Supplementary Material

Cross-tolerance evolution is driven by selection on heat tolerance in *Drosophila subobscura*

Figure S1. Diagram of the experimental design.

Table S1. Descriptive statistics of knockdown time of *Drosophila subobscura*.

Table S2. Results of the mixed-linear models on the knockdown time of *Drosophila subobscura.*

Table S3. Tukey's contrast analysis for the knockdown time of *Drosophila subobscura*.

Table S4. Thermal-death-time (TDT) curves for *Drosophila subobscura*.

Table S5. Results of the desiccation survival of *Drosophila subobscura*.

Table S6. Results of the starvation survival of *Drosophila subobscura*.

Figure S1. Diagram of the experimental design: 100 isofemale lines from *Drosophila subobscura* were used to establish an outbred population. The F1 of these isofemale lines were transferred to a population cage and the F2 flies were divided into three replicates: R1, R2, and R3. After 3 generations, each population cage was divided into four population cages, which were assigned to four different artificial selection protocols in triplicate: fast-ramping selection, fast-ramping control, slow-ramping selection, and slow-ramping control lines. During 16 generations, heat tolerance was selected for 33% highest values of knockdown temperature using two different selection protocols: slow ramping rate (0.08 ºC/min) and fast ramping rate (0.4 ºC/min). Traits were evaluated at generation 23 and 24.

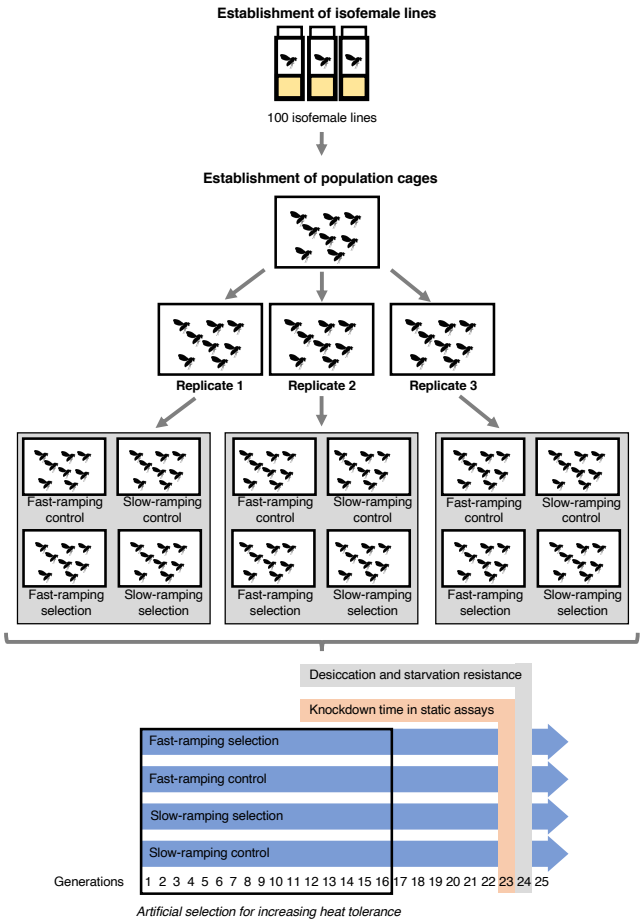

Table S1. Mean (SD) knockdown time of *Drosophila subobscura* flies assayed at four static temperatures. Values are organized by sex, selection protocol, and replicate cage.

		Females				Males			
Selection protocol	**Replicated cage**	**Knockdown time at 35ºC (min)**	**Knockdown time at 36ºC (min)**	**Knockdown time at 37ºC (min)**	**Knockdown time at 38ºC (min)**	**Knockdown time at 35ºC (min)**	**Knockdown time at 36ºC (min)**	**Knockdown time at 37ºC (min)**	**Knockdown time at 38ºC (min)**
Fast-ramping control	R1	28.91 (1.48)	14.66 (1.20)	7.34 (1.31)	6.17 (1.34)	25.39 (1.56)	14.09 (1.42)	7.95 (1.36)	5.73 (1.77)
	R2	33.71 (1.26)	13.86 (1.49)	6.94 (1.39)	5.95 (1.44)	30.02 (1.25)	15.00 (1.41)	6.49 (1.58)	5.55 (1.51)
	R3	30.13 (1.54)	15.51 (1.36)	7.88 (1.49)	6.75 (1.35)	33.64 (1.48)	15.38 (1.46)	5.60 (1.55)	5.33 (1.53)
Slow-ramping control	R1	34.30 (1.49)	15.80 (1.43)	6.91 (1.25)	5.74 (1.26)	25.43 (1.42)	12.07 (1.34)	5.86 (1.58)	5.65 (1.50)
	R2	38.16 (1.51)	10.86 (1.46)	7.31 (1.18)	5.82 (1.80)	28.15 (1.60)	13.75 (1.26)	7.25 (1.34)	5.63 (2.14)
	R3	33.84 (1.67)	13.80 (1.64)	7.09 (1.59)	5.42 (1.44)	31.18 (1.37)	13.34 (1.63)	7.62 (1.34)	5.04 (1.48)
Fast-ramping selection	R1	31.83 (1.35)	22.08 (1.55)	10.52 (1.64)	7.64 (1.41)	30.56 (1.58)	16.81 (1.43)	11.12 (1.52)	5.58 (1.24)
	R2	37.69 (1.21)	18.30 (1.43)	9.02 (1.30)	7.87 (1.34)	39.11 (1.37)	19.14 (1.30)	11.94 (1.33)	4.91 (1.69)
	R3	37.45 (1.46)	17.20 (1.89)	9.17 (1.38)	7.81 (1.57)	32.45 (1.21)	16.11 (1.39)	11.08 (1.54)	5.69 (1.69)
Slow-ramping selection	R1	34.72 (1.29)	19.95 (1.42)	9.48 (1.24)	6.54 (1.44)	29.76 (1.50)	18.17 (1.55)	9.73 (1.53)	7.11 (1.22)
	R2	32.06 (1.50)	18.09 (1.36)	10.01 (1.30)	7.09 (1.37)	26.38 (1.44)	16.98 (1.34)	6.49 (1.59)	6.82 (1.55)
	R3	41.97 (1.29)	18.40 (1.52)	8.36 (1.44)	6.53 (1.51)	33.08 (1.34)	13.81 (1.33)	9.34 (1.42)	5.85 (1.63)

Table S2. Results of the mixed-linear models on the knockdown time of *Drosophila subobscura*. Fixed effects were tested by a type III ANOVA, and the random effect was tested by a likelihood ratio test comparing the model with and without the replicate lines. Response variables were squared root transformed. Significant effects ($P < 0.05$) are indicated in bold.

Knockdown time at 35ºC

Fixed effect	*SS*	*DF_{num}, DF_{den}*	*F*	*P value*
Selection	1.82	3,170	0.62	0.602
Sex	8.44	1,170	8.64	**0.004**
Selection × Sex	1.89	3,170	0.64	0.588
Random effect	*Variance*	*Likelihood ratio test (df=1)*		*P value*
Replicate(Selection)	0.0000	0		1
Error	0.9767			

Knockdown time at 36ºC

Fixed effect	*SS*	*DF_{num}, DF_{den}*	*F*	*P value*
Selection	16.68	3,232	9.86	**3.8×10^{-6}**
Sex	1.49	1,232	2.65	0.10
Selection × Sex	1.25	3,232	0.74	0.53
Random effect	*Variance*	*Likelihood ratio test (df=1)*		*P value*
Replicate(Selection)	0.0000	0		1
Error	0.5639			

Knockdown time at 37ºC

Fixed effect	*SS*	*DF_{num}, DF_{den}*	*F*	*P value*
Selection	14.96	3,223	18.75	**7.0×10^{-11}**
Sex	0.002	1,223	0.009	0.93
Selection × Sex	1.58	3,223	1.99	0.12
Random effect	*Variance*	*Likelihood ratio test (df=1)*		*P value*
Replicate(Selection)	0.0000	0		1
Error	0.2656			

Knockdown time at 38ºC

Fixed effect	*SS*	*DF_{num}, DF_{den}*	*F*	*P value*
Selection	1.69	3,224	2.27	0.08
Sex	1.42	1,224	5.70	**0.02**
Selection × Sex	1.74	3,224	2.33	0.08
Random effect	*Variance*	*Likelihood ratio test (df=1)*		*P value*
Replicate(Selection)	0.0000	0		1
Error	0.2485			

Table S3. Tukey's contrast analysis for the knockdown time of *Drosophila subobscura* assayed in four static temperature assays. P values were corrected using the false discovery rate method. Significant differences ($P < 0.05$) are indicated in bold.

Knockdown time at 35ºC			
No selection effect			

Knockdown time at 36ºC			
Contrasts	*df*	*t*	*P value*
slow-control vs. fast-control	8	-1.426	0.230
slow-control vs. slow-selected	8	-3.994	**0.012**
slow-control vs. fast-selected	8	-4.773	**0.008**
fast-control vs. slow-selected	8	-2.568	0.050
fast-control vs. fast-selected	8	-3.347	**0.020**
slow-selected vs. fast-selected	8	-0.779	0.458

Knockdown time at 37ºC			
Contrasts	*df*	*t*	*P value*
slow-control vs. fast-control	7.91	-0.010	0.992
slow-control vs. slow-selected	8.06	-3.432	**0.013**
slow-control vs. fast-selected	7.64	-6.368	**0.001**
fast-control vs. slow-selected	8.33	-3.392	**0.013**
fast-control vs. fast-selected	7.91	-6.302	**0.001**
slow-selected vs. fast-selected	8.06	-2.841	**0.026**

Knockdown time at 38ºC			
No selection effect			

Table S4. Thermal-death-time (TDT) parameters calculated from heat tolerance measurements for *Drosophila subobscura*.

Selection regimen	Replicate	Sex	TDT curve	CT_{max} (ºC)	z (ºC)	r^2	Q_{10}
Fast-ramping control	R1	females	$\log_{10} t = 9.6218 - 0.2338\ T$	41.15	4.28	0.9425	217.84
	R2	females	$\log_{10} t = 10.3173 - 0.2527\ T$	40.83	3.96	0.9297	336.39
	R3	females	$\log_{10} t = 9.4471 - 0.2281\ T$	41.42	4.38	0.9469	190.81
	R1	males	$\log_{10} t = 8.8691 - 0.2132\ T$	41.61	4.69	0.9637	135.36
	R2	males	$\log_{10} t = 10.1218 - 0.2478\ T$	40.85	4.03	0.9484	300.91
	R3	males	$\log_{10} t = 11.3142 - 0.2803\ T$	40.36	3.57	0.9147	635.93
Slow-ramping control	R1	females	$\log_{10} t = 11.1809 - 0.2761\ T$	40.49	3.62	0.9455	577.29
	R2	females	$\log_{10} t = 10.2409 - 0.2506\ T$	40.87	3.99	0.8199	320.36
	R3	females	$\log_{10} t = 11.0648 - 0.2731\ T$	40.52	3.66	0.9572	538.05
	R1	males	$\log_{10} t = 9.1095 - 0.2214\ T$	41.15	4.52	0.8985	163.61
	R2	males	$\log_{10} t = 9.2449 - 0.2235\ T$	41.37	4.47	0.9132	171.69
	R3	males	$\log_{10} t = 10.6311 - 0.2618\ T$	40.61	3.82	0.9760	414.62
Fast-ramping selection	R1	females	$\log_{10} t = 9.0865 - 0.2155\ T$	42.16	4.64	0.9789	143.04
	R2	females	$\log_{10} t = 9.6911 - 0.2329\ T$	41.60	4.29	0.9357	213.53
	R3	females	$\log_{10} t = 9.7291 - 0.2336\ T$	41.65	4.28	0.9337	216.83
	R1	males	$\log_{10} t = 10.2040 - 0.2479\ T$	41.15	4.03	0.9875	301.61
	R2	males	$\log_{10} t = 10.1460 - 0.2463\ T$	41.20	4.06	0.9783	290.34
	R3	males	$\log_{10} t = 9.5585 - 0.2302\ T$	41.52	4.34	0.9863	200.49

Slow-ramping selection	R1	females	$\log_{10} t = 10.2076 - 0.2474\ T$	41.25	4.04	0.9797	298.12
	R2	females	$\log_{10} t = 9.4324 - 0.2263\ T$	41.69	4.42	0.9840	183.12
	R3	females	$\log_{10} t = 11.0759 - 0.2711\ T$	40.86	3.69	0.9551	513.69
	R1	males	$\log_{10} t = 9.2689 - 0.2219\ T$	41.77	4.51	0.9895	165.59
	R2	males	$\log_{10} t = 9.8391 - 0.2120\ T$	41.70	4.72	0.8784	131.73
	R3	males	$\log_{10} t = 9.6621 - 0.2339\ T$	41.31	4.27	0.9567	218.37

Table S5. Results of the desiccation survival analysis testing the effect of selection protocol, sex, and their interaction in *Drosophila subobscura*. Significant effects ($P < 0.05$) are indicated in bold.

Effect	**exp(coefficient)**	**z**	***P value***
Slow-ramping selection	0.422	–2.623	**0.009**
Fast-ramping selection	0.556	–1.797	0.072
Males	7.108	5.242	**1.6×10^{-7}**
Slow-ramping selection – males	1.773	1.264	0.206
Fast-ramping selection –males	1.391	0.731	0.465

Selection treatment	**vials**	**Median (h)**	**95% CI (h)**
Females			
Control	21	18.3	11.9 – 25.5
Slow-ramping selection	21	26.1	20.7 – 29.1
Fast-ramping selection	21	21.9	15.3 – 27.3
Males			
Control	21	5.81	4.51 – 7.50
Slow-ramping selection	21	5.81	4.51 – 10.49
Fast-ramping selection	21	5.81	5.10 – 9.23

Sex	**vials**	**Median (h)**	**95% CI (h)**
Females	63	21.87	19.5 – 25.5
Males	63	5.81	5.61 – 6.3

Table S6. Results of the starvation survival analysis testing the effect of selection protocol, sex, and their interaction in *Drosophila subobscura*. Significant effects ($P < 0.05$) are indicated in bold.

Effect	**exp(coefficient)**	**z**	***P value***
Slow-ramping selection	2.062	2.240	**0.025**
Fast-ramping selection	2.268	2.474	**0.013**
Males	22.749	8.212	**$<2\times10^{-16}$**
Slow-ramping selection – males	0.223	–3.305	**0.0009**
Fast-ramping selection –males	0.218	–3.273	**0.001**

Selection treatment	**vials**	**Median (h)**	**95% CI (h)**
Females			
Control	21	53.0	46.5 – 58.5
Slow-ramping selection	21	42.9	41.1 – 50.7
Fast-ramping selection	21	44.7	39.3 – 50.1
Males			
Control	21	25.5	24.9 – 28.5
Slow-ramping selection	21	30.3	27.9 – 33.3
Fast-ramping selection	21	26.7	24.9 – 32.7

Sex	**vials**	**Median (h)**	**95% CI (h)**
Females	63	46.5	42.9 – 50.1
Males	63	27.3	26.1 – 29.1