Supporting Online Material

Steroid control of longevity in *Drosophila melanogaster*

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1.Materials and methods

- Mutant lines:

EcR^{V559fs}, *EcR*^{A483T}, *EcR*^{F288Y} and *EcR*^{C300Y} were obtained through an EMS mutagenesis in a *cn bw* background (*1*); *EcR*^{M554fs} was induced in a Canton-S background (*1*). All EcR mutants, as well as the parental *cn bw* line, were kindly provided by M. Bender. *DTS-3* was induced by X-ray in the wild-type Samarkand (*2*); Samarkand was from the Bloomington Stock center. Canton-S, w_{1118}, and $w_{1118}Cs_{10}$ (w_{1118} outcrossed 10 times to Canton-S) were from our laboratory stocks.

- Longevity experiments:

EcR mutants: Crosses were made between 20 virgin +/+ females and 10 males of either *EcR*^{mutant}*/SM6G* (*SM6G* is a balancer of the second chromosome) or +/+. *EcR*^{mutant}/+ or +/+progeny from each cross were collected 2 to 3 days after adult emergence, allowing time for mating. Males and females were then separated under brief $C0_2$ anesthesia, and transferred every 2 to 3 days in vials containing fresh regular food (standard cornmeal agar medium) (*3*) at 25°C, 50% humidity, under a 12:12 hour light-dark cycle.

DTS-3 study: 10 males of *DTS-3/TM3* (TM3 is a balancer of the third chromosome) or 10 males of Samarkand were crossed with 20 virgin Samarkand females at 25°C. *DTS-3*/+ or +/+progeny from each cross were collected and longevity measured as for EcR mutants.

- Developmental time of EcR:

Two crosses were made, in triplicate: female and male *cn EcR*$^{+}$ *bw/cn EcR*$^{+}$ *bw* crossed to each other, and female *cn EcR*$^{+}$ *bw/cn EcR*$^{+}$ *bw* crossed to *cn EcR*^{V559fs} *bw/SM6G*)males. Bottles were seeded with 20 virgin females mated with 10 males, for 3 days, at 25°C, and the adult progeny counted until no more emerged.

- Weight

Flies were collected as for a longevity experiment, fed on regular food, transferred every 2 to 3 days, and samples of 10 flies weighed at various ages.

- Resistance to various stresses

Non-thermosensitive mutants:

Flies were raised at 25°C until emergence as adults, and 2 to 3 day old males and females (collected as in Fig. 1) were put into vials (40 flies/vial) with regular food overnight, then submitted to the different stresses. Oxidative stress: Flies were starved in empty vials for 6 hours at 25°C, then provided with 20 mM paraquat in 5% sucrose in water. Heat: Flies were put in vials with 3 ml of regular food, and placed in an incubator at 36°C. Dry starvation: Flies were placed in empty vials at 25°C.

Thermosensitive mutants: prior to testing the flies for stress, under the conditions described above, they were placed for a week at the various temperatures (permissive, restrictive).

- Phototaxis test

Tests for phototaxis were performed in a countercurrent apparatus, the flies being given 5 trials for moving toward a daylight fluorescent light within 15 sec. (*4*). 30 to 100 naive flies 3-5 days old were used for each run.

- Reproductive ability

All experiments were done in triplicate, each run consisting of 5 females and 5 males, mated in vials containing standard food, and transferred daily at 25°C. The number of eggs laid in each vial was counted, as a measure of fecundity, and the vials were kept until eclosion of all the adult progeny, to determine fertility.

- 20-OH-ecdysone feeding

Longevity experiment: the flies were collected as for a regular longevity experiment, but 3 ml of regular food was mixed with 150 µl of a solution of 20-OH-ecdysone (20-OH-E) in 1% ethanol, in water, resulting in final concentrations of 10^{-3}M to 10^{-5}M. Some food dye was added (0.4% by volumefinal of McCormick Red food color) to ensure homogeneity. Dry starvation test: prior to testing the flies for stress, as above, they were fed 20-OH-E for a week at 29°C.

2.Supporting text

Steroids, which are important for development and sex specification, also have diverse effects during adulthood in reproduction, digestion, and coping with stress. Estrogen has been claimed to protect the aging human brain, as well as neurons in culture (*5, 6*). However, high

levels of estrogen are associated with epileptic seizures (*5, 7, 8*), and exacerbate symptoms of Parkinson's disease (*7*). In addition, recent studies have shown negative secondary effects of estrogen replacement therapy during post-menopausal treatment (*9-11, 12*). Corticosteroids, while necessary for coping with stress, may have deleterious effects if present in excess (*13, 14*).

In long-lived *Drosophila* lines derived by selective breeding, the ecdysteroid level, measured at one day after eclosion, is reduced (*15*). The *EcR* gene has been genetically mapped by deletions to position 42A7-12 (*1*) on the second chromosome, and QTL (quantitative trait loci) studies for longevity also indicate the presence of a QTL in 42A (*16, 17*).

We investigated mutations of *EcR* in the region common to the three isoforms. They are developmental lethals as homozygotes, but have extended longevity when heterozygous.

EcR^{A483T} is a point mutation, in a *cn bw* background (*1*), affecting the domain of interaction with the SMTER co-repressor, and EcR^{A483T} females are thermosensitive (no data were given for males) (*18, 19*). We found that heterozygous females lived 22% longer than controls at 29°C (Fig. 1SA). Males showed longevity increases over controls: 37% at 25°C and 9% at 29°C. Increases in stress resistance followed a similar pattern for females, but did not depend on temperature for males (Fig. 1SA). As before, we tested this mutant in two different *cn bw* backgrounds, and the results, being similar, were pooled in Fig. 1SA. We tested the effect of exposure to higher temperature for a limited time in early adult life. Spending the first seven days of adulthood at the restrictive temperature (29°C) increased longevity during the reminder of life at 25°C (Fig. 1SB) despite the fact that higher temperature ordinarily shortens lifespan (*20*). As ecdysone is a sex hormone, EcR might be regulated differently in males and females, leading to different effects.

Another mutation in the predicted ligand-binding domain, EcR^{M554fs}, induced in a Canton-S background (*1*), also showed increased longevity. We tested the mutation in the offspring of crosses with our laboratory strain of Canton-S, as well as with w^{1118} crossed ten times to Canton-S ($w^{1118}CS_{10}$) (Fig. 1S, C and D). In addition, two mutations in the predicted DNA-binding domain, EcR^{F288Y} and EcR^{C300Y}, induced in a *cn bw* background, similarly showed an increased lifespan over controls (Fig. 1S, E and F). In summary, all the mutant alleles of *EcR* tested as heterozygotes, whether in the ligand-binding domain or in the DNA-binding domain, and whether in a *cn bw* or Canton-S background, showed increased longevity.

We focused a considerable part of the study on the question of genetic background. First, the experiments included studies of thermosensitive alleles in two different genetic backgrounds (*cn bw* and Samarkand). In each case, the genetic background was constant, only temperature was changed, and the mutants exerted their effects at the elevated restrictive temperature. Another experiment involved feeding various concentrations of ecdysone to the flies, the genotype of the flies again being constant. For the studies on the EcR mutations, we used as a control the parental *cn bw* line in which the mutagenesis had been done. Because five years had passed since the mutagenesis, we were concerned that the control strain might have acquired inbreeding defects. In that case, crossing to the strain carrying an EcR mutation might lead to hybrid vigor, and thus to an improvement in longevity not linked to the EcR mutation. We therefore tested the parental *cn bw* against another *cn bw* line, from the collection in our own laboratory. If the strains had accumulated different mutations affecting longevity, that should be revealed by crossing them with each other. The progeny of that cross showed no difference in longevity from flies homozygous for either of the control lines, indicating that hybrid vigor was not an issue. In contrast, the mutations EcR^{V559fs} and EcR^{A483T}, tested as heterozygotes over each of the two *cn bw* lines, showed the increased longevity. Since the *EcR* mutations were studied as heterozygotes, it was conceivable that the chromosomes carrying the mutations might also carry other, dominant mutations, the latter being the ones extending longevity. To check, we studied four alleles, obtained in the same mutagenesis, as well as another allele, obtained independently in a different (Canton-S) genetic background. We tested this last mutation in two different genetic backgrounds, our Canton-S and our w^{1118}. In all cases, increased longevity was observed.

Other important players in the aging process are molecular chaperones, such as heat-shock proteins (*21*). The interaction of EcR with a chaperone complex is necessary for activation of the heterodimeric receptor of ecdysone (*22*), and overexpression of such a chaperone, Hsp70 (*23*), leads to increased longevity (*24*). It is conceivable that, when overexpressed, Hsp70 might trap EcR in a non-functional state, thus leading to extended longevity.

Although no gene homologous to EcR has so far been described in *C. elegans*, the steroid pathway has been proposed to influence lifespan, as suggested by increased longevity in mutations of *daf-9*, a cytochrome P450 related to those involved in biosynthesis of steroid hormones, and in *daf-12*, a nuclear hormone receptor (*25-27*). Another hormonal pathway that has been demonstrated to improve longevity when down-regulated in *C. elegans* is the insulin

pathway (*28*). In *Drosophila*, mutants of the insulin receptor, *InR* (*29*), or of an insulin receptor substrate protein, *chico* (*30*), also have increased lifespan. The steroid and insulin pathways have very different roles; the insulin pathway is involved in growth and metabolism, while the ecdysone pathway is involved in developmental transitions and reproduction. However, there is evidence that they do interact, as shown in the mosquito, where oogenesis is dependent on blood intake (*31*). A reduction of ecdysone titer has been shown in the ovaries of *InR* mutants, but ovary size itself is reduced (*32*). Juvenile hormone (JH), a sesquiterpenoid, is also reduced in the long-lived *InR* mutant, but it is unclear whether that reduction is the reason for extension of longevity (*29*). A direct involvement of juvenile hormone in aging has been shown through studies on reproductive diapause in *Drosophila* and in the monarch butterfly (*33*). The involvement of JH in longevity is particularly interesting, as *ultraspiracle* (*USP*) has been proposed to be a receptor for JH (*23*). These two hormones might interact in regulating lifespan.

The effects of the mutations studied can be long-lasting, as indicated by our observations that expressing a mutant phenotype for only part of the adult lifespan can induce an increase in longevity. Extension of longevity by gene silencing has been also shown in yeast and *C. elegans* through mutations in *Sir* genes, which encode NAD histone deacetylases (*34*).

3.Supporting figures

Fig. 1S. Data on four additional alleles of *EcR*.

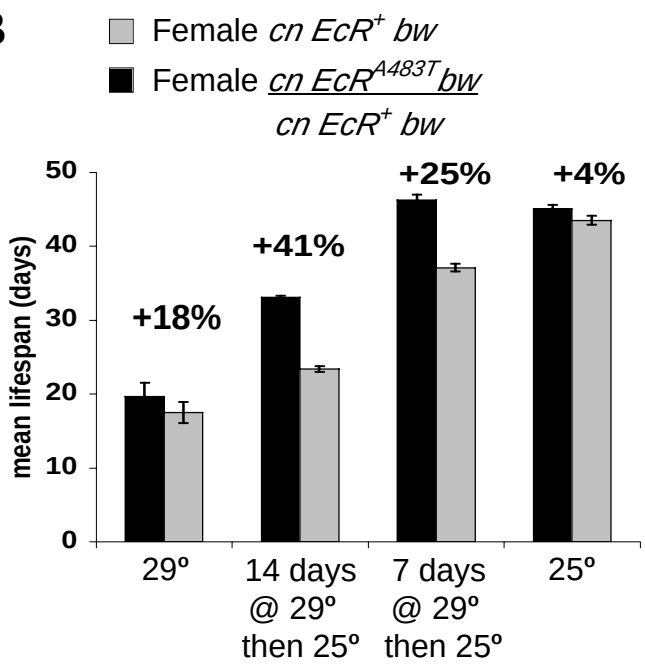
(A) and **(B)**: Thermosensitive mutant EcR^{A483T}/+ (permissive for development and oogenesis at 20°C, restrictive above 25°C). **(A)** Increases in lifespan and survival under dry starvation, compared to parental *cn bw* controls. Males *cn* EcR^{A483T} *bw/cn* EcR^{+} *bw* or *cn* EcR^{+} *bw* were crossed to *cn* EcR^{+} *bw* females at 20°C (permissive for development). Adult *cn* EcR^{A483T} *bw/cn* EcR^{+} *bw* or *cn* EcR^{+} *bw* progeny 2 to 3 days old were placed at either 25°C or 29°C for measurement of lifespan. For dry starvation tests, the 2-3 day old flies were first maintained for 7 days at 25°C or 29°C on regular food, then tested for dry starvation in empty vials at 25°C. Females showed increased longevity and stress resistance at 29°C; males showed the best effect at 25°C. (**B)** Effect of shifting females from 29°C to 25°C at various times during adulthood. Bars represent mean lifespan ± SD. Shifting to restrictive temperature at any of the times tested led to an increase in mean lifespan compared to controls. (**C)** through **(F)**: Survival curves for three *EcR* mutants, tested as heterozygotes, compared with appropriate controls. Experiments performed as in Fig. 1. (**C)** EcR^{M554fs}, a mutation in the predicted ligand-binding domain, induced in a Canton-S background, and tested in progeny of a cross with our laboratory strain of Canton-S, compared to the latter. (**D)** Same mutant tested in progeny of a cross with $w^{1118}CS_{10}$, compared to the latter. (**E)** and **(F)**: Two mutations in the predicted DNA-binding domain, induced in a *cn bw* background, tested in progeny of a cross with the parental *cn bw*, compared to the latter. (**E)** *cn* EcR^{C300Y} *bw* and (**F)** *cn* EcR^{F288Y} *bw*. *EcR* mutants, black lines; controls, gray lines. Error bars: ± SD, % increases in mean lifespan, and *P* values of the curves calculated by the Wilcoxon-rank test, one tail. Mated flies were collected as in fig. 1.

A

	Temperature (°C)	% Increase ± SD in Mean Lifespan	n*	Mean survival time under dry starvation	n*
Females *EcR*^{A483T}/+	25°C	4 +/- 12	3	12 +/- 12	2
	29°C	22 +/- 5	3	27 +/- 8	2
Males *EcR*^{A483T}/+	25°C	37 +/- 3	3	18 +/- 4	2
	29°C	9 +/- 3	3	17 +/- 12	2

n= replicates of 80 to 160 flies
SD: standard deviation to the mean

B



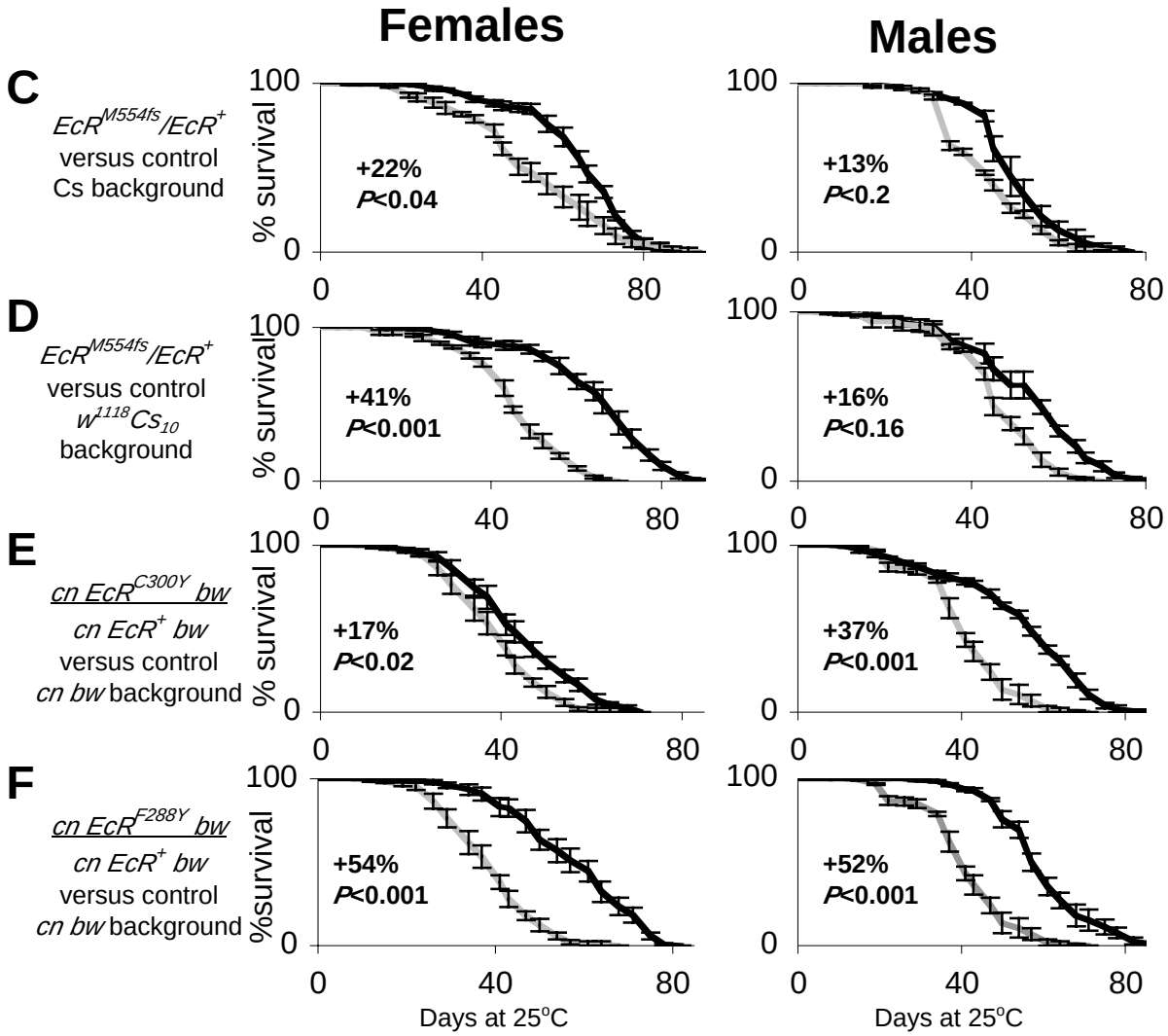


Fig. 2S. *DTS-3/+* longevity and stress resistance in males and females.

(A) Male longevity in Samarkand background. Consistent with the earlier observation that the ecdysteroid titer in this mutant is reduced only in females, males did not show increased lifespan at any of the temperatures tested. **(B)** Females DTS-3 longevity in w^{1118} background. To control for genetic background, *DTS-3* was outcrossed twice with our w^{1118} line. **(C)** Resistance to dry starvation of males and females DTS-3/+, in Samarkand and w^{1118} backgrounds. **(D)** Resistance to dry starvation at 25°C, after feeding 20-OH-ecdysone to DTS-3/+ and Sam females, for a week at the restrictive temperature of 29°C. Feeding 20-OH-ecdysone also reversed the increased survival under dry starvation. Mated flies were collected as in fig. 1.

A

Samarkand Background	Temperature (°C)	% Increase in mean lifespan (± SD)	n*
Males *DTS-3/+*	20°C	-16 ± 5	2
	25°C	-9 ± 14	2
	29°C	5 ± 4	2

n*= replicates of 40 to 160 flies
SD: standard deviation of the mean

B

w^{1118} Background	Temperature (°C)	% Increase in mean lifespan (± SD)	n*
Females *DTS-3/+*	20°C	-11 ± 9	2
	25°C	19 ± 5	2
	29°C	12 ± 2	3

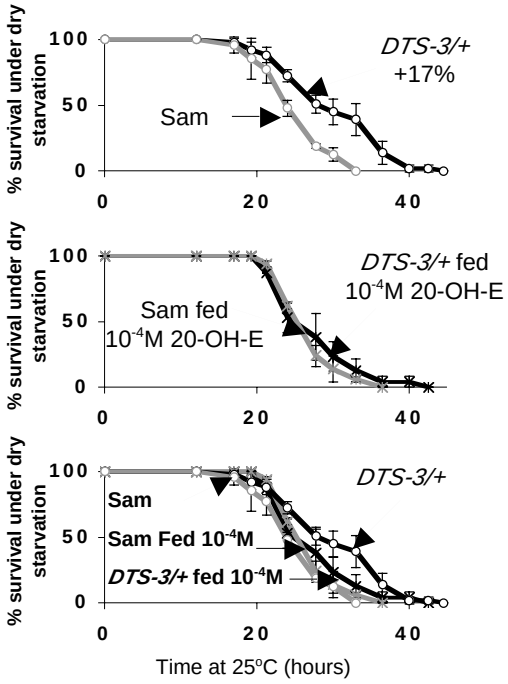
n*= replicates of 40 to 160 flies
SD: standard deviation of the mean

C

	Temperature (°C)	% Increase in mean survival time under dry starvation (± SD) Sam	n*	w^{1118}	n*
Females *DTS-3/+*	20°C	-18 ± 4	2	-16	1
	25°C	-23 ± 3	3	-16	1
	29°C	33 ± 14	3	12	1
Males *DTS-3/+*	20°C	-15 ± 3	2	-5	1
	25°C	-6 ± 13	2	-2	1
	29°C	-5 ± 9	2	-9	1

n*= replicates of 40 to 160 flies
SD: standard deviation of the mean

D



4. Supporting references

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