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Los Angeles

Elevated Sleep in a Stress-Resilient *Drosophila* Species

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of

Philosophy in Molecular, Cellular, and Integrative Physiology

by

Jessica Yano

2023

ABSTRACT OF THE DISSERTATION

Elevated Sleep in a Stress-Resilient *Drosophila* Species

by

Jessica Yano

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

University of California, Los Angeles, 2023

Professor Jeffrey M. Donlea, Chair

Sleep is broadly conserved across the animal kingdom but can vary widely across species. It is currently unclear which selective pressures and sleep regulatory mechanisms influence differences in sleep between species. The fruit fly *Drosophila melanogaster* has become a successful model system for examining sleep regulation and function, but little is known about the sleep patterns and need for sleep in many related fly species. Here, it is shown that fly species that have adapted to extreme desert environments, including *D. mojavensis*, exhibit strong increases in sleep compared to *D. melanogaster*. Long-sleeping *D. mojavensis* show intact sleep homeostasis, indicating that these flies may carry an elevated need for sleep. Additionally, *D. mojavensi*s exhibit altered abundance or distribution of several sleep/wake-related neuromodulators and neuropeptides that are consistent with their reduced locomotor activity and increased sleep. Finally, in a nutrient-deprived environment, the sleep patterns of individual *D. mojavensis* are strongly correlated with their survival time and disrupting sleep via constant light

stimulation renders *D. mojavensis* more sensitive to starvation. These results demonstrate that *D. mojavensis* is a novel model for studying organisms with high sleep need, and for exploring sleep strategies that provide resilience in extreme environments.

The dissertation of Jessica Yano is approved.

Mark A. Frye

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University of California, Los Angeles

2023

DEDICATION

To Derek, Dante, and Hazel: I never would have made it this far without our little family.

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ACKNOWLEDGMENTS

I further thank my advisor, Jeff Donlea, for his unfailing support, encouragement, and advice throughout the years.

I thank Shivan Bonanno for taking the time to teach me R and help me make sense of my sequencing data.

I thank Dr. Bruce Kagan for helping make it possible for me to get through grad school.

I thank my parents for their support.

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- Compositions and methods for inhibiting seizures (Publication date: 2021, US Patent number 11129858)

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INTRODUCTION

Sleep is widespread throughout the animal kingdom, and has been identified in species across diverse phyla. Indeed, it is often claimed that some form of sleep has been identified in every species which has been investigated, although not without some contention (Anafi et al., 2019; Jaggard et al., 2021; Siegel, 2008). It can at least be argued that proof of the existence of an animal that does not sleep has yet to be found (Cirelli and Tononi, 2008).

Defined behaviorally, sleep is a quiescent state to which the following criteria have been ascribed: 1) It is rapidly reversible (which distinguishes sleep from other quiescent states, such as coma or hibernation). 2) Responsiveness to arousing stimuli is decreased (distinguishing sleep from quiet wakefulness). 3) It is homeostatically regulated (i.e. lost sleep is recovered by subsequently sleeping longer and/or more deeply). Additional criteria, such as species-specific posture, have also been used (Campbell and Tobler, 1984; Hendricks et al., 2000). This behavioral state further correlates with altered brain activity, such as is seen electrophysiologically in the NREM and REM sleep stages in mammals.

Sleep can render an animal vulnerable to predation and diminishes the ability to respond to immediate changes in the environment. Furthermore, time spent asleep is time spent forgoing other crucial activities such as foraging, feeding, or mating (Capellini et al., 2008). That sleep is still pervasive across diverse species implicates some crucial benefit(s) which outweigh these potential costs.

The functions of sleep

Sleep's prevalence across multiple phyla implies ancient origins. This further implicates one or more functions of biological significance, but whether sleep has a core function universal to all animals is unknown. Studies in rodents and flies have reported that prolonged sleep deprivation leads to death (Rechtschaffen and Bergmann, 2002; Shaw et al., 2002). However, the interpretation that sleep serves a vital function has been disputed (Rial et al., 2007).

Nevertheless, sleep has been implicated in functions as numerous and varied as energy conservation, reduction of oxidative stress, metabolite clearance, memory consolidation, and synaptic regulation (Schmidt, 2014; Berger and Phillips, 1995; Siegel, 2009; Hill et al., 2018; Xie et al., 2013; Stickgold, 2005; Tononi and Cirelli, 2006; Liu et al., 2010). Chronic sleep loss in humans is considered a risk factor for metabolic syndrome, immune system dysregulation, cardiovascular disease, and psychiatric disorders (Chaput et al., 2013; Faraut et al., 2012; van Leeuwen et al., 2009; Freeman et al., 2020).

The evolution of sleep - sleep across the animal kingdom

An examination of sleep behavior across phyla shows its presence even in organisms with simple nervous systems. A study of jellyfish, an animal lacking a centralized nervous system, reported periods of decreased pulsation frequency which met behavioral criteria for sleep, including homeostatic regulation (Nath et al., 2017). The nematode *Caenorhabditis elegans*, which has a nervous system limited to a few hundred neurons, has a quiescent behavioral state during transitions between larval stages known as lethargus, which also meets behavioral criteria for sleep (Raizen et al., 2008). Flatworms sleep, and furthermore, their sleep

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is increased by melatonin, just as with mammals, indicating conservation of neurochemical regulatory mechanisms (Osmond et al., 2017). Sleep has also been reported in more neuroanatomically complex invertebrates of the phylum Mollusca: the common octopus and the common cuttlefish (Brown et al., 2006; Frank et al., 2012).

Among fish, the model organism zebrafish (*Danio rerio*) meet behavioral criteria for sleep in that they display periods of reversible immobility with increased arousal threshold. Additionally, disrupting these rest periods with electrical stimulation prompts a homeostatic rebound sleep response (Yokogawa et al., 2007). Melatonin also promotes sleep in zebrafish (Zhdanova et al., 2001).

Regarding the emergence of distinct sleep stages in more complex organisms, NREM and REM states are present in both mammals and birds, suggesting their presence in the most recent common ancestor at least 300 million years ago (Ungurean, 2020). However, while there have been reports on reptiles (the closest living relatives to birds) identifying sleep states somewhat resembling the NREM and REM sleep seen in mammals and birds, other reports on different reptile species failed to identify a similar two-state system (Libourel et al., 2018; Ungurean, 2020). Thus, there is some ambiguity as to whether the two sleep states in birds and mammals were in fact a holdover from a common ancestor or whether they arose separately through convergent evolution.

Comparing sleep across species - sleep is variable

While sleep may arguably be universal, characteristics and patterns of sleep vary significantly across species, even in species relatively closely related.. For example, some

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mammalian species sleep as little as 3-4 hours a day, whereas others sleep for up to 20 hours in a typical day (Siegel, 2005). In mammals and birds, there is also a great deal of variability in the proportion of time spent in NREM vs. REM sleep (Lesku et al., 2009; Siegel, 2009). In a survey of 20 primate species' daily sleep quotas, humans (at approximately 8 hours) slept the least while having the greatest proportion of total sleep time in REM. The authors speculate that humans have evolved to forgo the benefits of longer sleep times in order to increase time for other activities contributing to reproductive success, such as learning or social bonding (Nunn et al., 2016).

Some animals, such as aquatic mammals and birds, have the remarkable ability to sleep with only one hemisphere of the brain at a time. With only half the brain in a state of sleep, the other half remains awake, allowing the animal to continue surfacing to breathe (in the case of aquatic mammals) and to detect and respond to threats (Lyamin et al., 2008; Rattenborg et al., 2016).

Sleep differences across species may reflect variability in need for the core functions of sleep (if there are universal core functions). They may also reflect differing functions for different species. Interspecies comparative studies may provide valuable insights into the evolution of sleep and the impact of ecological factors on sleep, as well as sleep's functions and regulatory mechanisms. However, differences in conditions (e.g. laboratory vs. captivity vs. wild), protocols, and measurements make direct comparisons across the literature difficult (Campbell and Tobler, 1984; Siegel, 2005).

Drosophila melanogaster - a model organism for sleep research

Since the turn of the century, the fruit fly *Drosophila melanogaster* has served as a model organism for sleep research (Shaw et al., 2000; Hendricks et al., 2000). Earlier work had identified sleep in other insects such as honey bees and cockroaches, but none with the utility of *D. melanogaster* (Kaiser and Steiner-Kaiser, 1983; Tobler and Neuner-Jehle, 1992).

With its short life cycle and ease of husbandry, *Drosophila melanogaster* has been a favored model organism in biological research for over a hundred years. The ability to breed them quickly and in high abundance makes them ideal for high-throughput forward genetic screens. Additionally, there is a wide array of available tools and techniques for genetic manipulation (reviewed in Tomita et al., 2017).

Careful characterization of prolonged periods of quiescence in *D. melanogaster* has shown that these flies display all the behavioral hallmarks of sleep, with postural changes, reduced responsiveness to sensory stimuli, and homeostatic regulation in response to sleep deprivation (Shaw et al., 2000; Hendricks et al., 2000; Huber et al., 2004). Sleep in *D. melanogaster* follows a predictable pattern under conditions of 12 hours of light alternating with 12 hours of darkness. Flies show peaks of activity at lights-on and prior to lights-off with higher amounts of sleep mid-day and throughout the night. Arousal thresholds are higher during nighttime sleep, indicating increased sleep depth. Overall sleep is also sexually dimorphic, with males amassing more sleep over a 24-hr period than females (Huber et al., 2004). As in mammals, metabolic rate (as measured by carbon dioxide release rates) decreases during sleep, drugs such as caffeine and anti-histamines alter sleep behavior, and sleep fragments with aging (Stahl et al., 2017; Hendricks et al., 2000; Ho and Sehgal, 2005; Koh et al., 2006).

Besides the model species *D. melanogaster*, the *Drosophila* genus is diverse, consisting of at least 1600 species, many of which can be cultured and behaviorally monitored in standard laboratory settings (O'Grady and DeSalle, 2018). While focus on a model species like *D. melanogaster* permits the rapid development and proliferation of genetic tools and mechanistic frameworks, few studies have examined sleep in related species that are adapted to thrive in a variety of environmental conditions. Different *Drosophila* species thrive in a wide variety of environmental conditions across the planet, providing a diverse set of natural experiments to explore the physiological adaptations that might be associated with variations in sleep need. Exploiting this natural diversity to identify species with strongly elevated or reduced needs for sleep may provide new avenues to examine the fundamental functions fulfilled by sleep, neural signaling mechanisms that regulate sleep, and physiological tradeoffs that might be associated with different sleep strategies.

Drosophila mojavensis - a desert-adapted species

The species *Drosophila mojavensis* is found in desert regions of Mexico and the southwestern USA and includes four geographically segregated and genetically differentiated subspecies: *D. moj. mojavensis*, *D. moj. baja*, *D. moj. sonorensis*, and *D. moj. wrigleyi* from the Mojave Desert, Baja California, the Sonoran Desert, and Santa Catalina Island, respectively (Markow, 1991; Guillén et al., 2015; Etges, 2019). Genomes of all four subpopulations have been sequenced (Allan and Matzkin, 2019). They feed and breed on necrotic cactus tissue, with each subspecies specializing on a specific cactus species. They utilize detoxification enzymes,

such as cytochrome P450, to cope with the toxic alkaloids present in high concentrations within the cactus rots which serve as the plants' defense (Frank and Fogleman, 1992).

In order to thrive in harsh desert environments, *Drosophila mojavensis* display remarkable heat tolerance, exceeding even that of other *Drosophila* species endemic to the Sonoran Desert (Stratman and Markow, 1998). Microclimates within and around necrotic cacti expose flies to highly variable temperatures, from less than 5° C to over 40° C over a single 24-hr period (Gibbs et al., 2003). Under laboratory conditions, *D. mojavensis* decrease courtship behaviors with increased temperature at significantly slower rates than were seen in *D. melanogaster* (Patton and Krebs, 2001). Indeed, in the wild, *D. mojavensis* continues to reproduce even during the hottest summer months, rather than entering a state of estivation, as with some desert insects (Shaible and Matzkin, 2022). *D. mojavensis* is also highly desiccation-resistant, with low rates of water loss and low metabolic rates in dry conditions (Gibbs and Matzkin, 2001; Gibbs et al., 2003).

Here, we examined differences in sleep between the genetic model species *D. melanogaster* and the desert-adapted *D. mojavensis*.

METHODS

Fly Rearing and Stocks

Fly stocks were cultured on standard cornmeal molasses media (per 1L H_2O: 12 g agar, 29 g Red Star yeast, 71 g cornmeal, 92 g molasses, 16mL methyl paraben 10% in EtOH, 10mL propionic acid 50% in H_2O) at 25°C with 60% relative humidity and entrained to a daily 12h light, 12h dark schedule. Experiments with Banana-Opuntia media used a recipe from the National *Drosophila* Species Stock Center (NDSSC; Cornell University): per 1L H_2O: 14.16g agar, 27.5 g yeast, 2.23g methyl paraben, 137.5g blended bananas, 95g Karo Syrup, 30g Liquid Malt Extract, 22.33g 100% EtOH, 2.125g powdered opuntia cactus.

Canton-S were provided by Dr. Gero Miesenböck (University of Oxford) and *Pcf* were shared by Dr. Mark Frye (UCLA). Primary stocks of *D. moj. mojavensis, D. moj. baja, D. moj. wrigleyi*, and *D. moj. sonorensis* were a gift from Dr. Luciano Matzkin (University of Arizona), and additional stocks of *D. moj. mojavensis* and *D. moj. baja* were shared by Dr. Paul Garrity (Brandeis University). *D. arizonae* (SKU: 15081-1271.36), *D. mulleri* (SKU: 15081-1371.01), and *D. buzzatii* (SKU: 15081-1291.02) were ordered from the NDSSC. Wild caught *D. melanogaster* descended from a single pair of flies trapped in Los Angeles, CA in spring, 2020.

Behavior

4-8 day old female flies were housed individually in borosilicate glass tubes (65mm length, 5mm diameter) containing fly food coated with paraffin wax at one end and a foam plug in the other.

Locomotor activity was recorded using DAM5M or DAM5H multibeam Drosophila Activity Monitors from Trikinetics Inc. (Waltham MA, USA) and sleep was analyzed in Matlab (MathWorks Inc) with the SCAMP script package (Donelson et al., 2012). Locomotor activity was measured as the number of movements between beams per one-minute bins. Periods of sleep were defined by at least 5 minutes with no change in position within the multibeam activity monitors.

Sleep Deprivation and Arousability

Sleep deprivations were performed mechanically by mounting DAM5M activity monitors onto platform vortexers (VWR 58816-115). Individual tubes were plugged with food at one end and 3D-printed PLA plastic caps at the other. Monitors were vortexed at an intensity of 2.5g for 3-second pulses every minute through the duration of the 12-hour dark period. Arousability was tested in a darkened incubator with 60 seconds of blue light (luminance 0.048 Lv) every hour for 24 hours following sleep deprivation.

Food- and Water-Deprivation Assays

All flies were put in DAM5H activity monitors on standard food for baseline recording. After 2-3 days, control flies were transferred to tubes containing fresh food, food-deprived flies to tubes containing a 1% agar gel, and food-and-water-deprived flies to empty tubes plugged with foam at both ends. Flies immobile for at least 24 hours were defined as dead and data subsequent to their last full day alive was removed from sleep analysis.

Pharmacological Microinjections

4-8 day old female flies were loaded into behavior tubes and monitored in DAM5M Activity Monitors to obtain baseline sleep and locomotor activity under 12h light: 12h dark (25˚C). After 1-2 days of baseline in DAM5M monitors, flies housed in borosilicate tubes were placed on ice for anesthetization prior to injection using Drummond Nanoject II. For injection of exogenous neuromodulators, the anteriormost ocelli of D. mojavensis baja were injected with 18.4nl of 20mg/mL of Octopamine (Sigma-Aldrich, Catalog # O0250). For each round of injections, new OA is solubilized using Schneider's Drosophila Medium with L-Glutamine (Genesee Scientific, Catalog # 25-515). Following each individual injection, flies are returned back into individual borosilicate tubes, and placed in respective DAM5M Activity Monitors to continue sleep and activity surveillance for >48h.

Immunohistochemistry

Female D. melanogaster and D. mojavensis were reared in 12h light:12h dark schedule at 25˚ C in normal fly food. Individual fly brains were dissected 5-7 days post-eclosion between a ZT0-ZT3 window to minimize time-of-day variation to antibody targets. All dissections, antibody staining, and preparation for imaging were carried out in the exact same manner to minimize variability when comparing between species. Flies are anesthetized using ice. Brains were dissected in chilled 1X PBS then placed in 4.0% paraformaldehyde/1X PBS (PFA) for 30 mins. in room temperature on a benchtop rotator. PFA from brains were removed by washing with 1.0% Triton-X in 1X PBS 3 times for 10 mins. each. Once brains were free of PFA, the brains were placed in 1x Sodium Citrate (10mM, pH=6.0, 15 mins. at 80˚ C) for antigen retrieval.

Brains were then placed in a blocking buffer (5.0% normal goat serum in 0.5% Triton-X/1X PBS) and incubated at room temperature for 1.5h on a rotator. Brains were incubated with one the following primary antibodies (diluted using blocking buffer): 1:1000 Mouse anti-PDF (Developmental Studies Hybridoma Bank). Primary antibodies were incubated for two days in 4˚C. After incubation, brains we're washed using 0.5% Triton-X in 1X PBS five times, 10 mins. each. Fly brains were then incubated in AlexaFluor secondary antibodies (1:1000 Goat anti-Mouse AlexaFluor 633nm; Molecular Probes) overnight at 4˚C. Brains were washed using 0.5% Triton-X in 1X PBS five times, 10 mins. After washing, brains were mounted on glass slides in Vectashield mounting media, sealed with a coverslip and nail polish. Brains were imaged using a Zeiss LSM 880 laser scanning confocal microscope using a z-slice thickness of 1um and saved as CZI files. Maximum intensity projections were created from CZI files using FIJI/ImageJ (https://imagej.net/software/fiji/) (Schindelin et al., 2012).

Neurochemical Quantifications

Sample preparation protocol

Fly brain samples were stored at -80°C then treated with 99.9/1 Water/Formic Acid. An internal standard (IS) of each targeted compound was added to every sample to account for compound loss during sample processing. The samples are vortexed, homogenized for 30 sec in a bead beater using 2.0 mm zirconia beads, and centrifuged at 16.000xg for 5 min. The supernatant is transferred to new microcentrifuge test tubes and dried in a vacuum concentrator. The samples are reconstituted in 40 µl of water, vortexed, and centrifuged. The supernatant is transferred to

HPLC vials and 10 μl is injected to an HPLC - triple quadrupole mass spectrometer system for analysis.

Liquid Chromatography-Tandem Mass Spectrometry LC-MS

A targeted LC-MS/MS assay was developed for each compound using the multiple reaction monitoring (MRM) acquisition method on a triple quadrupole mass spectrometer (6460, Agilent Technologies) coupled to an HPLC system (1290 Infinity, Agilent Technologies) with an analytical reversed phase column (GL Sciences, Phenyl 2 μm 150 x 2.1 mm UP). The HPLC method utilized a mobile phase constituted of solvent A (100/0.1, v/v, Water/Formic Acid) and solvent B (100/0.1, v/v, Acetonitrile/Formic Acid) and a gradient was used for the elution of the compounds (min/%B: 0/0, 10/0, 25/75, 27/0, 35/0). The mass spectrometer was operated in positive ion mode and fragment ions originating from each compound was monitored at specific LC retention times to ensure specificity and accurate quantification in the complex biological samples (Octopamine OA 159-136, Histamine HA 112-95, Dopamine DA 154-137, Serotonin 5HT 177-160). The standard curve was made by plotting the known concentration for each analyte of interest (CDN Isotopes) against the ratio of measured chromatographic peak areas corresponding to the analyte over that of the labeled standards. The trendline equation was then used to calculate the absolute concentrations of each compound in fly brain tissue.

Statistical Analysis

Statistical tests were completed as described in the figure legends using Prism 9 (GraphPad Software, Boston MA, USA). Statistical comparisons primarily consist of one- or two-way

ANOVAs followed by pairwise Holm-Sidak's multiple comparisons test when experiments include at least three experimental groups or two-tailed Student's T-test for experiments that include two groups; specific tests used are described in each figure legend. All data figures pool individual data points from at least two independent replicates.

RESULTS

To begin sampling the sleep strategies of *Drosophila* species, we compared *D. melanogaster* to the cactophilic species *D. mojavensis* (See **Fig. 1A** for phylogenetic tree, based on Keesey et al., 2019). Desert-adapted *D. mojavensis* shows heightened resilience to heat, starvation, and desiccation stresses, which may contribute to their ability to thrive in harsh desert conditions, but behavioral adaptations that accompany stress resilience in *D. mojavensis* remain unexplored (Matzkin et al., 2007; Matzkin et al., 2009; Stratman and Markow, 1998).

We show that *D. mojavensis* exhibits increased sleep time across the day and night compared to *D. melanogaster*, and that desert-adapted *D. mojavensis* flies respond to sleep loss with a homeostatic increase in sleep drive. We observe several changes in sleep- or wake-related neuromodulator distribution: long-sleeping *D. mojavensis* flies exhibit high levels of serotonin, decreased abundance of wake-promoting octopamine, and reduced numbers of cells expressing the circadian output peptide Pigment Dispersing Factor (PDF). Finally, we examine contributions of elevated sleep to stress resilience in *D. mojavensis* by measuring starvation and dehydration responses. Long-sleeping *D. mojavensis* flies exhibit extended survival during food or food and water deprivation compared to *D. melanogaster*, and individual sleep time of *D. mojavensis* correlates positively with survival time while flies are starved and dehydrated. These results indicate that *D. mojavensis* exhibits an increased need for sleep relative to *D. melanogaster*, and that adaptations in sleep may contribute to increased stress resilience in desert-adapted flies.

Characterizing hypersomnolence in *Drosophila mojavensis*

We measured sleep in all four *D. mojavensis* subspecies and in two wild-type stocks of *D. melanogaster* (*Cs* and *Pcf*) using multibeam *Drosophila* activity monitors. Each *D. mojavensis* subspecies exhibits significantly elevated sleep throughout the day and night compared to *D. melanogaster* **(Figs. 1B-C)**. To test whether hypersomnolence in *D. mojavensis* can be attributed to an elevated pressure to maintain sleep and/or to an increased drive to initiate sleep episodes, we quantified the likelihood that a sleeping fly would awaken (P(wake); **Fig. 1D**) or that a waking fly would fall asleep (P(doze); **Fig. 1E**) (Wiggin et al., 2020). Each of the four *D. mojavensis* subspecies exhibits reduced P(wake) and elevated P(doze) compared to *D. melanogaster*, consistent with both strengthened sleep maintenance and an elevated pressure to fall asleep. Along with increased sleep time, *D. mojavensis* also exhibits reduced waking locomotor activity **(Fig. 1F)**, consistent with previous reports (Gibbs et al., 2003).

Additionally, we analyzed the cumulative distribution of sleep bout lengths during the day **(Fig. 1G)** and night **(Fig. 1H)**. We found that *D. mojavensis* flies exhibit an elevated frequency of longer sleep episodes than those observed in either wild-type line of *D. melanogaster*. Since *D. mojavensis* sleep consists of longer sleep bouts, *D. moj. moj.* and *D. moj. baja* continue to exhibit elevated sleep amounts when we increase the minimum period of quiescence scored for sleep from 5 min, as most commonly used for *D. melanogaster*, to at least 60 min **(Fig. 1I)** (Shaw et al., 2000; Hendricks et al., 2000). Together, these results indicate that hypersomnolence in *D. mojavensis* consists of increased drive to fall asleep and prolonged sleep episodes.

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To test for variations in sleep across days, we measured locomotion in *D. moj. mojavensis* flies across a 7-day period and found that daily sleep varies between individuals, but remains relatively stable over time for single flies **(Fig. 1J-K)**.

Increased sleep in cactophilic *Drosophila* across experimental conditions

The *D. mojavensis* stocks that we describe above were derived from wild populations more recently than either of our lab-reared wild-type *D. melanogaster* stocks. We tested whether fly lines isolated from the wild sleep more than those reared in lab conditions for longer periods of time. First, we found that independent stocks of *D. moj. moj.* and *D. moj. baja* (in addition to the lines shown in Fig.1) also show a strong increase in sleep time compared to wild-type *D. melanogaster* **(Fig. 2A)**. Next, we examined sleep in a *D. melanogaster* stock that originated from flies collected in the Westwood area of Los Angeles in 2020. *D. melanogaster* descended from flies caught in Westwood, Los Angeles, CA showed comparable sleep amounts to *Cs* and *Pcf* laboratory strains **(Fig. 2B)**.

To test the impact of diet on *D. mojavensis* sleep, we collected freshly eclosed flies and reared them on media that included extract of opuntia cactus, a natural host for desert-adapted *D. mojavensis*. Sleep in *D. mojavensis* remained elevated relative to *D. melanogaster* when both species were fed a banana-cactus diet **(Fig. 2C)**.

In addition to *D. mojavensis*, several other related fly species, including *D. arizonae, D. buzzatii,* and *D. mulleri,* also local to deserts and feed on cactus hosts (Reed et al., 2007; Ruiz and Heed, 1988; Barker and Mulley, 1976). As shown in **Fig. 2D**, the three additional cactophilic species sleep as much, or more than *D. mojavensis*, suggesting that elevated sleep is not

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exclusive to *D. mojavensis* and could be conserved across the Repleta species that localize to desert regions (Pfeiler, 2018).

In their desert habitats, *D. mojavensis* are exposed to environmental stressors that include temperature variations and periods of sparse food and/or water availability. To measure sleep during desert-like temperature fluctuations, we exposed both *D. melanogaster and D. mojavensis* flies to daytime temperature ramps. Flies were held at 25˚C overnight, then began to progressively increase the temperature across the first 6h of daytime to a peak of 35˚C before reducing back to 25˚C by lights-off at ZT12. While *D. mojavensis* maintained higher amounts of sleep than *D. melanogaster* across most of the day during these conditions **(Fig. 2E)**, both species showed a brief decrease in sleep when temperature peaked at 35˚C at mid-day. As the temperature decreased afterwards, *D. melanogaster* briefly increased their sleep to comparable levels as the desert-adapted *D. mojavensis* subspecies. These results indicate that sleep in both species can be altered by variations in temperature, but that *D. mojavensis* retain elevated levels of daily sleep under naturalistic daytime temperature conditions.

Sleep homeostasis remains intact in long-sleeping *Drosophila mojavensis*

Elevated sleep in a desert-adapted species could indicate at least two possibilities: first, that sleep provides a period of adaptive inactivity during which animals can store metabolic resources and avoid predation, or alternatively, that this species has adapted an elevated need for basic functions that are fulfilled by sleep (Siegel, 2009). To test whether desert-adapted *D. mojavensis* maintain an elevated sleep need, we tested whether they respond to mechanical sleep deprivation with a homeostatic rebound to recover lost sleep. Vortex stimuli delivered for 3s each

minute were sufficient to strongly suppress sleep in *D. moj. moj.* **(Fig. 3A)** and in *D. moj. baja* **(Fig. 3B)**. Following sleep deprivation, both *D. mojavensis* subspecies showed a recovery period of significantly increased sleep compared to baseline and regained approximately 20-40% of their lost sleep after 24 hours **(Fig. 3C)**. In the 24h following deprivation, P(wake) is decreased during daytime sleep on the first recovery day after deprivation, an indication of increased sleep depth **(Fig. S1A-B)**. Additionally, there was no decrease in locomotor activity per time awake **(Fig. S1C-D)**, indicating that waking locomotor activity was unimpaired by mechanical sleep deprivation. Following the first 24h of recovery, *D. moj. moj.* flies reduced their sleep nearly to baseline levels on the second recovery day **(Fig. S1E-F)**.

After finding that *D. mojavensis* exhibited a rebound in sleep time after overnight sleep loss, we next probed arousability to test for additional markers of increased sleep depth in recently deprived *D. moj. moj.* Flies were either left undisturbed, sleep-deprived for 12h overnight (SD), or sleep-deprived and permitted 24h of recovery (SD+24h) before they were exposed hourly to 60s pulses of blue light. Light pulses were less likely to awaken sleep-deprived flies than rested controls; arousability returned to control levels in SD+24h flies **(Fig. 3D)**. After each light pulse, *D. moj. mojavensis* flies in the SD group had a reduced latency to fall back asleep compared to both the control and SD+24h groups **(Fig. 3E)**. These results indicate that long-sleeping *D. mojavensis* responds to mechanical sleep loss with homeostatic increases both in sleep time and intensity, consistent with the hypothesis that *D. mojavensis* have adapted an increased need for sleep.

To further probe responses of *D. mojavensis* to acute sleep loss, we also exposed *D. moj. moj.* and *D. moj. baja* flies to arousing blue light for 12h overnight (ZT12-0). Overnight blue

light disrupted sleep in both desert subspecies and was followed by prolonged rebound during the first recovery day **(Fig. 3F-I)**. During light stimulation, *D. moj. moj.* lost 83.90±3.50% (mean ± SEM, n=35) of their sleep while *D. moj. baja* reduced their sleep by 42.89±3.74% (mean ± SEM, n=53) **(Fig. 3H)**.

Given that overnight light exposure significantly disrupted sleep, we next tested whether acute visual input bidirectionally influences sleep by housing *D. mojavensis* in two days of constant darkness. Both *D. moj. Moj.* **(Fig. 3J)** and *D. moj. baja* **(Fig. 3K)** significantly increased their sleep when transferred to constant darkness after entrainment in a 12h:12h light-dark schedule. We found that in the absence of day-night light signals, the immediate increase in subjective daytime sleep persists across at least two days **(Fig. 3L)**. Previous observations of *D. melanogaster* have found either reduced or unchanged sleep when flies were housed in constant darkness, indicating that light-dependent modulation of sleep could be a target of evolutionary adaptation (Cirelli et al., 2005; Joiner et al., 2006; Chung et al., 2009).

Interspecies variation in sleep/wake-related neuromodulators correlates with sleep patterns

Research over the past 20 years identified several neuromodulators and neuropeptides that influence sleep/wake regulation in *D. melanogaster*, but interspecies variation of these signals across fly species is not well-studied (Kume et al., 2005; Andretic et al., 2005; Yuan et al., 2006; Crocker and Sehgal, 2008; Parisky et al., 2008; Shang et al., 2013). In particular, we hypothesized that hypersomnia in *D. mojavensis* may be correlated with an upregulation of sleep-promoting signals and a decrease in arousal pathways.

To identify relevant neuromodulators, we conducted liquid chromatography-mass spectrometry (LC-MS) assays of fly heads from both *D. melanogaster* and *D. mojavensis*. We found that long-sleeping *D. mojavensis* flies from all four subspecies contain a significant increase in serotonin (5-HT) and decrease of octopamine (OA) **(Fig. 4A-B)**. No uniform change in dopamine (DA) or histamine (HA) was measured between species **(Fig. 4C-D)**. 5-HT signaling promotes sleep in *D. melanogaster* and in vertebrates, while OA, a paralog of norepinephrine, drives arousal (Yuan et al., 2006; Liu et al., 2019; Knapp et al., 2022; Qian et al., 2017; Frank et al., 2002; Oikonomou et al., 2019; Lee et al., 2020; Roeder, 1999; Crocker and Sehgal, 2008; Crocker et al., 2010).

Changes in the abundance of 5-HT and OA between species indicates either that altered numbers of neurons produce these modulators or that conserved populations of cells have changed their rates of 5-HT and OA synthesis and/or release. We observed the distribution of 5-HTergic cells by staining for the serotonin transporter (SERT) in *D. melanogaster* **(Fig. 4E)**, *D. moj. baja* **(Fig. 4F)**, and *D. moj. moj.* **(Fig. 4G)**. Images of the anterior and posterior cell bodies indicate that both species show similar overall patterns of 5-HTergic neurons, but it is possible that projection targets or cell numbers within specific clusters may vary. Similarly, we stained *D. melanogaster, D. moj. baja*, and *D. moj. moj.* brains for the OA synthesis enzyme Tdc2 **(Figs. 4H-J)** to observe the number and organization of OAergic cells. Our images reveal weak Tdc2-immunostaining in the ASM neurons of the anterior protocerebrum of *D. mojavensis* flies **(Figs. 4H, S2A-C)**, a population of cells that underlies the wake-promoting role of OA (Crocker et al., 2010). Together, these results indicate that the distribution of 5-HTergic neurons is similar

between species, but that *D. mojavensis* may contain either weak signal or only a subset of the OAergic cells that are observed in *D. melanogaster*.

We next sought to test whether arousal circuitry might retain sensitivity to OA in this hypersomniac species by microinjecting *D. moj. baja* females with 18.4 nL of either 20mM OA or vehicle control. During the first 24h after OA injections, we found that *D. moj. baja* females showed reduced sleep and increased locomotor activity **(Fig. 4K-M)** compared to vehicle-treated siblings. While octopamine abundance is decreased in *D. mojavensis*, the wake-promoting effect of OA injection suggests that octopamine-sensitive arousal circuitry is likely conserved in desert-adapted flies.

To examine whether the distribution of other wake-promoting signals might differ between these two fly species, we performed immunostaining for the arousing circadian output peptide Pigment Dispersing Factor (PDF) (Renn et al., 1999). While *D. melanogaster* brains contain eight PDF-positive neurons in each hemisphere, four s-LNvs and four l-LNvs **(Fig. 5A)**, careful analysis reveals inconsistent PDF-expression patterns between *D. melanogaster* and *D. mojavensis* (Helfrich-Förster and Homberg, 1993). Specifically, *D. mojavensis* retained three to four PDF-positive l-LNvs, but showed no s-LNv cell bodies or dorsal protocerebrum projections that were labeled with anti-PDF **(Fig. 5B-C)**. A loss of PDF-immunostaining in s-LNvs has also been reported in other *Drosophila* species, indicating that selective pressures may drive reconfiguration of clock circuits as species adapt to different environments (Beauchamp et al., 2018; Menegazzi et al., 2017; Hermann et al., 2013; Kauranen et al., 2012). Together, these results indicate that elevated sleep of desert-adapted *D. mojavensis* correlates with both an increase in sleep-promoting 5-HT and reductions of arousing OA and PDF.

Sleep in *Drosophila mojavensis* supports resilience to nutrient deprivation

D. mojavensis sleeps more than *D. melanogaster* and responds to prolonged waking with increased recovery sleep, indicating that this species may have an increased need for sleep relative to *D. melanogaster*. Increased sleep is a behavioral adaptation that is hypothesized to support resistance to nutrient scarcity, and artificial selection for starvation resistance in *D. melanogaster* can result in increased sleep time (Siegel, 2008; Masek et al., 2014). To further test the functional relevance of heightened sleep pressure in desert-adapted flies, we measured sleep and survival while flies were deprived of food alone or both food and water. Both Baja and Mojavensis subspecies of *D. mojavensis* survive longer than wild-type *D. melanogaster* when housed in glass tubes with non-nutritive agar media **(Fig. 6A)** or in empty, dry glass tubes **(Fig. 6B)**, as described previously (Matzkin et al., 2009).

While wild-type *D. melanogaster* suppress their sleep during food deprivation, *D. mojavensis* instead show subspecies-specific changes (Thimgan et al., 2010; Keene et al., 2010). *D. moj. baja* exhibit moderate increases in sleep time during several days of food deprivation and reduce sleep when both food and water are unavailable **(Fig. 6C)**. In contrast, *D. moj. moj.* show no significant sleep changes when food deprived and only a transient increase in sleep on the first day of food and water deprivation **(Fig. 6D)**. These trends are consistent with the hypothesis that hypersomnia in *D. mojavensis* is associated with prolonged survival during nutrient deprivation. We tested this relationship by depriving *D. mojavensis* females of food alone or both food and water, then housing them either in 12h:12h LD light or in constant blue light to disrupt

sleep **(Fig. S3A)**. Food-deprived *D. moj. baja* that were housed in constant blue light die from food deprivation more rapidly than siblings housed in LD **(Fig. 6E)**.

To more broadly test whether disrupted sleep might render *D. mojavensis* flies more sensitive to starvation, we tested the relationship between average daily sleep and survival time using the individuals shown in **Fig. 6E** and found a highly significant positive correlation **(Fig. 6F)**. Similar trends arose when *D. moj. baja* flies were exposed to constant light to suppress sleep while deprived of both food and water **(Fig. S3B)**; constant light exposure reduced survival time **(Fig. 6G)** and daily sleep strongly correlated with survival **(Fig. 6H)**. Although LL exposure significantly reduced survival time in food-deprived *D. moj. moj*. **(Fig. 6I)**, the effect of constant light was less pronounced than in *D. moj. baja*. Interestingly, individual *D. moj. moj*. flies showed a wide variety of sleep responses to constant light during starvation **(Fig. 6J)** and subdividing the *D. moj. moj*. flies that were housed in LL during food deprivation revealed that the half of that group with the lowest sleep time during starvation died earlier than the half with the weakest sleep disruption **(Fig. 6K)**.

To more broadly examine whether sleep loss might render *D. mojavensis* flies more sensitive to starvation, we tested the correlation between average daily sleep and survival time in *D. moj. moj*. **(Fig. 6L)** and, as with *D. moj. baja,* found highly significant positive correlations between daily sleep and starvation survival time. When *D. moj. moj*. were denied both food and water, LL exposure alone had no significant effect on survival time **(Fig. 6M)**. When the LL group of *D. moj. moj*. flies were sorted by daily sleep, we found that the half with the lowest amount of daily sleep during desiccation showed reduced survival time **(Fig. 6N-O)**. Further, plotting individual daily sleep against desiccation survival time for *D. moj. moj*. that were housed

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in LD (filled dots) or in constant light (open dots) revealed a significant positive correlation across both experimental groups **(Fig. 6P)**. These data indicate that high amounts of sleep may confer desert-adapted flies with resistance to periods of insufficient food or water.

We also detected significant negative correlations between survival and daily activity counts indicating that the effect of sleep could, in part, be linked with decreased energy consumption during locomotion **(Fig. S3)**. Because we detected a significant correlation between waking activity intensity (counts/waking minute) and survival only for food deprived *D. moj. baja* **(Fig. S3D)**, and not for food and water deprived *D. moj. baja* or either *D. moj. moj.* condition **(Fig. S3G, J, M)**, it is likely that the influence of activity on survival can be linked with sleep amount and not necessarily changes in intensity of waking activity.
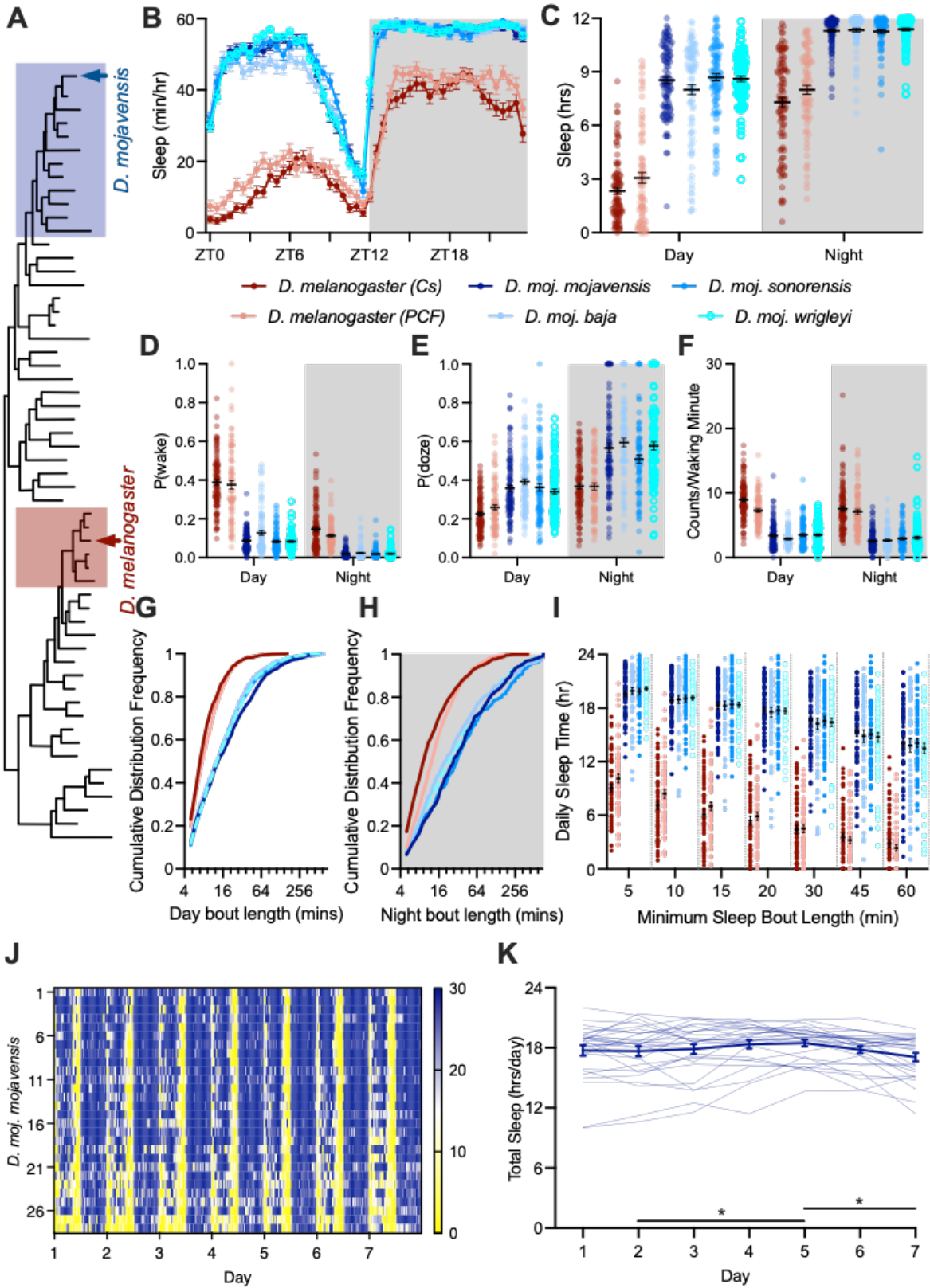
Figure 1 – Elevated sleep time in desert-dwelling *Drosophila mojavensis*

(A) Phylogenetic tree of *Drosophila* species, *D. melanogaster* (red) and *D. mojavensis* (blue) are marked with arrows. (Based on Keesey et al., 2019). Shading represents Melanogaster (red) and Repleta (blue) species groups that include *D. melanogaster* and *D. mojavensis,* respectively.

(B) 24h sleep time course for wild-type *D. melanogaster (Cs,* dark red; *Pcf,* light red) and four subspecies of *D. mojavensis* (blue). Two-way repeated measures ANOVA finds a significant genotype-by-time interaction ($F_{(235,27213)}$=16.99, p<0.0001).

(C) Day and night sleep totals for *D. melanogaster* (*Cs*, dark red*; Pcf,* light red) and *D. mojavensis* (blues). Two-way repeated measures ANOVA finds a significant genotype-by-time interaction ($F_{(5,577)}$=24.981, p<0.0001).

(D-E) P(wake) **(D)** and P(doze) **(E)** during the day and night for *D. melanogaster* (reds) and *D. mojavensis* (blues) stocks. Two-way repeated measures ANOVA detects a significant genotype-by-time interaction for P(wake) ($F_{(5,579)}$=75.43, p<0.0001) and for P(doze) ($F_{(5,553)}$=5.628, p<0.0001).

(F) Waking activity (position movements/waking minute) is decreased in *D. mojavensis* subspecies (blues) relative to *D. melanogaster* (reds). Two-way repeated measures ANOVA finds a significant main effect of genotype ($F_{(5,576)}$=139.4, p<0.0001).

For panels **(A-F)**, n= 101 *Cs,* 82 *Pcf,* 100 *D. moj. moj.*, 100 *D. moj. baja*, 93 *D. moj. sonorensis*, 106 *D. moj. wrigleyi*.

(G-I) Cumulative distributions for duration of sleep bouts of *D. melanogaster* (reds) and *D. mojavensis* (blues) during the day **(G)** and night **(H)**. **(I)** Depicts daily sleep for individuals from **(G-H)** using increasing periods for the minimum sleep bout threshold. Kruskal-Wallis tests find significant effects of genotype for day bout lengths (**G**; Kruskal-Wallis statistic = 467.7,

n=737-1204 sleep bouts/group from 60-64 flies/group, $p < 0.0001$) and night bout lengths (**H**; Kruskal-Wallis statistic = 499.7, n = 398-1380 sleep bouts/group from 58-64 flies/group, $p<0.0001$). Two-way repeated measures ANOVA finds a significant genotype-by-threshold interaction ($F_{(30,2244)}=6.142$, $p<0.0001$, n=62-64 flies/group).

(J-K) Sleep time course heatmap **(J)** and daily sleep totals **(K)** for *D. moj. moj.* female flies across a 7-day experiment (Friedman test statistic=18.47, $p=0.0051$, n=28 flies). * indicates $p<0.05$ by Dunn's pairwise test for **(K)**.
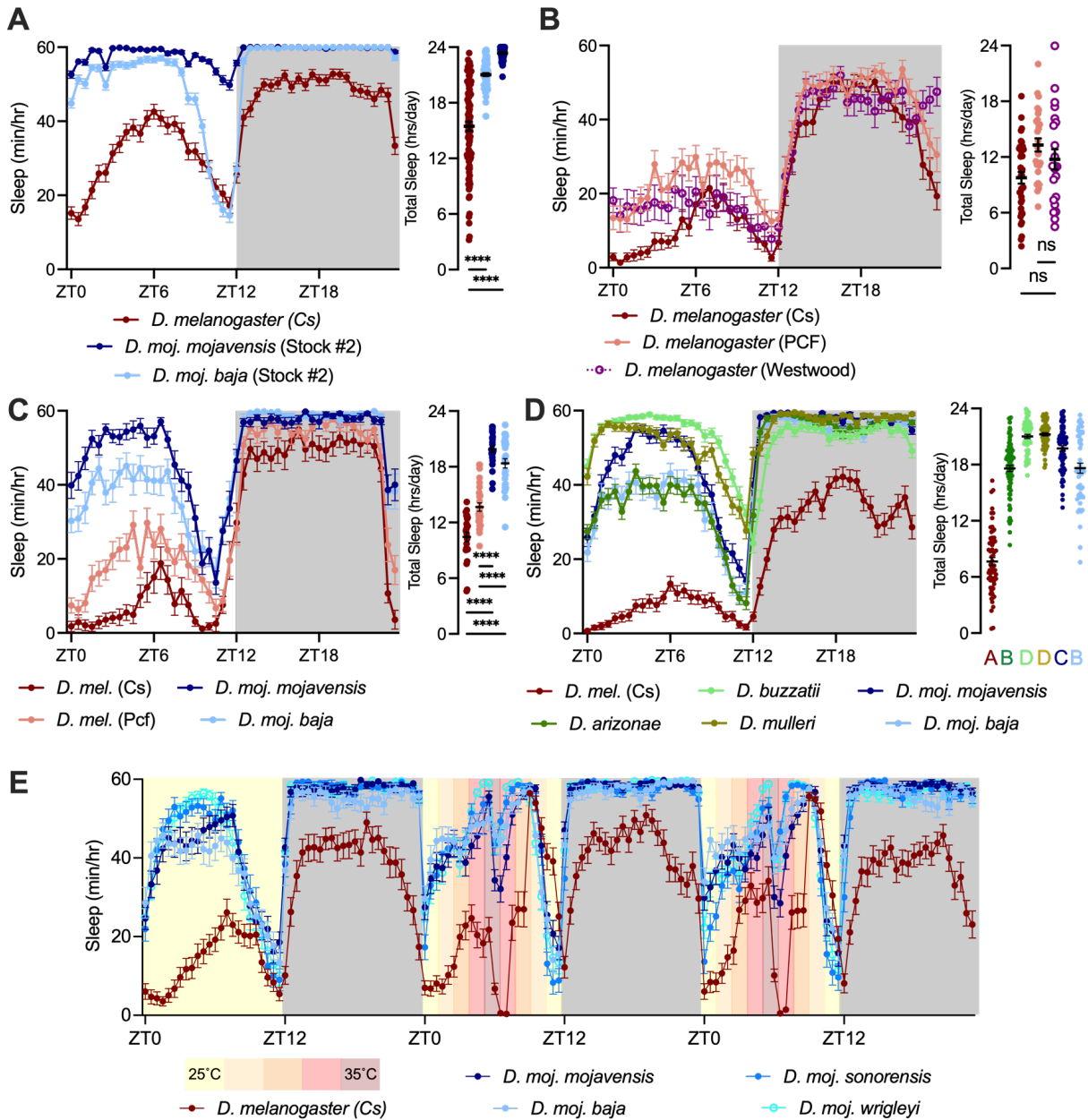
Figure 2 – Hypersomnolence in *D. mojavensis* across conditions

(A) Sleep time course (left) and total daily sleep (right) for *Canton-S* (red) and additional stocks of *D. moj. moj.* (dark blue) and *D. moj. baja* (light blue). ANOVAs find a significant genotype-by-time interaction in sleep time course ($F_{(94,13207)}$=40.94, p<0.0001) and main effect of genotype for total daily sleep ($F_{(2, 281)}$=178.9, p<0.0001, n= 96 *Canton*-S, 96 *D. moj. moj.,* and 92 *D. moj. baja*).

(B) Sleep time course (left) and total daily sleep (right) for *Canton-S* (dark red), *Pcf* (light red), and flies descended from *D. melanogaster* caught in Westwood, Los Angeles (open purple circles). ANOVAs detect a significant genotype-by-time interaction in sleep time course ($F_{(94,3995)}$=2.385, $p<0.0001$) and main effect of genotype for total daily sleep ($F_{(2,85)}$=5.793, $p=0.0044$, n= 38 *Canton-S,* 28 *Pcf*, and 22 wild-caught flies).

(C) Sleep time course (left) and total daily sleep (right) for *D. melanogaster* stocks (red) and two *D. mojavensis* subspecies (blue) from flies reared on Banana-Opuntia media. ANOVAs detect significant genotype-by-time interaction for the sleep time course ($F_{(141,4841)}$=8.838, $p<0.0001$) and a significant effect of genotype for total daily sleep ($F_{(3, 103)}$=91.08, $p<0.0001$, n= 24 *Canton-S*, 27 *Pcf*, 28 *D. moj. moj.,* and 28 *D. moj. baja*).

(D) Sleep time course (left) and total sleep (right) for *D. melanogaster* (red), *D. arizonae* (green), *D. buzzatii* (light green), *D. mulleri* (olive), and *D. mojavensis* (blues). ANOVA tests find significant genotype-by-time interaction for sleep time course ($F_{(235,17531)}$=18.07, $p<0.0001$) and a significant effect of genotype for total sleep ($F_{(5,373)}$=215.4, $p<0.0001$, n= 63 *Canton-S*, 78 *D. arizonae*, 62 *D. buzzatii, 57 D. mulleri,* 69 *D. moj. moj*, and 50 *D. moj. baja*). Letters below graph indicate statistically distinct groups by Holm-Sidak pairwise comparisons.

(E) Sleep time courses for *D. melanogaster* (Cs; red) and *D. mojavensis* (blues) flies that were housed at 25˚C for one baseline day, then exposed to a temperature ramp from 25˚C to 35˚C and back to 25˚C during the daytime (ZT0-12). Two-way repeated measures ANOVA finds a significant time-by-strain interaction ($F_{(568, 26412)}$=9.054, $p<0.0001$, n= 40 *Cs*, 39 *D. moj. moj*, 35 *D. moj. baja*, 35 *D. moj. sonorensis*, 42 *D. moj. wrigleyi*).
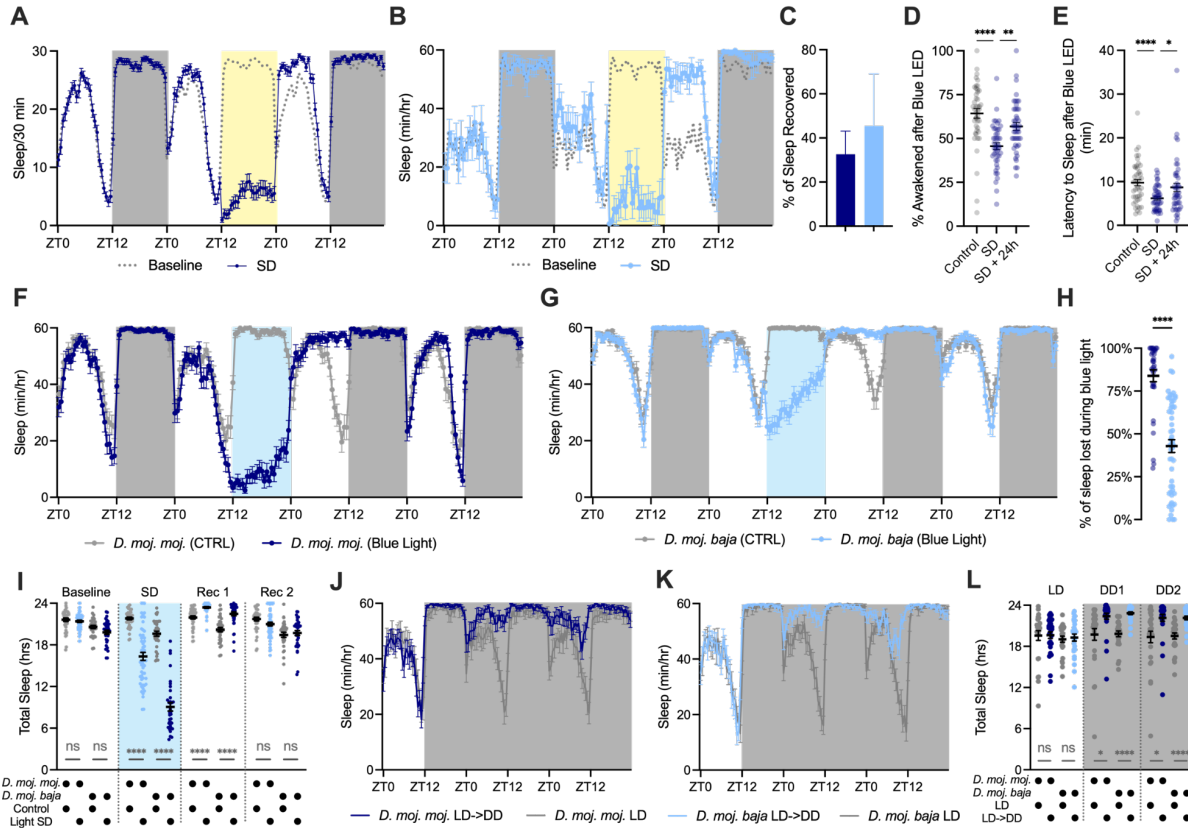
Figure 3 – Homeostatic regulation of sleep and arousability in *Drosophila mojavensis*

(A-B) Sleep time course of *D. moj. moj.* **(A)** and *D. moj. baja* **(B)** across baseline, overnight mechanical sleep deprivation, and recovery days. Yellow shading indicates time of sleep deprivation. Dotted gray lines show baseline sleep patterns replotted on deprivation and recovery days for visual comparison (n= 77 flies in **A**, 38 in **B**).

(C) Percentage of sleep recovered within 24h of recovery from mechanical sleep deprivation. *D. moj. moj.* shown in dark blue, *D. moj. baja* in light blue. (n= 77 for *D. moj. moj.* and 38 for *D. moj. baja* and flies/group).

(D) Portion of sleeping *D. moj. mojavensis* flies awakened by 60s pulses of blue light. Individual data points represent group mean response rate from individual hourly light exposure trials. One-

way repeated measures ANOVA finds a significant effect of condition ($F_{(1.874, 80.56)}$=15.41, p<0.0001, n=44 trials/group).

(E) Mean sleep latency of *D. moj. mojavensis* flies after hourly 60s pulses of blue light is reduced after mechanical sleep deprivation. Individual data points represent group mean sleep latency after individual hourly light exposure trials. One-way repeated measures ANOVA finds a significant effect of condition ($F_{(1.730,74.40)}$=7.342, p=0.002, n=44 trials/group).

(F-G) Sleep time course of *D. moj. mojavensis* **(F)** and *D. moj. baja* females **(G)** during baseline, overnight blue light exposure, and two recovery days. Blue shading shows the time of overnight light stimulation. Gray traces represent undisturbed controls and blues depict sleep for flies exposed to blue light from ZT12-24 on day 2. Two-way repeated measures ANOVAs find significant time-by-condition interactions for **(F)** ($F_{(191,12606)}$=33.98, p<0.0001, n=33 control, 35 Light SD) and for **(G)** ($F_{(191,16999)}$=15.98, p<0.0001, n=38 control, 53 Light SD).

(H) Percentage of sleep lost during 12h of overnight blue light exposure in *D. moj. moj.* (dark blue) and *D. moj. baja* (light blue). Unpaired T-test t=7.593, df=86, p<0.0001, n=35 *D. moj. moj.*, 53 *D. moj. baja*.

(I) Daily sleep totals for groups shown in **(F)** and **(G)**. Two-way repeated measures ANOVA finds a group-by-day interaction ($F_{(9,465)}$=97.60, p<0.0001, n=33 Control *D. moj. moj.*, 35 Light SD *D. moj. moj.*, 38 Control *D. moj. baja*, 53 Light SD *D. moj. baja*).

(J-K) Sleep time courses for *D. moj. moj.* **(J)** and *D. moj. baja* **(K)** during one day of 12h:12h light-dark followed by two days in constant darkness. Gray traces show controls that remain on 12h:12h LD schedule, groups transferred to darkness depicted in blues. Two-way ANOVAs find

significant group-by-time interactions for **(J)** ($F_{(143, 7436)}$=6.694, p<0.0001, n=26 LD, 28 LD->DD flies/group) and **(K)** ($F_{(143, 7293)}$=10.40, p<0.0001, n= 25 LD, 28 LD->DD flies/group).

(L) Total daily sleep for groups shown in **(J)** and **(K)**. Two-way repeated measures ANOVA finds a significant group-by-day interaction ($F_{(6, 206)}$=12.02, p<0.0001, n= 26 *D. moj. moj.* LD, 28 *D. moj. moj.* LD->DD, 25 LD *D. moj. baja*, 28 LD->DD *D. moj. baja*).
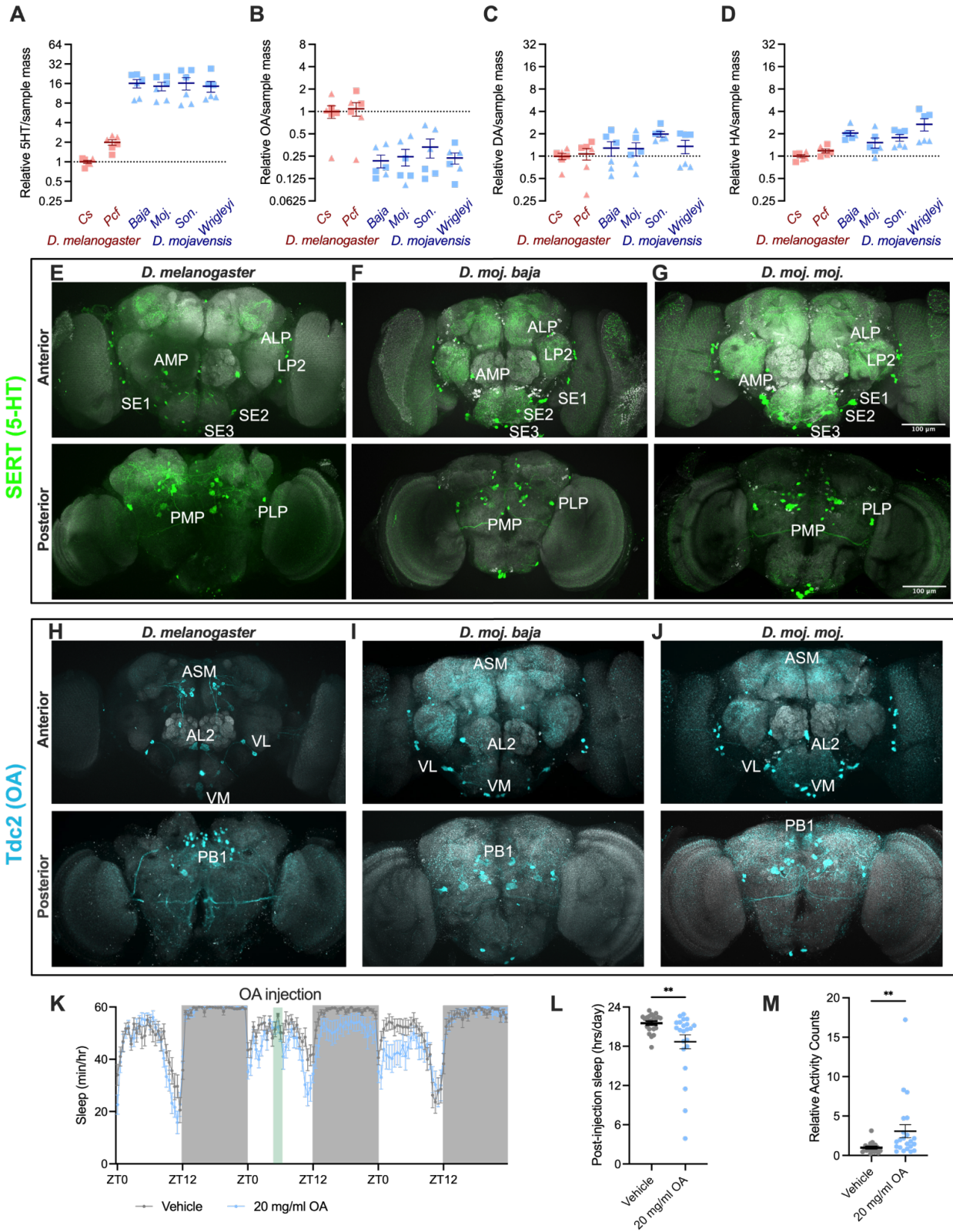
See also Figure S1.

Figure 4 – Interspecies variation of sleep- and wake-regulatory modulators between *D. melanogaster* and *D. mojavensis*

(A-D) Relative LC-MS/MS quantification of 5-HT **(A)**, octopamine **(B)**, dopamine **(C)**, and histamine **(D)** in heads of *D. melanogaster* wild-type stocks (reds) and *D. mojavensis* subspecies (blues). Data represent two independent experiments, each with three biological replicates per group (n=~100 heads/biological replicate; squares represent data from Expt #1, triangles are from Expt #2). One-way ANOVAs find a significant effect of genotype for 5-HT ($F_{(5,30)}$=10.26, p<0.0001), octopamine ($F_{(5,30)}$=9.488, p<0.0001), and histamine ($F_{(5,30)}$=5.950, p=0.0006), but no significant effect of genotype for dopamine ($F_{(5,30)}$=2.465, p=0.055).

(E-G) Immunostaining for SERT (green) in brains from *D. melanogaster* **(E)**, *D. moj. baja* **(F)**, and *D. moj. moj.* **(G)**. Z-projections showing cell bodies from anterior of brain in top row, from posterior in lower row. Scale bars in **(G)** also depict dimensions for **(E-F)**. Cell cluster labels based on Sitaraman et al., 2008.

(H-J) Immunostaining for Tdc2 (cyan) in whole brains from *D. melanogaster* **(H)**, *D. moj. baja* **(I)**, and *D. moj. moj.* **(J)**. Z-projections showing cell bodies from anterior of brain in top row, from posterior in lower row. Cell cluster labels based on Busch et al., 2009.

(K) Sleep time course for *D. moj. baja* flies that were microinjected with 18.4 nL of 20mM Octopamine (blue) or vehicle (gray). Green shading denotes the time of OA injection. Two-way repeated ANOVA finds a significant time-by-treatment interaction ($F_{(143,5863)}$=1.651, p<0.0001).

(L-M) Total sleep **(L)** and normalized activity counts **(M)** during 24h post-injection for groups shown in **(K)**. At least one distribution in **(L)** and **(M)** fail D'Agostino & Pearson test for Normality; Mann-Whitney tests find U= 125, p=0.0092 for **(L)** and U=110.5, p=0.0051 for **(M)**.

For **(K-M)**, n= 21 vehicle control and 22 OA-injected flies.

See also Figure S2.
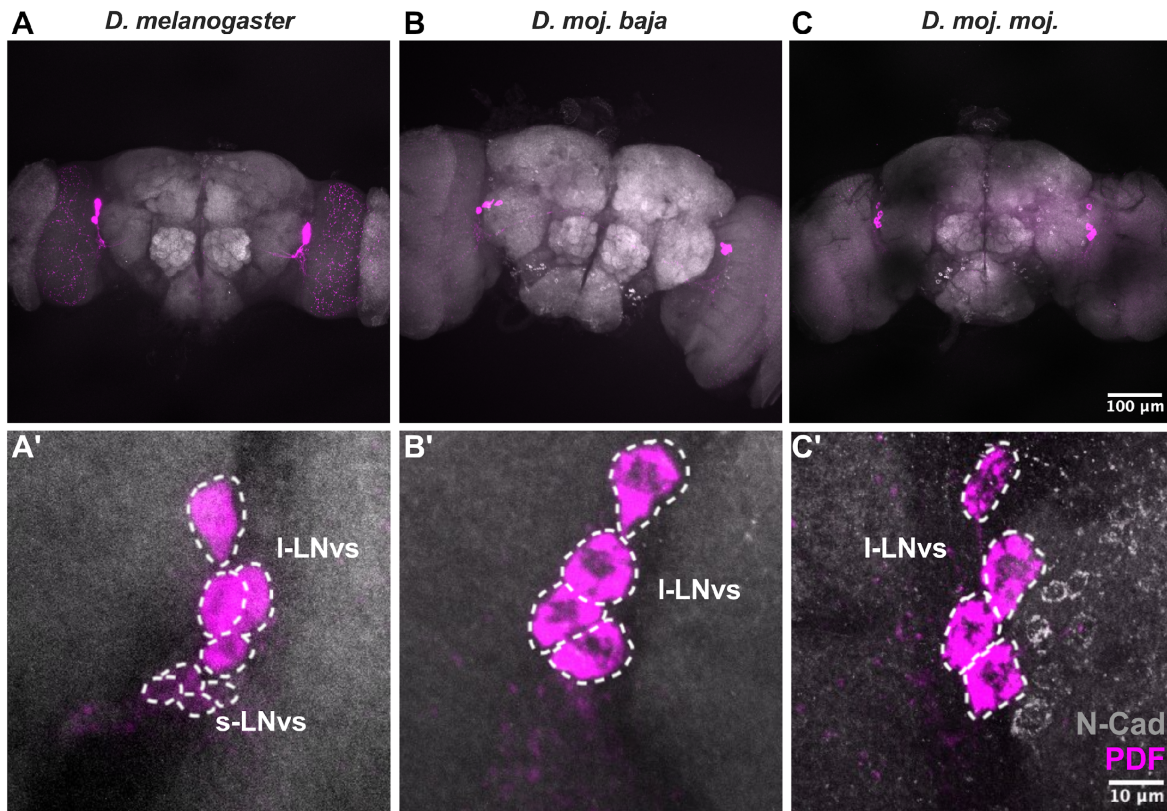
Figure 5 – Interspecies variability in PDF distribution

(A) Confocal projection of PDF immunostaining in a whole brain from *D. melanogaster*. **(A')** Higher magnification confocal micrograph of LNv cell bodies in *D. melanogaster*.

(B) PDF immunostaining in *D. moj. baja* brain, high magnification image of l-LNv soma in **(B').**

(C) Distribution of PDF in *D. moj. moj.* brain, **(C')** depicts high magnification view of l-LNv cell bodies.

Figures 5A-C use identical scales (see 100 μm scale bar in **(C)**), **Figures 5A'-C'** also use matching scales (see 10 μm bar in **(C')**).

Figure 6 – Sleep responses of *D. mojavensis* to nutrient deprivation correlate with survival time

(A-B) Survival times for *D. melanogaster* (*Cs*; red) and *D. mojavensis* (blues) females when housed on starvation agar **(A)** or dry tubes **(B)**. Mantel-Cox test finds significant effects for **(A)** (χ^2=250.6, df=2, p<0.0001, n= 70 *Cs*, 45 *D. moj. moj.*, 77 *D. moj. baja*) and for **(B)** (χ^2=232.6, df=2, p<0.0001, n= 64 *Cs*, 63 *D. moj. moj*, 64 *D. moj. baja*).

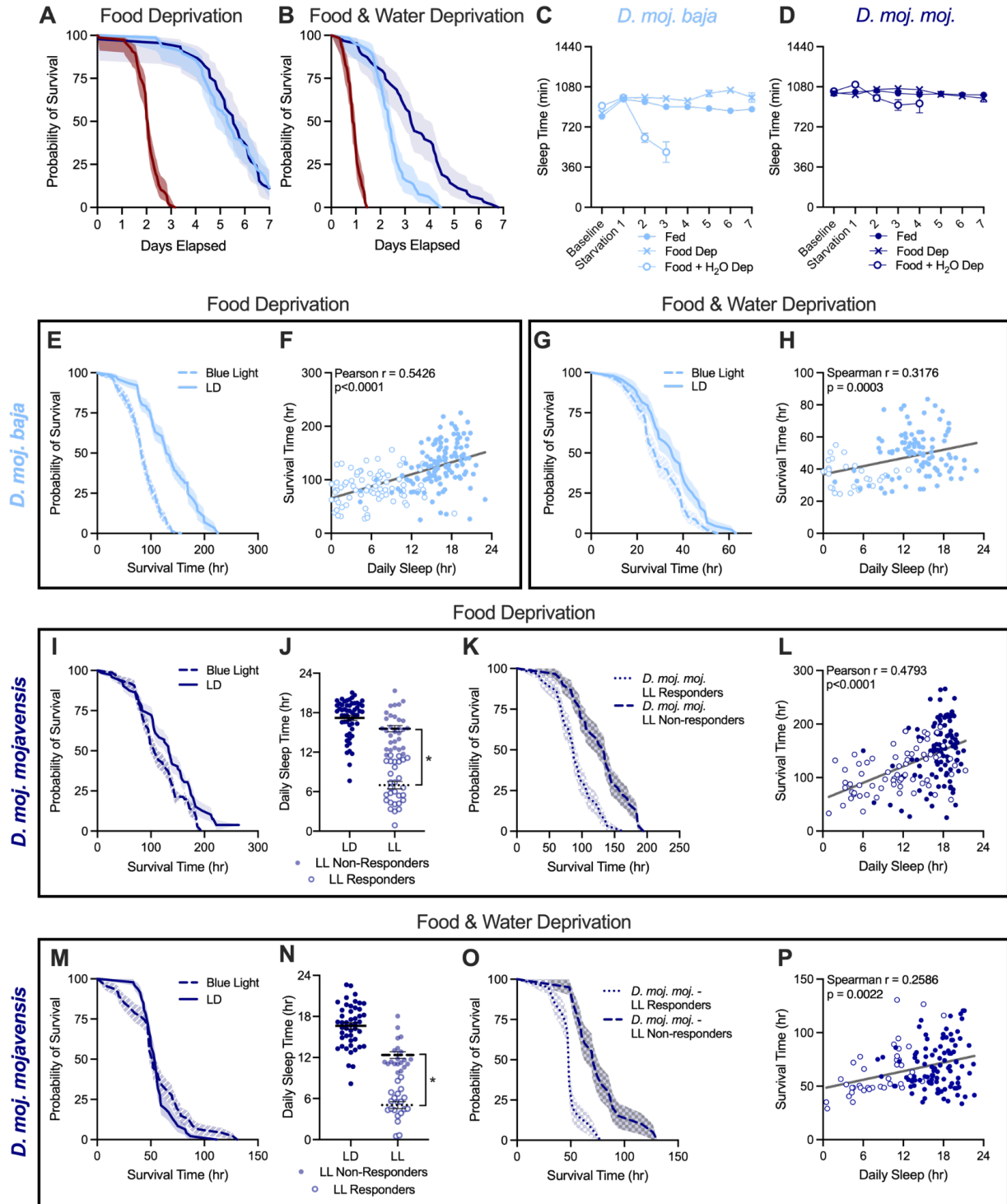**(C)** Daily sleep time for *D. moj. baja* flies during one day of baseline conditions that were then fed standard fly media (closed blue circles), 1% agar in water (crosses), or dry tubes (open circles). Mixed-effects analysis finds significant effect of time (p<0.0001), but not of condition (p=0.0966), n = 153 control, 108 food deprived, and 110 food and water deprived flies at beginning of experiment.

(D) Daily sleep time for *D. moj. moj.* flies during one day of baseline followed by feeding either standard fly media (closed circles), 1% agar in water (crosses), or dry tubes (open circles). Mixed-effects analysis detected no effect of condition (p=0.7460), but a significant effect of time (p=0.0011), n = 140 control, 95 food deprived, 100 food and water deprived flies at the beginning of the experiment.

(E) Survival time of food-deprived *D. moj. baja* was reduced for flies housed in constant blue light (dashed line) compared to siblings in 12h:12h LD (solid line). Log-rank (Mantel-Cox) test identifies a significant effect of constant light (χ^2=56.49, p<0.0001, n=76 flies/group).

(F) Mean sleep/day for food deprived *D. moj. baja* individuals that were housed in 12h:12h LD (filled dots) or in constant blue light (open dots) shows positive association with survival time. Pearson r = 0.5128, p<0.0001, n=60-76 flies/group).

(G) Survival time for *D. moj. baja* flies that were housed in LD (solid line) or constant blue light (dashed line) while deprived of both food and water. Mantel-Cox test χ^2=5.126, p=0.0236, n=44-48 flies/group.

(H) Average daily sleep plotted against survival time for individual *D. moj. baja* flies housed in LD (filled dots) or constant blue light (open dots). Spearman r=0.3176, p=0.0003, n=29 flies in constant light, 95 flies in LD.

(I) Survival time for food-deprived *D. moj. moj.* females that were housed in 12h:12h LD (solid line) or constant blue light (dashed line). Log-rank (Mantel-Cox) test finds a significant effect of light exposure (χ^2=4.977, p=0.0257, n=72-75 flies/group).

(J) Daily sleep of food deprived *D. moj. moj.* housed in LD or constant light. Flies exposed to LL that responded with daily sleep in lower half of the distribution marked with open circles, filled circles mark non-responders with highest sleep amounts. Mann-Whitney test between blue light responders vs non-responders p<0.0001, n=29 LL responders, 30 LL non-responders, 56 LD.

(K) Starvation survival time for *D. moj. moj.* that responded to constant light with lowest sleep (dotted line) was reduced compared to siblings with higher sleep amounts during light exposure (dashed line). Mantel-Cox test χ^2=28.16, p<0.0001, n=29-30 flies/group.

(L) Mean sleep/day during starvation for individual *D. moj. moj* females housed in 12h:12h LD (filled dots) or constant blue light (open dots) correlates positively with survival time. Pearson r = 0.4523, p<0.0001, n=56 flies in LD, 59 flies in constant blue light.

(M) Survival time for food and water deprived *D. moj. moj.* housed in LD (solid line) or constant blue light (dashed line). Mantel-Cox test χ^2=1.225, p=0.2684, n=47 flies/group.

(N) Daily sleep during food and water deprivation for *D. moj. moj.* flies housed in LD or constant blue light. Flies exposed to LL that responded with daily sleep in lower half of the distribution marked with open circles, filled circles mark non-responders with highest sleep amounts. Mann-Whitney test between blue light responders vs non-responders p<0.0001, n=20 LL responders, 20 LL non-responders, 47 LD.

(O) Survival time during food and water deprivation for *D. moj. moj.* females housed in constant blue light. Short-sleeping responders represented by dotted line, longer-sleeping non-responders by dashed line. Mantel-Cox test χ^2=21.39, p<0.0001, n=20 flies/group.

(P) Scatter plot of daily sleep vs survival time for *D. moj. moj.* that were housed in LD (filled dots) or constant blue light (open dots) during food and water deprivation. Spearman r=0.2586, p=0.0022, n=98 flies in LD, 40 flies in constant light.

See also Figure S2.
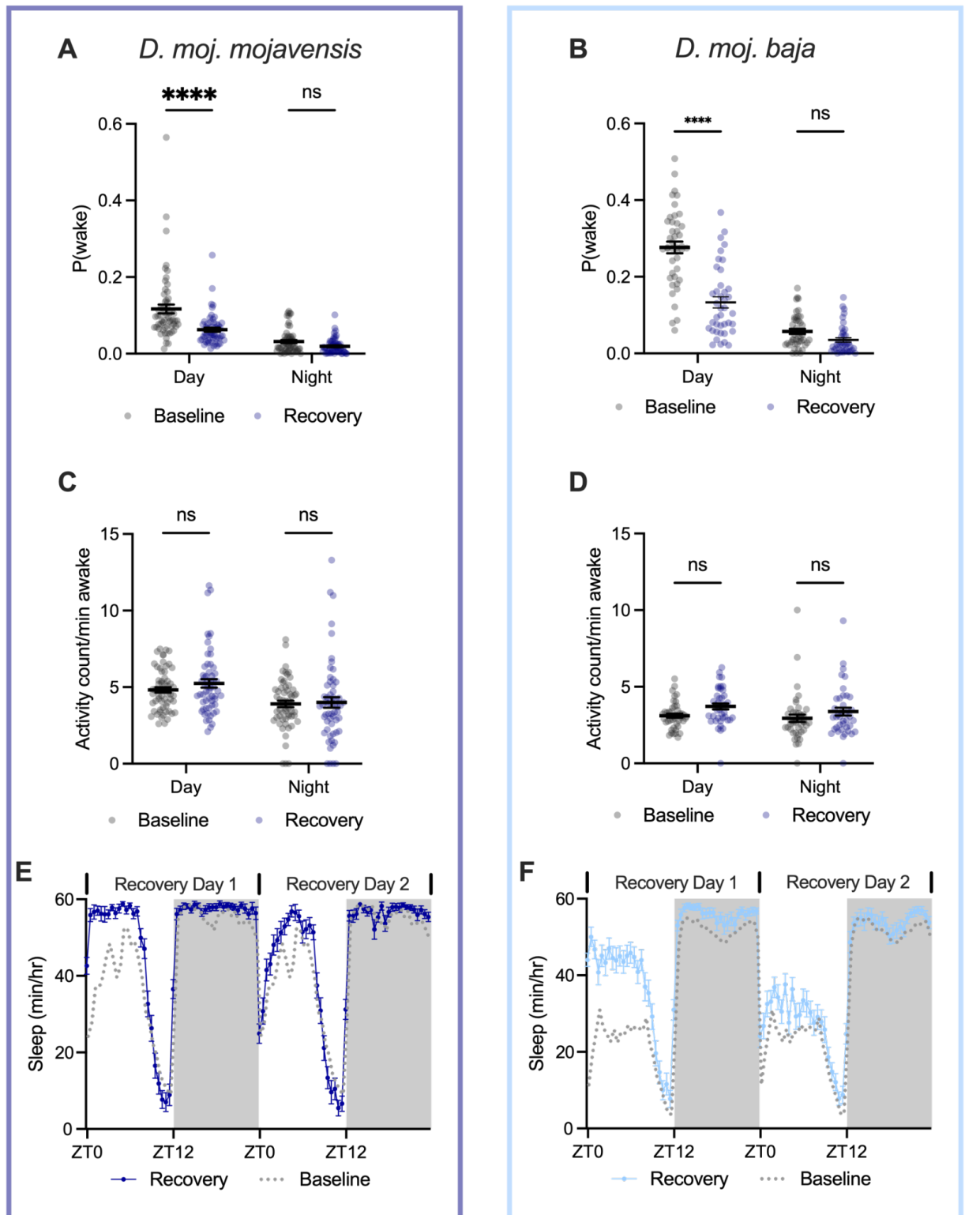
Figure S1 - Sleep and activity parameters following sleep deprivation.

Related to Figure 3.

(A-B) P(wake) for *D. moj. mojavensis* **(A)** and *D. moj. baja* **(B)** du—------ring baseline (gray) and first recovery day (blue) after overnight vortex sleep deprivation. Two-way repeated measures ANOVAs find significant day-by-time interaction for **(A)** $F_{(1,58)}$=17.08, p=0.0001, n=59 flies and **(B)** $F_{(1,36)}$=39.14, p<0.0001, n=40 flies/group.

(C-D) Activity counts/waking minute for *D. moj. mojavensis* **(C)** and *D. moj. baja* **(D)** during baseline (gray) and first recovery day after vortex sleep deprivation (blue). Two-way repeated measures ANOVA find no significant main effect of day for **(C)** $F_{(1,58)}$=1.061, p=0.31, n=59 flies, but do find a significant effect of day for **(D)** $F_{(1,42)}$=5.509, p=0.0237, n=40 flies/group.

(E-F) Sleep time courses during two days of recovery following overnight sleep deprivation in *D. moj. mojavensis* **(E)** or *D. moj. baja* **(F)**. Sleep traces from 24h of baseline replotted in gray, recovery days shown in blues.

| **A** *D. melanogaster* | **B** *D. moj. baja* | **C** *D. moj. moj.* |

Tdc2 N-Cad — Tdc2 N-Cad — Tdc2 N-Cad
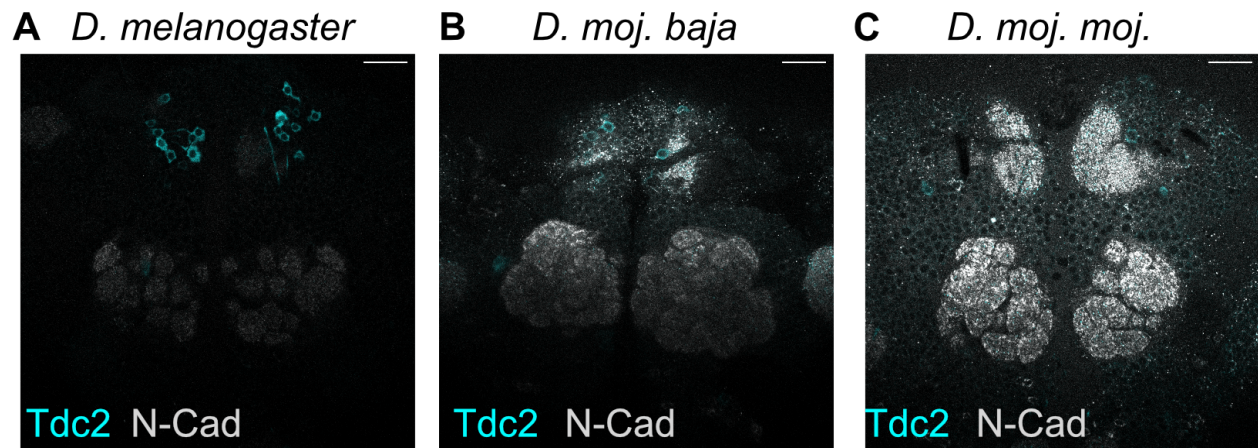
Figure S2 - Confocal images of Tdc2-positive ASM neurons in *D. melanogaster* and *D. mojavensis*.

Related to Figure 4.

(A-C) Single confocal slices showing Tdc2-positive ASM neurons in *D. melanogaster* **(A)**, *D. moj. baja* **(B)**, and *D. moj. moj.* **(C)**. Image acquisition and display settings are matched for all three panels; scale bars depict 25μm.

A Food Deprivation
B Food & Water Deprivation

Food Deprivation

C Pearson r = -0.4586, p<0.0001
D Pearson r = -0.3491, p<0.0001
E Pearson r = -0.7296, p < 0.0001

Food & Water Deprivation

F Spearman r = -0.2378, p = 0.0078
G Spearman r = -0.09686, p = 0.2845
H Spearman r = -0.8605, p < 0.0001

Food Deprivation

I Pearson r = -0.4041, p<0.0001
J Pearson r = -0.1451, p=0.0568
K Pearson r = -0.8192, p < 0.0001

Food & Water Deprivation

L Spearman r = -0.2202, p = 0.0094
M Spearman r = -0.08413, p = 0.3266
N Spearman r = -0.9464, p < 0.0001

Figure S3 - Sleep and activity parameters during nutrient deprivation.

Related to Figure 6.

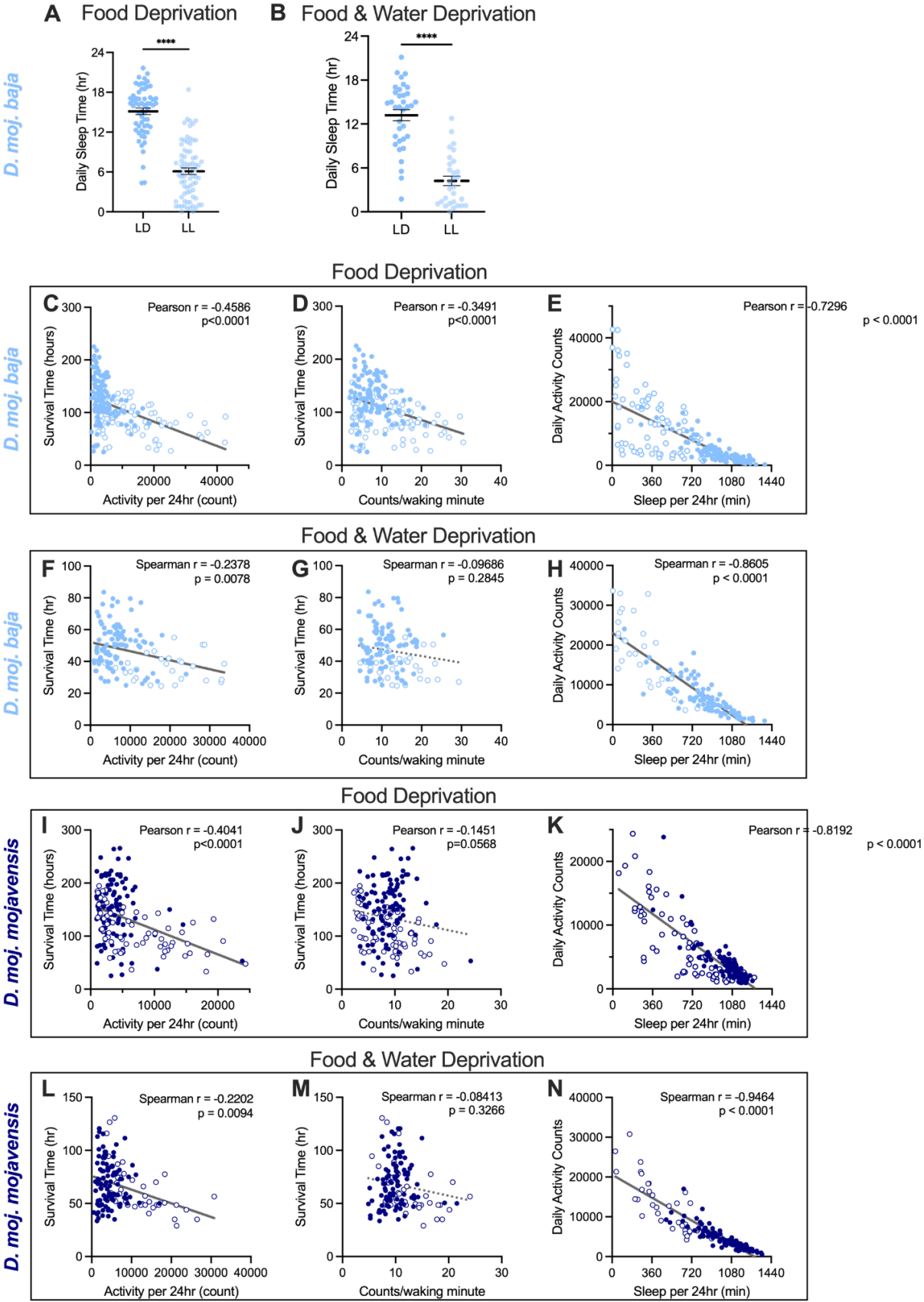**(A-B)** Daily sleep amounts for *D. moj. baja* flies that were deprived of food **(A)** or of food and water **(B)** while housed in LD or constant blue light. Food deprivation: Mann-Whitney U=305, p<0.0001, n=60-75 flies/group; Food and water deprivation: Mann-Whitney U=68.50, p<0.0001, n=29-35 flies/group.

(C-E) Plots showing relationships between daily activity and survival time **(C)**, counts/waking minute and survival time **(D)**, and daily sleep and daily activity counts **(E)** for *D. moj. baja* flies that were deprived of food. Filled dots represent individuals housed in LD, open dots show flies in constant blue light. Pearson correlation results shown for each panel.

(F-H) Daily activity versus survival time **(F)**, counts/waking minute versus survival time **(G)**, and daily sleep versus daily activity counts **(H)** for *D. moj. baja* flies that were deprived of food and water. Filled dots represent individuals housed in LD, open dots show flies in constant blue light. Pearson correlation results shown for each panel.

(I-K) Scatter plots showing daily activity and survival time **(I)**, counts/waking minute and survival time **(J)**, and daily sleep and daily activity counts **(K)** for food-deprived *D. moj. moj.* flies. Filled dots represent individuals housed in LD, open dots show flies in constant blue light. Spearman correlation results shown for each panel.

(L-N) Daily activity versus survival time **(F)**, counts/waking minute versus survival time **(G)**, and daily sleep versus daily activity counts **(H)** for *D. moj. moj.* flies that were deprived of food

and water. Filled dots represent individuals housed in LD, open dots show flies in constant blue

light. Spearman correlation results shown for each panel.

DISCUSSION

Periods of adaptive sleep loss have been reported in several vertebrate species, especially in birds and marine mammals (Lesku et al., 2012; Lyamin et al., 2018; Lyamin et al., 2005). During these periods, it is thought that animals can acutely defer or offset the costs that accumulate from sleep loss. Here, we find that *D. mojavensis* exhibits an opposing behavioral strategy: they chronically show elevated sleep time and consolidation, even during periods of insufficient food. This adaptive sleep strategy confers a survival advantage in conditions of hunger or thirst, supporting a functional role for sleep in maintaining efficient energy usage (Markwald et al., 2013; Stahl et al., 2017; Knutson et al., 2007; Lesku and Schmidt, 2022). While additional work is required to identify and characterize functional advantages that are fulfilled by elevated levels of sleep, it is also possible that hypersomnia may allow desert-adapted *D. mojavensis* to clear metabolic waste, or manage oxidative stress (Xie et al., 2013; Bedont et al., 2023; Hill et al., 2018; Vaccaro et al., 2020).

Alternatively, the increased need for sleep in *D. mojavensis* could offset costs of physiological adaptations made by desert-adapted flies that allow them to feed on cactus diets or to thrive in the desert environment (Matzkin et al., 2007; Stratman and Markow, 1998; Guillén et al., 2015; Krebs, 1999; Frank and Fogleman, 1992; Cázarez-García et al., 2017; Fellows and Heed, 1972). Interestingly, another recent study found that other *Drosophila* species exhibit a range of homeostatic responses to sleep loss, indicating that broad studies of *Drosophila* evolution could uncover interspecific adaptations in sleep need or function (Joyce et al., 2023). Our characterization of increased sleep time and need in stress-resilient *D. mojavensis* provide a

novel model species to examine the adaptive advantage(s) of elevated sleep and to investigate the evolution of sleep regulatory mechanisms across related species.

Recent efforts to sequence the genomes of many *Drosophila* species has enabled the analysis of genetic correlates to environmental adaptations, but the contributions of altered behavioral strategies as populations adapt to environmental niches remain to be explored (Guillén et al., 2015; Matzkin and Markow, 2009; Rane et al., 2019; Hasson et al., 2019; Parker et al., 2018; Moreyra et al., 2023). We anticipate that combining genomic approaches with behavioral phenotyping across many species could identify common mechanisms that drive changes in sleep regulation and in the underlying functions of sleep across the animal kingdom. While this approach may open broad possibilities to study species that have adapted to a variety of conditions, more specific insights may be gleaned by focusing on species adapted to particular conditions. In this case, examining flies that have evolved to withstand high desert temperatures and periods of nutrient deprivation could inform our understanding of the tolls of changing global climates on physiology and identify possible behavioral approaches for animals to cope with a warming world (Obradovich et al., 2017; Berger et al., 2023; Buchholz et al., 2019).

Our neurochemical and anatomical studies reveal differences in the abundance of two sleep/wake-related neuromodulators, 5HT and OA, between *D. melanogaster* and *D. mojavensis*, along with a restricted distribution of the wake-promoting circadian output peptide PDF in long-sleeping *D. mojavensis* flies. The similar distributions of serotonergic and octopaminergic neurons within *D. melanogaster* and *D. mojavensis* suggest that sleep circuit organization may be shared between the species, but that mechanisms governing neural activity or signaling dynamics may be differentially tuned as populations evolve. The availability of sequenced

genomes for many *Drosophila* species, including *D. mojavensis*, may enable future studies to dissect neuromodulator signaling components with precise genetic tools similar to those already applied in *D. melanogaster* (Clark et al., 2007; Myers et al., 2000; Miller et al., 2018; Kim et al., 2021). While the global organization of 5HT and Tdc2-expressing neurons appears largely similar between *D. melanogaster* and *D. mojavensis*, distribution of PDF expression in core circadian circuits differs. Similar to reports in several other *Drosophila* species[49–51], we did not detect immunostaining for PDF in soma or axonal projections from s-LNvs, indicating that circadian circuit organization may commonly differ between fly species (Menagazzi et al., 2017; Hermann et al., 2013; Kauranen et al., 2012). Future studies will be required to examine the precise contributions of changes in each neuromodulator system to behavioral variations between species, and precisely examining each of these components may provide more insight into the functional importance of high sleep drive in *D. mojavensis*.

APPENDIX - RNA-sequencing of body segments after sleep deprivation in *Drosophila melanogaster*

Introduction

The brain is not the only source of sleep-regulating signals. For example, whole-animal deletion of the circadian clock gene BMAL1 in mice display abnormalities in both sleep timing and sleep duration. Phenotypic rescue is seen when *Bmal1* expression is restored in skeletal muscle, but not in brain (Laposky et al., 2005; Ehlen et al., 2017). In *Drosophila,* an immune molecule, the NF-kappaB *Relish*, promotes sleep when expressed in the fat body (Williams et al., 2007).

The purpose of this experiment was to use RNA-sequencing of *D. melanogaster* body segments to identify candidate genes in non-brain tissues which may serve as homeostatic sleep-regulatory signals by prolonging time awake via mechanical overnight sleep deprivation.

Methods

Fly experiments

Canton-S flies were cultured at 25ºC at 60% humidity on a 12h:12h light:dark cycle and kept on standard cornmeal molasses media. At 4-7 days post-eclosion, they were loaded into Trikinetics activity monitors and given 2 days to acclimate. Flies were then mechanically deprived of sleep via the Sleep Nullifying Apparatus (SNAP) (see Melnattur et al., 2020) throughout the 12h dark/night period and collected at the onset of the next 12h light period.

Tissue collection and preparation

Live flies were frozen at -80ºC. Frozen flies were then vortexed to remove heads and legs. Bodies were segmented into thoraxes and abdomens over dry ice. Total RNA was isolated from body segments using Trizol and RNeasy Mini Kit from Qiagen according to the manufacturer's instructions.

RNA sequencing

RNA sequencing, quality control, and analysis were performed by the UCLA Neuroscience Genomic Core. Reads were aligned to the latest Drosophila melanogaster_dm6 reference genome using the STAR spliced read aligner. Total counts of read-fragments aligned to known gene regions within the Drosophila melanogaster dm6 refSeq reference annotation were used as the basis for quantification of gene expression. Differentially expressed genes were identified using edgeR and ranked based on FDR p-values of ≤ 0.05.

Heat maps and volcano plots were constructed with R. Heatmaps used the R package ComplexHeatmap.

Results

Significantly differentially expressed genes

[See Figures A1-A8]

Of the differentially expressed genes (DEGs) for which there was a statistically significant change in expression after sleep deprivation (of p-value<0.05), male thoraxes showed an increase in the expression levels of 37 genes and a decrease in 14 genes in response to sleep

deprivation. Female thoraxes showed an increase in 34 genes and a decrease in 18 genes. Male abdomens showed an increase in 8 genes and a decrease in 56 genes. Female abdomens showed an increase in 27 genes and a decrease in 16 genes.

DEGs in common between males and females

[See Tables A1-A2]

DEGs in common between males and females are of particular interest under the assumption that sleep regulatory signals might be shared between the sexes.

For thoraxes, there were a total of 37 DEGs in common between males and females (26 up-regulated, 11 down-regulated). For abdomens, there were a total of 13 DEGs in common (1 up-regulated in both males and females, 1 down-regulated in males and up-regulated in females, and 11 down-regulated in both males and females). Thus, DEGs identified in both males and females account for 71.2-72.5% of total DEGs identified in thorax and 20.3-30.2% of DEGs identified in abdomen.

The only gene identified as a DEG for males and females in both thorax and abdomen was the down-regulated gene Lsd-1, a lipid storage protein.
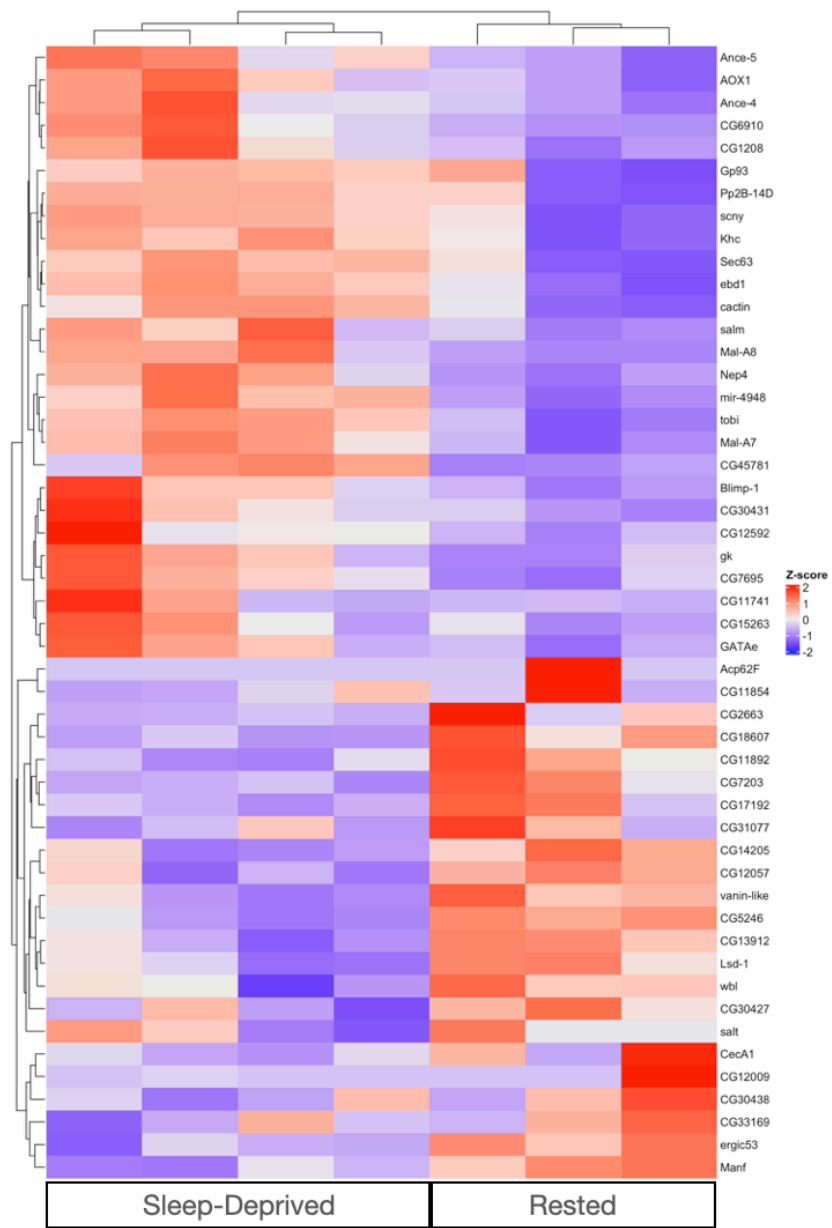
Figure A1: Heat map of top 50 differentially expressed genes - female abdomens
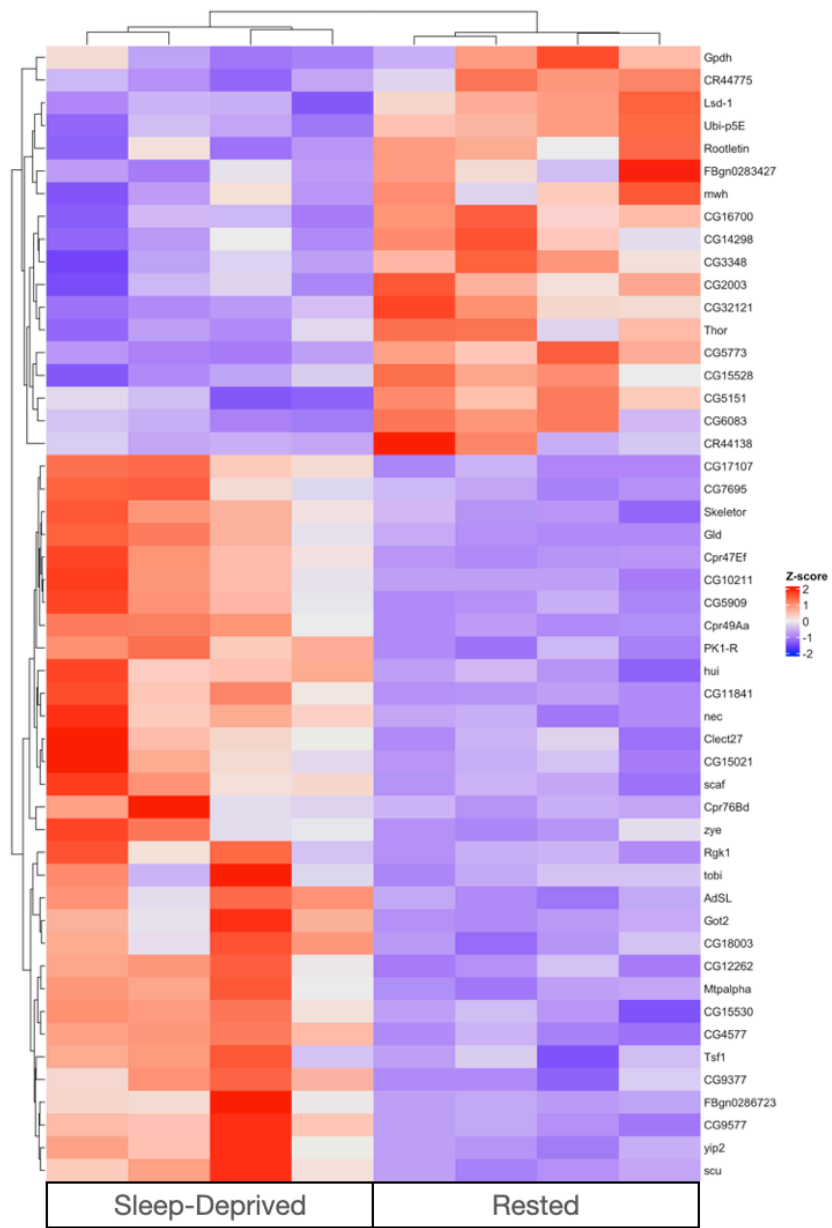
Figure A2: Heat map of top 50 differentially expressed genes - female thoraxes
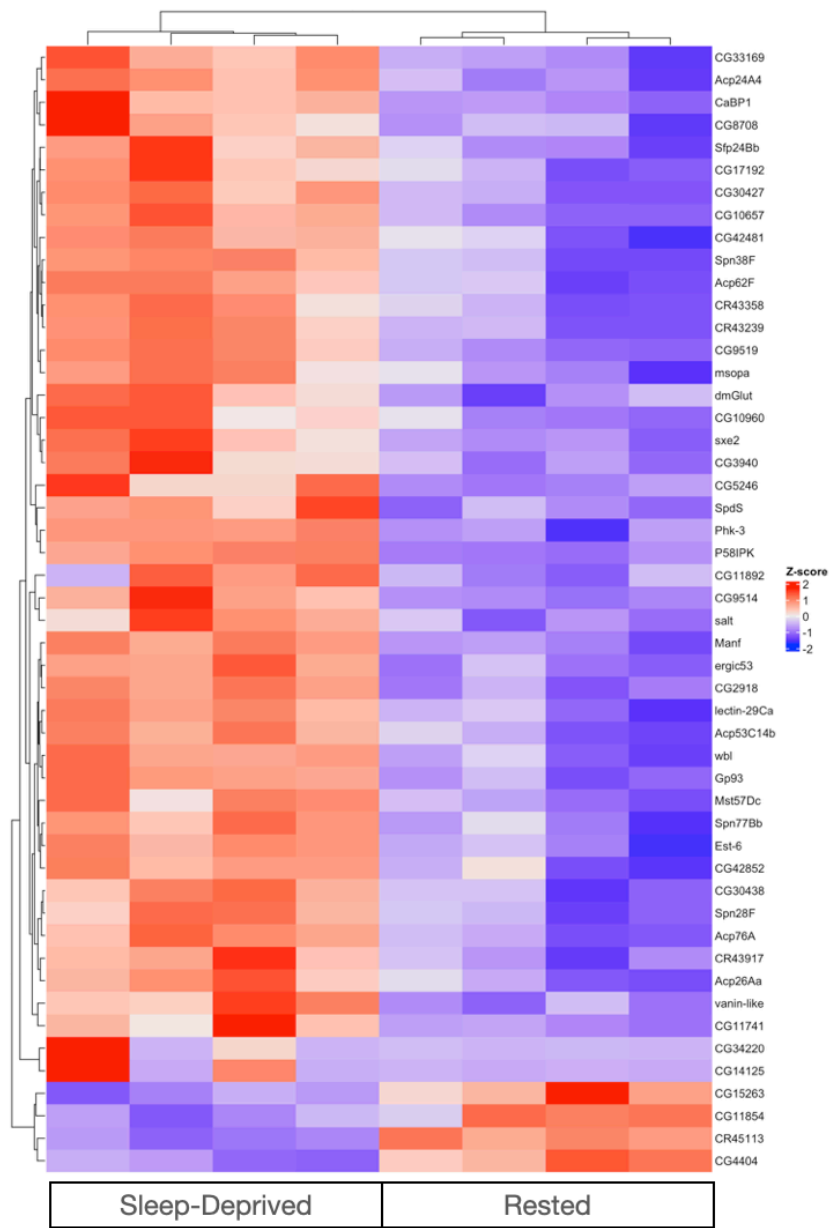
Figure A3: Heat map of top 50 differentially expressed genes - male abdomens
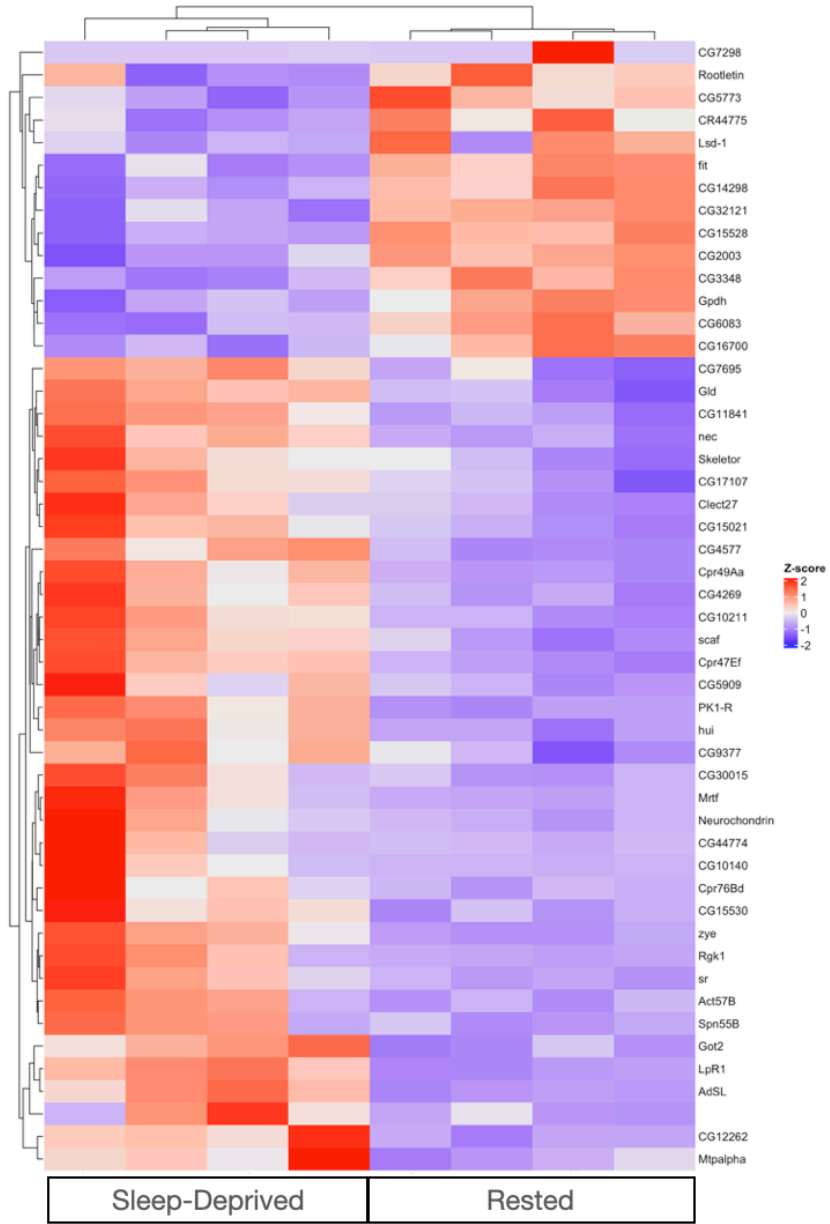
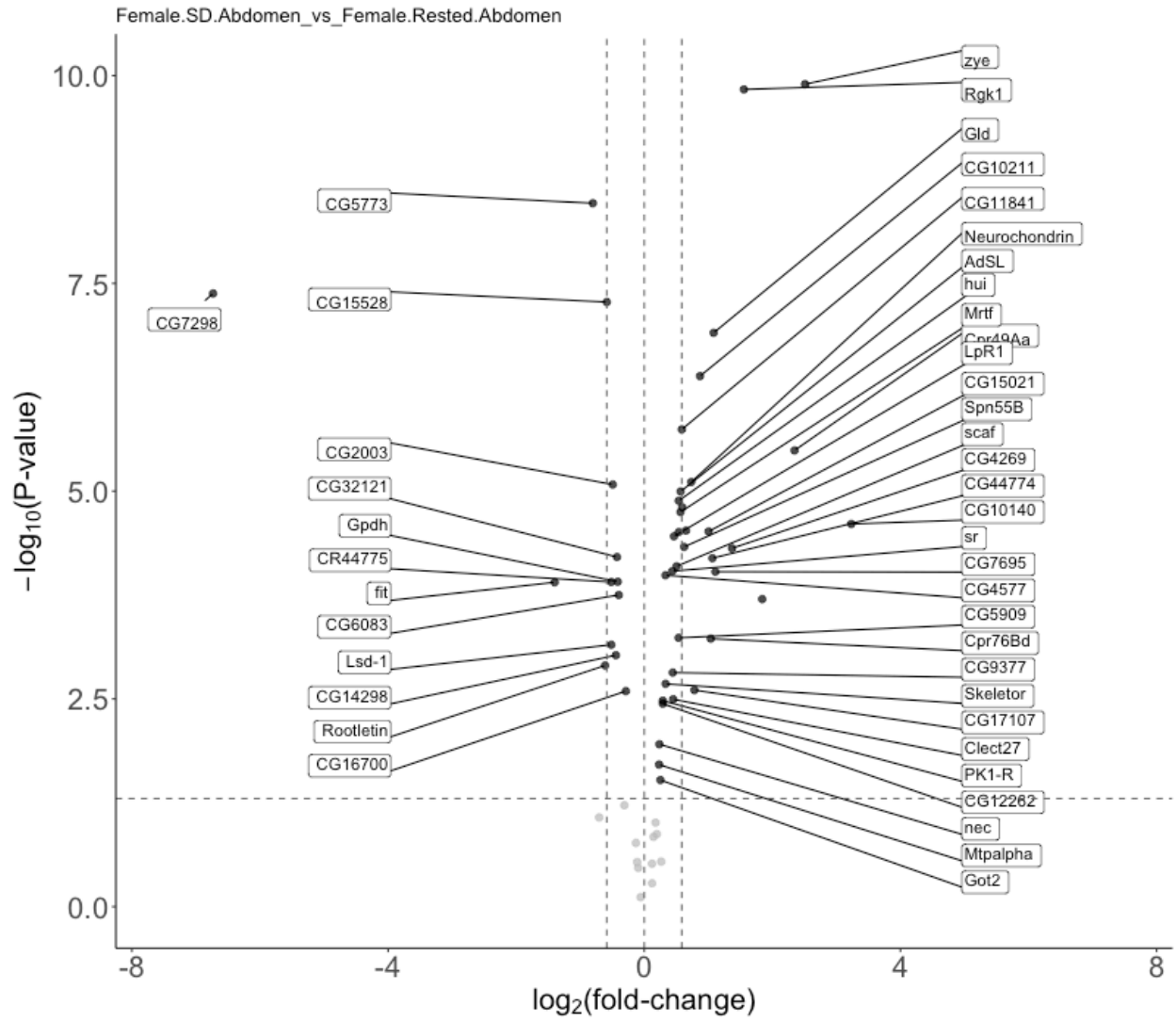**Figure A4: Heat map of top 50 differentially expressed genes - male thoraxes**

Figure A5: DEG volcano plot - female abdomens
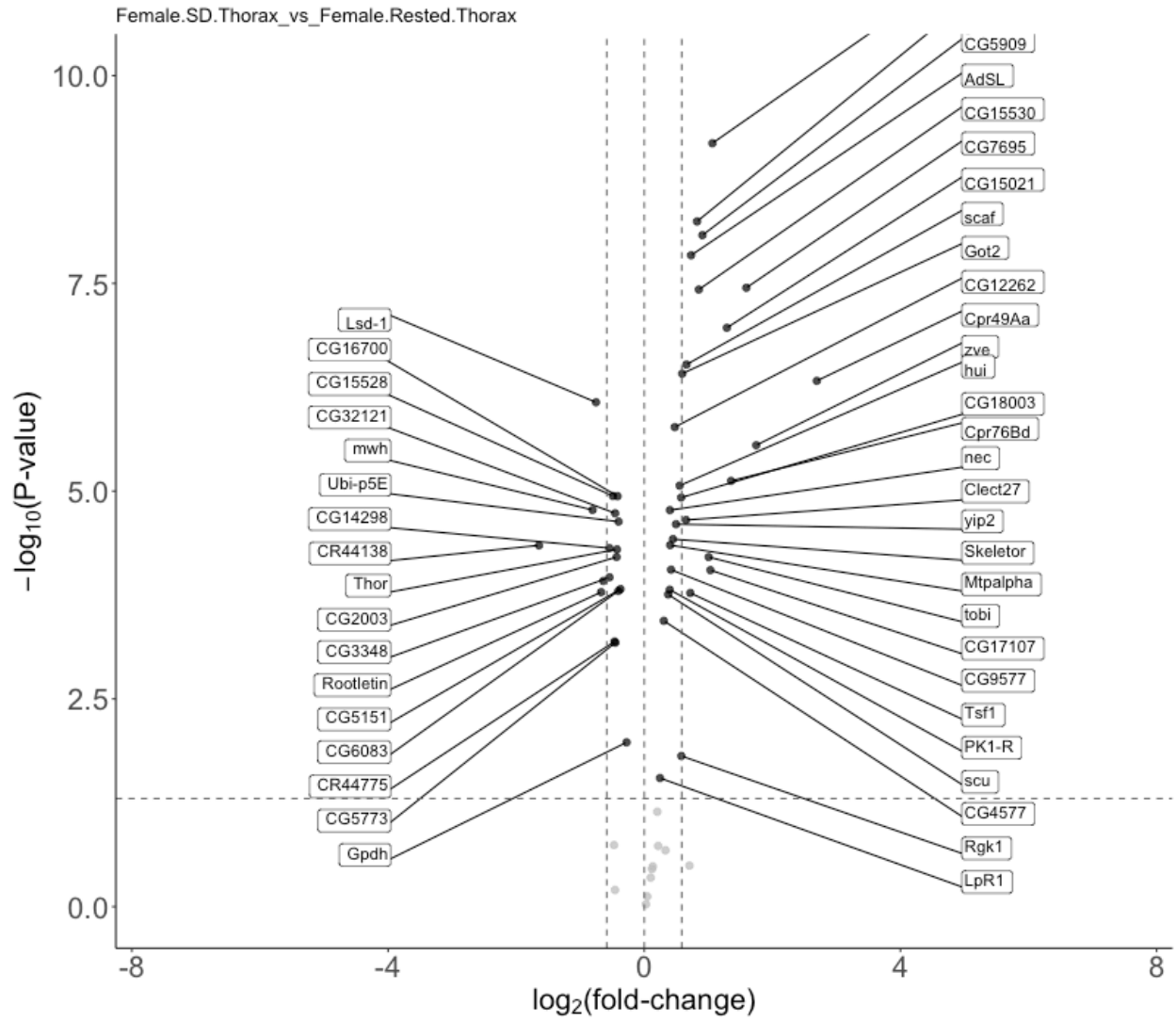
Figure A6: DEG volcano plot - female thoraxes
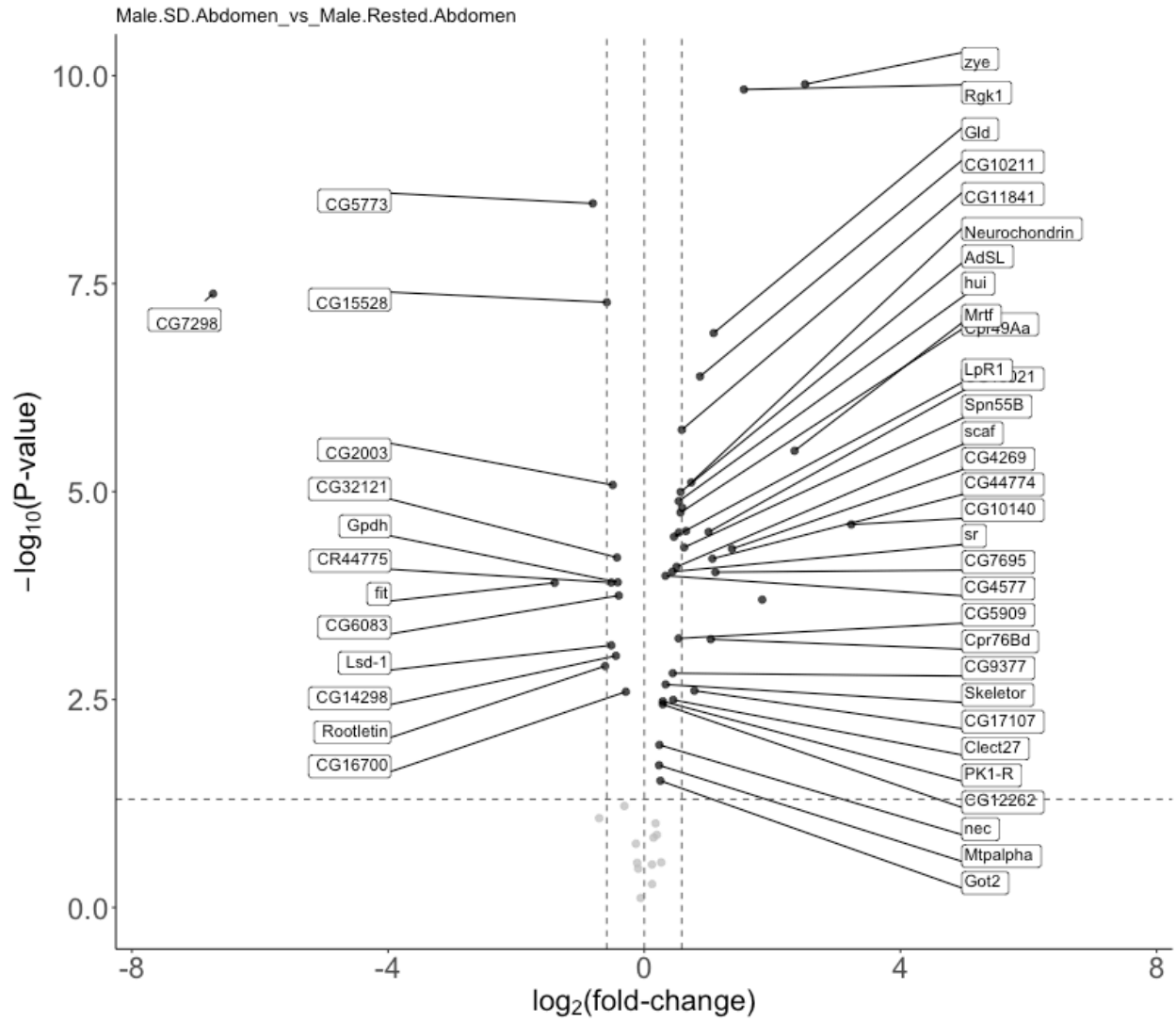
Figure A7: DEG volcano plot - male abdomens
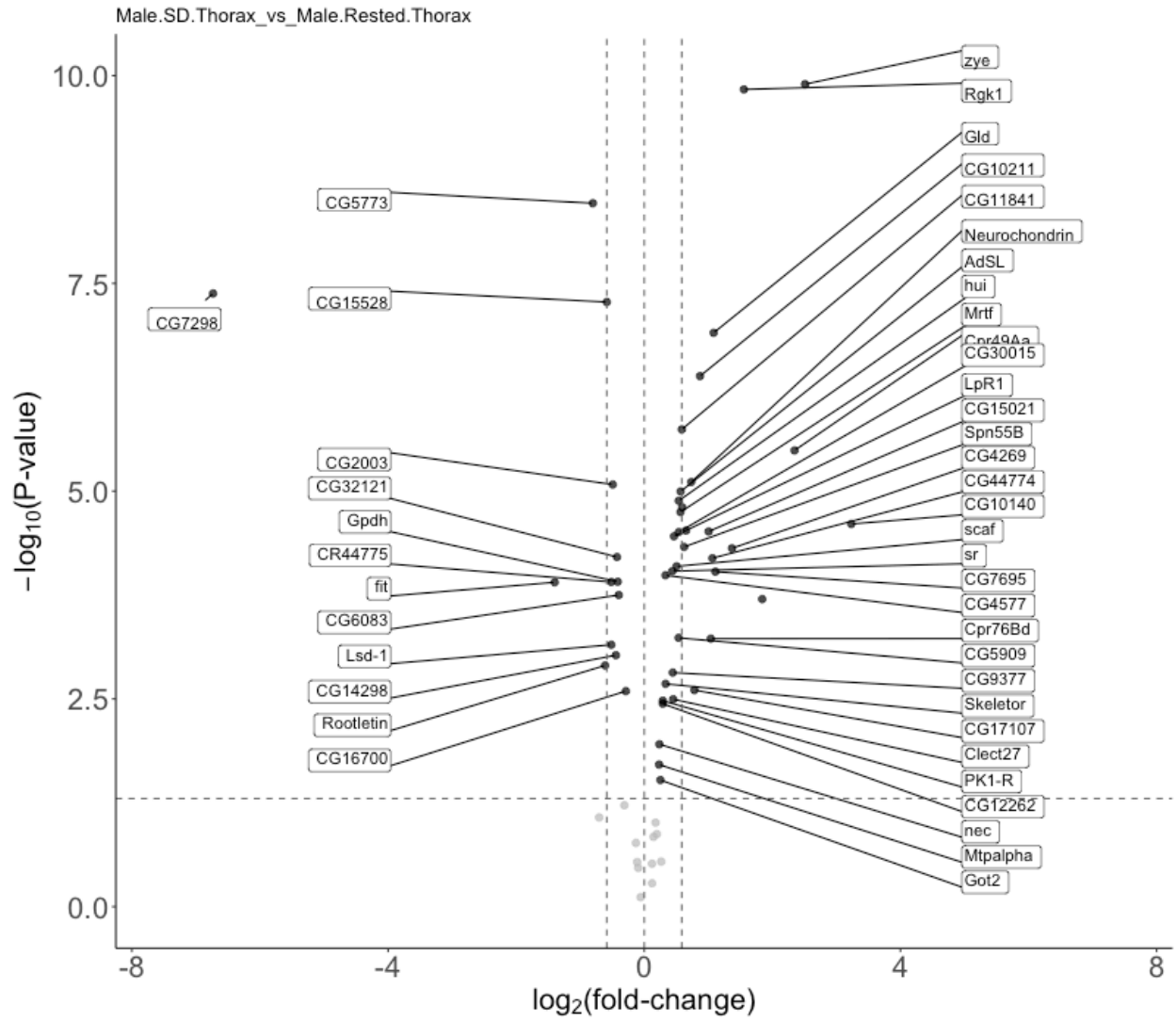
Figure A8: DEG volcano plot - male thoraxes

DEG	Male Log Fold Change	Female Log Fold Change
CG15263	0.9288932	1.207198069
Gp93	-0.4053066	0.247572683
Manf	-0.3616992	-0.16523069
CG30427	-0.5566686	-0.33151938
wbl	-0.6518532	-0.39734499
CG11892	-0.542109	-0.57813414
Lsd-1	-0.421041	-0.58867207
CG13912	-0.3185969	-0.61098624
CG17192	-1.059647	-0.89178786
vanin-like	-0.8110433	-0.90283597
CG5246	-0.749905	-1.12808419
CG18607	-0.4652528	-1.85798052
CG2663	-0.5562154	-2.23316517

Table A1: DEGs in common between males and females - abdomens

DEG	Male Log Fold Change	Female Log Fold Change
Cpr49Aa	2.346495967	2.693987099
Cpr47Ef	1.52573994	1.802215858
zye	2.511007198	1.747935239
CG7695	1.110048616	1.593264647
Gld	1.083387229	1.395537487
Cpr76Bd	1.038441947	1.356104744
CG15021	1.004970334	1.28974819
CG10211	0.86993417	1.064009023
CG17107	0.783424387	1.032991491
CG5909	0.536027246	0.907806519
CG15530	0.651618164	0.852271717
CG11841	0.5862445	0.835974317
CG9377	0.44421468	0.823615363
AdSL	0.567996916	0.730525294
scaf	0.504665828	0.658788997
Clect27	0.451610183	0.651490122
Got2	0.252254226	0.592421409
Rgk1	1.555671403	0.57912126
hui	0.538487433	0.552161398
CG12262	0.289146638	0.476560641
Skeletor	0.33351716	0.448018098
Mtpalpha	0.231187503	0.404538909
nec	0.236349979	0.401147649
PK1-R	0.289250944	0.397917379
CG4577	0.331505463	0.3053098
LpR1	0.463704511	0.245820861
Gpdh	-0.414141706	-0.275606265
CG6083	-0.398513093	-0.405092439
CG2003	-0.492594178	-0.431360451
CG5773	-0.802817052	-0.451657285
CG32121	-0.426145491	-0.455142676

CR44775	-0.514475339	-0.464204409
CG15528	-0.584052522	-0.489175353
CG3348	-0.988445255	-0.543163952
CG14298	-0.443448692	-0.544680135
Rootletin	-0.611461143	-0.673791802
Lsd-1	-0.515618218	-0.753866574

Table A2: DEGs in common between males and females - thoraxes

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