# Title: Humidity as a zeitgeber for circadian entrainment of insect systems

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**Abstract:** Humidity levels, like light and temperature, fluctuate daily yet are less predictable; however, whether humidity entrains circadian clocks and enables animals to synchronize behaviors to environmental variations remains unknown. Here, we investigate the circadian humidity entrainment in various insects. Multiple species robustly respond to humidity cycles, and when the humidity cue is removed, their rhythmic behaviors continue, suggesting that humidity-associated rhythmic activities are under circadian control. Moreover, the *Drosophila* clock and hygrosensation mutants lack rhythmic activities during and after humidity entrainment, indicating that the core clock components and hygrosensors are essential to circadian entrainment. Our findings identify that humidity serves as a novel zeitgeber for circadian entrainment for insects that could have broad applicability and importance among animal systems.

**One Sentence Summary:** Humidity entrainment of the circadian clock enables the synchronization of insect behaviors to environments.

Animals experience daily fluctuations in their surrounding environment that are mainly tied to daily changes in light levels and temperature shifts. These diurnal cycles of light and temperature are the main cues (zeitgeber) that entrain circadian clock and synchronize organismal biological functions (*1*, *2*). Circadian disruption and misalignment often lead to harmful effects on organisms (*3*, *4*). Recent studies on insects have shown that besides light and temperature, multiple biotic and abiotic factors play important roles in the synchronization of circadian rhythm (*5–8*). One specific abiotic factor that has not been directly examined is whether humidity could serve as a potential zeitgeber in animals. Of interest is that humidity has distinct daily cycles, but does show high variability based on precipitation, and terrestrial animals often show adaptive responses to humidity changes to avoid desiccation (*9*, *10*); however, whether humidity can entrain the circadian clock in animals remains unknown.

For terrestrial arthropods, humidity impacts the spatio-temporal dynamics of behaviors and physiology. Exposure to arid conditions alters the general activity and feeding behaviors of various pest species (*11*, *12*). Of particular interest is that several factors that underlie humidity sensing have been recently identified in insects (*13–21*). Although this growing evidence reveals how animals respond to humidity changes and locate water sources, interaction between hygrosensation and diel activities in insects, or other animals, has yet to be studied. This study aims to identify the role of daily humidity cycling in the entrainment of insect circadian clocks. Our identification and understanding of the mechanisms of a novel humidity-entrainable clock for insects establish that humidity, even though variable, could strongly influence circadian biology and will help us better predict how the behavior and distribution of animals may be altered due to environmental conditions.

## Results

### Humidity cycles can entrain insect locomotor activities

To establish if humidity cycling can entrain circadian clocks, the activity pattern of various insect species under arid (A, 40-50%RH)-humid (H, > 85%RH) cycles (12h:12h A:H) was examined with temperature (25°C) and air pressure (780 mm Hg; fig. S1 and S2). Previous studies showed that temperature cycles can synchronize behavioral activity and the expression of clock genes both under constant light (LL) and constant darkness (DD). We therefore examined whether activities of various arthropods can be synchronized to humidity cycles and humidity can entrain circadian clocks under LL and DD (*25*, *26*). In the present study, all insects examined robustly respond to the environmental humidity changes (AHLL) with two distinct behavioral patterns and various activity levels (Fig. 1A to C). Kissing bugs, spider beetles, and two mosquito species (daytime-active *Aedes aegypti* and nighttime-active *Culex pipiens*) showed higher activity levels in the dry phase than those in the humid phase (Fig. 1C). *Drosophila* exhibited a behavioral pattern with an activity peak at the dry-to-humid transition (Fig. 1B, near ZT 12). Shifting the humidity phase by 6 hours resulted in a shift in behavior to match the new cycle within 3 days, highlighting that individuals will be entrained to changes in this cue (fig. S3). We observed that flies raised for their entire life in LL under constant temperature also showed rhythmic behaviors in the humidity cycles (AH) and free-running (HH) in constant dark conditions (DD) (Fig. 1E-G: $w^{1118}$; Fig.1H-J: Oregon R; Fig. 1K and L: period and rhythmicity in the free-running period, FRP, HH).

To determine if humidity can serve as a zeitgeber to entrain the circadian clock, we examined the activities of insects in constant humidity (HHLL) following entrainment under humidity cycles (AHLL). Most species, except for a more muted effect in mosquito species in our study, displayed rhythmic activities with periods of ~24 hr in the absence of humidity cues in this

free-running period (FRP) (HHLL: Fig. 1D, table S1; HHDD: Fig. 1K-L, table S2). Rhythmic patterns were noted in *D. melanogaster* sleep under AHLL and HHLL (fig. S4). This indicates that terrestrial insects react to daily humidity fluctuations in a way that effectively synchronizes their behaviors to daily humidity fluctuations.

**Humidity-associated activities are under circadian control**

After establishing a distinct humidity cycle response and entrainment, we aimed to confirm if the humidity-associated activities of insects are under circadian regulation. We examined the activities of *Drosophila* mutants for key clock genes ($per^{01}$, $tim^{01}$, $cyc^{01}$, and $Clk^{Jrk}$) and the main circadian neuropeptide ($pdf^{01}$) under AH and HH conditions. Although these mutants responded to the humidity changes under AH to some extent (Fig. 2A), all lines exhibited weaker rhythmicity with more arrhythmic flies in the FRP compared to $w^{1118}$ (Fig. 2B; table S3). These results suggest that functional circadian clock molecular machinery is essential for humidity entrainment.

**Humidity sensing-associated molecules are essential for the circadian humidity entrainment**

Hygrosensation requires specific sensors (*13*, *14*, *16*), which we targeted to determine if these are critical to humidity entrainment. *Drosophila* hygrosensation mutants differentially responded to humidity cycles (Fig. 3A) and most showed lower rhythmicity in HH after entrainment under AH (Fig. 3B). Among them, the rhythmicity of *Ir40a* and *Obp59a* mutants along with *Ir76b* which is related to low salt and amino acid, sour sensing (*27–29*), was significantly lower than that of $w^{1118}$ flies (Fig. 3B; table S4). These results reveal that hygrosensation is essential for humidity entrainment of circadian clock and insects may use different repertoires of hygrosensing molecules for behavioral synchronization to daily humidity

changes. Lack of specific hygrosensing molecules like Ir40a and Obp59a may hinder the humidity signal input into clock which will dampen or weaken internal molecular oscillations, eventually leading to failure of behavioral synchronization.

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**Sugar-feeding behaviors are aligned with humidity-associated activity levels**

To verify these behaviors beyond activity (Fig. 4, A and B top), *Drosophila* food intake behaviors (*30*) were examined in relation to AH and HH conditions (Fig. 4, A and B bottom). Sugar-feeding behaviors for *Drosophila* are associated with the humidity conditions under AH
100 with higher food consumption beginning at ZT 12-15 (Fig. 4A) that corresponds to increased activity. A similar rhythmic pattern for feeding was observed under HH but was slightly advanced to CT9-12 compared to the feeding pattern under AH (Fig. 4, A and B). Together, these results suggest that insect sugar-feeding behaviors, like locomotor activities, are associated with environmental humidity changes and under circadian control.

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**Discussion**

This study reveals that humidity can serve as a zeitgeber to synchronize insects' behaviors to environmental changes. Outside of this study, the direct observation of humidity as a potential zeitgeber has only been observed in a single plant system (*31*). Previous literature has suggested
110 that humidity cycles may more closely match insect activity than light:dark or temperature cycles (*32*). In our studies, various insect species robustly respond to humidity cycles and their locomotor activities remain rhythmic in the FRP. *Drosophila* clock and hygrosensation mutants show abnormal phenotypes during humidity entrainment and low rhythmicity in the free-running period, suggesting humidity entrainment is under circadian control and requires hygrosensation-associated

115 receptors or molecules (Fig. S5). The humidity-sensing molecules are known to primarily be expressed in the antenna (*13–16*, *19*). Among them, we found that Ir40a and Obp59a may play an important role in the sensory input to this humidity-entrainable clock (Fig. 3). Since both Ir40a and Obp59a are selectively expressed in sacculus of the antenna (*14*, *15*, *15*, *16*) and the cells expressing Ir40a and Obp59a are adjacent to each other (*16*), it is likely that the external signal
120 input for circadian entrainment is through the neural circuit composed of hygrosensitive neurons in the second chamber of the sacculus, small glomerulus at posterior antennal lob (PAL), and projection neurons relaying the information to the higher brain (*15*).

Circadian clock governs a broad aspect of animals' lives through the synchronization of
125 organisms to environmental changes. While light and temperature are generally acknowledged as the most potent zeitgebers for circadian rhythms, humidity remains largely unknown in this regard and may also play a crucial role in the rhythmic patterns of biological processes, especially for terrestrial animals. On a warm, dry, sunny day, terrestrial animals gradually experience water loss, which threatens their survival if essential adaptations do not occur, such as rehydration from food
130 sources or reduction of water loss (*11*, *33*, *34*). An endogenous humidity-entrainable clock enables animals to better predict the time-of-day variation in humidity. Our data reveal that locomotor activity and feeding assays peak at similar times, suggesting these clock-driven behaviors could link to water seeking or feeding activities and play a critical role in dehydration avoidance. Unlike the well-studied sensory input for light and temperature entrainments and the interaction between
135 light- and temperature-entrained clocks (*26*, *35–38*), the humidity-input pathway for circadian oscillation of insects, along with other animals remains unknown (*31*). Moreover, understanding how humidity interacts with light and temperature could unveil new insights into circadian regulation in animal behavior and physiology under multiple mixed environmental cues in nature.

The humidity-entrainable oscillator is likely universal in various terrestrial arthropods. Synchronization of behaviors to environmental humidity changes is critical for animal fitness, especially for dehydration avoidance (*11*). This study paves the way to elaborate on the interplay between daily humidity fluctuation and activities of many pests. Our understanding of the underlying mechanism will help us not only predict the time-of-day behavioral patterns of many harmful agricultural and public health pests but also develop effective approaches to reduce vector-host interaction for disease intervention.

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**Competing interests:** The authors declare that they have no competing interests.

**Data and materials availability:** Data will be made available on Dryad at acceptance

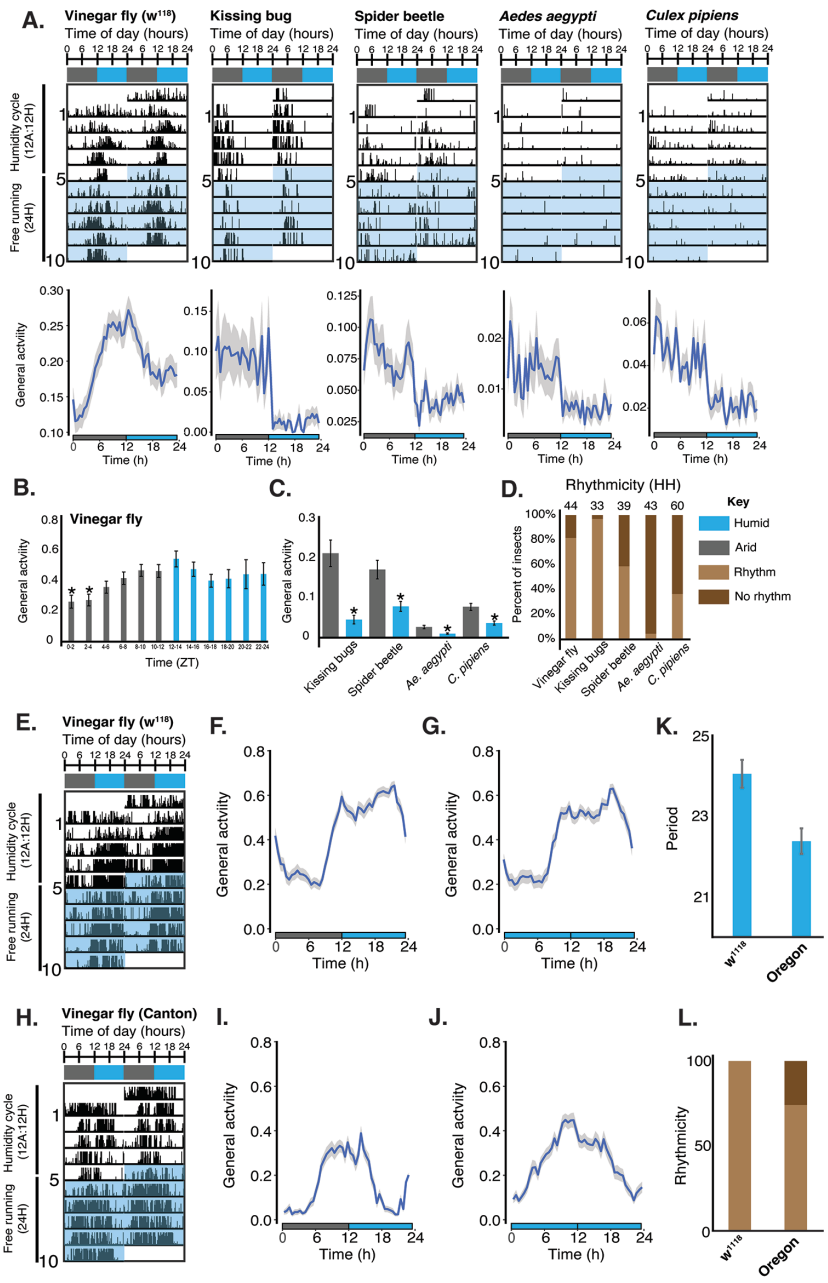


**Fig. 1. The humidity entrainment of circadian clocks of various insect species**

(A) Top: Double-plotted actograms of insects in the humidity cycles and free-running period. Bottom: General activity patterns of insects in the last two days of the humidity cycles. Numbers of animals examined (n): vinegar flies, *Drosophila melanogaster* ($w^{1118}$, ♂) =124, kissing bugs, *Rhodnius prolix*us (♀) =38, spider beetles, *Mezium affin*e (sex unknown) =71, yellow fever mosquitoes, *Ae. aegypt*i (♀) =48, northern house mosquitoes, *C. pipiens* (♀) =59. (B) Twenty-four-hour activity of *Drosophila* during the humidity entrainment (n=115). One-way ANOVA and post-hoc Tukey HSD test were used for comparison to ZT12–14. ∗ denotes $p < 0.05$. (C) Comparison of activities of multiple insects in the dry and humid phases during the humidity entrainment. n for each species: kissing bugs=19, spider beetles=71, *Ae. aegypt*i=50, *C. pipiens*=59. ∗ denotes $p < 0.05$ in Student's t-test. (D) Rhythmicity of various insects in the free-

running period. Numbers above the bar graph: n for each species. (E-G) $w^{1118}$: representative actogram (E), general activity patterns of humidity cycles (F, n=44) and FRP (G, n=47). (H-J) Oregon R: representative actogram (H), general activity patterns of humidity cycles (I, n=26) and FRP (J, n=23). (K,L) Period (K) and Rhythmicity (L) of $w^{1118}$ (n=47) and Oregon R (n=23) in the FRP. (E-L) Flies were raised for entire life under LL and moved to DD for humidity assay.

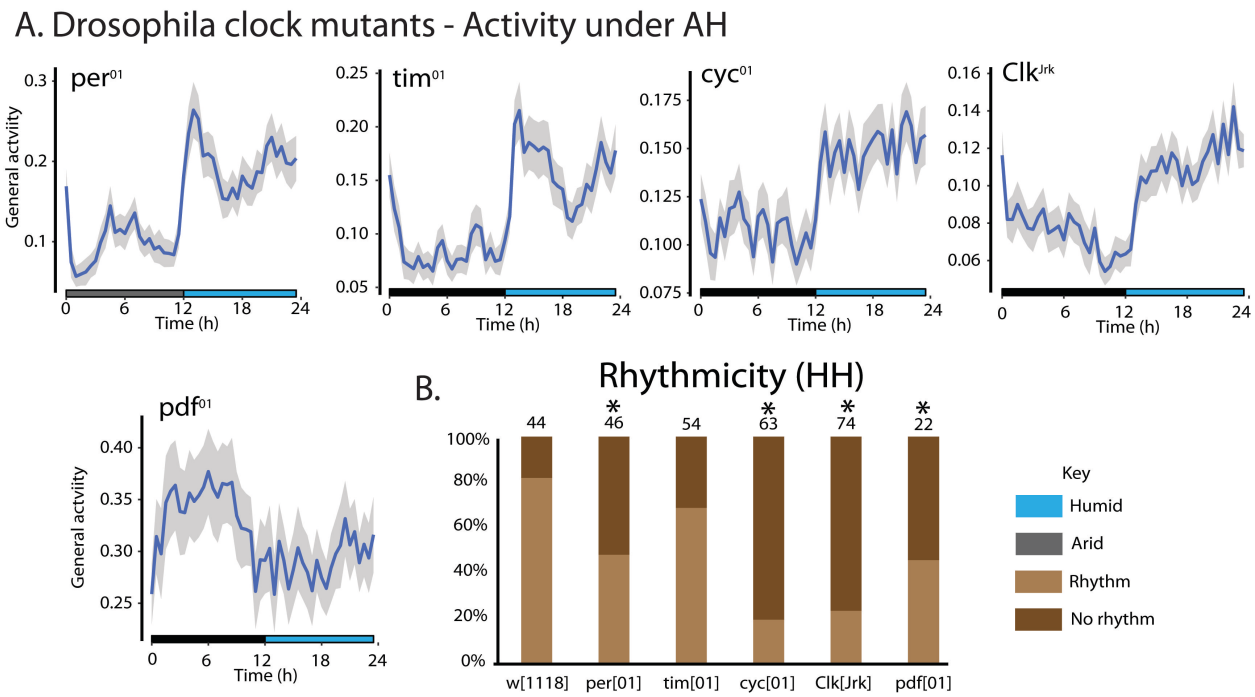


**Fig. 2. The rhythmic humidity-associated behaviors are under circadian control**
(A) General activity patterns of *Drosophila melanogaster* clock and *pdf*$^{01}$ mutants (♂) in the last two days of the humidity cycles. n: *per*$^{01}$=49, *tim*$^{01}$=58, *cyc*$^{01}$=62, *Clk*$^{Jrk}$ =75, *pdf*$^{01}$ =29. (B) Rhythmicity of clock and *pdf*$^{01}$ mutants in the free-running period. Numbers above the bar graph: n in each species. ∗ denotes significance ($p < 0.05$) in the Fisher exact test followed by the post-hoc Bonferroni test.

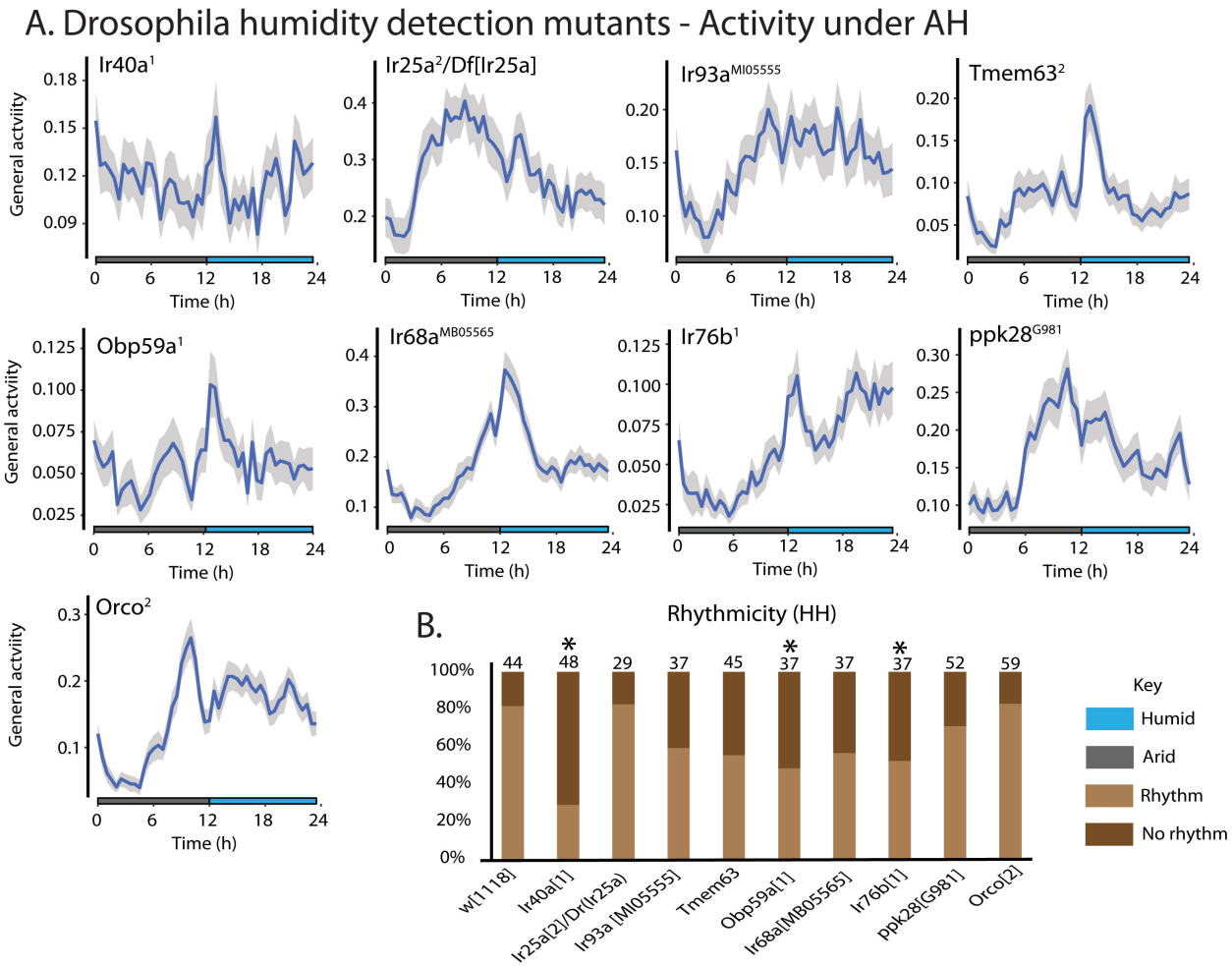


**Fig. 3. Hygrosensation is required for the humidity entrainment of circadian clock**
(A) General activity patterns of *Drosophila* hygrosensation mutants (♂) in the last two days of the humidity cycles. n: *Ir40a*$^{1}$=55, *Ir25a*$^{2}$*/Df(Ir25a)*=30, *Ir93a*$^{MI05555}$=57, *Tmem63*$^{2}$=45, *Obp59a*$^{1}$=46, *Ir68a*$^{MB05565}$=48, *Ir76b*$^{1}$=60, *ppk28*$^{G981}$=52, *Orco*$^{2}$=66. (B) Rhythmicity of hygrosensation mutants in the free-running period. Numbers above the bar graph: n in each species. ∗ denotes significance ($p < 0.05$) in the Fisher exact test followed by the post-hoc Bonferroni test.

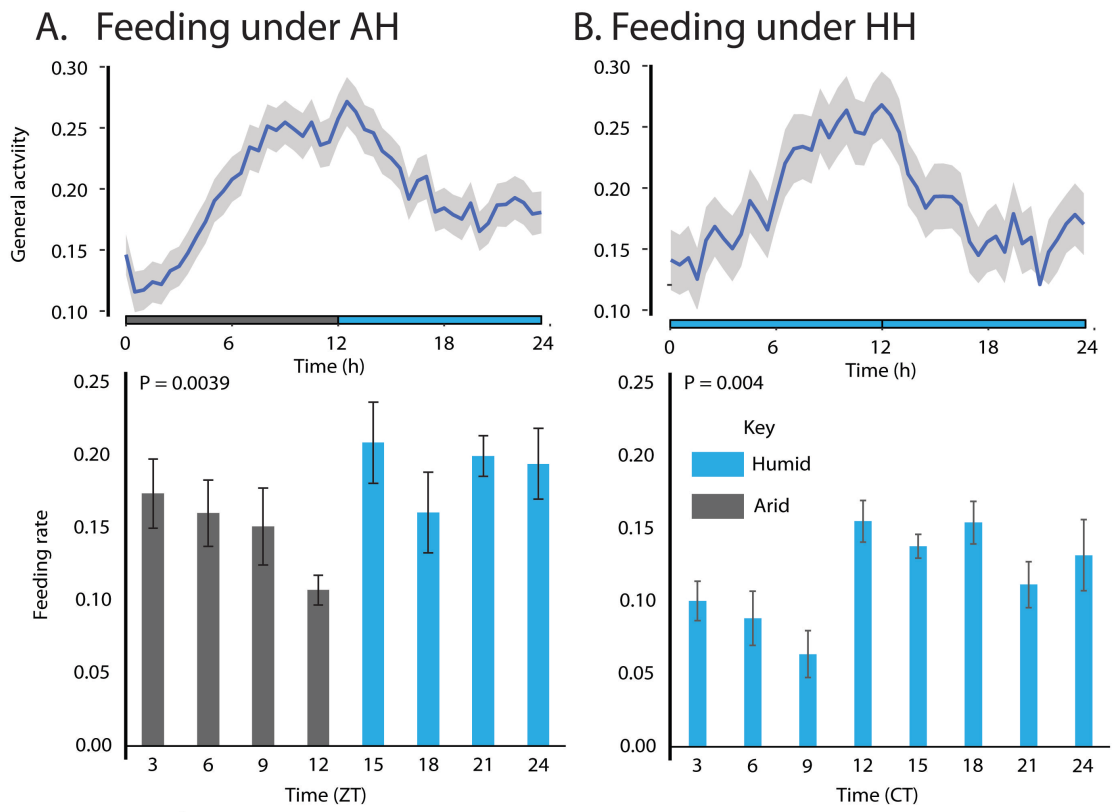


**Fig. 4. Sugar-feeding behavior of *Drosophila* is regulated by the humidity-entrainable clock** Rhythmic activity (top in A and B, n=124 and 121) and feeding behavior (bottom in A and B) of *Drosophila* w$^{1118}$ (♂) in DH and HH. (A) *Drosophila* sugar-feeding behavior in the humidity cycles (DH) was measured by CAFE assay at 3-hr intervals for 24 hours. Gray: dry phase; blue: humid phase. (B) CAFE assay in the free-running period (HH), same setup as in (A). The significance of the feeding patterns was examined with a general linear model. At least 5-13 replications for each group in DH and HH.

## References and Notes


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List of Supplementary Materials

Materials and Methods

Supplementary Text

Figs. S1 to S5

Tables S1 to S5

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