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RESEARCH ARTICLE

Hydrogen isotopes (δ^2H) of polyunsaturated fatty acids track bioconversion by zooplankton

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Abstract

1. Organisms at the base of aquatic food webs synthesize essential nutrients, such as omega-3 polyunsaturated fatty acids (n-3 PUFA), which are transferred to consumers at higher trophic levels. Many consumers, requiring n-3 long-chain (LC) PUFA, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have limited ability to biosynthesize them from the essential dietary precursor α-linolenic acid (ALA) and thus rely on dietary provision of LC-PUFA.
2. We investigated LC-PUFA metabolism in freshwater zooplankton using stable hydrogen isotopes (δ^2H) of fatty acids as tracers. We conducted feeding experiments with the freshwater keystone grazer *Daphnia* to quantify changes in the δ^2H value of body FA in response to the FA composition of their food and the δ^2H value of the ambient water.
3. The isotopic composition of LC-PUFA changed in *Daphnia*, depending on the integration of ^2H from ambient water during de novo synthesis or bioconversion from dietary precursors, allowing us to distinguish dietary from bioconverted EPA in body tissue. We tested the applicability of these laboratory findings in a field setting by analysing δ^2H values of PUFA in primary producers and consumers in eutrophic ponds to track EPA sources of zooplankton.
4. Multilinear regression models that included conversion of ALA to EPA correlated better with zooplankton δ^2H_{EPA} than seston δ^2H_{EPA} at low dietary EPA supply.
5. This study provides evidence that zooplankton can compensate for low dietary EPA supply by activating LC-PUFA biosynthesis and shows that herbivorous zooplankton play a crucial role in upgrading FA for higher trophic levels during low dietary EPA supply.

KEYWORDS
bioconversion, compound-specific stable isotopes, deuterium, ecophysiology, eutrophication, polyunsaturated fatty acids, trophic ecology, zooplankton

1 | INTRODUCTION

Tracing and quantifying the transfer of dietary energy and nutrients within aquatic food webs is critical for understanding trophic interactions among species. For decades, stable isotopes of carbon (^{13}C), nitrogen (^{15}N) and hydrogen (^{2}H) from bulk tissue samples have been routinely applied in trophic ecology as natural diet tracers (Doucett et al., 2007; Fry, 2006; Kling et al., 1992). Fatty acids (FA) are increasingly used as specific dietary source biomarkers in aquatic consumers and for disentangling autochthonous versus allochthonous diet sources (Nielsen et al., 2018). The FA content of organisms enables researchers to evaluate different diet sources (Brett et al., 2009, 2017; Galloway et al., 2014; Kainz & Mazumder, 2005; Nielsen et al., 2018), but relies on the assumption that dietary FA remain unaltered in consumers. Moreover, models from controlled experiments need to take food limitations into account, which might change the FA profiles of consumers (Taipale et al., 2015; Twining et al., 2020). Omega-3 polyunsaturated fatty acids (n-3 PUFA), such as α-linolenic acid (ALA; 18:3n-3), eicosapentaenoic acid (EPA; 20:5n-3) or docosahexaenoic acid (DHA; 22:6n-3), are particularly important for trophic studies (Carlson & Neuringer, 1999), since most consumers are unable to synthesize them de novo, that is, from low-molecular-weight precursors. However, various animals have been shown to be capable of elongating and desaturating dietary C18 PUFA to C20 PUFA to adapt their n-3 PUFA profile to their specific physiological needs, which is a complex process involving several enzymes and comes at higher energy costs than direct dietary acquisition (Taipale et al., 2011; Twining et al., 2016).

Compound-specific stable isotope analyses (CSIA) of FA allow natural tracing of FA sources and, compared to bulk stable isotope analysis, are more informative at disentangling food web interactions (Pilecky, Kämmer, et al., 2021; Pilecky, Winter, et al., 2021; Twining et al., 2020). The typically large $\delta^{2}H$ differences between aquatic versus terrestrial sources (Hobson et al., 2020; Solomon et al., 2009) offer a new and promising approach for ^{2}H-CSIA to better understand the sources and fate of FA in food webs. The C–H bonds of FA are stable and their H atoms do generally not exchange with ambient water, thus distinctive dietary H isotope source signals are retained (Sessions et al., 2004). Furthermore, the linking of C and H in molecules implies that reaction kinetics, for example, during bioconversion, and their alteration due to the introduction of heavier isotopes may affect the isotopic composition of both elements.

While a similar potential exists for ^{13}C of FA, one major disadvantage of using carbon isotopes is the relatively small isotope source signal differences (Taipale, Vuorio, Brett, et al., 2016; Taipale, Vuorio, Strandberg, et al., 2016). The large stable isotope fractionations of $^{2}H/H$ in nature compared to $^{13}C/^{12}C$ may provide better resolution for tracking ecophysiological processes and resolving FA sources in terrestrial and aquatic ecosystems. One key advantage is that lipid biosynthesis leads to large ^{2}H depletion in the formed products compared to the ambient water (Chikaraishi et al., 2004; Sessions et al., 1999). During FA synthesis, H isotope fractionation is induced by variations in the H isotope composition of cellular water, acetate and the co-factor NADPH, and by different isotopologue kinetics (a) during uptake and utilization of diet, (b) via enzymes involved in the lipid biosynthetic pathways and (c) during FA allocation and metabolism (Zhang et al., 2009). Similarly, FA elongation and saturation introduces new ^{2}H atoms into the molecule, altering its $\delta^{2}H$ composition depending on environmental factors, such as diet, the isotopic composition of ambient water and temperature (Chikaraishi et al., 2004; Zhang & Sachs, 2007). While isotope fractionation during FA synthesis in primary producers has been investigated, nothing is known about the alteration of $\delta^{2}H$ values by consumers, including processes such as bioconversion of ALA to LC-PUFA.

We hypothesized that ^{2}H-CSIA tracks the integration of ^{2}H into LC-PUFA and thus provides a basis for differentiation among dietary and biosynthesized LC-PUFA. To test this, we conducted controlled laboratory experiments varying the dietary quality and $\delta^{2}H$ of ambient water to track isotope integration pathways (i.e. dietary vs. metabolic) into EPA, which is a key n-3 PUFA in consumers, including *Daphnia*. We also evaluated the effect of introducing excess of ^{2}H into FA, on the $\delta^{13}C$ value of the linked C. Subsequently, we tested this hypothesis in the field by analysing water, seston and zooplankton from eutrophic ponds during the summer period to investigate, based on compound-specific $\delta^{2}H$ values, the origin of EPA in zooplankton by applying predictive models that tested for bioconversion or dietary allocation of FA.

2 | MATERIALS AND METHODS

2.1 | Controlled feeding experiments

The unicellular green alga *Acutodesmus obliquus* (Kützing, 1833, formally known *Scenedesmus obliquus*) and the cryptophyte *Cryptomonas ozolinii* (Skuja, 1939) were cultured separately in 1 L Wright's cryptophyte (WC) medium at 20°C (Guillard & Lorenzen, 1972). Both algae are easily ingestible for *Daphnia* but differ in their nutritional quality with respect to PUFA composition (Taipale et al., 2013). *A. obliquus* lacks PUFA with more than 18 C-atoms, whereas *C. ozolinii* is rich in EPA and DHA (Ahlgren et al., 1992; Masclaux et al., 2009; Table S1). WC medium was spiked above natural abundance levels by using $^{2}H_{2}O$ (99.8% $D_{2}O$; Sigma-Aldrich) at two concentrations (*c*. 25, or 50 mg/L) to achieve increased $\delta^{2}H$ values compared to standard culture water. The measured $\delta^{2}H$ composition of the water in these three treatments was −78.9‰ ± 0.7 (reference), +92.2‰ ± 0.1 (25 mg/L) and +273.1‰ ± 10.0 (50 mg/L), relative to the VSMOW standard. The $\delta^{2}H$ values of culture medium were monitored as described below.

Laboratory feeding experiments were performed using a clone of the herbivore *Daphnia magna* (obtained from the University of Clermont Auvergne, France), kept in filtered (<0.7 μm) water (20°C) supplemented with 5% ADaM medium (Klüttgen et al., 1994) and fed ad libitum with the respective algae species before the start of the experiment, which kept them in a fast growing stage. For each run and treatment (in triplicate), 30 neonates (F_{1} generation) were

transferred into a single jar (400 ml). Before each feeding, the total organic carbon content of the algal cultures was adjusted via OD to supply ~1 mg C L^{-1} day^{-1} to *D. magna* in all treatments to ensure non-limiting diet supply (Lampert, 1978). Water was replaced weekly in all jars. The F_1 generation was fed algae that were pre-cultured for at least one month with −78.9‰, +92.2‰ or +273.1‰ ^2H_2O as tracer. After 7 days of feeding, and before the onset of egg production, ~20 individuals (~1.5 mg dw) of *Daphnia* were collected from each jar, freeze-dried and subjected to lipid extraction. The remaining individuals were retained to produce F_2 neonates, which were transferred into new jars under the same feeding and water treatments. Additionally, a subset of the F_2 offspring was kept in +273.1‰ spiked lake water/ADaM while fed with non-deuterium enriched algae to investigate how the δ^2H of ambient water affected the H isotope composition of FA in *Daphnia* (Figure 1). Comparing data from both generations revealed minor differences regarding isotopic composition of FA (Table S5; Figure S6), suggesting reported data from the second generation were at or close to dietary isotopic steady state. Experiments with small invertebrates did not require ethical approval.

2.2 | Field sampling

Water, dietary plankton (seston) and zooplankton were collected monthly from nine shallow (1–2 m depth) fishponds located in northern Austria (48°49′N, 15°17′E, 510 m) from June to September 2020. Permission for sampling was granted by the private owners. Pond water samples were collected in triplicate, filtered through a 30-μm mesh and stored in plastic vials for δ^2H analysis of the water. The most edible seston size fraction for zooplankton (<30 μm; Brooks & Dodson, 1965; Vanderploeg & Paffenhöfer, 1985) was sampled in triplicate from each pond using a Schindler trap, pre-filtered through a 30-μm mesh and retained on pre-weighed and pre-combusted GF/C filters (0.7 μm), and subsequently stored at −80°C until further analysis.

Zooplankton samples were collected in triplicate from each pond by gently pulling an Apstein's zooplankton-net (500 μm; HydroBios GmbH) below the surface (~0.5 m). Zooplankton size fractions (>500, 500–250 and <250 μm particle size) were collected in sterile Falcon® tubes (50 ml). Both the filters and zooplankton were frozen (−80°C), freeze-dried (Virtis Genesis Freeze dryer, for minimum 24 hr) and subsequently stored frozen (−20°C) until further processing. Zooplankton samples (>500 μm) from one sampling (July) were separated into cladocerans, copepods and *Chaoborus* larvae for taxa-specific isotope analysis. Because no significant differences in δ^2H values of FA were detected, all other samples were processed as homogenized bulk samples.

2.3 | Gas chromatography (GC) and isotope ratio mass spectrometry (IRMS)

Lipids were extracted from all samples according to Heissenberger et al. (2010). Briefly, freeze-dried samples were homogenized and mixed with chloroform:methanol (2:1 V/V) following the addition of 0.9% NaCl, sonication, vortexing and centrifuging 3× to remove non-lipid materials. Extracted lipids were evaporated to a final volume of 1.5 ml under N_2 gas flow. For FAME formation, samples were incubated with sulphuric acid:methanol (1:100 V/V) for 16 hr at 50°C, following the addition of $KHCO_3$ and hexane. Samples were shaken, vortexed and centrifuged and the upper organic layers collected, pooled and concentrated under a N_2 gas flow.

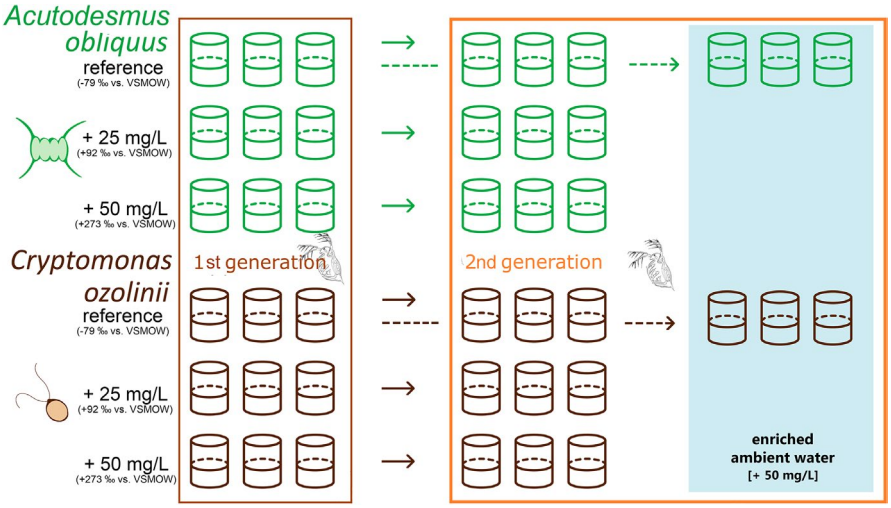


FIGURE 1 Outline of the controlled laboratory experiment. The two algae *Scenedesmus obliquus* and *Cryptomonas ozolinii* were grown in medium spiked with different ^2H_2O concentrations (−78.9‰, +92.2‰ or +273.1‰, VSMOW) and subsequently fed to the first generation of *Daphnia*. Neonates of the second generation were raised under the same conditions as their mothers, except for those receiving the non-deuterium enriched diets. This group was split with one half kept in ^2H enriched ambient water while still receiving non-enriched diet (blue square), while the other half was kept in a non-enriched environment and served as control. Data of first and second generation were compared and suggested dietary isotopic steady state for generation 2 (see Supporting Information)

Fatty acid methyl esters (FAME) were quantified using a gas chromatograph (TRACE GC ThermoFisher Scientific, Detector: 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 for FAME separation and quantification. Chromeleon 7 software (ThermoFisher) was used for peak integration. FAME were identified by comparison of retention times to known reference standards (37-component FAME mix, 47885-U, Supelco; Sigma-Aldrich). Fatty acid concentrations were quantified using calibration curves based on different known standard concentrations. FA are reported as μg/mg dry weight after applying a conversion factor for each individual FAME accounting for the mass fraction of the methyl group.

^{2}H- and ^{13}C-CSIA were performed according to Pilecky, Winter, et al. (2021), Pilecky, Kämmer, et al. (2021), using a Thermo Trace 1310 GC (ThermoFisher), coupled by a ConFlo IV interface (ThermoFisher) to a continuous-flow isotope-ratio mass spectrometer (DELTA V Advantage, ThermoFisher). FAME were separated using either a VF-WAXms 60 m column, 0.25 mm ID, film thickness 0.25 μm; or a VF-WAXms 30 m column, 0.32 mm ID, film thickness 1 μm (both Agilent). The temperature programme for the 60 m GC column started at 80°C, held for 2 min, after which the temperature was ramped 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 then maintained for 30 min. The temperature programme for the 30 m GC column started at 80°C, held for 2 min, after which the temperature was ramped by 30°C/min to 175°C, and then by 5°C/min to 240°C, which was held for 35 min. For δ^{13}C analysis, FAME were oxidized to CO_2 in an online combustion reactor filled with Ni, Pt and CuO wires, at a temperature of 1,000°C. For δ^2H analysis, FAME were pyrolysed to H_2 gas by passing them through a high temperature graphite conversion reactor at 1,200°C.

Samples were run against certified Me-C20:0 stable isotope reference material (USGS70: δ^{13}C = −30.53‰, δ^2H = −183.9‰, USGS71: δ^{13}C = −10.5‰, δ^2H = −4.9‰ and USGS72: δ^{13}C = −1.54‰, δ^2H = +348.3‰). Weighted-average combined δ^2H values of monounsaturated fatty acids (MUFA = C14:1, C16:1, C18:1) were obtained by integrating over all isoforms (e.g. C18:1n-7 + C18:1n-9) because no clear baseline separation could be achieved. FA δ^{13}C/δ^2H values (δI_{FA}) were corrected for the methyl group addition during methylation according to Pilecky, Winter, et al. (2021) and Pilecky, Kämmer, et al. (2021). Values for δ^{13}C are referenced to Vienna PeeDee Belemite (^{13}C:^{12}C = 0.01118).

$$\delta^{13}C_{FA} = \left(\frac{^{13}C/^{12}C_{Sample}}{^{13}C/^{12}C_{VPDB}} - 1 \right) \times 1,000.$$

Values for δ^2H are standardized against Vienna Standard Mean Ocean Water (^{2}H:^{1}H = 155.76 ppm).

$$\delta^{2}H_{FA} = \left(\frac{^{2}H/^{1}H_{Sample}}{^{2}H/^{1}H_{VSMOW}} - 1 \right) \times 1,000.$$

For δ^2H analysis of pond and experimental waters used, three replicates each sample were filtered before isotope analysis using a L2130-*I* (Picarro Inc.) and using the techniques described elsewhere (Coplen & Wassenaar, 2015). For waters with D_2O added, we used IAEA-604 (+799.0‰) and IAEA VSMOW2 (0.0‰) as our bracketing standards.

2.4 | Data analysis

Data analysis was conducted and plots were produced in R (Version 4.0.2, R Core Team, 2020) using the packages rstatix, ggplot2, ggpubr, lme4, relaimpo v2.2 and corrplot. Isotope data are summarized as the mean carbon or hydrogen isotope δ value ± *SD*. Unless indicated, values reported from the controlled experiment are from the second generation of *Daphnia* at dietary steady state. Paired *t* test and ANOVA were used for comparison of dietary and consumer compound-specific δ values. The Pearson method was used for linear correlations. Multiple linear regression models were developed by a stepwise backward elimination process (Ghani & Ahmad, 2010). Explanatory variables were accepted or eliminated based on the combination of *p*-values and Feldman's relative importance (Feldman, 2005; Grömping, 2006).

3 | RESULTS

3.1 | Fatty acid variation in δ^2H values

In the controlled laboratory feeding experiments, *Daphnia* fed *C. ozolinii* had significantly higher EPA contents than those fed *A. obliquus* (6.0 ± 1.9 and 1.1 ± 1.1 μg/mg, respectively, ANOVA, $F_1 = 110.5$, $p < 0.001$), but significantly lower ALA contents (18.0 ± 11.5 and 34.4 ± 5.7 μg/mg, respectively, ANOVA, $F_1 = 38.2$, $p < 0.001$; Table S1). The controlled feeding experiments using algae grown under various ^{2}H water treatments revealed a strictly dietary allocation pattern with no significant differences between diet and consumers in the δ^2H values of saturated FA (SFA = 14:0, 16:0, 18:0; ANOVA, $F_6 = 1.9$, $p = 0.172$), and the PUFA ALA (ANOVA, $F_3 = 2.4$, $p = 0.139$), stearidonic acid (SDA, 18:4n-3; ANOVA, $F_3 = 2.7$, $p = 0.117$) and gamma-linolenic acid (GLA, 18:3n-6; ANOVA, $F_3 = 1.42$, $p = 0.248$). Linoleic acid (LIN, 18:2n-6) was isotopically slightly ^{2}H-depleted in *Daphnia* (ANOVA, $F_3 = 5.5$, $p = 0.031$). *Daphnia* δ^2H values of MUFA showed diet-dependent ^{2}H enrichment; however, the δ^2H values of diet and consumer FA were different. EPA in *Daphnia* that were feeding on *A. obliquus*, which does not contain EPA, was significantly enriched in ^{2}H (−77.8‰ ± 43.5) compared to the δ^2H values of the dietary precursors ALA and SDA (−202.8‰ ± 3.07 and −219.7‰ ± 10.2; ANOVA, $F_5 = 7.8$, $p = 0.0017$). In contrast, *Daphnia* fed *C. ozolinii* showed similar δ^2H values compared to diet for 14:0 (ANOVA, $F_3 = 2.6$, $p = 0.130$), 16:0 (ANOVA, $F_3 = 5.2$, $p = 0.038$), 18:1 (ANOVA, $F_3 = 0.3$, $p = 0.586$), SDA (ANOVA, $F_3 = 1.9$, $p = 0.188$) and EPA (ANOVA, $F_3 = 0.4$, $p = 0.516$), whereas significant ^{2}H isotope

enrichment was detected in 18:0 (ANOVA, $F_3 = 21.7$, $p < 0.001$), 14:1 (ANOVA, $F_3 = 20.1$, $p < 0.001$), 16:1 (ANOVA, $F_3 = 9.0$, $p = 0.009$) and a slight but significant decrease in the δ^2H of LIN (ANOVA, $F_3 = 10.7$, $p = 0.006$) and ALA (ANOVA, $F_3 = 8.1$, $p = 0.013$; Figure 2).

The δ^2H composition of FA in *Daphnia* raised in ^{2}H-spiked water (+351.9‰ vs. reference treatment) showed no ^{2}H enrichment compared to the control for LIN (−85.1‰ ± 52.3 vs. −48.5‰ ± 55.8, *t* test, $p = 0.516$), GLA (−49.2‰ ± 42.8 vs. −66.5‰ ± 54.5, *t* test, $p = 0.736$) and ALA (−221.8‰ ± 8.4 vs. −219.5‰ ± 18.4, *t* test, $p = 0.886$), while MUFA were significantly ^{2}H enriched (−53.9‰ ± 8.0 vs. −110.7‰ ± 13.9, ANOVA, $F_3 = 54.3$, $p < 0.001$) with δ values close to those fed with 25 mg/L ^2H_2O-supplemented diet (−25.4‰ ± 16.7). ARA (−2.3‰ ± 11.1) and EPA (10.4‰ ± 10.7) of *Daphnia* fed *A. obliquus* were significantly enriched in ^{2}H compared to the control algae (−73.5‰ ± 18.8 and −77.8‰ ± 10.7, respectively; ANOVA, $F_2 = 21.7$, $p = 0.002$), while SDA was not significantly enriched in ^{2}H (−229.1‰ ± 40.0 vs. −246.9 ± 20.7, *t* test, $p = 0.641$). We found no effect of environmental water on δ^2H values of FA of interest for *Daphnia* fed with *C. ozolinii* raised in ^{2}H-enriched water compared to the control treatment (Figure 2).

3.2 | Fatty acid variation in δ^{13}C values

The introduction of ^{2}H via diet or ambient water did not change the δ^{13}C values for SFA (ANOVA, $F_3 = 0.1$, $p = 0.723$), ALA (ANOVA, $F_3 = 0.2$, $p = 0.628$) or LIN (ANOVA, $F_3 = 0.4$, $p = 0.527$). *Daphnia* raised in ^{2}H-enriched water were more depleted in ^{13}C compared to the control group with δ^{13}C values of 14:1 (−31.74‰ ± 0.98 vs. −27.10 ‰ ± 1.12; ANOVA, $F_3 = 26.0$, $p < 0.001$), 16:1 (−31.87‰ ± 2.88 vs. −28.89‰ ± 0.98; ANOVA, $F_3 = 6.5$, $p = 0.022$), GLA (−34.28‰ ± 2.04 vs. −30.57‰ ± 2.91; ANOVA, $F_3 = 4.5$, $p = 0.053$), SDA (−31.15‰ ± 1.94 vs. −29.22‰ ± 1.28; ANOVA, $F_3 = 4.1$, $p = 0.061$), ARA (−30.63‰ ± 1.68 vs. −27.89‰ ± 1.37; ANOVA, $F_3 = 8.05$, $p = 0.013$) and EPA (−32.73‰ ± 3.17 vs. −28.07‰ ± 0.53; ANOVA, $F_3 = 10.6$, $p = 0.006$; Figure 3).

3.3 | δ^2H and δ^{13}C analyses of LC-PUFA in pond zooplankton

The δ^2H values of water in the ponds varied only slightly over the sampling timeframe, although each pond was distinctive, which is

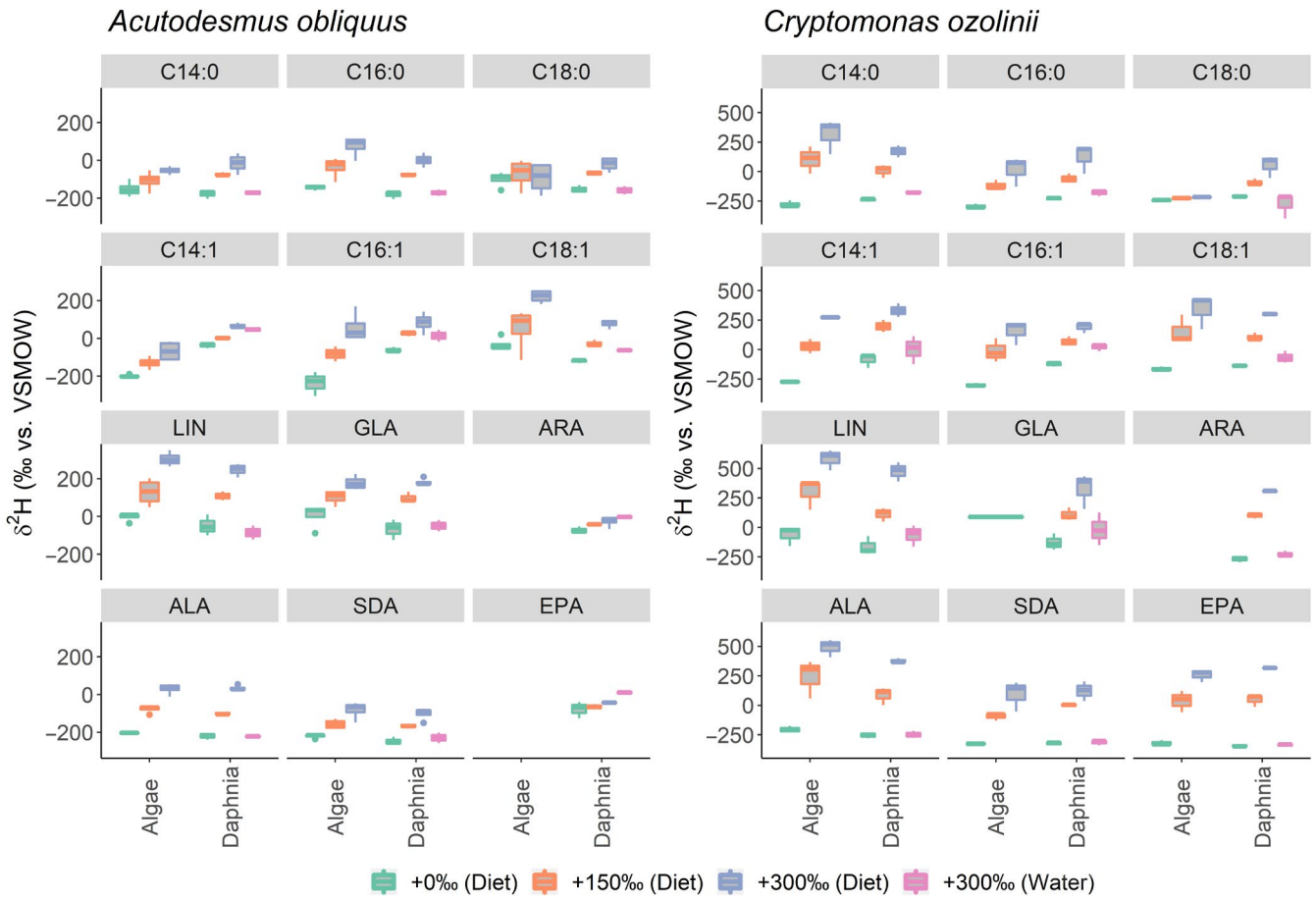


FIGURE 2 Compound-specific deuterium allocation into fatty acids of *Daphnia* via diet (green, orange and blue) or environmental water (purple). MUFA experienced significant changes in δ^2H values, as well as a significant enrichment in deuterium when *Daphnia* were raised in ^2H_2O, indicating FA synthesis. Strict dietary controlled allocation of fatty acids can be seen for LIN and ALA. Deuterium enrichment in ARA and EPA can be seen in *Daphnia* when raised in deuterium-enriched environmental water with poor diet quality (*Acutodesmus obliquus*) but not with high diet quality (*Cryptomonas ozolinii*) in which case EPA is also strictly dietary controlled

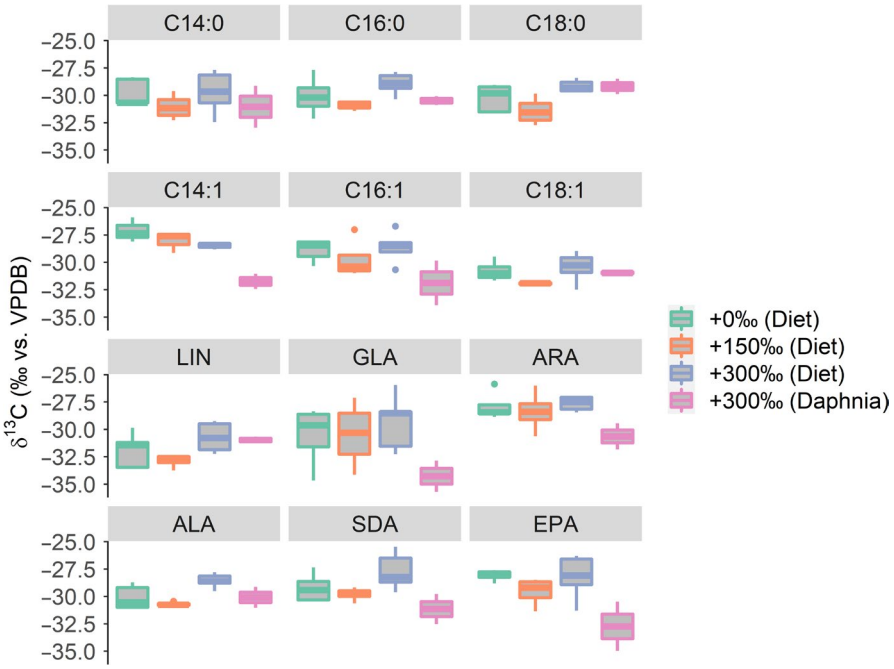


FIGURE 3 $\delta^{13}C$ values of FA detected in *Daphnia* fed *Acutodesmus obliquus*. No significant differences for FA could be seen for SFA, LIN and ALA. FA that are suspected to be de novo synthesized (14:1, 16:1), or had to be converted from a precursor (EPA, ARA) were significantly depleted in ^{13}C. A non-significant trend can be seen for the conversion intermediates GLA and SDA

relevant for establishing comparative H isotope baseline conditions (−48.9‰ ± 6.0; min: −59.1‰, max: −37.1‰). None of the ponds deviated from the local meteoric evaporation line, indicating a lack of progressive evaporation over the time frame of sampling. These eutrophic ponds had generally high chlorophyll-a concentrations (89 ± 38 μg/L), with the exception of two ponds (< 20 μg/L at any time; Table S4). Cladocerans were the predominant zooplankton taxa in all ponds (>50%). Six ponds also contained high numbers of calanoid copepods and *Chaoborus* larvae. Mass fractions of ALA, EPA and DHA varied in seston during the sampling period (Table S4; Figure 4c). EPA contents of zooplankton were independent of seston EPA (Pearson, $r = -0.039$, $p = 0.83$) and remained similar from June to August (7.6 mg/g ± 3.3; 6.5 mg/g ± 3.2; 6.7 mg/g ± 2.2) with a slight increase in September (10.5 mg/g ± 3.3; ANOVA, $F_{11} = 6.0$, $p < 0.001$).

A significant correlation was found between δ^2H composition of seston and zooplankton for all FA (Pearson; $p < 0.01$), except C14:1 ($p = 0.67$), C18:0 ($p = 0.05$), SDA ($p = 0.09$) and ARA ($p = 0.04$). Except for 14:0, all SFA were enriched in ^2H in zooplankton compared to seston (16:0: $t(31) = -9.44$, $p < 0.0001$; 18:0: $t(31) = -10.88$, $p < 0.0001$), while LC-PUFA were more depleted (ARA: $t(27) = 9.93$, $p < 0.0001$; EPA: $t(31) = 10.59$, $p < 0.0001$; DHA: $t(23) = 3.50$, $p = 0.0046$; Figure 4a). The ^2H depletion of EPA in zooplankton compared to seston was significantly correlated with a decrease in the EPA mass fraction in seston (Pearson, $r = 0.82$, $p < 0.001$). Similar correlations of $\Delta\delta^2H_{Zooplankton\text{-}Seston}$ and the respective FA mass fraction in seston were detected for ALA ($R = 0.61$, $p < 0.001$) and SDA ($R = 0.4$, $p = 0.025$; Figure 4b). Changes in δ^2H of the individual FA were independent of zooplankton taxa, and no significant differences between *Chaoborus* larvae, copepods or cladocerans were found (Figure S6; ANOVA, $df = 18$, $p = 0.053$).

The $\delta^{13}C$ composition did not significantly differ among n-3 PUFA (ALA, SDA, EPA; ANOVA, $F_3 = 0.4$, $p = 0.532$) or n-6 PUFA (LIN, ARA; ANOVA, $F_2 = 0.8$, $p = 0.380$) in both seston and zooplankton in all ponds (Figure S5).

3.4 | Conversion models for predicting EPA δ^2H values of zooplankton

To predict the δ^2H composition of zooplankton EPA, a multiple linear regression model was developed using stepwise backwards iteration to correlate δ^2H_{EPA} values of zooplankton to a combination of δ^2H values of other zooplankton and seston FA, as well as the δ^2H values of the environmental water. After iteration and convergence, the best fit model, using δ^2H of zooplankton FA and environmental water, was:

$$\delta^2H_{EPA} = 0.65\delta^2H_{ALA} + 0.40\delta^2H_{16:1} + 1.08\delta^2H_{H_2O} - 38.26.$$

Overall, predictions of δ^2H_{EPA} values using this model ($F_{4,27} = 16.42$, $p < 0.001$, $R^2 = 0.67$) were better than predictions based on correlations with dietary (i.e. seston) δ^2H_{EPA} ($F_{1,30} = 13.97$, $p < 0.001$, $R^2 = 0.32$). Other potential covariates, such as SFA or dietary and zooplankton SDA were insignificant and thus removed from the model iteration process. However, when considering only samples with high seston EPA (>0.6 mg/g), seston δ^2H_{EPA} values were a better predictor of zooplankton δ^2H_{EPA} than from the conversion model. The calculations of Feldman's relative importance revealed a shift in the explanatory value from somatic ALA, 16:1, and environmental water, associated with conversion to dietary EPA at values of approximately 0.3–0.5 mg/g dry weight of seston EPA (Figure 5a).

4 | DISCUSSION

The aim of this study was to assess the potential of using δ^2H to differentiate between dietary and bioconverted LC-PUFA, in particular EPA, of *Daphnia*. EPA of *Daphnia* was significantly enriched in ^2H when raised in ^2H-supplemented water on an EPA-free diet, showing

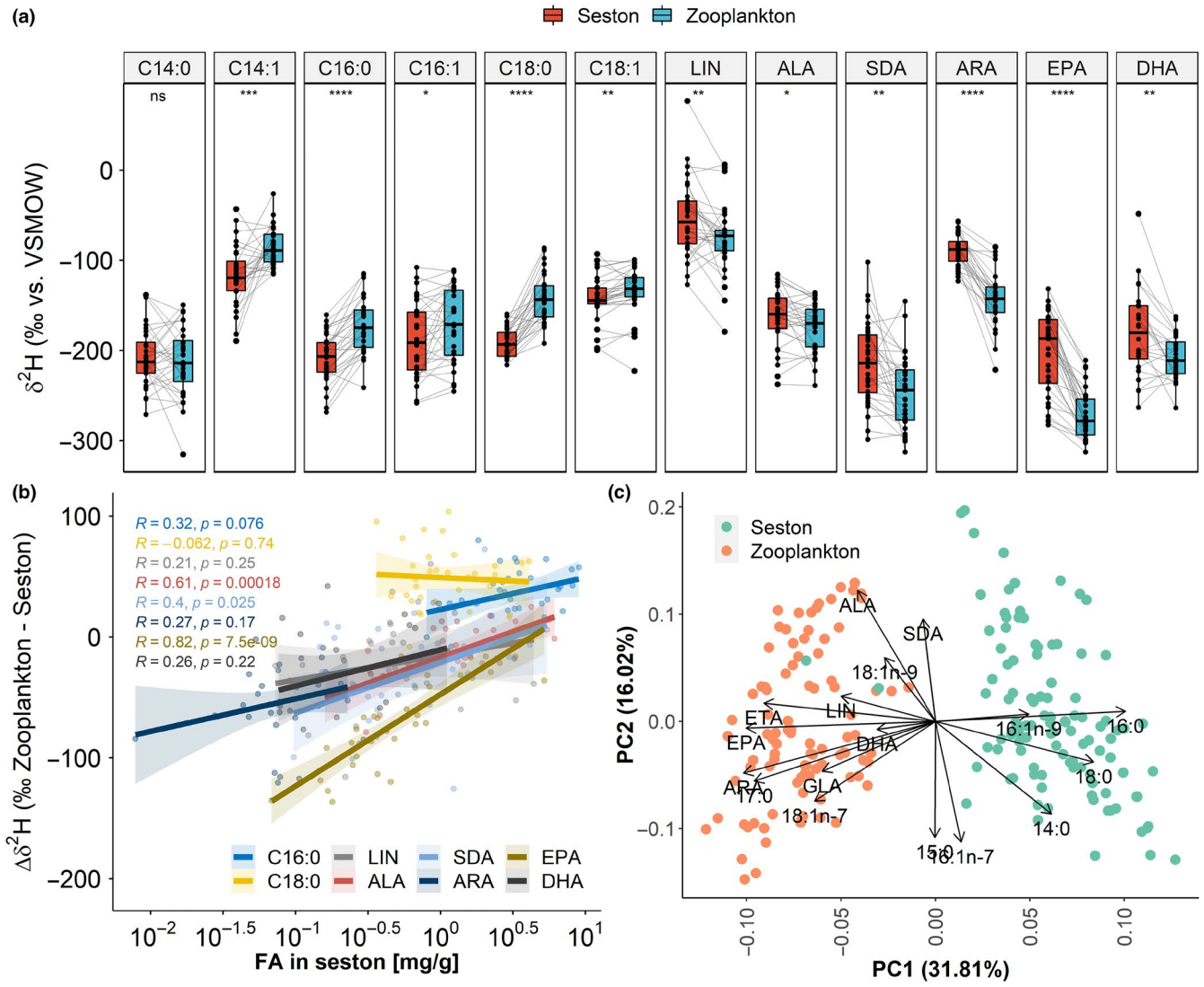
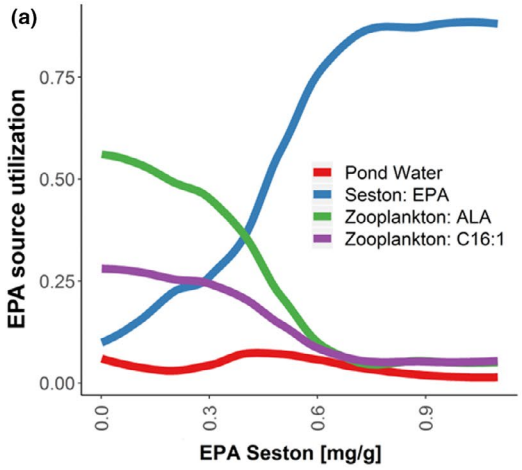


FIGURE 4 δ^2H and δ^{13}C in FA of seston (<30 μm size fraction) and zooplankton in nine different ponds sampled from June to September 2020. (a) Significant enrichment in ^{2}H of the saturated fatty acids C16 and C18 in zooplankton compared to seston, and significant depletion in ^{2}H of ARA and EPA. ns, not significant; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (b) Relationships between fatty acid mass fractions in seston and fractionation of hydrogen isotopes in fatty acids of zooplankton relative to seston; the isotopic fractionation of ^{2}H in EPA decreased strongly with increasing dietary EPA mass fractions (Pearson correlation values were calculated for differences in zooplankton to seston plotted vs. the FA concentration in the seston). (c) Principal component analysis of the relative FA composition of seston and zooplankton. Only FA contributing to more than 0.1% were considered

integration of H from ambient water during FA conversion and biosynthesis. Applying ^{2}H-CSIA to investigate FA bioconversion in consumers under field conditions revealed that biosynthesis of EPA in zooplankton increased with decreasing dietary EPA supply, whereby a critical threshold appeared to be at 0.5 mg EPA/g dry weight of seston. According to the environmental water δ^2H baseline values, bioconversion of ALA to EPA in *Daphnia* resulted in significant changes in δ^2H_{EPA} values. This study demonstrated that stable H isotopes of FA in aquatic organisms can be used in trophic ecology as a highly informative tracer to better understand how consumers use and/or convert dietary resources.

Integration of ^{2}H from environmental water into FA occurs during redox-reaction steps via NADH/NADPH, which is a common cofactor in many catabolic/anabolic processes (Yang & Sauve, 2016), leading to ^{2}H-enriched alkyl chains during FA synthesis and elongation (Baillif et al., 2009; Figure 5b). In general, the involvement of heavier isotopes decelerates reaction kinetics. Generally, if a reaction directly involves breaking a ^{12}C–^{2}H bond, it is seven times slower compared to a reaction breaking a ^{12}C–^{1}H bond (primary isotope effect; Westheimer, 1961). If the heavier isotope is not directly involved in a chemical reaction but is present in the non-reacting part of a heavier molecule ($M + 1$), the reaction rate constant (k) is also lower than the lighter isotopologue with mass (M). This results in reaction products with a lower ^{2}H isotopic content than their educts, that is, the originally used substrates (secondary isotope effect; Cleland, 2005; Maggi & Riley, 2010). In our case, we assume that k_{M+1} during FA elongation was constant for secondary isotope effects, regardless of whether the additional mass is introduced by substitution of ^{12}C with ^{13}C or ^{1}H with ^{2}H. Thus, if an



	Estimate	SE	t value	p-value	rel. imp.
Conversion Model: R^2 = 0.68, p = 4.85e-07					
(Intercept)	−38.26	35.21	−1.087	0.2865	
Zooplankton: ALA	0.65	0.11	5.74	<0.0001	0.57
Zooplankton: C16:1	0.40	0.08	5.09	<0.0001	0.33
Pond H_2O	1.08	0.52	2.04	0.0506	0.09
Dietary allocation Model: R^2 = 0.32, p = 0.0008					
(Intercept)	−203.28	18.89	−10.76	<0.0001	
Seston: EPA	0.34	0.09	3.74	<0.0001	

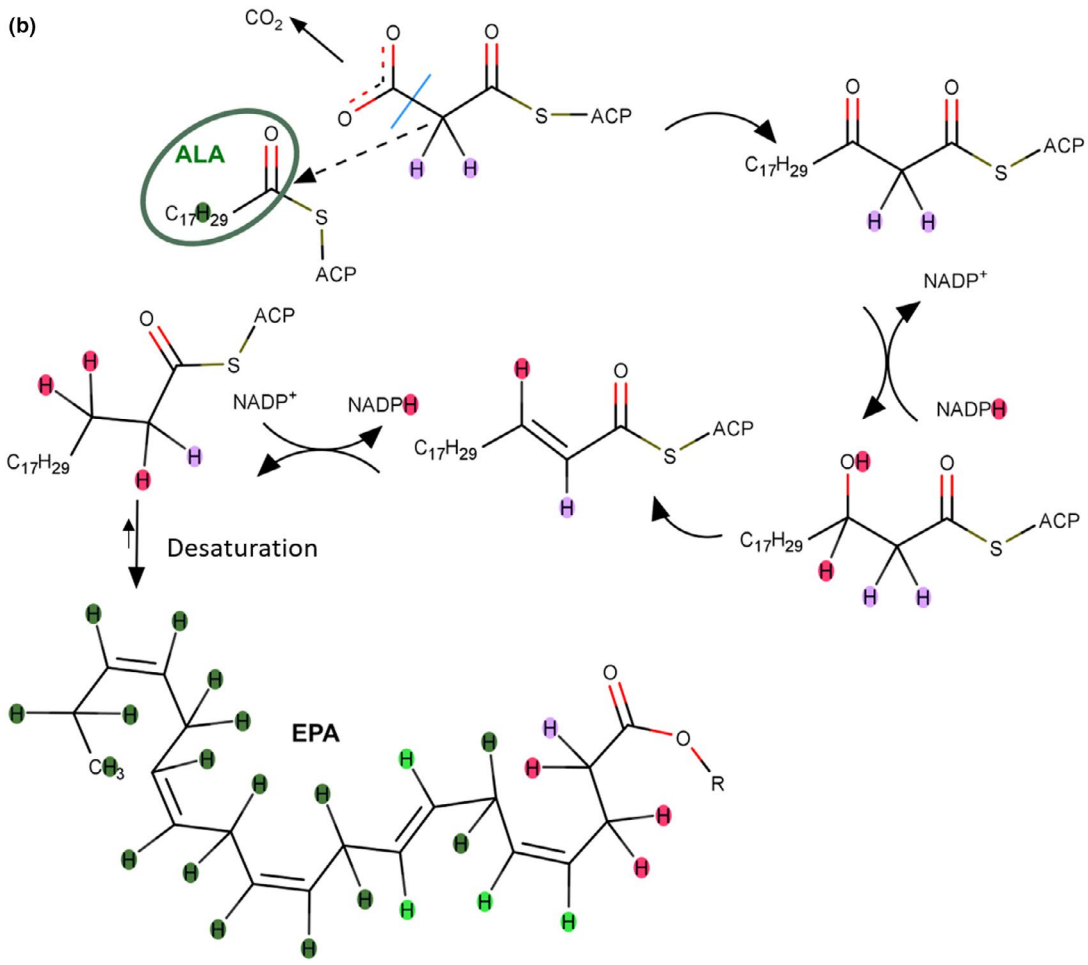


FIGURE 5 Multiple linear regression models (MLRM) based on δ^2H values of FA suggest significant conversion activity in zooplankton in eutrophic ponds. (a) In a MLRM using environmental water, zooplankton ALA (precursor), C16:1 (proxy for FA synthesis) and seston EPA δ^2H values, a switch in Feldman's relative importance, calculating their contribution to the model at the respective level, occur at mass fractions between 0.3 and 0.5 mg/L dry weight in seston indicating transition from conversion to dietary allocation at this threshold. (b) Tracking H atoms during biochemical conversion of ALA to EPA by fatty acid synthase and other involved enzymes displays the involvement of water H atoms (red) various energy sources (lilac) and H atoms subjected to isotopic fractionation during the potentially reversible desaturation processes (light green)

increasing amount of ^{2}H is introduced into the acyl chains, a correspondingly lower amount of ^{13}C-containing isotopomers is processed compared to the isotopically lighter ^{12}C-all-^{1}H-alkyl molecules, leading to an overall ^{13}C depleted isotopic composition of the FA. This means that, in isotope studies, ^{13}C- and ^{2}H-values of individual FA should not be considered as independent variables.

In our controlled feeding experiments, the integration of ^{2}H into FA from ambient water was clearly seen in case of MUFA, as the

MUFA in *Daphnia* raised in lake water spiked with ^2H_2O were enriched in ^2H, despite feeding on non-deuterium enriched algae. A corresponding decrease in $\delta^{13}C$ values suggested altered reaction kinetics, implying ongoing FA synthesis. In contrast, the δ^2H and $\delta^{13}C$ composition of LIN and ALA matched those of the diet, indicating direct assimilation since these FA cannot be synthesized de novo by most animals (Cook & McMaster, 2002). In the absence of dietary LC-PUFA, that is, *Daphnia* feeding on *A. obliquus*, δ^2H_{ARA} and δ^2H_{EPA} were hardly influenced by ^2H enrichment of the diet. However, significant ^2H enrichment occurred when the ambient water was spiked with ^2H, indicating ongoing PUFA conversion. In contrast, when feeding on high-quality diet containing long-chain PUFA (i.e. *C. ozolinii*), δ^2H_{ARA} and δ^2H_{EPA} values matched those of the algae, and environmental water had little influence, indicating a strictly dietary PUFA transfer to consumers. Although *A. obliquus* contains substantial amounts of the intermediate SDA (18:4n-3), a slight but insignificant increase in δ^2H_{SDA} values was detected, accompanied by a slight but insignificant decrease in $\delta^{13}C_{SDA}$, suggesting the use of ALA rather than SDA as precursor for EPA. This complements recent findings that algae containing high amounts of 20:3n-3 lead to significantly higher EPA levels in chironomids than those containing high amounts of SDA (Strandberg et al., 2020), suggesting the first step of bioconversion is elongation of ALA to 20:3n-3 rather than desaturation to SDA.

In contrast to controlled laboratory feeding experiments, zooplankton in natural aquatic ecosystems face a vast mixture of dietary sources containing varying amounts and sources of LC-PUFA. In our pond field study, we tested whether the δ^2H of FA provided better discrimination among dietary transferred versus converted EPA. This is ecologically and ecophysiologically important since LC-PUFA, particularly EPA and DHA, contributes significantly to consumer fitness (Brett et al., 2009; Marzetz et al., 2017), and improve predation or evasion skills (Pilecky, Kämmer, et al., 2021; Pilecky, Závorka, et al., 2021). Despite the large variations in seston FA composition and EPA and DHA mass fractions, the EPA mass fractions in zooplankton of these study ponds remained similar during the season. The differences in δ^2H_{EPA} values between seston and zooplankton correlated with decreasing EPA mass fraction in seston, indicating that a lack of dietary EPA supply was compensated for by increased PUFA conversion in zooplankton. However, there was no significant difference in δ^2H values of essential PUFA between copepods and cladocerans. The ability to biosynthesize EPA from ALA may vary greatly among zooplankton taxa and hence further studies are needed to extend our findings to other zooplankton. However, the patterns of δ^2H values of PUFA in zooplankton point to FA conversion and/or retention processes that may be applicable to zooplankton in general.

As demonstrated by our controlled laboratory experiment, the bioconversion of precursor-PUFA to LC-PUFA resulted in significant changes in δ^2H values of the products. We postulate that the resulting δ^2H values are determined by three factors: (a) the H isotopic composition of the precursor, (b) the H isotopic composition of the environmental water and (c) the taxa-specific H isotopic fractionation during lipid synthesis. In our field study, δ^2H values of ALA, 16:1, and environmental water were the best combination for predicting the δ^2H_{EPA} values of zooplankton and omitting of either factor led to a significant loss in predictive value. In this case, ALA was used as precursor for synthesis of EPA, and has to be dietary acquired, as most animals lack the required enzymes to synthesize fatty acids, which contain the omega-3 or omega-6 double bond, de novo (Cook & McMaster, 2002). SDA was excluded as irrelevant early in the iteration processes, supporting our controlled laboratory experiments that dietary SDA is of limited importance as precursor for EPA bioconversion. Regarding taxa-specific isotopic fractionation, results from the controlled feeding experiments suggest a high rate of de novo synthesis of *Daphnia* 16:1, in which case, the same C2 bodies—that is, malonyl-CoA—as for FA elongation during PUFA conversion are used. Thus, the δ^2H value of 16:1 is considered as taxa specific for the FA synthesis complex, which is additionally influenced by environmental factors including environmental water or bulk δ^2H values of the diet. Finally, desaturation can lead to a limited amount of H isotope exchange via possible back reactions for at least one of the two involved carbon positions (Baillif et al., 2009), which might also involve further H isotopic fractionation. The negative intercept of about −40‰ in the conversion model may indicate a net overall isotopic fractionation occurring when converting ALA to EPA in zooplankton, which also matches the difference in δ^2H values between ALA and EPA in seston. Overall, 6–8 of the 29 H-atoms are potentially affected during the conversion of ALA to EPA in contrast to only 2 of the 20 C-atoms of EPA. Combined with greater H isotopic fractionation dynamics due to altering precipitation and local evaporation effects, our findings suggest that δ^2H values of FA provided a better metric of bioconversion than $\delta^{13}C$ values of FA.

The threshold below which EPA is biosynthesized in zooplankton ranged between 0.3 and 0.5 mg EPA/g. This dietary threshold value might be critical, as conversion requires energy, potentially resulting in reduced offspring production (Taipale, Aalto, et al., 2019; Taipale, Vuorio, et al., 2019). Though our data hint at similar effects for n-6 PUFA, the lower contents of ARA compared to EPA and isotope analysis close to the limit of quantification made further elaboration difficult. Because the enzymes involved in elongation and desaturation do not distinguish between n-3 and n-6 PUFA and n--3/n-6 PUFA precursors can act as competitive inhibitors (Chen & Nilsson, 1993), it seemed likely that a similar mechanism exists for n-6 PUFA conversion in zooplankton.

The controlled feeding experiment in combination with the field study provided new insights into the role zooplankton play in biosynthesizing LC-PUFA in natural ecosystems. The eutrophication of aquatic ecosystems downgrades dietary quality in terms of LC-PUFA quantity and thus trophic transfer of essential FA to aquatic consumers and has the potential to decreases the dietary supply of dietary PUFA required by humans (Colombo et al., 2020; Schindler, 2009; Taipale, Vuorio, Brett, et al., 2016; Taipale, Vuorio, Strandberg, et al., 2016). For sustainable management of aquatic ecosystems, it is crucial to understand the ecophysiological processes that supply essential micronutrients to consumers, including their ability of

biosynthesis of precursor SC-PUFA to LC-PUFA. Fish rely more on dietary DHA than EPA (Pilecky, Kämmer, et al., 2021; Pilecky, Závorka, et al., 2021) and because the conversion rate of ALA to DHA is approximately 10 times lower than of EPA to DHA (Mourente, 1996; Mourente & Tocher, 1998), it is important to receive preformed dietary LC-PUFA. The 'buffering' role of zooplankton, that is, the capacity to dampen variations in LC-PUFA production by primary producers (Rasconi et al., 2015, 2017; Taipale, Aalto, et al., 2019; Taipale, Vuorio, et al., 2019) for higher consumers, might be of great importance since maintaining high-performance zooplankton populations eventually also benefits fish production.

5 | CONCLUSIONS

This study provides laboratory and field evidence that the δ^2H composition of fatty acids considerably improved the resolution of food web relationships and thus can help researchers to better understand FA bioconversion in zooplankton and likely also in other aquatic consumers. The δ^2H composition of FA revealed the extent to which zooplankton perform bioconversion in eutrophic aquatic ecosystems that are depleted in LC-PUFA, thereby providing 'stability' in dietary quality for higher consumers, such as fish. Higher EPA levels of seston replaced biosynthesis by zooplankton as the main source of EPA. Finally, the kinetics of ^{2}H and ^{13}C of FA, and probably other molecules, are linked in FA bioconversion processes.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

M.P. and S.K.K. conceived the ideas and designed the methodology; M.P., S.K.K. and M.M.-R. collected the data; M.P. and L.I.W. analysed the data; M.P., M.M.-R., S.J.T. and M.J.K. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data can be accessed online from the Dryad Digital Repository https://doi.org/10.5061/dryad.ns1rn8pv0 (Pilecky, Kämmer, et al., 2021).

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