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ORIGINAL RESEARCH

Intrinsic Skeletal Muscle Function and Contraction-Stimulated Glucose Uptake Do Not Vary by Time-of-Day in Mice

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Abstract

A growing body of data suggests that skeletal muscle contractile function and glucose metabolism vary by time-of-day, with chronobiological effects on intrinsic skeletal muscle properties being proposed as the underlying mediator. However, no studies have directly investigated intrinsic contractile function or glucose metabolism in skeletal muscle over a 24 h circadian cycle. To address this, we assessed intrinsic contractile function and endurance, as well as contraction-stimulated glucose uptake, in isolated extensor digitorum longus and soleus from mice at 4 times-of-day (zeitgeber times 1, 7, 13, 19). Significantly, though both muscles demonstrated circadian-related changes in gene expression, there were no differences between the 4 time points in intrinsic contractile function, endurance, and contraction-stimulated glucose uptake, regardless of sex. Overall, these results suggest that time-of-day variation in exercise performance and the glycemia-reducing benefits of exercise are not due to chronobiological effects on intrinsic muscle function or contraction-stimulated glucose uptake.

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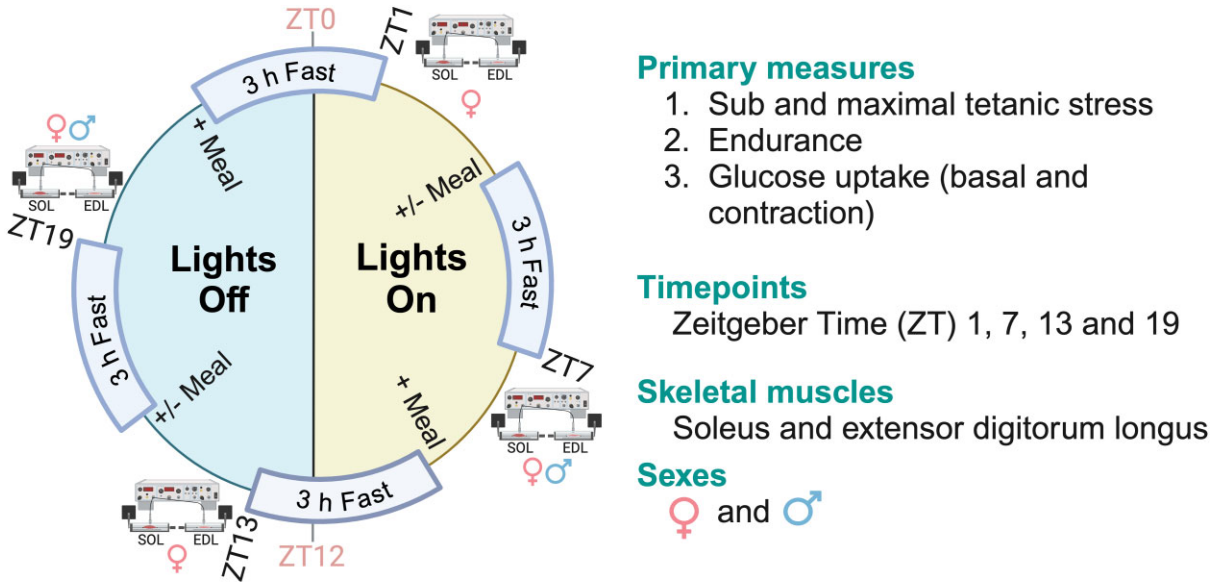

Primary measures

1. Sub and maximal tetanic stress
2. Endurance
3. Glucose uptake (basal and contraction)

Timepoints

Zeitgeber Time (ZT) 1, 7, 13 and 19

Skeletal muscles

Soleus and extensor digitorum longus

Sexes

♀ and ♂

Take home message: The intrinsic contractile properties of skeletal muscle, and contraction-stimulated glucose uptake, are not different by time-of-day, regardless of sex or muscle type.

Key words: physiology; 2-deoxyglucose; extensor digitorum longus; soleus; mechanics; chronobiology; circadian; metabolism; zeitgeber; fatigue

Introduction

Through circadian cycles, or rhythms, time-of-day impacts many aspects of mammalian physiology.[1–3] Recently, the field of exercise physiology has been focused on the effect of time-of-day on exercise capacity and athletic performance. On the whole, measures of both strength and endurance performance tend to be lower in early morning and higher in the afternoon/evening.[4–14] While a number of factors have been proposed to underlie these performance impacting effects of time-of-day, including body temperature,[15,16] motor unit recruitment,[17–19] and meal timing/muscle glycogen status,[20,21] an emphasized point in the field is that variation in exercise performance is due to circadian fluctuations in the intrinsic properties of skeletal muscle.[13] In contrast to these data, recent work found that the maximal intrinsic force-generating capacity of the mouse extensor digitorum longus (EDL) is not different between 2 different times of the light phase (ie, zeitgeber time [ZT] ZT1 and ZT9)[22] (personal communication with Dayanidhi S, Kahn RE, and Lieber RL). While this study is interesting, whether there are intrinsic changes in skeletal muscle physiology, such as submaximal contractile function, resistance to fatigue, or the ability to take up glucose from the blood during contractions, over the course of a 24 h circadian cycle or in other muscles, remains unknown.

Exercise is a cornerstone therapeutic for preventing or treating clinical hyperglycemia.[23–28] A fundamental reason for this beneficial effect of exercise is that muscle contraction potently stimulates glucose disposal from the blood into the exercising skeletal muscle,[29] and does so in an insulin-independent manner.[30–32] Interestingly, like exercise performance, a large focus of the field of exercise physiology, and more broadly, the field of diabetes care, has been on the effect of time-of-day on the glucose-controlling benefits of exercise.[33] Indeed, in humans, undertaking an exercise training intervention in the afternoon has been found to be superior at improving glycemic control and skeletal muscle insulin sensitivity to exercise training in the morning.[34–36] Similarly, in mice, a single exercise bout in the early part of the active (ie, dark) phase, as compared to the early rest (ie, light) phase, promoted a glycolytic transcriptional signature in skeletal muscle and reduced blood glucose concentration.[37] However, whether this time-of-day effect of exercise on glycemic control is due to chronobiological variation in the intrinsic capacity of contraction-stimulated glucose uptake by skeletal muscle has not been investigated.

To address these major gaps in knowledge, we used an *ex vivo* approach to assess intrinsic contractile function (sub-maximal and maximal) and fatigability, and contraction-stimulated glucose uptake at 4 different times of the 24 h cycle, ZT1, ZT7, ZT13, and ZT19; "zeitgeber time" is commonly used in circadian biology to describe different times of the light-dark cycle in an animal vivarium, with ZT0 representing the lights turning on and ZT12 representing the lights turning off for a standard 12 h/12 h light-dark cycle. Utilizing an *ex vivo* approach allowed us to study skeletal muscle in isolation, and thus, independent of other factors that might influence contractile function or glucose metabolism, such as muscle temperature, blood flow, nerve function, humoral factors (ie, glucocorticoids, catecholamines, androgens). We also studied 2 different muscles, the EDL and soleus (SOL), which have distinctly different myosin heavy chain compositions,[38] and both female and male mice, thus allowing us to address the potential role of both muscle fiber type and sex. Considering the literature, our hypothesis was that intrinsic skeletal muscle contractile function, fatigability, and contraction-stimulated glucose uptake would be higher in the afternoon or evening, as compared to morning, regardless of the muscle or sex studied.

Material and Methods

Animals

All studies in female mice were conducted in C57BL/6NJ mice (The Jackson Laboratory, stock #05304) at 13.0 ± 0.1 weeks of age. All studies in male mice were conducted in C57BL/6NJ mice (The Jackson Laboratory, stock #05304) at 12.0 ± 0.1 weeks of age. Female mice arrived at the vivarium at 10 weeks of age and male mice at 9 weeks of age and were housed in a conventional facility with a 12 h light/12 h dark cycle (Light: 0600 h [ZT0]; Dark: 1800 h [ZT12]) for 3.0 ± 0.2 weeks after arrival. All animal experiments were approved by and conducted in accordance with the Animal Care Program at the University of California, San Diego.

Experimental Groups

We undertook 3 separate studies. In Study 1, which was in female mice, there were 4 experimental groups: ZT1, ZT7, ZT13, and ZT19; the experimental timing is overviewed in Figure 1A. These times during the light (ZT1 and ZT7) and dark (ZT13 and ZT19) phases were chosen to represent "early" (ZT1 and ZT13) and "late" (ZT7 and ZT19) timepoints within each phase. If the experiment occurred during a dark phase timepoint, all animal handling before anesthetization was done under dim red light. After anesthetization, muscle dissections and *ex vivo* testing occurred under standard ambient light regardless of time point. Because eating patterns differ by time-of-day in mice and meal timing and carbohydrate intake impact exercise performance,[21,39,40] in Study 1, we controlled the last meal before tissue dissection.

Thus, all mice were orally gavaged with 50% dextrose (2 g/kg) 3 h before tissue dissection and were then fasted with *ad libitum* access to water. Because oral gavage can be a potentially confounding variable in animal studies[41], for example, through elevated glucocorticoids, which are major entrainment factors for circadian clocks in peripheral tissues,[42] it is possible that stress of the gavage could override, reset, or otherwise mask any potential 24 h differences in contractile parameters. To address this concern, in Study 2, the experimental design was identical to Study 1, except mice were not gavaged 3 h prior to tissue dissection; female mice were studied, and food was removed 3 h prior to tissue dissection. Moreover, only 2 times-of-day (ZT7 and ZT19) were studied. The experimental design for Study 2 is overviewed in Supplementary Figure S1A. In Study 2, intrinsic contractile properties, endurance capacity, and contraction-stimulated glucose uptake were studied. In Study 3, male mice were studied at ZT7 and ZT19, with the experimental timing overviewed in Supplementary Figure S2A; food was removed 3 h prior to tissue dissection, and there was no oral gavage. In Study 3, only intrinsic contractile properties and endurance capacity were studied.

Tissue Dissection

An overview of tissue dissection for Study 1 is presented in Figure 1B, Study 2 in Supplementary Figure S1B, and Study 3 in Supplementary Figure S2B. Fasted (3 h) mice were weighed (nearest 0.01 g), and blood glucose (tail vein; Contour® blood glucose meter) was measured. Mice were then administered an intraperitoneal injection of a pentobarbital/phenytoin-containing solution (300 mg/kg; EUTHASOL®; Virbac, ANADA # 200-071). For Study 1 and Study 2, paired EDL and SOL were rapidly dissected and incubated in "recovery" Krebs–Henseleit buffer (REC-KHB; 116 mM NaCl, 4.6 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$ at room temperature) for subsequent testing of contractile function and 2-deoxyglucose (2DOG) uptake (see "*Ex-vivo SOL and EDL incubation: Assessment of contractile function and 2DOG uptake*" below). For Study 3, immediately after dissection, the SOL and EDL were transferred to a custom chamber for mechanical testing only (see "*Contractile function in male mice*"). In Study 1, blood was collected from the inferior vena cava into ethylenediaminetetraacetic acid-containing tubes, centrifuged (14 167 × *g*, 20 min, 4°C), and the plasma was isolated for assessment of fasting insulin concentration. In Studies 1 and 2, tibialis anterior (TA), gastrocnemius (GA), liver and heart were dissected, weighed, and rapidly frozen in liquid nitrogen. For Study 1, in a separate cohort of identically treated mice, we collected EDL and SOL for gene expression analysis, with these muscles being rapidly frozen (liquid nitrogen) after dissection. For Study 3, only EDL and SOL were weighed. All samples were stored at −80°C.

Study 1 and Study 2: *Ex vivo* EDL and SOL Incubation and Assessment of Contractile Function and 2DOG Uptake in Female Mice

The experimental design for muscle testing was identical for Study 1 and Study 2 and is overviewed in Figure 1C. Immediately after dissection, each EDL and SOL recovered in room temperature (21.2 ± 0.2°C) REC-KHB, with each muscle incubated in a separate, oxygenated (95% O_2, 5% CO_2) flask. After 20 min, the SOL and EDL for contraction (ie, CXN group) were transferred to a custom chamber for mechanical testing and assessment of glucose uptake. Thus, the muscle origin was tied with 4-0 silk suture to a rigid post, and the insertion was secured to the arm of a dual-mode ergometer. Then, muscles were electrically stimulated (model S88; Astro-Med, West Warwick, RI, USA) via parallel platinum electrodes (35 V, 0.3 ms pulse duration) with single twitches (1 Hz) to set optimal muscle length. After, 30 min in REC-KHB, the CXN group EDL and SOL underwent a 2-step contraction protocol; the first step tested the stress–frequency relationship, and the second step tested fatigability. The stress–frequency testing was as follows: muscles were stimulated (EDL: 300 ms train, 0.3 ms pulse; SOL: 400 ms train, 0.3 ms pulse) at different frequencies (1, 10, 20, 40, 60, 80, 100, and 120 Hz), with 60 s between each contraction. After assessing stress–frequency, the muscles rested for 10 min, with the total time to complete the stress–frequency testing and rest being 17 min. Then, the REC-KHB was replaced with room temperature "incubation" KHB (INC-KHB: 116 mM NaCl, 4.6 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM Na-pyruvate, 8 mM mannitol, 1 mM 2DOG, 0.1 mCi/mL [^{14}C]-mannitol [American Radiolabeled Chemicals, Inc.], and 1 mCi/mL [^{3}H]-2-deoxyglucose [2DOG] [American Radiolabeled Chemicals, Inc.]) for the assessment of 2DOG uptake during contraction. Thus, immediately after switching to INC-KHB, each muscle underwent a fatiguing contraction protocol for 15 min (EDL: 1000 ms train, 0.3 ms pulse, 100 Hz every 15 s; SOL: 400 ms train, 0.3 ms pulse, 40 Hz every 2 s). The contralateral

SOL and EDL were in the "rest" group (Rest). After a comparable 47 min of incubation in REC-KHB, the "rest" EDL and SOL were transferred to flasks containing room temperature INC-KHB for the assessment of basal 2DOG uptake. After 15 min in INC-KHB, all CXN and rest muscles were rapidly blotted dry, trimmed, weighed, frozen in liquid nitrogen, and stored (−80°C). Muscle stresses were calculated by normalizing force in Newtons to the physiologic cross-sectional area (PCSA) of each muscle (Stress [kPa] = F [Newtons]/PCSA [mm^2]). Cumulative fatigue was measured by assessing area under the curve (units = kPa min).

Study 3: Assessment of Contractile Function in Male Mice

The experimental design for Study 3 is outlined in Supplementary Figure S2C. After dissection, paired EDL and SOL muscles were rapidly dissected and then transferred to a custom chamber containing oxygenated Ringer's solution (137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 24 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 1 mM $MgSO_4$, and 0.01% tubocurarine chloride [pH 7.5]) for mechanical testing. The muscle origin was tied with 4-0 silk suture to a rigid post, and the insertion was secured to the arm of a dual-mode ergometer. Then, muscles were electrically stimulated (model S88; Astro-Med, West Warwick, RI, USA) via parallel platinum electrodes (35 V, 0.3 ms pulse duration, EDL and SOL 400 ms train), with single twitches (1 Hz) to set optimal muscle length. After finding optimal muscle length, the muscles rested for 5 min and the force–frequency was assessed (1, 10, 20, 40, 60, 80, 100, and 120 Hz every 60 s), followed by a 10 min rest period. After the rest period, a fatiguing contraction protocol was conducted (EDL: 300 ms train, 0.3 ms pulse, 40 Hz every 4 s, for 10 min; SOL: 500 ms train, 0.3 ms pulse, 40 Hz every 4 s, for 20 min). Muscle stresses were calculated by normalizing forces to PCSA, as described above.

Muscle Homogenization for 2DOG Uptake Assessment and Immunoblotting

Muscles were transferred to 1.5 mL tubes on ice containing a 1/8 inch stainless steel bead and 500 μL of homogenization buffer (50 mM Tris [pH 7.5], 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 1 mM $Na_2(PO_4)_2$, and 0.1% DTT) containing 1 M nicotinamide (MilliporeSigma #N0636), 1 mM Pefabloc SC PLUS (MilliporeSigma #11873601001), 1 mM trichostatin A (Cell Signaling #9950S), Complete (MilliporeSigma #11836170001), phosphatase inhibitor cocktail (PIC) 2 (MilliporeSigma #P5726), and PIC3 (MilliporeSigma #P0044). The muscles were then homogenized (Bullet Blender, Next Advance #BT24M) and subsequently rotated for 2 h at 4°C. The homogenate was then centrifuged (14 489 × g) for 20 min at 4°C. The supernatant was collected and stored at −80°C for subsequent scintillation counting and determination of 2DOG uptake as previously described.[43] Immunoblotting was conducted using the Jess Automated Western Blot System (Protein Simple #004-650). Antibodies used were phospho-AMPKα (Thr172; pAMPK [T172]) Antibody (Cell Signaling #2531) and eEF2 (Cell Signaling #2332).

RNA Extraction, Reverse Transcription, and Real-Time PCR

RNA was extracted from tissues with TRIzol Reagent (Invitrogen™ #15596026). RNA concentration and quality of RNA were measured (NanoDrop™ 2000 spectrophotometer; Thermo Scientific™ #ND-2000), and 500 ng of RNA was used for cDNA synthesis (Applied Biosystems #4368814). Semi-quantitative real-time PCR analysis was conducted using PowerUp™ SYBR™ Green Master Mix (Thermo Scientific™ #A25741). Relative expression levels for each gene of interest were calculated with the ΔΔCt method, using *Rn18s* as the normalization control. Primers used were *Bmal1* (5′-CACTGTCCCAGGCATTCCA-3′ FWD, 5′-TTCCTCCGCGATCATTCG-3′ REV), *Dbp* (5′-CCTGAGGAACAGAAGGATGA-3′ FWD, 5′-ATCTGGTTCTCCTTGAGTCTT-3′ REV), *Nr1d1* (5′-TGGCCTCAGGCTTCCACTATG-3′ FWD, 5′-CCGTTGCTTCTCTCTCTTGGG-3′ REV), and *Rn18s* (5′-GCTTAATTTGACTCAACACGGGA-3′ FWD, 5′-AGCTATCAATCTGTCAATCCTGTC-3′ REV).

Plasma Insulin

Plasma insulin was analyzed using an ELISA kit, per the manufacturer's instructions (80-INSMS-E-01; ALPCO Diagnostics).

Statistics

Statistical analyses were performed using Prism v10.0.2 (GraphPad Software Inc., La Jolla, CA, USA). For Figures 1 and 2, data were analyzed by either a 1- or 2-way analysis of variance or mixed-effects model; details regarding specific statistical tests for each figure are detailed in corresponding figure legends. For Figure 3, statistical significance of circadian rhythmicity was determined by a zero-amplitude test on the Cosinor.Online web application.[44] All data are expressed as mean ± SD. Significant differences ($P < .05$) are marked with "#" in figures. Sample sizes to detect a 20% difference with an a of 0.05 and a β of 80% were estimated using G*Power v3.1.9.6 and were based on means and standard deviations from the literature for maximal tetanic tension,[45] fatigability,[46] and contraction-stimulated glucose uptake.[47]

Results and Discussion

Intrinsic Skeletal Muscle Contractile Function Does Not Vary Over a 24 h Circadian Cycle

Studies suggest that circadian variation in skeletal muscle contractile function, such as anaerobic power output, concentric force production, and maximal torque production, is due to diurnal variation in the intrinsic contractile properties of the skeletal muscle.[11,13,17,48,49] Others suggest that daily variance in exercise capacity in mice may be due to distinct diurnal transcriptomic and metabolic signatures (eg, NAD^+, ZMP) in skeletal muscle.[50–52] Certainly, the time-of-day that exercise is undertaken has differential effects on the gene expression response to exercise.[51,53–55] However, a key gap in the field is that, to our knowledge, no studies have investigated skeletal muscle contractile function at multiple points throughout both the light and dark phases of the circadian cycle (ie, over a 24 h period).

In Study 1, body mass, muscle mass (TA, SOL, and EDL), liver and heart mass, and fasting glucose and insulin concertations were not different across the 4 timepoints (Table 1). For both EDL and SOL, while there was the expected effect of stimulation frequency on muscle stress production, contrary to our hypothesis and current thinking in the field, the stress–frequency relationship at low, moderate, or maximal stimulation frequency was not different across a 24 h circadian cycle, in either mus-

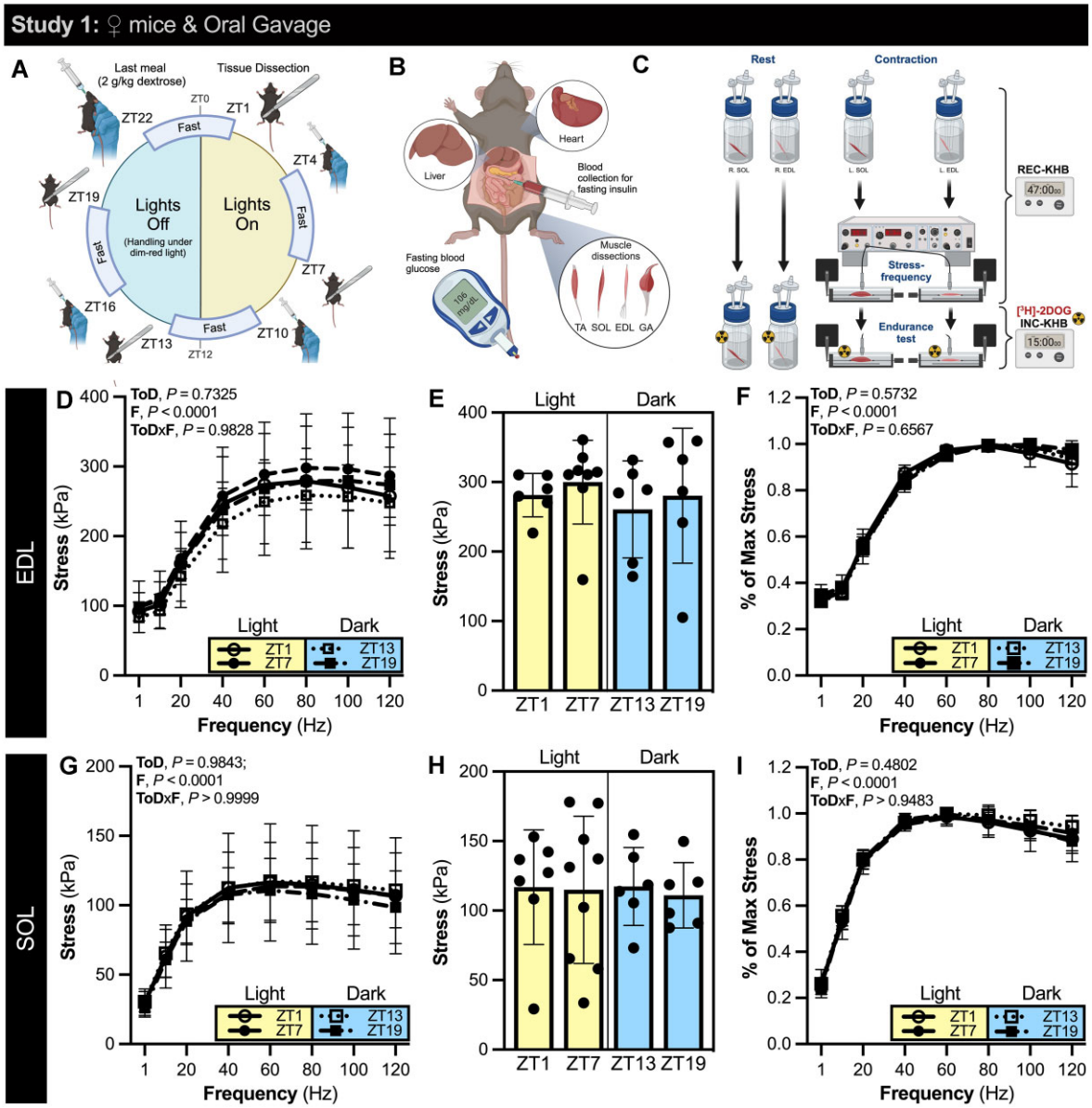

Figure 1. Intrinsic contractile function in 2 different skeletal muscles does not vary over a 24 h circadian cycle (Study 1). (A) Overview of the experimental timing for the 4 experimental groups for Study 1. Mice were orally gavaged with a standardized meal of 50% dextrose (2 g/kg) and then fasted for 3 h before tissue dissection, which occurred at zeitgeber time (ZT) 1, ZT7, ZT13, and ZT19. Vivarium lights were on at ZT0 (0600 h) and were off at ZT12 (1800 h). Thus, ZT1 and ZT7 were during the light/rest phase (yellow), while ZT13 and ZT19 were during the dark/active phase (blue). (B) Overview of the tissue dissection and collection procedure. TA, tibialis anterior; SOL, soleus; EDL, extensor digitorum longus; GA, gastrocnemius. (C) Overview of the *ex vivo* testing of intrinsic contractile function and endurance, and basal and contraction-stimulated [^{3}H]-2-deoxyglucose (2DOG) uptake in paired (R., Right; L., Left) SOL and EDL. KHB, Krebs–Henseleit buffer; REC-KHB, recovery KHB; INC-KHB, incubation KHB. (D) Stress–frequency relationship in the EDL (1-120 Hz, 300 ms train, 0.3 ms pulse, 35 V; $n = 6/8/6/6$ for ZT1/7/13/19, respectively). (E) Maximal tetanic stress in the EDL, extracted from the stress–frequency test ($n = 6/8/6/6$ for ZT1/7/13/19, respectively). (F) Stress–frequency relationship in the EDL, normalized to maximum tetanic stress ($n = 6/8/6/6$ for ZT1/7/13/19, respectively). (G) Stress–frequency relationship in the SOL (1-120 Hz, 400 ms train, 0.3 ms pulse, 35 V, $n = 7/9/6/6$ for ZT1/7/13/19, respectively). (H) Maximal tetanic stress for the SOL, extracted from the stress–frequency testing ($n = 7/9/6/6$ for ZT1/7/13/19, respectively). (I) Stress–frequency relationship in the SOL, normalized to maximum tetanic stress ($n = 7/9/6/6$ for ZT1/7/13/19, respectively). Data were reported as mean ± SD. Statistics: (D, E, G, and H) Repeated measures 2-way (ToD, time of day; F, stimulation frequency) ANOVA with Geisser–Greenhouse correction. (F and I) Ordinary 1-way ANOVA. Figures A, B, and C were created with BioRender.com (www.biorender.com), and confirmation of publication and licensing rights was obtained.

cle (EDL: Figure 1D and E, Supplementary Figure S3A-D [stress tracings], Table 1 [twitch parameters]; SOL: Figure 1G and H, Supplementary Figure S3E-H [stress tracings], Table 1 [twitch parameters]). Notably, while Kahn et al.[56] did not study contractile function across a range of stimulation frequencies, our findings of no difference in maximal tetanic stress in the EDL during the light phase are in line with their maximal tetanic stress data (personal communication with Dayanidhi S, Kahn RE, and Lieber RL). There was also no time-of-day effect in the EDL or SOL when expressing the stress frequency curve relative to maximum stress (Figure 1F and I, respectively). Additionally, contractile parameters, including peak twitch tension and time-to-peak tension, are indistinguishable between wild-type (WT) controls and mice with double knockout of core clock proteins, *Cry1* and

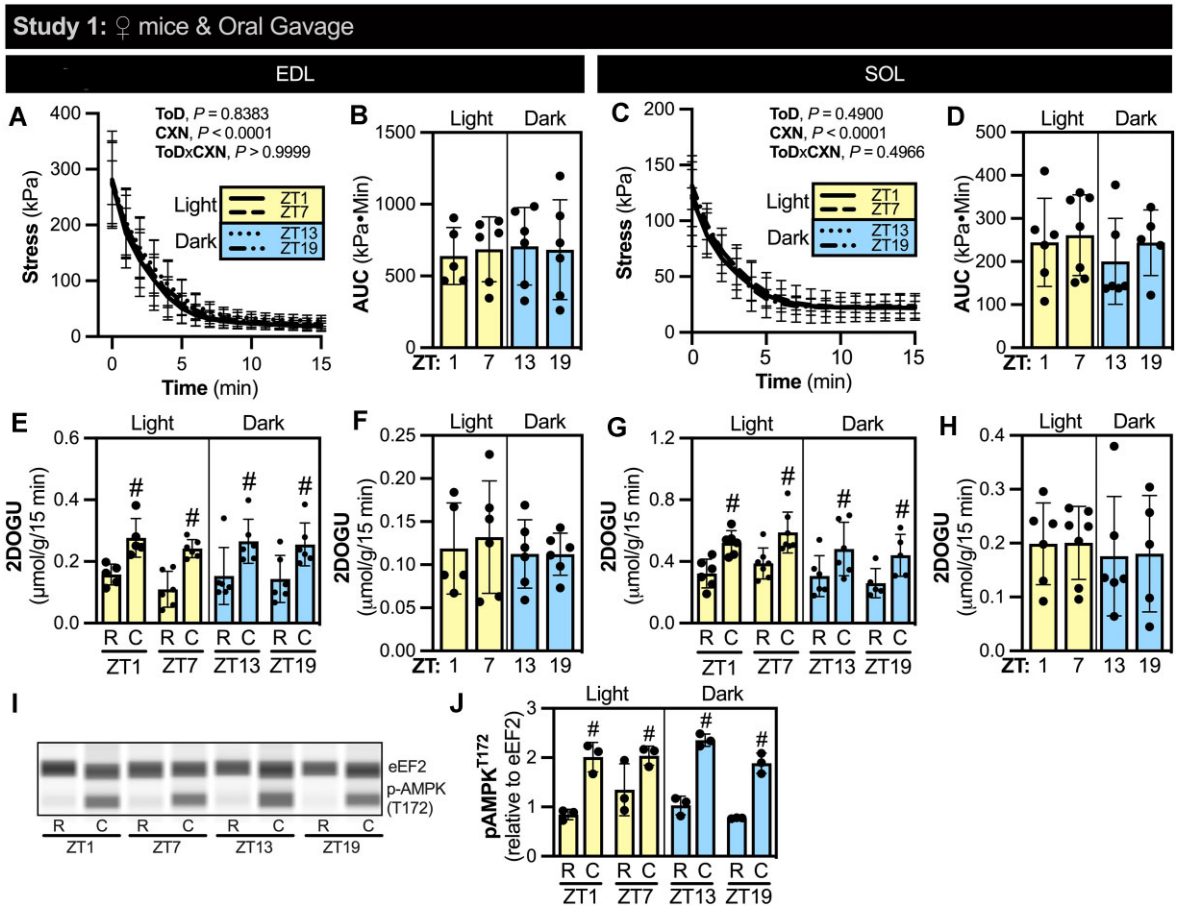

Figure 2. Muscle endurance and contraction-stimulated glucose uptake do not vary over a 24 h circadian cycle (Study 1). All measurements for all panels were conducted at zeitgeber time (ZT) 1, ZT7, ZT13, and ZT19, per the experimental design for Study 1. (A) Muscle stresses measured during the fatigue test for extensor digitorum longus (EDL) (n = 5/6/6/6 for ZT1/7/13/19, respectively). (B) Cumulative fatigue during the endurance test, measured by AUC (kPa Min) for EDL (n = 5/6/6/6 for ZT1/7/13/19, respectively). (C) Muscle stresses measured during the fatigue test for soleus (SOL) (n = 6/7/6/5 for ZT1/7/13/19, respectively). (D) Cumulative fatigue during the endurance test, measured by AUC (kPa Min), for SOL (n = 6/7/6/5 for ZT1/7/13/19, respectively). (E) 2-deoxyglucose (2DOG) uptake in rested (R) and contracted (C) EDL (n = 5/6/6/6 for ZT1/7/13/19, respectively). (F) Contraction-stimulated glucose uptake (Contraction 2DOG uptake—Rested 2DOG uptake) in EDL (n = 5/6/6/6 for ZT1/7/13/19, respectively). (G) 2DOG uptake in rested (R) and contracted (C) SOL (n = 6/7/6/5 for ZT1/7/13/19, respectively). (H) Contraction-stimulated glucose uptake in SOL (n = 6/7/6/5 for ZT1/7/13/19, respectively). (I) Representative immunoblot for eEF2 and pAMPK^{T172} in rested (R) and contracted (C) EDL, and, (J) quantification of pAMPK^{T172} in rested (R) vs. contracted (C) EDL, normalized to eEF2 (n = 3/3/3/3 for ZT1/7/13/19, respectively). Data were reported as mean ± SD. Statistics: (A and C) Repeated measures 2-way ANOVA with Geisser–Greenhouse correction, Tukey's multiple comparisons test. (B, D, F, and H) Ordinary 1-way ANOVA with Tukey's multiple comparison's test. (E, G, and J) Repeated measures 2-way ANOVA with Šídák's multiple comparison's test. #, $P < .05$ for R vs. C within each zeitgeber timepoint.

Cry2 (Cry1$^{-/-}$; Cry2$^{-/-}$ DKO), further supporting a lack of chronobiological/intrinsic muscle clock effect on skeletal muscle force-generating capacity.[57]

In Study 1, to control for potential effects of food intake on contractile function, all mice were orally gavaged with glucose 3 h before tissue dissection. However, oral gavage can be a stressful intervention in mice, and as such, it is possible that the stress of the oral gavage procedure overrides, resets, or otherwise masks any potential 24-h differences in contractile parameters. Addressing this possibility, Study 2 was identical to Study 1, except mice were not orally gavaged. Body and tissue masses and fasting glucose were not different between mice at the 2 timepoints studied, that is, ZT7 and ZT19 (Supplementary Table S1). Significantly, in line with our findings in Study 1, in both EDL and SOL we found no difference in the absolute or relative stress–frequency curves (EDL: Supplementary Figures S1C and S3I-J, and S1D, respectively; SOL: Supplementary Figure S1F and S3M-N, and S1G, respectively), twitch characteristics (Supplementary Table S1), or maximal tetanic stress (EDL: Supplementary Figure S1E; SOL: Supplementary Figure S1H]) between ZT7 and ZT19.

Furthermore, to address potential sex differences, in Study 3, we studied the EDL and SOL of male mice (which were not orally gavaged); body and muscle masses and fasting glucose were not different between mice at ZT7 versus ZT19 (Supplementary Table S2). Similar to our findings in female mice, the absolute and relative stress–frequency relationship (EDL: Supplementary Figure S2D and E, respectively; SOL: Supplementary Figure S2G and H, respectively), twitch characteristics (Supplementary Table S2), stress tracings (EDL, Supplementary Figure S3K and S3L; SOL, Supplementary Figure S3O and S3P), and maximal tetanic stress (Supplementary Figure S2F [EDL] and S2I [SOL]) were not different between ZT7 and ZT19 in either muscle. Taken together, these 3 independent studies demonstrate that the intrinsic contractile properties of skeletal muscle do not vary over the course of a 24 h circadian cycle in female or male mice, regardless of muscle type studied.

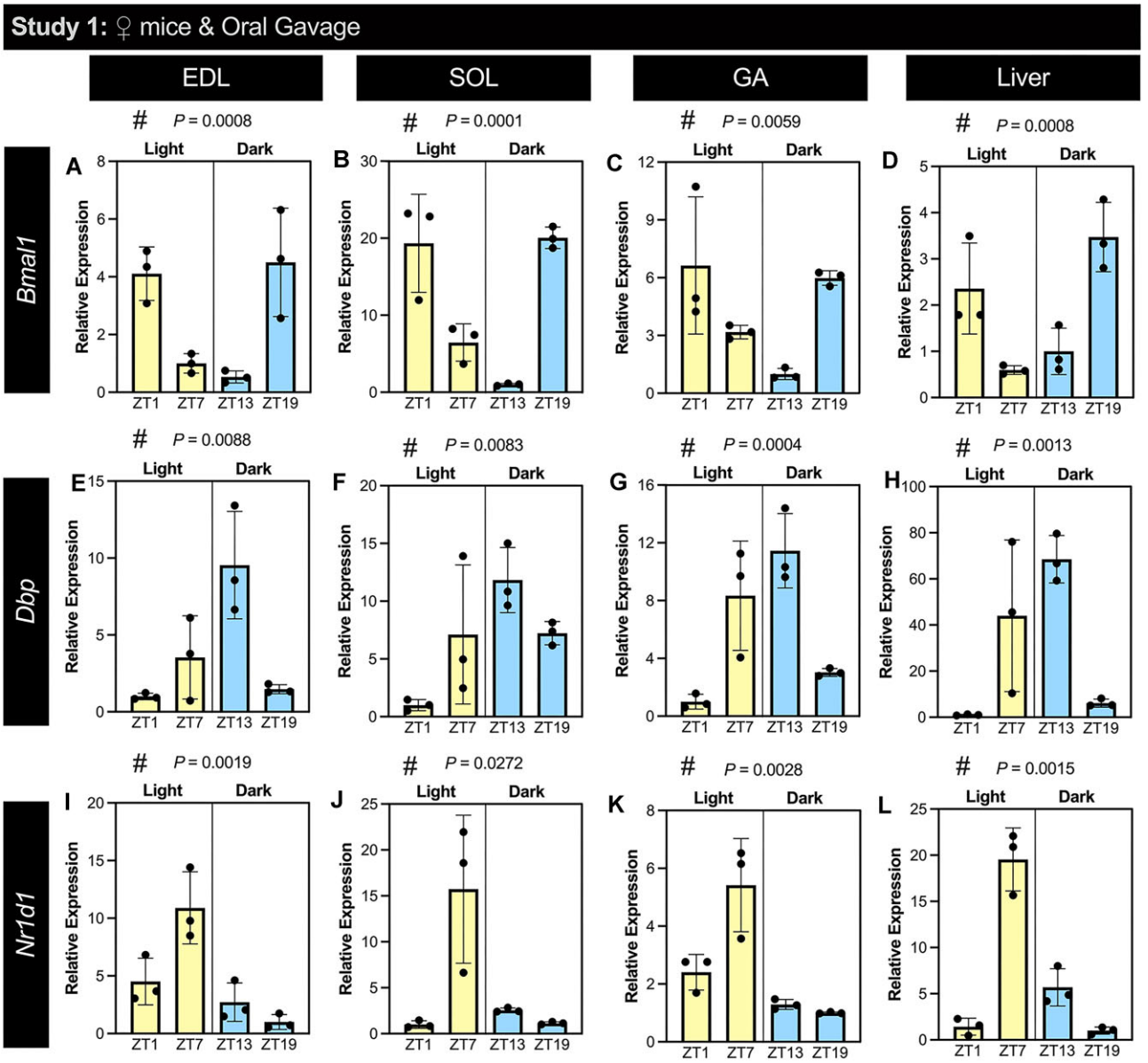

Figure 3. Normal circadian rhythmicity in the expression of core clock genes in skeletal muscle and liver. All measurements for all panels were conducted in extensor digitorum longus (EDL), soleus (SOL), gastrocnemius (GA), or liver that were collected at zeitgeber time (ZT) 1, ZT7, ZT13, and ZT19, per the experimental design for Study 1; $n = 3$ for all time points and tissues. mRNA expression of *Bmal1* (A-D), *Dbp* (E-H), and *Nr1d1* (I-L) normalized to *Rn18s* expression in EDL (A, E, and I), SOL (B, F, and J), GA (C, G, and K), and liver (D, H, and L). Data were reported as mean ± SD. Statistics: Zero amplitude test for circadian rhythmicity was reported for each gene-tissue combination above each panel; # indicates $P < .05$.

Muscle Endurance Does Not Vary Over a 24 h Circadian Cycle

Similar to the effects of time-of-day on muscle force-generating capacity, there is a documented effect of time-of-day on endurance exercise performance across species, including in humans[48,58–60] and rodents.[51,54,61,62] A potential role of the circadian clock in endurance performance is further substantiated by the fact that mouse models with disruption of the circadian clock (eg, *Per2*$^{-/-}$ and *Bmal1*$^{-/-}$ mice) do not demonstrate variability in exercise performance within and/or between the light and dark phases of the circadian cycle.[51,62–64] Nevertheless, while some studies demonstrate that treadmill running performance in mice differs between the light and dark phases in wild-type mice,[51,65] importantly, this is not a universal finding.[53] While variability in these results could be due to many factors, including the treadmill testing approach used (eg, electrical shock or not), as well as other common factors impacted by time-of-day, such as body temperature, motivation to exercise, food intake, muscle glycogen content, central nervous system arousal, and pain tolerance, intrinsic muscle properties have been emphasized as an underlying mediator,[13] although this concept has not been empirically tested.

Thus, to specifically study intrinsic skeletal muscle endurance over a 24 h circadian cycle, in Studies 1, 2, and 3, we conducted a fatiguing contraction protocol in the EDL and SOL. To confirm that the muscles were not fatigued before

Table 1. Study #1 (Females, Gavaged). Body and Tissue Masses, Fasting Glucose and Insulin, and Twitch Characteristics at Different Times-of-Day

		ZT1	ZT7	ZT13	ZT19	*P*-value	*n* (ZT1/7/13/19)
Body and tissue mass							
Body mass (g)		26.7 ± 1.7	25.8 ± 2.8	25.6 ± 1.2	25.6 ± 1.5	.9587	7/9/8/6
EDL mass (mg)		11.5 ± 1.5	11.0 ± 2.1	12.2 ± 0.9	12.0 ± 1.2	.3432	7/9/6/6
SOL mass (mg)		11.3 ± 1.4	11.2 ± 0.6	11.8 ± 1.3	11.5 ± 0.7	.6616	7/9/6/6
TA mass (mg)		51.6 ± 3.0	50.3 ± 5.2	51.4 ± 4.4	49.6 ± 3.4	.7709	7/9/8/6
Heart mass (mg)		121.1 ± 8.5	124.0 ± 18.1	128.3 ± 11.5	113.7 ± 12.2	.1995	7/9/8/6
Liver mass (g)		1.321 ± 0.148	1.198 ± 0.122	1.184 ± 0.174	1.167 ± 0.152	.9268	7/9/8/6
Fasting glucose and insulin							
Fasting glucose (mg/dL)		131 ± 14	119 ± 16	109 ± 14	112 ± 21	.4027	7/9/8/6
Fasting insulin (ng/mL)		0.85 ± 0.09	0.88 ± 0.12	0.82 ± 0.11	0.83 ± 0.07	.7686	4/4/4/4
Contractile function: twitch characteristics							
EDL	Peak twitch tension (kPa)	92.0 ± 13.3	98.7 ± 20.4	83.0 ± 21.1	98.7 ± 36.9	.6229	6/8/6/6
	Time-to-peak tension (ms)	25.3 ± 1.4	24.4 ± 2.2	25.3 ± 2.2	23.7 ± 2.1	.9691	6/8/6/6
SOL	Peak twitch tension (kPa)	29.6 ± 10.2	30.5 ± 14.4	30.5 ± 7.3	26.0 ± 5.4	.8507	7/9/6/6
	Time-to-peak tension (ms)	60.0 ± 4.5	55.8 ± 5.4	62.8 ± 7.1	64.3 ± 16.0	.2905	7/9/6/6

Abbreviations: EDL, extensor digitorum longus; SOL, soleus; TA, tibialis anterior; ZT, zeitgeber time.
Statistics: Within each row, data were analyzed by 1-way ANOVA with Tukey's multiple comparison's test. Data are mean + SD.

starting the fatiguing protocol, we compared the stresses for the first contraction of the fatigue protocol to the corresponding stimulation frequency from the stress–frequency curve. Importantly, there was no significant difference in the stresses between these time points in the EDL or SOL (Study 1: Supplementary Figure S4A and S4B, respectively; Study 2: Supplementary Figure S4C and S4D, respectively; Supplementary Figure S4E and S4F, respectively). During the endurance protocol in Study 1 (in which mice were orally gavaged), muscle stresses robustly decreased in response to repeated contractions in the EDL (Figure 2A and B) and SOL (Figure 2C and D). Significantly, however, the rate of fatigability did not differ within the light or dark phases, or between the light and dark phases, in either muscle. Similarly, in Study 2 (female, no gavage) and Study 3 (male, no gavage), there was no difference in the rate of fatigability of the EDL or SOL when comparing ZT7 and ZT19 (EDL—Study 2: Supplementary Figure S5A and S5B, Study 3: Supplementary Figure S6A and S6B; SOL—Study 2: Supplementary Figure S5C and S5D, Study 3: Supplementary Figure S6C and S6D). Interestingly, these findings, which suggest no time-of-day effect on fatigability, are supported by the fact that intrinsic endurance of the SOL and EDL is not different between *Cry1*$^{-/-}$; *Cry2*$^{-/-}$ *DKO* mice and WT controls.[57]

As expected, pAMPK (T172) was significantly increased by the fatiguing protocol, but there was no time-of-day variability within the resting or contracting groups (Figure 2I and J). This lack of an effect of time-of-day on basal or exercise-mediated activation of pAMPK (T172) in skeletal muscle is in line with work by others,[51] but differs from findings in murine hypothalamus and embryonic fibroblasts, which demonstrate circadian oscillations in pAMPK (T172).[66] In summary, contrary to our hypothesis and current thinking in the field, intrinsic skeletal muscle endurance does not vary over the course of a 24-h circadian cycle in female or male mice, regardless of muscle type.

Contraction-Stimulated Glucose Uptake by Skeletal Muscle Does Not Vary By Time-of-Day

To meet the energetic demands of contraction, skeletal muscle glucose uptake increases over time and/or with increasing intensity of contraction.[67–70] In addition to meeting the energetic demands of exercise, contraction-stimulated glucose uptake is important to glycemic control, with exercise being a cornerstone intervention for treating or preventing clinical hyperglycemia.[23] Nevertheless, while studies[34–36] demonstrate time-of-day effects of exercise training on glycemic control, we studied whether this might be due to intrinsic changes contraction-stimulated glucose uptake by muscle. As expected, in Study 1, there was a robust effect of contraction to increase muscle glucose uptake as compared to the contralateral rested muscle in the EDL (~85% higher in CXN vs. Rest; Figure 2E) and SOL (~59% higher in CXN vs. Rest; Figure 2G). However, there was no time-of-day difference in 2DOG uptake when comparing within the rested or contracted muscles. As a result, contraction-stimulated 2DOG uptake (calculated as: CXN 2DOG uptake—Rest 2DOG uptake) was not different across the 24 h circadian cycle in the EDL or SOL (Figure 2F and H, respectively). Notably, this lack of difference in contraction-stimulated glucose uptake over the 24 h period was not due to the gavage protocol in Study 1, as contraction-stimulated glucose uptake in Study 2 (ie, no oral gavage) was comparable when comparing ZT7 and ZT19 (EDL: Supplementary Figure S5E and S5F; SOL: Supplementary Figure S5G and S5H). Thus, contrary to current thinking in the field, contraction-stimulated glucose uptake does not vary over the course of a 24 h circadian cycle in mice, regardless of muscle type.

Skeletal Muscle and Liver Exhibit Circadian Rhythmicity in Gene Expression

Food intake and carbohydrate content of meals are important contributors to exercise performance[21,39,71–73] and a powerful zeitgeber for the circadian cycle.[40,74] Moreover, systemic glucose or insulin (through carbohydrate intake) availability can profoundly impact muscle gene expression.[75] To date, to our knowledge, all studies that have focused on the role of time-of-day on exercise performance have not controlled for the potential effect of food/carbohydrate availability, which may underlie some of the variability in findings in the field. Addressing this, in our study, all mice received a standardized glucose meal 3 h before tissue dissection. Accordingly, we wanted to validate that there was circadian rhythmicity in skeletal muscle and the liver (as an

example of another important metabolic tissue). Thus, we measured mRNA expression levels of the core clock regulator *Bmal1*, and the clock output genes *Dbp* and *Nr1d1* in EDL (Figure 3A, E, and I, respectively), SOL (Figure 3B, F, and J, respectively), GA (Figure 3C, G, and K, respectively), and liver (Figure 3D, H, and L, respectively). In these 3 muscles and the liver, there was significant circadian rhythmicity of the 3 genes (as measured by zero-amplitude testing), with circadian changes in these genes being consistent with previous studies in skeletal muscle[50] and liver.[76] These data demonstrate that circadian rhythmicity, regardless of prior food intake, is strongly entrained in skeletal muscle and liver; this finding is in line with a recent study showing that circadian rhythmicity in the core clock genes even when subjects were fed hourly isocaloric meals.[77] It also demonstrates that the lack of effect of time-of-day on skeletal muscle contractile function, fatigability, or contraction-stimulated glucose uptake is not due to a lack of circadian rhythmicity in skeletal muscle or liver gene expression.

Summary

Contrary to our hypothesis and current thinking in the field, we found that the intrinsic contractile functionality and capacity for contraction simulated glucose uptake of 2 skeletal muscles with robustly different myosin heavy chain compositions does not differ over a 24 h circadian cycle in mice. Thus, circadian variation in exercise performance and the glycemia-reducing benefits of exercise do not appear to be due to chronobiological variation in intrinsic muscle function or contraction-stimulated glucose uptake, respectively. Notably, while *ex vivo* contractions are a useful approach to assess intrinsic skeletal muscle mechanical properties, these contractions are inherently different from those that occur in vivo during exercise. By extension, future studies may use a more physiologically relevant experimental set-up (eg, *in situ* contractions) to study time-of-effects on contractile function and contraction-stimulated glucose uptake. Finally, it will be interesting in future work to further define the underlying factor(s) (eg, body or muscle temperature, meal timing, muscle glycogen, motor unit recruitment, humoral factors, etc.) that mediate chronobiological variation in exercise performance or how exercise modulates systemic glycemia.

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Author Contributions

Liam S. Fitzgerald; conception and design, acquisition of data, analysis and interpretation of data, drafting and revising the article. Shannon N. Bremner; acquisition, analysis and interpretation of data, revising the article. Samuel R. Ward; analysis and interpretation of data, revising the article and funding acquisition. Yoshitake Cho: acquisition of data, analysis and interpretation of data, revising the article. Simon Schenk; conception and design, acquisition of data, analysis and interpretation of data, drafting and revising the article and funding acquisition.

Supplementary Material

Supplementary material is available at the *APS Function* online.

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Conflict of Interest

The authors declare they have no competing interests.

Data Availability

All data are available upon a request to the corresponding author.

References

1. Logan RW, McClung CA. Rhythms of life: circadian disruption and brain disorders across the lifespan. *Nat Rev Neurosci* 2018;**20**(1):49–65.
2. Millar-Craig MW, Bishop CN, Raftery EB. Circadian variation of blood-pressure. *Lancet* 1978;**1**(8068):795–797.
3. Krauchi K, Wirz-Justice A. Circadian rhythm of heat production, heart rate, and skin and core temperature under unmasking conditions in men. *Am J Physiol* 1994;**267**(3 Pt 2):R819–R829.
4. Reilly T, Atkinson G, Edwards B, Waterhouse J, Farrelly K, Fairhurst E. Diurnal variation in temperature, mental and physical performance, and tasks specifically related to football (soccer). *Chronobiol Int* 2007;**24**(3):507–519.
5. Atkinson G, Speirs L. Diurnal variation in tennis service. *Percept Mot Skills* 1998;**86**(3 Pt 2):1335–1338.
6. Reilly T, Down A. Circadian variation in the standing broad jump. *Percept Mot Skills* 1986;**62**(3):830–830.
7. Edwards BJ, Lindsay K, Waterhouse J. Effect of time of day on the accuracy and consistency of the badminton serve. *Ergonomics* 2005;**48**(11-14):1488–1498.
8. Atkinson G, Reilly T. Circadian variation in sports performance. *Sports Med* 1996;**21**(4):292–312.

9. Baxter C, Reilly T. Influence of time of day on all-out swimming. *Br J Sports Med* 1983;**17**(2):122.
10. Rodahl A, O'Brien M, Firth RGR. Diurnal variation in performance of competitive swimmers. *J Sports Med Phys Fitness* 1976;**16**(1):72–76.
11. Martin A, Carpentier A, Guissard N, Van Hoecke J, Duchateau J. Effect of time of day on force variation in human muscle. *Muscle Nerve* 1999;**22**:10 1380–1387.
12. Czelusniak O, Favreau E, Ives SJ. Effects of warm-up on sprint swimming performance, rating of perceived exertion, and blood lactate concentration: a systematic review. *J Funct Morphol Kinesiol* 2021;**6**(4):85.
13. Douglas CM, Hesketh SJ, Esser KA. Time of day and muscle strength: a circadian output? *Physiology* 2021;**36**(1):44–51.
14. Bessot N, Nicolas A, Moussay S, Gauthier A, Sesboüé B, Davenne D. The effect of pedal rate and time of day on the time to exhaustion from high-intensity exercise. *Chronobiol Int* 2006;**23**(5):1009–1024.
15. Bergh U, Ekblom B. Influence of muscle temperature on maximal muscle strength and power output in human skeletal muscles. *Acta Physiol Scand* 1979;**107**(1):33–37.
16. Harrison SM, Bers DM. Influence of temperature on the calcium sensitivity of the myofilaments of skinned ventricular muscle from the rabbit. *J Gen Physiol* 1989;**93**(3):411–428.
17. Nicolas A, Gauthier A, Michaut A, Davenne D. Effect of circadian rhythm of neuromuscular properties on muscle fatigue during concentric and eccentric isokinetic actions. *Isokinet Exerc Sci* 2007;**15**(2):117–129.
18. Gueldich H, Zarrouk N, Chtourou H, Zghal F, Sahli S, Rebai H. Electrostimulation training effects on diurnal fluctuations of neuromuscular performance. *Int J Sports Med* 2017;**38**(1):41–47.
19. Sedliak M, Finni T, Peltonen J, Hakkinen K. Effect of time-of-day-specific strength training on maximum strength and EMG activity of the leg extensors in men. *J Sports Sci* 2008;**26**(10):1005–1014.
20. Koch CE, Begemann K, Kiehn JT, et al. Circadian regulation of hedonic appetite in mice by clocks in dopaminergic neurons of the VTA. *Nat Commun* 2020;**11**(1):1–11.
21. Kerksick CM, Arent S, Schoenfeld BJ, et al. International society of sports nutrition position stand: nutrient timing. *J Int Soc Sports Nutr* 2017;**14**(1):33.
22. Kahn RE, Lieber RL, Meza G, et al. Time-of-day effects on ex vivo muscle contractility following short-term satellite cell ablation. *Am J Physiol Cell Physiol* 2024;**327**(1):C213–C219.
23. Knowler WC, Barrett-Connor E, Fowler SE, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 2002;**346**(6):393–403.
24. Evans EM, Racette SB, Peterson LE, Villareal DT, Greiwe JS, Holloszy JO. Aerobic power and insulin action improve in response to endurance exercise training in healthy 77-87 yr olds. *J Appl Physiol (1985)* 2005;**98**(1):40–45.
25. Bajpeyi S, Tanner CJ, Slentz CA, et al. Effect of exercise intensity and volume on persistence of insulin sensitivity during training cessation. *J Appl Physiol (1985)* 2009;**106**(4):1079–1085.
26. Loimaala A, Huikuri HV, Kööbi T, Rinne M, Nenonen A, Vuori I. Exercise training improves baroreflex sensitivity in type 2 diabetes. *Diabetes* 2003;**52**(7):1837–1842.
27. Cohen ND, Dunstan DW, Robinson C, Vulikh E, Zimmet PZ, Shaw JE. Improved endothelial function following a 14-month resistance exercise training program in adults with type 2 diabetes. *Diabetes Res Clin Pract* 2008;**79**(3):405–411.
28. Balducci S, Iacobellis G, Parisi L, et al. Exercise training can modify the natural history of diabetic peripheral neuropathy. *J Diabetes Complications* 2006;**20**(4):216–223.
29. Constable SH, Favier RJ, Cartee GD, Young DA, Holloszy JO. Muscle glucose transport: interactions of in vitro contractions, insulin, and exercise. *J Appl Physiol* 1988;**64**(6):2329–2332. https://doi.org/10.1152/JAPPL.1988.64.6.2329.
30. Goodyear LJ, Kahn BB. Exercise, glucose transport, and insulin sensitivity. *Annu Rev Med* 1998;**49**:235–261.
31. Goodyear LJ, King PA, Hirshman MF, Thompson CM, Horton ED, Horton ES. Contractile activity increases plasma membrane glucose transporters in absence of insulin. *Am J Physiol* 1990;**258**(4 Pt 1):E667–72. https://doi.org/10.1152/AJPENDO.1990.258.4.E667
32. Ploug T, Galbo H, Richter EA. Increased muscle glucose uptake during contractions: no need for insulin. *Am J Physiol* 1984;**247**(6 Pt 1):E726–E731.
33. Riddell MC, Turner LV, Patton SR. Is there an optimal time of day for exercise? A commentary on when to exercise for people living with type 1 or type 2 diabetes. *Diabetes Spectr* 2023;**36**(2):146.
34. Qian J, Xiao Q, Walkup MP, et al. Association of timing of moderate-to-vigorous physical activity with changes in glycemic control over 4 years in adults with type 2 diabetes from the look AHEAD trial. *Diabetes Care* 2023;**46**(7):1417–1424.
35. Mancilla R, Brouwers B, Schrauwen-Hinderling VB, Hesselink MKC, Hoeks J, Schrauwen P. Exercise training elicits superior metabolic effects when performed in the afternoon compared to morning in metabolically compromised humans. *Physiol Rep* 2021;**8**(24):e14669. https://doi.org/10.14814/PHY2.14669
36. Savikj M, Gabriel BM, Alm PS, et al. Afternoon exercise is more efficacious than morning exercise at improving blood glucose levels in individuals with type 2 diabetes: a randomised crossover trial. *Diabetologia* 2019;**62**(2):233–237.
37. Sato S, Basse AL, Schönke M, et al. Time of exercise specifies the impact on muscle metabolic pathways and systemic energy homeostasis. *Cell Metab* 2019;**30**(1):92–110.e4.
38. Burkholder TJ, Fingado B, Baron S, Lieber RL. Relationship between muscle fiber types and sizes and muscle architectural properties in the mouse hindlimb. *J Morphol* 1994;**221**(2):177–190.
39. Coyle EF, Coggan AR, Hemmert MK, Lowe RC, Walters TJ. Substrate usage during prolonged exercise following a preexercise meal. *J Appl Physiol* 1985;**59**(2):429–433.
40. Aoyama S, Shibata S. Time-of-day-dependent physiological responses to meal and exercise. *Front Nutr* 2020;**7**:18
41. Hoggatt, AF., Hoggatt, J., Honerlaw, M. and Pelus, LM. (2010) **49**, 329–34 20587165
42. Spencer RL, Chun LE, Hartsock MJ, Woodruff ER. Glucocorticoid hormones are both a major circadian signal and major stress signal: how this shared signal contributes to a dynamic relationship between the circadian and stress systems. *Front Neuroendocrinol* 2018;**49**:52–71.
43. Martins VF, LaBarge SA, Stanley A, et al. p300 or CBP is required for insulin-stimulated glucose uptake in skeletal muscle and adipocytes. *JCI Insight* 2022;**7**(1): e141344. https://doi.org/10.1172/JCI.INSIGHT.141344
44. Molcan L. Time distributed data analysis by Cosinor.Online application. *Biorxiv* 2023. Published online January 18. https://doi.org/10.1101/805960

45. Svensson K, LaBarge SA, Sathe A, et al. p300 and cAMP response element-binding protein-binding protein in skeletal muscle homeostasis, contractile function, and survival. *J Cachexia Sarcopenia Muscle* 2020;**11**(2):464.
46. Jensen TE, Sylow L, Rose AJ, et al. Contraction-stimulated glucose transport in muscle is controlled by AMPK and mechanical stress but not sarcoplasmatic reticulum Ca2+ release. *Mol Metab* 2014;**3**(7):742–753.
47. Kang JH, Park JE, Dagoon J, et al. Sirtuin 1 is not required for contraction-stimulated glucose uptake in mouse skeletal muscle. *J Appl Physiol* 2021;**130**(6):1893.
48. Chtourou H, Zarrouk N, Chaouachi A, et al. Diurnal variation in Wingate-test performance and associated electromyographic parameters. *Chronobiol Int* 2011;**28**(8):706–713.
49. Guette M, Gondin J, Martin A. Time-of-day effect on the torque and neuromuscular properties of dominant and non-dominant quadriceps femoris. *Chronobiol Int* 2005;**22**(3):541–558.
50. McCarthy JJ, Andrews JL, McDearmon EL, et al. Identification of the circadian transcriptome in adult mouse skeletal muscle. *Physiol Genomics* 2007;**31**(1):86.
51. Ezagouri S, Zwighaft Z, Sobel J, et al. Physiological and molecular dissection of daily variance in exercise capacity. *Cell Metab* 2019;**30**(1):78–91.e4.
52. Peek CB, Affinati AH, Ramsey KM, et al. Circadian clock NAD+ cycle drives mitochondrial oxidative metabolism in mice. *Science* 2013;**342**(6158). https://doi.org/10.1126/SCIENCE.1243417/SUPPL_FILE/PEEK.SM.REVISED.PDF
53. Casanova-Vallve N, Duglan D, Vaughan ME, et al. Daily running enhances molecular and physiological circadian rhythms in skeletal muscle. *Mol Metab* 2022;**61**:101504
54. Maier G, Delezie J, Westermark PO, Santos G, Ritz D, Handschin C. Transcriptomic, proteomic and phosphoproteomic underpinnings of daily exercise performance and zeitgeber activity of training in mouse muscle. *J Physiol* 2022;**600**(4):769.
55. Mirizio GG, Nunes RSM, Vargas DA, Foster C, Vieira E. Time-of-day effects on short-duration maximal exercise performance. *Sci Rep* 2020;**10**(1):1–17.
56. Kahn RE, Lieber RL, Meza G, et al. Time-of-day effects on *ex vivo* muscle contractility following short-term satellite cell ablation. *Am J Physiol-Cell Physiol* 2024. Published online April 8, 2024. doi:10.1152/AJPCELL.00157.2024.
57. Jordan SD, Kriebs A, Vaughan M, et al. CRY1/2 Selectively repress pparδ and limit exercise capacity. *Cell Metab* 2017;**26**(1):243–255.e6.
58. Souissi N, Gauthier A, Sesboüé B, Larue J, Davenne D. Effects of regular training at the same time of day on diurnal fluctuations in muscular performance. *J Sports Sci* 2002;**20**(11):929–937.
59. van Moorsel D, Hansen J, Havekes B, et al. Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity. *Mol Metab* 2016;**5**(8):635–645.
60. Gemmink A, Daemen S, Wefers J, et al. Twenty-four hour rhythmicity in mitochondrial network connectivity and mitochondrial respiration; a study in human skeletal muscle biopsies of young lean and older individuals with obesity. *Mol Metab* 2023;**72**:101727
61. Wolff G, Esser KA. Scheduled exercise phase shifts the circadian clock in skeletal muscle. *Med Sci Sports Exerc* 2012;**44**(9):1663–1670.
62. Adamovich Y, Dandavate V, Ezagouri S, et al. Clock proteins and training modify exercise capacity in a daytime-dependent manner. *Proc Natl Acad Sci USA* 2021;**118**(35): e2101115118. https://doi.org/10.1073/PNAS.2101115118/-/DCSUPPLEMENTAL
63. Xin H, Huang R, Zhou M, et al. Daytime-restricted feeding enhances running endurance without prior exercise in mice. *Nat Metab* 2023;**5**(7):1236–1251.
64. Hesketh SJ, Sexton CL, Wolff CA, Viggars MR, Esser KA. Early morning run-training results in enhanced endurance performance adaptations in mice. *bioRxiv*. 2023. https://doi.org/10.1101/2023.09.18.557933
65. Maier G, Delezie J, Westermark PO, Santos G, Ritz D, Handschin C. Transcriptomic, proteomic and phosphoproteomic underpinnings of daily exercise performance and zeitgeber activity of training in mouse muscle. *J Physiol* 2022;**600**(4):769–796.
66. Um JH, Pendergast JS, Springer DA, et al. AMPK regulates circadian rhythms in a tissue- and isoform-specific manner. *PLoS One* 2011;**6**(3):e18450.
67. Richter EA, Hargreaves M. Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev* 2013;**93**: 993–1017.
68. Fell RD, Terblanche SE, Ivy JL, Young JC, Holloszy JO. Effect of muscle glycogen content on glucose uptake following exercise. *J Appl Physiol Respir Environ Exerc Physiol* 1982;**52**(2):434–437.
69. Ploug T, Galbo H, Richter EA. Increased muscle glucose uptake during contractions: no need for insulin. *Am Physiol Soc* 1984;**10**(6):E726–E731. https://doi.org/10.1152/AJPENDO.1984.247.6.E726
70. Romijn JA, Coyle EF, Sidossis LS, et al. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol* 1993;**265**(3 Pt 1):E380–E391.
71. Fielding RA, Costill DL, Fink WJ, King DS, Hargreaves M, Kovaleski JE. Effect of carbohydrate feeding frequencies and dosage on muscle glycogen use during exercise. *Med Sci Sports Exerc* 1985;**17**(4):472–476.
72. Anantaraman R, Carmines AA, Gaesser GA, Weltman A. Effects of carbohydrate supplementation on performance during 1 hour of high-intensity exercise. *Int J Sports Med* 1995;**16**(7):461–465.
73. Costill DL, Sherman WM, Fink WJ, Maresh C, Witten M, Miller JM. The role of dietary carbohydrates in muscle glycogen resynthesis after strenuous running. *Am J Clin Nutr* 1981;**34**(9):1831–1836.
74. Stephan FK. The "other" circadian system: food as a Zeitgeber. *J Biol Rhythms* 2002;**17**(4):284–292.
75. Batista TM, Garcia-Martin R, Cai W, et al. Multi-dimensional transcriptional remodeling by physiological insulin In vivo. *Cell Rep* 2019;**26**(12):3429–3443.e3.
76. Guan D, Lazar MA. Circadian regulation of gene expression and metabolism in the liver. *Semin Liver Dis* 2022;**42**(2): 113.
77. Smith HA, Templeman I, Davis M, et al. Characterising 24-h skeletal muscle gene expression alongside metabolic & endocrine responses under diurnal conditions. *J Clin Endocrinol Metab* 2024:dgae350. https://doi.org/10.1210/CLINEM/DGAE350

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