Variation in sleep and metabolic function is associated with latitude and average temperature in *Drosophila melanogaster*

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**Abstract**

Regulation of sleep and metabolic homeostasis is critical to an animal’s survival and under stringent evolutionary pressure. Animals display remarkable diversity in sleep and metabolic phenotypes; however, an understanding of the ecological forces that select for, and maintain, these phenotypic differences remains poorly understood. The fruit fly, *Drosophila melanogaster*, is a powerful model for investigating the genetic regulation of sleep and metabolic function, and screening in inbred fly lines has led to the identification of novel genetic regulators of sleep. Nevertheless, little is known about the contributions of naturally occurring genetic differences to sleep, metabolic phenotypes, and their relationship with geographic or environmental gradients. Here, we quantified sleep and metabolic phenotypes in 24 *D. melanogaster* populations collected from diverse geographic localities. These studies reveal remarkable variation in sleep, starvation resistance, and energy stores. We found that increased sleep duration is associated with proximity to the equator and elevated average annual temperature, suggesting that environmental gradients strongly influence natural variation in sleep. Further, we found variation in metabolic regulation of sleep to be associated with free glucose levels, while starvation resistance associates with glycogen and triglyceride stores. Taken together, these findings reveal robust naturally occurring variation in sleep and metabolic traits in *D. melanogaster*, providing a model to investigate how evolutionary and ecological history modulate these complex traits.

**KEYWORDS**

clinality, evolution, metabolism, natural variation, sleep, starvation resistance

1 | **INTRODUCTION**

Species display robust differences in homeostatically regulated behaviors including sleep, feeding, and metabolic function, yet little is known about the ecological and functional relationship between these traits (Aulsebrook, Jones, Rattenborg, Roth, & Lesku, 2016; Eban-Rothschild, Giardino, & de Lecea, 2017). In mammals, sleep duration ranges from ~2 to 18 hr/day, suggesting that the environment and evolutionary history potently affect sleep regulation (Capellini, Barton, McNamara, Preston, & Nunn, 2008). While the ecological factors that drive differences in sleep remain largely unknown, a central hypothesis is that variation in sleep is linked to an animal’s metabolic and foraging needs (Capellini et al., 2008; Siegel 2005). Supporting this notion, variation in sleep duration in...
Drosophila is associated with latitudinal cline (Svetec, Zhao, Saelao, Chiu, & Begun, 2015), raising the possibility that temperature and food availability also impact sleep need. Determining the relationship between sleep and metabolic function, and how evolution shapes these processes, is critical for understanding the function of sleep and basis for sleep differences between and within species.

The fruit fly Drosophila melanogaster presents a powerful model for investigating genetic interactions between sleep and metabolic processes (Erion, DiAngelo, Crocker, & Sehgal, 2012; Yurgel, Masek, DiAngelo, & Keene, 2014). High throughput measurements of fly sleep and activity can be obtained using Drosophila activity monitors (DAMS), where infrared beam breaks are indicative of fly movement (Pfeifferberger, Lear, Keegan, & Allada, 2010a). Sleep is measured by 5 min of inactivity because it correlates with all other characteristics of sleep (Shaw, Cirelli, Greenspan, & Tononi, 2000).

Flies acutely modulate their sleep in accordance with nutrient availability, and starvation potently inhibits sleep and initiates foraging, thereby providing a system to investigate the relationship between sleep and metabolic regulation (Keene et al., 2010; Lee & Park, 2004; Linford, Chan, & Pletcher, 2012). Genetic evidence suggests sleep and metabolic function are highly conserved from flies to mammals at the molecular, pharmacological, and physiological levels (Allada & Siegel, 2008; Padmanabha & Baker, 2014), indicating that flies are an excellent system to examine the interactions between sleep and metabolic function.

While screens of inbred Drosophila lines have identified many regulators of sleep and metabolism (Cirelli et al., 2005; Koh et al., 2008; Rogulja & Young, 2012), naturally occurring variation has also been leveraged to identify the genetic architecture regulating these processes (Harbison, Carbone, et al., 2009; Harbison, McCoy, & Mackay, 2013; Hardy et al., 2018). Quantitative genetic approaches in fully sequenced lines have provided insight into the genetic basis for resistance to environmental and physiological stressors including starvation resistance, and identified novel regulators of sleep (Harbison, Yamamoto, Fanara, Norga, & Mackay, 2004; Vieira et al., 2000). Further, experimental evolution and artificial selection approaches have revealed a relationship between sleep, feeding, and starvation resistance (Masek et al., 2014; Slocumb et al., 2015). For example, selection for short-sleeping flies results in reduced energy stores and sensitivity to starvation, while selecting for starvation resistance increases sleep duration (Masek et al., 2014; Seugnet et al., 2009). These studies have provided insight into the genetic and functional relationship between sleep and metabolic regulation; yet, the ecological factors that shape the diversity of these traits, such as temperature, lighting, humidity, and distribution of food resources in naturally occurring populations remain poorly understood.

Inspired by previous work investigating the relationship between geographic locality and sleep regulation (Svetec et al., 2015), we examined the relationship between sleep, metabolic function, and environmental localities. Here, we describe the analysis of sleep regulation, starvation resistance, the effects of starvation on sleep, and measurements of nutrient storage in D. melanogaster collected from geographically distinct localities that differ in latitude, longitude, temperature, and altitude to determine the environmental and geographic factors that associate with sleep regulation. We tested 24 populations of outbred D. melanogaster for these behavioral and physiological variables, providing insight into the relationship between these traits and their association with geographic locality. Our findings reveal highly significant variation in all traits measured, suggesting these traits are influenced by their environmental and evolutionary history.

2 | METHODS

2.1 | Drosophila maintenance

All populations were obtained from the Drosophila Species Stock Center (University of California, San Diego) with stock numbers provided in Table 1. They were maintained at this stock center on standard Drosophila media at 18–25°C for between 8 and 63 years. In the laboratory, flies were reared and maintained on a 12:12 light–dark cycle in humidified incubators at 25°C and 65% humidity (Percival Scientific, Perry, IA). Unless otherwise noted, all flies were maintained and tested on a standard cornmeal/agar medium used by the Bloomington Drosophila Stock Center, consisting of yeast, soy flour, cornmeal, malt extract, agar, light corn syrup, and propionic acid (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm).

2.2 | Behavioral analysis

Upon eclosion, male and flies were transferred to empty vials containing standard cornmeal/agar medium for 24–48 hr to age and mate. At 3–5 days of age, flies were then briefly anesthetized using CO₂; females were sorted and then individually placed into plastic tubes containing standard food media for behavioral monitoring. Flies were then acclimated to these conditions for at least 24 hr prior to testing. Fly activity was monitored using DAM2 DAMS (Trikinetics, Waltham, MA) as previously described (Hendricks et al., 2000; Shaw et al., 2000). The DAM system measures activity by monitoring the number of infrared beam crossings for each fly. These data were then used to calculate sleep-related traits by extracting immobility bouts of 5 min or more using the Drosophila Sleep Counting Macro (Pfeifferberger, Lear, Keegan, & Allada, 2010b). Multiple variables of sleep were analyzed, including total sleep duration, waking activity, sleep bout number, and average sleep bout length as previously described (Pfeifferberger et al., 2010b; Pitman, McGill, Keegan, & Allada, 2006). For experiments examining the effects of starvation on sleep, activity was recorded for 1 day on standard food media prior to transferring flies into tubes containing 1% agar (Fisher Scientific, Hampton, NH) at the start of lights on at zeitgeber time (ZT) 0. To calculate starvation-induced sleep suppression, the within-fly percentage change in sleep was calculated as follows: \( \frac{(\% \text{ sleep starved} - \% \text{ sleep baseline})}{(\% \text{ sleep baseline})} \times 100 \) (Keene et al., 2010; Murakami et al., 2016). This calculation accounts for differences in baseline sleep and accurately reflects the response...
to starvation. The same flies used to measure sleep and starvation-induced sleep suppression were also used in measurements of starvation resistance, which was assessed once flies were transferred to tubes containing 1% agar. The time of death was manually determined as the very last bout of waking activity for each individual fly.

2.3 | Climate data

Latitude, longitude, and altitude measurements for each locality were obtained from Google Earth (https://www.google.com/earth/). Latitude measurements were adjusted to represent the absolute distance from the equator. Longitude measurements to the east of the Prime Meridian were assigned a positive value, while measurements to the west were assigned a negative value. Altitude measurements were obtained from the latitudinal and longitudinal coordinates of each locality. As these measurements were not normally distributed, the Log10 of altitude was used. Temperature data were obtained from Berkeley Earth (http://berkeleyearth.org/). Monthly temperature measurements were obtained from a 1° × 1° latitude-longitude grid covering surface of the Earth. Average temperature was calculated as the average of the monthly temperature measurements.

2.4 | Triglyceride, glucose, and glycogen measurements

Whole flies, aged 3–5 days, were collected and flash-frozen on dry ice at ZT 0 for subsequent analyses. Protein, glucose, glycogen, and triglyceride measurements were then performed as previously

| Line | Stock     | Locality            | Collection date | Latitude   | Longitude   | Altitude | Average temperature |
|------|-----------|---------------------|-----------------|------------|------------|----------|---------------------|
| 1    | 14021-0231.58 | Bermuda             | 1954            | 32°18′28.08″N | 64°45′1.80″W | 14.16    | 23.20              |
| 2    | 14021-0231.134 | American Samoa     | 2009            | 14°18′5.90″S   | 170°41′46.25″W | 238.29  | 26.55              |
| 3    | 14021-0231.132 | Southwest Harbor, Maine | 2009        | 44°16′47.37″N   | 68°19′29.97″W | 11.35    | 6.58               |
| 4    | 14021-0231.59 | Bogota, Colombia    | 1962            | 4°42′39.56″N   | 74°4′19.53″W  | 2.581.03 | 20.51              |
| 5    | 14021-0231.69 | Athens, Greece      | 1965            | 37°59′1.71″N   | 23°43′39.14″E | 71.08    | 16.95              |
| 6    | 14021-0231.64 | Kariba Dam, Zimbabwe | 1963         | 16°31′19.58″S   | 28°45′42.01″E | 664.14  | 23.34              |
| 7    | 14021-0231.131 | La Jolla, California | 2009        | 32°49′58.12″N   | 117°16′16.58″W | 51.89    | 16.49              |
| 8    | 14021-0231.136 | Fukushima, Japan    | 2009            | 37°45′39.00″N   | 140°28′29.02″E | 68.24    | 11.72              |
| 9    | 14021-0231.67 | Pyrenees, Spain     | 1965            | 42°40′5.45″N   | 1°0′4.28″E    | 2,314.12 | 8.43               |
| 10   | 14021-0231.129 | Cebu, Philippines   | 2008            | 10°18′56.52″N   | 123°53′7.57″E | 56.31    | 27.19              |
| 11   | 14021-0231.137 | Ogasawara Islands, Japan | 2009 | 27°4′30.19″N   | 142°12′41.77″E | 223.45  | 24.38              |
| 12   | 14021-0231.61 | Blacksburg, Virginia| 1954            | 37°13′46.46″N   | 80°24′50.18″W | 634.67   | 11.58              |
| 13   | 14021-0231.23 | Crete, Greece       | 2002            | 35°14′24.42″N   | 24°48′33.37″E | 1,586.60 | 18.30              |
| 14   | 14021-0231.03 | Queensland, Australia| Not Listed | 20°55′3.27″S   | 142°42′10.06″E | 321.11  | 25.09              |
| 15   | 14021-0231.43 | San Luis Potosi, Mexico | 2005      | 22°9′23.29″N   | 100°59′7.95″W  | 1,877.06 | 18.85              |
| 16   | 14021-0231.15 | Bahia, Brazil       | Not Listed      | 12°34′47.06″S   | 41°42′2.62″W  | 715.73   | 22.13              |
| 17   | 14021-0231.130 | Queensferry, Scotland | 2009       | 55°59′24.01″N   | 3°23′56.56″W  | 19.71    | 7.45               |
| 18   | 14021-0231.22 | Chiapas, Mexico     | 2002            | 16°45′24.95″N   | 93°7′45.25″W  | 549.63   | 24.23              |
| 19   | 14021-0231.01 | Ica, Peru           | 1956            | 14°4′31.66″S   | 75°44′3.05″W  | 399.25   | 19.25              |
| 20   | 14021-0231.35 | Monkey Hill, St. Kitts | 2005       | 17°19′26.30″N   | 62°43′30.14″W | 120.42   | 27.24              |
| 21   | 14021-0231.56 | Plainville, Connecticut | 2007      | 41°40′32.68″N   | 72°51′48.11″W | 52.86    | 9.74               |
| 22   | 14021-0231.62 | Cape Town, South Africa | 1954      | 33°55′29.53″S   | 18°25′26.60″E | 52.57    | 16.35              |
| 23   | 14021-0231.68 | Israel              | 1954            | 31°2′45.78″N   | 34°51′5.80″E  | 287.34   | 19.93              |
| 24   | 14021-0231.66 | Madeira, Portugal   | 1965            | 32°45′38.55″N   | 16°57′34.10″W | 1,581.31 | 19.73              |
described (Gingras, Warren, Nagengast, & Diangelo, 2014; Sassu et al., 2012). Briefly, two headless female flies aged 3–5 days were homogenized in 50 mmol/L Tris-HCl, pH 7.4, 140 mmol/L NaCl, 0.1% Triton-X, and 1× protease inhibitor cocktail (Sigma Aldrich, St Louis, MO). Triglyceride levels were measured using the Infinity Triglycerides Kit (Fisher Scientific, Hampton, NH), while protein levels were measured using the Pierce BCA Protein Assay Kit (Fisher Scientific, Hampton, NH). Total glucose levels were determined using the Glucose Oxidase Reagent (Pointe Scientific, Canton, MI) in samples previously treated with 8 mg/mL amyloglucosidase in 0.2 mol/L Sodium Citrate buffer, pH 5.0 (Boston BioProducts, Ashland, MA) for 2 hr. Glycogen levels were determined by measuring free glucose in samples not treated with amylglucosidase and then subtracting the free glucose from total glucose concentration. For each sample, triglyceride, glycogen, and free glucose levels were standardized to the total protein content.

2.5 | Statistics

First, to assess the normality of each trait for each population, we performed a Shapiro–Wilk test. For several traits, there was at least one non-normally distributed population, including: waking activity, bout length, % change in sleep, and measurements of glycogen levels. To assess variation among populations in traits where not all populations were normally distributed, we performed the nonparametric Wilcoxon rank-sum test. To assess variation among populations in traits in which all populations were normally distributed, we performed a one-way analysis of variance (ANOVA): $Y = \mu + \text{Line} + \varepsilon$, where Line represents the fixed effect of population from a given locality and $\varepsilon$ indicates error. In order to determine whether a given population differed from the global average, we performed a one-sample t-test and then adjusted for multiple testing using Bonferroni’s correction. A one-sample t-test was also performed to determine whether sleep responses as a result of starvation were significantly different from zero. Differences in survival upon starvation were assessed using the nonparametric log-rank test. Linear regressions were used to determine the relationship between a given trait and geographic variable as well as between two traits. For each regression analysis, with the exception of measurements of starvation resistance, the population average of each trait was used. For starvation resistance, the median time until death (LT50) was used. To account for multiple testing, Bonferroni correction was performed for each correlation between a given continental unit and geographic variable. All data were analyzed using JMP 12.0 software (SAS Institute Inc., Cary, NC).

3 | RESULTS

To determine the contribution of geographic variation on sleep and metabolic regulation, we obtained 24 populations of D. melanogaster from diverse localities throughout the world (Figure 1a; Table 1). To assess differences in sleep and metabolic function within individual flies, sleep was measured in 3- to 5-day-old female flies on food. Following 24 hr of sleep acquisition, flies were transferred to starvation tubes containing agar alone and maintained on this substrate to measure starvation-induced changes in sleep and starvation resistance (Figure 1b), providing multiple metrics of sleep and metabolic function within an individual animal.
3.1 Variation in sleep traits

Quantification of sleep duration in fed flies revealed remarkable diversity in sleep duration and architecture between fly populations (Figure 2; Table 2). The average sleep duration of all 24 lines tested for 24 hr on food was 855 min. The shortest sleeping populations include flies from Fukushima, Japan, and Israel, sleeping on average 413 and 570 min, respectively (Figure 2a; Table 2). In addition, several long-sleeping populations were identified, including flies from Bogota, Colombia (1,100 min), and Bermuda (1,117 min) (Figure 2a, Table 2). The differences between short- and long-sleeping flies were present during both the day and night, thereby suggesting that the observed phenotypes are not the result of altered circadian regulation (Figure 2b).

Given that these populations have been stored in laboratory conditions for between 8 and 63 years, we next assessed whether the duration of time spent in these conditions may have influenced the extensive variation in sleep duration we observed.
### Table 2

Mean responses of measurements of sleep and starvation resistance for each locality. The standard error (SE) is shown to the right of each mean, while the sample sizes are depicted on the far right.

| Locality                        | Sleep duration Average | Sleep duration SE | Waking activity Average | Waking activity SE | Bout number Average | Bout number SE | Bout length Average | Bout length SE | % Change in sleep Average | % Change in sleep SE | Starvation resistance LT50 | Sample size |
|---------------------------------|------------------------|-------------------|-------------------------|-------------------|--------------------|-------------------|--------------------|-------------------|--------------------------|---------------------|-----------------------------|-------------|
| Bermuda                         | 1,117.22               | 19.08             | 0.69                    | 0.04              | 28.42              | 1.63              | 48.31              | 6.17              | -12.33                   | 2.43                | 1.86                        | 32          |
| American Samoa                  | 1,083.00               | 18.95             | 0.59                    | 0.04              | 27.26              | 1.50              | 45.50              | 3.65              | -11.58                   | 2.13                | 3.44                        | 34          |
| Southwest Harbor, Maine         | 660.25                 | 29.46             | 0.94                    | 0.03              | 28.30              | 1.72              | 31.92              | 5.77              | -6.22                    | 7.23                | 3.87                        | 40          |
| Bogota, Colombia                | 1,100.31               | 23.85             | 0.90                    | 0.06              | 27.59              | 1.80              | 46.61              | 3.96              | -8.95                    | 1.95                | 2.62                        | 40          |
| Athens, Greece                  | 842.90                 | 33.53             | 1.16                    | 0.08              | 40.90              | 2.13              | 23.10              | 1.87              | -32.90                   | 7.31                | 2.02                        | 33          |
| Kariba Dam, Zimbabwe            | 1,096.35               | 22.06             | 0.63                    | 0.04              | 32.11              | 1.41              | 37.77              | 2.60              | -41.64                   | 5.64                | 2.44                        | 39          |
| La Jolla, California            | 771.00                 | 44.35             | 1.04                    | 0.05              | 34.47              | 2.10              | 26.95              | 3.77              | 5.03                     | 5.30                | 3.69                        | 36          |
| Fukushima, Japan                | 413.08                 | 30.36             | 0.87                    | 0.03              | 35.64              | 1.46              | 11.80              | 0.95              | -28.38                   | 7.95                | 3.00                        | 32          |
| Pyrenees, Spain                 | 781.58                 | 41.04             | 0.92                    | 0.04              | 41.24              | 1.66              | 20.64              | 1.81              | -24.64                   | 6.53                | 3.07                        | 40          |
| Cebu, Philippines               | 927.34                 | 31.24             | 0.76                    | 0.02              | 40.09              | 1.76              | 25.61              | 2.15              | 1.22                     | 3.53                | 4.01                        | 36          |
| Ogasawara Islands, Japan         | 1,043.28               | 21.12             | 0.77                    | 0.03              | 26.59              | 1.40              | 43.79              | 3.08              | -14.28                   | 2.14                | 3.90                        | 32          |
| Blacksburg, Virginia            | 830.90                 | 35.61             | 1.04                    | 0.06              | 33.36              | 2.08              | 30.58              | 2.96              | 1.67                     | 5.30                | 2.61                        | 40          |
| Crete, Greece                   | 964.58                 | 30.65             | 1.11                    | 0.06              | 33.75              | 1.93              | 34.06              | 3.42              | -14.19                   | 3.51                | 3.67                        | 40          |
| Queensland, Australia           | 762.12                 | 24.85             | 0.68                    | 0.03              | 39.61              | 1.77              | 20.56              | 1.13              | -19.29                   | 6.51                | 1.94                        | 32          |
| San Luis Potosi, Mexico         | 809.13                 | 31.61             | 0.85                    | 0.03              | 37.88              | 1.74              | 24.14              | 1.87              | -7.23                    | 5.43                | 3.85                        | 40          |
| Bahia, Brazil                   | 880.31                 | 33.90             | 0.75                    | 0.02              | 32.00              | 1.92              | 34.56              | 4.55              | -0.90                    | 5.75                | 3.68                        | 32          |
| Queensferry, Scotland           | 925.31                 | 33.84             | 0.87                    | 0.05              | 33.47              | 1.54              | 30.58              | 2.50              | -19.66                   | 3.99                | 3.59                        | 32          |
| Chiapas, Mexico                 | 820.32                 | 25.32             | 0.79                    | 0.03              | 23.94              | 1.40              | 36.98              | 1.89              | -8.37                    | 2.66                | 3.23                        | 32          |
| Ica, Peru                       | 766.61                 | 35.14             | 0.85                    | 0.04              | 47.94              | 2.28              | 17.78              | 1.56              | -36.79                   | 4.30                | 6.00                        | 32          |
| Monkey Hill, St. Kitts          | 899.08                 | 19.76             | 0.81                    | 0.02              | 26.21              | 1.28              | 38.56              | 2.73              | 0.05                     | 3.30                | 3.72                        | 32          |
| Plainville, Connecticut         | 851.25                 | 23.76             | 0.78                    | 0.03              | 32.03              | 1.82              | 31.05              | 2.69              | 9.99                     | 4.16                | 3.29                        | 32          |
| Cape Town, South Africa         | 1,005.29               | 39.30             | 1.09                    | 0.06              | 36.56              | 2.45              | 35.76              | 4.10              | -43.59                   | 4.82                | 1.97                        | 39          |
| Israel                          | 569.86                 | 29.09             | 0.90                    | 0.05              | 33.66              | 1.72              | 17.69              | 0.90              | 6.80                     | 5.60                | 2.97                        | 32          |
| Madeira, Portugal               | 605.56                 | 33.41             | 0.82                    | 0.04              | 44.78              | 1.80              | 14.29              | 1.14              | 39.31                    | 11.79               | 3.90                        | 40          |
We found no association between the date a given population was collected and either the average sleep duration or the coefficient of variation of sleep duration (Figure S1), suggesting that variation in sleep regulation across populations is not associated with the length of time animals were housed in laboratory. It is also possible that differences in sleep duration are reflective of lethargy or hyperactivity rather than sleep per se. To investigate this, we measured the relationship between sleep duration and waking activity. We observed no difference in waking activity between the two shortest sleeping populations and the global average, while a moderate reduction was observed in one of the long sleeping populations (Figure 2c). A regression analysis of the 24 populations tested revealed a lack of correlation between sleep duration and waking activity (Figure 2d), suggesting that these traits are independently regulated and that the differences in sleep across geographic localities are not the result of hyperactivity or lethargy.

Sleep duration is composed of individual sleep bouts and may be enhanced by increasing the number of bouts or the length of each individual sleep bout within a given day (Pfeiffenberger et al., 2010b). To differentiate between these possibilities, we calculated the average number of bouts for each population. Overall, the average number of bouts ranged from 24 to 48 per 24 hr (Figure 2e; Table 2), and there was a moderate but significant correlation between bout number and sleep duration (Figure 2f), suggesting that sleep initiation only mildly associates with variation in sleep duration. In addition, the length of individual sleep bouts was shorter in the Fukushima, Japan and Israel populations, and longer in the Bogota, Columbia and Bermuda populations compared to the global average (Figure 2g). Furthermore, a regression analysis revealed a highly significant correlation between sleep duration and bout length (Figure 2h). Taken together, these findings suggest that the diversity in sleep duration is primarily conferred by increasing the length of individual sleep bouts.

A previous study examining D. melanogaster collected from five North American localities suggested that sleep is increased in equatorial regions (Svetec et al., 2015). To determine how sleep and activity relate to variation in geographic and environmental locality, we performed linear regressions on four geographic variables including latitude, longitude, altitude, and temperature. This analysis revealed an association between sleep duration and both latitude and temperature, while no relationship was observed between sleep duration and longitude or altitude (Figure 3; Appendix S1). When divided into continental units, we observed a significant negative correlation between sleep duration and temperature in Asia/Pacific as well as the Americas, while no correlation was observed between populations originating from Europe/Middle East/Africa (Figure 3a). We also observed a significant positive correlation between waking activity and temperature (Appendix S1). Although this association includes all localities tested, this trend is most strongly evident in populations from the Americas. Taken together, these results suggest that close proximity to the equator and increased temperature are both associated with increased sleep duration and reduced waking activity.

### 3.2 Variation in the metabolic regulation of sleep

Flies, like mammals, suppress sleep when starved, presumably to initiate a foraging response (Dangui & Nicolaidis, 1979; Keene et al., 2010). This phenotype has been extensively investigated in inbred fly lines; however, it has not been studied in outbred populations of Drosophila (Yurgel et al., 2014). To determine how sleep is modulated by nutrient deprivation, flies were starved following the 24-hr sleep recordings on food by being transferred to tubes containing

![Figure 3](link-to-figure3)

**Figure 3** Linear regression analyses highlight the relationship between sleep duration and geographic variables. There is a significant relationship between (a) sleep duration and distance from the equator as well as (b) sleep duration and temperature. There is no relationship between (c) sleep duration and longitude nor between (d) sleep duration and altitude. Each population is color coded to represent their respective continental unit. Statistical analyses are reported in Table 2.
agar alone and the change in sleep between the two housing conditions was then determined. After 24 hr of starvation, twelve lines significantly suppressed sleep in response to starvation, eleven lines exhibited no change in sleep, and a single line significantly increased sleep (Appendix S2). Further, after 48 hr, the number of lines that significantly suppressed sleep in response to starvation increased to twenty. Flies from Cape Town, South Africa (44%) and Kariba Dam, Zimbabwe (42%) displayed the greatest sleep suppression, while flies Madeira, Spain (39%) showed the greatest increase (Figure 4a). There was no correlation between sleep duration on food and starvation-induced sleep suppression (Figure 4b), suggesting that sleep duration and changes in sleep resulting from starvation are independently regulated.

It is possible that sleep suppression in response to starvation represents a specific sensitivity to sleep regulation or provides a more general indicator of starvation tolerance. To measure starvation resistance, we maintained flies on agar in activity monitors and measured time of death. The mean starvation resistance for all 24 lines tested was 3.26 days. The least resistant populations include flies from Bermuda and Queensland, Australia, living on average 1.86 and 1.93 days, respectively. The most resistant populations include flies from Cebu, Philippines, living 4.01 days and Ica, Peru, living 6.00 days (Figure 4c). We observed no correlation between starvation-induced sleep suppression and starvation resistance when all flies were included in the regression (blue line; Figure 4d). Given that flies from Ica, Peru live approximately 2 days longer than the next longest-living population and over three standard deviations above the global average, we next performed a linear regression in the absence of this outlier locality. We found a strong positive correlation between starvation-induced sleep suppression and starvation resistance (black line: Figure 4d), suggesting that sleep duration and changes in sleep resulting from starvation are not independently regulated. To determine whether starvation-induced sleep suppression and/or starvation resistance is associated with variation in geographic variables, we performed linear regression analyses between these traits and the geographic variables. For both traits, we did not observe any correlation with the geographic variables measured (Appendix S3). Taken together, these findings demonstrate dramatic variation in starvation resistance and the metabolic regulation of sleep in different fly populations, indicating that the metabolic regulation of sleep is coregulated with starvation resistance.

### 3.3 Variation in nutrient storage

Energy stores and circulating nutrients potently affect both sleep and starvation resistance. To investigate the relationship between energy stores and these processes, we measured triglyceride levels, glycogen levels, and free glucose across all 24 populations. We identified at least a fourfold difference in triglyceride, glycogen, and free glucose levels in the populations tested (Figure 5a–c; Appendix S4). Across all three measurements, energy stores were lowest in the Queensland, Australia population. A linear regression analysis of the relationship between energy levels and starvation-induced sleep suppression revealed no significant correlation between...
starvation-induced changes in sleep and triglyceride or glycogen levels (Figure 5d,e). However, a significant correlation was observed between starvation-induced regulation of sleep and free glucose, suggesting that flies with lower levels of free glucose during the fed state display a greater reduction in sleep upon starvation (Figure 5f). Conversely, increased starvation resistance correlated with elevated levels of triglycerides and glycogen, but not free glucose (Figure 5g–i), indicating elevated energy stores in fed flies are indicators of starvation resistance. Taken together, these findings suggest that energy storage molecules (glycogen and triglycerides) and free glucose have distinct effects on the metabolic regulation of sleep and starvation resistance.

To determine the relationship between energy stores and free glucose with geographic variables, we again performed linear regression analyses between these traits and the geographic variables. These analyses revealed no correlation between triglyceride or glycogen levels and any geographic variables measured (Appendix S5). However, we did observe a correlation between free glucose levels and two of the geographic variables (Figure 6). We found that free glucose levels were significantly correlated with increased distance from the equator and decreased mean annual temperatures, and that these associations are strongest in populations from the Americas (Figure 6a,b; Appendix S5). Therefore, variation in both latitude and temperature appear to associate with the regulation of sleep duration and free glucose.

4 | DISCUSSION

The expansive radiation of D. melanogaster provides an excellent opportunity to examine the interrelationship between natural variation
in complex behaviors, physiological traits, and the environmental factors that may act as selective forces to shape such variation. Here we examined natural variation in sleep and metabolic function, as well as their relationship with each other, and with several geographic and environmental gradients, by investigating 24 *D. melanogaster* populations from localities across the globe. Our assessment of sleep and metabolic traits over a wide geographic range has made it possible to identify general trends in sleep-related traits, as well as evaluate their degree of variation. The vast majority of sleep studies in *Drosophila* have used inbred or isogenic *D. melanogaster* strains. Inbred populations of laboratory *Drosophila* strains typically sleep ~600–800 min daily (Zimmerman, Chan, Jackson, Maislin, & Pack, 2012), while the sleep duration for strains in this study ranged from 413 to 1,117 min. Similarly, starvation resistance in inbred strains is ~36–49 hr, while flies in this study survived up to 144 hr (Gáliková et al., 2015; Mattaliano, Montana, Parisky, Littleton, & Griffith, 2007). Therefore, naturally occurring variability encompasses the sleep and starvation resistant phenotypes that extend well beyond the phenotypes of the commonly used laboratory strains. This remarkable variability in natural populations of *Drosophila* is suggestive of maintenance of genetic and phenotypic variation associated with geographically independent populations of *Drosophila* that persists through years or decades of maintenance. The extreme phenotypes observed in natural populations of *Drosophila* suggest differences between populations could be used to uncover novel genetic architecture associated with natural variation in sleep and metabolic function.

Sleep is regulated by complex genetic architecture and is highly influenced by genetic variation (Cirelli, 2009; Wu, Kumar, Serrano Negron, & Harbison, 2017). While many genes have been identified using mutagenesis approaches, much less is known about the modulation of sleep via naturally occurring genetic variation. For most humans, sleep need is estimated to range from 7 to 9 hr, and single alleles have been identified that robustly influence sleep, a number of which are conserved in *Drosophila* (Allebrandt et al., 2013; He et al., 2009; Shi, Wu, Ptáček, & Fu, 2017). Genome-wide association studies have identified loci associated with sleep variability, but assessing the contributions of loci to sleep variation is difficult (Kalmbach et al., 2017). Sequenced inbred *Drosophila* lines derived from a wild-caught population have been previously characterized for variation in sleep, revealing numerous differentially expressed genes and molecular polymorphisms that associate with sleep duration and architecture (Harbison, Mackay, & Anholt, 2009; Harbison et al., 2013). The identification of differences in sleep and metabolic phenotypes from geographically diverse regions presents a complementary approach to investigate the genetic architecture underlying variation in sleep regulation.

Clinal variation has been observed in diverse traits including body size, fecundity, and temperature resistance, but much less is known about the relationship between behavior and clinality [for review, see Adrion, Hahn, and Cooper (2015)]. Here, we observed a moderate association between distance from the equator and sleep duration; however, this was below the threshold of significance after a Bonferroni correction was applied. We also identified a relationship between sleep duration and average temperature. Specifically, we found that increased sleep duration is associated with increased average temperature in Asia/Pacific and the Americas, suggesting that sleep duration is a convergent adaptation to temperature. We did not observe an association with temperature in Europe/Middle East/Africa. It is possible that clinal variation in sleep duration is restricted to specific geographic regions. However, given the limited number of populations within each continental unit (four in the case of Asia/Pacific), we have limited ability to discern this in the present study. Given that there are several temperature-associated metrics that contribute to the average yearly temperature, including daily/monthly temperature, minimum/maximum temperature, as well as temperature range, another possibility is that a combination of such variables may more robustly
associate with variation in sleep duration. Furthermore, the conti-
teinal units allocated cover vast transects of geography, making it possible
that more geographically restricted clines will shed additional light
on these geographic associations with sleep duration. These findings
are for the most part of modest effect, which may in part be due to
the duration of time several of the lines have spent in the laboratory
or the lack of sampling depth at any particular locality. Despite these
caveats, our worldwide sampling scheme revealed geographic-scale
trend in sleep and metabolic traits.

A previous study using five North American populations found
clinal variation in nighttime bout length and sunrise anticipation (Svetic
et al., 2015). The moderate clinal relationship between latitude and
sleep was observed during the nighttime only, suggesting that ecologi-
cal variables have differential effects on daytime and nighttime sleep
(Svetic et al., 2015). Similarly, we observed no relationship between
latitude and daytime sleep, further supporting the notion that clinal
variation in sleep duration is nighttime specific. In contrast to Svetic
et al., 2015; our analyses include not only sleep behavior, but also
traits associated with the metabolic regulation of sleep. Additionally,
our analyses take a more global approach to assess variation in sleep
and metabolic traits by testing populations from multiple continents.
Together, these analyses provide the framework for a more detailed
investigation of behavioral adaptations to environmental gradients.
Unsurprisingly, there are many additional traits associated with lati-
dudal clines. Sunrise anticipation, where flies become active prior to
the onset of the light cycle, was also associated with latitude (Svetic
et al., 2015). Morning anticipation, sleep duration, and tempera-
dependent modulation of sleep are regulated by the circadian clock
in Drosophila (Agosto et al., 2008; Guo et al., 2016; Parisky, Agosto
Rivera, Donelson, Kotecha, & Griffith, 2016). Therefore, it is possible
that alterations in circadian neural circuitry or the molecular machin-
ery governing the circadian clock may contribute to cline-associated
differences in sleep among Drosophila populations.

Flies robustly suppress sleep and increase activity in response to
starvation, and this is mediated by both chemosensory and hormonal
factors (Keene et al., 2010; Lee & Park, 2004; Linford et al., 2012;
Murakami et al., 2016). Although we did not observe an association
between sleep and starvation-induced sleep suppression, differ-
ent genetic factors have been previously implicated in these traits
(Keene et al., 2010; Yurgel, Masek, DiAngelo, & Keene, 2015). While
this phenotype has been reported in diverse genetic backgrounds
of D. melanogaster (McDonald & Keene, 2010), we found that 10 of
the 24 populations tested do not suppress sleep when starved for
24 hr. It is possible that this is associated with a delayed response
to starvation. As such, an analysis of sleep during later periods of
starvation (48 hr) identified starvation-induced sleep suppression in
eight additional populations. This is further supported by the cor-
relation between starvation induced sleep suppression and starva-
tion resistance. Surprisingly, we identified a single population that
significantly increased sleep during starvation, revealing opposing
responses to starvation. Although enhanced sleep during starvation
has not been reported in Drosophila, we have previously identified
increased sleep during starvation in the Mexican blind cavefish, and
many animals enter hibernation during winter seasons when food
availability is scarce (Jaggard et al., 2017; Schmidt, 2014). Therefore,
there are likely multiple strategies that are implemented in response
to food shortage, including induction of foraging behavior and con-
sequently sleep suppression, or increasing sleep to conserve energy.
A better understanding of how environmental factors shape the
evolution of these opposing strategies is of particular interest.

In this study, we did not find an association between starvation
resistance and starvation-induced sleep suppression with any envi-
ronmental gradient, raising the possibility that (1) variation in these traits
may be due to an environmental gradient not measured in this study
or (2) these traits do not correlate with clinal/geographic variables.
Nevertheless, in the case of starvation resistance, several previous stud-
ies have also failed to find a relationship with latitudinal cline (Goenaga,
Jose Fanara, & Hasson, 2010; Hoffmann, Shirriffs, & Scott, 2005;
Robinson, Zwaan, & Partridge, 2000), while others reported negative
latitudinal clines (Arthur, Weeks, & Sgro, 2008; Karan & Parkash, 1998;
Karan et al., 1998). In the case of starvation-induced sleep suppression,
the absence of an association with an environmental gradient persisted
even when flies were starved for 48 hr. To our knowledge, no similar in-
vestigation has been previously performed on the relationship between
environmental gradients and starvation-induced sleep suppression.

Although we did not identify clinal variation in starvation resis-
tance or starvation-induced sleep suppression, our analysis does not
take into account additional variables such as ultraviolet light intensity,
seasonality, day length, or other factors that may potently affect food
availability and influence selection. It is also possible that there are
environmental factors specific to individual localities that shape the
behavioral and metabolic responses we observed. One population in
which this may indeed be the case is Drosophila from Ica, Peru, the
most starvation resistant population in our analysis. The city of Ica
borders the Atacama Desert, which is classified as a hot desert climate
according to the Köppen-Geiger climate classification system (Peel,
Finlayson, & McMahon, 2007). Therefore, it would be informative to
assess starvation resistance in additional populations from this region,
as well as from other desert climates. Given that this line was collected
in 1956, investigating the behavior and physiology of wild-caught flies
from this region, as well as flies from nearby more humid regions, may
be informative. We posit that the arid climate of this region would
select for Drosophila with an increased resistance to starvation, as this
is the case with several Drosophila species that are found in more xeric
habitats (Matzkin & Markow, 2009). Nevertheless, our finding that av-
erage temperature is associated with differences in sleep duration and
activity across continents suggests a relationship between geographic
conditions and sleep regulation.

Upon investigation into the relationship between sleep regula-
tion and energy stores, we found that populations with lower levels
of free glucose display a greater increase in sleep suppression after
starvation. Limiting glucose utilization pharmacologically suppresses
sleep, presumably by mimicking the starvation state (Murakami
et al., 2016). These findings suggest natural variation in free glucose
associate with how flies modulate sleep in accordance with nutri-
ten shortage. We also found a positive correlation between energy
storage measurements (glycogen and triglyceride levels) and starvation resistance. Previous work has shown that artificial selection for increased starvation resistance results in a correlated increase in energy stores (Slocumb et al., 2015), raising the possibility that increased energy storage is an adaptation to areas with limited or sporadic food availability. Of these metabolic traits, we found that free glucose is associated with both equatorial proximity and average temperature, and that clinal variation in populations from the Americas primarily drives this global pattern. Interestingly, allozymes of glucose-6-phosphate dehydrogenase (G6PD), an enzyme involved in the breakdown of glucose, also display latitudinal clinality, such that the allozyme with low activity is found at higher frequencies at higher latitudes (Bublí, Kalabushkin, & Imashova, 1999; Oakeshott, Chambers, Gibson, Eanes, & Willcocks, 1983; Singh, Hickey, & David, 1982). We can speculate that this may contribute to the higher levels of free glucose; however, functional tests remain an important next step. Overall, this suggests that geographic and environmental gradients are among the selective forces that mediate genetic variation in glucose metabolism and its impact on behavior.

Clinal patterns have been found for numerous phenotypes and their underlying genetic architecture, including allozyme variants, sequence variants, and chromosome inversions (Fabian et al., 2012; Knibb, 1982; Kolaczkowski, Kern, Holloway, & Begun, 2011; Oakeshott et al., 1983; Sezgin et al., 2004), suggesting that convergent evolution in similar environments may have shaped variation in these traits, albeit in different parts of the world. The expansion to northern habitats is suggested to be a relatively recent phenomenon in the history of D. melanogaster. This species is thought to have migrated from equatorial zones to northern latitudes 10,000–20,000 years ago (Begun & Aquadro, 1993; David & Capy, 1988). Migration to certain geographic areas, such as North America and Australia, is thought to be much more recent, and as late as the last 200 years (Hoffmann & Weeks, 2007; Keller, 2007; Knibb, 1982). While the recent migration into these areas may be indicative of a population bottleneck, thereby reducing genetic heterogeneity, gene expression analyses of low and high latitude North American populations revealed significant differences in gene expression between these populations (Svetc et al., 2015; Zhao, Wit, Svetic, & Begun, 2015), suggesting that these populations are indeed genetically dissimilar. It is possible that reduced sleep is associated with this migration and is an evolutionary adaptation to seasonal changes in temperature and light cycle (Adrion et al., 2015; Li & Stephan, 2006). Achieving a better understanding of the evolution and biogeography of D. melanogaster in the geographic regions investigated in this study will help inform the relationship between sleep and the metabolic traits measured here. Overall, our results reveal dramatic variation in sleep and metabolic regulation. Although our findings revealed that the associations between sleep and metabolic traits with the geographic variables examined were moderate, our observations associating clinal variation with naturally occurring differences in sleep-related traits suggests that environmental gradients are potent selective forces that can shape behavior. In addition to the variables examined here, there are numerous additional factors that have been previously shown to influence sleep behavior, including lighting (Menegazzi et al., 2017), daily changes in temperature (Parisky et al., 2016), and composition of food resources (Catterson et al., 2010). Future studies examining the response of these and similar populations to changes in these environmental factors, and how they associate with sleep and metabolic function, may shed light on the environmental factors that modulate variation in sleep regulation.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

A.C.K. and E.B.B. designed the study and wrote the manuscript. E.B.B., J.T., V.R., and A.K. conducted the sleep and starvation experiments. R.A.B. and J.R.D. performed the nutrient storage measurements. E.B.B. analyzed the data. All authors read and approved of the manuscript.

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