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Ann-Marie Martin,[1] Jeffrey A. Elliott,[2] Pat Duffy,[1] Catriona M. Blake,[1] Sarra Ben Attia,[1] Lisa M. Katz,[1] John A. Browne,[1] Vivian Gath,[1] Beatrice A. McGivney,[1] Emmeline W. Hill,[1] and Barbara A. Murphy[1]

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Martin A, Elliott JA, Duffy P, Blake CM, Attia SB, Katz LM, Browne JA, Gath V, McGivney BA, Hill EW, Murphy BA. Circadian regulation of locomotor activity and skeletal muscle gene expression in the horse. *J Appl Physiol* 109: 1328–1336, 2010. First published September 16, 2010; doi:10.1152/japplphysiol.01327.2009.—Circadian rhythms are innate 24-h cycles in behavioral and biochemical processes that permit physiological anticipation of daily environmental changes. Elucidating the relationship between activity rhythms and circadian patterns of gene expression may contribute to improved human and equine athletic performance. Six healthy, untrained mares were studied to determine whether locomotor activity behavior and skeletal muscle gene expression reflect endogenous circadian regulation. Activity was recorded for three consecutive 48-h periods: as a group at pasture (P), and individually stabled under a light-dark (LD) cycle and in constant darkness (DD). Halter-mounted Actiwatch-L data-loggers recorded light exposure and motor activity. Analysis of mean activity (average counts/min, activity bouts/day, average bout length) and cosinor parameters (acrophase, amplitude, mesor, goodness of fit) revealed a predominantly ultradian (8.9 $\pm$ 0.7 bouts/24 h) and weakly circadian pattern of activity in all three conditions (P, LD, DD). A more robust circadian pattern was observed during LD and DD. Muscle biopsies were obtained from the middle gluteal muscles every 4 h for 24 h under DD. One-way qRT-PCR results confirmed the circadian expression ($P < 0.05$) of six core clock genes (*Arntl, Per1, Per2, Nr1d1, Nr1d2, Dbp*) and the muscle-specific transcript, *Myf6.* Additional genes, *Ucp3*, *Nrip1,* and *Vegfa,* demonstrated *P* values approaching significance. These findings demonstrate circadian regulation of muscle function and imply that human management regimes may strengthen, or unmask, equine circadian behavioral outputs. As exercise synchronizes circadian rhythms, our findings provide a basis for future work determining peak times for training and competing horses, to reduce injury and to achieve optimal performance.

equine; circadian; muscle; clock genes; activity rhythms

CIRCADIAN RHYTHMS are approximate 24-h cycles in the behavioral, physiological, and biochemical processes of organisms. Daily oscillations of physiological variables such as locomotor activity, body temperature, heart rate, blood pressure, and plasma hormone concentration have been described in a multitude of species (11, 39). These endogenous, self-sustaining rhythms are ubiquitous across mammals and can be entrained to external cues (zeitgebers) such as light, temperature, feeding, and social interactions (4, 18, 43, 52). Of these zeitgebers, the environmental light-dark (LD) cycle is the primary signal for coordination of internal time to the earth's 24-h rotation (43). Synchronization between endogenous daily rhythms and the environment ensures optimum survival of organisms by permitting physiological anticipation of rhythmic environmental changes in light, temperature, humidity, food availability, and predation pressure (34, 59).

Mammalian circadian rhythms are ultimately regulated by internal molecular clocks that exist as a hierarchy within the mammalian circadian system. The central pacemaker or "master clock" is situated in the suprachiasmatic nucleus (SCN) of the hypothalamus and orchestrates, via neural and humoral signals, numerous semiautonomous peripheral clocks located in tissues throughout the organism (26). The molecular clockwork mechanism is composed of a series of gene-protein-gene autoregulatory feedback loops (46). The primary feedback loop comprises a specific set of core clock genes: *Arntl* (aryl hydrocarbon receptor nuclear translocator-like), *Clock* (circadian locomotor output control kaput*)*, *Per1* (period homolog 1), *Per2* (period homolog 2), *Per3* (period homolog 3), *Cry1* [cryptochrome 1 (photolyase-like)], and *Cry2* [cryptochrome 2 (photolyase-like)] (24). The positive axis of this loop is generated by transcription and translation of the basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded) transcription factors, *Clock* and *Arntl* (46). CLOCK/ARNTL proteins heterodimerize and bind to E-box enhancers upstream of *Per* and *Cry* genes to activate their transcription (16, 21). In turn, PER and CRY proteins form a complex that inhibits CLOCK/ARNTL activity, thus repressing their own transcription and completing the negative axis of the primary feedback loop (26). The orphan nuclear receptors, *Rora* (RAR-related orphan receptor A)*, Nr1d1* (nuclear receptor subfamily 1, group D, member 1), and *Nr1d2* (nuclear receptor subfamily 1, group D, member 2), constitute a secondary feedback loop in the clockwork mechanism. *Rora* acts to initiate *Arntl* transcription while *Nr1d1* and *Nr1d2* repress its expression (19, 50).

In addition to the core clock genes, rodent studies have revealed that up to 10% of a peripheral tissue's transcriptome undergoes circadian regulation (38, 53). These clock-controlled genes (CCGs) are largely responsible for the specificity and temporal variation in individual tissue function. For example, the demonstration that metabolic genes undergo circadian regulation in mouse skeletal muscle substantiates numerous reports of daily variations in athletic performance parameters such as muscle force, strength, and power (64). A circadian rhythm in human athletic performance has recently been demonstrated in a swim study (23). It is considered likely that diurnal variation in muscle transcription may contribute to this rhythm in performance, in addition to 24-h rhythmicity in cardiorespiratory factors (17, 31, 51).

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The evidence that exercise acts as a synchronizer of circadian rhythms (6, 13) lends support to the hypothesis that enhanced performance occurs when times of training and competition coincide (20). This theory is especially important for equine athletes, particularly racehorses that are trained in the early morning hours and are then expected to perform optimally in the late afternoon. It could be postulated that there is increased risk of musculoskeletal injury on racetracks if strenuous exercise occurs at times that conflict with entrained rhythms.

In rodent skeletal muscle, a high proportion of cycling transcripts peak midway through the dark phase, a time period of high physical activity and feeding in nocturnal species. Diurnal variation in equine locomotor activity has been documented in a variety of studies (9, 10, 41). Evidence from these studies suggests that stabled horses display diurnal (day active) patterns of activity. However, horses in these previous studies were routinely exercised and fed concentrates during daylight hours, as is common equine husbandry practice. It is possible this observed diurnality in equine activity was influenced by management factors (e.g., light, feeding and exercise) independent of endogenous circadian regulation. In contrast to the diurnal activity patterns recorded in stabled horses, a group of feral Przewalski mares maintained in their natural environment demonstrated ultradian rhythms (period $<$ 24 h) expressing multiple bouts (peaks) of locomotor activity per 24 h (9). For a rhythm to be identified as circadian it must persist with a period of ~24 h under constant environmental conditions lacking 24-h time cues. Therefore, the aims of this study were *1*) to determine the activity patterns of horses in their natural environment; *2*) to determine for the first time under constant conditions whether equine behavioral activity is circadian, ultradian or both; *3*) to investigate circadian regulation of gene expression in equine skeletal muscle; and *4*) to correlate skeletal muscle gene expression patterns with activity patterns in the horse. Understanding the relationship between the temporal patterns of locomotor activity and patterns of circadian gene expression in skeletal muscle ultimately provides a foundation for future studies investigating circadian regulation of performance in the equine athlete.

MATERIALS AND METHODS

All animal procedures were approved by the University College Dublin Animal Research Ethics Committee.

Experimental design. Six healthy, nonpregnant mares (*Equus caballus*) of various lightweight breeds, untrained and unaccustomed to any form of exercise routine, were used in this study. Two experiments were conducted over consecutive weeks beginning in late August, a time of year that corresponded to approximately 14 h light and 10 h dark at longitude W6.8, latitude N53.2 (County Kildare, Ireland). Before commencement of the study, mares were maintained together at pasture for 1 mo under natural photoperiod conditions. The same pasture was used for activity recording for the P condition (see *Activity experiment* described below). For both experiments, mares were fitted with halter-mounted Actiwatch-L (Respironics, Bend, OR) actigraphy-based monitors that record a digitally integrated measure of motor activity and light exposure at 1-min epochs (Respironics). Actiwatch data loggers are sensitive to 0.05 g pressure/movement in any direction. This activity data-acquisition system has been used previously to measure activity rhythms in humans (7) and horses (10, 39, 41).

Activity experiment. Mares were maintained outdoors in a large 4-acre pasture (P) for 48 h to determine equine activity rhythms under natural conditions. Mares were then individually stabled in standard 12 ft $\times$ 12 ft stalls within a light-proofed barn for 48 h under a light-dark (LD) cycle that mimicked the environmental photoperiod (sunrise at 06:18 and sunset at 20:36 on August 22nd, 2008) (eye-level intensity of light in LD was ~120 lx). Finally, the horses were maintained in the light-proofed barn for a further 48 h under constant darkness (DD, $<$1 lx). Each animal was housed in an individual stall and visually isolated from herd companions. While stabled, access to hay and water was ad libitum and topped up at 4-h intervals to avoid a conspicuous 24-h temporal cue (40). Temperature inside the barn remained relatively constant for the duration of the study (16–18°C). Following the activity experiment, mares were released into the same pasture under natural environmental photoperiod until again barn-housed the subsequent week (for the LD cycle and subsequent muscle biopsy experiment).

Muscle biopsy experiment. The following week, mares were housed in individual stalls in the light-proofed barn for 48 h under an LD cycle, followed by 48 h of continuous darkness. For the first 24-h period under DD, percutaneous muscle biopsies were obtained from the right and left middle gluteal muscles at 4-h intervals (beginning at the time corresponding to dawn of the previously entrained photoschedule) using a 6-mm-diameter, modified Bergstrom biopsy needle (Jørgen Kruuse, Veterinary Supplies). Biopsies were taken ~15 cm caudodorsal to the tuber coxae on an imaginary line drawn from the tuber coxae to the head of the tail at a depth of 80 mm. Each biopsy site was shaved, scrubbed with an antiseptic, and desensitized by injection of 5 ml of 2% mepivicaine (Intra-Epicaine, Arnolds Veterinary Products, Shrewsbury, Shropshire, UK) subcutaneously using a 25-gauge needle. Samples were cleaned by multiple washes in sterile RNAlater (Ambion, Austin, TX), and immediately preserved in RNAlater for 24 h at 4° C (as per manufacturer recommendations), followed by long-term storage at −20°C. Biopsies were conducted with the aid of infrared torches (peak wavelength $>$ 600 nm) with a light intensity $<$ 5 lx, as determined by a light meter (Handsun Enterprise, Shanghai, China). Extreme caution was exercised so as to avoid shining light into the mares' eyes.

Activity data analysis. A general measure of movement activity (counts/min) was recorded using Actiwatch-L devices which reflected behaviors such as locomotion, feeding, and drinking. Raw Actiwatch data were displayed as actograms (graphs of activity plotted against time) (Fig. 1) using Vital View software (Minimitter, Bend OR). Additionally, the raw activity data files were converted to Minimitter AWD format for display and analysis in ClockLab (Actimetrics, Evanston, IL). ClockLab's batch analysis function was used to compute average activity counts per minute for each mare in each treatment interval (P, LD, DD). The ultradian structure of the Actiwatch data was examined by first averaging the 1-min epoch data into 5-min bins, which were subsequently subjected to bout analysis to identify distinct bouts of elevated activity. The threshold for this bout analysis was defined individually as 75% of the average counts per minute. Bouts were defined as periods of higher activity beginning with a rise of counts per minute above the threshold level and ending at the time that counts per minute subsequently fell below this level and remained low for at least 30 min. One-way repeated-measures ANOVA, followed by Bonferrroni post hoc tests (GraphPad Prism Version 4.0 for Windows, GraphPad Software, San Diego, CA; http://www.graphpad.com) were conducted on the following parameters (averages): counts per minute, bouts per day, counts per bout; bout length, and percentage of activity counts per light phase (subjective day in DD) across the three treatments (P, LD, and DD). Additionally, paired Student's *t*-tests set at a confidence interval of 95% were performed on average counts per day during the light phase vs. the dark phase (scotophase). Data are presented as means ± SE with $P < 0.05$ deemed significant.

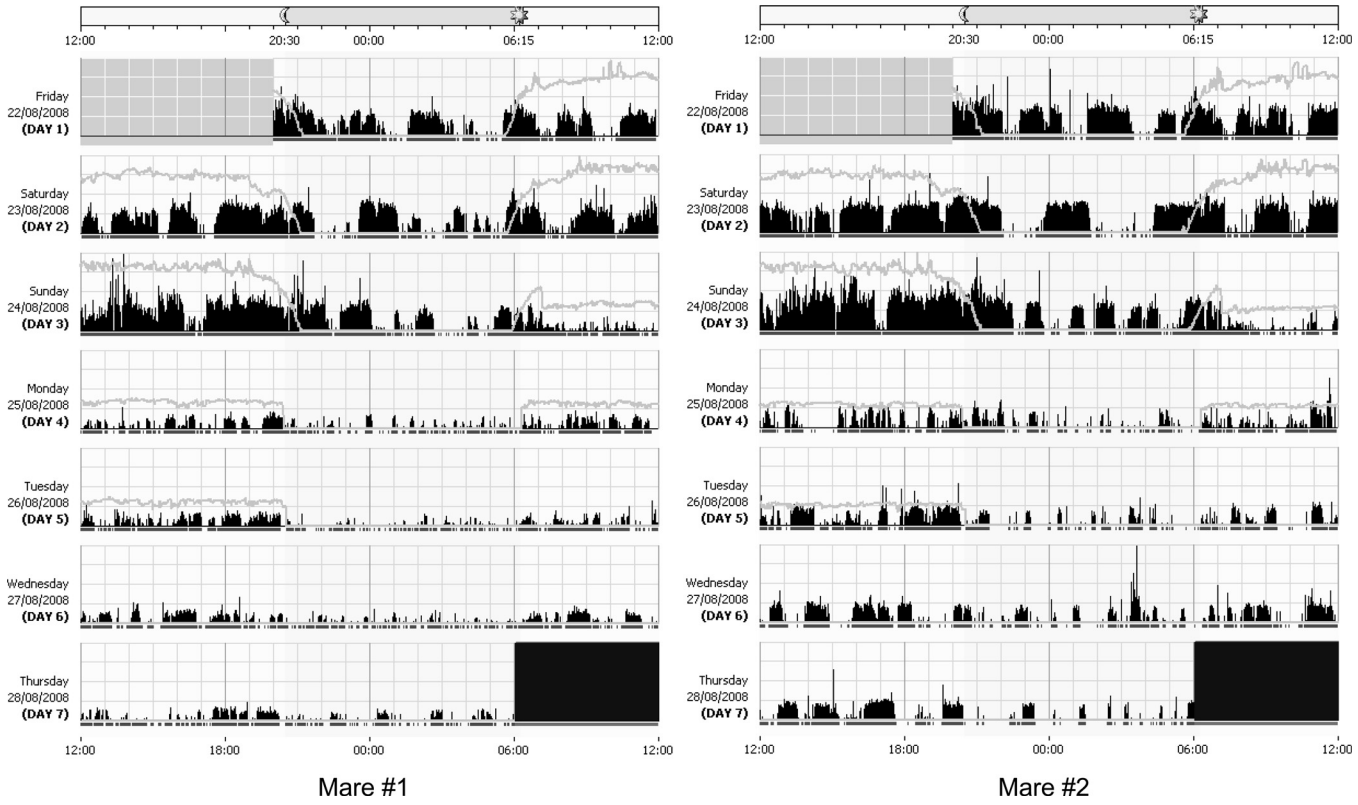

Fig. 1. Actograms representing Actiwatch-L recorded activity from 2 representative mares. Black vertical lines represent activity (counts/min) and superimposed curves indicate light intensity. *Days 1–7* (*y*-axis label) represent successive 24-h periods (noon to noon). Mares were at pasture (P) on *days 1–3*, moved into the barn on the morning of *day 4* (0700) where they remained on an artificial light-dark (LD) cycle (14 h:10 h) until lights out on *day 5*. Thereafter (*days 6–7*), they remained in continuous darkness (DD). White and gray bars above each actograph represent light and dark periods, respectively, of the environmental LD cycle present naturally at P and artificially in Barn LD conditions. The shaded dark area represents excluded data following removal of the Actiwatches. Note the prominence of ultradian activity bouts (multiple peaks/24 h) when horses are outdoors and the subsequent emergence of a 24-h rhythm when mares are stabled both in LD and DD.

Time series activity data were further analyzed (Action3, Ambulatory Monitoring, Ardsley, NY) using the least-squares cosine-fit method of Nelson et al. (36) to detect 24-h periodicity. For each mare and each treatment interval (P, LD, DD), this cosinor method gave estimates of four rhythm parameters: acrophase (time of peak value of the fitted cosine function), mesor (middle value of the fitted cosine curve representing the rhythm adjusted mean), amplitude (difference between maximum and mesor of the fitted cosine function), and Q value (goodness of fit). One-way repeated-measures ANOVA was used to assess significant changes in the above cosinor parameters over time between the three treatments (GraphPad Prism, $P < 0.05$, as above). Finally, for graphic illustration of ultradian and circadian temporal variation (Fig. 2), 5-min activity data were averaged into 30-min bins, normalized separately for each mare (bin value/condition mean $\times$ 100%) for each condition (P, LD, DD), and a matching 24-h period plotted (mean $\pm$ SE) for comparison.

RNA isolation and cDNA synthesis. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA), subsequently DNase-treated with the RNase-free DNase Set (Qiagen, Hilden, Germany), and purified with the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA was quantified using a NanoDrop ND1000 spectrophotometer V 3.5.2 (NanoDrop Technologies, Wilmington, DE). RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). For each sample, 500 ng RNA was converted to complementary (c) DNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The reaction volume for each sample was corrected to 20 μl using nuclease-free water (Sigma-Aldrich, Irvine, Ayrshire, UK) and stored at −20°C.

Real-time quantitative reverse transcription-polymerase chain reaction. Real-time quantitative reverse transcription PCR (qRT-PCR) assays were performed using the ABI Fast Real-Time PCR System and Fast SYBR Green Master Mix (Applied Biosystems). A candidate gene approach was employed to identify circadianly regulated genes in equine skeletal muscle. A panel of 36 genes was selected that included core clock genes, previously identified circadianly regulated genes involved in muscle metabolism in mice, and equine exercise-associated genes. GenBank (NCBI)-published equine sequences were used to design oligonucleotide primers for candidate genes using a combination of Primer3 version 4.0 [http://frodo.wi.mit.edu/primer3/ (48)] and PrimerQuest (Integrated DNA Technologies, http://eu.idtdna.com/Scitools/Applications/Primerquest/) software (primer sequences provided as Supplemental Material, available with the online version of this article). Sequence specificity was confirmed using NCBI Blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Oligonucleotide primers were commercially synthesized by Eurofins MWG Operon (Ebersberg, Germany). Each 20-μl reaction contained 5 μl cDNA (0.83 ng/μl), 2.4 μl forward and reverse primer mix (150 nM), 10 μl Fast SYBR Green Master Mix (Applied Biosystems), and 2.6 μl nuclease-free H_2O (Sigma-Aldrich). For each qRT-PCR assay, a standard curve was generated using twofold serial dilutions of pooled cDNA. Thermal cycling consisted of one cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Dissociation curves were examined for each gene to ensure specificity of amplification. A panel of four putative housekeeping genes, *GAPDH, Gusb* (glucuronidase, beta), *Actb* (actin, beta), and *Ttn* (titin), were evaluated for their suitability as a stable internal reference gene in equine muscle tissue over time. *Ttn* was

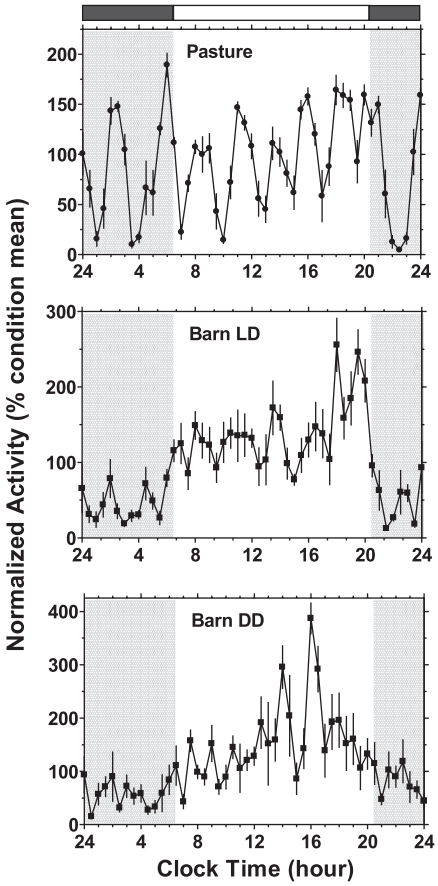

Fig. 2. Equine behavioral activity displays 24-h and circadian variation. Averaged temporal patterns of equine behavioral activity in 3 environments: at pasture (P), and stabled in a light-controlled barn with an artificial light-dark cycle (LD) and in continuous dark (DD) (see MATERIALS AND METHODS). Raw activity for each mare (counts/min) were averaged in 30-min bins and normalized relative to the mean activity level in each condition (bin/mean × 100%). Each panel plots group mean ± SE ($n = 6$) for each 30-min bin (midnight to midnight) in relation to sidereal clock time (h). Bars at top and internal shading represent the natural light cycle at P, and its simulation in LD. At P, mares displayed synchronous well-defined ultradian bouts evidenced by multiple peaks and small SE error bars. In LD, a clear 24-h rhythm emerges with increased activity throughout the day. Although raw activity levels were lowest in DD, the amplitude (peak-100%) of the endogenous circadian rhythm in DD is greater than that for the 24-h rhythm in LD or the multiple ultradian peaks in P.

selected for its greater stability by both geNorm (56)and NormFinder (3) software programs. Transcript abundance was determined relative to *Ttn* using the standard curve method according to the manufacturer's instructions (Applied Biosystems, ABI Prism 7700 Sequence Detection System User Bulletin 5). One-way ANOVA and Bonferroni post hoc tests (GraphPad Prism Version 4.0) were used to determine whether the temporal pattern of expression for each transcript varied significantly over the 24-h period. The relative abundance of mRNA is presented as mean ± SE, with a P value of < 0.05 considered significant.

RESULTS

Activity experiment. Figure 1 shows representative actograms displaying temporal patterns of activity (counts/min, Actiwatch L) and light exposure for two mares (*1* and *2*). Consistent with these examples, visual inspection of the actograms of all six mares, together with quantitative analysis (see

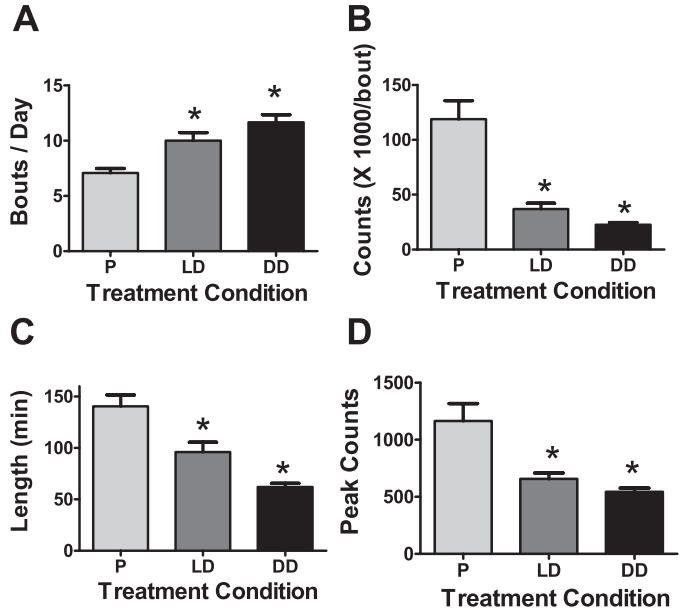

Fig. 3. Bout analysis of ultradian rhythmicity. Panels *A–D* present bar graphs illustrating differences in bout analysis parameters (bouts/day, counts, length, and peak counts, respectively) (means ± SE) comparing horses observed sequentially in 3 contrasting environments: at pasture (P), and while stabled in light-controlled barn, first in a light cycle (LD), and second, in continuous darkness (DD). *Group means for LD or DD that differ from P (see text).

below and Figs. 2–4), supports the following subjective summary. In P the temporal variation in activity was predominantly ultradian with multiple bouts of elevated activity almost equally distributed over day and night; in contrast, while ultradian fluctuations persist in the barn, there was a substantial decrease in activity levels in LD and DD along with the emergence of diurnality, exemplified by increased activity during daytime hours (compare ultradian vs. circadian profiles plotted in the panels of Fig. 2).

Quantitatively, mares demonstrated an average of 8.9 bouts of elevated activity per day (SE ± 0.7) across the three treatments (P, LD, and DD). By definition (see MATERIALS AND

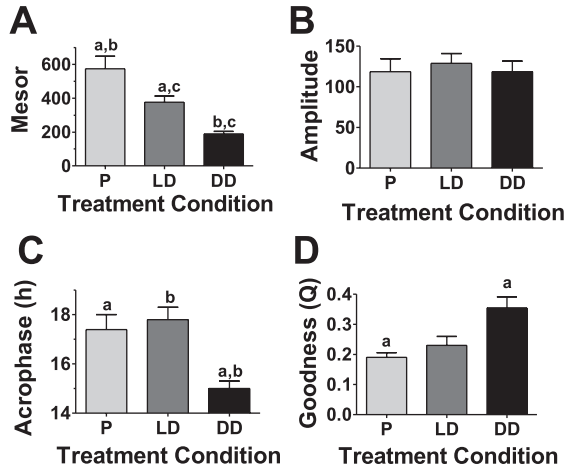

Fig. 4. Cosinor analysis for 24-h and circadian rhythmicity. Panels *A–D* plot bar graphs illustrating differences in cosine analysis parameters (means ± SE) comparing treatment conditions (conventions as in Fig. 2). *A*: mesor. *B*: amplitude. *C*: acrophase. *D*: goodness of fit. Shared letters (a, b, c) indicate group means that differ from each other (see text).

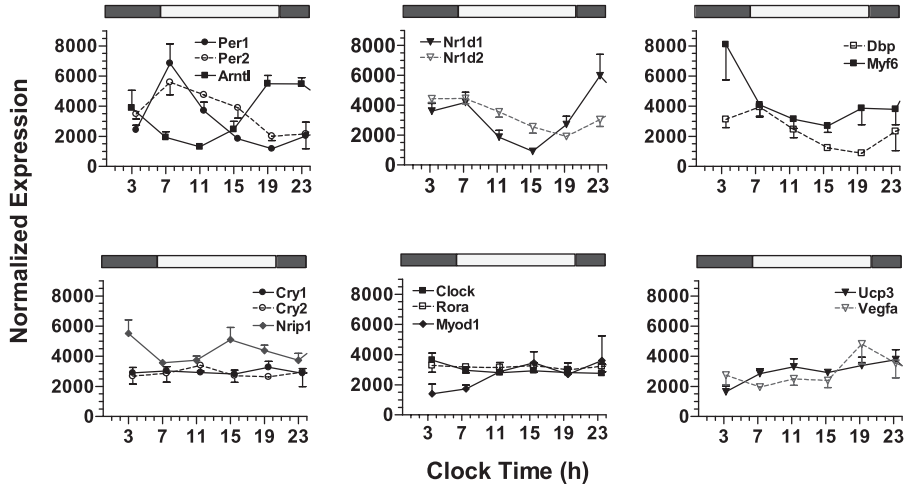

Fig. 5. Twenty-four-hour profiles of skeletal muscle gene expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *Ttn*, in equine skeletal muscle over 24 h in constant darkness. *Top*: genes that displayed a significant variation over time: *Per1, Per2, Arntl, Nr1d1, Nr1d2, Dbp,*and *Myf6* ($P < 0.05$). *Bottom*: nonsignificant core clock genes *Cry1, Cry2, Clock* and *Rora* ($P > 0.05$); and potential clock-controlled genes *Nrip1, Myod1, Ucp3*, and *Vegfa* ($P > 0.05$). Each time point represents the mean ± SE ($n = 6$). The barn light cycle in effect before to entry into constant darkness (DD) is depicted above each graph with the dark gray shading representing subjective night (~CT14–CT24) and light gray shading representing subjective day (~CT0–CT14), corresponding to times of natural or simulated night and day existing before DD.

METHODS) these bouts were separated by gaps of ≥ 30 min of subthreshold activity. One-way repeated-measures ANOVA revealed a lower number of activity bouts per day at P compared with the stabled conditions of LD and DD ($P < 0.01$ and $P < 0.001$, respectively). Although mares at P demonstrated a reduced number of bouts per day (Fig. 3*A*), overall activity levels (average counts/min) and counts per bout in this outdoor environment were higher than both LD and DD ($P < 0.001$) (Fig. 3*B*). Additionally, the maximum intensity of activity as measured by peak counts per minute averaged for all bouts in each condition was higher at P [1,120 ± 355 (mean ± SE) counts/min] than either LD (657 ± 124 counts/min; $P < 0.05$) or DD (544 ± 80 counts/min; $P < 0.001$) (Fig. 3*D*). There was no significant difference between peak counts per minute comparing LD and DD ($P > 0.05$). Mares at P displayed greater bout length (118 ± 19 min) than DD (62 ± 9 min; $P < 0.01$) but not greater than LD (96 ± 23 min; $P > 0.05$) (Fig. 3*C*). Paired Student's *t*-tests comparing average counts per day during the photoperiod with average counts per day during the scotophase over the specified intervals did not vary significantly at P or in LD, but noticeably differed in DD ($P < 0.01$). Furthermore, the percentage of light counts, i.e., activity counts that occurred during the photophase (subjective day in conditions of constant darkness) was higher in DD compared with P ($P < 0.01$) and LD ($P < 0.001$), suggesting that horses have an innate circadian tendency for increased activity during subjective day. Thus from a circadian perspective it appears appropriate to consider horses to be a diurnally active species.

Consistent with the above, cosine curve fitting detected a weak but significant 24-h component in the temporal organization of activity across all three treatments ($P < 0.05$) with increased robustness, Q (goodness of fit values), associated with DD ($P < 0.05$) (Fig. 4*D*). As robust circadian rhythms often display Q values of ≥ 0.9, the mean Q values for equine activity patterns are comparatively low (0.36 in DD, 0.23 in LD, and 0.19 at P), indicating that the overall temporal variation in activity has a weak rather than a dominant 24-h component. Activity acrophase (peak time of the 24-h fitted cosine curve) occurred at a later time of day (1748 ± 0030 local time) in LD compared with P (1724 ± 0036) and DD (1454 ± 0018) (Fig. 4*C*). Acrophase values of P and LD did not vary significantly; however, they did differ between P and DD ($P < 0.01$) and between LD and DD ($P < 0.001$). Mesor (mean value of the 24-h fitted curve, expressed in counts/min) varied significantly between P and LD ($P < 0.05$), P and DD ($P < 0.001$), and LD and DD ($P < 0.05$) (Fig. 4*A*). Amplitude (difference between acrophase and mesor values, expressed in counts/min) did not vary significantly between treatments ($P > 0.05$) (Fig. 4*B*). The above cosine results concur with the switch from a predominately ultradian activity pattern to one with a stronger circadian component as depicted graphically in Fig. 2, which used normalized 24-h profiles of activity averaged into 30-min bins of sidereal time.

Muscle biopsy experiment. The expression patterns of nine core clock genes, *Arntl, Clock, Per1, Per2, Nr1d1, Nr1d2, Cry1, Cry2,* and *Rora,* and the clock controlled gene (CCG) *Dbp* (D-site of albumin promoter binding protein) were investigated in this study. We detected mRNA expression of all 10 aforementioned genes in equine skeletal muscle. Significant 24-h oscillations were observed for six core genes (*Arntl, Per1, Per2, Nr1d1, Nr1d2,* and *Dbp*; Fig. 5) but not in the remaining four (*Clock, Cry1, Cry2,* and *Rora*; Fig. 5). The *P* values of the six significant genes are provided in Table 1. *Nr1d1* displayed the highest peak-trough fold increase and *Nr1d2* the least (Table 1). Expression of the *Per* transcripts were observed to be antiphase to *Arntl*; *Per* acrophases occurred in the morning, whereas that of *Arntl* was seen 12 h later in the evening. Similarly, *Nr1d2* and *Dbp* displayed acrophases in the morning that were antiphase to *Arntl*. The peak and trough times of significant genes are also provided in Table 1.

Of the remaining candidate genes, *Myf6* (myogenic factor 6) was the only mRNA to display distinct oscillations ($P < 0.05$)

Table 1. *Peak times, trough times, and fold increases of genes found to significantly vary over time using one-way ANOVA*

Gene Symbol	Peak (Clock Time)	Trough (Clock Time)	Fold Increase	*P* Value
Arntl	1900	1100	4.2	<0.0001
Per1	0700	1900	5.8	<0.0001
Per2	0700	1900	2.8	0.0009
Nr1d1	2300	1500	6.5	0.0002
Nr1d2	0700	1900	2.3	<0.0001
Dbp	0700	1900	4.5	0.0045
Myf6	0300	1500	3.0	0.0406

(Fig. 5). However, a number of genes such as *Ucp3* (uncoupling protein 3), *Nrip1* (nuclear receptor interacting protein 1), and *Vegfa* (vascular endothelial growth factor A) yielded *P* values that approached significance ($P = 0.0699$; $P = 0.0889$; $P = 0.1119$; respectively) (Fig. 5). A list of remaining candidate genes and their associated *P* values are provided as Supplemental Material, available with the online version of this article.

DISCUSSION

Ultradian and circadian characteristics of equine activity rhythms. To our knowledge, this is the first study to report that under constant environmental conditions (notably in the absence of known or suspected 24-h zeitgebers), horses can display an endogenous circadian rhythm in locomotor activity (Figs. 1, 2, and 4). This observation is important because it implies that while horse activity and behavior may be greatly influenced by and in some cases driven by external environmental factors including interactions with humans, it is nonetheless importantly influenced by endogenous circadian rhythms (rhythms which represent the output of cellularly autonomous circadian oscillators, i.e., 24-h cellular clocks) that exist both centrally (brain) and peripherally. To further highlight the potential importance of circadian regulation of the behavior and physiology of the horse, we also demonstrate for the first time strong circadian oscillations in skeletal muscle transcription of several genes known for their central role in the generation of mammalian circadian rhythms.

In an effort to clarify apparently conflicting reports of ultradian/diurnal (9) and purely diurnal equine activity rhythms (10, 41), we investigated equine locomotor activity in the horse's natural environment, in LD and under DD. The latter (DD) condition was included to specifically address whether any 24-h component in activity reflected regulation by an endogenous circadian clock mechanism. Additionally, we anticipated that knowledge of the endogenous circadian rhythm of behavioral activity would be useful in interpreting equine muscle gene transcription profiles.

Mares at P demonstrated bouts of activity that were scattered across the 24-h period to form an overall ultradian pattern (Figs. 1–3) consistent with previous observations (9). These bouts of high activity at P are consistent with behavior of a continuous grazer that spends up to 18 h/day foraging and ~2.5 h/day sleeping (12). Accordingly, mares were found to be significantly more active outdoors than when stabled. It is noteworthy that at P, the six mares appeared to demonstrate ultradian activity bouts that were relatively synchronous among the individuals of the group (Fig. 2*A*). Thus, while the overall rhythmic pattern at P is both strongly ultradian and weakly circadian, the relative synchrony of the ultradian activity bouts suggests a strong influence of social cues on this rhythmicity (4). It is therefore possible that when horses are in a more natural environment, the temporal organization of activity, and perhaps additional aspects of equine physiology, rely relatively more on conspecific/social signals than on endogenous circadian regulation and/or external time cues, an idea we previously suggested as a possible explanation for the absence of 24-h clock gene oscillation in equine peripheral blood (35).

Interestingly, ultradian rhythms of activity persisted when the mares were stabled under both LD and DD conditions, albeit at a lower intensity (as measured by mean peak counts) and the activity was not noticeably synchronous among individuals. However, close visual inspection of the actigraphs generated during LD and DD clearly suggests the emergence of a 24-h periodicity in the overall activity patterns, as supported graphically and quantitatively in Figs. 3 and 4. Additionally, statistical tests comparing average counts per day during the photophase and scotophase periods confirmed the presence of a diurnal rhythm in DD, but surprisingly not in LD. Further analysis using cosine methods verified the existence of a weak 24-h component in LD and DD, and unexpectedly, also in horses at P. The identification of a weak circadian component in the time series data from all three treatment conditions supports previous observations of simultaneous ultradian and circadian rhythms of locomotor activity in horses at pasture (9). Our findings of circadian activity rhythms in stabled horses in DD also extend and clarify previous reports of diurnality in this species (10, 39, 41) by providing the first evidence of endogenous circadian (24 h) periodicity in the absence of diurnal light cues.

The acrophase of the equine activity rhythm in DD occurred at ~1500 local time, indicating the endogenous peak time of activity in untrained horses. Mares at P and stabled in LD conditions demonstrated much later mean acrophases (~1720 and ~1750 local time, respectively). The 2- to 3-h acrophase advance comparing horses housed in DD and LD/P potentially highlights how equine circadian rhythms may be shifted when exposed to human management regimes such as stabling. Humans have a tendency to encourage diurnal behavior among domesticated animals; dogs, for example, are naturally crepuscular (active at dawn and dusk), but they learn to become diurnal through human interactions (58). Similarly, human-imposed exercise schedules may also influence the overall phase timing (rhythm acrophase) and the time of peak activity in equine athletes.

Circadian regulation of muscle-specific genes. The identification of oscillating transcription of *Arntl, Per1, Per2, Nr1d1,* and *Nr1d2* in skeletal muscle of sedentary horses under DD is the first in vivo evidence of an endogenous circadian clock in an equine tissue and, moreover, the first confirmation of a synchronized molecular clock in the muscle tissue of a large mammal and important agricultural species. The observed antiphase expression profiles of *Per2* and *Arntl* with peaks at circadian time (CT) 0 and CT12, respectively, are characteristic of the clockwork mechanism that has been described in all studies of mammals to date (33). (In this study, CT0 represents dawn/lights on and CT12 is 12 circadian hours later). Interestingly, these temporal expression patterns are the inverse of those detected in rodent skeletal muscle, where multiple studies report a *Per2* peak between CT12 and CT16 and an *Arntl* peak between CT23 and CT2 (28, 61). The identification of antiphase expression in equine skeletal muscle occurring at almost the exact opposite time points to nocturnal rodent skeletal muscle further supports the opposing diurnal/nocturnal behavioral characteristics of these species.

Our finding of a constitutively expressed *Clock* transcript is consistent with findings in rodent skeletal muscle (1, 28). In addition, *Cry* family genes did not oscillate in the present study. This contrasts with a previous demonstration of *Cry1*

24-h oscillation in an equine fibroblast cell line and adipose tissue (35). Interestingly, a recent study of human heart muscle has revealed the absence of circadian rhythmicity in *Cry1* mRNA despite high expression levels of this gene in all samples (25). Almon et al. (1) also reported a tonic expression of *Cry2* in rat skeletal muscle, although they attribute this finding to a low signal intensity of the *Cry2* probe used in their microarray set. The microarray chip utilized by these authors did not contain a probe for *Cry1*, so it remains unclear whether any member of the *Cry* family cycles in a 24-h pattern in rat skeletal muscle. In mouse skeletal muscle, Yan et al. (61) reported that *Cry1* gene expression was circadian while McCarthy et al. (28) observed a distinct circadian oscillation of the *Cry2* transcript. Thus the detection of *Cry* family members as constitutively expressed genes in equine skeletal muscle is an intriguing finding that supports previous reports of nonrhythmic core clock genes in particular tissues (25, 55). Furthermore, the *Rora* transcript did not display temporal variation in the present study. It is important to note that activity in rodents is strongly circadian with activity largely confined to the scotophase; in this study we find that equine activity is only weakly circadian and displays a temporal structure that is highly ultradian, especially when animals are at pasture, but also somewhat diurnal when they are stabled. It is therefore conceivable that aspects of the equine muscle clock might display reduced oscillations in certain components compared with species exposed to greater partitioning of environmental pressures (i.e., foraging in nocturnal species). On the other hand, posttranscriptional and posttranslational modifications, such as phosphorylation, sumoylation, histone acetylation, and methylation, have recently been shown to play increasingly important roles in regulatory pathways and processes within the cell (15). It is feasible that this too is the case for *Cry* and *Rora* genes in equine muscle tissue. The use of proteomic techniques in future studies could expand our knowledge and help establish whether clock proteins oscillate in equine skeletal muscle. Moreover, there have been suggestions that significant redundancy exists within the clockwork mechanism, such that in certain tissues, oscillation of some identified clock genes are not essential for circadian periodicity (5).

Similarly to *Arntl* and *Per*, the phase of *Dbp* in this study (peak at 0700 local time, CT0) is inverse to that of mouse and rat studies (peaks at CT10 and CT12, respectively) (28, 61). CCGs are believed to be important for transducing core gene oscillations into rhythms of physiology (45, 46). *Dbp* encodes the regulatory protein DBP, a transcription factor that gives rise to a resulting network of circadian gene expression and ultimately to circadian rhythms of physiology (45). Thus our identification of *Dbp* as a member of the equine skeletal muscle circadian transcriptome strongly suggests that a significant subset of as yet unidentified genes exhibit a daily rhythm in this tissue.

Myf6 was the only exercise-relevant transcript to display a 24-h oscillation in our limited selection of candidate genes. *Myf6* is a member of the myogenic regulatory transcription factor (MRF) family, along with *Myf5* (myogenic factor 5), *Myod1* (myogenic differentiation 1), and myogenin (30). The identification of *Myf6* as a circadian-regulated transcript suggests that this MRF plays a role in the normal daily functioning of equine skeletal muscle. *Myf6* is the most abundantly expressed gene of the MRF family in adult muscle and is therefore purported to play a role in the maintenance of skeletal muscle phenotype (60). Recent studies have identified *Myf6* in newly developed myotubes of regenerating muscle in the amphibian *Xenopus* (8) and the rat (64). These observations are consistent with numerous reports of a role in myogenesis (32). Furthermore, elevated levels of *Myf6* mRNA have been detected in human skeletal muscle following heavy-resistance training, indicating that this gene may also play a role in skeletal muscle hypertrophy (44).

Although a number of genes examined in this study did not satisfy the criteria for 24-h oscillation, several did approach significance. In particular, *Ucp3*, *Vegfa,* and *Myod1* displayed noticeable circadian trends of expression. Conversely, the expression profile of *Nrip1* did not display convincing 24-h rhythmicity (Fig. 5). *Ucp3's* 24-h waveform pattern of expression was clearly evident in skeletal muscle from our untrained horses. This transcript is purported to play a role in the protection of muscle from reactive oxygen species (ROS) damage during oxidative stress (27). ROS are normal byproducts of mitochondrial respiration (27) that increase during physical exercise and may result in oxidative stress, a state in which ROS production exceeds the body's antioxidant defense mechanisms and subsequently induces lipid, protein, and DNA damage (22). Exercise-induced oxidative stress is associated with muscle damage and decreased muscle performance in horses (22), a concept that especially resonates with horse trainers as musculoskeletal injury is the most common reason for wastage in racehorses (47).

Vegfa is a fundamental regulator of angiogenesis (14) and has also been proposed to play a role in the maintenance of adult skeletal muscle microvasculature (37). *Vegfa* stimulates vascular endothelial cell growth, survival, and proliferation, and in addition promotes vascular permeability (14). Exercise-induced increases in *Vegfa* expression are thus associated with the formation of new capillaries within skeletal muscle (2).

Although *Myod1* failed to satisfy our criteria for circadian transcripts, this gene clearly displays a 24-h waveform when graphed (Fig. 5). A central player in skeletal myogenesis (57), *Myod1* specifies skeletal muscle lineage in mice (49, 54) and is required for proliferation of muscle satellite cells (62). Zhang et al. (64) suggest that *Myod1* is a CCG and thus regulated directly by the skeletal muscle molecular clock. These authors propose that the cellular clock contributes to maintenance of muscle structure via its direct effects on *Myod1* and consequent effects on *Myod1*-regulated genes. Our findings further suggest a role for this gene in daily regulation and maintenance of muscle tissue in the horse.

It is worth reemphasizing that all of the aforementioned genes were measured in muscle from sedentary, untrained horses that exhibited weak circadian activity rhythms compared with rodents and other species. This has important implications for the known ability of physical activity to act as an entrainment factor for peripheral circadian clocks (6, 13). Equine diurnal rhythms, such as those of platelet aggregation, shift in response to an exercise regime (42). Furthermore, resistance exercise has been suggested as a direct regulator of circadian rhythms in human skeletal muscle (63). It is likely that, similar to humans, synchronization of molecular clocks in equine skeletal muscle also occurs in response to training, with clock-regulated genes shifting their phases of peak expression to the most advantageous time of day. In strong support of this

hypothesis is a recent paper by McGiveney et al. (29) that identifies the clock genes *Per2* and *Per3* within a subset of genes uniquely upregulated in response to training in skeletal muscle from elite equine athletes. In the horse-racing industry, Thoroughbreds are commonly maintained under a strict routine and trained in the early morning hours. Accordingly, equine CCGs such as *Myf6* and potential CCGs (*Ucp3* and *Vegfa*) may be in phase with scheduled training times. Future studies that investigate the correlation between training times and the temporal profile of the equine muscle transcriptome may indicate the most favorable times of day for training and competition and thus help to minimize and/or avoid muscle injury and fatigue and to achieve maximal aerobic exercise response and performance from equine athletes.

Conclusions. This is the first study to investigate the natural activity patterns of horses under constant conditions. The mares in this study displayed predominantly ultradian rhythms of activity at pasture with a weak circadian component that was modestly enhanced when the animals were stabled (LD and DD). It is proposed that the DD condition permits greater unmasking of endogenous circadian periodicities in the absence of environmental stimuli such as light, and social and feeding cues. In addition, this study breaks new ground as it is the first of its kind to demonstrate the existence of an endogenous peripheral tissue clock in the horse. The implications are that muscle function undergoes rhythmical 24-h regulation, which, in turn, provides molecular support for a potential circadian variation in equine performance capacity and subsequent training specificity. In particular, daily variation in *Myf6*, and apparent diurnal variation in *Ucp3* and *Vegfa* transcripts, highlights the importance of continued investigation of the equine muscle transcriptome for exercise performance optimization. The weak but clearly 24-h periodicity of equine activity patterns at pasture and when stabled (LD and DD) coexists with 24-h oscillations in circadian clock genes and suggest exciting future opportunities to discover additional circadian clock-regulated genes in tissues from horses exposed to regular exercise at specified hours of the day. Finally, our results suggest the likelihood that temporal regulation of muscle metabolism also occurs in other important agricultural species such as sheep and cattle, with potential implications for the influence of, for example, time of day of slaughter on meat attributes such as tenderness.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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