Supplementary information

Micronutrient supplementation affects transcriptional and epigenetic regulations of lipid metabolism in a dose-dependent manner

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Supplementary Methods

Experimental diets and feeding trial

More comprehensive methods and materials regarding the feeding trial with experimental diets than the descriptions below are available[1] if needed.

Experimental diets

Feeds were formulated to reflect standard practice in commercial salmon feeds in terms of protein, oil and energy contents (Supplementary Table 2). Thus, feeds were initially formulated to contain 48% protein and 20% lipid (~22 MJ), with protein content decreasing and lipid content increasing with increasing pellet size to reach 36% protein and 34% lipid (~24 MJ) in the largest pellet size in seawater (Supplementary Table 3). The experimental feeds were a low FM (fish meal) / FO (fish oil) formulation (initially 15% and 8% in freshwater, decreasing to 5% and 3% respectively in seawater). Feeds were supplemented with a nutrient package (NP) at one of three inclusion levels to produce three dietary treatments: L1, 100% NP; L2, 200% NP; L3, 400% NP (Supplementary Table 1), the assumption being that the 100% NP package should contain 100 % of assumed requirement based on the given requirement levels reported for Atlantic salmon at the time[2] and modified according to earlier trials as part of the EU-funded ARRAINA project[3, 4]. Specifically, the NP contained 24 nutrients in total these being; vitamins (A, D3, E, K3, C, thiamine, riboflavin, B6, B12, niacin, pantothenic acid, folic acid and biotin), minerals (Ca, Co, I, Se, Fe, Mn, Cu and Zn), crystalline amino acids (L-histidine and taurine) and cholesterol. Total and available phosphorus were fixed in all diets at 12.0 g/kg and 9.0 g/kg respectively, and magnesium at 1.5 g/kg, and were not part of the NP. Pellet size was adjusted according to fish weight (2mm, 3.5mm, 5mm, 7mm, 9mm). All non-oil ingredients were mixed, and pellets produced by extrusion to produce three base pellets that had oil added by vacuum coating. All feeds were produced at the BioMar Tech-Centre (Brande, Denmark).

Micronutrient analysis of experimental diets

Biotin (B7), niacin (B3), folate (B9), pantothenic acid (B5) and cobalamin (B12) were all determined by microbiological methods[5, 6]. Thiamine (prEN 14122, CEN, 2003), vitamin B6 (prEN 14164, CEN, 2006) and riboflavin[7] as well as vitamins C[8], E, K (prEN 12822, CEN, 1999), D and, A[9] were

determined by high-performance liquid chromatography (HPLC). Multi-element determination of minerals was done by inductively coupled plasma mass spectrometry (ICP-MS).

Feeding trial

The freshwater phase was carried out at the Niall Bromage Freshwater Research Facility (Stirlingshire, UK). Initially, 500 diploid salmon parr (initial mean weight, 38.2 ± 5.8 g) were stocked (September 4th, 2014) into nine x 1.6 m^3 circular fibreglass tanks (three tanks/diet). Fish were acclimatised to the experimental conditions for two weeks before being fed the experimental diets. Fish were fed continuously during the light period of the light dark cycle by automatic feeders (Arvotec T2000, Arvotec, Finland) controlled by a PC system. Specific feeding rates (SFR; % tank biomass per day) were adjusted automatically according to predicted growth and daily temperature. An out-of-season photoperiod (LL – 4 weeks LD14:10 – 4 weeks LL) and ambient water temperature (12 - 16 ˚C) was applied to produce S0+ smolts, with lighting provided by two 28 W fluorescent daylight bulbs (4000 °K, RS Components, UK) mounted centrally within the tank lid. Water was supplied by an upstream reservoir under flow-through conditions (10 L/min). Oxygen levels were always higher than 8 mg/L. Uneaten feed recovery was not feasible during the freshwater phase.

Post-smolts were transferred (November 4th, 2014) to the Marine Harvest (Mowi) Feed Trial Unit, (Ardnish, Scotland) and on-grown for 12 months in 5 x 5 x 5 m sea pens under natural photoperiod and ambient water temperatures ranging from 6 to 16 ˚C. Triplicate groups of 250 post-smolts from respective tanks were stocked per pen and on-grown to a final size of ~3.0 kg. Fish were fed continuously during daylight by automatic feeders (Arvotec T2000) controlled by a PC system. Specific feeding rates (SFR; % pen biomass per day) were adjusted weekly according to predicted growth and water temperature. Waste feed was collected per pen by means of air uplifts following meal delivery ensuring satiation and allowing calculation of total daily feed intake (Fi).

DNA and RNA extraction

At the end of the feeding trial, liver tissue for both RNA and DNA extraction were dissected from 6 fish per diet at the same area of each individual, the ventral posterior lobe, and snap frozen in liquid nitrogen and stored at -80°C until further processing. DNA and RNA were extracted from same fish. For each feed group, liver samples were selected from the triplicate feeding tanks for RNA sequencing and RRBS. For both RNA and DNA extraction, tissue samples were homogenized using ceramic beads CK28 and a Precellys 24 homogenizer (Bertin Technologies).

RNA was extracted using the BioRobot EZ1 and EZ1 RNA Universal Tissue kit (Qiagen) and DNase treated with Ambion DNA-free DNA removal kit (Invitrogen, USA) according to the protocols. RNA quantity, which was 2460 ± 652 ng/ml on average, were assessed using NanoDrop

ND-1000 Spectrophotometer (Nanodrop Technologies). RNA integrity (RIN), which was 9.55 ± 0.15 on average, were analysed using an Agilent 2100 Bioanalyser (RNA 6000 Nano LabChip kit, Agilent Technologies).

Homogenate from six single male livers from each feed group were resuspended in lysis buffer and DNA isolation was performed using the DNeasy Blood &Tissue Kit (Qiagen, Cat. No. #69506) according to the manufacturers protocol, except that after homogenization, tissue for DNA extraction were pre-treated with RNase A (provided by the Qiagen kit, 50ng/µL, 10 min at room temperature) immediately followed by proteinase K treatment (New England biolabs, #8102S 20µg/µL, 1.5 h at 55°C). DNA was eluted in Milli Q H_2O. Quantification of double stranded genomic DNA was done using the Qubit High Sensitivity Assay (Life Technologies #Q32854).

RRBS library preparation and sequencing

For RRBS, 100 ng of genomic DNA were digested for 6h at 65°C with 20 U TaqI (New England Biolabs) and 6h hours at 37°C with 20 U of MspI (New England Biolabs) in 30 µl of 1x NEB buffer 2. To retain even the smallest fragments and to minimize the loss of material, end preparation and adaptor ligation were performed in a single-tube setup. End fill-in and A-tailing were performed by addition of Klenow Fragment 3′ -> 5′ exo- (New England Biolabs) and dNTP mix (10 mM dATP, 1 mM dCTP, 1 mM dGTP). After ligation to methylated Illumina TruSeq LT v2 adaptors using T4 DNA Ligase rapid (Enzymatics), the libraries were size selected by performing a 0.75x clean-up with AMPure XP beads (Beckman Coulter).

The libraries were pooled based on qPCR data and subjected to bisulfite conversion using the EZ DNA Methylation Direct Kit (Zymo Research) with changes to the manufacturer's protocol: conversion reagent was used at 0.9x concentration, incubation performed for 20 cycles of 1 min at 95°C, 10 min at 60°C and the desulphonation time was extended to 30 min. These changes increase the number of CpG dinucleotides covered, by reducing double-strand break formation in larger library fragments. Bisulfite-converted libraries were enriched KAPA HiFi HS Uracil+ RM (Roche). The minimum number of enrichment cycles was estimated based on a qPCR experiment. After a 1x AMPure XP clean-up, library concentrations were quantified with the Qubit Fluorometric Quantitation system (Life Technologies) and the size distribution was assessed using the Bioanalyzer High Sensitivity DNA Kit (Agilent).

RRBS libraries were sequenced on Illumina HiSeq 3000/4000 instruments in 50-base-pair-single-end mode or 60-base-pair-single-end mode, and base calls provided by the Illumina Real-Time Analysis (RTA) software were subsequently converted into BAM format (Illumina2bam) before de-multiplexing (BamIndexDecoder) into individual, sample-specific BAM files via Illumina2bam tools (1.17.3 https://github.com/wtsi-npg/illumina2bam).

Wilcoxon signed-rank test (Wilcox) to find enriched KEGG pathways by comparing methylation rates of CpG sites between two diet groups

Instead of using DMGs as for ORA, we directly used methylation rates at the mapped CpG sites with the Wilcoxon signed-rank test, which is a non-parametric version of paired test, to identify enriched KEGG pathways. We first linked a KEGG pathway with CpG sites through the genes where the mapped CpG sites reside, followed by the Wilcoxon signed-rank test to compare two samples of methylation rates in a pair-wised manner. The pathways associated less than 20 CpG sites were ignored. We defined enriched pathways when they had adjusted p-values < 0.05 after applying the Benjamini-Hochberg procedure. Moreover, we performed this procedure on several different regions, such as intron, exon, P250, and so on.

For example, 1074 CpG sites were associated with mTOR signalling pathway (sasa04150) for L1 and L3 in introns. The medians of methylation rates were 96.1% and 95.5% for L1 and L3, respectively. To find whether two samples were from the same distribution, the Wilcoxon signed-rank test was used to calculate a p-value by comparing all the 1074 methylation rates between L1 and L3. This pathway was identified as enriched in introns since its adjusted p-value was < 0.05.

Bootstrap Kolmogorov-Smirnov test (KS-boot) to find enriched KEGG pathways by comparing methylation differences against the background distribution of a region

We also compared methylation differences that belong to a specific KEGG pathway against the whole background methylation differences by using the Kolmogorov-Smirnov test. The p-value of the Kolmogorov-Smirnov test is known to be inaccurate when there are ties. We used a bootstrap version of the test (with 1000 bootstraps) to avoid the difficulties with ties since there were many CpG site with no methylation differences, whose values were simply 0%. We defined enriched pathways when they had adjusted p-values < 0.01 after applying the Benjamini-Hochberg procedure. We performed the same procedure on different regions.

For example, 1074 CpG sites were associated with mTOR signalling pathway (sasa04150) for L3:L1 in introns, whereas 110 395 CpG sites were identified in the whole introns. The medians of methylation differences were -0.18% for L3:L1 and 0% for the whole introns. To find whether the methylation differences of L3:L1 in introns were independent from the distribution of the methylation differences in the whole introns, the Bootstrap Kolmogorov-Smirnov test was used to calculate a p-value by comparing 1074 methylation differences of L3:L1 against 110 395 methylation differences. This pathway was identified as enriched since its adjusted p-value was < 0.01.

Combine ORA, Wilcox, and KS-boot to summarise enriched KEGG pathways

We made the final list of enriched KEGG pathways by combining all the results from the three methods as ORA OR (Wilcox AND KS-boos). The enriched functions identified by ORA were always considered, whereas only intersections of the pathways found in both Wilcox AND KS-boos were considered. The final list of the pathways was formed by the union of ORA and the intersection of Wilcox and KS-boos.

Supplementary tables

Supplementary Table 1. Added micronutrient concentrations (mg/kg) within the NP.

Nutrient group	Micronutrient	L1	L2	L3
Vitamin	Vitamin A	3.79	7.58	15.16
	Vitamin D3	0.05	0.1	0.2
	Vitamin E	102.4	204.9	409.8
	Vitamin K3	9.82	19.64	39.28
	Thiamine (B1)	2.67	5.34	10.68
	Riboflavin (B2)	8.3	16.6	33.2
	B6	4.77	9.54	19.08
	B12	0.25	0.5	1
	Niacin (B3)	24.8	49.6	99.2
	Pantothenic Acid (B5)	17.15	34.3	68.6
	Folic Acid (B9)	2.82	5.64	11.28
	Biotin (B7)	0.14	0.28	0.56
	Vitamin C	80	160	320
Micro-mineral	Cobalt (Co)	0.94	1.88	3.76
	Iodine	0.67	1.34	2.68
	Selenium	0.23	0.46	0.92
	Iron	32.64	65.28	130.6
	Manganese	12.03	24.06	48.12
	Copper	3.24	6.48	12.96
	Zinc	66.92	133.8	267.7
Macro-mineral	Calcium (Ca)	0.4	0.8	1.6
Amino acid	Taurine	2450	4900	9800
	Histidine	1400	2800	5600
Cholesterol	Cholesterol	1100	2200	4400

Supplementary Table 2. Ingredients and formulation (g/100g) of the experimental diets.

	Marine pellet size[1]		
Ingredients	**3.5 mm**	**5.0 / 7.0 mm**	**9.0 mm**
Fish Meal[2]	13.00	8.00	3.00
Krill Meal[3]	2.00	2.00	2.00
Soy Protein Concentrate[4]	15.58	15.40	12.58
Wheat Gluten[5]	12.46	13.09	10.70
Maize Gluten	3.12	3.08	2.52
Pea Protein Concentrate[6]	12.46	13.09	10.70
Wheat[7]	17.86	10.00	13.33
Sunflower Expeller	-	6.32	6.77
Fish Oil[8]	5.00	4.00	3.00
Rapeseed oil[5]	7.65	10.61	16.17
Linseed oil	1.91	2.55	3.84
Palm kernel oil	4.78	6.38	9.61
ARRAINA Nutrient Package[9,†] (L1 / L2 / L3)	**0.75 / 1.50 / 3.00**	**0.75 / 1.50 / 3.00**	**0.75 / 1.50 / 3.00**
Monosodium Phosphate	2.03	-	-
Monocalcium phosphate	-	2.05	1.46
Amino acid Premix[10,*] (L1 / L2 / L3)	2.14 / 2.34 / 2.32	2.07 / 2.11 / 2.16	2.15 / 2.18 / 2.24
Yttrium	0.05	0.05	0.05
Lucantin Pink	0.06	0.06	0.06
Water change	-1.98	-0.52	0.28

[1]Feed Services, Bremen, Germany; [2]Aker Biomarine, Norway; [3]Caramuru, Brazil; [4]Cargill, Germany; [5]Agrident, Germany; [6]WN Lindsey, UK; [7]ED & F Man, Germany; [8]DSM, Netherlands; [9]Evonik, Germany; [†]Added as components of the nutrient package (NP), and times requirement based on NRC (2011) minimum requirement for Atlantic salmon and modified according to Hemre et al., (2016), diet L1 achieving assumed 100 % minimum requirement; [*]Balanced for lysine, methionine, threonine and valine. Contains antioxidant.

Supplementary Table 3. Alternations of pellet sizes with different FM and FO inclusions

Pellet size	Stage	Fish size	Fish meal (FM)	Fish oil (FO)	Total FM and FO
2.0 mm	Fresh water	20-60 g	15%	8%	23%
3.5 mm	Salt water	75-250 g	15%	5%	20%
5.0 / 7.0 mm	Salt water	250-1000 g	10%	4%	14%
9.0 mm	Salt water	1000-2500 g	5%	3%	8%

Supplementary Table 4. Analysed concentrations of the experimental diets.

Nutrient group	Unit	Micronutrient	Graded level†	L1	L2	L3	NRC 2011‡
Vitamin	mg/kg	Vitamin A	Yes	3.73	5.15	12.16	0.75[a]
		Vitamin D3	Yes	0.15	0.19	0.19	0.04[a]
		Vitamin E	Yes	241.5	364	436.5	60[b]
		Vitamin K3	Yes	0.71	1.51	2.7	<10[b]
		Thiamin (B1)	Yes	4.5	7.1	8.8	1[a]
		Riboflavin (B2)	Yes	17.2	27.8	33.5	4[a]
		Vitamin B6	Yes	12.8	16.8	21.3	5[b]
		Vitamin B12	Yes	0.18	0.35	0.67	NT
		Niacin (B3)	Yes	73	112	148	10[a]
		Pantothenic acid (B5)	Yes	24	58	44	20[a]
		Folic acid (B9)	Yes	6.53	9.69	11.67	1[a]
		Biotin (B7)	Yes	0.51	0.72	0.74	0.15[a]
		Vitamin C	Yes	183	251	409	20[b]
Micro-mineral	mg/kg	Cobalt	Yes	0.18	0.22	0.32	NT
		Iodine	Yes	n.a	n.a	n.a	1.1[a]
		Selenium	Yes	1.13	1.48	1.65	0.15[a]
		Iron	Yes	330	358	403	30-60[b]
		Manganese	Yes	42	53	86	10[b]
		Copper	Yes	11.8	14.8	22.8	5[b]
		Zinc	Yes	94	156	330	37[b]
Macro-mineral	g/kg	Calcium	Yes	6.7	7.1	8.2	NR*[b]
		Magnesium	No	1.73	1.66	1.68	0.4[b]
		Phosphorus	No	12.7	12.5	12.5	8.0[b]
Amino acid	g/kg	Taurine	Yes	2.6	4.4	10.1	NR[b]
		Methionine	No	9.7	9.9	10.3	7.0[b]
		Histidine	Yes	11.4	13.1	17.1	8.0[b]
Cholesterol		Cholesterol	Yes	n.a	n.a	n.a	NR

†Nutrients added at graded levels to the feeds. ‡Current NRC, 2011, minimum requirement recommendations determined in [a] rainbow trout and [b]Atlantic salmon. Non-numeric values represent; n.a (not analysed), NR (no requirement), NR* (no requirement freshwater), and NT (not tested).

Supplementary Table 5. Growth measures (mean ± SEM) recorded at the smolt and final stages.

Stage	Measurement	Unit	L1	L2	L3
Smolt	Body weight	g	68.0 ± 0.5^{c}	77.4 ± 0.5^{a}	73.8 ± 0.8^{b}
	Condition Factor (K)	g/cm^3	1.21 ± 0.00	1.19 ± 0.00	1.19 ± 0.00
	Hepatosomatic index (HIS)	%	1.28 ± 0.06^{a}	1.04 ± 0.01^{b}	1.15 ± 0.06^{b}
	Mortality	%	0.2 ± 0.2	0.3 ± 0.1	0.0 ± 0.0
Final	Body weight	g	2127 ± 59^{b}	2381 ± 82^{a}	2385 ± 37^{a}
	Condition Factor (K)	g/cm^3	1.37 ± 0.01^{b}	1.43 ± 0.02^{ab}	1.46 ± 0.03^{a}
	Hepatosomatic index (HIS)	%	1.08 ± 0.06	1.02 ± 0.01	1.10 ± 0.04
	Mortality	%	0.95 ± 0.76	2.64 ± 2.25	0.54 ± 0.13

^{a,b,c}Superscripts denote significant differences between diets ($p < 0.05$, one-way ANOVA).

Supplementary Table 6. Read alignment of 18 RNA-seq samples on the Salmon genome.

No	Name	Diet	Sex	Total reads	Uniquely mapped	(%)	Multi-mapped	(%)	Non-mapped	(%)
1	L1	L2	M	23 930 797	19 047 920	(79.6)	3 773 848	(15.8)	1 109 029	(4.6)
2	L6	L3	M	25 782 928	20 489 194	(79.5)	3 988 812	(15.5)	1 304 922	(5.1)
3	L9	L3	M	24 514 536	18 671 845	(76.2)	4 725 509	(19.3)	1 117 182	(4.6)
4	L11	L1	M	35 224 641	28 126 452	(79.9)	5 379 803	(15.3)	1 718 386	(4.9)
5	L18	L2	M	26 963 177	21 310 739	(79.0)	4 436 696	(16.5)	1 215 742	(4.5)
6	L21	L1	M	18 941 648	15 002 865	(79.2)	2 951 549	(15.6)	987 234	(5.2)
7	L22	L1	M	23 088 225	18 320 836	(79.4)	3 680 166	(15.9)	1 087 223	(4.7)
8	L23	L1	M	25 797 353	20 597 043	(79.8)	3 946 915	(15.3)	1 253 395	(4.9)
9	L24	L1	M	24 137 905	18 864 956	(78.2)	4 142 372	(17.2)	1 130 577	(4.7)
10	L26	L3	M	22 122 866	17 141 205	(77.5)	3 815 675	(17.3)	1 165 986	(5.3)
11	L27	L3	M	23 379 331	18 435 658	(78.9)	3 806 566	(16.3)	1 137 107	(4.9)
12	L31	L2	M	22 903 741	17 406 367	(76.0)	3 765 846	(16.4)	1 731 528	(7.6)
13	L32	L2	M	24 847 074	19 675 370	(79.2)	3 952 409	(15.9)	1 219 295	(4.9)
14	L34	L2	M	25 061 137	19 505 737	(77.8	4 244 064	(16.9)	1 311 336	(5.2)
15	L35	L2	M	26 768 289	20 824 641	(77.8)	3 715 678	(13.9)	2 227 970	(8.3)
16	L36	L3	M	23 217 907	18 221 640	(78.5)	3 877 812	(16.7)	1 118 455	(4.8)
17	L40	L3	M	26 143 116	20 707 682	(79.2)	4 103 148	(15.7)	1 332 286	(5.1)
18	L41	L1	M	23 950 855	18 851 368	(78.7)	4 100 134	(17.1)	999 353	(4.2)

Supplementary Table 7. Common DEGs between L2:L1 and L3:L1.

Gene symbol	Gene ID	Gene name	LFC L1L2	LFC L1L3	Graded
LOC106605546	106605546	squalene monooxygenase-like	-3.5	-5.1	Yes
hmgcr	106570829	3-hydroxy-3-methylglutaryl-CoA reductase	-2.6	-4.5	Yes
LOC106579093	106579093	squalene monooxygenase-like	-1.8	-4.4	Yes
LOC106601335	106601335	solute carrier family 25 member 38-B-like	-1.8	-3.0	Yes
LOC106605924	106605924	farnesyl pyrophosphate synthase-like	-1.8	-2.1	Yes
LOC106604248	106604248	sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating-like	-1.6	-2.7	Yes
lss	106581851	lanosterol synthase	-1.5	-3.5	Yes
LOC106564571	106564571	glutaryl-CoA dehydrogenase, mitochondrial-like	-1.3	-2.0	Yes
LOC106591920	106591920	3-beta-hydroxysteroid-Delta(8),Delta(7)-isomerase-like	-1.1	-2.8	Yes
LOC106591002	106591002	probable ergosterol biosynthetic protein 28	-1.0	-1.4	Yes
LOC106587139	106587139	7-dehydrocholesterol reductase-like	-0.7	-1.5	Yes
tmem97	106581529	transmembrane protein 97	-0.3	-0.4	Yes
LOC106587488	106587488	fibrinogen-like protein 1-like protein	0.4	0.5	Yes
LOC106601178	106601178	NF-kappa-B inhibitor alpha-like	0.9	0.7	No
LOC106607496	106607496	nuclear receptor subfamily 1 group D member 1-like	1.0	1.1	Yes
LOC106582110	106582110	nuclear receptor subfamily 1 group D member 2-like	1.0	0.8	No
LOC106584490	106584490	cyclin-dependent kinase inhibitor 1B-like	1.2	1.2	No
LOC106602919	106602919	putative monooxygenase p33MONOX	1.2	1.1	No
LOC106601460	106601460	serine/threonine-protein kinase SBK1-like	1.4	0.9	No
LOC106580424	106580424	B-cell linker protein-like	1.6	2.2	Yes
igfbp-1b1	100136518	IGF binding protein 1	1.8	1.8	Yes
LOC106605529	106605529	Krueppel-like factor 10	2.1	1.9	No
LOC106608133	106608133	type III iodothyronine deiodinase-like	2.3	2.5	Yes
LOC106585060	106585060	B-cell linker protein-like	2.3	1.9	No
LOC106574392	106574392	ladderlectin-like	3.8	4.8	Yes
LOC106590962	106590962	ladderlectin-like	7.0	6.3	No

Supplementary Table 8. Enriched KEGG pathways for DEGs (adjusted p-value < 0.05).

Dataset	KEGG ID	Description	GeneRatio[a]	BgRatio[b]	Adjusted p-value[c]	Sig[d]
L2:L1	sasa00100	Steroid biosynthesis	9/38	32/13972	3.7E-15	**
L3:L1	sasa00100	Steroid biosynthesis	18/107	32/13972	6.7E-29	**
	sasa00900	Terpenoid backbone biosynthesis	9/107	34/13972	1.2E-10	**
	sasa03320	PPAR signaling pathway	10/107	173/13972	2.3E-05	**
	sasa01212	Fatty acid metabolism	8/107	137/13972	2.1E-04	**
	sasa01200	Carbon metabolism	11/107	303/13972	3.4E-04	**
	sasa00620	Pyruvate metabolism	5/107	89/13972	8.5E-03	**
	sasa04146	Peroxisome	6/107	173/13972	2.6E-02	*
	sasa00650	Butanoate metabolism	3/107	37/13972	2.8E-02	*
	sasa01040	Biosynthesis of unsaturated fatty acids	4/107	78/13972	2.8E-02	*
	sasa00061	Fatty acid biosynthesis	3/107	39/13972	2.8E-02	*
	sasa00260	Glycine, serine and threonine metabolism	4/107	82/13972	2.8E-02	*
	sasa00760	Nicotinate and nicotinamide metabolism	4/107	88/13972	3.1E-02	*
	sasa00072	Synthesis and degradation of ketone bodies	2/107	14/13972	3.1E-02	*
	sasa01230	Biosynthesis of amino acids	6/107	207/13972	3.1E-02	*
	sasa00740	Riboflavin metabolism	2/107	16/13972	3.7E-02	*
	sasa00480	Glutathione metabolism	4/107	107/13972	4.9E-02	*

[a]Gene ratio as (# of DEGs in the pathway) / (# of DEGs). [b]Background ratio as (# of genes in the pathway) / (# of genes in KEGG). [c]Calculated by hypergeometric test on odds ratio and adjusted by Benjamini-Hochberg. [d]Statistical significance as "*" indicating adjusted p-value < 0.05 and "**" indicating adjusted p-value < 0.01.

Supplementary Table 9. Classes and related pathways of the enriched KEGG pathways by ORA.

Pathway	KEGG ID	Class	Related pathways
Steroid biosynthesis	sasa00100	Metabolism/Lipid metabolism	sasa00120 Primary bile acid biosynthesis
			sasa00140 Steroid hormone biosynthesis
			sasa00900 Terpenoid backbone biosynthesis
Terpenoid backbone biosynthesis	sasa00900	Metabolism/Metabolism of terpenoids and polyketides	sasa00010 Glycolysis / Gluconeogenesis
			sasa00100 Steroid biosynthesis
			sasa00130 Ubiquinone and other terpenoid-quinone biosynthesis
			sasa00510 N-Glycan biosynthesis
PPAR signaling pathway	sasa03320	Organismal Systems/Endocrine system	sasa00071 Fatty acid degradation
			sasa00072 Synthesis and degradation of ketone bodies
			sasa00120 Primary bile acid biosynthesis
			sasa00564 Glycerophospholipid metabolism
			sasa04920 Adipocytokine signaling pathway
Fatty acid metabolism	sasa01212	Metabolism/Global and overview maps	sasa00020 Citrate cycle (TCA cycle)
Carbon metabolism	sasa01200	Metabolism/Global and overview maps	-
Pyruvate metabolism	sasa00620	Metabolism/Carbohydrate metabolism	sasa00010 Glycolysis / Gluconeogenesis
			sasa00020 Citrate cycle (TCA cycle)
			sasa00061 Fatty acid biosynthesis
			sasa00072 Synthesis and degradation of ketone bodies
			sasa00260 Glycine, serine and threonine metabolism
			sasa00290 Valine, leucine and isoleucine biosynthesis
			sasa00630 Glyoxylate and dicarboxylate metabolism
			sasa00640 Propanoate metabolism
			sasa00650 Butanoate metabolism
			sasa00760 Nicotinate and nicotinamide metabolism

Supplementary Table 10. Enriched GO terms for DEGs (adjusted p-value < 0.01, gene count > 4).

Dataset	Domain[a]	# Terms	Enriched terms[b]
L2:L1	BP	1	lipid biosynthetic process (GO:0008610)
	MF	2	monooxygenase activity (GO:0004497), oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (GO:0016705)
L3:L1	CC	4	endoplasmic reticulum membrane (GO:0005789), endoplasmic reticulum subcompartment (GO:0098827), nuclear outer membrane-endoplasmic reticulum membrane network (GO:0042175), endoplasmic reticulum part (GO:0044432)
	BP	39	steroid metabolic process (GO:0008202), lipid biosynthetic process (GO:0008610), sterol metabolic process (GO:0016125), steroid biosynthetic process (GO:0006694), sterol biosynthetic process (GO:0016126), organic hydroxy compound metabolic process (GO:1901615), isoprenoid biosynthetic process (GO:0008299), secondary alcohol metabolic process (GO:1902652), isoprenoid metabolic process (GO:0006720), organic hydroxy compound biosynthetic process (GO:1901617), cholesterol metabolic process (GO:0008203), cellular lipid metabolic process (GO:0044255), small molecule biosynthetic process (GO:0044283), alcohol metabolic process (GO:0006066), acetyl-CoA metabolic process (GO:0006084), coenzyme metabolic process (GO:0006732), monocarboxylic acid metabolic process (GO:0032787), nucleoside bisphosphate metabolic process (GO:0033865), ribonucleoside bisphosphate metabolic process (GO:0033875), purine nucleoside bisphosphate metabolic process (GO:0034032), cofactor metabolic process (GO:0051186), alcohol biosynthetic process (GO:0046165), nucleobase-containing small molecule metabolic process (GO:0055086), organic acid biosynthetic process (GO:0016053), carboxylic acid biosynthetic process (GO:0046394), nucleotide metabolic process (GO:0009117), nucleoside phosphate metabolic process (GO:0006753), fatty acid metabolic process (GO:0006631), acyl-CoA metabolic process (GO:0006637), thioester metabolic process (GO:0035383), phospholipid biosynthetic process (GO:0008654), monocarboxylic acid biosynthetic process (GO:0072330), organophosphate biosynthetic process (GO:0090407), fatty acid biosynthetic process (GO:0006633), ribose phosphate metabolic process (GO:0019693), pyridine nucleotide metabolic process (GO:0019362), nicotinamide nucleotide metabolic process (GO:0046496), pyridine-containing compound metabolic process (GO:0072524), oxidoreduction coenzyme metabolic process (GO:0006733)
	MF	9	coenzyme binding (GO:0050662), monooxygenase activity (GO:0004497), oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor (GO:0016616), oxidoreductase activity, acting on CH-OH group of donors (GO:0016614), oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (GO:0016705), iron ion binding (GO:0005506), flavin adenine dinucleotide binding (GO:0050660), heme binding (GO:0020037), tetrapyrrole binding (GO:0046906)

[a]CC (cell component), BP (biological process), MF (molecular function). [b]Enriched terms are sorted by adjusted p-values with the most significant one listed first.

Supplementary Table 11. Enriched pathways for DEGs by GSEA (adjusted p-value < 0.01).

Dataset	Down/Up	# Pathways	Enriched pathways
L2:L1	Down	23	Steroid biosynthesis (sasa00100), Terpenoid backbone biosynthesis (sasa00900), Glycine, serine and threonine metabolism (sasa00260), Aminoacyl-tRNA biosynthesis (sasa00970), Cysteine and methionine metabolism (sasa00270), Butanoate metabolism (sasa00650), Synthesis and degradation of ketone bodies (sasa00072), Ribosome biogenesis in eukaryotes (sasa03008), Arginine and proline metabolism (sasa00330), Alanine, aspartate and glutamate metabolism (sasa00250), Biosynthesis of amino acids (sasa01230), Valine, leucine and isoleucine degradation (sasa00280), Selenocompound metabolism (sasa00450), Fatty acid metabolism (sasa01212), Fatty acid degradation (sasa00071), Glutathione metabolism (sasa00480), Carbon metabolism (sasa01200), PPAR signaling pathway (sasa03320), Insulin signaling pathway (sasa04910), p53 signaling pathway (sasa04115), Peroxisome (sasa04146), Wnt signaling pathway (sasa04310), Lysosome (sasa04142)
	Up	7	Intestinal immune network for IgA production (sasa04672), Cytokine-cytokine receptor interaction (sasa04060), Ribosome (sasa03010), AGE-RAGE signaling pathway in diabetic complications (sasa04933), NOD-like receptor signaling pathway (sasa04621), C-type lectin receptor signaling pathway (sasa04625), Cell adhesion molecules (CAMs) (sasa04514)
L3:L1	Down	39	Steroid biosynthesis (sasa00100), DNA replication (sasa03030), Terpenoid backbone biosynthesis (sasa00900), Mismatch repair (sasa03430), Aminoacyl-tRNA biosynthesis (sasa00970), Ribosome biogenesis in eukaryotes (sasa03008), Fatty acid metabolism (sasa01212), Ribosome (sasa03010), Base excision repair (sasa03410), Oxidative phosphorylation (sasa00190), Biosynthesis of unsaturated fatty acids (sasa01040), Glyoxylate and dicarboxylate metabolism (sasa00630), Propanoate metabolism (sasa00640), Proteasome (sasa03050), Carbon metabolism (sasa01200), Fatty acid biosynthesis (sasa00061), Glutathione metabolism (sasa00480), Homologous recombination (sasa03440), alpha-Linolenic acid metabolism (sasa00592), Nucleotide excision repair (sasa03420), Butanoate metabolism (sasa00650), Pyruvate metabolism (sasa00620), Fanconi anemia pathway (sasa03460), Valine, leucine and isoleucine degradation (sasa00280), Citrate cycle (TCA cycle) (sasa00020), Synthesis and degradation of ketone bodies (sasa00072), Spliceosome (sasa03040), Glycine, serine and threonine metabolism (sasa00260), Fatty acid elongation (sasa00062), RNA transport (sasa03013), Arachidonic acid metabolism (sasa00590), Cell cycle (sasa04110), Arginine and proline metabolism (sasa00330), Fatty acid degradation (sasa00071), Biosynthesis of amino acids (sasa01230), Alanine, aspartate and glutamate metabolism (sasa00250), RNA polymerase (sasa03020), Cysteine and methionine metabolism (sasa00270), RNA degradation (sasa03018)
	Up	10	ECM-receptor interaction (sasa04512), Cell adhesion molecules (CAMs) (sasa04514), Cytokine-cytokine receptor interaction (sasa04060), Adherens junction (sasa04520), Intestinal immune network for IgA production (sasa04672), AGE-RAGE signaling pathway in diabetic complications (sasa04933), Phosphatidylinositol signaling system (sasa04070), Inositol phosphate metabolism (sasa00562), Vascular smooth muscle contraction (sasa04270), ErbB signaling pathway (sasa04012)

[a]Down (down-regulated) or Up (up-regulated) pathways. [b]Enriched pathways are sorted by adjusted p-values with the most significant one listed first.

Supplementary Table 12. GSEA enriched pathways common in both L2:L1 and L3:L1.

Enriched pathways	KEGG ID	NES (L2:L1)[a]	NES (L3:L1)[a]
Steroid biosynthesis	sasa00100	-2.53	-2.76
Terpenoid backbone biosynthesis	sasa00900	-2.31	-2.47
Glycine, serine and threonine metabolism	sasa00260	-2.24	-1.71
Aminoacyl-tRNA biosynthesis	sasa00970	-2.17	-2.24
Cysteine and methionine metabolism	sasa00270	-2.12	-1.53
Butanoate metabolism	sasa00650	-1.94	-1.87
Synthesis and degradation of ketone bodies	sasa00072	-1.85	-1.75
Ribosome biogenesis in eukaryotes	sasa03008	-1.82	-2.24
Arginine and proline metabolism	sasa00330	-1.81	-1.61
Alanine, aspartate and glutamate metabolism	sasa00250	-1.79	-1.55
Biosynthesis of amino acids	sasa01230	-1.75	-1.56
Valine, leucine and isoleucine degradation	sasa00280	-1.74	-1.79
Fatty acid metabolism	sasa01212	-1.72	-2.20
Fatty acid degradation	sasa00071	-1.65	-1.57
Glutathione metabolism	sasa00480	-1.61	-1.94
Carbon metabolism	sasa01200	-1.59	-1.97
Cell adhesion molecules (CAMs)	sasa04514	1.37	1.66
AGE-RAGE signaling pathway in diabetic complications	sasa04933	1.45	1.48
Ribosome	sasa03010	1.57	-2.19
Cytokine-cytokine receptor interaction	sasa04060	1.64	1.66
Intestinal immune network for IgA production	sasa04672	1.75	1.51

[a]Negative NES (normalized enrichment score) values indicate down-regulation, whereas positive NES values indicate up-regulation.

Supplementary Table 13. Read alignment of 21 RRBS samples on the Salmon genome.

No	Name	Diet	Sex	Total reads	% Aligned[a]	# Aligned Cs[b]	% CpGs[c]	Methylation rate[d]
1	L1	L2	M	64 938 299	47.67	326 750 266	21.05	84.77
2	L6	L3	M	61 742 432	46.87	307 091 088	20.93	84.51
3	L7	L3	F	52 579 647	48.64	272 484 899	20.92	84.6
4	L11	L1	M	68 986 028	48.76	353 798 160	20.89	83.85
5	L12	L1	F	73 106 174	47.7	369 892 480	21.01	84.69
6	L13	L1	F	65 180 703	47.26	325 973 342	20.91	84.25
7	L14	L1	F	42 920 963	47.91	213 558 541	20.9	83.73
8	L16	L2	F	68 898 332	46.74	345 631 982	20.88	84.28
9	L18	L2	M	70 070 490	47.67	353 591 304	20.87	84.34
10	L20	L2	F	52 527 471	48.55	268 129 597	20.86	83.57
11	L22	L1	M	53 584 633	45.99	262 714 857	20.86	84.68
12	L23	L1	M	93 411 879	48.67	476 236 067	21.09	85.12
13	L24	L1	M	49 281 151	47.74	247 448 574	21	83.79
14	L26	L3	M	55 156 176	47.16	276 699 136	20.94	84.07
15	L28	L3	F	70 063 826	47.9	349 040 205	21.13	84.73
16	L30	L3	F	74 423 657	47.36	371 927 481	20.97	84.37
17	L32	L2	M	58 784 680	48.12	297 408 787	21.02	84.51
18	L33	L2	F	56 337 641	46.95	279 726 633	20.95	84.81
19	L38	L3	F	58 371 657	47.76	293 090 760	20.96	84.5
20	L42	L1	F	61 552 817	47.62	312 335 351	20.84	84.58
21	L43	L1	F	46 490 239	47.69	236 010 085	20.71	83.43

[a]Percentage of uniquely aligned reads. [b]Number of Cs among the uniquely aligned regions. [c]Percentage of CpG sites among the aligned Cs. [d]Average methylation rate calculated from all mapped CpG sites

Supplementary Table 14. Distributions of the mapped CpGs in and around mRNAs.

Region	# Nucleotides[a]	(%)	# Mapped CpGs	(%)	Odds ratio[b]	P-value[c]
Exon	128 738 024	(2.2%)	26 899	(11.6%)	5.3	0
Intron	999 945 268	(16.9%)	92 681	(39.9%)	2.4	0
P250	11 628 692	(0.2%)	1 338	(0.6%)	2.9	5.8E-246
P1K	32 875 941	(1%)	2 260	(1%)	1.8	5.8E-133
P6K	187 371 613	(3.2%)	13 805	(5.9%)	1.9	0
Flanks	412 209 951	(7%)	23 199	(10.0%)	1.4	0
Intergenic	4 161 010 917	(70.1%)	72 348	(31.1%)	0.4	0

[a]Number of the nucleotides found in the whole genome. [b]Ratio calculated as A/B where A = (# nucleotides in a region) / (total # of nucleotides in all regions) and B = (# mapped CpGs of in a region) / (total # of mapped CpGs in all regions). [c]Calculated by hypergeometric test on odds ratio.

Supplementary Table 15. Distributions of DMCs in mRNAs and their surroundings.

Dataset	Region	# Mapped CpGs	(%)	# DMCs	(%)	Odds ratio[a]	P-value[b]	Sig[c]
L2:L1	Exon	32 424	(11.8%)	301	(8.0%)	0.68	2.0E-14	*
	Intron	108 703	(39.6%)	1 446	(38.6%)	0.97	1.1E-01	
	P250	1 625	(0.6%)	17	(0.5%)	0.77	1.6E-01	
	P1K	2 616	(1.0%)	62	(1.7%)	1.74	3.3E-05	**
	P6K	16 262	(5.9%)	276	(7.4%)	1.24	1.5E-04	**
	Flanks	27 576	(10.0%)	419	(11.2%)	1.11	1.1E-02	**
	Intergenic	85 359	(31.1%)	1 226	(32.7%)	1.05	1.6E-02	*
L3:L1	Exon	32 649	(11.7%)	338	(9.1%)	0.77	7.5E-08	*
	Intron	110 395	(39.6%)	1 401	(37.5%)	0.95	4.1E-03	**
	P250	1 652	(0.6%)	19	(0.5%)	0.86	2.9E-01	
	P1K	2 676	(1.0%)	68	(1.8%)	1.90	8.2E-07	**
	P6K	16 483	(5.9%)	265	(7.1%)	1.20	1.5E-03	**
	Flanks	27 923	(10.0%)	464	(12.4%)	1.24	1.0E-06	**
	Intergenic	86 796	(31.2%)	1 179	(31.6%)	1.01	3.0E-01	

[a]Ratio calculated as A/B where A = (# mapped CpGs of in region) / (total # of mapped CpGs in all regions) and B = (# DMCs in a region / (total # of DMCs in all regions). [b]Calculated by hypergeometric test on odds ratio of >1. [c]Statistically significant as * and ** indicating p-values < 0.05 and 0.01, respectively.

Supplementary Table 16. Enriched GO terms for DMGs in L2:L1 (adjusted p-value < 0.001, gene count > 9).

Domain[a]	GO ID	GO term	Region (gene ratio)
CC	GO:0044456	synapse part	Gene body (25/734), Intron (23/613), RS+GB (29/1031)
	GO:0045202	synapse	P+GB (32/880), Gene body (31/734), Intron (28/613), RS+GB (35/1031)
BP	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	Gene body (27/527)
	GO:0007268	chemical synaptic transmission	P+GB (20/628), Gene body (18/527)
	GO:0007399	nervous system development	P+GB (42/628), Gene body (37/527), Intron (31/423), RS+GB (46/741)
	GO:0032879	regulation of localization	P+GB (38/628), Gene body (33/527)
	GO:0051049	regulation of transport	P+GB (33/628), Gene body (29/527)
	GO:0098609	cell-cell adhesion	Gene body (31/527)
	GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	Gene body (28/527)
	GO:0098916	anterograde trans-synaptic signaling	P+GB (20/628), Gene body (18/527)
	GO:0099536	synaptic signaling	P+GB (20/628), Gene body (18/527)
	GO:0099537	trans-synaptic signaling	P+GB (20/628), Gene body (18/527)
MF	GO:0004970	ionotropic glutamate receptor activity	P+GB (13/1062), RS+GB (15/1245)
	GO:0005230	extracellular ligand-gated ion channel activity	RS+GB (26/1245)
	GO:0008066	glutamate receptor activity	P+GB (13/1062), RS+GB (15/1245)
	GO:0008146	sulfotransferase activity	RS+GB (18/1245)
	GO:0015276	ligand-gated ion channel activity	P+GB (30/1062), RS+GB (34/1245)
	GO:0022834	ligand-gated channel activity	P+GB (30/1062), RS+GB (34/1245)

[a]CC (cell component), BP (biological process), MF (molecular function).

Supplementary Table 17. Enriched GO terms for DMGs in L3:L1 (adjusted p-value < 0.001, gene count > 9).

Domain[a]	GO ID	GO term	Region (gene ratio)
CC	GO:0044456	synapse part	P+GB (26/854), Gene body (26/720), Intron (26/606)
	GO:0045202	synapse	P+GB (31/854), Gene body (31/720), Intron (31/606), RS+GB (32/1009)
	GO:0045211	postsynaptic membrane	P+GB (17/854), Gene body (17/720), Intron (17/606), RS+GB (18/1009)
	GO:0097060	synaptic membrane	P+GB (17/854), Gene body (17/720), Intron (17/606), RS+GB (18/1009)
	GO:0098590	plasma membrane region	P+GB (21/854), Gene body (21/720), Intron (20/606), RS+GB (23/1009)
	GO:0098794	postsynapse	Gene body (17/720), Intron (17/606)
BP	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	P+GB (33/627), Gene body (32/513), Intron (26/417)
	GO:0007267	cell-cell signaling	P+GB (34/627), Intron (27/417)
	GO:0007268	chemical synaptic transmission	P+GB (21/627), Gene body (19/513), Intron (18/417)
	GO:0098609	cell-cell adhesion	P+GB (36/627), Gene body (35/513), Intron (29/417)
	GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	P+GB (33/627), Gene body (32/513), Intron (26/417)
	GO:0098916	anterograde trans-synaptic signaling	P+GB (21/627), Gene body (19/513), Intron (18/417)
	GO:0099536	synaptic signaling	P+GB (21/627), Gene body (19/513), Intron (18/417)
	GO:0099537	trans-synaptic signaling	P+GB (21/627), Gene body (19/513), Intron (18/417)
MF	GO:0004970	ionotropic glutamate receptor activity	P+GB (16/1016), Gene body (16/847), Intron (16/703), RS+GB (17/1228)
	GO:0005230	extracellular ligand-gated ion channel activity	Intron (20/703)
	GO:0008066	glutamate receptor activity	P+GB (16/1016), Gene body (16/847), Intron (16/703), RS+GB (17/1228)
	GO:0015276	ligand-gated ion channel activity	Intron (23/703)
	GO:0022824	transmitter-gated ion channel activity	P+GB (18/1016), Gene body (18/847), Intron (18/703), RS+GB (19/1228)
	GO:0022834	ligand-gated channel activity	Intron (23/703)
	GO:0022835	transmitter-gated channel activity	P+GB (18/1016), Gene body (18/847), Intron (18/703), RS+GB (19/1228)
	GO:0030594	neurotransmitter receptor activity	P+GB (26/1016), Gene body (25/847), Intron (25/703), RS+GB (27/1228)

[a]CC (cell component), BP (biological process), MF (molecular function).

Supplementary Table 18. The number of common DMCs and DMGs between L2:L1 and L3:L1.

Region	# of common DMCs	# of common DMGs
Exon	43	38
Intron	194	176
P1K	9	9
P250	2	2
P6K	43	33
Flanks	70	61
Total	361	319

Supplementary Table 19. DMGs with at least two common DMCs between L2:L1 and L3:L1.

Gene ID	Symbol	Region	Gene name	# DMCs	Hypo/Hyper		Matched
					L2:L1	L3:L1	
106574316	LOC106574316	P6K	T-cell-specific surface glycoprotein CD28-like	5	0/5	0/5	Yes
106578606	LOC106578606	Exon	homeobox protein engrailed-2a-like	5	5/0	5/0	Yes
106590111	LOC106590111	Intron	disks large-associated protein 1-like	4	0/4	0/4	Yes
106560380	LOC106560380	Intron	rho GTPase-activating protein 39-like	3	1/2	1/2	Yes
106579972	LOC106579972	Flanks	calcium-binding mitochondrial carrier protein SCaMC-2-A-like	3	3/0	3/0	Yes
106586910	LOC106586910	Flanks	zinc finger CCCH domain-containing protein 15-like	3	3/0	3/0	Yes
100380475	nt5d1	P6K	5-nucleotidase domain-containing protein 1	2	0/2	0/2	Yes
106561885	kif6	Intron	kinesin family member 6	2	0/2	0/2	Yes
106562128	LOC106562128	P6K	meiotic recombination protein REC114-like	2	0/2	0/2	Yes
106562942	ankra2	P6K	ankyrin repeat family A member 2	2	0/2	0/2	Yes
106563039	LOC106563039	Flanks	homeobox protein orthopedia B-like	2	2/0	2/0	Yes
106563583	LOC106563583	Intron	MAP7 domain-containing protein 2-like	2	0/2	0/2	Yes
106566691	LOC106566691	Flanks	vesicle-associated membrane protein-associated protein B-like	2	0/2	0/2	Yes
106568947	LOC106568947	Exon	cAMP-specific 3',5'-cyclic phosphodiesterase 4D-like	2	0/2	0/2	Yes
106570019	LOC106570019	P6K	zinc finger MYM-type protein 4-like	2	2/0	2/0	Yes
106576329	LOC106576329	Intron	liprin-alpha-2-like	2	2/0	2/0	Yes
106576938	LOC106576938	Intron	potassium channel subfamily K member 18-like	2	0/2	0/2	Yes
106580649	LOC106580649	Flanks	60S ribosomal protein L35a	2	0/2	0/2	Yes
106580806	LOC106580806	Intron	limbic system-associated membrane protein-like	2	0/2	0/2	Yes
106580848	LOC106580848	Intron	sodium/calcium exchanger 2-like	2	2/0	2/0	Yes
106582848	LOC106582848	Intron	contactin-3-like	2	0/2	0/2	Yes
106585555	LOC106585555	Flanks	glial fibrillary acidic protein-like	2	0/2	0/2	Yes
106598174	grk7	P6K	G protein-coupled receptor kinase 7	2	0/2	0/2	Yes
106602730	map9	Flanks	microtubule associated protein 9	2	0/2	0/2	Yes
106603642	LOC106603642	Intron	cell adhesion molecule 1-like	2	2/0	2/0	Yes
106607780	LOC106607780	Intron	protein FAM184A-like	2	0/2	0/2	Yes
106607986	LOC106607986	Intron	uronyl 2-sulfotransferase-like	2	0/2	2/0	No
106608148	LOC106608148	P6K	disks large-associated protein 2-like	2	0/2	0/2	Yes
106610332	LOC106610332	Intron	hydroperoxide isomerase ALOXE3-like	2	0/2	0/2	Yes
106611463	pacs2	Intron	phosphofurin acidic cluster sorting protein 2	2	0/2	0/2	Yes
106613191	LOC106613191	Intron	roundabout homolog 2-like	2	2/0	2/0	Yes

Supplementary Table 20. Associated GO terms for common DMGs between L2:L1 and L3:L1.

Gene name	Signaling related†	Synapse related†	Immune response related†	Others†
T-cell-specific surface glycoprotein CD28-like	cytokine biosynthetic process, apoptotic signaling pathway, cell surface receptor signaling pathway, positive regulation of phosphatidylinositol 3-kinase signaling, positive regulation of protein kinase B signaling, T cell receptor signaling pathway	-	humoral immune response, immune system process, positive regulation of inflammatory response to antigenic stimulus, positive regulation of interleukin-10 production, positive regulation of interleukin-2 biosynthetic process, positive regulation of interleukin-4 production, positive regulation of isotype switching to IgG isotypes, positive regulation of viral genome replication, regulation of defense response to virus by virus	negative regulation of apoptotic process, negative regulation of gene expression, negative thymic T cell selection, positive regulation of alpha-beta T cell proliferation, positive regulation of gene expression, positive regulation of mitotic nuclear division, positive regulation of T cell proliferation, positive regulation of transcription by RNA polymerase II, positive regulation of translation, regulation of regulatory T cell differentiation, regulatory T cell differentiation, T cell activation, T cell costimulation
homeobox protein engrailed-2a-like	-	-	axonogenesis involved in innervation, neuron differentiation	anterior/posterior pattern specification, apoptotic process involved in morphogenesis, cell fate specification, midbrain development, midbrain-hindbrain boundary development, midbrain-hindbrain boundary morphogenesis, regulation of transcription by RNA polymerase II
disks large-associated protein 1-like	-	chemical synaptic transmission	-	-
rho GTPase-activating protein 39-like	regulation of small GTPase mediated signal transduction, signal transduction	postsynapse organization	-	-
calcium-binding mitochondrial carrier protein SCaMC-2-A-like	calcium ion transmembrane transport	-	-	adipose tissue development, ATP metabolic process, camera-type eye development, cellular respiration, multicellular organism growth, response to activity, response to dietary excess, response to food Source
zinc finger CCCH domain-containing protein 15-like	cytokine-mediated signaling pathway	-	-	cytoplasmic translation, positive regulation of GTPase activity

†GO terms of corresponding human orthologues were retrieved from UniProt (https://www.uniprot.org) and manually separated mainly by their name into four categories, signalling related, synapse related, immune response related, and others.

Supplementary Table 21. List of CpGs with methylation calls of the *acaca* gene common in L2:L1 and L3:L1.

Chromosome	Start	End	Strand	Region	L2:L1			L3:L1		
					Q-value	Mdiff[a]	Sig[b]	Q-value	Mdiff[a]	Sig[b]
NC_027312.1	83593310	83593310	+	Exon	1.06E-05	-3.02	*	3.70E-01	-0.09	
NC_027312.1	83593311	83593311	-	Exon	7.05E-05	6.15	*	2.24E-03	4.58	*
NC_027312.1	83593349	83593349	+	Exon	5.81E-02	1.71		4.36E-01	0.12	
NC_027312.1	83623210	83623210	+	Intron	7.42E-04	-2.41	*	8.35E-06	-4.81	*
NC_027312.1	83623218	83623218	+	Intron	3.44E-01	0.65		2.79E-01	1.09	
NC_027312.1	83623248	83623248	+	Intron	2.55E-06	-4.82	*	7.96E-05	-3.74	*
NC_027312.1	83623302	83623302	-	Exon	2.02E-02	1.86		4.03E-02	-2.88	
NC_027312.1	83652565	83652565	-	P250	1.26E-04	8.80	*	3.03E-02	4.51	
NC_027312.1	83652613	83652613	+	P250	1.56E-01	3.94		3.48E-01	1.43	
NC_027312.1	**83652614**	**83652614**	**-**	**P250**	**3.92E-17**	**19.83**	*	**1.15E-30**	**24.72**	**
NC_027312.1	83652620	83652620	+	P250	5.05E-04	-12.65	*	2.43E-03	-10.72	*
NC_027312.1	83652631	83652631	+	P250	1.27E-01	-3.98		1.69E-03	-10.53	*
NC_027312.1	83652654	83652654	+	P250	3.57E-01	-1.35		2.01E-01	3.29	
NC_027312.1	**83652756**	**83652756**	**-**	**P250**	**1.07E-02**	**10.26**		**8.45E-08**	**20.02**	**
NC_027312.1	**83652793**	**83652793**	**-**	**P1K**	**5.67E-07**	**18.87**	*	**6.88E-13**	**23.78**	**
NC_027312.1	**83652803**	**83652803**	**-**	**P1K**	**2.58E-05**	**17.54**	*	**4.02E-08**	**20.91**	**
NC_027312.1	83655043	83655043	-	P6K	1.92E-01	1.28		5.04E-03	-4.85	*
NC_027312.1	83655046	83655046	-	P6K	2.06E-03	1.59	*	4.03E-07	-6.47	*
NC_027312.1	83655054	83655054	-	P6K	1.30E-02	-2.94		4.36E-01	-0.12	
NC_027312.1	83655073	83655073	-	P6K	2.51E-01	0.72		4.08E-01	-0.23	
NC_027312.1	83655078	83655078	-	P6K	4.39E-01	0.05		1.09E-05	-4.80	*
NC_027312.1	83655395	83655395	-	P6K	3.18E-02	3.36		2.18E-01	-1.27	
NC_027312.1	83655424	83655424	-	P6K	1.68E-06	4.97	*	1.19E-10	4.97	*
NC_027312.1	83655440	83655440	-	P6K	1.46E-03	2.12	*	9.46E-04	-3.28	*
NC_027312.1	83655885	83655885	-	P6K	6.98E-02	2.88		1.15E-03	-9.11	*
NC_027312.1	83655890	83655890	-	P6K	4.47E-01	0.01		3.46E-01	-0.76	
NC_027312.1	83655915	83655915	-	P6K	1.47E-01	2.35		6.07E-02	-4.55	
NC_027312.1	83655930	83655930	-	P6K	1.64E-01	-1.56		9.67E-02	-2.33	
NC_027312.1	83656220	83656220	+	P6K	2.89E-01	1.27		1.22E-01	-3.14	

[a]Methylation difference in percentage. [a]Signicance level as "*" indicating q-value < 0.01 and "**" indicating q-value < 0.01 and methylation difference >20%.

Supplementary Table 22. Count comparisons of samples and DMCs between 9 and 21 samples.

Dataset	Diets	# Total[a]	# Treatment[b]	# Control[c]	# Total DMCs	# hypo DMCs	# hyper DMCs
9 samples	L2:L1	7	3	4	20 260	10 225	10 035
	L3:L1	6	2	4	33 099	19 162	13 937
21 samples	L2:L1	15	6	9	2 521	1 395	1 126
	L3:L1	15	6	9	2 555	1 311	1 244

[a]Number of samples. [b]Number of treatment (L2 or L3 diet) samples. [c]Number of control (L1 diet) samples.

Supplementary Table 23. Relationship of samples between RNA-seq and RRBS.

Diet	#Samples		Tank[a]	RNA-seq[b]	RRBS[b]
	RNA-seq	RRBS			
L1	6	9	16	**L11 (male)**	**L11 (male)**
					L12 (female)
					L13 (female)
					L14 (female)
			19	L21 (male)	
				L22 (male)	**L22 (male)**
				L23 (male)	**L23 (male)**
				L24 (male)	**L24 (male)**
			24	L41 (male)	
					L42 (female)
					L43 (female)
L2	6	6	13	**L1 (male)**	**L1 (male)**
			18		L16 (female)
				L18 (male)	**L18 (male)**
					L20 (female)
			21	L31 (male)	
				L32 (male)	**L32 (male)**
					L33 (female)
				L34 (male)	
				L35 (male)	
L3	6	6	15	**L6 (male)**	**L6 (male)**
					L7 (female)
				L9 (male)	
			20	**L26 (male)**	**L26 (male)**
				L27 (male)	
					L28 (female)
					L30 (female)
			23	L36 (male)	
					L38 (female)
				L40 (male)	

[a]Tank number used in our feeding trail. [b]Names of samples for RNA-seq and RRBS. Names of identical fish are emphasised in bold.

Supplementary Table 24. List of DEGs for L2:L1 and L3:L1.

File: Dataset_01_DEG.xlsx (provided in Excel format)

Sheets: L2L1, L3L1

Fields:

Gene ID	Entrez Gene ID
Base mean	Output of DESeq2
Log2 fold change	Output of DESeq2
P-value	Output of DESeq2
Adjusted p-value	Output of DESeq2
Gene symbol	Gene symbol
Gene name	Gene name

Supplementary Table 25. List of enriched KEGG pathways for DEGs.

File: Dataset_02_KEGG_DEG.xlsx (provided in Excel format)

Sheets: L2L1, L3L1

Fields:

KEGG ID	KEGG ID
Pathway	Enriched KEGG pathway
Gene ID	Entrez Gene ID
Log2 FC	Log2 fold change
Gene symbol	Gene symbol
Gene name	Gene name

Supplementary Table 26. List of CpGs with q-value < 0.01 for L2:L1.

File: Dataset_03_CpG_L2L1.xlsx (provided in Excel format)

Sheet: L1L2

Fields:

Chromosome	Salmon chromosome name
Start	1-base start
End	1-base end
Strand	+/-
Salmobase	Link to Salmobase genome browser
Region	Intron, Exon, P250, P1K, P6K, Flanks
Q value	Logistic regression and SLIM, < 0.01
Mdiff	Methylation difference
abs(Mdiff)	Absolute methylation difference
Gene ID	Entrez Gene ID
Gene symbol	Gene symbol
Gene name	Gene name
RefSeq id	RefSeq ID

Supplementary Table 27. List of CpGs with q-value < 0.01 for L3:L1.

File: Dataset_04_CpG_L3L1.xlsx (provided in Excel format)

Sheet: L3L1

Fields:

Chromosome	Salmon chromosome name
Start	1-base start
End	1-base end
Strand	+/-
Salmobase	Link to Salmobase genome browser
Region	Intron, Exon, P250, P1K, P6K, Flanks
Q value	Logistic regression and SLIM, < 0.01
Mdiff	Methylation difference
abs(Mdiff)	Absolute4 methylation difference
Gene ID	Entrez Gene ID
Gene symbol	Gene symbol
Gene name	Gene name
RefSeq id	RefSeq ID

Supplementary Table 28. List of DMGs for L2:L1 and L3:L1.

File: Dataset_05_DMG.xlsx (provided in Excel format)

Sheets: L2L1, L3L1

Fields:

Gene ID	Entrez Gene ID
Total	Total count of DMCs
GB	Total count of DMCs in gene body
Exon	Count of DMCs in exon
Intron	Count of DMCs in intron
P	Total count of DMCs in promoter
P250	Count of DMCs in promoter (1 - 250)
P1K	Count of DMCs in promoter (251 - 1K)
P6K	Count of DMCs in Promoter (1001 - 5K)
Gene symbol	Gene name
Gene description	Description of gene
RefSeq IDs	RefSeq IDs linked to the gene ID

Supplementary Table 29. List of enriched KEGG pathways with DMCs for L2:L1.

File: Dataset_06_KEGG_DMG_L2L1.xlsx (provided in Excel format)

Sheet: Enriched pathway

Fields:

Name	KEGG pathway
ID	KEGG ID
KEGG site	Link for KEGG pathway page
ORA (gene ratio)	Regions where enrichment is identified by over-representation analysis
Wilcox	Regions where enrichment is identified by Wilcoxon signed-rank test
KS boot	Regions where enrichment is identified by Kolmogorov-Smirnov + bootstrapping

Sheets: sasa04514, sasa03018, sasa00531, sasa04137, sasa04371, sasa00500

Fields:

Chromosome	Salmon chromosome name
Start	1-base start
End	1-base end
Strand	+/-
Salmobase	Link to Salmobase genome browser
Region	Intron, Exon, P250, P1K, P6K, Flanks
Q value	Logistic regression and SLIM, < 0.01
Mdiff	Methylation difference
abs(Mdiff)	Absolute4 methylation difference (>= 20%)
Gene ID	Entrez Gene ID
Gene symbol	Gene symbol
Gene name	Gene name
RefSeq id	RefSeq ID

Supplementary Table 30. List of enriched KEGG pathways with DMCs for L3:L1.

File: Dataset_07_KEGG_DMG_L3L1.xlsx (provided in Excel format)

Sheet: Enriched pathway

Fields:

Name	KEGG pathway
ID	KEGG ID
KEGG site	Link for KEGG pathway page
ORA (gene ratio)	Regions where enrichment is identified by over-representation analysis
Wilcox	Regions where enrichment is identified by Wilcoxon signed-rank test
KS boot	Regions where enrichment is identified by Kolmogorov-Smirnov + bootstrapping

Sheets: sasa04514, sasa03018, sasa00531, sasa04137, sasa04371, sasa00500

Fields:

Chromosome	Salmon chromosome name
Start	1-base start
End	1-base end
Strand	+/-
Salmobase	Link to Salmobase genome browser
Region	Intron, Exon, P250, P1K, P6K, Flanks
Q value	Logistic regression and SLIM, < 0.01
Mdiff	Methylation difference
abs(Mdiff)	Absolute4 methylation difference (>= 20%)
Gene ID	Entrez Gene ID
Gene symbol	Gene symbol
Gene name	Gene name
RefSeq id	RefSeq ID

Supplementary Table 31. List of common DEGs between L2:L1 and L3:L1.

File: Dataset_08_Common_DMG.xlsx (provided in Excel format)

Sheet: Common DEGs

Fields:

Gene ID	Entrez Gene ID
Symbol	Gene symbol
Gene name	Gene name
Region	Intron, Exon, P250, P1K, P6K, Flanks
# DMCs	Number of DMCs
L2:L1 (hypo/hyper)	Number of hypo and hyper DMCs for L2:L1
L3:L1 (hypo/hyper)	Number of hypo and hyper DMCs for L3:L1
Matched	Yes: The counts of hypo and hyper DMCs are equivalent between L2:L1 and L3:L1. No: the counts are different.

Supplementary Table 32. List of DEGs with DMCs.

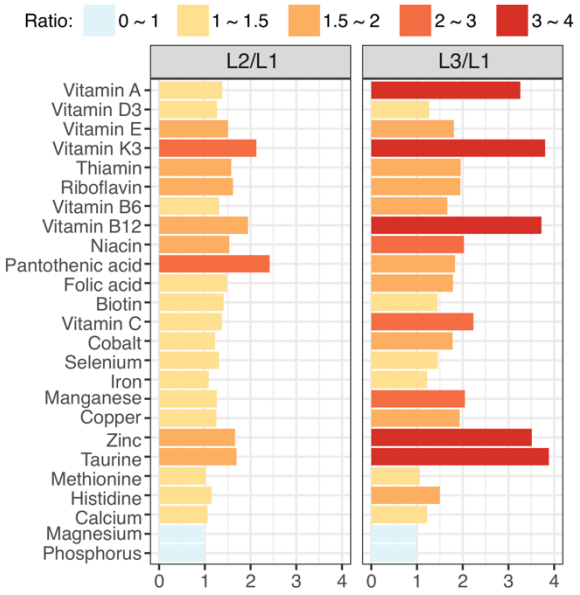
File: Dataset_09_DEG_DMC.xlsx (provided in Excel format)

Sheets: L2L1, L3L1, L2L1 (no mdiff filter), L3L1 (no mdiff filter), L2L1 (promoters), L3L1 (promoters)

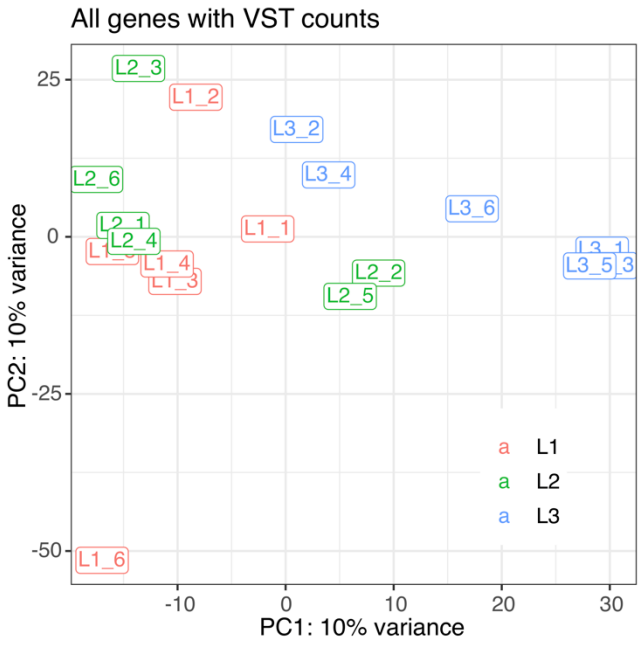
Fields:

Gene ID	Entrez Gene ID
Gene symbol	Gene symbol
Gene name	Gene name
Log2 FC	Output of DESeq2
Adj p-value	Output of DESeq2
Region	Intron, Exon, P250, P1K, P6K, Flanks
Mdiff	Methylation difference
Q value	Logistic regression and SLIM, < 0.01

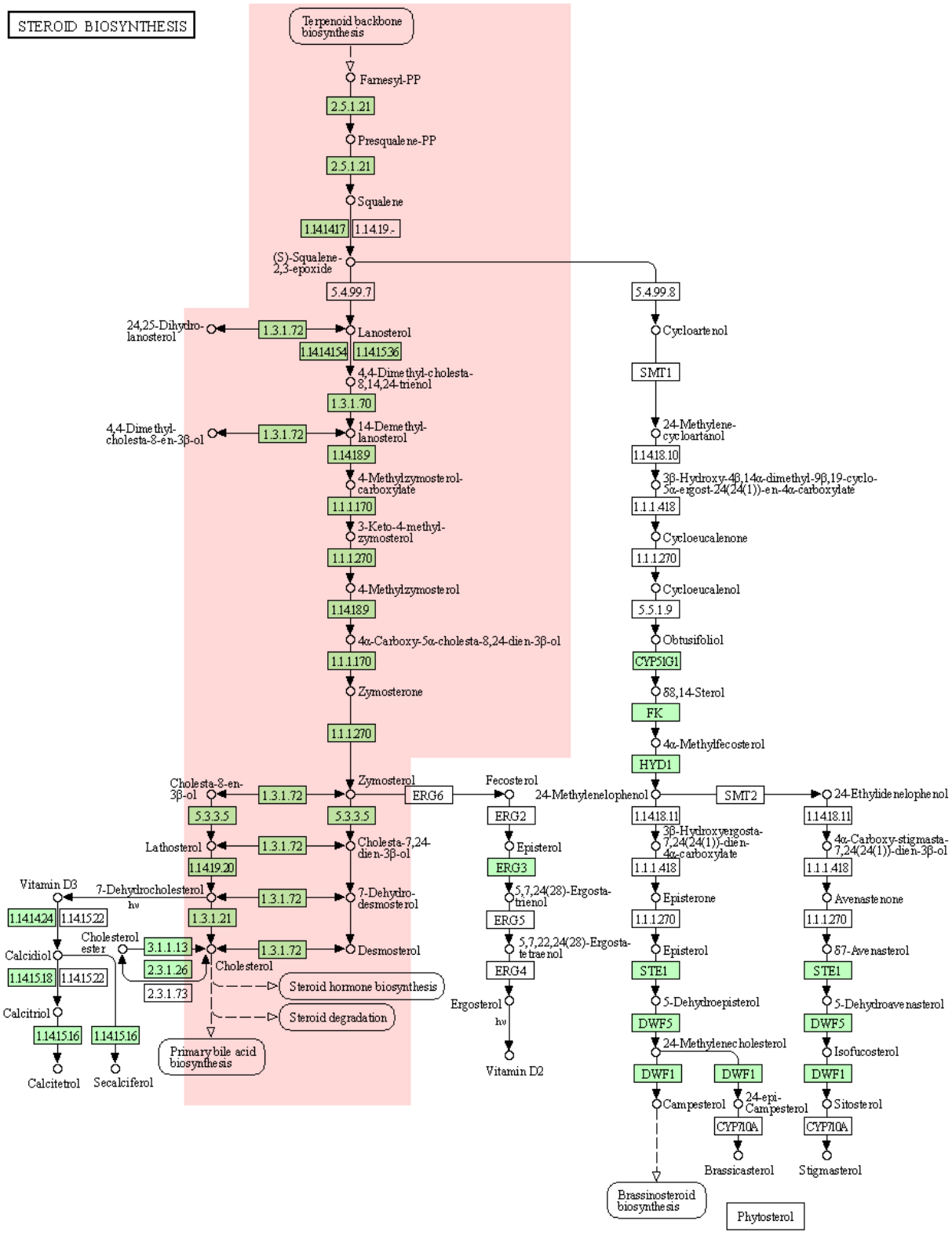
Supplementary figures



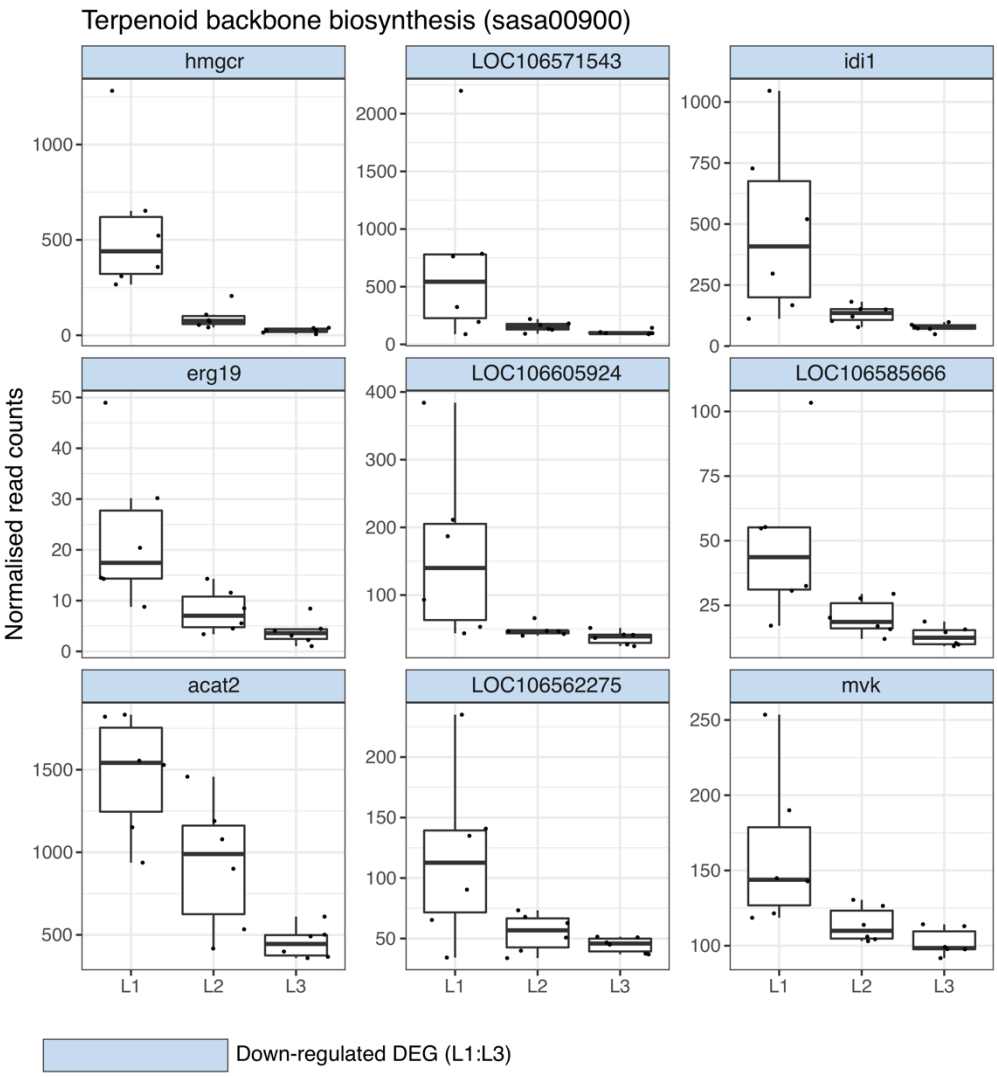
Supplementary Figure 1. Observed versus expected ratios of micronutrient concentrations for L2 and L3 based on L1.
The bar plot shows the ratios of L2 and L3 against L1 that were calculated with the analysed concentrations of selected vitamins, amino acids (taurine, methionine and histidine), and minerals (calcium, magnesium and phosphorous). The colours of the bars represent five different ranges of the ratio for L2/L1 and L3/L1.



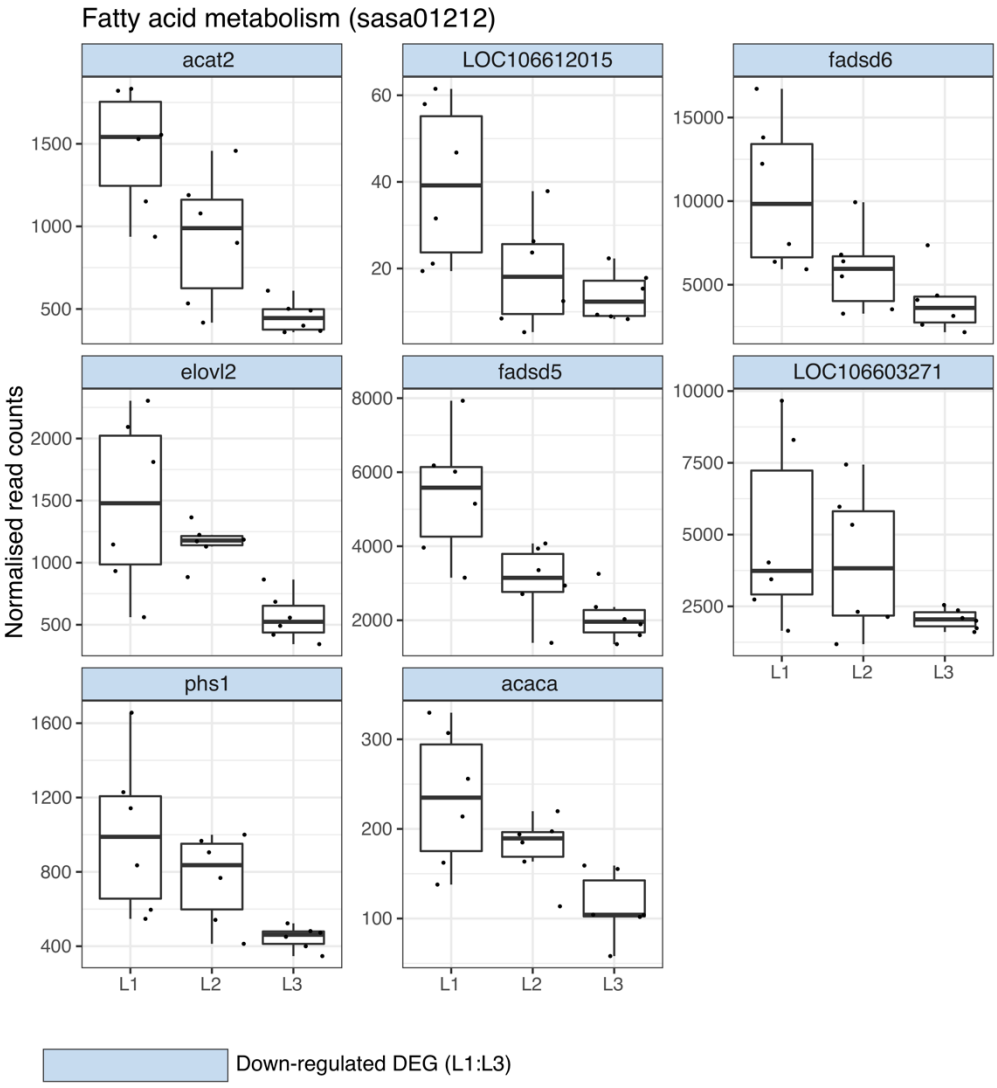
Supplementary Figure 2. Clustering of RNA-seq counts with all genes.
The PCA bi-plot shows all genes of RNA-seq counts with variance stabilization transformation. Samples are grouped with three different colours for L1 (red), L2 (green) and L3 (blue) diets.



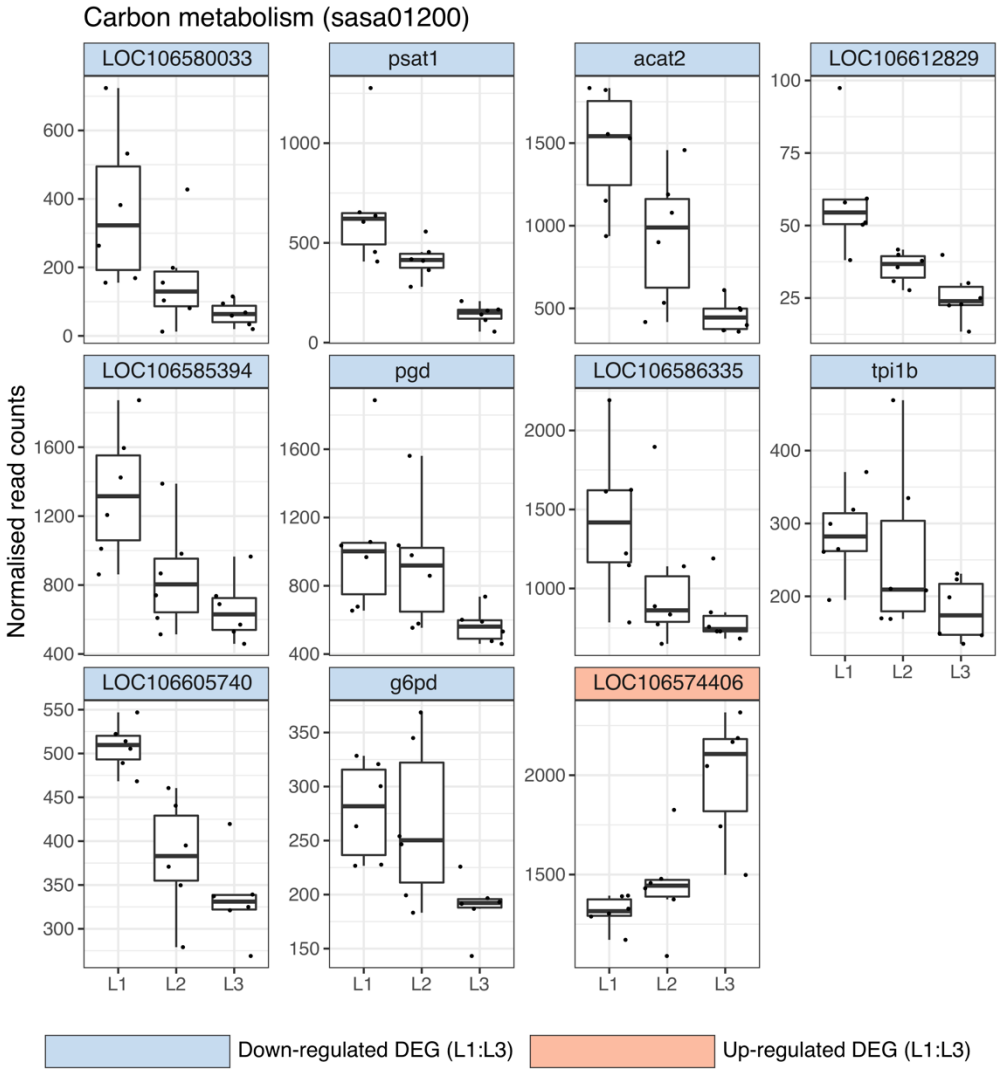
Supplementary Figure 3. Steroid biosynthesis pathway from KEGG.
The pathway diagram shows the KEGG steroid biosynthesis pathway (sasa00100) with a total of 59 genes. The part of the pathway highlighted by light red is used in the main text.



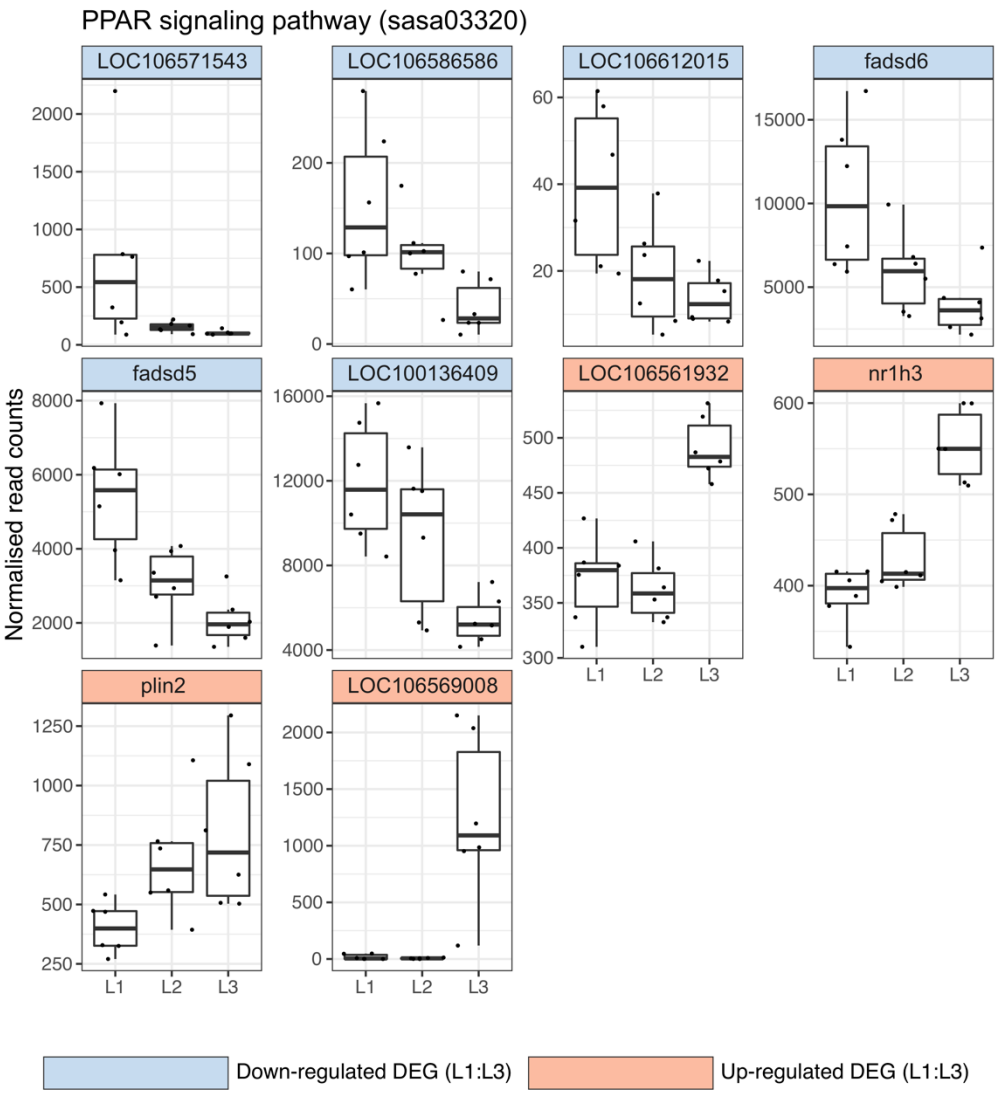
Supplementary Figure 4. Normalised read counts of terpenoid backbone biosynthesis.
Box plots show the normalised read counts of the nine down-regulated DEGs in L3:L1 that were associated with the terpenoid backbone biosynthesis pathway. The counts were normalised with the median ratio method provided by the DESeq2 package.



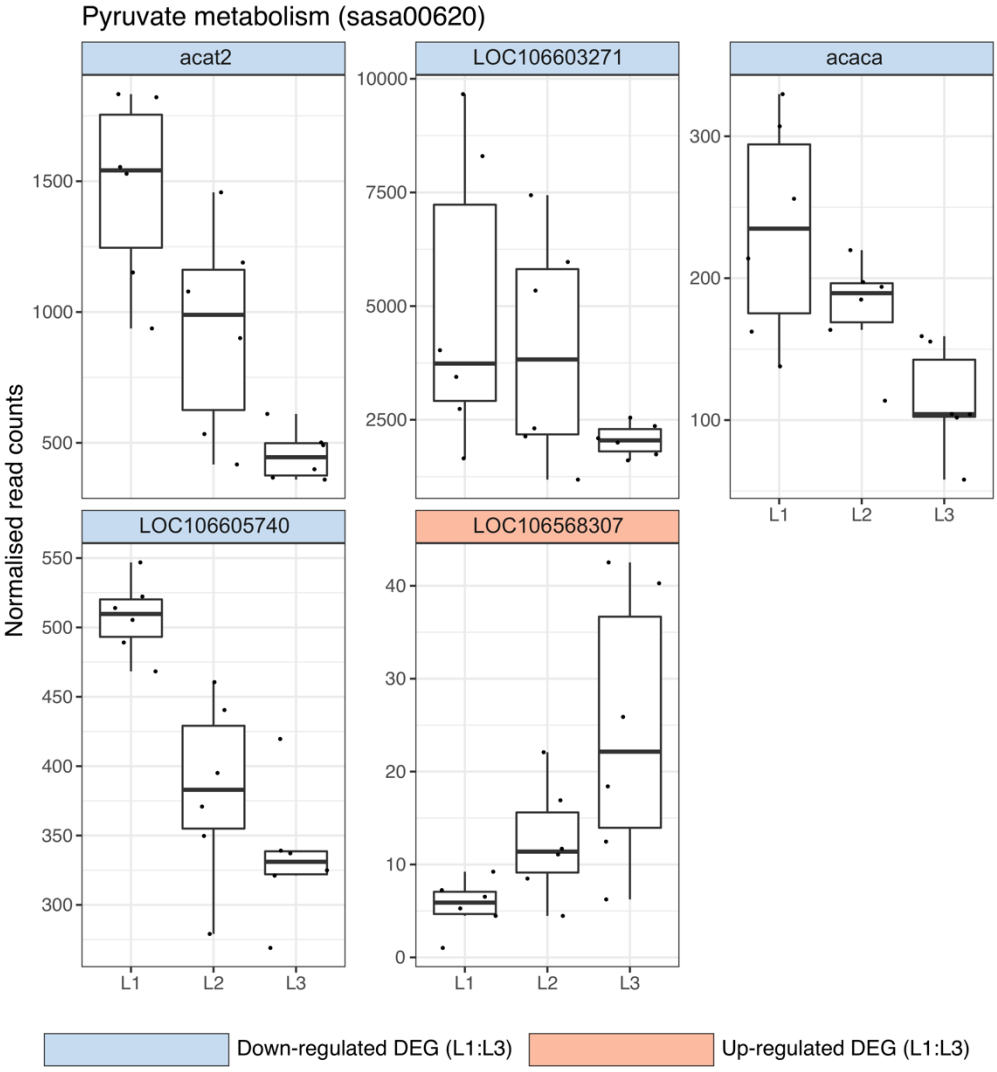
Supplementary Figure 5. Normalised read counts of fatty acid metabolism.
Box plots show the normalised read counts of the eight down-regulated DEGs in L3:L1 that were associated with the fatty acid metabolism pathway. The counts were normalised with the median ratio method provided by the DESeq2 package.



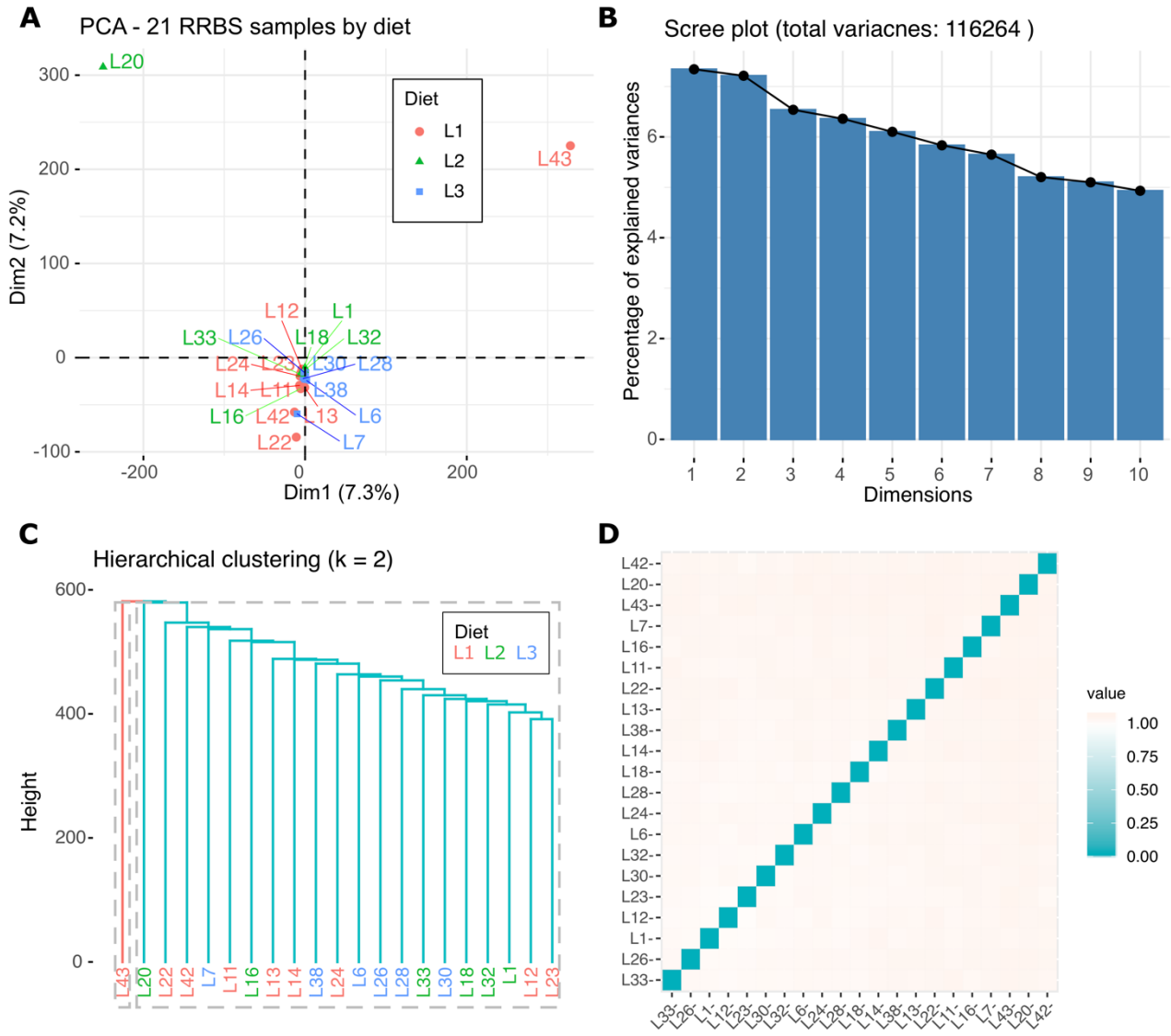
Supplementary Figure 6. Normalised read counts of carbon metabolism.
Box plots show the normalised read counts of 10 down-regulated and one up-regulated DEGs in L3:L1 that were associated with the fatty acid metabolism pathway. The counts were normalised with the median ratio method provided by the DESeq2 package.



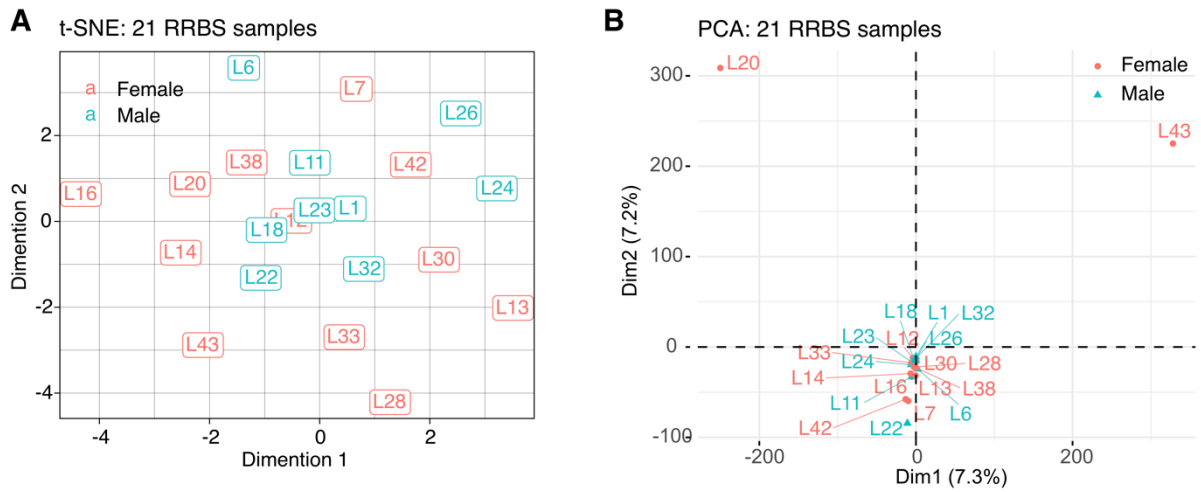
Supplementary Figure 7. Normalised read counts of PPAR signaling pathway.
Box plots show the normalised read counts of six down-regulated and four up-regulated DEGs in L3:L1 that were associated with the PPAR signaling pathway. The counts were normalised with the median ratio method provided by the DESeq2 package.



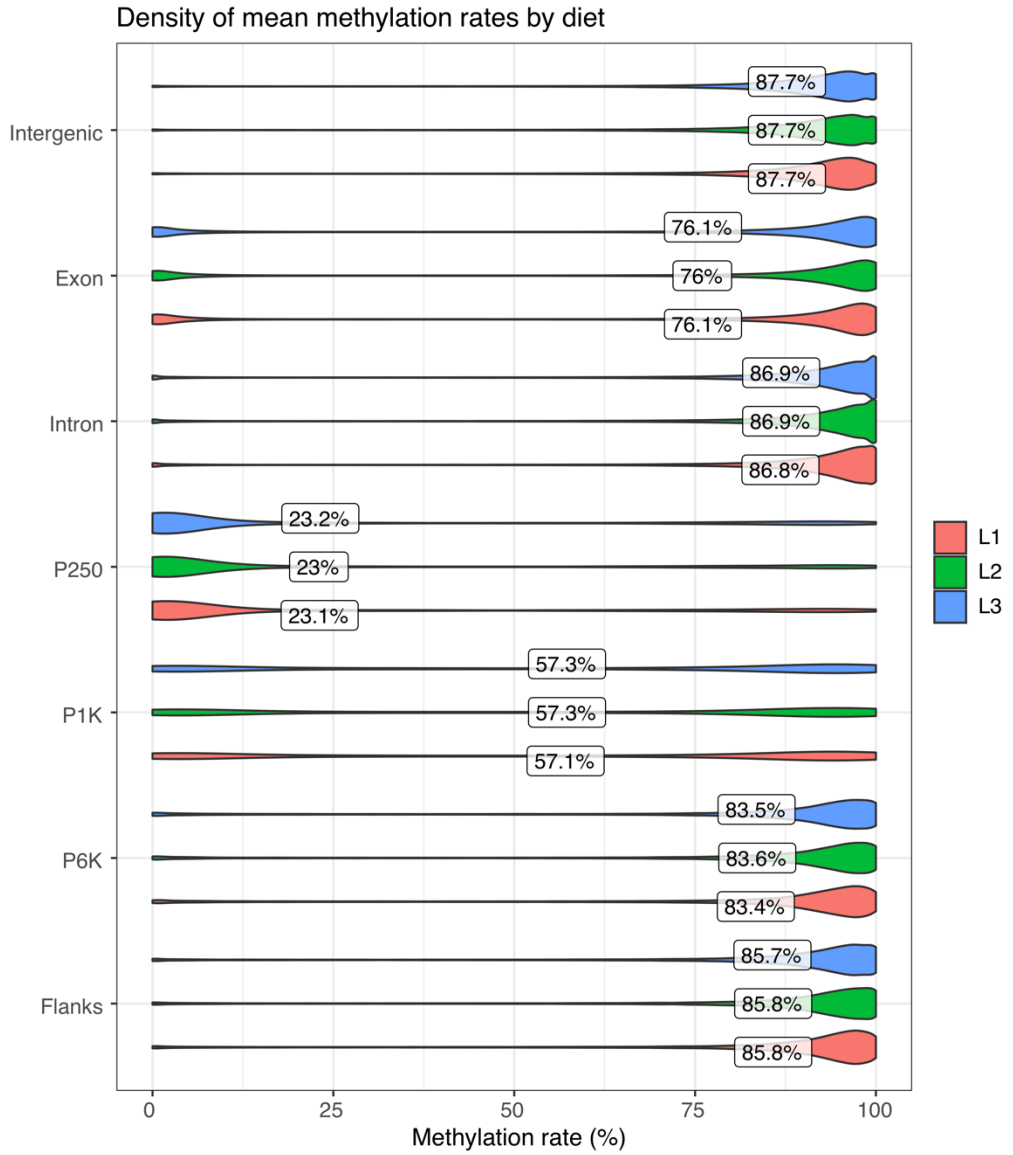
Supplementary Figure 8. Normalised read counts of pyruvate metabolism.
Box plots show the normalised read counts of four down-regulated and one up-regulated DEGs in L3:L1 that were associated with the pyruvate metabolism pathway. The counts were normalised with the median ratio method provided by the DESeq2 package.



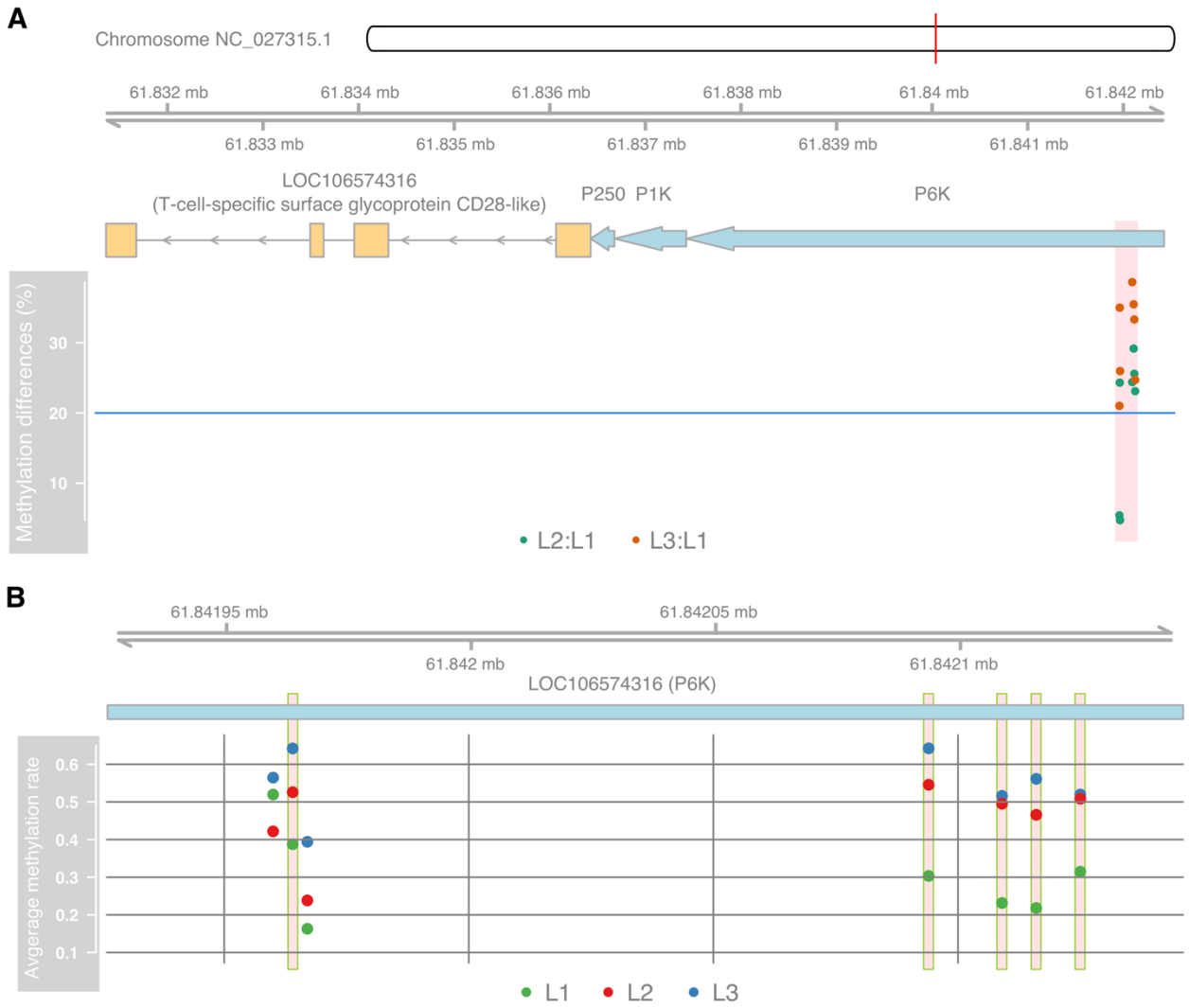
Supplementary Figure 9. Clustering 21 RRBS samples by three different algorithms.
(A) Biplot of principal component analysis (PCA) shows clustering of 21 samples with L1 (red), L2 (green) and L3 (blue). The percentages of explained variances for the 1st and 2nd dimensions are added to the axis title. (B) Scree plots shows the percentages of explained variances for the top 10 dimensions of the PCA. (C) Hierarchical clustering shows a dendrogram with two clusters (k = 2). The optimal number of clusters (k) was estimated by the gap statistics. (D) Heatmap shows sample-sample distances in a grid. The distance value, *d*, was calculated as normalized Pearson's correlation coefficient in a range between 0 and 1, with *d* = 0 as *r* = 1 and *d* = 1 as *r* = -1.



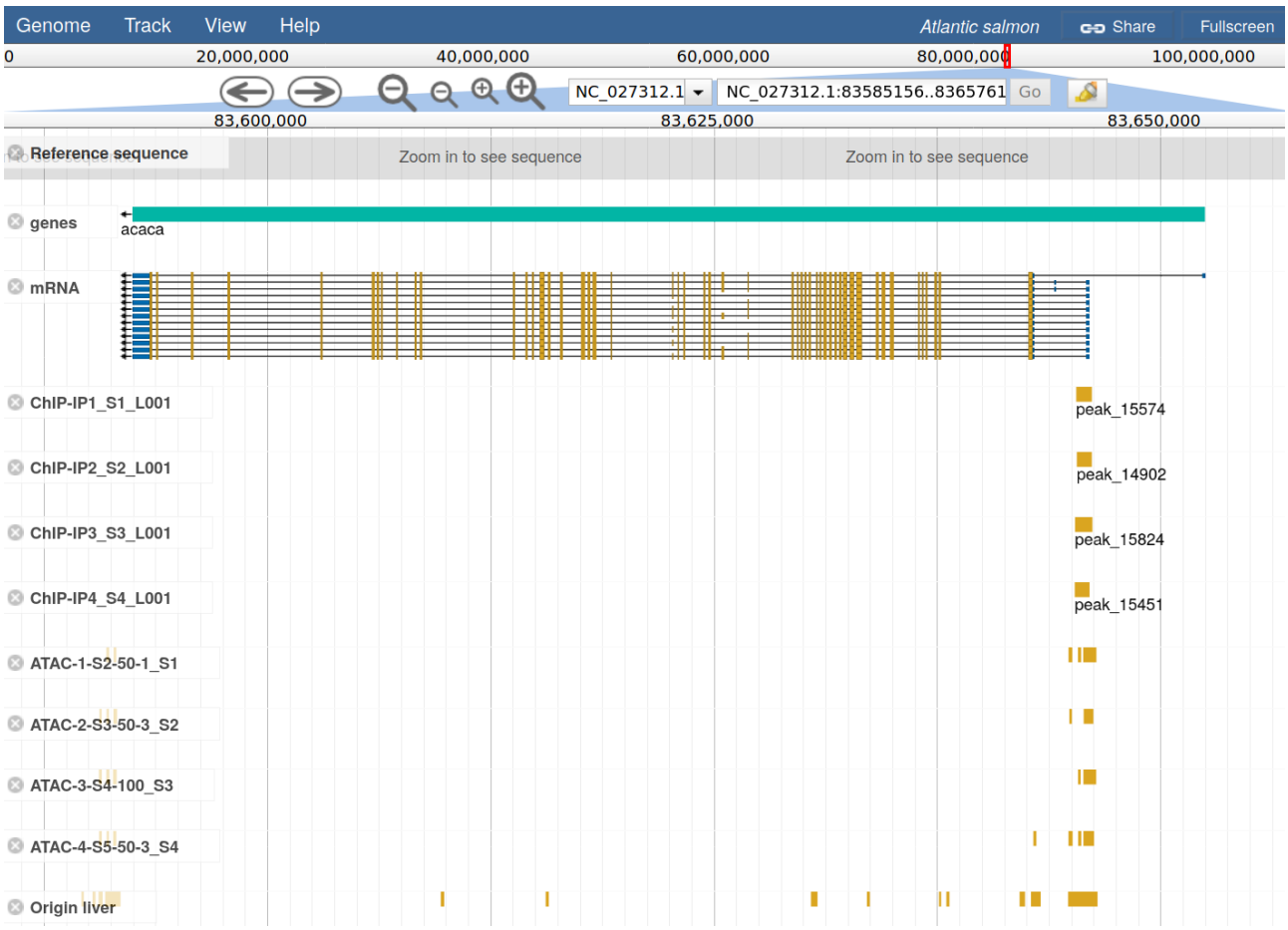
Supplementary Figure 10. Clustering 21 RRBS samples by sex.
(A) The t-SNE plot shows clustering of 21 RRBS samples with female (red) and male (blue). The perplexity used was 2. (B) Biplot of principal component analysis (PCA) shows clustering of 21 samples with female (red) and male (blue). The percentages of explained variances for the 1st and 2nd dimensions are added to the axis title.



Supplementary Figure 11. Regional methylation rates by diet.
The violin plots show the densities of methylation regions in seven different regions (intergenic, exon, intron, P250, P1K, P6K, and flanks) by diet.



Supplementary Figure 12. Genomic feature view of the *CD28* (*LOC106574316*).
Visualization of genomic data of the *CD28* (*T-cell-specific surface glycoprotein CD28-like*, *LOC106574316*) locus and its vicinity provides information about DNA methylation features: methylation differences and methylation rates. (A) The main track at the bottom shows differences of methylation rates for L2:L1 and L3:L1. The blue line indicates a threshold 20%. (B) An enlarged view shows the part of the genomic region that is indicated as red highlighted rectangle in (A). The main track at the bottom shows methylation rates of L1, L2, and L3. Five common DMCs between L2:L1 and L3:L1 are highlighted with vertical bars.



Supplementary Figure 13. Screenshot of the genomic region around *acaca* in SalmoBase.
The screenshot of genome bowser in SalmoBase shows a genomic region around the *acaca* gene with its isoforms, four ChiP IP peaks, four general ATAC peaks, and one liver specific ATATC peaks.

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