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Seasonal changes in European whitefish muscle and invertebrate prey fatty acid composition in a subarctic lake

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Summary:

1. Ambient light and temperature show extreme seasonal variation in subarctic lakes due to the mid-night sun period in summer and cold polar night period in winter. These changes have clear impacts on fish feeding and reproduction cycles, potentially affecting the fatty acid (FA) composition of muscle. Despite extensive research into fish FA over recent decades, we know little about intra-annual changes of fish FA profile and content.

2. We studied intra-annual changes in the FA profile (mol%) and content (mg g^{-1} DW) of sexually mature European whitefish (*Coregonus lavaretus*) muscle in a large and deep subarctic lake located in northern Fennoscandia. We collected fish, zooplankton and benthic macroinvertebrate samples during three ice-covered months, including December (during whitefish spawning), and three open-water months. Fish size, age, sex, stomach content and fullness, as well as gonadosomatic index were also assessed as co-variates.

3. Whitefish changed diet from benthic macroinvertebrates in winter to zooplankton in summer. Generally, whitefish somatic growth was slow and most energy was used for gonad growth. Zooplankton had higher total content and different profile of FA compared to benthic macroinvertebrates. Increased zooplanktivory in summer was detected with higher α-linolenic acid (ALA, 18:3n-3) and stearidonic acid (SDA, 18:4n-3) percentage and content as well as increased the ratio of polyunsaturated FAs (PUFAs) of n-3 and n-6 family (n-3/n-6 –ratio) in fish muscle.

4. Whitefish gonadal growth and development occurs during the summer growing season and continues until the initiation of spawning in early winter. We found that the content of physiologically crucial PUFA, eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and arachidonic acid (ARA, 20:4n-6) decreased by ca. 60% between late summer and the spawning period

in early winter. After spawning, total FA content of whitefish muscle increased rapidly reaching the maximum recorded level in mid-summer.

5. Intra-annual changes in whitefish muscle FA profiles and contents were modified both by available diet and reproductive phase, however, reproductive physiology was clearly a stronger driver of the changes in muscle FA composition. Results suggest marked changes in intra-annual FA composition of fish muscle, an important factor that should be considered in future studies and especially in long term monitoring programs. Future studies are needed to find out whether these inter-annual FA patterns revealed in this study can be extended to different regions and to e.g. adipose or spring spawning species.

Introduction

Seasonal variation of light and temperature in subarctic lakes has significant impacts on primary and secondary production but also the metabolism and feeding of animals (Christoffersen et al., 2008; Lizotte, 2008; Hayden et al., 2014, McMeans et al., 2015, Hampton et al., 2017). Light and temperature are key environmental cues for fish, inducing gonadal development and later spawning activities (e.g. Wanzenböck et al., 2012). Gonadal development places extremely high energetic demands on fish, and likely requires a high quality food supply prior to the gonads being grown (e.g. Jobling et al., 1998). At an annual scale, benthic macroinvertebrates are the most important prey for many salmonid species in subarctic lakes (Svenning et al., 2007; Amundsen and Knudsen, 2009; Eloranta et al., 2010; Hayden et al., 2014). However, salmonids (Salmonidae) show a dietary shift from benthic prey to zooplankton during the summer coinciding with the peak zooplankton abundance (Heikinheimo et al., 2000; Eloranta et al., 2010, 2013; Hayden et al., 2014). Such dietary change is visible in adult fish muscle fatty acid composition 1–2 months after a switch to a new diet (Jobling et al., 2002; Milardi et al., 2016). In general, zooplankton provide physiologically crucial highly unsaturated fatty acids (HUFA), such as eicosapentaenoic acid (EPA, 20:5n-3),

docosahexaenoic acid (DHA, 22:6n-3), while arachidonic acid (ARA, 20:4n-6) is typically derived more from benthic macroinvertebrates. Due to the trophic retention of HUFAs, a low n-3/n-6 ratio in freshwater fish is often used as an indicator for utilization of littoral or terrestrial resource (benthic macroinvertebrates), whereas a high ratio indicates the contribution of pelagic phytoplankton via zooplankton (Kuusipalo and Käkelä, 2000; Kainz et al., 2017; Strandberg et al., 2018). Fish are generally unable to synthesize these important biomolecules efficiently from precursor molecules: α-linolenic (ALA, 18:3n-3) and linoleic acid (LIN, 18:2n-6) (e.g. Henderson 1996; Tocher et al., 2003), and therefore tend to rely on lower trophic levels for their supply. The current paradigm in ecological fatty acid (FA) related studies is that EPA and DHA are synthesized only by some phytoplankton taxa (Ahlgren et al., 1992, Gladyshev et al. 2013; Taipale et al., 2013, 2016) and transferred to fish via zooplankton, allowing the growth and functions of delicate and complex organs of fishes, e.g. muscle, eye, brain and gonads (e.g. Watanabe et al., 1989; Arts et al., 2001; Tocher et al., 2003). Salmonids gain both somatic and gonadosomatic mass during summer period, and thus pelagic planktivory has been suggested to be essential for gonadal development (Eloranta et al., 2010; 2013; Hayden et al., 2014).

Fish white muscle is usually characterized by large absolute and relative content of EPA, DHA and ARA (Łuczyńska et al., 2008; Muir et al., 2014; Gladyshev et al., 2017; Strandberg et al., 2018). Adipose salmonid fish, such as Arctic charr (*Salvelinus alpinus* (L.)), have large lipid reserves in muscle and carcass (including skin), whereas in many lean species, such as cod (*Gadus morhua* L.), the liver is the most important lipid storage tissue (Jobling et al., 1998; 2008). Luzzana et al. (1996) reported that in Lake Maggiore (northern Italy), at the southernmost distribution of European whitefish (*Coregonus lavaretus* (L.)), perivisceral adipose tissue is an important energy source for gonad development. Gonadal development, spawning and overwintering are energetically expensive for fish (Jørgensen et al. 1997; Jobling et al., 1998). Whitefish invest HUFAs into gonad tissues by mobilizing FAs from perivisceral lipids, and to a lesser degree, from muscle lipids (Luzzana et al.,

1996; Muir et al., 2014). Gonadal development in whitefish is rapid, and usually occurs in late autumn, just prior to spawning in early winter in subarctic lakes (e.g. Hayden et al., 2014, Keva et al., 2017). However, information on annual variation of FA in muscle tissue remains scarce for subarctic whitefish, which often dominates lake fish communities in the region. Whitefish hold a key role in food web dynamics, as they support important fisheries across their distribution and are sensitive to environmental stressors such as climatic and land use change (Hayden et al. 2017; Thomas et al. 2017).

Subarctic lakes provide excellent opportunities to study the relative importance of reproduction and pelagic dietary shifts on fish muscle FA composition, due to: i) the intense summer growth season and ii) the fact that benthic-derived energy dominates these systems for most of the year except for a short summer shift to pelagic-derived energy (Sierzen et al., 2003; Eloranta et al., 2010; Hayden et al., 2014). Such a shift could be especially important in autumn/winter spawning fishes that must develop their gonad tissues (which can reflect more than 20 % of somatic mass in females (e.g. Rösch 2000)) during summer and autumn. In the current study, we examined variation in annual dorsal muscle FA in whitefish inhabiting a well-studied subarctic lake (Hayden et al., 2014; Keva et al., 2017). The main motivation for the study was a lack of knowledge on how fish muscle FA composition and content vary intra-annually in subarctic lakes, and how dietary resource shifts, fish condition and the reproductive cycle may affect muscle FAs. To seek answers for these questions, we examined two hypotheses:

H1: The strong year-round reliance of whitefish on littoral benthic macroinvertebrates should be reflected in muscle FA composition, where littoral markers (e.g. ARA) should dominate FAs for most of the year. However, during, and shortly after the zooplankton dietary shift in late summer, pelagic markers (e.g. LIN, and EPA) should increase in whitefish muscle FA content and profiles (Hayden et al., 2014).

H2: n-3 HUFA (EPA and DHA) and n-6 HUFA (ARA) in whitefish muscle should be the lowest during, and after spawning due to their mobilization for reproduction. Moreover, the total FA content of muscle should be the lowest in midwinter, and that the subsequent recovery should be slow as whitefish generally feed at very low rates during winter, due to low water temperatures (Hayden et al., 2013, 2014; Keva et al., 2017).

Materials and methods

Sampling area and period

Samples for this year-round study were collected in 2011 and 2012 both during ice-covered winter months (December, February, May) and ice-free summer months (June, July, September) from a subarctic lake, Kilpisjärvi (hereafter Kilpis) located in northern Finland (Fig. S1). Kilpis is an oligotrophic lake with cold, clear and neutral water (detailed water chemistry in Hayden et al., 2014), with a surface area of 37.3 km^2, a shoreline length of 71.5 km, and maximum and mean depths of 57 m and 19.7 m, respectively. The catchment area (293 km^2) mainly consists of subarctic tundra and human population densities are low (e.g. Hayden et al., 2017).

Whitefish dominate the fish fauna of Kilpis: they comprise approximately 95% of the total fish biomass (Harrod et al., 2010; Malinen et al., 2014). In this region, whitefish populations are often polymorphic, but Kilpis has only a single generalist morph that is the most ubiquitous to the region; the large sparsely rakered (LSR) whitefish (Harrod et al., 2010; Kahilainen et al., 2017). Seven other fishes inhabit Kilpis: alpine bullhead (*Cottus poecilopus* Heckel), pike (*Esox lucius* L.), burbot (*Lota lota* (L.)), minnow (*Phoxinus phoxinus* L.), brown trout (*Salmo trutta* L.), Arctic charr and grayling (*Thymallus thymallus* L.) (Kahilainen et al., 2007). In Kilpis, copepods (*Cyclops scutifer* Sars and *Eudiaptomus graciloides* Liljeborg) dominate the pelagic zooplankton community year-round, whereas cladocerans (Cladocera, mainly *Bosmina* sp. and to a smaller degree *Daphnia* sp. and

Holopedium gibberum Zaddach) are apparent during the mid- to late-summer months (Kahilainen et al., 2007; Hayden et al., 2014). The pelagic zooplankton peak typically occurs in late July, whereas densities are lowest in mid-winter (Hayden et al., 2014). The profundal benthos of Kilpis largely consists of chironomid larvae (Chironomidae), oligochaetes (Oligochaeta) and *Pisidium* sp., whereas the shallower water littoral benthos is more diverse, and includes several insect larvae (Trichoptera, Plecoptera, Ephemeroptera, Megaloptera, Dytiscidae, Tabanidae) benthic crustaceans (*Eurycercus* sp. and *Megacyclops* sp.) and periphyton-grazing snails (*Lymnaea* sp. and *Valvata* sp.) (Hayden et al., 2014).

Sampling methods and measurements

Fish samples were collected with 240 m long benthic gill net series including seven panels of different mesh sizes (knot-to-knot mesh sizes: 12, 15, 20, 25, 30, 35, 45 mm; net height: 1.8 m) and one multi-mesh NORDIC-net (5.25–55 mm; net height 1.5 m) set overnight (10–12h) during the open-water sampling (Jun-12, Jul-12, Sep-12) or for up to two days (24–48h) during the under-ice sampling (Dec-11, Feb-12, May-12). On capture, all fish were immediately euthanized by cranial concussion, removed from nets, stored in ice and transported to the laboratory. Pelagic zooplankton were sampled through vertical hauls (from depth of 10 m) of a plankton net (diameter 25 cm, mesh size: 50 μm), benthic macroinvertebrates were sampled using an Ekman-grab (area: 272 cm^2), in shallow littoral areas benthic macroinvertebrates were also collected by a kick-net. All invertebrate and fish individuals were identified to the lowest practical taxonomic level.

Fish total length (±1mm) and blotted wet mass (±0.1g) were measured, and the Fulton's condition factor was derived from the formula (Nash et al., 2006): $K=M/TL^3 \times 100$, where K is condition factor, M is mass (g) and TL is total length of fish (cm). Age determination was performed under microscope using one clear and one burned-and-cracked sagittal otolith immersed under water in a petri-dish and using a microfiche to read ventral scales pressed on a polycarbonate slide (Kahilainen et al., 2003).

We used these different bony structures to improve the reliability of aging (Kahilainen et al., 2017). The first left gill arch was dissected and gill rakers were counted under preparation microscope. Gill raker number is a heritable trait in genus *Coregonus*: it commonly used for morph identification as it is related to diet, e.g. a high number of gill rakers facilitates dietary specialization to zooplankton (Kahilainen et al., 2011a, 2011b).

Sex and maturation level were visually determined from gonads using a 1-7 scale, where values between 1 and 3 represent juveniles and 4 and 7, mature individuals in different maturity stages. Gonads were weighed (±0.01g) and the gonadosomatic index calculated (Hayden et al., 2014): GSI=GM/SM×100, where GSI is gonadosomatic index, GM is the gonad mass (g) and SM is the somatic mass (g). Stomach contents where characterized using a points method (Hynes, 1950), where stomach fullness was visually estimated in scale of 0-10 (0=empty, 10=extended full). Prey items were first identified to the lowest feasible taxonomic level under a dissection microscope and their relative contribution to total fullness was estimated. A piece of dorsal muscle tissue and invertebrate samples were freeze-dried (–80°C for 48h), ground to fine powder and frozen (–80°C) for subsequent analysis. We took advantage from previously published stable isotope and total mercury studies (Hayden et al., 2014; Keva et al., 2017) to gain individual values for fish age, sex and maturity stage to select individuals to FA analyses. We selected six mature individuals (3 male, 3 female) per sampling month, all from the same dominant year class (2003), and from a similar size class where possible, to minimize potential effects of maturity, age and size on FA composition. Harsh ice-out conditions in Jun-12 resulted in limited sample size and was supplemented with some older and larger individual for FA analyses.

Fatty acid analysis

Freeze-dried samples were ground to fine powder and weighed (10±1 mg) into tin cups, which were subsequently placed into test tubes (10 ml). Each sample was spiked with an internal standard (free

FA 13:0) which was used in calculating FA content (µg mg^{-1}) in the sample (equation 1). The sample and internal standard were mixed into 2 ml of 1% methanolic $H_{S2}O_4$ supplemented with 1ml hexane, and the solution heated under nitrogen atmosphere in capped vials in a heat block at 95 ºC for 120 min. After cooling of the tubes, water (1.5 ml) and hexane (4 ml) were added, and subsequently generated FA methyl esters (FAMEs) were extracted into hexane. FAME solutions were dried on NaS_2O_4, concentrated under nitrogen flow, and the hexane volume adjusted to 1 ml. Samples were stored at -80 °C until analyzed with a GC-2010 Plus gas chromatograph (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with an auto injector (AOC-20i) and a flame ionization detector (FID). The quantification was based on the FID responses, and the peak areas were integrated using GCsolution software (version 2.41.00, Shimadzu). The structures of the 80 FAs detected were identified based on their mass spectrum recorded by Shimadzu GCMS-QP2010 Ultra (Shimadzu) with mass selective detector (MSD). In the GC-FID and GC-MSD, the FAMEs were chromatographed using a similar capillary column (Zebron XB-wax, length 30 m, diameter 0.25 mm, film thickness 0.25 µm; Phenomenex, Torrence CA, USA). FA molar percentages (mol%) were calculated as the ratio of FA peak area to the peak areas of all FAs adjusted with the theoretical correction factors for FID (Ackman, 1992). Sample FA content was calculated with the following equation (1) based on the assumption that the FID corrected ratio of each unknown FA amount to its peak area equals to the FID corrected ratio of the known amount of the standard FA to its peak area:

$$C_{FAi} = \frac{m_{st}}{m_{sample}} \times \frac{A_{FAi}}{A_{st}} \times \frac{M_{FAi}}{M_{st}} \times \frac{CF_{FAi}}{CF_{st}} \quad (1)$$

, where C_{FAi} is the content of individual FA (µg mg^{-1}) in the sample, m_{st} and m_{sample} are the masses of internal standard FA (13:0) and the dried sample weighed into the tin cup (mg) respectively. A_{FAi} and A_{st} are the integrated peak areas of FA_i and the standard FA, respectively. M_{FAi} and M_{st} represent the molecular mass of FA_i and the standard FA (13:0). CF_{FAi} and CF_{st} are the corresponding

theoretically calculated and experimentally confirmed correction factors for the slightly different FID responses of different FA structures. After these calculations we sorted FAs by their mean mol% contribution and selected FAs higher than 0.5 mol% for later analysis without normalizing the data to 100% (as done previously by Luzzana et al., 1996; Hessen and Leu, 2006). This subset of FAs was used in all further data analysis and cataloging. In addition, analyzed FAs were grouped into SFA, MUFA, PUFA, n-3 PUFA, n-6 PUFA, and also the dimethyl acetals derived from phospholipid alkenyl chains (DMAs) were included in the analyses. The ratios of n-3/n-6, unsaturated to saturated FAs (UFA/SFA) and the sum of all FAs (Tot-FA) were calculated.

Statistical analysis

Differences in fish background ecological data (variables described in *Sampling methods and measurements*) and FA between sexes were tested by month with T-test or Mann-Whitney U-test when appropriate. For the FA mol% data, we used permutational analysis of variance (PERMANOVA) based on a Bray-Curtis distance matrix to test the most important variables driving dissimilarities. We used non-metric multidimensional scaling (nMDS) ordinations based on the Bray-Curtis distance matrix to illustrate the PERMANOVA results. We used SIMPER (similarity percentage test) as a *post-hoc* means to characterize differences observed in the PERMANOVA results. Additionally, to test the differences of individual FA percentage (mol%) and content (mg g^{-1} DW) between sampling months in fish or between invertebrate habitats, we used Analysis of Variance (ANOVA) with Bonferroni corrected t-tests (here-after Bonferroni test) for *post-hoc* comparisons. If the assumption of normality (Shapiro-Wilk's test) or homogeneity (Levene's test) was violated, we used repeated Welch's t-test (W-ANOVA) with Games Howell *post-hoc* tests. For hypothesis 1, we examined the difference in FA quality and quantity between fish caught in September and fish from the previous months to reveal the effects of the shift from a benthic to pelagic diet on whitefish muscle FA composition. For hypothesis 2, we focused on the possible FA differences between the fish caught

in December and previous and following months to reveal how spawning, and subsequent physiological recovery affected whitefish muscle FA composition. In all statistical tests, we used an alpha level of 0.05 to test null hypothesis. All statistical analyses were conducted using R through RStudio version 3.4.1. with base and/or vegan packages (R Core Team, 2017; Oksanen et al., 2018).

Results

Basic ecological metrics

We first examined potential differences in background ecological data between sexes: we found that the only factor that differed was GSI, with females continually having GSI values 5-10 times higher than males (Table S1). In the pooled ecological background data, the whitefish we examined were similar in age (mean±sd: 9.2±1.6) and size (TL: 29.2±3.8 cm, mass: 197.1±110.0 g, condition factor: 0.74±0.07, gill rakers: 24±2) throughout the study (Table 1; Table S1), apart from the individuals caught in June. These individuals were older (11.1±3.4) and larger (TL: 34.7±6.7 cm, mass: 356.7±206.3 g, condition factor: 0.74±0.12, gill rakers: 25±1) compared to the fish caught in the other months, and reflect issues with limited sample sizes following sampling immediately after ice break-up. GSI was stable from February to July and increased progressively towards the December spawning period (Table 1). Gill raker number remained stable during the whole season (Table 1). Condition factor was highest in September and lowest in February, but we did not find statistical differences (Table 1). Stomach fullness was lowest under ice, *i.e.* during and after spawning (Dec-May: 1.2±1.5) and highest in the open-water season (Jun-Sep: 4.7±1.1) (Table 1). Whitefish largely consumed benthic prey, especially *Pisidium* sp. and chironomid larvae, which were present in the stomachs throughout the year. However, in June and September, littoral *Eurycercus* sp. and pelagic zooplankton (e.g. *Bosmina* sp. and Calanoida) made the largest relative contribution to whitefish diet (Table 1).

We found very small differences in FAs between whitefish sexes i.e. six differences out of 138 potential comparisons (Table S2). In addition, PERMANOVA indicated that sampling month was the only important variable (r^2=0.648, p<0.01) explaining dissimilarities among whitefish FA profiles. Sex (r^2=0.003, p=0.887) or the month*sex interaction (r^2=0.069, p=0.302) were clearly non-significant (Table S3). Therefore, we pooled the two sexes together in all subsequent statistical analysis.

Ordination of FA profiles showed that invertebrates (classified by both taxa and habitat) were clearly differentiated from fish (Fig. 1; PERMANOVA: Table S3). Due to low sample size of invertebrates by taxa and month, the invertebrate data was pooled into to three habitat groups (pelagic zooplankton, littoral benthic macroinvertebrates, profundal benthic macroinvertebrates). Habitat (r^2=0.240, p=0.003) was the most important variable for explaining the dissimilarities between invertebrate FA profiles, with neither month (r^2=0.087, p=0.880), nor the habitat*month interaction (r^2=0.089, p=1.0) affecting FA profiles (Table S3). SIMPER, which indicated that 70-80% of the FA dissimilarity within fish and invertebrates was associated with habitat (Table 2) was explained by: 14:0, 16:0, 16:1n-7, 18:1n-7 18:1n-9, ARA, EPA and DHA. Similarly, SIMPER results for Fish FA profile data based on sampling month (Table 2) indicated that 70-80% of the dissimilarity was explained by: 14:0, 16:0, 18:0, 16:1n-7, 18:1n-9 ARA, EPA, DHA, but in some cases both LIN and SDA also contributed to dissimilarities.

H1 Late summer dietary shift towards pelagic zooplankton affects whitefish muscle FA composition

Invertebrate groups showed differences in mol% among the FA structural categories (ANOVA/W-ANOVA: SFA, $F_{2,12.3}$=4.2, p=0.004; MUFA, $F_{2,19.9}$=14.5, p<0.001; PUFA, $F_{2,36}$=4.4, p=0.02). SFA and PUFA were highest in pelagic zooplankton (*post-hoc* tests: p<0.05; Table S4), whereas MUFA showed lower contribution in pelagic zooplankton (15.2±3.8 mol%) than in littoral benthic macroinvertebrates (30.2±10.4 mol%) (*post-hoc* tests: p<0.05; Table S4). The FA profile of benthic

macroinvertebrates was relatively similar between habitats, but MUFA was higher in littoral benthic macroinvertebrates compared to profundal benthic macroinvertebrate (19.9±5.6 mol%) (*post-hoc* test: $p<0.05$; Table S4). DHA percentage was clearly highest in pelagic zooplankton (5.9±4.5% mol%), and ARA contribution was the highest in profundal benthic macroinvertebrates (2.2±1.2 mol%), a difference highlighted by SIMPER (Table 2; Table 4S).

The n-3/n-6 ratio was the most important FA marker highlighting differences among invertebrate groups (Fig. S2; Table S6), being around 80% higher in pelagic zooplankton (2.43) compared to littoral and profundal benthic macroinvertebrates (1.38). The mean of Tot-FA (171.5 mg g^{-1} DW) was >100%, SFA (61.1 mg g^{-1} DW) and n-6 PUFA (22.1 mg g^{-1} DW) were >200% higher, PUFA (75.6 mg g^{-1} DW) was >300% higher and n-3 PUFA (53.5 mg g^{-1} DW) was >400% higher in pelagic zooplankton compared to benthic macroinvertebrate habitat groups. SIMPER and ANOVA results showed that EPA, DHA, ARA, 14:0, ALA, and 22:5n-6 were also clearly higher in zooplankton (Table 2; Fig. S2; Table S6). However, variation (±SD) in pelagic zooplankton was relatively high due to seasonal changes in the FA content (Fig. S3-S5; Table S6), and therefore statistical differences in ANOVA was not found besides in n-3/n-6 -ratio.

In pelagic zooplankton, ALA, SDA and n-3/n-6 ratios all varied among months (ANOVA/W-ANOVA: ALA, $F_{5,30}=5.5$, $p=0.001$; SDA, $F_{5,30}=4.2$, $p=0.005$; n-3/n-6 $F_{5,30}=8.5$, $p<0.001$), and all were highest in September (Fig. S2). Moreover, whitefish muscle SDA content varied among months (ANOVA: $F_{5,30}=6.8$, $p<0.001$; Fig. S2; Table S7) and were ca. twice as high in September (0.32±0.14 mg g^{-1} DW) than in the other months (pooled average: 0.13±0.04 mg g^{-1} DW). Moreover, n-3/n-6 ratio in whitefish muscle was found to be highest in September (3.91±0.33) and lowest in December (2.72±0.48) (Fig. 2; Table S7).

H2 Whitefish muscle FA profile and content during the spawning at December

Whitefish FA profile varied seasonally: December was particularly distinct (Fig. 1; Fig. 2; Fig. S2; Table 2, Table S5). The relative percentages of each FA category differed considerably between months (ANOVA/W-ANOVA: SFA, $F_{5,13.5}$=11.4, p<0.001; MUFA, $F_{5,13.8}$=5.1, p=0.01; PUFA, $F_{5,13.7}$=8.1, p<0.001; n-3 PUFA, $F_{5,30}$=16.8, p<0.001; n-6 PUFA, $F_{5,30}$=18.9, p<0.001). SFA and MUFA percentages were highest in December (45.6±4.1 mol%) and decreased towards summer - reaching the lowest value recorded in June (31.9±1.3 mol%) (*post-hoc* tests: p<0.01; Table S5). Conversely, the lowest percentage of n-3 PUFA (17.9±6.8 mol%) and n-6 PUFA (7.0±1.4 mol%) was found in December, and both FA classes increased towards the following summer (*post-hoc* tests: p<0.01 in all cases). Only DMA 16:0 remained static (~0.6 mol%) across the whole sampling period (Fig. S2; Table S5). In addition, UFA/SFA and n-3/n-6 –ratios differed among the months (ANOVA/W-ANOVA: $F_{5,13.2}$=32.3, p<0.001; $F_{5,30}$=8.5, p<0.001, respectively) being highest in September (1.6±0.1 and 3.6±0.3) and lowest in December (0.9±0.2 and 2.5±0.5) (*post-hoc* tests: p<0.01; Table S5).

To summarize the detailed FA profile data of whitefish muscle, 16 of the 24 selected FAs showed differences in their percentages in December compared to the other months (Fig. S2; Table S5). SIMPER and ANOVA results showed that only some n-3 PUFAs (ALA, SDA, 20:4n-3, EPA, DHA) and individual n-6 PUFAs (ARA, 22:5n-6) decreased from September to December, after which they increased towards summer (Table 2; Fig. S2; Table S5). In contrast, SFAs (14:0, 16:0, 18:0) and n-7 and n-9 MUFAs (16:1n-9, 24:1n-9, 18:1n-9) increased in their percentages from September to December, and after that decreased towards February (Table 2; Fig. S2; Table S5).

Mean whitefish muscle total-FA content were almost 25% lower in December (15.36±3.02 mg g^{-1} DW) than in the other months (pooled: 22.31±5.92 mg g^{-1} DW), but the difference was not statistically significant (Fig. 2; Table S7). Moreover, PUFA, n-3 PUFA, n-6 PUFA content and UFA/SFA and n-

3/n-6 ratios (from content data) showed intra-annual variation (ANOVA/W-ANOVA: PUFA, $F_{5,30}$=7.2, p<0.001; n-3 PUFA, $F_{5,13.5}$=29.2, p<0.001; n-6 PUFA, $F_{5,30}$=5.7, p=0.001; UFA/SFA, $F_{5,12.7}$=15.4, p<0.001; n-3/n-6, $F_{5,30}$=7.9, p<0.001). In December, PUFA (5.2±1.0 mg g^{-1} DW), n-3 PUFA (3.9±0.9 mg g^{-1} DW), n-6 PUFA (1.4±0.1 mg g^{-1} DW) contents and UFA/SFA –ratio (1.3±0.3) were at the lowest levels recorded during the study (*post-hoc* tests in all cases p<0.05; Table S7). PUFA content were around 60% lower in December (5.2 mg g^{-1} DW) than in other months (pooled average: 10.8 mg g^{-1} DW). SFA and MUFA content in fish muscle were stable throughout the year, yet showing generally the lowest content in February, despite 24:1n-9 which was lowest in September (Fig. 2; Table S7). Eight of the most abundant FAs contributed >75 % of the total FAs, and of these, three (ARA, EPA, DHA) showed differences in content among months (ANOVA/W-ANOVA: p<0.05 in all cases; Fig. 2; Fig. S2; Table 2; Table S7), and had the lowest content in December (*post-hoc* tests in all cases p<0.05).

Discussion

Main results

In our study conducted across a single annual cycle, we found that phytoplankton-zooplankton related markers (ALA, SDA and n-3/n-6) reached their highest percentages and ratio (respectively) in whitefish muscle tissue in September, approximately one to two months after the whitefish underwent a dietary shift to zooplankton (H1). Whitefish muscle contained ~60% less of the physiologically bioactive HUFA (EPA, DHA and ARA) during spawning (mean±sd: 0.83±0.15, 2.25±0.71, 0.68±0.08 mg g^{-1} DW), compared to other months (pooled average: 1.97±0.01, 5.84±0.08, 1.51±0.02 mg g^{-1} DW) (H2). Seasonal variation in FA content was associated with annual dietary shifts and more closely with spawning.

H1 Whitefish dietary shift from benthic macroinvertebrate to zooplankton can be detected with FA biomarkers

Seasonal changes in zooplankton biomass volume and composition are associated with shifts in fish foraging behavior. Various empirical studies using stomach content and stable isotope analyses have shown that generalist salmonids undergo seasonal diet shifts in subarctic lakes (Amundsen and Knudsen, 2009; Eloranta et al., 2010; Kahilainen et al., 2016). During the ice-covered period, when pelagic zooplankton densities are low, generalist fishes typically feed on benthic macroinvertebrates. Moreover, feeding activity (stomach fullness) has been usually reported to be the highest in summer and the lowest in winter (Svenning et al., 2007; Hayden et al., 2015) – as seen here. However, feeding activity does continue during the long period of ice cover, but this has traditionally been related to maintenance metabolism only. Increased feeding activity and energy gain during summer result in a growing season for most fish, which is reflected in higher condition indices in summer than in winter (Le Cren, 1951; Tolonen, 1999). Eloranta et al. (2013) found in their snap-shot summer-winter field study, that Arctic charr muscle contained more FAs in summer than in winter, suggesting that it was caused by summer-time zooplanktivory and overall high feeding activity. Whitefish muscle tissue is much leaner than that of Arctic charr and we observed relatively stable Tot-FA, n-3 PUFA and n-6 PUFA content outside the spawning period. This highlights the conservative nature of muscle FA composition and the major energy demand of gonadal development. Studies from aquaculture (e.g. Turchini et al. 2003; Suomela et al. 2017) have revealed that the consumption of fish feed provided in excess can modify muscle FA composition over a period of 1-2 months during the growing season. We did not find similar FA signature turnover rates in the current study, most likely due to limited prey resources and slow growth rate of whitefish, which gain only minor somatic growth during the growing season (Hayden et al., 2014; Keva et al., 2017). Previous whitefish studies have shown a clear dietary shift from benthic macroinvertebrates to zooplankton using SCA, but with stable isotope analysis of whitefish muscle, the shift was undetectable suggesting a very long turnover-time of

muscle tissue in such cold-water lake (Hayden et al., 2014; Thomas and Crowther, 2015). Collectively, this may indicate that turnover-times of stable isotopes and FAs derived from aquaculture environments using optimal diets, excess feeding and lack of predation may not extend to wild populations in resource-limited subarctic lakes.

Despite the relative stability of FA composition outside the spawning period, increased zooplanktivory during late summer was highlighted by some FA markers. In this study pelagic zooplankton contributed less in whitefish stomach content than littoral zooplankton in summer (i.e. *Eurycercus* sp.). In literature, FA data of littoral *Eurucercus* is scarce, but some studies suggest it to contain significant amounts of ALA and HUFAs (Smirnov 2017), therefore being potentially nutritionally valuable for fish and closely similar to pelagic zooplankton. In the current study, dietary related changes in whitefish muscle FA composition were only observable during late summer – an observation that is consistent with the previous findings from slow growing subarctic fish (e.g. Milardi et al., 2016). SDA and ALA were at their highest percentages in whitefish muscle in September. This was in line with the dietary hypothesis, since zooplankton were also rich in these FAs which have been previously reported to be higher in zooplankton than in benthic macroinvertebrates (e.g. Eloranta et al., 2013). Moreover, SFA 14:0 increased from June, reaching the highest content in December, but this change was not statistically significant due to high variance. However, 14:0 is a potential pelagic biomarker, typically high in diatoms (Bacillariophyceae), which are digested by zooplankton and later by fish (e.g. Taipale et al., 2016; Thomas et al., 2019). The n-6 PUFAs reached their maximum content in June and decreased steadily towards December, while n-3 PUFA content was relatively stable before fast decline during the spawning season in December. These trends result in both the seasonal maximum in the n-3/n-6 ratio in September and the minimum in December, and may originate from the interacting and combined effects of the dietary shift in mid-summer and gonadal investment in late autumn–early winter (Kainz et al., 2017; Strandberg et al., 2018).

H2 Energy investment to gonads affects the quantity and quality of whitefish muscle FAs

Gonads include elevated content of lipids with HUFAs (especially EPA, DHA, ARA), which are essential for gonadal development, and are relocated from different tissues and organs to the gonads (Luzzana et al., 1996; Jobling et al., 1998; Muir et al., 2014). Muir et al. (2014) found that female lake whitefish (*Coregonus clupeaformis* Mitchill) condition did not affect egg FA content and therefore concluded that FA content of eggs is highly conserved. They demonstrated that total FA content in lake whitefish eggs were 3–4 times higher than in the muscle tissue. In addition, Strandberg et al. (2018) suggested that in the autumn-spawning pelagic zooplanktivore vendace (*Coregonus albula*), reproduction costs can be such to affect muscle FA composition up to the late spring in the following year. Sushchik et al. (2007) suggested that spawning was the main factor driving the seasonal changes i.e. EFA depletion during the spawning of riverine Siberian grayling (*Thymallys arcticus*).

In the present study, whitefish muscle Tot-FA, n-3 PUFA and n-6 PUFA content were lowest in December during spawning, but these were already recovered almost fully in February, reaching a maximum in mid-summer. Therefore, we conclude that gonadal development requires large amounts of energy and HUFAs (e.g. EPA, DHA and ARA), especially directly prior to spawning when most of the gain in gonadal mass is concentrated (Jobling et al., 1998). We did not find major differences in female and male muscle FA content nor profile seasonally, suggesting approximately similar qualitative gonad investment or high energy costs associated with spawning (or a combination of these factors). Surprisingly, the highest content of MUFA and SFA were observed during spawning and lowest right after, even more unexpectedly, a rapid increase in PUFAs was recorded after spawning. The high MUFAs and SFAs could be explained by the assimilation of perivisceral fat and translocation of storage fats from adipose tissue (mainly MUFAs and SFAs) to liver and muscular cells allowing the production of different lipid classes (Jobling et al., 1998). This is supported by the

observation of elevated C:N ratio in whitefish liver in December (Keva et al., 2017), as FAs are high in carbon and low in nitrogen. However, the reasons for the relatively rapid and major increment of important HUFAs (+100–200 %) after spawning and during the time of low feeding activity remains unclear. We suggest that this may reflect an increased rate of lipid mobilization from other tissues, increased HUFA synthesis, increased feeding activity beginning in the spring, or most likely a combination of these factors.

In addition to the hypothesis we examined, we found that whitefish FA profiles reacted incrementally (or decreased) relative to other FA content. In our data, this trivial mathematical phenomenon related on the dependency of proportional variables, is particularly seen in whitefish muscle 16:0 where mol% data showed significant increases from September to December while 16:0 content showed no significant variation. Without the content data, we would not be able to identify whether this trend was driven by the changes in other FA content or simply by changes in 16:0 content. Therefore, we argue that especially in FA studies recording temporal changes in FA quality, concentration-based analyses should be preferred as previously suggested (e.g. Gladyshev et al., 2018).

Conclusions

Annual changes in whitefish muscle FA were linked both diet shifts and reproduction, but the latter was much more important driving factor. Our results underlines a pressing need to include annual angle in future studies and monitoring programs using FAs, especially in any environments with seasonality. In future, we suggest that researchers undertake year-round FA comparisons of low and high lipid-content vertebrates with various reproduction times, preferably along a gradient of growth rates to test generality of current study results.

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Supplementary material

Open access supplementary data (Table 1S-7S & Fig. 1S-6S) related to this article can be found online at [TYPE THE URL HERE].

Author contributions

K.K.K. designed the study. K.K.K., B.H. and C.H. undertook field and laboratory work. R.K., P.T. and S.J.T. supervised O.K. in FA analyses. O.K. conducted the FA laboratory analyses, statistical analyses and wrote the first version of manuscript. All authors contributed in revising the manuscript and no conflict of interest occurs.

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Table 1. Ecological characteristics (sample size; age; body size; condition factor, gill raker number, gonadosomatic index; GSI and diet) of whitefish. For each continuous variable monthly mean ± SD values and ANOVA statistics are presented if ANOVA $p<0.05$. Bold superscript numbers before the mean values indicate statistical difference (Bonferroni corrected t-tests, adj.$p<0.05$) against the indicated months. Stomach fullness, number of empty stomachs and detailed stomach content for different prey groups are presented as mean percentage contributions. Abbreviations l., n. and p. after invertebrate orders indicates larva, nymph and pupa respectively.

	^{2}Feb-12	^{5}May-12	^{6}Jun-12	^{7}Jul-12	^{9}Sep-12	^{12}Dec-11	ANOVA
N	6	6	6	6	6	6	
Age	9±0	9±0	^{12}11.2±3.4	9±0	9±0	^{6}8±0	$F_{5,30}$=3.35
Total length (mm)	^{6}274±20	^{6}288±19	^{2,5,7,9,12}347±67	^{6}282±6	^{6}286±8	^{6}272±15	$F_{5,30}$=5.07
Total mass (g)	^{6}145±28	^{6}177±40	^{2,5,7,9,12}356±206	^{6}166.43±14	^{6}187±16	^{6}150.23±34	$F_{5,30}$=4.90
GSI	^{12}0.48±0.39	^{12}0.48±0.34	^{12}0.93±0.61	^{12}0.78±0.42	2.91±1.68	^{2,5,6,7}6.32±5.59	$F_{5,30}$=5.52
Gill raker count	23±2	24±1	25±1	24±2	25±1	27±2	$F_{5,30}$=2.91
Condition factor	0.70±0.04	0.73±0.04	0.74±0.13	0.74±0.04	0.80±0.04	0.74±0.04	$F_{5,30}$=1.45
Stomach fullness	^{6,7,9}0.83±1.17	^{7,9}2.00±2.10	^{2,12}4.33±0.52	^{2,12}4.50±0.55	^{2,5,12}5.17±1.72	^{6,7,9}0.83±0.98	$F_{5,30}$=13.39
Empty stomachs	3	2	0	0	0	3	
Pelagic zooplankton	0.0	0.0	0.0	3.7	4.8	0.0	
Bosmina sp.	0.0	0.0	0.0	3.7	0.0	0.0	
Calanoida	0.0	0.0	0.0	0.0	3.2	0.0	
Copepoda	0.0	0.0	0.0	0.0	1.6	0.0	
Benthic zooplankton	0.0	41.7	0.0	50.0	48.4	0.0	
Eurycercus sp.	0.0	0.0	0.0	50.0	48.4	0.0	
Megacyclops	0.0	41.7	0.0	0.0	0.0	0.0	
Benthic macroinvertebrates	100.0	26.7	100.0	31.5	12.9	100.0	
Chironomid l.	70.0	15.8	18.5	5.6	0.0	60.0	
Chironomid p.	0.0	0.0	50.0	9.3	0.0	0.0	
Ephemeroptera n.	0.0	0.0	0.0	0.0	0.0	20.0	
Lymnaea sp.	0.0	0.0	26.5	7.4	1.6	0.0	
Pisidium sp.	30.0	10.0	1.2	5.6	11.3	20.0	
Plecoptera n.	0.0	0.0	3.8	0.0	0.0	0.0	
Trichoptera l.	0.0	0.8	0.0	3.7	0.0	0.0	
Terrestrial insects	0.0	0.0	0.0	13.0	33.9	0.0	
Geometrid moth	0.0	0.0	0.0	0.0	11.3	0.0	
Other insects	0.0	0.0	0.0	13.0	22.6	0.0	
Fish	0.0	31.6	0.0	0.0	0.0	0.0	
Whitefish eggs	0.0	31.6	0.0	0.0	0.0	0.0	
Other	0.0	0.0	0.0	1.8	0.0	0.0	
Corixidae	0.0	0.0	0.0	1.8	0.0	0.0	
SUM (%)	100.0	100.0	100.0	100.0	100.0	100.0	

Table 2. SIMPER results of FA profile data. Columns separated with dashed lines indicate pairwise SIMPER tests between whitefish (LSR) and invertebrates: zooplankton (ZPL), benthic macroinvertebrates (BMI) grouped by habitats (upper section of the table) and subsequent months (lower section of the table). The total amount of dissimilarity (%) between groups is shown in the first underlined row in parentheses. FAs are ordered from the most to the least significant driver to total dissimilarity, dis.sum indicates cumulative sum in total dissimilarity. FA means from the tested groups are presented in the means column, corresponding with the group order in the underlined header.

LSR--ZPL (38.3%)			LSR--profundal BMI (44.3%)			LSR--littoral BMI (45.6%)			ZPL--profundal BMI (41.2%)			ZPL--littoral BMI (43.0%)			profundal--littoral BMI (36.4%)		
FA	means	dis.sum	FA	means	dis.sum	FA	means	dis.sum	FA	means	dis.sum	FA	means	dis.sum	FA	means	dis.sum
14:0	2.9--16.6	0.21	DHA	19.3--1.3	0.27	DHA	19.3--0.9	0.25	14:0	16.6--4.1	0.23	14:0	16.5--3.6	0.21	16:0	9.8--14.3	0.21
DHA	19.3--5.9	0.42	16:0	25.8--9.8	0.51	16:0	25.1--14.3	0.41	16:0	15.9--9.8	0.39	16:1n-7	6.0--14.0	0.35	16:1n-7	6.3--14.0	0.40
16:0	25.1--15.9	0.57	EPA	7.9--3.6	0.58	16:1n-7	2.6--14.0	0.56	DHA	5.9--1.3	0.48	16:0	15.9--14.3	0.47	18:1n-9	5.6--8.3	0.50
ARA	5.8--1.6	0.63	16:1n-7	2.6--6.3	0.63	EPA	7.9--3.3	0.63	18:1n-7	1.5--5.2	0.54	DHA	5.9--0.9	0.55	LIN	4.1--4.7	0.57
EPA	7.9--5.1	0.69	ARA	5.8--2.2	0.69	ARA	5.8--1.2	0.69	16:1n-7	6.0--6.3	0.6	18:1n-7	1.5--6.1	0.62	18:1n-7	5.2--6.1	0.63
16:1n-7	2.6--6.0	0.74	18:1n-9	5.2--5.6	0.73	18:1n-9	5.2--8.3	0.75	EPA	5.1--3.6	0.66	18:1n-9	5.2--8.3	0.69	EPA	3.6--3.3	0.69
ALA	1.8--4.1	0.78	18:1n-7	2.8--5.2	0.77	18:1n-7	2.8--6.1	0.80	18:1n-9	5.2--5.6	0.72	EPA	5.1--3.3	0.75	18:0	4.0--4.2	0.72
18:0	5.8--3.2	0.82	LIN	2.2--4.1	0.81	LIN	2.2--4.7	0.83	ALA	4.1--1.34	0.77	LIN	4.0--4.7	0.80	14:0*iso*	2.9--2.0	0.76
Dec-11-- Feb-12 (23.6%)			Feb-12--Mar-12 (6.9%)			Mar-12--Jun-12 (9.2%)			Jun-12--Jul-12 (8.9%)			Jul-12--Sep-12 (9.4%)			Sep-12-- Dec-11 (21.0%)		
FA	means	dis.sum	FA	means	dis.sum	FA	means	dis.sum	FA	means	dis.sum	FA	means	dis.sum	FA	means	dis.sum
DHA	9.9--23.0	0.31	DHA	23.0--24.5	0.26	DHA	24.5--21.0	0.25	DHA	21.0--19.7	0.17	DHA	19.7--17.4	0.20	DHA	17.4--9.9	0.22
16:0	30.6--24.5	0.45	EPA	8.1--8.4	0.38	18:1n-9	3.9--6.1	0.38	18:1n-9	6.1--4.9	0.28	16:0	24.8--22.8	0.34	16:0	22.8--30.6	0.43
EPA	4.2--8.1	0.54	16:0	24.5--24.3	0.48	EPA	8.4--8.7	0.47	16:0	23.5--24.8	0.37	EPA	9.4--8.4	0.43	EPA	8.4--4.2	0.55
18:1n-9	7.4--4.0	0.62	ARA	6.4--6.7	0.55	16:1n-7	1.8--3.0	0.57	16:1n-7	3.0--2.8	0.46	16:1n-7	2.8--3.6	0.51	18:1n-9	4.8--7.4	0.62
14:0	5.4--2.5	0.70	14:0	2.5--1.9	0.61	16:0	24.3--23.5	0.65	14:0	1.6--2.8	0.53	ARA	6.11--4.9	0.58	14:0	3.2--5.4	0.68
ARA	3.4--6.4	0.76	16:1n-7	1.5--1.8	0.67	LIN	1.8--2.7	0.70	EPA	8.7--9.4	0.60	18:1n-9	4.9--4.8	0.63	18:0	5.3--7.5	0.74
18:0	7.5--5.7	0.81	18:1n-7	2.2--2.5	0.71	14:0	1.9--1.6	0.74	ARA	7.0--6.1	0.66	SDA	0.9--1.5	0.68	ARA	4.9--3.4	0.79
16:1n-7	3.2--1.5	0.85	18:1n-9	4.0--3.9	0.75	ARA	6.7--7.0	0.78	LIN	2.7--2.2	0.71	18:1n-7	2.8--3.2	0.72	16:1n-7	3.6--3.2	0.82

Figure captions

Figure 1. nMDS plot of whitefish and invertebrate FA profile data. Whitefish are shown as circles and month by different intensity of shading by gray scale. Invertebrate groups and habitats are presented with different marker shapes, with the shading of smaller overlaying circles indicating sampling month. The most important fatty acids corresponding to 70-80% of the total dissimilarities between groups were identified using SIMPER results (Table 2) and they are presented as light gray text.

Figure 2. Boxplots of whitefish muscle Total FA and PUFA content (mg g^{-1} DW) (A–B), UFA/SFA and n-3/n-6 –ratios (C–D) and content of eight most abundant FAs from the lowest to the highest contribution (E–L). Note the differences in y-axis scales in figures A, B, C, D, E-J, K-L. Bold horizontal lines indicate median values, the box indicate first and third quartile and whiskers indicate present minimum and maximum values unless outliers (open circles) are displayed (distance from median > 1.5*interquartile range).

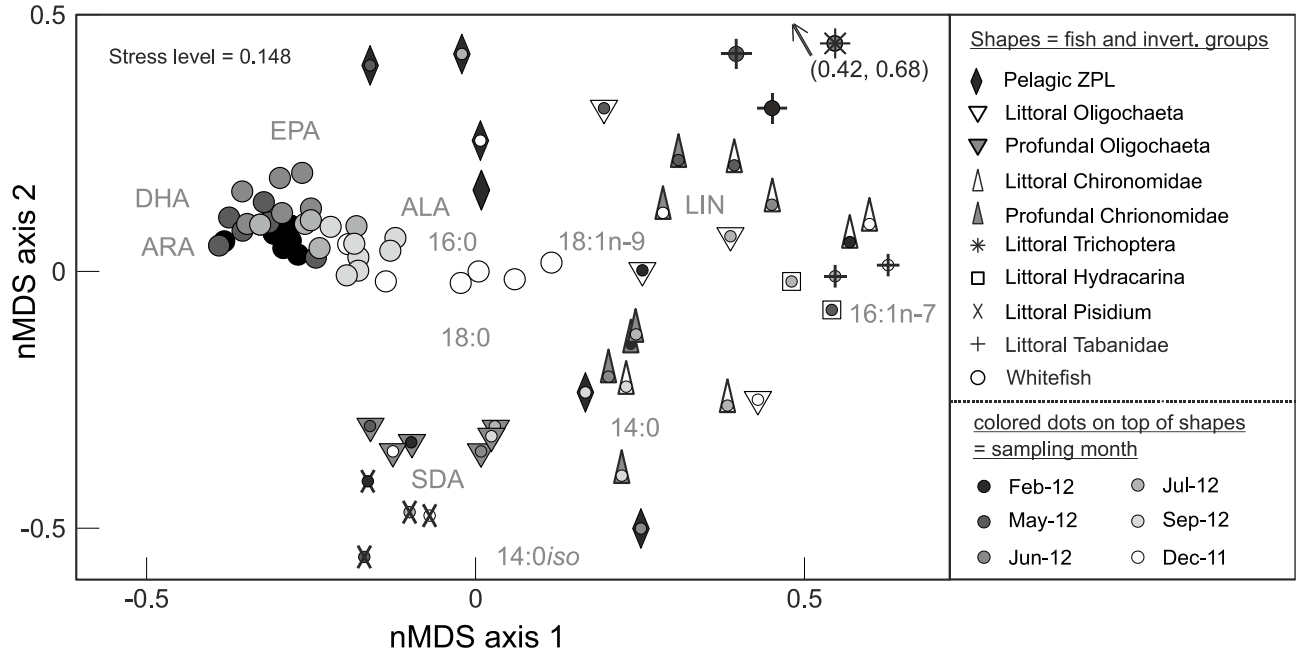
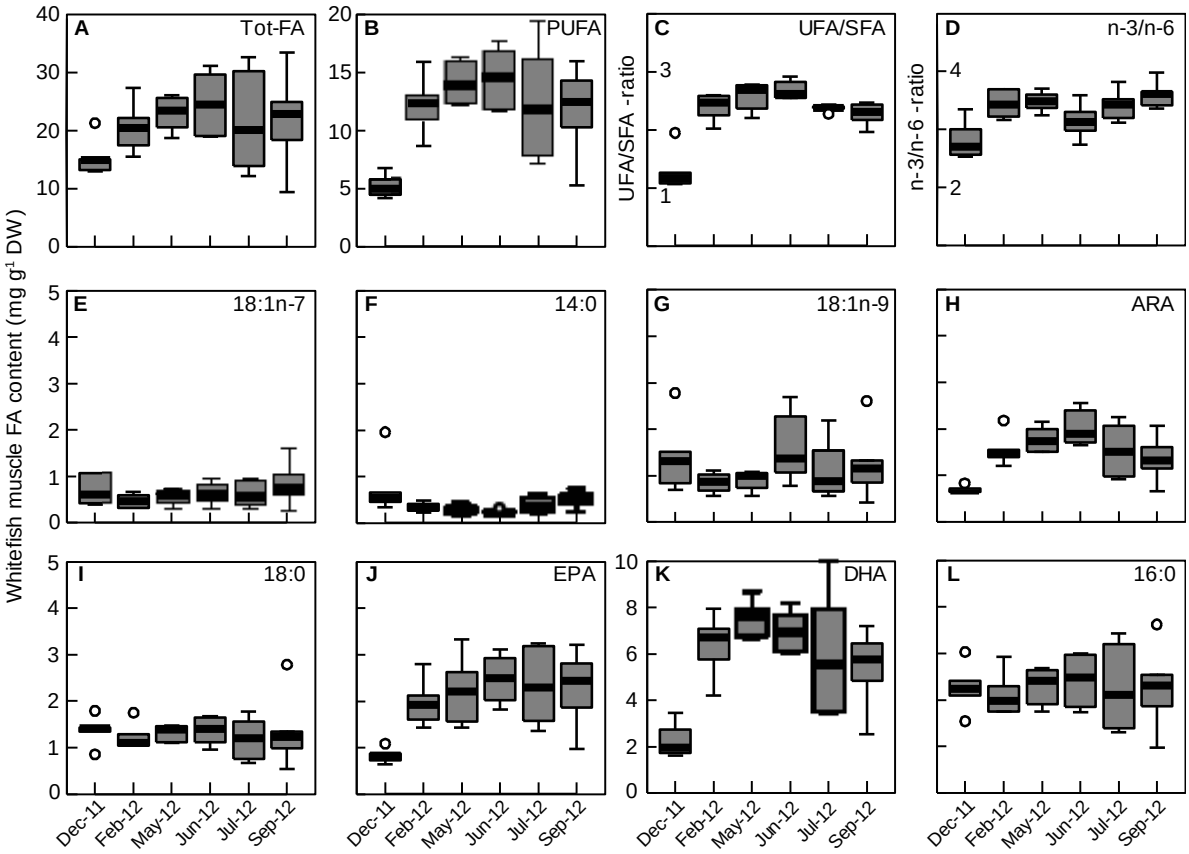

Stress level = 0.148
(0.42, 0.68)
EPA
DHA
ARA
ALA
16:0
18:1n-9
LIN
16:1n-7
18:0
14:0
SDA
14:0iso
nMDS axis 2
nMDS axis 1
0.5
0
-0.5
-0.5
0
0.5
Shapes = fish and invert. groups
Pelagic ZPL
Littoral Oligochaeta
Profundal Oligochaeta
Littoral Chironomidae
Profundal Chrionomidae
Littoral Trichoptera
Littoral Hydracarina
Littoral Pisidium
Littoral Tabanidae
Whitefish
colored dots on top of shapes = sampling month
Feb-12
May-12
Jun-12
Jul-12
Sep-12
Dec-11

Fig. 1.


A
Tot-FA
B
PUFA
C
UFA/SFA
D
n-3/n-6
E
18:1n-7
F
14:0
G
18:1n-9
H
ARA
I
18:0
J
EPA
K
DHA
L
16:0
Whitefish muscle FA content (mg g-1 DW)
UFA/SFA -ratio
n-3/n-6 -ratio
Dec-11
Feb-12
May-12
Jun-12
Jul-12
Sep-12

660

661 Fig. 2.