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Invited Review Article

The clockwork of champions: Influence of circadian biology on exercise performance[☆]Stuart J. Hesketh^{a,*}, Karyn A. Esser^b^a School of Medicine, University of Central Lancashire, 11 Victoria St, Preston, PR1 7DS, UK^b Department of Physiology and Aging, University of Florida, 1345 Centre Drive, Gainesville, FL, USA

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ABSTRACT

Exercise physiology and circadian biology are distinct and long-standing fields. Recently they have seen increased integration, largely due to the discovery of the molecular components of the circadian clock and recognition of human exercise performance differences over time-of-day. Circadian clocks, ubiquitous in cells, regulate a daily tissue specific program of gene expression that contribute to temporal patterns of physiological functions over a 24-h cycle. Understanding how circadian clock function in skeletal muscle, as well as other tissues contribute to exercise performance is still in the very early stages. This review provides background on this emerging field with a review of early exercise and time-of-day studies in both human and animals. We then move into the role of the circadian clock and its daily program of gene expression in skeletal muscle with a focus on specific metabolic and physiological outputs that vary over time-of-day. Lastly, we discuss the recognition that the timing of exercise communicates with the skeletal muscle circadian clock to adjust its phase settings and why this may be important for performance and health.

1. Introduction and background

The fields of exercise physiology and circadian biology are both long standing and well-established, the conception of each tracing back many thousands of years. The origins of exercise physiology and the importance of exercise for health and well-being can be followed back to many ancient cultures. However, Hippocrates in Ancient Greece is probably best known as the first to advocate moderate exercise to maintain and improve health status [1,2]. Similarly circadian biology can trace its roots back to the time of Alexander the Great, whose scribe documented that the leaves of certain plants opened and closed in synchrony with the rising and setting of the sun [3]. Despite these long-standing histories, integration of the fundamental principles of exercise physiology and circadian biology have been only a recent development; the field only really emerging properly in the late 1940s and experiencing significant expansion over the past decade. One of the first pioneering works in this field examined the impact of time-of-day on human physical work capacity reporting that some people have a consistent preference for daytime activities whilst others preferred the night-time [4]. Subsequent research has reported on athletic performance at specific times of day [5,

6], whilst the first experiments to exploring circadian variations in exercise tolerance were conducted in the late 1960s [7,8]. In contrast to our current understanding, these early studies did not determine exercise performance was different over the course of the day. However, the authors do comment that this could have been due to design issues resulting in large interindividual variability due to small sample sizes and human variation. Much later came work to establish differences in time-of-day exercise performance (See section 2). This initial convergence of circadian concepts into the study of exercise physiology were based on more systemic outcomes e.g., exercise capacity or heart rate, therefore not on a molecular scale.

In recent years the convergence of circadian biology and exercise physiology has attracted increasing attention, particularly since the discovery of the molecular components of the circadian timing mechanism, and the recognition of the molecular clock as the mechanism regulating circadian rhythms, culminating in the Nobel Prize in Physiology or Medicine in 2017. Circadian clocks are found within virtually all cells in the body and function to temporally orchestrate mammalian physiological function on a roughly 24-h cycle. In particular, the circadian clock is an essential mechanism that directs a daily program of gene

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expression to temporally regulate different aspects of cell physiology and metabolism. The hierarchical organisation of the circadian system begins with the suprachiasmatic nucleus (SCN) located in the hypothalamus of the brain, and serves as the master or central clock. The central clock is entrained by light and works to synchronise peripheral molecular clocks across tissues through neural and humoral mechanisms, underscoring its influence across diverse tissues and organs of the body e.g., skeletal muscle (Fig. 1A) [9]. Both central and peripheral clocks ensure perpetual 24-h rhythms of behavioural and physiological functions, such as sleep-wake cycles, feeding schedules, hormone secretion, cardiovascular function, and whole-body metabolism [10].

The circadian clock mechanism is defined as a transcriptional-translational feedback loop. The positive arm of the feedback loop consists of basic helix-loop-helix (bHLH)-PAS family of transcription factors, circadian locomotor output cycles kaput (CLOCK; [11] and Brain and Muscle ARNT-like 1 (BMAL1; [12], that heterodimerize and bind to E-box elements [13] within the promoters of the negative arm genes, *Period* (*Per 1/2/3*) and *Cryptochrome* (*Cry 1/2*), facilitating their transcription. Once translated, PERs and CRYs form multimers, translocate back to the nucleus, and inhibit the transcriptional activity of the CLOCK: BMAL1 complex [14,15]. Other regulatory proteins include the retinoic acid-related orphan nuclear receptors, REV-ERBs (Nr1d1, Nr1d2), and ROR, which compete for binding to retinoic acid-related orphan receptor response elements (ROREs) within the *BMAL1* promoter, either inhibiting or activating *BMAL1* transcription, respectively [16,17]. This self-sustaining mechanism cycles within approximately 24 h and is required for mammalian circadian rhythms (Fig. 1B). Although the core clock machinery is ubiquitous across all tissues and cell types, clock transcriptional output (e.g., clock output) is highly tissue-specific and can be impacted by physiological cues such as stress, physical activity, and food consumption [18]. As such, the circadian clock induces daily oscillations in substrate metabolism, glucose homeostasis [19], skeletal muscle insulin sensitivity [20], and oxidative capacity [21], all pathways that hold potential to influence exercise and sporting performance.

Since the convergence of circadian biology and exercise physiology, much human work has been done to establish that diurnal differences in exercise performance exist. However, the precise biological mechanisms responsible are yet to be elucidated. The discovery of the intrinsic molecular clock has led subsequent work in this field down a line of

investigation that is on the molecular scale. Due to technical constraints, preclinical models play a vital role in this line of enquiry. Of note, studies utilizing mice have demonstrated significant differences in strength and endurance over time-of-day. Use of mouse models in which the clock genes are knocked out in all tissues have confirmed that an intact circadian clock mechanism is required for the time-of-day performance differences. Thus, confirming that the molecular circadian clock system is required for orchestrating diurnal exercise performance. It is reasonable to think that the circadian clock in muscle is important for time-of-day performance, although the specific tissues and tissue clocks required have not been delineated.

Herein, the aim of this review is to present the intrinsic physiological and metabolic processes governed by circadian biology and highlight their connection to human performance across different times of the day. We will first explore the influence of the time-of-day on human exercise performance, and then move on to discuss how the timing of exercise informs the clocks in peripheral tissues. Finally, we will assess the potential role of the skeletal muscle molecular clock in shaping time-of-day exercise performance. By elucidating these connections, we hope to contribute to a deeper understanding of the interplay between exercise and circadian clocks with an impact on athletic performance. In addition, we outline the significant gaps in this new research area providing a basis for future research to clearly define the interactions between the circadian mechanism and the modulation of exercise performance. We suggest that understanding these relationships will ultimately pave the way for personalized and optimised strategies in enhancing human performance and overall well-being.

2. Human circadian physiology and exercise performance

There are many physiological processes that are subject to circadian control e.g., core temperature, heart rate, blood pressure etc. Human exercise performance has also been observed to exhibit diurnal patterns, which extends across various performance measures e.g., strength, and endurance capacity, implicating the influence of circadian biology on exercise outcomes. However, despite these observations, the precise mechanisms underlying the interaction between circadian rhythms and exercise performance, particularly in skeletal muscle, remain incompletely understood. This section aims to present some of the mammalian physiological systems that display circadian rhythms and examine how

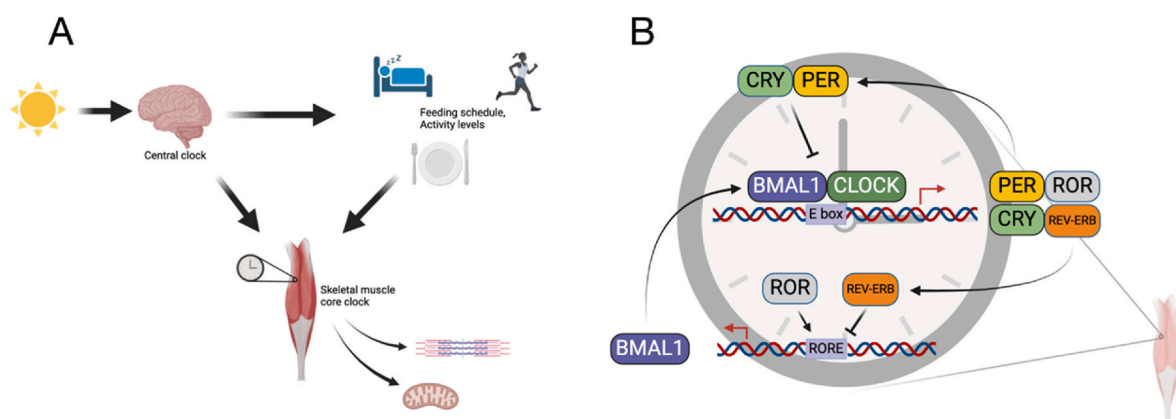


Fig. 1. Central and peripheral core clock circadian mechanisms. A) Circadian regulation of activity and muscle function. The network of circadian regulators encompass the synchronisation of sleep-wake cycles, feeding patterns, physical activity, and other external cues by both the central master clock located in the suprachiasmatic nucleus and peripheral clocks, found in tissues throughout the human body (e.g. skeletal muscle). Circadian coordination extends to influence diverse physiological processes, including the modulation of muscle cell metabolism and contractile performance, highlighting the pervasive impact of circadian rhythms on human physiology. B) Basic mechanism of the transcriptional-translational feedback loop of the molecular clock. The constituents of the positive arm (BAML1 and CLOCK) bind to E-box elements, facilitating the transcription of repressive genes of the negative arm (PER and CRY), which repress their own transcription by inhibiting the BMAL1: CLOCK heterodimer. BMAL1 and CLOCK also facilitate the transcription of ROR and REV-ERB genes that promote or inhibit the expression of BMAL1, respectively. In addition, the BMAL1: CLOCK heterodimer binds to E-box elements of genes, often accompanied by tissue-specific transcription factors, facilitating the expression of clock-controlled genes, and contributing to circadian physiology.

these intrinsic rhythms may contribute to the differences in diurnal human performance.

There are numerous studies that have demonstrated physiological processes in the human body, such as heart rate, hormonal patterns, core body temperature, and blood pressure all exhibit robust circadian rhythms [22–30]. Further, evidence supports that these daily oscillations are not acute responses to the immediate environment but are regulated by intrinsic circadian clocks. For example, hormonal levels of cortisol and melatonin, heart rate, blood pressure, and body temperature all exhibit well defined circadian variation. To this end, it has long been established that typically both cortisol [31] and body temperature [25,32] are at peak levels during the mid-afternoon/evening, while levels of melatonin display higher values during the nocturnal period preceding the transition to sleep [33].

It is also now acknowledged that human exercise performance follows a diurnal pattern that coincides with some of the physiological parameters discussed above. For instance, peak levels of performance typically occur in the late afternoon/evening (16:00–19:00 h) compared with the morning hours (Fig. 2). Interestingly, it seems that this circadian variation is independent of specific exercise modality or intensity. For example, measures of power output, strength, endurance capacity, and maximal uptake of VO_2 have all been shown to be elevated during the evening compared to the morning [21,34–38]. This evening superiority of muscle force production and power output are also displayed regardless of the muscle group measured [25,29]. Short-term maximal power output (often referred to as anaerobic performance) i.e., activities lasting less than 6 s have previously been shown to peak between 17:00 to 19:00 h [39,40]. Similarly, anaerobic capacity, further defined as activities lasting between 30 s and 2 min in duration show a peak in performance with greater values detected during afternoon/evening hours between 16:00 to 19:00 h [41,42].

Despite the variety in testing methodology and fitness components, significant diurnal performance differences remain [43–47]. For example, sprint tests using a cycle ergometer [46,48], swimming [49], overground running [50], jump tests [39,51], and force-velocity tests [52]. Whereas to assess anaerobic capacity, many tests ranging from the Wingate test [53], repeated sprint performance/ability [32,50,54], and running based anaerobic sprint tests [55] have previously been used in the literature to assess time-of-day variation of exercise performance. For example, classical Wingate tests have shown differences in mean and peak power by 11 % and 14 % respectively during evening time compared to morning efforts [29]. Similarly with variables related to

repeated sprint performance have shown to peak between 17:00 h and 19:00 h with differences ranging from 3 to 10 %.

More recently, literature from preclinical studies have used genetic mouse models to demonstrate that the core circadian clock mechanism is required for time-of-day differences in exercise performance [56–58]. Specifically, Ezagouri et al., [56], assigned wild-type or *Per1/2* double knockout male mice (loss of clock mechanism in all cells) to either an early (ZT14) or late (ZT22) active phase acute exercise group and subjected them to moderate-intensity exercise (~55 % intensity of maximal running). Interestingly, wild-type mice ran ~67 % longer in the late ZT22 group compared to the early ZT14 exercise group. In contrast, when the clock mechanism was knocked out in the *Per1/2* knockouts, there was no time-of-day difference in performance. These findings demonstrate that a functional circadian clock mechanism is required for the differences in time-of-day endurance performance in mice.

Adamovich et al., [58], performed a very short-term (2 weeks) exercise training study, with training sessions conducted during either an early (ZT14) or late (ZT22) time of the active phase. They found that after two weeks of training the late group runners still maintained a greater performance compared to the early exercisers. In addition, the *Per1/Per2* knockout mice did not show any daytime variance in exercise performance after two weeks of training. Additional analyses suggest that one key factors for improved endurance performance is the higher glycogen storage in the liver in the late exercise vs. early exercise group. This early vs. late differential in liver glycogen is not seen in the *Per1/Per2* knock out mice and the authors suggest that either loss of normal time-of-day feeding behaviour or altered capacity in the liver to store glycogen are the circadian clock factors necessary for time-of-day performance. In contrast, Souissi et al. [47], performed a 6-week resistance exercise training study with early and late training and this was sufficient to overcome time-of-day maximal strength differences and these effects were still apparent at 2 weeks post training. Together, these data suggest that time-of-day performance differences can be overcome, but it requires greater than two weeks of training at the same time-of-day to be realised. Further, this is evidence that circadian clocks directly underpin time-of-day differences in exercise performance. However, it is important to note here that both murine models [56,58] where global knockouts of clock genes, and therefore it is difficult to pinpoint what tissue(s) are required for the differences observed in daily exercise performance. It is also important to acknowledge that the integration of exercise information and its effects on the central clock and on peripheral clocks *in vivo* requires further investigation. Despite

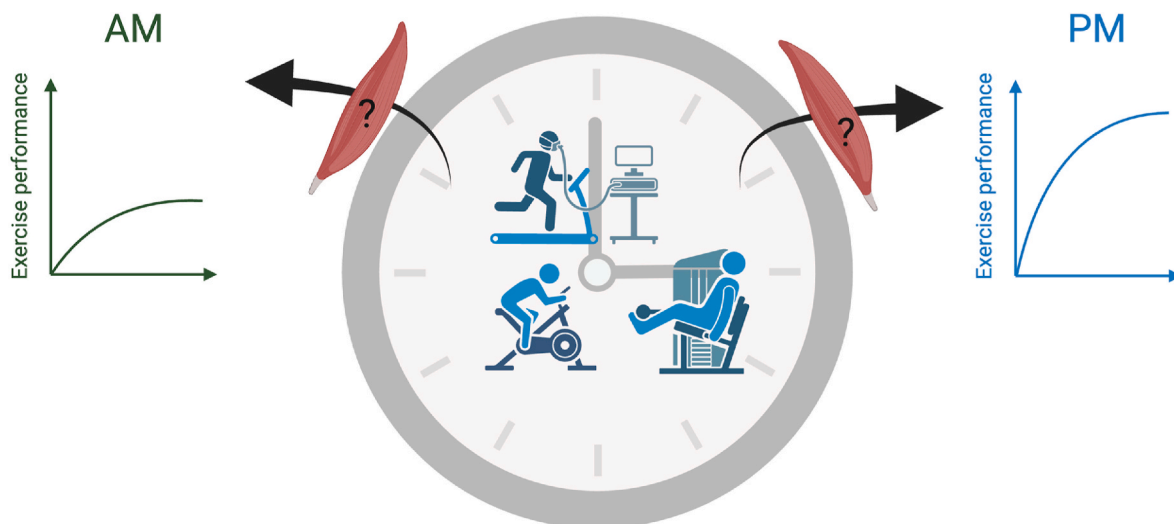


Fig. 2. The muscle clock maybe responsible for diurnal exercise performance.

Schematic illustrating exercise performance, including aerobic, anaerobic, power or strength output is different depending on what time of the day it is performed. The current mechanism is not fully resolved but it is possible the muscle clock maybe responsible for these diurnal changes in exercise performance.

these advances, the underpinning mechanism(s) downstream of the circadian clock across tissues that govern the temporal regulation of exercise performance are in the early stages with much still to be learned. The diurnal variation in human exercise performance likely requires coordination among many tissues and organs in the body. To discuss the impact of all these central and peripheral clocks and their contribution to performance is beyond the scope of this work. However, we do predict that clocks in different parts of the brain e.g., hippocampus, hypothalamus, and other tissues e.g., heart, liver etc. will all be required for optimal exercise performance.

The daily changes in muscle performance have been extensively researched and partially attributed to the daily oscillations in core body and muscle temperatures [59]. At this time the potential mechanisms contributing to muscle performance include central (neurological factors [60–64], systemic (hormonal factors, [31], and intrinsic (muscle specific factors, [65]). Several of these studies were done prior to recognition of the endogenous clock mechanism in skeletal muscle fibres. Moving forward the designs for these studies need to include considerations of the potential sources for intrinsic time-of-day contributions to changes in muscle physiology. In addition, inclusion of subject chronotype, i.e., morning larks vs. night owls, would be helpful for interpreting differences in exercise performance. As these fields come closer together, it will be exciting to learn about the contributions of circadian biology as they impact exercise performance and health.

3. The effect of exercise timing on circadian biology

In this section, we will review the evidence highlighting that the timing of exercise can inform the circadian clock system to modify or entrain the settings of the clock mechanism. Specifically, studies are emerging that indicate that time of exercise can act as a time-giver (zeitgeber) with the ability to shift the clock phase, which implies that clock output also shifts to promote temporal alterations in substrate metabolism and clock output [66–68]. However, studying the influence of exercise as a zeitgeber poses fundamental challenges in research methodology. For instance, it is necessary to maintain meticulous control over both photic (such as light, whether solar or ambient) and non-photoc zeitgebers, encompassing factors like timing of feeding and engagement in physical activity [69]. Despite this, directly measuring the activity of the circadian clock mechanism in human muscle presents inherent difficulties as it requires repeat sampling/biopsies. However, there are several studies in humans that have demonstrated that time of exercise can result in phase shifts through changes in circulating hormones, core temperature, and/or muscular contraction (Fig. 3) (see Fig. 4).

Prior studies in humans have shown that depending on the time-of-day of an exercise bout, phase-shifts have been shown to occur in the

pattern of melatonin, thyroid stimulating hormone, cortisol rhythms as well as physiological variables such as blood pressure and body temperature rhythms [71–76]. For example, it is reported that body temperature can be phase shifted (up to 6 h delay) in response night-time cycling in humans [77]. Likewise, cortisol rhythms demonstrate phase delays in response to nocturnal exercise but not to the same magnitude as temperature [72,78]. However, whether this effect is the result of masking or whether it persists after stimulus removal is unclear. Masking is the ability of an organism to act immediately in response to changes in its environment, thus meaning its response is not due to the impact of an internal oscillator [79]. For example, in one study the authors report no significant effect from the first exercise stimulus, rather multiple bouts were required to manifest melatonin phase changes. This would suggest a zeitgeber effect rather than a masking effect. Further, Buxton et al., [72], report bimodal phase-shifting in response to exercise at different times of the day with morning exercise eliciting phase delays and evening exercise eliciting phase advances in plasma melatonin secretion. The authors plotted phase response curves based on their data and showed that the responsiveness to exercise followed a different timing pattern than would be expected for light, strongly suggesting that the phase shifts were indeed the result of exercise and not a result of light exposure [80]. This concept is an important one, as any shifts in the temporal phase of the central clock will have downstream effects on the temporal phasing of factors such as performance outcomes and substrate metabolism.

Much of this work has inspired recent investigations to study the effect of exercise on circadian rhythms. Youngstedt et al., [81], demonstrated in humans that just three days of scheduled moderate exercise for 1 h induced significant shifts in the onset of key metabolites reflecting human circadian rhythms (e.g., urinary 6-sulphatoxymelatonin). Interestingly, the timing of exercise appeared to modulate these effects, with morning exercise inducing a phase advance while evening exercise resulted in a phase delay, suggesting potential clock alignment with anticipated activity periods. Moreover, Thomas et al., [82], observed phase advances in dim light melatonin onset (DLMO) following morning exercise in young sedentary adults, further supporting the role of exercise as a non-photoc zeitgeber in humans. These observations are also supported from preclinical studies. For example, Sasaki et al., [83], found that treadmill running of mice during the inactive phase led to pronounced shifts in cage activity behaviours but with subsequent removal of the exercise stimulus the activity behaviours gradually returned to innate rhythms. More recently, Hughes et al., [84], used *Vipr2* knock out mice that displayed reduced behavioural rhythmicity and were able to show that regular scheduled exercise was able to restore robust circadian rhythms in behaviour, further indicating exercise serves as a potential zeitgeber for activity behaviours. It is important to note here that these output measures of behaviour changes and/or

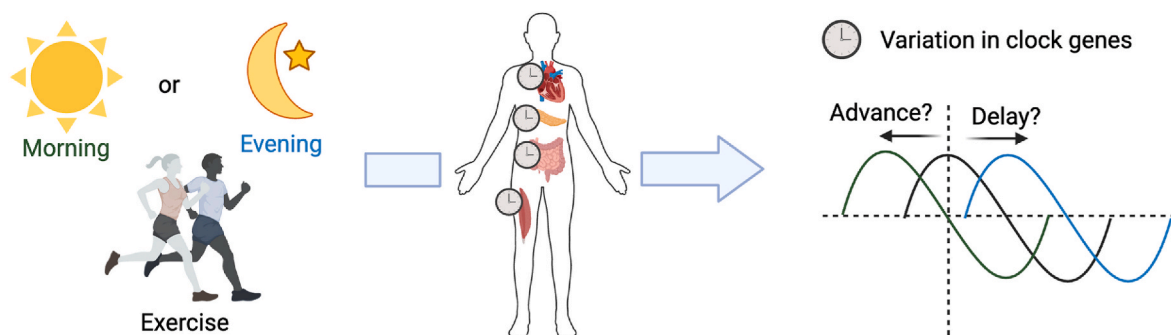


Fig. 3. – Exercise induced phase shift of the intrinsic circadian clock.

Exercise timing plays a crucial role in inducing phase shifts in the skeletal muscle clock, as illustrated by the schematic representation. The timing of exercise session, either during the early active phase (Morning) or the late active phase (Evening) influence the direction of the phase shift of the molecular clock. Arrows indicate the potential directional shift of the muscle clock. Phase shifting of the muscle clock is important as there are many genes downstream of the muscle clock that will influence the transcriptional landscape.

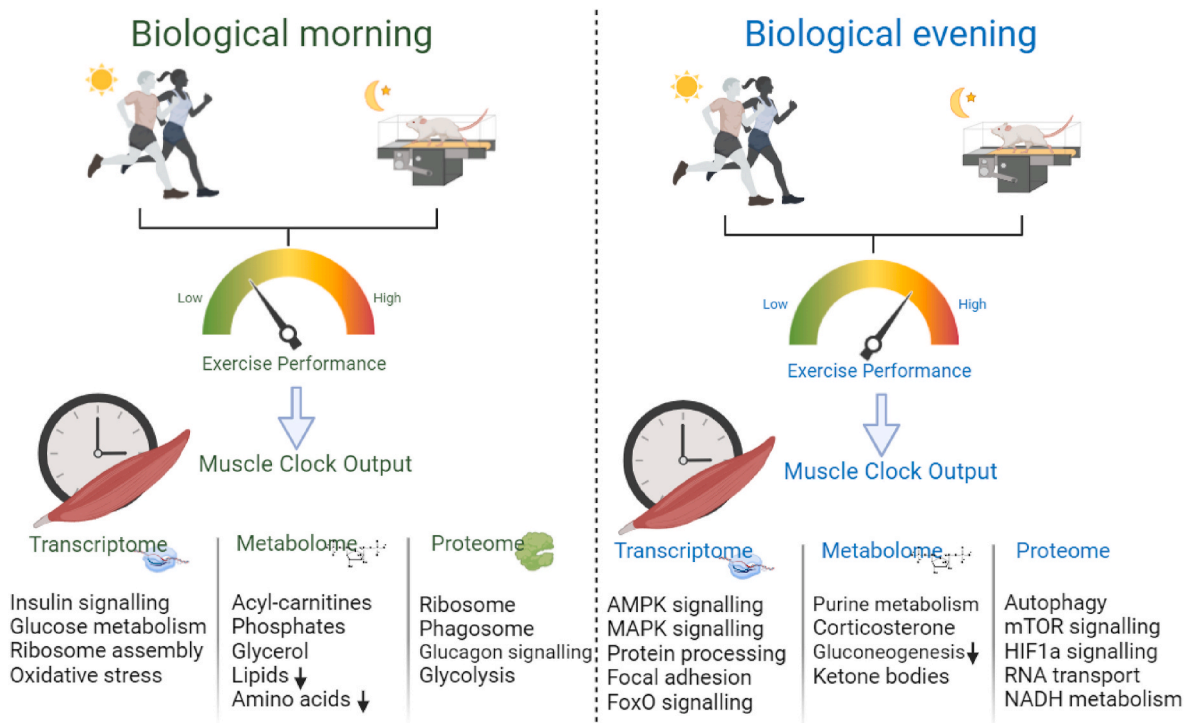


Fig. 4. – Muscle clock output is different depending on the time of exercise.

Both human and rodent exercise performance is greater in the biological evening compared to the biological morning. Studies using rodent models have been able to use integrated multi-omic analysis of skeletal muscle to measure the transcriptome, metabolome, and proteome in response to acute exercise bouts at both the biological morning and evening to demonstrate that skeletal muscle clock output is different depending on the time-of-day exercise is undertaken. The functionally annotated groups under each '-omic' heading represent groups of genes, metabolites and proteins that are enriched in skeletal muscle unique to the exercising group at each respective time-of-day. Data taken from Ezagouri et al., [56]; Sato et al., [67]; Adamovich et al., [58]; Maier et al., [70].

serum or urinary melatonin levels suggests that time of exercise can modulate the central clock mechanism in both mice and humans.

To further investigate exercise as a zeitgeber and study circadian phase-shifting effects of time of exercise in humans, [85], were among the first to utilise a constant routine paradigm in their experimental design. Constant routine protocols have been more recently defined by Duffy et al. [86], whereby conditions are standardised, so subjects maintain constant light, temperature, and a semi-recumbent posture for at least 24 h in order to study endogenous circadian rhythms without any unique external input. In the Vanreeth et al. study, participants adhered to a strict constant routine for 7 days preceding each experimental trial with food intake also controlled. The timing of exercise was standardised relative to the nadir in core body temperature, with exercise sessions scheduled to occur either 3 h before, or at, or 2 h after the minimum core body temperature. Results indicated that nocturnal exercise was associated with phase delays in serum melatonin and thyroid-stimulating hormone secretion, with the magnitude of delays generally smaller when exercise was performed later in the night (at nadir of core temperature) or early morning hours (before nadir of core temperature). Other studies employing constant routine protocols have demonstrated consistent findings, with serum melatonin phase delays observed predominantly following evening or overnight exercise. These studies are incredibly difficult to perform and the features that are held constant are different such as, some regulate sleep-wake cycles, or physical activity, or use infusion to control nutritional intake. With these variations all these studies have reported robust melatonin phase shifts associated with night-time exercise sessions [71,72,87].

More recently, investigations have started to directly address whether time of exercise can modify the expression of the core clock mechanism in different tissues, but this requires working with preclinical models. One of the challenges in studying circadian clock phase is to find a read out for the clock that can be tracked longitudinally and at a

frequency that will provide robustness and temporal precision for analysis of clock function. The development of the PERIOD2:LUCIFERASE (PER2:LUC) reporter mouse model has provided the most common mouse model for these types of studies, as researchers can monitor core clock function by tracking PER2 protein levels through real-time bioluminescence recording (data collected every 10 or 15 min) over several days [88]. Notably, Wolff and Esser, [89], conducted a comprehensive training study incorporating both treadmill running and voluntary wheel running during the inactive/light phase to investigate the impact of scheduled exercise training on PER2:LUC rhythms in tissues. This unique design allowed for a comparative analysis of clock outcomes between voluntary wheel running and treadmill running, controlling for potential stressors induced by forced treadmill exposure. Following 4 weeks of training, tissues collected 24 h post-exercise revealed significant alterations in PER2:LUC rhythms in lung and various skeletal muscles, indicating a pronounced phase shift compared to the non-exercised counterparts. Intriguingly, no discernible change was detected in PER2:LUC rhythms in the SCN with exercise, suggesting the possibility of exercise exerting direct effects on skeletal muscle independently of the central SCN clock [89]. While the absence of a phase shift in the SCN may be attributed to light-induced resetting during the light: dark cycle, it underscores the potential of exercise as a potent zeitgeber. Similarly, Dudek and colleagues, [90], exposed mice to 12 days of incremental treadmill running 2 h after the beginning of the normal resting phase, leading to a significant phase advance of the skeletal circadian clock of up to 8 h, but no detected phase shift was observed for SCN. Taken together, these studies provide strong evidence for the systemic influence of exercise on peripheral clocks, as documented by the observed effects on both the skeletal muscle, bone, and lung clocks. However, despite these advancements, the exploration of the effect of exercise on other peripheral tissues remains relatively limited, necessitating further investigation to comprehensively

understand the impact of exercise on systemic clock modulation.

The timing of a single exercise bout to study the acute effects of exercise on the phase of the muscle clock has also been investigated. Kemler et al., [91], used PER2:LUC mice and subjected them to a single bout of moderate treadmill exercise lasting 60 min at 3 different times of day corresponding to various phases of rest/light and activity/dark. Notably, exercise during the middle of the resting phase resulted in an advance in PER2:LUC rhythms, while exercise at the end of the rest phase led to a delay in PER2:LUC rhythms in muscle. Conversely, exercise at the midpoint of the active phase did not result in a significant phase shift [91]. These data are novel in that they demonstrate that a single bout of exercise has the capacity to shift the phase of the muscle clock according to the time-of-day the exercise bout it administered.

While acute changes in phase response suggest a strong influence of exercise timing on the muscle clock, it does not definitively distinguish between the possibility of the SCN regulating the muscle clock imposing a direct effect of exercise on these peripheral clocks. Emerging evidence from both *ex vivo* and *in vitro* studies supports the notion of exercise as a potent time cue capable of directly influencing the muscle clock. *In vitro* experiments conducted by Small et al., [92], demonstrate that electrical stimulation of mouse skeletal muscle in an *ex vivo* system induces an increase in *Per2* gene expression, demonstrating that muscle contractions alone can elicit changes in this core clock gene. Furthermore, Kemler et al., [91], utilised synchronised muscle cell lines transfected with *Bmal1*-luciferase reporter, with electrical induced contractions to reveal that a 60-min bout of muscle contractions *in vitro* produced sustained phase advances or delays (or no change) in the circadian muscle clock that mirror findings from *in vivo* treadmill running. These *in vitro* and *ex vivo* investigations provide compelling evidence that the circadian clock in muscle can receive direct time-setting cues from exercise and/or muscle contractions.

It is also important to note here, that the way exercise is administered to small rodents may impact circadian oscillators. Sasaki et al., [83], investigated whether peripheral clocks are entrained to the same degree in both voluntary or forced exercise in PER2:LUC mice. The authors demonstrated that forced running rather than voluntary running entrained peripheral clocks more strongly, even though distance, speed, and body temperature increases were all comparable. Thus, suggesting exercise-induced phase shifts in peripheral oscillators may be due to differences in type rather than intensity of exercise. Further analysis revealed that corticosterone and noradrenaline in peripheral tissues were significantly higher in the forced running group compared to the voluntary group, further suggesting forced running represents a more stressful situation to the animals, consequently resulting in larger phase shifts. However, specifically in skeletal muscle Wolff and Esser, [89], demonstrated that both voluntary and forced treadmill exercise administered during the rest phase induced similar phase shifts of the muscle clock, revealing the potential for an exercise specific impact of type of exercise, but currently data are equivocal.

4. The skeletal muscle circadian clock

This section will deal with skeletal muscle clock-controlled output and how functional and metabolic changes occur in accordance with time-of-day. We will also discuss how clock output is influenced by the timing of exercise, before finally discussing how certain signaling pathways implicated in exercise are modulated over time-of-day and may impact performance. Intrinsic to skeletal muscle is the molecular clock which mediates transcriptional, metabolomic, and proteomic programs that exhibit daily rhythmic oscillations, therefore potentially influencing metabolism, physiology, and exercise performance in a time-of-day manner.

As noted earlier, the core clock mechanism regulates a daily program of gene expression in all tissues, including skeletal muscle. Consistent with the circadian modulation of muscle physiology, hundreds of transcripts in skeletal muscle oscillate with a 24 h periodicity in both

humans [93] and rodents [94]. Moreover, insulin sensitivity, mitochondrial respiration, glucose, and lipid-related metabolites likewise follow similar patterns in muscle tissue [95,96]. In line with this, the daily variations in exercise peak performance have been reported to fluctuate during the active/feeding phase in humans [65,97] and rodents [56,58]. This notion suggests that circadian clock-controlled output, specifically in skeletal muscle, is likely indicative of the specific temporal responses to exercise. For example, exercise during the early active/feeding phase, when hepatic glycogen content is reduced, rather than exercise at the early rest/fasting phase, when hepatic glycogen content is increased, results in the rapid depletion of carbohydrate energy stores in skeletal muscle, and a shift toward utilisation of fatty acid metabolism. Therefore, exercise induces a major physiological systems perturbation but the clock output in skeletal muscle is highly dependent on the position of the clock, directly impacting on exercise response and exercise performance.

Previous data demonstrates that human muscle mitochondrial oxidative capacity exhibits a time-of-day pattern with highest capacity in the late afternoon (e.g., Ref. [21]). More recently, these observations have been followed by data from Gemmink et al. [98], demonstrating time-of-day fluctuations in mitochondrial morphology that mirrors oxidative capacity. Additional papers have also highlighted various aspects of mitochondrial structure and function with muscle circadian clocks [99–102], indicating that circadian clock output in skeletal muscle contributes to the daily variations in mitochondrial oxidative capacity. There is now growing evidence to support this notion with recent findings from Xin et al., [103], who demonstrated that the use of time restricted feeding as a method to advance the muscle clocks, is sufficient to improve rest phase maximal endurance capacity, even in the absence of exercise training. Xin et al., [103], found that the increased performance was associated with the alignment of the expression profile of the oxidative metabolism gene network in muscle to time of feeding. Importantly, this effect was not observed in mice without a functional whole-body or muscle-specific clock mechanism, illustrating that the muscle clock directed the changes in endurance performance observed with rest-phase-restricted feeding. The implications of this body of work suggests that when implementing an exercise training regimen, it will be important to consider time-of-day to support alignment of the muscle clock and metabolic profile to the time of performance.

Transcriptional and metabolite profiles in skeletal muscle have previously been shown to alter in response to acute exercise at different times of the day. Ezagouri et al., [56], conducted a study on the skeletal muscle of animal exercisers from the early (ZT14) versus the late (ZT22) active phase. The authors identified a greater number of significantly regulated transcripts that were unique to exercise at the early active phase (343 genes), rather than those unique to the late active phase (125 genes), with an overlap of 160 genes. A similar study was reported in Sato et al. [67], in which they subjected mice to a 1 h acute bout of treadmill exercise at the early resting/fasting phase (ZT3) or during the early active/feeding phase (ZT15) with respective control groups. The analysis revealed significant changes in numerous transcripts and metabolites, particularly evident after exercise during the early active phase (biological morning) compared to the early rest phase (biological night). Both these studies demonstrate that the molecular and metabolomic acute exercise response varies a lot based on the time-of-day exercise is undertaken. Importantly, the same exercise bout matched for intensity and duration elicits very different gene expression changes and metabolomic outcomes depending on the time-of-day. This suggests that the transcriptional and metabolomic response to exercise maybe interacting through the circadian clock influencing clock output. However, as these studies where in response to acute bouts of exercise we do not currently know what this means for exercise training.

It is not just transcriptional and metabolite alterations that are apparent over time-of-day. Proteomic and phosphoproteomic profiles have also been shown to change upon acute exercise at different daily

times which also point towards differential biological responses in skeletal muscle unique to the exercise timing [70]. Proteins involved in energy provision and catabolic pathways have been shown to be preferentially enriched after morning and evening exercise. Providing strong evidence that suggests specific time-of-day exercise imparts very different molecular responses depending on the time of execution. Together these multi-omics layers of evidence underscore the time-of-day-dependent impact of exercise on energy metabolism followed by endurance exercise performance [56,58,67,70], demonstrating the timing of exercise impacts the skeletal muscle clock by modulating transcriptional, metabolomic and proteomic profiles downstream of the clock (clock output). While this does not address potential changes with long-term training, it does highlight the interaction of exercise and the circadian clock, providing evidence that exercise can affect the muscle clock independently and downstream factors of the clock are different over time-of-day.

Exercise can also function to enhance clock output. A recent circadian time course study assessed this notion measuring the circadian transcriptome (clock output) after exercise training in mice. Casanova-Vallve et al., [104], compared sedentary mice with those provided with access to a running wheel for six weeks for voluntary exercise training. Strikingly, mice with access to the running wheel exhibited approximately a 50 % increase in the number of rhythmically expressed genes compared to sedentary counterparts (1446 versus 938). This enhancement of clock output with exercise training is similar to findings from Acosta-Rodriguez et al., [105], demonstrating the time restricted feeding can also enhance clock output in the liver in aging mice. There is still very little research in this area, but these two studies support the concept that environmental non-photic time cues, such as exercise and feeding, might be considered as chronotherapeutic strategies to support and enhance clock output in peripheral tissues. Despite this, further investigations are imperative to ascertain whether the heightened number of rhythmically expressed genes in response to exercise training stems from increased calcium signaling, alterations in transcription factor abundance, or enhanced chromatin accessibility. Notwithstanding these uncertainties, the above findings propose that regular physical activity may serve to bolster the functionality of the skeletal muscle clock by amplifying clock output, encompassing the rhythmic expression of transcription factors, cofactors, and genes that orchestrate metabolic processes and can in turn effect exercise performance.

Exercise responses within signalling pathways have also been shown to exhibit time-of-day specificity, implying a nuanced interaction between circadian timing and molecular pathways. Well characterised pathways in muscle physiology, such as mechanistic target of rapamycin complex 1 (mTORC1) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1a), have been reported to be downstream of the molecular clock, suggesting a mechanism through which circadian timing influences exercise outcomes [106,107]. Moreover, evidence indicates potential interactions between core clock components and these pathways. For instance, Wu et al. (2019) found that liver PER2, peaking at the end of the resting/fasting phase, suppresses mTORC1 activity. However, whether such interactions are conserved across tissues, particularly skeletal muscle, remains uncertain.

Key pathways have emerged in skeletal muscle which are now known to influence the circadian muscle clock. These pathways are involved in mitochondria and the electron transport chain; for example, the regulation of nicotinamide adenine dinucleotide (NAD⁺) is under clock control [108]. In turn, NAD⁺ impacts mitochondrial respiration through the NAD-dependent deacetylases the Sirtuins [109], which are involved in chromatin remodelling, suggesting a connection between metabolism and transcriptional regulation [110,111]. Additionally, pathways like AMP-activated protein kinase, known for its responsiveness to exercise [112], have been implicated in the regulation of circadian genes [113].

Nonetheless, the molecular responses to exercise in skeletal muscle display time-dependent effects, especially in the context of acute

exercise. Resistance exercise performed during the early active/feeding phase induces acute mTORC1 activation in skeletal muscle via p70S6K phosphorylation, contrasting with a more subdued response observed with late active/feeding phase exercise in humans [114]. Despite these acute variations, consistent timing of resistance exercise training (over 12 weeks) appears to have no significant impact on the magnitude of skeletal muscle hypertrophy [36,115]. However, it is crucial to recognise that the clock output operates akin to a chemical rheostat, sensitive to time-of-day variations. Consequently, the timing of exercise is likely to modulate the sensitivity, and potentially the amplitude and duration, of exercise signalling pathways. Nonetheless, it remains to be determined whether time-of-day differences observed in acute exercise responses or exercise performance can be mediated through such pathways by the intrinsic muscle clock. Further investigations are warranted to unravel the biological relationship between circadian timing, molecular pathways, and exercise outcomes, potentially offering future insights into the optimisation of exercise timing for enhanced physiological adaptations and exercise performance.

5. Conclusions and future Perspectives

In summary, the well documented diurnal changes in muscle and exercise performance have been extensively researched and evidenced. Attempts to attribute these changes to circadian physiological changes have been robust, but the evidence suggests that the mechanisms for such changes are likely to be at the molecular scale. Current research goes a long way in attempt to elucidate these underlying mechanisms, but the precise biological machinery responsible for diurnal oscillations in muscle function, performance, and exercise adaptation remains elusive. In reality, the interaction between exercise, performance factors, and the molecular clock is likely to be confounded by many factors, including mealtime, sleep, psychological stress, and other external zeitgebers [116] making the study of this interaction challenging. However, animal experiments with voluntary wheel running and scheduled exercise suggest that skeletal muscle clock oscillations are robust, even when subject to perturbations induced by daytime running and feeding [58,70].

What is clear, is that studies of the transcriptome, metabolome, proteome, and signalling changes agree that circadian clocks in skeletal muscle, and other tissues, function to regulate clock output in a time-of-day manner. This daily expression pattern downstream of the clock is part of our predictive homeostasis and plays a critical role in modulating cell physiology over time-of-day, and potentially exercise performance. These findings are supported by studies in both humans and rodents, highlighting that the physiological response to an acute bout of exercise will differ based on time-of-day. While these findings are quite clear there are still many fundamental questions to be addressed as to the impact of specific time-of-day training. For example, it is currently unclear what impact chronic exercise training at different times of the day has on the muscle clock and clock output, and thus the potential to impact adaptation. Furthermore, it is still unknown whether these daily molecular programs contribute towards a muscle phase shift, or if the molecular profile significantly differs after a muscle phase shift. Despite these knowledge gaps, the emerging understanding strongly indicates that exercise acts as a zeitgeber, synchronising our internal circadian system. Whether this mechanism can optimise athletic potential within the circadian framework remains to be seen. However, the interaction between exercise and circadian rhythms, particularly in the context of the muscle clock, demonstrates that time-of-day exercise imparts very different molecular responses depending on the time of execution, but further work is required to understand if this is responsible or can be utilised to enhance exercise performance.

CRedit authorship contribution statement

Stuart J. Hesketh: Writing – review & editing, Writing – original

draft, Visualization, Conceptualization. **Karyn A. Esser:** Writing – review & editing.

Declaration of competing interest

Stuart J. Hesketh and Karyn A. Esser declare they have no conflicts of interest.

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