RESEARCH PAPER

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The circadian clock: an important factor modifying the effect of physical activity on the lifespan of *Drosophila melanogaster*

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It is well established that the circadian clock has pronounced effects on functioning of cells, tissues and body organs. Little, however, is known regarding its role in the body's adaptation to physical activity. The aim of the present study was to establish the effect of physical activity—performed at either the activity peak (ZT1) in the morning or during 'siesta' time (ZT6) in the middle of the day—on the lifespan of male and female *Drosophila melanogaster*. Three-day-old virgin flies (separated by sex) were allocated to control or exercise groups. Flies in the exercise groups were subjected to exercise every day by applying motor stimulation at ZT1 or at ZT6. Overall lifetime physical activity of controls was lower in male *D. melanogaster* compared to females. Forced physical activity at ZT1 prolonged the male lifespan by ~35%, whereas females in the same group exhibited a lifespan reduction of ~84%. In contrast, physical activity performed at 'siesta' time shortened the lifespan of both male and female flies, by ~20% and ~67%, respectively. Forced locomotor activity performed during the time of peak natural daily activity prolonged life expectancy in males but had a negative effect on female longevity. Interestingly, when physical activity was applied during the 'siesta' time, it shortened the lifespan regardless of sex. Therefore, our study suggests that synchronizing physical activity to the daily rhythm of activity and rest dictated by the circadian clock could be a new strategy for optimizing the effects of physical training. However, this interpretation requires further research involving human subjects of different chronotypes.

Key words: circadian rhythms, fruit flies, Zeitgeber, life expectancy, locomotor activity, rest/activity cycle

INTRODUCTION

Regular physical activity induces many adaptive responses in various organs of the human body (Zoladz et al., 2016, 2022; Jarmuszkiewicz et al., 2020; Chow et al., 2022; Majerczak et al., 2024) and can enhance health status (Pedersen & Saltin, 2015; Warburton & Bredin, 2019). However, to what extent the magnitude of exercise-induced adaptive responses is dependent on the daily timing of training is a question of increasing relevance, referred to as chrono-exercise.

The maintenance of the body's homeostasis, including the proper functioning, physiology, and metabolism of organs strictly depends on the rhythm of environmental conditions determined primarily by the rotation of the Earth (Green et al., 2008). This 24-hour (h) cycle of light and darkness induced the evolution of efficient mechanisms to adapt organisms to changes occurring over the course of the day,

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called the circadian clock: an endogenous timekeeping system that regulates many behavioral and physiological processes (Bargiello & Young, 1984; Reddy et al., 1984; Bautista et al., 2025). The circadian clock and its role in regulating behavior are particularly well described in *Drosophila melanogaster*, with locomotor activity the most extensively studied output. Insects maintained under standard laboratory conditions of 12 h of light and 12 h of darkness (LD 12:12) at 25°C are characterized by two activity peaks: the morning peak associated with the onset of light and the evening peak associated with the onset of darkness. These peaks are separated by two periods of reduced locomotor activity: at noon (the 'siesta') and at midnight (sleep) (Aschoff, 1966; Hendricks et al., 2000; Shaw et al., 2000). The morning peak is mostly driven by light; in conditions of constant darkness (DD), it is weak or not present, while the evening peak is under more endogenous circadian control.

The circadian clock regulates a wide range of physiological processes in humans, including those related to physical activity (Kim et al., 2023). Interestingly, there is a growing body of evidence that the impact of physical exercise on adaptive and maladaptive changes in the human body is under the control of the circadian clock. Consequently, appropriate timing of exercise may help to better maintain and improve human health. The full extent of the importance of so-called chrono-exercise is still poorly understood; however, it seems that designing training plans in accordance with circadian rhythms—i.e., consistent with the diurnal fluctuations in the secretion of various hormones involved in energy metabolism that are important for proper body adaptations—could lead to improved benefits from physical training (Kim et al., 2023).

The main aim of the present study was to establish the effect of forced physical activity performed at either the peak of activity in the morning or during 'siesta' time in the middle of the day on the life expectancy of male and female *D. melanogaster* flies. *D. melanogaster* is an excellent model organism for studying physical activity in relation to circadian regulation, as the species exhibits various circadian rhythms at the cellular and organismal levels, and is easy to maintain and study in laboratory conditions (Bargiello & Young, 1984; Reddy et al., 1984). We hypothesized that the effect of forced exercise on life expectancy depends on sex and the timing of exercise. Specifically, we predicted that forced exercise would be more beneficial for male flies. Furthermore, we expected that exercise performed at activity peaks during the day would prolong life, whereas exercise performed in 'siesta' time would not be beneficial for life expectancy in either sex.

METHODS

Animals

A total of 539 female and 519 male *D. melanogaster* wild-type flies (Canton S) were used in the present study, divided into two separate experiments. The effect of physical activity on lifespan was examined in 475 females and 455 males. Two experimental groups, exercised and control unexercised insects, were obtained from synchronous breeding: the insect eggs, laid within one day on a fresh medium (sugar and agar dissolved in distilled water; surface covered with dry yeast), were collected and distributed to vials filled with a standard cornmeal medium for development. Immediately after eclosion from pupae, the virgin females and males were collected. Then the flies, separated by sex, were randomly allocated into control and exercise groups for each time point (ZT1 or ZT6). Flies were placed in separate culture vials with standard cornmeal medium, which was changed every three days. The precise number of fruit flies in each studied group is shown in Fig. 1.

To assess the baseline life expectancy in males and females, we analyzed the control groups, which simultaneously served as reference cohorts for the forced physical activity experiment.

Canton S *D. melanogaster* stocks were reared on a standard cornmeal medium of agar (BioShop), artificial honey, yeast, cornmeal, and 0.1% methyl 4-hydroxybenzoate as an antifungal agent (Sigma-Aldrich) dissolved in 90% ethanol (Linegal Chemicals), and maintained at 25°C under LD 12:12 conditions and a relative humidity of ~60% throughout the experiment. The flies had *ad libitum* access to food, except at the time of daily motor stimulation in the exercise groups and corresponding time points in control groups, and could move freely in the culture vials.

Locomotor activity analysis

The locomotor activity of the flies was recorded using a *Drosophila* Activity Monitoring System (DAMS; Trikinetics). Young males and females (1–3 days old) were placed in glass tubes (5 mm in diameter and 65 mm in length) with a small portion of rearing medium (water, sugar, and agar) and inserted into the monitors (DAM2). For this experiment, 64 females and 64 males at 1–3 days old were used. During the experiment, 3 males and 2 females died and were excluded from further analysis.

Locomotor activity monitors are equipped with infrared light transmitters and receivers. When an

insect interferes with the infrared light beam by moving inside the tube, an event is recorded. Locomotor activity was monitored over 6 consecutive days. The activity monitors were kept in an incubator with constant temperature (25°C) and humidity, under LD 12:12 conditions (Rosato & Kyriacou, 2006). Locomotor activity analysis was conducted using ShinyR-DAM web application (Cichewicz & Hirsh, 2018).

Forced physical activity

We developed a protocol of forced physical activity for flies, based on negative geotaxis triggered by the rotating movements of vials placed in a HulaMixer Sample Mixer (Invitrogen/Thermo Fisher Scientific). We determined the effects of daily physical activity on the lifespan of male and female fruit flies at two different time points: ZT1—one hour after lights-on, the morning peak of activity—or ZT6, six hours after lights-on, representing the 'siesta' period. Prior to daily behavioral experiments, flies from the exercise and control groups were anesthetized with CO_2, using the minimal exposure necessary. Following anesthesia, flies were allowed to recover in empty vials before being subjected to the physical activity protocol. The control group was left for 15 min in place of forced physical activity.

Motor stimulation (daily physical activity) was performed at ZT1 or ZT6 (Fig. 1). Virgin females and males (3 days old) were exercised separately in empty plastic vials (22 mm diameter, 63 mm length; number of individuals per vial: 39 ± 9; min–max: 17–60).

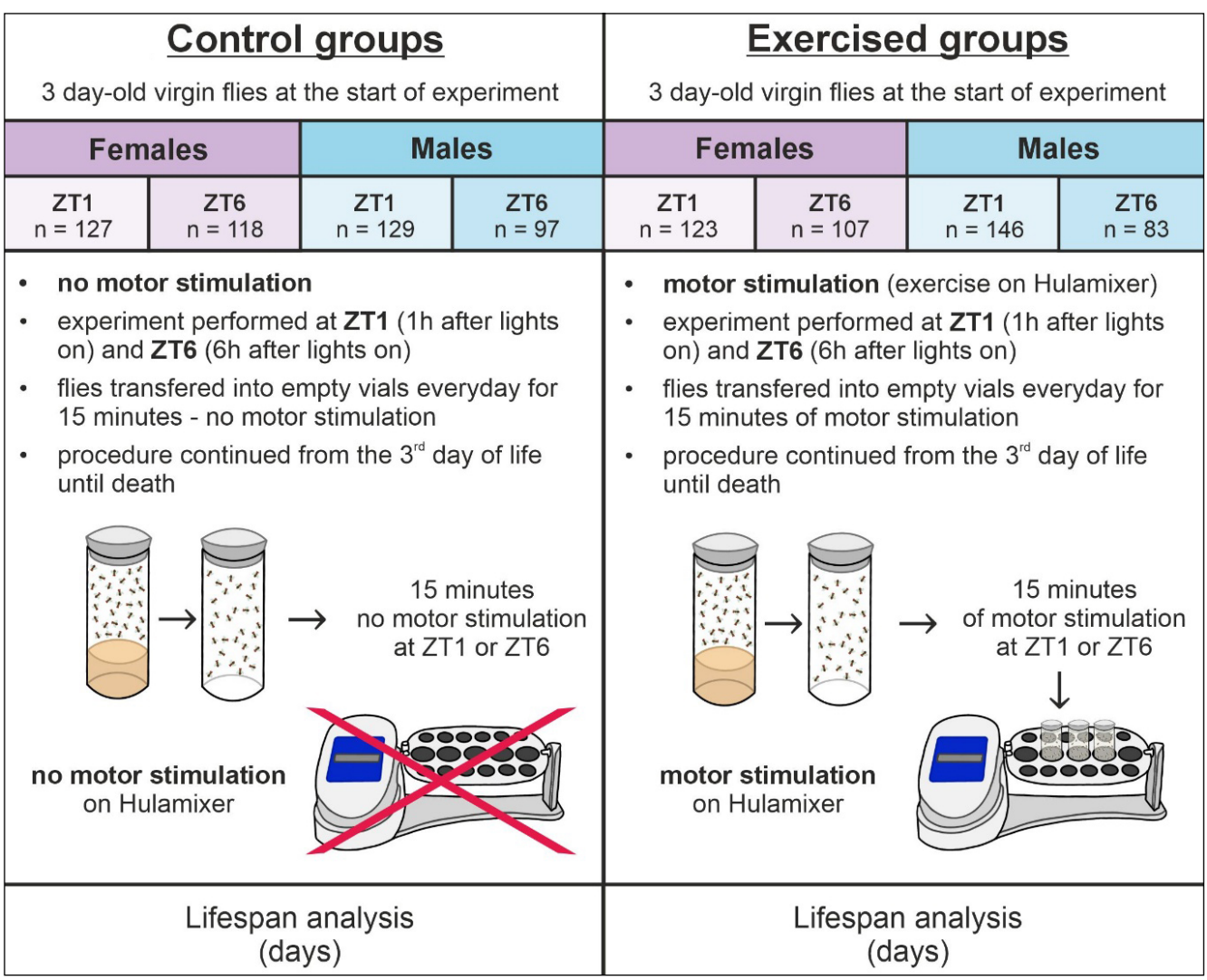

Fig. 1. The experimental procedure. ZT, Zeitgeber time; ZT1, one hour after lights-on; ZT6, 6 hours after lights-on.

The 15-minute-long stimulation was applied daily until the last day of life. Flies were forced to move by inducing a repetitive, innate negative geotaxis response to sequential rotary, turning, and vibratory motions. The rotary motion speed was 60 rpm (1 rotation around the long axis of the platform), with a duration of 1 second. This was followed by turning movement, during which the platform revolved by 180° (90° angle between the maximum inclination and vertical position of the platform). Full rotation of the platform was performed over 1 second. During a 5-second pause in the rotation and turning motion, vibration occurred at an amplitude of 1°. Each experimental group was accompanied by a control group of untrained female and male flies. The number of flies for each group, both trained and control, is given in the figure legends. Control flies were placed in empty vials for 15 minutes per day at the corresponding time points (ZT1 or ZT6).

The locomotor stimulation settings described above were chosen experimentally. A number of trials were carried out to observe conditions under which flies were "forced" or "encouraged" to move. The combination of settings in the applied program allowed exercised flies to move freely to the top of the vial. This takes advantage of the fact that *D. melanogaster* exhibits behavior commonly referred to as negative geotaxis, referring to moving against gravity when they are at the bottom of a vial (Miquel et al., 1976).

Lifespan analysis

The lifespans of males and females from control and exercise groups were observed daily (before the daily bout of physical training). The number of dead individuals was recorded in spreadsheets. Three repetitions of the experiment were performed for each experimental group.

Statistical analysis

The locomotor activity data of the unexercised flies were examined for the presence of outliers using the Grubbs' test. Due to the varying number of males and females that survived during the experiment, the Mann–Whitney U test was performed to compare the total level of locomotor activity of flies in LD 12:12. The progressive decline in locomotor activity with age from the 1st until the 6th day of recording was analyzed using Friedman ANOVA followed by a Dunn's *post hoc* test. Statistical analysis was carried out using Origin2022 v 9.9 software (OriginLab Corporation, Northampton, MA, USA) and Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA).

Survival probabilities were estimated using the Kaplan–Meier method, and survival curves were generated to visualize the differences between groups. Statistical significance of differences between survival curves was assessed using the log-rank test in Statistica 13.3.

RESULTS

Basal locomotor activity and basal life expectancy of females and males

The daily locomotor activity of male and female flies was recorded over six consecutive days (LD 12:12) using the DAMS (Fig. 2A).

The mean locomotor activity per day (Fig. 2B-C) and the total activity over 6 days (Fig. 2D) of males were lower by ~28% (P<0.0001) than that of females. We also found a progressive decline in locomotor activity with age from the 1st until the 6th day of recording in both females (1st *vs.* 5th day P=0.0002; 1st *vs.* 6th day P<0.0001; 2nd *vs.* 5th day P<0.0001; 2nd *vs.* 6th day P<0.0001; 3rd *vs.* 6th day P<0.0001; 4th *vs.* 6th day P<0.0001; 5th *vs.* 6th day P=0.02) (Fig. 2B) and males (1st *vs.* 4th day P=0.03; 1st *vs.* 5th day P<0.0001; 1st *vs.* 6th day P<0.0001; 2nd *vs.* 5th day P<0.0001; 2nd *vs.* 6th day P<0.0001; 3rd *vs.* 5th day P<0.0001; 3rd *vs.* 6th day P<0.0001; 4th *vs.* 5th day P=0.0002; 4th *vs.* 6th day P<0.0001) (Fig. 2C). Females showed a 29.3% decrease in locomotor activity, while males displayed a 36.5% decrease between the first and last days of recording.

The basal life expectancy of males and females

When we compared survival rates between untrained female controls at ZT1 (median lifespan 43 days) and ZT6 (median lifespan 42 days), there were no statistically significant differences (P=0.155; Fig. 3A). In contrast, we observed statistically significant differences in the survival curves for untrained male controls at ZT1 (median lifespan 40 days) and ZT6 (median lifespan 51 days) (P<0.0001; Fig. 3B), with the ZT6 control group living longer than the ZT1 group.

Survival rates were also different between untrained males (combined controls for ZT1 and ZT6) and untrained females (combined controls for ZT1 and ZT6) (P=0.001; Fig. 3C), with a median lifespan of 45 days for males and 43 for females.

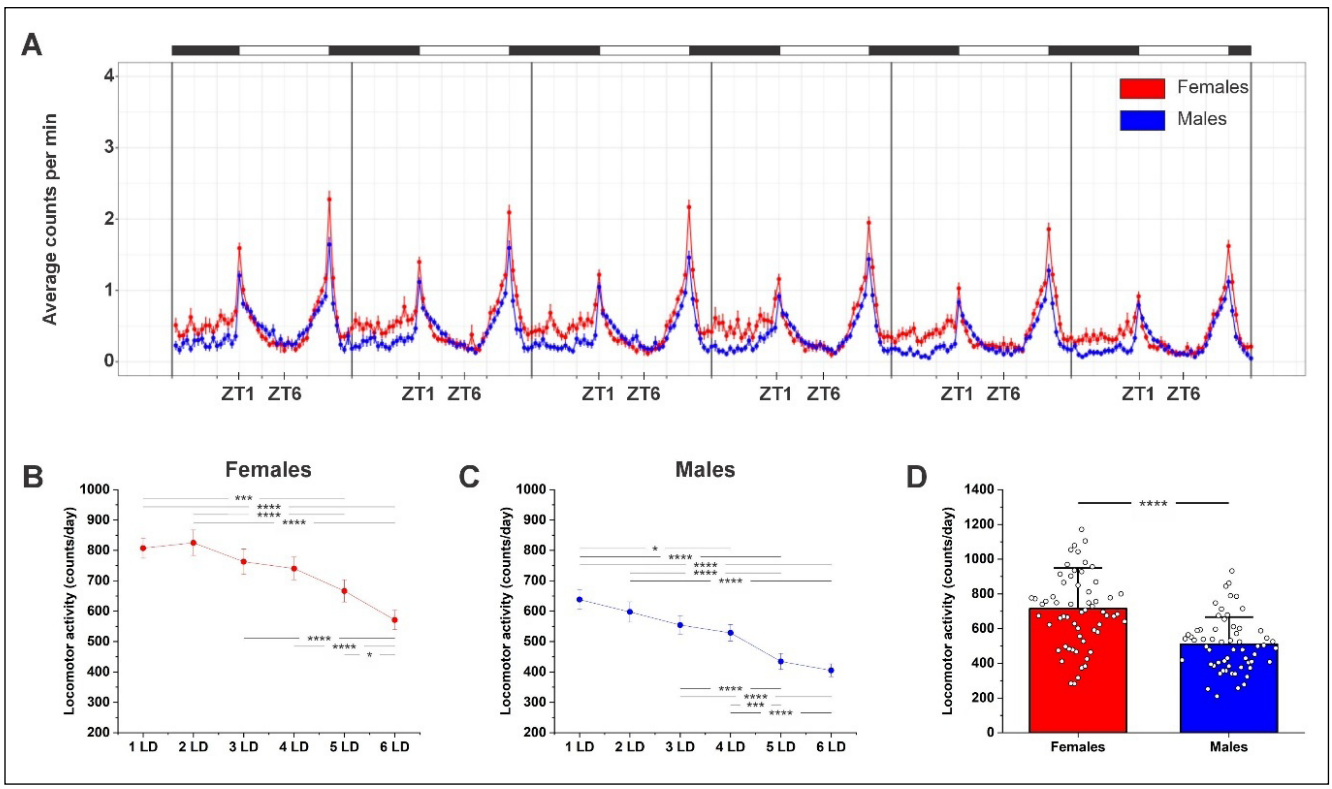

Fig. 2. The basal locomotor activity of unexercised female and male flies in LD 12:12. The daily activity of female (red) and male (blue) flies recorded over 6 consecutive days (presented as the average number of counts per minute). The black and white horizontal bars represent the duration of the dark and light periods, respectively (A); the mean locomotor activity of females (B) and males (C) per day recorded over 6 consecutive days. The data are presented as the mean + SEM. Friedman ANOVA followed by a Dunn's *post hoc* test was used (B and C); total level of locomotor activity (presented as the mean of 6 days of locomotor activity) of females (red) and males (blue). The data are presented as the mean + SD. Each data point in the dot plot represents one individual fly sample. The Mann–Whitney U test was used (D). Statistically significant changes (P<0.05) are plotted on the graphs B–D: * P<0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001. N_{Female}=61, N_{Male}=60. ZT, Zeitgeber time; ZT1, one hour after lights-on; ZT6, 6 hours after lights-on.

The impact of physical training performed at different times of the day on the lifespan of *Drosophila melanogaster*

Fifteen minutes of daily motor stimulation performed during the peak of morning activity (ZT1) prolonged the lifespan of males by ~35% when compared to the sedentary control group (P<0.0001; Fig. 4A). The median lifespan of exercised males increased to 54 days, whereas the lifespan of unexercised males was 40 days.

Interestingly, forced motor activity performed at the morning peak of activity during the day (ZT1) reduced survival of female flies by ~84% compared to unexercised control females (P<0.0001; Fig. 4B). The median life expectancy of exercised females was 7 days, compared to 43 days in sedentary, unexercised female flies.

Forced motor activity at 'siesta' time (ZT6) had a negative impact on the life expectancy in flies of both sexes. Life expectancy was reduced in males by ~20% (P<0.0078; Fig. 4C) and in females by ~67% (P<0.0001; Fig. 4D) compared to their control counterparts. The group of males exercising at ZT6 had a median lifespan of 41 days, compared to 51 days in control males. The median lifespan of females exercising at ZT6 was 14 days, while the sedentary females had a median lifespan of 42 days.

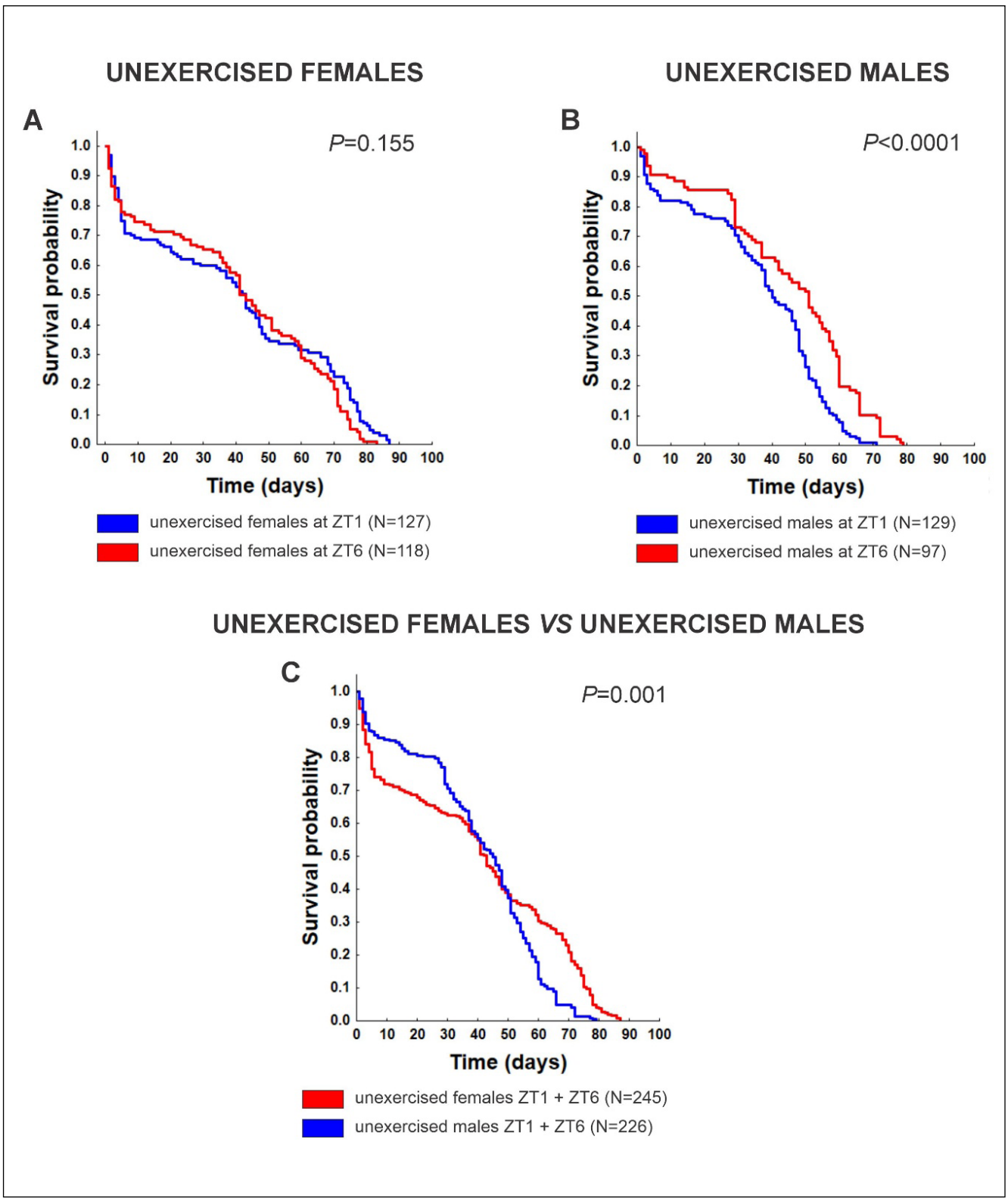

Fig. 3. The survival of *Drosophila melanogaster* of untrained (control) groups of females and males. Kaplan–Meier estimation and the log-rank test were used. Statistically significant differences: *P*=0.001. ZT, Zeitgeber time; ZT1, 1 hour after lights-on; ZT6, 6 hours after lights-on.

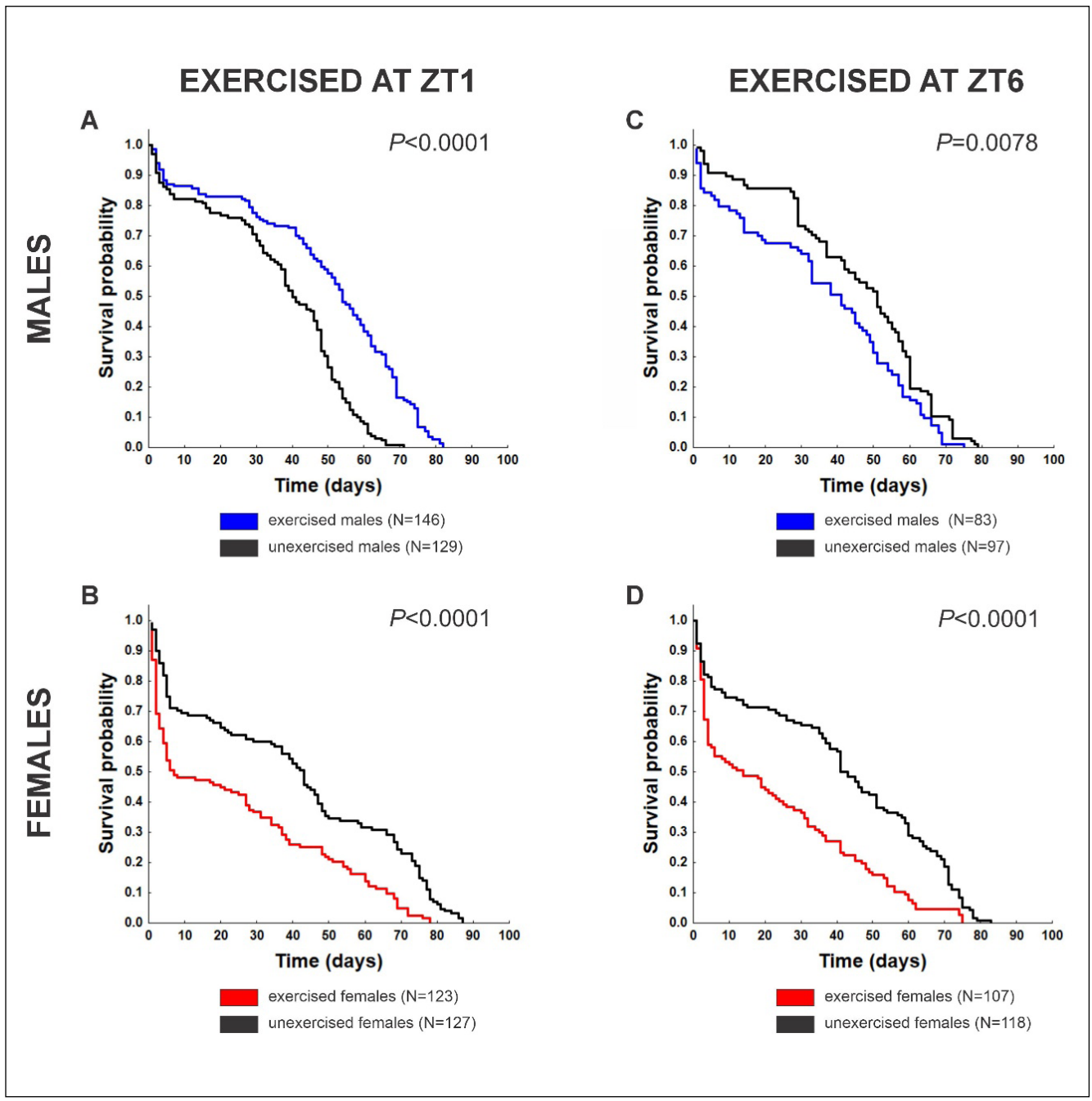

Fig. 4. The survival of *Drosophila melanogaster* after lifelong daily motor stimulation at different times of the day. The lifespan of control (black) and exercised at ZT1 (blue) male flies (A); the lifespan of control (black) and exercised at ZT1 (red) female flies (B); the lifespan of control (black) and exercised at ZT6 (blue) male flies (C); the lifespan of control (black) and exercised at ZT6 (red) female flies (D). Kaplan–Meier estimation and the log-rank test were used. Statistically significant differences: *P*-values are shown in individual graphs. ZT, Zeitgeber time; ZT1, 1 hour after lights-on; ZT6, 6 hours after lights-on.

DISCUSSION

The main finding of this study is that forced motor activity during the morning peak of locomotor activity increased both life expectancy and median lifespan in male *D. melanogaster* flies, whereas in females, the same physical activity had an opposite effect, shortening the median lifespan. Interestingly, daily forced motor activity during the insects' 'siesta' time (ZT6) decreased the longevity of both female and male flies, as shown by shortened median lifespan. Therefore, our results in this simple model organism show that aligning physical activity with the pattern of daily locomotor activity generated by the circadian clock and light may influence the lifespan.

Basal locomotor activity of *Drosophila* females and males

The daily locomotor activity of *D. melanogaster*, maintained under standard laboratory conditions in LD 12:12, is characterized by two peaks of activity, in the morning and evening, and reduced activity at noon—the siesta or day sleep—and in the middle of the night (Aschoff, 1966; Hendricks et al., 2000; Shaw et al., 2000). Peaks of activity are preceded by a gradual increase in activity, called morning anticipation (before the onset of light) and evening anticipation (before the onset of darkness). These rhythmic changes in activity and sleep are driven by the circadian clock, composed of ~150 clock neurons in the lateral and dorsal regions of the *D. melanogaster* brain (Özkaya & Rosato, 2012). The so-called ventral lateral neurons constitute the morning oscillators, while dorsal lateral and some dorsal neurons represent the evening oscillators, which control morning and evening anticipation, respectively (Grima et al., 2004; Stoleru et al., 2004). The morning activity peak is mostly driven by light, as it is weak or not present in constant darkness conditions (DD), while the evening peak is under endogenous circadian control.

In agreement with previous studies, we found that both females and males showed morning and evening anticipation, with two peaks of activity in LD 12:12 separated by two periods of day and night sleep. This indicates the proper functioning of the circadian clock in the insects used in the study.

Moreover, virgin females exhibited higher locomotor activity than their male counterparts, consistent with previous studies (Fernández et al., 1999; Helfrich-Förster, 2000; Isaac et al., 2010; Anderson et al., 2022). We also demonstrated that virgin females are especially active during the dark period. In addition, we confirmed that in both females and males, locomotor activity decreased with age, aligning with previous reports (Fernández et al., 1999; Ridgel & Ritzmann, 2005; Yusuf et al., 2024).

The life expectancy of *D. melanogaster* females and males

Sexual dimorphism in life expectancy is well established, with females living longer in most animal taxa (Lemaître et al., 2020). *D. melanogaster,* cultured under laboratory conditions, also manifests this phenomenon (Lehtovaara et al., 2013; Tower & Arbeitman, 2009; Yusuf et al., 2024), as we confirmed in the present study. Nevertheless, wider studies showing sex differences in the fruit fly lifespan are inconclusive, and results from a number of strains indicate that males have a longer lifespan than females (Nuzhdin et al., 2005; Huang et al., 2020; Lin et al., 2023).

Timing of physical activity and life expectancy in fruit flies

Using the simple model organism of the fruit fly, in which the first clock genes were described (Konopka & Benzer, 1971; Bargiello et al., 1984; Reddy et al., 1984; Bargiello & Young, 1984), we have demonstrated a chrono-exercise effect for the first time, showing that synchronization of physical activity with the circadian clock directly affects *D. melanogaster* life expectancy. In males, when the exercise intervention was applied according to their natural daily activity rhythm—i.e., the insects were stimulated to move in the morning, at the time of their highest locomotor activity—an extended life expectancy was observed. On the contrary, if forced motor activity was applied to males during their 'siesta' time, their life expectancy was lowered.

The effect of synchronizing exercise to the circadian clock on life expectancy may vary, however, according to sex. In females, both morning and evening exercise interventions lowered life expectancy. An effect of sexual dimorphism in relation to exercise has also been observed in humans (Arciero et al., 2022). In women, morning exercise reduced abdominal fat and blood pressure, while evening exercise enhanced muscular performance; meanwhile, in men, evening exercise increased fat oxidation and reduced systolic blood pressure and fatigue (Arciero et al., 2022). Therefore, the timing of exercise appears important for optimizing individual training plans in both females and males.

Sex differences in exercise hormesis of *D. melanogaster*

Daily physical activity is unquestionably a form of stress for flies, but may also trigger hormetic adaptations; that is, low-level stress may have a beneficial effect on an organism, enhancing insects' health and longevity (Krebs & Loeschcke, 1994; Calabrese, 2008). In most cases, mild stress has been shown to have a positive effect on *D. melanogaster* males and a negative or neutral effect on females (Sujkowski et al., 2017; Le Bourg, 2020). According to Sujkowski et al. (2017), female flies respond to exercise stimuli, but do not exhibit the adaptive training response seen in males.

Consistent with this, we observed increased survival probability and extended maximum lifespan in males exercised at ZT1 (morning activity); however, in females, morning activity accelerated aging and shortened life expectancy. One possible explanation of the observed sex differences could be the varied energy cost of maintaining biological functions. In the case of *D. melanogaster*, the higher energy expenditure observed in females compared to males may be explained by the additional energy required for egg production (Laturney et al., 2023). Another likely factor contributing to higher energy consumption in females is their higher overall locomotor activity compared to males (Fig. 2; Anderson et al., 2022), further intensified by their larger body size (Mathews et al., 2017). This could explain the observed negative effect of physical activity on life expectancy in females exercised in morning hours when compared to males. Therefore, although females are thought to be more stress-resistant (Pomatto et al., 2018; Lin et al., 2023), the additional loss of energy associated with morning activity could result in the shortening of their lifespans.

Flies in our locomotor training protocol were not assessed for typical markers of stress. Nevertheless, several aspects of our experimental paradigm suggest that the stress induced was likely minimal. The induction of negative geotaxis relied primarily on rotational motion, thereby avoiding the repeated falls and mechanical trauma associated with other methods, such as the Power Tower (Lowman et al., 2018). More importantly—as demonstrated by Ghimire and Kim (2015)—short bouts of locomotor stimulation are generally associated with an improvement of metabolic health rather than with stress. Finally, although food restriction was also incorporated into our experimental paradigm, time-restricted feeding on 12-hour cycles has been reported to improve motor performance and increase brain levels of primary antioxidant enzymes in aging flies (Abdulwahab et al., 2025).

The effect of morning endurance physical activity in males on life expectancy

In the present study, we developed a new exercise protocol, different from those previously published (see e.g., Wen et al., 2018, Peng et al., 2024), employing a HulaMixer Sample Mixer device to train the flies. The applied physical activity involved bouts of exercise lasting 15 minutes, performed every day until the end of life. Since the exercise schedule also used vibratory motion, there was no medium in the training flasks (to avoid insects becoming stuck to the medium), which also contributed to the reduction of the daily training time. The protocol design was also specifically related to the use of females, who have higher basal energy demands than males. Therefore, we aimed not to over-intensify the females' energy expenditure, which could lead to acute effects. Nevertheless, we observed a shortened lifespan in females as a result of training during both peak activity and 'siesta' periods.

The direction of further studies on chrono-exercise effect

The fruit fly is broadly used as a model species for studying human diseases and the effects of various stimuli, including forced activity, on fitness and lifespan. Many genes in the *D. melanogaster* genome are orthologous to human genes, and 77% are involved in human diseases (Reiter et al., 2001). It has also been reported that flies are able to adapt to physical training and exhibit phenotypic responses similar to those in humans (Wen et al., 2018; Damschroder et al., 2018). However, it must be kept in mind that our results only provide a suggestion for further investigation into the impact of chronobiology on the effects of controlled physical exercise in vertebrates, including humans.

CONCLUSIONS

Our study provides evidence from a *D. melanogaster* model that following the circadian clock when performing exercise may impact the outcome of the physical activity and its effect on the lifespan. Namely, forced physical activity in males synchronized to the circadian peak of locomotor activity resulted in an extended lifespan in males when compared to activity performed in 'siesta' time. Surprisingly, when physical activity was applied during the 'siesta' time, the lifespan was shortened regardless of sex. To the best of our knowledge, this is the first observation on the impor-

tance of the circadian clock for the effects of lifelong physical training in flies. Unexpectedly, we also found that forced physical activity in female flies, regardless of the time of the day, had negative effects on their lifespan when compared to sedentary controls.

Overall, our results indicate that physical activity applied according to the circadian clock's regulation of activity and sleep could be a new strategy to potentiate the effects of lifelong physical training on the body. Moreover, we have shown that forced physical activity during day sleep ('siesta') may negatively affect the lifespan. Further studies are needed, however, to extend our knowledge regarding the influence of lifelong physical activity in relation to natural daily rhythms in other species. Additionally, despite the importance of circadian rhythm on the effect of regular physical activity, sex should be taken into consideration when preparing and optimizing training plans. It is also possible that mild physical activity correlated with daily peaks of activity may be beneficial for females.

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