SUPPLEMENTARY INFORMATION

A proteome-driven elucidation of adaptive responses to combined vitamin E and C deficiency in zebrafish

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Supplementary Table S1. Pipetting scheme used for sample lysis and preparation of digests.

Supplementary Table S2. Synapt G2 HDMS instrument settings used for Ion-Mobility-MS^E acquisitions.

Supplementary Table S3. The number of peptides and proteins found in each of the biological samples subjected to LC-IM-MS^E.

Supplementary Table S4. Proteins identified in the E-C+ and the E-C- groups, GO annotations and fold-changes observed in the vitamin C deficient fish (E-C-/E-C+ transition).

Excel file: Supplementary Table S5. Protein Table for Study (A) E+C+ vs E+C- and (B) E-C+ vs E-C- The compilation of proteins found in each samples, unique peptides identified per protein and respective sequence coverage.

Supplementary Figure S1. Design of the feeding experiment. Briefly, at 42 day of age, 40 fish were divided in two groups and fed diets low in vitamin C with vitamin E at sufficient or deficient levels. Thus, two vitamin groups were created: E+C- and E-C- with 20 fish in each group. Supplementation continued for 56 days, after which half of the fish population in each group was harvested. The diet of remaining fish was altered to have a high amount of vitamin C, thus creating another two vitamin groups: E+C+ and E-C+. After an additional 21 days, the fish were harvested. Thus, four groups with 10 fish in each were created, each having different vitamins levels that varied with supplementation: E+C+, E-C+, E+C- and E-C-.

Supplemental Figure S2. Workflow used for preparing the samples for the label-free "bottom up" quantitative proteomics study.

Fish was individually ground under liquid nitrogen using a mortar and pestle. While still dry, the fish powder was transferred to a pre-weighted microcentrifuge tube and the mass of the tube with fish powder was recorded. The mass of the fish was calculated by the difference. A PBS solution with ProteaseMAX (0.04%) was added to the micro tubes for the extraction of proteins; its volume was adjusted to account for fish mass variability. Assuming the density of extraction solution 1 mg/mL, its mass was calculated as 6 times that of a fish. Tubes of fish powder solution were frozen in liquid nitrogen for 2 minutes, thawed for 5 min, and sonicated for 15 minutes. Last three steps were repeated three times. After lysis, tubes were centrifuged at 4 ºC for 10 minutes at 15,000 relative centrifugal force (rcf) and the supernatants were transferred to new microcentrifuge tubes (1.3 mL). After protein digestion, 9 µL of digest was mixed with 1 µL of the internal standard and the mixture was subjected to LC- ion mobility – MS^E analysis.

Supplementary Figure S3. Fish characteristics dependent on the vitamin regime fed.

(A) Boxplots of distribution of fish body weight. Fish from vitamin groups were harvested at preselected age (98 days vs. 119 days) and had a different vitamin supplementation. Therefore it was expected that there will be a difference between the average fish mass between groups. The median fish mass is lower for groups with a shorter life span, but the difference was statistically significant only between E+C+ and E-C- groups (p-value=0.006).

(B) The ratios of median growth rate between vitamins groups. Ratios representing the effect of vitamin E deficiency are colored in green and those representing the effect of vitamin C are

in blue. The growth rate (median body mass / age) of fish adequate in both vitamins (E+C+ group) was approximately 3-times higher compared to the group deficient in both vitamins (E-C- group). Vitamin C deficiency seemed to have a more drastic effect on the growth rate compared to inadequate vitamin E levels.

Supplemental Figure S4. A boxplot of the distribution of the total protein concentration after extraction and lysis.

Total protein concentrations were distributed in a narrow range, as was expected with the sample preparation that accounts for differences in fish mass. The median of total protein concentration was 2.382 mg/mL, sample size=37.

Supplementary Figure S5. Gradient profiles with respective base peak chromatograms.

To yield the maximum number of eluted peptides, a LC gradient was optimized. For this, a sample was run using different LC gradients and the number of eluted peptides was compared. By changing the mobile phase composition over time, the gradient was optimized after each run to make the elution of peptides even across a chromatogram and to reduce peptide coelution. The gradients were named “Gradient1”, “Gradient2”, “Gradient3” and “Gradient4”. The comparison of the total ion chromatograms illustrates how different clusters of eluting compounds are moving across the chromatogram allowing better separation of peptides dependent on the gradient design.

Supplementary Figure S6. Venn’s diagrams comparing the number of peptides eluting from a column and identified with LC-IM-MS^{E}.

More identified peptides means more identified proteins with greater probability. Gradient 4 (3% to 40% B in 90 minutes) was chosen for further peptide separation.

Supplementary Figure S7. Correlation plot between E-C+ sample 9 and E-C+ sample 8.
On the axis is log2 of the concentration of proteins found in both groups. 95% confidence band is drawn with short-dashed line, 95% prediction band is long-dashed line. Adjusted R^2 shows some variability between biological replicates.

Supplementary Figure S8. Correlation plot matrix of the E-C+ group.
As expected, variation between fish was observed and the correlation is stronger between samples, where the number of proteins found is higher.

Supplementary Figure S9. The responsive protein network for the vitamin transition C+/C- and both groups deficient in vitamin E status (E-C+ vs E-C+). The network was constructed using proteins observed in the quantitative proteomics screens of fish adequate in vitamin C versus fish deficient in vitamin C both with deficient vitamin E status (transition E-C+ /E-C-). In the network each node represents a protein found in either group E-C+ or E-C-. The change in protein amounts between vitamin C adequate and deficient fish is color coded: green nodes represent proteins that increased their amounts, red represent those that decreased, and grey nodes show proteins that were not quantified. The size of a node represents an average of the protein abundance estimate in E-C- group. The width of a line connecting proteins represents the strength of proteins interaction, as extracted from STRING. Proteins involved in glycolysis, the TCA cycle, stress response and heat shock proteins are highlighted.

Supplementary Table S1. Protocol for sample lysis and preparation of digests.

		Solubilization/Lysis				Preparation of Digests		
Sample	Fish weight, mg	Volume of pMAX, µL	Volume of PBS, µL	Total volume, µL	Concen-tration, µg/mL	Volume for digestion, µL	Mass of proteins for digestion, µg	Volume of NH_4HCO_3, µl
C+E+ #3	62.0	74	298	372	2080.0	24.0	49.9	69.5
C+E+ #4	115.0	138	552	690	2642.6	18.9	49.9	74.6
C+E+ #5	29.2	35	140	175	1682.1	29.7	50.0	63.8
C+E+ #6	143.4	172	688	860	845.1	59.2	50.0	34.3
C+E+ #7	231.0	277	1109	1386	2423.0	20.6	49.9	72.9
C+E+ #8	207.3	249	995	1244	2217.2	22.6	50.1	70.9
C+E+ #9	242.7	291	1165	1456	2148.6	23.3	50.1	70.2
C+E+ #10	183.5	220	881	1101	1942.8	25.7	49.9	67.8
C+E- #1	127.6	153	612	765	2217.2	22.6	50.1	70.9
C+E- #2	81.2	97	390	487	707.9	70.6	50.0	22.9
C+E- #3	58.9	71	283	354	2477.9	20.2	50.1	73.3
C+E- #4	103.1	124	495	619	2587.7	19.3	49.9	74.2
C+E- #5	124.1	149	596	745	2464.2	20.3	50.0	73.2
C+E- #6	71.8	86	345	431	2697.5	18.5	49.9	75.0
C+E- #7	65.8	79	316	395	2656.3	18.8	49.9	74.7
C+E- #8	207.6	249	996	1245	2258.8	22.1	49.9	71.4
C+E- #9	123.8	149	594	743	2299.5	21.7	49.9	71.8
C+E- #10	215.3	258	1033	1291	2381.9	21.0	50.0	72.5
C-E+ #1	32.5	39	156	195	2450.5	20.4	50.0	73.1
C-E+ #2	201.9	242	969	1211	2313.3	21.6	50.0	71.9
C-E+ #3	168.8	203	810	1013	2258.4	22.1	49.9	71.4
C-E+ #4	41.1	49	197	246	2409.3	20.8	50.1	72.7
C-E+ #5	70.1	84	336	420	2464.2	20.3	50.0	73.2
C-E+ #6	168.2	202	807	1009	2327.0	21.5	50.0	72.0
C-E+ #7	51.1	61	245	306	2519.1	19.8	49.9	73.7
C-E+ #8	42.7	51	205	256	2656.3	18.8	49.9	74.7
C-E+ #9	73.1	88	351	439	2327.0	21.5	50.0	72.0
C-E+ #10	76.9	92	369	461	2368.2	21.1	50.0	72.4
C-E- #1	72.4	87	348	435	1983.9	25.2	50.0	68.3
C-E- #2	14.9	18	72	90	2711.2	18.4	49.9	75.1
C-E- #3	87.8	105	421	526	2409.3	20.8	50.1	72.7

C-E- #4	34.8	42	167	209	2464.2	20.3	50.0	73.2
C-E- #5	40.0	48	192	240	2423.0	20.6	49.9	72.9
C-E- #6	19.7	24	95	119	2327.0	21.5	50.0	72.0
C-E- #7	73.4	88	352	440	2532.8	19.7	49.9	73.8
C-E- #8	31.7	38	152	190	2834.7	17.6	49.9	75.9
C-E- #9	111.4	134	535	669	2299.5	21.7	49.9	71.8

Supplementary Table S2. Synapt G2 HDMS instrument settings used for the ion-mobility-MS^{E} acquisitions.

Capillary voltage, kV	2.5
Sampling cone	40
Extraction cone	4.2
Source temperature, ºC	40
Desolvation temperature, ºC	300
Cone gas flow, L/Hr	20
Nanoflow gas pressure, Bar	1
Purge gas flow, mL/Hr	100
Desolvation gas flow, L/Hr	100
IMS gas flow, mL/min	75.30
Reference scan frequency, sec	60
LockSpray capillary, kV	3.0
Use manual trap DC	FALSE
Use manual IMS DC	FALSE
Use manual transfer DC	FALSE
Source manual control	OFF
Trap manual control	OFF
IMS manual control	OFF
Transfer manual control	OFF
Function parameters – Function 1	
Trap collision energy, eV	6.0
Transfer collision energy, eV	0.0
Maintain mobility separation	YES
Function parameters – Function 2	
Using auto trap MS collision energy, eV	4.0
Transfer MS collision energy low, eV	27.0
Transfer MS collision energy high, eV	50.0
Maintain mobility separation	YES
Function parameters – Function 3 (Lock Mass)	
Using auto trap MS collision energy, eV	4.0
Using auto trnsfer MS collision energy, eV	0.0
Maintain mobility separation	YES

Supplementary Table S3. The number of proteins, peptides and spectra found in biological samples, as extracted from Scaffold software.

Category	Bio Sample	#Prot	#Pept	#Spec
E-C+	B1	50	138	3341
E-C+	B2	197	1263	37888
E-C+	B3	185	1164	26128
E-C+	B4	109	373	8439
E-C+	B5	74	217	5770
E-C+	B6	63	306	3156
E-C+	B7	99	345	8833
E-C+	B8	167	1016	16434
E-C+	B9	159	845	11886
E-C+	B10	140	793	10109
E-C-	D1	110	441	11595
E-C-	D2	104	389	7832
E-C-	D3	94	509	6751
E-C-	D4	99	431	9345
E-C-	D5	138	797	9668
E-C-	D6	134	842	8257
E-C-	D7	72	255	13220
E-C-	D8	74	357	3632
E-C-	D9	133	827	8665
E+C+	A3	153	811	8920
E+C+	A4	64	273	4447
E+C+	A5	162	735	12179
E+C+	A6	102	239	12038
E+C+	A7	183	1000	17021
E+C+	A8	178	821	16849
E+C+	A9	127	405	9551
E+C+	A10	61	44	1581
E+C-	C1	101	401	12201
E+C-	C2	152	536	14657
E+C-	C3	68	93	2717
E+C-	C4	125	340	12040
E+C-	C5	125	488	9672
E+C-	C6	112	295	7091
E+C-	C7	151	596	27808
E+C-	C8	143	568	10600
E+C-	C9	129	441	8682

E+C-	C10	49	48	648

Supplementary Table S4. Proteins identified in the E-C+ and the E-C- groups, GO annotations and fold-changes observed in the vitamin C deficient fish (E-C-/E-C+ transition).

Accession number	Protein name	Gene name	Ave E-C+, fmol	Fold change
	Glycolysis cycle			
Q6DG54	Pyruvate kinase	pkm2b	45.29	**0.43
Q6P043	Fructose-bisphosphate aldolase	aldoab	19.00	*1.57
Q7ZW73	Fructose-bisphosphate aldolase	aldob	39.04	0.85
Q803Q7	Fructose-bisphosphate aldolase	aldoaa	78.28	0.44
Q7SXW7	Phosphoglucomutase 1	pgm1	23.81	0.53
Q7ZV29	Phosphoglycerate kinase	pgk1	27.86	0.85
	TCA cycle			
Q6PEI6	Aconitase 2, mitochondrial	aco2	10.48	1.05
Q7T334	Malate dehydrogenase	mdh2	28.84	0.77
Q801E6	Malate dehydrogenase	mdh1a	19.01	1.13
Q7ZUP6	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	idh2	37.54	0.73
B2GTW6	Citrate synthase	cs	22.32	0.89
	Glutaminolysis			
Q6P3L9	Glutamate dehydrogenase 1	glud1b	15.59	*2.12
Q7ZVJ4	L-lactate dehydrogenase	ldha	24.75	1.13
Q803U5	L-lactate dehydrogenase	ldhba	13.76	0.97
	Structural molecule activity			
Q8JIY5	Glial fibrillary acidic protein	gfap	1.19	**27.82
Q90441	Desmin	desma	12.24	1.62
A8WGN0	Keratin 8	krt8	36.64	1.72
Q9PUB5	Type II cytokeratin	krt5	13.41	0.68
Q9PUB6	Type I cytokeratin	cki	4.75	1.42
Q9PWD8	Type I cytokeratin	cyt1	6.42	0.58
Q1LXJ1	Novel protein similar to type I cytokeratin, enveloping layer	krt17	19.37	2.34

Q6DHB6	Zgc:92533	zgc:92533	53.76	0.40
	ATP binding (not functional)			
Q9PTF5	Nucleoside diphosphate kinase	nme2b.1	28.46	1.80
Q7SXL4	Nucleoside diphosphate kinase	nme2b.2	535.05	1.11
A0JMC2	Myhz2 protein (Fragment)	myhz2	2.44	0.73
A2BGX8	Novel protein similar to myosin heavy chain 4 (Myhc4) (Fragment)	CH211-251M3.3-001	4.93	2.95
A7E2L9	LOC100002040 protein (Fragment)	myhb	1.93	*5.96
A8WBZ8	Slow myosin heavy chain 3 (Fragment)	smyhc3	2.98	1.07
B6IDE1	Slow myosin heavy chain 2	smyhc2	33.18	1.23
B8A559	Novel myosin family protein (Fragment)	CH211-158M24.10-001	10.63	*5.90
B8A561	Novel myosin family protein	myhz1.2	2.42	4.48
O93409	Myosin light chain 2	mylz2	363.78	1.33
Q508P7	Fast myosin heavy chain 4	myhc4	65.68	0.25
Q9I8U7	Fast skeletal muscle myosin light polypeptide 3	mylz3	89.46	1.56
Q7T1B8	Fast skeletal myosin heavy chain 3 (Fragment)	myhz1.2	4.39	1.30
Q6P0G6	Novel protein similar to vertebrate myosin, light chain 6, alkali, smooth muscle and non-muscle (MYL6, zgc:77231)	myl1	39.60	1.17
Q9PVE1	Ventricular myosin heavy chain	vmhc	17.21	**4.19
A7E2H1	Zgc:152732 protein (Fragment)	vmhcl	22.92	0.80
A0JMP4	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 like	atp2a1l	29.87	0.82
Q08BA1	ATP synthase subunit alpha	atp5a1	42.54	0.61
Q4VBK0	ATP synthase subunit beta	atp5b	14.01	1.26
Q6ZM60	Novel protein similar to vertebrate ATPase, Ca++ transporting, cardiac muscle, slow twitch 2 (ATP2A2)	atp2a2b	2.61	1.81
Q7ZW18	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2a	atp2a2a	10.65	0.62
Q8UUJ8	Stress protein HSP70	hsp70	7.19	1.74
Q6P3L3	Heat shock protein 5	hspa5	18.91	0.52
Q6PGX4	Heat shock cognate 71 kDa protein	hsc70	21.12	1.31

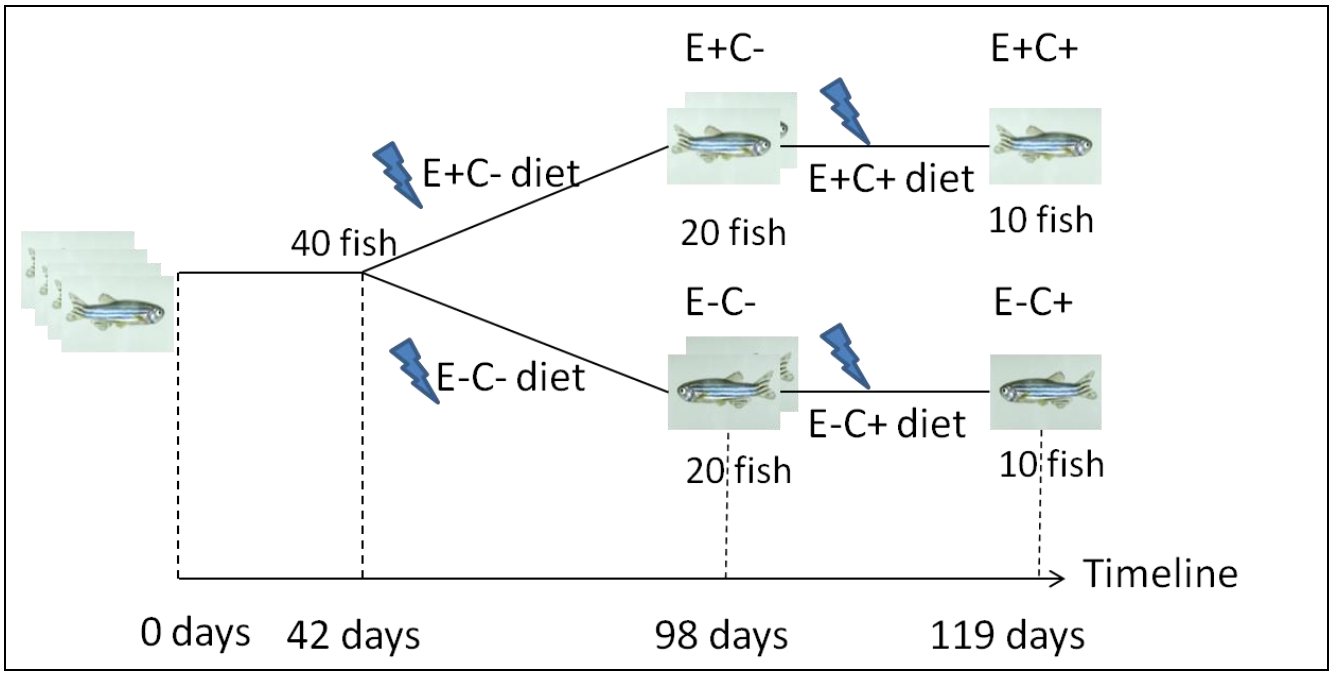
A5WWG8	Heat shock protein 90kDa alpha (Cytosolic), class B member 1	hsp90ab1	7.55	*2.32
A8WG05	Bactin2 protein	bactin2	186.24	1.31
Q08BV6	Novel protein (Zgc:92079)	ckba	10.83	0.15
Q68EH2	Zgc:91930	ak1	35.98	0.51
Q7T306	Ckmb protein	ckmb	128.37	1.51
Q802Z4	Zgc:66156 protein (Fragment)	zgc:66156	47.02	0.86
	Cardiac muscle contraction			
A7E2K1	Tropomyosin alpha-1 chain	tpm1	9.10	1.04
Q5SPK5	Alpha-tropomyosin	tpma	3.78	0.94
Q5SPK6	Alpha-tropomyosin	tpma	122.33	1.53
Q5U3J6	Tropomyosin 4	tpm4	4.04	2.09
Q6P0W3	Novel protein similar to vertebrate tropomyosin 1 (Alpha) (TPM1, zgc:77592)	tpm3	7.72	1.41
Q7ZVK9	Tpm1 protein	tpm1	20.75	1.24
Q7SXW1	Zgc:63734	tpm4b	28.49	0.61
Q7T3F0	Tropomyosin 4	tpm4	21.16	0.99
Q803M1	Novel protein similar to vertebrate tropomyosin 1 (Alpha) (TPM1, zgc:77592)	tpm3	23.85	0.40
Q6IQD7	Zgc:86810	tpm2	18.26	0.53
	Monooxygenase activity			
A3KNI9	Ywhab1 protein	ywhaba	23.26	1.29
B8JLJ3	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, iota polypeptide	ywhai	11.79	0.55
Q5CZQ1	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide 2	ywhae2	15.11	1.16
Q6P102	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide like	ywhabl	71.98	0.42
Q7ZW20	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide 1	ywhae1	22.16	0.47
Q803M8	Tryosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	ywhaqb	18.02	0.65
	Oxidoreductase activity			
B0S7W4	Aldehyde dehydrogenase 9 family, member A1 like 1	aldh9a1a	14.03	0.60
Q4KME8	Vesicle amine transport protein 1 homolog (T californica)	vat1	26.08	0.71

	DNA binding			
A7E2M8	Histone H2B	zgc:173585	67.48	1.85
O93584	Y box binding protein 1	ybx1	12.31	0.75
	Calcium ion binding			
Q2YDR5	Actinin, alpha 2	actn2	0.76	5.48
Q7SYE2	Actinin, alpha 4	actn4	22.56	3.61
Q8AX99	Actn3b	actn3b	44.21	0.88
A2BHA3	Creatine kinase, muscle	ckm	54.23	0.47
D1GJ54	Actn1 isoform c (Fragment)	actn1	5.98	2.34
Q804W0	Parvalbumin 1	pvalb1	198.64	0.57
Q7ZT36	Parvalbumin 3	pvalb3	538.72	0.81
Q6IMW7	Parvalbumin 4	pvalb4	39.32	0.39
Q6XG62	Ictacalcin	icn	61.70	0.73
Q9I8U8	Fast skeletal muscle troponin C	tnnc	31.42	1.14
	Lipid binding			
B2ZHD8	Fatty acid-binding protein 6	fabp6	18.52	1.31
Q66I80	Fatty acid binding protein 11a	fabp11a	19.26	*0.36
Q9I8N9	Brain-type fatty acid binding protein	fabp7a	11.22	0.39
A2BGB3	Apolipoprotein A-I	apoa1	13.83	1.98
Q567B9	Apolipoprotein A-IV (Fragment)	apoa4	17.21	2.72
	Other or unknown activity			
A0JMJ1	Scinderin like a	scinla	16.08	2.22
B0S564	Novel protein similar to vertebrate procollagen-proline	p4hb	16.20	0.85
B0UYN4	Novel protein (Zgc:162944)	DKEY-57A22.8	4.02	2.29
B3DFP9	Zgc:193613	apoa2	51.57	*1.72
B3DG37	Ba1 protein	ba1	50.50	1.65
Q1JQ69	Hbaa1 protein	hbaa1	93.74	0.69
B7ZVL6	Putative uncharacterized protein	tnnt3b	79.49	0.58
B8JL43	Transferrin-a	tfa	37.09	1.32
Q32PU7	Zgc:123194	zgc:123194	21.88	0.96

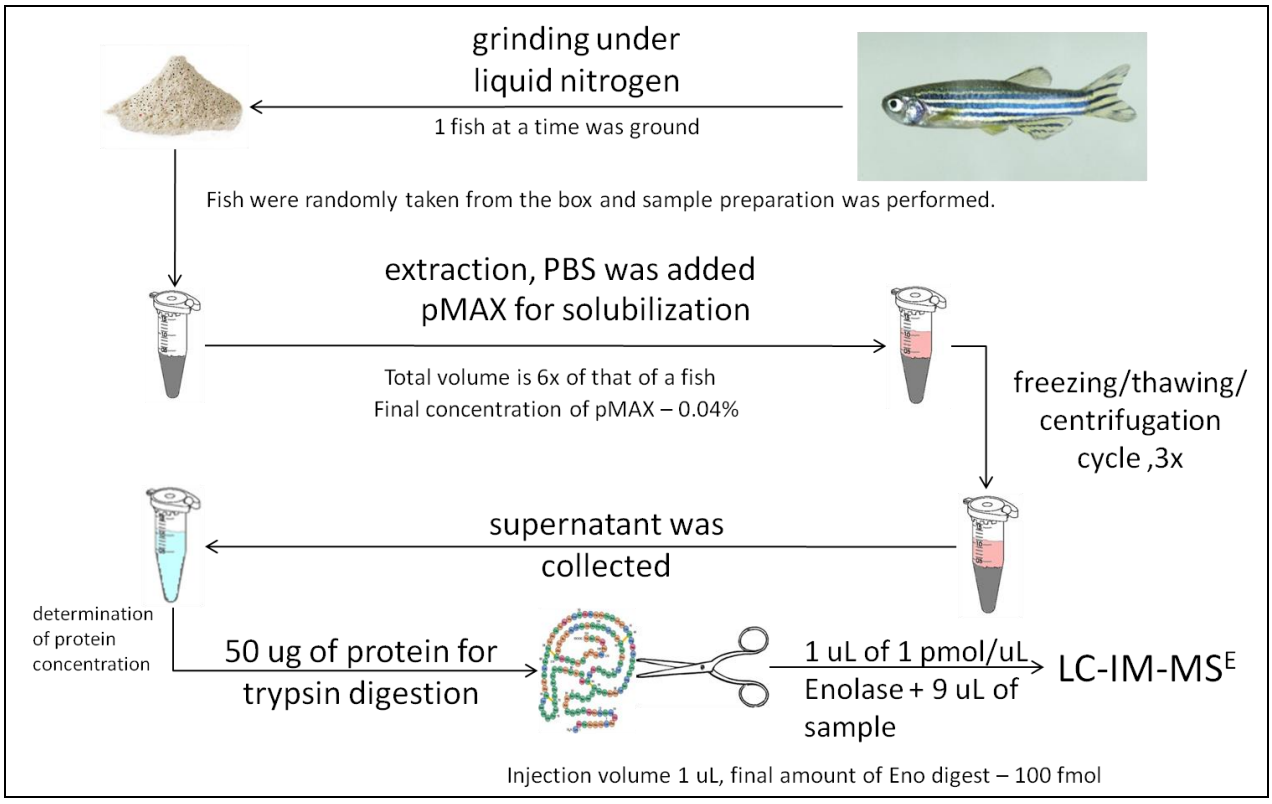
Q4JIY5	Elongation factor 1-alpha	ef1a	15.86	2.46
Q503C7	Phosphorylase	pygma	31.55	0.76
Q5RKM9	Phosphorylase	pygl	7.56	4.45
Q8UUZ6	Alpha A crystallin	cryaa	13.04	0.93
Q52JI3	Beta B3-crystallin	crybb3	15.22	0.56
Q52JI4	Beta B2-crystallin	crybb2	11.43	*1.82
Q5BJ14	Zgc:110761	mybpc2b	24.50	1.38
Q5PR62	Zgc:103458	tnni1b	5.50	1.89
Q6DHP2	Troponin I, skeletal, fast 2b.2	tnni2b.2	23.01	2.78
Q6DHU6	Troponin I, skeletal, fast 2a.3	tnni2a.3	50.41	0.67
Q6IQ92	Troponin 1	tnni1al	4.48	1.27
Q6PBJ8	Peptidyl-prolyl cis-trans isomerase	fkbp1aa	22.04	**0.19
Q6PC53	Peptidyl-prolyl cis-trans isomerase	ppial	47.77	0.74
Q6TLG8	Ribosomal protein S3	rps3	6.40	1.32
Q7ZUI4	Thioredoxin	zgc:56493	12.73	0.81
Q9DDU5	Glutathione S-transferase pi	gstp1	17.87	0.69

* p-value<0.05

** p-value<0.01

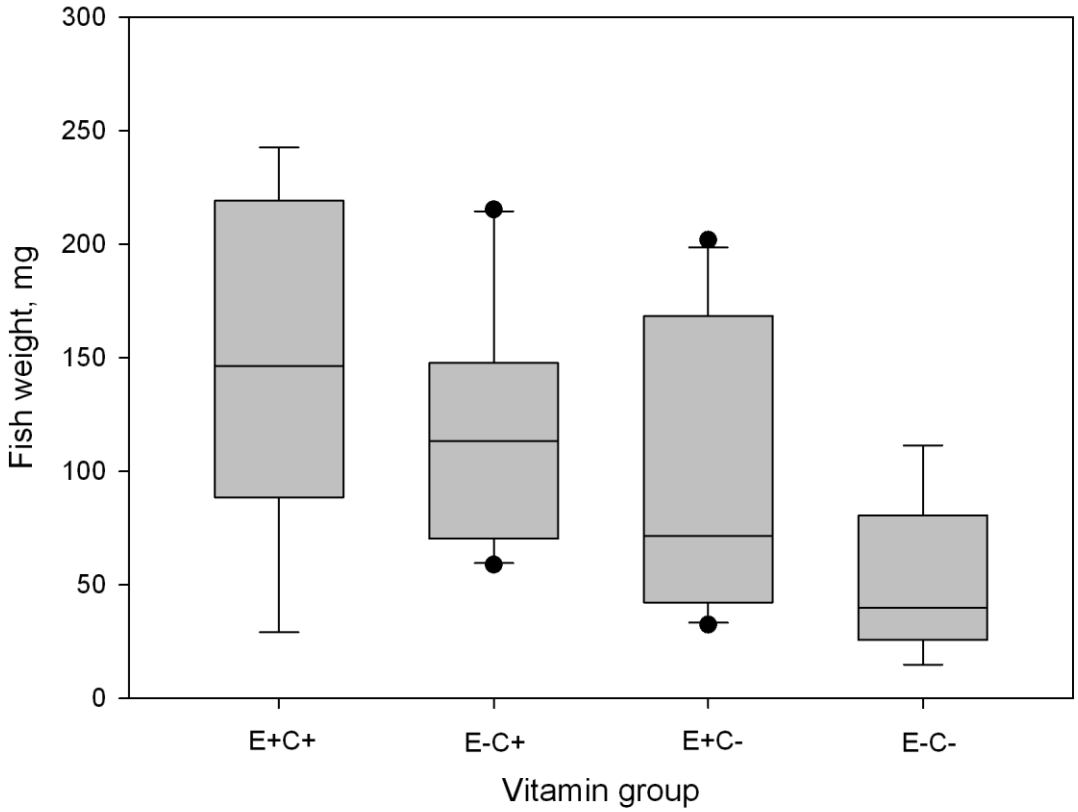


Supplementary Figure S1. Design of the feeding experiment.



Supplemental Figure S2. Workflow used for preparing the samples for the label-free "bottom up" quantitative proteomics study.

A



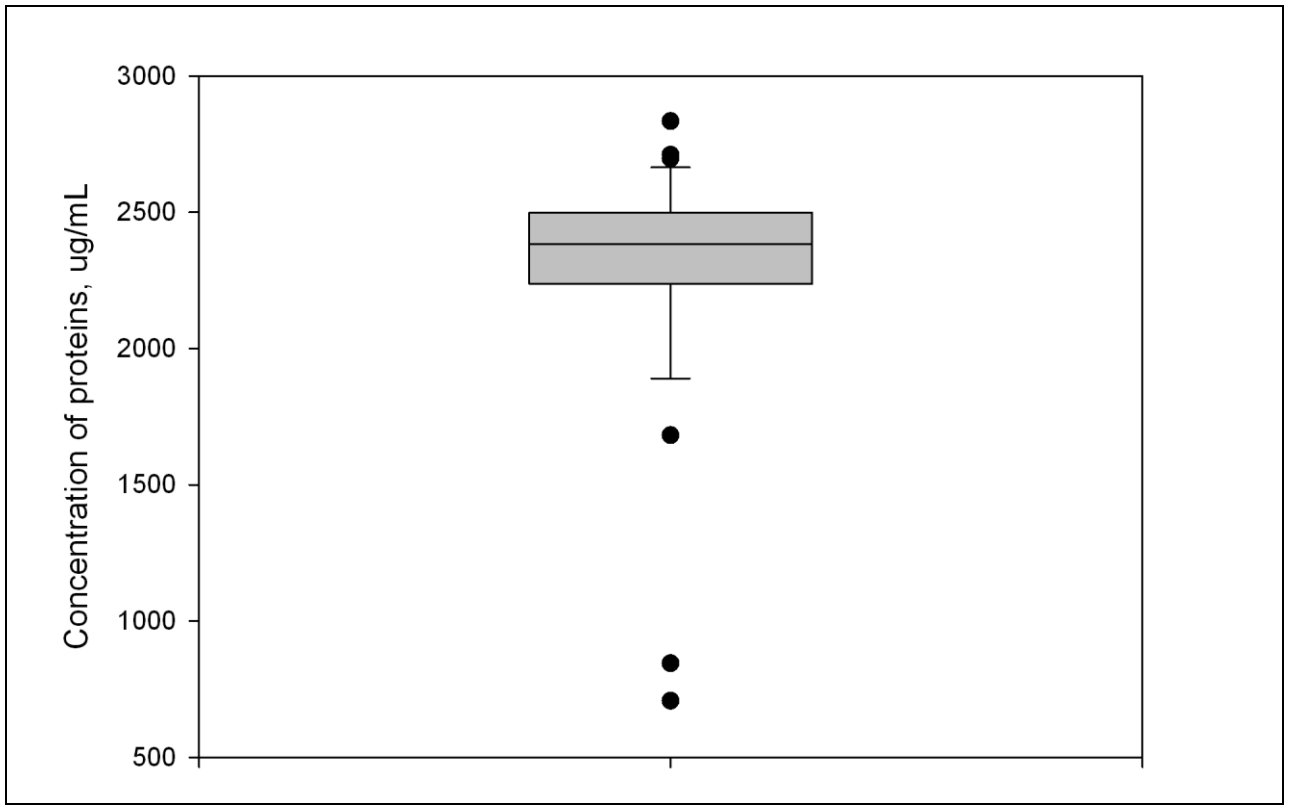
B

	E+C+	E-C+	E+C-	E-C-
E+C+	1.00			
E-C+	1.29	1.00		
E+C-	1.68	1.30	1.00	
E-C-	3.02	2.34	1.79	1.00

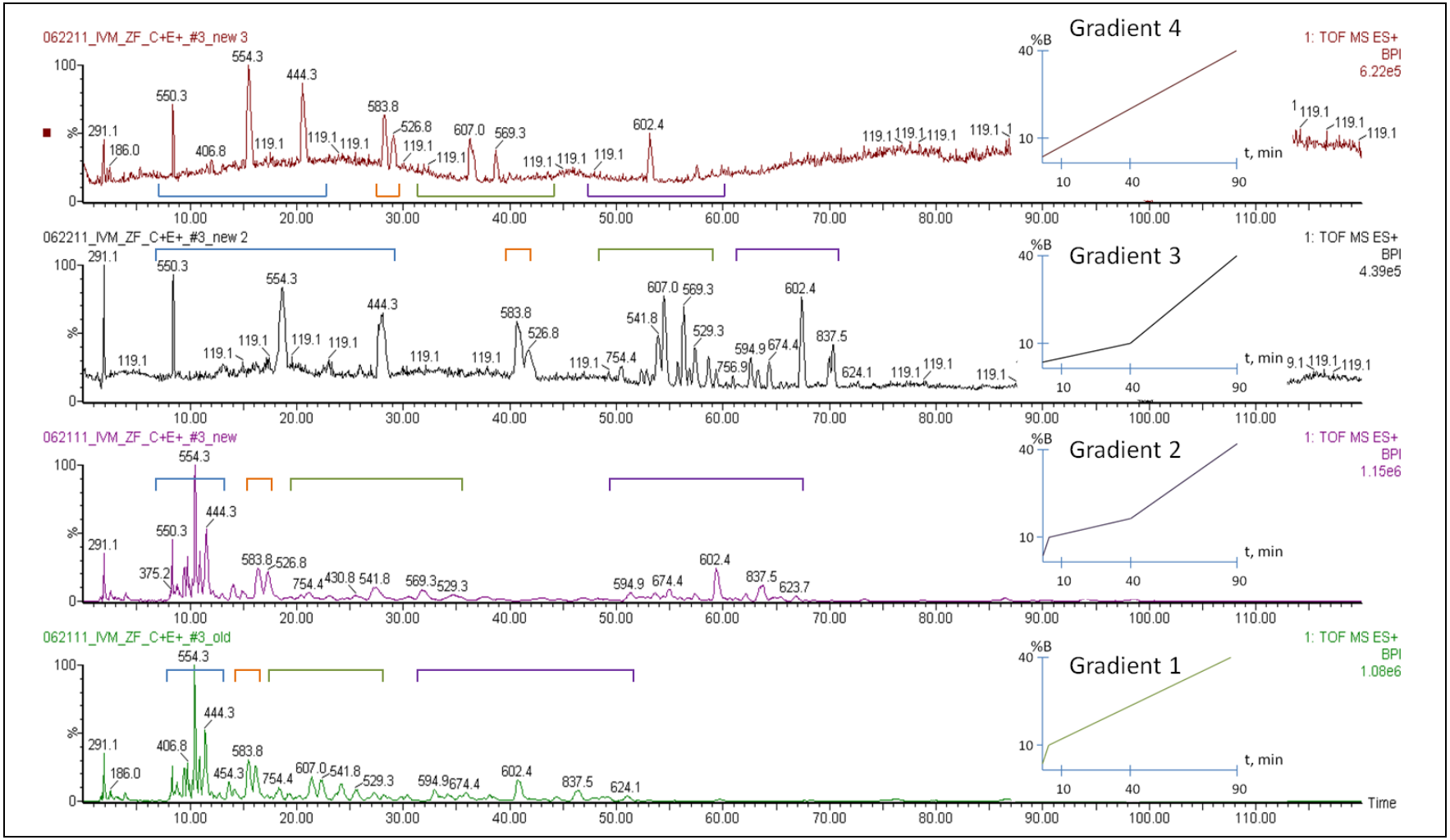
Supplementary Figure S3. Fish characteristics dependent on the vitamin regime fed.

A) Boxplots of distribution of fish body weight.

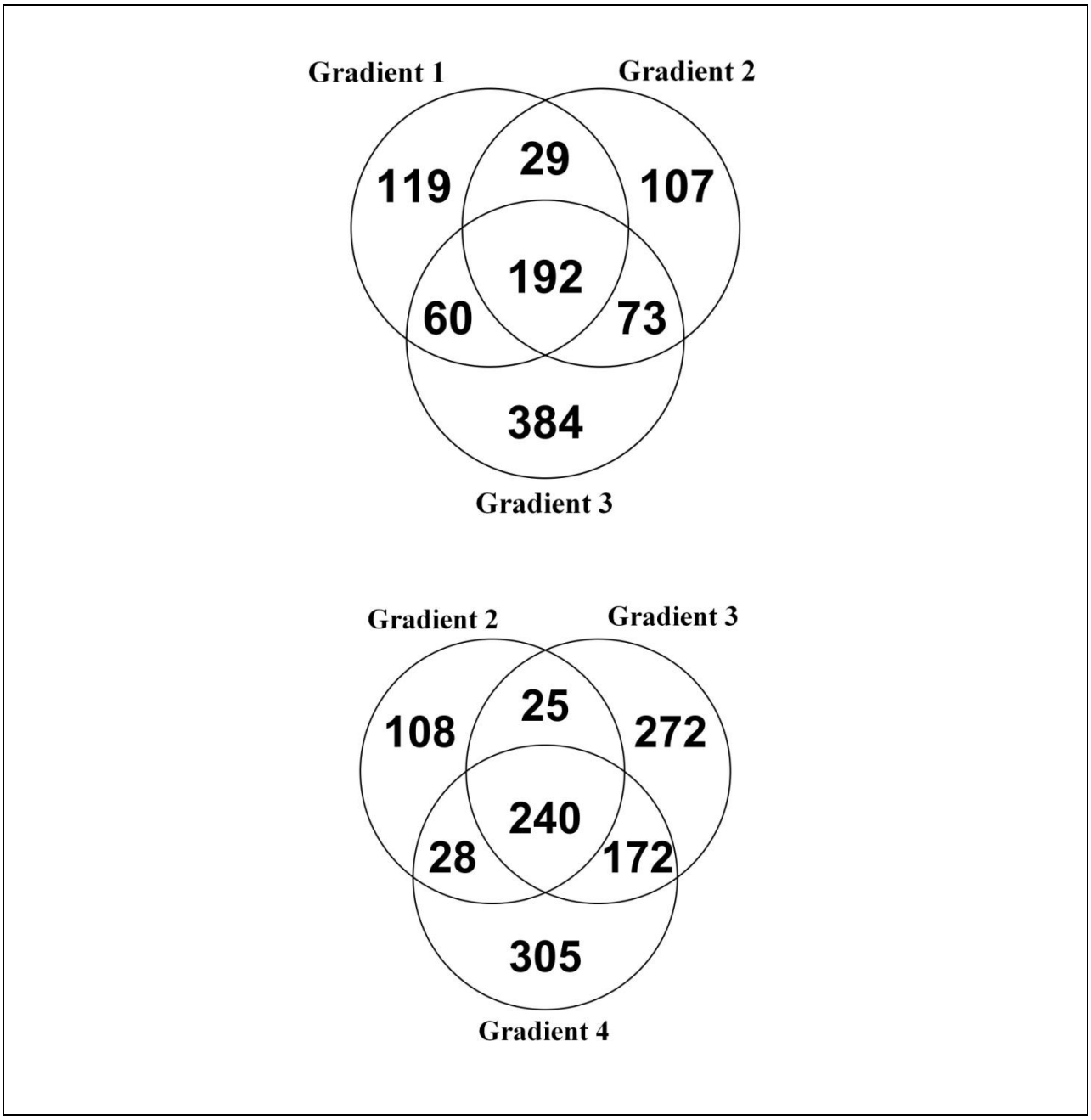
B) Ratios of median growth rate between vitamin groups.



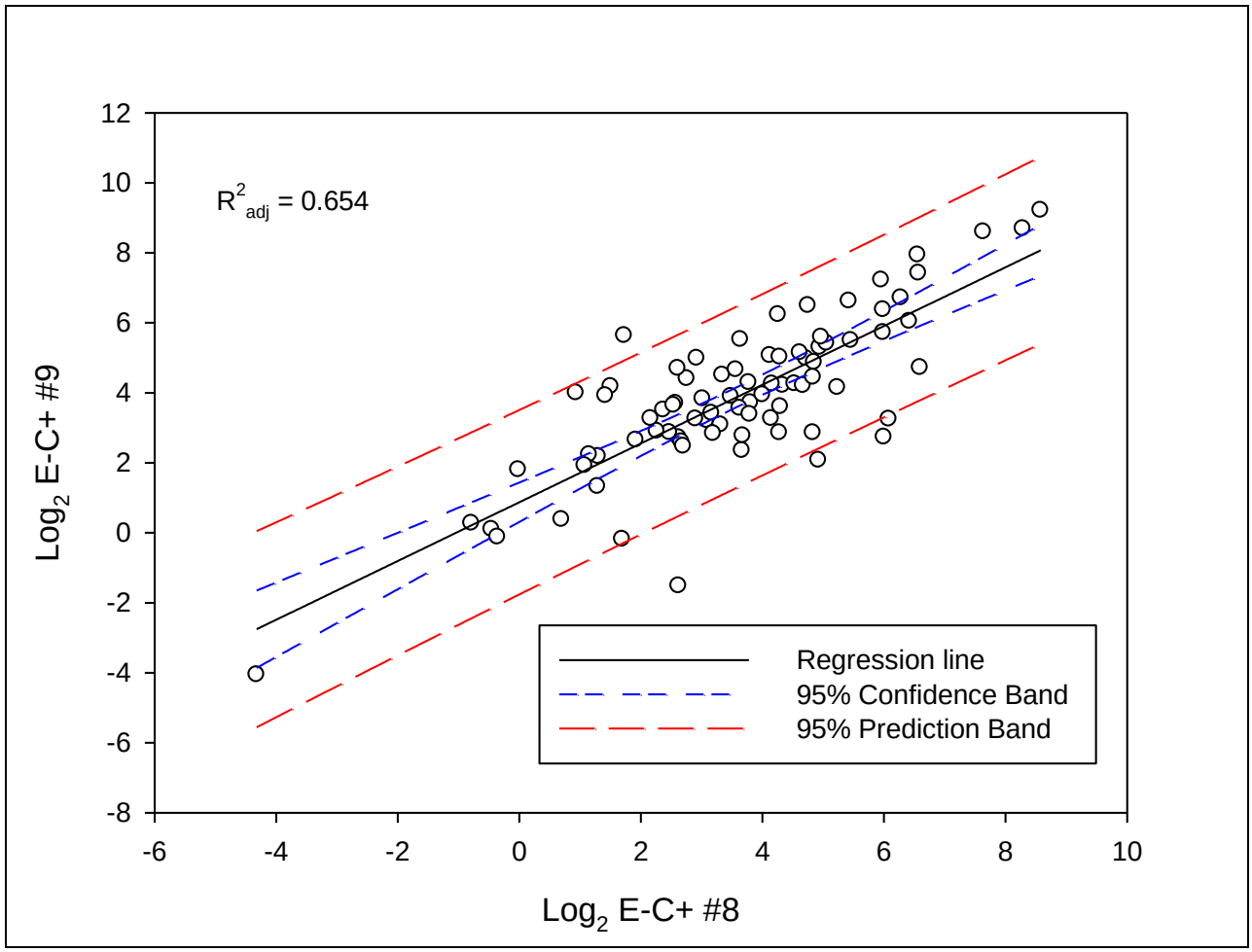
Supplemental Figure S4. Boxplot of the distribution of the total protein concentration after extraction and lysis.



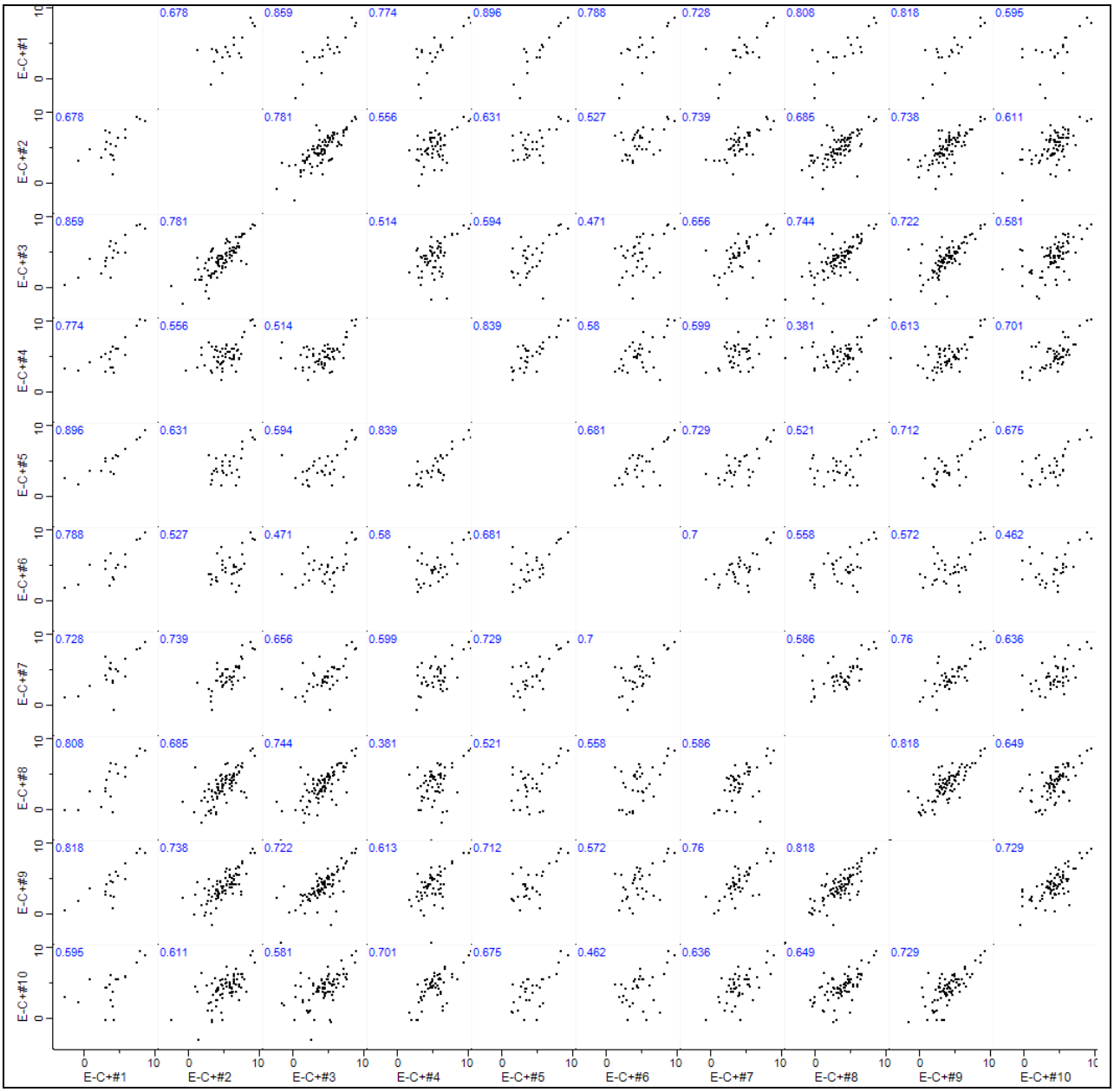
Supplementary Figure S5. Gradient profiles with respective base peak chromatograms.



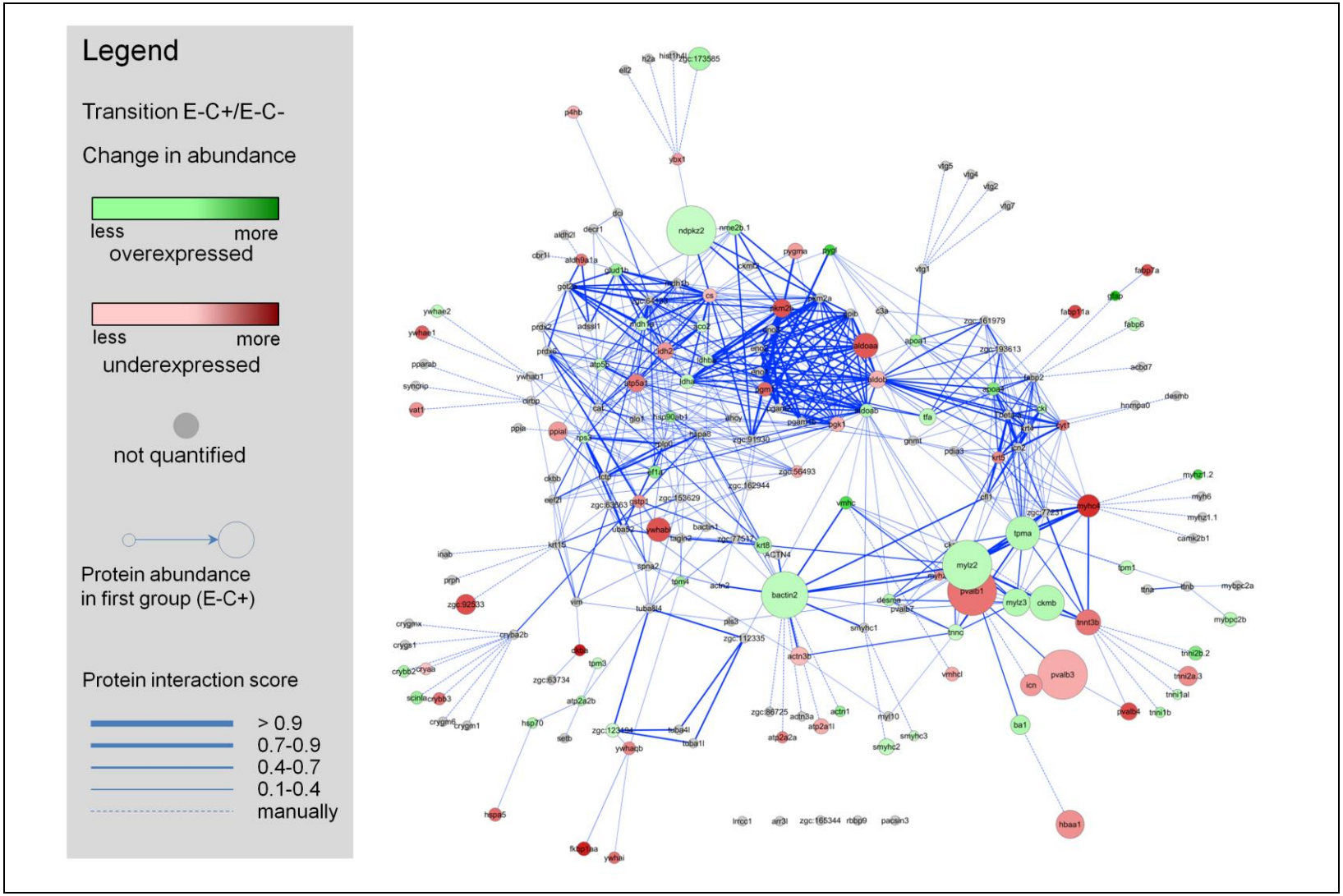
Supplementary Figure S6. Venn's diagrams comparing the number of peptides identified by LC-IM-MS^{E}.



SupplementaryFigure S7. Correlation plot between E-C+ sample 9 and E-C+ sample 8.



Supplementary Figure S8. Correlation plot matrix of the E-C+ group.



Supplementary Figure S9. The responsive protein network for the vitamin transition C+/C- with deficient vitamin E status.