Throughout history, people have tried to accurately measure time to predict the rhythmic changes caused by the sun. Dating back to earliest days of civilization, by using the position of the moon relative to the stars, people could plan their agriculture and religious ceremonies throughout the year. Around 3500 BC, ancient Egyptians would coordinate their daily activities by using an obelisk as a sundial to track time. Later, methods that do not depend on the sun where developed, such as the clepsydra, candle clock and hourglass, as shown in Fig. 1.1. However, for longer time intervals lasting days or even months, these methods are too inaccurate to measure time reliably. It was not before 1656, that the first precise mechanical clock was invented by the famous Dutch physicist Christiaan Huygens. His design for a pendulum clock reduced the error in the period to less than 10 seconds a day or one ten thousands of a second.

Given humanities long strive to accurately measure time, it is remarkable that precise clocks have been on earth for billions of years already. Cyanobacteria, single cellular organisms that use sunlight for their growth, are likely to have mastered accurate time measurement at least 2 billion years ago. They contain a 24 hour rhythm, resulting from chemical reactions between proteins, which is called a circadian (Latin: *circa* for “around” and *dies* “day”) clock. Nowadays, circadian clocks are found in organisms ranging from bacteria and fungi, to plants, insects and animals and allow them to anticipate the changes between day and night. Remarkably, these clocks can often maintain stable rhythms for months or even years in the absence of any daily cue from the environment, such as light-dark or temperature cycles [1]. This robustness is surprising, given the fact that experiments in recent years have vividly demonstrated that protein synthesis, which is required to sustain the clock, is highly stochastic [2]. Clearly, circadian clocks tend to be designed in such a way that they have become resilient to the intrinsic stochasticity of the underlying biochemical reactions.

Next to the biological clock, all living cells maintain another rhythm that is essential to their existence: The continuous repetition of growth and division called the cell cycle. Be it bacterial cells, yeast cells, or cells in growing tissues; they all have to duplicate all their components during the cell cycle. Interestingly, experiments in recent years
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Figure 1.1: Different methods for keeping track of time. (A) A shadow cast by a sundial, tracking the sun's position, can be used to tell the time of the day. However, it only works when the sun shines. A candle clock (B) and a clepsydra or water clock (C) do not have this problem. The regular burning of candle wax or the constant filling of a vat with water can be used to measure time. However, these methods are too inaccurate to be able to tell time over the course of months. This problem was finally solved by Christiaan Huygens' design of the escapement mechanism of the pendulum clock (D). In his design, the driving of the pendulum by the falling weight (to compensate friction), did not affect the period of the swing.

have shown that the clock of one of the best characterized model systems in biology, the cyanobacterium *S. elongatus*, is not only robust to biochemical noise, but is also insensitive to variations in the growth rate: the circadian clock maintains a stable period of 24 hours, even when the cell cycle (i.e. the time between successive cell divisions) is reduced from 24 hours to 10 hours [3]. Since the cell cycle inevitably leads to oscillations in the protein synthesis rate [4], which one expects to affect the clock, the question is how it can be that the clock's rhythm is unaffected by the cell cycle.

A fascinating property of circadian clocks is that they continue to tick with a nearly 24 hr period even when cues in the environment, like temperature, change, a phenomenon known as input compensation [5]. Yet, while these clocks are robust to a permanent change in the environment, they are still sensitive to transient changes in the environment [6, 7]. The latter phenomenon is known as entrainment, and it is necessary to keep the clock in phase with the day-night rhythm despite the presence of inevitable biochemical noise. How clocks can exhibit both input compensation and entrainability, is still poorly understood. Changes in the environment such as those in temperature typically lead to changes in the reaction rates, which tend to affect the period of the clock. Input compensation means that the clock is designed such that it has become insensitive to permanent changes in the biochemical reaction rates. Entrainability means, however, that the clock is still sensitive to periodic changes in these rates. How can the clock pick up transient changes yet ignore permanent changes in reaction rates?

In this thesis, I use mathematical modeling to study the robustness of cellular oscillators in the chaotic environment of the growing and dividing cell and in vitro. I will focus on the Kai system of the cyanobacterium *S. elongatus*, although I will also study the implications of our findings for the design of synthetic oscillators. First, in the remainder of this chapter, I provide a short overview of the history of the field followed by my view on the role of modeling in systems biology. I end with a summary of the chapters in this
1.1. A BRIEF HISTORY OF THE RESEARCH OF CIRCADIAN CLOCKS

The research of circadian rhythms started by the 18th century astronomer Jean-Jacques d’Ortous, who discovered that the leaves of heliotrope plans show a rhythmic motion of their leaves in response to light. More importantly, even when the plants were placed in continuous darkness, their leaves continued to move with a 24 hour period [8]. This was the first indication that some organisms have an internal rhythm, or clock, which is not simply the result of the daily rhythm in light and temperature. In the early 1970’s the research in circadian rhythms took a major leap when Ronald Konopka and Seymour Benzer revealed the genetic basis of the circadian activity of the fruit fly *Drosophila melanogaster*. By studying the chromosomes of mutants, which have a different rhythm in their activity compared to wild-type flies, they could track down the difference in behavior to a single genetic locus, termed the *period* gene [9]. Flies with mutations in this gene had a different period in their activity than normal flies.

For long, scientists thought that circadian clocks can only exist in eukaryotic cells because a cell nucleus, intercellular communication and cell division times longer than 24 hours were necessary for a biological clock to function. However, in the mid 1980’s, it was shown that cyanobacteria have clear circadian rhythms in their nitrogen fixation, amino acid uptake and cell division. In 1993, Kondo and coworkers were able to implement a luciferase reporter gene on the chromosome of the cyanobacterium *Synechococcus elongatus* PCC7942, “to demonstrate unequivocally that cyanobacteria exhibit circadian behaviors that are fundamentally the same as circadian rhythms in eukaryotes” [5]. Shortly after, the genes that form the internal clock of *S. elongatus* were identified by Ishiura et al. and denoted KaiA, KaiB and KaiC, after the Japanese word for cycle [10]. They found a negative control loop where KaiC suppresses its own expression which generates the circadian oscillations in cyanobacteria. KaiC can be phosphorylated, which is regulated positively by KaiA and negatively by KaiB [11]. Finally, by a series of groundbreaking experiments, Nakajima and coworkers showed that the circadian oscillation of KaiC phosphorylation could be reconstituted in a test tube with only KaiA, KaiB, KaiC and ATP [12]. This is the only known system that shows circadian oscillations without a transcription-translation cycle. Isolated from all the other processes inside the cell, the Kai circadian clock of *S. elongatus* has become the ultimate system for studying the mechanisms involved in generating robust post-translational oscillations, both for experiments and theoretical modeling.

1.2. THE ROLE OF MODELING IN BIOLOGY

The natural world can be studied through experiments, theoretical analysis and, recently, also through computational modeling. In physics, theoretical analysis has been very successful in making quantitative predictions and explaining physical phenomena. In biology, on the other hand, most discoveries are made through experimental exploration, as the systems studied are often regarded as too complex to explain through the-
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Theoretical analysis [13]. However, over the past decades experiments in biology have improved to such a high level that we now have very quantitative data, down to the level of single cells and even proteins [2]. This quantitative data has made it possible to verify the predictions from theoretical models. Therefore, improvements in experimental techniques in biology has made the use of modeling in biology to answer questions more common. Because models of biological systems are often very complex, computers are used to simulate the models and make predictions.

In this thesis, I will use the quantitative data gathered on the cell cycle and the circadian clock of *S. elongatus* to understand how a biological clock can be so robust in its chaotic environment. However, the behavior of circadian clocks, and of biological oscillators in general, is notoriously hard to grasp intuitively or analytically, because of the many non-linear effects, feedback loops and delays that are present in their reaction networks. This makes computational modeling an essential tool for a systematic study on the robustness of biochemical oscillators, and their complex interaction with the cell cycle. Previously, modeling has been very successful by illuminating several mechanisms in the Kai circadian clockwork of *S. elongatus*, such as the role of synchronization of the phosphorylation of KaiC [14–18], the necessity of having both a phosphorylation cycle and a transcription-translation cycle [15, 19–21], the function of having two phosphorylation sites per monomer [18, 22–25], and the presence of a long temporal delay in the cycle [15, 19, 26, 27].

I will use different models of the circadian clock of *S. elongatus* to investigate the clock’s robustness. This might sound strange –why not use the ‘best’ or most detailed model of the system? The reason is that one wants to use the simplest possible model to answer a particular question. Using a too complex model might obscure a researchers view from gaining insight into the system's inner workings. The motive for modeling, in general, is not to make the most detailed possible description of the system —since the best model is the biological system itself— but to illuminate and understand a particular mechanisms at work. Furthermore, by using a simplified model of the clock, we emphasize that a certain mechanism does not critically depend on the details of the particular system studied like *S. elongatus*. We expect to find the same mechanism in biological oscillators of different organisms as well. Therefore, in the first chapter we used the relatively simple model of the phosphorylation cycle by Van Zon *et al.* [15]. In chapter 4 we develop a new model to reveal the thermodynamic drive behind the Kai circadian clock. To this end, we had to design a model that includes all hydrolysis, phosphorylation and dephosphorylation reactions of the Kai system, and make sure that all these reactions fulfill detailed balance. This required us to make a model that describes the clockwork in a much more detail than the models we employed in previous chapters.

1.3. Scope of this Thesis

First, in chapter 2, we address the question of how the circadian clock can maintain its 24 hour period inside a growing and dividing cell. Cells that grow and divide, be it bacterial cells, yeast cells, or cells in growing tissues, all have to duplicate all their components during the cell cycle. Interestingly, experiments in recent years have shown that the clock of *S. elongatus* is insensitive to variations in the growth rate: the circadian clock main-
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tains a stable period of 24 hours, even when the cell cycle (i.e. the time between successive cell divisions) is reduced from 24 hours to 10 hours [3]. Previous studies focused on the effect of intrinsic number fluctuations in the concentration of clock proteins and the noise in gene expression and their effect on the clock’s period. However, in these studies, the direct effects of the cell cycle on the clock were not taken into account. Here we include the effect of chromosome replication which causes a doubling of the transcription rate of clock related proteins, with a period of that of the cell division cycle.

We find that circadian oscillators are particularly sensitive, as they can phase-lock to the cell cycle, so that the clock period tracks the cell division time, or exhibits erratic behavior. Circadian clocks employ two general mechanisms that insulate them from the cell cycle: i) A phosphorylation-based protein modification oscillator, together with its accompanying push–pull read-out circuit that responds primarily to the ratios of different phosphoform concentrations, which makes the clock less susceptible to perturbations in protein synthesis rate; ii) the presence of multiple, asynchronously replicating copies of the same chromosome diminishes the effect of replicating any single copy of a gene.

We extend our analysis of the effects of the cell cycle in chapter 3, to two famous synthetic oscillators: the repressilator by Elowitz and Leibler [28] and the dual-feedback oscillator by Stricker et al [29]. Given that the networks of these oscillators have a simpler design than biological oscillators, as the reaction networks contain fewer genes and do not involve post-translation modification, we want to know how sensitive synthetic oscillators are to the effects of the cell cycle. We find that the period of both oscillators is strongly perturbed by the cell cycle, and that the effect critically depends on the position of the genes of the oscillator on the chromosome. Even in the limit of high levels of noise in the replication times of the genes, which reduces the coupling between the clock and the cell cycle, both oscillators show clear signatures of locking. We argue that the study of synthetic oscillators can be used to test which mechanisms are critically important for a robust biological oscillator inside growing and diving cells.

In chapter 4 we present a new, thermodynamically consistent, statistical-mechanical model of the Kai circadian clock. We were inspired by the remarkable recent observation that ATP is regenerated during the dephosphorylation of KaiC, which suggests KaiC goes through its phosphorylation cycle without consuming ATP —something that clearly violates the second law of thermodynamics. We set out to answer the question of how the clock is thermodynamically driven. In our new model, building on ideas from previous mathematical models, KaiC consists of the CI and the CII domain. Hydrolysis of ATP in the CI domain provides the thermodynamic driving force for the conformational switch in KaiC, which determines whether the phosphorylation level of KaiC increases or decreases. Phosphorylation of the CII domain acts as a timer for when the hexamer switches confirmation. Using a dedicated kinetic Monte Carlo algorithm, which makes it possible to efficiently simulate the system which contains over a billion reactions, we show that our model can describe the majority of experimental results. Remarkably, the model shows very strong input compensation: When we lower the ATP level in the buffer from 100 to 50%, which slows down the rates at which KaiC phosphorylates, the period in the phosphorylation fraction only slightly changes. Because phosphorylation is the slowest reactions in the biochemical network, and therefore determines its period, we want to know how the period can be constant under changing ATP levels.
This motivated us to test how well our new model fulfills two essential criteria for a functional circadian clock: The clock should be entrainable, such that it is always in phase with the time of the day, and should exhibit input compensation, such that the period remains 24 hours in different environments. Experiments show that the daily fluctuation of the ATP fraction inside the cyanobacterium is the main cue for entrainment, while the period of the clock almost does not depend on the bulk ATP fraction [7, 26, 30]. In chapter 5 we compare our new model with the models by Van Zon et al. [15] and Rust et al. [22], and find that the new model exhibits the best trade-off between input compensation and entrainability under a change in the ATP fraction of the bulk. Performing stochastic simulations at the level of individual hexamers allows us to identify a new mechanism for input compensation: At lower ATP fraction, the individual hexamers make a shorter cycle in the phosphorylation state space, which compensates for the lower pace at which they traverse the cycle.
BIBLIOGRAPHY


