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Supplementary Materials for

A deep sleep stage in *Drosophila* with a functional role in waste clearance

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at advances.sciencemag.org/cgi/content/full/7/4/eabc2999/DC1)

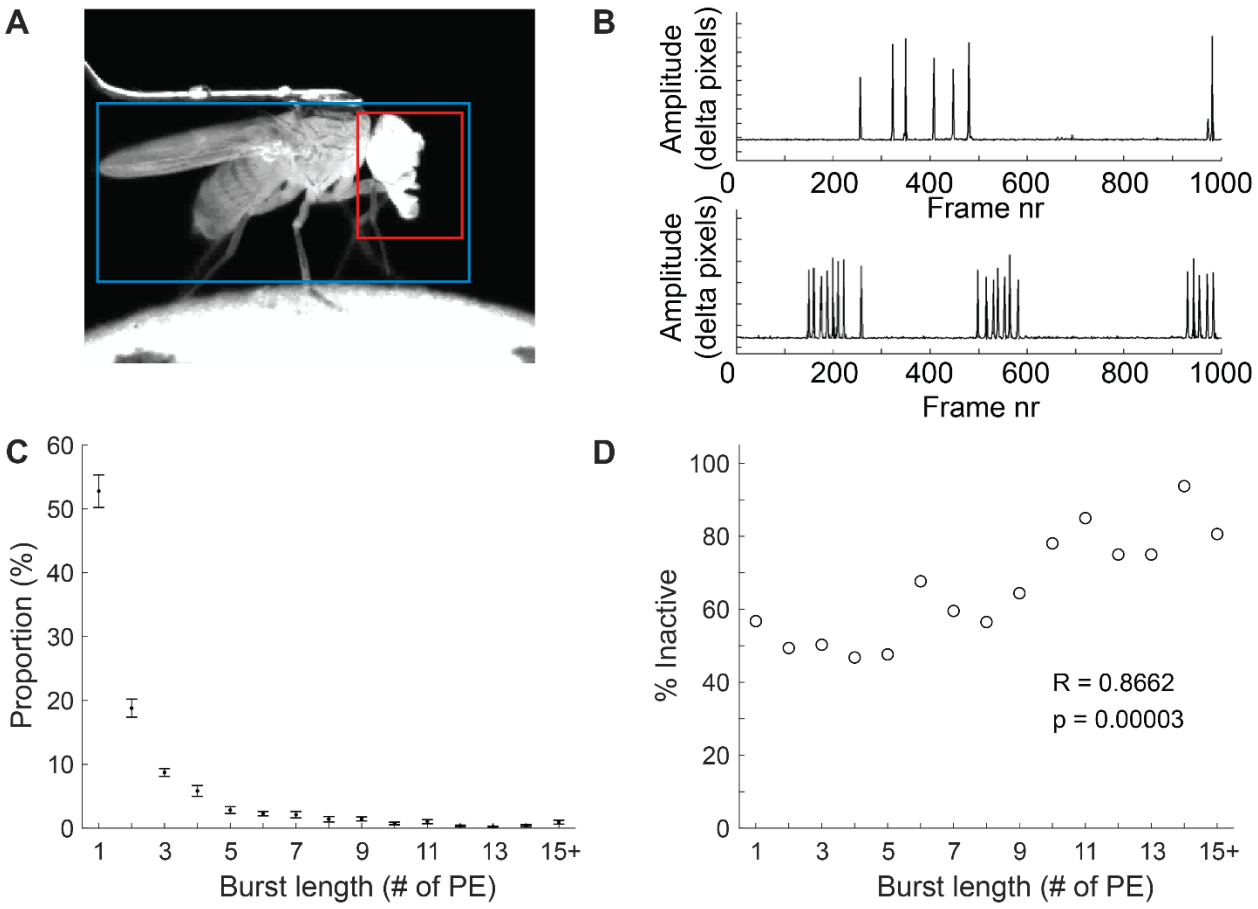

Fig. S1 – PE detection in tethered flies

(A) Fly activity and PE were monitored using a pixel subtraction approach (c.f. (*27*)) in two regions of interest to determine fly movement (blue area) and proboscis movement (red area).

(B) PE occur either as single events or as bursts, where each new PE starts soon after the previous one is completed

(C) 53% of all PE occur as a single PE, the remainder consists of burst of 2-15+ consecutive PEs

(D) There is a positive correlation between burst length and inactivity, where flies are more likely to be inactive during longer bursts (R = 0.8662, p = 0.00003)

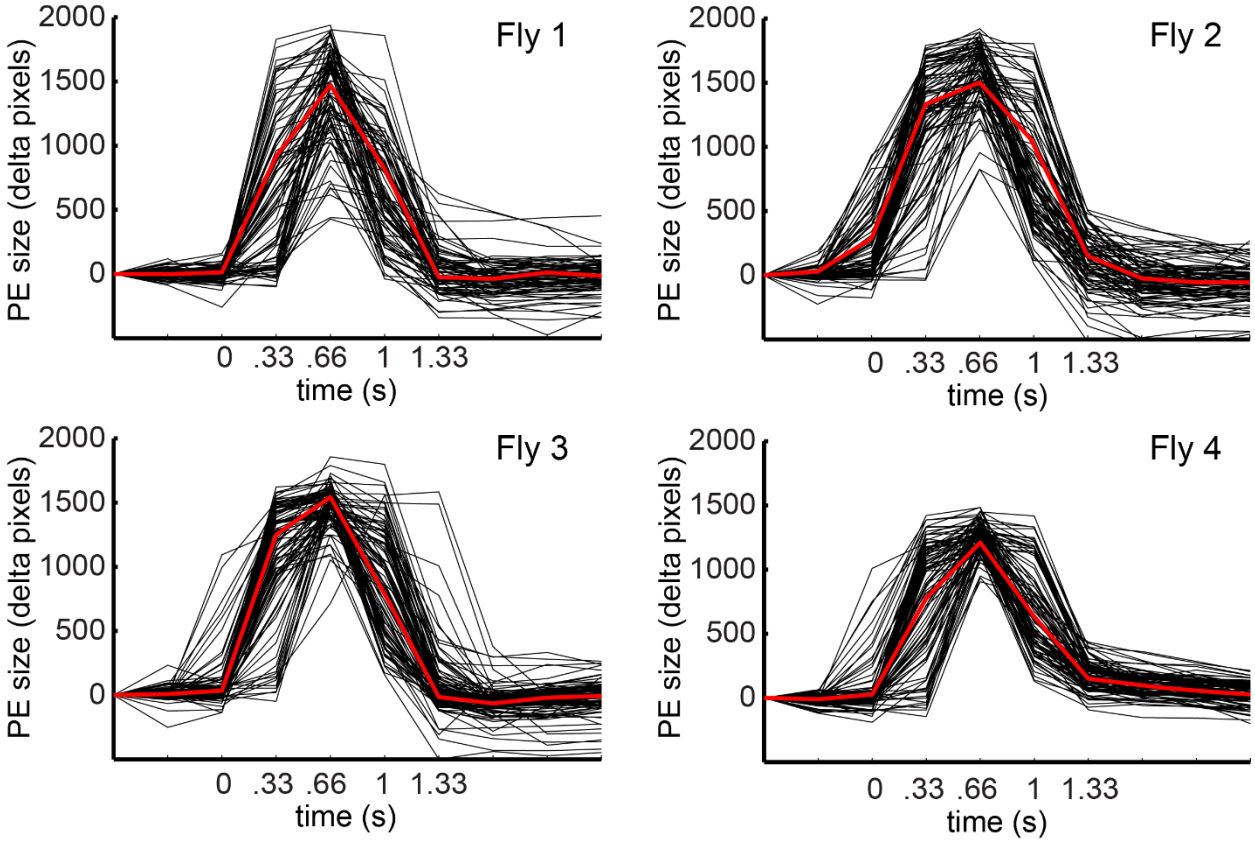

Fig. S2 – Proboscis extensions resemble a fixed action pattern

PE for four flies were aligned by peak amplitude (max delta pixels). The red trace shows the average trace. PE resemble a fixed action pattern where an extension of the proboscis (of variable amplitude) is immediately followed by a retraction. This process takes ~1.3s

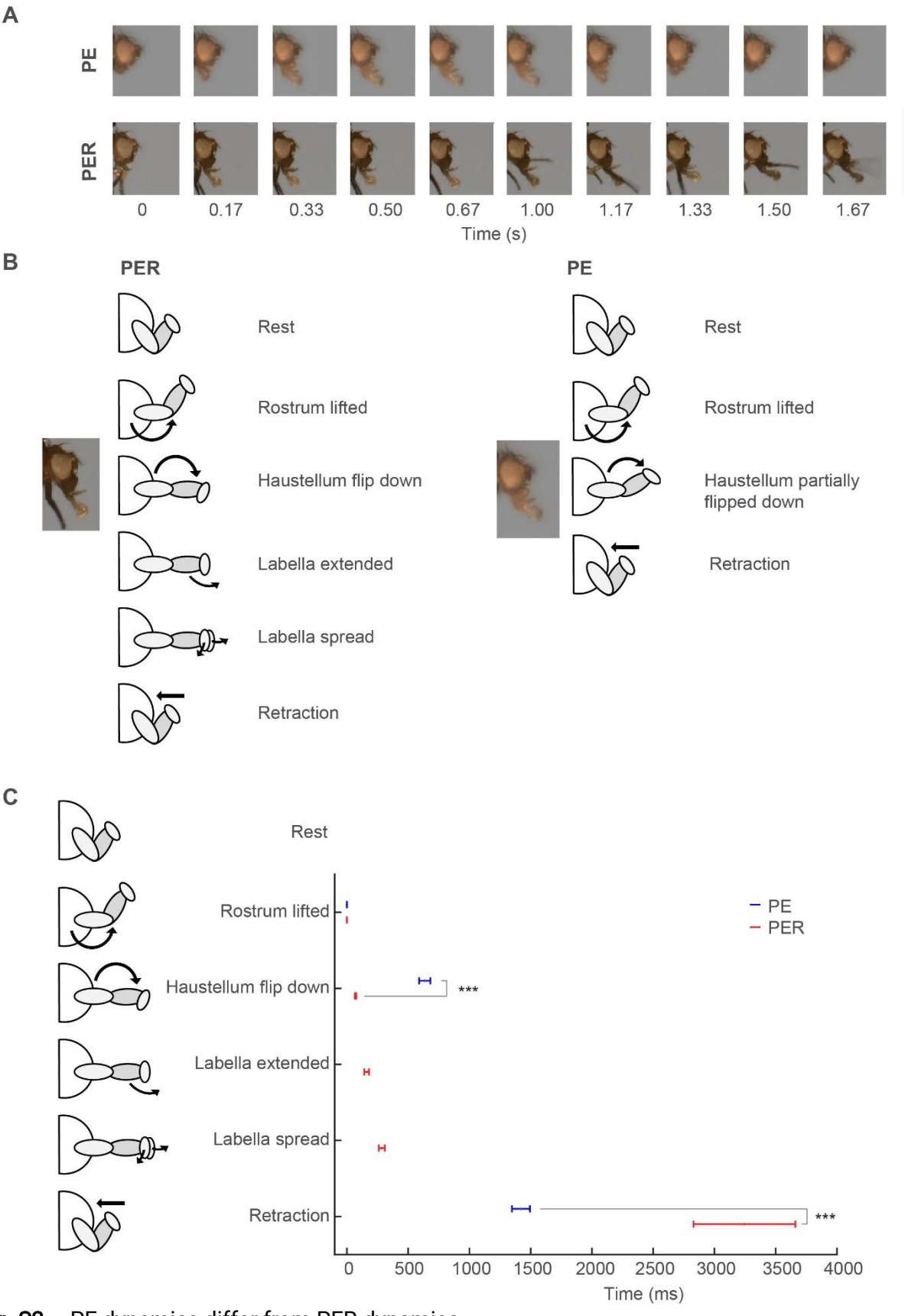

Fig. S3 – PE dynamics differ from PER dynamics

A) Spontaneous PE during sleep follow a shorter time course than sugar-induced PER, where the movement is initiated slower, but retracted faster.

B) During PER, the proboscis is extended and retracted through a series of discrete steps. First the rostrum is lifted, then the haustellum is flipped down, followed by the labella being extended and spread. Eventually, the proboscis is retracted if no food source is detected. PE follow some of these steps. Initially, the rostrum is lifted, but the haustellum is only partially flipped down and the proboscis is retracted soon afterwards

C) PE and PER occur on different time scales. In PER, the haustellum is flipped down sooner than during PE but the proboscis stays extended much longer

PE: n = 7 flies, average of 5 PE/fly

PER n = 7 flies, average of 3-5 PER/fly

*** $p < 0.001$, two tailed t-test.

Errorbars indicate SEM

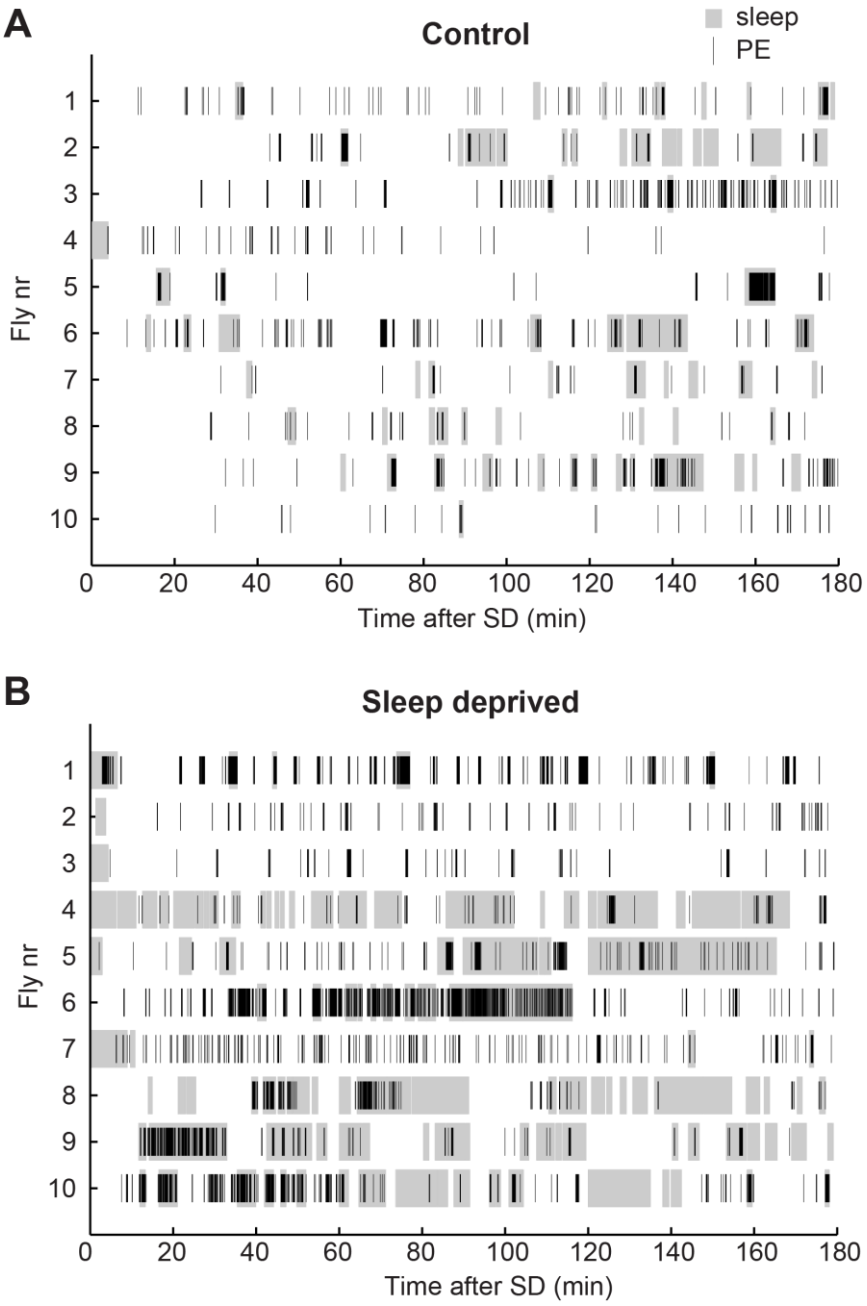

Fig. S4 – PE and inactivity after sleep deprivation

Raster plots show PE and inactivity for tethered controls (A) and flies after 12 hours of sleep deprivation (B).

After sleep deprivation, flies sleep sooner, longer and show a dramatic increase in PE

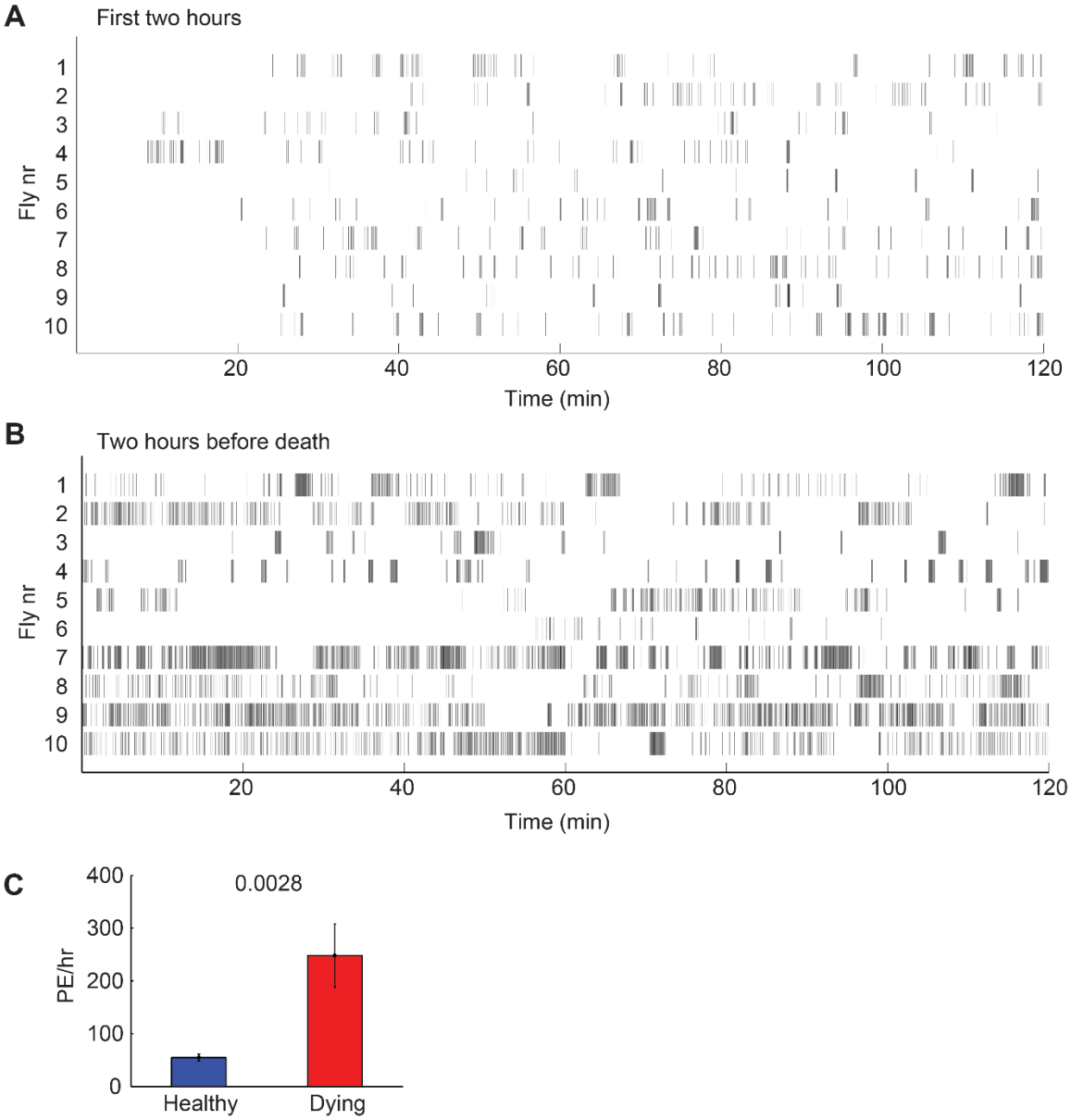

Fig. S5 - PE increase in dying flies

(A) Raster plots showing PE timing in the first two hours after being tethered.

(B) PE greatly increase in the two hours leading up to fly deaths.

(C) The number of proboscis extensions per hour is greatly increased shortly before death (p = 0.028; two tailed t-test)

n = 10/group. Errorbars indicate SEM.

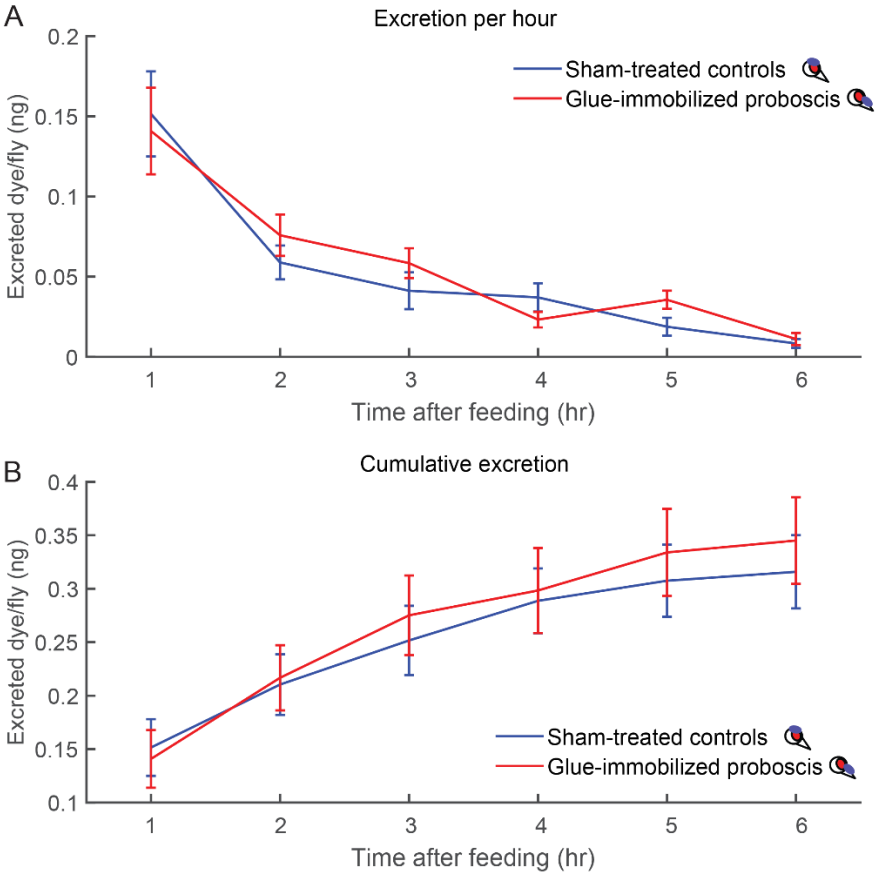

Fig. S6 - Immobilizing the proboscis does not affect feeding or gut function

(A) To test whether immobilizing the proboscis affects food consumption, we immobilized the proboscis three days before the experiment, after which flies were placed on agar sucrose food containing 1% Blue #1. After 24 hours, flies were transferred hourly to glass vials. Afterwards, the amount of excreted dye in each vial was quantified. In both sham-treated and proboscis immobilized flies, excretion is highest in the first hour after being taken off Blue#1 food and decreases every hour afterwards. There is no significant different between both groups (n= 6/group; ANOVA).

(B) Likewise, there is no difference in cumulative excretion (n= 6/group; ANOVA).

Error bars indicate SEM

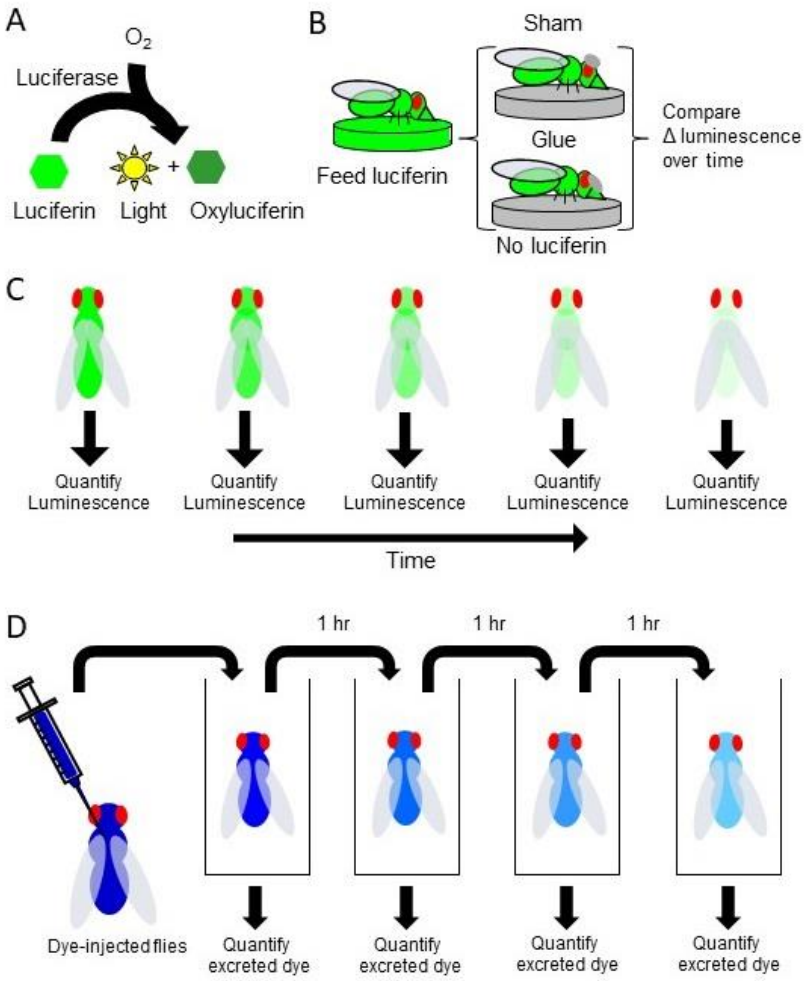

Fig. S7 – Clearance assays

(A) Luciferase reaction, where luciferase converts luciferin and oxygen into oxyluciferin and light. Flies do not produce luciferin, but adding luciferin to food allows the reaction to occur, as long as luciferin is present.

(B) This allows us to use luciferase activity as a proxy for how much luciferin is present in the fly. OK107>Luc flies were fed food with 5mM luciferin for 24 hours. Afterwards, their proboscis was immobilized and flies were transferred to a plate reader containing food without luciferin. Luciferin will be converted to oxyluciferin, metabolized or excreted.

(C) As the amount of luciferin in the fly decreases, the probability of luciferin being close enough to luciferase to feed the conversion reaction, and the number of photons per time unit will decrease.

(D) Dye injection paradigm. 50nL of 1% Blue#1 in PBS was injected into lateral thorax. Injected flies are transferred to glass vials every hour. Afterwards, the amount of excreted dye in each vial is quantified.

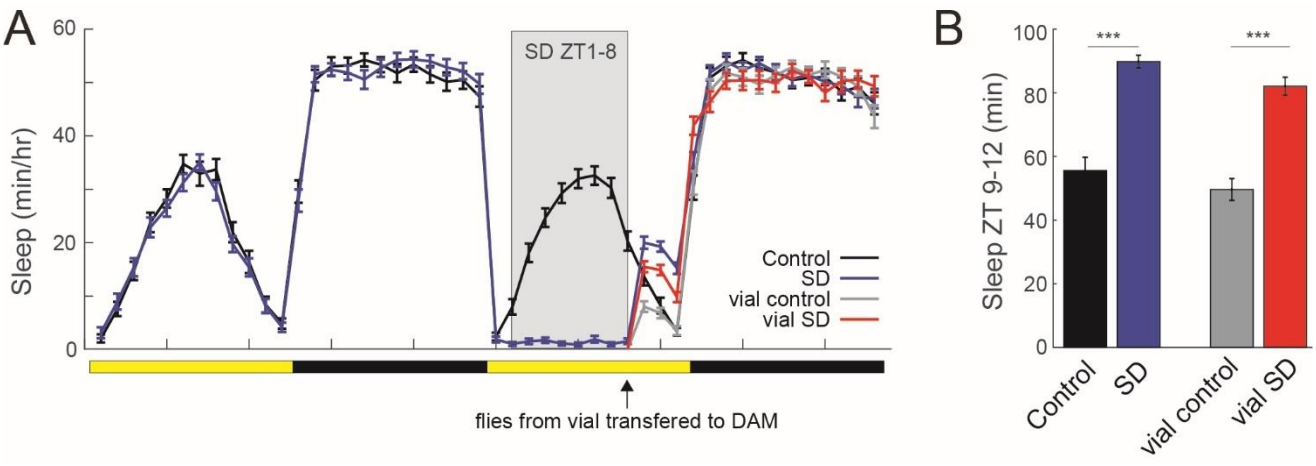

Fig S8 – sleep deprivation in vials results in robust rebound sleep

(A) Flies were sleep deprived in Drosophila Activity Monitors (blue trace) in the SNAP device or vials placed in the SNAP device (red trace). Controls were kept in activity monitors (black trace) or in vials (grey trace) in the same incubator. After sleep deprivation ended, flies were transferred from vials to activity monitors

(B) Total sleep was quantified for all groups in ZT9-12. Sleep deprived flies in activity monitors (blue) and vials (red) show robust rebound sleep (*** $p < 0.001$, t-test). Errorbars indicate SEM.

Movie S1.
PE in freely moving flies

Movie S2.
PE, PER, drinking and feeding occur on different timescales

Movie S3.
Limb relaxation during PE

Movie S4.
PE during LFP recording

Movie S5.
Glue-immobilization reduces PE amplitude

Movie S6.
Model of how proboscis extensions drive waste clearance from the hemolymph