



Food for Thought

Chronobiology and the design of marine biology experiments

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Marine habitats are shaped by several geophysical cycles ranging from a few hours (tidal and solar cycles) to a year (seasons). These cycles have favoured the selection of endogenous biological clocks. Such a clock is a molecular time-keeping mechanism that consists of a set of core clock genes whose expression oscillates. The clocks produce biological rhythms and influence virtually all metabolic, physiological, and behavioural functions in organisms. This work highlights the importance to take chronobiology into account in experimental marine biology to avoid faulty results, misinterpretation of results, and/or to strengthen observations and conclusion. A literature survey, based on 150 articles, was conducted and showed that, despite the pervasive imprint of biological rhythms in marine species, environmental cycles such as the 24 h-light/dark cycle and the seasonality are rarely considered in experimental designs. This work emphasizes that better integrating the temporal organization and regulation of marine species within the marine biology community is essential for obtaining representative results.

Keywords: biological rhythms, environmental cycles, experimental settings, marine biology, marine chronobiology.

Introduction

The Sun, the Moon, and the Earth's immutable rotation on their orbits deeply influence our living world. The pervasive alternation of nights and days has favoured the selection of an endogenous circadian clock. The genetic basis of the circadian clockwork mechanism was first discovered in fruit flies (Hardin *et al.*, 1990; Sehgal *et al.*, 1995; Allada *et al.*, 1998; Rutala *et al.*, 1998): translation and transcription feedback loops of core clock genes set the tempo for cells, tissues, and ultimately the whole organism (Chaix *et al.*, 2016; Kumar, 2017). Mechanistically, the clock varies between species, but its formal principle is ubiquitous from cyanobacteria, to plants, and animals (Young and Kay, 2001). Although ubiquitous among taxa, biological clocks probably evolved independently at least twice (Rosbash, 2009). The clock is synchronized to the Earth's 24 h-revolution by external cues called zeitgebers (time-givers), such as the light/dark cycle. Without zeitgeber, the clock free-run at its endogenous period that is *circa* 24 h, meaning approximately 24 h. Endogenous periods can vary between individuals (Aschoff, 1981; Johnson *et al.*, 2004). The circadian clock drives organisms' biological rhythms, including the sleep/wake or hormonal cycles (Kumar, 2017).

Biological rhythms are considered to be adaptive (Woelfle *et al.*, 2004) in that they allow the anticipation of cyclic environmental changes, and ensure consistency in organisms' physiology, metabolism, and behaviour (Rosbash, 2009). An example of synchronization and anticipation is the daily rhythm of body temperature in human: the trough occurs at night, it starts rising in anticipation of waking, reaches a peak in the early evening, and drops in anticipation of sleep (Refinetti and Menaker, 1992).

Marine species are influenced not only by environmental cycles associated with the solar day, but also by moon-related environmental cycles, which include the tidal cycle (with period of 12.4 h), the lunar day (24.8 h, the time it takes for the moon to complete an orbit around the earth), and the semi-lunar/lunar cycles (14.8/29.5 days; Tessmar-Raible *et al.*, 2011). The seasons also deeply influence both terrestrial and marine habitats; in *Crassostrea gigas*, for example, temperature and photoperiod (i.e. the duration of the light phase of a light/dark cycle) regulate oyster annual reproduction (Fabioux *et al.*, 2005). All these cycles make the marine biotope a very complex, yet predictable, cyclic environment. Whereas a deep understanding of the mechanisms underlying the circadian clockwork has been provided in

terrestrial species, the timing mechanisms in marine organisms still need to be deciphered (Tessmar-Raible *et al.*, 2011; de la Iglesia and Johnson, 2013). However, endogenous rhythms corresponding to all of these environmental cycles have been described in a variety of marine organisms including annelids (Last *et al.*, 2009), molluscs (Connor and Gracey, 2011), arthropods (Zhang *et al.*, 2013), chordates (Vera *et al.*, 2013), phytoplankton (Bouget *et al.*, 2014), and manifest in many phenotypes including locomotor and feeding activity, as well as metabolism and reproduction.

In the laboratory, incubation conditions affect biological rhythms. However, despite the importance of environmental cycles in driving major rhythms of marine organisms, they are often neglected in the design of marine biology experiments. Such a practice may lead to desynchrony within the group under study if no environmental cycle is implemented, add some undesired variance among individuals if irregular sampling is performed, or alter the parameter studied if unnatural incubation conditions are implemented. These can potentially alter all observations. Therefore, the objective of the present study was twofold: (1) to evaluate in which way chronobiological information was mentioned or rather neglected, and (2) to show examples of misinterpretation of results when not considering the biological rhythmicity in marine organisms.

Material and methods

A literature survey of experimental laboratory work ($n=150$ articles; Supplementary Table S1) on marine and brackish species (according to the World Register of Marine Species, <http://www.marinespecies.org>) was performed, using articles which were published between July and December 2017 in the top 20 journals in the “marine and freshwater biology” category of the 2016 Thomson Reuters Ranking. Articles were selected in the last published issues of the journals at the time of reading, in decreasing ranking. It was reported if and in which way environmental cycles were integrated into the experimental design. As simulating tidal cycles in the laboratory is technically demanding and thus rarely done, they were neglected from the search. In the published articles, to find out how organisms were synchronized to an environmental cycle and at which stage of the cycle they were studied, I focused on the following questions:

- (1) Were the light conditions mentioned? This include the presence/absence of a day/night cycle, the photoperiod, the light intensity or spectrum, and whether there were abrupt or gradual changes in light intensity at light on and light off. For the light intensity or spectrum, I considered any information including either natural illumination, the type of lamp, the irradiance ($\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), or the photon flux (lux).
- (2) Was the temperature mentioned and was it kept constant or cyclic?
- (3) For animals, was there a regular feeding procedure implemented?
- (4) Was the time of the year at which the experiment was performed reported, either in terms of month(s) or season(s)?
- (5) Was the origin of the biological material provided and was the experimental location mentioned?

I searched for these elements in the Material and methods section, and scanned the text according to keywords (“light”, “dark”, “cycle”, “photo*” for either photoperiod or photons, “°C”, “temperature”, “fed”, “feed”, “food”, each month and season, “lab*” for lab or laboratory, “center”, “instit*” for either institution or institute, “hous*” for housed). The purpose of this analysis was to assess the integration of biological rhythms within marine biology.

Results

The results are summarized in Table 1; detailed information is gathered in Supplementary Table S1. Out of the 150 articles reviewed, 32% did not provide information about the presence/absence of a light/dark cycle in the experiment. Another 10% provided only partial information such as for a specific time period during the experiment (i.e. acclimation or incubation), a particular development stage (for adults but not for their progeny) or for a subset of the studied organisms. Information on a diel cycle was rarely missing for plants (13% and 8%) or chromists (2% and 9%) while it was often missing for animals (52% and 9%), revealing a strong difference in treatment between photosynthetic and non-photosynthetic organisms. Of all articles analysed, 52% reported experiments run under a light/dark cycle while 6% of the experiments were explicitly run either under constant light or constant darkness. Only 1 article provided an explicit reason to work under constant conditions. The photoperiod was given in only 47% of the 150 articles: some articles specified the presence of a light/dark cycle but only mentioned a natural photoperiod without detail, for example. The information was not relevant for studies conducted under continuous conditions. The type of light, either in terms of intensity or spectrum and when continuous light or light/dark cycles were implemented, was mentioned in only 43% of the 150 articles considered. Unless implicit when working with outdoor or with natural daylight, the information on whether light on and off were abrupt or gradual was rarely mentioned (7% of the articles).

Temperature conditions were mentioned in 67% of the articles. The 20% of articles without information on a temperature cycle are distributed as follow: 6% did not mention any temperature, and 14% did mention either a range of temperature or a mean \pm standard deviation/standard error where the latter is equal or superior to 2°C. Without further clarification, it cannot be determined whether there was a planned or unplanned temperature cycle. Again, temperature conditions were less often mentioned in studies conducted on animals (56%) than on plants (75%) or chromists (83%). In the vast majority of cases, the temperature was kept constant, not cyclic.

Feeding cycles were considered for animals only. Out of the 86 articles involving animals, 40% did not provide information about the presence/absence of a feeding cycle, another 34% provided only partial information. Most studies that contained partial information on the feeding cycle mentioned that animals were fed either once or twice daily, but did not provide additional information such as: was the feeding implemented at the same time(s) each day? Fourteen percent of the articles provided full information on the feeding procedure, but only those that were on a daily basis were considered as cyclic, not those involving weekly or every other day feeding.

The time of year at which the experiment was performed was lacking in 70% of the work reported. Another 11% included only

Table 1. Summary of the literature survey.

	Information				Parameter	
	None	Partial	Full	NA	Cyclic	Constant
Light/dark conditions (all phylla, n = 150)	32	10%	58%	/	52%	6%
Plants (n = 24)	13%	8%	79%	/	75%	4%
Chromists (n = 46)	2%	9%	89%	/	78%	11%
Animals (n = 86)	52%	9%	38%	/	36%	2%
Photoperiod (all phylla, n = 150)	32%	15%	47%	6%	/	/
Plants (n = 24)	13%	21%	58%	8%	/	/
Chromists (n = 46)	2%	11%	76%	11%	/	/
Animals (n = 86)	52%	19%	27%	2%	/	/
Lighting conditions (all phylla, n = 150)	48%	6%	43%	3%	/	/
Plants (n = 24)	13%	8%	75%	4%	/	/
Chromists (n = 46)	2%	7%	89%	2%	/	/
Animals (n = 86)	81%	7%	10%	1%	/	/
Abrupt/gradual change (all phylla, n = 150)	85%	3%	7%	5%	/	/
Temperature cycle (all phylla, n = 150)	20%	13%	67%	/	1%	66%
Plants (n = 24)	17%	8%	75%	/	4%	71%
Chromists (n = 46)	11%	7%	83%	/	0%	83%
Animals (n = 86)	27%	17%	56%	/	0%	56%
Feeding cycle (all phylla, n = 150)	23%	20%	9%	48%	1%	5%
Plants (n = 24)	/	/	/	/	/	/
Chromists (n = 46)	/	/	/	/	/	/
Animals (n = 86)	40%	34%	14%	13%	8%	1%
Time of year (all phylla, n = 150)	70%	11%	19%	/	/	/
Experimental facility (all phylla, n = 150)	46%	11%	43%	/	/	/
Origin of the biological material (all phylla, n = 150)	4%	5%	91%	/	/	/

Percentages of articles providing information on the presence/absence of a light/dark cycle, the photoperiod, the light conditions (either in terms of intensity or spectrum), the setting of the light/dark change, whether the temperature was constant or cyclic, the feeding procedure and whether it was regular or not, the time of year (either in terms of month(s) or season), the experimental facility used for the experimental work, and the origin of the biological material (collection place or strain). Articles (n = 150) involving the following: plants (n = 24), chromists (n = 46), animals (n = 86), bacteria (n = 4), and fungi (n = 1). Percentages calculated for those n. Some articles may include several experiments and phylla, some run under cyclic conditions, others under constant conditions, and will therefore be counted in both categories, potentially giving a total for a line slightly different than 100%. NA: not applicable.

partial information, such as the collection time of animals from the field but not the experimental time or for only part of the experiments. Collection time and experimental time are not necessary the same, as animals might be kept in the lab as brood-stock, or for acclimation.

Compared to temporal information, spatial information was mostly provided in the literature: the origin of the organisms studied was detailed in the majority of cases (91%). However, the location of where the experiment took place was given in only 43% of the articles analysed (Table 1).

Discussion

The importance of biological rhythms is valid for potentially all biological functions as biological clocks deeply influence organisms' physiology and behaviour. In the mouse genome, almost half of all genes show circadian rhythms in transcription in at least one organ (Zhang *et al.*, 2014). Similarly, >40% of *Mytilus californianus* gill transcriptome is cyclic under light/dark and tidal entrainment (Connor and Gracey, 2011), and ~23% of the transcripts are rhythmic in the sea anemone *Aiptasia diaphana* (Sorek *et al.*, 2018). This is valid at all levels of organization, from the molecular to the behavioural level, and can occur even in cells as self-sustained circadian oscillations persisting for over 20 cycles have been reported in isolated tissues of mice (Yoo *et al.*, 2004). The observation has been extended to other organisms and there

is potentially a clock in each cell and each tissue (Mohawk *et al.*, 2012). Rhythms are also important for full-length cDNA analysis as differentially spliced mRNA isoforms may show rhythmic oscillations in relative abundance (Preußner *et al.*, 2014). No matter what we study, biological rhythms may thus influence virtually all major biological functions. Environmental cycles and biological rhythms should therefore be taken into consideration in the experimental strategy, as neglecting them may generate several problems.

Problem induced when working with desynchronized organisms

When an organism possessing a biological clock is kept under free-running conditions, i.e. without zeitgeber, it cycles with its own endogenous period. Due to inter-individual differences in this period, each animal may be in a different phase to its neighbour with obvious implications for variation in the phenotype (Figure 1a). For an experiment that aims at measuring a phenotype, this can artefactually increase the variance and the inter-individual variability. For experiments that aim at studying the effect of a treatment, for example, it might similarly alter reported observations. If the amplitude of the effect analysed is within the range of cyclic variation for the parameter studied, similarities or differences between those organisms could artefactually result

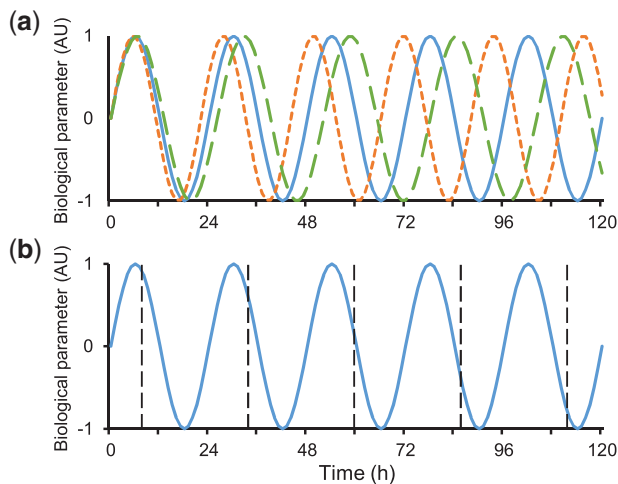


Figure 1. (a) Shift of a biological parameter under circadian control for 3 organisms whose internal period are 22h (short dotted line), 24h (continuous line), and 26h (long dotted line), respectively, over a 5-day experiment under free-running conditions. The desynchronization would involve different values for the parameter studied, even if organisms are sampled at the same time. (b) Effect of sampling at different times over the diel cycle in synchronized organisms. The value of the parameter studied would here be 0.9, 0.5, 0, -0.5, or -0.9 AU for sampling performed at 8, 10, 12, 14, or 16h, respectively. AU: arbitrary units.

from their intrinsic biological rhythm. The sand hopper *Talitrus saltator*, for example, exhibits a daily locomotor activity rhythm, being active at night. This activity rhythm is under circadian control (Bregazzi and Naylor, 1972). Without zeitgeber, a group of *T. saltator* won't be synchronized anymore and at the same time of day, one might have both active and resting animals, depending on their individual endogenous time. This would have an effect on the measure of locomotor activity. Rhythms are not only important for the absolute value of what we measure, but also for the nature of the measure itself. Indeed, they also influence how organisms cope with the same circumstances, either favourable or unfavourable, at different times of the day or seasons. For example, olfactory responses to food-related odours and pheromones in the cockroach *Leucophaea maderae* is under circadian control, with a 5–10-fold change in sensitivity to food-related odours between night and day (Page and Koelling, 2003; Rymer *et al.*, 2007). One might similarly expect clock-regulated responses in the marine environment and these rhythmic changes would again influence our observations. It is thus essential to work with synchronized organisms as working with desynchronized ones might highly disturb the parameter analysed, rendering it potentially unusable.

Problems occurring when ignoring the influence of entrainment in experimental design

The influence of entrainment should also be carefully considered in the experimental design as omitting it can introduce two types of bias. First, it should be included in the sampling strategy; rhythmic changes present in all treatments could be interpreted as differences between treatments if comparing samples collected at different times (Figure 1b). Second, the technical setup and maintenance of an experiment also needs to be considered within

a cyclic frame. Besides light, temperature and food are known potential and powerful zeitgebers for the circadian and circatidal clocks. If the temperature in the experimental setting is intended to be constant and measured once a day at the same time, the parameter may indeed appear constant over the course of the experiment while there might actually be an undesired daily cycle. While the controlled chambers or continuous recording inherently overcome this bias, manual measures should be planned at different phases of the implemented environmental cycle(s). Similarly, any maintenance operation like feeding that is operated daily should occur at the same time(s), both during the week and the weekend. It is also globally important to avoid unplanned entrainment like turning the light on during the dark phase, or an unmonitored change of the temperature of the water because zeitgebers synchronize the clock(s). In both nocturnal and diurnal organisms, light applied during the dark phase can reset the circadian clock and advance or delay the observed rhythm, depending on its administration time (Johnson *et al.*, 2004). In flying squirrels, a 1-s light pulse is sufficient to provide proper photoentrainment (Johnson *et al.*, 2004). Marine species may similarly show great sensitivity to different environmental cycles and proper control of experimental conditions is essential to gather relevant results.

Problems occurring when working with unnatural conditions

Beyond their constant or cyclic aspect, biological rhythms and clocks are affected by environmental and laboratory conditions. In the seabream *Sparus aurata*, mealtime determined whether locomotor activity was diurnal or nocturnal and influenced clock gene expression in the liver, while clock gene expression in the brain was determined by the light/dark cycle (Vera *et al.*, 2013). The light intensity and spectrum are also relevant as they can influence organisms' physiology or behaviour. For example, *Nephrops norvegicus* lobsters exposed to light/dark cycles showed a nocturnal burrow emergence activity under 10 lux, but a diurnal one under 0.1 lux (Chiesa *et al.*, 2010). Additionally, *N. norvegicus*' eyes are very sensitive to light-induced damage (Gaten *et al.*, 2013); in the laboratory, using a light intensity consistent with the animal's natural environment and working with progressive lights on and off allows avoidance of eye damage (Sbragaglia *et al.*, 2013). Photoperiod and temperature also provide temporal information to organisms on an annual scale, and seasons influence organisms' morphology, physiology, and behaviour (Helm *et al.*, 2013). They drive the lifecycle of the toxic dinoflagellate *Gonyaulax tamarensis* (Andersen and Keafer, 1987) and determine the diel valve activity pattern in the oyster *Crassostrea gigas* that is rather diurnal in spring and summer and nocturnal in autumn and winter (Mat *et al.*, 2012). Seasons also control major life traits like gametogenesis and spawning in several marine species including bivalves (Fabioux *et al.*, 2005), corals (Sorek and Levy, 2014), and worms (Naylor, 2010). They influence the response of daily locomotor activity to temperature changes in the crab *Uca pugilator* (Mat *et al.*, 2017), and affect the chemical composition in the kelp *Eisenia arborea* (Landa-Cansigno *et al.*, 2017). The photoperiod, temperature, and time of year therefore have several implications in laboratory experiments. First, an unnatural photoperiod can cause behavioural, physiological, or metabolic changes related to seasonal phenology based on photoperiod measurement. Second, it is also

critical to simulate a time that is suitable to investigate the scientific question. Photoperiodic induction of diapause has been reported in the marine copepod *Labidocera aestiva* (Marcus, 1980). If one wants to study the hatching success of eggs, animals should not be exposed to a photoperiod that triggers diapause. Working with relevant environmental incubation conditions is thus crucial to obtain realistic results. Controlling and informing about the timing of an experiment is also important for comparing experimental results with the existing literature, and to improve reproducibility.

Finally, both the origin of the biological material and the experimental facility are important information to understand and compare both the natural and laboratory conditions of the studied species. Organisms' collection and transfer can strongly affect rhythmicity due to stress, changing conditions, or transport conditions.

Guide for future studies

The present literature search demonstrates that the consideration of biological rhythms in marine species tend to stay confined to the field of chronobiology and are not yet well integrated into the broader field of marine experimental biology: 32%, 20%, and 23% of the articles analysed did not provide information about the light/dark, temperature, and feeding cycles in the experimental setup, respectively. Similarly, the time of year was not provided in 70% of the manuscripts. The following are suggestions to improve our experimental setups and practices, allowing greater potential for realistic observations and inter-study comparisons:

- (1) If there is no specific need or relevance for constant darkness or illumination for the experiment (e.g. work on cave species, photoinhibition), organisms should be under a light/dark cycles, either one mimicking the natural condition or a 12:12 light/dark cycle; ideally, using a light intensity and spectrum that are relevant for the studied species. For experiments which run over several weeks or months, mimicking the change in photoperiod would more closely reflect natural conditions. Gradual changes in light intensity would ideally be more realistic than abrupt changes, but are more difficult to implement; one should however be aware that this can influence experimental results.
- (2) Realistic environmental temperatures should be tightly controlled and monitored for the studied species. This includes frequent monitoring of the both constant or cycling temperatures throughout the various phases of the experimental cycle.
- (3) Daily feeding should occur at the same time(s), both during the week and the weekend. If the organisms are not fed but kept with running seawater, monitoring chlorophyll a might be one way to control for the absence of an undesired cycle.
- (4) If several cycles are implemented such as temperature and light/dark cycles for example, they should be consistent with each other. Other environmental cues can also act as zeitgebers for the circadian and circatidal clocks including salinity, pH, and turbulence cycles (Naylor, 2010). The most exhaustive control and monitoring of experimental conditions are therefore globally important to avoid either unwanted cycles or erratic patterns.

- (5) Under light/dark cycles, sampling per day should be performed at the same time to compare data acquired at the same phase of the cycle. If several cycles are included in the experimental setting, sampling per day should be performed at the most appropriate time to compare data acquired at the same phase of the cycle.
- (6) Simulating tidal cycles in the laboratory can be challenging, as many parameters can act as a tidal zeitgeber (e.g. salinity, air/water exposure, and temperature) and the organism might be specific in their responsiveness to these parameters. However, tidal cycles could be implemented when possible to reflect more closely the natural conditions marine species encounter in the field.
- (7) The awareness of biological rhythm should also be extended to field studies, where it can influence sampling time. Sampling at noon on week 1 and 2 correspond to different phases of a tidal cycle as high and low tide drift by ~ 48 min every day. Conversely, sampling specifically at low tide might result in sampling occurring both during the day and the night over several weeks.
- (8) Thoroughly document in the Material and methods section the light conditions (photoperiod, intensity, spectrum, abrupt or gradual light transition), temperature (mean \pm standard deviation), feeding protocol if any, the month(s) during which the work was performed, the collection place or strain used, as well as the experimental facility. Consider their potential influence on the obtained results in the Discussion section.

In conclusion, integrating the temporal organization and regulation of marine species within the marine biology community is essential for obtaining representative results, strengthening the validity of our observations, and improving reproducibility.

Supplementary data

Supplementary material is available at the ICESJMS online version of the manuscript.

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