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Blood-derived dietary protein promotes sleep in the mosquito *Aedes aegypti*

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eLife Assessment

This **important** study links blood-derived dietary content to sustained increases in sleep in the mosquito *Aedes aegypti*. Using multiple independent approaches, the authors provide **convincing** evidence for blood-induced changes in sleep. These findings have broad implications for understanding how specialized diets regulate sleep across species and for mosquito vector biology.

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Abstract

Sleep is a ubiquitous, yet highly variable, behavior across species. The duration and timing of sleep are influenced by ecological demands and dietary context. In the mosquito *Aedes aegypti*, a blood-feeding insect with specialized nutritional requirements, the relationship between feeding and sleep remains poorly understood. Here, we investigate how blood-derived dietary protein influences sleep regulation. Using postural analysis, videography, and arousal-threshold assays, we establish that immobility bouts of ≥ 10 minutes reliably define sleep in *Ae. aegypti*. Infrared activity monitoring revealed that blood-fed females exhibit a marked increase in sleep beginning immediately after feeding and persisting for several days, accompanied by reduced locomotor activity. Notably, this sleep elevation lasts well beyond the cessation of host-seeking behavior, suggesting distinct phases of opportunistic versus determined host pursuit. To determine the dietary basis of this effect, we tested mosquitoes fed a bovine serum albumin (BSA)-based diet. BSA feeding alone was sufficient to mimic the sleep-promoting and hypoactive effects of blood, identifying dietary protein as the key nutritional regulator. Moreover, RNAi-mediated knockdown of the leucokinin receptor (lkr), previously implicated in fluid regulation and feeding, enhanced sleep and reduced activity, implicating LK signaling in postprandial sleep modulation. Together, these findings demonstrate that blood-derived proteins drive sustained increases in sleep and reduced locomotion in *Ae. aegypti*. This work positions *Ae. aegypti* as a model for dissecting nutrient-specific regulation of sleep and highlights potential adaptive functions of protein-induced quiescence, such as energy conservation and predator avoidance. More broadly, it provides insight into how specialized diets shape the neural and behavioral architecture of sleep.

Introduction

Sleep duration and timing varies significantly across the animal kingdom [1]. In diverse species, sleep is modified by an animal's internal and external environment, including social cues, early-life development, stress, and food availability [2,3]. Moreover, evolutionary pressures have shaped species-specific sleep strategies, balancing the restorative benefits of sleep with ecological demands such as predation risk, foraging requirements, and reproductive behaviors [4]. While the evolutionary basis for interspecies variation in sleep remains poorly understood, there is mounting evidence that context-dependent regulation of sleep is adaptive [5,6]. Understanding

how these factors interact to regulate sleep not only provides insight into the adaptive significance of sleep but also informs broader questions about the links between sleep, health, and survival across taxa.

Food availability appears to be a potent regulator of sleep [7]. Many species of animals have been shown to forgo sleep to forage under food-deprived conditions [3]. Conversely, flies, fish, and mammals all display post-prandial sleep, demonstrating that sleep is bidirectionally modified by feeding state [8–10]. In insects, both total caloric content and specific dietary nutrients have been shown to influence sleep [11–13]. While this phenomenon is highly conserved, most studies have been performed in *Drosophila*, a dietary generalist, and far less is known about how diet contributes to sleep regulation in animals that consume more specialized diets [14].

Blood-feeding insects provide a unique opportunity to investigate the effects of diet on behavior [15]. Their obligate dependence on vertebrate blood imposes distinct nutritional constraints that differ markedly from the diets of generalist feeders. In addition, the dramatic physiological changes that occur following a blood meal, such as gut distension and shifts in metabolic state, are likely to influence neural circuits that govern sleep and activity. The mosquito, *Aedes aegypti*, is a model for understanding the effects of blood feeding on behavior [16,17]. These insects rely exclusively on blood to complete their reproductive cycle, making them an ideal system to study the behavioral consequences of this specialized diet. While sleep has been described in this system, the effects of blood feeding on sleep regulation have not been investigated [18,19].

There is evidence to suggest that blood feeding and dietary protein influence sleep in *Ae. aegypti*. Following a blood meal, females suppress both host seeking and feeding for several days [20]. Moreover, an artificial protein-based diet composed of γ -globulins, hemoglobin, and albumin is sufficient to stimulate egg production [21], revealing dietary protein as a critical factor in reproduction and in the suppression of feeding. This suggests that dietary proteins, rather than other components of blood, underlie these behavioral shifts. Nevertheless, the specific amino acids that mediate the regulation of reproductive and feeding behaviors remain unidentified. Examining sleep across different feeding conditions therefore provides an opportunity to determine which dietary macronutrients regulate sleep in *Ae. aegypti* and to test whether these macronutrients overlap with those that regulate other blood-feeding-associated behaviors.

Here, we systematically defined sleep behavior in mosquitoes and tested the effects of blood feeding and dietary protein on sleep. We found that both blood meals and a bovine serum albumin (BSA)-based protein diet increase sleep for several days. Critically, we found that the period of increased sleep is substantially longer than the cessation of host seeking, suggesting the existence of discreet opportunistic vs determined host-seeking phases. These findings establish *Ae. aegypti* as a model for investigating the effects of diet on sleep regulation and imply that methods to disrupt mosquito host-seeking must consider both of these behavioral systems.

Results

Sleep has previously been defined as inactivity bouts that are greater than two hours based on postural analysis [19]. We sought to further define periods of immobility used to define sleep using multiple established criteria including arousal threshold, posture, and circadian regulation [22]. To identify species-specific postures during the day and night, individual mated female mosquitos were recorded at high resolution. Key postural differences were identified with inactive and active mosquitoes that were consistent with those previously used to define sleep [19]. Shortly following activity, the hind legs extend upward while the proboscis points downward, often contacting the ground revealing a posture associated with waking activity (Fig. 1A–B [23]). Following immobility lasting on average 20 min during the day, and 11 min in the evening, the hind legs are in contact with the tube and the proboscis becomes elevated (Fig. 1C–E [24]). These findings suggest a shorter duration of immobility is associated with sleep than has previously been reported.

To define further the period of immobility associated with sleep in *Ae. aegypti* we tested mosquitoes in the *Drosophila* ARousal Threshold (DART) that has been widely used to measure sleep duration and intensity in flies (Fig 1F [25]) [23–25]. The responsiveness to stimuli was

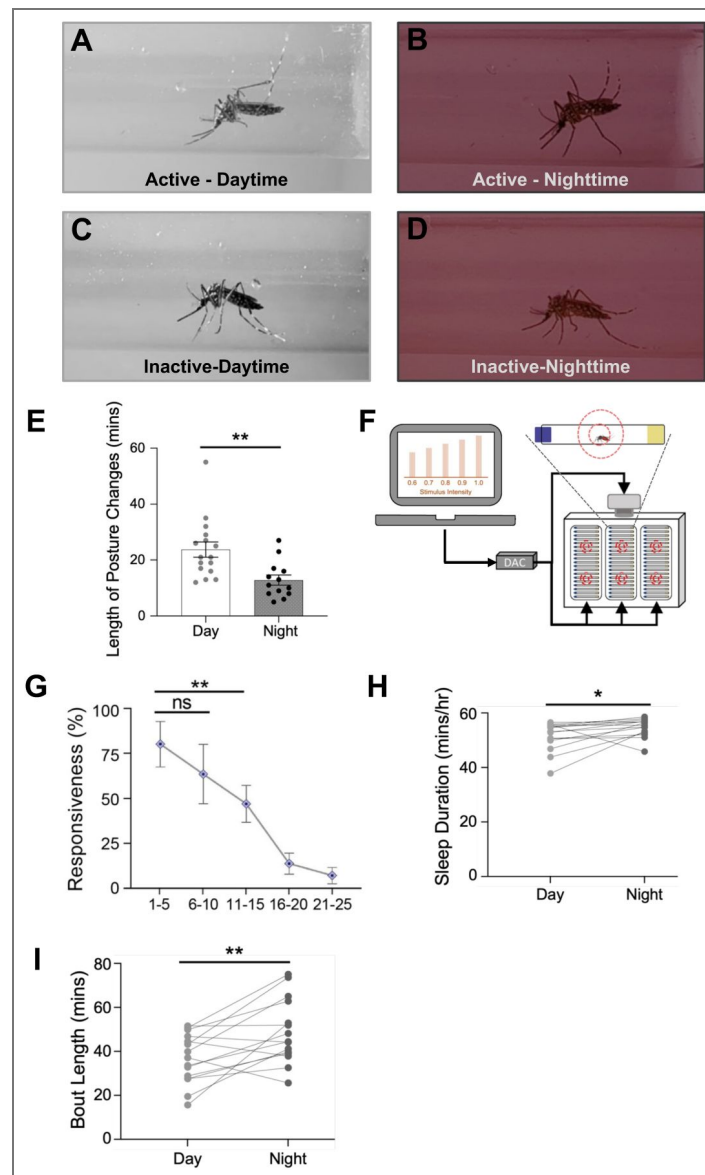


Figure 1. Characterization of sleep and arousal threshold in female *Ae. aegypti*.

(A–D) Representative images of adult female *Ae. aegypti* either in active state or during sleeping. (E) Quantification of sleep onset latency in mosquitoes using postural recordings from an iPhone 16 Pro. Sleep onset latency was significantly longer during the day than at night (ANOVA with Mann-Whitney test, $F_{1,12}=7.792$, $P=0.0013$). Individual data points are shown as gray (day) and black (night) dots. (F–I) A modified version of *Drosophila* Arousal Tracking (DART) system was used to assess sleep responsiveness in mosquitoes. The setup records mosquito movements while simultaneously delivering mechanical stimuli through a digital analog converter (DAC). All measurements were taken from sleeping mosquitoes and recorded hourly, beginning at ZT0. (F) Schematic of the vibratory stimulus setup used to assess percent of sleep responsiveness. (G) Arousal threshold measurements in the DART system. The response probability was greater for animals that had been inactive for 1–9 minutes than that had been inactive for (≥ 10 minutes) One-way ANOVA $F_{4,145}=6.817$, (1-5) vs. (6-10) $P=0.2460$; (1-5) vs. (11-15) $P=0.0051$, $N=14$. (H–I) Individual mosquito trajectories showing increased sleep duration (H, paired t test, $P=0.0478$) and sleep bout length (I, paired t test, $P=0.0041$). Error bars represent the standard error of the mean ($\pm$ SEM). Here and in subsequent figures, asterisks indicate the level of significance: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

significantly reduced at periods of 10 minutes of immobility or longer (Fig 1G). Sensory responsiveness decreased further for bout lengths greater than 15 min, raising the possibility of light and deep sleep (Fig 1G). Together, these findings suggest that bout lengths of 10 min or longer can be used to define sleep.

Using the definition of 10 min of immobility to define sleep, we then sought to determine whether sleep is under circadian regulation. Periods of immobility were quantified using videography from the DART system. Both total sleep duration, and the average length of each bout were greater in the nighttime as compared to the daytime (Fig 1H-I). Therefore, behavioral posture, and arousal threshold, and circadian regulation support a 10 min immobility threshold in *Ae. aegypti*.

We applied infrared-based activity monitors that have been widely used to measure sleep in *Drosophila*, as well as *Ae. aegypti*, to analysis sleep [19,26]. To determine if diet impacts sleep, we compared differences between sugar or blood fed females. Female mosquitoes aged 3-5 days after eclosion were blood-fed with defibrinated sheep blood, and after three days were allowed to lay eggs (Fig 2A). We focused on the period after oviposition as we were primarily interested in behavioral differences induced by blood feeding that were independent of host-seeking, which is restored by 72hr after feeding [27]. Following oviposition, mosquitoes were lightly anesthetized on ice and loaded into custom-building infrared-based activity monitors, like those regularly used to measure sleep and activity in fruit flies. A sleep profile beginning on day 4 following feeding reveals persistently elevated sleep for blood-fed mosquitoes across the day and night periods (Fig 2B and Fig 2C). Analysis over time reveals that sleep was significantly elevated in blood-fed mosquitos for Days 4 and 5, before returning to sugar-fed levels (Fig 2D). We applied a Markov model that determines propensity to remain asleep, which serves as an indicator of sleep depth [28]. On Day 4 following blood feeding, mosquitoes displayed reduced wake propensity and increased sleep propensity (Fig 2E,F). These findings suggest blood feeding induces long-lasting increases in sleep that extend far beyond the window where host responsiveness/seeking is restored.

To determine whether diet also influences activity, we normalized activity to account for sleep differences between conditions, as has previously been described in fruit flies [29]. Validated the increased sleep in blood fed mosquitoes with 30min interval analysis (Fig S1A), the waking activity (activity per waking minute) was reduced compared to sugar fed controls, indicating less intensity of movement (Fig 2G). Analysis through nine-days post-blood feeding reveals that waking activity was reduced in blood-fed mosquitoes through Day 5, before returning to normal (Fig S1B-E). Therefore, blood fed mosquitoes are hypoactive for a time course that mirrors the sleep increases [24].

To confirm that the effects observed are not due specifically to recent oviposition only and further define the time-course associated with dietary changes in sleep, we sought to measure sleep immediately following a blood meal. Mosquito behavior was recorded in 6-well tissue culture plates containing 1% agar and 10% sucrose for the first day following bloodfeeding (Fig 3A). Behavior was then tracked using Ethovision as we have previously described in *Drosophila* [26]. An increase in sleep was detected immediately after blood feeding and this persisted over the 24 hr recording (Fig 3B-C). This was associated with reduced total locomotor activity (3D,E). These findings confirm that blood feeding has both immediate and prolonged effect on sleep and locomotor activity.

Protein represents a primary dietary component that contributes to sleep regulation [11,30]. Feeding mosquitoes bovine serum albumin (BSA) has previously been shown induce egg laying indicating that proteins represent the dietary cue in blood that modulates behavior [31]. To determine whether dietary proteins alone increase sleep, we compared sleep in sugar fed mosquitoes and those fed BSA beginning at Day 4 following bloodfeeding using the same time-course applied to quantify the effects of blood feeding on sleep (Fig 4A). Sleep was elevated in BSA-fed mosquitos on Day 4 following feeding compared to sugar-fed controls, and was persistently elevated during the day and the night periods (Fig 4B,C). Sleep measurements over six days revealed that sleep in BSA-fed mosquitos was elevated in BSA-fed mosquitoes for Day 4 and 5, then returned to sugar-fed levels (Fig 4D). The increase in sleep duration was associated

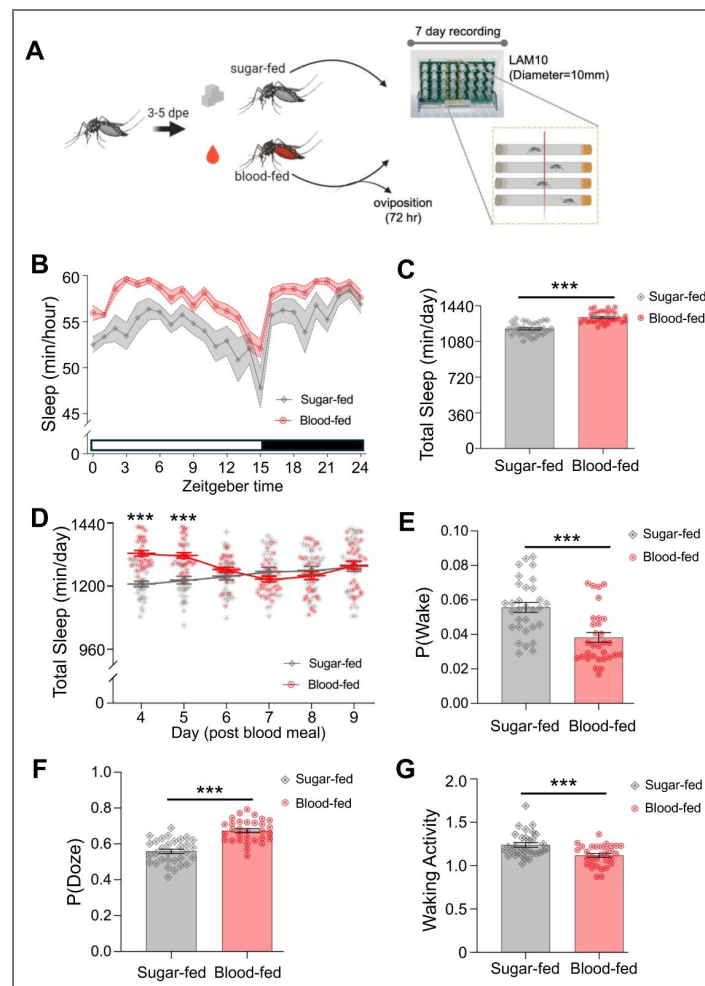


Figure 2. Effects of blood feeding on sleep patterns in *Ae. aegypti*.

(A) Schematic representation of the experimental design. Female mosquitoes, aged 3-5 days post-eclosion (dpe), were either sugar-fed or blood-fed. Following a 72-hour period of oviposition, individual animals were loaded to the infrared-based activity monitors (LAM10, diameter=10mm) used for tracking sleep and activity. (B) Sleep profile (minutes per hour) across the 24-hour cycle on Day 4 post-oviposition. Blood-fed mosquitoes (red line) displayed a consistently elevated sleep profile compared to sugar-fed counterparts (gray line). Light-colored shadows indicate the $\pm$ SEM error bar, and white and black boxes indicate the daytime and nighttime, respectively. (C) Comparison of total sleep of blood-fed mosquitoes (red cycles) on Day 4 post blood meal to sugar-fed controls (gray diamonds). Blood-fed mosquitoes showed a significant increase in total sleep compared to sugar-fed mosquitoes ($F_{1,30}=135.8$, $P<0.0001$). (D) Total sleep duration across Day 4 to 9 post-blood meal. Blood-fed mosquitoes (red cycles) exhibited significantly increased sleep compared to sugar-fed controls (gray diamonds) on Days 4 and 5 (Day 4: $F_{1,30}=135.8$, $P<0.0001$; Day 5: $F_{1,30}=79.00$, $P<0.0001$). (E-G) Changes of sleep architecture of blood-fed mosquitoes (red cycles) on Day 4 post blood meal compared to sugar-fed controls (gray diamonds). Blood-fed mosquitoes demonstrated a significantly lower waking propensity, $P(\text{Wake})$, compared to sugar-fed mosquitoes (E, $F_{1,30}=29.79$, $P<0.0001$). Blood-fed mosquitoes exhibited a significantly higher sleeping propensity, $P(\text{Doze})$, compared to sugar-fed controls on Day 4 post-blood feeding (F, $F_{1,30}=64.44$, $P<0.0001$). Error bars represent the standard error of the mean ($\pm$ SEM). Data points in the bar graphs represent individual animals. Waking activity (activity per waking minute) was reduced on Day 4 post blood meal compared to that of sugar-fed animals, indicating lower movement intensity after blood feeding (G, $F_{1,30}=19.98$, $P<0.0001$).

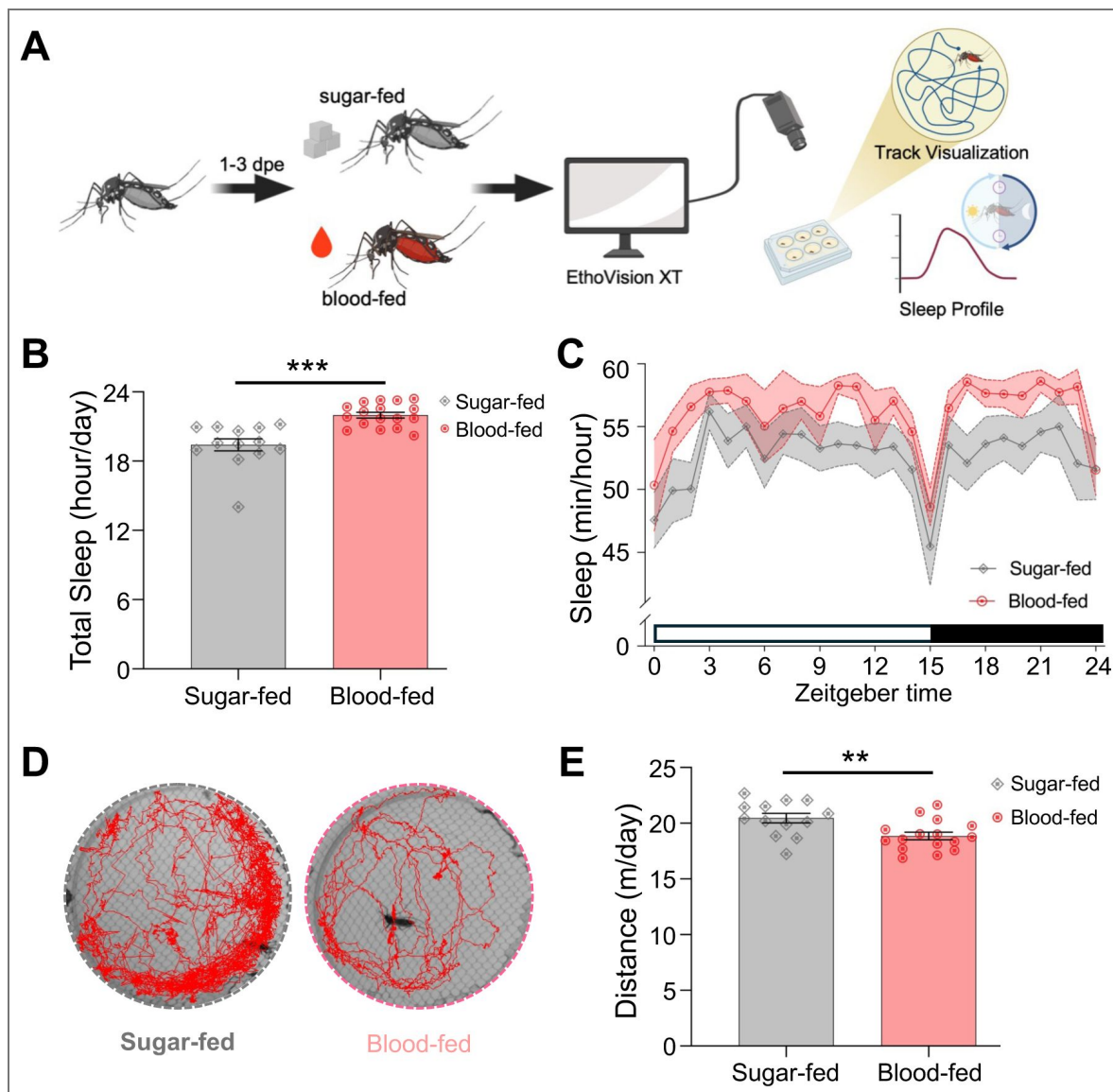


Figure 3. Validation of blood feeding induced sleep in female *Ae. aegypti* using EthoVision.

(A) Schematic of the setup for behavioral recording via EthoVision XT. Female mosquitoes, at the age of 1-3 dpe, were either sugar-fed or blood-fed, and immediately transferred to a 6-well plate after meal for monitoring activities over one LD cycle using EthoVision XT, which allowed for track visualization and analysis of sleep patterns. One animal was housed in one well of the plate. **(B)** Total daily sleep duration in blood-fed mosquitoes (red) compared to sugar-fed controls (gray). A significant increase of daily sleep exhibited in blood-fed group compared to sugar-fed ($F_{1,12}=24.01$, $P<0.0001$). **(C)** Sleep profiles across the 24-hour cycle for sugar-fed and blood-fed mosquitoes via EthoVision recording. Blood-fed mosquitoes (red) showed a distinct sleep pattern, with increased sleep duration observed during both day and night periods compared to sugar-fed counterparts (gray). Light-colored shadows indicate the $\pm$ SEM error bar, and white and black boxes indicate the light and dark, respectively. **(D)** Representative tracks of sugar-fed (left) and blood-fed (right) mosquitoes under EthoVision recording. The red lines indicate the movement paths of the mosquitoes, illustrating differences in activity levels between the two feeding groups. **(E)** Total traveled distance (meters per day) by sugar-fed and blood-fed mosquitoes. Blood-fed mosquitoes (red cycles) showed a significantly shorter distance compared to sugar-fed controls (gray diamonds, $F_{1,12}=13.06$, $P=0.0058$), indicating reduced activity levels. Error bars represent the standard error of the mean ($\pm$ SEM) and each dot on the bar graphs represents individual animal.

with reduced wake propensity and increased sleep propensity supporting deeper sleep (Fig 4E-F). Furthermore, waking activity was reduced in BSA fed animals, suggesting BSA alone is sufficient to induce hypolocomotion (Fig 4G). Together, these findings reveal that the effects of BSA on sleep phenocopy blood feeding, suggesting it is specifically dietary protein that promotes sleep.

The *Ae. aegypti leucokinin receptor (lkr)* (VectorBase ID: AAEL006636), has been previously shown to modulate fluid excretion following a blood meal and sugar perception [32–38], and has been implicated in regulating sleep in *Drosophila* [39], raising the possibility that it also impacts sleep in mosquitoes. To test for the role of LK signaling in sleep regulation, we targeted *lkr* in blood fed animals and measured the effects on sleep. Briefly, mosquitoes were blood fed, then injected with dsRNA targeted to either *lkr* or *eGFP* and tested for sleep immediately following injection and oviposition period (Fig 5A). Surprisingly, loss of *lkr* increased sleep over control mosquitoes injected with dsRNA targeted to *eGFP* for Days 4 and 5 following blood feeding (Fig 5B–D). This was accompanied by a reduction in wake propensity and an increase in sleep propensity (Fig 5E,F). Furthermore, waking activity was reduced in *lkr* knockdown mosquitoes compared to *dsEGFP* injected mosquitoes (Fig 5G). Therefore, silencing *lkr* promotes sleep and reduces locomotion similarly to blood feeding or BSA feeding. This raises the possibility that blood-feeding inhibits LK signaling to promote sleep.

Discussion

Standardized behavioral characteristics of sleep including elevated arousal threshold, rebound following deprivation and species-specific posture, have been used to characterize sleep in species ranging from the jellyfish *Cassiopeia* through humans. Sleep can be characterized by physiological changes in brain activity or by monitoring the behavioral correlates that accompany these changes [40,41]. Here, we apply posture analysis, videography, and measurement of arousal threshold to define sleep as periods of immobility lasting greater than 10 minutes. This is longer than the five-minutes of immobility that is widely used to define sleep in *Drosophila*, and shorter than the 120 minutes that has previously been used to define sleep in *Ae. aegypti* [19,42]. While the 10-minute definition appears to meet existing criteria, further work could be used to define sleep regulation.

Additional approaches have been used in other models to refine the definition of sleep. For example, indirect calorimetry has been applied in flies and rodents to measure sleep-associated changes in whole-body metabolic rate [43,44]. Application of this approach could define whether the graded changes in arousal threshold observed in mosquitos represent light and deep sleep. In addition, analysis of microbehaviors such as haltere movements associated that are associated with sleep in flies could be applied to mosquitos to better define sleep duration and quality [45,46]. Finally, sleep in flies is associated with changes in local-field potentials and neural activity that could be measured in mosquitos [47,48]. Therefore, while our findings provide support for using 10 minutes of immobility to define sleep, the application of additional methods will further our understanding of sleep quality.

Here, we identify a critical role for dietary protein in regulation of mosquito sleep. A central question is whether specific protein components within blood promote sleep. Prior work in fruit flies has shown that tryptone protein alone is sufficient to induce post prandial sleep [11]. In addition, feeding flies the amino acids D-Serine or D-Glutamine alone increases sleep, suggesting that individual amino acids are sufficient to promote sleep [49]. Studies in mosquitos have attempted to identify dietary components associated with egg production and refeeding. An artificial protein-based diet consisting of gamma-globulins, hemoglobin and albumin was able to stimulate egg production in *Ae. aegypti*, revealing dietary protein to be a critical factor in reproduction [50–52]. One consistent theme amongst these studies is that blood plasma or BSA can trigger ecdysteroid production and vitellogenesis, but equivalent proportions of amino acids cannot, suggesting diverse amino acids are required. [53,54]. Applying similar approaches of minimal diets and individual amino acid supplementation may help identify wake-promoting dietary amino acids.

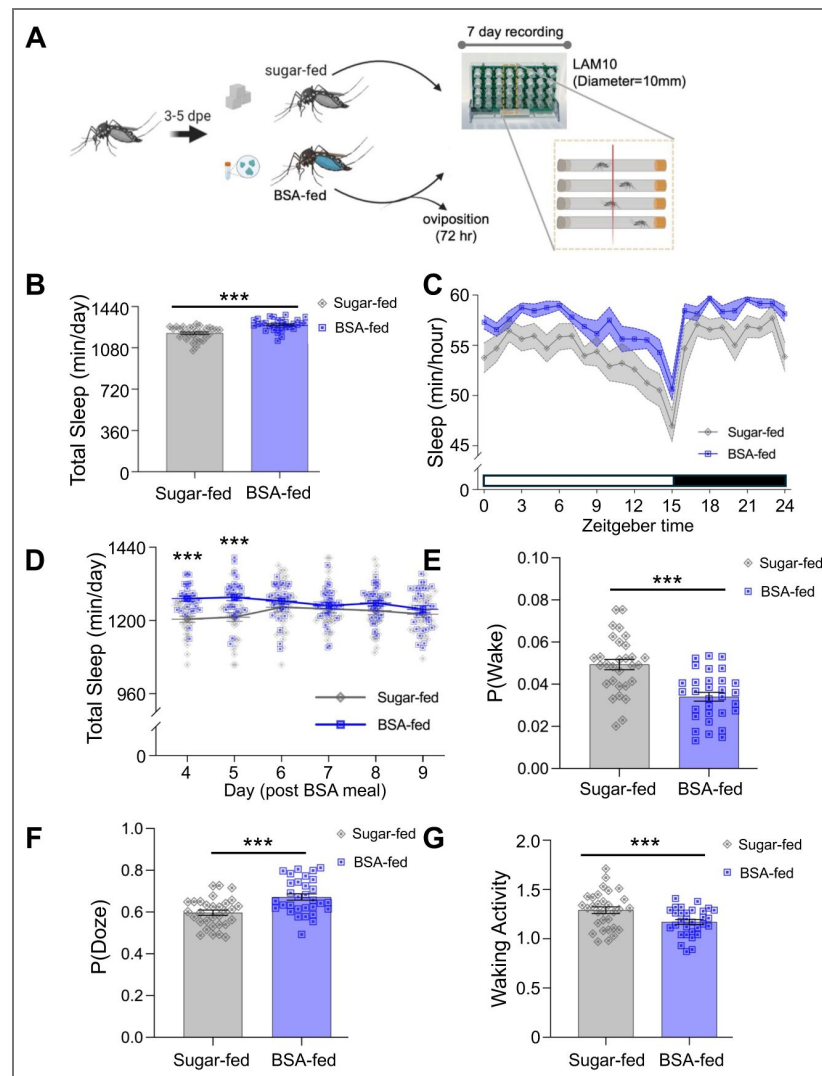


Figure 4. Dietary protein increases sleep in female *Ae. aegypti* via BSA feeding.

(A) Schematic overview of the experimental design. Female mosquitoes, aged 3-5 dpe, were either sugar-fed or fed Bovine Serum Albumin (BSA). Following a 72-hour period of oviposition, sleep was monitored using custom-built infrared activity monitors over a 7-day recording period. (B) Comparison of total sleep of BSA-fed mosquitoes (blue squares) on Day 4 post blood meal to sugar-fed controls (gray diamonds). Total sleep duration of BSA-fed mosquitoes showed a significant increase compared to sugar-fed mosquitoes ($F_{1,30}=26.68$, $P<0.0001$). (C) Sleep distribution across one LD 15hr:9hr cycle on Day 4 post-BSA meal. BSA-fed mosquitoes (blue line) demonstrated elevated sleep levels during both day and night periods compared to sugar-fed mosquitoes (gray line). Light-colored shadows indicate the $\pm$ SEM error bar, and white and black boxes indicate the daytime and nighttime, respectively. (D) Total sleep duration across Days 4 to 9 post-BSA feeding. BSA-fed mosquitoes (blue) exhibited significantly increased sleep compared to sugar-fed controls (gray) on Days 4 and 5 post-BSA feeding (Day 4: $F_{1,30}=26.68$, $P<0.0001$; Day 5: $F_{1,30}=15.17$, $P=0.0005$). (E-G) Changes of sleep architecture of BSA-fed mosquitoes (blue) on Day 4 compared to sugar-fed mosquitoes (gray). Significantly lower P(Wake) (E, $F_{1,30}=22.90$, $P<0.0001$) and higher P(Doze) (F, $F_{1,30}=12.91$, $P=0.0003$) was showed in BSA-fed population compared to sugar-fed group on Day 4 post-BSA feeding. Data points in the bar graphs represent individual animals and error bars represent the standard error of the mean ($\pm$ SEM). BSA-fed mosquitoes exhibited reduced waking activity compared to sugar-fed controls on Day 4 post-BSA feeding (G, $F_{1,30}=23.63$, $P<0.0001$).

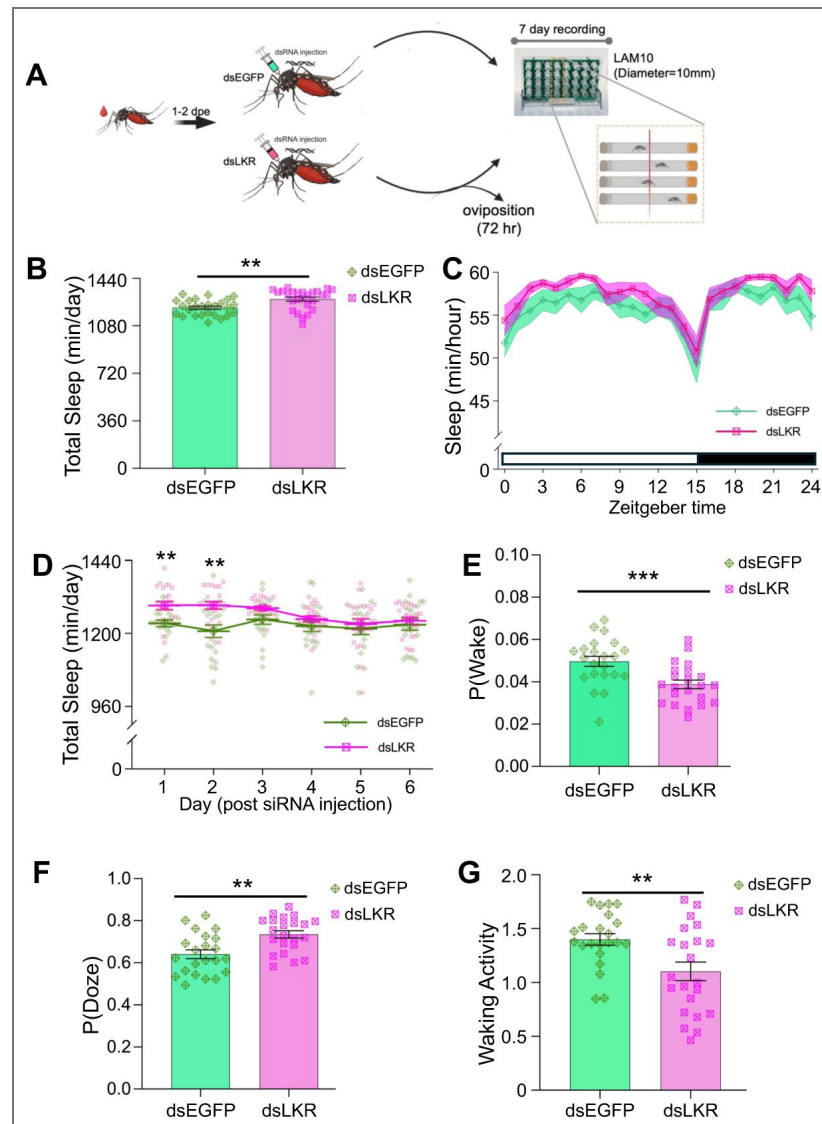


Figure 5. Role of the leucokinin receptor (*lkr*) in sleep regulation of female *Ae. aegypti*.

(A) Schematic overview of the experimental design for sleep monitoring in dsRNA-injected mosquitoes. (B) Daily sleep duration was significantly induced in *dsLKR*-injected mosquitoes (magenta cross markers) compared to *dsEGFP* controls (green diamonds) ($F_{1,25}=15.89$, $P=0.0012$). (C) Sleep profiles of female mosquitoes injected with either *dsLKR* or *dsEGFP* across the 24-hour cycle of Day one post injection. Mosquitoes with *LKR* knockdown (magenta) displayed a distinct sleep pattern, with increased sleep observed during both day and night periods compared to *dsEGFP* group (green). Light-colored shadows indicate the $\pm$ SEM error bar, and white and black boxes indicate the light and dark periods, respectively. (D) Total sleep duration across Days 1 to 6 post-siRNA injection. Mosquitoes injected with *dsLKR* (magenta line with cross markers) exhibited significantly increased sleep compared to controls injected with *dsEGFP* (green line with diamond markers) on Days 1 and 2 ($F_{1,25}=15.89$, $P=0.0012$ for Day 1 and $F_{1,25}=15.18$, $P=0.0015$ for Day 2). (E-G) Comparisons of P(Wake), P(Doze) and waking activity of mosquitoes injected with *dsLKR* to *dsEGFP* controls on Day 1 post injection. After knockdown of *LKR*, female mosquitoes demonstrated a significantly lower probability of waking compared to *dsEGFP* controls (E, $F_{1,25}=13.19$, $P=0.0002$). In contrast, the sleeping propensity, P(Doze), was significantly induced in *dsLKR*-injected mosquitoes compared to *dsEGFP* controls (F, $F_{1,25}=9.48$, $P=0.0012$). *dsLKR* mosquitoes (magenta cross markers) exhibited significantly reduced waking activity compared to *dsEGFP* controls (green diamonds, G, $F_{1,25}=11.71$, $P=0.0053$). Error bars represent the standard error of the mean ($\pm$ SEM), and unpaired t-test was used for significance: ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

Genetic studies in the fruit fly *Drosophila melanogaster* have led to the identification of highly conserved pathways that regulate both sleep and feeding [40,41,55,56]. Many genes and signaling molecules required for sleep and metabolic regulation are conserved across phyla, including those that regulate the circadian clock, energy stores, and neuropeptide regulation [10,39,57]. For example, *Lk* or the *Lkr* are essential for both postprandial sleep and starvation-induced sleep suppression, suggesting the *Lk* signaling mediates both sleep-feeding interactions [11,39]. Here, we find that targeted knockdown of *Lkr* increases sleep in amino acid fed flies, suggested a wake-promoting role that may be similar to what has been observed for starvation-induced sleep suppression in flies [39]. Additional analysis of the effects of *lkr* on sleep across different feeding conditions, as well as the generation of stable mutant loss-of-function will support further dissection of its role in feeding regulation. In addition, the use of dsRNA to identify regulators of sleep in *Ae. aegypti* provides proof-of-principle for the application of screening approaches in this model that would support the identification of novel sleep regulations.

Our findings raise the possibility that increased sleep and reduced activity following a meal is evolutionary adaptive. There are multiple possible explanations for how these physiological changes might improve survival or reproduction. For example, sleep is proposed to be a mechanism of predator avoidance [58], increasing the likelihood that the female mosquito survives when neither mating nor feeding is required. Second, sleep provides a mechanism of energy conservation that may increase the efficiency of time to the subsequent egg-laying cycle or the likelihood of survival. In the case of *Ae. aegypti*, we found increased sleep and reduced activity for up to 5 day after blood feeding, long after responsiveness to host cues has been restored. This suggests the existence of an opportunistic phase of host-seeking, whereby a bloodmeal provides sufficient nutrients to not only produce eggs but which allows *Ae. aegypti* to act more as an ambush predator that can respond to cues and seek hosts when they stray too close to the chosen resting sites without expending much effort/risking predation (Fig 5 [↗](#)). Once these resources are exhausted, sleep levels decrease, activity increases in a more determined host seeking mode, where *Ae. aegypti* may take on greater risks in long-distance host pursuit. Identifying whether this effect is specific to *Ae. aegypti* or generalizable across other mosquito species and blood-feeding insects will help inform its adaptive function.

Together, these results position *Ae. aegypti* as a powerful new model for dissecting how diet shapes sleep. By linking blood feeding and dietary protein to sustained changes in sleep and activity, this work establishes a foundation for exploring the molecular and neural mechanisms that couple feeding state to sleep regulation in a highly specialized dietary context. The availability of sophisticated behavioral assays, alongside emerging genetic tools in *Ae. aegypti*, opens the door to mechanistic studies that can parse the contributions of specific nutrients, signaling pathways, and neuronal circuits to sleep regulation. Looking forward, this system offers an unparalleled opportunity to address broad questions about the adaptive functions of sleep, the evolutionary diversity of sleep-feeding interactions, and the conserved pathways that integrate metabolism and behavior across species.

Materials and methods

Mosquito Husbandry and Maintenance

For all experiments, *Aedes aegypti* mosquitoes (Liverpool strain, *Lvp*, originally obtained from Virginia Tech) were cultured at Adelman Lab, Texas A&M University, under controlled conditions. Adult mosquitoes were maintained in mixed-sex groups at a temperature of 26-28°C and 70% ($\pm 10\%$) relative humidity, with a photoperiod of 15 hours of light followed by 9 hours of darkness (LD: 15:9 hr). Eggs were hatched in deionized water; larvae were fed ground TetraMin® fish food daily. Pupae were transferred to mesh-covered cups for adult emergence. For adults, mated females were fed defibrinated sheep blood (Colorado Serum Company, CO) via a Parafilm® membrane feeder at 37°C and allowed for oviposition for 72 hours prior to behavioral experiments. Unless otherwise noted, mated females 3-5 days post-eclosion (dpe) were selected for all experiments.

Sleep, Activity and Arousal Measurement

Analysis of postural changes

The device for recording mosquito posture changes consists of two main components. The first component is an iPhone 16 Pro (Apple Inc., CA, USA), utilized for close-range, high-definition monitoring of posture. The iPhone is securely mounted on a tripod, allowing for easy adjustment of height and distance. The second component is a custom-designed chamber for housing the animals and light sources. This chamber features a compact white exterior with a cut-out front panel, facilitating the placement and adjustment of the iPhone 16 Pro while ensuring clear visibility of the interior. Inside, there is a flat surface made of non-reflective material, serving as a base for placing experimental samples. The subjects are housed in a transparent Pyrex glass tube (length: 100 mm, diameter: 10 mm; TriKinetics Inc., USA), which is vertically fixed to the base. At the upper interior of the device, two adjustable light sources (LD: 15:9 hr) are installed, positioned to uniformly illuminate the sample area. The overall structure is robustly designed to minimize external interference and create a controlled environment for precise measurements and observations. When introducing vibrational mechanical stimuli, the glass tube is placed horizontally on a 3D-printed base, with the monitoring camera positioned directly above it. Unless otherwise specified, mosquitoes aged 3-5 dpe were used for behavioral monitoring following blood feeding. The recorded videos were used for quantification of the length from when mosquitoes, which were in a state of quiescence, initiated putting down the elevated hind leg to the point when the hind leg was fully contact with the substrate. This period is defined as the duration of time required for a mosquito to enter a sleep state.

Activity monitoring system

Sleep and activity were quantified using custom-built infrared activity monitors (LAM10, Trikinetics, Waltham, MA), which allows for infra-red based tracking of behavior. Mosquitoes at the age of 3-5 dpe were briefly anesthetized with ice and loaded individually in to 10mm diameter glass tubes containing sugar food (10% sucrose with 1% agar) following a period of oviposition for 72 hours. Tubes with animals were placed to the monitoring system for recording movement and inactivity, enabling the determination of sleep duration and activity patterns over the subsequent six days. Data were collected as previously described [59], and total sleep duration and activity were then calculated for each mosquito. Sleep was defined as periods of inactivity lasting more than 10 minutes, consistent with the data collected for analysis of postural changes.

Video tracking

Ethovision XT 15 (Noldus Information Technology Inc., VA, USA) system was used for video tracking. In brief, one mosquito was placed in one well of the 6-well plates following blood meal. Videos were recorded for 24 hr (one LD cycle) at 15 frames per second using a USB webcam (LifeCam Studio 1080p HDWebcam, Microsoft) through the video processing software VirtualDub (v1.10.4). To enable recording in darkness and maintain uniform illumination, the cameras' stock IR-cut filters were removed and swapped for IR long-pass filters (Edmund Optics Worldwide). After acquisition, videos were imported into EthoVision XT 15 to generate x-y coordinates for each mosquito across the entire 24 h recording; subsequently positional data were exported from Ethovision and analyzed using a custom-made Perl script (v5.10.0) and Excel Macro (Microsoft). To acquire the tiny action or movement performed on the same spot, a threshold of 1.8 mm per second was used to compute velocity and locomotor activity metrics as previously described [60].

Arousal threshold

A modified *Drosophila* Arousal Tracking (DART) system was used for mosquitos [24]. In brief, at 3-5-day-old, sugar-fed female mosquitoes were placed individually in 100 mm glass tubes (Trikinetics, Waltham, MA), which were mounted on a vibration-capable platform. The setup was kept in an environmentally controlled chamber with constant temperature (26-28°C) and humidity (70% ±10%) under a 15:9 h LD cycle. Mosquito behavior was continuously recorded using a high-resolution USB camera (Logitech, 800x600, 5 frames per second) for 24 hours. To measure arousal threshold, vibratory stimuli of increasing intensity [0.6g-1g (acceleration)] were delivered once per

hour for 24 hours, beginning at ZT0. Vibrational stimulation, video tracking and analyzing were performed using a custom MATLAB pipeline (MathWorks, Natick, Massachusetts) as described by Faville *et al.* (2015) [24]. To quantify activity, videos were subsampled at 1 frame per second, and pixel differences between consecutive frames were calculated by subtracting a background reference created from 20 randomly selected frames. Movement was defined as a positional change greater than a specified pixel threshold. Inactivity periods of 10 minutes or more were classified as sleep bouts. For each stimulus, the duration of the mosquito's ongoing inactivity (bout length) was recorded just before stimulation. A behavioral response was defined as visible movement within 15 seconds after the onset of vibration. The proportion of mosquitoes responding was then calculated for each bout length bin (e.g., 1 min, 3 min, 5 min, etc.).

Sugar, Blood, and BSA Feeding Assay

To assess the effects of different feeding regimens on sleep and activity, female mosquitoes were provided with either a 10% sucrose solution, defibrinated sheep blood (Colorado Serum Company), or Bovine Serum Albumin (BSA, 150 mg/mL in PBS with 1 mM ATP) for 72 hours of feeding. Simultaneously, these females were allowed to acclimate and oviposit for 72 hours prior to the experimental test. For the blood feeding assays, mosquitoes were fed using an artificial membrane feeder (Ref). The BSA feeding was administered by placing the protein solution (150 mg/mL in PBS with 1 mM ATP) in small containers accessible to the mosquitoes. Following feeding, mosquitoes were loaded into activity monitoring system for the subsequent six days.

Double-stranded RNA (dsRNA) synthesis and injection

To knock down AaLKR (AAEL006636), we used the dsRNA sequence against the AaLKR from the Kwon *et al.* study, which targets a 382 nt region of the transcript [61]. We amplified the template for dsRNA using the primers containing the T7 RNA polymerase promoter on the 5' ends with Phusion DNA polymerase (NEB) and female adult cDNA as a template. The PCR product (dsRNA template) was integrity-verified by agarose gel electrophoresis and purified using the Nucleospin Gel and PCR cleanup kit (Macherey-Nagel). dsRNA was synthesized using the MEGAscript T7 transcription kit (Thermo Fisher) with 500 ng of template DNA for an overnight reaction at 37 °C. The in-vitro synthesis reaction was treated with RNase-free DNase (TURBO DNase, Thermo Fisher) for 30 min at 37 °C and purified using the MEGAclear transcription cleanup kit (Thermo Fisher). Purified dsRNA was confirmed by agarose gel electrophoresis, quantified by spectrophotometry (NanoDrop One, Thermo Fisher), and stored in aliquots at −80 °C until use. We also made dsRNA against EGFP as a negative control. All primer sequences are found in Table A. 3-5 days old female adult mosquitoes were fed with defibrinated sheep blood (Colorado Serum Company) using an artificial membrane feeder, and only engorged individuals were kept with male mosquitoes (to ensure mating). At about 24 h post blood-feeding, the female mosquitoes were anesthetized on ice and were injected in the thorax with dsRNA (1 µg per individual) using Nanoject II microinjector (Drummond). After the injection, the mosquitoes were maintained on a sucrose solution (30%) for another 48 h, and were provided a cup with damp paper for egg laying. The next day, the mosquitoes were loaded on the DAM instrument.

Table A: primers used to amplify dsRNA templates. Capital letters indicate annealing sequence and lower case letters T7 promoter.

Primer ID 5'-3'

AaKR_T7F taatacgactcactatagggTGTATCAACACGGATCATTCATGGAATGGC

AaKR_T7R taatacgactcactatagggCATCGCTGCCGTTCAAGTATTGTTGTTGC

dsEGFP_F taatacgactcactatagggATGGTGAGCAAGGGCGAGGAGC

dsEGFP_R taatacgactcactat

Statistical Analysis

Comparisons between two groups, either blood feeding or BSA feeding versus sugar feeding controls, were conducted in all cases with a one-way ANOVA followed by Mann-Whitney tests and Welch's correction where applicable. In the knockdown experiment, sleep duration and architectures were compared between dsRNA targeting *lkr* and dsEGFP animals. Apart from the data plotted by the paired dot graphs, all the other data were analyzed using the unpaired t-test, and statistical significance was set at $P < 0.05$. All analyses were performed using InStat software GraphPad Prism (version 10.3.0 for Windows, Boston, Massachusetts, USA), and results are presented as mean $\pm$ standard error of the mean (SEM).

Supplementary Figures

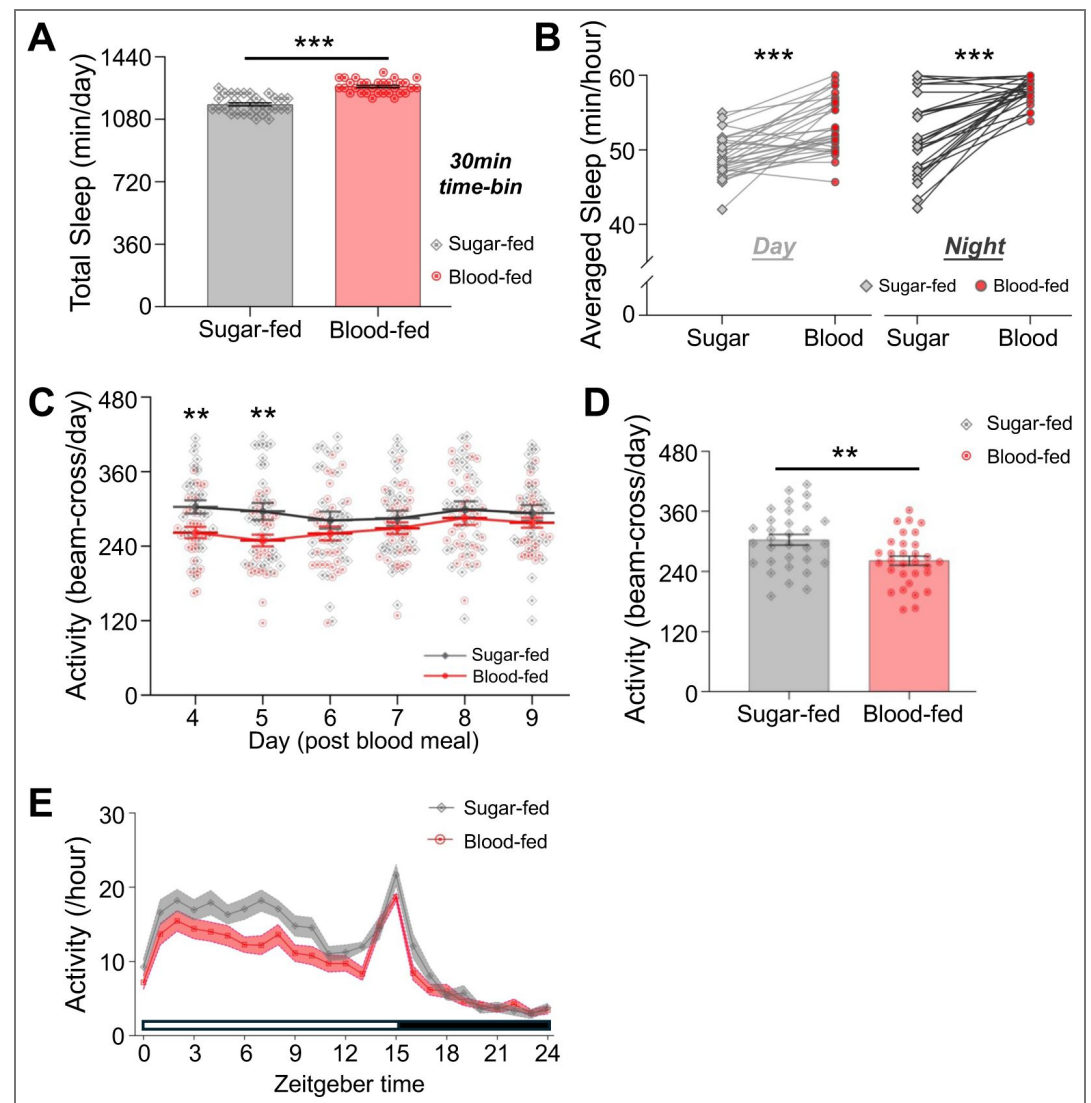


Figure S1. Effects of blood feeding on sleep and activity in *Ae. aegypti* females. (A) Total sleep duration in sugar-fed *Ae. aegypti* versus blood-fed with analysis in 30-min intervals. Sleep of blood-fed mosquitoes (red cycles) was significantly increased compared to sugar-fed controls (gray diamonds, $F_{1,30}=111.5$, $P<0.0001$) (B) Paired dot plot displayed the averaged sleep for either sugar-fed or blood-fed individual subjects during day and night periods. Blood-fed mosquitoes (red dots) showed significantly higher sleep levels during both day and night compared to sugar-fed controls (light gray diamonds, $F_{1,30}=36.00$, $P<0.0001$ for day and $F_{1,30}=44.44$, $P<0.0001$ for night). (C) Daily activity across Days 4 to 9 post-blood meal. Blood-fed mosquitoes (red line) exhibited significantly reduced activity compared to sugar-fed controls (dark gray line) on Days 4 and 5 ($F_{1,30}=10.75$, $P=0.0045$ for Day 4

and $F_{1,30}=10.08$, $P=0.0061$ for Day 5). **(D)** Total daily activity for sugar-fed (gray diamonds) and blood-fed (red cycles) mosquitoes on Day 4 post blood meal. Blood-fed mosquitoes displayed significantly lower daily activity levels compared to sugar-fed controls ($F_{1,30}=10.75$, $P=0.0045$). **(E)** Hourly activity profiles across the 24-hour cycle for sugar-fed (gray line) and blood-fed (red line) mosquitoes. Blood-fed mosquitoes showed a distinct reduced activity during both day and night periods. Light-colored shadows indicate the $\pm$ SEM error bar, and white and black boxes indicate the daytime and nighttime, respectively. Error bars represent the standard error of the mean ($\pm$ SEM), and asterisks indicate the level of significance: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

Data availability

All raw data will be provided upon request. The compiled data will be published as supplemental information with this manuscript.

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Peer reviews

Reviewer #1 (Public review):

Summary:

The presented investigation aims to expand the sleep definition and its relationship with blood meal and/or circadian clock in the mosquito, *Aedes aegypti*. The authors exhausted the established sleep analytical paradigm and three behaviour toolkits: LAM10, EthoVision, and DART. They also investigated the potential underlying molecular mechanism by using dsRNA injection (LkR) and a KO mosquito (Cyc^{-/-}).

Strengths:

The authors presented a very solid dataset showing posture changes and an increase in the arousal threshold of the mosquito after 10 minutes of immobility. This is a major clarification and extension to our understanding of insect sleep beyond *Drosophila*. Inclusion of analytical parameters such as bout length, waking activity and pDoze/Wake provide critical reminder for other investigators of the steps needed for defining sleep in a new species. The investigation, with its technical span in behaviour assays, therefore establishes a good standard for mosquito sleep analysis to the same quality seen in the landmark studies (Shaw et al 2000 and Hendricks et al 2000) for *Drosophila* sleep. The pioneering data showing a clear effect of blood meal and LkR reduction on locomotion and sleep provides an entry point for further investigations.

Weaknesses:

Despite the versatility of the behaviour and transgenic methods in this manuscript, there are two logical gaps in the conclusion, which are related to the effect of blood meal/BSA/Lkr KD on *A. aegypti* sleep:

(1) Conventionally, a coincidence of sleep increase and locomotion reduction would weaken the certainty of a sleep increase assessment. The authors implied this concurrence observed after blood meal is derived from internal "drowsy" neural state instead of physical "cripple", but they did not use their two high-resolution video tracking velocity or pDoze/Wake to clarify this.

(2) The major molecular component underlying blood meal effect on sleep/locomotion is less certain, because the BSA solution used for feeding contains ATP, which itself is able to enter haemolymph and potentially exerts sleep/locomotion effect. Additionally, the basal or control sleep recording is done after sucrose feeding. It is, however, unclear from the method if this is 10% too? And if the observed sleep level increase after a blood meal is a result of sugar level reduction in the blood (~0.1%).

<https://doi.org/10.7554/eLife.110245.1.sa1>

Reviewer #2 (Public review):

Zhang et al. investigate how blood feeding and dietary protein influence sleep in the mosquito *Aedes aegypti*. The authors first establish a behavioural definition of sleep using postural analysis and arousal threshold measurements, then demonstrate that both blood meals and a bovine serum albumin (BSA)-based protein diet increase sleep for several days. They further show that RNAi-mediated knockdown of the leucokinin receptor (Lkr) enhances sleep, implicating neuropeptide signalling in the regulation of postprandial sleep. The authors propose that elevated sleep persists well beyond the restoration of host-seeking behaviour, suggesting the existence of distinct "opportunistic" versus "determined" host-seeking phases.

Strengths

The central question is well-motivated, and the experimental approach is systematic. The use of multiple independent methods to characterise sleep - postural analysis, infrared activity monitoring, videography, and arousal threshold - provides converging evidence. The BSA feeding experiment is a particularly effective demonstration that dietary protein, rather than other blood components, is the key regulator of the sleep increase. The conservation of leucokinin signalling in sleep regulation between *Drosophila* and *Ae. aegypti* is a noteworthy finding that adds comparative depth.

Weaknesses

(1) Sleep definition.

The authors settle on a 10-minute immobility threshold, but their own data do not convincingly support this choice. The arousal threshold data (Figure 1G) show no significant difference between the 1-5 min and 6-10 min bins ($P=0.246$), with significance emerging only at the 11-15 min bin. The postural analysis likewise indicates that sleep-associated postures appear at ~20 min during the day and ~11 min at night. A 15-minute threshold would be better supported by the data as presented. The previous literature used 120 minutes for this species (Ajayi et al. 2022), making this a dramatic shift.

(2) Confound of reproduction and sleep.

The primary experimental paradigm measures sleep beginning at Day 4 post-blood feeding, immediately after oviposition. Animals have undergone gut distension, vitellogenesis, and

oviposition, and what is being measured as "sleep" could reflect post-reproductive quiescence or recovery rather than diet-induced sleep per se. The BSA experiment partially addresses this, but since BSA also triggers vitellogenesis and egg production (as the authors note), the confound persists.

(3) Opportunistic vs. determined host-seeking hypothesis.

This framework is presented as a key conceptual contribution, but the paper contains no data on host-seeking behaviour. The authors infer two phases from the temporal mismatch between a 72-hour host-seeking suppression window (from prior studies) and elevated sleep through Day 5 (~120 hours). While this is an interesting hypothesis, it requires actual measurement of host-seeking alongside sleep to be substantiated, or at least the caveats need to be discussed more explicitly.

(4) Statistical approach.

The methods describe "one-way ANOVA, followed by Mann-Whitney tests with Welch's correction," which is an internally inconsistent combination: Mann-Whitney is non-parametric and does not use Welch's correction (which applies to t-tests). Throughout the figures, F-statistics (parametric) are reported alongside what appear to be non-parametric tests. The statistical framework needs to be clarified and made consistent. Exact sample sizes per group should also be stated explicitly in the methods for all experiments.

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