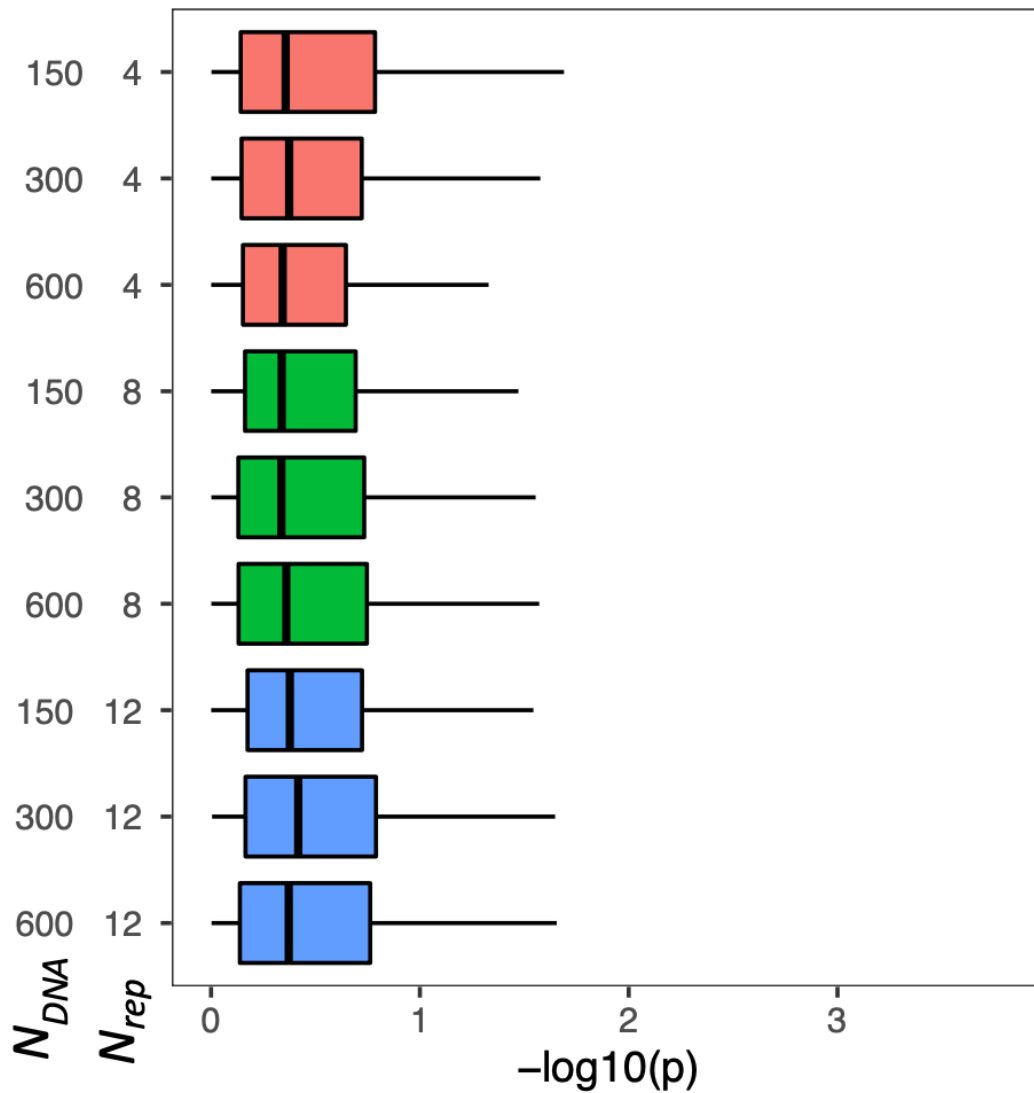
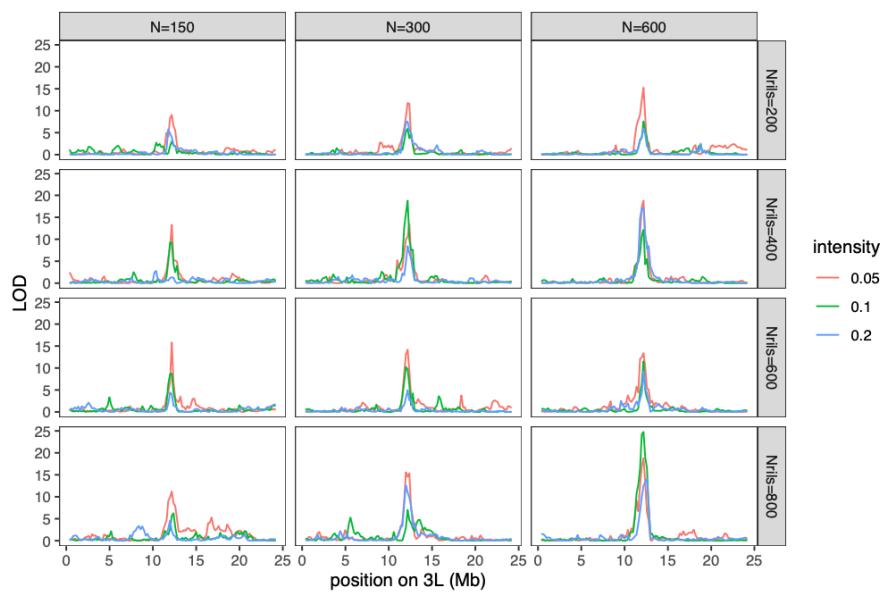


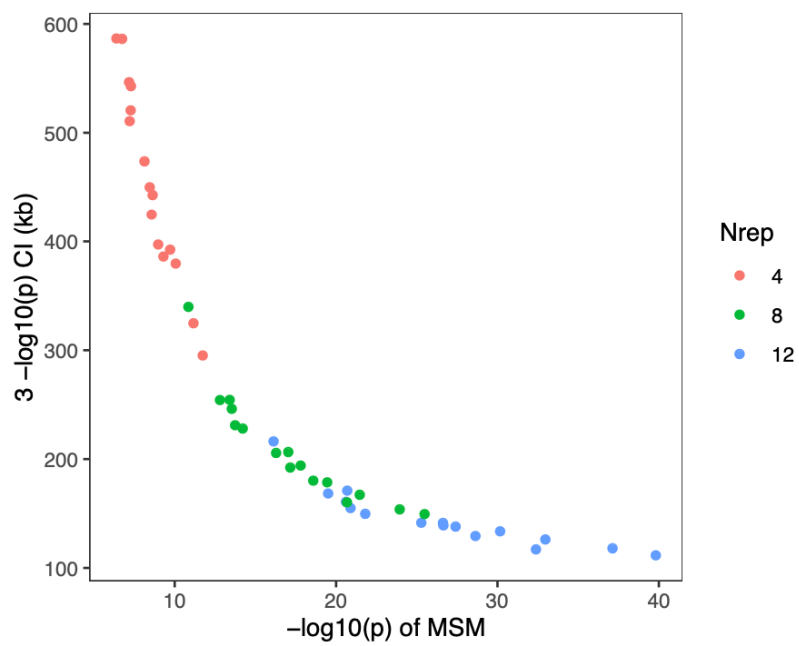
## Supplementary Figures



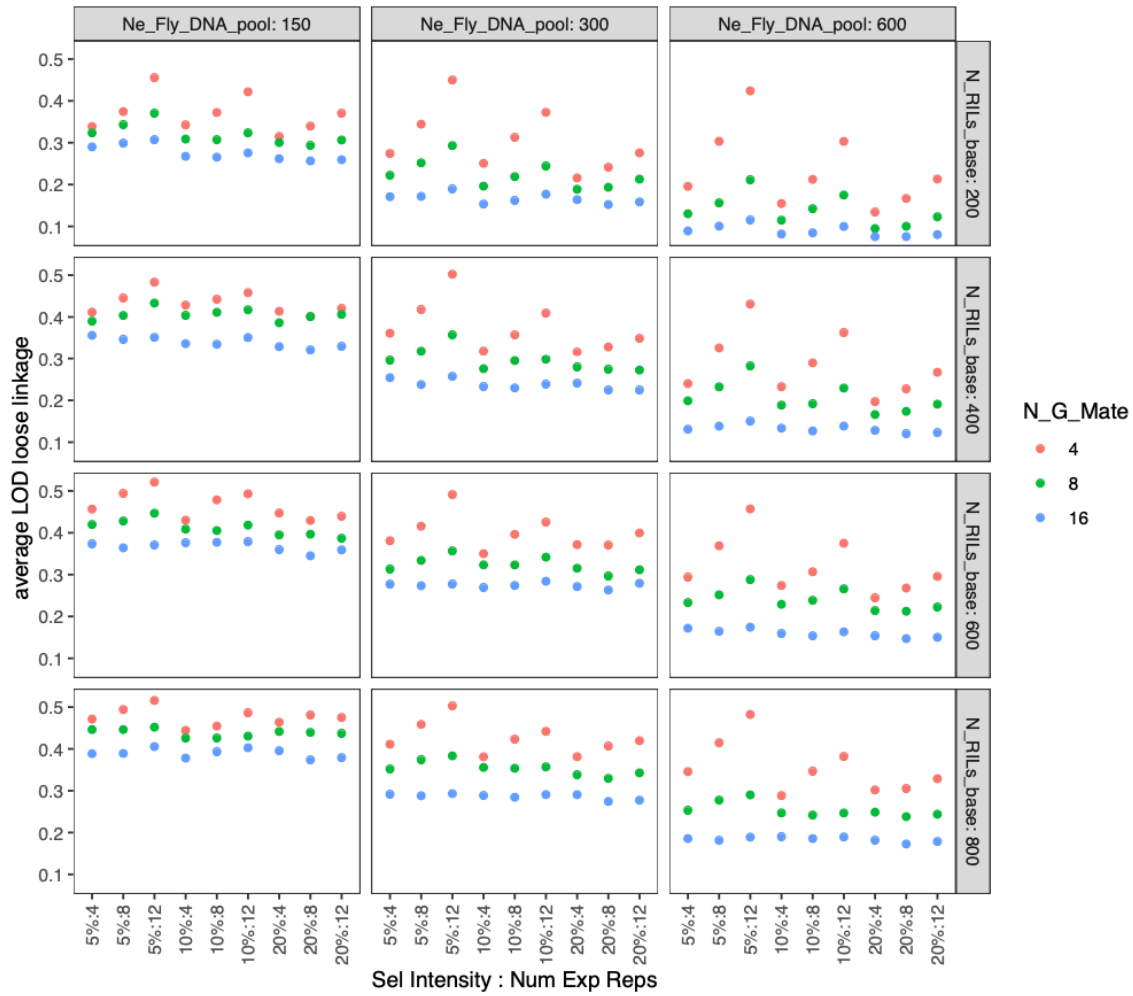
**Figure S1: Null distribution of  $-\log_{10}(p)$  test statistics with X-QTL mapping.** The expected distribution of the test statistic when comparing haplotype frequencies between two, equally sized draws ( $N = 150, 300$ , or  $600$  individuals) from the base population for different numbers of replicates of the experiment ( $N_{rep} = 4, 8$ , or  $12$ ).



A

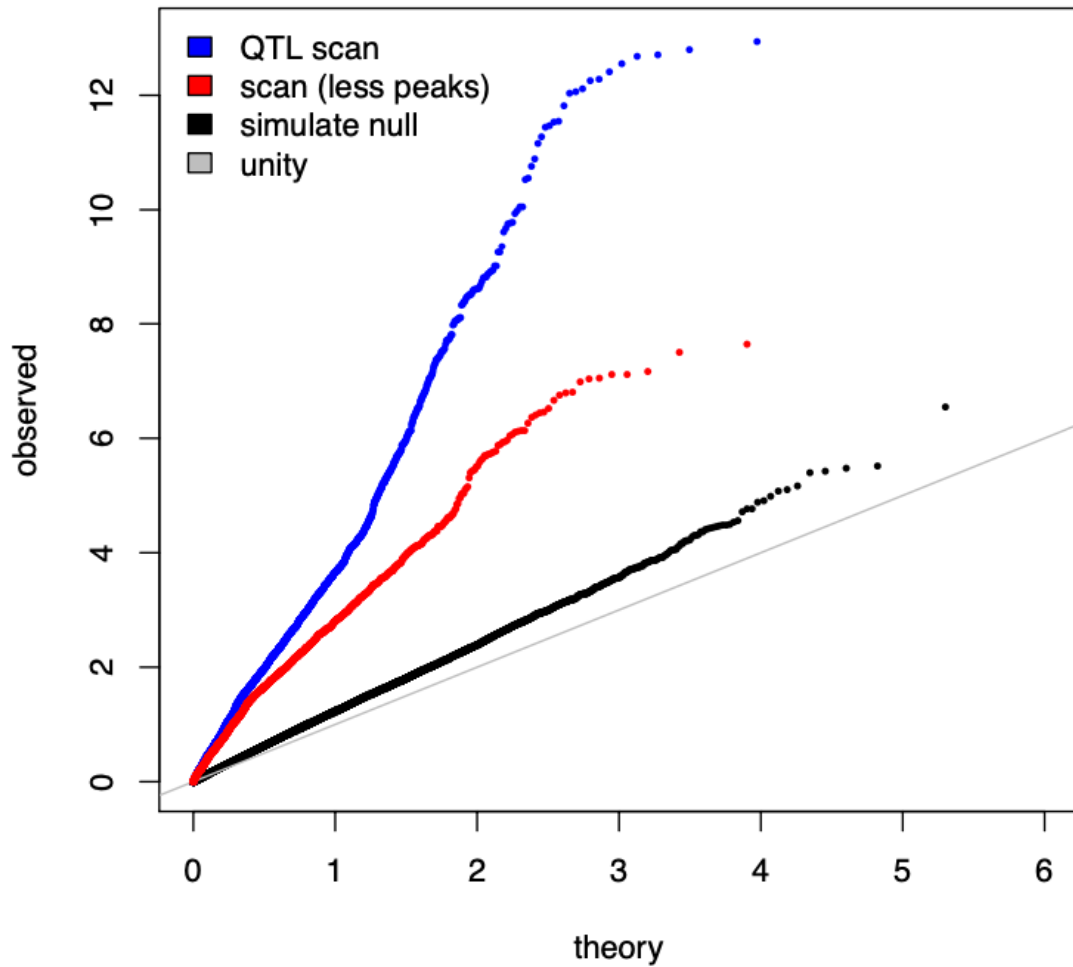


B

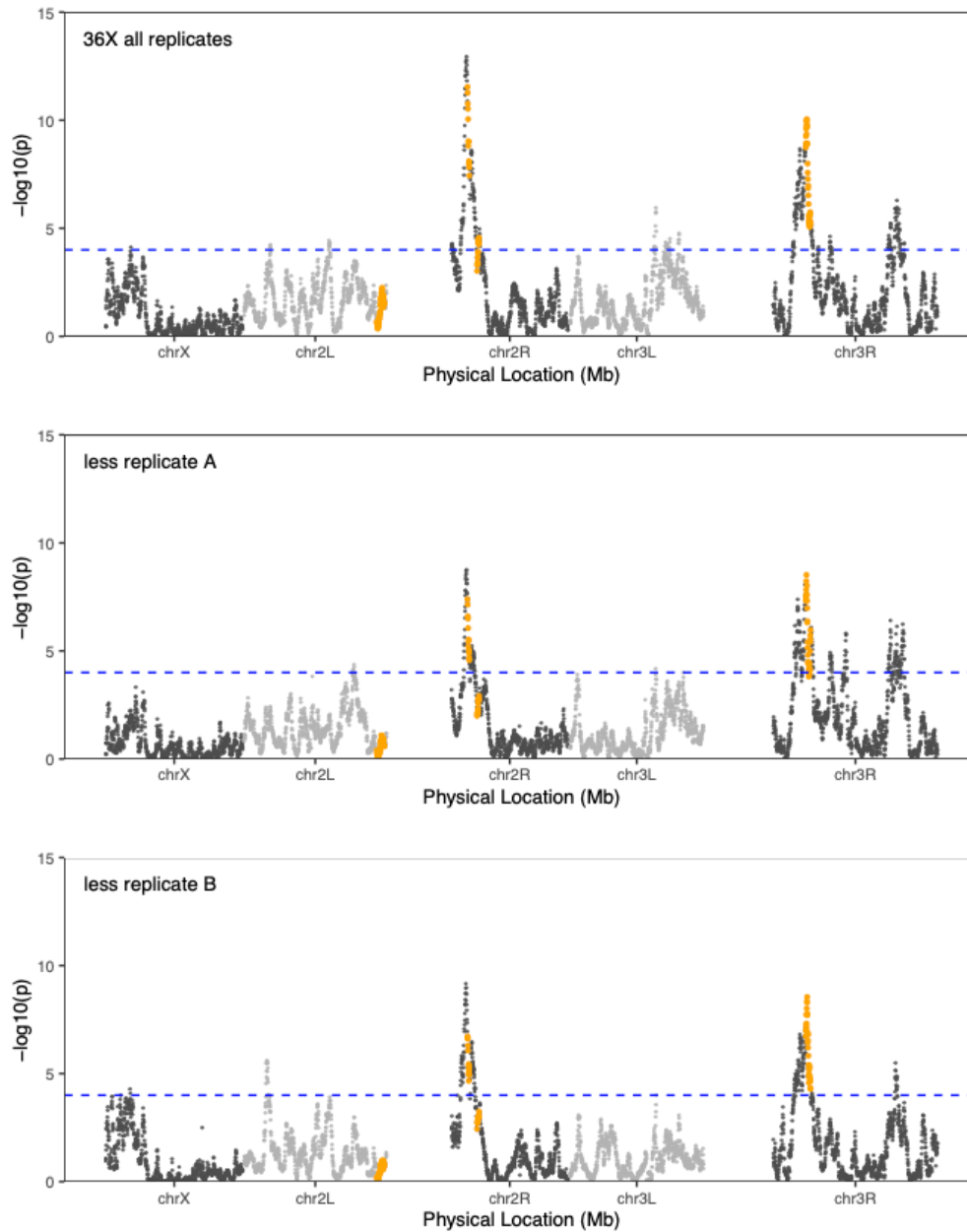


C

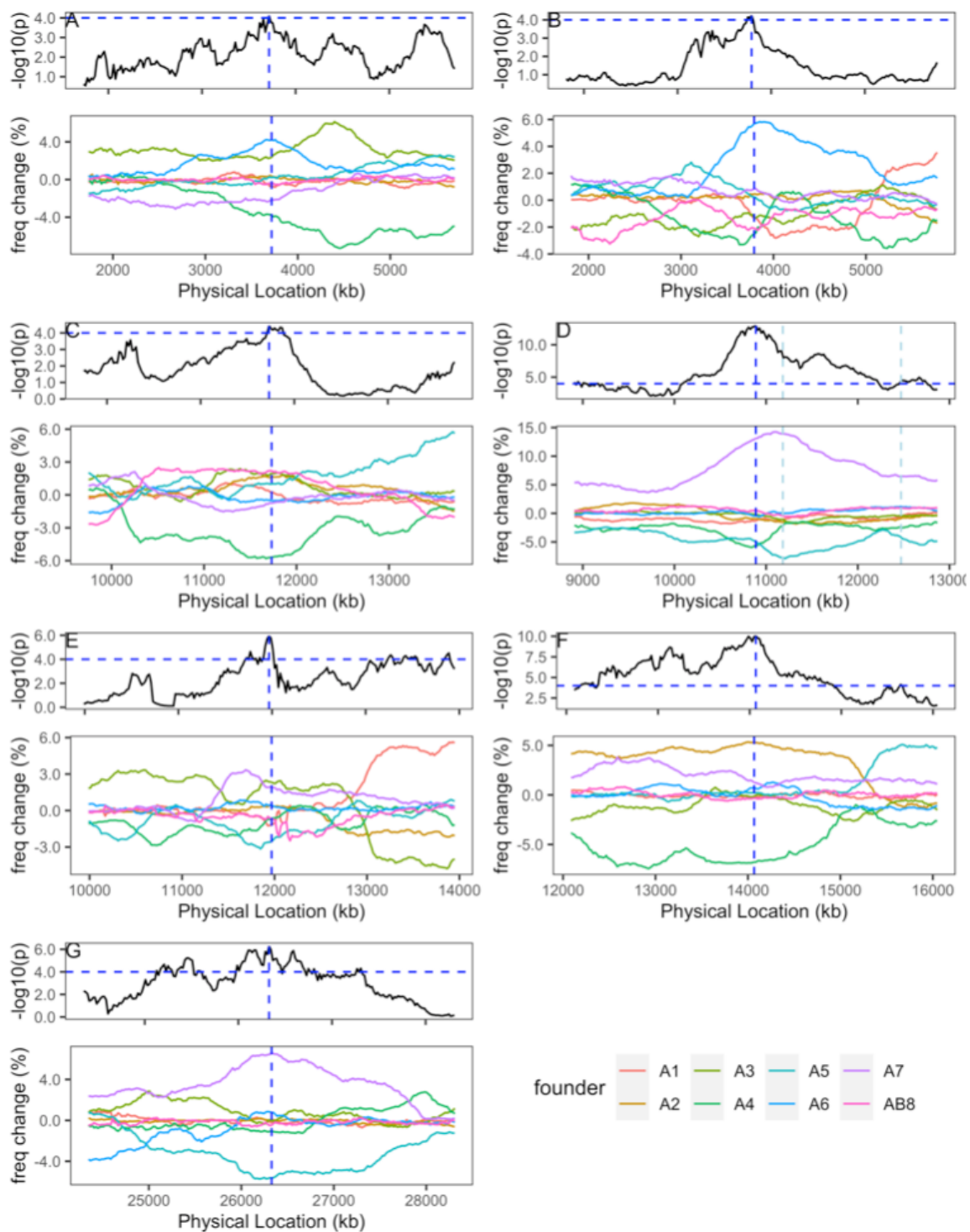
**Figure S2: (A) QTL localization for an X-QTL experiment for a single replicate.** All parameters identical to Figure 3, except we only present a single realization of the X-QTL experiment. This figure highlights the strong correlation in test statistics at adjacent markers. **(B) Average size of  $3 - \log_{10}(P)$  support interval as a function of  $-\log_{10}(P)$  score at the most significant marker.** Experiment simulates four generations of random mating following base population establishment for different numbers of experimental replicates ( $N_{rep}$ ). Different points are different combinations of design parameters ( $N_{DNA} = 300, 600$ ;  $N_{RIL} = 200, 400, 600, 800$ ;  $i = 5\%, 10\%$ ). **(C) Average  $-\log_{10}(P)$  score at markers loosely linked to a causative region.** Data suggests some inflation of  $-\log_{10}(P)$  scores as a function of the number of generations of random mating following base population establishment,  $N_{DNA}$ ,  $N_{RIL}$ ,  $i$ , and the number of experimental replicates.



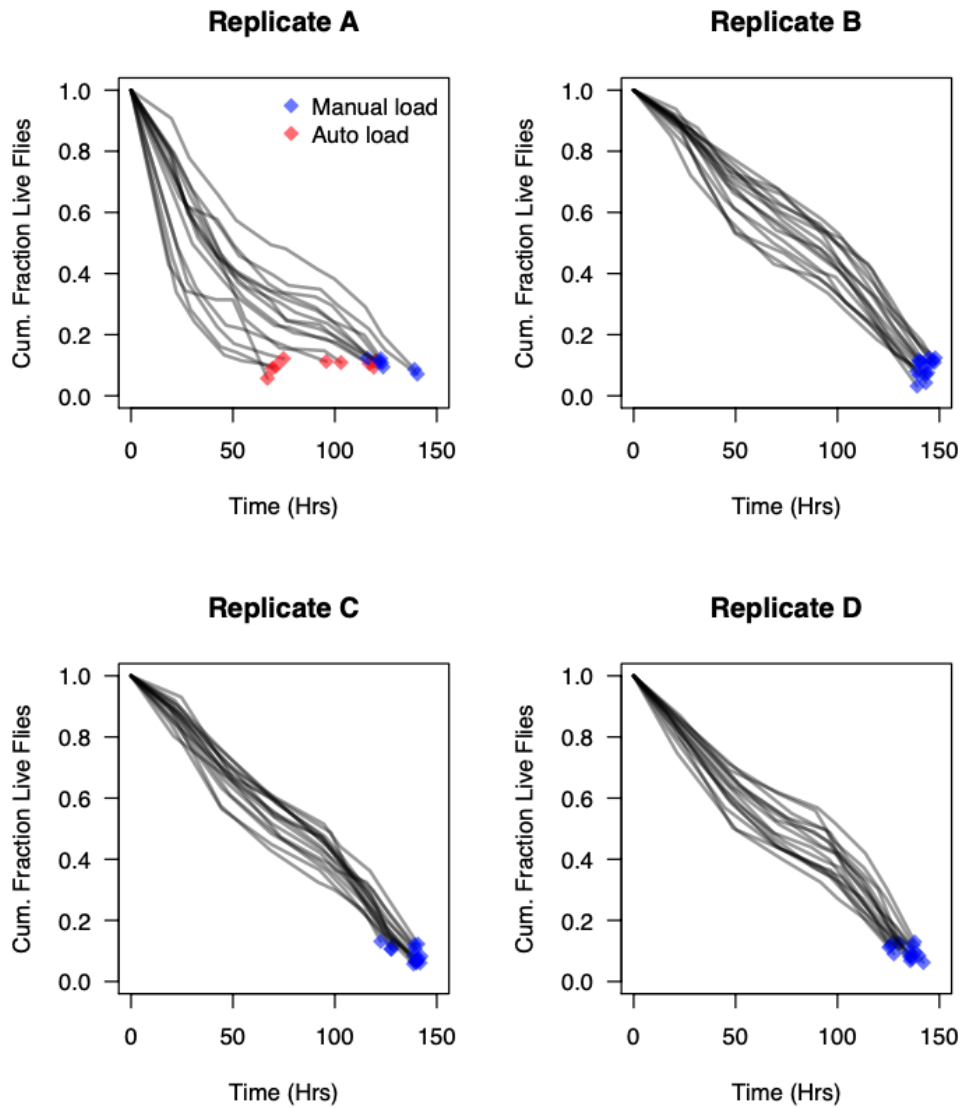
**Figure S3: QQ-plots:** Theoretical  $-\log_{10}(P)$  quantiles on the x-axis, and simulated quantiles (black) or observed quantiles (red and blue) from the caffeine resistance mapping experiment on the y-axis. The grey line is unity, and would be expected if observed quantiles matched theory under a model with no QTL. The black points are observed quantiles under a simulated experiment comparing two control draws of the same size from the base population of size  $N_{DNA} = 150$  from a 4-fold replicated simulated experiment. There is a slight inflation of the test statistic due to sampling variation. The blue points are observed quantiles under the empirical full coverage X-QTL scan for caffeine resistance loci, and red points are observed quantiles from the same scan with 2-Mb intervals centered on the seven detected peaks (Table 1) removed.



**Figure S4: A QTL scan at ~36X coverage with some replicates dropped.** The upper panel is for the entire dataset (all 4 replicates), while the two lower panels drop either replicate “A” (where a fraction of the animals were dispensed into phenotyping assay tubes automatically) or replicate “B” (where, similar to replicates “C” and “D”, all animals were dispensed via manual aspiration). The similarity of the  $-\log_{10}(P)$  profiles in the lower two panels implies that the method of dispensing does not markedly contribute to the outcome.



**Figure S5: Founder haplotype frequencies at caffeine X-QTL.** This figure is identical to [Figure 5](#) except the  $-\log_{10}(P)$  scores and frequencies are calculated from the lower coverage, ~35X, sequencing data.



**Figure S6: Mortality trajectory of flies from each replicate.** Each curve in each replicate-specific plot represents the fraction of flies alive per tray at a series of timepoints throughout the experiment. Trays typically contained 160 flies at the start of each replicate experiment, each fly held singly in an activity monitor tube. Dead flies were typically counted twice per day, and the mortality trajectory is roughly consistent across trays. For replicate A, a fraction of the flies were automatically loaded into monitor tubes (red) and a fraction of the flies were manually loaded (blue), as they were for replicates B-D. Automatic loading led to a reduced lifespan on average, although this variation in experimental method appears not to have had a dramatic effect on the mapping results (see [Figure S4](#)).

**Table S1.** Details of each replicate caffeine resistance experiment using the mixed population of DSPR pA RILs.

Activity	Replicate A	Replicate B	Replicate C	Replicate D
Generations since population founding	1	3	4	5
Egg collection date <sup>a</sup>	25-26/7/19	8-9/8/19	30-31/8/19	5-6/9/19
Number of vials of eggs collected	105	73	84	54
First flies evident in rearing vials	3/8/19	17-18/8/19	7-8/9/19	14-15/9/19
Transfer adults from each rearing vial to a mixed-sex housing vial	5/8/19	19/8/19	9/9/19	16/9/19
Experimental female collection date	6/8/19	20/8/19	10/9/19	17/9/19
Housing of experimental females	1 vial of 30 per rearing vial	2 vials of 22 per rearing vial	2 vials of 19 per rearing vial	2 vials of 28 per rearing vial
Date flies loaded into activity monitor tubes	8/8/19	22/8/19	12/9/2019	19/9/19
Estimated age of experimental females on experiment start	3-5 days old	3-5 days old	3-5 days old	3-5 days old
Total number of control flies collected	250	250	250	250
Total number of flies assayed	2,337	2,572	2,535	2,563
Total number of selected flies collected	241 (10.3 %)	235 (9.1 %)	228 (9.0 %)	254 (9.9 %)

<sup>a</sup> day\_start-day\_end/month/year



**Table S2.** Counts of CyO balancer-containing animals per Gal4 × UAS cross.

UAS (VDRG ID, Gene Symbol)	Gal4 Stock ID <sup>a</sup>	UAS CyO Status	Gal4 CyO Status	Cross Direction (F × M)	Vial 1 <sup>b</sup>		Vial 2 <sup>b</sup>		Vial 3 <sup>b</sup>		Overall CyO %
					WT	CyO	WT	CyO	WT	CyO	
2620, <i>E23</i>	BDSC 25374	Fixed	Fixed	UAS × Gal4	16	30	14	39	-	-	69.7 <sup>d</sup>
				Gal4 × UAS	10	40	25	35	26	29	63.0 <sup>d</sup>
	Flygut 1099	Absent	Fixed	UAS × Gal4	17	21	21	23	-	-	53.7
				Gal4 × UAS	18	18	22	34	25	24	53.9
6121, <i>Vha100-5</i>	BDSC 25374	Fixed	Absent	UAS × Gal4	21	18	29	29	-	-	48.5
				Gal4 × UAS	35	23	15	20	37	32	46.3
9489, <i>Ugt36A1</i>	BDSC 25374	Fixed	Absent	UAS × Gal4	17	17	22	26	-	-	52.4
				Gal4 × UAS	40	32	21	33	42	28	47.4
12138, <i>Cyp6d5</i>	BDSC 25374	Fixed	Absent	UAS × Gal4	29	30	24	35	-	-	55.1
				Gal4 × UAS	45	29	34	32	-	-	43.6
37736, <i>Crys</i>	BDSC 25374	Fixed	Absent	UAS × Gal4	27	26	23	25	-	-	50.5
				Gal4 × UAS	32	38	31	28	43	40	50.0
38661, <i>osy</i>	BDSC 25374	Fixed	Absent	UAS × Gal4	0	34	0	12	-	-	100 <sup>e</sup>
				Gal4 × UAS <sup>c</sup>	NA	NA	NA	NA	NA	NA	NA
46424, <i>Tlk</i>	BDSC 25374	Fixed	Absent	UAS × Gal4	0	24	0	12	-	-	100 <sup>e</sup>
				Gal4 × UAS <sup>c</sup>	NA	NA	NA	NA	NA	NA	NA
50507, <i>Cyp12d1</i>	BDSC 25374	Fixed	Absent	UAS × Gal4	31	30	17	3	-	-	40.7
				Gal4 × UAS	28	16	12	15	15	13	44.4
60000, Control	BDSC 25374	Fixed	Absent	UAS × Gal4	18	16	40	46	-	-	51.7
				Gal4 × UAS	29	24	24	29	34	36	50.6

<sup>a</sup> Strain 25374 expresses Gal4 ubiquitously under the control of an *Act5C* promoter. Strain 1099 expresses Gal4 in the anterior region of the adult midgut.

<sup>b</sup> Per vial counts of the numbers of wildtype (WT) or CyO-carrying F1 females. Cells containing "-" values indicate that only 2 replicate cross vials were utilized.

<sup>c</sup> Cross was not attempted since reciprocal cross yielded no non-CyO, Gal4-UAS-RNAi F1 females.

<sup>d</sup> Both parental strains are fixed for CyO, so >50% F1 animals carrying CyO is expected.

<sup>e</sup> Zero wildtype (WT) F1 females were observed; presumably ubiquitous gene knockdown is pre-adult lethal.

**Table S3.** *D. melanogaster* release 6 euchromatic boundaries

<b>Arm</b>	<b>Left boundary</b>	<b>Right boundary</b>
X	277,911	18,930,000
2L	82,455	19,570,000
2R	8,860,000	24,684,540
3L	158,639	18,438,500
3R	9,497,000	31,845,060

## **SUPPLEMENTARY TEXT**

**Text S1.** Media recipes employed in the study.

### APPLE JUICE AGAR

For 1-liter of apple juice agar:

750-ml water

20-g agar (Genesee Scientific; 66-111)

Mix using stirring hotplate until mix boils

250-ml apple juice (store bought)

25-g sugar (store bought)

Mix in a beaker

Add to boiling water/agar mix

Lower temperature and continue to heat/stir for ~15-min

Remove from stirring hotplate to orbital shaker to cool

5-ml 95% ethanol

1.5-g tegosept (Genesee Scientific; 20-258)

Dissolve tegosept in ethanol in 50-ml centrifuge tube

Add to water/agar/juice/sugar mix when it has reached ~60°C

Pour into petri dishes

Avoid generating bubbles, but if some form, use bunsen burner flame to remove them

### LIVE YEAST PASTE

Mix approximately equal volumes of water and active dry yeast (Genesee Scientific; 62-103) until it is smooth, and achieves the consistency of toothpaste.

### CORNMEAL-YEAST-MOLASSES REARING/HOUSING MEDIA

28.5-liters water

280-g agar (Genesee Scientific; 66-111. This is based on a gel strength of 1,090 g/cm<sup>2</sup>, and will change depending the batch)

Add water to steam kettle, turn on electric mixer, and slowly add agar

Bring mix to a boil

3,200-ml molasses (Genesee Scientific; 62-117)

Reduce the kettle pressure to reduce the heat slightly

Add molasses, and bring mix back to a boil

4-liters water

1,460-g inactive dry yeast (Genesee Scientific; 62-107)

Mix in bucket using paint-stirring drill attachment

4-liters water

2,600-g yellow cornmeal (Genesee Scientific; 62-101)

Mix in bucket using paint-stirring drill attachment  
Add both the water/yeast and water/cornmeal mixes to the steam kettle  
Bring mix back to boil, and simmer for ~15-min  
Release pressure from steam kettle, but continue to stir with electric mixer

330-ml water  
259-ml propionic acid (ThermoFisher; A258-500)  
31-ml phosphoric acid (85%; ThermoFisher; A242-500)  
Pour mix into kettle

400-ml 95% ethanol  
1.5-g tegosept (Genesee Scientific; 20-258)  
Dissolve tegosept in ethanol  
Pour mix into kettle  
Fill vials/bottles

### CORNMEAL-YEAST-DEXTROSE ASSAY MEDIA

For ~1.5-liters of media:

1,028-ml water  
7.5-g agar (Genesee Scientific; 66-111)  
Mix using stirring hotplate until mix boils  
Reduce heat and continue boiling for ~15-min until mix is clear

180-ml water  
45-g inactive dry yeast (Genesee Scientific; 62-107)  
81-g yellow cornmeal (Genesee Scientific; 62-101)  
96-g dextrose (Fisher Scientific; D16-1)  
Mix in a beaker  
Stir into agar/water mix  
Boil for ~10-min (manually stir frequently to avoid burning)  
Remove from stirring hotplate to orbital shaker to cool

18-ml acid mix (see below\*)  
30-ml tegosept/ethanol mix (see below\*\*)  
Add to water/agar/yeast/cornmeal/dextrose mix when it is ~65°C  
Move 1-liter of media to fresh beaker

10-g caffeine (SigmaAldrich, C0750)  
Add to mix when it is ~55°C

\* Acid mix:

418-ml propionic acid (ThermoFisher; A258-500), 50-ml phosphoric acid (85%; ThermoFisher; A242-500), 532-ml water

\*\* Tegosept / ethanol mix:

3-g tegosept (Genesee Scientific; 20-258) dissolved in 30-ml 95% ethanol

**Text S2.** Pooled fly DNA isolation protocol.

- (1) Homogenize pool of ~250 flies in 2-ml of 1X PBS using glass beads with a Mini-BeadBeater-96 (Biospec).
- (2) Add 4-ml of cold cell lysis buffer\* to sample, and subject to 3-4 strokes of both the "loose" and "tight" pestles of a glass dounce tissue grinder (Wheaton, 7-ml).
- (3) Using a wide-bore pipet tip, move 600- $\mu$ l of the resulting slurry to a 1.7-ml microcentrifuge tube, incubate at 65°C for 25-min, and cool to room temperature.
- (4) Add 3- $\mu$ l of RNase A solution\* to lysate, mix the tube by inverting 25 times, incubate at 37°C for 40-min, and rapidly cool to room temperature by placing sample on ice.
- (5) Add 200- $\mu$ l of protein precipitation solution\* to the lysate, vortex on high speed for 20 seconds, place sample on ice for 5-min, and centrifuge at 14,000-rpm\*\* for 3-min.
- (6) Move supernatant to new 1.7-ml microcentrifuge tube, and centrifuge at 14,000-rpm\*\* for 1-min.
- (7) Move supernatant to a 1.7-ml microcentrifuge tube containing 600- $\mu$ l of isopropanol (avoiding any remaining detritus), mix tube by inverting 50 times, centrifuge at 14,000-rpm\*\* for 1-min, and gently pour off supernatant.
- (8) Add 600- $\mu$ l of 70% ethanol, invert tube 2-3 times to wash pellet, centrifuge at 14,000-rpm\*\* for 1-min, pipette off supernatant, leave tube inverted to air dry for 15-min, and resuspend with 50- $\mu$ l of Qiagen EB buffer\*\*\*.

\* Cell lysis buffer, RNase A solution, and protein precipitation solution are part of the Gentra Puregene Cell Kit (Qiagen, 158767).

\*\* This is >20,000-g (rcf) in our Eppendorf instrument.

\*\*\* Qiagen, 19086.