

Is the circalunar clock derived from photoperiodic diapause – or vice versa?

Dissertation

in fulfillment of the requirements for the degree of

Doctor rerum naturalium

of the Faculty of Mathematics and Natural Sciences

at Kiel University

submitted by

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Research Group Biological Clocks

Max Planck Institute for Evolutionary Biology

Kiel, February 2024

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Date of oral examination: 30th of April 2024

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Kurzfassung

Das Leben auf der Erde hat sich in der Gegenwart wiederkehrender Umweltzyklen entwickelt. Diese umfassen die Gezeiten, den Tag-Nacht-Zyklus, die Jahreszeiten und den Mondzyklus. In Anpassung an diese Zyklen haben Organismen endogene Zeitmessersysteme, so genannte biologische Uhren, entwickelt. Die zirkadiane Uhr antizipiert den Tag-Nacht-Zyklus und ihr Mechanismus ist auf physiologischer und molekularer Ebene gut untersucht und verstanden, was 2017 mit dem Nobelpreis für Physiologie oder Medizin gewürdigt wurde. Im Gegensatz dazu sind die nicht-zirkadianen Uhren, einschließlich der zirkalunaren Uhr, die den Mondzyklus antizipiert, noch weitgehend unerforscht. Dies ist bemerkenswert, wenn man bedenkt, dass Mondrhythmen in marinen Lebensräumen allgegenwärtig sind und die zirkalunare Uhr dort eine weit verbreitete Rolle für die Fortpflanzung bei Eukaryonten spielt - von den Algen bis zu den Fischen. Im Rahmen dieser Arbeit haben wir eine umfangreiche Literaturrecherche über zirkatidale und zirkalunare Uhren bei Insekten (**Kapitel 1**) und über zirkalunare Uhren im gesamten Stammbaum des Lebens (**Kapitel 2**) durchgeführt. Wir legten dar, wie die zirkalunaren Uhren in verschiedenen Organismen auf unterschiedlichen Funktionsprinzipien beruhen und dies möglicherweise auf mehrere evolutionäre Ursprünge der zirkalunaren Uhren hindeutet. Des Weiteren heben wir den Mangel an experimentellen Studien hervor, die für die Untersuchung der zugrundeliegenden Mechanismen der zirkalunaren Uhren unerlässlich sind. Handelt es sich um einen Zähler, einen Oszillator oder ist eine Kombination aus mehreren biologischen Uhren erforderlich? Folglich untersuchten wir experimentell das

Funktionsprinzip der zirkalunaren Uhr in der marinen Mücke *Clunio marinus* (**Kapitel 3**). Wir fanden heraus, dass die zirkalunare Uhr in *C. marinus*, im Gegensatz zu früheren Annahmen, ein zirkalunarer Zähler ist, der auf zirkadianen Zyklen basiert. Dieses Ergebnis bestärkt die Annahme, dass sich die zirkalunare Uhr in marinen Mücken mechanistisch von der mariner Anneliden unterscheidet. Auf dieser Grundlage stellten wir die Hypothese auf, dass der zirkalunare Zähler in marinen Mücken von einem photoperiodischen Zähler abgeleitet sein könnte. Wir verglichen daher die Transkriptome (mRNA) zweier Phänotypen, die der zirkalunaren Uhr und dem photoperiodischen Zähler nachgeschaltet sind, d. h. des zirkalunaren Entwicklungsstillstands und der Diapause, in der marinen Mücke *C. marinus* (**Kapitel 4**). Wir fanden heraus, dass beide Entwicklungsstillstände im selben Larvenstadium auftreten und sich möglicherweise im Glukosestoffwechsel unterscheiden. Schließlich präsentieren wir das erste annotierte Genom auf Chromosomenebene für die marine Mücke *Clunio tsushimensis* (**Kapitel 5**). Diese Ressource bildet die Grundlage für vergleichende Genomanalysen, um die Anpassungen der zirkalunaren Uhr bei verschiedenen Arten zu untersuchen und des Weiteren, um zu verstehen, warum die Genome von *Clunio* so hochkompakt sind.

Abstract

Life on Earth evolved in the presence of recurring environmental cycles, e.g. the tides, the day-night cycle, the seasons and the lunar cycle. In adaptation to those, organisms evolved endogenous time keeping systems - so called biological clocks. The circadian clock anticipates the day-night cycle and its mechanism is well understood on the physiological and molecular level, which was appreciated by the Nobel prize in Physiology or Medicine in 2017. In contrast, non-circadian clocks, including the circalunar clock which anticipates the lunar cycle, remain largely understudied. This is striking considering the omnipresence of moon-related rhythms in marine habitats and the widespread role of the circalunar clock for reproduction across Eukaryotes, ranging from algae to fish. In the frame work of this thesis we conducted a comprehensive literature review of circatidal and circalunar clocks in insects (**Chapter 1**) and circalunar clocks across the tree of life (**Chapter 2**). We demonstrate that circalunar clocks in different organisms rely on different functional principles, potentially indicating multiple evolutionary origins of circalunar clocks. We highlight the lack of experimental evidence that is indispensable to study the underlying mechanisms of circalunar clocks. Is it a counter, an oscillator or does it require the combination of multiple biological clocks? Consequently, we investigated the functional principle of the circalunar clock experimentally in the marine midge *Clunio marinus* (**Chapter 3**). We found, that in contrast to previous assumptions, the circalunar clock in *C. marinus* is a circalunar counter based on circadian cycles. This result strengthens the idea that the circalunar clock in marine midges is mechanistically different compared to

that of marine annelids. We hypothesized that the circalunar counter in marine midges may be derived from a photoperiodic counter. Therefore, we compared the transcriptomes (mRNA) of two phenotypes which are downstream of the circalunar clock and the photoperiodic counter, i.e. the circalunar developmental arrest and diapause, respectively, in the marine midge *C. marinus* (**Chapter 4**). We found that both developmental arrests occur in the same larval sub-stage and potentially differ in their glucose metabolism. Finally, we present the first annotated genome on chromosome scale for the marine midge *Clunio tsushimensis* (**Chapter 5**). This resource constitutes the basis for comparative genomic analysis in order to study adaptations of the circalunar clock in different species and to understand why *Clunio* genomes are so highly compact.

Scope of the thesis

This thesis comprises five chapters, of which two are published (**Chapter 1 & 2**) and one submitted (**Chapter 3**). **Chapter 1** and **Chapter 2** are literature reviews. **Chapter 3** and **Chapter 4** contain experimental work. **Chapter 5** presents a new genomic resource.

The overall objective of the research presented in this thesis was to examine the functional principle and evolutionary context of the circalunar clock in the marine midge *Clunio*.

The chapter-specific objectives of the research presented here are:

- I. summarize the current state of knowledge about circalunar clocks and their functional principles within an evolutionary framework. (**Chapter 1 & 2**)
- II. study the functional principle of the circalunar clock in the marine insect *Clunio marinus*. (**Chapter 3**)
- III. characterize the circalunar developmental arrest and diapause in *C. marinus* on the transcriptomic level. (**Chapter 4**)
- IV. obtain an annotated genome on chromosome scale for the marine midge *Clunio tsushimensis*. (**Chapter 5**)

I specifically aimed to test the following hypotheses:

- i. The functional principles of the circalunar clock differ in organisms depending on the phylum (or group). Comparing behavioral and molecular studies in different species may unravel common evolutionary trajectories of circalunar clocks between or within phyla (or groups). (**Chapter 1 & 2**)
- ii. The functional principle of the circalunar clock in *Clunio marinus* is either based on an independent circalunar oscillator, a circalunar counter or a superposition of a circadian and a circatidal rhythm. All three hypothesis can be distinguished experimentally. (**Chapter 3**)
- iii. The transcriptomes of larvae of *C. marinus* undergoing a circalunar developmental arrest (CDA) and of larvae undergoing the overwintering dormancy arrest (diapause) share similarities in mRNA levels of metabolism-related genes and are different from the transcriptome of developing, non-arresting larvae, i.e. CDA may be a mini-diapause. (**Chapter 4**)
- iv. The exceptionally small size of *Clunio* genomes may be explained by the number of rearrangements or control of transposable elements. To address these hypotheses, a second *Clunio* genome is required. The annotated, chromosome scale genome of the marine midge *C. tsushimensis* is suitable for downstream comparative genome analysis with *C. marinus*. (**Chapter 5**)

Contents and contributions of individual chapters

Chapter 1: Organisms have adapted to environmental cycles by evolving endogenous time keeping systems, so called biological clocks. In this chapter we summarize the current state of knowledge on moon-related clocks - the circatidal and the circalunar clock - with a focus on insects. By providing a detailed overview of the experimental evidence available for insects with lunar and tidal rhythms, we highlight that the ubiquity of these rhythms in marine habitats stands in stark contrast to the current lack of understanding their underlying molecular mechanism. We argue that insects are excellent candidates to study not only the molecular mechanisms of circalunar clocks but also their evolutionary significance, e.g. the adaptive value of these clocks has never been experimentally confirmed. Investigating the mechanisms, functional principle, adaptive benefits and diversity of circatidal and circalunar clocks will be indispensable to understand time keeping and temporal organization on the circalunar and tidal scale.

Jule Neumann & Tobias S. Kaiser (2023)

Lunar and tidal rhythms and clocks

In: *Insect Chronobiology*. Ed. Hideharu Numata & Kenji Tomioka

Singapore: Springer Nature Singapore, Pages 203–227

https://doi.org/10.1007/978-981-99-0726-7_10

Conceptualization	JN, TSK
Manuscript writing	JN, TSK
Manuscript editing	JN, TSK

* The authors are listed in descending order of their respective contributions. Equal contributions are indicated by the & symbol.

Chapter 2: In this chapter we focus on the circalunar clock, which is the main study object of this thesis. We review the literature for organisms that exhibit (semi)lunar rhythms and evaluate their experimentally confirmed biological clock criteria. As new evidence, we present and discuss a previously unpublished phase response curve for the circalunar clock of *C. tsushimensis*. Strikingly, to this date not only the molecular mechanism but also the overall functional principle of circalunar clocks is still poorly understood. The functional principle here refers to the underlying principle of the clock: is the circalunar clock, like the circadian clock, an oscillatory system or is it rather based on a counting mechanism? Alternatively, does the circalunar clock require the superposition of several rhythms, requiring the circatidal clock or the circadian clock? And how can this be tested experimentally? Ultimately, we hypothesize that these divergent clock principles found in different branches of eukaryotes may indicate multiple evolutionary origins of circasemilunar and circalunar clocks. This is in contrast to circadian clocks, which likely also have multiple evolutionary origins but seem to be built along similar principles.

Tobias S. Kaiser & **Jule Neumann** (2021)

Circalunar clocks — Old experiments for a new era

BioEssays, 43(8), 2100074.

<https://doi.org/10.1002/bies.202100074>

Conceptualization	TSK, JN
Manuscript writing	TSK, JN
Manuscript editing	JN, TSK

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Chapter 3: In this chapter we address the lack of experimental evidence for the functional principles of circalunar clocks, as highlighted in Chapter 2. In fact, we distinguish experimentally between three hypotheses (oscillator, counter and beat, see Chapter 2) of the circasemilunar clock in the marine midge *Clunio marinus*. By conducting three different chronobiological assays, we demonstrate that the circasemilunar clock of *Clunio* is based on a counting mechanism which integrates endogenous circadian oscillations. This result opposes the long-held view that *C. marinus*' free-running circasemilunar clock operates independently of the circadian clock. In a broader evolutionary context, this study adds experimental evidence to our hypothesis suggested in Chapter 2; that the circasemilunar clocks of dipterous insects are based on different functional principles compared to the circa(semi)lunar clocks of marine annelids and algae. The functional principle of the clock might therefore depend on the evolutionary history of the organism. We hypothesize that the circalunar counter of *Clunio* could even be derived from a photoperiodic counter.

Jule Neumann, Dharanish Rajendra & Tobias S. Kaiser (under revision)

*The free-running circasemilunar period is determined by counting circadian clock cycles in the marine midge *Clunio marinus**

submitted in JBR (Journal of Biological Rhythms) with ID JBR-23-0138,

Conceptualization	TSK & JN
Experimental Design	TSK & JN
Execution of Experiments	JN, DR, TSK
Data Analysis	JN
Manuscript writing	JN
Manuscript editing	JN, TSK, DR

* The authors are listed in descending order of their respective contributions. Equal contributions are indicated by the & symbol.

Chapter 4: We address the hypothesis formulated in Chapter 3, that the circalunar counter may be derived from a photoperiodic counter, by examining two developmental arrests in *Clunio marinus* with a combination of behavioral assays and transcriptomics. The circalunar clock synchronizes development in *Clunio* on the population level with the semilunar phase during the 4th larval instar. Synchronization is achieved via a short arrest in development we termed circalunar developmental arrest (CDA), which is reminiscent of a very short diapause or quiescence. If the photoperiodic counter and downstream regulated developmental arrest (diapause) were coopted by the circalunar clock for synchronization of development, we expect similar physiological processes to be at play. Hence, using a transcriptomics approach, we characterized the differences in mRNA levels between larvae in CDA, larvae in non-CDA (committed in development) and larvae undergoing diapause. To do this, we re-established a protocol which allowed us to substage the 4th instar of *Clunio* according to imaginal disc development and obtained a distribution of the substages throughout the semilunar cycle under winter and summer conditions. We provide the first experimental evidence that CDA happens at exactly the same larval stage as the regular photoperiodic diapause that northern European *Clunio* populations undergo in winter.

Jule Neumann, Danila Voronov & Tobias S. Kaiser (in preparation)

Transcriptomic profiling of a circasemilunar developmental arrest and diapause in the marine midge Clunio

Conceptualization	TSK & JN
Experimental Design	TSK & JN, DV
Execution of Experiments	JN, DV
Data Analysis	JN
Chapter writing	JN
Chapter editing	JN, DV, DD

* The authors are listed in descending order of their respective contributions. Equal contributions are indicated by the & symbol. DD: David Doležal

Chapter 5: Genomic resources are indispensable for studying biological phenomena. The generation of such is a crucial step for investigating a phenotype of interest. The high-quality reference genome of *C. marinus* has highlighted several interesting genomic features, e.g. a very small size but yet comparable number of genes as other *Dipterans* and a high degree of gene duplications and structural variation. Some of these features might be linked to its adaptation to marine habitats and/or its peculiar circalunar timing. In order to conduct comparative genomics analysis and identify common genomic features and mechanisms which allow *Clunio* to maintain consistently compact genomes, an annotated reference genome for a second *Clunio* species is required. Based on linkage map analysis of two mapping families, we obtained an automatically annotated genome assembly on chromosome scale for *C. tsushimensis*. This chapter describes and focuses on how the reference genome was obtained.

Conceptualization	TSK
Experimental Design	TSK & JN
Execution of Experiments	JN
Data Analysis	JN, CP
Chapter writing	JN
Chapter editing	JN, EHS

* The authors are listed in descending order of their respective contributions. Equal contributions are indicated by the & symbol. EHS: Eva H Stukenbrock

The following additional publications have been developed within the framework of this thesis and are currently in preparation:

Celine Prakash, **Jule Neumann**, Dušica Briševac, Tjorben Nawroth, Ewan Harney, Vaishnavi Dandavate, Nico Fuhrmann, Carolina Peralta, Susanne Mentz, Kerstin Schaefer, Sina Schirmer, Kimaya Tekade, Kristian Ullrich, Seung-Joon Ahn, Andrea J. Betancourt & Tobias S. Kaiser (in preparation for submission in BMC Genomics)

Chromosomal level Clunio assemblies provide insights into compact insect genomes.

Dharanish Ranjendra, **Jule Neumann**, Tobias S. Kaiser & Chaitanya S. Gokhale (in preparation for submission in The American Naturalist)

On the evolution of emergence patterns driven by environmental cycles.

Danila Voronov, **Jule Neumann** & Tobias S. Kaiser (in preparation)

Time series analysis of transcriptomic changes over the lunar cycle in the marine midge Clunio

Introduction

Environmental cycles have inevitably shaped life on earth

Recurring environmental cycles including the day-night cycle, the tides, the lunar cycle and the seasons are as old as Earth itself. The origin of life can be traced back to at least 3.7 billion years ago [1, 2], a time when the days were much shorter, than our 24 hours today [3]. The place where life originated is highly debated and current hypothesis include hydro-thermal vents in the sea [4] and warm shallow waters on land [5]. While tides are most apparent in the shallow waters of the coast, they also affect the waters in the deep ocean including the life around hydro-thermal vents [6, 7, 8]. We do not know which role the recurring alternation of day and night or the relentless back and forth of the tides played for the genesis of life back then. However, with the evolution of primitive photosynthesis 3.5 billion years ago, the harvest of light began [9]. About 2.7 billion years ago, about the same time as unicellular Eukaryotes appeared [10], prokaryotic Cyanobacteria came to light. Their appearance inevitably changed the trajectory of life, as they were the pioneers to produce oxygen on a monumental scale [11].

The light-dependent reaction of photosynthesis, in which water is converted to oxygen, can only occur during the day. Life and life processes began to be organized in time. In Cyanobacteria, nitrogen fixation has to be separated in either space or time from photosynthesis, because the enzyme nitrogenase, which is required to fix nitrogen, is sensitive to oxygen [12, 13]. In contrast to temporal organization, the compartmentalization within cells, i.e. their organization in space, has been given a great deal of scientific attention. In fact, a plethora of endosymbiotic theories exist for the origin of eukaryotic cells, with their nucleus, mitochondrion and chloroplasts [14]. Further, the formation of internal membranes, associated with the increase of cell size, has been established as the basis for the separation of functions within an organism [15]. Life compartmentalizes in space, among others to enable redox control of metabolic processes [16] or to regulate gene expression even within the nucleus [17]. But how and when did organisms start to use temporal organization? Between 2 and 0.6 billion years ago the day-night alternation reached a stable 19-20 hour cycle and since then has increased steadily to the 24 hour solar day we have today [3]. It was under these conditions that, that multi-cellular Eukaryotes evolved more than 1.6 billion years ago during the Paloproterozoic [18]. Recurring environmental cycles inevitably affected organisms and to say it with the words of one great chronobiologist "[...] the major environmental cycles of day, tide, month, and year have con-

fronted natural selection with windows of opportunity and hazard that recur with precisely predictable frequency [...]" [19].

Organisms evolved biological clocks to anticipate environmental cycles

Organisms have evolved endogenous time keepers - so called *biological clocks* - to time metabolic processes, gene expression and behavior to predictable environmental conditions. Biological clocks are internal, molecular mechanisms which can keep track of time even in the absence of the environmental cycle. Experimental evidence for the circadian clock is already found in Prokaryotes, in no other than the Cyanobacteria [20]. Their photosynthetic and nitrogen-fixation rhythms persist even in the absence of the light-dark cycle, providing evidence for the truly endogenous regulation of these rhythms from early on in the history of life [20]. Since then, great advances have been made to understand the underlying genes and molecules being essential to maintain endogenous circadian rhythms in these organisms. In fact the circadian clock in the cyanobacterium *Synechococcus elongatus* has been reconstructed *in vitro* [21]. It is noteworthy that evidence for circadian clocks in Prokaryotes also include the non-photosynthetic bacteria *Bacillus subtilis* [22] and *Klebsiella aerogenes* [23], insinuating that biological clocks may have been present in the earliest life forms of this planet.

On the importance of biological clock research

Except for Archaea, circadian clocks are found throughout the tree of life. In the Eukarya domain, this includes euglenoids [24], dinoflagellates [25, 26], ciliates [27, 28], diatoms [29, 30], brown, green and red algae [30, 31, 32], plants [33], fungi [34], sponges [35, 36] (tentative evidence), insects [37], birds [38, 39], fish [40] and mammals [41]. This omnipresence may suggest that endogenous time keeping is an essential feature of life, but to substantiate its adaptive value is not trivial [42, 43]. Chronobiologists, i.e. researchers studying biological time keeping, distinguish between the intrinsic advantage and extrinsic advantage hypotheses, i.e. biological clocks may confer advantages to organisms by synchronizing internal physiological processes or by synchronizing their physiology to the optimal external condition, respectively [44]. Tentative, but incomplete evidence exists for both [44]. Why circadian clocks came into being in the first place is another uncharted question, which is hard to answer. Pittendrigh suggested that circadian clocks evolved essentially to escape from light, i.e. some light-sensitive reactions may not take place at certain times of the day (see Cyanobacteria) and organisms may protect their DNA during cell division from UV radiation [19, 45]. While the questions surrounding the evolution of circadian clocks remain to be answered, major advances have been achieved

in unraveling its molecular architecture. This entails identification of core clock genes [46], the discovery of the involvement of transcriptional-translational feedback loops [26] and the role of epigenetic processes [47, 48]. These advances and their impact were acknowledged in 2017 by the Nobel Prize for Physiology or Medicine awarded to Jeffrey C. Hall, Michael Rosbash and Michael W. Young for their discoveries of molecular mechanisms controlling the circadian rhythm [49]. Notably, research conducted in the field of Chronobiology impacts our everyday lives. This includes recommendations on when school should start [50], optimizing the timing of chemotherapy treatment [51] or developing guidelines on how experimental studies should be designed, conducted and interpreted [52]. Consequently, Chronobiology, the study of endogenous timekeeping, has revealed the immense importance of endogenous timekeeping for life on Earth, including humans.

The quest for deciphering non-24 clocks

I wrote the last few paragraphs to emphasize how intimately biological clocks are intertwined with the evolution of life and how significant the understanding of the circadian clock has been. However, there lies great potential in understanding biological time keeping on other, non-24 hour scales. Lets take for example the phenomenon of photoperiodism, which is widespread among plants, fungi and animals [53]. Photoperiodism encompass physiological and developmental reactions of organisms towards changes in the length of the light or dark period, i.e. the seasons [53]. Examples for photoperiodic responses include the flowering time in plants, insect diapause, migration in birds or reproduction in mammals [53]. As diverse the perception of photoperiodic cues and the behavioral output is, common features of photoperiodism in plants and animals exist, including the role of the circadian clock [54, 55, 56]. These common features are important to understand challenges like the potential of populations to adapt to rapid climate change [57, 58]. In the case of insect photoperiodism, few molecular studies exist and theoretical models have been developed to link photoperiod as a cue with a perception mechanism and a core-clock mechanism to the output behavior [59, 60]. It is known that insects usually require a defined number of nights of a certain length to initiate a photoperiodic response [61, 62]. However, to this day no one understands how such a *photoperiodic counter* works on the neuronal or molecular level. Deciphering the photoperiodic counter will be essential to ultimately link seasonal rhythms with the circadian clock. However, what about the link of such a counting mechanism to other, non-24 hour clocks? This knowledge gap is certainly driven by the fact that knowledge about other non-24 hour clocks is scarce.

The extensive knowledge that has been acquired about the circadian clock is in stark con-

trast to what is known about the non-24-hour clocks, including the circatidal, circalunar and the circannual clocks. This is surprising considering that knowledge about the existence of tidal, lunar and annual rhythms is as old as the field of Chronobiology itself [63]. Traits associated with the yearly migration of birds are controlled by a circannual clock [64]. However, migration is not only controlled by the circannual clock but further by the circadian clock as well as photoperiodic time keeping [53]. How can we understand what selection is acting upon when we miss a lot of temporal dimensions in the trait itself? Hence, studying non-24 hour clocks and organisms with phenotypes that comprise many different timing dimensions and systems is necessary to advance the field of Chronobiology.

The Moon is the first milestone on the road to the stars. [65]

The moon tremendously influences marine habitats, i.e. by causing tides, and tidal and lunar rhythms are widespread and prevalent in marine habitats. Many of these biological rhythms are controlled by circatidal or circalunar clocks [66]. Because the first two chapters of this thesis give a comprehensive overview of lunar and tidal rhythms and clocks, I will not go into much detail here. In summary, these rhythms have been mostly studied on the behavioral level. Often, because they are pronounced in non-model species for which molecular techniques are scarce. In fact, the circalunar clock has been studied in the laboratory only in a few organisms, mainly in the marine annelid *Platynereis dumerilii* and marine insects of the genus *Clunio* [67]. Understanding how the circalunar clock functions at the molecular and conceptual level offers enormous potential for understanding how life is organized at more than just the circadian scale. And it raises the possibility to address why and how endogenous time keeping evolved in the first place. Because what is ultimately under selection is the cooperation of the entire temporal and spatial organization of an organisms - not only the circadian clock. Studying circalunar clocks is therefore essential and addressed in the framework of this thesis. In particular, I studied the interaction of the circadian with the circalunar clock and investigate the potential involvement of a counting mechanism in circalunar time keeping, in the marine midge *Clunio marinus*.

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Chapter 1

1 Lunar and tidal rhythms and clocks

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Neumann J & Kaiser TS (2023). Lunar and tidal rhythms and clocks. In: *Insect Chronobiology* (pp. 203–227). Ed. Numata H & Tomioka K, Singapore: Springer Nature Singapore. https://doi.org/10.1007/978-981-99-0726-7_10

Abstract

The presence of the moon results in tidal and lunar cycles that particularly affect life in the intertidal zone through tidal motion. A few insect species managed to colonize this dynamic interface between marine and terrestrial habitats, displaying tidal and lunar rhythms. These rhythms are caused by endogenous time-keeping mechanisms, namely, circatidal and circalunar clocks, but to date, the molecular mechanisms of lunar and tidal time-keeping remain largely unknown. Extensive chronobiological behavioral experiments conducted in a few insect species have identified the basic properties of circatidal and circalunar clocks, such as the free- running period, zeitgebers for entrainment, phase response curves, and temperature compensation. First molecular insights have been obtained for the circatidal clock of the mangrove cricket. Further development of molecular manipulation methods, as well as of genetic screens and omics experiments, will pave the way to unravel the mechanisms of both circatidal and circalunar clocks.

Keywords *Apteronemobius asahinai* · Circalunar clock · Circatidal clock · *Clunio*

Introduction

The moon has been our planet's most loyal companion. Its presence inevitably shaped the appearance of Earth, most evidently through tidal motion – the greatest synchronized movement of matter on our planet (Bowers and Roberts 2019). Both the 29.5-day lunar cycle and the 12.4-h tidal cycle are induced by the moon and influence life in the oceans. Consequently, lunar and tidal rhythms are found in the life cycles and synchronized reproduction of many marine species. This is especially true for coastal habitats, where the intertidal zone alternates between marine and terrestrial conditions. Historically, causal relationships between lunar and tidal cycles and their related biological rhythms have been difficult to demonstrate by experimental verification. At the same time, a plethora of pseudoscientific publications debase the authenticity of such phenomena. Today, not only the existence but also the endogenous nature of circalunar and circatidal clocks have been experimentally confirmed in organisms across eukaryotes. Such prevalence underlines the ecological and evolutionary significance of moon-related biological rhythms. Even though only a few insect species have adapted to the marine environment, they provide us with the unique opportunity to study the acquisition of circalunar and circatidal clocks in organisms which colonized the ocean secondarily. Today, we are only at the beginning of an exciting journey to unravel the molecular mechanisms of lunar and tidal time-keeping, their operating principles, and their underlying genes.

The moon causes environmental cycles with different periods

The combined gravitational pull of the moon and sun on large water bodies, along with the rotation of the Earth, results in the phenomenon of tides, i.e., the periodic rise and fall of the water surface at a particular place (Figure 1a,b). Along most coasts, tides rise and fall twice a day (every 12.4 h) and are called semidiurnal tides (Figure 1b). In a few locations, tides occur only once a day (every 24.8 h) and are therefore referred to as diurnal tides. When sun, moon, and Earth align every 14.77 days during full and new moon, their gravitational pull interferes constructively, and the tidal amplitude is highest, resulting in so-called spring tides (Figure 1a). The synodic lunar cycle is completed when all visible phases of the moon in relation to the Earth and sun have passed after 29.53 days (Figure 1c). There are two spring tide events in a full synodic lunar cycle, i.e., spring tides occur in a semilunar rhythm (Figure 1c). Tidal (12.4 h), semilunar (14.77 days), and lunar (29.53 days) rhythms are to be found in many marine organisms inhabiting the intertidal zone, including insects.

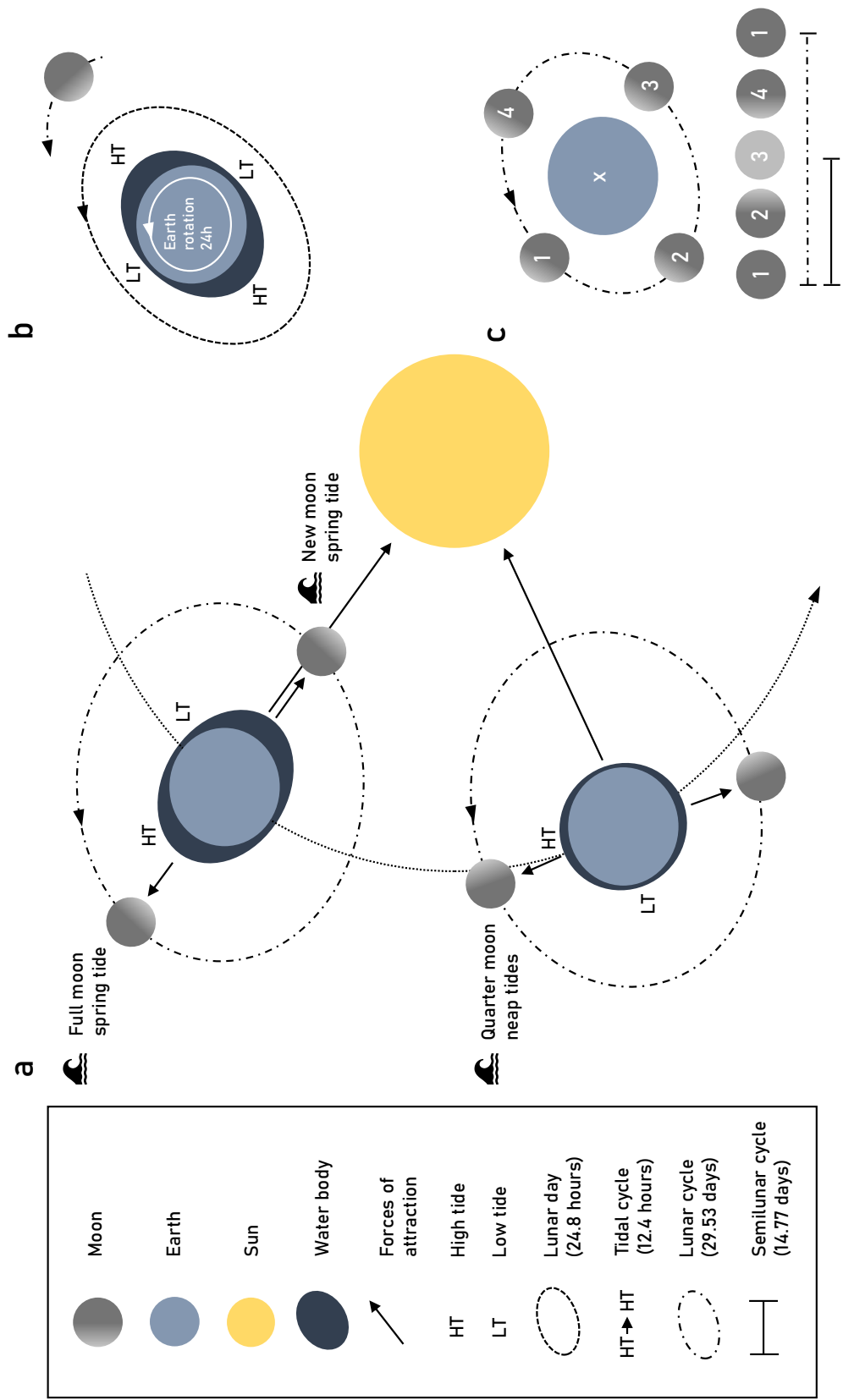


Figure 1: The interaction of the sun, moon, and Earth results in tidal and lunar cycles. (a) Forces of attraction between the sun, moon, and Earth on Earth's water body result in the occurrence of tides. When all celestial bodies align during the full and new moon, the forces add up, and the tidal amplitude is highest (spring tides), while when the sun and moon pull on Earth's water body at a 90° angle, forces cancel each other out, and the tidal amplitude is lowest (neap tides). (b) The moon orbits Earth in the same direction as Earth rotates around its own axis. To regain its exact position in relation to the moon, every location on Earth has to (continued) catch up with the 50-min discrepancy between both trajectories. Therefore, a lunar day from Earth's perspective is 24 h and 50 min. Hence, a location experiences two low and high tides per day, resulting in a tidal cycle of 12.4 h. (c) From Earth's perspective (x), the moon passes through all lunar phases every 29.53 days (synodic lunar cycle). A synodic semilunar cycle corresponds to half a synodic lunar cycle (14.77 days)

Organisms anticipate moon-related cycles by endogenous circasemilunar, circalunar, and circatidal clocks

Lunar and tidal rhythms could either be merely induced by external stimuli or could result from an endogenous biological clock. This is a fundamental difference, and hence, the first question with respect to an observed biological rhythm is always: Is there an endogenous (molecular) time-keeping mechanism that can tell time even in the absence of environmental cycles? The matter has been a long-standing debate in chronobiology. Today, the criteria characterizing an endogenous biological clock are well defined: The clock must be free-running, be temperature-compensated, and be subject to entrainment by specific stimuli (Pittendrigh 1993). The following section will explain these basic features. Biological clocks are described in the terminology of physics and mathematics. They are viewed as oscillatory systems (limit-cycle oscillators) with a natural, endogenous period τ which is slightly different from the period T of the corresponding exogenous environmental cycle. To test for the existence of an internal oscillator with period τ , the organism displaying the rhythm of interest is transferred into constant conditions (e.g., for a tidal rhythm, none of the environmental conditions should have a 12.4-h period). If the organism still displays rhythmicity with τ close to T over several cycles in this constant environment, the rhythm is free-running. Furthermore, the free-running period of the rhythm must be temperature-compensated, meaning that within a given range of temperatures, the period τ does not change. This is in stark contrast to most other chemical and metabolic processes, which increase at a rate of 2 to 3 times with every 10 °C increase in temperature (Q_{10} temperature coefficient = 2–3). Temperature compensation is important for reliably keeping track of time independent of the naturally fluctuating temperature of the environment. Finally, it must be possible to synchronize the endogenous biological clock to the exogenous environmental cycle via specific environmental cues, which are called zeitgebers. The process of synchronization is called entrainment, during which the observed biological rhythm assumes a specific phase relationship to the environmental cycle. Zeitgebers therefore set the phase of the displayed rhythm. For lunar and tidal rhythms, tidal vibration, tidal temperature cycles, and moonlight have all been identified as zeitgebers for entraining the circatidal, circasemilunar, and circalunar clocks in insects (Table 1).

Evolutionary significance of circatidal, circasemilunar, and circalunar clocks: Why should we study lunar and tidal rhythms in Insects?

Studying the evolutionary causes and history of circatidal and circalunar clocks will be valuable to understand biological temporal organization. In this section, we will explore three

evolutionary concepts:

- (a) The adaptive benefits of circalunar and circatidal clocks: Why did circatidal and circasemilunar clocks evolve? In other words, what is the ultimate or evolutionary advantage for keeping track of the tides and the lunar cycle?
- (b) The diversity of time-keeping mechanisms: Do organisms find different solutions for circalunar and circatidal time-keeping mechanisms? This will help to understand how tidal and lunar biological rhythms evolved or how plastic time-keeping can be organized.
- (c) The temporal organization within an organism: How do organisms track different geophysical cycles simultaneously?

The adaptive benefits of circalunar and circatidal clocks

The intertidal zone is defined as the area lying within the tidal range. This means that during low tides, the seafloor is exposed, while at high tides, it is submerged. Changes in the water level are accompanied by considerable differences in temperature, salinity, UV exposure, nutrient availability, or predator abundance. While the adaptive value of the circadian clock has been demonstrated (Abhilash and Sharma 2016), such evidence is entirely lacking for circatidal or circa(semi)lunar clocks. However, there are two main hypotheses for the adaptive benefits of these moon-related rhythms. The first is that they may synchronize important life cycle events, such as reproduction or hatching, as well as locomotor and feeding behavior, with favorable tidal conditions. This is known as the extrinsic advantage hypothesis (Naylor 2010). Second, they may serve to synchronize these same life cycle events within a population (Naylor 1976). In particular, lunar rhythms may serve to synchronize reproduction within a population, especially in marine or tropical habitats where seasonality is often not very pronounced (Hartland-Rowe 1958). In a sparse population, it will be harder to find a suitable mate, and therefore, reproductive success decreases, which is known as the Allee effect (Courchamp et al. 1999). Precise synchronization of reproduction reduces the Allee effect. Despite the lack of experimental evidence, the omnipresence of (semi)lunar and tidal rhythms across the tree of life suggests that moon-related rhythms are adaptive for many organisms, particularly in marine environments.

Diversity of circatidal and circalunar clocks

As insects first evolved in terrestrial habitats, all marine insect species must have colonized the ocean secondarily (Andersen 1999; Misof et al. 2014). These insects provide us with an

excellent opportunity to study recently acquired endogenous timing systems as adaptations to the tidal environment. The principles of these timing mechanisms can then be compared to those that have evolved in primordial marine organisms, like those of marine annelids. First evidence suggests that the circa(semi)lunar clocks of annelids, insects, and algae may follow different functional principles (Kaiser and Neumann 2021). Unraveling these principles and the molecular clockwork of circatidal and circa(semi)lunar clocks across the tree of life will help to fully understand their divergent mechanisms and multiple evolutionary origins (Rock et al. 2022).

Temporal organization on different time scales

Organisms are simultaneously exposed to a variety of environmental cycles of different periods. The circadian clock might time the feeding behavior of an organism, while a circalunar clock times reproduction, and a photoperiodic response regulates overwinter dormancy. The interplay of time-keeping mechanisms, their interrelations, coordination, and dependencies are ultimately what ensures survival of an organism and is subject to selection. It remains an exciting challenge to unravel the complex interplay of time-keeping mechanisms. Only organisms that show robust phenotypes on all different time scales will allow us to understand their overall temporal organization. Insects displaying tidal and (semi)lunar rhythms are a starting point for addressing such complex questions.

Circatidal clocks in Hexapods

Tides and lunar cycles predominantly impact life in the ocean where insects are rare. Approximately 50% of all insect orders contain marine species, most notably the orders Collembola (springtails), Hemiptera (true bugs), Coleoptera (beetles), and Diptera (true flies) (Cheng 2009). The vast majority of marine insects inhabit the intertidal zone, and some of them display behavioral rhythms related to tides. For example, hemipteran bugs of the genus *Halovelina* and members of the family Hermatobatidae leave their refuge of the upper tidal zone to forage during low tide (Cheng 2009). The common tiger beetle *Cicindela* inhabits sandy shores and populates the intertidal zone when the tide recedes. Larvae of the beetles plug their burrow entrances shortly before the tide returns to avoid flooding of their refuge (Cheng 2009). Similar burrow-plugging behavior was also observed in the mangrove forests of Japan for the tiger beetle *Callytron yuasai* (Satoh and Hayaishi 2007). Interestingly, a circaseptan activity rhythm was recorded in the laboratory for the beach beetle *Chaerodes trachyscelides* (Meyer-Rochow and Brown 1998). The authors speculate that such weekly rhythms could be an adaptation to

the position of the wrack line, which is altered by weekly alternation of spring and neap tides and may serve as shelter and foraging grounds. Despite these observations, experimental confirmation of circatidal clocks in insects is scarce (Table 1). The criteria for endogenous circatidal rhythms have been investigated in only four hexapod species: the springtail *Anurida maritima* (Collembola) (Foster and Moreton 1981), the beetles *Callytron inspecularis* and *Thalassotrechus barbarae* (Coleoptera) (Evans 1976; Satoh et al. 2006), and the mangrove cricket *Apteronomobius asahinai* (Orthoptera) (Satoh et al. 2008). It appears that tidal rhythms generally dampen rapidly under constant conditions, which complicates their study in the laboratory. With respect to zeitgebers, artificial submergence given in a tidal pattern has been identified to entrain *C. inspecularis* and *A. asahinai* (Satoh et al. 2006; Satoh et al. 2009). Temperature compensation of the tidal rhythm has never been experimentally verified in any of the examined insect species (see Table 1). This may be due to the quick dampening, as well as a lack of efficient culturing procedures for most intertidal species.

Collembola: *Anurida maritima*

The small Collembola *Anurida maritima* inhabits rocky intertidal zones and salt marshes (Cheng 2009). During high tide, *A. maritima* can be found hiding among rocks, while during low tide, they come out to forage. This circatidal locomotor rhythm has been demonstrated to be free-running under constant light (LL) in the laboratory for at least 7 days (Mcmeechan et al. 2000; Foster and Moreton 1981). Early anticipation of the approaching tide is essential for survival of the Collembola because it physically cannot outrun the water (Foster and Moreton 1981). Some individuals inhabit the higher intertidal zone, which is not exposed to high tides every day. Interestingly, *A. maritima* there still displays a tidal activity rhythm, providing a rare opportunity to investigate an apparently free-running rhythm under field conditions (Foster and Moreton 1981). When *A. maritima* moves toward the upper shore around high tide, they often aggregate with hundreds of individuals (Joosse 1966). This aggregation behavior also persists rhythmically under LL, matching the phase of lowest locomotor activity (Mcmeechan et al. 2000). Additionally, it has been reported that in the field, the peak of activity occurring during the night low tide in *A. maritima* can be suppressed (Foster and Moreton 1981). This raises the question as to whether the circadian clock modulates the tidal activity pattern or whether darkness or temperature instantly suppresses the behavior (Foster and Moreton 1981; Mcmeechan et al. 2000). Interestingly, *A. maritima* is usually negatively phototactic, meaning they move away from light, consistent with the shelter searching behavior in the field observed during high tide. However, for 2–7 h after high tide, most of the population becomes positively

Species	Behavioral rhythm	(1) Free-run demonstrated in the laboratory	(2) Entrainment		(3) Temperature compensation
			Identified cue (zeitgeber)	Phase response curve	
Circatidal rhythms in hexapods					
<i>Anurida maritima</i> (Collembola)	Locomotor activity, aggregation behavior, phototaxis	[1] LL [2] LL, [2] LL, [3]	— — —	— — —	— — —
<i>Thalassotrechus barbarae</i> (Coleoptera)	Locomotor activity	[4] LL	—	—	—
<i>Callytron inspecularis</i> (Coleoptera)	Burrow plugging	[5] LL	[5] TSu	—	—
<i>Apteronomobius asahinai</i> (Orthoptera)	Locomotor activity	[6] DD [9] LL	[7] TSu [8] Wa	[7] [8]	—
Circalunar rhythms in insects					
<i>Clunio marinus</i> (Diptera)	Semilunar or lunar adult emergence	[10] LD [11] LL	[10] ML [12] TVi [13] TTe	—	—
<i>Clunio tsushimensis</i> (Diptera)	Semilunar adult emergence	[14] DD	[14] ML	[15]	[14]
<i>Pontomyia oceana</i> (Diptera)	Semilunar adult emergence	[16] LD, LL, and DD	[16] ML	—	[16]

ML moonlight, *TVi* tidal vibration, *TTe* tidal temperature, *TSu* tidal submergence, *DD* constant darkness, *LL* constant light, *LD* light-dark cycle, *Wa* water stimulus

[1] Foster and Moreton 1981, [2] Mcmeechan et al. 2000, [3] Manica et al. 2000, [4] Evans 1976, [5] Satoh et al. 2006, [6] Satoh et al. 2008, [7] Satoh et al. 2009, [8] Sakura and Numata 2017, [9] Satoh 2017, [10] Neumann 1966, [11] Neumann 1976, [12] Neumann 1978, [13] Neumann and Heimbach 1984, [14] Neumann 1988, [15] Kaiser and Neumann 2021, [16] Soong et al. 2011

Table 1: Insects displaying lunar and tidal rhythms for which endogenous biological clock criteria have been experimentally examined

phototactic, coinciding with *A. maritima* leaving their refuge to feed (Manica et al. 2000). This change in phototactic behavior has been shown to follow a circatidal rhythm, potentially using the horizon brightness as a guidepost (Manica et al. 2000). Water reflects light better than soil, and thus, the horizon toward the water is brighter, attracting *A. maritima* during their foraging excursions (Manica et al. 2000).

Coleoptera: *Thalassotrechus barbarae* and *Callytron inspecularis*

The carabid beetle *Thalassotrechus barbarae* was the first insect for which a circatidal locomotor rhythm had been investigated in the laboratory. The beetles inhabit the rocky tidal shore along the American continent. Adults forage during the night, but just at low tide. Hence, they display a lunidian (lunar-day) locomotor activity rhythm (24.8 h) during night tides. Consistently, under LD (light:dark) 15:9 in the laboratory, beetles were active only during the dark phase, suggesting either a circadian or circalunidian rhythm, which could not be distinguished statistically (Evans 1976). However, under LL, a significant correlation between the amplitude of the corresponding field night tide and the amount of activity was demonstrated for the first 3 days after beetles were brought to the laboratory (Evans 1976). Accordingly, it has been proposed that a circatidal oscillator might suppress the beetle's activity during high tide (Evans 1976). It should be noted, however, that there was never a follow-up publication investigating the endogenous nature of the tidal rhythm in *T. barbarae*.

Larvae of the tiger beetle *Callytron inspecularis* reside in sand burrows, which they plug shortly before high tide. It was found that this burrow-plugging behavior continues under LL in the laboratory (Satoh et al. 2006). When one larva was subjected to an artificial tidal cycle of submergence, the burrow-plugging rhythm was entrained and, after cessation of the cue, was free-running with a period of 12.45 ± 0.51 h (Satoh et al. 2006). Interestingly, larvae only start the plugging behavior after their burrows have been submerged by one to three high tides (Satoh et al. 2006). When approaching neap tide, the tides, and therefore tidal submergence, cannot reach the beetle's habitat, and the burrow-plugging behavior eventually ceases (Satoh et al. 2006). It has been hypothesized that the circatidal oscillator is entrained by tidal submergence but dampened with subsiding tides, in accordance with the observation that burrow plugging in the field has not been observed during neap tides.

Orthoptera: *Apteronemobius asahinai*

The circatidal activity rhythm in the mangrove cricket free-runs under LL and DD

By far, the best studied circatidal rhythm of an insect is found in the mangrove cricket *Apteronemobius asahinai*. These crickets are active during low tide but remain inactive around high tide, residing on mangrove roots. The locomotor rhythm of field-caught crickets has a free-running period of approximately 12.6 h under constant darkness (DD) (Satoh et al. 2008) as well as under LL (Satoh 2017). Notably, the recorded activity levels differed in amplitude under DD, alternating between strong and weak activity peaks (Figure 2a,a'). This can be interpreted as a diel modulation of the activity, with the crickets being less active during the night. Such diel modulation might be controlled by a circadian clock. Indeed, the difference in activity levels was absent in LL, and it has been hypothesized that under LL, the circadian modulation diminishes because of the suspension of the circadian clock (Satoh 2017). Under DD, the circadian oscillations can persist, and hence, the circadian modulation of the circatidal rhythm is reflected in an unequal activity rhythm. To further disentangle circatidal and circadian rhythms, locomotor activity of crickets that never experienced a natural tidal cue (raised from eggs in the laboratory) was monitored under dim red light (Sakura and Numata 2021). Some crickets showed significant circatidal rhythmicity for more than 20 days, while others showed circatidal rhythmicity only within the first 10 days but switched to diurnal rhythmicity afterward. Additionally, some crickets displayed a circatidal rhythm in the first 10 days but became arrhythmic thereafter. To investigate whether the diel rhythm was controlled by a circadian clock, the crickets were subjected to LL, as well as light-dark cycles (LD) with phase advances and delays. However, no consistent responses could be obtained, and more experiments are required to investigate whether the diel activity rhythm is controlled by a circadian clock.

Inundation stimuli entrain the circatidal rhythm in the mangrove cricket

Artificial inundation was found to be a zeitgeber for the circatidal clock of *Apteronemobius asahinai* and served to obtain the only circatidal phase response curve for insects (Satoh et al. 2009). Field-caught crickets were exposed to DD for approximately 10 days, and free-running circatidal locomotor rhythm was recorded. When a 30-min inundation stimulus was given at different times of the tidal cycle, the onset of activity was shifted depending on when the cue was given. If the stimulus was given during subjective high tide, only a small phase advance could be invoked, while a stimulus given during the subjective low tide resulted in noticeable

phase delays (first half) and advances (second half). Although inundation was shown to entrain the circatidal clock in the mangrove crickets, the particular sensory stimulus could not be clarified from the experiment. As a follow-up, it was tested whether contact with water was the cue (Sakura and Numata 2017). In cold-anesthetized and immobilized crickets, a water stimulus given during the middle phase of the subjective low tide resulted in a delay of locomotor rhythm, while a stimulus given during the later phase of the subjective low tide advanced the rhythm (Sakura and Numata 2017). Only a slight phase shift was observed when the water stimulus was given during the subjective high tide. The authors proposed that water receptors could perceive the water stimulus, as they have been described in the legs of another cricket, *Gryllus bimaculatus* (Kanou et al. 2007).

Involvement of the circadian clock in circatidal time-keeping

It has been a long-standing debate whether the circatidal clock in one way or the other involves the circadian clock's molecular machinery. There are three main hypotheses. Enright suggested that a single clock could govern both circatidal and circadian rhythms and be entrained by both tidal and circadian zeitgebers (Enright 1976). Palmer proposed the presence of two 24.8-h oscillators that run in antiphase and produce a rhythm that appears to be only 12.4 h apart, known as the circalunidian clock hypothesis (Palmer 1995; Palmer and Williams 1986). Circalunidian refers to the period between consecutive moonrises, equaling 24.8 h (Figure 1b). Given the period close to 24 h, such circalunidian oscillators could be based on the circadian clock machinery. Finally, Naylor proposed an independent circatidal clock mechanism with its own molecular machinery (Naylor 1996; Naylor 1958).

Given the inability of the circadian clockwork to adopt periods that deviate significantly from 24 h, Enright's idea of a single clock would only work if the overt 12.4-h tidal rhythm is generated as a submultiple of a 24.8-h circalunidian oscillator (Enright 1976; Goto and Takekata 2015). Additionally, the circalunidian instance of such a single multipurpose clockwork must not respond to light entrainment, as then night and day should force it to a 24-h period (Palmer 1995). There are no observations in insects that would support such a single clockwork mechanism, but it was demonstrated for fish (Gibson 1973) and crustaceans (Akiyama 1997). Interestingly, it is well documented that in *Drosophila* the morning (M) and evening (E) activity peaks are generated by two oscillators in antiphase (Helfrich-Förster 2009; Yoshii et al. 2012). Considering that marine insects evolved from terrestrial ancestors, it might be that by extending the M-E interval, an adaptation to the tides (a 12.4-h rhythm) could have been achieved (Zhang et al. 2013). The burrow-plugging behavior of the tiger beetle *Callytron inspecularis* has been hy-

pothesized to be explained by the circalunidian hypothesis because the time intervals between two consecutive burrow-plugging events vary in the field (Sato et al. 2006). This suggests that the two oscillators are slightly out of phase. In line with that, in free-run experiments with crabs, the two daily activity peaks sometimes seem to run with different free-running periods (Palmer 2000; Palmer and Williams 1986). However, based on experiments in *Carcinus maenas* (Naylor 1996; Naylor 1958), Naylor argued that the observed patterns under free-run conditions can rather be explained by the interplay of independent circatidal and circadian oscillators. Indeed, for the mangrove cricket, an independent circatidal clock has been proposed based on molecular knockdown of core circadian clock genes (see next paragraph). Additionally, recent molecular findings in the crustacean *Eurydice pulchra* suggest that the circatidal oscillator may use some molecules of the circadian clock, but not all of them (Zhang et al. 2013). This finding might support an independent circatidal oscillator with some circadian clock components reused.

Molecular and neurophysiological data support an independent circatidal pacemaker in the mangrove cricket

The circadian clock is by far the best studied biological clock at the molecular level. As a consequence, involvement of the circadian clock in other biological time-keeping systems – including photoperiodism, circatidal, circalunar, and circannual clocks – has been the focus of research for a long time. Knocking down known core circadian clock genes using RNA interference (RNAi) is one approach for investigating the role of the circadian clock. If the circadian phenotype is disrupted but the tidal, lunar, or photoperiodic rhythm persists, the underlying clock is assumed to run independently of the circadian system. Knockdown methods are often the only available tool for non-model species displaying circatidal, circalunar, or circannual rhythms. In the long run, not only knockdown but also knockout and gain of function studies need to be established and conducted to clarify the involvement of the circadian clock.

In the mangrove cricket *Apteronemobius asahinai*, knockdown experiments using RNAi against the core clock gene *period* and clock support the independence of the circatidal and circadian clocks (Takekata et al. 2012; Takekata et al. 2014b). The circadian modulation of the tidal locomotor rhythm was disrupted, while the tidal rhythm persisted (Figure 2b,b'). Neurophysiological ablation experiments were also conducted in the mangrove cricket to identify the pacemaker center of the circatidal clock. It is known that the optic lobe is the location of the circadian clock pacemaker in crickets (Shiga et al. 1999; Tomioka and Chiba 1992). Removal of the optic lobe in the mangrove cricket disrupted circadian but not circatidal rhythmicity, in-

dicating that this region is not important for circatidal time-keeping (Figure 2c) (Takekata et al. 2014a). When the pars intercerebralis (PI, another region relevant to the circadian clock (Sokolove and Loher 1975)) and/or surrounding regions were removed, the tidal rhythm was perturbed, i.e., more than half of the crickets became tidally arrhythmic. This suggests that the PI is important not only for the circadian clock but also for the circatidal clock (Figure 2c') (Takekata et al. 2018).

The possibility of global genomic and transcriptomic screens offered by next-generation sequencing methods can be key for getting hold of yet unknown components of circatidal and circalunar clocks. Unfortunately, such screens are still scarce for organisms with robust tidal rhythms. A pilot study (only one replicate per time point) analyzed RNA-sequencing (RNA-Seq) data over two tidal cycles sampled every 3 h under DD of the mangrove cricket's head (Satoh and Terai 2019). A total of 206 genes were found cycling with a circadian period. Arrhythmic expression of clock is in accordance with Takekata et al. (2012), in which mRNA of clock was found not cycling under LD in crickets with β -lactamase RNAi. However, the RNA-Seq data could not recapture the cycling pattern of *period* mRNA, probably because one replicate did not give enough power to detect all cycling transcripts. Nevertheless, more than 284 genes were found to cycle tidally in the cricket brain, with most of them peaking around either subjective high or low tide, indicating that their transcript abundance might be correlated with the tidal cycle. Moreover, genes involved in metabolic processes and molecular chaperons were upregulated at low tide (Satoh and Terai 2019). Because only half of all tidally cycling genes could be annotated, the authors speculated that some of them could potentially be noncoding RNAs. More sophisticated RNA-Seq experiments are required to understand tidal gene regulation in insects. As an important step on the way, an annotated genome has been recently published for the mangrove cricket (Satoh et al. 2021).

Tidal non-oscillatory timing mechanisms

Although a biological clock has been traditionally defined as an oscillatory system, it is worth noting that other ways of tidal time-keeping exist. Adults of an Arctic population of the marine midge *Clunio marinus* emerge every low tide during midsummer. The temperature increase associated with exposure of the substrate during low tide causes the start of an 11- to 13-h hourglass timer, which allows the midges to emerge during the subsequent low tide (Pflüger 1973). In the laboratory, no free-running emergence rhythm was observed, consistent with the idea that a non-oscillatory or highly dampened oscillatory system times emergence of the midge (Pflüger 1973).

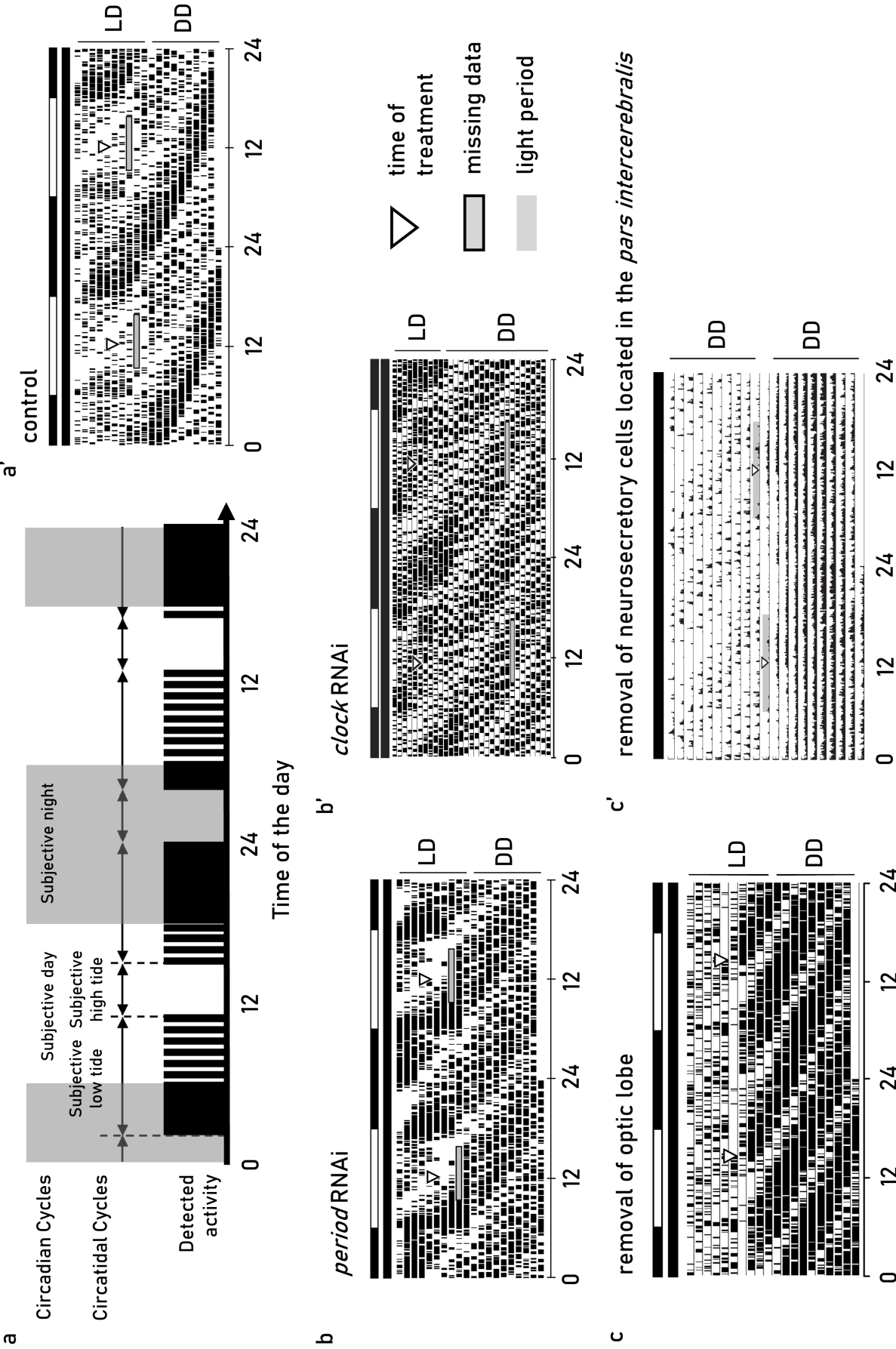


Figure 2: (a, a') The circadian clock modulates the circatidal activity rhythm of the mangrove cricket *Apteronomobius asahinai*. Crickets are active during subjective low tide, but this activity remains suppressed by the circadian clock during the subjective day. Schematic figure a modified from Goto and Takekata (2015), Copyright (2015), with permission from Elsevier. Experimental figure a' used with permission of The Royal Society from Takekata et al. (2012), Copyright (2012), permission conveyed through Copyright Clearance Center, Inc. (b, b') RNAi of the circadian clock gene period (b) and clock (b') abolishes the circadian modulation of the circatidal activity rhythm, indicating that both genes are essential for the circadian but not the circatidal clock. (b) used with permission of The Royal Society from Takekata et al. (2012), Copyright (2012), permission conveyed through Copyright Clearance Center, Inc. (b') Reprinted from Takekata et al. (2014b), Copyright (2014), with permission from Elsevier. (c) Removal of the optic lobe results in a circatidal activity rhythm without circadian modulation. Reprinted from Takekata et al. (2014a), Copyright (2014), with permission from SAGE. (c') More than half of the crickets display an arrhythmic activity pattern after removal of the neurosecretory cells in the pars intercerebralis (PI), indicating that this region is important for both circadian and circatidal time-keeping. Adapted from Takekata et al. (2018), with permission from Springer Nature: Copyright (2018)

Circalunar clocks in Insects

Lunar rhythms of insects in terrestrial habitats

Lunar periodicity of abundance in insects has been observed for various insect orders, including Ephemeroptera, Trichoptera, and Diptera (Hartland-Rowe 1955; Corbet 1958), as well as Lepidoptera and Coleoptera (Nowinszky et al. 2010). Lunar periodicity in flight behavior has also been observed for a few species of Hymenoptera, Heteroptera, Isoptera, and Orthoptera (Danthanarayana 1986). It should be noted, however, that some controversy among authors exists. Lunar phase and rhythmicity have been inconsistently reported for the same insect species (Danthanarayana 1986; Nowinszky 2004). Danthanarayana (1986) provides an extensive list of insect species for which flight activity has been correlated to lunar phases, noting that not only aquatic but also entirely terrestrial species show clear lunar rhythmicity in abundance. It is accepted that changes in insect abundance in relation to the lunar phase can be attributed to a reduction in the effectiveness of the trap (light of the trap essentially competes with moonlight) (Williams et al. 1956). However, some species truly vary in abundance due to changes in the lunar phase (Bowden and Church 1973). It has been hypothesized that such lunar periodicity in emergence may be an adaptation to wind-assisted dispersal (migration) (Danthanarayana 1976).

However, to date, there is no fully convincing evidence for an endogenous lunar time-keeping mechanism in a terrestrial insect. Ito et al. (1993) found that the catch size of adult Malayan black rice bugs *Scotinophara coarctata* was highest around full moon. However, when second instar nymphs were isolated and monitored in the laboratory, adult emergence was not synchronized with the lunar cycle. The mayfly *Povilla adusta* shows a lunar rhythm in emergence, peaking around full moon (Hartland-Rowe 1955). Although it was shown that this emer-

gence peak persists in DD and artificial LD (Hartland-Rowe 1955; Hartland-Rowe 1958), the very small number of individuals examined (<10) in a short time frame (<1 month) disqualifies the use of these experiments as evidence for an endogenous lunar free-run in the mayfly. The pit volume of the ant lion *Myrmeleon obscurus* is highest around full moon and was observed for two peaks subjected to daylight conditions as well as in DD in the laboratory (Youthed and Moran 1969). An interesting example of a semilunar rhythm comes from the cockroach *Periplaneta americana*, for which neurotransmitter-like substances in the central nervous system reach their highest levels around full and new moon (Rounds 1981).

Lunar rhythms of insects in marine habitats

Lunar rhythms are prevalent in the marine environment across the tree of life, and free-run criterion has been experimentally validated in many species, including algae (Bünning and Müller 1961), annelids (Hauenschild 1960; Franke 1985), mollusks (Yoshioka 1989), crustaceans (Saigusa 1980; Enright 1972), and fish (Hsiao and Meier 1992). Interesting cases of lunar rhythms can be found in marine midges of the genera *Clunio* and *Pontomyia* (Diptera, Chironomidae), which have extremely short adult life spans of only 1–2 h. Adult emergence is precisely timed to the lowest low tides around full and new moon by a circalunar clock. For both *Pontomyia* and *Clunio*, free-run, entrainment, and temperature compensation are well documented, making them attractive systems to study the circalunar clock (Table 1).

The case of Clunio

In marine nonbiting midges of the genus *Clunio*, development is tightly coupled with the lunar cycle (Neumann and Spindler 1991; Krüger and Neumann 1983). Chevrel (1894) was the first to notice that adults of *Clunio marinus* only swarm around full and new moon for mating. Enduring most of their life as larvae, adult emergence is restricted to only a few days around full and new moon, precisely matching the spring tides. *Clunio marinus* can be found along the entire rocky Atlantic coast of Europe from Portugal to Norway, wherever spring tides expose the seafloor and larval substrate becomes available to oviposition of the sticky egg clutches. To achieve this precise synchronization to the lunar cycle and the tides, a developmental waiting stage (CDA, circalunar developmental arrest) occurs during the early fourth instar in *Clunio* (Neumann and Spindler 1991). This developmental stage experiences a brief increase in ecdysone titer followed by temperature-compensated development (Neumann and Spindler 1991). It is the circa(semi)lunar clock that controls the developmental switching point in the fourth instar and ultimately synchronizes development of the marine midge with the lunar

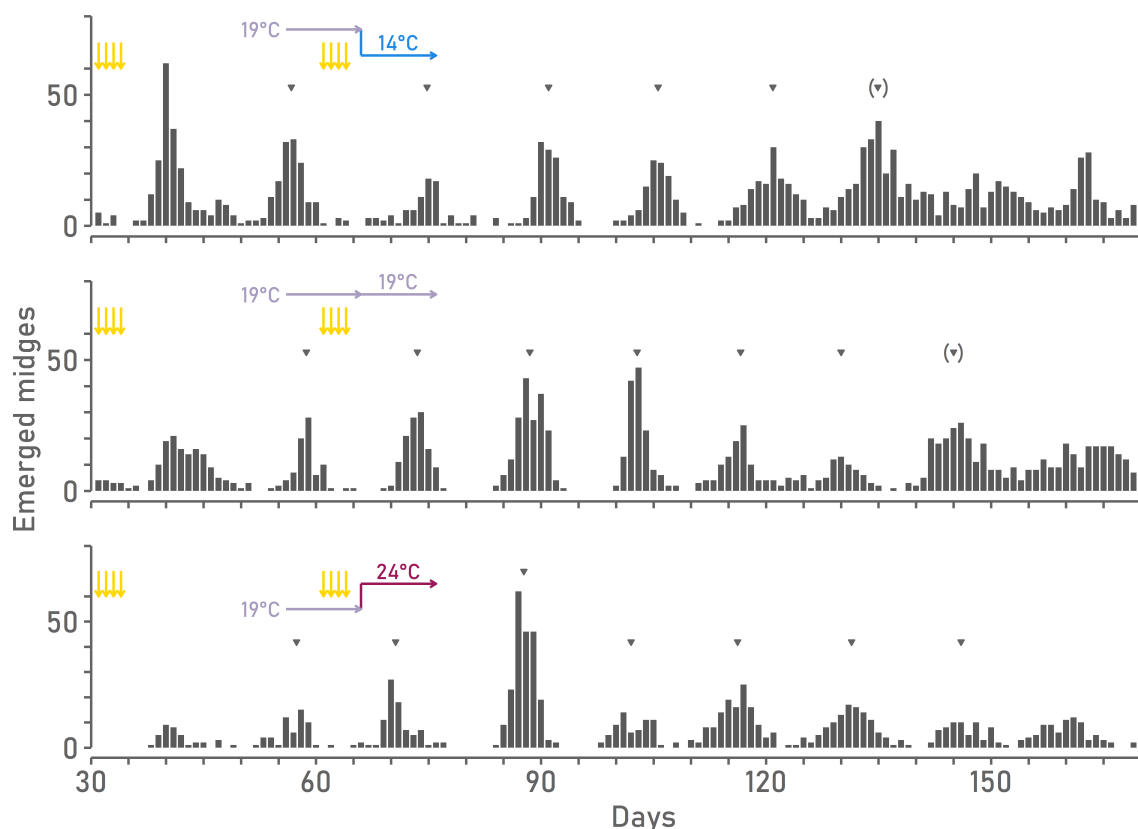


Figure 3: The free-running semilunar emergence rhythm of the marine midge *Clunio tsushimensis* is temperature-compensated. After entrainment by moonlight (four nights every 30 days; yellow arrows), the semilunar rhythm free-runs for several months with a period of approximately 15 days. From Kaiser and Neumann (2021) licensed under CC BY 4.0

cycle. During the 1960s, Dietrich Neumann established a culturing protocol for *Clunio* which allowed to systematically study endogenous clock criteria in the laboratory (Neumann 1966).

The semilunar emergence rhythm of Clunio is free-running and temperature-compensated

The (semi)lunar adult emergence rhythm of *Clunio* persists under constant conditions (LD, but no lunar time cues), verifying the endogenous nature of the rhythm (Neumann 1966; Neumann 1976). Free-running lunar and semilunar emergence rhythms also persist in DD (Neumann 1988) as well as in LL (Neumann 1976). Additionally, the free-running semilunar period has been found to be temperature-compensated between 14 °C and 24 °C in the Japanese midge *Clunio tsushimensis* (Figure 3) (Neumann 1988).

*Moonlight, tidal turbulence, and temperature cycles can entrain the circalunar clock of *Clunio**

When artificial moonlight (0.3 lux) was given for four consecutive nights every 30 days, a semilunar or lunar emergence rhythm can be evoked in southern populations of *Clunio marinus* (Neumann 1966) and in *Clunio tsushimensis* (Neumann 1988). Importantly, only light perceived during the subjective night synchronizes the phase of the lunar rhythm (Neumann 1995). It was shown that the circadian clock is important to set such a nocturnal light sensitivity window (Neumann 1995). As the rising and setting of the moon changes with the lunar cycle, moonlight is only available throughout the night around full moon. Moonlight is detected when the presence of moonlight during the night-time low tide and the nocturnal light sensitivity window coincide (Kaiser et al. 2011); hence, this is an instance of coincidence detection. At higher latitudes, the moon stays close to the horizon, and days become very long during summer, making it difficult to perceive “light at night” as a distinct cue (Neumann 1995). Correspondingly, the (semi)lunar rhythm of northern populations of *Clunio marinus* is often more precisely entrained by vibration (Neumann and Heimbach 1979) or temperature (Neumann and Heimbach 1984) given in a tidal pattern (Neumann 1968; Neumann 1978). Such tidal cycles of vibration or temperature can be used as a semilunar time cue because – just as the rising and setting of the moon – the 12.4-h tidal cycle advances every day by approximately 50 min relative to the 24-h LD. This unique phase relationship between LD and the tidal cycle recurring every 15 days serves as a semilunar cue. It is assumed that the detection of these tidal cues is also based on a coincidence detection mechanism, very similar to the detection of moonlight (Neumann 1995; Neumann and Heimbach 1985). A lunar phase response curve has been recently published for *Clunio tsushimensis* (Kaiser and Neumann 2021). Interestingly, there is a linear phase response without transient cycles, suggesting an immediate resetting of the circalunar clock. This implies a tight coupling of the overt rhythm to the pacemaker. *Clunio*'s lunar PRC is different from that of the marine annelid *Syllis prolifera*, which has a nonlinear phase response and characteristic transient cycles, suggesting that the two species may rely on different circalunar clock mechanisms (Franke 1986).

The case of *Pontomyia*

Midges of the genus *Pontomyia* inhabit tidal pools and shallow waters of the Western Pacific (Huang and Cheng 2011). The Taiwanese midge *Pontomyia oceana* has a well-studied semilunar rhythm of adult emergence (Soong et al. 1999). Midges emerge around full and new moon in the field. The semilunar rhythm has been shown to persist under LD as well as in DD in the laboratory for at least two peaks. Hence, it is free-running (Soong et al. 2011). The semilunar



Figure 4: Lunar rhythmicity, as well as phase and period are genetically determined in the marine midge *Clunio marinus* and differ between populations. *Clunio marinus* is therefore an attractive model to study the genetic architecture of lunar time-keeping using quantitative trait locus (QTL) mapping and genome-wide association screens (GWAS). (a) *Clunio marinus* inhabits the intertidal zone along the rocky Atlantic coast of Europe. Dots mark sampling locations from which lab strains were derived. Color coding denotes the emergence phenotype. (b) Under artificial moonlight (arrows, four nights of dim night light), populations emerge either around the new moon (NM) or the full moon (FM), both at the full and new moon (semilunar, SL) or arrhythmically (AR). (c) The phase of the lunar rhythm is genetically determined. When crossing a semilunar strain (Por) and a new moon strain (Jean), the F1 generation emerges in an intermediate phase and a semilunar period with unequal peaks. Based on Kaiser et al. (2011) licensed under CC BY 2.0

emergence rhythm is also temperature-compensated between 24 °C and 30 °C, although the Q₁₀ value of 0.86 is lower than the expected value of 1 (Soong et al. 2011).

The role of the circadian clock in circalunar time-keeping

Bünning and Müller proposed three hypotheses on how a lunar or semilunar period could be evoked by the circalunar clock (Bünning and Müller 1961). First, an independent semilunar or lunar oscillator of yet unknown molecular nature could run with a period of 15 or 30 days, respectively. Second, the beat hypothesis assumes superposition of a circadian rhythm (24 h) with either a circalunidian (24.8 h) or a circatidal rhythm (12.4 h). This would result in a beat wave with an amplitude changing in a lunar or semilunar rhythm. Third, there could be a counter mechanism based on either counting endogenous circadian oscillations or LD. Importantly, a circadian system is indispensable for the beat hypothesis as well as the counter hypothesis. In contrast, a circa(semi)lunar oscillator could hypothetically run independently of the circadian system. When exposing *Pontomyia* midges to different T-cycles between LD 11:11 and LD 14:14, the period of the semilunar emergence rhythm was found to change linearly with the change in LD, i.e., the free-running semilunar emergence rhythm has a shorter period under short T-cycles and a longer period under longer T-cycles (Soong and Chang 2012). However, the semilunar emergence rhythm was unchanged under multiples of 24 h (LD 3:3, LD 6:6, and LD 12:36). These experiments suggest that *Pontomyia* uses a mechanism based on counting endogenous circadian oscillations.

Persistence of rhythmic emergence of *Clunio* in experiments conducted in DD (Neumann 1988) and LD (Neumann 1976) has been taken as evidence that *Clunio*'s circalunar clock is an independent self-sustained oscillator. However, the existence of a counter mechanism based on endogenous circadian oscillations cannot be excluded. When reanalyzing the circadian phenotype under DD (Neumann 1988), a significant circadian period of approximately 25 h persisted for up to 17 days. This is long enough to explain the observed persistence of the lunar rhythm in DD, and thus, a counting mechanism might also exist in *C. marinus*. The fact that the marine midge *Pontomyia oceanica* was already found to count circadian cycles for lunar timekeeping (Soong and Chang 2012) allows one to speculate that marine midges in general could use a counter mechanism for lunar time-keeping. In an evolutionary context, this makes sense: Diptera evolved on land (Misof et al. 2014) and marine midges secondarily colonized the ocean. Many terrestrial insects possess a photoperiodic counting mechanism. It seems possible that marine insects coopted the photoperiodic counter for circalunar time-keeping, i.e., as a circalunar counter.

Molecular and genetic data on circalunar time-keeping in insects

To understand the mechanisms of lunar time-keeping, a combination of molecular and genetic methods is indispensable. Considering that *Clunio* and *Pontomyia* – the only insects with described robust lunar rhythms – are still non-model organisms, molecular methods often need to be established from scratch. Even annotated reference genomes cannot be taken for granted. Generating these resources will be necessary to unravel the pathways and genes underlying lunar rhythmicity. As a major asset, *Clunio marinus* has a highly complete and well-annotated reference genome on chromosomal scale (Kaiser et al. 2016). Additionally, there are many laboratory strains of *C. marinus* that differ in various timing phenotypes and can hence serve for comparative molecular analysis and genetic dissection of the circa(semi)lunar clockworks (Figure 4a). First, populations of *Clunio marinus* differ in whether they emerge only around full moon, only around new moon, or during both full and new moon (Figure 4b). The phase of emergence within the lunar cycle is genetically determined (Kaiser et al. 2011) (Figure 4c). Through crossing experiments and quantitative trait locus (QTL) mapping, it was found that the lunar emergence phase is determined by two major QTLs (Kaiser et al. 2016; Kaiser and Heckel 2012). Second, populations of *Clunio marinus* differ in whether they respond to moonlight or tidal cycles of vibration. These phenotypes offer a starting point to understand the entrainment pathways. The shielding pigment transparency changes over the lunar cycle in larval ocelli of *Clunio* (Fleissner et al. 2008; Falkenberg et al. 2013). Therefore, the ocelli might play a crucial role in the perception of moonlight. Finally, Arctic and Baltic populations of *C. marinus* have lost the lunar rhythm altogether, as the lunar rhythm has no adaptive value in the absence of tides in the Baltic Sea and detection of the lunar time cues is not possible during the polar day in the Arctic. Recent genome screens that compared the lunar-arrhythmic Baltic ecotype to the lunar-rhythmic Atlantic ecotype suggest that lunar arrhythmicity is primarily dependent on circadian clock genes as well as genes involved in nervous system development (Fuhrmann et al. 2023). These findings are in line with the involvement of the circadian clock in the perception of lunar zeitgebers (Neumann 1995; Neumann and Heimbach 1985; Neumann 1989), as well as a possible counting mechanism based on circadian clock cycles.

Conclusions

Despite their importance for numerous organisms, circalunar and circatidal clocks are still scarcely addressed in the field of chronobiology. Studying lunar and tidal rhythms provides us with the unique opportunity to understand time-keeping on multiple time scales as well as its evolution under multiple geophysical cycles. Tidal and lunar rhythms remain hard to

study because they are displayed by marine non-model organisms which are often difficult to culture in the laboratory and for which molecular methods usually still need to be established. However, insects might be the key to addressing such a task. They have adapted multiple times to the marine environment and are a very diverse group providing us with the opportunity to study potentially different tidal and lunar clock mechanisms. Knowledge about lunar and tidal rhythms is as old as the field of chronobiology, but advances in understanding them at the molecular level lag far behind those of the circadian clock. Thus, the fascinating world of lunar and tidal rhythms still offers ample opportunities for discovery.

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Chapter 2

2 Circalunar clocks – Old experiments for a new era

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Kaiser TS, & **Neumann J** (2021). Circalunar clocks — Old experiments for a new era. *BioEssays*, 43(8), 2100074. <https://doi.org/10.1002/bies.202100074>

Abstract

Circalunar clocks, which allow organisms to time reproduction to lunar phase, have been experimentally proven but are still not understood at the molecular level. Currently, a new generation of researchers with new tools is setting out to fill this gap. Our essay provides an overview of classic experiments on circalunar clocks. From the unpublished work of the late D. Neumann we also present a novel phase response curve for a circalunar clock. These experiments highlight avenues for molecular work and call for rigor in setting up and analyzing the logistically complex experiments on circalunar clocks. Re-evaluating classic experiments, we propose that (1) circalunar clocks in different organisms will have divergent mechanisms and physiological bases, (2) they may have properties very different from the well-studied circadian clocks and (3) they may have close mechanistic and molecular relations to seasonal rhythms and diapause.

Introduction

When in 2017 the Nobel Prize in Physiology or Medicine was awarded to Jeffrey C. Hall, Michael Rosbash and Michael W. Young for ‘discoveries of molecular mechanisms controlling the circadian rhythm’ (press release from the Nobel Assembly at Karolinska Institute), it underscored the growth and immense progress that the study of biological clocks has experienced in the past decades. However, the statement also highlighted how little we still know about the molecular clockworks underlying ‘non-circadian’ biological clocks. Circatidal, circalunar and circannual clocks are still ‘nature’s enigmatic clocks’. ^[1] Currently, a new generation of biologists is setting out to discover the molecular underpinnings of these clocks, supported by technological advances such as high throughput sequencing or genome editing. But while we depart for new ground, we should not abandon the many classic experiments that have been carried out on circatidal, circalunar and circannual clocks since the early 20th century. They not only remind us of the experimental rigor required to address complex phenomena such as long-term biological rhythms. Careful re-evaluation of this work also produces hypotheses along which we can design experiments to be conducted with today’s advanced technologies.

The little we know about the molecular basis of circalunar clocks has been comprehensively reviewed before. ^[2-4] In this review, we throw the spotlight on major mechanistic concepts which come from a large body of now classic, largely behavioral laboratory experiments. We underline the implications and prospects that these concepts and experiments have for guiding new molecular work. By integrating classic work, we cast hypotheses on the molecular basis of circalunar clockworks. Firstly, we suggest that circalunar clocks will not be all build along the same mechanistic principles, implying that they arose several times in the course of evolution. In contrast, circadian clocks likely also have multiple evolutionary origins, but seem to be built along similar principles. ^[5] Secondly, circalunar clocks may have some properties that differ markedly from circadian clocks. We illustrate this with an unpublished experiment by the late Dietrich Neumann, a pioneer of circalunar clock research. Thirdly, we propose that circalunar clocks may have close mechanistic and molecular relationships with seasonal timing mechanisms. In some species circalunar clocks may even be derived from a diapause-like mechanism. Formulation and evaluation of these hypotheses requires basic understanding of biological clock concepts and terms, which we develop in the following section.

Cycles, rhythms and clocks — Basic terminology and classification

Biological clocks are endogenous time-keeping mechanisms, which allow an organism to anticipate regular environmental cycles and adjust their physiology and behavior accordingly. There is an important distinction between *cycles*, *rhythms* and *biological clocks*. The term *cycle* is somewhat indiscriminately used for both environmental cycles, for example, night and day, as well as cycles in organisms' behavior or physiology, for example, the sleep-wake cycle. In contrast, the term *rhythm* is reserved for recurring behavioral or physiological events in organisms. Rhythms can be *direct responses* to environmental cycles. In this case they are exogenously driven and do not result from a biological clock. Alternatively, they can be the manifestation of an endogenous *biological clock*, allowing for anticipation of the exogenous cycle. This can be demonstrated in so-called *free-run experiments* (see below).

Biological clocks and rhythms are cyclic phenomena and borrowing from physics are described as *oscillators*, which among other characteristics have a specific *period* and *phase* (Figure 1A). The *period* of a clock or rhythm is the time that it takes to reach the same point in the oscillation again, for example, the time between one peak of activity and the next (Figure 1A). The *phase* of a clock or rhythm describes when the specific phenomenon happens relative to an external time frame (Figure 1A). For example, nocturnal and diurnal animals both have a rhythm with a 24-h period, but they differ in their phase of activity.

In nature there are four highly regular environmental cycles, which all result from the movements of earth and moon in relation to the sun: tides, night and day, the lunar cycle and the seasons (Figure 1B, C). For each of these cycles, corresponding biological clocks have evolved. As night and day and the seasons depend on the earth's movements related to the sun, the corresponding clocks can be considered 'sun-related' clocks (Figure 1D). The tides and the lunar cycle depend largely on the movement of the moon relative to earth. The corresponding clocks can therefore be considered 'moon-related' clocks (Figure 1D). Tides and the lunar cycle are closely intertwined. Most notably, the amplitude of the tides changes throughout the lunar cycle and is largest just after full moon and new moon during the so-called spring tides (Figure 1C). Organisms which require a specific tidal situation, such as spring tides, often have clocks that allow them to time half a lunar cycle—these are called circasemilunar clocks (Figure 1C, D).

The periods of circadian and circatidal clocks are in the range of hours (Figure 1D). These short-period clocks usually control an organism's 'daily business', for example its activity,

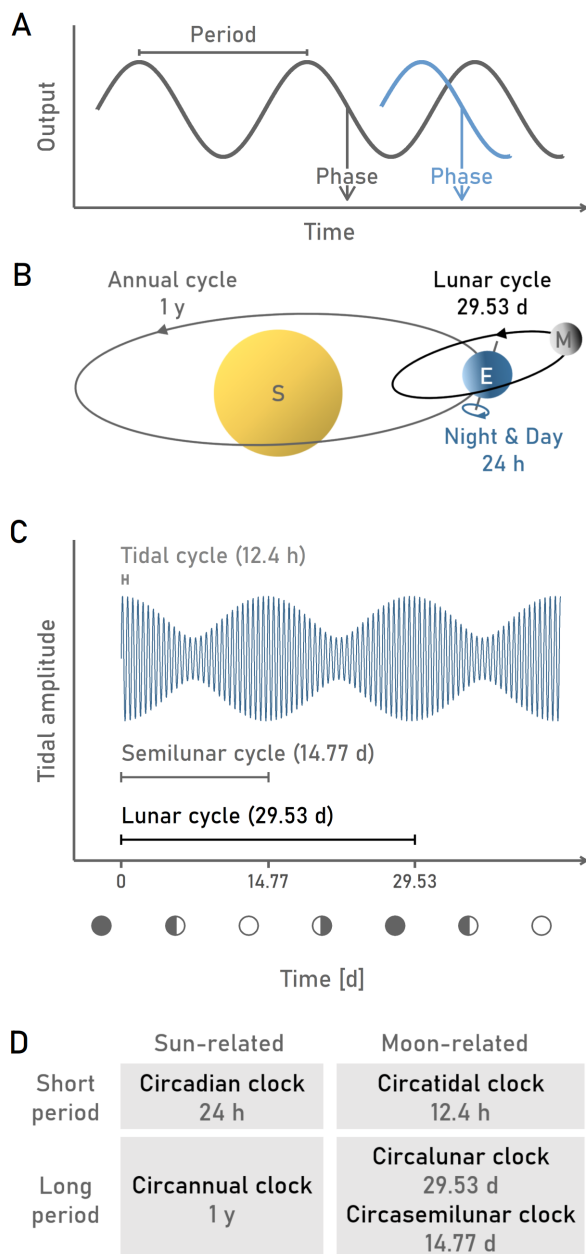


Figure 1: Features of biological rhythms and the tides. (A) Biological rhythms are described as oscillations. The time between two successive occurrences of the same state is the period of the rhythm. The time when a specific state occurs relative to an external time frame is the phase of the rhythm—for example, nocturnal versus diurnal activity. (B) All regular environmental cycles come about by the movements and positions of earth (E), moon (M) and sun (S). Revolution of the earth around the sun causes the annual cycle. Rotation of the earth around its own axis causes night and day. Revolution of the moon around the earth causes the lunar cycle. The tides come about by a complex interaction of the moon's and sun's gravitational influence on the earth's water bodies, combined with the earth's rotation around its axis. (C) The period of the tidal cycle is 12.4 h. The amplitude of the tides changes throughout the lunar cycle. It is particularly large twice a lunar cycle, namely during the spring tides just after full moon and new moon. Spring tides repeat in a semilunar cycle. (D) Biological clocks can be classified based on their period and their major geophysical influences.

feeding or UV protection. ^[6-8] Given that a circatidal period is very close to half a circadian period, there has been a long debate on whether or not these two clocks may rely on the same molecular clockwork, ^[9, 10] with a recent molecular twist that suggests both is partially true. ^[11] The long-period biological clocks—circalunar clocks and circannual clocks—usually control an organism's long-term investments, which are development, maturation and reproduction. Again, regulation of similar physiological processes may suggest the two clocks are related, an idea that we will discuss in more detail at the end of our essay.

In summary, biological clocks are experimentally confirmed endogenous time-keeping mechanisms. They time an organism's physiology and behavior to regular environmental cycles and

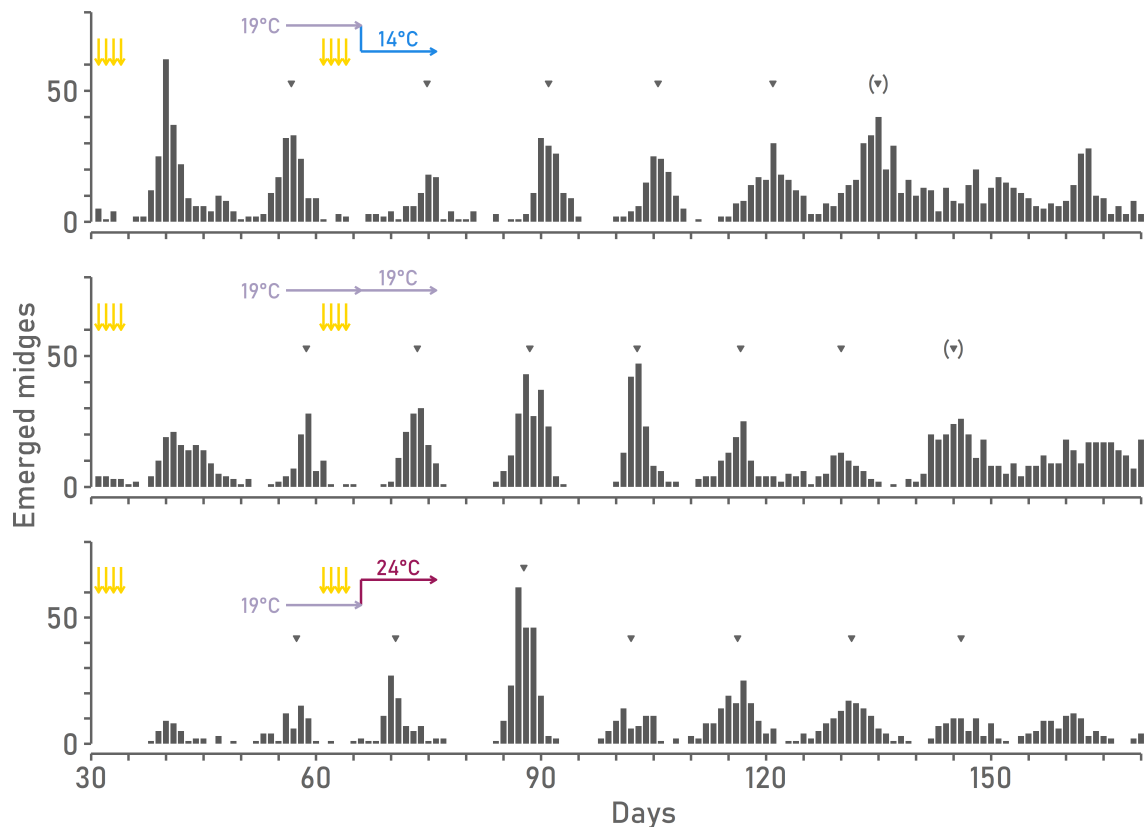


Figure 2: The circasemilunar rhythm of *Clunio tsushimensis*. After entrainment by moonlight (four nights every 30 days; yellow arrows) the semilunar rhythm persists (free-runs) for several months with a period of 15 days. Arrowheads indicate the median of individual emergence peaks. The free-running period is the same under 14°C, 19°C and 24°C, that is, the rhythm is temperature-compensated. Figure re-drawn according to ^[21] with permission from Springer Nature.

can be distinguished based on their underlying period. Circalunar and circasemilunar clocks have long periods and usually control reproduction.

Free-run experiments indicate that circalunar clocks are wide-spread in marine organisms

Proof of the endogenous nature of a biological rhythm comes from *free-run experiments*. In such an experiment the organism is kept under constant conditions without exogenous cycles. If the behavioral or physiological rhythm continues, it must be controlled by a biological clock. In a free-run experiment the rhythm must fulfill three major criteria, ^[12] which are illustrated in Figure 2.

First, the rhythm must free-run, that is, it must continue without any exogenous cues. Additionally, the *period of the free-running rhythm* (termed τ) must be close to the exogenous cycle that it is supposed to correspond to. The period of a free-running rhythm is rarely exactly the same as the period of the exogenous cycle and there is also individual variation within a

species. For this reason free-running rhythms are called ‘circa’-dian, ‘circa’-lunar and so on. The deviation in period from the exogenous cycle is crucial for the free-run experiment, as it rules out that the observed rhythm is controlled by exogenous cues that could not be excluded from the experiment (e.g., cycles in gravity or magnetic fields in the case of circalunar clocks).

Second, it must be possible to synchronize or reset the rhythm with specific environmental cues. Such specific cues are called *zeitgeber* (German; literally ‘time-giver’) and the process of synchronization is called *entrainment* of the clock. Entrainment ensures that the endogenous clock is always synchronized with the environmental cycle, despite the slight deviation of its free-running period.

Third, the free-running rhythm must be *temperature-compensated*, that is, the free-running period should be unaffected by ambient temperatures, in the range that is physiologically relevant to the organism. Usually, biochemical processes proceed 2–3 times faster if the temperature is raised by 10°C, which is expressed as them having Q10 values of 2 to 3. Biological clocks have Q10 values of close to 1 (compare Figure 2)—if they had not, they could not reliably tell time.

While lunar rhythms are reported for many organisms, ^[3, 13] most famously the palolo worm ^[14] or the pacific grunion, ^[15] experimental confirmation of an underlying circalunar clock is only available for a few (Table 1). First experimental proof of an endogenous circalunar clock came in 1960 for the marine annelid *Platynereis dumerilii* ^[16] (confirmed by ^[17]), followed by the brown alga *Dictyota dichotoma* ^[18] and the marine midge *Clunio marinus*. ^[19] To date endogenous circalunar or circasemilunar clocks have been demonstrated in about two dozen of species (Table 1). For most of these organisms only the first two criteria—free-running nature and entrainment—have been tested. The remarkable phenomenon of temperature compensation in these long-term developmental or reproductive cycles has only been demonstrated in the annelid *Syllis prolifera* (previously *Typosyllis prolifera*), ^[20] the midges *Clunio tsushimensis* ^[21] (see Figure 2) and *Pontomyia oceana*, ^[22] as well as the killifish *Fundulus grandis*. ^[23]

Circalunar clocks are found in a broad variety of taxa, most of which are marine (Table 1). The prevalence of circalunar clocks in marine organisms may be due to their two-fold adaptive value. Many intertidal organisms use circalunar clocks to time the delicate events of reproduction to ecologically suitable tides (see Figure 1C; e.g., in crabs ^[24, 25] or *Clunio* midges ^[26]). Additionally, circalunar clocks synchronize reproduction within a population, maximizing the chances of successful fertilization. This aspect is most important for broadcast spawners,

Organism	Nature of the rhythm	Free-run with appropriate period (Crit 1)	Entrainment (crit 2)	Temperature compensation (crit 3)
<i>Algae</i>				
<i>Dictyota dichotoma</i>	Semilunar rhythm of egg release	[18]	ML [18]	? [18]
<i>Fucus vesiculosus</i>	Semilunar rhythm of gamete release	? (period is ~7 days) [87]	–	–
<i>Ulva pseudocurvata</i>	Semilunar rhythm of gamete release	? (period is 7 days) [88]	ML? [88]	–
<i>Cnidarians</i>				
<i>Pocillopora damicornis</i>	Lunar rhythm of planula-larvae release	? [89]	ML? [89]	–
<i>Annelids</i>				
<i>Platynereis dumerilii</i>	Lunar rhythm in spawning	[16]	ML [16]	–
<i>Syllis prolifera</i> (previously <i>Typosyllis prolifera</i>)	Lunar rhythm in stolonization	[20,60]	ML [20]	[20]
<i>Molluscs</i>				
<i>Liolophura japonica</i> (previously <i>Acanthopleura japonica</i>)	Semilunar rhythm in spawning	[90]	Undetermined tidal factor [90]	–
<i>Haliotis asinina</i>	Semilunar rhythm in spawning	[91]	–	–
<i>Crustaceans</i>				
<i>Chiromantes haematocheir</i> (previously <i>Sesarma haematocheir</i>)	Semilunar rhythm of egg-laying and zoea-larvae release	[92]	ML [92]	–
<i>Leptuca pugilator</i> (previously <i>Uca pugilator</i>)	Semilunar rhythm of larval hatching	[93]	–	–
<i>Talitrus saltator</i>	Semilunar rhythm of locomotor activity and molt synchrony	[94]	–	–
<i>Eurydice pulchra</i>	Semilunar rhythm of swimming behavior	[95]	TTu [41,95,96]	–
<i>Excirolana chiltoni</i>	Semilunar swimming activity	[97]	–	–
<i>Scyphax ornatus</i>	Semilunar activity rhythm	[98]	–	–
<i>Bathyporeia pilosa</i>	Semilunar periodicity in swimming activity	[99]	–	–
<i>Gammarus chevreuxi</i>	Semilunar periodicity in swimming activity	[100]	–	–
<i>Insects</i>				
<i>Clunio marinus</i>	Lunar or semilunar rhythm in adult emergence	[19]	ML [19] TTu [47] TTe [40]	–
<i>Clunio tsushimensis</i>	Semilunar rhythm in adult emergence	[21]	ML [21]	[21]
<i>Pontomyia oceana</i>	Semilunar rhythm in adult emergence	[101]	ML [101]	[101]
<i>Myrmeleon obscurus</i>	Lunar rhythm in pit size	? [102]	–	–
<i>Fish</i>				
<i>Fundulus grandis</i>	Semilunar spawning activity	[23,103–104]	–	[23]
<i>Fundulus heteroclitus</i>	Semilunar spawning activity	[103]	–	–
<i>Oncorhynchus kisutch</i>	Semilunar growth rate and feeding rhythm	[105]	ML? (switch from LL to LD) [106]	–
<i>Oncorhynchus mykiss</i> (previously <i>Salmo gairdneri</i>)	Semilunar growth rate and feeding rhythm	[107–108]	–	–

Abbreviations: ML = moonlight, TTu = tidal turbulence, TTe = tidal temperature

Table 1: Organisms with experimental proof of a circalunar or circasemilunar clock

which release their gametes into the open water (e.g., corals ^[27] or annelids ^[28]). Particularly in tropical oceans, with no seasonality to synchronize reproduction, lunar rhythms are very common. In line with this idea, lunar reproductive rhythms have also been reported for a few tropical freshwater insects. ^[29, 30]

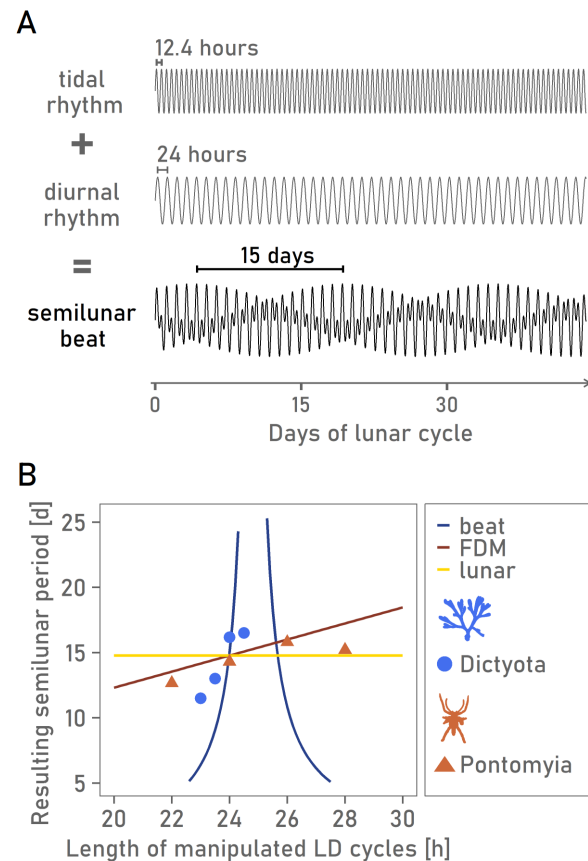
Experimental tests for the nature of the circalunar oscillator suggest different mechanisms and multiple evolutionary origins of the circalunar clock

Simplistically, biological clocks are described to consist of three parts. At the core of the clock is the oscillator, which maintains the free-running endogenous rhythm. The input pathways convey zeitgeber information to the oscillator for entrainment of the clock. The output pathways serve to orchestrate physiology and behavior according to the endogenous clock. In the following sections, we integrate what we know about these stylized compartments for the circalunar clock and we derive hypotheses and experiments for future research.

While the core molecular clockwork maintaining circalunar rhythms is still unknown, there are three main hypotheses for its mechanism. ^[18] The default assumption is that there is a circalunar oscillator with a 30-day period (or a 15-day period for circasemilunar clocks), based on a yet unknown physiological mechanism. Already in the 1960s Erwin Bünning developed the beat hypothesis as an alternative mechanism. ^[18, 31] Inspired by work on intertidal crabs, which show simultaneous circadian and circatidal rhythms (e.g., ^[32]), he proposed that lunar phase can be determined by superposition of a circadian and a circatidal clock. These two clocks would adopt the same phase relationship every 15 days and this ‘beat’ could serve to produce a circasemilunar rhythm (Figure 3A). By the same principle, superposition of a circadian with a circalunidian clock (‘lunar day clock’, 24.8 h) would produce a 30-day beat for a circalunar rhythm. As a third hypothesis, circalunar and circasemilunar clocks could rely on a counter mechanism counting 15 or 30 circadian cycles (also known as frequency demultiplication hypothesis, borrowing from circannual clock research ^[33–35]). In a narrow sense, the terms ‘circalunar clock’ and ‘circasemilunar clock’ can be understood to only refer to circalunar or circasemilunar oscillator mechanisms. However, throughout this essay we use the terms in a broad sense, including all mechanisms capable of producing free-running circalunar or circasemilunar rhythms.

Bünning also proposed an experiment to discriminate between the proposed clock mechanisms. ^[18, 31] Circadian clocks usually synchronize to light. Driven by exogenous light-dark (LD) cycles, they can adopt periods slightly longer or shorter than 24 h. Circatidal clocks usu-

Figure 3: Three possible mechanisms for circa(semi)lunar clocks. (A) The beat hypothesis suggests that a semilunar clock relies on superposition of a tidal rhythm with a diurnal rhythm, which produces a semilunar beat. (B) Alternatives to the beat hypothesis (blue) are an independent semilunar oscillator with a period of about 15 days (yellow) or the counting of days (frequency demultiplication hypothesis, FDH; red). Manipulation of daylength affects these mechanisms differently and is predicted to lead to discriminative changes in the period of the free-running semilunar rhythm (colored lines). Experimental evidence (colored dots) suggests that different mechanisms underlie the semilunar rhythms in the alga *Dictyota dichotoma*^[18, 36] and the midge *Pontomyia oceana*.^[35]



ally do not respond to light, as light is not a very good indicator of the tides. Thus, under a 23-h LD cycle, the circadian clock would assume a 23-h period whereas the circatidal clock would keep a 12.4-h period. This would change the beat dramatically in a non-linear way (Figure 3B). In contrast, a counter mechanism should change in a linear relationship to the change in the LD cycle, while a dedicated circalunar oscillator should be unaffected (Figure 3B).

In the brown alga *Dictyota dichotoma* the circasemilunar rhythm may rely on a beat phenomenon, but the effect is not as strong as expected (Figure 3B; ^[18, 36]). The crustacean *Scyphax ornatus* might also use a beat mechanism. ^[37] However, these experiments were performed under permanent entrainment by both LD cycles and tidal vibration. Under these conditions the two exogenous zeitgebers already produce a beat themselves, which the organism may respond to directly. For testing the beat hypothesis, one of the two clocks must free-run, to make sure the beat is produced endogenously. In contrast, the lunar rhythm in the marine midge *Pontomyia oceana* relies on counting circadian cycles (Figure 3B, ^[35]). Finally, evidence for a dedicated circalunar oscillator is available for the annelid *Platynereis dumerilii*. After early experiments with manipulated LD cycles, ^[16] chemical inhibition of the circadian clock left the circalunar rhythm unaffected. ^[17] As both the beat hypothesis and counting are ex-

Mechanism	Species	Publication
Circalunar oscillator	probably in <i>Platynereis dumerilii</i>	[16–17]
Beat hypothesis	<i>Dictyota dichotoma</i>	[18,36]
Counting/Frequency demultiplication hypothesis (FDH)	<i>Pontomyia oceana</i>	[35]

Table 2: Different mechanisms for circalunar and circasemilunar clocks and their experimental support

pected to involve the circadian clock, *P. dumerilii* is probably using an independent circalunar oscillator.

In summary, the circalunar and circasemilunar clocks of different organisms are likely based on different mechanisms (Table 2) and evolved multiple times independently. While circadian clocks rely largely on feedback loops in transcription, translation and protein modification,^[5] circalunar and circasemilunar clocks may rely on other physiological processes, which may more readily resonate with long periods. As these processes may well differ between organisms, we must be very cautious when transferring experimental insights on circalunar clocks from one organism to another.

Zeitgeber perception likely involves a circadian clock

Early experiments on the zeitgebers entraining circalunar clocks were performed in *Platynereis dumerilii*, showing that artificial moonlight—that is, dim light presented for few nights—can synchronize lunar spawning behavior.^[16] Ablation of the worm’s eyes did not alter periodic swarming behavior, suggesting that moonlight is perceived independent of the eyes.^[38] Entrainment of circalunar and circasemilunar clocks by moonlight was also demonstrated in various other taxa (Table 1), but the underlying photoreceptors and integration processes remain unclear. It was shown that circasemilunar rhythms can also be entrained by 12.4-h tidal cycles, which change over the 15-day spring-neap tide cycle. Semilunar rhythms in the marine midge *Clunio marinus* are synchronized by tidal turbulence cycles^[39] and tidal temperature cycles.^[40] Similarly, entrainment of semilunar rhythms in some crustaceans depends on wave agitation^[41] (Table 1). Such tidal zeitgebers might be of particular importance at high latitudes, where moonlight cannot be reliably perceived, or in locations where tides are very strong.^[42]

Mechanistic insights into how zeitgebers are perceived mainly come from the marine midges *Clunio marinus* and *Clunio tsushimensis*. In a very elegant experiment, ^[43] *C. tsushimensis* was subjected to constant darkness for four days once per lunar cycle. Constant darkness allowed for the presentation of artificial moonlight at different times of day. Moonlight only entrained the circalunar clock when it was presented around midnight, suggesting the moonlight receptor is insensitive during the day. Receptor sensitivity must be controlled by a circadian clock, as it is still regulated in constant darkness. ^[43] Muting the moonlight receptor during the day not only solves the problem of discriminating moonlight from sunlight, but also serves to obtain information on lunar phase. The daily timing of moonlight changes throughout the lunar cycle and in the intertidal zone can be further narrowed by the tides. ^[44] Therefore, a nocturnal light sensitivity window will only coincide with available moonlight during a few nights of the lunar cycle. Such a mechanism is called coincidence detection. It is closely related to the external coincidence model for photoperiodism, ^[45] which we will return to in the last section of this essay.

Coincidence detection is also related to the beat hypothesis. But importantly, the beat hypothesis describes the oscillator. The beat is fully internal to the organism, based on coincidence between two endogenous rhythms, producing an internal mechanism to measure time. In contrast, coincidence detection refers to synchronizing the oscillator with the outside world. It combines a single endogenous rhythm in sensitivity (usually a circadian rhythm) with an external environmental cycle. This constitutes a fundamental conceptual difference, which must be carefully discriminated in any experimental setup.

Coincidence detection is also at the heart of entraining circalunar clocks with tidal turbulence and temperature pulses, as was shown in *C. marinus*. ^[40, 46–48] In the intertidal zone, water turbulence, water temperature and many other factors fluctuate over the tidal cycle of 12.4 h. As an example, strong water turbulence, which corresponds to rising tide, ^[47] will due to its 12.4-h cycle gradually shift in daytime. Every 15 days it will occur at the same time of day. When a circadian clock tunes turbulence receptor sensitivity to a specific time of day, sensitivity and stimulus will only coincide every 15 days. In that way, coincidence detection can extract long-term information on lunar phase from two short-term cycles. Again, this mechanism can be experimentally assayed by manipulating the LD cycle. In *C. marinus* entrainment of the circalunar clock indeed changes with manipulations in the LD cycle ^[48] and is no longer possible when the length of the LD cycle is outside the ranges of entrainment of the circadian clock. ^[49] Experiments in the crustaceans *Eurydice pulchra* ^[41] and *Scyphax ornatus* ^[37]

suggest they may possess similar mechanisms. Finally, a tidal oscillation of melatonin receptor gene expression is reported for the puffer fish *Takifugu niphobles*.^[50] The combination of tidally modulated melatonin sensitivity with daily changes in melatonin levels might produce a coincidence detection system that entrains the semilunar rhythm.^[50]

In conclusion, all three well-studied lunar zeitgebers—moonlight, as well as mechanical and temperature stimuli associated with the tides—may have similar detection mechanisms, which involve a circadian clock. Notably, involvement of a circadian clock in entrainment (coincidence detection) does not imply that the circadian clock is also involved in the oscillator (beat hypothesis). These two aspects need to be carefully discriminated when designing behavioral or molecular experiments. Identification of cellular or molecular integration points between putative lunar zeitgeber receptors and the circadian clock seems a valuable target for future research.

A phase response curve for the circalunar clock of *C. marinus*

The way a clock entrains to a zeitgeber usually depends on when that zeitgeber is presented. At some points of a clock's oscillation, zeitgebers can strongly shift the clock, at other points they have basically no effect. This is captured in a so-called phase-response curve (PRC). The now classic PRC for circadian entrainment in the fly *Drosophila pseudoobscura*^[45] shows that light exposure during expected day barely shifts the clock, whereas light during expected darkness shifts the circadian clock to various extents. Light immediately resets the circadian oscillator,^[51–53] but the observed behavioral rhythm usually requires a few days to gradually follow the adjustment, showing so-called transient cycles.^[45]

A PRC for a circalunar clock has to date only been published for the annelid *Syllis prolifera*.^[54] Similar to circadian PRCs, it shows a non-linear response to the zeitgeber, as well as transient cycles (Figure 4A). The late Dietrich Neumann obtained unpublished phase response data for the circasemilunar clock of the midge *C. tsushimensis*, which we analyzed and present here and which reveals notable differences to the PRC of *Syllis prolifera* (Figure 4B, raw data in Figure S1). For interpreting these results, it is important to know that in *Clunio* the time point of adult emergence and thereby its circalunar rhythm is predetermined 15–20 days beforehand.^[55] As a consequence, all emergence peaks that occur within a window of 15–20 days after the resetting moonlight stimulus are unchanged in phase (Figure S1). Emergence peaks occurring after that are at once shifted to the new phase. There are no transient cycles (Figure 4B). The shift in adult emergence is proportional to the shift in the zeitgeber, so that there is a

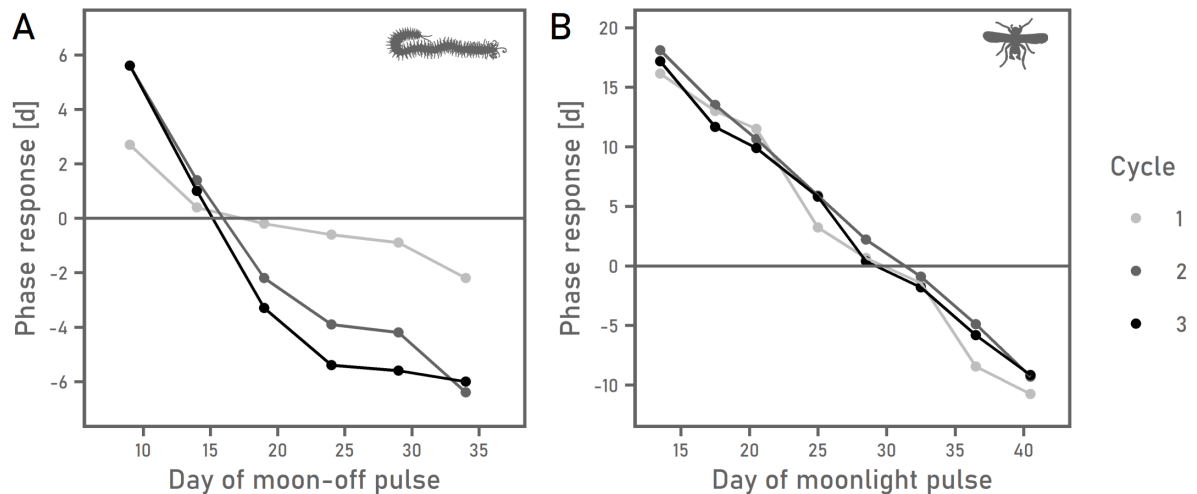


Figure 4: Phase response curves (PRCs) for moonlight entrainment of circalunar clocks. (A) The PRC for the circalunar clock of *Syllis prolifera* shows a non-linear response and transient cycles (data from [54]). (B) The PRC for the circasemilunar clock of *Clunio tsushimensis* shows a linear response and no transient cycles, consistent with an immediate resetting of the clock by moonlight (based on unpublished data by D. Neumann, Figure S1).

linear phase response (Figure 4B). This can be interpreted as an immediate resetting of the circalunar rhythm within the limits imposed by the required predetermination of development. The contrasting observations made in *Syllis prolifera* and *Clunio tsushimensis* underscore that circalunar clocks diverge in their properties and may follow different mechanistic principles.

Output pathways are complex to assess and likely divergent between species

As circalunar clocks control development and maturation, it is generally assumed that their outputs must at some point converge with the hormonal systems underlying these two processes, be it steroid hormones, gonadotropic hormones or other neuropeptides (for a recent overview see [4]). The classic demonstration of hormonal control of the lunar rhythm comes from the annelid *Platynereis dumerilii*. Annelids' large and soft bodies enable transplant experiments and made them model organisms for development and regeneration. [56] When in *P. dumerilii* the head is removed, the animal immediately undergoes maturation and reproduces irrespective of lunar phase. [57–59] If the head is re-implanted anywhere in the body, maturation is inhibited again. Similar results were obtained for *Syllis prolifera*. [60] Likely, the release of the brain hormone inhibiting maturation is controlled by a circalunar clock. Recently the inhibitory brain hormone of *P. dumerilii* was identified to be methylfarnesoate. [61] Interestingly, this molecule also acts as a juvenile hormone or juvenile hormone precursor in many crustaceans and insects. In the insect *C. marinus*, the levels of juvenile hormone's opponent ecdysone may fluctuate across the lunar cycle. [55]

These findings do not necessarily imply evolutionary conservation of direct circalunar clock outputs. The conserved hormonal pathways regulating development and reproduction can be far downstream of the circalunar clock, with circalunar clock outputs merely converging on them. Recent -omics studies on two main circalunar clock models, *C. marinus* and *P. dumerilii*, underline this call for caution. In *P. dumerilii*, a combined transcriptomic and proteomic approach, identified a number of lunar marker transcripts/proteins, which are differentially abundant during two different lunar phases. ^[62] But none of the corresponding genes is even present in the high-quality reference genome of *C. marinus*. ^[63] Thus, while both organisms likely share the use of juvenile hormone in suppressing development, other circalunar clock outputs will likely differ.

Nevertheless, global screening approaches such as transcriptomics, proteomics and metabolomics are undoubtedly promising to detect circalunar clock outputs. Transcriptomic changes have also been assessed over the lunar cycle in *Acropora* corals ^[64–66] and the limpet *Cellana rota*. ^[67] In many other organisms, expression of circadian clock genes has been studied at different lunar phases (reviewed in ^[2, 4]). But these experiments are usually very hard to interpret. From the varying experimental setups it is often not clear if differences in gene expression are driven by exogenous factors and at best serve as an input for setting the circalunar clock, if they are part of the oscillator according to the beat hypothesis or a counting mechanism, or if they are outputs of the circalunar clock (discussed in detail in ^[2, 4]). Precisely recording experimental and environmental conditions would be a first step to reach better comparability. ^[2] But for pinpointing circalunar clock effects we have to face the challenge of combining the experimental sophistication of classic behavioral studies with the standards set for circadian -omics experiments. ^[68]

First, experiments should be performed under free-running conditions, so that influences from exogenous zeitgeber cycles can be excluded. This is already difficult, as many organisms with circalunar clocks cannot be bred in the laboratory. Second, several lunar cycles should be assessed, which makes experiments very long and prone to technical failure. Third, a sufficiently large number of time points per cycle need to be assessed. If a large number of cycles are recorded, theoretically two time points per cycle are sufficient to detect rhythmicity. ^[69] However, in circadian time-series over few cycles the standard procedure of collecting six time points per cycle was already found to be statistically underpowered and 12 time points are recommended. ^[68] Fourth, as there likely are interactions between circadian and circalunar clocks at some level, ideally for every circalunar time point several circadian time points

are tested. Including the required biological replicates and possible experimental treatments quickly leads to enormous numbers of required samples. Fifth, in order to disentangle the usually overwhelming signals of development and maturation, which likely are merely circalunar clock outputs, from any signals of a circalunar oscillator, experiments would require testing a long-lived life stage that has a running circalunar clock, but does not (yet) develop and mature. For most species such a life stage might not exist.

In summary, circalunar control of maturation and development can be expected to generally involve some hormonal regulation, but possibly far downstream the circalunar clock. Circalunar clock outputs are accessible to current-day large-scale screening techniques, but these experiments are logistically very challenging. Circalunar clock outputs are not necessarily conserved across species, again arguing for caution when trying to transfer findings across taxa.

A circalunar clock derived from photoperiodic diapause induction?

Photoperiodism is the ability of organisms to anticipate seasonal changes by using daylength as a predictive cue. ^[70] Photoperiodism is not a circannual clock mechanism, as there is no yearly endogenous oscillation involved, but photoperiodism can serve to synchronize a circannual clock. The Bünning hypothesis, ^[71] later refined by Pittendrigh and Minis to become the external coincidence model, ^[45] suggests that detection of daylength depends on light sensitivity in the early or late night, regulated by a circadian clock. If light is perceived during that sensitivity window, days must be long. If there is no light, days must be short. As outlined above, this very mechanism has been experimentally shown to be at the core of moonlight entrainment of the circasemilunar clock of *Clunio tsushimensis*. ^[72] It has also been discussed for lunar phase detection in other organisms. ^[2, 73] In line with these findings, moonlight has been found to affect photoperiodism or seasonal features of circadian timing in beans, ^[74] hamsters, ^[75] fruit flies ^[76] and other insects. ^[77, 78] Thus, photoperiodism may not only be a way of entraining circannual clocks, ^[79, 80] but there are also hints that photoperiodism and circannual clocks might share mechanistic principles with circalunar clocks.

In marine midges of the genus *Clunio* there are additional lines of evidence, which combined make a compelling case that the circalunar clock of these insects might be directly derived from a photoperiodic diapause mechanism. Firstly, moonlight detection follows an external coincidence model as discussed above. Secondly, moonlight cues must be integrated over several nights, ^[19] just like most insects must integrate daylength cues over many days before

entering diapause. ^[81] Thirdly, the larval development of *Clunio* is only synchronized with lunar phase in the last larval instar. Synchronization is achieved via a short arrest in development, reminiscent of a very short diapause. ^[55] Even more, this circalunar developmental arrest happens at exactly the same larval stage as the regular photoperiodic diapause, that northern European *Clunio* populations undergo in winter. ^[82, 83]

Insects evolved on land, where seasonal rhythms are prominent, and the closest relatives of *Clunio* are terrestrial midges ^[84] with no known lunar rhythms. We must assume that *Clunio* secondarily evolved a circalunar clock only after colonizing the sea. It seems highly plausible that an existing mechanism such as photoperiodic diapause induction was re-used and complemented with some form of resonance with the lunar cycle. Involvement of the circadian clock in photoperiodism is well established, ^[85] and therefore circadian clocks might also play a role in such a derived circalunar time-keeping mechanism. In contrast, taxa with an ancient marine origin, for example, algae or annelids, likely evolved their circalunar clocks earlier and on a different molecular background. This underscores the idea of multiple evolutionary origins of circalunar clocks and various circalunar clock mechanisms, as outlined above. But as life originated in the sea, circalunar clocks can be expected to be ancient timing mechanisms. Could it be that photoperiodism or circannual rhythms are derived from an ancient circalunar clock? Could *Clunio*'s circalunar clock actually be reusing a photoperiodic timer which was reusing a circalunar clock? Such a scenario would well befit the tinkering nature of evolution. ^[86]

Conclusions

Organisms are adapted to regular environmental cycles through various time-keeping mechanisms (Figure 1). While the circadian clock is well understood, the molecular mechanisms underlying circalunar clocks and other long-term rhythms remain elusive. Classic behavioral experiments suggest that circalunar rhythms evolved several times based on distinct mechanisms, not all of which require a circalunar oscillator. Some circalunar time-keeping mechanisms may re-use existing molecular pathways involved in photoperiodic diapause or circannual, circadian and circatidal clocks. The next decade of investigations into the molecular basis of circalunar clocks, might reveal close mechanistic and evolutionary links between all of these time-keeping mechanisms.

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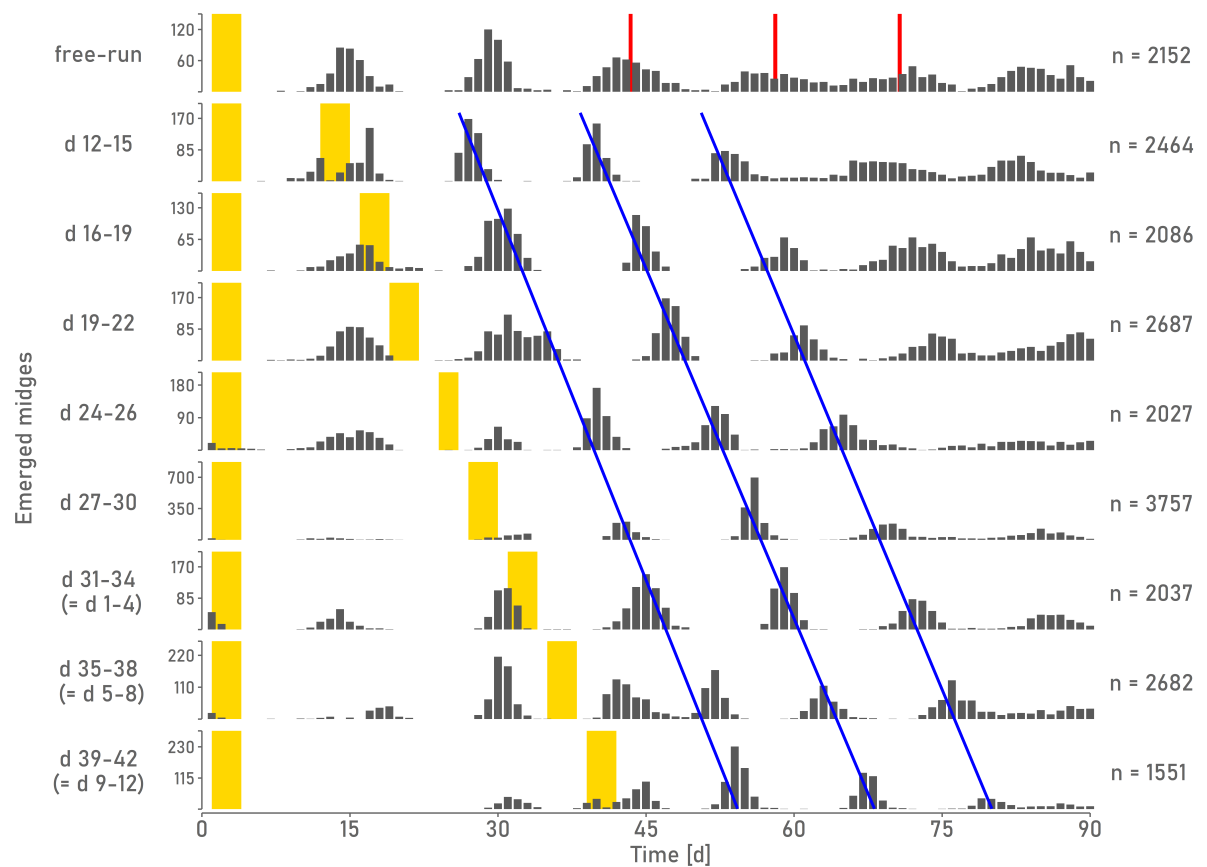
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Supplementary Material



Supplementary Figure 1: Phase response of the circasemilunar clock of *Clunio tsushimensis*. In unpublished experiments by D. Neumann, moonlight (yellow shading) was administered during 4-day windows at different points of a 30-day artificial moonlight cycle. Notably, a 15-day semilunar rhythm is assayed over a 30-day zeitgeber cycle. Red line indicates the reference emergence peak under free-run for calculation of phase shift. Blue line marks the corresponding response peaks resulting from lunar entrainment. The time point of adult emergence is predetermined 15-20 days earlier ^[55], resulting in a corresponding refractory period for entrainment.

Chapter 3

3 The free-running circasemilunar period is determined by counting circadian clock cycles in the marine midge *Clunio marinus*

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This part is submitted in JBR (Journal of Biological Rhythms) and currently under revision (ID JBR-23-0138).

Neumann J, Rajendra D & Kaiser TS (in revision). The free-running circasemilunar period is determined by counting circadian clock cycles in the marine midge *Clunio marinus*.

Abstract

Semilunar rhythms are found in numerous marine organisms, but the molecular mechanism and functional principles of endogenous circasemilunar clocks remain elusive. Here, we explore the connection between the free-running circasemilunar clock and the circadian clock in the marine midge *Clunio marinus* with three different chronobiological assays. First, we found that the free-running circasemilunar period of the adult emergence rhythm in *C. marinus* changes linearly with diel T-cycle length, supporting a day-counting mechanism. Second, under LD 6:6, periods of circasemilunar and circadian emergence were comparable to those under LD 12:12, indicating that the circasemilunar counter in *C. marinus* relies on endogenous circadian oscillations rather than external T-cycles. Finally, when desynchronizing the circadian clock with constant light, the free-running circasemilunar emergence rhythm disappeared as well, suggesting that it requires a synchronized circadian clock. These results oppose the long-held view that *C. marinus*' free-running circasemilunar clock operates inde-

pendently of the circadian clock. In a broader evolutionary context, our results strengthen the idea that the circasemilunar clocks of dipterous insects are based on different functional principles compared to the circasemilunar or circalunar clocks of marine annelids and algae. These divergent clock principles may indicate multiple evolutionary origins of circasemilunar and circalunar clocks.

Introduction

Life on Earth evolved under recurring environmental cycles, namely the tides ($T=12.4\text{h}$), night and day ($T=24\text{h}$), the lunar cycle ($T=29.5\text{d}$), and the seasons ($T=1\text{y}$). In anticipation of the predictable conditions that accompany these geophysical cycles, organisms have evolved corresponding endogenous time-keeping mechanisms – so-called biological clocks. In marine habitats, especially in the intertidal zone, organisms are constantly exposed to dramatic changes in the environment governed by the tides (e.g. salt concentration, UV radiation, temperature). Adaptation to those is reflected in the prevalence of circatidal clocks [1]. Additionally, the tidal amplitude is modulated over the semilunar cycle ($T=14.77\text{d}$), reaching its maximum during the spring tides around full moon and new moon. To synchronize reproduction, or behavioral rhythms such as locomotion and feeding to the most favorable tidal amplitude, organisms use circasemilunar and circalunar clocks [2]. The endogenous nature of circa(semi)lunar clocks has been validated across the eukaryote branch of the tree of life, in algae, cnidarians, annelids, mollusks, crustaceans, insects and fish [3]. However, their molecular mechanisms remain unknown [4]. Three hypotheses for the functional principle of circasemilunar clocks have been put forward [5]: a molecular oscillator with an intrinsic 15-day period (oscillator hypothesis), a day-counting mechanism (counter hypothesis) or superposition of a circadian and a circatidal rhythm, which results in a 15-day beat phenomenon (beat hypothesis). These hypotheses can be tested with specific chronobiological experiments, without the need for molecular readouts or tools. However, out of more than 20 species with a known circasemilunar clock, the functional principle has only been examined for four of them [3]. We argue that in order to understand the molecular mechanism, it is important to know the operating principle of the clock, because it guides the search for molecular readouts and candidate genes.

One experiment to distinguish between the beat, counter and oscillator hypotheses was proposed by [5] in the early 1960s. By experimentally manipulating the diel T-cycle length the free-running circasemilunar period is expected to change differently depending on the operating principle of the clock (Figure 1a). The free-running period of a 15-day circasemilunar oscillator that operates independently of the circadian system should be unaffected by changes

in T-cycle length. In contrast, a semilunar day-counting mechanism must go through a fixed number of "steps" and, therefore, depends on either the endogenous circadian period or the length of the exogenous environmental T-cycle. This is based on the assumption that the circadian period can be modified by the superimposed diel T-cycle because the circadian clock can maintain a fixed phase angle with the T-cycle within its ranges of entrainment [6]. Hence, the semilunar period is expected to change linearly with T-cycle length under the counter hypothesis. Finally, the pattern of the semilunar beat wave depends on the underlying circadian and circatidal periods. Because of the non-linear interaction of the two, slight changes in the T-cycle are expected to drastically change the semilunar period (Figure 1a).

We investigated the functional principle of the circasemilunar clock in the marine midge *Clunio marinus* (Diptera, Chironomidae). *C. marinus* inhabits the intertidal zones along the rocky shores of Europe [7]. A circasemilunar clock controls the semilunar adult emergence rhythm, which restricts the emergence and reproduction of the short-lived (few hours) adults to the spring tides, when eggs can be deposited on the exposed larval substrates [8]. The circadian clock both governs the time of the day when adults emerge, and sets a nocturnal sensitivity window for the perception of moonlight cues [9]. The beat hypothesis has been convincingly ruled out for *C. marinus* by the above proposed experiment conducted for T-cycles of 23.2, 24 and 24.4 hours [10]. However, these slight differences in T-cycle length are not informative for distinguishing between the counter and oscillator hypothesis. The oscillator hypothesis has been favored for the semilunar emergence rhythm of *Clunio*, based on the observation that the rhythm continues in constant light (LL) [10] and constant darkness (DD) [8], ruling out the counting of light-dark (LD) cycles [9]. However, these experiments do not exclude the possibility that *Clunio* counts endogenous circadian oscillations. Such a mechanism has been suggested for the marine midge *Pontomyia oceana* [11].

We monitored the free-running circasemilunar adult emergence rhythm of *C. marinus* under different diel T-cycle lengths ranging from 22h to 30h to capture the change in the circasemilunar period predicted by the counter hypothesis. We found that the circasemilunar period of *C. marinus*' emergence rhythm changes linearly with T-cycle length, supporting the counter hypothesis. In order to further distinguish a counting mechanism rooted in the circadian system from one based on external T-cycles, we exposed *C. marinus* to a T-cycle of 12h (LD 6:6). The circadian clock is an oscillatory system that, when exposed to an environmental cycle having a submultiple of its period, can entrain to every second or third (or fourth, etc.) cycle, essentially maintaining a 24h period. This phenomenon is known as frequency demultiplica-

tion [12]. The period of a circasemilunar counter based on endogenous circadian oscillations should be unaffected by diel T-cycles being a submultiple of 24h. In contrast, if the circasemilunar counter takes information directly from the environmental T-cycle, its period is expected to halve under LD 6:6 compared to LD 12:12. In our experiment, both the circasemilunar and the circadian period remained unchanged under LD 6:6 compared to LD 12:12, suggesting the circasemilunar rhythm in *C. marinus* is based on counting circadian clock cycles. Finally, in the experiment conducted under LL by [10], the emergence rhythm was monitored for less than 30 days. Hence, this is not enough time to capture the truly free-running circasemilunar period. To test if *C. marinus* requires a synchronized circadian clock for maintaining the semilunar emergence rhythm, we repeated the experiment and exposed midges to constant light (LL) for more than 80 days and monitored both the circadian and circasemilunar emergence rhythm. The results confirm that *C. marinus*' day-counter mechanism is based on counting endogenous circadian cycles.

Methods

Laboratory cultures & circasemilunar entrainment

A laboratory strain of *C. marinus* collected from Helgoland, Germany was reared at 18°C in plastic containers filled with natural North Sea water and deionized water (1:1) according to a standard protocol [7]. Animals were supplied twice a week with diatoms (*Phaeodactylum tricornutum*; strain UTEX646) and every two weeks with powdered nettles (*Urtica sp.*). The circasemilunar emergence rhythm was entrained by a 24h LD-cycle and mechanical vibrations given in a tidal pattern of T=12.4h using an unbalanced motor (50 Hz, ~ 30 dB above background noise, motor alternated between 6.2h on and 6.2h off) [13, 14]. Air temperature and light intensity were measured and recorded every 10 minutes using Onset HOB0 data loggers (UA-002-08) for all experiments.

T-cycle experiment

Three independent experimental runs were conducted in 2020, 2021 and 2023 with the Helgoland laboratory strain of *C. marinus*. The experiment consisted of two phases: During the *entrainment phase*, animals were reared from eggs under LD 12:12 and tidal vibration. During the subsequent *experimental phase*, the semilunar entrainment was concluded (no tidal vibration) and larvae were exposed to T-cycles of differing lengths: T=22h (LD 11:11), T=24h (LD 12:12), T=26h (LD 13:13), T=28h (LD 14:14) or T=30h (LD 15:15). Changing the diel T-cycle length while the

circasemilunar clock was free-running ensured that the introduced changes in T-cycle length did not re-entrain the circasemilunar clock. Larvae were between two and eight weeks old when entering the experimental phase. The mixed age structure was necessary because the circadian and circasemilunar phenotypes can only be observed at the population level. Emergence occurs only once during the insects' lifetime. However, multiple emergence peaks are required to calculate the circasemilunar period reliably. Hence, different age cohorts of the population are required. In total, five time series replicates were conducted for T=24h, three for T=22h & T=26 and one for T=28h & T=30h (Supplementary Figure S1, S2, S3, S4, S5). For the T=24h treatment in 2021, a phase shift in the emergence rhythm occurred (Figure S1b, gray arrow), which coincided with heavy vibrations due to construction works at our institute. We excluded the data point from downstream analysis. However, statistical conclusions remain the same when including the replicate. We included an additional time series of T=24h, which was the control to the constant light experiment for downstream analysis (see section "Constant light (LL) experiment", Figure 5, labeled 2023b in Figure 1). The circasemilunar phenotype is the number of emerged adults counted once during the first half of the light phase of each T-cycle treatment. Numbers were assigned to the 24h-day on which the preceding light-dark transition occurred because this is when midges emerged [7, 15]. For T-cycle lengths longer than 24h, this results in information ostensibly missing on some 24h-days. Circasemilunar emergence data can be found in Supplementary File S1 (2020, 2021, 2023a, 2023b). The time series for calculation of the circasemilunar period started on day 31 of the experimental phase (explanation see section "Time series analysis") and lasted until day 79 (49 days in total) for all experimental runs. The emergence phenotype was monitored for more than 79 days for 2021 and 2023 and continued to be rhythmic, but the time series were cut for comparison purposes.

LD 6:6 experiment

Both the circadian and circasemilunar phenotypes were assessed under LD 6:6 in *C. marinus*. Similar to the T-cycle experiment, the setup consists of two phases. For assessment of the circadian phenotype, *C. marinus* was entrained with tidal vibration under LD 16:8. Subsequently, four to six-week old animals were transferred into either LD 6:6 or LD 12:12 and semilunar free-run (no tidal vibration). Emergence was recorded hourly using a custom-made fraction collector [16]. The middle of the night was defined as zeitgeber time (ZT) 0, and data was adjusted accordingly. The transfer into the new light regime was defined as day 0 of the time series. Time series analysis started with the first full day in LD 6:6 (day 1). The length of the circadian time series was 91 days (hourly data, 2,184 bins). Circadian emergence data is provided

in Supplementary File S2 (2022). For assessment of the circasemilunar phenotype, a population of mixed age was entrained under LD 12:12 and tidal vibration as previously described. Two replicates were conducted in 2021 and 2023. Semilunar time series started with day 31 of the experimental phase and lasted until day 79 for *C. marinus* (49 days in total). Circasemilunar emergence data is provided in Supplementary File S1 (2021, 2023a).

Constant light (LL) experiment

C. marinus midges were entrained with tidal vibration and LD 12:12. Larvae were between four to six weeks when the circasemilunar clock was released into free-run conditions (no tidal vibration) and LD 12:12. During the experimental phase, midges were released either into constant light (LL) (~ 1000 lux) or kept in LD 12:12 as a control and the hourly number of emerged midges was monitored as described above. The transfer into constant light was defined as day 0 of the time series. Circadian time series analysis started with the first full day in LL (day 1). The length of the circadian time series was 83 days (hourly data, 1,992 bins). Circadian emergence data is provided in Supplementary File S2 (2023). The semilunar time series started on day 31 after being released into circasemilunar free-run and lasted until day 79 (49 days in total). Circasemilunar emergence data is provided in Supplementary File S1 (2023b).

Time series analysis

The first 30 days after midges were released into circasemilunar free-run were excluded for calculation of the circasemilunar period because eclosion behavior in *C. marinus* is developmentally predetermined up to 20 days in advance [17], i.e. midges emerging during the first 20 days in circasemilunar free-run were already developmentally bound to emerge at a specific time and were therefore not affected by the treatment. We removed the first two full semilunar cycles (30 days) in order not to include partial emergence peaks. All statistical analyses were conducted in R version 4.3.0 [18].

To visualize periodic patterns in the circasemilunar time series of the T-cycle experiment, auto-correlation was calculated using the `acf()` function with `na.action=na.pass`. Periods were visualized using Lomb-Scargle (LS) periodograms [19], calculated with `lsp()` and `alpha=0.05`, `type="period"`, `from=3`, `to=35` for circasemilunar time series and `alpha=0.05`, `type="period"`, `from=15`, `to=40` for circadian time series. Circasemilunar and circadian periods for downstream analyses were calculated using the `meta2d()` function combining the Jonckheere-Terpstra-Kendall

(JTK) and Lomb-Scargle (LS) algorithms as part of the MetaCycle package v 1.2.0 [20]. The integration of multiple algorithms allows a more robust estimation of the period compared to using a single one. The ARSER algorithm available in MetaCycle cannot be used for time series containing missing data and was therefore excluded. For circasemilunar periods, parameters were set to *minper=3*, *maxper=20*, *outIntegration="onlyIntegration"*, *cycMethod=c("LS", "JTK")*, *combinePvalue="fisher"*. The boundaries were chosen because they cover all periods expected under the different hypotheses. All circasemilunar time series were analysed together (n=16, day 31 to 79 of circasemilunar free-run) and can be found in Supplementary File S1. For circadian time series, parameters were set to *minper=20*, *maxper=28*, *cycMethod=c("LS", "JTK")*, *combinePvalue="fisher"*. For time series longer than 1,000 time points, JTK could not calculate a period. Hence, for the circadian time series of the constant light and LD 6:6 experiments, we only report the periods calculated by the Lomb-Scargle periodogram. All circadian time series can be found in Supplementary File 2. Circadian time series of the same length were analysed together. Integrated periods from *meta2d()* were considered significant when the p-value of *meta2d* was < 0.05 (corrected by the Benjamini-Hochberg procedure).

Model selection and fit

To test which of the hypotheses fit the observed circasemilunar periods best, we fit three different models, corresponding to the counter, oscillator and beat hypotheses and compared the residuals, Akaike information criterion for small sample sizes (AICc) and AICc weight for each of them. All calculations are based on the observed circasemilunar periods (τ) computed with *meta2d()*. The residuals were retrieved with *res()* and calculated as:

$$\tau_{observed} - \tau_{expected} \quad (1)$$

Under the counter hypothesis, we expect a linear relationship between T-cycle length (T) and circasemilunar period (τ). Hence, we fit a linear model for T-cycle lengths of 22h, 24h and 26h in R with:

$$lm(\tau \sim T) \quad (2)$$

Under the oscillator hypothesis, the circasemilunar period is *not* expected to change with T-cycle length. Hence, the slope of the regression line is 0 and the expected circasemilunar period is calculated as the mean of all observed periods. We therefore encoded the oscillator

model in R as:

$$lm(\tau \sim 1) \quad (3)$$

The expectation for the beat hypothesis of an entrained, semilunar 15-day period in hours is calculated as:

$$\tau_{beat} = \frac{T * k}{2 * |T - k|} \quad (4)$$

whereby T is T-cycle length in hours and k is the duration of the lunar day in hours ($k = 24.8h$). The semilunar period in days is calculated as:

$$\frac{\tau_{beat}}{24} \quad (5)$$

However, $k = 24.8h$ holds only true for the entrained semilunar period. The free-running circasemilunar period deviates from 15 days. Hence, the formula needed adjustment. We assume that the tidal parameter k takes a different, but fixed value. This is based on the assumption that a potential circatidal oscillator free-runs unaffected by T-cycles while the circadian period is modified by them. The best fitting value for k was calculated using `nls()` with `start=24.8`. Expected periods under the beat hypothesis were then calculated with the estimate $k = 25$ (estimate=25.0147, $p < 2e-16$) for obtaining the residuals. The beat model was encoded in R as:

$$beat.model <- function(T, k)((T * k / (2 * abs(T - k))) / 24) \quad (6)$$

$$nls(\tau \sim beat.model(T, k)) \quad (7)$$

To compare the fit of the models, the second-order Akaike information criterion (AICc) was calculated for all three models with `AICc()` of the MuMIn package version 1.47.5 [21]. The weights of the AICc values were calculated with `akaie.weights()` of the qPCR package version 1.4-1 [22].

Results

The free-running circasemilunar period increases linearly with T-cycle length in *C. marinus*

To distinguish between oscillator, counter and beat hypotheses as outlined in the introduction, we monitored the semilunar emergence rhythm of *C. marinus* in circasemilunar free-run and diel T-cycles of 22, 24, 26, 28 and 30 hours (Figure 1a). Circasemilunar rhythmicity was clearly

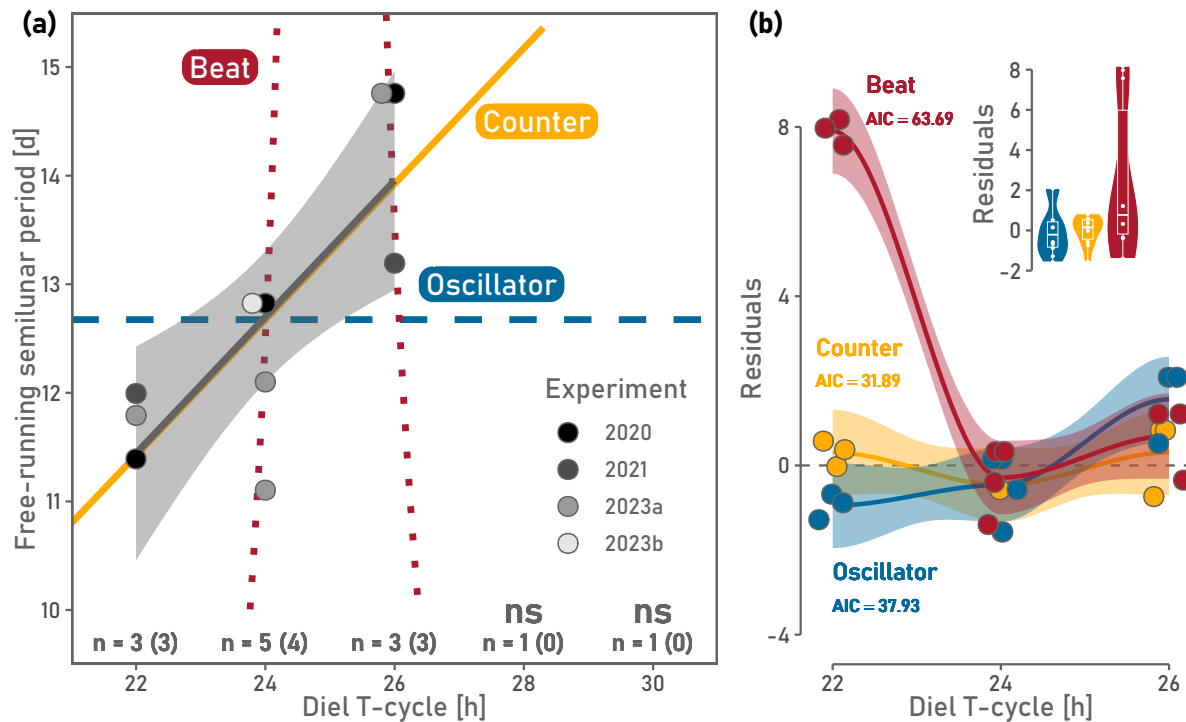


Figure 1: Observed circasemilunar periods of *C. marinus*' emergence rhythm under different T-cycle lengths are compared with periods as predicted by three hypotheses (counter=yellow, oscillator=blue and beat=red). (a) Period of the free-running circasemilunar emergence rhythm increased linearly with T-cycle length between 22 and 26 hours. Emergence is arrhythmic under T-cycles of 28 and 30 hours. *n* represents the number of replicates conducted. The number of significant values used for downstream analyses is depicted in brackets. The linear model fitted to the observed periods is depicted as the gray solid line ($ci=0.95$). Overlapping periods observed under T-cycles of 24 and 26 hours were jittered along the x-axis for better visualization. Replicates are labeled according to the year they were conducted. 2023b refers to the control of the constant light experiment. (b) The residuals (difference between observed and expected periods) are plotted for all three hypotheses. The AICc value was lowest for the counter hypothesis, indicating that it is the best fitting model. All residuals were jittered along the x-axis for visualization of overlapping data.

evident for T-cycles between 22h and 26h and only for those significant free-running periods were obtained with meta2d() (Figures S1, S2, S3). Thus, we assessed the different hypotheses based on the time points in Figure 1a.

The expectation for the circasemilunar period under the beat hypothesis does not match the observed periods. Under a T-cycle length of 22h, residuals for the beat hypothesis deviate vastly (Figure 1b). In line with that, the AICc value and residual standard error was the highest for the beat model compared to the counter and oscillator hypotheses (Table 1). The beat hypothesis can, therefore, be ruled out, which is in line with previous experiments [10]. This leaves the counter and the oscillator hypotheses for *C. marinus*. When plotting the residuals for the oscillator and counter hypotheses (Figure 1b), values are close to 0 for both. However, the residual standard error was lower for the counter model (0.81) compared to the oscillator (1.28), indicating that the former model fits better than the latter (Table 1). A higher AICc value and lower Akaike weight also supports the counter as the best-fitting model (Table 1; 31.89 vs

37.93). The linear model corresponding to the counter hypothesis fits the data significantly (estimate=0.628, $p=5.21e-03$, gray solid line, ci=0.95). The slope of the linear model (0.628) is very close to the expected slope under the counter hypothesis (0.625). These results favor the counter hypothesis over the oscillator hypothesis.

Hypothesis	Model	Residual standard error	AICc	A. weight
Counter	$lm(\tau \sim T)$	0.808	31.89	9.54e-01
Oscillator	$lm(\tau \sim 1)$	1.277	37.93	4.65e-02
Beat	$nls(\tau \sim beat.model(T, k))$	4.629	63.69	1.19e-07

Table 1: Selected models and parameters for the beat, counter and oscillator hypothesis. The smallest residual standard error, the lowest AICc value and the highest Akaike weight indicate that the counter hypothesis fits the observed circasemilunar periods better than the oscillator and beat hypothesis.

There is a second line of evidence supporting the counter hypothesis: If the molecular machinery underlying *C. marinus*' emergence rhythm was driven by an oscillator with an intrinsic 15-day period, it should be unaffected by changes in T-cycle length in its free-running state. We would, therefore, expect a detectable (and constant) circasemilunar period also under very long T-cycles of 28 or 30 hours. However, there was no significant rhythmicity under T-cycles of 28h and 30h, speaking against the oscillator hypothesis. Under the counter hypothesis, if the counter relied on external T-cycles, the circasemilunar period is also expected to persist regardless of T-cycle length. However, if the counter was based on information coming from the endogenous circadian system, essentially the circadian period, long T-cycles could lie outside the circadian clock's limits of entrainment, and therefore the circasemilunar rhythm would be expected to disappear. Arrhythmicity observed under T=28h and T=30h, therefore, supports the counting of endogenous circadian oscillations rather than the counting of external T-cycles. To further test the assumption of counting endogenous circadian periods, we performed experiments under a T-cycle of 12 hours (LD 6:6), as well as under constant light (LL).

The circadian clock frequency demultiplies under LD 6:6 and the circasemilunar period remains close to 15 days

To further investigate if *C. marinus*' circasemilunar counter is indeed counting circadian periods rather than T-cycles, we examined the circasemilunar and circadian emergence rhythm under a T-cycle of 12h (LD 6:6). Because T-cycle length of 12h is a submultiple of 24h, the circadian clock may frequency demultiply i.e. may continue to run with a period close to 24 hours. Indeed, the circadian emergence rhythm of *C. marinus* had the same period under

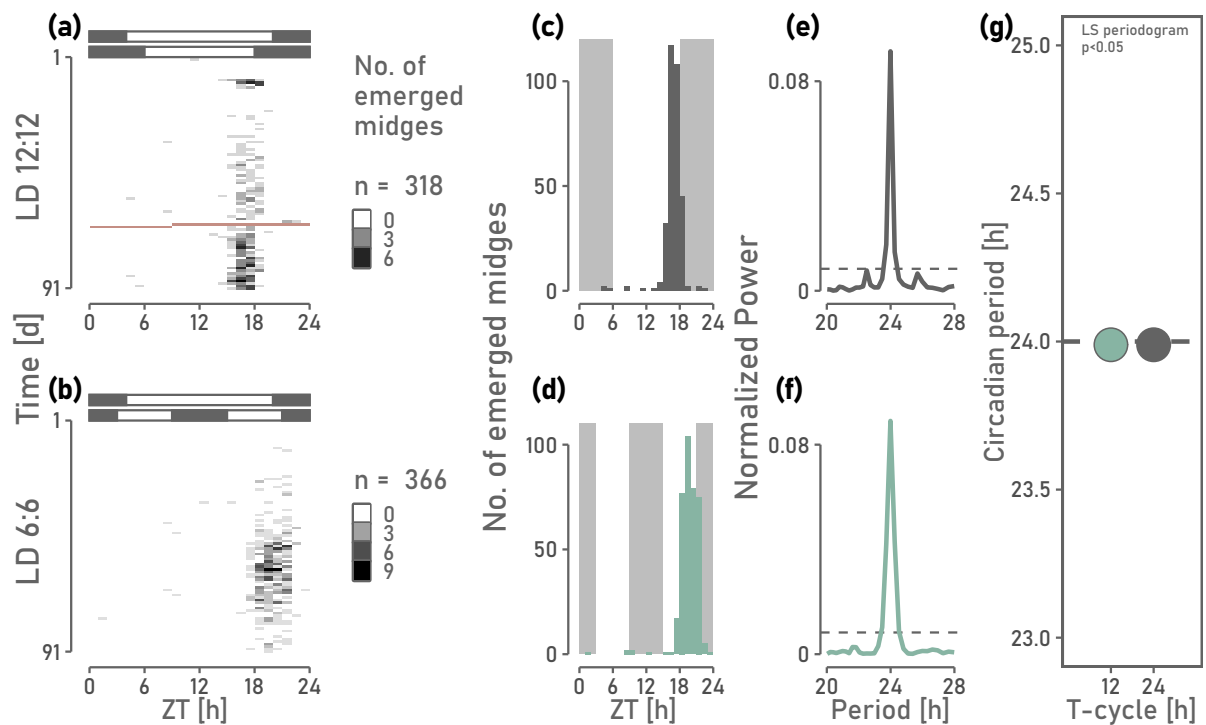


Figure 2: Circadian emergence phenotype of *C. marinus* under LD 12:12 and LD 6:6 after entrainment in LD 16:8. The experiment was conducted in 2022. (a,b) Hourly number of emerged midges for 91 days in (a) LD 12:12 and (b) LD 6:6. Missing values (NA) are displayed in red. (c-d) Numbers of emerged midges were summed per hour over all days. (c) Emergence occurred right before the light-dark transition in LD 12:12. (d) Emergence occurred only at one of the two light-dark transitions in LD 6:6, indicating frequency demultiplication of the circadian clock. (e-g) Lomb-Scargle periodogram analysis reveals a 24h period of emergence under both LD 12:12 and LD 6:6.

LD 12:12 ($\tau_{\text{circadian}}=23.99\text{h}$, $p=1.24\text{e-}45$) and LD 6:6 ($\tau_{\text{circadian}}=23.99\text{h}$, $p=6.61\text{e-}45$) (Figure 2e-g), supporting frequency demultiplication of the circadian oscillator.

If the counter receives information directly from the external T-cycle, the circasemilunar period is expected to halve under LD 6:6 compared to LD 12:12. However, if the circasemilunar counter relies on counting circadian periods, the circasemilunar period is expected to stay close to 15-days under LD 6:6. This based on the assumption that the circadian period remains 24 hours through frequency demultiplication. Indeed, the circasemilunar period departed not more than 2 days from the entrained 15-day period (Figure 3), indicating that the circasemilunar counter in *C. marinus* relies on counting the circadian period, i.e. endogenous information from the circadian system.

The circadian and circasemilunar clocks desynchronize in constant light

The preceding experiments suggest that *C. marinus* relies on a circasemilunar counter mechanism, which relies on counting circadian clock periods. Therefore, desynchronizing the circadian clock should result in arrhythmic semilunar emergence. Constant light can lead to arrhythmicity of behavioral rhythms governed by the circadian clock of mammals [23] and

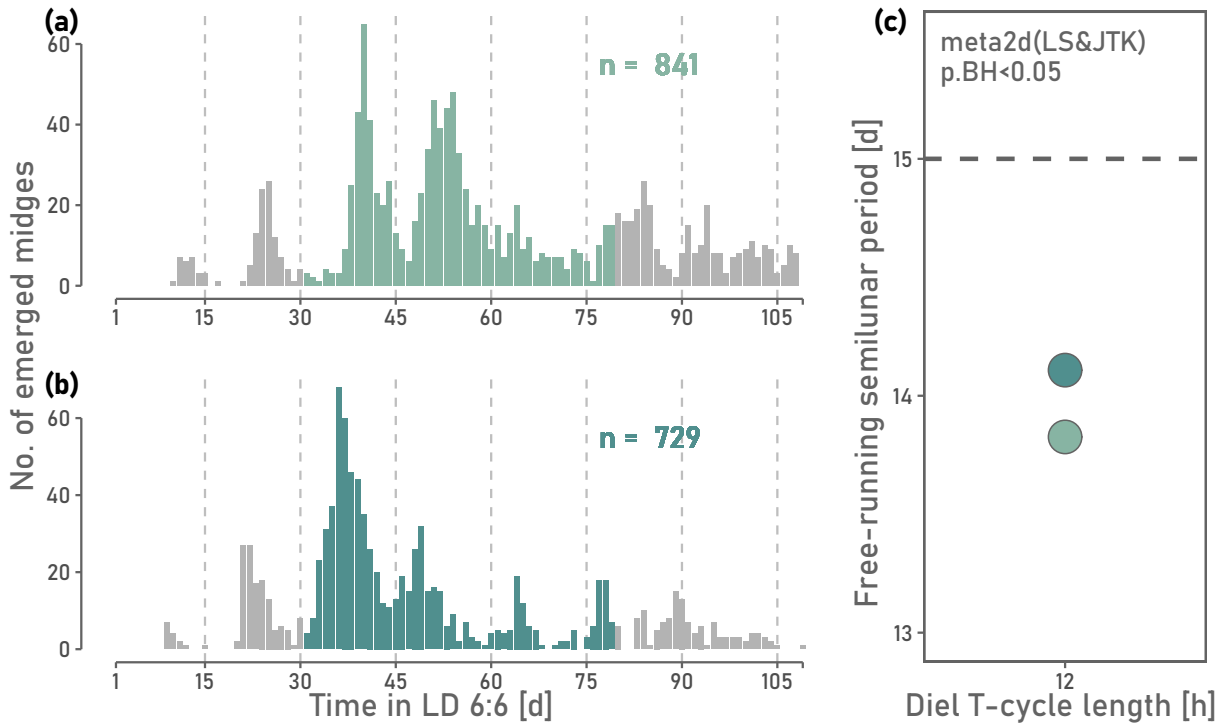


Figure 3: (a,b) Daily number of emerged midges for *C. marinus* under LD 6:6 and circasemilunar free-run after circasemilunar entrainment in LD 12:12. Experiments were conducted in 2021 (a) and 2023 (b). For time series analysis, only the colored bars were considered. (c) The circasemilunar period remains close to 15 days (dashed line) under LD 6:6 for both replicates (a): $\tau_{\text{circasemilunar}} = 13.83\text{d}$, $p = 6.34\text{e-}04$; (b): $\tau_{\text{circasemilunar}} = 14.11\text{d}$, $p = 4.26\text{e-}02$.

Drosophila [24], given sufficient intensity [25]. Therefore, we exposed *C. marinus* to constant, high-intensity light and monitored the circadian and circasemilunar emergence rhythm. Circadian emergence (Figure 4) was rhythmic under LD 12:12 ($\tau_{\text{circadian}} = 23.99\text{h}$, $p = 2.90\text{e-}27$) but became arrhythmic under constant light ($p = 7.76\text{e-}01$). Hence, the constant light conditions were sufficient to desynchronize the circadian clock. If a synchronized circadian clock is essential for the circasemilunar clock, the circasemilunar emergence rhythm is expected to disappear in constant light as well. Indeed, emergence was rhythmic in LD 12:12 ($\tau_{\text{circasemilunar}} = 12.82\text{d}$, $p = 2.26\text{e-}02$) but arrhythmic in LL ($p = 8.81\text{e-}01$), indicating that a synchronized circadian clock is required for maintaining a free-running circasemilunar rhythm.

Discussion

C. marinus counts endogenous circadian oscillations for semilunar time-keeping

This study characterizes the functional principle of the circasemilunar clock in the marine midge *C. marinus* by distinguishing between the beat, counter and oscillator hypotheses. The free-running period of circasemilunar emergence increased linearly with T-cycle length between 22 and 26 hours, following predictions by the counter hypothesis. Arrhythmicity under T-cycles of 28 and 30 hours indicates that the counter relies on the circadian system. Con-

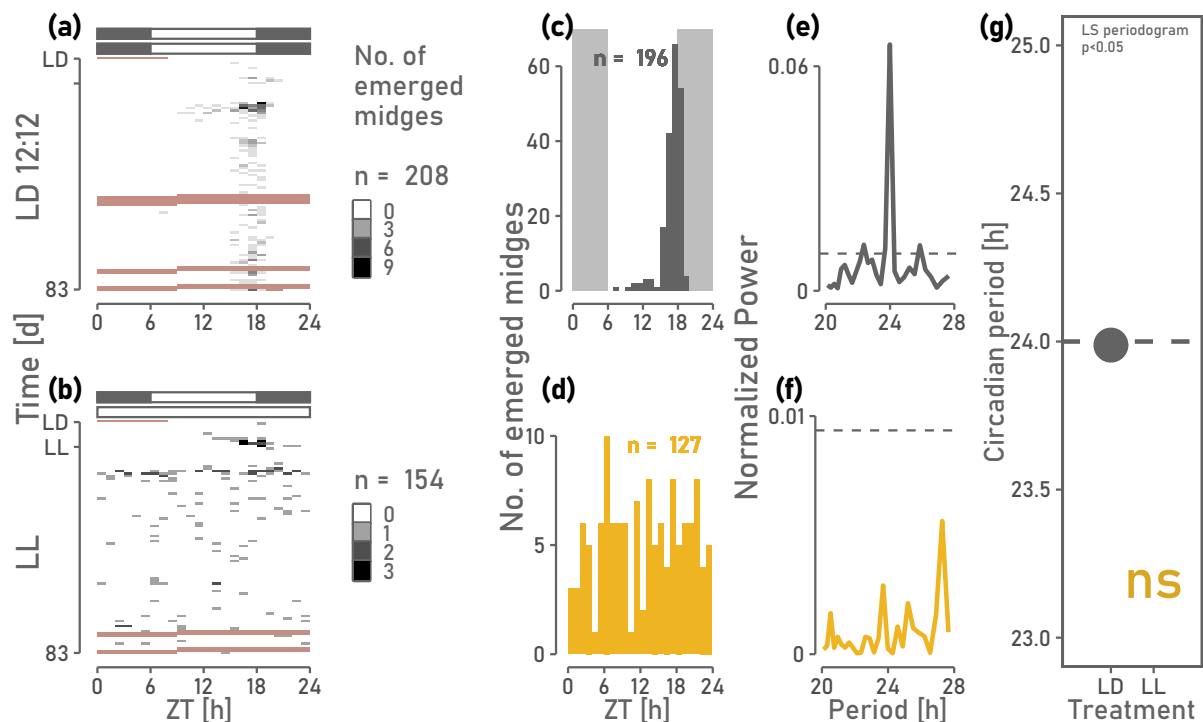


Figure 4: Circadian emergence of *C. marinus* in LD 12:12 and under constant light. (a) Under LD 12:12 midges emerge synchronized in phase around ZT 18, at the light-dark transition. (b) In constant light, midges emerge throughout the 24h-day. (c,d) Number of emerged midges per ZT was summed up over all 83 cycles to visualize the phase of emergence. (e-g) Lomb-Scargle periodogram analysis identifies a significant 24h-period under LD 12:12 (e) but not under LL (f), suggesting that *C. marinus*' circadian clock desynchronizes in constant light.

sistently, under LD 6:6 the circasemilunar period was comparable to the one under LD 12:12, suggesting that the circadian clock frequency demultiplies and that the counter depends on the circadian period rather than external T-cycles. In constant light, the circadian *and* circasemilunar periods fade, suggesting that a synchronized circadian clock is essential for circasemilunar time keeping in the marine midge.

Until this study, the free-running circasemilunar emergence rhythm of *Clunio* was postulated to run independently of the circadian system [8], favoring an independent circasemilunar oscillator as the functional principle of the circasemilunar clock. This assumption was based on two experiments in which the circasemilunar emergence rhythm continued for *C. marinus* in LL [10] and *C. tsushimensis* in DD [8]. The author concluded that external LD-cycles are not required for maintenance of the circasemilunar emergence rhythm and, therefore, reject the counter hypothesis. However, these experiments do not rule out that the circasemilunar counter could instead be based on endogenous circadian oscillations. If this was the case, we would have to assume that the circadian clock remains synchronized during LL and DD. For the experiment under LL, we were able to retrieve the circadian time series data from Neumann's unpublished data. We found that in his experiment indeed the free-running circadian period

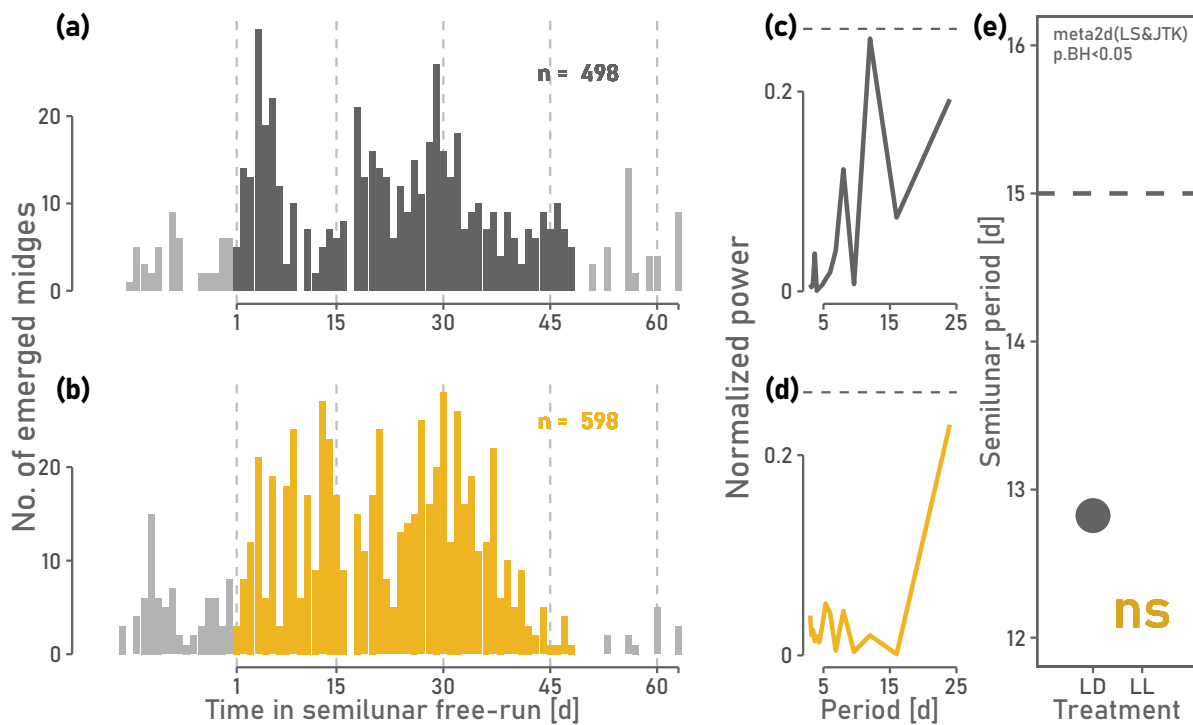


Figure 5: The circasemilunar emergence pattern of *C. marinus* becomes arrhythmic under constant light. (a) Number of emerged midges per day under LD 12:12. (b) Number of emerged midges under LL (c,d) Lomb-Scargle periodogram analysis reveals that free-running circasemilunar emergence was rhythmic under LD 12:12 but not in constant light. (e) meta2d() detects a significant circasemilunar period only under LD 12:12.

remained rhythmic during the first semilunar emergence peak ($\tau_{\text{circadian}}=25.10\text{h}$, $p=0$, day in LL 1-7, hourly data, 168 bins) as well as the second semilunar emergence peak ($\tau_{\text{circadian}}=26.83\text{h}$, $p=2.33\text{e-}06$, day in LL 13-19, hourly data, 168 bins) (Figure S6). Circadian emergence data is provided in Supplementary File S2 (1976). This LL experiment, therefore, does not rule out the counter hypothesis and does not contradict our results. Interestingly, in our own LL experiment, the circadian clock was already desynchronized after a few days, and so was the circasemilunar rhythm. We assume that this might be due to very high light intensities in our experiment.

In agreement with our study, the beat hypothesis has previously been rejected for *C. marinus* in two experiments. First, under shorter and longer T-cycles (LD 11.6:11.6 and LD 12.2:12.2) the circasemilunar period did not vary according to expectations of the beat hypothesis [10]. Second, a phase shift in the LD cycle did not affect the circasemilunar period [26]. In the latter experiment, the circasemilunar clock of *C. marinus* (Helgoland strain) was entrained by the superposition of an LD cycle with a tidal vibration pattern. Both cycles resume a unique recurring phase relationship every 15 days. Then, the tidal vibration pattern was concluded (circasemilunar free-run) and a phase shift in the LD-cycle was introduced. It was assumed that if *Clunio's* circasemilunar clock is explained by the beat hypothesis, the now free-running

circatidal oscillator would in combination with the shift in the LD-cycle, result in a phase shift of the circasemilunar emergence rhythm. However, the emergence rhythm continued without a phase shift, rejecting a beat mechanism. In fact, to this day, a tidal rhythm (and, for that matter, a circatidal clock) has never been observed in *C. marinus*, raising the question if it at all exists in the marine midge.

Connecting the oscillator, the counter mechanism and photoperiodism in *Clunio*

Distinguishing between a counter and an oscillator mechanism might be an oversimplification and might not be meaningful for the biology of every organism. The circasemilunar emergence rhythm of *Clunio* is, as a whole, clearly an oscillatory system, matching all criteria for a biological clock [6], including free-run, entrainment and temperature compensation [8, 7, 27, 14]. However, our study suggests that the circasemilunar oscillation is not simply based on an (molecular) oscillator with a corresponding period of 15 days. Instead, circadian periods are counted in a step-wise manner. We must assume that the timing-system can only move to the next step when a specific signal from the circadian system is received. The phase of the rhythm is set by semilunar cues. The phase response curve for the circasemilunar emergence rhythm of *C. tsushimensis* suggests an immediate resetting without transients, indicating a strong coupling between the pacemaker and the overt behavior, at least in the examined experimental conditions [3]. The absence of transient cycles also speaks against a 15-day oscillator mechanism and is in line with the idea of a counter, which can be reset immediately. Arrhythmicity of emergence in constant light further supports that the circasemilunar clock depends on regular input by the circadian system.

The finding of a counter mechanism rooted in a synchronized circadian system as the functional principle for circasemilunar time-keeping in the marine midge *C. marinus* prompts a series of exciting new questions: How many “steps” or “days” are counted (semilunar 15 vs lunar 30)? How is this number determined at the molecular level? And why is the free-running circasemilunar period shorter (12–13 days) than the semilunar period in its entrained state (15 days)? Resetting by semilunar time cues can make the entrained period shorter, but how can it be lengthened to 15 days? Do semilunar cues modulate the length of the “steps”? First insights into counter mechanisms come mainly from photoperiodism research. Numerous organisms measure day or night length and express a photoperiodic response after a defined number of cycles, that seems to be temperature compensated [28, 29, 30]. Different models exist on how a counter could work by accumulating a signal or substance, until ultimately crossing an internal threshold leading to the phenotype [31, 32, 33]. However, the molecular mechanism

of the photoperiodic counter is not understood. The role of the circadian clock for photoperiodism, on the other hand, has been proposed a long time ago [34, 35] and since then has been frequently investigated, revealing that it does play a role for some but not all organisms [36]. Hypotheses from research on photoperiodism might provide a starting point for understanding the circasemilunar counter in *C. marinus*. It could be a candidate to find accumulating or degrading molecules (transcripts, proteins, hormones) over the semilunar cycle.

The functional principle of *Clunio's* circasemilunar clock lays the foundation for unravelling its molecular mechanism and for studying time keeping across various time-scales

The evidence presented here establishes a link between the circadian clock and circasemilunar time-keeping in *C. marinus*. However, in order to understand the underlying molecular mechanism and substantiate a causal link between the circadian clock and the circasemilunar counter, it will be necessary to conduct functional validation studies. This requires establishing the molecular architecture of the circadian clock in *C. marinus* and subsequent genetic manipulation of core circadian clock genes. Another approach could be to chemically inhibit components of the circadian clock, as has been successfully undertaken in other non-model clock species [37, 38]. Finally, *Clunio's* diversity of circasemilunar and circalunar emergence phenotypes make it a unique model to study the circasemilunar counter using genomic approaches [39, 40]. In whole-genome screens, core circadian clock genes have been identified as associated with the presence and absence of circasemilunar rhythms [41]. The circadian clock gene *period* was identified as a candidate involved in determining the phase of a circalunar rhythm by a combination of QTL mapping and whole-genome screens [42]. The current study provides a mechanistic framework for those genomic findings, explaining how the circadian clock could be involved in circasemilunar time keeping of *C. marinus*.

To precisely coordinate physiological processes, organisms unite molecular mechanisms operating on different time scales, reaching from seconds to years. However, an organism's fitness depends on the accurate adjustment of all of them. Understanding the interrelation of time-keeping systems remains challenging. Because most organisms displaying short- and long-term non-24h rhythms are non-model organisms, bespoke molecular techniques are scarce. However, a robust hypothesis on the functional principle underlying each time keeping system is an important first step towards understanding temporal organization in its entirety. The marine midge *C. marinus* has an experimentally verified circadian clock, as well as a circasemilunar clock and a photoperiodic diapause response [43], providing an opportunity to address the complex organization of time keeping within the same organism.

Divergent clock principles may indicate multiple evolutionary origins of circasemilunar clocks

A systematic investigation of the functional principles of the circa(semi)lunar clock is challenging because only a few circasemilunar clock model organisms exist, and experimental evidence is scarce [3]. Weak evidence for the beat hypothesis has been established for the alga *Dictyota dichotoma* [5, 44]. The lunar maturation rhythm of the marine annelid *Platynereis dumerilii* continued when the circadian clock was chemically inhibited, suggesting that its circalunar clock does not depend on the circadian system and is, therefore, most likely based on a 30-day circalunar oscillator [38]. The lunar phase response curve of the marine annelid *Syllis prolifera* shows transient cycles, characteristic of an oscillatory system [45]. A circasemilunar counter based on the circadian system is evident for the marine midges *Clunio marinus* (this study) and *Pontomyia oceana* [11]. Thus, the circa(semi)lunar clock seems to rely on different functional principles in different organisms, which is in stark contrast to the well-conserved molecular mechanism of the circadian clock [46, 47]. Different functional principles of circa(semi)lunar time-keeping could be rooted in the different life histories of diverse organisms. *Polychaetes* are mainly marine organisms that originated during the Cambrian [48] and stayed in the ocean thereafter. They might have an evolutionarily ancient circa(semi)lunar clock. In contrast, marine midges (Diptera) seem to rely on a counting mechanism. Considering that the first Diptera appeared during the Middle Triassic (around 240 million years ago) in terrestrial habitats [49], they secondarily colonized the ocean. Hence, marine midges may have co-opted an already existing day-counting mechanism - the photoperiodic counter - in order to track the (semi)lunar cycle. Further investigations are required to test the idea that marine annelids and marine midges rely on different functional principles of their circa(semi)lunar clock.

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Supplementary Figures

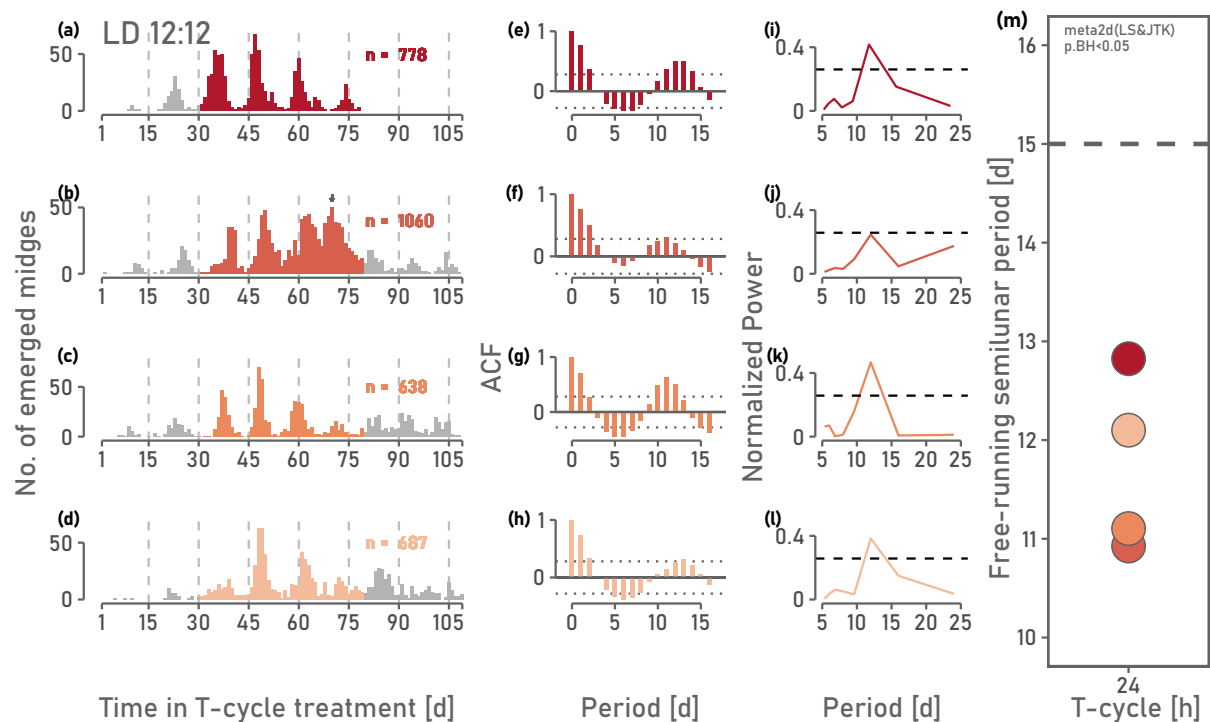


Figure S1: Time series data and analysis of the free-running circasemilunar emergence rhythm of *C. marinus* under LD 12:12. Experiments were conducted in 2020 (a,e,i), 2021 (b,f,j) and 2023 (c,g,k and d,h,l). For time series analysis, only the colored bars were considered. (a-d) Daily number of emerged midges after release into circasemilunar free-run and LD 12:12. In one replicate (b) a phase shift was observed (indicated by gray arrow), likely due to vibrations coming from construction works. (e-h) Auto-correlation of time series. The confidence interval was set to 0.95 and is indicated as the gray dashed line. (i-l) Lomb-Scargle periodograms with alpha of 0.05 indicated as gray dashed line. (m) Significant circasemilunar periods calculated with meta2d() are displayed. Colors of dots correspond to the colors of the time series in a-l.

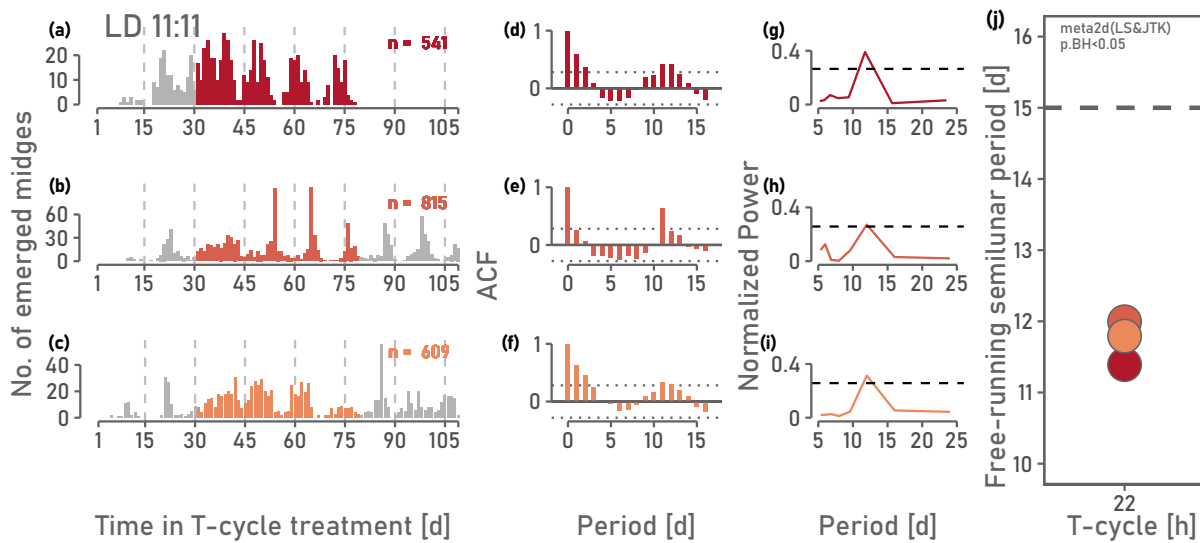


Figure S2: Time series data and analysis of the free-running circasemilunar emergence rhythm of *C. marinus* under LD 11:11. Experiments were conducted in 2020 (a,d,g), 2021 (b,e,h) and 2023 (c,f,i). For time series analysis, only the colored bars were considered. (a-d) Daily number of emerged midges after release into circasemilunar free-run and LD 11:11. (d-f) Auto-correlation of time series. The confidence interval was set to 0.95 and is indicated as the gray dashed line. (g-i) Lomb-Scargle periodograms with alpha of 0.05 indicated as gray dashed line. (j) Significant circasemilunar periods calculated with meta2d() are displayed. Colors of dots correspond to the colors of the time series in a-i.

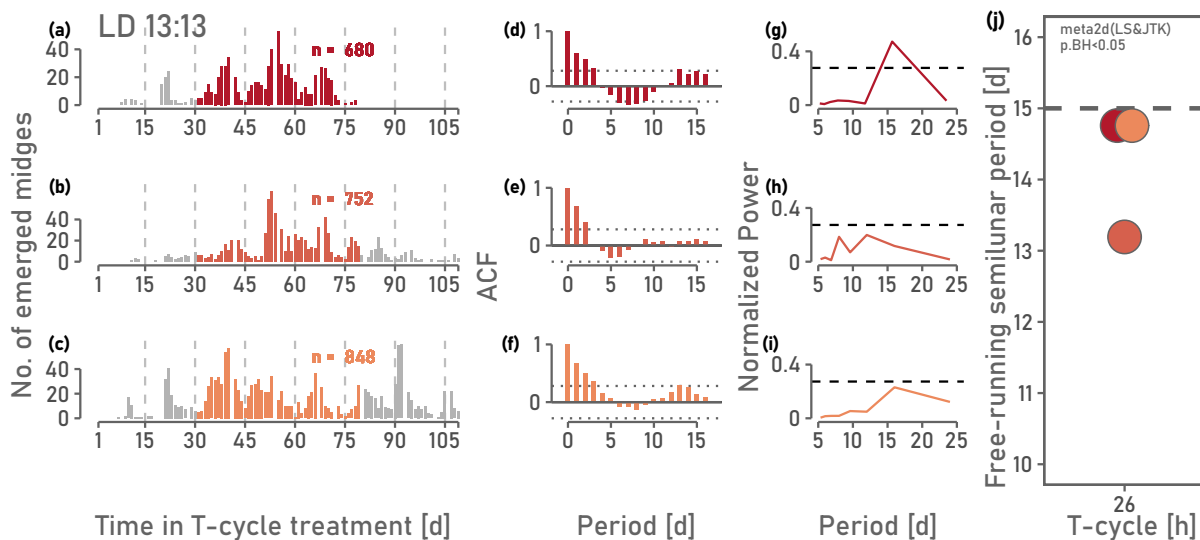


Figure S3: Time series data and analysis of the free-running circasemilunar emergence rhythm of *C. marinus* under LD 13:13. Experiments were conducted in 2020 (a,d,g), 2021 (b,e,h) and 2023 (c,f,i). For time series analysis, only the colored bars were considered. (a-d) Daily number of emerged midges after release into circasemilunar free-run and LD 13:13. (d-f) Auto-correlation of time series. The confidence interval was set to 0.95 and is indicated as the gray dashed line. (g-i) Lomb-Scargle periodograms with alpha of 0.05 indicated as gray dashed line. (j) Significant circasemilunar periods calculated with meta2d() are displayed. Colors of dots correspond to the colors of the time series in a-i.

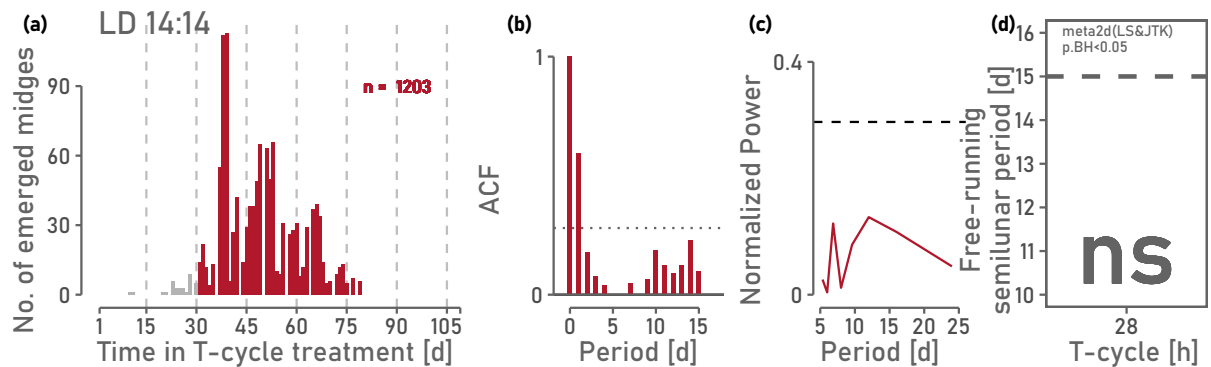


Figure S4: Time series data and analysis of the free-running circasemilunar emergence rhythm of *C. marinus* under LD 14:14. The experiment was conducted in 2020 (a-c). For time series analysis, only the colored bars were considered. (a) Daily number of emerged midges after release into circasemilunar free-run and LD 14:14. (b) Auto-correlation of time series. The confidence interval was set to 0.95 and is indicated as the gray dashed line. (c) Lomb-Scargle periodogram with alpha of 0.05 indicated as gray dashed line. (d) There is no significant circasemilunar period calculated with meta2d().

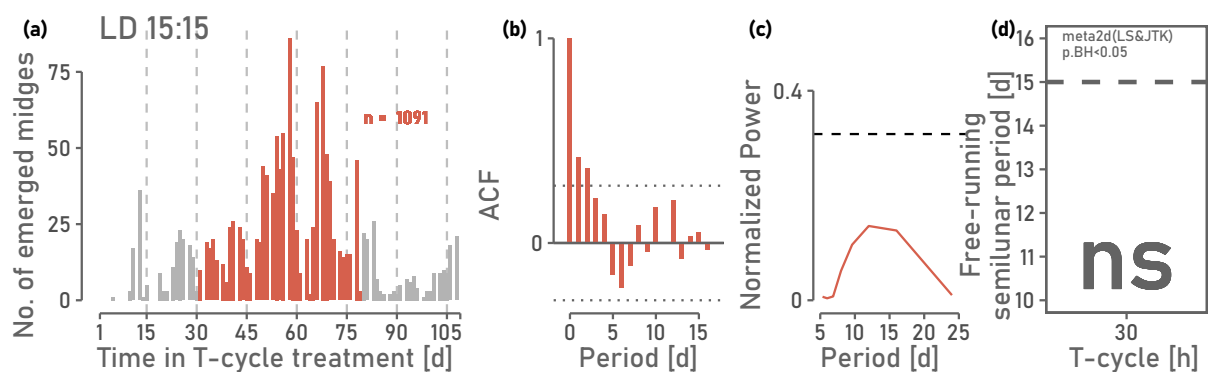


Figure S5: Time series data and analysis of the free-running circasemilunar emergence rhythm of *C. marinus* under LD 15:15. The experiment was conducted in 2021 (a-c). For time series analysis, only the colored bars were considered. (a) Daily number of emerged midges after release into circasemilunar free-run and LD 15:15. (b) Auto-correlation of time series. The confidence interval was set to 0.95 and is indicated as the gray dashed line. (c) Lomb-Scargle periodogram with alpha of 0.05 indicated as gray dashed line. (d) There is no significant circasemilunar period calculated with meta2d().

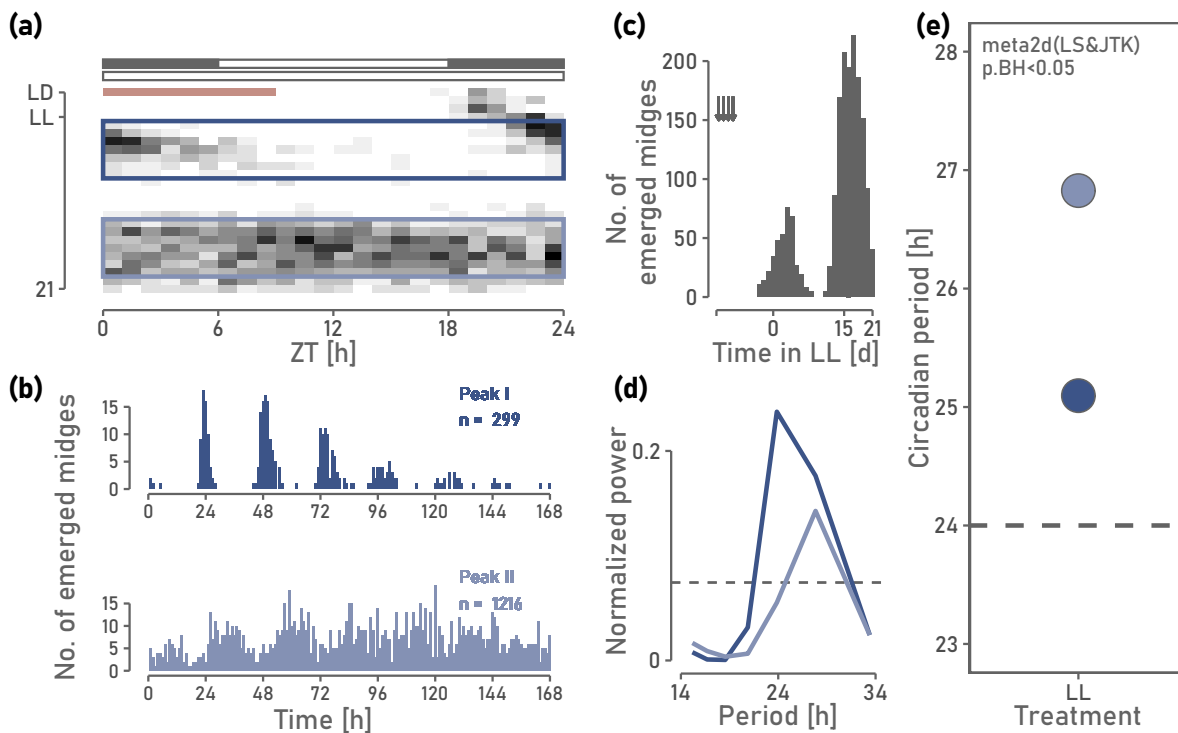


Figure S6: Circadian and circasemilunar emergence rhythm under constant light of a *C. marinus* population from Northern Spain. Data from [10]. (a) The circadian emergence rhythm free-runs under constant light with a period longer than 25 hours. Missing data (NA) is encoded in red. (b) The number of emerged midges per hour for two circasemilunar emergence peaks (peak I: day 1-7 and peak II day 13-19). (c) The circasemilunar emergence rhythm is synchronized under constant light for at least two peaks. Moonlight (dim night light indicated by arrows) was used to entrain the circasemilunar clock. (d) The LS periodogram indicates rhythmicity of circadian emergence for both emergence peaks (peak I: $\tau_{\text{circadian}} = 23.86\text{h}$, $p = 1.14\text{e-}08$; peak II: $\tau_{\text{circadian}} = 27.83\text{h}$, $p = 1.31\text{e-}04$). (e) meta2d() detects a significant circadian period for both emergence peaks.

Chapter 4

4 Transcriptomic profiling of a circasemilunar developmental arrest and diapause in the marine midge *Clunio*

Jule Neumann, Danila Voronov and Tobias S. Kaiser

This Chapter is in preparation for publication.

Abstract

Adult eclosion in the marine midge *Clunio marinus* (Helgoland strain) is synchronized with the semilunar cycle, matching precisely the lowest spring tides around full and new moon. A circasemilunar clock synchronizes development on the population level during the 4th larval instar. Depending on the semilunar day, larvae either undergo a circalunar developmental arrest (CDA) or progress in development (non-CDA). Based on an established protocol on imaginal disc morphology we found that the same developmental stage arrests its development during CDA and winter diapause. We further characterize the transcriptomes of this larval stage in CDA and non-CDA and compare it to larvae in diapause. Only 26 genes, mainly related to glucose metabolism and muscle constitution are differential expressed between CDA and non-CDA larvae. A set of genes, uniquely differential expressed in non-CDA samples compared to winter samples, contain genes involved in ecdysteroid metabolism while a set of genes, uniquely differential expressed in CDA samples compare to winter samples, is enriched for genes involved in glucose metabolism. We present here the first study which characterizes the transcriptome of a developmental arrest governed by a circalunar clock.

Introduction

Development is a key characteristic of life. Developmental synchronization refers to the synchronization of physiological processes constituting growth and development in organisms. Such coordination can occur on the sub cellular to the population level, including within and between organisms, as well as between organisms and their environment. In fact, being in a specific developmental stage at the right time can have evolutionary consequences, i.e. asynchrony of reproduction can result in decreased fitness [1, 2, 3]. Organisms evolved endogenous time keeping systems – so called *biological clocks* – to coordinate crucial life cycle events with environmental cycles. Anticipation of favorable or unfavorable conditions can provide advantages, e.g. through predator avoidance or coordination of mating availability [4, 5]. Involvement of the circadian (about 24 hour) clock into developmental synchronization has been intensively examined and studied up to the molecular level [6]. However, molecular mechanisms and physiological pathways underlying coordination of development by other, non-24 hour clocks, in particular the circalunar (about 15 or 30 days) clock, remain unidentified.

In insects, endogenous clocks coordinate crucial life cycle events on the population level, including egg-hatching, pupation and adult eclosion. Synchronized egg-hatching is a widespread phenomenon in insects [7, 8, 9] and involvement of the circadian clock has been demonstrated in moths [10, 11] and crickets [12]. Pupation is controlled by a circadian clock in some *Drosophila* flies [13, 14] and by a circannual (about 1 year) clock in carpet beetles *Anthrenus sp.* [15]. However, the arguably most studied case is eclosion [16, 17, 18, 19]. Eclosion refers to the emergence of the adult from the pupal case, constituting the last developmental transition in the life cycle of a holometabolous insect. The role of the circadian clock in eclosion rhythms of *Drosophila* flies is well established [20, 21, 22]. The circadian clock restricts or "gates" eclosion to a certain phase of the day. A similar mechanism was later demonstrated for larval ecdysis or molt. In a pioneering paper, experiments confirmed in the silkworm *Manduca sexta* that the circadian clock determines when developmental hormones, like the prothoracicotropic hormone (PTTH) are released [23]. Since then, the interplay of the circadian clock and hormonal cascades have been intensely discussed and studied [24, 25]. Many insects require a state of developmental competence that can be measured as absolute size or weight for metamorphosis [26, 27]. This developmental competence concedes the secretion of hormones required for ecdysis. However, availability of these hormones is time restricted by the circadian clock in many insects [28, 29, 22, 23]. Important hormones include PTTH and ecdysteroids, regulated through the brain–prothoracic gland axis as well as juvenile hormone which can alter ecdysteroidogenic

activity of the prothoracic gland [30].

Coordination of development can occur through the timing of phases of progression (as discussed above) but also by synchronizing phases of developmental arrest. Numerous insects overwinter in a hormonally programmed, alternative physiological state characterized by metabolic suppression in order to survive harsh conditions. This overwintering state is referred to as diapause [31] and many insects measure night length as a cue for diapause induction [32]. Consequently, molecular evidence for the involvement of the circadian clock in the photoperiodic dormancy response exist in some but not all insect species and a generalized model is still debated [33, 34]. Diapause is a dynamic physiological state, divided into defined phases including initiation, maintenance and termination [35]. These phases are characterized by distinct changes in gene expression, metabolic signatures and hormonal levels [36, 37, 38]. Understanding the regulation of diapause in insects has significant potential for pest management and for the study of anthropogenic change effects on insect including adaptation to climate change [39, 40, 41].

In contrast, less understood are physiological processes underlying the interplay of non-24 hour clocks and development. For example, though the circannual clock is known to be involved in pupation timing in carpet beetles, no studies have been conducted to unravel the molecular link [15, 42]. A circalunar clock controls the adult eclosion rhythm of marine midges of the genera *Clunio* and *Pontomyia* [43, 44]. However, its molecular components and the mechanisms underlying developmental synchronization remain unknown [45]. It is surprising that non-24 hour clocks have received so little attention considering that tidal, lunar and annual rhythms have been part of Chronobiology since its landmark meeting in 1960 [46] and are important for numerous organisms [47].

In this manuscript, we investigate for the first time a developmental arrest gated by a circasemilunar clock on the transcriptomic level. The marine midge *Clunio marinus* (Diptera, Chironomidae) is a holometabolous insect which has to undergo several developmental stages in order to progress from the egg to the reproductive adult stage [48]. These include four larval instars and a pupal stage. Strikingly, adult emergence in some populations of *Clunio*, including the here studied Helgoland population, is synchronized with the semilunar cycle, matching precisely the lowest spring tides during full and new moon [48, 49]. After eclosion, the short-lived adults have only a few hours before the approaching high water to mate, reproduce and oviposit the eggs on the exposed substrate. Their precise eclosion timing is coordinated by at least two endogenous time keeping systems, a circalunar clock and a circadian clock [48].

In order to synchronize adult emergence with the semilunar cycle, development in *Clunio* is synchronized on the population level in advance of eclosion. More specifically, in the European species *Clunio marinus* (Helgoland), a developmental switching point occurs during the early fourth larval stage [50, 51]. This observation was based on imaginal disc morphology in the last larval instar. Imaginal discs are clusters of cells which are already present in the larvae of holometabolous insects and will later develop into the adult appendages including leg, wing, haltere and reproductive organs [52]. The 4th larval stage of *C. marinus* can be subdivided into nine substages based on the progression of imaginal disc growth [50, 51]. Interestingly, imaginal disc development is synchronized with the semilunar cycle in the field and laboratory under summer conditions (long days and warm temperatures) in *C. marinus*, i.e. the distribution of 4th instar substages has a characteristic modulation over the semilunar cycle [50, 51]. Early 4th instar substages are present throughout the semilunar cycle. However, 4th instar larvae which are further progressed in their imaginal disc development, display a semilunar modulation. This indicates that while early 4th instar larvae are always present in equal numbers, the progression of the "older" 4th instar larvae is somehow coordinated by the circalunar clock. In fact, the early 4th instar contains a waiting stage which synchronizes the development on the population level via a physiological arrest. These larvae can either progress in development or wait till the next "window of opportunity" opens. We coin this waiting period *circalunar developmental arrest* or *CDA*. Notably, the correlation of imaginal disc development with the semilunar cycle is temperature compensated [51] - an uncommon feature of insect development. However, temperature-compensation is a characteristic feature of biological clocks. This highlights that once the "decision" to progress in development has been made, adult midges will reach the pupal stage in a defined amount of time, regardless of the outside temperature. This might ensure that the next spring tide is not missed, even when temperatures might fluctuate greatly in the intertidal zone. In summary, the circasemilunar clock gates development in *C. marinus* during the last larval stage. However, CDA has not been studied yet and its physiology remains unknown.

We hypothesize that CDA may be in fact a mini-quiescence, potentially derived from a photoperiodic diapause in *Clunio*. Because, field observation suggest that *C. marinus* overwinters in the same exact substage in which CDA was described, namely the early 4th larval instar [50]. In the laboratory, only a combination of short days and low temperature can induce the overwintering diapause [53]. But adult emergence can be reinstated by switching back only to long days (while keeping the temperature low). This suggests that *Clunio*'s overwintering dormancy is in fact a photoperiodic induced diapause [53]. We investigated the coding tran-

scriptomes of larvae in CDA, non-CDA and winter diapause. Therefore, we obtained the distributions of adult emergence and 4th instar substages in *C. marinus*, not only in summer, but also diapause-inducing winter conditions. We confirm the field observation that the early 4th instar is undergoing diapause under controlled laboratory condition [50]. We then sampled this exact substage at two semilunar phases in winter and summer conditions for RNA-Sequencing. We compared the transcriptomes of larvae in CDA and non-CDA under summer conditions to characterize the approximate physiological state of larvae in CDA. We identified a small set of 26 genes to be differential expressed between CDA and non-CDA larvae. Those genes involve key enzymes in glucose metabolism and genes involved in muscle constitution and reorganization of the cytoskeleton. We further identified two genes sets uniquely differential expressed in CDA or non-CDA samples compared to diapausing larvae, i.e. we compared transcriptomes of larvae undergoing a developmental arrest (CDA and diapause) with non-arresting larvae (non-CDA) to identify genes which are similar in gene expression within the first group but differential expressed to the latter and the other way around (non-CDA and diapause vs CDA).

Methods

Laboratory culture

A laboratory culture of *Clunio marinus* (Helgoland strain) was used for all experiments described in this manuscript. The population was originally obtained from Helgoland, Germany (Westwatt, 54°11'05"N 7°52'30"E) in 2005 by Dietrich Neumann. Larvae were cultured in 8x8x8cm acryl containers (Wenko, i30053670). 1.5g sterilized sand (collected in Roscoff, France and Praia da Duquesa, Cascais, Portugal, 1:1) was added to each container together with 200ml of a mix of sea and demineralized water (1:1). Containers were constantly ventilated with pressurized air. The midges were fed twice a week with 1 ml diatoms (*Phaeodactylum tricornutum*, strain UTEX646). Water was exchanged and 500µl nettle (*Urtica dioica*) powder added every two to four weeks. Light intensity and air temperature were measured every 10 minutes using Onset HOB0 data loggers (UA-002-08). Circasemilunar entrainment was induced throughout the entire experiment by mechanical vibrations given in a tidal pattern of T=12.4h using an unbalanced motor (50 Hz, ~ 30 dB above background noise, motor alternated between 6.2h on and 6.2h off) [54, 55]. Day 1 of the semilunar cycle is defined as the day when the onset of the vibration occurs right after the middle of the dark period. The middle of the dark period is defined historically for *Clunio* as ZT 0. Vibration was recorded using Onset HOB0 data loggers (UA-004-64).

Experimental Setup

The adult emergence rhythm and a distribution of substages of the 4th larval stage of *C. marinus* was monitored over the semilunar cycle in summer and winter conditions.

Summer conditions

In total two experiments were conducted under summer conditions. For technical reasons it was not possible to obtain enough larvae for staging the 4th instar substages while simultaneously monitoring the adult emergence phenotype. Hence, we conducted one experiment in which we monitored only adult emergence and one in which we sampled the larvae for the staging procedure after occurrence of the first emergence peak. However, environmental conditions were similar for both experiments. From the egg stage on, *C. marinus* was reared under long days (L:D 16:8) and high temperature (19°C). Fertilized eggs were added over multiple emergence peaks every 15 days when adult emergence occurred. Because eclosion occurs only once during an individual's life time, a continuous supply of eggs is crucial for obtaining overlapping generations. This is essential to get an unbiased distribution of 4th instar larval substages, i.e. the same number of 3rd instar larvae has to enter the 4th instar stage every day. *Clunio* takes at least 6 weeks from the embryonic stage to reach the adult stage [48]. Phenotyping of adult emergence therefore started during the sixth week after the first fertilized eggs were added to cover the first emergence peak. The number of emerged adults was counted every day and are visualized in Figure 1a (solid red line indicates the experiment conducted for adult phenotyping, dashed red line indicates the experiment conducted for the sampling of 4th instar larvae). Figure 1b displays the recorded environmental conditions for the first experiment.

Winter conditions

From eggs on, *C. marinus* was reared under summer conditions (L:D 16:8 & 19°C) for 30 days to ensure sufficient development of overlapping generations. Fertilized eggs were continuously supplied as described above only during the summer period. After six weeks, on day 1 of the semilunar cycle, conditions were switched to short day (L:D 8:16) and cold temperatures (10°C) to induce an overwintering diapause. Only the combination of short days and cold temperature can effectively induce diapause in *C. marinus* [53]. After 12 weeks in winter conditions, temperature and photoperiod were changed back to summer conditions in order to monitor the adult emergence phenotype of *Clunio* after breaking the dormancy-inducing conditions. Adult

eclosion was monitored during the second "summer period" for 8 weeks. Emergence data is displayed in Figure 1a and the monitored environmental conditions in Figure 1c.

Staging & sampling of 4th instar larvae

Clunio has four larval instars which can be distinguished by head capsule size [48]. However, the 4th instar can be divided into nine substages based on the development of the thoracic and genital imaginal discs [50, 51]. Imaginal discs are epithelial cell clusters found in the larval stage of holometabolous insects, which after metamorphosis turn into external structures (e.g. the wings or legs). While 3rd instar larvae of *Clunio* still lack imaginal discs, early 4th instar larvae have primordial imaginal discs that grow in size until the prepupal stage. Substaging of the 4th instar conducted for this study was based on previous work in *Clunio* and *Chironomus* [50, 51, 56] (Supplementary Figure 1). Larvae of both sexes were substaged every two days, consecutively over the semilunar cycle (semilunar days 4, 6, 8, 10, 12, 14, 1 and 3) in summer and winter conditions. In summer conditions, larvae were sampled during the second experiment after about 6 weeks (Figure 1b, circles). For the winter experiment, larvae were sampled after 8 weeks in winter conditions (Figure 1c, circles).

On all sampling days, larvae from six replicates (=containers/boxes) were preserved during the middle of the light period, i.e. four hours after light on in winter and 8 hours after light on in summer conditions. The middle of the photoperiod was chosen as a reference point to compare gene expression between long days in summer and short days in winter conditions. For sampling of larvae, the sea water was removed from the container and larvae briefly submerged in demineralised water. After removing all water, 30ml of absolute ethanol (>99.8 PERCENT, Carl Roth™, 9065.4) was added to the containers. The procedure took between 2 and 5 minutes per container. Larvae remained in their container (original environment) throughout the procedure and were incubated in ethanol on room temperature for 5 minutes before they were transferred on ice. Imaginal disc development of larvae submerged in ethanol was classified with a transmitted-light microscope (Leica DMLB, PH3) with 10x magnification ocular using LabCam™ microscope adapters for iPhone 6/6s and iPhone 7/8. Thoracic discs (wing, legs and haltere) were observed from lateral while the genital discs were observed from ventral. The nine substages of the 4th instar are from now on referred to as LIV-1, LIV-2 etc. til LIV-9. Males and females could only be distinguished from the LIV-2 stage onward. Females lack the haltere disc and have an underdeveloped wing disc. Males develop a characteristic hypopygium (last sternum of the abdominal segment containing the copulation organs) while females have a gallert gland (translation from the German word *Schleimdrüse* used by [56]) (Supplemen-

tary Figure 1). The staging procedure took 2.5 - 3 hours per sampling day (corresponding to the time larvae were in ethanol on ice). Afterwards, larvae were preserved in TRIzol Reagent (Thermo Fisher Scientific, 15596026) and stored at -70°C until RNA extraction.

For obtaining a distribution of the 4th instar substages over the semilunar cycle, the number of larvae per substage per day was divided by the total number of 4th instar larvae that were substaged that day multiplied by 100. Results are visualized in Figure 1d,e and raw data can be found in Supplementary Table S1 and Supplementary Table S2 for summer and winter conditions. Based on the distribution of 4th instar substages in summer conditions, the substage and semilunar phase in which larvae undergo CDA was identified. LIV-2 were collected at the same semilunar days (SLDs) in winter conditions to minimize the effect of the semilunar phase or circasemilunar clock on transcriptomic differences between conditions.

RNA extraction & sequencing

RNA of 48 individual LIV-2 larvae (24 in summer and 24 in winter conditions at two phases of the semilunar cycle, SLDs 14 to 1 (CDA) and SLDs 6 to 8 (non-CDA), respectively) was extracted using Direct-zol RNA Microprep (Zymo, 2060) with a modified protocol. 1.5ml tubes (Sarstedt, 72.706.400) were prepared with six steel beads (Biospec, 1.5mm, 11079115ss). TRIzol Reagent was added and individual larvae transferred into a tube. Single larvae were homogenized using a tissue lyser (30Hz, 2min, Qiagen, TissueLyser II, 85300) and incubated for 5 minutes at room temperature. This step was repeated three times. Samples were then centrifuged at 4°C for 5min (Eppendorf centrifuge 5417R, 14800rcf) and supernatant transferred. Protocol of the Zymo microprep kit was then followed including DNase digest. Centrifugation steps were conducted at 16,000rcf for 30sec (Eppendorf centrifuge 5418). During the last step (wash buffer removal) samples were centrifuged at 16,000rcf for 2 minutes. RNA was eluted in 14 µl DNase/RNase free water (2 times in 7µl).

All 48 samples were randomized for library preparation. Illumina stranded mRNA (150bp, paired end, polyA capture) of each sample was sequenced in a single run on a single chip across three lanes on a NovaSeq™ 6000 platform using Nextera adapters. Consequently, three data files, containing the forward and reverse reads per sample from different lanes, respectively, were concatenated before use for downstream analysis.

Read processing & differential gene expression analysis

Adapters and bad quality reads were trimmed with trimmomatic 0.39 [57] with HEADCROP:3, ILLUMINACLIP:NexteraPE -PE.fa:2:30:10:2:True, SLIDINGWINDOW:5:25 and MINLEN:50. The reads were mapped and counted with salmon 1.10.0 [58]. A reference transcriptome of *C. marinus* contained the exported transcript sequences from the manually curated CLUMA 2.0 genome [59] and was used to create a decoy aware salmon index with index -p 8, -k 31. The count files created by salmon were used for downstream analysis in R 4.3.0 [60].

Quality filtering of samples and genes

The raw data set consists of 48 individual male LIV-2 samples, 12 each belonging to one of the four groups: CDA (summer condition, SLD 14 & 1), non-CDA (summer condition, SLD 6 & 8), dormancy I (winter condition, same semilunar phase as non-CDA group, SLD 14 & 1) and dormancy II (winter condition, same semilunar phase as non-CDA SLD 6 & 8).

Samples with mapping rates with less than 50% and less than 5 Mio reads were excluded for downstream analysis. For outlier detection we ran DiscoRhythm [61] with `discoInterCorOutliers()` (`cor_method="pearson"`, `threshold=3`, `thresh_type="sd"`). DiscoRhythm was run on the normalized read counts obtained with the variance stabilizing transformation function `vst()` being blind to the experimental design (`blind = T`). However, DiscoRhythm was aware of the groups for outlier detection. No outliers were identified with DiscoRhythm. Crucially, one outlier was removed manually via PCA (Supplementary Figure 2). RNAseqQC [62] was used to filter out genes with a minimum count of 10 reads across at least five samples per group with `filter_genes` (`min_count = 10`, `min_rep = 5`). Sample size of the smallest group was five, ensuring that only genes with reads covering all samples in the smallest group were considered for downstream analysis. The final data set contained 12,311 genes and 37 samples (CDA: 5, non-CDA: 9, dormancy I: 11, dormancy II: 12).

Differential gene expression analysis with DESeq2

Differential gene expression analysis was conducted using DESeq2 [63]. Principle component analysis (PCA) and Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) were plotted based on `vst` transformed read counts (`blind = T`) with `plotPCA()` [64] and `umap()` with `random_state=125` [65]. DESeq2 was run in default mode as `DESeq()` with the design set to `~ group`. We conducted all possible six pairwise comparisons between groups with

the `results()` function (`independentFiltering = TRUE`, `alpha = 0.05`). Five genes were flagged as outliers and excluded from downstream analysis resulting in 12,306 genes for all comparisons. The log fold change per gene was calculated using the MLE method and the p-value calculated using the Wald test. The results tables were filtered for differential expressed genes by adjusted p-values (Benjamini Hochberg procedure) smaller than 0.05.

To verify that the data fits the model of DESeq2, several diagnostic plots were obtained. The `plotDispEsts()` function was used to plot dispersion estimates against the normalized read count to assess intragroup variability (low expressed genes tend to have a higher variance) and fit of the DESeq2 model. `plotMA()` was used to visualize the estimated log fold change against the normalized mean count per gene for each pairwise group comparison. The $-\log_{10}$ p-value was plotted against the mean normalized read count per gene. Finally the consequences of the independent filtering option for each pairwise group comparison was visualized by plotting a histogram of p-values passing and not passing the filter threshold of DESeq2 based on mean counts.

Obtaining lists of candidate genes

First, we focused on genes that are differential expressed between CDA and non-CDA samples. Due to the small number of genes found differential expressed, we established a permutation procedure in which we reshuffled the sample labels belonging to the two summer groups. The aim was to assess the certainty for the genes found to be differential expressed by DESeq2 using an alternative statistical approach. The number of permutations was 10,000. For the by DESeq2 identified differential expressed genes, we randomly sampled five of the by `deseq` normalized read counts, representing the CDA group and assigned the 9 remaining values to the non-CDA group. We then calculated the absolute difference of mean gene expression for both groups. The p-value was calculated as the number of permutations in which the absolute mean difference was higher than the observed absolute difference divided by the total number of permutations. Only genes with p-value smaller than 0.05 were kept for downstream analysis. The normalized number of counts per group was visualized using `plotCounts()`. Heatmaps were created by scaling the normalized read counts obtained with `scale()` and `vst()` transform.

Second, we were interested to detect gene sets in which genes display a distinct expression pattern in either the CDA or non-CDA group in comparison to the winter groups. Genes found in those gene sets could reveal biological processes which are shared between larvae in winter diapause and larvae undergoing CDA and between larvae in winter diapause and lar-

vae undergoing non-CDA. We therefore filtered the significantly differential or non-differential expressed genes of all pairwise group comparisons identified by DESeq2 as follows a) by intersecting genes that are differentially expressed between dormancy I and non-CDA samples with genes that are differentially expressed between dormancy II and non-CDA samples but non differentially expressed between CDA and both winter groups (gene set A) and b) by intersecting genes differentially expressed between dormancy I and CDA samples with genes differentially expressed between dormancy II and CDA samples but non differentially expressed between non-CDA and both winter groups (genes set B) (Table 1). The idea behind that was that genes having an intermediate mRNA level in one summer group (CDA or non-CDA) in comparison to the remaining groups may not be differential expressed between CDA and non-CDA samples, but differential expressed only between the winter and one of the summer groups.

Gene sets identified by intersecting:	Common set of DEGs	Common set of non-DEGs
A	intersect(dormancy I vs non-CDA, dormancy II vs non-CDA)	intersect(dormancy I & CDA, dormancy II & CDA)
B	intersect(dormancy I vs CDA, dormancy II vs CDA)	intersect(dormancy I & non-CDA, dormancy II & non-CDA)

Table 1: Pairwise group comparisons for all four groups were conducted with DESeq2 as described above. The resulting lists of differential expressed genes (DEGs, vs) and non-differential expressed genes (non-DEGs, &) were intersected as outlined in this table. E.g. *gene set A* contains the intersect of genes differential expressed between non-CDA and winter samples and genes that are non-differential expressed between CDA and winter samples. Hence, gene set A contains genes which have similar mRNA levels in CDA and winter samples but different mRNA levels in non-CDA compare to winter samples. Note that these genes are not necessarily differential expressed between the CDA and non-CDA group.

GO term analysis

GO term analysis of candidate genes was conducted with topGO [66]. Ontology was set to Biological Process (ontology = "BP") and the test statistic was Fisher test with the weight01 parameters set to topNodes=1000 and numChar=10000. Results were filtered for a Fisher p-value < 0.05. Further, rrvgo [67] was used to reduce the redundancy of GO terms using calculateSimMatrix() with ont = "BP", method = "Rel" and reduceSimMatrix() with threshold=0.7, scores = "size".

Results

The adult eclosion rhythm of *Clunio* is suppressed under dormancy-inducing winter conditions in the laboratory

We monitored the adult eclosion rhythm of *C. marinus* under summer and winter conditions (Figure 1a-c) as a control for the substage experiment (Figure 1d,e). The adult eclosion rhythm of the marine midge *C. marinus* is synchronized with full and new moon, matching the lowest low tides in the field [48]. The circalunar clock of a population of *C. marinus* from Helgoland is effectively entrained by superposition of a 24 hour light dark cycle with a 12.4h tidal pattern of vibration [55]. This superposition results in a unique phase relationship between the two cues, recurring every 15 days. Consequently, adults emerge synchronized with the semilunar cycle in the laboratory under summer conditions, i.e long days and warm temperature, when exposed to both the tidal and light-dark (LD) cycles (Figure 1a,b; red line). It has been demonstrated previously that the adult emergence rhythm of *C. marinus* is suppressed by a combination of short days and cold temperatures emulating winter conditions [53]. In our experiment, the first emergence peak was delayed under winter conditions compared to summer conditions, before eclosion was eventually suppressed (Figure 1b,c). When switching from summer to winter conditions, at least one emergence peak is expected in *Clunio*, because emergence is developmentally predetermined up to 20 days in advance [51], i.e. midges were destined to eclose regardless of the change in environmental conditions. The delay of the first emergence peak is caused by the temperature sensitivity of the pupal stage (the pupal stage is *not* temperature-compensated) [50], i.e. the pupal stage is prolonged by low temperatures and is therefore delaying the phase of adult emergence. Adult eclosion is completely suppressed 2 to 3 months after the switch to dormancy-inducing winter conditions (Figure 1a; blue line), which is in accordance with previous observations [53]. To confirm that larvae retained the developmental competence to emerge in favorable conditions, winter conditions were shifted back to summer for the remaining larvae after sampling of the substages (Figure 1c). A first cohort emerged about 2 weeks right after the shift (Figure 1a; blue line). This cohort is not synchronized to the semilunar cycle and potentially in a state of quiescence, i.e waiting for favorable conditions to occur in order to continuous development immediately. A second, smaller cohort emerged about one week after the first cohort in the correct semilunar phase (Figure 1a; blue line). After that, two subsequent emergence peaks were recorded, both correctly synchronized with the semilunar cycle. In the field, continuous emergence was observed throughout the first month in spring for the same population used in this study [50]. The results presented

here could therefore explain the field observation.

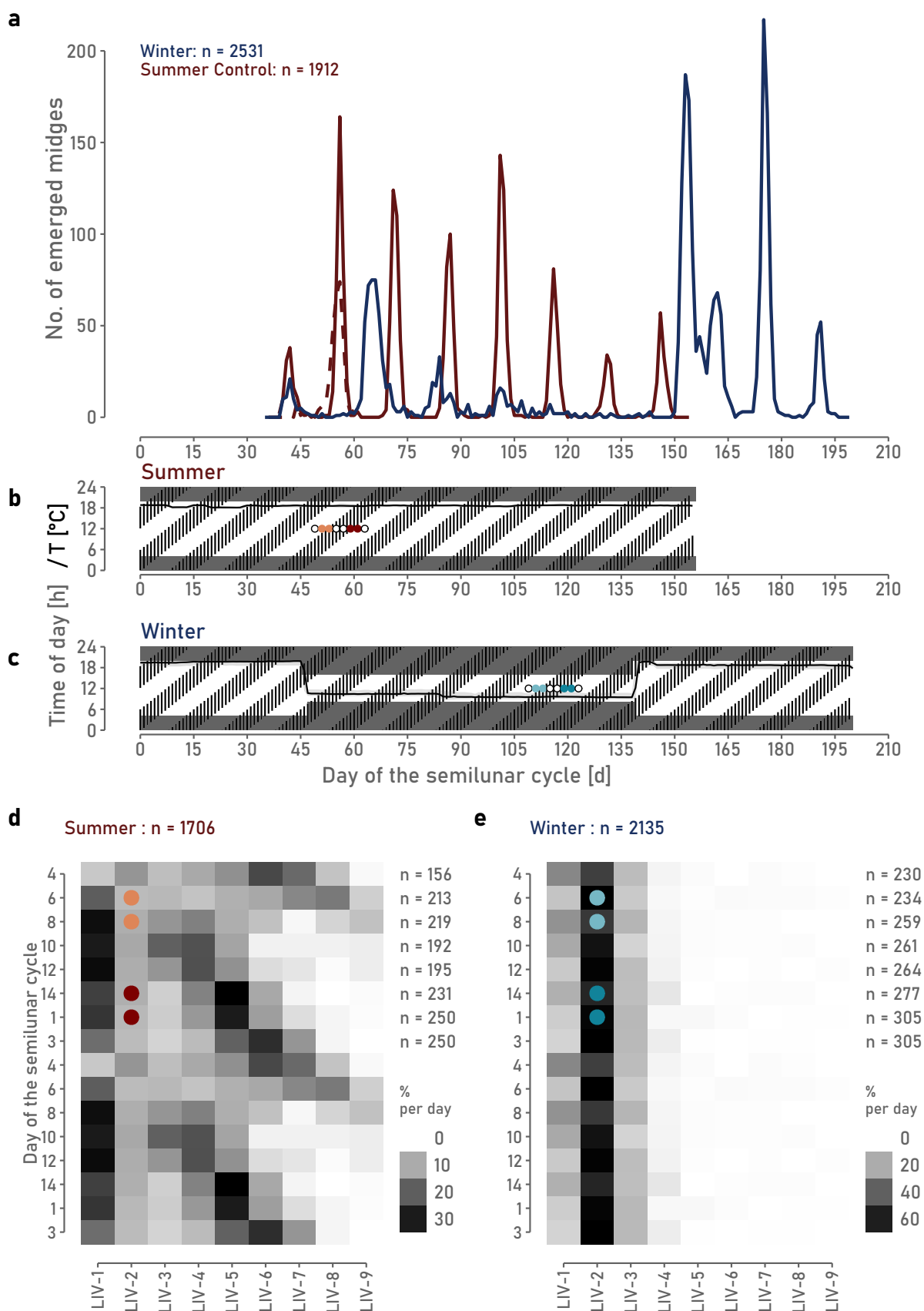


Figure 1: Adult emergence and distribution of 4th instar larval substages of *C. marinus* under summer (LD 16:8, 18°C, red) and winter conditions (LD 8:16, 10°C, blue). (a) The number of adults was monitored daily (solid lines). The dashed line indicates emergence recorded under summer conditions of the substage sampling experiment in (e) (see Methods section). (b,c) Measured experimental conditions. Vertical lines indicate exposure to tidal vibration. Dark shades indicate time of the day when light was off. The black line indicates the average temperature per day with maximum and minimum as gray shaded interval. Circles mark the days larvae were sampled for substaging. Colored dots indicate group affiliation and the semilunar days when samples, that were used for sequencing, were taken. (b) Environmental conditions for the summer emergence phenotype control (a, solid red line). (c) Environmental conditions for the winter experiment (a, blue line) (d, e) Double plotted distribution of 4th instar substages over the semilunar cycle under summer and winter conditions. The number of larvae per substage was normalized to the day and is plotted in percent. The colors of dots encode for larvae in non-CDA (orange), CDA (red), dormancy phase I (blue) and dormancy phase II (light blue) used for mRNA sequencing.

CDA and winter dormancy occur in the same 4th instar larval substage

In order to synchronize adult emergence to the semilunar cycle, development is already synchronized during the last larval stage on the population level in *Clunio* [50, 51]. The early 4th instar larvae act as a switching point, i.e. larvae wait in this developmental stage until progression in development is gated by the circasemilunar clock. This observation is based on the distribution of the nine developmental substages of the 4th instar obtained in summer condition [50, 51]. The substages can be distinguished by the morphology of the imaginal discs. We obtained a distribution of the 4th instar larvae in summer and for the first time in dormancy-inducing winter conditions (Figure 1d,e) to investigate if both developmental arrests occur at the same developmental stage. In summer conditions, we found similar results to [50] and [51], namely that the early LIV-1 and LIV-2 substages are present in equal numbers throughout the semilunar cycles (Figure 1d). However, starting with the LIV-3 stage, all subsequent substages have a clear semilunar distribution with their maxima delayed in phase depending on the developmental stage (Supplementary Figure 3). This distribution can be interpreted as follows: each day of the semilunar cycle, the same number of LIV-1 turns into LIV-2. However, only on certain days of the semilunar cycle do some larvae of the LIV-2 stage enter the LIV-3 stage. Hence, during day 6 until day 10 of the semilunar cycle, the LIV-2 stage is most likely physiologically set to continue in development (Figure 1d, orange dots). In other words, semilunar days 6 to 10 mark the beginning of a developmental progression diagonal in our experiment, i.e. development is continuous from the LIV-3 til the prepupal starting with those semilunar days. In contrast during days 14 until 3 of the semilunar cycle no or few larvae will be LIV-3 the next day, i.e. developmental stages that are further progressed in imaginal disc development than the LIV-2 were found only in low numbers on subsequent days. Hence, those larvae most likely undergo a developmental arrest controlled by the circalunar clock (Figure 1d, red dots). We termed this arrest circalunar developmental arrest (CDA). Field evidence suggested that

the same larvae stage is also the overwintering stage [50]. However, this was not examined in the laboratory. We found that indeed the vast majority of the population overwinters in the LIV-2 stage (Figure 1e). In accordance with the suppressed adult emergence phenotype, no developmentally progressed larvae (LIV-4 and higher) were found. Notably a constant but low number of LIV-3 is present throughout the semilunar cycle.

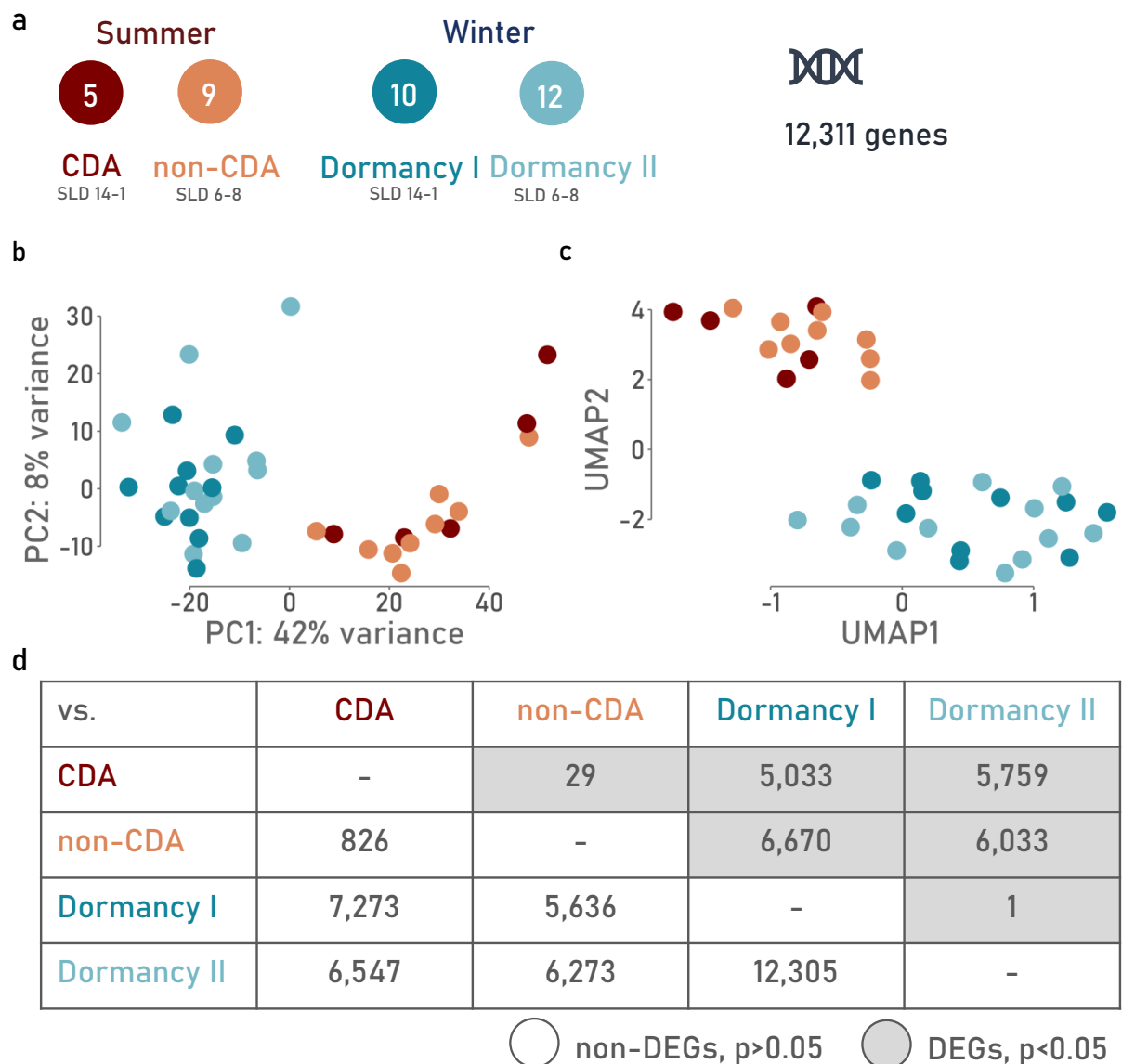


Figure 2: (a) 12 individual male LIV-2 were sampled in two conditions (summer and winter) at two different semilunar phases (SLD 14-1 and SLD 6-8). The numbers in circles indicate the number of samples remaining after quality filtering. For those, 12,311 genes were considered for differential gene expression analysis with DESeq2. (b-c) PCA and UMAP for the data set after applying a variance stabilizing transformation. (d) Pairwise comparisons of all four groups and by DESeq2 identified differential expressed genes (DEGs) and non differential expressed genes (non-DEGs) after removing 5 outlier genes (12,306 genes in total).

Transcriptomes are most different between summer and winter conditions rather than between groups within a condition

We sequenced mRNA from 12 individual male LIV-2 larvae sampled in two different semilunar phases under winter and summer conditions (Figure 1e,f, colored dots), i.e. four groups with 12 samples each. We conducted pairwise differential gene expression analysis for all four groups (CDA, non-CDA, dormancy I and dormancy II) using DESeq2. After filtering out samples with low raw read counts and low mapping rates (see Methods section), we kept 5 samples for CDA, 9 samples for non-CDA, 10 samples for dormancy I and 12 samples for dormancy II for downstream analysis (Figure 2a). The reference genome of *C. marinus* has 13,840 annotated gene models. We found reads mapping to 13,802 of them, indicating that the sequenced transcripts cover almost the entire transcriptome. 12,311 genes were kept for downstream analysis after filtering out genes with low sample coverage. Of those, five genes were identified as outliers by DESeq2, resulting in 12,306 genes either differential or non-differential expressed between groups. We verified that the DESeq2 model fits our data by plotting the dispersion parameter against the mean of normalized read counts for our data and the by DESeq2 estimated counts used for differential gene expression analysis (Supplementary Figure 4).

Samples are clearly separated by condition (PC1: 42% variance, PC2: 8%) based on PCA and UMAP (Figure 2b,c), suggesting that transcriptomes are different between summer and winter conditions (Figure 2d). Pairwise comparisons of mRNA levels between groups conducted with DESeq2 reveal that most genes between summer and winter conditions are differential expressed (Figure 2d, Supplementary Figure 5b-e, Supplementary Figure 6b-e) (5,033 to 6,670 genes depending on the comparison). Groups sampled in the same condition (CDA vs non-CDA in summer and dormancy I vs II in winter) are not clearly separated. In winter conditions we did not expect to see differences in the transcriptomes between semilunar phases, because we do not see a semilunar modulation of the substage distribution in winter and larvae are assumed to diapause regardless of the semilunar phase. PCA and UMAP confirm this assumption. The two winter groups dormancy I and II are essentially identical on the transcriptome level with only one gene found as differentially expressed (Figure 2d, Supplementary Figure 5f, Supplementary Figure 6f). This gene has no known function or annotation (10,057 of all genes in the *C. marinus* reference genome have annotated names predicted by homology [59]).

26 genes are differential expressed between CDA and non-CDA larvae

Under summer conditions, we expected to see a difference in the transcriptome between larvae in CDA and larvae in non-CDA, because the first is expected to arrest its development while the latter will physiologically progress in development. However, only 29 genes were found differential expressed with DESeq2 (Figure 2d, Supplementary Figure 5a, Supplementary Figure 6a). Notably only 860 genes were considered for statistical analysis by DESeq2. DESeq2 conducts independent filtering of genes, i.e. DESeq2 assumes that genes with low read counts usually have high dispersion and are excluded in order to maximize the statistical power for the remaining genes. For the pairwise comparisons CDA vs dormancy I, CDA vs dormancy, non-CDA vs dormancy I and non-CDA vs dormancy II, no genes were excluded (Supplementary Figure 7b-f). However, in the CDA vs non-CDA comparison 11,451 genes were removed as "low counts" (< 5,196 reads) based on the distribution of p-values (explanation in caption of Supplementary Figure 7a). Hence, only highly expressed genes were considered for differential gene expression analysis for this pairwise comparison. Interestingly, only 26 of those were also found significantly differential expressed by permutation procedure (Supplementary Figure 8). We conservatively therefore focus on only those for downstream analysis. When plotting the PCA based solely on the 26 candidate genes, 73% of variance is explained by PC1 and three main clusters, the winter, CDA and non-CDA cluster, can be distinguished (Figure 3a). The cluster containing all winter samples separates from the non-CDA cluster by PC2 with much lower variance (10% variance). Notably, larvae in CDA display the highest variance in gene expression and intragroup variance is higher in summer conditions than winter conditions.

Surprisingly, gene expression level is more similar between winter dormancy and non-CDA samples than between winter samples and CDA or CDA and non-CDA samples (Figure 3a-c). The vast majority of genes (18 of 26 candidates) are differential expressed between CDA and winter samples but not differential expressed between non-CDA and winter samples. 21 out of the 26 genes are down regulated in CDA samples compared to winter and non-CDA samples. All of the five in CDA samples up-regulated genes have an unknown function. One sample of the non-CDA group is more similar to the CDA group than to the other non-CDA samples (Figure 3b). Because we cannot track the individual fate of the larvae, it might be that this sample corresponds to a larvae in CDA, undergoing a developmental arrest. The distinction between CDA and non-CDA larvae is ultimately based on the population phenotype which clearly contains some noise (Figure 1e).

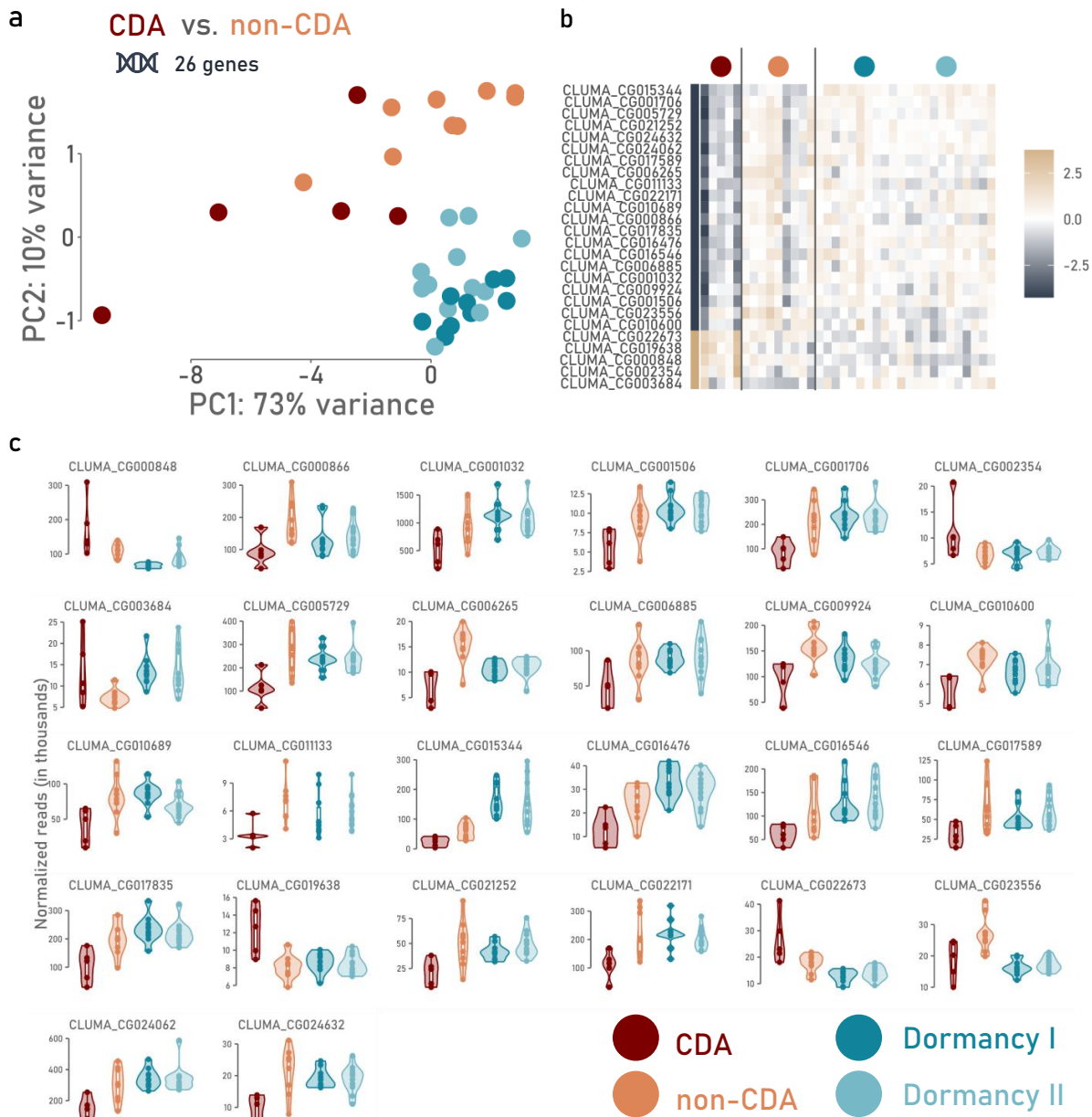


Figure 3: 26 genes were found differential expressed between LIV-2 in CDA and non-CDA by both permutation sampling procedure and DESeq2. (a) PCA of all four groups based on variance stabilizing transformed expression pattern of those 26 genes. (b) Heatmap of expression pattern scaled by gene for all 26 candidates. (c) Normalized number of reads in thousands for the 26 candidate genes for all four groups.

Candidate genes are mainly involved in muscle and glucose metabolism

To understand the biological role of the 26 candidate genes better, we conducted GO term analysis (Figure 4). Only 12 of the 26 candidate genes have an annotated GO term. Hence, we additionally consulted the annotated names of the CLUMA 2.0 reference genome. The GO terms were summarized further with rrvgo based on semantic similarity (Figure 4b, Supplementary Figure 9). Importantly, similar combinations of genes might have different annotated GO terms (Supplementary Figure 9). The parent GO terms with the highest number of genes associ-

ated with are related to nucleobase biosynthesis, glucose metabolism and muscles (Figure 4b). Eight genes (CG001032, CG001706, CG009924, CG016546, CG017589, CG024062, CG017835 and CG005729) have annotated functions in muscle constitution, development or contraction. Seven genes (CG001506, CG006265, CG006885, CG010600, CG016476, CG021252 and CG024632) are predicted to be involved in glucose and glycogen metabolism including connective pathways like glutamate biosynthesis to the citric acid cycle.

Intersect of pairwise group comparisons reveal gene sets unique to CDA and non-CDA samples

A set of 782 genes was found non differential expressed between CDA and winter samples but differential expressed with non-CDA samples (Figure 5a). Hence, these genes have a distinct normalized read count only in non-CDA samples. PCA based solely on this genes set reveals that winter, CDA and non-CDA samples can be mostly separated by PC1 with CDA samples in between non-CDA and winter samples (Figure 5c). Heatmaps of scaled normalized read counts indicate that the gene expression level for those genes are intermediate in the CDA group compared to the winter and non-CDA group (Figure 5a). GO term analysis identifies most genes to be involved in multi-organism reproductive processes and cellular macromolecule biosynthetic processes (Figure 5e). A smaller gene set of 288 genes are non differential expressed between winter and non-CDA samples but differential expressed with CDA samples (Figure 5b), i.e these genes are only differential expressed in CDA samples (note that this does not mean that the genes are differential expressed between non-CDA and CDA samples). PCA reveals that PC1 separates CDA samples from a cluster containing winter and non-CDA samples (Figure 5d). Hence, this gene sets distinguishes mainly CDA from winter and non-CDA samples but not clearly between winter and non-CDA samples. Again, one larvae belonging to the non-CDA group is transcriptomically most similar to the CDA group. GO term analysis reveals that most of these genes are involved in purine metabolism, aging and the break down of proteins (Figure 5f).

Discussion

In this manuscript we monitored the adult eclosion rhythm and the distribution of 4th instar substages of the marine midge *Clunio marinus* over the semilunar cycle under summer and dormancy-inducing winter conditions. *C. marinus* undergoes CDA in order to synchronize development on the population level with the semilunar cycle. Based on imaginal disc morphology, we found that the same developmental stage that arrests its development over the semilunar cycle under summer conditions also undergoes overwintering diapause. This ob-

a CDA vs. non-CDA

26 genes

* Annotated GO term

Gene ID	Annotation
CLUMA_CG000848	NA
CLUMA_CG000866	(mn)_Sarco/endoplasmic_reticulum_Ca(2+)-ATPase_(SERCA)
CLUMA_CG001032	(mn)_Actin_5_(Act5)
CLUMA_CG001506*	(sp)_putative_Aspartate_aminotransferase,_cytoplasmic
CLUMA_CG001706*	(sp)_putative_Myosin_light_chain_alkali
CLUMA_CG002354	NA
CLUMA_CG003684	NA
CLUMA_CG005729	(mn)_Tropomyosin_1_(Tm1)
CLUMA_CG006265*	(sp)_putative_Phosphoglucosyltransferase
CLUMA_CG006885*	(sp)_putative_Enolase
CLUMA_CG009924	(pfam)_Myofilin_domain_containing_protein
CLUMA_CG010600*	(sp)_putative_Succinate--CoA_ligase_[ADP/GDP-forming]_subunit_alpha,_mitochondrial
CLUMA_CG010689	(sp)_putative_Fructose-bisphosphate_aldolase
CLUMA_CG011133*	(sp)_putative_E3_ubiquitin-protein_ligase_lubel
CLUMA_CG015344	(pfam)_DUF243_domain_containing_protein
CLUMA_CG016476*	(sp)_putative_Triosephosphate_isomerase
CLUMA_CG016546*	(sp)_putative_Paramyosin,_long_form
CLUMA_CG017589*	(sp)_putative_Alpha-actinin,_sarcomeric
CLUMA_CG017835*	(sp)_putative_Troponin_I
CLUMA_CG019638	NA
CLUMA_CG021252*	(mn)_glycosyltransferase_glycogen_phosphorylase_(glyp)
CLUMA_CG022171	NA
CLUMA_CG022673	NA
CLUMA_CG023556	NA
CLUMA_CG024062	(sp)_putative_Troponin_T,_skeletal_muscle
CLUMA_CG024632*	(sp)_related_to_Pyruvate_kinase_CLUMA_CG024632

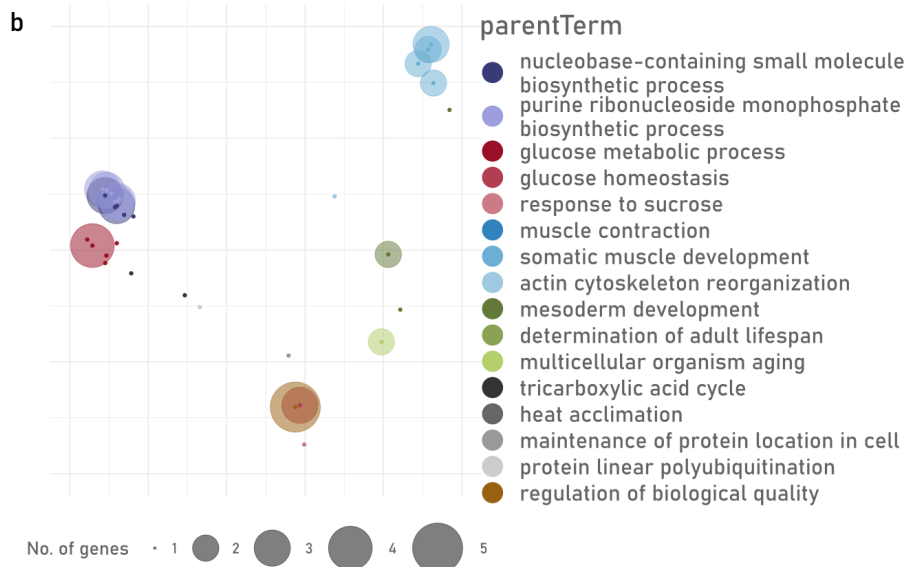


Figure 4: Annotation and GO term analysis of 26 candidate genes found to be differential expressed by permutation procedure and DESeq2. (a) Gene ID and annotation of the candidate genes based on the reference genome CLUMA 2.0. The gray stars mark genes with annotated gene ontology term which were considered by gene ontology term analysis. (b) Parent terms summarized with rrvgo for identified gene ontology terms. Transparent circles encode the number of genes belonging to the individual GO term called by topGO and colors encode to which parent term the individual terms belong.

servation was obtained in the laboratory for the first time and is in accordance with field observation [50]. Diapause is usually confined to a specific developmental stage [39]. Considering that CDA and diapause happen in exactly the same developmental stage in *C. marinus*, we examined if physiological similarities exist between the two arrests using a Transcriptomics approach. Similarities in gene expression patterns could reveal candidate genes which in the future may be tested with functional validation techniques including knockdown (RNAi) and knockout (CRISPR-Cas9). Further, diapause in *Clunio* has not yet been studied on the molecular level. Therefore, based on the obtained distribution of 4th instar substages, we sequenced mRNA of individual males in the LIV-2 stage in two conditions (summer and winter) and at two semilunar phases.

Key enzymes of glucose metabolism are down regulated in CDA compared to non-CDA larvae

Interestingly, transcriptomes of larvae in CDA and non-CDA are very similar with only 26 genes found as differential expressed. Strikingly, almost all of those genes are involved in either muscle or glucose metabolism. Notably, we sequenced the transcriptomes of whole larvae containing a mix of all tissues. We therefore expect that the identified transcriptomic signatures are mainly driven by highly expressed genes of tissues that constitute most parts of the larvae. These tissues include the fat body, the gut and muscles. Sequencing whole larvae points to the general transcriptomic state of CDA rather than to the underlying mechanism. The latter would require tissue specific sequencing of organs associated with the control of development, e.g. the brain or hormone producing glands. The observation that the coding transcriptome of CDA and non-CDA larvae are so similar could mean that physiological changes are not occurring on the transcriptional but rather post-transcriptional or translational level. However, the genes found as differential expressed encode key enzymes in the conversion of glucose to pyruvates (glycolysis) including the penultimate and ultimate step. This might indicate that differences in glucose metabolism and potentially energy demand exist between arresting and developing larvae. Candidate genes encode the enzymes *enolase*, which catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate and *pyruvate kinases* which catalyzes the phosphorylation of phosphoenolpyruvate to adenosine diphosphate. Further, two enzymes encoded by the *triosephosphate isomerase* and the *fructose-bisphosphate aldolase* genes, catalyze the conversion of D-glyceraldehyde 3-phosphate and fructose 1,6-bisphosphate, respectively, to Dihydroxyacetone phosphate. The latter is a substrate for triglyceride synthesis. Additionally, the gene encoding for *cytoplasmic aspartate aminotransferase* was found differential expressed. This enzyme catalyzes the interconver-

sion of aspartate to oxaloacetate, a substrate for the citric acid cycle. Finally, the enzyme encoded by *glycosyltransferase glycogen phosphorylase* converts glycogen during glycogenolysis to glucose.

Hence, most genes that are differentially expressed between CDA and non-CDA larvae are involved in carbohydrate metabolism and its intermediary with cellular respiration. Strikingly, we find all those enzymes less expressed in CDA compared to non-CDA larvae. Glycogen is the primary carbohydrate source of energy for insects and phosphorylase activity in the fatbody increases during larval development [68]. The higher expression level of the gene in non-CDA samples might be caused by the fact that those larvae have a different energy demand as they will progress in development shortly. Indeed, when comparing expression levels of the enzyme throughout development of the 4th instar, we see an increase in mRNA level with sub-stage (Supplementary Figure 10). Glycogen phosphorylase is also higher expressed in winter samples compared to the CDA group which could be because carbohydrate reserves might be mobilized by glycogen exploitation in diapause [68]. Hence, non differential mRNA levels of genes between non-CDA and winter samples might be caused by similar energy demands for different reasons. Indeed, in the gene set containing 288 genes that are differential expressed between CDA and winter samples but non differentially expressed between non-CDA and winter samples, enriched GO terms include "protein localization of lipid droplets", "gluconeogenesis", "glucose homeostasis" and pathways associated with the break down of proteins and peptides. As diapause in insects is fueled through lipid metabolism and maintenance of diapause characterized by exploitation of energy reserves [69, 31], this might indicate that diapausing and non-CDA larvae require similar carbohydrate metabolic substrates. However, measuring metabolites, including amino acids, proteins and lipids, for example with liquid or gas chromatography-mass spectrometry (LC-MS or GC-MS) are necessary to understand exactly if and which metabolic pathways are different in CDA compared to non-CDA and diapausing larvae.

Genes associated with muscle and cytoskeletal reorganization and calcium homeostasis have lower mRNA levels in CDA compared to non-CDA samples

About half of the genes that were found differential expressed between CDA and non-CDA samples are associated with cytoskeletal reorganization and calcium dependent muscle regulation. Genes encoding components of the thick filaments (*myosin alkali light chain* and *paramyosin*) as well as the thin filaments (*troponin I*, *troponin T*, *tropomyosin*, *actin*) are lower expressed in CDA larvae compared to non-CDA and winter samples. Further, the gene encod-

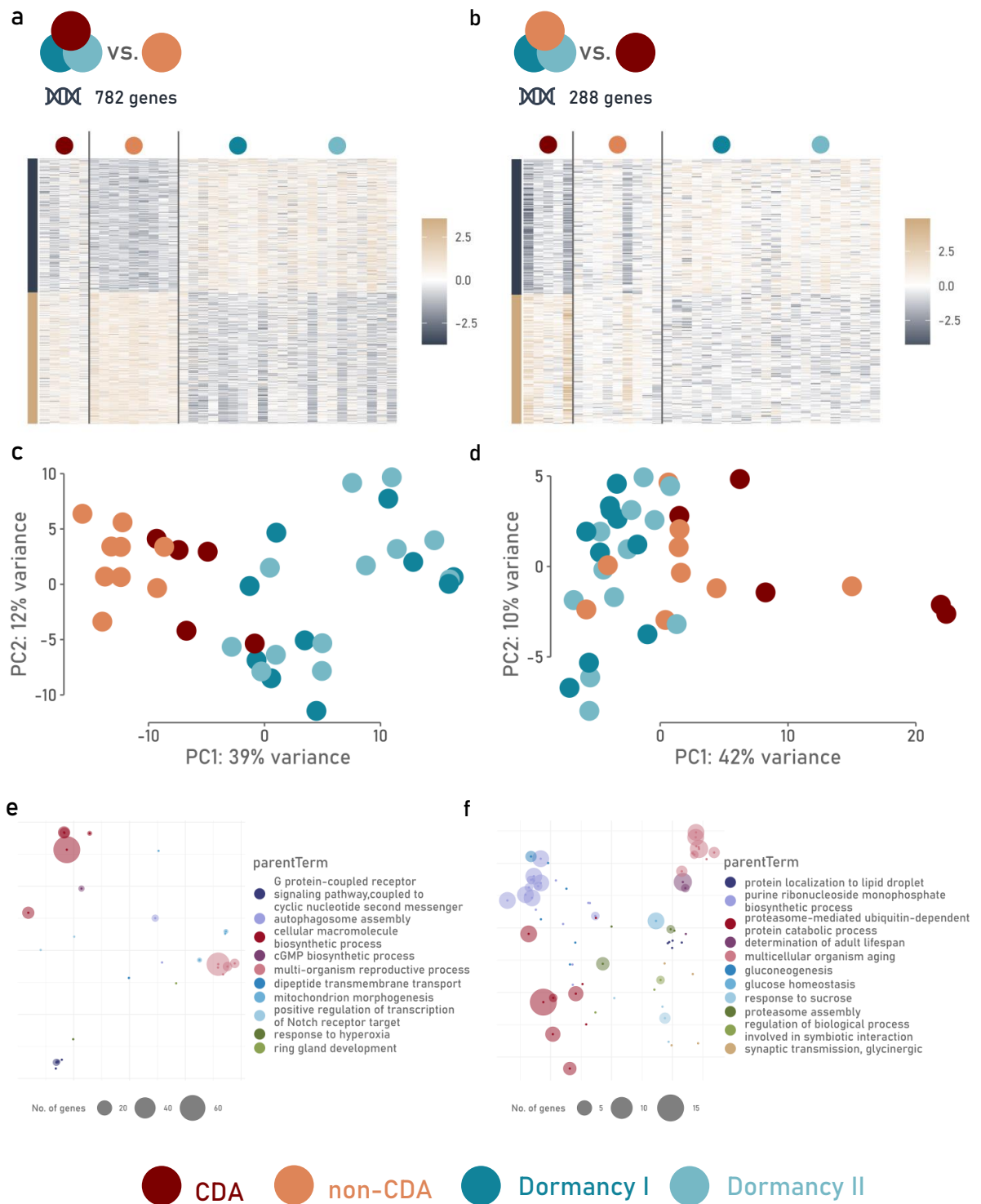


Figure 5: Two sets of genes with a unique expression pattern in either non-CDA (a,c,e) or CDA (b,d,f) samples were identified. The non-CDA specific set contains 782 genes similar in expression between CDA and winter samples but different to non-CDA samples. The second set contains 288 genes similar in expression between non-CDA and winter samples but different to CDA samples (b,d,f). (a-b) Heatmap of expression patterns scaled by gene both sets. (c-d) PCA of all four groups based on variance stabilizing transformed expression pattern for both gene sets. (e-f) GO term analysis for both sets of genes. Parent terms were summarized with rrvgo for the by topGO identified gene ontology terms. Transparent circles encode the number of genes belonging to the individual GO term called by topGO and colors encode to which parent term the individual terms belong.

ing for *actinin* is differential expressed between CDA and non-CDA larvae. Numerous transcriptomic screens identified genes associated with the cytoskeleton in diapausing insects [70, 71]. In fact, structural changes of the cytoskeleton in cold-acclimated crickets might combat the depolymerising effects of cold induced shock [72]. *Actin* genes are upregulated in diapausing adults of the house mosquito *Culex pipiens* [73, 74] and play different role throughout development in insects [75]. Tentatively, cytoskeletal depletion might save energy resources in diapausing *Daphnia* embryos [76].

Interestingly, the gene encoding for the Sarco/endoplasmic reticulum Ca(2+)-ATPase (SERCA) has an intermediate mRNA level in CDA samples compared to non-CDA and winter samples. The calcium pump is important for calcium homeostasis of the endoplasmatic reticulum and lipid storage in *Drosophila* [77, 78]. Impaired activity of SERCA reduced the fat storage in adipose tissue of *Drosophila* [79]. Calcium signalling plays a role in the reproductive diapause of the spider mite *Tetranychus urticae* [80] and calcium might act as a second messenger in the prothoracic glands, regulating development [81]. Further, SERCA activity was suppressed during overwintering conditions in the moth *Epiblema scudderiana* and gall fly *Eurosta solidaginis* [82]. In line with that, we found lower gene expression levels of SERCA in CDA and diapausing *C. marinus* larvae compared to non-CDA larvae. In summary, genes encoding key players in cytoskeleton constitution are differential expressed between CDA and non-CDA larvae but more similar between non-CDA and winter samples, i.e. they are differential expressed between CDA and non-CDA samples and between CDA and winter samples, but not differentially expressed between non-CDA and winter groups. SERCA might be a promising candidate to understand if and how development is regulated in the prothoracic gland of *C. marinus*.

Future work may focus on ecdysteroid metabolism and its regulation over the semilunar cycle

Insect development and metamorphosis is coordinated by hormones belonging to the ecdysteroids family, including ecdysone and its active form, 20-hydroxyecdysone, as well as sesquiterpenoids including juvenile hormones [83]. In fact, ecdysone is essential for progression of development through larval molt in all holometabolous insects [84]. Further, a link between ecdysteroid-related genes and diapause has been found in the drosophilid fly *Chymomyza costata* [85]. Ecdysone is synthesized from cholesterol via multiple conversion steps. These steps are catalyzed by conserved enzymes encoded by the so-called *halloween genes*. The initial signal for ecdysone release comes from four neurons which produce the prothoracicotrophic hormone (PTTH). PTTH then stimulates production of ecdysone in the prothoracis

gland. Ecdysone is subsequently converted into its active form in the hemolymph. In contrast, juvenile hormones are secreted by the corpus allatum and usually prevent larvae from undergoing metamorphosis [86].

Ecdysteroids play an important role in development of *C. marinus* peaking during the pupal stage [51] as seen in other insect species [84]. However, ecdysone is further regulated throughout the semilunar cycle in the early 4th instar [51]. In the particular substage (LIV-2) that undergoes CDA, ecdysteroids peak during specific days of the semilunar cycle. Further the ratio of 20-hydroxyecdysone to ecdysone is lower in early compared to late 4th larval instars [51]. Considering the highly conserved role of ecdysone throughout insect development and its correlation with the semilunar cycle in *Clunio*, pathways underlying its regulation may be promising candidates downstream of the circalunar clock to study. We find a handful genes related to ecdysteroid regulation in genes differential expressed between non-CDA and winter samples but not between CDA and winter samples suggesting that they might contribute to differences between CDA and non-CDA samples. These include genes encoding for *eclosion hormone*, which has been found to form a positive feedback loop with ecdysis triggering hormones [87] and the cytochrome P450 enzyme *spook*, which is involved in the black box reaction of ecdysteroid biosynthesis, i.e. the conversion of cholesterol to 5-beta-ketodiol. Interestingly, differences in gene expression levels were also found in *juvenile hormone acid O-methyltransferase*. The encoded enzyme converts the precursor of juvenile hormone into its active form [88], making it a strong candidate to study on the protein and enzymatic level. Additionally, two ecdysteroid 22-kinase domain containing proteins were found differential expressed. Both convert free ecdysteroids to physiologically inactive ecdysteroid 22-phosphates in ovaries of the silkworm *Bombyx mori* [89]. Strikingly, expression level of the gene encoding for CYP18A1 is more similar between non-CDA and winter samples than between CDA and winter samples. CYP18A1 is a key enzyme for steroid hormone inactivation in *Drosophila* [90]. Why it is lower expressed in the CDA group remains to be studied. Likewise, the gene encoding for *hormone receptor 4* has a higher mRNA count in CDA samples compared to all other groups. The receptor controls steroid hormone pulses in *Drosophila* [91] and is involved in ecdysteroidogenesis in coleoptera [92].

In general, GO terms highly relevant for larval development and metamorphosis were found enriched for the gene set that is uniquely differential expressed in non-CDA samples. "G protein-coupled receptor signaling pathway coupled to second messenger" is one of them and relevant because calcium was found as a second messenger regulating ecdysteroid synthesis

[83]. Some of the genes are associated with the GO term "positive regulation of transcription of notch receptor targets". Notch signaling plays a role in the development of imaginal discs [93]. Two genes *wnt-2* and *wnt-5* have similar mRNA levels in CDA and winter samples but are differentially expressed between winter and non-CDA samples. The Wnt pathway is a highly conserved developmental pathway and core components were found differentially expressed between diapausing and non-diapausing samples in multiple insect species [94].

Limitations of this study

The transcriptomic profiling of the circalunar developmental arrest and diapause in *C. marinus* conducted for this manuscript is only a starting point to characterize these physiological arrests comprehensively. In fact, although mRNA levels of some tissue-specific enzymes (e.g. halloween genes) correlate well with the level of ecdysteroids in insects [95] and gene expression levels of *krüppel homolog 1* can be used as a read-out for juvenile hormone presence [96], global mRNA levels may not be informative for characterizing the potential differences in hormone composition and quantity between the here examined groups. This is important considering that progression in development and developmental arrests in insects are mainly driven by hormones potentially activated or inactivated on the post-transcriptional level. Hence, techniques capturing those post-transcriptional changes may be conducted in larvae undergoing CDA, non-CDA and winter dormancy. This includes proteomic studies and mass spectrometry to measure ecdysteroids and juvenile hormones composition in the different developmental arrests. Further, many enzymes and peptides involved in ecdysteroid synthesis and signaling are locally expressed, sometimes in only a few cells (e.g. PTTH). Hence, tissue specific sequencing, e.g. of the prothoracic gland or the corpus allatum, are necessary to draw conclusions for the underlying regulatory mechanism. Further, recent advances have been made in uncovering the role of regulatory RNA (miRNA) in insect metamorphosis and diapause [97] and future efforts may include those.

Imaginal disc morphology was used to determine the development of larval stages in order to capture the switching point in which *Clunio* either progresses or arrests its development. However, progression in imaginal disc growth is a continuous process and morphological landmarks were used to essentially discretize development. E.g. this could mean that some of the early LIV-3 stages may actually be considered late LIV-2 or vice versa, adding unintended, biological variation to the data. Further, it is not known yet, if and to which extent the morphological distinction of imaginal discs in *Clunio* corresponds to transcriptomic differences caused by physiological changes. It will be therefore necessary to sequence the transcrip-

tomes of larvae by substage. Within the framework of the here presented study, we did obtain and sequenced these samples. However, the analysis has to be conducted yet. It will be interesting to examine how mRNA levels of genes differ between CDA and non-CDA samples in the context of their gene expression levels throughout the entire subsequent larval development. Can we identify genes which are correlated with development in *Clunio* but are uniquely different in larvae undergoing CDA? We could further track transcriptomic changes of the early 4th instar in *Clunio* throughout the semilunar cycle in a time series as well as the transcriptomes of the developmental diagonal, i.e. the progression of development from the LIV-3 to the LIV-9 stage. This could add a better resolution to how this developmental progression is correlated with the semilunar cycle and to ultimately disentangle genes involved in development vs genes that are controlled by the circalunar clock directly.

We further acknowledge that the probably more clean experimental design of the study would have been to run both summer and winter conditions, including the phenotyping of adult emergence and substaging of larvae, simultaneously. This means that the difference in mRNA levels between summer and winter conditions may be partially driven by the differences in the experimental run and therefore may decrease the differences between groups within a condition. Additionally, the high variance of samples within the CDA group needs to be investigated further. It may be problematic that this group (CDA), which was the focus of this study, has the smallest number of samples (five). Though, it was methodologically not possible to use pools of samples, i.e. multiple larvae per replicate, obtaining transcriptomes of *single* larvae may have introduced additional variation.

Is the circalunar counter derived from the photoperiodic counter in *Clunio*?

The functional principle of the circalunar clock in *Clunio* is a circalunar counter based on circadian oscillations (Chapter 3). This suggests that transcriptomic changes affecting the period of the circadian clock will influence the circalunar clock and therefore developmental timing, including CDA. It might be worth noting that one important core component of the circadian clock has been found differentially expressed between non-CDA and winter samples but not between CDA and winter samples. The gene *clockwork orange* encodes a protein that acts as a transcriptional repressor of CLOCK mediated gene expression activation which results in lengthening of the circadian period [98, 99, 100]. Interestingly, expression level of the core component *clock* is not differentially expressed between non-CDA and winter samples but differentially expressed between CDA and winter samples. Additionally, we found *casein kinase 1* differentially expressed between non-CDA and winter samples but not between CDA and win-

ter samples. CK1 or double-time can alter the circadian period in mammals and *Drosophila* [101, 102]. Tidal rhythms are likewise affected by pharmacological inhibition of CK1 [103] while the lunar spawning rhythm of the marine annelid *Platynereis dumerilii* is not [104]. Hence, *casein kinase 1* may play a central role in biological timing on various scales and is a candidate for functional validation of the circalunar clock in *Clunio*. Because marine midges evolved on land, where photoperiodic counters are prevalent, we hypothesized that the circalunar counter could be in fact derived from a photoperiodic counter [45]. However, a link can only be established when core components of the molecular mechanism of both, the circalunar clock and the photoperiodic counter in *Clunio*, are sufficiently established through functional validation techniques.

Diapause is arguably the most significant synchronizing element in the life cycles of insects and probably the most studied dormancy response [105]. We hypothesized in the beginning that the transcriptomic state of larvae undergoing CDA might be somewhat similar to a "mini-diapause" or state of quiescence. However, most genes that are significantly different in their mRNA level between CDA and non-CDA samples are not differential expressed between non-CDA and winter samples. Though we have some explanations for individual genes (see above), this overall trend remains to be explained. As pointed out before diapause is characterized by distinct phases that differ in transcriptomic profiles and metabolic footprint. We did not know in which diapause stage the larvae were when we sequenced them. Though most of them are probably in the maintenance phase, the immediate emergence peak after the switch to summer conditions might suggest that some of them could be in a quiescence already. Quiescence is the preparation phase before continuation of development. Hence, transcriptomically they could have similar energy demands. Further, CDA is occurring over a much shorter time period compared to diapause, i.e. two weeks is the minimum period larva undergo a developmental arrest considering that the duration of the semilunar cycle is 15 days. Finally, it is unknown if and how much larvae feed during CDA and diapause. Different food intakes between the arrest might result in vastly different energy demands and expression profiles. Notably, the variance in expression based on PCA for the 26 candidate genes found differential expressed between CDA and non-CDA samples is very high in CDA compared to the non-CDA and winter samples (3a). This could indicate that expression of those genes are less restricted in CDA samples and more tightly coordinated in non-CDA and winter samples. However, further experiments are required to confirm this observation.

Conclusion

Clunio marinus is an excellent model species to study a developmental arrest governed by a non-24 hour clock, namely the circalunar clock. In this manuscript, we examined the coding transcriptomes of both the circalunar developmental arrest (CDA) and diapause in the marine midge under the hypothesis that CDA could be physiologically a mini-diapause. Interestingly, the transcriptomes of developing vs CDA larvae are very similar with key differences in glucose metabolism and cytoskeleton constitution. A set of 782 genes is differentially expressed between developing and diapausing larvae but not differentially expressed between CDA and diapausing larvae and contains genes which have an intermediate mRNA level in CDA samples compared to all other groups. Tentatively, this could indicate that expression profiles of these genes may contribute to the physiology of the circalunar developmental and diapause in the marine midge compared to developing larvae.

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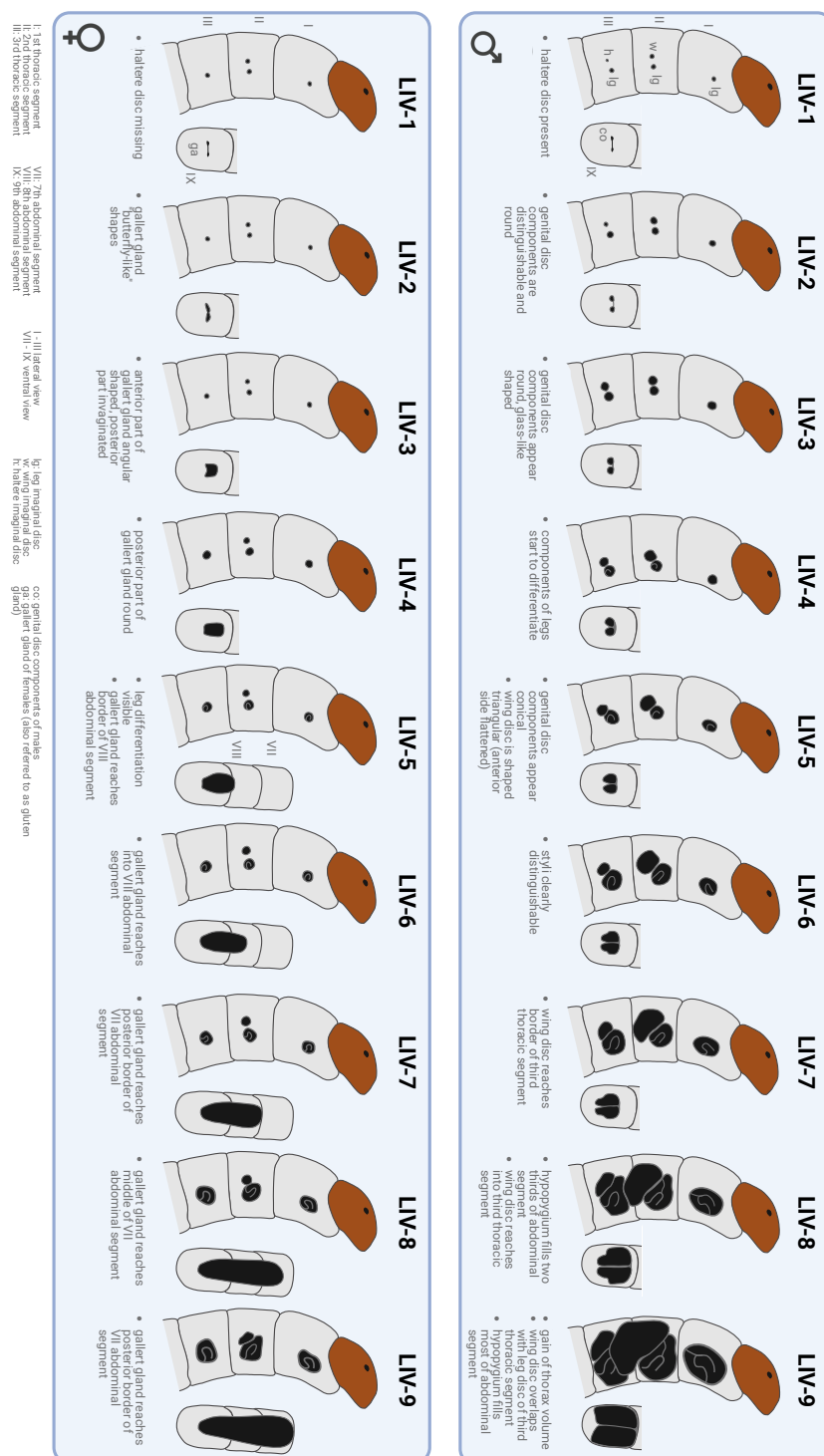
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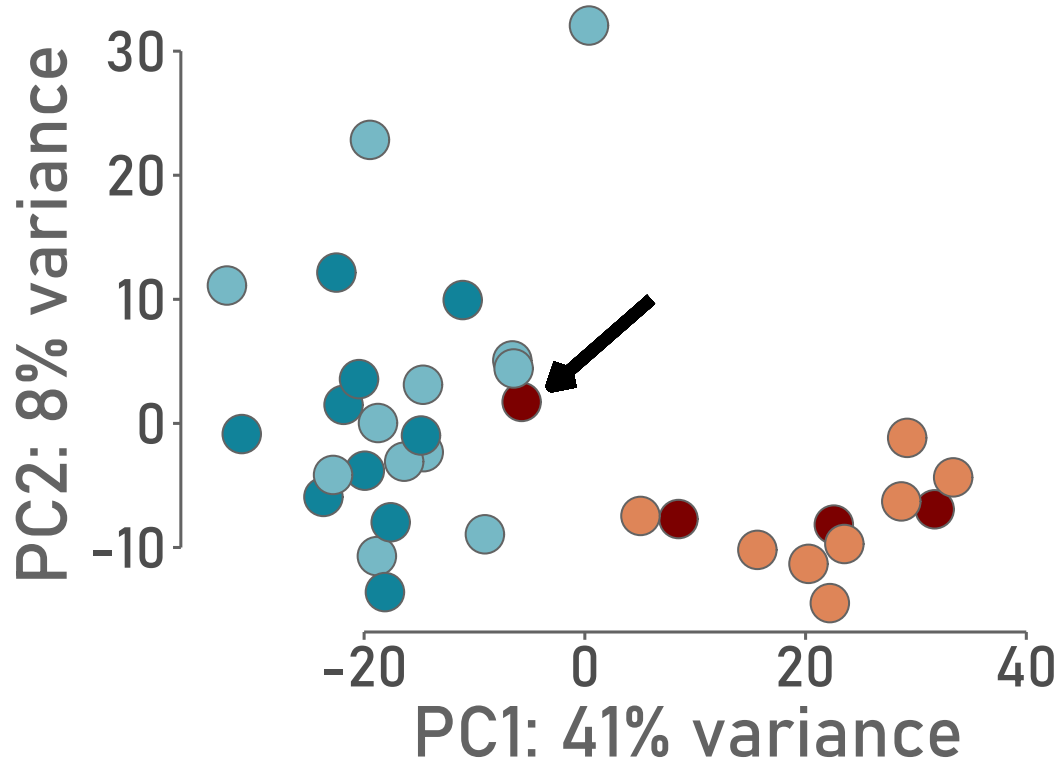
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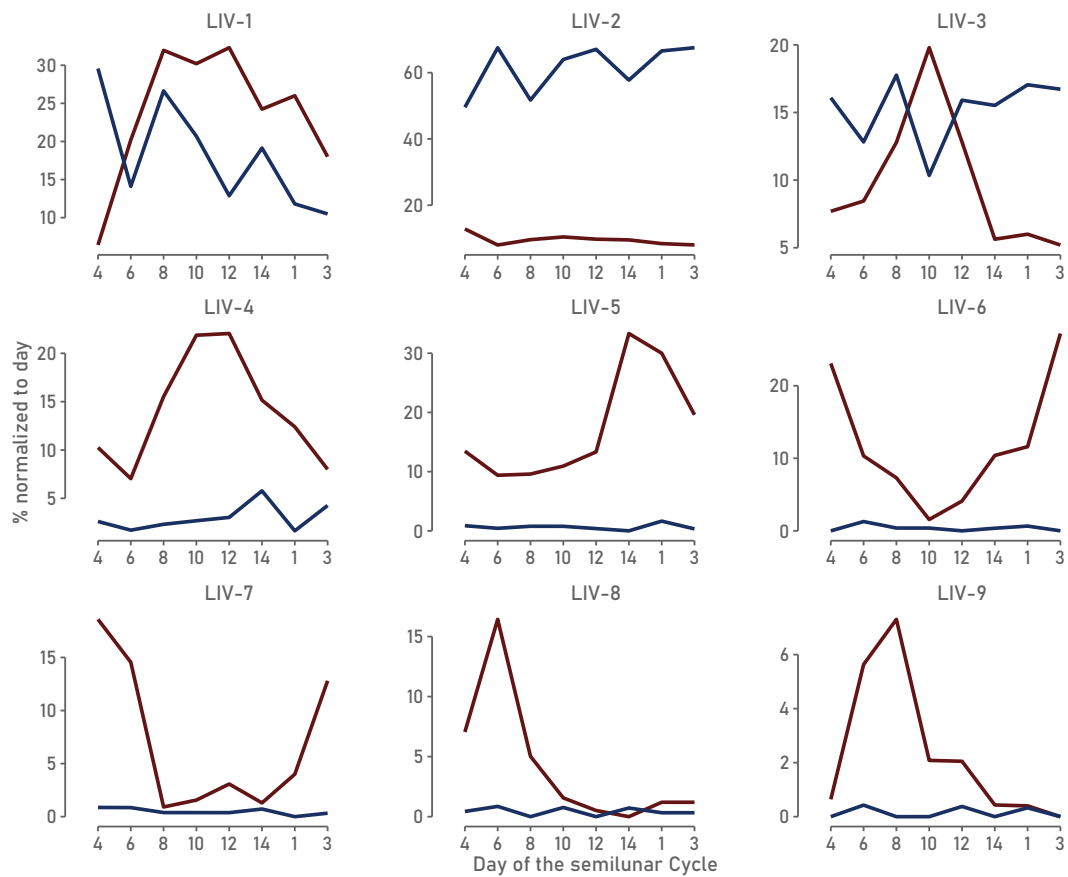
Supplementary Figures



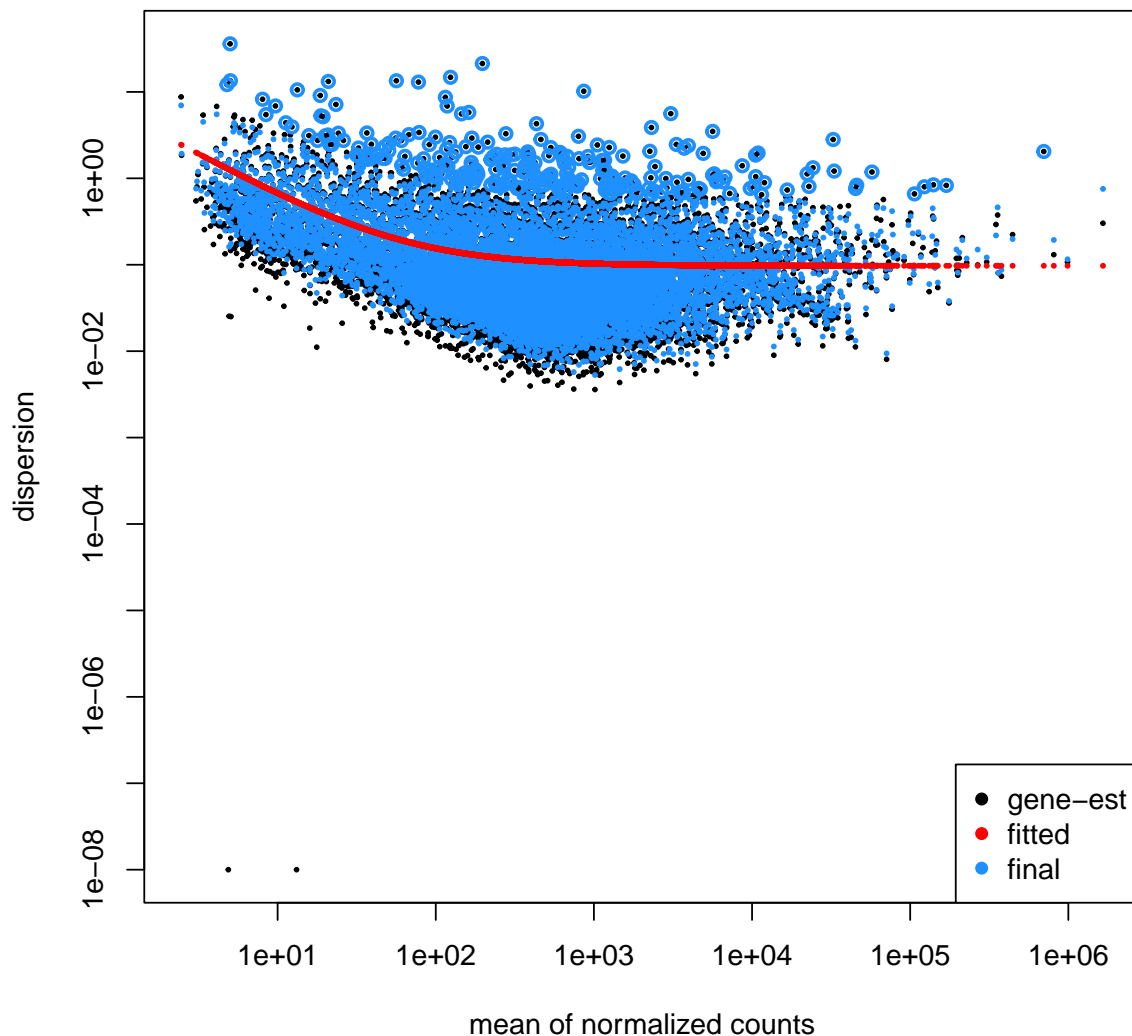
Supplementary Figure 1: 4th instar larvae of *C. marinus* were classified into nine substages (LIV-1 until LIV-9) based on imaginal disc development. The classification was based on [56, 51, 50]. Imaginal discs were fixated in absolute ethanol.



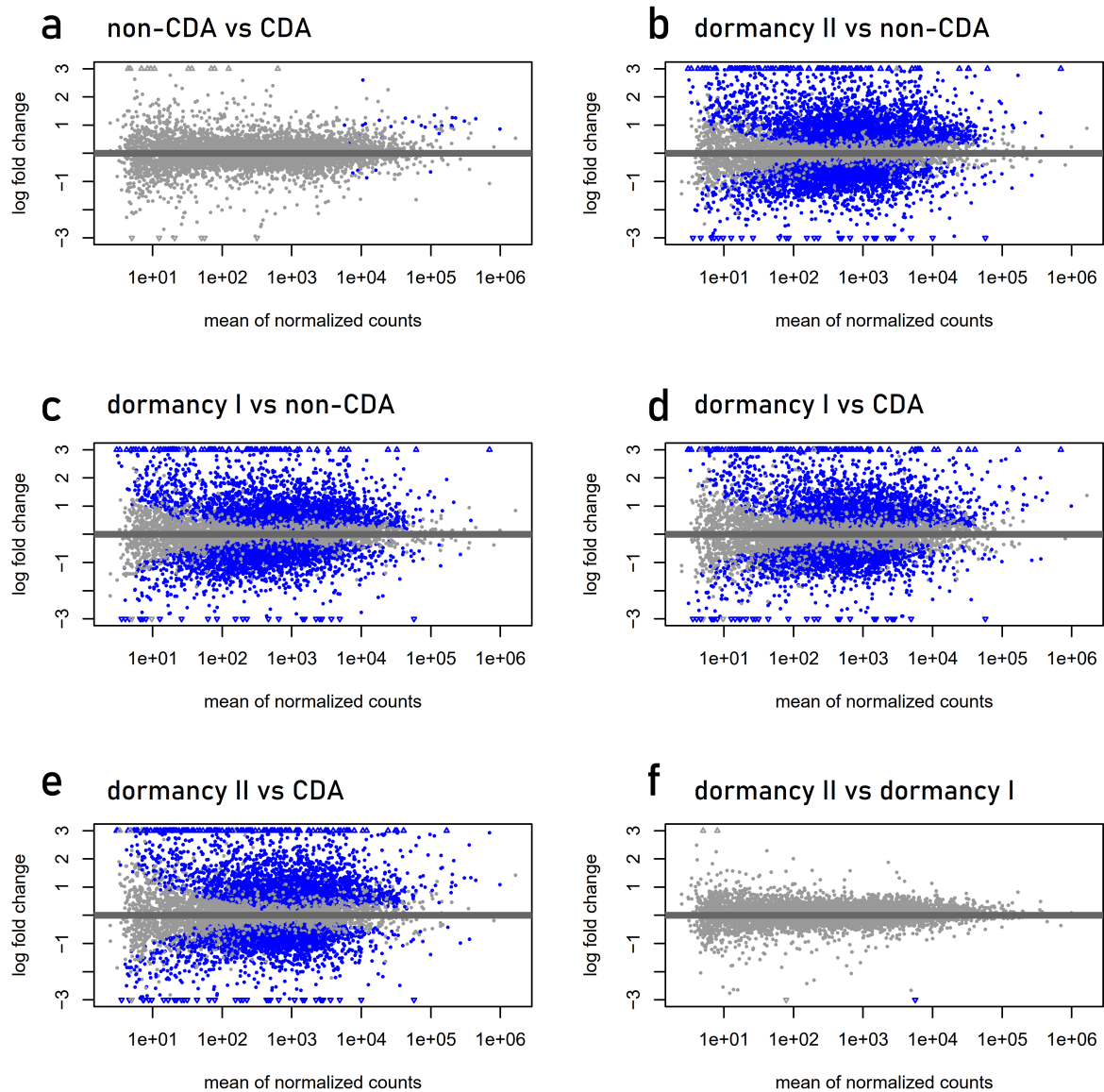
Supplementary Figure 2: PCA of 37 samples. Reads were normalized with the variance stabilizing transform. The arrow marks the sample which was removed as an outlier.



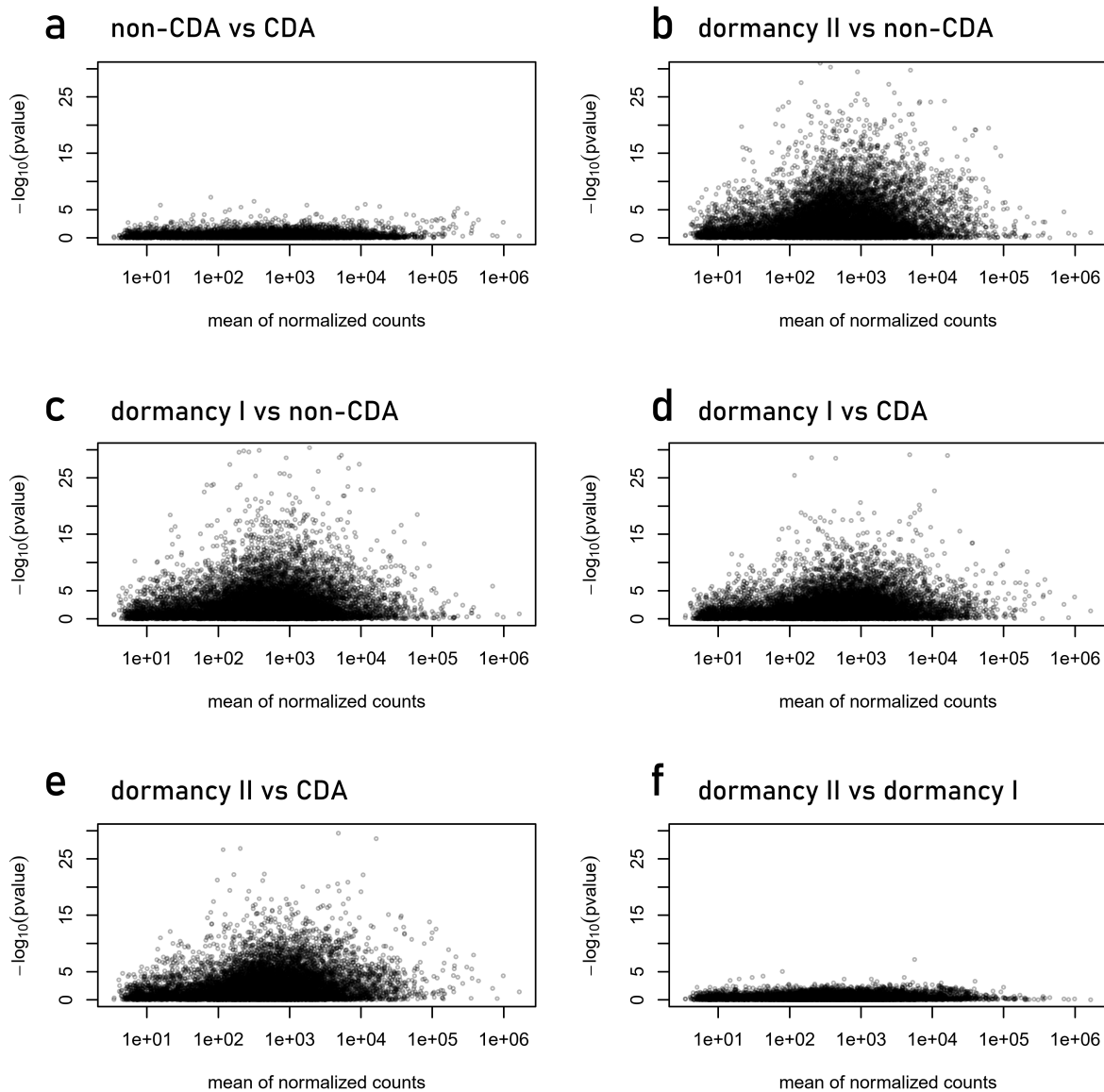
Supplementary Figure 3: Percentage of 4th instar substages of *C. marinus* over the semilunar cycle. The red line indicates the distribution obtained in summer conditions and blue indicates the distribution obtained under winter conditions.



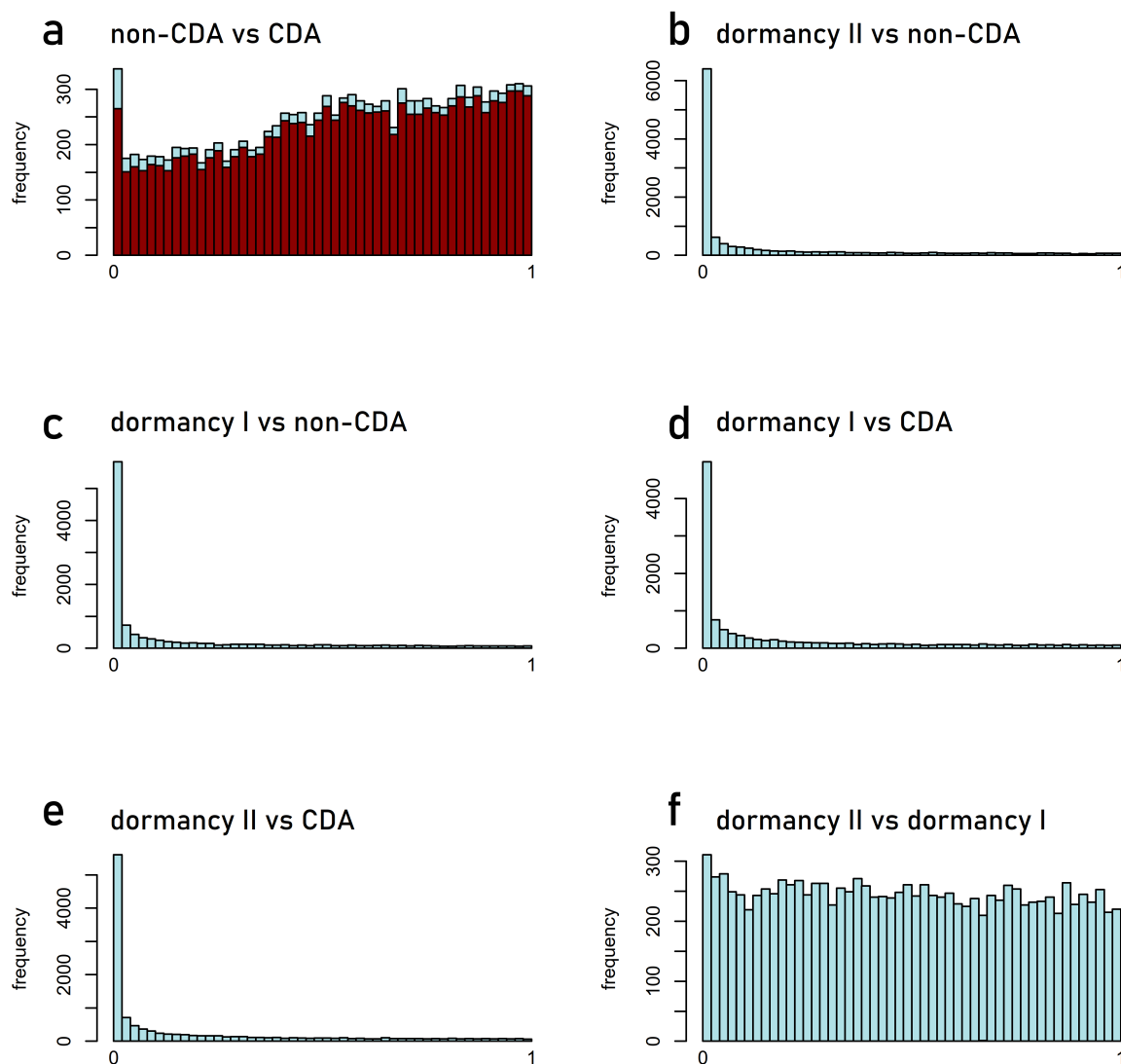
Supplementary Figure 4: Within group variability is modeled by a dispersion parameter in DESeq2. Black dots indicate from the data estimated gene dispersion (Maximum likelihood estimate). The red line is the fitted dispersion value based on the MLE and mean of normalized counts. DESeq2 generally assumes that variance in gene expression is correlated with the mean expression level. Blue indicates the final estimate of dispersion used by DESeq2 for further analysis. For genes with a high deviation from the fitted dispersion value, the MLE values is kept (blue circles). Overall, the data fits the DESeq2 model well.



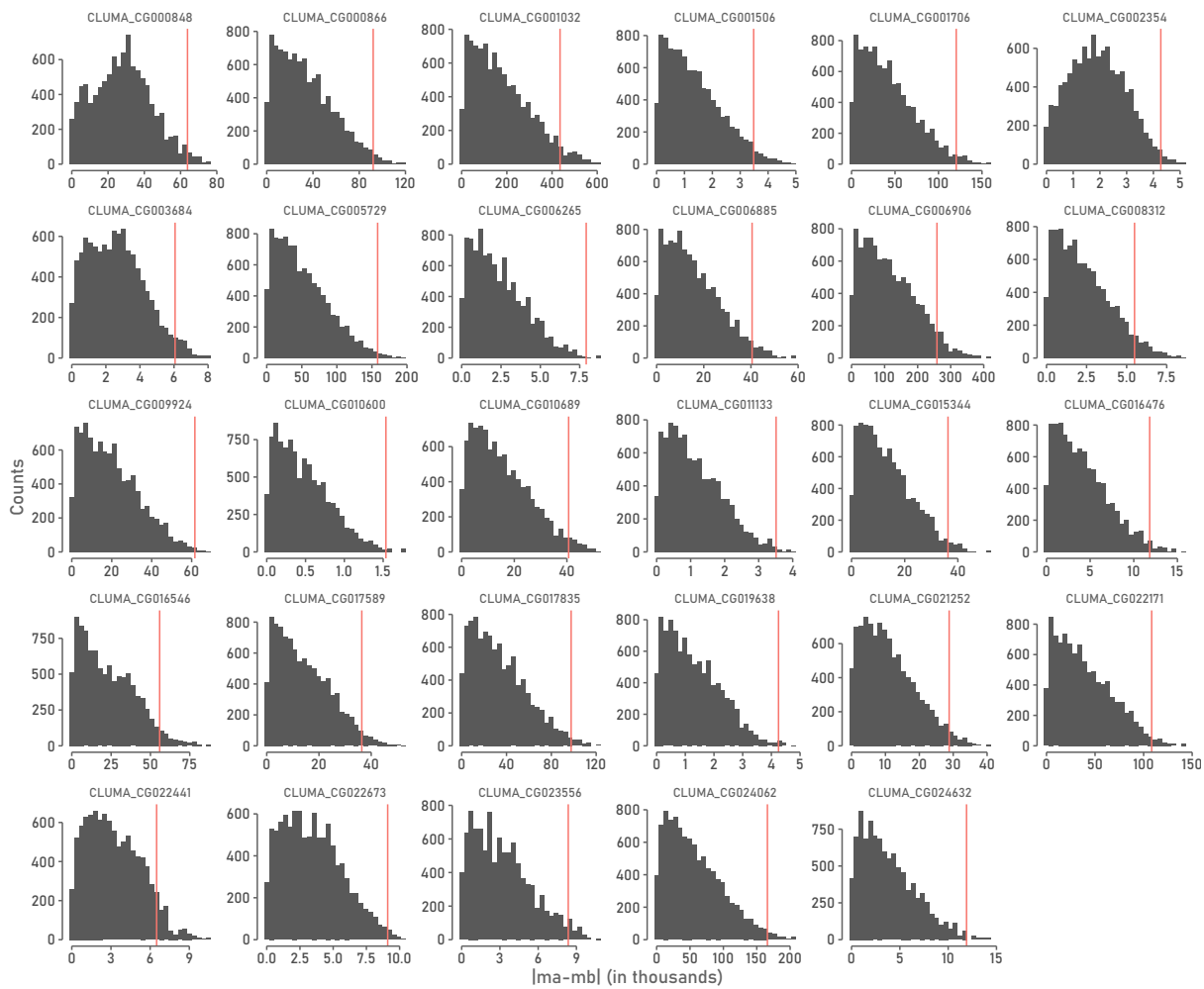
Supplementary Figure 5: By DESeq2 estimated log fold change plotted against the mean of normalized read counts for all pairwise group comparisons. Blue dots indicate genes which are found significantly differential expressed. (a,f) Comparisons between groups of the same condition (a: summer or f: winter) have only few differential expressed genes. (b,c,d,e) Comparisons of groups between conditions result in a large number of differential expressed genes.



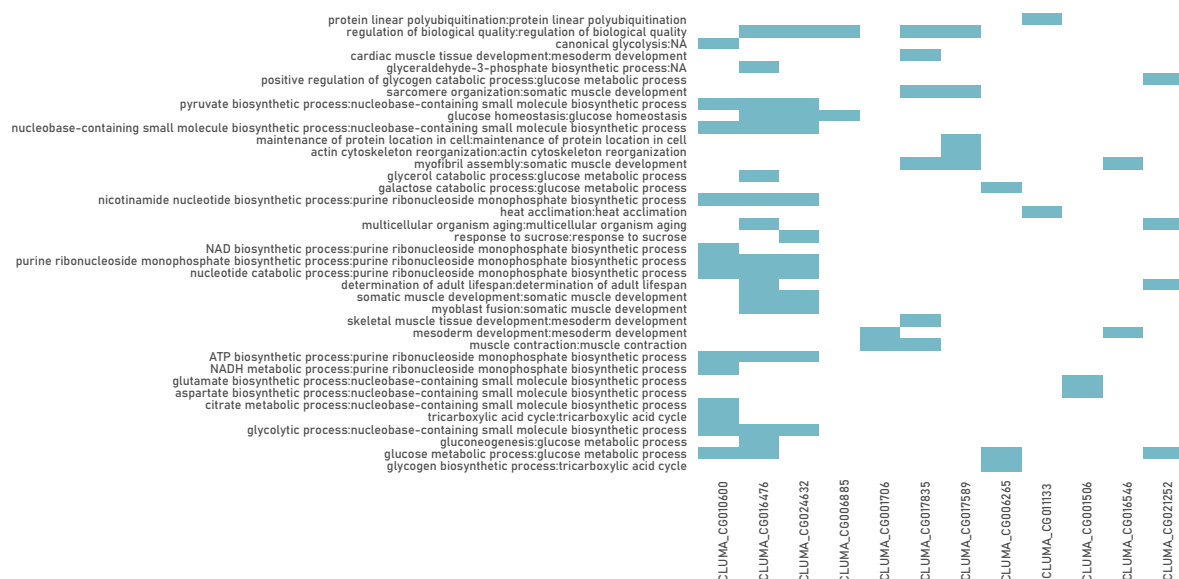
Supplementary Figure 6: The $-\log_{10}$ of the p-value is plotted against the mean of the by DESeq2 normalized counts. (b-e) P-values are highly significant for group comparisons between conditions. (a,f) P-values are not significant for groups of the same condition.



Supplementary Figure 7: Distribution of p-values for all pairwise group comparisons. Red indicates p-values of genes filtered out based on the independent filtering procedure of DESeq2. Blue indicates genes kept for differential gene expression analysis. (b-e) Between condition comparisons have a peak of p-values around 0 indicating that a very high fraction of genes is differential expressed. (a,f) Within condition comparisons show a rather uniform distribution of p-values indicating that only very few genes are differential expressed. For (a) the independent filtering option removes p-values for genes indicated in red and hence leaves a p-value distribution (in blue) comparable to (b-e).



Supplementary Figure 8: Candidate genes found differential expressed by DESeq2 for the comparison non-CDA versus CDA were additionally examined with a permutation procedure. By DESeq2 normalized read counts were randomly drawn in groups of size 5 and 9 (corresponding to original group sizes) and the absolute difference of mean calculated (10,000 permutations). The orange line indicates the observed difference in means between non-CDA and CDA samples. Of the 29 candidate genes, 26 were found differential expressed by permutation procedure ($p < 0.05$) and only those were used for downstream analysis. For readability, the read counts were



Supplementary Figure 9: 12 out of 26 candidate genes were considered for GO term analysis. rrvgo reduces the individual GO terms obtained with topGO by removing redundancy based on semantic similarity. Row names are encoded as individual GO term by topGO;parent term by rrvgo.



Supplementary Figure 10: By DESeq2 normalized number of reads in thousands for the 26 candidate genes differential expressed between non-CDA and CDA samples. Colors encode the four experimental groups as well as the 4th instar substages LIV-3 to LIV-8. Note that the absolute number of reads cannot be directly compared to 3 because normalization in DESeq2 depends on the median expression value which is different for a sample set containing all developmental stages.

Supplementary Tables

Table S1: Number of larvae per 4th instar substage collected in summer conditions. SLD: semilunar day, no.: Number of 4th instar larvae staged as this substage.

4th instar substage (LIV-)	sampling date	SLD	no.	rel abundance per day
1	4/17/2022	4	10	0.0641
2	4/17/2022	4	20	0.1282
3	4/17/2022	4	12	0.0769
4	4/17/2022	4	16	0.1026
5	4/17/2022	4	21	0.1346
6	4/17/2022	4	36	0.2308
7	4/17/2022	4	29	0.1859
8	4/17/2022	4	11	0.0705
9	4/17/2022	4	1	0.0064
total	4/17/2022	4	156	1
1	4/19/2022	6	43	0.2019
2	4/19/2022	6	17	0.0798
3	4/19/2022	6	18	0.0845
4	4/19/2022	6	15	0.0704
5	4/19/2022	6	20	0.0939
6	4/19/2022	6	22	0.1033
7	4/19/2022	6	31	0.1455
8	4/19/2022	6	35	0.1643
9	4/19/2022	6	12	0.0563
total	4/19/2022	6	213	1
1	4/21/2022	8	70	0.3196
2	4/21/2022	8	21	0.0959
3	4/21/2022	8	28	0.1279
4	4/21/2022	8	34	0.1553
5	4/21/2022	8	21	0.0959
6	4/21/2022	8	16	0.0731
7	4/21/20223	8	2	0.0091
8	4/21/2022	8	11	0.0502
9	4/21/2022	8	16	0.0731
total	4/21/2022	8	219	1
1	4/23/2022	10	58	0.3021
2	4/23/2022	10	20	0.1042
3	4/23/2022	10	38	0.1979
4	4/23/2022	10	42	0.2188
5	4/23/2022	10	21	0.1094
6	4/23/2022	10	3	0.0156
7	4/23/2022	10	3	0.0156
8	4/23/2022	10	3	0.0156
9	4/23/2022	10	4	0.0208
total	4/23/2022	10	192	1
1	4/25/2022	12	63	0.3231
2	4/25/2022	12	19	0.0974
3	4/25/2022	12	25	0.1282
4	4/25/2022	12	43	0.2205
5	4/25/2022	12	26	0.1333
6	4/25/2022	12	8	0.0410
7	4/25/2022	12	6	0.0308
8	4/25/2022	12	1	0.0051
9	4/25/2022	12	4	0.0205
total	4/25/2022	12	195	1
1	4/27/2022	14	56	0.2424
2	4/27/2022	14	22	0.0952
3	4/27/2022	14	13	0.0563
4	4/27/2022	14	35	0.1515

5	4/27/2022	14	77	0.3333
6	4/27/2022	14	24	0.1039
7	4/27/2022	14	3	0.0130
8	4/27/2022	14	0	0.0000
9	4/27/2022	14	1	0.0043
total	4/27/2022	14	231	1
1	4/29/2022	1	65	0.2600
2	4/29/2022	1	21	0.0840
3	4/29/2022	1	15	0.0600
4	4/29/2022	1	31	0.1240
5	4/29/2022	1	75	0.3000
6	4/29/2022	1	29	0.1160
7	4/29/2022	1	10	0.0400
8	4/29/2022	1	3	0.0120
9	4/29/2022	1	1	0.0040
total	4/29/2022	1	250	1
1	5/1/2022	3	45	0.1800
2	5/1/2022	3	20	0.0800
3	5/1/2022	3	13	0.0520
4	5/1/2022	3	20	0.0800
5	5/1/2022	3	49	0.1960
6	5/1/2022	3	68	0.2720
7	5/1/2022	3	32	0.1280
8	5/1/2022	3	3	0.0120
9	5/1/2022	3	0	0.0000
total	5/1/2022	3	250	1

Table S2: Number of larvae per 4th instar substage collected in winter conditions. SLD: semilunar day, no.: Number of 4th instar larvae staged as this substage.

4th instar substage (LIV-)	sampling date	SLD	no.	rel abundance per day
1	11/28/2022	4	68	0.2957
2	11/28/2022	4	114	0.4957
3	11/28/2022	4	37	0.1609
4	11/28/2022	4	6	0.0261
5	11/28/2022	4	2	0.0087
6	11/28/2022	4	0	0.0000
7	11/28/2022	4	2	0.0087
8	11/28/2022	4	1	0.0043
9	11/28/2022	4	0	0.0000
total	11/28/2022	4	230	1
1	11/30/2022	6	33	0.1410
2	11/30/2022	6	158	0.6752
3	11/30/2022	6	30	0.1282
4	11/30/2022	6	4	0.0171
5	11/30/2022	6	1	0.0043
6	11/30/2022	6	3	0.0128
7	11/30/2022	6	2	0.0085
8	11/30/2022	6	2	0.0085
9	11/30/2022	6	1	0.0043
total	11/30/2022	6	234	1
1	12/2/2022	8	69	0.2664
2	12/2/2022	8	134	0.5174
3	12/2/2022	8	46	0.1776
4	12/2/2022	8	6	0.0232
5	12/2/2022	8	2	0.0077
6	12/2/2022	8	1	0.0039
7	12/2/2022	8	1	0.0039
8	12/2/2022	8	0	0.0000
9	12/2/2022	8	0	0.0000
total	12/2/2022	8	259	1
1	12/4/2022	10	54	0.2069
2	12/4/2022	10	167	0.6398
3	12/4/2022	10	27	0.1034
4	12/4/2022	10	7	0.0268
5	12/4/2022	10	2	0.0077
6	12/4/2022	10	1	0.0038
7	12/4/2022	10	1	0.0038
8	12/4/2022	10	2	0.0077
9	12/4/2022	10	0	0.0000
total	12/4/2022	10	261	1
1	12/6/2022	12	34	0.1288
2	12/6/2022	12	177	0.6705
3	12/6/2022	12	42	0.1591
4	12/6/2022	12	8	0.0303
5	12/6/2022	12	1	0.0038
6	12/6/2022	12	0	0.0000
7	12/6/2022	12	1	0.0038
8	12/6/2022	12	0	0.0000
9	12/6/2022	12	1	0.0038
total	12/6/2022	12	264	1
1	12/8/2022	14	53	0.1913
2	12/8/2022	14	160	0.5776
3	12/8/2022	14	43	0.1552
4	12/8/2022	14	16	0.0578
5	12/8/2022	14	0	0.0000
6	12/8/2022	14	1	0.0036
7	12/8/2022	14	2	0.0072

8	12/8/2022	14	2	0.0072
9	12/8/2022	14	0	0.0000
total	12/8/2022	14	277	1
1	12/10/2022	1	36	0.1180
2	12/10/2022	1	203	0.6656
3	12/10/2022	1	52	0.1705
4	12/10/2022	1	5	0.0164
5	12/10/2022	1	5	0.0164
6	12/10/2022	1	2	0.0066
7	12/10/2022	1	0	0.0000
8	12/10/2022	1	1	0.0033
9	12/10/2022	1	1	0.0033
total	12/10/2022	1	305	1
1	12/12/2022	3	32	0.1049
2	12/12/2022	3	206	0.6754
3	12/12/2022	3	51	0.1672
4	12/12/2022	3	13	0.0426
5	12/12/2022	3	1	0.0033
6	12/12/2022	3	0	0.0000
7	12/12/2022	3	1	0.0033
8	12/12/2022	3	1	0.0033
9	12/12/2022	3	0	0.0000
total	12/12/2022	3	305	1

Chapter 5

5 Obtaining an annotated reference genome on chromosome scale for the marine midge *Clunio tsushimensis*

Jule Neumann, Celine Prakash and Tobias S. Kaiser

This chapter was written by Jule Neumann and describes exclusively her own work. Analysis which were conducted by others are indicated in the text. The genomic resources presented here contributes to the following paper, which is not included in this thesis:

Prakash C, **Neumann J**, Briševac D, Nawroth T, Harney E, Dandavate V, Fuhrmann N, Peralta C, Mentz S, Schaefer K, Schirmer S, Tekade K, Ullrich K, Ahn S-J, Betancourt AJ & Kaiser TS (in preparation for submission in *BMC Genomics*). Chromosomal level *Clunio* assemblies provide insights into compact insect genomes.

Abstract

Marine midges of the genus *Clunio* (Diptera, Chironomidae) are characterized by their small but compact genomes. The reference genome of the marine midge *C. marinus* has been invaluable to study the molecular underpinnings and genetic timing adaptations of the circalunar clock (Kaiser et al. 2016). However, to understand the common features underlying circalunar time-keeping in *Clunio* and to characterize the midges's unique genomic architecture, a genome for a second *Clunio* species is required. We therefore obtained an annotated reference genome on chromosome scale for the Japanese species *Clunio tsushimensis*. The genomic resource provided here constitutes the basis for comparative genomic analysis and ultimately for studying the evolution of these midges.

Introduction

Today more than 3,000 genomes across the Animal kingdom have been sequenced. However, arthropods, including Diptera remain underrepresented and only about a third of available genomes have annotations (Hotaling et al. 2021). The high-quality reference genome of *Clunio marinus* has highlighted several interesting genomic features, e.g. a very small size but yet comparable number of genes as other *Dipterans*, few transposable elements, a high degree of gene duplications and structural variation (Kaiser et al. 2016, further unpublished data). Some of these features might be linked to its adaptation to marine habitats and/or its peculiar circalunar timing. The genome of *C. marinus* is arranged into 3 chromosomes (Kaiser et al. 2016) and consists of roughly 80 Mbps (Prakash and Kaiser 2023). The current version of the reference genome of *C. marinus* is with 13,840 annotated gene models highly complete (96% according to BUSCO) (Prakash and Kaiser 2023). Hence, though the genome of *C. marinus* is small in size, most genes are present, i.e. the genome is highly compact. Interestingly, an extremely small genome size is in fact common to family members of *Chironomidae* (Cornette et al. 2015).

It has been known for a long time that the C-value, i.e. the amount of DNA in picogram present in a gamete of a cell (a measure for genome size), is highly variable across Eukaryotes and not correlated with genome complexity (Gregory 2005). The fact that large parts of the genome contain non-coding DNA explains the missing correlation of genome size with gene number (Gregory 2005). How then can differences in genome size be explained? One type of non-protein-coding DNA are transposable elements and their amount is highly correlated with genome size in plants (Lee and Kim 2014). The genome of the puffer fish *Fugu rubripes* is eight-fold smaller than the human genome but it contains a comparable number of genes (Aparicio et al. 2002). The compactness stems from introns being shorter and a lower abundance of repetitive DNA in the puffer fish compared to humans (Aparicio et al. 2002). Strikingly, the puffer fish genome contains some giant gene loci spread over much greater distance compared to homologous counter parts (Aparicio et al. 2002), highlighting that non model species genomes can give valuable insights into genome architecture. Genome size can also change because of the rate, structural variations including duplications, insertions or deletions occur (Lynch 2007). In summary, why some genomes are highly compact remains an open question and is likely based on the different selection and mutation pressures (Petrov 2001).

High quality, chromosome-level reference genomes are essential for unraveling the genomic basis of phenotypes and have been pivotal for entire fields including conservation bi-

ology (Formenti et al. 2022) and human disease research (Hofker et al. 2014). The reference genome of *C. marinus* provided the basis to study intraspecific timing adaptations of the circadian and the unexplored circalunar clock (Kaiser et al. 2016). However, a reference genome of a second *Clunio* species is required to study interspecific adaptations and to conduct comparative genomic analysis. *C. tsushimensis* is a marine midge inhabiting the intertidal zone of Japan (Saigusa and Akiyama 1995). Its genome is the smallest known insect genome (C-value 0.07pg DNA, roughly 68 Mbps) (Cornette et al. 2015), even smaller than the genome of *C. marinus*. Similarly to *C. marinus*, this species has a circalunar clock as an adaptation to the reoccurring timing of the tides (Neumann 1995).

We have sequenced the genome of *C. tsushimensis* using PacBio technology. Together, our two *Clunio* assemblies take us a step closer in understanding the mechanisms in these midges that maintain consistently compact genomes. In the paper mentioned above, which is not part of this thesis, we will explore two avenues:

- I. *C. marinus* was found to have an elevated rate of chromosomal rearrangements in comparison with other *Dipterans*, i.e. *Drosophila melanogaster* and *Anopheles gambiae* (Kaiser et al. 2016). Can we observe the same trend of high rearrangements in *C. tsushimensis*? Can we quantify the number of rearrangements between species pairs and develop hypothesis on how those could explain the exceptionally small genome size of the two *Clunio* species.
- II. Small genome sizes have been consistently associated with low proportions of transposable elements (TEs) (Cornette et al. 2015). Can we identify genes that may play a role in TE control, such as those involved in the piRNA pathway, uniquely present or duplicated in *Clunio*?

To answer the questions associated with (I), we require a highly contiguous genome assembly and for (II), a reliable annotation. Thus, within the scope of this thesis, our aim was to obtain an annotated genome assembly on chromosome scale for *C. tsushimensis* that will serve as a basis for downstream analyses. We previously assembled a very first transcriptome and automatically annotated the genome draft of *C. tsushimensis* using MAKER (Cantarel et al. 2008). The primary genome assembly was obtained by Tobias S. Kaiser. Within this thesis, we obtained an annotated genome of *C. tsushimensis* on chromosome scale based on linkage map analysis of two mapping families. The details of this are outlined in the Methods section. In the Results & Discussion section, I describe the conducted quality checks and provide a com-

parison between the original genome draft and the final genome on chromosome scale for *C. tsushimensis*. In addition, we then compared genome features including chromosome size, number of gene models and AT content to the high quality genome of *C. marinus*. Our quality checks and comparisons demonstrate that we now have a suitable chromosome-level, annotated assembly for *C. tsushimensis* that is appropriate for downstream comparative genomics analysis.

Methods

DNA extraction and sequencing for the primary genome assembly of *C. tsushimensis*

DNA was extracted with a salting out method (Reineke et al. 1998). Because of the small size of the animals, several 100 adult males from the Shi laboratory strain (established in 2015 from Shirahama, Chiba prefecture, Japan, by R. Cornette and T. Kaiser) were pooled to get sufficient yield, which, as for *C. marinus*, resulted in a considerable degree of polymorphism in the sequence data. A PacBio sequencing library was prepared at the Max Planck Genome Center in Cologne according to standard protocols, with size selection for 10–15 kbp fragments. Sequencing was done in one run of an PacBio RSII sequencer, resulting in 741,715 reads and 4.5 Gbp of sequence data or roughly 60-fold coverage of the genome (estimated genome size: 75 Mbp).

Primary assembly and correction

Based on the experience with the *C. marinus* reference assembly (Kaiser et al. 2016), we assembled the *C. tsushimensis* genome in Canu (Koren et al. 2017) with parameters corOutCoverage=100, MhapSensitivity=high, correctedErrorRate=0.2, rawErrorRate=0.25, corMinCoverage=3 and genomeSize=75m. The resulting contigs were error corrected as described above, based on Illumina sequencing data for a single female in one iteration of PILON (Walker et al. 2014). The resulting assembly has 103.6 Mbp in 1,504 contigs, a contig N50 of 322,376 bp and the longest scaffold is 2,333,522 bp long.

Crosses and ddRAD sequencing

Individuals of the Shi laboratory strain of *C. tsushimensis* were crossed and F1 families were raised individually until they exceeded the 2nd larval stage. Two families with 52 (Family ID 6) and 33 (Family ID 12) offspring, respectively were chosen for linkage mapping. DNA was extracted from the adult parents and the larval offspring with a salting out method (Reineke et

al. 1998). Genomic DNA was amplified using the REPLI-g Mini Kit QIAGEN (150025) according to the manufacturer's instructions. Two double-digest Restriction-site Associated DNA sequencing (ddRAD-seq) libraries with 46 and 43 individuals respectively were produced according to Peterson et al. (2012) and Yang et al. (2016). Briefly, 100 ng of DNA per individual was digested with BglII (FD0084 Thermo Fisher) and MspI (FD0544 Thermo Fisher) for 2h 37°C. Barcoded P1 and P2 adapters were ligated simultaneously with T4 ligase (EK0032 Thermo Fisher) for 2 hours at 22°C, before the T4 ligase was heat-inactivated at 70°C for 10min. Samples were pooled, precipitated and loaded on the 1 % TEB gel. DNA fragments sizes between 250 and 900 bp were extracted from the gel (Zymoclean™ Gel DNA Recovery Kit). Extracted fragments were amplified with Phusion Master Mix (HF) using P1/P2 amplification primers in 16 PCR cycles and products were again size-selected on an agarose gel as described above. Sequencing of the ddRAD libraries was performed according to standard protocols on an Illumina HiSeq3000 in two runs at the Max Planck Genome Center in Cologne with paired-end reads to obtain a total of 9 Gbp of sequence data, with an observed 0.3 to 2 million reads per individual.

Obtaining sex-informative and family specific linkage maps

Demultiplexing of samples, i.e. *C. tsushimensis* individuals belonging to the two linkage mapping families, was conducted using a python script. Adapters were trimmed using Trimmomatic (Bolger et al. 2014) with parameters 'ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:2:true', 'LEADING:20', 'TRAILING:20', 'MINLEN 50'. Overlapping paired end reads were merged with PEAR (Zhang et al. 2014) with parameters -n 50, -c 40, -k. Reads were mapped to the genome assembly draft of *C. tsushimensis*, sorted and indexed using NextGenMap (Sedlazeck et al. 2013) with minimum read identity = 0.9 and minimum fraction of mapped reads = 0.9. Variants were called with GATK (McKenna et al. 2010) against the original genome assembly of *C. tsushimensis*. Quality filtering of SNPs was conducted with vcftools (Danecek et al. 2011) with -maf 0.15, -max-missing 0.8 and -minGQ 50 resulting in 4,842 SNPs for family ID 6 and 5,780 SNPs for family ID 12. Those SNPs were then filtered for homozygosity in one parent but heterozygosity in the other parent, resulting in one male and one female informative map for each family (1,824 SNPs for male informative map ID 6, 1,334 SNPs for female informative map ID 6, 1,743 SNPs for male informative map ID 12 and 1,916 SNPs for female informative map ID 12). All four maps were handled independently for downstream analysis and are subsequently referred to as Map6M, Map6F, Map12M and Map12F.

Initial linkage grouping was performed using the R package qtl (Broman et al. 2003). First, a stringent filtered marker set was obtained using the functions ntyped() and drop.markers().

The data set contained only markers present in >70% of individuals. For Map6M and Map6F only individuals with more than 900 markers were kept and for Map12M and Map12F only individuals with more than 1,300 markers were kept. Markers were examined for deviations from Mendelian segregation pattern using `geno.table()` based on a chi-square test and Bonferroni correction. After filtering, 48 individuals with 1,709 and 1,165 markers, remained for Map6M and Map6F, respectively. All 33 individuals were kept with 1,644 and 1,799 markers for Map12M and Map12F, respectively. Duplicated markers were collapsed with `findDupMarkers()` resulting in 197, 132, 79 and 114 informative markers for Map6M, Map6F, Map12M and Map12F, respectively. Initial linkage groups (LGs) were calculated with `formLinkageGroups(reorgMarkers=TRUE)`, `switchAlleles()` and `est.rf()`. Possible number of linkage groups were investigated by systematically changing `max.rf` and `min.lod`. Based on expectations from *C. marinus*, three linkage groups were obtained containing 69, 67 and 61 collapsed markers for Map6M (`max.rf=0.3` & `min.lod=2.5`); 54, 53 and 25 markers for Map6F (`max.rf=0.25` & `min.lod=2.5`); 40, 35 and 4 markers for Map12M (`max.rf=0.3` & `min.lod=2`) and 44, 41 and 29 markers for Map12F (`max.rf=0.3` & `min.lod=2`). Marker order was obtained using the R package ASMap (Taylor and Butler 2017) implementing the Minimum Spanning Tree algorithm (Wu et al. 2008). R/qtl maps were imported to ASMap with `mstmap.cross()` not allowing for regrouping and reordering of markers with `bychr = FALSE`, `p.value = 0.001` for Map6M, Map6F and Map12F, and `p.value = 0.0015` for Map12M.

The duplicated markers were re-included into the data set to preserve information about contig locations. We thereupon went back to the quality filtered genotype tables which were based on backcross-like inheritance, and manually arranged contigs according to the extended marker order obtained with R/qtl and R/ASMap. If necessary, contig orientation and position was rearranged to reduce the number of recombination events per individual. For final linkage maps, manually curated marker orders were collapsed containing only one representative marker, i.e. the one with the least missing information and no deviation from Mendelian expectation, for each contig and recombination event. Genotyping errors (immediate forth and back flip of genotypes within a contig) were manually curated. The number of markers reflecting all contigs and recombination events was 255 (LG1=93, LG2=88, LG3=74) based on 48 individuals for Map6M; 220 (LG1=87, LG1=74, LG2=59) based on 49 individuals for Map6F, 209 (LG1=72, LG2=70, LG3=67) based on 33 individuals for Map12M and 246 (LG1=90, LG2=82, LG3=74) based on 33 individuals for Map12F. All four linkage maps were used to create a consensus linkage map.

Generating a consensus linkage map

For the consensus linkage map, all contigs bigger than 100,000 bps mapping to at least one linkage map and all contigs smaller than 100,000bps mapping to at least three out of four linkage maps were included. Contigs were oriented based on recombination events in relation to mapped neighboring contigs. No contradictions of orientation or order of contigs between the four linkage maps were allowed for. Five misassembled contigs were identified because contig parts mapped to different linkage groups and this was supported by at least two linkage maps. Misassembled contigs were divided into mapped and unmapped contig parts depending on the marker position in the linkage maps. Consequently, the five misassembled contigs were separated into 17 smaller contigs. The number of contigs (initially 1,504 from the reference genome draft) increased therefore to 1,516. Of those, 227 contigs (77,244,316bps) could be placed in the final consensus linkage map: 72 contigs (24,882,361bps), on LG1, 76 contigs (26,285,595bps) on LG2 and 79 contigs (26,076,360bps) on LG3 and are hence classified as mapped. The linkage groups were named based on synteny to *C. marinus*.

One contig contained the mitochondrial genome sequence and its size was manually curated, resulting in 19,296 bps. The mitochondrial sequence of *C. tsushimensis* was identified via BLAST (Altschul et al. 1990) of the mitochondrial sequence of *C. marinus* against the genome draft of *C. tsushimensis*. The size of the identified contig had to be curated manually because it contained multiple copies of the mitochondrial sequence. 28 contigs (395,597bps) were identified as contamination based on Kraken (Wood and Salzberg 2014, analysis by Celine Prakash). 476 contigs (6,444,084bps) were identified as polymorphisms of the mapped contigs based on the criteria >90% identity and >50% coverage with BLAST. None of the mapped contigs smaller than 100kbps were polymorphisms of the mapped contigs bigger than 100kbps. The mapped contigs which could not be placed and oriented unambiguously based on information from the four linkage maps, were placed and oriented based on synteny to *C. marinus* using MUMmer (Marçais et al. 2018) with parameters `nucmer -maxmatch -c 100 -p nucmer` and `delta-filter -r -q`. If a contig of interest in *C. tsushimensis* had the same adjacent contigs as *C. marinus*, i.e. contig order was preserved between species, the contig of interest was arranged and oriented based on *C. marinus*. Hence, synteny similarity between *C. marinus* and *C. tsushimensis* could be overestimated in downstream analysis.

Contigs of the *C. tsushimensis* reference genome, i.e. the consensus sequence, were classified in one of seven groups. A: position and orientation based solely on linkage maps (60 contigs, 36.4 Mbps), B: position based on linkage maps but orientation based on synteny (41

contigs, 9 Mbps), C: both position and orientation based on synteny (99 contigs, 24.8 Mbps), D: position based on linkage maps, but orientation could not be determined (2 contigs, 0.46 Mbps), E: position based on synteny, but orientation could not be determined (2 contigs, 0.35 Mbps), F: position determined by linkage maps, contigs could be relatively oriented within a contig cluster by synteny, but the cluster itself could not be oriented within the linkage group (8 contigs, 3 Mbps) and G: contig was mapped to a certain contig cluster by linkage maps or synteny but could not be oriented within the cluster (15 contigs, 3 Mbps) (Figure 1). The final consensus genome sequence was compiled with scaffolder (Barton and Barton 2012). The orientation of contigs was modified according to the consensus linkage map with the reverse option set to TRUE. We introduced 10,000 Ns between contigs because the continuity of sequence remains to be verified in the laboratory.

Annotation of the *C. tsushimensis* reference genome

For transcriptomic evidence, eggs, larval stages I to IV and adults of *C. tsushimensis* were preserved in TRIzol Reagent (Thermo fisher Scientific, 15596018) and total RNA extracted using a modified extraction protocol from Chomczynski and Sacchi (2006). For sequencing, the RNA concentration ratio of adult to larvae to egg was 1:1:1. Libraries were prepared for Illumina as well as PacBio sequencing at the Max Planck Genome Center in Cologne. Reads sequenced with Illumina HiSeq3000 were processed with Trimmomatic 0.11.5 (Bolger et al. 2014) to remove adapters and filter quality (Q20) with parameters 'TruSeq3-PE.fa:2:30:10' 'SLIDINGWINDOW:4:5' 'LEADING:5' 'TRAILING:5' 'MINLEN:75'. A de novo transcriptome assembly was obtained with Trinity 2.8.4 (Grabherr et al. 2011) with `-SS_lib_type RF` resulting in 59,297 transcripts.

Reads obtained with PacBio sequencing were processed using the Iso-Seq 3 Analysis pipeline in SMRT Link 6.0 (de novo identification of transcripts and splice variants) (Rhoads and Au 2015) generating 20,563 high quality isoforms. MAKER pipeline (Cantarel et al. 2008) was used for automatic annotation of the reference genome. Both Illumina and PacBio transcripts (79,860) were used as mRNA evidence for annotation. First, PacBio HQ transcripts and contigs larger than 20,000 bps of the *C. tsushimensis* genome draft were used to train AUGUSTUS by submitting files to the AUGUSTUS training web server application (<http://bioinf.uni-greifswald.de/webaugustus/training/create>). Protein sequences used as evidence for MAKER came from *Drosophila melanogaster* (30,506 proteins), *Tribolium castaneum* (16,422 proteins), *Apis mellifera* (15,314 proteins) and *Anopheles gambiae* (14,970 proteins) downloaded from Fly-Base, BeetleBase, BeeBase and VectorBase, respectively. MAKER behavior parameters were set to default except `max_dna_len=100000`, `min_contig=5000`, `red_flank=200`, `pred_stats=1`,

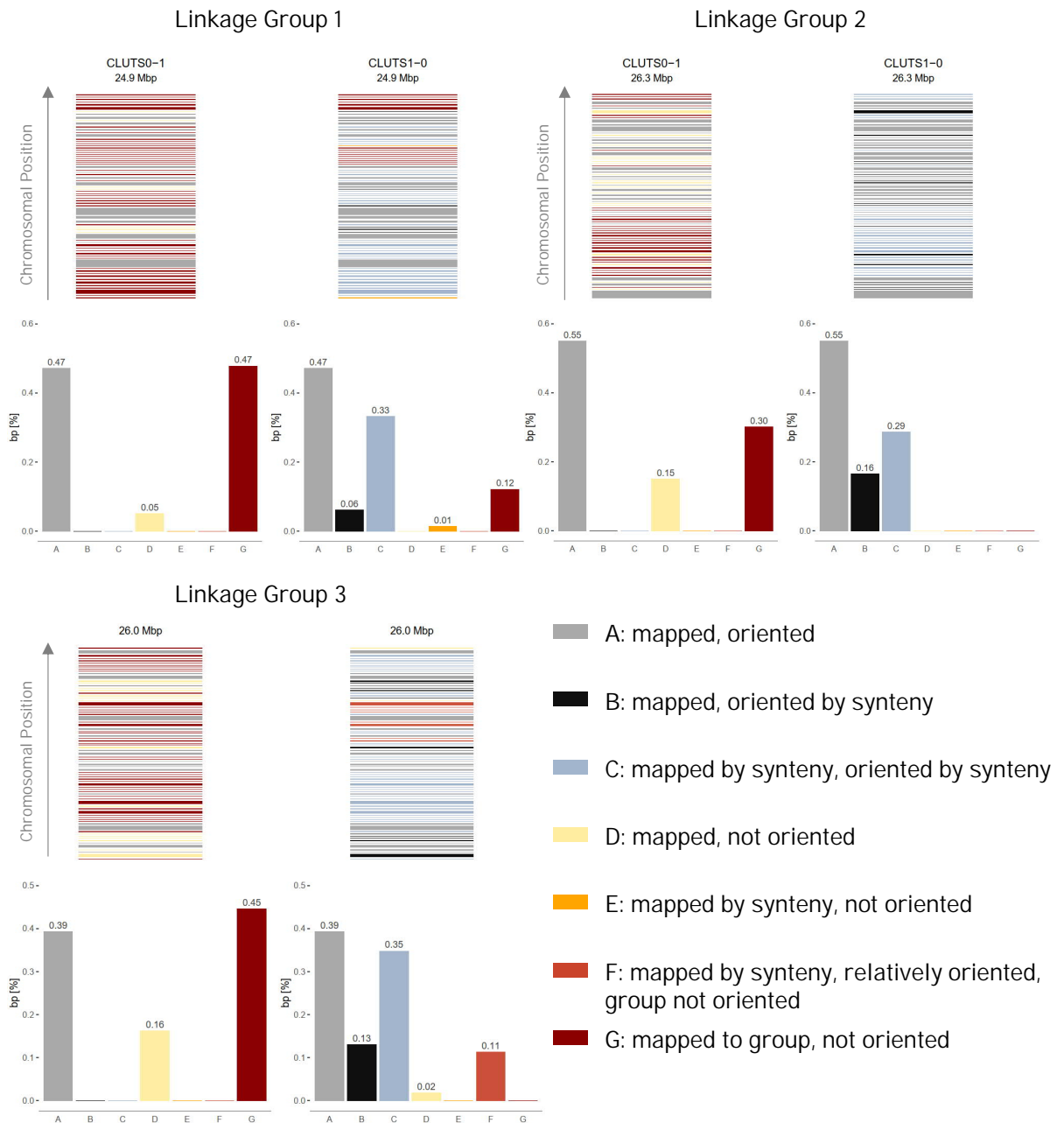


Figure 1: Quality of contig placement (color coded) before (CLUTS0-1) and after taking into account synteny information to *C. marinus* (CLUTS1-0) for all three identified linkage groups of the *C. tsushimensis* genome. Thickness of bars (corresponding to a contig) was weighted by their length in base pairs. Bar plots display percentage of bps falling into one of the colour coded categories.

AED_threshold=1, min_protein=0, alt_splice=1, always_complete=0, map_forward=0, keep_preds=0, split_hit=10000, min_intron=10, single_exon=1, single_length=250, correct_est_fusion=0, tries=2, clean_try=0, clean_up=0. The MAKER output was merged into one .gff file with gff3_merge. To assess the assembly quality, BUSCO version 3.0.2 was used (Simão et al. 2015) with parameters -m genome -c 1 -e 1e-05 -sp fly based on the dipt10 dataset.

Divergence time estimation

The divergence time between *C. marinus* and *C. tsushimensis* was estimated by Celine Prakash using IQ-TREE 2.0.3 (Minh et al. 2020) and r8s version 1.81 (Sanderson 2003). The analysis was based on multiple sequence alignment of amino acid sequences of 732 single-copy BUSCOs across seven species: *Clunio marinus*, *Clunio tsushimensis*, *Anopheles gambiae*, *Anopheles stephensi*, *Drosophila melanogaster*, *Drosophila triauraria* and *Danaus plexippus*, with *D. plexippus* as the outgroup. Alignment was conducted with MAFFT (Kato and Standley 2013) with all pairwise alignments computed with a local algorithm with the generalized affine gap cost (Altschul 1998) and maxiterate 1000 (E-INS-i). Tree reconstruction was done using IQ-TREE with 1000 ultrafast bootstrap replicates (Hoang et al. 2018) and an edge-linked-proportional partition model with separate substitution models and separate rates across sites (Chernomor et al. 2016). Best fit model for each partition was chosen using ModelFinder (Kalyaanamoorthy et al. 2017). The chronogram of divergence times was obtained with r8s calibrated with fossil data. Age constraints were: min age=195 max age=230 (Suvorov et al. 2022) for Diptera, min age 201 (Cranston et al. 2012) for Nematocera and min age=306.9 max age=311.4 (Misof et al. 2014) for Holometabola. For syntenic analysis, a set of 2,386 BUSCOs, shared by the mentioned species except for *D. plexippus*, was obtained.

Results & Discussion

Quality assessment of the *C. tsushimensis* genome

The final genome on chromosome scale of *C. tsushimensis* is with 77.2 Mbps still roughly 10 Mbps bigger than the 68 Mbps estimated by flow cytometry. This overestimation might be due to overlapping contig ends caused by sequence polymorphisms or repetitive sequences. As expected, the genome of *C. tsushimensis* is slightly smaller than the genome of *C. marinus* (79.4 Mbps). Notably, the current genome draft consists of 227 contigs which even though placed and oriented based on recombination events, is still scaffolded together with introduced gaps of fixed lengths. The continuity of the sequence, i.e. the sequence between two contigs, has been confirmed in the laboratory by PCR for *C. marinus*. This step remains for *C. tsushimensis*. The genome of *C. tsushimensis* is with 88.5% (estimated by BUSCO) highly complete. However, a discrepancy between the first genome draft which was 94.4% complete and the genome on chromosome scale exist (Figure 2). It might be that certain contigs could not be mapped to most of the linkage maps and therefore did not appear in the consensus map. In line with this, the number of gene models annotated by MAKER differs between the genome draft and the

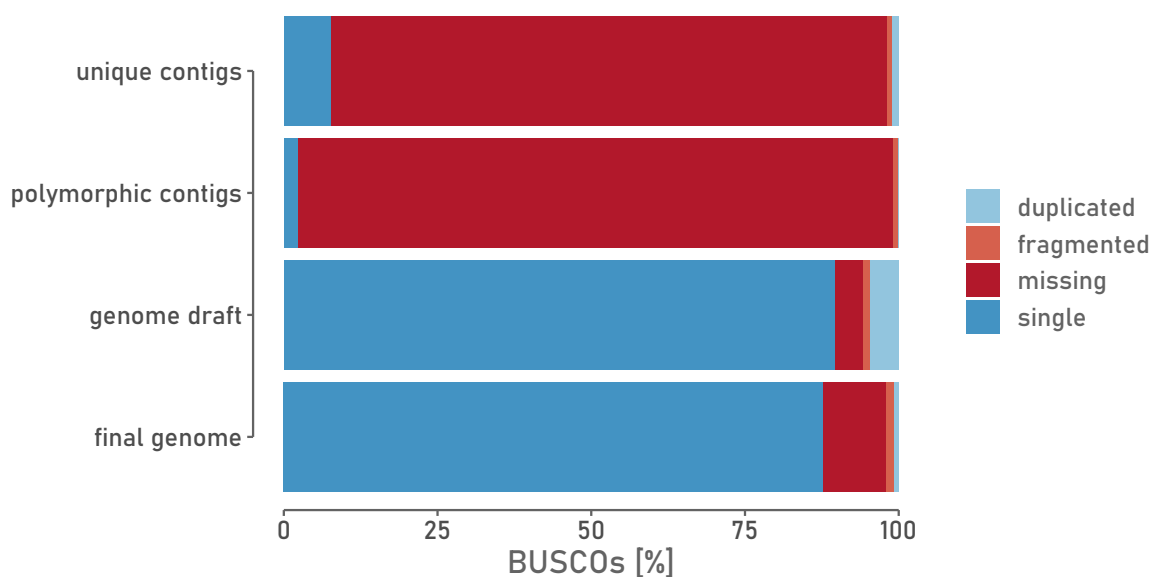


Figure 2: Percentage of BUSCOs (n=3285, e-value = 1E-05) falling in one of four categories: single, duplicated, fragmented or missing. Data is displayed for the original genome draft assembly, the final annotated genome on chromosome scale and contigs which were identified as either polymorphic or unique for *C. tsushimensis*.

final genome on chromosome scale (14,084 gene models vs 11,078 gene models). For comparison, the reference genome of *C. marinus* has 13,840 gene models. However, almost all single complete BUSCOs which were previously identified in the genome draft are also present in the new chromosome scale assembly (Figure 2). The number of duplicated BUSCOs decreased in the final genome compared to the assembly, which explains the reduced number of complete BUSCOs (Figure 2). Multiple high quality transcriptomes went into the MAKER annotation pipeline of *C. marinus* and all gene models were manually curated. Hence, the genome of *C. marinus* is of outstanding high quality.

Based on linkage group affiliation and recombination events we could identify five misassembled contigs. Further, we identified about 400,000 bps of contamination and 6.4 Mbps of polymorphic contigs, leaving 19 Mbps of unique, unmapped sequence (Figure 3). After taking into account synteny information to *C. marinus*, most of the contigs could be unambiguously placed and oriented (Figure 1). Overall, the genome of *C. tsushimensis* is comparable in chromosome size, AT content, genome completeness and number of gene models to *C. marinus* (Figure 3). Hence, *C. tsushimensis* is of sufficient quality and suitable for downstream analysis, including comparative genomics.

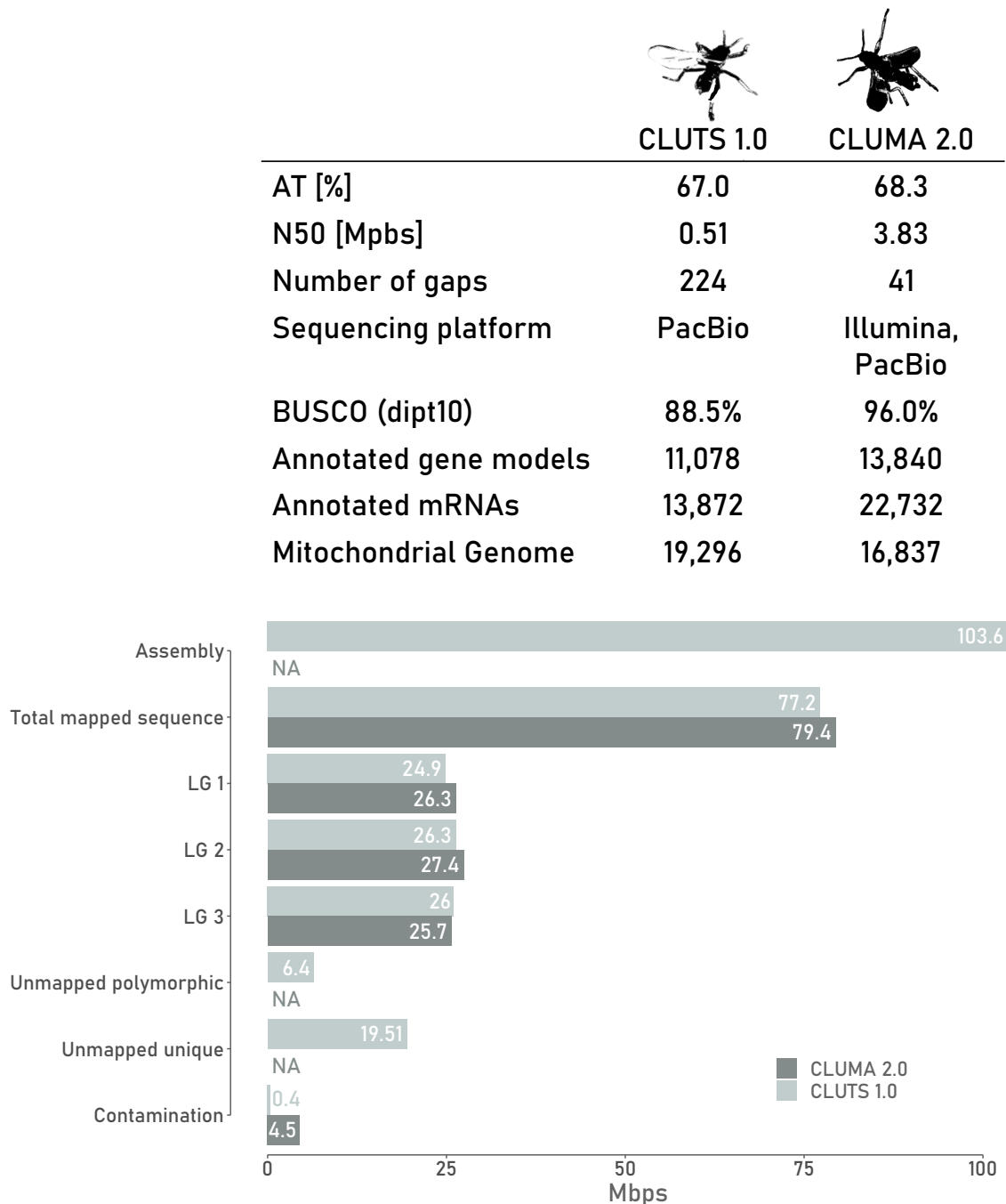


Figure 3: Comparison of genome size and quality of the two *Clunio* genomes. Most contigs in the *C. tsushimensis* genome could be ordered and oriented based on linkage maps and synteny to *C. marinus*. Assembly: genome draft after sequencing, Total mapped sequence: sequence included in the final genome, LG: Linkage group, Unmapped polymorphic: as polymorphic identified unmapped sequence of the assembly, Unmapped unique: as unique identified unmapped sequence of the assembly, Contamination: with Kraken identified contamination in the assembly.

Alignment and synteny analysis of the two *Clunio* genomes reveals conserved chromosomes

Next, we aligned the genome of *C. tsushimensis* to *C. marinus* using MUMmer (Figure 4). The alignment plots suggest that the linkage grouping algorithm based on linkage information for *C. tsushimensis* could convincingly recover the three chromosomes. Notably, all three linkage

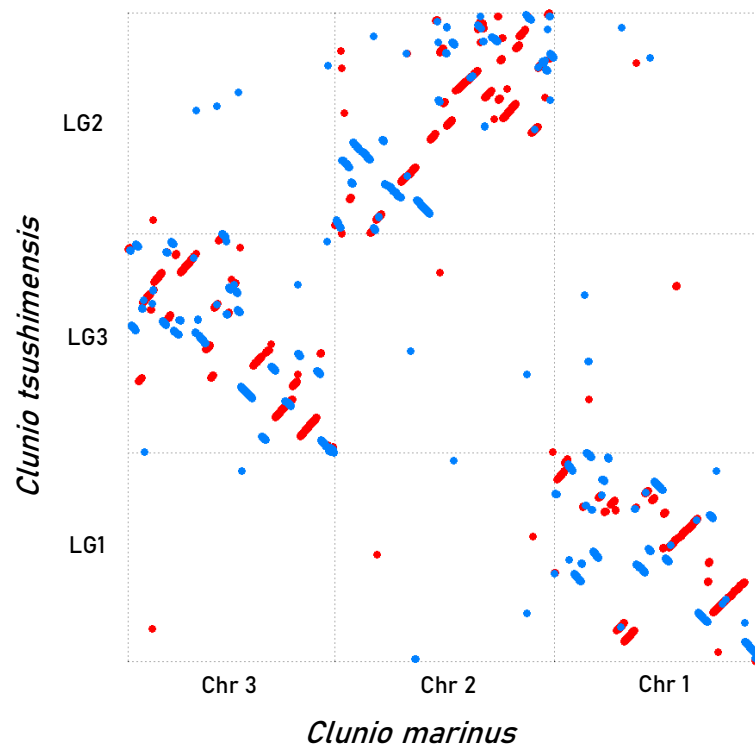


Figure 4: Mummer alignment of *C. marinus* versus *C. tsushimensis*. Colors correspond to forward (red) and reverse (blue) MUMs (matches between the reference and query).

groups are highly conserved between the two *Clunio* species. This result is additionally supported by synteny analysis conducted by Celine Prakash based on 2,386 single-copy BUSCOs between the two species (Figure 5; Note that the number of BUSCOs was constraint to 2,386 because for downstream analysis a set of BUSCOs likewise present in more diverged species, i.e. *Drosophila* and *Anopheles*, had to be obtained, see Methods section). The chromosome arms itself are conserved between the two species. However, within the chromosome arms, multiple re-arrangements exist between *C. marinus* and *C. tsushimensis*. If the number of re-arrangements is different in *Clunio* genomes compared to other species, will be examined in the paper mentioned above. Therefore, we finally estimated a divergence time of around 30 million years between *C. marinus* and *C. tsushimensis*.

Conclusion & Outlook

The reference genome of the marine midge *C. marinus* provides a valuable genomic resource to study why genomes of the marine midges are so highly compact. Manual curation of the gene models, multiple transcriptomic lines of evidence and confirmation of contingency with PCR (scaffolding) makes it a high quality resource. We obtained a reference genome on chromosome scale for a second *Clunio* species, namely *C. tsushimensis*. Four main criteria may

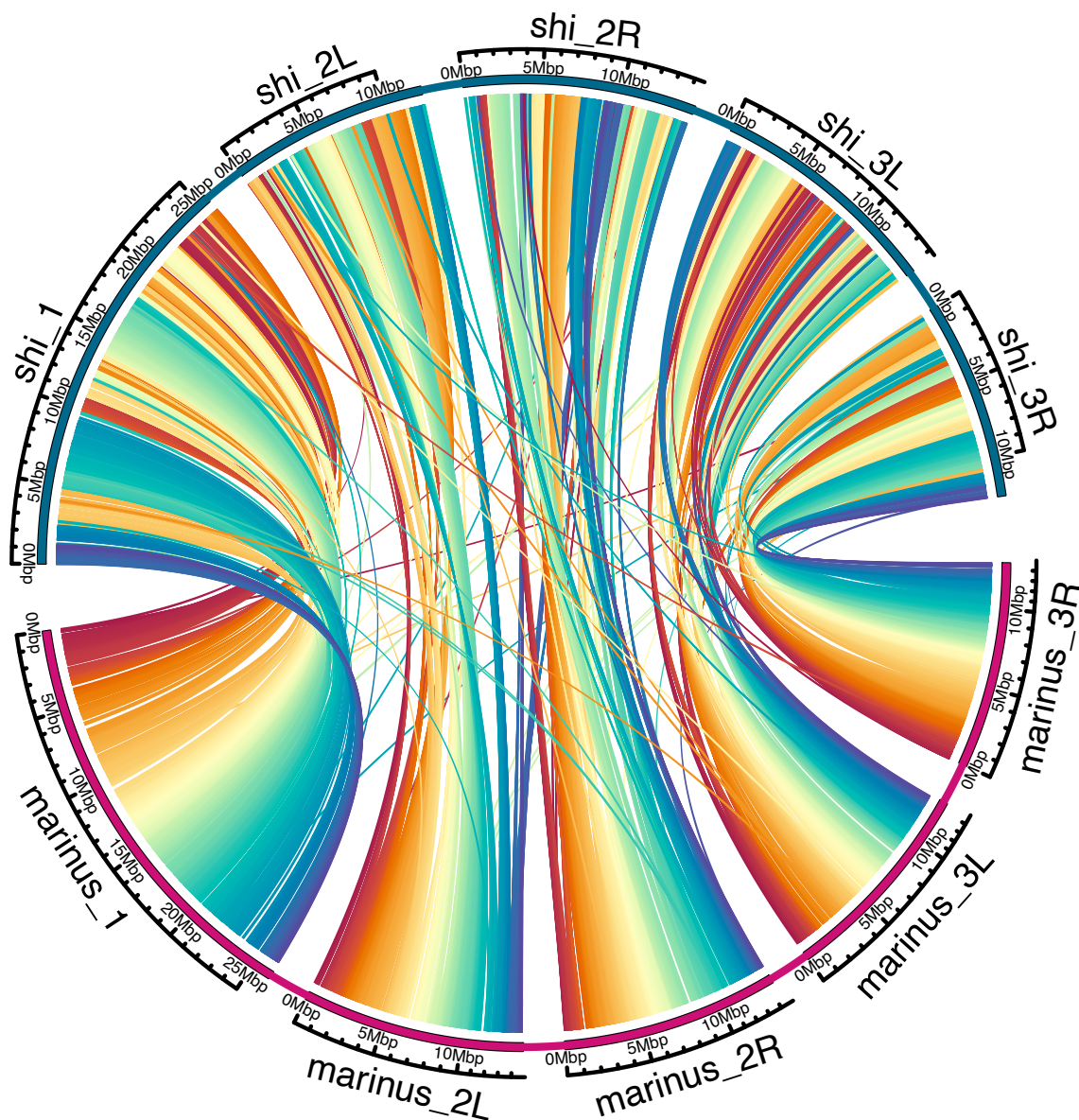


Figure 5: Synteny comparison based on 2,386 single copy Diptera BUSCOs to visually identify rearrangements between the two *Clunio* species (analysis by Celine Prakash). The genome sequence of the *C. tsushimensis* genome is indicated in blue (shi). The reference sequence is *C. marinus* indicated in magenta (marinus)

be used to evaluate the quality of a reference genome (Li et al. 2019), three of which can be applied here. First, the size of the genome assembly may be compared to another independent measure of size. The genome assembly of *C. tsushimensis* is with 77.2 Mbps larger than the by flow cytometry estimated 68 Mbps. This may be due to the presence of polymorphic sites, reflected in the independent assembly of haplotypes (Li et al. 2019). Second, the contiguity of a genome is reflected in its N50 value. N50 indicates the sorted contig length of which 50% of the assembly size is covered. The N50 value for the reference genome of *C. tsushimensis* is with

0.51 Mbps clearly smaller than the 3.38 Mps of *C. marinus* indicating much lower contiguity. This is not surprising considering that the *C. marinus* genome is scaffolded by PCR. However, the N50 is comparable to other non-model species (Hotaling et al. 2021) Thirdly, widely shared single-copy orthologous genes may be used to assess the completeness of the genome. The genome of *C. tsushimensis* is less complete than *C. marinus* but the numbers of single BUSCOs differ only marginally between the initial genome draft and the final genome on chromosome scale (89.7% and 87.7%, respectively) for *C. tsushimensis*.

Even though the mentioned manual curation steps were not performed yet for *C. tsushimensis*, we obtained a highly complete genomic resource in terms of conserved gene content on chromosomal scale. Why *Clunio* genomes are consistently compact over long evolutionary time scales is a question which remains to be answered. A second annotated *Clunio* genome on chromosome scale allows finally to conduct comparative genomics analysis within the same genus, which is bringing us a little closer towards understanding the smallest known insect genomes.

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***Clunio* research in the era of classic Chronobiology**

In 1894, René Chevrel walked along a beach in the Normandy in France, and noticed the mass swarming of tiny insects at the water line [1]. He quickly realized that this phenomenon only occurred at certain days of the lunar cycle and identified the insect as the marine midge *Clunio marinus* (Diptera, Chironomidae). About 70 years later, Dietrich Neumann, certainly impressed by the lunar modulation of the insect's eclosion rhythm, spent years to establish a culturing method to maintain these midges in the laboratory in order to systematically study their precisely timed behavior [2]. His publication in 1966 still constitutes the foundation of circalunar clock research in the marine midge [3]. In more than 40 publications, underpinned with hard-won experimental evidence, he gradually built up a comprehensive model for the temporal organization of the eclosion behavior in *Clunio*. His work truly pioneered circalunar clock research from the very beginnings of the field of Chronobiology itself. In fact, almost everything we know about *Clunio*'s particular life cycle, its ecology, physiology and behavior, we owe to his persistent research.

The life cycle of the marine midge *Clunio* is precisely adapted to the environmental cycles of the intertidal zone through a combination of different biological clocks and time keeping systems. A *photoperiodic timer* coordinates when larvae arrest their development (enter diapause) to survive the harsh conditions of winter in the larval stage [4, 5]. On the other hand it coordinates when larvae shall remain competent in their development in order to reach the adult stage and reproduce during the summer season [4, 5]. A *circalunar clock* synchronizes development on the population level in the last larval instar to match adult eclosion to the day of the lowest spring tides around full and new moon [5, 6]. Only, when a certain physiological threshold, gated by the circalunar clock, is crossed, larvae undergo a phase of continuous, temperature-compensated development until the pupal stage [6, 7, 5]. A *circadian clock* times emergence to one of the two daily low tides, by gating the diel time adults eclose from their pupal cases [3]. Hence, the interplay of seasonal, lunar and diel endogenous timing mechanisms throughout different developmental stages ultimately regulate the precise emergence timing of *Clunio*.

D. Neumann's findings on the life cycle of *Clunio* were mainly based upon the classical toolkit of Chronobiology available at the time. The experiments were predominately evidentiary

on behavioral observations conducted to establish the endogenous nature of the circadian and circalunar clock. This includes studying free-run [8, 3, 9], temperature compensation [8] and zeitgebers (environmental cues) [9, 10, 11] of both diel and lunar rhythms. To my knowledge there is only one publication before the year 2000 in which a physiological parameter, namely ecdysteroid hormones, were measured in *Clunio* [5]. With his unfortunate passing, D. Neumann left a stack of data, never published, carefully handwritten from which valuable insights into the mechanism of *Clunio*'s time keeping can be still drawn (see this thesis: circalunar phase response curve in Chapter 2 and circadian time series data in Chapter 3).

***Clunio* research in the -omics era**

Then, more than ten years ago, Tobias S. Kaiser, under whose supervision this thesis was carried out, lifted *Clunio* and its circalunar clock research into the omics era. He began to harvest *Clunio*'s diversity of (semi)lunar phenotypes to study the circalunar clock and its local adaptations on the genetic level by exploiting the tools of genetics and genomics [12, 13, 14, 15, 16, 17]. He assembled and annotated the first *Clunio* reference genome and identified loci in the genome which are potentially involved into lunar time keeping [14]. There is no other organisms for which the genomic basis of a lunar rhythm is as thoroughly described and systematically investigated as for the marine midge, which makes it truly unique to study the circalunar clock. Genomic resources constitute a powerful basis to study any complex phenotype [18], and within this thesis we contribute an annotated reference genome on chromosomal scale for a second *Clunio* species (Chapter 5). The second reference genome may allow future comparative genomics studies not only on the population but also on the interspecific level.

Why we should revisit the classic Chronobiology for *Clunio* research

I view this thesis dwelling in between *Clunio*'s classic chronobiological era established by D. Neumann and the omics era introduced by T. Kaiser. Understanding the circalunar clock principle from a conceptual point of view, is important as it guides the search for the molecular mechanism. While D. Neumann always favored the independent oscillator hypothesis for *Clunio*'s circalunar clock [9, 7], our experiments support a circalunar counter dependent on the circadian clock. The discrepancy is partially explained by the fact that we disagree with the way he defined *free-run* for *Clunio* in his experiments [9]. The first two emergence peaks observed under constant conditions (constant in terms of the circalunar clock) should according to our opinion not be considered truly free-running yet because development in *Clunio* is pre-determined up to 20 days in advance. Hence, the first two emergence peaks in a semilunar

population under constant conditions may not be considered for analysis of the truly free-running state of the circasemilunar clock. While we agree that the circalunar clock is not based on counting external LD-cycles, a statement put forward by D. Neumann [9], we argue that he did not consider the possible counting of *endogenous* circadian cycles. The T-cycle experiment conducted in this thesis (Chapter 3) to distinguish between the counter, oscillator and beat hypothesis has been executed for only a few marine species with partially ambiguous results (Chapter 2). Further, a study exists in which diel and tidal T-cycles were changed to reveal the underlying functional principle of a circasemilunar clock in a crustacean, but it was conducted under entrained conditions [25]. Hence, the truly free-running state of the clock was not heeded in this study and the observed phenotype may have been driven rather exogenously by the superposition of the two environmental cycles. In the presence of entraining cues, rhythms may be masked and it is only under free-running conditions, that the behavior of the underlying time keeping mechanism is revealed. Hence, by thoroughly re-evaluating the experimental data gathered for the functional principle of the circalunar clock in *Clunio* and by conducting additional, refined experiments, we contribute further to revealing its operating principle, namely a counter mechanism relying on the circadian clock. Notably, core circadian clock genes were identified to be associated with lunar phenotypes in *Clunio* [14, 16, 17].

To understand the potential causal connection between the circadian and the circalunar clock in *Clunio*, several lines of experiments can be performed. A crucial next step is to characterize the circadian clock on its molecular level. This includes its neuronal location and characterizing the interaction of core circadian clock components (genes, proteins, phosphorylation pattern, epigenetic make up). The first step, namely the identification of core clock genes known from other insects is already provided by the plethora of genomes available for *Clunio* [14, 16, 17] and their molecular functional validation and location may be performed by the suggest molecular tools below, including cell culture assays. To understand the molecular link between the circadian and circalunar clock, the circadian period may be manipulated using pharmacological inhibition as demonstrated successfully in other non-model species displaying tidal and lunar rhythms [26, 20]. Further, circadian clock genes of known molecular role may be knocked down (RNAi) or knocked out (CRISPR-Cas9) and the circadian and circalunar emergence phenotype subsequently monitored. Activity assays of the calcium/calmodulin-dependent kinase II.1 (CaMKII.1) of *Clunio* in *D. melanogaster* S2 cells revealed that CaMKII.1 regulates transcriptional activity of the core clock genes *clock* and *cycle* and circadian period length was correlated with splice variants of CaMKII.1 [14]. Hence, the enzyme may be an interesting candidate to study modulation of the circadian period in *Clunio*.

Future *Clunio* research may focus on the establishment of functional validation techniques

However, there is one important line of research yet missing for the circalunar clock model *Clunio* and this is the study of the molecular architecture of the lunar emergence rhythm [19]. Studies conducted on the transcriptomic and proteomic level in other organisms displaying a lunar rhythm, including the marine annelid *Platynereis dumerilii*, revealed important insights into the clock's molecular architecture [20, 21, 19]. However, most of these studies were conducted in a semi- or natural environment and not in controlled laboratory conditions [19]. Availability of molecular analyses techniques is most advanced for the marine annelid *P. dumerilii*, which can be cultured in the laboratory [22]. However, the circalunar phenotype is much less precise compared to *Clunio*. For *Clunio* there has been so far only one publication which addresses the circalunar clock mechanism with non-genomic, molecular tools. The paper investigates the lunar-cyclic change in transparency of a screening pigment (melanin) in the larval ocelli of *C. marinus* using micro-X-ray fluorescence [23]. This study essentially examines the potential input pathway of the clock (perception). However, to understand the clock location and core mechanism, establishment of a comprehensive map of the nervous system [24] as well as immunohistochemistry techniques, including *in situ* and antibody staining protocols are on the way to lay the foundation to study the circalunar clock in a tissue specific manner. A big caveat at the moment is that there are no genetic manipulation techniques available for *Clunio*. Establishing knock-down (RNAi), knock-out (CRISPR-Cas9) and gain of function techniques need to be the focus of the near future in order to test the long list of candidate genes identified by genomic screens. In this thesis, we examined transcriptomic profiles of the developmental arrests governed by the circalunar clock and photoperiodic counter in *Clunio* (Chapter 4) and thereby aim to address the lack of transcriptomic studies for physiological processes that are downstream of the circalunar clock in the marine midge. The identified candidate genes may be tested with the functional validation techniques outlined above.

The extended phenotype of the circalunar clock in *Clunio*

Currently, the examined circalunar and circadian phenotypes are only observed on the population level in the adult stage. Considering, that eclosion is far downstream of the circalunar clock, future research may focus on identifying behavioural and molecular rhythms on the circadian and circalunar scale in the 3rd and 4th larval stage. Because it is during those developmental stages, that the circalunar clock is entrained, oscillates and ultimately gates the physiological switching point. Further, it may be interesting to identify diel phenotypes in the

larval stage, e.g. larvae may leave their tubes only at certain times of the day or feeding behavior may be under control of the circadian clock, and to establish an autonomous tracking system which allows to monitor rhythms on the individual scale. This provides a behavioral read-out on the circadian scale, in the developmental stage for which the circalunar clock is crucial. When manipulating the circadian clock by one of the proposed functional validation techniques, these protocols will help to see those effects immediately and understand the link of the circadian clock with circalunar time keeping in *Clunio*. Though at the moment there are no indications for the necessity of behavioural rhythms on the semilunar or lunar scale in the larval stages of *Clunio*, time series analysis over the (semi)lunar cycle on the transcriptomic, proteomic or metabolomic level may give new insights into the architecture of the circalunar clock in *Clunio*.

Studying *Clunio's* circalunar clock in an evolutionary context

Finally, the evolutionary context of circalunar clocks may be considered. Given that there are functional connections between circadian, circalunar and seasonal timing in *Clunio* (see Chapters 3 and 4 of this thesis), some targets of selection may affect several phenotypes at a time. This is in line with the finding that circadian and circalunar timing adaptations are not inherited independently [13]. Studying precise phenotypes on different time scales is essentially impossible in most other species with a known circalunar clock. Hence, future lines of research may focus on studying the interaction of time-keeping on different scales in the marine midge. However, for that it is important to describe the molecular basis for all known timing systems in *Clunio* first. And I acknowledge that it requires extreme measures, resources and time to experimentally examine long-term rhythms, e.g. to reach sufficient statistical power to identify underlying periods, multiple cycles have to be recorded.

Chapter 1 and Chapter 2 of this thesis give an extensive overview where in the tree of life circatidal and circalunar clocks can be found and what is known about their operating principle. To understand the evolution of circalunar time keeping, it will be important to study lunar and tidal rhythms in other species, across the Eukaryotic branch. The diversity of operating principles in circalunar clocks are in stark contrast to the unifying principle of the circadian clock and are therefore important to study. Why are circalunar clocks so diverse? Why do some depend on the circadian system and others do not? Can we identify a pattern in certain branches of Eukaryotes? To answer these questions, more scientific attention needs to be drawn on circalunar clock research in general, because it remains to be the outsider in the field of Chronobiology. Unjustly so, in my opinion.

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Acknowledgments

This thesis would not have been possible without the love and care of a great number of people.

First and foremost I would like to thank **Dr. Tobias S. Kaiser**. Tobias, you took me on as your first intern, your first master student and allowed me to stay on as a PhD candidate. You were the first to call me a scientist and always gave me trust and independence to work on myself as well as my scientific career. You greatly shaped who I am as a scientist and I am utterly grateful for that. Whenever you could, you were there to support me and you gave me the opportunity on countless occasions to present your work and your group. I will not forget that.

I deeply thank my thesis advisory committee members, **Dr. David Doležal**, **Prof. Dr. Miriam Liedvogel** and **Prof. Dr. Eva Stukenbrock**. In times of uncertainty you were there to carry this thesis further regardless. Your open doors encouraged me to carry on and all of you shaped the way I think about the projects today. Eva, in the greatest need you took time out of your schedule to help me move on and your encouragement was what let me take the final steps. Thank you.

I am grateful for the **Max Planck Institute for Evolutionary Biology** and the **International Max Planck Research School (IMPRS)** who have been hosting me since 2017. The communal support you can provide is truly inspiring and it has been my absolute pleasure to learn under you. Thank you **Karel Jankovskis** and **Haustechnik** for the immense technical help you have been providing including building a shed in which the experiments could run undisturbed by vibration. I additionally thank the **Studienstiftung des Deutschen Volkes**, who awarded me a four-years doctoral stipend.

I am also grateful to the **Research Group Biological Clocks**. From the beginning you gave me a safe space to explore what science is and means and what part I want to play in it. Though things have been tough in many moments and we disagreed occasionally in how to shape this group, I respect and admire you all and I wish you only the best and that we may meet again. Thank you. **Nico Fuhrmann**, for agreeing to take me on as an intern back then. **Kerstin Schäfer**, you carry this lab on your shoulders – we would be truly lost without you. **Alexander Jacobsen**, your optimism made me feel warm and welcome on so many occasions and the way you think is truly inspiring. **Carolina Peralta**, you have been my best friend in a very difficult time and

I will not forget that. **Danila Voronov**, we shared an office and with it most of our emotions. **Duška Briševac**, you shaped my earliest scientific career almost more than Tobias has. I also thank all the previous and current members of the group I could not mention here.

I am grateful for the support and inspiration the research community has given me. Yes, academia is full of flaws and toxicity, but there are people with an indescribable amount of selflessness and I was so lucky they shared their gift with me. They are the lanterns in a moonless night and without them I might have gotten lost on the way. Among others, this include **Daniel Unterweger**, **Chaitanya Gokhale**, **Alexander Klimovich**, **Bernhard Haubold**, **Charlotte Helfrich-Förster** and the Chronobiology community I met at **SRBR 2022** in Florida.

I am further immensely moved by the trust and support my family has been providing throughout this years. **Ida**, the thesis is dedicated to you, because you are my inspiration for happiness. I love you so much and you are the sun of my universe. **Skywalker**, my sister, we have been through so much together and most people will never understand why life is so difficult for us. But you remind me of why it is worth to carry on. I love you. **Papa**, you introduced me to academia even before I could walk and your passion for science has been a great motivator throughout my career. I love you too. I also like to thank my family of choice: **Levke**, **Maja**, **Öfi** and **Miri**, I have no words to describe the amount of love I have for you. You have been the steady environmental cycle throughout my entire adult life. And thank you, **Finn** and **Phili** for always cheering me on. I could mention so many more members of this crazy world I call my family but this requires to write another thesis.

And then there is you **my love**. I never imagined I would find you here. I met you in a time of chaos and fragility. And you carried me without asking, effortlessly and selflessly. Thank you for being yourself and for allowing me to share my life with you.

Declaration

I hereby declare,

- that apart from my supervisors, Dr. Tobias S. Kaiser's and Prof. Dr. Eva H. Stukenbrock's guidance, the content and design of the thesis is my own work and written using only the specified sources.
- that the thesis has not been submitted partially or wholly as part of a doctoral degree to any other body as part of an examination procedure. Apart from the included published papers and the submitted papers, no part of the thesis has been published or submitted for publication. The section ***Contents and contributions of individual chapters*** states the author contributions.
- that the thesis has been prepared according to the Rules of Good Scientific Practice of the German Research Foundation.
- that an academic degree has never been withdrawn.

Jule Neumann

Kiel, 2nd of February 2024