

foraging alters resilience/vulnerability to sleep disruption and starvation in *Drosophila*

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Recent human studies suggest that genetic polymorphisms allow an individual to maintain optimal cognitive functioning during sleep deprivation. If such polymorphisms were not associated with additional costs, selective pressures would allow these alleles to spread through the population such that an evolutionary alternative to sleep would emerge. To determine whether there are indeed costs associated with resiliency to sleep loss, we challenged natural allelic variants of the *foraging* gene (*for*) with either sleep deprivation or starvation. Flies with high levels of Protein Kinase G (PKG) (*for^R*) do not display deficits in short-term memory following 12 h of sleep deprivation. However, short-term memory is significantly disrupted when *for^R* flies are starved overnight. In contrast, flies with low levels of PKG (*for^S*, *for^{S2}*) show substantial deficits in short-term memory following sleep deprivation but retain their ability to learn after 12 h of starvation. We found that *for^R* phenotypes could be largely recapitulated in *for^S* flies by selectively increasing the level of PKG in the α/β lobes of the mushroom bodies, a structure known to regulate both sleep and memory. Together, these data indicate that whereas the expression of *for* may appear to provide resilience in one environmental context, it may confer an unexpected vulnerability in other situations. Understanding how these tradeoffs confer resilience or vulnerability to specific environmental challenges may provide additional clues as to why an evolutionary alternative to sleep has not emerged.

Although sleep is a behavioral state that is conserved across a diverse range of species, the biological functions of sleep remain unknown. Sleep deprivation (SD) has been shown to negatively impact cognition, but individual responses to sleep loss can vary significantly within a population (1). Recent studies suggest that a portion of this variability may be influenced by genetic factors (2). For example, polymorphisms for PERIOD 3 (*PER3*), a circadian clock gene, can predict the magnitude of cognitive impairment and sleep homeostasis in response to a night of SD in humans (2). Although these genetic contributions may attenuate impairments following SD, the tradeoffs associated with resistance to sleep loss remain unknown. Presumably, the potential costs must be substantial. Thus, it is likely that the price of protection from sleep loss that can be conferred by allelic variation in one environment may induce a cost when manifested in a different environment. To date, putative costs of resiliency to sleep loss have not been identified in humans or any model organism.

foraging (*for*), which codes for Protein Kinase G (PKG), is maintained in wild-type populations as a genetic polymorphism that results in either higher or lower levels of PKG activity (3). The allele associated with higher levels of PKG (“rover”; *for^R*) results in larvae with longer foraging trails between food patches, whereas the allele associated with lower levels of PKG (“sitter”; *for^S*) results in larvae with shorter foraging trails; a mutant of *for* (*for^{S2}*) generated in the *for^R* background also displays shorter foraging trails. Different foraging patterns appear beneficial in discrete situations, so neither allele has achieved a consistent advantage, suggesting an explanation for their persistence over time (4). Interestingly, *for* is highly pleiotropic and is known to influence many behaviors in multiple species (5), including sleep (6, 7) and learning and memory (8), to name only a few. With

respect to learning and memory, recent studies have shown that *for^R* flies perform better on short-term memory tasks than *for^S* flies, whereas *for^S* flies have better long-term memory acquisition (4). These differences suggest that the *for* alleles may confer strikingly different strategies for survival, with clear advantages and disadvantages in distinct environments (8, 9).

SD is known to result in robust cognitive impairments in humans (10), rodents (11), bees (12), and flies (13, 14). However, the extent to which prolonged waking will result in cognitive impairments is strongly influenced by the environmental context. For example, although starvation is known to induce wakefulness in many animals (15, 16), including flies (17, 18), recent studies from our laboratory indicate that wakefulness induced by starvation is not accompanied by cognitive impairments (18). Given that the *foraging* gene has been implicated in memory and sleep as well as energy storage and responses to food deprivation, it is likely that flies with the naturally existing *foraging* polymorphisms will differ in their ability to maintain cognitive functioning during sleep loss. Indeed, a recent study has reported that *foraging* alters the amount of waking observed during starvation (17). However, neither sleep homeostasis, survival, nor cognitive behaviors were evaluated in *for^R* and *for^S* flies following starvation. As a consequence, it remains unclear whether the alternate waking strategies exhibited by *for^R* and *for^S* flies result in functional outcomes that may provide a selective advantage or disadvantage during food loss. Because the physiological demands of SD are likely to differ from those observed during starvation, it is unlikely that the molecular mechanisms that allow the animal to succeed in one environment will be effective in the other. Thus, we hypothesized that behavioral responses of *foraging* allelic variants that may confer an advantage to SD would be deleterious during starvation.

Results

We hypothesized that polymorphisms in *foraging* would influence the response to SD as measured by both sleep homeostasis, the increase in sleep seen following sleep loss, and short-term memory. Because diet strongly modulates the behavior of the *foraging* alleles, we first asked whether *for^R* flies would sleep significantly longer than *for^{S2}* mutants when tested under our laboratory conditions as described previously (7). As seen in Fig. S1, under our dietary conditions, *for^R* flies sleep significantly longer than *for^{S2}* mutants.

Next, we exposed *for^R*, *for^S*, and *for^{S2}* flies to 12 h of SD during their primary sleep period using the sleep-nullifying apparatus. As seen in Fig. 1A, *for^R* flies did not compensate for lost sleep during 48 h of recovery, whereas both *for^S* flies and *for^{S2}* mutants

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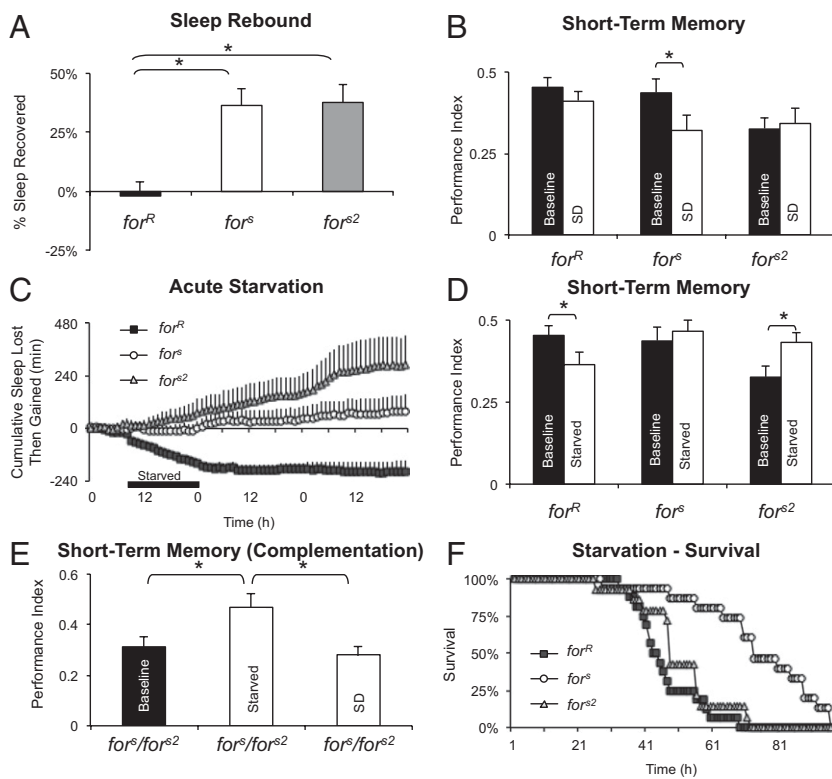


Fig. 1. *foraging* differentially confers resilience/vulnerability to SD and starvation. (A) *for^R* flies do not compensate for 12 h of SD with a subsequent increase in sleep, whereas both *for^S* and *for^{S2}* mutants exhibit a sleep rebound typical of *Cs* flies. % sleep recovered is calculated for each individual as a ratio of the minutes of sleep gained above baseline during 48 h of recovery divided by the minutes of sleep lost during SD; * $P < 0.05$, modified Bonferroni test. Data are presented as mean \pm SEM. (B) STM is impaired in *for^S* flies following 12 h of SD, whereas *for^{S2}* mutants display impairments both during baseline and following SD. In contrast, *for^R* flies maintain normal STM following SD. (C) When placed in starvation before lights out, *for^R* flies display significantly less sleep than during the previous baseline night and do not exhibit a sleep rebound when placed back onto food the following morning. Neither *for^S* flies nor *for^{S2}* mutants respond to starvation with an increase in waking. Cumulative sleep lost or gained during starvation. (D) STM is impaired in *for^R* flies when waking is induced by starvation. *for^S* flies maintain STM following a night of starvation. Surprisingly, *for^{S2}* mutants have normal STM following a night of starvation. (E) STM is impaired in *for^S/for^{S2}* flies under baseline conditions compared with starved siblings. Sleep-deprived *for^S/for^{S2}* flies show no further STM impairments relative to baseline. (F) *for^S* flies survive longer than *for^R* and *for^{S2}* flies during chronic starvation ($n > 27$ per group).

displayed a sleep rebound similar to that previously seen in *Canton-s* (*Cs*) flies (19–21). The lack of a homeostatic response seen in *for^R* flies may represent either an adaptation that allows animals to better withstand the negative effects of waking, or it may indicate that *foraging* disrupts regulatory processes, thereby preventing flies from obtaining needed sleep. Because deficits in short-term memory are a robust consequence of sleep loss (13, 22, 23), we evaluated short-term memory (STM) using aversive phototaxic suppression (APS) in *for^R*, *for^S*, and *for^{S2}* flies following 12 h of SD. In the APS, flies are individually placed in a T maze and allowed to choose between a lighted and darkened chamber (13, 24). During 16 trials, flies learn to avoid the lighted chamber, which is paired with an aversive stimulus (quinine/humidity). The performance index is calculated as the percentage of times the fly chooses the dark vial during the last 4 trials of the 16-trial test (13, 25). As seen in Fig. 1B, *for^R* flies maintain their ability to learn following SD, whereas *for^S* flies are significantly impaired; *for^{S2}* mutants showed impaired performance in the APS both under baseline conditions and following SD. *for^R*, *for^S*, and *for^{S2}* flies did not differ in sensory thresholds as measured by either the photosensitivity index (PI; percentage of photopositive choices in 10 trials in the absence of quinine) or the quinine sensitivity index (QSI; time in seconds flies reside on the non-quinine side of a chamber) (Table S1) (13, 25). Given that *for^R* flies maintain their ability to learn following 12 h of SD and do not appear to be sleepy, as indicated by the absence of a sleep rebound, *for^R* flies are considered to be resistant to SD. In contrast, both *for^S* and *for^{S2}* flies remain vulnerable to the negative effects of extended waking as measured by learning deficits and an increased sleep rebound.

Interestingly, the deleterious effects of extended waking are absent when waking is induced by starvation (18). Given that *foraging* alters the response to food deprivation, we hypothesized that *for^R*, *for^S*, and *for^{S2}* mutants would show different vulnerabilities to waking induced by starvation. As seen in Fig. 1C, when *for^R* flies are placed in recording tubes with agar and water (starvation), they exhibit an immediate and sustained increase in waking behavior and show no evidence of a sleep rebound when placed back on

their standard diet 12 h later. Interestingly, whereas the wake-promoting effects of starvation are absent in *for^R* flies as previously described (17, 18), *for^{S2}* mutants respond to starvation with a significant increase in sleep (Fig. 1C). If *for^R* flies are resistant to the negative effects of waking induced by starvation, they should maintain their ability to learn in the APS as they did following SD. However, in contrast to waking induced by SD, *for^R* flies are impaired following waking induced by starvation (Fig. 1D). Surprisingly, *for^{S2}* mutants, which exhibit impaired short-term memory both under baseline conditions and after SD, recover their ability to learn when starved; previous studies have shown that neither SD nor starvation alters PI or QSI (13, 18, 25, 26). Thus, *for^{S2}* mutants sleep more and display normal cognitive behavior following starvation, whereas *for^R* flies display an unexpected vulnerability in short-term memory when waking is induced by the absence of food. Consistent with previous results (17, 18), SD and starvation likely invoke distinct physiological responses even though each manipulation produces an increase in waking.

Resilience to sleep loss is indicated by the ability to maintain optimal performance after sleep disruption. Thus, although baseline learning in *for^{S2}* mutants is at the level observed in memory mutants (13, 25), neither *for^{S2}* mutants nor *for^R* flies, which are in the same genetic background, show performance decrements following sleep loss. With that in mind, we conducted a complementation test to examine learning in *for^S/for^{S2}* flies under baseline and in response to both SD and starvation. As seen in Fig. 1E, *for^S/for^{S2}* flies do not learn under baseline or after SD, but display STM after starvation. These results, along with the gain- and loss-of-function data presented below, indicate that it is the levels of *foraging*, rather than genetic background, which most likely account for differences in learning following SD and starvation.

Although waking up to forage during starvation would enhance the opportunity to find food, it requires additional energy expenditure. In contrast, sleeping would minimize the ability to find food but would likely conserve energy. Thus, we asked whether the alternate behavioral strategies exhibited by *for^R* and *for^{S2}* mutants would be associated with changes in survival during starvation. The average difference in the LD₅₀ in hours to

starvation between *for^R* and *for^{s2}* mutants was 10.25 ± 3.19 ($t = 2.306$, $P = 0.01$, one-sample t test, $n = 4$ replicates); a representative example of survival during starvation is shown in Fig. 1E. Thus, whereas *for^R* flies appear resistant to the behavioral consequences of SD, *for^{s2}* flies appear more suited to withstand the challenge of overnight starvation.

Given that sleep plays a role in memory consolidation (27) and that the *foraging* polymorphism has been shown to independently alter both sleep and memory (7, 8), we examined the relationship between sleep and plasticity in *for^R*, *for^s*, and *for^{s2}* flies. Previous studies have shown that enriched social environments induce synaptic elaboration in mammals and flies and that these changes are followed by several days of increased sleep (28–30). Thus, we evaluated sleep in *for^R*, *for^s*, and *for^{s2}* flies after they had been exposed to either social enrichment, which consists of ~60 flies maintained in a 50-mL vial, or social isolation, which consists of flies being housed individually in TriKinetics tubes, for 5 d (31, 32). Surprisingly, neither *for^s* nor *for^{s2}* flies, which have long-term memory (LTM) using olfactory conditioning (8), display an increase in sleep following social enrichment (Fig. 2A). In contrast, *for^R* flies, which have impaired LTM using olfactory conditioning, maintain their ability to increase sleep following social enrichment (Fig. 2A). Together, these data extend results with STM and LTM by showing that *foraging* plays a role in an additional type of plasticity, and further suggest that *foraging* may play a unique role when plasticity is induced in a social context.

To further test this hypothesis, we evaluated LTM in male flies using a spaced training protocol in a courtship conditioning assay that results in decreased courtship behavior for at least 48 h after

training (31, 32). As seen in Fig. 2B, *for^R* and *for^s* flies display a significant reduction in courtship 48 h following spaced training (T) compared with their naïve siblings (N), indicating that they developed LTM. In contrast, *for^{s2}* males show no reduction in courtship, indicating that they have impaired memory consolidation (Fig. 2B, Right). Note that whereas naïve courtship was low in *for^s* males, it was not so low as to preclude the development of LTM. Moreover, naïve courtship was also low in *for^{s2}* mutants, which are in the same genetic background as *for^R*. Thus, it is likely that the reduced level of naïve courtship is due to the *foraging* gene and not due to genetic background. The observation that *for^R* flies show both an increase in sleep in response to social enrichment and LTM following courtship conditioning suggests that *foraging* may be particularly relevant for plasticity induced in a social context.

We have previously shown that sleep is increased following courtship conditioning in *Cs* flies and that LTM is disrupted if flies are sleep-deprived immediately following training (31). As seen in Fig. 2C, male *for^R* flies sleep significantly more following spaced training than their naïve siblings. Similarly, *for^{s2}* mutants, which did not develop LTM, did not increase their sleep following training, consistent with previous reports that courtship behavior in the absence of LTM formation does not alter sleep (31). Given that *for^s* flies developed an LTM, it is unclear why they did not show an increase in posttraining sleep (Fig. 2C). However, one explanation may be that the changes in the behavior of *for^s* flies shown in Fig. 2B were too small to effectively induce changes in sleep. We next asked whether posttraining SD would disrupt memory consolidation in *for^R* and *for^s* flies. Interestingly, 4 h of SD immediately following spaced training (T+SD) did not disrupt LTM in *for^R* flies, whereas LTM was disrupted in *for^s* flies. Thus, *foraging* appears to allow memory consolidation to proceed in the absence of sleep.

Given that the mushroom bodies (MBs) modulate both sleep and memory (13, 33, 34), we hypothesized that *for* signaling in the MBs would phenocopy *for^R* and confer resistance to SD. Sleep homeostasis and performance in the APS were evaluated following 12 h of SD in flies overexpressing *for* in the MBs of otherwise *for^s* homozygous background. As seen in Fig. 3A and B, when *for* is overexpressed primarily in the α/β lobes of the MB using the *c739* or *30y* GAL4 drivers, sleep rebound is significantly attenuated. Thus, expressing *for* in the MBs recapitulates the sleep rebound phenotype observed in *for^R* flies. Interestingly, overexpression of *for* using the *201y* GAL4 driver, which expresses predominantly in the λ lobes and only weakly in the α/β lobes, does not significantly alter sleep rebound (Fig. 3C). As mentioned above, a low sleep rebound could represent either an adaptation that allows animals to better withstand the negative effects of waking, or a disruption in regulatory processes that prevent flies from obtaining needed sleep. Consistent with the *for^R* phenotype described above, overexpressing *for* using *c739* or *30y* in an otherwise *for^s* background also prevented deficits in short-term memory following SD (Fig. 3D and E). *201y/+; for^s* control flies were altered in the APS under baseline conditions, such that the effect of MB γ -lobe overexpression using *201y* on short-term memory after SD could not be assessed. No differences in sensory thresholds were observed between genotypes. To determine whether reducing *foraging* within the MBs would phenocopy *for^{s2}* mutants, we expressed *UAS-for^{RNAi}* using *UAS-dicer²;30y-GAL4*. As seen in Fig. 3F, *UAS-dicer²;30y-GAL4/+>UAS-for^{RNAi}* flies, but not parental controls, showed disrupted STM during baseline and after SD, whereas STM was restored following starvation. These results are similar to those observed in *for^{s2}* mutants. These data indicate that *for* activity in the mushroom bodies, particularly the MB α/β lobes, recapitulates the sleep phenotypes observed in *for^R* and *for^{s2}* flies.

A previous report indicates that *for^R* flies and flies overexpressing *for* within the MBs using *c739*, *30y*, and *201y* GAL4 drivers have impaired LTM following olfactory conditioning (8). However, the data presented above indicate that *for^R* flies can generate LTM following courtship conditioning. To further define

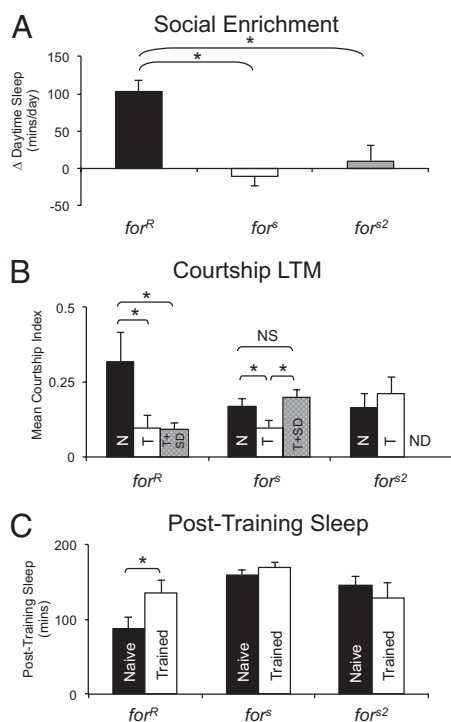


Fig. 2. Sleep deprivation does not block LTM in *for^R* flies. (A) *for^R* females increase sleep following social enrichment relative to isolated siblings, whereas *for^s* and *for^{s2}* females do not. $*P < 0.05$, modified Bonferroni test. Data are presented as mean \pm SEM. (B) *for^R* flies have intact LTM that is not disrupted when sleep-deprived for 4 h immediately after training (T+SD). Trained *for^s* flies have intact LTM during baseline but are impaired when sleep-deprived following training. *for^{s2}* mutants have impaired LTM; courtship was not evaluated in *for^{s2}* flies following SD (ND). NS, nonsignificant. $*P < 0.05$, modified Bonferroni test. (C) Following spaced training for courtship conditioning, *for^R* males, but not *for^s* or *for^{s2}* flies, sleep significantly more than their naïve siblings; $*P = 0.03$.

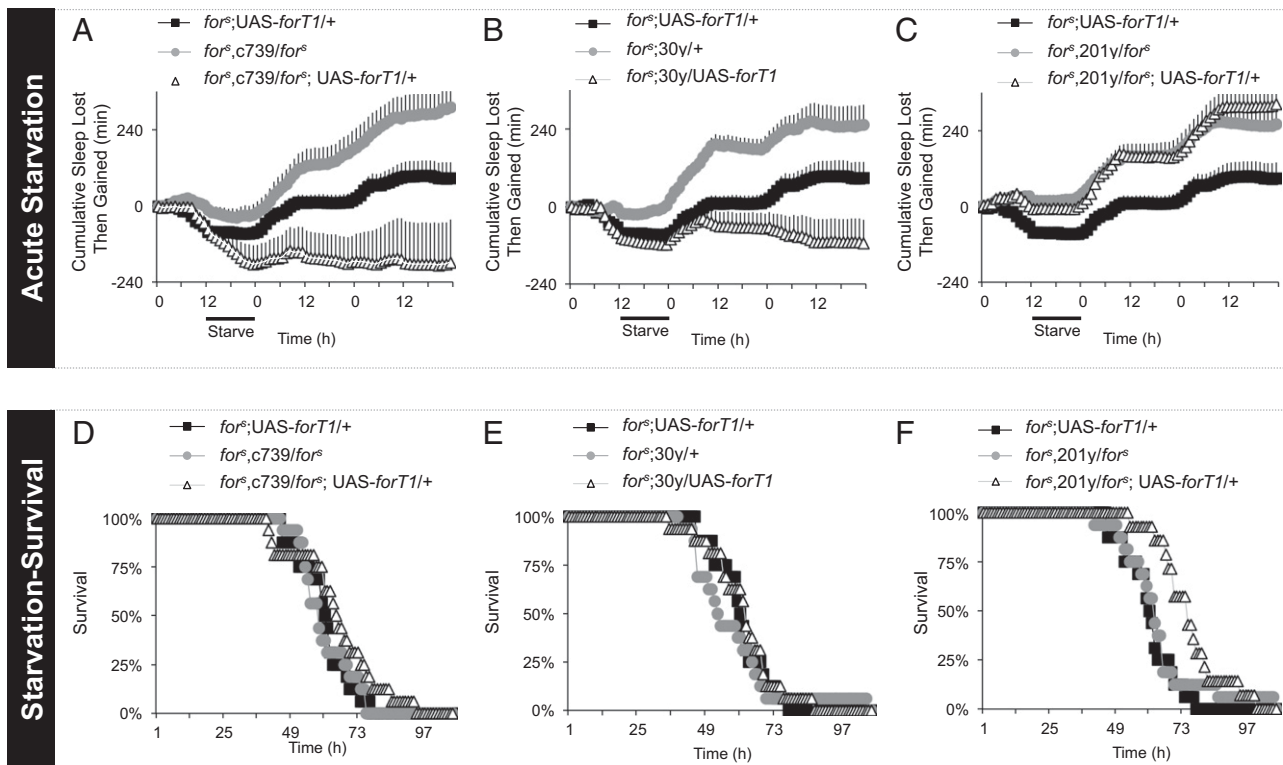


Fig. 4. *for* overexpression in the MBs alters response to starvation. (A–C) *w*; $for^s;c739/for^s;UAS-for/+$ and *w*; $for^s;30y/UAS-for$ flies exhibit a for^s -like response to 15 h of starvation, whereas the parental lines retain the for^s phenotype; $for^s;UAS-for/+$ data are replotted in B and C to facilitate comparisons. In contrast, *w*; $for^s;201y/for^s;UAS-for/+$ flies and their parental controls (*w*; $for^s;201y/for^s$) exhibit a for^s response to starvation (C). Data are presented as mean \pm SEM. (D–F) Survival during chronic starvation is not altered when *UAS-for* is expressed in MB α and β lobes but is increased when *UAS-for* is expressed in MB γ lobes.

resistance to starvation and a corresponding tradeoff, as indicated by an increased vulnerability to SD. Importantly, the phenotypes seen in *foraging* alleles can be largely recapitulated by overexpressing or reducing *foraging* in the α/β lobes of the MBs.

Does the variability in resilience to sleep loss that is conferred by the *for* polymorphism have ecological relevance? Currently, it is not clear whether the ability to withstand sleep loss can confer an advantage in reproductive fitness and, thus, influence natural selection at the *for* locus. Furthermore, the *for* locus is notably pleiotropic and has been implicated in modulation of learning and memory (8) as well as metabolic plasticity (35), making it difficult to specify which phenotype might respond to a given selection pressure aimed at changing the allelic variation at the *for* locus. It has been established, however, that flies carrying a given *for* allele have a relative fitness advantage when that allele is more rare (4). This finding is consistent with the idea that flies might exploit the resiliencies conferred by their *for* genotype to increase their chances of reproduction. For example, if a rover fly lives in a population where the for^s allele is most frequent, it might increase its reproductive fitness by forgoing sleep to mate at night while its sitter neighbors must rest. This strategy may allow the rover to reduce the competition for a mate yet still maintain optimal functioning the following day. Conversely, a sitter fly might outcompete rover rivals by forgoing a feeding to mate. Under this hypothesis, both natural *for* alleles (along with their associated resiliencies) could be maintained within a given population of flies.

Ecological pressures have been shown to affect cavefish, which have moved from living near the surface of lakes to deeper inside caves (36). Shifting ecological pressures have independently led each of these populations to sleep less than their surface-dwelling ancestors and, importantly, all three have converged upon similar genetic adaptations to adapt to a decrease in sleep time (36). It is possible that the polymorphism in *for* evolved in response to such ecological conditions, such as a prolonged food

shortage that might select for animals able to withstand starvation or to seasonal changes in the length of nights that might place constraints on sleep time.

Ultimately, the extent to which resiliency to sleep loss contributes to the frequency of *for* alleles in clinically varying natural populations of flies remains to be determined. It is important to note, however, that roles for PKG in sleep regulation have been identified in *Caenorhabditis elegans* (7) and in mice (6), indicating that the influence of PKG on sleep regulation is likely to be evolutionarily conserved.

Human studies indicate that individuals vary greatly in their vulnerability to sleep loss (1). With that in mind, several laboratories have begun to examine naturally occurring polymorphisms in humans to determine their role in this differential sensitivity (2, 37, 38). For example, a polymorphism in PERIOD3 (PER3) is associated with larger cognitive deficits following SD (2). Similarly, a functional polymorphism in adenosine deaminase results in increased sleep pressure and increased sensitivity to SD (37). Moreover, a functional polymorphism in brain-derived neurotrophic factor alters EEG slow-wave activity (0.75–4.5 Hz) during both baseline and recovery following SD (38). Pharmaceuticals are commonly used to offset the negative results of SD (e.g., caffeine, Modafinil, etc.). Not surprisingly, polymorphisms also influence the efficacy of drugs to improve performance during SD (39). In this context, the present study suggests that cGMP signaling and PKG are a candidate pathway for sleep resilience. Single-nucleotide polymorphism in the human *for* ortholog PRKG1 could be investigated for association with sleep loss.

Thus, human studies have begun to identify molecular pathways that alter not only sleep time but resilience to sleep loss. Unfortunately, determining whether a polymorphism in humans is also associated with unexpected tradeoffs is time-consuming and costly. However, such experiments are tractable in the fly. Indeed, a previous report has found natural genetic variants that contribute

to baseline sleep time during the fly's primary waking period (40). Our data extend these findings to show that naturally occurring polymorphisms alter sleep homeostasis and, importantly, can confer resilience to sleep loss. In addition, our data suggest that the power of *Drosophila* genetics can be applied to these questions to determine the mechanism and extent to which a polymorphism has unexpected tradeoffs. Understanding how these tradeoffs confer resilience or vulnerability to specific environmental challenges is highly relevant for understanding both the importance of sleep during evolution and translational sleep research.

Materials and Methods

Flies. Flies were cultured at 25 °C with 50–60% relative humidity and kept on a diet of yeast, dark corn syrup, and agar under a 12-h light/12-h dark cycle with lights on at 0800 h. Sleep and activity patterns were assessed as described previously (13). Locomotor activity was measured in 1-min bins, and sleep was defined as periods of quiescence lasting at least 5 min.

Sleep Deprivation. Four- to seven-day-old females were sleep-deprived for 12 h during the dark phase using the sleep-nullifying apparatus as previously described (13). All sleep and activity were monitored using the TriKinetics *Drosophila* Activity Monitoring System.

Starvation. Four- to seven-day-old females were transferred to individual tubes containing a 1% agar gel before lights out and returned to normal fly media the next morning at lights on as previously described (18) or, during survival experiments, kept on agar until death. Cumulative sleep lost and then gained was calculated for the acute starvation experiments by comparing sleep during baseline to the starvation day and two subsequent recovery days.

APS Short-Term Memory. One-week-old female flies were placed in a T maze and allowed to choose between a lighted and a dark chamber. Filter paper was wetted with 10^{-1} M quinine hydrochloride solution and placed in the lighted chamber; the percentage of times the fly visits the dark vial was tabulated during 16 trials. The performance index is calculated as the percentage of times the fly chooses the dark vial during the last four trials.

Courtship Conditioning. Four- to six-day-old males were trained as previously described (31). The males were exposed to pheromonally feminized *Tai2* males in a spaced training protocol consisting of three 1-h training sessions, each separated by 1 h. Forty-eight hours later, trained and naïve males were exposed to *Tai2* males for a 10-min testing period. The courtship index is defined as the percentage of time that each subject fly spends in courtship behavior during a 10-min testing period.

Social Enrichment. Three- to four-day-old flies were divided into a socially isolated group, which were individually housed in 65-mm glass tubes, and a socially enriched group, consisting of 40–45 female flies housed in a single vial as previously described (31). After 5 d of social enrichment/isolation, flies were placed in clean 65-mm glass tubes and sleep was recorded for 3 d. The difference in daytime sleep between isolated and enriched flies was averaged over 3 d and referred to as "ΔSleep."

Statistics. All comparisons were done using a Student's *t* test or, if appropriate, ANOVA and subsequent modified Bonferroni tests unless otherwise stated. Statistical tests for data shown in Figs. 1–4 are presented in Tables S2–S5. An asterisk represents $P < 0.05$ by modified Bonferroni test in all figures unless otherwise described. All statistically different groups are defined as $P < 0.05$.

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