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The Perilipin Homologue, Lipid Storage Droplet 2, Regulates Sleep Homeostasis and Prevents Learning Impairments Following Sleep Loss

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Abstract
Extended periods of waking result in physiological impairments in humans, rats, and flies. Sleep homeostasis, the increase in sleep observed following sleep loss, is believed to counter the negative effects of prolonged waking by restoring vital biological processes that are degraded during sleep deprivation. Sleep homeostasis, as with other behaviors, is influenced by both genes and environment. We report here that during periods of starvation, flies remain spontaneously awake but, in contrast to sleep deprivation, do not accrue any of the negative consequences of prolonged waking. Specifically, the homeostatic response and learning impairments that are a characteristic of sleep loss are not observed following prolonged waking induced by starvation. Recently, two genes, brummer (bmm) and Lipid storage droplet 2 (Lsd2), have been shown to modulate the response to starvation. bmm mutants have excess fat and are resistant to starvation, whereas Lsd2 mutants are lean and sensitive to starvation. Thus, we hypothesized that bmm and Lsd2 may play a role in sleep regulation. Indeed, bmm mutant flies display a large homeostatic response following sleep deprivation. In contrast, Lsd2 mutant flies, which phenocopy aspects of starvation as measured by low triglyceride stores, do not exhibit a homeostatic response following sleep loss. Importantly, Lsd2 mutant flies are not learning impaired after sleep deprivation. These results provide the first genetic evidence, to our knowledge, that lipid metabolism plays an important role in regulating the homeostatic response and can protect against neuronal impairments induced by prolonged waking.

Introduction
Insufficient sleep adversely affects both endocrine and metabolic processes, resulting in glucose intolerance, insulin resistance, and obesity [1]. Moreover, sleep deprivation results in extensive physiological impairments in both vertebrates and invertebrates, including, but not limited to, cognitive impairments and death [2–6]. Sleep homeostasis is defined as the increase in sleep observed following sleep loss. It is theorized that sleep homeostasis restores vital biological functions degraded during sleep deprivation. Unfortunately, precisely which processes need restoration remains a matter of speculation and debate. A common strategy to identify pathways that regulate sleep homeostasis has been to compare animals that have been sleep deprived with a control group that has been sleeping or has had the opportunity to sleep. While many processes have been identified with this approach [7–10], the extent to which they play a role in sleep homeostasis is largely unknown.

An alternative strategy for identifying pathways associated with sleep homeostasis is to take advantage of the observation that environmental conditions influence the response to prolonged waking [11–13]. Indeed, many behaviors are influenced by interactions between genes and the environment [6,14–19]. That is, an individual will respond to the specific demands/constraints of their current environment by altering their physiological response in order to optimize their chances of success. Thus a similar challenge that occurs in two distinct environments may differentially activate specific pathways such that contrasting outcomes are observed. For example, at room temperature, flies mutant for the canonical clock gene cycle (cyc*01) show an exaggerated sleep rebound (~10-fold greater than wild-type flies) and begin to die if kept awake for 10 h [6]. However, if cyc*01 mutants are exposed to an environment with a higher temperature, they no longer exhibit an exaggerated sleep rebound after sleep deprivation, and they do not die when kept awake for 10 h [6,20]. Thus, it may be possible to more efficiently identify...
pathways that protect flies from the negative effects of waking by evaluating the differential responses to sleep loss that occur in two distinct environments.

One such environmental condition that may be particularly useful for contrasting with sleep deprivation is starvation. It has long been recognized that, in several species, the lack of food availability increases the duration of waking [21–25]. Moreover, rats respond to chronic total food deprivation with a linear increase in wakefulness, and when allowed access to food, they do not show increases in non-rapid eye movement sleep [26]. Similarly, fasting humans show a reduction in sleep time and increased sleep latency [27]. It has been suggested that animals that are able to remain alert and vigilant in the absence of food might have a selective advantage over animals that accrue sleep debt at a normal rate [24]. Thus, identifying the unique physiological responses to waking induced by starvation as compared to waking induced by sleep deprivation may provide insights into mechanisms underlying sleep homeostasis.

We evaluated the consequences of waking induced by starvation and contrasted them to an equivalent amount of waking induced by sleep deprivation. Our results indicate that while sleep deprivation robustly activates sleep homeostasis and results in learning impairments, these negative consequences are not observed following waking induced by starvation. Although cry01 flies die from sleep loss in 10 h, they can withstand ~28 h of waking induced by starvation [6]. We demonstrate that cry01 mutants have increased triglyceride stores compared to background controls, suggesting that genes involved in lipid metabolism may influence the response to extended waking. Two likely candidates are bmm (bmm), a homologue of adipose triglyceride lipase, and Lipid storage droplet 2 (Lsd2), a homologue of Perilipin. bmm mutants exhibit increased triglycerides and resistance to starvation [28], while Lsd2 mutants are lean and sensitive to starvation [29]. We show here that bmm and Lsd2 mutants play a role in sleep regulation. That is, bmm mutants displayed a large homeostatic response following sleep deprivation, while Lsd2 mutants had a suppressed sleep rebound. Importantly Lsd2 mutants maintain their ability to learn even in the face of sleep loss, indicating that they are protected from the negative effects of waking. To our knowledge, these results provide the first genetic evidence that lipid metabolism plays a role in regulating sleep homeostasis.

Results

Waking Induced by Starvation Does Not Initiate a Sleep Rebound

To begin, we investigated the effects of starvation on sleep homeostasis in flies mutant for cry01 and period (per01). We chose to evaluate cry01 mutants first due to their extreme sensitivity to sleep loss.

That is, in contrast to wild-type flies and other clock mutants, cry01 flies show an exaggerated homeostatic response after short-term sleep deprivation and, as a group, begin to die if kept awake for 10 h [6]. When cry01 flies are placed into recording tubes with agar and water (starvation), they exhibit an immediate and sustained increase in waking behavior (Figure 1A, Figure S1); when placed back on to their standard diet 7 h later, sleep simply returned to baseline with no evidence of a sleep rebound (Figure 1A, squares). The effects of starvation were contrasted with an equivalent amount of sleep deprivation induced using the sleep nullifying apparatus (SNAP), an automated sleep deprivation device that has been found to keep flies awake without nonspecifically activating stress response genes [6]. Sleep deprivation, starvation, and control treatments were conducted concurrently in arrhythmic cry01 siblings maintained in constant darkness (DD). Consistent with our previous results, cry01 flies that were sleep deprived using the SNAP displayed an exaggerated homeostatic response (Figure 1A, diamonds); locomotor activity levels between sleep deprived and starved siblings were not significantly different (unpublished data). To confirm that sleep deprived flies could eat during the deprivation protocol and were not starved, we assessed food intake by placing flies on food with a blue dye. Following 7 h of sleep deprivation, 13 out of 14 cry01 flies clearly exhibited evidence of blue dye in their abdomen while 11 out of 13 untreated baseline controls exhibited blue dye as rated by an observer blind to condition. Spectrophotometric data confirmed these results (absorbance at 625 λ: 8.7×10^-3/fly and 13.5×10^-3/ fly, respectively). Thus, both the untreated and sleep deprived flies have access to and consume food throughout the treatment period. Activity, sleep, and feeding behavior of flies immediately following sleep deprivation and starvation can be seen in Video S1. It is important to note that with longer durations of starvation, flies begin to display sleep homeostasis indicating that the cost of waking does indeed accrue in the absence of food (Figure S2). However, our data indicate that cry01 flies have a qualitatively different response to ~7 h of waking induced by starvation versus 7 h of waking induced by sleep deprivation.

The lack of a homeostatic response following starvation may represent either an adaptation that allows animals to better withstand the negative effects of waking, or it may simply reflect a physiological impairment that globally disrupts several regulatory processes, including sleep homeostasis. To distinguish between these two possibilities, we evaluated Amylase transcript levels, a known biomarker of sleepiness in flies, to determine if it was elevated following starvation. We have previously shown that, in flies, Amylase levels are only elevated following waking conditions that are associated with increased sleep homeostasis and are not induced by stress [30]. As seen in Figure 1B, cry01 flies that are sleep deprived for 7 h exhibit a large increase in Amylase mRNA while Amylase mRNA levels remain unchanged in cry01 siblings starved for 7 h. Thus, both Amylase mRNA levels and the absence
of a sleep rebound indicate that, although starvation increases waking, it may not increase sleep drive.

Given that starvation is a metabolic challenge, waking induced by starvation could potentially disrupt the normally tight association that is typically observed between Amylase and sleepiness [30]. Therefore we utilized a second, independent behavioral assay to evaluate the functional consequences of waking induced by starvation in cycD flies. We chose to evaluate learning, since deficits in learning and memory are well-conserved consequences of sleep deprivation [4,5,31]. Learning was examined using Aversive Phototaxic Suppression (APS) [32]. In this task, flies are individually placed in a T-maze and allowed to choose between a lighted and darkened chamber. During 16 trials, flies learn to avoid the lighted chamber that 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 [5,33]. Consistent with our previous results, 7 h of sleep deprivation resulted in a significant reduction in performance (Figure 1C, black). However, no learning deficits were observed following an equivalent amount of waking induced by starvation (Figure 1C, gray). Our previous studies have shown that the mechanical stimulus used to keep the animals awake does not disrupt performance [5]. Importantly, the time required for the fly to complete the 16 trials (TCT) was not modified by either sleep deprivation (15.5 ± 0.55 min) or starvation (14.5 ± 0.76 min) compared to controls (14.4 ± 0.63 min), indicating that differences in performance are unlikely due to alterations in motivation. Moreover, starvation did not alter sensory thresholds (Table S1) 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) consistent with our previous results indicating that sleep deprivation does not alter PI or QSI [5]. The magnitude of the learning deficit observed in cycD flies following sleep deprivation is similar to that previously reported for sleep-deprived wild-type flies, flies lacking Mushroom Bodies and classic memory mutants [5,33].

Figure 1. Starvation produces a waking state that does not activate sleep homeostasis. (A) cycD flies show a large homeostatic response after 7 h of sleep deprivation (SD, black) but do not display a homeostatic response following waking induced by 7 h of starvation (stv, gray). The sleep deprivation and starvation experiments were conducted in parallel on female siblings whose baseline sleep was monitored for 3 d; experiments were conducted in constant darkness (DD). Cumulative sleep lost or gained during SD or starvation. A negative slope indicates sleep lost, and a positive slope indicates sleep gained; when the slope is zero, recovery is complete. Stippled bar indicates treatment and white bar indicates recovery (n = 72 for each group, data are presented as mean ± SEM). (B) Amylase mRNA for sleep deprived and starved cycD flies expressed as a percentage change from age-matched untreated controls as measured by QPCR (n = 3 replicates of 20 heads/group; *p < .05 Student’s t-test). (C) APS performance is significantly disrupted by SD but is unchanged following starvation in cycD flies. The performance index is the number of photonegative choices during the last 4 trials of a 16-trial test; a higher score indicates learning. One way ANOVA F(2,30) = 5.26; n = 10/group; *p < .05 modified Bonferroni Test. (D) per01 flies show a large sleep rebound following 7 h of sleep deprivation but do not display a homeostatic response following 7 h of starvation (n = 72 for each group). (E) Amylase mRNA for sleep deprived and starved per01 flies expressed as a percentage change from age-matched untreated controls as measured by QPCR (n = 3 replicates of 20 heads/group; *p < .05 Student’s t-test). (F) APS performance is significantly disrupted by SD but is unchanged following starvation in per01 flies. One way ANOVA F(2,26) = 2.6; *p < .05 modified Bonferroni Test. doi:10.1371/journal.pbio.1000466.g001

er, no learning deficits were observed following an equivalent amount of waking induced by starvation (Figure 1C, gray). Our previous studies have shown that the mechanical stimulus used to keep the animals awake does not disrupt performance [5]. Importantly, the time required for the fly to complete the 16 trials (TCT) was not modified by either sleep deprivation (15.5 ± 0.55 min) or starvation (14.5 ± 0.76 min) compared to controls (14.4 ± 0.63 min), indicating that differences in performance are unlikely due to alterations in motivation. Moreover, starvation did not alter sensory thresholds (Table S1) 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) consistent with our previous results indicating that sleep deprivation does not alter PI or QSI [5]. The magnitude of the learning deficit observed in cycD flies following sleep deprivation is similar to that previously reported for sleep-deprived wild-type flies, flies lacking Mushroom Bodies and classic memory mutants [5,33]. Moreover, the deficits in
learning following sleep loss in flies are within the range of effect
sizes observed following sleep loss in humans and rodents across a
number of cognitive domains [34–37]. Thus, in contrast to 7 h of
waking induced by sleep deprivation, 7 h of waking induced by
starvation does not induce (1) a homeostatic response, (2) an
increase in the expression of a biomarker of sleepiness, or (3)
learning impairments.

We next tested whether starvation would alter behavior in per::fl
flies. We chose to evaluate per::fl mutants since our previous results
suggested that they are more sensitive to the lethal effects of
starvation [6]. As seen in Figure 1D starvation resulted in an
immediate and sustained increase in waking that was not
compensated for by a homeostatic response when flies were
placed back on to their normal diet. Similar to the effects observed
in cyo::fl mutants, Amylase levels were not elevated in per::fl mutants
following waking induced by starvation but were elevated by an
equivalent amount of waking induced by sleep deprivation
(Figure 1E). Finally, while 7 h of waking induced by sleep
deprivation resulted in significant decrements in performance in
the APS, 7 h of waking induced by starvation did not result in
learning deficits (Figure 1F). Starvation did not alter TCT in per::fl
mutants compared to untreated controls (14.7±0.06 versus
13±0.30 min, respectively) and did not alter PI or QSI. Thus,
two different clock mutants exhibit similar response to sleep
deprivation and starvation as measured by sleep homeostasis,
Amylase and learning.

The ability of starvation to increase locomotion in Drosophila is
well documented [38–42], suggesting that its effects are likely to
extend beyond clock mutants. However, since the effects of
starvation on waking have not been quantified directly, we
evaluated waking following starvation in flies maintained on a
typical light-dark schedule. Wild-type Canton-S (CS) flies and flies
mutant for Clock (Clk<sup>CS</sup>) were exposed to starvation for 12 h during
the dark period. As seen in Figure 2, (CS) flies and Clk<sup>CS</sup> mutants
also displayed an immediate increase in waking in the absence
of food that is not compensated by a homeostatic response. In CS
flies, starvation began at a time of day when feeding is normally
low [43] and the amount of waking induced by this duration of
starvation is lower than in the clock mutants. Since transgenic
flies are frequently generated in a white mutant background
(w<sup>1118</sup>), we further examined the effects of starvation in w<sup>1118</sup>
mutants as means to assess whether available transgenic tools can
be applied to this question. As seen in Figure 2B, 12 h of sleep
deprivation in w<sup>1118</sup> mutants produces a sleep rebound that is
similar to that observed for other wild-type flies [44–46].
Importantly, waking induced by 12 h of starvation was not
compensated by a sleep rebound in w<sup>1118</sup> flies (Figure 2B, gray)
even though the amount of sleep lost during starvation did not
differ from that induced by sleep deprivation (p = .22). Next, we
determined whether learning would remain intact in w<sup>1118</sup>
mutants following waking induced by starvation as was observed
in cyo::fl and per::fl flies. Performance in the APS was evaluated in
w<sup>1118</sup> mutants following 12 h of sleep deprivation and 12 h of
starvation. As seen in Figure 2C, waking induced by sleep
deprivation resulted in a significant reduction in learning while
siblings that experienced a similar amount of waking induced by
starvation performed at baseline levels; the amount of sleep lost
did not differ between sleep deprived and starved flies (p = .23).
Thus, starvation increases waking in wild-type flies and three
independent clock mutants, suggesting that the effects of
starvation are not limited to a specific genotype or genetic
background. Together these data indicate that starvation may be
a practical environmental intervention that can be used to
identify genes underlying sleep homeostasis.

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![Figure 2](https://www.plosbiology.org/article/fetchObject/v1/10.1371/journal.pbio.1000466.g002)

**Figure 2.** Waking induced by starvation does not activate sleep homeostasis in wild-type flies or mutants for Clock. (A) Starvation induces waking in Wild-type Canton-S flies (squares, n = 16) and flies mutant for the canonical clock gene Clock (Clk<sup>CS</sup>, diamonds, n = 16). CS flies were kept on a 12:12 LD schedule and starved for 12 h during the dark period (stippled). Clk<sup>CS</sup> mutants were maintained under DD and starved for 12 h. At the end of the starvation period, flies were then placed back on to normal food into LD or DD, respectively, for recovery (black). Cumulative sleep lost over 48 h was calculated. Data are presented as mean ± SEM. (B) Recovery sleep measured in w<sup>1118</sup> flies following 12 h of sleep deprivation (n = 18) or 12 h of starvation (n = 9) during the dark period. % sleep recovered is calculated for each individual as a ratio of the minutes of sleep gained above baseline during recovery divided by the total min of sleep lost during sleep deprivation. Sleep homeostasis was larger in sleep deprived flies versus their starved siblings (* p = 0.018 by Student’s t test). (C) Starved w<sup>1118</sup> flies (gray bar, n = 8) exhibit similar learning scores after extended waking versus untreated circadian matched controls (white bar, n = 10) while their sleep deprived siblings (black bar, n = 8) display impaired learning; One way ANOVA F<sub>2,23</sub> = 2.1; *p<.05 modified Bonferroni Test.

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cyc01 Flies Have Increased Triglycerides

We have previously shown that while cyc01 flies die from sleep loss in 10 h, they can maintain ~28 h of waking induced by starvation [6]. This result suggested to us the possibility that cyc01 flies might have increased lipid stores. This hypothesis is consistent with recent studies showing that mice mutant for mammalian homologue of cycle, Bmal1−/−, show increased total fat content [47]. Thus, we evaluated lipids in cyc01 mutants under baseline conditions. As seen in Figure 3A–Aii, cyc01 mutants had higher levels of lipid stores in their abdomen compared to genetic background controls, ry506 (Figure 3B–Bii). This included increased lipid droplets in gut epithelial cells (Figure 3Ai versus Bi) and the abdominal fat bodies (Figure 3Aii versus Figure 3Bii). Since the cyc01 mutation was generated in a ry506 background (i.e., its full genotype is cyc01, ry506) [48], ry506 is the appropriate background control for these experiments. To confirm that this phenotype maps to the cyc locus, we crossed cyc01 homozygotes with flies carrying the appropriate deficiency

Figure 3. cyc01 flies have increased adiposity. (A) Representative Oil Red O staining, which stains lipids red, reveals high levels of fat stores in the abdomen of female cyc01 flies. Boxed areas (Ai and Aii) are presented at higher magnification. * denotes developing eggs, and arrowheads are placed in the lumen of the gut and point to gut epithelial cells. Arrows point to the location of the fat bodies which abut the cuticle (9 flies were examined). (B) Representative section of ry506 abdomen, the background control for cyc01. ry506 have little accumulation of lipid droplets in the abdomen in similar areas to (A) as revealed by Oil Red O staining. Boxed areas (Bi and Bii) are presented at higher magnification (9 flies were examined). (C) Representative section of Oil Red O stained cyc01/Df abdomen reveals high levels of fat stores. (Ci and Cii) are presented at higher magnification (9 flies were examined). (D) Organismal triglyceride (TG) levels are elevated in cyc01, cyc01/Df, and cyc01/ry506 flies compared to ry506 controls; one-way ANOVA for Genotype $F_{[4,38]} = 3.2$; *p < .05 modified Bonferroni Test; n = 10 flies/condition, a group was comprised of 10 flies. (E) An elevated homeostatic response is observed in cyc01 mutants that were starved for 7 h (n = 45) on 1% agar containing 25 μM etomoxir. No further increase in homeostasis is found in cyc01 sleep deprived for 7 h on food containing 25 μM etomoxir (n = 72); compare with Figure 1A. Siblings starved in the absence of etomoxir are shown for comparison (n = 92); all experiments were conducted in parallel.

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DJ(3)Lk62/TM6B, Tb. The resulting cyo1/Df transheterozygote flies showed an Oil Red O staining pattern that was qualitatively similar to that seen in cyo1 (Figure 3C–Cii). The increased adiposity in cyo1 mutants was confirmed using biochemical measurements of organismal triglyceride (TG) levels [7]. As seen in Figure 3D, both cyo506 and cyo1/Df show significantly elevated TG levels compared to y506 controls, whereas the cyo1/cyo506 heterozygote displayed an intermediate phenotype. Future experiments will be required to determine whether the observed adiposity is due to a direct effect of the cyo1 mutation on lipid stores or whether the elevated TG levels are an indirect consequence of a disrupted circadian clock.

These observations led us to speculate that starvation might protect flies from sleep loss by diverting lipids towards β-oxidation in mitochondria (Figure S3). To test this hypothesis, we reduced long chain free fatty acid (FFA) entry into the mitochondria by feeding cyo1 mutants the carnitine palmitoyltransferase antagonist, etomoxir [49]. Sleep was not modified in control flies fed 25 μM etomoxir for 7 h either during or after administration (unpublished data). Thus, cyo1 flies were fed etomoxir for 7 h during sleep deprivation or 7 h during starvation and then placed back onto their normal food. Etoximox administration during sleep deprivation did not alter the size of the subsequent homeostatic response (Figure 3E). However, starved cyo1 flies fed etomoxir showed a dramatic increase in sleep homeostasis, which resembled that normally seen after sleep deprivation (Figure 3E). Thus, the mobilization of FFAs may play a role in sleep homeostasis.

The Adipose Triglyceride Lipase Homologue, Brummer, Modulates Sleep Homeostasis

A Drosophila mutant, bmm1, has recently been described, which has large triglyceride stores and is resistant to starvation due to its inability to efficiently liberate FFA from triglyceride stores. To determine whether transcript levels of bmm are responsive to either sleep deprivation or starvation, we evaluated relative changes of bmm mRNA from sleep deprived and starved cyo1 mutants. As seen in Figure 4A, sleep deprived cyo1 flies that display a large sleep rebound also exhibit a >5-fold increase in bmm transcript levels. However, bmm transcripts were increased by <2-fold in cyo1 flies following a similar amount of waking induced by starvation. Together these data suggest that bmm may play a role in modulating the response to extended waking. To test this hypothesis, we obtained a deletion mutant, bmm1, and its genetic background control (bmm00). Before evaluating sleep, we wished to confirm that the previously reported lipid phenotype was present in flies maintained under our dietary conditions. Evaluation of organismal TG levels established that bmm1 mutants exhibited increased TG in their hands; 58±6 μg TG/mg fly; n = 5 groups of 10 compared to their genetic background controls bmm00 = 18±2 μg TG/mg fly; n = 4 groups of 10 (p < 0.001 by Student’s t test). Although Gronke and colleagues reported increased fat stores in the abdominal fat body, the effects of the bmm1 deletion on the head fat body were not evaluated. As seen in Figure 4B,C, Oil Red O staining of lipids reveals that bmm1 mutants show lipid droplets that are so large that they are hard to discriminate while lipid droplets in the heads of genetic controls (bmm00) are small and well defined. Thus bmm1 mutants, like cyo1 mutants, display increased adiposity.

Although bmm1 mutants display increased lipids, their baseline sleep parameters were not severely altered (see Table S2 for baseline sleep characteristics). However, bmm1 mutants had a large sleep rebound following 12 h of sleep deprivation compared to their background controls, bmm00 (Figure 4D). To determine whether the large sleep rebound in bmm1 mutants was due to an elevated sleep drive, we evaluated Amylase mRNA levels following sleep deprivation. As seen in Figure 4E, sleep deprived bmm1 mutant exhibit an elevation in Amylase mRNA while their genetic background controls, bmm00, showed a less dramatic increase in Amylase mRNA. Given that Amylase levels are not induced by either paraquat [30] or starvation (Figure 1B) and are not a necessary response to sleep deprivation (see below), the increase in Amylase seen in bmm1 is unlikely due to increased sensitivity to stress. Finally, we conducted a rescue experiment to re-introduce wild-type bmm (bmm+) into an otherwise bmm1 mutant fly. Since lipases are likely to be ubiquitously expressed, we drove bmm+ using an Act-GAL4 driver. As seen in Figure 4F, no sleep rebound was observed following 12 h of sleep deprivation in the rescue line (Act-GAL4/UAS-bmm+/; bmm1/bmm1) while both parental lines (Act-GAL4/+; bmm1/bmm1 and UAS-bmm+/; bmm1/bmm1) displayed a robust homeostatic response (Figure 4F). Together, these data indicate that bmm can influence the response to sleep deprivation.

Lsd2 Regulates Sleep Homeostasis and Prevents Learning Impairments Following Sleep Loss

Lsd2 is a lipid droplet associated protein with peroxin/DRP7/TIP47 domain (PAT). PAT proteins regulate lipolysis by either blocking lipase access to droplets and by promoting access when phosphorylated [50]. Thus while Lsd2 mutants may release and re-esterify fatty acids, they would also be expected to show reduced lipolysis upon stimulation. Interestingly, Lsd2 mutants (Lsd251) display lower levels of TG while bmm mutants have higher levels of TG than their respective background controls [29]. This relationship between Lsd2 and bmm makes it of particular interest for further investigation. Indeed, whereas bmm1 mutants are fat and readily survive starvation, Lsd251 are lean and die rapidly when starved [28,29]. Together with the observation that loss-of-function mutants for Lsd2 exhibit phenotypes that share aspects with starvation, we hypothesized that normally fed sleep-deprived Lsd2 mutants would behave as starved flies and would not compensate for lost sleep with a subsequent sleep rebound. To begin, we confirmed that Lsd251 mutants were lean (Lsd251 = 24±2 μg TG/mg fly; n = 4 groups of 10) compared to their genetic controls in which the P-element had been excised (Lsd200 = 34±6 μg TG/mg fly; n = 4 groups of 10; p = 0.014 by Student’s t test) [29,31]. In addition, we evaluated lipids in the head fat body. Although not as dramatic as the change seen in the head of bmm1 mutants, lipid droplets were qualitatively smaller in Lsd251 mutants compared to genetic background controls, Lsd200 (Figure 5A,B). As predicted, Lsd251 flies did not compensate for lost sleep with a significant increase in sleep over baseline during 48 h recovery from sleep deprivation while genetic controls showed a sleep rebound during this time (Figure 5C). Importantly, Lsd251 flies did not respond to sleep deprivation with an increase in Amylase mRNA levels (Figure 5D), suggesting that they were not sleepy. These data are consistent with our previous results demonstrating that, in flies, Amylase levels are responsive to conditions of high sleep drive, do not depend upon the method used to keep the animal awake, and are not simply activated by stress. Thus, Lsd251 flies showed opposite phenotypes to those seen in bmm1 mutants as measured by lower TG stores, no sleep rebound, and no induction of Amylase after sleep deprivation.

As mentioned above, the failure to observe a sleep rebound may simply reflect a physiological impairment that globally disrupts several regulatory processes, including sleep homeostasis. If the genetic lesion associated with Lsd2 simply disrupts the ability to initiate a homeostatic response, then Lsd2 flies should be learning impaired following sleep deprivation. However, if disrupting Lsd2 protects a fly from sleep loss, they should learn following sleep deprivation. A direct test of this hypothesis is achieved within a genotype by determining if performance is reduced following sleep deprivation in comparison with untreated siblings [33]. If a fly is
learning impaired following sleep loss, they are considered wild-type. With this in mind, we evaluated learning in Lsd251 mutants following 12 h of sleep deprivation using APS. As seen in Figure 5E, Lsd251 mutants maintained normal levels of learning even after being kept awake for 12 h and thus do not display a wild-type response. In contrast, their genetic background controls, Lsd2rev, responded to 12 h of sleep deprivation with a significant reduction in learning (Figure 5F) and are considered to have a wild-type response to sleep loss. It is worth noting that while the performance in Lsd251 flies appears to be slightly lower than that observed in other lines, including genetic controls, the learning scores are well within the range observed for wild-type flies [5]. Moreover, flies that obtain similar performance levels can achieve lower learning scores following sleep deprivation [33]. Thus Lsd2251 mutants phenocopy starvation as measured by their ability to withstand waking without initiating a homeostatic response or becoming learning impaired.

**Discussion**

We have developed a novel strategy to identify pathways involved in sleep homeostasis. This strategy takes advantage of the
Figure 5. Lsd2 mutant flies mimic the phenotypes seen in starved cye<sup>29</sup> flies. (A) A representative section of Lsd2<sup>2wv</sup> head stained with Oil Red O. Head fat bodies are denoted by arrowheads. (B) A representative section of Lsd2<sup>2</sup> head stained with Oil Red O. (C) Lsd2<sup>2</sup> mutants (n = 22) do not exhibit a homeostatic response following 12 h of sleep deprivation while their genetic background control, Lsd2<sup>2wv</sup> (n = 41), do exhibit a sleep rebound following sleep loss. *p = 0.012; Student’s t test. (D) Amylase mRNA levels of Lsd2<sup>51</sup> mutants are significantly lower than genetic background controls, Lsd2<sup>2</sup>, following 12 h of sleep deprivation. Data are presented as percentage from untreated controls (2 replicates of n = 20 heads/group). *p < 0.05, Student’s t test. (E) Learning is not impaired in Lsd2<sup>2</sup> mutants (n = 7) following 12 h of sleep deprivation compared to untreated controls (n = 8); p = 0.37 Student’s t test. (F) Learning is impaired in Lsd2<sup>2wv</sup> flies (n = 15) following 12 h of sleep deprivation compared to untreated controls (n = 15); * p = 0.036 Student’s t test. doi:10.1371/journal.pbio.1000466.g005

observation that many behaviors are influenced by interactions between genes and the environment [6,14–19]. We chose to examine starvation because it is common in nature and therefore the response to the absence of food is likely to be evolutionarily conserved. More importantly, starvation is a simple manipulation that can be readily placed under experimental control. We report that starvation induces episodes of waking that are not compensated for by a sleep rebound and do not result in learning deficits. Based upon these results, we then evaluated two genes, brummer (bmm) and Lsd2, which have been shown to modulate the response to starvation [28,29]. bmm<sup>1</sup> mutants, which have increased lipid stores, display an exaggerated sleep rebound. In contrast, mutants for Lsd2, which has been reported to mimic some aspects of starvation, are able to withstand the negative effects of waking without compensating for lost sleep or exhibiting the learning deficits that are typically observed after 12 h of sleep deprivation. These data suggest that proper lipid handling is important for modulating an organism’s response to sleep loss. Although the precise mechanisms by which these genes alter sleep regulation remains to be determined, these data represent a first step in the molecular dissection of sleep homeostasis.

It is interesting to note that gene profiling studies in several species have consistently identified genes involved in metabolism as being modified by behavioral state [7,9,10,52,53]. Indeed, the first gene found to be modified by behavioral state in flies was fatty acid synthase [44]. Although many of the specific genes are not identical across studies, it is important to recognize that the categories and pathways are consistent, thereby reinforcing the view that sleep regulatory pathways and lipid metabolism are intimately involved. The impact of sleep deficits on metabolism is now well documented [3,54]. In humans, sleep deficits are known to result in metabolic disruption and increased adiposity [1,55]. Similarly, long-term chronic total-sleep deprivation in rodents is also associated with severe metabolic disruption [56]. Thus while our data confirm previous observations that sleep loss activates metabolic genes, we also present data demonstrating that metabolic genes, in turn, can influence sleep regulatory centers as measured by sleep homeostasis. Together these data imply a bidirectional relationship between sleep and metabolism.

It should be noted that lipids are not just a source of energy but are important modulators of cell signaling, gene transcription, metabolism, and appetite [57]. They modify the functional responses of ion channels, synaptic function, and cellular signaling cascades [58,59]. Lipids also activate G-Protein coupled receptors suggesting that they have an extracellular mode of action [60]. Determining which lipid is able to influence sleep homeostasis is a considerable challenge that cannot be solved using genetic strategies alone. Thus, while our genetic studies have identified important lipid metabolism pathways, additional work will be required to fully elucidate the precise molecular mechanisms that impact the sleep regulatory centers. It is highly likely that future studies will turn to lipidomic analysis. The genes and genetic tools we have identified here may be particularly useful in guiding future lipidomic studies.

We began by contrasting waking induced by sleep deprivation with waking induced by starvation. Interestingly the mutant Lsd2<sup>51</sup>, which phenocopies aspects of starvation as measured by low triglyceride stores [28,29], also phenocopies starvation at the behavioral level. That is, Lsd2<sup>51</sup> mutants can withstand 12 h of sleep deprivation without exhibiting any evidence of a compensatory sleep rebound as is seen with starved flies. It is unlikely that mutations in Lsd2 disrupted the ability of the fly to recover needed sleep since they did not appear sleepy as measured by Amylase mRNA. This interpretation is bolstered by the observation that 12 h of waking in Lsd2<sup>51</sup> mutants did not result in learning impairments. Learning impairments are a robust consequence of sleep deprivation in mammals and in flies [4,5,37]. We have previously shown that neither Amylase mRNA levels nor learning impairments can be explained by the method used to keep the animals awake or stress [30]. The observations obtained in both Lsd2<sup>51</sup> mutants and starved flies provide additional confirmation of these conclusions. Moreover, these results emphasize the utility in evaluating Amylase and learning in addition to sleep homeostasis when interpreting results from genetic studies. Given that sleep homeostasis, Amylase, and learning all suggest that Lsd2 mutants are resilient in the face of sleep loss, understanding the underlying mechanisms may have clinical utility.

At this stage, most of our knowledge about the role of lipid regulation, in general, and Perilipin, in particular, has been derived from mammalian studies, although great strides are being made with Drosophila [28,29,51,61–63]. The protein product of Perilipin, the mammalian homolog of Lsd2, surrounds the lipid droplet, thereby preventing access of lipases to the TGs. In addition, Perilipin is able to sequester proteins that activate lipolysis [50]. Mice lacking a functional Perilipin gene (PLIN<sup>−/−</sup>) display higher levels of basal lipolysis in white adipose tissue (WAT). However, PLIN<sup>−/−</sup> mice do not show the typical increase in lipolysis upon β-adrenergic receptor stimulation [64,65]. In contrast, mice lacking a functional Adipose triglyceride lipase gene (Atg<sup>−/−</sup>), the mammalian homolog of bmm, have decreased basal

PLoS Biology | www.plosbiology.org 8 August 2010 | Volume 8 | Issue 8 | e1000466
lipolysis. Yet like PLIN<sup>−/−</sup> mice, Atgl<sup>−/−</sup> do not increase lipolysis when stimulated by a β-adrenergic agonist; Atgl<sup>−/−</sup> mice also show reduced lipolysis when stimulated by starvation or cold-stress [66]. These data suggest the possibility that deficits in Perilipin may protect against the negative effects of waking, in part, via a sustained release of FFAs. In any event, future studies will be needed to determine whether the response to sleep deprivation observed in bnn<sup>−/−</sup> and Lsd2<sup>−/−</sup> mutant flies will be observed in Perilipin and Atgl null mutant mice.

Although the mechanisms underlying sleep homeostasis are largely unknown, adenosine has been implicated as playing a role observed in needed to determine whether the response to sleep deprivation or conditionally knocking out the gene largely unknown, adenosine has been implicated as playing a role observed in needed to determine whether the response to sleep deprivation observed in bnn<sup>−/−</sup> and Lsd2<sup>−/−</sup> mutant flies will be observed in Perilipin and Atgl null mutant mice.

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Materials and Methods

Flies and Husbandry

Flies were reared in standard laboratory conditions, 12:12 light:dark schedule, standard food (yeast, sucrose, corn syrup, molasses, and agar), 25°C, and 50% humidity. The cycle<sup>−/−</sup> (cy<sup>−/−</sup>) and period<sup>−/−</sup> (per<sup>−/−</sup>) mutant flies were obtained from Dr. Jeff Hall [48]. This mutation was originally generated in a ry<sup>506</sup> background (i.e., the full genotype would be +;+;cy<sup>−/−</sup>, ry<sup>506</sup>), thus ry<sup>506</sup> was used as its background control [48]. Actin-GAL4/CyO (Act-GAL4), ry506 (ry<sup>506</sup>), and Df(3L)kto2/TMB,Tb1 were obtained from the Bloomington Stock Center (Bloomington, Indiana). The null mutation for brummer (bnn<sup>−/−</sup>) and the background control (bnn<sup>+/−</sup>) as well as the Lsd2 mutant (Lsd2<sup>−/−</sup>) and its background control (Lsd2<sup>+/−</sup>) were obtained as a generous gift from Dr. Ronald Kuhnlein.

Sleep Recording

Three-day-old flies were placed into 65 mm glass tubes containing standard lab food and monitored with the Trikinetics activity-monitoring system (Waltham, MA) as previously described [6,44]. Briefly, activity was recorded in 1 min bins and episodes of quiescence ≤5 min were considered sleep. Total sleep time, sleep architecture, and sleep homeostasis were calculated using an in-house program according to criteria previously established [6,44,83].

Sleep Deprivation

Flies were reared using the sleep-nullifying apparatus (SNAP), which asymmetrically tilted −60° to +60° such that the sleeping flies were displaced during the downward movement 6 times/minute [6,44]. Flies were deprived of sleep for 12 h between ZT12 (lights out) to ZT0 (lights on) at which point flies were released into recovery where they remained unperturbed for 48 h. The clock mutants cy<sup>−/−</sup> and per<sup>−/−</sup> were maintained and sleep deprived under constant darkness; sleep deprivation occurred for 7 h during the day between ZT0 and CT12. Sleep homeostasis was calculated for each individual as a ratio of the minutes of sleep gained above baseline during recovery divided by the total minutes of sleep lost during sleep deprivation (min gained/min lost). Cumulative difference plots were calculated for each individual fly first by subtracting the minutes of sleep during deprivation and recovery from the corresponding baseline value and summing the difference score with the preceding hour. A negative slope indicates that sleep is being lost; a positive slope indicates sleep gained; and a slope of zero indicates that recovery is complete.

Starvation and Ectomoxir

Starvation is operationally defined as a condition in which the animal has no access to food and during which energy intake drops below levels that the animals would normally experience at that time. Flies were placed into Trikinetics tubes containing 1% agar solution and then switched back to their normal food at the end of the starvation period. For all genotypes, starvation was carried out in constant darkness at the same time, for the same duration, and under the same conditions as for sleep deprived flies. Durations for starvation and sleep deprivation were based on Figure S2 and [6].
For the clock mutants, *cyo*¹ and *per*¹°, a 7 h treatment was chosen because it maximized the difference in behavioral responses to sleep deprivation and starvation but did not result in lethality. CS flies were housed under DD for 3 d. On day 4, starvation was carried out for 12 h during the primary sleep period. The primary sleep period was identified from the previous days’ data based upon the average time that the CS flies initiated their longest sleep bout. 2 1/2 experiments were carried out under LD conditions. Flies were transferred to starvation media prior to lights out, where they remained for the 12 h dark period. The following morning, flies were placed back on to normal food to evaluate sleep homeostasis or their performance was evaluated in the APS. For etomoxir experiments, flies were placed into tubes containing either 1% agar or standard laboratory food with a final concentration of 25 μM etomoxir. At the end of the manipulation, flies were placed back on to standard laboratory food without etomoxir.

**Learning Assay**

The learning paradigm requires flies to inhibit a prepotent attraction towards light and has been previously described [32]. Both dark and lighted vials are covered with filter paper. The filter paper in the lighted vial is wetted with 320 μl of a 10⁻¹ M Quinine hydrochloride solution (Sigma, St. Louis, MO). After entering the dark or lighted vial, the choice is recorded and the fly is quickly removed from the vial and placed back at the entrance of the maze. The number of times the fly enters the dark vial is tabulated during 4 blocks of 4 trials. During the test, the light and quinine/humidity appear equally on both the right and left. For an experiment, learning was evaluated by the same experimenter who was blind to genotype and condition. Unless otherwise stated, all flies were tested in the morning between ZT0 and ZT4. For sleep deprived and starved flies, they remained in their respective conditions until tested. Learning scores are normally distributed and are analyzed by a signed rank test or analyses of variance (ANOVAs), which were followed by a modified Bonferroni test; unless stated otherwise, all experiments are n≥7.

**Photosensitivity**

Photosensitivity was evaluated in the T-maze over 10 trials in the absence of filter paper. The lightened and darkened chambers appeared equally on both the left and right. P1 is the average of the scores obtained for 5–6 flies ± SEM.

**Quinine/Humidity Sensitivity**

Five flies were individually placed at the bottom of a 14 cm cylindrical tube (Becton-Dickson, Franklin Lakes, NJ), which was uniformly lighted. Each half of the apparatus contained separate pieces of filter paper, which could be wetted with quinine or kept dry. The QSI was determined by calculating the time that the fly spent on the dry side of the tube when the other side had been wetted with quinine, during a 5 min period.

**QPCR**

Total RNA was isolated from 20 fly heads with Trizol (Invitrogen, Carlsbad, CA) and DNase I digested. In the case of whole flies, 3–5 flies were frozen and homogenized. cDNA synthesis was performed in triplicate using Superscript III (Invitrogen, Carlsbad, CA), according to manufacturer protocol. In order to evaluate the efficiency of each reverse transcription, equal amounts of cDNA were used as a starting material to amplify RP49 as previously described [6]. cDNA from comparable reverse transcription reactions were pooled and used as a starting material to run three QPCR replicates. Expression values for RP49 were used to normalize results between groups. For flies maintained on an LD schedule, both experimental and untreated controls were collected at the exact same circadian time ZT0-1. For clock mutants, the control, sleep deprivation, and starvation experiments were run in parallel and the flies were collected at the same time.

**Triglyceride Measurements**

For each genotype, 10 female flies were frozen and stored at ~80°C. Lipid measurements were carried out at the Clinical Nutrition Research Unit at Washington University. Flies were weighed and homogenized in a 2:1 (methanol:chloroform) solution to extract the lipids [84]. The MeOH:chloroform is evaporated using the speed vac, and the lipids were re-suspended in the starting reagent for Infinity (ThermoElectron, Waltham, MA) triglyceride reagent and triglyceride levels detected using the colorometric detection according to the manufacturer’s specifications. Lipid levels are quantified using a standard curve of known triglyceride run in parallel.

**Oil Red O Staining**

Flies were immobilized with CO₂, submerged in Optimal Cutting Temperature (Tissue-Tek, Torrance, CA) and frozen on dry ice. 12–15 μm frozen sections were collected on Histobond slides (VWR, West Chester, PA). Sections were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and subsequently rinsed in PBS. Slides were then rinsed in 60% isopropanol for 5 min. The solution was changed to Oil Red O stain (Sigma, St. Louis, MO) in 60% isopropanol. Sections were stained for 5–10 min. Slides were rinsed several times in distilled water to get rid of excess stain. Slides were dried and mounted using Glycergel (Wako, Carpinteria, CA). Brightfield Images were taken on a Nikon Eclipse 80i microscope (Belmont, CA) using a Micropublisher 5.0 RTV camera (Q imaging, Surrey, British Columbia, Canada) and visualized with the software package Metamorph (Universal Imaging, Downingtown, PA). Images were optimized for visualization and publication using Adobe Photoshop (Adobe, San Jose, CA).

**Feeding Assay**

Flies were transferred onto normal food with 1% (v/v) blue food dye (F D & C Blue Dye no. 1, Durekce). At the end of the measurement period, flies were anesthetized by CO₂ and the appearance of blue dye in the abdomen through the cuticle was evaluated by an observer blinded to conditions using a Vista Vision dissecting microscope (VWR, West Chester, PA).

Spectrophotometric measurement of feeding was based on [43,85,86]. Flies from the visual confirmation of blue dye were frozen on dry ice. Heads were then removed to prevent eye pigment from interfering with the absorbance spectrum of the dye. Fly bodies were homogenized in 200 μL PBS buffer and centrifuged (13,000 rpm) for 25 min. The supernatants were transferred to a new tube, again centrifuged at 13,000 rpm for 25 min, and absorbance was measured at 625 nm. Absorbance per fly was determined by taking the total absorbance from the group and dividing it by the total number of flies, then subtracting the absorbance per fly from control flies fed non-dyed normal food to give a final absorbance reading per fly.

**Supporting Information**

**Figure S1 Flies respond rapidly to starvation.** (A) Although *cyo*¹° flies sleep normally during the 30 min prior to...
starvation, once starvation begins no cyc01 fly is quiescent for ≥5 min (paired t test, $p = 4.91 \times 10^{-9}$, $n = 3$). Inset: % of flies sleeping during 30 min of baseline and 30 min of starvation. (B) Counts/waking minute are significantly elevated in cyc01 flies during the first 30 min of starvation compared to waking activity in the preceding 30 min (paired t test, $p = 6.36 \times 10^{-8}$, $n = 3$). Inset: % of flies that display an increase in counts/waking minute compared to baseline.

Found at: doi:10.1371/journal.pbio.1000466.s001 (0.46 MB TIF)

Figure S2 Starvation reduces the cost of waking for a short time. Sleep homeostasis was assessed after 7, 14, and 21 h of extended waking induced by starvation in cyc01 flies. At time 0, flies were moved from standard laboratory food to agar and water. After the designated starvation period, flies were placed back on standard food for recovery. For cumulative sleep lost then gained, a negative slope indicates sleep lost, a positive slope indicates sleep gained; when the slope is zero, recovery is complete. After 7 and 14 h of starvation, no rebound was observed. In contrast, a rebound was observed after 21 h of waking induced by starvation.

Found at: doi:10.1371/journal.pbio.1000466.s002 (0.51 MB TIF)

Table S1 Control metrics for the APS. Control metrics consisting of: Phototaxis index (PI) and Quinine Sensitivity Index (QSI) for each experiment are within a previously observed range of scores that sustain normal learning [5,33]. No statistically significant differences were found for QSI and PI. Since we have previously shown that neither PI nor QSI are modified by sleep deprivation [5] and that even large changes to PI do not impede the identification of learning impairments following sleep deprivation [33], we only show PI and QSI for starved flies, not sleep deprived flies. The failure to observe changes in TCT in learning-impairde sleep deprived cyc01 flies and starved cyc01 flies that learn is consistent with our previous report that TCT provides no explanatory value for predicting the final performance index, and thus we do not report TCT for other genotypes. * values are shown in [5].

Found at: doi:10.1371/journal.pbio.1000466.s003 (0.50 MB TIF)

Table S2 Sleep parameters for flies used for sleep homeostasis. Baseline sleep characteristics. Total sleep time (TST), daytime sleep (DTS), nighttime sleep (NTS), latency to the first sleep bout after lights off (latency), average sleep bout duration at night (NBD), and the number of flies evaluated for each genotype (n). All data are presented in minutes and represent mean ± SEM. With the exception of the values in bold-type font, sleep parameters fall well within the normal variability found in wild-type populations of flies [93]. The sleep characteristics shown are intended to be descriptive and thus to simplify the presentation of these data; no statistical comparisons are presented.

Found at: doi:10.1371/journal.pbio.1000466.s004 (0.02 MB XLS)

Video S1 Fly behavior after waking induced by starvation and sleep deprivation. Three minutes of behavior for flies after 7 h of starvation (left, $n = 4$), 7 h of sleep deprivation (right, $n = 4$), or untreated controls (middle, $n = 4$). cyc01 siblings were maintained in constant darkness prior to recording, which subsequently took place in the light. Flies were removed from their respective conditions, placed into Trikinetics tubes containing blue dye, allowed to acclimate for a few minutes, and were then recorded for 3 min. The video has been compressed to conserve space; however, the location of the fly in proximity to the food can be assessed. Given the transfer of flies to new tubes, the amount of sleep is relatively low. The flies depicted are a subset of flies that were used to quantify food intake for the first hour immediately after sleep deprivation and starvation. After an hour on blue dye, 13/13 flies that had been sleep deprived showed blue dye in their abdomen compared to 14/14 untreated controls. Spectrophotometric data confirmed these results (absorbance at 625 nm: 8.57 ± 10−3/fly and 11.57 ± 10−3/fly, respectively). Moreover, flies that were starved for 7 h and placed onto food containing blue dye displayed results similar to that seen in untreated controls (14/14 with blue dye and absorbance at 625 nm: 12.89 ± 10−3/fly). Given the similarities in feeding behavior, it is unlikely that food intake post-treatment is responsible for ameliorating the negative effects of waking.

Found at: doi:10.1371/journal.pbio.1000466.s005 (0.02 MB XLS)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: MST PJS. Performed the experiments: MST VS LS LG PJS. Analyzed the data: MST VS LS LG PJS. Wrote the paper: MST PJS.

References


