PNAS OPINION

Researchers need to better address time-of-day as a critical biological variable

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More explicit consideration of time-of-day information in experimental designs, analyses, and reporting will improve the rigor and reproducibility of scientific research. Image credit: Getty/Eleonora Grigorjeva.

Nearly all physiological and behavioral processes display daily rhythms driven by endogenous circadian clocks—molecular representations of the external solar day. Circadian rhythms therefore influence the outcome of experiments—that is, the answer to an experimental question may depend in part on the time-of-day when the question is asked. Yet, the time-of-day is rarely reported in scientific correspondence. It is a neglected, yet crucial, methodological variable in research on living systems. We argue that more explicit consideration of time-of-day information in experimental designs, analyses, and reporting will improve the rigor and reproducibility of scientific research.

Biological responses to stimuli vary significantly across the day. For example, fruit flies (*Drosophila melanogaster*) express a circadian rhythm in antennae electrical responses to ethyl acetate, a food odorant and attractant, and benzaldehyde, an odorant that causes behavioral avoidance (1). Demonstration of a diurnal rhythm in neural responses to olfactory stimuli has implications not just for natural behaviors that rely on olfaction, such as foraging and courtship, but also the olfactory-based learning paradigms commonly used in fly behavioral research. The biological responses of the outcomes that researchers have quantified often differ 50–100% across the day.

For instance, mice tested during the night (their active phase) display better performance (ranging from 20 to 80% fewer errors depending on the test) in

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Author contributions: R.J.N., A.C.D., and B.J.P. wrote the paper.

The authors declare no competing interest.

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Published July 17, 2024.

memory tasks compared to those tested during the day (their inactive phase) (2). Similarly, tests of balance and coordination of neuronal nitric oxide knockout (nNOS$^{-/-}$) mice revealed no effects when tested during the day; this lack of effect was unexpected because the cerebellum, where balance and coordination are regulated, is richly stained with nNOS. However, testing during the night (their active phase) revealed striking differences in balance and coordination (~40% impairment in nNOS$^{-/-}$ mice compared to wild-type [WT] mice), suggesting that the null results from daytime testing reflected a floor effect and that the true contribution of nNOS to balance and coordination was only observed during the active period of the mice (3).

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Too often, circadian rhythms are not taken into consideration in the practice of medicine, even though their role is well known (4). For example, the effectiveness of chemotherapies for colorectal cancer varies based on the time-of-day at which they are administered (5). Similarly, several drugs work best when administered at certain times-of-day, but reports of these temporal effects during clinical trials have had limited influence in medical treatment (4–6). Indeed, only 4 of the 50 most prescribed drugs in the United States have time-of-day dosing recommendations from the Food and Drug Administration; the World Health Organization Model List of Essential Medicines (23rd list) does not mention dosing times for medications or vaccines (6).

Most physiological systems are regulated by circadian rhythms. For example, blood pressure (BP) and heart rate decline during the inactive phase and rise in anticipation of the active phase (7), even among bedridden individuals. The risk of myocardial infarction (MI) follows a similar pattern to BP in humans; there is a threefold increase in MIs at the 9 AM BP peak compared to the typical BP low point around 11 PM (8). Increased central nervous system damage is observed after MIs that occur early in the active phase compared to later in the day (9). These data could inform the optimal timing of treatments with antihypertensives and cardiac medications. Although it has been established that medications work optimally at specific times of day, again, little of this temporal knowledge has been translated to clinical practice (6, 10).

Daily rhythms in immune function are ubiquitous and reflect complex interactions among circadian rhythms in the host and rhythms within immune cells themselves (11). For example, innate immune responses to pathogens or pathogen-associated molecules vary over the course of a day; in nocturnal rodents, symptoms of infection display strong diurnal patterns, with more severe responses observed when animals are challenged during their rest phase (12–14). Since 1960, we have known that mice treated with the same dose of *Escherichia coli* at different times of day display vastly different outcomes. Only about 15% of mice receiving the *E. coli* during their active periods (the middle of the night) perished from the bacterial infection, whereas nearly 90% of the mice died if injected at the end of their rest period (the transition between day and night) (12, 13).

Unexpected circadian patterns also may emerge in datasets. For example, the toxicity of a poison depends on the time of day at which it is ingested. One study of ~15,000 patients with intentional self-poisoning (either by oleander seed [*Cascabela thevetia*] ingestion or pesticide ingestion, viz., organophosphorus) reported that fatalities were reduced by up to 50% if the poisoning occurred during the evening compared to late morning (15). The difference in survival did not reflect differences in treatment, but the researchers hypothesize that daily rhythms in metabolism (P-glycoprotein and hepatic cytochrome P450 3A4) contributed to poisoning outcomes (16).

In sum, chronobiologists have compiled extensive lists of basic and clinical biological processes that vary over the circadian cycle (17), and, although these strong daily fluctuations are now well-known, recent reviews of the literature suggest that they are largely ignored by researchers (18, 19).

A Neglected Variable

An analysis of the literature underscores the extent to which time-of-day is ignored. In one study (18), we reported that most behavioral neuroscience studies conducted behavioral testing during the light phase. This may not produce optimal performance for nocturnal rodents because daytime is typically their less active period. This would be akin to waking up people to administer a memory test at 3 AM. Moreover, we discovered that there was little consistency across studies. In some cases, researchers housed nocturnal rodents on a reversed light–dark cycle to facilitate testing during the active period. In other studies, they housed animals in a light–dark cycle similar to the external environment, with testing during the dark phase. Still others tested either early or late during the active phase. Moreover, most papers surveyed failed to include methodological descriptions that clearly indicated the clock time at which key manipulations were performed, and those that tested subjects in the dark rarely reported how circadian rhythms were protected from unwanted light exposure en route to or in the testing environment. Our analysis suggested that time-of-day information is rarely considered in the reporting of behavioral neuroscience research.

To determine whether the neglect of time-of-day reporting extended to other research fields, we analyzed the top 50 cited papers across 10 major domains of the biological sciences in the calendar year 2015 (19). We repeated this analysis for the year 2019, hypothesizing that the awarding of a Nobel Prize in 2017 for achievements in the field of circadian biology would highlight the importance and awareness of circadian rhythms for scientists across many disciplines and that awareness might translate into improved attention to circadian issues such as time-of-day reporting. We reported that of these 1,000 empirical papers, the vast majority failed to include sufficient temporal details when describing experimental methods. Overall, only 6.1% of reports between 2015 and 2019 included time-of-day information about experimental measures and manipulations, and no significant improvement in reporting was evident across years.

Rigor and Research Quality

Circadian rhythms are a defining feature of most living systems, and innumerable biological processes fluctuate over the circadian cycle. Thus, information on when procedures and data collection are performed provides critical biological context to a research report. Including time-of-day information can also contribute positively to experimental rigor by allowing more accurate methodological replication. Moreover, the absence of time-of-day information stands to hamper reproducibility across laboratories and complicate interpretation of results; this may be especially important in studies of nocturnal animals that are extrapolated to diurnal humans. Finally, even if a given trait or biological process does not change substantially over the circadian cycle, reporting such information is intrinsically valuable and stands to inform other scientists in unforeseeable ways.

In an effort to improve the reproducibility of biomedical research, journals are increasingly requiring that submissions involving animal research adhere to minimum standards of methodological reporting. One such set of guidelines, Animal Research: Reporting of In Vivo Experiments (ARRIVE), includes information on sample sizes, randomization, blinding, statistical models, and results reporting, along with experimental procedures (20). In this latter category, ARRIVE guidelines recommend information on what, how, where, why, and (critically) when procedures were performed. The guidelines recommend that "Methods" sections "clearly report the frequency and timing of experimental procedures and measurements, including the light and dark cycle, circadian time cues, and experimental time sequence" (20). Although our meta-analyses spanned intervals that preceded the publication of ARRIVE 2.0 guidelines, it appears that very few journals have historically (prior to 2020) required reporting of time-of-day information.

It is too early to see whether the adoption of the ARRIVE 2.0 guidelines are having an effect on reporting time-of-day information. Nevertheless, going forward, we recommend that this component of the ARRIVE guidelines should be enforced by both journals and reviewers. Much as the inclusion of sex as a biological variable has become required, time-of-day information should be included for all published experimental methods. The NIH may need to provide guidance on this issue, as they have done previously to increase awareness of sex as a biological variable.

Finally, because time-of-day is a critical and controllable biological factor, researchers should consider a priori incorporating time-of-day into the design, implementation, and analyses of experimental data (whether animal testing, sample collection, or in vitro procedures). Considering the hundreds or even thousands of genes that are circadian-regulated in a tissue-specific manner (21, 22), it makes sense to note a clear rationale for when data are collected. For traits that vary markedly over the circadian cycle, such as those noted here, defined circadian windows for data collection may be warranted. In some cases, it may be necessary to test nocturnal animals during the light phase (e.g., some behavioral tests require animals to be tracked in visible light); when researchers do so, they should indicate as such. If testing occurs during the nighttime, then researchers should describe methods for protecting circadian rhythms (dim red lighting, night vision goggles, etc.). Nonetheless, to improve time-of-day reporting, details regarding time-of-day, photocycles (e.g., 12:12 light–dark cycles or 14:10 light–dark cycles) used for animal housing, time of testing, and whether testing occurred during the dark or light should all be clearly accessible in a "Methods" section.

Life on Earth is adapted to the 24-h solar day, and adaptations to temporal niches have shaped virtually all aspects of biology during evolution to increase fitness. Ignoring these temporal influences during the conduct of animal studies will influence the collected data and muddle interpretations. Taken together, evidence-based decision-making in the timing of data collection, protection against exposure to extraneous light during dark-phase testing, incorporation of temporal factors in data analysis and interpretation, and meticulous reporting of temporal factors in publications have the potential to improve experimental rigor and reproducibility across all fields in biology.

ACKNOWLEDGMENTS. This work was supported by NIH Grants R21 AT011238 (to R.J.N.) and R21 R21CA276027 (to A.C.D. and R.J.N.) and NSF Grant 2242771 (to R.J.N.).

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