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RESEARCH ARTICLE

Sleep deprivation negatively impacts reproductive output in *Drosophila melanogaster*

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ABSTRACT

Most animals sleep or exhibit a sleep-like state, yet the adaptive significance of this phenomenon remains unclear. Although reproductive deficits are associated with lifestyle-induced sleep deficiencies, how sleep loss affects reproductive physiology is poorly understood, even in model organisms. We aimed to bridge this mechanistic gap by impairing sleep in female fruit flies and testing its effect on egg output. We found that sleep deprivation by feeding caffeine or by mechanical perturbation resulted in decreased egg output. Transient activation of wake-promoting dopaminergic neurons decreased egg output in addition to sleep levels, thus demonstrating a direct negative impact of sleep deficit on reproductive output. Similarly, loss-of-function mutation in dopamine transporter *fumin* (*fmn*) led to both significant sleep loss and lowered fecundity. This demonstration of a direct relationship between sleep and reproductive fitness indicates a strong driving force for the evolution of sleep.

KEY WORDS: Sleep loss, Egg output, Fitness, Caffeine, Dopamine, Fecundity

INTRODUCTION

Almost all animals show activity/rest cycles in response to daily solar cycles of light, temperature and other environmental cues. The rest phase of sleep is remarkably ubiquitous in animals, suggesting that sleep is important. While we humans spend a third of our lives sleeping, we do not know why sleep is indispensable. Several studies link sleep levels to cognition, mood and emotional states (Krause et al., 2017), as well as physiological health in humans (Mahoney, 2010). When rats are chronically deprived of sleep, there are detrimental effects on longevity (Rechtschaffen et al., 1983), skin condition (Everson et al., 1989) and body weight (Everson and Szabo, 2011) accompanied by physiological changes in internal organs (Everson and Szabo, 2009). Thus, sleep positively influences many organ systems in addition to the nervous system.

The genetically tractable model organism *Drosophila melanogaster* exhibits several characteristics of mammalian sleep – increased arousal threshold, site specificity, regulation by homeostatic and circadian clock mechanisms and even sleep-specific electrophysiological signatures (Hendricks et al., 2000; Nitz et al., 2002; Shaw et al., 2000; van Alphen et al., 2013). Sleep deprivation in flies results in deleterious effects similar to those seen in mammals. Mechanically depriving flies of sleep decreases their lifespan (Seugnet et al., 2009; Shaw et al., 2002) and short-sleeping mutants of the Shaker potassium channel have reduced lifespan (Bushey et al., 2010; Cirelli et al., 2005). However, lifespan by itself is an insufficient indicator of overall fitness of an organism as it can be radically influenced by reproductive output (Sheeba et al., 2000). As reproductive success is a strong evolutionary driving force, we focused on possible mechanistic links between sleep and reproductive output.

In humans, infertility is often associated with sleep disturbances; however, the complexity of the reproductive system and sleep characteristics in humans makes the analysis of sleep disruption affecting reproductive processes difficult (Kloss et al., 2015). Shift-workers and women who experience frequent jet lag conditions report sleep disturbances and abnormal menstrual cycles and are at a higher risk of developing pregnancy related complications (Mahoney, 2010). Chronic sleep deprivation in rats increases spontaneous ejaculations (Andersen and Tufik, 2002) and reduces the number of live sperm (Alvarenga et al., 2015). In mice subjected to light protocols mimicking jet lag and circadian misalignment, reproductive success is hampered (Summa et al., 2012). Circadian clock mutants with defective timing and consolidation of sleep also have reduced reproductive output in flies (Beaver et al., 2002) and mice (Loh et al., 2014). In a recent study in *Caenorhabditis elegans*, it was found that depriving worms of the developmentally regulated sleep-like lethargus state activated a protective response in the endoplasmic reticulum. Blocking the response resulted in apoptosis of sperm as well as defects in muscular activity of egg-laying circuit (Sanders et al., 2017). Sleep deprivation alters aggressive behaviour in flies and hampers the chances of mating (Kayser et al., 2015). Most studies show that sleep and reproductive output are associated with one another, without testing the direct effects of sleep on reproductive success. Here, we addressed this question by impairing sleep in female fruit flies and testing its effect on reproductive output. We found that feeding flies with caffeine or depriving them of sleep by mechanical perturbation, or by decreasing sleep by genetic activation of wake-promoting dopamine neurons all resulted in decreased egg output. Decreased sleep was associated with decreased egg output for all manipulations. Thus, our study established a model system to study the mechanisms underlying relationships between sleep and reproductive processes that underlie fitness.

MATERIALS AND METHODS

Fly strains

Fly strains used for both activity/rest and egg output assays were w^{1118} (Bloomington *Drosophila* Stock Center no. 5905), *fumin* (*fmn*), *2202CS* (background control for *fmn* flies, henceforth referred to as *fmn-bg*), *TH Gal4*, *UAS dTrpA1* and a previously described outbreeding population Chrono Control Merged [*CCM* (Gogna et al., 2015)]. *fmn* and *fmn-bg* flies were gifts from

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Dr Kazuhiko Kume, Nagoya City University, Nagoya, Japan. Other fly lines were obtained from the Bloomington *Drosophila* Stock Center, Bloomington, IN, USA. All the transgenic flies used were back-crossed into the standard w^{1118} background for at least seven generations.

Activity/rest and egg output assays

For the activity/rest assays, 4–5 day old virgin female flies were initially allowed to mate for 1 day and were then individually housed in tubes (65 mm length, 3 mm diameter) with standard cornmeal food at one end and a cotton plug at the other, and activity was recorded in DAM2 monitors (*Drosophila* activity monitoring system, TriKinetics, Waltham, MA, USA). The DAM system works on the standard beam-breaking principle where a fly cuts an infra-red beam whenever it moves in the middle portion of the tube, thereby generating activity counts. Activity counts were binned at 1 min intervals to obtain sleep parameters using the software PySolo (Gilestro and Cirelli, 2009). Flies were housed in light- and temperature-controlled environments under a 12 h:12 h light:dark photoperiod at 25°C using incubators (MIR-273, Sanyo, Osaka Japan; DR-36VLC8 Percival Scientific Inc., Perry, IA, USA). Flies were transferred into tubes containing either standard food or food containing different concentrations of caffeine (Hi-Media, Bengaluru, KA, India) every 12 h depending upon their treatment. The activity recording assays were run for a period of 6–7 days. The first two days represented baseline days of recording, the next three days (days 3–5) were the days during which sleep deprivation was given either by caffeine treatment or temperature increase, and the last two days represented the recovery days during which sleep rebound was expected to occur. For specific assays, flies were fed with caffeine either during the day or night for a period of 6 days.

The egg output assays were conducted simultaneously along with the activity/rest assays, on a parallel set of flies housed in glass vials (10 cm length, 2.5 cm diameter) containing ∼3 ml of cornmeal food with or without caffeine depending upon the treatment. For the egg output assays, a small amount of charcoal (0.8 g l^{-1}) was added to cornmeal food to increase the contrast between eggs and food surface, thereby aiding in egg counting. As before, flies were transferred into fresh food vials every 12 h and the number of eggs laid was counted with the help of a stereo-microscope (Olympus, SZ160, Tokyo, Japan). In the experiment for sleep deprivation by mechanical means, individual flies were housed in tubes (65 mm in length, 5 mm in diameter) placed in DAM5 monitors (TriKinetics), which were then mounted on a vortexer (VWR, Radnor, PA, USA) that was used to mechanically disturb the flies either during the day or night. Eggs laid by flies in these tubes as well as by flies that remained undisturbed throughout the day or night were then counted for a period of 5 days. Oviposition preference assays were performed by introducing five female w^{1118} flies for a period of 2 or 12 h to Petri dishes that contained standard cornmeal food in one-half and cornmeal food with specific concentrations of caffeine in the other.

The capillary feeder (CAFÉ) assay was carried out for a period of 24 h as described in Ja et al. (2007). Briefly, individual flies were housed in vials containing 0.5% agar and 5 µl microcapillaries containing a solution of 5% sucrose, 1% food dye (blue) and either 0.5 or 1 mg ml^{-1} caffeine as the food source. Fresh microcapillaries were provided after 12 h and the level of food was noted to indicate food consumption for the 12 h duration. Filled microcapillaries in vials with no flies served as evaporation controls. The final consumption values were obtained after correcting for evaporation and adding the values for both day and night durations.

Statistical analysis

Oviposition preference for a given food was defined as the percentage of total eggs laid on that food surface. Percentage sleep loss was calculated as percentage decrease in sleep during sleep-deprivation days with reference to sleep levels during baseline days. Sleep measurements of control and sleep-deprived flies were compared using one-way ANOVA with treatment or genotype as a fixed factor followed by *post hoc* Tukey's honest significant difference (HSD) test with a *P*-level set at 0.05. Egg output data were first tested for normality using a Shapiro–Wilk's *W*-test. One-way ANOVA followed by *post hoc* Tukey's HSD test were conducted if all datasets under consideration were normally distributed. However, even if one of the datasets was not normally distributed, a Kruskal–Wallis test was conducted with a *P*-level set at 0.05.

RESULTS

Effect of sleep deprivation on egg output of inbred w^{1118} flies

To assess the impact of sleep deprivation on reproductive output, we first used caffeine to deprive female flies of sleep. Flies were given caffeinated food during the day only (D_{caf}), or during the night only (N_{caf}) or standard cornmeal food during both the day and night that acted as controls (Ctrl). To estimate the appropriate concentration of caffeine for our egg output assay, we quantified the amount of sleep loss in flies with two concentrations (0.5 and 1 mg ml^{-1}) based on previous studies (Andretic et al., 2008; Wu et al., 2009) and our pilot experiments. Flies that were fed food containing 0.5 mg ml^{-1} caffeine only during the day (D_{caf}) tend to exhibit less sleep during the day as compared with their own baseline (BS) as well as compared with control flies during caffeine (CAF) days (Fig. 1A, BS and CAF), although this reduction was not statistically significant (Fig. 1Bi, day). However, these flies showed a rebound increase in daytime sleep upon removal from caffeinated food (Fig. 1A, RC), which was significantly higher than daytime sleep during BS and CAF (Fig. 1Bi, day). Similarly, when flies were provided food containing 0.5 mg ml^{-1} caffeine only during the night (N_{caf}, Fig. 1A,Bi), their night-time sleep was significantly reduced as compared with their own BS days as well as control flies during CAF days (Fig. 1A, BS and CAF; Fig. 1Bi, night). These data show that caffeine has an immediate effect on sleep – D_{caf} flies show reduced daytime sleep whereas N_{caf} flies show reduced night-time sleep. We found similar trends of reduced daytime sleep of D_{caf} and reduced night-time sleep of N_{caf} with respect to BS when flies were fed food containing 1 mg ml^{-1} caffeine (Fig. S1). Importantly, 0.5 mg ml^{-1} of caffeine is more efficient in decreasing sleep levels (53% day and 49% night sleep loss) compared with 1.0 mg ml^{-1} of caffeine (38% day and 4% night sleep loss; Fig. 1Bii). This is likely due to reduced food intake with increasing caffeine content, as a CAFÉ assay (Ja et al., 2007) conducted for a period of 24 h showed that flies consumed a lower quantity of 5% sucrose solution containing 1 mg ml^{-1} caffeine (0.55±0.07 µl, N=8 flies) as compared with that containing 0.5 mg ml^{-1} caffeine (1.05±0.12 µl, N=8 flies, Mann–Whitney U-test, P<0.005), which could in turn result in reduced sleep loss.

As providing flies with food containing 0.5 mg ml^{-1} caffeine during the day or night leads to ∼50% reduction in both daytime and night-time sleep, we next determined how this affects their reproductive output. We subjected 5 day old female flies (mated for one day prior to the start of the experiment) to caffeine treatment only during the day (D_{caf}) or only during the night (N_{caf}). We found that both D_{caf} and N_{caf} flies laid a lower number of eggs as compared with the control flies during the day as well as the night (Fig. 1Ci), even though on the first day and night the number of eggs laid were comparable, suggesting a cumulative effect of caffeine-mediated

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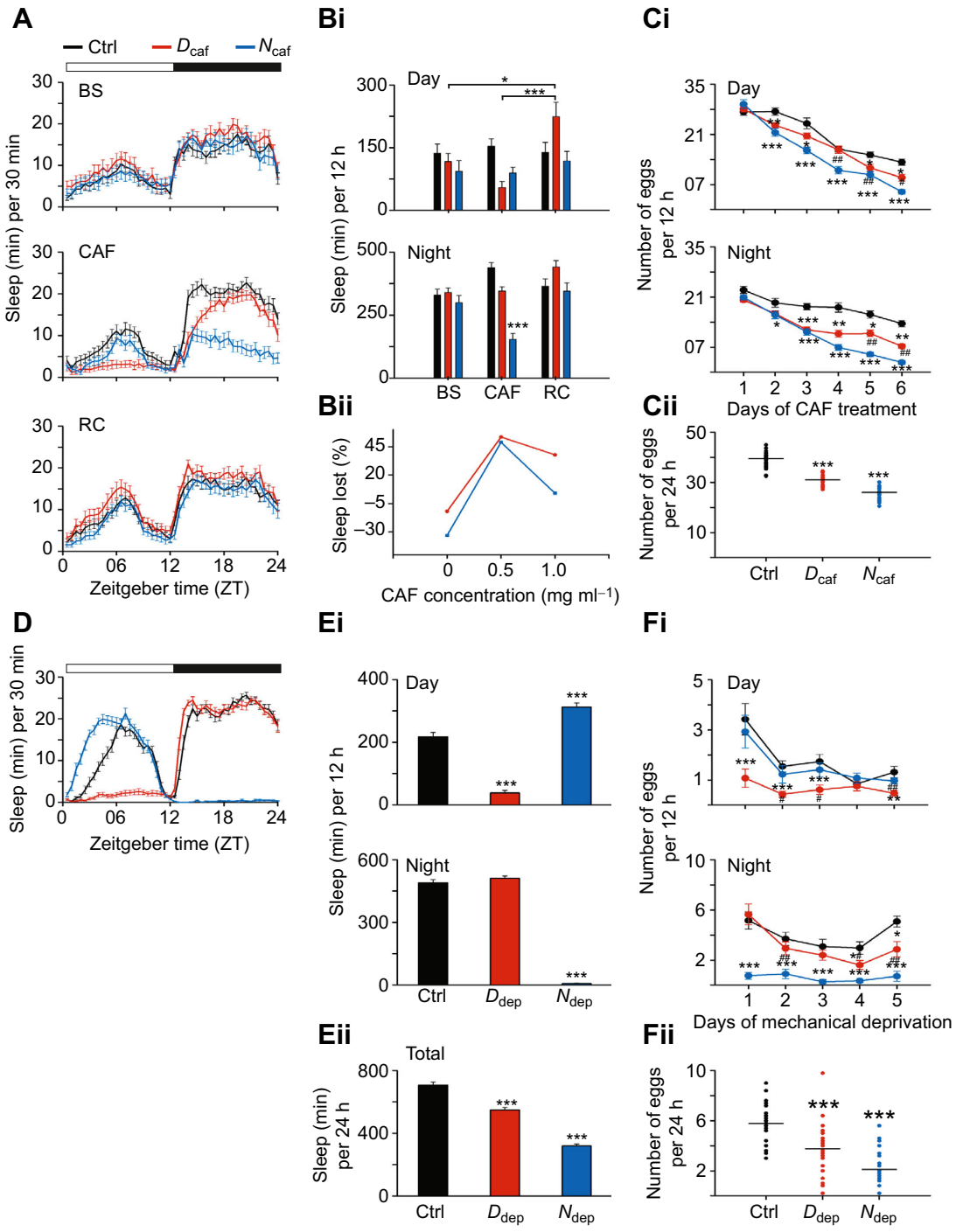

Fig. 1. See next page for legend.

sleep loss on egg output. N_{caf} flies laid a lower number of eggs as compared with D_{caf} flies also, which was statistically significant on the later days of the treatment (Fig. 1Ci). When we compared the total number of eggs averaged over the 6 days of treatment, D_{caf} flies laid a significantly lower number of eggs as compared with control flies, and N_{caf} flies laid a significantly lower number of eggs as compared with both control and D_{caf} flies (Fig. 1Cii).

As it is likely that flies fed with caffeine laid fewer eggs simply because oviposition was inhibited by food containing caffeine, we carried out an oviposition preference assay, where flies were allowed to lay eggs for 2 h on a Petri dish with half the plate containing standard food and the other half containing 0.5 mg ml^{-1} caffeinated food. We found that flies laid an almost equal number of eggs in both halves, suggesting that, for food containing caffeine at a concentration of 0.5 mg ml^{-1}, flies do not have any ovipositional avoidance (Preference Index$_{caf}$=0.49±0.11, chi-square test, χ^2=0.049, P=0.82). However, because our egg output assays lasted for a period of 5–6 days and flies had access to fresh food every 12 h, yet another oviposition preference assay was conducted for a longer time course of 12 h. We found that when given a choice for a longer period of time, flies tend to lay more eggs on food containing 0.5 mg ml^{-1} caffeine as compared with standard food.

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Fig. 1. Sleep deprivation by caffeine and mechanical disturbance of w^{1118} flies results in a decrease in egg output. (A) Sleep in minutes for every 30 min over a period of 24 h is shown for w^{1118} flies fed with standard food (Ctrl, N=28), flies fed with 0.5 mg ml^{-1} caffeine only during the day (D_{caf}, N=25) and only during the night (N_{caf}, N=24) averaged across two baseline (BS), three caffeine-feeding (CAF) and two recovery (RC) days. Horizontal white and black bars at the top represent day and night, respectively. (Bi) Daytime (top) and night-time (bottom) sleep of control, D_{caf} and N_{caf} flies are compared across BS, CAF and RC days. D_{caf} flies show a significant increase in daytime sleep during RC days as compared with that during BS and CAF days. N_{caf} flies show significantly lower levels of night-time sleep during CAF days as compared with that during BS and RC days, as well as night-time sleep of controls during CAF days (two-way ANOVA with treatment and days as fixed factors followed by *post hoc* Tukey's HSD test; *P<0.05; ***P<0.001). (Bii) Percentage total sleep loss during CAF days with respect to BS days plotted as a function of caffeine concentration shows that sleep loss is higher for a caffeine concentration of 0.5 mg ml^{-1} during both the day and night as compared with a concentration of 1.0 mg ml^{-1}. (Ci) Number of eggs laid by control (N=25), D_{caf} (N=24) and N_{caf} (N=25) flies both during the day and night over a period of 6 days of caffeine (0.5 mg ml^{-1}) treatment. * denotes significant differences between either D_{caf} or N_{caf} with control flies (*P<0.05; **P<0.01; ***P<0.001), whereas # indicates significant differences between D_{caf} and N_{caf} flies (Kruskal–Wallis test, $^{\#}P$<0.05; $^{\#\#}P$<0.01; $^{\#\#\#}P$<0.001). (Cii) Total number of eggs laid averaged across 6 days of caffeine treatment. D_{caf} flies laid a significantly lower number of eggs as compared with control flies, whereas N_{caf} flies laid a significantly lower number of eggs as compared with both control and D_{caf} flies (one-way ANOVA with treatment as fixed factor followed by *post hoc* Tukey's HSD test; ***P<0.001). The experiment was repeated with similar results (data not shown). (D) Sleep in minutes for every 30 min over a period of 24 h averaged across 5 days is shown for control w^{1118} flies (Ctrl, N=26), flies receiving mechanical disturbance only during the day (D_{dep}, N=28) and only during the night (N_{dep}, N=27). (Ei) Daytime sleep (top) of D_{dep} flies is significantly reduced as compared with Ctrl and N_{dep}, whereas that of N_{dep} flies is significantly higher than that of Ctrl and D_{dep}. Night-time sleep (bottom) of N_{dep} flies is significantly lower than Ctrl and D_{dep} flies. (Eii) Total sleep of D_{dep} flies is significantly lower than Ctrl and that of N_{dep} flies is significantly lower than Ctrl and D_{dep} flies (one-way ANOVA with treatment as fixed factor followed by *post hoc* Tukey's HSD test for Ei and Eii; ***P<0.001). (Fi) Number of eggs laid by control, D_{dep} and N_{dep} flies during the day and night over a period of 5 days of mechanical deprivation protocol. * denotes significant differences between either D_{dep} or N_{dep} with control flies (*P<0.05; **P<0.01; ***P<0.001), whereas # indicates significant differences between D_{dep} and N_{dep} flies (Kruskal–Wallis test, $^{\#}P$<0.05, $^{\#\#}P$<0.01). (Fii) Total number of eggs laid by Ctrl, D_{dep} and N_{dep} flies averaged across 5 days. D_{dep} flies show a significant reduction in the number of eggs laid as compared with Ctrl; N_{dep} flies laid even lower number of eggs significantly reduced as compared with both Ctrl and D_{dep} flies (Kruskal–Wallis test, ***P<0.001). Error bars are s.e.m.

Therefore, flies tend to show a significant preference towards caffeine-containing food in conditions resembling the egg output assays (Preference Index$_{caf}$=0.75±0.1, chi-square test, χ^2=99.75, P<0.0005). Thus, these results suggest that flies lay a lower number of eggs when exposed to caffeine in spite of a preference towards it. Overall, caffeine decreases egg output and flies that lose night-time sleep tend to lay a lower number of eggs than flies that lose daytime sleep.

To confirm the effect of sleep loss on egg output, we used a completely different sleep deprivation method. We substituted caffeine with a vortexer-based mechanical perturbation protocol. As this assay was done in DAM5 monitors with flies housed in glass tubes (65 mm in length, 5 mm in diameter) as opposed to the caffeine-fed flies that were housed in standard glass vials (10 cm length, 2.5 cm diameter), the overall number of eggs laid was expected to be significantly fewer in the glass tubes (39 versus 6 for control flies in Fig. 1Cii and 1Fii). Three sets of flies received either of the following treatments: exposure to mechanical disturbance only during the day (D_{dep}), or only during the night (N_{dep}), or the control (Ctrl) condition with no mechanical perturbation. For the same sets of flies, we obtained both sleep levels and egg counts by transferring flies to fresh tubes every 12 h for 5 days. As expected, mechanical disturbance during the day reduced daytime sleep and mechanical disturbance during the night reduced night-time sleep drastically (Fig. 1D,Ei). However, only N_{dep} flies recovered this lost night-time sleep during the subsequent days (Fig. 1Ei, top) whereas D_{dep} flies did not recover the lost daytime sleep during subsequent nights (Fig. 1Ei, bottom). Nevertheless, N_{dep} flies lost a greater amount of overall sleep as compared with D_{dep} flies (Fig. 1Eii). Importantly, the number of eggs laid by D_{dep} flies was lower than the controls especially during the daytime (Fig. 1Fi, top) and that of N_{dep} flies was significantly lower than the controls during the night (Fig. 1Fi, bottom). Unlike the caffeine-fed flies, the effect of sleep loss owing to mechanical perturbation on egg output was evident from the first day of treatment (Fig. 1Fi). Moreover, the average egg output in both D_{dep} and N_{dep} flies was significantly lowered as compared with the control flies (Fig. 1Fii). Furthermore, N_{dep} flies, which on average lost more sleep, also laid a significantly lower number of eggs as compared with D_{dep} flies (Fig. 1Eii,Fii). Thus, these results along with similar results obtained with sleep deprivation using caffeine suggest that sleep loss results in a reduction in egg output and that sleep loss during the night has a greater detrimental effect on egg output.

Effect of sleep deprivation on the reproductive fitness of outbred flies

In the studies described above, we used a strain of w^{1118} flies that has been maintained in our laboratory for several years and is likely to harbour loci that have been fixed for certain traits, which may have resulted in the above phenotype by chance. Given that reproductive output is a major Darwinian fitness trait, we asked how sleep loss might affect reproductive output in a large, random mating and therefore outbred population of flies, which is unlikely to have suffered from similar genetic bottlenecks (*CCM*; Gogna et al., 2015). We subjected flies to three different concentrations of caffeine (0.5, 1.0 and 1.5 mg ml^{-1}) either only during the day or only during the night and found that none of the D_{caf} flies lost daytime sleep whereas all of the N_{caf} flies lost similar amounts of night sleep (Fig. 2A,B). However, D_{caf} (1.5 mg ml^{-1}) flies laid a significantly lower number of eggs than the control flies, suggesting that caffeine at a relatively higher concentration can affect egg output even without an effect on daytime sleep (Fig. 2C). Moreover, N_{caf} flies receiving 0.5 and 1.5 mg ml^{-1} caffeine also showed reduced egg outputs as compared with control flies (Fig. 2C). These results point towards a direct effect of caffeine on egg output independent of its effect on sleep as well as an indirect effect on egg output through sleep loss. Alternatively, this could also indicate the inability of infrared beam-break-based methods, such as DAM system, to detect subtle effects of caffeine treatment and also that immobility may not always be the best measure for sleep. Nevertheless, we next increased caffeine concentration and found that even higher caffeine concentrations of 4.0 mg ml^{-1} fed during the day did not affect daytime sleep (Fig. S2A, BS and CAF; Fig. S2B, day); however, when fed during the night, it decreased night-time sleep (Fig. S2B, night). With respect to egg output, we found that the total number of eggs laid by D_{caf} and N_{caf} flies was significantly lower than that of the control flies; however, the number of eggs laid by D_{caf} and N_{caf} flies was not statistically different from each other (Fig. S2C,C′) similar to what was found for lower concentrations of caffeine. Caffeine treatment does not affect the viability of the eggs laid as seen from the egg-to-adult survivorship of eggs laid by D_{caf}, N_{caf} (0.5 mg ml^{-1}) and Ctrl flies (data not shown). Taken together, these results suggest that caffeine

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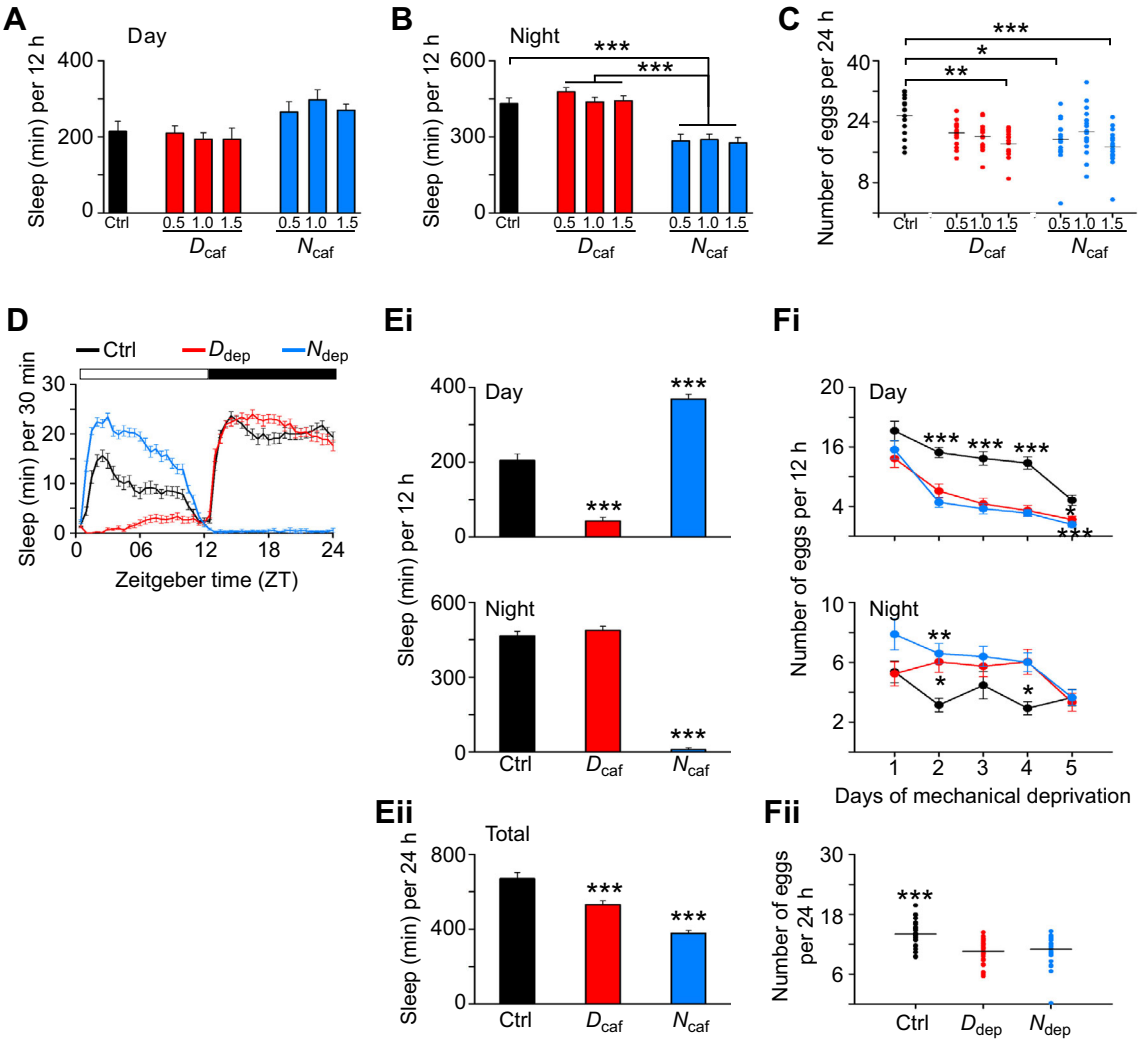

Fig. 2. Sleep deprivation by caffeine and mechanical disturbance of outbred *CCM* flies results in egg output reduction. (A) Daytime and (B) night-time sleep of flies of the outbred *CCM* population fed with standard food or food containing different concentrations of caffeine (0.5, 1.0 and 1.5 mg ml^{-1}) either only during the day (D_{caf}) or only during the night (N_{caf}). Daytime sleep of flies receiving all the treatments is similar, whereas night-time sleep of N_{caf} flies of all caffeine concentrations is significantly reduced as compared with control and D_{caf} flies of all caffeine concentrations (one-way ANOVA with treatment as fixed factor followed by *post hoc* Tukey's HSD test; ***P<0.001). N≥21 for all treatments. (C) The total number of eggs laid averaged across 6 days by $D_{caf1.5}$ (N=13), $N_{caf0.5}$ (N=19) and $N_{caf1.5}$ (N=17) flies is significantly reduced as compared with the control (N=16) flies. D_{caf} and N_{caf} flies of any caffeine concentration do not differ in the total number of eggs laid from each other. $D_{caf0.5}$ (N=17), $D_{caf1.0}$ (N=17) and $N_{caf1.0}$ (N=18) do not differ from the control flies in the number of eggs laid (Kruskal–Wallis test; *P<0.05; **P<0.01; ***P<0.001). (D) Sleep in minutes for every 30 min over a period of 24 h averaged across 5 days is shown for control (N=28) flies of the outbred *CCM* population, flies mechanically disturbed during the day (D_{dep}, N=30) and during the night (N_{dep}, N=31). (Ei) During the day (top), D_{dep} flies sleep significantly less than both the control and N_{dep} flies due to mechanical disturbance, N_{dep} flies sleep significantly longer than the control and D_{dep} flies, indicating sleep rebound due to sleep deprivation during the previous night. During the night (bottom), N_{dep} flies sleep significantly less than the control and D_{dep} flies due to mechanical perturbation. (Eii) Total sleep averaged across 5 days of D_{dep} flies is significantly lower than control flies, whereas that of N_{dep} is significantly lower than both the control and D_{dep} flies (one-way ANOVA with treatment as fixed factor followed by *post hoc* Tukey's HSD test for Ei and Eii; ***P<0.001). (Fi) The number of eggs laid by both D_{dep} and N_{dep} flies is significantly lower than the controls during days 2–5 (top) and they show a trend of increased egg output during the night (bottom), which is significantly different from the controls on the second and fourth nights (Kruskal–Wallis test; *P<0.05; **P<0.01; ***P<0.001). (Fii) The total number of eggs laid averaged across 5 days by both D_{dep} and N_{dep} flies is significantly lower as compared with control flies (Kruskal–Wallis test; ***P<0.001). All other details as in Fig. 1. A similar experiment with higher levels of deprivation yielded similar results (data not shown).

treatment may affect reproductive fitness directly or indirectly through sleep loss.

We next subjected the *CCM* flies to the sleep deprivation protocol using mechanical perturbation either during the day only (D_{dep}) or during the night only (N_{dep}). As expected, D_{dep} flies lost daytime sleep and N_{dep} flies lost night-time sleep, which they could recover during subsequent days (Fig. 2D,Ei). Nevertheless, N_{dep} flies lost an overall greater amount of sleep as compared with D_{dep} flies (Fig. 2Eii). Here too, because the assay was conducted in tubes (see Materials and methods), as expected all flies laid a lower number of eggs owing to the decreased surface area of food as compared with the caffeine-feeding experiment where flies were housed in vials. Unlike the mechanically disturbed inbred flies (Fig. 1Fi), in the case of outbred flies, both D_{dep} and N_{dep} flies laid a significantly lower number of eggs as compared with controls during the day (Fig. 2Fi, top) whereas they both laid a higher number of eggs compared with controls during the night (Fig. 2Fi, bottom) starting from day two. Again, as in the case of caffeine-fed outbred flies, with mechanical

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Table 1. Sleep loss and rebound characteristics of night-time sleep

	% Sleep lost		% Sleep rebound	
Genotype	Caffeine	Depriver	Caffeine	Depriver
Inbred (w^{1118})	48.9	98.8	26.1	45.9
Outbred (*CCM*)	34.3	98	23.7	80.2

Percentage sleep lost during the night and rebound during the subsequent days after caffeine treatment (0.5 mg ml^{-1}) and mechanical deprivation for inbred and outbred flies. These values were calculated on the basis of baseline sleep levels for w^{1118}-caffeine flies. For the rest, these values are calculated with respect to sleep levels of control flies set as baseline.

disturbance also we found that there was a reduction in egg output in D_{dep} and N_{dep} flies as compared with control flies, although there was no difference in egg output between flies experiencing daytime versus night-time sleep disturbance (Fig. 2Fii). This difference among inbred and outbred flies could be due to different levels of sleep rebound, at least in the case of mechanical deprivation (Table 1). However, in yet another assay with mechanically sleep-deprived flies, the egg output of N_{dep} flies averaged across 3 days after the deprivation protocol was still significantly reduced whereas that of D_{dep} flies was comparable with control flies (Fig. S3). Therefore, with both caffeine and mechanical disturbance, the resultant sleep deprivation contributed in part to the decrease in egg output of outbred flies. Furthermore, as seen in inbred flies, night-time sleep loss had a greater impact on egg output as compared with daytime sleep loss, although this difference was less discernible and the effect much more subtle in outbred flies. Nevertheless, the finding that flies deprived of sleep during the night showed high levels of daytime sleep rebound, yet it does not lead to a concomitant rescue of harmful effects on egg output to levels mimicking undisturbed flies, further highlights the notion that sleep during the night is more important.

Transient sleep reduction is accompanied by a transient reduction in egg output

It is possible that both caffeine feeding and mechanical perturbation could have broad effects on the general physiology of the fly. Therefore, we used a third method (genetic) whereby sleep reduction was transient and measured egg output following neural-circuit-driven sleep loss. We used the GAL4–UAS system to express a temperature-sensitive cation channel *Drosophila* Transient Receptor Potential 1 (*dTRPA1*, which opens above temperatures of 27°C and causes hyper-excitation; Hamada et al., 2008), in dopaminergic neurons that have previously been shown to be wake promoting (Liu et al., 2012; Shang et al., 2011; Ueno et al., 2012). We recorded the sleep levels of flies in tubes and egg output in vials exposed to the following regime: two days at 21°C, followed by 3 days at 28°C, followed by 1 day at 21°C under a 12 h:12 h light:dark photoperiod. As expected, at the higher temperature, sleep was reduced both during daytime and night-time when dopaminergic neurons were activated, whereas the baseline sleep levels of these experimental flies were not different from that of the parental controls at the lower temperature (Fig. 3A,B). The number of eggs laid by the experimental flies was significantly lower than that of the controls (Fig. 3Ci,Cii). Indeed, these differences in egg output between experimental and control flies were not seen at the lower temperature of 21°C (Fig. 3Ci,Cii) when sleep levels were not

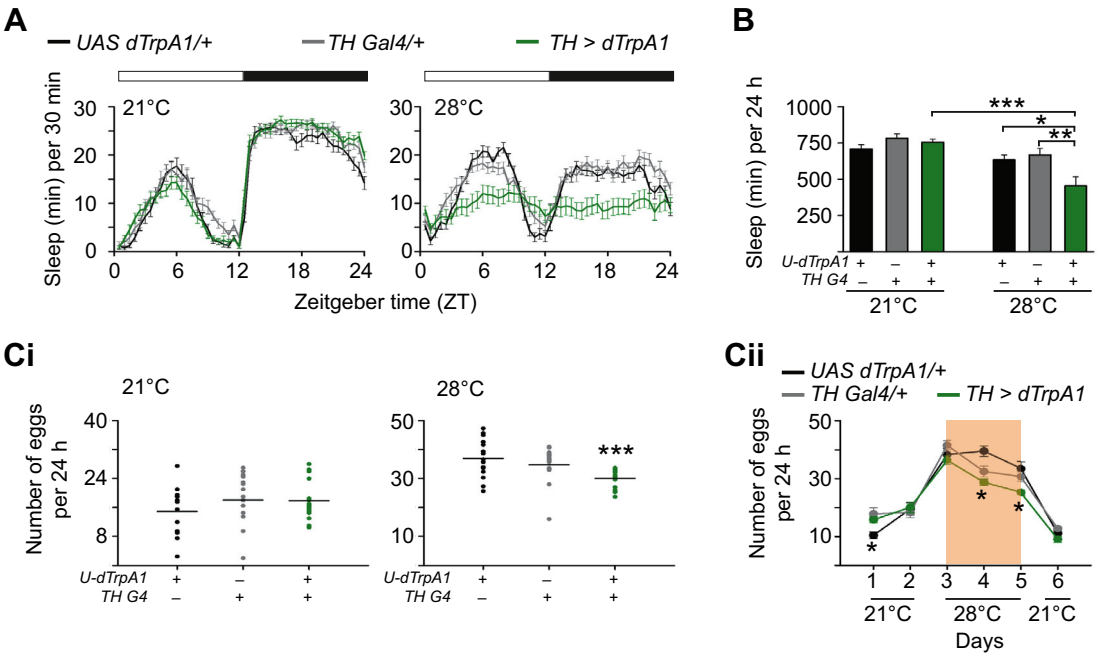

Fig. 3. Decreasing sleep levels using dTRPA1-based reversible activation of dopaminergic neurons reversibly decreases egg output. (A) Sleep in minutes for every 30 min over a period of 24 h averaged across 2 days at 21°C (left) and 3 days at 28°C (right) is shown for *UAS dTrpA1/+* (N=29), *TH Gal4/+* (N=28) and *TH Gal4>UAS dTrpA1* (N=32) flies. (B) At 21°C, total sleep levels of all three genotypes is similar, whereas at 28°C, *TH Gal4>UAS dTrpA1* flies sleep significantly less than *UAS dTrpA1/+* and *TH Gal4/+* flies (two-way ANOVA with genotype and temperature as fixed factors followed by *post hoc* Tukey's HSD test; *P<0.05; **P<0.01; ***P<0.001). (Ci) The total number of eggs laid averaged across 2 days at 21°C (left) is similar across all genotypes, whereas the average number of eggs laid by *TH Gal4>UAS dTrpA1* (N=16) flies is significantly lower than *UAS dTrpA1/+* (N=16) and *TH Gal4/+* (N=19) flies during the 3 days at 28°C (right, Kruskal–Wallis test; ***P<0.001). (Cii) Total number of eggs laid on all 6 days of the assay at the different temperatures as indicated. The highlighted region represents the high temperature of 28°C. *TH Gal4>UAS dTrpA1* flies laid a significantly lower number of eggs as compared with *UAS dTrpA1/+* and *TH Gal4/+* especially on the final 2 days of 28°C (Kruskal–Wallis test; *P<0.05), whereas all flies laid a similar number of eggs at 21°C except on day 1, when *UAS dTrpA1/+* flies laid a slightly, but significantly, lower number of eggs. All other details as in Fig. 1.

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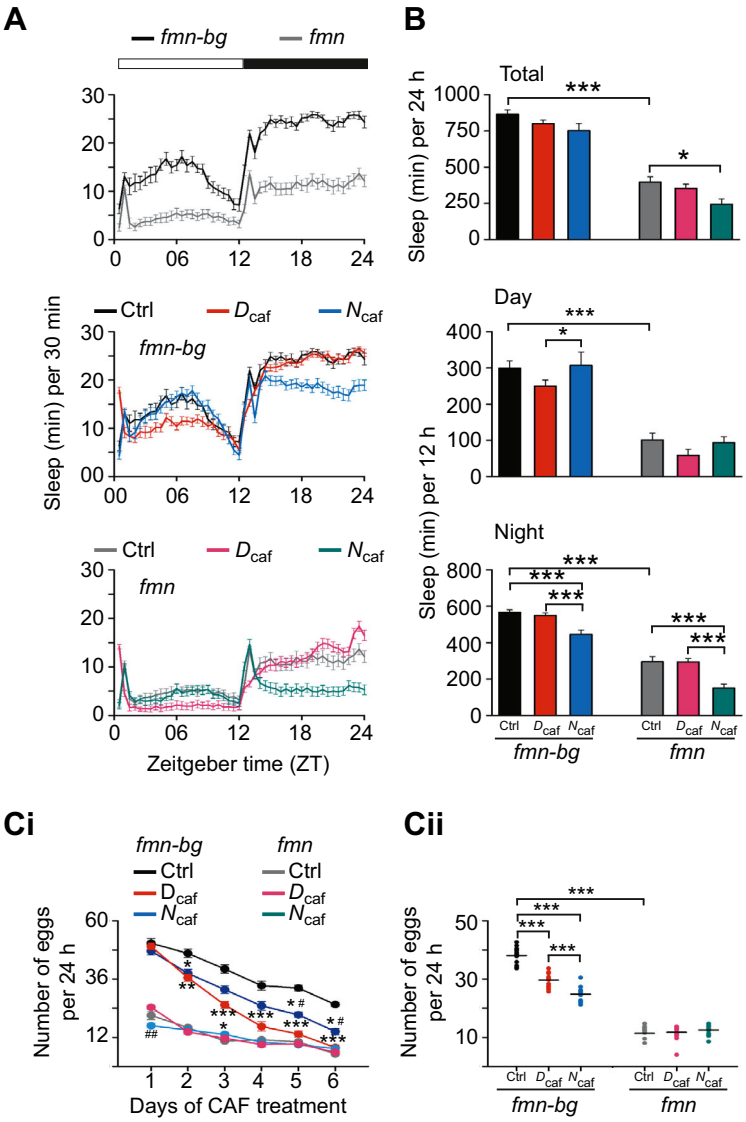

Fig. 4. *fmn* flies reduce sleep but not egg output in response to caffeine. (A) Sleep in minutes for every 30 min over a period of 24 h averaged across 6 days of *fmn* and *fmn* background control (*fmn-bg*) flies (top), *fmn-bg* flies fed with standard food (*N*=17), caffeine food (0.5 mg ml^{-1}) only during the day (D_{caf}, *N*=28) and only during the night (N_{caf}, *N*=26) (middle) and *fmn* receiving control (*N*=22), D_{caf} (*N*=24) and N_{caf} (*N*=28) treatments (bottom). (B) Total sleep levels of *fmn-bg* and *fmn* flies, compared with that of D_{caf} and N_{caf} flies of each genotype (top), daytime sleep (middle) and night-time sleep (bottom). *fmn* flies sleep significantly less than the *fmn-bg* flies both during the day and night, thereby leading to overall reduced levels of sleep. Daytime sleep of D_{caf} and N_{caf} flies of the control genotype are significantly different from one another, whereas night-time sleep of N_{caf} flies is significantly lower than D_{caf} and control flies of the *fmn-bg* genotype. Night-time sleep of N_{caf} flies is significantly lower than both the control and D_{caf} flies of the *fmn* genotype (two-way ANOVA with genotype and treatment as fixed factors followed by *post hoc* Tukey's HSD test; **P*<0.05; ****P*<0.001). (Ci) Total number of eggs laid on all 6 days of CAF treatment shows that *fmn-bg* D_{caf} and N_{caf} flies laid a significantly lower number of eggs than their controls from days 2–6, whereas there was no difference in the number of eggs laid by D_{caf}, N_{caf} and control flies of *fmn* genotype on any of the days except day 1 (**P*<0.05 D_{caf}/N_{caf} less than Ctrl; $^{\#}$*P*<0.05 D_{caf}/N_{caf} different from each other). (Cii) The total number of eggs laid averaged over 6 days by *fmn* flies is significantly lower than that of *fmn-bg* flies (Student's two-tailed *t*-test). D_{caf} flies of the *fmn-bg* genotype (*N*=14) laid a significantly lower number of eggs as compared with its controls (*N*=14), whereas N_{caf} flies of the *fmn-bg* genotype (*N*=16) laid a significantly lower number of eggs as compared with both control and D_{caf} flies (****P*<0.001). Control (*N*=15), D_{caf} (*N*=17) and N_{caf} (*N*=17) flies of the *fmn* genotype laid a similar number of eggs (two-way ANOVA with genotype and treatment as fixed factors followed by *post hoc* Tukey's HSD test). All other details as in Fig. 1.

affected (Fig. 3A,B), suggesting that transiently reducing sleep levels by activating wake-promoting neurons also resulted in a transient reduction of egg output. As the *TH Gal4* that we have used drives expression in ∼130 dopaminergic neurons (Friggi-Grelin et al., 2003; Mao and Davis, 2009) which could likely comprise a combination of neurons that independently regulate sleep and egg output, we asked whether decreasing sleep levels by using a more restricted driver also leads to a decrease in the egg output. We used the *TH-F2 Gal4* driver, which targets expression in a restricted subset of ∼20 dopaminergic neurons and hyper-excitation of these neurons results in a decrease in sleep levels (Liu et al., 2012) (Fig. S4A,B). Reducing sleep using this driver has a somewhat less dramatic effect on egg output as compared with the broader driver; nonetheless, the number of eggs laid by flies with reduced sleep due to hyper-excited TH-F2^{+} neurons is still less than its parental controls (Fig. S4C,C′), although it reaches statistical significance only when compared with the *Gal4* control flies. This suggests that perhaps the *TH Gal4* driver may still drive expression in dopaminergic neurons that affect egg output without necessarily affecting sleep, even though to date no study has shown a direct role for dopamine on egg laying. Nevertheless, the trend of reduced egg output with reducing sleep occurs even with targeting a smaller subset of neurons and thus taken together, our results suggest that sleep loss leads to a reduction in egg output, irrespective of the method of sleep deprivation.

Dopamine transporter mutants show reduced sleep but not reduced egg output in response to caffeine

Given that increasing dopaminergic activity increases wakefulness and decreases egg output, we asked whether increasing the amount of dopamine in synaptic clefts also led to decreased egg output. We used flies with loss-of-function mutation in the *fumin* (*fmn*) gene, which codes for dopamine transporter. Mutant *fmn* flies have been reported to show overall reduced sleep and no reduction in lifespan but the authors did not measure fertility in their study (Kume et al., 2005). We quantified their egg output along with sleep levels and found that the *fmn* flies expectedly showed reduced sleep levels both during the day and night (Fig. 4A,B, top), and the egg output of *fmn* flies was drastically reduced as compared with that of the background control flies (*fmn-bg*, Fig. 4Ci). As *fmn* flies carry a mutation in the dopamine transporter gene throughout the body, it is likely that this mutation can have fecundity defects independent of sleep. A previous study has demonstrated that *fmn* mutants show a further reduction in sleep when fed with caffeine (Andretic et al.,

2008). We asked whether the egg output is also further reduced in *fmn* flies fed with caffeine compared with those fed with standard food. We fed *fmn* and *fmn-bg* flies with 0.5 mg ml^{-1} caffeine either only during the day or night and found that N_{caf} flies of both *fmn* and *fmn-bg* genotypes showed reduced levels of night-time sleep as compared with their respective controls (Fig. 4B, night), whereas D_{caf} flies of both genotypes showed reduced levels of daytime sleep (Fig. 4B, day), even though it does not reach statistical significance. Interestingly, just like the previously used inbred flies of the w^{1118} genotype, the *fmn-bg*, which are flies from another inbred line, showed a statistically significant trend of a decreasing number of eggs laid by Ctrl, D_{caf} and N_{caf} flies, in that order (Fig. 4Ci,Cii). However, flies of the *fmn* genotype receiving the Ctrl, D_{caf} or N_{caf} treatments did not differ in the average number of eggs laid (Fig. 4Ci,Cii). This suggests that although sleep is affected by caffeine treatment in *fmn* flies, egg output is not, suggesting that egg output cannot be reduced by caffeine beyond a threshold due to a floor effect. Alternatively, the *fmn* gene may be involved in caffeine-mediated egg output reduction independent of the caffeine-mediated sleep loss.

DISCUSSION

Our study aimed to understand how sleep affects reproductive output in female fruit flies *D. melanogaster*. We found that feeding flies with caffeine such that it reduced sleep also reduces egg output in both inbred and outbred strains of flies (Figs 1 and 2). We note that reduced night-time sleep can be seen consistently across two 'wild-type' strains and perhaps the milder effects of caffeine on daytime sleep result in the inconsistent effects across strains. Depriving flies of sleep via mechanical perturbation also reduced egg output considerably (Figs 1 and 2). A loss-of-function mutation in the dopamine transporter gene that results in reduced sleep (Kume et al., 2005) also resulted in reduced egg output (Fig. 4). Most importantly, reducing sleep by transient dopaminergic neuronal activation reduced egg output; removal of this activation resulted in wild-type levels of sleep and egg output (Fig. 3). Thus, these results strongly indicate that it is sleep loss that has a direct detrimental impact on reproductive output. While it is possible that three distinct methods of sleep deprivation all cause a direct negative impact on egg output independent of sleep loss, we feel that it is unlikely, especially considering the transient nature of the genetic manipulation-induced sleep loss. To our knowledge, this is the first study to establish a direct link between sleep and reproductive physiology in *D. melanogaster*.

Egg laying in *Drosophila* is the final step in a sequence of processes that occur in a co-ordinated manner, which include ovulation of eggs into the uterus, mating and subsequent sperm storage in a pair of spermathecae and the seminal receptacle as well as fertilization in the uterus (reviewed in Bloch Qazi et al., 2003). Thus, mechanistically, sleep could influence egg output by modulating any combination of some or all of the above processes. Virgin females also lay a small quantity of unfertilized eggs; therefore, by quantifying the egg numbers laid by sleep-deprived virgin flies, the question of whether sperm storage gets modulated by sleep levels could be addressed. We found that the fraction of virgin flies laying eggs was reduced when they were deprived of sleep either during the day or night (19% of D_{dep} flies, N=21, 17.4% of N_{dep} flies, N=23) as compared with control flies that slept normally (40.7% of control flies, N=27). This indicates that sleep modulates egg output by affecting steps other than sperm storage, as virgin flies do not store sperm and yet their egg output is reduced upon sleep deprivation. However, a more detailed analysis of ovulation rates, egg hatchability, mature and immature egg numbers and amount of stored sperm will aid in the finer dissection of the relationship between sleep and the reproductive system.

Reproduction in *Drosophila* is regulated by an array of hormones and fecundity crucially depends upon a balance in the amounts of juvenile hormone (JH) and ecdysone (20E; Soller et al., 1999). Dopamine regulates levels of JH in *Drosophila viridis* (Rauschenbach et al., 2007), thereby indirectly affecting fecundity. Indeed, dopaminergic neuronal circuits are involved in governing oviposition choice, specifically to media containing favourable levels of alcohol (Azanchi et al., 2013). Moreover, it has been also shown that dopamine acts to promote adaptation of *Drosophila sechelia* to a specialist diet of an otherwise toxic fruit, *Morinda citrifolia*, by boosting its fecundity (Lavista-Llanos et al., 2014). In a recent study using genome-wide association methods, two genes encoding dopamine receptors (*Dop1R1* and *DopEcR*) in *D. melanogaster* were shown to have pleiotrophic effects on traits associated with ovariole number and sleep parameters (Lobell et al., 2017). Importantly, lowered levels of dopamine during larval stages or immediately after eclosion both have far reaching consequences in terms of decreased egg output and stalled ovarian development, respectively (Neckameyer, 1996). In contrast, we show that a loss-of-function mutation in the dopamine transporter gene, which retains dopamine in synaptic clefts, reduces sleep and reduces egg output whereas a transient increase in dopaminergic activity causes a transient decrease in both sleep and egg output (Fig. 3). Together, these results demonstrate that levels of neuromodulatory substances can have strong dose-dependent effects such that both low and high titres can lead to sub-optimal outcomes for the organism (Berridge and Arnsten, 2013).

Caffeine is one of the most widely used psychostimulants in the world and it promotes wakefulness and causes sleep deprivation. With increased precedence in shift work and a general lifestyle favouring delayed bedtimes and decreased night-time sleep levels, the consumption of caffeine specifically during the night is bound to increase. Here, we showed that caffeine consumption and increased night activity decreases sleep and negatively alters egg output in *Drosophila*. While we have shown this effect with female flies, similar trends may also be found in male reproductive output. In conclusion, our results unequivocally show that each method of sleep deprivation, be it chemical, mechanical or genetic, results in sleep loss accompanied by a reduction in egg output. For animals that invest in parental care, sleep deprivation may be an inevitable consequence resulting in lowered reproductive output, thereby potentially giving rise to a subtle level of parent–offspring conflict or co-adaptation. We conclude that sleep may contribute to the reproductive success of organisms, thereby amplifying its propensity to be selected for, over evolutionary timescales.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.P., V.S.; Methodology: S.P.; Formal analysis: S.P.; Investigation: S.P., D.K.D., F.A.T., S.L.; Writing - original draft: S.P.; Writing - review & editing: S.P., V.S.; Supervision: V.S.; Project administration: V.S.; Funding acquisition: V.S.

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Supplementary information
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Supplementary Information

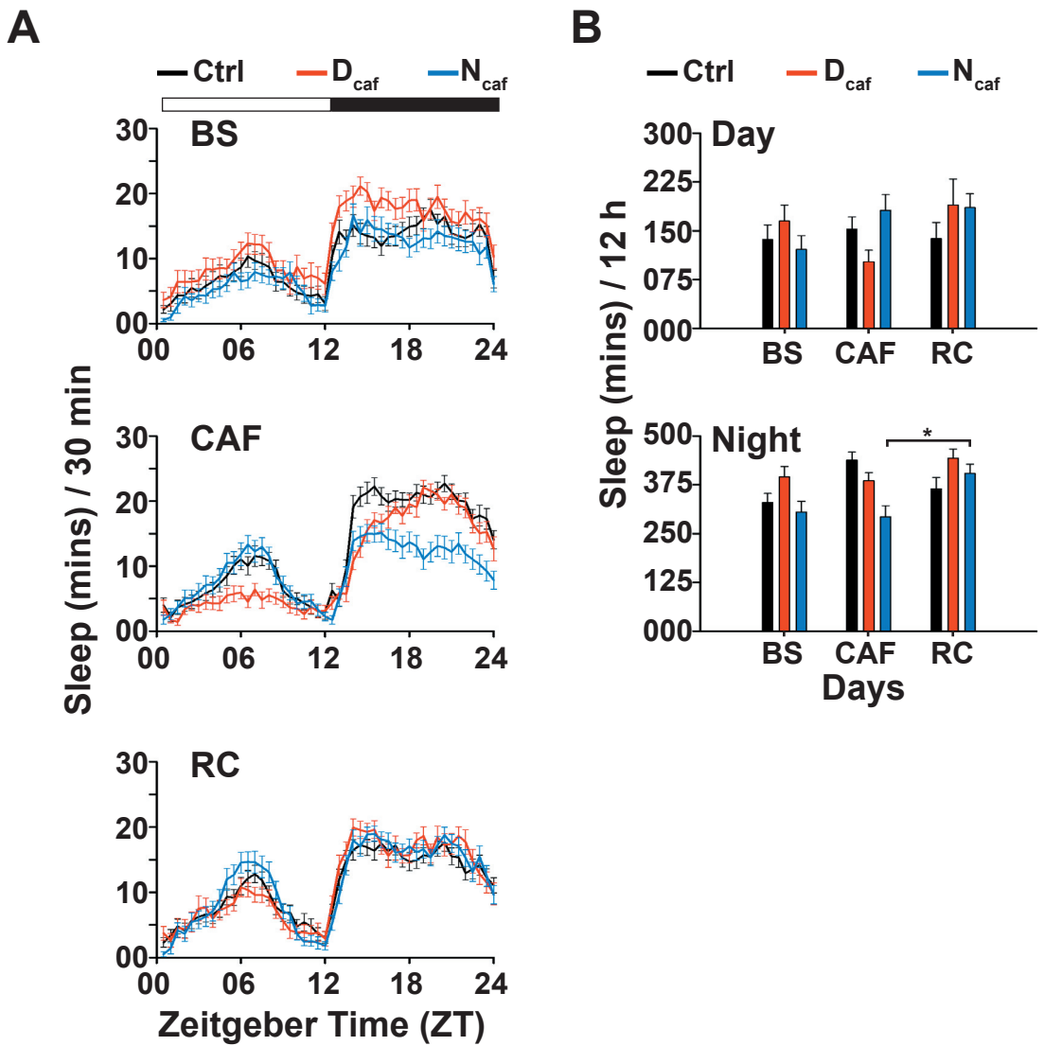

Fig. S1. (A) Sleep in minutes for every half hour over a period of 24 h is shown for w^{1118} flies fed with standard food (Ctrl, n = 28), flies fed with 1.0 mg/ml caffeine only during the day (D_{caf}, n = 29) and only during the night (N_{caf}, n = 28) averaged across two baseline (BS), three caffeine feeding (CAF) and two recovery (RC) days. (B) Daytime (top) and night (bottom) sleep of control, D_{caf} and N_{caf} flies are compared across BS, CAF and RC days. Only night sleep of N_{caf} flies during CAF and RC days is significantly different from each other (two-way ANOVA with treatment and days as fixed factors followed by post-hoc Tukey's HSD test). All other details as in Figure 1.

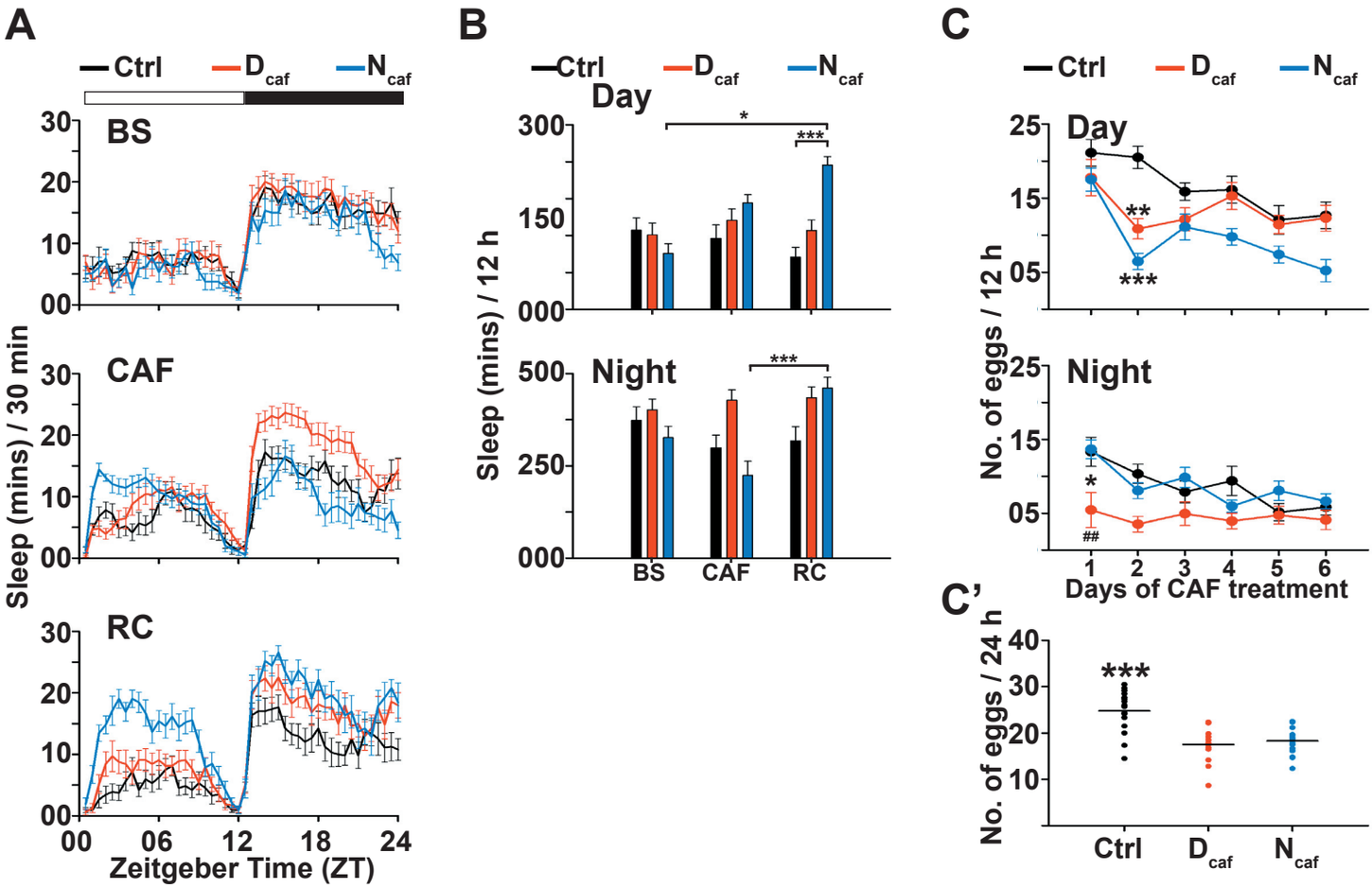

Fig. S2. (A) Sleep in minutes for every half hour over a period of 24 h is shown for control flies of outbred *CCM* population fed with standard food (Ctrl, n = 16), flies fed with caffeine only during the day (D_{caf}, n = 16) and only during the night (N_{caf}, n = 14) for caffeine concentration of 4.0 mg/ml averaged across two baseline (BS), three caffeine feeding (CAF) and two recovery (RC) days. Night sleep of N_{caf} flies during CAF days is lower than that of controls, and both daytime and night sleep of N_{caf} flies is higher than the controls during RC. (B) Daytime sleep levels of control and D_{caf} flies show no differences across different days, whereas those of control and N_{caf} flies significantly differ from each other during RC. Daytime sleep of N_{caf} flies during RC is significantly higher than that during BS. Night sleep of N_{caf} flies during CAF and RC days are significantly different from each other other (two-way ANOVA with treatment and days as fixed factors followed by post-hoc Tukey's HSD test). (C) N_{caf} flies showed a trend of laying lower number of eggs than controls during the daytime (top), which was significant on day 2, while D_{caf} flies showed a trend of laying lower number of eggs during the night (bottom) which was significant on night 1 (Kruskal-Wallis test). (C') Total eggs laid by control (n = 16), D_{caf} (n = 14) and N_{caf} (n = 18) flies averaged across six days of caffeine feeding. Control flies laid higher number of eggs as compared to both D_{caf} and N_{caf} flies (Kruskal-Wallis test). All other details as in Figure 1.

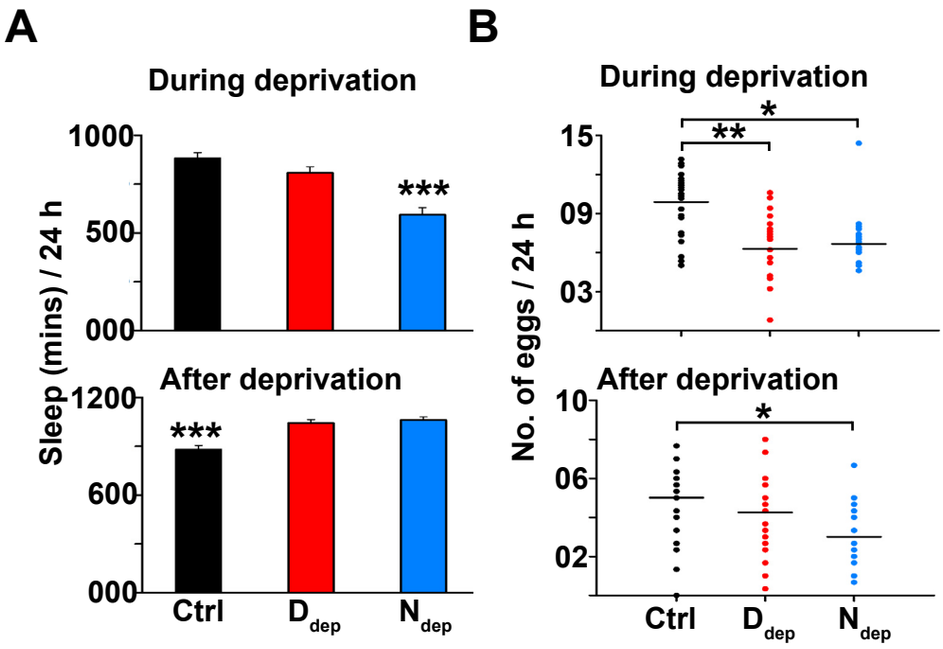

Fig. S3. (A) Total sleep (top) during 6 days of sleep deprivation and (bottom) averaged for 3 days post-deprivation. Sleep of N_{dep} (n = 16) flies is significantly lower than both control (n = 29) and D_{dep} (n = 21) flies during sleep deprivation, whereas both D_{dep} and N_{dep} flies sleep more after deprivation (one-way ANOVA followed by post-hoc Tukey's HSD test). (B) Average number of eggs laid (top) during sleep deprivation and (bottom) after sleep deprivation. D_{dep} and N_{dep} flies lay lesser number of eggs as compared to control flies during deprivation, but only N_{dep} flies lay lower number of eggs compared to control flies after deprivation (Kruskal-Wallis tests). All other details as in Figure 1.

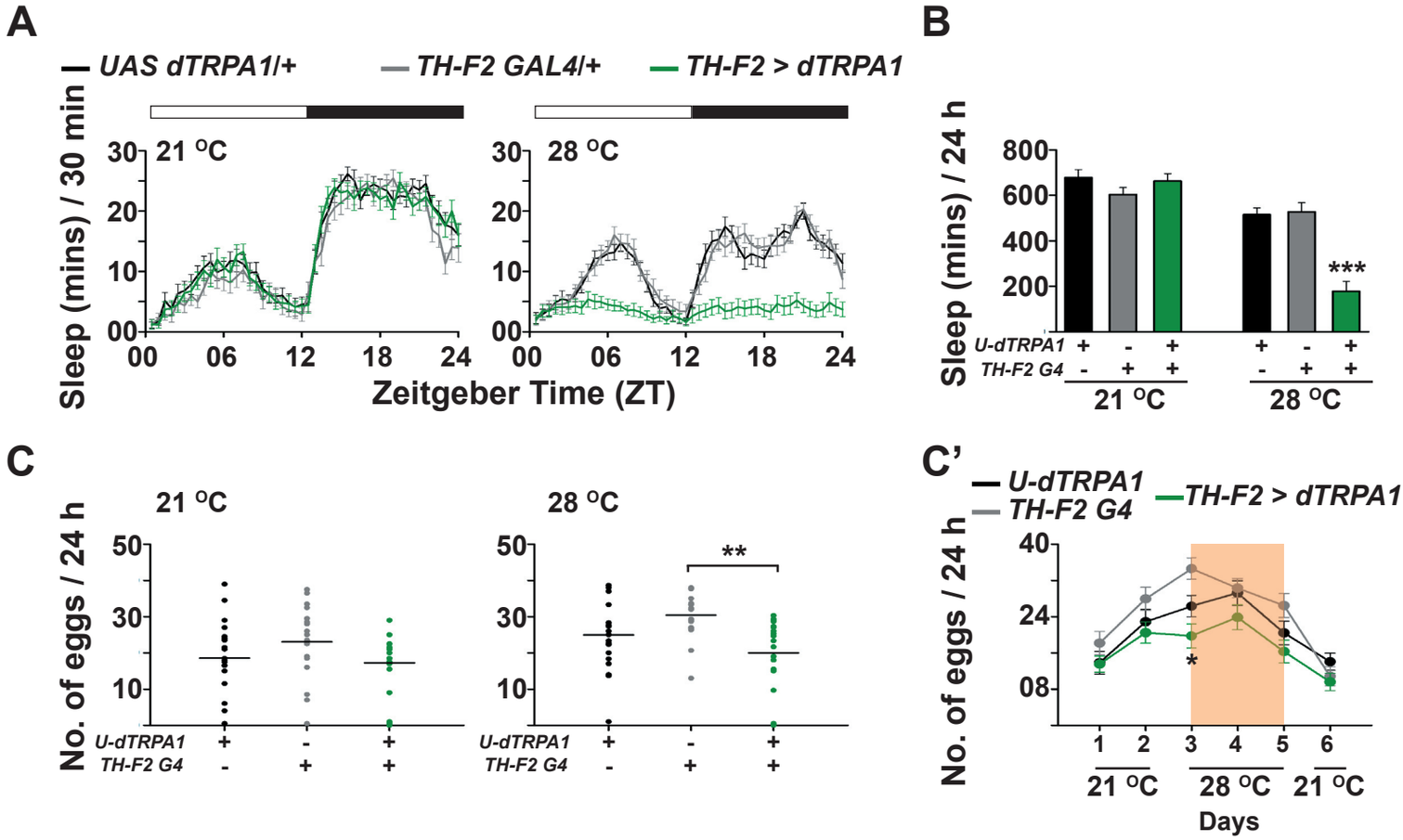

Fig. S4. (A) Sleep in minutes for every half hour over a period of 24 h averaged across two days at 21 °C (left) and three days at 28 °C (right) is shown for *UAS dTRPA1/+*(*n* = 22), *TH-F2 GAL4/+* (*n* = 23) and *TH-F2 GAL4 > UAS dTRPA1* (*n* = 23) flies. (B) At 21 °C, total sleep levels of all three genotypes is similar, whereas at 28 °C, *TH-F2 GAL4 > UAS dTRPA1* flies sleep significantly less than *UAS dTRPA1/+* and *TH-F2 GAL4/+* flies (two-way ANOVA with genotype and temperature as fixed factors followed by post-hoc Tukey's HSD test). (C) Total number of eggs laid averaged across two days at 21 °C (left) is similar across all genotypes, while average number of eggs laid by *TH-F2 GAL4 > UAS dTRPA1* (*n* = 20) flies is significantly lower than *TH-F2 GAL4/+* (*n* = 18) flies during the three days at 28 °C, but not from *UAS dTRPA1/+* flies (*n* = 20) (right, Kruskal-Wallis test). (C') Total number of eggs laid on all six days of the assay at different temperatures as indicated. *TH-F2 GAL4 > UAS dTRPA1* flies laid significantly lower number of eggs as compared to *UAS dTRPA1/+* and *TH-F2 GAL4/+* on the first day of 28 °C (Kruskal-Wallis test), while it showed a decreasing non-significant trend on the other two days of 28 °C. All flies laid similar number of eggs at 21 °C. All other details as in Figure 3.