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Fatty Acids in Natural Ecosystems and Human Nutrition

Edited by
Michail I. Gladyshev

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Special Issue Editor

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About the Special Issue Editor

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Preface to "Fatty Acids in Natural Ecosystems and Human Nutrition"

Fatty acids (FAs), including polyunsaturated fatty acids (PUFAs) of the omega-3 family, have been the focus of attention of biochemists, physiologists, and ecologists in recent decades. On the one hand, fatty acids, especially long-chain PUFAs, are of great physiological importance for animals, including humans, as i) biochemical precursors of signaling molecules, e.g., oxylipins; ii) structural components of the cell membranes of neural tissues; and iii) "pacemakers" for the metabolism of animal cells, i.e., activators of membrane-bound enzymes. For humans, the consumption of PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is recommended by the World Health Organization to prevent cardiovascular diseases and neural disorders. On the other hand, FAs are considered the most reliable biomarkers in tracing fluxes of matter and energy in trophic webs of natural ecosystems and, thereby, to evaluate their structure and functions. Among the natural ecosystems, water bodies were found to play a peculiar role concerning long-chain PUFAs. Indeed, vascular plants, the main primary producers in terrestrial ecosystems, do not synthesize EPA and DHA. By contrast, some taxa of microalgae produce substantial amounts of EPA and DHA. Once synthesized by microalgae, PUFAs are transferred through trophic webs to organisms of higher trophic levels, i.e., invertebrates and fish. Thus, aquatic ecosystems are the main source of physiologically important EPA and DHA for many animals, including some omnivorous terrestrial animals and humans. At present, the ability of aquatic ecosystems to produce long-chain PUFAs, including those for human nutrition, is threatened by numerous anthropogenic factors, such as pollution, eutrophication and climate change, and biological invasions. Thus, the evaluation of fatty acids in natural ecosystems are of considerable importance for pure and applied sciences.

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Review

Long-chain Omega-3 Polyunsaturated Fatty Acids in Natural Ecosystems and the Human Diet: Assumptions and Challenges

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Abstract: Over the past three decades, studies of essential biomolecules, long-chain polyunsaturated fatty acids of the omega-3 family (LC-PUFAs), namely eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), have made considerable progress, resulting in several important assumptions. However, new data, which continue to appear, challenge these assumptions. Based on the current literature, an attempt is made to reconsider the following assumptions: 1. There are algal classes of high and low nutritive quality. 2. EPA and DHA decrease with increasing eutrophication in aquatic ecosystems. 3. Animals need EPA and DHA. 4. Fish are the main food source of EPA and DHA for humans. 5. Culinary treatment decreases EPA and DHA in products. As demonstrated, some of the above assumptions need to be substantially specified and changed.

Keywords: eicosapentaenoic acid; docosahexaenoic acid; nutritive quality; eutrophication; fish; culinary treatments

1. Introduction

Polyunsaturated fatty acids in the omega-3 family (PUFAs) are a focus of interest in many fields of science: biochemistry, physiology, dietetics, pharmacology, agriculture, aquaculture, ecology, etc. [1–7]. For many animals and humans, long-chain polyunsaturated fatty acids (LC-PUFAs), namely eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), are precursors of signaling molecules (bioactive lipid mediators) and essential components of cell membranes in neural and muscle tissues [8–11]. The number of publications on EPA and DHA in scientific journals has substantially increased since the 1970s (Figure 1). It is impossible to equally review LC-PUFA studies in all the fields of science; therefore, we will primarily take into consideration environmental issues, because natural and agricultural ecosystems are the source of EPA and DHA for human nutrition. There are many papers that report the fatty acid (FA) profiles of diverse microorganisms, plants, and animals, and address EPA and DHA as components of these profiles. However, we will consider only those studies that emphasize the role of these LC-PUFAs as well as their precursors.

In the field of ecology, even when studies have focused on primary producers (microalgae), the importance of EPA and DHA for human health was the rationale for the study of the content and composition of LC-PUFAs [12]. Indeed, only some taxa of microalgae can synthesize large amounts of EPA and DHA, while animals, including humans, have a comparatively low ability for such synthesis via conversion of the precursor, a short-chain PUFA, alpha-linolenic acid (18:3n-3, ALA) [13–15]. ALA, which is synthesized by plants, can be obtained by most animals only from food [16,17]. It should be noted that in contrast to EPA and DHA, which are synthesized by some algae, ALA is synthesized by terrestrial vascular plants and is the main component of the photosynthetic membranes

of chloroplasts [18–21]. Since EPA and DHA can be efficiently synthesized *de novo* only by some taxa of algae, aquatic ecosystems are recognized as the main source of these LC-PUFAs in the biosphere [22]. The algae-synthesized EPA and DHA are transferred through trophic chains to organisms at higher trophic levels, invertebrates, and fish, and then to terrestrial consumers, including humans.

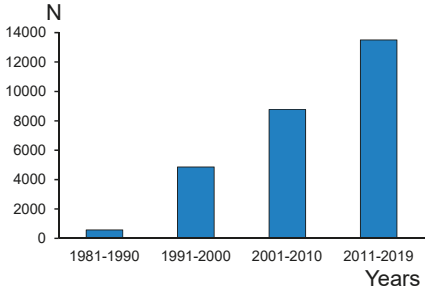

Figure 1. Number of publications containing (*N*) the terms 'eicosapentaenoic' or 'docosahexaenoic' in the Web of Science Core Collection during the last four decades.

In studies of EPA and DHA transfer from microalgae to humans, which inevitably included the culinary treatment of aquatic products for human nutrition, considerable progress has been made in recent decades. Many important findings have been summarized in several keynote statements [1,2,6,12,22], and a number of assumptions have appeared. New data appear continuously, and new questions arise, which naturally challenge some parts of the former assumptions. The aim of this paper is to consider and specify, if necessary, some important assumptions in the field of LC-PUFA production and regarding LC-PUFA transfer from natural ecosystems to the human diet.

2. Assumption 1: There Are Algal Classes of High and Low Nutritive Quality

In the nutrition ecology of zooplankton and other microalgaevorous organisms, microalgae have been subdivided into groups representing good and poor nutritive value according to their size and shape, and later to their carbon:nitrogen:phosphorus (C:N:P) ratio [23]. Starting with the milestone work of Ahlgren et al. [24], the PUFA content of microalgae became an important indicator of the nutritive quality of algae for consumers. In this work, it was found that Cryptophyceae and Dinophyceae (Peridinea) had high levels of EPA and DHA, and were the best food for zooplankton [24]. In turn, Chlorophyceae (green algae) and cyanobacteria contained no LC-PUFAs but certain levels of PUFAs, namely ALA, and had comparatively lower nutritive value [24]. Then, in another milestone paper, Muller-Navarra [25] demonstrated that Bacillariophyceae (diatoms), which had a high content of EPA, were a higher-quality food source for *Daphnia* than chlorophytes.

Since then, depending on their content of LC-PUFAs, microalgae have been subdivided into classes representing high and low nutritive quality, Cryptophyceae, Dinophyceae, and Bacillariophyceae vs. Chlorophyceae and cyanobacteria, respectively [26]. Some other classes of microalgae, Eustigmatophyceae, Prymnesiophyceae, Prasinophyceae, Chrysophyceae (golden algae), and Euglenophyceae, have also been shown to have a high content (mg g^{-1} C) of EPA and especially DHA [27–30]. Based on FA percentages, Trebouxiophyceae and Raphidophyceae were classified as intermediate and excellent food resources for zooplankton, respectively [31]. Recently, the division of phytoplankton classes into four categories was suggested based on the content of several biomolecules that included EPA and DHA: poor, medium, high, and superior quality food [30].

On the one hand, the division of microalgae into classes of high and low nutritive value in terms of their EPA and DHA content is reasonable. Indeed, Chlorophyceae and cyanobacteria (blue-green algae) do not produce these LC-PUFAs [32,33]. On the other hand, species from the classes lacking LC-PUFAs can have a high content of ALA, as mentioned above, and thereby provide a high growth

rate for consumers that can efficiently convert ALA to EPA and DHA (e.g., [34–37] see also Assumption 3 below). For instance, there were no statistically significant differences among the growth rates of *Daphnia magna* when feeding on chlorophytes, chrysophytes, and diatoms [30]. The cited authors emphasized that the reproduction of *D. magna* in feeding experiments was dependent on total n-3 FAs rather than only on EPA.

Moreover, within algal classes that can synthesize EPA and/or DHA, there are species with low contents of these LC-PUFAs and thereby with low nutritive value. Indeed, EPA levels (as a percent of total FA) in 17 marine diatom species used in aquaculture ranged from 5 to 30% [38]. The EPA content in diatoms per gram of organic carbon varied from 1.7 mg g^{-1} C in *Cyclotella meneghiniana* [30] to 45.9 mg g^{-1} C in *Thalassiosira oceanica* [39]. Moreover, in addition to inter-species variability, high variation in EPA content within one species occurs. Indeed, despite the lowest value for *C. meneghiniana* given above, a considerably higher content of EPA, 40.8 mg g^{-1} C, was reported for this species [25]. In natural ecosystems, it has been shown that some marine plankton diatoms have little EPA [40]. In a freshwater reservoir, *Cyclotella* was not associated with the EPA content in seston, while there was a significant correlation between *Stephanodiscus* and the content of EPA [41]. Similar results demonstrating contrasting levels of EPA in different diatom taxa were obtained for river littoral epilithic microalgae [42]. Evidently, diatom species can differ strongly in EPA content, and the common point of view that all diatoms are the superior quality food should be revised.

Consequently, dividing microalgae on the basis of their LC-PUFA content into classes appears to be too coarse for assessing nutritional value for consumers. It is worth noting that 'nutritional value' is not characteristic of a food item only, but indicates the demands of the consumer as well. Indeed, if a consumer does not need considerable amounts of EPA and DHA (see also Assumption 3 below), the above subdivision of microalgae into classes of high and low nutritive value is irrelevant. As found in a study of a freshwater plankton community, "there were no phytoplankton species of clearly high or low nutritive value. All phytoplankters, or at least detritus, that originated from them may meet the specific elemental and biochemical requirements of specific groups of zooplankton" [43]. Thus, Assumption 1 should be improved in future studies.

3. Assumption 2: EPA and DHA Decrease with the Increasing Eutrophication of Aquatic Ecosystems

The primary producers of EPA and DHA, Bacillariophyceae, Cryptophyceae and Dinophyceae are known to mainly inhabit oligotrophic aquatic ecosystems with low concentrations of total phosphorus (TP), while eutrophic (high TP) aquatic ecosystems are dominated by green algae and cyanobacteria, which do not produce LC-PUFAs (e.g., [44,45]). Thus, a high TP concentration decreases the contents of EPA and DHA in seston due to an increase of cyanobacteria, and thereby decreases the transfer of these LC-PUFAs to higher trophic levels [45,46]. Indeed, the EPA(DHA)-to-carbon content ratio in lake seston had a statistically significant negative relationship with TP concentration in lake water [46], and the EPA + DHA content in perch (per unit muscle mass) also had a statistically significant negative relationship with lake TP [45]. Thus, the nutritive quality of fish for humans becomes lower with eutrophication [45]. An increase in lake eutrophication, measured via the chlorophyll *a* concentration, also resulted in a significant decrease in the EPA content in bighead carp [47].

However, in the above publications, the relative content of LC-PUFAs per unit of organic carbon, C, or fish mass was estimated. These measures of relative content definitely indicate food quality, namely, the nutritive value of seston for zooplankton [46] and fish for humans [45,47]. In addition, if the quantification of the LC-PUFA supply for human nutrition is to be regarded as the paramount aim of relevant ecological studies, it is necessary to quantify this supply as the EPA and DHA yield, $Y_{LC\text{-}PUFA}$ value, which has the units μg or mg of LC-PUFA per m^2 or m^3 in an aquatic ecosystem per day or year. Thus, to convert food quality, i.e., the relative content of LC-PUFAs (μg mg C^{-1}), to the

quantity, $Y_{LC\text{-}PUFA}$ (μg m^{-3} day^{-1}), it is necessary to take into account the production of organic carbon in an aquatic ecosystem, V (mg C m^{-3} day^{-1}):

$$Y_{LC\text{-}PUFA} = LC\text{-}PUFA \cdot V. \quad (1)$$

For instance, Muller-Navarra et al. [46] provided the following relation of the content of EPA (μg mg^{-1} C) in lake seston to the concentration of total phosphorus (TP; μg L^{-1}) in lake water:

$$\ln EPA = -0.69 \cdot \ln TP + 2.78. \quad (2)$$

From Equation (2), the following dependence can be obtained:

$$EPA = e^{(-0.69 \cdot \ln TP + 2.78)} = 16.12 \cdot TP^{-0.69}. \quad (3)$$

The dependence of the rate of photosynthesis (primary production), V (mg C m^{-3} day^{-1}), in lake phytoplankton on TP was given in [48]:

$$V = 10.4 \cdot TP - 79. \quad (4)$$

To determine the value of the yield of EPA, Y_{EPA} (μg m^{-3} day^{-1}) (Equation (1)), i.e., its amount produced in a lake, it is necessary to multiply Equation (3) by Equation (4):

$$Y_{EPA} = 16.12 \cdot TP^{-0.69} \cdot (10.4\ TP - 79) = 167.6 \cdot TP^{0.31} - 1273.5 \cdot TP^{-0.69}. \quad (5)$$

Graphs of Equations (3)–(5) are given in Figure 2. Evidently, with a decrease in the relative content of EPA in seston, there is an increase in EPA yield with increasing TP, i.e., with increasing lake eutrophication. The same is true for DHA if the analogue for Equation (1) is taken from Muller-Navarra et al. [46].

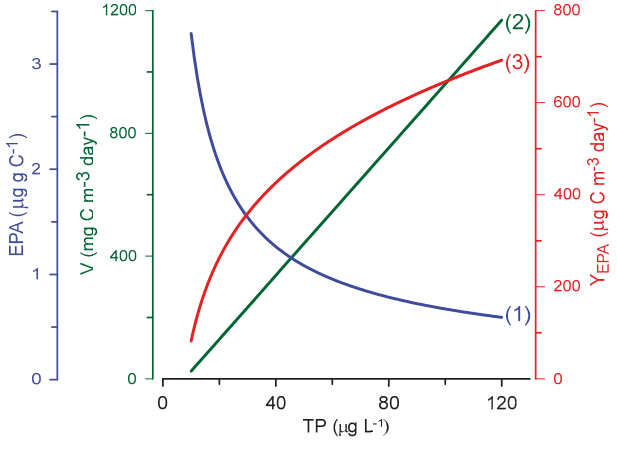

Figure 2. Dependence of the content of eicosapentaenoic acid in lake seston, EPA (1), rate of photosynthesis (primary production), V (2), and the yield of EPA, Y_{EPA} (3), on the total phosphorus concentration in water, TP.

It is worth noting that according to Equation (4), the LC-PUFA yield produced by microalgae (lake phytoplankton) increases gradually with increasing TP (Figure 2). This relationship contradicts taxonomic changes in phytoplankton that usually occur during eutrophication. If primary production in a water body increases only at the expense of cyanobacterial or green algal growth, there would be

no increase in LC-PUFA yield. In contrast, the EPA + DHA yield obtained from lakes where perch were caught increased up to a TP concentration of ~40 μg L^{-1} and then decreased with increasing eutrophication [49]. Thus, the maximum LC-PUFA yield (g km^{-2} $year^{-1}$) associated with fish catches occurred in mesotrophic rather than oligotrophic or eutrophic aquatic ecosystems [49].

Moreover, in eutrophic temperate lakes, cyanobacteria dominate in the phytoplankton community only in summer, while in spring, at low water temperatures, "blooms" of psychrophilic diatoms with high contents of EPA often occur. The above equation describing the relation between EPA and TP (Equation (2)) was obtained by Muller-Navarra et al. [46] for summer phytoplankton only. However, if a whole year is taken into consideration, in a eutrophic reservoir, a spring "bloom" of psychrophilic diatoms can produce a large pool of EPA, which can then be transferred through the trophic chain and peak in zooplankton and fish biomass with a time lag during summer [50]. It should also be noted that with decreasing phosphate availability, the proportion of EPA in some freshwater algae significantly decreased [51].

Thus, the common assumption that EPA and DHA decrease with increasing eutrophication of aquatic ecosystems should be improved. Such a decrease occurs in the relative contents of these LC-PUFAs per unit of sestonic organic carbon or fish biomass. However, with regard to EPA and DHA yield, including that available for human nutrition, measured as LC-PUFA quantity per unit area or volume of an aquatic ecosystem per unit time, such a decrease may not occur. At present, mesotrophic aquatic ecosystems are believed to provide a maximum supply of EPA + DHA for humans via fish catches. Ranking aquatic ecosystems on the basis of their ability to produce LC-PUFAs for human nutrition is an important challenge for future research.

4. Assumption 3: Animals Need EPA and DHA

As mentioned above, EPA and DHA play important biochemical and physiological roles in humans and many animals, and the common point of view is that all vertebrates and most invertebrate groups require these LC-PUFAs [52]. The low ability of animals to synthesize EPA and DHA from short-chain ALA necessitates them to obtain these LC-PUFAs from food. However, the commonly used terms of "many animals" and "most invertebrates" as well as "low ability" and "necessity" have not been specified or quantified yet. Furthermore, the hypothesis that in natural ecosystems "many" animals can be limited by a low EPA and DHA supply is the important premise of a number of studies [22,52]. Thus, the specification and quantification of the above terms seems to be an important challenge for relevant ecological studies [22,53].

First, it should be noted that a large group of invertebrates, terrestrial insects, practically do not have EPA and DHA [54–59]. Indeed, terrestrial insects use EPA only as the precursor of lipid mediators, eicosanoids, and thereby synthesize it from consumed ALA in small quantities, at the level of vitamins [60]. In contrast to terrestrial insects, aquatic (amphibiotic) insects have high levels of EPA in their biomass, but contain very low, if any, DHA [61–64]. As mentioned above, DHA is the main component of the phospholipids of the cell membranes of vertebrate neural tissues, including retinal photoreceptors [3,4,65,66] (Figure 3). However, instead of DHA, there are 18C PUFAs in the eyes of terrestrial insects [3,67] (Figure 3), and EPAs in the eyes of amphibiotic insects [68] (Figure 3). Thus, terrestrial insects evidently do not need EPA or DHA, and aquatic insects do not need DHA in their food or in their biomass in considerable amounts.

Other terrestrial invertebrates, earthworms (*Lumbricus terrestris*), likely need EPA since they have a comparatively high content of this LC-PUFA in their biomass [69]. However, earthworms do not need to obtain these biomolecules from their food, since they likely obtain EPA from their gut microflora [69]. According to our unpublished data obtained using GC-MS and internal standards as in [61,70], Californian worms (*Eisenia foetida*) from a laboratory culture [71] contain 0.37 ± 0.02 and 0.02 ± 0.02 (n = 3) mg g^{-1} WW EPA and DHA, respectively. Moreover, some species of soil nematodes can *de novo* synthesize omega-3 PUFAs, namely ALA and EPA [72].

Herbivorous terrestrial vertebrates that consume green parts of plants can satisfy their physiological needs for EPA and DHA through the conversion of ALA [5,15,73]. Some omnivorous terrestrial vertebrates with high metabolic rates, such as the rattlesnake (*Crotalus atrox*), hummingbird (*Archilochus colubris*), white-throated sparrow (*Zonotrichia albicollis*), deer mouse (*Peromyscus maniculatus*), and bank vole (*Myodes glareolus*), have high proportions of DHA in their muscle phospholipids, accounting for up to 33% of the total fatty acids [74–77]. However, these animals evidently have no dietary source of this LC-PUFA, because even aquatic insects or earthworms, if presented in the diet, could provide them with only EPA rather than DHA.

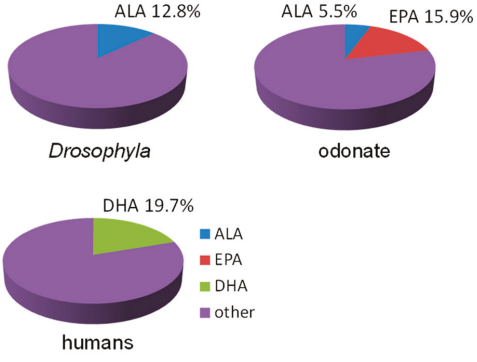

Figure 3. Levels (% of total fatty acids) of alpha-linolenic (ALA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in the heads of *Drosophila* [67], the eyes of odonates [68] and human retinas [3].

The high levels of n-3 LC-PUFAs in the functional lipids and organs of some consumers, such as those mentioned above, certainly indicate the physiological significance of these compounds. Recently, new promising approaches have been used to confirm that a consumer has a dietary need for EPA and DHA. One of them combines the elucidation of a dietary source of LC-PUFAs for a consumer and possible physiological consequences of deprivation of this dietary source. For instance, wolf spiders (*Tigrosa georgicola*) that inhabited wetlands and consumed aquatic insects had higher tissue levels of aquatically derived LC-PUFAs and elevated immune function in comparison to upland spiders [78]. Another way to obtain evidence of a dietary need for EPA and DHA is to measure tissue LC-PUFA pools formed due to direct incorporation from an aquatic diet versus conversion from dietary ALA of terrestrial origin. For instance, Twining and colleagues [79] showed that the ALA content of terrestrial insects, and the ALA-to-EPA conversion efficiency, are insufficient to supply insectivorous tree swallow chicks with the n-3 LC-PUFAs that they require. The authors concluded that EPA-rich aquatic insects are ecologically essential resources during a critical ontogenetic period in this bird.

Thus, the statement that "many" animals need LC-PUFAs and must consume them can be challenged by the statement that "many" animals do not need EPA and DHA or do not need to obtain them from food. Evidently, more work is needed to specify species of aquatic and terrestrial animals that truly depend on dietary sources of LC-PUFAs.

5. Assumption 4: Fish Are the Main Food Source of EPA and DHA for Humans

Fish and aquatic invertebrates (shellfish, crayfish, etc.) are known to be the main source of LC-PUFAs for humans [7,80–84]. In general, i.e., on a global scale, this statement is absolutely true. However, in some cases, it should be used with caution. First, the contents of the sum of EPA + DHA in the edible parts (muscle tissue) of fish species varied ~400-fold, from 32.78 mg g^{-1} wet weight (WW) in the boganid char (*Salvelinus boganidae*) [85] to 0.08 mg g^{-1} WW in red hybrid tilapia (*Oreochromis* sp.) [86].

To obtain health benefits, the World Health Organization and some national nutrition and health organizations recommend daily personal consumption of 0.5–1.0 g of EPA + DHA [6,7,81,87,88] and even >1.0 g for a Western-type diet [7,88]. Thus, the recommended personal daily dose of EPA + DHA is contained in 15–30 g of meat of boganid char or in 6250–12500 g of meat of the tilapia. Naturally, a question arises: is the fish red hybrid tilapia a real source of LC-PUFAs for humans if 6–12 kg of its meat should be consumed daily to obtain the recommended healthy dose? If 1.0 g of EPA and DHA is considered as the daily dose for the reliable prevention of heart diseases and 1 kg as the maximum portion of fish per serving, the lower threshold value of EPA+DHA content in edible fish biomass is 1.0 mg g^{-1} [85]. All fish species with LC-PUFA contents below this threshold cannot be regarded as a "main source" of EPA and DHA for humans. Nevertheless, these "low LC-PUFA fish" are valuable sources of protein, vitamins, and other nutrients.

Second, some people do not eat fish, and so fish naturally cannot be the "main source" of EPA and DHA for them. For such people, there are alternative dietary LC-PUFA sources, for instance, the livers of terrestrial production animals, which contain EPA + DHA at a level close to the above threshold value of 1.0 mg g^{-1} [83]. However, as mentioned above, on a global scale, the livers of production animals, such as cattle, pigs, and chickens, cannot be alternatives to fish but represent an additional source only. Indeed, the global EPA + DHA supply through the wild fish catch is ~180 10^6 kg $year^{-1}$, while the global production of both of these LC-PUFAs together in animal livers is ~4 10^6 kg $year^{-1}$ [83]. In addition, some game birds from the order Passeriformes, which have EPA + DHA contents in their meat above the threshold value, from 1.8 to 3.7 mg g^{-1} [89], may also be an alternative source of these LC-PUFAs for humans who do not eat fish.

Human populations have different diets, e.g., the vegetarian diet in South and Eastern Asia and omnivorous (Western-type) diet in Europe and North America. As a rule, the LC-PUFA status of individuals from populations with a vegetarian diet, i.e., the content of EPA and DHA in various tissues and organs, is significantly lower than that of their omnivorous counterparts [90]. They commonly have a very low intake of preformed LC-PUFAs, ca. 10–74 mg per day, and most EPA and DHA is obtained as a result of the conversion of the dietary short-chain PUFA, ALA [90,91]. The following question arises: how does this conversion meet the demands for LC-PUFAs in vegetarian humans, since its rate is known to be low? Recent studies have found marked global polymorphism in the FADS (fatty acid desaturase) gene cluster, which is strongly associated with the efficiency of the conversion of linoleic and linolenic acids to LC-PUFAs of the corresponding family [92–94]. Human populations that have moved to more vegetarian diets are adapted to a low intake of LC-PUFAs, and these alleles provide the more efficient conversion of ALA to EPA and DHA. The selective patterns in FADS genes have been suggested to be driven by a change in the dietary composition of fatty acids following the transition to agriculture. Overall, there is a premise that vegetarian humans can function adequately with the found LC-PUFA status. More studies are necessary to assess the physiological and pathological outcomes of a vegetarian diet in terms of individual- and population-based genetic differences in the metabolism of dietary 18C-PUFAs [91,94].

When discussing fish as the main source of EPA and DHA for humans, the following paradox should be mentioned. Since the growing human population requires an increasing supply of essential LC-PUFAs, and wild catch fisheries are at exploitable limits, a greater proportion of food fish are obtained from aquaculture [95]. However, farmed fish, such as one of the most popular and valuable species, Atlantic salmon (*Salmo salar*), need high levels of EPA and DHA in their diet, which are obtained from the limited wild catch fisheries in fishmeal and fish oil [95]. Thus, in aquaculture oilseed, plant sources are increasingly used in feed to substitute the finite fishmeal and oil. This replacement of fish oil with the sustainable alternative, vegetable oils, has no detrimental effect on fish growth but results in a dramatic decrease in EPA and DHA in their flesh. For instance, the contents of EPA+DHA in the flesh of farmed Atlantic salmon in Scotland decreased from 27.4 mg g^{-1} in 2006 to 13.6 mg g^{-1} in 2015 [95]. Evidently, sustainable sources of EPA and DHA, in addition to fish, must inevitably satisfy the growing human population.

6. Assumption 5: Culinary Treatments Decrease EPA and DHA in Products

Polyunsaturated fatty acids are known to be preferentially affected by oxidation during heating [96–98]. Thus, the degradation of LC-PUFAs in food during cooking and other culinary treatments has been reported by many authors [99–111]. However, other authors reported no decrease in EPA and DHA during cooking [70,83,112–123]. Indeed, products such as raw fish and production animals do not contain EPA and DHA in pure chemical form, but as components of phospholipids integrated into cell membranes, which have comparatively low susceptibility to degradation [82,124].

It is important to note that the above data on the degradation of LC-PUFAs during culinary treatments are based on measurements of their relative levels as a percentage of total FA. Furthermore, it has been demonstrated that the use of relative values (%) instead of the absolute content of EPA and DHA (mg g^{-1} WW) for the estimation of nutritive value gives erroneous conclusions regarding the nutritive values of fish and other products for humans (e.g., [83,95,117,125–128]). For instance, there are many fish species with high EPA+DHA contents, >8 mg g^{-1}, but a low percentage, <20%, e.g., chum salmon (*Oncorhynchus keta*), coho salmon (*Oncorhynchus kisutch*), and lake trout (*Salvelinus namaycush*), while there are species with a high percentage, >40%, and low content, <3 mg g^{-1}, e.g., Atlantic cod (*Gadus morhua*) and whiting (*Merlangius merlangus*) [129].

Another striking example is related to edible macroalgae (seaweeds): red algae (Rhodophyta), (*Palmaria palmata*), have extremely high levels of EPA, ca. 50% of the total fatty acids, but because of their very low total lipid contents, at realistic daily consumption levels, they cannot satisfy the LC-PUFA requirements of humans [126].

There is no correlation, or even a negative correlation, between EPA and DHA levels (%) and contents (mg g^{-1} WW) in fish [129,130]. The explanation for the above phenomenon is believed to be as follows: EPA and DHA are mostly contained in phospholipids (PLs), i.e., in the structural lipids of cell membranes, which should remain nearly constant in proportion to functional muscle tissues, while many other fatty acids are contained in reserve neutral lipids, triacylglycerols (TAGs), whose composition is highly variable in fish biomass [125,130–132]. For this reason, in fish which are considered to be fatty, i.e., accumulating comparatively more TAG [133], EPA and DHA are "diluted", and their relative levels decrease.

Thus, the nutritive value of products for human nutrition should be estimated on the basis of the contents of EPA and DHA, mg per g of product, which can be obtained using internal standards during chromatography, rather than levels, or the % of total FAs [83,117,127,134,135]. Furthermore, content estimates based on internal standards are scarce, and most data are published as the level, or %, of total FAs [100–102,105–111]. Here, we provide data obtained using internal standards, which allows us to compare the real nutritive value of fish and production animal products (Table 1). As noted in Assumption 4, some products prepared from terrestrial animals are comparable in their EPA and DHA contents with those of some fish (Table 1). In general, according to the quantitative data, mg per g of product, there is no decrease in LC-PUFA contents following most culinary treatments, and cooked products prepared from relevant raw biomass are good sources of EPA and DHA for humans.

Table 1. Contents of the sum of eicosapentaenoic and docosahexaenoic fatty acids (EPA+DHA, mg g^{-1} of product) in cooked fish and the daily portion of products (DP, g) that need to be consumed to obtain the recommended intake of EPA+DHA for humans, 1 g day^{-1}.

Product	EPA + DHA	DP	Reference
Atlantic salmon *Salmo salar* (fried)	40.1	25	[121]
Pacific saury *Cololabis saira* (canned, brand H)	37.9	26	[136]
Atlantic salmon *Salmo salar* (fried)	19.6	51	[114]
Pacific herring *Clupea harengus* (canned)	17.9	56	[118]
Atlantic salmon *Salmo salar* (fried)	17.0	59	[112]
Baltic sprat *Sprattus sprattus* (canned)	14.3	70	[118]
Pacific saury *Cololabis saira* (canned, brand N)	13.1	76	[136]
King salmon *Oncorhynchus tshawytscha* (baked)	12.4	81	[137]
Lake trout *Salvelinus namaycush* (baked)	12.4	81	[122]
Lake trout *Salvelinus namaycush* (fried)	12.4	81	[122]
Lake trout *Salvelinus namaycush* (broiled)	12.3	81	[122]
King salmon *Oncorhynchus tshawytscha* (steamed)	11.9	84	[137]
King salmon *Oncorhynchus tshawytscha* (fried)	11.5	87	[137]
King salmon *Oncorhynchus tshawytscha* (microwaved)	10.4	96	[137]
King salmon *Oncorhynchus tshawytscha* (poached)	10.0	100	[137]
Sardine *Sardina pilchardus* (fried)	8.8	114	[112]
Humpback salmon *Oncorhynchus gorbuscha* (boiled)	6.0	167	[116]
Brown trout *Salmo trutta* (boiled)	5.7	175	[117]
Humpback salmon *Oncorhynchus gorbuscha* (stewed)	5.3	189	[116]
Humpback salmon *Oncorhynchus gorbuscha* (roasted)	5.0	200	[116]
Humpback salmon *Oncorhynchus gorbuscha* (fried)	4.3	233	[116]
Brown trout *Salmo trutta* (fried)	4.1	244	[117]
Cod *Gadus morhua* (fried)	4.1	244	[114]
Spanish mackerel *Scomberomorus commerson* (fried)	3.9	256	[112]
Pacific herring *Clupea harengus* (boiled)	3.9	256	[117]
Pacific herring *Clupea harengus* (fried)	3.8	263	[117]
Rock sole *Lepidopsetta bilineata* (boiled)	3.6	278	[117]
Chinook salmon *Oncorhynchus tshawytscha* (fried)	3.2	313	[122]
Rock sole *Lepidopsetta bilineata* (fried)	3.1	323	[117]
Chinook salmon *Oncorhynchus tshawytscha* (baked)	3.1	323	[122]
White sucker *Catostomus commersonii* (baked)	2.3	435	[122]
Cod *Gadus morhua* (boiled)	2.4	417	[117]
Chinook salmon *Oncorhynchus tshawytscha* (fried)	2.8	357	[122]
Cod *Gadus morhua* (fried)	2.2	455	[121]
Walleye (*Sander vitreus*) (baked)	2.1	476	[122]
White sucker *Catostomus commersonii* (broiled)	2.1	476	[122]
White sucker *Catostomus commersonii* (fried)	2.0	500	[122]
Walleye (*Sander vitreus*) (broiled)	1.9	526	[122]
Walleye (*Sander vitreus*) (fried)	1.9	526	[122]
Prawn *Macrobrachium acanthurus* (fried)	1.8	556	[138]
Beef liver (boiled)	1.3	769	[83]
Zander *Sander lucioperca* (boiled)	1.1	909	[70]
Pork liver (boiled)	1.0	1000	[83]
Zander *Sander lucioperca* (stewed)	1.0	1000	[70]
Zander *Sander lucioperca* (fried)	1.0	1000	[70]
Common carp *Cyprinus carpio* (fried)	1.0	1000	[122]
Chicken liver (boiled)	0.7	1429	[83]
Common carp *Cyprinus carpio* (baked)	0.7	1429	[122]
Gilthead sea bream *Sparus aurata* (fried)	0.6	1667	[139]
Common carp *Cyprinus carpio* (broiled)	0.5	2000	[122]
Pork (fried)	0.3	3333	[119]
White rabbit (baked)	0.1	10000	[140]

7. Conclusions

A number of assumptions important for developing LC-PUFA studies in the ecological and food sciences should be improved:

1. Dividing microalgae on the basis of their LC-PUFA content into classes of high and low nutritive value appeared to be too coarse. Although there are no Chlorophyceae (green algae) that contain EPA and DHA, there are Bacillariophyceae (diatoms) with low contents of LC-PUFAs.
2. The maximum LC-PUFA yield (g km^{-2} $year^{-1}$) that can be ultimately obtained by humans occurs in mesotrophic rather than oligotrophic aquatic ecosystems.
3. Many animals and terrestrial insects do not need EPA, and aquatic insects do not need DHA in any considerable quantity. Many other animals do not need LC-PUFAs in their food: some worms can obtain these biomolecules from their intestine microflora, and strictly herbivorous terrestrial mammals can synthesize required quantities of EPA and DHA from ALA obtained from the green parts of consumed plants.
4. There are many fish species that are not adequate sources of EPA and DHA for humans, especially for those with a Western-type diet. In turn, there are products of terrestrial animals that can be a source of LC-PUFAs for persons who do not eat fish. In human populations with a vegetarian diet, the conversion of dietary C18-PUFAs is considered to be sufficient to meet the demands for LC-PUFAs based on the found genetic patterns; however, this statement requires further study.
5. Most common culinary treatments do not decrease the EPA and DHA contents in fish and other animal products.

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Impact of Water Pollution on Trophic Transfer of Fatty Acids in Fish, Microalgae, and Zoobenthos in the Food Web of a Freshwater Ecosystem

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Abstract: This research work was carried out to determine the effects of water contamination on the fatty acid (FA) profile of periphyton, zoobenthos, two Chinese carps and a common carp (*Hypophthalmichthys molitrix*, *Ctenopharygodon idella* and *Cyprinus carpio*), captured from highly polluted (HP), less polluted (LP), and non-polluted (NP) sites of the Indus river. We found that the concentration of heavy metals in the river water from the polluted locations exceeded the permissible limits suggested by the World Health Organization (WHO) and the US Environmental Protection Agency (EPA). Fatty acid profiles in periphyton, zoobenthos, *H. molitrix*, *C. idella*, and *C. carpio* in the food web of river ecosystems with different pollution levels were assessed. Lauric acid and arachidic acids were not detected in the biomass of periphyton and zoobenthos from HP and LP sites compared to NP sites. Alpha-linolenic acid (ALA), eicosadienoic acid and docosapentaenoic acid were not recorded in the biomass samples of periphyton and zoobenthos in both HP and LP sites. Caprylic acid, lauric acid, and arachidic acid were not found in *H. molitrix*, *C. idella*, and *C. carpio* captured from HP. In this study, 6 and 9 omega series FAs were identified in the muscle samples of *H. molitrix*, *C. idella* and *C. carpio* captured from HP and LP sites compared to NP sites, respectively. Less polyunsaturated fatty acids were observed in the muscle samples of *H. molitrix*, *C. idella*, and *C. carpio* collected from HP than from LP. The heavy metals showed significant negative correlations with the total FAs in periphyton, zoobenthos, and fish samples.

Keywords: Fatty acid; fish; food web; periphyton; trophic transfer; water pollution; zoobenthos

1. Introduction

The aquatic fauna and flora of river ecosystems comprise a complex assemblage of different communities and are biologically important because of the interlinking between different trophic levels. These aquatic food chains are very feeble and sensitive to contaminants, especially to the toxicity of exogenous chemicals and heavy metals that are discharged into freshwater reservoirs due to various human activities. Heterotrophic aquatic organisms in food chains consume a variety of metalloids and xenobiotic compounds, which usually cause immutable degradation of the planktonic life at higher concentrations [1,2]. The toxic response in freshwater fish species to contaminated environments has been reported on a global scale [3,4]. The uptake of heavy metals into the aquatic food chain can occur either by dietary or non-dietary routes [5]. Therefore, the concentration of heavy metals in fish normally indicates levels present in sediment and water that is specifically in freshwater reservoirs

where the fish is captured from [6], as well as the time of exposure [7]. The concentration of essential metals, if increased above the normal metabolic needs of fish, may become toxic for the fish and for the ultimate consumer, humans [8]. Heavy metals may accumulate in primary producers such as microalgae, where diatoms ultimately transfer them to other trophic levels [9]. Heavy metals are ingested by fish and bio accumulate in the liver, kidneys, and other vital organs through adsorption and absorption [7].

Lipids are considered to be one of the most essential nutrients, which affect the growth and development of fish and other organisms [10], and alleviate immune competence [11]. Essential lipids are nutritionally important for the consumers in the food chain because they promote the growth and development and overall health of aquatic fauna and flora of aquatic communities in freshwater ecosystems [12–14]. Kainz et al. [15] proposed that the trophic movement of fatty acid (FA) in the food chain may be used as a physiological biomarker for monitoring the status of contamination in freshwater ecosystems. Kainz et al. [15] further mentioned that this trophic movement of FA in the food chain may be used as a physiological biomarker for observation of the status of contamination in freshwater ecosystems. Thus, it is necessary to ensure the abundance of microalgae and zoobenthos for trophic transfer into higher levels in the food web to ensure the transfer of FA and polyunsaturated fatty acid (PUFA) to the fish [16]. Currently, there are no comprehensive reports in the literature describing the interlinking trophic movements of PUFA and the impact of water pollution in the river ecosystems. The latter is still poorly understood with reference to FA profiles of periphyton, zoobenthos, and fish, and effect of contaminants. periphyton and zoobenthos can be used as valuable indicators to determine the effect of contamination and the synthesis of FA in freshwater ecosystems [17].

Aquaculture plays an important role in providing good quality animal protein and provides sustainable livelihood opportunities and food security for the ever increasing world population [18,19]. Fish are recognized as an important part of the human diet, owing to its balanced ratio proteins/PUFAs, including omega series FAs [20] which may reduce the risk of heart diseases. Because of the nutritional and pharmaceutical importance of PUFAs, researchers in the discipline of fishery sciences have been paying them increasing attention [21,22].

The bioaccumulation of metals in fish is triggered by the accumulation of these elements in phyto- and zooplankton; however, this event ceases to be the most relevant as long as biomagnification takes place. Biomagnification can have serious impacts on the food chain [23]. Fish has the potential to accumulate more metals from food and water [24]. Kainz and Fisk [25] mentioned that most of the FAs and pollutants move trophically through the food chain, ultimately having effects on the final consumer. This situation warrants an understanding of the fate of FAs and the impact of heavy metals contamination on the variability of FA on the food chain in river ecosystems. Variation in FAs dynamics in the food web is linked to increases in environmental stress and habitat destruction due to water pollution within a freshwater ecosystem [26]. Moreover, the disparity in FAs and pollutant trophic movement in the food chain may give insights into ecological functions and their impact on habitat and environmental stress. Keeping this in mind, it is necessary to investigate the interlink and biotransformation of FAs in the food web and the relationship with water pollution. This requires assessing and contrasting the trophic movement of lipids and pollutants in the aquatic food chain. The main aim of research was (i) to assess the fatty acid profiles in periphyton, zoobenthos, *Hyphpthalmichthys molitrix*, *Ctenopharyngodon idella*, and *Cyprinus carpio* in the food web of river ecosystems with different pollution levels; (ii) to assess their flow in aquatic ecosystems; (iii) to explore their potential for evaluating and monitoring the health of aquatic habitats; (iv) and to apply FA profiles as a possible physical biomarker of environmental stress from heavy metal pollution.

2. Materials and Methods

2.1. Study Area

The Indus river is the longest river in Pakistan. The Indus River originates on the Tibetan Plateau, enters into towards Gilgit-Baltistan from Ladakh, and then flows from Punjab Province and joins into

the Arabian Sea. It is the largest river in Pakistan with a total catchment basin of about 1,165,000 km^2 (450,000 m^2) https://en.wikipedia.org/wiki/Indus_River).

The Mianwali District is situated in the province of the Punjab and is about 200 m above sea level (Figure 1; 25). The Mianwali is one of the districts in the province of the Punjab and is about 200 m above sea level [27]. This district is rich in minerals, clay, coal, gypsum, limestone, etc., which are excavated for commercial purposes. In this district there is also a nuclear power plant and the Chashma Hydel power plant, which are adding electricity into the national electricity grid. The temperature ranged between –2 °C and 51 °C with 255 mm of rainfall [28]. The experimental sites were selected in Area 1 (Kalabagh; high pollution (HP) site), Area 2 (Chashma; low pollution (LP) site), and non-polluted (NP) site (Area 3; Attock) along the River, and these sites were 35 km apart from each other.

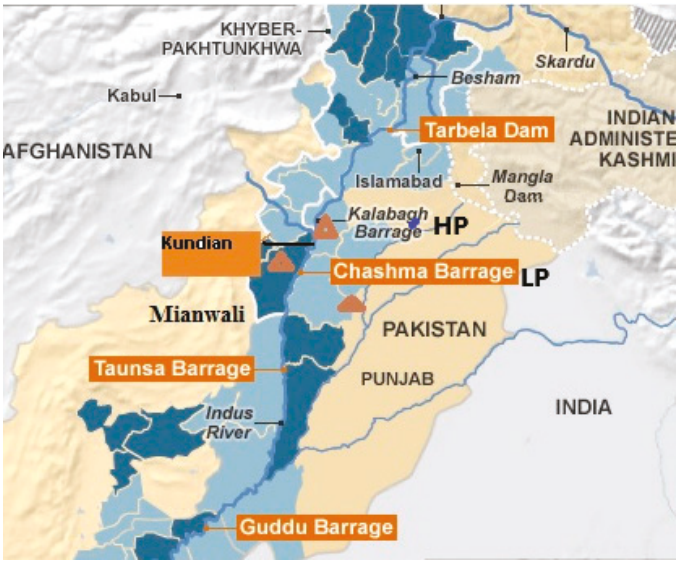

Figure 1. Map of the locations of sampling sites on the Indus River (Kundian Barrage, Kalabagh Barrage, and Chashma Barrage) [25]. Source: OCHA (United Nation Office for the Coordination Humanitarian Affairs).

2.2. Collection and Preparation of Fish Samples

Hypophthalmichthys molitrix is planktivorous and consumes the organisms within lower multiple lower trophic levels across a range of habitats. Grass carp (*Ctenopharyngodon idella*) is a large cyprinid and is a voracious feeder. Small grass carp consume planktonic crustaceans, rotifers, and insect larvae, while the adults are completely vegetarian. *Cyprinus carpio* is a popular benthivorous fish that has larger bottom–up effects than other benthivorous fish. The bottom–up effects of *C. carpio* mainly depend on the incorporation of benthos-derived nutrients and the release of nutrients from bottom sediment during grazing on benthos. Twenty-one specimens of *H. molitrix*, *C. idella*, and *C. carpio* each were captured from HP, LP and NP sites for an evaluation of the fatty acid profiles. A total of 63 fish specimens were procured with the help of fishermen. The average weight ranged from 900 to 1200 g. Fish specimens were transferred live in polyethylene bags to the laboratory. Muscle samples were processed as per the method mentioned by reference [29]. This study was approved by the Ethics and Animal Welfare Committee of the Department of Zoology, GC University, Faisalabad (Ethical code number: GCUF/Zool/EAWC/34).

2.3. Analysis of Water Samples

Water samples were collected in hydrographic bottles of 32 oz capacity at the depth of 30 cm below the surface from the three determined sampling sites for the determination of selected physiochemical parameters and selected heavy metals through an atomic absorption spectrophotometer ("Hitachi polarized Zeeman AAS, 2000 series") by following the procedure as mentioned by reference [30]. The water samples were collected in the morning and these were stored in iceboxes before being taken to the laboratory for analysis. Different dilutions of Hg, Sn, Cr, Pb, Zn, Mn, Cu, and Cd were made to check the accurateness of the equipment during the analysis of samples. The quality control and quality assurance protocol was followed as mentioned in our previous published work [9]. Calibration curves were plotted and validated with their corresponding R^2 values for the detection of each metal. The values of R^2 of the curves were 0.99983, 0.99981, 0.99951, 0.99984, 0.99926, 0.99987, and 0.99982 for Hg, Sn, Cr, Pb, Zn, Mn, Cu, and Cd, respectively.

2.4. Periphyton Sampling

Periphyton samples were obtained from the three experimental locations by following the methodology of references [28,29]. "A 10 × 10 cm steel frame was fixed at the bottom at three points of each location and composite them to collect the periphyton, then the pebble was removed. The periphyton samples was cleaned from the pebble surface using brushes, after which it was washed with river water. Aliquots from this volume were centrifuged at 2500 g for 15 min for the further analysis of metals and fatty acids" [31].

2.5. Zoobenthos Sampling

Zoobenthos samples were obtained from the experimental locations at three points and composite them through a Samples Surber-type kick-bottom sampler as mentioned by reference [31].

2.6. Fatty Acid Profiling

The lipid components were obtained from the fish muscle, periphyton, and zoobenthos samples with help of Soxhlet extractor (Electrothermal EME6 England), as described by reference [9]. "The extracted lipids were transformed to fatty acid methyl esters using methanolic sulfuric acid by an esterification procedure", as described by references [32,33]. The fatty acid profiling was carried out by following the methods of reference [34], through gas chromatograph (Perkin Elmer Model 3920) with flame ionization detector (FID) column 2 m in length and 2 mm in diameter. The chromatograms recorded from all samples were used to observe the retention time of each fatty acid (Fatty acid methyl esters (FAMEs)) and these were compared to the chromatogram of a standard (mixture of pure FAMEs) as described by reference [35].

2.7. Statistical Analysis

The data obtained was processed using Minitab software for analysis of variance (ANOVA) to assess the dissimilarity between various parameters of this study between the three sampling sites. Duncan's multiple range test (DMR test) ($p < 0.05$) was used to compare the means. "Shapiro-Wilk's W test and Levene's test" was used for normality and homogeneity of the data when necessary [36]. Correlation coefficients were calculated to determine the relationship between the concentration of heavy metals and the total FAs profile in fish and planktonic life from three sampling sites.

3. Results

3.1. Physico-Chemical Factors and Heavy Metals

The physico-chemical parameters of the water samples from sampling sites (HP, LP and NP) are presented in Table 1. The level of salinity of HP was about 2%, found to be close to the level of salinity

of the open ocean (normally about 3%). pH levels were 12.1 ± 0.36, 8.6 ± 0.12, and 8.1 ± 0.08 in HP, LP, and NP sites, respectively. The pH level was very high at the HP site. The highest biochemical oxygen demand (81.2 ± 1.10 mg/L) and chemical oxygen demand (195.8 ± 1.16 mg/L) were recorded at the HP site. The concentration of total dissolved solids (2445.5 ± 8.41 mg/L) and total suspended solids (329.6 ± 6.41 mg/L) were very high at the HP site. The concentration of phenols and sulfates were highest at the HP site, closely followed by the LP site. The level of phenols at the HP site was 15 times higher than at the NP site (Table 1).

Table 1. Mean Physico-Chemical parameters and metal concentrations (± SE) at different sampling locations of Indus River.

Water Quality Characteristics	HP Site	LP Site	NP Site	Permissible Limits
pH	12.1 ± 0.36 a	8.5 ± 0.12 b	8.1 ± 0.08 b	D: 6.5–8.5, P: **
BOD (mg/L)	81.2 ± 1.10 a	48.8 ± 0.41 b	36.7 ± 0.77 c	†D: 30 mg/L, P: **
COD (mg/L)	195.8 ± 1.16 a	71.2 ± 0.90 b	65.5 ± 0.58 c	†D: 250 mg/L, P: **
TDS (mg/L)	2444.5 ± 8.41 a	1319.8 ±10.62 b	340.3 ± 7.24 c	D: 500 mg/L, P: 2000 mg/L
TSS (mg/L)	329.6 ± 6.41 a	218.6 ± 5.15 b	190.6 ± 4.24 c	D: 100mg/L, P: **
Salinity (mg/L)	1951.2 ±18.31 a	458.5 ± 7.22 b	242.3± 4.90 b	P: <100 mg/L
Conductivity µS/cm)	4.1 ± 0.22 a	1.55 ± 0.11 b	0.42 ± 0.051 c	D:650 µS/cm, P: 1055 µS/cm
Phenols (mg/L)	2.49 ± 0.18 a	0.84 ± 0.04 b	0.21 ± 0.01 c	D: 0.001 mg/L, P: 0.002 mg/L
Sulfates (mg/L)	452.3 ± 7.62 a	341.21± 0.08 b	97.4 ± 3.90 c	D: 0.001 mg/L, P: 0.002 mg/L
Heavy Metal Contamination				
Sn (mg/L)	0.54 ± 0.02 a	0.03 ± 0.0 b	0.01 ± 0.0 b	D: 0.01 mg/L, P: **
Cr (mg/L)	0.72 ± 0.03 a	0.36 ± 0.02 b	0.05 ± 0.00 c	D: 0.05 mg/L, P: **
Pb (mg/L)	3.02 ± 0.07 a	0.21 ± 0.02 b	0.14 ± 0.01 c	D: 0.05 mg/L, P: **
Zn (mg/L)	0.56 ± 0.02 a	0.251 ± 0.03 b	0.05 ±0.00 a	D: 5 mg/L, P: 15 mg/L
Mn (mg/L)	2.81 ± 0.12 a	2.05 ± 0.06 a	0.41 ± 0.01 c	D: 0.1 mg/L, P: 0.3 mg/L
Cu (mg/L)	2.05 ± 0.05 a	0.99 ± 0.11 b	0.08 ± 0.00 c	D: 0.05 mg/L, P: 1.5 mg/L
Cd (mg/L)	0.29 ± 0.02 a	0.03 ± 0.00 b	0.00 ± 0.00 b	D: 0.01 mg/L, P: **
Hg (mg/L0	1.51 ± 0.04 b	0.05 ± 0.01 c	< 0.001	D: 0.001 mg/L, P: **

BOD: Biological oxygen demand, COD: Chemical oxygen demand. TDS: Total dissolved solids, TSS: Total suspended solids. Different letters (a, b, c) in the same row represent significant ($p < 0.05$) differences. D; Desirable limits. P; Permissible limits. †; Effluent inland surface water quality standards. ** No relaxation.

The concentration of studied heavy metals are presented in Table 1. These concentrations exhibited significant variations between the three sites. The level of Sn, Cr, Pb, Mn, Cu, Cd, and Hg at the HP in fish muscle, periphyton, and zoobenthos biomass were highest at HP compared to LP and NP site and was above the upper limits stated by reference [34] (Tables 1 and 2). The highest level of Cu in muscle samples was detected in *C. carpio* and plankton from HP, followed by LP sites. The maximum level of metals was recorded in the muscle samples of *C. carpio* captured from HP, followed by LP and NP sites. The lowest concentration of these metals was recorded in the muscles of *C. idella* (Table 2).

3.2. Fatty Acids Profile

The saturated fatty acids (SFAs) were low in the biomass of periphyton and zoobenthos obtained from HP and LP sites, compared to NP sites (Table 3). Lauric acid and arachidic acids were not detected in the biomass of periphyton and zoobenthos from HP and LP. The Environmental Protection Agency (EPA) value was significantly higher in the biomass sampled from NP, compared to HP and LP. The number of monounsaturated fatty acids (MUFAs) was higher in samples of periphyton and zoobenthos from NP (Table 3). Palmitoleic acid, vaccenic acid, oleic acid, eicosenic acid and erucic acid were not detected in periphyton biomass samples from HP and LP. PUFAs level was greater in periphyton and zoobenthos biomass from NP, compared to HP and LP sites. Alpha-linolenic acid (ALA), eicosadienoic acid, docosapentaenoic acid and docosapentaenoic acid were not detected in the

biomass of periphyton and zoobenthos sampled from HP and LP. The percentage of EPA and DHA were higher in the periphyton biomass from HP, compared to NP (Table 3).

Table 2. Heavy metal concentrations (mg/kg) in the biomass of periphyton, zoobenthos and in the muscle of fish species from different sampling locations of the Indus River.

Parameter	HP Site	LP Site	NP Site
Periphyton			
Sn	17.61 ± 0.90 a	11.34 ± 0.77 b	4.88 ± 0.61 c
Cr	6.10 ± 0.70 a	1.98 ± 0.55 b	0.15 ± 0.23 c
P b	1.68 ± 0.67 a	0.41 ± 0.10 b	0.15 ± 0.03 c
Zn	14.46 ± 1.35 b	23.11 ± 1.66 a	8.19 ± 0.1.05 c
Mn	20.66 ± 1.41 a	11.44 ± 1.33 a	5.90 ± 0.88 c
Cu	18.43 ± 1.44 a	6.97 ± 0.92 b	4.02 ± 0.55 c
Cd	2.41 ± 0.31 a	0.62 ± 0.12 b	0.16 ± 0.02 c
Hg	3.78 ± 0.40 b	1.70 ± 0.18 c	0.17 ± 0.00 c
Zoobenthos			
Sn	3.43 ± 0.31 a	2.90 ± 0.70 b	1.11 ± 0.40 c
Cr	2.80 ± 0.42 a	1.22 ± 0.18 b	0.26 ± 0.03 c
P b	1.21 ± 0.05 a	0.38 ± 0.01 b	0.15 ± 0.02 c
Zn	6.94 ± 0.77 b	8.06 ± 1.0 a	3.80 ±0.22 c
Mn	3.91 ± 0.48 a	1.30 ± 0.26 b	0.92 ± 0.01 c
Cu	6.02 ± 0.72 a	1.40 ± 0.21 b	0.33 ± 0.03 c
Cd	1.99 ± 0.18 a	0.89 ± 0.02 b	0.36 ± 0.07 c
Hg	1.92 ± 0.31 b	0.86 ± 0.18 c	0.09 ± 0.00 c
Hypophthalmichthys molitrix			
Sn	1.95 ± 0.41 a	1.32 ± 0.36 b	0.67± 0.16 c
Cr	3.01 ± 0.60 a	1.71 ± 0.22 b	0.82 ± 0.15 c
P b	0.81 ± 0.16 a	0.49 ± 0.08 b	0.21 ± 0.01 c
Zn	5.67 ± 0.67 a	3.62 ± 0.41 b	1.62 ± 0.33 c
Mn	2.70 ± 0.41 a	1.92 ± 0.38 b	0.79 ± 0.08 c
Cu	4.93 ± 0.62 a	1.08 ± 0.15 b	0.49 ± 0.05 c
Cd	1.41 ± 0.28 a	0.76 ± 0.12 b	0.35 ± 0.05 c
Hg	1.37 ± 0.22 a	0.87 ± 0.09 b	0.06 ± 0.00 c
Ctenopharyngodon idella			
Sn	1.81 ± 0.3 a	1.12 ± 0.18 b	0.61 ± 0.06 c
Cr	2.28 ± 0.27 a	1.57 ± 0.20 b	0.71 ± 0.15 c
P b	0.74 ± 0.10 a	0.49 ± 0.06 b	0.23 ± 0.01 c
Zn	5.62 ± 0.72 a	3.01 ± 0.47 b	1.52 ± 0.31 c
Mn	2.50 ± 0.38 a	1.62 ± 0.21 b	0.69 ± 0.17 c
Cu	4.70 ± 0.52 a	1.02 ± 0.20 b	0.42 ± 0.05 c
Cd	1.34 ± 0.31 a	0.70 ± 0.21 b	0.31 ± 0.06 c
Hg	1.41 ± 0.22 a	0.87 ± 0.16 b	0.09 ± 0.00 c
Cyprinus carpio			
Sn	2.41 ± 0.4 a	1.61 ± 0.17 b	0.72 ± 0.06 c
Cr	3.44 ± 0.40 a	1.90 ± 0.23 b	0.95 ± 0.16 c
P b	0.98 ± 0.12 a	0.69± 0.07 b	0.40 ± 0.03 c
Zn	6.75 ± 0.88 a	3.96 ± 0.60 b	1.88 ± 0.44 c
Mn	2.90 ± 0.40 a	1.80 ± 0.24 b	0.77 ± 0.18 c
Cu	5.48 ± 0.80 a	1.57 ± 0.28 b	0.58 ± 0.02 c
Cd	1.62 ± 0.40 a	0.88 ± 0.16 b	0.45 ± 0.05 c
Hg	1.69 ± 0.21 a	0.92 ± 0.18 b	0.22 ± 0.01 c

Values (Mean ± SE) are averages of five samples analyzed in triplicate. Different letters (a, b, c) in the same row represent significant ($p < 0.05$) differences.

Table 3. Fatty acids (% ±SE) in periphyton and zoobenthos from three sampling sites at different pollution levels in the Indus River.

Pytoperiphyton			
Fatty Acids	**HP Site**	**LP Site**	**NP Site**
SFAs			
C8:0	———	———	———
C10:0	3.33 ± 0.11 c	6.78 ± 0.88 b	8.12 ± 0.98 a
C12:0	———	———	———
C14:0	1.67 ± 0.08 c	4.41 ± 0.16 b	5.69 ± 0.77 a
C16:0	16.69 ± 2.77 c	19.63 ± 2.55 b	21.89 ± 2.44 a
C18:0	12.47 ± 1.63 c	13.12 ± 1.20 b	15.76 ± 2.89 a
C20:0	———	———	0.67 ± 0.11
MUFAs			
C16:1(n-7)	———	———	———
C16:1(n-9)	———	———	———
C18:1(n-7)	———	———	1.99± 0.16 a
C18:1(n-9)	———	———	0.97 ± 0.11
C20:1(n-9)	———	———	———
C22:1(n-9)	3.11± 0.13 c	5.12 ± 0.34 b	7.89 ± 0.25 a
PUFAs			
C18:2(n-6)	0.43 ± 0.04 c	0.66 ± 0.19 b	1.15 ± 0.22 a
C18:3(n-3)	8.31 ± 0.70 b	9.11 ± 0.90 a	9.44 ± 0.88 a
C18:4(n-3)	———	———	———
C20:2(n-6)	———	———	2.24 ± 0.05 a
C20:4(n-6)	6.11 ± 0.55 c	8.02 ± 0.71 b	9.98 ± 0.41 a
C20:5(n-6)	———	4.11 ± 0.22 b	5.23 ± 0.28 a
C20:5(n-3)	6.78 ± 0.90 a	5.97 ± 0.60 a	4.66 ± 0.70 c
C22:4(n-6)	———	———	———
C22:5(n-6)	———	———	2.79 ± 0.24 a
C22:5(n-3)	6.57 ± 0.41 c	7.33 ± 0.70 b	8.95± 0.66 a
C22:6(n-3)	5.44 ± 0.25 a	4.76 ± 0.41 a	3.77 ± 0.33 b
Zoobenthos			
Fatty acids	**Highly polluted water**	**Less polluted water**	**Non-polluted site**
SFAs			
C8:0	———	———	———
C10:0	1.44 ± 0.23 c	4.11 ± 0.71 b	6.32 ± 0.71 a
C12:0	———	———	———
C14:0	0.99 ± 0.08 c	2.98 ± 0.27 b	4.22 ± 0.55 a
C16:0	13.22 ± 1.66 c	15.38 ± 2.80 b	18.45 ± 2.88 a
C18:0	7.23 ± 1.66 b	10.45 ± 1.76 b	14.77 ± 2.18 a
C20:0	———	———	0.1 ± 0.11
MUFAs			
C16:1(n-7)	———	———	———
C16:1(n-9)	———	———	———
C18:1(n-7)	———	———	1.66 ± 0.07 a
C18:1(n-9)	———	———	0.77 ± 0.08
C20:1(n-9)	———	———	———
C22:1(n-9)	1.72 ± 0.14 c	3.77 ± 0.53 b	4.99 ± 0.66 a
PUFAs			
C18:2(n-6)	0.20 ± 0.02 c	0.41 ± 0.28 b	0.81 ± 0.30 a
C18:3(n-3)	4.10 ± 0.7 c	6.22 ± 0.41 b	7.69 ± 0.44 a
C18:4(n-3)	———	———	———
C20:2(n-6)	———	———	2.89 ± 0.14
C20:4(n-6)	3.00 ± 0.49 c	4.77 ± 0.79 b	6.67 ± 0.51 a

Table 3. *Cont.*

Pytoperiphyton			
Fatty Acids	**HP Site**	**LP Site**	**NP Site**
C20:5(n-6)	———	1.90 ± 0.27 b	3.69 ± 0.47 a
C20:5(n-3)	4.89 ± 0.71 a	3.81 ± 0.20 a	2.44 ± 0.22 b
C22:4(n-6)	———	———	———
C22:5(n-6)	0.60 ± 0.09 b	1.00 ± 0.11 b	1.89 ± 0.47 a
C22:5(n-3)	4.12 ± 0.70 c	6.22 ± 0.60 b	7.52 ± 0.70 a
C22:6(n-3)	4.78 ± 0.31 a	3.83 ± 0.54 a	2.77 ± 0.40 c

SFAs: Saturated fatty acids; MUFAs; Monounsaturated fatty acids; PUFAs: Polyunsaturated fatty acids; Values (Mean ± SE) are averages of five samples for each fish species analyzed in triplicate. Different letters (a, b, c) in the same row represent significant ($p < 0.05$) differences.

The fish captured from HP exhibited lower FAs and SFAs compared to the fish captured from LP (Table 4). The percentage of PUFAs in *H. molitrix*, *C. idella*, and *C. carpio* captured from HP was 32.32 ± 0.65, 7.19 ± 0.35, and 26.13 ± 0.82%, respectively. The percentage of PUFAs in *H. molitrix*, *C. idella*, and *C. carpio* captured from NP was 48.65 ± 1.11, 41.55 ± 0.97, and 44.15 ± 1.90%, respectively. The percentage of MUFAs and SFAs in *H. molitrix*, *C. idella*, and *C. carpio* captured from HP were 6.74 ± 0.29, 5.14 ± 0.17, and 5.46 ± 0.54 and 43.38 ± 2.45, 62.94 ± 3.05, and 74.07 ± 4.14%, respectively. The total MUFAs and SFA profiles in *H. molitrix*, *C. idella*, and *C. carpio*, captured from LP showed a similar trend of fluctuations to fish from HP (Table 4).

Table 4. Fatty acid profile % (±SE) of fish muscle from three sites at different pollution levels.

Less Polluted Site (LP)			
Fatty Acids	***H. molitrix***	***C. idella***	***C. carpio***
SFAs			
C8:0	———	———	———
C10:0	1.02 ± 0.07 a	0.41 ± 0.01a b	1.44 ± 0.03 a
C12:0	0.01 ± 0.00 a b	0.01± 0.03 a	0.74 ± 0.06 a
C14:0	1.54 ± 0.04 a	1.37 ± 0.03 a	0.53 ± 0.02 b
C16:0	41.22 ± 2.70 a	37.66 ± 2.61 a	43.98 ± 2.14 a b
C18:0	0.47 ± 0.05 b	24.66 ± 3.40 a b	29.51 ± 2.67 b
C20:0	———	———	1.45 ± 0.03
MUFAs			
C16:1(n-7)	0.44 ± 0.04 a	———	———
C16:1(n-9)	0.71 ± 0.10 a b	0.79 ± 0.02 b	0.29 ± 0.01 a b
C18:1(n-7)	0.14 ± 0.08 b	0.08 ± 0.01a b	0.40 ± 0.04 b
C18:1(n-9)	3.95 ± 0.33 b	4.79 ± 0.04 a	0.11 ± 0.02 a b
C20:1(n-9)	0.77 ± 0.03	———	———
C22:1(n-9)	0.90 ± 0.06 b	0.41 ± 0.03 a	0.32 ± 0.04 a b
PUFAs			
C18:2(n-6)	0.70 ± 0.06 a b	1.29 ± 0.3 a	0.41 ± 0.07 a b
C18:3(n-3)	12.66 ± 0.79 a	3.82 ± 0.04 a	0.003 ± 0.00 a b
C18:4(n-3)	3.24 ± 0.19 a	0.44 ± 0.05 a	2.60 ± 0.02 a b
C20:2(n-6)	———	———	———
C20:4(n-6)	0.87 ± 0.09 a	0.39 ± 0.02 a	0.82 ± 0.01 a
C20:5(n-6)	12.06 ± 0.54 a	8.02 ± 0.24 a	9.16 ± 0.42 a
C20:5(n-3)	2.42± 0.30 b	0.37 ± 0.18 c	3.61 ± 0.22 a
C22:4(n-6)	———	———	———
C22:5(n-6)	16.77 ± 0.66 a	4.70 ± 0.12 b	5.44 ± 0.22 b
C22:5(n-3)	1.17± 0.10 b	0.02 ± 0.00 c	1.88 ± 0.06 b
C22:6(n-3)	4.02 ± 0.33 a b	2.94 ± 0.22 a	3.71 ± 0.14 a b

Table 4. *Cont.*

Highly polluted site (HP)			
Fatty acids	***H. moiltrix***	***C. idella***	***C. carpio***
SFAs			
C8:0	———	———	———
C10:0	0.18 ± 0.03 c	1.97 ± 0.11 b	6.93 ± 0.77 a
C12:0	———	———	———
C14:0	0.44 ± 0.05 c	1.83 ± 0.21 b	2.65 ± 0.22 a
C16:0	34.25 ± 4.66 c	44.25 ±5.26 b	50.12 ± 4.77 a
C18:0	27.18 ± 3.16 b	35.69 ± 3.75 a	12.24 ± 1.77 c
C20:0	———	0.25 ± 0.01	———
MUFAs			
C16:1(n-7)	———	———	———
C16:1(n-9)	———	———	———
C18:1(n-7)	5.22 ± 0.52	———	———
C18:1(n-9)	0.62 ± 0.06 a	———	0.49 ± 0.01 a
C20:1(n-9)	———	———	———
C22:1(n-9)	0.32 ± 0.02 c	5.61 ± 0.17 a	4.11 ± 0.22 b
PUFAs			
C18:2(n-6)	———	———	———
C18:3(n-3)	16.55 ± 0.54 a	3.66 ± 0.32 b	0.24 ± 0.01 c
C18:4(n-3)	———	———	———
C20:2(n-6)	———	0.87 ± 0.11 a	0.59 ± 0.02 b
C20:4(n-6)	4.72 ± 0.33 b	7.42 ± 0.22 a	3.13 ± 0.07 c
C20:5(n-6)	———	———	0.92 ± 0.11
C20:5(n-3)	0.68± 0.07	———	———
C22:4(n-6)	———	———	———
C22:5(n-6)	0.52 ± 0.11 b	0.17 ± 0.01 c	0.88 ± 0.11 a
C22:5(n-3)	6.12 ± 0.44 a	4.12 ± 0.22 b	———
C22:6(n-3)	3.62 ± 0.11 a	0.21 ± 0.01 c	1.76 ± 0.22 b
Non-polluted site (NP)			
Fatty acids	***H. moiltrix***	***C. idella***	***C. carpio***
SFAs			
C8:0	0.80 ± 0.05 a	0.01 ± 0.01 a	———
C10:0	0.31 ± 0.00 b	3.15 ± 0.03 a	3.67 ± 0.08 a
C12:0	1.21± 0.15 c	3.10 ± 0.40 a	2.16 ± 0.05 b
C14:0	0.45 ± 0.01 c	4.68 ± 0.55 b	7.79± 0.44 a
C16:0	17.10 ± 0.61 a	14.78 ± 1.12 b	12.42 ± 0.55 b
C18:0	6.01 ± 0.41 a	3.80 ± 0.25 c	8.90 ± 0.75 a
C20:0	1.42 ± 0.17 a	0.32 ± 0.01 b	0.38 ± 0.01 b
MUFAs			
C16:1(n-7)	———	———	0.72 ± 0.04
C16:1(n-9)	4.75 ± 0.16 a	2.41 ± 0.04 b	2.77 ± 0.05 b
C18:1(n-7)	4.81 ± 0.45 a	3.60 ± 0.02 b	1.81 ± 0.11 b
C18:1(n-9)	13.12 ± 0.82 a	12.87 ± 0.66 a	10.94 ± 0.42 a
C20:1(n-9)	1.44 ± 0.07 b	4.14 ± 0.51 a	3.88 ± 0.22 a
C22:1(n-9)	0.01 ± 0.00 c	0.61 ± 0.01 a	0.70 ± 0.02 a
PUFAs			
C18:2(n-6)	4.07 ± 0.06 a	3.42 ± 0.11 a	0.01 ± 0.00 b
C18:3(n-3)	3.44 ± 0.23 b	3.22 ± 0.22 b	5.94 ± 0.41 a
C18:4(n-3)	2.41 ± 0.11 a	2.05 ± 0.07 a	0.71 ± 0.02 b
C20:2(n-6)	0.91 ± 0.02 a	0.88 ± 0.01 a	0.57 ± 0.02 b
C20:4(n-6)	14.26 ± 0.87 a	12.14 ± 0.28 b	9.87 ± 0.71 c

Table 4. *Cont.*

Highly polluted site (HP)			
Fatty acids	***H. moiltrix***	***C. idella***	***C. carpio***
C20:5(n-6)	6.44 ± 0.28 a	4.12 ± 0.20 b	0.21 ± 0.01 c
C20:5(n-3)	0.40 ± 0.01 c	5.06 ± 0.31 b	8.23 ± 0.60 a
C22:4(n-6)	1.21 ± 0.02 a	0.60 ± 0.00 b	0.50 ± 0.00 b
C22:5(n-6)	3.44 ± 0.32 b	4.02 ± 0.09 b	6.68 ± 0.25 a
C22:5(n-3)	5.05 ± 0.33 a	3.60 ± 0.22 b	2.39 ± 0.04 c

SFAs: Saturated fatty acids; MUFAs; Monounsaturated fatty acids; PFAs: Polyunsaturated fatty acids; Values (Mean ± SE) are averages of five samples for each fish species analyzed in triplicate. Different letters (a, b, c) in the same row represent significant ($p < 0.05$) differences.

The maximum percentage of SFAs in *C. carpio* was observed in HP. A decrease in the abundance of *C. carpio* was noticed during the study period (Table 4). Caprylic acid (C8:0), lauric acid (C12:0) and C20:0 arachidic acid were not found in *H. molitrix, C. idella*, and *C. carpio* from HP. A very small amount of lauric acid (C12:0) and C20:0 arachidic acid was recorded in *H. molitrix, C. idella*, and *C. carpio* from LP. Eicosapentaenoic acid was not detected in any of the fish species collected from HP sites. In this study, 6 and 9 omega series FAs were found in muscle samples of *H. molitrix, C. idella*, and *C. carpio* from HP and LP, respectively. Linoleic acid (C18:4(n-3), eicosadienoic acid (C20:2 (n-6), and docosapentaenoic acid (C22:4 (n-6) were not recorded in fish from HP. Eicosapentaenoic acid (C20:5 (n-3) was detected only in the muscle samples of *H. molitrix* from HP. Total 11 omega series FA were recorded in muscle of *H. molitrix, C. idella*, and *C. carpio* from NP sites. Caprylic acid was not detected in *H. molitrix, C. idella*, and *C. carpio* from LP sites (Table 4). Myristic acid (C14:0) was recorded as 0.44 ± 0.05, 1.826 ± 0.21, and 2.651 ± 0.22% in *H. molitrix, C. idella*, and *C. carpio*, respectively, from HP. Myristic acid was determined as 1.54 ± 0.04, 1.37 ± 0.03, and 0.53 ± 0.02 and 0.14 ± 0.01, 4.68 ± 0.55, and 7.79 ± 0.44% in *H. molitrix, C. idella*, and *C. carpio* from LP and NP sites, respectively. Arachidic acid was not found in *H. molitrix* and *C. carpio* from HP sites. Oleic acid (C18:1 (n-9) was not detected in *C. idella* collected from HP sites. C16:1 (n-7) (palmitoleic acid), C16:1 (n-9) (Cis-7 hexadecenoic acid), and C20:1 (N-9) (Eicosenoic acid) were not found in these fish species captured from HP. C18:1 (n-7) (cis-vaccenic acid) was detected only in the muscle sample of *H. molitrix* from HP. C16:1 (n-7) was detected only in the muscle samples of *H. molitrix* from LP sites. The concentration of C16:1 (n-7) was only determined as 0.72 ± 0.04 in *C. carpio* from NP sites (Table 4).

Correlation indices that were calculated among the concentrations of total FAs in periphyton, zoobenthos, fish muscle, and heavy metals in water samples are presented in Table 5. It has been observed that Sn, Cr, Pb, Zn, Mn, Cu, Cd, and Hg indicated significantly negative correlations with total FA profile in periphyton, zoobenthos, and fish samples from HP and LP (Table 5). Highly significant negative correlations were observed among Cr, Zn, Mn, and Cu and the total fatty acid profile samples of periphyton, zoobenthos, and fish collected from the HP site. The variation in FA found positively correlated with the level of contamination of these heavy metals in the food web. The health of the aquatic system was found to be significantly affected by the water quality of the HP and LP sites of the river compared to the NP site, which possibly causes decreases in the abundance of periphyton and fish populations in the aquatic system.

Table 5. Correlation matrix for metal concentrations with total fatty acids in periphyton, zoobenthos, and fish in three sites at different pollution levels.

Metals	HP-PP	LP-PP	NP-PP	HP-ZB	LP-ZB	NP-ZB	HP-HM	LP-HM	NP-HM	HP-GC	LP-GC	NP-GC	HP-CP	LP-CP	NP-CP
Sn	−0.24 *	−0.13	0.07	−0.37 *	−0.28 *	0.05	−0.46 **	−0.24 *	0.06	−0.30 *	−0.31 *	0.08	−0.60 **	−0.27 *	0.05
Cr	−0.56 **	−0.33 *	0.10	−0.58 **	−0.33 *	0.02	−0.33 *	−0.24 *	0.04	−0.58 **	−0.31 *	−0.07	−0.54 **	−0.28 *	−0.11
P b	−0.37 *	−0.26 *	−0.06	−0.58 **	−0.28 *	−0.05	−0.44 **	−0.25 *	0.03	−0.40 **	−0.22 *	0.01	−0.560 **	−0.27 *	−0.01
Zn	−0.51 **	−0.23 *	0.03	−0.53 **	−0.25 *	−0.07	−0.34 *	−0.23 *	0.01	−0.28 *	−0.27 *	−0.03	−0.57 **	−0.24 *	0.09
Mn	−0.60 **	−0.28 *	0.02	0.56 **	−0.27 *	−0.09	−0.48 **	−0.24 *	−0.02	−0.64 **	−0.26 *	0.04	−0.57 **	0.28 *	−0.05
Cu	−0.60 **	−0.27 *	0.05	−0.57 **	−0.44 **	−0.08	−0.53 **	−0.25 *	0.03	−0.37 *	−0.28 *	−0.06	−0.57 **	−0.29 *	0.04
Cd	−0.27 *	−0.24 *	0.10	−0.33 *	−0.25 *	−0.07	−0.41 *	−0.27 *	−0.12	−0.42 **	−0.28 *	0.02	−0.51 **	−0.25 *	−0.05
Hg	−0.34 *	−0.22 *	0.01	−0.27 *	0.12	0.01	−0.28 *	−0.22 *	0.004	−0.35 *	−0.27 *	−0.01	−0.48 **	−0.26 *	0.003

*significant at 0.05 level; ** significant at 0.01 level; HP: highly polluted site; LP: low polluted site; NP: Non-polluted site; PP: periphyton; ZB: zoobenthos; HM: *H. moiltrix*; CI: *C. idella*; CP: *C. carpio*.

4. Discussion

The trophic transfer of FAs from periphyton to the organisms at higher trophic levels is important for their health and growth [14,37]. This movement of important nutrients in the food chain may be affected by different contaminants in the freshwater ecosystem [15]. The metals and metalloids, phenols, and organic contaminants in freshwater ecosystems enter the food of aquatic animals from various sources, including anthropogenic activities, and accumulate in planktonic life and fish. The heavy metals which accumulate can cause physiological stress on FA at different trophic levels in the food chain, and ultimately in humans [16,37].

Fish are used as a bioindicator for different organic and inorganic pollutants in freshwater ecosystems due to their presence in different trophic levels, because of their age, size, and mode of nutrition [15]. Various factors have effects on the distribution of aquatic fauna and flora in freshwater reservoirs [38,39]. Abiotic parameters are considered to mostly affect the pattern of distribution and richness of planktonic life [39,40]. The metals assessed in this study accumulated in fish directly from the water and planktonic life in the Indus River in the study area. In this study, higher concentrations of salinity, sulfates, phenol and heavy metals were the driving force which decreased the abundance of phytoplankton and zooplankton, and their FA profile. The phenol, sulfate, total dissolved solids (TDS), and TS values clearly indicated difference in their concentration at HP and LP sites. The level of salinity at HP was very close to that of brackish waters. The higher concentration of total TDS and TS at HP may be due to high turbidity. The presence of different metals in freshwater ecosystems varied with the physico-chemical factors of the corresponding ecosystem, particularly the pH and redox state. Reference [41] reported that the decrease in pH at high river discharges may release metals from complexes in the river and streams, which may be toxic to the aquatic fauna and flora in the ecosystem.

The levels of heavy metals in the water samples collected from HP passed the upper limits recommended by reference [37]. The heavy metals level in the water samples and in the muscles of *H. molitrix*, *C. idella*, and *C. carpio*, and planktonic biomass collected from HP. The bioaccumulation of heavy metals is known to influence the FA profile of fish. Reference [42,43] mentioned that metals stimulate cellular synthesis and metabolism of FA through β-oxidation, while pharmaceutical products act as peroxisomal proliferators [43–45]. Very limited information is available about the influence of heavy metals on Proliferation Activate Receptors (PPARs) expression and the transcription factors of FA metabolism in fish [46]. Elements in these fish species captured from HP and LP sites were accumulated by bio-concentration, and through food and water [16,37]. The increased concentration of heavy metals along with salinity and phenols at HP and LP sites compared to N P site probably are major factors which caused physiological variation in the food web and disturb the biosynthesis of FAs in *H. molitrix*, *C. idella*, and *C. carpio* [16,38]. The concentration of many heavy metals decreased in higher trophic levels in the food web [45]. In this study, similar results were obtained for most of the heavy metals, except for Hg [47,48]. This was particularly so in the higher trophic levels, and ultimately affected terrestrial ecosystems through fish [16,38].

PUFAs enter at the first trophic level of the food chain via FA synthesis in periphyton. Reference [49] has mentioned that light causes multiple effects on periphyton lipid metabolism and FA profiles. In general, higher light intensity normally causes oxidative damage to PUFA. In addition to the contamination, low light intensity and poor water quality at HP and LP sites influenced the abundance of periphyton producing high quality FA, thereby affecting PUFAs. The movement of FAs from periphyton to the fish level was found to be increasing with the pH at HP and LP sites. Thus, alkaline pH stress promoted an accumulation of TAG (Triacylglycerols) and a proportionally decrease in membrane lipids [50] In this research work, the changes in physio-chemical factors influenced the production of lipids in the planktonic life at HP and LP sites. The current findings seem to agree with the results of reference [51]. They had mentioned that phytoplanktonic abundance and their diversity were affected by eutrophication, which influence the FAs production due to interspecific variations in periphyton FA levels. However, there remains very little information on the molecular mechanisms involved in these abiotic environmental stressors.

The concentration of EPA, DHA and PUFAs was greater in the microalgae at LP compared to HP, which may due to the higher biomass of microalgae. The increase in microalgae growth is promoted by the higher concentration of nutrients, which might have promoted the synthesis of EPA [31,52]. Reference [53] reported that fluctuations in nutrient availability in the food chain affect on FAs profiles of periphyton. The fluctuations in FA profiles in the trophic levels of the aquatic food chain are probably due variations in the periphyton community composition. Our results of increases in the percentage of EPA were not in line with the results of reference [54]. Total PUFA and PUFA:SAFA ratios were reduced in periphyton and zoobenthos with increase in pollution at the HP site compared to the NP site. The level of PUFA in zoobenthos relies on various biotic and abiotic factors [50] such as food types and levels of contamination [31]. Reference [55] reported that increases in the concentration of Cd decreased the production FA profiles in *Chlorella vulgaris*. However, more Cd accumulated under N stress, which reduced the production of triglycerols in algae. DHA is necessary for the good growth of these freshwater fish species in aquatic ecosystems. The low level of DHA in planktonic food may affect the growth and development of different organs in freshwater fish species [31,56]. Here the reduction in the level of DHA was detrimental to the fatty acid profiles of *H. molitrix, C. idella*, and *C. carpio* from HP sites. The accumulation of PUFA in zoobenthos depends on various biotic and abiotic factors [52,57], food types [58] and pollution levels [37].

Fish are considered to be the best source of animal protein, globally. However, deterioration in their quality and losses in FAs cannot be recouped. Differences were non-significant for the FA profile in *C. carpio* sampled from HP and LP sites compared to NP sites, which exhibit an identical response to the chemical pollutants. The maximum percentage of SFAs in *C. carpio* was recorded in the fish procured from HP. Fish with a high concentration of SFAs need more energy for their movement and to search for food [59]. The SFAs C8:0, C12:0 and C20:0 were not recorded for *H. molitrix, C. idella*, and *C. carpio* captured from HP sites. The higher levels of heavy metals at HP and LP sites in the river adversely affected the synthesis of FAs in the three fish species. The higher SFA levels are probably due to de novo synthesis within these fish species. The heavy metals accumulate towards the bottom of the river, and *C. carpio* feeds on a variety of benthic organisms and macrophytes, thereby exposing it to high proportions of heavy metals [60]. The total MUFA concentrations recorded were supported by the findings of reference [61].

A significant lower percentage of PUFAs was noticed in *H. molitrix*, *C. idella*, and *C. carpio* from HP and LP. However, H. molitrix exhibited higher levels of ω-3 FAs and a large loss of ω-6 fatty acids. Eicosapentaenoic acid (C20:5n3) was not recorded in *C. idella* and *C. carpio* from HP. The concentrations of EP and C20: 5n3 were lower than those of menhaden oil. Identical results were reported by reference [62]. Linoleic acid, eicosadienoic acid and docosapentaenoic acid were not detected in fish procured from HP. Eicosapentaenoic acid was detected only in the muscle samples of H. molitrix from HP. The reduction in the production of PUFAs in *H. molitrix*, *C. idella*, and *C. carpio* from HP and LP may be due to increased levels of metals in the water at these locations in the river [63]. The zooplankton is a source of EPA and DHA for fish in the aquatic ecosystem [26]. The alterations in the food web, linked with an increase in environmental stress in freshwater ecosystems, invasive species, and habitat deterioration, may cause a significant variation in pollutant and lipid trophic transfer [26,64]. *C. idella* captured from HP and LP sites surprisingly responded to the general environment for FA profiles, although they feed on aquatic vegetation. We did not work on the FA profile of aquatic vegetation, and suspect that the alteration in the FA profile was due to an increased water pollution. The higher metal concentration might have affected the FA profile of the aquatic vegetation. This aspect may be verified in future studies. The alterations in the food web, linked with an increase in environmental stress in freshwater ecosystems, invasive species, and habitat deterioration, may cause a significant variation in pollutant and lipid trophic transfer [16]. The variations in FA and heavy metals trophic transfers in the food chain can provide insights into ecological functioning and the fallout of environmental stressors on the FA profile of different organisms in freshwater food webs.

5. Conclusions

Lipids play a significant role in the bioaccumulation of lipophilic pollutants in freshwater fish. The increase of heavy metals in the waters of the Indus River has produced trophic transfers to periphyton, zoobenthos, and fish in highly polluted (HP) and less polluted (LP) sites. Polyunsaturated Fatty Acids (PUFAs) level was greater in periphyton and zoobenthos biomass from non-polluted (NP) sites, compared to HP and LP sites. Fatty acids in the fish muscles were affected by the level of contamination due to the alterations in the food web, linked with an increase in environmental stress, invasive species, and habitat deterioration. It has been inferred that abiotic factors and chemical pollutants induced the trophic transfer in the food, and ultimately the loss of essential fatty acids (FAs) in fish meat. The variations in FA and heavy metals trophic transfers in the food chain can provide insights into ecological functioning and the fallout of environmental stressors on the FA profile of different organisms in freshwater food webs.

It is proposed that FAs may be used to evaluate trophic relationships among water, planktonic life forms, and fish in the food web in order to provide information to consumers about the safety of fish meat. Thus, the variation in FA profiles may be used as a biomarker to assess the status of the health of the ecosystem, and possibly to identify the causes of decreases in the abundance of fish populations.

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Origin of Carbon and Essential Fatty Acids in Higher Trophic Level Fish in Headwater Stream Food Webs

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Abstract: Dietary carbon sources in headwater stream food webs are divided into allochthonous and autochthonous organic matters. We hypothesized that: 1) the dietary allochthonous contribution for fish in headwater stream food webs positively relate with canopy cover; and 2) essential fatty acids originate from autochthonous organic matter regardless of canopy covers, because essential fatty acids, such as 20:5ω3 and 22:6ω3, are normally absent in allochthonous organic matters. We investigated predatory fish *Salvelinus leucomaenis* stomach contents in four headwater stream systems, which are located in subarctic region in northern Japan. In addition, stable carbon and nitrogen isotope ratios, fatty acid profile, and stable carbon isotope ratios of essential fatty acids were analyzed. Bulk stable carbon analysis showed the major contribution of autochthonous sources to assimilated carbon in *S. leucomaenis.* Surface baits in the stomach had intermediate stable carbon isotope ratios between autochthonous and allochthonous organic matter, indicating aquatic carbon was partly assimilated by surface baits. Stable carbon isotope ratios of essential fatty acids showed a positive relationship between autochthonous sources and *S. leucomaenis* across four study sites. This study demonstrated that the main supplier of dietary carbon and essential fatty acids was autochthonous organic matter even in headwater stream ecosystems under high canopy cover.

Keywords: fatty acids; dietary sources; allochthonous; *Salvelinus leucomaenis*

1. Introduction

Aquatic animals are supported by two basal organic carbon sources, autochthonous (aquatic primary producers) and allochthonous sources (fallen leaf litter and insects from surrounding terrestrial ecosystems) [1]. Contributions of these basal organic carbon sources to aquatic food webs depend on the proportion of microalgal and phytoplankton productivity, and the number of terrestrial subsidies [2]. For lower order headwater streams, as high canopy cover promotes abundant inputs of litter falls [3] and limited productivity of attached algae due to shading effects [4], the main carbon sources for aquatic consumers are predicted as allochthonous by the river continuum concept (RCC) [5]. However, food web studies in headwater streams have demonstrated that the dominant dietary contribution for macroinvertebrates is both autochthonous [6,7] and allochthonous [8,9], indicating that the predominant carbon sources for headwater stream food webs are unclear.

Previous studies on the dietary contribution of these basal organic carbon sources have mainly focused on quantitative contribution; however, studies focusing on dietary quality are relatively rare in headwater stream food webs [10,11]. For instance, essential fatty acids are known to be important nutrition for fish health [12–14]. In particular, the roles of 20:5ω3 and 22:6ω3 in fish growth, survival, and reproduction have been studied in many species [15–17]. These studies have demonstrated that dietary essential fatty acids could improve fish condition. Freshwater fish can synthesize 20:5ω3 and 22:6ω3 if 18:3ω3, which is precursor of these two essential fatty acids, is available from dietary sources [13]. However, the conversion efficiency in aquatic animals is generally very low. Consequently, direct intake of these fatty acids from dietary sources is required [18]. However, terrestrial organic matter contains only 18:3ω3 but not 20:5ω3 and 22:6ω3, indicating that terrestrial organic matter is nutritionally poor [18]. 20:5ω3 is present at high levels in diatoms [19], which are sometimes the dominant algae attached to the surface of substrates in headstream ecosystems [20]. This implies that autochthonous organic carbon (i.e., attached algae) may be the main essential fatty acid source for consumers, although other carbon components are derived from allochthonous inputs in headwater streams.

Stable isotope ratios of bulk carbon in consumers reflect those of assimilated diet with only minor fractionation (<1‰), which enable us to infer its dietary carbon sources [21]. In addition, stable carbon isotope ratios of autochthonous organic sources (e.g., attached algae) and allochthonous organic sources are distinguishable in many cases [8,22]. Accordingly, the contribution of allochthonous organic matter to stream food webs has been evaluated by bulk carbon stable isotope ratios [8]. Stable isotope ratios of bulk nitrogen (δ^{15}N) have also been used in food web studies, because of its usefulness to evaluate the trophic position of animals owing to the substantial enrichment of approximately 3‰ relative to that of assimilated diet [23–25].

For tracing the dietary essential fatty acid origin, although their carbon stable isotope ratios would be helpful, information about isotopic fractionation of essential fatty acids between diet and consumers is limited. For instance, Budge et al. [26] demonstrated that there was no isotopic fractionation in several essential fatty acids between the diet and serum of fish in a feeding experiment with Atlantic Pollock. Moreover, an almost isotopically unchanged transfer of 18:2ω6 and 18:3ω3 between diet and zebrafish *Danio rerio* was observed in a 100-day feeding experiment with constant dietary sources [27]. Several aquatic food web studies have already assumed no fractionation of isotopic value of essential fatty acids [28,29]. On the contrary Gradyshev et al. [30] found gradual depletion of stable carbon isotope ratios of essential fatty acids, including 18:2ω6 and 18:3ω3, through higher trophic levels in a stream food chain, suggesting that fractionation was negative. Depleted fractionation in 18:3ω3 was also reported in zooplankton in a feeding experiment [31]. Depleted fractionation in other essential fatty acids have been also reported by a feeding experiments with Daphnia [32]. This can be explained by lighter compounds being assimilated preferentially. As above, the information on fractionation of fatty acids are not sufficient and they were conflicting (i.e., no or small fractionation and negative fractionation). Nielsen et al. [33] pointed out more information on the fractionation of fatty acids are required for diet tracing study. Thus, we did not apply a constant value of isotopic fractionation of essential fatty acids in a food chain in this study. We applied a correlation analysis of stable carbon isotope ratios of essential fatty acids between diet and consumer among the study sites. If organic sources consistently contribute to consumers and have wider isotopic differences, one would expect to detect a significant and positive relationship in stable carbon isotope ratios of essential fatty acids between consumers and assimilated food source regardless of isotopic fractionation [34–36].

Here, we tested the following two hypotheses that: 1) the dietary allochthonous contribution for fish in headwater stream food webs positively relates with canopy cover; and 2) essential fatty acids originate from autochthonous organic matter regardless of canopy covers. To test these hypotheses, we analyzed fatty acid compositions and bulk carbon and compound-specific isotope ratios in fish from four headstream ecosystems.

2. Materials and Methods

2.1. Sampling

We conducted field surveys in four headwater streams, located in subarctic area in the northern part of Japan from July to September 2016 (Table 1). Canopy cover was calculated from a hemispherical photography taken from the center of each stream using CanopOn2 program [37].

Table 1. Description of study sites in this study.

Study Site	GPS	Order	Sampling Date	Canopy (%)	Water Temperature (°C)
Babame	N39.8678°, E140.2552°	1	10 July	93.5	15.9
Hayakuchi	N40.4227°, E140.3470°	3	20 July	67.6	15.6
Kurikoma	N38.9169°, E140.7356°	1	2 September	91.1	14.2
Naruse	N 39.0716°, E 140.7187°	3	18 September	63.5	18.5

Salvelinus leucomaenis is a dominant predatory fish in these four study sites. The main dietary sources of *S. leucomaenis* are larvae and adults of aquatic insects and terrestrial insects [38,39]. *S. leucomaenis* was sampled by fishing. The total length and whole wet weight were measured and a muscle near the pelvic fins was dissected for further analyses. The stomach was preserved in 90% ethanol. For autochthonous organic sources analyses, epilithic biofilm, which was mainly composed of attached algae, was removed using a brush from several randomly selected stones. The collected epilithic biofilm was placed in a plastic sampling bottle with distilled water. The bottle containing algae was filtered through two glass filters (GFF; Whatman, Little Chalfont, UK) in the laboratory for fatty acid and bulk stable carbon and nitrogen isotope analyses. For allochthonous organic sources analyses, decomposed immersed leaf litter was sampled into a plastic bag. Both autochthonous and allochthonous organic sources were sampled in triplicate. The larvae of aquatic insects including Ephemeroptera, Trichoptera, and Plecoptera, which are potentially a direct dietary source for *S. leucomaenis*, were collected using D-frame nets (250 μm mesh) and sorted in the laboratory. Heptageniidae and Ephemerellidae were used for further analysis as they were dominant and commonly detected across all four study sites. All collected samples were transported to the laboratory in a cooler box. All samples were placed in a plastic bag separately and stored in a freezer at −20 °C until further analysis.

Additional sampling for analyses of bulk stable isotope ratios of *S. leucomaenis*, its stomach contents, epilithic biofilms, and leaf litter were conducted in the same location of Babame in July 2018. The same sampling procedure was applied except for stomach contents. The stomach of *S. leucomaenis* was placed in a plastic bottle with distilled water and moved to laboratory in a cooler. The stomach contents were identified and separated. *S. leucomaenis*, epilithic biofilms, and leaf litter were treated following the method mentioned above. All samples were preserved in a plastic bag and stored in a freezer at −20 °C until further analysis.

2.2. Analyses

The stomach contents of each *S. leucomaenis* individual were divided into four groups based on the morphological characteristic using a stereoscopic microscope: water baits (larvae of aquatic insects) and surface baits (adults of aquatic insects and terrestrial insects) according to the definition of Tsuda [38], terrestrial plants, and unknown. Each group was weighted and the contribution of each group was calculated.

Freeze-dried samples of *S. leucomaenis*, both organic sources, and aquatic insects were used for the 'one-step method' [40] for fatty acid analysis. For aquatic insects, two or three individuals were pooled as one sample, and prepared three samples in each study sites. Freeze-dried samples were moved to a 10 mL glass tube. For *S. leucomaenis*, aquatic insects, and leaf litter, approximately 50 mg of homogenized sample was used. For epilithic biofilms, one sheet of GFF was used in the analysis. One milliliter of an internal standard (0.1 mg of tricosanoic acid per 1 mL of hexane), 1 mL of hexane,

and 0.8 mL of 14% boron trifluoride methanol were added to the 10 mL glass tube. Nitrogen gas was then used to fill the head space. The glass tube was placed in a 100 °C dry bath for 2 h, followed by cooling to room temperature, and 0.5 mL of hexane and 1 mL of ultrapure water were added. The glass tube was vigorously shaken manually and centrifuged for 3 min at 2,500 rpm. The upper layer of hexane, containing fatty acid methyl esters (FAMEs), was transferred to a 1.5 mL gas chromatography (GC) vial. Solid residues of *S. leucomaenis* were used for bulk carbon and nitrogen isotope ratio analysis.

One microliter of FAME solution was injected in a gas chromatograph (Trace GC, Thermo Fisher Scientific, Bremen, Germany) equipped with a capillary column (Select FAME, 100 m × 0.25 mm i.d.; Agilent Technologies, Santa Clara, CA, USA). The GC analysis was carried out under the analytical conditions described by Fujibayashi et al. [28]. Each fatty acid peak was identified by comparing their retention times with those of commercial authentic standard mixtures (Supelco, Inc., Bellefonte, PA, USA). The peak area was used for calculating the contribution of each fatty acid to total fatty acids.

After fatty acid analysis by GC, the remaining hexane sample was used to analyze the essential fatty acids stable carbon isotope ratio. FAMEs in the hexane solution were injected into a GC–isotope ratio mass spectroscopy instrument (Trace GC Ultra/Delta-V Advantage; Thermo Fisher Scientific, Bremen, Germany), which was equipped with a capillary column (SP2560, 100 m × 0.25 mm i.d.; Supelco, Inc., Bellefonte, PA, USA). The operating conditions were as described by Fujibayashi et al. [29]. Each essential fatty acid peak was identified as described above for the GC-FID analysis. Stable carbon isotope ratios of fatty acids were determined using the following formula:

$$\delta^{13}\text{C or N (‰)} = (R_{sample}/R_{standard} - 1) \times 1000 \quad (1)$$

where R_{sample} is the $^{13}C/^{12}C$ ratio of the sample, and $R_{standard}$ is the $^{13}C/^{12}C$ ratio of the international isotopic standard (i.e., Vienna Pee Dee Belemnite).

Correction for the effect of additional carbon from boron trifluoride methanol on $\delta^{13}C$ was conducted according to Fujibayashi et al. [29]. The stable carbon isotopes of fatty acids in *S. leucomaenis*, epilithic biofilms, and terrestrial litter samples were analyzed.

Dried solid residues of *S. leucomaenis*, subsamples of freeze-dried terrestrial litter, GFFs (epilithic biofilms), and aquatic insects were used for bulk stable carbon and nitrogen isotope ratio analysis. Utilization of solid residues after a one-step method potentially changes the isotopic value. Therefore, the relationship between the stable isotope ratios of carbon and nitrogen in original samples and those in the corresponding dried solid residue after the one-step method were checked in advance with freshwater fish muscle samples (Supplementary file Figure S1). For nitrogen, while a significant positive relationship was detected, variation was relatively high. Thus, estimation of trophic position of *S. leucomaenis* using solid resides may include some extents of error. However, stable carbon isotope rations of solid residues well reflected that of the original samples, and the utilization of solid resides for stable carbon isotope analysis was applied in this study. For the sampling of aquatic insects, one individual was used for one sample, and we prepared three samples for each study site. All samples were weighed in microcapsules and injected into an elemental analyzer (Flash EA; Thermo Fisher Scientific, Bremen, Germany) linked to a mass spectrometer (Delta-V Advantage; Thermo Fisher Scientific, Bremen, Germany). Stable isotope ratios of bulk carbon and nitrogen were expressed as Equation (1); where R_{sample} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the sample, and $R_{standard}$ is the $^{13}C/^{12}C$ and the $^{15}N/^{14}N$ ratio of the international isotopic standard (Vienna Pee Dee Belemnite, and atmospheric N_2, respectively).

3. Results

Although canopy cover was high in both the first order rivers, Babame and Kurikoma, with 93.5% and 91.1%, respectively; the third-order rivers, Hayakuchi and Naruse, had relatively open canopy with 67.6% and 63.5%, respectively.

Ten and 11 individuals of *S. leucomaenis* were caught in Babame and Kurikoma by fishing; however, just one individual was caught in Hayakuchi and Naruse. There was a relatively high proportion, ranging from 31% to 68%, of unknown components in the stomach contents that could not be identified because of decomposition (Figure 1). Terrestrial plants were almost not detected in the stomach contents, while water and surface baits were dominant in the stomach contents in *S. leucomaenis*. There was no obvious relationship between water and surface bait contribution and canopy cover.

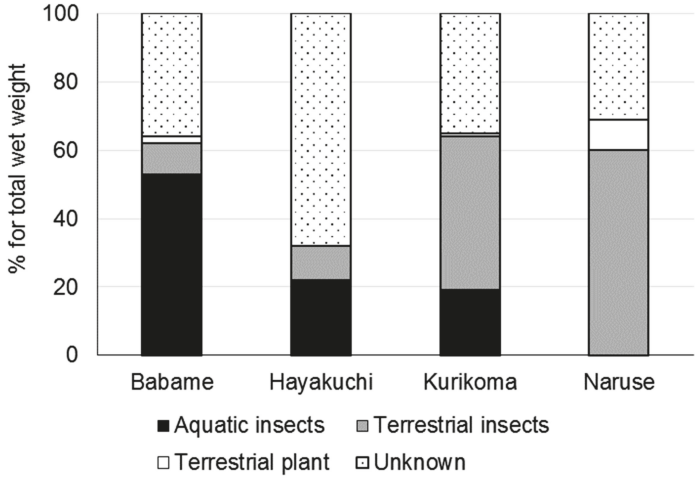

Figure 1. Stomach contents (wet weight %) of *S. leucomaenis* from four study sites.

Although stable carbon and nitrogen isotope ratios of *S. leucomaenis* varied among study sites, the trophic position of *S. leucomaenis* was always the highest (Figure 2). Aquatic insects were generally at a lower position than *S. leucomaenis* with similar carbon isotopic values. Leaf litter showed the most depleted isotopic value for both carbon and nitrogen in all study sites. The range of stable carbon isotope ratios of leaf litter was relatively narrow, from −31.5‰ in Hayakuchi to −29.8‰ in Kurikoma. For epilithic biofilms, stable carbon isotope ratios were more enriched than terrestrial litter and showed a wider range, from −27.2‰ in Kurikoma to −23.8‰ in Hayakuchi.

All essential fatty acids were detected in *S. leucomaenis* (Figure 3). The major essential fatty acid was 22:6ω3. The average contribution of 22:6ω3 was the highest in *S. leucomaenis* from Kurikoma. With respect to other essential fatty acids, the other omega-3 fatty acids, such as 18:3ω3 and 20:5ω3, presented a higher contribution than that of omega-6 fatty acids. In both aquatic insects, essential fatty acid distribution was similar, with no 22:6ω3. The major fatty acids in both aquatic insects were 18:3ω3 and 20:5ω3. This essential fatty acid pattern was similar among study sites. Epilithic biofilms mainly consisted of 18:3ω3 and 20:5ω3. The contribution of 20:5ω3 was relatively constant in all study sites, while 18:3ω3 contribution varied widely among study sites. Only small amounts of 22:6ω3 were detected from epilithic biofilms. Terrestrial litter only contained 18:2ω6 and 18:3ω3. Other C20 essential fatty acids were only detected at low percentages.

The stable carbon isotope ratios of 18:2ω6, 20:4ω6, and 20:5ω3 in *S. leucomaenis* and epilithic biofilms were almost the same across study sites (Supplementary file Figure S2); consequently, a significant or marginally positive relationship was detected between them (correlation analysis: 18:2ω6, n = 4, r = 0.98, $p < 0.01$; 20:5ω3, n = 4, r = 0.99, $p < 0.001$; 20:4ω6, n = 4, r = 0.94, $p = 0.063$) (Figure 4). Although there was no statistical significance, a positive trend was detected between the stable carbon isotope ratios of 18:3ω3 in epilithic biofilms and that in *S. leucomaenis*. The stable carbon isotope ratios of 18:3ω3 were generally lower in the epilithic biofilms than in *S. leucomaenis*

(Supplementary file Figure S2). Contrarily, the stable carbon isotope ratios of 18:2ω6 and 18:3ω3 in leaf litter were not positively correlated with those of in *S. leucomaenis*.

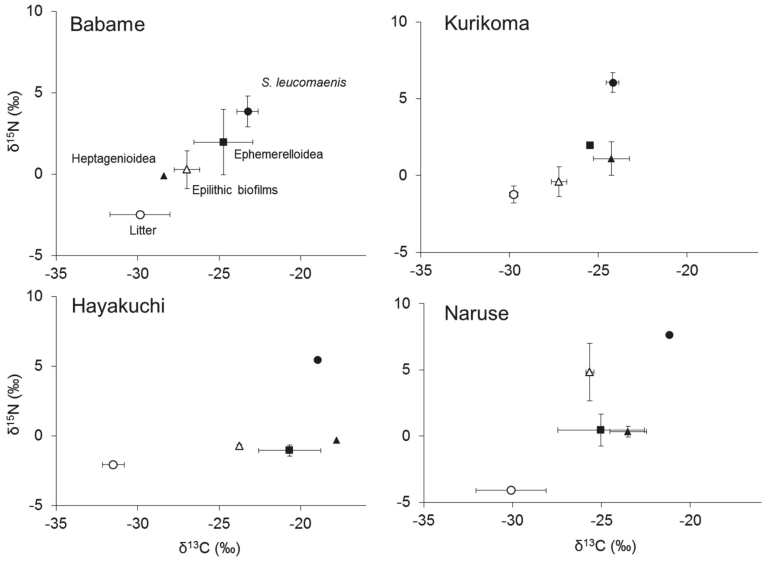

Figure 2. Stable isotope ratios biplot for bulk carbon and nitrogen in basal organic carbon sources and consumers in four study sites. Error bars represent standard deviation.

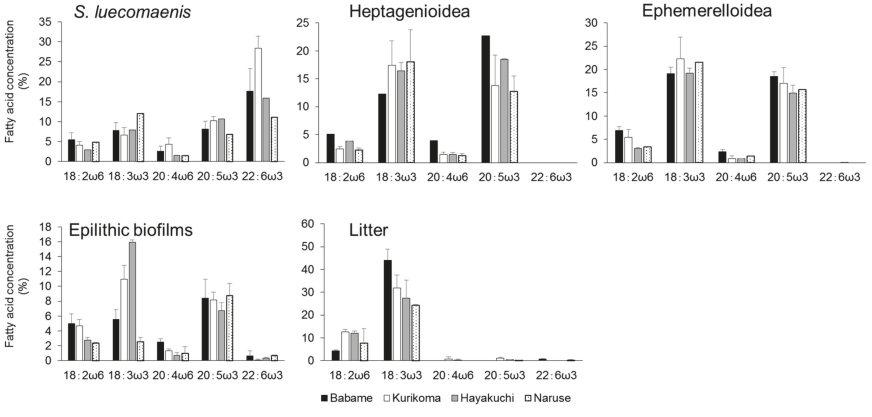

Figure 3. Contribution of essential fatty acids in basal organic carbon sources and consumers in the four study sites. Error bars represent standard deviation.

The bulk carbon and nitrogen isotope ratios of leaf litter in Babame in July 2018 showed values similar to those in July 2016, −29.3‰ for carbon, and −2.1‰ for nitrogen (Figure 5). The carbon and nitrogen stable isotope ratios of epilithic biofilms were −25.1‰ and 4.3‰, respectively. Terrestrial insects in the stomach of *S. leucomaenis* were between leaf litters and epilithic biofilms for carbon and nitrogen stable isotope ratios, with a mean value of −26.7‰ for carbon and 2.2‰ for nitrogen. The bulk stable carbon and nitrogen isotope ratios of *S. leucomaenis* were the most enriched among all samples, and close to those of epilithic biofilms.

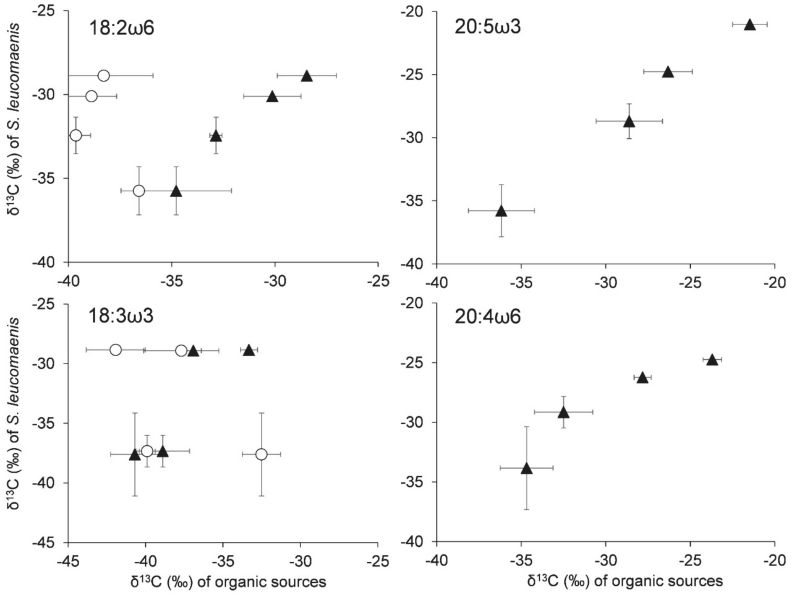

Figure 4. Relationship between stable isotope ratios of essential fatty acid in *S. leucomaenis* and basal organic sources from the four study sites. The black triangle and open circle represent autochthonous organic sources (epilithic biofilms) and allochthonous organic sources (leaf litters), respectively. Error bars represent standard deviation.

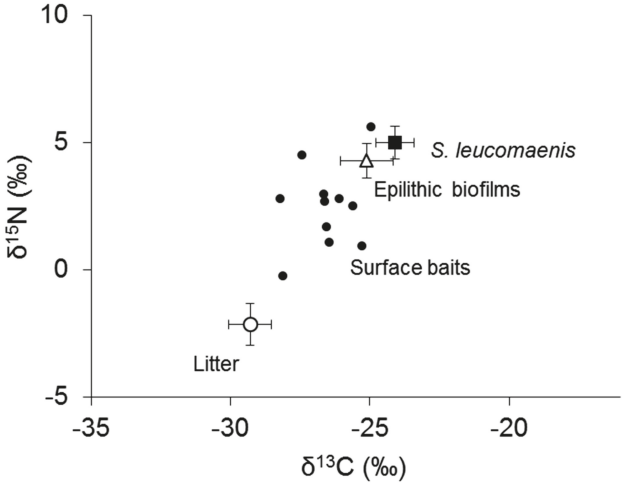

Figure 5. Biplot for stable isotope ratios of bulk carbon and nitrogen of basal organic carbon sources, *S. leucomaenis*, and surface baits from *S. leucomaenis* stomachs in Babame, 2018. Error bars represent standard deviation.

4. Discussion

4.1. Origin of Organic Sources

Canopy cover is an important factor for biogeochemical and biological processes in headwater streams [41]. High leaf litter input and limited primary production are expected in headwater streams. Therefore, a positive relationship between allochthonous contribution and canopy cover was expected [42]. For instance, dietary inputs of surface baits, such as emerged aquatic insects and terrestrial insects, can be expected to increase as the canopy cover increased. However, the stomach contents did not show the expected patterns. Furthermore, our results of bulk carbon stable isotope ratios demonstrated that allochthonous contribution was very rare in *S. leucomaenis*, even in the Babame and Kurikoma study sites, where the canopy cover was very high (>90%). The isotopic positions of *S. leucomaenis* were relatively close to aquatic insects for carbon and higher for nitrogen, indicating that aquatic insects were diet items for *S. leucomaenis*. There were isotopic differences of 2–3‰ in carbon between epilithic biofilms and aquatic insects. The differences seem to be relatively high if a dietary relationship was assumed between epilithic biofilms and aquatic insects, because 0–1‰ fractionations have generally been assumed [21]. These relatively high differences may be explained under some assumptions. First, epilithic biofilms are the mixture of various organic sources with not only algal species, but also terrestrial organic matter [43]. According to our fatty acid analysis of epilithic biofilms, 24:0, which is a fatty acid biomarker of higher plants [44], was detected at 1–2% (data not shown), indicating that the analyzed epilithic biofilms contained terrestrial organic matter. Therefore, the stable carbon isotope ratios are the average of all contained organic matter [43]. The stable carbon isotope ratios of leaf litter showed generally low values in our study sites, indicating pure attached algae stable isotope values were likely more enriched than the analyzed values. If aquatic grazers can selectively utilize specific preferred carbon sources from periphyton [45], the observed wider fractionation between aquatic insects and epilithic biofilms is explainable. This wider fractionation could also be explained by temporal variation of stable carbon isotope ratios in epilithic biofilms. Although the leaf litter stable carbon isotope ratios were relatively constant among the study sites, those of epilithic biofilms widely varied, even in the same study sites between 2016 and 2018 in Babame. It is known that algal stable carbon ratios varied along the gradient of some environmental factors such as isotopic value of dissolved inorganic carbon [8,46] and growth stage [46,47]. This potentially high variability made it difficult to infer the algae dietary contribution for consumers under one-time sampling of stable carbon isotope. Contrastingly, stable carbon isotope ratios of animals were considered to integrate relatively long times [22]. The stable carbon isotope ratios of aquatic insects were generally higher than those of leaf litter and relatively similar to those of attached algae. Thus, the main organic source for *S. leucomaenis* seems to be autochthonous, that is, epilithic biofilms transferred through aquatic insects, regardless of canopy cover in the study sites.

Major dietary contribution of autochthonous sources for headstream consumers has been reported for tropical [6], subtropical [48], and temperate regions [7]. For instance, Lewis et al. [49] showed a major contribution of autochthonous dietary input even under the dominant input of allochthonous organic matters. These contrary observations against the predictions of RCC can be attributed to poor food quality of terrestrial organic matters and high quality of algae [18,50]. However, as observed from the stomach contents analysis, there was a substantial contribution of fallen insects to *S. leucomaenis* diet, in accordance to previous studies on *S. leucomaenis* stomach content [51]. In this study, we could not identify each species. However, according to the stable carbon isotope ratio analysis of surface baits in stomach contents, the carbon sources of these surface baits must have been derived partly from aquatic algae. Some riparian insects spend larval life in aquatic ecosystems and have been known to play an important role transferring highly unsaturated fatty acids from aquatic to terrestrial ecosystems by emerging [52]. This indicated that parts of surface baits can also be a vector of autochthonous carbon going back to aquatic ecosystems. Several researchers pointed out that dietary utilization of riparian

insects is one of the pathways to acquire autochthonous organic sources [18]. Therefore, to evaluate organic matter origin, assimilation-based methods (e.g., stable isotope and fatty acids) are required.

4.2. Origin of Essential Fatty Acids

It is well known that lipids have more negative $\delta^{13}C$ values than that of other biochemical compounds because lighter carbons tend to be used for conversion of pyruvate to acetyl coenzyme A in lipid synthesis [53]. Therefore, several studies have reported more depleted isotopic values in essential fatty acids than that of bulk carbon [27,30]. Similarly, stable isotope ratios of essential fatty acids were substantially depleted compared with that of bulk carbon in the current study. Furthermore, spatial difference was also observed in both bulk and essential fatty acids isotope. Enriched isotopic value was observed in Hayakuchi and Naruse Rivers where canopy cover was relatively low, indicating high availability of sunlight for photosynthesis. It is known that high growth rate makes algal isotopic value enriched due to the increase of contribution of heavy CO_2 [46]. The observed wider difference in isotopic ratios of epilithic biofilm can be reflected in the photosynthetic activity in each study site.

S. leucomaenis contained all essential fatty acids. 18:2ω6 and 18:3ω3 were detected in both organic carbon sources, leaf litters and epilithic biofilms. These fatty acids are not synthesized by animals [54], indicating the origin of these fatty acids in *S. leucomaenis* was either or both of them. We observed a significant positive relationship of stable carbon isotope ratios of 18:2ω6 between epilithic biofilms and *S. leucomaenis* indicating that this essential fatty acids in *S. leucomaenis* was mainly of autochthonous origin. For 18:3ω3, although a positive tendency was observed between epilithic biofilms and *S. leucomaenis*, this relationship was not statistically significant. However, since 18:3ω3 cannot be synthesized by *S. leucomaenis*, 18:3ω3 must come from either epilithic biofilms or leaf litter. Epilithic biofilms seem to be a probable candidate for the origin in 18:3ω3 for *S. leucomaenis* because isotopic values of leaf litter were highly depleted compared to that of *S. leucomaenis*, which cannot be explained by the previously reported isotopic fractionation, small [27] or depleted and inconsistent [32].

The origin of 20:5ω3 and 20:4ω6 seems to be epilithic biofilms or biosynthesis from their corresponding precursors, namely 18:3ω3 and 18:2ω6, respectively [13]. The stable carbon isotope ratios of both essential fatty acids showed positive relationships between *S. leucomaenis* and epilithic biofilm, indicating that these essential fatty acids were also of autochthonous origin. If we assume the origin of essential fatty acids to be epilithic biofilm, isotopic fractionation through two trophic levels, namely epilithic biofilm, aquatic insects, and *S. leucomaenis* for 18:2ω6, 20:5ω3, and 20:4ω6 was −0.9–0.4‰, 0–1.5‰, and −1.0–3.3‰, respectively. On the contrary, for 18:3ω3, the expected isotopic fractionation of epilithic biofilm to *S. leucomaenis* via aquatic insects was 1.6–8.0‰. Fujibayashi et al. [29] found no significant difference of isotopic value of 18:3ω3 between freshwater fish and blooming cyanobacteria. However, the mechanism of this inconsistent and variable fractionation for 18:3ω3 was not explainable in this study. Further research is required for isotopic fractionation of essential fatty acids in food chains.

While 22:6ω3 was the most abundant essential fatty in *S. leucomaenis*, both organic sources and aquatic insects did not contain 22:6ω3. However, we only analyzed the fatty acid content in two ephemeral groups. Moreover, the absence or very small contribution of 22:6ω3 in aquatic insects has been reported for a wide range of aquatic insect taxa [55–57]. As 22:6ω3 was less available from dietary sources, 22:6ω3 detected in *S. leucomaenis* must be biosynthesized from its precursor [11]. During elongation from 20:5ω3 to 22:6ω3, lighter carbon may be preferentially added. Consequently, 22:6ω3 isotopic value depleted compared with that of 20:5ω3 [27]. However, we found that almost the same or slightly enriched isotopic values in 22:6ω3 compared with that of 20:5ω3 (Supplementary file Figure S2). The same tendency was observed in several aquatic consumers, including fish, in Yenisei River [30]. Gladyshev et al. [30] pointed out the possibility that the acetate pool, which is required for elongation of fatty acid, is significantly enriched in ^{13}C compared with fatty acids. While further study is needed to comprehensively understand essential fatty acid dynamics in aquatic ecosystems,

our results demonstrated that the main source of essential fatty acids in headstream food webs was autochthonous organic matter.

5. Conclusions

Dietary origin of total organic carbon and essential fatty acids for the predatory fish *S. leucomaenis* was investigated in the four headwater streams with the two hypotheses: (1) the dietary allochthonous contribution for fish in headwater stream food webs positively relate with canopy cover; and (2) essential fatty acids originate from autochthonous organic matter regardless of canopy cover. Our results indicated that autochthonous organic matters were the main dietary origin of not only essential fatty acids, but also total organic carbon regardless of canopy cover.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/9/9/487/s1. Figure S1 Relationship between stable isotope ratios of bulk and lipid free fish muscle samples (n = 137). Figure S2 Isotopic value of essential fatty acids in collected samples from each study stream. Error bars represent standard deviation.

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Preliminary Estimations of Insect Mediated Transfers of Mercury and Physiologically Important Fatty Acids from Water to Land

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Abstract: Aquatic insects provide an energy subsidy to riparian food webs. However, most empirical studies have considered the role of subsidies only in terms of magnitude (using biomass measurements) and quality (using physiologically important fatty acids), negating an aspect of subsidies that may affect their impact on recipient food webs: the potential of insects to transport contaminants (e.g., mercury) to terrestrial ecosystems. To this end, I used empirical data to estimate the magnitude of nutrients (using physiologically important fatty acids as a proxy) and contaminants (total mercury (Hg) and methylmercury (MeHg)) exported by insects from rivers and lacustrine systems in each continent. The results reveal that North American rivers may export more physiologically important fatty acids per unit area (93.0 ± 32.6 Kg Km^{-2} $year^{-1}$) than other continents. Owing to the amount of variation in Hg and MeHg, there were no significant differences in MeHg and Hg among continents in lakes (Hg: 1.5×10^{-4} to 1.0×10^{-3} Kg Km^{-2} $year^{-1}$; MeHg: 7.7×10^{-5} to 1.0×10^{-4} Kg Km^{-2} $year^{-1}$) and rivers (Hg: 3.2×10^{-4} to 1.1×10^{-3} Kg Km^{-2} $year^{-1}$; MeHg: 3.3×10^{-4} to 8.9×10^{-4} Kg Km^{-2} $year^{-1}$), with rivers exporting significantly larger quantities of mercury across all continents than lakes. Globally, insect export of physiologically important fatty acids by insect was estimated to be ~43.9×10^{6} Kg $year^{-1}$ while MeHg was ~649.6 Kg $year^{-1}$. The calculated estimates add to the growing body of literature, which suggests that emerging aquatic insects are important in supplying essential nutrients to terrestrial consumers; however, with the increase of pollutants in freshwater systems, emergent aquatic insect may also be sentinels of organic contaminants to terrestrial consumers.

Keywords: aquatic ecosystems; subsidies; eicosapentaenoic acid; docosahexaenoic acid; food webs

1. Introduction

The movement of materials between juxtaposed habitats has received much attention by food web and landscape ecologists in the last four decades (reviewed by Richardson and Sato [1]). Freshwater ecologists have long documented that exogenous organic matter (e.g., terrestrial leaves) fuels rivers via inputs of nutrients and organic matter [2], but in recent decades, the importance of aquatic insect subsidies to riparian predators (e.g., bats; [3]) has been emphasized [4–6]. These aquatic subsidies are known to affect the behaviour, productivity, and diversity of riparian predators [7,8].

One such subsidy is in the form of physiologically important fatty acids (eicosapentaenoic acid (EPA; 20:5ω3) and docosahexaenoic acid (DHA; 22:6ω3)), both of which are of fundamental physiological importance to all organisms [5,9] because most consumers do not possess the necessary enzymes to synthesize them in the required quantities, so they must obtain them from their diet. These physiologically important fatty acids are required for the maintenance of cell membrane structure and function [10,11], regulating hormonal processes and preventing cardiovascular diseases [12].

Aquatic insects are one group of organisms known to be key exporters of physiologically important fatty acids to terrestrial systems [13], and because many adult insects do not return to the water [14],

they represent a net loss of organic nutrients from the aquatic system, and potential food for consumers in adjacent terrestrial ecosystems. A plethora of studies on fatty acids in aquatic systems generally support the premise that aquatic insects are richer in physiologically important fatty acids [15–17] than their terrestrial counterparts [13]. Aquatic insects lay their eggs in freshwaters, where the larvae then develop and accumulate physiologically important fatty acids [18]. Subsequently, owing to their complex life cycles, aquatic insects can effectively transfer physiologically important fatty acids to the terrestrial system when they emerge and fall prey to terrestrial predators [19]. As such, knowledge of fatty acids in food sources and consumers is important both for obtaining basic dietary information on consumers within one habitat and for assessing the nutritional implications of reciprocal fluxes in juxtaposed habitats.

Further to providing critical nutrients to terrestrial consumers, aquatic insects can also supply unwanted contaminants to recipient food webs [20]. One such contaminant is mercury, a metal that has become a global concern because of its toxicity. Specifically, methylmercury (MeHg) is of concern as it concentrates at the base of aquatic food webs (e.g., algae) and is subsequently biomagnified, resulting in high concentrations of MeHg in the tissues of predators (e.g., spiders; [21]). The potential of MeHg to be biomagnified presents a health hazard to aquatic organisms and terrestrial wildlife with trophic linkages to aquatic food webs (e.g., those that consume emergent aquatic insects; [22–24]). While many studies have examined the movement of contaminants between habitats (e.g., Du et al. [25]), few studies have concurrently measured the fluxes of contaminants and fatty acids from streams to riparian zones; even though stream contamination is widespread [26].

Great strides have been made by individual researchers on the potential export of fatty acids from water to land (e.g., [13,27]), however, studies looking into the potential export by insects are scanty. Furthermore, our current knowledge of transfer of fatty acids and contaminants extends only to site-specific studies (with many being biased toward the Northern Hemisphere), effectively limiting our ability to understand the global effects of stream-derived contaminants and nutrients across aquatic–terrestrial boundaries.

Through the seminal works of Gladyshev and others [18], the first global estimate of physiologically important fatty acids by emerging insects was estimated to be between 0.1 Kg km^{-2} $year^{-1}$ to as high as 672.2 Kg km^{-2} $year^{-1}$. One would expect that with new studies documenting fatty acids in insects, these estimates may have changed significantly. To date, no global estimates are available for the global estimate of mercury from water to land. To this end, the aim of this study was to build on past works by Gladyshev et al. [18] and estimate the continental and global export of contaminants (methylmercury) and nutrients (physiologically important fatty acids) from freshwater systems to land and to determine the extent of coupling between contaminants and nutrients.

2. Material and Methods

2.1. Literature Search and Data Extraction

To quantify export of physiologically important fatty acids and mercury (Aim: estimate continental and global export of mercury and physiologically important fatty acids via insects) from freshwater systems to land, I quantified subsidies (using physiologically important fatty acids; DHA + EPA) and the potential export of contaminants (methylmercury and total mercury) from freshwater to terrestrial ecosystems by carrying out an extensive search of the scientific literature. To identify relevant studies, a comprehensive literature search was conducted using papers from scientific databases (Google Scholar©, Scholars Portal© and Thomson Reuters Web of Science©) using the search algorithm: fatty acids OR mercury*AND benthic invert*aquatic insects* OR insect emergence. I also included papers from the first global estimates of insect emergence and fatty acids listed in works by Gladyshev et al. [18]. These initial searches yielded >400 articles published up to October 2019. From this initial set, the final dataset (Tables 1–5) was chosen based on the following criteria: (1) emergence reported in mg m^{-2} year (or comparable units) for the year, (2) fatty acids and mercury were reported in mg g^{-1} and ng g^{-1},

respectively (or comparable units e.g., ug g^{-1}), for benthic insects, (3) only emergence traps were used to collect emergent insects, (4) studies that did not use allometric equations (length-weight regressions) to estimate the dry weight of emergent insects (e.g., [13]) that may overestimate emergence rates [28], and (5) only studies published in English, were included in literature surveys. Criterion 2 excluded studies that reported fatty acid and mercury data as relative proportions or percentages (%).

In several cases, fatty acid, mercury and emergence data were available for different seasons or from different locations. Within a single location, a grand mean was calculated from the fatty acid data from that location, regardless of season; thus, the values represented the average values for a location. Data from different studies were combined to provide a grand mean for each type of data (fatty acid, methylmercury, total mercury, emergence).

To standardise values with those reported in the broader scientific literature, I ensured that all units were converted to match those reported in the literature by other authors [27].

2.2. Calculation of Surface Area

Total surface area (Km^2) was estimated by calculating areas of lakes and rivers for six of the world's continents (Africa, Asia, Australia, Europe, North America and South America; Table S1 in Supporting information). I used estimates from the Global Lakes and Wetlands Database (GLWD; [29]), Digital Chart of the World (DCW; [30]), HydroSheds (basins and stream networks; [31]) and HydroK1 (US Geological Survey. [32], empirical data supplied by authors [33]) to calculate the total surface area of lakes and rivers. All Shapefiles (.shp) were visualized and surface areas measured using GRASS GIS [34] and QGIS (version 3.10, [35]). For global estimates of surface areas of lakes and rivers, theoretical calculations from several models in the literature were used (see Supplementary information; Table S2).

Aquatic insects develop and live in only a small portion of aquatic habitats. For instance, over 72% of insects only live in the littoral area of lakes near the shore [36]. Similarly, littoral zones can make up anywhere from 3.4% to 30.3% of the surface area of lakes [36]. As such, I adjusted the measurements of all areas to account for the littoral zone to be between 3.4% to 30.3% (average of 18.6% for all Lakes).

2.3. Emergence of Insects

Data for emerging aquatic insects (dry weight; g m^{-2} $year^{-1}$) were extracted from diverse literature data (Figure 1; Tables 1 and 2). Because only a very small percentage of emergent aquatic return to the stream, I used the average calculations of return of insect to freshwaters. For instance, Jackson and Fisher [14] enumerated the return of adult aquatic insects to be only 3.1% of the emerged biomass returned to the stream. Elsewhere, Gray [37] found that less than 1% of aquatic insects in a prairie stream returned to the aquatic system, whereas other researchers have documented larger (9.2%) returns by biomass in lacustrine systems [38,39]. As such, I corrected the net export to account for the return of between 1% to 9.2% for lakes and rivers (average of 4.43% return rate).

2.4. Estimates of Physiologically Important Fatty Acids in Aquatic Insects

Available data on physiologically important fatty acids (Figure 1; mg g^{-1} of dry mass) were obtained based on studies that quantitatively determined the fatty acids content of insects using standard fatty acid extraction methods (e.g., [40,41]). Some data reported were for aquatic insect larvae and these were included in the analysed dataset. Fatty acid content of insect differs with life stages from larvae to adults [41], however, the life-stage differences in physiologically fatty acids are minor. For example, some mosquito (Culicidae) larvae and adults have been observed to contain approximately similar quantities of physiologically important fatty acids [41]. Where data were reported as wet weight, I used the moisture content given by the authors to calculate the dry mass. Taxa included were from Europe and Asia (Table 3). Most data collected indicated that Diptera are the most dominant order in most emergence data sets.

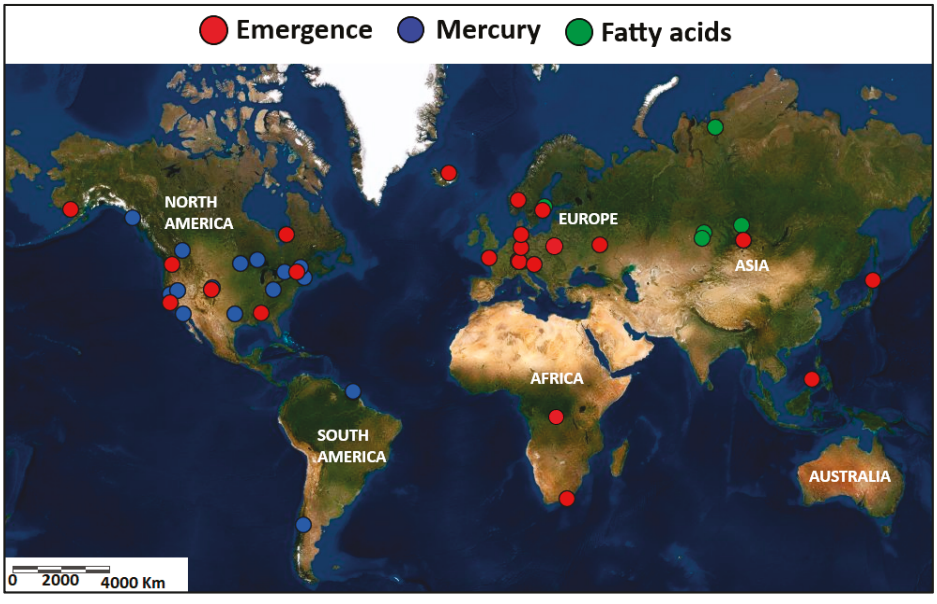

Figure 1. Map showing locality of studies documenting the emergence fatty acids and mercury content in six continents.

Table 1. Insect emergence from lakes (g DM m^{-2} $year^{-1}$) for available continents. 'Community' denotes instances where whole taxa values are reported. Average and coefficient of variation in bold represents the grand average that was used to calculate emergence for Africa, South America, Asia, Australia.

Continent	Taxa	Emergence	Reference
Europe			
	Chironomidae, Ephemeroptera, Trichoptera	4.0	[42]
	Community	1.8	[43]
	Community	1.4	[43]
	Community	1.1	[43]
	Community	2.4	[44]
	Chironomidae	1.9	[45]
	Chironomidae	0.2	[46]
	Community	0.2 [a]	[47]
Average ± SD		1.6 ± 1.2	
Coefficient of variation (%)		70.9	
North America			
	Chironomidae	1.5 [b]	[38]
	Community	1.1 [c]	[48]
	Chironomidae	0.2 [d]	[49]
	Chironomidae	1.9	[14]
Average ± SD		1.2 ± 0.6	
Coefficient of variation (%)		53.8	
Average ± SD		**1.5 ± 1.0**	
Coefficient of variation (%)		**70**	

[a] average values calculated from Table 3 of the reference. [b] averaged author's data. [c] Recalculated from authors data. [d] average value calculated from Table 2 of the reference.

Table 2. Insect emergence from rivers (g DM m^{-2} $year^{-1}$) for available continents. 'Community' denotes instances where whole taxa values are reported. Values in 'bold' denote the grand means and standard deviation for all available data. Average and coefficient of variation in bold represents the grand average that was used to calculate emergence for Australia and South America.

Continent	Taxa	Emergence	Reference
Africa			
	Trichoptera	0.5 [e]	[14]
	Community	4.0 [e]	[14]
Average ± SD		2.2 ± 1.7	
Coefficient of variation (%)		78.6	
Asia			
	Community	2.1 [f]	[50]
	Community	1.2 [g]	[51]
Average		1.7 ± 0.5	
Coefficient of variation (%)		27.3	
Europe			
	Diptera, Trichoptera, Ephemeroptera	1.7	[52]
	Ephemeroptera, Plecoptera, Trichoptera	3.6 [h]	[14]
	Ephemeroptera, Plecoptera, Trichoptera	5.0 [h]	[14]
	Community	5.4 [h]	[14]
	Community	2.6 [h]	[14]
	Community	2.6 [h]	[14]
	Community	3.7 [h]	[14]
	Community	3.7 [h]	[14]
	Community	2.0 [h]	[14]
	Community	2.6 [h]	[14]
	Community	3.2 [h]	[14]
	Chironomidae	1.9 [h]	[14]
Average		3.2 ± 1.1	
Coefficient of variation (%)		35.7	
North America			
	Diptera, Chironomidae	1.2 [i]	[53]
	Trichoptera, Ephemeroptera, Plecoptera, Diptera	6.6 [j]	[54]
	Ephemeroptera, Plecoptera, Trichoptera	0.3	[39]
	Chironomidae, Ephemeroptera, Trichopetra	23.1 [h]	[14]
	Community	5.3	[14]
	Community	7.1	[14]
Average ± SD		7.8 ± 9.2	
Coefficient of variation (%)		117.4	
Average ± SD		**4.5 ± 4.5**	
Coefficient of variation (%)		**100.4**	

[e] data for Democratic republic of Congo (formerly Zaire) stream from Table 5 of the reference; [f] averaged from using average weight of insect specimen dry mass 150 µg; [g] recalculated from Figure 1C of the reference; [h] data for Europe from Table 5 of the reference; [i] averaged author's data; [j] recalculated from authors data.

Table 3. Physiologically important fatty acids (EPA+DHA, mg g^{-1} of dry mass) in emergent aquatic insects in lakes and rivers. Taxa in italics represent fatty acids measured in insect larvae. Average and coefficient of variation in bold represents the grand average that was used to calculate emergence for all six continents.

Continent	Taxa	EPA +DHA	Reference
Lentic			
	Odonata	8.27 [k]	[55]
	Chironomidae	11.9	[46]
	Community	17.8 [l]	[56]
	Chironomidae	4.0	[40]
	Chironomidae	7.0	[40]
	Ephemeroptera	11.3	[27]
	Chironomidae	10.1	[57]
	Culicidae	6.77	[41]
Average ± SD		**9.6 ± 3.9**	
Coefficient of variation (%)		**41**	
Lotic			
	Trichoptera [m]	11.6	[58]
	Ephemeroptera [m]	12.8	[58]
	Chironomidae [m]	7.7	[58]
	Chironomidae	18.1	[18]
	Trichoptera	9.4	[27]
Average ± SD		**11.9 ± 3.6**	
Coefficient of variation (%)		**30**	

[k] converted wet weight to dry weight based on authors data of moisture of ~71.7%; [l] average estimated from Figure 3 of the reference; [m] dry weight estimated from the reference using moisture contents of 83.8% Trichoptera, Chironomidae 78.0%, Ephemeroptera (80%).

Table 4. Total mercury (Hg, mg g^{-1} of dry mass) and methylmercury (MeHg, mg g^{-1}) in emergent aquatic insects in lakes. 'Community' denotes instances where whole taxa values are reported. Average and coefficient of variation in bold represents the grand average that was used to calculate emergence for Africa, Asia, Australia, Europe.

Continent	Taxa	Total Mercury	Methylmercury	Reference
Lentic				
North America				
	Trichoptera, Diptera	[n] 4.2×10^{-4}	[n] 1.6×10^{-4}	[48]
	Coleoptera	1.8×10^{-4}	1.1×10^{-4}	[59]
	Ephemeroptera	1.3×10^{-4}	1.4×10^{-5}	[59]
	Hemiptera	2.6×10^{-4}	1.2×10^{-4}	[59]
	Odonata	1.4×10^{-4}	1.0×10^{-4}	[59]
	Trichoptera	1.3×10^{-4}	4.9×10^{-5}	[59]
	Trichoptera	4.9×10^{-4}	2.5×10^{-5}	[60]
	Odonata	1.1×10^{-4}	5.7×10^{-5}	[60]
	Ephemeroptera	1.1×10^{-4}	2.1×10^{-5}	[60]
	Coleoptera	1.5×10^{-4}	2.0×10^{-5}	[60]
	Trichoptera	3.8×10^{-5}	1.6×10^{-5}	[60]
	Odonata	7.1×10^{-5}	4.8×10^{-5}	[60]
	Ephemeroptera	7.5×10^{-5}	1.9×10^{-5}	[60]
	Odonata	9.7×10^{-5}	1.1×10^{-4}	[61]
	Ephemeroptera	1.1×10^{-4}	7.9×10^{-5}	[61]
	Trichoptera	5.0×10^{-5}	3.7×10^{-5}	[61]
	Diptera	6.9×10^{-5}	3.6×10^{-5}	[61]
	Odonata	-	1.3×10^{-4}	[62]
	Diptera	-	7.9×10^{-5}	[62]
	Trichoptera	-	8.9×10^{-5}	[62]
Average ± SD		$1.3 \times 10^{-4} \pm 8.9 \times 10^{-5}$	$6.6 \times 10^{-5} \pm 4.3 \times 10^{-5}$	

Table 4. *Cont.*

Continent	Taxa	Total Mercury	Methylmercury	Reference
Coefficient of variation (%)		70	65	
South America				
	Diptera	[o] 1.3×10^{-3}	-	[63]
	Ephemeroptera	5.7×10^{-4}	-	[63]
	Odonata	1.7×10^{-4}	-	[63]
	Plecoptera	2.0×10^{-3}	-	[63]
	Trichoptera	3.1×10^{-4}	-	[63]
	Community	2.0×10^{-4}	3.4×10^{-5}	[64]
	Community	2.8×10^{-4}	1.9×10^{-4}	[65]
Average ± SD		$6.9 \times 10^{-4} \pm 6.4 \times 10^{-4}$	$7.0 \times 10^{-5} \pm 4.9 \times 10^{-5}$	
Coefficient of variation (%)		93	68	
Average ± SD		**$2.9 \times 10^{-4} \pm 4.4 \times 10^{-4}$**	**$7.0 \times 10^{-5} \pm 4.9 \times 10^{-5}$**	
Coefficient of variation (%)		**150**	**70**	

[n] mean from data presented in Table 3 in authors data; [o] units converted from ug g to mg g^{-1}.

Table 5. Total mercury (Hg, mg g^{-1} of dry mass) and methylmercury (MeHg, mg g^{-1}) in emergent aquatic insects in rivers. 'Community' denotes instances where whole taxa values are reported. Average and coefficient of variation (in bold) represents the grand average that was used to calculate emergence for Africa, Asia, Australia, Europe, and South America.

Continent	Taxa	Total Mercury	Methylmercury	Reference
Lotic				
North America				
	Diptera	[p] 4.5×10^{-4}	[p] 2.0×10^{-4}	[66]
	Ephemeroptera	[q] 3.4×10^{-5}	[q] 1.8×10^{-5}	[67]
	Trichoptera	5.1×10^{-5}	*	[68]
	Community	2.7×10^{-4}	*	[69]
	Ephemeroptera	8.1×10^{-5}	*	[70]
	Plecoptera	6.1×10^{-5}	7.3×10^{-5}	
	Diptera	2.0×10^{-5}	*	[22]
Average ± SD		$1.4 \times 10^{-4} \pm 1.5 \times 10^{-4}$	$9.6 \times 10^{-5} \pm 7.5 \times 10^{-5}$	
Coefficient of variation (%)		108	78	
South America				
	Community	5.7×10^{-4}	5.0×10^{-4}	[65]
Average ± SD		**$1.9 \times 10^{-4} \pm 2.0 \times 10^{-4}$**	**$2.0 \times 10^{-4} \pm 1.9 \times 10^{-4}$**	
Coefficient of variation (%)		**104**	**95**	

[p] based on average from authors data; [q] based on means of authors data. * Asterisks denote instance where data were not recorded cited reference.

2.5. Estimates of Mercury and Methylmercury Content in Aquatic Insects

Data on Hg and MeHg (mg g^{-1} of dry mass; Tables 4 and 5) were obtained based on studies that quantitatively determined the content of the two forms of mercury in aquatic insects using advanced mercury analyzers like amalgamation-thermal atomic absorption spectrometers [48,66]. While original data were presented by most authors in ng g^{-1}, I converted the values to mg g^{-1} (by multiplying all ng g^{-1} values by 1×10^{-6}) for all analyses to match the values reported for emergence data.

2.6. Data Analyses

Initially, content for fatty acids (mg g^{-1}) was multiplied by the emergence to obtain the export of fatty acids in (Kg Km^{-2} $year^{-1}$). Mercury content data were converted from ug g^{-1} to mg g^{-1} and subsequently multiplied by emergence to obtain methylmercury (MeHg) and total mercury (Hg) as Kg Km^{-2} $year^{-1}$.

To estimate the total net export (Kg $year^{-1}$) of mercury and fatty acids from water to land, export of mercury and fatty acids (Kg km^{-2} $year^{-1}$) were multiplied by the estimate of areas of lakes and rivers (Km^2) globally and by continent. Because some continents had no available emergence and

mercury data for lakes (e.g., Africa, Australia, Asia and South America) and rivers (e.g., South America, I used the grand mean calculated for all available data for each ecosystem type (Lake or River).

All means and coefficients to variations (CV) were calculated for each data type. All mean values for data were compared using MedCalc® (statistical software version 14.8.1, software bvba, Ostend, Belgium; http://www.medcalc.org; 2018) and following procedures described in Altman [71].

3. Results

All literature survey data for fatty acids, Hg, MeHg are presented in Tables 1–5. Overall, the data, as evidenced by high coefficients of variation depict that there is a lot of variation in fatty acid and mercury data recorded in the literature. For example, Hg (Table 5) has a coefficient of variation of over 100 percent. Similarly, the grand means for fatty acids and mercury also show large variations across datasets.

3.1. Continental Exports of Physiologically Important Fatty Acids

Considering export of physiologically important fatty acids per unit area, lentic systems export similar quantities of fatty acids across all six continents in this study (range: 11.3 to 14.2 Kg Km^{-2} $year^{-1}$; Figure 2).

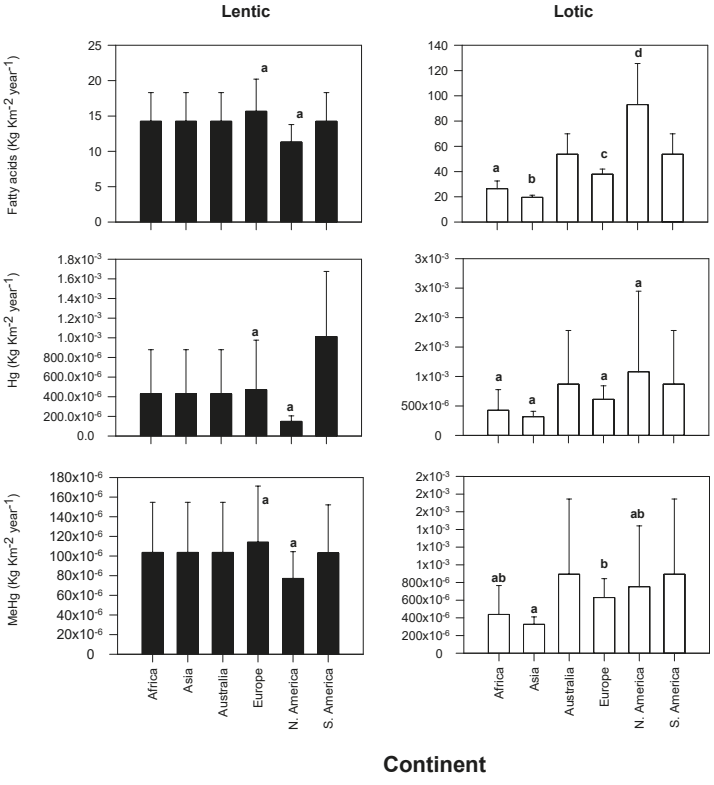

Figure 2. Estimate (±SD) of physiologically important fatty acids, methylmercury (MeHg) and total mercury (Hg) calculated for each continent. The letters depict results from Medcalc® comparison of means calculator within each continent, where values with the same letters depict no significant difference between the export values. Note that only continents where emergence data are available are statistically compared.

In rivers (Figure 2), North America exports a larger amount of fatty acids (93.0 ± 32.6 Kg Km^{-2} $year^{-1}$; Figure 2) compared to all other continents (range: 19.7 to 53.8 Kg Km^{-2} $year^{-1}$) per unit area. The lowest exports of fatty acids per unit area exported from river to land by aquatic insects were in Asia (19.7 Kg Km^{-2} $year^{-1}$).

Considering the total area of rivers and lakes by continent reveals that the quantity of fatty acids (Kg $year^{-1}$) exported from lakes to land are highest in Asia (2.2 × 10^6 Kg $year^{-1}$; Figure 3) and North America (2.2 × 10^6 Kg $year^{-1}$), with Australia exporting the lowest amount of fatty acids (3.4 × 10^4 Kg $year^{-1}$; Figure 3).

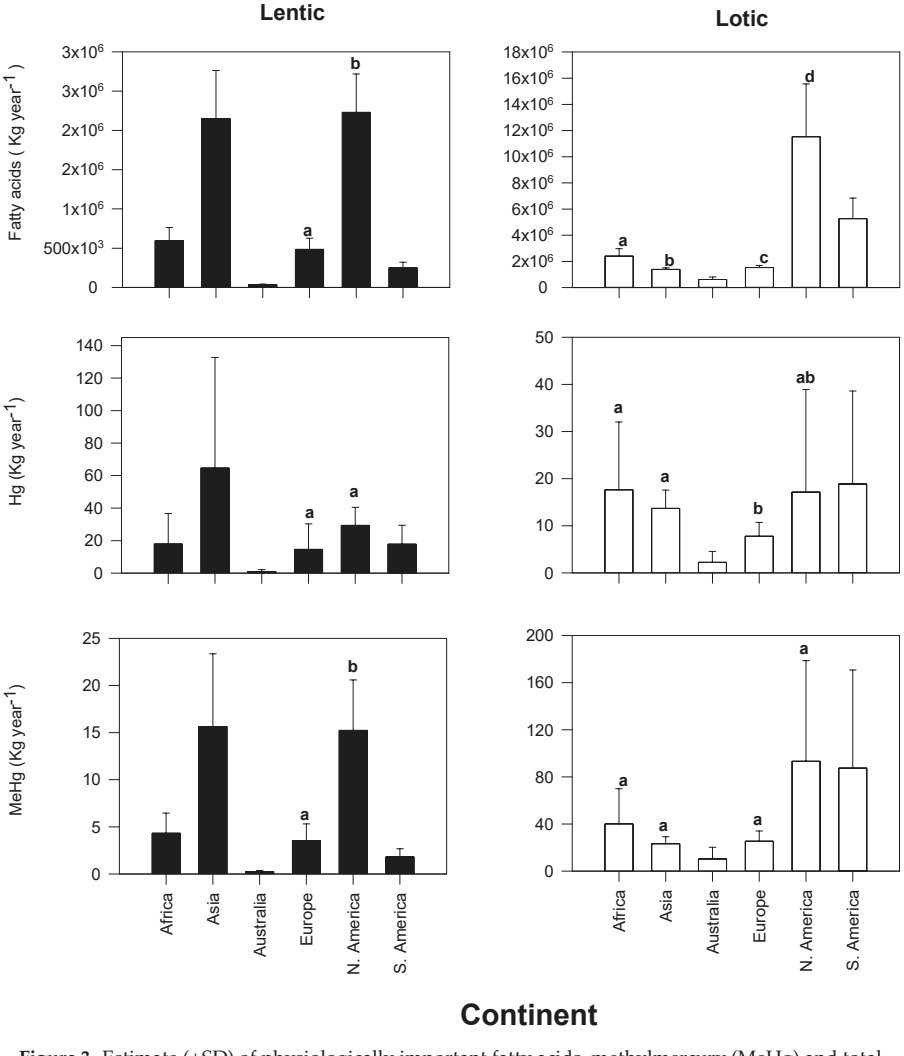

Figure 3. Estimate (±SD) of physiologically important fatty acids, methylmercury (MeHg) and total mercury (Hg) calculated for each continent. The letters depict results from Medcalc® comparison of means calculator within each continent, where values with the same letters depict no significant difference between the export values. Note that only continents were emergence data are available are statistically compared.

In rivers, North America contributes more to the export of fatty acids (11.5×10^6 Kg year^{-1}) than all the other continents (range: 62.4×10^4 to 52.7×10^5 Kg year^{-1}). South America is the second largest exporter of fatty acids from river to land (52.7×105 Kg year^{-1}), with Australia exporting the lowest (62.4×10^4 Kg year^{-1}). Overall, rivers across all continents contribute more to export of fatty acids than lakes.

3.2. Continental Exports of Mercury and Methylmercury

Regarding the export of Hg and MeHg from lakes to land per unit area, there are no significant differences among the exports of Hg (range: 1.5×10^{-4} to 1.0×10^{-3} Kg Km^{-2} year^{-1}; Figure 2) and MeHg (range: 77.2×10^{-6} to 103×10^{-6} Kg Km^{-2} year^{-1}) in lentic systems.

In rivers, there were no significant differences in flow of Hg from water to land among continents per unit area (mean range: 3.2×10^{-4} to 1.1×10^{-3} Kg Km^{-2} year^{-1}; $p > 0.05$). Similarly, there were no significant differences among exports of MeHg by continent. The only exception was between Europe and Asia, where Europe (6.4×10^{-4} Kg Km^{-2} year^{-1}) exported more MeHg per unit area from land to water than Asia (3.3×10^{-4} Kg Km^{-2} year^{-1}).

By considering the total area of rivers and lakes at each continent, I was able to calculate the amount of Hg and MeHg exported from water to land per year (Kg year^{-1}). The results from these calculations reveal that there are no significant differences in export of Hg from lakes (Figure 3). Australia was the only exception as it had significantly lower exports of Hg (2 Kg year^{-1}) from lake compared to all the other continents. Methylmercury exported from lake to land is greatest in Asia (15.6 Kg year^{-1}) and North America (15.2 Kg year^{-1}) compared to the other continents (mean range: 0.3 to 4.33 Kg year^{-1}).

In rivers, there were no significant difference in exports of Hg and MeHg from river to land, with exceptions occurring between some continents (e.g., export of Hg is significantly higher in Europe than in Australia).

3.3. Global Exports of Physiologically Important Fatty Acids and Mercury

Global export of fatty acids per year are higher in rivers (35.4×10^6 Kg year^{-1}) than in lakes (85.1×10^5 Kg year^{-1}; Figure 4; $p < 0.001$). Similarly, MeHg exports are higher in rivers (572.1 Kg year^{-1}) than in lakes (255.9 Kg year^{-1}; Figure 4). Congruent to MeHg exports, Hg differs significantly between rivers and lakes globally (587.7 Kg year^{-1} for rivers versus 61.9 Kg year^{-1} for lakes; Figure 4; $p < 0.05$).

Overall, global estimates reveal that there is some coupling between mercury and fatty acid exports; when fatty export and emergence are high, the values are synchronous to mercury exports by insects (Figures 2–4).

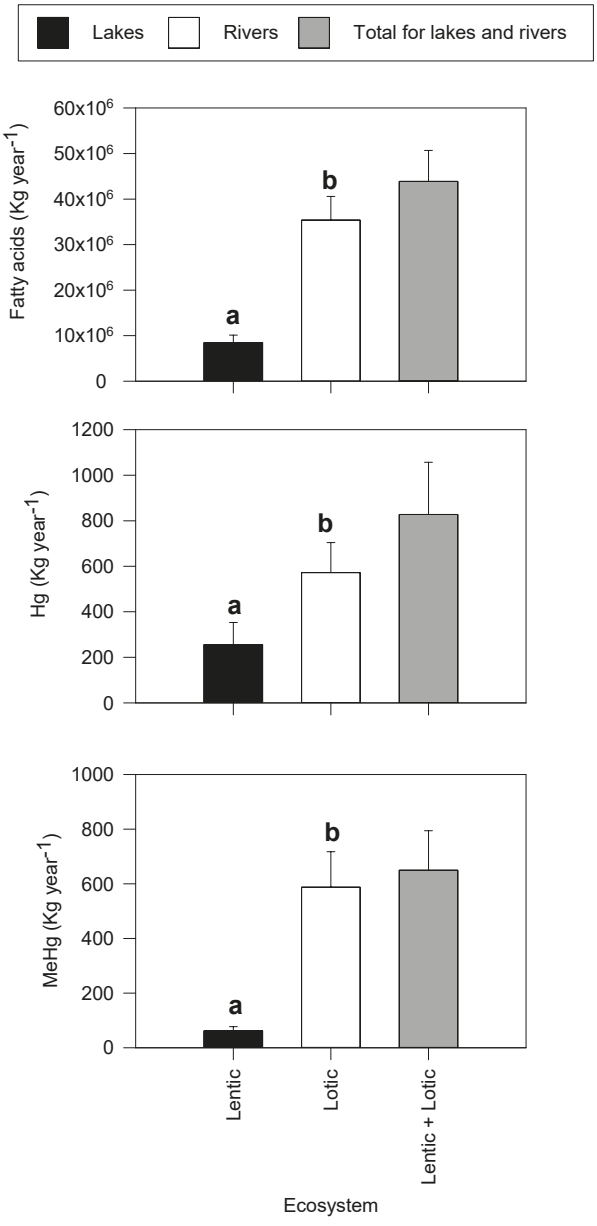

Figure 4. Global estimate (±SD) of physiologically important fatty acids, methylmercury (MeHg) and total mercury (Hg) calculated from diverse ecosystems. The letters depict results from Medcalc® comparison of means calculator between lentic and lotic systems, where values with the same letters depict no significant difference between the export values.

4. Discussion

Subsidies are known to affect terrestrial consumers in recipient systems, but these cross-boundary fluxes also transport persistent mercury [26]. Here, the first global perspective of the potential

synchrony between export of physiologically fatty acids is presented using a plethora of data from different systems. The estimates build on general ideas originally formulated for rivers and lakes as donors of aquatic subsidies via emergent insects [18,72], which have demonstrated the importance of exports of nutrients from water to adjacent land [18,51,72]. One key finding from this this work is that there is synchrony between physiologically important fatty acids and mercury; because of emergence rates. Congruent to previous research (e.g., [54,73]), the results also demonstrate how the export of physiologically important fatty acids and mercury values vary spatially (by continents), with the North American continent exporting more fatty acids from water to land than all other continents.

The estimate of fatty acids exported from water to land (11.3–93.0 Kg km^{-2} $year^{-1}$; Figure 2) are within the range of the first estimate documented to date (0.1 to 672 Kg km^{-2} $year^{-1}$) [18]. The differences in the values obtained may be driven by the availability of more emergence data from other ecosystems. Presently, there are no estimates for export of mercury by aquatic insects to compare with these findings (Figure 1), mainly as a result of prior studies being focused on one aspect on the export of subsidies (nutrients). More studies on the potential export are thus warranted and should yield more fascinating results on the effects of subsidy type on consumers. Considering that hundreds of thousands of miles of streams and lakes are impaired by persistent mercury [74], the results suggest that aquatic insects are likely key movers of mercury from freshwater to terrestrial systems at a global scale. While these estimates are cursory, they may have huge implications for the ecology of terrestrial consumers and humans.

5. Implications

5.1. Wildlife

Terrestrial consumers are known to benefit from aquatic subsidies [7]. For example , quality of fatty acids can affect the fitness of tree swallows [75]. Assuming the trophic transfer efficiency of physiologically important fatty acids through the food web to be 10% (i.e., 90% of energy lost at each trophic level; Figure 5) [76,77] in a presumed three-trophic-level food web, aquatic insects can contribute between 0.4×10^6 to 4×10^6 Kg $year^{-1}$ to terrestrial consumers. It is worth noting that while there may be a 10% dissipation with increased trophic level, other researchers have shown that physiologically important fatty acids are retained and are not dissipated by changing trophic positions [78]. To this end, assuming no dissipation of fatty acids happens up the terrestrial food chain implies that fatty acid production of the third level consumers may be equated to the initial contribution of physiologically important fatty acids with insect emergence (Figure 5). The no dissipation scenario is also tenable considering that physiologically important fatty acids are moved through trophic chains at about double the efficiency of biomolecules such as organic carbon and are effectively bioaccumulated (with no dilution) in higher trophic level consumers [79]. However, it must be emphasized that demand by terrestrial consumers for physiological fatty acids is sparse and further studies are warranted to assess terrestrial consumer dietary needs [80].

Terrestrial consumers that depend on aquatic subsidies may suffer irreversible behavioral, physiological, and reproductive effects [81,82] from exposure to MeHg. For example, some birds (e.g., belted kingfisher (*Ceryle alcyon*) and bald eagle (*Haliaeetus leucocephalus*)) and small mammals (e.g., American mink; *Neovison vison*) have been observed to suffer from visual, cognitive, and neurobehavioral effects [82], and even death within a year when exposed to MeHg concentrations of 1 μg g^{-1} [74]. Because MeHg increases in concentration as it progresses up the food chain, one can predict that organisms consuming prey at higher trophic levels are exposed to higher concentrations of total Hg and MeHg (Figure 4; [83,84]). Assuming that MeHg does not change significantly up the food chain suggests that consumers accumulate 649.6 Kg $year^{-1}$. However, the absolute assimilation efficiencies of MeHg vary with trophic level, uptake pathway, and water chemistry conditions; therefore, the estimates need to be interpreted with caution.

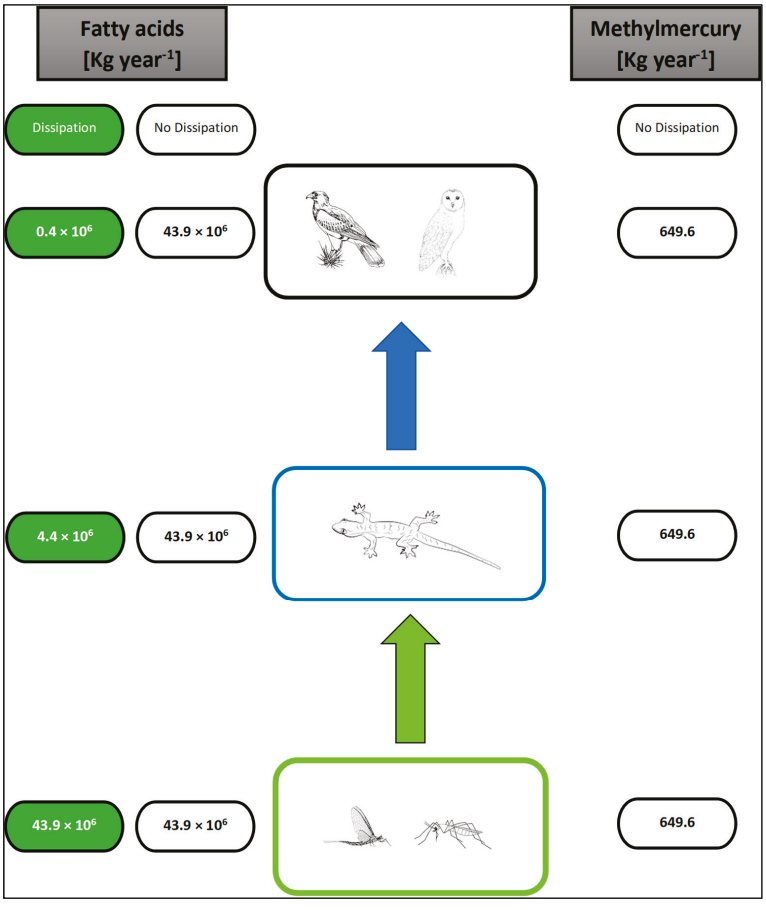

Figure 5. Depiction of the movement of physiologically important fatty acids and methylmercury (MeHg) as mediated my aquatic insects. Transfer efficiency based on traditional trophic pyramid concept of 10% dissipation at each trophic level.

5.2. Climate Change

Climate warming decreases the production of physiologically important fatty acids by decreasing polyunsaturated fatty acid membrane content while simultaneously increasing saturated fatty acids via homeoviscous adaptation [85]. Specifically, climate warming of 2.5 °C is predicted to reduce physiologically important fatty acid in algae by 8.2% to 27.8% (estimated to reduce physiologically important fatty acids from 240 to 225 tonnes [9]. This reduction under climate change will result in many aquatic insects receiving fewer fatty acids and this may subsequently have major effects on terrestrial consumers that often rely on aquatic subsidies to meet their dietary needs. However, some studies show that temperature does not have an effect on the quantity of physiologically fatty acids in consumers. For instance, Gladyshev et al. [86] found that contrasting temperatures have no effect on physiologically important fatty acids (EPA and DHA) with significant effects only observable in C18 saturated and polyenoic acids. As such, it is plausible that the temperature-dependent decrease in EPA and DHA quantities happens mostly due to changes in the taxonomic composition of aquatic communities as a response to temperature changes [86].

6. Additional Considerations and Conclusions

In any study, there are caveats in protocols that can include trap design and other collection tools [87], so some caution is necessary for interpreting any results. I investigated fluxes from river to land using data collected by several authors in different ecosystems, as such, some variation can be expected in these estimates. For example, Different collection methods and traps may overestimate or underestimate fluxes for a variety of reasons [88,89]. Specifically, emergence traps may underestimate the fluxes of odonates from rivers, as some odonates crawl onto vegetation and rocks rather than fly out [87,90]. Additionally, Odonates, individually, have very high biomasses relative to other aquatic insects [90], and their contributions to outward subsidies may be underestimated in all our calculations. I recommend that additional studies incorporate the capture of crawling insects, as this aspect would improve the estimates of aquatic invertebrate flow from water to land.

Additionally, it is worth noting that the values expressed here for annual export of physiologically important fatty acids and MeHg via insects are preliminary estimates, based on averaging data from different ecosystems, and merely represent an initial attempt to calculate the order of magnitude of exports that are mediated my insects. I am cognisant that there are many limitations and sources of error in this type of global extrapolation, including the fact that fatty acids and mercury concentrations may vary depending on region, growth phase, climate, light regime and local nutrient conditions. For example, various authors have shown that mercury varies substantially over space and time [91,92]. Nevertheless, these kinds of data using a global perspective are needed to give a broader scale (*sensu* Gladyshev et al. [18]), which, in the future, may be refined further to create models to predict how environmental perturbations like climate change may affect the spatial and temporal dynamics of subsidies and methylmercury exported from water to land.

Summarily, these results underscore the need to view freshwater systems as just not nutrient exporters but lateral exporters of harmful contaminants [64] that can potentially be biomagnified within the food web. This view departs from the traditional viewpoint of streams being exporters of nutrients alone. Riparian insectivores (e.g., birds and small mammals) facilitate the transfer of aquatic mercury to higher trophic levels, thus serving as conduits in the dispersal of aquatic contaminants to the broader terrestrial food web [82]. Given the widespread contamination of streams, the ubiquity of stream insects, and the importance of insect subsidies to riparian predators, more research is needed to quantify the magnitude and risk of exposure to riparian food webs.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/10/1/129/s1, **Table S1**: Continental estimates of surface areas of lakes and rivers based on shapefiles. All data measured and analysed in qGis, **Table S2**: Global estimates of surface area of lakes and rivers from diverse datasets.

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Variation in ω-3 and ω-6 Polyunsaturated Fatty Acids Produced by Different Phytoplankton Taxa at Early and Late Growth Phas

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Abstract: Phytoplankton synthesizes essential ω-3 and ω-6 polyunsaturated fatty acids (PUFA) for consumers in the aquatic food webs. Only certain phytoplankton taxa can synthesize eicosapentaenoic (EPA; 20:5ω3) and docosahexaenoic acid (DHA; 22:6ω3), whereas all phytoplankton taxa can synthesize shorter-chain ω-3 and ω-6 PUFA. Here, we experimentally studied how the proportion, concentration (per DW and cell-specific), and production (μg FA L^{-1} day^{-1}) of ω-3 and ω-6 PUFA varied among six different phytoplankton main groups (16 freshwater strains) and between exponential and stationary growth phase. EPA and DHA concentrations, as dry weight, were similar among cryptophytes and diatoms. However, *Cryptomonas erosa* had two–27 times higher EPA and DHA content per cell than the other tested cryptophytes, diatoms, or golden algae. The growth was fastest with diatoms, green algae, and cyanobacteria, resulting in high production of medium chain ω-3 and ω-6 PUFA. Even though the dinoflagellate *Peridinium cinctum* grew slowly, the content of EPA and DHA per cell was high, resulting in a three- and 40-times higher production rate of EPA and DHA than in cryptophytes or diatoms. However, the production of EPA and DHA was 40 and three times higher in cryptophytes and diatoms than in golden algae (chrysophytes and synyrophytes), respectively. Our results show that phytoplankton taxon explains 56–84% and growth phase explains ~1% of variation in the cell-specific concentration and production of ω-3 and ω-6 PUFA, supporting understanding that certain phytoplankton taxa play major roles in the synthesis of essential fatty acids. Based on the average proportion of PUFA of dry weight during growth, we extrapolated the seasonal availability of PUFA during phytoplankton succession in a clear water lake. This extrapolation demonstrated notable seasonal and interannual variation, the availability of EPA and DHA being prominent in early and late summer, when dinoflagellates or diatoms increased.

Keywords: polyunsaturated fatty acids; phytoplankton; freshwater; nutritional value

1. Introduction

Phytoplankton, the microscopic primary producers, are central transformers and cyclers of energy and biomolecules in aquatic food webs [1]. The ability of phytoplankton to synthesize different biomolecules influences their nutritional values and reflects their productivity throughout the aquatic food web [2–4]. Among all biomolecules synthesized by phytoplankton, alfa-linolenic acid (ALA; 18:3ω3) and linoleic acid (LIN, 18:2ω6) can be considered as essential polyunsaturated fatty acids

(PUFAs) since consumers cannot synthesize these de novo [5]. These medium-chain ω-3 and ω-6 PUFA are precursors for eicosapentaenoic acid (EPA, 20:5ω-3), docosahexanoic acid (DHA, 22:6ω-3), and arachidonic acid (ARA, 20:4w6), but due to the consumers' limited ability to bioconvert them from ALA or LIN, they can be considered as physiologically essential [5,6]. The physiological importance of long-chain ω-3 and ω-6 PUFA varies by consumers. Usually, DHA appears to be the most retained FA for copepods and many fish, whereas EPA is the most retained FA for *Daphnia* and some benthic invertebrates [6–10]. However, *Daphnia* can grow and reproduce without EPA, whereas total ω-6 availability may negatively affect somatic growth of *Daphnia* [11]. The egg production and hatching success of marine copepods from the genus *Acartia* have been reported to be highly positively correlated with ALA, EPA, and DHA and negatively correlated with SDA and LIN [12,13]. More precisely, ALA had less effect on egg production and hatching success than EPA and DHA, and DHA had higher effect than EPA [13]. Nevertheless, EPA and DHA are not the only important PUFA for zooplankton, and thus, production of medium chain ω-3 and ω-6 PUFA can promote consumers' optimal health.

Although phytoplankton can synthesize many different biomolecules (e.g., amino acids, sterols, carotenoids) [11], species containing high amounts of EPA and DHA are considered high-quality food for zooplankton [2,14]. Among freshwater phytoplankton, cryptophytes, dinoflagellates, golden algae, diatoms, and raphidophytes have been identified as EPA-synthesizing taxa and cryptophytes, dinoflagellates, golden algae, and euglenoids as DHA-synthesizing taxa [15–17]. In addition, some marine green algae and eustigmatophytes can synthesize EPA, and cryptophytes synthesize DHA [12]. Even though cyanobacteria and freshwater green algae cannot synthesize EPA or DHA, some cyanobacteria strains and all green algae can synthesize ALA and stearidonic acid (SDA, 18:4ω3) [16,18] and can contribute much or all their FA. In addition to long-chain and medium-chain PUFA, green algae and diatoms can synthesize 16 PUFA, which does not have physiological importance for aquatic consumers [19].

There is a gap in the knowledge on how efficient different phytoplankton groups are in producing different PUFAs and on how much PUFA content per cell varies among phytoplankton species and within phytoplankton groups. Current knowledge of production efficiency comes from biofuel studies and other applications and majorly focuses on fast growing taxa, e.g., non-EPA- and DHA-producing green algae, or in the optimization of PUFA production of specific species in certain growth conditions, utilizing, for example, industrial side streams [20,21]. These results are therefore not directly applicable when implemented to phytoplankton field data. Studies on laboratory cultures have shed light on the effects of environmental conditions on different phytoplankton taxa to synthesize PUFA [22]. The nutritional value of phytoplankton has shown to be dependent on growth rate regulated by ambient temperature and irradiance [23,24] or on nutrient stress experienced by the phytoplankton. Mitchell et al. [25] reported three–four times higher importance of phytoplankton taxa in relation to environmental conditions on PUFA contributions. However, they were not able to define how much the PUFA content (per biomass or cell) varied within phytoplankton groups or by environmental conditions. Taipale et al. [26] studied the nutritional values of natural phytoplankton communities in 107 boreal lakes sampled once for two summers. They found a negative pattern along nutrient concentration and nutritional value of phytoplankton; however, the variation in the predictability was rather high, suggesting that there are other factors influencing phytoplankton PUFA content.

The main aim of the current research was to study the connections between phytoplankton taxa and the production of ω-3 and ω-6 PUFA along their growth. Furthermore, we wanted to study how the nutritional value of phytoplankton changes when ω-3 and ω-6 PUFA content is calculated per cell instead of per biomass. For this experimental study, we cultured 16 strains from six phytoplankton main groups isolated from boreal and temperate freshwaters. We also studied how the abundance of certain phytoplankton groups influences the production of ω-3 and ω-6 PUFA in eutrophic lake by a calculation of PUFA concentrations based on phytoplankton biomasses. We hypothesized that strains belonging to cryptophytes, dinoflagellates, chrysophytes, and diatoms display higher concentrations—both proportion and cell specific—of ω-3 and ω-6 PUFAs than green algae and cyanobacteria both in early

and late growth phases. Additionally, we hypothesized that production rates of the former algae group were higher than that of the latter.

2. Materials and Methods

2.1. Phytoplankton Culturing

To study how phytoplankton taxa and growth influence the contribution, content, and production of ω-3 and ω-6 PUFAs, we cultured 16 freshwater phytoplankton strains belonging to six phytoplankton main groups (Table 1). From now on, we refer to the strains by their main groups or genus for readability. Each phytoplankton strain was pre-cultured using MWC medium [27,28] with AF6 vitamins [29] at a temperature of 18 ± 1 °C, under 14 h:10 h light:dark cycle with a light intensity of 50–70 μmol m^{-2} s^{-1}. For the actual experiment, we used 200 mL tissue tubes with 75 mL inoculum of pre-cultured algae and 125 mL of fresh MWC with AF6 vitamins. Each strain was cultured in three replicates. Cell density of phytoplankton cultures were measured prior and during the experiment by using an electronic cell counter (Casy, Omni Life Science, Bremen, Germany) with 60 μm capillary (measurement range 1.2–40 μm). Samples for fatty acid analyses were harvested by filtering 20–100 mL of phytoplankton culture onto cellulose nitrate membrane filters (pore size 3 μm, Whatman, Maidstone, Kent, UK).

The specific rates of increase (r_n, divisions day^{-1}) for all strains were calculated for the exponential growth phase using Equation (1):

$$r_n = \ln(N_t/N_0)/t \quad (1)$$

where N_0 is a population at the beginning of the experiment, N_t is the population size at the time t that was determined as the exponential growth phase at the time when the first fatty acid samples were harvested.

2.2. Lipid Extraction and Fatty Acid Methylation

Lipids were extracted from the filters using a chloroform:methanol 2:1 mixture and then sonicated for 10 min, after which 0.75 mL of distilled water was added. Samples were mixed by vortexing and centrifuged (2000 rpm) in Kimax glass tubes, after which the lower phase was transferred to a new Kimax tube. The solvent was evaporated to dryness. Fatty acids of total fraction were methylated using acidic conditions. Toluene and sulfuric acid were used for the transesterification of fatty acid methyl esters (FAMEs) at 50 °C for 16 h, which is the optimal method for methylation PUFA [30]. FAMEs were analyzed with a gas chromatograph (Shimadzu Ultra, Kyoto, Japan) equipped with mass detector (GC-MS) and using helium as a carrier gas (linear velocity = 36.3 cm s^{-1}). The temperature of the injector was 270 °C and we used a splitless injection mode (for 1 min). Temperatures of the interface and ion source were 250 °C and 220 °C, respectively. Phenomenex® (Torrance, CA, USA) ZB-FAME column (30 m × 0.25 mm × 0.20 μm) with 5 m Guardian was used with the following temperature program: 50 °C was maintained for 1 min, then the temperature was increased at 10 °C min^{-1} to 130 °C, followed by 7 °C min^{-1} to 180 °C, and 2 °C min^{-1} to 200 °C. This temperature was held for 3 min, and finally, the temperature increased 10 °C min^{-1} to 260 °C. The total program time was 35.14 minutes and solvent cut time was 9 minutes. Fatty acids were identified by the retention times (RT) and using specific ions [18], which were also used for quantification. Fatty acid concentrations were calculated using calibration curves based on known standard solutions (15 ng, 50 ng, 100 ng and 250 ng) of a FAME standard mixture (GLC standard mixture 566c, Nu-Chek Prep, Elysian, MI, USA) and using recovery percentage of internal standards. The Pearson correlation coefficient was >0.99 for each individual fatty acid calibration curve. Additionally, we used 1,2-dinonadecanoyl-sn-glycero-3-phosphatidylcholine (Larodan, Malmö, Sweden) and free fatty acid of $C_{23:0}$ (Larodan, Malmö, Sweden) as internal standards and for the calculation of the recovery percentages.

Table 1. Cultured phytoplankton strains (taxa, order, species, and strain number), their mean size (diameter by electronic cell counter, μm), and growth phase rate (divisions d^{-1}) for the exponential phase (P1, sampling point 1) and stationary phase (P2, sampling point 2)

Taxa	Order	Species	Strain	Nr.	Size (μm)	Growth P1	Growth P2
Chlorophyceae (green algae)	Chlamydomonadales	*Chlamydomonas reinhardtii*	NIVA K-1016	1	6.1	0.14 ± 0.00	−0.02 ± 0.01
	Chlamydomonadales	*Haematococcus pluvialis*	NIVA K-0084	2	17	0.38 ± 0.10	0.07 ± 0.03
	Sphaeropleales	*Acutodesmus* sp.	University of Basel	3	5	0.11 ± 0.00	−0.12 ± 0.00
	Sphaeropleales	*Monoraphidium griffithii*	NIVA-CHL 8	4	4.6	0.11 ± 0.04	0.07 ± 0.03
Cyanophyceae (cyanobacteria)	Chroococcales	*Microcystis* sp.	NIVA-CYA 642	5	4.1	0.21 ± 0.01	−0.06 ± 0.02
	Synechococcales	*Snowella lacustris*	NIVA-CYA 339	6	2	0.05 ± 0.00	−0.08 ± 0.00
Cryptophyceae (cryptophytes)	Cryptomonadales	*Cryptomonas erosa*	CPCC 446	7	6.14	0.09 ± 0.04	0.06 ± 0.00
	Pyrenomonadales	*Rhodomonas lacustris*	NIVA 8/82	8	11.04	0.12 ± 0.03	0.08 ± 0.00
Synyrophyceae (golden algae)	Synurales	*Mallomonas caudata*	CCAP 929/8	9	12.5	0.05 ± 0.00	−0.08 ± 0.00
	Synurales	*Synura petersenii*	CCAP 960/3	10	8.8	0.05 ± 0.00	0.07 ± 0.01
Chrysophyceae (golden algae)		*Dinobryon bavaricum*	CCAC 2950B	11	5.6	0.12 ± 0.02	0.09 ± 0.00
	Chromulinales	*Uroglena* sp.	CPCC 278	12	8.3	0.14 ± 0.00	0.02 ± 0.01
Bacillariophyceae	Bacillariales	*Nitzchia* sp.		13	6.09	0.56 ± 0.02	0.04 ± 0.01
	Tabellariales	*Diatoma tenuis*	CPCC 62	14	6.14	0.45 ± 0.01	0.10 ± 0.02
	Tabellariales	*Tabellaria fenestrata*	CPCC 619	15	5.94	0.50 ± 0.07	0.07 ± 0.04
Dinophyceae (dinoflagellates)	Peridianales	*Peridinium cinctum*	SCCAP K-1721	16	32.29	0.13 ± 0.00	0.04 ± 0.00

2.3. Quantitation of Fatty Acids

Here, we focused on two medium chain ω-3 (ALA, SDA) and two ω-6 (LIN, GLA) PUFA and two long-chain ω-3 (EPA, DHA) and ω-6 (ARA, DPA) PUFA. However, we calculated the contribution of these PUFA from all quantified fatty acids. In addition to the contribution of PUFA, we calculated their content per phytoplankton dry weight biomass and per cell. The fatty acid content (µg in mg) was calculated based on the following Equation (2):

$$\frac{Q_{FA} \times V_{vial}}{DW_1 \times R_p} \tag{2}$$

where Q_{FA} is the concentration of the fatty acid (µg µL^{-1}) based on calibration curves of GLC-566C (Nu-Chek Prep, Elysian, MN, USA) for each fatty acid, V_{vial} denotes the running volume of the samples (µL), DW_1 is dry weight of the sample, and R_p denotes the recovery percentage based on internal standards.

We calculated ω-3 and ω-6 PUFA content per phytoplankton carbon biomass. The fatty acid content (µg in mg C) was calculated based on Equation (3):

$$\frac{Q_{FA} \times V_{vial}}{V_{filtered} \times TCBM \times R_p} \tag{3}$$

where Q_{FA} is the concentration of the fatty acid (µg µL^{-1}), V_{vial} denotes the running volume of the samples (µL), $V_{filtered}$ is the total volume of filtered lake water (L), $TCBM$ denotes the total phytoplankton carbon biomass (µg C L^{-1}) of the corresponding sample, and R_p denotes the recovery percentage based on internal standards.

The cell-specific fatty acid concentration (pg in cell) was calculated based on Equation (4):

$$\frac{Q_{FA} \times V_{vial}}{V_{filtered} \times Cell \times R_p} \tag{4}$$

where Q_{FA} is the concentration of the fatty acid (µg µL^{-1}), V_{vial} denotes the running volume of the samples (µL), $V_{filtered}$ is the total volume of filtered of cultured phytoplankton (L), $Cell$ is the number of cells of the culture, and R_p denotes the recovery percentage based on internal standards.

Additionally, daily production of PUFA (µg L^{-1} Day^{-1}) was calculated based on Equation (5):

$$\frac{Q_{FA} * V_{vial}}{DW_1 * R_p} \times \frac{DW_2 / V_{filtered}}{Days} \tag{5}$$

where Q_{FA} is the concentration of the fatty acid (µg µL^{-1}), V_{vial} denotes the running volume of the samples (µL), DW_1 is dry weight of the sample, and R_p denotes the recovery percentage based on internal standards. DW_2 is dry weight of the phytoplankton samples between time 1 (e.g., initial) and 2 (e.g., exponential phase), $V_{filtered}$ is the total volume of filtered of cultured phytoplankton (L), and Days cites to the number of culturing days between time 1 and 2.

2.4. Statistical Methods

Bray Curtis similarity matrix of fatty acid data was created using Primer 7[81] (Plymouth Routines In Multivariate Ecological Research, Primer E) of which a non-metric multidimensional scaling (NMDS) plot was created. CLUSTER analysis (Hierarchical Cluster analysis) was used to create 70% similarities in the NMDS ordination. PERMANOVA (Permutational multivariate analysis of variance [31]) was used to test if differences in the ω-3 and ω-6 PUFA composition, biomass, and cell content and production were statistically significant between phytoplantkon groups and growth phase. PERMANOVA was run with unrestricted permutation of raw data and type III sums of squares.

Similarity percentages (SIMPER) were used to detect how different units influence the similarity within phytoplankton group and to identify the characteristic fatty acids of each phytoplankton group. We used PERMDISP (Distance-based test for homogeneity of multivariate dispersions [32]) to investigate the within-class variation in ω-3 and ω-6 PUFA composition, biomass, and cell content and production.

2.5. Implementing Laboratory Culturing Data on Field Data

To scrutinize the phenology of PUFA availability in a well-studied urban lake, phytoplankton data from the Enonselkä basin of Lake Vesijärvi, Central Finland (WGS84 61°2.2′N, 25°31.7′E), were taken from the Hertta database of the Finnish Environment Institute (requires registration, https://www.syke.fi/avointieto). Phytoplankton countings saved in the database were done using accredited method (EN 16695, 2015) by the Finnish Environment Institute. Lake Vesijärvi is a eutrophic, clear water lake (total phosphorus 27 μg L^{-1} and water color 10 mg Pt L^{-1}, Finnish Environment Institute, Water Framework Directive classification and status assessment) regularly experiencing blooms of cyanobacteria and diatoms.

Phytoplankton biomasses (mg C L^{-1}) from open water seasons 2015–2018 (five–six samplings in May–November), including contrasting cyanobacteria-dominant years and years without cyanobacteria blooms, were used to form comparisons with the experimental design. For this, the counted phytoplankton taxa were divided into main taxa: cryptophytes, cyanobacteria, diatoms, dinoflagellates, golden algae, and green algae that included also conjugatophytes. Other reported algae were classified as "other." Phytoplankton biomasses were converted to PUFA availabilities by using the amount of each compound in the experimental study as an average dry weight per mg in exponential and stationary phase. A coefficient of 0.45 was used to convert dry weight to carbon biomass based on our previous measurements [33]. If the experimentally studied main taxon included several tested strains, such as cryptophytes, included the *Cryptomonas* and *Rhodomonas* species, the average of the two strains was used. This was based on the analysis of experimental data, illustrating that the main taxa explained most of the variation in the fatty acid composition as μg FA per mg dry weight.

3. Results

3.1. Growth Rate

Cell abundance was highest (2.5×10^7) with cultured cyanobacteria strains but remained low ($<2.5 \times 10^4$ cell mL^{-1}) throughout 22 days in cultures of *Mallomonas*. Growth rate (Table 1, Figure 1) between initial and the middle of exponential growth phase was highest with all three strains of diatoms (*Nitzchia*, *Tabellaria* and *Diatoma*) and second-highest with *Haematococcus* (green algae; 0.38 divisions d^{-1}) and *Microcystis* (cyanobacteria; 0.21 divisions d^{-1}), even though *Haematococcus* culture did not reach high density. Growth rates were slowest with strains of golden algae of *Synura*, *Mallomonas*, and *Uroglena*, and then with dinoflagellate *Peridinium*. Diatoms reached stationary phase already in eight–13 days, whereas it took 51 days for *Uroglena* to reach the stationary phase.

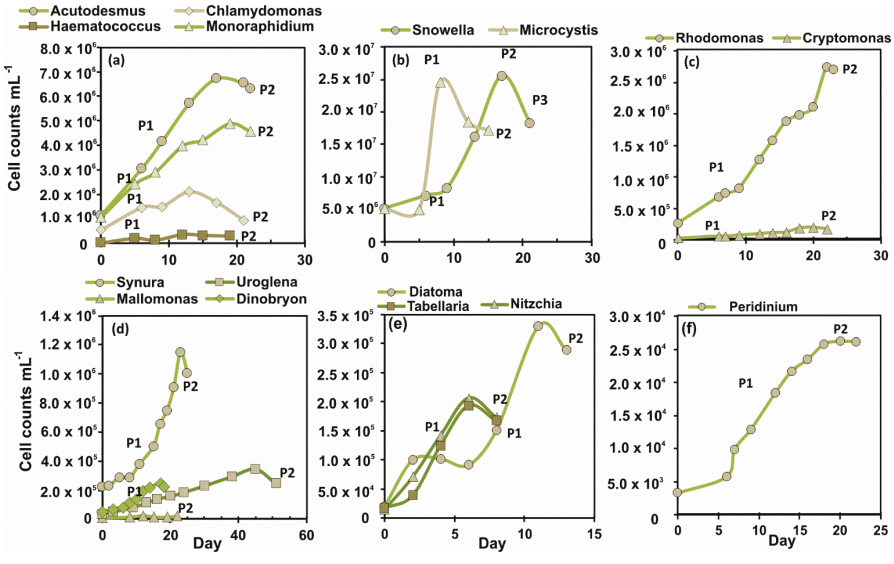

Figure 1. Growth curves for 16 cultures of phytoplankton strains classified by phytoplankton groups: (**a**) green algae, (**b**) cyanobacteria, (**c**) cryptophytes, (**d**) golden algae including chrysophytes and synyrophytes, (**e**) diatoms, and (**f**) dinoflagellate. P1 cites to sampling point during exponential growth phase and P2 cites to the sampling point in stationary phase.

3.2. Phytoplankton Taxa and Growth Phase Impact on the Contribution of ω-3 and ω-6 PUFA

The contribution of ω-3 and ω-6 PUFA of 16 phytoplankton strains varied by the phytoplankton group (Figure 2), but also by growth phase (Figure 3). All strains of green algae and cyanobacteria contained ALA, SDA, and LIN, excluding *Snowella* that did not contain any SDA. The contribution of GLA was highest in *Microcystis*, whereas trace amounts were found among golden algae, diatoms, and green algae. In addition to medium-chain ω-3 and ω-6 PUFA, diatoms, golden algae, and the dinoflagellate contained also EPA and DHA. The absolute contribution of ALA was highest in green algae and *Snowella* (~30% of all FA), whereas cryptophytes and *Dinobryon* had the highest (~26% of all FA) contribution of SDA among all phytoplankton strains. Octadecapentaenoic acid (OPA, 18:5ω3) was found only from the dinoflagellate *Peridinium cinctum* (~4% of all FA). The contribution of LIN was highest (~10% of all FA) in *Haematococcus*, *Uroglena*, *Mallomonas*, and *Synura*, whereas diatoms and the dinoflagellate had only a minor contribution of LIN (<1% of all FA). All strains of cryptophytes, diatoms, and the dinoflagellate had equal contribution of EPA (~13% of all FA), whereas the contribution of DHA was highest (18.4 ± 0.2 % of all FA) in *Peridinium*. Additionally, cryptophytes and golden algae contained also docosapentaenoic acid (ω-6 DPA).

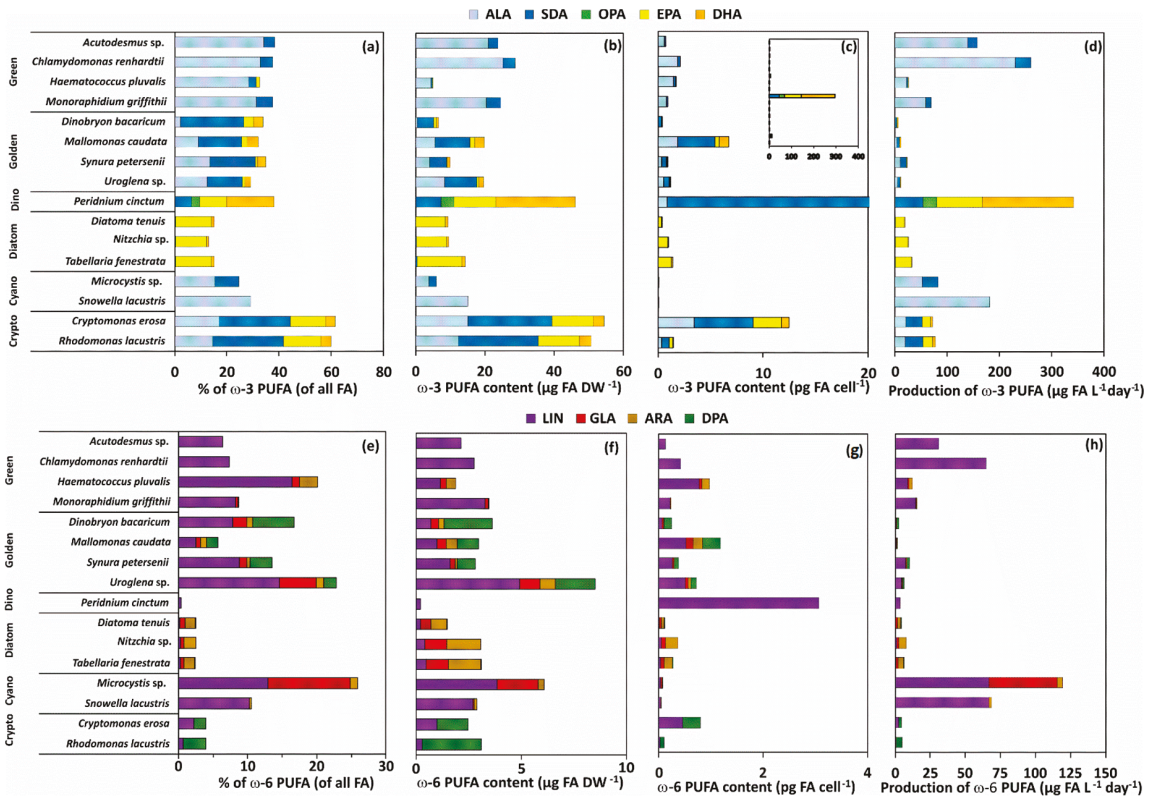

Figure 2. The proportion of all fatty acids (**a**,**e**), per biomass content (**b**,**f**), per cell content (**c**,**g**), and daily production (**d**,**h**) of ω-3 (ALA, SDA, OPA, EPA, DHA) and ω-6 (LIN, GLA, ARA, DPA) PUFA in cultured 16 phytoplankton strains.

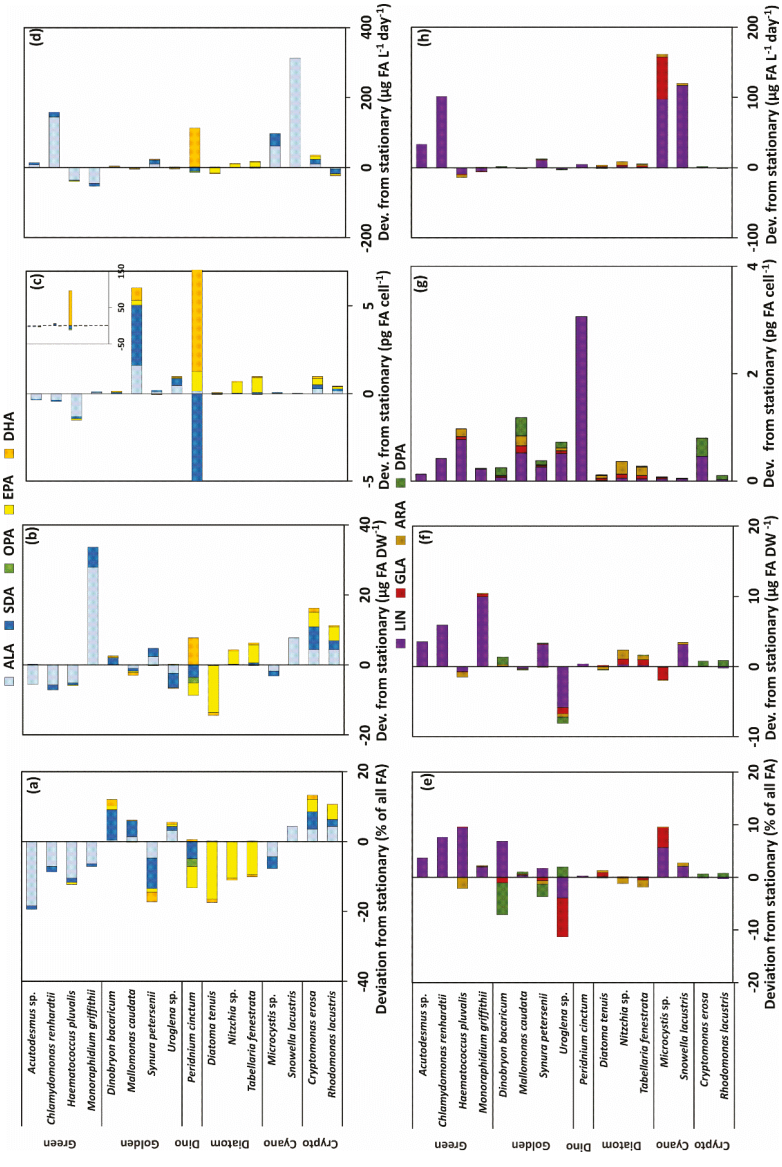

Figure 3. The deviation of ω-3 (ALA, SDA, OPA, EPA, DHA) and ω-6 (LIN, GLA, ARA, DPA) PUFA between exponential and stationary phases: proportion of all fatty acids (**a**,**e**), per biomass content (**b**,**f**), per cell content (**c**,**g**), and daily production (**d**,**h**).

According to the PERMANOVA (Table 2) the contribution of ω-3 and ω-6 PUFA differed between strains by the taxa, but also by the growth phase. Taxa explained 84% of all variation, but growth phase explained only 1% of the variation. Pairwise PERMANOVA (t = 2.58–27.8, P(MC) < 0.003) showed that the contribution of ω-3 and ω-6 PUFA differed among phytoplankton main groups. However, non-metric multidimensional scaling analysis (Figure 4) clustered (CLUSTER analysis) *Snowella* with green algae and *Microcystis* with exponential phase of *Uroglena* together by 70% similarity excluding. Furthermore, NMDS output of percentages of ω-3 and ω-6 PUFA separated strains by growth phase. Pairwise PERMANOVA (t = 3.7–7.1, P(MC) = 0.001) showed statistical difference between exponential and stationary phase for green algae, diatoms, dinoflagellates, and cryptophytes, but not for cyanobacteria or golden algae (t = 0.75–1.01, P(MC) = 0.35–0.55). The contribution of ω-3 PUFA was higher in exponential phase in green algae, dinoflagellates, and diatoms, whereas cryptophytes and chrysophytes (excluding *Synura*) had higher contribution of different ω-3 PUFA in stationary phase (Figure 3). The contribution of LIN in green algae and cyanobacteria was higher in stationary phase than in exponential phase. Otherwise, similar clear trends were not seen in the contribution of ω-6 PUFA with other taxa. Permutational analysis of multivariate dispersions (PERMDISP) showed lowest dispersion among cryptophytes and green algae, whereas dispersion was highest within cyanobacteria (Figure 5) reflecting high variation among these phytoplankton classes (Figure 3a).

Table 2. Pseudo-F and Monte Carlo p-values (P(MC) for PERMANOVA analysis of ω-3 and ω-6 PUFA of phytoplankton strains by the phytoplankton group and phase and mix of them as factors.

			PERMANOVA		
Unit	**Factors**	**Df**	**Pseudo-F**	**exp %**	**P(MC)**
Contribution	Group	5	141.46	84	**0.001 ***
	Phase	1	7.2967	1	**0.001**
	GroupxPhase	5	7.8303	5	**0.001**
Biomass content	Group	5	39.307	69	**0.001**
	Phase	1	1.6199	1	0.154
	GroupxPhase	5	1.075	2	0.345
Cell content	Group	5	33.402	65	**0.001**
	Phase	1	1.2592	0	0.233
	GroupxPhase	5	1.3961	2	0.12
Production	Group	5	40.176	66	**0.001**
	Phase	1	2.9217	1	0.019
	GroupxPhase	5	3.7874	6	**0.001**

* bold value means statistically significant different.

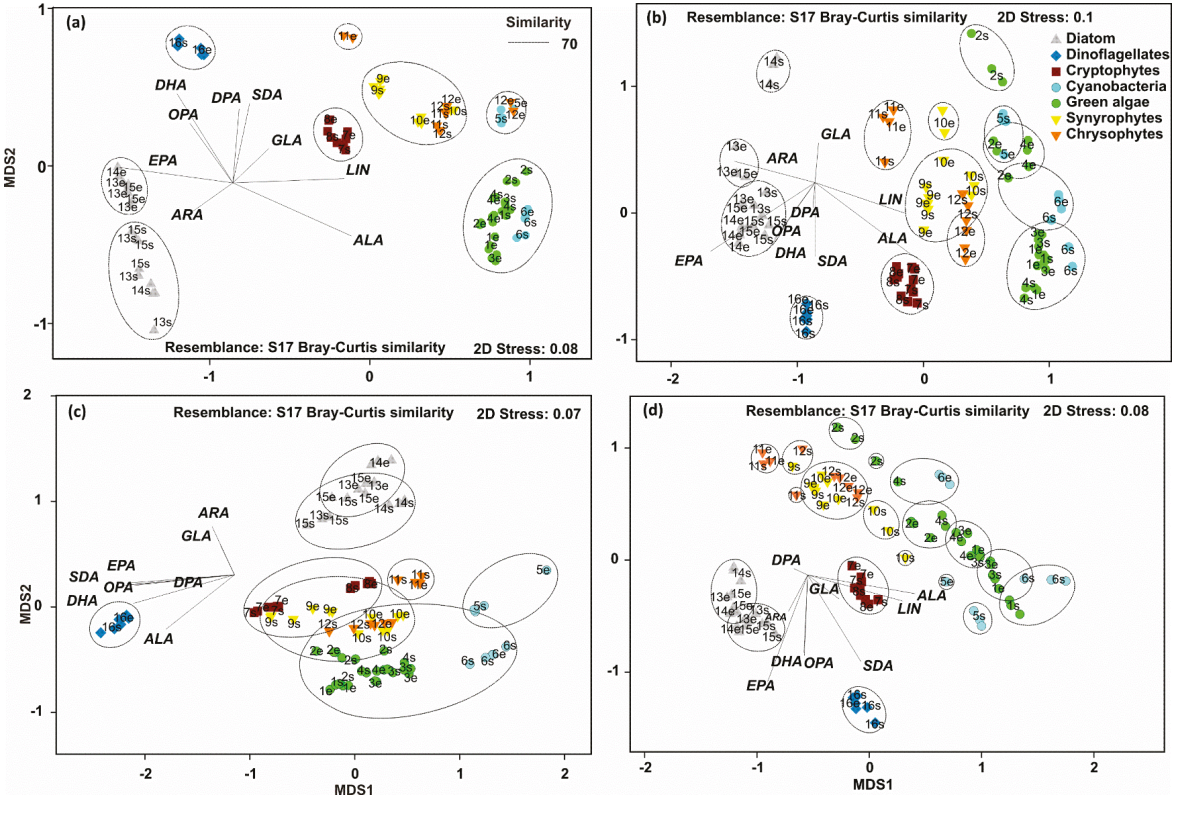

Figure 4. Non-metric multidimensional scaling plots of Bray Curtis similarity of percentages (**a**), biomass content (**b**), cell content (**c**), and daily production (**d**) of ω-3 (ALA, SDA, EPA, DHA) and ω-6 (LIN, GLA, ARA, DPA) PUFA in cultured 16 phytoplankton strains (see Table 1). Golden algae are divided here into Synyrophytes and Chrysophytes to demonstrate the difference in these classes. Abbreviations after strain number: e = exponential phase and s = stationary phase.

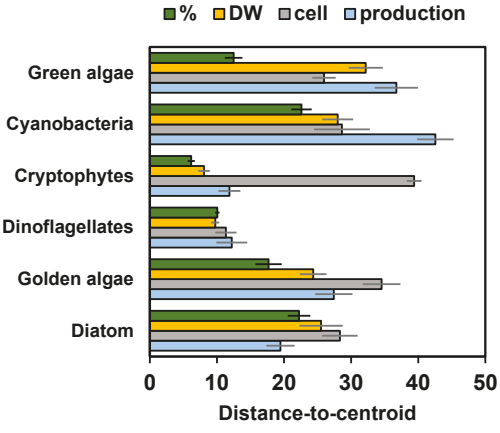

Figure 5. Permutational Analysis of Multivariate dispersion (PERMDISP) of ω-3 and ω-6 PUFA across each phytoplankton class (contribution (%), biomass content (DW), cell content (cell), and production.

3.3. Phytoplankton Taxa and Growth Phase Impact on the Content of ω-3 and ω-6 PUFA

The biomass (DW) and cell content of individual ω-3 and ω-6 PUFA varied greatly among 16 phytoplankton strains (Figure 2). According to the PERMANOVA (Table 2) the content (per biomass and cell) of ω-3 and ω-6 PUFA differed by the phytoplankton group, but not by the growth phase. Phytoplankton taxa explained 69% and 65% of all variation for biomass and cell contents, respectively. Pairwise PERMANOVA (t = 2.3–9.6, P(MC) = 0.001–0.008) comparison showed that all phytoplankton groups differed from each other when PUFA content was calculated per cell but not between cyanobacteria and green algae when PUFA content was calculated per biomass (t = 1.615, P(MC) = 0.071). Total biomass content of ω-3 PUFA was highest in cryptophytes (Figure 2), but when ω-3 PUFA content was calculated per cell the dinoflagellate *Peridinium* had 24-fold content of ω-3 PUFA in relation to any phytoplankton strain (Figure 2). More specifically, green algae excluding *Haematococcus* had highest ALA content per biomass, cryptophytes had the highest SDA content and cryptophytes, diatoms, and dinoflagellates had the highest EPA content. *Peridinium* had seven times higher DHA content than in any other phytoplankton strains. Total ω-6 PUFA biomass content was highest among *Uroglena* and *Microcystis*, which had both especially high LIN and GLA. Additionally, all cryptophytes and golden algae had relatively high ω-6 DPA content.

Dispersion (PERMDISP) of ω-3 and ω-6 PUFA per DW was low (Figure 5) and group similarity was high (SIMPER; Table 3) only among cryptophytes and dinoflagellates (including only one species of exponential and stationary). When using per cell PUFA concentrations in PERMDISP analysis, dispersion was high and similarity low among all phytoplankton. This trend was especially seen with golden algae and cryptophytes: cell ω-3 and ω-6 PUFA content was relatively higher in *Mallomonas* and *Cryptomonas* than in other species of golden algae or cryptophytes, respectively. The output of non-metric multidimensional scaling of ω-3 and ω-6 PUFA content (Figure 4b,c) also showed that dissimilarity within phytoplankton group is higher when PUFA content is calculated per cell than per biomass. This was especially seen between golden algae and cryptophytes that clustered separately in NMDS when using per biomass content but did not differ in NMDS when per cell content was used. We found logarithmic regression ($y = 2.9093\ln(x) + 8.0141$; $r^2 = 0.645$) between cell size and ω-3 PUFA content per cell. The per biomass content of ω-3 and ω-6 PUFA of phytoplankton strains in exponential and stationary phase varied greatly within phytoplankton groups, and cryptophytes were the only group in which both strains had higher PUFA content in stationary than in exponential phase. When the ω-3 PUFA content was calculated per cell, all cultured strains excluding *Acutodesmus*,

Chlamydomonas, and *Haematococcus* had equal or higher ω-3 PUFA content per cell in stationary than in exponential phase (Figure 3).

Table 3. Similarity percentages of SIMPER analysis used to assess similarity within phytoplankton class/group by the different units of the ω-3 and ω-6 PUFA abundance and main PUFAs, explaining most of the similarity. *n* = strain number within taxa + number of growth phases.

		SIMPER	
Taxa	**Unit**	**Average Sim. (%)**	**Main PUFA**
Diatom	Contribution	70.7	EPA
(*n* = 3 + 2)	Biomass content	64.8	EPA
	Cell content	60.7	EPA
	Production	72.7	EPA
Golden algae	Contribution	74.6	SDA, ALA, LIN
(*n* = 4 + 2)	Biomass content	65.8	SDA, ALA, LIN
	Cell content	52.3	SDA, ALA, LIN
	Production	60.7	SDA, ALA, LIN
Dinoflagellate	Contribution	86.5	DHA, EPA
(*n* = 1 + 2)	Biomass content	85.8	DHA, EPA
	Cell content	83.1	DHA, EPA
	Production	81.3	DHA, EPA, SDA
Cryptophytes	Contribution	91.3	SDA, ALA, EPA
(*n* = 2 + 2)	Biomass content	88.5	SDA, ALA, EPA
	Cell content	51.8	SDA, ALA, EPA
	Production	83.3	SDA, ALA, EPA
Cyanobacteria	Contribution	70.7	ALA, LIN
(*n* = 2 + 2)	Biomass content	62.3	ALA, LIN
	Cell content	51.8	ALA, LIN
	Production	41.3	ALA, LIN
Green algae	Contribution	82.3	ALA
(*n* = 4 + 2)	Biomass content	57.1	ALA
	Cell content	64.6	ALA
	Production	49.2	ALA

3.4. Phytoplankton Taxa and Growth Phase Impact on the Production of ω-3 and ω-6 PUFA

The production of medium-chain and long-chain ω-3 and ω-6 PUFA differed (PERMANOVA, Table 2) according to phytoplankton class (Figure 2d,h), within the phytoplankton main group (PERMDISP and SIMPER; Figure 5, Table 3), and by the growth phase (Table 2). However, growth phase explained only 1% of the variation, whereas phytoplankton taxa explained 66% of all PUFA variation. Pairwise PERMANOVA (t = 4.80–10.37; P(MC) = 0.001) showed that all phytoplankton groups, excluding cyanobacteria and green algae, differed from each other (t = 1.39, P(MC) = 0.124). Production of ω-3 and ω-6 PUFA differed by growth phase among diatoms and cyanobacteria (Pairwise PERMANOVA: t = 1.93–3.38; P(MC) = 0.001–0.041). The production of ALA was highest with green algae (*Chlamydomonas*, *Acutodesmus*) and cyanobacteria (*Snowella*), whereas dinoflagellate (*Peridinium*) and cryptophytes had the highest production of SDA per day. The dinoflagellate *Peridinium* produced three and 33 times more EPA and DHA per day (μg PUFA L^{-1} day^{-1}), respectively, than any other phytoplankton strain. Diatoms had highest production values for EPA and cryptophytes for DHA after *Peridinium*. Furthermore, diatoms and cryptophytes had 87 and 34 times higher production of EPA than chrysophytes, respectively. Production of LIN was highest in cyanobacteria and *Chlamydomonas* and *Acutodesmus*, whereas *Microcystis* alone had highest production of GLA. Cryptophytes and golden algae produced highest amount of ω-6 DPA in a day, even though it was relatively low in comparison with the production of LIN produced by green algae and cyanobacteria. Similarity analysis (SIMPER) showed that similarity in the production ω-3 and ω-6 PUFA was highest among cryptophytes and diatoms, whereas the similarity (SIMPER) was lowest with green algae and cyanobacteria. Green algae and cyanobacteria also clustered together in the NMDS plot. Production of ω-3 and ω-6 PUFA did not

differ statistically between the exponential and stationary growth phase at the main group level, but some strains, e.g., *Chlamydomonas*, *Microcystis*, and *Snowella*, had a relatively higher production of ALA and LIN at the stationary phase (Figure 4d,h).

3.5. Extrapolation to Field Data

The community composition in Lake Vesijärvi had no clear pattern during the study years (Figure 6, Figures S1 and S2). However, the proportion of dinoflagellates was generally highest in spring. In June 2015, cryptophytes and golden algae increased and were followed by diatoms and cyanobacteria in autumn. On the contrary, years 2016 and 2018 were dominated by cyanobacteria from June until autumn, whereas in 2017, cryptophytes and diatoms increased in mid-summer and cyanobacteria in autumn.

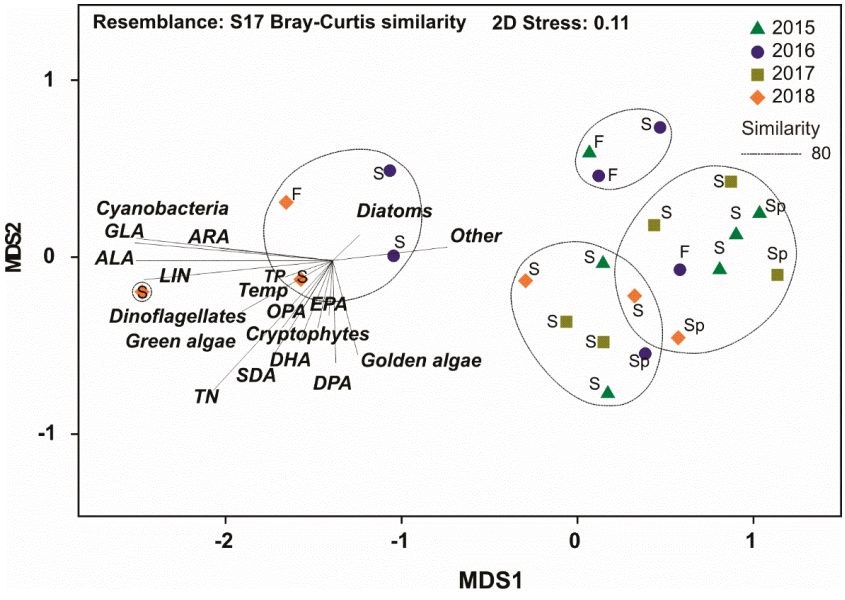

Figure 6. Non-metric multidimensional scaling plots of Bray Curtis similarity of ω-3 (ALA, SDA, EPA, DHA) and ω-6 (LIN, GLA, ARA, DPA) PUFA concentration of phytoplankton (μg PUFA L^{-1}), main phytoplankton groups and physico-chemical parameters in Lake Vesijärvi in years 2015–2018. TN—Total Nitrogen, TP—Total Phosphorus, Temp—temperature in the epilimnion. S = summer, F = fall, Sp = Spring.

Converted to fatty-acid availabilities, the concentration of ω-3 and ω-6 PUFA did not differ between years (PERMANOVA: Pseudo-F = 1.49, p = 0.195), but field data demonstrated notable seasonal and interannual variation (PERMANOVA: Pseudo-F = 4.36, p = 0.007). According to the two factor PERMANOVA, the season explained 24% of all variation in the PUFA concentrations. Generally, non-metric multidimensional scaling clustered phytoplankton and corresponding PUFA concentrations in four groups with 80% similarity (Figure 6). NMDS1 correlated strongly negatively (r = −0.98) with cyanobacteria. One point was close with cyanobacteria, and four points related closely with diatoms and all other sampling points were in the right side of the NMDS output. Different PUFA showed a strong relationship with certain phytoplankton groups. Cyanobacteria-dominance was reflected as the high concentration and proportion of ALA, LIN, and GLA throughout the growing season (Pearson correlation: r = 0.93–0.97, p < 0.001), which peaked after midsummer. The relative proportion of DHA was highest in early summer, when biomass of dinoflagellates was relatively high

(Figure 6, Figure S1). The concentration of DHA showed a strong correlation with the biomass of dinoflagellates (Pearson correlation: $r = 0.957$, $p < 0.001$), whereas the concentration of EPA was most closely related with diatoms (Pearson correlation: $r = 0.648$, $p < 0.001$) and cryptophytes (Pearson correlation: $r = 0.671$, $p < 0.001$). However, NMDS output separated diatoms as their own group, and EPA was more closely related with golden algae and cryptophytes than with diatoms. The abundance of green algae showed strong correlation with biomass of cryptophytes and dinoflagellates (Pearson correlation: $r = 0.47$–0.59, $p < 0.004$–0.022), as can be seen in the NMDS output (Figure 6), resulting in a strong inter-correlation with the concentration of EPA and DHA (Pearson correlation: $r = 0.57$–0.72, $p < 0.0001$–0.005). Whereas total phosphorus (TP) was positively related with cyanobacteria in NMDS output, temperature was positively related with dinoflagellates, cryptophytes, golden algae, and green algae. However, a negative relationship between cyanobacteria and TP was not statistically significant ($r = 0.406$, $p = 0.055$).

4. Discussion

The experimental setup of this study consisted of six main groups of phytoplankton (cryptophytes, dinoflagellates, golden algae, diatoms, green algae, and cyanobacteria), which were sampled at early and late growth phase to understand how phytoplankton nutritional value and production of ω-3 and ω-6 PUFA may vary along phytoplankton growth. Inclusion of one–four different strains in each main group facilitated scrutinization of variation inside taxonomic main groups. Briefly, even though the ability to synthesize different ω-3 and ω-6 PUFA follows strictly phylogenetical groups [15,16,22,34], the PUFA content per cell and the production of PUFA can vary greatly within phytoplankton groups.

Typically, the studies on phytoplankton fatty acids report the contribution of different PUFA together with the total concentrations of PUFA (e.g., per dry weight or carbon) [15,16]. Deviating from the previous studies, we determined the cell-specific fatty acid content and production rates for the main freshwater phytoplankton groups. Proportions, dry weights, and cell-specific concentrations were calculated for both exponential and stationary growth phase. Our results revealed that cell-specific PUFA content differed greatly from biomass-specific PUFA content and the variation in cell PUFA content within phytoplankton group was high likely due to the variable size of phytoplankton. Comparison of the different metrics demonstrated risk of being misled if scrutinizing only one type of concentration and making ecological extrapolation. Proportion and concentration as dry weight can give only restricted amount of information on PUFA and might be of more interest in biofuel production [19]. However, information of the cell-specificity is important, because in plankton communities, secondary consumers feed on a diverse phytoplankton community, and the size of the animal is proportional to the size of the phytoplankton that it can ingest [1,15].

In this study, *Peridium* had a large cell diameter and relatively slow specific growth rate, both characteristics typical of *K*-strategists displaying resource-efficiency in traditional r/K classification [1]. In Lake Vesijärvi, dinoflagellates occurred at the time typical for cells displaying these functional traits. However, DHA content per DW was seven times higher in *Peridium* than in any other phytoplankton strain, whereas DHA content per cell in *Peridium* was ~200 fold in relation to any other phytoplankton strain. This makes a many-fold difference for filter-feeding zooplankton grazers, and explains why dinoflagellates are the preferable diet for copepods [35]. *Daphnia* do not grow well with *Peridinium*, maybe due to the armoring and low amounts of sterols [11]. However, according to the fatty acid modeling, *Daphnia*'s diet consisted of ~20% dinoflagellates in Lake Vesijärvi in year 2016 [26]. Therefore, it seems that dinoflagellates can fuel EPA and DHA demand of both zooplankton groups and the whole food web as seen earlier in a strong correlation between the biomass of dinoflagellates and DHA content of perch [17]. Even though *Peridinium* grew slowly, we found that the production of DHA was 40 times higher with *Peridinium* than any other phytoplankton strain, which emphasizes the role of this non-toxic freshwater dinoflagellate in the synthesis of DHA. Therefore, even a small increase in the biomass of *Peridinium* can significantly increase the production of DHA in boreal lakes. However,

some dinoflagellates species, e.g., *Ceratium*, are too large for zooplankton to ingest, and thus, high DHA content in them is not available for zooplankton.

Herbivorous cladoceran can have a high proportion of EPA, whereas DHA is nearly absent in them [10,36,37]. Therefore, the production of EPA is important for herbivorous cladocerans (e.g., *Daphnia*). Diatoms and cryptophytes are crucial producers of EPA in freshwaters [38]. Meanwhile, the percentage and biomass content of EPA is similar with cryptophytes and diatoms. Our results showed that cell content of EPA varies greatly between these two phytoplankton groups. Meanwhile, *Cryptomonas* had a higher EPA content per cell than any of the studied diatoms. We found the lowest cell EPA content in *Rhodomonas*. Moreover, since diatoms grow faster than cryptophytes, we found 2.3 times higher production of EPA with diatoms than with cryptophytes. These two phytoplankton groups equally influenced the concentration of EPA in Lake Vesijärvi, showing the importance of diatoms, especially in spring and autumn, while cryptophytes' importance was largely shown in summer. Furthermore, our previous fatty acid-based modeling on the composition of *Daphnia* diets also showed that cryptophytes and diatoms are the two main dietary sources of this key herbivorous zooplankton in Lake Vesijärvi [26]. However, the size and form of diatoms vary greatly, and they have silica frustules that might be difficult for *Daphnia* to ingest. Therefore, digestibility of diatoms varies greatly. Furthermore, diatoms can form large colonies and blooms, which are not ingestible for zooplankton, resulting in poor utilization of the diatom-produced EPA. Moreover, previous studies have shown that the EPA content of different species and by habitat is highly variable [13,39,40].

Even though ω-3 and ω-6 PUFA profiles of cyanobacteria and green algae differ at some level, the biomass and cell content of these PUFA did not differ markedly but were clustered together in NMDS output. This results from the fact that both phytoplankton groups grow fast and have a high ALA and LIN content. Our results also showed that these two groups were superior in producing ALA and LIN, which is one reason why they have been used for biofuel production. However, in terms of efficient transfer of these medium-chain PUFA in aquatic food webs, phytoplankton need to be digestible for zooplankton, and zooplankton need to have the ability to bioconvert EPA or DHA from ALA or ARA from LIN. Generally, it has been assumed that zooplankton does not feed on especially large-sized cyanobacteria, whereas other studies suggest that zooplankton can feed on cyanobacteria [34,35]. In Lake Vesijärvi, cyanobacteria (e.g., *Planktothrix*, *Snowella*, *Aphanizomenon*, *Microcystis*) can form blooms that can last throughout summer, as were seen in 2016. According to the fatty acid-based modeling [26], cyanobacteria formed less than 10% of the diet of *Daphnia,* and when the model uncertainties were considered, it could be noted that cyanobacteria were an insignificant diet source for *Daphnia*. Therefore, it seems that cyanobacteria may contain much of ALA and LIN but remain an inaccessible resource for zooplankton. Secondly, it should be noted that *Daphnia* has a poor ability to bioconvert EPA from ALA [41,42].

Here, we focused on phytoplankton phylogeny and growth phase and were unable to extrapolate the environmental conditions' impact on production of ω-3 and ω-6 PUFA, since we converted phytoplankton biomass to fatty-acid availabilities in our field data. However, our field data showed a positive relationship between total phosphorus and ALA, LIN, and GLA production by cyanobacteria, whereas increased temperature and total nitrogen was related with the production of SDA, EPA, DHA, and ω-6 DPA by cryptophytes, golden algae, and dinoflagellates. In addition to changes in phytoplankton composition, environmental conditions can affect phytoplankton PUFA content [43–45], and thus, potentially, also their production. Our recent study [46] with 107 boreal lakes showed that intensified eutrophication decreases the nutritional value of phytoplankton. The high difference in temperature between freshwater and brackish and marine phytoplankton strains resulted in a 10-fold difference in the production of EPA [39]. Another study [43] with green algae, cryptophytes, and diatoms showed that the light and temperature increase (from 10 to 25 °C) have a relatively minor impact on PUFA content in green algae. Surprisingly, in our study, slow growing cryptophytes and golden algae had higher EPA contribution in stationary phase, whereas fast growing diatoms and slower growing dinoflagellates and synurophytes had higher EPA contribution in the exponential

than in the stationary phase. The same trend was also seen in the biomass and cell PUFA content of cryptophytes, dinoflagellate, and diatoms, excluding *Diatoma*, which had minimal PUFA content in the stationary phase. However, the effect of the growth phase on the EPA production of cryptophytes, golden algae, dinoflagellate, and diatoms was ambiguous, showing that the production of EPA can vary within phytoplankton groups. The growth phase had a small impact on the *Peridinium* biomass and cell EPA content, but *Peridinium* had two times higher DHA content per cell and production of DHA in stationary than in exponential phase. The contribution of ALA and SDA of green algae was higher in stationary than in exponential phase; however, the biomass and cell content and the production of ALA and SDA varied greatly by green algae strains. The growth phase affected the contribution, content, and production of ALA and SDA differently. Altogether, it seemed that the growth phase together with the environmental parameters could affect PUFA content and production of freshwater phytoplankton.

Based on their capability to overcome and adapt to environmental constrains, phytoplankton can be categorized into functional groups [44,45,47]. Functional classification may include growth and morphometric traits that determine how easily a phytoplankter is eaten by a consumer [47]. This could be an important approach, because it includes both environmental conditions and phytoplankton physiological traits, and modern food web models typically use functional rather than phylogenetic phytoplankton inputs [48]. Here, we focused on growth rate and cell size; however, future studies might benefit from using phytoplankton strains from different functional groups.

5. Conclusions

In conclusion, for understanding the synthesis and transfer of ω-3 and ω-6 PUFA, calculations of PUFA content per phytoplankton cell are beneficial in addition to biomass content. Our results showed that phytoplankton PUFA per biomass content varies from the cell PUFA content due to the positive impact of cell size on PUFA content. Therefore, larger cells have a higher PUFA content than smaller cells, but too large cells are not digestible for herbivorous zooplankton, and subsequently, are not utilized or transferred in the freshwater food web. Our laboratory culturing emphasized that different ω-3 and ω-6 PUFA are synthesized by certain phytoplankton taxa. Extrapolation on field phytoplankton data demonstrated how the availability of PUFA differed inter- and intra-annually. Dinoflagellates were superior producers of DHA, whereas diatoms and cryptophytes were crucial producers of EPA in boreal lakes. Our results also demonstrated that phytoplankton PUFA content and production varied by growth phase; however, this change is difficult to predict due to the high variation between strains within the same phytoplankton groups.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/10/4/559/s1, Figure S1: Development of phytoplankton biomass (as mg C L^{-1}) in L. Vesijärvi (data from the database of Finnish Environment Institute) and derived PUFA availability per liter. Figure S2: Development of phytoplankton biomass (as mg C L^{-1}) in L. Vesijärvi (data from the database of Finnish Environment Institute) and the derived PUFA availability per phytoplankton carbon content. Table S1: FA profiles of cultured phytoplankton strains.

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Review

Fatty Acids of Marine Mollusks: Impact of Diet, Bacterial Symbiosis and Biosynthetic Potential

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Abstract: The n-3 and n-6 polyunsaturated fatty acid (PUFA) families are essential for important physiological processes. Their major source are marine ecosystems. The fatty acids (FAs) from phytoplankton, which are the primary producer of organic matter and PUFAs, are transferred into consumers via food webs. Mollusk FAs have attracted the attention of researchers that has been driven by their critical roles in aquatic ecology and their importance as sources of essential PUFAs. The main objective of this review is to focus on the most important factors and causes determining the biodiversity of the mollusk FAs, with an emphasis on the key relationship of these FAs with the food spectrum and trophic preference. The marker FAs of trophic sources are also of particular interest. The discovery of new symbioses involving invertebrates and bacteria, which are responsible for nutrition of the host, deserves special attention. The present paper also highlights recent research into the molecular and biochemical mechanisms of PUFA biosynthesis in marine mollusks. The biosynthetic capacities of marine mollusks require a well-grounded evaluation.

Keywords: fatty acids; mollusks; symbiotic bacteria; biosynthesis

1. Introduction

The importance of fatty acids (FAs) in marine environments commonly focus on polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and, to a lesser extent, arachidonic acid (ARA, 20:4n-6), which are vitally important not only to human health but also to health and survival of marine and terrestrial organisms. They are derived from two metabolically distinct n-3 and n-6 FA families. The metabolic precursor of EPA and DHA is α-linolenic acid (ALA, 18:3n-3), whereas linoleic acid (LA, 18:2n-6) is the metabolic precursor of ARA. It is common knowledge that animals and humans cannot synthesize both n-3 and n-6 PUFAs de novo. Nevertheless, they are required for normal development, growth and optimal health. They can be produced endogenously by humans, but the rate of their biosynthesis is too low to satisfy the physiological requirements. Thus, n-3 and n-6 PUFAs are considered as essential for important physiological processes and must be supplied in the diet. The beneficial effects of n-3 and n-6 PUFA supplementation in diets have been well established both for humans and for marine animals.

The major sources of n-3 PUFAs are aquatic food webs [1–3]. They play a key role in biological processes and are among the most important molecules transferred via the plant–animal interface in aquatic food webs. According to generally accepted views, PUFAs are produced de novo mainly by unicellular phytoplankton and seaweeds and further transferred from primary producers to consumers on the following trophic levels of the marine food chains [4]. The most physiologically important EPA and DHA are accumulated within aquatic ecosystems, as they are transferred to animals that can be consumed by humans. Numerous studies have shown the relationship of the FA composition of consumers and food consumed, and, therefore, FA can be used as efficient and useful biomarkers for the study of trophic interactions between organisms in aquatic ecosystems [5,6].

However, information about the endogenous mechanisms of marine invertebrates responsible for synthesis of n-3 and n-6 PUFAs is still being accumulated. Recent researches have shown the potential of some marine mollusks for endogenous synthesis of long chain PUFAs (LC-PUFAs) [7–9]. Based on the transcriptome and genome sequences, as well as various publicly available databases, a number of novel fatty acyl desaturases (*Fad*) and elongations of very long-chain fatty acid (*Elovl*) genes have been identified from the major orders of the phylum Mollusca, suggesting that many mollusks possess most of the required enzymes for the synthesis of long chain LC-PUFAs [10]. The question whether these findings of the desaturase sequences in invertebrate species really cast doubt on the idea that the organic matter is transferred along the food chains, and thus the existence of trophic links between primary producers and consumers and the relationship of the FA composition of animals and the FA composition of food, are currently under discussion [8].

Mollusk FA have attracted the attention of researchers that has been driven by their critical roles in aquatic ecology and in trophic food webs, as well as by their importance as sources of essential FAs with important impacts on human health [11]. Among marine animals, mollusks are especially important as a source of PUFAs (after fish). Many members of the phylum Mollusca, commonly known as clams and snails, are traditional seafood items in human diets, and rich in essential PUFAs. The edible mollusks are commercially harvested and cultured [12]. Marine bivalve mollusks are highly appreciated, partly because of their positive effects on human health arising from their constituents—highly valued n-3 LC-PUFA—and so their consumption is increasing every year [13]. The mollusks represent different trophic levels, trophic groups, and differ by various dietary habits. To date, extensive data has been accumulated on mollusk FAs. The great diversity of mollusks is accompanied by their wide chemodiversity because of their trophic preferences and defense modes, as well as the biosynthetic capacities that influence their chemical composition.

The main objective of this review is to focus on the most important factors and causes determining the biodiversity of the mollusk FAs, with an emphasis on the key relationship of these FAs with the trophic sources and the food spectrum, rather than to make a complete description of the FA composition of the known mollusk species. The marker FAs of the trophic sources are also of particular interest. The discovery of new symbioses involving invertebrates and bacteria, which are responsible for nutrition of the host, deserves special attention. The present paper also highlights recent research into the molecular and biochemical mechanisms of PUFA biosynthesis in marine mollusks. The biosynthetic capacities of marine mollusks require a well-grounded assessment.

2. Importance of Essential Polyunsaturated Fatty Acids for Human Health

FAs are involved in several biochemical pathways and, being an important source of energy and components of cell membranes, are responsible for determining their structure, functions and cell signaling [14]. They ensure fluidity of the lipid bilayer, selective permeability and flexibility of cellular membranes, and are responsible for the mobility and function of embedded proteins and membrane-associated enzymatic activities [15].

Many biological actions of PUFAs are mediated via bioactive lipid mediators produced by fatty acid oxygenases and serve as endogenous mediators of cell signaling and gene expression that regulate inflammatory and immune responses, platelet aggregation, blood pressure and neurotransmission [16]. They support the physiological functions as homeostatic mediator [17]. PUFAs n-6 and n-3 are precursors of signaling molecules with opposing effects. ARA is converted to prostaglandins, leukotrienes and lipoxins, whose effect is predominantly pro-inflammatory. In contrast, EPA- and DHA-derived eicosanoids have chiefly an anti-inflammatory effect. PUFAs n-3 exhibit the most potent anti-inflammatory effects that helps to control inflammation underlying many chronic diseases, including atherosclerosis, coronary heart disease, diabetes, rheumatoid arthritis, cancer and mental health [18]. A large number of epidemiological studies and clinical trials suggest a beneficial relationship between n-3 PUFA consumption and reduced inflammatory symptoms. So, EPA and DHA are capable of partly inhibiting inflammation reactions, including leukocyte chemotaxis, adhesion molecule expression, leucocyte–endothelial adhesive interactions, production of

inflammatory cytokines, and T cell reactivity [19]. Low intake of dietary EPA and DHA is associated with increased inflammatory processes, general cardiovascular health and risk of the development of Alzheimer's disease, as well as with poor fetal development, including neuronal, retinal and immune function [11,20,21]. Low maternal DHA intake may also cause increased risk of early preterm birth and asthma in children [22,23].

Many beneficial cardiovascular effects have been ascribed to PUFAs, including hypolipidemic, antithrombotic, antihypertensive, anti-inflammatory and antiarrhythmic properties, as well as the reduction of blood pressure [24]. The effectiveness of n-3 PUFAs for the prevention of cardiovascular diseases (CVD) is based on multiple molecular mechanisms, including membrane modification [25,26] where n-3 PUFAs are incorporated into lipid bilayer and affect membrane fluidity, formation of lipid micro-domains and also mechanisms such as attenuation of ion channels, regulation of pro-inflammatory gene expression and production of lipid mediators [27,28]. The use of n-3 PUFAs is recommended for ameliorating the CVD risk factors [11,29].

DHA, the dominant n-3 FA in the brain and retina, plays an important role in neural function, exhibits neuroprotective properties and represents a potential remedy against a variety of neurodegenerative and neurological disorders [30,31]. The potentially beneficial effect of DHA in preventing or ameliorating age-related cognitive decline has been revealed in a clinical study [30]. The n-3 LC-PUFAs exert positive effects on memory functions in healthy elderly adults [21] and support the neurological development of the infant brain [32,33]. Consumption of n-3 LC-PUFAs, particularly DHA, may enhance cognitive performance relating to learning, cognitive development, memory and rate of fulfilling cognitive tasks [34]. EPA and DHA play a critical role in neuronal cell functions and neurotransmission, as well as in inflammatory and immune reactions that are involved in neuropsychiatric disease states. Most experimental and epidemiological studies show the beneficial effect of n-3 PUFAs in various neurological and psychiatric disorders [35]. A diet supplemented with n-3 PUFAs exerts positive effects on brain structure and function in healthy elderly adults [36].

Several studies have confirmed that n-3 PUFAs possess a potential for prevention and therapy of several types of cancers and, moreover, they can improve the efficacy and tolerability of chemotherapy [37,38]. According to other studies, n-6 PUFAs, vice versa, induce progression in certain types of cancer [38]. Epidemiological and experimental studies have found a relationship between a PUFA-supplemented diet and the development of some types of cancer, including colon and colorectal carcinoma, breast cancer, prostate cancer, as well as lung cancer and neuroblastoma [38]. The promising effect of n-3 PUFAs on certain types of cancer is explained by their ability to modulate membrane-associated signal transductions and gene expression involved in cancer pathogenesis, as well as to suppress systemic inflammation [39].

Dietary intake of these essential components, as substances with therapeutic action, may maintain health, prevent the development of many diseases and mitigate a number of pathological conditions. Supplementation of PUFAs at a rate of at least 1 g per day, either in capsules or by marine products, demonstrated a protective effect against cardiovascular disorders, hyper-triglyceridemia, hyperlipidemia, metabolic syndrome or type 2 diabetes [29].

3. Primary Producers of Polyunsaturated Fatty Acids in Marine Ecosystems

3.1. Microalgae

Each algal class is characterized by a specific FA profile. The occurrence of certain compounds can be used as an FA signature for different algal classes. Chemotaxonomic differences in FA may be useful in the estimation of the input of specific microalgae in the tracing of these components on marine food webs.

Members of Bacillariophyceae are abundant in aquatic habitats and are considered as the most important primary producers of n-3 LC-PUFAs in marine food chains. Diatoms frequently dominate in seasonal phytoplankton blooms and, accordingly, these algae are the most studied classes of microalgae in terms of their lipids and FAs. The FAs reported for different species of Bacillariophyceae are typical

for diatoms. The most abundant FAs are 20:5n-3 (it averages at 20–40% of total FA), 16:1n-7, 16:0, 14:0 and C16 PUFAs, 16:2n-4, 16:3n-4 and 16:4n-1, which account for about 80% of total FAs [40–44]. Hence, reliable markers of Bacillariophyceae have a high percentage of EPA, the predominance of 16:1n-7 over 16:0 and the presence of 16:2n-4, 16:3n-4 and 16:4n-1 along with low amounts of C18 PUFAs and DHA.

Dinophyceae species are major contributors to marine food webs and are second to diatoms as primary producers of organic matter in the oceans. They are especially abundant in coastal waters worldwide, where their exuberant growth, named algal bloom, is often observed. They are known as the main supplier of n-3 LC-PUFAs to marine animals. The more prominent FAs found in dinoflagellates are 16:0, 18:4n-3 (2.3–15.3% of total FA), 18:5n-3 (6.4–43.1%), 20:5n-3 (ranged from 1.8 to 20.9% in different species), and 22:6n-3, DHA (9.5–26.3%) [42,45–47]. Summing up the information on the FAs of this algal class, the high contents of 18:4n-3 and 22:6n-3 have generally been considered as useful signature compounds of dinoflagellates.

Green algae are classified into two classes, Chlorophyceae and Prasinophyceae, with their FA composition varying considerably. The most abundant FAs of the class Chlorophyceae are C18 and C16 PUFA isomers n-3 and n-6, of which, for example, 18:3n-3 reaches 43% of the total FAs [40,42,48]. The distinctive C16 PUFA isomers, 16:2n-6, 16:3n-3 and 16:4n-3, can be used in ecological studies as signature lipids to estimate abundance of these algae in phytoplankton or their input in the diet of invertebrates or transfer of these compounds into food webs. In general, the specific features of green algae are high concentrations of C16 PUFAs consisting of 16:2n-6, 16:3n-3 and 16:4n-3, and C18 PUFAs, such as 18:2n-6 and 18:3n-3, which are essential FAs and the precursors of metabolically distinct families of n-3 and n-6 PUFAs.

Eustigmatophyceae species contribute significantly to the organic matter of coastal waters in the Northern and Southern Hemispheres. Their FAs are dominated by three components, 16:0, 16:1n-7 and 20:5n-3, which together account for about 75% of the total FAs. In addition, an appreciable percentage of 20:4n-6 is detected (4–8.8%), whereas C18 PUFAs are present as minor components [42,49].

Cryptophyceae species are small marine or freshwater flagellates, which are abundant in some seasons and, hence, play an important role as food for invertebrates. A common characteristic of many cryptomonads is a very high proportion of n-3 PUFAs (up to 60–81.1% of total FAs) [42,50,51]. Among them, 18:4n-3 and 18:3n-3 are the most pronounced (together making up 40–50% of total FAs), but a high concentration of 20:5n-3 is also common (13–26%) [42,50,51]. Thus, the high percentage of 16:0, 18:4n-3, 18:3n-3 and 20:5n-3, along with a very low abundance of C16 PUFAs, is typical of most cryptomonads, which are considered as a highly valuable food source rich in n-3 PUFAs in aquatic ecosystems.

The class Prymnesiophyceae is divided into four orders, which have essential differences in lipid composition [42,52,53]. In general, the members of this class, similarly to diatoms, contain 14:0, 16:0, 16:1n-7 and 20:5n-3 as main components, but their distinguishing feature is the abundance of 18:4n-3 and 22:6n-3.

The FA profile of members of the Rhodophyceae is dominated by three major FAs, 16:0, 20:4n-6 and 20:5n-3, which together account for about 80% of the total FA [42,50]. It is worth noting that only red microalgae show a significant concentration of 20:4n-6 (up to 28%), which is a relatively rare or minor component in other classes.

Thus, the taxonomic differences in the FA composition between microalgae classes are obvious and each class is characterized by its specific FA profile. An FA analysis of microalgae has revealed signature compounds that may be useful to evaluate them as sources of different PUFAs. Uncommon FAs or groups of FAs may serve useful biochemical indicators in ecological studies. Chemotaxonomic differences, particularly those in terms of FAs, may be used for assessing the input of specific microalgae in the diet of animals.

3.2. Heterotrophic Protists

Another important source of PUFAs for marine mollusks is heterotrophic protists, zooflagellates and ciliates, constituting the links in the food web named the "microbial loop". Among marine heterotrophic nanoplankton, flagellates are the dominant group in terms of abundance, biomass and diversity [54], while flagellates, in turn, are consumed by ciliates in the food chain. Heterotrophic protists, flagellates and ciliates, similarly to microalgae, are responsible for the production of LC-PUFAs, which are essential for organisms at higher trophic levels in marine ecosystems. The marine ciliate *Parauronema acutum* is reported to contain a significant level of PUFAs: 18:4n-3 (9% of total FAs), 20:5n-3 (10%) and 22:6n-3 (5%) [55]. A similar pattern exists for marine free-living heterotrophic flagellates [56]. The ability of zooflagellates and ciliates to efficiently produce n-3 PUFAs, 20:5n-3, 22:6n-3 and 20:4n-6, was proven experimentally [56,57]. Thus, zooflagellates and ciliates that constitute links of the microbial loop can be a source of PUFAs for suspension- and deposit-feeding mollusks in marine ecosystems [56–58].

4. Biochemical Markers for Identification of Mollusk Feeding Patterns

Due to the great structural diversity of FAs and their substantial taxonomic specificity, the identification of characteristic FA patterns at different trophic levels allows estimation of relationships between primary producers and consumers of different trophic levels of a food web [5,59]. The current trend in lipid biochemistry is the use of FAs as biochemical markers for determination of animals' food sources and trophic relationships between species in aquatic communities [6,59–61]. The specificity of the FA composition of algae and microorganisms, which serve as food for consumers, are well documented (references for Section 3), and many of these FAs are transferred from prey to predators without modification [5,6,59]. This approach is based on the limited ability of animals to synthesize FAs, much of them animals receive from consumed food, particularly PUFAs, which can only be biosynthesized by microalgae and protozoa and become an essential dietary component for higher trophic levels. Potential food sources, such as diatoms, dinoflagellates, zooplankton and bacteria, have a distinctive FA composition with unique FAs or a specific FA ratio used as dietary tracers of mollusks (Table 1). For this reason, FAs are considered as biochemical markers, a very efficient and useful tool to provide information on the food spectrum and diversity of food sources for marine organisms and for studying food chains in marine ecosystems.

Table 1. Fatty acids as biomarkers of food sources for mollusks.

Fatty Acid Markers	Food Source	References
20:5n-3, 16:1n-7/16:0 > 1, 14:0, 16:2n-4, 16:3n-4, 16:4n-1	Diatoms	[41,42]
18:4n-3, 22:6n-3	Dinoflagellates	[46]
18:2n-6, 20:4n-6, 22:6n-3	Heterotrophic flagellates	[56,57]
22:6n-3, 18:1n-9	Animal material Meiobenthos	[59,62]
15:0, 15:1, *iso*-15:0, *anteiso*-15:0, *iso*-16:0, 17:0, *iso*-17:0, *anteiso*-17:0	Heterotrophic bacteria	[63–66]
16:0, 18:0, 22:0	Detritus	[67,68]
18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3, 20:4n-6, 20:5n-3	Brown algae	[69]
Very long-chain FAs: *iso*-5,9-25:2; 25:2Δ5,9; 26:2Δ5,9; 27:2Δ5,9; 26:3Δ5,9,19; 26:3Δ5,9,17; 27:3Δ5,9,19	Sponges	[70–72]
Tetracosapolyenoic acids: 24:5n-6, 24:6n-3	Soft corals	[73]

5. Fatty Acids of Marine Mollusks

Mollusks are extremely widely represented in the oceans, both in number of species and in density of populations. Of the seven classes of this phylum, Gastropoda, Bivalvia and Cephalopoda account for more than 95% of the mollusk species and are a major marine fishery resource. Plenty of information on the lipids and FAs of these classes, their commercial importance and, particularly, on their nutritional value as sources of n-3 PUFAs has been accumulated to date. In her review, Joseph emphasizes the

important influence of environmental and biological factors on FA for members of this phylum [74]. Currently, new data are collected, which make it possible to review the features of the mollusks' FAs and the impact of different factors on FA profiles. In this Section, we focus on the different diets of members of these classes, determining the principal differences in FAs between their species.

5.1. Gastropoda

The diet of gastropods, which are represented by the greatest number of species, differs according to the trophic group considered. According to the type of food, gastropods are generally divided into two groups: herbivorous and predators [75]. Their dietary specialization and trophic relationships are reflected in the FA composition of the species. Their trophic habits and food preferences influence the composition of their FAs, which can differ fundamentally for species with different diets (Figure 1).

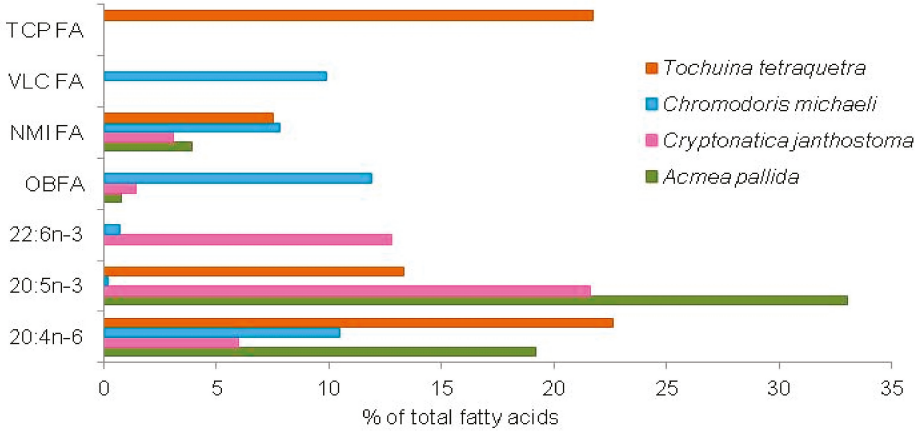

Figure 1. Distribution of fatty acids in gastropods with different types of feeding: herbivorous and carnivorous. Results are expressed as the mean [73,76,77]. TCP FA, tetracosapolyenoic fatty acid; VLC FA, very long chain fatty acid; NMI, non-methylene-interrupted; OBFA, odd-chain and branched fatty acids.

Evidently, the most primitive type of gastropod feeding involves browsing and grazing of algae from rocks. High percentages of 16:1n-7 and 20:5n-3, typical of diatoms, have been found in the pelagic pteropod *Limacina helicina* that inhabits Arctic and Antarctic waters, indicating a strong evidence of diatom ingestion [78]. The limpet *Acmaea pallida* feeds most frequently on brown algae, while *Lottia dorsuosa* feeding on filamentous and unicellular algae, scraping them from the surface of stones. Consequently, FAs of algal origin found in snails, such as 18:3n-3, 18:4n-3, 20:4n-6 and 20:5n-3, reflects a herbivorous feeding strategy (Table 2) [76]. Two intertidal grazers, *Patella aspera* and *P. candei*, also exhibit high levels of EPA and ARA [79]. Meanwhile, in lipids of carnivores, *Cryptonatica janthostoma* and *Nucella heyseana*, 22:6n-3 is dominant, as a result of their animal diet (Table 2). These species are known as consumers of mollusks, mainly bivalves [75]. The FA composition of limpets and snails is characterized generally by predominance of 20:5n-3 and 22:6n-3, which constitute usually 25%–35% of total FAs, being a rich source of n-3 PUFAs.

In contrast, sea slugs dot not have this specific feature; these two marine PUFAs are minor components and in sum do not exceed 1–3% of total FA. FA profiles of nudibranchs differ principally from those of other mollusks in the abundance of numerous very long chain FAs (VLC FAs) specific for marine sponges [70–72] or by the high portion of tetracosapolyenoic acids (TCP FAs), produced by octocorals (Figure 1) [73]. The opisthobranchs, including sea slugs, are predators on sessile animals, such as sponges, corals, bryazoans and ascidians. The majority of nudibranchs are predators on

sponges, and the occurrence of VLC FAs with double bonds at Δ5, 9, including 5,9–24:2, 5,9–25:2, 5,9–26:2 and *iso*-5,9–25:2, are certainly a result of feeding on sponges [77,80]. TCP FAs, 24:5n-6 and 24:6n-3, found in high proportions (each is more than 10% of total FAs) in the tritonid nudibranch *Tochuina tetraquetra*, originate undoubtedly from soft corals of the subclass Alcyonaria, which it feeds on [73]. FAs of the nudibranch *Armina maculate*, which feeds on a pennatulacean commonly named "Sea Pen", *Veretillum cynomorium*, constituted predominantly 16:0, 18:0, 20:4n-6 and 20:5n-3 (62% of total FA); thus, evidencing a similarity with the FA profile of "Sea Pen" represented by the same major compounds, whereas FAs of the cephalaspidean *Aglaja tricolorata*, presumably feeding on foraminiferans from sandy bottoms, is rich in EPA and DHA (27% of total FAs) [81]. Moreover, the studied nudibranchs exhibit one more specific feature: their lipids are rich in n-6 PUFA, and their level is much higher than that of n-3 PUFAs. Dorid nudibranchs, besides 20:4n-6, contain also 22:4n-6 and 18:2n-6 [77]. High values of n-6 relative to n-3 PUFAs are unusual for marine organisms and are reported mostly for snails grazing on brown algae, being rich in 20:4n-6 (Table 1) [74,76].

Table 2. Fatty acid composition of gastropod mollusks from the East Pacific (% of total FAs) [76].

Fatty Acids	*Acmea pallida*	*Lottia dorsuosa*	*Ischnochiton hakodadensis*	*Cryptonatica janthostoma*
14:0	0.4	4.6	4.5	3.4
15:0	0.3	1.0	0.5	0.6
16:0	5.9	13.9	13.2	6.4
16:1	0.8	5.8	3.8	2.8
17:0	–	0.5	–	–
16:3n-4	0.8	2.9	0.4	1.7
17:1n-8	0.4	–	0.4	0.8
18:0	6.3	4.5	–	8.8
18:1	13.2	15.9	15.7	3.6
18:2n-6	–	4.9	2.0	2.6
18:3n-6	–	–	0.5	0.2
18:3n-3	1.1	–	4.4	0.6
20:1	10.5	9.2	3.9	7.3
18:4n-3	1.1	2.2	1.7	0.5
20:2NMI	1.5	1.2	–	8.1
20:3n-6	1.6	0.9	3.5	4.2
20:4n-6	19.2	15.5	6.8	6.0
22:2NMI	3.9	4.2	3.6	3.1
20:5n-3	33.0	11.8	13.3	21.6
22:4n-6	–	0.7	4.2	1.1
22:5n-6	–	–	0.8	0.5
22:5n-3	–	0.9	4.4	2.6
22:6n-3	–	–	0.8	12.8

5.2. Bivalvia

Most mollusks from the phylum Bivalvia are known to be suspension-feeders, their diet consisting mainly of plankton from the water column, protists from the near-bottom water layer and deposit-feeders collecting food from the surface of bottom sediments. Thus, planktonic and benthic microalgae, zooplankton, protozoans, including heterotrophic flagellates and ciliates, and also bacteria from detritus are the main components of diet of filter-feeding bivalves [75]. This feeding mode and, consequently, the diet primarily impact the composition of the mollusk FAs (Table 3), which exhibit an abundance of EPA, DHA, and quite often, ARA [61,74].

Table 3. Fatty acid composition of bivalve mollusks from the East Pacific (% of total FA) [76].

Fatty Acids	Arcidae			Mytilidae			Ostreidae	Cardiidae	Veneridae				Mactridae		Pectinidae	
	Scapharca broughtoni	*Arca boucardi*	*Anadara maculosa*	*Mytilus edulis*	*Crenomitilus grayanus*	*Modiolus difficilus*	*Crassostrea gigas*	*Clinocardium californiense*	*Callista brevisiphonata*	*Saxidomus purpuratus*	*Protothaca jedoensis*	*Mercenaria stimpsoni*	*Spisula voyi*	*Mactra chinensis*	*Patinopecten yessoensis*	*Chlamys swifti*
14:0	0.6	0.6	2.7	2.1	3.9	2.7	3.0	2.0	0.6	1.8	2.8	7.8	6.9	3.1	3.0	4.2
15:0	1.1	0.4	0.3	0.7	0.5	0.5	0.8	0.9	0.5	0.4	0.4	0.6	0.6	0.6	0.5	0.4
16:0	10.2	9.2	13.3	14.8	16.6	14.9	14.9	12.9	13.5	11.0	10.9	14.8	12.4	15.0	11.2	13.7
16:1n-7	1.5	3.2	2.4	5.0	8.3	5.5	4.9	4.9	2.4	3.3	6.6	9.2	5.6	9.6	5.0	4.9
16:3n-4	3.1	1.2	4.2	0.8	1.5	2.0	2.1	1.8	1.0	0.1	2.4	1.4	1.9	1.8	1.1	0.3
17:1n-8	2.2	1.3	0.2	0.2	0.6	1.4	1.0	2.4	2.0	0.6	0.8	0.6	0.8	0.8	08	0.9
18:0	10.6	6.6	13.5	3.5	2.8	5.8	3.6	5.5	0.5	5.8	4.0	6.4	6.8	4.0	6.1	5.3
18:1n-7	5.7	2.8	4.7	3.6	5.7	6.6	12.1	7.1	0.3	4.2	6.8	3.2	4.9	6.0	6.1	8.2
18:2n-6	2.2	1.6	3.8	1.7	2.2	2.1	2.2	1.2	0.5	0.8	1.3	1.4	1.3	0.4	9.0	1.6
18:3n-6	0.1	–	0.5	0.1	–	–	0.3	0.6	0.4	0.1	0.2	0.6	0.2	0.3	0.2	0.5
18:3n-3	1.0	2.1	1.5	1.4	1.5	1.5	1.8	1.5	0.2	0.7	0.3	0.7	0.8	0.4	0.4	0.9
20:1	10.8	12.5	8.9	12.3	5.6	7.4	6.8	3.2	12.5	17.3	5.8	3.6	6.5	6.8	3.9	5.5
18:4n-3	0.6	1.4	0.8	1.8	2.7	2.1	3.1	1.2	1.3	2.7	1.0	4.5	3.7	2.7	2.6	4.5
20:2NMI	0.1	0.7	0.1	0.8	3.9	1.2	1.3	3.4	0.2	0.3	0.2	1.9	1.0	–	0.9	0.2
20:3n-6	0.1	–		0.8	1.7	–	–	1.2	0.5	0.7	–	1.1	1.5	1.4	0.1	0.7
20:4n-6	5.8	6.4	7.8	3.9	2.5	3.3	2.1	4.3	3.6	2.9	3.5	1.5	2.7	2.1	3.0	3.8
22:2NMI	20.7	12.8	12.7	4.6	4.6	3.8	4.7	6.1	6.0	0.7	6.5	1.9	1.7	0.6	0.7	0.6
20:5n-3	6.1	10.3	4.0	14.5	16.2	22.9	16.7	13.4	18.3	22.3	14.4	24.5	17.3	21.2	19.7	20.2
22:3n-6	1.2	1.3	0.4	1.5	0.8	1.3	1.0	1.4	1.6	1.5	1.6	1.6	1.4	1.4	0.9	1.1
22:4n-6	0.9	0.8	1.3	0.1	0.2	0.4	0.1	2.5	1.1	2.2	1.4	0.4	0.8	0.8	0.1	0.2
22:5n-6	1.3	1.4	2.3	0.7	0.4	0.6	0.4	1.1	1.6	1.1	1.1	0.3	0.9	0.9	0.6	0.6
22:5n-3	1.0	1.3	0.7	1.1	0.8	1.1	0.1	3.0	2.0	3.3	1.5	0.1	2.0	2.0	0.5	0.9
22:6n-3	14.2	22.4	13.1	23.0	15.3	12.0	16.0	16.7	19.5	15.2	24.2	12.3	17.4	17.4	18.5	21.2

Variations in the trophic environment and also the food selectivity of the species result in the dominance of the FA biomarkers of diatoms or dinoflagellates, zooplankton or detritus, or a combination of these sources. The DHA to EPA ratio reflects the proportion of zooplankton, diatoms and dinoflagellates in the bivalve's diet [6,82,83]. DHA often dominates in FAs of zooplankton and dinoflagellates [6,46,56,57], whereas EPA originates from diatoms [40–42]. A high concentration of 16:1n-7 and 20:5n-3, as well as a higher EPA/DHA ratio, suggests the importance of diatoms in the diet of the mollusks, whereas an elevated level of 18:2n-6, 20:4n-6 and DHA indicates the important contribution of microheterotrophs (flagellates and ciliates) in the diet. A higher proportion of odd-chain and branched FAs (OBFAs) is the evidence of the presence of bacteria in the diet of bivalves [84,85].

FA composition of the different taxa of marine bivalves from temperate waters of the East Pacific shows that their characteristic feature is a high abundance of n-3 PUFAs (Table 3). The concentration of both EPA and DHA reaches 25%, and ARA extends to about 8% of total FAs. FA composition varies from species to species, but n-3 PUFA are usually dominant. Furthermore, the high content of EPA and DHA shown in Table 3 is similar to the values obtained for the other species from different regions, for example, *Crassostrea angulata, Mytilus edulis, C. edule* and *Venerupis pullastra* from the coastal and estuarine systems of Portugal [86]; the oyster *Crassostrea virginica* [87] and sea scallop *Placopecten magellanicus* from the coast of Canada [88]; and the pod razor clam *Ensis siliqua* [89]. PUFAs, especially EPA (19–22% of total FAs) and DHA (20–32% of total FAs) were found to account for the majority of total FAs in tissues of the scallops *Patinopecten yessoensis* and *Chlamys farreri*, which provides an opportunity to use them as a potentially health-promoting food for human consumption [90]. Previous studies also reported the dominance of these PUFAs in tissues of *P. yessoensis* [84] and *Pecten maximus* [91].

In addition, spatio-temporal intraspecific variations in mollusk FAs are observed. So, FAs of *Pecten maximus* showed strong differences between individuals from shallow and deep-water habitats. This trend was driven by the content of marker FAs of diatoms, which are abundant near coasts. Scallops from deeper habitats are characterized by higher contents of flagellate FA markers compared with scallops from shallow habitats that emphasize the variability of the FA content according to the diet of this species along its distribution range [91]. FA biomarkers (Table 1) explain the spatial and temporal heterogeneity in nutrient sources for mollusks. The pattern of spatial and temporal variations of the biomarker FAs in the bivalve *Spondylus crassisquama* [83] and *Mytilus galloprovincialis* [92] revealed the nature and origins of food sources for these bivalves. Species-specific feeding adaptations to environmental variability of two bivalves, the clam *Callista chione* and the cockle *Glycymeris bimaculate*, from two shallow sites of the coastal oligotrophic Mediterranean Sea are revealed. The species demonstrate the differences in FAs mainly due to EPA and DHA percentage during the seasons. FA markers revealed a mixed diet where *Callista chione* fed more upon fresh material (diatoms and zooplankton) than *Glycymeris bimaculate*, which relied largely on bacteria-derived detritus [85].

Pinna nobilis, endemic to the Mediterranean Sea, is known to ingest different food items depending on its shell size. As a result, small-sized *P. nobilis* are associated with a detrital food chain characterize by saturated FAs (38%) and OBFAs (9.9%), while the diet of large- and medium-sized individuals have a greater proportion of PUFAs (EPA from 13% to 22% and DHA from 13 to 44% of total FAs). Thus, FA composition of the species reflects a lower contribution by markers of detritus and an increasing contribution of phytoplankton and zooplankton with increasing shell size [93].

5.3. Cephalopoda

Compared to data on lipids of gastropods and bivalves, information on cephalopods is not as abundant. Nevertheless, it is evident that their FA composition, similarly to that in gastropods and bivalves, is dietary dependent [94,95]. They inhabit pelagic ecosystems and are active predators preying on a variety of fish and invertebrates, such as crustaceans and mollusks. Their diet varies between species and is affected by gender, size, sexual maturity and season of year [96]. Cephalopods are generally known to be consumers of higher trophic levels, or top predators, actively accumulating n-3 PUFAs, EPA and, in particular, DHA in their tissues, which, are transferred up food chains from primary producers

and ingested with their food [94]. They are excellent sources of n-3 PUFAs, especially EPA and DHA. An FA analysis of the most commonly consumed cephalopods, such as common cuttlefish *Sepia officinalis*, European squid *Loligo vulgaris*, common octopus *Octopus vulgaris* and musky octopus *Eledone moschata*, showed the dominance of DHA (21–39% of total FA), EPA (8–17%), ARA (1.5–12%), as well as saturated 16:0 (16–25%) and 18:0 (4–10%) during the seasons [97].

The FA composition of the mantle and digestive gland differed markedly between the squid species. The digestive gland is rich in monounsaturated FAs whereas the mantle contains high concentrations of PUFAs, particularly DHA (about 40% of total FAs) (Figures 2 and 3). These findings imply that the squid, as a top predator, actively concentrates EPA and, in particular, DHA in the tissues from the diets. Published data show a similarity in FA of mantle tissue between various species from the different geographic regions (Figure 3), including *Nototodarus gouldi*, inhabiting the tropical and temperate waters of Australia and New Zealand [98], and *Moroteuthis ingens*, an endemic species to the Southern Ocean, having a circumpolar distribution in the sub-Antarctic [99]. Meanwhile, FAs of the digestive gland of squids differ significantly between species (Figure 3), largely reflecting the variety of diet consumed, e.g., [95]. An FA analysis, frequently applied in dietary studies of cephalopods, indicates that the digestive gland is an accurate source of dietary tracers [95], thus revealing a recent history of dietary intake [94,99].

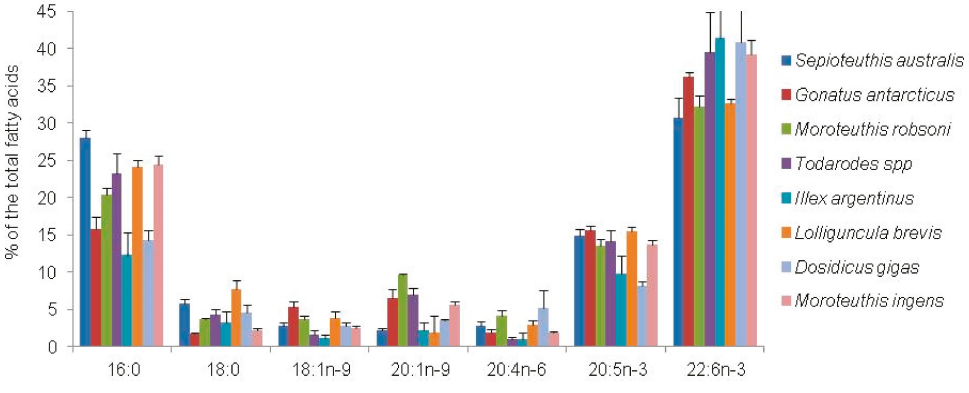

Figure 2. Major fatty acids (% of total FAs) in the mantle of squids. Values are mean ± standard deviation (SD) [94,95,99–101].

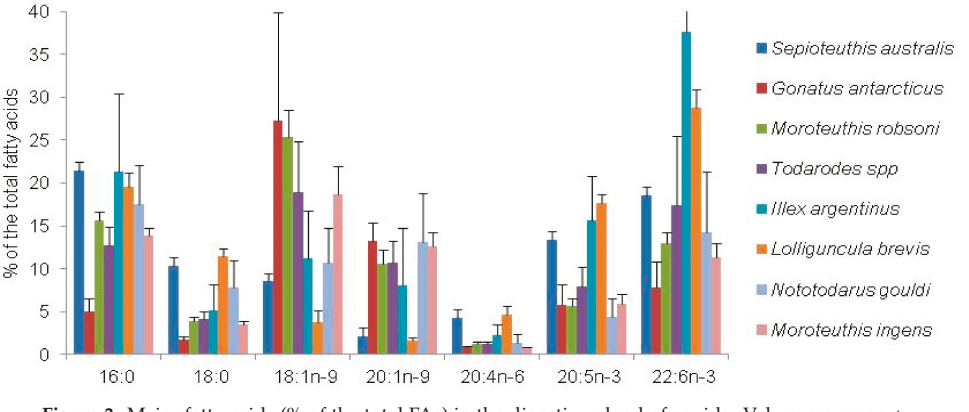

Figure 3. Major fatty acids (% of the total FAs) in the digestive gland of squids. Values are mean ± SD [94,95,98,99,101].

A combination of stomach content and FA signature analyses provided clear evidence of seasonal shifts in prey composition of the arrow squid *Nototodarus gouldi* and suggested temporal variations in its diet. Additionally, FA analyses show dietary differences related with gender, size and maturity of females. According to these relationships, the diet of *N. gouldi* is closely associated with prey size, abundance, availability and, possibly, to life-history stages [98]. The spatial variations in diet are believed to result in the differences in FA profiles of the digestive gland of the onychoteuthid squid, *Moroteuthis ingens*, from four different areas of the Southern Ocean. The FA analysis indicates that crustaceans are an important prey for smaller squid, whereas fish constitute a major portion of prey of larger squid [94]. Moreover, on the example of the jumbo squid, *Dosidicus gigas*, it was shown that an FA analysis can trace the geographic origin of squid individuals [100].

The FA biomarker concept has proven to be useful in the study of energy sources for reproduction in the squid *Illex argentines*. It was found that the FA composition of ovaries shows a more pronounced correlation with that of digestive glands than with the mantle, an energy reserved organ that reflects the dietary intake. The similarity in FA composition between the ovaries and the mantle during the early maturation and spawning period indicates that during these two periods, the somatic energy reserve is involved in reproductive growth. Thus, the potential implication of FAs is useful to provide insights into the breeding strategies among cephalopods [101].

Octopus species consume mainly mollusks, crustaceans, fishes and, sometimes, smaller species of *Octopus* as supplementary dietary components. Significant differences between FAs of the tissues of common octopus *Octopus vulgaris* are evident [102]. Among PUFAs, ARA, EPA and DHA are present at high concentrations in all tissues. ARA is more abundant in the digestive gland compared to muscles (11.4 and 7.9%, respectively), DHA dominates muscle tissues (20.7 and 14.0%) and the percentage of EPA is similar in these tissues (about 15% of total FAs). In contrast to squids, monoenoic FAs are not the main components in octopus, but saturated FAs, 16:0 and 18:0, are prominent in all tissues. Furthermore, the muscles contain more 16:0 compared with the digestive gland (20.1 and 12.4%, respectively) and 18:0 content is similar in these tissues (about 12%) [102].

Cuttlefish is an inhabitant of the seafloor that ambushes small animals such as crabs, shrimps, fishes and small mollusks. Feeding experiments have demonstrated that the FA profile of the digestive gland of the cuttlefish, *Sepia officinalis*, reflects the FA profile of its prey. Cuttlefish that had been fed fish showed comparatively high levels of fish-derived signatures, and this dietary dependence was also found for cuttlefish fed on crustaceans [103]. The proportions of the specific prey FAs are mirrored on the animal FAs. The major FAs in the cuttlefish mantle are DHA, EPA, 16:0 and 18:0 [97].

Thus, cephalopods, being top predators, actively accumulate n-3 PUFAs, EPA and, in particular, DHA in their tissues, coming from their prey, and therefore are valuable marine sources containing high levels of DHA, EPA and a noticeable level of ARA.

6. Contribution of Symbionts to the Fatty Acid Pool of Mollusks

Symbiotic associations between mollusks and microorganisms are widespread; they result in unique ecological strategies and increased metabolic diversity of the partners (Table 4). Symbiotic microbes typically supply nutrients to host animals that provide the microbes with shelter.

Table 4. Symbiotic microbes in marine mollusks.

Type of Nutrition	Symbionts	Function	Host	References
Chemotrophic	Bacteria	Nutritional	Bivalves and gastropods	[104]
Phototrophic	Zooxanthellae	Nutritional	Giant clam *Tridacna squamosa*, Gastropod *Strombus gigas*	[105]
	Algal chloroplasts	Nutritional	Sea slug *Elysia chlorotica*	[106]
	Chlorella	Nutritional	Clams, e.g., *Anodonta*	[107]
Heterotrophic	Bacteria	Nutritional	Bivalve shipworm *Bankia setacea*	[108]
		Light production	Squid *Euprymna scolopes*	[109]
		Chemical defense	Sacoglossan *Elysia rufescens*	[110]

Mollusks inhabit a variety of marine ecosystems. In environments characterized by poor nutrient contents, alternative strategies for nutrition have evolved. For example, some marine invertebrates, including mollusks living near deep-sea hydrothermal vents, cold seeps, on whales, wood falls on the deep-sea floor and shallow-water coastal sediments, derive their nutrition from chemoautotrophic microbes housed in their tissues and specialized structures [104]. The occurrence of symbiotic microbes with invertebrates that fix carbon dioxide autotrophically and synthesize organic compounds that are passed on to the host, play a critical role in establishing the lipid composition of the animals. Bacteria are known to produce various odd and branched FAs (OBFAs) named "bacterial acids" (Table 1). Additionally, *cis*-vaccenic acid, 18:1n-7, is biosynthesized by the anaerobic pathway unique for bacteria [111]. These FAs are widely offered as an indicator of the bacterial input in marine environment. Accordingly, elevated concentrations of the specific bacterial FAs, such as OBFAs, 16:1n-7 and 18:1n-7, coupled with a considerable reduction in n-3 and n-6 PUFAs produced by algae in lipids of the animals suggest a contribution of bacteria to the mollusk nutrition.

FAs have been used as a biomarker to reveal symbiotic relationships between bacteria and the bivalve mollusks *Solemya velum* [112], *Pillucina picidium* [113] and *Axinopsida orbiculata* from a shallow-water hydrothermal vent ecosystem of Kraternaya Bay [114], as well as the nudibranch *Dendrodoris nigra* [115]. These animals exhibit a high percentage of monoenoic FAs (about 40% of total FAs) mainly due to 18:1n-7, low concentrations of n-3 and n-6 PUFAs and an increased level of dienoic NMI FAs (Figure 4). In contrast, lipids of filter-feeding mollusks are dominated by 20:5n-3 and 22:6n-3, accounting for one-third of total FAs (Figure 4) [113]. A gastropod species, *Ifremeria nautilei*, from the deep-sea hydrothermal vent systems of the West Pacific, harbors two types of bacterial symbionts: a high abundance of sulphide-oxidizing and a low abundance of methane-oxidizing bacteria. It results in the dominance of 18:1n-7 (about 25% of the total FAs), 16:1n-7 (20–40%), and 16:0 (up to 15%) in its lipids and a low content of EPA and DHA [116].

Unlike bivalves from hydrothermal vents, deep-sea species living near cold seeps contain neither n-3 nor n-6 PUFAs. Two cold-seep bathymodiolin mussels, *Bathymodiolus japonicus* and *B. platifrons*, contain n-4 and n-7 PUFAs (25–27% of total FAs), including 18:3n-7,10,13; 18:4n-4,7,10,13; 20:3n-7,10,13; and 20:4n-4,7,10,13 with the main 16:1n-7 and 18:1n-7 (up to 25% in sum) [117], because they host methane-oxidizing bacteria and survive independently of photosynthetic products. A unique FA composition was reported for the cold-seep vesicomyid clam, *Calyptogena phaseoliformis*, which houses sulfur-oxidizing bacteria. The major FAs found in this clam belong to the novel n-4 and n-1 NMI PUFAs, such as 20:3n-4,7,15; 21:3n-4,7,16, and 20:4n-1,4,7,15, with significant levels of 20:2n-7,15 and 21:2n-7,16 as n-7 NMI FAs [118]. Similar traits exhibit another species of vesicomyid clams, *Phreagena* (synonym *Calyptogena*) *soyoae* and *Archivesica gigas*, harboring symbiotic sulphide-oxidizing chemoautotrophic bacteria in their gills. They are common in deep-sea chemosynthesis-based communities in the North Pacific. An FA analysis confirmed the lack of n-3 and n-6 PUFAs in their composition and revealed

a high percentage of n-7, n-4 and n-1 NMI PUFAs. A comparison of FA compositions of various organs of the clams showed that the content of these NMI FAs in gills was much lower than that of other organs, it suggests that the biosynthesis of n-7, n-4 and n-1 NMI PUFAs occurs in tissues of vesicomyid clams [119].

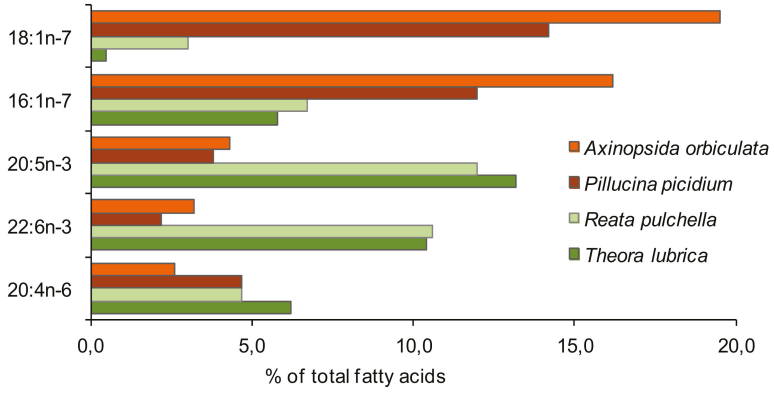

Figure 4. Distribution of the most remarkable of marker fatty acids of bacterial symbionts in the bivalve mollusks *Axinopsida orbiculata* [114], *Pillucina picidium* [113], containing sulfate-reducing symbiotic bacteria, and the symbiont-free bivalves, *Reata pulchaella* and *Theara lubrica* [113].

Thus, in contrast to shallow-water filter-feeding or grazing mollusks, which contain photosynthetic n-3 and n-6 PUFAs as the main components, mollusks with symbiotic chemoautotrophic bacteria show significant modification of FA composition: A decline or complete lack of essential n-3 and n-6 PUFAs and appearance of significant amounts and a variety of NMI FAs, which can probably by synthesized by mollusks using the bacterial FAs as precursors.

The specific FA composition of symbiont-containing species may give a hint of the character of symbiosis. Photosynthetic symbionts, such as dinoflagellates from the genus *Symbiodinium*, settle on corals and giant clams *Tridacna* and supply photosynthetically fixed carbon to their hosts, which contribute to their lipid composition [120,121]. Some of these compounds, such as 18:4n-3, 18:3n-3, 18:5n-3, 22:6n-3 and 16:0, being biomarkers of symbiotic dinoflagellates [46,122], are detected in host organisms. Metabolic interactions consist in exchange of nutrients between the host and its symbionts [120], providing them with a competitive advantage in tropical waters poor in nutrients.

By comparing FAs of the herbivorous limpet *Acmea pallida* and nudibranch species, it is obvious that a striking feature of the nudibranchs is the unusually high level of OBFAs specific for bacteria (Figure 5). It is evident that the share of total OBFAs, predominantly 15:0, 17:0, 17:1n-8 and *iso*- and *anteiso*-C15, C16, C17, C18, and C19, in *A. pallida* is 0.7% of total FAs, whereas in the *D. nigra* it reaches 18.6%, in *Chromodoris* sp. 15.8% and in *Phyllidia coelestis* exceeds 30% [80]. They are normally minor metabolites in marine invertebrates, but the level of these bacterial acids recorded from nudibranchs proved to be extraordinary. A high level of bacterial FAs in nudibranchs may serve, in our opinion, as an indicator that the symbiotic bacteria provide the host with nutrients. Indeed, transmission electron microscopy (TEM) confirmed the presence of rod-shaped Gram-negative symbiotic bacteria in the cytoplasm of epithelial cells and the glycocalyx layer covering the epithelium of the notum and the mantle of *D. nigra* [115]. Moreover, some bacterial OBFAs, such as 17:1n-8 and 19:1n-8, evidently, serve as potential precursors for the biosynthesis of odd-chain PUFA, such as 21:2Δ7,13 identified in nudibranchs [80].

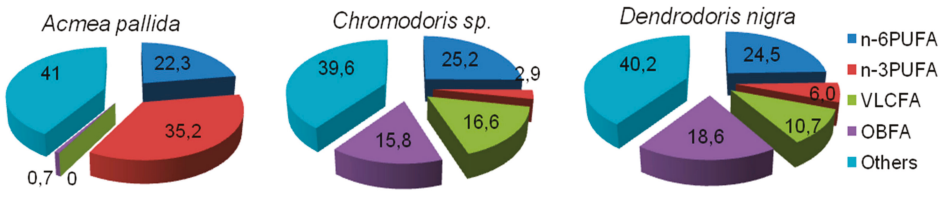

Figure 5. Distribution of fatty acids in the carnivorous nudibranch *Chromodoris* sp. [77], in *Dendrodoris nigra* [115] feeding on sponges and in the herbivorous limpet *Acmea pallida* [76] feeding on brown algae. *D. nigra* is known to harbor symbiotic intracellular bacteria [115].

7. Biosynthesis of Fatty Acids in Mollusks

Biosynthetic pathways of PUFAs are described in detail in many articles and reviews. In brief, monoenoic FAs, such as 18:1n-9 and 16:1n-7, are produced through the action of Δ9 fatty acyl desaturases. This activity is ubiquitous and found in all living organisms. Oleic acid, 18:1n-9, can be further desaturated through the action of n-6 or Δ12 desaturase to form, first, LA; then n-3 or Δ15 desaturase converts LA into ALA. Until recently it was believed that only plants are able to produce de novo LA and ALA, which are essential for animals [1,4,8]. The enzymes involved in LC-PUFA biosynthesis, namely, Δ8, Δ6, Δ5 and Δ4 desaturases, necessary for the production of EPA and DHA from 18:3n-3, have been described in algae [123].

Hitherto, there was a concept that the mollusks, as well as other marine invertebrates, are not able to synthesize n-3 and n-6 PUFAs de novo to satisfy physiological needs, and PUFAs in the marine invertebrates are derived exclusively from phyto- and zooplankton. This opinion was confirmed by experimental data on the incorporation of ^{14}C-acetate in FAs of the yellow clam *Mesodesma macroides* [124,125]. Similarly, in the experiments with juvenile oysters, *C. gigas* fed algae that had previously been cultured with labeled ^{14}C-acetate confirmed that dietary FAs are incorporated directly into oyster lipids, mostly in a unaltered form, and only less than 1% of the ^{14}C-label was found in 20:5n-3 and 22:6n-3 [126]. Recently, it was shown experimentally that the majority of radioactivity from ^{14}C-FAs incorporates into lipids of *Octopus vulgaris* paralarvae [127] and *Sepia officinalis* hatchlings [7], found as unmodified FAs with elongation being the only metabolism detected, and no desaturation activity towards the FAs was recorded. Moreover, in the study of the FA biosynthesis by bivalves, *Scapharca broughtoni*, *Callista brevisiphonata* and *M. edulis*, the active incorporation of ^{14}C-acetate was found in saturated, monoenoic and dienoic NMI FAs, whereas the radioactive label in LC-PUFAs n-3 and n-6 families, as well as in their precursors, LA and ALA, was not detected. It has been found that NMI dienoic acids, 20:2Δ5, 11, 20:2Δ5, 13 and 22:2Δ7,13, 22:2Δ7,15, are the only PUFAs that mollusks are able to synthesize [128,129]. These FAs with isolated double bonds were suggested to be derived as a result of the action of Δ5desaturase and elongations. To produce these dienoic FAs, Δ5desaturase mediates the insertion of the double bond in 20:1n-7 and 20:1n-9, common for invertebrates [128]. Indeed, a fatty acyl desaturase (*Fad*) gene with Δ5 activity has been characterized both molecularly and functionally from the octopus *Octopus vulgaris* [102], the gastropod *Patella vulgata* [8], the abalone *Haliotis discus* [130], the noble scallop *Chlamys nobilis* [131] and the bivalve *Sinonovacula constricta* [132].

Thus, returning to the issue of the biosynthesis of FAs in mollusks, it should be notde that relying on the experimental data on radioactive acetate or FA incorporation it is proved that marine mollusks are not capable to synthesize de novo LA and ALA, and their long-chain homologues, EPA and DHA, which are essential acids and PUFAs, must be considered essential dietary nutrients.

With the development of genetic methods, interest in the issue of the capability of marine invertebrates to biosynthesize n-3 and n-6 PUFAs has risen and many noteworthy findings in this field have been made recently. The availability of gene sequences databases of multitude species of invertebrates contributes to understanding of the biochemical mechanisms of PUFA biosynthesis in marine invertebrates at a molecular level [8,9,133,134]. Using molecular genetics approaches, the *Fad* and elongation of very

long-chain FA (*Elovl*) genes have been characterized both molecularly and functionally, namely, isolated, identified, and expressed in the yeast heterologous hosts. It has been proven that multiple invertebrates, including representative of mollusks, the gastropod *Patella vulgata*, possess the endogenous capability to produce n-3 PUFAs de novo and further biosynthesize physiologically essential n-3 LC-PUFAs [8]. Among aquatic invertebrates, the biosynthesis of LC-PUFAs has been more extensively investigated in marine mollusks [10,102,132,133]. Cloning and characterization of functional diversity of *Fad* and *Elovl* involved in the PUFA biosynthetic pathway was carried out in the cephalopods *Octopus vulgaris* and *Sepia officinalis*, abalone *Haliotis discus* and bivalves, *Chlamys nobilis* and *C. angulata*, and the achievements were reviewed [9]. The capability of LC-PUFA biosynthesis of particular species has been established to depend upon the complement of the key enzymes required, *Fad* and *Elovl* [9].

Thus, the presence of the *Fad* and *Elovl* genes coding the critical enzymes participating in the PUFA biosynthesis in invertebrates can be considered as the biosynthetic potential of mollusks to endogenously produce PUFAs.

8. Dietary Source of PUFAs Versus Own Biosynthetic Capability of Mollusks

The use of genetic methods contributed to a significant advance in the search of genes of the enzymes involved in the PUFA biosynthesis. Does the discovery of desaturase genes in a number of metazoans that enable them to endogenously produce PUFAs, actually mean that we should revise the concept of microalgae as primary producers of PUFAs and the subsequent transport of organic matter to invertebrates up the food chains and dietary origin of the PUFAs? It is worth clarification whether data on the presence of *Fad* and *Elovl* genes in mollusks can contradict the concept of the dietary dependence of fatty acids of animals on the fatty acid composition of their food.

The number of studied mollusk species, which possess genes encoding enzymes important in the LC-PUFA biosynthesis, is quite limited. Furthermore, the distribution of ωx desaturase genes within particular taxonomic groups is non-uniform, and the ability of the production of LC-PUFAs has been established to vary greatly between different mollusk species and heavily depend on the complementation of the desaturase and elongase genes, as well as on their enzymatic activity [9]. For example, ωx desaturase genes are found in the freshwater mussel *Elliptio complanate* and the common octopus *O. vulgaris* [102,134], but not in the marine oyster *C. gigas* nor the mussel *M. galloprovincialis* [8]. The presence of different types of biosynthetic enzymes, as well as their different enzymatic specificity, suggests that the abilities of mollusks for PUFA biosynthesis vary among species. A similar conclusion follows from the studies of biosynthetic capacity of mollusks using radio-labelled precursors [7,126–129].

The mechanism of gene expression is known to be complex and depend on various factors. Genes usually interact with and respond to the organism's environment. *Fad* and *Elovl* genes are usually identified through a search for available sequence databases, and through an analysis of the distribution of ωx desaturase genes across the Phylum and functional characterization of the enzymes using a yeast heterologous expression system [8]. Marine ecosystems are highly rich in n-3 PUFAs produced by planktonic microalgae and, therefore, it can be assumed that the abundance of PUFA in the diet of mollusks may be among the environmental factors that can determine ωx desaturase gene expression. Probably, the genetically incorporated mechanism of PUFA n-3 synthesis is not implemented in mollusks, at least not to the full extent, since the amount of these vital components coming from food is sufficient for animals to provide their physiological and biochemical requirements.

However, in conditions of a PUFA deficiency in animals living in extreme environmental conditions, for example, in deep-sea ecosystems, there is no compensation for this deficiency by endogenous synthesis of PUFAs. As a result, a low content of PUFAs in mollusks or their absence is observed with the simultaneous compensatory increase in the number of NMI FAs [116,135–139]. There is ample evidence of the increase in NMI FAs with a shortage of PUFA [71,76,119,140]. A similar pattern is observed with the symbiosis of bivalves and chemoautotrophic bacteria, leading to a decrease in the PUFA and an increase in the NMI FA levels in the host [113,119,141]. This emphasizes the importance

of understanding the biosynthetic capabilities of invertebrates, as well as the importance of combining dietary and biosynthetic approaches to understanding the origin of mollusk fatty acids.

Thus, some mollusk species, similarly to the majority of other invertebrates, possess genes encoding desaturases and elongases involved in pathways of biosynthetic pathways of LC-PUFAs [8,9,132,133], although both their potential and functions remain to be clarified. However, the most species of marine mollusks are apparently not capable to carry out the synthesis of these FA to a sufficient extent to satisfy the physiological requirements. The dependence of the FA composition of the mollusks on food consumed shows that n-3 PUFAs should be considered essential FAs, since their endogenous production appears to be limited.

9. Variations in Fatty Acids in Response to Environmental Factors

Numerous studies explore the influence of biotic and abiotic factors on their FA composition. The majority of studies focus on the seasonal fluctuations in the lipid and FA compositions that are found to directly relate with the reproductive cycle [142] or correlate with changes in the mollusk diet which followed the seston dynamics during the seasons [82,143]. Other studies assess the relationship between FAs and water temperature fluctuations [144,145]. Meanwhile, the species differ in their sensitivity to environmental factors. Since the importance of nutrient quality in terms of FA composition has already been addressed above, in this section the emphasis is on the environmental factors, such as bottom sediments, water salinity, temperature and water pollution.

For benthic animals, the structure and composition of bottom sediments are the important characteristics of their habitat, being one of the key factors that determine the trophic potential of benthic epifauna. In muddy areas, a benthic community is composed mainly of diatoms, heterotrophic nanoflagellates and oligotrich ciliates, whereas in sandy areas, heterotrophic nanoflagellates, euglenoid flagellates, oligotrich ciliates and scuticociliates are dominant among protists [146]. For example, the FA compositions of the scallop *P. yessoensis* from two habitats, muddy and sandy, show pronounced differences pointing to different food availabilities. An elevated content of diatom biomarkers, 20:5n-3, 16:1n-7 and C16 PUFAs, indicates that scallops from a muddy bottom are more reliant on diatom sources. Scallops inhabiting a sandy site have higher amounts of biomarkers of flagellates, ciliates and invertebrate larvae, such as 18:2n-6, 20:4n-6 and 22:6n-3, compared to individuals from the muddy site. This suggests that a scallop's diet depends on food availability [84].

Water salinity is one of the most influential environmental factors especially in estuarine systems and intertidal zone, where its variations cause major physiological and biochemical stress for aquatic organisms. Various species show different tolerance to water salinity. Under salinity stress, the bivalves *Cerastoderma edule* and *Scrobicularia plana* reduce food consumption and physiological pathways; a decrease in PUFA content is observed in *C. edule*. They can store the FAs which are of high physiological importance by reducing their activity and energy consumption [147]. The authors attribute the observed variations in the FA composition, particularly the contents of n-3 PUFAs, NMI FAs and n-6 PUFAs in the littoral mussel *M. edulis* from two different habitats, presumably to the necessity to survive the frequent fluctuations in such environmental factors as water temperature and salinity [148]. The observed modifications in the membrane lipids of the mussel gills lead to a change in the physical state of the membranes, their fluidity and permeability, the functions of ion channels, enzymes and receptors, which ensures the normal functioning of the organism under fluctuations in sea water salinity [148].

Intraspecific variations in FAs are also found to occur in response to water temperature variations. Negative relationship was observed between the acclimation temperature and the unsaturation index of membrane lipids in the oyster *C. virginica*, according to the homeoviscous adaptation theory. These temperature-related changes are mainly due to the variations in EPA content in fast-growing oysters, and in DHA and EPA contents in slow-growing animals [145]. The blue mussel *M. edulis* and the oyster *C. virginica* showed an increased 20:4n-6 level in their tissues as temperature rose, suggesting an increased availability of this FA for eicosanoid biosynthesis during stress response [144].

Contaminants, including the wide variety of pesticides and heavy metal pollutants, increase in marine ecosystems as the results of development of industrial and agricultural activities, being a stress factor for marine organisms. Such contaminants, like heavy metals, were reported to influence feeding, growth, reproduction, cardiac activity, and maturation of bivalve mollusks [149]. These physiological changes lead to changes in the lipid and FA metabolism, while the EPA level is reduced due to exposure to metals and organic pollutants [150,151]. A stress response, manifested as a decrease in PUFA and NMI FA content, was observed in the mussel *M. galloprovinciales* exposed to cadmium and copper [149], as well as to polycyclic aromatic hydrocarbon contamination [152]. A lower value of EPA compared to the control was recorded from the bivalves *Cerastoderma edule* and *Scrobicularia plana* exposed to copper sulphate [153], *Mizuhopecten yessoensis* exposed to cadmium [150] and *Scrobicularia plana* from a habitat contaminated by dioxin and polycyclic aromatic hydrocarbons [154], which can be considered a possible biochemical and physiological consequence for these animals.

Thus, the influence of some factors is not as evident; it is explained by the masking effect of a more significant contribution of food to the FA composition of the organisms. It should be noted that despite the variations in the FA composition of the filter-feeding mollusks in response to biotic and abiotic factors, their specific features, such as EPA and DHA dominance, are retained unchanged.

10. Conclusions

PUFAs, especially EPA and DHA, are fundamental to the health and survival of marine and terrestrial organisms. Mollusk FAs play critical roles in aquatic ecology and trophic food webs, and also play an important role as sources of essential PUFAs, significantly contributing to human health. This review highlights that the extensive taxonomic biodiversity of mollusks accompanies a wide chemical diversity, since the trophic preferences, biosynthetic abilities and physiological requirements of mollusks effect their chemical composition. The review provides evidence of a trophic transfer of FAs from various food sources to marine mollusks, which further emphasizes the nutritional contribution of the FA composition of animals. The variation in FA distribution and abundance between mollusks of different taxonomic and trophic groups is estimated. Mollusks differ in their feeding strategy, divided into the following trophic groups: filter-feeding, gathering, carnivorous, and the symbiont contribution. Some mollusks give shelter to microbes that provide them with nutrients, and these enable animals to settle in nutrient poor environments. Mollusks, which rely completely on bacterial endosymbionts in their diet, have specific FA profiles rich in "bacterial FAs" and poor in PUFAs. In addition, the biosynthetic potential of mollusks influences the FA composition. Mollusks are capable of producing numerous NMI FAs, which are especially important in low nutrient environments. Based on experimental data on incorporation of radioactive acetate or FA into marine mollusks, it is proved that they are not able to synthesize de novo LA and ALA and their long-chain homologues, EPA and DHA. However, it has recently been shown that some mollusk species possess genes encoding desaturases and elongases involved in pathways of biosynthetic pathways of LC-PUFAs. The biosynthetic capacities of marine mollusks require a well-grounded evaluation.

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Comparison of Fatty Acid Contents in Major Lipid Classes of Seven Salmonid Species from Siberian Arctic Lakes

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Abstract: Long-chain omega-3 polyunsaturated fatty acids (LC-PUFA) essential for human nutrition are mostly obtained from wild-caught fish. To sustain the LC-PUFA supply from natural populations, one needs to know how environmental and intrinsic factors affect fish fatty acid (FA) profiles and contents. We studied seven Salmoniformes species from two arctic lakes. We aimed to estimate differences in the FA composition of total lipids and two major lipid classes, polar lipids (PL) and triacylglycerols (TAG), among the species and to evaluate LC-PUFA contents corresponding to PL and TAG in muscles. Fatty acid profiles of PL and TAG in all species were characterized by the prevalence of omega-3 LC-PUFA and C16-C18 monoenoic FA, respectively. Fish with similar feeding spectra were identified similarly in multivariate analyses of total lipids, TAG and PL, due to differences in levels of mostly the same FA. Thus, the suitability of both TAG and total lipids for the identification of the feeding spectra of fish was confirmed. All species had similar content of LC-PUFA esterified as PL, 1.9–3.5 mg g^{-1}, while the content of the TAG form strongly varied, from 0.9 to 9.8 mg g^{-1}. The LC-PUFA-rich fish species accumulated these valuable compounds predominately in the TAG form.

Keywords: arctic; Salmoniformes; long-chain polyunsaturated fatty acids; polar lipids; triacylglycerols; eicosapentaenoic acid; docosahexaenoic acid

1. Introduction

Long-chain omega-3 polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are known to be essential compounds for human nutrition, since they can modulate the functioning of cardiovascular and neural systems and general metabolism, being the precursors for the synthesis of diverse lipid mediators and directly affecting membrane properties [1–5]. Most international and national health agencies and foundations recommended personal consumption of 0.5–1.0 g of EPA+DHA per day for reducing the risk of cardiovascular diseases and other metabolic disorders [6–8]. Although a lot of potential sources of LC-PUFA are now being considered, natural fish populations are still the major source of these compounds for human nutrition [9,10]. Recent reviews showed the deficiency of the LC-PUFA supply with fish caught from natural populations and emphasized the potential negative influence of some global threats, like climate change, pollution,

eutrophication, etc. [10–13]. To challenge above threats and to sustain the LC-PUFA supply from natural populations, one needs to know how environmental and intrinsic factors affect fish fatty acid profiles and content, including those of EPA and DHA. Causes of variations of fatty acid composition and content in wild fish are still incompletely understood [10,14].

The ability of fish to deposit fat (lipids) in muscles varies from species to species and may be a crucial intrinsic factor [15]. According to their functions, lipids in fish, like in other animals, could roughly be divided into energy-reserve and membrane-structural groups [16,17]. Fish reserve lipids are primarily represented by triacylglycerols (TAG) and include mostly fatty acids that come from food sources. Fatty acid profiles of TAG are generally considered as valuable trophic markers due to their resemblance with fatty acid profiles of particular food sources [18]. In addition, the TAG fraction in fish can also contain high levels of monoenoic C16-C18 fatty acids that are intensively synthesized in so called "fatty" fish species to provide energy reserves. TAG molecules are accumulated either directly in muscle cells as droplets or in specific adipocytes which may be integrated in muscle tissues or form separate layers of adipose tissue.

The structural polar lipids (PL) that form fish cellular and intracellular membranes mostly comprise phospholipids [19,20]. As known, fatty acid composition of PL affects physico-chemical properties of cellular membranes. Hence, PL are considered to have conservative fatty acid profiles which slightly reflect that of diet. The essential omega-3 LC-PUFA are preferentially accumulated in PL fraction of muscle tissues due to their strong membrane-modulating properties. Thus, fatty acid profiles of the major lipid classes, TAG and PL, in fish muscles are different in general [19].

TAG content per mass unit of fish muscles is highly variable due to influence of many factors [15]. In contrast, PL content per mass unit of muscles is fairly constant [21]. Thereby, we hypothesize that PL specific content has a putative upper threshold, because amounts of PL molecules in tissue are likely determined by a volume of membranes.

Contents of omega-3 LC-PUFA in muscle tissue of diverse fish species greatly vary, approximately ~400-fold [22]. The question arises what part of this variation in total EPA and DHA contents is provided by TAG or PL variability? There is a basic assumption in the current literature that a major part of omega-3 LC-PUFA presents as acyl groups of membrane phospholipid molecules [11].

To evaluate contribution of the two major lipid fractions in total content of LC-PUFA in edible muscle tissue (filets) we studied seven commercial species of the order Salmoniformes that inhabit oligotrophic non-polluted lakes in Arctic Siberia. The fish species vary in their feeding habits and habitats and have different fat content in filets. Using data on these fish we aimed to compare distribution of fatty acids, including omega-3 LC-PUFA in total lipids and two major lipid classes: TAG and PL. Specifically, we aimed (i) to check if the fish species with various feeding spectra can be differentiated basing on FA profiles of total lipids, TAG or PL, (ii) to evaluate LC-PUFA content corresponded to TAG and PL classes in muscles, (iii) to range species according to their nutritive value for humans in respect of LC-PUFA content.

2. Materials and Methods

2.1. Sampling

Fish specimen of commercial sizes were collected during July 2017 from catches of local authorized fishers. Following sampling was done in accordance with the BioEthics Protocol on Animal Care approved by Siberian Federal University. The catches were from two oligotrophic arctic lakes, Sobachye and Pyasino. Sobachye Lake was previously characterized elsewhere [23]. Briefly, it is located at 69°01′ N 91°05′ E and has maximum depth of 162 m and area equal to 99 km^2. Pyasino Lake situates at 69°40′ N 87°51′ E, has average depth of 4 m and an area equal to 735 km^2 [24].

Whitefish *Coregonus lavaretus* (Linnaeus, 1758), non-identified form of whitefish *C. lavaretus*, round whitefish *Prosopium cylindraceum* (Pennant, 1784) and charr *Salvelinus drjagini* Logashev, 1940 were caught in Sobachye Lake; broad whitefish *Coregonus nasus* (Pallas, 1776), muksun *Coregonus muksun*

(Pallas, 1814) and inconnu *Stenodus leucichthys nelma* (Guldenstadt, 1772) were caught in Pyasino Lake. All studied fish species are of the Salmoniformes order. Numbers of samples, average individual sizes and weights, and main food sources for the studied fish species are given in Table 1. Stomach contents of all specimen were studied under a light microscope, and main food items were identified to a possible taxon level.

Table 1. The basic biological and sampling information on fish species (Salmoniformes order) from Siberian arctic lakes, 2017: *n*—number of sampled individuals; L—total length, cm (mean ± SE); W—total weight, g (mean ± SE); Food—items found in stomachs.

Common and Species Name	Lake	*n*	L	W	Food
Charr *Salvelinus drjagini*	Sobachye	9	608 ± 17	2371 ± 271	Fish (salmonids)
Whitefish *Coregonus lavaretus*	Sobachye	7	480 ± 23	1153 ± 167	Amphipods, mollusks, chironomid larvae
Muksun *Coregonus muksun*	Pyasino	8	492 ± 14	1271 ± 160	Ostracods, mollusks, chironomid larvae, detritus
Inconnu *Stenodus leucichthys nelma*	Pyasino	5	675 ± 86	3239 ± 1581	Fish
Broad whitefish *Coregonus nasus*	Pyasino	10	563 ± 17	1916 ± 183	Gastropods, detritus
Round whitefish *Prosopium cylindraceum*	Sobachye	7	409 ± 7	488 ± 27	Caddisfly and chironomid larvae, filamentous algae
Whitefish *Coregonus lavaretus* non-identified form	Sobachye	7	402 ± 12	568 ± 80	Chironomid and other insect pupa and adults

For biochemical analyses, we cut slices of fish white muscles of approximately 2–3 g, 2–3 cm below the dorsal fin. The samples were subdivided into two parts: for FA and moisture analyses. For FA analyses, ca. 1 g of muscle tissues was immediately placed into a volume of 3 mL of chloroform/methanol (2:1, by vol.) and kept until further analysis at −20 °C. Another subsample of ca. 1–2 g of wet weight was weighed, dried at 105 °C until constant weight, and weighed dry for moisture calculation.

2.2. Lipid and Fatty Acid Analyses

In laboratory, lipids were extracted with chloroform/methanol (2:1, by vol.) in triplicate, simultaneously with homogenizing tissues with glass beads in a mortar. Prior to the extraction, an aliquot of 19:0-fatty acid methyl ester (FAME) chloroform solution, as the internal standard, was added to samples for quantification of chromatographic peaks. The extracts were combined and dried with anhydrous Na_2SO_4 and the solvents were roto-evaporated under vacuum at 35 °C. The extracted lipids were redissolved in a 1 mL portion of chloroform and separated in two equal parts. To analyze the fatty acid composition of total lipids, one part of the lipid extract was methylated in the following way. The lipids were hydrolysed under reflux at 90° C for 10 min in 0.8 mL of methanolic sodium hydroxide solution (8 g/L). Then the mixture was cooled for 5 min at room temperature. Next, 1 mL of methanol/sulphuric acid (97:3, by vol.) was added and the mixture was heated under reflux at 90 °C for 10 min to methylate free fatty acids. At the end, 5 mL of saturated solution of NaCl and 3 mL of hexane were added. The FAMEs were extracted for 1 min, the mixture was transferred to a separatory funnel, and the lower aquatic layer was discarded. The hexane layer was additionally washed once with an aliquot of the NaCl solution and twice with 5 mL of distilled water. Then the hexane solution of FAMEs was dried with anhydrous Na_2SO_4, and hexane was removed by roto-evaporating at 30 °C.

We fractionated another part of the lipid extracts by thin layer chromatography (TLC) on silica gel G with hexane-diethyl ether-acetic acid mixture (85:15:1, by vol.). We prepared a reference mixture containing triolein, oleic acid, cholesterol, and phosphatidylcholine (Sigma, St. Louis, MO, USA), which was applied on the side lanes of the silica gel plates. To identify the lipid composition of fish species, we applied aliquots of samples to the plates, and then developed them as described above. After developing, the plates were sprayed with mixture of ethanol/sulphuric acid (90:10, by vol.) and gently heated until grey spots of lipid classes appeared. To evaluate fatty acid profiles and quantify

dominant lipid classes, we separated a main portion of lipid extracts on the silica gel plates, and then visualized only the side lanes corresponding to the reference–compound mixture by a reaction with phosphomolybdic acid ethanolic solution. Lipid spots of the fish samples on the plates were blind detected according to positions of the reference compounds. The lipid spots containing TAG and PL fractions were scraped off from the silica gel plates and placed in flasks. Aliquots of 19:0-FAME hexane solution (t internal standard) were added into the flasks containing silica gel powder with the lipid fractions. To prepare FAMEs, 1ml of hexane and 0.2 mL of fresh 3 M sodium methoxide methanolic solution was added, and the mixture was shaken vigorously for 1 min. Subsequently, the mixture was kept calm at room temperature for 5 min, and finally 3 mL of hexane and 5 mL of a saturated solution of NaCl were added. The next procedure of FAME extraction and washing was the same as for those prepared from total lipids.

Analyses of all FAMEs were done with a gas chromatograph equipped with a mass spectrometer detector (model 6890/5975C; Agilent Technologies, Santa Clara, CA, USA) and with a 30-m long, 0.25-mm internal diameter capillary HP-FFAP column. Detailed descriptions of the chromatographic and mass-spectrometric conditions are given in [23].

2.3. Statistical Analysis

The Kolmogorov–Smirnov one-sample test for normality $D_{K\text{-}S}$, standard errors (SE), Student's *t*-tests, one-way ANOVA with post hoc Tukey HSD test, Kruskal–Wallis test (in the absence of normal distribution) and canonical correspondence analysis (CCA) were calculated conventionally, using STATISTICA software, version 9.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results

According to gut content analysis, *C. lavaretus* in Sobachye Lake was benthivorous (Table 1). Whitefish of a non-identified form in this lake consumed mostly pupa and adult insects, i.e., foraged near the water surface. Round whitefish fed on benthic invertebrates and algae (Table 1). Both broad whitefish and muksun in Pyasino Lake consumed benthic food items, including detritus. Charr in Sobachye Lake and inconnu in Pyasino Lake were piscivorous (Table 1).

Average values of moisture content, lipid content and sum of fatty acid content for total lipids in the studied fish are given in Table 2. Lower moisture values were characteristic of the species with higher values of lipid and sum FA content, charr and whitefish (Table 2). In contrast, round whitefish and whitefish of the non-identified form had the maximum moisture content and the minimal contents of lipids and sum FA. Sum FA content of total lipids significantly varied ~7-fold among the studied species (Table 2). Based on the averages of lipid and sum FA contents, charr and whitefish are further considered as "fatty" fish, muksun, inconnu and broad whitefish as "medium fat" fish, and round whitefish and the non-identified form of whitefish as "lean" fish (Table 2).

Table 2. Average (± SE—standard errors) moisture content (% wet weight), lipid content (mg g^{-1} wet weight) and sum fatty acid content for total lipids (mg g^{-1} wet weight) in muscle tissues of fish species caught in Siberian arctic lakes, 2017. Means of total fatty acids labeled with the same letter are not significantly different at $P < 0.05$ after ANOVA post hoc Tukey HSD test.

Common and Species Name	Moisture	Lipids	Total Fatty Acids
Charr *Salvelinus drjagini*	69.8 ± 1.3	155.8 ± 7.4	78.8 ± 5.1 ^{D}
Whitefish *Coregonus lavaretus*	69.8 ± 1.4	82.0 ± 1.9	62.7 ± 7.2 ^{CD}
Muksun *Coregonus muksun*	74.4 ± 1.0	n.d.	45.4 ± 8.7 ^{BC}
Inconnu *Stenodus leucichthys nelma*	72.1 ± 2.6	68.4 ± 11.4	36.5 ± 10.0 ^{ABC}
Broad whitefish *Coregonus nasus*	73.8 ± 1.4	n.d.	31.9 ± 5.1 ^{AB}
Round whitefish *Prosopium cylindraceum*	76.1 ± 0.6	39.1 ± 3.3	13.8 ± 1.3 ^{A}
Whitefish *Coregonus lavaretus* non-identified form	76.0 ± 0.9	41.1 ± 2.1	11.5 ± 1.9 ^{A}

n.d.—no data.

Levels of 25 prominent individual FA and their structural groups in total lipids are showed in Table 3. Charr had the highest levels of 20:2n-6, 20:3n-3, 20:4n-3, 22:4n-3 and C24 PUFA among the studied species (Table 3). Whitefish had the significantly highest levels of 16:1n-7 and C16 PUFA, and tended to be higher in levels of 18:1n-9 and 20:5n-3. Muksun tended to have higher levels of 14:0, 20:1 and 22:5n-6 (Table 3). Broad whitefish had higher levels of 16:1n-9, C15-17 BFA (branched-chain fatty acids), 18:0, 18:1n-7, 18:2n-6, 18:3n-3 (Table 3). Whitefish of the non-identified form had higher levels of 16:0 and 22:6n-3 compared to those of the other fish. Inconnu and round whitefish had intermediate levels of all FA in total lipids (Table 3).

Table 3. Mean levels of fatty acids (% of the total) in total lipids of species of Salmoniformes order: charr—*S. drjagini* from Sobachye Lake; whitefish—*C. lavaretus* from Sobachye Lake; muksun—*C. muksun* from Pyasino Lake; inconnu—*S. leucichthys nelma* from Pyasino Lake; broad whitefish—*C. nasus* from Pyasino Lake; round whitefish—*P. cylindraceum* from Sobachye Lake; whitefish nd—the non-identified form of *C. lavaretus* from Sobachye Lake. Cases (fatty acids) with normal distribution are given in bold. Means labeled with the same letter are not significantly different at $P < 0.05$ after ANOVA post hoc Tukey HSD test (cases with normal distribution) or Kruskal–Wallis test. If ANOVA is insignificant, letters are absent.

Fatty Acid	Charr	Whitefish	Muksun	Inconnu	Broad Whitefish	Round Whitefish	Whitefish nd
14:0	4.0 ± 0.1 ^{A}	2.9 ± 0.1 ^{B}	4.2 ± 0.2 ^{A}	3.4 ± 0.1 ^{AC}	2.5 ± 0.2 ^{B}	2.2 ± 0.2 ^{B}	2.3 ± 0.2 ^{B}
15:0	0.3 ± 0.0 ^{A}	0.2 ± 0.0 ^{CD}	0.5 ± 0.0 ^{B}	0.4 ± 0.0 ^{A}	0.5 ± 0.0 ^{B}	0.2 ± 0.0 ^{D}	0.3 ± 0.0 ^{C}
16:0	15.4 ± 0.2 ^{C}	14.9 ± 0.2 ^{C}	15.2 ± 0.2 ^{C}	16.3 ± 0.7 ^{AC}	18.2 ± 0.4 ^{AB}	16.6 ± 0.2 ^{AC}	18.9 ± 0.8 ^{B}
16:1n-9	0.4 ± 0.0 ^{ABD}	0.2 ± 0.0 ^{C}	0.3 ± 0.0 ^{CD}	0.4 ± 0.0 ^{AC}	0.5 ± 0.1 ^{B}	0.2 ± 0.0 ^{C}	0.4 ± 0.1 ^{ABD}
16:1n-7	6.6 ± 0.2 ^{B}	17.4 ± 0.5 ^{D}	10.1 ± 0.3 ^{AC}	13.4 ± 1.0 ^{ACE}	11.8 ± 0.8 ^{C}	15.2 ± 0.4 ^{DE}	9.7 ± 1.4 ^{BC}
15-17BFA	1.8 ± 0.0 ^{A}	1.0 ± 0.0 ^{CD}	1.8 ± 0.1 ^{A}	1.3 ± 0.0 ^{AC}	2.5 ± 0.2 ^{B}	0.6 ± 0.0 ^{D}	1.0 ± 0.1 ^{CD}
16PUFA	0.2 ± 0.0 ^{B}	4.6 ± 0.2 ^{C}	2.3 ± 0.2 ^{DE}	1.5 ± 0.2 ^{AD}	1.3 ± 0.1 ^{A}	2.9 ± 0.1 ^{E}	1.5 ± 0.4 ^{AD}
17:0	0.2 ± 0.0 ^{A}	0.3 ± 0.0 ^{D}	0.4 ± 0.0 ^{B}	0.2 ± 0.0 ^{AD}	0.4 ± 0.0 ^{B}	0.1 ± 0.0 ^{C}	0.2 ± 0.0 ^{A}
18:0	3.2 ± 0.1 ^{C}	1.9 ± 0.0 ^{D}	2.4 ± 0.1 ^{A}	2.5 ± 0.2 ^{AB}	3.0 ± 0.1 ^{BC}	2.8 ± 0.0 ^{AB}	2.5 ± 0.2 ^{A}
18:1n-9	17.9 ± 0.1 ^{AC}	19.8 ± 0.2 ^{C}	16.1 ± 0.4 ^{A}	16.8 ± 0.8 ^{AC}	16.3 ± 0.7 ^{A}	11.7 ± 0.6 ^{B}	12.3 ± 1.0 ^{B}
18:1n-7	3.1 ± 0.0 ^{C}	3.9 ± 0.1 ^{AC}	4.0 ± 0.3 ^{AC}	4.3 ± 0.2 ^{AB}	5.1 ± 0.1 ^{B}	4.6 ± 0.1 ^{AB}	3.7 ± 0.5 ^{AC}
18:2n-6	3.0 ± 0.1 ^{AC}	2.1 ± 0.1 ^{A}	2.8 ± 0.1 ^{AC}	2.7 ± 0.2 ^{AC}	4.7 ± 0.6 ^{B}	3.7 ± 0.1 ^{BC}	2.7 ± 0.3 ^{AC}
18:3n-3	2.6 ± 0.1 ^{AC}	1.3 ± 0.0 ^{A}	2.9 ± 0.2 ^{BC}	2.2 ± 0.2 ^{AC}	3.8 ± 0.6 ^{B}	2.7 ± 0.2 ^{AB}	1.6 ± 0.1 ^{AC}
18:4n-3	1.7 ± 0.0	1.8 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	1.5 ± 0.2	1.4 ± 0.2	1.3 ± 0.2
∑20:1 *	1.6 ± 0.0 ^{AD}	1.2 ± 0.1 ^{BCD}	2.3 ± 0.1 ^{A}	2.1 ± 0.9 ^{ABC}	1.7 ± 0.3 ^{AB}	0.6 ± 0.0 ^{C}	0.8 ± 0.1 ^{BC}
20:2n-6	1.0 ± 0.0 ^{C}	0.3 ± 0.0 ^{A}	0.6 ± 0.0 ^{B}	0.4 ± 0.0 ^{A}	0.6 ± 0.0 ^{B}	0.3 ± 0.0 ^{A}	0.4 ± 0.1 ^{A}
20:4n-6	1.9 ± 0.0 ^{AC}	1.6 ± 0.0 ^{CD}	2.4 ± 0.1 ^{B}	2.3 ± 0.3 ^{AB}	2.8 ± 0.1 ^{B}	1.4 ± 0.1 ^{D}	2.6 ± 0.2 ^{B}
20:3n-3	1.5 ± 0.0 ^{B}	0.2 ± 0.0 ^{C}	0.6 ± 0.0 ^{AB}	0.4 ± 0.0 ^{ABC}	0.3 ± 0.0 ^{AC}	0.2 ± 0.0 ^{C}	0.4 ± 0.1 ^{AC}
20:4n-3	2.9 ± 0.0 ^{A}	0.7 ± 0.0 ^{BC}	1.2 ± 0.1 ^{AC}	1.1 ± 0.1 ^{AC}	0.6 ± 0.0 ^{B}	0.8 ± 0.0 ^{BC}	0.8 ± 0.1 ^{BC}
20:5n-3	4.8 ± 0.1 ^{C}	10.4 ± 0.2 ^{A}	9.6 ± 0.2 ^{AB}	7.4 ± 0.7 ^{ABC}	6.5 ± 0.3 ^{BC}	10.2 ± 0.1 ^{A}	10.3 ± 0.3 ^{A}
22:5n-6	1.2 ± 0.0 ^{B}	0.3 ± 0.0 ^{C}	1.3 ± 0.1 ^{B}	1.0 ± 0.1 ^{AB}	0.8 ± 0.1 ^{A}	0.3 ± 0.0 ^{C}	0.8 ± 0.1 ^{A}
22:4n-3	1.5 ± 0.1 ^{C}	0.0 ± 0.0 ^{AB}	0.1 ± 0.0 ^{AC}	0.2 ± 0.1 ^{AC}	0.0 ± 0.0 ^{B}	0.0 ± 0.0 ^{AB}	0.1 ± 0.0 ^{ABC}
22:5n-3	3.0 ± 0.1 ^{CD}	2.5 ± 0.1 ^{AC}	2.5 ± 0.1 ^{AC}	2.3 ± 0.1 ^{A}	1.7 ± 0.1 ^{B}	3.0 ± 0.0 ^{D}	2.4 ± 0.1 ^{A}
22:6n-3	12.1 ± 0.1 ^{AC}	6.3 ± 0.2 ^{D}	9.6 ± 0.6 ^{ADE}	11.3 ± 1.1 ^{ACD}	7.7 ± 0.9 ^{AD}	14.4 ± 1.5 ^{BCE}	20.1 ± 3.0 ^{B}
24PUFA	4.3 ± 0.3 ^{B}	1.0 ± 0.1 ^{AB}	1.1 ± 0.1 ^{BC}	1.0 ± 0.2 ^{AC}	0.7 ± 0.0 ^{A}	0.8 ± 0.0 ^{A}	0.6 ± 0.1 ^{AC}

* sum of 20:1n-11, 20:1n-9 and 20:1n-7, here and in other Tables.

We performed CCA of FA profiles of total lipids of the fish species to find out their overall differences (Figure 1). Along Dimension 1, most difference in FA composition was observed between charr, on the one hand, and whitefish, on the other hand. The differences were primarily caused by higher percentages of 22:4n-3, 20:3n-3 and C24 PUFA in charr, and higher percentages of C16 PUFA and 16:1n-7 in whitefish. In Dimension 2, whitefish of a non-identified form and round whitefish located at the one end and broad whitefish was at the other end (Figure 1). Whitefish of the non-identified form and round whitefish were separated due to higher levels of 22:6n-3 and 20:5n-3, and partial separation of broad whitefish was due to higher levels of C15-17 BFA (Table 3). Samples of the non-identified whitefish were markedly scattered (Figure 1) due to the high variability in percentages of 22:6n-3 (Table 3).

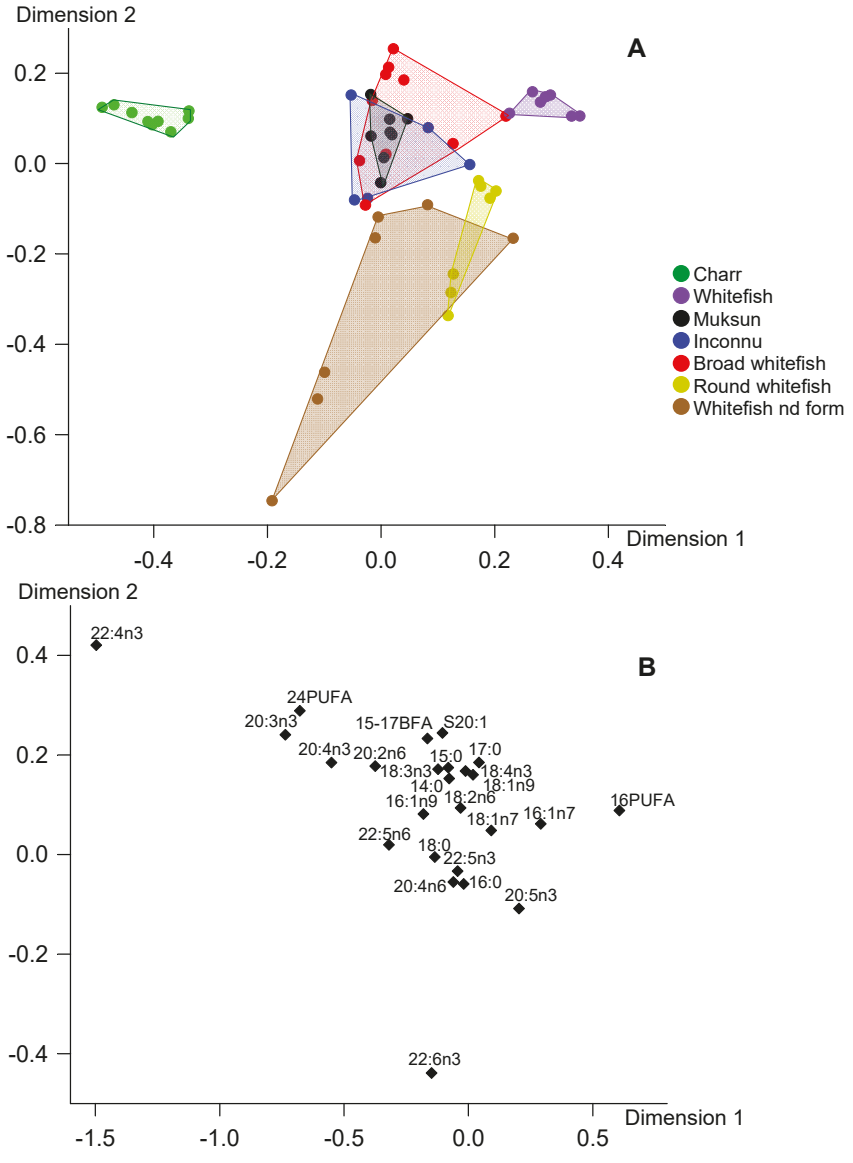

Figure 1. Canonical correspondence analysis of fatty acid percentages (% of FA sum) in total lipids of muscles of seven fish species from arctic lakes (Siberia, Russia). **A**—individual variables, **B**—factor structure coefficients for fatty acids. Dimension 1 and Dimension 2 represented 33.9% and 21.2% of inertia, respectively.

In PL of all studied species, 22:6n-3, 16:0 and 20:5n-3 were dominant fatty acids (Table 4). Charr had the highest levels of 22:6n-3, 22:4n-3, 22:5n-6, 20:4n-3 among the studied species (Table 4). Whitefish had a significantly higher level of 20:5n-3 compared to that of the other fish. Inconnu tended to be higher in 18:1n-9 level. Broad whitefish had the highest levels of 20:4n-6 and 18:2n-6 (Table 4). Round whitefish had significantly higher levels of 16:1n-7, 18:1n-7 and C16 PUFA compared to those of the

other fish. Muksun and whitefish of the non-identified form had intermediate levels of most FA in PL (Table 4).

Table 4. Mean levels of fatty acids (% of the total) and total content of fatty acids (Sum FA, mg g^{-1} wet weight) in polar lipids of Salmoniformes species: charr—*S. drjagini* from Sobachye Lake; whitefish—*C. lavaretus* from Sobachye Lake; muksun—*C. muksun* from Pyasino Lake; inconnu—*S. leucichthys nelma* from Pyasino Lake; broad whitefish—*C. nasus* from Pyasino Lake; round whitefish—*P. cylindraceum* from Sobachye Lake; whitefish nd—the non-identified form of *C. lavaretus* from Sobachye Lake. Cases (fatty acids) with normal distribution are given in bold. Means labeled with the same letter are not significantly different at $P < 0.05$ after ANOVA post hoc Tukey HSD test (cases with normal distribution) or Kruskal–Wallis test. If ANOVA is insignificant, letters are absent.

Fatty Acid	Charr	Whitefish	Muksun	Inconnu	Broad Whitefish	Round Whitefish	Whitefish nd
14:0	1.2 ± 0.1 ^{AB}	0.9 ± 0.0 ^{A}	1.1 ± 0.1 ^{AB}	1.5 ± 0.2 ^{B}	1.2 ± 0.1 ^{AB}	0.9 ± 0.1 ^{A}	1.4 ± 0.1 ^{B}
15:0	0.2 ± 0.0 ^{A}	0.2 ± 0.0 ^{A}	0.4 ± 0.0 ^{CD}	0.3 ± 0.0 ^{BC}	0.5 ± 0.0 ^{D}	0.2 ± 0.0 ^{A}	0.3 ± 0.0 ^{AB}
16:0	24.2 ± 0.4 ^{A}	29.4 ± 0.5 ^{BC}	26.3 ± 0.6 ^{AB}	25.9 ± 0.5 ^{AB}	27.3 ± 0.8 ^{AB}	29.1 ± 1.3 ^{BC}	31.7 ± 1.1 ^{C}
16:1n-9	0.3 ± 0.0 ^{AB}	0.1 ± 0.0 ^{A}	0.3 ± 0.0 ^{BC}	0.2 ± 0.1 ^{AB}	0.5 ± 0.1 ^{C}	0.2 ± 0.0 ^{AB}	0.3 ± 0.1 ^{BC}
16:1n-7	1.3 ± 0.1 ^{A}	2.2 ± 0.1 ^{BC}	1.9 ± 0.2 ^{AB}	2.3 ± 0.1 ^{B}	2.9 ± 0.2 ^{B}	4.1 ± 0.2 ^{D}	3.0 ± 0.2 ^{C}
15-17BFA	0.6 ± 0.0 ^{C}	0.1 ± 0.0 ^{A}	0.4 ± 0.0 ^{BC}	0.3 ± 0.0 ^{AB}	0.6 ± 0.1 ^{C}	0.2 ± 0.0 ^{AB}	0.3 ± 0.1 ^{AB}
16PUFA	0.0 ± 0.0 ^{A}	0.2 ± 0.0 ^{B}	0.1 ± 0.1 ^{AB}	0.1 ± 0.0 ^{AB}	0.1 ± 0.0 ^{AB}	0.3 ± 0.0 ^{C}	0.1 ± 0.0 ^{AB}
17:0	0.2 ± 0.0 ^{A}	0.2 ± 0.0 ^{AB}	0.3 ± 0.0 ^{B}	0.2 ± 0.0 ^{AB}	0.3 ± 0.0 ^{B}	0.1 ± 0.0 ^{A}	0.2 ± 0.0 ^{A}
18:0	2.8 ± 0.1 ^{AB}	2.1 ± 0.1 ^{A}	3.0 ± 0.1 ^{B}	2.9 ± 0.3 ^{AB}	2.2 ± 0.2 ^{A}	3.4 ± 0.2 ^{B}	2.8 ± 0.3 ^{AB}
18:1n-9	6.2 ± 0.3 ^{AB}	6.5 ± 0.2 ^{AB}	6.6 ± 0.4 ^{AB}	7.8 ± 0.4 ^{B}	6.1 ± 0.5 ^{A}	5.6 ± 0.3 ^{A}	6.9 ± 0.3 ^{AB}
18:1n-7	1.6 ± 0.1 ^{A}	1.7 ± 0.1 ^{AB}	2.2 ± 0.2 ^{BC}	2.4 ± 0.1 ^{C}	2.5 ± 0.1 ^{C}	3.3 ± 0.2 ^{D}	2.4 ± 0.3 ^{C}
18:2n-6	0.7 ± 0.0 ^{A}	0.8 ± 0.0 ^{A}	1.0 ± 0.0 ^{A}	1.1 ± 0.1 ^{A}	2.5 ± 0.4 ^{B}	1.6 ± 0.0 ^{A}	1.4 ± 0.3 ^{A}
18:3n-3	0.6 ± 0.0 ^{AB}	0.4 ± 0.0 ^{A}	1.0 ± 0.1 ^{ABC}	1.0 ± 0.2 ^{ABC}	2.1 ± 0.4 ^{C}	1.3 ± 0.1 ^{BC}	0.7 ± 0.1 ^{ABC}
18:4n-3	0.1 ± 0.0 ^{AB}	0.0 ± 0.0 ^{A}	0.2 ± 0.0 ^{B}	0.2 ± 0.0 ^{AB}	0.1 ± 0.0 ^{AB}	0.3 ± 0.0 ^{B}	0.2 ± 0.1 ^{AB}
∑20:1	0.2 ± 0.0 ^{BC}	0.1 ± 0.0 ^{A}	0.3 ± 0.0 ^{C}	0.1 ± 0.0 ^{ABC}	0.1 ± 0.0 ^{AB}	0.2 ± 0.0 ^{ABC}	0.2 ± 0.1 ^{AC}
20:2n-6	0.2 ± 0.0 ^{BC}	0.0 ± 0.0 ^{A}	0.1 ± 0.0 ^{AB}	0.1 ± 0.0 ^{A}	0.2 ± 0.0 ^{C}	0.1 ± 0.0 ^{AC}	0.1 ± 0.0 ^{AB}
20:4n-6	3.9 ± 0.1 ^{BC}	3.0 ± 0.1 ^{AB}	4.1 ± 0.2 ^{C}	3.8 ± 0.3 ^{BC}	5.6 ± 0.3 ^{D}	2.4 ± 0.1 ^{A}	3.2 ± 0.2 ^{ABC}
20:3n-3	0.5 ± 0.0 ^{C}	0.1 ± 0.0 ^{A}	0.2 ± 0.0 ^{B}	0.1 ± 0.0 ^{AB}	0.2 ± 0.0 ^{B}	0.1 ± 0.0 ^{AB}	0.1 ± 0.0 ^{AB}
20:4n-3	1.1 ± 0.0 ^{B}	0.3 ± 0.0 ^{A}	0.5 ± 0.0 ^{AB}	0.5 ± 0.1 ^{AB}	0.4 ± 0.1 ^{A}	0.5 ± 0.0 ^{AB}	0.4 ± 0.0 ^{A}
20:5n-3	7.9 ± 0.2 ^{A}	16.6 ± 0.6 ^{D}	12.5 ± 0.3 ^{BC}	11.2 ± 1.1 ^{BC}	10.9 ± 0.4 ^{B}	13.6 ± 1.0 ^{C}	10.0 ± 0.5 ^{AB}
22:5n-6	2.4 ± 0.1 ^{C}	0.6 ± 0.0 ^{A}	2.0 ± 0.3 ^{C}	1.7 ± 0.2 ^{BC}	2.2 ± 0.2 ^{C}	0.4 ± 0.0 ^{A}	0.9 ± 0.1 ^{AB}
22:4n-3	0.2 ± 0.0 ^{B}	0.0 ± 0.0 ^{A}	0.0 ± 0.0 ^{A}	0.0 ± 0.0 ^{A}	0.0 ± 0.0 ^{A}	0.0 ± 0.0 ^{A}	0.0 ± 0.0 ^{A}
22:5n-3	2.5 ± 0.0 ^{A}	2.7 ± 0.2 ^{AB}	2.5 ± 0.1 ^{A}	2.4 ± 0.3 ^{A}	2.6 ± 0.1 ^{AB}	3.2 ± 0.3 ^{B}	2.1 ± 0.1 ^{A}
22:6n-3	39.9 ± 0.5 ^{D}	31.1 ± 0.7 ^{BC}	31.8 ± 0.5 ^{C}	32.4 ± 0.9 ^{C}	26.4 ± 0.6 ^{A}	27.9 ± 0.7 ^{AB}	30.1 ± 1.1 ^{BC}
24PUFA	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.4	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sum FA	*3.0 ± 0.2* ^{AB}	*3.3 ± 0.5* ^{AB}	*3.2 ± 0.9* ^{AB}	*2.6 ± 0.2* ^{AB}	*3.4 ± 0.2* ^{B}	*3.2 ± 0.7* ^{AB}	*2.1 ± 0.2* ^{A}

To reveal overall differences in PL FA, CCA was performed (Figure 2). In the first dimension, a conspicuous difference of round whitefish versus charr was found. This difference was provided mostly by the greater levels of C16 PUFA, 16:1n-7 and 18:2n-6 in PL of round whitefish and by greater levels of C24 PUFA and 22:4n-3 in that of charr (Figure 2). The variation in the second dimension of CCA was related to differences between whitefish and broad whitefish due to levels of 15:0 and 14:0 versus levels of C16 PUFA, 18:2n-6 and 18:3n-3.

In FA composition of triacylglycerols of all the studied arctic fish, 18:1n-9, 16:1n-7, 16:0 and 20:5n-3 dominated (Table 5). Charr had the highest levels of 22:6n-3, C24 PUFA, 22:5n-3, 20:4n-3 and 20:3n-3 compared to that of the other studied species. Whitefish had the maximum levels of 18:1n-9 and 20:5n-3 (Table 5). Muksun had the significantly higher level of 14:0 than the other fish. Inconnu and whitefish of the non-identified form had intermediate FA levels in TAG (Table 5). Broad whitefish had the maximum levels of 16:0, C15-17 BFA, 17:0, 18:2n-6, 18:3n-3, and 20:4n-6 among the studied fish. Round whitefish had the significantly higher percentages of 16:1n-7 and C16 PUFA in TAG (Table 5).

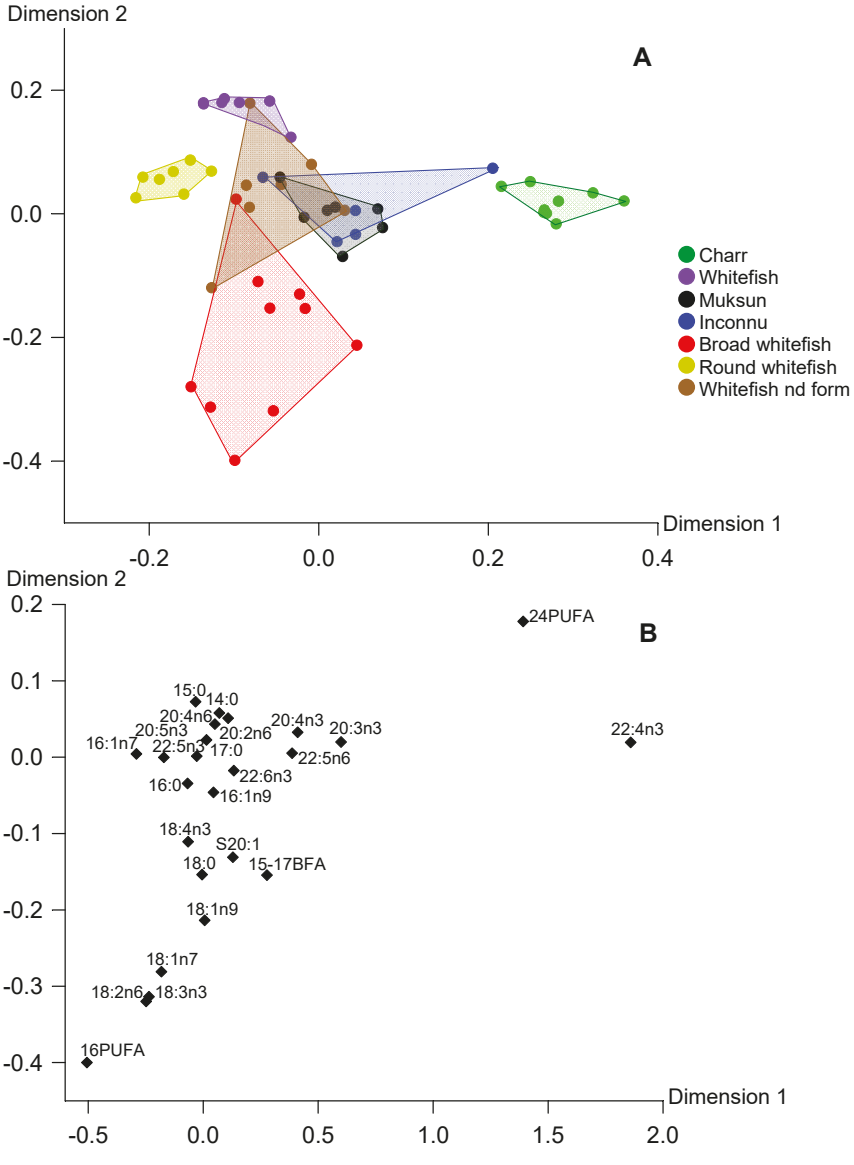

Figure 2. Canonical correspondence analysis of fatty acid percentages (% of FA sum) in polar lipids of muscles of seven fish species from arctic lakes (Siberia, Russia). **A**—individual variables, **B**—factor structure coefficients for fatty acids. Dimension 1 and Dimension 2 represented 30.9% and 22.5% of inertia, respectively.

Table 5. Mean levels of fatty acids (% of the total) and total content of fatty acids (Sum FA, mg g^{-1} wet weight) in triacylglycerols of Salmoniformes species: charr—*S. drjagini* from Sobachye Lake; whitefish—*C. lavaretus* from Sobachye Lake; muksun—*C. muksun* from Pyasino Lake; inconnu—*S. leucichthys nelma* from Pyasino Lake; broad whitefish—*C. nasus* from Pyasino Lake; round whitefish—*P. cylindraceum* from Sobachye Lake; whitefish nd—the non-identified form of *C. lavaretus* from Sobachye Lake. Cases (fatty acids) with normal distribution are given in bold. Means labeled with the same letter are not significantly different at $P < 0.05$ after ANOVA post hoc Tukey HSD test (cases with normal distribution) or Kruskal–Wallis test. If ANOVA is insignificant, letters are absent.

Fatty Acid	Charr	Whitefish	Muksun	Inconnu	Broad Whitefish	Round Whitefish	Whitefish nd
14:0	4.4 ± 0.1 ^{CD}	3.3 ± 0.1 ^{B}	4.8 ± 0.3 ^{D}	3.9 ± 0.0 ^{BC}	2.7 ± 0.2 ^{A}	3.1 ± 0.2 ^{B}	3.5 ± 0.4 ^{ABC}
15:0	0.3 ± 0.0 ^{B}	0.2 ± 0.0 ^{AB}	0.5 ± 0.0 ^{C}	0.4 ± 0.0 ^{BC}	0.5 ± 0.1 ^{C}	0.2 ± 0.0 ^{A}	0.3 ± 0.0 ^{AB}
16:0	16.7 ± 0.4 ^{C}	14.6 ± 0.2 ^{AB}	14.6 ± 0.8 ^{AB}	15.9 ± 0.7 ^{B}	17.4 ± 0.3 ^{C}	12.9 ± 0.4 ^{A}	15.7 ± 0.5 ^{B}
16:1n-9	0.8 ± 0.3 ^{B}	0.3 ± 0.0 ^{A}	0.4 ± 0.0 ^{AB}	0.4 ± 0.1 ^{AB}	0.8 ± 0.1 ^{B}	0.3 ± 0.0 ^{A}	0.5 ± 0.1 ^{AB}
16:1n-7	7.9 ± 0.4 ^{A}	19.4 ± 0.8 ^{C}	12.6 ± 1.3 ^{B}	15.9 ± 1.4 ^{BC}	13.2 ± 0.7 ^{B}	24.5 ± 1.2 ^{D}	15.7 ± 0.9 ^{BC}
15-17BFA	2.0 ± 0.2 ^{BCD}	1.0 ± 0.0 ^{AB}	1.9 ± 0.1 ^{CD}	1.1 ± 0.1 ^{ABC}	2.8 ± 0.4 ^{D}	0.7 ± 0.1 ^{A}	1.4 ± 0.1 ^{AB}
16PUFA	0.4 ± 0.1 ^{A}	4.8 ± 0.2 ^{C}	3.0 ± 0.6 ^{B}	1.6 ± 0.4 ^{AB}	1.3 ± 0.2 ^{AB}	5.4 ± 0.7 ^{C}	2.2 ± 0.5 ^{B}
17:0	0.2 ± 0.0 ^{AB}	0.3 ± 0.0 ^{BC}	0.3 ± 0.0 ^{C}	0.2 ± 0.0 ^{AB}	0.5 ± 0.0 ^{D}	0.1 ± 0.0 ^{A}	0.3 ± 0.0 ^{BC}
18:0	3.4 ± 0.1 ^{C}	1.9 ± 0.0 ^{A}	2.4 ± 0.1 ^{A}	2.5 ± 0.3 ^{AB}	3.2 ± 0.1 ^{BC}	2.5 ± 0.1 ^{A}	2.6 ± 0.3 ^{A}
18:1n-9	19.2 ± 0.6 ^{BC}	21.5 ± 0.4 ^{C}	16.8 ± 1.0 ^{AB}	18.9 ± 1.4 ^{B}	17.8 ± 0.8 ^{AB}	14.6 ± 0.8 ^{A}	18.3 ± 0.6 ^{B}
18:1n-7	2.9 ± 0.4 ^{A}	4.2 ± 0.1 ^{AB}	4.6 ± 0.4 ^{BC}	4.9 ± 0.2 ^{BC}	5.9 ± 0.3 ^{C}	5.9 ± 0.4 ^{C}	4.8 ± 0.5 ^{BC}
18:2n-6	3.2 ± 0.1 ^{AB}	2.2 ± 0.1 ^{A}	3.0 ± 0.1 ^{AB}	3.0 ± 0.4 ^{AB}	5.4 ± 0.6 ^{D}	5.1 ± 0.1 ^{CD}	3.9 ± 0.3 ^{BC}
18:3n-3	2.6 ± 0.1 ^{AB}	1.4 ± 0.1 ^{A}	3.0 ± 0.2 ^{BC}	2.4 ± 0.3 ^{A}	4.0 ± 0.5 ^{C}	3.6 ± 0.4 ^{BC}	2.2 ± 0.2 ^{AB}
18:4n-3	1.6 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.7 ± 0.2	1.5 ± 0.2	1.9 ± 0.3	2.1 ± 0.4
∑20:1	1.3 ± 0.0 ^{BC}	1.0 ± 0.1 ^{AB}	1.9 ± 0.2 ^{C}	2.0 ± 0.9 ^{ABC}	1.7 ± 0.3 ^{BC}	0.7 ± 0.1 ^{A}	1.3 ± 0.1 ^{ABC}
20:2n-6	0.9 ± 0.0 ^{C}	0.2 ± 0.0 ^{A}	0.6 ± 0.0 ^{B}	0.4 ± 0.0 ^{AB}	0.4 ± 0.1 ^{AB}	0.3 ± 0.0 ^{AB}	0.5 ± 0.1 ^{B}
20:4n-6	1.7 ± 0.0 ^{BC}	1.4 ± 0.0 ^{B}	2.1 ± 0.1 ^{D}	1.9 ± 0.2 ^{CD}	2.3 ± 0.1 ^{D}	0.6 ± 0.0 ^{A}	1.7 ± 0.1 ^{BCD}
20:3n-3	1.4 ± 0.0 ^{C}	0.1 ± 0.0 ^{A}	0.5 ± 0.1 ^{BC}	0.4 ± 0.0 ^{ABC}	0.3 ± 0.0 ^{AB}	0.2 ± 0.0 ^{AB}	0.4 ± 0.1 ^{AB}
20:4n-3	2.7 ± 0.1 ^{C}	0.7 ± 0.1 ^{AB}	1.2 ± 0.1 ^{BC}	1.2 ± 0.1 ^{ABC}	0.6 ± 0.0 ^{A}	0.9 ± 0.1 ^{AB}	0.9 ± 0.1 ^{AB}
20:5n-3	4.4 ± 0.1 ^{A}	9.4 ± 0.2 ^{D}	9.0 ± 0.3 ^{D}	6.5 ± 1.0 ^{BC}	5.3 ± 0.4 ^{AB}	7.0 ± 0.5 ^{BC}	8.6 ± 0.5 ^{CD}
22:5n-6	0.9 ± 0.0 ^{D}	0.2 ± 0.0 ^{AB}	1.0 ± 0.1 ^{D}	0.7 ± 0.1 ^{CD}	0.5 ± 0.1 ^{BC}	0.0 ± 0.0 ^{A}	0.4 ± 0.1 ^{AB}
22:4n-3	1.3 ± 0.0 ^{B}	0.0 ± 0.0 ^{A}	0.1 ± 0.0 ^{A}	0.2 ± 0.1 ^{A}	0.0 ± 0.0 ^{A}	0.0 ± 0.0 ^{A}	0.1 ± 0.0 ^{A}
22:5n-3	2.7 ± 0.1 ^{B}	2.2 ± 0.1 ^{B}	2.4 ± 0.2 ^{B}	2.1 ± 0.2 ^{B}	1.4 ± 0.1 ^{A}	2.3 ± 0.1 ^{B}	2.1 ± 0.2 ^{B}
22:6n-3	9.9 ± 0.2 ^{C}	4.1 ± 0.2 ^{B}	6.4 ± 0.3 ^{C}	7.8 ± 0.8 ^{C}	4.1 ± 0.3 ^{B}	2.4 ± 0.2 ^{A}	6.6 ± 0.5 ^{C}
24PUFA	2.9 ± 0.1 ^{B}	0.7 ± 0.1 ^{A}	0.9 ± 0.1 ^{AB}	0.6 ± 0.3 ^{A}	0.7 ± 0.0 ^{A}	0.6 ± 0.1 ^{A}	0.7 ± 0.1 ^{A}
Sum FA	*30.3 ± 4.7* ^{AB}	*33.2 ± 6.9* ^{AB}	*41.6 ± 15.0* ^{B}	*18.7 ± 7.0* ^{AB}	*19.5 ± 7.0* ^{AB}	*4.9 ± 1.2* ^{A}	*2.0 ± 0.5* ^{A}

To study differences in fish reserve lipids, we performed CCA of FA in TAG (Figure 3). Like the multidimensional analysis for PL, Dimension 1 showed a marked difference of round whitefish versus charr. This difference was provided mostly by the greater levels of C16 PUFA and 16:1n-7 in TAG of round whitefish and by greater levels of 22:4n-3 and 20:3n-3 in that of charr (Figure 3). The second dimension of CCA for TAG also showed a similar trend to that observed in CCA of PL (Figures 2 and 3). In this dimension, most prominent difference was found between whitefish and broad whitefish due to variability in levels of C15-17 BFA, 17:0 and C16 PUFA (Figure 3).

In general, positioning of fish species in the biplot for reserve TAG well corresponded to that in biplot for structural PL (Figures 2 and 3). It should be also noted that physiologically significant EPA and DHA were not found among the FA markers responsible for separation of fish species in CCA for TAG and PL (Figures 2 and 3). Positioning of fish species in the biplot for total lipids generally corresponded to that in biplots for the lipid classes, with exception of whitefish of the non-identified form (Figures 1–3). The fatty acid markers responsible for separation of the fish samples in CCA were generally similar for total lipids, TAG and PL with exception of DHA and EPA in CCA of total lipids (Figures 1–3).

A visual analysis of all thin-layer chromatograms showed a marked dominance of TAG, PL and sterols as major lipid fractions. Spots that corresponded to other lipid classes were negligible. Therefore, we considered TAG and PL as major acyl-containing fractions, summarized their FA contents per mass unit (Tables 4 and 5) and calculated their parts in the sum of FA in the fish muscles (Figure 4A). Polar lipids constituted from 10.3 to 57.0% of the acyl-containing lipid sum, being the highest in whitefish of

the non-identified form (Figure 4A). Triacylglycerols were the dominant acyl-containing lipid fraction for majority of the studied fish and exceeded 85% in charr, whitefish and muksun (Figure 4A). Note that increase in lipid content and total fatty acids for the studied species well corresponded with the increase in the TAG proportion of the acyl-containing lipids (Table 2, Figure 4A).

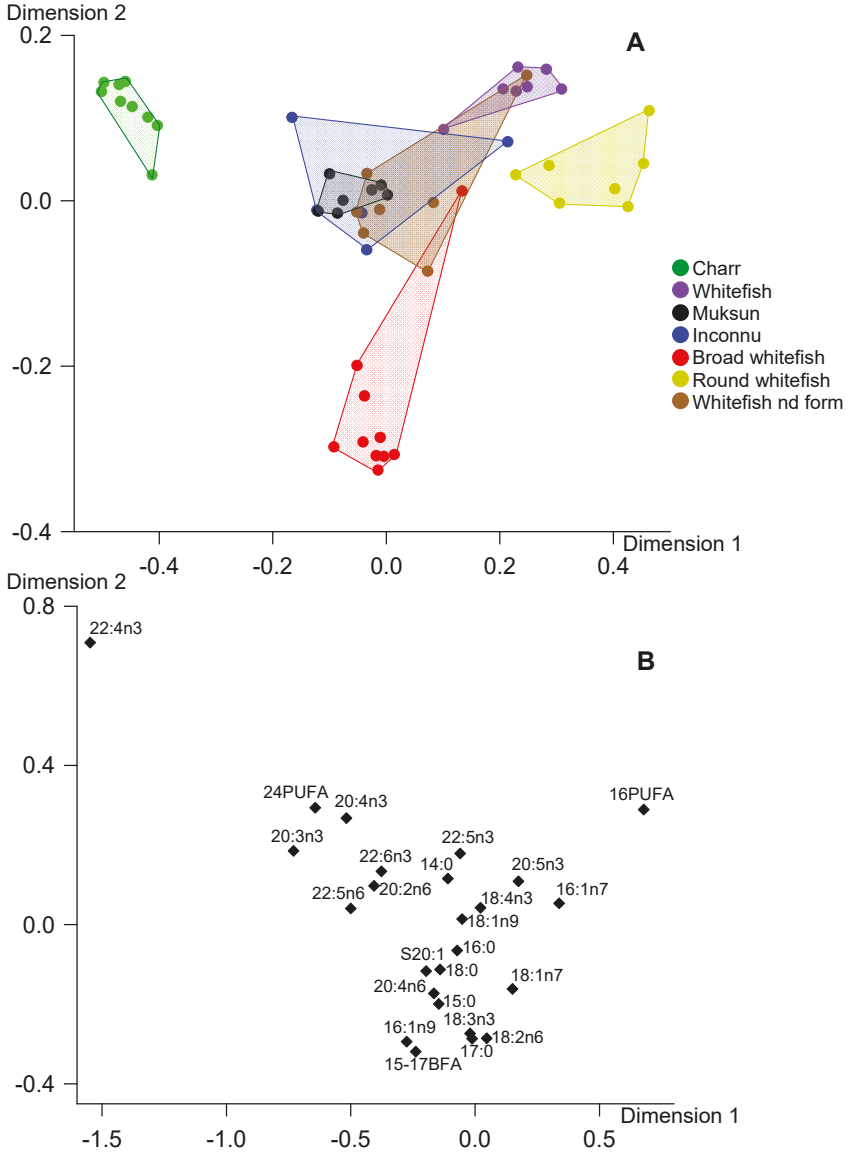

Figure 3. Canonical correspondence analysis of fatty acid percentages (% of FA sum) in triacylglycerols of muscles of seven fish species from arctic lakes (Siberia, Russia). **A**—individual variables, **B**—factor structure coefficients for fatty acids. Dimension 1 and Dimension 2 represented 51.1% and 15.6% of inertia, respectively.

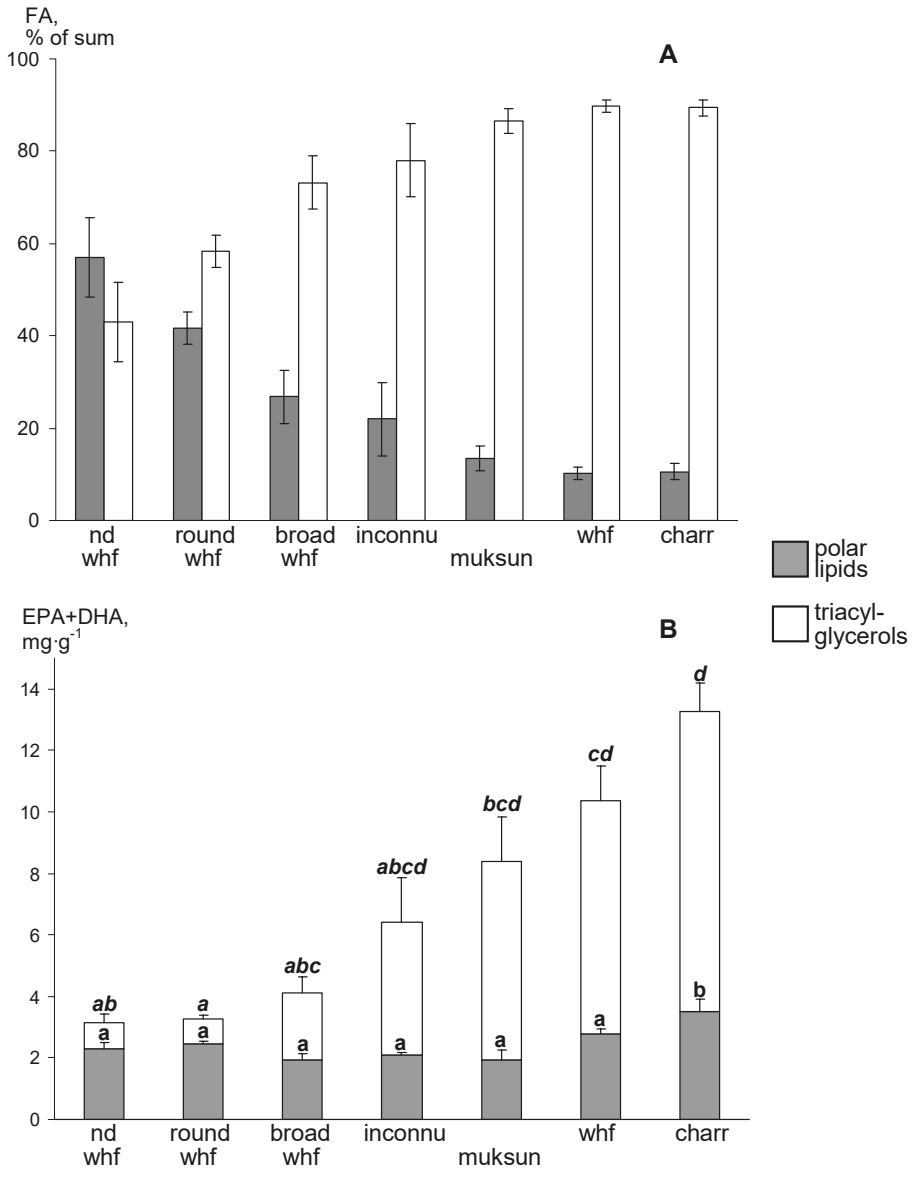

Figure 4. Mean percentages of polar lipids and triacylglycerols of their sum—**A**, and mean contents (mg g^{-1} wet weight) of sum of eicosapentaenoic and docosahexaenoic acids that corresponded to polar lipids and triacylglycerols—**B**, in muscles of seven fish species from arctic lakes (Siberia, Russia): nd whf—the non-identified form of *C. lavaretus* from Sobachye Lake, round whf—*P. cylindraceum* from Sobachye Lake, broad whf—*C. nasus* from Pyasino Lake, inconnu—*S. leucichthys nelma* from Pyasino Lake, muksun—*C. muksun* from Pyasino Lake, whf—*C. lavaretus* from Sobachye Lake, charr—*S. drjagini* from Sobachye Lake. Bars represent standard errors. Means labelled with the same letter are not significantly different at $P < 0.05$ after Tukey HSD post hoc test.

Using the PL and TAG percentages of the acyl-containing lipid sum and content of EPA and DHA of total lipids per mass unit, we calculated parts of EPA + DHA that provided by polar lipids versus triacylglycerols and expressed them as mg g^{-1} wet weight of muscle tissue (Figure 4B). Contents of EPA + DHA provided by PL fraction varied from 1.9 to 3.5 mg g^{-1} (Figure 4B). The average value for the seven fish species was 2.4 ± 0.2 mg g^{-1}, and coefficient of variation, CV, was 8.7%. Contents of EPA + DHA in TAG were of a greater range, from 0.9 to 9.8 mg g^{-1}; the average value was to 4.4 ± 0.2 mg g^{-1}, and CV was 28.9% (Figure 4B).

4. Discussion

4.1. Main Finding

All the taxonomically related species of order Salmoniformes had nearly similar content of EPA+DHA in PL, 2.4 ± 0.2 mg g^{-1}, in average. In contrast, content of EPA + DHA esterified as TAG varied ~10-fold among the studied salmonids. Thus, all variations of nutritive value, i.e., EPA + DHA content per mass unit of filet, were caused by TAG fraction, while PL had a constant species (taxon—specific physiologically optimum content. As a result, fatty fish, charr, whitefish and muksun, contained most amounts of nutritionally valuable EPA and DHA in TAG fraction of muscles. Conversely, the lean species, round whitefish and whitefish of the non-identified form, had the omega-3 LC-PUFA contained mostly in polar lipids. Regarding nutritive value, the fatty species with higher proportion of TAG in muscles, charr, whitefish and muksun, appeared to be most valuable and had 13.3 ± 0.8, 10.4 ± 1.1 and 8.4 ± 1.4 mg g^{-1}, respectively.

4.2. Fatty Acid Markers in Fish Total Lipids, TAG and PL

In our snap-shot field study of salmonids from arctic lakes we found significant differences between fatty acid profiles of the main lipid classes, with prevalence of n-3 LC-PUFA and monoenoic 16-18 FA in structural PL and storage TAG, respectively. In addition, both FA profiles of TAG and PL, as well as profiles of total lipids, had distinct peculiarities among the studied fish that allowed separating the most species in the CCA biplots (Figures 1–3). It should be emphasized that the separations of the fish in the multivariate analyses of all three lipid fractions were provided mainly by the same marker FA: C24 PUFA, 22:4n-3, 20:3n-3, C16 PUFA, 16:1n-7 and C15-17 BFA. The conspicuous exception was 22:6n-3 and 20:5n-3 in total lipid CCA that separated whitefish of the non-identified form and round whitefish from the other species (Figure 1). PL had a predominant contribution to total lipids of these lean fish (Figure 4A). Therefore, FA profiles of total lipids of whitefish of the non-identified form and round whitefish mostly reflected FA composition of PL which were considerably rich in DHA and EPA (Table 4). As a result, DHA and EPA, dominant FA of PL, played as markers for the lean fish in CCA of total lipids.

Similar patterns of biomarker FA, characteristic of zoobenthic, algal, terrestrial and other food sources, within TAG and PL fractions of the studied species allowed to use both these fractions, as well as their sum, total lipids, for identification of food sources of wild fish (Figures 1–3). In ecological studies, analysis of FA trophic markers of various consumers is often performed for TAG assuming that they generally deposit fatty acid molecules coming from food assimilation [18,25,26]. Meanwhile, many studies used FA composition of total lipids to elucidate trophic relations of various fish species, e.g., [27–31]. In overall, both approaches base on a premise that biochemical composition of food sources is reflected by FA profiles of TAG or total lipids.

Another lipid class of high concern is polar lipids comprised mostly phospholipids and glycolipids that are main constituents of cell membranes. As known, the specific FA composition of PL provides proper membrane structure and functions. As a result, FA composition of PL are considered to be highly conserved relative to diet and tended to reflect FA biosynthetic capacities of an organism [18].

In contrast to the common opinion on conserved PL composition, in some studies FA profiles of PL were successfully used as trophic markers. For instance, fatty acid profiles of both polar and neutral

lipids conspicuously differed among three species, benthivorous whitefish *Coregonus clupeaformis*, and piscivorous walleye *Sander vitreus* and northern pike *Esox lucius*, due to different feeding habits of the fish [32]. We also confirmed that PL FA profiles of muscles allow to identify feeding spectra of fish similarly that TAG profiles do (Figures 2 and 3). For instance, round whitefish was one of the most separated species in the both multivariate analyses of TAG and PL due to the greatest levels of C16 PUFA and 16:1n-7 which originated from diatoms and green algae [33]. Indeed, the algae were one of the dominant items in stomach content of this species (Table 1).

In CCA biplots of total lipids, PL and TAG, charr had a particular position due to higher levels of minor n-3 PUFA, like 22:4-3, C24 PUFA and 20:3n-3. These FA were not assigned as trophic markers, whereas some of them, C24 PUFA, were considered as intermediate compounds indicative for conversion of C20 to C22 PUFA [34,35]. In TAG of charr the percentage of 22:4n-3 accounted for 1.3% of FA sum, being absent or found in traces in other studied fish. The presence of this PUFA was previously reported for least cisco *Coregonus sardinella*, small-sized pelagic fish inhabited Sobachye Lake [23]. The studied charr from Sobachye Lake was piscivorous (Table 1), thus, it could obtain this PUFA from the consumed least cisco. Like in our study, species of the same genus and its prey, lake trout *Salvelinus namaycush* and cisco from Great Bear Lake, were together separated from other hydrobionts in a multivariate analysis due to higher levels of 22:4-3 and 20:3n-3 [31]. Alternative explanation based on coincidence between 22:4n-3 and C24 PUFA levels is that the fatty acid 22:4n-3 may be a marker of LC-PUFA conversion in fish. Anyway, we suppose that considerable levels of 22:4n-3, 20:3n-3 and C24 PUFA might be a characteristic feature of FA profiles of *Salvelinus* genus.

In both CCA analyses of TAG and PL, broad whitefish well separated from the other species due to higher levels of C15-17 BFA, 17:0, 18:2n-6 and 18:3n-3. The two former FA are known to be markers of bacterial organic matter, while the two latter are considered as markers of terrestrial organic matter [31,33]. Broad whitefish is a typical benthivorous species that likely got these marker fatty acids from detritus enriched with bacterial and terrestrial organic matter. The species had the highest levels of 18:2n-6 in TAG, and 20:4n-6 in PL, relatively. This finding likely indicates for the initial storage of dietary 18:2n-6 in TAG and its consequent conversion to 20:4n-6 with further transfer to PL.

The similarity of FA sets that are markers for food sources between TAG and PL classes likely indicates that the studied wild fish are able to directly incorporate dietary biochemical components, i.e., fatty acyl groups, into membrane PL. Besides, FA originated from food assimilation, fish are able to include in lipid molecules fatty acyl groups obtained due to biosynthesis or conversion from precursors. Freshwater fish are known to have capacity to synthesize LC-PUFA from the shorter chain precursors [35,36]. Indeed, some studied fish, e.g., charr, contained in TAG and PL certain amounts of C24 PUFA and 20:4n-3 that likely were intermediates of DHA and EPA synthesis.

4.3. Content of Essential LC-PUFA in Fish PL and TAG

The studied seven salmonid species varied ~ 7-fold in total lipid and fatty acid contents per a mass unit of muscle tissues. Most of this variation was related with different TAG content in muscles (Table 5, Figure 4), whereas PL content evaluated as their FA sum varied slightly (Table 4). The observed variation in lipid class contents in the studied fish is in agreement with well-known notion that polar lipids comprise cellular membranes and, as a result, have a relatively constant content in muscle cells, in contrast to that of triacylglycerols [19,21,37]. For instance, an absence of relation between total lipid and phospholipid contents and a strong relation between total lipid and triacylglycerol contents expressed as percentages of muscle mass were previously shown for a number of marine myctophid species [38].

Fish polar lipids are commonly considered as a physiologically crucial lipid class that are rich in LC-PUFA, mostly in DHA and, to a lesser extent, in EPA [19,37,39]. Indeed, percentages of DHA and EPA in PL of various wild marine and freshwater species ranged as 11.5–55.7% and 2.6–14.6%, with average values of 31.4% and 7.4%, respectively [40–46]. The average levels of EPA and DHA in

the fish species from our study coincided with the above ranges, except the EPA value of whitefish, 16.6%, which was a bit higher than the known values.

Triacylglycerols are considered to be relatively poor in LC-PUFA and preferably accumulate monoenoic C16-22 FA [18,19,37]. According to the available data, levels of DHA and EPA in fish TAG varied in intervals of 2.3%-23.3% and 1.1%–14.1%, with average values of 8.8% and 5.3%, respectively [40–46]. The percentages of both EPA and DHA of TAG in the fish species from our study well coincided with the above ranges (Table 5).

Triacylglycerols commonly comprise a large part of total lipids in muscles of medium-fat and fatty fish species. For instance, TAG achieved 80%, 90% and 51.5% in marine species arrow-tooth flounder (*Atheresthes stomias)* and golden pompano (*Trachinotus blochii*) and freshwater whitefish (*Coregonus lavaretus*), respectively [43,47,48]. In the studied freshwater salmonids, TAG percentages varied from 43.4% to 89.7% of the sum of two acyl-containing lipid classes (Figure 4A). Such high TAG levels may be explained by adaptation of the fish species to low-temperature conditions in the studied arctic lakes [49,50].

Regarding the relatively high contents of TAG per mass unit and percentages of EPA and DHA in TAG, we hypothesized that content of EPA and DHA in TAG would appreciably contribute to total content of EPA and DHA and would increase along with lipid content in muscles of the studied fish. Hence, we compared content of EPA and DHA esterified as TAG versus that esterified as PL. Content of EPA+DHA in PL per mass unit of muscle tissues were similar among the studied salmonids, moderately varying in the interval of 1.9–3.5 mg g^{-1} (Figure 4B). In contrast, values of EPA+DHA in TAG of the fish species greatly varied, ~10-fold. Lean fish, i.e., whitefish of the non-identified form, round whitefish and broad whitefish, contained only 25%–47% of EPA + DHA of total content of these PUFA in the muscles esterified as TAG molecules. In contrast, the medium-fat and fatty fish, inconnu, muksun, whitefish and charr, had more than half of their muscle EPA and DHA content as TAG molecules, up to 72%. Thus, the wild salmonids that had relatively high content of n-3 LC-PUFA in muscles (~ > 5 mg g^{-1}) contained the major portion of these nutritionally valuable compounds in the storage lipids. Our finding evidently contradicts a common notion that lean and medium-fat fish that have PL as a main lipid class in the muscles are the best dietary sources of n-3 LC-PUFA for humans [19,51]. Wild fatty fish which are able to deposit large amounts of storage lipids in their muscles appear to be the most valuable sources of n-3 LC-PUFA in human diet.

Further, our results are in a good accordance with many studies showed strong relationship between total lipid and EPA, DHA or their sum contents in fish muscles. Such relation was found for marine species, sprat *Sprattus sprattus* and herring *Clupea harengus* from Baltic Sea [52], for five marine species from the northeast Pacific [38] and for several freshwater species from a subalpine lake [53]. The significant relation was also found across farmed families of Atlantic salmon *Salmo salar* [54].

It is interesting to note that percentages of the n-3 LC-PUFA and lipid (or total FA as its proxy) content were negatively correlated in aforementioned and other studies [10]. The reported negative correlation was explained by the fact that total lipids increase preferably at the expense of TAG, whereas content of the membrane phospholipids, which are rich in n-3 PUFA remains fairly constant [21,37]. As a result, the proportions of EPA and DHA in muscle total lipids become diluted due to the accumulation of neutral lipids, which have high levels of monounsaturated FA. Although increase of total lipid content at the expense of TAG in fish muscles, as a rule, leads to decrease of n-3 LC-PUFA percentage, this does not mean that a concomitant decrease of nutritional quality of a fish occurs. Nutritional quality of fish products must be estimated on quantitative base expressed as mg FA per gram of tissue rather than percentage base [10].

Quantitative (mg per gram of tissue) measurements of TAG versus PL contribution in n-3 LC-PUFA of fish muscles are very scarce. Some studies gave indirect evidence of significant TAG contribution. For instance, among four fish species commercially harvested in Alaskan waters, arrow-tooth flounder *A. stomias* had maximum contents of EPA and DHA, 7.0 mg g^{-1}, as well as maximum levels of TAG,

80% of total lipids in edible muscles [47]. Myctophid fish species with higher total lipid content (proxy for TAG content) also had higher contents of EPA and DHA esterified as TAG [38].

Some direct measurements showed that lean fish contained less than half of EPA and DHA in their muscles esterified as TAG, e.g., wild white seabream *Diplodus sargus* [40], whitefish *Coregonus lavaretus* [43], six commercial Chilean marine species [46]. In contrast, the only studied medium-fat fish (2–4% lipid content of wet mass), Pacific sandperch *Prolatilus jugularis*, had approximately 60% of EPA+DHA esterified as TAG [46]. Similar to latter finding, farmed *C. lavaretus* which had one of the highest known values of EPA and DHA in muscles, 18.6 mg g^{-1} wet weight, had 61% of that value in TAG [43]. In our study, the fish species were strongly variable in lipid and total FA content and, as a result, in n-3 LC-PUFA content esterified as TAG. Like in the abovementioned studies, the fatty fish, muksun, whitefish and charr, had relatively higher content of EPA + DHA per mass unit that were mostly esterified as TAG (Figure 4B).

If we take the threshold of the recommended personal daily dose of EPA + DHA as 1 g and the average per serve portion of fish as 200 g [55,56], a fish of proper nutritional value should contain EPA + DHA nearly or more than 5 mg g^{-1} of filet [57]. The obtained data on lipid class composition and content mean that when such fish is consumed, nearly or more than half of the essential n-3 LC-PUFA comes as TAG form. Recent studies showed that bioavailability of FA, including LC-PUFA esterified as TAG may be lower than that esterified as PL [58,59], but see [60]. Thus, distribution of LC-PUFA in major lipid classes should be further addressed in studies of nutritional quality of various fish products.

5. Conclusions

The studied fish with similar feeding spectra were identified similarly by a multivariate analysis of FA profiles of total lipids, TAG and PL. Marker FA characteristic of diverse food sources (benthic, terrestrial, etc.), accumulated in nearly similar proportions within TAG and PL, and thereby allow to use both these fractions, as well as total lipids, for identification of food sources of wild fish. The found incorporation of the fatty acid trophic markers in structural polar lipids similarly to that in reserve TAG deserves further studies. Regarding contribution of TAG and PL into content of essential LC-PUFA of the taxonomically closely related fish species of order Salmoniformes, we found that content of EPA+DHA esterified as PL was nearly invariable, presenting presumably a species/taxon-specific optimal level. In contrast, content of EPA+DHA esterified as TAG greatly varied among the studied fish and provided most contribution to total EPA+DHA content in the fatty fish species, charr, whitefish and muksun. We can conclude that EPA+DHA-rich fish species likely accumulate these nutritionally valuable compounds predominately in the TAG form.

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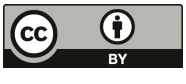

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Tiny but Fatty: Lipids and Fatty Acids in the Daubed Shanny (*Leptoclinus maculatus*), a Small Fish in Svalbard Waters

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Abstract: The seasonal dynamic of lipids and their fatty acid constituents in the lipid sac and muscles of pelagic postlarval *Leptoclinus maculatus*, an ecologically important fish species in the Arctic food nets, in Kongsfjord, Svalbard waters was studied. The determination of the qualitative and quantitative content of the total lipids (TLs), total phospholipids (PLs), triacylglycerols (TAGs), cholesterol (Chol), cholesterol esters (Chol esters) and wax esters was analyzed by TLC, the phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyelin (SM) were determined by HPLC, and fatty acids of total lipids using GC. The lipid sac is a system of cavities filled with lipids, and it is not directly connected to organs of the digestive system. The wall's inner layer is a multinuclear symplast that has a trophic function. The results provide additional knowledge on the role of lipids in the biochemical and physiological adaptation of fish to specific environments and clarify the relationship between fatty acids and the food specialization of postlarvae. Analysis of the fatty acid (FA) profile of TLs in the muscles and lipid sac of daubed shanny pelagic postlarvae showed it to be tissue- and organ-specific, and tightly associated with seasonal variations of environmental factors (temperature conditions and trophic resources).

Keywords: lipids; fatty acids; *Leptoclinus maculatus*; ontogenesis; adaptations; trophic nets; Arctic

1. Introduction

Lipids are one of the diverse, multifunctional, and basic groups of biochemical molecules. Among the numerous functions of lipids in the body, we can distinguish the following primary functions: in any cell, lipids are the basis of biological membranes, where proteins also act (lipids as structural molecules); lipids provide energy for metabolic reactions and processes (acting as energy producers or providing a storage function); and lipids provide a regulatory function that is carried out by biologically active molecules (eicosanoids). The diverse functions of lipids are carried out both intra- and intercellularly [1–6]. Individual lipid classes in the body, particularly those of fish, perform several functions, each of which is crucial in specific ecological and physiological conditions [7]. An organism's stability and sensitivity to various external influences or fluctuations of environmental factors is largely determined by peculiarities of lipid metabolism.

Fatty acids (FAs), which are mainly in a bound state in the body, are the most mobile components of lipid molecules and are characterized by multifunctionality [8]. Fatty acid constituents of lipids are incorporated into adaptive reactions relatively quickly, and under both normal and stress-induced conditions, various FAs provide the body with the choice of alternative ways (mechanisms) of response: regulation of the physical–chemical state of biomembranes, changes in enzyme activity without changing protein concentration, and the synthesis of biologically active mediators [9]. A major proportion of the fish lipid FA complexes are long-chain and highly unsaturated FAs, which emphasizes their important functional role in the body [8]. Moreover, Arctic marine organisms, including fish, accumulate high amounts of monounsaturated long-chain FAs (such as C20 and C22) that, together with polyunsaturated FAs (PUFAs), have significant biological effects on organisms, including humans.

Research on the composition and role of lipids and their FA constituents in fish inhabiting northern seas, as well as the processes of lipid transformation and transfer along the food chains of aquatic ecosystems, is essential for understanding the optimal functioning of all metabolic systems of the body in a changing environment (for example, daily, seasonal, and interannual). With the accumulation of lipids, and their dynamics and expenditure peculiarities for northern latitudes, aquatic organisms ensure the maintenance of vital activity and determine an individual survival rate under changing environmental factors and their combination, taking into account the life cycle of an organism.

Despite the severe environment in the Arctic, ichthyofauna is characterized by high plasticity and variability in life strategies and, in general, Arctic boreal marine ecosystems are very productive. The Arctic ichthyofauna is poorly studied in comparison to that of the Antarctic. There is a lack of up-to-date data on the study of lipids in ecological and biochemical adaptations of marine organisms, especially fish in the Arctic, taking into account that Arctic marine ecosystems are lipid-dependent, and lipids are considered vital molecules in northern latitudes.

The Stichaeidae family is an evolutionarily relatively young, extensive, and extremely diverse taxon [10–12] characterized by high adaptive capacities as well as plasticity to environmental factors and their fluctuations. The main directions of the research on representatives of this family around the world are aimed at obtaining new data in the fields of taxonomy, general biology, physiology, and biochemistry, studying the processes by which high-latitude aquatic organisms adapt to their environment [13,14]. Fish of the Stichaeidae family dwell in the bottom of the continental shelf in the coastal waters of marine ecosystems. The depth of their habitat varies greatly, and some species live in tidal currents. Thus, fish belong to the eurigaline group. Specific habitat conditions and a wide range of variations in environmental factors for fish from this family determine adaptability and plasticity.

Prolonged spatial continental isolation has led to the formation of suborder endemic species, including the *Lumpenus* and *Leptoclinus* genera that, among other things, define the northwestern Atlantic region of this suborder endemism [12,15,16]. The daubed shanny *Leptoclinus maculatus* (Fries, 1838) is a marine demersal species belonging to the Arctic boreal [17–19] zoogeographical group. Information about the biology of these species from the Stichaeidae family is not abundant [20–26]. Earlier [27–29], it was found that pelagic daubed shanny juveniles have a unique formation, namely, a lipid sac in the ventral part of their body. The lipid sac is a depot of lipids that ensures buoyancy and,

to some extent, phenotypic adaptation to pelagic conditions. It consists of large, densely packed lipid drops [27,28,30]. Later [28,29], it was found that postlarvae inhabit the pelagic zone until the age of 3–5 years, after which juveniles switch to a demersal mode of life. Several postlarval stages (L1–L5) have been distinguished for *L. maculatus* on the basis of age, size and weight, body color, and pigmentation, as well as the presence and parameters of the so-called lipid sac [28,29]. The lipid sac is resorbed after the fish become demersal, whereas in the pelagic young, it is distinct and formed by accumulating lipids from a diet of zooplankton (*Calanus*), which is their principal source of food [27,29,31–33].

The ecological role of *L. maculatus* becomes clearer upon a detailed examination of the trophic structure of Arctic and boreal waters, in which these species are among the main food sources for marine fish, mammals, and birds [34–36]. Studied fish species occupy a double niche in Arctic trophic chains, acting as both predator and prey [37]. The only detailed study of the contribution of the Stichaeidae family, including *L. maculatus* and *Lumpenus fabricii*, to the nutrition of northern birds [38] are results of the study of behavioral reactions of *Cepphus columba*, or pigeon guillemot, when feeding chicks in the Alaskan region, and includes an analysis of the composition and selection of objects as food. Despite the fact that some birds specialize in catching lipid-rich fish (for example, the northern Pacific sand lance *Ammodytes hexapterus* and Pacific herring *Clupea pallasii*), most prefer to produce food for chicks with fish species that are available and constant in the coastal zone, despite their lower calorie content, such as adults of Stichaeidae family.

The aim of the present study was to study the seasonal dynamics of lipids and fatty acids in the lipid sac and muscles of pelagic postlarval *L. maculatus* in Kongsfjord, Svalbard waters. The results can clarify the role of lipids in the adaptation of young fish to specific environments, and the relationship between fatty acids and postlarval food specialization.

2. Material and Methods

2.1. Sample Collection and Description

Leptoclinus maculatus pelagic postlarvae were collected in summer, winter, and spring in Kongsfjord (79°01′, 11°21′), Svalbard, using pelagic and bottom trawls on board the R/V *Helmer Hanssen* (UiT, Norway). The hydrological characteristics of the sampling sites are presented in Table 1.

Table 1. Hydrological characteristics of the sampling sites of *L. maculatus* postlarvae in Kongsfjord.

Sampling Fjord	**Kongsfjord**		
Season/Parameter	**T (°C)**	**Salinity (%)**	**Depth (M)**
Summer (end of July-beginning of August)	4.2	34.7	100
Winter (mid. January)	1	34.8	125
Spring (mid. April)	0.5	34.95	100

Daubed shanny juveniles are characterized by long-term development in the pelagic zone and relatively recently established multistage; stages are indicated by a Latin letter and number in the proposed system, and range from L1 to L5 [28,39] (Figure 1). Stages L1–L3 were mainly collected by pelagic trawl, and L4–L4 * by bottom trawl.

Postlarval stages of the daubed shanny differ in their morphological and physiological characteristics (length, weight, body color, pigmentation, and the presence and state of the lipid sac), and are also divided according to belonging to ecological groups (pelagic, transitional, demersal) [29,32,39] (Figure 1). The data on postembryonic growth and the developmental features of the juvenile *L. maculatus* and, in particular, changes in the length and state of the lipid sac during larval development, were presented for the first time in the work of S. N. Pekkoeva [29]. These data are unique for this species as they largely clarify some features of its ecology.

Figure 1. Stages of development of *L. maculatus* by Pekkoeva S.N., 2018 [29].

In the present research, lipid profiling was performed on postlarvae in the L3 developmental stage (Figure 2).

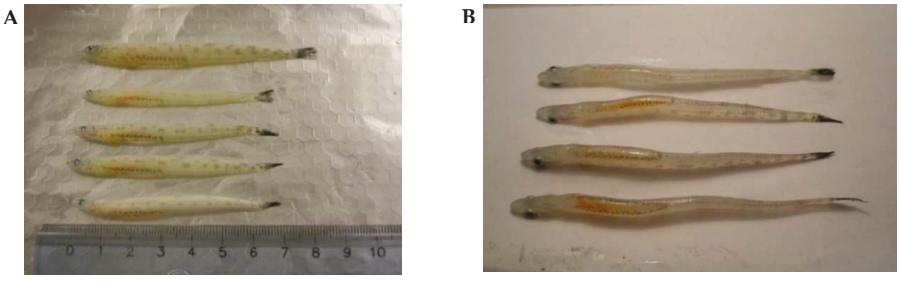

Figure 2. Pelagic postlarvae of *L. maculatus* (L3 stage of development) in summer. (**A**) A side view, (**B**) A view down the lipid sac.

Histomorphological studies of the lipid sac were presented on postlarvae in L2–L5 developmental stages.

All work was performed according to and within the regulations enforced by the Norwegian Animal welfare authorities and no specific permissions were required. The R/V Helmer Hanssen is owned by the University of Tromsø and has all necessary authorization from the Norwegian Fisheries Directorate to use a bottom trawl to collect fish for scientific purposes. Furthermore, the organisms are neither protected nor endangered in the coastal waters of the Svalbard Archipelago. Upon trawling, the fish were sacrificed by a sharp blow to the head and immediately dissected as specified below.

2.2. Lipid Extraction

The lipid sac and muscle of postlarval *L. maculatus* (L3 developmental stage) were immediately dissected from fresh fish aboard the vessel. Samples were homogenized in glass vials in a chloroform/methanol (2:1, v/v) solution (10 mL per 1–3 g wet weight). Total lipids (TLs) were extracted using the Folch method [40]. The homogenate was filtered, and the residue, retained on the paper filter, was rinsed with 30 mL of extractive mixture. The extract was then mixed after the addition chloroform and deionized water, and left to settle in the separatory glass funnel (Schott

Duran, Hamburg, Germany) until complete separation of organic phases. Lipids remained in the lower chloroform layer, whereas non-lipid substances moved to the upper aqueous methanol phase. The chloroform layer was then withdrawn to be evaporated under vacuum on rotary evaporator Hei-VAP Advantage HL/G3 (Heidolph, Schwabach, Germany), and dried in a vacuum exicator over phosphoric anhydride to constant weight. Total lipids were dissolved in chloroform/methanol and stored at −80 °C.

2.3. Lipid Class Analysis

Thin-layer chromatography (TLC) was used to identify the lipid classes as total phospholipids (PLs), triacylglycerols (TAGs), cholesterol (Chol), cholesterol esters (Chol esters), and wax esters. Fractionation of total lipids was performed on ultrapure glass HPTLC Silica gel 60 F_{254} Premium Purity plates (Merck, Darmstadt, Germany). Certain lipids, namely, PLs, TAGs, Chol esters, and wax esters, were quantified using the hydroxamate method that was modified by [41], which involves the formation of dark brown complexes of trivalent iron ions with hydroxamic acid through ester bonding between lipids and hydroxylamine [42]. Stain intensity was measured using a spectrophotometer (SF-2000, OKB "Spectr", St. Petersburg, Russia) at a wavelength of 540 nm. The quantitative determination of Chol was made on the basis of the method described in [43] by using trichloroacetic iron dissolved in perchloric acid. Stain intensity was measured using a spectrophotometer at a wavelength of 550 nm. Standard references and analytical standards for TLC (Sigma Aldrich, St. Louis, MO, USA; Avanti Polar Lipids Co., Alabaster, AL, USA) were used to distinguish the lipid classes.

The spectra of individual phospholipid fractions were determined by high-performance liquid chromatography (HPLC) using Aquilon Stayer HPLC (Aquilon LLC, Moscow, Russia) according to the method of Arduini [44], using a Nucleosil 100-7 C18 HPLC column with a acetonitrile/hexane/methanol/phosphorus acid (918:30:30:17.5, by volume) mobile phase, the rate of movement was 540 mkl/h, the volume of the injected sample was 5 µL, and the injector was Rheodyne 7725i. Detection was performed using a spectrophotometer (UV light, 206 nm), and the method was isocratic. Samples were manually injected using Rheodyne Valco Beckman and SSI Valves syringes (Hamilton, Reno, NV, USA). Phospholipid standards (Sigma Aldrich, St. Louis, MO, USA) were used for the identification and quantification of the phospholipid compounds in the sample. We identified six phospholipids: phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and sphingomyelin (SM).

2.4. Fatty Acid Analysis

The fatty acid profile of the total lipid extracts was analyzed by gas chromatography (GC). The methylation of fatty acids from the lipid extract was made in a glass retort in which 0.1 mL of a solution containing 20 mg/10 mL (behenic FA, C22:0) (Sigma Aldrich, St. Louis, MO, USA) in methanol was added as internal standard; then, we carried out transesterification in methanol (2 mL) containing chlorate acetyl (0.2 mL) at 70 °C for 90 min (using a Schott Duran glass serpentine condenser). After extraction, cooling with hexane was carried out in glass serpentine condensers that were rinsed with 5 mL of hexane for each sample. Then, 2 mL of deionized water was added to each glass retort for phase separation in the separatory glass funnels for 15 min. Fatty acid methyl esters (FAMEs) remained in the upper hexane layer, whereas other substances moved to the lower aqueous phase. The hexane layer was then withdrawn to be evaporated under vacuum on rotary evaporator Hei-VAP Advantage HL/G3 (Heidolph, Schwabach, Germany). Then, 0.9 mL of hexane for GC (Sigma Aldrich, St. Louis, MO, USA) was added to each glass retort, and the content was moved to the glass GC vials for the following GC analysis.

FAMEs were identified using a Chromatek-Crystall-5000.2 (Chromatek, Yoshkar-Ola, Russia) gas chromatograph with a flame-ionization detector (FID) and a Zebron ZB-FFAP capillary gas chromatographic column (Phenomenex, Torrance, CA, USA). An isothermal column configuration was used (200 °C); the temperatures of the detector and evaporator were 250 and 240 °C, respectively.

Chromatek-Analytik-5000.2 software (Chromatek, Yoshkar-Ola, Russia) was used for recording and integrating the data. FAMEs were identified with standards of Supelco 37 Component FAME mix, bacterial acid methyl ester (BAME), and PUFA No. 1 (Sigma Aldrich, St. Louis, MO, USA), and by comparing the equivalent lengths of carbon chains and table constants according to Jamieson [45].

2.5. Statistical Analysis (Lipid Study)

Data were analyzed to determine whether they exhibited normal distribution. Significant differences ($p \leq 0.05$) in the mean of the studied lipids and fatty acids between fish collected in different seasons were tested by one-way ANOVA. To perform statistical analysis, StatGraphics (Statpoints technologies, Inc., The Plains, VA, USA) and Microsoft Excel 10 (Windows 7, USA) were used.

2.6. Histological Analysis

The lipid sac of postlarval *L. maculatus* (L2–L5 stages) was dissected from fresh material and fixed in 4% formaldehyde (buffered) onboard the vessel. The tails were cut off from postlarval *L. maculatus* (L2 and L3 stages). In the postlarvae at the L4, L4 *, and L5 stages, the heads, pectoral fins, the dorsal part of the body, and tails were removed. In one case, the lipid sac was dissected from the body cavity, but it was found to be dissatisfactory. The material was washed in PBS, dehydrated, and embedded in paraplast (Leica, Wetzlar, Germany) according to the standard procedure. Serial parasagittal sections of 6–7 μm were cut using the sleigh microtome Leica SM 2010R (Leica Microsystems, Wetzlar, Germany). The sections were stained with Carazzi's hematoxylin and eosin (Biovitrum, St. Petersburg, Russia). Histological sections were studied using the light microscope Leica DMI6000 (Leica, Wetzlar, Germany). Measurements were made using Fiji software [46]. To minimize the risk of remeasuring the same nucleus, at least four sections were left between the measured ones.

2.7. Statistical Analysis (Histological Analysis)

Differences in the LSN lengths between L2 and L5 were evaluated using the Mann–Whitney U-test, with $p < 0.05$ indicating significant difference. All data are presented as mean ± SE.

3. Results

3.1. Histomorphology of the Lipid Sac of Postlarval L. Maculatus (L2–L5 Developmental Stages)

The histomorphological study of the lipid sac (LS) of postlarval *L. maculatus* confirmed that the LS is a complex of lipid-filled compartments that is not directly connected to the digestive system (Figures 3–5). The inner layer of these compartment walls is a multinucleate symplast, and the outer layer is a connective tissue [27]. Several layers of connective tissue and blood vessels are found in the walls. We named the LS symplast the lipid syncytial layer (LSL) by analogy with the yolk syncytial layer (YSL). Unstained lipid inclusions were seen in the LSL cytoplasm. The LSL nuclei (LSN) were generally round, elliptical, or elongated in sections. The LSN could be stained weakly or intensively with hematoxylin as a likely indication of differing eu- and heterochromatin content. The linear sizes of the LSN increase significantly by stage L5 (average length of LSN rose from 8.12 ± 0.17 μm at L2 (n = 185 where n is the number of nuclei) to 15.94 ± 0.3 at L5 (n = 250)).

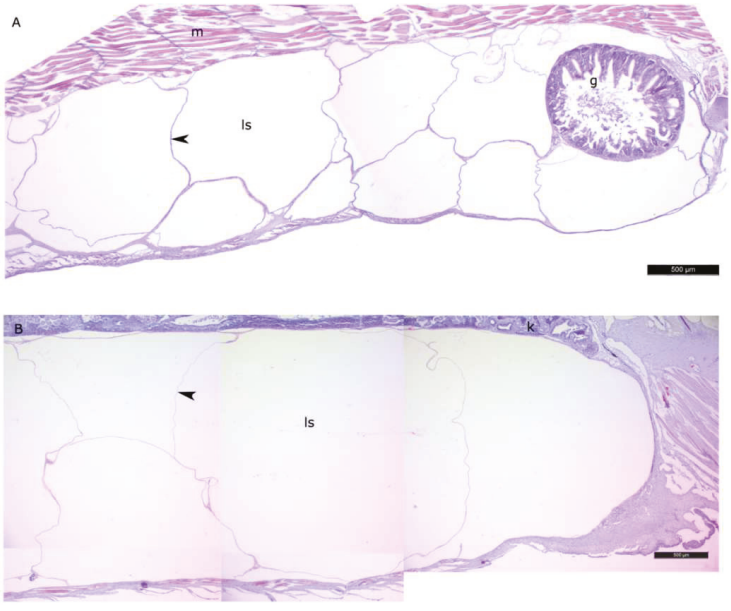

Figure 3. The general view of the lipid sac of postlarval daubed shanny. Parasagittal sections. (**A**)—L2 stage, (**B**)—L4 * stage. Figure legend: g—gut, ls—lipid sac, m—myomere, k—kidney. The walls of the lipid sac cavity are indicated with arrowheads. Staining: Carazzi's hematoxylin and eosin. Bar: 500 µm.

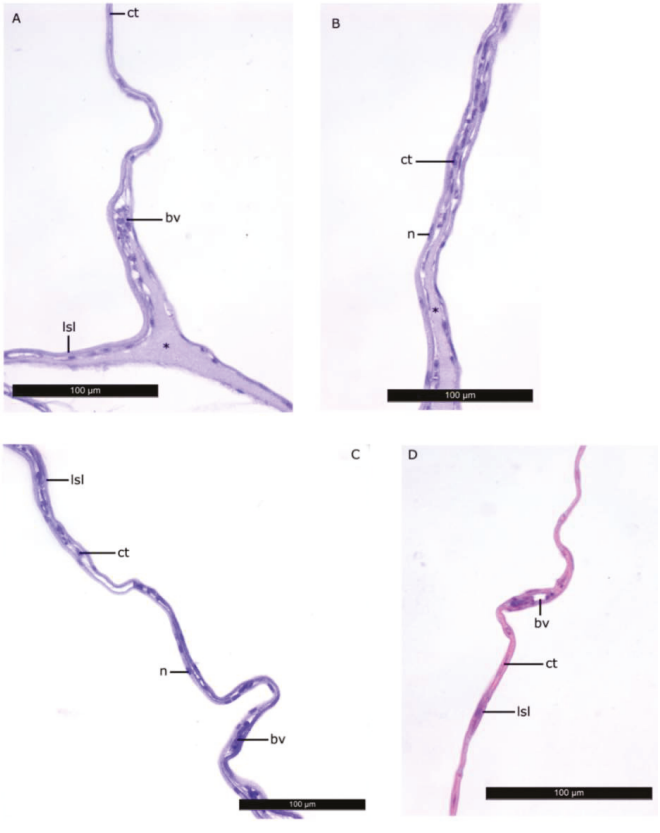

Figure 4. The walls of the lipid sac compartments. Parasagittal sections. (**A**–**C**)—L2 stage, (**D**)—L4 * stage. Figure legend: bv—blood vessel, lsl—lipid syncytial layer, ct—connective tissue, n—nucleus of LSL. The homogenous matrix between the compartments of LS. Staining: Carazzi's hematoxylin and eosin. Bar: 100 µm.

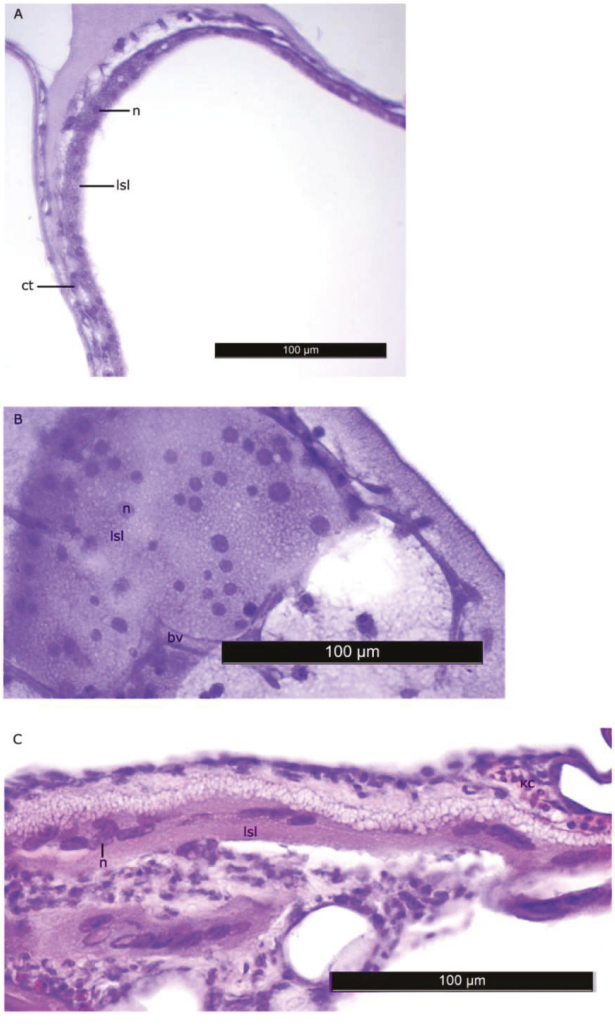

Figure 5. Nuclei of the LSL of LS at the L2 stage (**A**,**B**) and L5 stage (**C**). Nuclei generally have regular shapes, their linear sizes increase by the stage L5. Figure legend: bv—blood vessel, lsl– lipid syncytial layer, ct—connective tissue, n—nucleus of LSL. The homogenous matrix between the compartments of LS. Staining: Carazzi's hematoxylin and eosin. Bar: 100 µm.

3.2. Seasonal Dynamics of Total Lipids in the Lipid Sac and Muscles of Pelagic Postlarval (L3 Developmental Stage) L. Maculatus

The seasonal variation of total lipid (TL) content in the muscles and lipid sac of pelagic postlarval daubed shanny from Kongsfjord was determined in spring, summer and winter.

TL level was the lowest in muscles in the winter and spring (13.9% and 13.4% dry weight, respectively; no significant differences), whereas the content in muscles in the summer was significantly higher—27.9% dry weight. A different pattern of dynamic in TLs was observed in the lipid sac: the content increased significantly from summer to winter—64.4% and 75.9%, and decreased to 46.96% dry weight by spring (Table 2).

Table 2. Seasonal dynamics of total lipids and their individual classes (% of dry weight; % of total phospholipids for individual phospholipid classes) in the muscle and lipid sac of postlarval daubed shanny from Kongsfjord.

Season	Summer		Winter *		Spring	
Tissue/Organ	**Muscle**	**Lipid Sac**	**Muscle**	**Lipid Sac**	**Muscle**	**Lipid Sac**
n	15	15	40	40	12	12
TL	27.9 ± 3.00^{b}	64.4 ± 2.1^{a}	13.9 ± 0.5^{a}	75.9 ± 2.8^{b}	13.4 ± 0.4^{a}	46.9 ± 2.9^{c}
TAG	12.7 ± 1.8^{a}	58.3 ± 3.1^{a}	2.4 ± 0.2^{b}	68.8 ± 2.5^{b}	6.1 ± 1.0^{c}	28.1 ± 1.3^{c}
Chol esters+wax esters	1.7 ± 0.4^{a}	0	0.9 ± 0.1^{b}	3.02. ± 0.4	1.7 ± 0.2^{c}	2.6 ± 1.0
PL	9.6 ± 1.4^{a}	6.1 ± 1.7^{a}	7.2 ± 0.3^{b}	0.8 ± 0.3^{b}	2.5 ± 0.3^{c}	6.3 ± 1.3^{c}
PI	2.3 ± 1.0^{a}	1.1 ± 0.1^{a}	3.8 ± 0.2^{a}	2.4 ± 0.6^{b}	3.0 ± 0.2^{a}	2.3 ± 0.5^{b}
PS	1.0 ± 0.1^{a}	2.0 ± 0.1^{a}	3.4 ± 0.2^{b}	3.3 ± 0.2^{b}	3.1 ± 0.2^{b}	1.2 ± 0.4^{c}
PE	20.0 ± 1.8^{a}	18.1 ± 1.3^{a}	26.7 ± 0.8^{b}	22.2 ± 1.0^{b}	24.6 ± 1.0^{c}	20.1 ± 1.4^{c}
PC	74.5 ± 2.7^{a}	75.2 ± 2.1^{a}	64.5 ± 1.1^{b}	68.2 ± 1.5^{b}	62.3 ± 2.0^{b}	66.2 ± 2.0^{c}
LPC	0.3 ± 0.1^{a}	0.8 ± 0.2^{a}	0.1 ± 0.0^{b}	0.9 ± 0.2^{b}	0.2 ± 0.1^{b}	1.0 ± 0.8^{b}
SM	0.4 ± 0.0^{a}	0.5 ± 0.1^{a}	1.5 ± 0.1^{b}	2.5 ± 0.5^{b}	1.0 ± 0.1^{c}	2.0 ± 0.2^{b}
Chol	3.9 ± 1.2^{a}	0^{a}	3.5 ± 0.2^{a}	3.4 ± 0.4^{b}	3.1 ± 0.1^{a}	10.0 ± 2.5^{c}
Chol/PL	0.4 ± 0.1^{a}	0.01^{a}	0.5 ± 0.2^{b}	4.1 ± 1.2^{b}	1.3 ± 0.1^{c}	1.6 ± 0.8^{c}

TL—total lipid, TAG—triacylglycerol, Chol esters—cholesterol esters, PL—total phospholipids, PI—phosphatidylinositol, PS—phosphatidylserine, PE—phosphatidylethanolamine, PC—phosphatidylcholine, LPC—lysophosphatidylcholine, SM—sphingomyeline, Chol—cholesterol. Different uppercase letters indicate significant ($p \leq 0.05$) differences when comparing the values in the same type of tissue; n—number of tissues; *—values are given according to [29].

3.3. Seasonal Dynamics of Certain Lipid Classes in Lipid Sac and Muscles of Pelagic Postlarval L. Maculatus

The content of storage lipids in the form of triacylglycerols (TAGs), cholesterol esters (Chol esters) and wax esters, and membrane lipids in the form of phospholipids (PLs) in the muscles of the daubed shanny decreased significantly by winter compared to its levels in summer (2.4%, 0.9%, and 7.2% dry weight versus 12.7%, 1.7%, and 9.6%, respectively) (Table 2). The level of TAGs in the fish increased significantly in spring compared to winter (6.1% vs. 2.4% dry weight) but remained lower than in the summer (12.7% dry weight). The level of Chol esters and wax esters in the fish in spring rose to levels observed in the muscles in the summer (1.7% and 1.7% dry weight). The PL content in postlarval muscles was the lowest in spring (2.5% dry weight).

The level of total PLs in *L. maculatus* muscles was associated with variations in both the dominant phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (the former tended to decline while the latter increased from summer towards winter), as well as minor phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM), which arose in the fish from winter towards spring (Table 2). In the muscles of the postlarval daubed shanny, the seasonal variation of total PLs consisted of slight but statistically significant variations of PC and PE, as well as minor PI, PS, and SM, especially in the winter season (Table 2).

The lipid sac of daubed shanny postlarvae had the following changes from summer towards winter: storage TAGs increased from 58.3% to 68.8% dry weight, and trace amounts of Chol esters and wax esters increased from 0.1% to 3.0%, remaining at this level towards spring (2.6% dry weight). Total PL levels declined towards winter (from 6.1% in summer to 0.8% in winter), and by spring, PL content reached a level observed in the young in summer (6.3% vs. 6.1% dry weight). The Chol content in the lipid sac of postlarvae tended to rise from summer towards spring from trace amounts (0.1%) in summer to 3.4% in winter and 10.0% dry weight in spring. The Chol/PL ratio was the highest in the lipid sac of postlarvae in the winter season (4.1), the lowest in summer (0.01), and intermediate in spring (1.6) (Table 2). In spring, when the trend was a general decrease in TLs and TAGs in the lipid sac, the Chol/PL ratio (1.6) could be considered as an indicator of lipid components being utilized for maintaining the viability of postlarval fish (and, hence, the extraction of lipids from the sac).

3.4. Seasonal Dynamics of Certain Fatty Acids in the Lipid Sac and Muscles of Pelagic Postlarval L. Maculatus

Among FAs in the muscles of the studied fish in summer, monounsaturated FAs (MUFAs) were dominant (51.3% of the FA sum) due to 20:1(n-9) and 22:1(n-11) FAs (18.3% and 13.9% of the FA sum, respectively), which pointed to the prevalence of *Calanus* copepods in the diet (Figures 6 and 7). The muscles were also supplied with 16:1(n-7) and 18:1(n-9) FAs (6.2% and 6.2% of the FA sum, respectively), which are of phytoplanktonic origin (Figure 7). The second position in the FA profile in the muscles belonged to PUFAs (27.1% of the sum of FA), due to the (n-3)PUFA (23.3% of the sum of FAs) (Figure 6), including the essential 20:5 (n-3) and 22:6 (n-3), as well as another FA 18:4(n-3) (6.6%, 8.3%, and 4.6% of the FA sum, respectively) (Figure 8). The contribution of the (n-6) PUFA was not high, with 3.3% of the sum of FAs (Figure 6), where 18:2(n-6) and 20:4(n-6) accounted for 1.5% and 0.4% of the sum of the FAs, respectively (Figure 9). The saturated fatty acid (SFA) level was 21.6% of the sum of FAs due to 16:0, 14:0, and 18:0 FAs (12.5%, 6.5%, and 2.1% of the sum of FAs, respectively) (Figures 6 and 10).

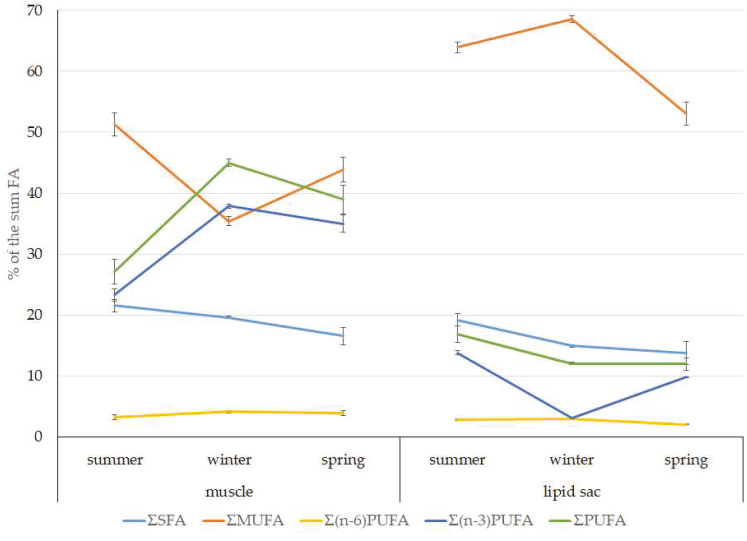

Figure 6. Seasonal dynamic of the saturated FA (SFA), monounsaturated FA (MUFA), polyunsaturated FA (PUFA) levels, including (n-3)PUFA and (n-6)PUFA (% of the sum of FAs) in muscle and the lipid sac in postlarval (L3 developmental stage) daubed shanny from Kongsfjord. The data presented as M ± SE.

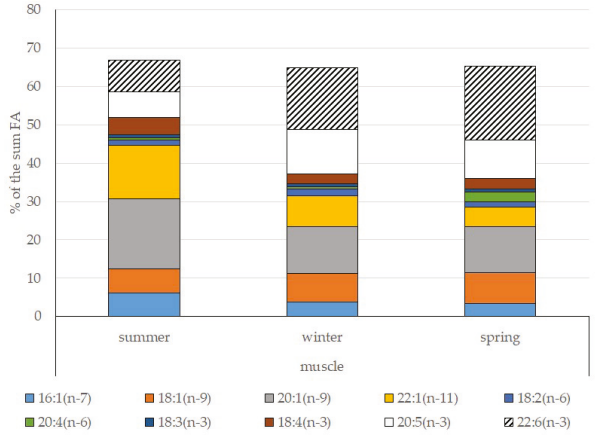

Figure 7. Seasonal dynamic of certain MUFA and PUFA levels (% of the sum of FAs) in muscle in postlarval (L3 developmental stage) daubed shanny from Kongsfjord.

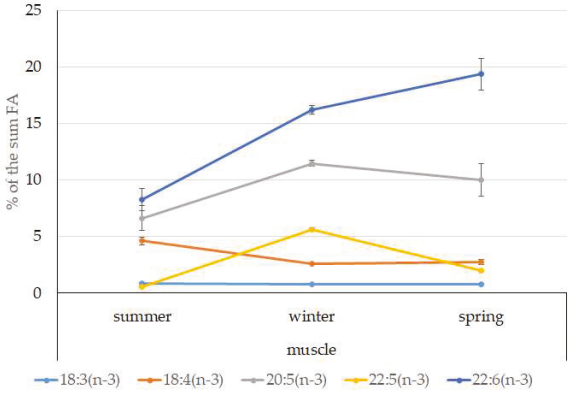

Figure 8. Seasonal dynamic of certain (n-3)PUFA levels (% of the sum of FAs) in muscle in postlarval (L3 developmental stage) daubed shanny from Kongsfjord. The data presented as M ± SE.

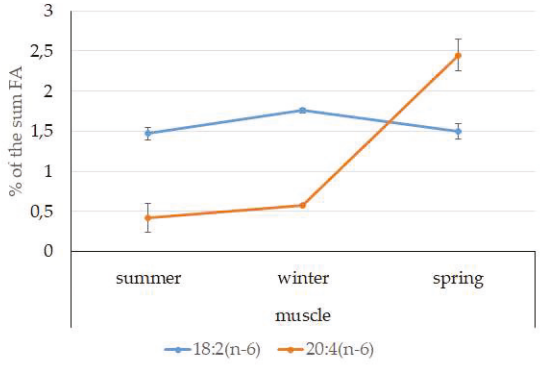

Figure 9. Seasonal dynamic of certain (n-6)PUFA levels (% of the sum of FAs) in muscle in postlarval (L3 developmental stage) daubed shanny from Kongsfjord. The data presented as M ± SE.

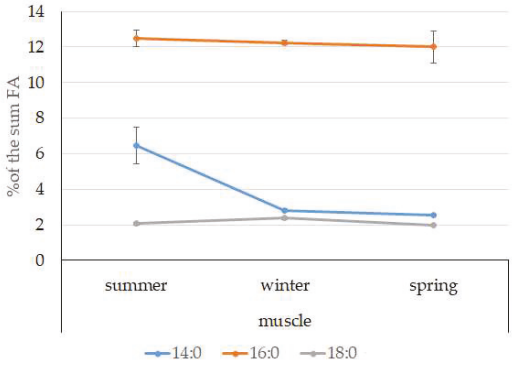

Figure 10. Seasonal dynamic of certain SFA levels (% of the sum of FAs) in muscle in postlarval (L3 developmental stage) daubed shanny from Kongsfjord. The data presented as M ± SE.

The lipid profile of muscles in winter had a different quantitative FA composition with the dominance of PUFAs, in which the level increased, compared to summer, to 45% of the sum of FAs due to (n-3)PUFA (37.9% of the sum of FAs), among which the essential 20:5(n-3), 22:6(n-3), and 22:5(n-3) FAs increased in proportion (11.5%, 16.2%, and 5.6% of the sum of FAs, respectively) (Figure 8). The level of (n-6)PUFA had also increased since summer, to 4.2% of the sum of FAs due to increased contents of 18:2(n-6) and 20:4(n-6) FAs (1.8% and 0.6% of the sum of FAs, respectively). MUFA amounts in the muscles of *L. maculatus* in winter decreased to 35.4% of the sum of FAs, mainly at the expense of 20:1(n-9), 22:1(n-11) FAs (12.2% and 8.1% of the sum of FAs, respectively). The decline in 16:1(n-7) and 18:1(n-9) FAs in fish muscles in winter was the least pronounced, but reliable compared to summer levels (3.8% and 7.5% vs. 6.2% and 2.1% of the sum of FAs, respectively).

MUFAs dominated in muscles in daubed shanny in spring, but their amounts were reliably lower than in summer (31.6% vs. 51.3% of the sum of FAs). The springtime FA profile of muscles featured a PUFA level that was significantly lower than that in summer or winter at the expense of the essential 20:5(n-3) and 22:6(n-3) FAs, with 11.2% and 19.3% of the sum of FAs, respectively. Curiously, it was in spring that the quantities of another essential FA of the (n-6)PUFA (arachidonic FA) reached a maximum in *L. maculatus*, at 2.5% of the sum of FAs. Variations in PUFAs and their individual FAs were correlated with changes in the content of some minor PL classes, namely, PI and PS.

The dominant group in the lipid sac (all seasons) was MUFAs (Figure 11), mainly due to 20:1(n-9) and 22:1(n-11) FAs (respectively 63.9%, 23.7%, and 19.8% of the sum of FAs (Figure 8)), derived from a copepod diet of *Calanus* spp. *Calanus* copepods synthesize these FAs de novo and are the main food for postlarvae at the L3 stage. Another MUFA present was 16:1(n-7), amounting to 8.4% of the sum of FAs. It is produced by diatoms and consumed by postlarvae through food. The second largest FA class in the lipid sac of the daubed shanny was SFAs, due to 16:0, 14:0, and 20:0 (19.2%, 7.9%, 5.5%, and 4.1% of the sum of FAs, respectively), which are the main constituents of the biomembranes of the lipid sac (Figure 12). Another component of membrane structures, alongside SFAs, are PUFAs (16.9%), due to (n-3)PUFA 20:5(n-3) and 22:6(n-3) FAs, which contributed 13.8%, 4.6%, and 3.9% of the sum of FAs, respectively (Figure 13). The FA profile of the lipid sac of postlarvae in winter was noted for its elevated MUFA content (68.6% of the sum of FAs), due to the dietary 20:1(n-9) and 22:1(n-11) FAs.

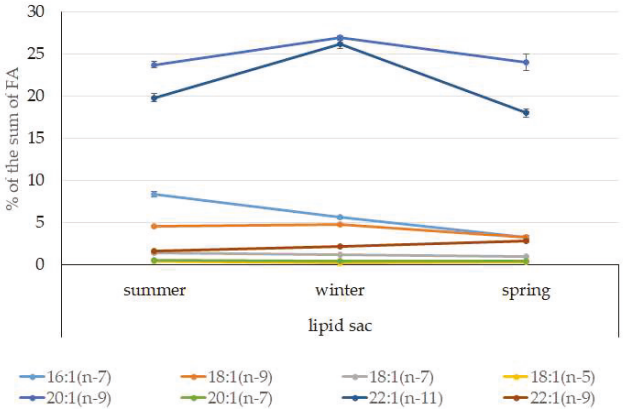

Figure 11. Seasonal dynamic of certain MUFA levels (% of the sum of FAs) in the lipid sac in postlarval (L3 developmental stage) daubed shanny from Kongsfjord. The data presented as M ± SE.

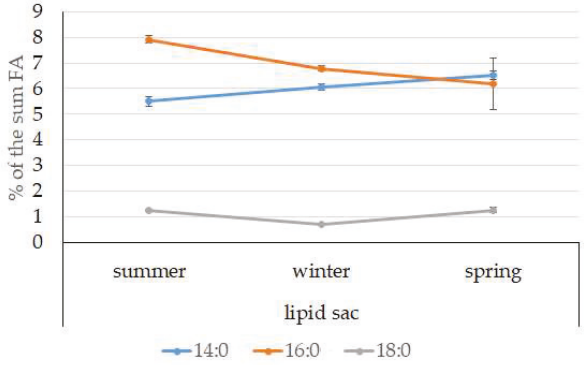

Figure 12. Seasonal dynamic of certain SFA levels (% of the sum of FAs) in the lipid sac in postlarval (L3 developmental stage) daubed shanny from Kongsfjord. The data presented as M ± SE.

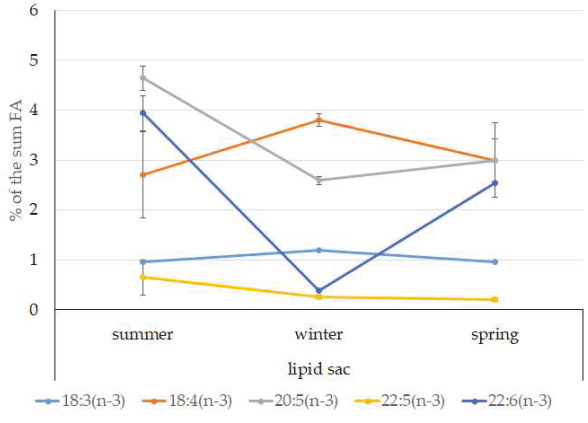

Figure 13. Seasonal dynamic of certain (n-3)PUFA levels (% of the sum of FAs) in the lipid sac in postlarval (L3 developmental stage) daubed shanny from Kongsfjord. The data presented as M ± SE.

According to the 18:1(n-9)/18:1(n-7) ratio, juveniles belong to the group of zooplanktonophages. This is also indicated by the dominance of 20:1(n-9) and 22:1(n-11) MUFAs, which are copepod biomarkers. Seasonal variations in the ratio of these FAs allow for tracking of seasonal variations in the species composition of copepods, the main food objects of juveniles, and their availability in different seasons of the year (Figure 14).

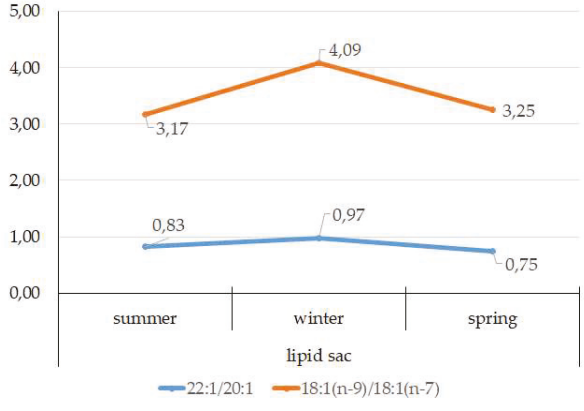

Figure 14. Seasonal dynamic of 22:1/20:1 and 18:1(n-9)/18:1(n-7) ratios in the lipid sac in postlarval (L3 developmental stage) daubed shanny from Kongsfjord. The data presented as M±SE.

4. Discussion

4.1. Histomorphology of the Lipid Sac of Postlarval L. maculatus

The lipid sac (LS) is found only in the circumpolar fish *L. maculatus* of the Stichaeidae family. The LS is a unique temporary structure and acts as one of the mechanisms for physiological and biochemical adaptation, helping young fish to survive and develop in northern latitudes [27–29,31–33].

Interestingly, the LSL and the YSL of bony fish have several important similarities, although these structures are not analogous or homologous: during development, their nuclei grow and their shape becomes more complex with different heterochromatin content throughout, and the functioning of symplasts intensifies during transitory developmental periods. A symplast with polyploid nuclei is one of the most widespread organizational variants of the extraembryonic system.

4.2. Dynamics of Total Lipid and Certain Lipid Classes in the Lipid Sac and Muscles of Postlarval L. maculatus

The lipid sac serves as a lipid depot, securing the viability and resilience of pelagic postlarvae in the severe environment of Svalbard waters, including prolonged winter and conditions of polar night. In addition, we found a significant decrease in TL content in the lipid sac by spring.

Pelagic postlarvae accumulate lipids during summer, which is the most productive but the shortest period in the Arctic. Typically, pelagic juveniles of *L. maculatus* at the L3 developmental stage are zooplanktivourous, fattening in the summer by consuming high energy food such as copepods, most abundantly represented by *Calanus finmarchicus* and *Canalus glacialis*. Copepods are the principal food components for the daubed shanny at this stage of development [29,32].

It has been presented [47,48] that copepods account for over 60% of the total number of species in the zooplankton community in Svalbard waters, the dominant ones being boreal oceanic *C. finmarchicus*; Arctic neritic *C. glacialis*; arctoboreal *Pseudocalanus* sp.; cosmopolitan *Oithona similis*; Arctic bathypelagic *Metridia longa*; oceanic cold-water *Microcalanus* sp.; and arctoboreal neritic *Acartia longiremis*. *C. finmarchicus* was shown to be abundantly present in Kongsfjord [47] due to the specific hydrobiological characteristics of this fjord. It appears that, like some other Arctic aquatic

organisms (e.g., zooplankton) that accumulate lipids in the body or in its special formations, deposition of a certain amount of lipids is a prerequisite for overwinter survival, as well as for providing energy for the growth and development of larvae and postlarvae of *L. maculatus*, as well as processes of metamorphoses. Furthermore, lipid accumulation in the lipid sac improves the buoyancy of postlarvae, and enables them to stay in the high-productive pelagic zone.

TLs in the muscles of young *L. maculatus* are accumulated and utilized exclusively for their direct function. The highest range of storage-lipid variation in the muscles of postlarval *L. maculatus* proves that these lipids are utilized to maintain energy-intensive metabolic processes that provide relevant postlarval locomotion. The decrease of storage lipids in TAGs that form from summer to winter points to their preferred utilization by the organism, as well as to a reduction of their amount due to limited nutrition in winter. The increase of TAGs and minor classes of storage lipids—represented by Chol esters and wax esters—in the muscles of the postlarval daubed shanny from winter towards spring can be regarded as an indicator of active feeding resumption, mainly on zooplankton. In muscles, the content of Chol, which is a major structural component of biomembranes, did not vary among seasons.

The resistance of aquatic organisms' biomembranes to various environmental factors largely depends on their lipid components. Changes in PL content and the ratios of their individual classes (mainly in muscles and the lipid sac) are key compensatory mechanisms in organisms that secure optimal performance of a large number of membrane-bound enzymes and their complexes [49] under a range of temperature conditions in the studied seasons. Furthermore, variations in PL quantities (mainly in PE, PS, and PI) and in FA components maintain and ensure required membrane fluidity, which is a property associated with adaptation to variable temperature conditions in the muscles of the daubed shanny [50–52]. Seasonal variations of water temperature in Kongsfjord (4.2 °C in summer, 1 °C in winter, and 0.5 °C in spring) activate the adaptive modifications of lipid components and influence the physical and chemical properties of cellular and subcellular membranes, as indicated by the PC to PE ratio in the muscles of postlarvae, as previously demonstrated for other aquatic organisms [53–55]. The mechanism of biomembrane adaptation to temperature reduction demonstrated for the muscles and gills of dark flounder *Pseudopleuronectes obscurus* [54] is based on oppositely directed variations of FAs within PC and PE: a rise in saturated FAs to polyunsaturated FA (SFA/PUFA) molecular form and a reduction in the ratio MUFA/PUFA, and PUFA/SFA in PC with the opposite trend observed for PE. Such changes in a certain set of the molecular forms of major membrane PLs are meant to maintain functional activity of the inner lipid monolayer and do not affect transformations in the outer monolayer of biomembranes, i.e., FA components of PLs are redistributed without a change in PL composition.

Functional biomembrane activity in the lipid sac, similar to the muscles of *L. maculatus* postlarvae exposed to variable temperature and foraging conditions in the studied seasons, is maintained both by changes in the quantities and ratios of individual PL classes, as well as through other biochemical mechanisms. One is a change in the Chol/PL ratio, which indicates variations in the microviscosity of biomembranes and their permeability mediated by key membrane lipids. A lower Chol/PL ratio in summer points to a higher permeability of biomembranes in the lipid sac as a result of utilization of lipid components for maintaining metabolic activity in this period. Thus, the lipid sac is a unique, metabolically active organ exemplifying successful physiological and biochemical adaptation of the young daubed shanny to living in high-latitude Arctic waters. Lipid sac formation, which begins with exogenous feeding in the larvae, is considered as an example of physiological and biochemical adaptation to fish habitats in the pelagic zone, contributing to their successful growth [29,56].

4.3. Seasonal Dynamic of Certain Fatty Acids of Total Lipids in Postlarval L. maculatus

The qualitative and quantitative composition of FA components of TLs in the muscles and lipid sac of *L. maculatus* postlarvae inhabiting Kongsfjord in summer reflects the dietary regime and specialization in this period, as well as tissue- and organ-specificity, which is defined both by metabolic features as well as by physiological and biochemical functions performed by these organs under given

environmental conditions. A manifestation of the latter is the selective integration of FAs derived from food into the lipid structure of muscles and the lipid sac, thus providing the fish with energy and structural components that the organism needs. This adaptive modification satisfies the organism's physiological demand for the accumulation of lipids with a certain structure in order to maintain a relevant metabolic level during long winters. Thus, the inclusion of 20:1(n-9) and 22:1(n-11) into the structure of energetic lipids (mainly TAGs) renders them metabolically active owing to the physical and chemical properties of these FAs, and helps satisfy the energy demand of daubed shanny postlarvae, even when water temperatures are low. Stage CV copepodites and adult copepods of the *Calanus* species have quite a high share of 20:1 and 22:1 FAs within the dominant energetic lipids class, namely, wax esters.

It was previously demonstrated that phytoplanktonic PUFAs can be partially transformed by zooplankton into SFAs and MUFAs, and can be deposited in their body as waxes, a long-term energy depot [57]. Thus, postlarval *L. maculatus* obtains essential phytoplankton-derived 20:5(n-3) and 22:6(n-3) FAs while feeding on zooplankton, which is a link for the transfer of essential FAs from primary producers to zooplankton feeders and higher-order consumers in high-latitude marine ecosystems, especially during the most productive summer period, when fish actively forage to accumulate lipid reserves.

Previously [29,58], it was shown that the dominance of young individuals (L2 and L3 developmental stages) in winter in Kongsfjord and older-age individuals (L5 stage) in the near-bottom layers of the water column corresponds with the division of the young daubed shanny into pelagic and demersal ecological groups. Similar changes in lifestyle and living conditions are also found in Antarctic fish [59,60]. In addition, studies [29,57] of the growth and early development patterns of the daubed shanny showed an increase in the length and weight of juveniles with age, increased growth at the age of 2+ (L2 stage), which is connected with the transition from phytoplankton feeding to high-energy zooplankton, while energy in the form of lipids is used on active growth. Thus, nutrition basis at the L1 development stage was phytoplankton-dominated by dinophyte algae, while a high level of biomarker 20:1(n-9) and 22:1(n-11) FAs from stage L2 reflects the nutrition of high-energy zooplankton of the *Calanus* genus, which is able to synthesize these FAs de novo [29,61]. Thus, the unique role of the postlarval daubed shanny in the transfer of matter and energy in the Arctic pelagic food chain with the participation of key chain links (phyto- and zooplankton), even in winter conditions, was also determined in this study. Interestingly, these results support the hypothesis about peculiarities of marine ecosystems functioning as a whole during polar nights in terms of the fact that the interaction of ecosystem components is determined by the season, e.g., despite the specific photoperiod, they actively interact with each other in winter, and zooplankton continue to make up a certain share in pelagic community structure, providing food to pelagic fish. The change of food objects and, accordingly, the type of food from phyto- to zooplankton in the juvenile daubed shanny, and then to carnivorous in adult individuals, was also shown for Antarctic silverfish *Pleurogramma antarcticum* [62], which is most numerous in East Antarctic waters [63].

By accumulating PUFAs in the TLs of its muscles, the postlarval daubed shanny ensures that membrane-bound enzymes and their complexes can function optimally when the functional load on the organ (e.g., swimming function and rheotaxis) or environmental conditions (e.g., temperature and salinity) changes. Polyunsaturated FAs largely define the inner structure of biological membranes and conditions for the activity of integral membrane proteins [64]. Some previously reported experiment data [64,65] suggested that if lipids with polyunsaturated chains are involved in the formation of the specific microenvironment of integral proteins then, by virtue of their physical and chemical properties, they can contribute to maintaining the relevant conformational mobility of these proteins and mitigate the negative effects of temperature changes on their activity, thus helping them to function normally. Furthermore, a sufficient supply of PUFAs during the development of *L. maculatus* postlarvae is necessary for their normal growth and development, since these compounds play important roles in regulating the activity of nerve cells in the formation of the vision system in fish,

which is particularly crucial for the pelagic young. Young fish suffering a deficit of PUFAs may exhibit abnormal behavioral reactions.

The elevated PUFA level in the muscles of postlarval *L. maculatus* in winter is most probably due to their accumulation during the fattening season, which is a necessary precondition for the optimal functioning of integral membrane enzymes under low temperatures (the temperature in Kongsfjord in winter is 1 °C) and for these PUFAs to co-maintain their general membrane structure together with the basic MUFAs in the PL structure, 18:1(n-9) FAs, whose level remains constant in the winter season. With regard to temperature adaptations, one should always keep in mind the important role of biomembranes and their sensitivity to changes in ambient temperature. Many processes going on in cellular and subcellular membranes are essential for the organism, including the biosynthesis of the membrane itself. It is membranes that set the temperature limits within which all systems can function normally and an organism remains viable.

An elevated content of the essential 20:4(n-6) FA in the muscles of postlarvae may point to the ongoing synthesis of lipid signal mediators, which are synthesized in a majority of tissues and are involved in the regulation of a series of physiological processes: immune response, inflammatory reactions, and functioning of vision and the nervous system [66,67].

The FA profile of the lipid sac of the postlarvae in winter was noted for its elevated MUFA content (68.6% of the sum of FAs) due to dietary 20:1(n-9) and 22:1(n-11) FAs, corroborating its key role as an energy storage organ and demonstrating a successful accumulation of lipids, both in quality and quantity, during the fattening season. Studies showed [47] that the wintering stocks of *C. glacialis* are chiefly made up of stage IV–V copepodites, concentrated at 150 m depth, and for *C. finmarchicus*, this is copepodite stage V, living at 50–150 m depths. An 18:1(n-9)/18:1(n-7) ratio allowed us to categorize the daubed shanny as a carnivorous group of animals. Juveniles belong to the group of zooplanktonophages according to the 18:1(n-9)/18:1(n-7) ratio (above 1).

The lipid sac of the postlarvae contained lower levels of PUFAs in winter, with a simultaneous rise in Chol content compared to that in summer. This combination may point to an increase in biomembrane viscosity and a corresponding reduction in the activity of lipid transport from the lipid sac to preserve them for utilization later during the winter.

There probably exists some threshold metabolic level in this age group (L3 developmental stage) of *L. maculatus*, tightly linked to the stressfulness of environmental conditions in winter, and until this threshold is reached, lipids stored in the lipid sac are moderately utilized to support basic functions. This statement is confirmed by the reduced MUFA level (to 53% of the sum of FAs), including 20:1(n-9) and 22:1(n-11), in the lipid sac of postlarvae. However, the spring sampling of the young daubed shanny in Kongsfjord took place on 21 April, when phytoplankton was blooming (*Chl a* level was within 1.6–1.9 μg/L; 51% due to diatoms), and *Calanus* spp. copepods were present in the area [68]. Phytophagous copepods with pronounced ontogenetic (seasonal) migrations display a distinct correlation with the onset of the high-productivity period, when the vernal phytoplankton outburst takes place. At this point, the overwintered stage V–VI copepodites concentrate close to the surface for the start of the breeding season [47]. Meteorological conditions in this period of spring strongly fluctuated (wind speed up to 8.2 m/s and its directions), causing the *Chl a* level to fall below 0.5 μg/L and resulting in vertical water mixing and cooling, especially down to a depth of 60 m. The vertical redistribution of the phytoplankton biomass happening in the process caused transient breaks in its blooming, and a redistribution of phytoplankton species ratios and its low biomass were therefore observed [69,70]. Following these events, in the period from 28 April to 12 May, the conditions at our study site in the fjord were shown to have stabilized [68]. Thus, the lowest MUFA level in the lipid sac was detected in springtime, possibly due to their utilization within lipids to support the vitality of postlarval fish under the specific conditions of this period.

Observed quantitative MUFA and PUFA variations testified to their particular roles and evidence that the FA composition of lipids is specific to the studied organs, in accordance with the function they perform under given and variable environmental conditions. Certain FAs are retained in

the structure of lipids to enhance their metabolic value, both to facilitate a general adaptation of postlarvae to overwintering, and to provide specific compensatory reactions adjusting an organism to seasonal environment.

MUFAs dominated in muscles in the studied fish in spring, but their amounts were reliably lower than those in summer (31.56% vs. 51.30% of the sum of FAs). The springtime FA profile of the muscles of the postlarvae featured a PUFA level that was reliably lower than that in summer or winter at the expense of the essential 20:5 (n-3) and 22:6 (n-3) FAs—11.15% and 19.34% of the sum of FAs, respectively. Curiously, the quantities of another essential FA of the n-6 family (arachidonic FA) reached a maximum in *L. maculatus* (2.45% of the sum of FAs) in spring. Variations in PUFAs and their individual FAs were correlated with changes in the content of some minor PL classes, namely, PI and PS. This resulted in an alteration in the activity of biomembrane enzymes due to modification of their lipid environment. Furthermore, in a stressful environment, FA components perform the function of lipid mediators, becoming integrated in the structure of their molecules. As mentioned above, the water temperature in Kongsfjord is 0.5 °C in spring. The observed lipid and FA profile of postlarval muscles in springtime is indicative of their role in the adaptation to the highly variable meteorological and hydrological conditions in the habitat in this season, which most probably demand intensified locomotion, orientation in the water column, and relevant provision for corresponding metabolic functions (energy supply and signal transduction in tissue by means of lipid-type mediators). In addition to their structural function, the minor PI and its physiologically active metabolites (inositol, triphosphate, diacylglycerols) are known to modulate the activity of the phosphotransferase protein kinase C, which is particularly important for cell growth and differentiation in young fish [71].

Thus, compensatory modifications in the composition of lipids and their FA components in the muscles and lipid sac of typically pelagic L3 stage postlarvae of the daubed shanny living under highly variable environment (primarily temperature and foraging conditions, as well as photoperiod) secure the viability of this Arctic fish species. Owing to some of its specific physiological and biochemical traits (e.g., the lipid sac in its postlarvae), the daubed shanny has a marked ecological role and significance in the marine ecosystem's food chains [72].

5. Conclusions

This paper presents results on the seasonal dynamics of lipids and their FA components in the muscles and the lipid sac of postlarval *L. maculatus* inhabiting Kongsfjord in the summer, winter, and spring in the Arctic, with particular attention paid to the role of lipids in the adaptation of young fish to seasonal variations in temperature and photoperiods (abiotic factors), as well as an important biotic factors associated with the number, species composition, and availability of food objects. Biochemical analysis was carried out for the typically pelagic L3 developmental stage of the juvenile *L. maculatus*, which is predominantly zooplanktovorous.

Both general and numerous specific biochemical adaptation mechanisms of the compensatory and exploitative variety, which were found in daubed shanny juveniles, involve lipid participation, and their high plasticity can be explained by the evolutionary "youth" of this species, formed due to the movement of its Pacific ancestors to the North Atlantic about 3.0–3.5 million years ago [12], in addition to prolonged spatial continental isolation, which led to formation of an endemic species of the *Leptoclinus* genus [12].

Among specific adaptations, we have to highlight:

- The presence of a unique formation, in the form of the *lipid sac*, is a specific ecological and biochemical adaptation in the early development of daubed shanny. The lipid sac symplast of the juvenile daubed shanny is a structure that was discovered and described for the first time;
- The lipid sac accumulates a large amount of lipids, mainly TAGs and specific MUFAs of food origin, 20:1(n-11) and 22:1(n-9), indicating that *Calanus* spp., which form the basis of the Arctic zooplankton biomass, dominate in the diet of juveniles. The established seasonal dynamics and changes in the ratios of these FAs indicate different species composition and accessibility of

copepods in the studied fjords (mainly Kongsfjord, Svalbard) for the pelagic juveniles of the daubed shanny. Tracking the movement of food-derived FAs determines the basis for the chemoindication of qualitative and quantitative relationships between zooplankton and zooplanktophages in Arctic marine food chains;

- A slight but significant increase in the level of derived from food FAs in the lipid sac in spring is synchronized with the beginning of the spring bloom of phytoplankton and the appearance of stage V–VI copepods in the pelagic water layers to start a reproductive period. Interestingly, the inclusion of long-chain 20:1(n-9) and 22:1(n-11) FAs in the structure of energy lipids (mainly TAGs) of the lipid sac of the daubed shanny maintains their energy value, which brings on the corresponding needs of juveniles, even at low temperatures during the long winter period in the Arctic;
- A minor class of energy lipids in the form of Chol esters and waxes is only found in the lipid sac of juveniles in the winter–spring period, which reflects the peculiarities of their nutrition and lipid accumulation.

Among the general adaptation mechanisms, a high unsaturation of lipids was shown for daubed shanny juveniles due to their FA components, characteristic of inhabitants of marine ecosystems of northern latitudes. A high degree of lipid unsaturation is supported, first, due to the dominance of MUFAs, among which oleic acid 18:1(n-9), that is present in the structure of almost all lipids, both structural and primarily storage lipids, is one of the main biochemical component. The second position is often competitively occupied by PUFAs and/or SFAs. In this case, we should mention the relationship between the results of this work and theoretical studies of the properties of FA chains (the shape and flexibility of FA chains) in an unperturbed state, obtained by computer simulation with the Monte Carlo method [63,64,73,74]. In particular, it was found that FA chains (16:0, 18:1(n-9)*cis*, 18:2(n-6)*cis*, 18:3(n-3)*cis*, 20:3(n-6)*cis*, 20:4(n-6) *cis*, 20:5(n-3)*cis*, 22:5(n-3)*cis*, 22:6(n-3)*cis*), i.e., key FAs for the studied fish, have equal longitudinal sizes due to which molecular substitutions, manipulations, and complementarity (in the properties) of these FAs in the PL structure achieve constant thickness of the membrane hydrocarbon layer and adjust its fluidity, which ensures the functionality of the lipid bilayer under changing environmental factors.

In addition, for fish of the Stichaeidae family, both juveniles and adults [75], lipid unsaturation is achieved due to PUFAs, especially EPA and DHA, in muscle tissue, which is consistent with the increased motor activity of fish in specific habitat conditions (currents, turbulent flows, etc.) and, ultimately, is directed to ensure homeostasis of the internal environment to maintain optimal activity of membrane-bound enzymes with a general increase in the level of metabolism. At the same time, attention should be drawn to the fact that this phenomenon is apparently based on a mechanism that is associated with certain properties: increased flexibility (which is associated with the fluidity of the biomembrane) for PUFAs, i.e., EPA and DHA, compared to SFAs. For example, with an increase in the metabolic load in fish muscle tissue in a specific environment (low temperatures, increased concentration of dissolved oxygen, fluctuations in salinity, etc.), the full functioning of biomembranes is maintained due to the control of such signs as thickness and resistance to mechanical action (with the active work of proteins) and the provision of a thermally insulating function for membrane-bound enzymes in the individual domains of the lipid bilayer. For young marine fish living at low temperatures, the increased level of EPA and DHA is due to their adaptive significance of overcoming the negative impact of fluctuations of this factor on the growth and development of juveniles. Thus, the experimental results of FA status in fish of the northern seas obtained in this work are consistent with the previously formulated concept of the functions of polyunsaturated chains [63,64,73,76].

The conducted studies allowed us to identify the general and specific features of the biology of the daubed shanny, an ecologically important representative of the Arctic ichthyofauna. Further research on the daubed shanny as a component of the Arctic trophic net will be focused on the study of the abilities and/or limitations of biosynthesis of certain MUFAs and PUFAs, including food-derived ones,

that can compensate environmental changes and maintain the presence of the species in the northern marine ecosystems.

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Review

Assessment of Fatty Acid Desaturase (Fads2) Structure-Function Properties in Fish in the Context of Environmental Adaptations and as a Target for Genetic Engineering

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Abstract: Fatty acid desaturase 2 (Fads2) is the key enzyme of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis. Endogenous production of these biomolecules in vertebrates, if present, is insufficient to meet demand. Hence, LC-PUFA are considered as conditionally essential. At present, however, LC-PUFA are globally limited nutrients due to anthropogenic factors. Research attention has therefore been paid to finding ways to maximize endogenous LC-PUFA production, especially in production species, whereby deeper knowledge on molecular mechanisms of enzymatic steps involved is being generated. This review first briefly informs about the milestones in the history of LC-PUFA essentiality exploration before it focuses on the main aim—to highlight the fascinating Fads2 potential to play roles fundamental to adaptation to novel environmental conditions. Investigations are summarized to elucidate on the evolutionary history of fish Fads2, providing an explanation for the remarkable plasticity of this enzyme in fish. Furthermore, structural implications of Fads2 substrate specificity are discussed and some relevant studies performed on organisms other than fish are mentioned in cases when such studies have to date not been conducted on fish models. The importance of Fads2 in the context of growing aquaculture demand and dwindling LC-PUFA supply is depicted and a few remedies in the form of genetic engineering to improve endogenous production of these biomolecules are outlined.

Keywords: fatty acyl desaturase; Δ6 - desaturase; long-chain polyunsaturated fatty acid; LC-PUFA; ω3; ω6; EPA; DHA; AA; essential fatty acid; health; fish; transgene

1. Introduction

Fatty acid desaturase 2 (Fads2) is an endoplasmic reticulum membrane bound protein which acts as the first enzyme in the biosynthesis of long chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA). This pathway includes physiologically important eicosapentaenoic acid (EPA, ω3-20:5^{5,8,11,14,17}), docosahexaenoic acid (DHA, ω3-22:6^{4,7,10,13,16,19}), and arachidonic acid (AA, ω6-20:4^{5,8,11,14}) which are produced from the shorter and lower level polyunsaturated fatty acids (PUFA) α-linolenic acid (ALA, ω3-18:3^{6,9,12}) and linoleic acid (LA, ω6-18:2^{9,12}). Human and many fish genomes encode for Fads2 as well as for some other enzymes acting in the LC-PUFA biosynthetic pathway, namely Fads1, elongase 5 (Elovl5), elongase 4 (Elovl4) or elongase 2 (Elovl2). LC-PUFA are often referred as conditionally essential nutrients, meaning that however the organism could be capable to produce them, this endogenous production is insufficient to meet demand, hence, LC-PUFA biomolecules must

be obtained through the diet. Endogenous production is hypothesised to serve as a compensation apparatus, which helps the organisms to maintain homeostasis under fluctuating environmental conditions and LC-PUFA availability. This could be the reason why marine fish, unlike freshwater fish, do not have the capability to produce LC-PUFA at a significant level [1] as a consequence of living in nutritionally rich oceans. In contrast to the conditional-essentiality of LC-PUFA, precursors of LC-PUFA, LA and ALA, cannot be created de novo in nearly any living animal, since their genomes do not encode for enzymes capable to create them (such would be methyl-end desaturases with Δ12 and Δ15 activity converting oleic acid (18:1n-9) into LA (18:2n-6) and ALA (18:3n-3)) [2]. Hence, animals are usually dependent on plants for providing double bonds in the Δ12 and Δ15 positions of the two major precursors of the ω6 and ω3 fatty acids LA and ALA [3]. These two fatty acids, therefore, are called essential fatty acids. In the literature, however, most often, the conditional-essentiality of AA, EPA and DHA for vertebrates and humans is not considered and with LA and ALA, these biomolecules are altogether called essential fatty acids (EFA).

The exogenous supply of EFA for many animals, including some omnivorous terrestrial animals and humans, is from aquatic ecosystems. In aquatic ecosystems, substantial amounts of EPA and DHA are provided by primary producers. Historically, the primary production of these biomolecules has been associated exclusively to single-cell microorganisms such as photosynthetic microalgae, heterotrophic protists and bacteria. Recently, however, multiple invertebrates, many of them representing abundant groups in aquatic ecosystems, have been confirmed to be able to produce PUFA de novo and farther biosynthesize them into ω3 LC-PUFA similarly to single-cell microorganisms [4,5]. Once synthesized by microalgae or invertebrates, these biomolecules are transferred through trophic webs to organisms of higher trophic levels. Fish are considered as the best source of ω3 LC-PUFA for humans. However, anthropogenic factors such as pollution, eutrophication, climate change or biological invasions threaten the LC-PUFA production by primary producers at present. World capture fishery production cannot be increased and aquaculture is expected to be continuously growing to deliver food to humans. Here, this could be seen a paradox. While aquaculture has increasingly become the major source of EPA and DHA for humans, it has also, at the same time, become the greatest consumer of the world's available supply of EPA and DHA. The problem of bridging the gap between supply and demand of LC-PUFA was very recently excellently reviewed by Tocher et al. [1]. Since LC-PUFA have been identified as globally limited nutrients, the ability of an organism to compensate for dietary deficiencies of LC-PUFA by enhanced activity of its endogenous biosynthesis is of great importance for human and animal health as well as for the maintenance of fish as an EFA source for the human diet and aquaculture food.

Although it has been confirmed that alterations in activities of elongases Elovl4 and Elovl5 catalysing subsequent steps in LC-PUFA biosynthesis can alter EPA, DHA and AA production by promoting various disease states [6], Fads2 is still commonly considered as the rate-limiting and the most important enzyme of LC-PUFA biosynthesis. Moreover, in light of some recent significant publications, Fads2 appears as an enzyme with far-reaching implications for environmental sustainability.

2. Significance and Essentiality of LC-PUFA Biomolecules

LC-PUFA are important components of fat and in higher eukaryotes confer fluidity, flexibility and selective permeability to cellular membranes. They greatly influence many physiological processes. Participation of LC-PUFA in several major human pathologies (inflammatory-autoimmune diseases, cardiovascular diseases, cancer and neurodegenerative disorders) has been reviewed recently by Zárate et al. [7]. The pivotal role of lipids as an essential dietary component is now widely accepted; however, many decades of research have gone into this conclusion, which has recently been very well reviewed by Spector and Kim [8]. Fish played an essential role in coming to this conclusion. The essentiality of any fatty acid biomolecule was, for the very first time, reported in what was at the time highly controversial scientific work of George Oswald Burr in 1929 [9,10]. He and co-workers demonstrated that LA and ALA rescued the growth retardance phenotype in rats fed fat-free diet and found the first clue that LA is a precursor of AA. The concept of essential fatty acids appeared [11]. But it was before

chain desaturation or elongation of fatty acids had been demonstrated and the authors wondered how two double bonds + two double bonds could equal four [12], as was elucidated later in series of studies conducted by Mead et al. [13]. The linkage between LA, its ω6 fatty acid desaturation and elongation products, and the formation of prostaglandins as biomediators was reported by Bergström et al. [14,15], who stated" ... *the symptoms of essential fatty acid deficiency at least partly are due to an inadequate biosynthesis of the various members of the prostaglandin hormone system.*" The pathway through which ALA is converted into EPA and DHA was determined in 1960 by Klenk et al. [16]. Noteworthily, no important functions were attributed to ω3 fatty acids for a long time. However, this changed in 1968 when Dr. Jørn Dyerberg made the remarkable discovery that fats in the diet of Greenland Eskimos comprised mainly of fish were associated with a lowered risk of cardiovascular diseases, concretely, that plasma of these people contained large amount of ω3 fatty acids and their phospholipids contained high levels of EPA, but very little AA [17]. Their conclusion was that EPA protects against cardiovascular diseases [18]. Without a doubt, the paradigm had been changed and fatty acids were no more considered to fulfil the only function in energy storage. Widespread interest was awoken in investigations of unsaturated fatty acids as biomolecules indispensable for health. Numerous global and national health agencies and associations and government bodies have produced many recommendations for EFA intake for a healthy human diet through fish consumption. With the advent of molecular and genetic technologies, there appeared much evidence that a balanced abundance of EFA is a prerequisite for health and disease prevention in humans [19–21]. Meeting the dietary demands of a burgeoning human population with a correct dietary balance of EFA and at levels required for normal health and development has become a major challenge. It has been clear that understanding the molecular basis of LC-PUFA biosynthesis would underpin efforts to meet this challenge. Various strategies of human populations regarding EFA metabolism have been shown by Gladyshev and Sushchik this year [22]. Studies performed in fish are advantageous mainly because there is wide variation between fish species in their ability to biosynthesize LC-PUFA, probably as a consequence of inhabiting widely different environments. Comparisons of their genomes and expression levels of genes encoding key elements in the LC-PUFA biosynthetic pathway between species have been promising to increase knowledge of the molecular components of the pathway and of the molecular genetic basis of phenotypic variation in LC-PUFA biosynthesis.

3. Fads2 in LC-PUFA Biosynthesis

The biosynthesis of $C_{20\text{-}22}$ LC-PUFA involves alternating steps of desaturation (introduction of an additional double bond) and elongation (addition of two carbons) of the dietary essential C_{18} fatty acids LA and ALA [1]. Firstly, in the biosynthesis of LC-PUFA, Δ6 Fads2 desaturase converts dietary obtained LA (18:2n-6) and ALA (18:3n-3) into gamma-linoleic acid (GLA) and stearidonic acid, respectively. Subsequently, in the biosynthesis of LC-PUFAs of ω3 series, it converts tetracosapentaenoic acid into tetracosahexaenoic acid which is then converted to DHA. Enzymatic steps in the biosynthesis of LC-PUFAs in vertebrates are shown in Figure 1 [23–30]. AA and EPA are biosynthesized in the same pathway in which LA and ALA substrates compete of the same enzymes, respectively. The pathway revealed from studies in vertebrates are the so-called "Δ6 pathway" (Δ6 desaturation–elongation–Δ5 desaturation) and the "Δ8 pathway" (elongation–Δ8 desaturation–Δ5 desaturation). DHA is achieved downstream in the biosynthesis of LC-PUFA from EPA via two alternative routes. Either, two consecutive elongations of EPA produce tetracosapentaenoic acid (TPA, 24:5n-3), which then undergoes a Δ6 desaturation to tetracosahexaenoic acid (THA, 24:6n-3), the latter being β-oxidised to DHA in peroxisome organelles following the translocation from endoplasmic reticulum, the so-called "Sprecher pathway" identified in mammals [24,31], or, the direct Δ4 desaturation of docosapentaenoic acid (DPA, 22:5n-3) into DHA via the 'Δ4 route´. The first Fads2 gene with Δ4 activity was identified in the marine herbivorous fish *Siganus canaliculatus* [32]. It was not only the first enzyme with this activity among fish, but it was the first discovered case in all vertebrates. The discovery indicated that there exists another possible mechanism for DHA biosynthesis, a direct route involving elongation of EPA

to 22:5n-3 followed by Δ4 desaturation. If both DHA routes were coexist, this would represent a clear advantage for satisfying DHA requirements through endogenous production. After further identification of 11 teleost species having a putative Δ4 Fads2 by Oboh et al. [33], it was made clear that the direct Δ4 pathway is more widespread among teleost fish than initially believed.

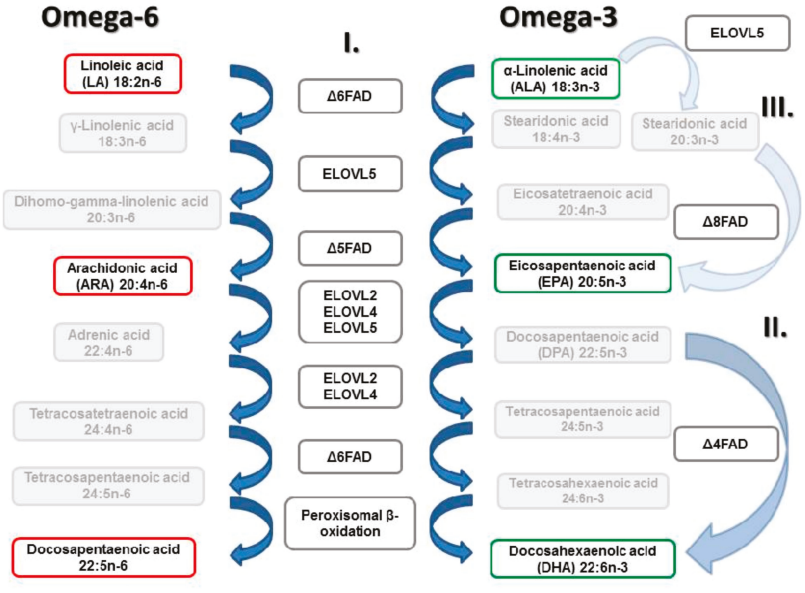

Figure 1. Biosynthetic pathways of long-chain (≥ C_{20}) polyunsaturated fatty acids (LC-PUFAs) of ω3 and ω6 families from dietary essential α-linolenic (ALA, 18:3n-3) and linoleic (LA, 18:2n-6) acids, respectively, by elongation and desaturation reactions. Adapted from (Carmona-Antoñanzas et al., 2011; Monroig et al., 2011; Sprecher, 2000; Voss et al., 1991); modified after (Trattner, 2009; Vestergeren, 2014; Yan, 2016).

4. Fads Gene Repertoire in Fish

There is a fundamental difference between fish and mammals regarding the gene repertoire encoding for enzymes performing desaturation activities needed for LC-PUFA biomolecules production. In contrast to mammals, where distinct separate genes *Fads1* and *Fads2* encode enzymes Fads1 and Fads2 with appropriate specificities Δ5 and Δ6 [34], respectively, in fish, *Fads1* gene has been lost during the evolution. As a result, all desaturation steps of the LC-PUFA biosynthetic pathway in fish are catalysed by Fads2 enzymes, exhibiting different Δ activities which can be overlapping to some extent.

Until recently, this scenario was generally accepted with no exceptions. Accordingly, one single Δ6 Fads2 appears most often [35–38]. Less often, a separate Δ6 Fads2 and Δ5 Fads2 paralogues appeared, such as in Atlantic salmon (*Salmo salar)* [39,40], or more than one single Δ6 Fads2 paralog are present such as in common carp (*Cyprinus carpio*) [41] and recently confirmed in numerous Osteoglossomorpha species [42]. In some teleosts studied, Δ6 Fads2 had measurable levels of Δ5 activity [37] or Δ8 activity [42,43]. A single bifunctional Δ6/Δ5 Fads2 acts in zebrafish (*Danio rerio*) [44] which was the first functionally characterized fish desaturase and for some time, it has been considered as an exception, not only in fish but in vertebrates in general. Later, two desaturases from marine rabbitfish (*Siganus canaliculatus*) were functionally characterized, one of which was shown to be Δ6/Δ5 bifunctional and the other Δ5/Δ4 bifunctional [32]. There exist extreme exceptions as well, represented by teleosts lacking *Fads*-like genes in their genomes, namely pufferfish *Takifugu rubripes* and *Tetraodon nigroviridis* [42]. The Atlantic salmon Δ6 and Δ5 *Fads2* cDNAs are very similar, sharing greater than 95% nucleic acid

identity, indicating the presence of a recently duplicated locus, probably as the result of the recent salmonid whole genome duplication event [40].

The property of fish Fads2 exhibiting a more varied spectrum of Δ activities towards substrates has been hypothesized by Castro et al. [45] as a result of a functionalization process that occurred in response to dietary availability in natural pray. Functionally characterized Fads2 in numerous teleosts and all their activities determined by heterologous expression in yeast are listed in recent review of Kabeya et al. [46]. However, the persisting lack of information in some teleost lineages, such as Elopomorpha, and other nonteleost lineages, such as Lepisosteiformes, Polypteriformes or Cyclosomata, has hampered the full comprehension of Fads enzymes function in fish for a long time. Current novel insights into the fish LC-PUFA biosynthesis have provided a study on Fads desaturases published by Lopes-Marques et al. [42]. Accordingly, two types of desaturase repertoire are confirmed to appear in teleost fish, separating Elopomorpha from the other living teleost lineages. The orthologous gene to *Fads1* has been found in Japanese eel (*Anguilla* japonica), an Elopomorpha teleost specie, and confirmed by heterologous expression approach in yeast that desaturates the corresponding fatty acid substrates in the Δ5 position as well as sharing the common structural features to mammalian Fads1 enzymes. Farther Fads1 have been identified in some representatives of ancient fish lineages such as the Senegal bichir (*Polypterus senegalus*) and spotted gar (*Lepisosteus oculatus*) by these authors [42].

Based on sequence and phylogenetic data, *Fads2* and *Fads1* genes have been deduced to originate from the vertebrate ancestor and *Fads1* seems to be lost in Teleostei lineages except in Elopomorpha. It could be hypothesized that some teleosts have generated a mechanism to overcome the bottleneck caused by the loss of Δ5 Fads1, since otherwise, they would not be able to convert PUFA to LC-PUFA. Such a mechanism would be *Fads2* gene duplication followed by the process of functionalization as most probably was the case in salmonids, whereby acquisition of Δ5 *Fads2* occurred in one of the several *Fads2* gene copies. Another example would be the zebrafish (*Danio rerio*) in which Δ6 Fads2 acquired the ability to desaturate even in the Δ5 position [42,44]. The loss of canonical *Fads1* gene followed by *Fads2* subfunctionalization that teleosts have undergone during evolution could be linked to and explain the higher plasticity with which fish produce LC-PUFA biomolecules in comparison to other vertebrates.

5. Fads2 Structure and Structural Implications of Substrate Specificity

Fads2 are modular proteins which characteristically have a cytochrome *b5*-like domain on the N-terminus and the main desaturation domain with three histidine-rich regions on the C-terminus [47–49]. The fusion of the cytochrome *b5*-like domain to the main desaturase protein domain enables the NADH cytochrome *b5* reductase to directly transfer electrons to the catalytic site of Fads2 via the cytochrome *b5*-like domain without the requirement for an independent cytochrome *b5* [50,51]. However, solid evidence has been provided that both the cytochrome *b5*-like domain of Fads2 and microsomal cytochrome *b5* are necessary in the process of Δ6 desaturation and that the microsomal cytochrome *b5* does not compensate for the role of cytochrome *b5*-like domain of Fads2, which is accompanied by highly conserved heme-binding HPGG motif of the cytochrome *b5*-like domain. Moreover, protein–protein interactions between Fads2 and microsomal cytochrome *b5* are required for proper Fads2 function [52]. Phylogenetic studies have shown that cytochrome *b5* domain from Δ6 Fads2 proteins form a single cluster which points to a single ancient fusion event that took place in the common ancestor of all eukaryotes [53].

As a hydrophobic membrane-bound protein, Fads2 is extremely recalcitrant to characterization by conventional biochemical methods. A three-dimensional structure of Fads2 by X-ray crystallography is missing to date. The only animal desaturase whose structure is known is the stearoyl-CoA desaturase with Δ9 desaturation activity for which crystal structures have been published in humans [54] and rats [55]. There are some characteristic features common to all desaturases. The amino acid sequences within the substrate binding channel in all membrane desaturases contain the three His-boxes which histidine residues hold two irons in the active site. These histidine residues are of high evolutional

conservation [56,57] and take place in very close proximity to the fatty acid substrate, referred to as "contact residues" [51]. Hydropathy analyses have shown that desaturases contain up to three long hydrophobic domains which are long enough to span the membrane bilayer twice whereas the His-boxes have a consistent positioning with respect to these potential membrane spanning domains. Sayanova et al. [56] undertook a massive motif analysis in more than fifty eukaryotic genomes, obtaining 275 desaturases, and reported the sequence logo representations of conserved histidine regions shown in Figure 2.

Figure 2. Sequence logo of histidine boxes in membrane associated front-end desaturases such as Δ6 Fads2. The high of letters corresponds to the occurrence probability. Adapted from (Sayanova, 2001). Modified after (Hashimoto, 2007).

The first report on the structural basis of the substrate specificity of a mammalian front-end fatty acid desaturase was published by Watanabe et al. in 2016 [58]. Using the crystal structure modelling of the human soluble stearoyl-CoA (Δ9) desaturase [54,55], these authors performed homology modelling and revealed that Arg216, Trp244, Gln245, and Leu323 are located near the substrate-binding site. They applied site-directed mutagenesis to create mutations in rat Δ6 Fads2 at those sites they had predicted to influence the enzymatic function. Then, they exchanged these amino acids accordingly to be the same as in the unique bifunctional Δ6/Δ5 Fads2 from zebrafish. They determined amino acid residues responsible for both switching and adding the substrate specificity of rat Δ6 Fads2. Additionally, they predicted tertiary structure of rat Δ6 Fads2. There is a very important outcome from their publication—that one single amino acid in the Δ6 Fads2 desaturase enzyme, when changed, has the potential to switch the specificity towards substrates.

This corresponded to results from investigations on sex pheromones in moths [59], where similarly, a change as small as a single amino acid substitution in a fatty acid desaturase enzyme was sufficient to change the enzymatic function of the whole enzyme, moreover, resulting in huge consequences in reproduction. According to their data delivered, MsexD2 desaturase gene in *Manduca sexta* duplicated during the evolution whereby one copy acquired one amino acid change. Then, in the process of neofunctionalization, this novel gene acquired the ability to introduce another double bond and produce an uncommon sex pheromone with significant implication in species reproduction.

Corresponding data were obtained by the study of Δ6 Fads2 from marine algae *Thalassiosira pseudonana* [60]. Mutation sites in Δ6 Fads2 from *T. pseudonana* were determined which appeared to induce a propensity for the enzyme to favor binding of a particular fatty acid, suggesting that these may be associated with substrate specificity. The focused primarily on desaturation kinetics and assessed molecular mechanisms underlying the catalytic activity of Fads2 in *T. pseudonana*, because this model organism offers the advantage of exhibiting a very high desaturase catalytic activity suitable for such studies. They divided the amino acid sequence of Fads2 into sections and at the same time, they have used Fads2 from *Glossomastix chrysoplasta*, which in the opposite has very low enzymatic activity, and divided it in the same sections. To determine the catalytic activity of each region, the corresponding regions of both Fads2 enzymes were systematically exchanged to construct recombinant swap genes, which were expressed in yeast. Kinetics of enzymatic catalytic activity of recombinant desaturase were measured as well as amino acid residues important for catalytic activity by the use of site-directed mutagenesis were determined. As a result, topology prediction was created depictured in Figure 3. Amino acid substitutions significantly impacted the desaturation catalytic efficiency providing a solid basis for in-depth understanding of catalytic efficiency of Δ6 Fads2 enzyme.

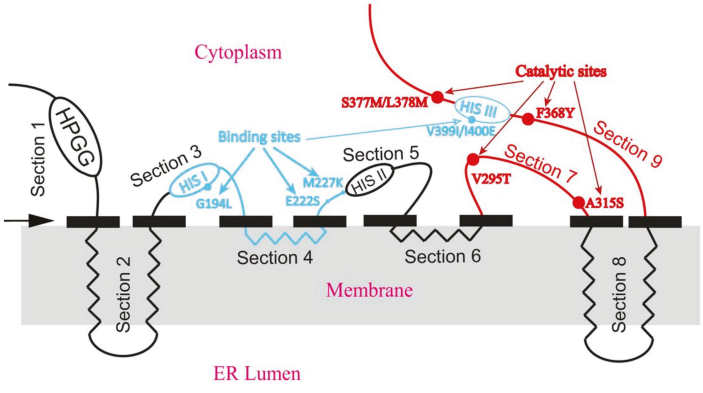

Figure 3. The predicted topology model of Δ6 Fads2. The black solid rectangles indicate boundaries of domains. Four alpha-helices span the membrane. The blue lines and blue dots indicate the areas and sites implicated in substrate specificity, red lines and red dots indicate the areas and sites important for catalytic activity, respectively. ER lumen: endoplasmic reticulum lumen. HIS I, HIS II and HIS III: histidine rich motifs. Modified from (Shi et al., 2018).

The abovementioned studies clearly demonstrated that the strictness of structure-function relationship of Fads2 enzymes might be enormous and acquired changes as small as one single amino acid of enzyme primary structure might have significant consequences for the organism studied which could be a general feature extrapolatable even to more diverse taxa such as fish. Comparative studies of highly effective and minimally effective LC-PUFA biosynthetic machineries either between more or less related species or occurring in one single species (as typically studied in salmonids [29,40,61] have justified, that this is a promising strategy with great potential to gain insight into the challenging LC-PUFA biomolecules research in fish.

The question why some species can survive in EPA and DHA poor environment and other even closely related species not, has been an attractive research topic in the very recent past. An interesting structure-function study performed by Xie et al. [62] has addressed that question by studying the *Fads2* promoter sequence. The binding site for stimulatory protein Sp1 has been found as lacking in the promoter of *Fads2* gene in marine teleost *Epinephelus coioides*. The authors speculated therefore, that the Sp1-binding site absence might be the main cause of the very low Fads2 expression in marine carnivorous teleost species. To test this hypothesis, they inserted the Sp1-binding site from the *Fads2* promoter sequence of the herbivorous *Siganus canaliculatus*, the first marine teleost demonstrated to have LC-PUFA biosynthetic ability, into the corresponding region of *E. coioides Fads2* promoter sequence. As expected, Δ6 desaturation activity increased significantly. The results provided direct evidence for the importance of the Sp1-binding site in determining *Fads2* promoter activity and indicated that its lack may be a reason for very low expression of Fads2 and poor LC-PUFA biosynthetic ability in *E. coioides*. The Sp1-binding site has been found as lacking in marine carnivorous fish *Gadus morhua* [36] as well as in *Dicentrarchus labrax* [63], while in *Oncorhynchus mykiss* its promoter activity was weaker [64].

6. Fads2 Copy Number Variation

The best was yet to come regarding studying marine vs. freshwater fish dealing with LC-PUFA poor food sources. Just a few months ago, Science released an exciting paper from Ishikawa et al. [65]. In this comprehensive study, the authors compared three-spined stickleback (*Gasterosteus aculeatus* species complex) which successfully colonized newly emerged freshwater bodies after glacial retreat with closely related marine Japan Sea stickleback (*G. nipponicus*) which had failed to colonize freshwater. They linked the colonization success to *Fads2* gene copy number, being higher in Pacific Ocean stickleback from the *G. acuelatus* complex. When transgenic Japan Sea stickleback overexpressing

Fads2 was made and fed only DHA-free *Artemia*, the Fads2 transgenics showed a higher survival rate and higher DHA content at 40 days after fertilization than the control GFP-transgenics. Moreover, *Fads2* gene linkage to X chromosome was confirmed, resulting in higher copy number in females. That fact is consistent with higher female survival observed. These results suggested that lower *Fads2* copy number may be a constraint to colonization of DHA-deficient freshwater niches by Japan Sea stickleback. These authors went deeper into the copy number assessing in the context of phylogeny and deduced a general mechanism: Higher *Fads2* copy number contributes to survival with DHA-free diets. Hence, *Fads2* was the metabolic gene important for overcoming the nutritional constraints associated with freshwater colonization in fishes. The authors mentioned the intriguing feature of the *Fads2* gene to make strong signatures of selection even in human such as in Greenland Eskimos which might be farther extrapolated to even more diverse taxa.

7. Fads2 Transgenes

The limited availability of LC-PUFA derived from fish represents the critical bottleneck in food production systems, one that numerous research institutions and aquafeed companies in this field are trying to overcome. Attempts to replace fish-derived LC-PUFA by plant derived alternatives often resulted in low quality products lacking the original content of these health promoting biomolecules [66]. This problem could be minimized by either feeding fish genetically modified plants for enhanced EPA and DHA production or by gene editing fish to be capable to produce endogenous LC-PUFA more effectively. Genetic engineering has been long been utilized as a strategy to increase natural productivity. Genetically engineered organisms could have the potential to reduce pressure on current LC-PUFA natural resources. The efforts and progress to develop transgenic plants as terrestrial sources of ω3 fish oils as well as advances in the field have been reviewed recently by Napier [67]. Transgenic fish have many potential applications in aquaculture, but the research also raised concerns regarding the possible risks to the environment associated with release and escape. A tabulated balance sheet of likely benefits and risks have been published by Maclean and Laight [68]. In this review, we focused on attempts to produce genetically modified fish with an enhanced content of ω3 LC-PUFA.

The first step to modifying the LC-PUFA biomolecules production pathway using genetic engineering was done in zebrafish [69], into which a gene for Δ5 Fads2 from masu salmon was introduced. The result demonstrated that masu salmon (*Oncorhynchus masou)* Δ5 Fads2 is functional in zebrafish and modifies its LC-PUFA metabolic pathway; hence, the technique could be applied to farmed fish to generate a nutritionally richer product for human consumption. The closely relative to zebrafish, the common carp (*Cyprinus carpio*) accounts for about 40% of the total global aquaculture production and could therefore deliver a significant amount of LC-PUFA if they were produced in their body. However, the content of EPA and DHA (mg g^{-1}) in muscle tissue of common carp is relatively low when compared to many other fish species, as revealed by recent meta-analysis data [70].

Some pioneering transgenesis experiments were carried out which reported trends towards increased ω3 LC-PUFA content in muscle of transgenic progeny—Δ5 *Fads2* from masou salmon driven by a β-actin promoter was introduced into common carp [71] and channel catfish (*Ictalurus punctatus*) [72] with the aim to improve ω3 LC-PUFA production. The results have shown promise for future work in this area, when utilizing homozygous transgenic individuals in contrast to the heterozygous individuals utilized in these studies. The effects of the transgene varied between common carp and channel catfish, being higher in common carp [72].

However, only a few month ago, *´Haiyouli´* construction was published [73], which, in Chinese, means "advantageous carp-like marine fish". This common carp was genetically modified with the aim to elevate production of ω3 LC-PUFA. The transgene used was a fish-codon optimized fatty acid desaturase (*fat1*) coding sequence originally from *Caenorhabditis elegans* driven by the 5´upstream regulatory region of common carp β-actin. Unexpectedly for the authors, under transgene expression fat accumulation of the internal organs decreased and in the liver tissues, *fat1*-transgenic common carp showed less accumulation of lipid droplets when compared with wildtype. However, the quantitative

RT-PCR results showed a 10.5-fold increase in Fads2 expression, a 6.5-fold increase in elongase 5 expression and a 3-fold increase in elongase 2 expression in the transgenic tissue, indicating stimulation of LC-PUFA biosynthesis by the expression of exogenous *fat1* desaturase. Interestingly, the transcription of acyl-CoA oxidase 3 increased by 8.2 in transgenic tissue, which perfectly explains the lipid content decrease in internal organs of genetically modified common carp. Intriguingly, the authors stated that the ω6 to ω3 ratio of their transgenic common carp (0.4) was even lower than that of the Atlantic salmon (0.58) reported by Henderson and Tocher [74]. For this reason, *Haiyouli* has been presented as a potentially ideal fish produced in modern society to balance the high ω6 to ω3 ratio of human diets. However, such a conclusion is questionable since they stimulate LC-PUFA biosynthesis by the expression of exogenous *fat1* at the expense of overall lipid biosynthesis. When total mass unit of LC-PUFA per mass unit of filet is calculated and compared to salmon, there may, in fact, be no relative advantage to consuming *Haiyouli*. Hence, it is rather a step forward on the way to constructing the ideal fish.

Successful production of DHA using *Fads2* transgenes has been reported in mammals. In Chinese hamster ovary cells, LC-PUFA-elevated production was achieved by heterologous expression of fish Δ4 Fads2 from *Siganus canaliculatus* with concomitant overexpression of Δ6 Fads2 and Δ5 Fads1 from mice. The authors stated that this new technology has been confirmed as very effective in high-level production of DHA from dietary ALA and provided a potential for the creation of new land animal breeds who could produce DHA abundantly in their related products [75].

If such solutions come into practice, this will have a positive effect in sufficient delivery of health promoting LC-PUFA to humans while at the same time, preserving wild fish populations.

8. Conclusions

Fads2 is a fascinating enzyme with far-reaching implications for both human health and environmental sustainability. It is clear that Fads2 has played an important role in the adaptations to novel environments throughout evolutionary history as differences in both gene expression and copy number have been reported across freshwater and seawater dwelling species. We have demonstrated the importance of this enzyme in the context of growing aquaculture demand and dwindling LC-PUFA supply and outlined a few remedies in the form of genetic engineering to improve endogenous PUFA production. By improving our understanding of Fads2, we can address major environmental concerns and break out of the cycle of exploitation that currently strains our wild fish reserves to feed the growing aquaculture sector.

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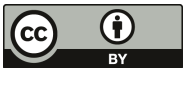

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Limited Antioxidant Effect of Rosemary in Lipid Oxidation of Pan-Fried Salmon

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Abstract: Consumption of omega-3 polyunsaturated fatty acids (n-3 PUFAs) rich fatty fish is known to provide an array of health benefits. However, high temperature in food preparation, such as pan-frying, potentially degrades eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) of the n-3 PUFAs by heat oxidation. The addition of antioxidant condiments, and herbs in particular, may retard PUFA peroxidation and preserve EPA and DHA during pan-frying. In this study, different types of antioxidant condiments (sage, rosemary, black peppercorn, thyme, basil, and garlic) were tested for antioxidant capacity, and the condiment with the highest capacity was selected for its effect on lipid oxidation of salmon. The changes in fatty acids and lipid peroxidation of salmon, during pan-frying with the selected condiment (olive oil infused with rosemary, $RO_{(infused)}$), were compared with salmon prepared in extra virgin olive oil, olive oil, or without oil. The total saturated fatty acid was found to be less in pan fried salmon with $RO_{(infused)}$. None of the oil type conserved EPA- and DHA-content in salmon. However, $RO_{(infused)}$ lowered lipid peroxidation by lessening hydroperoxide and 4-HNE formation, but not the other related products (HDHA, HETE, isoprostanes). Our observation indicates that the antioxidant capacity of $RO_{(infused)}$, when it is incorporated with food, becomes limited.

Keywords: salmon; pan-frying; rosemary; lipid oxidation; polyunsaturated fatty acids; aldehydes; isoprostanes

1. Introduction

Fatty fish, such as salmon, is rich in long chain omega-3 polyunsaturated fatty acids (n-3 PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Numerous researches evidently showed that EPA and DHA are beneficial to the human body, such as in lowering cardiovascular disease, aiding brain development, preventing neurodegenerative diseases, and alleviating the symptoms of rheumatoid arthritis [1–6]. EPA and DHA can be converted from plant-based n-3 PUFA e.g., α-linolenic acid (ALA) but the conversion efficacy is low in human metabolism, therefore, it is essential for one to consume sufficient fatty fish for health benefits [7].

Because of food safety reasons, salmon is commonly cooked before consumption. Pan-frying is a popular method of preparing fatty fish, since it is fast, convenient, and can generate typical characteristics of color, flavor, texture, and palatability, which is highly appreciated by consumers [8]. However, pan frying salmon may also cause some undesirable changes [9,10]. The high temperatures generated from pan-frying may breakdown EPA and DHA through lipid oxidation [11–13]. Also, studies suggest that the heat from pan-frying, not only increase the oxidation of fatty acids on the surface, but also inside fish meat [8,9,14,15].

It is well-studied that thermal treatment increases the rate of non-enzymatic lipid oxidation in food, and that it generates a mixture of products. For example, F_2-isoprostanes are formed from arachidonic acid (ARA), which is a known biomarker for oxidative stress [16]. Similarly, F_3-isoprotanes and F_4-neuroprostanes are formed from EPA, and DHA, respectively [17,18]. Although increasing numbers of research suggest that F_3-isoprostanes and F_4-neuroprostanes are beneficial to human health, due to their cardio-protective and anti-inflammatory properties [19,20], it is not known whether they can be absorbed or provide health benefits when consumed. Furthermore, the production of 4-hydroxy-2-hexenal (4-HHE) from DHA is claimed to be neurotoxic in neuronal cells by augmenting ROS activity and down-regulating the antioxidant enzyme glutathione (GSH) levels [21]. To our understanding the production of these oxidized products will exhaust the salmon EPA and DHA, and consequently affect the nutritional quality of salmon.

Vegetable oils are used to prevent food sticking onto the pan during cooking. However, depending on the type of cooking oil, the fatty acid content is different where monounsaturated fatty acids, such as oleic acid, are predominant in olive oil, whereas n-6 PUFAs are the main fatty acids in corn oil. Aside from heat, the rate of oxidative degradation is greatly affected by the level of fatty acid unsaturation, where the skipped diene structure increases the vulnerability to oxidation [9,22]. Nonetheless, the presence of natural antioxidants in the oil e.g., extra virgin olive oil, may potentially reduce the susceptibilities towards lipid oxidation during pan-frying [22]. It is suggested that herbs and spices are rich in different types of antioxidants and potentially lower lipid oxidation when added to cooking oil. For example, piperine in black pepper was observed to protect against oxidative damage, in several in vivo studies, by inhibiting or quenching free radicals and reactive oxygen species [23–25]. Rosemary consists of carnosic acid and carnosol that are shown to inhibit lipid peroxidation in cell culture [26], lower DNA damage [27], and improve antioxidant status in aged rats [28]. Altogether, it is possible that pan-frying salmon with suitable cooking oils and condiments may protect EPA and DHA degradation by lowering the rate of lipid oxidation [29,30].

Although many studies on lipid oxidation in cooked salmon have been reported, the information on cooking salmon with condiments-infused oil is limited. In this study, we (1) investigated the best condiments to infuse into the cooking oil, based on their antioxidant properties, (2) evaluated the changes in fatty acids and lipid oxidation in salmon pan-fried with different cooking oils, and (3) elucidated whether the infusion of condiments in cooking oil preserved EPA and DHA in pan-fried salmon.

2. Materials and Methods

2.1. Chemicals and Reagents

All organic solvents used were at least analytical grade. Methanol and acetic acid, used in LC-MS/MS analysis, were HPLC grade. Boron trifluoride-methanol solution, used in the sample preparation of gss chromatography-mass spectrometry (GC-MS), was GC grade. The organic solvents and 37-FAME mix were purchased from Sigma Aldrich (St. Louis, MO, USA). Isoprostanoids, hydroxy-docosahexaenoic acid (HDHA), hydroxyeicosatetraenoic aicd (HETE) and resolvin standards were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) and dihomo-isoprostanes, dihomo-isofurans, neuroprostanes, and neurofurans were synthesized in-house by Institut des Biomolécules Max Mousseron (IBMM, Montpellier, France).

2.2. Fish Samples, Cooking Oils, and Condiments

Frozen salmon fillets (with skin) were purchased from a local supermarket and stored at −20 °C before use. Extra virgin olive oil (EVOO) and olive oil (OO) (Bertolli, Italy) were also purchased from a local supermarket. Six different fresh condiments, including sage, rosemary, black peppercorn, thyme, basil, and garlic were selected for the experiment, due to their popularity in common salmon recipes. They were all purchased from a local supermarket, stored in 4 °C, and used for the experiments within 2 days.

2.3. Sample Preparation and Analysis

2.3.1. Condiments and Cooking Oils

The condiments were extracted according to a previous study [31] to measure the level of antioxidant activity (Supplementary S1), and the antioxidants in the oils were extracted, according to Ninfali et al. [32] (Supplementary S2). The antioxidant capacity was analyzed by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay, according to Re et al. [33] (Supplementary S3) and the total phenolic content of the condiment extracts was determined by Folin–Ciocalteu assay [34] (Supplementary S4).

2.3.2. Condiment-Infused Oil

The highest lipophilic antioxidant capacity was rosemary, while black pepper showed the greatest hydrophilic antioxidant capacity (Figure 1). Therefore, rosemary and black peppercorn were chosen for condiment-infused oil production. Rosemary was cut into 0.5 cm^2 and blended into semi-paste by a kitchen blender (BL227, Kenwood, NT, Hong Kong). Black peppercorn was milled by the grinder into powder. Either the rosemary or black pepper was added into olive oil in a ratio of 1:4 (g of condiment to ml of olive oil), heated at low heat (80 ± 5 °C) for 10 min. A mass of 0.5 g oil was collected at 3 time points: Before heating ($RO_{(initial)}$), immediately after heating ($RO_{(heated)}$), and after resting with the rosemary or black peppercorn for 1 hour in room temperature ($RO_{(infused)}$). The collected oils were filtered with 0.45 µm PTFE filter and analyzed for their antioxidant capacity by ABTS assay [33].

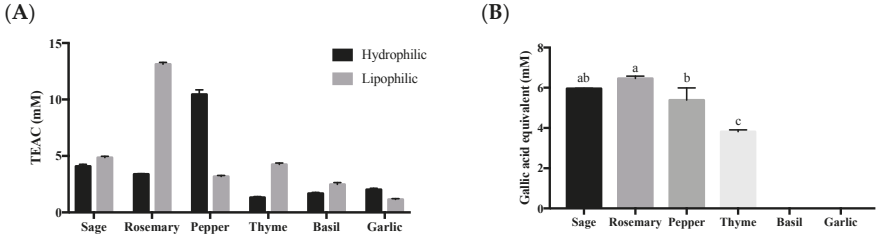

Figure 1. Antioxidant capacity (**A**) and total phenolic content of fresh condiment extract (1:1, hydrophilic: lipophilic) (**B**). Data presented are mean ± S.D. (n = 3). Kruskal-Wallis (non-parametric ANOVA) test ranks black pepper and rosemary to be the highest in hydrophilic, and lipophilic extracts, respectively. Columns sharing different alphabets are significantly different at least $p < 0.05$.

2.3.3. Oil from Salmon Meat

Before cooking, the salmon meat (50 g each) was thawed at 4 °C overnight and divided randomly into 4 groups: (i) Pan-frying without oil as control; (ii) pan-frying with 5 ml EVOO as a positive control; (iii) pan-frying with 5 mL OO; (iv) pan-frying with 5 mL rosemary-infused oil ($RO_{(infused)}$). Rosemary was chosen as it had the highest antioxidant capacity among the condiments (as shown in Figures 1 and 2A). The fillets were pan-fried on a medium-sized frying pan at 200 °C for 4 min and then flipped for another 4 min cooking. The samples were cooled, finely chopped, and stored at −80 °C until further analysis.

Oil was extracted from a portion of the cooked salmon samples (50 g) with 500 mL of n-hexane/diethyl ether (80:20, vol/vol) for 8 hours using a Soxhlet extractor. The oil collected was cooled to room temperature and dried completely using nitrogen gas. The dried oil was purged with nitrogen and stored at −80 °C until further analysis. The extracted fish oil was used for PV test to measure hydroperoxides, according to Takagi et al. method [35] (Supplementary S5).

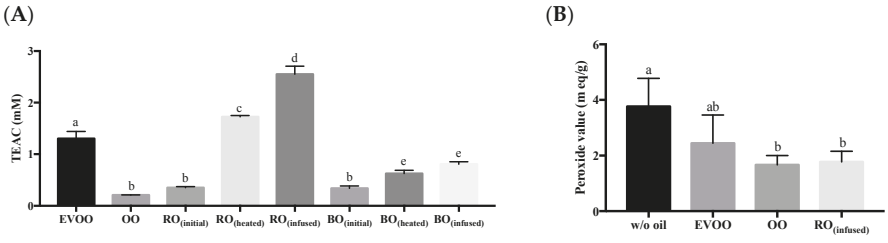

Figure 2. Antioxidant capacity of the cooking oils (**A**) and peroxide value of pan-fried salmon in cooking oils (**B**). Data presented are mean ± S.D. (n = 3). EVOO: Extra virgin olive oil; OO: Olive oil; $RO_{(infused)}$: Rosemary-infused oil; BO: Black pepper-infused oil; (initial): Oil collected immediately after addition of condiments; (heated): Oil collected after addition of condiments and heating for 10 min; (infused): Oil collected after addition of condiments, heating for 10 min, and infusion for 1 h. Columns sharing different alphabets are significantly different at least $p < 0.05$.

2.3.4. Salmon Meat

The antioxidant component of the salmon meat was extracted according to previous study with modifications [36] (Supplementary S2). All extracts were analyzed for the antioxidant capacity by ABTS assay [33]. The fatty acid content in the four groups of treated salmon fillet samples (n = 6) were extracted and analyzed by gas chromatography mass spectrometry (GC-MS), according to Quehenberger et al., with modifications [37] (Supplementary S6). Oxidized PUFA products were extracted from the salmon meat samples (n = 6) and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), according to Dupuy et al. method [38] (Supplementary S7), while the concentrations of 4-HHE and 4-HNE in salmon meat samples were analyzed by LC-MS/MS, as reported previously with modifications [39] (Supplementary S8).

2.4. Statistical Analysis

All data were analyzed by GraphPad Prism (version 6 for Mac, USA) and reported as mean ± S.D. For the differences between groups, significances were analyzed by one-way analysis of variance (ANOVA) and p-value <0.05 was considered statistically significant.

3. Results and Discussion

Six condiments, that are commonly used for pan-frying salmon, were chosen to test antioxidant capacity [30,40–45]. Rosemary showed the highest lipophilic antioxidant capacity (Figure 1A) and phenolic compound levels (Figure 1B), while black pepper had the highest hydrophilic antioxidant capacity and the second highest phenolic compound content. They were, accordingly, selected to be infused in olive oil, considering the hypothetical fact that they may enhance the antioxidant capacity of oil.

Infusing rosemary in OO enriched the antioxidant capacity. The polyphenols, namely lipid soluble carnosic acid of the fresh rosemary, potentially enriched OO for this antioxidant effect [40]. Although our study did not determine carnosic acid in the $RO_{(infused)}$, we observed that rosemary required low temperature heating for adequate infusion of the antioxidant component (as measured by antioxidant capacity) to be absorbed by the olive oil (Figure 2A). On the other hand, as anticipated, black pepper did not increase the antioxidant capacity in olive oil as the potent antioxidant portion was hydrophilic.

Unexpectedly, pan-frying salmon with $RO_{(infused)}$ reduced total fatty acids, when compared to the control group (Table 1). It is plausible the fatty acids from salmon leached into the cooking oil [46] or degraded into volatile compounds. Notwithstanding this, the fatty acid profile of the remaining cooking oil was not measured as a minute amount of cooking oil remained on the pan after frying. Nonetheless, % saturated fatty acids in pan-fried salmon with $RO_{(infused)}$ was significantly lowered than those cooked without oil, while the % n-6 PUFAs in pan-fried salmon with EVOO significantly

decreased. Moreover, both OO and EVOO are rich in oleic acid (C18:1n9) but the levels were not elevated in salmon pan-fried with OO, EVOO or $RO_{(infused)}$. It was observed by Sioen et al. [46], OO formed a crust on the fish during cooking and, thus, prevents the salmon meat form absorbing exogenous oil that could affect the fatty acid composition. In line with the findings in this study, the group also reported pan-frying salmon with OO did not significantly alter the fatty acid profile.

Table 1. Fatty acids content in pan-fried salmon (μg of analytes per g of salmon meat) cooked with or without extra virgin olive oil (EVOO), olive oil (OO), or rosemary-infused oil ($RO_{(infused)}$).

	Salmon (n = 6)			
	w/o oil	**EVOO**	**OO**	**$RO_{(infused)}$**
Total	14034.2 ± 1856.9 ^{a}	14651.7 ± 3588.7 ^{a}	12140.9 ± 1059.4 ^{a}	9869.2 ± 1714.8 ^{b}
∑ SFA (%)	8.69 ± 0.14 ^{a}	8.76 ± 0.18 ^{a}	8.45 ± 0.30 ^{ab}	7.94 ± 0.73 ^{b}
∑ MUFA (%)	25.50 ± 1.14 ^{a}	24.91 ± 1.09 ^{a}	24.75 ± 2.01 ^{a}	23.38 ± 1.10 ^{a}
∑ n-6 PUFA (%)	20.74 ± 0.65 ^{a}	19.11 ± 0.95 ^{b}	20.00 ± 0.52 ^{ab}	19.95 ± 1.33 ^{ab}
∑ n-3 PUFA (%)	45.06 ± 1.67 ^{a}	47.22 ± 1.82 ^{a}	46.81 ± 2.51 ^{a}	48.72 ± 3.13 ^{a}

Data presented are mean ± S.D. w/o oil: Salmon pan-fried without oil; EVOO: Salmon pan-fried with EVOO; OO: Salmon pan-fried with olive oil; $RO_{(infused)}$: Salmon pan-fried with rosemary-infused oil. Values sharing different alphabets are significantly different at least $p < 0.05$.

During pan-frying, the high temperature accelerates the lipid peroxidation process and generates an array of mixed lipid oxidized products. In this study, both primary, intermediate, and secondary oxidation products were evaluated. A remarkable suppression in primary lipid peroxidation was observed in pan-fried salmons, prepared in OO and $RO_{(infused)}$, where the peroxide values were significantly lower, compared to the control group (Figure 2B). Since the peroxide values of salmon pan-fried with OO and $RO_{(infused)}$ were similar, the reduction in primary lipid peroxidation did not appear to be attributed to the antioxidant component.

Hydroperoxides are a mix of primary and intermediate lipid peroxidation products that are unstable and decompose into a wide range of volatile flavor compounds and non-volatile products, or further oxidize into secondary oxidation products, such as HNE and HHE [47]. In correspondence, $RO_{(infused)}$ reduced the formation of aldehydes, especially 4-HNE during pan-frying of salmon (Figure 3). 4-HHE and 4-HNE are some of the end products of n-3, and n-6 PUFAs oxidation, respectively. Both are considered toxic lipid peroxidation products, however, in low concentrations, they showed cardioprotective effects. Low concentrations of 4-HHE protected endothelial cells through antioxidant activation of Nrf2 mediated HO-1 expression [48], while low concentrations of 4-HNE prevented cardiac ischemia-reperfusion injury by activating Nrf2 and the subsequent stimulation of glutathione biosynthesis [49]. Nevertheless, these aldehydes in excess, promote cross-linking with protein, form adducts in vivo, cause mitochondrial DNA mutation, as well as mitochondrial dysfunction [50,51]. Moreover, augmented levels of these adducts were found in human atherosclerotic lesions and in the spinal cords of patients with amyotrophic lateral sclerosis [50], and HNE has been associated to neurological diseases, cancer, diabetes, and CVD [51].

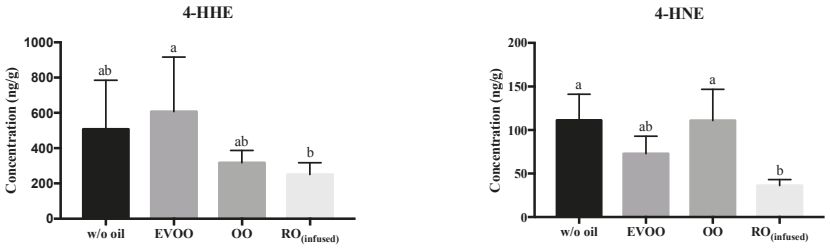

Figure 3. Level of 4-HHE and 4-HNE in pan-fried salmon (ng of analytes per g of salmon meat). Data presented are mean ± S.D. (n = 6). w/o oil: without oil; EVOO: extra virgin olive oil; OO: olive oil; $RO_{(infused)}$: rosemary-infused oil. Columns sharing different alphabets are significantly different at least $p < 0.05$.

As indicated, carnosic acid in rosemary is a potent lipophilic antioxidant. In an in vitro study using carnosic acid, Masuda et al. [52] explicitly reported that the two reactive phenolic groups in the aromatic ring of the structure were responsible for the antioxidant activity. When salmon was pan-fried with OO, two non-enzymatic n-3 PUFAs intermediate oxidized products; 8-F_{3t}-Isoprostane derived from EPA, and 8-HDHA derived from DHA, were significantly reduced (Supplementary Figure S1). Pan-frying salmon with OO also showed a suppressing effect on two non-enzymatic n-6 PUFAs intermediate oxidized products; 9-HETE and 11-HETE from ARA (Figure 4). However, this does not imply that the formation of free radicals or reactive oxygen species (ROS) are inhibited because the oxidative stress biomarker, i.e., the level of F_{2t}-Isoprostane (although low in concentration) [14], was not substantially reduced. Nonetheless, $RO_{(infused)}$ did not protect against lipid peroxidation in pan-fried salmon, and the lipid peroxidation products were at similar levels as the control. During the frying process, temperature-sensitive lipoxygenase enzyme was expected to be inactivated by the high temperatures [21]. Despite this presumption, it was surprising that the lipoxygenase-mediated intermediate oxidized lipid products, including 8-, 12- and 15-HETE in pan-fried salmon, while OO and $RO_{(infused)}$ were reduced (Figure 5). It is possible that the formation of HETE racemics may have reduced non-enzymatic metabolite generation, and/or 12- and 15-HETE were further oxidized into down-stream metabolites that are not cytotoxic [53].

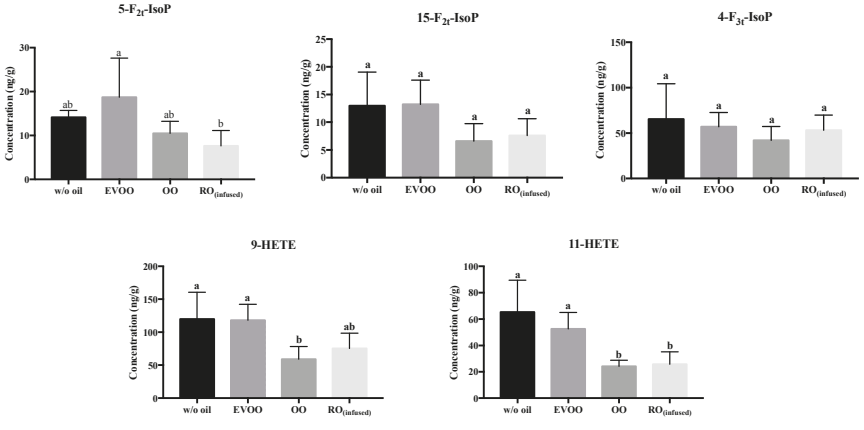

Figure 4. Concentration of non-enzymatic oxidized products of n-6 polyunsaturated fatty acid (PUFA) in pan-fried salmon samples (ng of analytes per g of salmon meat). F_2-isoprostanes and HETEs are derived from arachidonic acid (ARA) and 4-F_{3t}-IsoP is derived from n-6 DPA. Data presented are mean ± S.D. (n = 6). w/o oil: without oil; EVOO: extra virgin olive oil; OO: olive oil; $RO_{(infused)}$: rosemary-infused oil. Columns sharing different alphabets are significantly different at least $p < 0.05$.

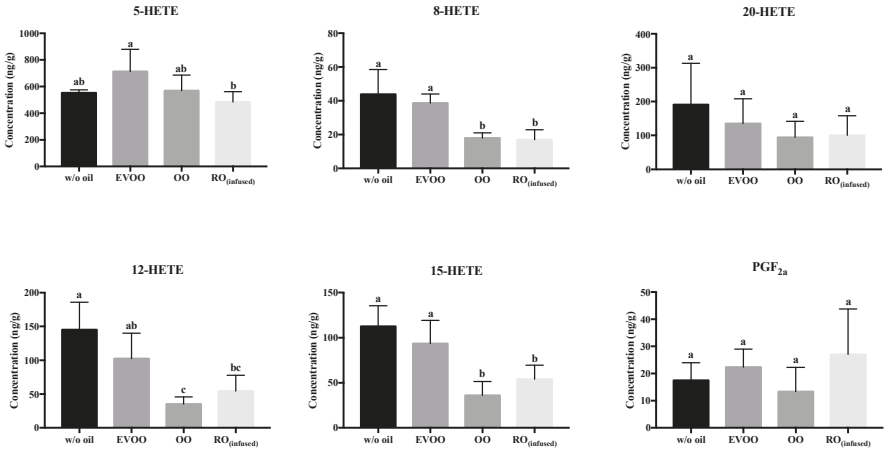

Figure 5. Concentration of enzymatic oxidized products of ARA in pan-fried salmon samples (ng of analytes per g of salmon meat). Data are presented in mean ± S.D. (n = 6). w/o oil: without oil; EVOO: extra virgin olive oil; OO: olive oil; $RO_{(infused)}$: rosemary-infused oil. Columns sharing different alphabets are significantly different at least $p < 0.05$.

It was anticipated that the effect of $RO_{(infused)}$ in reducing lipid peroxidation would be more distinguishable. In previous studies, it showed excellent antioxidant properties and prevented lipid peroxidation. Some studies reported that rosemary delays n-3 PUFA peroxidation of salmon in frozen storage [41,54]. However, the addition of carnosic acid to virgin olive oil did not inhibit lipid oxidation, and in fact augmented both primary and secondary oxidation products when used for deep-frying food [55]. In this study, the addition of $RO_{(infused)}$ did not effectively inhibit lipid oxidation in salmon, yet, primary, intermediate, and secondary oxidation products did not elevate either. The difference in cooking temperature and time resulted in contrasting findings with other research. Further, it is likely that carnosic acid underwent thermal degradation during the cooking process, thereby reducing the protection mechanism of lipid peroxidation by $RO_{(infused)}$.

In conclusion, $RO_{(infused)}$ reduced the saturated fatty acids of the salmon meat. It did not alter the intermediate oxidized products (F_3-isoprostanes, F_4-neuroprostanes, HDHA) in pan-fried salmon that are health benefiting, instead, it lowered the development of toxic aldehydes of ARA and DHA.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/9/8/313/s1.

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Fish Oil, but Not Olive Oil, Ameliorates Depressive-Like Behavior and Gut Microbiota Dysbiosis in Rats under Chronic Mild Stress

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Abstract: Background: This study investigated the effects of fish oil and olive oil in improving dysbiosis and depressive-like symptoms. Methods and results: Male rats were fed normal, fish oil-rich or olive oil-rich diets for 14 weeks. Chronic mild stress (CMS) was administered from week 2. The sucrose preference test (SPT) and forced swimming test (FST) were used to determine depressive-like behavior. The SPT results revealed that the CMS, CMS with imipramine (CMS+P) treatment, and CMS with olive oil diet (CMS+O) groups exhibited significantly reduced sucrose intake from week 8, whereas the fish oil diet (CMS+F) group exhibited significantly reduced sucrose intake from week 10. The FST results showed that the immobile time of the CMS+F group was significantly less than that of the CMS-only group. Next generation sequencing (NGS) results showed CMS significantly reduced the abundance of *Lactobacillus* and increased that of *Marvinbryantia* and *Ruminiclostridium_6*. However, the CMS+F group showed an increase in the abundance of *Eisenbergiella*, *Ruminococcaceae_UCG_009*, and *Holdemania*, whereas the CMS+O group showed an increase in the abundance of *Akkermansia*. Conclusions: CMS stimuli altered the gut microbiome in depressed rats. Fish oil and olive oil exerted part of a prebiotic-like effect to ameliorate dysbiosis induced by CMS. However, only fish oil ameliorated depressive-like symptoms.

Keywords: chronic mild stress; depression; gut microbiota; fish oil; olive oil

1. Introduction

The World Health Organization has reported that more than 350 million people worldwide have depression. Furthermore, depressive disorder is predicted to be the second leading cause of disability in 2020 [1]. Various therapies have been introduced for treating depression, however, antidepressants have severe side effects that cause low compliance among patients and even patient resistance to regular medical therapy. Consequently, discovering new therapies is extremely urgent.

Some adjunctive therapies have been discovered for the treatment of depression. Probiotic and prebiotic supplements are one potential approach. Kelly et al. [2] reported that depression can be induced in healthy rats by transplanting gut microbiota from major depressive disorder (MDD) patients. This suggests that gut microbiota might modulate brain activity and behavior. Among clinical trials relevant to depression, one randomized controlled study linked treatment with multispecies probiotics

to emotional reactions of sad moods, particularly rumination and aggressive thoughts in non-depressed people [3]. Another study proved that prebiotics, which are non-digestible fibers that promote the growth or activity of beneficial microorganisms, have potential antidepressive effects [4]. The role of the microbiome–gut–brain axis in the pathology of depression has been discussed by scholars.

Associations between various types of diet and the pathology of depression have been discovered in the last two decades [5–8]. For instance, evidence has emerged that the Mediterranean-style diet has beneficial effects on neurological disorders, including stroke, depression, and cognitive impairment [9,10]. Fish oil, one of the main lipids in the Mediterranean diet, contains a high percentage of n-3 polyunsaturated fatty acid (PUFA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). A clinical trial that implemented a Mediterranean-style dietary intervention with fish oil supplementation demonstrated that an increase in n-3 PUFA intake reduced the severity of depression symptoms and improved quality of life [11]. The large amount of olive oil used in the Mediterranean diet has been considered to have health benefits, particularly regarding depression risk [12]. However, the association between the effects of olive oil on gut microorganisms and depressive-like behavior has not yet been elucidated in basic and clinical studies.

Scholars have concluded that the amounts and types of lipids in the diet affect the occurrence of depression [5,10,11]. C57BL/6 mice fed a high-fat diet (60% of energy (kJ) obtained from lipids, with refined palm oil as the main lipid source) for 8 weeks showed significantly decreased sociability and sucrose preference [5]. In another study, a lard-based high-fat and high-sugar diet (36% of energy (kJ) obtained from lipids) significantly reduced the frequency of social behaviors, impaired memory, and altered microbiome composition [13]. However, whether dysbiosis caused by an unhealthy saturated fatty acids-rich diet results in neurobehavioral alteration remains unclear. Therefore, we investigated whether fish oil and olive oil interventions exerted an antidepressive effect in a chronic mild stress (CMS) model and explored the potential effects of these two lipids on the intestinal dysbiosis induced by CMS.

2. Materials and Methods

2.1. Animals and Diets

In this study, male Sprague–Dawley rats (n = 43, 6 weeks old; Bio-LASCO, Taiwan) were used. The rats were housed in a temperature- and humidity-controlled room (22 °C ± 2 °C; humidity: 60%) under a 12 h light–dark cycle (light period: 08:00–20:00) and had free access to food and water. After 2 weeks of acclimation, the rats were divided into five groups (n = 8 or 9 per group): the normal control (N), CMS, CMS treated with a drug (imipramine) (CMS+P), CMS treated with a fish oil diet (CMS+F), and CMS treated with olive oil (CMS+O) groups (Figure 1). The study was conducted in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of Taipei Medical University (LAC-2016-0405).

The animal diets were prepared on the basis of the AIN-93M semi-purified diet composition. Three oil-based diets were used, specifically, the diets of the N, CMS, and CMS+P groups contained 4% (*w*/*w*) soybean oil, the diet of the CMS+F group contained 2% fish oil and 2% soybean oil, and the CMS+O group's diet contained 2% olive oil and 2% soybean oil. The fish oil (Chueh Hsin Co., Taipei, Taiwan) contained 20.5% (*w*/*w*) EPA and 11.2% DHA, whereas the extra virgin olive oil (EVOO) (Laconia Greece S.A., Sparta, Greece) contained 65% oleic acid.

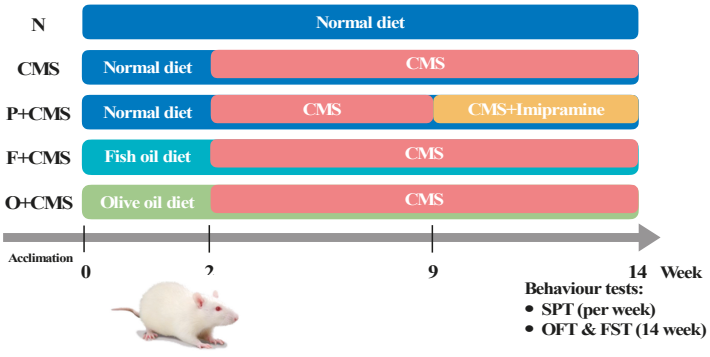

Figure 1. Experimental flow chart. Experimental animals were divided into five groups. CMS—chronic mild stress; SPT—sucrose preference test; OFT:—open field test; FST—forced swimming test.

2.2. Experimental Protocols

The experiment was conducted over 14 weeks (Figure 1). Briefly, the diets containing different dietary oils were administrated during the experimental period. Except for the N group, all groups were subjected to CMS from week 2 to week 14. CMS was exerted every week by randomly applying six out of nine possible stresses. The chronic mild stresses were as follows: (1) Water and food deprivation for 12 h, (2) a 30° cage tilt for 6 h, (3) damp sawdust (250 mL water in sawdust bedding) for 24 h, (4) physical restraint for 1 h, (5) cold swimming for 1 h, (6) blank cages without sawdust for 24 h, (7) reversed rhythm circadian for 2 days, (8) living space limitation for 8 h, and (9) social stress for 12 h. The N group lived normally without being placed under any stress. Food and water were freely available. Imipramine (Sigma-Aldrich Co., Ltd., Taiwan) was administered daily to the CMS+P group through drinking water (20 mg/kg) from week 8 to week 14, as described elsewhere [14].

2.3. Sucrose Preference Test

The sucrose preference test (SPT) is a measure of CMS-induced anhedonia, a key depressive behavior. Briefly, the rats were fasted for 12 h and then given two bottles of water, one containing reverse osmosis water and the other containing 1% sucrose solution. Sucrose preference was calculated as the percentage of the 1% sucrose solution consumed relative to the total liquid intake.

2.4. Open Field Test and Forced Swimming Test

The apparatus for the open field test (OFT) consisted of a square area (50 × 50 cm^2) with walls 40 cm high constructed from black polyvinyl chloride plastic board. The arena was lit by lights placed 145 cm above the arena and was divided into a central area (25 cm × 25 cm) and an outer area, which included the peripheral region of the arena and the wall area. During a test session, the total distance traveled and central visit duration were measured. Each test session lasted 5 min and was recorded using a video camera placed 145 cm above the arena. The videos were analyzed using ActualTrackTM software (ActualAnalytics Co., Ltd., UK).

The forced swimming test (FST) is a model of behavioral despair that is considered effective for predicting antidepressant efficacy. The study was conducted using a previously reported method with slight modification [15]. In brief, each rat was placed in a Plexiglas cylinder (37 cm in diameter and 70 cm in height) containing 50 cm of water (24 °C ± 1 °C). In the pretest session, a rat was placed in the water for 15 min to induce a state of despair and then dried with a towel and warmed in a plastic cage under a heat lamp. After 24 h, the rat was exposed to the same experimental conditions for a 5 min test session, which was recorded by HDR-SR1 (SONY, Tokyo, Japan). The videos were analyzed using

software (Forced Swim Scan 2.0, CleverSys, Reston, VA, USA) to determine the time each rat spent immobile, swimming, and struggling (including climbing, escaping, and diving)).

2.5. Corticosterone Assay

After finishing the FST, the rats were immediately anesthetized. Blood was collected directly from the abdominal aorta, stored in prechilled ethylenediaminetetraacetic acid (EDTA)-coated blood collection tubes, and centrifuged (3000 rpm, 10 min, 4 °C). Plasma was taken and immediately stored at −80 °C until analysis. Plasma corticosterone was measured using the AssayMax™ Corticosterone ELISA kit (AssayPro, St. Charles, MO, USA), according to the manufacturer's instructions.

2.6. Lipid Extraction and Fatty Acid Profile Analysis

Selected tissue samples were extracted using a modified Folch method, as previously described [16]. Twenty milligrams of prefrontal cortex (PFC) or hippocampus and 1 mL of phosphate buffered saline (PBS) were completely homogenized, and 1 mL of red blood cells was mixed with 1 mL of water. Methanol (1.5 mL) was added to 200 μL of the sample aliquot and vortexed. Subsequently, 3 mL of chloroform was added, the mixture was left for 1 h at room temperature with gentle shaking, and the liquid was then separated by adding 1.25 mL of water. The extract was incubated for 10 min at room temperature and centrifuged at 3000 rpm for 10 min. The lower (chloroform) phase was collected. Phospholipids were separated using a HybridSPE®-Phospholipid column (Supelco, St. Louis, MO, USA). Fatty acid methylation was performed by heating the samples at 90 °C for 1 h with boron trifluoride–methanol reagent (15%, 0.3 mL) to form fatty acid methyl ether (FAME), and the solvent was then removed using a vacuum pump. The FAME was analyzed using a TRACE™ gas chromatograph (Thermo Fisher Scientific Inc., Milan, Italy) equipped with a 30 m × 0.32 mm inner diameter (I.D.) × 0.20 μm df Rtx-2330 column (Restek, Bellefonte, PA USA) and flame ionization detector. The gas chromatograph oven temperature was initially maintained at 160 °C and then increased at 5 °C/min to 250 °C, where it was maintained for 5 min. The injector and detector were both maintained at 260 °C. Results were obtained according to the retention time of the appropriate standard GLC-455 (Supelco, St. Louis, MO, USA), and the percentage of fatty acid profiles was calculated based on the 12 different fatty acids (Table S1).

2.7. DNA Extraction, Amplification, and Sequencing

Faeces were collected before the rats were sacrificed and immediately stored at −80 °C until analysis. DNA was extracted from 200 mg of faeces by using the PowerSoil® DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), according to the manufacturer's instructions.

The Illumina MiSeq system and the MiSeq Reagent Kit v2 500-cycle (San Diego, CA, USA) was used to sequence the V3–V4 regions of the 16s rRNA gene extracted from rat faeces. Universal primers were removed, and low-quality reads were trimmed using cutadapt (v1.15). The paired reads were then processed using the DADA2/phyloseq workflow in the R environment. Briefly, filtering, trimming, dereplication, and denoising of the forward and reversed reads were performed using DADA2 (v1.6.0). The paired reads were then merged, and chimeras were subsequently removed. The inferred amplicon sequence variants were subjected to taxonomy assignment using the SILVA database (v132) as the reference, with a minimum bootstrap confidence of 80. Multiple sequence alignment of the amplicon sequence variants was performed using DECIPHER (v2.6.0), and a phylogenetic tree was constructed from the alignment using RAxML (v8.2.11). The frequency table, taxonomy, and phylogenetic tree information were used to create a phyloseq object, and bacterial community analyses were performed using phyloseq (v1.19.1).

2.8. Statistics

All data were presented as mean ± SD. Statistical analyses were performed using GraphPad Prism software, version 7.0 (San Diego, CA, USA). The data were analyzed using analysis of variance followed

by Tukey's post hoc test. The correlation between microbiota abundance and the fatty acid profile was analyzed using Pearson's correlation coefficient; $p < 0.05$ was considered significant. Microbiota enrichment analysis was conducted using the linear discriminant analysis effect size (LEfSe) method and visualized through cladograms obtained using GraPhlAn.

3. Results

3.1. Weight Change under CMS

At the beginning of the experiment, the groups did not differ significantly in body weight [$F(4,38) = 2.003$, $p > 0.05$]. CMS stimuli were administered to the rats from week 2, and the body weight gain of the CMS-treated rats (CMS, CMS+P, CMS+F, and CMS+O groups) slowed during week 4–8 compared with that of the N group (Figure 2). A similar result was observed in week 8–14. In addition, the weight change of the CMS+P group was significantly smaller than that of the other groups during week 8–14.

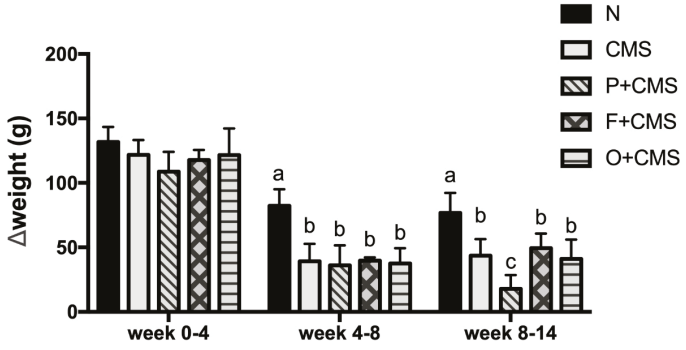

Figure 2. Weight change groups. The entire experimental period was divided into three parts. The group differences are displayed for each part. CMS was administered from week 2, and CMS significantly reduced the increase in weight during week 5–8. Data are expressed as mean ± SD (n = 8 or 9 per group). Values with different superscript letters are significantly different at $p < 0.05$.

3.2. Anxiety-Like Behavior Test

The OFT was used to identify anxiety-like symptoms in this research. We determined that the CMS rats traveled significantly smaller total distances than the N group. Fish oil and olive oil intervention restored this anxiety-like behavior, but imipramine had no effect on anxiety-like behavior in the OFT (Figure 3A). Nonetheless, no significant differences in central visit duration were discovered between all groups (Figure 3B).

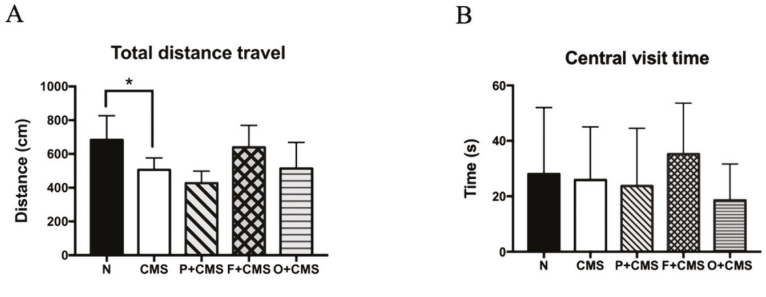

Figure 3. *Cont.*

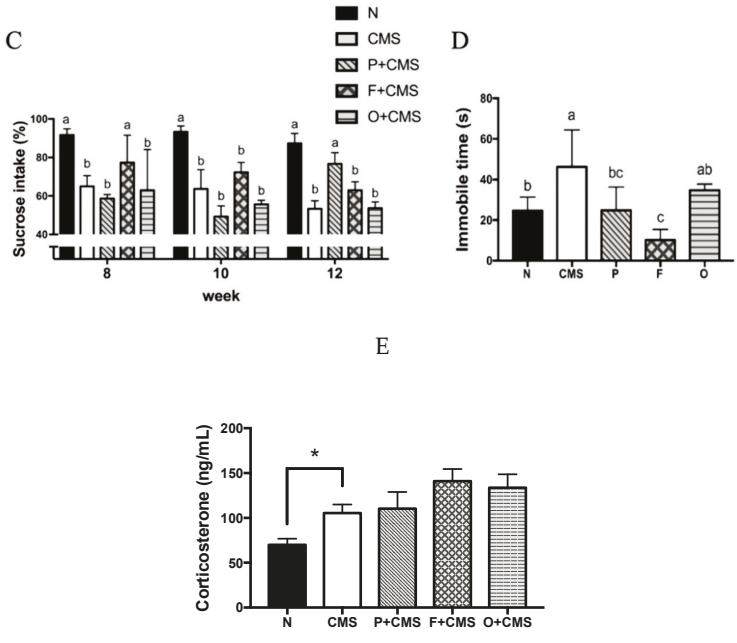

Figure 3. Anxiety-like and depressive-like behavioral tests. (**A**) Total distance traveled and (**B**) central visit duration in the OFT. CMS significantly reduced the total distance traveled. (**C**) Percentage of sucrose water consumed in the SPT. The CMS groups drank significantly less sucrose water. (**D**) Immobile time in the FST and (**E**) corticosterone levels in plasma. CMS significantly elevated the immobile time. Data are expressed as mean ± SD (n = 6 per group). * Significantly different using Student's t test ($p < 0.05$). Values with different superscript letters are significantly different at $p < 0.05$.

3.3. Depressive-Like Behavior Tests

Anhedonia, a depressive-like behavior, was represented by the percentage of sucrose solution intake in the SPT. Compared with the N group, the CMS rats had a significantly lower percentage of sucrose solution intake in week 8, except for the CMS+F group. We also observed that fish oil intervention delayed the onset of depressive-like behavior in the SPT, because the CMS+F group showed significantly reduced sucrose intake at week 10. The antidepressant imipramine was administered to the CMS+P group from week 8 to week 14, and after 4 weeks (at week 12), sucrose intake trend was significantly reversed (Figure 3C). Thus, six weeks of CMS successfully induced depressive-like symptoms in this study.

As illustrated in Figure 3D, the time the rats spent immobile in the FST was analyzed and represented depressive-like symptoms. Compared with the N group, the immobile time of the CMS group significantly increased during the test ($p = 0.001$). Fish oil intervention and imipramine treatment reversed the stress-induced abnormal depressive-like behavior exhibited in the FST ($p < 0.001$).

Overactivity of the hypothalamic-pituitary-adrenal (HPA) axis was discovered in long-term corticosterone levels after the FST (Figure 3). A significant difference was revealed in the corticosterone levels between the N and CMS groups ($p = 0.02$). Fish oil, olive oil, and imipramine did not recover the abnormally high corticosterone under acute stress (Figure 3D).

3.4. Fatty Acid Profiles of Brain and Red Blood Cells

Different dietary lipids, such as fish oil and olive oil, can result in significantly differing fatty acid profiles in both brain regions (the PFC and hippocampus) and red blood cells. The percentage of EPA

(C20:5) in the prefrontal cortex (PFC) was significantly higher in the CMS+F group than the other groups, whereas the percentage of DHA (C22:6) was unaffected by the fish oil intervention (Figure 4). We also discovered that the C18:0 in the PFC was significantly higher in the CMS+O group than the N group (Table S1). Moreover, the EPA percentage in the hippocampus of the CMS+F group was significantly higher than that of the other groups, except for the N group. The DHA percentage in the hippocampus of the CMS+F group was significantly higher than that of the N group. We also found that the EPA percentage in the red blood cells of the CMS+F group was significantly higher than that of the other groups (Table S2).

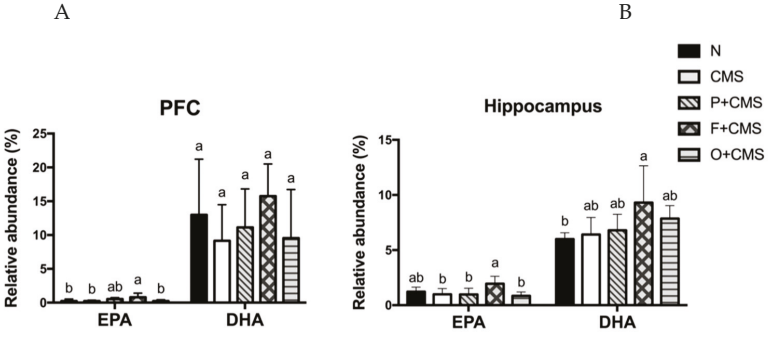

Figure 4. Percentages of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the (**A**) prefrontal cortex (PFC) and (**B**) hippocampus. Data are expressed as mean ± SD (n = 8 or 9 per group). Values with different superscript letters are significantly different at $p < 0.05$.

3.5. Microbiota Alteration after Different Dietary Lipid Interventions

After removing bias from variation in the sample read number, sequencing of the microbiota performed using the Miseq led to 2.2 million sequenced reads. In the N and CMS group samples, distinct clustering and separation of the CMS group from the N group were observed in the nonmetric multidimensional scaling plot within the Bray–Curtis distance methods. The results revealed clear separation of the CMS+F and CMS+O groups from the other groups (Figure 5A).

Analysis of relative abundance using the linear discriminant analysis (LDA) effect size (LEfSe) method indicated that the faecal microbiota composition differed significantly between all groups. Bacteroidaceae, Prevotellaceae, and Lactobacillaceae were significantly more abundant in the N group. *Bacteroides*, *Lactobacillus*, *Terrisporobacter*, *Candidatus_Stoquefichus*, and *Proteus* were significantly more abundant in the N group, whereas *Marvinbryantia*, *Ruminiclostridium_6*, *Ruminococcaceae_NK4A214*, and *Erysipelotrichaceae_ge* were significantly more abundant in the CMS group. Different dietary lipids resulted in differing microbiota compositions. At the genus level, fish oil (CMS+F group) significantly elevated the relative abundance of *Eisenbergilla*, *Ruminococcaceae_UCG_009*, and *Holdemania*. Nevertheless, the CMS+O group had significantly higher abundances of *Romboutsia*, *Akkermansia*, and *Ruminococcaceae_UCG_003* (Figure 5B,C).

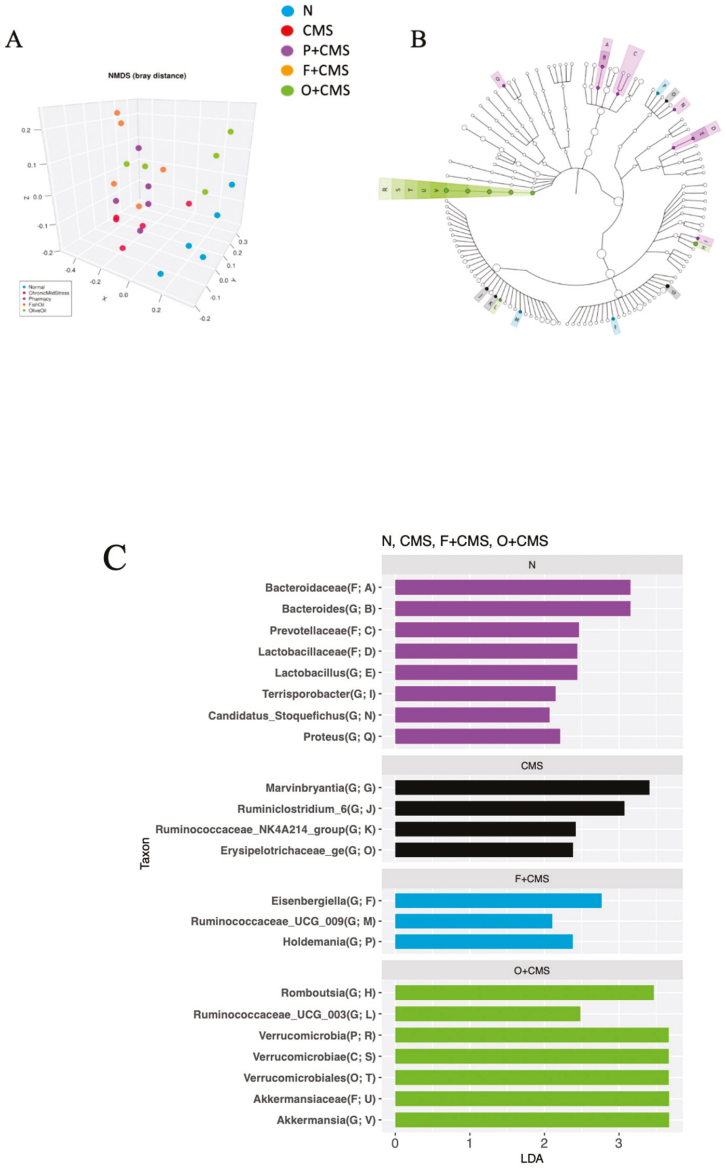

Figure 5. Taxonomic differences in faecal microbiota. Comparison of relative abundance across all groups. (**A**) Non-metric multidimensional scaling Bray distance methods were used to discriminate between the five groups. (**B**) LEfSe was used to identify the most differentially abundant bacteria in all groups, except the CMS+P group. The brightness of each dot is proportional to the effect size. (**C**) Only bacteria meeting a linear discriminant analysis threshold of >2 are shown (n = 5 per group).

3.6. Correlation between Fatty Acid Profiles and Microbiota

We evaluated the correlations between the relative abundance of microbiota and EPA, DHA, and arachidonic acid (AA) percentage by using Pearson coefficient correlation analysis. The results indicated that some bacterial genera were significantly correlated with the percentage of specific fatty

acids in the hippocampus. *Lachnospiraceare_UCG006* was positively correlated with EPA and DHA percentages (Figure 6A,B $p = 0.0198$ and 0.04, respectively). We also determined that *Eryspelatoclostridium* was negatively correlated with EPA percentage (Figure 6C, $p = 0.04$). Notably, *Ruminiclostridium_5*, which belongs to Ruminococcaceae, negatively correlated with AA percentage (Figure 6D, $p = 0.029$) and positively correlated with EPA percentage (Figure 6E, $p = 0.006$).

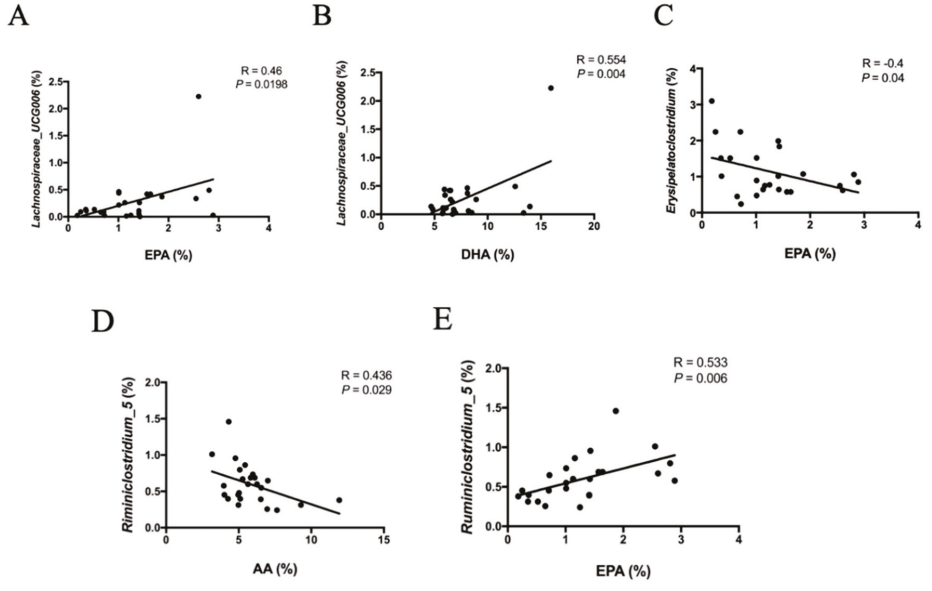

Figure 6. (**A**) and (**B**) Pearson correlation between relative abundance of *Lachnoapiraceae_UCG006* and percentage of EPA and DHA in the hippocampus, respectively. (**C**) Pearson correlation between relative abundance of *Erysipelatoclostridium* and percentage of EPA in the hippocampus. (**D**) and (**E**) Pearson correlation between relative abundance of *Ruminiclostridium_5* and percentage of EPA and arachidonic acid (AA) in the hippocampus, respectively. A value of $p < 0.05$ indicates a significant correlation.

4. Discussion

The CMS model is recommended as an effective animal model that induces depressive-like symptoms such as anhedonia, hopelessness, and despair [17–19]. This depression animal model simulates the progression of human depression, which is induced by continuous psychological stress. The present data demonstrated that CMS induced a depressive-like state characterized by a decrease in sucrose solution intake in the SPT and an increase in immobile time in the FST.

The CMS model also resulted in lower body weight gain in the experimental rats than the control rats. Cavigelli et al. demonstrated that four weeks of CMS reduced the body weight of rats [20]. Another study reported that six weeks of CMS significantly reduced body weight, and fluoxetine antidepressant treatment improved depressive-like behaviors without causing body weight gain [21]. Our study demonstrated that persistent psychological stress for more than 12 weeks strongly influenced body weight gain. Given that the food consumption was significantly lower after two weeks of CMS, persistent psychological stress may attenuate the appetite of rats. Notably, food consumption slowly recovered from week 10 in all CMS groups except the CMS+P group; however, the body weight of the CMS groups remained lower than that of the N group. Our data revealed that imipramine may have adverse effects such as decreased appetite [22]. In addition, the CMS intervention may have impaired the rats' physiological metabolism [23]. Therefore, the food consumption and body weight of the CMS+P group were significantly lower than those of the other groups (Figure S1).

Various neurobehaviors, such as despair, anhedonia, loss of willingness to explore, and general locomotor activity, are considered to represent depressive-like and anxiety-like behaviors caused by various types of stress [1,21,24,25]. In this study, CMS strongly induced anxiety-like and depressive-like behaviors, including decreased total distance traveled in the OFT, percentage of sucrose solution intake in the SPT, and increased immobile time in the FST. Dietary fish oil intake increased the total distance traveled and percentage intake of sucrose solution and reduced the immobile time; however, the other main lipid source in the Mediterranean diet, olive oil, did not improve depressive-like behaviors. Notably, the total distance traveled and central visit duration of the CMS+P group did not improve at all, indicating that imipramine had a weak anxiolytic effect [26]. However, several studies reported that imipramine can improve anxiety-like behaviors in the elevated-plus-maze test [27,28]. Therefore, the anxiolytic effect of imipramine remains controversial and requires additional research.

Fish oil, which contains large amounts of n-3 PUFA, has been demonstrated to exert antidepressant effects in both preclinical and clinical studies [25,29–31]. Recently, a meta-analysis of 13 randomized clinical trials indicated that fish oil exhibited beneficial effects in patients with major depressive disorder [32]. In the present study, the fish oil intervention slowed the progression of depression characterized by significantly decreased sucrose intake until week 10 for the CMS+F group compared with the N group, which was two weeks later than the other CMS groups. Thus, the fish oil intervention exerted an antidepressant effect.

The most well-known pathogenesis of depression is HPA axis dysregulation [33,34]. Because of a potent endocrine mechanism, the HPA axis influences numerous other parameters related to the pathophysiology of depression, such as monoaminergic neurotransmission, synaptic plasticity, and neuropeptide activity [35]. Furthermore, HPA axis dysregulation in patients with depression was found to be associated with immune function and age-related disease [36]. In the present study, corticosterone, which is a reliable biochemical marker of HPA axis dysregulation, was detected after the FST. The results indicated that after 12 weeks of CMS, the ability of the rats to adapt to acute stress decreased significantly because the corticosterone levels of the CMS-related groups were significantly higher than those of the N group. We also discovered that the antidepressant effects of n-3 PUFA was not associated with corticosterone level, because different dietary lipids could not relieve overactivity of the HPA axis. However, we only measured the corticosterone level of the rats. Whether the various dietary lipids modulate other parts of the HPA axis, such as the functions of glucocorticoid receptors or signal transduction of the central nervous system, remains unclear.

To determine whether the intake of different lipids altered the phospholipid composition of rat cell membranes, we analyzed the fatty acid profile of phospholipids in the PFC and hippocampus. The results revealed that fish oil intake not only ameliorated depressive-like behaviors in the FST and SPT, but also changed the composition of phospholipids in the rats. This suggests that EPA may play a more crucial role than DHA in the antidepressive effect of fish oil. A meta-analysis reported that EPA exerted a stronger effect on patients with depressive disorder than those without [37]. Studies have demonstrated that more than 50% of DHA supplements do not significantly reduce the severity of depression symptoms, whereas supplements containing pure EPA or more than 50% EPA significantly improve these symptoms [30,38]. Notably, although DHA is a critical and fundamental component of the brain, only EPA was elevated significantly in the hippocampus and PFC. Conversely, DHA has various essential functions in the brain and exerted an antidepressant effect on women with postpartum depression [30]. In recent years, some scholars have mentioned that intake of free DHA and triglyceride (TG)-form DHA did not increase DHA levels in the brain or improve depression symptoms because these forms of DHA could not completely cross the blood–brain barrier and simply elevated DHA levels in adipose tissue and the heart, whereas lysophosphatidylcholine-form DHA significantly increased DHA levels in the brain [39,40].

Furthermore, our data suggest that behavior alterations may be regulated by gut microbiota. Recently, microbiota have been proven to contribute to depression through several mechanisms. For example, scholars proved that the gut microbiota directly affects the immune system through activation

of the vagus nerve [41,42]. In our study, linear discriminant analysis with effect size measurement was used to determine the major bacteria in the different treatment groups. Intestinal dysbiosis in the CMS group was characterized by identifying significant taxonomical differences compared with the N group. Well-known probiotics, namely *Lactobacillus* and Lactobacillaceae, were significantly decreased in the CMS-exposed rats. The abundances of Bacteroidaceae, *Bacteroides*, and Prevotellaceae were significantly higher in the N group than in those rats with dysbiosis. *Ruminiclostridium_6* was significantly higher in the CMS group in the LEfSe analysis and was previously reported to be increased in both early-diabetic and diabetic mice [43]. The relative abundance of *Ruminiclostridium_6* was positively correlated with the levels of pro-inflammatory factors IL-17A, TNF-α, and lipopolysaccharides (LPS), but negatively correlated with the level of anti-inflammatory cytokine IL-10 in the plasma [43]. Dietary prebiotics significantly improved the inflammatory response and reduced the abundance of *Riminiclostridium_6* [43,44].

In the present study, dietary fish oil prevented depressive-like status by improving gut dysbiosis. Prebiotic chrysanthemum polysaccharide intervention was previously reported to boost the abundance of *Ruminococcaceae UCG_009* [44]. In this research, *Ruminococcaceae UCG_009* increased with the fish oil intervention in the CMS+F group. In clinical trials, high dietary fiber intake increased the abundance of *Holdemania*, which is involved in butyrate production [45]. *Holdemania* abundance also increased in the CMS+F group. Moreover, dietary olive oil changed the composition of the microbiota considerably by significantly increasing the relative abundance of *Akkermansia*, which was demonstrated to have a positive effect on obesity, insulin resistance, and diabetes [46,47]. In the present study, however, olive oil did not exhibit a positive effect on psychological abnormalities.

Pearson's correlation analysis was performed to elucidate the associations between types of fatty acids in the hippocampus and the relative abundance of microbiota to understand the potential effects of dietary lipids on the gut microbiota. Our results indicated that *Lachnospiraceae_UCG006* abundance was positively associated with both EPA and DHA percentages in the hippocampus. We also discovered that *Ruminiclostridium_5* was significantly positively associated with EPA percentage and significantly negatively associated with AA percentage. EPA is an n-3 fatty acid and exerts an anti-inflammatory effect, whereas AA is an n-6 fatty acid and has a pro-inflammatory effect.

Although our study revealed that different dietary lipids resulted in differing gut microbiota composition, which may involve improvement of CMS-induced depression, there were still some limitations in our experiment. First, the olive oil we used only contained 65% oleic acid. Considering that oleic acid and polyphenols are two main beneficial factors of EVOO, we could have used EVOO, which contains higher levels of oleic acid. Also, more studies should be conducted to elucidate the effects of dietary lipids on the composition of microbiota, especially clinical trials. Whether dietary lipids affect the composition of the intestinal microbiota in humans still needs to be demonstrated.

5. Conclusions

In summary, fish oil improved psychiatric status by ameliorating gut dysbiosis in the CMS rat model. Additionally, the two main lipids in the Mediterranean diet, namely, fish oil and olive oil, were discovered to exert part of the effect exerted by prebiotics, preventing CMS-induced dysbiosis. Fish oil exerted a mild preventive effect on depression and improved the severity of depressive-like symptoms, but olive oil exhibited weaker effects than fish oil. Additional studies involving the alteration of lipid metabolites may elucidate the temporal and causal relationships between gut microbiota, depression, and dietary lipids.

Supplementary Materials: The supplementary materials are available online at http://www.mdpi.com/2218-273X/9/10/516/s1.

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the manuscript. T.-H.T., Y.-T.T., and S.-Y.H. prepared the initial draft and finalized the manuscript. All authors participated in the analytical discussion of the results and approved the final version of the manuscript.

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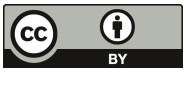

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Natural CLA-Enriched Lamb Meat Fat Modifies Tissue Fatty Acid Profile and Increases n-3 HUFA Score in Obese Zucker Rats

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Abstract: Ruminant fats are characterized by different levels of conjugated linoleic acid (CLA) and α-linolenic acid (18:3n-3, ALA), according to animal diet. Tissue fatty acids and their N-acylethanolamides were analyzed in male obese Zucker rats fed diets containing lamb meat fat with different fatty acid profiles: (A) enriched in CLA; (B) enriched in ALA and low in CLA; (C) low in ALA and CLA; and one containing a mixture of olive and corn oils: (D) high in linoleic acid (18:2n-6, LA) and ALA, in order to evaluate early lipid metabolism markers. No changes in body and liver weights were observed. CLA and ALA were incorporated into most tissues, mirroring the dietary content; eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) increased according to dietary ALA, which was strongly influenced by CLA. The n-3 highly-unsaturated fatty acid (HUFA) score, biomarker of the n-3/n-6 fatty acid ratio, was increased in tissues of rats fed animal fats high in CLA and/or ALA compared to those fed vegetable fat. DHA and CLA were associated with a significant increase in oleoylethanolamide and decrease in anandamide in subcutaneous fat. The results showed that meat fat nutritional values are strongly influenced by their CLA and ALA contents, modulating the tissue n-3 HUFA score.

Keywords: CLA; conjugated linoleic acid; ALA; α-linolenic acid; n-3 HUFA score; meat fat; vegetable fat

1. Introduction

Dietary fats are often associated with diet-derived health problems such as obesity, coronary heart disease, diabetes, and tumors. Nevertheless, some dietary fatty acids (FAs) have been found to act as preventing factors against cardiovascular disease (CVD) [1] and certain types of tumors [2,3]. The unusual fatty acid, conjugated linoleic acid (CLA) (CD18:2), and n-3 polyunsaturated fatty acids (PUFAs) are considered the major preventing factors that are naturally present in food derived from milk and meat ruminant fats, and from fish, respectively. Consequently, since ruminant products rich in saturated fatty acids (SFAs) are not entirely acceptable to consumers [4], strategies to manipulate the fat content and fatty acid (FA) composition of ruminant meat and milk have been proposed [5,6]. Natural CLA, mainly cis-9, trans-11 CLA (18:2c9,t11), a conjugated dienoic isomer of linoleic acid (18:2n-6, LA), derives from the incomplete biohydrogenation of LA in the rumen, and/or by the action of stearoyl-CoA desaturase (Δ9 desaturase) on vaccenic acid (trans-11 18:1, VA) within the mammary gland [7,8]. Natural CLA and its isomers constitute a special category of trans FAs that have been

shown to exert anti-carcinogenic, anti-obesity, and anti-inflammatory effects, among others [2,9,10], by interfering with the metabolism of n-6 PUFAs [11,12]. It is possible to increase the content of CLA in meat and milk from ruminants when animals graze fresh pastures [13], and through supplementation of the diet with oils or seeds [14,15], like safflower oil, which is rich in LA [16], or linseed oil, which is high in α-linolenic acid (18:3n-3, ALA) [17]. CLA is accumulated in a similar fashion as oleic acid (18:1, OA), and desaturated and elongated in tissues to conjugated linolenic acid (CD18:3) and conjugated eicosatrienoic acid (CD20:3), retaining an unaltered conjugated diene structure [18]. CLA is also efficiently β-oxidized in peroxisomes, and acts as avid ligand of peroxisome proliferator-activated receptors type α (PPAR-α) [19], which regulate the expression of genes involved in peroxisomal β-oxidation [20].

The main n-3 long-chain, highly-unsaturated FAs (HUFAs) are represented by eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), which exert anti-inflammatory and hypolipidemic effects through increased PPAR-α-mediated β-oxidation of FAs [21,22]. EPA and DHA may derive in tissues from seafood products or from the elongation and desaturation process of ALA. However, the conversion rate of ALA to DHA is too low to be considered efficient from a nutritional point of view [23]. Therefore, there is a need to increase the intake of foods with n-3 PUFAs [24].

The balance of LA and ALA, as precursors of the n-6 and n-3 FA families, is critical for the formation of n-3 HUFA [25]. It has been shown that a maximal incorporation of DHA into tissues can be achieved using diets with LA/ALA ratios between 4:1 and 2:1 [26–28]. Lands et al. measured the n-3 HUFAs content of red blood cells and tissues in rats consuming different LA/ALA ratios, and developed the concept that diets high in LA would inhibit the synthesis of n-3 HUFAs by simple competitive inhibition of the Δ6 desaturase (Δ6-D) enzyme and other enzymes [29,30].

The n-3 HUFA score that is obtained as the percentage of n-3 highly-unsaturated fatty acids (HUFA ≥ 20 carbons and ≥ 3 double bonds) in the total HUFAs pool is a potential blood biomarker of n-3 FAs intake and tissue status. HUFAs are mainly incorporated into phospholipids (PLs), and are potential precursors of biologically-active eicosanoids and docosahexaenoids [31]. Because the n-3 HUFA score has been shown to be less variable than n-3 FAs in the blood and tissues of rats, it could serve as a modifiable risk factor for CVD [31].

The ratio between the sum of n-3 HUFAs (EPA, DHA) + dihomo-γ-linolenic acid (20:3n-6, DGLA) and arachidonic acid (20:4n-6, AA), i.e., the anti-inflammatory FA index (AIFAI), provides a marker of the ability to decrease the formation of n-6 eicosanoids [32,33]. In fact, AIFAI has been reported to increase in association with a significant decrease in the formation of PGE2, 6-keto-PGF, prostanoids, and TNFα [34].

Many studies have found that a high fat diet can induce obesity, implying the obesogenic role of dietary fat; however, most of these studies did not take into account the fact that dietary FA composition is crucial in the regulation of body fat deposition and distribution. Some authors have described obesity resistance and reduced hypertrophy of visceral fat pads when employing fish oil-based diets. This might be related to increased lipid oxidation in these animals due to the n-3 HUFA-induced activation of PPAR-α [35,36]. Similarly, dietary CLA has been shown to decrease body fat in animals and humans [37]. Some of the effects of dietary FAs have been shown to be mediated by endocannabinoids (EC), namely anandamide (AEA) and 2-arachidonoyl–glycerol (2-AG), both of which are derived from AA, and by AEA congeners such as *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA), which are avid ligands of PPAR-α. An overactive endocannabinoid system may favor visceral fat deposition and thereby obesity, while the activation of PPAR-α has been shown to reduce body weight [35,36].

In the present study, we aimed to evaluate whether the peculiar nutritional effect of CLA in combination with ALA on increasing n-3 HUFA score, found in humans with dietary CLA-enriched cheese [38,39], would also be confirmed with dietary lamb meat fats which were differentially enriched in CLA and ALA compared to vegetable fats in Zucker rats, a rat model of obesity [40].

2. Materials and Methods

2.1. Reagents

The acetonitrile, methanol, chloroform, n-hexane, ethanol, acetic acid, and fatty acids standards were HPLC grade, and like deferoxamine mesylate, were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Ascorbic acid, potassium hydroxide, and hydrochloric acid were purchased from Carlo Erba (Milano, Italy). Internal deuterated standards for the AEA, 2-AG, and OEA quantification by isotope dilution ($[^2H]_8$ AEA, $[^2H]_5$ 2-AG, $[^2H]_2$ OEA) were purchased from Cayman Chemicals (MI, USA).

2.2. Animals and Diets

Twenty-four male obese Zucker rats (Harlan) four weeks of age with an initial weight of 200 ± 15 g were randomly assigned to four groups, and fed for four weeks with different diets containing 6% total fat, which provided 14% of the total energy (%en). The diets, based on the AIN-93G formulation with the substitution of soybean oil with experimental fats, differed only for FAs composition: (A) fat enriched in CLA, 1.3 g/kg of total diet, obtained from the meat of suckling milk from grazing ewes; (B) fat enriched in ALA and low in CLA, respectively 0.9 and 0.6 g/kg of the total diet, obtained from the meat of heavy lambs fed a diet based on cereal grains and integrated with rolled linseed + stoned olive cake; (C) fat containing LA, ALA, and CLA, respectively 2.3, 0.3, and 0.4 g/kg of total diet, obtained from the meat of lambs fed a diet based on cereal grains; (D) fat high in LA, and ALA, respectively 13.1 and 0.6 g/kg of total diet, of vegetable origin from a mixture of olive and corn oils, as depicted in Table 1. Diets A and B were characterized by a high content of trans FAs (VA and CLA), and ALA. The SFA content was higher in animal fat, while unsaturated fatty acids were higher in the diet with vegetable fat. Animal fat for the diets was obtained from the carcasses of lambs produced according to the feeding protocols described by Serra et al. [41] for diet A and Mele et al. [42] for diets B and C. The diets were prepared by Harlan.

Table 1. Principal fatty acids in experimental diets. [1]

FA	A [2]	B [2]	C [2]	D [2]
		g/kg diet		
14:0	4.3	2.6	2.4	0.3
16:0	13.1	11.6	12.6	5.9
18:0	8.6	9.5	10.8	1.4
VA	1.4	1.0	0.6	-
OA	22.2	19.7	21.6	33.7
LA	0.8	2.0	2.3	13.1
ALA	0.4	0.9	0.3	0.6
CLA	1.30	0.61	0.41	0.03
EPA	0.09	0.06	0.05	-
DHA	0.13	0.05	0.05	-
SFA	27.9	25.4	27.7	7.6
UFA	30.8	32.9	31.2	47.3
n-6/n-3	1.1	1.1	4.2	22.0
ALA/CLA	0.3	1.5	0.8	22.2
LA/ALA	2.2	2.2	7.0	22.0
total FA	58.7	58.3	58.8	54.9

[1] Diets were AIN-93G by Harlan; standard fat formulation was substituted with experimental fats. [2] Fats in the diets were: (A) enriched in conjugated linoleic acid (CLA); (B) enriched in α-linolenic acid (ALA) and low in CLA; (C) low in ALA and CLA; (D) high in linoleic acid (LA) and trace levels of CLA. Vaccenic acid (VA), oleic acid (OA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), saturated fatty acid (SFA), unsaturated fatty acid (UFA).

Body weight and food intake were measured weekly across the study. Body length, from tip of nose to the base of the tail, was measured at baseline (week 0), and at the study endpoint (week 4).

All experiments were performed according to the guidelines and protocols approved by the European Union (EU Council 86/609; D.L. 27.01.1992, no. 116) and by the Animal Research Ethics Committee of the University of Cagliari, Italy. The authorization number from the Italian Ethical Committee, approved on 28 September 2018, is 733/2018-P.

2.3. Tissues and Blood Sampling

Before sacrifice, rats were fasted for 12 h. After Fentanyl treatment (100 μg/kg of body weight) rats were euthanized without any further anesthesia by decapitation. Immediately after death, liver, heart, hypothalamus, visceral and subcutaneous adipose tissues were taken and stored at −80 °C. Blood was taken and centrifuged at 2000× *g* for 15 min at room temperature, plasma was stored at −80 °C for future lipid analyses.

2.4. Lipid Analyses

Fatty acid analysis was conducted from the total lipids previously extracted from tissues by the method of Folch [43]. Aliquots of chloroform were dried and mildly saponified as previously described [44] in order to obtain free fatty acids for HPLC analysis. The separation of unsaturated fatty acids was carried out with an Agilent 1100 HPLC system (Palo Alto, CA, USA) equipped with a diode array detector, as previously reported [45]. Since SFAs are transparent to UV detection, they were measured, after methylation, by Agilent 6890 gas chromatography (Palo Alto, CA, USA), as described in [46].

Endocannabinoid and congener quantification is described in [47]. Deuterated EC and congeners were added as internal standards to the samples before extraction. Analyses were carried out by liquid chromatography, atmospheric pressure chemical ionization, and MS (LC–APCI–MS) (Palo Alto, CA, USA), using selected ion monitoring (SIM) at M+1 values for the compounds and their deuterated homologs.

The n-3 HUFA score was calculated as the percentage of the sum of n-3 FAs with 20 or more carbon atoms and three or more double bonds, divided by the sum of total FAs with 20 or more carbon atoms and more than three double bonds [31]:

$$\text{n-3 HUFA score} = (\text{EPA} + \text{DHA} + \text{docosapentaenoic acid (22:5n-3, DPAn-3)})/(\text{EPA} + \text{DHA} + \text{DPAn-3} + \text{DGLA} + \text{AA} + \text{22:4n-6} + \text{DPAn-6} + \text{20:3n-9}) \times 100 \quad (1)$$

The anti-inflammatory FA index is obtained as [33]:

$$\text{AIFAI} = (\text{EPA} + \text{DHA} + \text{DGLA})/(\text{AA} \times 100) \quad (2)$$

2.5. Statistical Analysis

Data are expressed as the mean ± SEM, specifically, fatty acids as nmoles per gram of tissue or ml of plasma, or g/kg diet. EC and congeners are expressed as Mol% compared to total FAs. Multiple unpaired comparison tests were performed by ordinary one-way ANOVA followed by a Tukey's posthoc multiple comparison test in order to check the effect of specific dietary lipids on lipid metabolism in an animal model of obesity. The statistical analyses were performed using the GraphPad Prism 6.01 Software (La Jolla, CA, USA).

3. Results

No variations of food intake, body and liver weights, or liver total lipid concentration were detected in the obese Zucker rats in relation to different dietary fat sources. The BMI of rats fed diet D (0.96 ± 0.022), containing vegetable fat, was slightly, but not significantly increased compared to rats fed diet C (0.89 ± 0.023).

3.1. Tissue FA Profile

An analysis of FAs revealed that the tissue FA profiles, except in the hypothalamus, were strongly influenced by dietary FAs (Table 2). As expected, CLA concentrations mirrored the diet CLA content in the following order A > B > C > D in liver, subcutaneous adipose tissue (SAT), and visceral adipose tissue (VAT) (Table 2). The same pattern was observed in plasma, even though there were not significant differences between diets B and C or C and D; in heart, CLA reached similar concentrations in the A and B groups, i.e., higher than diets C and D, while, as anticipated, no changes were observed in the hypothalamus. The pattern of VA, the other rumen-derived trans FA, did not mirror its dietary concentration, and higher values were observed in all tissues except the hypothalamus in rats fed diet B. Moreover, in liver and both adipose tissues, rats on diet C, displayed higher amount than those fed diet A (Table 2).

Table 2. FA concentrations in obese Zucker rats fed diets A, B, C, or D [1] for 4 wk.

Diet Group	Liver (nmol/g)	Heart (nmol/g)	VAT (nmol/g)	SAT (mol/g)	Plasma (nmol/mL)	Hypothalamus (nmol/g)
			ALA			
A1	898.7 ± 128.3 ^{ab}	102.5 ± 11.8 ^{a}	23926.7 ± 1248.4 ^{ab}	24589.5 ± 1560.4 ^{b}	97.7 ± 13.5 ^{a}	ND
B1	1190.2 ± 100.1 ^{b}	208.6 ± 18.9 ^{b}	27737.5 ± 978.7 ^{a}	31367.0 ± 1096.6 ^{a}	112.1 ± 17.2 ^{a}	ND
C1	969.6 ± 61.6 ^{ab}	126.2 ± 10.1 ^{a}	22779.2 ± 1710.2 ^{b}	26599.3 ± 1326.9 ^{b}	103.1 ± 7.1 ^{a}	ND
D1	596.8 ± 163.3 ^{a}	103.7 ± 15.9 ^{a}	20194.1 ± 1017.2 ^{b}	25711.5 ± 1746.5 ^{ab}	80.8 ± 5.2 ^{a}	ND
			EPA			
A1	541.0 ± 92.3 ^{a}	59.4 ± 3.3 ^{a}	407.8 ± 35.6 ^{ab}	506.8 ± 22.6 ^{ab}	64.5 ± 2.9 ^{a}	ND
B1	555.9 ± 70.6 ^{a}	61.9 ± 2.9 ^{a}	495.9 ± 38.3 ^{a}	605.9 ± 42.1 ^{a}	65.5 ± 7.2 ^{a}	ND
C1	377.1 ± 34.0 ^{ab}	42.7 ± 1.0 ^{b}	284.3 ± 17.9 ^{bc}	386.6 ± 20.6 ^{bc}	55.1 ± 3.1 ^{ab}	ND
D1	185.1 ± 59.7 ^{b}	22.2 ± 1.1 ^{c}	169.0 ± 21.8 ^{c}	262.1 ± 28.5 ^{c}	32.3 ± 2.0 ^{b}	ND
			DHA			
A1	8242.1 ± 407.5 ^{a}	4318.2 ± 161.8 ^{a}	2604.9 ± 372.5 ^{a}	2403.9 ± 111.3 ^{a}	480.6 ± 16.9 ^{a}	11791.5 ± 881.7 ^{ab}
B1	8101.5 ± 514.4 ^{a}	3709.7 ± 115.7 ^{b}	2124.2 ± 187.6 ^{a}	2014.8 ± 134.8 ^{ab}	428.3 ± 48.9 ^{a}	11687.5 ± 349.5 ^{ab}
C1	7752.8 ± 394.0 ^{a}	3506.4 ± 95.7 ^{bc}	2307.0 ± 189.3 ^{a}	1986.7 ± 106.9 ^{ab}	439.4 ± 26.1 ^{a}	11331.8 ± 369.2 ^{b}
D1	6720.0 ± 671.9 ^{a}	3068.7 ± 201.6 ^{c}	1500.0 ± 264.6 ^{a}	1664.0 ± 23.0 ^{b}	410.4 ± 45.2 ^{a}	13612.8 ± 519.5 ^{a}
			n-3 HUFA score			
A1	32.0 ± 0.8 ^{a}	34.6 ± 0.3 ^{a}	22.8 ± 2.4 ^{ab}	23.7 ± 1.0 ^{a}	22.8 ± 0.91 ^{a}	49.8 ± 0.7 ^{a}
B1	30.3 ± 0.6 ^{a}	31.9 ± 1.1 ^{a}	23.5 ± 0.8 ^{a}	22.2 ± 0.9 ^{a}	21.2 ± 0.72 ^{ab}	48.9 ± 0.1 ^{ab}
C1	28.1 ± 0.2 ^{b}	32.0 ± 1.1 ^{a}	19.3 ± 1.0 ^{b}	18.9 ± 0.7 ^{b}	20.2 ± 0.33 ^{b}	48.3 ± 0.2 ^{b}
D1	24.4 ± 0.23 ^{c}	25.1 ± 0.7 ^{b}	14.2 ± 1.1 ^{c}	12.8 ± 0.7 ^{c}	16.2 ± 0.02 ^{c}	47.6 ± 0.2 ^{b}
			AA			
A1	19396.2 ± 994.3 ^{a}	9694.9 ± 74.9 ^{a}	8253.3 ± 277.1 ^{a}	8558.3 ± 319.4 ^{b}	2058.7 ± 57.1 ^{a}	8670.8 ± 648.7 ^{b}
B1	20722.6 ± 1212.4 ^{a}	9762.2 ± 117.0 ^{a}	8138.1 ± 581.0 ^{a}	8820.2 ± 457.9 ^{b}	2089.9 ± 152.1 ^{a}	9066.6 ± 252.8 ^{b}
C1	21505.6 ± 1048.2 ^{a}	9638.7 ± 102.4 ^{a}	9364.8 ± 645.9 ^{a}	10015.1 ± 418.8 ^{b}	2237.5 ± 90.7 ^{a}	9036.5 ± 244.4 ^{b}
D1	22810.6 ± 2082.8 ^{a}	9885.1 ± 236.5 ^{a}	10188.2 ± 889.6 ^{a}	12291.9 ± 566.0 ^{a}	2575.2 ± 213.2 ^{a}	11128.7 ± 436.4 ^{a}
			CLA			
A1	622.6 ± 66.7 ^{a}	84.1 ± 10.4 ^{a}	19892.6 ± 473.5 ^{a}	17208.7 ± 931.2 ^{a}	58.9 ± 7.3 ^{a}	13.4 ± 0.5 ^{a}
B1	416.2 ± 27.9 ^{b}	75.8 ± 8.7 ^{a}	12108.4 ± 396.1 ^{b}	11697.7 ± 424.9 ^{b}	35.6 ± 5.3 ^{b}	17.7 ± 1.3 ^{a}
C1	280.7 ± 17.4 ^{c}	43.2 ± 2.9 ^{b}	8218.0 ± 577.4 ^{c}	7911.8 ± 251.0 ^{c}	29.1 ± 1.8 ^{bc}	11.7 ± 1.5 ^{a}
D1	58.2 ± 11.1 ^{d}	12.7 ± 2.8 ^{c}	1585.7 ± 171.0 ^{d}	1669.5 ± 181.7 ^{d}	10.9 ± 1.8 ^{c}	11.2 ± 4.8 ^{a}
			VA			
A1	529.7 ± 109.7 ^{b}	274.1 ± 5.7 ^{b}	14613.7 ± 200.3 ^{b}	12199.2 ± 968.5 ^{b}	76.6 ± 5.79 ^{a}	656.8 ± 152.5 ^{a}
B1	1248.4 ± 249.9 ^{a}	389.7 ± 14.1 ^{a}	35216.2 ± 384.0 ^{a}	30441.6 ± 1602.2 ^{a}	122.5 ± 17.93 ^{a}	693.5 ± 54.7 ^{a}
C1	732.1 ± 151.1 ^{bc}	219.6 ± 11.1 ^{c}	19793.3 ± 1407.8 ^{bc}	17789.3 ± 1032.3 ^{bc}	96.3 ± 6.52 ^{ab}	707.0 ± 47.3 ^{a}
D1	214.7 ± 82.3 ^{bd}	124.8 ± 5.6 ^{d}	2962.6 ± 342.8 ^{d}	2844.9 ± 212.1 ^{d}	47.5 ± 7.87 ^{b}	744.3 ± 14.3 ^{a}

Values are means ± SEMs, n = 6/group. Within tissue, labelled means in a variable without a common superscript letter differ, as determined by Tukey's post hoc test after a significant one-way ANOVA, $p < 0.05$; the maximum value is labeled as 'a', the smaller value with difference is marked as 'b', the smaller value than 'b' with difference is marked as 'c', and the smallest value with difference is marked as 'd'. ND not detected. Subcutaneous (SAT), visceral adipose tissue (VAT); arachidonic acid (AA), n-3 highly-unsaturated fatty acids (n-3 HUFA score). [1] Fats in the diets were: (A) enriched in CLA; (B) enriched in ALA and low in CLA; (C) low in ALA and CLA; (D) high in LA and trace level of CLA.

Table 3 shows that CLA was efficiently desaturated to CD18:3 and elongated to CD20:3 in the liver. A similar pattern was observed in other tissues (data not shown), and despite the higher concentration of CLA in diet B compared to diet C, CD20:3 in the B group was not significantly different from the concentrations found in the C group.

Table 3. CLA and its metabolites in liver of obese Zucker rats fed diets A, B, C, or D for 4 wk[1].

	nmol/g Liver		
Diet Groups	**CLA**	**CD18:3**	**CD20:3**
A[1]	622.6 ± 66.7 [a]	34.1 ± 4.5 [a]	59.1 ± 3.1 [a]
B[1]	416.2 ± 27.9 [b]	28.0 ± 4.4 [a]	28.6 ± 1.8 [b]
C[1]	280.7 ± 17.4 [c]	10.3 ± 1.5 [b]	24.2 ± 1.9 [b]
D[1]	58.2 ± 11.1 [d]	6.7 ± 1.3 [b]	7.7 ± 0.6 [c]

Values are means ± SEMs, n = 6/group. Within a variable, labelled means without a common superscript letter differ as determined by Tukey's post hoc test after a significant one-way ANOVA, $p < 0.05$; the maximum value is labeled as 'a', the smaller value with difference is marked as 'b', the smaller value than 'b' with difference is marked as 'c', and the smallest value with difference is marked as 'd'. Conjugated dienes (CD). [1] Fats in the diets were: (A) enriched in CLA; (B) enriched in ALA and low in CLA; (C) low in ALA and CLA; (D) high in LA and trace level of CLA.

As shown in Table 2, ALA concentrations were significantly increased in diet B compared to diet D in the liver, and in diet B compared to all the other groups in heart; in VAT in B compared to C and D; in SAT in B compared to the A and C groups. No significant differences were detected in plasma, while ALA was not detectable in the hypothalamus.

EPA was not detectable in the hypothalamus, and was lower in the tissues of animals fed vegetable fat compared to those fed meat fats. EPA was significantly increased mainly with diets A and B compared to the other groups (Table 2). Among all the dietary groups, DHA significantly increased in SAT compared to D, while in heart, DHA was significantly higher in A compared to all other groups; no changes were observed in liver, plasma, and VAT. In the hypothalamus, DHA levels were significantly reduced by diet C compared to diet D (Table 2). Interestingly, DHA concentrations seem to vary according to the dietary amount of CLA, rather than in relation to the dietary content of its putative precursor, ALA, in liver, heart, and plasma. The maximum yield of DHA was obtained at the lowest ALA/CLA ratio in the diet (0.3 in A). DHA concentrations, except in the hypothalamus, were higher in group A, though significantly, only in heart (Tables 1 and 2).

The n-3 HUFA score was significantly increased in tissues of meat-fat-fed rats compared to those fed vegetable fat, particularly with diets A and B, except in the hypothalamus, in which the level increased significantly only with diet A (Table 2). Interestingly, the n-3 HUFA score was higher in the tissues of rats fed diets with high LA/ALA ratios (diets A and B) (see Tables 1 and 2).

The main SFA concentrations did not change among the groups in liver, heart, SAT, plasma, and hypothalamus, while they were slightly, but significantly, increased in VAT by diets B and C compared to diet D (data not shown).

AIFAI was significantly increased with meat fat diets in liver, heart, and VAT. Specifically, diets A and B induced the highest increase in liver and SAT, while in the heart, A was significantly higher than B, C, and D, while B and C were higher than D. In the hypothalamus, this index was raised only in A compared to the C and D groups, and in plasma in A and B compared to D (Table 4).

Table 4. Anti-inflammatory FA index (AIFAI) obtained as (EPA + DHA + DGLA)/AA in tissues of obese Zucker rats fed diets A, B, C, or D for 4 wk [1].

	Anti-Inflammatory Index				
Diet Groups	**Heart**	**VAT**	**SAT**	**Plasma**	**Hypothalamus**
A1	52.8 ± 1.3 [a]	78.8 ± 6.7 [a]	84.3 ± 5.3 [a]	31.3 ± 1.9 [a]	139.9 ± 6.2 [a]
B1	49.1 ± 0.7 [b]	70.3 ± 2.2 [a b]	77.5 ± 4.8 [a]	28 ± 1.7 [a]	131.5 ± 0.6 [ab]
C1	44 ± 0.3 [c]	64.8 ± 1.2 [b]	62.2 ± 2.0 [bc]	26.2 ± 0.7 [ab]	127.6 ± 1.2 [b]
D1	34 ± 0.6 [d]	45.4 ± 1.2 [c]	46.4 ± 5.7 [c]	20.6 ± 0.8 [b]	124.1 ± 1.2 [b]

Values are means ± SEMs, n = 6/group. Within a tissue, labelled means without a common superscript letter differ as determined by Tukey's post hoc test after a significant One-way ANOVA, Tukey's multiple comparisons test, $p < 0.05$; the maximum value is labeled as 'a', the smaller value with difference is marked as 'b', and the smallest value with difference is marked as 'c'. Subcutaneous (SAT) and visceral adipose tissue (VAT). [1] Fats in the diets were: (A) enriched in CLA; (B) enriched in ALA and low in CLA; (C) low in ALA and CLA; (D) high in LA and trace level of CLA.

3.2. Tissue Endocannabinoids and Congeners

An analysis of EC and congeners, OEA, and PEA, was performed in all tissues except plasma; these compounds were only marginally influenced by diet. Specifically, AEA in SAT was significantly decreased in rats fed diet A compared to those fed diet C, while OEA was significantly increased in A compared to B (Figure 1). In hypothalamus, 2-AG was significantly increased in A compared to B; AEA in liver was slightly reduced with diet A.

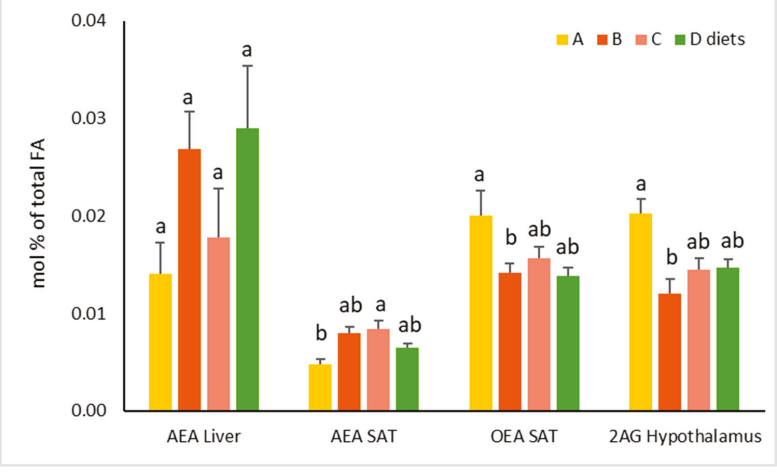

Figure 1. AEA, OEA, and 2-AG in Liver, subcutaneous adipose tissue (SAT), and hypothalamus from rats fed diets A, B, C, or D for 4 wk. Fats in the diets were: (A) enriched in CLA; (B) enriched in ALA and low in CLA; (C) low in ALA and CLA; D) high in LA and trace level of CLA. Values are expressed as mol% of total fatty acids and represent means ± SEMs, n = 6/group. Within a tissue, labelled means in a variable without a common superscript letter differ as determined by Tukey's post hoc test after a significant one-way ANOVA, $p < 0.05$; the maximum value is labeled as 'a', the smaller value with difference is marked as 'b'.

4. Discussion

The fatty acid composition of ruminant meat is strongly influenced by the diet of animals. In the present study, we compared three kinds of meat fats obtained from lambs under different feeding regimens: meat from light lambs fed only milk from grazing ewes, meat from heavy lambs maintained under a typical intensive feeding regimen based on cereal grains, integrated or not with rolled linseed as

a source of ALA. It is well known that meat from suckling lambs is usually rich in CLA, especially when lactating ewes are fed on pasture [41]. The use of linseed in the diet of intensive rearing heavy lambs has been associated with increasing amounts of ALA in intramuscular fat. At the same time, the ratio n-6/n-3 FA is lower in the meat fat from lambs fed diets integrated with linseed compared to feeding regimens based on cereal grains [42].

The results of the present study suggest that the FA composition of dietary fat does not always anticipate its metabolic impact in tissues. This study confirms that the CLA naturally found in ruminant fat is able to significantly increase n-3 HUFA score. In fact, previously, we found that the intake of CLA naturally incorporated into 90 grams of enriched cheese for four weeks, or 50 grams for two months, significantly increased plasma DHA in humans, suggesting that amount and duration are key aspects of CLA intake to induce DHA biosynthesis [38]. In that study, we observed that enriched cheese intake increased PPAR-α gene expression, which is responsible for the induction of key enzymes of peroxisomal β-oxidation [19,48], which is involved in DHA biosynthesis [49]. Our data indicated that irrespective of the matrix, natural CLA is able to increase the n-3 HUFA score. Most of the effects attributed to the n-3 PUFA family are mainly related to dietary EPA and DHA, while ALA seems to have other beneficial effects which are unconnected to its putative property as precursor of EPA and DHA [50]. In fact, the biosynthesis of DHA requires a crucial step in peroxisome for a partial β-oxidation [51]. Moreover, ALA might act as inhibitor of Δ-6-desaturase, which is essential for DHA synthesis [52]. Therefore, any other event that increases desaturase activities and peroxisomal β-oxidation may also favor DHA biosynthesis.

Our results have shown that, in all tissues except in hypothalamus, ALA and CLA incorporation and metabolization is proportional to their concentration in the diet. On the other hand, DHA concentrations changed mostly depending on the relative amount of CLA and only slightly according to their parent availability in the diet. In our earlier human studies, we found that dietary CLA, in a specific, very low range of ALA/CLA ratio (1:3), was able to significantly increase n-3 HUFA biosynthesis [39], while in a previous study, dietary ALA/CLA in a ratio 11/3.2 failed to enhance DHA biosynthesis [53], suggesting that CLA rather than ALA is crucial to enhance DHA biosynthesis. Therefore, on the basis of the data available in the literature, it seems that CLA products could be an unexpected source of DHA. Diet A, enriched in CLA, contains more DHA and EPA than the other diets; however, the concentration of EPA and DHA are extremely low and, for example, while EPA has similar concentration in diets B and C, and about 1/3 lower than diet A, EPA levels in tissues of rats fed diets A and B are in general significantly higher than in tissues of rats fed the diet C. As a matter of fact, the n-3 UFA score was found to be similarly increased in tissues of rats fed diets A and B, irrespective of EPA and DHA differences in the diets.

The plasma n-3 HUFA score is widely used to evaluate the impact of a nutritional treatment on the balance of n-3/n-6 HUFAs [54]. Our data showed that the highest increase in n-3 HUFA score was induced by A and B meat fat diets which were enriched in ALA and CLA (Table 2).

Interestingly, in group A, characterized by high levels of CLA and an ALA/CLA ratio of 0.3, the increase of n-3 HUFA score was mainly attributed to an increase of DHA, while in diet B, it was characterized by a higher ALA/CLA ratio, i.e., 1.5. The n-3 HUFA score increase was due to a greater tissue concentration of EPA. Since data from human studies are usually limited to plasma analyses, we also evaluated changes in FA metabolism in different tissues.

Interestingly, the higher incorporation in tissues of DHA and CLA induced by diet A was associated with a significant increase of OEA and a concomitant decrease of AEA in some tissues like SAT (Figure 1). Dietary CLA supplementation may increase OEA levels in the livers of obese Zucker rats, possibly by activating PPAR-α [55], which may also contribute to the higher DHA biosynthesis via enhanced peroxisomal β-oxidation. These data are in agreement with what we previously found, i.e., that in obese rats, a diet enriched with n-3 HUFA resulted in the reduction of EC biosynthesis as a result of a decrease in their precursor concentration in membrane PLs, which may account for the reduction of ectopic fat and inflammatory mediators [43], and imply that DHA and CLA may

exert a direct effect on EC and the biosynthesis of congeners. Conversely, diet B, with a relatively high dietary ratio ALA/CLA, may result in a lower PPAR-α activation, which may explain the significantly higher accumulation of VA in tissues due to a reduced peroxisomal β-oxidation. In fact, it has been demonstrated that trans FAs are preferentially β-oxidized in peroxisomes [56], which are regulated by PPAR-α [57].

The n-3 HUFA score showed a pattern similar to CLA or VA in liver and plasma, but not in the hypothalamus, as expected, which appeared to be more resistant to FA profile modification by dietary means, with early administration in life and duration of exposure, as well as dietary concentrations, being key factors in the detection of significant alterations [58]. Nevertheless, in hypothalamus, we found a significant increase in the AA concentration with the vegetable oil-based diet D, rich in LA, a precursor of AA; meanwhile, with diet A, enriched in CLA, we found an increase in the n-3 HUFA score. Our data suggest that the n-3 HUFA score change in the hypothalamus was not due to an increased biosynthesis of DHA and EPA in this tissue, but rather, to an increased transportation of these n-3 PUFAs from plasma. One can speculate that changes of FAs in peripheral tissue can directly influence FA concentrations in specific brain areas.

These modest changes in the hypothalamus were in the order of 15–20%, and may not be sufficient to exert significant effects on feeding behavior. Accordingly, the increase of 2-AG found in the hypothalamus of rats fed diet A was not associated with changes in food intake.

We previously demonstrated that CLA passes the blood brain barrier [59]; in the present experimental setting dietary, the CLA level was probably too low to be incorporated into the hypothalamus. Future studies should aim at evaluating whether dietary meat fat higher in CLA and/or longer feeding periods are able to modify hypothalamus CLA levels and influence feeding behavior through PPAR-α activation.

Another remarkable feature which may influence the n-3 HUFA score is the LA/ALA ratio, based on the concept that diets high in LA would inhibit the synthesis of n-3 HUFA by simple competitive inhibition [29,30]. In chickens, it has been found that when the LA/ALA ratio in the diet was above 5, liver PLs were rich in AA and poor in EPA; meanwhile, when the ratio dropped below 5, there was an exchange of AA for EPA [60]. The DHA status increased with a dietary level of ALA of around 1%en in rats, after which DHA accumulation was inhibited and then declined [60]. The LA/ALA ratio in our experimental diets was 2.1 (ALA 0.09%en) in A, 2.2 (ALA 0.21%en) in B, 7.0 (ALA 0.081%en) in C, and 22.0 (ALA 0.1%en) in D (Table 1). We found a significantly reduced n-3 HUFA score with C, and particularly with D in all tissues, while in hypothalamus, as reported, we observed an increase for diet A.

Since obesity is regarded as a low-grade chronic inflammatory condition characterized by increased proinflammatory cytokines in the white adipose tissue [61], it had been suggested that diets that can enhance n-3 PUFA could reduce the synthesis of PGE2 and enhance the production of PG involved in the resolution of inflammatory disorders [34]. We found that AIFAI index was significantly increased in the tissues of obese rats fed meat fat diets compared to obese rats fed vegetable fat diets (Table 4).

Interestingly, as observed in hypothalamus and in the other tissues, our data revealed that AA in SAT was significantly decreased with diets based on meat fat compared to those with vegetable fat. It is possible that increased CLA intake may interfere with the further metabolism of LA. We have previously seen that even though there was no perturbation in tissue LA, LA metabolites (including 18:3n-6, DGLA, and in particular AA) were consistently depressed in tissues by up to 1% CLA in the diet [11]. Consequently, CLA might further enhance the AIFAI index probably by inducing a decrease in AA and an increase in DHA biosynthesis. It is possible that CLA, or its relative metabolites, might differentially modulate the distribution of AA in various PLs [62], competing with AA for incorporation; therefore, this scenario may affect the eicosanoid signaling mechanism.

The consumption of meat fat rich in CLA-ALA resulted in significantly increased accumulation of DHA and depression of AA synthesis, which may have therapeutic potential to ameliorate clinical symptoms and complications that are secondary to the excessive production of proinflammatory

mediators. Our data clearly indicate that metabolic changes by dietary FAs seem to be tissue specific and affected by other factors such as background diet, energy and lipid metabolism. However, in our model of obesity, we didn't find any changes in parameters of metabolic syndrome such as dyslipidemia or fatty liver, probably due to the relative short-term feeding period or to the relatively low CLA concentration.

5. Conclusions

Our data put in evidence that the feeding system of livestock may play an important role in modulating the effect of meat fat on lipid metabolism, as some FAs, like CLA and ALA, improve the tissue FA profile, as shown with the increased n-3 HUFA score. While not being comparable to the direct intake of EPA and DHA through fish products, it seems that meat that is naturally enriched with CLA could be an unexpected source of DHA, provided that a specific ratio of FAs in the pool of total FAs is respected.

These results are promising, especially regarding individuals for whom the intake of fish products is quite low, i.e., far below the recommended daily dose. Future studies are envisaged to evaluate whether dietary fat of different origin and composition is able to modify these parameters in humans.

Author Contributions: S.B., M.M., G.C.: conceived the study, participated in its design; G.C., E.M.: carried out the fatty acid and endocannabinoid and congeners analysis; G.C., S.B., E.M.: performed the statistical analyses, interpreted the data; G.C.: drafted the manuscript; G.C., E.M., C.M., A.S.: contributed to the intellectual content; and all authors: were involved in conducting the research and critically evaluated and revised the final manuscript, read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Fatty Acid Content and Composition of the Yakutian Horses and Their Main Food Source: Living in Extreme Winter Conditions

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Abstract: For the first time, seasonal changes in the content of total lipids (TLs) and phospholipids (PLs) were studied in fodder plants growing in Central Yakutia—a perennial cereal, smooth brome (*Bromopsis inermis* L.), and an annual cereal, common oat (*Avena sativa* L.). Both species have concentrated TLs and PLs in autumn under cold hardening. In addition, a significant increase in the content of fatty acids (FAs) of *B. inermis* was observed during the autumn decrease in temperature. The Yakutian horses, which fed on cereals enriched with nutrients preserved by natural cold (green cryo-fodder), accumulated significant amounts of 18:2n-6 and 18:3n-3, the total content of which in cereals was 75% of the total FA content. We found differences in the distribution of these two FAs in different tissues of the horses. Thus, liver was rich in 18:2n-6, while muscle and adipose tissues accumulated mainly 18:3n-3. Such a distribution may indicate different roles of these FAs in the metabolism of the horses. According to FA content, meat of the Yakutian horses is a valuable dietary product.

Keywords: essential polyunsaturated fatty acids; linoleic acid; alpha-linolenic acid; food quality; muscle tissue; subcutaneous adipose tissue; liver; green cryo-fodder

1. Introduction

The Republic of Sakha (Yakutia), located between 105°32′–162°55′ E and 55°29′–76°46′ N, occupies the territory of 3103.2 thousand km^2 and lies completely in the permafrost zone in Russia. During the short growing season, plants are exposed to high activity of solar radiation, moisture deficiency, and short-term frosts on the soil surface in early summer and autumn. Native plant species growing in such extreme conditions adapt to going through all the stages of ontogenesis in a shorter time period [1,2]. At different stages of ontogenesis, the ability of plants to adapt to cold hardening is not the same: the closer the plant is to the reproductive phase, the lower its ability to adapt to cold [3]. More than 2000 species of higher vascular plants grow in the permafrost zone of Yakutia, which is an unusual phenomenon [4]. Some of them play an important role as a food source for herbivores.

A specific feature of the seasonal growth and development of the bulk of vegetation in the permafrost zone is that its intensive growth occurs in the first half of summer. However, at this time, northern meadow plant communities are often covered with floodwaters and are also subjected to

grazing and haying. After traumatic regeneration, the plants do not have time to go through the full cycle of growth and development, produce fully developed seeds, and stay in a green frozen state under the snow cover in winter (green cryo-fodder). The basis of cool-season grass is cereals, which preserve up to 80% of herbage under snow, as well as sedge, cotton grass, and some horsetails [5,6]. The wintergreen parts of the above families of fodder plants retain higher contents of proteins, carbohydrates, and fats for the winter compared to warm-season grass [7,8].

Green cryo-fodder is the basis of nutrition for many animals, including the Yakutian horses. This breed is considered a direct successor and descendant of the horses brought from the Baikal region in the 13th–15th centuries AD [9–11]. The origin of the horses was confirmed by molecular genetic methods [12–15].

The Yakutian horse demonstrates unique adaptation to long-term low-temperature stress, which has been achieved in a short evolutionary period [16]. The reasons for such good adaptation have not been fully studied. Feeding on green cryo-fodder may help animals survive in extremely cold winters [16].

The aim of the present work was to study lipid accumulation in a perennial cereal (*Bromopsis inermis* L.) and an annual cereal (*Avena sativa* L.) cultivated at different temperatures. Additionally, we aimed to study the content and composition of fatty acids (FAs) in liver, muscle, and subcutaneous adipose tissues of Yakutian horses, which have *B. inermis* and *A. sativa* as part of their staple diet.

2. Materials and Methods

The annual cereal common oat (*Avena sativa* L., Nyurbinsky type) was sown on 31 May 2014 (control) and on 15 July 2014 (treatment). The perennial cereal smooth brome (*Bromopsis inermis* L., Ammachaan type) was mowed after spring growth to allow the aftergrowth in the middle of summer (15 July 2014)—the treatment, and it was compared with the unmown plants—the control. The experiments were carried out in field plots in the conditions of Central Yakutia (near Yakutsk, 62° N, 130° E). Samples of the control and treatment plants were taken, depending on the phases of development and hardening, 4–5 times during the growing season.

For the analysis of total lipids of the common oat, the control samples were taken 4 times from July 7 to July 25, 2014; and the treatment samples were taken 4 times from July 25 to September 30, 2014. For the analysis of total lipids of the smooth brome, the control samples were collected 4 times from June 6 to July 25, 2014; and the treatment samples were collected 5 times from July 25 to September 30, 2014. To analyze phospholipids of the common oat and the smooth brome, the controls were sampled on July 25 and June 16, respectively; and the treatments of both plants were sampled on October 3, 2014. To analyze FA composition of the smooth brome, samples of the control were collected on July 7, 2013 and those of the treatment on September 25, 2013. The FA composition of the common oat (the control and the treatment) was reported in a study by Petrov et al. (2016) [17].

Sampling took place in the first half of the day in three biological replicates. Samples were fixed with liquid nitrogen immediately after their collection, in situ, and transported in Dewar vessels to the laboratory.

The samples of liver, muscle and subcutaneous adipose tissues were collected in November 2017 and 2018, from female and male Yakutian horses, most of which were less than 1 year old. Four female horses were seven, eight, and eighteen months old and five years old; and two male horses were eight months old, and one male horse was seven months old. The horses were feeding on green cryo-fodder during three months before sampling. Muscle and adipose tissues were carved from the costal part of the animals. The samples were collected from horses inhabiting Oymyakonsky, Verhoyansky, Megino-Kangalassky, Churapchinsky, Olekminsky, and Suntarsky districts of Yakutia.

Large pieces of horse tissues (200–300 g) were immediately frozen and kept at −20 °C at the slaughter site. Then, in approximately 2 weeks, frozen tissues were transported to the laboratory. In the laboratory, samples were taken from the frozen horse tissues, placed into vials with chloroform and methanol (2:1, *v*/*v*), and kept at −20 °C for further analysis.

2.1. Conditions of Keeping and Feeding the Horses

The absolute annual temperature difference in the breeding area of Yakutian horses exceeds 100 °C (the maximum summer and winter temperatures are +38 °C and −70 °C, respectively). The frost period lasts 7–8 months a year. In such conditions, the herds of Yakutian horses (12–15 individuals) are kept in the open. The horses are mainly fed on cereal grains and sedge frozen by natural cold. Horse breeders feed only weakened, emaciated individuals and mares. The weight of a breeding stallion reaches 430–520 kg and the weight of a mare 415–480 kg. In our study, we mainly used tissue samples from 6–8-month-old horses taken from local horse breeders. At this age, horse's tissues have a high nutritional value. Mass slaughter was conducted in November, when horses reached an average of 120–150 kg of live weight, having accumulated the largest amount of fat. For most of their lives, horses fed exclusively on warm-season grass and green cryo-fodder. The biochemical content and the composition of blood of Yakutian horses are described in detail in the literature [18].

2.2. Analyses of Lipids and FAs of Plants

A weighed portion of plant material (0.5 g) was ground to obtain homogeneous mass [19]. Then, it was supplemented with 10 mL of the chloroform: methanol mixture (2:1, *v*/*v*), and ionol was added as antioxidant (0.00125 g per 100 mL of the chloroform: methanol mixture). The resulting mixture was thoroughly stirred and left for 30 min until the lipids completely diffused into the solvent. The solution was transferred quantitatively to a separatory funnel through a paper skim filter (9 cm in diameter, Khimreaktivkomplekt); the mortar was washed three times using the same solvent mixture. For better delamination, water was added.

For the analysis of total lipids, the chloroform fraction was separated. Chloroform was removed from the lipid extract using an RVO-64 rotary vacuum evaporator (Czech Republic). Nonadecanoic acid (C19:0) was used to control the extractability of lipids (%), with its known amount added at the stage of homogenization. Methyl ethers of fatty acids (FAMEs) were obtained using the method [20]. For additional purification of FAMEs, TLC method was used in a chromatographic chamber with benzene as the mobile phase (R_f = 0.71–0.73) on glass plates with silica gel. The FAME zone was removed from the plate with a spatula and eluted from the silica gel with (*n*)-hexane. The FAME analysis was performed on the gas chromatograph Agilent-6890N coupled to an Agilent-5973 quadrupole mass spectrometric detector (Agilent Technologies, USA). The ionization method used was electron impact; the ionization energy was 70 eV. The analysis was carried out in the recording mode of the total ion current. An HP-INNOWAX capillary column (30 m × 250 μm × 0.50 μm) with a polyethylene glycol stationary phase was used to separate the FAME mixture. The carrier gas was helium, the rate of gas flow was 1 mL/min.

The temperature of injection port was 250 °C, the temperature of the ion source was 230°C and that of a quadrupole was 150 °C. Scanning was performed in the range of 41–450 atomic mass units. The volume of the injected sample was one μL, the flow divider was 5:1. The separation of the FAME mixture was carried out in isothermal mode at 200 °C. The duration of the chromatographic course was 60 min. For identification of FAs, the NIST 08 and WILEY7 mass spectral libraries were used. The relative content of FAs was determined by the method of internal normalization, i.e., as weight percent (wt.%) of their total content in the sample, taking into account the response factor of FAs. The absolute content of total lipids and FAMEs was determined by weighing them on GR-120 electronic scale (A&N Company Ltd., Japan) after drying the samples to constant weight.

Separation of PL fractions into individual lipids was carried out by thin layer chromatography (TLC) on Sorbfil PTLC-AF-V-UV chromatographic plates (10 × 10 cm, Russia). For the detection and identification of phospholipids in plant material, specific reagents were used: molybdenum blue for phosphorus-containing components [21], Dragendorf reagent prepared according to the method described by Wagner et al. [22] for choline-containing lipids, and a 0.2% solution of ninhydrin in acetone for amino-containing lipids [23].

Quantitative determination of phospholipid content was carried out according to the Vaskovsky method [21]. The polar lipids were separated using a two-dimensional system: the mobile phase in the first direction—chloroform—methanol—benzene—28% NH_4OH, 65:30:10:6, and the mobile phase in the second direction—chloroform—methanol—acetic acid—acetone—benzene—water, 70:30:4:5:10:1. To determine the phosphorus content in phospholipids separated by TLC, the silica gel from the zones containing separated phospholipids was transferred with a micro spatula into the tubes; 0.05 mL of 72% perchloric acid was added to each and heated at 180–200 °C for 15–20 min, placing the tubes in a heated aluminum block so that the top of the tube served as an air cooler for perchloric acid vapors. After cooling, 0.45 mL of working reagent was added to the tubes: a mixture of 5.5 mL of universal molybdate reagent, 26 mL of 1N sulfuric acid, and 68.5 mL of distilled water. The reagent was used for one week. The mixture in the tube was thoroughly mixed using a shaker. The tubes were placed in boiling water bath for 15 min and then cooled; the absorbance value was measured at 815 nm. An aliquot of the solvent containing the lipid extract was taken as a blank sample [21]. The air temperature in the experimental area was recorded using a DS 1922L iButton thermograph (Dallas Semiconductor, USA).

2.3. Analyses of FAs in Animal Tissues

The samples (0.2–1.3 g) of intercostal muscle, subcutaneous adipose tissue, and liver were homogenized, and lipids were extracted with chloroform and methanol (2:1, *v*/*v*). Dry lipids were then supplemented with 1 mL of sodium methylate solution in methanol (8 g/L). The mixture was heated for 15 min at 90 °C. The tubes were cooled, supplemented with 1.3 mL of methanol: H_2SO_4 (97:3, *v*/*v*), and methylated for 10 min at 90 °C. The FAMEs were extracted from the mixture with 2 mL hexane and washed three times with 5 mL of saturated NaCl solution. The hexane extract containing FAMEs was dried by passing it through a layer of anhydrous Na_2SO_4, and then the layer of anhydrous Na_2SO_4 was washed with 6 mL of hexane. Hexane was evaporated on a rotary vacuum evaporator. FAMEs were resuspended in 0.1 to 0.3 mL hexane prior to chromatographic analysis.

Analysis of fatty acid methyl esters was conducted using a gas chromatograph with a mass spectrometric detector (Model 7000 QQQ, Agilent Technologies, USA), which was equipped with a 30 m capillary HP-FFAP column with the internal diameter of 0.25 mm. The conditions of the analysis were as follows: the velocity of the helium carrier gas was 1.2 mL/min; the temperature of the injection port was 250 °C; the temperature of the heater was programmed from 120 to 180 °C at a rate of 5 °C/min for 10 min isothermally, then to 220 °C with a rate of 3 °C/min for 5 min isothermally, and then to 230 °C at a rate of 10 °C/min for 20 min isothermally; the temperature of the chromatography/mass interface was 270 °C; the temperature of the ion source was 230 °C and that of the quadrupole was 180 °C; the ionization energy of the detector was 70 eV; and scanning was performed in the range of 45–500 atomic units with a rate of 0.5 sec/scan [24]. The data were analyzed and counted by the MassHunter Software (Agilent Technologies). The peaks of fatty acid methyl esters were identified by the mass spectra obtained. The content of fatty acids in the biomass was quantified based on the peak value of the internal standard, nonadecanoic acid (Sigma-Aldrich, USA), a certain amount of which was supplemented to the samples before the extraction of lipids.

2.4. Desaturase and Elongase Activity Indices

Desaturase and elongase activity indices were calculated using the product/precursor ratio of the percentages of individual FAs according to the following notation: 16:1n-7/16:0 = Δ9-desaturase, 18:1n-9/18:0 = Δ9-desaturase, 20:4n-6/20:3n-6 and 20:5n-3/20:4n-3 = Δ5-desaturase and 18:0/16:0 = elongase [25]. Additionally, we measured a conversion efficiency of 18:2n-6 to 20:4n-6 (20:4n-6/18:2n-6) and a conversion efficiency of 18:3n-3 to 20:5n-3 (20:5n-3/18:3n-3).

2.5. Statistical Analysis

The tables and figures show the averages of three to six biological replicates and their standard errors. Statistical processing of experimental data was carried out using the statistical analysis package in Microsoft Office Excel 2010 and STATISTICA-9 software (Stat Soft Inc., USA). The normality of the distribution of the data obtained was checked using the Kolmogorov–Smirnov one-sample test for normality $D_{K\text{-}S}$.

3. Results

The contents of total lipids in oat leaves of both the control and the treatment gradually increased as they grew and developed (Table 1). With the decrease in the average daily air temperature from 9 to 1 and −3 °C (periods of the first and second hardening phases), the content of total lipids in oat leaves increased by a factor of 1.2 compared with the control plants of the same stage of development (*t*-test Student's = 3.34) (Table 1).

Table 1. Contents of total lipids (TL, mg/g dry weight ± standard error) in the leaves of the annual cereal *Avena sativa* sown on May 31 and July 15, 2014 at different stages of development and growing at different temperatures.

Date	t, °C *		Stages of Development	TL, mg/g DW
	min	Average		
Control (sown on May 31, 2014)				
07.07	14	18	Stem elongation	99 ± 4
11.07	13	21	Stem elongation	114 ± 4
14.07	17	23	Ear emergence	127 ± 5
25.07	16	21	Dough development	129 ± 5
Treatment (sown on July 15, 2014)				
25.07	16	21	Germination	73 ± 3
11.09	1	9	Stem elongation, ear emergence	128 ± 5
25.09	−4	1	Dough development (cold hardening phase I)	154 ± 6
30.09	−7	−3	Dough development (cold hardening phase II)	155 ± 6

*—daily air temperature.

In the summertime (June–July), the perennial smooth brome grown without mowing demonstrated lower absolute content of total lipids at all stages of development, i.e., below 60 mg/g dry weight, compared to the aftergrass (Table 2).

Cool-season cereals growing after mowing, which were hardened by low positive temperatures, i.e., when the average daily air temperature reached 1 °C, showed the amount of total lipids 2.4 times higher (Student's *t*-test = 14.93) compared to the control plants in the same stage of development (Table 2).

Table 2. Total contents of lipids (TLs, mg/g dry weight ± standard error) in the leaves of the perennial cereal *Bromopsis inermis* growing at different temperatures, at different stages of development.

Date	t, °C *		Stages of Development	TLs, mg/g DW
	min	Average		
Control—grass without mowing				
06.06	3	12	Tillering	26 ± 2
16.06	12	16	Stem elongation	30 ± 2
11.07	13	21	Ear emergence	44 ± 2
25.07	16	21	Dough development	57 ± 3
Treatment—grass after mowing (July 15, 2014)				
25.07	16	21	Aftergrass	93 ± 3
18.08	7	16	Stem elongation	89 ± 3
11.09	1	9	Ear emergence	124 ± 5
25.09	−4	1	Dough development (cold hardening phase I)	134 ± 4
30.09	−7	−3	Dough development (cold hardening phase II)	137 ± 4

*—daily air temperature.

The following phospholipids (PLs) were found in the cereal plants: phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA), and diphosphatidylglycerol (DPG). The dominant PLs were PC and PG (Figures 1 and 2).

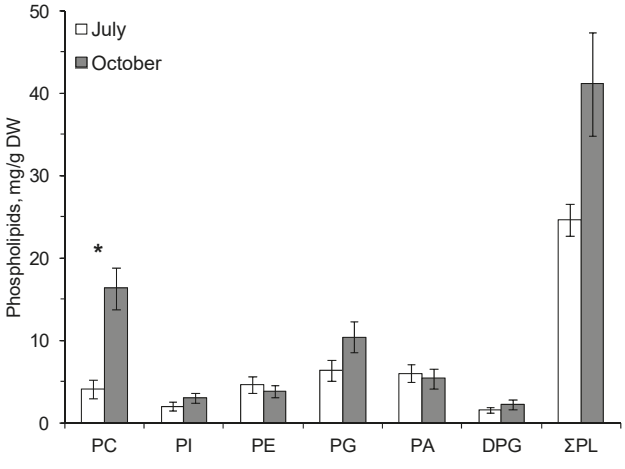

Figure 1. The contents (mg/g dry weight, standard error) of total phospholipids (ΣPL), phosphatidylcholine—PC, phosphatidylinositol—PI, phosphatidylethanolamine—PE, phosphatidylglycerol—PG, phosphatidic acid—PA and diphosphatidylglycerol—DPG in the leaves of *Avena sativa* on 25.07.2014 (July) and 3.10.2014 (October). *—Significant differences according to Student's *t*-test.

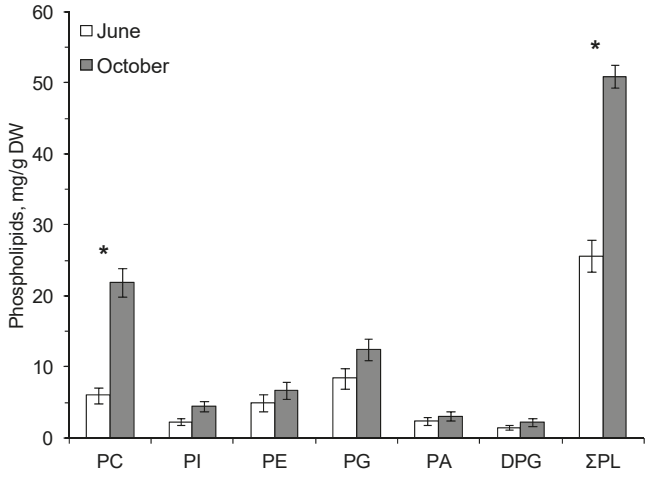

Figure 2. The contents (mg/g dry weight, standard error) of total phospholipids (ΣPL), phosphatidylcholine—PC, phosphatidylinositol—PI, phosphatidylethanolamine—PE, phosphatidylglycerol—PG, phosphatidic acid—PA and diphosphatidylglycerol—DPG in the leaves of *Bromopsis inermis* on 16.06.2014 (June) and 3.10.2014 (October). *—Significant differences according to Student's *t*-test.

In autumn, at the onset of low positive temperatures, the amount of PC increased in common oats by a factor of 4 and in the smooth brome by a factor of 3.7 compared with the content of these phospholipids in summer (Figures 1 and 2). The content of membrane PLs in the leaves of the smooth brome hardened by low positive temperatures significantly increased compared to summer values (Figure 2).

Sixteen fatty acids were identified in all samples of the smooth brome. The quantitatively and qualitatively prominent FAs are shown in Table 3. Among FAs, 18:3n-3, 16:0 and 18:2n-6 dominated, their total content reaching 85–90%. The total content of FAs in the leaves of the brome in the autumn period was significantly (1.8 times) higher than in the summer period (Table 3).

Table 3. Contents of fatty acids (mg/g of dry weight and % of total FA ± standard error) in the leaves of the perennial cereal *Bromopsis inermis* before mowing—07.07.2013 (July) and after mowing—25.09.2013 (September), and values of Student's *t*-test (*t*).

Fatty Acids	July *	September	*t*	July	September	*t*
	mg/g	mg/g		%	%	
14:0	0.10 ± 0.06	0.14 ± 0.01	0.62	0.6 ± 0.3	0.45 ± 0.02	−0.57
16:0	3.5 ± 0.4	4.9 ± 0.3	**2.83**	20 ± 1	15.8 ± 0.3	**−2.80**
18:0	0.5 ± 0.1	0.6 ± 0.1	1.12	2.7 ± 0.3	1.8 ± 0.1	−2.56
20:0	0.19 ± 0.01	0.25 ± 0.03	1.76	1.10 ± 0.04	0.8 ± 0.1	**−3.66**
22:0	0.23 ± 0.03	0.28 ± 0.02	1.57	1.3 ± 0.1	0.91 ± 0.04	**−3.33**
16:1n-9+n-7	0.1 ± 0.1	0.27 ± 0.03	**2.91**	0.3 ± 0.3	0.9 ± 0.2	1.60
16:1n-5	0.36 ± 0.03	0.6 ± 0.1	**4.90**	2.1 ± 0.1	2.02 ± 0.01	−0.43
18:1n-9	0.6 ± 0.3	0.6 ± 0.1	−0.28	3.6 ± 1.6	1.81 ± 0.04	−1.11
18:2n-6	2.0 ± 0.2	4.1 ± 0.4	**4.87**	11.5 ± 0.4	13.0 ± 0.3	**3.12**
18:3n-3	9.7 ± 1.2	19 ± 1	**5.37**	55 ± 4	61 ± 1	1.52
SFAs	4.7 ± 0.5	6.4 ± 0.5	2.54	27 ± 2	21 ± 1	**−2.79**
MUFAs	1.2 ± 0.3	1.6 ± 0.2	1.15	6.8 ± 1.7	5.2 ± 0.2	−0.90
PUFAs	12 ± 1	23 ± 2	**5.45**	67 ± 4	74.2 ± 0.4	2.07
ΣFAs	18 ± 2	31 ± 2	**4.93**	-	-	-

* the average air temperature in July = 10.9 °C, the average air temperature in September = −0.6°C; ΣFAs—total fatty acids, SFAs—saturated fatty acids, MUFAs—monounsaturated fatty acids, PUFAs—polyunsaturated fatty acids, bold font—significant differences according to Student's *t*-test.

The content of polyunsaturated fatty acids (PUFAs) in the smooth brome leaves significantly increased, while the content of total saturated fatty acids (SFAs) did not change with the decrease in air temperatures (Table 3). The content of 16:0, 16:1 isomers, 18:2n-6, and 18:3n-3 in the leaves of brome in autumn was significantly higher than in summer (Table 3).

The percentage of SFAs in brome leaves was lower in September compared with July. The decrease in SFAs was due to a decrease in the percentage of 16:0, 20:0, and 22:0 (Table 3). The percentage of PUFAs did not change with the decrease in air temperatures while the percentage of 18:2n-6 significantly increased (Table 3).

Fifty-three FAs were identified in the samples of liver, muscle and subcutaneous adipose tissues of the Yakutian horses. The percentages of important and quantitatively significant FAs are shown in Figure 3. The percentage of SFAs in the liver of the animals was significantly higher than in the muscle and adipose tissues (Figure 3a). Among the SFAs in the liver, 18:0 dominated. Its percentage was about 4 and 6 times higher than that in the muscle and adipose tissues, respectively. Shorter-chain SFAs, such as 14:0 and 16:0, dominated in the muscle and adipose tissues, and their percentages were significantly higher than in the liver (Figure 3a). The percentages of monounsaturated FAs (MUFAs) in the muscle and adipose tissues of the horses were twice higher than in the liver (Figure 3b). Among MUFAs, 18:1n-9 dominated in all types of tissues. Nevertheless, its percentage in the liver was significantly lower than in the other tissues (Figure 3b). The percentage of PUFAs was significantly higher in the animal liver than in the muscle and adipose tissues (Figure 3c). Among PUFAs in the liver, omega-6 PUFA, namely 18:2n-6, dominated. Its percentage was more than twice higher than in the muscle and adipose tissues. In contrast, the muscle and adipose tissues were dominated by omega-3 PUFA, namely 18:3n-3. Its percentage was more than twice as high as the percentage of this FA in the liver (Figure 3c). In total, 70% of all FAs in the muscle and adipose tissues were represented by 18:1n-9, 16:0, 18:3n-3, and 18:2n-6 and in the liver by 18:2n-6, 18:0, 16:0, and 18:1n-9 (Figure 3). No trans-FAs were found in the FA tissue of the horses, and the percentage of branched FAs was less than 1% of the total FAs. The percentages of many FAs were similar in the muscle and adipose tissues of the horses. In adipose tissues, however, the percentages of 18:3n-3 and short-chain SFA (12:0 and 14:0) were significantly higher than in muscles, but almost all long-chain PUFAs, including physiologically important arachidonic (ARA, 20:4n-6), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids, were absent (Figure 3). The percentages of EPA and DHA in the liver and muscle tissues were insignificant and ranged from 0.1% to 0.3% of the total FAs.

The contents of physiologically important EPA and DHA in the muscle tissue and liver of the horses were similar (Table 4). The n-6/n-3 ratio in the muscle tissue was about 7 times lower than in the liver, but did not differ significantly from that in the adipose tissue. The total content of FAs in the adipose tissue was 30 times higher than that in the muscle tissue and in the liver (Table 4).

Table 4. Content of EPA+DHA and total fatty acids (mg/100g and mg/g of wet weight, respectively) and the ratio of total omega-6 and omega-3 PUFAs in the muscle, liver, and subcutaneous adipose tissue of Yakutian horses. Means in lines labeled with the same letters are not significantly different at $p < 0.05$ after Tukey's HSD *post hoc* test (normal distribution, standard errors are given) or Kruskal–Wallis test with multiple comparisons of mean ranks (non-normal distribution standard errors are omitted).

	Muscle	**Liver**	**SCfat**
EPA+DHA, mg/100 g ww	11 ± 1 ^{A}	11 ±1 ^{A}	-
Total FA, mg/g ww	31 ^{A}	28 ^{A}	854 ^{B}
n-6/n-3	1.1 ± 0.2 ^{A}	5.5 ± 1.2 ^{B}	0.44 ± 0.03 ^{A}

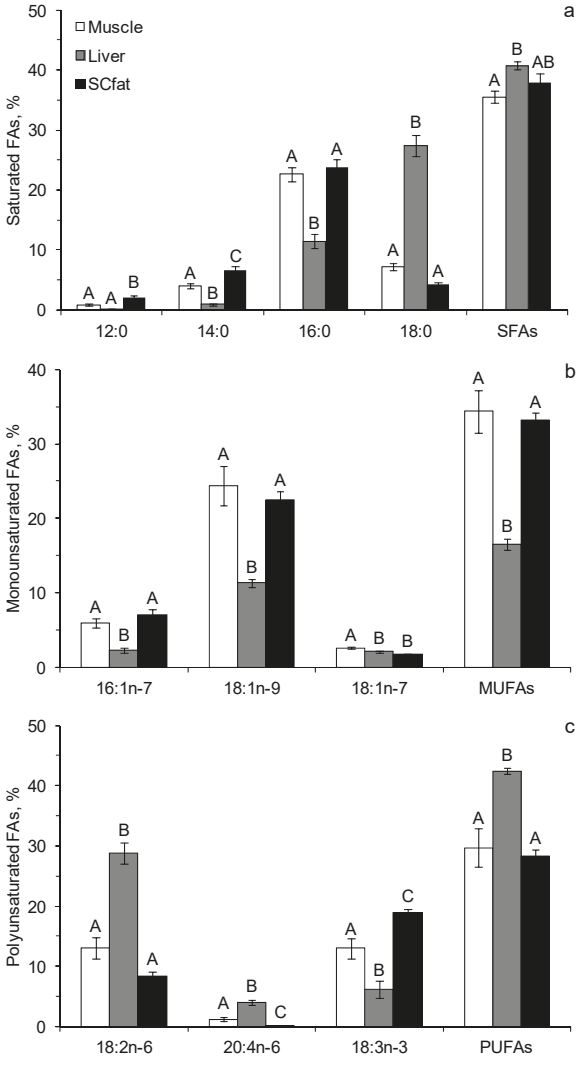

Figure 3. Contents of the prominent saturated fatty acids (**a**), monounsaturated fatty acids (**b**) and polyunsaturated fatty acids (**c**) (% of total FAs, standard error) in liver, muscle and subcutaneous adipose tissues of the Yakutian horses. Means for the same FAs labeled with the same letters are not significantly different at $p < 0.05$ after Tukey's HSD *post hoc* test.

The desaturase and elongase activity, estimated by an indirect method (by product/precursor ratio), were significantly different between the adipose tissue and the liver (Table 5).

Table 5. Calculated desaturase and elongase activity indices.

Product/Precursor Ratio	Liver	SCfat	*t*
18:0/16:0	2.5 ± 0.3	0.19 ± 0.02	**7.48**
16:1n-7/16:0	0.20 ± 0.02	0.3 ± 0.1	**−3.85**
18:1n-9/18:0	0.42 ± 0.04	5.3 ± 0.4	**−12.81**
20:4n-6/20:3n-6	7.9 ± 0.7	-	-
20:5n-3/20:4n-3	8.8 ± 0.8	0.5 ± 0.1	**10.19**
20:5n-3/18:3n-3	0.06 ± 0.03	0.0006 ± 0.0002	**2.34**
20:4n-6/18:2n-6	0.14 ± 0.01	0.003 ± 0.001	**11.80**

Bold font—significant differences according to Student's *t*-test.

The conversion efficiencies of 16:0 and 18:0 to 16:1n-7 and 18:1n-9, respectively, were higher in the adipose tissue and the conversion efficiencies of 16:0 to 18:0, 20:4n-3 to 20:5n-3, 18:3n-3 to 20:5n-3, and 18:2n-6 to 20:4n-6 were higher in the liver (Table 5). The conversion efficiency of 18:2n-6 to 20:4n-6 was higher than the conversion efficiency of 18:3n-3 to 20:5n-3 both in the liver and in the adipose tissue (Student's *t*-test = 2.75 and *t* = 2.66, respectively). The conversion efficiency of 20:4n-3 to 20:5n-3 and 20:3n-6 to 20:4n-6 in the liver did not differ significantly (Student's *t*-test = 1.09).

4. Discussion

Lower ambient temperatures significantly affect the 'liquidity' of plant membranes, reducing their fluidity. This leads to the increased expression of the genes responsible for FA desaturation [26]. The increased fraction of unsaturated FAs in plants with the temperature decrease stabilizes membrane fluidity and restores physiological activities of the associated enzyme and electron transport systems, photosynthesis in particular [27–29]. Affected by low temperatures, the genes that encode the synthesis of desaturases involved in the formation of 18:2n-6 and 18:3n-3 are activated in the plants [26,30]. The increase in total lipids, phospholipids, and total FAs that we detected in the cereals showed that these substances along with sugars, proteins, antioxidants, and carotenoids [1,31] are involved in the cold adaptation of cool-season plants in the cryolithozone of Central Yakutia. In the same way as *B. inermis* studied in our work, other herbaceous plants (*Avena sativa, Elytrigia repens, Equisetum variegatum*, and *Equisetum scirpoides*) accumulated significantly more FAs in their vegetative organs during the period of winter cold adaptation than in summer [17,32–34].

During cold adaptation of plants, the contents of phospholipids and polyunsaturated fatty acids increase in their tissues [35–37]. However, in contrast to most published data, we found a significant increase in the content of phosphatidylcholine but not in the other phospholipids. This finding probably shows the key role of phosphatidylcholine in temperature adaptation of the cereals.

Along with *Bromopsis inermis* and *Avena sativa*, the ability to cryopreserve green mass was found in many other plants in Central Yakutia, for example, cereals—*Arctophila fulva*, *Deschampsia borealis*, *Puccinellia jacutica*, *Poa alpigena*, hydrophytic sedges—*Carex rhynchophysa*, *C. atherodes*, *C. vesicata*, *C. enervis*, and most cotton grasses—*Eriophorum scheuchzeri*, *E. vaginatum*, *E. russeolumsubsp. leiocarpum*, *E. angustifolium* [6]. In the pre-winter period of fat accumulation, herbivores actively consume cool-season and winter-green parts of these fodder plants with the high contents of nutrients [2].

The main consumer of green cryo-fodder in plant ecosystems of cryolithozone in Central and North-Eastern Yakutia is the Yakutian horse. In autumn, from August to the beginning of October, the horses feed on green cryo-fodder. In favorable years, the accumulation of fat on green cryo-fodder by the Yakutian horses lasts up to mid-November [38,39].

The tissues of the Yakutian horses and their fodder were rich in two PUFAs, namely 18:3n-3 and 18:2n-6. These FAs are essential for the majority of animals [40–42]. Vertebrates can synthesize physiologically important long-chain PUFAs—20:5n-3, 22:6n-3, and 20:4n-6—from their dietary precursors 18:3n-3 and 18:2n-6, respectively, but the rate of synthesis is generally ineffective [40,43]. Suagee et al. found that mesenteric adipose tissue in horses had a high lipogenic capacity followed

by subcutaneous adipose tissue and then liver [44]. Very low percentages of 20:5n-3 and 22:6n-3 (the average value = 0.4% of total FAs) as well as a low 20:5n-3/18:3n-3 ratio in the tissues probably indicated a low conversion efficiency of omega-3 PUFAs in the Yakutian horses. The conversion of omega-6 PUFAs seemed to be more efficient than conversion of omega-3 PUFAs, at least in the liver. The literature data and our results suggest that dietary sources of 20:5n-3, 22:6n-3 and 20:4n-6 were absent from the diet of the Yakutian horses [17]. Thus, we suppose that these long-chain PUFAs were synthesized in horses' tissues. According to our data, the efficiency of elongation of 16:0 to 18:0 was significantly higher in the liver while the conversion efficiency of SFAs to MUFAs was higher in the subcutaneous adipose tissue. Similar trends in the conversion efficiency of SFAs to MUFAs and elongation of 16:0 to 18:0 in liver and subcutaneous adipose tissue were reported by Adolph et al. for Warmblood horses [25].

Different contents of C18, C20, and C22 PUFAs in the tissues of the horses may indicate different functions of these PUFAs. Unlike Warmblood horses, the subcutaneous adipose tissue of the Yakutian horses was rich in PUFAs, especially in the omega-3 family [25]. Mordovskaya et al. and Slobodchikova et al. also noted enrichment of the Yakutian horses' adipose tissue with 18:3n-3 [45,46]. High percentages of 18:3n-3 in the subcutaneous fat of the Yakutian horses may increase fluidity (liquidity) of adipose tissue during the winter period of extremely low temperatures. This may be a reason for high mitochondrial activity in adipocytes, which increases energy production at low ambient temperatures. The beneficial effects of omega-3 PUFAs on the thermogenic function of adipocytes have recently been demonstrated [47–49]. Thus, we suppose that 18:3n-3 served as an energy-related component in the horses. By contrast, omega-6 PUFAs, namely 18:2n-6 and 20:4n-6, which were accumulated in liver and muscle tissue, likely served as important structural components or precursors of lipid mediators. Similar results were reported in a study of the FA composition of different lipid classes in Iberian horses [50]. In the muscle tissue, 18:2n-6 and 20:4n-6 accumulated in polar lipids, apparently performing a building function, and 18:3n-3, on the contrary, accumulated in neutral lipids, performing an energy function [50].

The PUFA content in the horse muscle and adipose tissues varies greatly depending on the diet, breed, and age of the animals [51–53]. For example, the content of 18:2n-6 in horse muscles varied from 12% to 32%, and the content of 18:3n-3 varied from 0.43% to 23.9% [53]. Along with individual fatty acids, the total contents of SFAs, MUFAs and PUFAs can also vary greatly in the horse muscle tissue: 34.2–47.8%, 16.4–50.2% and 15.6–46%, respectively [53]. The muscle tissue of the horses we studied contained equal proportions of SFAs, MUFAs, and PUFAs, which corresponded to the minimum SFA values and the average MUFA and PUFA values available in the literature. Similar to the Yakutian horses, horses that were fed on the native grass pasture had the same percentages of SFAs, MUFAs, and PUFAs, as well as high percentages of 18:3n-3, in subcutaneous adipose tissue [54]. The total FA content of the subcutaneous adipose tissue of the Yakutian horse corresponded to the high values known for horses, varying between 457 and 904 mg/g wet weight [53,54]. Obviously, nutrition has a significant effect on the variability of FA percentages in horses. Horses eating fresh plant food, but living in a mild climate, had similar contents and distribution of FAs, including 18:2n-6 and 18:3n-3, to those in the Yakutian horses. High contents of lipids and FAs such as 18:3n-3 and 18:2n-6 in green cryo-fodder probably help the Yakutian horses successfully survive the extreme temperatures of Central Yakutia. However, the results obtained only indirectly indicate this and do not allow us to clarify the subject.

In contrast to many farm animals, horses are able to efficiently assimilate PUFAs from plant food owing to the structure of their gastrointestinal tract, activity of microorganisms, and the presence of specific pancreatic lipases related with protein 2 (PLRP2) [53,55]. Thus, horse meat is considered as a useful dietary product, i.e., a source of essential PUFAs, namely 18:2n-6 and 18:3n-3 [56–58], and can be potentially enriched with long-chain omega-3 PUFAs, 20:5n-3 and 22:6n-3. However, our data suggest that meat, subcutaneous fat, and liver of the Yakutian horses are not rich in 20:5n-3 and 22:6n-3 if their food does not contain these PUFAs. This may indicate limitation in PUFA synthesis in the horses' tissues. The contents of 20:5n-3 and 22:6n-3 (% and mg/g wet weight) in the liver of the Yakutian

horses and other horse breeds were significantly lower than in the liver of cows, pigs, and chickens [59]. The contents of 20:5n-3 and 22:6n-3 (mg/g wet weight) in the meat of horses were the same as in beef and higher than in pork [53,60]. In general, because of the high content of 18:3n-3 and the optimal ratio of n-6 to n-3 PUFAs, the Yakutian horse meat is a more valuable and health food product compared to beef, pork, and chicken, which is consistent with the data of many authors [45,60–62].

5. Conclusions

The cereal plants studied (*B. inermis* and *A. sativa*) accumulate lipids, phosphatidylcholine and fatty acids, in particular, during the period of natural cold hardening in extremely cold climates of the permafrost zone. Cereals enriched with nutrients are the basis for the Yakutian horse feeding during pre-winter fat accumulation. The muscle and adipose tissues and liver of the horses contained high percentages of 18:2n-6 and 18:3n-3, which were abundant in the cereals studied in this work. A likely reason for the diverse distribution of these FAs in tissues is that these FAs perform different functions in the animals. 18:2n-6 is probably used as a precursor in the synthesis of physiologically valuable 20:4n-6, while 18:3n-3 mainly performs an energy-related function. Such a high content of 18:2n-6 and 18:3n-3 in the tissues of horses of the Yakutian breed apparently helps animals successfully survive the extreme temperatures of Central Yakutia, although more research is needed. Additionally, the Yakutian horse meat has proved to be a valuable dietary product due to its low n-6/n-3 ratio.

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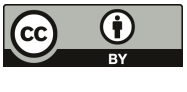

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