



Regulation of biological clock through circadian rhythm in insects

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Abstract

Circadian rhythms are particularly ubiquitous in insects and, as in other organisms, have been the best analysed. Physiological circadian rhythms in insects have been well- documented in relation to hormone production, particularly hormones controlling postembryonic development. The expression of rhythmicity in insects has been largely documented at the cellular level, in organs, as changes in the physiology and behaviour of individuals, as well as in population synchrony. Fruit flies, cockroaches, butterflies, honey bees, and other insects cast much light on the physiology and molecular basis of the circadian clock. Insect adaptation and success in the colonisation of the most diverse environments is also associated with the temporal organisation of their daily life. Circadian locomotor rhythms of the adult flies reflect endogenous, self-sustained oscillations with a temperature compensated period. The free-running rhythms become synchronised (entrained) to daily light:dark cycles, but become arrhythmic in constant light above a certain intensity. Some flies show fragmented rhythms (internal desynchronisation) suggesting that overt rhythmicity is the product of a multioscillator (multicellular) system.

Keywords: Circadian rhythms, rhythmicity, multioscillator, synchronized

Introduction

Insects, like other organisms, evolved in an environment dominated by daily periodicity. Their presence on the earth for longer than vertebrates allowed selective forces the time for fine tuning in time for several processes not only at the cell, organ, and individual levels but beyond at the population and multitrophic interactions. Their ectothermic condition together with their small size render insects particularly sensitive to the environmental temperature, and their high surface-to-volume ratio facilitates water loss. Additionally, the design of their eyes does not permit sufficient sensitivity to see at night. These facts, along with others originated in their corporal and functional design, acted as selection forces to predict changes in their environment. Thus, it is not surprising that insect life is temporally strongly and finely organized, and that they express many physiological and behavioural daily rhythms at many different levels. Chronobiologists some time ago realized the suitability of insects for experiments and made them one of their favourite subjects of study. Indeed, insects are closely associated with the history of the study of biological rhythms. They allowed unravelling many fundamental questions to persons as Colin Pittendrigh, Jürgen Aschoff, Edwin Bünning, Tony Lees, and several other personalities in Chronobiology.

Definition

A circadian rhythm is any biological process that displays an endogenous, entrainable oscillation of about 24 hours. These 24hour rhythms are driven by a circadian clock, and they have been widely observed in plants, animals, fungi, and cyanobacteria ^[1]. The term circadian comes from the Latin *circa*, meaning "around" (or "approximately"), and *diēm*, meaning "day". HISTORY AND ORIGIN :- The

earliest recorded account of a circadian process dates from the 4th century B.C.E., when Androstrhenes, a ship captain serving under Alexander the Great, described diurnal leaf movements of the tamarind tree ^[2]. The observation of a circadian or diurnal process in humans is mentioned in Chinese medical texts dated to around the 13th century, including the Noon and Midnight Manual and the Mnemonic Rhyme to Aid in the Selection of Acupoints According to the Diurnal Cycle, the Day of the Month and the Season of the Year ^[3]. The first recorded observation of an endogenous circadian oscillation was by the French scientist Jean Jacques 'Ortous de Mairan in 1729. He noted that 24hour patterns in the movement of the leaves of the plant *Mimosa pudica* continued even when the plants were kept in constant darkness, in the first experiment to attempt to distinguish an endogenous clock from responses to daily stimuli ^[4, 5]. In 1896, Patrick and Gilbert observed that during a prolonged period of sleep deprivation, sleepiness increases and decreases with a period of approximately 24 hours ^[6]. In 1918, J.S. Szymanski showed that animals are capable of maintaining 24hour activity patterns in the absence of external cues such as light and changes in temperature ^[7]. In the early 20th century, circadian rhythms were noticed in the rhythmic feeding times of bees. Extensive experiments were done by Auguste Forel, Ingeborg Beling, and Oskar Wahl to see whether this rhythm was due to an endogenous clock. Ron Konopka and Seymour Benzer isolated the first clock mutant in *Drosophila* in the early 1970s and mapped the "period" gene, the first discovered genetic determinant of behavioral rhythmicity ^[8]. Joseph Takahashi discovered the first mammalian circadian clock mutation (clock Δ 19) using mice in 1994 ^[9, 10]. However, recent studies show that deletion of clock does not lead to a behavioral phenotype (the animals

still have normal circadian rhythms), which questions its importance in rhythm generation^[11, 12]. The term circadian was coined by Franz Halberg in the 1950s. The simplest known circadian clock is that of the prokaryotic cyanobacteria. Recent research has demonstrated that the circadian clock of *Synechococcus elongatus* can be reconstituted in vitro with just the three proteins (KaiA, KaiB, KaiC)^[13] of their central oscillator. This clock has been shown to sustain a 22-hour rhythm over several days upon the addition of ATP. Previous explanations of the prokaryotic circadian timekeeper were dependent upon a DNA transcription/translation feedback mechanism. A defect in the human homologue of the *Drosophila* "period" gene was identified as a cause of the sleep disorder FASPS (Familial advanced sleep phase syndrome), underscoring the conserved nature of the molecular circadian clock through evolution. Many more genetic components of the biological clock are now known. Their interactions result in an interlocked feedback loop of gene products resulting in periodic fluctuations that the cells of the body interpret as a specific time of the day. It is now known that the molecular circadian clock can function within a single cell; i.e., it is cell-autonomous^[14]. This was shown by Gene Block in isolated mollusk BRNs^[15]. At the same time, different cells may communicate with each other resulting in a synchronised output of electrical signaling. These may interface with endocrine glands of the brain to result in periodic release of hormones. The receptors for these hormones may be located far across the body and synchronise the peripheral clocks of various organs. Thus, the information of the time of the day as relayed by the eyes travels to the clock in the brain, and, through that, clocks in the rest of the body may be synchronised. This is how the timing of, for example, sleep/wake, body temperature, thirst, and appetite are coordinately controlled by the biological clock.

Criteria of circadian clock

To be called circadian, a biological rhythm must meet these three general criteria^[16]: 1. The rhythm has an endogenous free-running period that lasts approximately 24 hours. The rhythm persists in constant conditions, (i.e., constant darkness) with a period of about 24 hours. The period of the rhythm in constant conditions is called the free-running period and is denoted by the Greek letter τ (tau). The rationale for this criterion is to distinguish circadian rhythms from simple responses to daily external cues. A rhythm cannot be said to be endogenous unless it has been tested and persists in conditions without external periodic input. In diurnal animals (active during daylight hours), in general τ is slightly greater than 24 hours, whereas, in nocturnal animals (active at night), in general τ is shorter than 24 hours. 2. The rhythms are entrainable. The rhythm can be reset by exposure to external stimuli (such as light and heat), a process called entrainment. The external stimulus used to entrain a rhythm is called the Zeitgeber, or "time giver". Travel across time zones illustrates the ability of the human biological clock to adjust to the local time; a person will usually experience jet lag before entrainment of their circadian clock has brought it into sync with local time. 3. The rhythms exhibit temperature compensation. In other words, they maintain circadian periodicity over a range of physiological temperatures. Many organisms live at a broad range of temperatures, and differences in thermal energy

will affect the kinetics of all molecular processes in their cell(s). In order to keep track of time, the organism's circadian clock must maintain roughly a 24-hour periodicity despite the changing kinetics, a property known as temperature compensation. The Q10 Temperature Coefficient is a measure of this compensating effect. If the Q10 coefficient remains approximately 1 as temperature increases, the rhythm is considered to be temperature compensated.

Location of circadian clock

Drosophila's clock is comprised of just 150 neurons per hemisphere. These clock neurons are divided into seven major groups, named after their anatomical position. Three neuronal groups are located more dorsally and are thus called dorsal neurons 1–3 (DN1–3), the other four groups are located more laterally and are therefore called lateral neurons (LN_d, l-LN_v, LPN, and s-LN_v). Additionally there are a few hundred glia cells expressing clock proteins like Per or Tim in the fly's brain^[17, 19]. This classification is of course very crude and does not always reflect functional unity among one cluster. Therefore clock neurons can be further subdivided into different subgroups according to their protein content size and/or function. The DN1 cells consist of about 16 cells. Two of those cells, the DN1a do not express the transcription factor Glass, but do express the neuropeptide IPN-amide and the blue light photoreceptor Cryptochrome^[20]. Cryptochrome is expressed in two to six other DN1 cells, that are located more posterior, namely the DN1p^[21]. With only two cells, the DN2 cluster is the smallest among the clock neurons, while the 40 DN3 neurons form the largest group. Again DN3 neurons show a variety in cell body sizes and can be subgrouped as well^[20]. The about four l-LN_vs and four of the five s-LN_vs are expressing the neuropeptide pigment-dispersing factor (PDF). A 5th s-LN_v is located in close proximity to the l-LN_vs and is lacking PDF. The LN_ds are located more dorsally. This heterogeneous cell cluster comprises six cells, all expressing different neurotransmitters, like acetylcholine (as judged from the presence of the choline acetyltransferase), ion transporter peptide, the long or the short form of neuropeptide F. The last lateral neuronal group is the LPN cluster. Those neurons seem to be tightly connected with the temperature entrainment of the circadian clock.

Adaption to the environment

If a circadian clock would not be flexible to adapt to different environments or photoperiods it would lack an important ability. Therefore circadian clocks evolved sophisticated molecular mechanisms to react to new environments. Several input factors – so called "Zeitgebers" – harmonize the clock neurons to the environment^[22]. Light, temperature, social cues^[23] can influence the circadian clock, whereupon light is the most important input factor. The response to daily or seasonal changes in light is mainly caused by the degradation of Tim protein^[24]. Timeless protein itself is not responsive to light. So another protein must act as circadian photoreceptor. The identity of this photoreceptor was unknown for a long time, until it was shown that animals with a mutated cryptochrome (cry) gene, like *cry⁰* nulls still behave rhythmic in LL (constant light) and show abnormalities in their behavioral responses to light. Cry is a

blue-light photopigment that is expressed in specific subsets of the clock neurons (Fig. 2) and in the compound eyes. This protein is activated in the light, most probably because of a conformational change. Light activated Cryptochrome can bind to Tim and thus triggers Tim degradation (Fig. 3). As a consequence to the lack of Tim, Period is now vulnerable to Dbt phosphorylation and subsequently degraded (and the clock reset). Tyrosine phosphorylated Tim protein is recognized by light-activated Cry. This complex is detected by the F-Box protein Jetlag (Jet) and as a consequence Tim is ubiquitinated and degraded in the proteasome [25]. Not only Tim is target of Jet, but Cry as well. Jet binds light-dependently to Cry and promotes Cry's degradation but only after Tim is present at very low level, thus allowing a new start of a circadian cycle [26]. Another interesting part in the light dependent degradation of Tim, and the adaptation of the fly to its environment assumes the GSK3-beta ortholog Shaggy (Sgg). Overexpression of Sgg causes a similar phenotype under LL, like cryb. Furthermore it was shown, that Sgg can bind to Cry and thus dramatically stabilizes Cryptochrome and hinders somehow Cry from triggering Tim degradation. Even though the direct interaction between Sgg and Tim was never revealed, Sgg seems to phosphorylate Tim, thus allowing Tim to enter the nucleus. Protein Phosphatase 1 (PP1) is dephosphorylating Tim, but this dephosphorylation is not affecting the nuclear entry of Tim. Hence it is possible that Sgg and PP1 target different phosphorylation sites. Another important Zeitgeber is temperature. The daily cycle of cold and warm can properly entrain the fly, while a change of about 3C temperature is enough to adapt *Drosophila*'s circadian clock to the environment [27]. Temperature cycles entrain the clock under constant dark or constant light conditions, implying that there must be an "override" of light dependent degradation of Tim – normally LL renders fruit flies arrhythmic. Temperature can be perceived in cultures of isolated body parts, like wings, legs or heads – indicating that the circadian temperature sensor is tissue-autonomous. The circadian photoreceptor Cry can be found in many clock neurons in the brain, thus directly responding to light stimuli. The circadian thermoreceptor is not located in the brain or clock neurons. Isolated brains are not able to synchronize to temperature cycles, showing that the brain is dependent on temperature information from the periphery [28]. Two genes are known so far to influence the circadian temperature reception. One is the *norpA* gene that encodes for the Phospholipase C. Animals carrying a mutated *norpA* gene are not able to synchronize to temperature cycles [29]. This indicates that a G-protein mediated signal transduction might be involved in circadian temperature reception. The second gene is *nocte*, which encodes a large glutamine-rich protein with unknown function. A mutated *nocte* gene does not allow proper entrainment of the *Drosophila* circadian clock by temperature [28, 29]. Interestingly, downregulation of *nocte* in peripheral tissues is enough to prevent circadian temperature entrainment in the fly. Further narrowing of the peripheral tissue revealed that specific sensory structures called chordotonal organs are necessary for behavioral temperature entrainment [28]. Chordotonal organ neurons, which do not possess a functional clock, send temperature information to peripheral clock neurons in the thoracic CNS, or directly to the clock neurons within the brain. Which clock neurons receive the temperature information from the periphery first or if there are some specific temperature

neurons in the brain is not revealed yet. But it was shown, that some Crynegative neurons are very sensitive to temperature, namely the DN2s and the LPNs [30]. It is still under debate how the temperature information from the periphery is causing a change in the expression of Per and Tim in the clock neurons. What is known so far is that an 89 bp sized intron of period is spliced alternatively, depending on the temperature. At low temperatures, more of the spliced per variant is produced (type B) than of the unspliced variant (type A). As a consequence to an up regulation of type B spliced per, Per level is increasing earlier and so is the locomotor activity. Different levels of spliced per cannot be observed in *norpA* mutants – here type B is always high. *nocte* mutants on the other hand display a wildtype like splicing [29]. All those results are obtained from experiments performed under laboratory conditions, i.e., for example a change of temperature, while the light conditions are constant. Under natural conditions light and temperature interact in entraining the clock. Under temperature cycles solely some clock neurons were not entrained at all, and under LD cycles solely others were only slightly entrained and cycled with low amplitude.

Morning vs. Evening

Most animals display a bimodal peak of their activity, e.g., *Drosophila* shows one peak in the morning and one in the evening [31]. Therefore Pittendrigh and Daan proposed, back in the seventies, a model explaining this bimodal activity pattern. They predicted that there is not only one circadian oscillator, but two. One oscillator – the so called morning oscillator (M) – is responsible for the activity in the morning and is accelerated by light. The other oscillator the evening oscillator (E) – is inducing activity in the evening and is slowed down by light. Both oscillators are coupled. The reason for this bimodal activity is the adaptation to different photoperiods. In summertime the sun rises each day slightly earlier and sets slightly later. The M-oscillator responds to this changed environment with an advanced activity – whilst the E-oscillator delays the onset of activity. As mentioned above wild-type *Drosophila* is rendered arrhythmic in LL. But when cryb flies – or occasionally even wildtype animals – were exposed to very low levels of constant light they started to display a split activity rhythm. One rhythm was faster than normal, i.e., about 22 h and one rhythm was slower i.e., 25 h. This phenomenon can be explained by the two oscillator model and the claim that light is causing acceleration (M-oscillator) or deceleration (E-oscillator) depending on the oscillator. Two further studies unraveled the location of the E- and M oscillator. By manipulating different subsets of clock neurons they showed that the LNvs function as M-oscillator while the LNds and DN2s function as E-oscillator. Further studies restricted the location of the M-oscillator to the s-LNvs and revealed that the 5th, Pdf-negative, s-LNv is part of the E-oscillator. The s-LNvs are considered to be the main circadian pacemaker cells, because they are mandatory to sustain rhythmic locomotor behavior under constant darkness (DD). Recent studies revealed though that under certain conditions the E-oscillator cells drive circadian behavior in constant light (LL). This was possible after reducing the lightsensitivity of the neurons or under dim LL even in the absence of a functional clock in the s-LNvs. A current model predicts that the E-oscillator cells rather maintain circadian rhythm in LL or under long summerday conditions, while the M-oscillator

cells maintain circadian rhythm under DD conditions or in short winterdays.

Process of Circadian Clockgenes Responsible for Rhythm

The current model (Fig. 1) predicts that the helix-loop-helix transcription factors clock (CLK) and cycle (Cyc) bind as heterodimers to E-Box sequences (CACGTG) at midday in the genome of the fly [32, 33]. E-Boxes are found in the promoter region of many circadian regulated genes like period (*per*), timeless (TIM), vrille (VRI) or PAR domain protein 1e (*pdp1e*), but the very center of the clock represents the activation of *per* and *tim*. The subsequent rise of the *per* and *tim* mRNA in the evening/night leads to accumulation of Per and Tim in the cytoplasm – but only after dark, because of the light sensitivity of Tim and the fact that Tim stabilizes Per. Without Tim's protection Per is phosphorylated by the Double Time (DBT) kinase and afterward ubiquitinated by the F-Box protein Slimb and then degraded in the proteasome [34]. Period's degradation is counterbalanced by the protein phosphatase 2A (PP2A). Tim and Per then enter the nucleus alone or as a heterodimer [16, 17] thus allowing Per associated Dbt to coenter the nucleus. This transport is mediated through Per phosphorylation by Casein Kinase 2 (CK2). Inside the nucleus the Per-Dbt-Tim complex accumulates and binds to Clk/Cyc dimers via a Per-Clk interaction. This interaction causes hyperphosphorylation of clock and thus prevents Clk/Cyc dimers from binding to the DNA and inhibits the transcription of *tim* and *per* [35, 36]. Period's inhibition of its own transcription generates a negative feedback loop and thus cyclic expression of Period and Timeless protein and mRNA. A second feedback loop regulates the transcription of Clk and thereby of course interlocks with the first loop. Clk mRNA is transcribed in a reciprocal way to *tim/per* mRNA, showing peak times in the late evening, early morning. As already mentioned Clk/Cyc dimers activate the transcription of two basic leucine zipper proteins, Vrille and Pdp1e [37]. Both proteins bind to so called V/P boxes in the promoter region of Clk. While Vri is inhibiting the transcription of Clk, Pdp1e activates Clk's transcription.

Physiological Rhythm

Physiological circadian rhythms in insects have been well-documented in relation to hormone production, particularly hormones controlling postembryonic development. Further analysis revealed that, in addition to their purely endocrine functions, certain hormones, such as the prothoracicotrophic hormone (PTTH), ecdysteroids, and juvenile hormones (JHs), the main hormones responsible for insect moult and metamorphosis, form a key component of the circadian system and may represent the central timekeeping system [38].

Figure 1 represents the hormonal axis controlling moulting in insects, as well temporal control and synchronization by light. Figure 1. Control of the rhythmic release of hormones controlling moult in *Rhodnius prolixus*. In insect brain, cells possessing clock-gene cycling expression are entrained by light (open arrows). These cells regulate the rhythmic release of PTTH (prothoracicotrophic hormone) through the corpora cardiaca (CC). PTTH stimulates and acts as a Zeitgeber for release of ecdysteroids by prothoracic glands (PG), which also possess light cycle-entrainable autonomous oscillators. Ecdysteroid action triggers the

rhythmic expression of genes on different tissues. Insect moult involves replacement of the entire exoskeleton by a larger one. This process, under the control of ecdysteroids, implies deposition of proteins and chitin (n-acetyl glucosamine) in the newly formed tegument, which continues between moults. This deposition takes place in a precise manner, with chitin fibres organized in layers with different orientations and thicknesses [39]. These layers alternate regularly, giving rise to a lamellate cuticle, which is deposited during one part of each day, and non-lamellate cuticle that is formed during the remainder of each day, representing daily growth layers whose formation is controlled by a circadian clock [40]. Other hormones, such as the juvenile hormone (JH), also appear to be produced in a rhythmic fashion. JH has multiple targets and is concerned with different physiological processes, such as control of ovarian activity and control of sensitivity to sexual pheromones in moths, eventually inducing rhythmicity in their expression. A variation in the sensitivity of antennal chemoreceptors was also verified in the cockroach *Leucophaeamaderae* [41]. As in *Drosophila*, this variation renders the insect more sensitive to several odours, probably to all of these. It means that the animal is more or less sensitive to chemical signals at given moments of the day. Paradoxically, in both cases maximal sensitivity occurs during resting periods and not when the animal is active making use of olfactory information. The bugs evinced a narrow temporal window of responsiveness, but in contrast to the previously described experiences this window perfectly matched the activity period when the insect searches for food [42]. Furthermore, these bugs evince a bimodal activity pattern. They search for food at the beginning of the night, and for refuge at dawn. Because both behaviours are guided by odours (i.e., volatiles released by the host and aggregation pheromones, respectively), this does not appear as adaptive for sensitivity to every odour during only a moment each day, as predicted by results *Drosophila* and *Leucophaea*. Even when both host odours and pheromones are always present in their habitat, they should only respond to the odour that is relevant at a particular moment. The example of blood-sucking bugs has interesting consequences beyond chronobiology. Actually, the chemical ecology of disease vectors is a field in which much effort is concentrated in order to seek novel tools to control pests. It is clear from the previously presented examples that the response of an insect to a given odour will be influenced by temporal matching between the moment of the experiment and the temporal context in which such an odour is relevant in nature. Nevertheless, a review of the recent literature an interesting example of both circadian control and integration of endogenous and exogenous rhythms comprises adaptation to light intensities of the haematophagous bug *Triatoma infestans*. Nectar concentrations are highest at the onset of nectar secretion and exhibit a gradual dilution through the morning. These diel patterns show little day-to-day variability under greenhouse conditions: date was not a significant factor determining either volume or concentration in two separate trials. The relative changes of nectar volumes and concentrations through anthesis yield the greatest nectar sugar production rates at midanthesis, with relatively low sugar production rates at the beginning and end. The diel nectar patterns were found to be quite robust. Both the volume and concentration patterns continued, in two

separate trials, under simulated drought conditions in the greenhouse (Edge *et al.*, 2011).

Behavioural Rhythm Activity Patterns

The easiest behavioural rhythm observable in insects comprises the daily pattern of spontaneous locomotion as representative of activity and resting periods. It is relatively easy to measure and to test for its endogenous or exogenous nature. The majority of activity rhythms in individual insects possess a strong endogenous component, although they may be, to a variable extent, modulated by the direct effects of the environment. The pattern of rhythm varies across species, depending, as in other animals, on specific adaptation for exploiting particular resources. Therefore, unimodal and bimodal activity distributions can be observed in both diurnal and nocturnal insects. It should be noted that the daily pattern of spontaneous locomotion represents the summation of all the different activities performed by the animals. Conversely, not every behavioural activity is carried out at every moment the insect is in motion (Figure 2). For instance, several haematophagous insects feed at the moment of the day when their vertebrate hosts are less active. If the host is diurnal, these insects' main biting activity is concentrated during the night, and vice-versa. This minimizes the risk of being detected by the host, which in many cases may become a predator when active [43].

Triatominae (Heteroptera: Reduviidae), a nocturnal insect. Insect activity patterns also provide some interesting insights concerning the adaptive organisation of behavioural and physiological rhythms. For instance, the ant *Cataglyphis* habits in the Sahara desert and is one of the most thermo-tolerant land animals known (19). Their bodies reaches temperatures $>50^{\circ}\text{C}$ during foraging trips over the desert's sandy surface at midday. At this time, predators hide in the shadow to avoid excessive heating. Each day, foraging activity is preceded by the synthesis and accumulation of heat-shock proteins, which protect the animal from the excessive heat. The crepuscular activity of certain insects appears to contain a strong exogenous component, because the period of day at which activity can occur is restricted by environmental factors, particularly light intensity. Mating in the fruit-fly *Dacstryoni* occurs at an optimal light intensity of ca 0.8 lux. Under a constant low light of this intensity, mating followed a circadian rhythm that free-ran with a period ca 28 h for ca 4 days; nonetheless, under either DD or LL conditions mating is strongly depressed. Under natural conditions, given that both high light intensity and darkness suppress mating, mating activity is restricted to a remarkably short period of 30 min daily. The adaptive value of limiting mating to dusk is presumably because this synchronises the sexual behaviour of all individuals in the population, thereby increasing mating efficiency [44]. When the coordinates were connected in sequence, a series of walking-paths were produced, describing the bed bugs' movement. Walking-paths could best be described as meandering movement with frequent changes in direction. Results demonstrates the difference in walking-paths between bed bugs in the presence and absence of olfactory cues. Unapparent from these walking-paths were the short but frequent periods of motionlessness that interrupted longer periods of movement. While a majority moved away from the harborage area during the experiments, some bugs only moved inside of the harborage area, while others didn't move at all (Suchy and Lewis, 2011) [3].

The ability of honey-bees (*Apis mellifera*) to return to a food source at the same time each day was known nearly 100 years ago, when the Swiss naturalist August Forel observed that bees arrived at his breakfast table for food. Because the bees always came at the same time even when food was not present—Forel [45] proposed that bees possess a memory for time. Later, Beling [46] trained bees by offering them a sugar solution in an artificial feeding set in a certain place at the same time each day. During subsequent days (the test period), the feeding place was without sugar, but each visiting bee and its time of arrival was recorded. Beling demonstrated that bees do indeed return at the same time each day (Figure 4). The adaptive value of this ability is related to the fact that different plant species bloom at different times throughout the day and this is another consequence of the co-evolution plant-pollinator.

The unequivocal test for the endogeneity of bee time-memory came during the 1950s. Renner [47] trained bees to a food source in Paris between 8.15 and 10.15 a.m. local time in a closed chamber under LL and constant temperature. The bees were then transported overnight to New York and tested the following day under identical conditions. In this now classical translocation experiment, the bees had been transported over 76° of longitude, or a difference of ca 5 h in real local time. If an endogenous circadian rhythm were involved, bees should have come to the test dish 24 h after the training period; if, on the other hand, the bees were responding to subtle local influences they should forage at the same local or sun time. The results showed that the former alternative was the case: bees came to the feeding dish at 3.00 a.m. Eastern daylight time, exactly 24 h after their last feeding period in Paris. The reciprocal experiment involving translocation from New York to Paris yielded an analogous result. LEARNING

Ability Rhythm

Decker and co-workers [48] have recently shown that olfactory learning in the cockroach *Leucophaeamaderae* is regulated by the circadian system. Insects were trained and tested at different circadian phases for their performance in odour discrimination 30 min after training (short-term memory) or 48 h after training (long-term memory). After differential conditioning in which one odour was associated with a positive reward (sugar solution) and another different odour with a negative one (saline solution), cockroaches conditioned in the early subjective night exhibited a strong preference for the former and retained the memory for at least 2 days. Animals trained and tested at other circadian phases demonstrated significant deficits in performance for both short- and long-term memory. Performance depended on circadian time (CT) of training, not CT of testing, and results indicate that memory acquisition rather than retention or recall is modulated by the circadian system. The question arises concerning the adaptive value of circadian learning and memory regulation. In the majority of instances in which circadian control of memory formation has been demonstrated, humans and other animals perform better with training during the active phase. Decker and coworkers proposed that memories are only profitable when formed within the environmental context in which they will be utilized. Because the environment is periodic and because cockroaches are active at night and spend the daytime hidden in dark shelters, little would be gained by forming memories during the daytime. Memories based on

information obtained at a time and in an environment in which the animal is unlikely to be foraging could interfere with successful foraging during its normal foraging at nighttime hours.

Conclusion

Insects have contributed much to unravelling many big questions in chronobiology and continue highlight many relevant aspects. They are excellent experimental models, as demonstrated in seminal works conducted by the pioneers of the study of biological rhythms. They can be easily reared in the laboratory, have short generation times, and are robust for experimental surgical manipulation (e.g., ligatures, decapitation, transplantation, parabiosis). Conversely, chronobiological studies on pest insects can greatly improve our knowledge concerning their biology, providing basic knowledge on the temporal organisation of pest life. This information possesses a large applied value, because it can aid in improving their control by applying control measures during the time of higher susceptibility, for example, when ecdysis or hatching take place and the body cuticle is most permeable.

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