Clock proteins and training modify exercise capacity in a daytime-dependent manner

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Exercise and circadian biology are closely intertwined with physiology and metabolism, yet the functional interaction between circadian clocks and exercise capacity is only partially characterized. Here, we tested different clock mutant mouse models to examine the effect of the circadian clock and clock proteins, namely PERIODs and BMAL1, on exercise capacity. We found that daytime variance in endurance exercise capacity is circadian clock controlled. Unlike wild-type mice, which outperform in the late compared with the early part of their active phase, PERIODs- and BMAL1-null mice do not show daytime variance in exercise capacity. It appears that BMAL1 impairs and PERIODs enhance exercise capacity in a daytime-dependent manner. An analysis of liver and muscle glycogen stores as well as muscle lipid utilization suggested that these daytime effects mostly relate to liver glycogen levels and correspond to the animals' feeding behavior. Furthermore, given that exercise capacity responds to training, we tested the effect of training at different times of the day and found that training in the late compared with the early part of the active phase improves exercise performance. Overall, our findings suggest that clock proteins shape exercise capacity in a daytime-dependent manner through changes in liver glycogen levels, likely due to their effect on animals' feeding behavior.

circadian clocks | exercise | metabolism | training | glycogen

Physical activity elicits an integrative systemic response to the rise in metabolic demands (1, 2). Exercise capacity, therefore, relies on the coordinated physiologic and metabolic response of various tissues (2). Regular exercise, compared with a sedentary lifestyle, carries major health benefits and has been widely recommended as a therapeutic and preventive intervention for a wide variety of pathologies (e.g., obesity and metabolic syndrome) (3–5). Consequently, there is growing interest in exercise biology in general and specifically in its interaction with other processes that govern whole-body physiology and metabolism.

Circadian clocks are present in most cells of the body and oscillate with a period of ∼24 h. These clocks coordinate a wide variety of behavioral, physiological, and molecular functions with the geophysical time (6, 7). Disruption of circadian rhythmicity through environmental and genetic perturbations has been implicated in various diseases (8). The molecular clockwork consists of transcription–translation feedback loops generated by "clock components" [e.g., PERIOD (PERs), CRYPTOCHROMEs (CRYs), CLOCK, BMAL1, REV-ERB, and RORs (9, 10)]. These proteins also participate in the regulation of various metabolic pathways alongside their role within the molecular oscillator (11–14).

Growing evidence points toward a reciprocal interaction between exercise and circadian clocks in both mice and humans (15, 16). Scheduled exercise can alter behavioral circadian rhythms (i.e., rest–activity profiles) and affect clock gene expression (17–21). Notably, several studies showed daily variance in exercise performance (22–24) and suggested that some clock components affect exercise capacity (15, 24). Yet our knowledge regarding the role of the circadian clock and specific clock proteins in control of exercise capacity is rudimentary. Furthermore, previous studies in rodents reported that scheduled training has a daytime effect on weight management (25, 26), while the daytime effect of scheduled training on exercise capacity is relatively uncharacterized.

In this study, we employed various clock mutant mouse models in conjunction with different light regimens to characterize the role of the circadian clock and clock proteins in the control of exercise capacity. We show that exercise capacity is circadian clock controlled and is differently regulated by PERs and BMAL1 in a time-dependent manner. An analysis of liver and muscle glycogen stores as well as muscle lipid utilization suggested that these daytime effects mostly relate to liver glycogen levels. In addition, using time-controlled running wheels, we show that scheduled training exhibits a daytime effect on exercise capacity. In summary, we propose that exercise capacity is circadian clock controlled and that clock proteins shape exercise capacity in a daytime-dependent manner through changes in liver glycogen levels, likely due to their effect on feeding behavior.

Results

Daytime Difference in Exercise Capacity Is Sex Independent. Recent findings from both human and animal models point toward sexual dimorphism in daily rhythms and circadian clocks (27). In

Significance

Studies in mice and humans have revealed daytime variance in exercise performance, yet it is still unclear whether and how these daytime differences are regulated by the circadian clock. Here, we show that exercise performance is circadian clock controlled and that specific clock proteins modify exercise capacity in a time-dependent manner, likely through differences in food consumption and liver glycogen stores. We also demonstrate that the time of day at which training is performed plays a role in improving exercise capacity. Our study suggests that exercise capacity is shaped by clock proteins and training in a daytime-dependent manner.

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addition, it is well documented that females differ from males in various physiologic and metabolic parameters under basal conditions as well as in response to metabolic challenges (28–31). Hence, we examined the exercise capacity of males versus females in conjunction with time of day. Since exercise is typically performed during the active phase, and mice are nocturnal animals, we examined the difference in exercise capacity at ZT (Zeitgeber Time) 14 and ZT22, which correspond to the early and late parts of their active phase, respectively (hereafter termed as Early and Late groups) (Fig. 1*A*). The exercise capacity of sedentary C57BL/6 male mice was tested in response to a 5.5-h moderate-intensity treadmill test, as previously described (32) and detailed in *Materials and Methods*. In agreement with our previous work (24), the Late group outperformed the Early group, maintaining higher blood glucose levels (i.e., above 70 mg/dL) and running for a longer duration (Fig. 1*B*). This daytime difference in exercise capacity was preserved even with a 1-h prerun food deprivation (*SI Appendix*, Fig. S1) as a means to impersonate nutrient control as is often done in humans (33, 34). Similar to males, in females, the Late group outperformed the Early group (Fig. 1*C*). Females showed lower blood glucose levels compared with males at rest (115 versus 140 mg/dL; Student's *t* test $P < 0.01$), consistent with previous reports that showed sex differences for glycemic parameters (35, 36), and overall performed less well than males. The female Early group reached blood glucose levels below 70 mg/dL already after 1.5 h, whereas the male Early group was able to sustain blood glucose levels above 70 mg/dL up to 3.5 h (Student's *t* test $P < 0.05$). Both males and females from the Late group were able to maintain blood glucose levels above 70 mg/dL throughout a 5.5-h run, yet females had lower blood glucose levels compared with males throughout the run (repeated measures two-way ANOVA: sex effect $P < 0.01$).

We also analyzed behavioral and physiologic parameters prior to the treadmill protocol (*SI Appendix*, Figs. S2*A* and S3*A* and Fig. 1*D*), as these factors are implicated in exercise performance and are indicative of the basal conditions at the start of the run (37). The respiratory exchange rate (RER), which is indicative of the nutrient selection as an energy source (i.e., lipids versus carbohydrates), was higher in females compared with males, in line with a previous report (38), yet it did not show daytime difference (*SI Appendix*, Fig. S2*A*). Total daily food intake per body weight was higher in females compared with males, as expected from their smaller body size (*SI Appendix*, Fig. S3*A*). We did observe differences in food consumption per body weight 12 h prior to the run between the Early and Late group both in males and females (Fig. 1*D*), which was related to higher liver

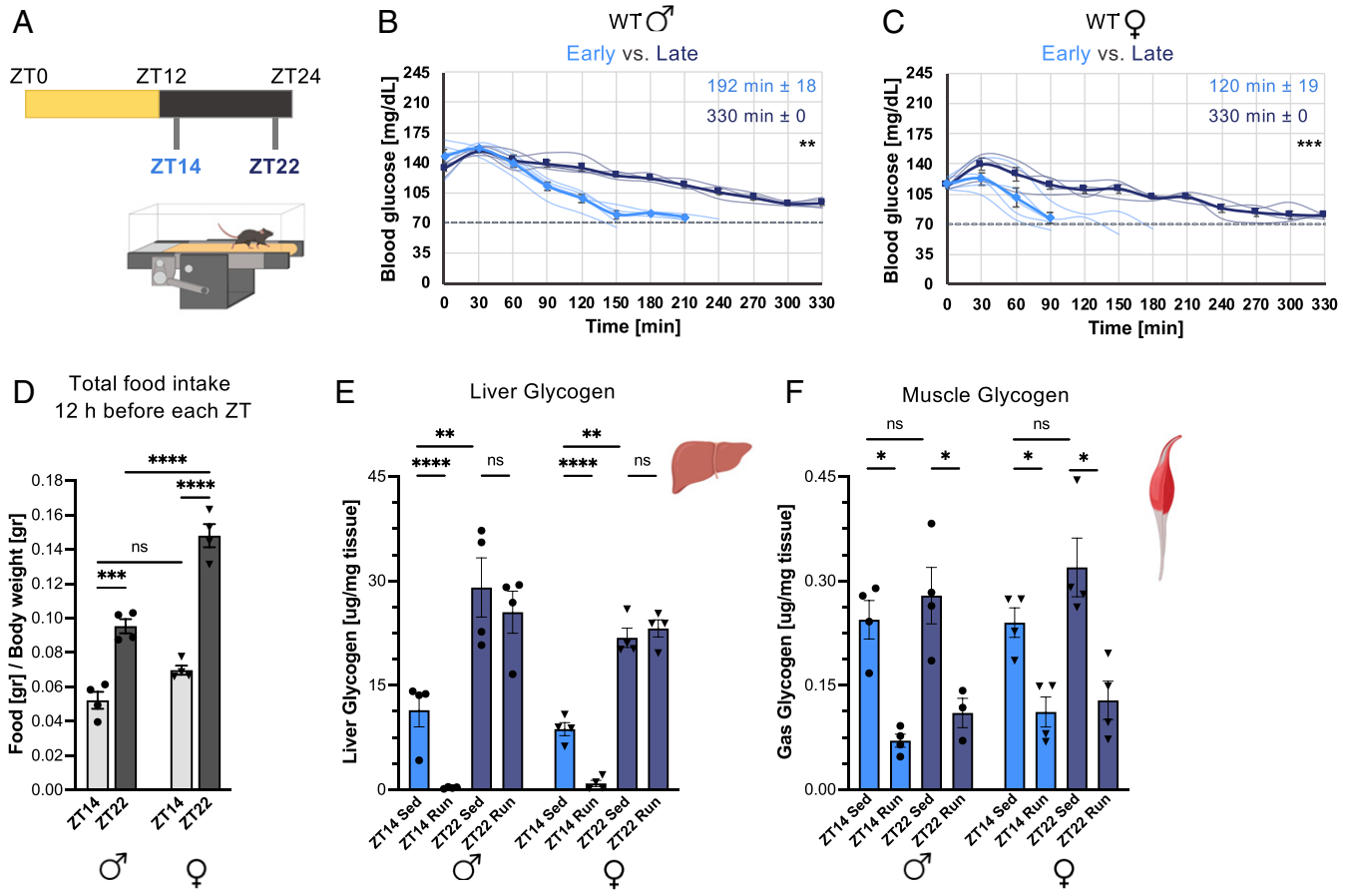

Fig. 1. Male and female mice show a daytime difference in their exercise capacity. Mice were housed under a 12-h light–12-h dark regimen, and a moderate-intensity treadmill test was performed either at the Early (ZT14) or Late (ZT22) part of the active phase. (*A*) Schematic representation of the experimental design. (*B* and *C*) Blood glucose profiles of individual WT mice during the test (thin lines) and mean ± SEM (thick line) for each group for males (Early, $n = 5$; Late, $n = 4$) and females (Early, $n = 5$; Late, $n = 4$). End point for each group, at which either blood glucose levels reached ≤70 mg/dL or the animal finished the 5.5-h test, is presented as average time ± SEM. (*D*) Food intake was monitored using metabolic cages. Data are presented as mean ± SEM of average food consumed during 12 h per body weight either before ZT14 or ZT22 from an average of two consecutive days of four male and four female mice. See also *SI Appendix*, Fig. S3*A*. (*E* and *F*) Liver and gastrocnemius (Gas) glycogen content ($n = 4$ per group) of Early (ZT14) and Late (ZT22) sedentary (Sed) mice or after a 90-min run (Run). Student's *t* test (*B* and *C*); two-way ANOVA with Tukey's post hoc test (*D*); three-way ANOVA with Tukey's post hoc test (*E* and *F*; for panel *E*, test was done on log-transformed values); ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, nonsignificant (ns).

glycogen content in the Late compared with the Early group in both sexes (Fig. 1*E*). Moreover, liver glycogen stores were depleted already after 90 min only in the Early group in both sexes (Fig. 1*E*). Muscle glycogen levels, however, were similar in the Early and Late groups in both sexes and were equally consumed (Fig. 1*F*).

We concluded that daytime difference in exercise capacity is preserved both in male and female mice and corresponds to daytime difference in food consumption and liver glycogen levels.

Time Difference in Exercise Capacity Is Circadian Clock Controlled. Daytime differences can stem from the circadian clock or in response to rhythmic environment. To distinguish between these scenarios, we performed the moderate-intensity running tests in the respective time points in constant dark. Hence, lights were turned off, and male mice were tested at Circadian Time (CT) 14 and CT22, (i.e., Early and Late, respectively) (Fig. 2*A*). Under these conditions, the Late group still performed substantially better than the Early group (Fig. 2*B*). These findings suggested that the observed time-dependent differences in exercise capacity are endogenously controlled, likely through the circadian clock. To specifically test the role of the circadian clock, we employed the clock mutant *Per1,2*$^{-/-}$ mice. Upon the 12-h light–dark regimen, *Per1,2*$^{-/-}$ mice show rhythms in behavior and physiology as well as gene expression, which are likely to be driven by the rhythmic environment. Yet, once these mice are shifted to constant dark, they become arrhythmic (38–40). In contrast to wild-type (WT) mice, clock mutant *Per1,2*$^{-/-}$ mice did not show time difference in exercise capacity, and glucose profiles of the Early and Late groups upon running were similar (Fig. 2*C*). Average RER values before the early and late time points were similar in both WT and *Per1,2*$^{-/-}$ mice (*SI Appendix*, Fig. S2*B*), suggesting that their nutrient utilization before the run is similar. We did not observe differences in food consumption per body weight in *Per1,2*$^{-/-}$ mice between the Early and Late groups (Fig. 2*D*). Consistently, liver glycogen content was similar in the two groups (Fig. 2*E*), which may explain the lack of time difference in exercise capacity in these mice. Muscle glycogen levels were similar in the two time points for both genotypes (Fig. 2*F*).

Taken together, the presence of time difference in WT mice and its absence in *Per1,2*$^{-/-}$ mice in constant dark indicated that the time dependency in exercise capacity is circadian clock controlled.

The Effect of Different Clock Proteins on Daytime Variance in Exercise Capacity. As mentioned in the previous section, *Per1,2*$^{-/-}$ mice maintain rhythmic behavior and physiology in response to light–dark cycles (38, 39) even though their molecular clock is nonfunctional. Nevertheless, our current (Fig. 3*A*) and previous findings (24) showed no daytime difference in their exercise capacity under a 12-h light–dark regimen. We therefore concluded

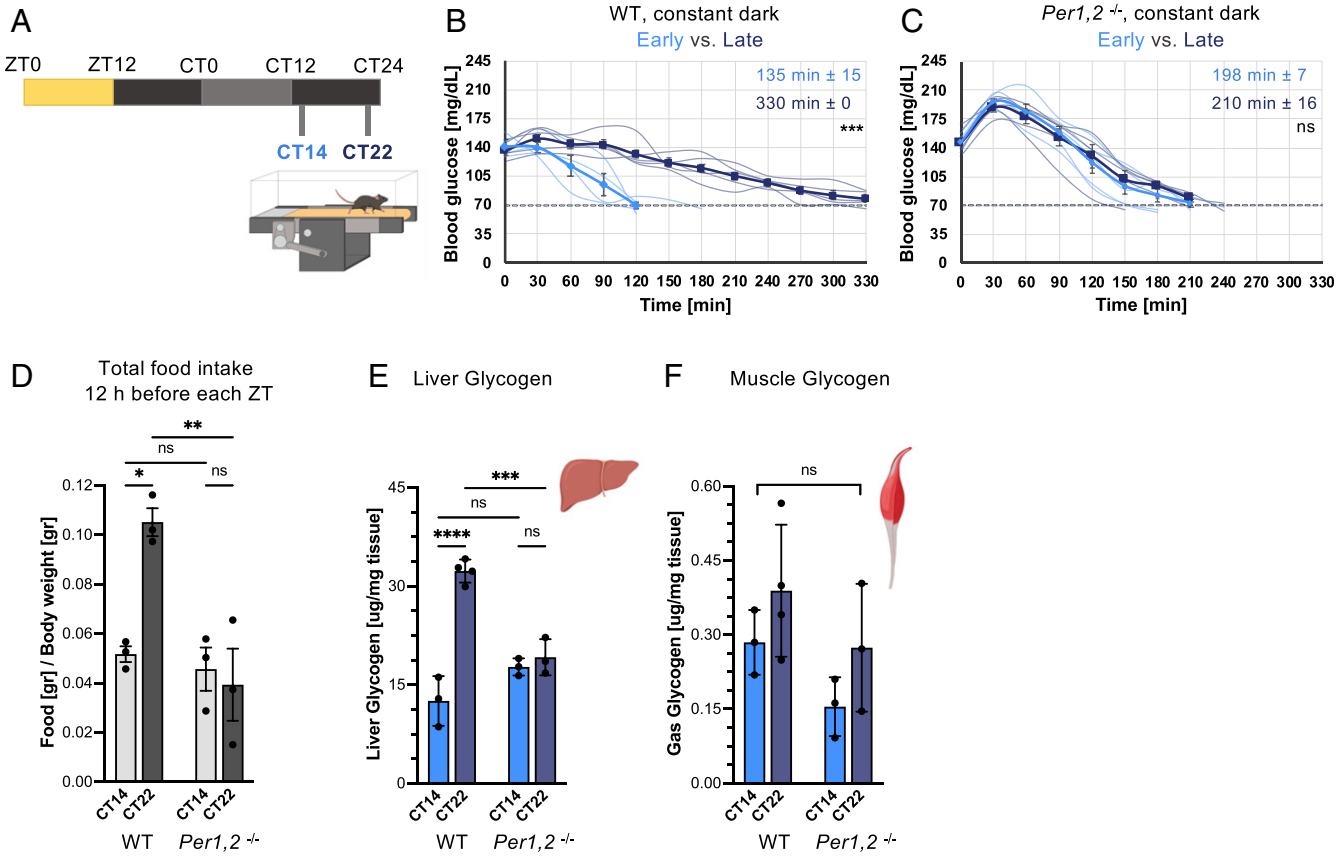

Fig. 2. Time difference in exercise capacity is circadian clock controlled. Mice were housed under a 12-h light–12-h dark regimen, and lights were not turned on at ZT0 on the day of the test. Moderate-intensity treadmill test was performed in constant dark conditions either at the Early (CT14) or Late (CT22) part of the respective active phase. (*A*) Schematic representation of the experimental design. (*B* and *C*) Blood glucose profiles of individual mice during the test (thin lines) and mean ± SEM (thick line) for each group for WT (Early, $n = 4$; Late, $n = 5$) and *Per1,2*$^{-/-}$ (Early, $n = 5$; Late, $n = 5$). End point for each group, at which either blood glucose levels reached ≤70 mg/dL or the animal finished the 5.5-h test, is presented as average time ± SEM. (*D*) Food intake was monitored using metabolic cages. Data are presented as mean ± SEM of average food consumed during 12 h per body weight either before CT14 or CT22 from an average of three WT and three *Per1,2*$^{-/-}$ mice. See also *SI Appendix*, Fig. S3*B*. Reanalysis of data presented in ref. 38. (*E* and *F*) Liver and gastrocnemius (Gas) glycogen content ($n = 3$ to 4 per group). Student's *t* test (*B* and *C*), two-way ANOVA (*F*) with Tukey's post hoc test (*D* and *E*); ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, nonsignificant (ns).

that in the absence of a functional clock, rhythmic environment is not sufficient to maintain a daytime difference in exercise capacity. It does not exclude, however, an effect of one or both PER proteins on exercise capacity per se.

To specifically study the effect of PER proteins on daytime variance in exercise capacity, we tested *Per1*$^{-/-}$ and *Per2*$^{-/-}$ single-knockout male mice. The circadian clock of *Per1*$^{-/-}$ and *Per2*$^{-/-}$ is functional, albeit with a shorter period length (39–41). *Per1*$^{-/-}$ mice exhibited daytime difference in exercise capacity, with the Late group outperforming the Early group (Fig. 3*B*), very similar to WT mice (Fig. 1*B*). By contrast, *Per2*$^{-/-}$ mice showed no time difference in their exercise capacity (Fig. 3*C*) and highly resembled *Per1,2*$^{-/-}$ mice (Fig. 3*A*). The RER prior to the run did not correspond to the daytime difference in exercise capacity of the different genotypes (*SI Appendix*, Fig. S2*C*). In contrast to RER and in line with the results described in the two previous sections, daytime differences in exercise capacity correlated with food intake per body weight (Fig. 3*D*) and with liver glycogen content (Fig. 3*E*). Muscle glycogen levels, however, were similar in both Early and Late groups for all genotypes (Fig. 3*F*). We concluded that the lack of PER2 is sufficient to abolish daytime variance in exercise capacity, which might stem from its role in metabolic control (42).

The specific effect of PER2 on daytime difference in exercise capacity led us to test whether other clock proteins might act similarly. Previously, it was shown that in addition to BMAL1's function as a clock component, it regulates a variety of transcriptional programs associated with carbohydrate and lipid metabolism (13, 14). This prompted us to examine the effect of BMAL1 on exercise capacity. *Bmal1*$^{-/-}$ animals are arrhythmic in constant dark, yet akin to *Per1,2*$^{-/-}$ animals, they maintain rhythmic behavior under light–dark cycles, albeit with reduced amplitudes (38, 43). It is noteworthy that BMAL1-null mice exhibit reduced lifespan and display various phenotypes that are associated with premature aging, such as sarcopenia, and reduced subcutaneous fat; however, they are indistinguishable from their WT littermates until 16 wk of age (44). Therefore, we tested 10- to 12-wk-old male *Bmal1*$^{+/+}$, *Bmal1*$^{-/+}$, and *Bmal1*$^{-/-}$ littermates for their exercise capacity either at ZT14 or ZT22 (Fig. 3 *G*–*I*). Heterozygous *Bmal1*$^{-/+}$ resembled their *Bmal1*$^{+/+}$ homozygous littermates and showed daytime difference in exercise capacity (Fig. 3 *G* and *H*). However, we did not observe significant differences in exercise capacity between the Early and Late *Bmal1*$^{-/-}$ mice (Fig. 3*I*). Hence, complete loss of both *Bmal1* alleles obliterates daytime difference in exercise capacity. All three genotypes showed similar RER values prior to the run irrespective of the time of day (*SI Appendix*, Fig. S2*D*). Unlike *Bmal1*$^{+/+}$ and *Bmal1*$^{-/+}$, *Bmal1*$^{-/-}$ mice did not show time difference in their food intake per body weight (Fig. 3*J*). Here as well, liver glycogen content correlated with feeding behavior and exercise performance (Fig. 3*K*), and muscle glycogen did not show daytime difference (Fig. 3*L*). Notably, *Bmal1*$^{-/-}$ mice had overall higher levels of glycogen in both liver and muscle compared with their *Bmal1*$^{+/+}$ and *Bmal1*$^{-/+}$ littermates.

Overall, our data suggested that the lack of either PER2 or BMAL1 abolishes daytime differences in exercise capacity.

Clock Proteins Modify Exercise Capacity in a Daytime-Dependent Manner. Our finding that daytime variance in exercise capacity is diminished in some clock mutant mouse models implied that these proteins affect exercise capacity in a time-dependent manner.

A lack of daytime difference in exercise capacity can stem from either an increase in exercise capacity in the Early group, a decrease in the Late group, or both. We therefore compared the time to fatigue, namely, reaching blood glucose levels below 70 mg/dL, for each genotype at the Early or Late time point. At the Early time point, *Per1*$^{-/-}$ mice showed the lowest exercise capacity (Fig. 4*A*). Interestingly, the exercise capacity of *Per1,2*$^{-/-}$ mice at the Early time point resembled that of WT mice, even though they lack *Per1*, hinting toward a potential compensatory mechanism in these animals. At the Late time point, *Per1,2*$^{-/-}$ closely followed by *Per2*$^{-/-}$ mice were ranked last (Fig. 4*A*).

A similar analysis of the different *Bmal1* mutant mice showed that at the Early time point, *Bmal1*$^{-/-}$ performed better than both *Bmal1*$^{+/+}$ and *Bmal1*$^{-/+}$ littermates (Fig. 4*B*). At the Late time point, the three genotypes were able to complete the 5.5-h run close to the 70 mg/dL threshold (Fig. 4*B*). Since all exercise tests were routinely terminated after 5.5 h, we were unable to experimentally determine which genotype shows the best exercise capacity (e.g., at the Late time point, both WT and *Per 1*$^{-/-}$ mice did not cross the 70 mg/dL threshold within the 5.5-h run). Altogether, our experimental data showed that for the Early run, the absence of PER1 reduces exercise capacity, whereas complete loss of BMAL1 increases it. However, for the Late run, lack of both PER1 and PER2 or even PER2 alone tempers exercise capacity.

To better characterize the differences in exercise performance and identify the best performers, we analyzed the glucose profiles of the different mice throughout their run. Under the premise that animals reach metabolic steady state and maintain it under constant running intensity (45), time to fatigue can be modeled based on the empirical data using a linear fit extrapolation. The ability of mice to maintain glucose levels above 70 mg/dL during an exercise test depends both on their peak glucose levels at the beginning of the run (after 30 min) and their glucose consumption rate (GCR) throughout the run. A comparison of peak glucose levels of WT and *Per* knockout mice revealed slight differences and, overall, were similar between the genotypes both for the Early and Late groups (Fig. 4*C*), whereas *Bmal1*$^{-/-}$ mice showed elevated peak glucose levels at both time points (Fig. 4*D*). To determine the GCR, we applied a linear regression on the glucose consumption profiles of WT and different *Per* mutant mice (Fig. 4*E*) as well as the different *Bmal1* mutant littermates (Fig. 4*F*) either at the Early or Late time point. WT and *Per* mutant mice did not significantly differ in their calculated GCR at the Early time point (Fig. 4*G*). However, at the Late time point, loss of either *Per2* or both *Per1* and *Per2* resulted in higher GCR compared with WT mice (Fig. 4*G*). Notably, the GCR of *Per1,2*$^{-/-}$ was more than twice that of WT mice (Fig. 4*G*). In the case of *Bmal1* mutant mice, no significant differences were observed at the Early time point, while at the Late time point, *Bmal1*$^{-/-}$ showed significantly higher GCR compared with their *Bmal1*$^{-/+}$ and *Bmal1*$^{+/+}$ littermates (Fig. 4*H*). Thus, both *Per1,2*$^{-/-}$ and *Bmal1*$^{-/-}$ exhibited higher GCR during the run, specifically at the Late time point (Fig. 4 *G* and *H*). Taking both these parameters into account, namely, peak glucose levels and GCR, we could now calculate the time animals will reach fatigue and cross the 70 mg/dL threshold. As shown in Fig. 4 *I* and *J*, the predicted fatigue time for the Early time point was in line with the empirical data for both *Per* and *Bmal1* mutant mice (compare Fig. 4 *A* and *B* with Fig. 4 *I* and *J*). Interestingly, for the Late time point, we found that WT mice are expected to perform significantly better than the different *Per* mutant mice and reach fatigue much later, in line with their lower GCR. By contrast, *Bmal1* mice did not exhibit differences in their predicted fatigue time, and it seems that although *Bmal1*$^{-/-}$ mice showed significantly higher GCR (Fig. 4*H*), it was counterbalanced by their elevated peak glucose levels (Fig. 4*D*).

Importantly, the above-detailed analyses uncovered time-dependent effects of different clock proteins on peak glucose levels and GCR upon exercise. These differences are likely to play a role in the observed variance in exercise capacity of the different clock mutant mice. Furthermore, our analyses revealed that lack of *Per1,2* impairs, whereas absence of *Bmal1* enhances, exercise capacity in a time-dependent manner.

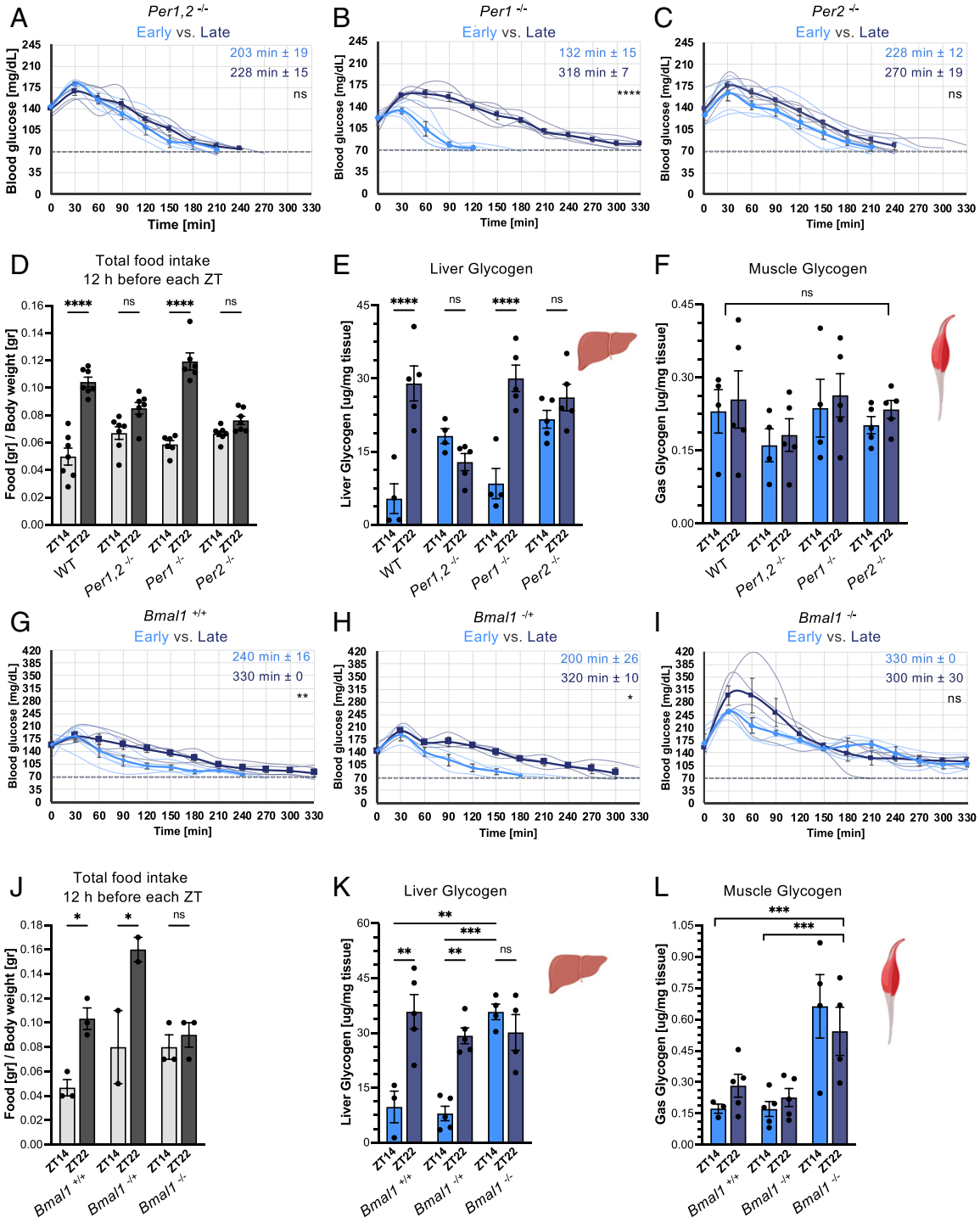

Fig. 3. The effect of clock proteins on daytime variance in exercise capacity. Mice were housed under a 12-h light–12-h dark regimen, and a moderate-intensity treadmill test was performed either at the Early (ZT14) or Late part (ZT22) of the active phase. Early and Late group blood glucose profiles of individual mice during a moderate-intensity run (thin lines) and mean ± SEM for each time point (thick line) of (*A*) *Per1,2*$^{-/-}$ mice (Early [$n = 4$] and Late [$n = 5$]), (*B*) *Per1*$^{-/-}$ mice (Early [$n = 5$] and Late [$n = 5$]), and (*C*) *Per2*$^{-/-}$ mice (Early [$n = 5$] and Late [$n = 5$]). End point for each group, at which either blood glucose levels reached ≤70 mg/dL or the animal finished the 5.5-h test, is presented as average time ± SEM. (*D*) Food intake was monitored using metabolic cages. Data are presented as mean ± SEM of average food consumed during 12 h per body weight either before ZT14 or ZT22 from an average of two consecutive days of six to seven mice from each genotype. See also *SI Appendix*, Fig. S3*C*. (*E* and *F*) Liver and gastrocnemius (Gas) glycogen content ($n = 4$ to 5 per group). Early and Late group blood glucose profiles of individual mice during a moderate-intensity run (thin lines) and mean ± SEM for each time point (thick line) of (*G*) *Bmal1*$^{+/+}$ mice (Early [$n = 5$] and Late [$n = 4$]), (*H*) *Bmal1*$^{-/+}$ mice (Early [$n = 3$] and Late [$n = 3$]), and (*I*) *Bmal1*$^{-/-}$ mice (Early [$n = 4$] and Late $n = 4$]). End point for each group, at which either blood glucose levels reached ≤70 mg/dL or the animal finished the 5.5-h test, is presented as average time ± SEM. (*J*) Food intake was monitored using metabolic cages. Data are presented as mean ± SEM of average food consumed during 12 h per body weight either before ZT14 or ZT22 from an average of two consecutive days of two to three mice from each genotype. See also *SI Appendix*, Fig. S3*D*. Reanalysis of data presented in ref. 38. (*K* and *L*) Liver and gastrocnemius (Gas) glycogen content ($n = 3$ to 5 per group). Student's *t* test (*A*–*C* and *G*–*I*), two-way ANOVA (*F*) with Tukey's post hoc test (*D*, *E*, *J*, *K*, and *L*); ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, nonsignificant (ns).

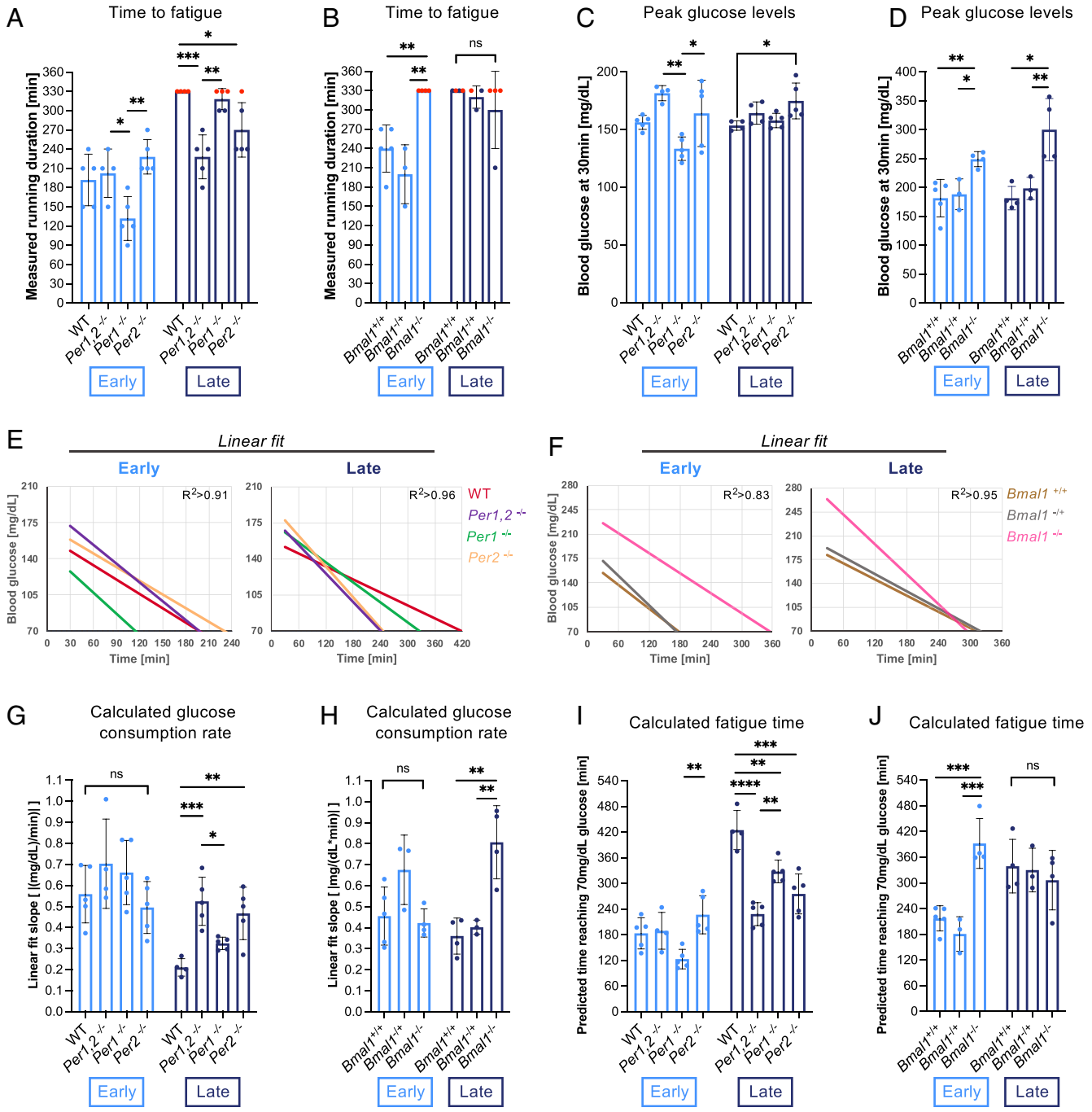

Fig. 4. Clock proteins modify exercise capacity in a time-dependent manner. (*A* and *B*) Time until reaching blood glucose levels ≤70 mg/dL (time to fatigue) upon moderate-intensity treadmill test for *Per* and *Bmal1* mutant mice, respectively. Red dots represent mice that finished the 5.5-h run without reaching blood glucose levels ≤70 mg/dL. (*C* and *D*) Peak blood glucose levels upon moderate-intensity treadmill test for *Per* and *Bmal1* mutant mice, respectively. (*E* and *F*) Representative linear fits for glucose consumption during the test for the average profiles of WT, *Per*, and *Bmal1* mutant mice. (*G* and *H*) Calculated GCRs (slopes) based on the linear fits for individual WT ($R^2 > 0.90$), *Per* ($R^2 > 0.78$), and *Bmal1* ($R^2 > 0.73$) mutant mice. (*I* and *J*) Calculated time to fatigue (intercept with $y = 70$) based on the linear fits for individual WT, *Per*, and *Bmal1* mutant mice. The analysis is based on data presented in Figs. 1*B* and 3 *A*–*C* and *G*–*I*. One-way ANOVA for each Late and Early groups (nonsignificant [ns] for this test is marked on the graphs) with Tukey's post hoc test (*A*–*D* and *G*–*J*); ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; multiple comparisons that were nonsignificant are not marked.

Muscle Lipid Composition of Sedentary and Exercised Clock Mutant Mice. Unlike WT, both *Per1,2*$^{-/-}$ and *Bmal1*$^{-/-}$ mice did not show daytime difference in their exercise capacity (Fig. 3). Daily rhythms in skeletal muscle lipid metabolism have been reported (46, 47), and both PERs and BMAL1 were found to regulate lipid metabolism and mitochondrial lipid utilization in sedentary mice (11, 47–49). Hence, we hypothesized that differences in muscle lipid metabolism might relate to exercise capacity of *Per1,2*$^{-/-}$ or *Bmal1*$^{-/-}$ mice. To test this, we characterized lipids that serve as energy sources, namely, triglyceride and acylcarnitines, in muscle (Fig. 5 and *SI Appendix*, Fig. S4 and Dataset S1). We compared the gastrocnemius triglyceride and acylcarnitine levels of WT versus *Per 1,2*$^{-/-}$ in sedentary and upon 1 h of moderate-intensity run at the Late time point. A similar comparison

was performed for *Bmal1*$^{-/+}$ that phenotypically resemble *Bmal1*$^{+/+}$ versus *Bmal1*$^{-/-}$ mice.

We did not observe significant differences in total muscle triglycerides between WT and *Per1,2*$^{-/-}$ mice in sedentary and exercised animals (Fig. 5*A*). Yet, the triglyceride composition differed between the genotypes in sedentary and exercised mice (Fig. 5*B*). We observed two main clusters. Cluster 1 consisted of triglycerides that were elevated in sedentary *Per1,2*$^{-/-}$ compared with WT mice and were strongly consumed upon exercise, whereas Cluster 2 consisted of triglycerides that were high in sedentary WT compared with *Per1,2*$^{-/-}$ mice and consumed upon exercise in WT but not in *Per1,2*$^{-/-}$ mice. Notably, Cluster 1 mainly consisted of saturated fatty acids compared with Cluster 2, further highlighting genotype-dependent differences in triglyceride metabolism both under sedentary and exercise conditions (*SI Appendix*, Fig. S4*A*). Next, we characterized the acylcarnitine levels and found, in line with previous reports (24, 50), that they tend to accumulate upon exercise (Fig. 5 *C* and *D*). Moreover, acylcarnitines reached higher levels in exercised *Per1,2*$^{-/-}$ compared with WT mice. The accumulation of high levels of acylcarnitines is associated with inefficient fatty acid oxidation and is consistent with lower exercise capacity (50), as observed for the Late group of *Per1,2*$^{-/-}$ mice.

In the case of *Bmal1* mice, total muscle triglyceride levels were similar (Fig. 5*E*), yet their composition differed between the genotypes both in sedentary and exercised mice (Fig. 5*F*) and showed two main clusters. Cluster 1 consisted of triglycerides that were elevated in sedentary *Bmal1*$^{-/-}$ compared with control mice and were strongly consumed upon exercise, whereas Cluster 2 consisted of triglycerides that were elevated upon exercise in control mice and consumed in exercised *Bmal1*$^{-/-}$ mice. Cluster 1 mostly consisted of saturated fatty acids, whereas Cluster 2 contained unsaturated fatty acids (*SI Appendix*, Fig. S4*B*).

Importantly, similar to control mice and in contrast to exercised *Per1,2*$^{-/-}$ mice, acylcarnitine levels did not show significant accumulation upon exercise in *Bmal1*$^{-/-}$ mice (Fig. 5 *G* and *H*). This is consistent with our observation that exercise capacity of these mice at the Late time point is similar to their control littermates.

Recently, we proposed that 5-Aminoimidazole-4-carboxamide ribonucleotide (ZMP), an endogenous AMPK activator, is induced by exercise to regulate fatty acid oxidation and promote

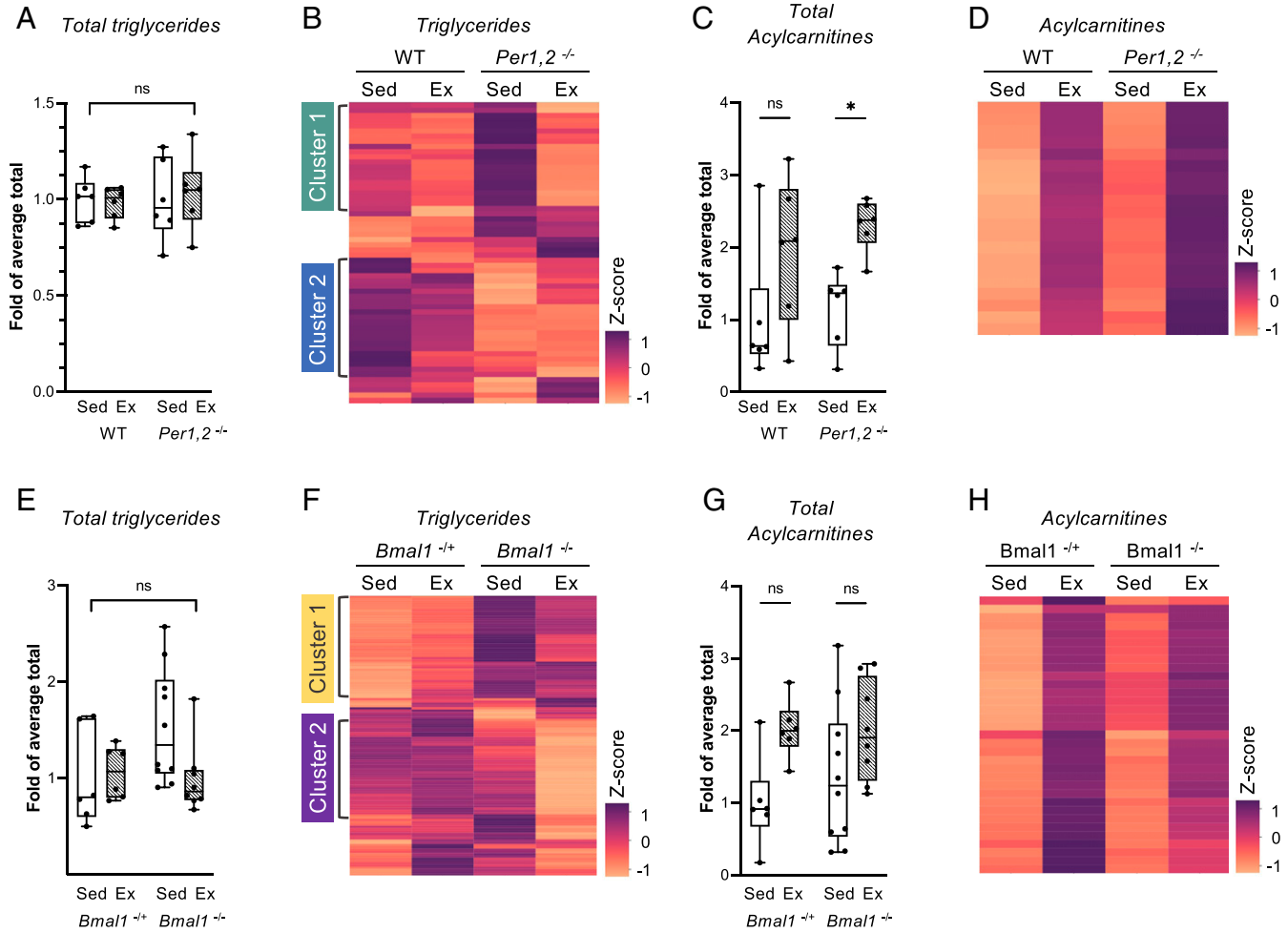

Fig. 5. Muscle lipid composition in sedentary and exercised *Per1,2*$^{-/-}$ and *Bmal1*$^{-/-}$ mice. Mice were housed under a 12-h light–12-h dark regimen and performed the moderate-intensity treadmill test at ZT22 for 1 h. Sedentary (Sed) and Exercised (Ex) mice were killed, and the gastrocnemius muscle was harvested and analyzed for its lipids content. Fold induction of mean total triglycerides identified in WT ($n = 6$) and *Per1,2*$^{-/-}$ ($n = 6$) mice (*A*) and in *Bmal1*$^{-/+}$ ($n = 8$) and *Bmal1*$^{-/-}$ ($n = 10$) (*E*). Heatmap representation of triglyceride levels for WT and *Per1,2*$^{-/-}$ (*B*) and for *Bmal1*$^{-/+}$ and *Bmal1*$^{-/-}$ (*F*). Fold induction of mean total acylcarnitines identified in WT and *Per1,2*$^{-/-}$ mice (*C*) and in *Bmal1*$^{-/+}$ and *Bmal1*$^{-/-}$ (*G*). Heatmap representation of acylcarnitine levels for WT and *Per1,2*$^{-/-}$ (*D*) and for *Bmal1*$^{-/+}$ and *Bmal1*$^{-/-}$ (*H*). Triglycerides and acylcarnitines with positive and negative Z scores are depicted in purple and orange, respectively. Clustering was performed using Python's Seaborn clustermap function on log2-transformed data. Two-way ANOVA (*A*, *E*, and *G*) with Bonferroni's post hoc test (*C*). *$P < 0.05$, nonsignificant (ns).

exercise capacity (24). This prompted us to examine muscle ZMP levels in sedentary and exercised *Per1,2*$^{-/-}$ and *Bmal1*$^{-/-}$ mice. In line with our previous report, moderate-intensity run increased muscle ZMP levels (*SI Appendix*, Fig. S5 *A* and *B*). Muscle ZMP levels were elevated upon the 1-h run both in *Per1,2*$^{-/-}$ and *Bmal1*$^{-/-}$ mice, similar to their control mice (*SI Appendix*, Fig. S5 *A* and *B*). We observed a trend toward lower induction in *Per1,2*$^{-/-}$ compared with control mice that is consistent with their reduced exercise capacity, yet it was not statistically significant.

Overall, we identified differences in the skeletal muscle lipid profiles that are genotype and exercise dependent, which point toward differences in muscle lipid utilization in the different genotypes and potentially relate to their exercise capacity.

The Effect of Daytime Scheduled Training on Exercise Capacity. Hitherto, we showed that exercise capacity exhibits daytime variance and can be differentially modified by clock components in a time-dependent manner. Training improves exercise capacity through various multiorgan physiologic and metabolic alterations (51). This prompted us to test the daytime effect of scheduled training on exercise capacity. To this end, we designed and built fully automated time-controlled running wheels (TRW) (Fig. 6*A*). TRW is a programmable system that can be integrated with commercial wheel-running cages and is compatible with wheel-running recordings. Using this system, the wheels can be programmed in advance to be in locked or unlocked positions for designated times and for different durations. The system is modular and can be easily scaled up or adapted to different cages/running wheels. It enables scheduled training of animals without manual interventions and can handle several different training regimens simultaneously.

To test the effect of daytime scheduled training on exercise capacity in mice, running wheels were programmed to be either constantly locked (Untrained) or unlocked for 6 h in the first or second half of the active phase (i.e., ZT12-18 and ZT18-24, Early and Late trained, respectively) (Fig. 6*B*). After a 2-wk acclimatization to the running wheels, we activated the training schedule for two subsequent weeks (Fig. 6*C*), during which both Early- and Late-trained animals ran similar distances per day (Fig. 6*D*). Next, mice were tested for their moderate-intensity exercise capacity at the Early time point (ZT14). As expected, training improved exercise capacity, as both the Early- and Late-trained animals performed significantly better than the Untrained group (Fig. 6*E*). Remarkably, the Late-trained outperformed the Early-trained mice, maintaining higher blood glucose levels and running for a longer duration (Fig. 6*E*). There were no significant differences in food consumption and liver glycogen levels prior to the run among Untrained, Early-, or Late-trained animals (*SI Appendix*, Fig. S6). Of note, Late-trained animals showed higher muscle glycogen levels, which might explain their increased exercise capacity.

An analysis of behavioral rhythms following 2 wk of scheduled training showed, in line with previous reports (18–21), that scheduled exercise shifts the clock (Fig. 6*F*). Yet, we did not observe significant differences in the phase or the period of Early- versus Late-trained mice (Fig. 6 *F* and *G*). Previously, it was shown that exercise can phase shift the circadian clock in skeletal muscle (17). To test whether our scheduled training protocol shifts the muscle clock, we used PER2::LUC mice, which are widely used to monitor circadian clock dynamics in mice (52). These mice were subjected to the same scheduled training, and muscle organotypic slices were used to monitor the effect on their muscle endogenous clocks. We did observe ∼2-h phase delay in the Late-trained compared with the Early and Untrained mice (Fig. 6*H*), with no significant effect on the period length (Fig. 6*I*). The observed shift in the muscle clock in the Late-trained animals might play a role in their increased exercise capacity.

In summary, we concluded that training in the late compared with the early active phase better improves exercise capacity.

Discussion

Daily variance in exercise performance of rodents and humans has been previously shown (22–24), yet it is still unclear how these effects are related to the circadian clock control of physiology (e.g., feeding behavior) and metabolism (e.g., lipid and glucose homeostasis). Here, we show that endurance running capacity is controlled by the circadian clock and differentially shaped by clock proteins in a daytime-dependent manner. These effects strongly relate to liver glycogen stores, which are widely accepted to play a critical role in endurance exercise (53, 54). We also found that the altered feeding behavior of the different clock mutant mouse models corresponds to liver glycogen stores. Together, this puts forward the idea that circadian clock control of feeding behavior affects liver glycogen stores and consequently shapes endurance exercise capacity. Yet, the picture is likely to be more complicated, as previous reports (55–58) suggest that clock components directly regulate the expression of rate-limiting enzymes in glycogen metabolism and affect liver glycogen levels irrespective of food intake. For example, liver-specific *Bmal1* mutant mice show similar feeding behavior as WT mice, yet their liver glycogen profiles differ (57). Nonetheless, our study suggests that clock control of liver glycogen stores is likely to play a central role in the daily variance in exercise performance.

We found that at the Early active phase, BMAL1 impairs and PER1 enhances exercise capacity, while at the Late active phase, both PER1 and PER2 improve exercise capacity. It was reported that *Cry1,2*$^{-/-}$ but not *Cry1*$^{-/-}$ or *Cry2*$^{-/-}$ mice perform better than their WT littermates, specifically under high- but not moderate-intensity exercise protocols (59). These exercise protocols differ from the protocol used here (e.g., different slope, speed increment, and running stimulus), and animals were tested at a single time point during the rest phase (i.e., ZT6-8), yet they further support the role of clock proteins as modifiers of exercise capacity. It would be interesting to further test whether the effect of clock proteins on exercise capacity varies with exercise intensity, namely, high- versus moderate- or low-intensity exercise protocols, as they rely on different physiologic and metabolic requirements. Furthermore, it would be important to address in future studies whether the effect of clock proteins on exercise capacity is dependent on their activity in specific tissues, such as the liver, muscle, and heart. Experiments with different tissue-specific clock mutant mouse models are expected to be informative in this conjunction.

Carbohydrates and fat are recognized as the main substrates utilized during prolonged endurance exercises (53). Consistent with its well-established role in glucose homeostasis in sedentary animals (55, 57, 60, 61), we show that BMAL1 affects glucose levels upon exercise. Similarly, the differences in the glucose profiles of *Per* mutant mice hint toward altered glucose metabolism in these mice upon exercise. Furthermore, we found that both *Per1,2*$^{-/-}$ and *Bmal1*$^{-/-}$ mice differ in their muscle lipid profiles both in sedentary animals and upon exercise. Notably, *Per1,2*$^{-/-}$ mice show higher accumulation of acylcarnitines upon exercise. These findings in muscle are in line with previous reports on the role of PER proteins in lipid metabolism in the liver (11, 48, 49). The accumulation of acylcarnitines is associated with inefficient fatty acid oxidation (50) and may contribute to the reduced exercise capacity of these mice. We also observed a tendency toward lower induction of muscle ZMP levels upon exercise in *Per1,2*$^{-/-}$ mice. ZMP as well as its exogenously administrated analog AICAR were reported to regulate FAO (24, 62, 63) and consequently affect exercise capacity. Hence, it

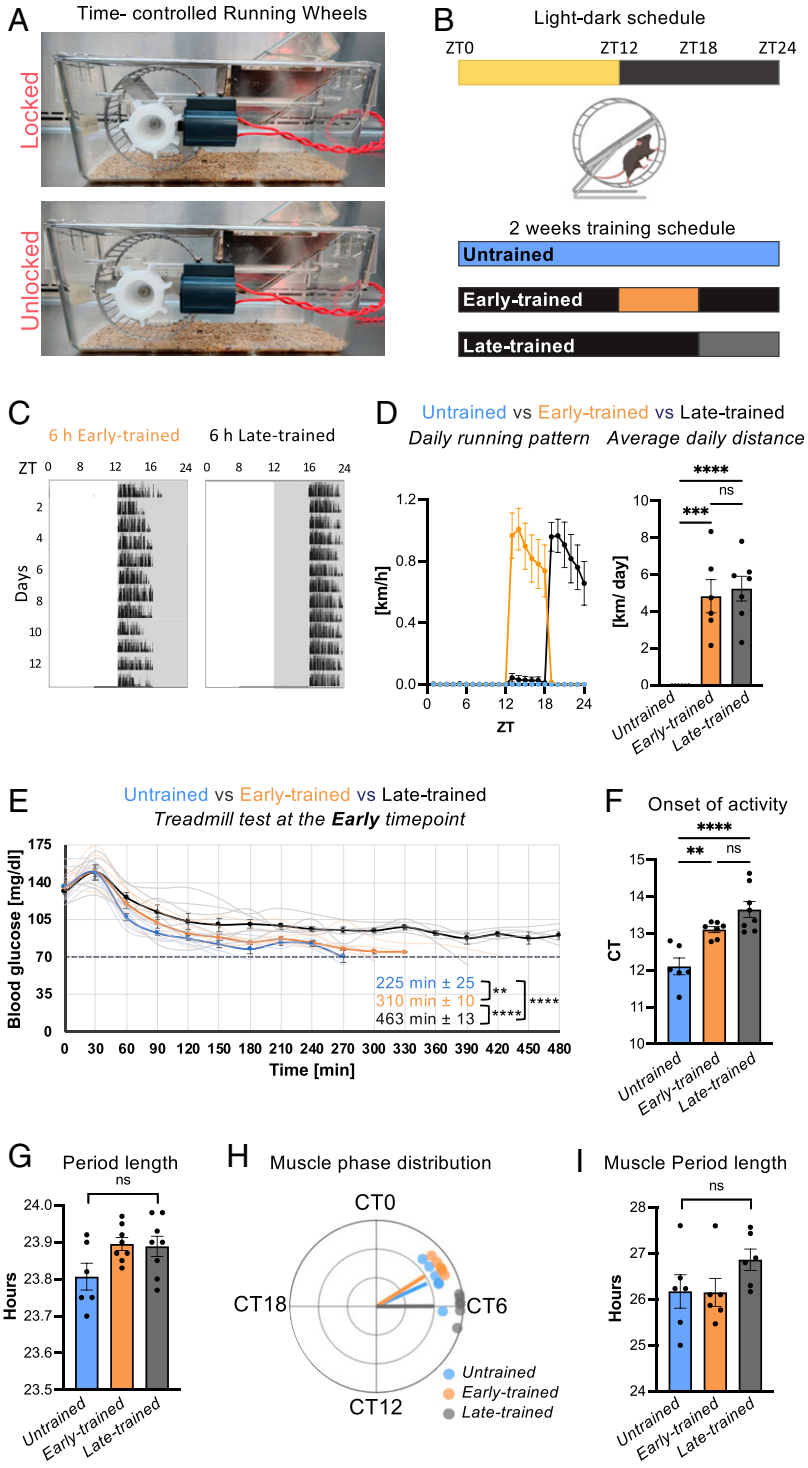

Fig. 6. The effect of daytime scheduled training on endurance capacity. (*A*) An image of the TRW. (*B*) A schematic representation of the experimental design. (*C*) Representative actograms of Early- and Late-trained mice throughout their scheduled training period. (*D*) The daily running pattern and the mean daily distance covered by the Early- and Late-trained mice throughout the scheduled training period (n = 5 to 7 in each group). (*E*) Blood glucose profiles of individual mice (thin lines) during an extended moderate-intensity treadmill test performed at ZT14 of Untrained (n = 5), Early- (n = 6), or Late-trained mice (n = 7) and mean ± SEM (thick line) for each time point. End point for each group, at which either blood glucose levels reached ≤70 mg/dL or the animal finished the 8-h test, is presented as average time ± SEM. (*F* and *G*) Running wheels of Early- and Late-trained mice were locked for a washout period of 24 h, after which at ZT0 the wheels were freely opened to all groups, and mice were kept in constant dark. Onset of activity of the first day and period lengths were analyzed from the recorded actograms. (*H*) Polar plot of the phase distribution of gastrocnemius muscle based on gastrocnemius organotypic slice from PER2::LUC mice that were subjected to the training protocol in *B* (n = 6 for each group). Each point represents the CT value of the first peak of the second day of recordings from a single mouse (mean of 3 to 5 technical replicates). The line's angle represents the circular mean of each condition, and line's radius anticorrelates with the circular variance (n = 6; $P < 0.05$, Watson–Williams test). (*I*) Period length based on gastrocnemius muscle PER2::LUC bioluminescence recordings (n = 6). One-way ANOVA (*G* and *I*), with Tukey's post hoc test (*D*, *E*, and *F*); ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, nonsignificant (ns).

would be interesting to test the levels/activity of enzymes implicated in FAO and mitochondrial lipid transport in these animals upon exercise. It is noteworthy that our single time-point measurements provide a limited snapshot. Future time series measurements or flux analyses of muscle ZMP levels and lipid profiles are expected to capture dynamic changes in the levels of these metabolites and provide vital information on muscle energy fuels upon exercise. Finally, we do not exclude the involvement of other physiologic and metabolic parameters that may involve other tissues as well and are responsible for differences in exercise capacity of the different clock mutant animals.

We found that time of training differentially affects exercise capacity, as Late-trained perform better than Early-trained animals. Here, too, the results refer to a moderate-intensity treadmill protocol and may vary between different exercise intensities, especially in view of their specific metabolic and physiological requirements. The same applies to the training protocol; running wheels are considered low-intensity training, and more intense training or different duration may have a different outcome. Finally, following the training period, mouse performance was tested during the Early active phase. This raises the question of whether the effect is also preserved if animals were to perform the treadmill test at the Late active phase. It is therefore still unclear whether Late training is better than Early training in general or if it depends on the intensity or daytime of the run. This type of time/intensity compatibility between training and the actual run is particularly critical for professional athletes who are scheduled to compete at a certain hour and need to optimize their performance to this specific time.

We show that exercise capacity is better in the late part compared with the early part of the active phase and that training is more efficient in the late part of the active phase in mice. Although this study cannot be directly translated to humans, there is similar evidence in humans that shows that exercise capacity is higher in the evening than morning (64, 65). Training has also been shown to have a higher impact if carried out in the evening rather than in the morning (66, 67). Altogether, exercise can vary depending on age, health status of the individual, chronotype, duration and intensity of exercise, and meal timing. Hence, it is unlikely that there will ever be a "one-size-fits-all" recommendation for the timing of exercise training.

In summary, our findings highlight the interaction between daytime, circadian clocks, and clock proteins with exercise biology, and although currently done in animal models, they are expected to have implications on exercise in humans as well.

Materials and Methods

Animals. All animal experiments and procedures were conducted in conformity with the Weizmann Institute Animal Care and Use Committee guidelines. We used C57BL/6J mice (Envigo) and PER2::LUCIFERASE mice (52). *Per1,2*$^{-/-}$ [*mPer1,2*^{Brdm1} (39)] were back crossed to C57BL/6J for 10 generations to establish a C57BL/6J *Per1,2*$^{-/-}$ colony (38). This colony was used to generate C57BL/6J single *Per1*$^{-/-}$ and *Per2*$^{-/-}$ knockout strains. *Bmal1*$^{-/+}$ heterozygous mice [Jackson Laboratory, B6.129-Arntl^{tm1Bra}/J (43)] were used to generate homozygous *Bmal1*$^{-/-}$ and *Bmal1*$^{+/+}$. All mice were 10 to 12 wk old with an average weight of 25 ± 3 g, maintained in SPF (Specific Pathogen Free) animal facility, and group housed (at least two animals in a cage) at a temperature of 22 °C. Unless stated otherwise, we used male animals and kept them under a 12-h light–12-h dark schedule (LD). ZT0 corresponds to the time lights were turned on and ZT12 to the time lights were turned off. Experiments in constant dark (DD) were performed on the first day of DD, following an LD regimen. Animals were given ad libitum access to food (regular chow, Envigo 2018 Teklad) and water.

Data Availability. All study data are included in the article and/or supporting information.

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