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The time is now: accounting for time-of-day effects to improve reproducibility and translation of metabolism research

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The constant expansion of the field of metabolic research has led to more nuanced and sophisticated understanding of the complex mechanisms that underlie metabolic functions and diseases. Collaborations with scientists of various fields such as neuroscience, immunology and drug discovery have further enhanced the ability to probe the role of metabolism in physiological processes. However, many behaviours, endocrine and biochemical processes, and the expression of genes, proteins and metabolites have daily ~24-h biological rhythms and thus peak only at specific times of the day. This daily variation can lead to incorrect interpretations, lack of reproducibility across laboratories and challenges in translating preclinical studies to humans. In this Review, we discuss the biological, environmental and experimental factors affecting circadian rhythms in rodents, which can in turn alter their metabolic pathways and the outcomes of experiments. We recommend that these variables be duly considered and suggest best practices for designing, analysing and reporting metabolic experiments in a circadian context.

Metabolism encompasses life-sustaining chemical reactions under basal or everyday conditions and in response to changes in the cellular or organismal environment. These chemical reactions include but are not limited to energy production, synthesis and breakdown of biological building blocks and elimination of cellular and metabolic wastes. Most organisms live under a 24-h cycle of light and darkness and have anticipatory rhythms in rest–activity and feeding–fasting. The metabolic states of organisms or cells also exhibit time-of-day-dependent adaptive changes. Consequently, the molecular underpinnings of metabolism including epigenetics, transcription, translation, post-translational modifications and metabolites exhibit relevant temporal changes. In parallel, cellular and organismal responses to acute changes in nutrients or stress also have time-of-day-dependent effects. Ideally, time series assessments of metabolism across 24 h would paint a thorough picture. However, most metabolic research often perturbs and/or measures metabolism at one or few time points. Hence, monitoring and reporting factors that affect metabolism across a 24-h day increase reproducibility, reduce cost, facilitate a deeper understanding of metabolic regulation and improve translational value in diurnal humans.

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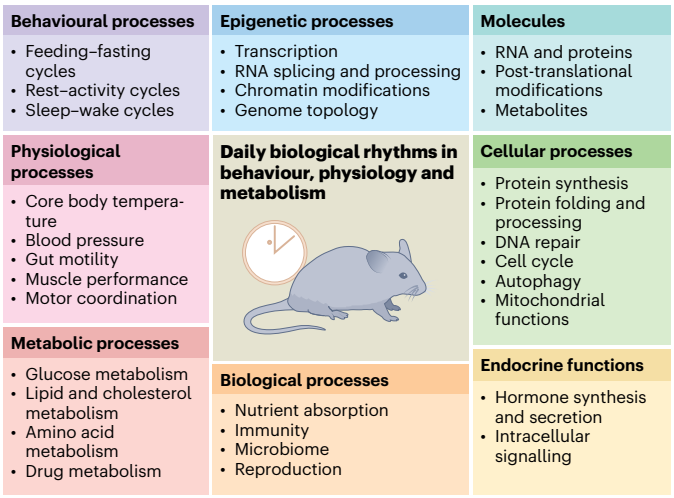

Fig. 1 | Daily biological rhythms at various organizational levels. The circadian clock and food availability regulate daily biological rhythms in various behavioural, physiological and molecular processes. These rhythms lead to diurnal fluctuations in hormones, intracellular signalling, metabolic pathways, cellular housekeeping functions and in the levels of RNA, proteins (and their modifications) and metabolites. Figure created using BioRender.com.

The importance of documenting and reporting the timing of experiments with animals is well accepted. The experimental procedure section of the ARRIVE 2.0 (Animal Research: Reporting In Vivo Experiments) guidelines requires reporting the time when the experiment was performed. Although the ARRIVE 2.0 guidelines have been adopted by several funding agencies, universities, scientific societies and journal publishers[1], a recent analysis of 1,000 research articles spanning immunology, neuroscience, oncology, physiology, metabolism, pharmacology and reproductive biology found that only 6% of reports included time-of-day information about experimental procedures[2]. This suggests that most researchers may not consider the timing of experimentation and data collection or properly report this information.

In this Review, we discuss the importance of considering circadian rhythms and time of day in metabolic research. Factoring circadian timing in experiments is critical, as most behavioural, physiological and molecular processes display daily fluctuations that are regulated by endogenous biological rhythms, ambient lighting conditions and timing of food availability, sleep and physical activity (Fig. 1). Daily rhythms in behaviour, physiology or metabolism are altered in many animal models of metabolic diseases as well as in human metabolic disorders. Behavioural interventions, such as time-restricted feeding, which increase the amplitude and synchronization of diurnal rhythms across tissues, also attenuate disease severity, thus suggesting rhythm disruption as a contributing factor to the disease.

Translational metabolic studies are complicated by the fact that mice are nocturnal while humans are diurnal. Most aspects of their physiology are inverted with respect to the light–dark and day–night cycle but are similar with respect to the rest–activity cycle. For example, after administering a fixed bolus of glucose, there is faster glucose clearance from blood during the early active phase (early dark phase Zeitgeber time (ZT)14 in rodents and morning in humans) than in the late active phase (late dark phase ZT22 in rodents and afternoon in humans)[3,4]. Thus, the difference in circadian timing of physiology between rodent models and humans is a critical parameter that needs consideration for proper experimental design and the translation of discoveries based on animal research to human application and/or clinical trials[5]. Here, we detail the biological, environmental and experimental factors that can affect circadian rhythms of mice and provide recommendations for designing and reporting experiments for in vivo metabolic studies. While this Review specifically focuses on metabolic research in mice, most of the points raised here are applicable to other fields of biomedical research and to other model organisms.

Plasticity of daily rhythms in metabolism, physiology and behaviour

A common misconception about circadian rhythms is that they relate only to sleep–wake cycles and are relevant only when studying sleep or experimental models of shift work. Circadian rhythms are regulated by cell-autonomous circadian clocks that are present in almost every cell type of animals and also in cultured cells. In mammals, the circadian timekeeping system is based on transcription–translation feedback loops in which more than a dozen transcriptional regulators (CLOCK, BMAL1, NPAS2, PER1, PER2, PER3, CRY1, CRY2, NR1D1, NR1D2, RORa, RORb, RORc) form activator and repressor complexes. The levels, macromolecular composition and nucleocytoplasmic localization of these complexes are regulated by transcription, translation, post-translational modifications and protein degradation to produce a ~24-h rhythm in their own functions and in the transcription of their downstream targets. In several cell types, circadian clock components and their downstream targets also interact with cell type-specific factors to drive rhythmic expression and functions of hundreds to thousands of genes. These molecular rhythms give rise to tissue-specific rhythms in cellular functions, intercellular communication and ultimately in metabolism, physiology and behaviour[6].

To adapt to seasonal changes in day length and associated changes in the timing of food availability, circadian clocks are also adaptable or entrainable. The main or central circadian oscillator in the suprachiasmatic nucleus (SCN) receives input from intrinsically photosensitive and melanopsin-expressing retinal ganglion cells[7]. Changes in ambient light sensed by the intrinsically photosensitive and melanopsin-expressing retinal ganglion cells entrain the SCN clock to new lighting conditions. The SCN, in turn, uses diffusible, systemic and as yet unknown factors to coordinate the timing of circadian clocks in other brain regions and the rest of the body. SCN neurons are strongly coupled, and the molecular clock in the SCN is highly responsive to changes in environmental light–dark cycles as compared to other peripheral organs[8]. However, in response to an abrupt change in light–dark cycle, as in experimental jet lag, the activity–rest cycle can take several days to reestablish a stable daily rhythm with the new light–dark cycle. Moreover, mice with a perturbed SCN synchronize more rapidly to jet lag or to alternate feeding schedules than SCN-intact mice, indicating that light-induced SCN-derived signals prevent aberrant synchronization of peripheral clocks to alternate Zeitgebers[9,10]. Under constant light, circadian rhythms are dampened but not abolished.

Circadian clock components, either directly or indirectly, reciprocally regulate several nutrient-sensing pathways, including but not limited to mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK) and nicotinamide adenine dinucleotide (NAD)-sensing components[11]. Several clock components also sense the redox state of the cell. Consequently, the circadian clock in the peripheral organs[12,13] as well as in most of the brain regions outside the SCN is influenced by the timing of feeding[14]. As a result, experimental conditions, such as calorie restriction (CR), paired feeding and meal feeding, in which food is delivered to animals at specific times of the 24-h day, also inadvertently affect the phase and robustness of the circadian clock in peripheral organs and the synchrony of peripheral organs with the SCN clock.

Light–dark and feeding–fasting cycles also have clock-independent effects on behaviour and metabolism. For example, mice carrying loss-of-function alleles of *Bmal1* or deficient in the products of *Per1* and *Per2* or *Cry1* and *Cry2* (CDKO) exhibit no rhythm in activity–rest under constant darkness. Yet under a light–dark cycle, *Per1*- and *Per2*-knockout and CDKO mice show an apparent rest–activity pattern

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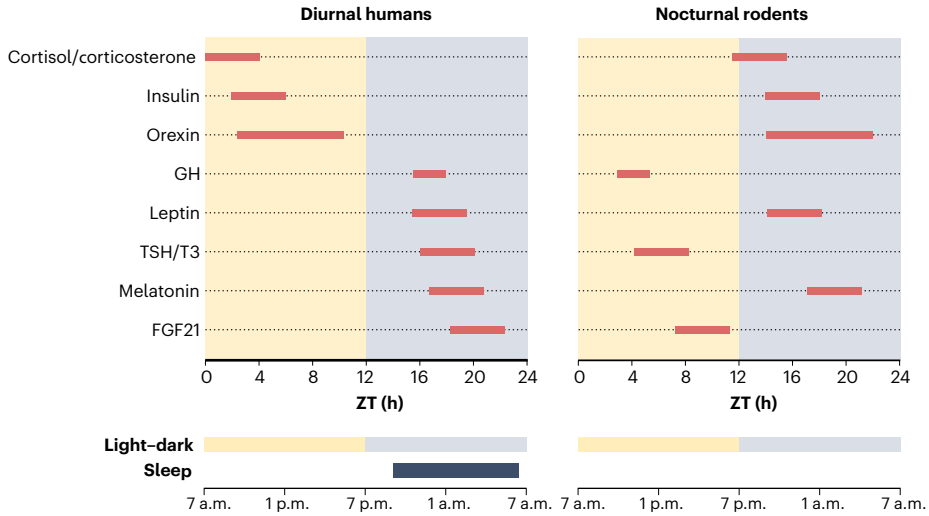

Fig. 2 | Schematic representation of the peaks of various hormones in the serum in diurnal humans and nocturnal rodents. TSH, thyroid-stimulating hormone; T3, triiodothyronine; GH, growth hormone; FGF21, fibroblast growth factor 21 (refs. 154–160).

that appears rhythmic, with low activity during the day (light) and higher activity at night (dark). *Bmal1*-knockout or CDKO mice show no apparent feeding rhythm under such light–dark conditions. Similarly, subjecting some of the clock mutant mice to time-restricted feeding during the dark phase bolsters rhythms in the respiratory exchange ratio and in the expression of several metabolic regulators in the liver[15,16]. Additionally, there is circadian-independent light regulation of metabolism involving melanopsin-based signalling via non-SCN brain regions and direct stimulation of other opsins in peripheral organs, which can affect thermogenesis, food intake, blood pressure, glucose uptake and mitochondrial functions[17]. In summary, the genetic makeup, ambient light conditions and timing of nutrition shape the daily rhythm in activity and metabolism.

Daily rhythms in nutrient fluxes

Metabolic flux in tissues is strongly influenced by the availability of nutrients. After feeding, nutrients are digested and absorbed in the gut through an intricate interaction of host factors (pancreatic secretion and bile acids) and gut microbiome-derived factors. Thus, the fed state is characterized by increased plasma levels of monosaccharides, amino acids and fatty acids, which stimulate the release of insulin from pancreatic beta cells to coordinate energy production and storage (as glycogen and triglycerides), cellular repair and metabolic homeostasis. These feeding-induced changes in nutrient fluxes are brought about by allosteric changes in metabolic enzymes, signalling pathways downstream of hormones and metabolic sensors, and transcription of genes involved in anabolic processes such as nucleotide metabolism, ribosomal RNA synthesis, protein synthesis and glycosylation.

In the post-prandial state, levels of metabolites and insulin drop, which signal glucagon release and induction of a fasting state. This transition reorganizes nutrient flux and induces catabolic pathways such as lipolysis and fatty acid oxidation, amino acid breakdown and gluconeogenesis, which are driven by cellular signalling pathways downstream of hormones and metabolic sensors, and the induction of catabolic gene expression. Upon the next meal, these catabolic processes are suppressed and anabolic processes are induced. Tissue and organ system responses to daily feeding and fasting are tuned by autonomous and cell and tissue non-autonomous mechanisms, including neuroendocrine inputs, circadian rhythms, body temperature and metabolite exchange[18]. These alternating feeding–fasting physiological states are causal as well as consequential for changes in nutrient fluxes, intracellular signalling and transcription[19–21].

Daily rhythms in hormones and intracellular signalling

Many hormones and signalling pathways have daily rhythms that are controlled by circadian clocks or their outputs, such as rhythms of sleep, activity and feeding (Fig. 2). For example, daily rhythms in glucagon and insulin secretion are generated by the transcription of genes involved in their secretion at specific times of the day, and these rhythms require an intact circadian clock in pancreatic alpha and beta cells. Moreover, ablation of beta cells does not induce a compensatory proliferation and regeneration response in mice lacking *Bmal1* (ref. 22). Furthermore, there are daily rhythms in insulin sensitivity and glucose tolerance in several peripheral tissues including liver and muscle, which are modulated by the circadian clock[23].

Levels of several other hormones, such as cortisol, glucagon, adiponectin and ghrelin, also exhibit daily rhythms[24]. Moreover, the gut exhibits substantial time-of-day variation at multiple levels, including alteration in cell proliferation and regeneration, gut motility, nutrient absorption, barrier function, susceptibility to inflammation, and secretion of enteroendocrine hormones such as GLP-1 (refs. 25,26). Furthermore, rhythms have been demonstrated for multiple processes, including hormone secretion, in gut organoids studied ex vivo.

These daily rhythms in hormones and metabolites cause downstream intracellular signalling to peak at specific times of the day and temporally regulate gene expression. For example, daily changes in glucose, amino acid and insulin secretion cause rhythmic mTOR complex 1 (mTORC1) signalling, which regulates nucleotide synthesis, ribosomal biogenesis, protein translation and DNA replication and the cell cycle[27]. Moreover, diurnal rhythms in free fatty acids and their derivatives activate peroxisome proliferator-activated receptors (PPARs) at specific times of the day[28,29]. Changes in plasma bile acid levels also affect signalling patterns through nuclear (for example, FXR and VDR) and membrane (for example, TGR5) bile acid receptors[30].

Daily rhythms in cellular omics

Global transcriptomic and proteomic studies in the past two decades have found that expression levels of ~85% of protein-coding genes have daily rhythms. Likewise, expression of most metabolic enzymes is elevated at specific times of the day[31,32]. Feeding–fasting and rest–activity rhythms regulate gene expression by driving daily variations in histone modifications, RNA polymerase II phosphorylation, chromatin accessibility and genome organization[33]. Additionally,

post-transcriptional and post-translational mechanisms coordinate daily rhythms in gene expression, protein synthesis, cell signalling and functions[34]. The mechanisms described above are regulated by dynamic changes in gene expression mechanisms and signalling networks, and endogenous clock proteins provide time-of-day information by gating their peak activity levels. Moreover, such regulatory mechanisms ensure anticipation of the feeding–activity phase by providing rapid physiological state transitions through changes in metabolites, signalling cascades and microRNAs.

These circadian rhythms in behaviour and physiology ultimately cause rhythms in 20–50% of metabolites across tissues[35]. Additionally, blood and tissue oxygenation can exhibit daily rhythms[36]. Together, these daily rhythms in gene expression, protein and post-translational modification levels and metabolites drive the compartmentalization of catabolic and anabolic processes throughout the day–night cycle to achieve an optimum balance in energy homeostasis.

Feedback mechanisms entrain the circadian clock

Daily rhythms in hormones, intracellular signalling, gene expression and metabolites also feed back to the core clock timekeeping mechanism to regulate circadian rhythms and modulate entrainment to changing environmental cues[18]. For example, changes in metabolic flux can affect core clock gene activity or stability via post-translational modifications. O-linked glycosylation by *N*-acetylglucosamine regulates the stability and transcriptional activity of the regulators CLOCK, BMAL1 and PER2 (ref. 37). NAD^+ levels regulate the poly-ADP ribosylation activity of PARPs and the deacetylation activity of sirtuins on CLOCK, BMAL1 and PER2. Cellular AMP/ATP ratios affect AMPK activity and phosphorylation of CRY1 to regulate its stability. In addition, O_2 rhythms can regulate hypoxia-inducible factor 1 (HIF1) stability and dimerization with BMAL1 for transcription activation[36]. Thus, there is cooperation among nutrient-sensitive transcription factors, signalling mechanisms and clock genes to coordinate gene expression with daily cycling of nutrients.

Daily rhythms in cellular processes

The molecular, structural and functional properties of several intracellular organelles, including nuclei, ribosomes, mitochondria, endoplasmic reticulum, lysosomes and autophagosomes, exhibit daily rhythms[38,39]. It is hypothesized that these rhythms in individual organelles are synchronized to maintain cellular energetics and to compartmentalize organelle functions. Various mechanisms such as intracellular signalling, proximity and contact sites, and shuttling of proteins and metabolites between various organelles have been proposed for maintaining this synchrony. Nevertheless, many questions remain unanswered in this field, and recent advances in spatial technologies may address many of these unresolved questions in the future.

Mitochondrial functions such as respiration and nutrient utilization show daily rhythms in rodents and humans, partly due to rhythmic changes in mitochondrial gene expression. This is corroborated by changes in mitochondrial biogenesis, morphology (fission and fusion) and mitophagy during day and night[40]. Additionally, more than one-third of the mitochondrial proteome and acetyl-proteome is rhythmic including rate-limiting metabolic enzymes, thus compartmentalizing the utilization of particular nutrients (for example, glucose versus fatty acids) to specific times of the day[41,42]. Mitochondrial metabolite flux is highly influenced by diurnal rhythms. For example, under a fed state (during the nighttime for mice), liver mitochondria actively uptake malate into the matrix as a part of the malate–aspartate shuttle. By contrast, under a fasted state (often the daytime for mice), mitochondrial malate and oxaloacetate are exported to the cytosolic compartment as gluconeogenic precursors in response to fasting-related hormones such as glucagon. Differential mitochondrial flux or utilization of fatty acids, acetyl-coenzyme A (CoA) and other tricarboxylic acid intermediates also shows fed–fasted-dependent changes and has been shown to influence metabolite-driven protein modifications such as acylation and glycosylation, which feed back onto clock proteins[40,43].

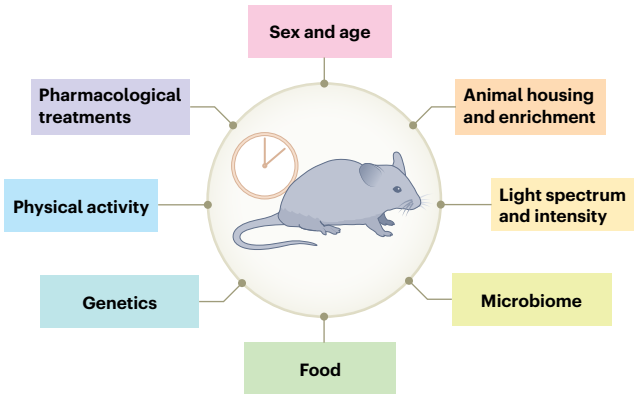

Fig. 3 | Factors affecting circadian rhythms and metabolic outcomes. The circadian rhythms of mice are altered by various biological, environmental and experimental factors. These alterations can significantly impact behaviour and physiology, consequently modifying metabolism and the outcomes of biological experiments. Figure created using BioRender.com.

Daily rhythms in autophagy have been observed in several organs including liver, skeletal muscle, kidney and heart, potentially contributing to rhythms in cellular repair, recycling and nutrient utilization. Cyclic autophagy flux is also associated with rhythmic expression of several autophagy genes across tissues and is co-regulated by signalling through metabolic sensors, hormones and transcription regulators such as C/EBPβ, PGC1α and PGC1β, TFEB and TFE3, and FOXO3 and the clock proteins CLOCK, BMAL1 and NR1D1. On the other hand, levels of clock proteins such as CLOCK, BMAL1, CRY1 and NR1D1 are themselves regulated by autophagy[44,45].

Circadian rhythms in cellular and molecular processes in animal models also extend to cells in culture, albeit with some modifications. Each cell in culture has its own circadian rhythm, and the bulk population is asynchronous unless the cells are synchronized[46]. Various external agents such as dexamethasone, forskolin, serum shock, temperature and even regular serum change can synchronize cells and restore coherent circadian rhythms in the culture dish before the circadian rhythms in individual cells disperse[47–50]. These synchronous cell culture systems that mimic in vivo circadian rhythms are better than the conventional unsynchronized cell culture systems for screening and testing drug metabolism and toxicity. Moreover, primary fibroblast cultures and derived induced pluripotent stem cells from human patients retain several host physiological properties and thus can be useful for basic as well as clinical research on understanding the interplay between circadian rhythms and disease pathology[51]. However, with recent advances in single-cell imaging techniques, it is possible to determine the circadian properties of individual cells in a bulk culture[52].

Factors affecting circadian rhythms

Below are the biological, environmental and experimental factors that can affect circadian rhythms of mice. These factors should be considered when designing experiments and reporting metabolic studies (Fig. 3).

Strain and genotype

Inbred mouse strains differ in their intrinsic circadian rhythms, activity levels, activity rhythms and melatonin production. Strain differences in the free-running circadian period under constant darkness can range from 22.9 h in the BALB/cByJ strain to 23.93 h in the 129/J strain and 23.77 h in the C57BL/6J strain[53].

The circadian rhythm or sensitivity of circadian rhythms to light also differs by strain. The number of days it takes mouse strains to adapt to a new light–dark cycle by establishing a stable activity pattern alone can vary by several days between different strains[54].

Strain differences also exist in total daily activity levels[55] as well as the daily pattern of activity. In C57BL/6 mice, exploratory cage activity levels (not wheel running) can be only half of those in BALB/c mice, and C3H and CBA mice have nearly half of B6 activity levels at night. Even within a 4-h time window during the nighttime, activity levels can change significantly, thus potentially affecting energy expenditure and the metabolic state. Therefore, any molecular or physiological measurements on a group of isogenic animals spread over a 4-h time window can have more biological noise than the same measurements collected within a 1-h or 2-h window. In other words, accounting for circadian patterns of activity and behaviour of the specific strain in use and taking measurements within a defined narrow time window can reduce biological variability, increase statistical power and reduce the number of animals needed for hypothesis testing.

Melatonin in the systemic circulation is produced in the pineal gland, and, in mice and humans, its levels rise during the night, reaching their peak 3–5 h before the onset of light or wake time[56]. The daily rhythm in melatonin secretion is a reliable measure of circadian clock output, and it is minimally influenced by stress, diet or exercise. In humans and rodents, melatonin has minimal impact on circadian rhythm in the activity–rest cycle. However, melatonin may affect entrainment of locomotor activity to shifted light–dark cycles (jet lag-like condition)[57]. Furthermore, human genome-wide association studies have linked the melatonin receptor to obesity and diabetes[58]. In mice, melatonin regulates systemic insulin production and sensitivity[59]. The melatonin content of pineal glands of 36 different strains of mice of US, European and Japanese origins revealed that only a few produce melatonin, including CBA and C3H/He[60]. The commonly used C57BL/6J mice do not produce melatonin in the pineal gland due to mutations in melatonin biosynthesis genes (arylalkylamine-*N*-acetyltransferase (*Aanat*) and *N*-acetylserotonin-*O*-methyltransferase (*Asmt*))[61]. However, while studying glucose homeostasis in a mixed genetic background or in strains that produce melatonin, the potential for melatonin production in such experiments should be considered.

The susceptibility to become obese and develop metabolic syndrome or steatotic liver disease upon consuming a high-calorie diet is also strain specific[62]. Inbred mouse strains resistant to diet-induced obesity maintain robust daily feeding rhythms as compared to strains that become obese and lose their daily feeding rhythms[63]. Additionally, genetic mouse models of metabolic diseases, developmental disorders, neuropsychiatric disorders and neurodegeneration often have a circadian disruption component, and, hence, even when sampling is done at the same time of the day, sampling with respect to the phase of the internal clock may be different. For example, *db*/*db* mice deficient in leptin receptor show disrupted circadian activity–rest rhythms, with more activity and feeding during the daytime. Additionally, the 24-h blood pressure rhythm in *db*/*db* mice is blunted, creating a nondipping phenotype, such that the nighttime blood pressure does not differ, while the daytime blood pressure (that is, during sleep in nocturnal mice) is higher, a risk factor for developing cardiovascular disease[64]. This phenotype worsens with age, so that *db*/*db* mice that are >3 months old are almost arrhythmic[65]. Nighttime-restricted feeding normalizes some of the aberrant circadian rhythms in *db*/*db* mice and can attenuate the cardiometabolic defects studied in this mouse model[66].

Beyond the core clock genes, it is now recognized that other well-known genes that influence metabolism can modify components of the circadian clock. Thus, caution has to be exercised in comparing results from constitutive and spatiotemporal genetic perturbations to account for secondary or unrelated phenotypic outputs, which may potentially lead to misinterpretation. The knockout or overexpression of such genes can cause unintended changes in circadian rhythms in sleep, activity, feeding and physiology[67]. For example. PPARα, PPARγ, PGC1α and nuclear factor κB (NF-κB) are not core components of the molecular clock, but disabling their function alters clock-controlled gene expression and affects downstream physiological outputs in different tissues in mice[68–72]. Moreover, mouse models of several diseases can have disrupted daily rhythms. For example, APP × PS1 mice, a model for Alzheimer's disease, display delayed activity onset and wakefulness as compared to control mice, at both younger and older ages[73].

Sex

Sex is a key biological variable for metabolic research and affects circadian rhythms[74]. More than three dozen brain regions either influence or are influenced by the central circadian clock in the SCN, and many of these brain regions express receptors for sex hormones[8]. Sex influences daily rhythms of activity–rest and feeding as well. The daily locomotor activity rhythm is stable across days in males. By contrast, the onset of daily activity is earlier (in hamsters and rats) and the duration of activity is longer (in rats and mice) in proestrus (when estradiol levels are high) than in other oestrus cycle stages (when estradiol levels are low)[75]. Additionally, the amplitude of daily rhythms in locomotor activity and feeding peaks in proestrus compared to other oestrus cycle stages in mice[76]. Changes in daily rhythms related to metabolic dysfunction also vary by sex. Unlike males, C57BL/6J female mice are resistant to developing diet-induced obesity and metabolic syndrome and maintain their daily feeding rhythms upon short-term high-fat diet (HFD) feeding[77]. Female mice also have a higher number of rhythmically expressed genes in the liver and are more resistant to developing cardiometabolic defects upon chronic circadian rhythm misalignment than male mice[78].

The molecular circadian clock and its outputs are also modulated by the oestrous cycle[79]. It is well known that stress can modulate metabolism and the stress response is affected by the oestrous cycle. Female rats in proestrus and dioestrus respond more robustly to foot shock and psychological stress than male mice, which is reflected in plasma levels of corticosterone and adrenocorticotropic hormone (ACTH) by as much as fourfold to fivefold[80].

Age

Age is an important variable in metabolic and circadian experiments. As mice age, daily rhythms of locomotor activity, sleep, feeding and several hormones dampen[81,82]. Moreover, the ability of behavioural and hormonal rhythms to entrain to external environmental factors becomes impaired[83,84]. For example, mortality was increased in aged mice (27–31 months old) but not in young mice (8–12 months old) when exposed to shifting light–dark cycles[85]. Additionally, there is a reduction in the number of rhythmic genes and a reorganization of rhythmic pathways in several metabolic organs during aging, with only a modest reduction in the expression and rhythmicity of core clock genes[86].

Housing conditions

Temperature. Housing temperature substantially affects metabolism in rodents. While the thermoneutral zone for mice is around 28–32 °C, mice are typically housed at 20–22 °C[87,88]. At these low temperatures, energy expenditure is ~30% higher than the basal metabolic rate. The cold-associated increase in energy expenditure is via enhanced shivering thermogenesis by skeletal muscle and nonshivering thermogenesis by brown and beige fat. One way to reduce the contribution of shivering thermogenesis to whole-body energy expenditure is to house mice at 30 °C. At this temperature, mice develop phenotypes of metabolic disease, including insulin resistance, pro-inflammatory responses and hepatic steatosis, in response to a high caloric diet at a faster rate than at temperatures below thermoneutrality[89].

Although thermoneutral conditions are often considered a 'humanized' condition for mice, several caveats are noteworthy, including that chronic thermoneutral acclimation impairs brown and beige

fat biogenesis[90]. Housing mice at 26 °C as compared to 21 °C also delays the onset of food intake, increases locomotor activity during the dark phase, reduces serum levels of glucose during the light phase and increases overall cholesterol and triglyceride levels. Housing mice at 26 °C reduces expression of gluconeogenesis, fatty acid oxidation and fatty acid synthesis genes while increasing glycogen synthesis genes in the liver[91]. Additionally, there is a diurnal rhythm in mouse body temperature, oxygen consumption and energy expenditure, and the mouse thermoneutral point changes by 4 °C from 29 °C in the light phase to 33 °C in the dark phase[92]. Although daily cycling of housing temperature from ~30 °C in the light phase to ~26 °C in the dark phase does produce more robust rhythms in oxygen consumption[93], it is not practical to implement it globally. Based on these studies, it is now recommended that mice be housed at temperatures between 26 °C and 28 °C, which is slightly below thermoneutral temperatures[93–95]. However, it is important to note that most of these studies have been performed with singly housed mice, and optimal housing temperatures must be reduced even further when mice are group housed and provided with bedding and nesting materials[87,93,95]. Thus, attention to the temperature at which mice are housed is an important consideration.

Running wheel. Voluntary wheel running is frequently used as a tool to monitor daily activity and behaviour in rodents. Most wheel-running activity in mice happens in the dark phase, with peak activity observed during the early dark phase. Mice run a total distance of ~4–20 km in ~3–7 h of wheel-running activity per day, and wheel running has also been used as a stress-free model of voluntary long-term endurance training[96]. Mice will voluntarily run on wheels during the light phase if they are locked during the dark (night) phase.

Studies that limited mouse wheel running only to the light phase found that the circadian clocks in skeletal muscles and lungs had shifted in phase, but the SCN clock had not[97]. Moreover, running wheel access changes the peak phase of clock gene expression in multiple organs and affects the phases and amplitudes of several genes involved in glucose and lipid metabolism in the liver[98,99]. Thus, the timing of voluntary wheel activity has systemic effects on circadian clocks. Additionally, male mice fed an HFD with running wheel access have blunted body weight gain and partially retain daily feeding rhythms compared to mice with locked running wheels[100]. Voluntary wheel running also increases food consumption, locomotor activity and body temperature during the dark phase, induces corticosterone to peak before the light-to-dark transition and accelerates reentrainment to changing light–dark cycles[98,99].

Home cage environment. Mice are social animals and form a social dominance hierarchy when group housed. Dominant or 'alpha' mice have priority over food and display increased activity and reduced sleep during daytime, which is lost upon individual housing[101,102]. Additionally, levels of the stress hormone corticosterone, which is known to influence circadian rhythms, are different in group-housed mice than in mice housed individually[102]. In line with this, mice subjected to chronic mild stress protocols have altered clock gene expression in multiple peripheral organs such as the liver, lungs and kidneys, possibly due to changes in daily rhythms of corticosterone, feeding, activity and sleep[103,104]. Additionally, providing environmental enrichment to mouse cages increases nighttime activity, improves functional connectivity and neuronal firing rate in the hippocampus during the dusk-to-night transition and increases slow-wave sleep[105]. Thus, the housing status of mice can affect daily rhythms in sleep, behaviour and physiology.

Light. Mice are night-active or nocturnal, while most studies aim to model diseases of day-active or diurnal humans. Thus, it is critical to consider the timing of experiments and tissue sampling with respect to the light–dark cycle[106]. Conventionally, the time of lights on is considered as ZT0 if mice are housed in light–dark conditions. However, if the experiments are performed under constant conditions, then circadian time (CT) should be used. In experiments in mice, CT12 is typically defined as the onset of locomotor activity. Generally, mice are kept under a 12-h light–12-h dark regimen. However, the use of different photoperiods must be indicated because of their impact on physiology and feeding rhythms[107]. Dim light at night can impact metabolism and lead to increased weight gain and associated diseases in rodents fed an obesogenic diet[108].

Most studies do not provide information regarding light quality or intensity in animal-holding facilities, and, when provided, it is usually reported in lux units. Lux-based measurements report perceived light intensity for humans, but they are not relevant for rodent studies due to differences in light sensitivity across the spectrum. Ideally, an unweighted power spectrum measurement ($\mu W\ cm^{-2}\ s^{-1}$) should be reported to provide the intensity of different wavelengths. This information is relevant because physiological or behavioural responses in mice can vary with changes in the light spectrum. For example, the extent of weight gain in a diet-induced obesity mouse model varies based on the wavelength of light[109]. However, the spectroradiometers required to measure light spectrum intensity data are considerably more expensive than lux metres. Thus, if a spectroradiometer is not available, then reporting the type of lighting (fluorescent, incandescent, warm or cool light LEDs, etc.) and its lux intensity is acceptable[110].

It would be ideal for some metabolic assays and exercise performance or behaviour tasks to be measured in the dark phase when mice are normally awake and active. This is because physiological and behavioural differences exist during day and night conditions, such as daily circadian rhythms in glucose and insulin tolerance[3]. The best way to achieve this would be to have animal rooms with reversed light–dark schedules so that mice can be tested in the dark phase during the researcher's normal working day. However, this approach still poses several operational issues. Collecting measurements in the dark requires using infra-red night vision devices, and it is more difficult to monitor animal welfare. Moreover, the light intensity should be carefully selected, as some dark room-style red lights are detected by mice and can elicit biological responses[106]. While light is a strong entrainer of the circadian clock, other sensory stimuli, such as sound in the rodent auditory range, can also indirectly modulate animal behaviour or, in some cases, stress animals. Hence, background tonic white noise may be considered.

Diet and feeding method

As mice are nocturnal, most (75–80%) of their food consumption is consolidated during the dark phase and 20–25% occurs during the light phase. Interestingly, obesity abrogates this daily circadian feeding pattern, and mice consume more food (30–35%) during the light phase[111,112]. Additionally, obese mice have damped daily rhythms of core body temperature and corticosterone and an impaired ability to synchronize their endogenous circadian rhythms to changing light–dark cycles[111–113]. However, young female mice are protected from diet-induced obesity and do not lose their daily eating rhythm during HFD feeding, at least upon short-term dietary treatment[77,114].

Interestingly, the effect of diet-induced obesity on daily eating rhythms is strain specific, and male mice from strains that do not become obese after being fed an HFD present preserved daily rhythms as compared to strains that become obese[115–117]. These results indicate that damping of daily eating rhythms and mistimed eating, as observed under dim light at night, play a key role in developing obesity and metabolic syndrome. Moreover, studies have shown that the loss of daily eating rhythms in diet-induced obese mice also rewires diurnal rhythms in gene expression and the metabolome across multiple organs, which can be partially corrected by restricting food intake only during the active (dark) phase[118]. In addition to HFDs, high-protein and ketogenic diets also affect daily feeding rhythms, entrainment

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to alternating light–dark cycles and the expression of clock genes in several organs[119–121].

Daily feeding rhythms have a strong impact on liver physiology and gene expression: at least 30% of liver rhythmic genes depend only on rhythmic feeding[15]. Additionally, the timing of feeding affects the outcomes of metabolic experiments that study interventions such as CR. Typically, when mice are subjected to CR, they are fed once a day in the morning. Due to the 20–40% reduction in calories, mice will consume most of this food during the first 2 h and fast for the remaining 22 h[122]. Thus, molecular changes caused by CR in such an experiment are due to a combination of reduced calories, an extended fasting interval and extreme daytime restricted feeding[122,123].

Mice also develop anticipatory behaviour and display elevated locomotor activity before feeding time due to the food-entrainable oscillator[122,124]. Even when CR is absent, daytime restricted feeding causes an 8–12-h shift in the peak phases of clock genes in some brain regions and peripheral tissues[12,13]. The rate of entrainment of gene expression varies by tissue. For example, clock and metabolic gene expression are altered by daytime feeding within a single day in the liver, but other tissues such as brown adipose tissue, heart and muscle are more resilient to changes[12,13,125] This is also true for intermittent fasting protocols, such as every-other-day feeding, in which mice are fasting for 1 d and have access to food the next day. In most cases, food is provided during the day, with profound impact on rhythmic physiology and the circadian clock[126].

Gut microbiome

The gut microbiome has diurnal variation in composition and function. This daily rhythm affects the timing of nutrient uptake and utilization, xenobiotic metabolism and redox balance, and its maintenance requires an intact molecular clock in gut epithelial cells[127]. An intricate signalling network between gut microbes, immune cells and epithelial cells maintains microbial rhythms. This daily rhythm in the gut microbiota is driven by the host epithelial circadian rhythm and daily feeding–fasting cycles, which affect local nutrient availability and dictate the release of bile acids and pancreatic secretion[128].

Conversely, gut microbes also regulate host rhythms. Secondary metabolites released by microbes, along with contact-based mechanisms, regulate daily rhythms in cellular signalling, histone modifications and gene expression in gut-resident immune cells, epithelial cells, the liver and the brain[78,129–132], indicating the possible circadian regulation of gut–liver and gut–brain axes. Importantly, the microbiome and secondary metabolite oscillations are dependent on the composition and timing of diet[127]. Thus, collecting samples at similar times of the day is critical for reproducibility and to reduce variability in gut microbiome data[133].

Studies investigating the effects of the gut microbiome on host metabolism often employ antibiotics or use germ-free mice for experiments. However, these strategies can have physiological effects on the host that may confound experimental conclusions. Antibiotic-induced microbial depletion increases glucose tolerance by improving GLP-1 rhythms and glucose metabolism in the gut[134] and also remodels diurnal metabolic cycles in different brain regions[132]. Moreover, germ-free mice have decreased sex-specific gene expression and an altered liver metabolome due to reduced sex and growth hormone signalling[78]. Additionally, antibiotic-treated and germ-free mice have distinct alterations in chromatin and gene expression networks regulated by the LXR and PPAR transcription factors due to changes in microbial metabolites such as short-chain fatty acids and secondary bile acids[129,130].

Treatments and interventions

Behavioural interventions and drug treatments substantially impact circadian rhythms of mice, and the response to these treatments often varies across the day–night cycle. For example, exercise training can affect the phases and amplitudes of several clock genes in muscles.

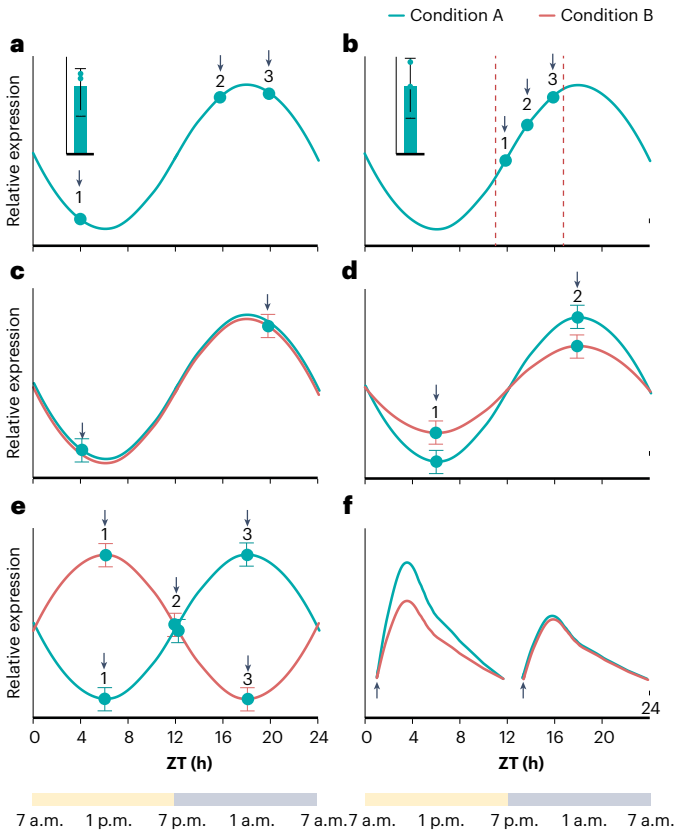

Fig. 4 | Various scenarios demonstrating how the timing of experiments or sample collection can affect conclusions. a, Performing measurements at random times throughout the day can reduce the reproducibility of results. Example: an experiment performed at ZT4 may not be reproduced at ZT16 or ZT20. **b**, Expanding the time window for measurements may increase variance. Example: collecting biological replicates spread over 4 h (ZT12–ZT16) can increase variance compared to that of samples collected within a 1-h window in the same interval. **c**, When comparing different treatments, erroneous conclusions can arise from measuring the outcomes of both groups at different time points, regardless of the daily rhythm remaining unchanged. One may erroneously conclude that condition B > A, if samples for A were collected at ZT4 and samples for B were collected at ZT20. **d**,**e**, Changes in amplitude (**d**) and phase (**e**) of the daily rhythm between conditions can lead to differing conclusions when measurements are taken at different times of the day. In **d**, one may conclude that condition B > A or condition A > B if samples were collected at only one time point (ZT6 or ZT18, respectively). In **e**, one may erroneously conclude that B > A, A = B or A > B if samples are collected at only one time point (ZT6, ZT12 or ZT18, respectively). **f**, Circadian gating of response to perturbations or challenges can significantly impact the outcome based on the time of day. A stimulus given at ZT0 may reveal a difference in responses between A and B, but the same stimulus at ZT12 may not reveal any difference. The black arrow (**a**–**f**) indicates time of experiment, measurement or sample collection. The numbers are technical or biological replicates.

Reciprocally, the molecular and physiological response to exercise varies depending on the time of day[135]. These differences could be mediated by diurnal changes in body temperature, neuromuscular junctions and substrate availability and utilization as well as other unidentified factors[135,136]. Moreover, drugs targeting cellular proteins affect feedback signalling to the core clock and clock-controlled gene expression[48,137–140]. In addition, pharmacokinetic and pharmacodynamic properties of drugs are influenced by the circadian clock and circadian physiology, and the time of administration could have a huge impact on the efficacy and the toxicity of the treatment or drug[141].

Interventions affecting sleep–wake cycles of mice can cause changes in daily physiological rhythms. For example, chronic sleep

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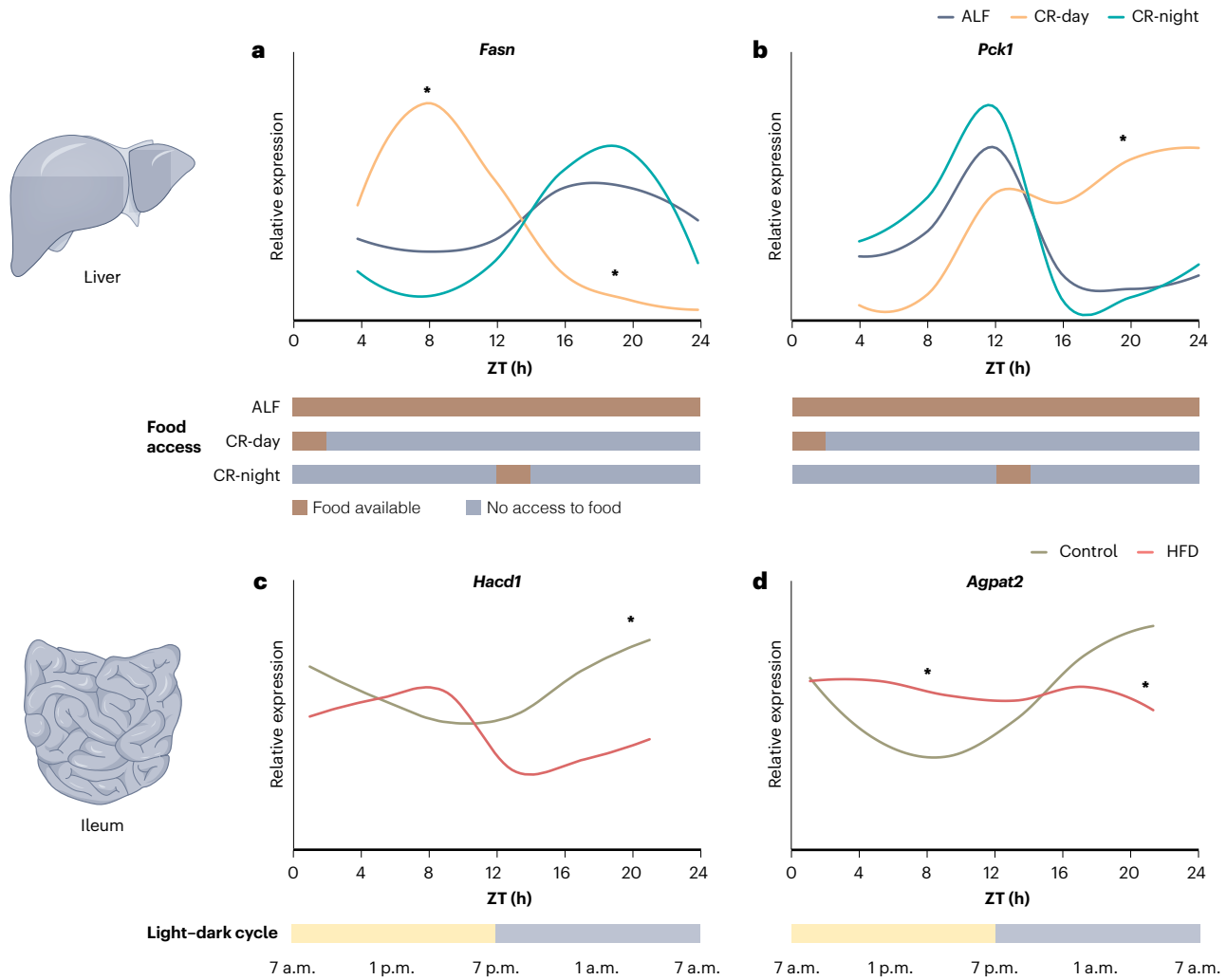

Fig. 5 | Examples of how ignoring the reporting of sample collection time (or using single-time point measurements) can lead to incorrect experimental conclusions. a,**b**, The mRNA levels of fatty acid synthase (*Fasn*) (**a**) and phosphoenolpyruvate carboxykinase 1 (*Pck1*) (**b**) in the liver show significant differences between CR-day mice and ALF or CR-night fed mice only at specific times of the day. **c**,**d**, Similarly, expression of genes such as those encoding 3-hydroxyacyl-CoA dehydratase 1 (*Hacd1*) (**c**) and 1-acylglycerol-3-phosphate *O*-acyltransferase 2 (*Agpat2*) (**d**) in the ileum exhibits time-of-day effects between mice fed a control diet or an HFD. In all the above scenarios, sampling at only one time of the day can lead to incorrect conclusions. Asterisks highlight notable difference in gene expression. Figures generated from the publicly available RNA-seq datasets from Acosta-Rodríguez et al.[123] and Dantas Machado et al.[152]. Figure created using BioRender.com.

deprivation impairs learning and memory in mouse models of Alzheimer's disease. Furthermore, intermittent hypoxia increases core body temperature, decreases heart rate and reduces rhythmic gene expression in the kidney and the liver[142–145]. Several mouse models of chronic mild stress also display disrupted sleep–wake and locomotor activity rhythms and altered circadian clock and metabolic gene expression in several organs[146–148].

Confounding factors in interpreting single-time point experiments

Most acute metabolic experiments such as glucose or insulin tolerance tests, nutrient uptake or utilization assays with heavy isotope labelling, hormone or cytokine profiling and cold tolerance experiments are performed only at one time of the day. Moreover, tissue samples are collected from mice at only a single time point. But the timing of these measurements can have profound effects on results and interpretation (Fig. 4). If experiments are performed at different times of the day or in a broad time window, there can be high variability and lack of reproducibility across laboratories or even within the same laboratory (Fig. 4a,b).

Table 1 | Summary of experimental design checklist

Technical aspect	Recommended approach
Transgenic strains and disease models	If developing a new strain or disease model, assess daily rhythms in feeding, locomotor activity and sleep to check for secondary effects.
Sample collection	Perform all collections within a narrow time window (~2h) to reduce variability. If the mice are collected in the dark phase, perform dissections under red light or house them in dark boxes until collection.
Timing of sample collection or measurement	For initial assessment, sample at least two time points: fasted (light) phase and fed (dark) phase to check for phenotype consistency.
Overnight fasting	Unless investigating the effect of reverse feeding, restricting food access during fasting should be performed during the light phase.
Behaviour and physiology assays	Perform tests in the dark phase when mice are active and in the fed state. This also avoids disrupting sleep when tests are performed in the light phase.

Table 2 | Summary of reporting checklist for rodent experiments in metabolic research

Type of variable	Parameters to report	Description
Biological	Strain	Specific animal strain and the original source; include number of generations of backcrossing
	Genotype	Genetic mutations, mode of creating the transgenic animal and the background strain
	Age	Age at which the experiment was initiated and timeline for experiments performed
	Sex	Experiments in males and/or females and whether the reported results are from pooled or stratified analysis
Environmental	Animal housing	Single or group housing (number of animals housed per cage)
	Enrichment	Access to home cage enrichment sources
	Temperature, humidity	Daily range of temperature and humidity
	Light–dark cycle	Light cycle length with timing of lights on and lights off
	Light quality	Light intensity and source/spectrum
	Wheel running	Voluntary access to running wheel
	Noise and human presence	Presence of white noise or music in animal rooms
Experimental	Diet	Composition, supplier and catalogue number of commercially available diets. Detailed composition of diets that are not commercially available
	Feeding method	Solid or liquid food, ad libitum or restricted feeding, time of feeding (ZT), pairing with activity/exercise or behaviour task
	Interventions	Type of intervention, duration, intensity, time of day (ZT), pairing with feeding
	Treatments	Nature of compound (biologics/chemical), mode of delivery, type of vehicle, time of day (ZT) and frequency of treatments
	Microbiome status	Housed in conventional or SPF facility, treated with antibiotics or raised germ free
	Preexperiment conditions	Status of food access before the experiment
	Time of sampling	Time of day (ZT/CT) at which the experiment was performed and samples were collected

SPF, specific pathogen free.

Moreover, if the experiment involves two or more groups and if the measurements in each group are performed at different times of the day, it can lead to erroneous conclusions despite having no difference in phenotype (Fig. 4c). Additionally, if there is a change in the amplitude or phase of the rhythmic parameter between different conditions, measurements taken at different times of the day can lead to opposing conclusions (Fig. 4d,e). Furthermore, circadian gating of the response to perturbations or challenges at specific times of the day can significantly impact the outcome of the experiment depending on the time of the day (Fig. 4f).

For example, glucose-induced GLP-1 secretion has a daily rhythm that peaks around ZT10 (before habitual food intake beginning at ZT12) and parallels that of glucose-induced insulin secretion in the plasma of rats[149,150]. The resulting area-under-the-curve delta response after a 4-h fast and a subsequent oral glucose tolerance test can show substantial differences, with lowest values for GLP-1, insulin and glucose observed at the end of nocturnal habitual feeding (ZT0) and peak values in mid-day–afternoon (ZT6–ZT10). The change in area under the curve for GLP-1 can be as much as fivefold, that for insulin, twofold and that for glucose, 1.8-fold. The proglucagon (*Gcg*) transcript in the intestinal mucosa also shows a strong rhythm and peaks at the end of the night (not midday).

We recognize that experiments and tissues are collected at a single time point for practical purposes. However, the above example of the impact of fasting time and circadian gating of the response to the oral glucose tolerance test highlights factors to be considered and reported for these single-time point experiments[151]. To mitigate these confounding effects, all experiments within a single study should be performed at the same time of day. This time of day should be reported as a ZT, which describes the time of the experiment or tissue collection relative to when lights are turned on, where ZT0 is lights on and ZT12 is lights off in standard 12-h light–12-h dark housing conditions. For example, if the lights were turned on in the animal facility from 07:00 to 19:00, and the experiment was performed at 12:00, this would equate to ZT5. It is acceptable to report the local times of experiments as long as the local times of lights on and lights off in the animal room are also reported.

Additionally, the time of day when experiments are conducted should be carefully considered. For example, overnight fasting is common in metabolic studies. This design is convenient for researchers but can have unintentional effects of imposing very long fasts on the mice, as they eat very little during the day and then are fasted overnight. Thus, overnight fasting (removing food for ~12 h) equates to an 18–24-h fast when the preceding daytime natural fast is considered.

Moreover, CR or restricted feeding in experiments performed during the day cannot be directly compared with ad libitum feeding (ALF) in control mice that predominantly eat during the night, as there will be effects due to when the mice are eating that may be independent of the effect of CR or restricted feeding[122,151]. As an example (Fig. 5a), fatty acid synthase (*Fasn*) messenger RNA (mRNA) levels are very low in the liver from mice fed a CR diet during the day (CR-day mice) as compared to ALF mice or mice fed a CR diet at night (CR-night mice) when analysed just before lights-on at 7 a.m. (~ZT24 or ZT0). However, if samples are collected at 11 a.m. (ZT4), there is no difference in gene expression between the groups. Importantly, *Fasn* levels are much higher in CR-day mice at 3 p.m. (ZT8) than in ALF or CR-night mice. A similar time-of-day effect is observed in expression of the gluconeogenic gene encoding phosphoenolpyruvate carboxykinase 1 (*Pck1*)[123] (Fig. 5b).

Even in mice fed an HFD, expression of genes such as those encoding 3-hydroxyacyl-CoA dehydratase 1 (*Hacd1*) and 1-acylglycerol-3-phosphate *O*-acyltransferase 2 (*Agpat2*) shows time-of-day effects in the ileum: analysing gene expression during daytime shows no change or upregulation of *Hacd1* and *Agpat2* mRNA levels, respectively, but downregulation of both genes at nighttime as compared to expression in control chow-fed mice[152] (Fig. 5c,d). Thus, depending on the time of day when samples are collected, we may find opposite results.

To mitigate this problem, it is advisable to perform experiments or collect samples at multiple time points every 2–4 h throughout the day–night cycle to identify time-of-day effects. This approach will

BOX 1

Terminology

Circadian rhythm: any metabolic, physiological or behavioural process repeating in cycles of approximately 24 h. The rhythmic process should be (1) generated endogenously, (2) self-sustained in the absence of external stimuli and (3) entrainable to external cycles that have periods of 24 h. Circadian is derived from the Latin 'circa', meaning about, and 'diem', meaning day.

Diurnal rhythm: any metabolic, physiological or behavioural process that cycles every ~24 h in a light–dark cycle. The rhythmic process can be called circadian if it follows the above listed criteria.

Diurnal: activity or event taking place between dawn and dusk (during the light phase in light–dark conditions) and during subjective day (in constant conditions).

Nocturnal: activity or event taking place between dusk and dawn (during the dark phase in light–dark conditions) and during subjective night (in constant conditions).

Zeitgeber: from the German noun 'time giver'. A periodic environmental stimulus that entrains a rhythmic process.

Photoperiod: the duration of the light phase in an external light–dark cycle.

Entrainment: coupling of an endogenous biological rhythm to an external cue or Zeitgeber (for example, light, food, etc.). Under conditions of steady entrainment, the period of the biological rhythm conforms to that of the Zeitgeber, and there is a stable phase relationship between the two.

Free running: a rhythm that is self-sustained in the absence of any external Zeitgeber or environmental factors that can affect the period of the oscillation.

Zeitgeber time ZT: a phase of an external cycle, the Zeitgeber. Under a standard 12-h light–12-h dark cycle, the time of lights on is defined as ZT0 and the time of lights off is defined as ZT12.

Circadian time CT: a standard of time that tracks the phase of an organism's endogenous biological clock or free-running rhythm. Conventionally, the onset of activity of diurnal organisms is defined as CT0 and the onset of activity of nocturnal organisms is defined as CT12.

Calorie restriction CR: a dietary intervention that involves reducing the total daily amount of calories consumed by 20–40% without causing malnutrition or deprivation of essential nutrients.

relieve the concern that performing experiments at only one time of the day may bias the results. In fact, observing consistent results at multiple times of the day will provide more information about the behaviour or physiology being assessed. If this approach is not feasible, then we recommend at least one time point each during fed (ZT12–ZT24 in the dark phase) and fasted (ZT0–ZT12 in the light phase) states in mice.

Proposed recommendations and guidelines for designing and reporting experiments

As there are several factors that affect endogenous biological rhythms, we have divided them into three broad categories: biological, environmental and experimental (Fig. 3). Biological variables, including strain, genotype, background, age and sex, should be reported as well as litter size, an underestimated confounder in metabolic research[153]. Next, environmental factors such as single or group housing, the daily range of temperature and humidity, the light–dark cycle, light quality and intensity (spectrum information or light intensity and source) and enrichments such as running wheel or nesting and/or sheltering access should be reported. In the experimental section, parameters such as diet composition and source, feeding method and the use of antibiotics or germ-free mice should be reported.

When any feeding interventions or pharmacological treatments are performed, it is critical to report the time of day in ZT or CT format as well as the duration and frequency of treatments. Moreover, any preexperimental conditions such as fasting, housing in a dark–dark cycle, etc. should be reported, along with information about the time of day when the condition was initiated (in ZT) and the duration. Finally, the time of day at which the experiment was started and completed and when the samples were collected should be reported in ZT or CT. Ideally, if mice are in the night phase of the light–dark cycle when tissues are collected, they should remain in the dark until euthanized because light exposure can affect hormonal profiles and gene expression. This can be achieved by either performing blood and tissue collection using infra-red night vision viewers in a dark room, under red light in a dark room or temporarily housing the mice in a light-tight box in the laboratory until euthanasia.

While the above listed variables should be considered when designing and performing experiments in the future, it does not necessarily invalidate the results published so far. Moreover, it may not be feasible for researchers to address all the variables. Thus, it is important to provide more details in the method section for better transparency and reproducibility and to comprehend variability in data across laboratories. Below are summary tables listing the proposed recommendations for designing and reporting experiments (Tables 1 and 2).

Conclusions

Acute and chronic disruption of circadian rhythms in activity–rest, feeding–fasting and light exposure patterns contribute to the onset, severity or prognosis of numerous diseases. Conversely, abnormal diurnal rhythms are emerging as hallmarks of various diseases. Causal links between circadian disruption and disease are at least partly demonstrated by behavioural interventions such as timed exercise, timed feeding or pharmacological interventions targeting clock components. These recent advances have emphasized the untapped potential of advancing basic and translational biomedical research by highlighting factors that affect circadian rhythms, controlling for timing in perturbation experiments and considering timing in measurements and result interpretation. It is therefore essential to consider and accurately report the biological, environmental and experimental factors that influence circadian rhythms. We hope that the effects of these variables will be considered during the design and analysis of future experiments and that the field will adopt the recommendations for experimental design and reporting methodologies outlined in this Review to improve reproducibility and translation of metabolic research (Box 1).

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Author contributions

S.D. and S.P. wrote the initial draft of the manuscript and led the revisions. All authors contributed to the content and organization of this Review; contributed to writing, editing and/or revising the manuscript; and approved the final version.

Competing interests

S.A.B. is a cofounder and scientific advisor of Ona Therapeutics. G.S.H. is a scientific advisory board member of Crescenta Biosciences. J.S.T. is a founder and scientific advisory board member of Synchronicity Pharma. J.D.R. is a paid adviser and/or stockholder in Colorado Research Partners, L.E.A.F. Pharmaceuticals, Faeth Therapeutics and Empress Therapeutics; a paid consultant of Pfizer; a founder and stockholder in Marea Therapeutics; and a founder, director and stockholder of Farber Partners, Raze Therapeutics and Sofro Pharmaceuticals. S.K. serves as a scientific advisory board member of Moonwalk Biosciences. V.D.L. has equity interest in L-Nutra, a company making medical food, and has filed patents related to fasting-mimicking diets and their medical use. M.A.L. is on the advisory board of Pfizer and serves on the advisory board and is a cofounder of Flare Therapeutics. E.V. is a scientific cofounder of Napa Therapeutics and BHB Therapeutics and serves on the scientific advisory board of Seneque. J.A. is a board member of NOV Metapharma, a founder and/or consultant for Vandria and Amprenta Therapeutics and consults for OrsoBio, MetroBiotech and Amazentis (now Timeline); none of these companies develop products regulating circadian activity. D.J.D. has served as a consultant or speaker within

the past 12 months for Amgen, AstraZeneca, Boehringer Ingelheim, Kallyope, Novo Nordisk and Pfizer. S.P. is the author of the books *the Circadian Code* and *the Circadian Diabetes Code* and is a scientific advisor to Hooke London, Avadel and WndrHlth. Other authors have no conflict of interests to declare.

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