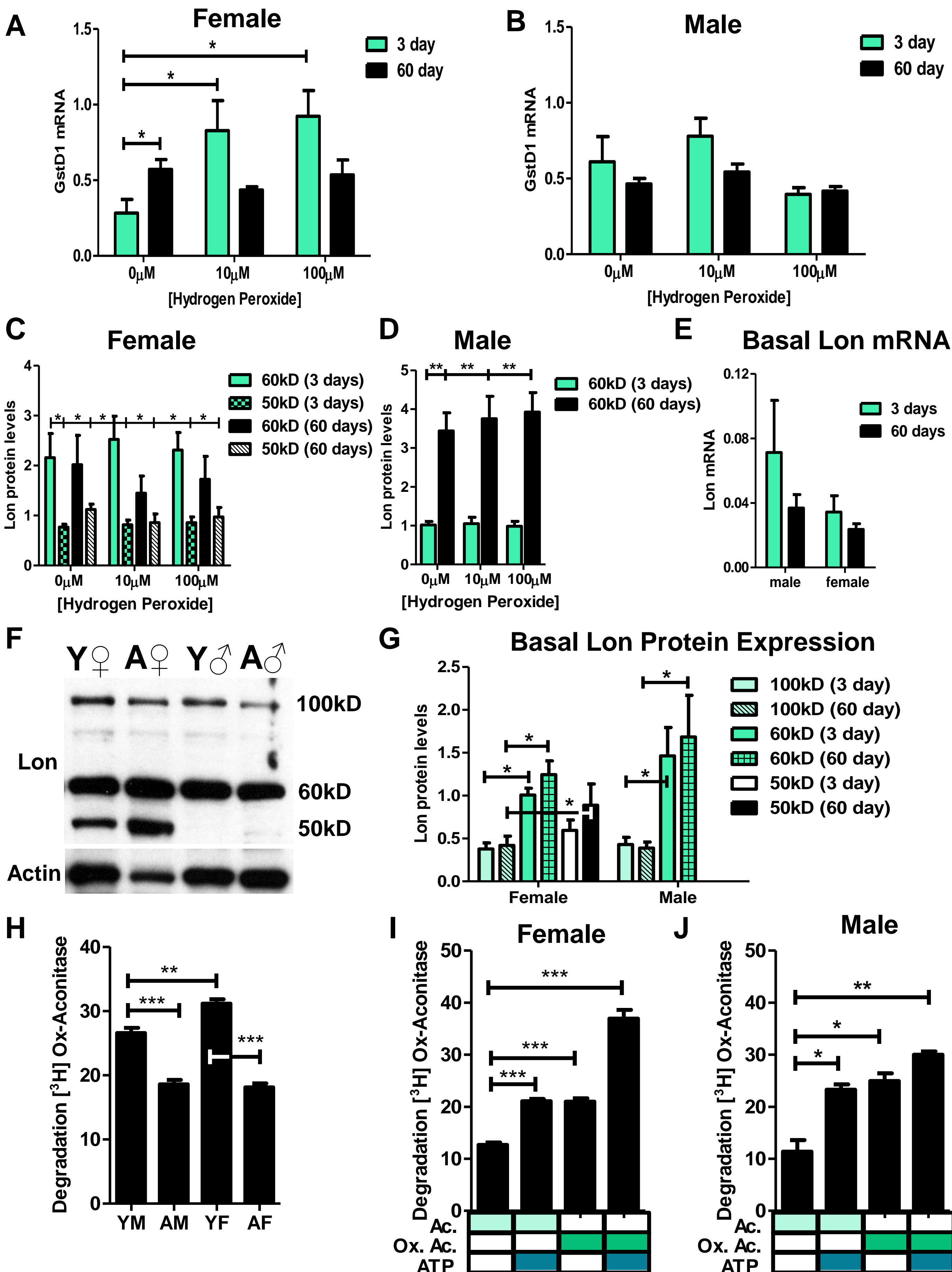
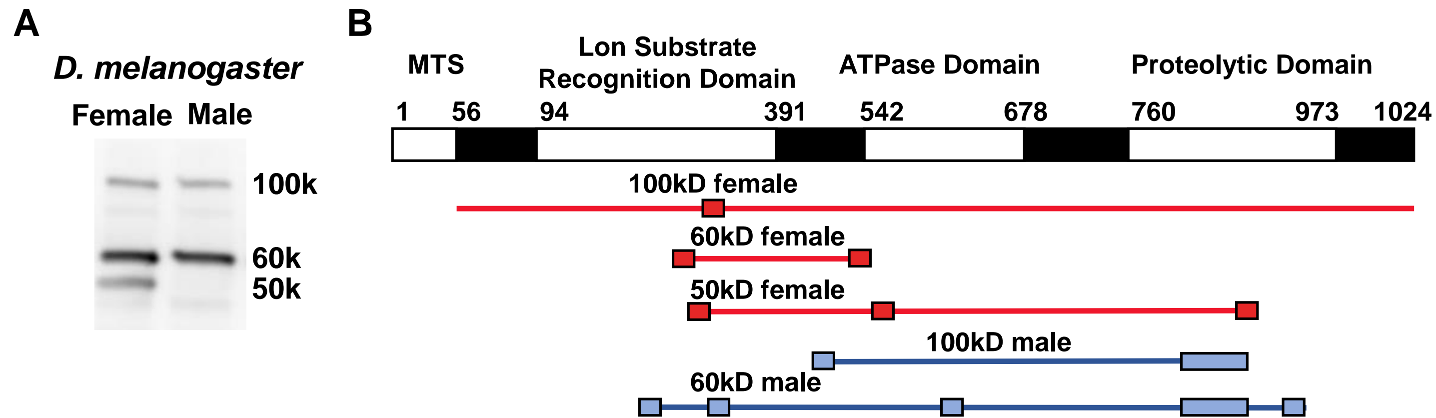
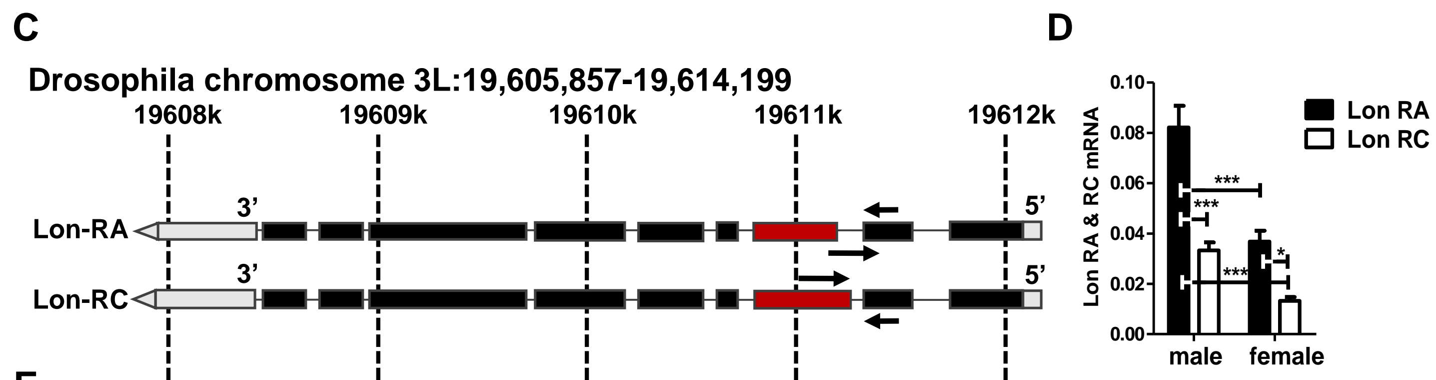
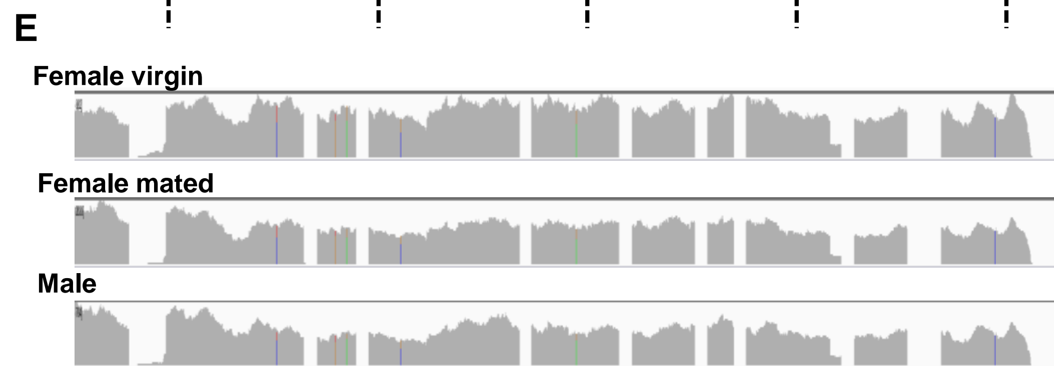

A
Female
GstD1 mRNA
3 day
60 day
0μM
10μM
100μM
[Hydrogen Peroxide]
B
Male
GstD1 mRNA
3 day
60 day
0μM
10μM
100μM
[Hydrogen Peroxide]
C
Female
Lon protein levels
60kD (3 days)
50kD (3 days)
60kD (60 days)
50kD (60 days)
0μM
10μM
100μM
[Hydrogen Peroxide]
D
Male
Lon protein levels
60kD (3 days)
60kD (60 days)
0μM
10μM
100μM
[Hydrogen Peroxide]
E
Basal Lon mRNA
Lon mRNA
3 days
60 days
male
female
F
Y♀ A♀ Y♂ A♂
Lon
100kD
60kD
50kD
Actin
G
Basal Lon Protein Expression
Lon protein levels
100kD (3 day)
100kD (60 day)
60kD (3 day)
60kD (60 day)
50kD (3 day)
50kD (60 day)
Female
Male
H
Degradation [3H] Ox-Aconitase
YM
AM
YF
AF
I
Female
Degradation [3H] Ox-Aconitase
Ac.
Ox. Ac.
ATP
J
Male
Degradation [3H] Ox-Aconitase
Ac.
Ox. Ac.
ATP

Figure S1, Relates to Figures 1 & 2. Age-related Changes Following Hydrogen Peroxide Pre-treatment.
(A,B) Glutathione S-transferase D1 (GstD1) mRNA levels were compared between 3 day and 60 day old w[1118] x Actin-GS-255B females and males following hydrogen peroxide pretreatment. (A) Females. (B) Males. Statistical significance was calculated using one-way ANOVA, with statistically significant differences ($p < 0.05$) indicated with asterisk. (C) Quantification of Lon protein bands (60kD and 50kD) following hydrogen peroxide pretreatment in 3 day old and 60 day old females. (D) Quantification of 60kD bands in 3 day old and 60 day old males pretreated with hydrogen peroxide. (E) Basal levels of *lon* mRNA were compared between 3 day and 60 day old w[1118] x Actin-GS-255B males and females. (F) Basal protein expression of Lon was compared between 3 day and 60 day old w[1118] x Actin-GS-255B males and females, protein content was normalized to anti-Actin-HRP antibody. (G) Quantification of Lon bands (100kD, 60kD, and 50kD) in 3 day (Y♀) and 60 day (A♀) females, and 3 day males (Y♂), and 60 day males (A♂) using ImageJ. (H) Basal activity was measured in mitochondria isolated from 3 day or 60 day old w[1118] x Actin-GS-255B females and males. (I,J) Basal activity was measured in mitochondria isolated from 3 day old w[1118] x Actin-GS-255B females and males, using the substrate [^{3}H] aconitase (Ac.) or oxidized [^{3}H] aconitase (Ox Ac.), in the absence or presence of 5mM ATP. (I) Females. (J) Males. Statistical significance was calculated using one-way ANOVA, with statistically significant difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) indicated with asterisk.







F
w[1118] x Actin-GS-255B
Female (3 days)
% survival
time (h)
0M
1M
2M
4M
4.4M
8M

G
w[1118] x Actin-GS-255B
Male (3 days)
% survival
time (h)
0M
1M
2M
4M
4.4M
8M

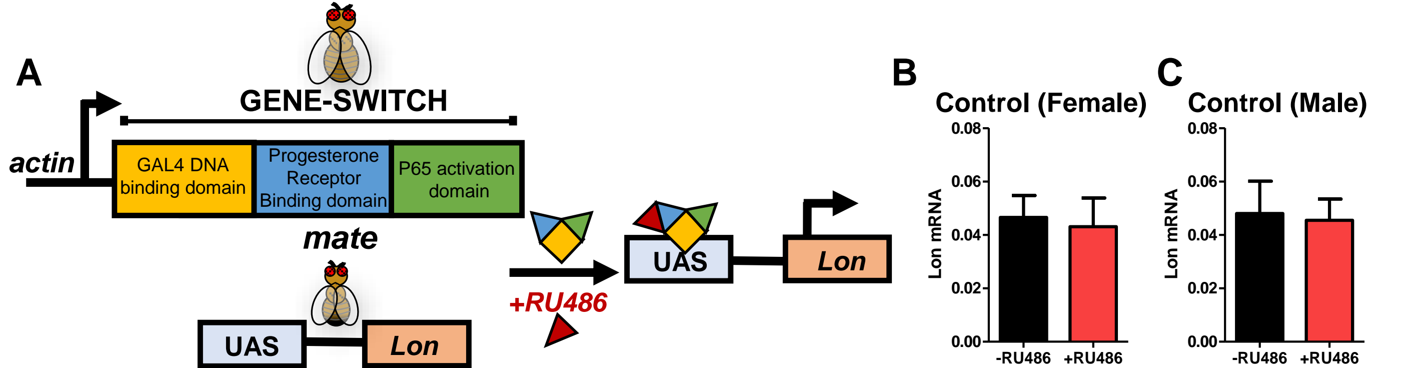
H
w[1118] x Actin-GS-255B
Female
% survival
time (h)
0uM (3 days)
10uM (3 days)*
100uM (3 days) *
0μM (35 days)
10μM (35 days)
100μM (35 days)

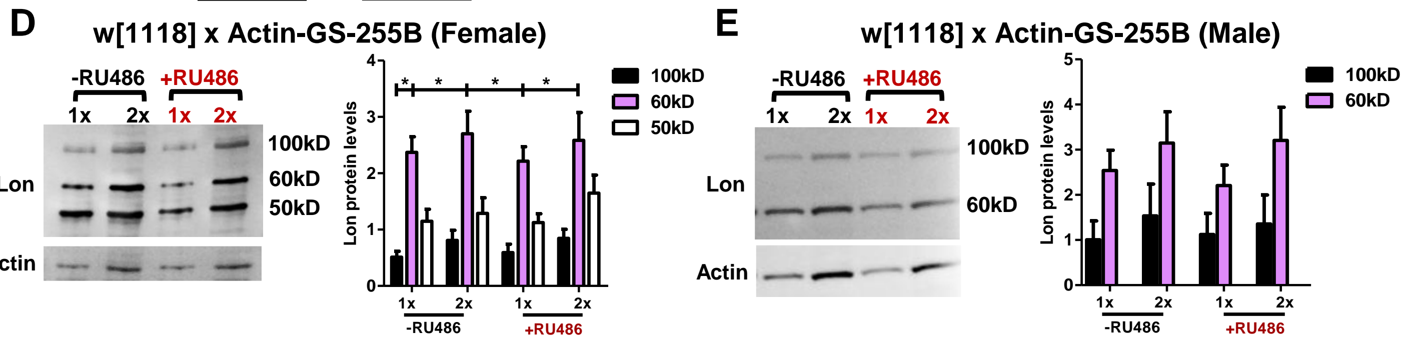
I
w[1118] x Actin-GS-255B
Female (35 days)
% survival
time (h)
0M
1M
2M
4M
4.4M
8M

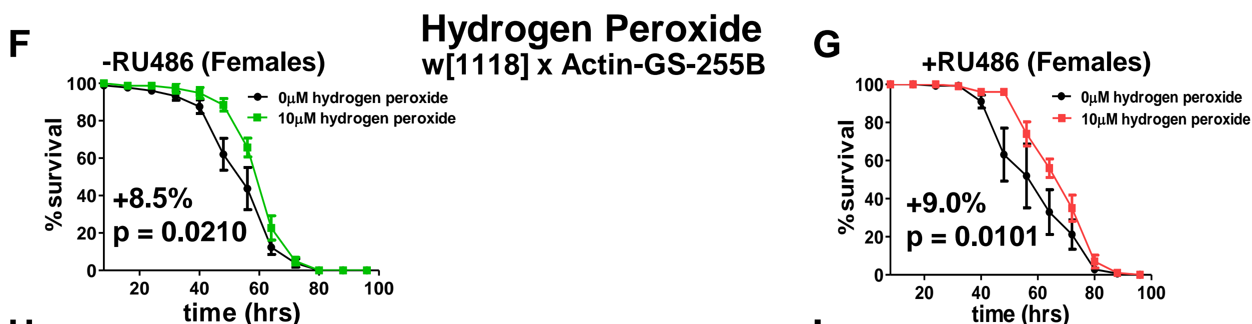
J
w[1118] x Actin-GS-255B
Male (35 days)
% survival
time (h)
0M
1M
2M
4M
4.4M
8M

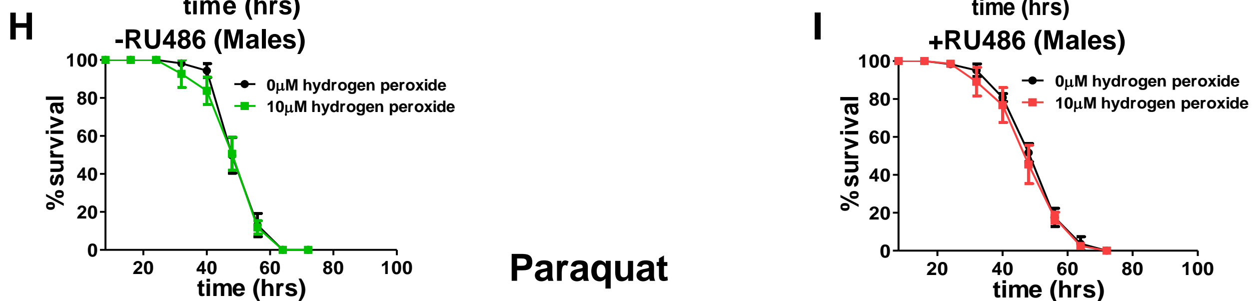
K
w[1118] x Actin-GS-255B
Male
% survival
time (h)
0uM (3 days)
10uM (3 days)
100uM (3 days)
0μM (35 days)
10μM (35 days)
100μM (35 days)

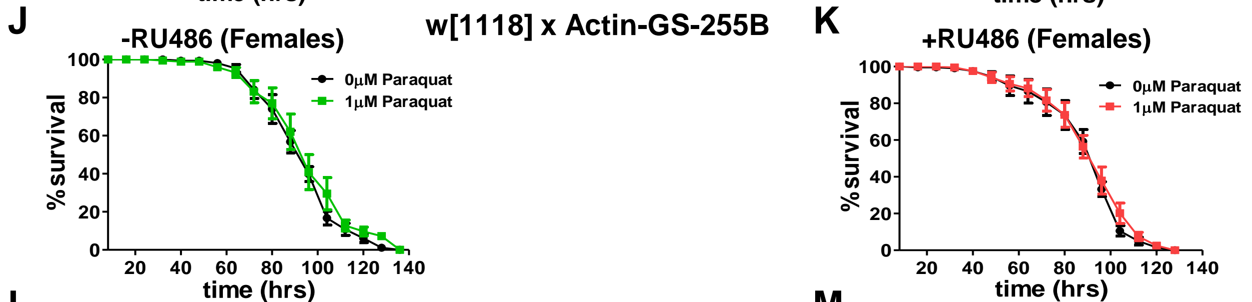
Figure S2, Relates to Figures 1, 2 & 5. Sex-dependent Variation of Lon Expression with Equal Sex Sensitivity to Hydrogen Peroxide, But Only Females Adapt.
(A) Western blot of w[1118] x Actin-GS-255B females and males probed with the polyclonal anti-Lon antibody. The sex-specific bands are marked at the corresponding molecular weights. (B) Depiction of the domains of *D. melanogaster* Lon. The colored boxes indicate the detected peptide fragments, pink corresponding to those found in females and blue indicating those found in males. The lines indicate the expected peptide length. (C) Diagram of known *D. melanogaster* Lon-RA and Lon-RC intronic and exonic transcript regions (FlyBase gene consortium). Black arrows indicate primers unique for each isoform. (D) Transcript levels of Lon RA and Lon RC in 3 day old w[1118] x Actin-GS-255B males and females measured by qPCR. (E) Exonic regions (gray) and intronic regions (white) of the total Lon transcript detected from RNA sequencing from 12 day old virgin females, mated females, and males. Statistical significance was calculated using one-way ANOVA, with statistically significant difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) indicated with asterisk. (F,G,I,J) Survival curves for w[1118] x Actin-GS-255B (control cross) exposed to increasing hydrogen peroxide concentrations. (F) 3 day old females. (G) 3 day old males. (I) 35 day old females. (J) 35 day old males. (H,K) Survival curves for control flies fed various adaptive doses of hydrogen peroxide [0μM-100μM] prior to exposure to challenge dose [4.4M]. (H) Females. (K) Males. Statistically significant difference in survival relative to control ($p < 0.05$) was calculated using the Log-Rank test, and is indicated by asterisk. Statistical summary is located in Table S1.


A
GENE-SWITCH
actin
GAL4 DNA binding domain
Progesterone Receptor Binding domain
P65 activation domain
mate
UAS
Lon
+RU486
UAS
Lon
B
Control (Female)
Lon mRNA
-RU486
+RU486
C
Control (Male)
Lon mRNA
-RU486
+RU486


D
w[1118] x Actin-GS-255B (Female)
-RU486
+RU486
1x 2x 1x 2x
Lon
Actin
100kD
60kD
50kD
Lon protein levels
100kD
60kD
50kD
-RU486
+RU486
E
w[1118] x Actin-GS-255B (Male)
-RU486
+RU486
1x 2x 1x 2x
Lon
Actin
100kD
60kD
Lon protein levels
100kD
60kD
-RU486
+RU486


Hydrogen Peroxide
w[1118] x Actin-GS-255B
F
-RU486 (Females)
0µM hydrogen peroxide
10µM hydrogen peroxide
+8.5%
p = 0.0210
%survival
time (hrs)
G
+RU486 (Females)
0µM hydrogen peroxide
10µM hydrogen peroxide
+9.0%
p = 0.0101
%survival
time (hrs)


H
-RU486 (Males)
0µM hydrogen peroxide
10µM hydrogen peroxide
%survival
time (hrs)
I
+RU486 (Males)
0µM hydrogen peroxide
10µM hydrogen peroxide
%survival
time (hrs)


Paraquat
w[1118] x Actin-GS-255B
J
-RU486 (Females)
0µM Paraquat
1µM Paraquat
%survival
time (hrs)
K
+RU486 (Females)
0µM Paraquat
1µM Paraquat
%survival
time (hrs)

L
-RU486 (Males)
0µM Paraquat
1µM Paraquat
+9.2%
p = 0.0028
%survival
time (hrs)
M
+RU486 (Males)
0µM Paraquat
1µM Paraquat
+10.7%
p = 0.0002
%survival
time (hrs)

Figure S3, Relates to Figure 2 & 5. RU486 Does Not Impact Lon Expression or Sex-specific Adaptation

(A) Diagram of the *Drosophila* Gene-Switch system. Females of the Actin-'Gene-Switch'-255B (Actin-GS-255B) driver strain are mated to males containing the target gene. Upon the addition of RU486, it binds to the progesterone receptor domain, causing the GAL4 DNA binding domain to interact with the upstream activation site to modulate the expression of the target gene. (B,C) The Actin-GS-255B strain was crossed to w[1118] control strain. Progeny were fed ±RU486 for 10 days prior to mRNA isolation. Transcript levels of *lon* mRNA in ±RU486 controls (B) Females. (C) Males. (D,E) Western blot of 10 day old w[1118] x 255B (control) flies were run on a 10% gel at a concentration of 5μg (1x) or 10μg (2x), with the molecular weights of the Lon bands marked. (D) Females. (E) Males. Protein content was normalized to anti-HRP-actin antibody and quantified using ImageJ. Statistical significance was calculated using one-way ANOVA, with statistically significant difference ($p < 0.05$) indicated with asterisk and compared to the 1x –RU486 control. (F-M) Adaptation curves for progeny of the w[1118] x Actin-GS-255B cross. (F,H,J,L) Progeny fed ethanol or (G,I,K,M) RU486 for 9 days prior to pretreatment. (F,G) Female pretreated with hydrogen peroxide. (H,I) Males pretreated with hydrogen peroxide. (J,K) Females pretreated with paraquat. (L,M) Males pretreated with paraquat. Statistical difference in survival ($p < 0.05$) was calculated using the Log-Rank test. The p-value and percent change of the median is indicated. Statistical Summary is located in Table S2.

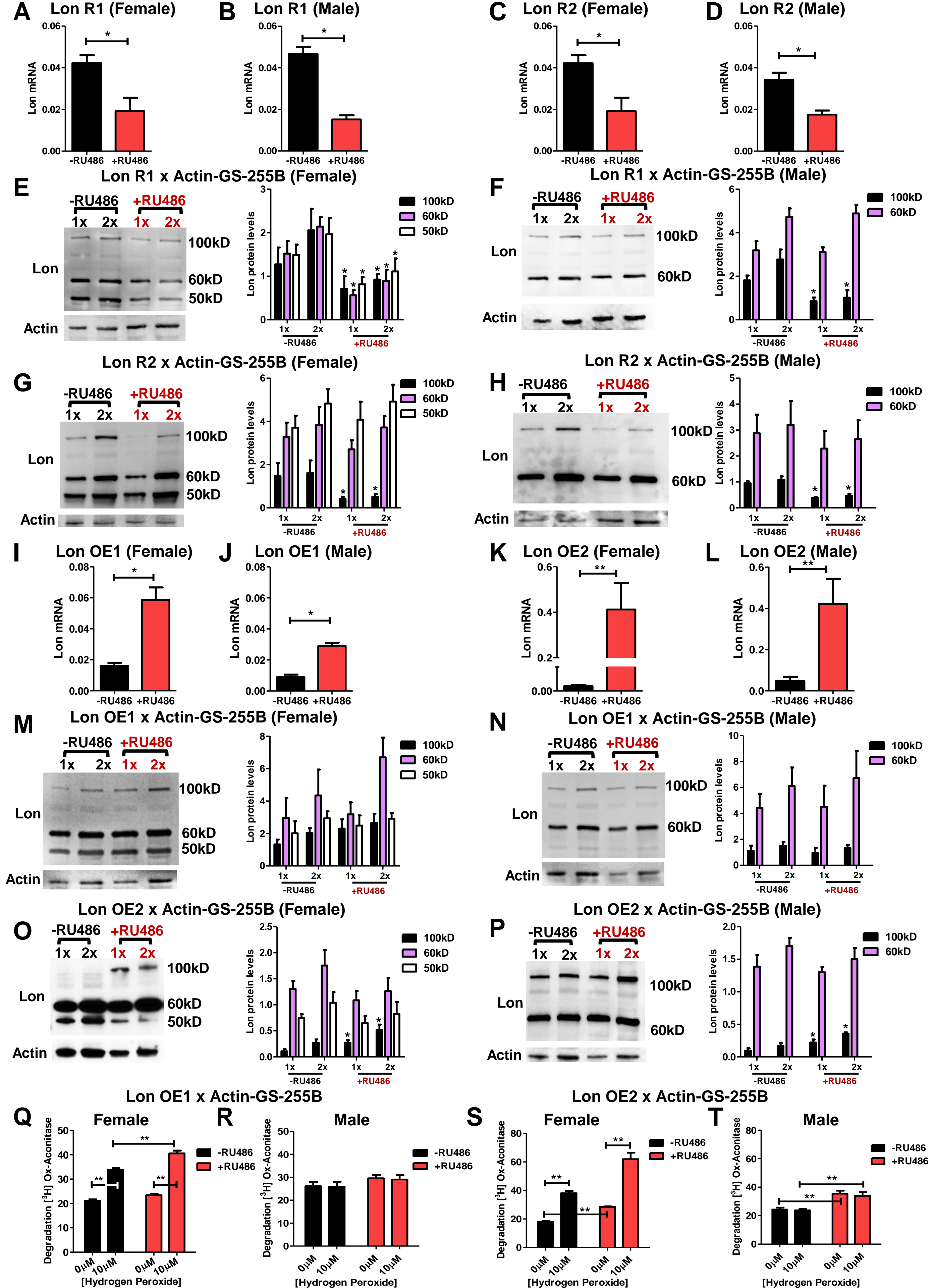
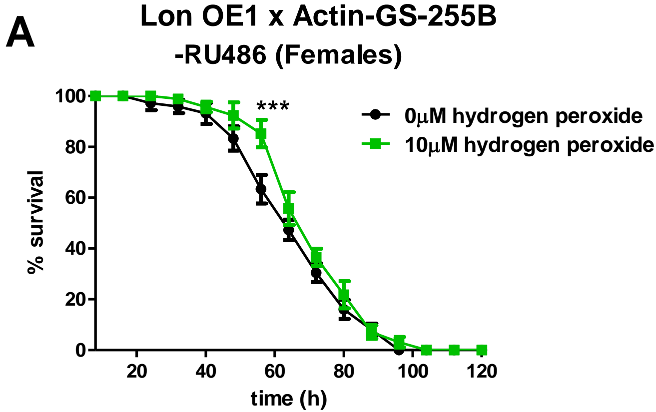
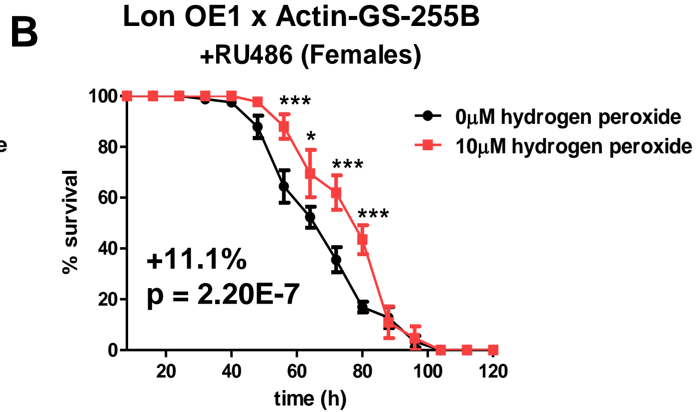

A Lon R1 (Female)
B Lon R1 (Male)
C Lon R2 (Female)
D Lon R2 (Male)
Lon mRNA
-RU486 +RU486
E Lon R1 x Actin-GS-255B (Female)
F Lon R1 x Actin-GS-255B (Male)
G Lon R2 x Actin-GS-255B (Female)
H Lon R2 x Actin-GS-255B (Male)
Lon
Actin
100kD
60kD
50kD
1x 2x
Lon protein levels
I Lon OE1 (Female)
J Lon OE1 (Male)
K Lon OE2 (Female)
L Lon OE2 (Male)
M Lon OE1 x Actin-GS-255B (Female)
N Lon OE1 x Actin-GS-255B (Male)
O Lon OE2 x Actin-GS-255B (Female)
P Lon OE2 x Actin-GS-255B (Male)
Lon OE1 x Actin-GS-255B
Lon OE2 x Actin-GS-255B
Q Female
R Male
S Female
T Male
Degradation [3H] Ox-Aconitase
0μM 10μM
[Hydrogen Peroxide]

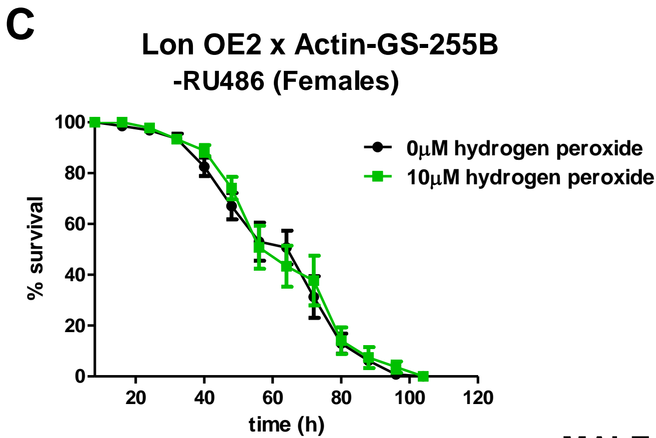
Figure S4, Relates to Figure 2 & 5. Modulation of Lon expression Using the Gene-Switch System

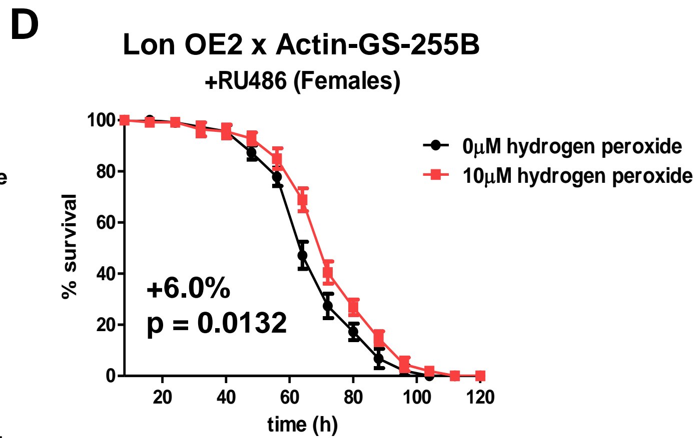
(A-T) Flies were fed ±RU486 for 10 days before assays were performed. The Lon transcript amount of RU486 fed Lon R1 and R2 (RNAi strains) were assessed to determine efficiency of the Gene-Switch system (A,C) Females. (B,D) Males. (E,F) Western blot of 10 day old Lon R1 RNAi flies run on a 10% gel at a concentration of 5μg (1x) or 10μg (2x). (E) Females showed banding of Lon marked at 100kD, 60kD, and 50kD. (F) Males showed Lon banding marked at 100kD and 60kD. Blots were normalized to anti-HRP-actin antibody. (G,H) Western blot of 10 day old Lon R2 RNAi flies. (G) Females showed three Lon bands marked at 100kD, 60kD, and 50kD. (H) Males showed two Lon bands at 100kD and 60kD. (I-L) Transcript levels of *lon* mRNA in Lon OE1 and OE2 flies (Lon Over-expression strains). (I,K) Females. (J,L) Males. (M,N) Western blot of 10 day old Lon OE1 Over-expression flies run on a 10% gel with Lon bands indicated. (M) Females. (N) Males. (O,P) Western blot of 10 day old Lon OE2 Over-expression flies. (O) Females showed three Lon bands at 100kD, 60kD, and 50kD. (P) Males showed two Lon bands at 100kD and 60kD. (Q-T) Proteolytic capacity in isolated mitochondria from flies of the Lon OE1 and OE2 strains, pretreated with hydrogen peroxide (Q) Lon OE1 females (R) Lon OE1 males (S) Lon OE2 females (T) Lon OE2 males. Protein was quantified using ImageJ. Statistical significance was calculated using one-way ANOVA, with statistically significant difference ($p < 0.05$) indicated with asterisk.

FEMALE

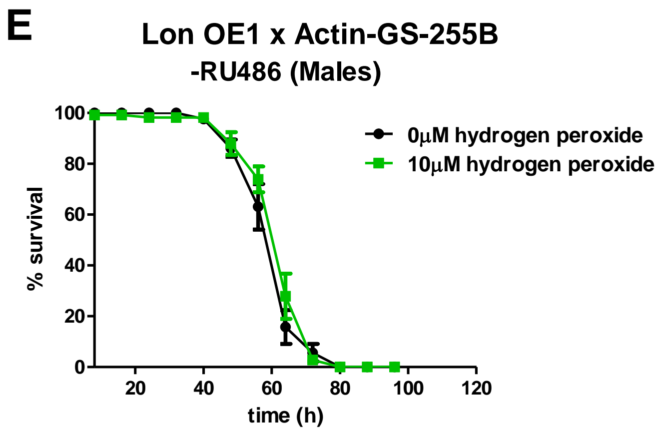


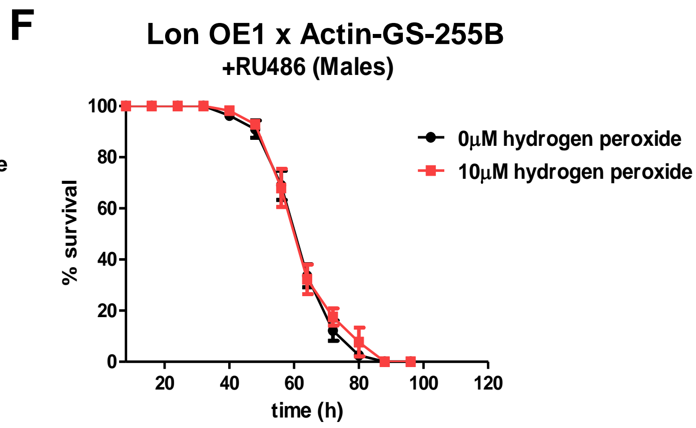


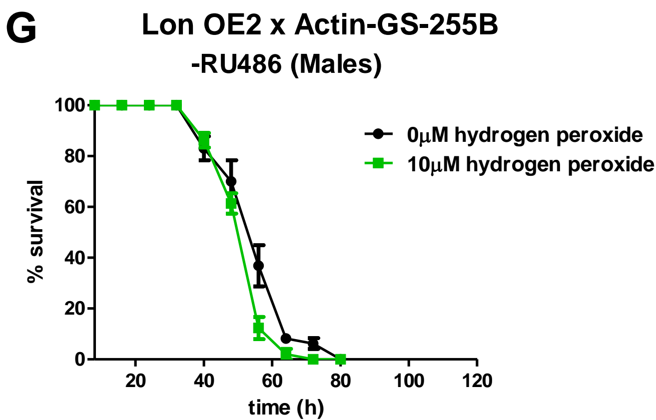




MALE







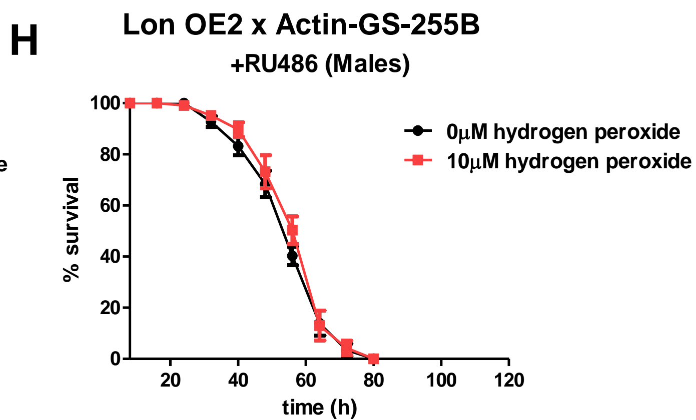
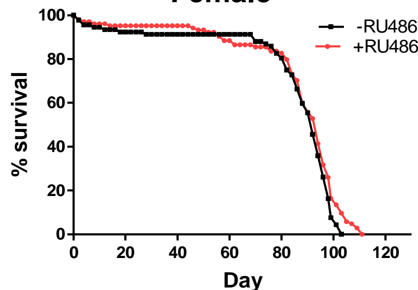


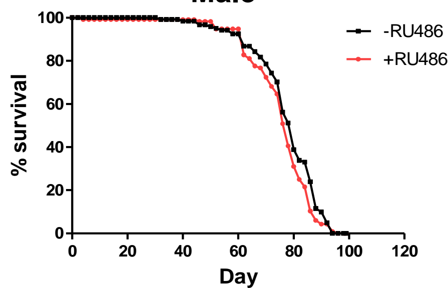
Figure S5, Relates to Figure 2. Over-expression of Lon is Beneficial to Hydrogen Peroxide Adaptation in a Female-specific Manner

Over-expression of Lon was generated by the Gene-Switch system. The Actin-GS-255B strain was crossed to two different Lon OE strains (Lon OE1 and Lon OE2). In all cases flies were cultured on either ethanol (vehicle) or mifepristone (RU486) for 9 days prior to pre-treatment with hydrogen peroxide. (A, C) Females cultured in the absence of RU486. (B, D) Females cultured in the presence of RU486. (E, G) Males cultured in the absence of RU486. (F, H) Males cultured in the presence of RU486. Statistical difference in survival ($p < 0.05$) was calculated using the Log-Rank test. The p-value and percent change of the median is indicated. Asterisks indicate two-sided t-test comparison that showed statistically significant differences ($p < 0.05$) at individual time points. Statistical Summary is located in Table S4.

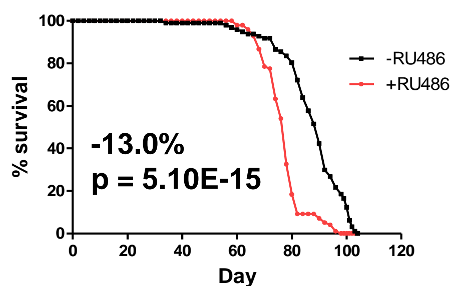
A w[1118] x Actin-GS-255B Female



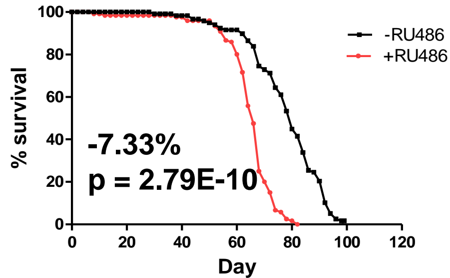
B w[1118] x Actin-GS-255B Male



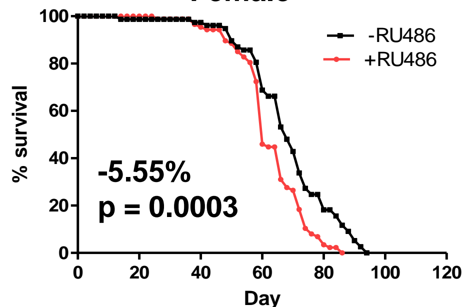
C Lon R1 x Actin-GS-255B Female



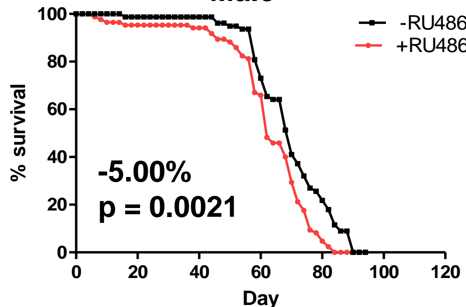
D Lon R1 x Actin-GS-255B Male



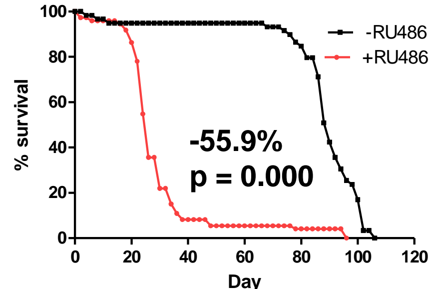
E Lon R2 x Actin-GS-255B Female



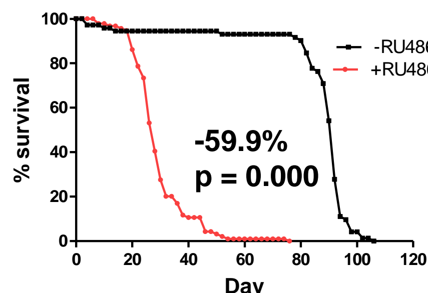
F Lon R2 x Actin-GS-255B Male



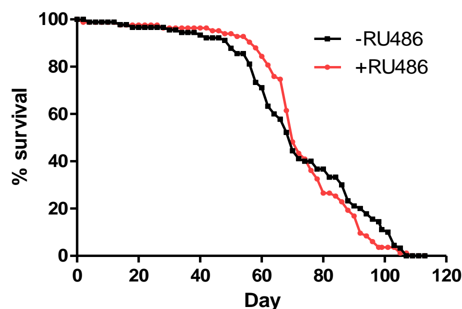
G Lon OE2 x Actin-GS-255B Female



H Lon OE2 x Actin-GS-255B Male



I Lon OE1 x Actin-GS-255B Female



J Lon OE1 x Actin-GS-255B Male

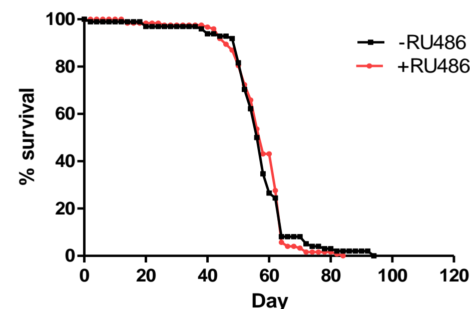


Figure S6, Relates to Figures 2 & 5. Constitutive Over-expression or RNAi Knockdown of Lon is Detrimental to Lifespan

(A,B) Controls for effect of RU486 drug on males and females. The Actin-GS-255B strain was crossed to w[1118] control strain and the progeny were assayed for life span in the presence and absence of RU486, as indicated. (A) Females. (B) Males. (C-F) Effect of Lon RNAi on life span. The Actin-GS-255B strain was crossed to Lon R1 RNAi or Lon R2 RNAi and the progeny were assayed for life span in presence and absence of RU486, as indicated. (C) Lon R1 Females. (D) Lon R1 Males. (E) Lon R2 Females. (F) Lon R2 Males. (G-J) Effect of Lon Over-expression on life span. The Actin-GS-255B strain was crossed to Lon OE2 or Lon OE1 Over-expression and the progeny were assayed for life span in presence and absence of RU486, as indicated (G) Lon OE2 Females. (H) Lon OE2 Males. (I) Lon OE1 Females. (J) Lon OE1 Males. Statistical difference in survival ($p < 0.05$) was calculated using the Log-Rank test. The p-value and percent change of the median is indicated. Statistical Summary is located in Table S6.

A

w[111]8 x Actin-GS-255B

B

TraF x Actin-GS-255B
Development (Hydrogen Peroxide)

C

TraF x Actin-GS-255B
Development (Paraquat)

D

TraF x Actin-GS-255B
Adult (Hydrogen Peroxide)

E

Tra RNAi x Actin-GS-255B
Development (Hydrogen Peroxide)

w[111]8 x Actin-GS-255B
Challenge dose (Hydrogen Peroxide)

F Female 4.4M

G Male 4.4M

TraF x Actin-GS-255B
Challenge dose (Hydrogen Peroxide)

H Female 4.4M

I Pseudo Female 4.4M

w[111]8 x Actin-GS-255B
Challenge dose (Paraquat)

J Female 30mM

K Male 30mM

TraF x Actin-GS-255B
Challenge dose (Paraquat)

L Female 30mM

M Male 30mM

Figure S7, Relates to Figures 6 & 7. *TraF* Transformation Using the Gene-Switch System
(A) Western blot of male and female progeny of w[1118] x Actin-GS-255B, raised in the absence (-RU486) or presence of various concentrations of RU486: 160µg/mL (1x RU486), 320µg/mL (2x RU486), and 640µg/mL (4x RU486). The increasing concentrations of RU486 does not impact male or female Lon specific banding patterns. (B-C). Progeny from the *TraF* x Actin-GS-255B cultured on either ethanol (-RU486) or 320µg/mL mifepristone (+RU486) throughout development. Western blots of flies' pretreated with (B) hydrogen peroxide [0µM-10µM] (C) paraquat [0µM-1µM]. (D) Adult progeny of the *TraF* x Actin-GS-255B were fed ±RU486 for 9 days prior to hydrogen peroxide [0µM-10µM] pretreatment. (E) Progeny of the *Tra* RNAi x Actin-GS-255B were cultured on ±RU486 throughout development prior to hydrogen peroxide [0µM-10µM] pretreatment. The concentration of pretreatment and the presence of RU486 is indicated with "+" above blots. Western blots were performed in triplicate and protein content was normalized to anti-Actin-HRP antibody. Quantification of Lon bands (100kD, 60kD, and 50kD) was completed using ImageJ and indicated in bar graphs. Statistical significance was calculated using one-way ANOVA, with statistically significant difference (*$p < 0.05$, **$p < 0.01$) indicated with asterisk. (F,G) Hydrogen peroxide [4.4M] challenge dose comparison in female and male progeny from w[1118] x Actin-GS-255B cultured on either ethanol (-RU486) or 320µg/mL mifepristone (+RU486) to demonstrate no drug affect. (H,I) Progeny of *TraF* x Actin-GS-255B exposed to hydrogen peroxide [4.4M] challenge dose to show no survival difference. (J,K) Paraquat [30mM] challenge dose in female and male progeny from the w[1118] x Actin-GS-255B, show no drug effect. (L,M) Progeny of the *TraF* x Actin-GS-255B exposed to paraquat [30mM] challenge dose, show no drug effect.

Table S1, Relates to Figure 3 and S2. Hydrogen Peroxide and Paraquat Adaptation Statistical Summary

Genotype	Sex	$[H_2O_2]$	N	Mean(SD)	Median	90%	Δ Mean %	Δ Median %	(p)
Control Strain (Hydrogen Peroxide)									
w[1118] x Actin-GS-255B Cohort 1 (3 days)	F	0μM	54	92 (16)	96	112			
		10μM	56	101 (16)	104	120	8.17	7.70	6.94E-12
		100μM	59	97 (19)	104	120	4.98	7.70	2.57E-08
w[1118] x Actin-GS-255B Cohort 2 (3 days)	F	0μM	59	77 (16)	80	96			
		10μM	57	85 (17)	88	104	9.71	9.10	1.96E-11
		100μM	60	82 (19)	88	104	6.13	9.10	2.10E-08
w[1118] x Actin-GS-255B Cohort 1 (3 days)	M	0μM	59	80 (13)	80	96			
		10μM	57	81 (12)	80	96	1.33	0.00	0.6360
		100μM	59	81 (12)	80	96	1.38	0.00	0.5971
w[1118] x Actin-GS-255B Cohort 2 (3 days)	M	0μM	58	72 (13)	72	80			
		10μM	59	73 (12)	72	80	1.31	0.00	0.6216
		100μM	56	73 (13)	72	80	1.22	0.00	0.4878
w[1118] x Actin-GS-255B Cohort 1 (35 days)	F	0μM	121	51 (14)	55	65			
		10μM	98	53 (16)	55	70	0.77	0.00	0.6348
		100μM	103	53 (15)	55	70	1.83	0.00	0.3828
w[1118] x Actin-GS-255B Cohort 2 (35 days)	F	0μM	110	50(16)	54	67			
		10μM	101	51 (15)	54	67	0.68	0.00	0.7747
		100μM	99	50 (15)	54	67	0.25	0.00	0.9695
w[1118] x Actin-GS-255B Cohort 1 (35 days)	M	0μM	120	37 (10)	42	50			
		10μM	100	36 (9.8)	40	50	-0.65	-1.09	0.5561
		100μM	100	37 (10)	42	50	0.35	0.00	0.8417
w[1118] x Actin-GS-255B Cohort 1 (35 days)	M	0μM	120	36 (10)	38	45			
		10μM	104	36 (10)	38	45	-0.58	0.00	0.8664
		100μM	101	36 (10)	38	45	-0.56	0.00	0.8208
Control Strain (Paraquat)									
w[1118] x Actin-GS-255B Cohort 1 (3 days)	F	0μM	56	61 (15)	64	80			
		1μM	58	61 (15)	64	80	0.21	0.00	0.8322
		10μM	59	61 (15)	64	80	0.21	0.00	0.8310
w[1118] x Actin-GS-255B Cohort 2 (3 days)	F	0μM	59	109 (10)	112	120			
		1μM	59	110 (9)	112	120	1.70	0.00	0.1904
		10μM	60	108 (12)	112	120	1.03	0.00	0.6271
w[1118] x Actin-GS-255B Cohort 1 (3 days)	M	0μM	59	79 (18)	80	96			
		1μM	60	81 (20)	88	104	2.75	9.09	0.0112
		10μM	58	84 (17)	88	104	6.12	9.09	0.0005
w[1118] x Actin-GS-255B Cohort 2 (3 days)	M	0μM	60	104 (8)	104	112			
		1μM	59	108 (17)	112	120	3.49	7.14	0.0000
		10μM	60	108 (9)	112	112	3.60	7.12	2.94E-6
w[1118] x Actin-GS-255B Cohort 1 (35 days)	F	0μM	107	30 (15)	34	50			
		1μM	100	30 (15)	34	50	0.04	0.00	0.9604
		10μM	100	29 (15)	34	50	-1.38	0.00	0.6229
w[1118] x Actin-GS-255B Cohort 2 (35 days)	F	0μM	110	29 (15)	30	48			
		1μM	101	27 (14)	30	48	1.16	0.00	0.5875
		10μM	100	29 (15)	30	48	-0.72	0.00	0.7436
w[1118] x Actin-GS-255B Cohort 1 (35 days)	M	0μM	123	26 (11)	27	38			
		1μM	100	26 (11)	27	38	0.02	0.00	0.9880
		10μM	101	28 (10)	27	38	1.14	0.00	0.5562
w[1118] x Actin-GS-255B Cohort 2 (35 days)	M	0μM	117	26 (11)	28	36			
		1μM	101	25 (11)	28	35	-0.58	0.00	0.6547
		10μM	100	26 (11)	28	36	0.68	0.00	0.8708

Table S2, Relates to Figure 2 and S3. Presence or Absence of RU486 during adaptation statistical summary

Genotype	Sex	RU486	pretreated	N	Mean(SD)	Median	90%	Δ Mean %	Δ Median %	(p)
Control Strain (Hydrogen Peroxide)										
w[1118] x Actin-GS-255B Cohort 1	F	No	No	120	54 (13)	56	72			
			Yes	120	60 (11)	64	72	5.88	8.5	0.0210
w[1118] x Actin-GS-255B Cohort 1	M	No	No	140	50 (7)	48	56			
			Yes	140	50 (9)	48	56	-0.05	0.00	0.6532
w[1118] x Actin-GS-255B Cohort 1	F	Yes	No	119	58 (14)	56	80			
			Yes	140	66 (13)	64	80	10.2	9.0	0.0101
w[1118] x Actin-GS-255B Cohort 1	M	Yes	No	100	50 (7)	48	56			
			Yes	98	45 (9)	44	56	-11.6	-8.01	0.0117
w[1118 x Actin-GS-255B Cohort 2	F	No	No	176	56 (13)	56	72			
			Yes	178	64 (11)	64	80	12.9	12.5	6.18E-06
w[1118 x Actin-GS-255B Cohort 2	M	No	No	101	51 (8)	50	58			
			Yes	103	51 (11)	50	58	-0.34	0.00	0.7475
w[1118 x Actin-GS-255B Cohort 2	F	Yes	No	140	51 (14)	48	72			
			Yes	138	59 (11)	56	80	13.5	14.2	0.0036
w[1118 x Actin-GS-255B Cohort 2	M	Yes	No	120	51 (6)	50	60			
			Yes	121	51 (10)	50	60	-0.48	0.00	0.7897
Control Strain (Paraquat)										
w[1118 x Actin-GS-255B Cohort 1	F	No	No	159	111 (9)	112	120			
			Yes	140	110 (9)	112	120	1.55	0.00	0.1415
w[1118 x Actin-GS-255B Cohort 1	M	No	No	117	71 (20)	72	88			
			Yes	116	82 (22)	81	108	10.2	9.2	0.0028
w[1118 x Actin-GS-255B Cohort 1	F	Yes	No	162	111 (11)	112	120			
			Yes	178	111 (11)	112	120	-0.27	0.00	0.5791
w[1118 x Actin-GS-255B Cohort 1	M	Yes	No	120	78 (26)	80	104			
			Yes	118	88 (17)	96	112	10.3	16.7	0.0022
w[1118 x Actin-GS-255B Cohort 2	F	No	No	119	88 (20)	80	120			
			Yes	118	90 (21)	80	128	1.50	0.00	0.6704
w[1118 x Actin-GS-255B Cohort 2	M	No	No	120	77 (21)	80	104			
			Yes	120	84 (19)	89	104	6.11	9.10	0.0046
w[1118 x Actin-GS-255B Cohort 2	F	Yes	No	198	87 (15)	88	111			
			Yes	204	87 (15)	88	112	0.21	0.00	0.9123
w[1118 x Actin-GS-255B Cohort 2	M	Yes	No	117	76 (23)	80	96			
			Yes	118	91 (21)	96	106	12.1	10.7	0.0002

Table S3, Relates to Figure 2. Hydrogen peroxide adaptation Lon RNAi strains statistical summary

Genotype	Sex	RU486	Pretreated	N	Mean(SD)	Median	90%	Δ Mean %	Δ Median %	(p)
Lon R1 x Actin-GS-255B			No	178	60 (13)	59	72			
Cohort 1	F	No	Yes	194	71 (13)	72	88	12.2	10.1	2.34E-05
Lon R1 x Actin-GS-255B			No	199	76 (9)	80	88			
Cohort 1	M	No	Yes	176	75 (9)	72	88	-2.97	-11.1	0.2419
Lon R1 x Actin-GS-255B			No	159	60 (10)	64	72			
Cohort 1	F	Yes	Yes	162	60 (12)	64	72	-0.49	0.00	0.5300
Lon R1 x Actin-GS-255B			No	136	78 (9)	80	88			
Cohort 1	M	Yes	Yes	158	76 (9)	72	88	-3.10	-10.0	0.1987
Lon R2 x Actin-GS-255B			No	159	65 (17)	64	88			
Cohort 1	F	No	Yes	166	77 (18)	88	96	13.3	15.7	2.21E-10
Lon R2 x Actin-GS-255B			No	195	63 (12)	64	80			
Cohort 1	M	No	Yes	165	63 (12)	64	80	-0.84	0.00	0.8069
Lon R2 x Actin-GS-255B			No	118	59 (14)	60	80			
Cohort 1	F	Yes	Yes	100	60 (14)	60	80	-3.41	0.00	0.5603
Lon R2 x Actin-GS-255B			No	179	75 (11)	76	88			
Cohort 1	M	Yes	Yes	178	74 (11)	72	88	-1.41	-5.56	0.3991
Lon R1 x Actin-GS-255B			No	120	66 (15)	64	85			
Cohort 2	F	No	Yes	118	77 (13)	80	96	14.3	20.0	6.88E-05
Lon R1 x Actin-GS-255B			No	188	55 (5)	56	56			
Cohort 2	M	No	Yes	196	52 (8)	56	56	-4.42	0.00	0.1801
Lon R1 x Actin-GS-255B			No	118	68 (11)	64	80			
Cohort 2	F	Yes	Yes	119	65 (13)	64	80	-3.77	0.00	0.5618
Lon R1 x Actin-GS-255B			No	182	55 (8)	56	64			
Cohort 2	M	Yes	Yes	186	56 (9)	56	64	2.55	0.00	0.3007
Lon R2 x Actin-GS-255B			No	160	76 (11)	77	88			
Cohort 2	F	No	Yes	159	81 (16)	80	96	5.91	3.21	0.0009
Lon R2 x Actin-GS-255B			No	167	59 (10)	64	64			
Cohort 2	M	No	Yes	158	61 (8)	64	67	3.34	0.00	0.3014
Lon R2 x Actin-GS-255B			No	167	74 (14)	80	88			
Cohort 2	F	Yes	Yes	159	74 (13)	80	88	-0.31	0.00	0.9872
Lon R2 x Actin-GS-255B			No	219	71 (8)	72	80			
Cohort 2	M	Yes	Yes	216	70 (9)	64	80	-2.63	-12.5	0.2358

Table S4, Relates to Figure 2 and S5. Hydrogen peroxide adaptation Lon OE strains statistical summary

Genotype	Sex	RU486	pretreated	N	Mean(SD)	Median	90%	Δ Mean %	Δ Median %	(p)
Lon OE1 x Actin-GS-255B Cohort 1	F	No	No	140	56 (9)	56	64			
Lon OE1 x Actin-GS-255B Cohort 1	F	No	Yes	155	57 (9)	56	68	1.82	0.00	0.7686
Lon OE1 x Actin-GS-255B Cohort 1	M	No	No	156	55 (6)	56	64			
Lon OE1 x Actin-GS-255B Cohort 1	M	No	Yes	158	55 (6)	56	64	-0.72	0.00	0.7372
Lon OE1 x Actin-GS-255B Cohort 1	F	Yes	No	178	51 (7)	48	56			
Lon OE1 x Actin-GS-255B Cohort 1	F	Yes	Yes	185	58 (12)	56	72	12.5	14.3	3.02E-05
Lon OE1 x Actin-GS-255B Cohort 1	M	Yes	No	182	55 (5)	56	64			
Lon OE1 x Actin-GS-255B Cohort 1	M	Yes	Yes	176	55 (6)	56	64	0.90	0.00	0.8744
Lon OE2 x Actin-GS-255B Cohort 1	F	No	No	219	69 (21)	72	96			
Lon OE2 x Actin-GS-255B Cohort 1	F	No	Yes	180	72 (20)	72	96	5.84	0.00	0.1506
Lon OE2 x Actin-GS-255B Cohort 1	M	No	No	227	53 (12)	50	56			
Lon OE2 x Actin-GS-255B Cohort 1	M	No	Yes	229	50 (10)	50	56	-7.89	0.00	0.1670
Lon OE2 x Actin-GS-255B Cohort 1	F	Yes	No	120	69 (15)	72	88			
Lon OE2 x Actin-GS-255B Cohort 1	F	Yes	Yes	123	74 (16)	72	96	5.55	6.00	0.0132
Lon OE2 x Actin-GS-255B Cohort 1	M	Yes	No	140	59 (12)	56	72			
Lon OE2 x Actin-GS-255B Cohort 1	M	Yes	Yes	142	59 (12)	64	72	2.20	12.5	0.5771
Lon OE1 x Actin-GS-255B Cohort 2	F	No	No	196	57 (17)	56	80			
Lon OE1 x Actin-GS-255B Cohort 2	F	No	Yes	199	64 (14)	64	80	11.3	12.5	0.0524
Lon OE1 x Actin-GS-255B Cohort 2	M	No	No	324	62 (9)	64	72			
Lon OE1 x Actin-GS-255B Cohort 2	M	No	Yes	318	61 (8)	64	72	2.42	0.00	0.2641
Lon OE1 x Actin-GS-255B Cohort 2	F	Yes	No	200	61 (13)	64	73			
Lon OE1 x Actin-GS-255B Cohort 2	F	Yes	Yes	198	74 (15)	72	96	18.1	11.1	2.20E-07
Lon OE1 x Actin-GS-255B Cohort 2	M	Yes	No	159	64 (10)	64	80			
Lon OE1 x Actin-GS-255B Cohort 2	M	Yes	Yes	160	64 (10	64	80	0.95	0.00	0.6619
Lon OE2 x Actin-GS-255B Cohort 2	F	No	No	297	58 (16)	56	80			
Lon OE2 x Actin-GS-255B Cohort 2	F	No	Yes	302	58 (16)	56	80	0.04	0.00	0.8280
Lon OE2 x Actin-GS-255B Cohort 2	M	No	No	196	58 (14)	56	70			
Lon OE2 x Actin-GS-255B Cohort 2	M	No	Yes	176	54 (8.8)	56	60.8	-8.47	0.00	0.7800
Lon OE2 x Actin-GS-255B Cohort 2	F	Yes	No	299	62 (18)	58	88			
Lon OE2 x Actin-GS-255B Cohort 2	F	Yes	Yes	319	75 (15)	72	96	7.70	9.20	9.62E-12
Lon OE2 x Actin-GS-255B Cohort 2	M	Yes	No	300	52 (9)	56	64			
Lon OE2 x Actin-GS-255B Cohort 2	M	Yes	Yes	300	52 (8)	56	64	0.77	0.00	0.6221

Table S5, Relates to Figure 5. Paraquat adaptation Lon RNAi strains statistical summary

Genotype	Sex	RU486	Pretreated	N	Mean(SD)	Median	90%	Δ Mean %	Δ Median %	(p)
Lon R1 x Actin-GS-255B			No	102	76 (19)	72	96			
Cohort 1	F	No	Yes	99	79 (22)	72	106	3.92	0.00	0.3232
Lon R1 x Actin-GS-255B			No	139	73 (25)	80	104			
Cohort 1	M	No	Yes	138	84 (21)	88	112	12.6	9.09	0.0265
Lon R1 x Actin-GS-255B			No	98	77 (23)	88	104			
Cohort 1	F	Yes	Yes	101	76 (21)	72	96	-2.40	-16.2	0.4405
Lon R1 x Actin-GS-255B			No	118	83 (20)	80	107			
Cohort 1	M	Yes	Yes	120	79 (22)	80	105	-3.51	0.00	0.7139
Lon R2 x Actin-GS-255B			No	124	105 (23)	112	120			
Cohort 1	F	No	Yes	132	103 (25)	112	120	-2.38	0.00	0.8875
Lon R2 x Actin-GS-255B			No	163	77 (12)	80	88			
Cohort 1	M	No	Yes	159	80 (11)	84	96	3.40	4.74	0.0335
Lon R2 x Actin-GS-255B			No	139	104 (21)	112	120			
Cohort 1	F	Yes	Yes	141	101 (18)	104	120	-2.98	-7.69	0.0077
Lon R2 x Actin-GS-255B			No	142	72 (11)	72	88			
Cohort 1	M	Yes	Yes	136	71 (11)	72	88	-1.51	0.00	0.3928
Lon R1 x Actin-GS-255B			No	100	80 (22)	88	105			
Cohort 2	F	No	Yes	102	83 (22)	88	112	4.04	0.00	0.4190
Lon R1 x Actin-GS-255B			No	120	73 (22)	72	97			
Cohort 2	M	No	Yes	119	79 (22)	80	112	7.85	10.0	0.0216
Lon R1 x Actin-GS-255B			No	122	76 (23)	80	104			
Cohort 2	F	Yes	Yes	100	75 (21)	72	104	-1.71	-10.0	0.6230
Lon R1 x Actin-GS-255B			No	120	73 (22)	76	104			
Cohort 2	M	Yes	Yes	122	71 (25)	72	104	-3.45	-5.55	0.8536
Lon R2 x Actin-GS-255B			No	183	86 (19)	80	112			
Cohort 2	F	No	Yes	156	83 (17)	80	112	-3.78	0.00	0.1412
Lon R2 x Actin-GS-255B			No	160	102 (21)	108	116			
Cohort 2	M	No	Yes	173	110 (16)	112	120	5.33	3.33	0.0026
Lon R2 x Actin-GS-255B			No	217	89 (19)	88	112			
Cohort 2	F	Yes	Yes	192	87 (18)	80	108	-2.61	-10.0	0.3962
Lon R2 x Actin-GS-255B			No	196	111 (18)	112	128			
Cohort 2	M	Yes	Yes	183	109 (21)	112	128	-1.16	0.00	0.3933

Table S6, Relates to Figure 3 and S6. Lifespan Statistical Summary

Genotype	Sex	RU486	N	Mean(SD)	Median	90%	Δ Mean %	Δ Median %	(p)
w[1118] x Actin-GS-255B Cohort 1	F	-	96	81 (22)	85	99			
		+	99	87 (17)	94	98	1.95	2.6	0.1400
w[1118] x Actin-GS-255B Cohort 1	M	-	123	80 (11)	80	94			
		+	114	82 (11)	80	94	1.62	0.00	0.4500
LonR1 x Actin-GS-255B Cohort 1	F	-	97	90 (13)	92	106			
		+	98	79 (9.4)	80	84	-12.2	-13.0	5.11E-15
LonR1 x Actin-GS-255B Cohort 1	M	-	118	80 (14)	82	95			
		+	120	67 (10)	68	76	-16.6	-17.1	0.0000
LonR2 x Actin-GS-255B Cohort 1	F	-	89	68 (23)	67	80			
		+	87	65 (24)	64	74	-3.60	-5.00	0.0021
LonR2 x Actin-GS-255B Cohort 1	M	-	116	74 (22)	74	90			
		+	113	68 (20)	66	80	-7.61	-5.55	0.0003
LonOE1 x Actin-GS-255B Cohort 1	F	-	91	79 (24)	72	96			
		+	86	79 (24)	76	104	0.62	5.55	0.5178
LonOE1 x Actin-GS-255B Cohort 1	M	-	121	62 (8.5)	64	67			
		+	122	59 (9.9)	64	66	-3.08	0.00	0.6949
LonOE2 x Actin-GS-255B Cohort 1	F	-	83	92 (27)	92	103			
		+	88	27 (18)	32	38	-65.2	-55.9	0.0000
LonOE2 x Actin-GS-255B Cohort 1	M	-	106	72 (23)	74	86			
		+	104	28 (9)	30	32	-58.8	-59.9	0.0000
w[1118] x Actin-GS-255B Cohort 2	F	-	83	95 (27)	92	106			
		+	79	95 (27)	92	105	0.721	0.00	0.5191
w[1118] x Actin-GS-255B Cohort 2	M	-	121	80 (12)	82	92			
		+	116	77 (12)	80	89	-2.63	-2.44	0.0990
LonR1 x Actin-GS-255B Cohort 2	F	-	82	87 (16)	88	100			
		+	95	80 (9.7)	80	89	-7.54	-9.09	9.631E-9
LonR1 x Actin-GS-255B Cohort 2	M	-	115	76 (16)	76	94			
		+	114	71 (7.3)	71	79	-6.58	-7.33	2.792E-10
LonR2 x Actin-GS-255B Cohort 2	F	-	95	75 (22)	70	90			
		+	89	67 (20)	62	76	-10.3	-11.1	0.0002
LonR2 x Actin-GS-255B Cohort 2	M	-	106	88 (26)	88	96			
		+	118	86 (25)	84	94	-2.65	-4.54	0.0129
LonOE1 x Actin-GS-255B Cohort 2	F	-	95	62 (21)	66	83			
		+	98	68 (13)	69	80	9.93	4.55	0.8078
LonOE1 x Actin-GS-255B Cohort 2	M	-	98	59 (12)	59	66			
		+	123	59 (9.6)	60	66	0.26	1.69	0.8741
LonOE2 x Actin-GS-255B Cohort 2	F	-	83	82 (24)	80	96			
		+	94	27 (8.1)	24	32	-67.5	-70	0.0000
LonOE2 x Actin-GS-255B Cohort 2	M	-	92	92 (24)	92	100			
		+	96	34 (11)	32	40	-62.6	-65.2	0.0000

Table S7, Relates to Figure 7. Adaptation sex transformation statistical summary

		Sex	Pretreated	N	Mean(SD)	Median	90%	Δ Mean %	Δ Median %	(p)
TraF x Actin-GS-255B (Development)	Cohort 1 (Hydrogen Peroxide)	Pseudo-female +2X RU486	No	160	52 (14)	49	72			
			Yes	200	56 (13)	56	72	7.75	14.3	0.0333
		Female +2X RU486	No	597	63 (19)	64	88			
			Yes	661	78 (21)	80	104	18.6	20.0	0.0000
		Female –RU486	No	640	81 (24)	84	112			
			Yes	657	86 (24)	88	112	6.07	4.10	0.0006
		Male –RU486	No	581	55 (14)	56	72			
			Yes	602	55 (13)	56	72	1.33	0.00	0.9434
	Cohort 2 (Hydrogen Peroxide)	Pseudo-female +2x RU486	No	464	50 (11)	48	64			
			Yes	439	59 (13)	56	72	14.2	15.4	0.0002
		Female +2x RU486	No	620	65 (20)	64	88			
			Yes	458	77 (17)	80	96	16.0	15.7	2.98E-11
		Female –RU486	No	191	64 (16)	64	80			
			Yes	201	76 (18)	80	96	17.0	15.4	5.38E-11
		Male –RU486	No	191	44 (11)	40	56			
			Yes	198	45 (12)	40	56	2.07	0.00	0.5557
	Cohort 1 (Paraquat)	Pseudo-female +2X RU486	No	80	42 (28)	44	88			
			Yes	82	43 (26)	44	88	-2.82	0.00	0.4070
		Female +2X RU486	No	108	95 (23)	101	128			
			Yes	106	94 (24)	100	127	-0.62	-0.23	0.6511
		Female -RU486	No	148	104 (27)	106	134			
			Yes	153	102 (24)	106	130	-1.03	0.00	0.3961
		Male -RU486	No	118	68 (27)	73	105			
			Yes	120	88 (26)	97	130	11.1	13.3	6.75E-08
	Cohoort 2 (Paraquat)	Pseudo-female +2X RU486	No	142	64 (29)	68	115			
			Yes	126	62 (24)	68	115	-2.97	0.00	0.1683
		Female +2X RU486	No	207	104 (28)	106	140			
			Yes	231	97 (28)	100	136	-1.91	-5.67	0.1797
		Female -RU486	No	158	95 (23)	101	130			
			Yes	162	95 (24)	102	130	0.62	0.00	0.6299
		Male -RU486	No	140	75 (27)	78	105			
			Yes	137	86 (21)	88	120	10.9	9.81	5.31E-09
TraF x Actin-GS-255B (Adult)	Cohort 1 (Hydrogen Peroxide)	Male +2X RU486	No	104	30 (13)	29	48			
			Yes	110	35 (12)	35	54	4.65	5.14	0.0271
		Female +2X RU486	No	158	55 (16)	57	74			
			Yes	161	65 (18)	66	85	8.36	9.76	0.0027
		Female –RU486	No	138	55 (20)	53	73			
			Yes	165	64 (19)	65	87	8.41	9.38	0.0024
		Male –RU486	No	112	33 (9)	31	47			
			Yes	116	33 (9)	31	47	0.29	0.00	0.9294
	Cohort 2 (Hydrogen Peroxide)	Male +2X RU486	No	109	27 (12)	26	35			
			Yes	106	33 (11)	32	45	5.53	5.98	0.0163
		Female +2X RU486	No	119	53 (16)	53	75			
			Yes	162	65 (15)	67	85	11.49	12.51	6.17E-05
		Female –RU486	No	161	56 (18)	57	77			
			Yes	155	75 (16)	78	100	15.62	16.09	7.80E-07
		Male –RU486	No	130	35 (9)	37	48			
			Yes	134	35 (8)	37	48	1.06	0.00	0.8360
Tra RNAi x Actin-GS-255B (Development)	Cohort 1 (Hydrogen Peroxide)	Female -RU486	No	178	81 (18)	88	102			
			Yes	183	95 (19)	96	116	11.9	7.13	0.0017
		Pseudo Male +2X RU486	No	103	70 (19)	71	87			
			Yes	116	70 (17)	71	90	0.52	0.00	0.2964
		Male –RU486	No	127	75 (15)	78	95			
			Yes	138	75 (14)	78	96	0.13	0.00	0.6883
		Male +2X RU486	No	101	70 (17)	72	85			
			Yes	109	71 (17)	74	85	1.06	1.47	0.4039
	Cohort 2 (Hydrogen Peroxide)	Female -RU486	No	160	60 (19)	60	80			
			Yes	179	70 (17)	74	93	8.93	13.09	2.57E-09
		Pseudo Male +2X RU486	No	97	66 (20)	68	79			
			Yes	99	67 (18)	68	84	1.58	0.00	0.2319
		Male –RU486	No	96	63 (14)	64	80			
			Yes	98	64 (14)	64	80	-1.35	0.00	0.6187
		Male +2X RU486	No	95	57 (20)	60	75			
			Yes	100	57 (18)	60	75	0.43	0.00	0.8755

Supplemental Experimental Procedures

Drosophila Strains and Culture

Flies were cultured on standard agar/dextrose/corn meal/yeast media at 25°C as previously described [S1]. Lon RNAi strains were obtained from Bloomington Drosophila Stock Center, *y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01060}cinattP2* (abbreviated Lon R1 RNAi), and the Vienna Drosophila RNAi Center, *w[1118]; P{GD14030}v36035* (abbreviated Lon R2 RNAi). Lon Over-expression were purchased from Bloomington Drosophila Stock Center, *y[1] w[*]; P{w[+mC]=EP}Lon[G3998]* (abbreviated Lon OE1), and the Drosophila Genetic Resource Center, *w[*]; P{w[+mC]=GSV2}Lon[GS5186]/TM3, Sb[1] Ser[1]* (abbreviated Lon OE2). Males from these lines (or *w[1118]* as a control) were crossed to virgin females of the Actin-Gene-Switch-255B (Actin-GS-255B) driver strain [S2]. Virgin male and female progeny were collected over 48 hours following eclosion. Adult flies were transferred to fresh media every other day. For changes in Lon gene expression, flies were maintained on media adjusted to final concentration of 160μg/ml mifepristone (RU486, no. M8046, Sigma-Aldrich) [S1], and transferred to fresh media every other day. Flies were incubated in vials for 10 days prior to adaptation experiments.

Treatment with Hydrogen Peroxide and Paraquat

Pretreatment experiments consisted of flies exposed to only an adaptive dose of an oxidant and allowed to recover prior to collection, whereas adaptation experiments included an additional exposure to a semi-lethal dose of the oxidant and survival was recorded. Both pretreatment and adaption experiments were conducted in the same manner as previously described [S3]. Briefly, for pretreatment, groups of 20 flies were transferred to vials containing a Kimwipe soaked in 1mL of 5% sucrose with various concentrations of hydrogen peroxide (no. H3410, Sigma-Aldrich) for 8 hours. Afterwards, flies were transferred to vials containing a Kimwipe soaked in 1mL of 5% sucrose for an additional 16 hours and then collected for downstream processing. For adaptation experiments, flies were transferred to vials containing a Kimwipe soaked in 1mL of 5% sucrose with various concentrations of hydrogen peroxide or methyl violgen dichloride (paraquat, no. 856177, Sigma-Aldrich) for 8 hours. Subsequently, flies were transferred to vials containing 5% sucrose for a 16 hour recovery. Afterwards, flies were transferred to vials containing a semi-lethal dose (4.4M hydrogen peroxide and 30mM paraquat). Flies were scored for survival every 8 hours until all flies had died.

Western Blot

20 flies were collected for each treatment group and frozen. Tissue was re-suspended in 200μL of tissue protein extraction buffer (no. 78501, Thermo-Scientific), supplemented with protease inhibitors (no. 04693159001, Roche), and homogenized using an electric pestle. Afterwards, to maximize lysis, samples underwent a 'freeze-thaw' cycle, consisting of a 5min incubation on dry-ice followed with a 5min incubation in water, and then vortexed, and repeated 2 additional times. Samples were then centrifuged at 10,000g to remove cuticle fragments and unlysed cells. Protein content was quantified with the Bicinchoninic acid assay (BCA) reducing agent compatible kit (no. 23252, Thermo-Scientific). Protein samples were run on a 10% SDS-PAGE gel and transferred to a PVDF membrane. Custom rabbit polyclonal Lon antibody directed against the Drosophila Lon (CG8798) peptide at amino acids Asp613 to Ser838 (1:200 dilution) was generously provided by Dr. Laurie Kaguni [S4]. The goat polyclonal anti-Actin-HRP antibody, conjugated to horseradish peroxidase (1:1000 dilution, no. sc-1616, Santa Cruz Biotechnology) was used for all loading controls. Mouse tissue from 3 month old male and

female black C57Bl/6 purchased from Jackson labs was generously provided by Dr. Valter Longo. 20µg of protein, for each sex, were run on 10% SDS-PAGE gels and transferred as described above. Mouse Lon protein was detected using a commercially available rabbit polyclonal anti-Lon antibody (1:200 dilution) (no.1-81734, Novus Biologicals).

RNA Extraction and Quantitative RT-PCR

Flies were collected in 500µL TRIzol (no. 15596-026, Life Technologies) and frozen. RNA extraction was performed following manufacturer's instructions with slight modification. Flies were homogenized in 500µL TRIzol, followed with the addition of 500µL TRIzol and incubated at room temperature for 5min. Samples were centrifuged at 12,000g for 10min at 4°C to remove cuticle fragments. Supernatant was decanted and 200µL of chloroform was added, and samples were vigorously shaken for 15 seconds, and then incubated at room temperature for 5min. Samples were centrifuged at 12,000g for 15min at 4°C. Aqueous phase was collected, and 500µL ice-cold 100% isopropanol was added, and samples were incubated at room temperature for 10min. Samples were centrifuged at 12,000g for 10min at 4°C, and RNA pellet was retained. To the RNA pellet, 1mL of ice-cold 70% ethanol was added, briefly vortexed, and centrifuged at 7,500g for 5min at 4°C. Pellet was dried and re-suspended in DEPC-treated water, and RNA concentration was assessed using a Nanodrop spectrophotometer (Thermo-Scientific).

RNA was reverse transcribed to cDNA using TaqMan® Reverse Transcription Reagents (no. N8080234, Life Technologies) and quantitative PCR was performed using iTaq SYBR Green (no. 1725120, Bio-Rad). Amplification for Lon was carried out with the primer sequences (Forward: 5' GAAGATAGTGGAGGTATCCA Reverse: 5' TGATGGCGAAGAGGAGCTTA). Primers for Rp49 were used as an internal control (Forward: 5'CGGATCGATATGCTAAGCTGT Reverse: 5' GCGCTTGTTCGATCCGTA). The primer sequence used for glutathione S-transferase D1 (Forward: 5'GACTCCCTGTACCCTAAGTGC Reverse: 5'TCGGCTACGGTAAGGGAGTCA). To detect the two isoforms of Lon (Lon RC and Lon RA) primers were designed to uniquely detect these two exon variants using the following primer sequences (Lon RA Forward: 5'CCAGTCTCAGGTTCCACTATC Reverse: 5'CTAAGCCCGCTGAAGATCAAA Lon RC Forward: 5'TGACAACTTTGCATTATCCTCT Reverse: 5'GACTCGACTTTGCCTGATTT). Primers were designed using the NCBI Primer-Blast software [S5].

Mitochondrial Isolation

Mitochondrial isolation was conducted as previously described with slight modification [S6, S7]. Following pretreatment experiments, 200 flies were collected per replicate for each treatment group. Flies were transferred into homogenization buffer (0.32M sucrose, 10mM EDTA, 10mM Tris/HCl, 2% BSA) and gently pressed using pre-chilled mortar and pestle. Samples were then centrifuged at 200g for 3min to remove cuticle fragments. Lysate was then centrifuged for 10min at 2200g, supernatant was removed and the pellet was re-suspended in non-BSA containing homogenization buffer. Pellets were then lysed by passing through a 21 gauge needle, followed with 3 cycles of freeze-thaw and an additional centrifugation. Protein content was quantified using the BCA protein assay reducing agent compatible kit (Thermo-Scientific), 15µg of protein were used for the activity assay.

Substrate Preparation

Protein substrates for activity assay, tritium-tagged aconitase and tritium-tagged oxidized-aconitase, were labeled as previously described [S8]. Briefly, 5mg of aconitase was dissolved in 0.1M Hepes buffer with the addition of 6.6uCi [H3]Formaldehyde and 20mM sodium cyanoborohydride. Mixture was incubated at room temperature on an end-over-end shaker for 1 hr. To one mixture, hydrogen peroxide was added at a final concentration of 5mM, and mixtures were rocked for an additional hour. Mixtures were dialyzed through a 10,000 MWCO filter (Millipore) at 15,000g for 30min, eluent was discarded, and slurry re-suspended in Hepes buffer. This was repeated for an additional 7 washes. Protein content was quantified with BCA assay kit (Thermo-Scientific).

Activity Assay

Isolated mitochondrial lysate was incubated in the presence of 5μg of protein substrate, with ± 5mM ATP and 2mM MgCl2. Samples were incubated at 37°C on a tube shaker for 2 hrs. Afterwards, 10μL of 20% BSA and 20μL of Trichloroacetic Acid (TCA) was added to the sample and centrifuged at 14,000g for 10min to quench the reaction. Supernatant was transferred to 5mL of scintillation fluid, and counts were read and calculated as acid-soluble counts minus background counts on a liquid scintillation counter (Wallace 1410).

Lifespan Assays

Lifespan assays were performed as previously described [S9]. Briefly, to generate age-synchronized cohorts of flies, virgin males and virgin females were collected from culture bottles over a 48 hour period following eclosion. The flies were maintained separately at 20 females per vial and 25 males per vial. Flies were transferred to fresh media every other day and deaths were recorded. The mean, median, percent change in the mean and median, and the log-rank p value were calculated using the R statistical software [S10].

RNA-sequencing Analysis

The RNA-sequencing data used in this study is previously described in detail [S11]. Briefly, the raw RNA sequence data was processed by trimming the reads using Trimmomatic [S12] to remove any remaining adapters and low quality bases. The processed reads were then mapped to the Ensembl BDGP5.25 build of the D. melanogaster (downloaded from the Illumina iGenomes website) reference genome using Tophat2 (version 2.0.12) [S13]. The abundance of total reads were plotted over the Lon locus using IGV (version 2.3) [S14], for virgin females, mated females and mated males.

Mass Spectrometry

200 flies were collected from 3 day old w[1118] x Actin-GS-255B males and females and used for immunoprecipitation with the custom D. melanogaster rabbit polyclonal anti-Lon antibody, as previously described [S15]. Samples were run on a 10% SDS-PAGE gel and bands were visualized with Coomassie Brilliant Blue. Bands were excised from gel and subjected to an in-gel trypsin digestion and subsequently dehydrated with acetonitrile before being prepared for LC-MS/MS analysis of fragments carried out by the USC Core Facility.

Sex Transformation

To generate transformed flies (pseudo-females) *y-ac-w; UAS-Tra[F]/SM5,CyO* [S16] or pseudo-males, *y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02830}attP40* (abbreviated Tra RNAi) were used. Virgin females were mated to males of the Actin-GS-255B driver strain and allowed to mate for

2 days on standard media containing ethanol vehicle, 160µg/mL (1x), or 320µg/mL (2x) RU486 [S17]. Virgin progeny were collected over a 48 hour period following eclosion.

Gonadal Isolation

Tissue was isolated as previously described [S18, S19]. Briefly, flies were fed yeast 1 day prior to extraction to increase ovary size. Ovaries, testes, and gonads were extracted from chromosomal females, chromosomal males, and pseudo-females. Following extraction, whole flies, carcasses, and reproductive organs were placed on ice. Tissue was prepared for western blot as described above.

Statistical Analysis

Data was expressed as the mean with S.E.M. and p values were calculated using a two-tailed Student's t-test for pairwise comparisons. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used for multiple comparisons. Statistical analysis were performed using GraphPad Prism v.6 software. Survival curves were analyzed by the Kaplan-Meier procedure and log-rank test.

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