Flying Clocks
The clocks of Drosophila

Wolfgang Engelmann
Institut für Botanik, Tübingen

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as a representative of the
insect world I have often wondered
on what man bases his claims
to superiority
everything he knows he has had
to learn whereas we insects are born
knowing everything
we need to know

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Only a fly ...

Autumn. The foliage is taking on their fall colors. Time of harvesting. The fruits have ripened and the kitchen swarms with little flies. Entire clouds of fruitflies may fly up from the waste bin if opened to get rid of a new load of garbage. Much to the annoyance of the housewife these *Drosophila* flies hang around allover in these days. But what wonders hide behind these little flight artists! Just a fly?

I invite you to a time journey. We will see, how from the egg which was layed on a fruit a fly develops, which mates with a male fly and produces eggs again, out of which already two weeks later a new generation comes into being. How maggots develop from the embryos in the egg, which molt and pupate. Early in the morning, while it is still cool and humid, the flies emerge, because an internal clock tells them its time to crawl out of the pupal casing.

The emerged fly obeys also an internal clock. This clock gives the measure for sleeping and waking. The light-dark cycle sets the clock for correct running. The environmental temperature can’t harm the clock: It has at higher and lower temperatures always the same speed.

Only a fly. Fly-clocks, flying clocks: Where do they tick in the fly, how do they tick and what for do they do it? A time journey, which shall show you the wonders in such a tiny fly.
Only a fly ...
1 The fruitfly *Drosophila* as a model-system of geneticists

*The generation cycle of the fruitfly Drosophila is described. The flies are suited for genetic studies. Numerous mutants exist, in which among others the daily clock has changed.*

In biology, the study of life, genetics plays a central role. Geneticists are studying heredity. The Augustinian monk Gregor Mendel (1822-1884) discovered already in 1866, that the genotype of an organism consists of individual factors (Mendel (1866)). He had crossed in the garden of the monastery in Brünn two pea varieties with each other which differed in just one trait such as the color of the flowers. The color of the flowers of the offsprings (F1-generation) was intermediate in respect to the colors of the parents. He observed, that by crossing their offsprings among each other plants developed in which the original color of the parent flowers segregated again in one fourth of the second generation (F2-generation).

The Mendel laws were rediscovered in 1900 by Correns (1864-1933), de Vries (1848-1935) and Tschermak (1871-1896) (Correns (1900), Vries (1900), Tschermak (1900)). Chromosomes were assigned to these abstract genetic factors as a concret substrate. Bateson (1902) showed, that the Mendel laws followed from the behavior of the chromosomes (called before coupling groups) during cell division and fertilization.

F. Miescher (1844-1895), a contemporary of Mendel, discovered in 1869 in Tübingen the nucleic acids (Miescher (1871)). It took almost 80 years, until they were identified as the carriers of the genetic information (Watson and Crick 1953, Watson (1969)). As DNA-double helix it fulfills all requirements which the genetic substance needs. In mutants particular genetic factors, called genes, have changed. The sequence of nucleic acids of the corresponding gene has varied. Thus, if in a red flowering pea by mutation the gene for the red color of the flower has changed, the pigment is not produced anymore and the flower stays white.

Nowadays much is known about genes, how they are built, how they function and how the gene leads to the phen, the external expression of the genetic information (such as the red color of the flower). Morgan (1866-1945, Nobel price 1933) found in his studies, that the fruitfly *Drosophila* is particularly well suited for studies (Morgan (1928)). There are in the meantime numerous mutants. Even certain behavioral patterns of these flies can be traced back to certain genes. More about it in section 1.2.

1.1 Vita of a fruitfly

The fruitfly *Drosophila* is often found on wrotten fruit. The males display in front of the females in a characteristic pattern ¹ which might occur by chance or induced by irradiation with UV-light or by mutagenic substances
Figure 1.2: Life cycle of the fruitfly Drosophila. Female fly (top) deposits eggs (one shown), out of which a larva emerges (three stages, third stage shown bottom left). It pupates in a puparium (center left) and transforms into a fly (metamorphosis). After completion and after the right time has been reached, a lid is pressed open and the fly emerges out of the pupal case. After Geibel (1987)
1.2 Drosophila - the ideal organism for scientists

Drosophila is an ideal object for geneticists, biologists, chronobiologists and molecular biologists. The animals are small, allowing to rear them in glass bottles in the laboratory in large numbers on a simple food mixture. They are, on the other hand, large enough to observe their behavior. Mutants are obtained by using mutagenic substances, and there are in the meantime many of them available. Even behavioral mutants have been found, especially by the pioneering studies of Benzer (Benzer, 1967). One of his students, Konopka, isolated mutants, in which the daily clocks had changed (Konopka and Benzer, 1971). In one of these mutants the daily clock ran faster as compared to the wild type, in another one more slowly, and in a third one the clock was defect. In the meantime the molecular biologist have studied these mutants intensively, trying to

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2 in chronobiology the significance of time and periodic timing for organisms is studied
3 molecular biologists try to understand the molecular basis of biological processes. How is, for instance, a gene composed and how is the genetic information coded and passed to the offsprings.
The fruitfly Drosophila as a model-system of geneticists

find out how these clocks might function on
the molecular basis. More about it in chap-
ter 5.
2 Eclosing in time windows

In the fruitfly Drosophila different rhythms can be observed. If in the pupal stage a fly is formed out of the maggots, it eclose at a certain time of the day out of the pupal case. An internal clock opens a time window. Only during this time span a fly can eclose, and this is advantageous for the animals. The eclosion clock is synchronized by the light-dark cycle of the day. We will get to know also the light receptors involved.

The fruitfly Drosophila pseudoobscura is found in the southern states of the USA. Especially Pittendrigh and his students studied in this species, at what time the animals eclose (Pittendrigh (1993), figure 2.1, Winfree (1986), figure 2.2). This species lives in arid areas. The larva crawl at the end of the fourth larval stage into the soil. In a depth of about 4 to 10 cm they pupate. No light penetrates in this depth and temperature differences between day and night are small. The larvae transform into a fly in about 7 days. It emerges out of the pupal case and crawls up to the surface of the soil. There the outer layer of its body (cuticula) hardens in the first hours and the animals are now able to fly.

The not yet hardened cuticle is water permeable. To avoid drying out, the animals have to eclose at a time, when the air is still humid. This is the case in the early morning. An internal clock tells the newly formed fly somehow, that now the most favorable time has come for eclosion. The animals open in the pupal case a preformed lid and can eclose. They make their

Figure 2.1: Colin S. Pittendrigh, the ‘Darwinian clock watcher’ (self-confession). University of Durham (England), Columbia university (New York, USA), Princeton university (New Jersey), Stanford university (California). Photography from 1967
2 Eclosing in time windows

way through the soil to the surface. Since the pupae are in the soil for about 7 days, the clock has to be quite accurate and has to open a time window every 24 hours, in which the animals can eclose as soon as the flies are formed.

If we observe many pupae, the eclosion from the pupal case is not uniformly distributed over the day. It occurs, instead, in the early morning briefly after sun rise. Each fly can, of course, eclose only once. It uses a time window of about 4 hours. If it is not yet ready to eclose, it will wait until the next time window on the following day for eclosion. Figure 2.3 shows in the second curve the number of eclosed animals per hour during one week at a temperature of 21\(^\circ\)C. Each day we observe during the first four hours after onset of light a high eclosion rate. During the afternoon and at night no animals eclose.

2.1 Time for eclosion

How do the animals know that it is time for eclosion? First of all they have to be developed far enough, so that the fly is completed. In addition, they have to know, at what time the daily time window for eclosion is open (early in the morning).

In the same way as a detective tries to think of varies possibilities how a crime might have occurred, we too might consider, how the flies have managed the trick to eclose at the right moment and to crawl to the surface whilst the air is still humid, thus avoiding to dry out. Scientist put forward hypotheses for solving a problem (here: How do the flies find the right time for eclosion). We will do the same. Like a detective we too have to test our hypotheses critically. Scientist do it usually by performing experiments.

Figure 2.2: Arthur Winfree studied technical physics at the Cornell university, took part in a sea voyage of the research vessel RV Chain, PhD thesis 1970 at Princeton university (New Jersey) under the guidance of Pittendrigh, afterward professor at the Purdue university, at the university of California in San Diego, and at the university of Arizona. Here with his son Erik at the authors home in Tübingen
Hypothesis 1: With the morning sun it becomes warmer. The rising temperature causes those animals, which have developed sufficiently, to eclose (upper diagram in figure 2.4). We test this hypothesis in a room with constant temperature (figure 2.5). The sunlight is replaced by a white fluorescent tube. It is switched on by an electric timer for 12 hours and afterward switched off for 12 hours. Result: In spite of the constant temperature the animals continue to eclose in the first hours after onset of light. This means, the animals do not eclose in the morning, because the temperature rises.

Hypothesis 2: The light in the morning is responsible for the increased ecolision rate (second bar chart in in figure 2.4). This hypothesis can also be tested (figure 2.5). We transfer pupae of varies ages, which are ready to eclose in the next days, after a 12 hour light period not into darkness, but in weak red light. The light stays on during the entire observation period for several days. For the animals red light is like darkness (it is also called safelight). The red light allows us to observe the ecolision of the animals. Astonishingly many flies eclose again in a four hour time window briefly after the time at which normally the white light would have started. And if we watch for another day, we find 24 hours after ecolision a further ecolision maximum. It looks like the animals are able to use the time window for ecolision even without an external indicator (or time cue).

we have to admit though that in nature the animals have pupated in an average depth of 7 cm, and it will surely take some time, until the soil has warmed up by the morning sun. It is therefore unlikely, that this hypothesis is true for animals outdoors. In spite of this it has been shown, that a temperature change with higher degrees during the days and lower temperatures in the night synchronizes ecolision.
Figure 2.5: Test of hypothesis 1 (top diagram): If the temperature fluctuations during the course of the day would be responsible for the eclosion time, eclosion should not occur anymore only at a certain time span under constant temperature. The animals continue, however, to eclose in a small time window at the onset of the light period.

Test of hypothesis 2 (second diagram): If the light period would be responsible for eclosion, it should without a light-dark cycle not be restricted anymore to certain time spans (the grey curve indicates the time span, at which the day before the light period was given). It turns out, however, that the animals continue to eclose at the same time and 24 hours later (between the 30. and 33. hour).

Test of hypothesis 3 (lower diagram): The light period was administered 12 hours later as usual (yellow line). It turns out, that this shifts the eclosion rhythm by 12 hours. If a factor X (green curve) would have been responsible for eclosion, the animals should have eclosed at the usual time.

We conclude, that the animals eclose at certain times, which are determined by an internal clock. This clock can be shifted by light (as shown by the lower curve).
Hypothesis 3: The animals could use an environmental factor (‘factor X’) as a time cue, which continues to occur in a 24 hour measure in our chamber in spite of constant temperature and continuous red light (lower diagram in figure 2.4). For instance the magnetic field of the earth fluctuates in daily cycles. To test this hypothesis, we keep differently aged pupae before eclosion in an inverted light-dark cycle: The 12 hour light period becomes a dark period, the night will be daytime. Now the animals eclose preferentially during the first hours after onset of the artificial light period, that is, a time, when it is night in nature (figure 2.5). If we now offer after the inverted lightening continuous weak red light, the animals eclose 24 hours after the increased eclosion rates of the inverted light-dark change. Would external time cues be responsible for the four hour eclosion maximum, they should shift eclosion under the continuous red light back to the normal eclosion time. We see thus, that a light-dark cycle is a time cue for eclosion: The rhythmic eclosion can be shifted to other times of the day. But under constant conditions the rhythm continues to run. There are no hidden external factors such as the periodic fluctuation of the magnetic field of the earth, which influence the eclosion time.

From these experiments we have to conclude, that the animals eclose at certain times of the day, which are determined by an internal clock. The clock can be shifted by light.

2.2 A clock for eclosion

In the last section we have seen, that Drosophila-flies possess internal clocks, which restrict eclosion to a certain time.
window, which is repeated every 24-hours. These clocks can be synchronized by the light-dark cycle. The animals need this internal clock, because they pupate in the soil in a depth, where no light can be seen and where temperature differences are small. Since it takes 7 days, until a larva has changed into a fly, the clock must be quite precise and open the time window every 24 hours. By the way, the eclosion clock of *Drosophila pseudoobscura* is one of the very rare cases, in which the period is exactly 24 hours long. We will get to know later other daily clocks. Most of those clocks run under constant conditions not exactly in a 24-hour-measure, but somewhat faster (for instance 23.4 hours) or somewhat slower (for instance 24.8 hours). These clocks are therefore called circadian clocks.

We have also seen, that there are no hidden time cues, which cause the rhythmic eclosion. If namely the animals are kept in an invers light-dark cycle, the eclosion rhythm is likewise inverted and continues to be inverted, if the animals are kept in the dark (or weak red light as safelight). In a station at the south pole circadian eclosion was also observed, although there are practically no time cues present for the day-night changes during the summer or winter time. Instead there is continuous light, and in the winter continuous darkness, and all other physical quantities, which change in a daily fashion on other places of the earth, are constant.

### 2.2.1 Mutants: Fast and slow ones

Although the eclosion rhythm has a precise 24-hour measure, there are even in *Drosophila* indications, that we are dealing with an internal clock (if the period length is exactly 24 hours, it is very likely that a hidden time cue drives the rhythm): In *Drosophila* mutants are known, the clocks of which run either faster (*per^s*), or slower (*per^l*) as compared to the wild type. This substantiates in a shorter than 24 hour (*per^s*) and a longer than 24 hour rhythm (*per^l*) of the daily eclosion. Thus, eclosion is indeed controled by an internal clock. The activity rhythms of the mutant flies will also show a shorter (for *per^s*), longer (for *per^l*) and an arhythmic (for *per^0*) pattern (figure 2.6). If the clock gene of the mutant is repaired by introducing the functional gene of the wild type, the eclosion rhythm is again exactly 24 hours respectively close to 24 hours in the case of the activity rhythm.

By the way, there are also mutants available which eclose earlier than normal (early) or later than normal (late), although the period length of the eclosion rhythm under constant conditions amounts to exactly 24 hours and is thus normal (figure 2.7). In this case the mutation has changed the coupling of the clock to the light-dark cycle.

### 2.2.2 Eyes of the eclosion clock: Time-taker and time-giver

There are employers and employees. The employer has a company and offers work to people who want to earn money. He is so to speak a work-giver. The workers are the employees or work-taker. For organisms with internal clocks there is a similar relation. The internal clocks require a time giver or time cue of the environment, which allows them to run with the right measure in a 24-hour cycling world. Without time cue these clocks would loose synchronization, since no clock is precise enough, to stay in synchrony without corrections. Furthermore, a clock has also to be started and needs to be synchronized.
2.2 A clock for eclosion

On the other hand clocks need also time-taker, that is, a device which allows them to recognize the time cue. The time cue signals have to be used to set the clock, if it is not running properly (figure 2.8).

We have seen on page 17, that the eclosion rhythm can be shifted by a light-dark cycle. Even a single light pulse can shift the rhythm of eclosion, if offered for instance to flies in pupae which are ready to emerge and are kept under continuous darkness. The light–dark cycle as well as a single light pulse are thus time giver or time cues. There must, however, also be time taker, which transfer the light signal to the clock, which times eclosion. How do we find out the type of light receptors which are involved?

The usual method is to obtain an action spectrum and to compare it with the known absorption spectra of various pigments. An action spectrum is obtained in the following way: Coloured light is used to irradiate the organism and the effect (‘action’) is measured. The more effective a particular color (scientifically: Wavelength) is, the fewer light energy is needed to obtain this effect.

How do we obtain an action spectrum in the case of the eclosion rhythm? We know, that a light pulse can shift the eclosion rhythm. We use now various light colors with different intensities and determine for each wavelength the amount of phase shift (figure 2.11). The higher the light intensity, the larger the phase shift of the rhythms. We can now determine the amount of light of a certain color, which shifts the rhythm for instance by four hours. If we do this for many wavelengths and plot the values in a diagram, we obtain an action spectrum (figure 2.12). In the next step we com-
Figure 2.9: A light pulse shifts the circadian eclosion rhythm of Drosophila pseudoobscura. Bottom curve: Control without light pulse. Top curve: A light pulse given at a certain time (*) shifts the eclosion maxima in respect to the corresponding maxima of the controls (broken blue lines), as shown by the red arrows (by 1, 2, 4, 4, 4, 4 hours)
2.2 A clock for eclosion

Figure 2.6: Fast (per<sup>s</sup>), slow (per<sup>l</sup>) and arrhythmic (per<sup>0</sup>) Drosophila-mutants. Activity pattern of a representative fly recorded first for a week in a light-dark cycle (LD, first line above the corresponding actogram with black bar for darkness and red bar for light) and afterward from day 8 to 30 in weak red light (DD, second bar above the corresponding actogram with black bar). The actograms show the daily activity of the flies from 0 to 24 o’clock. For better recognition the actogram block was plotted again at the right and displaced by one day upward, in order to have after day 1 day 2, after day 2 day 3 and so forth. On the y-axis the days are plotted. After Helfrich-Förster (2002)

Figure 2.7: Early and late eclosing Drosophila-mutants. Plotted is the eclosion pattern of fly populations in the course of a day. The early eclosing mutant (‘early’) has its eclosion maximum of flies out of the pupal case earlier as compared to the wild type, the late eclosing mutant (‘late’) later. After Pittendrigh (1981)
pare it with absorption spectra of various known pigments. If we find now, that our action spectrum corresponds to the absorption spectrum of flavin, it is likely, that this pigment is responsible for the shift of the eclosion rhythm by light.

The effect of the light depends on the strength of the pulse and on the time at which it hits the fly (figure 2.9, figure 2.10). At the subjective midnight\(^2\) the phase shift is strongest, provided the light pulse is strong.

Since the effect of a temperature pulse depends on the phase of the daily rhythm, a certain time point has to be chosen (for instance one hour before subjective midnight). If this is not done and different time points are used, the pulses lead to different effects, although the light intensity was not changed.

### 2.2.3 Temperature-compensation of the eclosion clock

Clocks are driven by a clock work and they must be set (synchronized). They need, however, a further property: They must not run faster at higher and slower at lower temperatures. This is indeed the case in the eclosion rhythm (figure 2.3): The rhythmic eclosion of flies occurs independently of the environmental temperature always in a 24-hours measure (Pittendrigh (1954)).

In spite of it the temperature has an effect on eclosion. If pupae in continuous red light are exposed to a 12:12 hours temperature-pulse in the light-dark cycle with a light period from 6 to 18 o’clock and a dark period from 18 to 6 o’clock midnight would be at 24 o’clock. If the organism, the circadian rhythm of which we study, is under constant conditions, the midnight-time point could well lie at another time. If, for instance, an inverse light-dark cycle is offered with a dark period at the day, the subjective midnight would be at 12 o’clock noon.

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\(^2\)in the light-dark cycle with a light period from 6 to 18 o’clock and a dark period from 18 to 6 o’clock midnight would be at 24 o’clock. If the organism, the circadian rhythm of which we study, is under constant conditions, the midnight-time point could well lie at another time. If, for instance, an inverse light-dark cycle is offered with a dark period at the day, the subjective midnight would be at 12 o’clock noon.
Figure 2.11: Dosis-effect curves of the phase shift of eclosion of Drosophila: Various light intensities (x-axis, number of light quanta per square meter and second) of different wavelengths (see the numbers at the curves, in nanometer) were used for irradiating groups of pupae at a certain phase of their circadian rhythm (CT 17). At this particular phase phase delays are induced, and higher intensities (more quantae) shift stronger, as shown by the slope of the curves. Certain wavelengths such as 459 and 451 nm are more effective as others (for instance 384 and 395 nm). This is reflected in the lower number of light quanta needed for phase shifting. The x-axis is logarithmic. Such data are the basis of the action spectrum in figure 2.12. Details in Helfrich-Förster and Engelmann (2002)
2 Eclosing in time windows

Figure 2.12: Action spectrum of phase shifting light in the eclosion rhythm of Drosophila: The number of quanta needed to shift the rhythm by four hours was obtained from figure 2.11. The reciprocal values (that is 1/value) was plotted as a measure for the sensitivity on the y-axis against the wavelengths (x-axis). 360 to 390 nm: UV-light, 400 to 480 nm: blue light, 510: green light. Longer wavelengths (red light) are without effect. Details in Helfrich-Förster and Engelmann (2002)

Figure 2.13: A 12:12 hour temperature change of 25°/20°C (red/white) synchronizes the eclosion rhythm (black bars) of Drosophila pseudoobscura. The larvae and pupae were kept under continuous red light. x-axis: time in hours. After Zimmerman et al. (1968)

light pulse can shift the rhythm, a single temperature pulse (for instance of 3 hours duration) is able to shift the rhythm under constant conditions.

2.3 Maggot or fly?

In contrast to the vertebrates insects do not possess an internal skeleton, but an external one. It consists of chitin, is hard and shock-resistant and protects the animals from desicating. However, it is not able to expand much. Therefore insects have to molt, if they have eaten for some
time and have grown. They pass through several larval stages. In the primary insects the larval stages and the adult animals are quite alike. Only the outer sexual organs and in some insects the wings are formed during the molt from the last larval stage to the grown up insect. Bugs and grasshoppers are examples for these hemimetabolic insects.

In holometabolic insects the larval stages are alike, but after the last molt they change completely. This occurs in a special pupal stage. In this stage a maggot changes into a fly, the caterpillar into a butterfly, the beetle larva to a beetle. There are thus two different processes occurring during the development of a fly: Larval growth and metamorphosis. The larvae grow, molt, grow, molt, until after the forth larval stage a pupa forms, which does not feed anymore. In the pupae the maggot changes into a fly. This is called metamorphosis.

How is the larval molt taking place? And why is in the last molt a pupa formed and the larva in the puparium changes into a fly which is so dramatically different in shape and structure from a maggot?

During molt many different tasks have to be coordinated. All cells underneath the cuticle (epidermal cells), the uppermost cell layer of the anterior and posterior intestine (epithelium) and the internal lining of the respiratory system (trachea) have to be renewed and the old one lifted up and stripped off (molted). Certain yield lines (molt lines) exist, at which the old skin bursts.

Even more complicated is the metamorphosis from the maggot to the fly in the pupal stage. Out of the imaginal disks and imaginal anlagen develop in the last larva stage the legs, wings, antennae and sexual organs. The second last larval skin is not stripped off, but converted into a puparium. Figure 2.14 shows this change in a series of illustrations. There are furthermore numerous changes in the body. Thus, certain muscles of the maggot are transformed into fly muscles. The fly becomes dark and ecloses finally from the pupal case with the help of a head balloon: A preformed lid in the pupal case is lifted and the fly emerges through the opening. It pumps hemolymph into the wings, thereby extending them. The cuticle hardens and the fly is now able to fly.

During the coordination of all these processes the neighborhood plays a role (for instance muscles and nerves), furthermore hormones of particular glands and neuroendocrine cells in the brain. Neural activities are also involved.

Depending on the interplay of the hormones involved, a larval molt or metamorphosis results. We should have a closer look at these events.

First of all the maggots have to molt (called ecdysis), because the old cuticle has become too tight. Quite a number of events occur in the body of the animals. Hormones play also an important role. Involved are 20E, PTTH, EH, ETH and CCAP (overview: Gade et al. (1997)). The names of these hormones are abbreviated. The control of all these events occurs in the brain of the maggots by special cells.

The brain of a larva with brain cells, which express PER and TIM -four small LN\textsubscript{v}, two DN\textsubscript{2}, and two DN\textsubscript{2} and their projections are illustrated in figure 2.15.

\footnote{\textsuperscript{3}the head balloon is a small vesicle at the front head and disappears after eclosion}

\footnote{\textsuperscript{4}20E: 20-hydroxy-ecdyson (Riddiford (1983)), PTTH: prothoracic tropic hormone, EH: molting- or eclosion-hormone (Truman (1992)), ETH: Ecdysis-inducing hormone (Zitnan et al. (1993)) and CCAP: Crustacean-cardioactive peptide (Ewer and Truman (1996), Green and Tobin (1999)).}
2 Eclosing in time windows

Figure 2.14: Conversion of a maggot of the fruitfly Rhagoletis pomonella into a fly. From left to right: 1. Fourth larva stage (La4), wrapped up by the cuticle of the third stage. 2. Pupa is formed (lateral view). 3. Pupa seen ventrally. 4. Head of the fly briefly before breaking forth and 5. after breaking forth. 6. Pupa completely developed. In 5. and 6. the head balloon is visible, which is used to penetrate the pupal case. It disappears after eclosion. After Weber (1954)

2.3.1 A fly flies off the skin

After the last larval stage the animals pupate and transform during metamorphosis into a fly. This is again a complicated interplay of diverse hormones and neurotransmitters in the head and body. It is not yet cleared up in all details.

Once the fly has formed, it has to eclose from the pupal case. Again this is a complicated game and as well not completely understood. The brain is schematically shown in figure 2.16. The players are pictured and the game, which leads to a successful eclosion of the fly out of the pupal case. On the one hand eclosion has to be prepared and performed, on the other hand it has to be coordinated by a circadian clock. First the preparation for eclosion:

In the Pars intercerebralis and the Pars lateralis of the central nervous system neurosecretory cells are present. They have a neuronal as well as a secretory function. PTTH and EH\(^5\) is secreted. Due to PTTH less 20E is produced in the prothoracic glands. Together with ETH of the Inka-cells of the epitracheal glands\(^6\) in the abdomen (Zitnan et al. (1993)) PTTH and EH induce the molting. EH and ETH stimulate the neurosecretory CCAP cells. They are located in the subesophageal ganglion and in the nervous system of the segments of the thorax and of the abdomen. CCAP is secreted at certain times only and modulates the muscle contractions in such a way that the animals finally eclose (Gammie and Truman (1997)).

The most important events are presented in figure 2.17. The figure shows also, where the circadian clock is working (red circle with oscillation sign). It controls the brain twofold:

1. the secretion of the prothoracotropic hormone by the PTTH-cells

\(^5\)EH exists as three different neuropeptides

\(^6\)glands which lie on the tracheae. The tracheae form the respiratory system of insects
Control of eclosion of Drosophila by cell groups in the brain. Decisive for the control of eclosion are PTTH and EH in the brain, CCAP in the subesophageal ganglion USG (dotted ellipse), PG, CA and CC in the ring gland. The circadian system consists of the sLNv (red) and the lLNv (violet). It is connected with the LNd-cell groups, the DN1, DN2 and the DN3-cell groups, and also with the structures, which are responsible for eclosion (PTTH, EH, CCAP). Light can synchronize via photoreceptors (compound eye, ocelli and Hofbauer-Buchner-eyelets HBA, golden and yellow) the eclosion rhythm (see figure 4.1). Since the scheme can be confusing, only the most important steps in the control of eclosion are presented in figure 2.17. After Myers et al. (2003) and Helfrich-Förster (2003a)
2. Eclosing in time windows

The PTTH-cells and the EH-cells of *Drosophila* are localized in the central brain (figure 2.16). The circadian clock is localized in the sLNv (red cells in figure 2.16). The exiting nerves of the circadian clock cells in the s-LNv overlap with fibers of the EH-cells (Blanchardon et al. (2001)) and innervate probably also the PTTH-cells (Zitnan et al. (1993)). The CCAP-neurons in the subesophageal ganglion can also contact the clock cells (figure 6A,B in Zhang et al. (2000)). Furthermore the fibers of the DN2 overlap with the CCAP-fibers (Park et al. (2003)). There are, however, further paths involved, since the flies eclose still rhythmically, even after the EH-neurons were removed (McNabb et al. (1997)). The prothoracic glands PG are possible candidates, since they exhibit a circadian rhythm (Emery et al. (1997)). Apparently eclosion is controlled in a daily (circadian) way by several parallel paths.

The result of all these events is, that the flies eclose only in a certain time window after onset of the light period in the morning (figure 2.17). The males eclose somewhat earlier than the females. We have seen already before, that it is not the onset of light, which induces the eclosion of the completely developed flies out of the pupal case, but an internal clock (figure 2.3).

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**Figure 2.15:** Nervous system of a 13 hour old larva of *Drosophila* seen from above with the two hemispheres (brain halves) and the ring gland (top) and behind it the elongate ventral cord with neurosecretory cells at the hind end. Brain cells, which express PER and TIM-four small LNv, red, two DN2, lila, and two DN2, light blue- and its projections. Light signals from the larval eyes travel via the Bolwig-nerve (yellow) to the sLNv. After Helfrich-Förster (1997) and Helfrich-Förster (2003a).
Figure 2.17: Events, which lead to molting and eclosion, and their control by a circadian clock (red circle with oscillation and dashed arrows, since uncertain). Two models are shown. The continuous red pathes are common to both models. Specialties of model 1 are dashed in red, specialties of model 2 are blue. A pair of neurosecretory PTTH-cells in the Pars intercerebralis of the fly brain in the pupa secret PTTH (a neuropeptid). It causes the prothoracic gland to secrete ecdysteroides in the hemolymph. Before eclosion the concentration is at first high, and then it declines considerably. Molt and eclosion can not begin, before the ecdysteroides have reached a low concentration in the animal. The eclosion-inducing hormone ETH of the Inka-cells of the epitracheal glands in the abdomen and the eclosion hormone EH stimulate the neurosecretory CCAP-cells in the subesophageal ganglion and in the nervous system of the thorax and the abdomen in such a way, that CCAP is secreted. It terminates the behavior before eclosion and affects the muscle contractions, which finally end up in the eclosion of the animals. After Myers et al. (2003)
2 Eclosing in time windows
3 How is the sleep-wake-cycle controlled?

Like eclosion, the locomotor activity is also controlled by an internal clock and synchronized by various light receptors. Under constant temperature- and light conditions the rhythm continues to run. The period length is, however, in the individual flies not any more exactly 24 hours, but either somewhat shorter or longer. Furthermore the period length depends also on the light intensity. Different mutants were isolated, the daily rhythm of which is changed. Genetical and molecular biological methods were applied in order to find out, how the circadian mechanisms function. Certain neurosecretory cells in the brain are the centers, which control the locomotor activity. Sleep-like behavior was observed and studied in Drosophila.

We will see, how the eclosion rhythm and the locomotor activity is recorded and analyzed.

3.1 From the internal clock to the legs

In the last section we have seen, that fruitflies eclose at certain times of the day and that a clock is responsible for it. Once the fly has emerged and its cuticle has hardened, it is able to run around and to fly. Fruitflies are eminent visual animals and therefore day active. In this respect they resemble humans (at least most of them). During the night they rest. It has even been shown, that they sleep (Shaw (2003), Hendricks et al. (2000)). The locomotor activity (running around and flying) of Drosophila is also controlled by a circadian clock. It can be recorded with an infrared light beam. Figure 3.1 shows the activity of one fly. It has been kept first for three days in a change of 12 hours light and 12 hours darkness (LD 12:12). Flies are active only during the light period. The light-dark cycle has synchronized the clock, which controls the locomotor activity. Afterward the fly was kept in weak continuous red light. Now the endogenous nature of the clock becomes visible: Since the period length is not exactly 24 hours (in contrast to the eclosion rhythm), the activity onset begins each day somewhat later (in this particular fly; in another case the clock could have been faster and therefore the fly would become active each day somewhat earlier). The period length of the rhythm in this particular case was 24.6 hours.

The period length can be influenced, if the light intensity is changed\(^1\) (Konopka et al. (1989)). This was in the case of temperature different: If the temperature was varied, the period length stayed (almost) constant. This temperature compensation has been discussed already while looking at the eclosion rhythm and is a characteristic property of circadian rhythms (see page 24 and figure 2.13). A clock, which would run with a different speed at various environmental temperatures would be quite useless.

Does the clock need a kick from a time cue of the environment, to start running? This

\(^{1}\text{the intensity has to be, however, low: In continuous light above 10 lux the rhythm of the locomotor activity disappears.}\)
3 How is the sleep-wake-cycle controlled?

Figure 3.1: Left: A Drosophila fly is kept in a small transparent cuvette with a plastic lid on the opening (situated right). A wick supplies drinking water from a container below the cuvette, a piece of sugar serves as food. If the fly interrupts the infrared light beam (red arrow in cuvette) of a light emitting diode (IR-LED, top), a signal is produced by the phototransistor underneath the cuvette, which is amplified (>>>) and stored in a PC. Recorded for many days an actogram (right) of the locomotor activity of a single fly is obtained. In an actogram the activity is displayed as strokes and, if following each other tightly, as bars. Successive days are plotted underneath each other. For the first 3 days the fly stayed in a light-dark cycle (LD 12:12) and afterward in constant weak red light. Under these conditions ‘freerun’ occurs, as shown by the red line: It connects the activity onset of the flies in days with weak red light. From the slope of this red line the period length can be determined. It amounts to 24.6 hours in this particular fly. Actually the activity from the eighth day onward behind 24 o’clock should have been plotted behind 0 o’clock. But then the freerun is less well visible.
was studied by rearing *Drosophila* from the egg stage onward in continuous darkness and under constant temperature. It turned out, that the flies possessed a circadian locomotor activity rhythm. It is, however, not synchronous among the individual flies. It is like in a clock shop, in which the watchmaker has displayed many clocks, which he had forgotten to set to the correct time. They do run, but display quite different times. The rhythm can thus be induce without light and other time cue. If light is offered during the first larval stage, the rhythm of the animals is synchronized. Light given during the embryo stage is not able to do that (Sehgal et al. (1992)).

### 3.2 Eyes for setting the activity clock

Circadian rhythms have certain properties such as freerun and temperature compensation. They can be synchronized by time cues, whereby the light-dark cycle is the most important one. The light is perceived by light receptors and transformed into signals, which reach the oscillator and influence it. We have learned already about photoreceptors and how light synchronizes eclosion while talking about eclosion (see subsection 2.2.2).

How light synchronizes the **locomotor activity rhythm** of *Drosophila*, is shown schematically in figure 4.1. Flies possess not only the two large lateral compound eyes for viewing, but also a number of other eyes and structures, which we normally do not associate with eyes. These light receptors are all involved in synchronizing the activity clock. How was this found out? Mutants were used, in which one or several of these receptors were missing. In spite of this the rhythm was still synchronized (Helfrich and Engelmann (1983), Helfrich (1986), Dushay et al. (1989), Wheeler et al. (1993)). Not before all photoreceptors were missing or lost their function, the flies are not synchronized anymore by a light-dark cycle (Helfrich-Förster et al. (2001)). We will have a more detailed look at it.

**Compound eyes** contribute to the synchronization of the circadian activity rhythm by light. Although the activity clock of mutants without compound eyes is still synchronizable by a light-dark cycle, there are differences: In one mutant without compound eyes (*sine oculis*) the circadian sensitivity to light is about thousand times smaller as compared to the wildtype with intact eyes. If the larvae of the wild type do not obtain carotin\(^2\) in the food, they are not able to see with the compound eyes, because no visual pigment is produced anymore. The animals are namely unable to synthesize carotin compounds by themselves. The sensitivity of the circadian rhythm to light is reduced by a factor of ten thousand without carotin. By the same amount the **visual sensitivity** of the compound eyes is decreased in such carotin impoverished flies.

Furthermore the action spectrum (as a reminder: It indicates, which color of the light can be seen well) differs from that of the wild type (Blaschke et al. (1996), Ohata et al. (1998)): The animals without functional compound eyes are not able to see red light (Helfrich-Förster (1996)). In addition the activity rhythm can not be synchronized by red light. The compound eyes play thus a role in synchronizing the daily clock.

The **ocelli** (= front eyes) seem to be important in connection with the compound eyes in synchronizing the activity rhythm, if the light-dark cycle deviates strongly from the normal one, such as for instance

\(^2\)carotin ist a pigment. It is, for instance, responsible for the yellow-red colour of carrots
3 How is the sleep-wake-cycle controlled?

in rather short or long dark periods.

There are, however, further photoreceptors involved in synchronizing the locomotor activity rhythm\(^3\). The Hofbauer-Buchner eyelets (Hofbauer and Buchner (1989)) belong to it. They consist of four light receptor cells and its neuronal fibers which project to the brain region, where the circadian clocks are located (Helfrich-Förster (1996), Kaneko (1998), Yasuyama and Meinertzhagen (1999)). If the clocks are synchronized by light signals, a connection to the light receptors has to be present.

But even the glass-mutants, which possess neither internal nor external eye structures, can still be synchronized by light (Helfrich-Förster (1996), Hall (1998)). In searching for these receptors it was finally shown, that the circadian clock cells (LNV) themselves were light-sensitive. They use the blue light sensitive cryptochrome as an absorbing molecule (Emery et al. (2000), Ceriani et al. (1999)).

Drosophila uses thus various light receptors for synchronizing its daily rhythms. Larvae and adults use cryptochrome as a pigment in the clock neurons. Extraretinal (Hofbauer-Buchner eyelets) and retinal eyes (compound eyes and ocelli) help, to adjust the activity to light intensity changes. One was wondering, why so many photoreceptors are involved. It might perhaps allow the circadian system to sense the various qualities of the natural light.

Natural light-dark cycles consist not only of white light, which suddenly switches on and off. Instead the light intensities in the morning and evening change slowly during the times of twilight in its intensity, spectral composition and in the position of the sun in respect to the horizon. Organisms are able to use these attributes, and to collect light according to their ecology and strategy (see Roenneberg and Foster (1997)). In all animals tested so far including man the morning- and evening twilight synchronizes more effectively as if light would suddenly come or disappear (Fleissnerg and Fleissner (2001)). Bünning (1936) has proposed, that organisms use the low light intensities of the twilight (during civil twilight) for measuring the daylength. In this way the seasons can be determined, since the daylength depends in a causal way on it. Furthermore the noise of the environmental signals is reduced, if several inputs are used. This applies also to light receptors.

Finally the various photoreceptors seem to have a different significance for the synchronization of the circadian rhythm and for direct light effects (Emery et al. (2000), Helfrich-Förster et al. (2001), Rieger (2002)). The compound eyes affect the activity pattern of the adults directly and synchronize the circadian rhythm especially under extreme long days and short days. The same effect is shown by the ocelli. They might perhaps affect a second rhythm, which is responsible for the second morning peak (see page 42), whereas the compound eyes are responsible for synchronizing the morning peak 1 (Rieger (2002)). Cryptochrome affects as a light receptor molecule in cells circadian rhythms, is however not obligatory for synchronizing the activity rhythm. The significance of the

\(^{3}\)Thus, Blaschke et al. (1996) found two maxima in the action spectrum of the wild type and eyeless flies. Furthermore the sloped lines, which represent in dose-response curves the percentage of synchronized flies as a function of the light intensity (see figure 2.11), do for several of the tested wavelengths not parallel each other. Interestingly even in eyeless flies not all of these lines were parallel to each other, which suggest, that even these flies use different photoreceptors for synchronization.
Hofbauer-Buchner eyelets for synchronizing the activity rhythms and for direct light effects is not yet known. There are namely no mutants yet available, which lack these photoreceptors or in which they are defective. Perhaps experiments with suicide-genes could be of use, which would specifically knock out these receptors.

3.3 Control of sleep in Drosophila

Sleep-like behavior has been described in many animals (Campbell and Tobler (1984)) and also in Drosophila (Hendricks et al. (2000), Shaw et al. (2000), Shaw (2003), Cirelli (2006)). The signals involved and the mechanisms are described by (Foltenyi et al. (2007) and there further literature). A limited number of cells in the Pars intercerebralis is involved. They project to the tritocerebrum. Epidermal growth factor receptors (extracellular growth factor receptor EGFR) and other factors activate the extracellular signal-regulating kinase ERK, which modulates in mammals and flies the synaptic plasticity. If EGFR is overexpressed, the sleep duration is increased. Locomotor activity, period length and phase of the activity rhythm are, however, not influenced.

Furthermore the dopaminergic system does not seem to be involved. Pharmacological and genetic manipulations show, that a reduced dopamin concentration increases the sleep duration and that an increased concentration shortens the sleep duration. Some prominent dopamin projections end in the mushroom bodies (see figure 3.2). They are responsible for reactions which induce resting behavior, associative conditioning and sleep modulation. Both systems are localized in the dorsal midbrain, where also PDF-containing cells are found which are relevant for the locomotor activity (see figure 3.2).
How is the sleep-wake-cycle controlled?

Figure 3.2: Location of brain areas involved in the control of sleep in Drosophila melanogaster and neurosecretory cells. PK mushroom body (golden), DA dopaminergic system (pink), PI Pars intercerebralis- neurons (light green), Hofbauer-Buchner eyelets (red), dorsal (DN1, 2, 3, green) and ventral lateral neurons (sLNv, red, and ILNv, dark). See also figure 4.1. After Helfrich-Förster et al. (2000) and Foltenyi et al. (2007)
4 Where is the internal clock of the fly hidden?

Rhythmic locomotor activity and eclosion are controlled by various cells in the brain of the flies. It will be shown how they are wired among each other and with the target organs. They are furthermore connected with photoreceptors, which receive light and synchronize the clocks with the environment. Rhythmic behavior is controlled by these central clocks, whereas local clocks are at work in the various tissues and organs. The circadian system of flies is thus composed of many clocks.

Where are the centers, which control eclosion and locomotor activity of the flies in a circadian manner? This has been studied in the last years intensively (overview: Helfrich-Förster (2001), Helfrich-Förster et al. (1998), Kaneko (1998)). The results are presented in the next section. The various photoreceptors for light, which synchronize the circadian rhythms, were discussed already (see section 3.2). Further questions such as, whether eclosion rhythm and locomotor activity rhythm are controlled by the same circadian clock and whether besides the central clocks in the brain local clocks exist in the tissues and in the organs, are answered in further section.

4.1 Looking into the fly brain

We have to look now into the brain of a fly, since it is there where not only the divers photoreceptors for light are found, which synchronize the rhythms (see section 3.2), but also the centers, which control the circadian behavior (figure 4.1, Veleri et al. (2003), Helfrich-Förster (2003b)).

The centers for daily eclosion and circadian activity are certain neurosecretory cells. They lie in the lateral parts of the brain hemispheres and are therefore called lateral neurons (LN). On each side there is a more dorsally lying group (LNd, 5 to 8 cells) and a more ventrally positioned (LNv). The ventral group consists of neurons with large cell bodies (ILNv, 4 bis 6 cells) and of neurons with small cell bodies (sLNv, 5 cells). The small LNv are the decisive cells for circadian behavior. They send fibers to the dorsal part of the central brain. In the upper dorsal brain are furthermore three groups of dorsal neurons (DN1, about 15 cells, two DN2 cells, and about 40 rather small DN3 cells) located.

In the brain of the larvae only four small LNv are found, two cells of the DN2 and two cells of the DN1 (see figure 2.15). The connections between the neurosecretory cells and the photoreceptors were demonstrated by staining characteristic substances. The lateral neurons project mainly to the dorsal protocerebrum. There the terminals overlap. Of the DN-cell groups only the fibers of the DN1 project to other brain areas such as the accessory medulla aMe and perhaps to the optical lobe. Other DN1-cells project to the esophagus. The most important connection of the DN1 is the dorsal commissure. They project to the opposite side of the dorsal brain. The DN2 neurons run in the same commissure. The fibers of the DN3 cells are more ventrally located and terminate at the same side of the dorsal brain. Here they overlap with some fibers of the LNd and of the DN2. The LNd fibers project to the surface
Figure 4.1: Position of the clock-cells and the photoreceptors for synchronizing the circadian activity clock in the brain of Drosophila melanogaster. Section from top to bottom (‘frontal’) through the head with central brain, optical lobe with lamina, medulla including lobula (not shown), various photoreceptors and neurosecretory cells. Light synchronizes the rhythm (yellow arrows) via the compound eyes, the Hofbauer-Buchner eyelets (HBE, red), and the ventral lateral neurons (sLNv, red, and ILNv, dark). The rhythm can be affected additionally via the ocelli and the dorsal lateral neurons (LNd, lilac). After Helfrich-Förster et al. (2000)
4.2 One clock for circadian eclosion and for locomotor activity?

of the lateral horns. They form a dorsal branch, which runs parallel to the DN3 fibers, and a ventral branch, which crosses the midline below the DN1/DN2 commissure.

The fibers of the clock cells terminate in the upper protocerebrum. This part of the brain is connected with most of the brain areas and contains furthermore additional neurosecretory cells. Circadian signals of the clock cells can thus reach electrically or via hormones the target organs. The neurons of all clock cells seem to be connected with each other. They are thus in a position, to send the circadian time to the dorsal protocerebrum and perhaps also via the large LN to the optical lobe. But the various cells play different roles in the circadian system.

The small LNv and LNd are the cell groups, which are responsible for the circadian rhythm of eclosion and of locomotor activity (Helfrich-Förster and Engelmann (2002)). Even a single functional LN cell is able to control these rhythms. That was shown in disco-mutants. Normally the LN cells are absent in these mutants, but occasionally one or a few of the cells are still present (Helfrich-Förster (1998)). In this case the animals display a rhythmic activity, whereas the usual disco-mutants are arhythmic. But the DN cells are also involved in the circadian system. For the circadian eclosion the small LNv cells are responsible. In the eclosed fly the LNd, the large LNv and the small LNv participate in the control of the circadian locomotor activity. These groups use PDF as a signal.

1These exceptional mutants were found, when Charlotte Förster recorded the locomotor activity of a few hundred disco-flies and discovered occasionally a rhythmic activity pattern. She checked in the rhythmic animals the head and found, that one or a few of the LNv-cells were still present.

2Pigment dispersing factor, a neuropeptide. It was for further downward positioned neurons. The large LN do not project to the dorsal protocerebrum and have perhaps a special function in coupling the small LNv and LNd of the two hemispheres.

4.2 Are eclosion and locomotor activity controled by the same circadian clock?

Of the numerous events which are in Drosophila controled in a circadian way, we got to know two examples, the eclosion- and the locomotor activity rhythm. Are we dealing with one clock, which controls the two behavioral rhythms or more? If more than one is involved, how are they related to each other and how do they interact? And where are they localized? Finally we would like to know, how these clocks function (chapter 5).

The eclosion rhythms tick even under constant conditions in a precise 24-hours measure, and it was already explained, why it is so (chapter 2). The activity clocks, on the other hand, run in a constant environment in the single flies in most cases either somewhat faster or somewhat slower than 24 hours. That speaks in the first instance against a single clock controlling both events. But not necessarily: The clock could have changed its speed after eclosion. Eclosion and activity would in this case still be controled by one clock. It was, however, shown, that the activity clock runs already in the pupa, but it happens to run with a different speed as compared to the eclosion clock (Mack and Engelmann (1982)).

On the other hand the mutants of Drosophila melanogaster, the eclosion clock of which is faster or slower as that of the
wild type, possess also faster or slower activity clocks (Konopka and Benzer (1971)). If the mutation has affected the oscillator, this observation would speak for one clock, which controls both events in a circadian manner. There is, however, also a mutant (ebony), in which the activity rhythm is changed, but the eclosion rhythm not (Newby and Jackson (1991)). Something like that was also observed in Drosophila pseudoobscura (Engelmann and Mack (1978)). As a further example in the mutant lark the phase of the eclosion rhythm is influenced, but the activity rhythm was normal in respect to phase and period. This would speak against one clock and in favour of a multioscillatory system.

For more than one clocks speaks also the following: Mutants of Drosophila melanogaster, the locomotor activity of which is not any more controled in a circadian way, do still react photoperiodically: \(^3\) They can be brought by shortday conditions into a resting state (diapause). It is known, that in photoperiodic timing a circadian clock is involved (Saunders et al. (1989)). That would mean, that a circadian clock controls the photoperiodic timing, which is not identical with the clock for the eclosion- and the activity rhythms.

Even the locomotor activity can be controled by more than one oscillator in a circadian way. Mutants, the optical lobes of which are heavily reduced, show in the actogram under constant conditions two circadian components. They differ in period length somewhat from each other. Therefore the two rhythms drift slowly apart from each other, until they meet again after a certain time (figure 4.2, Helfrich (1986), Helfrich-Förster (2001)). One of these components is the morning oscillator. Even this component seems to consist of two oscillations. This can be seen in actograms of animals, the locomotor activity of which has been recorded under extreme light-dark-cycles with either much shorter or much longer light periods (Rieger (2002)).

Drosophila thus possesses, incidently like other insects, a multioscillator system. It controls various events on different levels in a circadian way. This system consists of autonomous clocks in cells, tissues and organs, which are directly synchronized by light-dark cycles. Additionally it consists also of central clocks in the brain, which are synchronized by various photoreceptors, but also directly by light (see section 3.2). How these components of the circadian system cooperate, is not yet well understood.

4.3 Central and local clocks

Eclosion and locomotor activity of Drosophila are controled in a circadian way, as we have seen. Both events are behavioral rhythms and they are steered by central clocks in the brain.

But this does not describe the clock shop of Drosophila completely, because peripheral tissue and various organs are controled by local clocks. They were found in isolated and cultured compound eyes (Christopher and Hoffer (1998)), in chemosensory cells of the antennae (they are used by the flies for smelling, Plautz et al. (1997), Krishnan et al. (1999)), Malphigian tubes (the kidneys of the flies, Hege et al. (1997), Giebultowicz et al. (2001)) prothoracic glands (glands in the thorax, which are influenced by the brain, Emery et al. (1997)) (figure 4.3) and other tissues. These cells and organs are, however, not important for the

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\(^3\) the critical daylength is, however, by 2 hours shorter
Figure 4.2: Actogram of a Drosophila fly with two circadian components in the pattern of the locomotor activity. In the first seven days the fly was kept in a 12:12 hour light-dark cycle (see black line above the actogram; the second, third and so on day was plotted additionally at the right side, which allows to recognize the activity pattern better). The locomotor activity consists of a morning- and an evening part and the activity is synchronized with the external 24 hour rhythm. From day eight onward the fly was in continuous darkness. The activity shows a freerun with two components: The period of the shorter one is about 22 hours and runs from top right to bottom left, the period of the longer one is 25 hours and runs from top left to bottom right. Both components diverge first and meet again after some time. After Helfrich-Förster et al. (2000)
4 Where is the internal clock of the fly hidden?

Figure 4.3: *Luminescence rhythms of Drosophila-tissues:* per-gene driven green-fluorescent-protein (GFP) is produced only in cells, in which the clock-gene *per* is active. With a sensitive camera the green luminescence of the GFP can be monitored (after UV irradiation) for several days. In the light-dark cycle the rhythm is well expressed (day 0 and 1), in continuous darkness it damps out (day 2 to 5 in the two upper curves, day 2 to 6 in the two lower curves). A new light-dark cycle (day 6 and 7 respectively day 7 to 9) increases the amplitude and shifts the rhythm. Top (green) proboscis, below (brown) antenna, below (red) leg, bottom (blue) wing. After Plautz et al. (1997)
eclosion- and activity rhythm.

How has it been shown, that clocks are running also in tissues and organs? For this purpose a reporter-gene of an enzyme, the luciferase, was introduced into the body cells. Luciferase is responsible for the glowing of female fireflies, which lure in this way males in the dark. The gene was put behind a promotor, which was under the control of the circadian clock. Promotors are able to influence genes, for instance by switching them on or off. As a result the flies were capable to emit light from the tissues and organs. Since the circadian clock affects the promotor in such a way, that the luciferase gene is expressed at certain times of the day only, circadian rhythms of luminescence were obtained in the varies tissues and organs. These rhythms could also be observed in tissue cultures. In that case a per-gene driven green-fluorescent protein (GFP) was used, which is only produced in cells, where the clock gene per is active. Using a sensitive camera the green luminescence of the GFP after UV irradiation can be recorded for several days. Light pulses shifted the phase of these rhythms (Plautz et al. (1997)).

Drosophila and probably many other insects thus possess besides central clocks, which control behavioral events such as eclosion and activity in a circadian manner, also clocks in peripheral organs and tissues, which are autonomous and directly synchronizable by the day-night cycle.

4.4 Mutants

Various mutants with changes in the brain differ from the wild type by their activity patterns. They were used for clarifying the mechanism, on which the circadian clocks are based. The most important mutants are compiled in the upper part of table 4.1. Eye mutants were used, to find out, which photoreceptors are responsible for synchronizing the locomotor activity rhythm (Helfrich-Förster et al. (2001), lower part of table 4.1; see also section 3.2).
Table 4.1: The most important mutants of *Drosophila melanogaster* with changed properties of the circadian clock (upper part of table) and some eye mutants, which affect synchronization of the circadian system of *Drosophila melanogaster* (lower part of table). From Saunders (2002).

<table>
<thead>
<tr>
<th>mutant</th>
<th>name</th>
<th>protein in wild type</th>
<th>properties</th>
<th>remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>per⁰</td>
<td>period 0</td>
<td>PER</td>
<td>negative regulator</td>
<td></td>
</tr>
<tr>
<td>per⁰</td>
<td>period s</td>
<td>PER</td>
<td>negative regulator</td>
<td></td>
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<tr>
<td>per⁰</td>
<td>period l</td>
<td>PER</td>
<td>positive regulator</td>
<td></td>
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<tr>
<td>clk</td>
<td>clock</td>
<td>CLK</td>
<td>positive regulator</td>
<td></td>
</tr>
<tr>
<td>tim</td>
<td>timeless</td>
<td>TIM</td>
<td>negative regulator</td>
<td>light-sensitive</td>
</tr>
<tr>
<td>cyc</td>
<td>cycle</td>
<td>CYC</td>
<td>positive regulator</td>
<td></td>
</tr>
<tr>
<td>dbt</td>
<td>doubletime</td>
<td>DBT</td>
<td>phosphorylated PER</td>
<td>dbt</td>
</tr>
<tr>
<td>rigui</td>
<td>rigui</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vri</td>
<td>vrielle</td>
<td>VRI</td>
<td>inhibits per- and tim-expression</td>
<td></td>
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<tr>
<td>pdp₁ₑ</td>
<td>PDP₁ₑ</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**eyes-mutants**

<table>
<thead>
<tr>
<th>mutant</th>
<th>name</th>
<th>protein</th>
<th>properties</th>
<th>remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>so</td>
<td>sine oculis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>sol</td>
<td>small optic lobe</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>eya</td>
<td>eyes-absent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oc</td>
<td>ocelli-less</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dcry</td>
<td><em>Drosophila</em> cryptochrome</td>
<td>DCRY</td>
<td>photoreceptor; inputs</td>
<td></td>
</tr>
<tr>
<td>cry⁰</td>
<td>crybaby</td>
<td>CRYB</td>
<td>no functional cryptochrome</td>
<td></td>
</tr>
<tr>
<td>norpA</td>
<td>no-receptor-potential</td>
<td>phospholipase C</td>
<td>light transfer in comp.eye and ocelli</td>
<td></td>
</tr>
<tr>
<td>glass</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>disco</td>
<td>disconnected</td>
<td></td>
<td></td>
<td>optical ganglia blind, AR in DD</td>
</tr>
</tbody>
</table>
Most of the results of section 3.2 rely on experiments, in which eye-mutants were used.
4 Where is the internal clock of the fly hidden?
5 The clockwork

The mechanisms of the circadian clocks, which control eclosion and activity of flies, are presented. They are molecular feedback loops, in which certain gene products play decisive roles. The players and the rules of the game are to some extent known. Likewise the effect of light and temperature is quite well understood on the molecular level. For instance, one can explain, why the clock has the same speed at different constant environmental temperatures, but why the clock in spite of it reacts to an increase or decrease of the temperature. If due to mutations some of the players of these oscillatory events are gone or if they are too strong, the duration of the oscillation changes or the clocks stop running.

It is now time to have a closer look at the clocks, which are responsible for the various daily rhythms. We have seen, what they control. We do, however, not yet know, how they function. This will be done in the next sections.

5.1 A feedback model for oscillations: The game

Which mechanisms are the basis of the circadian clocks in the brain of *Drosophila*, that control eclosion and locomotor activity? This was studied intensively and successfully in the last years. There are, however, still many unanswered questions. Here only the most important foundations are described. Details are found in more recent overviews (Hall (1982), Edery (2000), Giebultowicz (2000), Stanewsky (2002), Williams and Sehgal (2001), Young and Kay (2001), Foltenyi et al. (2007)).

Both rhythms - activity and eclosion - can be described by molecular feedback loops. They are so to speak the principle of the game we are trying to spot. In the game there are players, namely a number of clock-genes. They are period (per), timeless (tim), clock (clk), cycle (cyc), doubletime (dbh), vrille (vri) and pdp1. Genes code for proteins, which often control as enzymes certain metabolic steps. The corresponding proteins of the clock-genes are PER, TIM, CLK, CYC, and DBT, VRI and PDP1 (see 5.1 table 4.1). Mutations of these genes influence both rhythms strongly, the flies become aperiodic or the period length of the freerun rhythms is altered.

How does the game look like, in which the mentioned players interact with each other? In figure 5.1 both feedback loops, -RKK and +RKK, are described, on which the circadian clock is supposed to be based (according to results of Cyran et al. (2003) and Glossop et al. (2003)). They are not easy to understand, and several points have to be explained first. I will proceed in the following way: The game will be described without explaining in-depth the used specialist terms first. This will be done not before the following section.

CLK and CYC are basic helix-loop-Helix-PAS transcription factors. They form heterodimeres and bind to an E-box element1 in the promotors of the *per* and

1 with the nucleotide sequence CACGTG
Figure 5.1: Model with two feedback loops. In the negative feedback loop -RKK (red hemmed) PER and TIM together with DBT inhibit CYC and CLK after being phospho-
ylated (by CK2 and SGG) via a kinase. As a result less TIM and PER is expressed 
by the tim- and per-genes. In a positive feedback loop +RKK (red hemmed) CYC and 
CLK increase the expression of the genes vri and pdp₁e. VRI inhibits and 
PDP₁e promotes the expression of clk and cyc. In the negative feedback loop -RKK PER and TIM 
inhibit the transcription factors CYC and CLK (short dash instead of an arrow), and 
the transcription factors CYC and CLK promote the expression of per and tim (marked 
by arrows). TIM and PER are phosphorylated by DBT, CK2 and SGG and degraded by 
proteasomes. In the positive feedback loop +RKK CYC and CLK promote the expres-
sion of vri and pdp₁e. The products of these genes, VRI and PDP₁e, promote (VRI) 
respectively inhibit (PDP₁e) the expression of the clk- and the cyc-gene. 
Light (yellow zigzag arrow) is absorbed by cryptochrome (yellow) and acts via a signal 
on TIM, which is as thereby destroyed. Exact details are not yet known. 
Both feedback loops act together and mRNA of clock controled genes such as PDF is 
produced in a circadian manner. The products can act on clock components or are af-
footed by these (PDF can influence PER, VRI inhibits the PDF-formation). The events 
occur in the cytoplasma and in the nucleus, as shown in the figure. After Cyran et al. 
(2003), Glossop et al. (2003) and Mackey (2007)
5.2 The players and the rules of the game

Thereby the transcription of both genes is activated (Darlington et al. (1998), Rutila et al. (1998), Hao et al. (1997)). The amount of mRNA of per and tim increases continuously, until in the early evening a maximum is reached. The products PER and TIM reach their maximum not before the late evening (Zerr et al. (1990), Edery et al. (1994)). The delay seems to be due to post-transcriptional regulation of the per mRNA (Chen et al. (1998)) and the PER (Dembinska et al. (1997), Stanewsky et al. (1997)). One of the regulatory mechanisms destabilizes PER by DBT-depending phosphorylation (Kloss et al. (1998), Price et al. (1998)). PER is, however, stabilized by dimerization with TIM. According to an earlier model these PER-TIM dimers should enter the nucleus after having reached a critical level and inhibit their own transcription by inactivating the transcriptional activator CLK-CYC (Darlington et al. (1998)).

This model has in the meantime become more complicated by new studies and results (see page 52). Due to time delay between mRNA and protein this negative feedback leads to a stable oscillation of per and tim mRNA and the proteins.

Whew, that was tough. That seems to be quite a dodgy situation and an eccentric game. Now, dead slow, what is the matter here? First of all have a look at a short animation, which shows in simple terms, what has been presented just now in such a complicated way (http://www.hhmi.org/biointeractive/clocks/animations.html). The molecular model of the Drosophila clock shown in figure 5.1 is shown here in an animation. You can see, how the clock gene per and tim produce the clock proteins PER and TIM. They travel from the nucleus in the cytoplasm and form there a dimer. This dimer can pass the nuclear membrane and inhibits the transcription factors CLK and CYC. As a result the transcription of PER and TIM is stopped. The amount of PER and TIM decreases, because they are degraded. Light is perceived via photoreceptors and it changed the Drosophila-cryptochrome CRY. Consequently the PER/TIM dimer is dismantled (Helfrich-Förster (2002)).

Now we should become more familiar with the players we got to know before (in section 5.1) and try to understand the rules of the game. The players are essential parts of the circadian clock (parts of the clockwork) and have to be distinguished from the clock controlled parts (hands of the clock).

Two of the players are PER and TIM, which are coded by the genes period (per) and timeless (tim). PER forms a dimer with TIM. It is stabilized by this dimerization. PER and TIM have the following tasks: If a critical level of the PER-TIM dimer has been reached, it can enter the nucleus and inhibit there the transcription of PER and TIM (that is, its own production) by inactivating the transcriptional activator CLK-CYC. The per- and tim genes are regulated by CLK and CYC, which are expressed by the genes clock (clk) and cycle (cyc). They are basic helix-loop-Helix-PAS transcription factors. They form heterodimers and bind to an E-box-element in the promotors of the per- and tim genes. That activates the transcription of both genes. DBT is responsible for the degradation of PER. DBT is coded by the gene doubletime (dbt). It promotes the phosphorylation of PER and this leads to PER’s destruction by ubiquitin in proteasomes. A
further clock protein is the cAMP response element binding protein (CREB). It is also involved in the feedback loop and promotes the oscillation of PER and TIM. Further clock-factors might exist.

The timing of these events are described by Saez et al. (2007) and shown in figure 5.2. It was clarified by preparing an old cell line of a Drosophila-embryo from 1972 in a special way (PER was marked with a yellow, TIM with a green fluorescent protein). Both markers could be induced by a heat shock. It turned out (see figure 5.2), that PER and TIM was, to begin with, for 4 hours uniformly distributed in the cytoplasm. After 5-6 hours it accumulated rapidly in the course of an hour in groups (focus, plural foci) around the nucleus. This occurred independently of the amount of PER- and TIM. Now PER and TIM entered the nucleus, again independent of the amount of PER- and TIM and independent from each other (PER as a rule faster). This was found out by using the FRET-method (fluorescence-resonance-energy-transfer), in which resonance occurs, if PER and TIM were close together. Afterward a PER/TIM-complex is formed rapidly (whereby the FRET-signals decrease).

In the mutant \textit{per}^{L2} with a circadian period of 28 instead of 24 hours the transition of PER and TIM into the nucleus is delayed by 4 hours, without affecting the formation of the complex. The accumulation in the nucleus is delayed by 8.5 hours. Association and dissociation of the complexes are not altered, but the hourglass clock (interval-timer), which runs in the cytoplasm, before PER and TIM enter the nucleus, is affected.

DBT interacts physically with PER, after it has formed a complex with TIM. Without the complex PER is phosphorylated by DBT. It is thereby destabilized and dismantled in proteasomes. TIM stabilizes also PER by inhibiting its phosphorylation through DBT.

5.3 If light comes into play

A model is only useful, if it explains the situation in the real world. In the case of the circadian clock it is first of all the cyclic order of events in eclosion and in locomotor activity of the flies. How this occurs at the molecular level was shown in section 5.2. Circadian clocks are, however, also affected by environmental factors such as light and temperature. If the feedback loop described before is the basis of the rhythms, the model must also be able to describe synchronisation of the clock by the day-night cycle. The model must furthermore explain, how light pulses shift the clock. Finally it must show, how continuous light stops the clock. This will be checked in this section.

What is the molecular basis of synchronisation by light? It is achieved by degradation of TIM, one of the players, in light; other players, that is clock-proteins, are not degraded by light (Hunter-Ensor et al. (1996), Lee et al. (1996)). Therefore in continuous light the amount of TIM is small. Since the player PER has to form a dimer with TIM, in order to prevent its degradation, the amount of PER is also low (Price et al. (1995)). The producers of PER and TIM, namely \textit{per} and \textit{tim}-mRNA, do not show circadian fluctuations, but has an average concentration. Therefore their proteins do not oscillate in continuous light. That would explain on a molecular biological level, why the circadian rhythm disap-
Focus degradation by proteasomes
definition

development

Figure 5.2: Model of the accumulation of PER, DBT and TIM in the nucleus of S2-cells after Saez et al. (2007). PER (blue) and TIM (green) are expressed by the per and tim-genes in the nucleus and arrive in the cytoplasm, where they are either dismantled, after phosphorylation by DBT (red), by proteasomes, or TIM and PER/DBT form a complex (green/blue/red). These complexes accumulate in foci close to the nucleus (Focus). After about 5.5 hours the complex falls apart and the parts enter the nucleus. There they combine with CYC and CLC to a complex, which prevents the expression of CYC- and CLC-controled genes such as tim, per, vri and pdp (dashed arrows). Compare this scheme with that in figure 5.1 and note the differences of the transport into the nucleus: Here not the complex PER/DBT/TIM is transported into the nucleus, but the individual proteines. The braking apart of the complexes PER/DBT/TIM in the cytoplasm is controled by a kind of hourglass and takes 5.5 hours. In the mutante per it takes 8.5 hours. In the nucleus a complex between PER/DBT respectively between TIM and CYC/CLK is formed, which inhibits the expression of tim, per, vri and pdp. After Mackey (2007) and Saez et al. (2007)
The clockwork

pears in continuous light and arhythmicity is found instead. The per and tim mutants are arhythmic, because this condition is always prevailing, even in light-dark cycles (Qiu and Hardin (1996)). That explains the question, why continuous light stops the clock.

Why do light pulses shift rhythm? Again the degradation of TIM by light is responsible. If the amount of TIM is measured after short light pulses in the LN cells (the clock cells), a strong correlation to the phase shift of the circadian rhythm turns up: The stronger the rhythm has been shifted by light, the more TIM was degraded (Young (1998)). Blue light was especially effective. And TIM is strongly degraded by blue light. If the effects of other wavelengths of the light are compared, the reactions to colored light in the TIM degradation parallel the phase shift. Both events are apparently tightly coupled. That would answer the question, why a light pulse shifts the rhythm.

TIM is degraded in light also, if PER is lacking. Without PER, however, the circadian clock does not work. TIM is therefore degraded also without a functional clock (Suri et al. (1998)). Furthermore, the compound eyes do not need to be present for the degradation of TIM in light (Yang et al. (1998)). That shows, that the degradation of TIM in light uses an extraretinal path. This was already known from behavioural studies.

All these results show, that the degradation of TIM is decisive for the circadian light perception. TIM is, however, not directly light sensitive. It is also not always degraded after onset of light. For instance the amount of TIM fluctuates in the larval stage in the dorsal neurons (DN) in opposite direction to that in the LN, that is, it is high during the day and low during the night (Kaneko et al. (1997)). What it means biologically is unclear. But it shows, that TIM can be regulated differently and that it is not light sensitive per se. Somehow the light signal has to be transferred to TIM biochemically, after having been absorbed by pigments.

Such a pigment is the Drosophila-cryptochrome (DCRY)\(^3\). It is involved in the transfer of light to TIM (Emery et al. (1998), Stanewsky et al. (1998)). The absorption spectrum of cryptochrome corresponds to the action spectrum of the behavioral rhythms of Drosophila (Selby and Sancar (1999)). DCRY plays thus an important role. Apparently TIM is a direct target of DCRY. It has indeed been shown recently, that DCRY alters its state after illumination. It can now enter the nucleus and interact there with the PER/TIM complex (Ceriani et al. (1999)). Thereby the PER/TIM complex is inactivated. It is unable to participate in the negative feedback loop. The degradation of TIM is thus not the first step after illumination. Instead, the first step is the inactivation of PER/TIM by DCRY.

DCry is expressed in the LNv, the circadian pacemakers (Egan et al. (1999)). That is true also for Drosophila-larvae. The small LNv are present already from the first larva stage onward and its PER and TIM content fluctuates in a circadian manner (Kaneko et al. (1997)). The small LNv of the larvae are synchronized by cryptochrome and by the larval eyes (Stanewsky et al. (1998), Helfrich-Förster et al. (2002)). If both photoreceptors are lacking, the animals can not be synchronized anymore. It is not yet known, whether this applies also to the eclosion rhythm.

5.4 Why temperature does not play a role

Remind you: The environmental temperature has almost no influence on the circadian eclosion rhythm and on the locomotion.
tor activity rhythm of the flies. And this has to be expected from a decent clock. It should not run faster at higher temperatures or to dawdle at lower temperatures. But how is this achieved at the molecular level? What are the foundations of the temperature compensation of the circadian rhythms of Drosophila?

A model of Goodwin (Leloup and Goldbeter (1997)) was extended by Ruoff and Rensing (1996). They assume a monomeric and an oligomeric form of the PER protein. The equilibrium between the two forms depends on the temperature. In the per\textsuperscript{L} mutant the equilibrium is shifted by an increased temperature towards the monomeric form and both kinds are more slowly degraded as compared to per\textsuperscript{+}. In the per\textsuperscript{S} mutants the equilibrium is unaffected, but PER\textsuperscript{S} is faster degraded (Ruoff et al. (1997)). In the model the temperature influence is controled by the clock protein degradation.

5.5 Why temperature pulses play a role

Although the environmental temperature does not influence the clock in its speed, it does react to sudden changes in temperature. If for instance the temperature is lowered, the rhythm is shifted. The period stays, however, in the now lower temperature constant, as described in the previous section. The same can be observed, if the temperature is increased. A short-term temperature decrease or -increase shifts likewise the rhythm.

5.6 Clock-driven events

We saw already in section 3.1, that the eclosion of the flies and their locomotor activity

Figure 5.3: The temperature compensation of the locomotor activity rhythm of Drosophila can be explained by the Goodwin-oscillator (Ruoff et al. (1997)). The temperature dependency of the Drosophila mutants per\textsuperscript{L} (green) and per\textsuperscript{S} (blue) are successfully predicted (circles are predictions, triangles experimental findings) and the temperature compensation of the wild type respectively of per\textsuperscript{+} (red) is valid. After Ruoff et al. (1997)
is controlled by a circadian clock. Further events in behavior, in tissues and in cells are also driven in a circadian manner. How does the clock affect these events, how is the situation at the molecular level? There must be outputs of the clock, which are rhythmic.

One of these outputs is the neuropeptide pigment dispersing factor PDF. It was originally found as the pigment dispersing hormone PDH in crustaceans and controls the coloration in these animals. In insects it has a different function. It transfers the rhythm of the circadian clock in the pacemaker cells $LN_v$ to the behavior (for instance eclosion and locomotor activity, Renn et al. (1999)).
6 How is eclosion and activity of the flies recorded?

How can we record the eclosion rhythm and the locomotor activity of Drosophila? Various methods and devices are presented, which allow to record the eclosion rhythm and the locomotor activity. It will also be mentioned, how to collect and analyze the data.

I am sure you would like to know by now, how the eclosion of the flies out of the puparium and the running around of the eclosed animals can be measured. One could of course observe the animals during these activities and make notes, but at least after the second night without sleep you will think about ways to automate the procedure. Some proposals are made in the following sections.

6.1 Recording the eclosion rhythm

The eclosion rhythm of Drosophila (and of other insects) can only be recorded by using a population. Early methods used a bang box. The pupae were pasted with wall paper glue to a metal plate. Each hour the plates were lifted mechanically and dropped, so that the eclosed flies fell through a flat funnel down into a container with water. A drop of detergent reduced the surface tension and the flies could not safe themselves at the wall of the container and drowned. Each hour with the help of a turning device a new container was positioned underneath the funnel (figure 6.1).

After 24 hours a new turning table with 24 new vials is placed underneath the bang box. In the vials of the turning table of the previous day the number of animals eclosed per hour was counted. The number of flies was plotted graphically (figure 6.2).

Later this elaborate and susceptible to faults method was improved. The pupae were glued to glass plates and layed on top of a teflon funnel. Beneath the end of the funnel an infrared light beam was mounted. An eclosed fly could not climb to the teflon (on glass walls they are able to walk!) and fell through the light beam into a water vial (figure 6.3).

In another method the pupae are individually placed in a hole of a plate, and a delicate net at the underside prevented them from falling out. Above the plate with holes a sooted glass plate was positioned. The eclosed flies try to escape and to scrape off the soot. They die rapidly and dry out. Red light from below penetrates the scraped off parts of the sooted glass and fall upon a photocell. The more animals have eclosed, the more soot is removed and the more light falls upon the photocell. The voltage of the photocell is thus a measure for the number of eclosed animals (figure 6.4, Engelmann (1999)).

Instead of a photocell a video camera looking at the glass plate from above can be used and the number of scraped off holes and thus the number of eclosed flies determined by using an imaging program.
6 How is eclosion and activity of the flies recorded?

Figure 6.4: Registrieren des eclosion rhythm von Drosophila-flies mit der Ruß-Methode. Oben: pupae (braun) werden einzeln in Löcher einer Lochplatte gebracht. Ein feines Netz auf der Unterseite verhindert, dass sie herausfallen. Über der Lochplatte liegt eine Glasplatte, deren Unterseite mit Ruß bedeckt ist. Beim eclosion kratzen die animals den Ruß ab (helle Stellen im Ruß des oberen Bildes), sterben and vertrocknen. red light von unten (red arrows) fällt durch die Ruß-freien Stellen auf eine Photozelle (lila), deren Spannung mit einem Voltmeter (yellow) or über einen Computer recorded wird and ein Maß für die Zahl der geschlüpften animals ist. Links unten: Vier berußte Glasplatten mit hellen Kreisen an Stellen, unter denen geschlüpfte flies den Ruß abgekratzt haben. Rechts unten: Diagramm des Schlüpfens im Laufe von sechs days. Je mehr animals schlüpfen, umso mehr Ruß wird abgekratzt and umso mehr light fällt auf die darüber liegende Photozelle. Damit steigt die Spannung, die somit die Zahl der geschlüpften flies wiederspiegelt.
6.1 Recording the eclosion rhythm

A flat funnel contains a plate, at which pupae are pasted with a wall paper glue. Front side and Top of the funnel are closed. Hourly the funnel is mechanically lifted and dropped several times (red arrow very top). Thereby the flies eclosed during this hour fall into a water vial and drown. In the next hour a turning mechanism shifts the next vial underneath the funnel opening and at the end of the hour again all eclosed flies are shaken out. The drowned flies are counted (see figure 6.2)

Figure 6.1: Recording of the eclosion rhythm of Drosophila flies with a bang box

Figure 6.2: With the bang box (figure 6.1) the eclosed flies were hourly collected in water filled vials and the number of male flies (plotted upward) and female flies (plotted downward) determined. Eclosion occurs in the early morning hours. The males eclose somewhat earlier as compared to the females (that is, not ladies first)
6 How is eclosion and activity of the flies recorded?

6.2 Measuring the locomotor activity

To record the locomotor activity of Drosophila, individual flies are kept in the kind of small cuvettes used for instance in spectral photometers. A small piece of sugar and the wick into a water bottle suffice to keep the animals for several weeks alive. The locomotor activity is recorded by using an infrared lightbeam (Engelmann (1999)). If the fly passes the light beam, an electrical signal is produced and stored. From the data actograms can be derived. Often the obtained information is reduced. One would thus not determine, how often an animal has interrupted the light beam in a certain time interval. Instead it is measured, whether an animal was active at least once (or at least n times) in a certain time interval (for instance in 4 minutes). Instead of obtaining a histogram a bar-actogram is produced (figure 4.2).

Especially versatile can imaging programs be used. With a video camera the behavior of the insects is recorded and the images analyzed with such an imaging program. Much more information can be obtained in this way as compared to the method described before using a light beam. For instance, it can be found out, at which place in the cuvette the fly is located, whether it is feeding or drinking (figure 6.5).
Figure 6.5: The behavior of individual Drosophila flies can be recorded with a video-camera and analyzed with a PC. Each of the (spectral photometer-) cuvettes contain a fly, a small piece of sugar (as food) and a wick to a water bottle (for drinking). Furthermore the cuvettes contain a lateral opening with a net for airation. Light of red fluorescence tubes (which are additionally wrapped with a red foil) illuminate the animals from the side, allowing the video camera to see them. The images are digitized in regular intervals and transformed in a program. In this way the position of the animals can be traced and the behavior (drinking, feeding, cleaning) recognized. After Schuster 1988 and Engelmann, unpublished.
6 How is eclosion and activity of the flies recorded?
7 Significance of the rhythms

If flies are kept in non-24-hours days (21- or 27-hours days) or if the internal clock is often shifted, they die earlier. Flies, which have a well expressed circadian rhythm, live longer. Arhythmic Drosophila-mutants have fewer progenies.

We have seen already in chapter 2, what a daily rhythm means for a fly, which leaves its protecting pupal case in the soil and enters a dangerous environment. The right time of eclosion can decide on Life or death. It is thus of high value, to possess a precise internal clock. There are, however further advantages of a daily clock.

Such an advantage was shown in experiments of Pittendrigh and Minis (1972) using *Drosophila melanogaster*-flies. A part of the animals was kept in artificial days, the period of which was not 24 hours, but 27 hours. Another part of the flies lived in a 21-hour-day. As controls animals were used, which stayed in a normal 24-hour-day. For each of the three groups every day the number of dead animals was determined. Figure 7.1 shows, that the animals in a normal 24-hour-day lived longest. Perhaps the internal clock in the shorter or in the longer artificial days had problems to adapt and that therefore the animals died earlier as their happier conspecifics in a normal day.

In other flies a similar experiment was performed: Aschoff et al. (1971) kept *Phormia terrae novae*-flies in a 12:12 hour light-dark cycle. One group was delayed each day by six hours in a way, they would experience if flown from Europa to the
Significance of the rhythms

USA. In another group the rhythm was advanced by six hours (as if flown from the USA to Europa). In a third group the rhythm was delayed in one week, in the next one advanced, afterward again delayed. A fourth group stayed as a control always in the same rhythm. In contrast to the control group the life expectancy of the animals in the three experimental groups was shortened (figure 7.2). It might well be, that here too the flies had problems to shift their internal clock again and again, in order to adapt to the shift in time, which was connected with the ‘travelling through time zones’.

In another experiment Musca domestica-flies were kept after eclosion in weak continuous red light and the locomotor activity recorded. They showed for some time a circadian rhythm, but most of the animals became arhythmic after some time. It turned out, that those animals lived longest, which were rhythmic until the end (figure 7.3). That could mean, that flies with a stable circadian rhythm live longer.

![Figure 7.2: Life duration of Phormia terrae novae flies, the light-dark-cycle of which was once a week advanced by 6 hours ('east', because advance corresponds to a flight eastward) or delayed ('west', because delay corresponds to a flight westward). One group ('both') was 'flown' to the east as well as to the west. Control animals, which did not undergo shifts in the light-dark cycle, lived as an average 125 days (blue curve). After Aschoff et al. (1971)](image1)

![Figure 7.3: Percentage of flies with a circadian rhythm under constant temperature in weak red light. With time (x-axis, days) more and more animals lose their circadian rhythm (become arhythmic). However, from the sixtieth day onward the percentage does not decline and increases to 50% at the end, when almost all animals had died (Engelmann, unpublished)](image2)

It would be interesting, to find out by experiments, whether the life expectancy is reduced, when the circadian rhythm is completely prevented.

Klarsfeld and Rouyer (1998) studied the life span of a short periodic (per^T, pe-
LD-cycle | per$^+$ | per$^T$ | per$^L$
---|---|---|---
8:8 | 50.9 ± 1.3 (9) | 46.9 ± 0.5 (7) | 48.5 ± 0.5 (8)
12:12 | 52.4 ± 0.8 (8) | 44.4 ± 0.9 (8) | 47.6 ± 0.9 (5)

Table 7.1: Life span of the wild type per$^+$ and of a short- (per$^T$) and a long-periodic (per$^L$) mutant of Drosophila melanogaster in days, with standard error and number of experiments in parenthesis. From Klarsfeld and Rouyer (1998)

period length 16 hours) and a long periodic mutant (per$^L$, period length 29 hours) of Drosophila melanogaster in a 12:12 hours light-dark cycle and in a 8:8 hour light-dark cycle. As shown in Table 7.1, the life span of the per$^+$-animals (corresponding to the wild type) was longer than that of the per-mutants, and that independent of the light-dark cycle. See also Allemand et al. (1973).

Arhythmic mutants (per$^0$) of Drosophila melanogaster spend less sperms during copulation. The females lay fewer eggs and more of them are unfertile (Beaver et al. (2002)). The number of offsprings is thus smaller, they have a reduced reproductive fitness. However, in the per$^+$-animals (in which the mutated gene is repaired) the figures are identical to those of the wild type (table 7.2). Clock-genes are thus important for the reproductive fitness.

$^1$Konopka et al. (1994) did not find any difference in the life expectancy, but their mutants possessed less extreme period lengths.
Table 7.2: Reproductive fitness in the wild type and in an arhythmic mutant of
Drosophila melanogaster. The eggs per pair, the number of unfertilized eggs and the
number of offsprings per pair is reduced in the arhythmic mutant per° as compared to the
wild type. ‘Repaired’ mutants per+ show the same values as the wild type. clock-genes
are thus important for reproductive fitness. From Beaver et al. (2002)
8 Annual calendar of flies

Flies overcome the strong winters in higher latitudes by entering a special resting stage. In this stage of diapause the metabolism is reduced, the cold resistance increased and the propagation stopped. The flies notice, whether the winter is approaching by the shorter days. They must somehow be able to measure the daylength and to enter after experiencing a certain number of shortdays diapause. The end of winter is noticed either by the days getting longer in the spring, or usually by the higher temperatures in the spring after the coldness in the winter.

Flies enter like many other insects of higher latitudes a special resting stage, if winter approaches. This stage is called diapause. What happens in this stage, and how the flies notice, that winter is coming, and how the end of the winter is recognized, shall be explained in the following sections. We will see, that flies like many other organisms possess an internal calendar, which is synchronized to the seasons by using the daylength. This has been studied especially well in Drosophila littoralis.

8.1 How does a fly overcomes the winter?

If you look at a fruitfly, its hard to imagine, how such a delicate structure can survive the winter in the higher latitudes. But it is able to, as shown by its sheer presence. How does the fly manage it? In the book ‘Biocalendar’ (Engelmann (2003)) I have shown, how insects overcome the winter in a special stage, in which they are protected against the inclemencies of the weathers. Development and propagation of the animals stops during the winter. This diapause state can occur in various developmental stages of the insects.

In Drosophila-species too diapause was described in several cases. Depending on the species it might occur in the adult stage, in the pupal stage or in the larval stage. As a fly overwinters for instance Drosophila nitens (Burla (1951)), Drosophila robusta (Carson and Stalker (1948)), Drosophila subobscura (Basden (1954)), Drosophila phalerata, Drosophila littoralis, Drosophila transversa and Drosophila subobscura. Drosophila alpina spends the winter in the pupal stage. In the larval stage overwinters Drosophila deflexa and pupates not before spring (Basden (1954)).

In Japan the overwintering was studied in numerous Drosophila-species by Toda (1979) and Beppu et al. (1996)). In Drosophila aurelia the geographic races of the japanese islands differ from each other by their temperature dependency (Pittendrigh and Takamura (1987)). It is likely that diapause in Drosophila-species of moderate and especially higher latitudes is much more common than known so far.

8.2 How does a fly notice, that winter is coming?

If a Drosophila would start to adapt to the winter conditions, when the first frost has
come, it would freeze to death. It has to take other tokens from the environment, which herald the coming winter. The safest token is the daylength (photoperiod). It is reliable even under an unusually warm fall. As a matter of fact many organisms use photoperiodic reaction to prepare for the winter. That is true also for insects, and Drosophila is no exception. If the days in the autumn are getting shorter, the animals enter diapause.

As an example for diapause in fruitflies Drosophila littoralis is presented. This species is found in different geographical varieties in Europe from Northern Finland to Italy. The females are photoperiodically responsive. We are thus dealing with a diapause in the adult stage. In shortdays no eggs are produced. The gonads stay small. At a certain time in the fall the animals experience a critical daylength, which induces diapause. Depending on the geographic latitude, in which the various varieties live, this critical daylength differs (figure 8.1). It amounts to 20 hours in animals from Oulu in Northern Finland (65.0°N), to 18.8 hours in animals from the lake Inari, that is even further north (68.8°N), to 12.3 hours in animals from Paltamo in Finland (65.0°N), to 18 hours in animals from Batumi in Aserbeidschan (41.6°N). Animals from the Tessin in Swizzerland (46.2°N) do not undergo a Diapause (Lankinen and Lumme (1984)).

8.3 How does a fly measure the daylength?

To react to the daylength for starting diapause the light period has to be measured. It was Bünning’s idea (Bünning (1936)), that the organisms use an internal daily clock for it. He proposed a model, which explained how the time measurement might occur. It is shon in figure 8.2 and explained.

Light has according to this model two functions: It synchronizes the circadian clock, and it induces the photoperiodic reaction, if the photoperiodic constellation of the season is adequate for the organism in question. The internal oscillation with its alternating circadian phases (photophilic and skotophilic) coincides with the external rhythm of the light-dark-cycle in different ways, depending on the daylength. This determines, whether diapause is entered in shortdays or development of the animals in longdays.

Bünning’s hypothesis was later modified. Instead of a skotophilic phase, which according to Bünning takes half of the day, it is only a short section of the oscillation which is light sensitive. This time is called light-inducible phase \( \Phi_l \). To induce diapause, the external light-dark-cycle has to coincide with \( \Phi_l \) in the right way (see figure 8.3). This model was called external coincidence model (Pittendrigh (1964)).

According to the Bünning-hypothesis as well as according to the modified model organisms use thus for photoperiodic reactions their circadian clocks to measure the daylength. However, it turned out that the story is more complicated as first assumed. That resulted from studies of the diapause of female Drosophila melanogaster-flies.

In these flies kept in shortday diapause is induced, and the female stop producing eggs. Mutants without daily eclosion rhythm and without a rhythmic activity enter diapause, although the circadian clock is absent, which is supposed to measure the daylength (see figure 8.4, Saunders et al. (1989)). However, the critical light period is in the arhythmic mutants by several hours shorter. The \( per \) gene does thus influence the photoperiodic time measure-
8.3 How does a fly measure the daylength?

Figure 8.1: *The critical daylength of diapause induction increases in various geographical varieties of Drosophila littoralis (location see markings in the map of Europe, top) with higher latitudes (40 to 70°N, below). After Lankinen and Lumme (1984).*
Figure 8.2: Bünning-model for the photoperiodic induction of diapause. Light has two functions: It synchronizes the circadian clock with the light-dark-cycle. The upper curve shows a freerun oscillation under constant conditions without time cue (That is, without light-dark-cycle and without temperature cycle). The two lower curves show rhythms, which are synchronized to the light-dark-cycle of the 24 hour day. The middle curve shows the situation for a longday, the lowest curve for shortday. Secondly light influences the photoperiodic system differently, depending on whether shortday or longday prevails. In a longday the long light period (white are above the x-axis) coincides not only with the so-called ‘photophilic phase’ (light-loving, red part of curve), but partly also with the skotophilic phase (‘dark-loving’, grey part of curve). In this case diapause would be prevented. In short day the skotophilic phase is not illuminated and diapause is induced. After Bünning (1983)
8.3 How does a fly measure the daylength?

...ment, but it is not imperative for inducing diapause.

This shows: There are apparently at least two different circadian clocks, one of which controls activity of the animals, whereas the other one measures daylength. The per gene is not causaly involved in measuring the daylength by the photoperiodic clock. It influences, however, the photoperiodic time measurement, since the critical daylength is altered in flies wit a defect (per$^{01}$) or missing (per$^{-}$) per-locus.

In the measurement of the daylength neural and hormonal events are involved, as shown in Drosophila grisea (Kambysellis and Heed (1971)).
8.4 How are the shortdays seen and counted?

To measure the daylength, receptors for light are needed and a signal has to be sent to centers, in which the photoperiodic decision for or against diapause is made. We have seen already (section 3.2), that for the synchronization of the circadian clock neither the compound eyes nor the ocelli are obligatory. This is true also for the photoperiodic time measurement. The responsible extraretinal receptors and the circadian diapause-clock are in the central brain, and there possibly in the Pars intercerebralis of the middle brain.

As a rule more than one inductive day is needed for the induction of diapause and other photoperiodic events. A model of Lewis and Saunders (1987) explains, why the number of cycles is important, and how this happens. It is the result of a damped circadian oscillator, as explained in figure 8.5. If the amplitudes of the oscillations are large enough, each day a factor is added, until after a certain number of days a threshold is reached. It allows the induction of diapause. Neurosecretory cells in the central brain seem to be involved.

8.5 When is the winter gone?

To enter diapause early enough is one story. As important is, however, to terminate diapause at the end of the winter and to continue active life. The Drosophila has to use time cues of the environment as signals, in order to terminate the diapause. One could imagine, that for this purpose again the daylength is used. The daylength increases again in the spring, and it is a very reliable time cue of the season. That is indeed occasionaly so, but in most cases another strategy is used for terminating the diapause. It consists of two parts. First, the animals need a certain number of days with low temperature (‘winter’), secondly the temperature in the environment has to be favorable for development. This strategy makes sense for flies, which overwinter in hidden places, where light can not be seen.

8.6 Diapause and heredity

The genetics of diapause of various varieties of Drosophila littoralis was studied by a group in Oulu (Finland) (figure 8.6, Lankinen and Lumme (1984)). The genetic factor for diapause segregates as a single Mendelian unit. It is at the X-chromosome close to the white-locus and is variable enough, to be able to explain the different critical daylengths (12 to 18 hours) of the various geographic races. Diapause dominates over non-diapause (Lumme and Lakovaara (1983)).
Figure 8.5: Model of the photoperiodic counter of Lewis and Saunders. Top left: Control system of a feedback oscillator. Synthesis of the oscillating substance \( c_t \) is determined by the difference between the reference value \( c_{\text{ref}} \) and the time-delayed value of \( c_t \left( c(t-t_0) \right) \). Light increases the concentration of \( c_t \), whereas a part of the substance is permanently lost. Top right: Depending on the synthesis rate \( SR \) simulations lead to the blue curves. Lower rates increase the damping. Horizontal lines are threshold values, and \( c_t \) values above these threshold are summed up with time (INDSUM formation, bottom right). Dynamic of the synthesis rate in the bottom left diagram as a function of the \( c(t-t_0) \) values. Synthesis rate is limited by an upper value, which prevents the amplitude of the oscillation to become too large (bottom left). After Lewis and Saunders (1987), Saunders and Lewis (1987a), Saunders and Lewis (1987b)
Figure 8.6: Two races of Drosophila littoralis, one from Kutaisi, the other one from Oulu, were crossed with each other and the critical daylength for the photoperiodic induction of diapause of the offsprings (F1, green curve) compared with those of the parents (Oulu blue, Kutaisi red). They lie between the one of the parents. After Lankinen and Lumme (1984)
9 Further books

I have written further books or am in the process of writing. They are also concerned with topics, which have to do with rhythmic events in organisms - my specialty as a scientist (Engelmann (2007), Engelmann (2004c), Engelmann (2009a), Engelmann (2009b), Engelmann (2009c), Engelmann (2009d), Engelmann (2008), Engelmann (2004a), Engelmann (2004d), Engelmann (2004b)).
9 Further books


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