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Original Article

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Terrestrial fatty acids from feed oil in feed for farmed salmonids are transferred to the liver, gonads, and muscle of wild Atlantic cod (*Gadus morhua*)

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Wild fish attracted to salmon farms feed on waste feed that presently contain high levels of fatty acids of terrestrial origin. This study examines whether mature Atlantic cod (Gadus morhua) caught at spawning grounds has eaten salmon waste feed. Cod were caught at four spawning grounds around Smøla (Norway), an area with multiple salmon farms, during the spawning season in 2018 (n = 327) and 2019 (n = 488). The fatty acid (FA) profile of their livers, gonads (ovary and testis), and muscles (2019) were determined. Multivariate k-mean cluster analysis of liver FA profiles revealed three main clusters, which could be allocated to trophic niches using known fatty acid trophic markers (FATMs). Of the sampled cod in 2018 and 2019, 13 % and 20 % respectively had high liver concentrations of terrestrial FATMs (18:1n-9, 18:2n-6, and 18:3n-3), indicating waste feed feeding. The remaining cod could be assigned to either the pelagic or benthic food chain. The cod identified as feeding on waste feed had large, fatty livers. The terrestrial FAs were also transferred to the muscle and gonad lipids. It is postulated that the latter may result in gametes with sub-optimal lipid composition, potentially impacting fitness, which warrants further investigation.

Keywords: diet composition, dietary biomarkers, fish farms, fish reproduction, lipids.

Introduction

Marine fish farming is an important and expanding industry in many coastal countries. In Norway, marine fish farming is dominated by salmonid rearing with a total production of 1.66 million metric tons in 2021 (Directorate of Fisheries, Norway; www.fiskeridir.no). Salmonid farming occurs in open net pens at over 990 locations dispersed along the coast and within fjords, causing concerns about the negative impacts Norwegian salmon farming may have on the coastal ecosystem (Taranger *et al.*, 2015). A substantial amount of feed pellets is lost from Norwegian salmon farms, with an estimated 3–5% of the pellets passing through the open net pens uneaten (Uglem *et al.*, 2020). Since the annual feed sale are >1.6 million tonnes in the Norwegian salmonid farming industry (Aas *et al.*, 2019), >48000 tonnes of salmon feed may be lost to the marine environment yearly.

Wild fish are attracted to the fish farms to feed directly on waste feed or prey on fish attracted to the farms (Callier *et al.*, 2018). Along the Norwegian coast, large numbers of saithe (*Pollachius virens*) and Atlantic cod (*Gadus morhua*) aggregate around salmon farms (Skog *et al.*, 2003; Dempster *et al.*, 2009; Uglem *et al.*, 2014). However, it is uncertain if and how feeding on salmon waste pellets impacts wild fish populations, e.g. by increasing the energy stores and condition of fish, or affects reproduction and recruitment (Dempster *et al.*, 2011; Barrett *et al.*, 2018).

The composition of the feed used in Norwegian aquaculture of salmonid species has changed markedly over time. The proportion of protein has decreased and fat increased, and the proportion of marine ingredients has decreased from 90 to 25% from 1990 to 2016, and concurrently the proportion of plant-based ingredients has increased from nearly 0 to 71% (Ytrestøyl *et al.*, 2015; Aas *et al.*, 2019). Fish oils have been primarily replaced by rapeseed (*Brassica napus* var. *napus*) oil (Aas *et al.*, 2019). Rapeseed oil contains ca. 44% oleic acid (18:1n-9), ca. 15% linoleic acid (18:2n-6), and 8% α-linolenic acid (18:3n-3), but does not contain the long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA), and arachidonic acid (20:4n-6) (ARA) present in marine fish oils (Bell *et al.*, 2003).

The LC-PUFAs are considered essential FAs (EFAs) for cold-water marine fish, such as cod, because they cannot be biosynthesized in sufficient quantities to support normal development (Glencross, 2009). Egg production and larval survival are influenced by the availability of LC-PUFAs (Pickova *et al.*, 1997; Salze *et al.*, 2005; Røjbek *et al.*, 2014; Norberg *et al.*, 2017), with optimum levels of DHA and ARA, as well as the ratio between ARA and other LC-PUFAs being important. Cod feeding on oil-rich diets develops large livers because surplus lipids are stored in the liver (Karlsen *et al.*, 2006), with the FA composition of the liver lipids reflecting the FA profile of the diet (Lie *et al.*, 1986; Jobling *et al.*, 2008; Jobling and Leknes, 2010). Although farmed cod does not seem to be harmed by being fed plant-based diets (Hansen and Hemre, 2013), it is unclear whether feed sources of non-marine origin can impact reproduction of wild fish (Gonzalez-Silvera *et al.*, 2020).

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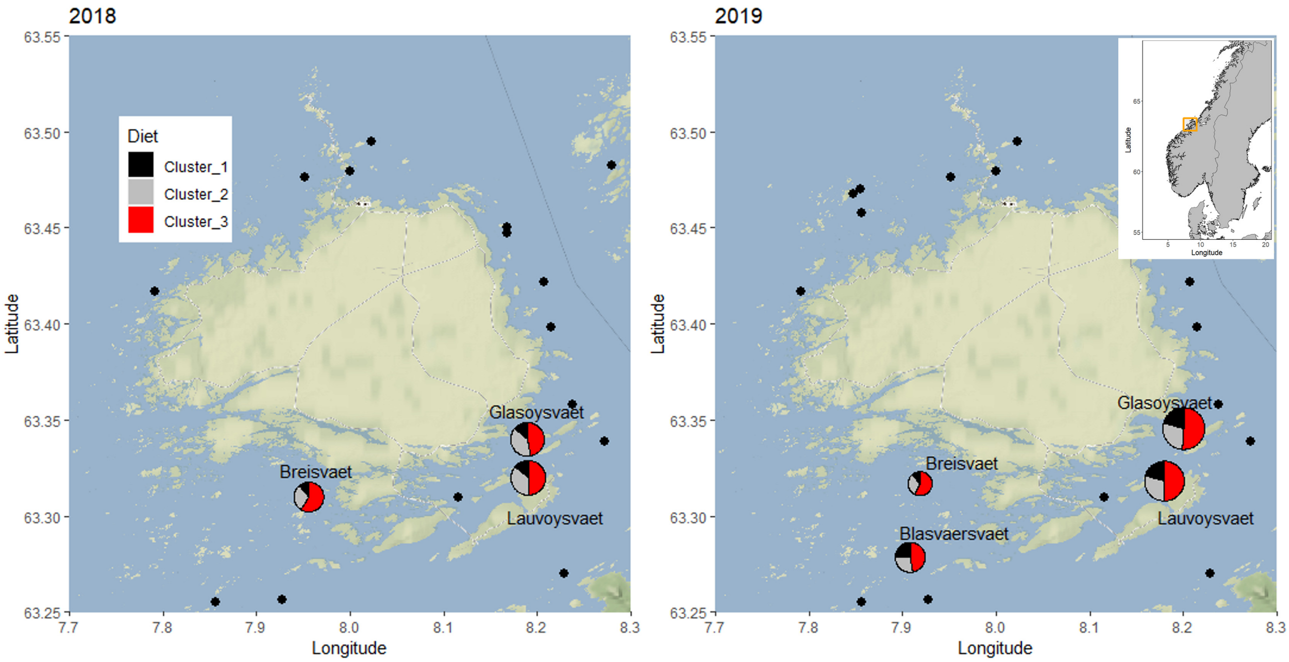

Figure 1. Maps showing fish capture locations in 2018 and 2019. Colour depicts spawning ground, and size of points are scaled to catch size. The colours in the "cake" diagram represent the proportion of fish from the three different diet clusters: black = cluster 1 (salmon waste pellets), grey = cluster 2 (pelagic diet), red = cluster 3 (benthic diet), and black circles represent salmon farm location. Inlet shows the study area on the Norwegian coast.

Analysis of specific FAs is a well-established method for studying trophic interactions and "who eats whom" in ecosystems (Dalsgaard *et al.*, 2003). The Fatty Acid Trophic Marker (FATM) method utilize that most marine FAs are synthesized at the lowest trophic levels (bacteria, phytoplankton, and zooplankton) and accumulate through the food chain with only minor biochemical modifications. Due to the high terrestrial lipid composition in salmon feed, FATM analyses are a powerful tool for determining to what extent wild fish has eaten salmon feed (Fernandez-Jover *et al.*, 2011; Skilbrei *et al.*, 2015).

Long chain monounsaturated FAs (MUFAs) as gadoleic acid (20:1n-11), gondoic acid (20:1n-9), erucic acid (22:1n-9), and cetoleic acid (22:1n-11) are synthesized by copepods, such as *Calanus* sp., and accumulate in pelagic fish such as sandeel (*Ammodytes* sp.), capelin (*Mallotus villosus*), herring (*Clupea harengus*), and mackerel (*Scomber scombrus*). Thus, these FAs are useful FATMs for pelagic food web feeding (Petursdottir *et al.*, 2008). Similarly, the levels of vaccenic acid (18:1n-7) are high in benthic organism such as decapods shrimps, starfish, and brittle stars (Graeve *et al.*, 1997; Howell *et al.*, 2003), and the levels of 20:4n-6 are very high in shore crabs (*Carcinus maenas*) (Styrishave and Andersen, 2000; Jungblut *et al.*, 2018) and brown crab (*Cancer pagurus*) (Barrento *et al.*, 2010). Feeding experiments where Atlantic cod has been fed with herring and shrimps (*Pandalus borealis*) (dos Santos *et al.*, 1993) or herring and shore crabs (van Deurs *et al.*, 2016) confirm that long-chain MUFAs increase in cod liver lipids for the herring diet, and that 18:1n-7 and 20:4n-6 are good biomarkers for shrimps and crabs, resembling a benthic diet.

The present study examines diet preferences of mature cod caught on local spawning grounds during the spawning season, using FATMs as indicators, and discusses potential influence of waste feed from salmon farms. The study was conducted around the island Smøla in mid-Norway, a region holding multiple salmon farms (Figure 1). In contrast to previous studies examining the biochemical composition of wild cod caught underneath the farms (Dempster *et al.*, 2009), cod were caught on spawning grounds some distance (2–5 km) from the closest farms. Our data thus shed light on the diet of cod before appearing on local spawning grounds, and the proportion of spawners feeding underneath fish farms. The FATM method was used to assign the cod's dietary preference prior to spawning to either (i) salmon waste pellets, (ii) pelagic diet, or (iii) benthic diet. The FA profiles in liver, gonads (ovary and testis), and muscle were all examined. Finally, the potential effects of continuous intake of salmon feed on wild cod reproduction, is discussed.

Material and methods

Fish capture

The cod used for analysis were caught by a local gillnet in the putative spawning season from February to April (Skjæraasen *et al.*, 2021) by a local gillnet fisherman. Fish were caught at the four spawning grounds Breisvaet, Blåsvaet, Lauvøysvaet, and Glasøysvaet around the island Smøla in mid-Norway (Figure 1) in 2018 ($n = 327$) and 2019 ($n = 488$). The length, sex, catch location, and date were recorded for each sampled fish (Table 1). Subsequently, the head, liver, and gonads were packed individually in plastic bags and frozen at −20°C and sent to the Institute of Marine Research's facility at Austevoll for processing. Otoliths were obtained for age determination. Tissue samples of liver, ovary, testis, and muscle (2019 only) for FA analyses were frozen in liquid nitrogen and stored at −80°C until analysed. All liver samples were analysed for FAs, in addition to a selection of gonads/muscle samples (129 samples in 2018 and 253 samples in 2019). Salmon feed of the brand Polarfeed Marin (Polarfeed AS, Leknes, Norway) and Mowi RI (Mowi ASA, Bergen, Norway), used at two facilities near the spawning areas, was analysed for comparison.

Table 1. Biological data for the three diet groups from 2018 and 2019; number of fish, sex, age, fish-length, gonad and liver weight, amount of FA (%), total amount of FA in the liver [TFA_L = liver weight × FA (%)].

Year	Sex	*N*	Cluster	Diet Class	Age (year)	Length (cm)	Gonad weight (g)	Liver weight (g)	FA (%)	TFA_L (g)	FATM (%) Terrestrial	Pelagic	Benthic
	Female	8	1	Salmon feed	6 ± 1	68 ± 4^{ab}	356 ± 185^{ab}	200 ± 85^{a}	39 ± 7^{ab}	82 ± 44^{a}	50 ± 7^{a}	6 ± 2^{b}	4 ± 0.5^{bc}
	Male	35	1	Salmon feed	7 ± 1	69 ± 5^{ab}	241 ± 129^{bc}	164 ± 95^{a}	44 ± 15^{a}	80 ± 64^{a}	56 ± 8^{a}	5 ± 3^{b}	4 ± 0.4^{c}
2018	Female	21	2	Pelagic	6 ± 1	71 ± 6^{a}	437 ± 243^{a}	145 ± 75^{bc}	31 ± 11^{b}	52 ± 38^{b}	16 ± 4^{c}	17 ± 6^{a}	5 ± 1^{bc}
	Male	96	2	Pelagic	6 ± 1	69 ± 7^{ab}	188 ± 112^{c}	83 ± 63^{b}	40 ± 14^{a}	38 ± 38^{bc}	16 ± 4^{c}	15 ± 6^{a}	5 ± 1^{b}
	Female	62	3	Benthic	6 ± 1	65 ± 6^{c}	372 ± 185^{a}	88 ± 53^{b}	22 ± 11^{c}	23 ± 23^{c}	20 ± 7^{b}	4 ± 1^{b}	9 ± 2^{a}
	Male	119	3	Benthic	6 ± 1	66 ± 5^{bc}	189 ± 100^{c}	54 ± 30^{c}	35 ± 13^{b}	21 ± 16^{c}	21 ± 5^{b}	4 ± 1^{b}	9 ± 2^{a}
	Female	26	1	Salmon feed	6 ± 1	68 ± 5^{bc}	491 ± 212^{a}	357 ± 139^{a}	54 ± 9^{b}	202 ± 98^{a}	54 ± 8^{a}	5 ± 2^{b}	5 ± 1^{c}
	Male	87	1	Salmon feed	6 ± 1	69 ± 5^{ab}	282 ± 127^{c}	285 ± 127^{b}	63 ± 9^{a}	185 ± 94^{a}	55 ± 7^{a}	5 ± 3^{b}	4 ± 1^{c}
2019	Female	22	2	Pelagic	5 ± 1	70 ± 6^{a}	380 ± 214^{b}	104 ± 82^{c}	28 ± 13^{e}	35 ± 58^{b}	15 ± 3^{d}	15 ± 6^{a}	6 ± 2^{b}
	Male	77	2	Pelagic	6 ± 1	67 ± 5^{bc}	235 ± 126^{c}	76 ± 54^{cd}	44 ± 14^{c}	38 ± 38^{b}	16 ± 5^{d}	15 ± 6^{a}	6 ± 2^{b}
	Female	64	3	Benthic	6 ± 1	67 ± 4^{bc}	474 ± 225^{a}	95 ± 42^{c}	22 ± 11^{e}	24 ± 19^{b}	19 ± 6^{c}	5 ± 2^{b}	11 ± 2^{a}
	Male	212	3	Benthic	6 ± 1	65 ± 4^{c}	229 ± 104^{c}	59 ± 28^{d}	40 ± 13^{d}	26 ± 17^{b}	22 ± 5^{b}	5 ± 2^{b}	10 ± 2^{a}

Sum of FATM for each diet group (FATM as % of total FAs). The data are shown as mean (± *SD*). The letters indicate statistical difference (one-way ANOVA) between groups for each year (sexes were tested together). $p < 0.05$. The grey sections show levels of FATMs that are associated with the respective diet groups.

Fatty acids analysis

The amount and composition of FAs were determined by direct methanolysis and gas chromatography following Meier *et al.* (2006). Briefly, a subsample of tissue (liver, muscle, gonad, or salmon feed) (50–100 mg) was weighed into 16 ml glass tubes. The liver subsamples were taken while the livers were still frozen to avoid "lipid bleeding". A known amount of nonadecanoic acid (19:0) was added as an internal standard, and all FAs were converted to fatty acid methyl esters (FAME) with the methanolysis reagent (2.5 M HCl in anhydrous methanol). The methyl esters were extracted with hexane before being analysed on a HP-7890A gas chromatograph (Agilent, USA) with a flame ionization detector (GC-FID).

The column was a CP-WAX 52CB with length 25 m, internal diameter 0.25 mm, and film thickness of 0.2 µm (Agilent p/n CP7713I). The temperature program was: total run time 75 min, initial temperature 90°C, increased to 150°C (30°C min^{-1}), hold time 0 min, increased to 240°C (2.5°C min^{-1}), and hold time 35 min. Helium (99.9999%) was used as mobile phase at 1 mL min^{-1} for 45 min, followed by a flow increase to 3 mL min^{-1}, which was held for 30 min. The method can distinguish between 97 different FAs that are identified based on comparison of retention time with standard mixtures of FAME, retention index card, and mass spectrum library (GC–MS) (http://www.chrombox.org). However, only the 51 FAs that contribute with >0.1% of the total FAs are included in the data analysis. The data are presented either as FA profiles (% of total FAs) or normalized to tissue weight (mg/100 mg wet weight) (Supplementary Tables S3 to S8). The total amount of FAs in the liver is calculated by multiplying FA content with liver weight TFA_L = liver FA (mg/100 mg) × liver weight (g)/100 (Table 1).

Classification of fatty acid data and statistical analysis

Classification into diet groups based on fatty acid data

The classification into different diet groups were performed from analysis of the FAs profiles of the liver samples. Principal component analysis (PCA) was executed in the software SIRIUS 11.5 (Pattern Recognition Systems, Bergen, Norway). The FA values were log-transformed, mean centering, and standardized by dividing by *SD* before performing the PCA. This transformation methods level out the quantitative differences among FAs and ensure that the variation in the minor FAs counts as much as for the dominating FAs.

Cluster analysis was done using partitioning methods (k-means) in Qlucore omics explorer (ver. 3.8). The optimum number of clusters for the k-mean clustering was determined to be three using the package NbClust (vers. 3.0.1) (Charrad *et al.*, 2014); in R (ver. 4.0.3, https://www.R-project.org/) running in R-Studio (ver. 1.4, http://www.rstudio.com/). The k-mean clustering method was used for grouping the fish into three main diet groups: (i) salmon waste pellets, (ii) pelagic, or (iii) benthic prey items. Data were visualized using the R-package ComplexHeatmap (ver. 2.6.2) (Gu *et al.*, 2016).

One-way ANOVA and Tukey–Kramer multiple comparison *post-hoc* tests were subsequently performed to test if the salmon waste pellets group differed significantly from the other two "natural" groups in terms of fish length, liver and gonad weight, and FA content. Statistical differences were examined using the XLSTAT software (Addinsoft, USA), and significance was assigned at *p*-values < 0.05. The correlation of terrestrial FATM levels between liver and gonads and between liver and muscle, was analysed using linear regressions. For comparisons failing to comply with assumptions for parametric tests (i.e. non-normal and/or heteroscedastic data), robust versions of ANOVA and *post-hoc* tests were performed using the R-package WRS2 (ver. 1.1–3) as described in Mair and Wilcox (2020).

Cod liver and gonad size and terrestrial FATM content

We also wanted to examine if there were any generic associations between the size of liver and gonads and the amount of terrestrial FATMs without any prior classification into different diet types. To do this, we employed regression analyses, where liver or gonad weight was treated as the dependent variable, and fish length (cm), FATM, and sampling year were treated as independent explanatory variables. Apart from the categorical variable sampling year, all dependent response variables and the independent explanatory variables were log-transformed. The regressions were done separately for each sex. The dredge command of the MuMIn library (Barton, 2020) of R was used for model selection to arrive at the most parsimonious model. All variables included in a model with an AICc score < 2 from the top model were retained given that these models have been given similar empirical support (Hurvich and Chih-Ling, 1989).

Fish diet as a function of length, sex, spawning ground, and sampling year

To evaluate if the continuous variable fish length and the categorical variables spawning ground, year, and sex had an influence on the probability of a fish having a salmon waste pellets diet, a logistical regression with a binomial response variable was employed, using the statistical software R studio version 4.0.2 (R Core Team, 2020; https://www.r-project.org). Fish diet was coded as 1 if the FA composition indicated salmon waste pellets diet, and 0 otherwise. All categorical variables were included as additive effects in the initial model. The same model selection procedure as described previously (2.3.2) was used to arrive at the final model.

Results

Grouping of fish according to FATM

The PCAs (Figure 2) show liver samples and salmon pellets from 2018/2019 using all the 51 FAs in the analysis. The first two axes in the PCA explained 60% of the total variance (axis 1 = 38%, axis 2 = 22%). The clustering of the samples was very similar both years, with a group of fish clearly separated from the others along PC1, and two further groups separating along PC2. In the k-mean cluster analysis (Figure 3), the heat plot shows the separation of the samples into three clusters, representative of the diet groups: (1) salmon waste pellets, (2) pelagic, or (3) benthic diet. The k-mean clustering was very robust, yielding significant differences between two or more of the clusters for all FAs ($p < 0.0001$, Supplementary Tables S1 and S2). The fish likely to feed on salmon waste pellet (cluster 1) had very high levels of terrestrial FATMs (18:1n-9, 18:2n-6, 18:3n-3) compared to the other clusters. The fish in cluster 2 are high in *Calanus* sp. FATMs (16:1n-11, 18:1n-11, 20:1n-9, 20:1n-11, 22:1n-9, 22:1n-11) indicating a pelagic diet. Additionally, these fish had significantly higher levels of 14:0, 24:1n-9, 18:4n-1, 16:2n-4, 18:4n-3, 20:4n-3, 21:5n-3, 22:6n-3, and 24:5n-3 than cod in the other two clusters. Fish in cluster 3 had relatively higher levels of branched saturated FAs (SFAs) (i-15:0, i-16:0, i-17:0, and ai-17:0) and SFAs with unequal carbon numbers (15:0 and 17:0), which all are FATMs for bacteria. Cluster 3 also have relatively higher levels of other SFAs (16:0, 18:0, 20:0), MUFAs in the n-7 family (16:1n-7, 18:1n-7, 20:1n-7, 22:1n-7), n-6 PUFAs (18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6), and n-3 PUFAs (20:3n-3, 20:5n-3, 22:5n-3) compared to the other clusters (Supplementary Table S3 and S4). Altogether the FA profiles of cluster 3 indicate a domination of benthic animals. Importantly, the classification does not identify exclusive diets or food chains, and many fish will prey on items from all food webs. However, these dietary groups can arguably be used to show individual cod's preferred prey or feed type.

Based on the cluster analysis, 13 and 20% of the cod caught was feeding on salmon waste pellets, 36 and 29% of the cod were assigned to the pelagic diet group in 2018 and 2019, respectively (Figure 1), and ~51% were assigned to the benthic diet group in both years. Bivariate plots of the most important FATMs in each cluster (Figure 4) show that in both cluster 2 (12 out of 258 fish) and cluster 3 (15 out of 316 fish), there are fish with high levels of terrestrial FATMs (18:2n-6 > 4%), likely indicating occasional feeding on salmon waste pellets. The average level of 18:2n-6 was 13.4 ± 2.2% (range: 7.7–16.9%) in the salmon waste pellet diet group, 1.8 ± 1.2% (1.1–8.5%) in the pelagic diet group and 1.9 ± 0.5% (0.5–9.9%) in the benthic diet group.

The commercial feeds had high relative levels (% of total FAs) of the terrestrial FAs 18:1n-9 (31 and 37%), 18:2n-6 (17 and 16%), and 18:3n-3 (5 and 6%) (Supplementary Table S8). The feeds differed in fish oil content, which affected the levels of the pelagic FATMs 20:1n-9 (5 and 2%) and 22:1n-11 (8 and 2%) (Supplementary Table S8).

Table 1 shows the morphological measurements of the cod from the different diet groups. The most significant differences between diet groups are that the cod in the salmon waste pellets diet group had larger livers with a higher FA content, and therefore significantly higher energy reserves (total liver FA content, TFA_L) compared to the other groups. In 2018, the benthic and pelagic diet groups had 27 and 51%, respectively, of the liver FA content of cod found in the salmon waste pellets diet group. In 2019, the salmon pellets diet group had very large livers with high lipid content, more than twice the amount observed in 2018, while the TFA_L from the benthic and pelagic diet groups were similar in 2018 and 2019. Accordingly, the benthic and pelagic diet groups had only 13 and 20%, respectively, of the TFA_L observed for the salmon waste pellets diet group in 2019. The differences in the FA content in the liver between the different diet groups are also seen in frequency distribution plots (Supplementary Figure S1), especially for the cod on the benthic diet, where a significant proportion of females (39%) and males (11%) had low FA levels (<20% of wet weight) in their livers. In contrast, for the salmon waste pellets group 13 and 73% of the females and 46 and 92% of the males had >50% FAs (% of wet weight) in 2018 and 2019, respectively.

Terrestrial fatty acids in the gonads and muscle

The terrestrial FAs that accumulate in the storage fat in the liver of cod feeding on salmon waste pellets were also transferred to both gonads and muscle as shown in Figure 5. There was a strong correlation between

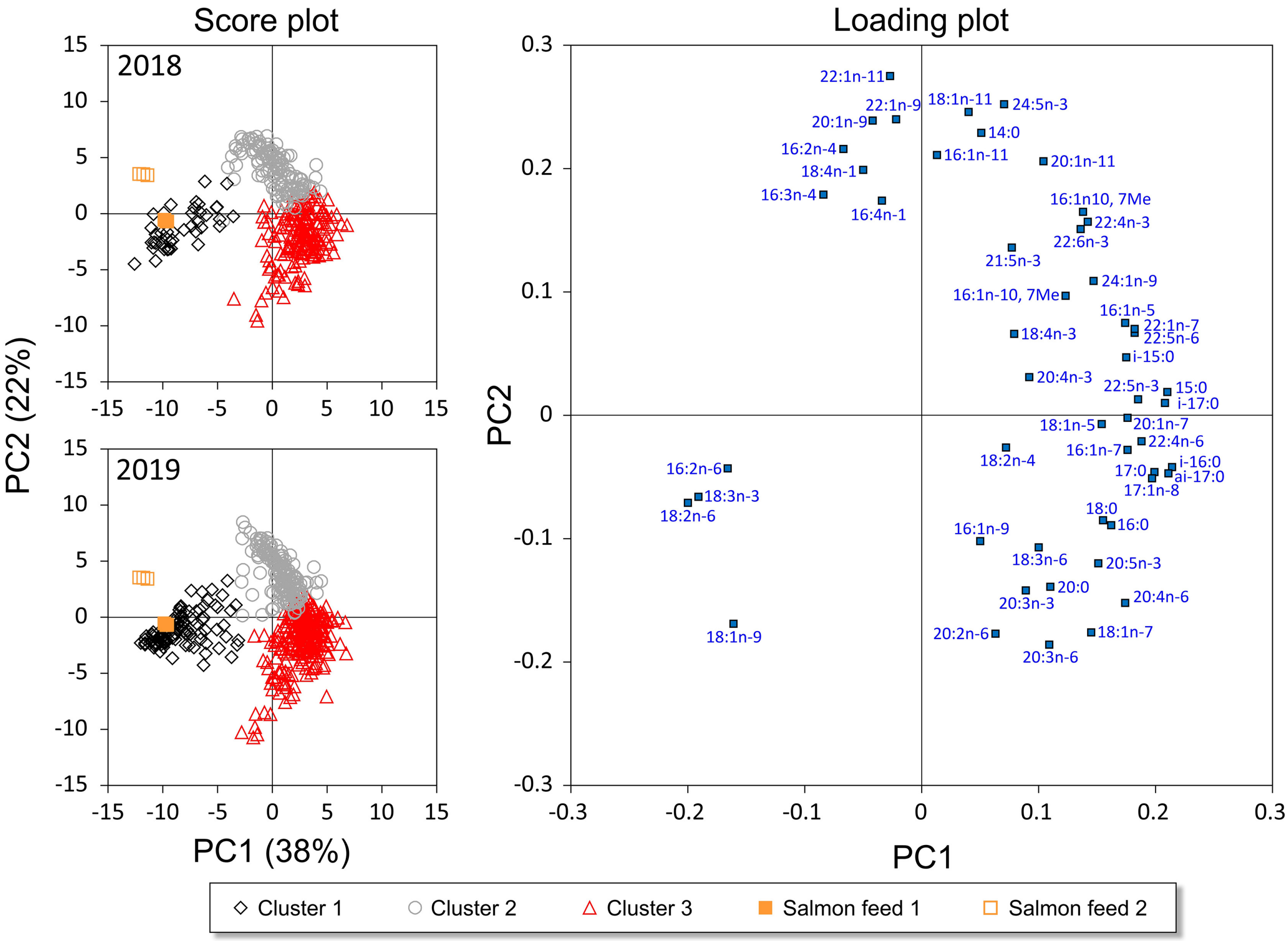

Figure 2. PCA of 51 FAs from liver samples of all cod from both 2018 and 2019 and the salmon pellets. The score plots show the grouping of the cod, the data from 2018 and 2019 are shown in each figure for clarity but are from the same PCA. The first two axes explain 60% of the total variance (axis 1 = 38%, axis 2 = 22%). The samples are marked according to the cluster analysis in Figure 3; cluster 1 (black diamond) are eating salmon waste pellets, cluster 2 (grey circle) have a dominating pelagic diet, and cluster 3 (red triangle) have a dominating benthic diet. The orange square shows the FATMs from the waste salmon feed used on two salmon farms in the immediate area.

the terrestrial FATM 18:2n-6 in the liver and ovary ($R^2 = 0.98$, $p < 0.0001$), liver and testis ($R^2 = 0.85$, $p < 0.0001$), and in the liver and muscle ($R^2 = 0.95$, $p < 0.0001$).

Multivariate PCA (Supplementary Figure S3) for the ovary, testis, and muscle show a similar picture as for the liver samples (Figure 3), with the salmon waste pellets diet group separating from the other two diet groups along PC1. The benthic and pelagic diet groups show some separation along the PC2 axis, but not as clear as for the liver samples. The loading plots grouped the terrestrial FAs 18:1n-9, 18:2n-6, and 18:3n-3 to the far left along PC1, which corresponds to the salmon waste pellets diet group. To the far right in this plot are ARA (20:4n-6) and DHA (22:6n-3), which indicate that these FAs are found in higher proportions in the benthic and pelagic food webs than in the salmon waste pellets diet group. The FAs separating the samples along the PC2 indicate that the relative levels of ARA are higher in the gonad and muscle of the benthic feeders, and that DHA is relatively higher in the pelagic diet group. The results show that terrestrial FAs are transferred to phospholipids and transported to both gonads and muscle.

Cod liver and gonad size and terrestrial FATM content

Liver size was positively associated with the terrestrial FAs (sum of 18:1n9, 18:2n6, 18:3n3) for both males and females ($p < 0.001$, Table 2) when all data were included without any *a priori* classification into diet groups. The final fitted models explained 55 and 60% of the variation in liver size for males and females, respectively. The terrestrial FATMs were also positively associated with gonad size for both males and females, but the variation explained by the final models was considerably lower than that of the liver size models, with 6 and 16% of the variation explained for males and females, respectively (Table 2).

Fish diet as a function of length, sex, spawning ground, and sampling year

The explanatory variables sampling year, length, and sex were retained in the final model on fish diet. No effect of spawning ground was found. There were more fish feeding on salmon waste pellets in 2019 than 2018 ($p < 0.01$, Table 3), and fish feeding on salmon waste pellets were larger than those not

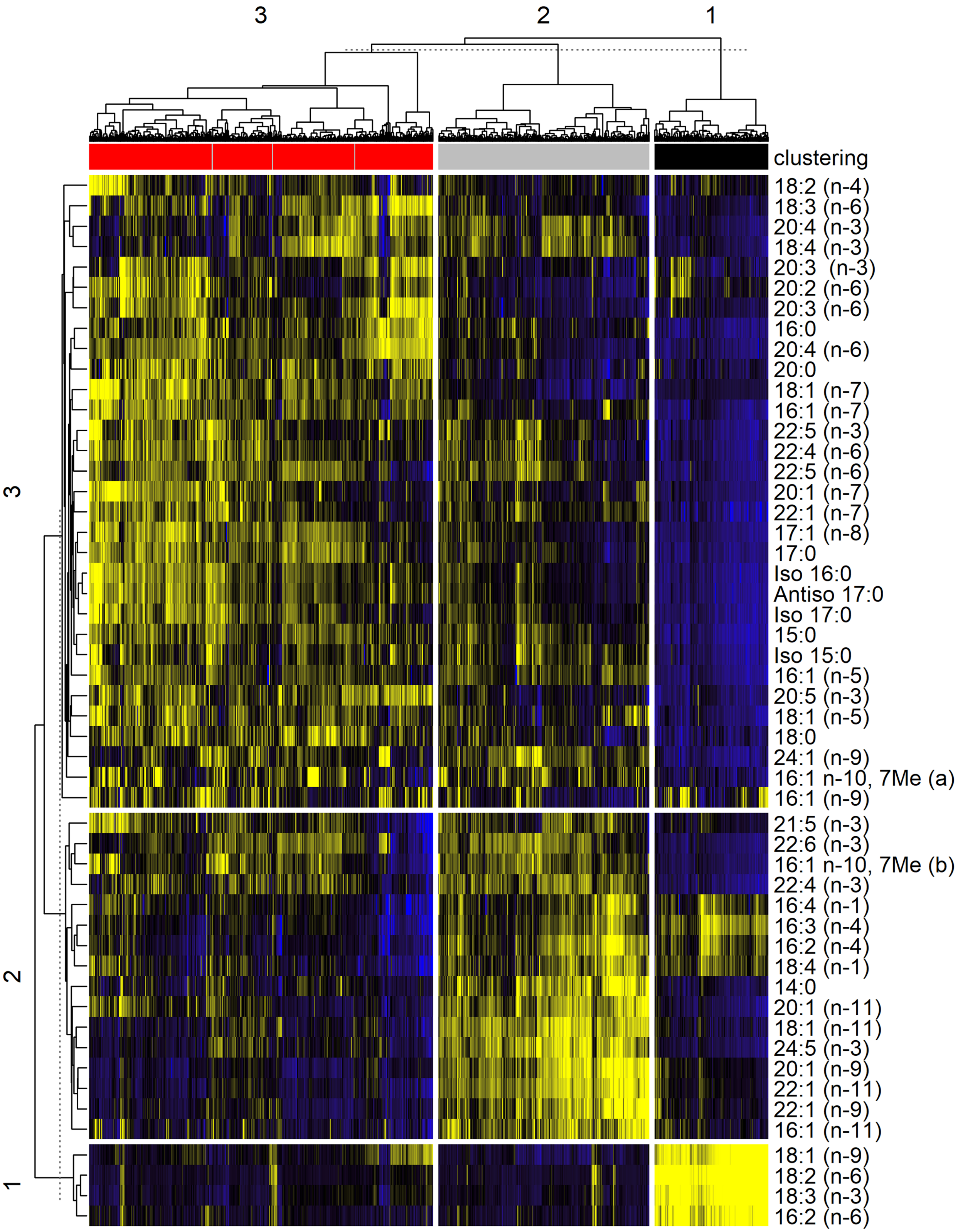

Figure 3. Heat plot from k-mean cluster analysis from the FAs profiles of the livers (all fish included, $n = 815$). Three clusters were found to be the optimum number of clusters. Cluster 1 (black) are very high in terrestrial FATMs and are classified as salmon waste pellets eaters, cluster 2 (grey) are high in *Calanus* FATMs and are classified as fish that had a dominating diet from the pelagic food web, and cluster 3 (red) are high in FATMs associated with benthic organisms.

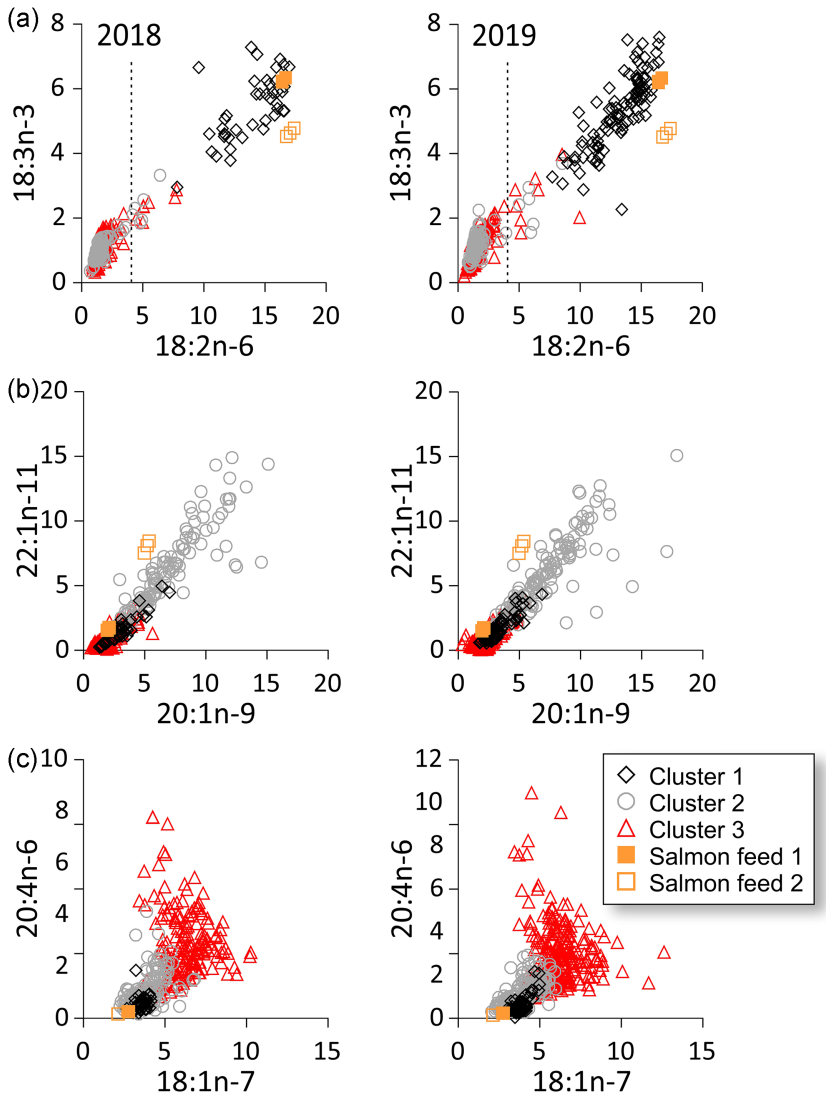

Figure 4. Bivariate plots of FATM from cod liver collected from fish caught at the spawning grounds of Smøla in 2018 and 2019. The data are given as % of total FAs. (a) Terrestrial FATM; 18:2n-6 and 18:3n-3; (b) Pelagic FATM; 20:1n-9 and 22:1n-11; and (c) Benthic FATM; 18:1n-7 and 20:4n-6. The samples are classified from cluster analysis; cluster 1 (black diamonds) are eating salmon pellets waste, cluster 2 (grey circles) have a dominating pelagic diet, and cluster 3 (red squares) have a dominating benthic diet. The orange squares show the FATMs from the salmon pellets used on two salmon farms in the close vicinity of the spawning grounds. The black lines in (a)A show 18:2n-6 = 4% (see text for explanation).

feeding on salmon waste pellets ($p < 0.0001$). Sex was also retained in the final model although this was far from significant ($p = 0.249$, Table 3). The final model only explained around 3% of the variation in diet. Investigating the difference in size between the fish consuming the different diets, it was clear that differences observed were caused by cod feeding on the benthic diet being smaller than the cod feeding on the salmon waste pellets. Fish that had a pelagic diet were of similar size as cod feeding on salmon waste pellets (Supplementary Figure S2).

Discussion

The FATM analysis indicated that ~20% of wild mature Atlantic cod of both sexes caught on spawning grounds close to salmon farms in a coastal area in Norway had been feeding on waste feed from the fish farms. Further, cod not feeding on salmon waste pellets could be classified into cod feeding either mainly in the pelagic food web, or mainly in the benthic food web.

The different diet groups

The present study demonstrates clearly indirect of direct feeding on waste pellet feed by mature cod and shows the general utility of FATM analyses for identifying trophic niches and assigning wild caught teleosts into different diet groups. Here, cod were caught on the spawning grounds and not underneath the farms. The results therefore reflects the proportion of the fish utilizing these spawning grounds that visit fish farms to feed on waste feed. Some of these cod may be feeding on salmon waste pellets indirectly through the consumption of prey that are feeding on salmon waste pellets. For instance, both polychaetes (*Capitella* sp.) and saithe sampled under fish farms contain relatively high levels of the terrestrial FATMs18:1n-9 (22 and 26%), 18:2n-6 (7 and 8%), and 18:3n-3 (2 and 3%), respectively (Arechavala-Lopez *et*

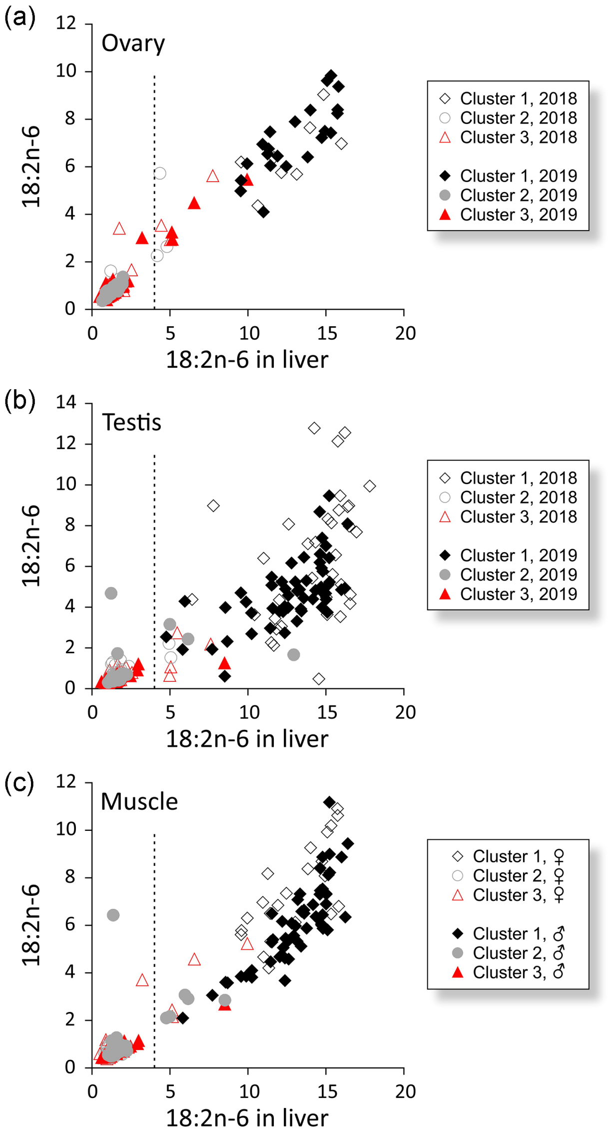

Figure 5. Bivariate plots of terrestrial FATM (given as % of total FAs); 18:2n-6 in the liver against the ovary (a), testis (b), and muscle (c) from fish caught at the spawning grounds of Smøla in 2018 and 2019 and the muscle (only data from 2019). The samples are classified from their liver FA composition from Figure 3 as salmon waste pellets diet (black diamonds), pelagic diet (grey circles), and benthic diet (red triangles).

al., 2015; Woodcock *et al.*, 2019). The levels of terrestrial FATMs in the two feeds analysed in this study were 53 and 60% of the total FAs. The very high levels of terrestrial FATMs (>50%) found in the liver of some of the cod indicate that a substantial proportion likely feeds almost exclusively directly on salmon waste pellets. Indeed on several occasions, the local fishermen at Smøla observed that stomachs of cod were filled with salmon pellets (pers. comm.). Telemetry studies overlapping in time and space with the present study confirmed that many of the local spawning cod regularly visited fish farms in the area (Skjæraasen *et al.*, 2022). Along the Norwegian coast, in particular gadoids like saithe and Atlantic cod are known to feed on salmon waste pellets under the nets (Skog *et al.*, 2003; Dempster *et al.*, 2009).

We did not find any difference between the different spawning grounds in the proportion of salmon pellets feeding cod. This is likely caused by the rather uniform distance to the

Table 2. Final models for how liver and gonad size were influenced by fish length (cm), FATM (%), and the categorical variable sampling year.

	Liver size—females			Liver size—males			Gonad size—females			Gonad size—males		
Predictors	*Estimates*	*Statistic*	*p*	*Estimates*	*Statistic*	*p*	*Estimates*	*Statistic*	*p*	*Estimates*	*Statistic*	*p*
(Intercept)	−9.91	−6.65	<0.001	−14.99	−12.27	<0.001	−2.95	−1.18	0.239	−5.88	−4.56	<0.001
Length	2.79	7.89	<0.001	3.85	13.17	<0.001	1.94	3.27	0.001	2.48	8.02	<0.001
FATM	0.93	14.95	<0.001	0.95	21.57	<0.001	0.22	2.15	0.033	0.18	3.98	<0.001
Year [2019]	0.05	0.74	0.460	0.19	4.27	<0.001				0.27	5.72	<0.001
Observations		204			611			204			609	
R^2/R^2-adjusted		0.599/0.593			0.552/0.550			0.072/0.063			0.164/0.159	

The response variable and the continuous dependent variables were log-transformed in the analyses. The treatment contrast of *R* was used for the analyses, with the intercept value depicting the estimate for 2018. Brackets indicate a categorical variable with the level inside the bracket showing the level being compared to the reference level of 2018. Significance was assigned at a level of $p < 0.05$, given in bold.

Table 3. Final model for the logistic regression on what affects the likelihood of cod being a salmon feeder.

	Salmon feeders		
Predictors	*Log-odds*	*CI*	*p*
(Intercept)	− 6.76	−9.20–(−4.36)	**<0.001**
Year [2019]	0.60	0.20–1.01	**0.004**
Sex [Male]	0.26	−0.17–0.72	0.249
Length	0.07	0.03–0.10	**<0.001**
Observations		814	
R^2 Tjur		0.029	

The treatment contrast of *R* was used for the analyses, with the intercept value depicting the estimate for 2018 and females as the reference levels. Brackets indicate a categorical variable with the level inside the bracket showing the level being compared to the reference levels of 2018 and females. Significance was assigned at a level of $p < 0.05$, given in bold.

nearest farms at all investigated spawning grounds. If cod also had been caught at spawning grounds further away from any farm, e.g. >20 km to the nearest farm, distance effects would be more likely to occur. Given the density of salmon farms along large parts of the Norwegian coast, this indicates that a substantial proportion of locally spawning wild cod in farm intensive areas along the Norwegian coast may have dietary inputs from waste feed.

The FATM analysis indicated that ~51% of the cod at Smøla had a diet dominated by benthic prey, with the high levels of 20:4n-6 (ARA) indicating that crabs are particularly important prey (Styrishave and Andersen, 2000). The FATM analysis also indicated that 29–36% of the cod had a diet dominated by lipid-rich pelagic fish species such as herring. Atlantic cod are a generalist feeder on both pelagic and benthic prey items (Link *et al*., 2009). For instance, crabs, hermit crabs and galatheoids, coastal fish and euphausiids are important prey at the west coast of Norway (Svåsand *et al*., 2000). In northern Norway, coastal cod are relying more on pelagic fish like Atlantic herring and capelin (Svåsand *et al*., 2000; Michalsen *et al*., 2008). Further south, like the present study site, it is believed that both stationery local fjord, i.e. costal cod, and so called bank-cod, which migrate along the coast (Johansen *et al*., 2020) utilize local spawning grounds. However, these latter phenotypes are not genetically distinguishable (Johansen *et al*., 2020). Therfore, we cannot be sure if the pelagic diet groups are local cod shifting their feeding preferences from benthic to pelagic prey during, e.g. the annual herring migration (Varpe *et al*., 2005) or bank-cod coming into to the spawning grounds at the time of spawning.

Notably, most of the fish caught in the vicinity of the spawning grounds were males, 72 and 77% in 2018 and 2019, respectively. Sex-bias in catch numbers have also previously been reported for gadoid fishes including cod (Morgan and Trippel, 1996). For cod, this pattern has been ascribed to their lek-like mating system (Hutchings *et al*., 1999; Skjæraasen *et al*., 2012), whereby males are believed to form a shoal during spawning, with females being more dispersed on the spawning grounds (Meager *et al*., 2010).

The transfer of terrestrial fatty acids into the liver, ovaries, and muscles of cod

Here, we have shown that terrestrial fatty acids may be transferred from salmon farms to not only the liver, but also the muscle and gonads of mature Atlantic cod. However, it is not clear whether such intake of salmon waste pellets has negative effects on the population dynamics of the farm-aggregated wild fishes, or whether it has a positive effect on gadoid reproduction by increasing energy levels and condition of the fish (Dempster *et al*., 2011; Barrett *et al*., 2018).

Females had significantly larger gonads and livers than the males. Apparently, females thus invested more in reproduction than males, and females prioritized energy stores (liver weights) more than males in line with previous findings (Karlsen *et al*., 1995). The females had a lower FA concentration in the liver than males, but due to their larger livers, the total amounts of lipid relative to fish length was similar between the sexes. Female liver energy content (liver weights and FA content) is correlated with fecundity (Marshall *et al*., 1999; Skjæraasen *et al*., 2006, 2010), indicating that the large increase in energy acquisition and condition factor (liver weights and FA content) observed in cod feeding on salmon waste pellets may increase gamete production (Dempster *et al*., 2011; Barrett *et al*., 2018; McAllister *et al*., 2021). However, while we do find a positive overall association between terrestrial FATM and gonad size, the variation explained was limited to compare the relationship between terrestrial FATM and liver size, especially so for females. This may indicate that the liver energy content was well above what is needed for oocyte production and other metabolic requirements, and that the high liver lipid content found in cod feeding on salmon waste pellets do not necessarily lead to greatly enhanced reproductive success.

Ovary growth occurs through the production of the lipoprotein vitellogenin in the liver, which is transported to the ovaries where it is incorporated into the maturing oocytes. Vitellogenin is composed of proteins, phospholipids, and storage lipids (triacylglycerols) (Silversand and Haux, 1995). In male fish, lipids supporting testis growth are controlled by both uptake of low-density lipoproteins (VLDL) from plasma and *de novo* synthesized in the testis (Bogevik *et al*., 2020). Our results confirm previous results for cod that feeding on salmon waste pellets leads to a modified liver FA profile due to accumulation of substantial proportions of terrestrial FAs (Jobling *et al*., 2008). As the terrestrial FAs to a large extent were found also in the lean tissues (muscle, and gonads), this shows that terrestrial FAs are also incorporated into phospholipids (Lie *et al*., 1992). Similar diet effects, with accumulation of terrestrial FAs into gonad lipids, have been reported for other fish species feeding on plant-based diets (Bell *et al*., 1996; Vassallo-Agius *et al*., 2001; Wassef *et al*., 2012).

The benthic diet group had higher levels of 20:4n-6 in both liver and gonads compared to the salmon waste pellets and the pelagic diet group. While the pelagic diet group had higher levels of 22:6n-3 in both liver and gonads compared to the salmon waste pellets and benthic diet group. Differences in micro-nutritional requirements for optimal reproductive performance of wild cod have been given some attention (Stoltenberg *et al.*, 2021; van Deurs *et al.*, 2022), but there is still a lack of experimental data to elucidate how different trophic niches may affect cod reproduction.

In conclusion, the highly modified FA profile in ovary and testis of the salmon waste pellet group may have negative effects on reproductive success (Gonzalez-Silvera *et al.*, 2020). Optimization of lipid and fatty acid composition of broodstock diets are very important for successful reproduction and offspring survival in fish (Izquierdo *et al.*, 2001; Tocher, 2010). Experiments investigating the effect of broodstock diet of cod (Salze *et al.*, 2005; Røjbek *et al.*, 2014; Norberg *et al.*, 2017) and field experiments using wild cod (Pickova *et al.*, 1997) show that fecundity and survival of cod larvae are positively correlated with high content of EFAs (20:4n-6, 20:5n-3, and 22:6n-3). These studies were conducted with variations in dietary marine FAs, and the reproductive success of cod fed a dietary lipid composition as found in salmon feed has not yet been fully examined experimentally. There is thus a lack of experimental data to determine if diets with high levels of terrestrial FAs affect sexual maturation, fecundity, or quality of gametes in cod, or the condition, viability, normal development, and health of the offspring. This should be a topic for further studies, as this information is imperative to predict and eventually mitigate effects on the reproduction of wild cod feeding on waste salmon feed released from fish farms.

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Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interests.

Author contributions

SM designed the study, analysed the fatty acids, analyzed, and interpreted the data, made figures, and wrote the paper. TVDM designed the study, collected the samples, made figures, and reviewed the paper. JES designed the study, analysed, and interpreted the data, and wrote the paper. RJB designed the study, reviewed the paper, and funded the research. JDR modelled the cluster analysis, made figures, and reviewed the paper. ØK designed the study and wrote the paper.

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Data availability statement

The data underlying this article are available in the article and in its online supplementary material.

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