Estrogen-mediated individual differences in female rat voluntary running behavior

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Running Head: Estrogen's impact on voluntary exercise behavior in female rats

Abstract:

Regular exercise has numerous health benefits, but the human population displays significant variability in exercise participation. Rodent models, such as voluntary wheel running (VWR) in rats, can provide insight into the underlying mechanisms of exercise behavior and its regulation. In this study, we focused on the role of estrogen on VWR in female rats. Female rats run more than males, and we aimed to determine to what extent running levels in females were regulated by estrogen signaling. The running behavior of rats (duration, speed, and total distance run) was measured under normal physiological conditions, ovariectomy (OVX), and estrogen replacement in an OVX background. Results show cyclic variations in running linked to the estrus cycle. Ovariectomy markedly reduced running and eliminated the cyclic pattern. Estrogen replacement through estradiol benzoate (EB) injections and osmotic mini-pumps reinstated running activity to pre-OVX levels and restored the cyclic pattern. Importantly, individual differences and ranking are preserved such that high vs. low runners before OVX remain high and low runners after treatment. Further analysis revealed that individual variation in running distance was primarily caused by rats running different speeds, but rats also varied in running duration. However, it is noteworthy that this model also displays features distinct from estrogen-driven running behavior under physiological conditions, notably a delayed onset and a broader duration of running activity. Collectively, this estrogen causality VWR model presents a unique opportunity to investigate sex-specific mechanisms that control voluntary physical activity.

New & Noteworthy

This study investigates estrogen's role in voluntary wheel running (VWR) behavior in female rats. Female rats exhibit greater running than males, with estrogen signaling regulating this activity. The estrous cycle influences running, while ovariectomy reduces it, and estrogen replacement restores it, maintaining individual differences under all conditions. Both running speed and duration contribute to VWR variations. These findings emphasize individual estrogen regulation in female exercise and provide an estrogen replacement animal model for investigating neurobiological underpinnings that drive voluntary exercise behavior.

Keywords:

wheel running, exercise motivation, ovariectomy, estrogen replacement, estrogen receptor, running speed, running time

Introduction

Regular exercise is essential for maintaining physical and mental health. Voluntary exercise, in particular, is beneficial for human health, including cardiovascular fitness, reducing the risk of chronic diseases, and promoting overall well-being (1, 2). On the other hand, a sedentary lifestyle characterized by insufficient exercise is a major risk factor for chronic disease (3, 4). Despite the plentiful benefits of exercise, the human population displays a range of participation levels (5).

The human heterogeneity in voluntary exercise can be recapitulated in a rodent model of wheel running (6-11), which allows for the isolation of physiological factors underlying exercise motivation from its social/health aspects. Indeed, voluntary wheel running (VWR) is natural and rewarding to rodents (12, 13), and selective breeding experiments have shown that individual differences in running behavior in rodents are mainly caused by individual differences in motivation to run rather than constraints of exercise physiology (14-16) . Thus, the voluntary running behavior of rodents can provide important insights into the underlying mechanisms of exercise behavior and the factors that regulate it.

Human and animal studies have identified molecular events, systems, and mechanisms that are involved in exercise's beneficial effects (17, 18). However, it is not clear yet what regulates exercise behavior itself or serves to maintain prolonged chronic exercise behavior. The cumulative data support the hypothesis that physical activity is genetically regulated (19), and a host of genetic factors have been identified from animal models recapitulating individual differences (20), including high vs. low activity lines of inbred mice (21, 22), selective breeding of high voluntary wheel running mice (15, 23), and high-capacity vs. low-capacity runners (24). However, connections between specific genetic features and the function of the reward/motivational circuits involved in regulating running remain unclear. It has been speculated that euphoria induced by exercise (e.g., "runner's high") serves as an intrinsic motivator (25), and some evidence supports the involvement of BDNF, dopamine, opioids, and endocannabinoids in the brain (17). A recent study suggests a connection between the motivation for exercise and certain products from gut microbiota (26). Clearly, the genetics of individual variation in physical activity levels is complex. It is not possible to isolate the neurological factors that motivate VWR by merely studying individual differences in VWR. Even when studying genetically defined lines from a selective breeding experiment, the act of running induces major changes in physiology, confounding cause from effect.

Animal models that connect a specific molecular feature to variation in voluntary running would be useful for piecing together the mechanisms. Among other factors, sex serves as a unique biological variable in rodent VWR behavior. Female rats and mice run significantly more than males under the same conditions and exhibit estrus cycle-dependent variations (27-30). This difference is linked to the female sex hormone estrogen and its receptors (31, 32). Ovariectomy (OVX) significantly reduces running activity and eliminates the rhythm. Replacing estrogen in an OVX background restores running levels, establishing a causal link between estrogen, and running (29, 32-37). Female rodents have a 4~5-day estrus cycle resulting in a natural fluctuation in the female sex hormone estradiol. Abundant evidence supports that estradiol exerts rapid effects on motivational behaviors, including food, sex, and drugs of abuse (38, 39). These effects

of estradiol appear to be mediated by specific limbic regions, including the dorsal and ventral striatum, and ventral tegmental area (40-42). Overall, studying the effects of estradiol in regulating voluntary wheel running behavior in female rats can provide insights into the underlying mechanisms of exercise behavior regulation.

This study aims to enhance our understanding of the causal link between estrogen and voluntary running in female rats, with a particular focus on individual performance. We conducted a thorough analysis of individual running parameters - such as running time, speed, and distance - correlating them across distinct conditions, including pre-ovariectomy (pre-OVX), OVX, and estrogen replacement in an OVX background. Additionally, we investigated the dynamics, encompassing onset and duration, of VWR induced by estrogen replacement. Our findings collectively contribute new evidence supporting the hypothesis that the individual variations in voluntary physical activity exhibited by female rats are directly influenced by estrogen and estrogen-dependent signaling cascades. Notably, we identified a specific time window associated with the estrogen causality VWR model. This temporal insight has the potential to guide future experimental designs, allowing the isolation of estrogen-mediated molecular events that drive running behavior, independent of the effect of running itself.

Material and Methods

Animals

5-8-week-old male and female Sprague Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) and were housed in temperature- (22 °C) and light- (12/12 h dark/light) controlled animal quarters. The rats had free access to standard laboratory rat chow and drinking water. Experimental procedures were conducted in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the National Research Council of the (U.S.) National Academies and were approved by Des Moines University Institutional Animal Care and Use Committee.

Voluntary wheel running (VWR)

After a 7-day acclimation period, rats were housed individually in polycarbonate living chambers (40.64 x 50.80 x 20.96 cm) equipped with stainless steel lids and running wheels with a circumference of 1.10 meters (Scurry Rat Activity Wheel with Living Chamber, Lafayette Instrument, Lafayette, IN). Scurry Rat Activity Counters were mounted to the wheels and connected to the Scurry Interface for Animal Activity. The interface was connected to a computer, and the use of each running wheel was reported in real time and stored in the Scurry Activity Monitoring Software (Lafayette Instrument, Lafayette IN). Wheel revolutions were recorded continuously throughout the experiment and translated to running distance based on the wheel size. The duration of running (or time spent on running) is determined as the total number of minutes with at least 1 wheel rotation. Speed is the average distance per minute across all minutes with at least 1 wheel revolution.

Vaginal swabbing and cytology

Vaginal canals of naïve female rats were swabbed daily using sterile cotton swabs moistened with normal saline. The swabs were inserted into the vaginal orifice at a depth of approximately 5-10 mm and rotated gently. The swabs were then removed, and vaginal epithelial cells were smeared onto a microscope slide and allowed to dry. Dried slides were stained using 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO). The slides were inspected using a light microscope at 10x objective to discern cell types. The estrous cycle stage at the time of swabbing was determined based on the cell types present in each sample in a method previously described by Cora et al. (43).

Surgical procedure

Ovariectomy and sham surgeries. Female rats were given free wheel access for 9-22 days before undergoing ovariectomy (OVX). VWR prior to OVX served to establish a habit of daily running, and to observe individual differences in running behavior to determine if these differences persisted after OVX and subsequent estrogen replacement. OVX was performed by placing rats in the prone position and making bilateral midline incisions approximately 1-2 cm in length on the dorsal flank. The skin and abdominal wall were opened, the ovaries were located, the ovarian vessels were ligated, and the ovaries excised. The sham surgeries were performed in the same manner, without ligation of vessels or excision of the ovaries. Rats were placed in standard cages without running wheels for two weeks following surgery to allow for healing and estrogen depletion in OVX rats. Post-operative care included 1/day triple antibiotic ointment applied to incisions and carprofen (5mg/kg) subcutaneous injection (Zoetis, Parsippany-Troy Hills, NJ) administered immediately after recovery from anesthesia and for the 2 days following surgery.

Rats were monitored closely while recovering from surgeries.

Osmotic mini pump implantation. Mini-pump implantation surgery took place 4-5 weeks after OVX/sham recovery and reintroduction to wheels (6-7 weeks after OVX/sham procedure). The day before implantation, pumps (Model 2004; Alzet, Cupertino, CA) were prepared according to manufacturer instructions. The surgical setup was the same as the OVX and sham surgeries but with a single horizontal midline incision of approximately 2cm on the upper dorsal surface of the rat. Using blunt dissection, a pocket was formed between the skin and muscle and an osmotic mini-pump was inserted into the pocket. The rats were returned to running wheel cages immediately after surgery recovery, and their running data was collected for another two weeks.

Estradiol administration

Subcutaneous injections After returning to running wheel cages for two weeks following surgery recovery, OVX rats were given subcutaneous injections of 1.5 µg of β-estradiol 3-benzoate (EB) (Sigma-Aldrich, St. Louis, MO) in 0.1mL of sesame oil (Sigma-Aldrich, St. Louis, MO) vehicle. Control groups were given 0.1mL sesame oil vehicle only. After receiving the injection, rats were immediately returned to their running wheel cages. The same protocol was followed for any additional EB injections, with at least one week between injections.

E2 osmotic mini pumps One week after the final injection of EB, the OVX rats underwent surgery to implant osmotic mini pumps to deliver EB (630ng per 6µl/day in polyethylene glycol). According to the manufacturer's claim, the pumps have a fixed delivery rate of 0.25µl/hour to provide subcutaneous and continuous substance release for up to 28 days. The rats were returned to running wheel cages immediately after recovering from anesthesia.

Serum estradiol measurement

Trunk blood was collected in serum collection tubes (BD Bioscience) and allowed to clot at room temperature for 15-30 minutes before undergoing centrifugation for 15 minutes at 3400rpm to separate serum from the cellular components. The total level of estradiol in the serum, including the free form and the conjugated estradiol, was measured using an ELISA kit (ALPCO) at the Ligand Assay and Analysis Core of the University of Virginia Center for Research in Reproduction. The sample preparation instructions provided by the kit were followed to ensure accuracy in measuring serum estradiol levels. Samples were run in singlet on the same plate. Estradiol (mouse, rat) intra-assay coefficients of variation: 8.6; inter-assay coefficients of variation: 11.3.

Data collection and analysis

Data are expressed as mean +/- SEM. VWR output data were collected from the Scurry Activity Monitoring Software. Statistical analysis and figures were completed in GraphPad Prism. Statistical significance was determined by Student's t-test when two groups were compared and one-way or two-way ANOVA when more than two groups were compared. Significance was defined as $P \leq 0.05$. Average running distances, time, and speed were calculated by taking the average of the 4 daily values directly preceding any surgery or EB treatment. This process is to ensure the values encompass a full estrous cycle for intact female rats and to remain consistent when comparing values between sexes or treatment groups. Rats were ranked according to their average daily running distance during the last 4 days of the experiment. High runners were

defined as those rats that fall within the top 25% of 4-day average running distances, and low runners were defined as those rats that fall within the bottom 25% of 4-day average running distances. Middle runners were defined as those rats that fall between the two categories.

Results

Individual and sex differences revealed by a voluntary wheel running (VWR) program

Similar to what has been observed in other studies, (44, 45), male rats with free access to running wheels began with low running activity (300-3,000 meters/day), as measured by daily running distance. However, two weeks of wheel exposure escalated their running and resulted in a 10-fold difference between the highest and the lowest running rats within a cohort (n=10) (**Fig. 1A**). Total running distance during the days 1-4 was strongly correlated with total distance run on days 11-14 (n=10, r=0.7714, p=0.0090) (**Fig. 1B**).

When conducting the same experiment with female rats, we observed a similar level of heterogeneity in VWR activity resulting in a range of ~5-25 km on the final day (n=16) (**Fig. 1C**). Like the male cohort, we found that total running distance on days 1-4 exhibited a strong positive correlation with outcomes on days 11-14 (n=16, r=0.7999, p=0.0002) (**Fig. 1D**).

As expected, a circadian rhythm in voluntary running was observed in both male and female rats when tracking their wheel-running activity by hour (**Fig. 1E**), and peak running, as measured by distance, occurred within the first 5 hours of the "lights off" phase (female: n=16, male: n=16) (**Fig. 1F**). The running speed exhibited a similar pattern (**Fig. S1**).

Further analysis of the daily VWR activity of female rats revealed a distinct qualitative difference in the pattern of activity. Female rats displayed a peak-valley pattern of daily running in which peaks occurred every 4-5 days. This pattern can be clearly observed when peaks for each rat are manually aligned (n=11) (**Fig. 2A**). This pattern is present in the daily running activity of every sexually mature female rat, regardless of individual differences in activity rate. To identify additional sex differences in VWR activity, we compared average daily running distances per week during the acquisition phase and found that female rats ran significantly further than males each week for 3 weeks (Female: week 1 n=54, week 2 n=32, week 3 n=8. Male: week 1 n=17, week 2 n=18, week 3 n= 13, $p<0.0001$) (**Fig. 2B**). Furthermore, when comparing the daily running distances of individual male and female rats, the difference in pattern becomes even more apparent (**Fig. 2C**). Through a method of estrous cycle staging previously described (43), we observed that the peaks in running activity coincided with proestrus, the stage with the highest estrogen levels. This pattern holds true for all female rats as a group. Over the course of one estrous cycle (4 days), all females exhibited increased running distances during proestrus, while average male running distances varied less day to day (**Fig. 2D**). Females ran significantly further per day than males (Female: n=16, male: n=16; p=0.0014). To establish this rhythm in daily running distances, we identified the 4 main stages of the estrous cycle by analyzing cell types and abundance in the vaginal epithelium (**Fig 2E**).

Contributions of running speed and time to running distance of female rats

Individual differences in average running distance could result from individuals running different durations (e.g., hours per day), and/or because individuals run at different speeds when running. We found a strong positive correlation between total running distance and running speed on days 11-14 (n=16, r=0.9443, $p<0.0001$) (**Fig. 3A**). A positive correlation was also observed between

average distance and duration (n=16, r=0.6384, p=0.0078) (**Fig. 3B**). No significant correlation was found between running speed and running duration (n=16, r=0.3647, p=0.1649) (**Fig. 3C**). To illustrate the fluctuation of running activity during the estrus cycle, we plotted daily distance, speed, and running time for groups of high, middle, and low runners (**Fig. 3D-F**). Daily running distance, speed, and time all peak during proestrus regardless of running performance (**Fig. S2A-C**). Additionally, we identified a positive correlation between the magnitude of running distance increase from diestrus to proestrus and the average running speed (**Fig. S3**). This indicates that those rats with higher average running speeds also express the greatest response to estrogen fluctuations.

Ovariectomy (OVX) diminished sex difference and eliminated female-specific running pattern

Having established a correlation between daily running behaviors and estrogen level fluctuations, we conducted ovariectomies (OVX) to examine the impact of reduced circulating estrogen on running activity. As shown in **Fig. 4A**, the VWR of female rats was monitored for 22 days before undergoing OVX, followed by a 2-week recovery period with no wheel access. On day 36, the rats regained wheel access, and their daily running activity was recorded for another 2 weeks. Inspection of the running activity of individual rats showed that not only were the peaks and valleys in running activity eliminated after OVX, but the average running distances also appeared much lower than before. Comparatively, the daily running distances of those rats in the sham group remained consistent with pre-surgery activity and retained a peak-valley running pattern (**Fig. S4A**) (n=4).

Quantitative comparisons between pre- and post-surgery running revealed that OVX significantly reduced average daily running distance, but sham did not (Sham: n=9, p=0.5817; OVX: n=20, p<0.0001; two-way ANOVA) (**Fig. 4B**). Individual plots disclosed that some rats decreased more than others by OVX (**Fig. 4C**) (p<0.0001). In addition, individual plots detail the similarities in average running distances pre and post sham (**Fig. 4D**) (p=0.3830). After establishing how OVX affects running distance, we sought to determine how OVX affects other running variables. OVX resulted in a significant decrease in both running duration and speed, as quantified by the average of the last 4 days (n=20, p<0.0001) (**Fig. 4E&F**). To assess the extent of influence attributed to each variable on running distance, we performed correlational analyses between each variable and distance. Both variables demonstrated a positive correlation (p<0.05); however, speed emerged as a more robust predictor (r=0.9212, p<0.0001, **Fig. 4G**) compared to duration (r=0.9134, p<0.0001, **Fig. 4H**). Altogether, these results suggest that OVX results in decreased overall running activity.

Estrogen replacement restored OVX-induced reduction in running activity

We next tested if replacing estrogen in an OVX background would restore VWR activity to pre-OVX levels. To do this, we tracked daily running distances of rats pre- and post-OVX and throughout estrogen manipulation, as shown in **Fig. 5A** with representative low, medium, and high runners. Following post-OVX VWR, subcutaneous injections of estradiol benzoate (EB) (1.5 µg EB in 0.1mL sesame oil) were administered on days 46 and 54. Following the EB doses, we observed acute increases in VWR activity manifesting over 24 hours after injections.

Responses to the estrogen replacement varied, with the high runners showing the most prominent response. On day 61, osmotic mini-pumps containing 17β-estradiol (E2) were implanted subcutaneously. The mini-pumps are designed to provide a steady infusion of E2 (750 ng per 6 µl/day) to the rats circulation for about 2 weeks. This steady infusion of estradiol was meant to simulate a baseline level of estrogen circulation, similar to what intact female rats experience between proestrus surges. The E2 mini-pumps resulted in a sustained increase in daily running for about a week before a final EB injection was administered on day 67. The final EB injection, in conjunction with the mini-pumps was meant to simulate the surge in estrogen levels intact female rats experience during proestrus. This arrangement resulted in an acute increase in VWR activity that mimicked the activity levels observed in intact females. The rats in the sham group maintained consistent daily running activity and regular peak-valley pattern (**Fig. S4B**) (n=3).

To illustrate the effects of estrogen replacement on individual VWR activity, we compared the 4-day average running distances during pre-OVX, post-OVX, and EB-evoked acute individual running peaks (n=16, pre-OVX vs. post-OVX: $p<0.0001$, post-OVX vs. EB peak: $p=0.0001$, pre OVX vs. EB peak: $p=0.1609$; one-way ANOVA with repeated measures) (**Fig. 5B**). While OVX decreased running levels, estrogen replacement via EB injections restored running to pre-OVX levels. The peak running activity induced by EB treatment exhibited a positive correlation with the daily running distance before OVX (n=16, $r=0.7418$, $p=0.0010$) (**Fig. 5C**), implying that EB reinstated individual differences in running. Similar results were found when estrogen was replaced via E2 osmotic mini-pumps (n=8) (**Fig. 5D:** pre-OVX vs. post-OVX: $p=0.0002$, post-OVX vs. mini-pump: $p=0.0009$, pre-OVX vs. mini-pump: $p=0.0102$; one-way ANOVA with repeated measures. **Fig. 5E:** $r=0.7724$, $p=0.0247$).

Estrogen-dependent increase in running speed and duration

Because individual differences in running distance persist after EB treatment, we investigated whether this was true for running speed and duration as well. Individual differences in running speed and duration were reinstated by the EB treatment, with a stronger positive correlation between pre-OVX and OVX+EB conditions for running speed ($r=0.9802$, $p=0.0006$) than duration ($r=0.4974$, $p=0.3154$) (n=6) (**Fig. 6AB**). We further evaluated the relative contributions of speed and running duration to estrogen-enhanced running distance. Both variables showed a positive correlation with EB-induced running distance; however, the correlation was stronger for speed ($r=0.9854$, $p=0.0003$), than duration ($r=0.7304$, $p=0.0993$) (n=6) (**Fig. 6CD**). Lastly, we explored the relationship of VWR activity between the OVX and OVX+EB conditions. We identified positive correlations in both speed ($r=0.9727$, $p=0.0011$) and running duration ($r=0.8714$, $p=0.0237$) (n=16) (**Fig. 6EF**). This set of results indicate that, in addition to distance, individual differences in running time and speed can also be recapitulated by EB treatment.

Estrogen replacement induces time-dependent physiological and behavioral changes

Estrogen exerts both short and long-term effects on various physiological processes through binding to estrogen receptors (ERs) (46). Because the behavioral change evoked by estrogen replacement takes over 24 hours to manifest (**Fig 7A**), we sought to determine physiological changes that occur prior to this behavioral response. We administered EB (1.5 µg) or vehicle (sesame oil) via subcutaneous injections 4 hours and 24+ hours before blood collection. The rats

receiving the injections 24+ hours before blood collection were allowed to run for 5 hours before sacrifice. Serum was collected, and total serum E2 was measured using enzyme-linked immunoassay (ELISA). We found that the serum E2 of EB-treated rats was significantly higher than controls after 4 hours (EB: n=4, vehicle: n=4, p=0.0380) but not after 24+ hours (EB: n=6, vehicle: n=4, p=0.0803) (**Fig 7B**).

To determine the impact of OVX on vaginal epithelial cells, we obtained samples from OVX rats (**Fig. 7C**). The samples contained an elevated presence of neutrophils and very few, if any, nucleated epithelial cells. Within 4 hours of EB treatment, there were no noticeable changes to the vaginal epithelium (**Fig. 7D**). Notably, 24+ hours after EB treatment, vaginal epithelial samples changed in cell type and density (**Fig 7E**). Instead of dense neutrophils, the samples contained primarily nucleated epithelial cells in clumps, more closely resembling samples obtained from intact females during the proestrus stage of the estrous cycle.

Timing of OVX in relation to wheel exposure

To investigate whether the acquisition phase of VWR prior to OVX is necessary to observe the same level of activity and response to estrogen replacement, we compared the post-OVX running activity of two groups of rats. One group had 2 weeks of wheel access before undergoing OVX and a 2-week recovery period before post-OVX running (pre-run group: n=6); the other had no access to wheels before undergoing OVX and a 2-week recovery period before post-OVX running (no pre-run group: n=6) (**Fig. 8A**). In comparing their daily running distances during the post-OVX running period, there was no distinct contrast in the pattern or level of activity between the groups. A comparison of the average daily running distances of the last 4 days of the post-OVX running period yielded no significant difference between the two groups (pre-run: n=6, no pre-run: n=6, p=0.7263) (**Fig. 8B**). In a longitudinal study of post-OVX running behavior of the pre-run group (**Fig. 8C**), subcutaneous EB (1.5 μg EB in 0.1mL sesame oil) injections were administered to each individual on day 13. The injections resulted in increased running distances for 3-4 days starting on day 15 (n=6). The no pre-run group underwent a similar study (n=6) (**Fig. 8D**). The rats received subcutaneous EB injections on day 15 and responded to the injections with increased running activity for 4-6 days starting on day 17.

Discussion

In rodents, females in the laboratory and in the wild are more active than males. This has been attributed to mate seeking behaviors and foraging to provide for young. In humans it is widely perceived that men are more active than women, and exercise motivators vary between genders (30). Humans encounter many life stressors that can impact their ability to participate in voluntary physical activity that animals do not. Some of those barriers may contribute to the gender difference like inconvenience, safety concerns, cost, location, and socioeconomic status (47, 48). It's worth noting that the studies reporting women as less active than men are largely sourced from surveys in which individuals report how often and to what extent they engage in exercise outside of work or normal daily activities. While women may participate in dedicated exercise outside of the home less often, that doesn't necessarily mean that they are less active overall. Some of the occupations most commonly held by women are those that require employees to be on their feet for most of the day like registered nurses, elementary and middle school teachers, and retail supervisors (49). Additionally, women who work in the home are likely spending most of their day completing household duties and caring for children. The accuracy of reporting these statistics is another confounding factor in determining gender differences in voluntary activities. Ultimately, we do not have a clear answer as to why women appear to be less physically active than men. This contrast between species justifies the need for a better understanding of the factors that drive running behavior in an exercise model. By doing so, we may be able to exploit these factors to enhance exercise motivation in humans. This underscores the strength of a causality animal model in voluntary exercise.

The primary finding of this study is that individual differences in VWR activity in female rats are strongly influenced by estradiol signaling. This validates a causality animal model for identifying factors regulating voluntary exercise participation and performance. Consistent with previous work in mice and rats (31, 37, 50, 51), estrogen replacement through estradiol benzoate (EB) injections and osmotic mini-pumps restored VWR activity to pre-OVX levels and reinstated the peak-valley pattern. Importantly, the estrogen treatments also recovered individual differences in levels of running. Our results are consistent with a large number of studies that show female rats run more than males, that individual differences are strongly repeatable across days, and that levels of running increase over the first few weeks of access (reviewed by (29, 30, 45). While several other studies have established that OVX decreases running in rodents and that estradiol replacement recovers running (37, 52), this is the first study to demonstrate that the running-induced from estradiol treatments in OVX rats recapitulates the individual level of running displayed by the rats before OVX treatment. This is important because it implies that estradiol is part of a molecular event for motivational and reward circuits capable of generating individual differences in levels of activity. The estrogen replacement model, therefore, provides a unique opportunity to investigate the molecular mechanisms serving estrogen's role in VWR regulation.

Estrogen exerts its effects through ERs, transcription factors that regulate gene transcription, as well as membrane-bound receptors. The effects on gene expression depend on the brain regions and cell types that express ERs (53). Notably, the EB-evoked running responses exhibited individual differences similar to those observed before OVX, resulting in a significant positive correlation between EB-evoked and natural running behavior (**Fig. 5C**). These results suggest a temporal arrangement of estrogen/ER-mediated genomic signaling cascades with VWR

responses. Blood estradiol levels rise 4 hours after EB injections (**Fig. 7B**) yet changes in VWR behavior and morphological changes in vaginal epithelial cells do not occur until 24 hours later (**Fig. 7DE**). Thus, while the molecular responses to EB at 4 hours are not sufficient to induce VWR, their downstream events are. This specific time window associated with the estrogen causality VWR model has the potential to guide future research to isolate estrogen-mediated molecular events that drive changes in running behavior, independent of the influence of running behaviors themselves. Indeed, an estrogen-sensitive node has been identified in which melanocortin-4 receptor (MC4R), a direct transcriptional target of ERα, modulates spontaneous physical activity in female mice following EB treatment (35). Many other transcriptional targets of ERα have been identified in the brain (54), providing a unique opportunity for future research to further explore these targets and their possible involvement in central mechanisms regulating VWR activity.

The metabolism dynamics of 17beta-estradiol 3-benzoate (EB), used in the estrogen replacement model, also contribute to the time window discussed above. EB is a synthetic derivative of estradiol, the main endogenous female sex hormone. The structural difference between estradiol and EB lies in the addition of a benzoate ester group to estradiol. The ester group is attached to the hydroxyl group at the 17-beta position of estradiol (PubChem, n.d. https://pubchem.ncbi.nlm.nih.gov/compound/222757). This modification increases the lipophilicity of estradiol by increasing its resistance to first pass metabolism and allowing for longer result duration when administered subcutaneously (55). After subcutaneous injection, EB enters the bloodstream, where it rapidly undergoes hydrolyzation into estradiol by esterases (PubChem, n.d.). This rapid hydrolyzation is likely why we found a significant increase in serum estradiol levels 4 hours after EB treatment (**Fig. 7B**). However, over 24 hours after EB treatment, serum estradiol levels were no longer significantly higher than controls, which is likely due to the rapid metabolization of estradiol. This process occurs primarily in the liver and intestine and results in a later excretion in the urine (56). In addition to lower average serum estradiol, we observed a wider range of levels between individuals compared to just 4 hours after treatment (4 hours: 12.6 – 15.6pg/mL, 24+ hours: 6.5 – 19.3pg/mL). This difference suggests that metabolism rates vary among individual rats. Overall, the extended time required to metabolize EB is a plausible explanation for the delayed behavior manifestation following estrogen replacement.

A previous study exploring individual differences in voluntary wheel running in rodents suggests that variation in running distances is mostly attributable to differences in running speed rather than duration (15). Rodents are nocturnal and thus are most active during the night, but what differs is the intensity of the activity during this period. For example, mice that were selectively bred for increased voluntary wheel running distance accomplished the increase in running mainly by running faster rather than more time per day. Similarly, we found that the estradiol replacement also increased the total distance run mainly by increasing running speed rather than minutes per day. Taken together with the selective breeding studies, this result further supports the idea that estradiol influenced motivational circuits to regulate individual differences in wheel-running behavior in female rats.

Our results suggest that estrogen acts as a master switch in regulating VWR in female rats. However, we observed that running activity evoked by a single injection of EB in the OVX background (**Fig. 5A, 7A, 8C&D**) persisted beyond the duration of running peaks observed in

naive female rats under physiological conditions (**Fig. 2A&C**). This observation suggests the involvement of other factors in the regulation of running behavior. Indeed, progesterone is the other major hormone produced by ovaries, and the interplay of estradiol, progesterone, and those factors might be responsible for shaping periodic peaks of running activity in female rats. Further investigation into the role of progesterone could provide valuable insights into the complex hormonal and neurobiological mechanisms that underlie the regulation of running activity.

In conclusion, our study provides compelling evidence that individual differences in voluntary wheel-running behavior in female rats are strongly influenced by estradiol signaling. The observed variability in running activity, the impact of estrogen on exercise behavior, and the estrogen replacement model promise to enhance our understanding of the complex nature of voluntary exercise engagement. In future studies, it may be possible to uncover the molecular cascade that begins with estrogen receptor signaling and ends with an alteration in the reward circuit in such a way that motivates voluntary exercise. Understanding how to motivate physical activity has important translational value for general wellness, physical and mental health.

Supplemental material

Supplemental Figs. S1-S4: https://doi.org/10.6084/m9.figshare.24952995

Figure Legends

Figure 1. Individual differences in voluntary wheel running (VWR). **(A)** Daily running distances of 10 representative male rats over a 2-week period. **(B)** The average running distance on days 1-4 demonstrated a positive correlation with the average running distance on days 11-14 (n=10, r=0.7714, p=0.0090). **(C)** Daily running distances of 16 representative female rats over a 2-week period. **(D)** Average running distance on days 1-4 demonstrated a positive correlation with average running distance on days 11-14 (n=16, r=0.7999, p=0.0002). **(E)** Circadian rhythm influences VWR activity in both male and female rats. **(F)** Dynamics of running activity taking place during the dark phase, with peak activity occurring during the first 5 hours for both male and female rats (n=16).

Figure 2. Sex difference in VWR behavior. (**A**) Daily distance pattern of female rats. Each line represents an individual rat. Running peaks are artificially aligned (indicated by the dashed lines). (**B**) Average daily running distance by week of male and female rats (Female: week 1 n=54, week 2 n=32, week 3 n=8. Male: week 1 n=17, week 2 n=18, week 3 n= 13; p<0.0001). (**C**) Running pattern of a representative female vs. male rat. Arrows denote running peaks coincident with the proestrus stage. **(D)** Comparison of female and male average daily running distances throughout one estrous cycle. Female rats ran significantly further than males and average running distances peaked during the proestrus stage (Female: n=16, male: n=16; p=0.0014). **(E)** Vaginal epithelium samples denoting each stage of the estrous cycle.

Figure 3. Individual differences in VWR speed, distance, and time. **(A)** Correlational analysis between 4-day average running distance and 4-day average running speed of intact female rats (n=16, r=0.9443, p<0.0001). **(B)** Correlational analysis between 4-day average running distance and 4-day average running time of intact female rats (n=16, r=0.6384, p=0.0078). **(C)** Correlational analysis between 4-day average running speed and 4-day average running time of intact female rats (n=16, r=0.3647, p=0.1649). **(D, E, F)** Degree of the synchronicity occurring between daily running speed, distance, and time with the stages of the estrous cycle in high, middle, and low runners (high: n=4, middle: n=8, low: n=4).

Figure 4. Ovariectomy (OVX) induces behavioral changes in VWR. **(A)** Longitudinal representation of VWR activity of 4 representative female rats pre- and post-OVX. **(B)** OVX resulted in a significant decrease in average running distance (Sham: n=9, p=0.5817; OVX: n=20, p<0.0001). **(C)** Individual representations of OVX induced a significant decrease in VWR activity (n=20, p<0.0001). **(D)** Individual representations of sham rats running activity before and after surgery (n=9, p=0.3830). **(E)** Comparison of 4-day average running time pre- and post-OVX (n=20, p<0.0001). **(F)** Comparison of 4-day average running speed pre- and post-OVX (n=20, p<0.0001). **(G)** Correlational analysis of post-OVX 4-day average running distance and speed (n=20, r=0.9212, p<0.0001). **(H)** Correlational analysis of post-OVX 4-day average running distance and running time (n=20, r=0.9134, p<0.0001).

Figure 5. Estrogen replacement reinstates female voluntary running activity. (**A**) Running activity of representative high, medium, and low-performance female rats before and after OVX, as well as estrogen replacement. Arrows denote acute estradiol benzoate (EB) supply (1.5 μg EB in 0.1mL sesame oil) to an OVX background via subcutaneous (s.c.) injections evoked running

peaks. (**B**) OVX significantly diminished the running level, and EB replacement via s.c. injection rescued the running to the pre-OVX level (pre OVX vs post OVX: $p<0.0001$, post OVX vs EB peak: $p=0.0001$, pre OVX vs EB peak: $p=0.1609$; $n=16$). (**C**) Positive correlation between EB-evoked responses (peak value) and the running activity prior to OVX (4-day average) ($n=16$, $r=0.7418$, $p=0.0010$). (**D**) OVX significantly diminished the running level, and E2 replacement via an osmotic pump significantly increased the running level (pre OVX vs post OVX: $p=0.0002$, post OVX vs mini-pump: $p=0.0009$, pre OVX vs mini pump: $p=0.0102$; $n=8$). (**E**) Positive correlation between mini-pump evoked responses (4-day average) and physiological running activity prior to OVX (4-day average) ($n=8$, $r=0.7724$, $p=0.0247$).

Figure 6. Contribution of running speed and time to estrogen evoked running. Correlational analyses of **(A)** 4-day average speed pre-OVX vs. OVX+EB ($n=6$, $r=0.9802$, $p=0.0006$), **(B)** 4-day average running time pre-OVX vs. OVX+EB ($n=6$, $r=0.4974$, $p=0.3154$), **(C)** 4-day average speed vs. distance induced by EB ($n=6$, $r=9854$, $p=0.0003$), **(D)** 4-day average running time vs. distance induced by EB ($n=6$, $r=0.7304$, $p=0.9993$), **(E)** 4-day average speed before and after EB treatment ($n=6$, $r=0.9727$, $p=0.0011$), **(F)** 4-day average running time before and after EB treatment ($n=6$, $r=0.8714$, $p=0.0237$).

Figure 7. Dynamics of EB-induced physiological and behavioral changes. (**A**) Representative running response to subcutaneous injection of 1.5 µg EB in OVX female rats. The arrow denotes the timing of an injection. (**B**) Total serum levels of estradiol (E2) at 4 vs. 24+ hours post-EB treatment measured by ELISA (enzyme-linked immunoassay) (4 hours EB: $n=4$, vehicle: $n=4$, $p=0.0380$; 24+ hours EB: $n=6$, vehicle: $n=4$, $p=0.0803$). (**C**) Representative vaginal epithelial cytology of an OVX rat. High neutrophil concentration and few, if any, nucleated epithelial cells. (**D**) Vaginal epithelial cytology representative of an OVX rat 4 hours after EB treatment. No evident change in cellularity from the previous sample. (**E**) Vaginal epithelial cytology representative of an OVX rat 24+ hours after EB treatment. Samples at this time point exhibited similarity to proestrus in intact females with primarily nucleated epithelial cells present in clumps.

Figure 8. (Lack of) effects of wheel exposure prior to OVX on subsequent VWR performance **(A)** Post-OVX daily running distances of rats with wheel running experience prior to OVX (pre-run group) and rats with no wheel running experience prior to OVX (no pre-run) (pre-run: $n=6$, no pre-run: $n=6$). **(B)** The average daily running distances of the last 4 days of post-OVX running were compared between the pre-run group and no pre-run group. There was no significant difference in daily running distances between the groups ($p=0.7263$). **(C)** Post-OVX daily running distances of the pre-run group. The arrow denotes the time of subcutaneous estradiol benzoate (EB) injection of 1.5 µg. Rats responded to the injection with increased running for 3-4 days ($n=6$) **(D)** Post OVX daily running distances of the no pre-run group. Arrow denotes the time of EB injection. Rats responded to the injection with a similar increase in running for 4-6 days ($n=6$).

Acknowledgments

We thank Drs. Jacob Meyer and Maria Barnes for their insightful discussions throughout this project. We would also like to express our appreciation to Lori Winter and Kristin Tefft for their excellent technical support. This project was supported by funding from the Iowa Osteopathic Education and Research (IOER) Foundation. Additionally, we acknowledge the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core, supported by the Eunice Kennedy Shriver NICHD/NIH Grant R24HD102061, for their assistance.

Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Research design: L-LY, VM, and SC. Conducted experiments: VM, LP, BP, C-M L, MM, SC, and L-LY. Data analysis: VM, LP, and L-L Y. Figure design and editing: VM and L-L Y. Manuscript preparation and editing: L-LY, VM, JSR, LP, and PC. All authors have given final approval for the version to be published.

References

1. **Dishman RK, Berthoud HR, Booth FW, Cotman CW, Edgerton VR, Fleshner MR, Gandevia SC, Gomez-Pinilla F, Greenwood BN, Hillman CH, Kramer AF, Levin BE, Moran TH, Russo-Neustadt AA, Salamone JD, Van Hoomissen JD, Wade CE, York DA, and Zigmond MJ**. Neurobiology of exercise. *Obesity (Silver Spring)* 14: 345-356, 2006.
2. **Matta Mello Portugal E, Cevada T, Sobral Monteiro-Junior R, Teixeira Guimaraes T, da Cruz Rubini E, Lattari E, Blois C, and Camaz Deslandes A**. Neuroscience of exercise: from neurobiology mechanisms to mental health. *Neuropsychobiology* 68: 1-14, 2013.
3. **Ding D, Lawson KD, Kolbe-Alexander TL, Finkelstein EA, Katzmarzyk PT, van Mechelen W, Pratt M, and Lancet Physical Activity Series 2 Executive C**. The economic burden of physical inactivity: a global analysis of major non-communicable diseases. *Lancet* 388: 1311-1324, 2016.
4. **Booth FW, Roberts CK, Thyfault JP, Ruegsegger GN, and Toedebusch RG**. Role of Inactivity in Chronic Diseases: Evolutionary Insight and Pathophysiological Mechanisms. *Physiol Rev* 97: 1351-1402, 2017.
5. **Sparks LM**. Exercise training response heterogeneity: physiological and molecular insights. *Diabetologia* 60: 2329-2336, 2017.
6. **Knab AM, Bowen RS, Moore-Harrison T, Hamilton AT, Turner MJ, and Lightfoot JT**. Repeatability of exercise behaviors in mice. *Physiol Behav* 98: 433-440, 2009.
7. **Friedman WA, Garland T, Jr., and Dohm MR**. Individual variation in locomotor behavior and maximal oxygen consumption in mice. *Physiol Behav* 52: 97-104, 1992.
8. **Lambert MI, and Noakes TD**. Spontaneous running increases VO2max and running performance in rats. *J Appl Physiol (1985)* 68: 400-403, 1990.
9. **Novak CM, Burghardt PR, and Levine JA**. The use of a running wheel to measure activity in rodents: relationship to energy balance, general activity, and reward. *Neurosci Biobehav Rev* 36: 1001-1014, 2012.
10. **Richter SH, Gass P, and Fuss J**. Resting Is Rusting: A Critical View on Rodent Wheel-Running Behavior. *Neuroscientist* 20: 313-325, 2014.
11. **Rodnick KJ, Reaven GM, Haskell WL, Sims CR, and Mondon CE**. Variations in running activity and enzymatic adaptations in voluntary running rats. *J Appl Physiol (1985)* 66: 1250-1257, 1989.
12. **Meijer JH, and Robbers Y**. Wheel running in the wild. *Proc Biol Sci* 281: 2014.
13. **Greenwood BN, Foley TE, Le TV, Strong PV, Loughridge AB, Day HE, and Fleshner M**. Long-term voluntary wheel running is rewarding and produces plasticity in the mesolimbic reward pathway. *Behav Brain Res* 217: 354-362, 2011.
14. **Rhodes JS, Garland T, Jr., and Gammie SC**. Patterns of brain activity associated with variation in voluntary wheel-running behavior. *Behav Neurosci* 117: 1243-1256, 2003.
15. **Rhodes JS, Gammie SC, and Garland T, Jr.** Neurobiology of Mice Selected for High Voluntary Wheel-running Activity. *Integr Comp Biol* 45: 438-455, 2005.
16. **Saul MC, Majdak P, Perez S, Reilly M, Garland T, Jr., and Rhodes JS**. High motivation for exercise is associated with altered chromatin regulators of monoamine receptor gene expression in the striatum of selectively bred mice. *Genes Brain Behav* 16: 328-341, 2017.
17. **Heijnen S, Hommel B, Kibele A, and Colzato LS**. Neuromodulation of Aerobic Exercise-A Review. *Front Psychol* 6: 1890, 2015.

18. **Basso JC, and Suzuki WA**. The Effects of Acute Exercise on Mood, Cognition, Neurophysiology, and Neurochemical Pathways: A Review. *Brain Plast* 2: 127-152, 2017.
19. **Lightfoot JT, EJC DEG, Booth FW, Bray MS, M DENH, Kaprio J, Kelly SA, Pomp D, Saul MC, Thomis MA, Garland T, Jr., and Bouchard C**. Biological/Genetic Regulation of Physical Activity Level: Consensus from GenBioPAC. *Med Sci Sports Exerc* 50: 863-873, 2018.
20. **de Geus EJ, Bartels M, Kaprio J, Lightfoot JT, and Thomis M**. Genetics of regular exercise and sedentary behaviors. *Twin Res Hum Genet* 17: 262-271, 2014.
21. **Lightfoot JT, Turner MJ, Daves M, Vordermark A, and Kleeberger SR**. Genetic influence on daily wheel running activity level. *Physiol Genomics* 19: 270-276, 2004.
22. **Turner MJ, Kleeberger SR, and Lightfoot JT**. Influence of genetic background on daily running-wheel activity differs with aging. *Physiol Genomics* 22: 76-85, 2005.
23. **Swallow JG, Carter PA, and Garland T, Jr.** Artificial selection for increased wheel-running behavior in house mice. *Behav Genet* 28: 227-237, 1998.
24. **Meek TH, Lonquich BP, Hannon RM, and Garland T, Jr.** Endurance capacity of mice selectively bred for high voluntary wheel running. *J Exp Biol* 212: 2908-2917, 2009.
25. **Fuss J, Steinle J, Bindila L, Auer MK, Kirchherr H, Lutz B, and Gass P**. A runner's high depends on cannabinoid receptors in mice. *Proc Natl Acad Sci U S A* 112: 13105-13108, 2015.
26. **Dohnalová L, Lundgren P, Carty JRE, Goldstein N, Wenski SL, Nanudorn P, Thiengmag S, Huang K-P, Litichevskiy L, Descamps HC, Chellappa K, Glassman A, Kessler S, Kim J, Cox TO, Dmitrieva-Posocco O, Wong AC, Allman EL, Ghosh S, Sharma N, Sengupta K, Cornes B, Dean N, Churchill GA, Khurana TS, Sellmyer MA, FitzGerald GA, Patterson AD, Baur JA, Alhadeff AL, Helfrich EJN, Levy M, Betley JN, and Thaiss CA**. A microbiome-dependent gut–brain pathway regulates motivation for exercise. *Nature* 612: 739-747, 2022.
27. **Jones LC, Bellingham WP, and Ward LC**. Sex differences in voluntary locomotor activity of food-restricted and ad libitum-fed rats. Implications for the maintenance of a body weight set-point. *Comp Biochem Physiol A Comp Physiol* 96: 287-290, 1990.
28. **Konhilas JP, Chen H, Luczak E, McKee LA, Regan J, Watson PA, Stauffer BL, Khalpey ZI, McKinsey TA, Horn T, LaFleur B, and Leinwand LA**. Diet and sex modify exercise and cardiac adaptation in the mouse. *Am J Physiol Heart Circ Physiol* 308: H135-145, 2015.
29. **Lightfoot JT**. Sex hormones' regulation of rodent physical activity: a review. *Int J Biol Sci* 4: 126-132, 2008.
30. **Rosenfeld CS**. Sex-dependent differences in voluntary physical activity. *J Neurosci Res* 95: 279-290, 2017.
31. **Gorzek JF, Hendrickson KC, Forstner JP, Rixen JL, Moran AL, and Lowe DA**. Estradiol and tamoxifen reverse ovariectomy-induced physical inactivity in mice. *Med Sci Sports Exerc* 39: 248-256, 2007.
32. **Ogawa S, Chan J, Gustafsson JA, Korach KS, and Pfaff DW**. Estrogen increases locomotor activity in mice through estrogen receptor alpha: specificity for the type of activity. *Endocrinology* 144: 230-239, 2003.
33. **Espinosa E, and Curtis KS**. Increased locomotor activity in estrogen-treated ovariectomized rats is associated with nucleus accumbens dopamine and is not reduced by dietary sodium deprivation. *Integr Zool* 13: 783-794, 2018.

34. **Garey J, Morgan MA, Frohlich J, McEwen BS, and Pfaff DW**. Effects of the phytoestrogen coumestrol on locomotor and fear-related behaviors in female mice. *Horm Behav* 40: 65-76, 2001.
35. **Krause WC, Rodriguez R, Gegenhuber B, Matharu N, Rodriguez AN, Padilla-Roger AM, Toma K, Herber CB, Correa SM, Duan X, Ahituv N, Tollkuhn J, and Ingraham HA**. Oestrogen engages brain MC4R signalling to drive physical activity in female mice. *Nature* 599: 131-135, 2021.
36. **Krentzel AA, Proano S, Patisaul HB, and Meitzen J**. Temporal and bidirectional influences of estradiol on voluntary wheel running in adult female and male rats. *Horm Behav* 120: 104694, 2020.
37. **Muller ST, Keiler AM, Kraker K, Zierau O, and Bernhardt R**. Influence of estrogen on individual exercise motivation and bone protection in ovariectomized rats. *Lab Anim* 52: 479-489, 2018.
38. **Yoest KE, Cummings JA, and Becker JB**. Estradiol, dopamine and motivation. *Cent Nerv Syst Agents Med Chem* 14: 83-89, 2014.
39. **Kokane SS, and Perrotti LI**. Sex Differences and the Role of Estradiol in Mesolimbic Reward Circuits and Vulnerability to Cocaine and Opiate Addiction. *Front Behav Neurosci* 14: 74, 2020.
40. **Knowlton BJ, and Patterson TK**. Habit Formation and the Striatum. *Curr Top Behav Neurosci* 37: 275-295, 2018.
41. **Lipton DM, Gonzales BJ, and Citri A**. Dorsal Striatal Circuits for Habits, Compulsions and Addictions. *Front Syst Neurosci* 13: 28, 2019.
42. **Yoest KE, Quigley JA, and Becker JB**. Rapid effects of ovarian hormones in dorsal striatum and nucleus accumbens. *Horm Behav* 104: 119-129, 2018.
43. **Cora MC, Kooistra L, and Travlos G**. Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. *Toxicol Pathol* 43: 776-793, 2015.
44. **Yamanaka Y, Honma S, and Honma K**. Daily exposure to a running wheel entrains circadian rhythms in mice in parallel with development of an increase in spontaneous movement prior to running-wheel access. *Am J Physiol Regul Integr Comp Physiol* 305: R1367-1375, 2013.
45. **Greenwood BN, and Fleshner M**. Voluntary Wheel Running: A Useful Rodent Model for Investigating the Mechanisms of Stress Robustness and Neural Circuits of Exercise Motivation. *Curr Opin Behav Sci* 28: 78-84, 2019.
46. **Lee HR, Kim TH, and Choi KC**. Functions and physiological roles of two types of estrogen receptors, ERalpha and ERbeta, identified by estrogen receptor knockout mouse. *Lab Anim Res* 28: 71-76, 2012.
47. **Costello E, Kafchinski M, Vrazel J, and Sullivan P**. Motivators, barriers, and beliefs regarding physical activity in an older adult population. *J Geriatr Phys Ther* 34: 138-147, 2011.
48. **Guthold R, Stevens GA, Riley LM, and Bull FC**. Worldwide trends in insufficient physical activity from 2001 to 2016: a pooled analysis of 358 population-based surveys with 1.9 million participants. *Lancet Glob Health* 6: e1077-e1086, 2018.
49. **Labor UDo**. Most common occupations for women in the labor force http://www.dol.gov/agencies/wb/data/occupations/most-common-occupations-women-labor-force.
50. **Stern JJ, and Murphy M**. The effects of thyroxine and estradiol benzoate on wheel running activity in female rats. *Physiol Behav* 9: 79-82, 1972.

51. **Fahrbach SE, Meisel RL, and Pfaff DW**. Preoptic implants of estradiol increase wheel running but not the open field activity of female rats. *Physiol Behav* 35: 985-992, 1985.
52. **Gentry RT, Wade GN, and Roy EJ**. Individual differences in estradiol-induced behaviors and in neural 3H-estradiol uptake in rats. *Physiol Behav* 17: 195-200, 1976.
53. **Vrtacnik P, Ostanek B, Mencej-Bedrac S, and Marc J**. The many faces of estrogen signaling. *Biochem Med (Zagreb)* 24: 329-342, 2014.
54. **Gegenhuber B, Wu MV, Bronstein R, and Tollkuhn J**. Gene regulation by gonadal hormone receptors underlies brain sex differences. *Nature* 606: 153-159, 2022.
55. **Kuhl H**. Pharmacology of estrogens and progestogens: influence of different routes of administration. *Climacteric* 8 Suppl 1: 3-63, 2005.
56. **Tsuchiya Y, Nakajima M, and Yokoi T**. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. *Cancer Lett* 227: 115-124, 2005.

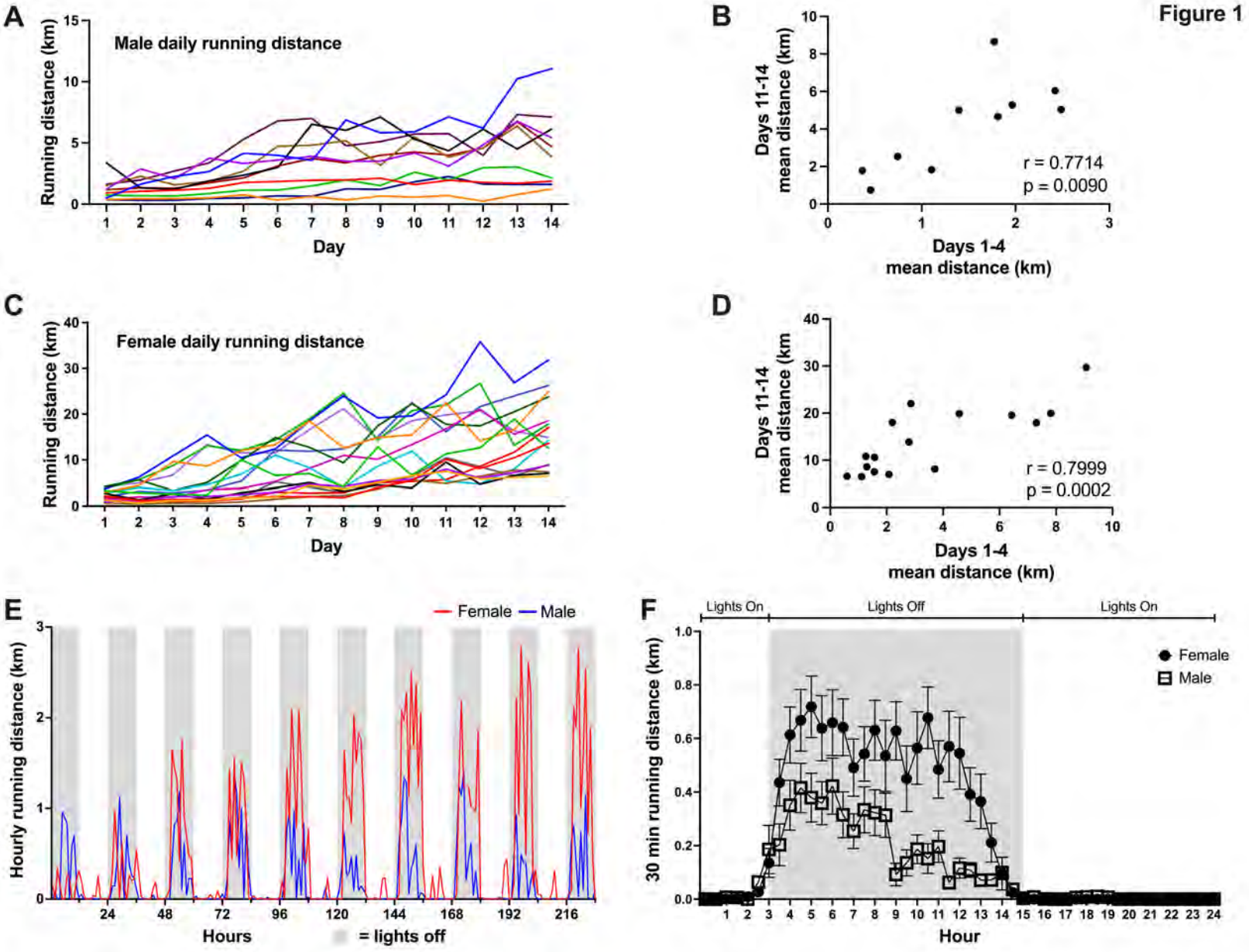
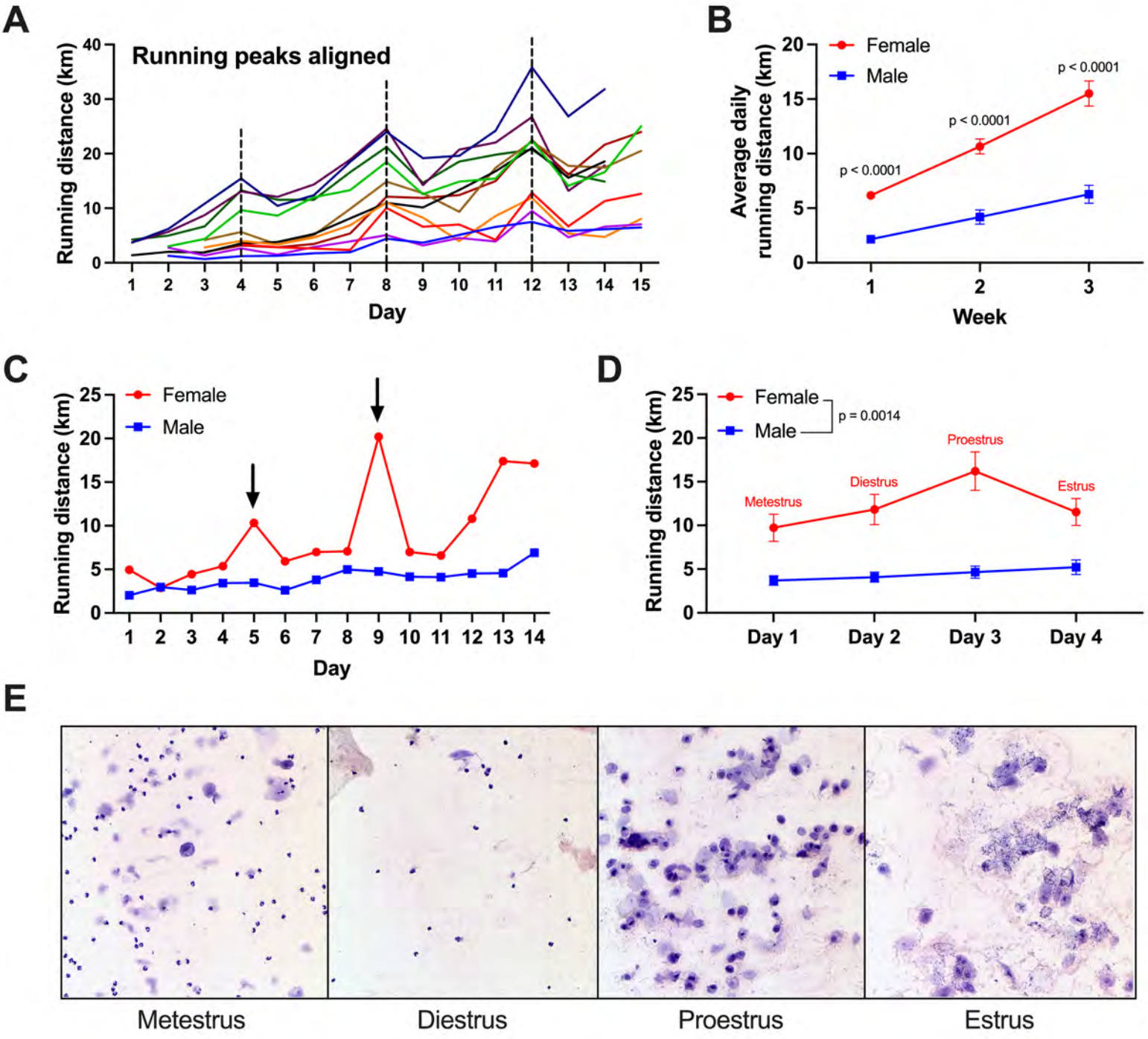
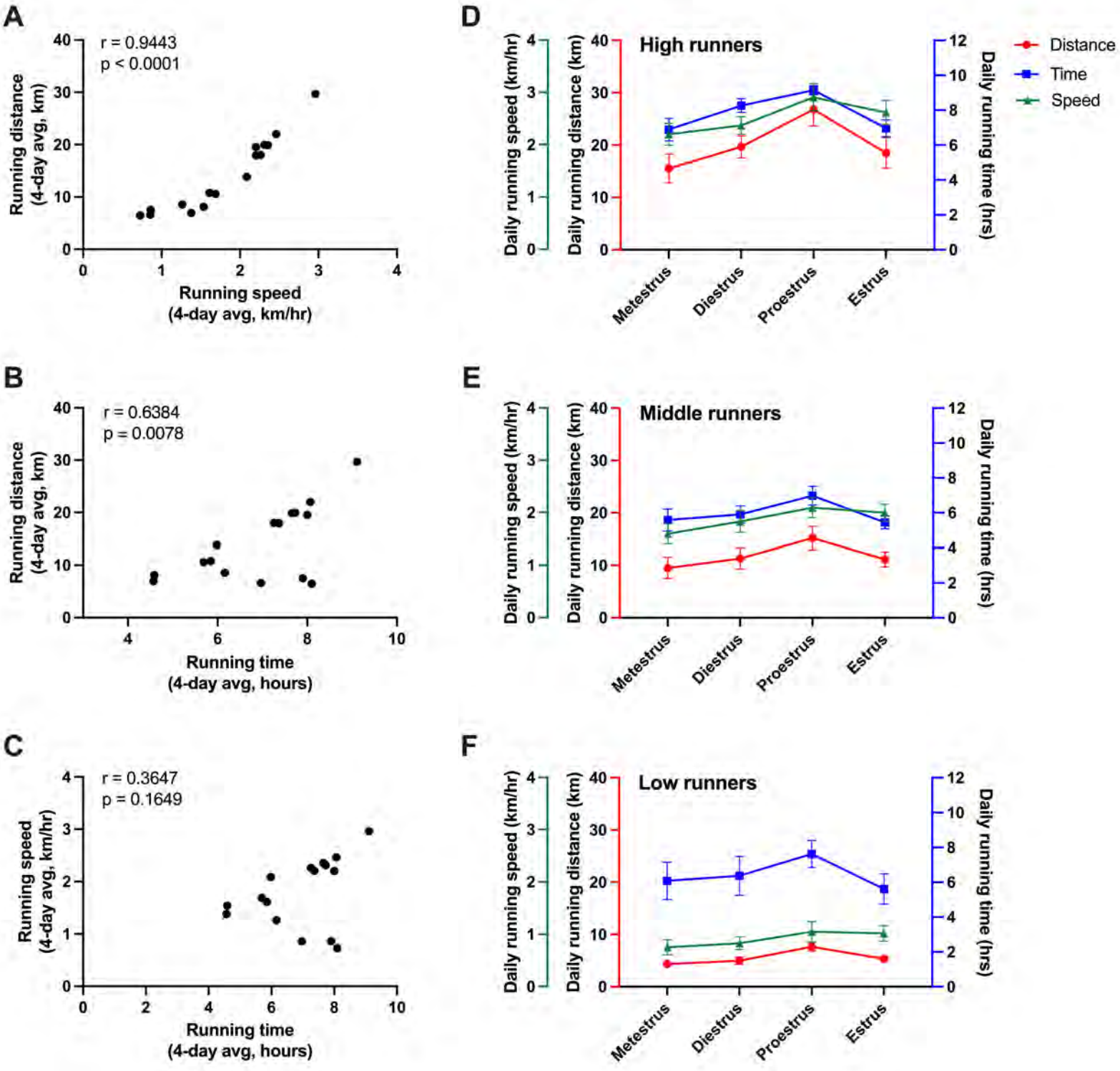


Figure 1

Figure 2





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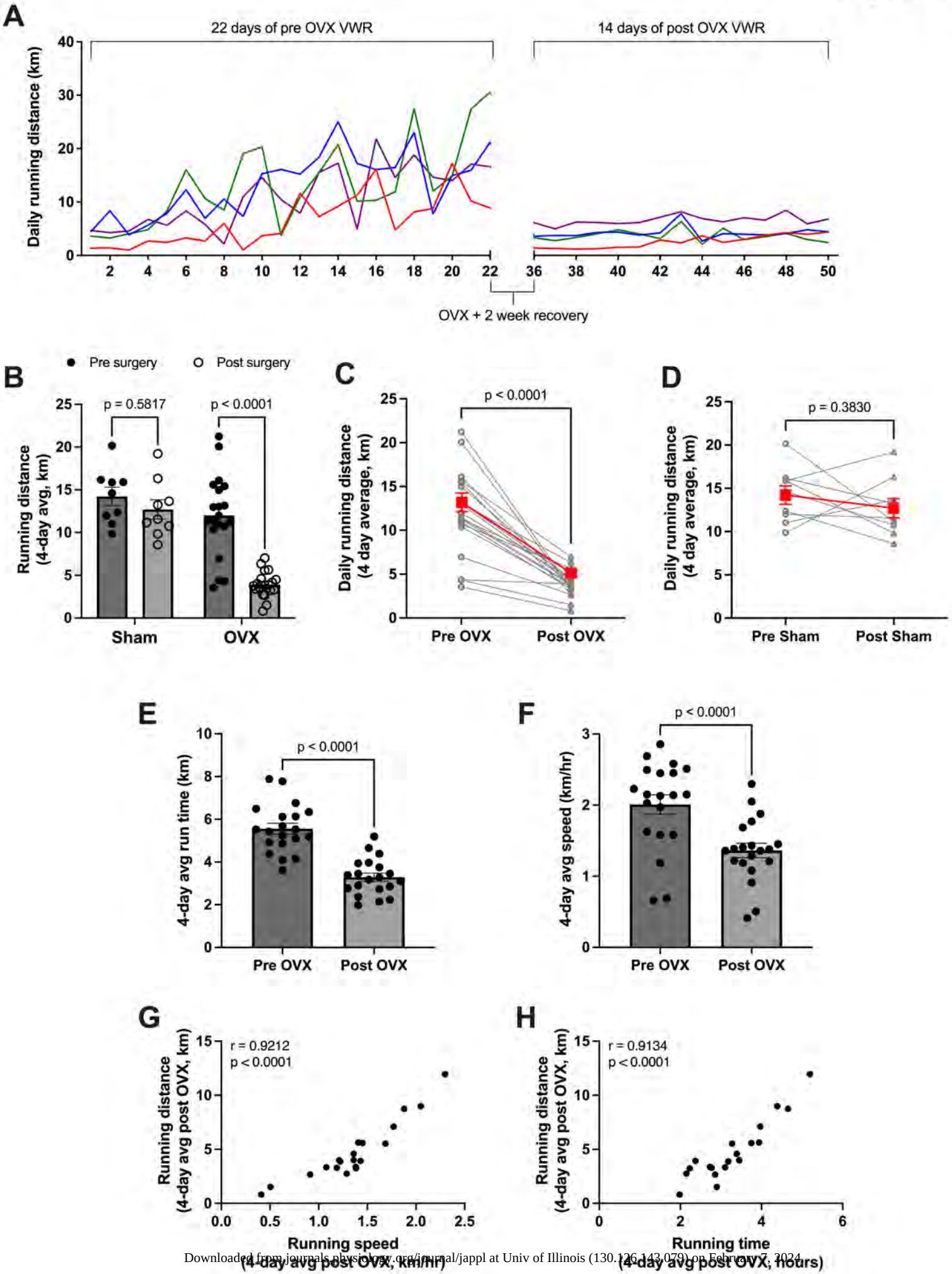


Figure 5

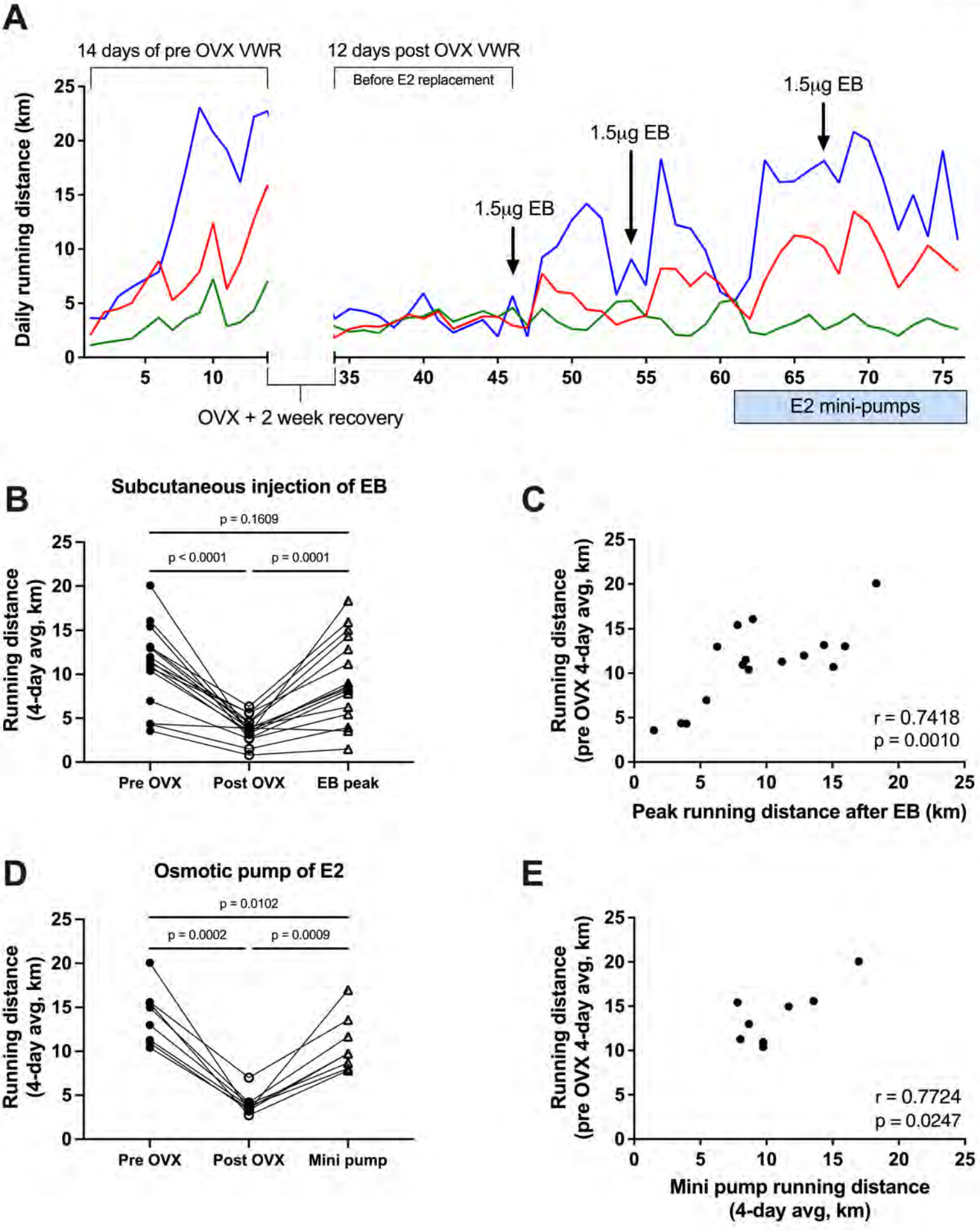


Figure 6

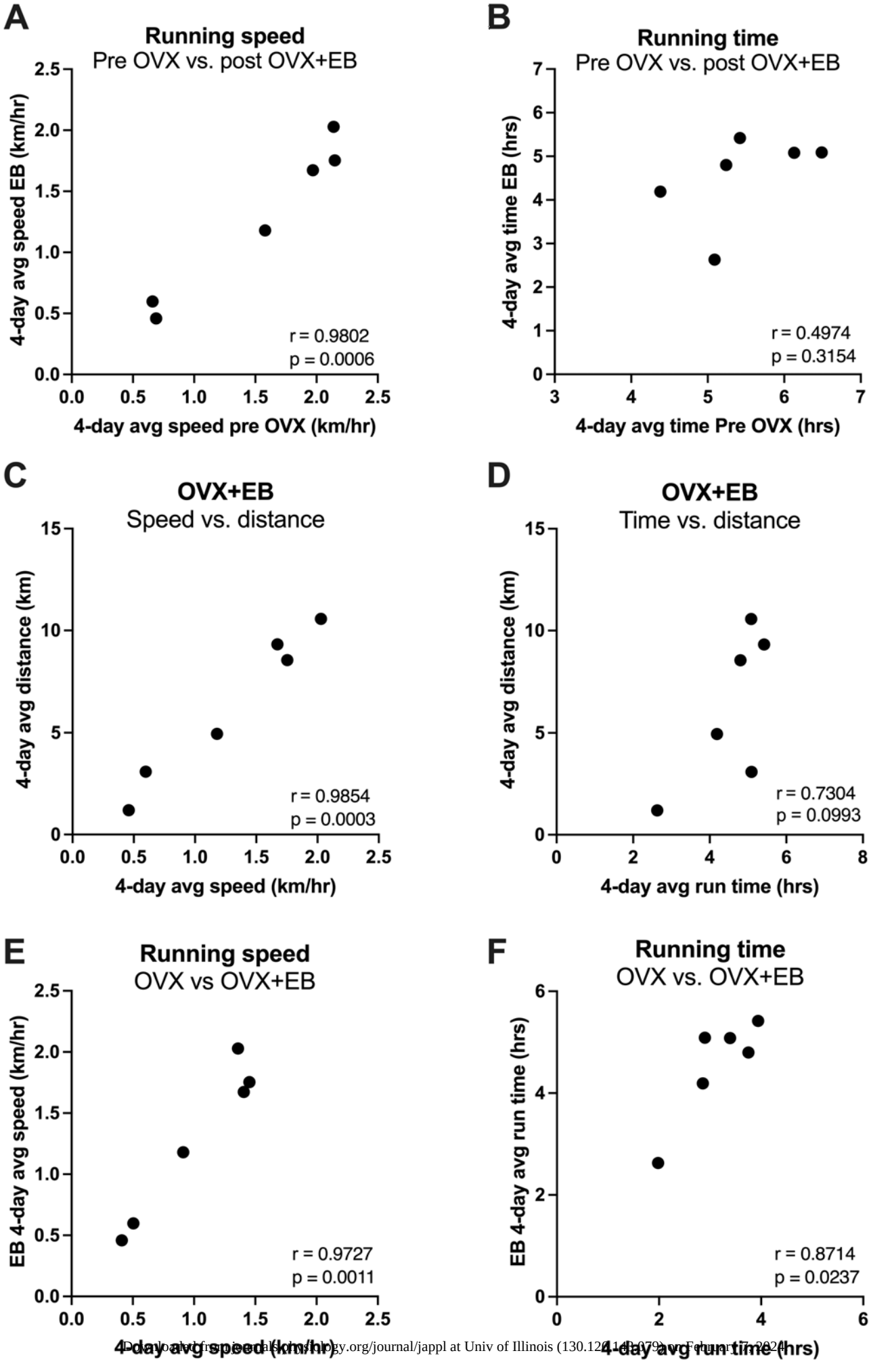


Figure 7

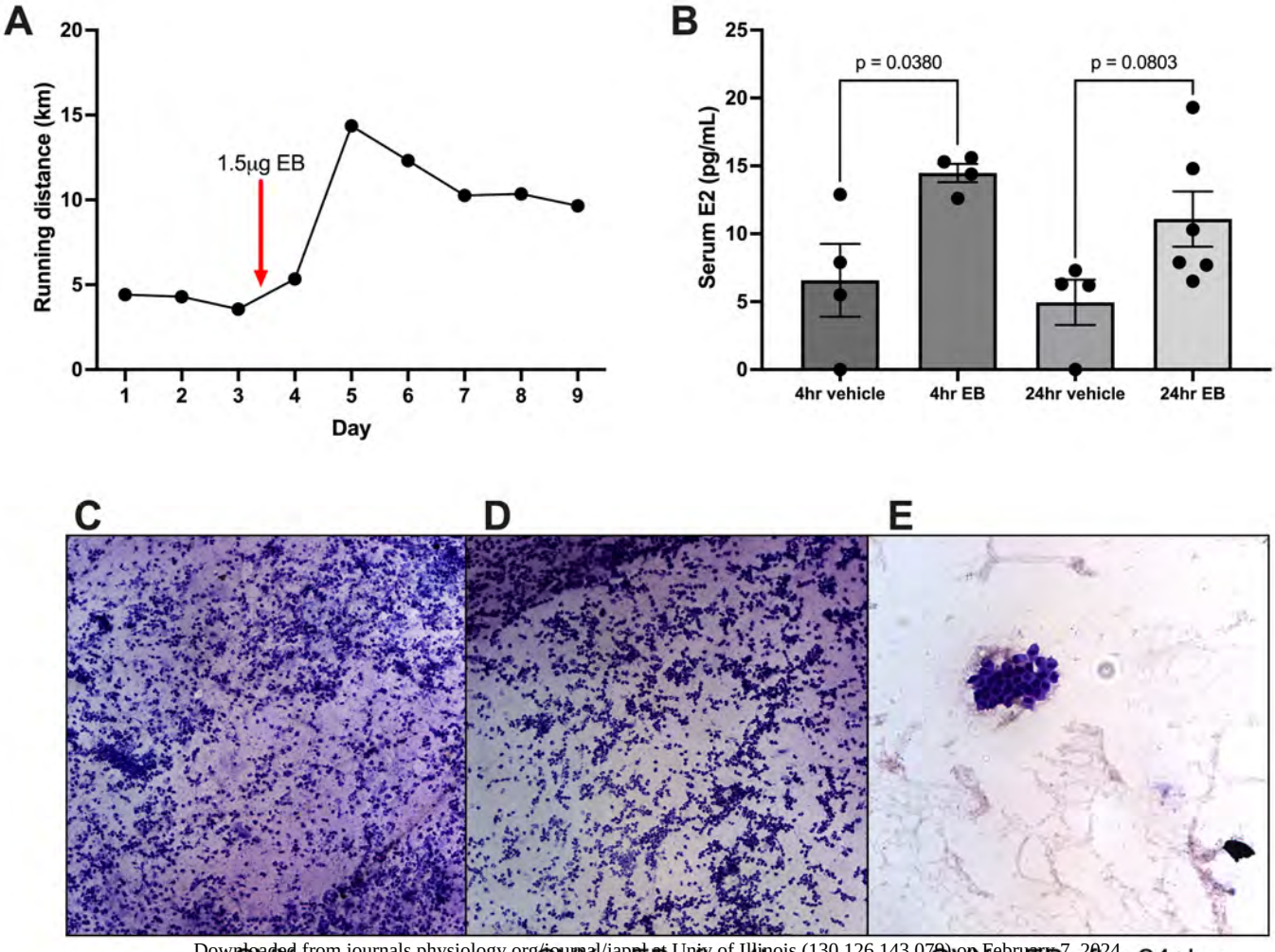
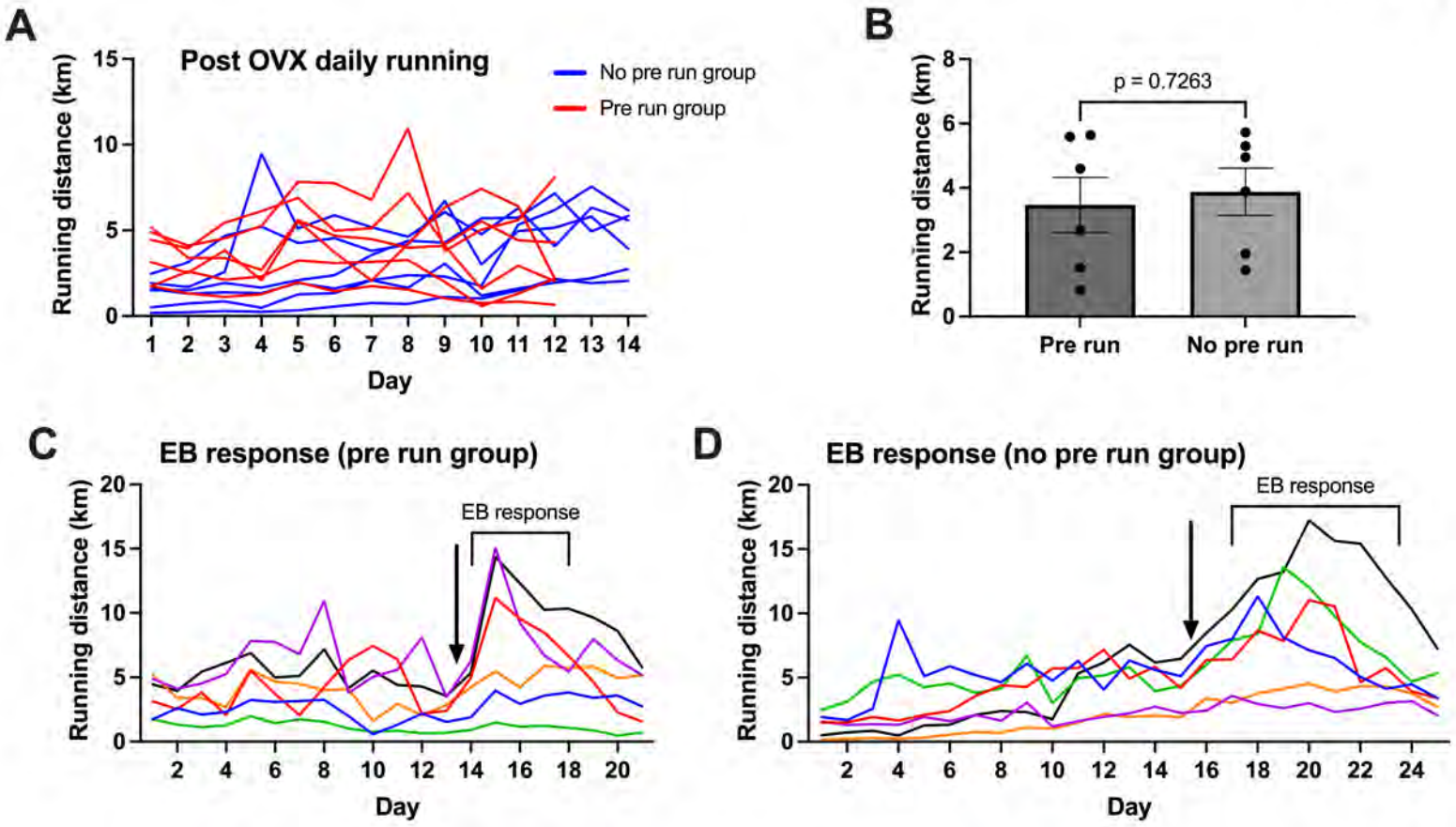
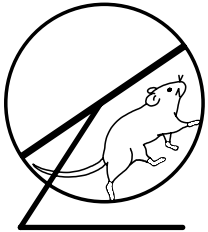


Figure 8

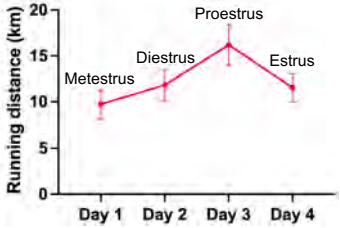


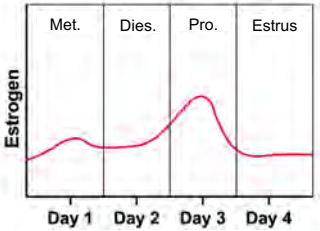
Estrogen-mediated individual differences in female rat voluntary running behavior



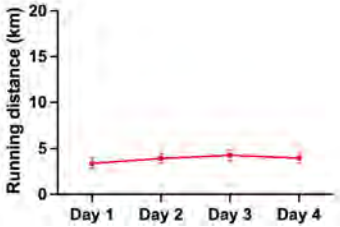
Voluntary wheel running (VWR)

Running peaks when estrogen peaks



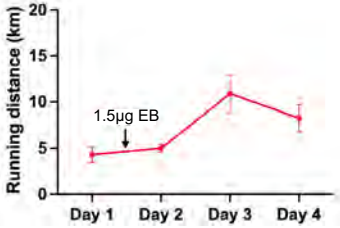


Ovariectomy (OVX) ↓**E2**



↓ speed
↓ duration
↓ distance

Estrogen replacement ↑**E2**



↑ speed
↑ duration
↑ distance

↑E2 = ↑VWR
↓E2 = ↓VWR

Individual differences retained through E2 manipulation

High runner before OVX = high runner after OVX + E2

Low runner before OVX = low runner after OVX + E2