Electronic Supplementary Information (ESI)

Pyrroloquinoline quinone and imidazopyrroloquinoline intake diminish mortality risk during midlife and improve muscular dysfunctions with age in mice

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Contents

Tables and Figures

Table S1 Number of male SAMP8 mice used in Experiments 1 and 2. 3

Table S2 List of the primary antibodies used for western blot analysis. 4

Fig. S1 Age-related changes of body weight and food intake of male SAMP8 mice in control, PQQ, and IPQ groups. 5

Fig. S2 Age-related change of food intake of male SAMP8 mice in control, PQQ, and IPQ groups. 6

Fig. S3 Age-related changes of blood glucose, triglyceride, β-ketone, HDL-cholesterol and plasma total cholesterol in control, PQQ and IPQ groups. 7

Fig. S4 Age-related change of the appearance of control, PQQ and IPQ groups. 8

Fig. S5 Age-related changes of the integument grading score in control, PQQ and IPQ groups. 9

Fig. S6 Effect of PQQ and IPQ intake from middle-age on the body weight and amount of food intake during the experimental period in male SAMP8. 10

Fig. S7 Effect of PQQ and IPQ intake from middle-age (7.6 months old) on survival of male SAMP8 mice. 11

Fig. S8 Effect of PQQ and IPQ intake from middle-age on the levels of blood glucose, triglyceride, β-ketone, HDL-cholesterol and plasma total cholesterol in male SAMP8 mice. 12

Fig. S9 Relationship between muscle function and muscle weight in male SAMP8 mice that began taking PQQ and IPQ from middle age. 13

Fig. S10 Western blot analysis of first- and slow-switch myosin heavy chain isoforms in different muscles of male SAMP8 mice. 14

Fig. S11 Western blot analysis of mitochondrial proteins in different muscles of male SAMP8 mice. 15

Fig. S12 Effects of PQQ and IPQ intake initiated from middle-age on adipose tissue weight in male SAMP8mice. 16

Fig. S13 Histochemical analysis of the number and size of adipocyte cells in subcutaneous fat (SUB) and epididymal fat of control, PQQ and IPQ groups. 17

Fig. S14 Liver appearance and histochemical analysis of the number and size of lipid droplets in the liver control, PQQ and IPQ groups. 18

Fig. S15 Effects of PQQ and IPQ intake initiated from middle-age on the mitochondrial proteins and the activity of fatty acid oxidation in liver. 19

Tables and Figures

Table S1 Number of male SAMP8 mice used in Experiments 1 and 2.

The number and age of male mice used in each experiment are shown in the table below.

	Experiments	Age	Control	PQQ	IPQ
Exp. 1	Survival	4 wk.	15	16	15
	Muscle function evaluation (Four and two limb hanging tests, Four limb grip strength test)	3 mo.	14	16	15
		6 mo.	13	16	15
		9 mo.	11	16	15
		12 mo.	9	14	11
	Blood test (Glucose, Triglyceride, β-ketone, HDL-cholesterol, Plasma total cholesterol)	3 mo.	15	16	15
		6 mo.	14	16	15
		9 mo.	11	16	15
		12 mo.	10	14	12
		15 mo.	5	11	6
	Appearance evaluation	2 mo.	15	15	13
		6 mo.	15	15	13
		9 mo.	11	14	13
		12 mo.	8	13	8
		15 mo.	5	7	6
Exp. 2	Survival	33 wk.	12	11	13
	Muscle function evaluation (Four and two limb hanging tests, Four limb grip strength test)	Before (33 wk.)	11	11	13
		6th wk. (39 wk.)	10	11	11
		12th wk. (45 wk.)	8	8	10
	Blood test (Glucose, Triglyceride, β-ketone, HDL-cholesterol, Plasma total cholesterol)	Before (33 wk.)	12	11	12
		6th wk. (39 wk.)	10	11	11
		12th wk. (45 wk.)	10	8	10
	Tissue weight	12th wk. (45 wk.)	8	8	10
	Western blot analysis	12th wk. (45 wk.)	5	5	5
	Adipocyte cell size analysis	12th wk. (45 wk.)	3	3	3
	Liver lipid droplet analysis	12th wk. (45 wk.)	3	3	3
	Liver β-oxidation assay	12th wk. (45 wk.)	5	5	5

Table S2 List of the primary antibodies used for western blot analysis.

Antibody	Mo/Po	Source/Isotype	Cat. No	Vendor
Myosin heavy chain 4 (MYH4)	Po	Rabbit/IgG	20140-1-AP	Proteintech
Myosin heavy chain 7 (MYH7)	Po	Rabbit/IgG	2280-1-AP	Proteintech
Succinate dehydrogenase complex, subunit A (SDHA)	Po	Rabbit/IgG	14865-1-AP	Proteintech
ATP synthase F1 subunit alpha (ATP5A1)	Po	Rabbit/IgG	14676-1AP	Proteintech
β-actin (D6A8)	Mo	Rabbit/IgG	#8457	Cell Signaling

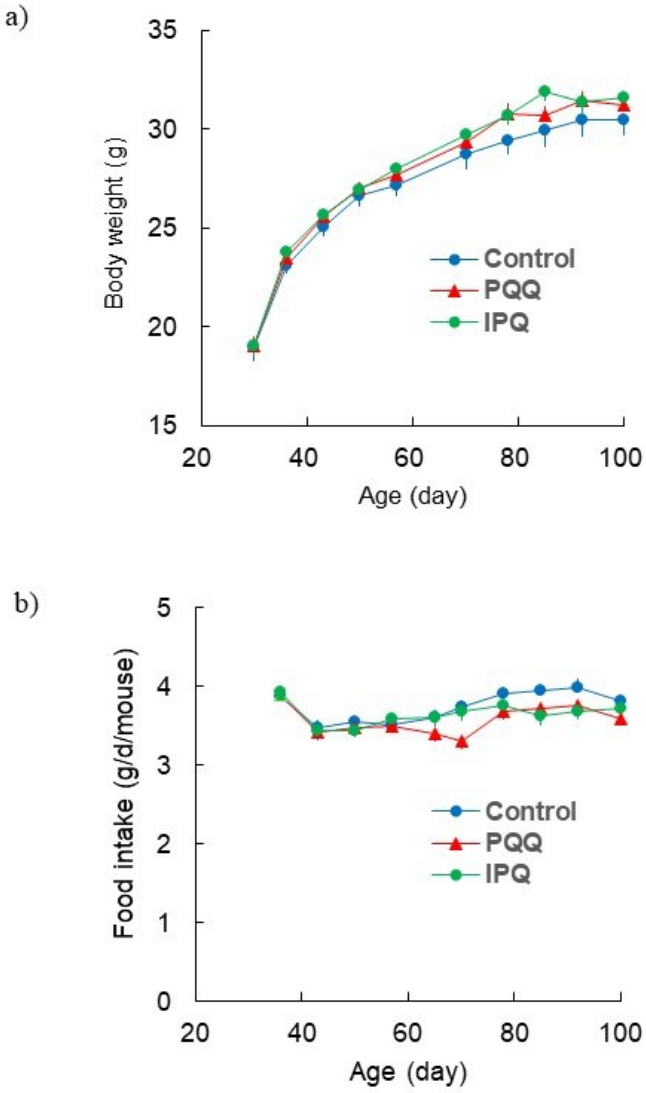


Fig. S1 Age-related changes of body weight and food intake of male SAMP8 mice in control, PQQ, and IPQ groups.
(a) Age-related change of body weight in control (blue, n=15), PQQ (red, n=16) and IPQ (green, n=15) groups. (b) The amount of food intake in in control (blue, n=15), PQQ (red, n=16) and IPQ (green, n=15) groups. Each value is the mean±SEM.

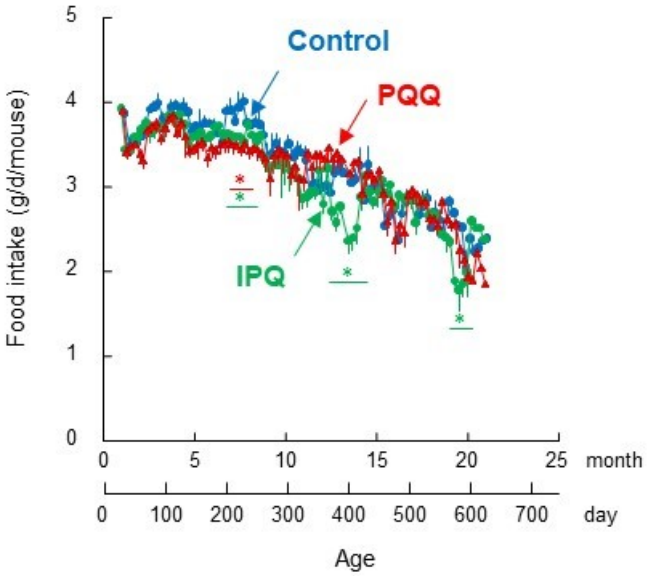


Fig. S2 Age-related change of food intake of male SAMP8 mice in control, PQQ, and IPQ groups.

Asterisks denote a statistically significant difference ($p < 0.05$) when compared with the control as determined using one-way ANOVA followed by Dunnett's post-hoc test.

Each value is the mean ± SEM. Number of mice （at the start, 1 month old) / (at the end, 21 months old): Control group (15 mice / 3 mice), PQQ group (16 mice / 3 mice), IPQ group (15 mice / 4 mice)

There was statistical significant differences in food intake between the control group and the PQQ and IPQ groups at about 7, 14 and 19 months of age. The significant temporary drops in food intake are due to an increase in the proportion of individuals whose food intake have decreased dramatically and who are close to death.

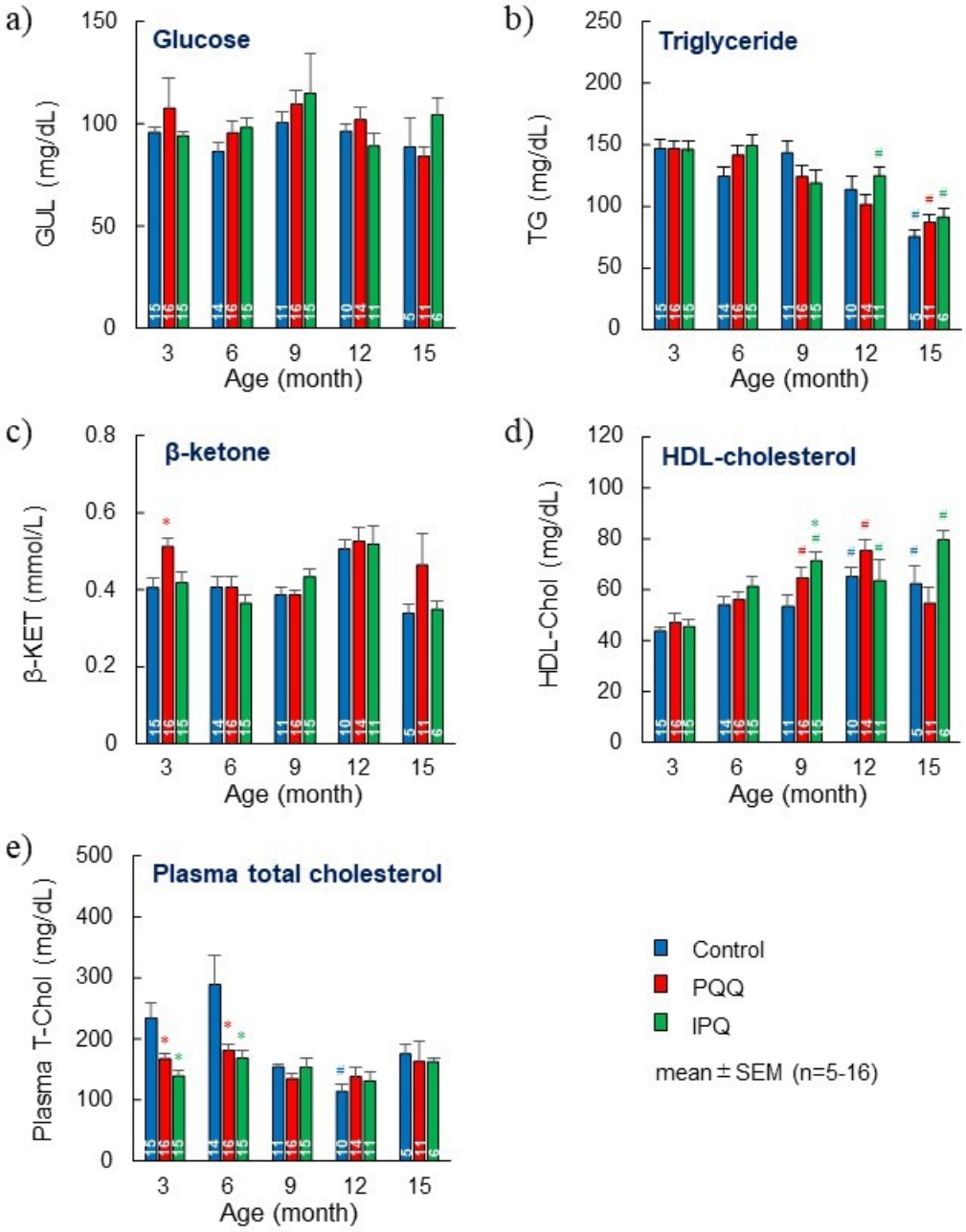


Fig. S3 Age-related changes in blood glucose, triglyceride, β-ketone, HDL-cholesterol, and plasma total cholesterol in control, PQQ, and IPQ groups.

Whole blood from the tail tip of a mouse was used to measure (a) glucose, (b) triglyceride, (c) β-ketone, and (d) HDL cholesterol levels. (e) Plasma prepared from blood from the tail tip was used to measure the total cholesterol level. # compared with each 3 month-old group, $p<0.05$ (paired t-test), * compared with the control group at the same age, $p<0.05$ (Dunnett's post-hoc test). Each value is the mean±SEM. The number of mice used in each group is shown on each bar.

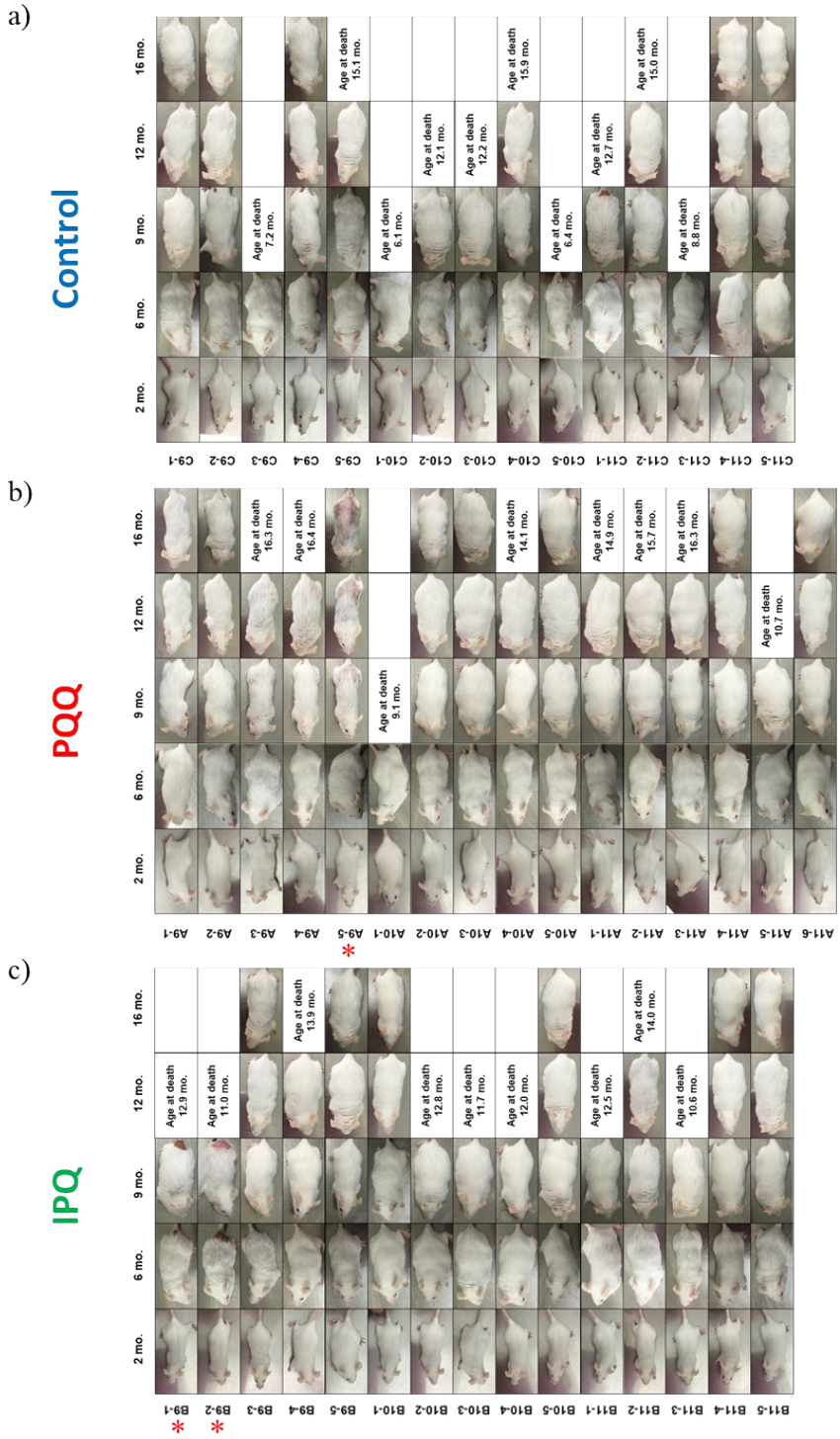
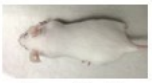


Fig. S4 Age-related changes in the appearance of control, PQQ, and IPQ groups.

Photographs of the backs of all mice in (a) control, (b) PQQ, and (c) IPQ groups were used to assess appearance aging. Photographs were taken at 2, 6, 9, 12, and 16 months of age. Blank spaces indicate the age at death for each mouse.

* Mice that lost hair due to fighting were excluded from assessment of the degree of appearance aging.

a)

	A	B	C
			
Age	**2 month-old**	**14 month-old**	**14 month-old**
Grading Scores			
Glossiness	0	3	2
Coarseness	0	1	2
Hair Loss	0	0	2
Skin Ulcer	0	0	0
Total score	**0**	**4**	**6**

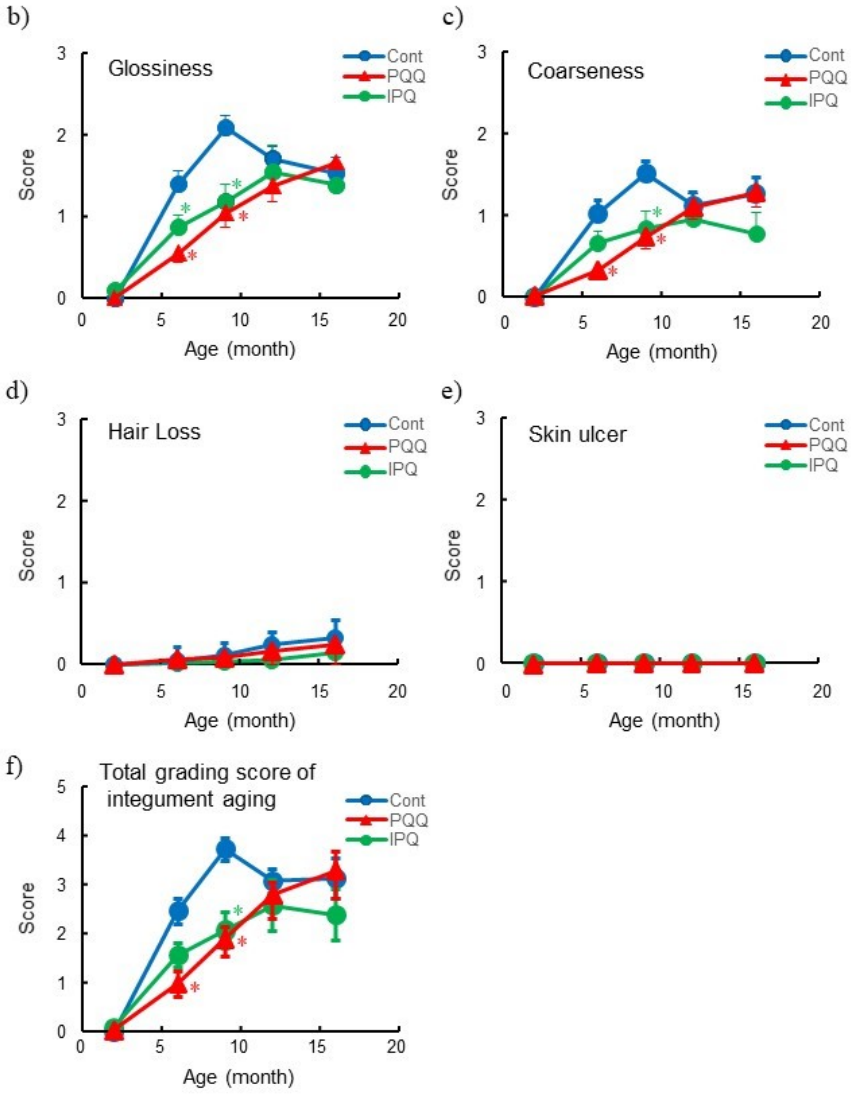


Fig. S5 Age-related changes of the integument grading score in control, PQQ and IPQ groups.

(a) Representative dorsal photographs of mice of different ages and their grading scores for glossiness, coarseness, hair loss, and skin ulcers. Age-related alteration of the integument grading score of (b) glossiness, (c) coarseness, (d) hair loss, (e) skin ulcer and (f) total grading score of integument aging. * Compared with the control group at the same age, $p<0.05$ (Dunnett's post-hoc test). Control group (n=5-15), PQQ group (n=7-15), IPQ group (n=6-13). Details of the number of mice used at each age in the experiment are shown in Table S1, Each value is the mean±SEM.

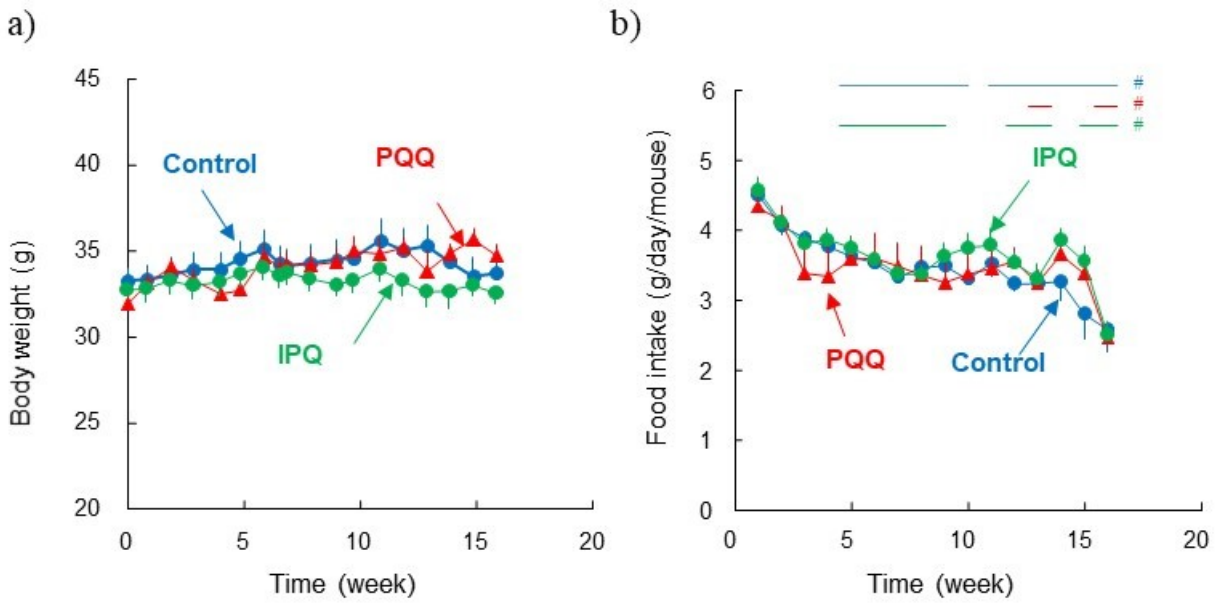
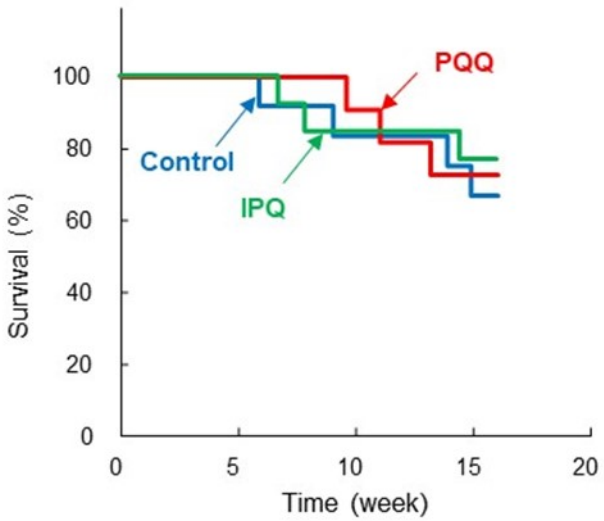


Fig. S6 Effect of PQQ and IPQ intake from middle-age on the body weight and amount of food intake during the experimental period in male SAMP8.

(a) Change of body weights in the control (blue, n=8-12), PQQ (red, n=8-11) and IPQ (green, n=10-13) groups. b) Change in food intake (g/day/mouse) in the control (blue, n=8-12), PQQ (red, n=8-11), and IPQ (green, n=10-13) groups. Bar with #: Comparison of food intake in the first week of each group, $p<0.05$ (paired t-test). The control, PQQ, and IPQ diets were administered to mice from 7.6 months (33 weeks). Each value is the mean± SEM.

a)



b)

	n	Log-rank *p* -value	Gehan *p* -value
Control	12	-	-
PQQ	11	0.7673	0.7632
IPQ	13	0.6047	0.6313

Fig. S7 Effect of PQQ and IPQ intake from middle-age (7.6 months old) on survival of male SAMP8 mice

(a) Kaplan-Meier survival curves of control (blue), PQQ (red) and IPQ (green) groups. (b) Statistical results of log-rank and Gehan tests for Kaplan-Meier survival curves. There was no significant differences among the three groups.

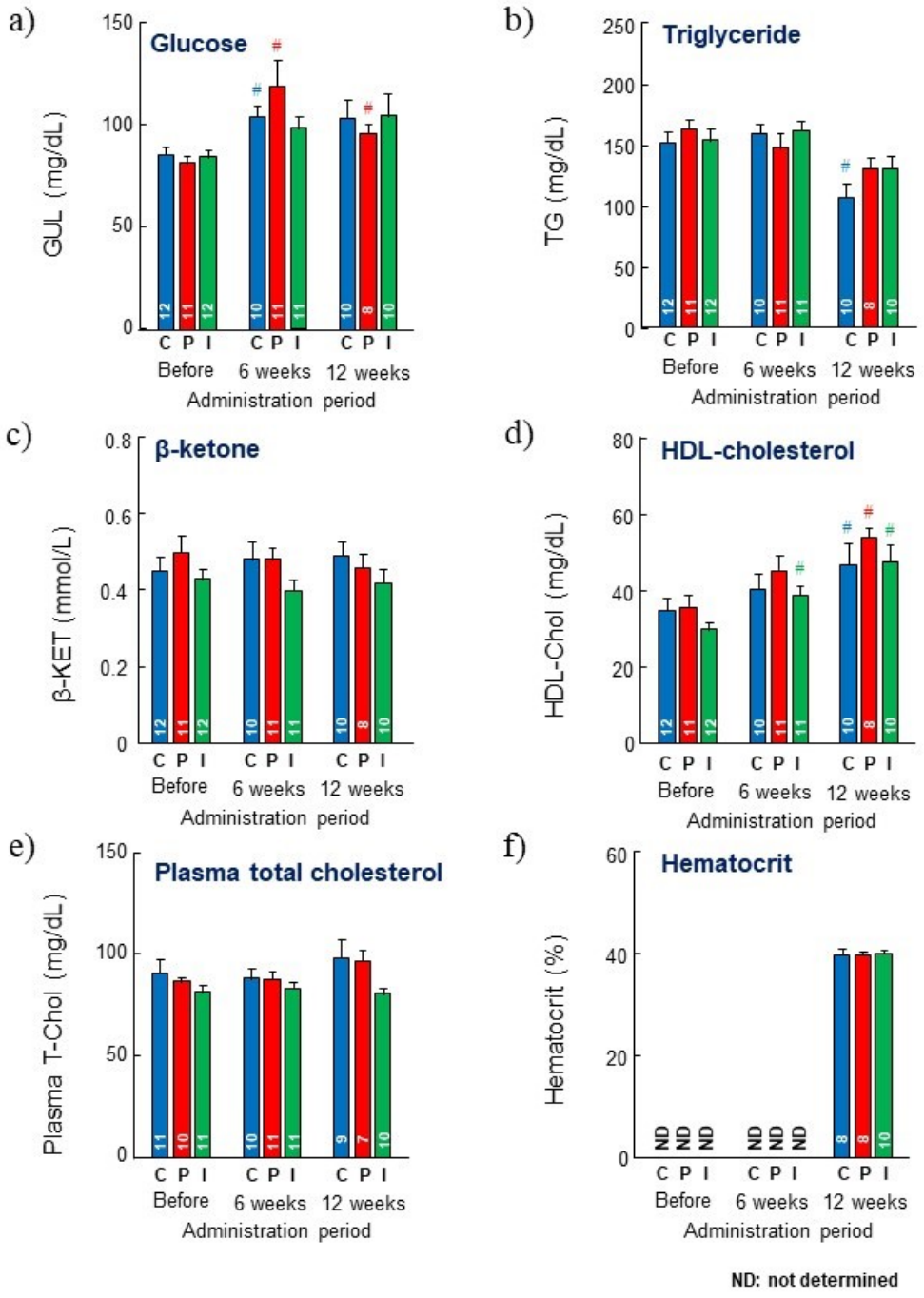


Fig. S8 Effect of PQQ and IPQ intake from middle-age on the levels of blood glucose, triglyceride, β-ketone, HDL-cholesterol and plasma total cholesterol in male SAMP8 mice.

Whole blood from the tail tip of a mouse was used to measure (a) glucose, (b) triglyceride, (c) β-ketone, and (d) HDL cholesterol levels. (e) Plasma prepared from blood from the tail tip was used to measure the total cholesterol level. (f) The hematocrit value was determined using blood obtained from the inferior vena cava at the time of dissection after completion of the 16-week administration experiment. ND: not determined. #: compared with each 3 month-old group, $p<0.05$ (paired t-test). Each value is the mean± SEM.

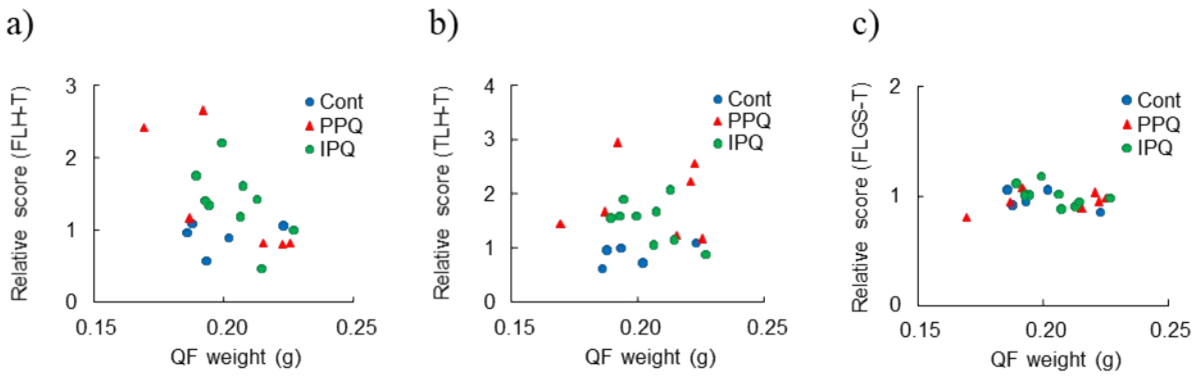


Fig. S9 Relationship between muscle function and muscle weight in male SAMP8 mice that began taking PQQ and IPQ from middle age.

Quadriceps femoris (QF) muscle weight vs. relative scores of (a) the four-limbed hanging test (FLH-T), (b) two-limbed hanging test (TLH-T), and (c) four-limbed grip strength test (FLGS-T) performed in the control (blue), PQQ (red), and IPQ (green) groups at week 12. No significant correlation was observed between the three test scores and the QF weight. Furthermore, no significant correlation was observed between muscle function and other muscles (tibialis anterior [TA], soleus [SOL], plantaris [PL], and gastrocnemius [GC]) (data not shown).

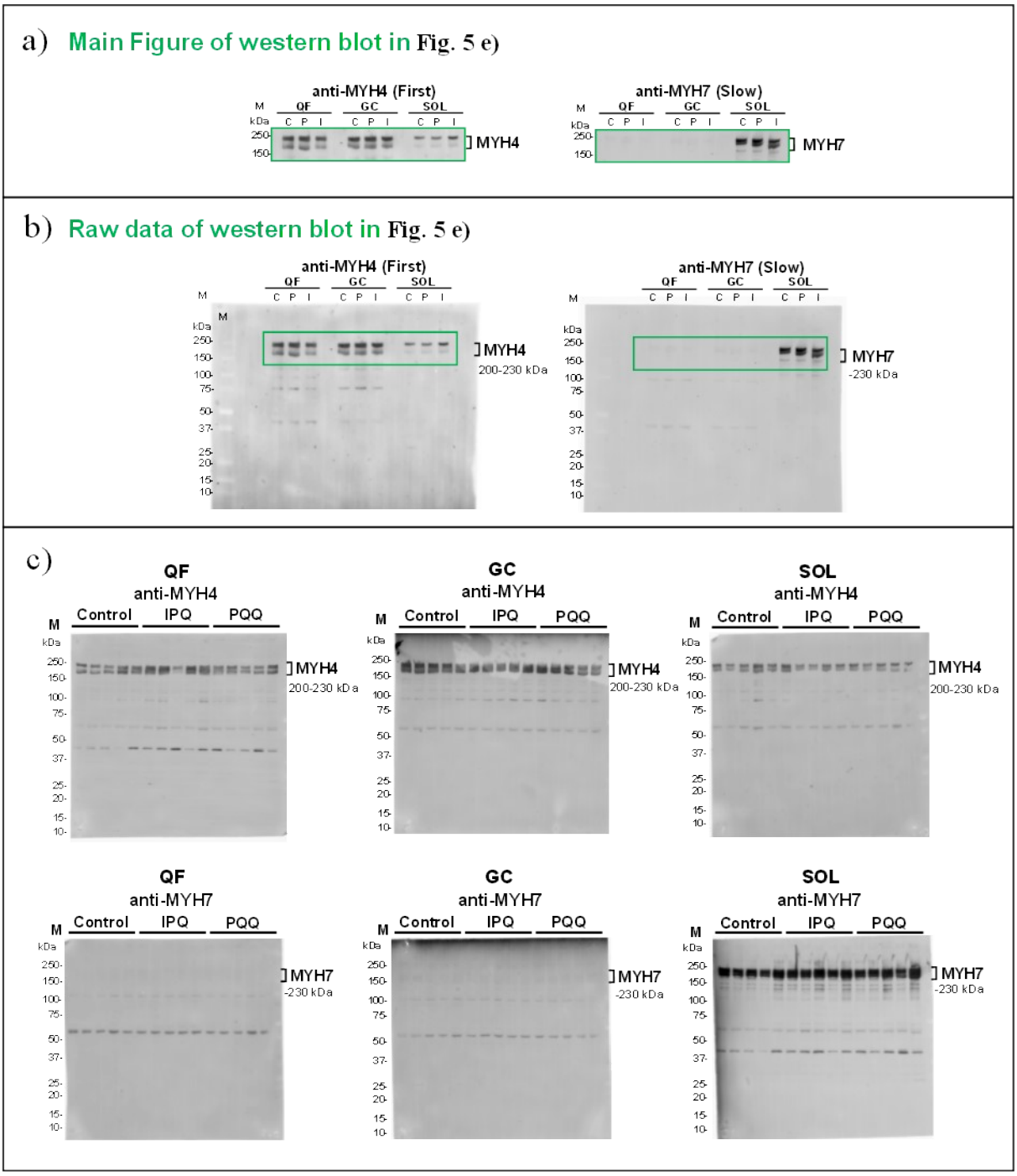


Fig. S10 Western blot analysis of first- and slow-switch myosin heavy chain isoforms in different muscles of male SAMP8 mice.

Western blot analysis of first-switch myosin heavy chain 4 (MYH4, 200-230 kDa) and slow-switch myosin heavy chain 7 (MYH7, 230 kDa) was conducted in the quadriceps femoris (QF), soleus (SOL), and gastrocnemius (GC) muscles of mice administered PQQ and IPQ for 12 weeks. (a) Western blot of MYH4 and MYH7 in an equal amount of mixtures of muscle homogenate from five mice in each group shown in Fig. 5c and (b) its raw data. (c) Western blot of MYH4 and MYH7 in homogenate from individual animals. M: Molecular weight marker (10-250 kDa).

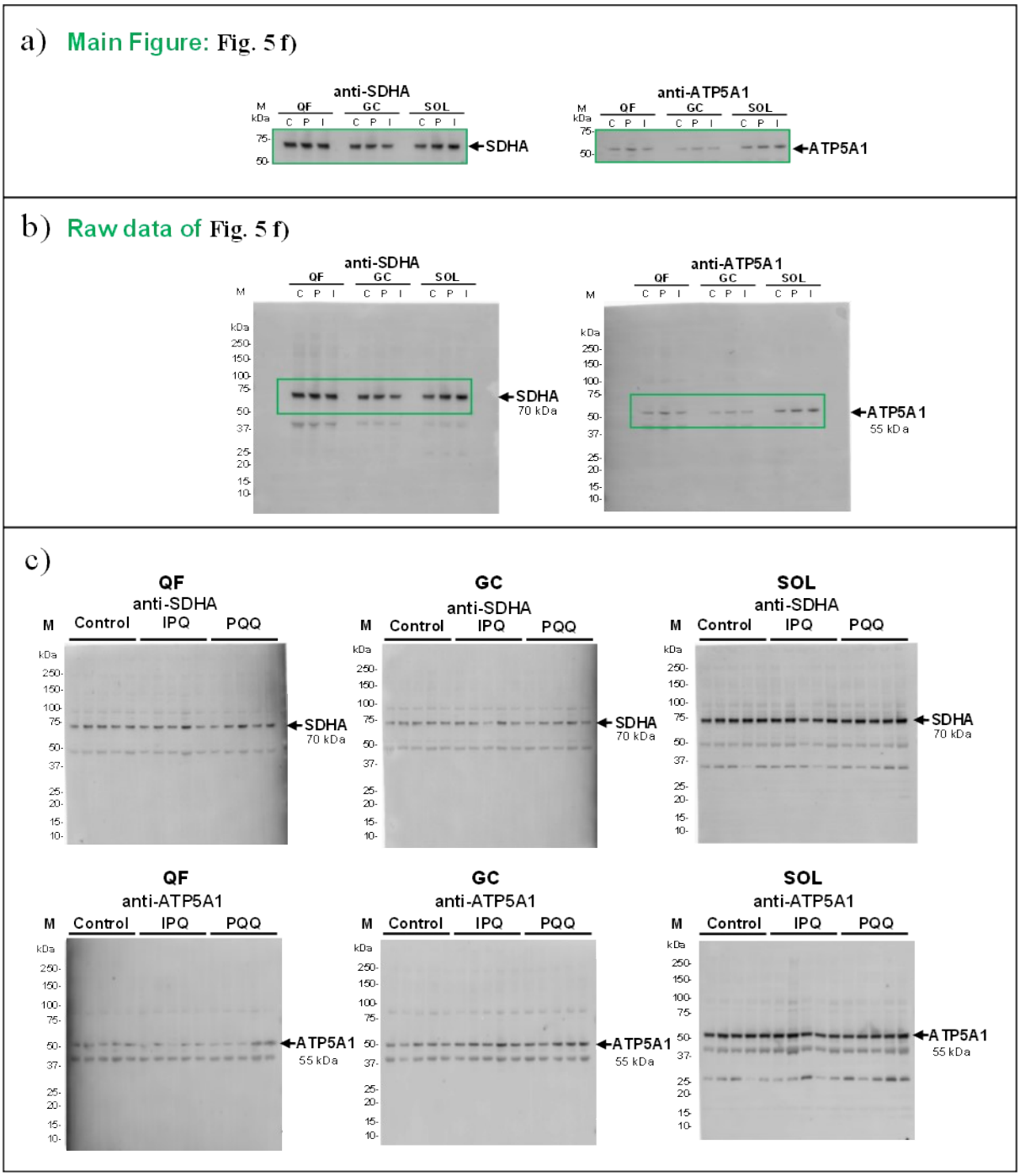


Fig. S11 Western blot analysis of mitochondrial proteins in different muscles of male SAMP8 mice.

Western blot analysis of succinate dehydrogenase complex, subunit A (SDHA, 70 kDa) and ATP synthase F1 subunit alpha (ATP5A1, 55 kDa) was conducted in the quadriceps femoris (QF), soleus (SOL), and gastrocnemius (GC) muscles of mice administered PQQ and IPQ for 12 weeks. (a) Western blot of SDHA and ATP5A1 in an equal amount of mixtures of muscle homogenate from five mice in each group shown in Fig. 5f, and (b) its raw data. (c) Western blot of SDHA and ATP5A1 in homogenate from individual animals. M: Molecular weight marker (10-250 kDa).

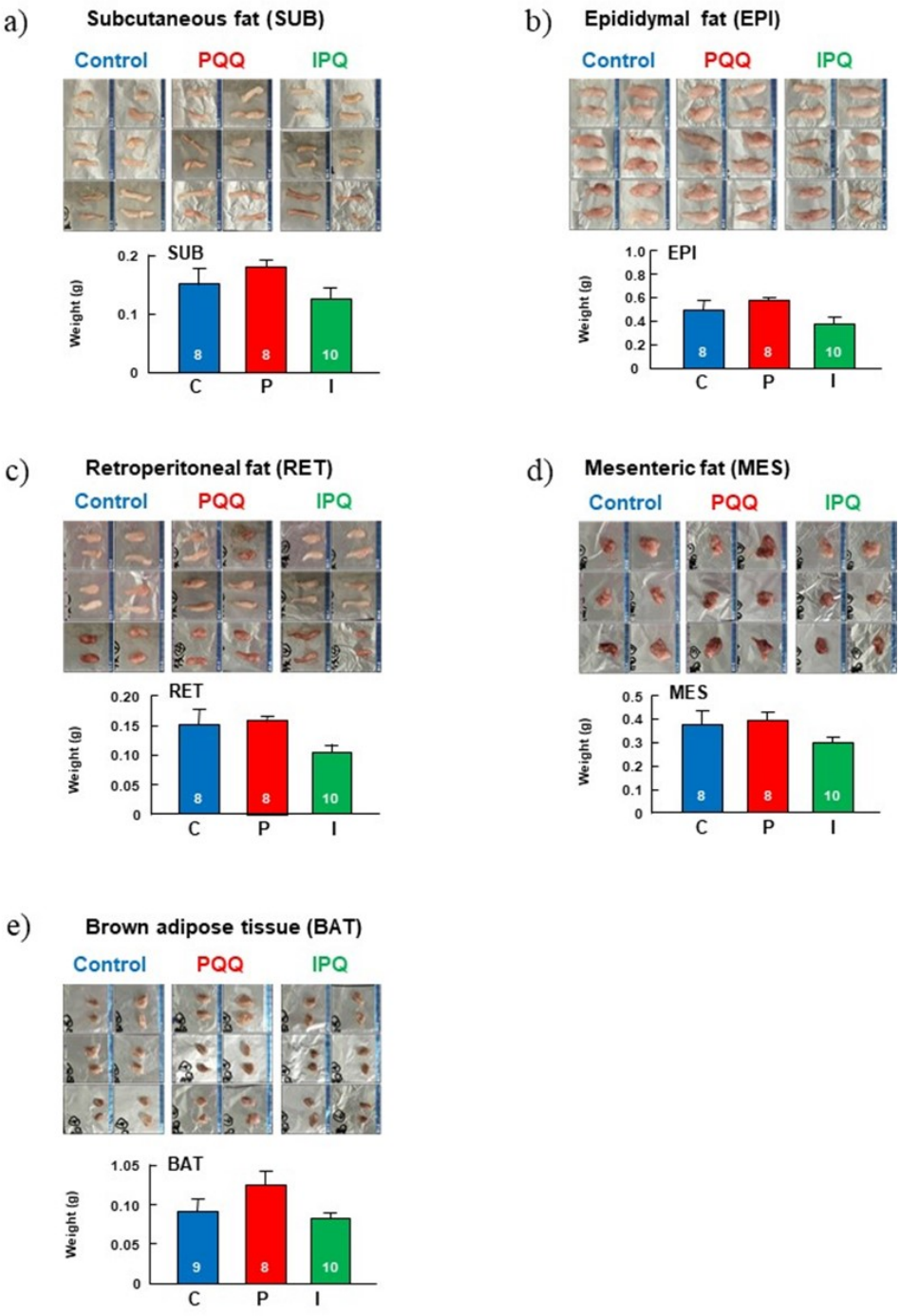
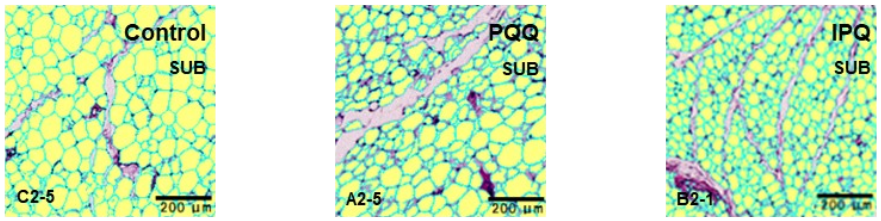


Fig. S12 Effects of PQQ and IPQ intake initiated from middle-age on adipose tissue weight in male SAMP8 mice.
Appearance and weights of different adipose tissues from the control (C), PQQ (P) and IPQ (I) groups.
(a) Subcutaneous fat (SUB), (b) Epididymal fat (EPI), (c) Retroperitoneal fat (RET), (d) Mesenteric fat (MES), (e) Brown adipose tissue (BAT). No Significant difference was observed compared with Control group (Dunnett's post-hoc test). The number of animals used is shown on each bar. Each value is the mean± SEM.

a)

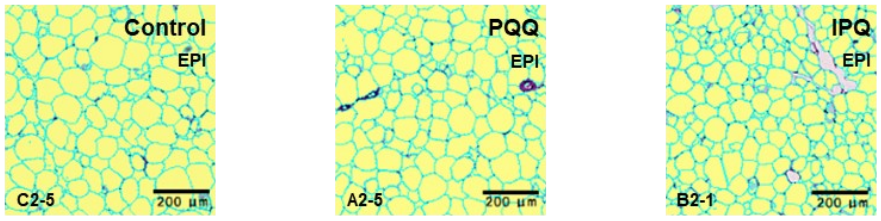


SUB cell size analysis

	Control	PQQ	IPQ
Size（μm^2）			
Mean	1482 ± 382	1333 ± 183	768 ± 28
Minimum [a)]	214	214	214
Quartile (25%)	760 ± 133	628 ± 28	437 ± 7
Median	1266 ± 262	1092 ± 40	688 ± 24
Quartile(75%)	1946 ± 537	1775 ± 251	1040 ± 50
Maximum	3704 ± 1096	3441 ± 571	1923 ± 140
Quantity (cells)	2556 ± 187	1853 ± 217	2805 ± 204

[a)] Lower analysis size limit : 200μm^2

b)



EPI cell size analysis

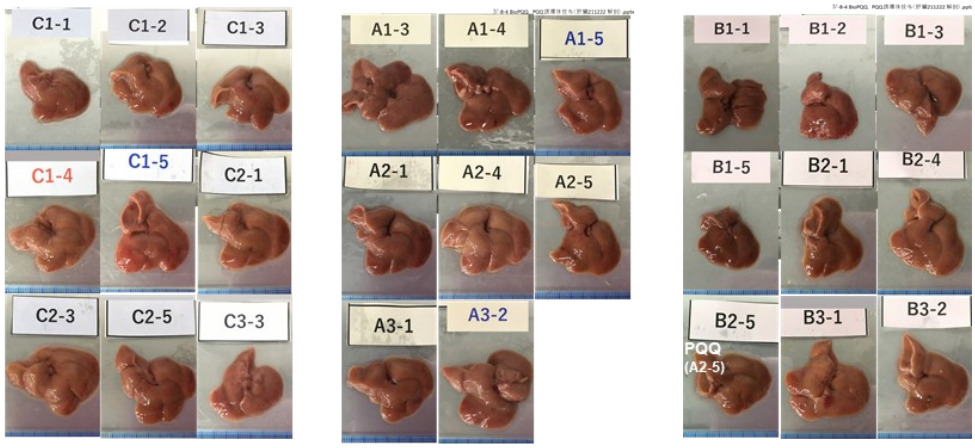
	Control	PQQ	IPQ
Size（μm^2）			
Mean	2788 ± 237	2703 ± 246	1899 ± 102
Minimum [a)]	214	214	214
Quartile（25%）	1130 ± 87	1097 ± 80	841 ± 82
Median	2463 ± 49	2489 ± 33	1690 ± 163
Quartile (75%)	4055 ± 382	3985 ± 458	2707 ± 184
Maximum	3704 ± 1096	3441 ± 571	1923 ± 140
Quantity (cells)	5480 ± 516	3480 ± 62	3536 ± 85

[a)] Lower analysis size limit : 200μm^2

Fig. S13 Histochemical analysis of the number and size of adipocyte cells in subcutaneous fat (SUB) and epididymal fat of control, PQQ and IPQ groups.

Analysis of the number and size of adipocytes was performed using H&E stained adipose tissues of subcutaneous fat, SUB (a) and epididymal fat, EPI (b) Representative images of the section of adipocytes (yellow) surrounded by green lines created using Hybrid cell count analysis software (KEYENCE) and the analysis results are shown in the table. Each value is the mean± SD (n=3).

a)



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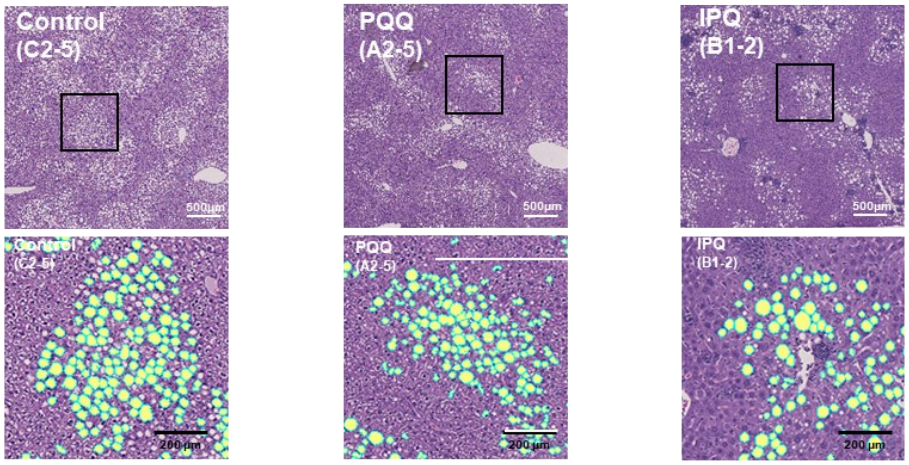

c)

	Control	PQQ	IPQ
Size (μm^2)			
Mean	460 ±39	433 ±31	508 ±43
Minimum (*)	223 ±24	199 ±0	199 ±0
Quartile (25%)	295 ±24	261 ±17	285 ±20
Median	389 ±24	351 ±44	427 ±40
Quaritile (75%)	541 ±50	513 ±61	617 ±64
Maximum	902 ±111	883 ±123	1092 ±158
Quantity (Lipid droplets)	2076 ±530	1948 ±270	1632 ±1025

*Lower analysis size limit : <199μm^2

Fig. S14 Liver appearance and histochemical analysis of the number and size of lipid droplets in the liver control, PQQ, and IPQ groups.

(a) Liver appearance, (b) Representative images of the liver lipid droplets (yellow) surrounded by green lines created using Hybrid cell count analysis software (KEYENCE). c) Summary of lipid droplet size analysis. Each value is the mean± SD (n=3).

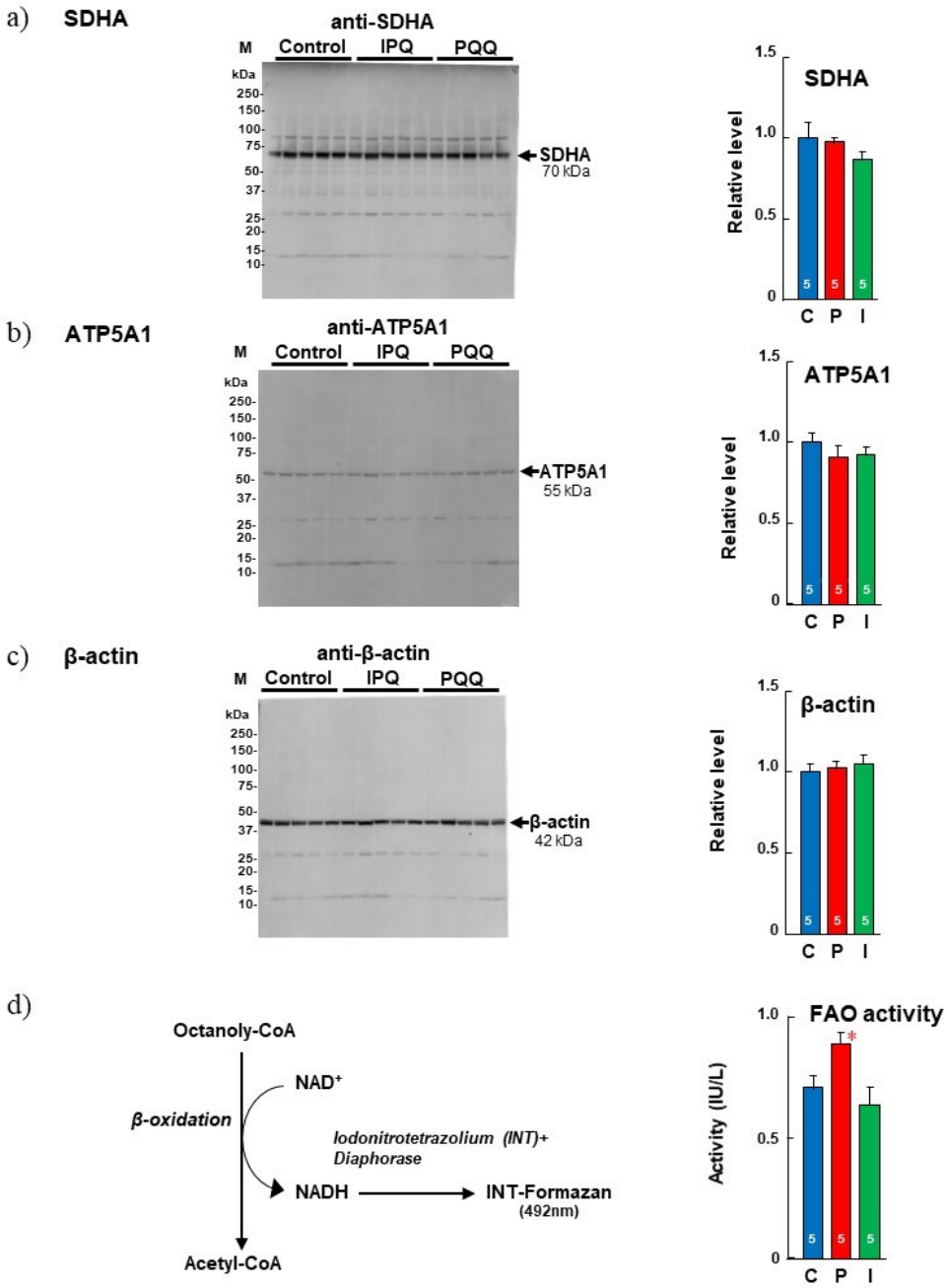

Fig. S15 Effects of PQQ and IPQ intake initiated from middle-age on the mitochondrial proteins and the activity of fatty acid oxidation in liver.

Western blot analysis of (a) SDHA, (b) ATP5A1, and (c) β-actin was performed using liver homogenates prepared individually from each of the five mice in the control, PQQ, and IPQ group, respectively. M: Molecular weight marker (10-250 kDa). No significant differences were observed in the expression levels of any of the three proteins among the control (C), PQQ (P), and IPQ (I) groups. (d) Principle of fatty acid β-oxidation activity assay (modified from the instruction manual of the Fatty Acid Oxidation Assay Kit, Assay Genie) and fatty acid oxidase activity in the liver of control (C), PQQ (P), and IPQ (I) groups. * Significantly different from control group, $p<0.05$ (Dunnett's post-hoc test). Each value is the mean± SEM (n=5).