kelly & Scheibling, 2012). Eight individual FA or groups of FA which represented essential FAs and important FA functional groups in stream ecosystems (Guo et al., 2017) were used for data analysis, including DHA, EPA, ARA, ALA, LIN, sum of saturated FA (SAFA), sum of monounsaturated FA (MUFA) and sum of bacterial FA (BAFA). Bacterial FAs included 15:0, 17:0 and their branched iso- and anteiso-homologues, and 18:1ω7.

TABLE 1 Environmental characteristics of nine study streams in the River Ybbs catchment, Austria

Location	Stream name	Latitude	Longitude	Canopy	Altitude (m)	Velocity (m/s)	Temperature (°C)	pH	DIN (mg/L)	SRP (mg/L)
Upstream	Weiße Ois unterhalb Faltl (WO)	47.7657	15.1785	Open	1,061	0.44	8.20	8.19	0.65	0.00
Upstream	Weiße Ois Rehberghütte (RB)	47.7684	15.1574	Open	1,050	0.33	8.00	8.42	0.67	0.00
Upstream	Taschlbach (TB)	47.7892	15.1915	Shade	1,018	0.35	8.20	8.32	0.61	0.17
Upstream	Tagles Schelchen (SCH)	47.8196	15.1122	Shade	880	0.35	7.80	8.50	0.54	0.67
Midstream	Tagles unten (TG)	47.8319	15.1245	Open	720	0.33	8.70	7.90	0.59	1.27
Midstream	Oberer Seebach Ritrodat (OS)	47.8524	15.0655	Open	618	0.25	8.80	8.31	0.81	1.83
Downstream	Ybbs Lunz – Kläranlage (YLK)	47.8550	15.0209	Open	595	0.52	8.60	8.37	0.92	1.87
Downstream	Ybbs Lunz – Großau (YLG)	47.8292	15.0022	Open	570	0.62	8.60	8.55	0.82	1.30
Downstream	Ybbs Göstling – Lagerhaus (YGL)	47.8079	14.9370	Open	532	0.65	9.00	8.53	0.81	1.07

3 | RESULTS

3.1 | Environmental characteristics of study streams

The first two principal components (PCs) accounted for 77.6% of the variation in physical and chemical attributes of nine study streams (Figure 1B; Table 1), and both components were related to a gradient of environmental factors from forested headwaters to downstream. PC1 explained 56.4% of the total variance and was negatively correlated with altitude (correlation coefficient: −0.93) and canopy (−0.66), but positively correlated with DIN (0.89), SRP (0.82) and temperature (0.88). Altitude decreased from upstream to downstream, whereas water temperature and DIN and SRP concentrations increased from upstream to downstream. Furthermore, PC1 arranged the nine stream sites into four groups (Figure 1B): upstream with shaded canopy and low nutrients (up_shade), upstream with open canopy and low nutrients (up_open), midstream with open canopy and relatively high nutrients (midstream) and downstream with open canopy and high nutrients (downstream). PC2 accounted for only 21.2% of the total variance and is not considered further here.

3.2 | Differences in the FA composition of basal food sources and consumers

The NMDS analysis illustrates the dissimilarity in the FA profiles of consumers and their basal food sources (Figure 2). NMDS1 was correlated positively with DHA (correlation coefficients: 0.87), EPA (0.74) and ARA (0.79) but negatively with LIN (−0.92), whereas NMDS2 was significantly correlated with ALA (−0.92), SAFA (0.90) and MUFA (0.97). All samples were classified into three distinct groups, namely submerged leaves (high in ALA and LIN), invertebrates and periphyton (high in EPA) and *Salmo trutta* (high in DHA, EPA and ARA).

Consistent with the NMDS analysis, the PERMANOVA showed significant differences in FA profiles among the above three groups. The FA profiles of leaves were significantly dissimilar to those of fish (F-value = 236.66, $p < 0.001$), invertebrates (F-value = 63.37, $p < 0.001$) and periphyton (F-value = 95.46, $p < 0.001$). The FAs of fish were also significantly dissimilar to those of invertebrates (F-value = 192.92, $p < 0.001$) and periphyton (F-value = 156.44, $p < 0.001$).

3.3 | Longitudinal variations in the FA profiles of basal sources

Submerged leaves and periphyton both had very low DHA and ARA, but leaves were also comparatively low in EPA (Figure 3, Table 2). Although submerged leaves contained high proportions of ALA, LIN, BAFA, MUFA and SAFA, their proportions did not change significantly among sites (Figure 3). Detailed statistical results are provided in the supplementary document.

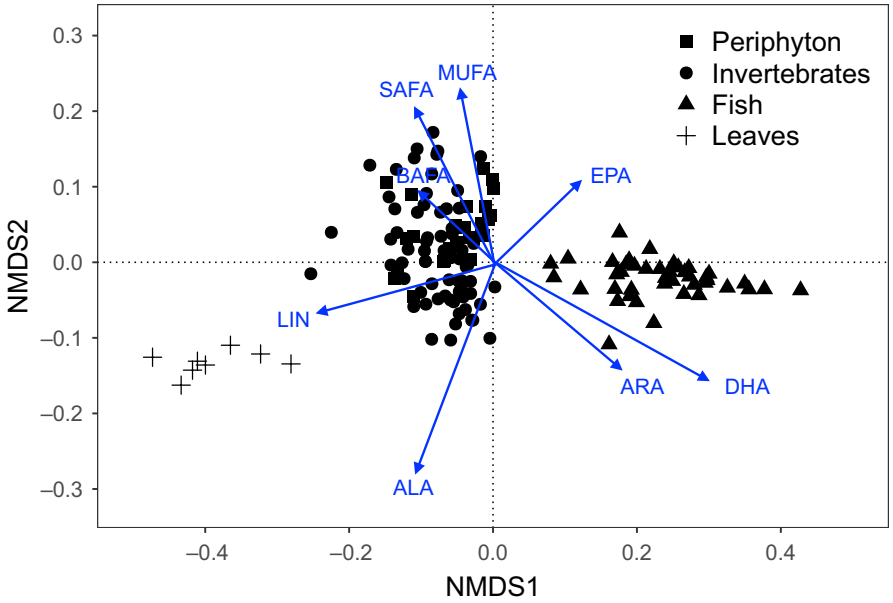

FIGURE 2 Non-metric multidimensional scaling (NMDS) of fatty acid compositions (% relative to total fatty acids) of basal food sources, invertebrates and fish from nine study sites in the River Ybbs catchment, Austria (two-dimensional, stress = 0.12)

Unlike submerged leaves, periphyton FAs showed pronounced variations from upstream to downstream (Figure 3, Table 2). Periphyton EPA was significantly lower in up_shade and up_open sites than in downstream sites (F-value = 15.1, $p < 0.001$; Tukey's HSD, up_shade vs. downstream, $p = 0.01$; up_open vs. downstream, $p < 0.001$). In contrast to EPA, periphyton ALA was significantly higher in up_shade and up_open sites than in downstream sites (F-value = 9.8, $p < 0.001$; Tukey's HSD, up_shade vs. downstream, $p < 0.001$; up_open vs. downstream, $p < 0.045$). Periphyton LIN did not significantly vary among sites (F-value = 0.37, $p = 0.77$). Additionally, periphyton BAFA was higher in up_shade than that in up_open and downstream sites (F-value = 5.5, $p = 0.006$; Tukey's HSD, up_shade vs. up_open, $p = 0.006$; up_shade vs. downstream, $p = 0.01$), whereas periphyton MUFA in up_shade sites was lower than that in up_open and downstream sites (F-value = 7.8, $p = 0.001$; Tukey's HSD, up_shade vs. up_open, $p < 0.001$; up_shade vs. downstream, $p = 0.04$).

3.4 | Longitudinal variations in consumer FA profiles

Variation in the FA profiles of invertebrate primary consumers, that is grazers, shredders and filterers, followed similar patterns as the periphyton FAs from up- to downstream sites, but only the variation in grazer EPA was significant (Figure 3, Table 2). Specifically, grazer EPA was significantly lower in up_shade and up_open sites than that in downstream sites (F-value = 7.5, $p = 0.004$; Tukey's HSD, up_shade vs. downstream, $p = 0.05$; up_open vs. downstream, $p = 0.003$). Additionally, consistent with the basal food sources, grazers, shredders and filterers all had low proportions of DHA and ARA (Table 2). Detailed statistical results are provided in the supplementary document.

The spatial variation of ALA in invertebrate predators, and ALA and EPA in fish, tracked changes in grazer FAs and periphyton FAs (Figure 3, Table 2). Predator ALA was higher in up_shade compared to downstream sites (F-value = 4.2, $p = 0.02$; Tukey's HSD, up_shade vs. downstream, $p = 0.03$). Similarly, fish ALA was higher in up_shade and up_open sites than that in downstream sites (F-value = 4.5, $p = 0.01$; Tukey's HSD, up_shade vs. downstream, $p = 0.04$; up_open vs. downstream, $p = 0.004$). Further, fish EPA was lower in up_shade and up_open sites than at the midstream sites (F-value = 6.5, $p = 0.001$; Tukey's HSD, up_shade vs. midstream, $p = 0.04$; up_open vs. midstream, $p = 0.001$). Fish DHA was similar among the sampling sites (F-value = 1.04, $p = 0.38$). In addition, invertebrate predators were low in DHA, while the predatory *Salmo trutta* was rich in DHA (Table 2).

3.5 | The trophic transfer of LC-PUFAs in river food webs

Piecewise SEM yielded final path models for ω3 LC-PUFAs (Figure 4). The results of directed separation tests, Fisher's $C = 11.61$ and $p = 0.48$, showed that all variables in the final causal model were conditionally independent. The direct influence of basal food sources on brown trout was not significant, but this effect indirectly occurred through invertebrate primary consumers and predators. Submerged leaves had no significant effect on grazer ω3 LC-PUFAs ($r = 0.60$, $p = 0.16$) and shredder ω3 LC-PUFAs ($r = 0.66$, $p = 0.11$). In contrast, periphyton FAs significantly predicted grazer ω3 LC-PUFAs ($r = 0.75$, $p = 0.048$) and shredder ω3 LC-PUFAs ($r = 0.78$, $p = 0.04$), and the effect on grazers was significantly transferred up to invertebrate predators ($r = 0.79$, $p = 0.04$), and then to fish ($r = 0.78$, $p = 0.04$). In addition to invertebrate predators, fish ω3 LC-PUFAs were also significantly affected by grazers ($r = 0.82$, $p = 0.02$) but not by shredders ($r = 0.60$, $p = 0.16$).

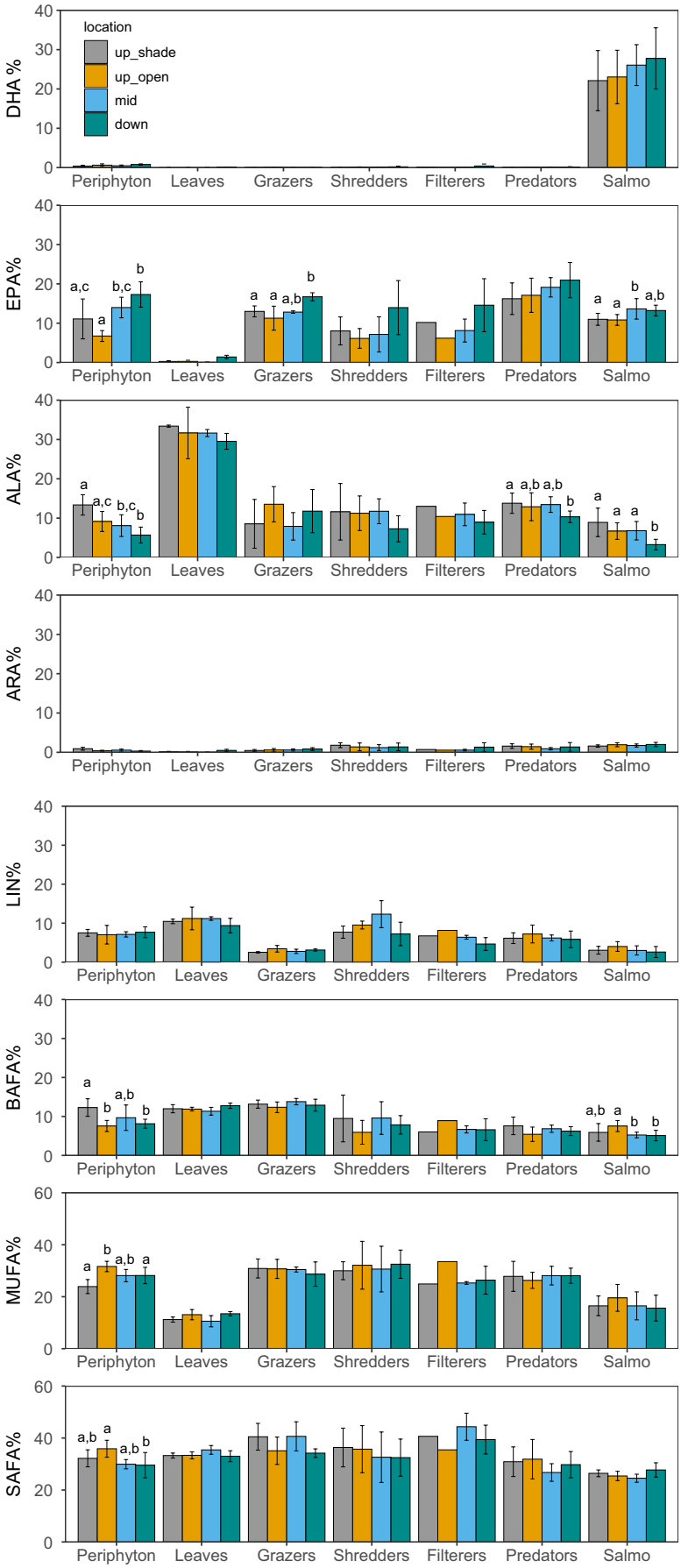

FIGURE 3 Variations in the fatty acid profiles (% relative to total fatty acids, mean ± *SD*) in basal food sources, invertebrate functional feeding groups and fish among stream locations in the River Ybbs catchment, Austria. Salmo, *Salmo trutta*. Letters indicate significant differences among stream locations ($p < 0.05$). Due to the very low proportions of DHA, EPA and ARA in submerged leaves, and DHA and ARA in periphyton, the differences of these FAs among sites were not examined. Due to the low sample size of invertebrate filterers, their FA differences among sites were not examined. Detailed statistical results were provided as the supplementary document. ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid

TABLE 2 Fatty acid composition (%, mean ± *SD*) in basal food sources, invertebrates and fish in different stream locations in the River Ybbs catchment, Austria

Location	Group	DHA	EPA	ALA	ARA	LIN	BAFA	MUFA	SAFA
Up_shade	Submerged leaves	0.00	0.27 ± 0.22	33.4 ± 0.26	0.14 ± 0.11	10.45 ± 0.59	11.98 ± 1.05	11.21 ± 0.99	33.26 ± 0.97
Up_shade	Periphyton	0.37 ± 0.23	11.08 ± 5.07	13.36 ± 2.59	0.91 ± 0.38	7.51 ± 0.88	12.28 ± 2.25	23.9 ± 2.71	32.18 ± 3.28
Up_shade	Grazers	0.02 ± 0.02	13.01 ± 1.39	8.54 ± 6.21	0.47 ± 0.25	2.52 ± 0.17	13.16 ± 1.03	30.88 ± 3.66	40.49 ± 5.15
Up_shade	Shredders	0.04 ± 0.09	8.03 ± 3.56	11.61 ± 7.19	1.8 ± 0.62	7.7 ± 1.56	9.49 ± 6	30 ± 3.45	36.35 ± 7.46
Up_shade	Filterers	0.08	10.19	12.99	0.72	6.74	6.03	24.91	40.66
Up_shade	Predators	0.05 ± 0.03	16.22 ± 4.02	13.79 ± 2.58	1.59 ± 0.59	6.14 ± 1.38	7.59 ± 2.26	27.84 ± 5.77	30.89 ± 5.71
Up_shade	*Salmo trutta*	22.12 ± 7.65	10.99 ± 1.53	8.91 ± 3.62	1.6 ± 0.3	3.05 ± 1	5.91 ± 2.27	16.49 ± 3.79	26.42 ± 1.32
Up_open	Submerged leaves	0 ± 0	0.29 ± 0.29	31.66 ± 6.55	0.1 ± 0.14	11.21 ± 2.91	11.88 ± 0.48	13.1 ± 1.98	33.33 ± 1.34
Up_open	Periphyton	0.61 ± 0.34	6.73 ± 1.39	9.15 ± 2.54	0.39 ± 0.14	7.04 ± 2.39	7.55 ± 1.43	31.64 ± 2	35.87 ± 3.26
Up_open	Grazers	0.06 ± 0.06	11.27 ± 3.05	13.53 ± 4.49	0.61 ± 0.35	3.44 ± 0.84	12.35 ± 1.36	30.71 ± 3.67	35.1 ± 5.28
Up_open	Shredders	0.09 ± 0.1	6.13 ± 2.51	11.24 ± 4.39	1.38 ± 1	9.52 ± 1.01	5.93 ± 3.06	32.1 ± 9.2	35.71 ± 9.05
Up_open	Filterers	0.04	6.19	10.42	0.56	8.15	8.92	33.52	35.45
Up_open	Predators	0.07 ± 0.05	17.1 ± 4.35	12.89 ± 3.54	1.45 ± 0.65	7.22 ± 2.3	5.43 ± 1.84	26.3 ± 3.08	31.86 ± 7.58
Up_open	*Salmo trutta*	23.05 ± 6.8	10.82 ± 1.38	6.7 ± 2.1	1.94 ± 0.51	4.02 ± 1.24	7.54 ± 1.42	19.57 ± 5.17	25.39 ± 1.79
Mid	Submerged leaves	0 ± 0	0.09 ± 0.03	31.64 ± 0.89	0.04 ± 0.06	11.18 ± 0.47	11.34 ± 0.99	10.58 ± 2.17	35.42 ± 1.69
Mid	Periphyton	0.46 ± 0.19	13.98 ± 2.62	8.09 ± 2.75	0.56 ± 0.27	7.12 ± 0.68	9.67 ± 3.27	28.14 ± 2.37	29.93 ± 1.8
Mid	Grazers	0.02 ± 0.02	12.82 ± 0.31	7.91 ± 3.49	0.6 ± 0.27	2.77 ± 0.55	13.8 ± 0.82	30.45 ± 0.97	40.64 ± 5.62
Mid	Shredders	0.09 ± 0.08	7.15 ± 4.48	11.74 ± 3.17	1.2 ± 0.74	12.3 ± 3.46	9.59 ± 4.19	30.64 ± 8.79	32.61 ± 9.7
Mid	Filterers	0.08 ± 0	8.13 ± 2.91	10.95 ± 2.88	0.57 ± 0.21	6.39 ± 0.5	6.67 ± 0.9	25.29 ± 0.52	44.35 ± 5.22
Mid	Predators	0.1 ± 0.06	19.13 ± 2.47	13.44 ± 1.98	0.92 ± 0.3	6.2 ± 0.76	6.85 ± 0.93	28.12 ± 3.59	26.74 ± 3.39
Mid	*Salmo trutta*	26.07 ± 5.2	13.64 ± 2.61	6.79 ± 2.35	1.74 ± 0.39	3.01 ± 1.18	5.24 ± 0.72	16.48 ± 5.38	24.53 ± 1.54
Down	Submerged leaves	0.12 ± 0.04	1.41 ± 0.43	29.53 ± 2.03	0.49 ± 0.28	9.38 ± 1.86	12.75 ± 0.67	13.45 ± 0.83	33 ± 2.11
Down	Periphyton	0.78 ± 0.16	17.29 ± 3.22	5.69 ± 2.03	0.32 ± 0.14	7.68 ± 1.38	8.13 ± 1.16	28.17 ± 3.14	29.57 ± 4.87
Down	Grazers	0.02 ± 0.03	16.72 ± 1.03	11.77 ± 5.48	0.85 ± 0.35	3.11 ± 0.27	12.89 ± 1.53	28.7 ± 4.7	34.18 ± 1.65
Down	Shredders	0.19 ± 0.17	13.96 ± 6.88	7.28 ± 3.3	1.38 ± 0.98	7.23 ± 3.01	7.84 ± 2.37	32.52 ± 5.44	32.46 ± 7.15
Down	Filterers	0.4 ± 0.51	14.58 ± 6.74	8.95 ± 3	1.33 ± 1.12	4.66 ± 1.65	6.59 ± 2.8	26.36 ± 5.36	39.39 ± 5.55
Down	Predators	0.11 ± 0.13	20.95 ± 4.48	10.33 ± 1.47	1.36 ± 1.12	5.85 ± 2.13	6.23 ± 1.14	28.09 ± 2.9	29.75 ± 5.05
Down	*Salmo trutta*	27.77 ± 7.79	13.22 ± 1.36	3.27 ± 1.34	2 ± 0.53	2.6 ± 1.42	5.1 ± 1.35	15.62 ± 4.97	27.71 ± 2.76

Abbreviations: 'Up_shade' = upstream with shaded canopy; 'Up_open' = upstream with open canopy; 'Mid' = midstream with open canopy; 'Down' = downstream with open canopy; ALA, alpha-linolenic acid; ARA, arachidonic acid; BAFA, bacterial fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LIN, linoleic acid; MUFA, sum of monounsaturated fatty acids; SAFA, sum of saturated fatty acids.

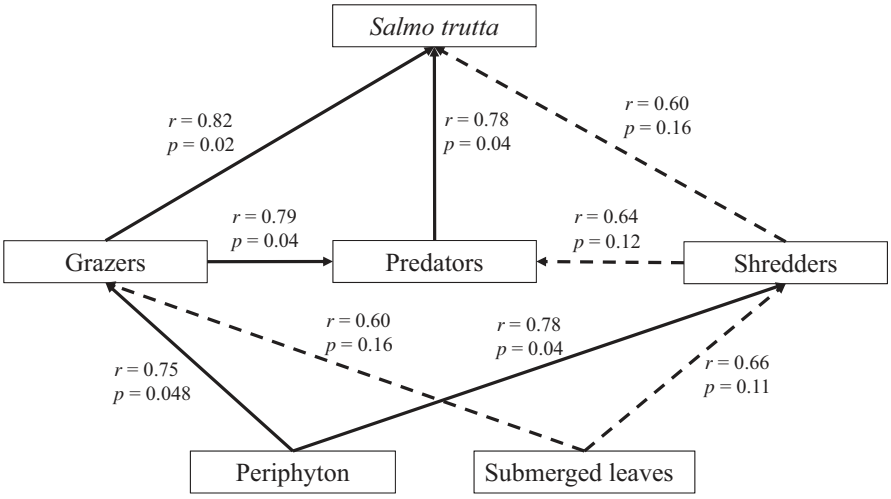

FIGURE 4 The pathways of ω3 long-chain polyunsaturated fatty acids from basal food sources to consumers. The pathways were examined by piecewise structural equation models. Solid paths are statistically different from 0 at $p < 0.05$, whereas dashed paths are not. Invertebrate filterers were not included in the analysis because of their small sample size

TABLE 3 Partial RDA results of the contributions of fish body size, spatial environmental factors and dietary invertebrate prey to the fatty acid profiles of *Salmo trutta* collected in the River Ybbs catchment, Austria

	Salmo trutta
Total effects (%)	33.90
Dietary invertebrate prey	23.80
Dietary invertebrate prey + Spatial environmental factors	31.80
Dietary invertebrate prey + Fish body size	26.30
Spatial environmental factors	27.40
Spatial environmental factors + Fish body size	29.60
Fish body size	2.20

3.6 | Partitioning effects of dietary invertebrate prey, fish body size and spatial environmental factors on fish FA composition

The summed dietary invertebrate prey, fish body size and spatial environmental factors together explained only 33.9% of the FA variation in *Salmo trutta* (Table 3). Dietary invertebrate prey contributed 23.8% of the explained FA variation in fish, which was 10.8 times higher than the effect of fish body size (2.2%) but similar to the contribution of spatial environmental factors (27.4%). The joint effects of dietary invertebrate prey and fish body size explained 26.3% of FA variations in fish, while the joint effects of dietary invertebrate prey and spatial environmental factors explained 31.8%.

4 | DISCUSSION

This field study provides evidence that there are longitudinal patterns in the food quality (ω3 LC-PUFAs) of periphyton that are reflected in the FA composition of invertebrate grazers and shredders, and further tracked by invertebrate predators and fish. Consistent with previous studies, the proportion of ω3 LC-PUFA EPA in periphyton varied with environmental conditions (Cashman et al., 2013; Guo et al., 2017; Whorley & Wehr, 2018), and EPA was highly accumulated in invertebrates (Kühmayer et al., 2020). The other important ω3 LC-PUFA, DHA, was high in *Salmo trutta*, but almost entirely missing in basal resources and invertebrates. The fish accumulated EPA from invertebrate prey and likely use this FA to synthesize DHA.

Strong differences were observed between the FA composition of terrestrial leaves and invertebrates and fish, confirming that allochthonous inputs are not the source of LC-PUFAs that are essential for growth, reproduction and maintenance of aquatic consumers in river ecosystems (Brett et al., 2017; Guo et al., 2017). Submerged leaves likely play important roles in stream systems as a reactive surface on which biofilms grow or as habitat for macroinvertebrates (Cummins & Klug, 1979), but offer only weak dietary contributions to consumer LC-PUFAs. This is evident because leaves are totally devoid of the ω3 LC-PUFA EPA for invertebrates and DHA for fish. Although biofilms growing on submerged leaves contain LC-PUFAs, their amount may be too low to cause larger changes in the FA composition of their consumers, which is supported by our path analysis results that submerged leaves did not have a significant effect on shredder FAs. In contrast, invertebrate overall FAs largely overlapped with periphyton FAs, indicating invertebrates may obtain their LC-PUFAs mainly from this source. For the study fish, *Salmo trutta*, its overall FA composition differed

from periphyton and invertebrates mainly because it contained a high proportion of DHA, which is probably obtained via the bioconversion of EPA contained within the trout's prey resources (Barry & Trushenski, 2020).

The diet quality of periphyton increased longitudinally from upto downstream sites, which is largely attributed to the DIN and SRP gradients rather than riparian canopy and temperature. Riparian canopy cover showed limited effects on periphyton EPA, as periphyton EPA was just slightly higher in upstream sites with shaded canopy than that in upstream sites with open canopy. In previous studies which reported strong effects of canopy cover on periphyton FAs (Guo et al., 2015; Hill et al., 2011), their canopy cover was either associated with large differences in light intensities (Hill et al., 2011) or ranged from 20% to 86% (Guo et al., 2015). We chose only two canopy cover levels, that is open canopy and partly shaded canopy, for which the differences may be not large enough to induce variations in periphyton species composition and FAs. Additionally, we sampled in late autumn, when most riparian leaves fall and more light could penetrate and reach the streams, partly reducing the differences between the two canopy cover levels. Meanwhile, the observed temperature effect on periphyton EPA also was rather small, likely due to the small scale of the study subalpine river catchment with small temperature variations. Therefore, the differences in periphyton EPA between upstream and downstream habitats are likely induced by increased DIN and SRP concentrations. Moderate nutrient levels are reported to lead to an increase in periphyton EPA, but decreases in the short-chain PUFA ALA (Guschina & Harwood, 2006; Hill et al., 2011), evidenced by the converse patterns of periphyton EPA and ALA in response to nutrient gradients from upstream to downstream in our study.

The path analysis showed that longitudinal variations in periphyton diet quality were tracked by grazers and shredders, consistent with previous findings that stream invertebrates have a FA composition that largely reflects their dietary algal FAs (Guo et al., 2018; Torres-Ruiz et al., 2010). Grazers and shredders are frequently used in studies when examining the importance of autochthonous and allochthonous diet sources for stream food webs, since these consumers have contrasting feeding behaviours on algae and terrestrial leaves respectively. It is predictable that grazer EPA varied with periphyton EPA from upstream to downstream, since grazers primarily feed on periphyton and retain large amounts of periphyton FAs (Guo et al., 2018; Guo, Bunn, et al., 2021). Surprisingly, shredders, which feed on allochthonous sources, also tracked the variations in periphyton EPA, although the pattern was not significant from upstream to downstream. Shredders feed on submerged leaves which may have algae growing on the surfaces, and these algae can boost shredder growth (Guo, Kainz, Valdez, et al., 2016), but few studies have shown how these algae influence shredder FA composition. Shredders are found to preferentially retain EPA from periphyton rather than submerged leaves (Kühmayer et al., 2020), probably due to the large amount of EPA in periphyton. Together with our results, we propose that shredder EPA is largely determined by periphyton EPA, provided via autochthonous diet sources.

Although predator FAs did not show longitudinal patterns, path analysis showed their ω3 LC-PUFAs were significantly influenced by grazers. This may be partly caused by the relatively high abundance of grazers (e.g. *Baetis* sp. and *Ecdyonurus* sp.) in our study streams, which potentially are the most abundant diet for predators. Further, the significant transfer of periphyton ω3 LC-PUFAs to predators via grazers confirms the physiological importance of ω3 LC-PUFAs for invertebrates (Stanley-Samuelson, 1994), and the increasing retention of ω3 LC-PUFAs in body tissues from primary consumers to predators indicates the more efficient transfer of autochthonous than allochthonous carbon in stream food webs (Gladyshev et al., 2011; Guo et al., 2018; Lau et al., 2012). Invertebrates feeding on and retaining dietary ω3 LC-PUFAs will be consequently high-quality food for their consumers, such as fish.

The ω3 LC-PUFA proportion in *Salmo trutta* was significantly influenced by that of invertebrates, suggesting that invertebrates supply EPA to this trout species. However, the contribution of invertebrate prey to the overall FA profiles of brown trout was low (23.8%), suggesting the fish may feed on other sources, and/or be capable of synthesizing LC-PUFAs, especially DHA. DHA in this trout species represented 24% of the total FA, similar to the average DHA levels in stream salmonids (~24%) but much higher than that in Cypriniformes (~5%), Siluriformes (~8%) and their food sources periphyton and invertebrates (typically <1%; Guo et al., 2017). The high DHA in salmonids in such a low-DHA environment challenges their DHA acquisition strategies between external dietary sources and internal conversion. Based on the gut content analyses, many studies have reported that stream-dwelling *Salmo trutta* primarily feed on aquatic and terrestrial invertebrates, >90% of the diet, with smaller contributions from fish and fish eggs (Becer Ozvarol et al., 2011; Elliott, 1967; Kara & Alp, 2005). However, *Salmo trutta* in our study catchment were mostly juveniles, which mainly consume benthic invertebrates (Sánchez-Hernández et al., 2012). Stream invertebrates are low in DHA, supported by our data, and thus the study fish likely converted dietary precursors (e.g. invertebrate EPA) to DHA.

In our study, the DHA proportion of the study fish remained constant among sites, indicating their strong ability to regulate DHA at a relatively constant level for physiological requirements regardless of stream locations and food sources. Moreover, our study fish accumulated EPA from invertebrate prey and may use this EPA to synthesize DHA. It is likely that our study fish may have a stronger ability to synthesize DHA from EPA compared with other riverine fishes whose FA profiles varied with algal FAs along the river continuum, such as bluegill (DHA, ~7.4%) in Illinois River (Rude et al., 2016). Recent feeding trials also show that the freshwater salmonids Arctic charr *Salvelinus alpinus* and rainbow trout *Oncorhynchus mykiss* are physiologically able to synthesize sufficient DHA from dietary precursors (Barry & Trushenski, 2020; Murray et al., 2014). Therefore, the converted DHA in the study salmonid may be sufficient to account for the high DHA proportion observed from stream ecosystems.

In summary, our study provides a nutritional perspective into river food web studies from a FA view and defines a more holistic

perspective of the trophic transfer of LC-PUFAs, from the base of the food web to carnivorous fish. Our results confirm that stream consumers derive their LC-PUFAs from periphyton, which is irrespective of the functional feeding modes of consumers and despite marked longitudinal differences in terrestrial litter inputs or riparian shading. There are, however, longitudinal variations in the diet quality of periphyton that are reflected in consumers. Therefore, we propose a longitudinal upstream to downstream high-quality periphyton-dominated energy pathway.

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CONFLICT OF INTEREST

None of the authors have a conflict of interest.

AUTHORS' CONTRIBUTIONS

F.G., M.J.K., S.E.B. and M.T.B. conceived the ideas and designed the methodology; F.G., N.E. and H.H. collected the data; F.G. analysed the data and led the writing of the manuscript. All authors contributed to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.4f4qrfjcm (Guo, Ebm, et al., 2021).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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PRIMARY RESEARCH PAPER

Polyunsaturated fatty acids in fish tissues more closely resemble algal than terrestrial diet sources

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Abstract The River Continuum Concept implies that consumers in headwater streams have greater dietary access to terrestrial basal resources, but recent studies have highlighted the dietary importance of high-quality algae. Algae provide consumers with physiologically important omega-3 (n-3) polyunsaturated fatty acids (PUFA), particularly eicosapentaenoic acid (EPA). However, terrestrial plants and most benthic stream algae lack the long-chain (LC) n-3 PUFA docosahexaenoic acid (DHA, 22:6n-3), which is essential for neural development in fish and other vertebrates. We sampled subalpine streams to investigate how the PUFA composition of neural (brain and eyes), muscle, and liver tissues of freshwater fish is related to their potential diets (macroinvertebrates, epilithon, fresh and conditioned terrestrial leaves). The PUFA composition of consumers was more similar to epilithon than to terrestrial leaves. Storage lipids of eyes most closely resembled dietary PUFA (aquatic invertebrates and algae). However, DHA and arachidonic acid (ARA, 20:4n-6) were not directly available in the diet but abundant in organs. This implies that algal PUFA were selectively retained or were produced internally via enzymatic PUFA conversion by aquatic consumers. This field study demonstrates the nutritional importance of algal

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PUFA for neural organs in aquatic consumers of headwater regions.

Keywords Stream food webs · Food quality · Headwaters · Fish brain · Fish eyes · Docosahexaenoic acid

Introduction

At the base of aquatic food webs, primary producers, e.g., microalgae, provide consumers with dietary nutrients, including essential polyunsaturated fatty acids (PUFA), that are crucial constituents of cell membranes (e.g., phospholipids). A steady supply of dietary PUFA to aquatic consumers, particularly omega-3 (n-3) long-chain (LC) PUFA, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), is critical as these fatty acids (FA) support somatic growth and reproduction (Müller-Navarra et al., 2000; Brett et al., 2009). However, most aquatic consumers have limited abilities to synthesize EPA and DHA and thus have to take up these biomolecules directly from their diets (Brett & Müller-Navarra, 1997; Arts et al., 2009; Torres-Ruiz et al., 2010). Vascular plants synthesize the short-chain PUFA: alpha-linolenic acid (ALA; 18:3n-3) and the n-6 PUFA linoleic acid (LA, 18:2n-6), but generally lack EPA and DHA (Brett et al., 2009; but see Napier, 2006). Although epilithic algae (especially diatoms) in freshwater ecosystems are comparatively rich in EPA, they are low in DHA (Brett et al., 2009; Hixson et al., 2015; Guo et al., 2018). However, DHA is essential for all vertebrates including freshwater fishes (Ahlgren et al., 1994; Guo et al., 2017) where it is associated with high membrane fluidity (e.g., signal transduction, neurotransmission or hormone regulation, Farkas et al., 2000), anti-inflammatory and—oxidative effects (e.g., neuroprotection, Bazan, 2005), proper neural development (cognitive performance, Lund et al., 2014), and sensory functioning (visual acuity, Bell et al., 1995).

In contrast to marine fish (Agaba et al., 2005; Tocher et al., 2006; Mohd-Yusof et al., 2010), previous studies have reported that enzymatic conversions to LC-PUFA are functional in some freshwater fish (e.g., *Oncorhynchus mykiss* (Walbaum, 1792), *Salvelinus alpinus* (Linnaeus, 1758), Buzzi et al., 1996; Tocher et al., 2001; Murray et al., 2014). These fish are able to transform dietary ALA via stearidonic acid (SDA) and EPA to DHA, but direct incorporation of dietary DHA is preferred by fish as it reduces the more energy-demanding endogenous production pathway. However, in contrast to such experimental studies, little is known about the PUFA pathways to and within fish in natural ecosystems deprived in DHA (Gladyshev et al., 2018), and especially how fish in streams acquire their PUFA and subsequently allocate these FA to different organs, including neural organs (Guo et al., 2017).

Neural organs, e.g., brain and eyes, are rich in DHA (Mourente & Tocher, 1998; Farkas et al., 2000; Stoknes et al., 2004), which is key for proper functioning of these organs and eventually optimal fish physiological performance. Common stream fish in alpine headwaters (e.g., salmonids and European bullhead) are visual predators that prey mostly on moving or drifting invertebrates oftentimes at night (Elliott, 1973; Mills & Mann, 1983). A reduced visual performance (Bell et al., 1995) or learning ability (Lund et al., 2014) of fish due to LC-PUFA-poor diets can reduce feeding success, predator avoidance (e.g., escape latency, swimming speed) and other behavioral traits, e.g., schooling behavior (Ishizaki et al., 2001). Despite the fact that neural organs have the highest lipid contents and are the most energy-demanding tissues in animals, our current knowledge about energy flows in aquatic food webs is primarily derived from studies on fish muscle and liver tissues (Volk & Kiffney, 2012; Kainz et al., 2017; Sushchik et al., 2018). Previous research indicated that FA in neutral lipids (NL) of fish muscles, such as triacylglycerols (TAG), reflect those from the diet, while FA in the polar lipids (PL) of fish muscles are driven by taxonomy (Iverson 2009; Sushchik et al., 2020). Total lipids and TAG contents vary among fish organs (Tocher & Harvie, 1988; Budge et al., 2006; Hong et al., 2014) and thus different organs may vary in correspondence to dietary FA.

Most vertebrate and fish brains consist mainly of PL (Soengas & Aldegunde, 2002), which are rich in DHA (Tocher & Harvie, 1988; Iverson, 2009), making them indispensable for cell functionality and relatively stable to dietary lipid intake. In contrast, fish eyes are rich in TAG (Mourente et al., 1991; Geurden et al., 1998; Stoknes et al., 2004) and thus eye lipids may reflect dietary PUFA sources better than brain lipids

(Brodtkorb et al., 1997). Bell et al. (1995) demonstrated that DHA-poor diets resulted in DHA-deficit retina membranes with impaired vision under natural light intensities. It is thus important to understand how lipid classes in neural organs are affected by dietary FA. However, this has not been tested yet for stream fish which receive very little DHA directly from organisms at the base of the food chain.

Motivated by the ambiguities outlined above, we designed a field study to investigate the PUFA distribution in freshwater fish and their potential diet sources in subalpine streams. We compared the PUFA composition of various fish organs, i.e., muscle, liver, brain, and eyes, with PUFA of potential diet sources (terrestrial plants, benthic algae and macroinvertebrates), and also explored the FA distribution of salmonid brain and eyes in an effort to discern how different lipid classes (NL versus PL) of these neural organs are affected by dietary PUFA sources. We tested the hypotheses that, (1) the PUFA composition of stream consumers (macroinvertebrates and fish) is generally more similar to epilithon than to the leaves of terrestrial vascular plants, and, (2) fish eyes have a greater similarity with dietary PUFA than fish brains due to differences in lipid class composition.

Methods

Study streams

We sampled 17 sites in 9 oligotrophic streams (1st-5th order, total length ~ 42 km) within the headwater region ('Weiße Ois') of the subalpine River Ybbs catchment, Austria (Online resource 1). The study catchment (550–1000 m.a.s.l.) has a nivo-pluvial flow regime that drains 254 km^2 and is characterized by glaciokarst and dolomite (82%), with forests (82%) and alpine meadows (11%) the predominant land cover forms (Besemer et al., 2013). The river substratum is dominated by gravel and cobble with intense bed-load transport during peak flows. The typical fish species in these study streams are Brown trout (*Salmo trutta fario* Linnaeus, 1758), European bullhead (*Cottus gobio* Linnaeus, 1758), European grayling (*Thymallus thymallus* Linnaeus, 1758) and Rainbow trout (*Oncorhynchus mykiss*). Stocking of Brown and Rainbow trout is reported but only at the downstream study sites (YKL, YLG, and YGL). Chemical parameter ($N\text{-}NO_3$, $N\text{-}NH_4$, $P\text{-}PO_4$), soluble reactive phosphorus (SRP), and dissolved organic matter (DOM) concentrations in these study streams were reported in a previous study (Guo et al., 2018).

Sample collection

Potential dietary resources for fishes, including epilithon, conditioned terrestrial leaves, and aquatic invertebrates were collected in October 2016. Conditioned leaves (submerged, senescent, and brownish leaves of various vascular plant species) were collected from streams). Epilithon from each of the sampling sites was transferred into sample containers from small cobbles using soft brushes and invertebrates were picked from rocks. The taxonomic composition of epilithon and periphyton (biofilm on submerged leaves) was not examined because we were primarily interested in their dietary and biochemical composition (Guo et al., 2018). Fresh leaves were picked directly from riparian vegetation in summer (July 2016). These samples included angiosperms (*Acer*, *Alnus*, *Corylus avellana* L., *Fagus sylvatica* L., *Hedera helix* L., *Phragmites*, *Salix* and gymnosperms (*Picea* and *Pinus*) and each plant species was analyzed separately.

Macroinvertebrates were identified to the genus level using a stereomicroscope and pooled by species for lipid analyses. Macroinvertebrate samples included Ephemeroptera (*Baetis*, *Ecdyonurus*, *Rhithrogena*), Plecoptera (*Leuctra*, *Nemoura*, *Perla*, *Perlodes*, *Isoperla*), Trichoptera (*Allogamus*, Odontoceridae, *Plectronemia*, *Potamophylax, Rhyacophila*, *Hydropsyche*), Platyhelminthes and the amphipod *Gammarus*.

Fish capture and collection for this research was permitted by the local fisheries authorities for these study streams. Individuals of three fish (*O. mykiss*, *S. trutta*, and *C. gobio*) were collected by electrofishing in November 2016, anesthetized and subsequently killed in accordance with the Federal Act on the Protection of Animals, Austria (http://www.ris.bka.gv.at). Fish total body length (mm) and weight (g) were recorded (Online Resource 2). No fish were found at the Schelchen (SCH) sampling site. Invertebrate samples were kept on ice in a portable cooling box and stored at cryogenic temperature (− 80°C) in the lab. Fishes were dissected and samples of the dorsal muscle and the entire liver of each specimen

were collected. Dermis and subcutaneous tissue of each muscle sample were removed and excluded from further biochemical analyses. Entire brains and eyeballs together, with the optic nerve, were removed from each fish. In some cases, in particular for European bullhead, some eye or brain samples had to be pooled prior to lipid extraction (1–5 individuals matched for similar size from each sampling site) to obtain sufficient biomass for lipid analysis. All samples were stored on ice during dissection, placed into Eppendorf tubes (brain and eyes) or scintillation vials (muscle and liver) and stored at cryogenic temperature (− 80°C) until lyophilization (Virtis Genesis^{TM} freeze dryer).

Lipid analyses

Total lipid extraction

Lipids and their FA were analyzed as described by Guo et al. (2015). Briefly, total lipids from freeze-dried (i.e., all lipids and FA were reported as dry weight; DW) and homogenized samples (fresh and conditioned terrestrial leaves: ~ 50 mg, epilithon: ~ 10 mg, invertebrates: ~ 5 to 7 mg, brain and eyes: ~ 5 mg, liver: ~ 10 to 15 mg, muscle: ~ 15 to 20 mg) were dissolved in ice-cold chloroform (2 mL) and stored under N_2 atmosphere over night at − 80°C to improve lipid extraction efficiency. Samples were then further extracted in chloroform–methanol (2:1) and NaCl (0.8 mL; salt wash), vortexed and sonicated, and subsequently analyzed gravimetrically in pre-weighed tin capsules (total lipid content determination).

Lipid class separation

Only salmonids (*S. trutta* and *O. mykiss*) were used for the analysis of FA in lipid classes in neural organs because of limited material for lipid class separation for European bullhead brain and eye samples. Lipid extracts of selected salmonid brain and eye samples were separated into lipid classes by thin-layer chromatography (TLC). Mass ratios of lipid extracts were adjusted after gravimetry with chloroform to obtain similar lipid amounts (150–200 µg) in the volume (50 µL) applied to the TLC plates for salmonid brain and eye samples only. Polar (membrane lipids, PL) and neutral lipids (storage lipids, NL) were separated by one-dimensional TLC on 10 × 10 cm silica gel plates (Merck TLC silica gel 60) using a hexane:diethylether:methanol:formic acid (90:20:3:2, v/v/v/v) solvent solution. After development, plates were sprayed with 0.05% (wt/vol) 8-anilino-4-naphthalenesulfonic acid in methanol and viewed under UV light to detect lipid fractions (Online resource 3). An internal standard (5 µL; non-adecanoic acid in chloroform; 4 mg mL) was added to each lipid fraction before individual lipid fractions were scraped from the TLC plates and transferred into separate solvent-rinsed vials.

Fatty acid methylation and analysis

Fatty acids from total lipid extracts and after lipid class separation were derivatized to fatty acid methyl esters (FAME) in a H_2SO_4 methanol solution for 16 h at 50°C. All FAME were stored at − 80°C until being separated using gas chromatography (THERMO^{TM} Trace GC) and detected using flame ionization detection (FID). FAME were separated by a Supelco^{TM} SP-2560 column (100 m, 25 mm i.d., 0.2 µm film thickness), identified by comparison to the retention times of known standards (37-component FAME Mix, Supelco 47885-U; Bacterial Acid Methyl Ester Mix, Supelco 47080-U). The FAME concentrations were quantified using calibration curves based on known standard concentrations. All FAME analyses were replicated within the study design (e.g., 5 epilithon samples per sample site). Results for each FAME were reported as relative values (% of total FAME).

Data analysis

Before multivariate analysis, all PUFA (%) data were arcsine-square-root-transformed for normal distribution approximation and variance stabilization. Multivariate outliers were removed by the minimum volume ellipsoid-based robust distance (Mahalanobis, *R* package "mvoutlier", Filzmoser & Gschwandtner, 2018).

Six individual PUFA (LA, ARA, ALA, SDA, EPA, and DHA) were selected for non-metric multidimensional scaling (nMDS) to ordinate the PUFA composition among fish and their food sources (leaves, algae and invertebrates) in two-dimensional space as a function of rank-order dissimilarity (Bray–Curtis). Analysis of similarity (ANOSIM, *R* package "vegan",

Oksanen et al., 2019) with 999 permutations was then used to assess similarities in the PUFA composition between food sources (leaves, algae and invertebrates), fish or their organs by ANOSIM global R value. This value ranges from − 1 to + 1. A global R statistic close to 1 indicates that the pair (or a set of samples) is different; an R statistic close to 0 implies that the pair is similar. In greater detail, a global R value of < 0.25 indicates that groups are hardly separated, $R < 0.5$ shows that groups differ but with some overlapping, and $R > 0.75$ implies groups are very well separated (Jaschinski et al., 2011). Although not commonly reported, negative values can occur and indicate greater dissimilarity within a group than among groups (Chapman & Underwood, 1999). In addition, a regression tree (R package "tree", Ripley, 2019) was performed with untransformed proportion data (Online resource 4) to explore patterns between individual PUFA and organisms at different trophic levels and among fish organs or lipid classes. The acceptable level of misclassification was set at < 0.30. The significance threshold for all data analyses was set at $P < 0.05$. For visualization of differences in the PUFA composition between salmonid brain and eye lipid classes, abundant FA other than PUFA: i.e., saturated FA (14:0 = myristic acid, 16:0 = palmitic acid, 18:0 = stearic acid) and monounsaturated FA, oleic acid (18:1n-9) and the diatom biomarker, palmitoleic acid (16:1n-7), were added to the previously selected 6 PUFA to better interpret the position of samples in the ordination space. For all presented ordination plots stress values < 0.1 were achieved.

Results

PUFA composition

The PUFA composition of muscle, liver, brain and eye samples in sedentary (European bullhead) and potentially migratory (Brown and Rainbow trout) fishes differed from that of their potential food sources (macroinvertebrates, epilithon, fresh and conditioned terrestrial leaves) (Fig. 1, Table 1). Basal resources (epilithon, fresh and conditioned leaves) in streams differed significantly from each other in their PUFA composition (ANOSIM; $R = 0.84$, $P < 0.001$, $n = 84$). Fresh leaves contained predominantly the short-chain PUFA ALA and LA, whereas conditioned leaves additionally contained traces of SDA and EPA (Table 2). Compared to leaves, epilithon and benthic invertebrates contained high levels of EPA and traces of DHA, which led to a further split in the classification tree between the latter two (online resource 4). Further, epilithon samples contained the highest levels of the diatom biomarker 16:1n-7 (Table 2). Fish differed mostly from their potential resources (macroinvertebrates, epilithon, fresh and conditioned leaves) by their high DHA content, particularly in brain tissue. Nonetheless, the PUFA composition of consumers (benthic macroinvertebrates and fish) was more similar to algal than to terrestrial resources (fresh and conditioned leaves, Table 3).

The organs of both salmonid taxa (*S. trutta* and *O. mykiss*) did not differ fundamentally in their PUFA composition and will subsequently be referred to as salmonids. The PUFA composition of salmonid organs grouped distinctly from each other (ANOSIM: $R = 0.68$, $P < 0.001$, $n = 284$), but those of European bullhead overlapped extensively (ANOSIM $R = 0.09$, $P = 0.002$, $n = 166$). However, the FA profiles of the organs still differed from each other in both the salmonids and European bullhead. In both fish taxa, brains had the lowest LA and ALA and the highest DHA content (Table 2). Compared to brains, the eyes contained more LA and ALA, but less EPA than liver and muscle samples, and livers contained the greatest levels of ARA. European bullhead had higher brain ARA and lower liver DHA levels than in salmonids, but muscle PUFA did not differ between the two fish taxa (ANOSIM R = 0.18, $P < 0.001$, $n = 147$). Brain and eye samples of European bullhead (ANOSIM: $R = 0.19$, $P = 0.002$, $n = 61$) and salmonids (ANOSIM: $R = 0.93$, $P < 0.001$, $n = 134$) were the most dissimilar organs. In both fish taxa, eye PUFA had the highest similarity with dietary PUFA (Table 3) and the highest levels of 16:1n-7 (Table 2).

FA composition in lipid classes of fish brain and eyes

Salmonid eyes contained 4 times more NL than PL, but their brains had 5 times more PL than NL (online resource 5). The nMDS (Fig. 2) of the FA in the lipid classes of the salmonid brain and eye samples showed that PL differed significantly from NL in both organs (ANOSIM $R = 0.89$, $P < 0.001$, $n = 70$, Fig. 2). Axis 1 of the nMDS separated brain and eye PL from brain NL on the basis of their LC-PUFA (correlation

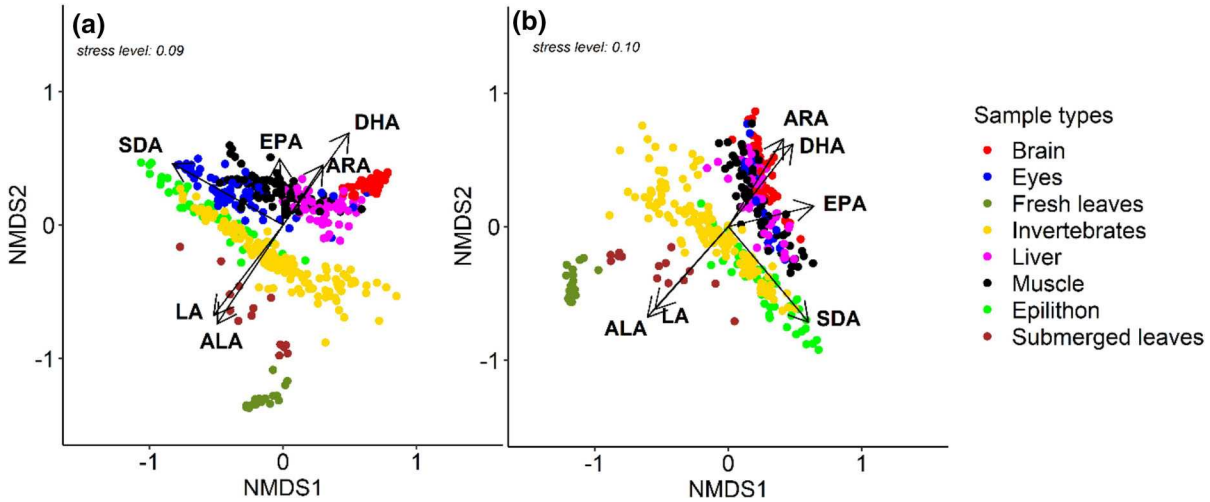

Fig. 1 Non-metric multidimensional scaling (nMDS) of arcsine-square-root-transformed PUFA (% of total FAME) of basal resources, benthic invertebrates and **a** salmonids (*S. trutta* and *O. mykiss*) and **b** European Bullhead (*C. gobio*). *ALA* alpha-linolenic acid, *SDA* stearidonic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *LA* linoleic acid, *ARA* arachidonic acid

Table 1 Key statistical parameters of non-metric multidimensional scaling (nMDS) of arcsine-square-root-transformed PUFA (% of total FAME) of basal resources, benthic invertebrates and (a) salmonids (*S. trutta* and *O. mykiss*) and (b) European Bullhead (*C. gobio*)

	FA	nMDS scores		*r*	*P*
		Axis 1	Axis 2		
Salmonids	EPA	− 0.05161	0.99867	0.50	< 0.001
	ARA	0.5554	0.83159	0.54	< 0.001
	DHA	0.5837	0.81197	0.85	< 0.001
	LA	− 0.60997	− 0.79243	0.85	< 0.001
	ALA	− 0.55755	− 0.83014	0.89	< 0.001
	SDA	− 0.87338	0.48704	0.95	< 0.001
European bullhead	EPA	0.97165	0.23644	0.66	< 0.001
	ARA	0.5331	0.84605	0.78	< 0.001
	DHA	0.61576	0.78794	0.79	< 0.001
	LA	− 0.66207	− 0.74944	0.83	< 0.001
	ALA	− 0.66585	− 0.74608	0.91	< 0.001
	SDA	0.64262	− 0.76619	0.93	< 0.001

Vectors (fatty acids, FA) are sorted ascending according to its correlation coefficient (*r*). *ALA* alpha-linolenic acid, *SDA* stearidonic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *LA* linoleic acid, *ARA* arachidonic acid

coefficients: DHA = 0.96, ARA = 0.84, EPA = 0.48), 18:1n-9 ($r = 0.89$) and SAFA (14 : 0 = 0.95, 18 : 0 = 0.95 , 16 : 0 = 0.76) content. Axis 2 of the nMDS segregated eye NL from brain and eye PL correlated strongly with C_{18}-PUFA (ALA = 0.92, LA = 0.90, SDA = 0.78) and the diatom marker 16:1n-7 ($r = 0.91$).

Brain- and eye PL were more similar (ANOSIM; $R = 0.70, P < 0.001, n = 38$) to each other than NL of both these organs (ANOSIM; $R = 0.97$, $P < 0.001$, $n = 32$). Generally, PL in both organs differed from

Table 2 Individual PUFA (% of total FAME) among different food-web components in streams (mean ± 1 standard deviation)

Samples		ALA	SDA	LA	EPA	ARA	DHA	16:1n-7	*n*
Terrestrial resources	Fresh leaves	37 ± 20	0 ± 0	16 ± 8	0 ± 0	0 ± 0	0 ± 0	1 ± 0	22
	Conditioned leaves	31 ± 4	1 ± 1	10 ± 1	1 ± 1	0 ± 0	0 ± 0	1 ± 1	14
Aquatic resources	Epilithon	9 ± 3	3 ± 2	7 ± 2	12 ± 5	1 ± 0	1 ± 0	15 ± 5	48
	Invertebrates	10 ± 4	1 ± 1	6 ± 3	14 ± 6	1 ± 1	0 ± 0	8 ± 4	146
European bullhead	Brain	2 ± 2	1 ± 0	1 ± 1	11 ± 3	4 ± 1	25 ± 4	5 ± 1	37
	Eyes	6 ± 4	1 ± 1	3 ± 1	8 ± 2	2 ± 1	19 ± 7	7 ± 3	24
	Liver	4 ± 2	1 ± 1	3 ± 1	12 ± 4	5 ± 2	19 ± 6	5 ± 2	48
	Muscle	5 ± 3	1 ± 1	3 ± 1	18 ± 4	3 ± 2	15 ± 5	5 ± 3	57
Salmonids	Brain	1 ± 0	0 ± 0	1 ± 0	8 ± 1	1 ± 0	32 ± 5	2 ± 1	70
	Eyes	10 ± 4	2 ± 1	5 ± 2	7 ± 2	1 ± 1	11 ± 7	12 ± 4	64
	Liver	4 ± 1	1 ± 0	3 ± 1	10 ± 3	4 ± 2	26 ± 7	4 ± 1	60
	Muscle	7 ± 3	1 ± 1	3 ± 1	13 ± 3	2 ± 1	24 ± 6	5 ± 2	90

ALA alpha-linolenic acid, *SDA* stearidonic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *LA* linoleic acid, *ARA* arachidonic acid, *n* sample size

Table 3 Pairwise similarity (ANOSIM; R) of FA compositions among basal resources, benthic invertebrates and fish (brain, eyes, liver and muscle)

Sample types	Basal resources			Invertebrates
	Fresh leaves	Conditioned leaves	Epilithon	
Fish (all organs)	0.9974***	0.8171***	0.475***	0.4891***
	n = 472	*n* = 464	*n* = 498	*n* = 596
Invertebrates	0.8899***	0.396***	0.235***	–
	n = 168	*n* = 160	*n* = 194	
European bullhead				
Brain	1***	0.9932***	0.8148***	0.5109***
	n = 59	*n* = 51	*n* = 85	*n* = 183
Eyes	1***	0.9278***	0.555***	0.3379***
	n = 92	*n* = 38	*n* = 72	*n* = 72
Liver	1***	0.9541***	0.617***	0.3214***
	n = 70	*n* = 62	*n* = 96	*n* = 96
Muscle	1***	0.9103***	0.5438***	0.3195***
	n = 79	*n* = 71	*n* = 105	*n* = 105
Salmonids				
Brain	1***	1***	0.9923***	0.8398***
	n = 92	*n* = 92	*n* = 118	*n* = 216
Eyes	0.9989**	0.8222***	0.1869***	0.3901***
	n = 86	*n* = 78	*n* = 112	*n* = 210
Liver	1***	0.9973***	0.8943***	0.4348***
	n = 82	*n* = 74	*n* = 108	*n* = 206
Muscle	1***	0.9607***	0.5857***	0.3974***
	n = 112	*n* = 104	*n* = 138	*n* = 236

Higher Global *R* values indicate higher dissimilarity between two or more groups. Asterisks indicate significance levels: ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$

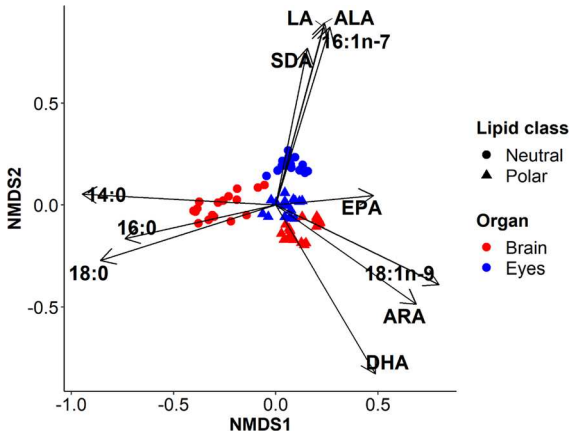

Fig. 2 Non-metric multidimensional scaling (nMDS) of arc-sine-square-root-transformed fatty acids (% of total FAME) of salmonid (*S. trutta* and *O. mykiss*) polar and neutral lipids from the brain and eyes. 14:0 = myristic acid, 16:0 = palmitic acid, 18:0 = stearic acid, 18:1n-9 = oleic acid, *ALA* alpha-linolenic acid, *SDA* stearidonic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *LA* linoleic acid, *ARA* arachidonic acid

NL by having a higher DHA content (Table 4). Brain PL were characterized by very low levels of LA compared to eyes. Neutral lipids of eyes contained higher contents of ARA than brain NL. Finally, NL of eyes had high contents of ALA, LA, SDA and the diatom biomarker (16:1n-7). Additionally, brain NL were rich in saturated fatty acids (SAFA).

Discussion

Earlier studies on dietary lipid and transfer along freshwater food webs focused on how dietary energy is passed from sources to whole consumers or selected parts thereof, e.g., dorsal muscle tissue of fish (Ballantyne et al., 2003; Kainz et al., 2004, 2017). By looking at selected parts of fish only, inferences about dietary energy transfer are limited to these tissues, and cannot be extended to whole organisms, thus limiting comprehensive assessments. This is the first study that shows the dietary transfer of LC-PUFA from epilithon to stream invertebrates and subsequently to various fish tissues, including neural organs.

At the base of stream food webs, we detected a substantial lack of LC-PUFA in terrestrial leaves (submerged and fresh leaves) compared to high EPA contents in epilithon samples. High EPA levels found in biofilms are an important dietary resource supporting somatic growth and reproduction of aquatic invertebrates (Goedkoop et al., 2007; Lau et al., 2008). Conditioned terrestrial leaves contained only traces of EPA, perhaps because of associated microalgae but, similar to earlier studies (Torres-Ruiz et al., 2007; Guo et al., 2016) still provided food of higher nutritional quality than fresh leaves for invertebrates. Naturally occurring terrestrial vascular plants lack the enzymes necessary to synthesize EPA or other LC-PUFA and are difficult to digest due to their high lignocellulose content (Brett et al., 2017). Consequently, EPA retention from epilithic biofilms is more beneficial instead of energy-demanding internal bioconversion from precursors. Stream invertebrates have a limited innate ability to transform ALA to EPA ('trophic upgrading') and thus their PUFA composition mostly resembles dietary PUFA (Torres-Ruiz et al., 2007; Masclaux et al., 2012). The PUFA profiles of benthic invertebrates were more similar to epilithon rather than to leaves, confirming that invertebrates mainly depend on algae (Lau et al., 2009; Guo et al., 2017, 2018). As a result, benthic biofilms colonizing stream rocks with a high content of physiologically required EPA convey nutrients of

Table 4 Fatty acid content (% of total FAME) in brain and eye lipid classes of *S. trutta* and *O. mykiss* combined (mean ± 1 standard deviation)

Organ	Lipid class	14:0	16:0	18:0	18:1n-9	16:1n-7	ALA	SDA	LA	ARA	EPA	DHA
Brain	Neutral	22 ± 6	29 ± 5	24 ± 7	5 ± 3	2 ± 2	1 ± 2	0 ± 1	1 ± 1	0 ± 0	1 ± 2	3 ± 3
	Polar	3 ± 2	20 ± 2	9 ± 2	18 ± 3	2 ± 1	1 ± 0	0 ± 0	0 ± 0	1 ± 0	3 ± 4	23 ± 4
Eyes	Neutral	5 ± 3	19 ± 2	6 ± 3	12 ± 3	12 ± 2	10 ± 3	3 ± 2	5 ± 1	1 ± 0	2 ± 4	3 ± 0
	Polar	6 ± 4	23 ± 4	13 ± 4	12 ± 3	6 ± 2	3 ± 1	1 ± 0	2 ± 1	1 ± 0	2 ± 4	15 ± 4

14:0 = myristic acid, 16:0 = palmitic acid, 18:0 = stearic acid, 18:1n-9 = oleic acid, *ALA* alpha-linolenic acid, *SDA* stearidonic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *LA* linoleic acid, *ARA* arachidonic acid

higher dietary high-quality conducive for consumer growth, than does terrestrial vegetation.

There was an evident mismatch between dietary LC-PUFA (terrestrial leaves, epilithon and aquatic invertebrates) and riverine fish, as previously noted by Guo et al. (2017). Fish need long-chain PUFA for optimal somatic growth and reproduction (Bell & Sargent, 2003; Ahlgren et al., 2009). Several feeding experiments have shown negative effects of LC-PUFA deficient diets on the growth rates of fish (Murray et al., 2014) or metabolic rates in birds (Twining et al., 2016) that may be related to the metabolic costs associated with converting dietary precursor PUFA (e.g., ALA or LA) to LC-PUFA. In late fall, the input of terrestrial insects to streams plays only a minor role in salmonid diets (Nakano & Murakami, 2001). Consequently, in this study only benthic invertebrates offered an optimal source of algal n-3 PUFA, in particular EPA (Ghioni et al., 1996), for further conversion, at levels that are sufficient for proper functioning in freshwater fish, even though past experimental studies have claimed low conversion rates (Buzzi et al., 1996). Previous research indicated a substantial contribution of algal carbon to consumer biomass is by stable isotope data, ranging from small tropical headwater streams (Lau et al., 2009) to large arid rivers (Bunn et al., 2013). Furthermore, isotopic evidence suggests most of the nitrogen assimilated by stream consumers is of algal origin, even in systems where there appears to be a significant terrestrial carbon contribution (Brett et al., 2017; Thorp & Bowes, 2017).

The high PUFA similarity between liver and muscle suggests that livers retained or endogenously transformed dietary PUFA and allocated them to muscles (Martin et al., 1994). Higher DHA levels in salmonid than in European bullhead livers could be the result of higher conversion rates or greater incorporation of dietary DHA from prey fish. However, given the fact that salmonids in this study were small and piscivory is not the primary mode of feeding for this size class (Elliott, 1973; Behnke et al., 2002), the high similarity between liver PUFA of European bullhead and both salmonid taxa (*S. trutta* and *O. mykiss*) implies that both were equally able to retain ALA and LA and further minimize potential dietary deficiencies in long-chain PUFA by endogenous conversion.

The ANOSIM results indicate that the PUFA composition in organs of both salmonid taxa (*S. trutta* and *O. mykiss*) were more similar to each other than to European bullhead. Gladyshev et al. (2018) reported that phylogeny (order identity) outweighs food or habitat as factors in fish muscle FA. In line with this argument, we argue that species identity could explain the high PUFA similarities between all sampled organs of Rainbow and Brown trout. Despite similar brain DHA content among both fish groups, the brain, and eyes of European bullhead contained more ARA than salmonids. This is also indicated by the more similar vector scores of DHA and ARA in the ordination plots of European bullhead than salmonids. Both ARA and EPA are associated with eicosanoid synthesis (e.g., prostaglandins) and act as hormone precursors (Sargent et al., 1995). Increased ARA content in brains and eyes of European bullhead could be related to spawning activities (Stacey & Goetz, 1982) as suggested by the presence of egg-bearing females observed during our sampling. Higher reproductive activities in European bullhead compared to salmonids are likely to have resulted in higher PUFA allocation and could explain higher similarities among organ PUFA. Changes in the FA composition due to reproductive activities were reported for gonadal, muscle, and liver tissues (Nogueira et al., 2017; Keva et al., 2019), but impacts on neural tissues have not been assessed yet. Other factors related to foraging behavior, eye anatomy or visual capabilities (Elliott, 1973; Mills & Mann, 1983; Guthrie, 1986; Wagner, 1990) could lead to differences in PUFA composition in brain and eye between European bullhead and salmonids, but further studies investigating such functional differences are still warranted.

The high dissimilarity in PUFA of salmonid brain and eyes was unexpected, but largely accounted for by differences in the lipid classes between these neural organs with direct implications for their PUFA composition: higher resemblance of dietary FA in eyes than brains (Bell & Dick, 1991; Tocher, 1993; Buzzi et al., 1996). This is consistent with other studies which showed that dietary inputs can significantly affect eye lipids (Bell et al., 1995; Brodtkorb et al., 1997; Navarro et al., 1997). Eyes store dietary FA for reasons that are still not fully understood. For example, apart from being major energy store, NL in eyes may serve as reservoir for membrane FA synthesis (Linares & Henderson, 1991). Similar to Brodtkorb et al. (1997), brain NL were composed predominantly of SAFA and when required, these

could serve as energy source (De Roos, 1994) even if most energy is mobilized from glycogen in teleost brains (Soengas & Aldegunde, 2002). The high abundance of the monosaturated FA 18:1n-9 was reported in total lipids (Tocher & Harvie, 1988; Stoknes et al., 2004; Amlund et al., 2012) and the membrane lipids (Brodtkorb et al., 1997) of neural tissues (brain and eyes) of trout, salmon and marine fish species (Geurden et al., 1998). Farkas et al. (2000) suggested that high levels of 18:1n-9 and 22:6n-3 in fish membranes are required for proper fusion and signal transduction rates in cold-adapted animals. Although entire eyeballs were analyzed, the high similarity between PL of eyes and brain imply the provision of LC-PUFA to the retina may suffice even if basal diet sources in stream food webs are deficient in n-3 LC-PUFA. Such dietary shortage of n-3 LC-PUFA is, to varying degrees, compensated in stream fish via dietary PUFA conversion to target PUFA, such as DHA, that are required for neural organs. Consequently, the low dietary availability of DHA in the trophic base of these subalpine streams might not have impaired the visual acuity or proper functioning of sensory systems of fish.

Our large-scale field study provides further evidence that algal PUFA support fish in headwater stream habitats. Contrary to the implications of the River Continuum Concept, algae rather than terrestrial sources supplied aquatic vertebrates with high-quality biomolecules (e.g., PUFA) required for neurogenesis. Algal PUFA that were first incorporated by benthic invertebrates serve subsequently as precursors for critical PUFA required for fish organs, in particular for DHA in fish brains and eyes. Based on these data, it is not possible to differentiate whether DHA was allocated from livers to respective organs or if DHA production occurred locally, e.g., in brain astrocytes (Mourente et al., 1991; Bell et al., 2001; Tocher et al., 2006). Future efforts to elucidate trophic and allocation pathways of individual n-3 PUFA in fish organs and dietary sources require compound-specific stable isotope analyses of individual FA (Kühmayer et al., 2020).

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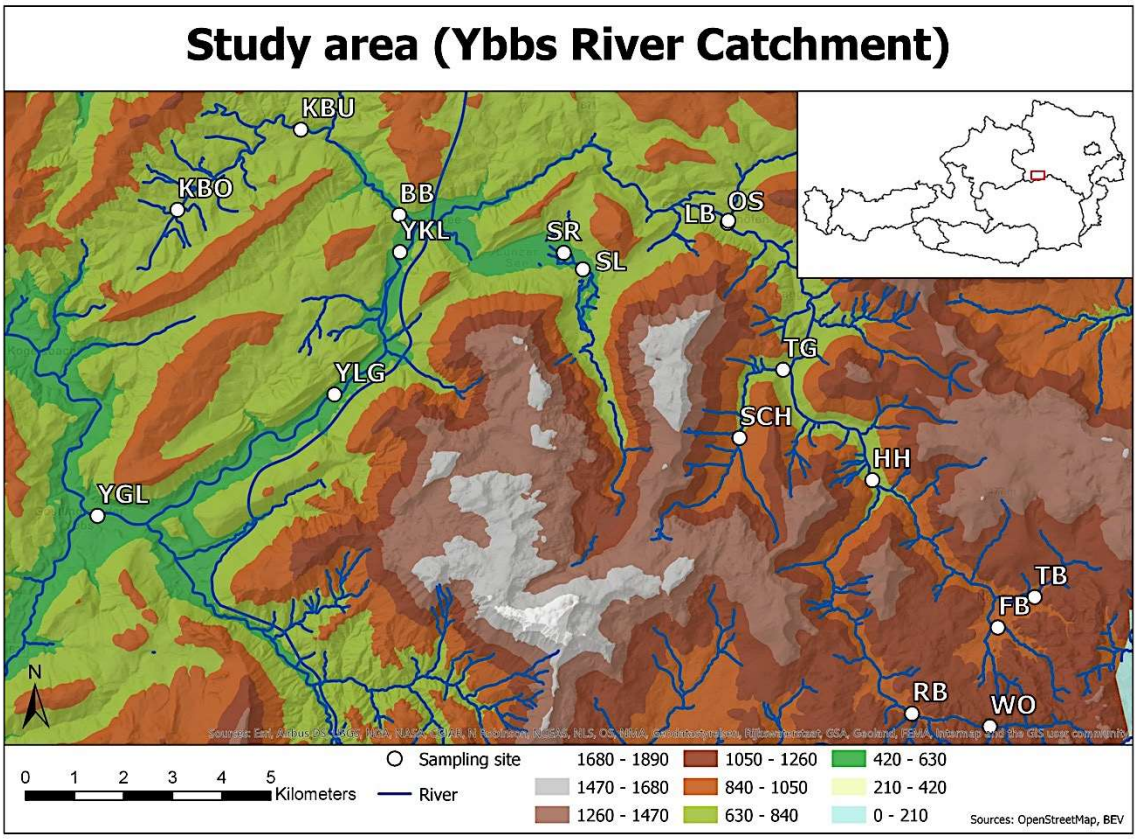
Supplementary data

Online resource 1.

Study area (Ybbs River catchment, 254km², 550- 1000 m.a.s.l) with 17 sampling sites (dots) at the 9 oligotrophic rivers (blue lines) of different stream orders (1-5). Bodingbach, BB (4), Faltlbach, FB (1), Kothbergbach oben, KBO (3), Kothbergbach unten, KBU (3); Lackenbach, LB (2); Oberer Seebach Lend, SL (2); Oberer Seebach Ritrodat, SR (2), Ois alte Säge, OS (4);Holzhüttenboden, HH (4); Schlechen, SCH (1); Tagles, TG (3); Taschlbach (1); Rehberghütte, RB (3), Weiße Ois, WO (3); Göstling Lagerhaus, YGL (5), Kläranlage Lunz, YKL (5), Lunz Großau, YLG (5).



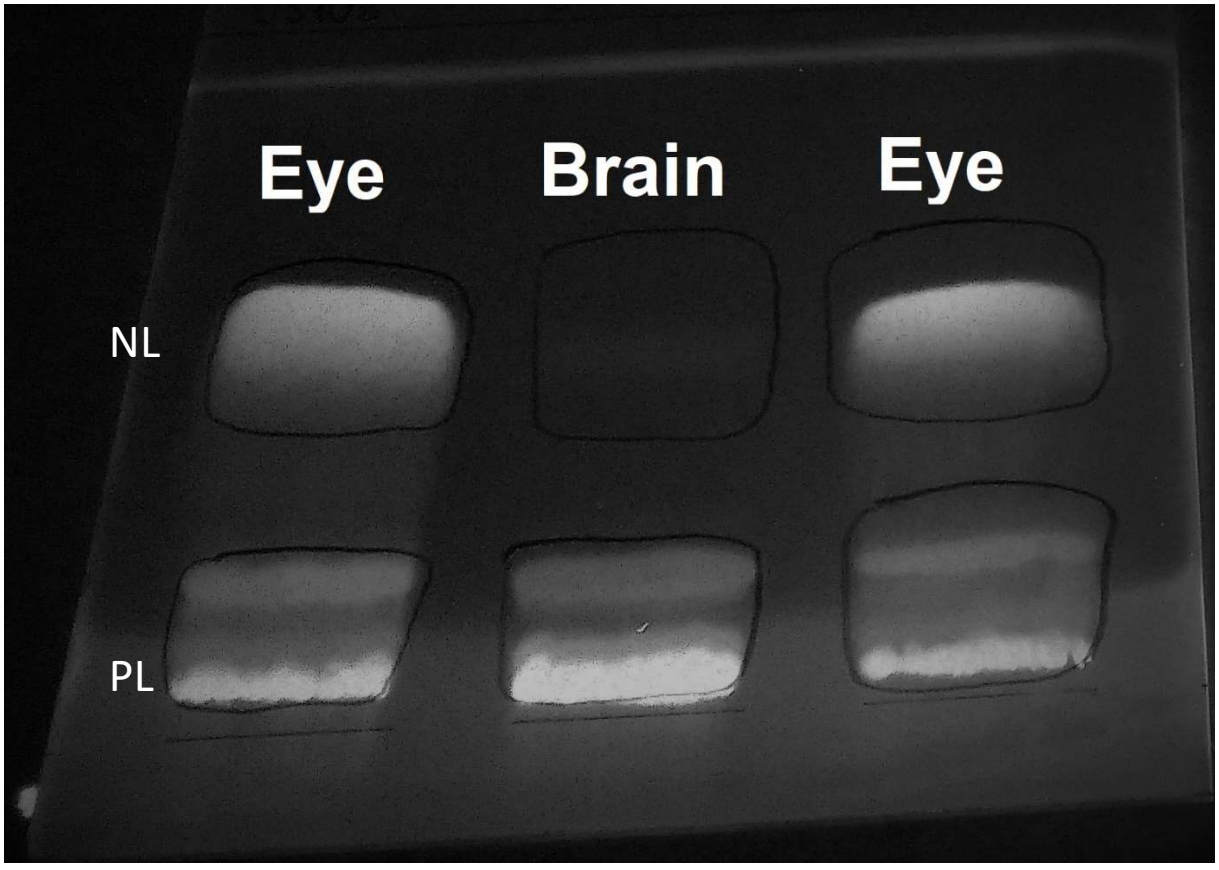
Online resource 2.

Mean body length (mm) and weight (g) of all three fish taxa (*Cottus gobio, Oncorhynchus mykiss, Salmo trutta fario*). n = sample size.

Taxa	n	Body length [mm]	Body weight [g]
Cottus gobio	64	90.9 ± 11.1	10.0 ± 4.1
Oncorhynchus mykiss	32	109.2 ± 35.2	17.5 ± 24.7
Salmo trutta fario	62	128.6 ± 49.5	28.9 ± 30.4

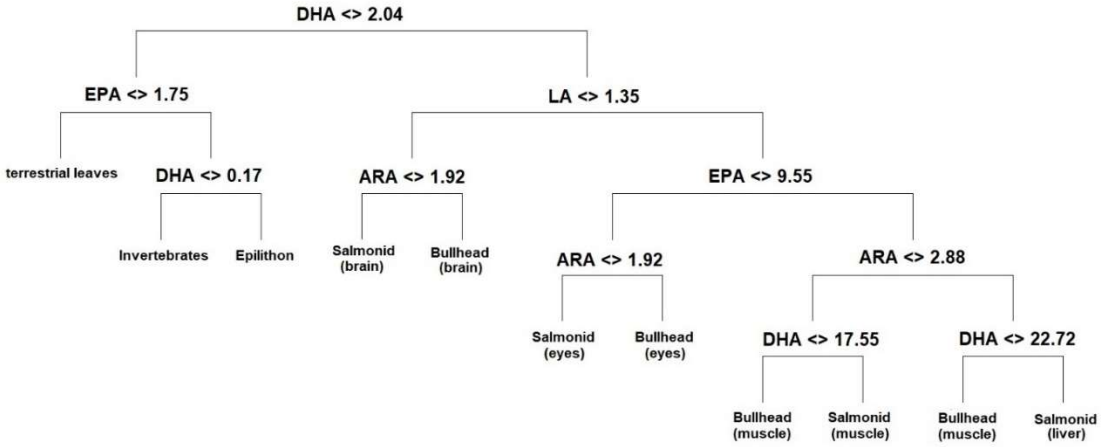
Online resource 3.

Lipid classes in salmonid brain and eyes after thin-layer-chromatography. Lipids in salmonid fish eyes contained neutral (NL) and polar (PL) lipids, brain lipids contained only polar lipids (PL).



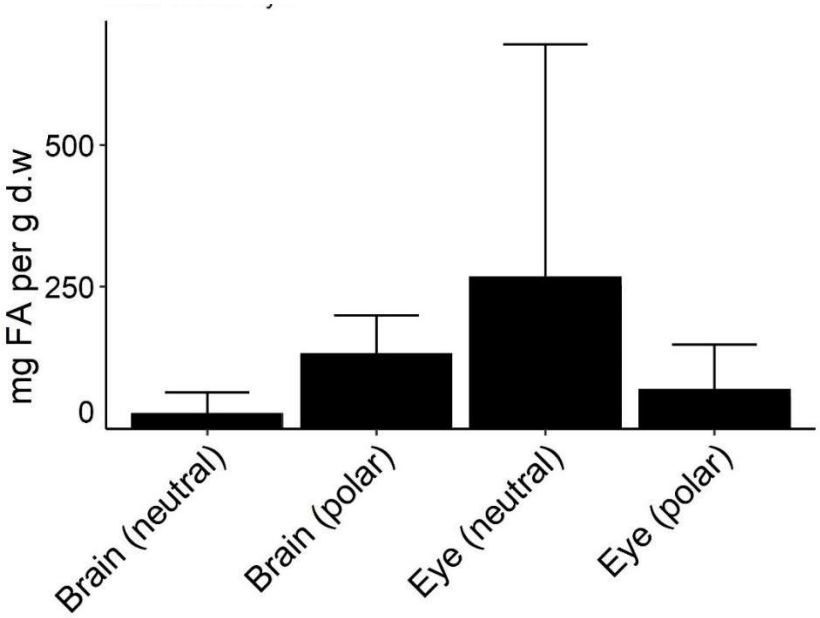
Online resource 4.

Regression tree for exploring patterns based on untransformed percentage data of individual fatty acids (ALA, SDA, LA, EPA, ARA, DHA) among food-web components (epilithon, Conditioned and fresh leaves, benthic invertebrates, salmonid and European Bullhead tissues). Misclassification was <30%.



Online resource 5.

Mean (± 1 standard deviation mg g d w-1) polar and neutral lipid content of salmonid brain and eyes (n=70).



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Basal resources of river food webs largely affect the fatty acid composition of freshwater fish

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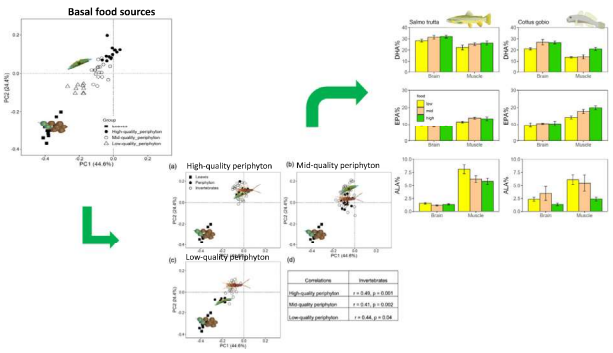
HIGHLIGHTS

- Little is known how changes in the nutritional quality at the base of freshwater food webs are reflected in fish.
- Periphyton was a high-quality food for all consumers that provides essential fatty acids to riverine fish.
- Changes in periphyton fatty acids across river ecosystems were tracked in fish prey macroinvertebrates.
- Reduced nutritional quality of periphyton reduced essential fatty acids in muscle tissues of bullhead and brown trout.
- Subtle human disturbances to streams can affect fish through changes to the nutritional quality of their basal food sources.

GRAPHICAL ABSTRACT



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ABSTRACT

1. Overfishing, altered flow regimes, loss of connectivity, pollution and other direct human disturbances have had a significant impact on freshwater fish biodiversity. While direct effects of these disturbances are well documented, some can also lead to changes in the nutritional composition at the base of freshwater food webs and may affect fish growth and reproduction. Thus far, little is known about how changes in the nutritional composition at the base of freshwater food webs are reflected in fish production and fatty acid composition.
2. We conducted a field study in subalpine rivers in Austria to examine how variations in the food quality of basal food sources affect the biochemical composition of freshwater fish. Basal food sources (terrestrial leaves and periphyton), the abundant fish taxa brown trout (*Salmo trutta*) and European bullhead (*Cottus gobio*) and their macroinvertebrate prey were collected and their stable carbon isotopes ($\delta^{13}C$) and fatty acid (FA) compositions were measured.
3. Our results show that periphyton was the major source of dietary carbon for fish and most invertebrates, except shredders, and a high-quality food for all consumers that provides long-chain polyunsaturated FA (LC-PUFA). In contrast, submerged leaves contained very low content of LC-PUFA and were low-quality food for consumers.
4. Changes in periphyton FA across these river ecosystems were tracked in macroinvertebrate FA profiles and in the FA composition of muscle tissues in both fish species. In contrast, the FA composition of fish brain remained unaltered across the study streams.

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5. The docosahexaenoic acid (DHA, 22:6ω3) content in muscle tissues of *S. trutta* remained stable and did not correspond to changes of periphyton FA, indicating strong regulation of DHA in this species which is independent of aquatic food sources. In contrast, muscle DHA and eicosapentaenoic acid (EPA, 20:5ω3) content of *C. gobio* significantly declined when periphyton quality decreased, reflecting the strong impact of the nutritional quality of periphyton on the FA composition of this bottom-dwelling fish.
6. Reduced nutritional quality of periphyton led to reduced LC-PUFA in muscle tissues of bottom-dwelling bullhead and even piscivorous brown trout, suggesting that changes of FA composition at the base of river food webs cannot be compensated by intermediate consumers, such as benthic invertebrates, that also convey dietary FA to fishes. This study highlights how subtle human disturbance to streams can affect freshwater fish species through changes to the nutritional quality of their basal food sources.

1. Introduction

Freshwater fish are an important component of aquatic biodiversity, help sustain the functioning of aquatic ecosystems, and contribute significantly to the nutrition, recreation and economies of humans (Dudgeon et al., 2006). However, fish food sources at the base of freshwater food webs are threatened by increasing human disturbances to freshwater ecosystems (Müller-Navarra et al., 2004), which decrease the transfer of required dietary biomolecules, in particular long-chain polyunsaturated fatty acids (LC-PUFA), to fish (Guo et al., 2021c; Taipale et al., 2018). The LC-PUFA docosahexaenoic acid (DHA, 22:6ω3), eicosapentaenoic acid (EPA, 20:5ω3) and arachidonic acid (ARA, 20:4ω6) are essential for fish growth, reproduction, behaviour, vision, osmoregulation, membrane fluidity (homeoviscous adaptation) and immune responses (Arts et al., 2009; Pilecky et al., 2021). However, many fish are unable, or have a limited ability to synthesize these essential compounds and must thus obtain them directly via their food sources. To date, it remains unclear how the retention of essential FA in fish is affected by food sources at the base of freshwater food webs.

The relative importance of basal food sources (i.e., terrestrial matter and aquatic algae) for freshwater fish and other consumers has been debated for decades. While earlier studies emphasized the importance of terrestrial matter due to their large inputs in some freshwater ecosystems (Cole et al., 2011; Solomon et al., 2008), recent evidence suggests that algae are the dominant carbon source for consumers (Bunn et al., 2003; Guo et al., 2018, 2021a; Lau et al., 2009), partly because of their higher LC-PUFA content (Brett et al., 2017). Algae rich in DHA, EPA and ARA, especially diatoms and cryptophytes, are considered high-quality food for consumers, and these high-quality algae enhance the energy transfer efficiencies from algae to higher trophic consumers (Lau et al., 2012; Müller-Navarra et al., 2004). Conversely, terrestrial organic matter and cyanobacteria generally lack LC-PUFA, and are considered low-quality food, with very low energy transfer efficiency to consumers (Müller-Navarra et al., 2004).

However, algal LC-PUFA composition is also sensitive to environmental changes (Guo et al., 2021b; Hill et al., 2011), varying across seasons and locations, which in turn affects the nutritional availability of invertebrates fed on by fish. Environmental conditions (e.g., nutrient, temperature, light and dissolved organic matter availability, etc.) can modify the species composition of periphyton communities and to a lesser extent the biochemical composition within algal taxa (Galloway and Winder, 2015). When feeding primarily on high-quality algae, invertebrate primary consumers become enriched in LC-PUFA and provide high-quality food for predators, including fish (Brett et al., 2017; Guo et al., 2018). In contrast, when experiencing low-quality food, such as leaves or cyanobacteria, the LC-PUFA content of primary consumers declines (Kühmayer et al., 2020; Müller-Navarra et al., 2004), resulting in low-quality food for their predators. Prior studies suggest that invertebrate predators selectively feed on and/or assimilate LC-PUFA from high-quality primary consumers, and can actively control their LC-PUFA in response to very low availability of high-quality food (Guo et al., 2018; Kühmayer et al., 2020). Yet, it is still controversial how fish are affected by variations in the nutritional quality of their invertebrate prey and basal food sources in freshwater ecosystems.

Fish LC-PUFA composition, an indicator often used to assess fish nutrition and health, is largely determined by the quality of their diets (Arts and Kohler, 2009). In aquaculture and laboratory feeding experiments, the incorporation of LC-PUFA into fish eyes, brains, livers, eggs and muscles reflected the availability of these FA in fish diets (Estévez et al., 1999; Tocher, 2010). In freshwater ecosystems, the FA profiles in the brains, eyes and muscles of brown trout (*Salmo trutta*), European bullhead (*Cottus gobio*) and rainbow trout (*Oncorhynchus mykiss*) were more similar to epilithon than to terrestrial leaves (Ebm et al., 2021). The FA profiles of a range of riverine fish species have been suggested to reveal the concurrent differences in the FA composition of primary producers (i.e., benthic algae and phytoplankton) longitudinally among reaches and laterally among floodplain habitats; e.g., Australian lungfish in the Brisbane River (Tao et al., 2020), bluegill across Illinois River reaches (Rude et al., 2016), channel catfish in the Kaskaskia River (Young et al., 2016), and sauger and white bass in the Ohio River (Dayhuff, 2004). However, a recent study conducted in Australian lowland rivers reported that, despite distinct differences in algal nutritional quality between floodplains and river channels, the FA profiles of the Eastern mosquitofish (*Gambusia holbrooki*) and their prey benthic zooplankton, pelagic zooplankton and the backswimmer (*Anisops thienemanni*) were similar between these habitats (McInerney et al., 2020). It remains unknown to what extent fish FA profiles are generally affected by varying dietary quality in freshwater ecosystems.

In addition to dietary supply, the FA composition of fish is also affected by internal conversion of LC-PUFA, particularly DHA. Compared with other LC-PUFA, DHA is more important in maintaining membrane fluidity in fish (Niebylski and Salem Jr., 1994), and strongly associated with fish neural tissues, playing an important role in brain development and vision (Farkas et al., 2000; Pilecky et al., 2021; Tocher, 2015). While marine fish generally obtain DHA directly from their food sources, freshwater fish, especially riverine fish, inhabit an environment that is very low in dietary DHA. DHA is generally absent in riverine basal food sources, i.e., leaves and algae (Guo et al., 2018). The precursors for fish to synthesize DHA include EPA, which is abundant in high-food quality algae and invertebrates, and alpha-linolenic acid (ALA, 18:3ω3), which is abundant in leaves and low-quality algae. Fish may use dietary EPA and ALA to synthesize DHA for physiological requirements. For example, when experiencing low-quality food, Arctic charr (*Salvelinus alpinus*) can convert ALA to DHA (Murray et al., 2014). Internal conversion from ALA to DHA is more energy intensive and less efficient than directly from EPA to DHA, but which precursor is used by fish to synthesize DHA probably depends on dietary availability. However, little is known about how dietary supply of EPA and ALA from the base of food webs affects the endogenous FA conversion in freshwater fish.

Additionally, in freshwater ecosystems, most previous field studies measured the FA composition of fish muscle tissues, with few considering the FA content of other organs, such as brain, but see Ebm et al. (2021), and Makhutova and Stoyanov (2021). Fish brain are rich in DHA (Nieminen et al., 2014), which can also be synthesized in vivo (Mourente and Tocher, 1998). While brain FA in marine fish vary with dietary supply (Mourente, 2003), it is not clear how brain FA in freshwater fish would

be affected by dietary FA, especially from food sources at the base of freshwater food webs.

We conducted a field investigation in subalpine rivers of Austria to examine how the dietary quality of basal food sources affects the FA composition of fish. Basal food sources of these streams, i.e., terrestrial leaf litter and periphyton, and common fish taxa and their potential prey macroinvertebrates were collected and their stable isotopes of carbon ($\delta^{13}C$) and FA composition were analysed. We hypothesized that: (1) periphyton is the main carbon source for riverine invertebrates and fish; (2) regardless of variations in periphyton food quality, invertebrates always selectively retain LC-PUFA from high-quality periphyton; (3) the LC-PUFA content in fish muscle tissues also vary with periphyton PUFA, but the PUFA composition of fish brain remains constant, and; (4) under low quality periphyton conditions, freshwater fish may be capable of synthesizing DHA from EPA and ALA in their invertebrate prey.

2. Methods

2.1. Study catchment

We conducted this study in the subalpine River Ybbs catchment, Austria (47°45′N, 15°12′E). This catchment has a drainage area 254 km^2, and the main geology is dolomite and karst. The climate is temperate with distinct seasons, but evenly distributed precipitation over the year. The dominant land use is forestry, with very few alpine meadows and agricultural areas (Guo et al., 2018). We selected 15 stream sites in the upper catchment of the *Weiße Ois*. Brown trout (*Salmo trutta*) and European bullhead (*Cottus gobio*) are the most abundant fish taxa in the catchment (Ebm et al., 2021).

2.2. Field collection

Fish, macroinvertebrates, periphyton and submerged leaves were collected from 15 streams in October 2016. Fish were collected by electrofishing, anesthetized and killed immediately after collection according to the Federal Act on the Protection of Animals, Austria (http://www.ris.bka.gv.at). The total length (mm) of each individual fish was measured. Fish with sufficient brain and muscle materials for FA and SI analysis were selected. In total, 38 individuals of *Salmo trutta* were sampled, with a body length ranging from 69 to 237 mm, while 22 individuals of *Cottus gobio* were used, with a body length from 70 to 124 mm.

Macroinvertebrates and periphyton were sampled in three 1.5 m ∗ 1.5 m quadrats along a 20-m reach at each stream, with one paired replicate from each quadrat. Within each quadrat, rocks were randomly collected, and macroinvertebrates clinging to the rocks were washed into a white tray and identified to Order or genus when possible. The same rocks were scraped with brushes to collect periphyton. Submerged leaves were hand-picked from each stream.

All samples were placed in zip-lock plastic bags, kept in a cool and dark portable freezer in the field, and brought to the laboratory within 4 h of collection. All samples were placed in a −80 °C freezer until further processing.

2.3. Lab analysis

The dorsal muscle tissue and brain of each individual fish were extracted immediately for stable isotope (SI) and FA analyses. Macroinvertebrates were assigned to functional feeding groups (FFG) (Cummins and Klug, 1979). The main grazers found in our 15 streams were *Baetis* sp., and *Ecdyonurus* sp., and shredders included *Leuctra* sp., *Nemoura* sp., *Potamophylax* sp., and *Allogamus* sp. The only filterer collected was *Hydropsyche* sp. Predators mainly included *Perla* sp., *Plectrocnemia* sp., *Perlodes* sp., *Rhyacophila* sp., and *Isoperla* sp.

Samples of fish brain and muscle tissues, macroinvertebrates, periphyton and submerged leaves were freeze-dried (Virtis Genesis Freeze Dryer). After freeze-drying, each sample of fish brain and muscle, invertebrates and periphyton was homogenized with a glass rod, whereas leaf samples were ground to powder and homogenized with a food processor. Additionally, for SI analysis, periphyton samples were fumigated with HCl (37%) to remove carbonates (Komada et al., 2008), then freeze-dried to constant weight and homogenized to powder with a glass rod.

Around 2 mg (dry mass) of each plant sample (periphyton or leaves) and 1 mg (dry mass) of each animal sample (macroinvertebrates, fish brain or muscle tissue) were weighed into small tin capsules. Carbon isotopes were measured with an isotope ratio mass spectrometer (IRMS; Delta V Adv MS, Thermo Fisher Scientific™). All stable isotope values were expressed as the relative per mil (‰) differences between the sample and a conventional standard (Vienna Pee Dee Belemnite carbonate) and reported as $\delta^{13}C = (R_{sample} / R_{standard} - 1) \times 1000$ (‰); where $R = {}^{13}C/{}^{12}C$.

Sample weights for lipid extraction were 5–6 mg (dry mass) for each fish brain sample, 15 mg for each fish muscle sample, 7 mg for each macroinvertebrate sample, 10 mg for each periphyton sample, and 30 mg for each leaf sample. Lipids were extracted and methylated according to the methods described in Guo et al. (2016b). Fatty acid methyl esters (FAME) were analysed using a gas chromatograph (Thermo Trace; FID 260 °C, Carrier gas: He: 1 ml/min, Detector gases: H_2: 40 mL/min, N_2: 45 mL/min, air: 450 mL/min, temperature ramp: 140 °C (5 min)–4 °C/min–240 °C (20 min) = 50 min) equipped with a temperature-programmable injector and an autosampler. A Supelco™ SP-2560 column (100 m, 25 mm i.d., 0.2 μm film thickness) was used to separate FAME, and the standards 37-component FAME mix (Supelco 47885-U) and Bacterial Acid Methyl Ester Mix (Supelco 47080-U) were used to identify each FAME. FAME were quantified with reference to seven-point calibration curves based on known standard concentrations. FA compositions were expressed as percentages relative to total FA (FA%).

2.4. Data analysis

The regression slope of consumer $\delta^{13}C$ vs periphyton $\delta^{13}C$ was used as an indicator of the reliance of each consumer group (invertebrate grazers, shredders, filterers and predators, and the fish *Salmo trutta* and *Cottus gobio*) on local resources (Bunn et al., 2013; Rasmussen, 2010). The strength and significance (R^2 and p values) of regressions between source and consumer $\delta^{13}C$ were firstly tested by ordinary least squares regression (OSL), and then the slope estimates were examined by reduced major axis regression (RMA). RMA was applied here, since $\delta^{13}C$ values of basal food sources and all consumer groups were measured with error and, as such, OLS could underestimate the slope of linear regressions. Slope = 1 suggests that the consumer organic C was entirely from that of the explanatory variable in the regression (e.g., periphyton), whereas 0 < slope < 1 indicates the explanatory resource contributed in part to the consumer organic C, and slope = 0 suggests that no contribution from the explanatory resource to consumer organic C (Jardine et al., 2012).

To better distinguish the nutritional differences between basal food sources, i.e., periphyton and submerged leaves, two types of principal component analysis (PCA) were conducted. One included DHA and the other excluded DHA, since previous studies found that DHA is only abundant in fish, but deficient in riverine invertebrates, periphyton and submerged leaves (Guo et al., 2018). The significance of the resulting eigenvalues was tested using Monte Carlo randomization with 1000 permutations.

Dissimilarity in the FA composition between periphyton and submerged leaves was further examined by permutational multivariate analysis of variance (PERMANOVA) and the test of multivariate homogeneity of group dispersions (PERMDISP) (Anderson and Walsh, 2013). PERMANOVA was run with taxa (periphyton and submerged leaves) as the fixed factor and sites as the blocking term. Then PERMDISP was applied to test if the effect of taxa on FA profiles was confounded by differences in dispersion. Furthermore, spatial variation in periphyton FA composition was plotted as the individual samples of periphyton on the first two axes (PC1 and PC2) from the PCA, and periphyton was then assigned to different nutritional groups.

Variations in invertebrate FA in response to each periphyton nutritional group were first plotted as individual samples of invertebrates and periphyton on the first two axes (PC1 and PC2) from the PCA. Then Procrustes

analysis was performed to assess the overall degree of association in the FA profiles between each periphyton nutritional group and its corresponding invertebrates. Procrustes analysis is known as the analysis of congruence between two multivariate data sets (Peres-Neto and Jackson, 2001), and produces an m^2-statistic that can be transformed into an r-statistic (r = square root of (1-m^2)), indicating the match between two ordinations (Peres-Neto and Jackson, 2001).

Variations in the FA profiles of fish brain and muscle tissues in response to periphyton nutritional groups were examined by two steps: (1) to estimate the correlations between fish body length and the FA content in brain and muscle tissues for two fish taxa, respectively; (2) to examine variations in the FA content of brain and muscles in response to each periphyton nutritional group by linear mixed-effect models. The protocol for linear mixed-effect model establishment, fit and validation followed Zuur et al. (2009) and Guo et al. (2016a). In our study, the response variables were individual FA% in fish brain or muscle tissue, with periphyton nutritional groups (low, mid, and high) as the fixed factor, and fish individuals (i.e., 38 individuals of *Salmo trutta* and 22 individuals of *Cottus gobio*) as the random factors. Three models were defined: a linear model using generalized least squares (without random effect) to investigate the fixed factor effects (M1), and two mixed-effect models, namely a random intercept model (with fish individual as random factors) (M2) and a random intercept and slope model (with periphyton FA groups as the fixed factor and fish individual as the random factor) (M3). Model fit was evaluated by restricted maximum likelihood estimation, and model selection was conducted by the likelihood ratio test. Post-hoc multi-comparison among groups was applied when the fixed factor (i.e., periphyton nutritional groups) showed a significant effect on the response variable (e.g., DHA in fish brain).

All FA (%) data were arcsine-square-root-transformed for normal distribution before analysis (Kelly and Scheibling, 2012). Eight FA groups were used for data analyses, i.e., DHA, EPA, ALA, ARA, linoleic acid (LIN, 18:2ω6), sum of bacterial FA (BAFA), sum of monounsaturated FA (MUFA) and sum of saturated FA (SAFA). These FA groups represented essential FA and important FA functional groups in riverine fish, macroinvertebrates and basal food sources (Guo et al., 2016b). All statistical analyses were conducted in the statistical software R version 4.0.2 (R Core Team, 2020), using the extension package *vegan* for PCA, PERMANOVA, PERMDISP and Procrustes analysis (Oksanen et al., 2013), *lme4* for linear mixed-effect models (Bates et al., 2014) and *multcomp* for post-hoc multi-comparison (Hothorn et al., 2014).

3. Results

3.1. The contribution of basal food sources to invertebrates and fish

Results of the carbon isotope regressions indicated that invertebrates depended on periphyton, with the exception of shredders (Table 1). The strength of these significant relationships was higher for grazers (R^2 = 0.70) than for filterers (R^2 = 0.57) and predators (R^2 = 0.59). The high slope estimates of these regressions ranged between 0.8 for filterers, 0.9 for grazers, and 1.0 for predators. These relationships suggest that these FFGs derive most of their biomass from periphyton. Periphyton was also the most likely basal resource for the two fish taxa (Table 1), with the slope estimates of 0.8 for *Salmo trutta* and 0.7 for *Cottus gobio*. The strength of these significant relationships (R^2) was 0.47 for *Salmo trutta* and 0.43 for *Cottus gobio*.

Table 1
Tests of the strength (R^2) and significance (p) of association between periphyton and consumer $\delta^{13}C$ and their slope estimates and 95% CIs, for the data collected from 15 subalpine streams in Austria, 2016.

Consumers	n	R^2	*p*	Slope estimates	95% CI	Dominant carbon sources
Invertebrates						
Grazers	89	0.70	<0.001	0.9	0.6–1.3	Periphyton
Shredders	16	0.25	0.14			Unresolved
Filterers	23	0.57	0.01	0.8	0.3–2.1	Periphyton
Predators	61	0.59	<0.001	1.0	0.6–1.7	Periphyton
Fish						
Salmo trutta	57	0.47	0.004	0.8	0.4–1.6	Periphyton
Cottus gobio	63	0.43	0.01	0.7	0.2–1.6	Periphyton

3.2. Nutritional quality of basal food sources

The results of the two PCA differed in their ability to distinguish differences between basal food sources, i.e., leaves and periphyton (Fig. 1). The PCA including DHA (Fig. 1a) showed a better ability to separate fish from all other organisms, with PC1 accounting for 72.2% of the total FA variation and strongly correlated with DHA (correlation coefficient: $r = 0.995$). PC2 explained only 12.4% of the total FA variation and was strongly associated with EPA ($r = 0.882$), separating leaves from all other organisms, i.e., periphyton, invertebrates and fish. In contrast, for the PCA excluding DHA (Fig. 1b), PC1 and PC2 clearly separated leaves from all aquatic organisms. PC1 accounted for 44.6% of the total FA variation and negatively correlated with ALA ($r = -0.951$), with leaves containing more ALA than all aquatic organisms. PC2 explained 24.4% of the total FA variation and was strongly associated with EPA ($r = 0.887$), with aquatic organisms containing much more EPA than leaves. Results of the PCA excluding DHA distinguished leaves and periphyton from both PC1 and PC2, and thus were used for further analyses.

Consistent with results of PCA, results of PERMANOVA indicated that the FA profiles of periphyton and leaves were significantly different from each other (F-value = 109.58, $p < 0.001$). Despite that, the result of PERMDISP was significant (F-value = 5.41, $p = 0.02$) and two completely separated groups of FA were found, suggesting that the differences in the FA profiles of periphyton and leaves were not confounded by sampling sites.

Clear spatial variation in periphyton EPA and ALA content was observed (Fig. 2). Along the PC1 from left to right, periphyton ALA% gradually decreased, whereas along PC2 from bottom to top, periphyton EPA% gradually increased. Based on this, periphyton from the 15 sampling sites was classified into three nutritional groups: low-quality periphyton (high in ALA%, but low EPA%), medium-quality periphyton (medium levels of ALA% and EPA%), and high-quality periphyton (high in EPA%, but low ALA%).

3.3. Variation in invertebrate FA in response to periphyton nutritional groups

Results of Procrustes analysis show significant positive correlations of overall FA profiles between each periphyton nutritional group and its corresponding invertebrates (Fig. 3) (High-quality periphyton vs invertebrates: $p = 0.001$, $r = 0.49$; Mid-quality periphyton vs invertebrates: $p = 0.002$, $r = 0.41$; Low-quality periphyton vs invertebrates: $p = 0.04$, $r = 0.44$). Regardless of changes in periphyton FA (Fig. 3, Table 2), invertebrates always selectively tracked periphyton FA, and contained a high proportion of EPA (15.84 ± 5.71%), and similar ALA content (10.74 ± 3.44%) with periphyton.

3.4. Variations in fish FA in response to periphyton nutritional groups

Both brain FA and muscle FA of the two fish taxa were characterised by a very high content of DHA, but less LIN and ALA compared with periphyton, leaves, and invertebrates (Fig. 1; Table 2). In addition, fish body length was not correlated with most FA in brain and muscle tissues of the two study fish taxa (Table 3).

The FA proportions in fish brain and muscle tissues showed contrasting patterns in response to different periphyton groups (Figs. 4 and 5). The contents of DHA, EPA and ALA remained constant in fish brain (Fig. 4) under different periphyton FA profiles but showed significant variations in muscle tissues. For *Salmo trutta*, the muscle DHA did not change across periphyton groups, but muscle EPA% was significantly lower under low-quality than

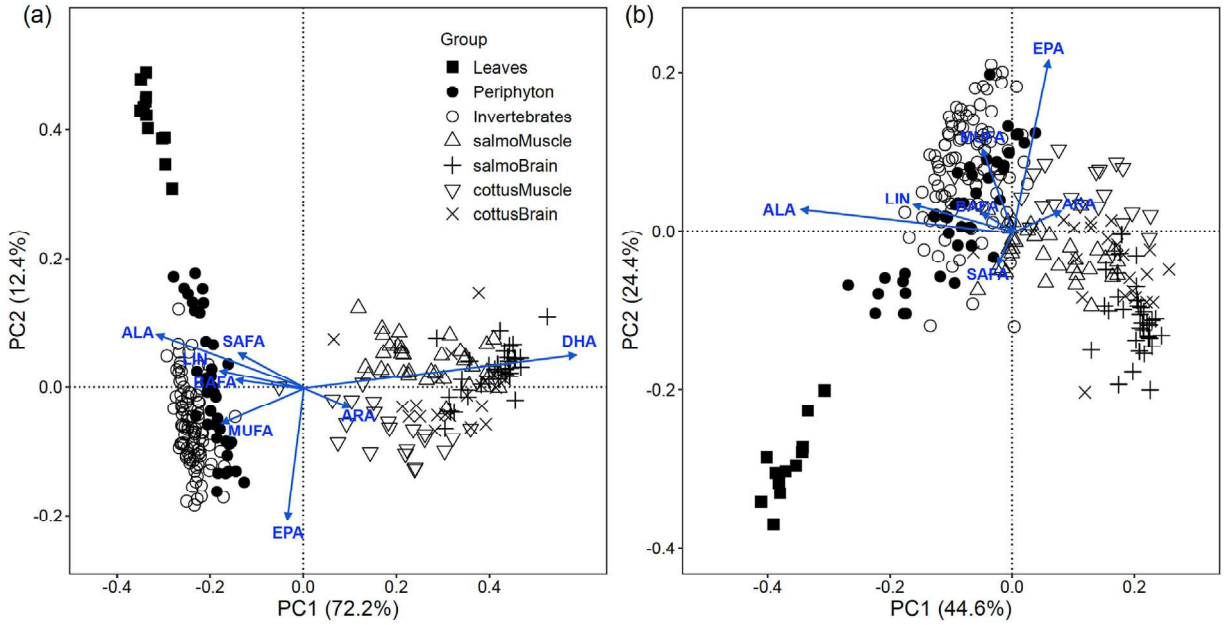

Fig. 1. Principal component analysis (PCA) on all fatty acid samples of fish, invertebrates, periphyton and submerged leaves from 15 study streams in the Ybbs catchment, Austria. (a) Results of PCA based on all identified FA; (b) Results of PCA based on FA profiles excluding DHA. *Salmo* Muscle: muscle tissues of *Salmo trutta*; *Salmo* Brain: brain tissues of *Salmo trutta*; *Cottus* Muscle: muscle tissues of *Cottus gobio*; *Cottus* Brain: brain tissues of *Cottus gobio*.

mid-quality periphyton (F-value = 3.64, p = 0.04). Trout muscle ALA% was significantly higher under low-quality than mid- and high-quality periphyton (F-value = 4.69, p = 0.03). For *Cottus gobio*, both muscle DHA % and EPA% were significantly lower under low-quality than high-quality periphyton (DHA: F-value = 6.94, p = 0.006; EPA: F-value = 6.22, p = 0.009), whereas muscle ALA% was significantly higher under low-quality than high-quality periphyton (F-value = 3.74, p = 0.04).

The contents of brain ARA, LIN and BAFA in both fish taxa did not change under different periphyton nutritional quality (Fig. 5). For *Salmo trutta*, muscle ARA% was significantly lower under low-quality than mid-quality periphyton (F-value = 5.96, p = 0.006), and muscle SAFA% was significantly higher under low- and mid-quality than under high-quality periphyton (F-value = 5.46, p = 0.008). For *Cottus gobio*, brain SAFA was significantly lower under low-quality than high-quality periphyton (F-value = 3.92, p = 0.04). Their muscle LIN% and MUFA% were significantly higher in the low-quality than in high-quality periphyton (LIN: F-value = 4.54, p = 0.03; MUFA: F-value = 5.53, p = 0.01).

4. Discussion

This field study demonstrates that fish FA composition is strongly associated with the dietary quality of periphyton at the base of river food webs. Our results complement previous findings that periphyton is not only the source of bulk carbon for fish and invertebrates (Bunn et al., 2003;

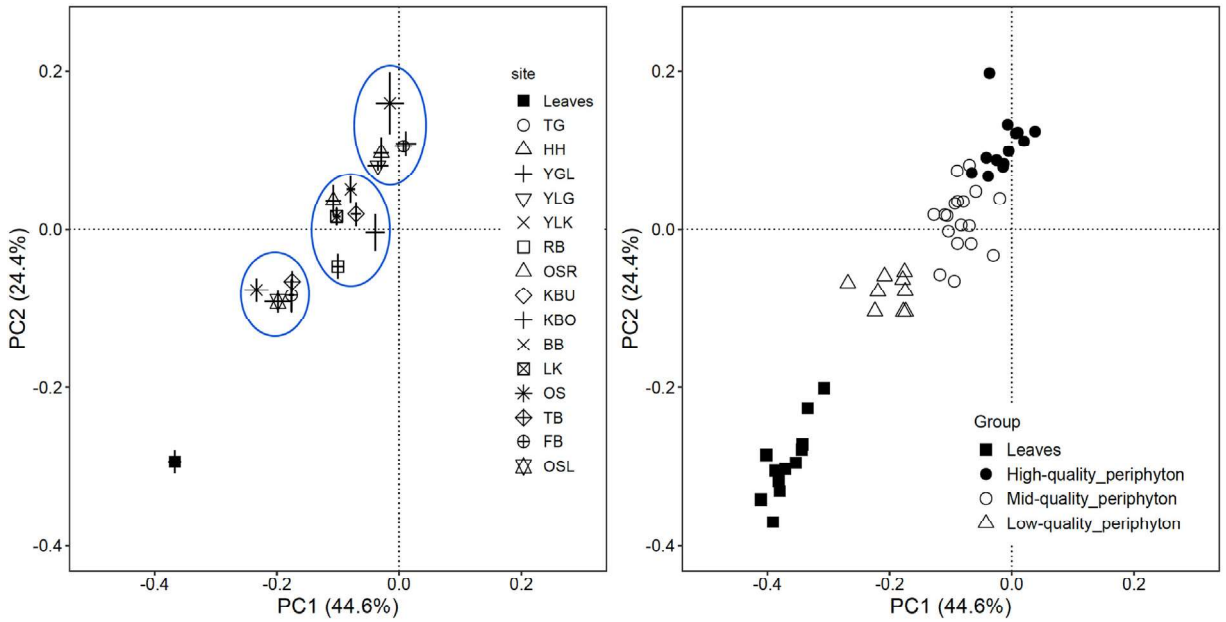

Fig. 2. Position of each study stream (centroid ± se) and replicates of periphyton on the first two axes (PC1 and PC2) from the principal component analysis (PCA) on all identified fatty acid of fish, invertebrates, periphyton and submerged leaves from 15 study streams, Austria. (a) 15 sampling streams; (b) all replicates of periphyton and submerged leaves.

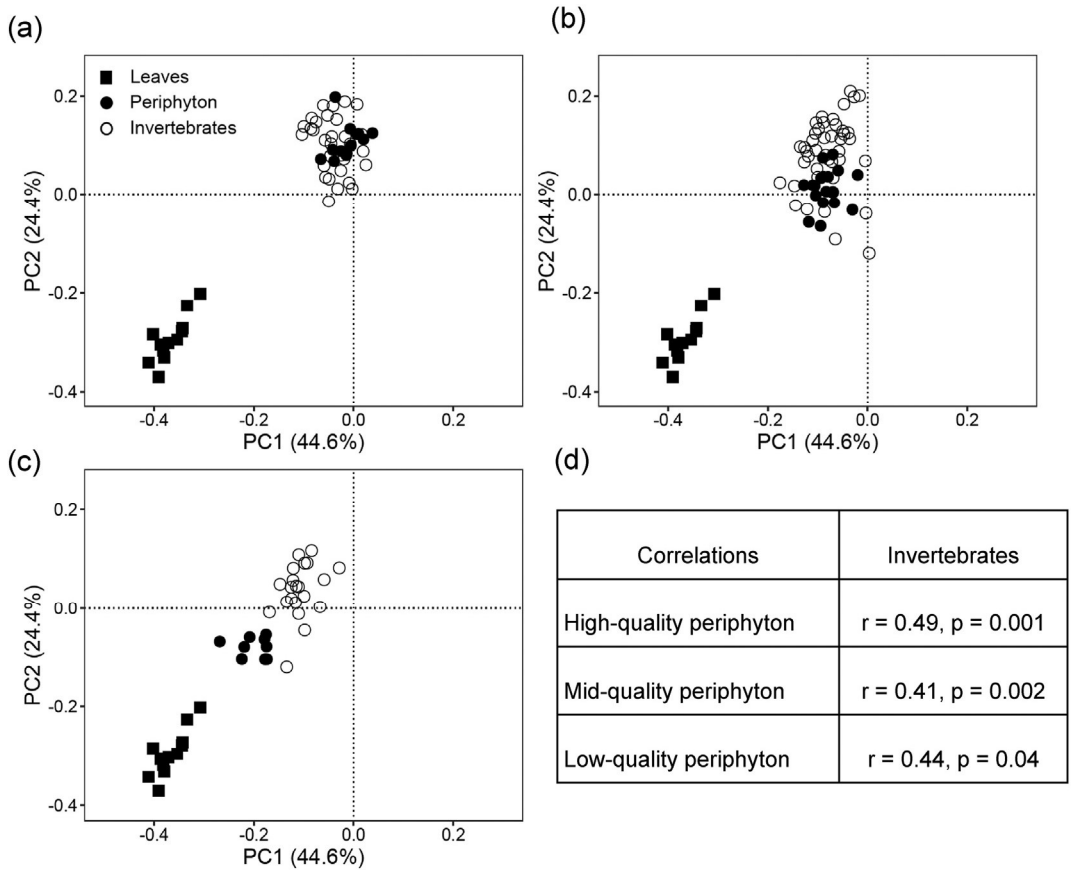

Fig. 3. Variations in invertebrate fatty acid content across three periphyton nutritional quality groups. (a) High-quality periphyton group; (b) Mid-quality periphyton group; (c) Low-quality periphyton group; (d) Correlation coefficients and associated levels of significance for relationships of fatty acid compositions between each periphyton nutritional group and corresponding invertebrates.

Table 2
FA compositions (% relative to total FAs, mean ± sd) of fish brain and muscle tissues, invertebrates, periphyton and leaf samples from 15 study streams in the Ybbs catchment, Austria.

Periphyton nutritional groups	Taxa/feeding groups	LIN	ALA	ARA	EPA	DHA	BAFA	MUFA	SAFA
High	Periphyton	7.21 ± 1.36	5.61 ± 1.6	0.42 ± 0.19	17.9 ± 1.83	0.68 ± 0.19	8.19 ± 1.47	28.48 ± 2.7	28.96 ± 3.55
High	Grazers	2.54 ± 0.24	6.01 ± 1.9	0.53 ± 0.25	14 ± 2.13	0.01 ± 0.02	13.03 ± 1.25	31.46 ± 3.74	41.67 ± 4.62
High	Shredders	6.01 ± 2.15	6.33 ± 3.3	1.45 ± 1.19	16.28 ± 6.21	0.26 ± 0.13	8.81 ± 1.68	33.74 ± 5.95	30.67 ± 7.57
High	Filterers	5.37 ± 1.53	10.66 ± 0.79	1.84 ± 0.97	18.47 ± 0.38	0.57 ± 0.6	5.13 ± 1.7	23.56 ± 3.21	36.24 ± 1.42
High	Predators	5.52 ± 1.81	10.92 ± 2.22	1.15 ± 0.93	20.18 ± 4.48	0.1 ± 0.11	6.57 ± 1.3	29.01 ± 4.18	29.4 ± 5.13
High	*Salmo* Brain	0.53 ± 0.22	1.35 ± 0.38	1.3 ± 0.35	8.75 ± 0.91	31.94 ± 3.64	4.69 ± 0.37	16.83 ± 3.18	30.37 ± 4.9
High	*Salmo* Muscle	3.32 ± 1.31	5.76 ± 1.71	1.55 ± 0.33	13.43 ± 3.16	26.17 ± 5.25	5.42 ± 0.95	17.9 ± 6.14	23.77 ± 1.17
High	*Cottus* Brain	1 ± 0.59	1.36 ± 0.54	3.47 ± 0.74	10.3 ± 4.26	26.64 ± 3.16	5.05 ± 0.89	19.58 ± 1.8	31.46 ± 1.96
High	*Cottus* Muscle	2.25 ± 0.98	2.37 ± 0.88	3.56 ± 0.92	19.8 ± 2.89	20.89 ± 3.32	5.06 ± 0.83	16.02 ± 2.27	25.74 ± 2.16
Mid	Periphyton	6.97 ± 1.47	8.69 ± 1.42	0.98 ± 0.56	11.54 ± 2.94	0.46 ± 0.25	10.95 ± 2.4	26.52 ± 2.35	34.84 ± 4.09
Mid	Grazers	3.49 ± 0.89	11.13 ± 3.37	1.05 ± 0.77	18.04 ± 6.17	0.04 ± 0.04	13.18 ± 1.06	29.11 ± 3.46	32.51 ± 5.35
Mid	Shredders	10.81 ± 1.95	9.92 ± 3.79	2.35 ± 1.13	8.74 ± 1.76	0.13 ± 0.12	8.07 ± 4.74	32.27 ± 9.05	31.37 ± 9.08
Mid	Filterers	5.01 ± 2.61	8.35 ± 3.8	0.75 ± 0.44	8.3 ± 2.9	0.03 ± 0.03	8.23 ± 1.24	29.59 ± 3.43	43.57 ± 6.83
Mid	Predators	7.27 ± 2.03	11.4 ± 1.96	1.92 ± 1.05	18.53 ± 3.8	0.13 ± 0.18	7.73 ± 2.55	28.44 ± 5.23	28.27 ± 3.49
Mid	*Salmo* Brain	0.5 ± 0.19	1.18 ± 0.32	1.49 ± 0.32	8.56 ± 1.47	31.37 ± 6.18	4.74 ± 0.92	15.67 ± 2.64	33.77 ± 5.52
Mid	*Salmo* Muscle	3.08 ± 1.28	6.22 ± 2.49	2.2 ± 0.89	13.82 ± 2.34	25.26 ± 4.87	5.85 ± 1.82	15.77 ± 4.44	26.05 ± 1.93
Mid	*Cottus* Brain	1.58 ± 1.04	3.45 ± 3.74	3.9 ± 1.43	10.47 ± 1.07	26.85 ± 6.83	6.11 ± 1.51	17.02 ± 2.45	30.62 ± 1.17
Mid	*Cottus* Muscle	3.49 ± 1.44	5.45 ± 4.4	3.87 ± 1.41	17.71 ± 3.52	13.95 ± 4.85	6.9 ± 1.5	21.55 ± 4.98	24.42 ± 2.39
Low	Periphyton	9.88 ± 3.42	14.54 ± 1.4	0.74 ± 0.38	5.95 ± 1.43	0.48 ± 0.18	12.84 ± 1.96	23.42 ± 2.23	34.92 ± 3.01
Low	Grazers	3.2 ± 0.5	14.23 ± 3.08	0.77 ± 0.45	15.27 ± 1.81	0.03 ± 0.02	12.53 ± 1.23	27.85 ± 4.46	33.68 ± 3.2
Low	Shredders	8.28 ± 2.54	11.92 ± 4.47	1.71 ± 0.45	9.99 ± 5.97	0 ± 0	6.78 ± 3.5	27.36 ± 5.11	36.65 ± 5.09
Low	Filterers	4.51	12.86	1.08	24.05	0.03	3.71	19.14	36.39
Low	Predators	5.85 ± 0.89	13.89 ± 3.08	0.9 ± 0.48	13.57 ± 3.15	0.07 ± 0.03	6.51 ± 1.99	28.93 ± 4.9	33.02 ± 4.69
Low	*Salmo* Brain	0.54 ± 0.18	1.56 ± 0.51	1.24 ± 0.18	8.75 ± 1.08	28.37 ± 4.07	4.65 ± 0.44	21.67 ± 7.44	29.84 ± 3.4
Low	*Salmo* Muscle	3.09 ± 0.95	8.11 ± 2.94	1.46 ± 0.29	11.48 ± 1.46	22.26 ± 7.23	6.26 ± 1.83	18.5 ± 5.42	25.8 ± 1.52
Low	*Cottus* Brain	1.93 ± 0.47	2.33 ± 1.04	4.14 ± 0.14	9.54 ± 3.95	21.02 ± 2.46	6.27 ± 1.17	29 ± 6.42	25.54 ± 2.5
Low	*Cottus* Muscle	4.15 ± 1.03	6.09 ± 2.5	4.94 ± 1.31	14.21 ± 2.3	13.57 ± 1.71	8.62 ± 1.09	21.7 ± 2.4	24.35 ± 1.38

LIN, linoleic acid (18:2ω6); ALA, α-linolenic acid (18:3ω3); ARA, arachidonic acid (20:4ω6); EPA, eicosapentaenoic acid (20:5ω-3); DHA, docosahexaenoic acid (22:6ω-3); BAFA, bacterial fatty acids; MUFA, sum of monounsaturated fatty acids; SAFA, sum of saturated fatty acids; *Salmo* Muscle: muscle tissues of *Salmo trutta*; *Salmo* Brain: brain tissues of *Salmo trutta*; *Cottus* Muscle: muscle tissues of *Cottus gobio*; *Cottus* Brain: brain tissues of *Cottus gobio*.

Table 3
Correlation coefficients and associated levels of significance for relationships between fish body length (mm) and their fatty acid compositions in brain and muscle tissues.

	Salmo trutta (n = 38)		*Cottus gobio* (n = 22)	
	Body length vs Muscle FA	Body length vs Brain FA	Body length vs Muscle FA	Body length vs Brain FA
LIN	0.19	0.23	0.07	0.07
ALA	−0.29	0.08	0.25	0.25
ARA	0.13	0.45**	0.14	0.14
EPA	0.00	0.30	0.25	0.25
DHA	−0.04	−0.2	−0.07	−0.07
BAFA	−0.21	0.11	−0.56**	−0.03
MUFA	0.29	0.22	−0.06	−0.45*
SAFA	−0.34	−0.14	−0.1	0.47*

n = sample size.
** $p < 0.01$.
* $p < 0.05$.

Jardine et al., 2012; Lau et al., 2009), but also supplies and predicts the LC-PUFA content of aquatic consumers (Brett et al., 2017; Guo et al., 2016b). Furthermore, our data provide new field evidence that changes in the FA composition of periphyton in river food webs can be transferred to fish via invertebrates and highlight primary producer-dependent variations in fish FA.

Food sources at the base of river food webs showed distinct differences in their nutritional quality, partly explaining why most invertebrates and two study fish taxa mainly depended on periphyton as the main carbon source (supporting hypothesis 1). Periphyton is rich in EPA, which is not synthesized by terrestrial vegetation, yet essential for invertebrate growth, emergence and reproduction (Stanley-Samuelson, 1994). Invertebrates living in stream and river ecosystems have a limited ability to synthesize EPA, and they must therefore obtain EPA from their food sources (Torres-Ruiz et al., 2010). In our study, $\delta^{13}C$ values indicate that invertebrate shredders did not use periphyton as the bulk carbon source, which is probably due to the fact that shredders mainly feed on leaf litter and can be responsible for up to 70% of the leaf litter reduction in stream ecosystems (Cuffney et al., 1990). Biofilms growing on leaf surfaces are an important source of EPA for shredders (Guo et al., 2016c), but the quantity of these biofilms can be very low (Kühmayer et al., 2020). Shredders are also found to mainly assimilate EPA from periphyton, even when submerged leaves are abundant (Guo et al., 2018; Kühmayer et al., 2020). Therefore, we propose that invertebrate shredders obtain bulk carbon mainly from submerged leaves, but actively assimilate FA as high-quality carbon mainly from periphyton.

No matter how the nutritional quality of periphyton has changed across river ecosystems, macroinvertebrate FA profiles clearly tracked periphyton FA, consistent with previous findings that macroinvertebrates obtain essential FA primarily from periphyton (Guo et al., 2018) (supporting hypothesis 2). In response to spatial and seasonal variations in periphyton FA content in river ecosystems, macroinvertebrates are able to adapt by feeding

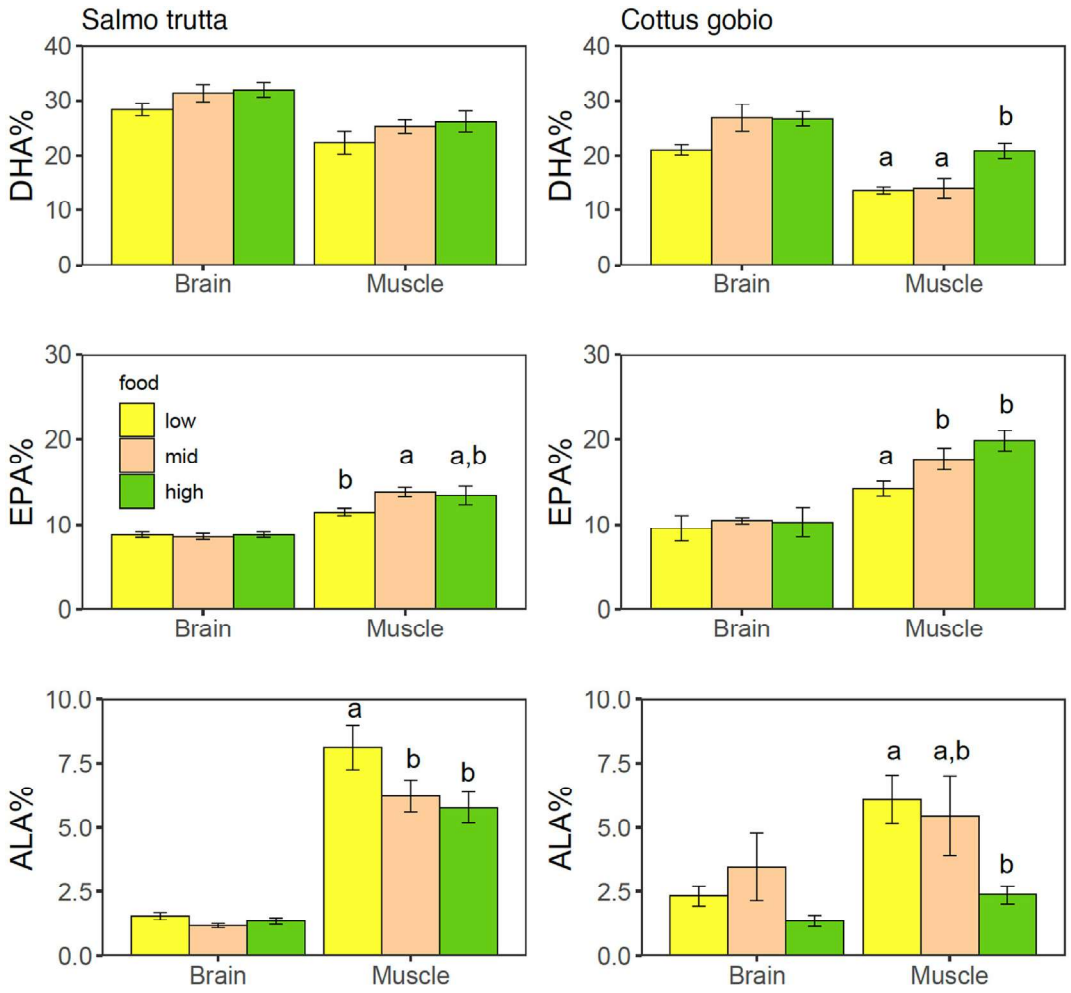

Fig. 4. Differences in omega-3 fatty acid compositions of brain and muscle tissues of *Salmo trutta* and *Cottus gobio* under different periphyton nutritional conditions (low-, mid- and high-quality groups). Letters indicate significant differences among groups ($p < 0.05$). Colours: yellow, low-quality periphyton group; pink, mid-quality periphyton group; green, high-quality periphyton group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

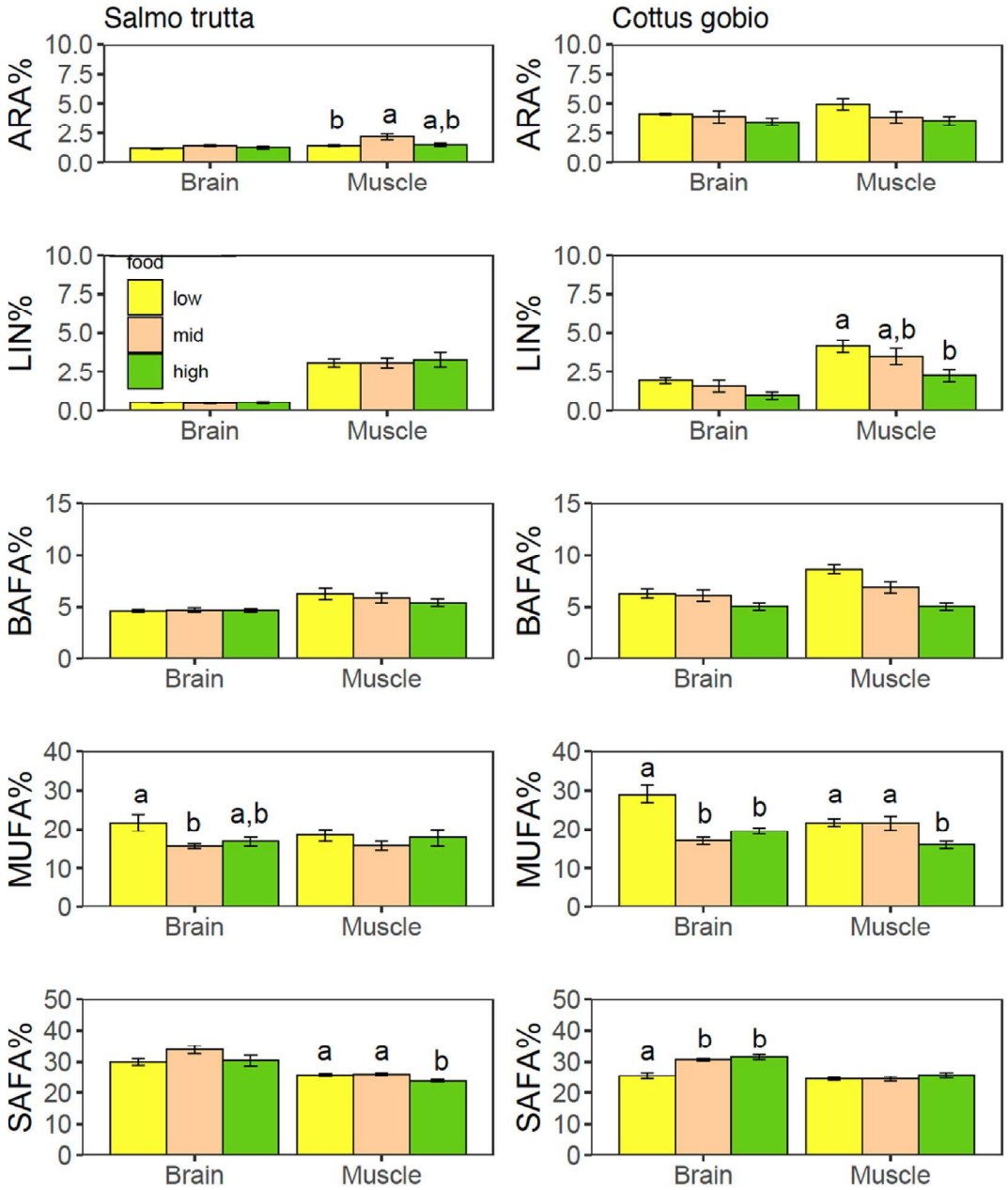

Fig. 5. Differences in the omega-6 polyunsaturated fatty acids arachidonic acid (ARA) and linoleic acid (LIN), bacterial fatty acids (BAFA), monounsaturated fatty acids (MUFA) and saturated fatty acids (SAFA) of brain and muscle tissues of *Salmo trutta* and *Cottus gobio* under different nutritional conditions of periphyton (low-, mid- and high-quality groups). Letters indicate significant differences among groups ($p < 0.05$). Colours: yellow, low-quality periphyton group; pink, mid-quality periphyton group; green, high-quality periphyton group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

selectively on, assimilating, and/or actively controlling their LC-PUFA, in particular EPA (Guo et al., 2018). In our study, macroinvertebrates always highly retained periphyton EPA, but less so ALA, which is supported by results from a recent flume experiment (Kühmayer et al., 2020). The EPA accumulation by macroinvertebrates reflects both the physiological importance of EPA and the general ecological response of invertebrates to varying nutritional conditions of periphyton in river and stream ecosystems (Brett et al., 2017; Gladyshev et al., 2011; Lau et al., 2012). Invertebrates rich in EPA would be consequently high-quality food for fish and may provide more dietary EPA for fish for subsequent DHA synthesis.

Although muscle LC-PUFA composition in both study fish taxa *Salmo trutta* and *Cottus gobio* varied with periphyton nutritional quality, brain LC-PUFA remained stable despite changes in basal food quality (supporting hypothesis 3), which may be attributed to different lipid classes in fish brain and muscle tissues. Fish brains are mainly composed of polar lipids, which are rich in DHA (Brodtkorb et al., 1997; Ebm et al., 2021), making them essential for cell functionality and relatively stable compared to dietary FA variations. Conversely, fish muscle tissues contain large amounts of neutral lipids, such as triacylglycerols, and more closely reflect dietary FA (Tocher, 2010).

The relative importance of dietary supply and internal conversion in determining the FA composition varied between the study fish taxa *Salmo trutta* and *Cottus gobio*. For *Salmo trutta*, muscle DHA remained similar under different nutritional conditions of periphyton, indicating their strong ability to regulate DHA at a relatively constant level regardless of dietary supply. The constant DHA in this fish even with varying FA composition in periphyton could be achieved by two mechanisms. First, fish may feed opportunistically on prey which are rich in DHA. Although the stream-dwelling salmonid *Salmo trutta* feed primarily on terrestrial and aquatic invertebrates, they also feed on lesser amounts of fish and fish eggs, which are

rich in DHA (Becer Ozvarol et al., 2011; Elliott, 1967). However, our study fish were mostly juveniles (body length < 237 mm), and mainly feed on invertebrates (Sánchez-Hernández et al., 2012), which are very low in DHA (Guo et al., 2018). Second, fish may internally convert dietary precursors to DHA, which is supported by our data. Fish EPA significantly declined under nutritionally low-quality periphyton compared with high-quality periphyton, suggesting that fish may have used dietary EPA to synthesize DHA. Decreased EPA% consequently led to the increase in the relative proportion of ALA. Our study fish may accumulate EPA from invertebrate prey, which selectively retained EPA from periphyton, and use the EPA to synthesize DHA. Our field evidence complements prior feeding experiments (Barry and Trushenski, 2020; Murray et al., 2014) which showed freshwater salmonids can synthesize DHA from dietary EPA (supporting hypothesis 4), which may be selectively retained from periphyton.

In contrast, *Cottus gobio* muscle DHA and EPA both significantly declined, but ALA increased under low-quality periphyton (low EPA, high ALA) compared with high-quality periphyton (high EPA, low ALA), reflecting the strong impact of periphyton nutritional quality on the FA composition of the study fish. *Cottus gobio* is a small bottom-dwelling fish and its diet is almost exclusively composed of benthic invertebrates (Mills and Mann, 1983). Therefore, their FA composition may be mainly affected by dietary invertebrate FA, which varied with periphyton FA content. However, both periphyton and invertebrates in river ecosystems are low in DHA, and the question remains how these benthivorous fish obtain physiologically important DHA. DHA in muscle tissues of *Cottus gobio* (15.8 ± 4.7%) was lower than that in *Salmo trutta* (24.4 ± 5.8%), but similar to the average level (~17%) in freshwater fish (Hixson et al., 2015). Cold-water fish, like salmonids, require more DHA than warm-water fish, since DHA has a greater effect on membrane fluidity than other LC-PUFA (i.e., EPA and ARA) (Niebylski and Salem Jr., 1994), especially at low temperatures, acting as an 'anti-freeze-like' compound in membrane phospholipids (Stillwell and Wassall, 2003). *Cottus gobio* can adapt to a wide range of environmental conditions (Mills and Mann, 1983), including a wide temperature range (Elliott and Elliott, 1995). Their relatively low DHA content may suggest a low DHA requirement, and they may thus synthesize DHA to a lesser extent compared to *Salmo trutta*. To date, very few studies have analysed the FA profiles of *Cottus gobio*, and/or assessed their ability to synthesize DHA. Combined with the feeding habits of *Cottus gobio* and related FA studies on freshwater fish, our results suggest that *Cottus gobio* may have lower ability to synthesize DHA and/or lower physiological requirement for DHA in their muscle tissues compared to *Salmo trutta*. Additionally, and their FA composition is largely associated with the nutritional quality of periphyton.

Our study shows that the nutritional quality of periphyton exerts a significant effect on fish LC-PUFA composition in river ecosystems. When periphyton nutritional quality declined, EPA and/or DHA in muscle tissues of the two study fish taxa *Salmo trutta* and *Cottus gobio* also decreased significantly. Decreased LC-PUFA content reflects declining conditions of fish nutrition and health (Arts and Kohler, 2009), which may negatively affect the functioning of freshwater ecosystems that fish sustain, such as food web dynamics and biodiversity. We suggest that the nutritional importance of periphyton and the factors that influence periphyton quality should be considered in future management plans for freshwater fish.

CRediT authorship contribution statement

FG: Conceptualization, Methodology, Field collection, Lab analysis, Data analysis, Writing – Original draft preparation.

NE: Methodology, Field collection, Lab analysis, Writing – Reviewing and Editing.

BF: Conceptualization, Writing – Reviewing and Editing.

SEB: Conceptualization, Writing – Reviewing and Editing.

MTB: Conceptualization, Writing – Reviewing and Editing.

XO: Methodology, Field collection, Writing – Reviewing and Editing.

HH: Methodology, Field collection, Writing – Reviewing and Editing.

MJK: Conceptualization, Methodology, Writing – Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.152450.

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Functions for PCA: prcomp, pca.eigenvec, pca.structure, env.scores, biplot, ordiplot

Functions for NMDS: metaMDS, envfit, ordiplot

Functions for PERMANOVA: adonis

Functions for ANOVA: anova, TukeyHSD

Functions for partial RDA: varpart, showvarparts, plot

Functions for SEM: DAG, basiSet, shipley.test

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Supporting information

TABLE 1 *Differences among four sampling locations in the River Ybbs catchment, Austria (up-stream shaded canopy and low nutrients, up-stream open canopy with low nutrients, midstream with open canopy and low nutrients and downstream with open canopy with high nutrients) were tested in basal food sources (periphyton, submerged leaves), invertebrate functional feeding groups (grazer, shredder, predators) and fish (Salmo trutta) by one- way ANOVA for alpha- linolenic acid (ALA), linoleic acid (LIN), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), bacterial fatty acids (BAFA), saturated fatty acids (SAFA) and monounsaturated fatty aicds (MUFA). Asterisks indicate significant differences (*p < 0.05; **p < 0.01; ***p < 0.001) in specific PUFA levels (% of total FA methyl esters) across locations.*

Sample	FA	factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Periphyton	EPA ***	location	3	0.105554	0.035185	15.119	0.00001461
		Residuals	22	0.051197	0.002327		
	ALA ***	location	3	0.060722	0.0202405	9.804	0.0002656
		Residuals	22	0.04542	0.0020645		
	LIN	location	3	0.0008787	0.0002929	0.3742	0.7724
		Residuals	22	0.0172198	0.00078272		
	BAFA **	location	3	0.020838	0.006946	5.5187	0.005571
		Residuals	22	0.027689	0.0012586		
	SAFA *	location	3	0.019222	0.0064073	3.7673	0.02536
		Residuals	22	0.037418	0.0017008		
	MUFA **	location	3	0.020592	0.0068641	7.7769	0.001013
		Residuals	22	0.019418	0.0008826		
Submerged leaves	ALA	location	3	0.0017576	0.00058587	0.4208	0.7485
		Residuals	4	0.0055692	0.00139229		
	LIN	location	3	0.0011882	0.00039607	0.479	0.7142
		Residuals	4	0.0033074	0.00082686		
	BAFA	location	3	0.00048404	0.00016135	0.9688	0.4898
		Residuals	4	0.00066619	0.00016655		
	SAFA	location	3	0.0008408	0.00028027	0.9999	0.479
		Residuals	4	0.0011212	0.00028029		
	MUFA	location	3	0.002851	0.00095035	1.5191	0.3388
		Residuals	4	0.0025024	0.0006256		
Grazer	EPA **	location	3	0.0156782	0.0052261	7.4821	0.004388
		Residuals	12	0.0083817	0.0006985		
	DHA	location	3	0.00045969	0.00015323	1.4279	0.2831
		Residuals	12	0.00128769	0.00010731		
	ALA	location	3	0.02367	0.0078901	1.0979	0.3878
		Residuals	12	0.086241	0.0071868		
	LIN	location	3	0.001558	0.00051933	2.4555	0.1133
		Residuals	12	0.002538	0.0002115		
	ARA	location	3	0.0013696	0.00045652	1.1765	0.3595
		Residuals	12	0.0046563	0.00038802		
	BAFA	location	3	0.0008463	0.00028209	0.7593	0.5382
		Residuals	12	0.0044583	0.00037153		
	SAFA	location	3	0.015096	0.005032	2.3863	0.1201
		Residuals	12	0.025305	0.0021087		
	MUFA	location	3	0.0017859	0.0005953	0.3509	0.7893
		Residuals	12	0.0203605	0.0016967		

Sample	FA	factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Shredder	EPA	location	3	0.040058	0.0133527	2.0018	0.1722
		Residuals	11	0.073373	0.0066702		
	DHA	location	3	0.0014812	0.00049373	0.8843	0.4791
		Residuals	11	0.0061415	0.00055832		
	ALA	location	3	0.015587	0.0051958	0.7843	0.5272
		Residuals	11	0.072869	0.0066244		
	LIN	location	3	0.015779	0.0052597	2.9408	0.0804
		Residuals	11	0.019674	0.0017885		
	ARA	location	3	0.0016429	0.00054764	0.3966	0.7581
		Residuals	11	0.0151879	0.00138072		
	BAFA	location	3	0.010918	0.0036394	0.6576	0.5949
		Residuals	11	0.060876	0.0055341		
	SAFA	location	3	0.00529	0.0017632	0.2283	0.8748
		Residuals	11	0.084955	0.0077232		
	MUFA	location	3	0.001835	0.0006118	0.1046	0.9556
		Residuals	11	0.064322	0.0058474		
Predator	EPA	location	3	0.01887	0.0062899	2.1943	0.1138
		Residuals	25	0.071662	0.0028665		
	DHA	location	3	0.0003405	0.0001135	0.5079	0.6804
		Residuals	25	0.0055861	0.00022345		
	ALA *	location	3	0.015718	0.0052395	4.2247	0.01512
		Residuals	25	0.031005	0.0012402		
	LIN	location	3	0.003336	0.001112	0.7519	0.5316
		Residuals	25	0.036975	0.001479		
	ARA	location	3	0.0027118	0.00090394	0.806	0.5024
		Residuals	25	0.0280385	0.00112154		
	BAFA	location	3	0.0063714	0.00212379	2.3239	0.09928
		Residuals	25	0.0228469	0.00091388		
	SAFA	location	3	0.009134	0.0030448	0.8302	0.4898
		Residuals	25	0.091687	0.0036675		
	MUFA	location	3	0.001831	0.00061034	0.3433	0.7942
		Residuals	25	0.044448	0.00177794		
Salmo trutta	EPA **	location	3	0.015937	0.0053123	6.5237	0.001142
		Residuals	38	0.030944	0.0008143		
	DHA	location	3	0.020295	0.0067651	1.0427	0.3848
		Residuals	38	0.246547	0.0064881		
	ALA **	location	3	0.028321	0.0094405	4.4737	0.008726
		Residuals	38	0.080188	0.0021102		
	LIN *	location	3	0.009745	0.0032482	3.118	0.03725
		Residuals	38	0.039587	0.0010418		
	ARA	location	3	0.0009263	0.00030877	1.1088	0.3575
		Residuals	38	0.0105817	0.00027846		
	BAFA ***	location	3	0.02109	0.0070299	8.1736	0.0002537
		Residuals	38	0.032683	0.0008601		
	SAFA	location	3	0.0040647	0.0013549	3.4171	0.02685

		Residuals	38	0.0150673	0.00039651		
	MUFA	location	3	0.018892	0.0062975	1.488	0.2333
		Residuals	38	0.160828	0.0042323		

TABLE 2 *For post-hoc comparisons, Tukey's honestly significant difference (HSD) test with Bonferroni correction was performed for each relevant fatty acid when the one-way ANOVA (see Table 1) indicated significant differences ($p < 0.05$) among the locations.*

Sample	FA	Location sites	diff	lwr	upr	p adj
Periphyton	**EPA**	mid-down	-0.04521634	-0.11581689	0.0253842	0.3098309
		up_open-down***	-0.16589803	-0.23649857	-0.09529748	0.0000082
		up_shade-down*	-0.09391524	-0.16863183	-0.01919864	0.0103442
		up_open-mid**	-0.12068168	-0.19802071	-0.04334266	0.0014196
		up_shade-mid	-0.04869889	-0.12981275	0.03241496	0.3639977
		up_shade-up_open	0.07198279	-0.00913106	0.15309664	0.0940951
	ALA	mid-down	0.04745973	-0.01903837	0.11395783	0.2249817
		up_open-down*	0.06759029	0.00109219	0.13408839	0.0454321
		up_shade-down***	0.13544197	0.065067	0.20581694	0.0001268
		up_open-mid	0.02013056	-0.05271446	0.09297557	0.8682208
		up_shade-mid*	0.08798224	0.01158175	0.16438274	0.0200856
		up_shade-up_open	0.06785168	-0.00854881	0.14425218	0.0937515
	BAFA	mid-down	0.02433568	-0.02758559	0.07625695	0.5716607
		up_open-down	-0.01104046	-0.06296174	0.04088081	0.9339007
		up_shade-down*	0.06840454	0.01345623	0.12335285	0.0111716
		up_open-mid	-0.03537614	-0.09225305	0.02150076	0.3341504
		up_shade-mid	0.04406885	-0.01558415	0.10372186	0.2002427
		up_shade-up_open**	0.079445	0.019792	0.139098	0.0063893
	SAFA	mid-down	0.00520466	-0.05515199	0.06556131	0.9950254
		up_open-down*	0.06839238	0.00803573	0.12874903	0.0225119
		up_shade-down	0.02935227	-0.03452321	0.09322774	0.5871212
		up_open-mid	0.06318772	-0.00292967	0.12930512	0.0644707
		up_shade-mid	0.02414761	-0.0451969	0.09349212	0.769275
		up_shade-up_open	-0.03904012	-0.10838462	0.03030439	0.4190037
	MUFA	mid-down	-0.00011782	-0.04359774	0.0433621	0.9999998
		up_open-down	0.03820615	-0.00527377	0.08168606	0.0986251
		up_shade-down*	-0.04866725	-0.09468207	-0.00265243	0.0355989
		up_open-mid	0.03832396	-0.0093059	0.08595383	0.145139
		up_shade-mid	-0.04854943	-0.09850405	0.00140519	0.0587997
		up_shade-up_open***	-0.08687339	-0.13682802	-0.03691877	0.0004328
Grazer	**EPA**	mid-down	-0.05490549	-0.11220751	0.00239653	0.0618956
		up_open-down	-0.08058453	-0.1332198	-0.02794926	0.0032414
		up_shade-down	-0.05252449	-0.10515976	0.00011078	0.0505422
		up_open-mid	-0.02567904	-0.08560693	0.03424885	0.5962322
		up_shade-mid	0.002381	-0.05754689	0.06230889	0.9993786
		up_shade-up_open	0.02806004	-0.0274224	0.08354248	0.4663486

Sample	FA	Location sites	diff	lwr	upr	p adj
Predator	**ALA**	mid-down	0.04815983	-0.00340183	0.09972149	0.07329
		up_open-down	0.03819569	-0.01023806	0.08662943	0.1595551
		up_shade-down	0.05275546	0.00432171	0.1011892	0.029105
		up_open-mid	-0.00996414	-0.06862032	0.04869204	0.9655334
		up_shade-mid	0.00459563	-0.05406055	0.0632518	0.9963697
		up_shade-up_open	0.01455977	-0.0413667	0.07048624	0.8897604
Salmo trutta	**EPA**	mid-down	0.00482828	-0.04465651	0.05431307	0.9935848
		up_open-down	-0.03724987	-0.08456638	0.01006664	0.1667477
		up_shade-down	-0.03468678	-0.08889465	0.01952109	0.328179
		up_open-mid	-0.04207815	-0.06981991	-0.01433639	0.0012445
		up_shade-mid	-0.03951507	-0.07784582	-0.00118431	0.0411866
		up_shade-up_open	0.00256309	-0.0329243	0.03805047	0.9973652
	ALA	mid-down	0.08141665	0.00175692	0.16107638	0.0435559
		up_open-down	0.08015688	0.00398761	0.15632615	0.0359077
		up_shade-down	0.11894755	0.03168468	0.20621041	0.0040573
		up_open-mid	-0.00125977	-0.04591797	0.04339842	0.999841
		up_shade-mid	0.03753089	-0.02417327	0.09923505	0.3722914
		up_shade-up_open	0.03879066	-0.01833629	0.09591762	0.2780531
	LIN	mid-down	0.01389328	-0.04207773	0.06986428	0.9088908
		up_open-down	0.04212049	-0.01139802	0.09563901	0.1669357
		up_shade-down	0.01619848	-0.04511468	0.07751165	0.8926124
		up_open-mid	0.02822722	-0.0031508	0.05960523	0.090976
		up_shade-mid	0.0023052	-0.04104975	0.04566016	0.9989415
		up_shade-up_open	-0.02592201	-0.0660609	0.01421688	0.3202691
	BAFA	mid-down	0.00407903	-0.04677735	0.05493541	0.9964044
		up_open-down	0.05071788	0.00208987	0.09934588	0.0381351
		up_shade-down	0.01477098	-0.0409394	0.07048135	0.8916045
		up_open-mid	0.04663885	0.01812815	0.07514954	0.0004823
		up_shade-mid	0.01069194	-0.02870124	0.05008513	0.8847962
		up_shade-up_open	-0.0359469	-0.0724179	0.0005241	0.054637

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ORIGINAL ARTICLE

Compound-specific stable isotopes resolve sources and fate of polyunsaturated fatty acids in biota of headwater streams

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Abstract

1. Organisms at the base of stream food webs are typically poor in long-chain polyunsaturated fatty acids (LC-PUFA), especially in docosahexaenoic acid (DHA), whereas consumers at higher trophic levels are often rich in LC-PUFA. For example, fish tissues, especially the brain, are DHA-rich. This obvious mismatch between consumer LC-PUFA and their basal dietary supply may result from selective retention and/or endogenous conversion of dietary precursors to LC-PUFA.
2. To determine which is more likely, we investigated compound-specific carbon stable isotopes in PUFA ($\delta^{13}C_{PUFA}$) of potential basal resources (stream epilithon, leaf litter) and consumers (invertebrates, European bullhead, and two salmonid species and their brain, eye, liver, and muscle tissues). We predicted that consumer-PUFA, depleted in ^{13}C values relative to their dietary sources, would indicate internal de novo PUFA synthesis. Alternatively, higher consumer-$\delta^{13}C_{PUFA}$ values would imply selective retention of aquatic (epilithon and conditioned leaves) rather than terrestrial resources or internal production, irrespective of trophic levels.
3. Invertebrate grazers and predators resembled $\delta^{13}C$ values of essential fatty acids ($\delta^{13}C_{EFA}$) of benthic algae, while shredder-$\delta^{13}C_{EFA}$ values reflected those of conditioned rather than fresh leaves. Lower eye-$\delta^{13}C_{EFA}$ values of salmonids than in livers indicated high retention of dietary PUFA sources (invertebrates and epilithon). Stable isotope values of eicosapentaenoic acid suggest that all consumers retained algal EPA, while insectivorous fish produced DHA in their liver. There is no further evidence from carbon stable isotopes for local PUFA conversion within neural tissues.
4. Our study demonstrates that $\delta^{13}C_{PUFA}$ can be used to track sources of these highly functional molecules in aquatic consumers and highlights the importance of algal derived-PUFA for these consumers in oligotrophic headwater streams.

KEYWORDS
benthic invertebrates, bioconversion, docosahexaenoic acid, epilithon, neural tissues

1 | INTRODUCTION

Stream biota obtain fatty acids (FA) from allochthonous (terrestrial) and autochthonous (e.g., algae or mosses) sources, yet retain FA profiles that differ from either dietary source (Ebm et al., 2021; Guo et al., 2017; O'Mara et al., 2022). Whether this mismatch between consumers and their food sources is due to selective retention and/or endogenous FA conversion is subject of ongoing research (Fujibayashi et al., 2019; Scharnweber et al., 2021). The identification of sources and metabolic fate of FA in consumers across stream food webs remains challenging. So far, it is very difficult to discern whether highly required FA in stream fish, e.g., the omega-3 long-chain polyunsaturated FA (n-3 LC-PUFA) docosahexaenoic acid (DHA; 22:6n-3), are directly acquired from the diet or converted from precursor FA (Chiapella et al., 2021; Hixson et al., 2014; Murray et al., 2014). Neural organs (i.e., brain and eyes) in fish differ substantially in their PUFA-composition and in their tendency to reflect dietary PUFA-sources (Brodtkorb et al., 1997; Ebm et al., 2021). Dietary lipids are more strongly reflected by organs rich in neutral (e.g., salmonid eyes) than polar lipids (e.g., brain; Iverson, 2009; Sushchik et al., 2020). Neutral lipids of salmonid eyes predominantly consist of short chain PUFA, i.e., α-linolenic (ALA, 18:3n-3), linoleic acid (LIN, 18:2n-6), and stearidonic acid (SDA, 18:4n-3; Ebm et al., 2021). The PUFA status of brains is subject to hepatic (Henderson et al., 1984) and/or astrocytic conversion (Bell et al., 2001; Tocher, 1993; Xue et al., 2014). Thus, it is important to understand how fish use, convert, and consequently transfer FA to their neural tissues to optimise their fitness (Bell et al., 1995).

The two essential PUFA (EFA), ALA and LIN, are present at high proportions (% FA) in terrestrial plants (e.g., fresh or conditioned leaves; Ebm et al., 2021) and less in aquatic primary producers (algae; Ebm et al., 2021; Honeyfield & Maloney, 2015; Twining et al., 2021) but they cannot be produced by animals (Cook & McMaster, 2002; Cunnane, 2003; but see Kabeya et al., 2018). However, FA similarity analyses indicate that aquatic consumers rely more on autochthonous than allochthonous resources (Ebm et al., 2021; Lau et al., 2009). Low digestibility and nutritional quality (low fat, but high phenolic or lignin contents) make terrestrial organic matter less valuable food sources for aquatic consumers (Brett et al., 2017; Breznak & Brune, 1994; Geib et al., 2008). Despite this, the large quantity of easily available allochthonous inputs (e.g., leaf litter) provides an additional dietary energy source to some macroinvertebrates (e.g., omnivores, detritivores or shredders) and this organic matter can be transferred across stream food webs and further support consumers at higher trophic levels in low-productivity systems (Crenier et al., 2017; Kühmayer et al., 2020; Polis & Strong, 1996).

The high accessibility of EFA contrasts with the limited dietary availability of LC-PUFA at the trophic base of terrestrial and freshwater ecosystems (Hixson et al., 2015; O'Mara et al., 2022; Twining et al., 2016, 2019, 2021). Non-genetically modified vascular plants lack the enzymes to synthesise n-3 LC-PUFA; such as eicosapentaenoic (EPA, 20:5n-3) and DHA and their n-6 analogues (i.e., arachidonic, ARA, 20:4n-6; and docosapentaenoic acid, DPA, 22:5n-6) (Twining et al., 2016; West et al., 2021). In contrast, diatom-dominated biofilms on rocks (epilithon) can be rich in LC-PUFA, with EPA being prevalent, whereas those on leaf litter (epiphyton) contain at least traces of LC-PUFA (Guo et al., 2016; Hixson et al., 2015; Torres-Ruiz & Wehr, 2010). Although many aquatic consumers can convert dietary precursors to LC-PUFA to compensate for these dietary deficiencies, most of our current knowledge of endogenous PUFA conversion relies on controlled feeding experiments (Goedkoop et al., 2007; Kühmayer et al., 2020; Murray et al., 2014) but field evidence for LC-PUFA production by consumers in stream ecosystems is scarce (Fujibayashi et al., 2019; Gladyshev et al., 2012; Scharnweber et al., 2021). Even less is known about the conversion of dietary PUFA, their trophic transfer, their isotopic fractionation from basal stream resources to top-predators (e.g., fish) and how they nourish their most energy-demanding organs (brain and eyes).

One way to overcome the problem of co-occurring FA in diet sources is to use source-specific diet biomarkers in compound-specific stable isotopes analysis (Burian et al., 2020; Scott Lacombe & Bazinet, 2020; Twining et al., 2020). Twining et al. (2020) demonstrated distinctive isotopic differences between allochthonous and autochthonous PUFA in primary producers and provided evidence of algae-derived EFA being retained in stream consumers. During conversion of dietary PUFA within consumers, FA can undergo a series of elongation and desaturation steps (e.g., from EPA to DHA), which result in isotopic fractionation (Budge et al., 2016; Hixson et al., 2014; Lacombe et al., 2017). Kinetic isotope effects result in ^{13}C depletion of highly unsaturated FA (e.g., DHA) relative to their dietary precursors (Gladyshev et al., 2012). Furthermore, if isotopic carbon values of PUFA ($\delta^{13}C_{PUFA}$) differ substantially among basal food sources it is possible to trace the dietary FA sources in consumers (Lacombe et al., 2017; Mathieu-Resuge et al., 2022).

The objective of this field study was to investigate the trophic transfer of allochthonous and autochthonous PUFA to benthic stream invertebrates and insectivorous fish in subalpine headwater streams. We used $\delta^{13}C_{PUFA}$ to assess the retention of allochthonous and autochthonous PUFA and to explore PUFA bioconversion in stream consumers. We hypothesised that:

1. Consumers preferentially retain ALA and LIN from epilithon rather than terrestrial sources (fresh or conditioned leaves) and expected that $\delta^{13}C$ values of these two essential PUFA ($\delta^{13}C_{EFA}$) in invertebrates and fishes reflect those of biofilms more strongly than those of terrestrial resources.

2. Insufficient dietary supply of LC-PUFA (e.g., ARA, EPA, and DHA) triggers endogenous production of these organic compounds in consumers (invertebrates and fish). Insectivorous fish rely mainly on DHA-low invertebrates (Ebm et al., 2021; Guo et al., 2018, 2021) while piscivorous fish (e.g., *Perca fluviatilis*) have access to dietary DHA (Scharnweber et al., 2021). Thus, internal PUFA production is expected to involve isotopic fractionation, where synthesised PUFA in consumers become ^{13}C-depleted relative to their diets. As the primary site of PUFA synthesis in fish are hepatocytes (James & Tocher, 1987) we expect that ^{13}C-depleted non-essential PUFA (e.g., ARA, EPA, and DHA) values in fish livers compared to potential

dietary sources indicate endogenous PUFA production. Similarly, ^{13}C-depleted non-essential PUFA values in invertebrates compared to potential diet sources (epilithon or conditioned leaf litter) indicate endogenous PUFA production.

3. Fish also convert precursor FA to LC-PUFA in neural organs (brains and eyes) with high contents of LC-PUFA (e.g., brain and eyes). Such tissue-specific production is expected to result in further ^{13}C-depletion of LC-PUFA values while similar or higher δ^{13}C-liver values would indicate hepatic allocation. We predict that neural organs are important sites of LC-PUFA-synthesis, and thus their $\delta^{13}C_{PUFA}$ will be significantly more negative compared to $\delta^{13}C_{PUFA}$ values in the fish liver.

2 | MATERIALS AND METHODS

2.1 | Study streams and sampling

We sampled 17 sites in nine oligotrophic streams (first to fifth order, Figure 1) within the headwater region (Weiße Ois) of the subalpine River Ybbs catchment, Austria (532–1000 m above sea level; see Ebm et al., 2021 for details) in three separated sampling campaigns.

In July 2016, numerous fresh leaves were picked directly from single plant individuals ($n = 28$) from multiple sites along the riparian vegetation in summer. These samples covered herbaceous (*Petasites*, *Urtica dioica*), or lignifying angiosperms (*Acer*, *Corylus*, *Fagus sylvatica*, *Hedera helix*, *Phragmites*, *Salix*, *Clematis vitalba*) and gymnosperms (*Picea*). At each site, every plant species was analysed separately.

In October 2016, five different cobbles were collected randomly from three quadrants (1.5 × 1.5 m) at each site. From these, aquatic macroinvertebrates were picked and pooled by genus level for further FA analysis using a stereomicroscope. Pooled samples of macroinvertebrates ($n = 149$) included Ephemeroptera (*Baetis*, *Ecdyonurus*, *Rhithrogena*), Plecoptera (*Leuctra*, *Nemoura*, *Perla*, *Perlodes*, *Isoperla*), Trichoptera (*Allogamus*, *Odontoceridae*, *Plectronemia*, *Potamophylax*, *Rhyacophila*, *Hydropsyche*), Platyhelminthes, and amphipods (*Gammarus*). They were then assigned to functional feeding guilds (FFGs) in accordance with Moog (2002).

At every site, epilithon samples ($n = 51$) were scraped with soft brushes from the same cobbles that have been collected for macroinvertebrates previously and were washed into small containers. Then, these samples were stored immediately on ice in the dark (portable freezer). Other potential terrestrial resources included conditioned leaves (submerged, senescent, and brownish leaves of various vascular plant species from the surrounding riparian vegetation, and fresh leaves). Conditioned leaf litter was collected at each site from the streambed in October 2016 ($n = 14$). All submerged and fresh leaf samples were immediately stored on ice in zip-lock plastic bags and kept in the dark (portable freezer). These samples contain whole biofilm community with autotrophs, heterotrophs, and detritus. These community structures were not examined because we were primarily interested in dietary and biochemical composition of potential food sources and their consumers (Ebm et al., 2021; Guo et al., 2018).

We needed to include additional fresh leaf ($n = 8$; *Ribes alpinuum*, *Lonicera alpigena*, *Tussilago farfara*), conditioned leaf ($n = 6$), and epilithon samples ($n = 5$) from August 2018 because some samples from 2016 had weak peak intensity and could not be remeasured. All these additional samples were collected in accordance with the procedure in 2016.

Fish capture and collection for this research was undertaken with permission from local fisheries authorities in November 2016. We decided to collect three insectivorous fish taxa that were abundant and self-sustaining in the chosen study area (metarhithral): two taxa from the family of salmonids (*Oncorhynchus mykiss*, $n = 32$; *Salmo trutta*, $n = 65$) and demersal European bullhead (*Cottus gobio*;

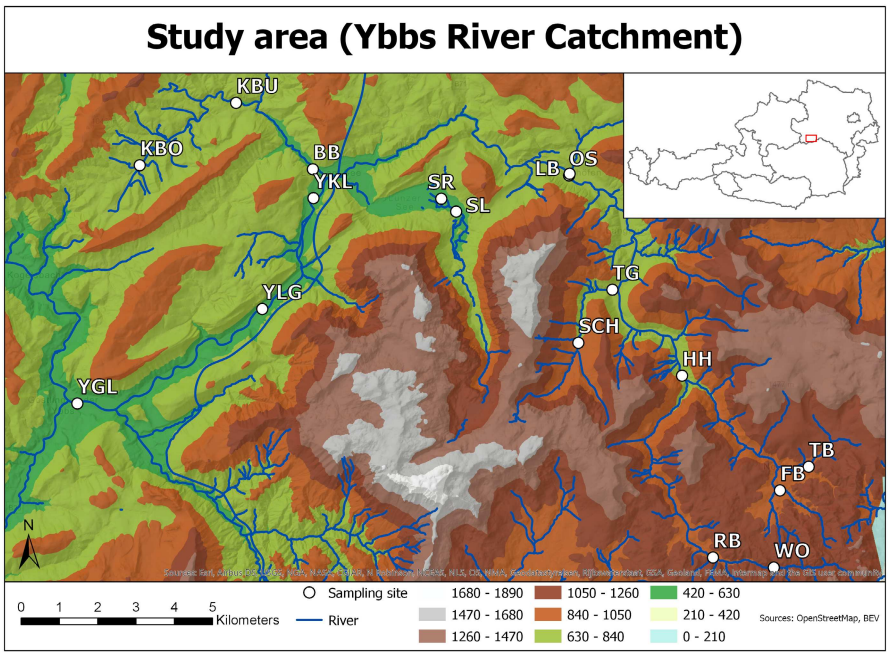

FIGURE 1 Sampling area (Ybbs River catchment, 256 km^2, 550–1000 m above sea level) with 17 sampling sites (dots) at nine oligotrophic rivers (blue lines) of different stream orders (1–5). Bodingbach, BB (4); Faltlbach, FB (1); Kothbergbach oben, KBO (3); Kothbergbach unten, KBU (3); Lackenbach, LB (2); Oberer Seebach Lend, SL (2); Oberer Seebach Ritrodat, SR (2); Ois alte Säge, OS (4); Holzhüttenboden, HH (4); Schlechen, SCH (1); Tagles, TG (3); Taschlbach, TB (1); Rehberghütte, RB (3); Weiße Ois, WO (3); Göstling Lagerhaus, YGL (5); Kläranlage Lunz, YKL (5); Lunz Großau, YLG (5).

$n = 61$). At each sampling site, we aimed to collect up to five individuals per fish taxa by electrofishing. Fish were anaesthetised and subsequently killed in accordance with the Federal Act on the Protection of Animals, Austria (http://www.ris.bka.gv.at). Before dissection, fish total body length (mm) and weight (g) were recorded. Brain, eyes, dorsal muscle tissues, and the entire liver were then collected from each specimen (for details, see Ebm et al., 2021).

All samples were kept on ice in a portable cooling box during field work and lab processing (identification or dissection), placed into Eppendorf tubes (brain and eyes) or scintillation vials (epilithon, leaf litter, invertebrate, muscle, and liver samples) and stored at −80°C until lyophilisation (Virtis Genesis™ freeze dryer).

2.2 | Lipid analyses

Total lipids from freeze-dried and homogenised samples (fresh and conditioned terrestrial leaves: *c*. 50 mg; epilithon: *c*. 10 mg; invertebrates: *c*. 5–7 mg, brain and eyes: *c*. 5 mg, liver: *c*. 10–15 mg, muscle: *c*. 15–20 mg) were dissolved in ice-cold chloroform (2 ml) and stored under N_2 atmosphere at −80°C overnight to improve lipid extraction efficiency. Samples were extracted in chloroform–methanol (2:1) and sodium chloride (0.8 ml; salt wash), vortexed, and sonicated and subsequently analysed gravimetrically in pre-weighed tin capsules. FA were derivatised to FA methyl esters (FAME) in an esterification reagent (methanolic sulfuric acid) for 16 hr at 50°C. All FAME were stored at −80°C until gas chromatography (THERMO™ Trace GC) as described previously (Ebm et al., 2021). Fatty acids were reported as relative values (% of total FAME).

2.3 | Compound-specific stable isotopes of FA

For compound-specific stable isotope analysis, we prepared a subset of lipid analysed samples ($n = 219$). We selected one replicate for each species of abovementioned spermatophyte genera ($n = 9$) and for each site for epilithon ($n = 17$). After sample pooling for increased peak quality in the gas chromatography–isotope ratio mass spectrometer (IRMS), only nine samples of submerged leaves were left. We chose only the macroinvertebrate genera that were present across most of our study sites ($n = 40$). This subset of stream invertebrates covered grazing Ephemeroptera (*Ecdyonurus*, $n = 9$), Plecoptera (shredder: *Leuctra*, $n = 8$ and predator: *Perla*, $n = 7$), and the amphipod *Gammarus* (shredder, $n = 8$). We considered only fish individuals (*O. mykiss*, $n = 8$; *S. trutta*, $n = 16$; and *C. gobio*, $n = 10$) that had a complete set of analysed organs (liver, muscle, brain, and eye tissue), which had not been pooled previously. FAME were separated using gas chromatography linked to an isotope ratio mass spectrometer (Delta V Advantage IRMS via Isolink 2 and Conflo IV, see Mathieu-Resuge et al., 2022). A split/split-less liner with single taper ($4 \times 6.3 \times 78.5$ mm, vat. No. 453A1355) was used, the injector temperature was kept at 250°C and all samples were injected in split-less mode. Split-less injection time is always 1 min. For $\delta^{13}C$, FAME were separated on a VF-WAX ms 60 m/0.25 mm i.d./0.25 µm film thickness column (Agilent Technologies) at a flow rate of 1.2 ml/min, followed by oxidation to CO_2 in a combustion reactor, filled with Ni, Pt and Cu wires, at 1000°C. The temperature gradient for $\delta^{13}C$ analysis started at 80°C, which was kept for 2 min, then the temperature was raised by 30°C/min to 175°C, by 5°C/min to 200°C and finally by 2.4°C/min to 250°C, which was maintained for 30 min. Finally, all CO_2 molecules were analysed in an isotope ratio mass spectrometer. Raw isotopic values were processed by Isodat 3.0.0.83 software (Thermo Fisher Scientific) and were normalised using validated 20-carbon FAME reference material (USGS70 and USGS71). Then they became expressed in δ units with respect to international standards for $\delta^{13}C$ (Vienna Pee Dee Belemnite, VPDB) $= \left[\left(R_{sample} / R_{standard}\right) - 1\right] \times 10^3$ (expressed in‰), where R is $^{13}C/^{12}C$. Analytical uncertainty was determined to be 0.7‰ based und repeated measurement of the isotope standards. In some samples, naturally occurring small FA concentrations were close to the detection limit and could not be precisely measured.

2.4 | Data analysis

All data analyses were performed using R version 4.1.3 (R Core Team, 2022) with the RStudio version 2022.07.2 (RStudio Team, 2022). For selected $\delta^{13}C_{PUFA}$ (ALA, LIN or EPA, ARA, and DHA) multivariate outliers were removed based on exceedance of adjusted quantiles according to Mahalanobis distance (Korkmaz et al., 2014) or interquartile range for univariate outliers. Significant differences in $\delta^{13}C_{PUFA}$-profiles among groups (season, sample type, taxa, etc.) were assessed by permutational multivariate analysis of variance (non-parametric equivalent of MANOVA, ADONIS) with 1000 permutations based on Bray–Curtis distance matrices (Oksanen, 2011) and with prior analysis of requested variance homogeneity (Anderson & Walsh, 2013). For testing hypothesis 1, we used a pairwise ADONIS for contrasting defined groups of consumers (e.g., invertebrate FFGs) with their potential food sources (e.g., epilithon). Pairwise ADONIS tests included *p*-value adjustment (Bonferroni correction) to maintain an overall α-value of 0.05. Summary statistics of the relative abundance of relevant FA (% FA, mean ± 1 *SD*) are included as supplementary material (Table S3).

To test whether mean isotopic values in LC-PUFA ($\delta^{13}C_{PUFA}$) in consumers differ from potential food sources (hypothesis 2), univariate tests were applied. Prior to this, normality distribution (Shapiro-Wilk test) and variance homogeneity (Levene's test) within each factor were evaluated (Hervé, 2021). When both prerequisites were fulfilled, Student *t*-tests (Effect size: absolute Cohen's D) or analysis of variance (ANOVA, Type II Sums of Squares[2]) with pairwise *t*-tests post hoc analysis with Bonferroni correction were performed (Ben-Shachar et al., 2020; John & Weisberg, 2021; Kassambara, 2021). If only variance homogeneity was violated, Welch-approximations were applied to further adjust these parametric tests. We performed non-parametric equivalents (e.g., Kruskal–Wallis, or Wilcoxon rank-sum test) in case of non-normal sample distributions and Dunn-tests

with Bonferroni correction for post hoc analysis (effect size: absolute value of Cliff's Delta, Mangiafic, 2021; Ogle et al., 2021). Effect size values were classified as small (<0.2), medium (<0.5), and large (≥0.8, Sullivan & Feinn, 2012).

For evaluating differences in $\delta^{13}C_{PUFA}$ between fish organs and their respective livers we aimed for paired two-sample *t*-tests (hypothesis 3) and selected only individuals for which LC-PUFA were measured in all organ samples. Before that, normal distribution in each PUFA and fish organ sample (brain, eye, and muscle), and the isotopic difference relative to its respective liver ($\Delta\delta^{13}C$), was assessed. In case of violated normality, paired two-samples Wilcoxon rank-sum tests were chosen. Significant *p*-values were reported together with aforementioned effect sizes.

3 | RESULTS

Temporal factors (e.g., season or year) had no effect on compound-specific stable isotopes of EFA or LC-PUFA in basal food sources (epilithon, conditioned, and fresh leaves; Tables S1 and S2). Thus, basal food sources were summarised irrespective of time.

The shredders *Leuctra* (ALA: −40.5 ±3.1‰, LIN: −36.2 ±2.3‰, $n=7$) and *Gammarus* (ALA: −40.0 ±1.4‰, LIN: −35.8 ±1.0‰, $n=6$) did not differ from each other in their $\delta^{13}C_{EFA}$ values (ADONIS (1) = −0.07, $p=0.975$, $n=13$) and thus were grouped. Their $\delta^{13}C_{EFA}$ were significantly ^{13}C-enriched compared to epilithon (ALA: −42.3 ±1.9‰, LIN: −39.2 ±2.0‰, $n=12$) and fresh leaves (ALA: −39.7 ±2.0‰, LIN: 40.2 ±2.1‰, $n=13$) but were similar to conditioned leaves (ALA: −39.3 ±0.6‰, LIN: −37.2 ±0.9‰, $n=9$; see Table 1 and Figure 2). Predator-$\delta^{13}C_{EFA}$ values (ALA: −42.8 ±2.5‰, LIN: −36.7 ±1.8‰, $n=7$) were similar to those of epilithon but their EFA were significantly more ^{13}C-depleted than conditioned and fresh leaves. Grazer-$\delta^{13}C_{EFA}$ values (ALA: −45.7 ±3.3‰, LIN: −40.8 ±2.8‰, $n=9$) were significantly lower than those of fresh and conditioned leaves but similar to those of epilithon samples. Values of $\delta^{13}C_{PUFA}$ (EFA and LC-PUFA) measured in fish organs were similar between *S. trutta* and *O. mykiss* (Tables S4–S6); therefore, measurements from these species (hereafter, salmonids) were grouped, by tissue, for subsequent data analysis. Permutational multivariate analysis of variance revealed no significant differences in $\delta^{13}C_{EFA}$ between epilithon and salmonid eyes (see Table 1 and Figure 2).

Invertebrate-$\delta^{13}C_{EPA}$ values (−39.8 ±3.4‰, $n=38$) and $\delta^{13}C_{ARA}$ values (38.9 ±3.9‰, $n=32$) were similar across FFGs (ANOVA (1) = 0.9264, $p=0.4055$; ANOVA (1) = 1.3055, $p=0.6585$, respectively). Invertebrate-$\delta^{13}C_{EPA}$ values were significantly depleted compared to conditioned leaf-$\delta^{13}C_{EPA}$ values (−36.1 ±0.8‰, $n=13$) but did not vary from epilithon-$\delta^{13}C_{EPA}$ (−39.9 ±2.9‰, $n=13$; Table 2). However, invertebrate-$\delta^{13}C_{ARA}$ values were similar to both potential resources; epilithon-$\delta^{13}C_{ARA}$ values (−40.5 ±3.7‰, $n=5$) and conditioned leaf-$\delta^{13}C_{ARA}$ values (−41.4 ±1.1‰, $n=14$). Gammarid-$\delta^{13}C_{PUFA}$ values did not vary seasonally and thus, were grouped (ADONIS (1) = 0.30609, $p=0.733$, $n=16$) for further subsequent data analysis, but differed from all other benthic invertebrates by the presence of DHA. Nevertheless, their $\delta^{13}C_{DHA}$ values (−36.5 ±2.5‰, $n=11$) were similar to epilithon (−39.2 ±2.1‰, $n=6$) and conditioned leaves (−37.0 ±4.1‰, $n=3$). SDA was detected only in grazers consistently and their isotope values (−45.9 ±2.6‰, $n=7$) were significantly depleted compared to that of epilithon $\delta^{13}C_{SDA}$ values (−37.6 ±2.3‰, $n=9$). In both fish groups (Figure 3), liver-EPA (−40.2 ±2.3‰, $n=34$) and ARA (−37.5 ±2.1‰, $n=34$) had similar isotopic ratios as stream invertebrates (EPA: 39.8 ±3.4‰, ARA: 38.9 ±3.9‰, DHA: −36.5 ±2.4‰)

TABLE 1 Pairwise permutated multivariate analysis of variation (ADONIS) in $\delta^{13}C_{ALA}$ and $\delta^{13}C_{LIN}$ among basal resources (epilithon, conditioned and fresh leaf samples, $n=36$) and consumers (invertebrate functional feeding guilds and salmonid eye samples, $n=47$).

Biota	Invertebrate – grazers	Invertebrate – shredders	Invertebrate – predators	Salmonid (eyes)
Epilithon	$F=6.26$ adj. $p=0.20$ $n=21$	$F=10.98$ **adj. $p=0.03$** $n=25$	$F=2.45$ adj. $p=0.999$ $n=19$	$F=1.95$ adj. $p=0.999$ $n=29$
Conditioned leaves	$F=26.78$	$F=1.29$	$F=7.59$	$F=35.02$
	adj. $p=0.02$ $n=18$	adj. $p=0.999$ $n=22$	**adj. $p=0.01$** $n=16$	**adj. $p=0.01$** $n=26$
Fresh leaves	$F=12.20$	$F=10.39$	$F=7.76$	$F=15.57$
	adj. $p=0.03$ $n=22$	**adj. $p=0.020$** $n=26$	**adj. $p=0.01$** $n=20$	**adj. $p=0.01$** $n=30$
Non-predatory invertebrates			$F=0.19$ adj. $p=0.999$ $n=29$	
*Invertebrates				$F=0.03$ **adj. $p=0.999$** $n=46$

Note: Reported statistical parameters include *F*-statistics (*F*), Bonferroni-corrected *p*-values (adj. *p*.). In all cases degrees of freedom were 1. Non-predatory invertebrates include all invertebrate taxa except predators. As result, intraguild predation is not considered in our data analysis. Asterisks (*) indicate that for this comparison all invertebrates were grouped and compared with fish eyes. Shaded cells indicate significance (adj.$p<0.05$) written in bold.

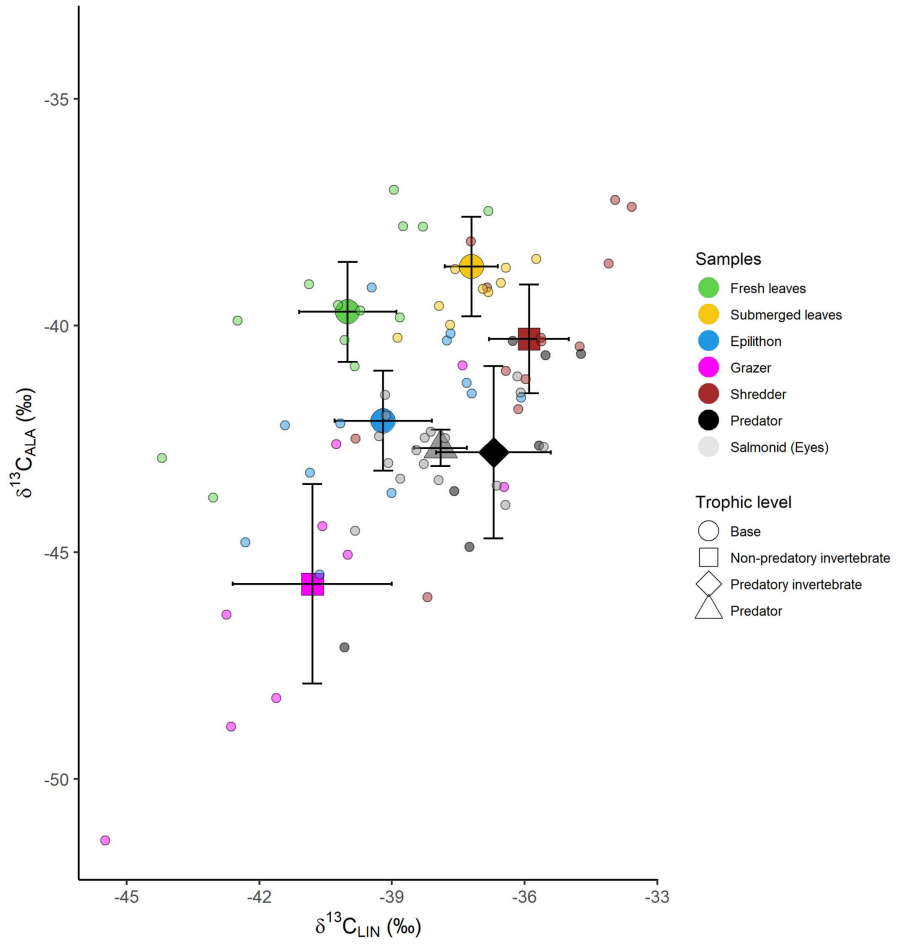

FIGURE 2 Mean with 95% confidence interval (colour- and shape-coded data points with error crosses) of compound-specific stable isotope values (‰) of essential fatty acids (ALA, alpha-linolenic acid; LIN, linoleic acid) of measured basal stream resource samples (colour-coded circles), benthic invertebrates (grazers, shredders, and predators) and salmonid eyes. Samples were summarised irrespective of sampling sites and season after testing ($n = 83$). Stable isotope values were expressed in δ notation with respect to international standards for $\delta^{13}C$ (Vienna Pee Dee Belemnite, VPDB) = $\left[\left(\frac{R_{sample}}{R_{standard}}\right) - \neg 1\right] \times 10^3$ (expressed in‰), where R is $^{13}C/^{12}C$.

TABLE 2 Univariate difference tests of compound-specific stable isotope values of polyunsaturated fatty acids ($\delta^{13}C_{PUFA}$) between consumers (invertebrates of various functional feeding guilds, fish) and potential resources (epilithon, conditioned leaves and invertebrates for fish).

Food source	PUFA	$\delta^{13}C_{PUFA}$ (‰)	*n*	Consumer	$\delta^{13}C_{PUFA}$ (‰)	*n*	Method	Statistic	adj. *p*	Effect size
Conditioned leaves	EPA	−36.1 ± 0.8	13	Invertebrates	−39.8 ± 3.4	38	Dunn-test	−2.693	0.007	0.71
	ARA	−41.4 ± 1.1	14		−38.9 ± 3.9	32	*t*-test	1.026	0.847	–
	DHA	−37.0 ± 4.1	3	Gammarus (shredder)	−36.5 ± 2.5	11	*t*-test	0.211	0.999	–
Epilithon	EPA	−39.9 ± 2.9	12	Invertebrates	−39.8 ± 3.4	38	Dunn-test	−0.293	0.999	–
	ARA	−40.5 ± 3.7	5		−38.9 ± 3.9	32	*t*-test	−0.784	0.999	–
	SDA	−37.6 ± 2.3	9	Ephemeroptera (grazer)	−45.9 ± 2.6	7	*t*-test	3.179	0.005	1.48
	DHA	−39.2 ± 2.1	6	Gammarus (shredder)	−36.5 ± 2.4	13	*t*-test	-2.364	0.138	–

Note: Summary statistics of $\delta^{13}C_{PUFA}$ are reported as mean ± 1 *SD*‰, sample size (*n*). For significant *p*-values we reported adequate effect size value where ecological significant effects were classified as small (<0.2), medium (<0.5), or large (≥0.8, Sullivan & Feinn, 2012).

Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SDA, stearidonic acid.

but differed from them by significantly lower liver-DHA (−40.5 ± 2.1‰, $n = 33$; Table S7).

Irrespective of LC-PUFA, isotopic values in sampled organs in European bullhead (ARA: −39.2 ± 1.4‰, EPA: −40.7 ± 1.8‰, DHA: −43.2 ± 0.9‰) were similar with their respective livers (ARA: −39.2 ± 2.3, EPA: −40.7 ± 2.6‰, DHA: −43.0 ± 1.2‰, Figure 4). However, salmonid brain-$\delta^{13}C_{LC\text{-}PUFA}$ values (ARA: −35.2 ± 2.2‰, EPA: −38.1 ± 1.3, DHA: −38.5 ± 1.9) were significantly ^{13}C-enriched compared

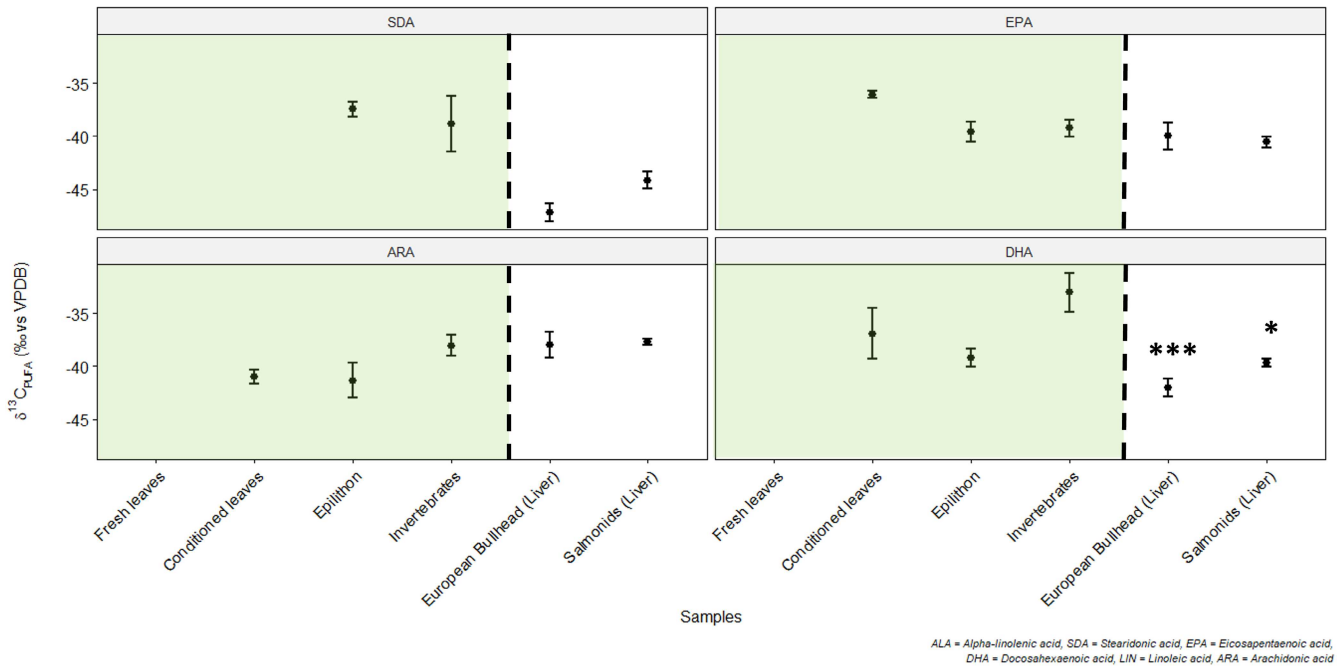

FIGURE 3 Similar mean (with 95% confidence interval) of compound-specific stable isotope values (‰) of stearidonic acid (SDA) and long-chain polyunsaturated fatty acids ($\delta^{13}C_{PUFA}$) between fish livers (Salmonids: *Oncorhynchus mykiss*, *Salmo trutta* vs. European bullhead) and basal stream resources (epilithon, conditioned and fresh leaves invertebrate prey) indicated dietary origin of fish-eicosapentaenoic acid (EPA) and endogenous production of fish-docosahexaenoic acid (DHA). Non-genetically modified vascular plants lack SDA and long-chain polyunsaturated fatty acids, thus these fresh leave $\delta^{13}C_{PUFA}$ were expected to be missing (empty slots). The δ notation expresses the variation of an isotopic ratio of $^{13}C/^{12}C$ relative to the isotopic ratio of an international standard (VPDB, Vienna Pee Dee Belemnite). Asterisks indicates significant differences ($p < 0.05$) in specific PUFA isotope ratios between invertebrate prey and livers only (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

with isotopic values of livers (ARA: −36.9 ±1.6‰, EPA: −40.5 ±1.9‰, DHA: −39.4 ±1.5‰, Table 3).

4 | DISCUSSION

Compared with traditional analytical techniques such as bulk stable isotope or FA analysis (Ebm et al., 2021; Hayden et al., 2016) or laboratory experiments (Hixson, 2014; Kühmayer et al., 2020), the application of $\delta^{13}C_{PUFA}$ to natural $^{13}C/^{12}C$ abundance levels could resolve the dietary origin of specific PUFA in stream consumers (e.g., invertebrates and fish) in their natural habitats. We provided field-evidence for greater importance of algal derived-PUFA to higher trophic levels, including fish and their neural organs (i.e., eyes) over terrestrial organic matter, indicating that trophically transferred algae-derived PUFA became integral parts of fish neural tissues. Most consumer-$\delta^{13}C_{EFA}$ values reflected basal aquatic foods (i.e., epilithon) rather than terrestrial resources. The predominance of catabolic processes during leaf litter decomposition may have resulted in higher conditioned leaf-$\delta^{13}C_{EFA}$ and -$\delta^{13}C_{EPA}$ values, which made them isotopically distinguishable from epilithon or fresh leaves. However, it is very unlikely that degradation alone can cause such large isotopic shifts. High metabolic rates in microbes colonising leaf detritus (Figure 5) may have increased $\delta^{13}C_{PUFA}$ values, while lower mass-specific metabolic rates in fresh leaves and consumers could account for their comparatively decreased $\delta^{13}C_{PUFA}$, as observed by Gladyshev et al. (2012). Among EFA, $\delta^{13}C_{ALA}$ values separated terrestrial and aquatic resources more distinctly than $\delta^{13}C_{LIN}$ values. Most grazer-$\delta^{13}C_{EFA}$ values were similar to those of epilithon while shredder-$\delta^{13}C_{EFA}$ values (i.e., *Leuctra* and *Gammarus*) were isotopically indistinguishable from conditioned leaves only. Predatory invertebrate-$\delta^{13}C_{EFA}$ values (i.e., *Perla*) did not differ from epilithon- or from other non-predatory invertebrate-$\delta^{13}C_{EFA}$ values, indicating that epilithon-EFA were assimilated indirectly via consumption of other invertebrate prey (i.e., grazers). However, it is possible that we have missed other potentially important food sources for grazers (e.g., bryophytes; Gladyshev et al., 2012; Hayden et al., 2016; McWilliam-Hughes et al., 2009) or have underestimated their capability in LIN-synthesis (Malcicka et al., 2018) as indicated by lower grazer-$\delta^{13}C_{EFA}$ values. Further, the significant isotopic difference of consumer-$\delta^{13}C_{EFA}$ values from fresh leaf-$\delta^{13}C_{EFA}$ values suggests that consumers fully reliant on terrestrial subsidies were absent in streams (Brett et al., 2017). The high phenol and lignin content of terrestrial matter and the lack of ligninolytic enzymes in most aquatic insects (Breznak & Brune, 1994; Geib et al., 2008) make fresh leaves a poor diet but conditioned leaf litter (overgrown with biofilms) can serve as an additional energy and food source for stream consumers (Guo et al., 2016; Hayden et al., 2016; Kühmayer et al., 2020).

Previously, we showed high FA similarity between epilithon and salmonid-eyes that contained high levels of ALA and palmitoleic

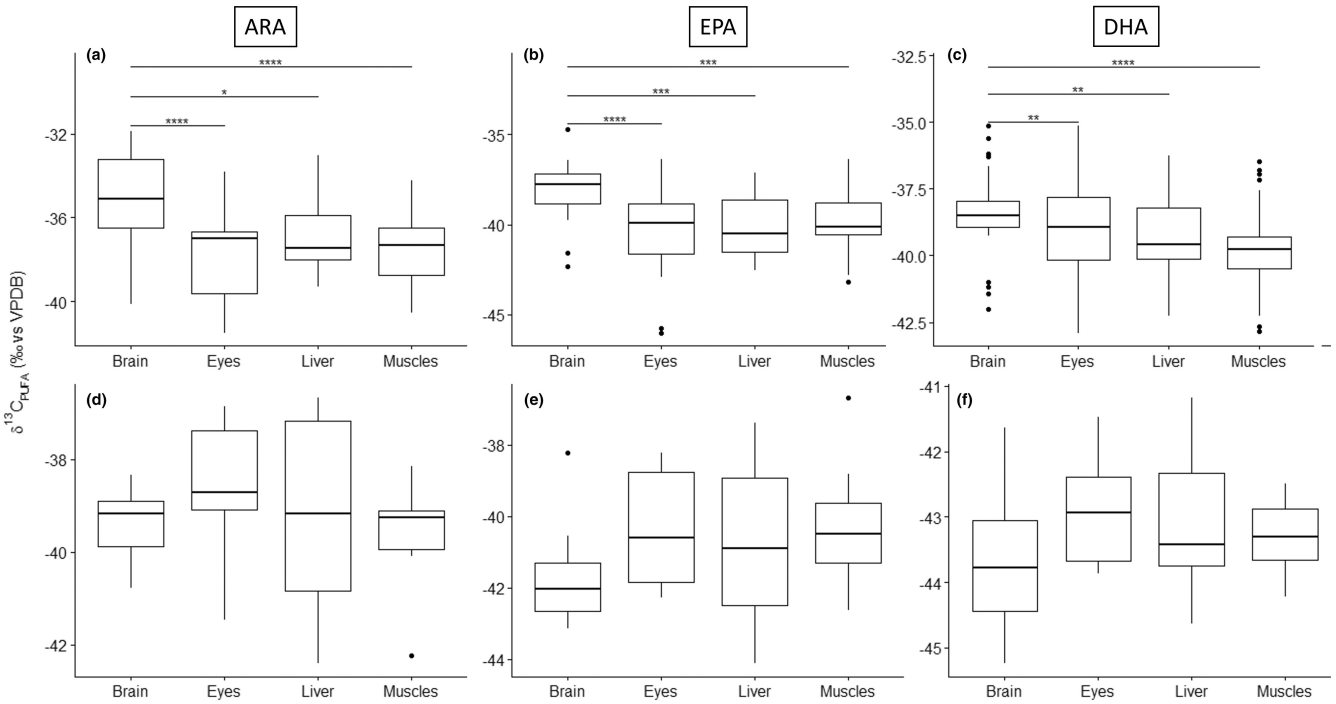

FIGURE 4 Boxplots (line = median; box = interquartile range centred at the median; whiskers = 3 × interquartile range; circles = outliers) show the variation in different compound-specific stable isotope values (‰) of long-chain polyunsaturated fatty acids ($\delta^{13}C_{LC\text{-}PUFA}$) across organs of (a–c) Salmonids, (d–f) European bullhead. The mean isotopic difference in each LC-PUFA (ARA, arachidonic acid, A + D; DHA, docosahexaenoic acid, C + F; EPA, eicosapentaenoic acid, B + E) between organs of European bullhead ($n = 9$) and respective livers was close to zero but were were significantly ^{13}C enriched salmonid brains ($n = 22$) compared to respective livers. Stable isotope values were expressed in δ notation with respect to international standards for $\delta^{13}C$ (Vienna Pee Dee Belemnite, VPDB) $= \left[\left(R_{sample} / R_{standard}\right) - 1\right] \times 10^3$ (expressed in‰), where R is $^{13}C/^{12}C$. Asterisks indicates significant differences ($p < 0.05$) in specific PUFA isotope ratios between livers and respective livers (paired tests, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

acid (16:1n-7) within their neutral lipids (Ebm et al., 2021). Tissues rich in neutral lipids are useful for trophic studies because they resemble their diet well. However, the coelution of 16:1 isomer prevented further isotopic analysis for tracking the origin of 16:1n-7. Consequently, we decided to use other prevalent eye FA (i.e., ALA and LIN) in this current study. Isotopic values of EFA in salmonid-eyes were significantly ^{13}C-depleted than their respective livers- or terrestrial-$\delta^{13}C_{EFA}$ (conditioned and fresh leaves) values but showed high similarities with potential dietary-EFA sources (i.e., invertebrates and epilithon), suggesting that algal rather than terrestrial PUFA directly and/or indirectly support fish, including their metabolically most active organs (i.e., eyes and brains).

Compound-specific stable isotopes confirmed that all consumers retained algal LC-PUFA (EPA and ARA), irrespective of their trophic levels. Although algae grew on both substrates (cobbles and leaf litter) in streams, epilithon-$\delta^{13}C_{EPA}$ values were significantly lower compared to those of conditioned leaves. Such isotopic differences enabled us to further differentiate both potential EPA-sources from each other in consumers. For other LC-PUFA (ARA and DHA) isotopic values of possible food sources (epilithon vs. conditioned leaf litter) were indiscriminate. Consumer-$\delta^{13}C_{EPA}$ values were significantly lower compared to conditioned leave-$\delta^{13}C_{EPA}$ values but close to epilithon-$\delta^{13}C_{EPA}$ values without any trophic fractionation, which contrasts with previous studies (Gladyshev et al., 2012). Our data suggest that most consumers assimilated epilithon-EPA with high efficacy and routed it directly or indirectly (i.e., predatory invertebrates and fish) into animal tissues without any further modification. By this strategy, consumers may save metabolic costs that would otherwise be required for internal PUFA production (Scharnweber et al., 2021; Twining et al., 2016; Vesterinen et al., 2022). Furthermore, higher invertebrate-$\delta^{13}C_{ARA}$ and $\delta^{13}C_{DHA}$ values relative to potential food sources imply that the majority of LC-PUFA are probably derived directly from autochthonous basal resources but not from endogenous PUFA conversion.

Although *Gammarus*-$\delta^{13}C_{EPA}$ did not vary significantly from other collected invertebrates, their slightly lower $\delta^{13}C_{EPA}$ values suggested EPA acquisition from sources other than epilithon, e.g., consumption of EPA-containing organic sediment particles (Ahlgren et al., 2003; Kolanowski et al., 2007; Makhutova et al., 2003) or by internal LC-PUFA biosynthesis. These alternative strategies may be necessary for gammarids because of low EPA availability in biofilms on submerged leaves (<1%) compared to epilithon (7%–17%) (Ebm et al., 2021). Lower invertebrate- and fish liver-$\delta^{13}C_{SDA}$ values could further indicate that at least SDA and small amounts of EPA were of endogenous origin in some consumers. Even though genetic analyses demonstrated that many of them are capable of PUFA-synthesis (Monroig & Kabeya, 2018), feeding experiments suggest this process is very limited on adequate diets (Crenier et al., 2017; Hixson

TABLE 3 Univariate difference tests of compound-specific stable isotope values of polyunsaturated fatty acids ($\delta^{13}C_{PUFA}$) between fish livers (salmonids and European bullhead) and their respective livers.

Fish group	PUFA	Organ	$\delta^{13}C_{PUFA}$ (‰)	liver-$\delta^{13}C_{PUFA}$ (‰)	*n*	Method	Statistic	*df*	*p*. adj	Effect size
Salmonid	ARA	Brain	−35.2 ± 2.2	−36.9 ± 1.6	19	*t*-test	3.392	18	0.020	0.78
		Eyes	−37.8 ± 2.2				−1.585	18	0.780	–
		Muscle	−37.4 ± 1.8				1.249	18	0.999	–
	EPA	Brain	−38.1 ± 1.7	−40.0 ± 1.9	20	*t*-test	5.042	19	0.000	0.90
		Eyes	−40.4 ± 2.6				−0.782	19	0.999	–
		Muscle	−39.9 ± 1.9				−0.413	19	0.999	–
	DHA	Brain	−38.5 ± 1.9	−39.4 ± 1.5	21	*t*-test	4.129	20	0.003	1.13
		Eyes	−39.2 ± 2.2				0.762	20	0.999	–
		Muscle	−39.7 ± 1.9				1.201	20	0.999	–
European bullhead	ARA	Brain	−39.4 ± 0.8	−39.2 ± 2.3	7	Wilcoxon rank-sum test	12.000	6	0.999	–
		Eyes	−38.6 ± 1.6				17.000	6	0.999	–
		Muscle	−39.7 ± 1.3				18.000	6	0.999	–
	EPA	Brain	−41.6 ± 1.6	−40.7 ± 2.5	8	*t*-test	−0.877	7	0.999	–
		Eyes	−40.4 ± 1.7				0.377	7	0.999	–
		Muscle	−40.3 ± 1.9				−0.362	7	0.999	–
	DHA	Brain	−43.7 ± 1.1	−43.0 ± 1.2	8	Wilcoxon rank-sum test	11.000	7	0.999	–
		Eyes	−42.9 ± 0.8				19.000	7	0.999	–
		Muscle	−43.3 ± 0.6				21.000	7	0.999	–

Note: All statistical tests (*t*- or Wilcoxon rank-sum tests) used were based on paired samples (each subject has a pair of measurements) where the alternative hypothesis (H1) assumes that mean differences are not equal to zero (organs differ from respective livers). Summary statistics of $\delta^{13}C_{PUFA}$ are reported as mean ± 1 *SD*‰, sample size (*n*). In addition, we reported adequate effect size values (Sullivan & Feinn, 2012) where significant *p*-values were observed. Effect size was classified as small (<0.2), medium (<0.5), or large effect (≥0.8).

Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

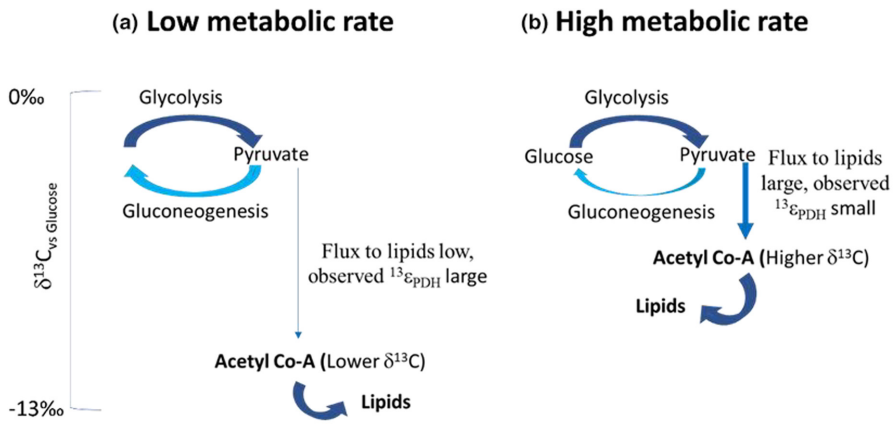

Figure 5

FIGURE 5 Biosynthetic control of $\delta^{13}C$ variation in lipids based on Fang et al. (2006), Fry (2013), Melzer & Schmidt (1987), and Hayes (2001). (a) Low metabolic rate: decarboxylation of pyruvate by pyruvate dehydrogenase involves a kinetic isotope effect ($^{13}\varepsilon_{PDH}$) that imparts low $\delta^{13}C$ values to acetyl CoA (i.e., values are lower than the value of dietary pyruvate and its parent glucose, here set at 0‰). Under conditions of low growth most pyruvate is reconverted to glucose via gluconeogenesis, this fractionation is larger, with −13‰ at the extreme expected for acetyl CoA. (b) high metabolic rate: The $^{13}\varepsilon_{PDH}$ is small and near 0‰ when pyruvate fluxes are mostly towards acetyl CoA, when all carbon regardless of isotopic composition is being used. With this scheme, high growth rates in microbes on leaf detritus may increase $\delta^{13}C_{PUFA}$, while lower mass-specific growth rates in consumers could account for their decreased $\delta^{13}C_{PUFA}$, as observed by Gladyshev et al. (2012) and in this study.

et al., 2014; Kühmayer et al., 2020). Thus, benthic macroinvertebrates in headwater streams provide an important PUFA link between algae and higher trophic levels, such as fish, and also terrestrial consumers, including spiders, birds, and bears via their emergence (Gladyshev et al., 2009; Koussoroplis et al., 2008; Martin-Creuzburg et al., 2017; Mathieu-Resuge et al., 2022).

In contrast to invertebrates that selectively retained all their PUFA from the diet, substantial levels of fish-DHA were produced endogenously by both fish groups (Salmonids and European bullhead) as indicated by their ^{13}C-depleted liver-DHA. Previous research demonstrated that DHA is abundant in all fish organs but is virtually absent in most stream invertebrates (Ebm et al., 2021; Guo et al., 2017; O'Mara et al., 2022). To overcome DHA-deficiency, dietary precursor-PUFA (i.e., ALA, SDA, or EPA) are transformed in serial desaturation and elongation steps inducing isotopic fractionation. The ability of salmonids and other fish species to convert precursor-PUFA enzymatically under PUFA-poor conditions has been shown experimentally before (Garrido et al., 2020; Murray et al., 2014; Xu & Kestemont, 2002). However, only a few studies have used $\delta^{13}C_{PUFA}$ at natural abundance levels to assess PUFA conversion rates (Hixson et al., 2014; Tocher & Sargent, 1990) or have shown that *de novo* DHA synthesis occurs under natural conditions in wild fish (Fujibayashi et al., 2019; Scharnweber et al., 2021).

Most salmonid brain-$\delta^{13}C_{LC\text{-}PUFA}$ values were significantly ^{13}C-enriched compared to their respective liver-$\delta^{13}C_{LC\text{-}PUFA}$ values. This implies that LC-PUFA might not originate from internal production within salmonid brains but were allocated from their livers and retained for long periods. However, local LC-PUFA conversion probably happened within salmonid eyes or in European bullhead brains, as indicated by the absence of clear differences between livers and respective organs. Our results contrast with previous *in vitro* studies on salmonid astrocytes (Mourente & Tocher, 1998; Tocher, 1993) and gene expression analysis (Carvalho et al., 2022; Tocher et al., 2006), which hypothesised that PUFA were converted in brains cells as well as in hepatocytes. However, such strong compensatory reactions in cod (Carvalho et al., 2022; Tocher et al., 2006) were generated under chronic PUFA-malnutrition and might derive from a series of metabolic reactions (i.e., prolonged DHA-retention times, increased hepatic DHA-synthesis and allocation) rather than by local PUFA conversion in brains only. The current study supports the D_5-ALA labelling experiment of Bell et al. (2001) where the highest D_5-DHA concentrations were recovered from eye and liver samples of *O. mykiss* but only small quantities of ^{13}C-depleted or recently produced DHA were detected in brains. The organ-$\delta^{13}C_{LC\text{-}PUFA}$ values of European bullhead were all lower than those of salmonids, which may be explained by phylogenetic differences in lipid metabolism or by increased PUFA conversions during gonadal development to meet requirements for prospective reproduction in *C. gobio* (Henrotte et al., 2011; Nogueira et al., 2017; Tocher, 2003, 2010). Another explanation for such strong differences between both fish groups may relate to fish age. Both salmonid taxa consisted predominately of juvenile fish (4–114 g) while the majority of European bullhead specimen were reproductive adults. Young salmonids may still show signals from yolks, particularly in terms of LC-PUFA due to high retention times. Relatively slow growth of fish brains, as stated by Bell et al. (2001), might even reinforce their ^{13}C-enrichment in LC-PUFA. The transgenerational transfer of maternal PUFA to offspring brains remains as yet vague (Fuiman & Perez, 2015; Steinberg, 2018). However, under increased PUFA-demand (i.e., reproductive activities), such local PUFA conversion in fish brains may occur to support normal brain function.

Although carbon stable isotope analysis provides a cost-effective and non-invasive method to track small quantities of important organic compounds across habitats, ecosystems and multiple organs within a single individual, its interpretation is complex and potentially biased without specifying discrimination factors resulting from the sum of various enzymatic processes during PUFA metabolism (e.g., β-oxidation, hydrolysis, or esterification reactions) in consumers (Budge et al., 2016). So far, much of our knowledge about isotopic fractionation of PUFA during trophic transfer, assimilation, and conversion remains insufficient and conflicting (Burian et al., 2020; Gladyshev et al., 2016; Twining et al., 2020) and further research must consider factors such as dietary quality, PUFA species, consumer identity and sampled tissue (Bec et al., 2011; Gladyshev et al., 2016; Taipale et al., 2014). Our study was undertaken in late autumn/winter when algal-PUFA could easily satisfy slowly growing consumers, which may not be the case in warmer months. To address these issues, future research needs to include field measurements at different times of the year, and laboratory experiments with different species, feeds, and conditions to quantify estimates of trophic dynamics (e.g., conversion rates) of critical dietary PUFA in aquatic consumers.

We conclude that the application of carbon stable isotope analysis at natural abundance levels provides evidence that consumers in oligotrophic, temperate, and dietary DHA-limited aquatic systems in winter, preferentially retained EFA from epilithon or conditioned leaves rather than allochthonous PUFA (i.e., fresh leaves) without significant PUFA production through internal conversion (high retention of algal-EPA). Among LC-PUFA, only fish-DHA was produced endogenously in substantial amounts by livers. There is, however, only weak isotopic evidence for extra de novo LC-PUFA synthesis in fish brains or eyes under natural conditions.

AUTHOR CONTRIBUTIONS

Conceptualisation and developing methods: M.T.B., S.E.B., B.F., and M.J.K. Conducting the research: N.E., F.G., and M.J.K. Data analysis and preparation of figures and tables: N.E. Data interpretation: N.E., B.F., and M.J.K. Writing: N.E., F.G., M.T.B., S.E.B., B.F., and M.J.K.

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CONFLICT OF INTEREST STATEMENT

We have no conflicts of interest to disclose and confirm that we abide by the statement of publication ethics.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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Zusammenfassung

Diese Dissertation untersuchte die ökologische Bedeutung von ***benthischen Algen*** in kleinen und beschatteten Flüssen, wobei der Schwerpunkt auf den aus Algen stammenden ***mehrfach ungesättigten Fettsäuren (PUFAs)*** und deren Beitrag zur Sekundärproduktion in Flussökosystemen lag. Diese Forschungsarbeit befasste sich nicht nur mit der Frage, ob Konsumenten in Flüssen (z. B. benthische Makroinvertebraten und Fische) essenzielle Nährstoffe wie langkettige PUFAs (LC-PUFAs) hauptsächlich aus autochthonen (benthischen Algen, Epilithon) oder allochthonen (terrestrischen) Nahrungsressourcen beziehen, sondern berücksichtigte auch interne Faktoren wie die endogene Synthese von LC-PUFAs aus Vorläufer-Fettsäuren. Diese sind entscheidend für den Aufbau und die Zusammensetzung von Nervengewebe bei Vertebraten (z. B. Gehirn und Augen). Diese Thematik ist von zentraler Bedeutung, da sie langjährige ökologische Paradigmen wie das ***River Continuum Concept*** (RCC) anzweifelt, das traditionell den terrestrischen Eintrag in Fließgewässern höher bewertet als die Eigenproduktion und damit die ökosystemare Funktion und folglich die ökologische Bedeutung von Fließgewässern systematisch unterschätzt.

Die vorliegenden Studien verfolgten den Ursprung und den Fluss essenzieller Fettsäuren in Nahrungsnetzen mithilfe von Freilanduntersuchungen, Fütterungsexperimenten und/oder Isotopenanalysen. Alle durchgeführten Untersuchungen zeigten, dass Epilithon – und nicht, terrestrische Einträge aus dem Umland – die Hauptquelle von LC-PUFAs für Wirbellose als auch Fische sind. Diese Erkenntnisse tragen erheblich zum Verständnis über Nährstoff- und Energiekreisläufe aquatischer Ökosysteme bei und heben die Rolle von Algen in Fließgewässern hinsichtlich der Versorgung von Konsumenten mit ***Eicosapentaensäure*** (EPA) entscheidend hervor.

Diese Forschungsarbeit überarbeitete aktuelle ökologische Modelle zur ***Produktivität von Fließgewässern*** und unterstreicht die Notwendigkeit, Algen als Nahrungsressourcen in Flüssen zu schützen und zu erhalten, insbesondere in Anbetracht der erwartet nachteiligen Auswirkungen des Klimawandels auf die Nährstoffqualität und dessen Gehalt in Algen aus temperatur-empfindlichen