PNAS RESEARCH ARTICLE | GENETICS

Time series transcriptome analysis implicates the circadian clock in the *Drosophila melanogaster* female's response to sex peptide

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Contributed by Andrew G. Clark; received September 1, 2022; accepted December 28, 2022; reviewed by Yael Heifetz, David E. Krantz, and Gene E. Robinson

Sex peptide (SP), a seminal fluid protein of *Drosophila melanogaster* males, has been described as driving a virgin-to-mated switch in females, through eliciting an array of responses including increased egg laying, activity, and food intake and a decreased remating rate. While it is known that SP achieves this, at least in part, by altering neuronal signaling in females, the genetic architecture and temporal dynamics of the female's response to SP remain elusive. We used a high-resolution time series RNA-sequencing dataset of female heads at 10 time points within the first 24 h after mating to learn about the genetic architecture, at the gene and exon levels, of the female's response to SP. We find that SP is not essential to trigger early aspects of a virgin-to-mated transcriptional switch, which includes changes in a metabolic gene regulatory network. However, SP is needed to maintain and diversify metabolic changes and to trigger changes in a neuronal gene regulatory network. We further find that SP alters rhythmic gene expression in females and suggests that SP's disruption of the female's circadian rhythm might be key to its widespread effects.

time-course RNA-seq | gene regulatory network | *Drosophila* | sex peptide | circadian rhythm

Plasticity is essential for animals to respond to changing environments. In response to change, animals must adjust and coordinate behavior and physiology through the regulation of hormone levels, protein abundance or gene expression. How animals regulate and orchestrate complex behaviors and physiological processes on a molecular level is a field of active study. Often, molecules with pleiotropic effects are key to the plasticity of complex traits, to coordinate changes, and potentially trade-offs, across tissues. For example, the vertebrate hormone vasopressin regulates blood pressure as well as behaviors related to communication and aggression (1). Juvenile hormone, the insect hormone that is essential during metamorphosis, also regulates adult immunity, reproduction, and aging (2). Nonhormonal molecules can also exert pleiotropic effects. The yolk protein precursor vitellogenin is expressed in sterile honeybee workers, where it stimulates foraging rather than nursing behavior (3). The *foraging* gene, which encodes a protein kinase conserved from insects to vertebrates, affects traits ranging from food searching behavior to sleep and memory (4). Another example of a pleiotropic regulator is sex peptide (SP), a seminal fluid protein of *Drosophila melanogaster* males, which is transferred to females during mating. Unlike the examples above, SP acts as a pheromone, i.e., a molecule from one organism that elicits responses in another organism. SP has extensive pleiotropic, well-characterized effects on female physiology and behavior.

SP-mediated responses include increased egg laying and reduced receptivity to additional matings (5, 6), and SP is needed to release sperm from storage organs (7, 8). SP leads to an increase in juvenile hormone titers (9, 10), immunity changes (11, 12), alterations to the female's metabolism, gut morphology and excretion (13–15), changes in activity levels (16, 17), an increased appetite, with a preference for high-protein food (18–21), increased aggression (22), and an enhanced long-term memory (23). Many of these responses extend long-term due to SP's ability to bind to stored sperm, followed by its gradual release via active proteolytic cleavage. This allows SP to be stably maintained in the female and exert its effects for multiple days (24). The large number of changes induced by SP makes this an ideal model to study how a master regulator of physiology and innate behavior executes its pleiotropic effects.

Studies have shown that most SP-mediated responses require binding of SP to the SP Receptor (SPR), a G protein-coupled receptor that is expressed in the female's reproductive tract and central nervous system (25). Several studies have started mapping the neuronal circuits that are required to sense SP and to relay SP/SPR signaling. These studies have shown that a common set of neurons, sensory ppk^{+}/fru^{+} neurons that innervate the reproductive tract (26–28), which connect to neurons in the abdominal ganglion (SP Abdominal

Significance

Regulation of *Drosophila* female postmating responses by the seminal fluid protein sex peptide forms an excellent model to study the orchestration and plasticity of complex behavioral and physiological changes. We combine longitudinal transcriptomics with a systems biology approach to construct regulatory networks that respond to sex peptide in female heads. Our approach gives insight into the molecular architecture and regulation of the postmating response. We find that changes in the female's circadian gene expression connect individual networks that likely underlie distinct physiological processes and behaviors. These results suggest that a male seminal fluid protein can alter the female's circadian rhythm to coordinate behavioral and physiological changes postmating. This finding has implications for our understanding of both reproductive and circadian processes.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2214883120/-/DCSupplemental.

Published January 27, 2023.

Ganglion neurons), which in turn project to the pC1 region in the brain (29–35), are all required for SP's effects on female receptivity (36), oviposition (29), sleep (37), and feeding behavior (18). Together, these studies show that at least some of the postmating responses induced by SP share a common neuronal basis, and they show that although SP is stored in the female reproductive tract, its signals result in a profound impact on the female's brain.

In addition to changes in neuronal signaling, SP causes changes in RNA abundance throughout the female body (12, 13, 38). However, knowledge of when and how SP taps into gene regulatory networks to mediate these expression changes across tissues remains elusive. Temporal aspects are clearly important in the female's response to mating. Gene expression in mated females changes over time (38–44), and distinct aspects of mating, such as seminal fluid proteins, sperm, or pheromones, exert their effects within different time windows after mating (45–49). Related to how SP alters regulatory networks, we do not know whether gene networks that are regulated by SP act independently of each other, or whether they are under the control of a shared molecular regulator that exerts pleiotropic effects downstream of SP. Fine-scale temporal transcriptomics analysis provides a powerful tool to investigate the genetic architecture of a response, as it can distinguish primary from secondary (and later) responses, pinpoint potential regulators and coregulated targets, and can be used to construct gene interaction networks (50–52). Thus, to address gaps in our knowledge regarding the genetic architecture of the complex postmating response, we analyzed time series gene expression data of the female head at 10 time points within the first 24 h after mating with a control or a SP null male.

Results

We sampled heads of females that were virgin (V) and at 0.5, 1, 2, 3, 4, 5, 6, 8, 12, and 24 h after mating with either a control male (SP+) or a SP null male (SP–) (Fig. 1*A*). We sampled more densely at early time points to capture the earliest regulators that act downstream of SP. Aside from neuronal tissue, heads include fat body, a multipurpose tissue that regulates metabolism, reproduction (through the synthesis of yolk proteins), immunity (through the synthesis of antimicrobial peptides), and feeding (through signaling to insulin-producing cells in the brain) (53). RNA-seq of heads provided us with the opportunity to interrogate the contribution of both neuronal and fat body tissues to SP's pleiotropic effects.

Mating and SP Alter RNA Abundance at the Gene and Exon levels. We first performed analyses to detect differential gene expression and differential exon use in response to SP and mating. Changes in

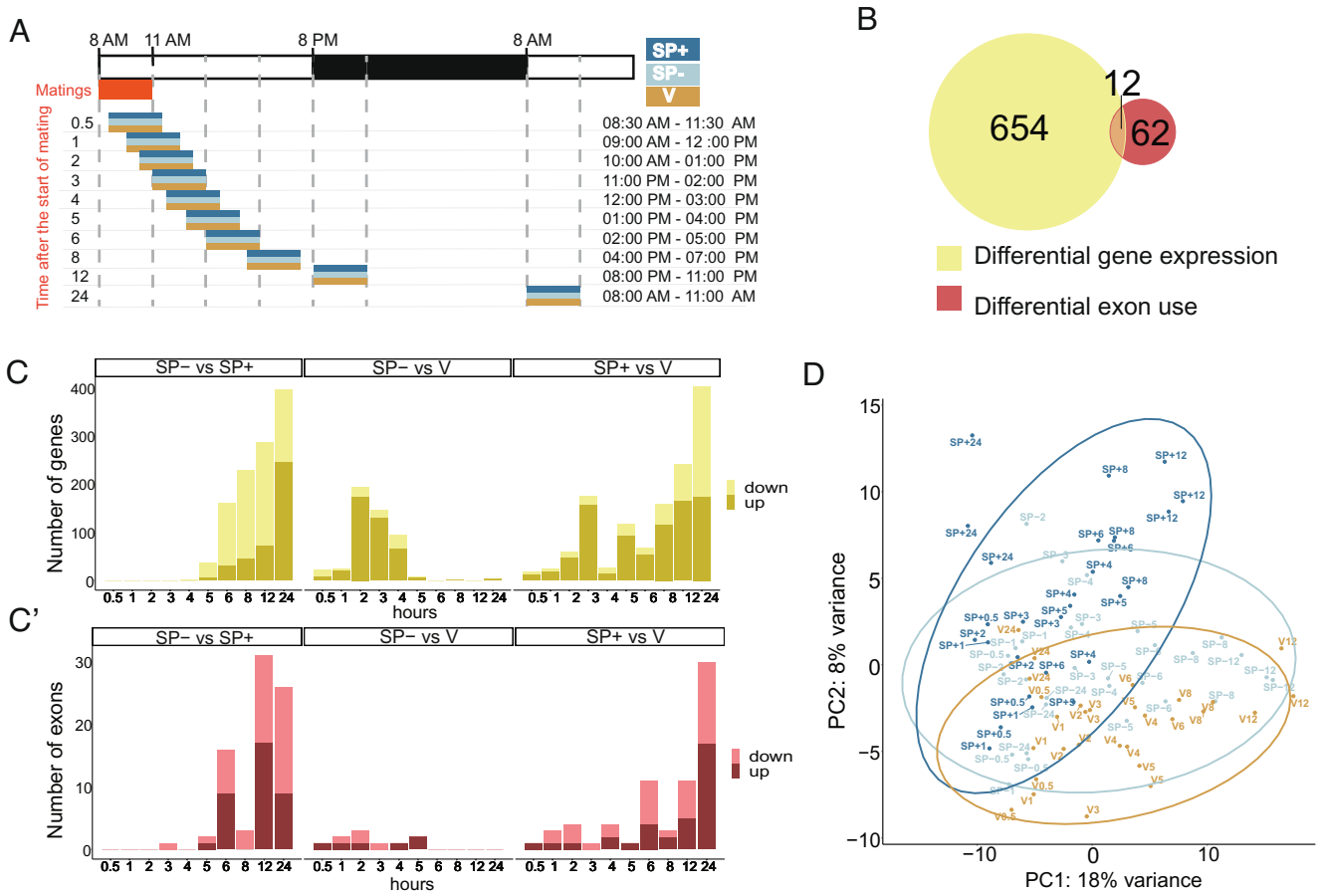

Fig. 1. A time series RNA-sequencing dataset of the female head's response to SP and mating. (*A*) Design of the time series mating assay. Three replicates of the time series were run on female heads of virgins (V), females mated to wild-type males (SP+) and females mates to SP null males (SP-). (*B*) Number of differentially expressed genes, and number of genes with significant differential exon use at different time intervals after mating, with or without SP. (*C*) Number of differentially expressed genes at each time point, for each pairwise contrast. (*q-value* <0.05 at the indicated time point; each gene has at least a 1.5-fold change in expression at minimum one of the time points.) (*C'*) Number of differentially used exons at each time point, for each pairwise contrast. (*q-value* <0.05 at the indicated time point; each exon has at least a 1.5-fold change in expression at minimum one of the time points.) (*D*) Principal component plot generated using gene-level normalized counts for the 500 most variable genes in the dataset. Samples in the plot are indicated using treatment (V = virgin, SP+ = mated with WT male, SP− = mated with SP null male) and time point.

exon usage suggest that an alternative splice form or transcription start site is used after mating, which might impact the function of the gene product or drive expression changes in specific tissues or cell types. A complex molecular organization with multiple promoters and/or splice forms can be fundamental to genes' pleiotropic effects (4, 54). We hypothesize that such genes could play an important role in relaying signals from, or responding to, SP. The use of alternative transcript isoforms has been reported for specific genes in the postmating response (55–57), but has not previously been investigated at a genome-wide level in *D. melanogaster*. Using pairwise comparisons between the three treatments, with a *q-value* threshold of 0.05 and requiring at least a 1.5-fold change in expression at minimum one time point, we found 666 genes and 86 exons with differential read counts across treatments (Datasets S1 and S2). Of these differentially expressed features, 223 genes and 20 exons underwent at least one twofold change in expression between the treatments. The 86 exons mapped to a total of 74 genes. We found little overlap between genes that are differentially expressed across the gene body, and genes that contain one or more differentially used exons. Only 12 genes were found in both categories (Fig. 1*B*), and genes that are differentially expressed across the gene body, and genes with differential exon use, were enriched for distinct Gene Ontology (GO) terms (*SI Appendix*, Fig. S1). Among the genes with differential exon use we detected *foraging* (differential use of exon *009*) and *Desat1* (differential use of exons *002* and *005*; *SI Appendix*, Fig. S6*B*). *E002* and *E005* make up two of a total of five alternative first exons of the *Desat1* gene. *Desat1* has roles in both synthesis and perception of pheromones through its use of different promoters and expression in different cell types (54). While the *foraging* gene influences several behaviors, *foraging* exon *009* is part of a subset of transcripts of *foraging,* which are all under the control of the fourth promoter of this gene, and which have been implicated in adult foraging behavior (58). Quantitative PCR confirmed that indeed only transcripts of *foraging*'s fourth promoter are up-regulated in the head after mating, and not transcripts controlled by promoters 1, 2, or 3, at least with a bulk approach (*SI Appendix*, Fig. S2). Taken together, mating and SP alter RNA abundance at both the gene- and individual exon-levels, and analyses at both levels yield complementary information.

Mating and SP Alter RNA Abundance within Distinct Time Frames. Since we expect that important regulators downstream of SP will show the earliest expression changes, we investigated the temporal dynamics of the transcriptome postmating. We examined the number of significant expression changes at each time point for each pairwise contrast, and strikingly similar results were found for differentially expressed genes and differentially used exons (Fig. 1 *C* and *C'*). In mated females that received SP, two waves of transcriptome changes occur: a smaller one before 4 h and a larger one after 4 h postmating. Only the first, smaller wave is also seen in SP- vs. V, indicating that it occurs independently of SP. The second, larger wave of transcriptome changes is fully dependent on SP. Similar patterns were observed in a principal component plot (Fig. 1*D*). Thus, contrary to our expectations, SP is not required to trigger the earliest transcriptome changes in the female head. Instead, SP is essential to maintain and diversify transcriptome changes, starting from around 5 h after mating. At this time, unstored sperm and most seminal fluid proteins have been ejected (59, 60), but SP remains abundantly present in the female through its binding to stored sperm (24). Given a report that males that lack SP demonstrate altered transfer of a subset of seminal fluid proteins (61), it is possible that these proteins also contribute to the transcriptome differences of females mated to SP-null vs. control males.

Metabolic and Neuronal Regulatory Networks Respond to Mating and SP. To investigate temporal expression changes in more detail and understand how SP coordinates changes across functional classes of genes, we selected a larger set of differentially expressed genes and differentially used exons (*SI Appendix, Materials and Methods*), and assigned these to one of three groups, guided by the patterns observed in the principal component plot. First, we grouped 368 features whose first significant change in expression was induced by a non-SP aspect of mating and occurred within 30 min to 4 h postmating ("group 1"). Second, we grouped 369 features whose first significant change in expression was induced by SP, and occurred within 5 to 12 h postmating ("group 2"). The final group contained 568 features whose first significant change in expression was induced by SP at 24 h postmating ("group 3"; Fig. 2*A*). We then used a computational approach to identify groups of coregulated features, and the tissues or cell types in which they might undergo changes in response to SP. Specifically, we constructed gene regulatory networks using hierarchical clustering based on lead-lag correlation (LLR^2) values as a similarity measure [(62) and *Materials and Methods*]. We used motif enrichment analysis to link differentially expressed transcription factors with potential target genes, and we deconvoluted our bulk RNA-seq dataset using publicly available single-nucleus RNA-seq data of the fly head to predict in which cell types expression changes might occur (63, 64).

Within group 1 (features with initial expression changes 30 min to 4 h postmating), we found a network that consists of mostly up-regulated genes with roles in amino acid metabolism, ribosome biogenesis, and DNA replication (Fig. 2*B*). Expression changes in this network were initiated in the absence of SP, but maintained past 5 h postmating only in the presence of SP. The transcriptional regulators in this network, *CrebA, Clk, Myc,* and *tj*, underwent expression changes 1 to 2 h before expression changes in their predicted targets were visible (Fig. 3*A* and *SI Appendix*, Fig. S3). *CrebA* is a regulator of genes involved in the canonical secretory pathway (65). *Clk* (*Clock*) encodes a regulator of the circadian clock (66), and the conserved protooncogene *Myc* stimulates ribosome biogenesis (67). Within group 2 (features with initial expression changes 5 to 12 h postmating), we identified networks of mostly up-regulated genes with roles in amino acid metabolism, and mostly down-regulated genes with roles in glycogen biosynthesis (Fig. 2*B* and *SI Appendix*, Fig. S5). Expression changes in these networks were maximal at 12 h after mating—nighttime in our time series. Of the differentially expressed genes in groups 1 and 2 that could be assigned to a specific cell type based on deconvolution analysis, most were assigned to fat cells (Fig. 2*C*). This suggests that mating and SP stimulate cell growth and protein synthesis and down-regulate carbohydrate metabolism in the fat body, an important metabolic tissue and site for yolk protein synthesis and secretion after mating (68). Indeed, *Yolk protein 2* and its known transcriptional regulator *dsx* (69, 70), and a *dsx* exon (*dsx:E007*), were also found to be part of groups 1 and 2 (*SI Appendix*, Fig. S4).

Within group 3 (features with significant changes only at 24 h postmating), we identified a network of genes with neuronal functions and functions in translation and cellular respiration (Figs. 2*B* and 3*B*). These genes were mostly down-regulated at 24 h after mating, in a SP-dependent manner. Deconvolution of the bulk RNA-seq data in group 3 assigned the largest number of genes (63 genes) to "fat cell", but in second place is "sensory neuron" with 43 genes (Fig. 2*C*). We were able to link a total of 45 transcription factors to group 3, either because they were themselves part of group 3, or because they were predicted to regulate genes in group 3 based on motif enrichment. These 45

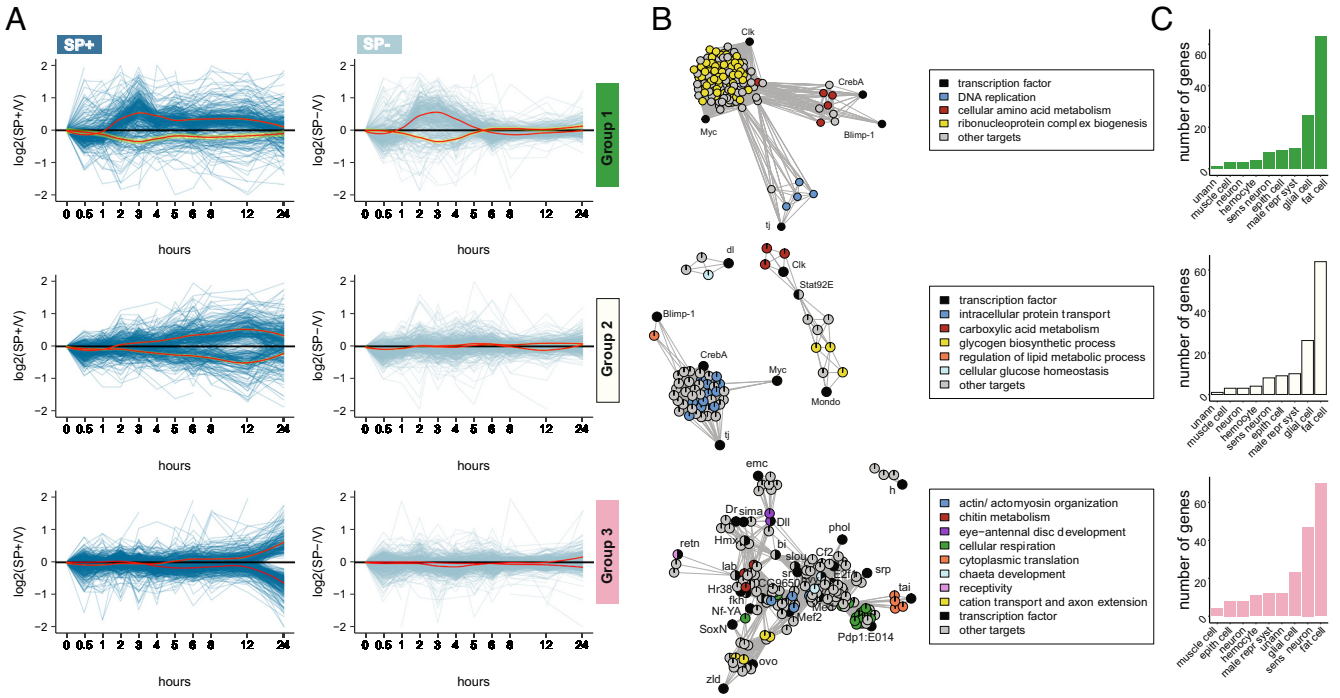

Fig. 2. Mating and SP alter metabolic and neuronal regulatory networks. (*A*) Temporal expression changes induced by mating and SP. Y-axis limits were set to −2 and +2 to show representative expression changes more clearly. Red trendlines were obtained using lowess smoothing. Yellow shaded areas show 95% CIs. Within each group, lowess smoothing was applied separately to genes with a mostly up- or downward trend in SP+ females (for group 1 based on up- vs. downregulation at 3 h postmating; for group 2 based on up- vs. downregulation at 12 h postmating; for group 3 based on up- vs. downregulation at 24 h postmating). (*B*) Networks showing transcription factors and predicted target genes, constructed using temporal expression differences between SP+ and virgin females. Edges represent distances between features based on LLR^2 values. Only edges are shown between transcription factors and predicted targets, and among predicted targets of the same transcription factor. Nodes are colored based on significant GO term enrichment (*q*-value < 0.05). (*C*) Assignment of differentially expressed genes to annotated cell types according to scMappR deconvolution analysis (sens neuron = sensory neuron; epith cell = epithelial cell; unann = unannotated; male repr syst = male reproductive system).

transcription factors are enriched for the GO term "nervous system development" (*SI Appendix*, Fig. S6*A*), and 33/45 transcription factors are markers of neuronal cell types based on single-nucleus RNA-seq (64) (Fig. 3*C*). In addition to genes with neuronal functions, this group also contains genes with roles in pheromone biosynthesis and detection (*dare, Desat1, Desat1:E002* and *Desat1:E005, FASN2, Obp19b, Obp19c, Obp56h, Obp99a*; *SI Appendix*, Fig. S6*B*) (54, 71–73). We speculate that the downregulation of neuronal transcription factors and predicted targets might reflect a decreased function of certain neurons in the mated female's head. This could also explain the coordinated downregulation of genes involved in translation and respiration (also

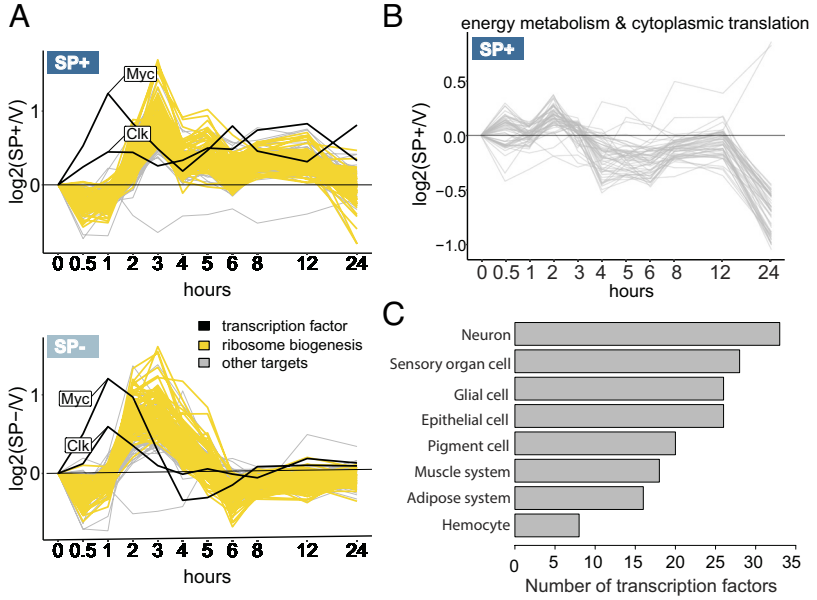

Fig. 3. Cluster and motif enrichment analyses identify genes and transcription factors with distinct temporal patterns and roles in the female's response to SP. (*A*) Log_2 fold changes of mated (SP+ or SP−) vs. virgin (V) females for transcription factors and predicted targets, for a subset of features in group 1. Predicted targets are colored based on significant GO term enrichment (*q-value* < 0.05). (*B*) Log_2 fold changes of mated (SP+) vs. V females for genes in group 3 involved in cytoplasmic translation and energy generation. (*C*) Intersection between 45 transcription factors linked with group 3 and known marker genes for indicated head cell types based on single-nucleus RNA-seq data from (64).

observed by ref. 74), since energy metabolism is often used as a reflection of a neuron's activity (75). A decreased activity of certain neurons in the head could contribute to altered sensory and behavioral responses in mated females that received SP.

Overall, our results show that SP alters the expression of multiple transcription factors, likely to exert local effects on metabolism or neuronal function in specific tissues. The in silico assignment of genes to specific cell types based on integration with single-nucleus RNA-seq data (64) is approximate but useful to highlight which networks are likely regulated in the fat body vs. neurons. These results can help inform single-cell studies with regard to the best time point to sample depending on whether researchers are interested in metabolic vs. neuronal changes postmating.

Mating and SP Alter the Expression of Features that Have a Circadian Rhythm in V Females. So far our analysis identified networks that respond to SP, but it did not give information about how SP coordinates changes across networks. Because a non-SP aspect of mating (in the first 4 h postmating) and SP (after 4 h) up-regulated the expression of *Clk,* and because SP influences behaviors that are under circadian control (16, 17), we investigated whether additional features with circadian rhythms were differentially expressed after mating. We found that of the 1,305 differentially expressed features from the network analysis, 18% (232 features) follow a circadian rhythm in V females (*q-value* <0.3, Fig. 4*A*). These include known regulators of the clock [*Clk* and *tim* (Fig. 4*B*)*, cwo, CG31324/Cipc* and an exon of *Pdp1*, *Pdp1:E014*], and two genes that were found to be putative regulators of circadian rhythms by ref. 76 (*CG14688* and *Gclm*). Some genes, such as *tim* or *Clk*, are up- or down-regulated by SP but maintain their circadian expression pattern across 24 h; many other genes lose their circadian rhythm after receipt of SP (Fig. 4 *A* and *B*).

SP affects rhythmic expression across networks: 20% of features in group 1, 25% of features in group 2, and 12% of features in group 3 have altered circadian expression patterns due to SP. Both metabolic and neuronal features are impacted, including candidates that might be involved in the receptivity postmating response [*retn*; part of group 3; Fig. 4*B*; (77)], and metabolic or feeding-related postmating responses [*fit*; part of group 2 (Fig. 4*B*; (78)) and *for:E009*; part of group 1 (58)].

Other genes whose circadian expression pattern is altered by SP are involved in ribosome biogenesis and glycogen biosynthesis. Ribosome biogenesis genes are up-regulated at night in all females but are also up-regulated during the day in SP+ females (Fig. 3*A*). Features involved in glycogen biosynthesis and the transcription factor *Lime*, previously found to play a role in maintaining levels of glycogen (79), have lower expression in SP+ vs. V females at night (*SI Appendix*, Fig. S5). SP alters the expression of two additional genes of interest, *CNMa* (part of group 1) and *sand* (part of group 2; Fig. 4*B*), which were not among the genes with a circadian rhythm in V females based on the MetaCycle analysis. However, *sand* is involved in sleep regulation (80), and *CNMa* is expressed in clock neurons and stimulates wakefulness (81, 82). Both genes' upregulation throughout the afternoon might contribute to the SP-induced loss of siesta sleep described by (16).

A possible mechanism for how SP can alter females' circadian expression patterns, is via connections between neurons known to respond to SP and neurons that regulate circadian behaviors in the brain. To investigate this possibility, we used the neuPRINT+ connectome database's "Shortest Path" search (83). We used this tool to determine whether there is a direct synaptic connection between two neurons of interest, an indirect connection via one or more other neurons, or no connection at all. We examined connections from neurons that respond to SP (pC1) to neurons involved in the regulation of circadian behaviors. pC1 neurons are silenced downstream of SP binding to the SPR, and this silencing is essential for multiple postmating responses (18, 29–37). Several types of neurons in the brain have been shown to influence circadian behaviors. Among these are central clock neurons, which function as core circadian pacemakers (84). In addition, there are other brain regions that act downstream of these pacemakers to regulate circadian processes: P2 neurons regulate sleep and feeding behavior (85); and neurons of the pars intercerebralis and dorsal fan-shaped body influence among others rest:activity cycles (86, 87). We identified a direct synaptic connection between a pC1 neuron and a dorsal lateral (LNd) neuron of the core clock system. We identified connections with one connecting neuron between pC1 neurons and subsets of lateral posterior LPN neurons, LNd and dorsal DN1 neurons of the clock, and a subset of neurons of the dorsal fan-shaped body and the pars intercerebralis (*SI Appendix*, Fig. S7 and Dataset S6). We further found that superior medial protocerebrum SMP454 neurons and oviposition inhibitory neurons (oviIN) were frequently used to connect pC1 neurons to neurons that regulate circadian behaviors. Of the paths with 1 or 2 connecting neurons, oviIN neurons were used in the path in 43% of cases, and SMP454 neurons in 52% of cases (Dataset S6). Not much is known about SMP454 neurons. The oviIN neurons are female-specific neurons that regulate oviposition (29). Our observations suggest that oviIN neurons might have a more general role in relaying SP signaling, not only targeted to oviposition regulation, but more generally allowing SP to alter signaling in neurons that regulate circadian behaviors.

In summary, SP alters the transcript abundances of circadian genes, including core molecular regulators of the clock. The postmating change in circadian expression is present across networks and likely plays an important role in SP's ability to coordinate behavioral and physiological changes in the female. The connectome database search indicates that the neuronal infrastructure needed for SP to influence the female's clock exists and brings up candidate neurons to investigate in functional assays.

Discussion

Reproduction is only possible by successfully completing a set of defined temporal events. In *D. melanogaster,* these events include complex stereotypical, timed behaviors that occur during courtship (88), and postcopulatory events, which include sperm storage, the ejection of unstored sperm and seminal fluid proteins, the start of increased egg production and ovulation (59, 89), and changes in behavior and physiology, many of which require the receipt of SP (reviewed in refs. 90 and 91). This makes the female's response to SP a convenient system to investigate how dynamic changes in molecular pathways are coordinated across tissues. There is evidence that for SP to exert its pleiotropic effects, it makes use of molecules within the female that have pleiotropic effects themselves. For example, SP increases levels of juvenile hormone in females (10). Juvenile hormone in turn stimulates both yolk production and midgut growth (92, 93). Our fine-grained time series analysis brings up additional genes, such as *foraging*, that have pleiotropic roles, that were not previously implicated in the postmating response, and that SP might use to coordinate changes postmating. The striking alterations in circadian expression patterns suggest that SP might also use the female's circadian rhythm to exert its pleiotropic effects. Surprisingly, we also found that SP is not the only molecule that can initiate changes in circadian genes. For example, the core circadian regulator *Clk* was differentially expressed in the absence of SP within the first 4 h after

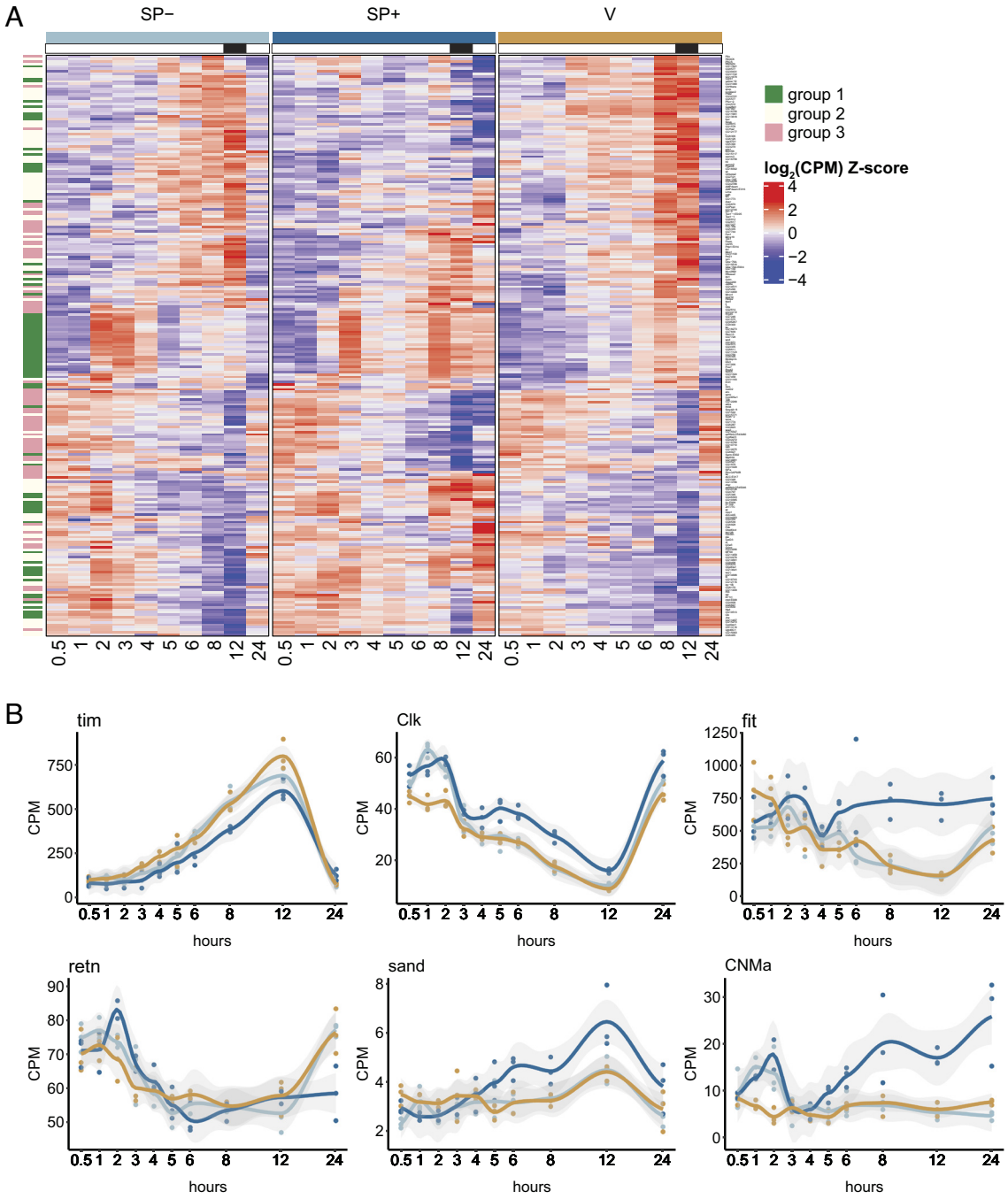

Fig. 4. Mating and SP alter the expression of genes and exons that follow a circadian rhythm in virgin females, or that influence circadian activity. (*A*) Row-normalized Z- scores of median $\log_2$(CPM) (counts per million) across available biological replicates, for genes and exons that have a circadian rhythm in virgin females according to MetaCycle analysis (*q-value* < 0.3). (Genes names are legible by zooming). (*B*) CPM values for selected genes. At each time point, biological replicates are indicated with dots. Lines are obtained using lowess smoothing. Gray shaded area shows the 95% CI.

mating, highlighting additional regulatory complexity of the female postmating response.

Rapid Postmating Transcriptome Changes Occur in the Absence of SP. SP is often described as a "master regulator" of female postmating responses, needed to "switch" a female from a V into a mated state (91). However, during the first 24 h after mating, we found that gene- and exon-level changes show a two-phase response, undergoing SP-independent changes within the first 4 h after mating, and SP-dependent changes after 4 h. The absence of SP-dependent changes before 4 h postmating could indicate that SP is dispensable for the earliest changes, but we cannot rule out the possibility that SP might elicit small, cell type-specific changes that are not easily detected using bulk RNA-seq. A single-cell RNA-seq experiment with 10 time points and three treatments comes with multiple challenges, including logistics, cost, and less power to look at alternative splice forms. We propose instead that our bulk RNA-seq time series can be used to inform the design of future single-cell RNA-seq experiments to investigate if SP is required for any transcriptome changes in specific cell populations within the first 4 h after mating.

Our finding that there are early transcriptome changes that do not require SP shows that, despite being crucial for long-term postmating responses and increased reproductive output, SP does

not solely control the initial V-to-mated switch. This observation brings up more questions. Why do at least some SP-specific transcriptome changes show up only after 4 h? In addition, which molecules are responsible for the early transcriptome changes? We propose two different but not exclusive mechanisms to potentially explain the two-phase response.

First, like several other seminal fluid proteins, SP enters the female hemolymph (94–99). Via the hemolymph, SP could exert rapid and direct effects on head tissues, but only briefly, since SP gets degraded in the hemolymph (94). Why then do we not see rapid SP-specific effects in the transcriptome data? It is possible that other male molecules that are in the female short-term act redundantly with SP and compensate for the lack of SP in the first 4 h after mating with a SP-deficient male. A candidate is the seminal fluid protein Dup99B, which has a high sequence similarity to SP (100), can bind the SPR (25), but is not found on sperm for longer than 5 h after mating (24, 101).

Alternatively, it could take longer for SP to influence the female's head transcriptome. The major way by which SP influences the female is via neuronal signaling (25–35). This requires binding of SP to sperm, sperm storage, proteolytic cleavage to release SP's active region from sperm, followed by its receptor binding, and neuronal signaling to the brain (91). These steps likely take time, which might be why other molecules need to "cover" for SP to regulate transcriptome changes in the first 4 h postmating. There is some evidence for this model. For example, female attractiveness is reduced by male-derived pheromones within the first few hours after mating, but later depends on sperm and accessory gland proteins (a subset of seminal fluid proteins that includes SP) (48, 49, 102). Thus, males make use of pheromones to prevent female remating until SP-induced responses kick in. A similar principle might apply to early head transcriptome changes, which affect predominantly metabolic genes: Males might use non-SP aspects of mating that are in or on the female short-term to prepare the female's fat body for increased egg production in advance of SP's effects.

SP Regulates the Female's Circadian Transcriptome. The time series sampling pattern allowed us to place the expression changes of genes and exons, and of the transcription factors that are predicted to orchestrate these changes, within the context of day and night. Across metabolic and neuronal regulatory networks, we identified marked time-of-day-dependent differences between mated and V females. Our results suggest that SP's influence on the female's circadian rhythm might be key to its varied and widespread effects. This hypothesis makes sense in light of our knowledge of postmating responses, since many of these affect circadian processes like sleep, feeding, receptivity, oogenesis, and egg laying (16, 20, 103–105).

If SP can alter circadian rhythms in females, the question becomes how it achieves this, and at what level SP signals are integrated in the female's clock. SP appears to override the clock, since circadian expression patterns are altered despite sustained light/dark cycles. SP may induce a "jetlag" in females since its effects do not simply shift circadian gene expression patterns. Instead, expression patterns are altered in different ways across different gene sets, suggesting a desynchronization of rhythms across neurons or peripheral tissues. Since synaptic connections exist between pC1 neurons and clock neurons, there is a possibility for SP to directly modulate the female's clock, thereby allowing SP to control many different postmating responses at once. Through *ppk*+ neurons that express SPR, SP alters neurotransmitter release from central clock ventral lateral (LNv) neurons, leading to loss of predawn elevated activity in mated females (17). LNv are called "morning neurons", and LNd (for which we found a direct connection with pC1) are called "evening neurons" (106). SP might make use of separate neuronal connections to influence both clock systems. These observations motivate follow-up studies to fully map the neuronal pathways that allow SP's integration into the female's clock system, and to determine which types of neurons and nonneuronal cell types exhibit clear shifts in circadian gene expression patterns.

This potential mode of action, through the female's clock, fits with an experimental evolution study that has shown that sexual conflict might drive males to manipulate the female's central nervous system (107). If SP truly acts on the female's circadian clock to establish postmating responses, this could have implications for the evolution of sexual conflict. If SP were to influence independent regulatory networks to trigger postmating responses, females might more easily adapt to escape one of its effects, such as the SP-induced reduction in receptivity to other males, which benefits the female's mate, but not necessarily the female herself. By acting on the female's circadian rhythm, SP could control many physiological and behavioral changes via one interconnected neuronal network, making it more difficult for the female to escape some of SP's costly effects without also losing access to beneficial effects. Population-level variation in female postmating circadian rhythm changes could be used to further investigate this hypothesis.

In conclusion, using a high-resolution time-course RNA-sequencing dataset of female heads, we have shown that a non-SP aspect of mating regulates a network of metabolic genes in females shortly after mating, and that SP is needed to sustain and diversify these changes. We further found that these regulatory networks themselves might be under shared circadian control. Future research opportunities lie in understanding mechanistically how signals from mating modulate the female's clock system, how that translates into expression changes in peripheral tissues, and what the significance of this is for the evolution of sexual conflict.

Materials and Methods

Fly Stocks and Husbandry. All flies were maintained on standard yeast/ glucose medium in a 12-h light/ dark cycle (lights on 8 AM, lights off 8 PM), at 25 °C. SP null mutants (SP−) and matched controls (SP+) were generated using the SP^0/*TM3,Sb* and *Δ130/TM3,Sb* lines (6). Females used in the experiment were from a *Canton-S* strain. All flies used in experiments were V and were aged for 3 to 5 d after eclosion. The absence of SP in SP- males was verified using western blot and a receptivity assay (*SI Appendix*).

Time Series Mating Assay. All matings were performed at room temperature (21 °C). We collected three independent biological replicates by repeating the entire experiment in three consecutive weeks. For each repeat experiment, flies were derived from independent parental flies. The afternoon before the day of the mating assay, females were isolated in individual vials. On the day of the mating assay, matings were set up starting at 7:30 AM, by adding one male (either SP null or control) to each vial with a female fly. Vials with females designated to remain V did not receive a male. Females were given three hours to mate, between 8 AM and 11 AM. Across all replicates, only two flies mated before 8 AM, and these were excluded from the experiment. All matings were observed at least once every 5 min to record the start time of mating and to ensure that each mating lasted at least 10 min. Females were frozen using dry ice at their designated time point after mating (30 min, 1, 2, 3, 4, 5, 6, 8, 12, or 24 h after mating), and V females were frozen in parallel, and stored at −80 °C.

Head Collection & Library Prep. To dislodge fly heads from the thorax, vials with female flies were dipped in liquid nitrogen and vortexed for 3× 5 s, dipping vials in liquid nitrogen in between each round of vortexing. Dislodged heads were collected manually in Trizol, homogenized using a mechanical rotor and plastic pestle, and stored at −80 °C. For each sample, 3 to 12 heads were collected, with a median of 6.5 heads. To extract RNA, samples in Trizol were thawed on ice. We extracted RNA and performed an on-column DNase treatment

using the Directzol RNA microprep kit (Zymo, CA), according to the manufacturer's instructions. Sample concentration and purity were determined using Qubit and Nanodrop (Thermo Fisher Scientific). RNA integrity was verified for a randomly selected subset of seven samples using the Fragment Bioanalyzer (Agilent) at the Cornell Genomics Facility. The minimum RNA Quality Number (RQN) obtained was 9.4. Poly(A)-selected, stranded libraries were made using the NEBnext Ultra II Directional RNA library prep kit and the Poly(A) messenger RNA magnetic isolation module (New England Biolabs). Library concentrations were determined using Qubit (Thermo Fisher Scientific) and a Fragment Bioanalyzer (Agilent) was used to inspect library size range and ensure absence of primer dimers. Libraries were sequenced using NextSeq) 500 (Illumina) at the Cornell Genomics Facility. All 90 samples were sequenced in the same lane, and this was repeated five times to obtain adequate read depth.

Read Quality Control, Processing, and Alignment. Quality control (QC) of sequenced libraries was performed using FastQC (version 0.11.8; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequencing adaptors were removed using Trimmomatic (version 0.39) (108), which was also used to trim low-quality bases at the 5′ end of reads (using parameters TRAILING:20 and SLIDINGWINDOW:5:20). Reads with a minimum length of 30 bases were retained. Reads were aligned to the *D. melanogaster* genome (dm6 version r6.24) using STAR [version 2.7.5a; (109)] and per-gene read counts were determined using HTSeq [version 0.11.2; (110)]. Because we obtained five fastq files for each of the 90 samples, we initially ran the QC, alignment, and read counting pipeline on each fastq file separately. We then performed a principal component analysis (PCA) to check for lane effects (*SI Appendix*, Fig. S9). Since no lane effects were observed, we merged the five fastq files for each sample. After merging, the median read count per sample was 22.4 million reads.

Read Count Filtering, PCA & Correction for Batch Effects. Raw counts were filtered to keep only genes with at least three counts per million [calculated using edgeR; (111, 112)] in at least three samples and genes encoded in mitochondrial DNA were removed. This left 9,027 expressed genes in the dataset. We performed a PCA to identify potential outlier samples and batch effects and corrected for batch effects using ComBat-seq [(113) and *SI Appendix*].

Differential Gene Expression and Differential Exon Use Analysis. To identify genes that were significantly differentially expressed between treatments across time, we used three methods [DESeq2 (114), Limma-Voom (115) and ImpulseDE2 (116); *SI Appendix*]. We selected genes with a *q*-value <0.05 [Benjamini–Hochberg correction; (117)] in at least two of the three methods, and with a minimal absolute shrunken (118) fold change of 1.5 or 1.3, at least at one time point (Dataset S1). While shrunken $\log_2$ fold changes were used to select differentially expressed genes, the original $\log_2$ fold changes were used in all other analyses and figures. We used DEXSeq (119) to quantify differential exon use (for details see *SI Appendix*). The final selection of differentially expressed exons is listed in Dataset S2. This table also contains the start and end location of each exon.

Cluster and Network Analysis. For all pairs of differentially expressed features (genes and exons), we determined the similarity of their expression profiles, represented as $log_2(SP+/V)$, using the LLR^2 method (62). This method uses empirical Bayesian regression and prior biological evidence of association to fit ordinary differential equation models to temporal feature profiles (for more details see *SI Appendix*). Gene and exon cluster assignment can be found in Dataset S4.

RcisTarget was used for transcription factor motif enrichment analysis (120). We applied default settings and the database "dm6-5kb-upstream-full-tx-11species.mc8nr" and used all 9,027 expressed genes in our dataset as background. RcisTarget was run on each cluster defined by the LLR^2 method. Of all transcription factors that were identified using RcisTarget (both using direct inference and based on homology), we only kept ones that were differentially expressed in our dataset, had at least 3 predicted targets, and whose targets had a median absolute LPWC (121) correlation ≥0.8. From the resulting set of transcription factors, we manually selected ones that are up- or down-regulated around the same time or earlier than their predicted targets.

Deconvolution of Bulk RNA-seq Using Single-Nucleus RNA-sequencing Data. To determine in which cell types differential expression might occur, we deconvoluted our bulk RNA-seq dataset using single-nucleus RNA-seq of the head as a reference. We downloaded single-nucleus RNA-seq of the *Drosophila* head from Scope [10×, stringent; (64)]. From this dataset, we selected only cells from females and used the available annotations ("annotation_broad") that assign cells to annotated cell types. Cells annotated with "artifact" were removed. We processed this dataset using Seurat V4 (122) and used the R package scMappR to assign differentially expressed genes or genes with differentially used exons to cell types (Dataset S5; (63); *SI Appendix*).

Functional Interpretation of Results. We ran GO enrichment analyses using ClusterProfiler and rrvgo (123–127). All GO terms listed in this study are significantly enriched with a *q*-value <0.05 (Benjamini–Hochberg correction) and based on a minimum gene set size of 10. We consulted Flybase [FB2021_01; (128)] to acquire additional information for differentially expressed genes of interest.

Identification of Genes with Circadian Rhythms. We identified genes with circadian rhythms in V females using the R package Metacycle (129). Minimum periodicity was set to 22 h; maximum periodicity was set to 26 h. We extracted results from the LS algorithm and classified genes with a *q-value* <0.3 (Benjamini–Hochberg correction) as having a circadian rhythm in V females.

R packages Used to Generate Figures. Figures were made using base R (R version 4.1.0) and the R packages ComplexHeatmap (130), igraph (131), eulerr (132), and ggplot2 (133).

Querying of the Fly Hemibrain Connectome. We investigated connections between pC1 (a, b, and c) neurons and neurons with roles in circadian behaviors using the "shortest path" tool in the connectome neuprint [hemibrain v1.2.1; (83)], using the default minimum of 10 synapses. We recorded the number of interneurons for each path as 0 (direct connection), 1 (1 interneuron), 2 (2 interneurons), or >2 (more than 2 interneurons). For paths with 2 or fewer interneurons, we registered whether SMP454 or oviIN neurons were used as interneurons.

Data, Materials, and Software Availability. Fastq files and raw gene- and exon-level count data tables are available on GEO (GSE198879). The R code used for analyses is available on GitHub (SDelb/Transcriptomics_SP_timecourse).

ACKNOWLEDGMENTS. We thank A. Jain for help with sample collection and library prep; the Cornell Genomics Facility for sequencing; N. Brown, D. Chen, Y. Hafezi, and A.-M. Jaksic for feedback regarding the experimental design; S. Misra for running the Western blot; E. Cosgrove for help with cluster analysis; I. Anreiter and M. Sokolowski for advice regarding *foraging* qPCR experiments. We thank the reviewers for their insightful suggestions and comments. A.G.C. and M.F.W. were supported by NIH award R01 HD059060. S.Y.N.D., S.V., A.G.C., M.T.W. and S.B. were supported by NIH award R01 GM135926. S.B. also acknowledges support from NIH award R21NS120227 and NSF award DMS-1812128.

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Reviewers: Y.H., Hebrew University of Jerusalem; D.E.K., University of California; and G.E.R., University of Illinois at Urbana-Champaign Institute for Genomic Biology.

Competing interest statement: The authors have additional information to disclose. Drs. Wolfner and Heifetz were members of separate consortia that published on fly single-nucleus sequence data (Li et al. 2022) or fly organ terminology (McQueen et al. (2022); they were in separate parts of those consortia. They cowrote an editorial that introduced a journal issue of review articles (Heifetz, Wolfner 2019). Drs. Wolfner and Robinson were part of a consortium that coauthored a review article (Sinha, et al. 2020).

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