

**Supporting Online Material to:****A cost of long-term memory in *Drosophila*****Materials and Methods****Fig. S1**

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**Materials and Methods***Conditioning procedure*

Conditioning and memory tests were performed on samples of 50 adult flies (sexes mixed), raised in standard conditions and aged 3-5 days. The conditioning procedure consisted of 5 training sessions separated by 20 min intervals (spaced protocol) or immediately following one another (massed protocol).

(A) Associative conditioning: In each training session flies were first exposed for 30 s to one odorant simultaneously with mechanical shock (2000 rpm vibration pulses of 1 s duration, delivered every 5 s by a test tube shaker). This period was followed by a 60 s rest period (no odor and no shock). Then, for 30 s another odorant was delivered, without shock. The training session ended with a second rest period of 60 s. 3-octanol and 4-methylcyclohexanol (both 0.6ml/l of paraffin) were used as odorants.

(B) Non-associative conditioning was similar except that the conditioned stimulus (odor) and the unconditioned stimulus (shock) were not presented simultaneously. Instead, in each training session the flies first received for 30 s shock only (on odors). Then one

odorant was provided for 30 s without shock, followed by 60 s rest period, 30 s of exposure to the second odorant, and 30 s of rest.

#### *Memory assay*

We tested 24 h memory retention after associative conditioning in the spaced and massed protocols. In order to verify that only the spaced protocol induces LTM (which is protein synthesis dependent), flies were fed 35 mM protein synthesis inhibitor cycloheximide (Sigma) in 4% sucrose (CMX+) or 4% sucrose alone (CXM-) for 15 h before training and for 24 h between training and memory assay. We also assayed 20 min memory retention after non-associative conditioning (without cycloheximide treatment).

For the assay the flies were transported to the choice point of a T-maze, in which they were exposed to two converging currents of air, one carrying octanol and the other methylcyclohexanol, and allowed to choose between the two odors for 60s. The memory score was calculated as the difference in the proportion of individuals choosing octanol between flies conditioned to avoid methylcyclohexanol and those conditioned to avoid octanol.

#### *Desiccation and starvation resistance*

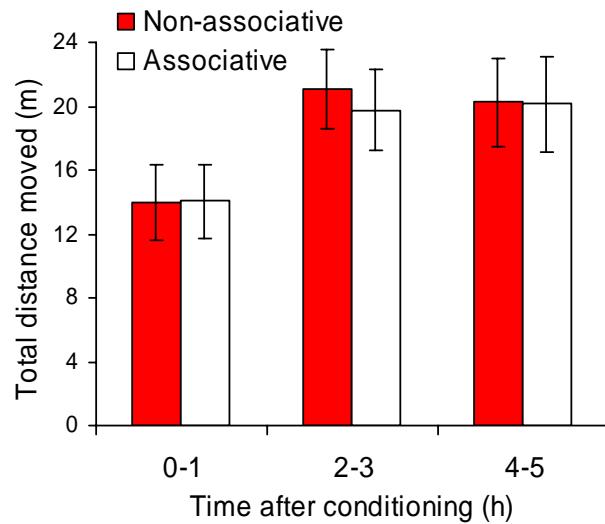
Three treatments were added to the four conditioning treatments described above (spaced versus massed protocol  $\times$  associative versus non-associative conditioning). *Shock only*: flies were exposed to a shock treatment, but no odors; the shock treatment followed either the spaced or the massed protocol. *Untreated*: flies were not exposed to odors or mechanical shock, but otherwise handled in the same way as in the other treatments.

Fifty flies (sexes mixed) were placed in an empty vial and subject to one of those seven treatments (four vials per treatment). After the longest treatment was completed, 30 flies per sex and treatment were individually transferred to an empty Petri dish (diameter 5.4 cm) and kept at 25°C and 70% humidity. Dead flies were counted and sexed 7, 8, 10, 11, 13, 15, 17, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 33, 35 and 39 h after being

removed from food. At 39h all flies were dead. Time till death was analyzed with a two-way ANOVA with treatment and sex as factors.

### *Locomotor activity*

Flies were subjected to associative or non-associative conditioning in the spaced protocol and then individually transferred to empty Petri dishes. The locomotion of each fly was recorded 0-1 h, 2-3 h and 4-5 h after conditioning using cameras (capture rate 4 images/s). Sixteen flies per sex and treatment were assayed over four days. Using VideoScript 3.0.12 we estimated the total distance covered by the fly during recording. There was no difference in locomotion between sexes (repeated-measures ANOVA,  $F_{1,123} = 1.6$ ;  $P = 0.23$ ), and between flies subject to associative and non-associative conditioning ( $F_{1,123} = 0.01$ ;  $P = 0.93$ ; Fig. S1), indicating that associative conditioning does not induce increased locomotor activity.



**Fig. S1.** Total distance moved by individual fly (mean  $\pm$  standard error, sex pooled) 0-1 h, 2-3 h and 4-5 h after being subjected to non-associative or associative spaced conditioning.