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Off-flavors and lipid components in rainbow trout (*Oncorhynchus mykiss*) reared in RAS: Differences in families of low and high lipid contents

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ABSTRACT

Recirculating aquaculture systems (RAS)s have become important in land-based fish production. Unfortunately, RAS-farmed fish accumulate off-flavors in their flesh. Off-flavor compounds geosmin (GSM) and 2-methylisoborneol (MIB) are lipophilic compounds and their concentrations need to be controlled in RAS. Therefore, a useful area of study would be the expected impact of total lipid contents and fatty acids in the fish on the accumulation of these off-flavor compounds. The objective of this research was to study the relationships between off-flavor compounds, lipid contents and fatty acids, both at individual and family levels, in rainbow trout (*Oncorhynchus mykiss*) reared in an experimental RAS.

This study showed that 1) the fish and families with high lipid contents accumulated high concentrations of off-flavor compounds, especially when the off-flavor compounds were expressed as contents of total lipids. The overall concentrations of off-flavors ranged from 0.1 to 3.9 ng g⁻¹ ww (GSM) and from 0.1 to 2.4 ng g⁻¹ ww (MIB). 2) The concentrations of polyunsaturated fatty acids (PUFA) and n-3, which are considered health-promoting groups of fatty acids, were lower in fish individuals and families with high lipid content and higher in fish individuals and families with low lipid contents. Therefore, excessive lipid contents may reduce the nutritional value of the fish. 3) Reduced levels of GSM correlated with increased levels of PUFA and n-3 and decreased levels of monounsaturated fatty acids (MUFA, a health-wise neutral effect), and n-6:n-3 ratio. These are highly favorable relationships for aquaculture: high concentrations of health-promoting fatty acids were related to lower off-flavor concentrations. The results imply that changes induced by selective breeding in lipid content may impact both the fatty acid profiles and off-flavor compounds in rainbow reared in a RAS, but large-scale genetic studies are needed to confirm the potential genetic basis of these relationships.

1. Introduction

Recirculating aquaculture systems (RAS)s have become increasingly important in land-based fish production, and when properly managed they are suitable for wide range of fish species (Rurangwa and Verdegem, 2015). Unfortunately, RAS-farmed fish often accumulate off-flavors in the muscle tissue of the fish, making it objectionable to consumers. Certain off-flavors in fish have a distinct musty, muddy, or earthy flavor, typically caused by semi-volatile, lipophilic compounds geosmin (GSM, *trans*-1,10-dimethyl-*trans*-9-decalol) and 2-methyl isoborneol (MIB, (1-*R*-exo)-1,2,7,7-tetramethyl-bicyclo[2.2.1]heptan-2-ol) (Gerber, 1968, 1969; Auffret et al., 2011), which are considered to be the most important compounds causing off-flavors in fish flesh.

However, many other unwanted off-flavor-inducing compounds have been identified in fish flesh and skin (Mahmoud and Buettner, 2017; Podduturi et al., 2017). GSM and MIB are formed as metabolites of a number of microorganisms, such as *Actinomycetales*, *Myxococcales*, and *Cyanobacteria* (Dickschat et al., 2005; Lukassen et al., 2017; Yamada et al., 2015).

Humans have very low odor detection limits for GSM and MIB. For example, human detection limits of 0.55–0.7 ng g⁻¹ for MIB and 0.9 ng g⁻¹ for GSM have been reported for rainbow trout (*Oncorhynchus mykiss*) and barramundi (*Lates calcarifer*) (Robertson et al., 2005; Petersen et al., 2011; Jones et al., 2013; reviewed in Lindholm-Lehto and Vielma, 2018), and even a 0.25 ng g⁻¹ limit value for GSM in channel catfish (*Ictalurus punctatus*) (Grimm et al., 2004). The detection limits depend

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not only on the fish species, but on the lipid content of the fish: an increased lipid content leads to a higher detection limit (Howgate, 2004; Moretto et al., 2022).

According to the current understanding, the off-flavor compounds seek an equilibrium between concentrations in the circulating water and in the lipids of fish flesh (Howgate, 2004). Concentrations above 5–10 ng L⁻¹ of GSM and MIB in water have been suggested to induce accumulation of off-flavors in fish flesh with objectionable flavor and odor (Petersen et al., 2011). Off-flavor compounds are rapidly adsorbed and accumulated in the fish flesh, typically within hours (Schram et al., 2018). On the other hand, depuration in clean water, in a flow-through mode to remove off-flavors is a slow process which takes several days in clean water, often 10–15 days, before the fish are suitable for sale (Davidson et al., 2014; Lindholm-Lehto et al., 2019). The lipophilic compounds accumulate in the fish flesh based on the lipid content of the fish and differences in concentrations can occur even in different parts of an individual fish. For example, Lindholm-Lehto et al. (2019) showed that the concentrations of GSM and MIB were higher in the belly and neck of European whitefish (*Coregonus lavaretus*) compared to the tail part with a lower lipid content.

Lipid content varies greatly between families in salmonid species. For example, the percentage of individual variation explained by genetics (i.e., heritability) can be up to 60% in rainbow trout (Tobin et al., 2006). In breeding programs of salmonid species, the aim has been to control the lipid content, and especially to avoid excess lipid contents. However, selection for fast growth often leads to a high lipid content (reviewed by Kause et al., 2007). The lipid content is related to product quality and leaner fish often have a better feed conversion ratio (Kause et al., 2016). The lipid content is also genetically related to the fatty acid profiles of the flesh, which show moderate levels of genetic variation (Leaver et al., 2011; Horn et al., 2018). The lipid content can be recorded on live fish from thousands of individuals which has helped to study family-differences in lipid content and to implement selection based on the lipid content in aquaculture breeding programs (Janhunen et al., 2017). Therefore, it is important to study the expected impact of lipids and fatty acids on off-flavor compounds, but so far there is limited knowledge on family differences, or correlations between the fatty acid composition and off-flavor accumulation (Lu et al., 2022).

The effects of feed compositions (Chaiyapechara et al., 2003; Rinchard et al., 2007; Horn et al., 2018), salinity (Haliloglu et al., 2004) and seasonal changes (Hong et al., 2015) on the lipid content and fatty acid composition in fish muscle have been studied, but as far as we are aware, family-based differences and family-based correlations between the fatty acid composition and off-flavor accumulation have not been established. The objective of this research was to study the relationships between off-flavor compounds, lipid contents and fatty acid composition, both at individual and family levels, in rainbow trout reared in a RAS. Specifically, we assessed 1) whether or not families have different levels of off-flavor compounds in the muscle, 2) whether the high-lipid fish and families have higher concentrations of off-flavor compounds, 3) which fatty acid groups and individual fatty acid compounds were connected to the highest lipid contents, and 4) to quantify the off-flavor compounds.

2. Materials and methods

2.1. Population structure and experimental design

The rainbow trout (*O. mykiss*) population originated from the Finnish national breeding program, maintained by Natural Resources Institute Finland (Luke) at the Enonkoski research station. The breeding program has been running since the late 80's (Kause et al., 2005). For the current study, eight full-sib families from the year class of 2014 (the 8th generation) were used. A subset of all families was used due to the laborious analysis procedure for off-flavor chemicals.

Among the year class for 2014, a total of 191 sires and 192 dams

were used to produce 376 full-sib families between 15th and 16th of May 2018. The egg batches of the families were incubated separately in horizontal incubators and in environmental water. At the eyed-stage, 200 families were moved for on-growing in family tanks. Each family was held in an indoor 150 L family tank at an ambient water temperature (at 0–20 °C) and fed with commercial feed (Raisioagro Ltd., Raisio, Finland; BioMar Group, Aarhus, Denmark).

Seven months after hatching, the fish of all families were individually tagged (in January–February 2019) by injecting a passive integrated transponder (PIT, Biomark GPT12, Biomarc, Inc., Boise, Idaho, USA) into a body cavity. A random sample of 50 full-sib families were chosen for an experiment carried out in the recirculating aquaculture system (RAS) facility at Laukaa fish farm. A total of 600 fish, on average weighing 70 g were delivered to Laukaa fish farm in May 2019. However, due to dysfunctional PIT tags, some fish were removed from the population.

In June 2019, the experiment started in a RAS. The fish were identified based on their PIT tags and evenly distributed in 10 round 450 L plastic rearing tanks. The trial began with a total of 50 families and 514 fish. Each tank contained a total of 51–52 fish, 1–2 fish from each family.

In the final sampling (on 2nd–6th December 2019), individual PIT tags were recorded and the fish wet weight, total length, carcass weight, and Distell lipid content values were measured and recorded. The Distell fat contents of live fish were measured by using the Distell Fish Fatmeter (FFM-692, Distell.com, Old Levenseat, Fauldhouse, Scotland) using the “trout-1” setting. Readings were taken from four points above the lateral line of the fish between the fish head and tail according to the manufacturer's recommendations.

The gender of the fish were visually identified based on the early signs of gonad development (17 males, 33 females, 14 immature fish). The fish were fasted for two days prior to sampling. The selected fish were humanly euthanized, instantly gutted, and filleted. Based on the previous Distell fat measurements (made in September 2019), four families with low fat (average 5.47% units) and four families high fat records (average 9.00% units) were chosen for more detailed analyses of the lipid content, fatty acid profile, and off-flavor compounds in the muscle. These eight families were the focus of this study.

2.2. Rearing conditions in RAS

The experimental RAS consisted of 20 identical 450 L bottom-drained rearing tanks made of fiberglass with a common water treatment system, a drum filter with a 60 µm mesh size (Faivre F2–80, France) for solids removal, and a moving-bed biofilter filled with plastic blocks. Another biofilter (CycloBio, Marine Biotech, USA) filled with fine sand as a carrier material was also used for nitrification. A cascade column, filled with plastic balls (polypropylene, 50 mm in diameter) was employed for gas exchange followed by an UV light disinfection unit (4 × 220 W UV light bulbs, Skjölstrup & Grönborg, Denmark). The tanks were lighted continuously (24-h white light) by led lamps on the tank inner cover. The oxygen content of the circulating water was maintained by aerating the water in the moving-bed biofilter and injecting pure oxygen into the cascade column. The pH of the circulating water was adjusted with a 2:1 mixture of NaHCO₃ (Solvay Chemicals International SA) and Microdol powder (CaMg(CO₃)₂, Talc As, Norway). Surface water from Lake Peurunka was used as the clean replacement water at a relative water renewal rate of about 750 L kg⁻¹ feed.

Ad libitum feeding was carried out for 6 h per day (03.00–06.00 and 11.00–14.00), using belt feeders and commercial feed developed for RAS (Circuit Red, Raisioaqua Ltd., Raisio, Finland). The main ingredients of feed consisted of fish meal made of Baltic herring and sprats, soya meal, and horse beans, fish and rapeseed oil being the lipid sources in the diets, including a total of 0.95–1.15% P and 7.52–7.84% N. The fish were fed with 3.5 mm (45% protein, 24% lipids), 5.0 mm (43% protein, 26% lipids), and 7 mm pellets (41% protein, 31% lipids), Circuit Red (Raisioaqua, Raisio Oy, Finland). During the experiment, the daily

feeding amount increased as the fish grew, but the share of waste feed was kept between 5% and 10% of the daily feeding and collected by feed traps in the tank outlets. At the beginning of the experiment, the fish were fed with 3.5 mm pellets, while from August 22nd 2019 they were fed with 5 mm pellets, and from September 27th 2019 until the end of the experiment with 7 mm pellets.

During the experiment, the oxygen saturation was maintained at $98.2 \pm 7.0\%$ in the tank inlet water, and $74.8 \pm 11.5\%$ - $94.8 \pm 12.2\%$ in the tank outlet. The water temperature was automatically hourly recorded and kept at 16.1 ± 0.8 °C. Water quality parameters, including the total ammonia, nitrite-N, and nitrate-N, were monitored weekly by quick spectrophotometric laboratory tests (Procedure 8038 Nessler, LCK340, LCK341, UN3316 9 II). The total ammonia nitrogen ($\text{NH}_3\text{-N} + \text{NH}_4\text{-N}$) was 0.27 ± 0.06 mg L⁻¹, unionized ammonia ($\text{NH}_3\text{-N}$) 0.001 mg L⁻¹, nitrite ($\text{NO}_2\text{-N}$) 0.03 ± 0.01 mg L⁻¹, nitrate ($\text{NO}_3\text{-N}$) 40.4 ± 5.0 mg L⁻¹, and the pH was 7.1 ± 0.3 .

The alkalinity was monitored using a standard titration method (ISO 9963 – 1:1994, TitraLab AT1000, Hach, Loveland, USA), resulting in 42.5 ± 16 mg L⁻¹ while the turbidity, measured using a Hach DR 3900 Turbidimeter (USA) was 4.6 ± 3.9 NTU. All the parameters were at suitable levels, indicating good water quality.

The study followed the protocols approved by the Luke Animal Care Committee, Helsinki, Finland, and EU Directive 2010/63/EU, n.d. for animal experiments.

2.3. Sampling for the analyses

A skinless sample of fish muscle ($20 \text{ g} \pm 1 \text{ g}$ per fish, 8 fish per family) was taken from between the pelvic fin and the lateral line, as defined by Hathurusingha and Davey (2016), for the analysis of the lipid content, off-flavors, and fatty acids. This was the same location as used for the Distell measurements. A piece of the fish sample was stored frozen at -24 °C until the analysis of the off-flavor compounds, and another similar piece was freeze-dried for the determination of the total lipid content and fatty acid composition.

2.4. Off-flavor analyses

Concentrations of the off-flavor compounds GSM and MIB were determined by a headspace solid phase micro extraction (HS-SPME) pretreatment followed by a gas chromatograph-mass spectrometer (GC-MS) analysis as described by Lindholm-Lehto et al. (2019). A manual SPME assembly with an extraction fiber coated with StableFlex divinylbenzene/ carboxene/ polydimethyl siloxane (DVB/CAR/PDMS), 1 cm, 50/30 µm (part no. 57328-U) in a manual holder was purchased from Supelco (Merck). Additionally, 10 mL headspace (HS) glass vials and polytetrafluoroethylene (PTFE) septum caps (both from VWR) were used.

For the quantification of GSM and MIB, 3-isobutyl-2-methoxypyridine (IBMP, $\text{C}_9\text{H}_{14}\text{N}_2\text{O}$, CAS 24683-00-9) (Sigma Aldrich, 99%, 0.99 g mL^{-1}) was used as an internal standard. A stock solution of 90 ng L^{-1} was prepared by diluting IBMP with high-performance liquid chromatography (HPLC) grade methanol (99%, J.T. Baker, WVR).

The extraction was performed using HS-SPME. A volume of 1 mL of water or 1 g of fish flesh was placed in a 10 mL HS vial with 750 µL of aqueous NaCl (98%, Merck). An internal standard solution of 30 µL was added to a sample vial. The sample vial was sealed and placed in a water bath at 60 °C. A DVB/CAR/PDMS fiber was exposed in the headspace and kept for 30 min to complete the extraction before introducing the fiber directly into the inlet of GC-MS (Agilent 6890 series/5973 N GC/MSD, Palo Alto, CA, USA).

The separation and quantification of GSM and MIB was carried out using a GC-MS equipped with a Phenomenex Zebron ZB-5MSi (Torrance, CA, USA) capillary column ($30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \text{ µm}$). The temperature of the injector was adjusted to 270 °C in the splitless mode. The carrier gas was helium at a flow rate of 0.7 mL min^{-1} . The

temperature of the oven started at 45 °C for 3 min and increased 30 °C min^{-1} to achieve 300 °C (total time 14.5 min).

The electron ionization (EI)-MS conditions were selected as 230 °C for the ion source with a 5 min delay time and ionizing voltage of 70 eV. The selected ion monitoring (SIM) mode with m/z 112, 126, and 182 was used for the detection of GSM, while m/z 95, 135, 168 were used for MIB, and the internal standard IBMP was identified at m/z 137. A detailed method description and the method validation data have previously been reported by Lindholm-Lehto et al. (2019). In this study, the concentrations of GSM and MIB were reported as the absolute content in fresh muscle tissue (ng g^{-1} muscle wet weight, ww), referred to as the absolute GSM/MIB content, and as the proportional content in the lipid fraction of a muscle (µg g^{-1} fat ww), referred to as the proportional GSM/MIB content.

2.5. Lipid content in fish muscle

The total lipid content in the muscle was determined by an accredited in-house method which is based on the AOAC Official Method 920.39 Fat (Crude) or Ether Extract in animal Feed, acid hydrolysis method, AOAC Method 954.02 (Association of Official Analytical Chemists, USA), and AACC method 30–25 Crude fat in Wheat, Corn, and Soy Flour, Feeds, and Mixed Feeds. The equipment used was a Foss Soxtec/Hydrotec 8000™ System for the total fat analysis, consisting of a Soxtec™ 8000 extraction unit and Hydrotec™ hydrolysis unit, (FOSS Analytical, Denmark). The test lab holds FINAS accreditation number T024 and follows the standard SFS-EN ISO/IEC 17025:2017. In this study, the muscle lipid content was reported in g kg^{-1} ww.

2.6. Fatty acid composition

Fatty acid methyl esters (FAME) of the lipids in the freeze-dried experimental diet samples (200 mg) were prepared in a one-step extraction-transesterification procedure using chloroform and 2% (v/v) sulfuric acid in methanol (Shingfield et al., 2003). The lipids in the freeze-dried trout samples (80 mg) were extracted with methanol, UHQ water and methyl tert-butyl ether and transesterified to FAME using acetyl chloride in methanol (1:9) (Ostermann et al., 2014). The fatty acid content was determined using tritridecanoin (T-135; Nu-Chek-Prep, Elysian, MN) as an internal standard and tripalmitin (T-5888; Sigma-Aldrich, St. Louis, MO) as an external standard. The FAME were quantified using a GC (model 6890 N; Agilent Technologies, Santa Clara, CA) fitted with a CP-Sil 88 column ($100 \text{ m} \times 0.25 \text{ mm i.d.}$, 0.2 µm film thickness; Agilent Technologies) and flame ionization detector (FID) by using a temperature gradient program (Shingfield et al., 2003) and hydrogen as the carrier gas operated at constant pressure (206.8 kPa) and a nominal initial flow rate of 2.1 mL min^{-1} . The fatty acid composition in weight percentages were calculated using theoretical response factors (Wolff et al., 1995). In this study, proportional fatty acids were reported in g kg^{-1} fish ww of the total weight of fatty acids.

Among the groups of fatty acids, the abbreviations used included monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and saturated fatty acids (SFA). Additionally, n-3 and n-6 were used for PUFAs with a double bond in the third or sixth carbon of fatty acid molecule chain from the terminal methyl group. The ratio of these two was n-6:n-3.

2.7. Statistical analyses

Statistical analyses were performed for the following: Distell lipid content, muscle lipid content, viscera percentage, absolute and proportional GSM and MIB. Individual and groups of fatty acids were recorded at final sampling (Supplementary Table S1).

2.7.1. Calculation of family differences

To evaluate the degree of family differences in the traits, an analysis

of variance (ANOVA) was performed in the SAS software package (version 9.4., SAS Institute inc.). The mixed model ANOVA included fish traits as a response variable, the family and experimental tank as random effects, and the gender of the fish as a fixed effect (Model 1). The model produced a statistical test for the family effect (proc GLM), and estimated the amount of variance explained by the family effect (proc varcomp). The proportion of individual variance explained by the family effect was calculated as: Family effect = $2 \times \text{family variance} \times \text{total individual variance}^{-1}$ (Falconer and Mackay, 1996). The family variance included non-genetic maternal and paternal effects, environmental effects common to full-sibs, parts of potential dominance effects, and the genetic effects.

2.7.2. Calculation of correlations

To calculate the family-mean correlations, the family-means were estimated by Model 1 and used as observations to calculate Pearson correlations between all traits. These family-mean correlations (r_{Fam}) contained a sample size of 8 families, and therefore only very high correlations were statistically significant. The value of the correlation coefficient itself was more informative than the p -value.

To quantify the relationships between individuals, phenotypic correlations (r_{Ind}) were calculated from the residuals of the mixed model ANOVA in which an experimental tank was a random effect and gender a fixed effect (Model 2). The trait mean was added to the residuals, to scale a trait back to the original mean. The sample size for these correlations was 63 or 64, depending on the trait combinations.

2.7.3. Bonferroni correlation for multiple testing of fatty acids

Within each fatty acid group, multiple statistical tests of correlations between individual fatty acids and off-flavor compounds were performed. Within each fatty acid group, many of the individual fatty acids were typically highly correlated, either positively or negatively, and these correlations were partly a result of a real underlying biological phenomenon. If one individual fatty acid was statistically significant, then many others were too. Moran (2003) has argued that in such cases a Bonferroni correction of p -values based on the number of tests performed is overly strict and illogical. As a compromise, we considered that the correlations of SFA, MUFA, and PUFA groups were three multiple tests that were repeatedly tested with the data on the individual fatty acids. Hence, the p -values of the correlations of individual fatty acids were divided by 3. This resulted in a significance level of $p = 0.0167$ to be statistically significant ($0.05/3 = 0.0167$).

2.7.4. Difference between correlations of GSM and MIB with individual fatty acids

To study the degree to which proportional MIB and GSM had different relationships with individual fatty acids, a regression equation between the correlations of proportional GSM with individual fatty acids, and the correlations of MIB with individual fatty acids were calculated (correlations of GSM on x-axis, and those of MIB on y-axis). The regressions were calculated separately for the phenotypic and family-mean correlations. A type II regression was used because both variables had measurement errors and either of the variables could be on the X-axis. The assumptions of the type I regression were not fulfilled (Sokal and Rohlf, 2012).

3. Results

3.1. Family differences

Between the two Distell recordings, the body weight of the fish increased from 620 g (± 151.7 g; SD) to 1178 g (± 386.8 g) ($n = 64$ fish) and the Distell lipid content increased from 7.36% (± 2.400) to 10.0% (± 3.145). The correlation of the first Distell lipid content with the second Distell lipid content was 0.82, and the individual correlation of first Distell lipid content with the second muscle lipid content was only

moderately positive at 0.53 ($n = 64$ fish). This shows that the first Distell lipid content reading was a moderate predictor of muscle lipid at the final sampling and that there was larger variation than expected under random sampling. This led to the analysis of both individual- and family-based correlations for the difficult-to-record traits, off-flavor compounds, and fatty acid profiles. The statistical power to observe family differences in lipids was increased by non-random sampling and the estimated correlations were expected to be stronger than under random sampling.

The Distell lipids and muscle lipids displayed a highly significant family effect, explaining 0.40 to 0.50 of the total individual variation (Table 1). The family effect for proportional GSM (0.42) was moderate and marginally significant, for proportional MIB the effect was moderate but non-significant (0.31), and for absolute MIB (0.0) and GSM (0.15) the effect was low and non-significant (Table 1).

The family effect was significant and ranged from moderate to high for proportional SFAs, PUFAs, and n-6 (0.30–0.49). The family effect was low and non-significant for MUFAs, n-3 and n-6:n-3 ratio (0.07–0.11) (Table 1).

3.2. Correlations of lipids

The Distell lipid content, viscera fat content, muscle lipid content, and total fatty acid content of the muscle were significantly and moderately positively correlated at the individual level ($r_{\text{Ind}} = 0.27$ – 1.0) (Table 2). The family-mean correlations were of the same magnitude ($r_{\text{Fam}} = 0.28$ – 1.0) but were non-significant due to the low statistical power. The Distell Fatmeter measured the whole fillet lipid content, while the chemically determined lipid content was analyzed more locally from a piece of fish muscle.

From here onwards, the results have been reported only for the Distell lipid content (as a useful non-destructive indicator of the fillet lipid %) and the muscle lipid content (real lipid concentration of a muscle tissue sample). The total fatty acids have also been excluded from further analysis which gave similar results to the muscle lipid content.

3.3. Correlation of off-flavor compounds

The absolute MIB and GSM contents were very weakly correlated between individuals ($r_{\text{Ind}} = 0.19$) and families ($r_{\text{Fam}} = -0.01$) (Table 3,

Table 1

Phenotypic variance and the proportion of variance explained by the family effect in rainbow trout families reared in RAS.

Trait	V_{ind}	Proportion explained by family effect	F	p
Body weight	128,105	0.130	1.97	0.088
Distell lipid content	12.35	0.502	3.36	0.008
Muscle lipid content	519.7	0.473	3.35	0.008
Absolute MIB content	0.2246	0.001	0.55	0.791
Absolute GSM content	0.6034	0.154	1.73	0.135
Proportional MIB	720.1	0.309	1.55	0.184
Proportional GSM	6061	0.416	2.09	0.071
SFA content	0.5215	0.349	2.69	0.026
MUFA content	3.686	0.106	1.49	0.203
PUFA content	3.482	0.298	2.48	0.035
n-3 content	3.428	0.107	1.53	0.189
n-6 content	0.2335	0.492	2.92	0.016
n-6:n-3 ratio	0.0124	0.065	1.43	0.226

V_{ind} - Phenotypic variance across individuals.

Proportion explained by family effect - Proportion of individual variance explained by the family effect.

F - F -statistics for the statistical significance of the family effect.

p - p value for the significance of the family effect. Significant when $p < 0.05$.

Table 2

Phenotypic (above diagonal) and family-mean correlations (below diagonal) between lipid contents.

	Parameter	Distell lipid content	Viscera %	Muscle lipid content	Total FA content
Distell lipid	<i>r</i>	–	0.39	0.65	0.65
	<i>p</i>	–	0.0015	<0.001	<0.0001
Viscera%	<i>r</i>	0.57	–	0.27	0.27
	<i>p</i>	0.1392	–	0.0347	0.0347
Muscle lipid content	<i>r</i>	0.58	0.28	–	1.00
	<i>p</i>	0.1347	0.4957	–	<0.0001
Total fatty acid content	<i>r</i>	0.58	0.28	1.00	–
	<i>p</i>	0.1348	0.4958	<0.0001	–

r - Correlation coefficient.

p - *p*-value for the significant difference from zero.

*Significant values in **bold**.

Table 3

Phenotypic (above diagonal) and family-mean correlations (below diagonal) between GSM and MIB defined as absolute content (MIB or GSM ng g⁻¹ tissue wet weight) or proportional content (MIB or GSM ng g⁻¹ lipid weight).

		Absolute MIB	Absolute GSM	Proportional MIB	Proportional GSM
Absolute MIB content	<i>r</i>	–	0.19	0.81*	0.035
	<i>p</i>	–	0.1355	<0.0001	0.7812
Absolute GSM content	<i>r</i>	–0.01	–	0.28*	0.87*
	<i>p</i>	0.9782	–	0.0265	<0.0001
Proportional MIB content	<i>r</i>	0.48	0.30	–	0.33*
	<i>p</i>	0.2301	0.4631	–	0.0078
Proportional GSM content	<i>r</i>	–0.09	0.86*	0.59	–
	<i>p</i>	0.8362	0.0065	0.1230	–

Significant correlations have been marked with bold *. Letters *r* and *p* stand for correlation coefficient and *p* value.

p > 0.05). However, between proportional MIB and GSM, there was a significant positive phenotypic correlation ($r_{\text{Ind}} = 0.33$) and a positive but non-significant family correlation ($r_{\text{Fam}} = 0.59$). These results indicated that only when the off-flavor compounds were defined as proportional to the lipid (in g kg⁻¹ per wet weight) in a muscle were MIB and GSM positively correlated. This emphasizes that the means of definition has a crucial importance in the interpretation of the results of this study.

3.4. Correlation of off-flavors with lipid contents

One out of the four phenotypic correlations of absolute MIB and GSM with the Distell lipid content and muscle lipid content were significantly positive (Table 4). This was in strong contrast to proportional MIB and GSM concentrations for which all the 4 phenotypic correlations were significantly positive, and 3 out of 4 family-mean correlations were higher than 0.61 with *p* values between 0.03 and 0.11 (Table 4; Fig. 1).

Table 4

Phenotypic (r_{Ind}) and family-mean correlations (r_{Fam}) of absolute and proportional GSM and MIB with Distell lipid content and lipid content in muscle.

		Absolute MIB		Absolute GSM		Proportional MIB		Proportional GSM	
		r_{Ind}	r_{Fam}	r_{Ind}	r_{Fam}	r_{Ind}	r_{Fam}	r_{Ind}	r_{Fam}
Distell lipid content	<i>r</i>	0.06	0.50	0.07	–0.22	0.32*	0.61	0.27*	0.15
	<i>p</i>	0.655	0.212	0.601	0.592	0.010	0.112	0.034	0.718
Lipid content in muscle	<i>r</i>	–0.20	–0.14	0.27*	0.35	0.30*	0.63	0.62*	0.76*
	<i>p</i>	0.107	0.7475	0.033	0.400	0.018	0.092	<0.0001	0.029

Significant positive correlations are marked with bold *. Letters *r* and *p* stand for correlation coefficient and *p* value.

3.5. Correlation of lipid contents with fatty acids

Both Distell and muscle lipids were similarly correlated with fatty acid groups (Table 5). The Distell lipid and muscle lipid correlated significantly at both the phenotypic and family level with MUFAs and PUFAs, but not with the SFAs. There was a positive correlation with MUFAs and negative with PUFAs. Furthermore, there was a significant negative phenotypic and family-mean correlation with the n-3 lipid fraction. In contrast, the correlations of n-6 with the two lipid traits were close-to-zero or non-significant, and accordingly the n-6:n-3 ratio had strongly significant positive correlations with the lipid contents (Table 5; Fig. 2). These results imply that increasing the lipid levels were related to reduced contents of health-promoting fatty acids (PUFAs, n-3) and to increasing levels of health-wise neutral fatty acids (MUFAs).

3.6. Correlation of off-flavors with fatty acids

When expressed proportionally to the lipid content, the proportional GSM was significantly positively correlated with MUFAs at both phenotypic and family level, and with the n-6:n-3 ratio, and with PUFAs and n-3 negatively at the phenotypic level (Table 6). Therefore, low levels of this off-flavor compound were related to increased levels of health-promoting fatty acids. Proportional MIB was significantly positively correlated only with MUFA at the family level (Table 6).

Of the phenotypic correlations of individual fatty acids with proportional MIB, only two were significant (Supplementary Table S2). These were the positive correlations of SFA 18:0 and MUFA *cis*-9 16:1.

There were 11 significant phenotypic correlations of individual fatty acids with proportional GSM (Supplementary Table S2). Saturated fatty acids 15:0 and 17:0 had negative correlations with proportional GSM, while MUFAs *cis*-9 16:1, *cis*-9 17:1, *cis*-9 18:1, *cis*-11 18:1 had positive correlations and *cis*-15 24:1 had a negative correlation. On the other hand, PUFAs 20:4n-6, 22:5n-6, 20:5n-3, and 22:6n-3 had negative correlations with proportional GSM. The family-mean correlation of proportional GSM with SFA 15:0 was significantly negative but other family-mean correlations were not significant.

The consistency of the correlations of proportional GSM and proportional MIB with individual fatty acids was quantified by regression slopes of 0.670 for the phenotypic correlations and 0.897 for the family-mean correlations (Fig. 3). This implied that the correlations of MIB increased at a lower rate than GSM. The phenotypic correlations of proportional MIB and GSM with individual fatty acids had an average of –0.041 and –0.016, respectively, but the standard deviation of the correlations was higher for GSM (std = 0.245) than for MIB (std = 0.164), again reflecting that GSM more frequently has more extreme correlations. A similar but weaker trend was observed for family-mean correlations with averages of –0.094 (std = 0.366) and –0.106 (std = 0.407) for MIB and GSM. These results reflected that GSM had more correlations with the fatty acid groups that were strongly positive and negative, as also visible in Table 6 and Supplementary Table S2, but otherwise MIB and GSM have very similar correlations with individual fatty acids.

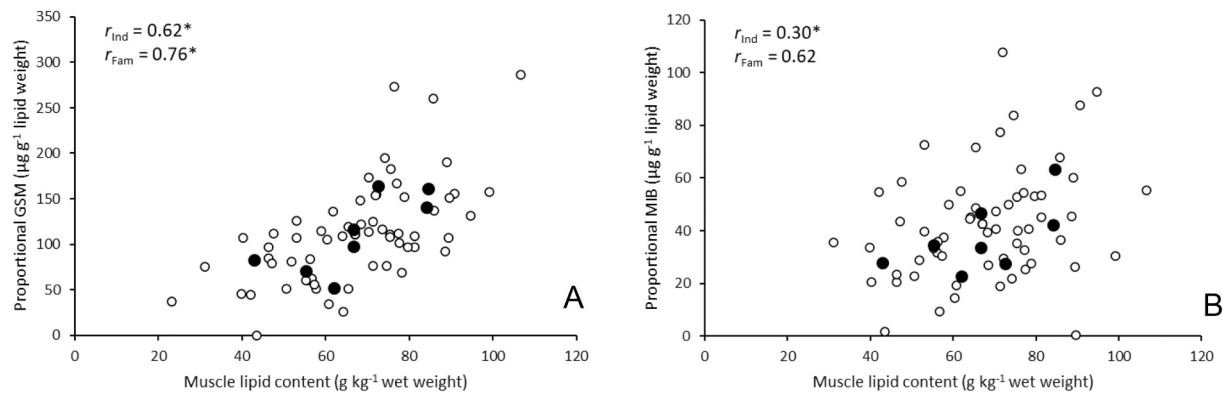


Fig. 1. Individual phenotypic (r_{ind} ; open circles) and family-mean correlations (r_{Fam} ; black dots) of proportional GSM (A, $\mu\text{g g}^{-1}$ lipid weight) and MIB (B, $\mu\text{g g}^{-1}$ lipid weight) with the muscle lipid content (g kg^{-1} wet weight).

Table 5

Phenotypic (r_{ind}) and family-mean correlations (r_{Fam}) of lipid traits with proportional fatty acids.

		Distell lipid		Muscle lipid	
		r_{ind}	r_{Fam}	r_{ind}	r_{Fam}
SFA	r	0.031	0.332	−0.123	−0.156
	p	0.809	0.421	0.332	0.713
MUFA	r	0.760*	0.740*	0.828*	0.907*
	p	<0.0001	0.036	<0.0001	0.002
PUFA	r	−0.791*	−0.793*	−0.799*	−0.707*
	p	<0.0001	0.019	<0.0001	0.050
n-3	r	−0.764*	−0.743*	−0.822*	−0.838*
	p	<0.0001	0.035	<0.0001	0.009
n-6	r	−0.098	−0.514	0.091	0.010
	p	0.439	0.193	0.474	0.981
n-6:n-3	r	0.722*	0.569*	0.846*	0.952*
	p	<0.0001	0.141	<0.0001	0.0003

Significant positive correlations are marked in bold *. Letters r and p stand for the correlation coefficient and p value.

4. Discussion

The comparison of individually tagged fish with the known family origin showed that there were family differences in the accumulation of lipids in the muscle of rainbow trout reared in RAS. A high lipid content was related to unfavorable changes in the amounts of off-flavors and the fatty acid profile, and simultaneously low concentrations of GSM and MIB were related to favorable fatty acid profiles. The two off-flavor compounds, MIB and GSM, were moderately correlated and their relationships with other traits can be different. These results encourage further studies on the genetic determination of the accumulation of individual off-flavor compounds, and on the way selective breeding and management practices could impact the lipid content and off-flavor compounds in RAS-farmed fish.

4.1. Family-differences in lipid contents, fatty acids, and off-flavor compounds

In this study, the first aim was to assess the degree of family differences in off-flavor compounds, fatty acids, and lipid contents in rainbow trout reared in RAS. The Distell Fatmeter device was successfully used to identify individual fish and families with different lipid levels during early growth. It should be noted that the Distell Fatmeter measures lipid content of the whole fillet. The fillet lipid, muscle lipid, and visceral lipid are known to be separate traits, with correlations lower than unity, or sometimes even with negative correlations (Kause et al., 2002, 2007, 2011). In this study, a high family effect was observed for lipid contents and the correlations of the lipid content between differently-sized fish

were positive which was in accordance with the large-scale pedigree-based analyses (reviewed by Kause et al., 2007). These results provided the basis for the experimental set up to identify families with different lipid contents during early growth.

A marginally significant family effect (0.42 of total variation explained) was observed for GSM, and a moderate but non-significant one was observed for MIB (0.31) when the compounds were defined as proportional (content per lipid weight in a sample), and non-existent or low (0.00–0.15) when defined as the absolute content (content per wet weight of a sample). When MIB and GSM were defined as concentrations in fresh muscle, the denominator consists of all the components of wet tissue, such as lipids, protein, inorganics, and water. It seems that these components confound the differences between families.

The results showed that proportional SFA, PUFA, and n-6 had major family differences, the family effect explaining 30–49% of the total individual variation. Horn et al. (2018) found significant heritability (0.09–0.26) in 194 families for the most abundant n-3 fatty acids, 18:3n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3, in Atlantic salmon. A study comparing groups of Atlantic salmon selected for either low or high lipid content measured using the Distell Fatmeter showed that only the total SFA (% of total fatty acids) was marginally significantly different between the fish groups (Bell et al., 2010). Leaver et al. (2011) used 48 families of Atlantic salmon and found that in Norwegian Quality Cuts the n-3 composition was a highly heritable trait (0.77) when expressed as the content of total fatty acids, but fell to 0.34 when expressed as $\text{mg } 100 \text{ g}^{-1}$ of wet tissue.

Overall, the results of this and previous studies (Bell et al., 2010; Leaver et al., 2011; Horn et al., 2018) showed that lipids and fatty acid profiles vary between families, while for the off-flavor compounds the family difference was only marginally significant. When family differences reflect underlying additive genetic variation, the variation can be exploited using selective breeding programs to change the fish traits. It should be noted that the family-based differences observed in our study did not prove that there was an additive genetic variation. To prove this, an experiment with several tens or hundreds of families with hierarchical or factorial matings would be required. Moreover, our study design aimed to ensure that we observed the potential family differences in traits related to lipid content, and hence the estimates of family differences for such traits were upward biased. This study design was chosen because there was no *a priori* information that family differences existed at all, and because only a small number of families could be analyzed.

4.2. High lipid content leads to increased off-flavors

The second main topic was to test the hypothesis that the high-lipid fish individuals and families had higher off-flavor compound concentrations. This was confirmed, especially when the off-flavor compounds

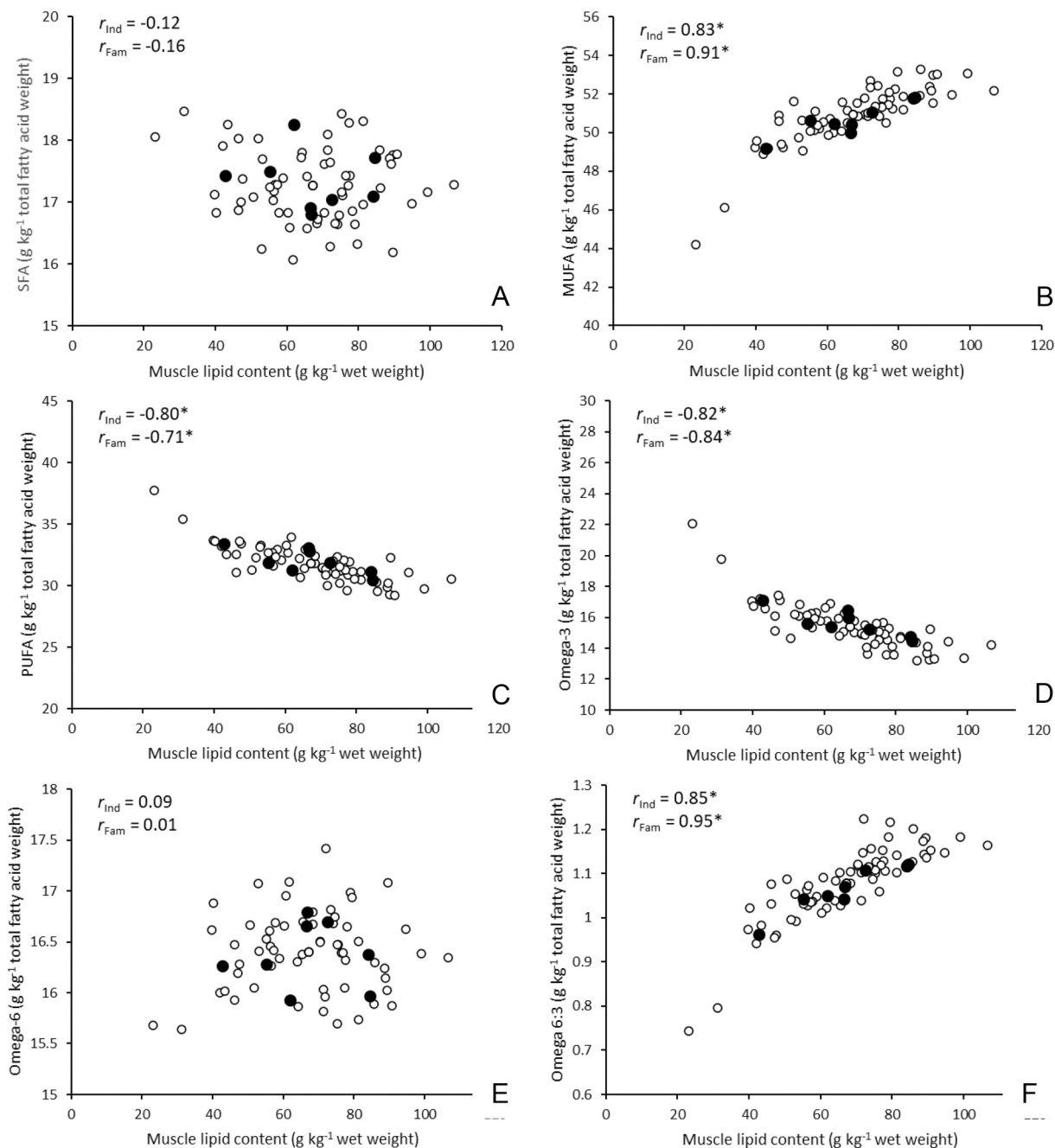


Fig. 2. Individual phenotypic (r_{Ind} ; open circles) and family-mean correlations (r_{Fam} ; black dots) of muscle lipid content (g kg⁻¹ wet weight) with the main fatty acid groups: SFA (A); MUFA (B); PUFA (C); n-3 (D); n-6 (E); and the n-6:n-3 ratio (F), g kg⁻¹ total fatty acid weight.

were expressed as a proportion of the total lipids in the muscle. The fish and families with a high lipid content in the muscle had higher concentrations of proportional MIB and GSM. This result was supported by the fact that GSM and MIB are lipophilic compounds (Howgate, 2004), which easily accumulate in the lipid tissue of fish flesh. Both compounds have similar octanol/water partition coefficients (Log K_{ow} 3.57 for GSM, and log K_{ow} 3.31 for MIB; Howgate, 2004). On the other hand, Schram et al. (2018) suggested that the GSM concentration may not be exclusively affected by the lipid content in tissues and suggested *in vivo* bio-concentration as an alternative mechanism.

The results showed that proportional MIB had moderate and positive correlation with proportional GSM. From a physiological point of view, the off-flavor compounds can be best defined based on the content in total lipids when analyzing their metabolism and the accumulation to

lipids. This was also reflected by the fact that the family differences were more visible for fatty acids and off-flavors when they were defined as contents in the total lipids.

A muddy taste sensation is influenced by the content of off-flavor compounds in the lipid tissue but also by the moisture and protein in the flesh (Howgate, 2004; Moretto et al., 2022). In contrast to proportional MIB and GSM, when measured as absolute content, the results suggested that GSM and MIB were very weakly correlated, and they may not have similar tendency in accumulating in fresh muscle tissue. Similarly, the results showed that the correlations of absolute GSM and MIB with other traits, including lipid contents, can be very different, even an opposite sign. Additionally, Bogdanović et al. (2012) stated that differences in off-flavor concentrations between individuals may be due to different feed intake, body composition, and fatty acid content,

Table 6

Phenotypic (r_{Ind}) and family-mean (r_{Fam}) correlations of proportional off-flavor compounds with proportional fatty acid groups.

		Proportional MIB		Proportional GSM	
		r_{Ind}	r_{Fam}	r_{Ind}	r_{Fam}
SFA	r	0.139	-0.116	-0.146	-0.432
	p	0.2759	0.7845	0.2487	0.2848
MUFA	r	0.141	0.527	0.437*	0.642
	p	0.2688	0.1797	0.0003	0.0864
PUFA	r	-0.202	-0.398	-0.390*	-0.348
	p	0.1120	0.3288	0.0014	0.3985
n-3	r	-0.141	-0.412	-0.417*	-0.510
	p	0.2716	0.3111	0.0006	0.1961
n-6	r	-0.229	-0.155	0.104	0.266
	p	0.0713	0.7133	0.4143	0.5239
n-6:n-3	r	0.101	0.479	0.455*	0.718*
	p	0.4324	0.2300	0.0002	0.0447

Significant correlations are marked in bold *. Letters r and p stand for correlation coefficient and p value.

leading to low correlations between absolute MIB and GSM.

4.3. High lipid contents and fatty acids

The third objective was to evaluate which groups of fatty acids and individual fatty acid compounds contributed to the elevated levels of lipid content, and especially, how the health-related fatty acids altered with different levels of lipid content. The results showed that the concentrations of fatty acids associated with favorable health effects, PUFA and n-3 were lower in the fish individuals and families with a high lipid content, and their concentration increased in fish with low lipid contents (Kheiri et al., 2022). Similarly, the n-6:n-3 ratio increased with increasing lipid contents. This showed that the contents of nutritionally beneficial n-3 fatty acids were lower in fish with increased lipid content. Similarly, Leaver et al. (2011) found that the concentrations of n-3 PUFA were lower with increasing levels of lipid contents in the flesh of Atlantic salmon. Horn et al. (2018) found that SFA 16:0 increased with an increasing lipid content, while simultaneously many abundant individual PUFAs decreased.

In our data, the fish and families with high lipid contents were associated with a significant increase in MUFAs, which are considered to have a neutral impact on human health. MUFAs are a typical form of storage for lipids in an animal body (Quillet et al., 2007). Therefore, it is logical that MUFAs increased with increasing lipid content levels. Concentrations of SFA and n-6 were very little or negatively influenced by the increase in the overall level of lipid content.

Our findings suggested that fatty acid composition might develop in a nutritionally more unfavorable direction if an increased lipid content is achieved by selection in rainbow trout. The restriction of excessive

lipid content could be of interest when selecting fish for rapid growth because lipid influences the quality of fish products and low levels of lipid contents can improve the feed conversion ratio (Kause et al., 2016; reviewed by Knap and Kause, 2018). In breeding programs, the lipid content can be recorded and selected on live fish using, e.g., the Distell Fatmeter as demonstrated by the current and the previous studies by Quillet et al. (2007), Kause et al. (2007), and Janhunen et al. (2017).

Aquatic animals are characterized by a great richness in highly unsaturated fatty acids, especially long-chain n-3 PUFA (LC n-3 PUFA) (Corraze, 2001). Changes in lipids are not reflected in the same way for the different classes of lipids: the concentration of phospholipids remains almost constant whereas neutral lipids are strongly influenced by dietary fatty acid composition (Corraze, 2001). Fish lipids are well known to be rich in LC n-3 PUFA, especially 20:5n-3 and 22:6n-3 (Haliloglu et al., 2004). Haliloglu et al. (2004) reported a total SFA value of 30% out of the total fatty acids, being mainly composed of 16:0, 18:0, and 14:0, and in a similar range with the results of the present study. Among MUFA, the most commonly found fatty acids were 18:1n-9, 16:1n-7, and 20:1n-9 (Haliloglu et al., 2004). Similarly, c9-18:1, c11-18:1, and c9-16:1 were the most abundant among MUFAs in the present study. In a study by Haliloglu et al. (2004) the content of n-3 fatty acids was nearly twice that of n-6 fatty acids, but our results showed that their proportions were almost equal. Although the most abundant fatty acids were partly similar, the different heritage of the rainbow trout might explain the differences.

Phospholipids are located in cell membranes and have relatively high levels of LC n-3 PUFA. The lipid content in the muscle increases as the fish grows (Jobling, 2004) leading to an increased proportion of triacylglycerols instead of phospholipids. Furthermore, the fatty acid composition is strongly influenced by the diet (Torstensen et al., 2004) and bioconversion. In salmonids, a conversion of 18:3n-3 to 22:6n-3 occurs in liver, intestinal, and muscle cells (Codabaccus et al., 2011).

4.4. Off-flavors correlated with fatty acids

The fourth object was to evaluate the relation between off-flavors and fatty acids. Off-flavor compounds GSM and MIB are lipophilic compounds which can accumulate into the lipid tissue of fish flesh (Schram et al., 2018). According to current understanding, the uptake proceeds via diffusion mainly through the gills and to a minor extent through the skin and gut (Howgate, 2004). Already Josephson et al. (1985) observed that the temperature and fatty acid composition in fish affected the concentration of volatile compounds, such as off-flavor compounds. When divided by the lipid contents in the muscle tissue, MIB and GSM correlated with several groups of fatty acids (SFA, MUFA, PUFA) (Fig. 2).

The comparison of the correlations of proportional MIB and GSM with fatty acids indicated that the off-flavor compounds may accumulate

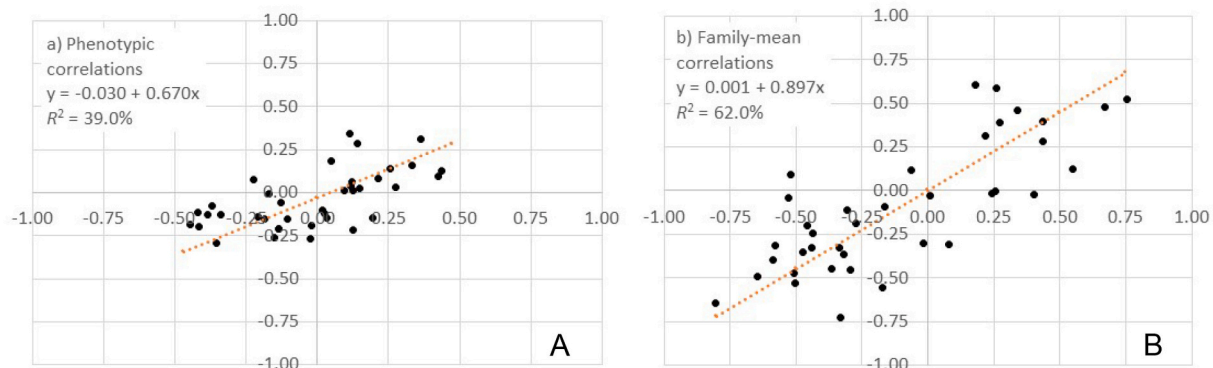


Fig. 3. Type II regression equations between (A) the correlations of proportional GSM with individual fatty acids and (B) the correlations of MIB with individual fatty acids. GSM correlations are shown on the x-axis, and MIB correlations on the y-axis.

similarly to other fatty acids. This is in accordance with the similar octanol/water partition coefficients of the two compounds (Howgate, 2004; Lindholm-Lehto and Vielma, 2018; Moretto et al., 2022) and the positive correlation between proportional MIB and GSM. The formulation of fish feeds and genetic selection can be used to change concentrations of individual fatty acids. Based on our study, changes in fatty acids are expected to change the levels of proportional MIB and GSM in a similar way.

Our results showed that decreased levels of proportional GSM correlated with increased levels of PUFA and n-3 which can be considered to be health-promoting groups of fatty acids, and with decreased levels of MUFA (health wise neutral effect), and a reduced n-6:n-3 ratio. These are very favorable relationships for aquaculture: high concentrations of health-promoting fatty acids were related to reduced off-flavor concentrations and previously reported for hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) (Phetsang et al., 2022). This means that individual fish and families can obtain their high lipid levels especially in the form of MUFAs at the expense of reduced PUFAs and n-3, associated with increased levels of GSM-induced off-flavor.

5. Conclusions

This study examined the relationships between lipid deposition, off-flavor compounds GSM and MIB, and fatty acids across rainbow trout individuals and families reared in RAS. There was limited previous information about the family-level relationships, and the results of the current study are the first step to understanding the genetic determination of these traits. The results imply that changes induced by selective breeding in lipid accumulation may impact both the fatty acid profiles and off-flavor compounds in rainbow trout reared in RAS. Large-scale genetic studies are needed to confirm the potentially additive genetic basis of these relationships. A deeper understanding of the accumulation of off-flavor compounds and the genetics related to the off-flavor compounds could act as a basis for innovations aiming to resolve off-flavor-related issues.

Disclosure statement

No potential conflict of interest was reported by the authors.

Author contributions

The experiment was planned by AK, JK, JV, and PLL. PLL conducted and organized the sample preparations and the chemical analyses of off-flavors. HL organized the analyses of the total lipid contents and fatty acid components. JK organized the fish sampling and collection of the data. The manuscript was drafted by PLL and AK, and critically examined and revised by JK, HL, and JV.

Author statement

Off-flavors and lipid components in rainbow trout (*Oncorhynchus mykiss*) reared in RAS: differences in families of low and high lipid contents by PC Lindholm-Lehto, J Koskela, H Leskinen, J Vielma, A Kause.

Petra Lindholm-Lehto: Provision of materials, reagents, laboratory equipment and methods (off-flavors), Data collection, Writing-original draft.

Juha Koskela: Conceptualization, Provision of materials (fish material, sampling), Performing the experiment, Data collection, Writing-review and editing.

Heidi Leskinen: Provision of materials, reagents, laboratory equipment and methods (total lipid contents and fatty acid components), Data collection, Writing-review and editing.

Jouni Vielma: Funding acquisition for the project, Performing the experiment, Supervision.

Antti Kause: Conceptualization, Formal analysis, Writing-review and editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2022.738418>.

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