Supplementary Information

Metabolic and molecular signatures of improved growth in Atlantic salmon (*Salmo salar*) fed surplus levels of methionine, folic acid, vitamin B6 and B12 throughout smoltification.

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Supplementary Methods

RNA library preparation and high-throughput sequencing

Total RNA quality was assessed using Agilent`s High Sensitivity RNA ScreenTape Analysis (Agilent Technologies, USA) before creating mRNA fragments for high-throughput sequencing. Libraries of 18 samples were simultaneously generated, and samples were balanced for gender, treatment group and stage. Libraries of approximately 200 nt insert size were generated following the protocol NEBNext® Ploy(A) mRNA Magnetic Isolation Module (NEB #E7490 New England BioLabs Inc., USA) using NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina® (NEB #E7760L, New England BioLabs Inc., USA). Libraries were barcoded with unique index primers using NEBNext® Multiplex Oligos for Illumina® (NEB #E6609, New England BioLabs Inc., USA) and PCR enriched. Library quality was assessed using High Sensitivity D1000 ScreenTapes (Agilent Technologies, USA). Barcoded libraries were normalized with KAPA library quantification kit (Kapa Biosystems, USA), pooled, and denatured according to the NextSeq System Denature and Dilute Libraries Guide (Illumina, USA). Pooled Libraries were spiked with a PhiX Control for HiSeq® and loaded on a NextSeq500 reagent cartridge (Illumina, USA) using NextSeq 500/550 High Output Kit v2 (Illumina, USA) to generate 76 bp single-end reads.

Analysis and bioinformatics environment

Quality control, mapping and quantification of the data has been completed on a Linux server via command-line operations. The program Fastqc v0.11.8 (Babraham Bioinformatics) was used for quality assessment. The program multiqc was used to collate QC reports for all samples. The subsequent analysis (outliers, differential expression, functional annotation) is done mostly in R using a variety of R packages. Bioconductor packages used in the downstream analysis are: DESeq2, foreign, AnnotationHub, clusterProfiler, geneplotter, pathview, Rgraphviz, GenomicFeatures, BiocParallel and other packages used include: tidyverse, httr, tidyr, pander, readxl, devtools, vegan, pheatmap, DT, RColorBrewer, rlang, openxlsx, igraph, ggraph, plotly, graphlayouts, ggpubr.

Supplementary Tables

Table S1. Experimental feed composition.

Re-used and modified table from Espe et al.[1].

	Base†	3 mm		4 mm	
		Control	1C+	Control	1C+
Feed composition (g/kg)					
Wheat		54.22	50.64	54.22	50.64
Wheat gluten	132.03	-	-	-	-
Sunflower meal	10.0	-	-	-	-
Dehulled faba beans	30.0	-	-	-	-
Pea concentrate	150.0	-	-	-	-
Soy protein concentrate	240.0	-	-	-	-
Krill meal	20.0	-	-	-	-
Fish meal	120.0	-	-	-	-
Rapeseed oil	81.24	-	-	-	-
Fish oil	126.9	-	-	-	-
Water		11.43	11.84	11.43	11.84
DL-methionine		0.05	3.12	0.05	3.12
choline	0.92	-	-	-	-
NRC mineral mix	2.0	-	-	-	-
NRC Vitamin mix	1.0	-	-	-	-
Vitamin B12		0.16	0.18	0.16	0.18
Folate		0.02	0.05	0.02	0.05
Vitamin B6		0.08	0.11	0.08	0.11
Taurine	2.8	-	-	-	-
Micronutrients	17.2[a]	-	-	-	-

†base value indicates that all values in the same row are equivalent/similar across the feeds and the pellet sizes.

[a] value is not equivalent but similar as determined by their relatively small SD: ±0.01, respectively.

[1] Espe M, Vikesa V, Thomsen TH et al. (2020) Atlantic salmon fed a nutrient package of surplus methionine, vitamin B12, folic acid and vitamin B6 improved growth and reduced the relative liver size, but when in excess growth reduced. Aquacult Nutr 26, 477-489.

Table S2. Sample information for RNA-sequencing analysis.

mRNA has been extracted from muscle tissue originated from individual fish. M: male, F: female, NA: not available, CF: condition factor, HSI: hepatosomatic index, RIN: RNA integrity number.

Sample ID	Analysis ID	Feed	Stage	Gender	Tank ID	Body weight (RW, g)	Body length (cm)	CF	Liver weight (g)	HSI	Flow cell	RIN
6	pre_L1C_5	Control	pre_smolt	M	t639	101	19.8	1.3	0.94	0.93	F1	9.4
7	pre_L1C_1	Control	pre_smolt	F	t639	92	19.8	1.19	0.82	0.89	F2	9.1
9	pre_L1C_2	Control	pre_smolt	M	t639	107	19.6	1.42	1.2	1.12	F2	9.3
22	pre_M1C_6	1C+	pre_smolt	M	t645	95	19.5	1.28	0.89	0.94	F1	9.3
23	pre_M1C_7	1C+	pre_smolt	F	t645	102	20.4	1.2	0.93	0.91	F1	9.1
24	pre_M1C_1	1C+	pre_smolt	M	t645	84	18.6	1.31	0.9	1.07	F2	9.4
26	pre_L1C_6	Control	pre_smolt	F	t648	103	20.5	1.2	1.42	1.38	F1	9.4
29	pre_L1C_7	Control	pre_smolt	M	t648	95	20.5	1.1	0.96	1.01	F1	9.4
30	pre_L1C_3	Control	pre_smolt	M	t648	72	27.8	0.34	9.83	13.65	F2	9.4
32	pre_M1C_2	1C+	pre_smolt	M	t649	NA	NA	NA	NA	NA	F2	9.4
33	pre_M1C_8	1C+	pre_smolt	M	t649	NA	NA	NA	NA	NA	F1	9.3
34	pre_M1C_3	1C+	pre_smolt	M	t649	NA	NA	NA	NA	NA	F2	9.3
37	pre_M1C_9	1C+	pre_smolt	F	t650	88	19	1.28	0.77	0.88	F1	9.4
39	pre_M1C_4	1C+	pre_smolt	F	t650	131	21.5	1.32	1.14	0.87	F2	9.3
40	pre_M1C_5	1C+	pre_smolt	F	t650	121	21	1.31	1.26	1.04	F2	9.3
41	pre_L1C_8	Control	pre_smolt	F	t651	88	18.5	1.39	0.95	1.08	F1	9.4
42	pre_L1C_4	Control	pre_smolt	F	t651	90	19.2	1.27	0.9	1	F2	9.3
44	pre_L1C_9	Control	pre_smolt	M	t651	102	20.2	1.24	1.14	1.12	F1	9.4
96	post_L1C_6	Control	post_smolt	F	t188	600	35	1.4	8.57	1.43	F1	9.1
98	post_L1C_1	Control	post_smolt	F	t188	489	33	1.36	7.25	1.48	F2	9.5
100	post_L1C_2	Control	post_smolt	M	t188	566	33.5	1.51	9	1.59	F2	9.1
111	post_M1C_5	1C+	post_smolt	M	t194	629	33.5	1.67	8.56	1.36	F1	9.5

114	post_M1C_6	1C+	post_smolt	M	t194	519	33	1.44	6.72	1.29	F1	9.5
115	post_M1C_1	1C+	post_smolt	M	t194	520	33	1.45	6.75	1.3	F2	9.5
118	post_L1C_7	Control	post_smolt	F	t197	540	33	1.5	7.63	1.41	F1	9.6
119	post_L1C_8	Control	post_smolt	M	t197	419	31	1.41	6.53	1.56	F1	9.6
120	post_L1C_3	Control	post_smolt	M	t197	449	32	1.37	6.63	1.48	F2	9.5
121	post_M1C_7	1C+	post_smolt	F	t198	583	34	1.48	5.99	1.03	F1	9.6
124	post_M1C_2	1C+	post_smolt	F	t198	531	32	1.62	5.93	1.12	F2	9.7
125	post_M1C_8	1C+	post_smolt	M	t198	798	36	1.71	12.04	1.51	F1	9.6
126	post_M1C_3	1C+	post_smolt	F	t199	577	33	1.61	6.52	1.13	F2	8.9
127	post_M1C_4	1C+	post_smolt	F	t199	513	32	1.57	6.24	1.22	F2	9.1
128	post_M1C_9	1C+	post_smolt	F	t199	509	32.5	1.48	5.79	1.14	F1	9.1
131	post_L1C_4	Control	post_smolt	F	t200	423	31	1.42	5.74	1.36	F2	9.2
133	post_L1C_9	Control	post_smolt	M	t200	572	34	1.46	9.59	1.68	F1	9.3
135	post_L1C_5	Control	post_smolt	F	t200	488	33	1.36	9.32	1.91	F2	8.2

Table S3. Sample information for global metabolic profiling and SAM/SAH analysis.

Muscle from 5 individual fish from the same tank were pooled for analysis.

Sample ID	Sample type	Feeds	Stage	tank ID
1794/2	Pooled	Control	pre-smolt	t639
1794/5	Pooled	1C+	pre-smolt	t645
1794/6	Pooled	Control	pre-smolt	t648
1794/7	Pooled	1C+	pre-smolt	t649
1794/8	Pooled	1C+	pre-smolt	t650
1794/9	Pooled	Control	pre-smolt	t651
1794/20	Pooled	Control	post-smolt	t188
1794/23	Pooled	1C+	post-smolt	t194
1794/24	Pooled	Control	post-smolt	t197
1794/25	Pooled	1C+	post-smolt	t198
1794/26	Pooled	1C+	post-smolt	t199
1794/27	Pooled	Control	post-smolt	t200

Table S4. Global metabolic profiling results and metabolite class enrichment of pre- and post-smolt Atlantic salmon muscle.

File:	Provided separately on figshare (DOI: 10.6084/m9.figshare.14484489)	
Sheets:	(1) Metabolite data	
	(2) Descriptive Statistics	
	(3) Inferential Statistics	
	(4) Enrichment pre-smolt	Metabolite class enrichment results
	(5) Enrichment post-smolt	Metabolite class enrichment results

Table S5. Changes in amino acid profiles discriminate both pre- and post-smolt muscle of salmon fed varying 1C nutrients.
Metabolite class enrichment analysis unravels the largest differences in the metabolic profiles between muscle from the 1C+ and Control group. Calculated enrichment scores underlie the ratio of significant altered metabolites (FDR<0.1) among detected ones in a metabolite class in relation to all significant altered metabolites among all detected ones in the global metabolic profiling. Significance of enrichment was assessed using Fisher`s exact test with Benjamini and Hochberg correction (Benjamini et al., 1995)[1]. Bold numbers designate significant class enrichment (adjusted p-value <0.05).

Metabolites		Pre-smolt muscle 1C+ vs. Control			Post-smolt muscle 1C+ vs. Control		
Metabolite class	Detected	Significant altered	Enrichment score	Adjusted P-value	Significant altered	Enrichment score	Adjusted P-value
Amino acids	19	6	13	**8.15E-04**	15	11.4	**8.69E-08**
Amino acids related	20	3	6.2	0.14	6	4.3	**0.04**
Bile acids	1	0	0	1	0	0	1
Carbohydrates and related	7	0	0	1	1	2.1	0.92
Complex lipids, fatty acids and related	201	0	0	1	5	0.4	1
Energy metabolism and related	15	0	0	1	0	0	1
Hormones, signal substances and related	1	0	0	1	0	0	1
Microbiome or alimentary	2	1	20.6	0.28	1	7.2	0.76
Miscellaneous	8	1	5.1	0.63	1	1.8	0.92
Nucleobases and related	12	0	0	1	0	0	1
Unknown	237	2	0.3	1	6	0.4	1
Vitamins, cofactors and related	13	0	0	1	2	2.2	0.78
Total metabolite count	536	13			37		

[1] Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. J R Stat Soc B 57, 289-300.

Table S6. RNA-sequencing mapping and quantification results.

Mapping results show the number and percentage of mapped reads to the entire reference genome, which includes introns, exons, intergenic regions, etc. Quantification results show how many of the successfully mapped reads fell within specific defined genomic regions - in this case genes.

Sample ID	Mapping results						Quantification results			
	Total reads	Single-map	Single-map	Multi-map	Multi-map	Total mapped	Assigned	Assigned	Unassigned	Unassigned
6	26 263 108	19 896 923	75.76 %	5 120 179	19.50 %	95.26 %	18 417 759	52.33 %	16 775 464	47.67 %
7	25 792 668	19 641 695	76.15 %	4 803 370	18.62 %	94.78 %	18 065 451	53.06 %	15 984 280	46.94 %
9	28 700 940	21 106 006	73.54 %	5 825 777	20.30 %	93.84 %	19 469 438	49.08 %	20 196 187	50.92 %
22	30 365 962	22 644 551	74.57 %	6 174 816	20.33 %	94.91 %	20 983 375	50.64 %	20 454 683	49.36 %
23	24 699 137	18 797 514	76.11 %	4 769 035	19.31 %	95.41 %	17 384 275	52.79 %	15 546 936	47.21 %
24	25 912 634	19 122 115	73.79 %	5 212 998	20.12 %	93.91 %	17 621 542	49.50 %	17 980 548	50.50 %
26	24 544 022	18 370 975	74.85 %	4 924 131	20.06 %	94.91 %	17 013 184	50.88 %	16 422 201	49.12 %
29	24 945 291	18 962 770	76.02 %	4 704 966	18.86 %	94.88 %	17 485 934	52.61 %	15 752 420	47.39 %
30	24 162 448	17 719 733	73.34 %	4 968 131	20.56 %	93.90 %	16 334 207	48.71 %	17 200 626	51.29 %
32	27 576 337	20 164 847	73.12 %	5 861 646	21.26 %	94.38 %	18 629 225	48.47 %	19 804 904	51.53 %
33	18 066 269	13 638 173	75.49 %	3 533 957	19.56 %	95.05 %	12 598 009	52.01 %	11 624 842	47.99 %
34	27 679 733	20 725 902	74.88 %	5 322 755	19.23 %	94.11 %	19 075 825	51.30 %	18 105 953	48.70 %
37	23 967 199	17 761 661	74.11 %	4 854 736	20.26 %	94.36 %	16 412 229	49.71 %	16 605 316	50.29 %
39	23 785 602	18 066 460	75.96 %	4 368 004	18.36 %	94.32 %	16 568 215	52.24 %	15 150 122	47.76 %
40	23 548 514	17 481 600	74.24 %	4 729 620	20.08 %	94.32 %	16 148 535	50.01 %	16 138 908	49.99 %
41	21 434 623	15 678 626	73.15 %	4 542 494	21.19 %	94.34 %	14 505 674	48.18 %	15 604 579	51.82 %
42	24 671 395	18 399 608	74.58 %	4 728 921	19.17 %	93.75 %	16 861 960	50.48 %	16 541 294	49.52 %
44	28 131 901	21 080 812	74.94 %	5 503 142	19.56 %	94.50 %	19 499 339	51.46 %	18 394 519	48.54 %
96	26 437 314	19 478 138	73.68 %	5 688 792	21.52 %	95.19 %	18 100 278	49.31 %	18 603 772	50.69 %
98	24 432 351	17 734 305	72.59 %	5 222 590	21.38 %	93.96 %	16 378 692	47.94 %	17 784 858	52.06 %
100	24 824 397	18 029 450	72.63 %	5 171 964	20.83 %	93.46 %	16 598 208	48.15 %	17 875 876	51.85 %

111	24 511 184	17 577 407	71.71 %	5 571 387	22.73 %	94.44 %	16 299 497	46.42 %	18 813 766	53.58 %
114	23 467 916	16 836 145	71.74 %	5 356 899	22.83 %	94.57 %	15 670 900	46.59 %	17 961 461	53.41 %
115	26 149 022	18 854 954	72.11 %	5 614 538	21.47 %	93.58 %	17 455 019	47.76 %	19 094 779	52.24 %
118	22 205 858	16 185 973	72.89 %	4 807 097	21.65 %	94.54 %	14 993 430	47.87 %	16 324 737	52.13 %
119	18 399 258	13 229 357	71.90 %	3 784 664	20.57 %	92.47 %	12 241 411	48.02 %	13 252 539	51.98 %
120	24 070 388	17 582 963	73.05 %	5 005 815	20.80 %	93.84 %	16 217 338	48.68 %	17 099 641	51.32 %
121	22 899 769	16 468 106	71.91 %	5 158 694	22.53 %	94.44 %	15 324 914	46.99 %	17 290 743	53.01 %
124	26 545 681	19 227 580	72.43 %	5 739 809	21.62 %	94.05 %	17 765 389	47.68 %	19 490 641	52.32 %
125	20 725 864	14 967 153	72.21 %	4 632 887	22.35 %	94.57 %	13 906 553	47.19 %	15 565 209	52.81 %
126	24 573 059	17 743 464	72.21 %	5 325 447	21.67 %	93.88 %	16 414 217	47.32 %	18 275 296	52.68 %
127	24 984 516	17 236 554	68.99 %	5 621 512	22.50 %	91.49 %	15 923 779	44.24 %	20 072 406	55.76 %
128	23 436 329	16 640 658	71.00 %	5 421 220	23.13 %	94.14 %	15 462 510	45.32 %	18 655 152	54.68 %
131	24 699 937	17 973 390	72.77 %	5 281 251	21.38 %	94.15 %	16 644 397	48.44 %	17 713 050	51.56 %
133	22 643 606	16 460 449	72.69 %	4 989 886	22.04 %	94.73 %	15 294 704	47.65 %	16 803 836	52.35 %
135	27 630 278	20 037 723	72.52 %	6 032 907	21.83 %	94.36 %	18 599 714	47.92 %	20 214 884	52.08 %
AVERAGE	24 635 681	18 097 882	73.43 %	5 121 557	20.81 %	94.24 %	16 732 365	49.08 %	17 366 012	50.92 %

Table S7. Lists of differentially expressed genes (DEG) in pre-smolt and post-smolt muscle and overlapping annotations.

File:	Provided separately on figshare (DOI: 10.6084/m9.figshare.14484489)
Sheets:	(1) Pre-smolt (2) Post-smolt (3) Overlap
Fields:	Entrez ID Entrez Gene ID

Fields details:

Entrez ID	Entrez Gene ID
Log2FC	Log2 fold change, output of DESeq2; Genes with negative log2FC are lower expressed in 1C+ than in Control muscle, and vice versa for positive log2FC
Pvalue	Output of DESeq2
Adjustedp	Adjusted p value, output DESeq2
Gene_symbol	Gene symbol
Description	Gene name
Stage	Pre-smolt or post-smolt data set

Table S8. Functional annotation of differentially expressed genes in pre-smolt muscle.

File:	Provided separately on figshare (DOI: 10.6084/m9.figshare.14484489)	
Sheets:	(1) KEGG	KEGG pathways
	(1) KEGG genes	List of DEGs with KEGG pathway IDs
	(2) CC terms	Gene Ontology term: cellular component
	(2) CC genes	List of DEGs with GO term (CC) IDs
	(3) BP terms	Gene Ontology term: biological process
	(3) BP genes	List of DEGs with GO term (BP) IDs
	(4) MF terms	Gene Ontology term: molecular function
	(4) MF genes	List of DEGs with GO term (MF) IDs
Fields:	Entrez ID	Entrez Gene ID
	Log2FC	Log2 fold change, output of DESeq2
	Gene_symbol	Gene symbol
	Gene_description	Gene name
	Pathway_ID	KEGG pathway ID
	Pathway_description	Description of the KEGG pathway
	GO_Term_ID	Gene Ontology term ID
	GO_Term_description	Description of the Gene Ontology term
	Adjusted_P	Adjusted p value by hypergeometric distribution test
	Gene_count	Gene count in a pathway or GO term

Table S9. Functional annotation of differentially expressed genes in post-smolt muscle.

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File:	Provided separately on figshare (DOI: 10.6084/m9.figshare.14484489)	
Sheets:	(1) KEGG	KEGG pathways
	(1) KEGG genes	List of DEGs with KEGG pathway IDs
	(2) GO_CC	Gene Ontology term: cellular component
	(2) GO_CC genes	List of DEGs with GO term (CC) IDs
	(3) GO_MF	Gene Ontology term: molecular function
	(3) GO_MF genes	List of DEGs with GO term (MF) IDs
	(4) GO_BP	Gene Ontology term: biological process
	(4) GO_BP genes	List of DEGs with GO term (BP) IDs
Fields:	Entrez ID	Entrez Gene ID
	Log2FC	Log2 fold change, output of DESeq2
	Gene_symbol	Gene symbol
	Gene_description	Gene name
	Pathway_ID	KEGG pathway ID
	Pathway_description	Description of the KEGG pathway
	GO_Term_ID	Gene Ontology term ID
	GO_Term_description	Description of the Gene Ontology term
	Adjusted_P	Adjusted p value by hypergeometric distribution test
	Gene_count	Gene count in a pathway or GO term

Supplementary Figures
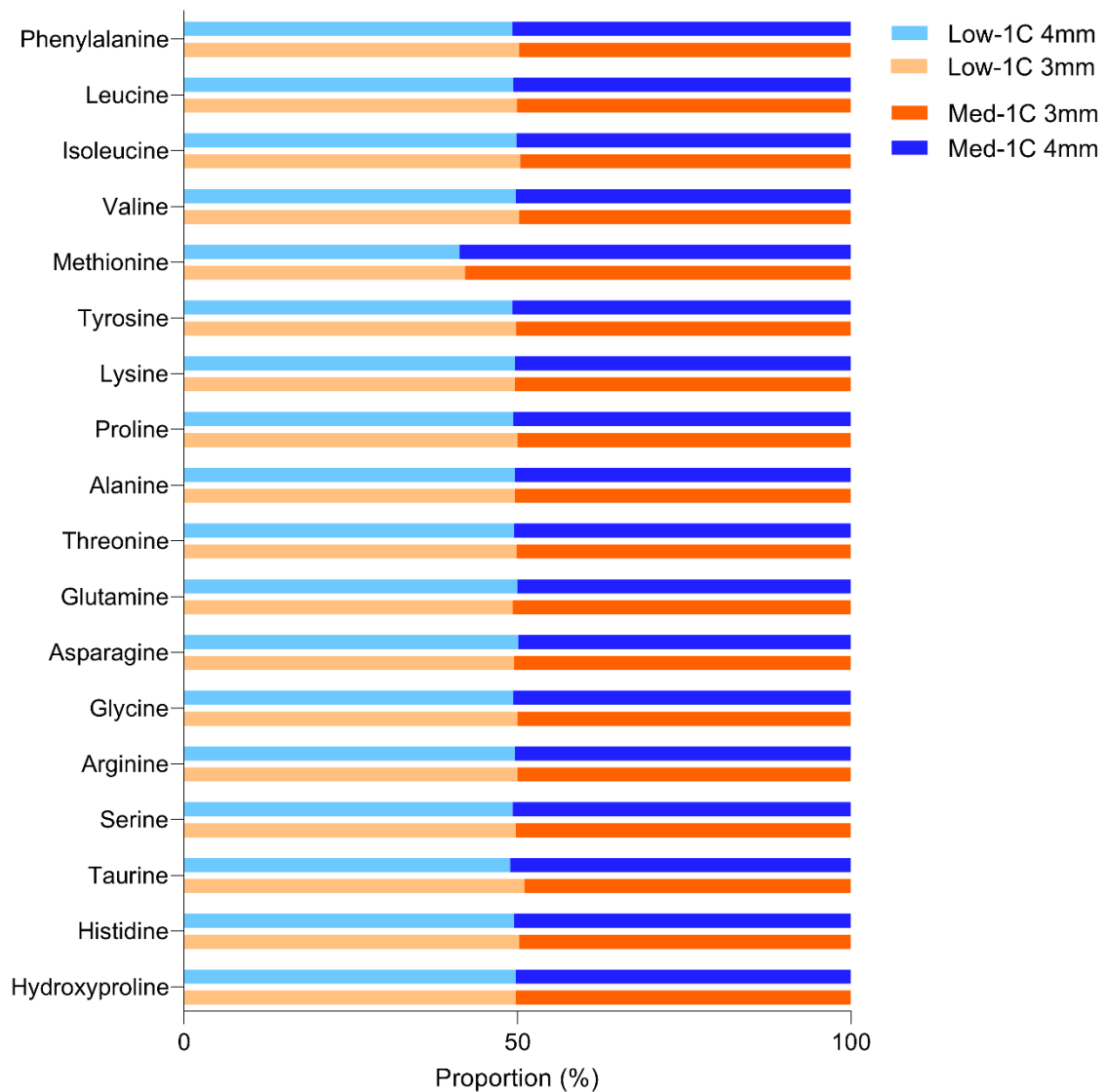
Figure S1. Amino acid composition in the Control and 1C+ feeds (pellet size 3 mm and 4 mm) illustrated as proportions.

Methionine is the amino acid different between the feeds, whereas other amino acids were the same. Modified graph from Espe et al. [1].

[1] Espe M, Vikesa V, Thomsen TH et al. (2020) Atlantic salmon fed a nutrient package of surplus methionine, vitamin B12, folic acid and vitamin B6 improved growth and reduced the relative liver size, but when in excess growth reduced. Aquacult Nutr 26, 477-489.
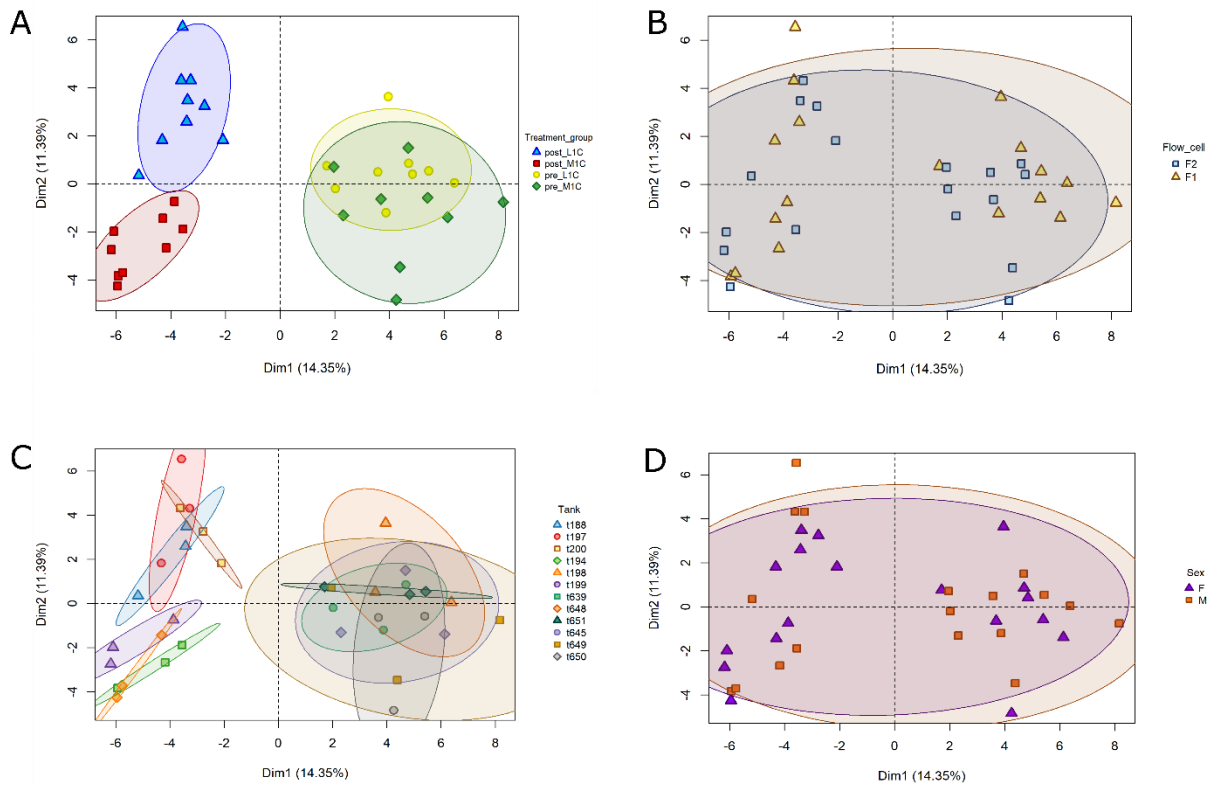
Figure S2. Principal component analysis (PCA) plots of variance stabilizing transformed (vst), normalized mean counts per sample.

Samples are colored by A: age (pre: pre-smolt and post: post-smolt) and dietary groups (L1C: control group, M1C: 1C+ group), B: flow cell (F: flow cell), C: tank ID (t: tank) and D: sex (F: female and M: male). Shaded areas represent 85% confidence interval of variance per treatment group.
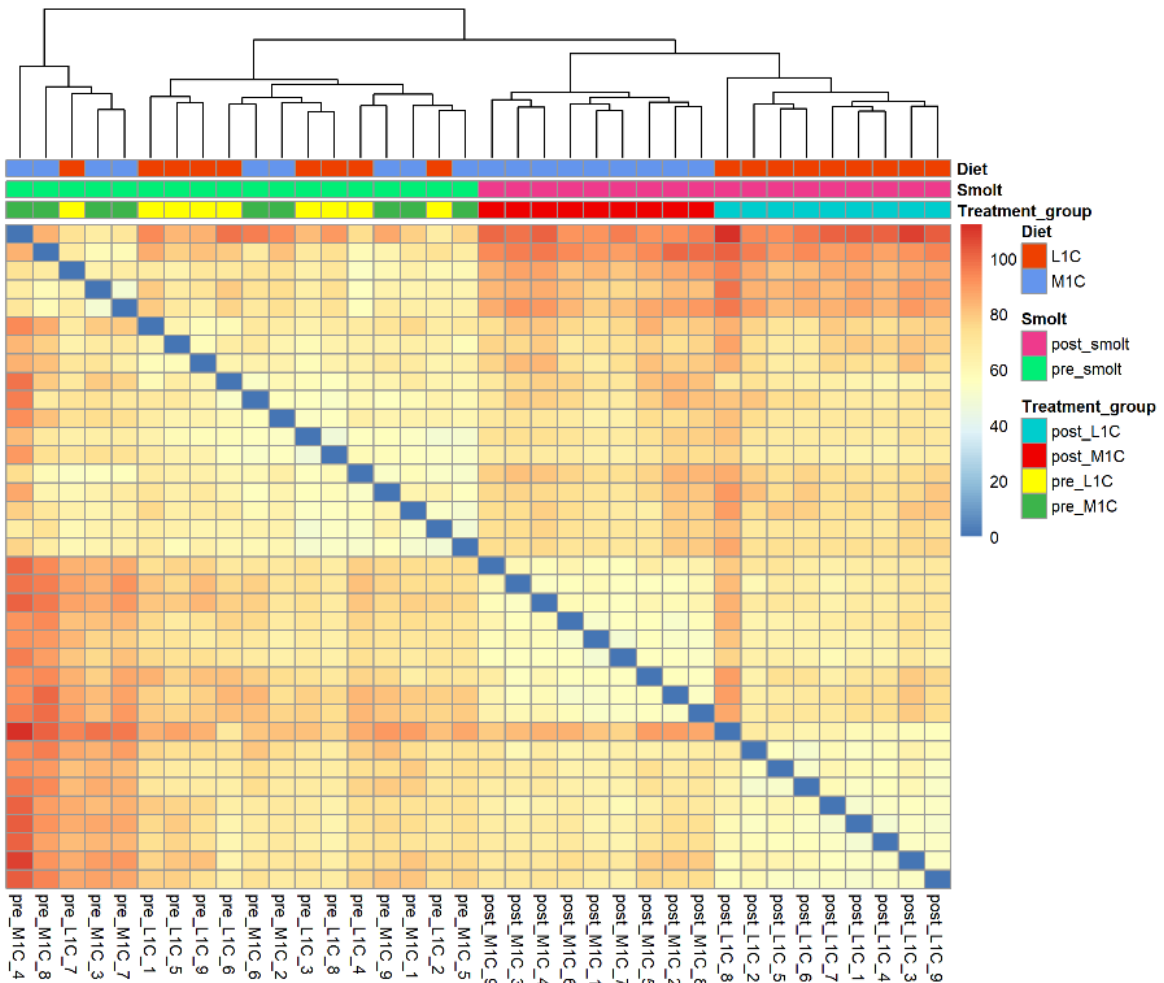
Figure S3. Heatmap and dendrogram of variance stabilizing transformed (vst), normalized mean counts per mRNA library sample.
Dendrograms show the hierarchical clustering of the samples, based on pairwise Euclidean distance. Heatmaps show, using a color gradient, pairwise Euclidean distance between individual samples. Pre: pre-smolt, post: post-smolt, L1C: control group, M1C: 1C+ group.
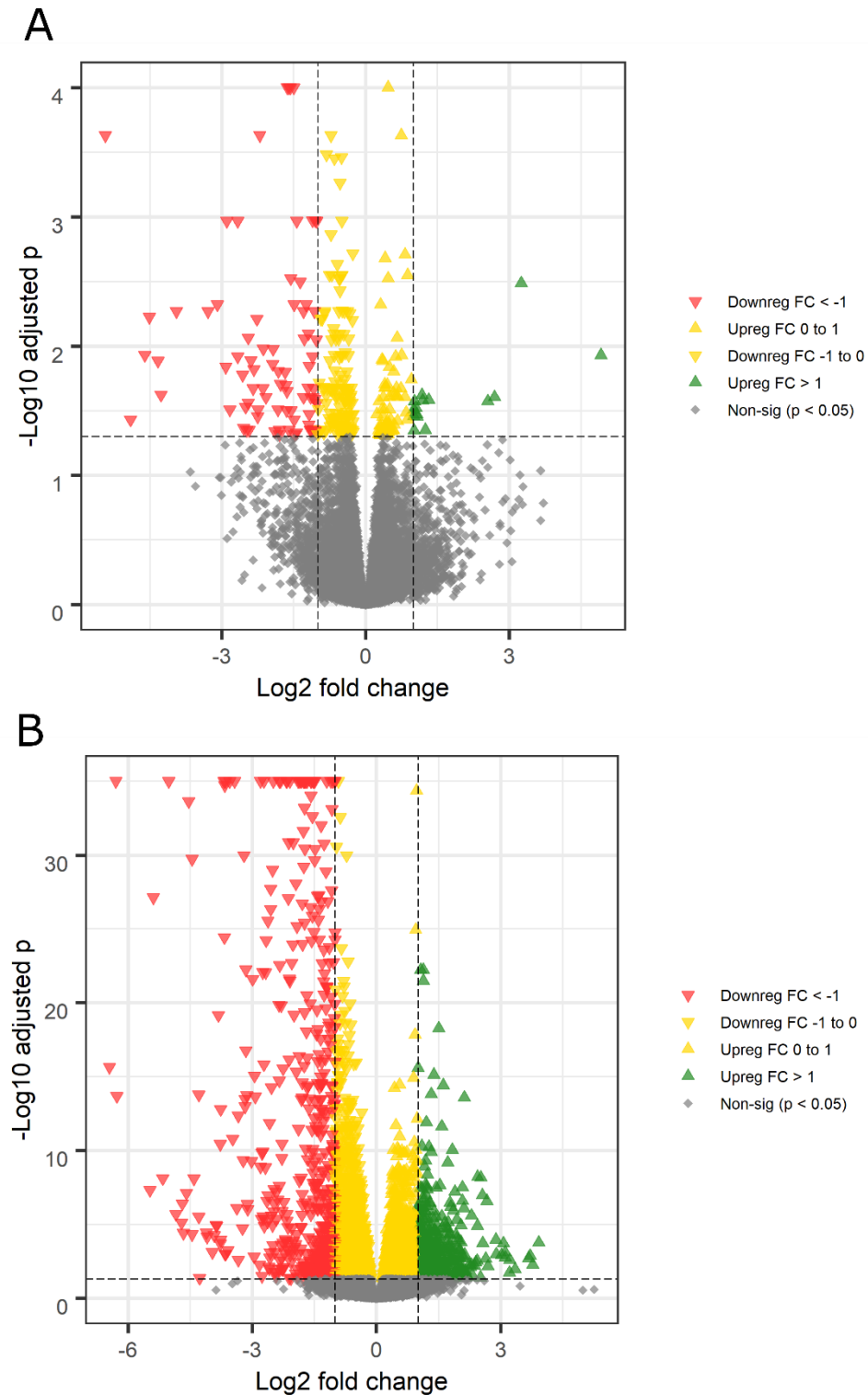
Figure S4. Volcano plot of differences in 1C+ vs. Control gene expression profiles for pre-smolt (A) and post-smolt (B) muscle.
5 DEGs (p<1e-04) for pre-smolt and 35 DEGs (p<1e-35) for post-smolt muscle are clustered at the top of the plot for plotting clarity. FC: log2-fold change.
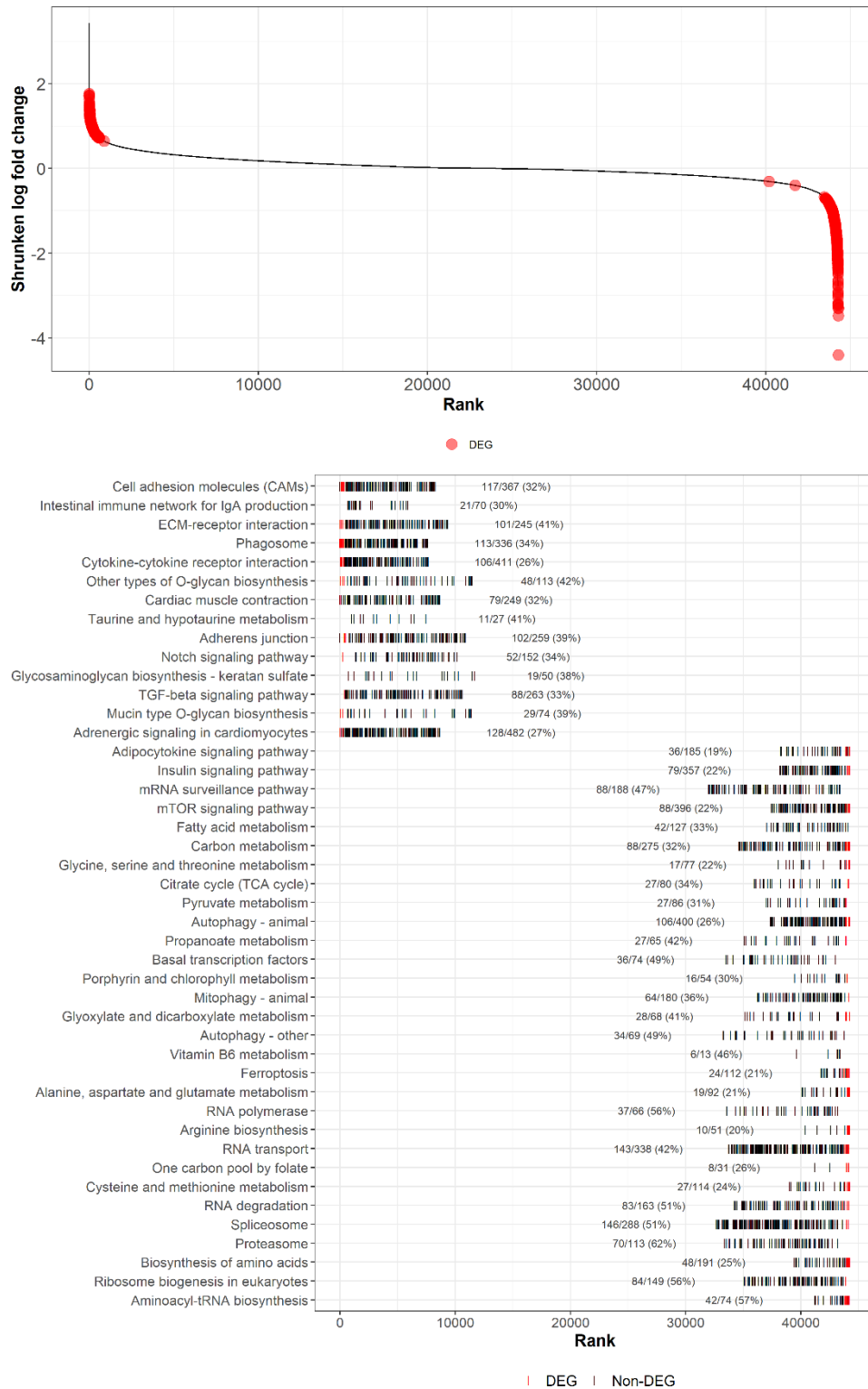
Figure S5. Gene Set Enrichment Analysis (GSEA) of KEGG pathways between 1C+ and Control post-smolt salmon muscle. GSEA is based on fold change differences for all differentially expressed genes between the 1C+ and the Control group.