Tissue-specific metabolic enzyme levels covary with whole-animal metabolic rates and life-history loci via epistatic effects

Jenni M. Prokkola, Kuan Kiat Chew, Katja Anttila, Katja S. Maamela, Atakan Yildiz, Eirik R. Åsheim, Craig R. Primmer, Tutku Aykanat

Supplementary Material

Supplementary Material and Methods

Enzymatic assays

Samples were homogenised soon after thawing, and all the downstream procedures were carried out on ice. Tissues were homogenised in 150 µL (liver), 300 µL (heart), 200 µL (muscle) and 400 µL (intestine) of ice-cold homogenization buffer (50mM Hepes, 1mM EDTA, 0.01% Triton X-100, pH 7.4). Homogenization was done in 2mL Eppendorf tubes with one steel bead (diameter 5 mm) in each tube using a Tissue Lyser II (Qiagen), shaking at 30 Hz speed for 60s for two rounds, by cooling samples on ice between rounds. Maximal activities of CS and LDH, i.e. the rate of substrate conversion in non-limiting concentrations of substrate and cofactor, were measured in triplicates for each sample using EnSpire Multimode plate reader (PerkinElmer, USA) at final protein concentrations shown in Table S1.

For enzymatic assays, 24 samples were analysed on each plate, i.e., in one run (384-well clear SpectraPlate, PerkinElmer). Samples were thawed on ice. Plates were prepared at room temperature and run at 25 °C. Muscle samples were centrifuged at 2200 x g for 1 min due to high viscosity, and the supernatants were used in making dilutions for the assays. Other tissues were not centrifuged. In the CS assays, 2.5 µL homogenate diluted in 50 mM Tris-buffer (pH 8.0) was mixed in 0.47 mM oxaloacetate, 0.17 mM DTNB, and 0.14 mM Acetyl Co-enzyme A in 50 mM Tris-buffer (pH 8.0) (total volume 53.5 µL) and the plate was shaken in the plate reader for 5 s before measurements. In the LDH assays, 2.5 µL of homogenate diluted in 50 mM Tris-buffer (pH 7.4) was used in each reaction. First, 24 µL of 0.25 mM NADH in 50 mM Tris (pH 7.4) was added on the samples, and the plate incubated at 25°C with gentle shaking for 6 min. Then, 24 µL of 0.25 mM NAHD with 25 mM Na-pyruvate was added on the samples and the plate was shaken in the plate reader for 5 s.

Absorbance was measured approx. once every 10 s at 412 nm for CS and at 320 nm for LDH. Assays were completed within 3 h after thawing the samples. The assays were rerun immediately if the coefficient of variation (CV) in the first run was >10%. After assays were completed, the data were filtered (in R) to optimize the analysed measurement duration in each assay (ranging from 80 seconds in samples with the highest activities to 9 min and 40 s

in samples with the lowest activities). The replicates with slope-R^2 <0.98 were excluded from analysis. Further, samples that failed to have at least two accepted replicates in which the CV of slopes was <10.5 % were excluded. The remaining samples were visually inspected for linearity. CS activity was determined as µmol citrate min-1 from the equation:

$$\frac{reaction\ slope\ OD\ min^{-1}\ \times\ reaction\ vol.\ (0.0535\ mL)}{optical\ path\ length\ (0.4\ cm)\ \times\ extinction\ coefficient\ (13.6\ OD\ \ mM^{-1}\ cm^{-1})\ \times\ sample\ vol.\ (0.0025\ mL)}$$

and LDH activity as µmol NADH used min-1 from the equation:

$$\frac{-1\ \times\ reaction\ slope\ OD\ min^{-1}\ \times\ reaction\ vol.\ (0.0505\ mL)}{optical\ path\ length\ (0.38\ cm)\ \times\ extinction\ coefficient\ (6.22\ OD\ \ mM^{-1}\ cm^{-1})\ \times\ sample\ vol.\ (0.0025\ mL)}$$

Enzymatic activities in replicated reactions were averaged for each individual. A parallel blank reaction was run for each sample without the substrate (Acetyl CoA for CS, Na-pyruvate for LDH); blank correction was not used in the data analysis, as activities were negligible.

Protein concentrations (summarised in Table S1) were determined from the same aliquots that were used in the enzymatic assays after one freeze-thaw cycle, except for aliquots used in Heart CS and white muscle LDH, for which concentration was determined from a less diluted aliquot and back-calculated to the same dilution as in the assay, and for Liver CS aliquots, which were further diluted five-fold before the BCA assay due to high concentrations. Standard curves for the BCA assays were prepared using bovine serum albumin (BSA, B14, Thermo Scientific) and run with six points in triplicates on each plate. Standard curves were fitted with polynomial fit (4PL) after obvious outliers were removed (two or more replicates for each concentration remaining). Protein concentrations (in mg/mL) were averaged across replicates for the same sample from each assay.

Supplementary Figures

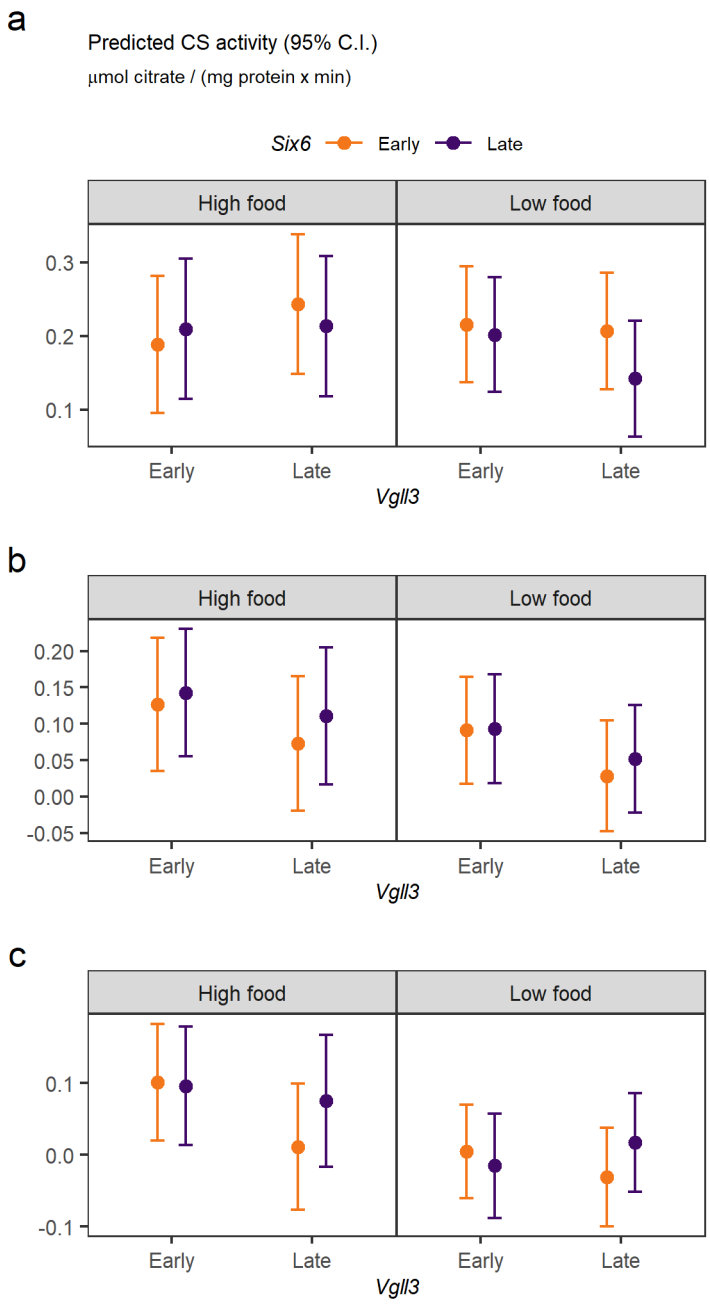

Fig. S1. Predicted means (95% confidence intervals) of CS activity in the a) intestine, b) white muscle, and c) liver of juvenile Atlantic salmon across different genotypes of *vgll3* and *six6* genomic regions related to early vs. late maturation. See tables S7-9 for the results of the models. No significant main effects or pairwise differences were found between genotypes. Negative activity values result from normalisation of activity with protein concentration and body mass, when the effect of normalisation is larger than the mean activity.

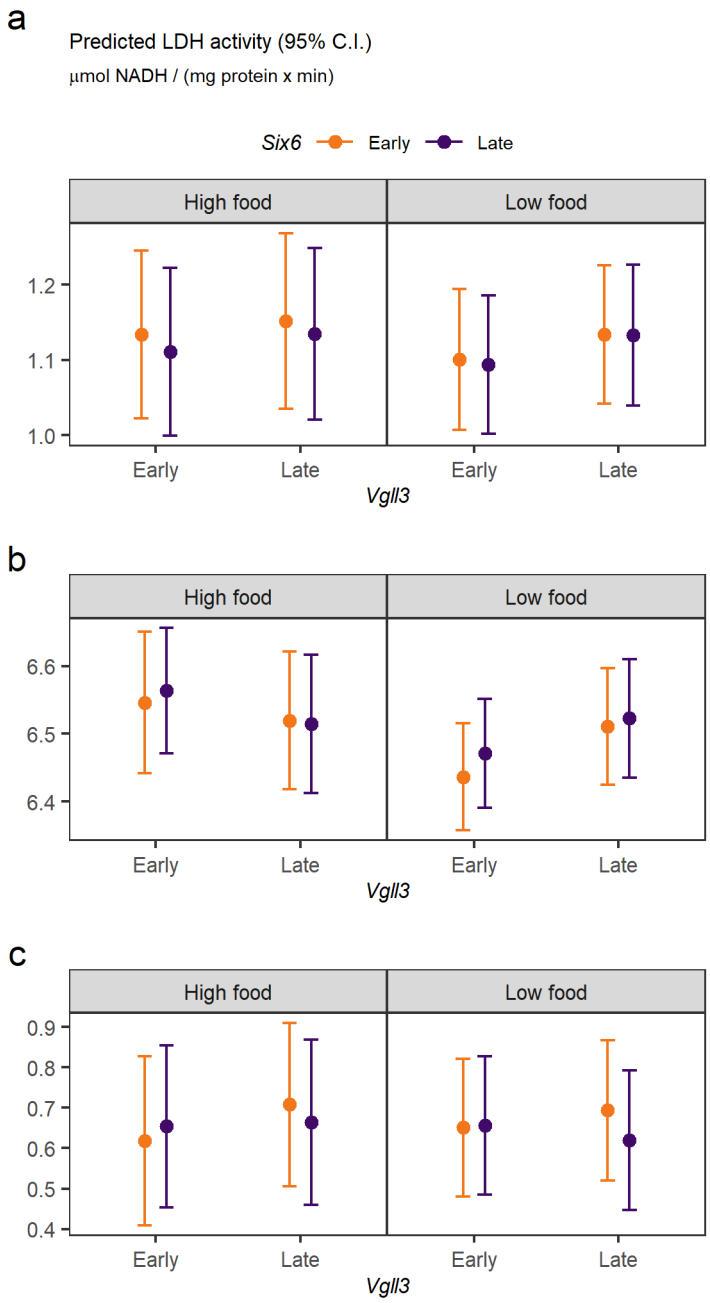

Fig. S2. Predicted means (95% confidence intervals) of LDH activity in the a) heart, b) muscle, and c) liver of juvenile Atlantic salmon across different genotypes of *vgll3* and *six6* genomic regions related to early vs. late maturation. See tables S10-S12 for the results of the models. No significant main effects or pairwise differences were found between genotypes.

Supplementary Tables

Table S1. Mean protein concentrations (± standard deviation) of homogenates analysed in each enzymatic assay.

Enzyme	Tissue	Prot. mg / ml	SD
CS	Heart	0.36	0.14
	Intestine	0.56	0.14
	Liver	16.63	6.68
	Muscle	0.92	0.32
LDH	Heart	0.45	0.22
	Intestine	0.86	0.25
	Liver	0.82	0.34
	Muscle	0.05	0.03

Table S2. Number of individuals analysed for activities of citrate synthase (CS) and lactate dehydrogenase (LDH) across high food and low food treatments and different tissue of juvenile salmon. Genotypes of *vgll3* and *six6* genomic regions are shown as E (homozygous early maturation) or L (homozygous late maturation genotype) within four families (three in high food, four in low food).

CS

Treatment	Tissue	Vgll3	Six6	F1	F3	F4	F8
High food	Heart	E	E	8	8	7	0
		E	L	9	6	7	0
		L	E	6	5	6	0
		L	L	7	6	7	0
	Intestine	E	E	8	9	8	0
		E	L	9	7	7	0
		L	E	8	7	8	0
		L	L	7	8	8	0
	Liver	E	E	7	9	7	0
		E	L	7	7	8	0
		L	E	7	6	6	0
		L	L	4	6	7	0
	Muscle	E	E	5	9	8	0
		E	L	9	8	8	0
		L	E	6	7	8	0
		L	L	6	7	7	0
Low food	Heart	E	E	10	13	11	6
		E	L	10	9	11	5
		L	E	11	11	10	6
		L	L	11	10	9	5
	Intestine	E	E	12	13	12	5
		E	L	12	11	13	6
		L	E	12	11	11	6
		L	L	12	12	11	6
	Liver	E	E	10	12	10	7
		E	L	11	8	6	5
		L	E	9	9	10	6
		L	L	8	11	11	4
	Muscle	E	E	11	12	11	5
		E	L	11	11	10	5
		L	E	12	11	7	5
		L	L	12	11	11	5

LDH

Treatment	Tissue	Vgll3	Six6	F1	F3	F4	F8
High food	Heart	E	E	5	5	8	0
		E	L	7	4	8	0
		L	E	4	5	5	0
		L	L	7	2	8	0
	Intestine	E	E	7	9	8	0
		E	L	7	8	8	0
		L	E	7	7	7	0
		L	L	6	8	6	0
	Liver	E	E	7	5	4	0

		E	L	9	7	6	0
		L	E	8	7	6	0
		L	L	6	8	5	0
	Muscle	E	E	4	8	6	0
		E	L	9	7	8	0
		L	E	6	5	8	0
		L	L	6	7	6	0
Low food	Heart	E	E	11	6	9	5
		E	L	11	8	12	4
		L	E	9	9	10	6
		L	L	11	7	9	4
	Intestine	E	E	10	10	12	3
		E	L	11	10	13	3
		L	E	11	11	11	6
		L	L	10	11	12	3
	Liver	E	E	10	11	10	6
		E	L	11	10	10	4
		L	E	10	7	9	6
		L	L	11	8	9	5
	Muscle	E	E	11	10	10	3
		E	L	9	11	8	5
		L	E	8	10	6	4
		L	L	8	8	8	3

Table S3. Mean (standard deviation) body mass and body length of fish in each treatment-by-genotype combination. E and L refer to early and late maturation related homozygous genotypes, respectively, of *vgll3* and *six6*.

Treatment	***Vgll3***	***Six6***	**Mass (g)**	**Length (mm)**
High food	E	E	4.1 ± 0.8	70 ± 5
	E	L	4 ± 0.7	70 ± 4
	L	E	4.3 ± 0.9	72 ± 4
	L	L	4 ± 0.8	71 ± 4
Low food	E	E	3.4 ± 0.7	67 ± 4
	E	L	3.4 ± 1	67 ± 6
	L	E	3.4 ± 0.9	67 ± 5
	L	L	3.1 ± 0.8	65 ± 5

Table S4. Linear mixed models used in this study.

Model nr	Model purpose	Figure nr	Table nr	Response variable	Fixed effects	Random effects
1-4	Effect of CS activity on rSMR	1	Table S5	rSMR	Normalised CS activity + Treatment + Normalised CS activity x Treatment	Treatment x Family + Date
5-8	Effect of LDH activity on rSMR	1	Table S5	rSMR	Normalised LDH activity + Treatment + Normalised LDH activity x Treatment	Treatment x Family + Date
9-12	Effect of CS activity on rMMR	1	Table S5	rMMR	Normalised CS activity + Treatment + Normalised CS activity x Treatment	Treatment x Family + Date
13-16	Effect of LDH activity on rMMR	1	Table S5	rMMR	Normalised LDH activity + Treatment + Normalised LDH activity x Treatment	Treatment x Family + Date
17	Effects of tissue and body mass on CS activity	2	Table S6	ln(CS activity (umol/min/mg protein))	Tissue + Treatment + Tissue x Treatment + Tissue x ln(Body mass)	Treatment x Family
18	Effects of tissue and body mass on LDH activity	2	Table S6	ln(LDH activity (umol/min/mg protein))	Tissue + Treatment + Tissue x Treatment + Tissue x ln(Body mass)	Treatment x Family
19-22	Genotype, treatment, and GxE effects on CS activity within tissues	3, S1	Table S7-9, Table 1	Normalised CS activity	Treatment + vgll3 + six6 + vgll3 x Treatment + six6 x Treatment + vgll3 x six6	Treatment x Family
23-26	Genotype, treatment, and GxE effects on LDH activity within tissues	4, S2	Table S10-12, Table 2	Normalised LDH activity	Treatment + vgll3 + six6 + vgll3 x Treatment + six6 x Treatment + vgll3 x six6	Treatment x Family

Table S5. Organismal metabolic rate variation (SMR and MMR) in relation to tissue-specific enzymatic activities for aerobic (CS) and anaerobic (LDH) metabolism across food availability treatments (high vs. low). CS denotes citrate synthase and LDH denotes lactate dehydrogenase. The fixed factors of the model, enzymatic activity and food treatment, were modelled with an interaction term. In all models, family/tank effect and sampling date were included as random terms.

			CS			**LDH**		
			Activity	Food	Activity x Food	Activity	Food	Activity x Food
SMR	Heart	DenDF	197	7	197	166	7	166
		F	0.000	0.313	5.775	0.121	0.171	0.004
		P	0.99	0.59	**0.02***	0.73	0.69	0.95
	Intestine	DenDF	216	7	216	199	7	199
		F	0.621	0.179	4.678	5.678	0.147	0.039
		P	0.43	0.68	**0.03***	**0.02***	0.71	0.84
	Liver	DenDF	183	7	183	185	7	185
		F	3.451	0.276	0.8	0.943	0.269	0.223
		P	0.06	0.62	0.37	0.33	0.62	0.64
	Muscle	DenDF	204	7	204	170	7	170
		F	0.385	0.092	0.411	0.319	0.124	0.35
		P	0.54	0.77	0.52	0.57	0.73	0.55
MMR	Heart	DenDF	204	7	204	174	7	174
		F	0.02	0.108	0.906	0.281	0.04	1.913
		P	0.89	0.75	0.34	0.6	0.85	0.17
	Intestine	DenDF	226	7	226	209	7	209
		F	4.518	0.266	0.546	2.512	0.131	0.024
		P	**0.03***	0.62	0.46	0.11	0.73	0.88
	Liver	DenDF	185	8	185	186	7	186
		F	0.055	0.162	0.886	0.602	0.166	0.058
		P	0.81	0.7	0.35	0.44	0.7	0.81
	Muscle	DenDF	170	7	170	161	7	161
		F	0.142	0.369	0.475	0.103	0.241	0.078
		P	0.71	0.56	0.49	0.75	0.64	0.78

Table S6. Results of linear mixed models of citrate synthase (CS) and lactate dehydrogenase (LDH) activities across treatments, tissues, and in relation to fish body mass nested within tissue.

CS			
Fixed effect	**Den df**	**F**	**p**
Tissue	937	87.35	<0.001***
Treatment	10	7.15	0.022*
Tissue x ln(body mass)	633	4.14	0.003**
Tissue x Treatment	937	0.05	0.986
Random effect	**Var**	**C.I. 2.5%**	**C.I. 97.5%**
Treatment x Family	0.0002	0	0.002
Residual	0.069		
LDH			
Fixed effect	**Den df**	**F**	**p**
Tissue	840	67.78	<0.001***
Treatment	7	0.63	0.453
Tissue x ln(body mass)	690	11.53	<0.001***
Tissue x Treatment	840	3.50	0.015*
Random effect	**Var**	**C.I. 2.5%**	**C.I. 97.5%**
Treatment x Family	0.0022	0.0004	0.0099
Residual	0.079		

Table S7. The results of linear mixed model with Type III test for CS activity in the intestine. CS activity (in µmol citrate/mg protein/min) was ln-transformed and normalised by ln(body mass) and ln(protein concentration) before the analysis. Intercept is shown with t-test value. Estimates show high vs. low food (Treatment) or EE vs. LL genotype (*vgll3* and *six6*).

Fixed effect	Estimate	95 % C.I.	SSq	Den df	F	p
Intercept	-0.014	-0.11, 0.087		16	-0.29	0.775
Treatment	0.027	-0.10, 0.15	0.0063	7	0.19	0.678
Vgll3	0.055	-0.031, 0.14	0.0004	250	0.01	0.913
Six6	0.021	-0.065, 0.11	0.029	249	0.86	0.356
Treatment x Vgll3	-0.064	-0.16, 0.029	0.061	250	1.82	0.179
Treatment x Six6	-0.035	-0.13, 0.058	0.018	249	0.55	0.460
Vgll3 x Six6	-0.051	-0.14, 0.039	0.042	250	1.25	0.264
Random effect	Var	C.I.low	C.I.high			
Treatment x Family	0.003	0.001	0.015			
Residual	0.033					

Table S8. The results of linear mixed model with Type III test for CS activity in the white muscle. CS activity (in µmol citrate/mg protein/min) was ln-transformed and normalised by ln(body mass) and ln(protein concentration) before the analysis. Intercept is shown with t-test value. Estimates show high vs. low food (Treatment) or EE vs. LL genotype (*vgll3* and *six6*).

Fixed effect	Estimate	95% C.I.	SSq	Den df	F	p
Intercept	0.049	-0.047, 0.14		35	1.05	0.303
Treatment	-0.035	-0.15, 0.084	0.06	7	1.35	0.284
Vgll3	-0.053	-0.16, 0.051	0.123	230	2.79	0.096
Six6	0.016	-0.086, 0.12	0.022	231	0.49	0.483
Treatment x Vgll3	-0.010	-0.12, 0.102	0.001	230	0.03	0.866
Treatment x Six6	-0.014	-0.13, 0.098	0.003	231	0.06	0.805
Vgll3 x Six6	0.022	-0.086, 0.13	0.007	231	0.15	0.695
Random effect	Var	C.I.low	C.I.high			
Treatment x Family	0.001	0.000	0.009			
Residual	0.044					

Table S9. The results of linear mixed model with Type III test for CS activity in the liver. CS activity (in µmol citrate/mg protein/min) was ln-transformed and normalised by ln(body mass) and ln(protein concentration) before the analysis. Intercept is shown with t-test value. Estimates show high vs. low food (Treatment) or EE vs. LL genotype (*vgll3* and *six6*).

Fixed effect	Estimate	95% C.I.	SSq	Den df	F	p
Intercept	0.082	0, 0.164		217	1.97	0.050
Treatment	-0.096	-0.12, 0.002	0.298	217	6.49	0.012*
Vgll3	-0.090	-0.20, 0.02	0.041	217	0.89	0.347
Six6	-0.005	-0.11, 0.102	0.024	217	0.53	0.469
Treatment x Vgll3	0.054	-0.065, 0.173	0.036	217	0.79	0.374
Treatment x Six6	-0.015	-0.13, 0.10	0.003	217	0.06	0.802
Vgll3 x Six6	0.068	-0.047, 0.18	0.063	217	1.37	0.243
Random effect	Var	C.I.low	C.I.high			
Treatment x Family	0.000	0.000	0.002			
Residual	0.046					

Table S10. The results of linear mixed model with Type III test for LDH activity in the heart. LDH activity (in µmol NADH/mg protein/min) was ln-transformed and normalised by ln(body mass) and ln(protein concentration) before the analysis. Intercept is shown with t-test value. Estimates show high vs. low food (Treatment) or EE vs. LL genotype (*vgll3* and *six6*).

Fixed effect	Estimate	95% C.I.	SSq	Den df	F	p
Intercept	0.020	-0.10, 0.14		14	0.34	0.736
Treatment	-0.033	-0.18, 0.12	0.002	7	0.08	0.790
Vgll3	0.018	-0.079, 0.12	0.036	192	1.25	0.266
Six6	-0.023	-0.12, 0.071	0.006	194	0.22	0.642
Treatment x Vgll3	0.016	-0.086, 0.12	0.003	192	0.09	0.761
Treatment x Six6	0.016	-0.086, 0.12	0.003	194	0.10	0.757
Vgll3 x Six6	0.006	-0.091, 0.10	0.000	192	0.01	0.908
Random effect	Var	C.I.low	C.I.high			
Treatment x Family	0.006	0.002	0.023			
Residual	0.029					

Table S11. The results of linear mixed model with Type III test for LDH activity in the white muscle. LDH activity (in µmol NADH/mg protein/min) was ln-transformed and normalised by ln(body mass) and ln(protein concentration) before the analysis. Intercept is shown with t-test value. Estimates show high vs. low food (Treatment) or EE vs. LL genotype (*vgll3* and *six6*).

Fixed effect	Estimate	95% C.I.	SSq	Den df	F	p
Intercept	0.042	-0.06, 0.15		202	0.79	0.428
Treatment	-0.110	-0.23, 0.01	0.123	202	2.02	0.157
Vgll3	-0.026	-0.16, 0.11	0.0075	202	0.12	0.725
Six6	0.017	-0.11, 0.15	0.010	202	0.17	0.682
Treatment x Vgll3	0.101	-0.04, 0.24	0.121	202	1.99	0.160
Treatment x Six6	0.017	-0.12, 0.16	0.004	202	0.06	0.807
Vgll3 x Six6	-0.023	-0.16, 0.11	0.007	202	0.11	0.745
Random effect	Var	C.I.low	C.I.high			
Treatment x Family	0.000	0.000	0.002			
Residual	0.061					

Table S12. The results of linear mixed model with Type III test for LDH activity in the liver. LDH activity (in µmol NADH/mg protein/min) was ln-transformed and normalised by ln(body mass) and ln(protein concentration) before the analysis. Intercept is shown with t-test value. Estimates show high vs. low food (Treatment) or EE vs. LL genotype (*vgll3* and *six6*).

Fixed effect	Estimate	95% C.I.	SSq	Den df	F	p
Intercept	-0.053	-0.28, 0.17		13	-0.50	0.626
Treatment	0.033	-0.26, 0.33	0.0002	7	0.00	0.962
Vgll3	0.090	-0.06, 0.24	0.0345	208	0.44	0.508
Six6	0.036	-0.11, 0.18	0.018	208	0.22	0.636
Treatment x Vgll3	-0.047	-0.2, 0.11	0.027	208	0.34	0.559
Treatment x Six6	-0.030	-0.19, 0.13	0.011	208	0.14	0.706
Vgll3 x Six6	-0.080	-0.23, 0.07	0.084	208	1.07	0.302
Random effect	Var	C.I.low	C.I.high			
Treatment x Family	0.022	0.008	0.085			
Residual	0.078					