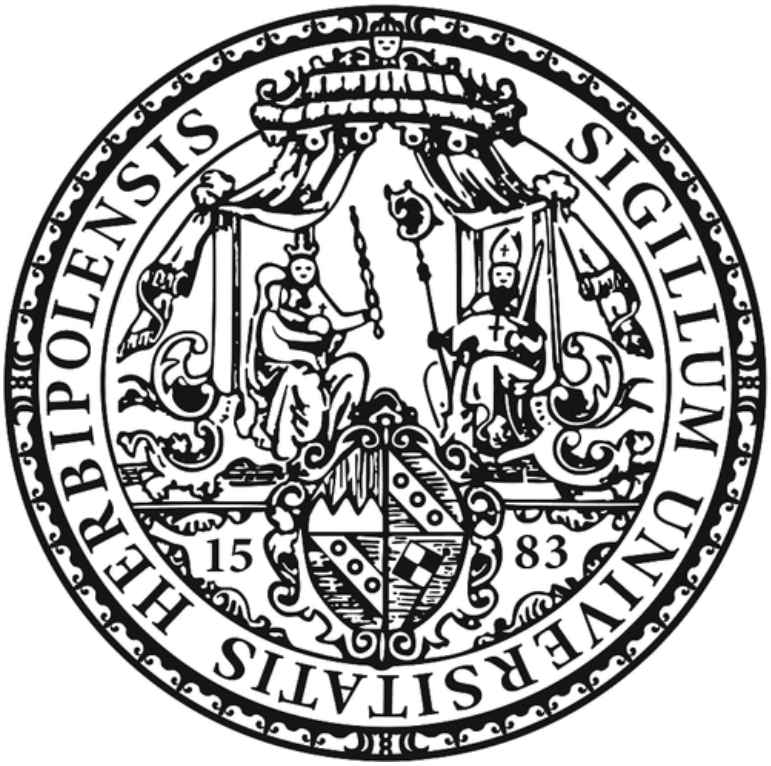
The impact of Drosophila melanogaster`s endogenous clock on fitness: Influence of day length, humidity and food composition

Auswirkungen von Drosophila melanogaster`s Innerer Uhr auf die Fitness: Einfluss von Tageslänge, Luftfeuchtigkeit und Ernährung



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“It is the time you have wasted for your rose that makes your rose so important.”

– Antoine de Saint-Exupéry, The Little Prince.

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Zusammenfassung

Wir leben in einem System, welches durch die Erdrotation permanenten Veränderungen der Umwelt unterliegt. Diese Veränderungen sind rhythmischer Natur, wobei die wichtigste Veränderung einen Rhythmus von circa 24 Stunden aufweist. Aber auch kürzere und längere Rhythmen charakterisieren unsere Umwelt. Um mit den permanenten Veränderungen klar zu kommen geht man davon aus, dass es von Vorteil ist wenn ein Organismus die Veränderungen wahrnehmen und vorausahnen kann. Die sogenannten Inneren Uhren ermöglichen dies und stellen möglicherweise einen Fitness Vorteil dar. Um den Mechanismus von Inneren Uhren zu untersuchen und aufzudecken benutzen Chronobiologen verschiedene Modellorganismen. In dieser Arbeit wurde *Drosophila melanogaster*, mit ihren etwa 150 Uhrneuronen welche die Innere Uhr im Zentralen Nervensystem darstellen, als Modellorganismus verwendet.

Der molekulare Mechanismus und die ineinandergreifenden Rückkopplungsschleifen mit den Hauptakteuren *period*, *timeless*, *clock* und *cycle* werden seit den 1970ern erforscht und wurden bisher recht gut charakterisiert. Aber der Einfluss einer funktionellen Inneren Uhr in Kombination mit diversen Faktoren und die daraus resultierenden Fitness Vorteile wurden in nur wenigen Studien untersucht und bleiben zu großen Teilen unbekannt. Deshalb war es das Ziel dieser Arbeit den Einfluss von *Drosophilas* Innere Uhr auf die Fitness der Taufliege aufzudecken. Um dieses Ziel zu erreichen wurden verschiedene Faktoren – wie z.B. Tageslänge, Luftfeuchtigkeit und Futterqualität – in Wildtyp CS und drei verschiedenen *period* Mutanten – namentlich per^{L}, per^{S} und per^{01}, welche alle eine Punktmutation tragen, welche die Freilauf-Periodenlänge verändert oder zu Arrhythmizität führt – sowie einem weiteren arrhythmischen Fliegenstamm, clk^{AR}, untersucht.

In Konkurrenzversuchen konkurrierten Wildtyp und Uhrmutanten über bis zu 63 Generationen unter normalen 24 Stunden Rhythmen mit jeweils 12 Stunden Licht/Tag und 12 Stunden Dunkelheit/Nacht oder unter T-Zyklen mit 19 oder 29 Stunden, entsprechend der Freilauf-Periodenlänge der Mutanten, oder Dauerlicht (LL) im Falle der arrhythmischen Mutante, sowie unter naturähnlichen Bedingungen im Feldversuch in zwei aufeinanderfolgenden Jahren. Im Gesamten war der Wildtyp den Uhrmutanten überlegen, unabhängig von den Umweltbedingungen. Da die per^{L} Mutanten Ihre Freilauf-Periodenlänge deutlich verlängerten, wurden die Konkurrenzexperimente mit auf natürlicher Weise mit dem Wildtyp CS rückgekreuzten Fliegenstämmen wiederholt. Mit diesen Experimenten konnte gezeigt werden, dass der genetische Hintergrund der Fliegenstämme – welche teils für Jahrzehnte im Labor

gehalten und nur wenige Male rückgekreuzt werden – sehr wichtig ist und die Fitness der Fliegen beeinflusst. Aber auch die Länge der Tage (19 h, 24 h oder 29 h) beeinflusst die Fitness der Fliegen und ermöglicht es Ihnen in höherem Anteil in einer Population unter Konkurrenz zu bestehen. Weitere Faktoren, welche das Überleben unter Konkurrenz möglicherweise beeinflussen können, wie z.B. eine Paarungspräferenz und Laufaktivität von homo- und heterozygoten Weibchen oder die Anzahl an Spermien, die pro Paarung übertragen werden, wurden untersucht. Diese Faktoren allein konnten jedoch die Ergebnisse der Konkurrenzversuche nicht erklären und spielen dabei keine oder nur geringfügige Rollen und stellen ein Beispiel für die Komplexität des ganzen Systems mit noch weiteren unbekannten Faktoren dar.

Im Weiteren wurde das Laufverhalten von ganzen Fliegenpopulationen aufgezeichnet, um zu erforschen, ob eine Fliegenpopulation einen gemeinsamen Freilauf an Laufaktivität aufweist oder nicht. Und tatsächlich konnte zum ersten Mal das Laufverhalten von ganzen Populationen aufgezeichnet werden und Sozialer Kontakt als Zeitgeber für *Drosophila melanogaster* bestätigt werden.

Zusätzlich wurde in dieser Arbeit relative Luftfeuchtigkeit und deren Auswirkung auf die Fitness der Fliegen, als auch als potentieller Zeitgeber untersucht. Die Fliegen wurden zum Schlupf und zur Entfaltung der Flügel unterschiedlichen Luftfeuchtigkeiten ausgesetzt und es wurden Phasenverschiebungsversuche mit Luftfeuchtigkeitszyklen durchgeführt, um diese zwei verschiedenen Fragen nach Fitness und potentiellem Zeitgeber zu beantworten. Die Fruchtfliege schlüpft normalerweise in den Morgenstunden, wenn die Luftfeuchtigkeit relativ hoch ist, weshalb im Allgemeinen angenommen wird, dass dies zu diesem Zeitpunkt des Tages geschieht, um eine Austrocknung zu verhindern. Die Ergebnisse dieser Arbeit waren sehr eindeutig und demonstrierten, dass die relative Luftfeuchtigkeit keinen großen Einfluss auf die Fitness der Fliegen in Bezug auf den Schlupferfolg und korrektes Entfalten der Flügel hat und dass die Temperatur wohl eher der ausschlaggebende Faktor sein könnte. In den Phasenverschiebungsversuchen mit Luftfeuchtigkeitszyklen konnte aufgedeckt werden, dass relative Luftfeuchtigkeit keinen Zeitgeber für *Drosophila melanogaster* darstellt, aber die Laufaktivität der Fliegen beeinflusst und maskiert, indem das Laufverhalten bei bestimmten relativen Luftfeuchtigkeiten zugelassen oder unterdrückt wird.

Außerdem wurde die Lebenserwartung der Wildtyp und Uhrmutanten Fliegenstämme unter verschiedenen Tageslängen und mit unterschiedlicher Futterqualität untersucht, um den

Einfluss dieser Faktoren auf die Fitness und somit das Überleben der Fliegen auf Dauer zu charakterisieren. Wie erwartet starben die Fliegen auf dem nährstoffarmen Minimalmedium früher als auf dem nährstoffreichen Maximalmedium, aber es konnte auch ein kleiner Effekt der Tageslänge gezeigt werden. Hierbei lebten die Fliegen etwas länger, wenn die Tageslänge die Freilauf-Periodenlänge der Fliegen widerspiegelte. Diese Versuche zeigten auch einen Fitness Vorteil der Wildtyp Fliegen gegenüber der Uhrmutanten auf lange Sicht, jedoch nicht zu Beginn (in den ersten ca. 2-3 Wochen).

Abschließend kann zusammengefasst werden, dass genetische Variation wichtig ist, um sich an Veränderungen in der Umwelt anzupassen und die eigene Fitness und somit Überleben zu steigern. Eine funktionelle Innere Uhr mit einer Periodenlänge von etwa 24 Stunden zu besitzen stellt einen Fitness Vorteil für die Fliegen dar, zumindest unter Konkurrenzbedingungen. Das ganze System ist sehr komplex und viele Faktoren – bekannte und noch unbekannte – spielen eine Rolle in diesem System, welches auf verschiedenen Ebenen interagiert, wie z.B. auf physiologischer, metabolistischer oder auf der Verhaltensebene.

Summary

We are living in a system that underlies permanent environmental changes due to the rotation of our planet. These changes are rhythmic with the most prominent one having a period of about 24 hours, but also shorter and longer rhythms characterize our environment. To cope with the ever-changing environmental conditions, it is thought to be beneficial if an organism can track and anticipate these changes. The so called endogenous clocks enable this and might provide a fitness advantage. To investigate and unravel the mechanism of endogenous clocks Chronobiologists have used different model organisms. In this thesis *Drosophila melanogaster* was used as model organism with its about 150 clock neurons representing the main endogenous clock of the fly in the central brain.

The molecular mechanisms and the interlocked feedback loops with the main circadian key players like *period*, *timeless*, *clock* or *cycle* are under investigation since the 1970s and are characterized quite well so far. But the impact of a functional endogenous clock in combination with diverse factors and the resulting fitness advantages were analysed in only a few studies and remains for the most part unknown. Therefore the aim of this thesis was to

unravel the impact of *Drosophila melanogaster*`s endogenous clock on the fitness of the fly. To achieve this goal different factors – like day length, humidity and food composition – were analyzed in wild type CS and three different *period* mutants, namely per^{L}, per^{S} and per^{01}, that carry a point mutation altering or abolishing the free-running period of the fruit fly as well as a second arrhythmic strain, clk^{AR}.

In competition assay experiments wild type and clock mutant flies competed for up to 63 generations under a normal 24 hour rhythm with 12 hours light/day and 12 hours darkness/night (LD12:12) or T-cycles with 19 or 29 hours, according to the mutants free-running period, or constant light (LL) in case of the arrhythmic mutant as well as under natural-like outdoor conditions in two consecutive years. Overall the wild type CS strain was outcompeting the clock mutant strains independent of the environmental conditions. As the per^{L} fly strain elongated their free-running period, the competition experiments were repeated with naturally cantonized new fly strains. With these experiments it could be shown that the genetic background of the fly strains – which are kept for decades in the lab, with backcrosses every few years – is very important and influences the fitness of flies. But also the day length impacts the fitness of the flies, enabling them to persist in higher percentage in a population under competition. Further factors that might influence the survival in a competing population were investigated, like e.g. mating preferences and locomotor activity of homo- and heterozygous females or sperm number of males transferred per mating. But these factors can still not explain the results in total and play no or only minor roles and show the complexity of the whole system with still unknown characteristics.

Furthermore populations of flies were recorded to see if the flies exhibit a common locomotor activity pattern or not and indeed a population activity pattern could be recorded for the first time and social contact as a Zeitgeber could be verified for *Drosophila melanogaster*.

In addition humidity and its impact on the flies´ fitness as well as a potential Zeitgeber was examined in this thesis. The flies experienced different relative humidities for eclosion and wing expansion and humidity cycle phase shifting experiments were performed to address these two different questions of fitness impact and potential Zeitgeber. The fruit fly usually ecloses in the morning hours when the relative humidity is quite high and the general assumption was that they do so to prevent desiccation. The results of this thesis were quite clear and demonstrate that the relative humidity has no great effect on the fitness of the flies according to successful eclosion or wing expansion and that temperature might be the more

important factor. In the humidity cycle phase shifting experiments it could be revealed that relative humidity cannot act as a Zeitgeber for *Drosophila melanogaster*, but it influences and therefore masks the activity of flies by allowing or surpressing activity at specific relative humidity values.

As final experiments the lifespan of wild type and clock mutant flies was investigated under different day length and with different food qualities to unravel the impact of these factors on the fitness and therefore survival of the flies on the long run. As expected the flies with nutrient-poor minimum medium died earlier than on the nutrient-rich maximum medium, but a small effect of day length could also be seen with flies living slightly longer when they experience environmental day length conditions resembling their free-running period. The experiments also showed a fitness advantage of the wild type fly strain against the clock mutant strains for long term, but not short term (about the first 2-3 weeks).

As a conclusion it can be said that genetic variation is important to be able to adapt to changing environmental conditions and to optimize fitness and therefore survival. Having a functional endogenous clock with a free-running period of about 24 hours provides fitness advantages for the fruit fly, at least under competition. The whole system is very complex and many factors – known and unknown ones – play a role in this system by interacting on different levels, e.g. physiology, metabolism and/or behavior.

1. Introduction

1.1 rhythms of nature

The Earth is just one of the planets of our solar system that is rotating around the sun. It is not only rotating around the sun, but it also rotates around its own axis. One rotation of the Earth around its own axis takes about 24 hours – 23 hours and 56 minutes – and is defined as one day. In about 365 days – or 366 days every fourth year in our calendar system to compensate the 4 minutes less than 24 hours each day – the Earth turns one round around the sun. Due to these rotations nearly all organisms experience rhythmic changes on a daily basis as well as seasonal changes of a year. The most prominent changes are on a daily basis in light and temperature conditions – with higher temperatures and light intensities during the day and lower temperatures and light intensities during the night. Therefore it is very important for most of the organisms and might represent a fitness advantage to not only sense and react, but to anticipate these changes. To accomplish this task endogenous clocks – generating a rhythm autonomously – evolved, running with different speeds or periods. Presumably the most important endogenous clock is the circadian one with a rhythm of about 24 hours (Latin: circa = approximately; dies = day). But besides a circadian clock organisms also evolved tidal (about 12,4 hours), semi-/lunar (about 14,5 and 29 days) or annual (about 1 year) rhythms of endogenous clocks (Engelmann, 2009). The endogenous clocks enable the organisms to anticipate the rhythmic changes of their environment as well as to adapt their behavior, metabolism and physiology to secure their survival.

Endogenous clocks can be found not only in mammals or humans (Aschoff, 1965; Wever, 1969), but for example also in insects (Beling, 1929; Hermann, 1962), fungi (Feldman and Hoyle, 1973), algae (Bruce, 1970; Roenneberg and Hastings, 1988), plants (Erwin, 1936) and bacteria (Johnson et al., 1996; Ishiura et al., 1998; Ouyang et al., 1998). Even organisms which live under constant environmental conditions exhibit rhythmic behavior (Espinasa and Jeffery, 2006; Imafuku and Haramura, 2011), pointing out the importance of an endogenous clock.

But the importance of a functional clock can not only be seen by the fact that nearly all organisms exhibit an endogenous clock. Endogenous clocks are involved in various behavioral, metabolic and physiological processes. For example the endogenous clock of the model organism *Drosophila melanogaster* is involved in or driving various behaviors and the underlying metabolic and physiological processes like locomotor activity, eclosion (Konopka and Benzer, 1971), egg-laying (Howlader and Sharma, 2006), mating (Fujii et al., 2007; Fujii

and Amrein, 2010), learning and memory (Lyons and Roman, 2009), olfactory and gustatory sensibility (Krishnan et al., 1999; Chatterjee et al., 2010). Endogenous clocks enable organisms not only to find a spatial ecological niche but also a temporal niche in the daily and seasonal changing environment. For most organisms being active during the day (diurnal), the night (nocturnal) or at twilight (crepuscular) is very important to find mating partners, food or to avoid natural enemies. An example for the fitness advantage of a functional clock – and therefore survival of the animals – for diurnal chipmunks is given by DeCoursey and colleagues (DeCoursey and Krulas, 1998; DeCoursey et al., 2000): The chipmunks with a non-functional endogenous clock due to suprachiasmatic nuclei (SCN) lesions survived significant less than the control groups with a functional clock. The SCN-lesioned chipmunks were not only active during the day like the control groups, but they were active throughout the day and night randomly and were exposed to natural enemies more often, which reduced the survival rate of the SCN-lesioned chipmunks.

The adaptation and synchronization of the endogenous clocks and the environment happens via external factors, so called Zeitgebers, like light and temperature (Pittendrigh et al., 1958; Zimmerman et al., 1968), feeding (Stephan et al., 1979) or social contacts (Levine et al., 2002). The process of synchronization is called entrainment. When external Zeitgebers are absent an endogenous clock persists to oscillate and free-runs with its own period length, but it can be reentrained to the environment by a Zeitgeber via resetting and/or shifting the clock. When we travel for long distances and across time zones we can experience such a re-entrainment ourselves. We call these for ourselves noticeable impacts of such a re-entrainment a jetlag.

The main Zeitgebers are light and temperature (Pittendrigh et al., 1958), which are also the most prominent environmental factors changing on a daily basis, allowing the organisms to anticipate and adapt their behavior for example. The endogenous clocks get an input via external environmental factors/Zeitgebers that can influence the core clock and/or peripheral clocks to generate specific output like for example behavior, metabolic and physiological processes (Fig. 1).

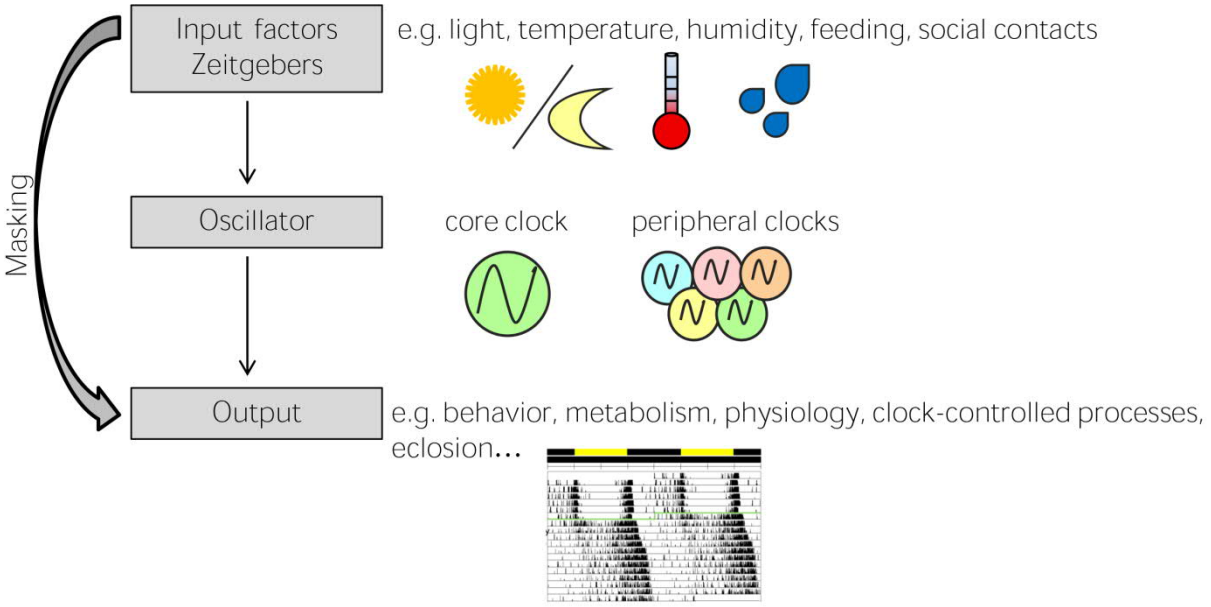

Figure 1: schematic overview of the clock system. Input factors and Zeitgebers, like light, temperature, humidity, feeding and social contacts influence the oscillator, which consists of the core clock as well as several peripheral clocks. The endogenous rhythm of the clock is synchronized to the environment and the entrained core clock regulates different output factors like behavior, metabolism, eclosion or other clock-controlled processes. This pathway via the synchronization of the oscillator/clocks can be circumvented and the input factors can evoke non clock-controlled output responses directly (masking).

But the input factors can also circumvent the clock and directly drive an output, which is called masking. The early Chronobiologists like Aschoff, Bünning and Pittendrigh (Bünning, 1935; Pittendrigh, 1954; Aschoff, 1960, 1979) defined three properties of circadian rhythms to separate clock driven and spontaneous masking output reactions: 1) a self-sustained oscillator has to generate the circadian rhythm, which persists and free-runs with a circadian period of about 24 hours under constant conditions without any Zeitgeber present. 2) The endogenous clock can be (re-)entrained via Zeitgebers. 3) The endogenous clock is temperature compensated and keeps its rhythm and period length over a large range of temperature values.

For example a typical masking effect in *Drosophila melanogaster*s behavior can be seen in the locomotor activity behavior recording. At the moment when the light turns on or off the flies simply react to this Zeitgeber stimuli and a bimodal locomotor behavior pattern can be recorded even in flies without a functional clock. However when the conditions are constant this bimodal activity pattern cannot be seen anymore, because the stimuli – or Zeitgeber light in this case – is not present anymore. Therefore the locomotor activity pattern in this case is a masking effect that was driven directly by light and not the endogenous clock with its master clock in the brain and the several peripheral clocks in different tissues of the fly`s body.

1.2 The circadian clock of Drosophila melanogaster

Drosophila melanogaster – with its high reproduction rate, short generation time, only four chromosomes, small size, relative neuronal simplicity and many genetic possibilities – turned out to be a perfect model organism to study the properties of the circadian clock. In the 1970s Seymour Benzer and Ron Konopka were the first ones to identify three different clock mutant fly strains. They used chemical mutagenesis and screened the flies for different behavior concerning the circadian clock. What they found were three different *Drosophila* clock mutants with altered period length or no rhythmic behavior in constant darkness (per^{01}). The ones with an altered rhythm exhibited a prolonged – about 29 hours, per^{L} – or shortened – about 19 hours, per^{S} – period length compared to the wild type. In fact all three mutant phenotypes were traced back to the same gene which was named *period* (*per*) (Konopka and Benzer, 1971). After this first clock gene *period* was discovered, several other core clock components were identified and functions unraveled (reviewed in Helfrich-Förster, 2014).

1.2.1 neuronal network of Drosophila`s clock

The central nervous system (CNS) of *Drosophila melanogaster* consists of about 100.000 neurons (Peng et al., 2011). About 150 of them – 75 per hemisphere – are oscillating cells (PERIOD and TIMELESS positive), the so-called pacemaker or clock neurons (Taghert and Shafer, 2006; Helfrich-Förster et al., 2007b). Spread throughout both hemispheres the clock neurons form a complex interacting neuronal network (Fig. 2). Within this network the clock neurons can be divided into several subgroups depending on size and location of the clock neurons in the brain, namely the dorsal (DNs) and lateral (LNs) neurons. The dorsal neurons are classified into three subgroups, the DN_1 (further subdivided into anterior (DN_{1a}) and posterior (DN_{1p}) located cell clusters), the DN_2 and the DN_3.

For the lateral neurons the cell clusters are classified as lateral posterior neurons (LPNs), six dorsolateral neurons (LN_ds), four Pigment Dispersing Factor positive (PDF^+) large ventrolateral neurons (l-LN_vs) and four PDF^+ with one PDF^- small ventrolateral neurons (s-LN_vs, 5^{th} s-LN_v) (Helfrich-Förster et al., 2007a; Helfrich-Förster et al., 2007b) (Fig. 2).

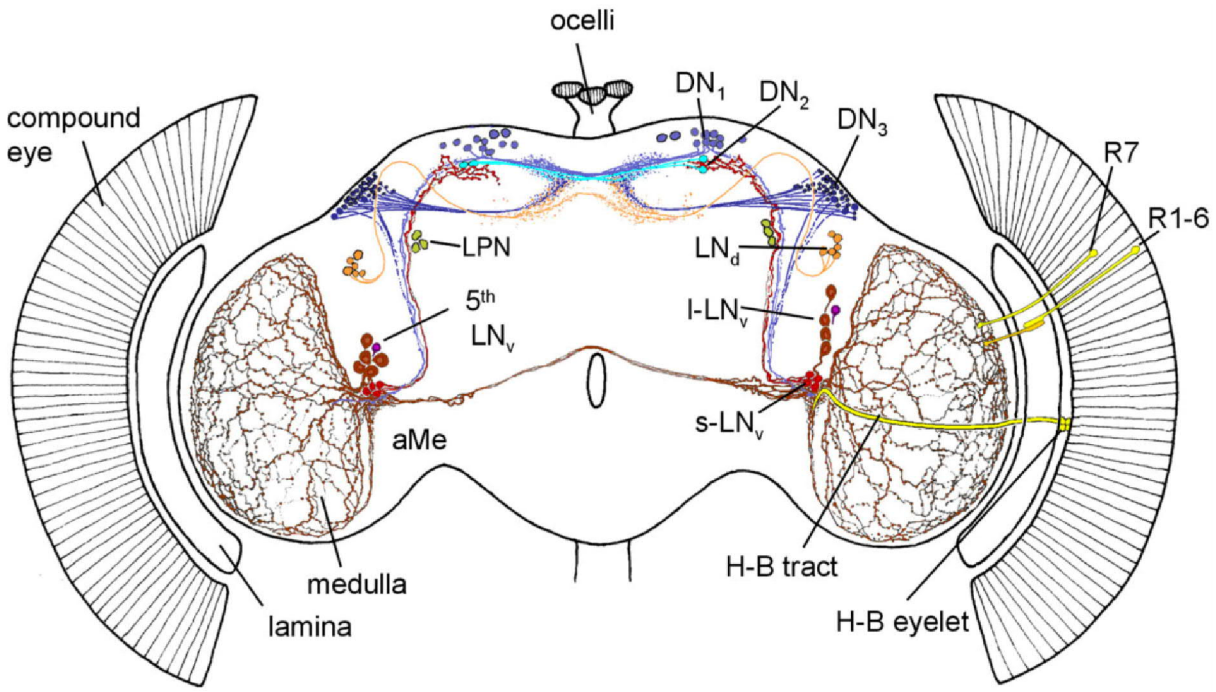

Figure 2: schematic overview of the neuronal clock network of an adult *Drosophila melanogaster* brain. The clock neurons are divided into two main groups and named due to their location in the central nervous system: the dorsal (DNs) and lateral (LNs) neurons. These two groups are subdivided into the DN_1, DN_2 and DN_3 as well as the LN_ds, LPNs and the s- and l-LN_vs with the 5^{th} LN_v. The projections of the cell clusters are also depicted in the color according to the cell cluster. Some of the light input pathways via the ocelli, the compound eyes and the H-B eyelet are implied as well (aMe = accessory medulla; more information in the text) (from Helfrich-Förster et al., 2007a; Schubert et al., 2018).

Not only the morphological clock network is well characterized so far, but also many functions of the clock neurons have been unraveled since the 1970s.

One of the functions of the clock neurons is to regulate the output behavior of the fly, the locomotor activity behavior for example. *Drosophila melanogaster* wild type flies show a typical bimodal locomotor activity profile under entrained light-dark conditions (LD) as well as in constant darkness (DD) with high locomotor activity in the morning and in the evening, separated by a time frame characterized by low locomotor activity during the midday, also called siesta, of the flies (Aschoff, 1966). Due to their observations in rodents Pittendrigh and Daan (1976) proposed a dual oscillator model, suggesting that two different oscillators are present in the CNS of rodents, driving the morning and evening activity of the animals. This model is also valid for *Drosophila* and therefore the clock cells that are involved in driving the morning activity are also called Morning cells (M cells) and the cells that drive the evening activity as Evening cells (E cells), respectively. The s-LN_vs seem to be the main pacemaker cells in DD and they belong to the M cells with some of the DN_1 cells that set the phase of the morning activity peak of the flies in LD (Helfrich-Förster, 1998; Renn et al., 1999; Peng et al.,

2003; Stoleru et al., 2004). Three of the LN_d (Cryptochrome positive, CRY^+) as well as some DN_1 and the 5^{th} LN_v represent the E cells of *Drosophila melanogaster*. The E cells are not only supposed to control the evening activity peak in LD but also seem to represent the main clock cells in constant light (LL) and these cells are thought to be involved in temperature entrainment as well (Grima et al., 2004; Stoleru et al., 2004; Rieger et al., 2006; Picot et al., 2007; Yoshii et al., 2012). But recent studies suggest that this regulation of morning and evening activity is a quiet complex and flexible system which also depends on environmental conditions (Rieger et al., 2009; Zhang et al., 2010).

The l-LN_vs project into the ventral elongation of the ipsilateral accessory Medulla (aMe) as well as innervate the outer layer of the ipsilateral and contralateral medulla, connecting the two brain hemispheres of the fly (Helfrich-Förster et al., 2007a). These PDF^+ cells express the blue-light sensitive protein CRYPTOCHROME (CRY) as well as most other clock cells in the brain of *Drosophila* (Emery et al., 1998; Yoshii et al., 2008), detect light and modulate behavioral arousal and sleep (Shang et al., 2008; Sheeba et al., 2008).

The DN_2 neurons seem to be involved in the temperature entrainment (Miyasako et al., 2007; Picot et al., 2009), but the function of the DN_3 neurons is mainly unknown so far.

Taken together the neuronal network with all subgroups of clock neurons and their projections throughout the brain of *Drosophila* is a quiet complex and flexible network that Chronobiologists try to understand and to unravel for the last about forty years. Due to their success we know much about the morphology and function of the circadian clock of *Drosophila* and other insects, but some secrets are still there waiting to be unraveled.

1.2.2 molecular clock mechanism

Drosophila melanogaster`s core molecular clock mechanism consists of two negative interlocked feedback loops – on the transcriptional and translational level – which exist in every single clock neuron (Hardin et al., 1990; Glossop, 1999; Hardin, 2005). Since Konopa and Benzer discovered the gene *period* the main players of these feedback loops, the clock proteins PER (Konopka and Benzer, 1971) and TIM (Sehgal et al., 1994) as well as Clock (CLK)(Allada et al., 1998) and Cycle (CYC) (Rutila et al., 1998), and further components like Doubletime (DBT) (Price et al., 1998), Par domain protein 1 (PDP1) (Cyran et al., 2003) and Vrille (VRI) (Blau and Young, 1999) could be identified over the last 40 years.

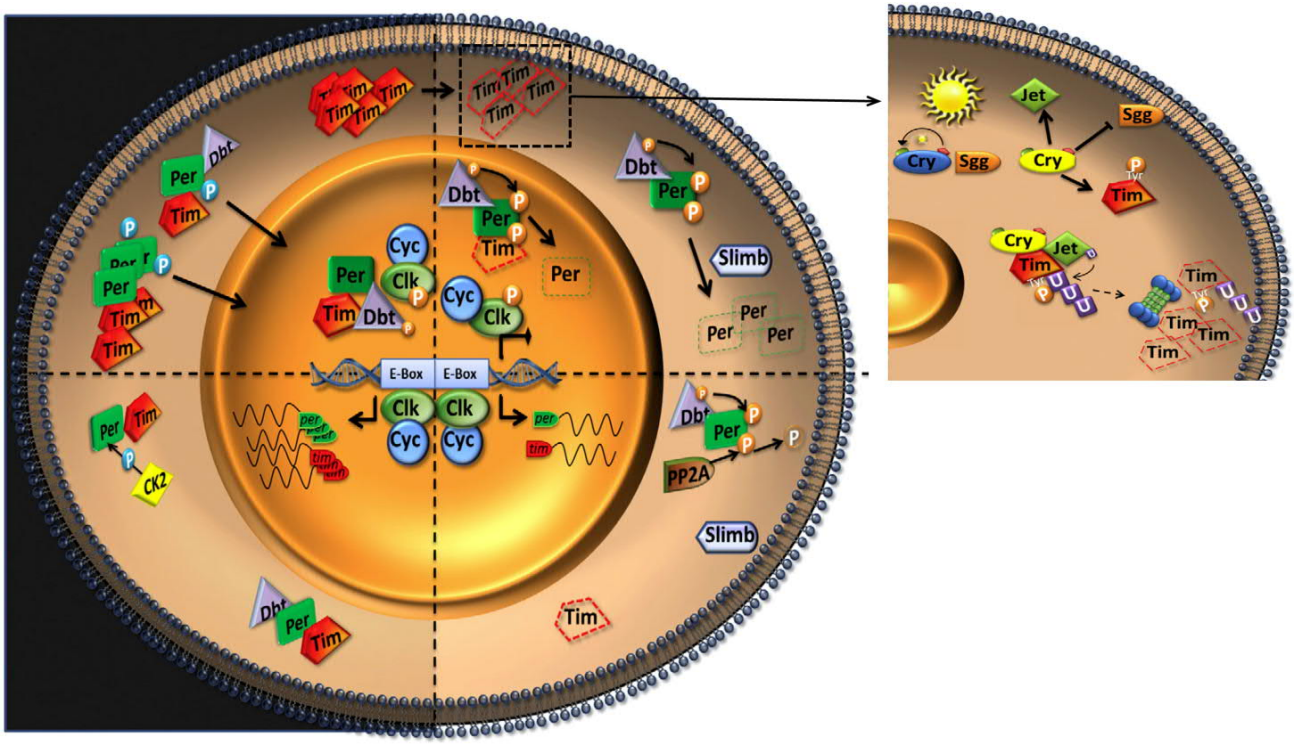

Figure 3: Molecular mechanism of *Drosophila melanogaster*`s circadian clock. The figure depicts one of the two feedback loops of a clock neuron in the course of a day with each quarter representing about 6 hours of the day. Black background represents the night/dark phase, white background the day/light phase. The main clock components are shown as mRNA (per and tim) and proteins expressed (full colored) or degraded (pointed lines without color filling) during a 24 hour cycle. Per = Period, Tim = Timeless, Clk = Clock, Cyc = Cycle, Dbt = Doubletime, P = Phosphate/phosphorylation, Cry = Cryptochrome, Jet = Jetlag, Sgg = Shaggy, U = Ubiquitin. The process of Tim degradation in the light phase mediated via Cry (inactive state in blue, active state in yellow) is depicted on the right upper corner. For details see text (from Peschel and Helfrich-Forster, 2011 with small adaption).

Within these interlocked feedback loops clock genes are transcribed rhythmically within every single clock neuron and the translated proteins influence their own transcription (Hardin, 2011). In one of the two feedback loops the key players are CLK, CYC, PER and TIM (Fig. 3). The transcription factors CLK and CYC form a heterodimer and act as transcriptional activator that recognizes a certain DNA motif – namely the E-box – in the promoter region of several clock controlled genes (*ccg*)(Allada et al., 1998; Darlington, 1998; Rutila et al., 1998). Due to the binding of the heterodimer CLK/CYC the transcription of the clock genes *per* and *tim* is activated (initiated around mid-day) and the mRNA is translocated to the cytoplasm where it accumulates during the evening. In the cytoplasm the *per* and *tim* mRNA is translated to the according PER and TIM proteins. In the night/darkness PER and TIM accumulate in the cytoplasm, form heterodimers and enter the nucleus. In the nucleus the protein interaction between PER and CLK inhibits the further binding of the CLK/CYC heterodimer to the E-boxes of the clock genes and thereby inhibit their own transcription (Lee et al., 1999). This oscillation

is synchronized to the surrounding environment, mainly the surrounding light condition, via the activity of the blue-light sensitive protein CRY that is expressed in most clock neurons (Emery et al., 1998; Yoshii et al., 2008). Light activates CRY which leads to the ubiquitination and degradation of TIM (Lin et al., 2001). Degradation of TIM leads to less PER/TIM heterodimers and the destabilization of PER, resulting also in PER degradation and the reduction of PER interaction with CLK. This increases CLK/CYC heterodimer binding to the E-boxes of the clock genes and therefore to the transcription of *per* and *tim* again. Without light, at night, CRY is inactive and PER and TIM proteins can accumulate again to keep the feedback loop running until light restarts the cycle (Fig. 3). This complex system of phosphorylation, dephosphorylation and translocation processes – between nucleus and cytoplasm – results in the cycling composition and degradation of the mentioned clock components and accounts for the 6-8 hours difference in maximum mRNA levels and PER and TIM protein cycling (Hardin et al., 1990; Zerr et al., 1990; Hardin et al., 1992). (Fig. 4)

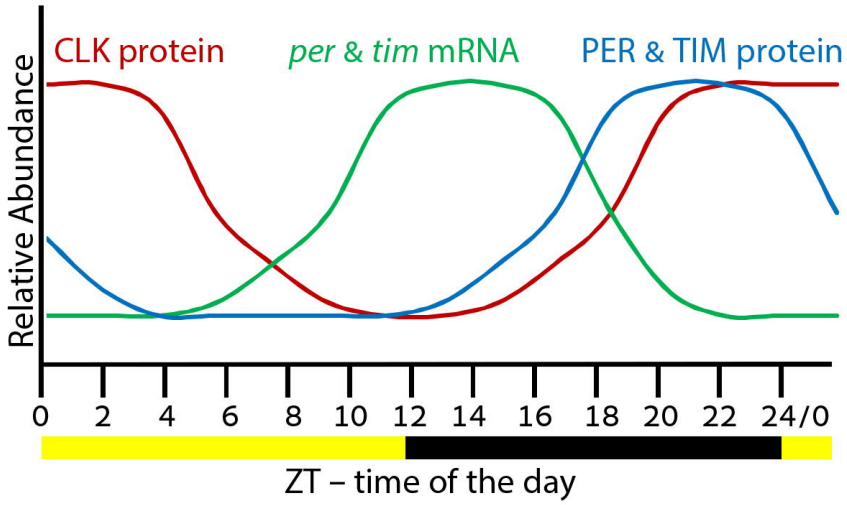

Figure 4: oscillations of clock proteins CLK, PER and TIM as well as *per* and *tim* mRNA. The core clock proteins and mRNA are cycling throughout the day. *per* and *tim* mRNA peak in the first half of the night, whereas the proteins PER and TIM reach the maximum levels about 6-8 hours later in the second half of the night, beginning of the day. With incoming light TIM is degradated and the PER and TIM levels decrease. CLK protein is regulated in the second feedback loop and is cycling in antiphase to *per* and *tim* mRNA (adapted from Peschel, 2008 ; reviewed e.g. in Helfrich-Förster, 2002; Peschel and Helfrich-Forster, 2011).

In the second feedback loop CLK is regulated and the heterodimer CLK/CYC is also one of the key players and therefore the connection of the two feedback loops. The heterodimer CLK/CYC also activates the transcription of the clock genes *vri* and *Pdp1*. Both proteins, VRI and PDP1 respectively, like PER and TIM, regulate their own transcription. The two proteins regulate the

transcription of the *clk* gene in the way that PDP1 activates and VRI represses the *clk* transcription (Blau and Young, 1999; Cyran et al., 2003).

But these few proteins are of course not the only ones regulating the feedback loops resulting in a self-sustaining oscillation. Many other factors are part of the system and form a complex network of interaction of phosphorylation, dephosphorylation and translocation processes (Price et al., 1998; Kloss et al., 2001; Lin et al., 2002; Sathyanarayanan et al., 2004; Peschel and Helfrich-Forster, 2011).

1.2.3 *period* – one of the main clock genes and its protein

As mentioned before (see 1.2) the *period* gene was the first of the clock genes to be discovered in the 1970s (Konopka and Benzer, 1971). Three point mutations were found in *Drosophila melanogaster* that changed the fly`s behavior in its free-running period. The gene – called *period* – and the mutant fly strains were named after the output of the specific point mutation under constant conditions, namely per^{Short} (per^{S}) – flies with a shortened free-running period of about 19 hours – , per^{Long} (per^{L}) – flies with a prolonged free-running period of about 29 hours – and per^{0} – being arrhythmic. The *period* gene is located on the X chromosome of *Drosophila melanogaster*, implicating that male flies possess only one copy and therefore show their genotype directly in the phenotypic behavior: in the locomotor activity (Fig. 5).

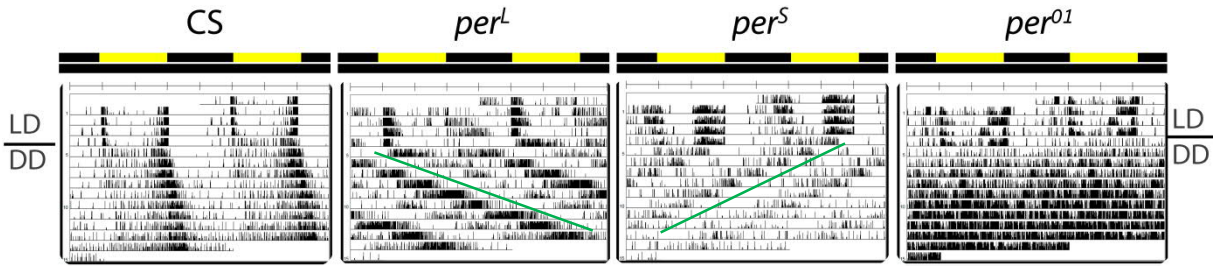

Figure 5: locomotor activity behavior of male wild type CS and the three *period* mutants, per^{L}, per^{S} and per^{01}. Example actograms (double plot) of a male single wild type CS or *period* mutant fly respectively recorded for 4 days in LD 12:12 (LD) followed by 10 days of constant darkness (DD). Black and yellow bars on top represent the light conditions. Small black lines of the graphs represent the activity of the flies. All flies showed the typical locomotor activity behavior under LD cycles with morning and evening activity. In DD wild type CS flies showed a continuous rhythm of about 24 hours. per^{L} and per^{S} flies with the prolonged or shortened free-running period start to be active a little bit later or earlier, respectively, every day resulting in the shifted black lines underlined by the green line. Per^{01} flies exhibit their arrhythmic behavior without any Zeitgeber being present.

Female flies on the other hand can be homozygous, with two identical copies of the gene, or heterozygous, with two different copies of the gene. Since *period* was discovered in 1971, many more details of the gene and its protein (PER) have been investigated.

In the *per*^{01} mutation a cytosine was replaced by a thymine, which changed the glutamine codon (CAG) at amino acid (aa) position 464 to a stop codon (TAG) resulting in a shortened and unfunctional PER protein of only 463 aa (Baylies et al., 1987; Yu et al., 1987) (Fig. 6). The wild type PER consists of a total of 1224 aa (Chang and Reppert, 2003) (Fig. 8). Due to this nonsense mutation and the truncated PER the interlocked feedback loops are impaired and account for the arrhythmic behavior of the *per*^{01} flies.

In the *per*^{S} mutation a guanine was exchanged by an adenine thus altering the serine aa at position 589 to asparagines (Baylies et al., 1987; Yu et al., 1987) (Fig. 6). This missense mutation leads to a too fast running clock in such a way that the *per*^{S} flies exhibit a shortened free-running period of only about 19 hours (Fig. 5).

In case of the *per*^{L} mutation a thymine was replaced by an adenine leading to the substitution of a valine with an aspartic acid (Baylies et al., 1987) (Fig. 6). This missense mutation causes a too slow running clock for which reason the *per*^{L} flies reveal a prolonged free running period of about 29 hours (Fig. 5).

										Asp								
per^{L}	Arg	Val	Lys	Glu	Asp	Ser	Phe	Cys	Cys	Val	Ile	Ser	Met	His	Asp	Gly	Ile	Val
	CGG	GTG	AAG	GAG	GAC	AGC	TTC	TGC	TGC	GTC	ATC	TCC	ATG	CAC	GAC	GGC	ATC	GTC
										A								
														AM				
per^{0}	Gly	Ala	Ser	Phe	Cys	Ser	Lys	Pro	Tyr	Arg	Phe	Leu	Ile	Gln	Asn	Gly	Cys	Tyr
	GGC	GCC	TCC	TTC	TGC	AGC	AAG	CCA	TAC	CGC	TTC	CTC	ATC	CAG	AAC	GGT	TGC	TAC
														T				
												Asn						
per^{S}	His	Glu	Asn	Glu	Leu	Thr	Val	Ser	Glu	Arg	Asp	Ser	Val	Met	Leu	Gly	Glu	Ile
	CAC	GAG	AAC	GAG	TTG	ACC	GTC	TCG	GAG	CGG	GAC	AGC	GTG	ATG	CTC	GGC	GAG	ATT
												A						

Figure 6: location of all three period point mutations and amino acids replacement. T to A substitution in *per*^{L}, C to T in *per*^{01} and G to A in *per*^{S} flies resulting in exchanged amino acids or a stop codon in case of *per*^{01}. For more details see text (from Baylies et al., 1987).

Besides the altered free-running period or arrhythmicity of the flies, the flies exhibit several further changes due to the point mutations. On the behavior level there are for example also changes in the mating behavior. The “love song” of *Drosophila melanogaster* male flies – produced by vibrations of their wings – consists of two parts, the pulse song and the sine song. The interval of the single pulses of the pulse song are shortened in the *per*^{S} flies, compared to the wild type flies, by about 25 %, prolonged in the *per*^{L} flies by about 50 % and in the arrhythmic *per*^{01} flies there is no rhythm detectable (Kyriacou and Hall, 1980, 1986).

On the transcriptional and translation level there are also alterations. The mRNA and protein synthesis is clock controlled and the maximum amount of *per* and *tim* mRNA is reached in the late evening and first half of the night under normal LD12:12 conditions. PER and TIM protein maximum levels are achieved about 6 to 8 hours later in the second half of the night before the proteins decline again in the morning due to light mediated degradation (Fig. 4). Under shortday and longday conditions – LDR 08:16 and LDR 16:08 respectively – the PER cycling in *Drosophila melanogaster* heads is altered (Bunz, 2013). It appears that PER accumulation seems to happen earlier in per^S flies compared to wild type CS flies under shortday conditions. Under longday conditions the PER accumulation seems to be later in per^L flies than in wild type CS flies. The duration of maximum PER levels appear to be dependent on the duration of the light phase with short PER peaking when the flies experienced a short light phase and prolonged PER maximum levels when the flies experienced a longer light phase (Bunz, 2013) (Fig. 7).

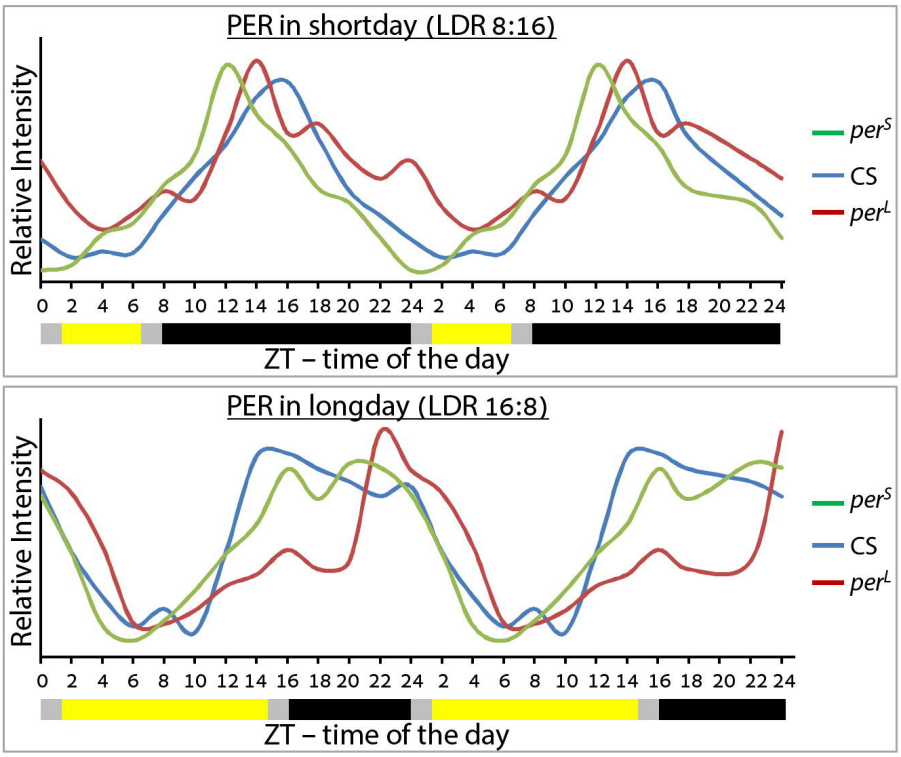

Figure 7: relative intensity of PER protein cycling levels under shortday and longday conditions, LDR 8:16 and LDR 16:8 respectively, of wild type CS and clock mutant per^S and per^L flies. Doubleplot of a 24 hour day. PER levels of wild type CS flies are depicted in blue, of per^S flies in green and of per^L flies in red. Under short days, PER levels seem to peak earlier in per^S than in wild type CS flies and under longday conditions PER accumulation and reaching the maximum level seem to be later in per^L flies than wild type CS flies. Black, yellow and gray bar underneath show the light conditions with light-phase in yellow, twilight in gray and darkness in black. For more details see text (adapted from Bunz, 2013).

Noticeable the PER accumulation happens already in the light phase in wild type CS and per^S flies under long day conditions (Fig. 7). This is different from the hypothesis that PER is

stabilized by TIM and degraded in the absence of TIM. But diverse studies could show – in vitro and in vivo – that PER can be transported to the nucleus and act as repressor without TIM (Rothenfluh et al., 2000; Shafer et al., 2002; Chang and Reppert, 2003; Cyran et al., 2005; Meyer et al., 2006). The stabilization of PER could be accomplished by PER:PER homodimerization (Landskron et al., 2009).

On the structural level there are modifications in the protein structure due to the *period* mutations in different domains (Fig. 8). The per^{L} mutation is located in the PAS domain of the protein, which is involved in the dimerization of PER and TIM. The efficiency of dimerization is reduced due to the per^{L} point mutation (Huang et al., 1993) which would also fit to the later accumulation of PER in Figure 7. Furthermore transport of cytoplasmic PER to the nucleus happens to be later in per^{L} than in the wild type CS flies (Curtin et al., 1995; Meyer et al., 2006) and seems to be dependent on the temperature (Curtin et al., 1995). In per^{S} flies on the other hand PER is phosphorylated earlier than in the wild type CS flies (Edery et al., 1994b). As the per^{01} mutation leads to a shortened protein, the protein structure is altered extremely with domains completely missing resulting in a unfunctional protein of only 463 aa instead of 1224 aa (Baylies et al., 1987; Yu et al., 1987).

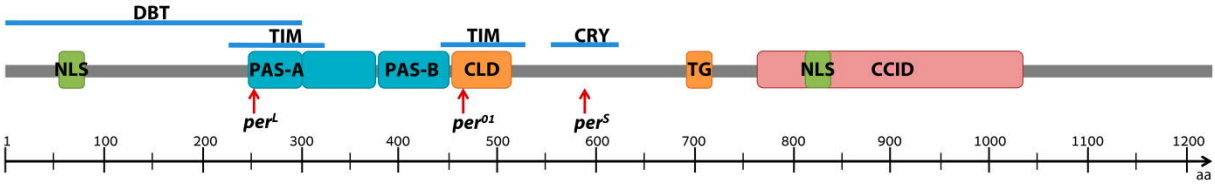

Figure 8: the PER protein with its domains, indicated binding partners and location of *period* mutations. Total length of the protein: 1224 aa. The areas of binding partner interactions is depicted as blue lines. Location of *period* mutations is indicated by red arrows. Ruler below the protein shows location of the domains in the protein amino acid sequence. DBT = Double-Time, TIM = Timeless, CRY = Cryptochrome, PAS-A and PAS-B = PAS domain, CLD = Cytoplamatic localization domain, TG = Threonin-Glycin repetition, NLS = nuclear localization signal, CCID = CLK:CYC inhibition domain; (adapted from Chang and Reppert, 2003).

To sum this up, there are many changes on divers levels due to the different *per* mutations and the examples given are not a complete list, but it demonstrates the complexity of the system and the impact of one key player of this system on the molecular, structural and behavioral level in the fruit fly *Drosophila melanogaster*.

1.3 input and output of *Drosophila melanogaster`s* circadian clock

Drosophila melanogaster`s circadian clock gets input from divers input factors like light, temperature, humidity, social contacts, food availability and others (Fig. 1). Some act as Zeitgebers that can actually (re-)entrain or shift the circadian clock and the resulting output of the circadian clock. Some act more as stimuli circumventing the clock to drive direct output, which is then also called masking effect or behavior due to a pure reaction to a stimuli with no or only very low impact on the circadian system. And to make the system more complex and flexible not every stimuli or input factor is equally strong integrated in general and at every timepoint of the day, enabling the fly to a broad range of variation and adaption by fine-tuning the system. All factors are perceived and integrated into the complex circadian system with the main clock in the brain of *Drosophila* as well as numerous peripheral clocks in other tissues, resulting in divers clock controlled output. This circadian clock controlled output can be seen on all levels from single mRNA or protein cycling in a single cell, to cascades of clock controlled processes in metabolism, physiological process or complex behavior like eclosion or the locomotor activity behavior. Presumably, to be able to integrate all input factors – dependent on the Zeitgeber type, intensity, duration and status or sensitivity of the clock – enables the fly to synchronize and properly adjust the circadian clock and all downstream processes perfectly to the environment to maximize fitness and survival in nature.

1.3.1 light

As mentioned above (see 1.1 and Fig. 1) light and temperature are input factors and Zeitgebers and they represent the two main Zeitgebers for *Drosophila melanogaster`s* clock for the entrainment of the circadian clock of the fly to the environment. Of these two Zeitgebers light is the stronger one.

Input of light to *Drosophila melanogaster`s* circadian clock is mainly mediated via the blue light photoreceptor cryptochrome (CRY) (Emery et al., 1998; Stanewsky et al., 1998; Helfrich-Förster et al., 2001) which is expressed in some $DN_1$ neurons, three $LN_d$s and in all $LN_v$s in *Drosophila`s* brain (see also 1.2.1) as well as in the cytoplasm of photoreceptor cells of the compound eyes (Yoshii et al., 2008). But the input of light is not exclusively mediated via CRY and even flies without CRY can synchronize to the environment (Kistenpfennig et al., 2012; Yoshii et al., 2015). *Drosophila melanogaster`s* visual system consists of the photoreceptive organs, namely the compound eyes, ocelli and the Hofbauer-Buchner eyelet (H-B eyelet) (Hofbauer and Buchner, 1989). The compound eyes – consisting of about 800 ommatidia – provide mainly

information about color, shape and motion vision (Menne and Spatz, 1977; Yamaguchi et al., 2010), the ocelli – three simple eyes on the head of adult *Drosophila melanogaster* – are involved in detection of small changes in light intensity (Goodman, 1970; Hu et al., 1978) and the H-B eyelet – made up of 4 photo receptor cells between the retina and the lamina of the compound eyes – projects to the aMe and takes part in circadian entrainment (Bünning, 1936; Hofbauer and Buchner, 1989; Helfrich-Förster, 2002; Helfrich-Förster et al., 2002b; Rieger et al., 2003).

Therefore the visual system contributes to light information mediation to the circadian clock of *Drosophila*. This enables the fly to recognize light to time and synchronize the circadian phase of the clock to the surrounding environment accurately (Hofbauer and Buchner, 1989; Helfrich-Förster, 2002; Helfrich-Förster et al., 2002b). Of the three photoreceptive organs – the compound eye, ocelli and H-B eyelet – the compound eyes are mainly responsible for adaption of the phase of activity and mediation of masking effects of light (Rieger et al., 2003). A very typical masking effect of light can be seen in locomotor activity recordings, when the light is turned on or off the flies just react to that stimuli and do not anticipate these changes. Therefore the behavior is driven by light directly and not by the circadian clock.

How important light is as a Zeitgeber to entrain the fly`s clock to surrounding environmental conditions can also be seen in phase shifting experiments. Depending on the time of the day, strength and intensity of a stimuli and status of the negative feedback loops in the clock neurons short light pulses are enough to reset the clock and/or to shift its phase by advancing or delaying the whole cycle. How the molecular clock with its negative feedback loop is organized and synchronized to the environment by light via CRY is already described in 1.2.2. To investigate the importance of light and how light changes the phase of *Drosophila melanogaster`s* circadian clock classical phase response curve experiments were performed, meaning that flies were kept in DD under free-running conditions and short light pulses were given to the flies at different timepoints of the subjective day or night of the fly. Analyzing the changes in the free-running period of the flies after those pulses – advancing, delaying or not shifting free-running locomotor activity behavior – gave insights to the status and sensitivity of the clock revealing the phase response curve, showing the non-parametric impact of light on the circadian clock as postulated by Pittendrigh (e.g. reviewed in Pittendrigh and Minis, 1964; Golombek and Rosenstein, 2010). For example an adequate light pulse given in the early subjective night (CT12-15) result in a delay of the clock, whereas an adequate light pulse given

in the late subjective night (CT19-23) results in an advance of the clock (e.g Suri et al., 1998; Kistenpfennig et al., 2012) . To summarize, light and its properties like intensity or duration is the most important Zeitgeber for the entrainment of *Drosophila melanogaster`s* circadian clock (e.g. reviewed in Schlichting and Helfrich-Forster, 2015).

1.3.2 temperature

Apart from light temperature is another important input factor and Zeitgeber for *Drosophila melanogaster`s* clock to be entrained to the environment. *Drosophila melanogaster`s* clock is very sensitive and recognizes changes of 1-3 °C which is already enough to entrain the clock and change locomotor activity behavior and eclosion of the flies (Zimmerman et al., 1968; Wheeler et al., 1993). Temperature changes can be sensed by *Drosophila melanogaster* in two ways. The first one is the perception of temperature via certain clock neurons like the LPNs or DN_2 (Yoshii et al., 2005; Busza et al., 2007; Miyasako et al., 2007; Picot et al., 2009). The second possible way for temperature sensation are the aristae and/or the chordotonal organs (Sayeed and Benzer, 1996; Gallio et al., 2011).

The clock can be entrained by light and temperature cycles (Yoshii et al., 2009a) but also in the absence of light in constant darkness and even under constant light conditions – which otherwise leads to arrhythmic behavior of the flies – temperature cycles are enough to entrain *Drosophila melanogaster`s* circadian clock (Yoshii et al., 2002; Glaser and Stanewsky, 2005; Yoshii et al., 2005). Temperature entrainment of *Drosophila`s* clock is clock-dependent with *period* as one of the main players. *Period* is not only expressed in the clock neurons, *per* and *tim* can be found in many tissues and organs (reviewed in Hall, 1995) and the oscillations can also be entrained by temperature cycles, representing a cell-autonomous mechanism of temperature entrainment (Glaser and Stanewsky, 2005). *Per* and *tim* are spliced temperature-dependent and therefore facilitate regulation of PER and TIM protein already on the transcriptional level due to changes in temperature (Boothroyd et al., 2007). Majercak and colleagues could show that *per* splicing is increased at low temperatures leading to an earlier accumulation of *per* mRNA and PER, resulting in an advance in molecular clock cycling (Majercak et al., 1999). At high temperatures flies exhibit a prolonged siesta – presumably to avoid the midday heat – due to the expression of alternative splicing variants (Low et al., 2008). *Per* splicing is also affected in *norpA* – encodes for phospholipase C – mutant flies, which are unable to entrain to temperature cycles as well as *nocte* mutant flies that show highly deformed chordotonal organs (Glaser and Stanewsky, 2005). Like light, temperature can shift

the phase of the clock when short temperature pulses are perceived in the early night, resulting in phase delays of the clock due to the downregulation of PER and TIM (Sidote et al., 1998).

The clock might be temperature compensated, but temperature can also induce specific behavior directly and therefore mask the actual clock output.

1.3.3 other factors

Besides light and temperature there are many other factors that *Drosophila* experiences and that can influence the circadian clock, but these factors were not studied as intense as light and temperature. Therefore there is less information available and known about the function and interactions of these factors in matters of the circadian system.

Social contacts or social experiences as input factor could be shown to be a Zeitgeber as well (Levine et al., 2002). Levine and colleagues also hypothesize that this Zeitgeber acts likely via chemosensory pathways, but they could not rule out the possibility of auditory or tactile involvement.

Another input factor that *Drosophila melanogaster* experiences on a regular and daily changing basis is humidity, which resembles the amount of water vapor that is present in the air. The relative humidity is coupled and influenced by the factors temperature and air pressure. It is expressed as a percentage, which tells the ratio of the partial pressure of water vapor in the air/water mixture to the vapor pressure of water. Therefore the amount of water vapor needed to obtain saturation decreases as the temperature decreases and vice versa, meaning an absolute amount of water vapor at 30 °C can resemble a quite low relative humidity of only about 20 %, whereas the same absolute amount might be 100% at 5 °C. For *Drosophila* it is assumed that the flies eclose in the early morning – due to a quite high relative humidity – to unfold their wings and to avoid desiccation (Pittendrigh, 1954).

Another factor that plays a role in influencing or interacting with the circadian clock system of *Drosophila* is the availability of food. As sleep is modulated by the circadian clock of *Drosophila melangaster*, Linford and colleagues found a connection between food availability and sleeping behavior in the means of altered arousal thresholds for sleep induced by different dietary cues (Linford et al., 2012). Furthermore starving flies respond with a high level of activity for the search of food and reduce their sleeping amount. Under such circumstances the circadian system comes to action and prevents the fly of too much sleep loss (Keene et al., 2010).

1.3.4 the dual oscillator model

The circadian clock of *Drosophila melanogaster* and its self-sustaining rhythmicity and regulation is a quite complex as well as flexible system which allows variation and adaption to a permanently changing environment. In the locomotor activity behavior *Drosophila* – as a crepuscular insect – exhibits a robust activity profile with two activity peaks, one in the morning and one in the evening separated by a phase of relative inactivity, the so-called midday siesta. In 1976 Pittendrigh and Daan proposed a dual oscillator model, suggesting that two distinct oscillators are active in nocturnal rodents regulating morning (M) and evening (E) activity (Pittendrigh and Daan, 1976). In *Drosophila melanogaster* with its bimodal activity pattern this dual oscillator model could be linked anatomically to different clusters of clock neurons. M activity is mainly driven by four PDF^+ s-LN_vs, which speed up the pace of the clock light-induced, advancing the phase of activity, whereas the E activity is controlled by three CRY^+ LN_d and the 5^{th}-LN_v , which slow down the pace of the clock light-induced, delaying the phase of activity to track dusk (Grima et al., 2004; Stoleru et al., 2004; Rieger et al., 2006; Picot et al., 2007; Yoshii et al., 2012). But as mentioned the circadian clock is a very complex system and it is flexible to be able to adapt to environmental changes. Therefore further studies show that this regulation of the dual oscillator model is rather flexible and depends on the environmental conditions (e.g. Rieger et al., 2009; Zhang et al., 2010). How flexible the system is could be shown in various studies. For example under long photoperiods – illustrating long summer days – *Drosophila melanogaster* tracks dusk and dawn by moving the two activity bouts further apart, which is mediated by acceleration of the clock in M cells and a slowdown of the clock in the E cells (Rieger et al., 2007; Yoshii et al., 2009b). Accordingly in short photoperiods the M and E activity peaks come closer together. This flexible connection of M and E activity to changing photoperiods or rather this change in phase angle between M and E activity peak is not unlimited. Under extreme long or short photoperiods dusk and dawn cannot be followed by the activity peaks anymore, suggesting that the M and E activity peaks are coupled somehow and are not running independently of each other (Rieger et al., 2003; Rieger et al., 2012).

1.4 fitness aspect and advantages of a functional clock

In general it is thought that the main task of endogenous clocks is to time the right behavior – like feeding, mating, egg laying, sleeping/resting etc. – or processes – like metabolic functions

– to the right time of the day, month or year (for insects see for example Sakai and Ishida, 2001; Howlader and Sharma, 2006; Xu et al., 2008; Chatterjee et al., 2010) to benefit of resulting fitness advantages compared to others of the same species without a functional clock. But only few studies investigated such fitness advantages due to a functional clock.

As mentioned above DeCoursey and colleagues investigated a possible fitness advantage of a functional clock in diurnal chipmunks. They compared the rate of survival of chipmunks with a disrupted and non-functional endogenous clock – due to SCN lesions – with a control group of chipmunks with a still intact and functional endogenous clock. They could show that the SCN-lesioned chipmunks survived significant less than the chipmunks with a functional clock (DeCoursey and Krulas, 1998; DeCoursey et al., 2000). They concluded that the SCN-lesioned chipmunks could not survive as long as the control group, because the animals were active throughout the day and night randomly which led to the exposure to natural enemies more often. Therefore the chipmunks with a functional clock benefitted from their functional clock. Another study – performed with SCN-lesioned squirrels in the lab – could show altered rhythms in body mass and hibernation duration (Ruby et al., 1997). A disrupted, shortened or prolonged hibernation or even hibernation at the wrong season of the year might be also crucial to survive in nature and therefore a functional clock resembles a great fitness advantage.

For plants (*Arabidopsis thaliana*) it could be demonstrated as well that a matching circadian period to the environment results in many fitness advantages (Dodd et al., 2005). Dodd and colleagues found that plants that endogenous rhythm fitted to the environmental cycles, could fix more carbon, grow faster, contained more chlorophyll and survived better than plants with a mismatch of their circadian period and the environmental cycles.

Another and very persuasive study was carried out with Cyanobateria by Ouyang and colleagues (Ouyang et al., 1998). They have grown Cyanobacteria strains for about 50 generations. The wild type strain and one of the clock mutant strains – possessing faster or slower or no clock at all – were grown in direct competition. Kept in a 24 hour day the wild type outcompeted the mutant strains. But interestingly when the competition assay was performed under time periods matching the ones of the mutant strain, the mutant strain could win over the wild type strain. This competition assay demonstrated a significant competitive advantage of those strains which endogenous free-running period matched the simulated T-cycle. Under such conditions the strains achieved an optimal phase relationship between the LD cycle and

their endogenous clock (Ouyang et al., 1998; Johnson et al., 2008). This is also known as resonance hypothesis, which was proposed decades ago by Pittendrigh and means that the fitness of an organism is enhanced in case the endogenous period is in synchrony with the period of the environmental cycles (Pittendrigh and Bruce, 1959).

The circadian resonance hypothesis was also revisited recently by Wyse and colleagues. They investigated the connection of lifespan and circadian free-running period of mammals (9 laboratory mouse strains, 24 other rodents and 13 primates), concluding that a misalignment of endogenous rhythms and the environmental cycles of about 24 hours effect the longevity negatively, presumably due to physiological costs that come along with permanent re-entrainment to the environment (Wyse et al., 2010).

Studies like the mentioned above cannot be performed in humans like in other species due to numerous reasons. Nevertheless in a huge number of mammalian cells there are oscillating circadian clock genes (Peirson et al., 2006), the transcription of about 10 percent of the genome is regulated by circadian clock genes (Storch et al., 2002) as well as many different and very important processes like the cell cycle, proliferation of cells and tumor suppression (all these studies mentioned were performed in mice) (Fu et al., 2002; Miller et al., 2007; Moriya et al., 2007). These facts in combination with many studies concerning shift workers – reviewed by Knutsson – do not explicitly name a fitness advantage in context of the resonance hypothesis – due to many factors playing a role in the extremely complex human system – but they show the tremendous significance of circadian control for human physiology and health (Knutsson, 2003; Wyse et al., 2010).

Among these studies concerning the fitness benefits of a functional clock there are also some studies for *Drosophila*. In those studies the reduced sperm production and fewer offspring – due to the lack of rhythmic sperm release in per^{01} flies – (Beaver et al., 2002) as well as the higher sensitivity of per^{01} flies to oxidative stress (Krishnan et al., 2008) or the lifespan of male flies in the lab (Klarsfeld and Rouyer, 1998) was investigated, pointing out fitness disadvantages of the non-functional clock in the arrhythmic per^{01} flies. But a recent study even suggested that a functional clock is not absolutely necessarily (Vanin et al., 2012). In this study the authors showed that the arrhythmic per^{01} flies are still able to time their daily activity like the wild type flies under natural-like conditions.

1.5 Aim of the thesis

As mentioned above there are only very few studies investigating potential fitness advantages due to a functional clock in general and also in the fruit fly *Drosophila melanogaster* although it is perfectly suited as model organism with its characteristics – like a short generation time, broad range of genetic tools and the already known clock mutant strains.

Therefore the aim of this thesis was to unravel the potential function and fitness advantages of *Drosophila melanogaster`s* endogenous clock related to day length, humidity and food composition. This was accomplished by a mixture of experiments separated into three parts according to the three examined factors day length, humidity and food composition.

The aim of the first and main part of this thesis was to determine the impact of *Drosophila melanogaster`s* endogenous clock and resulting potential fitness advantages in terms of day length. In this context competition assays were performed where wild type and clock mutant flies were kept together in the vials in direct competition as a mixed population. These vials were exposed to different light and therefore day length settings like natural-like conditions outdoors at the Biocenter and the Beestation of the University of Würzburg, where the flies experienced natural light, temperature and also humidity conditions. In the lab the vials were either kept under the normal LD12:12 condition, simulating a 24 hours day, or under T-cycles simulating a 19 or 29 hours day as well as under constant light conditions.

For the second part the interaction of *Drosophila melanogaster`s* endogenous clock and humidity was to be investigated. To unravel the question whether humidity can act as a Zeitgeber locomotor activity behavior of wild type and clock mutant flies was recorded with LD and humidity cycles, DD with humidity cycles – advanced or delayed – and under DD with constant humidity. And also eclosion and wing expansion success of wild type and clock mutant flies under different relative humidities were analysed to address the question whether the timing of eclosion to a specific range of relative humidity with a functional clock results in fitness advantages for the flies.

The third and last part of this thesis concentrated on the interplay of *Drosophila melanogaster`s* endogenous clock and different food compositions. In the lab the flies are supplied with food ad libitum, but in nature they probably will have to work harder to find appropriate food sources. Therefore the question whether flies with an intact endogenous clock can survive better than flies without an intact clock due to a fitness advantage aroused. To investigate this

question wild type and clock mutant flies were kept on two different food sources and the lifespan of flies as well as the locomotor activity of the flies was recorded.

To understand a potential function and fitness advantages of *Drosophila melanogaster`s* endogenous clock related to day length, humidity and food composition and the complex interplay of other factors some additional experiments were performed. These experiments concentrated on locomotor activity behavior and mating preferences of homozygous and heterozygous female flies, fertility of male flies by means of sperm production and copulation success of the flies as well as locomotor activity behavior in populations as social component.

2. Material and Methods

2.1 Material

2.1.1 Fly strains

wild type CantonS (CS)	Department of Neurobiology and Genetics, University of Würzburg
period^{Short} (*per*^{S})	Konopka, Benzer
period^{Long} (*per*^{L})	Konopka, Benzer
period^{01} (*per*^{01})	Konopka, Benzer
clk^{AR}	Michael Rosbach

2.1.2 technical equipment

Confocal laser scanning microscope (CLSM)	DM 5500 Q, Leica, Wetzlar, Germany
Drosophila Activity Monitoring (DAM) System	Trikinetics Inc., Waltham MA, USA
Diodenlaser 488nm, 532nm	Leica, Wetzlar, Germany
Forceps (fly handling), 5er Dumont	Neolab, Heidelberg, Germany
Incubator	DR-36NL with Percival Intellus Ultra Control System, Percival Scientific Inc., USA
Locomotor Activity Monitor (LAM25) System	Trikinetics Inc., Waltham MA, USA
LEDs (in light boxes)	Lumitronix, LED-Technik, Hechingen, Germany
Lichtorgel software	G. Stöckl, Regensburg
Light boxes	Department of Neurobiology and Genetics, University of Würzburg
Mating wheel	Department of Neurobiology and Genetics, University of Würzburg
Mesh vials	Department of Neurobiology and Genetics, University of Würzburg
Petri dish	Hartenstein, Würzburg, Germany
stereomicroscope Stemi SV6	Carl Zeiss, Germany

2.1.3 Solutions and others Materials

Agar-agar, Danish	Carl Roth
DAPI	Molecular Probes (Invitrogen)
Fixogum (Removable cover slip sealing)	Marabu
Phosphat-Puffer (PBS)	Sigma-Aldrich, Steinheim
standard cornmeal/agar medium	Department of Neurobiology and Genetics, University of Würzburg
Sucrose	Carl Roth

Further Material and solution compositions used in this thesis are listed in the Appendix.

2.2 Methods

2.2.1 Fly strains

For development and long-term maintenance all fly strains used were kept in a climate chamber at 25 °C ± 0.2 °C with 60 % ± 2 % of relative humidity (rH). As light condition a LD cycle of 12:12 hours was used. Food was prepared at the department and was a standard cornmeal/agar medium consisting of 0.8 % agar, 2.2 % sugar-beet syrup, 8.0 % malt extract, 1.8 % yeast, 1.0 % soy flour, 8.0 % corn flour, and 0.3 % hydroxybenzoic acid.

2.2.2 Direct competition assay

10 - 15 individual vials were used with 15 virgin female and male flies of wild type (CS) and *per* mutant flies to start the competition experiments. The mutant flies were either per^{L}, per^{S} or per^{01}. All vials were kept in a climate chamber at 25 °C ± 0.2 °C with 60 % ± 2 % of relative humidity. The light condition was either a light-dark cycle of 12:12 hours, a T-cycle of 29 or 19 hours (29 h = LD 14.5:14.5; 19 h = LD 9.5:9.5) or constant light (LL) depending on the *per* mutant flies used for the subset of the experiment, light intensity of about 100 lux (white light led, Lumitronix, LED-Technik). Two sets of the experiment were performed outdoors under natural-like conditions (1st floor, Biocenter, University of Würzburg in 2013; Beestation, University of Würzburg in 2014) (Fig. 9).

Figure 9: location of the vials during the outdoor experiment in the summer months. A) and B) show the location at the Biocenter, 1st floor, C) the sheltered location at the Bee station of the University of Würzburg.

Outdoors the flies were kept sheltered but they perceived all temperature and humidity changes as well as the natural light changes. All experiments of the competition assay are listed in Table 1.

Table 1: overview of all competition assay experiments performed under specification of subset name, genotype of flies, light condition, vial names and the location of the vials during the experiment

subset	genotypes	Light condition	Vial names	Location
ABC	CS x per^L	LD 12:12	A 1- A 10	climate chamber
	CS x per^S	LD 12:12	B 1 - B 10	
	CS x per^{01}	LD 12:12	C 1 - C 10	
GHI	CS x per^L	LD 14.5:14.5	G 1 - G 10	climate chamber
	CS x per^S	LD 9.5:9.5	H 1 - H 10	
	CS x per^{01}	LL	I 1 - I 10	
NOP	CS x per^L	LD 12:12	N 1 - N 10	climate chamber
	CS x per^S	LD 12:12	O 1 - O 10	
	CS x per^{01}	LD 12:12	P 1 - P 10	
QRS	CS x per^L	LD 14.5:14.5	Q 1 - Q 10	climate chamber
	CS x per^S	LD 9.5:9.5	R 1 - R 10	
	CS x per^{01}	LL	S 1 - S 10	
DEF	CS x per^L	Natural light	D 1 - D 15	outdoor
	CS x per^S		E 1 - E 15	
	CS x per^{01}		F 1 - F 15	
JKL	CS x per^L	Natural light	L 1 - L 12	outdoor
	CS x per^S		K 1 - K 12	
	CS x per^{01}		J 1 - J 12	

All vials kept in the climate chamber were flipped to fresh ones every 14 days to transfer the next generation onto fresh food. The experiments performed outside were flipped when the next generation had developed and hatched successfully. This was between 3 to 5 weeks due to the changing temperature and therefore slower development of the flies compared to the flies reared in constant 25°C in the climate chambers.

After transferring the next generation to fresh vials, the old vials were kept for another 2 to 3 days for more flies to hatch. The males of those flies where then used for locomotor activity recording to determine the genotype of the flies and the genotype distribution in the experimental vials. The locomotor activity recording was done for every third generation for all the subsets except for the flies kept outside. These flies were only tested once (F3) due to the fact, that the flies could be kept outside only in the summer months due to the climate in Würzburg (about June to September/October).

2.2.3 Sperm counts

For these experiments mating chambers were used with one female and one male fly per chamber (Fig. 11). The flies were allowed to mate and were separated after successful copulation. The females were dissected in PBS right after the mating process and the female reproductive organs – consisting of the uterus, seminal receptacle and the spermatheca (Fig. 10) – were transferred to a glass slide and stained with DAPI 1 µg/ml in PBS. The Leica confocal laser scanning microscope DM5500 was used to scan the tissue in 2 µm layer thickness. The counting of the sperm heads was performed with Fiji (as described in Garbaczewska et al., 2013), via defining ROIs and automated counting.

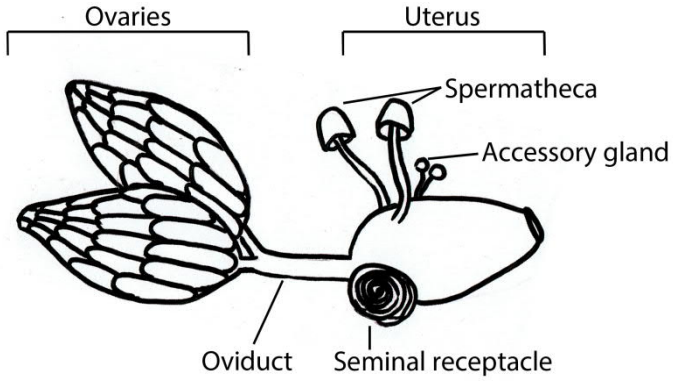

Figure 10: entire female reproductive organs consisting of the uterus with seminal receptacle, spermatheca and the accessory gland as well as the ovaries containing immature and mature eggs.

2.2.4 Female mating preferences

One female fly – heterozygous or homozygous – and two male flies of different genotypes (one male with the same genotype as the female and one male with another genotype) were placed in a mating chamber (Fig. 11). The mating process was monitored and the genotype of the male fly that copulated successfully was noted. To distinguish between the two males with different genotypes, one of the two flies was marked by a white spot on the thorax between the wings. This color marking was done for both tested males in two parallel subsets of the experiment to rule out the possibility of a color marking influence. The experiments were conducted at ZT 0 – 2 during the flies morning activity. Statistics were performed with R in cooperation with Thomas Hovestadt (for details see also Horn et al., 2019).

Figure 11: mating wheel with seven mating chambers filled with one female and two male flies each for mating preference testing. One of the two males is marked by a white spot to distinguish the flies. After successful copulation the genotype of the mated male was noted. This mating chamber was also used for the sperm count experiments with only one female and one male per chamber.

2.2.6 Locomotor Activity Recording

2.2.6.1 *Drosophila* Activity Monitoring (DAM) System

The recording of *Drosophila melanogaster*`s locomotor activity was performed with the *Drosophila* Activity Monitoring (DAM) System from Trikinetics. Flies – aged 3 to 5 days – were anesthetized with CO_2 and placed individually into glass tubes of 65 mm x 5 mm diameter. These glass tubes were filled by one third with nutrient-poor minimal medium (2 % agar, 4 % sucrose) as food source on the one side and closed with an air permeable plug on the other side after a single fly was placed inside (Fig. 12, A). Each monitor could hold 32 glass tubes for recording (Fig. 12, B). While the fly is moving back and forth inside the glass tube, it disrupts an infrared light beam approximately in the middle of the glass tube. The number of light beam disruptions is registered by the monitor and the DAMSystem Collection Software for every single fly in a minute interval. The raw data was read out as text files for further analysis.

The specific light conditions during the experiments were simulated by light-boxes manufactured at the workshop of the Biocenter (University of Würzburg), which used white LEDs (Lumitronix, LED-Technik, Hechingen, Germany) at an intensity of approximately 100 lux (Fig. 12, C). The light intensity as well as the light sequence were set using the software "Lichtorgel" (G. Stöckl, Regensburg). To record the free running behavior the flies were first entrained to a LD 12:12 light dark cycle for 4 to 7 days followed by constant darkness (DD) for at least 10 days. The temperature during the experiment was controlled by the climate chamber (25 °C ± 0.2 °C ; 60 % ± 2 % rH) where the light boxes were placed in.

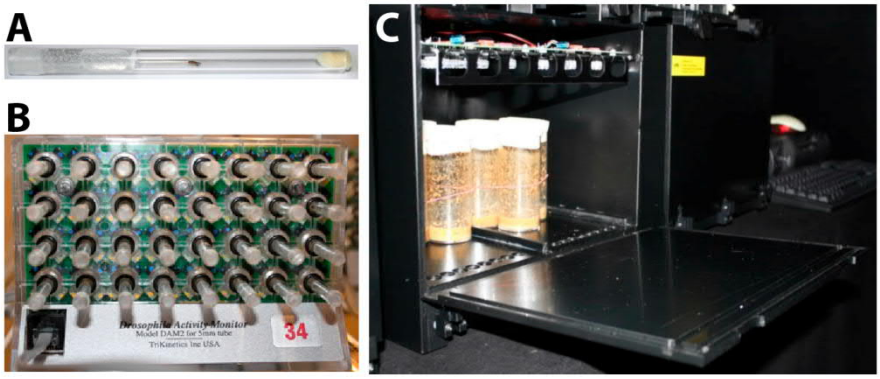

Figure 12: equipment for the locomotor activity recording of flies. A) single glass tube with food source on the left, fly sitting in the middle and air permeable plug on the right side of the tube. B) DAM System monitor filled with 32 glass tubes. Connection to the laptop and software via plug in on the left corner. C) light box for locomotor activity recording as well as simulation of T-cycles.

2.2.6.2 Locomotor Activity Monitoring (LAM25) System

For the recording of the locomotor activity of fly populations the Locomotor Activity Monitor (LAM25) System was used (see also DAM description above). The monitors were larger and the vials had a diameter of 25 mm to place several flies or other larger insects inside (Fig. 13). For the experiments 5 to 20 male or virgin female flies were placed in each vial. As food source the flies were provided with maximum medium (standard cornmeal/agar medium). The monitors were placed in an incubator (temperature and relative humidity set as in the climate chamber) in vertical or horizontal position to test which recording methods works best for *Drosophila melanogaster* populations. Data collection was also done in one minute intervals and read out as text files for further analysis.

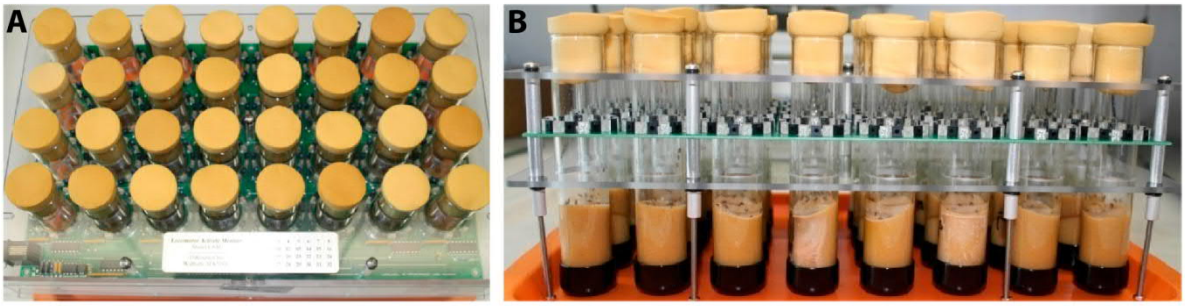

Figure 13: LAM System monitor filled with 32 glass tubes. B) Glass tubes filled with populations of flies feeding on standard cornmeal food.

2.2.6.3 locomotor activity pattern of flies with different food sources

The locomotor activity recording was performed as described above with the Drosophila Activity Monitoring (DAM) System, but the flies were either provided with nutrient-rich maximum medium (standard cornmeal/agar medium) or nutrient-poor minimal medium (sucrose/agar medium). Furthermore the monitors were placed in an incubator (25 °C ± 0.2 °C; 60 % ± 2 % rH) which was set identical to the climate chamber. Light conditions were also simulated using LED lights (IKEA lightbar warm white (2700 Kelvin) set to 100 lux) (Fig. 14).

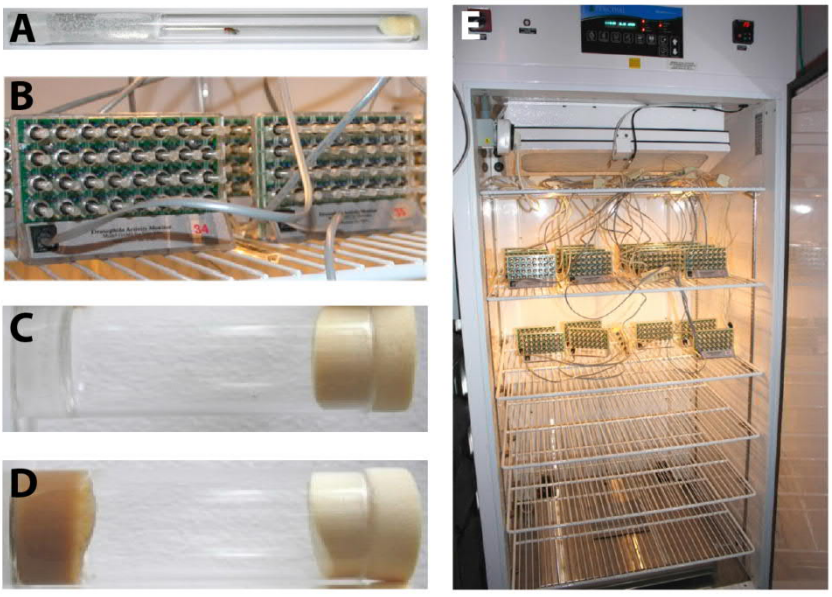

Figure 14: equipment for locomotor activity recording with different food sources in an incubator. A) single glass tube with fly in the inside, food on the left and air permeable plug on the right side. B) DAM system monitors filled with 32 glass tubes each and connected to the recording laptop. C) Minimum medium consisting of water, agar and sucrose. D) Maximum medium consisting of standard cornmeal/agar. E) Incubator used for simulation of light, temperature and humidity during the recording time.

2.2.6.4 Data analysis

The raw data was exported as text files by the DAM System Software. The data were displayed as actograms using a Fiji (https://fiji.sc/) plugin – ActogramJ (v0.9, Schmid et al., 2011) and saved as pdf files. An actogram displays the locomotor activity of single fly during the whole recording time. The activity is visualized via black bars on a time scale of 24 hours (single plot) or 48 hours (double plot). The actograms shown in this thesis are all displayed as double plots. The height of the black bar represents the amount of activity – namely the crosses of the infrared light beam – during the 1-min recording intervals. The recorded days are shown one below the other as well as side by side with a delay of 24 hours for better visual analysis (Fig. 15, A). Flies that died early in the course of the recording were excluded from further analysis.

To calculate the endogenous period of every single fly in DD, the text files were analyzed using a Microsoft Excel Macro (programmed by Taishi Yoshii) using χ^2-periodogram analysis with rhythmicity detected and free-running rhythm τ calculated at a p-value of $p \leq 0{,}05$. At $p > 0{,}05$ the flies were assumed to be arrhythmic with no free-running rhythm τ.

Average activity profiles of a group of flies were also calculated using a Microsoft Excel Macro. To create such an average activity profile of 24 hours (average day) the locomotor activity data of a group of flies in the first recording period (day 2-7) with light dark cycles was used. Day 1 was excluded due to the start of the experiment and to exclude any abnormal behavior of the flies caused by the CO_2-anesthetic treatment or handling of the flies. The data of every fly of the group was averaged and taken together to create an average day representing the average locomotor activity of a group or genotype. The red line represents the standard error of the mean (SEM) (Fig. 15, B).

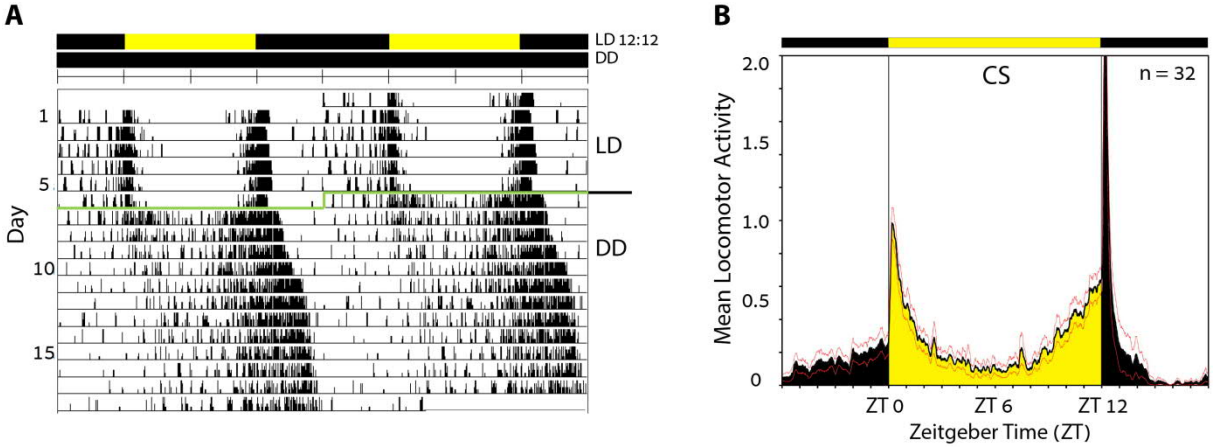

Figure 15: actogram and average day examples for data analysis. A) example actogram (double plot) of a single wild type CS fly recorded for 6 days in LD 12:12 (LD) followed by 12 days of constant darkness (DD). Green line indicates change of light condition with the graphical double plot delay B) example of an average day of

n = 32 wild type CS flies in LD12:12. Time scale is displayed as Zeitgeber Time with ZT 0 as light onset and ZT 12 as light offset. Red line indicates SEM. The black and yellow bars in A and B represent the light condition meaning darkness/lights-off (black) and light/lights-on (yellow) respectively.

2.2.7 Eclosion at different relative humidity values

Flies were allowed to lay eggs over night on standard food petri dishes in big egg-laying vials with mesh on top instead the normal vials with foam plugs (Fig. 16, A,B). Afterwards the adult flies were removed and the eggs/larvae were kept in the climate chamber (25 °C ± 0.2 °C; 60 % ± 2 % rH) for development with LD 12:12 as light regime. After the L3 larvae pupated at the side of the mesh-vials, the food source was removed and the mesh-vials were transferred to an incubator which was set at different relative humidity values (2 %, 60 % or 80 %). This transfer was done one or two days prior to eclosion of the flies. The light regime and temperature inside the incubator were kept the same as the climate chamber (LD 12:12, 25 °C ± 0.2 °C). After some days the number of successfully eclosed flies as well as the still closed pupae were counted (Fig. 16, C). All eclosed flies were also checked for successful wing expansion.

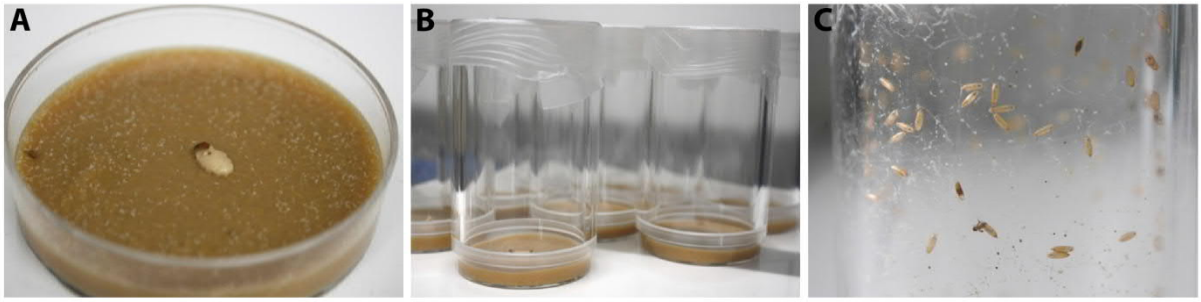

Figure 16: equipment for eclosion experiments at different relative humidity values. A) petri dish with standard cornmeal/agar and yeast in the middle surrounded by many eggs (small white dots). B) Vials with mesh instead a foam plug with additional parafilm on top to prevent desiccation. C) Vial with empty and full pupae after eclosion.

2.2.8 Humidity entrainment of flies

For calculation of the endogenous period of a fly, usually the locomotor activity is recorded with light entrainment to synchronize the fly`s clock. To have more possibilities in entrainment, humidity was tested as potential Zeitgeber for entrainment. For these experiments male flies were placed in the Drosophila Activity Monitoring (DAM) System like described above but the monitors were placed in an incubator simulating humidity cycles as entrainment phase and releasing the flies to constant conditions afterwards with no zeitgeber (constant rH and DD) as

free-running period. The humidity cycle was designed after a typical mean outdoor day recorded at the Beestation at the University of Würzburg. The simulated rH cycle is shown in Table 2 with the onset of light (ZT0) corresponding to the sunrise (07.00 CEST) and relative humidity values in nature.

Table 2: relative humidity (rH) cycle simulated in the incubator, calculated by relative humidity values measured outside the Beestation at the University of Würzburg. Central European summer Time (CEST) is given with correspondent Zeitgeber Time (ZT) and light condition used in entrainment. Light (L) was turned on from ZT0 to ZT12 and turned off (D) from ZT12 to ZT0, which corresponded to the natural lightphase (~07.00 to 19.00 CEST).

CEST	01.00	03.00	04.00	07.00	08.00	09.00	10.00	11.00	12.00	13.00	20.00	21.00	22.00	23.00	24.00
rH[%]	70	73	80	75	70	60	50	40	35	30	35	45	52	55	62
ZT	18	20	21	0	1	2	3	4	5	6	13	14	15	16	17
L/D	D	D	D	L	L	L	L	L	L	L	D	D	D	D	D

For entrainment not only wild type CS flies but also clock mutants (per^S, per^L and per^{01}) were used in two different subsets of the experiments. Both experiments started with a combination of light and humidity cycle coupled like in nature, followed by constant darkness – absence of light as synchronizing Zeitgeber – and a shifted (delayed or advanced) humidity cycle. After that the flies were released to constant conditions with no light or change in humidity. The exact entrainment conditions are shown in Table 3. This shift in the relative humidity cycle was done to see if the flies can use humidity as synchronizing Zeitgeber and are able to shift their activity as well according to the shifted relative humidity cycle.

Table 3: conditions of the humidity entrainment experiments. First week the flies were entrained with light and a relative humidity cycle before they experienced complete darkness – loss of light as Zeitgeber – as well as a shift in the relative humidity cycle. Afterwards the flies were released to constant condition with no Zeitgeber present.

experiment	light and humidity condition		
HE 01	6 days LD + rH cycle	7 days DD + rH cycle (8h delay)	10 days DD + constant rH
HE 02	6 days LD + rH cycle	9 days DD + rH cycle (8h advance)	9 days DD + constant rH

The raw data was recorded and read out as text files for further analysis like the normal locomotor activity recordings described above.

2.2.9 Lifespan of wild type and clock mutant flies

The lifespan of wild type (CS) and clock mutant (*per*^{S}, *per*^{L}, *per*^{01}, *clk*^{AR}) flies was tested in 10 vials per genotype with 50 males or 50 females in each vial. All vials were kept in a climate chamber (25 °C ± 0.2 °C ; 60 % ± 2 % rH) with LD 12:12. Every day the vials were checked for any dead flies and the number of dead flies was noted. Once per week the flies alive were transferred to fresh food vials. As food source there were two different compositions tested. One set of flies was provided with nutrient-rich maximum medium (standard cornmeal/agar medium) the other set of flies with nutrient-poor minimum medium (sucrose/agar medium) (Fig. 14, C,D).

2.2.10 cantonizing *per* strains via competition assay

To minimize the genetic background differences between the wild type CS and the *per* mutant fly strains, the *per* strains were cantonized. This was achieved by letting the *per* strains cross themselves to the wild type CS strain for several generations to get naturally backcrossed fly strains. That happened during the course of the competition assay experiment (subset ABC). After 54 generations of wild type CS and *per* mutant flies in the vials, virgin female and male flies were collected out of the vials and the flies locomotor activity was recorded for several days in constant darkness to determine the flies genotype. Afterwards the male *per* mutant flies were crossed to homozygous females or – if no homozygous females were available – to heterozygous females of the same *per* genotype. All these crosses were single crosses, i.e. one male was crossed with one female. In case the males were crossed to heterozygous females, the locomotor activity of the offspring was again recorded, the genotype determined and single crosses of male and female flies were set up. This process was repeated until a male fly could be crossed with a homozygous female. The genotype of the offspring was checked before the newly cantonized *per* fly strains were amplified for the stock collection and further experiments. The newly cantonized *period* fly strains were used for repeating the competition assay experiments under LD12:12 (subset NOP) and T-cycles (QRS) to rule out the possibility of side effects due to an altered genetic background of the flies.

2.2.11 Statistics

All statistics were performed with R. R version 3.2.4 Revised (2016-03-16 r70336) -- "Very Secure Dishes" Copyright (C) 2016 The R Foundation for Statistical Computing. Platform: i386-w64-mingw32/i386 (32-bit) (https://cran.r-project.org/mirrors.html). Significance levels are

indicated by asterisks within graphs, differentiating between significant (*, $p < 0,05$), highly significant (**, $p < 0,02$) and most significant (***, $p < 0,01$).

3. Results

3.1. survival competition assay

In the laboratory usually the fly strains are kept separately and all experiments are done separately or parallel for each genotype, but they are not mixed up together in direct competition. But in nature mutant flies have to compete with wild type flies directly to ensure the next generation. Therefore bringing the wild type and clock mutant fly strains together to see how the wild type and clock mutant flies cope with the situation and survive in direct competition under different conditions was exactly the aim of the competition assay experiments performed in this thesis. For the experiments in the laboratory not only a normal day with 24 hours was simulated, but also T-cycles of 19 hours or 29 hours corresponding to the free running period of the short *period* – per^S – and the long *period* – per^L – mutant flies. As constant light disturbs the clock of wild type flies resulting in arrhythmicity, constant light was used to create a suitable environment for the arrhythmic per^{01} flies in competition with wild type CS flies. To see how the flies react not only in the laboratory but also under natural-like conditions, the experiment was also performed outdoors under natural-like conditions, where the flies experienced not only the daily changes in light, but also the natural changes in temperature and relative humidity.

3.1.1 indoor experiments

For the experiments in the laboratory the flies were kept in light boxes in a climate chamber with regulated temperature and relative humidity. For an overview of all subsets performed see: Methods : 2.2.2 Direct competition assay. A detailed table containing all statistic values of the following experiments can be seen in the appendix (Table 9).

3.1.1.1 original fly strains in LD12:12 and T-cycles

The original fly strains were used to perform the competition assay experiments in a normal 24 hour day (LD12:12, ABC) or in T-cycles of 29 hours, 19 hours or LL (GHI) corresponding to the free running period of the *period* mutant strain used. The comparison of the two subsets of experiments provides information about the influence of the day length for the fitness of the flies. The results can be seen in Figure 17.

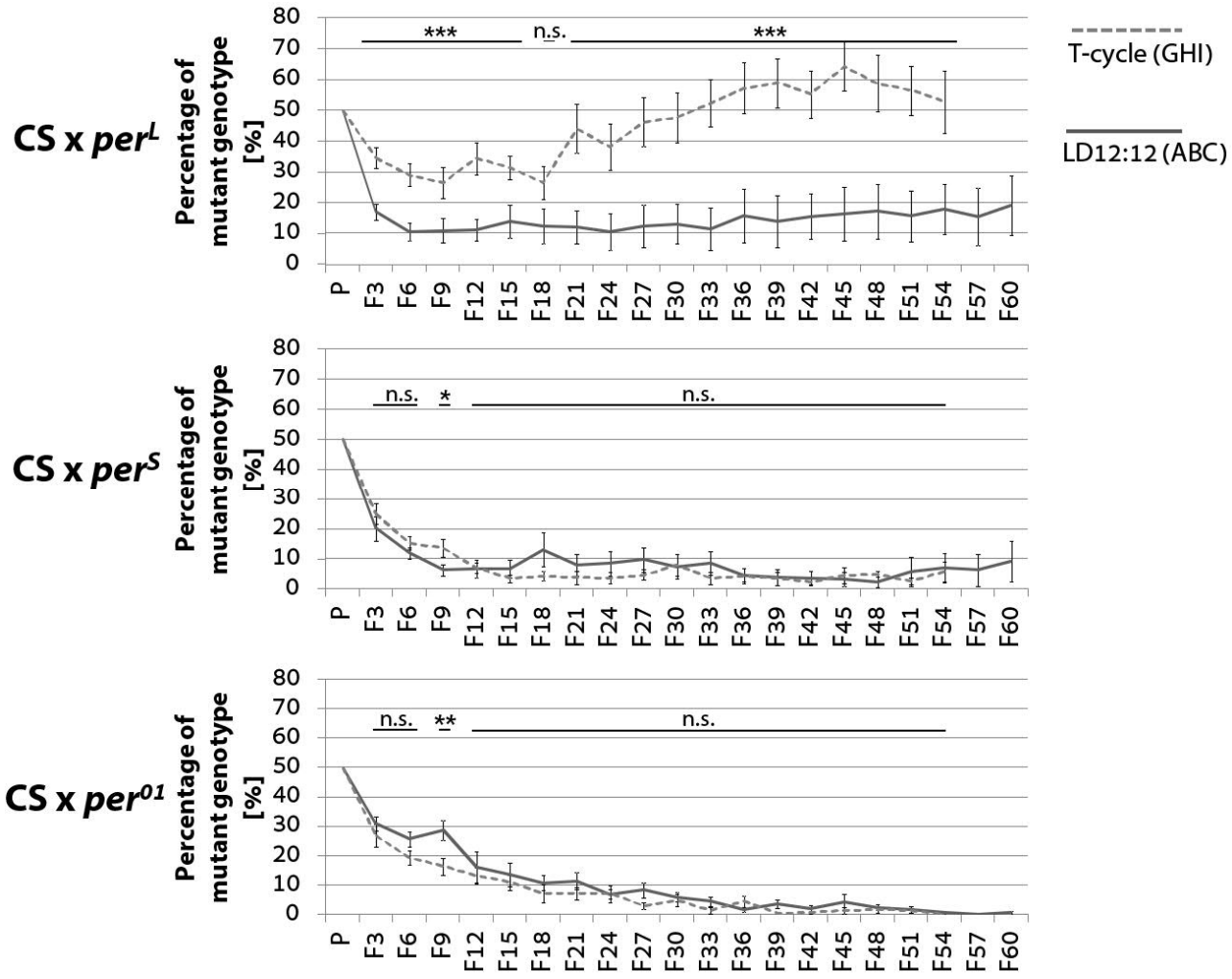

Figure 17: results of the competition assay experiment of the original fly strains in a light-dark cycle of 24 hours (LD12:12, ABC) and in T-cycles of 19/29 h or LL (GHI). The wild type CS flies dominated the *period* mutant flies in all experiments performed, except for the subset performed in a 29 hour T-cycle (CS x per^L). In this case the number of long *period* mutant flies was not declining steadily but declined for about the first 18 generations and increased in the following generations reaching up to about 65 %. Dashed line depicts the percentage of *period* mutant flies in the competition assay vials performed in T-cycles. Continuous line depicts the percentage of *period* mutant flies in the competition assay vials performed in LD12:12. Significant differences are shown by asterics (* = $p < 0,05$; ** = $p < 0,02$; *** = $p < 0,01$; n.s. = not significant). Black bars represent ± SEM.

In a normal 24 hours day (LD12:12) the wild type CS flies clearly dominated the per^L mutant flies already after the first three generations. This did not change in the course of the experiment and the number of per^L mutant flies in the competition vials stayed low but constant between 10 and 20 % (Fig. 17, CS x per^L). But when the flies were kept in a T-cycle of 29 hours – accordingly to the free running period of the per^L flies – the distribution of wild type CS and clock mutant per^L changed. During about the first 18 generations the number of per^L mutant flies declined, but after that the number of per^L flies increased and went up to more than 60 %. The number of clock mutant flies was not as constant as in LD12:12 but fluctuated

around the 50 % mark (Fig. 17, CS x *per*^{L}). The comparison of the 24 h/day and 29 h T-cycle experiment revealed a significant higher number of clock mutant flies in the 29 h T-cycle (Table 9). Therefore the wild type CS could not dominate the clock mutant flies like they did in the 24 hour day, showing that the day length influenced the fitness of the flies in favour of the clock mutants in this experiment, corresponding to the resonance hypothesis.

When the wild type and short *per*^{S} clock mutant flies were kept together in the competition vials there was the same trend in a 24 hour day as in the CS x *per*^{L} competition vials with a clear dominance of the wild type CS over the *per*^{S} flies already showing after the first three generations. The number of *per*^{S} flies declined quite fast during the first nine generations followed by a low but constant number of *per*^{S} flies during the rest of the experiment with fluctuating percentage of around 10 % clock mutant flies (Fig. 17, CS x *per*^{S}). This ratio of wild type and clock mutant flies did not change in a 19 h T-cycle. In the 19 h T-cycle the number of short clock mutant flies declined and stayed stable around about 10 % and there were no significant differences between the 19 h T-cycle and a normal 24 h day (Table 9).

Similar results could be seen in the experiments where wild type CS and the arrhythmic *per*^{01} flies were kept in direct competition. In a 24 h day as well as in constant light the number of *per*^{01} clock mutant flies declined steadily during the course of the experiment with almost extinction of *per*^{01} flies at the end of the experiments at generation 60 (Fig. 17, CS x *per*^{01}). And there were no significant differences between the 24 h day and constant light revealing no or a to small impact of the day length on the fitness of the flies (Table 9).

3.1.1.2 newly cantonized fly strains in LD12:12 and T-cycles

During the course of the first competition assay experiments the wild type CS and clock mutant flies mixed up and mated, resulting in flies with an improved conform genetic background compared to the original fly strains that were kept separately and cantonized a few years ago. Therefore the newly cantonized fly strains gathered from these experiments featured with a more conform genetic background differ – theoretically – in the *period* gene mutation only and point out the impact of day length on *Drosophila melanogaster*`s fitness in the survival competition assay experiments with less or even no side effects due to possible differences in the genetic background. The results of the experiments performed with the newly cantonized fly strains in either a normal 24 hour day (LD12:12, NOP) or a T-cycle of 19 h, 29 h or LL (QRS) are depicted in Figure 18.

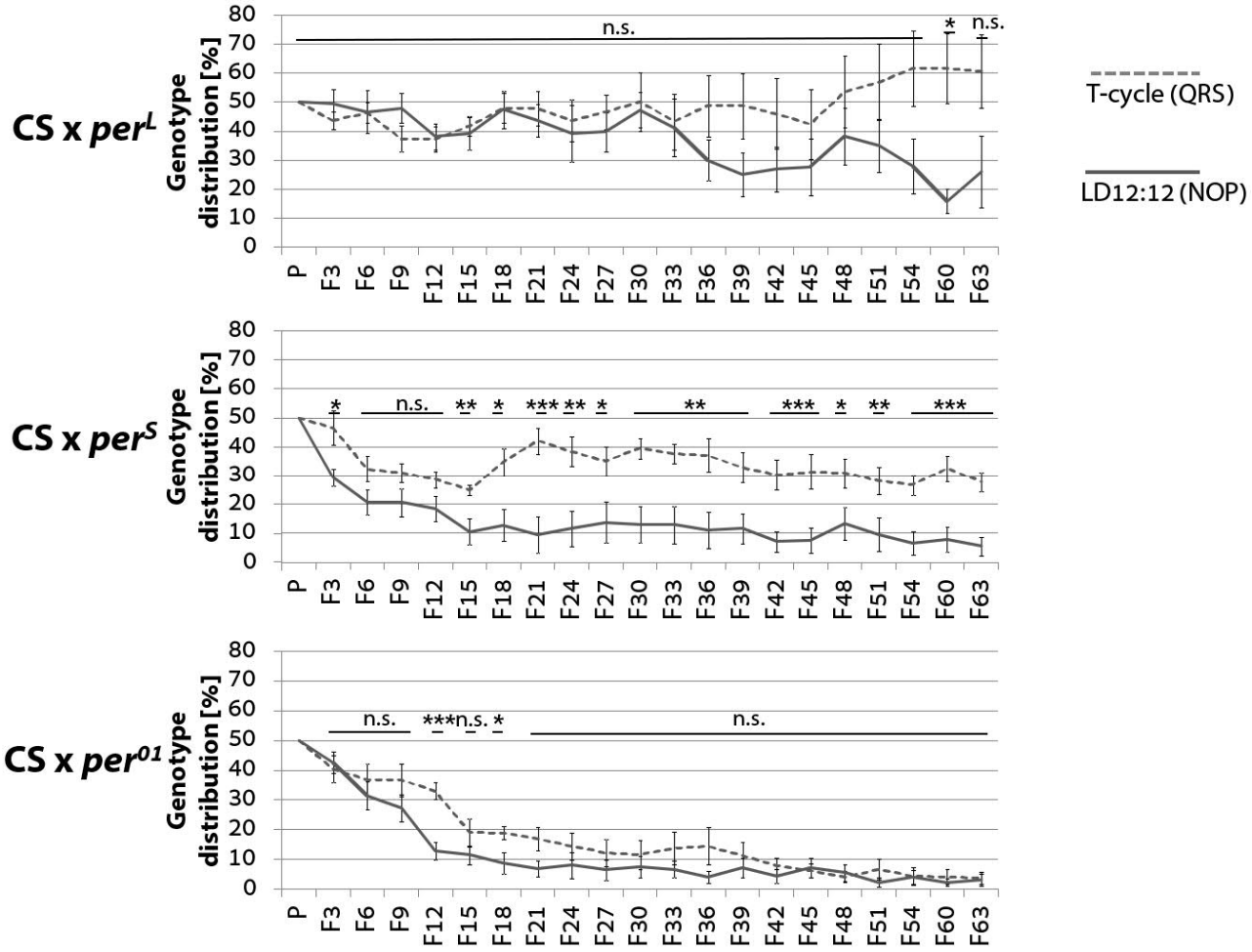

Figure 18: results of the competition assay experiment of the newly cantonized fly strains in a light-dark cycle of 24 hours (LD12:12, NOP) and in T-cycles of 19/29 h or LL (QRS). The wild type CS flies dominated the *period* mutant flies in all vials of CS x per^S and CS x per^{01} resulting in a decline of *period* mutant flies. For the subset performed in a 29 hour T-cycle (CS x per^L) the number of long period mutant flies was constantly fluctuating around about 45 % rising up to about 60% at the end, showing a tendency of better survival than in a 24 hour day, but overall there was no significant difference between a normal 24 hour day and the 29 hour T-cycle, except for the F60 generation. For CS x per^S and CS x per^{01} there were some significant differences between the different day length with higher number of mutant flies in the T-cycle corresponding to the free running period of the mutant used. For per^{01} flies this was only true for the first about 18 generations. For per^S flies the percentage of mutant flies was constantly higher in the T-cycle than in a 24 hour day, pointing out the fitness advantage of a circadian clock matching the environmental day length conditions. Dashed line depicts the percentage of *period* mutant flies in the competition assay vials performed in T-cycles. Continuous line depicts the percentage of *period* mutant flies in the competition assay vials performed in LD12:12, the 24 hour day. Significant differences are shown by asterics (* = $p < 0,05$; ** = $p < 0,02$; *** = $p < 0,01$; n.s. = not significant). Black bars represent ± SEM.

When wild type CS and per^L flies competed the number of mutant flies declined only a little bit at the beginning and stayed fluctuating around about 40-50 % of mutants under both day length conditions until about generation F33 (Fig. 18). After that the per^L mutant flies in the T-cycle experiment seemed to increase their number and the mutant flies in the 24 hour day decreased. This was not significant except for one of the final measured generation F60. This development was not significant – except for generation F60 – but the trend and relation to

the resonance hypothesis is visible, as the flies in the T-cycles seem to have a fitness advantage over time compared to the mutant flies under normal 24 hour day length conditions. For CS x per^S and CS x per^{01} the situation is different. In both cases the number of mutant flies in the competition vials declined under both day length conditions with wild type CS dominating the mutant strains. The number of mutants in the vials declined and stayed stable at about 8 % for per^{01} declining further over long time and at about 10-12 % for per^S in the normal 24 h day (LD12:12). In the T-cycles the course is the same but the number of mutant per^S as well as per^{01} flies is higher than in LD12:12 (Fig.18), at least at the beginning of the experiment in the case of the per^{01} flies. In those two direct comparisons of T-cycle and 24 h day there were significant differences in the tested generations with more mutant flies surviving under the T-cycle conditions that fit the mutants free running rhythm (Fig. 18, Table 9). These significant better survival of per^{01} mutant flies was only visible in the first about 18 generations, whereas for per^S mutant flies the percentage of mutant flies stayed constantly higher at about 30 % under T-cycles. These results lead to the assumption that for the per^L mutant flies the fitness of the flies was not effected as strongly as in the per^S mutant flies by the day length. The results support the resonance hypothesis, which can clearly be seen in the per^S mutant flies, but also in the other two subsets with per^L and per^{01} mutant flies as they seem to win over time – in case of per^L – or decline slower – in case of per^{01} – pointing out a fitness advantage for the flies when their endogenous clock matches the environmental day length conditions.

3.1.1.3 original and newly cantonized fly strains in LD12:12

The direct comparsion of the survival competition assay data performed with either the original or the newly cantonized fly strains under the same laboratory conditions of a normal 24 h day with LD12:12 points out the effects of the genetic background on *Drosophila melanogaster*`s fitness. The results can be seen in Figure 19.

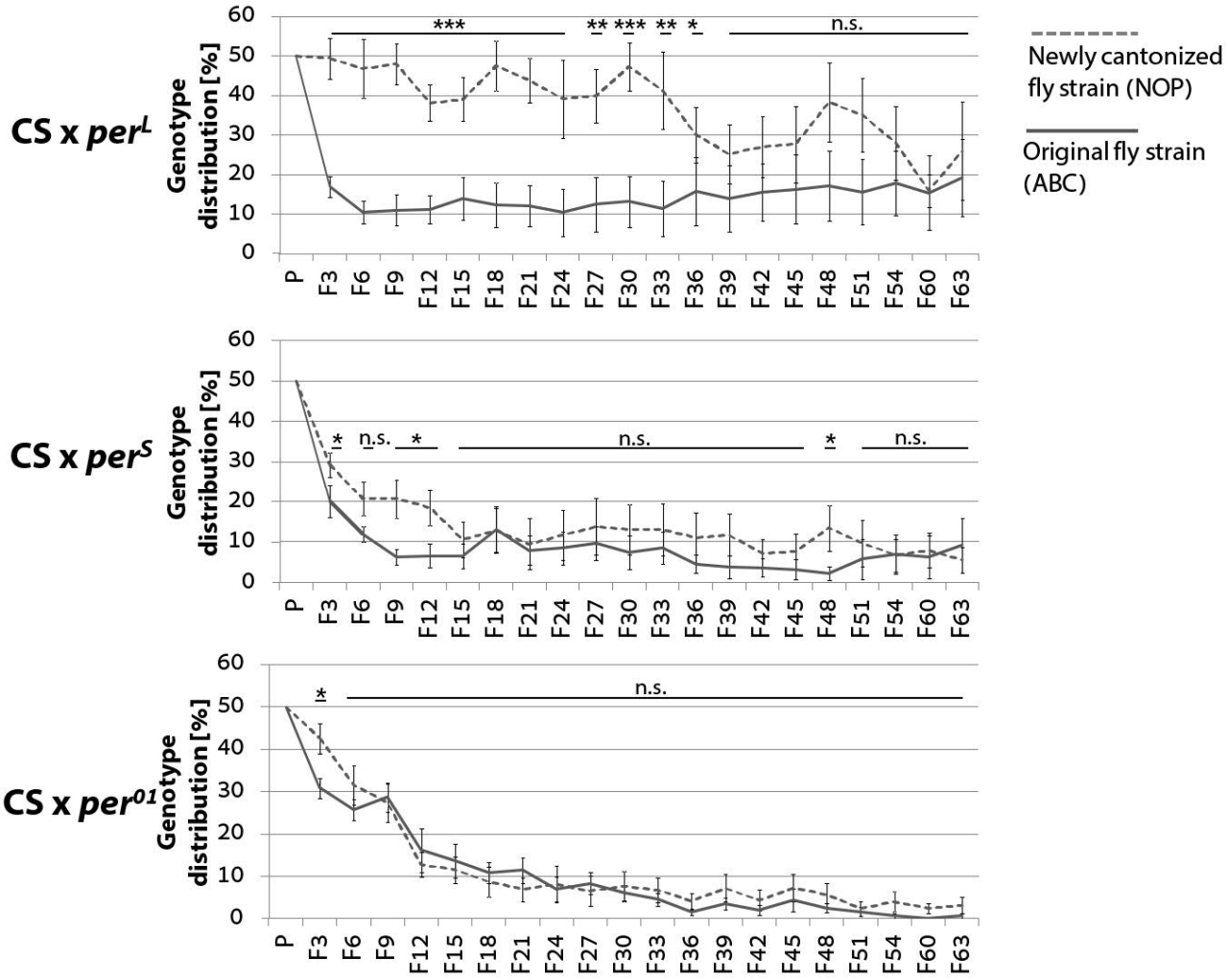

Figure 19: results of the competition assay experiment of original and newly cantonized fly strains in a light-dark cycle of 24 hours (LD12:12). The wild type CS flies dominated the *period* mutant flies in all experiments performed, except for the subset with newly cantonized flies (CS x per^L). In this case the number of long period mutant flies was not declining steadily but fluctuated around 45 % up to F36 and declined afterwards. Dashed line depicts the percentage of *period* mutant flies in the competition assay vials performed with newly cantonized fly strains. Continuous line depicts the percentage of *period* mutant flies in the competition assay vials performed with the original cantonized fly strains. Significant differences are shown by asterics (* = $p < 0,05$; *** = $p < 0,01$; n.s. = not significant). Black bars represent ± SEM.

The direct comparison of the old and the newly cantonized fly strains revealed that the number of newly cantonized per^L clock mutant flies did not extremely decline in the first few generations but stayed stable around 45 % up to generation F36 demonstrating that the newly cantonized per^L strain can cope significantly better with a 24 h day simulation (Fig 19, CS x per^L). This was not the case for the short *period* mutant per^S and the arrhythmic mutant per^{01}. Both clock mutant fly strains declined slightly slower at the beginning of the experiment – in the 19 h T-cycle and LL respectively – but reached the same level of mutant flies left in the competition vials in a 24 h day simulation after nine (per^{01}) or fifteen (per^S) generations. Per^S mutant flies stayed stable around 10 % whereas the per^{01} flies seemed to decline slowly but

constantly with a possible extinction (Fig. 19, CS x *per*^{S}, CS x *per*^{01}). Therefore there were no significant differences for *per*^{S} and *per*^{01} and consequently no great changes on the fitness level of the flies due to the genetic background under the simulated 24 hour day with LD12:12. On the other hand *per*^{L} mutants showed a clear and significant difference in the percentage of mutants in the vials demonstrating the impact of genetic background of the flies.

3.1.1.4 original and newly cantonized fly strains in T-cycles (19 h/29 h/LL)

After there were only obvious differences for the long *period* mutant *per*^{L} in a 24 h day dependent on the genetic background this was not true for the competition assay experiments performed in simulated T-cycles. The comparison of the original and the newly cantonized fly strains are shown in Figure 20.

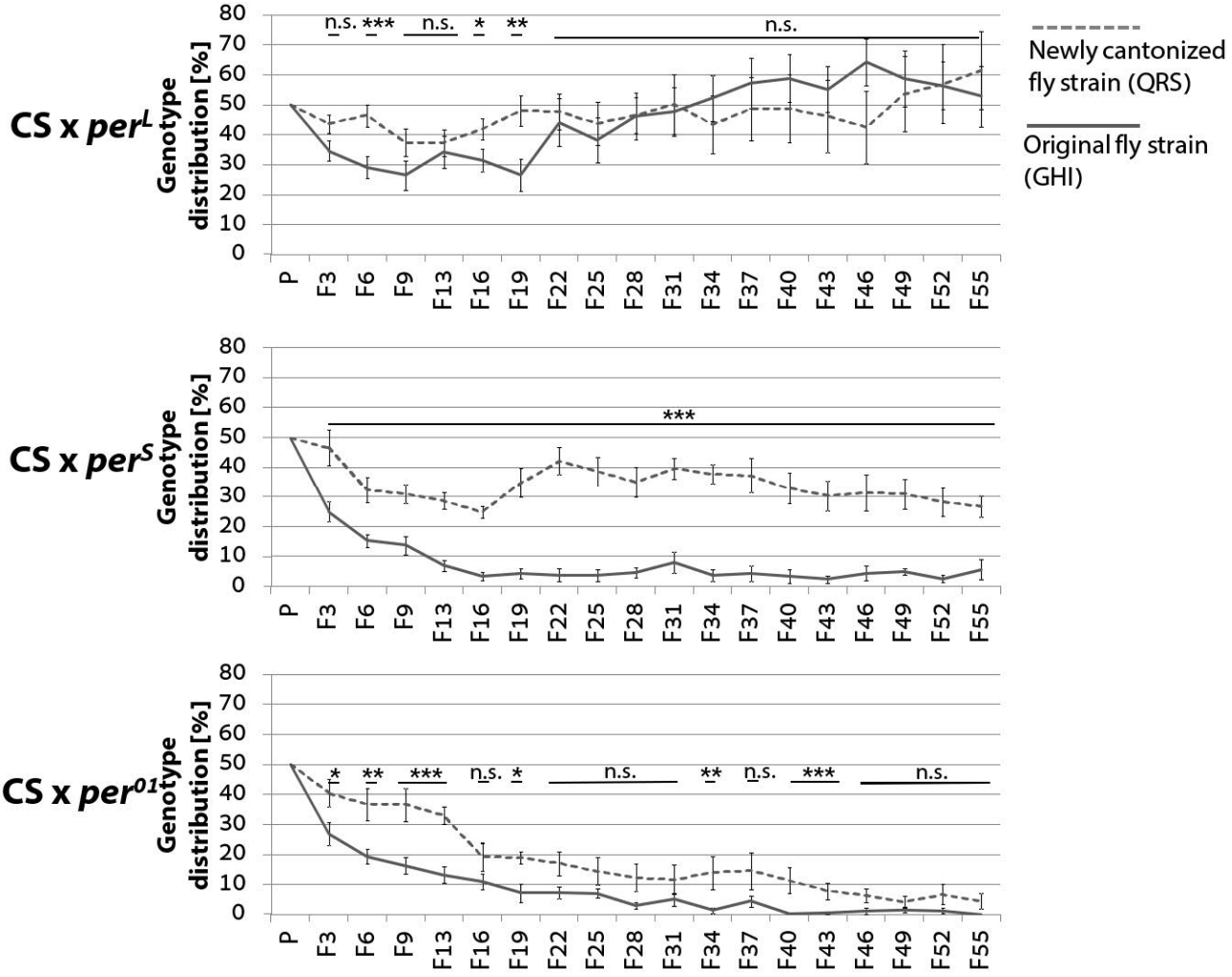

Figure 20: results of the competition assay experiment of original (GHI) and newly cantonized (QRS) fly strains in a T-cycle of 29 hours (CS x *per*^{L}), 19 hours (CS x *per*^{S}) and LL (CS x *per*^{01}). The wild type CS flies dominated the *period* mutant flies in a T-cycle of 19 hours and in LL where the number of *period* mutant flies declined in the first few generations and stayed constant afterwards (CS x *per*^{S}) or declined close to extinction (CS x *per*^{01}). For CS x *per*^{L} the percentage of mutant flies declined in the first part of the experiment but incresead

and stayed relative constant at around 50 %, keeping up with the wild type flies. In the case of CS x per^S the decline of short *period* mutant flies was less strong in the newly cantonized flies and fluctuated around 30 %. Dashed line depicts the percentage of *period* mutant flies in the competition assay vials performed with newly cantonized fly strains. Continuous line depicts the percentage of *period* mutant flies in the competition assay vials performed with the original cantonized fly strains. Significant differences are shown by asterics (* = $p < 0,05$; ** = $p < 0,02$; *** = $p < 0,01$; n.s. = not significant). Black bars represent ± SEM.

When the flies were kept in the T-cycles or constant light corresponding to their endogenous clock the frequency of mutant flies was significantly higher for all three tested *period* clock mutants – namely per^L, per^S and per^{01} – at least in parts of the experiment (Fig. 20). The number of *period* mutant flies declined significantly slower in case of all three mutant strains that were newly cantonized compared to the original fly strains. For per^L there were only significant differences in the first part of the experiment until about generation F19. Afterwards the newly cantonized flies as well as the original per^L flies increased in the percentage of flies compared to wild type CS flies, keeping the balance of mutant and wild type flies around 50 %. The CS x per^{01} experiments revealed also a slower decrease in mutant fly number when the flies where newly cantonized, although the number of flies decreased close to extinction in both strains. In case of the per^S mutant flies there were the most prominent changes visible. The per^S mutant flies – namely the newly cantonized per^S flies – survived much better under the T-cycle when the flies background was closer related to the wild type CS flies and were able to maintain a stable percentage of about 30 % of mutant flies in the experiment (Fig. 20). This direct comparison of competition assay results under same day length conditions demonstrates the importance of a similar genetic background.

3.1.2. outdoor experiments

The outdoor experiments were performed in 2013 and 2014 outside the biocenter and the bee-station of the University of Würzburg – sheltered from rain and direct sunlight – where the flies could sense all changes in light, temperature and relative humidity. Due to the changing temperature the flies needed far more time to develop and to generate the next generation. Therefore only the first three generations could be obtained and the third generation could be investigated for the genotype distribution of wild type CS and *period* clock mutant flies.

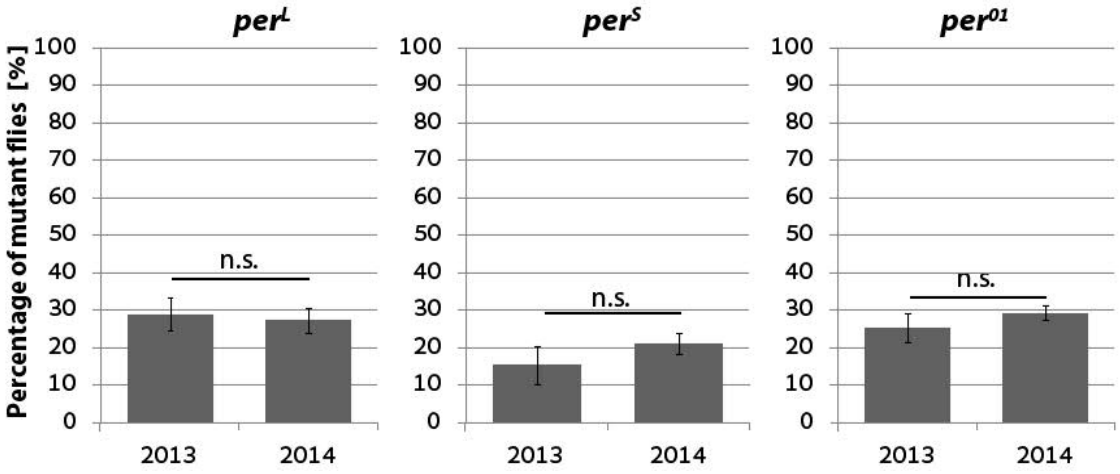

Figure 21: Percentage of mutant flies of the third generation of flies of the competition assay experiments performed outdoor in 2013 and 2014 in Würzburg. The number of *period* clock mutant flies in the competition vials decreased during the first three generations resulting in a low percentage of mutant flies that ranged from about 15 – 30 %. The results of 2013 could be reproduced in 2014. There were no significant (n.s.) differences in mutant fly numbers. Black bars represent ± SEM.

In all three cases where wild type and *period* clock mutant flies had to compete under natural-like outdoor conditions the wild type CS flies outnumbered the mutant strains already in the third generation. In the third generation there were 28,98 % (2013) and 27,42 % (2014) per^L clock mutant flies left in the competition vials. The arrhythmic per^{01} flies were reduced to about the same level with 25,42 % (2013) and 29,39 % (2014) per^{01} clock mutant flies left. The short *period* mutant per^S was reduced even further to only 15,42 % (2013) and 21,19 % (2014) (Fig. 21). The ratio of wild type CS and *period* clock mutant flies of 2013 could be reproduced and was consistent in 2014 with no significant changes (per^L, $p = 0{,}7236$; per^S, $p = 0{,}09462$; per^{01}, $p = 0{,}403$).

3.1.3 sperm counts

As shown above the wild type CS flies dominated the *period* mutants in the competition assay experiments in the laboratory under artificial as well as outdoors under natural-like conditions. To find out which factors contribute to these results different possibilities were analyzed. Not only external but also internal factors were considered. One of the internal factors is the number of sperm that is produced and transferred by the male flies. To rule out the possibility of less offspring of *per* mutants due to a lack of sperm production or problems during the mating process the number of sperms that is transferred during mating was investigated. Therefore a male and a female fly were allowed to mate in a mating chamber of a mating wheel. Directly after the successful copulation the female flies were dissected and the female

reproduction organs – uterus, seminal receptacle and spermatheca – were transferred to glass slides and the sperm heads containing the DNA were stained with DAPI. The following figure shows an example staining pattern of sperm heads surrounded by female tissue cell nuclei (Fig. 22).

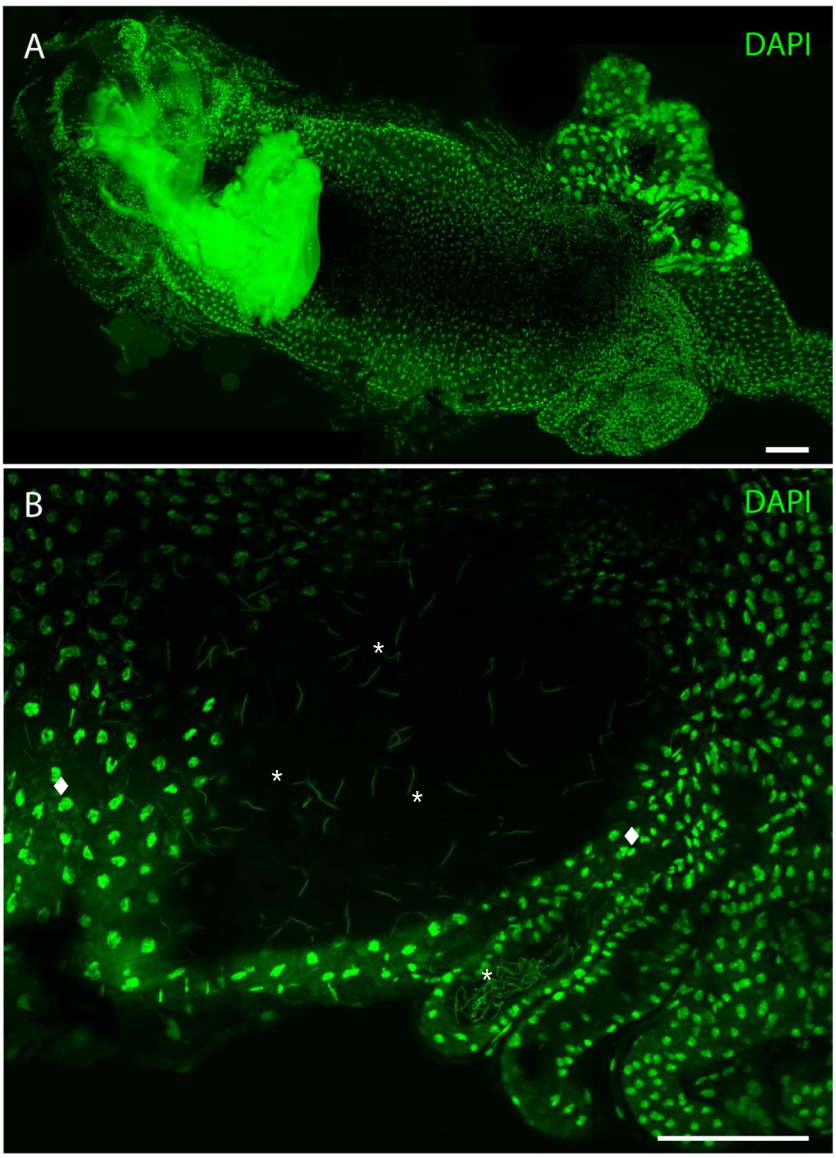

Figure 22: staining example of the female reproductive organs and the sperm heads with DAPI. A) DNA staining with DAPI of wild-type CS female reproductive organs consisting of uterus, seminal receptacle and spermatheca containing sperm after successful mating. B) sperm heads stained via DAPI (*) surrounded by female tissue nuclei (♦); scale bar indicates 50 µm.

The sperm heads were counted and there was no significant difference between the wild type CS and the three different *per* mutants, per^{L}, per^{S} and per^{01} (Fig. 23). The total range of transferred sperm lay between 1769 and 679 sperms. As the females can only store about 500 sperms (Miller and Pitnick, 2002; Manier et al., 2010) all male flies were able to transfer enough

sperm to secure the next generation. On average CS males transferred 1256, *per*^{L} 1213, *per*^{S} 948 and *per*^{01} 1188 sperms during copulation (Fig. 23).

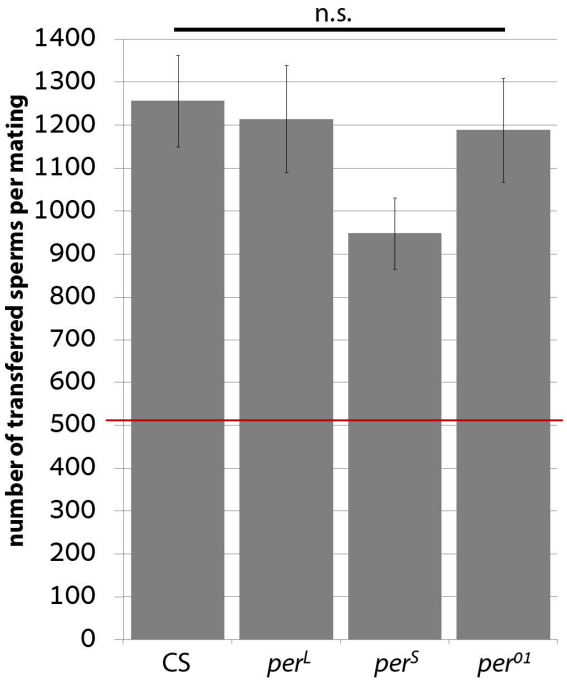

Figure 23: number of transferred sperms per mating of CS, *per*^{L}, *per*^{S} and *per*^{01} males flies. Mean of transferred sperm lies between around 950 and 1250 sperms. n = 8 for each genotype. Red line indicates sperm storage capacity of a female fly. n.s. = not significant; error bars depict ± SEM.

Therefore with only a storage capacity of about 500 sperms per female, which can lay less than 200 eggs at the end, all mutant flies as well as the wild type flies were able to secure the next generation with a sufficient number of sperms transferred during copulation.

3.1.4 female mating preference

In the course of the competition assay experiments wild type and mutant flies mated with each other – mixing up the genotypes – resulting not only in homozygous male and female flies but also in heterozygous female flies. The endogenous clock of a heterozygous female fly runs with a different period length than the homozygous ones (Fig. 29 and Tab. 4 in 3.2.2). For example a female CS/*per*^{S} fly has a free-running period of about 21.59 h, which lays in-between the wild type CS female (τ = 24.79 h) and the *per*^{S} female (τ = 19.41 h). For this reason female flies of all genotypes (homozygous and heterozygous) were tested for a mating preference to see whether they prefer a male fly with a similar activity pattern or one with a different free-running period. In case of heterozygous CS/*per*^{S} and CS/*per*^{L} female flies the heterozygous females preferred to mate with mutant *per*^{S} or *per*^{L} respectively (Fig. 24). Heterozygous CS/*per*^{01}

females showed no specific preferences for neither wild type nor *per*^{01} mutant males (Fig. 24). When the *per*^{S} female flies were tested, they showed a tendency to prefer wild type CS male flies. *Per*^{L} female flies on the other hand showed a tendency for mating with the same genotype, namely *per*^{L} male flies. But in both cases the mating choice was not statistically significant (Fig. 24). The arrhythmic *per*^{01} females showed no preference for *per*^{01} or CS male flies (Fig. 24).

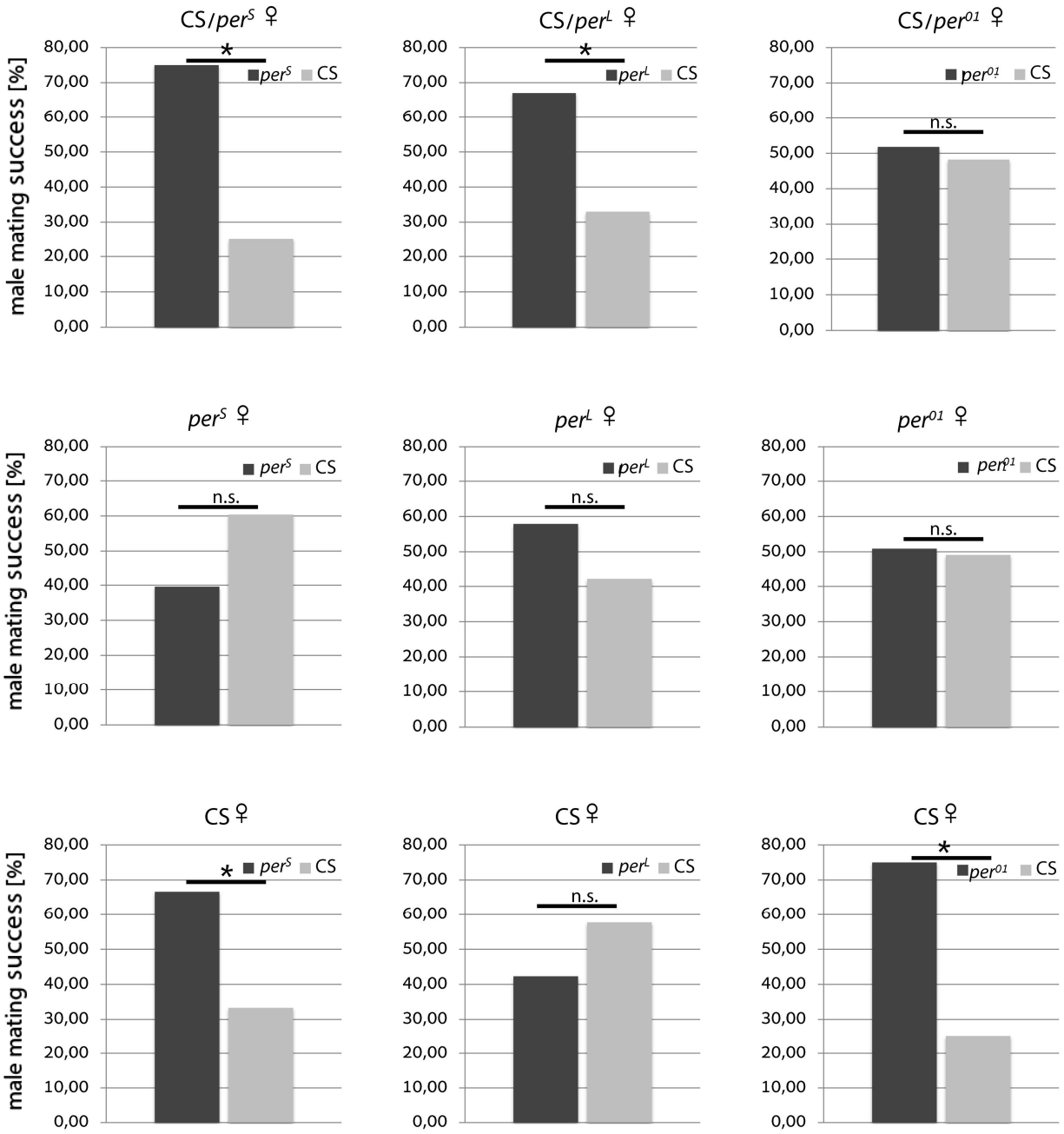

Figure 24: male mating success or rather mating preferences of heterozygous and homozygous female flies; first row shows the results of heterozygous female flies and their choices of the wild type or *period* mutant male in percent; second row shows the results of the *per* females and in the third row all results of wild type CS

females are shown. Wild type flies always had the choice between a wild type male or one of the *per* mutant males; significant differences in mating choice are indicated by asterisk (*, $p < 0,05$; n.s. = not significant)

Wild type CS females were tested in three different situations. The wild type females were presented always with one wild type CS male and one of the *period* mutant male fly. Thus the wild type females could choose between males of the genotype CS and per^S or CS and per^L or CS and per^{01}. In case per^S or per^{01} male flies were presented together with a wild type male fly, the wild type CS females significantly preferred to mate with the *per* mutant present in the mating chamber. When per^L and CS male flies were present in the mating chamber the female CS flies chose wild type CS males more often, but this was not statistically significant (Fig. 24).

3.2. locomotor activity pattern

3.2.1 of male flies feeding on maximum or minimum medium

To see whether the food source changes the behavior or influences the locomotor activity pattern of flies, the flies were provided with either the usually used nutrient-poor minimal medium – consisting of water, sucrose and agar – or nutrient-rich maximum medium – standard/cornmeal medium – for the locomotor activity recording. The results are shown as average days for analysis of the shape of the activity pattern and the total amount of activity was calculated to see differences in the amount of movements. The fly strains used were the original fly strains. In Figure 25 the locomotor activity profiles of male flies fed on maximum or minimum medium are shown as average days of wild type (CS) and clock mutant flies (per^S, per^L, per^{01}, clk^{AR}).

In all cases there was a reaction of the flies to the change of light condition – lights-on at ZT0 and lights-off at ZT12 – but for the flies with a functional clock the activity already changed before and/or after the transition of light due to the flies anticipation of changes in light, revealing the morning and evening activity peaks (Fig. 25: CS, per^S, per^L). The flies without a functional clock also reacted to light with a short peak in activity, but they exhibit relative constant movements and no such a change in activity before or after the transition of light (Fig. 25: per^{01}, clk^{AR}).

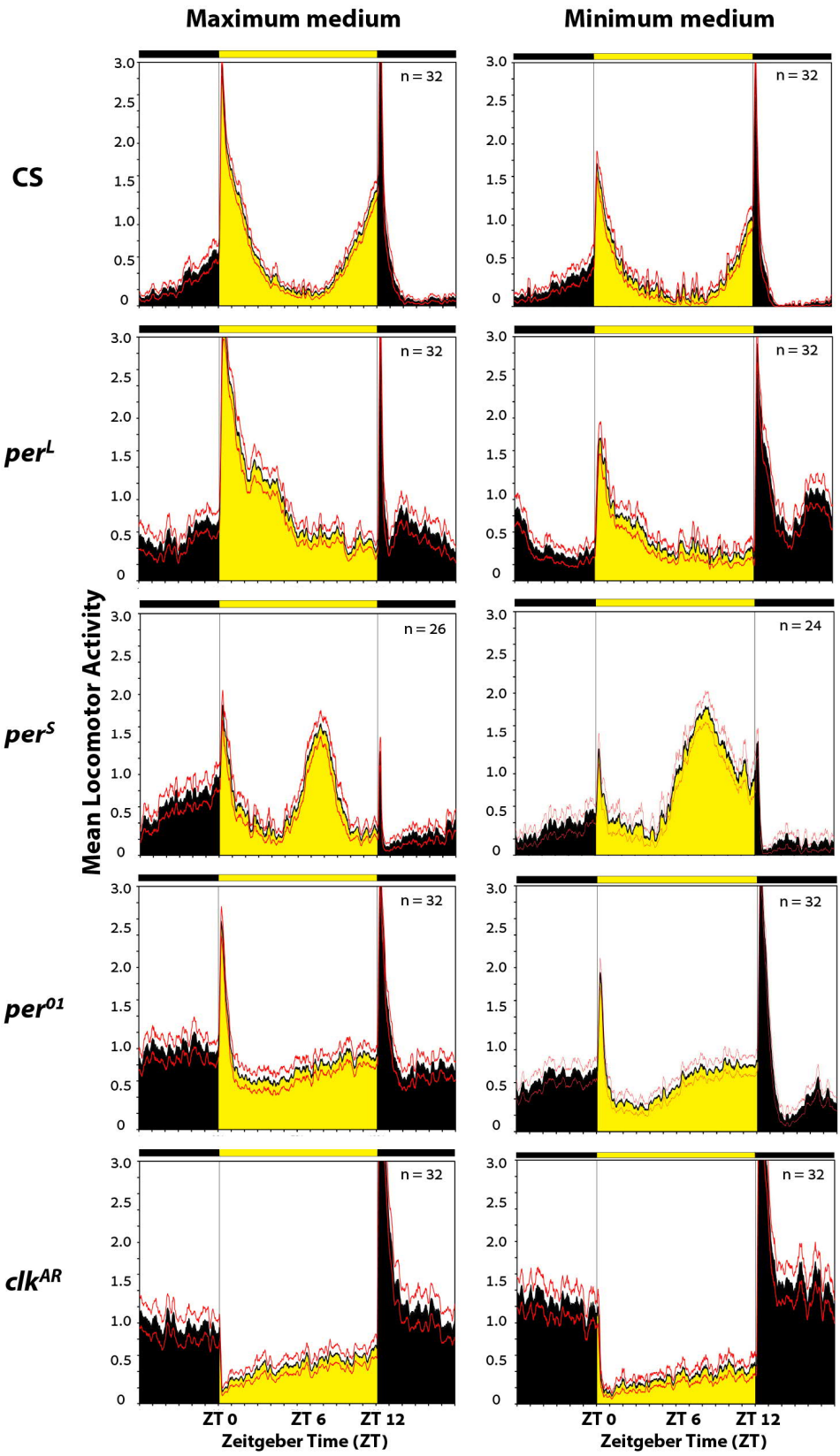

Figure 25: mean locomotor activity profiles of wild type and clock mutant flies displayed as average days. Left side shows the locomotor activity pattern of flies fed on nutrient-rich maximum medium, right side on nutrient-poor minimum medium, respectively. The numbers of flies used for calculation of the average day graphs are shown in each upper right corner (n). The black and yellow bars represent the light condition meaning darkness/lights-off (black) and light/lights-on (yellow) respectively. The red line indicates standard ±SEM.

CS males showed a typical bimodal activity pattern with a morning and evening peak in activity. For the wild type flies the shape of the activity pattern of the average days with the morning and evening peak was quite similar on both food sources. The flies anticipated the change of light and started to raise their activity already before the light turned on or off at ZT0 and ZT12 respectively (Fig. 25). In between the high activity phases the flies activity level dropped to a minimum. But the total activity of the wild type CS flies over a whole day was significantly lower on nutrient-poor food ($p = 0,00828$)(Fig. 26). The difference between night and day can also be seen in the total activity of the flies where it is more clearly and significantly different on nutrient-rich food ($p = 0,0000043$)(Fig. 27).

Per^{L} males exhibited broader morning activity on nutrient-rich food (Fig.25). The evening activity peak seemed to be split in both cases but it took place a little bit later on nutrient-rich food with the second half already taking place in the early morning around ZT22/23, whereas the flies on nutrient-poor food were active around ZT12 – when light was turned off – and around ZT17 with almost no increase of activity before lights-on at ZT0. On nutrient-rich food the flies were more active overall but the distribution of activity is different. The *per*^{L} flies were more active during day/light on maximum food (*, $p = 0,0307385$), whereas the flies spend more time being active in the night/darkness when they fed on the nutrient-poor minimum medium ($p = 0,005722$) (Fig. 27).

Per^{S} males demonstrated a slightly different locomotor activity pattern. The morning activity was a bit higher on nutrient-rich food, but the pattern and timing is the same in both cases (Fig. 25). But when the flies fed on nutrient-rich food they showed a more pronounced evening peak shifted to the afternoon due to the flies fast clock (Fig. 25). On nutrient-poor medium this activity peak was broader and the high activity lasted almost until the lights turned off. The distribution of activity at day and night varied a little bit dependent on the food source – in such a way that the flies were more active in the light and less active in the night phase on minimum medium – but on both food sources the flies were more active in the light than in the dark (max med, $p = 0,000752$; min med, $p = 0,0007039$)(Fig. 27) and the total amount of activity over a whole day with 24 hours stayed the same (Fig. 26).

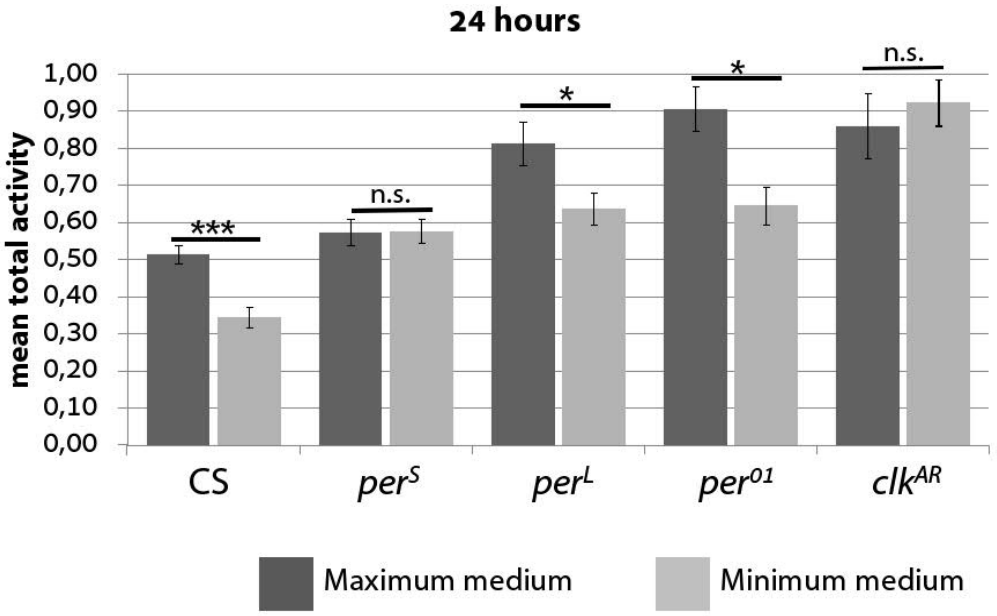

Figure 26: comparison of mean total activity of wild type and clock mutant flies in a 24 hour period/one day fed on maximum or minimum medium. Dark grey bars represent the flies fed on maximum medium (nutrient-rich medium), bright grey bars on minimum medium (nutrient-poor medium), respectively. Number of flies used for calculation was n = 32 each except for *per*^{S} (n = 26 max med, n = 24 min med). Significant differences are shown by asterics (* = $p < 0,05$; *** = $p < 0,01$; n.s. = not significant). Black bars represent ±SEM.

In case of *per*^{01} the flies had no functional clock and showed relative constant movements throughout the light/day and dark/night. They reacted to the change of light – being turned off or on – with a quick and short increase of activity followed by a trough and slow return to a specific level of activity which they kept constant until the next change of light happened (Fig. 25). They did not anticipate the change of light and thereby did not exhibit an increase of activity before the light situation changed, but just reacted to it. This is true for both types of food sources tested, however it is more pronounced on the nutrient-poor food. The total amount of activity over a whole day was higher on nutrient-rich maximum medium ($p = 0,02361$)(Fig. 26) where the flies distinguished between day and night with more activity in the dark ($p = 0,008373$) (Fig. 27). This is not true for flies on nutrient-poor minimum medium where the flies showed the same level of total activity in light and darkness (Fig. 27).

Clk^{AR} flies have no functional clock, which can be seen clearly in the locomotor activity pattern of an average day. The flies showed constant movements over the whole day with no anticipation of changes in the light conditions (Fig. 25), but the flies distinguished between night and day or lights-on and lights-off, respectively. They preferred to be active during darkness, which is especially obvious in the total activity of the flies showing significant differences on both food sources (max med, $p = 0,0009958$; min med, $p = 0,0000011$) (Fig. 27).

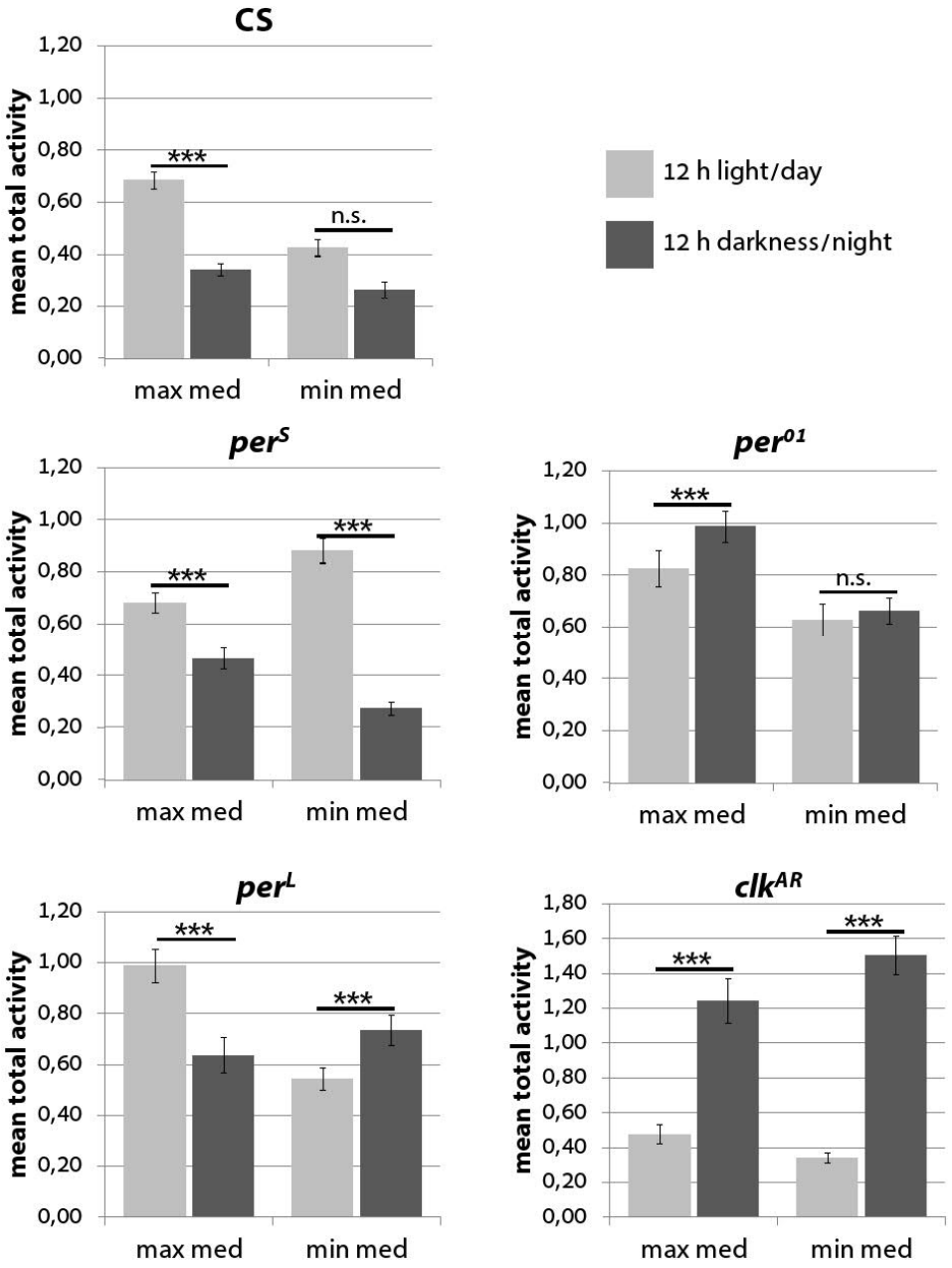

Figure 27: comparison of mean total activity in light/day and darkness/night of wild type and clock mutant flies fed on nutrient-rich maximum or nutrient-poor minimum medium. Dark grey bars represent the flies activity in the dark, bright grey bars in light, respectively. Number of flies used for calculation was n = 32 each except for *per*^{S} (n = 26 max med, n = 24 min med). Significant differences are shown by asterics (*** = $p < 0{,}01$; n.s. = not significant). Black bars represent ±SEM.

The comparison of the mean total activity of all genotypes revealed that the amount of movements of wild type CS flies was significantly lower on nutrient-poor medium, whereas the *per* mutants showed similar total activity on nutrient-poor medium with *clk*^{AR} significantly exhibiting most movements (Fig. 28, minimum medium).

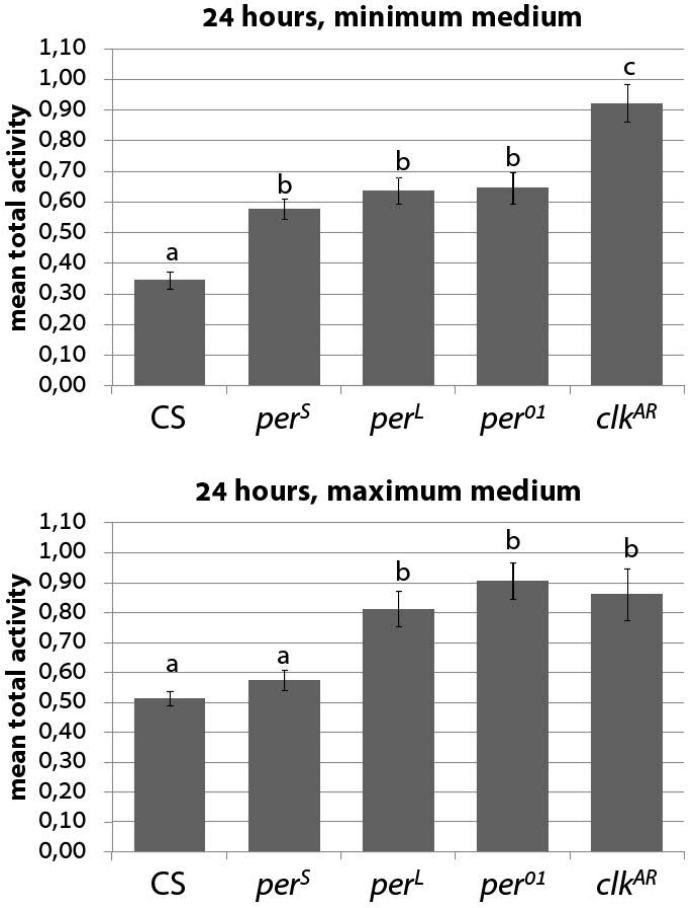

Figure 28: comparison of mean total activity of wild type and clock mutant flies in 24 hours on nutrient-poor minimum (upper graph) or nutrient-rich maximum (lower graph) medium. Number of flies used for calculation was n = 32 each except for per^S (n = 26 max med, n = 24 min med). Significant differences between the genotypes are shown by small letters (a, b, c). Black bars represent ±SEM.

On the nutrient-rich maximum medium the total activity pattern is similar with wild type flies showing significantly less total activity as well as per^S than the other clock mutant flies. Furthermore per^L, per^{01} and clk^{AR} showed no significant difference among each other displaying about the same level of mean total activity (Fig. 28).

To sum this up wild type flies showed the typical bimodal activity patter with less total activity than the clock mutant flies on both types of food sources. The short and long *per* mutants – per^S and per^L – also exhibited the bimodal activity pattern on nutrient-rich and nutrient-poor medium with anticipation of changes in light conditions, but they were not able to adjust their main activity (activity peaks) to ZT0/ZT12 due to their slow and fast running clocks. The arrhythmic clock mutant strains per^{01} and clk^{AR} reacted to the change of light, but could not anticipate these changes independent of the food source. But there is a trend that overall the flies were more active when they could feed on nutrient-rich food which was significant at least for CS, per^L and per^{01} (Fig. 26).

For a summarized and detailed list of all statistics of the total activity levels see Appendix.

3.2.2 of heterozygous and homozygous female flies

Usually only male flies are used for locomotor activity recording due to the fact that the females lay eggs and the developing larvae crawl inside the glass tubes crossing the infrared light beam and therefore falsify the recorded locomotor activity behavior of the female fly. But they do not only falsify the recorded locomotor activity, they also eat up the whole food in the tube and therefore the female fly will starve in only a few days. Nevertheless, to see if the females behave different from the males and to calculate the free running period of not only homozygous but also heterozygous female flies both homozygous and heterozygous female flies were recorded in the course of this thesis. To rule out any influence of developing larvae inside the locomotor activity glass tubes, only virgin female flies were used for the experiments. In Figure 29 the results of the locomotor activity recording of all female flies are shown.

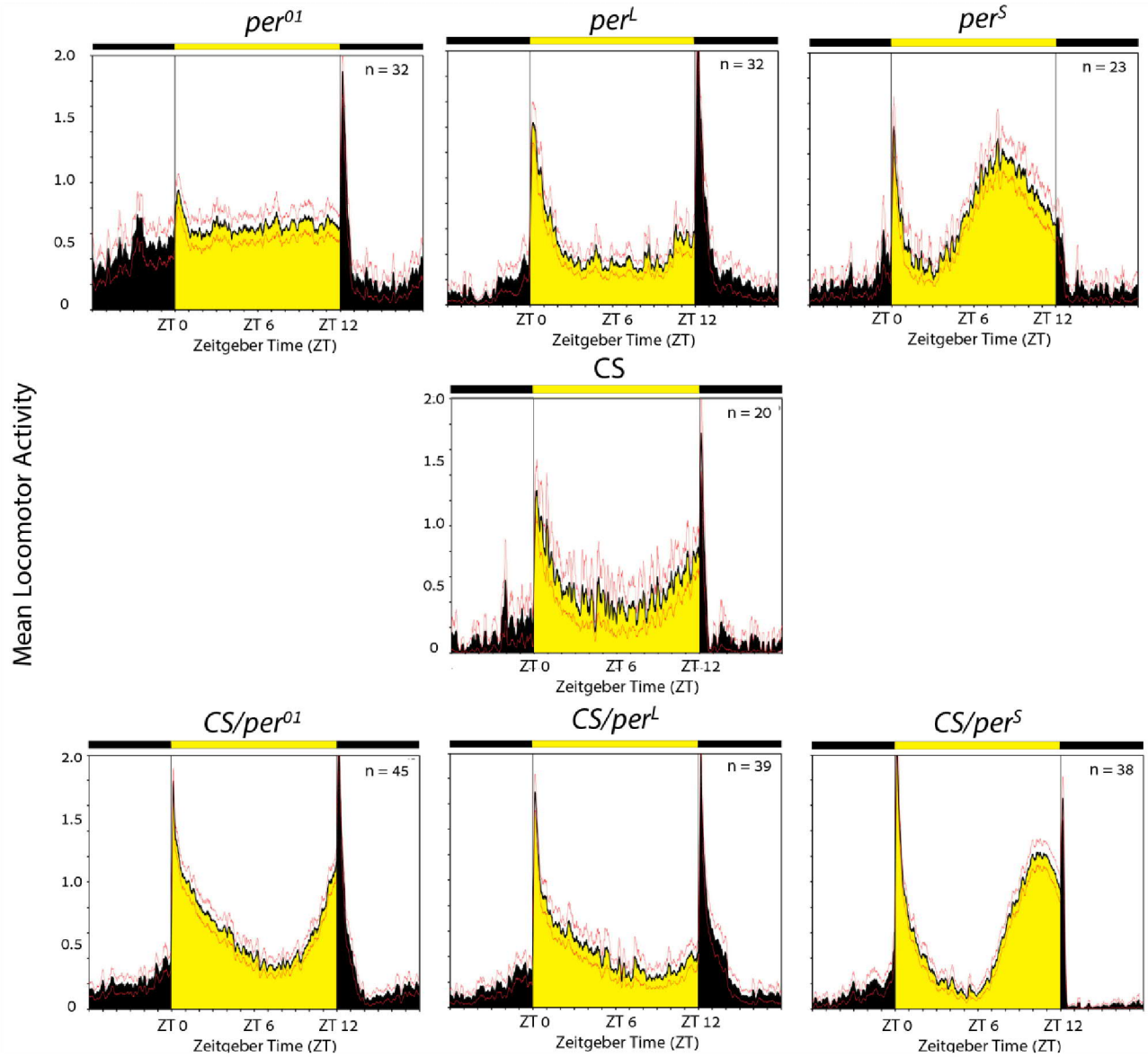

Figure 29: mean locomotor activity of homozygous *period* clock mutant (*per*^{S}, *per*^{L}, *per*^{01}) and wild type CS females as well as heterozygous (CS/*per*^{S}, CS/*per*^{L}, CS/*per*^{01}) females displayed as average days. The number of flies used for calculation of the average day graphs are shown in each upper right corner (n). The black and yellow bars represent the light condition meaning darkness/night (black) and light/day (yellow), respectively. The red line indicates standard ±SEM.

Wild type CS female flies as well as the homozygous clock mutants *per*^{L} and *per*^{S} exhibited the typical bimodal activity pattern with a morning and an evening peak of activity. The *per*^{S} females shifted their evening activity peak to the afternoon due to their fast running clock like the males did. In case of the *per*^{L} females the shift of the evening peak into the night was not that obvious like in the males average day pattern, but it is visible in the prolonged midday trough and later onset of evening activity and later offset after the lights were turned off compared to the wild type females (Fig. 29). The arrhythmic *per*^{01} females showed constant activity throughout the day/light with a period of rest in the first half of the night/darkness and

increasing activity during the second half of the night/darkness (Fig. 29). Overall the locomotor activity pattern of female flies matches the one of male flies.

This can also be seen in the calculation of the free running period of males and females (Tab. 04). The homozygous wild type CS females free running period with τ = 24,79 hours is about half an hour longer than the free running period of the males (τ = 24,24 hours). For per^S τ is almost the same with τ = 19,41 hours for the females and τ = 19,22 hours for the males (Tab. 04). In case of per^L the males exhibit a slightly longer free running period τ with τ = 29,24 hours in comparsion to the females with a τ of 28,28 hours. For per^{01} the males as well as the females are arrhythmic (Tab. 04).

Table 4: free running period of homozygous and heterozygous female as well as male wild type and clock mutant flies

	genotype	period [h+SEM]	power	nr of flies
heterozygous females	CS/per^S	21.59 ± 0.04	29.43 ± 1.16	n = 36
	CS/per^L	25.52 ± 0.13	25.61 ± 1.43	n = 34
	CS/per^{01}	24.89 ± 0.03	42.52 ± 2.15	n = 44
homozygous females	CS	24.79 ± 0.10	25.44 ± 1.86	n = 19
	per^S	19.41 ± 0.17	20.28 ± 1.65	n = 16
	per^L	28.28 ± 0.12	29.06 ± 2.84	n = 30
	per^{01}	arrhythmic	x	n = 30
males	CS	24.24 ± 0.02	48.90 ± 1.04	n = 242
	per^S	19.22 ± 0.05	27.47 ± 0.89	n = 211
	per^L	29.24 ± 0.06	29.40 ± 0.89	n = 202
	per^{01}	arrhythmic	x	

The locomotor activity pattern of an average day of the heterozygous females is quite interesting. All three genotypes – namely CS/per^S, CS/per^L as well as CS/per^{01} – exhibited a bimodal activity pattern. The CS/per^L female flies exhibited a broader morning activity after lights turned on with the midday rest trough shifted to the afternoon and the evening activity peak shifted to the first half of the night after the lights turned off (Fig. 29). The CS/per^S females were not able to time their evening activity peak to the evening and lights-off at ZT12 but shifted their midday trough and main evening activity a few hours earlier into the day. This is similar to the per^S females and males but the evening activity peak is taking place in the late afternoon instead of midday/early afternoon, expressing a mixture of the parental genotypes quite well (Fig. 29). CS/per^{01} females were able to show a typical bimodal activity pattern with

only one functional copy of the *period* gene. The free running period of the flies was only slightly longer with τ = 24,89 hours. The flies had a broader morning activity but could match the evening activity perfectly around ZT12, anticipating the change of light (Fig. 29).

The free running period of heterozygous females matches neither the τ of the homozygous female nor of the male flies. Instead the heterozygous female flies showed a free running period which lays in between the parental genotypes. That is a free running period τ = 21,59 hours for CS/per^S, τ = 25,52 hours for CS/per^L and τ = 24,89 hours for CS/per^{01} (Table 4).

3.2.3 of populations

The locomotor activity of flies is usually recorded for single individuals, but not for populations. However there are some interesting questions about the activity of a population or group of flies: How do the flies influence each other in a restricted area of space? Is it even possible to record rhythmic behavior of a group or population? And if yes, what does the locomotor activity pattern of a population look like? Does it differ from the locomotor activity pattern of a single fly?

Therefore to answer these questions populations of 20 individuals – males or virgin females – per vial were recorded in the LAM25 System in this thesis to investigate the behavior of a population of flies. Some actograms of the population locomotor activity recording can be seen in Figure 30.

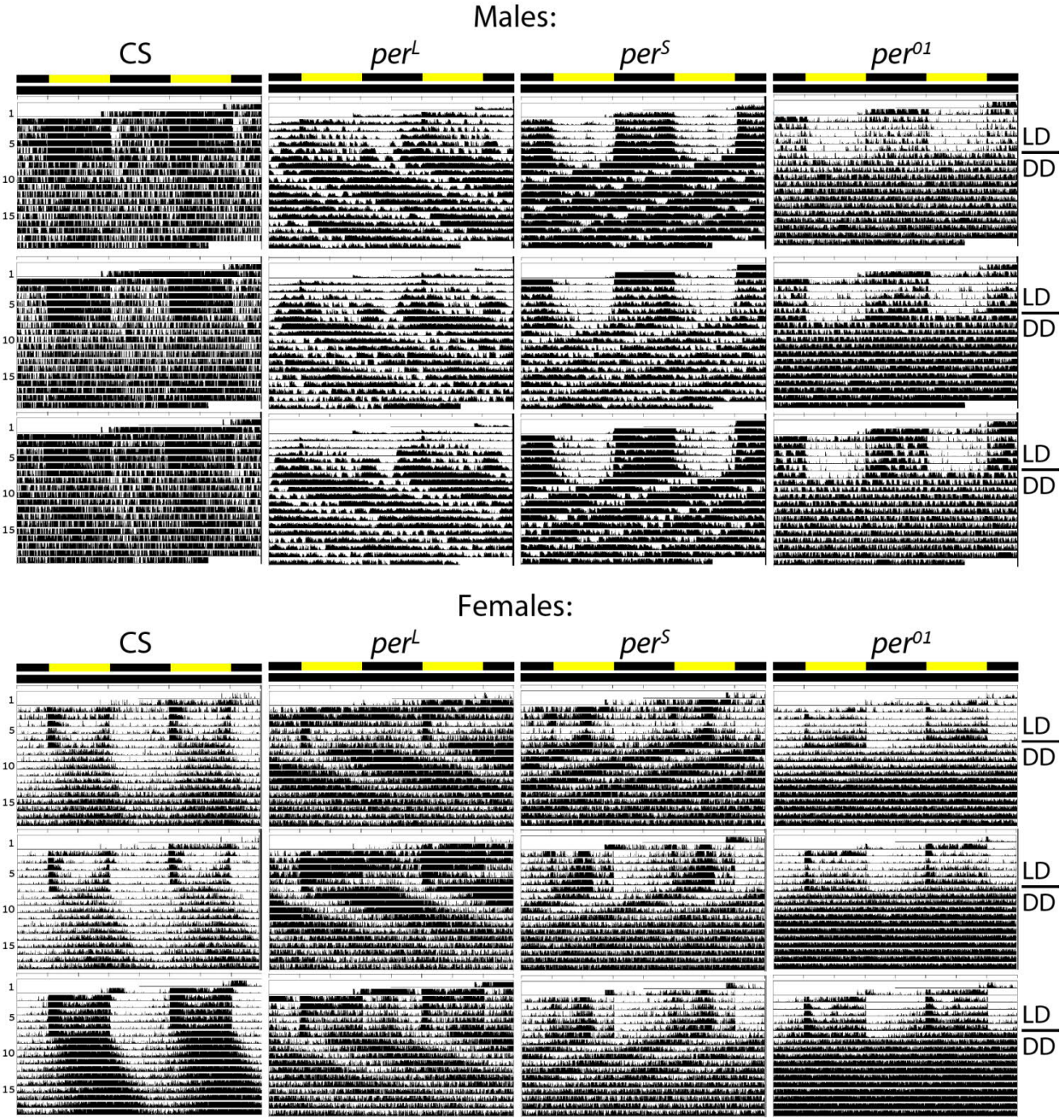

Figure 30: example actograms of populations of 20 males or 20 females each. Flies experienced a light-dark cycle for 7 days (LD 12:12) followed by constant darkness (DD) to record the free running behavior of a population of flies. The upper actogramms depict 20 males each, the lower actograms depict 20 females per actogram. The wild type CS as well as all three *period* mutant strains were recorded (per^S, per^{01}, per^L). Black and yellow bars represent darkness and light respectively.

Interestingly the flies in the wild type and *period* clock mutant populations were synchronized to each other showing the typical locomotor activity pattern that is expected from the single fly recording. In the first seven days of recording the male flies were active in synchrony due to light as Zeitgeber and the light-dark entrainment (LD12:12). The wild type CS and the long *period* mutant per^L exhibited more activity during the day/light than the night/darkness. For

the short and the arrhythmic – *per*^{S} and *per*^{01} respectively – *period* mutant male populations this was the other way round. The populations of male flies preferred to be active during the night/darkness instead of the day/light (males, Fig. 30). After light as Zeitgeber is dismissed and the flies were kept in constant darkness they began to free run. But they did not end up in an arrhythmic locomotor activity profile due to the flies being active with different free running periods. Instead the flies of the population synchronized to each other and free ran together at the same speed creating a rhythmic locomotor activity pattern. This is most obvious for the short and long *period* mutants, *per*^{S} and *per*^{L}, and less obvious but still visible in the wild type CS profiles (males, Fig. 30). The arrhythmic *per*^{01} flies exhibited an arrhythmic locomotor activity profile like expected due to the lack of a functional clock (males, Fig. 30). Due to the synchrony of the fly populations the free running period of the populations could be calculated. For the males the wild type CS populations were free running with a period of τ = 24,40 h, the *per*^{L} populations with τ = 28,37 h and the *per*^{S} populations with τ = 19,20 h. For the females the free running periods of the populations matched the ones of the males with τ = 24,42 h for CS, τ = 28,76 h for *per*^{L} and τ = 19,10 h for *per*^{S} (Tab. 05).

Like the males the females experienced an entrainment (LD12:12) during the first 7 days of recording before the flies were kept in constant conditions (DD) for recording of the free running behavior. The wild type CS activity profile of the females was clearer to see than the one of the male populations with clear distinguishable morning and evening activity. Furthermore the female *per*^{S} and *per*^{01} populations concentrated their main activity to the light/day like it is for a single fly and did not shift their activity into the dark/night like the male populations did (females, Fig. 30). After the LD entrainment in the first 7 days of the recording the flies were kept in DD and all females were free running in synchrony – arrhythmicity for *per*^{01} – according to their endogenous clocks. The about 24 h rhythm in the locomotor activity profiles of the female wild type CS populations were especially clear to see with the main activity taking place during the subjective day (females, Fig. 30).

Table 5: free running period of male and female fly populations. Each population consisted of 20 individual male/female flies

	genotype	period τ [h + SEM]	power	Rhythmic [%]
males	CS	24,40 ± 0,18	19,47 ±2,72	87,5
	per^{L}	28,37 ± 0,10	44,07 ± 1,71	100
	per^{S}	19,20 ± 0,06	55,61 ± 2,29	100
	per^{01}	x	x	
females	CS	24,42 ± 0,10	55,47 ± 8,55	100
	per^{L}	28,76 ± 0,21	38,58 ±3,40	100
	per^{S}	19,10 ± 0,14	37,68 ± 6,52	100
	per^{01}	x	x	

To sum this up the populations – consisting of 20 males or 20 females – were synchronized to the light-dark cycle in the first days of recording and they were synchronized to each other afterwards, resulting in a collective free running behavior with calculable period length τ with around 24 h, 19 h and 29 h according to the genotype or arrhythmicity in the none functional clock mutant per^{01} populations. The locomotor activity profiles of the populations were similar to the profiles of single male or female flies (Fig. 25, 29 and 30).

3.3 lifespan experiments

3.3.1 feeding on different food sources

The lifespan experiments in this thesis were performed to see how the nutritional value of different food sources effects the fitness of flies and therefore the lifespan of the flies. The flies were either fed on nutrient-rich maximum medium – standard cornmeal medium – or nutrient-poor minimum medium – water, agar, sucrose – during the whole experiment. The original fly strains were used for these experiments.

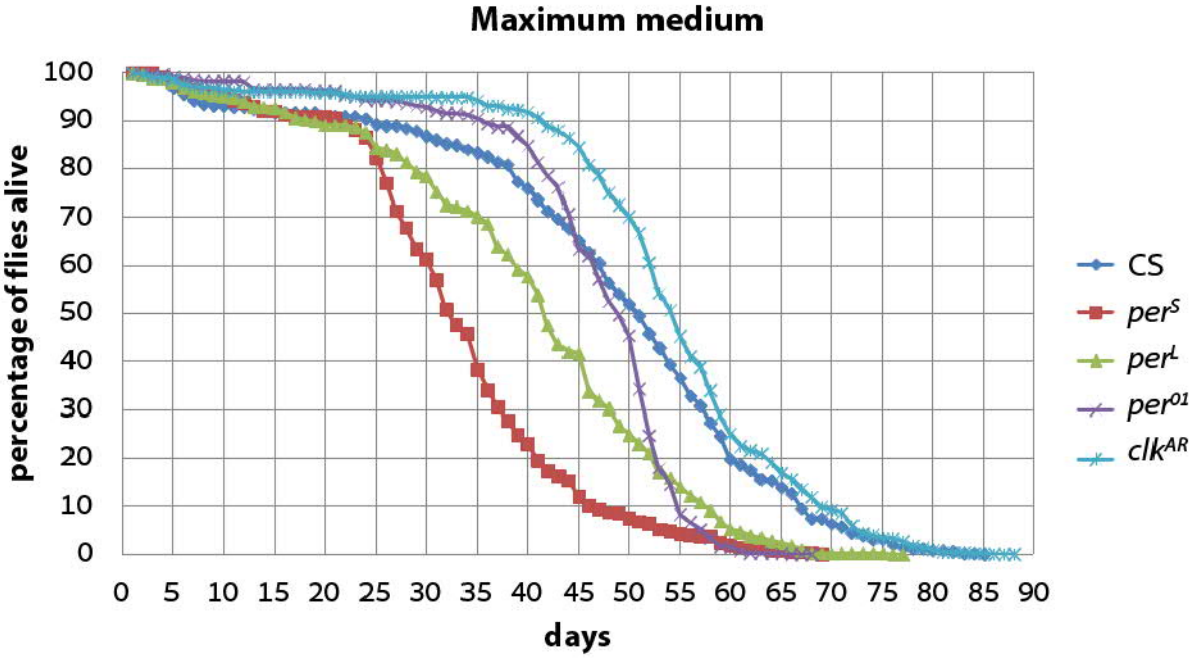

Figure 31: lifespan of wild type CS and clock mutant flies fed on nutrient-rich maximum medium as food source. The arrhythmic *clk*^{AR} and wild type CS flies survived the longest followed by *per*^{01}, *per*^{L} and *per*^{S}. In about the first 30 days only very few flies of all genotypes died. The flies lived up to 66 to 88 days.

On maximum medium the arrhythmic *clk*^{AR} flies survived longest followed by wild type CS, *per*^{L}, *per*^{01} and *per*^{S} flies (timepoint: 10% of flies alive). When 50% of the flies were still alive this pattern was the same except for *per*^{L} and *per*^{01}, where more of the *per*^{01} flies were alive than *per*^{L} (Fig. 31). For the competition assay experiments the flies were flipped every two weeks and therefore it was important to see whether there are any differences in the lifespan/survival of flies after 14 days. But this was not the case. All clock mutants tested on nutrient-rich maximum medium as food source were not significant different from wild type CS. This changed at the timepoint when 90 % of the flies were still alive. At that moment there were significant less *per*^{S} ($p = 0{,}02807$) and more *clk*^{AR} ($p = 0{,}02089$) flies alive. At 50 % flies alive there were less *per*^{S} ($p = 0{,}0003147$) and *per*^{L} ($p = 0{,}006322$) than wild type CS flies. Close to the end of the experiment – with only 10 % of the flies alive – there were significant less flies of all *period* mutants than the wild type CS (*per*^{S}, $p = 0{,}001478$; *per*^{L}, $p = 0{,}01713$; *per*^{01}, $p = 0{,}002432$). The oldest wild type CS fly was 85 days of age when it died. The oldest clock mutant fly of *per*^{S} died at the age of 69 days, of *per*^{L} at the age of 76 days, of *per*^{01} at the age of 66 days and of *clk*^{AR} at the age of 88 days. This means some of the flies lived almost up to three month in the laboratory. But the flies are usually only used for two or three weeks of age to make sure there are no aging effects influencing the results of the experiments.

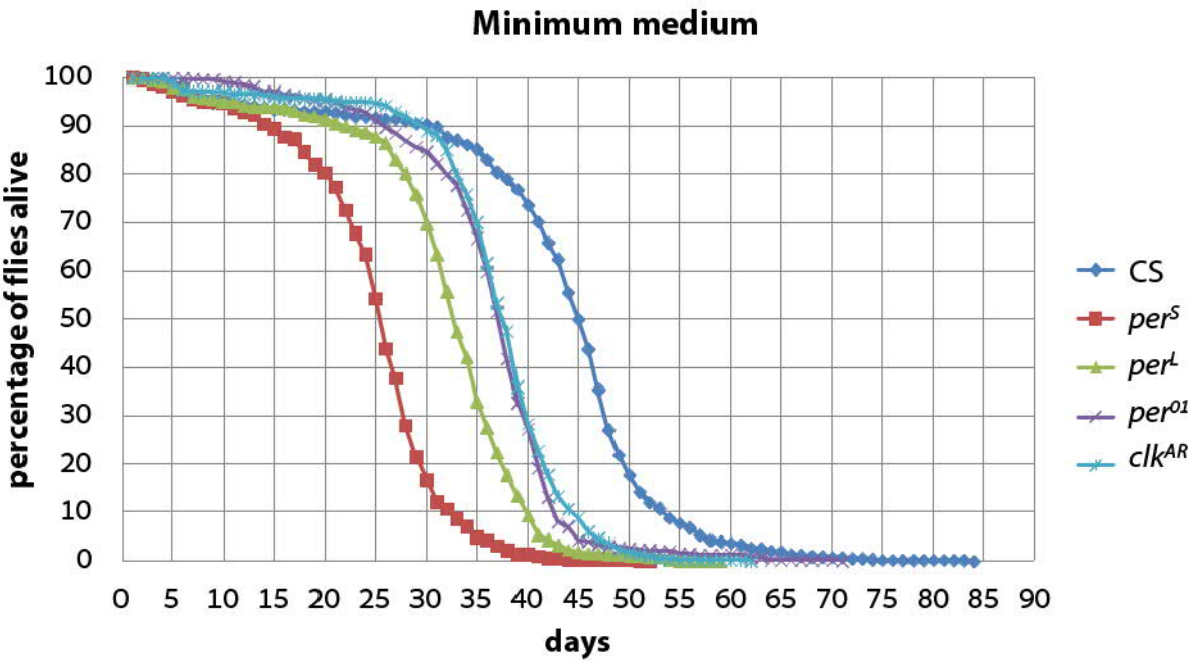

Figure 32: lifespan of wild type CS and clock mutant flies fed on nutrient-poor minimum medium as food source. Wild type CS survived best followed by both arrhythmic genotypes (per^{01}, clk^{AR}), per^{L} and per^{S}. The flies lived up to 53 to 84 days.

Although the nutrients for the flies were strongly limited on the nutrient-poor minimum medium the flies could survive quite long. On this minimum medium the wild type CS flies survived best, followed by the nearly identical arrhythmic per^{01} and clk^{AR} flies. They were followed by the per^{L} and per^{S} clock mutant flies (at 50 % and 10 % flies alive) (Fig. 32). When 90 % of the flies were still alive there were only significant less per^{S} than wild type CS flies ($p = 0{,}007001$). At 50 % alive there were significant less flies alive of all clock mutants than wild type CS flies (per^{S}, $p = 0{,}0002763$; per^{L}, $p = 0{,}0001766$; per^{01}, $p = 0{,}0004245$; clk^{AR}, $p = 0{,}0009004$) as well as at the timepoint of only 10 % flies alive (per^{S}, $p = 0{,}0002695$; per^{L}, $p = 0{,}0001766$; per^{01}, $p = 0{,}00314$; clk^{AR}, $p = 0{,}002151$). On the nutrient-poor minimum medium the wild type CS flies lived up to 84 days, per^{S} 53 days, per^{L} 59 days, per^{01} 71 days and clk^{AR} flies lived up to 62 days.

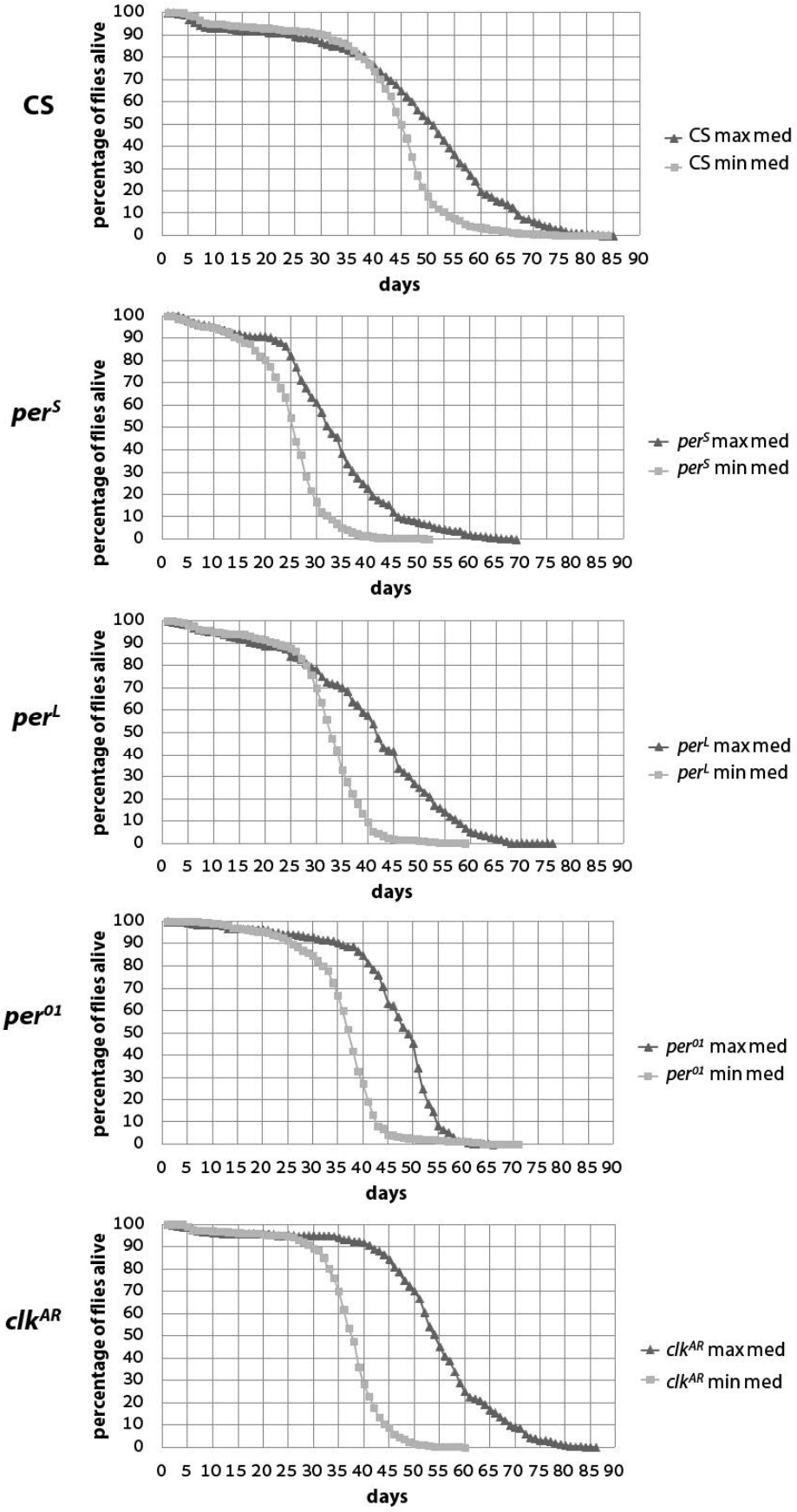

Figure 33: lifespan of wild type CS and clock mutant flies in direct comparison of the food source, namely nutrient-rich (max med) or nutrient-poor (min med) food. Flies of all genotypes fed on nutrient-rich food survived longer than the flies that were fed on nutrient-poor food.

The direct comparison of the flies fed on either maximum or minimum medium reveals that the flies survived longer when they were fed on the nutrient-rich maximum medium as expected (Fig. 33). But surprisingly there were no great differences for the clock mutants per^{L}, per^{01} and clk^{AR} until the age of about 30 days and about 20 days for per^{S}. For the wild type CS this was true for up to about 40 days. Significant differences between the two media could be seen for all genotypes when 50 % of the flies were still alive (CS, p = 0,0137; per^{S}, p = 0,001228; per^{L}, p = 0,000973; per^{01}, p = 0,02813; clk^{AR}, p = 0,003703).

For a list of all statistical values see appendix, Table 11.

3.3.2 at different day length

As the survival competition assays were performed not only in normal 24 hour days (LD12:12) but also in T-cycles of 19 hours or 29 hours or constant light, it was interesting to see how long the flies could survive under these conditions and whether the fitness of a fly is influenced by the day length and how it is affected. To investigate this, the wild type CS and clock mutant flies that are used for the specific day length experiments – per^{L}, per^{S} and per^{01} – were used for the lifespan experiments with the same simulated light conditions that the flies experienced during the survival competition assay experiments. Therefore CS and the short *period* mutant per^{S} were tested in a short-day T-cycle with only 19 hours (LD 9,5:9,5) and CS and the long *period* mutant per^{L} in a long-day T-cycle with 29 hours (LD 14,5:14,5), respectively. Due to the fact that the wild type CS flies become arrhythmic in constant light this was simulated for CS and the arrhythmic per^{01} mutant flies. All flies were fed on nutrient-rich maximum medium for these experiments as this was the same food as used for the competition assay experiments.

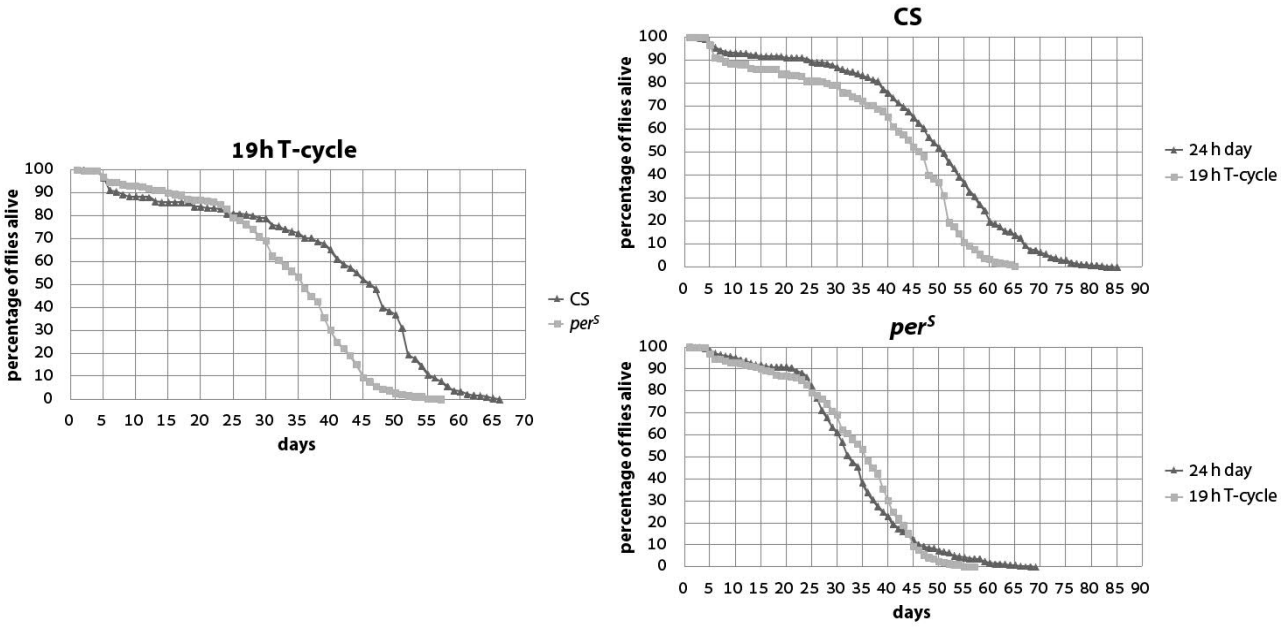

Figure 34: lifespan of wild type and clock mutant *per*^{S} flies in a 19 hour T-cycle (left) and comparison of lifespan of the flies exposed to 24 hour days and 19 hour T-cycles (right). Wild type CS flies survived longer than *per*^{S} mutants in a short 19 h T-cycle. The fitness of wild type CS flies was reduced in a 19 h T-cycle while the fitness of *per*^{S} flies was not impaired.

In the T-cycle of 19 hours the wild type CS flies survived significant longer than the short *period* mutant *per*^{S} when 50 % (p = 0,008032) and 90 % (p = 0,0002435) were still alive (Fig. 34). The direct comparison of a normal 24 h day and the short T-cycle of 19 h revealed that the wild type CS flies lived a significant shorter life when they were confronted with an abnormal short day, the 19 h T-cycle (14 days old p = 0,03051; 50 % alive p = 0,01696; 10 % alive p = 0,006441). This was not the case for the short *period* mutant. The *per*^{S} flies survived in a T-cycle of 19 hours as good as in a normal 24 hour day (Fig. 34).

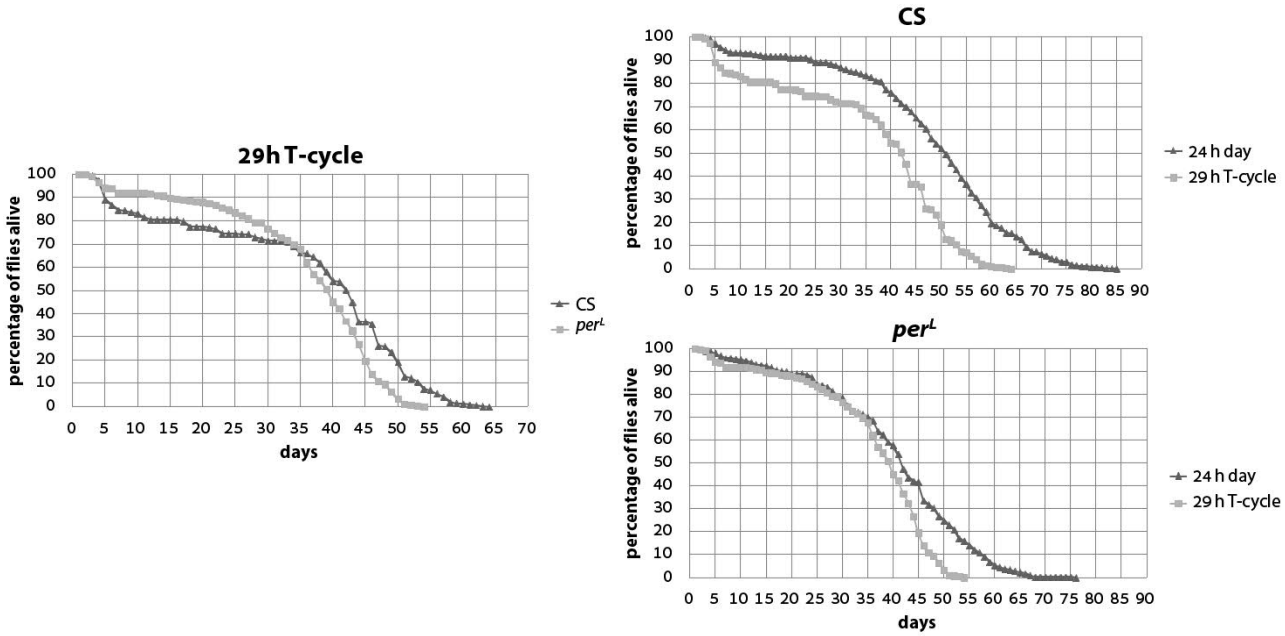

Figure 35:lifespan of wild type and clock mutant per^L flies in a 29 hour T-cycle (left) and comparison of lifespan of the flies exposed to 24 hour days and 29 hour T-cycles (right). Great aged wild type CS flies survived slightly longer than per^L mutants in a long 29 h T-cycle. The fitness of wild type CS flies was significantly reduced in a 29 h T-cycle while the fitness of per^L flies was only slightly impaired in the great aged flies.

Wild-type CS flies survived significant longer than the long *period* mutant per^L at the timepoint of 10 % alive in a T-cycle of 29 hours (p = 0,001438) but at the age of 14 days it is the other way round where the per^L mutants survived significantly better than the wild type CS flies (p = 0,04507) (Fig. 35, left). The wild type CS flies died significantly sooner in a 29 h T-cycle at all timepoints than in a normal 24 h day (p = 0,0007339) revealing fitness deficits due to the daylenght, whereas only the great aged per^L flies died significantly earlier in a 29 h T-cycle (10 % alive, p = 0,001672) (Fig. 35, right).

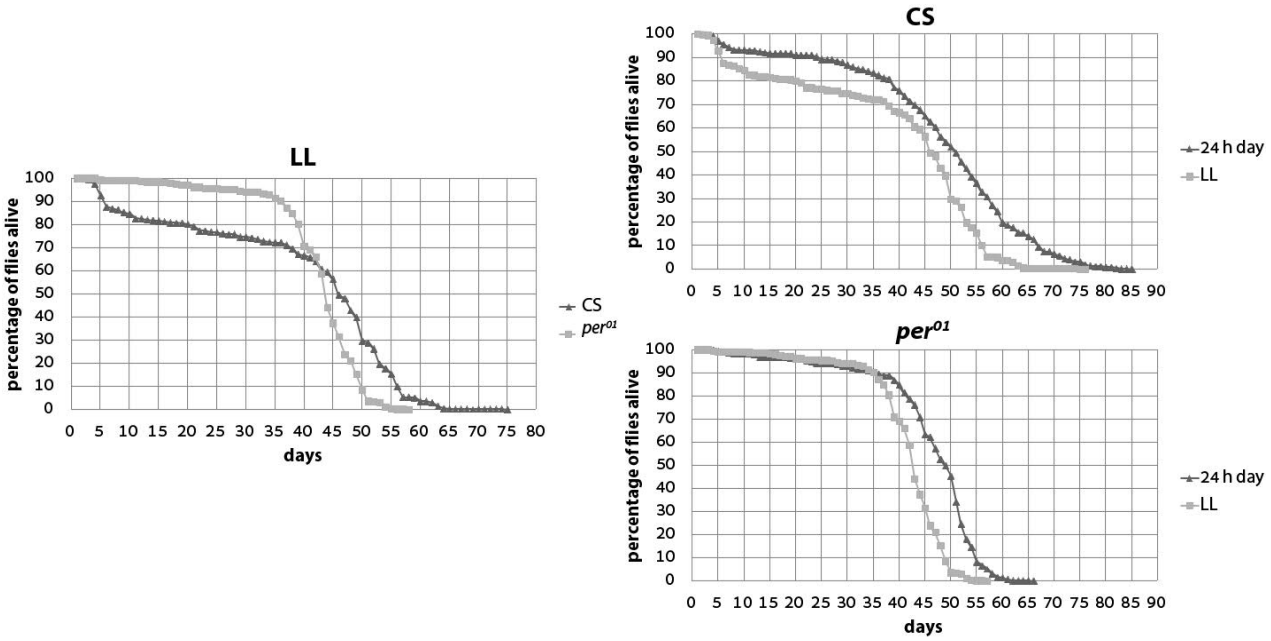

Figure 36: lifespan of wild type and clock mutant *per*^{01} flies in constant light (LL) (left) and comparison of lifespan of the flies exposed to 24 hour days and LL (right). Great aged wild type CS flies survived slightly longer than *per*^{01} mutants in LL. The fitness of wild type CS flies was significantly reduced in LL while the fitness of *per01* flies was only impaired in the second half of the flies lives.

Wild type CS and the arrhythmic *per*^{01} flies showed a similar pattern in LL as the wild type CS and *per*^{L} flies did in the 29 h T-cycle. In LL – in the first half of the flies lives – the *per*^{01} mutants survived significantly better than the wild type CS (90 % alive, p = 0,0002268), but this changed in the second half where the wild type CS flies survived longer (10 % alive, p = 0,0001736) (Fig. 36, left). Again the wild type CS flies survived significantly longer in a normal 24 h day compared to LL (p = 0,03039), whereas only the arrhythmic *per*^{01} flies – like *per*^{L} in the 29 h T-cycle – of great age died earlier in LL than a normal 24 h day (p = 0,0004224) (Fig. 36, right).

For an overview of all statistical values see Table 11 in the appendix.

3.4. newly cantonized fly strains

Fly strains are kept in stock collections in the laboratory for hundreds of generations and many years or decades. In the meantime it can happen that spontaneous mutations arise and stabilize in a fly strain population according to genetic drift. These mutations may influence the development, metabolism or behavior of the flies or just stay in the genome with no effects. Therefore this so called genetic background is very important and the experimental fly strains should in theory have the identical genetic background as the control strain(s) except for the investigated gene/mutation. To achieve the maximum possible conformity of control and

experimental fly strains the experimental fly strains are crossed with the corresponding control strains every now and then to minimize differences in the genetic background and therefore side effects during the experiments that are not due to the investigated mutation but to the genetic background. Based on that the *period* fly strains used in this thesis were naturally backcrossed to the wild type CS strain in the course of the first competition assay experiment performed (LD12:12, ABC) and the newly cantonized *period* mutation fly strains were selected for the *period* mutation around generation 54 as well as the wild type CS strain as corresponding control strain for further experiments.

The original and the newly cantonized fly strains did overall not differ in the phenotype and also the behavior of the flies was the same. There was only one exception for the long *period* mutant per^{L} which showed a lengthening of the endogenous free-running period in the course of the competition assay experiment.

The original wild type CS flies showed a free running period of 24,3 ± 0,04 h, the per^{S} flies a period of 19,0 ± 0,03 h and the per^{L} flies a period of 27,6 ± 0,06 h, as well as no rhythm for the arrhythmic per^{01} flies (Tab. 06). The newly cantonized fly strains were also analysed for their free running period, as well as the flies during the competition assay at every third generation when the genotype distribution was determined. This was done to see whether the period changes during the competition of flies due to the natural backcrosses and changes of genetic background or the competition situation.

Table 6: free running period τ [h ± SEM] of the original and newly cantonized *period* mutant fly strains as well as the corresponding wild type CS.

	genotype	period τ [h±SEM]
original fly strains	CS	24,3 ± 0,04
	per^{L}	27,6 ± 0,06
	per^{S}	19,0 ± 0,03
	per^{01}	x
newly cantonized fly strains	CS	24,23 ± 0,03
	per^{L} in 29h T-cycle	30,5 ± 0,71
	per^{L} in 24h T-cycle	31,1 ± 0,87
	per^{S}	19,43 ± 0,02
	per^{01}	x

The results showed clearly a lengthening of the free running period of the *per*^{L} flies. The *per*^{L} flies developed a period of 31,1 ± 0,87 h in the 24 hour simulation and a period of 30,5 ± 0,71 h in the long T-cycle simulation with 29 hours per day (Tab. 06). The period lengthening of *per*^{L} flies was visible in all vials measured (Fig. 37). The other strains and their free running period stayed rather constant and did not change as the long *period* mutant did (Tab. 06). The *per*^{L} flies were also analysed for their period lengthening kept alone without a competition environment. The results showed also a lengthening, but this lengthening appeared to be less compared to the effect of lengthening under competition (for more details see Horn et al., 2019).

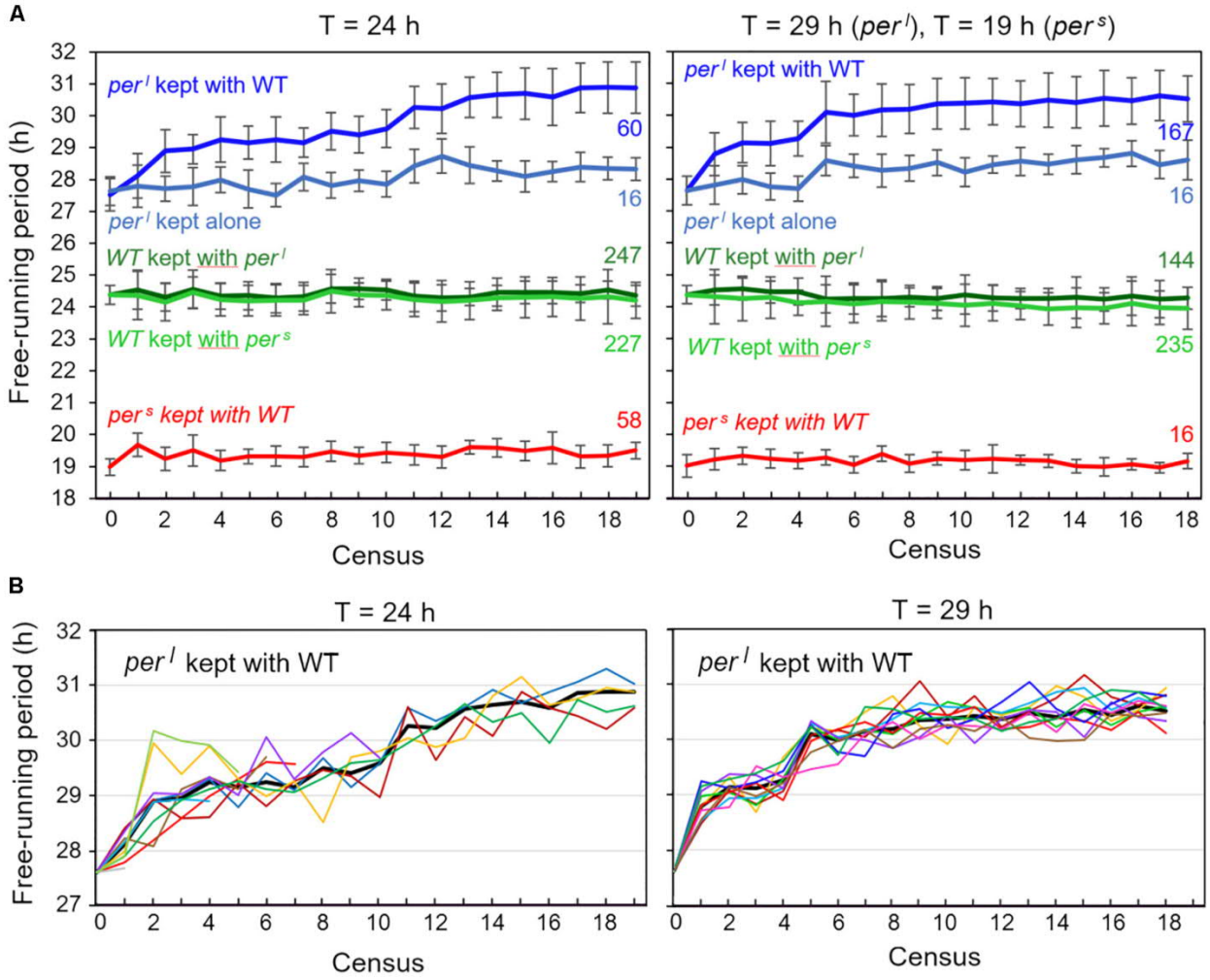

Figure 37: Free-running periods of flies recorded during the competition assay experiments started with the original fly strains at a T-cycle of 24 hours (left) or T-cycles of 19 or 29 hours (right). Every census, 32 flies of each of the 10 vials with the competing genotypes (*per*^{L} /WT, *per*^{S} /WT) were recorded in DD and the flies' free-running periods determined and plotted (mean of all flies from all 10 vials ± SD). A. The experiment started with equal numbers (~160 at census 1) for each genotype and ended with a lower number of mutants, except for *per*^{L} mutants kept under T = 29h (right diagram). The numbers at the right margin give the number of individuals for each genotype at the last census (19 under T=24h and 18 under T=19h/29h). In case of *per*^{S} the flies included

in the determination of period stem only from few vials (3 under T=24h, and 1 under T=19h). In case of *per*^{L}, the number of vials was larger (4 under T=24h, and 10 under T=29h). Wildtype flies were present in all 10 vials, except for the competition experiment with *per*^{L} mutants under T=29h. Here the calculated periods at census 18 stem from flies in 8 vials. Note that the sum of mutants and WT flies is always lower than 320, because some flies died during the recording. The pale blue curve give the mean period of *per*^{L} mutants that were kept separately (not in competition with wild type flies under the same environmental conditions as the experimental animals) and that were recorded in parallel to the other flies (16 flies per census). While the *per*^{L} mutants grown in competition with WT flies lengthened their period by ~3 h, the ones kept separately did so only by <1 h. B. Period lengthening of *per*^{L} mutants over the course of the competition studies in the single fly vials at T = 24h and T = 29h. The mean free-running periods of the flies for each of the 10 vials are shown as coloured lines. The thick black line shows the average period of all flies that is also depicted in Figure 9A. At T =24h, *per*^{L} mutants persisted only in 4 vials until the end of the experiment. Therefore, 6 coloured lines ended before census 19. This is different at T = 29h, where *per*^{L} mutants persisted in all 10 fly vials (graph and description from Horn et al., 2019).

The results indicated that the lengthening was not exclusively caused by founder effects or genetic drift, but were due to the competition situation with the wild type flies or a combination of competitive and genetic factors.

Some representative example actograms of the original and the newly cantonized *period* mutant fly strains as well as the correspondent wild type CS strains are shown in Figure 38.

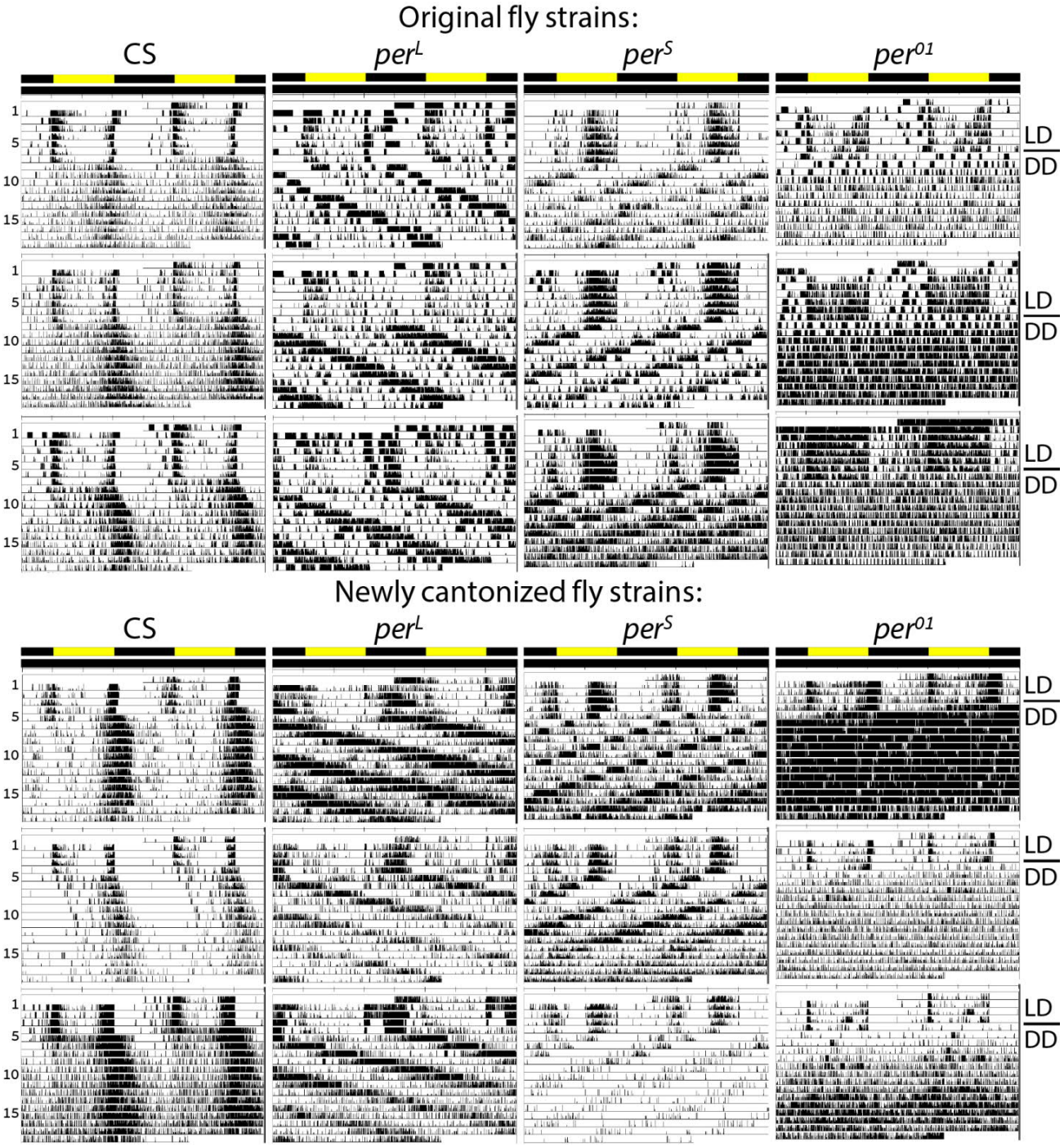

Figure 38: example actograms of the original and the newly cantonized fly strains used in this thesis. Flies experienced a light-dark cycle for 4 to 7 days (LD 12:12) followed by constant darkness (DD) to record the free running period of the flies. The original wild type CS as well as the short *period* (per^S) and the arrhythmic (per^{01}) mutant did not differ from the corresponding newly cantonized fly strain in the locomotor activity behavior. The newly cantonized long *period* mutant (per^L) showed a slightly longer free running period than the original per^L fly strain. Black and yellow bars represent darkness and light, respectively.

All flies of the original and the newly cantonized fly strains exhibited the typical locomotor activity pattern. The wild type CS flies showed a free running rhythm of about 24 hours in constant darkness. The per^L flies began to start their main activity of the day a little bit later and the per^S flies a little bit earlier every day in DD resulting in free running rhythms of about

29 hours and 19 hours, respectively. The *per*^{01} flies were arrhythmic in constant conditions (Fig. 38) and therefore no free running period could be calculated.

3.5. humidity and eclosion

3.5.1 eclosion at different relative humidity values

It is thought that *Drosophila melanogaster* ecloses in the morning hours of a day in nature due to the high relative humidity in the morning preventing the desiccation of newly eclosed flies and ensuring the proper wing expansion (e.g. Pittendrigh, 1954). But what happens if a fly`s clock is not working properly or at a different pace and the fly ecloses at the "wrong" time of the day? Do they really suffer from desiccation or are they not able to unfold their wings and therefore are unable to fly and meet and mate with others? Or does the fly eclose and unfold the wings without any problems? To answer these questions in this thesis the fly pupae were placed in an incubator at different relative humidity values for eclosion. The results of the wild type CS and *period* clock mutant flies that eclosed in 2 %, 60 % and 80 % rH can be seen in Figure 39.

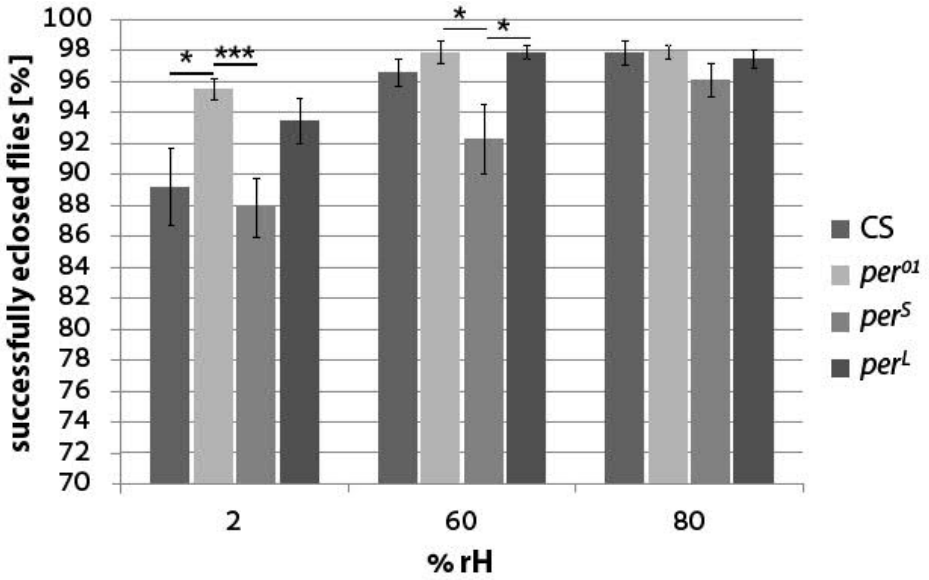

Figure 39: genotype comparison of the proportion of flies that eclosed successfully in different relative humidities (2/60/80 % rH). At 2 % rH significant more *per*^{01} flies eclosed successfully than wild type CS and *per*^{S} flies. At 60 % rH significant less *per*^{S} flies eclosed successfully than *per*^{01} and *per*^{L} flies. At 80 % rH there were no significant differences in eclosion success. Significant differences are indicated by asterics (* = $p < 0,05$; *** = $p < 0,01$). Black bars represent SEM.

At 2 % rH the eclosion success of the arrhythmic *per*^{01} flies was significantly higher than that of the wild type CS ($p = 0,04113$) and the *per*^{S} ($p = 0,004329$) clock mutant flies with 95,55 %

compared to 89,22 % (CS) and 87,89 % (*per*^{S}). Of *per*^{L} 93,46 % of the flies eclosed successfully which was not significant different from the other strains. At 60 % rH *per*^{S} flies had the lowest eclosion success with 92,31 % compared to the wild type CS (96,58 %), *per*^{01} (97,94 %) and *per*^{L} (97,91 %) flies which was significant different from *per*^{L} and *per*^{01} ($p = 0,02002$) (Fig. 39). At a relative humidity of 80 % there were no significant differences for all wild type CS and *period* clock mutant fly strains with very high eclosion success for all fly strains, namely 97,92 % for CS, 97,95 % for *per*^{01}, 96,13 % for *per*^{S} and 97,52 % for *per*^{L}.

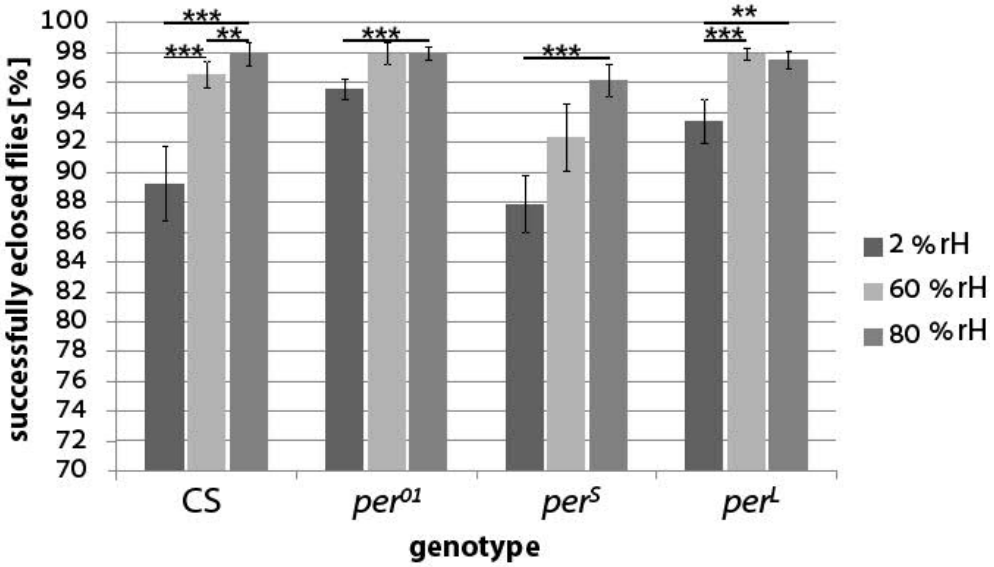

Figure 40: proportion of flies that eclosed successfully in different relative humidity values (2/60/80 % rH) for wild type CS and *period* clock mutant flies (*per*^{01}, *per*^{S} and *per*^{L}). In all genotypes the eclosion success of the flies was significant higher at 80 % than in 2 %. Significant differences are indicated by asterics (** = $p < 0,02$; *** = $p < 0,01$). Black bars represent ±SEM.

When the data is arranged for each genotype the differences in eclosion success at different relative humidity values becomes visible (Fig. 40). For all genotypes the eclosion success was significantly higher in 80 % than in 2 % (CS, $p = 0,001332$; *per*^{01}, $p = 0,007592$; *per*^{S}, $p = 0,003853$; *per*^{L}, $p = 0,01199$), but there was only a significant difference for wild type CS flies between 60 % and 80 % ($p = 0,01265$). Hence the eclosion success of the flies increased with rising relative humidity values.

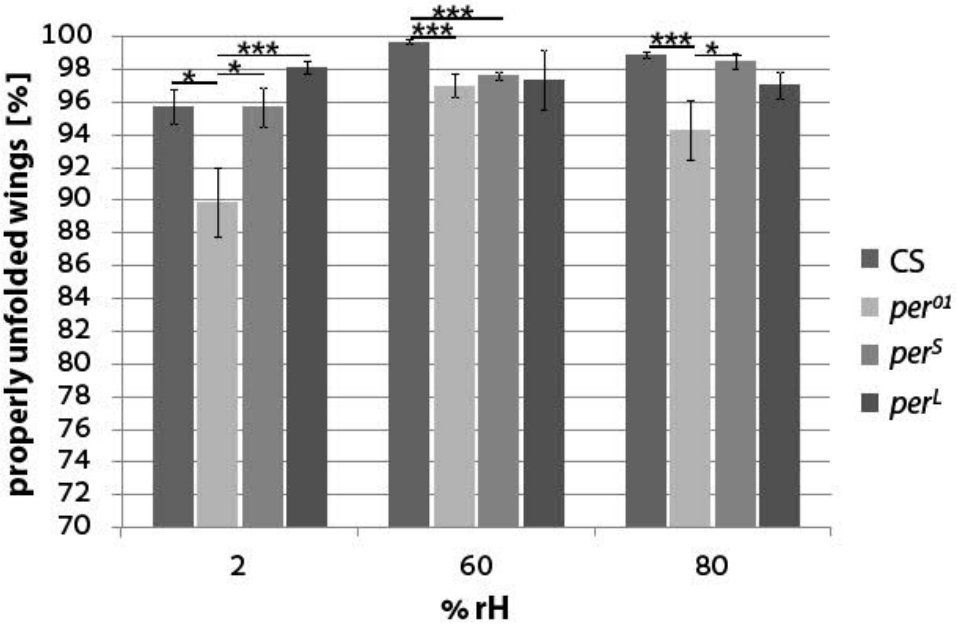

Figure 41: genotype comparison of the proportion of flies that unfolded their wings successfully after eclosion in different relative humidity values (2/60/80 % rH). There were significant differences in successful wing expansion especially for the arrhythmic *per*^{01} flies that were not able to unfold their wings properly compared to the wild type CS flies at all three relative humidity values measured. Significant differences are indicated by asterics (* = $p < 0,05$; *** = $p < 0,01$). Black bars represent ±SEM.

The successful or proper wing expansion at different relative humidity values caused more problems for the arrhythmic *per*^{01} flies than for the other wild type or *period* clock mutant fly strains *per*^{S} and *per*^{L} (Fig. 41). At 2 % rH there were significant differences for *per*^{01} compared to all other fly strains tested (CS, p = 0,04113; *per*^{S}, p = 0,02597; *per*^{L}, p = 0,002165). 89,88 % of the successfully eclosed *per*^{01} flies managed to unfold their wings properly compared to 95,72 % of CS, 95,70 % of *per*^{S} and 98,16 % of *per*^{L} flies (Fig. 41). At 60 % rH there were no significant differences between the three *period* mutant fly strains, but the wild type CS flies had significantly more success unfolding their wings properly than the arrhythmic *per*^{01} (p = 0,007796) and the *per*^{S} (p = 0,004772) flies. But overall between 97 and nearly 100 % of the flies succeeded in proper wing expansion at 60 % rH (CS : 99,70 %, *per*^{01} : 97,02 %, *per*^{S} : 97,61 %, *per*^{L} : 97,41 %). When the flies eclosed at 80 % rH, again almost all flies managed to unfold their wings properly with no significant differences between wild type CS and the short and long clock mutant flies, *per*^{S} and *per*^{L} respectively (Fig. 41). Overall 98,87 % of CS, 94,30 % of *per*^{01}, 98,53 % of *per*^{S} and 97,05 % of *per*^{L} flies that eclosed successfully at 80 % rH unfolded their wings properly, which indicated a significant lower wing expansion rate for *per*^{01} flies compared to wild type CS (p = 0,002468) and *per*^{S} mutant flies (p = 0,02162) (Fig. 41).

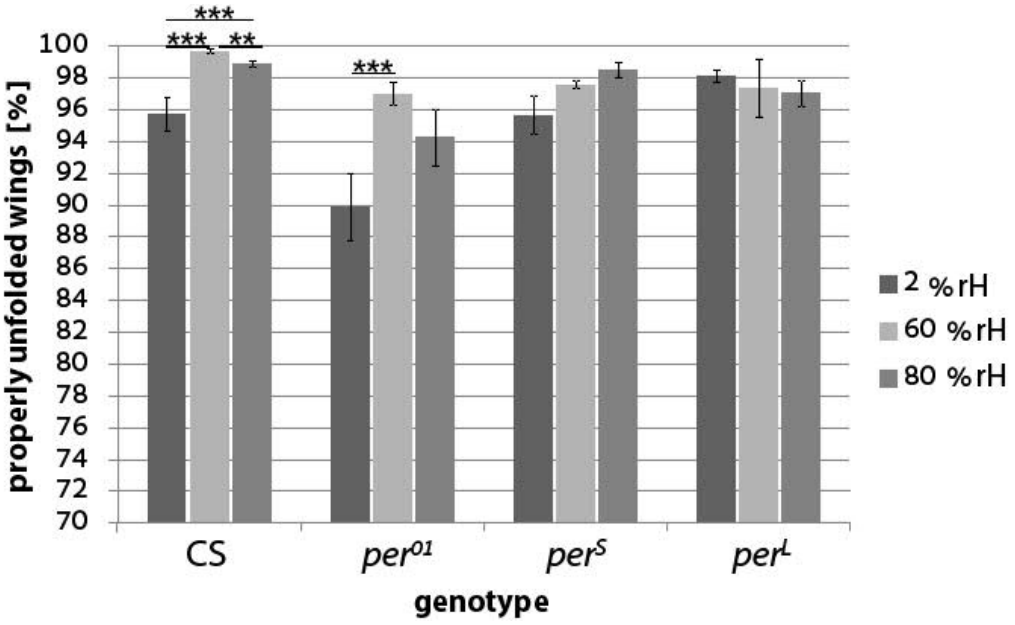

Figure 42: proportion of flies that unfolded their wings successfully after eclosion in different relative humiditiy values (2/60/80 % rH) for wild type CS and *period* mutant flies (per^{01}, per^{S} and per^{L}). Most significant differences were present for wild type CS flies, whereas there were no significant differences for per^{S} and per^{L} clock mutant flies. Significant differences are indicated by asterics (** = $p < 0,02$; *** = $p < 0,01$). Black bars represent ±SEM.

Again the different arranged data for each genotype visualizes the differences in successful wing expansion at different relative humidity values (Fig. 42).The *period* clock mutant fly strains unfolded their wings with similar success at 2 %, 60 % and 80 % rH except for the arrhythmic per^{01} flies that unfolded significant less wings properly at 2 % rH ($p = 0,0004329$) (Fig. 42). In case of the wild type CS flies there were significant differences between all three relative humidity values measured with the lowest success at 2 % rH compared to 60 % rH ($p = 0,004772$) or 80 % rH ($p = 0,007992$).

For a summary of all eclosion success and wing expansion success values as well as the statistics see Appendix, Table 12 and 13.

3.5.2 humidity as a potential Zeitgeber

Light is known to be a Zeitgeber to synchronize *Drosophila melanogaster´*s clock to the environment for a long time (e.g. Emery et al., 1998; Schlichting and Helfrich-Forster, 2015) and also other factors like temperature or social contacts are known to function as Zeitgebers (e.g. Pittendrigh et al., 1958; Wheeler et al., 1993; Levine et al., 2002; Yoshii et al., 2005). But it is not known if *Drosophila melanogaster* can be entrained via humidity as a Zeitgeber. Therefore humidity as a potential Zeitgeber was tested in this thesis. In the first few days of

locomotor activity recording the flies experienced a daily light dark change (LD12:12) as well as a relative humidity cycle (rH-cycle) according to an average day in nature (location used for calculation of an average day rH-cycle: University of Würzburg, Beestation). These conditions were followed by constant darkness – to exclude light as Zeitgeber – and a continuing rH-cycle that was shifted for 8 hours (delay in HE01 and advance in HE02) to see if the flies start to free run or if the flies shift and/or change their locomotor behavior due to the cycle of relative humidity. Some representative example actograms of both experiments can be seen for wild type CS and the three *period* clock mutant fly strains investigated in Figure 43 and Figure 44.

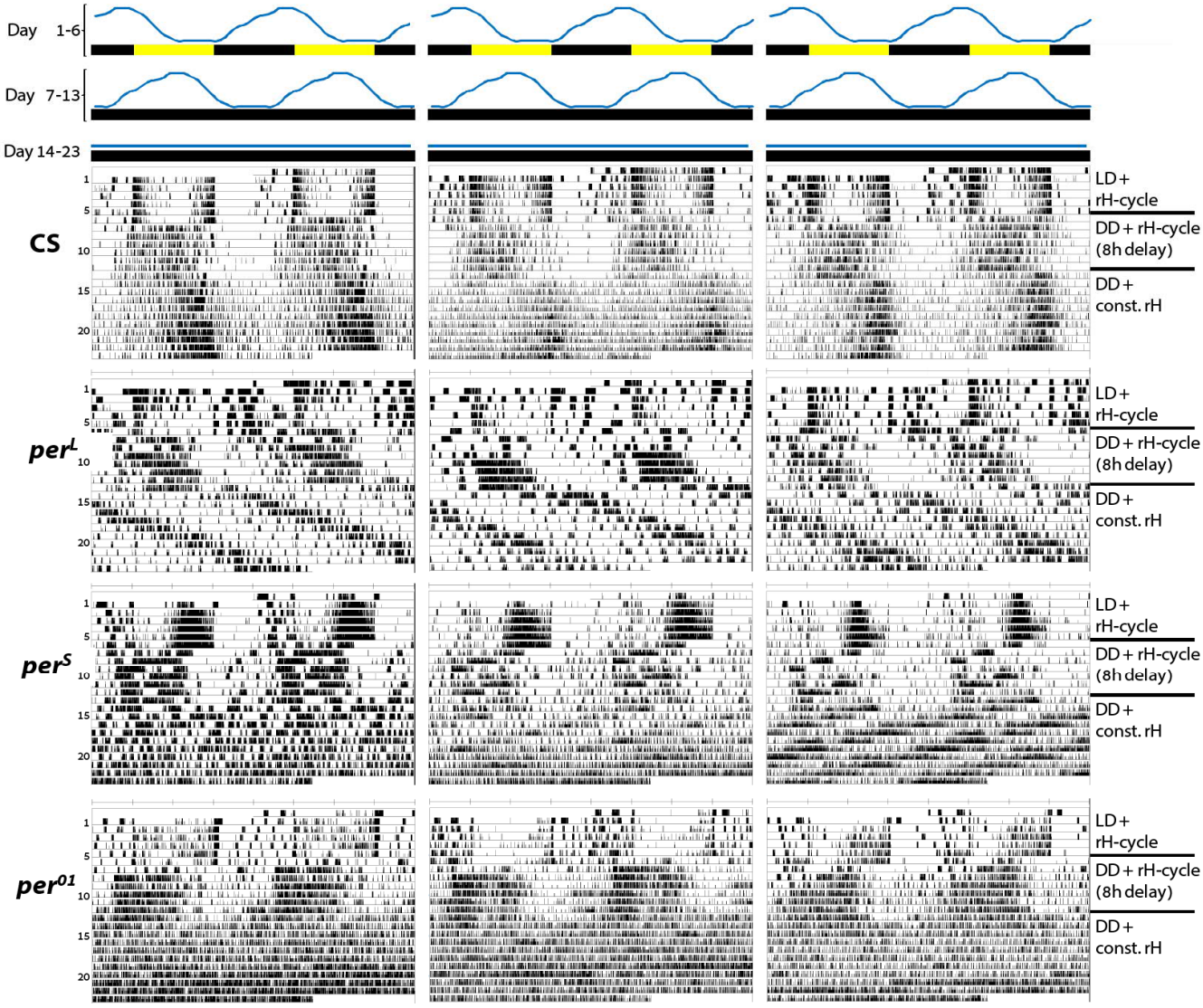

Figure 43: example actograms of wild type CS and *period* clock mutant flies in humidity entrainment 01 (HE01). First the flies experienced a light-dark cycle (LD12:12) combined with a humidity cycle like an average day in nature (LD + rH-cycle). After that light was turned off to constant darkness (DD) and the humidity cycle was delayed for 8 hours (DD + rH-cycle 8h delay). At the end the flies were recorded in constant darkness and constant humidity (DD + const. rH) to record the normal free running period of the flies. All genotypes reacted to the change in humidity and changed their locomotor activity behavior. More or less all genotypes tested presented the normal bimodal activity (day 1-6) with a later onset of evening activity in per^L and an earlier onset in per^S. In

constant darkness and the delayed rH-cycle (day 7-13) all genotypes reacted to the cycling relative humidity by concentrating their locomotor activity to the hours of rising rH values (30-80 %) during the subjective day but already showing free run behavior (*per*^{S} and *per*^{L}) within this time frame as well as rhythmic behavior for *per*^{01}. In constant condition (day 14-23) all flies were free running (CS, *per*^{S}, *per*^{L}) or arrhythmic (*per*^{01}) according to their endogenous clocks. Black and yellow bars represent darkness and light, respectively, blue lines represent cycling relative humidity or constant humidity.

Wild type CS flies exhibited a normal bimodal activity pattern during day 1-6 locomotor activity recording in LD12:12 with corresponding humidity cycling followed by constant but concentrated activity during the time frame of rising relative humidity values in DD with an 8 h delay (day 7-13) (Fig. 43). When the flies experienced constant conditions with no light and no cycling humidity the flies were in free run with most activity in the subjective evening according to the first few days of the recording.

The long *period* mutant *per*^{L} concentrated the main activity the same way as the wild type CS flies did during the daily rising rH values at day 7-13, but within this time slot it seems like the flies were already in free run with activity starting later on each following day ending in a normal free run in constant conditions at day 14-23 (Fig. 43). This became also obvious at the calculation of the free running period of the *per*^{L} flies, which was about 1,5 hours longer in DD + humidity cycle (τ = 25,57 h) than 24 hours but about 2 hours shorter than in constant conditions (τ = 27,67 h) (Table 07).

The situation for the short *period* mutant *per*^{S} was similar to *per*^{L}. The first days of recording the flies exhibited a bimodal activity pattern with a morning and a to the afternoon shifted evening activity peak according to the fast running endogenous clock (Fig. 43). In the second part of the recording – day 7-13 – the *per*^{S} flies concentrated their locomotor activity in the time frame of rising humidity but with free running elements like *per*^{L} flies did. In this time frame the *per*^{S} flies exhibited a free running period of τ = 20,76 h compared to τ = 19,04 h in constant conditions at day 14 to 23 (Tab. 07).

The arrhythmic *per*^{01} flies also concentrated their main activity of the day to the time frame of rising humidity at day 7 to 13 which appeared to be at the second half of the subjective night and early morning of the subjective day (Fig. 43) resulting in rhythmic behavior with a free running period of τ = 23,95 h matching the wild type CS (Tab. 07). After the change to constant humidity and DD the *per*^{01} flies were constantly active throughout the days presenting their arrhythmicity (Fig. 43).

Table 7: free running period τ of wild type CS and *period* clock mutant fly strains (*per*^{L}, *per*^{S} and *per*^{01}) during humidity entrainment HE01 and HE02.

Experiment / shift	genotype	DD + humidity cycle			DD + constant humidity			
		period [h±SEM]	power	rhythmic [%]	period [h±SEM]	power	rhythmic [%]	n
HE01/ 8 h delay	CS	23,96 ± 0,09	38,05 ± 1,60	100	24,18 ± 0,05	36,89 ± 2,47	100	31
	per^{L}	25,57 ± 0,39	32,51 ± 1,34	96,55	27,67 ± 0,13	31,21 ± 1,96	93,10	29
	per^{S}	20,76 ± 0,45	33,14 ± 1,53	96,67	19,04 ± 0,10	21,74 ± 1,75	80	30
	per^{01}	23,95 ± 0,08	35,91 ± 1,99	95,24	27,83 ± 1,19	19,75 ± 1,54	14,29	21
HE02/ 8 h advance	CS	24,35 ± 0,04	51,49 ± 2,47	100	24,32 ± 0,07	45,16 ± 3,83	100	31
	per^{L}	25,08 ± 0,44	28,89 ± 1,29	100	27,46 ± 0,14	28,12 ± 1,38	100	24
	per^{S}	20,03 ± 0,52	27,84 ± 1,47	100	18,88 ± 0,09	25,84 ± 2,34	80	20
	per^{01}	23,98 ± 0,04	36,21 ± 1,52	100	x	x	0	31

To see how the flies change their locomotor activity behavior due to cycling relative humidity vales or if they can be entrained by humidity cycles, I performed not only an experiment with a delay in the second part of the locomotor activity recording, but also an experiment with an advance of the humidity cycle. Example actograms are depicted in Figure 44.

Like the experiment HE01 with a delay of the rH-cycle the wild type CS flies exhibited a normal bimodal activity pattern during the first six days of recording in LD12:12 with corresponding humidity cycling in experiment HE02. In the second part of the recording the flies concentrated their locomotor activity to the time frame of rising and high relative humidity values, which appeared to be in the second half of the subjective day and the first half of the subjective night. After the main activity of the flies, the flies were hardly active at the subjective morning where their morning activity took place in the prior days recording. In the third part of the recording – with no light and no cycling humidity present – the flies showed their typical free running behavior continuing from the second part with no shift (Fig. 44).

The long *period* mutant *per*^{L} also concentrated the main activity in the time frame of rising humidity values at day 7 to 15 with a period of τ = 25,08 h but with elements of free run being present (*per*^{L}, red line, Fig. 44). Afterwards the flies continued to free run and started to be active a little bit later each day in the third part of recording (day 16 – 24) with a period of τ = 27,46 h (Tab. 07).

The short *period* mutant *per*^{S} exhibited a bimodal activity pattern during day 1 to 6 – in LD and corresponding humidity cycling – with the evening activity peak shifted to the afternoon due to their fast running endogenous clock. The flies started to free run right after light as

Zeitgeber was absent from day 7 onwards (per^S, above red line, Fig. 44) with a free running period of τ = 20,03 h in the second part (day 7-15) and τ = 18,88 h in the third part (day 16-24) of the recording (Tab. 07). But during day 7-15, when the flies experienced the advanced humidity cycle, the flies concentrated their activity in the time frame of rising/high humidity values and suppressed activity during the low humidity values like the wild type CS, per^L and per^{01} flies did (Fig. 44). This became visible in the actograms by an interruption of the free run (per^S, below red line, Fig. 44) which cannot be seen in constant darkness combined with constant humidity.

The arrhythmic per^{01} flies showed the typical locomotor activity pattern in the first 6 days of recording when the LD and rH-cycle was simulated. But the per^{01} flies exhibited a rhythmic locomotor activity pattern with the concentrated main activity during the rising and high relative humidity values with a period of τ = 23,98 h (Tab. 07) in the second part of the recording when light was absent and only the shifted humidity cycle was present. At the last days of the recording (day 16-24) the per^{01} flies were arrhythmic when no light and no humidity cycle was present (Fig. 44).

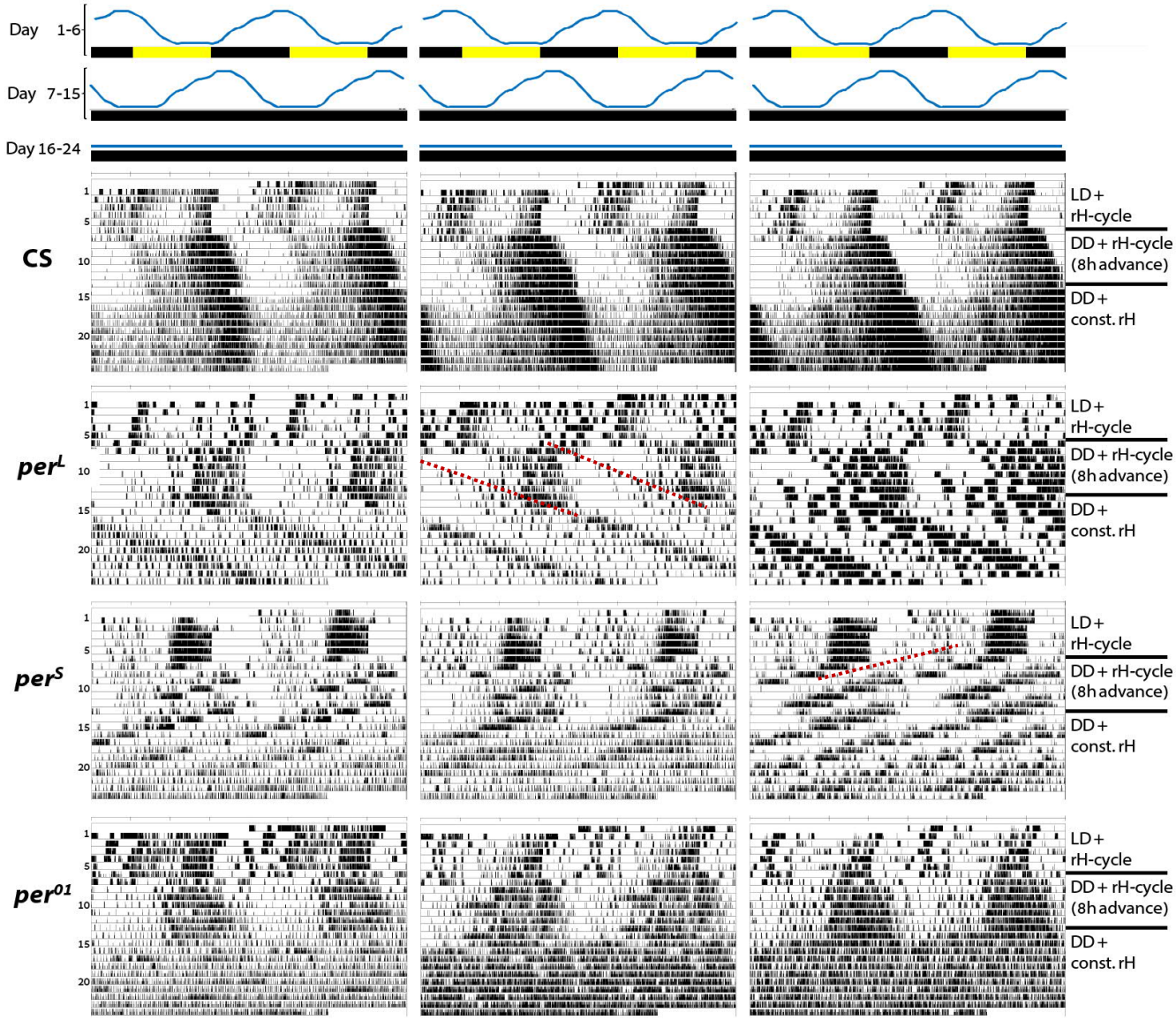

Figure 44: example actograms of wild type CS and *period* mutant flies in humidity entrainment 02 (HE02). First the flies experienced a light-dark cycle (LD12:12) combined with a humidity cycle like an average day in nature (LD + rH-cycle). After that light was turned off to constant darkness (DD) and the humidity cycle was advanced for 8 hours (DD + rH-cycle 8 h advance). At the end the flies were recorded in constant darkness and constant humidity (DD + const. rH) to record the normal free running period of the flies. All genotypes reacted to the change in humidity and changed their locomotor activity behavior. All genotypes tested presented the normal bimodal activity (day 1-6) with a later onset of evening activity in per^L and an earlier onset in per^S. In constant darkness and the advanced rH-cycle (day 7-15) all genotypes reacted to the cycling relative humidity by concentrating their locomotor activity to the hours of rising rH values (30-80 %) during the second half of the subjective day and the first half of the subjective night. Within this time frame per^S and per^L flies showed free running elements/behavior and the per^{01} flies were rhythmic. In constant conditions (day 16-24) all flies were free running (CS, per^S and per^L) or arrhythmic (per^{01}) according to their endogenous clocks. Black and yellow bars represent darkness and light, respectively, blue lines represent cycling relative humidity or constant humidity.

To sum up both experiments, the flies reacted to the humidity cycle by concentrating their main locomotor activity to the time frame of rising and high relative humidity values and showed hardly any activity at the lowest humidity values. The long and short *period* mutants,

per^{L} and *per*^{S} respectively, already began to free run or exhibited elements of free running behavior with different period length τ when light was absent but a shifted humidity cycle was present. The arrhythmic *per*^{01} flies were rhythmic during that period of recording. When no light and no humidity cycle were present all flies were free running or continued to free run. These results indicate that humidity can influence the fly`s behavior directly – therefore being a masking effect – but humidity did not act as a Zeitgeber and could not be used to shift or entrain the clock like other Zeitgeber – like light or temperature.

4. Discussion

In this thesis the *period* gene and three known mutants – per^S , per^{01} and per^L – were investigated in comparison to wild type CS flies to show the importance of the circadian clock of *Drosophila melanogaster* and the fitness advantages due to a functional and properly timed circadian clock under laboratory as well as under natural-like outdoor conditions. This was done by direct competition assay experiments, performed in the laboratory with a simulation of a 24 hour day or T-cycles of 19 hours or 29 hours corresponding to the per^S or per^L mutant or under LL for the arrhythmic per^{01} fly strain as well as outdoors. The resonance hypothesis, meaning the flies which endogenous period fits best to the environmental cycle profit from a fitness advantage, could be confirmed at least for the long *period* mutant, per^L.

But, as the *period* gene does not only play a very important role in the circadian system of *Drosophila melanogaster*, it was shown in divers studies that it has pleiotropic effects on the fly`s behavior, development and fecundity. For example it affects the frequency of the male courtship song (Kyriacou and Hall, 1980, 1986; Kyriacou et al., 1990b), developmental timing (Kyriacou et al., 1990a), sleep length (Shaw et al., 2000; Shaw et al., 2002), the fecundity of males as well as female flies (Beaver et al., 2002; Beaver et al., 2003), the activity of neurons involved in fast escape responses (Megighian et al., 2009), as well as the duration of copulating during the mating process (Beaver and Giebultowicz, 2004). Therefore other factors that could potentially result in fitness advantages or disadvantages were investigated as well. These experiments were performed with the original fly strains under a normal 24h day simulation due to the fact that the flies were kept under these conditions for decades.

Besides the competition assay experiments – and therefore mainly light and day length conditions – humidity and also food composition was examined in this thesis as potential factors influencing the fitness of *Drosophila melanogaster*. In the following paragraphs the different factors are discussed in further details.

4.1 impact of light and day length

To unravel the impact of light and day length on the fitness of *Drosophila melanogaster*, the activity of the flies was analysed and direct competition experiments – not only in the laboratory but also outdoors – were performed. For the analysis of the activity of the flies, not only male flies were investigated – as it is the usual procedure for experiments with *Drosophila melanogaster* – but also female flies – homozygous as well as heterozygous as they appear in

the competition experiments – and even larger groups of flies with social interaction (populations).

4.1.1 activity pattern of wild type and clock mutant flies

4.1.3.1 males (original and newly cantonized)

Locomotor activity recordings were performed for the male flies to see their activity profile under a normal 24 hour day (LD12:12) and to calculate the free running period of the wild type CS and the different clock mutant strains under constant conditions (DD).

Wild type CS as well as the short and long *period* mutant flies, per^S and per^L respectively, were able to anticipate the changes of light in the morning and in the evening and showed a typical bimodal activity pattern with a morning and evening activity peak (Fig. 25) during the first days with a LD12:12 light:dark cycle. But these morning and evening activity peaks were shifted for the *period* mutant flies according to their genotype and fast or slow running circadian clock. For example the per^S flies shifted their evening activity peak to the afternoon and the morning activity peak seemed to be even before lights on but might be suppressed by darkness. The per^L flies shifted their evening activity peak into the night what also seemed to be suppressed or reduced due to the darkness and their morning activity bout was extended until midday. The arrhythmic strains – per^{01} and clk^{AR} – exhibited an arrhythmic activity pattern as expected, with no anticipation of lights on/lights off and just reacted to the changes in light condition. Under DD only the arrhythmic strains – per^{01} and clk^{AR} – were arrhythmic (Fig. 38) and the free-running period of the other strains could be calculated.

These typical locomotor activity patterns were overall the same in the original and the newly cantonized fly strains. But it seemed like the actograms of the single flies were a little bit more robust and clear in case of the newly cantonized fly strains. An exception is the per^L fly strain. Here the flies exhibited a prolonged free-running period in the course of the competition assay experiments (Horn et al., 2019).

When the wild type CS and the *period* mutant fly strains were recorded under T-cycles, the situation changed a bit. In 19 hour T-cycles the wild type CS flies were not able to anticipate lights-on and exhibited a prolonged morning activity bout and the evening activity was shifted into the dark phase resembling very much the activity profile of per^L flies that were recorded under a 24 hour T-cycle (Horn et al., 2019). Furthermore in a 29 hour T-cycle the wild type CS flies activity profile with morning and evening activity peak resembled the profile of per^S flies

that were recorded under a 24 hour T-cycle but with a clear and not suppressed morning activity peak during the night (Horn et al., 2019). Whereas in the *per*^{S} and *per*^{L} mutant fly activity profiles, under 19 and 29 hour T-cycles respectively, the morning and evening activity peaks matched lights-on and lights-off looking like a wild type profile under a 24 hour T-cycle (Horn et al., 2019). As a summary the wild type flies were not able to track and shift their activity according to the given short or long T-cycle and the phase-relationship of activity peaks to the changes in light seemed to be mutant-like, whereas the mutants phase relationship seemed to be wild type-like in the according T-cycle matching the endogenous period of the flies (Horn et al., 2019).

From these results it can be concluded that the flies that endogenous period matches the given environmental conditions – T-cycles in this case – should have a fitness advantage in concern of the phasing of their activity and probably less stress induced that can arouse due to a mismatch of endogenous period and environmental cycles.

4.1.3.2 homozygous and heterozygous females

Commonly only male flies are used for locomotor activity recordings, because females lay eggs and the developing larvae crawl around the recording tube and eat up the food storage. Therefore larvae would falsify the results and eat up the food provided resulting in a starving and dying adult female fly. But as in the competition assay experiments females mate not only with males of their own genotype, but also with males of the competing genotype, resulting in heterozygous females, the locomotor activity pattern of these flies was investigated in this thesis. To manage that task only virgin females were used.

The locomotor activity pattern of homozygous females resembled the ones of the males. The wild type CS as well as the *per*^{L} and *per*^{S} homozygous females exhibited a bimodal activity pattern with anticipation of lights on/off and a typical morning and evening activity peak, with *per*^{S} flies shifting the evening activity peak to the late afternoon and *per*^{L} flies with a prolonged midday siesta and the evening activity peak shifted more towards late evening/beginning of the night, according to their endogenous free running period (Fig. 29). The arrhythmic *per*^{01} flies showed no distinct bimodal activity pattern with morning and evening activity peak, but a reaction to lights on/off. The calculated free running periods also resembled the ones of the males.

But interestingly the locomotor activity pattern of the heterozygous females was not showing the exact pattern like one of the parental strains, but rather showed a mixture of the profiles of

the parental strains that could also be seen in the length of the free running period of the females (Fig. 29, Tab. 04). The CS/*per*^{01} females exhibited a rhythmic bimodal activity pattern, the CS/*per*^{L} and CS/*per*^{S} heterozygous females showed evening activity peaks in between the pattern of the parental strains. These results show that the wild type and *period* mutant alleles are both expressed dominantly and they kind of add up to the final mixture of behavioral output of the females measured here as locomotor activity pattern and free running period. Furthermore one functional copy of the wild type *period* gene – in the case of the CS/*per*^{01} females – is enough for exhibition of robust rhythmic behavior.

4.1.3.3 larger groups - populations

As population dynamics are hardly investigated, but the flies in the direct competition assay experiments have to compete in a population, populations of flies – either males or virgin females – were also analysed in the Trikinetik LAM25 system to see whether a whole population shows a common locomotor activity pattern and to record this if the population does.

The results were quite amazing. Wild type CS as well as the *per*^{S} and *per*^{L} mutant flies – independent of the sex – exhibited a clear locomotor activity pattern like it is expected by a single fly (Fig. 25, 29 and 30) and the arrhythmic *per*^{01} flies showed no rhythm after the light as Zeitgeber was turned off. Even after 10 days of DD the populations were still running in synchrony and a collective free running period could be calculated for the populations (Tab. 05) except for the *per*^{01} populations, respectively. Interestingly the rhythmicity of the wild type CS populations was even clearer to see in female populations than in the male ones. This is also true for the *per*^{S} females, where the evening activity peak can be seen more clearly been shifted to the afternoon (Fig.30). Another interesting fact about the population recordings was the finding that the all populations exhibited more activity during the light phase in LD except for the *per*^{S} and *per*^{01} male populations. These concentrated their activity to the dark phase instead. Why they concentrated their activity more to the dark phase during LD is unknown and requires further investigation.

As a summary it can be said that a collective locomotor activity of populations can be recorded in the laboratory and that the flies keep synchronized to each other in DD even after 10 days with no other Zeitgeber – like light or temperature – present, confirming social contact as a Zeitgeber (Levine et al., 2002).

4.1.2 indoor competition experiments

4.1.1.1 original fly strains in LD12:12 and T-cycles

The original fly strains – per^S , per^{01} and per^L – were kept in direct competition assay experiments with wild type CS flies either under normal 24 hour day simulation (LD12:12), or according to the mutant under T-cycles of 19 hours (LD9,5:9,5) for per^S, 29 hours (LD14,5:14,5) for per^L or constant light (LL) in case of per^{01}. According to the resonance hypothesis, the flies which endogenous period fits best to the given environment should have a clear fitness advantage and should be able to outcompete the other fly strain in the direct competition experiments. Therefore a comparison of the results under the 24 hour day – where the wild type CS flies should hold the fitness advantage – with the results under the T-cycles – where the mutants should hold the fitness advantage – provides information about the difference and therefore the influence of a functional and properly timed circadian clock for the fitness of the flies.

But unexpectedly the results were different from what was expected. In all the competition assay experiments performed with the original fly strains, the wild type CS strain won against the mutants, except for the CS x per^L constellation under a 29 hour T-cycle where the flies seemed to keep in balance of around 50% of wild type and per^L mutant flies (Fig. 17). That the wild type flies dominated the *period* mutants under the 24 hour day was expected, but there was also no significant difference in the T-cycle experiments with per^S and per^{01}. The per^S flies could keep constant in the population in a low amount, but for per^{01} it seemed like the flies would go extinct after a certain time, declining during the whole experiment (Fig. 17), no matter of the lighting situation.

In case of the per^L mutants compared to 24 hour and a T-cycle of 29 hours, they declined significantly slower in the first few generations, but could also recover and stay in balance of about 50% in the population with the wild type CS flies. This shows that the flies actually have a fitness advantage according to the day length, confirming the resonance hypothesis for the per^L flies. But as the per^L flies declined also under the 29 hour T-cycle in the first few generations and recovered at a certain timepoint to a constant level in the population might also be a hint, that the genetic background of the flies had a great influence in the system and that the flies mixed up for several generation and assimilated their background with the wild type flies.

The experiments with the per^S and per^{01} mutants clearly showed, that here the resonance hypothesis and therefore the day length was not the main factor and that there must be

presumably several other factors that influence the fitness of the flies under these competition situation.

As the results indicated a possible different genetic background of the fly strains (although they were congenic and cantonized according to Vanin et al., 2012), probably due to the accumulation of smaller mutations according genetic drift during the long maintenance of the fly strains in the lab, newly cantonized flies were extracted out of the competition assay experiments after the flies assimilated their genetic background by mating and mixing in the population for more than 50 generations. The competition assay experiments were repeated with the now newly cantonized fly strains to rule out an influence by different genetic background.

4.1.1.2 newly cantonized fly strains in LD12:12 and T-cycles

As the first competition assay experiments showed a possible influence of the genetic background, the experiments were repeated with now newly cantonized fly strains that should hold the same genetic background. Again the wild type CS and *per* mutant fly strains competed under a normal 24 hour day (LD12:12) as well as under T-cycles of 19 hours, 29 hours or LL, according to the *period* mutant strain used.

When wild type CS and per^{L} mutant flies competed, the number of mutant flies was ranging between 40-50 % in the first half of the experiment under the 24 hour as well as under the T-cycle of 29 hours. Only in the second half of the experiment the number of per^{L} mutant flies was declining in the 24 hour day and increasing under the 29 hour day, as it was expected according to the resonance hypothesis, but due to the fluctuation of mutant numbers in the single vials this was not significant, but a clear trend was visible on the long run (Fig. 18).

In case of the wild type and per^{S} mutant competition assay experiments, the results were more clearly. Under the 24 hour day as well as the T-cycle of 19 hours, the shape of the graph seems to be the same with the mutant flies, declining in the first few generations but staying at a constant level in the population (Fig.18). But under the T-cycle of 19 hours the number of per^{S} mutant flies stayed constantly higher than the number of per^{S} mutants under the 24 hour day, which was also significant and points clearly towards the resonance hypothesis. Therefore this experiment showed the importance of a functional and properly timed circadian clock that results in a fitness advantage, enabling the flies to persist in a closed competitional population in higher number than under a mismatching photoperiod (24 hour day for per^{S}). But although

the experiment points out a fitness advantage of the *per*^{S} flies under a T-cycle of 19 hours compared to the 24 hour day, the *per*^{S} flies were still not able to outcompete the wild type CS flies, indicating that this is a complex system and also other factors play a role and influence the fitness of the flies.

This could be seen in the wild type CS and *per*^{01} competition assay experiments as well. There the *per*^{01} flies declined throughout the whole experiment, properly going extinct on the long run (Fig. 18). Under the 24 hour day and under LL there were only significant difference in two measured timepoints, but overall there were no significant differences visible, pointing out clearly that either LL is not the optimal condition for *per*^{01} flies – as constant light might also be a stress factor for the flies and the wild type CS might cope with this stress factor better or as well as the mutant flies – or that several other factors influence and regulate the whole system that had a greater impact on the fitness of the flies than the lighting situation.

4.1.1.3 comparison of original and newly cantonized fly strains

When the results of the competition assay experiments are directly compared between the original and the newly cantonized fly strains, the impact of the genetic background becomes quite clear.

In case of the *per*^{L} fly strain a big difference in mutant fly numbers can be seen. Under the 24 hour day simulation, the newly cantonized *per*^{L} flies do not decline as the original ones, but in contrast stay relative constant in a range of about 40-50 % compared to 10-20 % of the original *per*^{L} fly strain (Fig. 19). This difference is significant and only vanished towards the end of the experiment, properly due to the decline in mutant number also in the newly cantonized *per*^{L} flies and to a high fluctuation of the single vials measured. When the results of the 29 hour T-cycles are compared, the original and the newly cantonized *per*^{L} strain showed quite similar results and no bigger differences (Fig. 20). Therefore in case of the T-cycles where the *per*^{L} flies should hold a fitness advantage – according to their circadian clock fitting to the environment better than the clock of the wild type CS flies – the lighting situation and therefore day length simulation influenced the fitness of the flies in a greater amount than the genetic background.

The comparison of the original and newly cantonized *per*^{S} fly strains also revealed an influence of the genetic background of the flies like the results of the *per*^{L} fly strain. Here the original and the newly cantonized *per*^{S} flies showed hardly any difference under the 24 hour day condition (Fig. 19), showing that the genetic background did not influence the performance of the

mutant *per*^{S} flies very much. But under the T-cycle of 19 hours a highly significant difference in mutant *per*^{S} numbers in the population between the original and the newly cantonized flies was revealed. The newly cantonized *per*^{S} flies could still not outcompete the wild type CS flies, but stayed at a significantly higher constant level in the population than the original *per*^{S} fly strain (Fig. 20). Concluding that after the flies possessed a conform genetic background with the competing wild type CS flies, they had more reproductive success under the 19 hour T-cycle revealing the influence of the genetic background. But the fact that the *per*^{S} flies performed differently well under the 24 hour and the 19 hour T-Cycle after the genetic background was confirm with the wild type CS flies, showed that the day length is also influencing the fitness of flies. If the genetic background would be the only factor influencing the fitness of the flies, it would be expected that they perform better at the same amount under both day length conditions. Therefore the newly cantonized *per*^{S} flies did not perform better than the original flies, due to the mismatch of their endogenous clock with the given environment of a normal 24 hour day, almost regardless of the genetic background.

Similar results could be seen in the comparison of original and newly cantonized *per*^{01} fly strains under 24 hour or LL conditions. Under the normal 24 hour day simulation there was no significant difference in the performance of the *per*^{01} flies between the original and newly cantonized ones. But under the LL simulation a tendency could be seen that the newly cantonized *per*^{01} fly strain performed slightly better than the original one. This was significant at some timepoints of the experiments and sometimes not, but still under both day length simulation the fly numbers declined throughout the whole experiment.

To summarize these results, it can be said that the genetic background has an impact on the fitness of the *period* mutant flies. The amount of impact is different, which can be explained by the fact that the fly strains might have developed and accumulated different smaller mutations over time kept separated in the lab and therefore showed smaller or bigger differences after cantonizing when the original and the newly cantonized fly strains were compared to each other. *Per*^{S} and *per*^{01} flies showed the same trend and the effect was much higher in *per*^{S}, which might also be a hint towards a quite complex interplay of genetic background and day length simulation. The results of the *per*^{L} strains also point towards this direction and might point out the importance of a functional and properly timed circadian clock resulting in a fitness advantage that might even be able to balance smaller effects of a different genetic background.

4.1.3 outdoor competition experiments

Almost all experiments are performed in the laboratory under strictly defined and controlled parameters. But one of the most interesting questions – what happens outside under natural conditions when the flies have to compete? – cannot be answered in the laboratory. That`s why the competition assay experiments performed in this thesis were not only limited to the laboratory, but also performed outdoors under natural-like conditions. Due to the climate and weather conditions in Würzburg it was not possible to perform long term experiments outdoors. But still it was possible to keep the flies outdoors under natural-like conditions – flies perceived all changes in light, temperature and humidity – in the summer months to perform the competition assay experiment raising 3 generations each in two consecutive years that could be analysed for the genotype distribution of wild type and mutant flies. Like in the laboratory the wild type CS flies always had to compete with one of the three *per* mutant fly strains.

The outdoor experiments were performed with the original *period* fly strains and therefore can only be compared to the results obtained in the laboratory with the same original fly strains under a normal 24 hour day simulation. The results of the outdoor competition assay experiments were similar to the results in the laboratory. The wild type CS flies always remained in a higher percentage in the population and outcompeted the *period* mutants, suggesting a selection against the *period* mutant alleles.

When the results of the genotype distribution of the flies in the population of the vials was compared of the third generation after starting the experiments under laboratory and outdoor conditions, there where no significant differences in the percentage of *period* mutant flies. There was also no difference in the outdoor experiments in the comparison of the two consecutive years, concluding that the results were repeatable under the outdoor conditions (Fig. 21). The flies kept under natural-like outdoor conditions – with all natural changes in light, temperature and humidity – performed similar like the flies kept indoors under controlled laboratory conditions – with defined light/day length and constant temperature as well as humidity conditions. This result suggested that these 3 different conditions – light, temperature and humidity – might not have a great or the main impact on the survival and success and therefore fitness of the flies, pointing out that the system must be more complex with other factors influencing the fitness of the flies and a probably complex interplay of all factors.

4.2 impact of other factors

4.2.1 sperm counts

The results show no significant differences in the number of sperms transferred by the male flies during the mating process (Fig. 23). Therefore the possibility of fitness disadvantages concerning the number of sperms produced and transferred by the male *period* mutant flies can be ruled out. All *period* mutant males were able to provide a sufficient amount of sperms. Also the number of sperms transferred in the experiments of this thesis matches the numbers known from the literature. In two studies it could be shown that about 1500 sperms were transferred during the mating process to the female fly, but the female fly could only accommodate about 500 sperms in the specialized sperm-storage organs (Miller and Pitnick, 2002; Manier et al., 2010). It is also known from the literature that males adjust the number of sperm in their ejaculate to the presence of rival males (Garbaczewska et al., 2013). In this thesis there were no other males present during the actual mating process for the sperm count experiments, but before the mating experiments the males were kept in the normal vials with all developmental stages of *Drosophila melanogaster*, exposed to a number of rival males. In this thesis other sperm attributes – like size and motility – were not further investigated, but they might also influence the competition of sperm of multiple mated females, like they can arise in a population (Pizzari and Parker, 2009) and the whole system might be more complex (Tourmente et al., 2019). Therefore the hypothesis that a reduced fertility of *period* mutant flies – due to the production and transfer of sperms – might be a functional mechanism and explain the results of the competition assay experiments could not be confirmed in this thesis.

4.2.2 female mating preferences

The *period* gene influences not only circadian behavior of *Drosophila melanogaster*, but it is involved in many processes on the molecular, transcriptional and translational level. One of the influenced parameters on the behavioral level is the frequency of male courtship song, which also affects the mating process by influencing the willingness to mate of female flies (Kyriacou et al., 1990b; Kyriacou, 2017). It is thought, that the females prefer to mate with males that sing in the frequency of the females own genotype (Greenacre et al., 1993). Therefore the *period* mutant females should prefer the according *period* mutant males and wild type CS females the

wild type CS males. To test and maybe verify this hypothesis and as well as to find out a possible mating preference of heterozygous females – as the heterozygous females showed different free-running period lengths compared to the homozygous females – , mating preference tests were performed with not only homozygous, but also with heterozygous females.

Surprisingly the results were not as expected. In case of the homozygous *period* mutant females there were no significant preferences for the male with the same genotype (Fig. 24). For the wild type CS females also the wild type males were not preferred over the *period* mutant males. On the contrary even the per^{01} males were preferred significantly over the wild type CS males by the female wild type CS flies. Furthermore when the heterozygous female flies were tested for any preferences, the CS/per^{S} and the CS/per^{L} females significantly preferred to mate with the according per^{S} or per^{L} mutant males.

These results can therefore not verify the hypothesis of Greenacre and colleagues (1993) and showed a different mating preference pattern or rather no mating preference of the females in many cases. The results obtained in this thesis cannot explain the outcome and dominance of the wild type CS flies in the competition assay experiments, but they lead to the conclusion, that the mating preference of females influences the balance in the competition assay experiments and might be a possible explanation for the persistence of the *period* mutant flies in the population via persistence of the mutant alleles at a low frequency.

The mating success and also offspring per female fly was analysed by Horn and colleagues. They came to the conclusion that also male activity affects mating success and that the per^{L} mutation affected the number of offspring produced by a female fly, but not the per^{S} mutation in comparison to the wild type CS (Horn et al., 2019). But still the results of mating preference, mating success and offspring per female cannot explain the outcome of the competition experiments, but it shows that it is influencing the system and may account for the survival or persistence of the short and long *period* mutant genotype when the flies are kept in competition with the wild type CS.

4.2.3 lifespan of flies

As the impact of day length was investigated in this thesis lifespan experiments were used to analyse the fitness of flies. The lifespan experiments were performed under the normal LD12:12 day simulation, but also under T-cycles of 19 or 29 hours as well as under LL,

according to the three different *period* mutant strains. For lifespan experiments performed with different food sources see also chapter 4.4.

The comparison of the lifespan experiments performed under a 24 hour day and under the T-cycles/LL revealed that the wild type CS flies performed worse in all three cases (19 hours, 29 hours or LL compared to the 24 hour day), meaning the flies were less fit in all day length simulations that were different from their free running period of about 24 hour as well as constant light (Fig. 34, 35, 36). Hinting to the possibility that a day length deviating from the fly`s endogenous rhythm stresses the flies and results in reduced fitness.

In contrast the *period* mutants per^{S} and per^{L} showed no difference in survival and therefore fitness under a normal 24 hour and a 19 or 29 hour T-cycle respectively. The per^{01} flies showed also no differences in survival in the first two thirds of the experiment. Only at the end of the experiment the flies showed a reduced survival under LL compared to 24 hour day simulation with LD12:12. The flies do not perform better under the T-cycles or worse under the 24 hour day according to their endogenous period, as it would be expected by the results of the wild type CS flies. But the flies showed similar good survival and fitness under both conditions tested for each strain, meaning they might not be stressed by the 24 hour day as the wild type might be stressed by the T-cycles/LL, possible due to the fact that the fly strains are kept under LD12:12 for many decades in the lab and they might have adapted so far that they are not stressed by that 24 hour day length simulation. Concluding the day length is influencing the fitness of the flies on the long run. But in case of the competition assay experiment it is unlikely that it influences the balance of the genotypes in a great amount according to the relatively short time period of 14 days or even less due to the death of the parental generation in the sticky medium that softens during larval development.

The experiments for sperm counting, female mating preferences and lifespan of *Drosophila melanogaster* were conducted with the original fly strains and not with the newly cantonized fly strains, meaning in can not be completely excluded that the results might be influenced by the genetic background of the flies.

4.2.4 genetic background and overall fitness of the *period* fly strains

In the competition assay experiments performed for this thesis the per^{L} flies could remain in the highest percentage in the vials of all three *period* mutants with about ~20% when the original fly strains were used and about ~50% when the newly cantonized fly strains were competing.

The *per*^{L} flies showed a prolonged morning activity compared to the other strains and were quite successful in the mating preference test performed. Also the overall mating success of the flies seemed to be better than in the other *period* strains although the survival rate of *per*^{L} flies was lower than that of the *per*^{01} flies and they showed a reduced number of adult offspring per female (Horn et al., 2019). After the genetic background was assimilated due to cantonizing the flies naturally during the first competition assay experiment, the *per*^{L} flies demonstrated that they have a higher fitness than the original *per*^{L} strain by performing better in the competition assays, but they were still not able to outcompete the wild type CS flies.

But the most surprising effect found was the elongation of the free-running period of the *per*^{L} fly strain during the competition assay experiments. The flies elongated their free-running period during the competition assay experiments already after a few generations and led to the assumption that the genetic background of the *period* and wild type CS strain was not the same anymore as it was thought, probably due to the long inbreeding in the laboratory and the possible accumulation and manifestation of genetic mutations. To rule out this effect of genetic background variation between the wild type CS and *period* mutant fly strains, newly cantonized fly strains were obtained from the competition assay experiments and the experiments were repeated, revealing the impact of day length for the fly strains fitness.

The *per*^{S} fly strain could persist in the competition assay experiments at about ~10% independent of the day length – 24 hours or 19 hours T-cycle – and did not change the free-running period in the way the *per*^{L} strain did. But nevertheless the competition experiments with the newly cantonized *per*^{S} strain revealed a higher fitness of the flies and the impact of day length on the persistence of flies in competition – with *per*^{S} persisting at about ~30% in the 19 hour T-cycle but still only about ~10% in the 24 hour day – after cantonizing the fly strain. These results lead to the assumption that in the *per*^{S} strain, too, the genetic background was influencing the fitness of the mutant flies in the first competition experiments, surpressing or masking the effect of day length on the fitness of flies.

In case of the *per*^{01} strain the flies did not perform better or worse in the competition assay experiments on the long run after cantonizing and the effect of the genetic background could not be seen as clear as in the other two *period* mutant strains. But as the *period* gene does not only influence the circadian clock with e.g. the free-running behavior as visible output, the flies might be impaired due to the lack of a functional PER protein on many other levels. For example, recently it could be shown that the *period* gene affects intermediary lipid metabolism

by an impaired mitochondrial activity in *per*^{01} flies (Schäbler et al., 2020). As a result of the many involvements and functions of the *period* gene (see e.g. Kyriacou and Hall, 1980, 1986; Baylies et al., 1987; Yu et al., 1987; Huang et al., 1993; Edery et al., 1994a; Edery et al., 1994b; Curtin et al., 1995; Meyer et al., 2006; Krishnan et al., 2008; Megighian et al., 2009) it is not surprising that the *per*^{01} flies have a low level of fitness and loose against the wild type CS in the competition assay, because they might be impaired in many different ways.

Therefore these experiments could show that the genetic background impacts the fitness of the flies, but multiple factors like day length/lighting situation, mating preferences, level of activity, survival rate or further unknown factors also might influence this highly complex system. As a summary, on the one hand it seems that the *per*^{L} fly strain have a higher overall fitness than the other two *period* mutants, *per*^{S} and *per*^{01}. The *per*^{S} mutant is clearly less fit than wild type or *per*^{L} flies, but nevertheless they managed to persist in the population under competition with wild type CS flies. The *per*^{01} mutants on the other hand seemed to have the lowest overall fitness of the three *period* mutants and lost and even might go extinct in the competition assay experiments against the wild type independent of the lighting situation or the genetic background after cantonizing.

4.3 impact of humidity

In the eclosion experiments *Drosophila melanogaster* wild type CS and *period* clock mutant flies (*per*^{L}, *per*^{S} and *per*^{01}) were faced with different relative humidity values for eclosion and wing expansion. The lowest value of 2 % rH caused some problems for all genotypes in matters of eclosion compared to the highest value of 80 % rH (Fig. 40). When the genotypes are compared within and not with each other, significant lower eclosion rates of about 88 - 95 % successfully eclosed flies in 2 % rH compared to about 96 - 98 % successfully eclosed flies in 80 % rH can be seen. In matters of wing expansion only the wild type CS and the arrhythmic *per*^{01} flies unfolded their wings with a significantly lower success rate in 2 % rH compared to 60 % rH or 80 % rH (Fig. 42). The wing expansion process and success of the long and short *period* mutant, *per*^{L} and *per*^{S} respectively, was not affected by relative humidity and the flies were able to unfold their wings properly at very low as well as at high relative humidity. When the wild type CS and the *period* mutant strains are compared to each other, no effects of humidity can be seen in matters of successful eclosion at 80 % rH and only few significant

differences in 60 % rH and 80 % rH. At 60 % rH less per^S flies eclosed successfully than per^L and per^{01} and at 80 % rH more per^{01} flies could eclose successfully compared to wild type CS and per^S flies. The situation is a bit different in matters of wing expansion. There per^{01} flies were not able to unfold their wings as good as wild type CS and the long and short *period* mutant per^S and per^L at 2 % rH. Also at 80 % rH the arrhythmic per^{01} flies unfolded their wings with a lower success rate than wild type and per^S flies.

But in summary all flies eclosed with high success rates of 87,89 - 97,95 % and unfolded their wings with rates between 89,88 - 99,7 % meaning nearly all flies were able to eclose and to unfold their wings independent of the relative humidity (see also(Ruf et al., in submission). Furthermore *Drosophila melanogaster* will probably never experience the extremely low amount of only 2 % of relative humidity in nature. This leads to the conclusion that the relative humidity is not the most important or limiting factor for the flies for a successful eclosion and wing expansion. In fact temperature seems to be more important for the flies than the relative humidity itself, meaning that the flies struggle to eclose and unfold their wings properly with increasing temperature, especially at 30 degrees and higher (data not shown). This could also be seen in the eclosion of flies when wild type flies were tested for humidity as a potential Zeitgeber in the WEclMon (Ruf, 2016). *Drosophila melanogaster*`s eclosion could not be entrained to humidity and temperature was more important for the flies. Tanaka and colleagues studied the effects of humidity in the onion fly *Delia antiqua*. They also came to the result that high temperatures and not low relative humidity affect the viability and wing expansion of the flies in a negative way (Tanaka and Watari, 2009).

Humidity as Zeitgeber?

Light as a Zeitgeber is well known and used in the laboratory, but in some cases it is not appropriate to use due to the experimental setup or fly strain or investigated situation. In those cases usually temperature is chosen as the Zeitgeber to synchronize the flies, but even that might not always be possible. So to have another possible Zeitgeber to entrain the flies to, humidity was tested as potential Zeitgeber in this thesis.

Do humidity-cycles influence or change the locomotor activity behavior of *Drosophila melanogaster*? Is humidity a Zeitgeber and can be used for entrainment of the flies? Or is humidity influencing the fly`s behavior but not shifting and synchronizing the clock of *Drosophila melanogaster*, a so called masking effect? To answer these question wild type and

period clock mutant flies were placed in the Trikinetics DAM System for locomotor activity recording. During the first phase of recording the flies experienced the normal entrainment via a light-dark cycle (LD12:12) combined with a relative humidity cycle like it would be in nature. In the second phase light as Zeitgeber was dismissed and only the relative humidity cycle continued, but was shifted for 8 hours, either advanced or delayed, to see if the flies react to the shift of humidity and shift their main activity or if they do not react and start to free run due to the absence of light as a Zeitgeber. In the third phase of the recording the flies experienced constant conditions with no light and constant relative humidity to record the free running behavior of the flies. In the third phase it was interesting to see if the flies start to free run as they would expect the subjective day from the first phase or if they start to free run in accordance to the shifted humidity cycle of the second phase.

The flies behavior in the first phase was the normal locomotor activity behavior with the main activity peaks according to the genotype and LD entrainment. In the second phase of the recording the locomotor activity behavior of the flies changed. The flies shifted their main activity to the time frame of rising and high relative humidity values and were mainly inactive during the period of low relative humidity in both experiments with either an advance or a delay of the humidity cycle (Fig. 43 and 44). The short and long *period* mutant flies were still rhythmic, but with a free-running period of about 25 hours (per^L) and about 20 hours (per^S), as well as the per^{01} flies with a period length of about 24 hours (Tab. 07). The per^S and per^L flies already showed elements of free-running behavior expressed in the shortened or lengthened free-running period during constant darkness with humidity cycle. But the flies interrupted their activity during the time frame of low humidity values resulting in actograms with two rhythms visible, one of about 24 hours according to the humidity cycle and one according to their endogenous clocks within the 24 hour humidity rhythm (Fig. 43 and 44). In the third phase of the recording the flies experienced constant conditions and exhibited their normal free-running behavior according to their endogenous clock with a shortened or prolonged period length – for per^S and per^L respectively – as well as arrhythmic behavior for the per^{01} flies and a 24 hour rhythm of the wild type CS flies compared to the second recording phase with a humidity cycle only. It is not very obvious in all cases but the free-running behavior of the flies in the third phase looked like it continues from the conditions of the first phase with no real shift in between according to the shift in humidity. This might be due to the shift in humidity being not big enough and would be clarified in experiments with a greater shift in humidity.

But in some actograms it is visible that the flies start to free run right after light as Zeitgeber is absent (*per*^{S}, Fig. 43 and 44). As a conclusion the humidity cycle is influencing *Drosophila melanogaster´*s locomotor behavior and shifts or better limits the activity to a specific time frame, but a relative humidity cycle is not enough to reset the endogenous clock of the flies and therefore relative humidity is not an efficient Zeitgeber like light or temperature. Humidity is masking *Drosophila melanogaster`*s behavior. This result is consistent with the finding in eclosion and wing expansion experiments of this thesis as well as in the eclosion behavior of flies (Ruf, 2016; Ruf et al., in submission).

4.4 impact of food composition

4.4.1 lifespan of flies

Lifespan experiments were performed in this thesis to unravel the impact of nutritional value of different food sources on the fitness of flies. Flies are raised and kept on nutrient-rich maximum medium, but for most experiments – like the locomotor activity recordings – nutrient-poor minimum medium is usually used. It is assumed, that the flies benefit on the nutrient-rich maximum medium compared to the nutrient-poor minimum medium. The lifespan of flies was used as measured value of the fitness of flies. For the competition assay experiments it was interesting to see, whether the wild type CS and the *period* mutant flies are equally fit in means of survival in the first 14 days, because this was the timeframe after which the next generation was transferred to fresh medium vials.

The lifespan experiment results on maximum medium revealed no significant differences between the wild type CS and the *period* mutant as well as a second arrhythmic strain (*clk*^{AR}) that was included in the lifespan experiments, meaning all flies were equally fit (Fig. 31). This changed after about 25 days when the first differences became visible. In the course of the experiment first the *per*^{S} and *per*^{L} flies showed a reduced survival and therefore fitness compared to the wild type CS flies and later on also the *per*^{01} flies (Fig. 31).

On the nutrient-poor minimum medium the situation was similar to the nutrient-rich maximum medium. In the first few days there were no differences detectable, but in the course of the experiment clear and significant differences became visible with wild type CS flies showing the highest fitness according to the best survival, followed by the two arrhythmic strains – *per*^{01} and *clk*^{AR} – than *per*^{L} and last *per*^{S}, showing shortest survival and therefore the lowest fitness (Fig. 32). Direct comparison of the fly strains fed on nutrient-rich or nutrient-

poor medium revealed a reduced survival on the nutrient-poor medium and therefore fitness of the flies as expected. The underlying mechanisms or several other food sources in context of dietary restriction could not be further investigated in this thesis.

To sum this up the flies overall survival was longer on nutrient-rich maximum medium compared to the nutrient-poor minimum medium as expected and that the flies are equally fit up to the age of about 3 – 4 weeks, depending on the food source. Concluding that there is no effect measurable on the fitness level of the flies for the competition assay experiments. It is also astonishing how long *Drosophila melanogaster* can survive in total on such a low nutritional food source, pointing towards a take up and storage of all essential nutrients already during the larval stage as the minimum medium consisted of only water, agar and sugar.

4.4.2 activity pattern of wild type and clock mutant flies

To see any effects of the food source on the locomotor activity profile of the flies, the flies locomotor activity was recorded with nutrient-poor minimum as well as nutrient-rich maximum medium. The locomotor activity profiles of the wild type and the clock mutant fly strains showed overall the same pattern according to the flies endogenous clock, with morning and evening peaks or arrhythmic behavior in case of the per^{01} and clk^{AR} mutant flies. But there were some differences visible, like the more pronounced evening activity peak in the afternoon for the per^{S} flies fed on maximum medium or the higher total activity amount during the morning for the per^{L} flies (Fig. 25). The distribution of mean total activity spend in the light or dark phase was also similar in all cases except for per^{L} flies. They preferred to spend more activity in the light phase than in the dark when they were fed on the nutrient-rich maximum medium but on minimum medium it was the other way round with the flies being more active during the dark phase than the light (Fig. 27). In summary the mean total activity of wild type as well as per^{L} and per^{01} flies over a 24 hour period was significantly higher on maximum medium (Fig. 26).

More activity on the nutrient-rich maximum medium could be due to the higher energy content of the food source giving the flies more energy to be active. Another explanation could be that the flies need less time to eat due to the higher nutritional content of the maximum food and can spend additional time for other locomotor behavior.

As a summary it can be said that the food quality has only a minor impact on adult *Drosophila melanogaster`s* fitness. The fly can keep its overall normal activity pattern as usual with minor adaptions even if it does not feed on optimal food sources and can survive quite a long time on low nutritional food and therefore outlast periods of scarce food availability. For further experiments to address the impact of food composition/quality on the fitness of *Drosophila melanogaster* it would be interesting to analyze larval, pupal and adult stages of *Drosophila melanogaster* when the larvae were fed on different food qualities and in the context of dietary restriction.

4.5 Conclusion

As a conclusion the results obtained in this thesis showed that possessing a functional endogenous clock with a free-running period of about 24 hours – fitting the environmental condition of our planet – has a fitness advantage for the flies when they have to compete with others. The right timing of activity – like mating – might be crucial for survival in a population. The experiments also showed that multiple factors contribute to the fitness of *Drosophila melanogaster and* that the system is quite complex. There was no effect on the sperm number of the *period* mutant strains and the competition assay experiments results can also not be explained by the mating preferences of homo- or heterozygous female flies, but the mating preference might be a mechanism that enabled the mutant flies to remain stable in the population in case of per^{L} and per^{S} flies. Light and day length is influencing the fitness of the flies as shown for the per^{L} mutant strain according to the resonance hypothesis and also in the per^{S} mutant strain an effect of day length could be seen as well as for all *period* mutants and wild type CS on the long run in the lifespan experiments. The *period* mutants further seem to have different levels of fitness, with per^{S} and per^{01} being less fit than the per^{L} mutant. But also the genetic background plays an important role for the fitness of *period* mutant flies. And the results of the per^{01} mutants showed, that there must be other factors influencing the fitness of the flies under competition that were not investigated so far in this thesis. This confirms the knowledge that the *period* gene has pleiotropic effects on behavior, physiology and metabolism apart from its effect on the endogenous clock and timing.

Furthermore it could be shown that humidity is not acting as a Zeitgeber like light, but is influencing and masking *Drosophila melanogaster*`s behavior at least the locomotor activity of the fly. Humidity has further no effect on the fitness of *Drosophila melanogaster* wild type CS

and *period* mutants in the context of eclosion and wing expansion, which was thought to be timed to the morning hours to prevent desiccation. Food quality investigated in this thesis effected the flies survival on the long run but had no great effect on the fitness of the flies in the short term. To unravel the whole mechanism and impact of food further experiments with different food qualities and dietary restrictions would be necessary, which could not be realized in this thesis. Furthermore the experiments revealed the importance of genetic background variation and that genetic drift can lead to an accumulation of mutations in a rather short period of time, leading to the necessity of backcrosses and careful inspection of fly stocks kept for long periods in the laboratory. As a further result of this thesis the locomotor activity of fly populations could be recorded and social contact as Zeitgeber could be verified.

5. References

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6. Appendix

6.1 List of Figures

6.2 List of Tables

6.3 Additional Material and Data

Table 8: additional material and compositions

Buffer/Medium name	Ingredients/Source
Phosphate Buffered Saline (PBS)	1x, pH 7.4, SIGMA-Aldrich (10x stock)
standard cornmeal/agar medium (=nutrient-rich maximum medium)	0.8 % agar, 2.2 % sugar-beet syrup, 8.0 % malt extract, 1.8 % yeast, 1.0 % soy flour, 8.0 % corn flour and 0.3 % hydroxybenzoic acid
Nutrient-poor minimum medium	4 % sucrose; 2 % agar-agar; H_2O

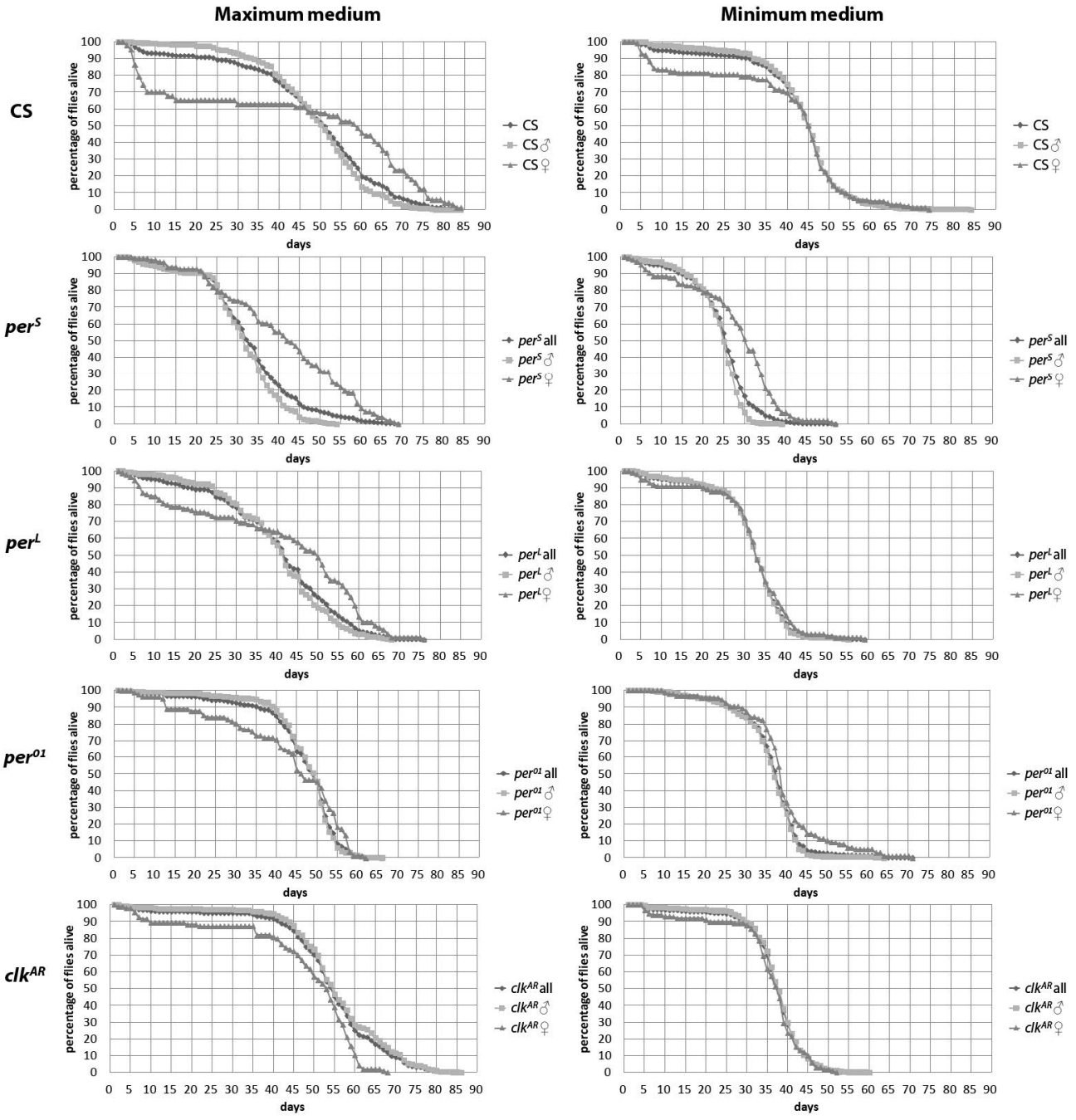

Figure 45: additional lifespan data; separated by male and female flies on nutrient-rich maximum (left) and nutrient-poor minimum (right) medium

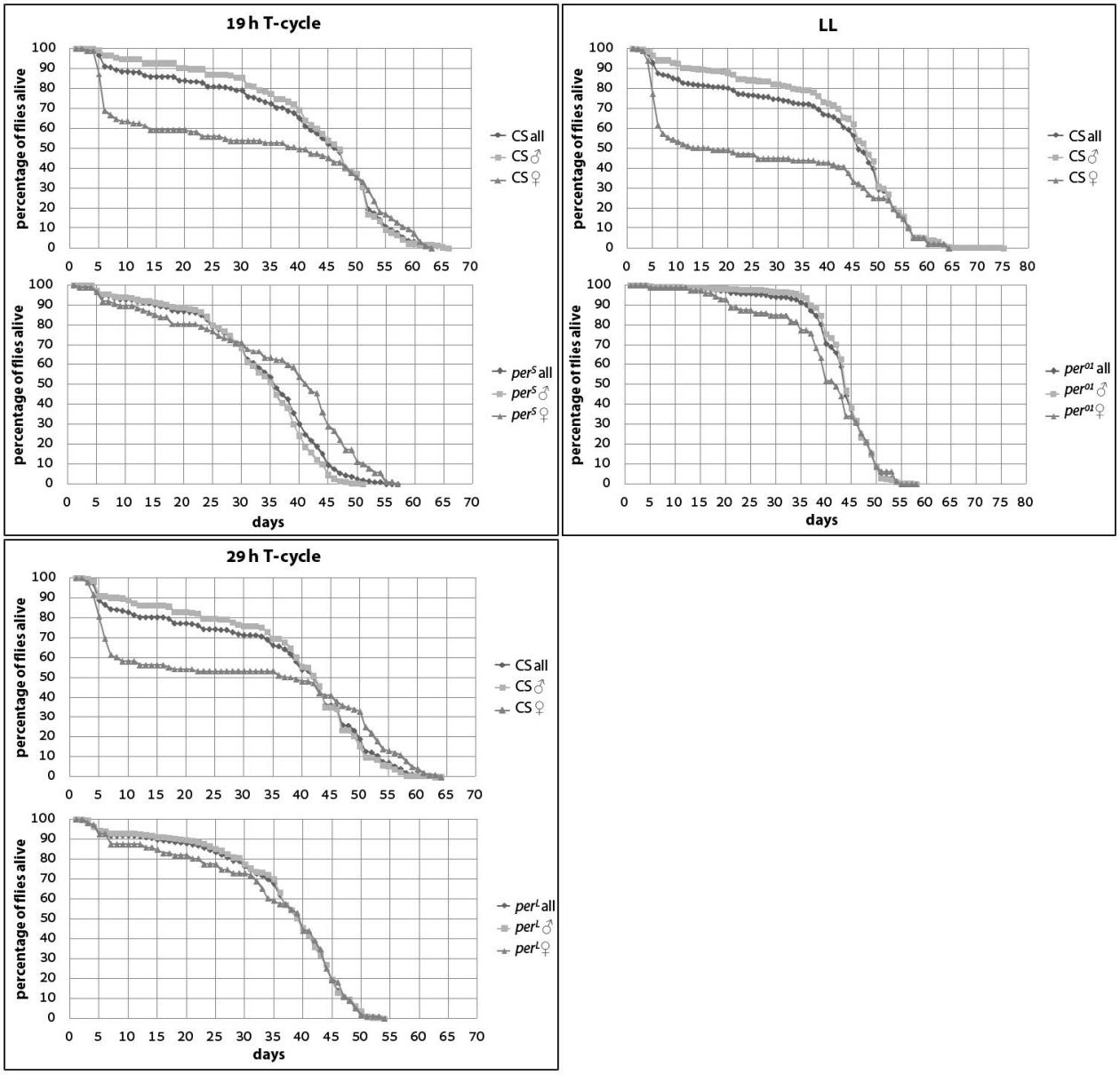

Figure 46: lifespan of males and females fed on maximum medium in different day length, namely 19 h T-cycle, 29 h T-cycle or LL

6.4 Statistics

In Table 9 all p-values of the competition assay experiments are listed in detail.

Table 9: statistic results of all competition assay experiments.

Ansatz			F3	F6	F9	F12	F15	F18	F21	F24	F27	F30	F33	F36	F39	F42	F45	F48	F51	F54
ABC (LD 12:12)																				
GHI (T-cycles)	CS x *per*^{l}		0.001293	0.002817	0.03096	0.002185	0.009004	0.05774	0.003072	0.01207	0.004286	0.002614	0.005154	0.004054	0.003589	0.005137	0.003172	0.007707	0.007312	0.02372
			***	***	*	***	***	n.s.	***	**	***	***	***	***	***	***	***	***	***	*
	CS x *per*^{s}		0.2401	0.2113	0.04758	0.4895	0.6108	0.5716	0.353	0.5078	0.7211	0.9356	0.4777	0.9319	0.9659	0.9646	0.4283	0.3278	0.7562	0.8038
			n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s	n.s.	n.s.
	CS x *per*^{ts}		0.4251	0.09555	0.01388	0.9394	0.9093	0.2347	0.3816	0.4664	0.1033	0.5318	0.096	0.1117	0.02364	0.4562	0.1692	0.564	0.7279	0.3681
			n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s	n.s.	n.s.

Ansatz			F3	F6	F9	F12	F15	F18	F21	F24	F27	F30	F33	F36	F39	F42	F45	F48	F51	F54	F57	F60
ABC (LD 12:12)																						
NOP (LD 12:12)	CS x *per*^{l}		0.0006123	0.0016	0.0004366	0.002105	0.005753	0.003682	0.003664	0.002807	0.0105	0.003523	0.02101	0.03325	0.08518	0.1695	0.131	0.06296	0.06002	0.2922	x	0.2925
			***	***	***	***	***	***	***	***	**	***	**	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	x	n.s.
	CS x *per*^{s}		0.04537	0.09037	0.02472	0.03467	0.4987	0.8525	0.7449	0.06554	0.7447	0.5454	0.6962	0.5261	0.405	0.3903	0.2232	0.04876	0.5112	0.2065	x	0.5783
			*	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	x	n.s.
	CS x *per*^{ts}		0.02283	0.3269	0.6887	0.9289	1	0.5928	0.3949	0.9635	0.4679	0.8179	0.7483	0.6484	0.5512	0.6721	0.7396	0.6493	1	0.1796	x	0.1019
			*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	x	n.s.

Ansatz			F3	F6	F9	F12	F15	F18	F21	F24	F27	F30	F33	F36	F39	F42	F45	F48	F51	F54	F57	F60	F63
NOP (LD 12:12)																							
QRS (T-cycles)	CS x *per*^{l}		0.4432	1	0.2128	0.7208	0.8589	0.9291	0.8585	0.8939	0.5726	0.8286	0.9654	0.1372	0.1812	0.4227	0.5335	0.3974	0.2265	0.1081	x	0.02882	0.1289
			n.s.	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	x	*	n.s.
	CS x *per*^{s}		0.02618	0.09987	0.09987	0.09121	0.0144	0.02311	0.003682	0.01445	0.04514	0.011	0.0123	0.01729	0.01794	0.008385	0.002743	0.03243	0.01394	0.002828	x	0.005578	0.002016
			*	n.s.	n.s	n.s.	**	*	***	**	*	**	**	**	**	***	***	*	**	***	x	***	***
	CS x *per*^{ts}		0.8235	0.2131	0.3066	0.002481	0.2662	0.03608	0.07309	0.1881	0.3423	0.5166	0.3063	0.3093	0.526	0.2724	1	0.9169	0.3984	0.955	x	0.8281	0.9141
			n.s.	n.s.	n.s	***	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	x	n.s.	n.s.

Ansatz			F3	F6	F9	F12	F15	F18	F21	F24	F27	F30	F33	F36	F39	F42	F45	F48	F51	F54
QRS (T-cycles)																				
GHI (T-cycles)	CS x *per*^{l}		0.06332	0.009056	0.1503	0.791	0.03546	0.01854	0.7959	0.6775	0.7335	0.9698	0.4272	0.3445	0.623	0.405	0.1209	0.9096	0.8498	0.5674
			n.s.	***	n.s	n.s.	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	CS x *per*^{s}		0.006421	0.01391	0.002089	0.0002409	0.0001621	0.0001612	0.0002017	0.0001485	0.0004181	0.0005264	0.0001309	0.001337	0.0003677	0.0001485	0.0006488	0.0005606	0.0001971	0.001981
			***	**	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
	CS x *per*^{ts}		0.03416	0.0113	0.007219	0.000865	0.1609	0.02875	0.06394	0.2029	0.125	0.329	0.01519	0.3206	0.005518	0.007027	0.09857	0.2173	0.2158	0.06207
			*	**	***	***	n.s.	*	n.s.	n.s.	n.s.	n.s.	**	n.s.	***	***	n.s.	n.s.	n.s.	n.s.

In Table 10 all p-values and the significance levels of the total activity data of the locomotor activity recordings (3.2.1 of male flies feeding on maximum or minimum medium) are summarized and listed in detail.

Table 10: statistic p-values and significance levels of all total activity data of the locomotor activity recordings

condition	tested variables (genotype/lightcondition/medium)	p-value	significance level
whole day / 24 hours	CS max med vs. CS min med	0,00828	***
	per^S max med vs. per^S min med	0,9005	ns
	per^L max med vs. per^L min med	0,04136	*
	per^{01} max med vs. per^{01} min med	0,02361	*
	clkARmaxWD <-> clkARminWD	0,3066	ns
CS	Light vs. Darkness on max med	0,0000044	***
	Light vs. Darkness on min med	0,0562416	ns
per^S	Light vs. Darkness on max med	0,0007520	***
	Light vs. Darkness on min med	0,0007039	***
per^L	Light vs. Darkness on max med	0,0307385	*
	Light vs. Darkness on min med	0,0057220	***
per^{01}	Light vs. Darkness on max med	0,0083730	***
	Light vs. Darkness on min med	0,3577000	ns
clk^{AR}	Light vs. Darkness on max med	0,0009957	***
	Light vs. Darkness on min med	0,0000011	***
Whole day & fed on minimal medium	CS vs. per^S	0.001283929	***
	CS vs. per^L	0.002961792	***
	CS vs. per^{01}	0.000851404	***
	CS vs. clk^{AR}	0.00005246	***
	per^S vs. per^L	0,2193	ns
	per^S vs. per^{01}	0,425	ns
	per^S vs. clk^{AR}	0.026048903	*
	per^L vs. per^{01}	0,6647	ns
	per^L vs. clk^{AR}	0,0005571	***
	per^{01} vs. clk^{AR}	0,0003026	***
Whole day & fed on maximal medium	CS vs. per^S	0,1362	ns
	CS vs. per^L	0.00728372	***
	CS vs. per^{01}	0.0001806	***
	CS vs. clk^{AR}	0,0007675	***
	per^S vs. per^L	0,001958	***
	per^S vs. per^{01}	0.006092283	***
	per^S vs. clk^{AR}	0,01361	**
	per^L vs. per^{01}	0,1576	ns
	per^L vs. clk^{AR}	0,9098	ns
	per^{01} vs. clk^{AR}	0,313	ns

Table 11 displays all statistics of all lifespan experiments with given information about light conditions, the food source, the genotype of flies and the comparison of the genotypes dependent on age or on the percentage of flies alive during the course of the experiment.

Table 11: statistic values of all lifespan experiments sorted by light conditions, type of food source (medium used) and genotype dependent on age or percentage of flies alive during the experiment

light condition	medium used	genotype of flies	p-values dependent on age or percentage of flies alive			
			14 days old	90 % alive	50 % alive	10 % alive
LD 12:12	maximum medium	CS vs. *per^{S}*	0,08753 (n.s.)	0,02807 (*)	0,0003147 (***)	0,001478 (***)
		CS vs. *per^{L}*	0,7016 (n.s.)	0,06943 (n.s.)	0,006322 (***)	0,01713 (**)
		CS vs. *per^{01}*	0,6961 (n.s.)	0,1036 (n.s.)	0,0953 (n.s.)	0,002432 (***)
		CS vs. *clk^{AR}*	1 (n.s.)	0,02089 (*)	0,1762 (n.s.)	0,1037 (n.s.)
	minimum medium	CS vs. *per^{S}*	0,08503 (n.s.)	0,007001 (***)	0,0002763 (***)	0,0002695 (***)
		CS vs. *per^{L}*	0,4233 (n.s.)	0,08198 (n.s.)	0,0001766 (***)	0,0001766 (***)
		CS vs. *per^{01}*	0,618 (n.s.)	0,2563 (n.s.)	0,0004245 (***)	0,00314 (***)
		CS vs. *clk^{AR}*	0,8357 (n.s.)	0,191 (n.s.)	0,0009004 (***)	0,002151 (***)
LD 12:12	maximum vs. minimum	CS	0,7869 (n.s.)	0,8798 (n.s.)	0,0137 (**)	0,002177 (***)
		per^{S}	0,5399 (n.s.)	0,07826 (n.s.)	0,001228 (***)	0,0009242 (***)
		per^{L}	0,8793 (n.s.)	0,9397 (n.s.)	0,00973 (***)	0,0001786 (***)
		per^{01}	0,9077 (n.s.)	0,02813 (*)	0,0001776 (***)	0,001293 (***)
		clk^{AR}	0,7695 (n.s.)	0,003703 (***)	0,000253 (***)	0,0002746 (***)
19 h T-cycle	maximum medium	CS vs. *per^{S}*	0,5702 (n.s.)	0,9696 (n.s.)	0,008032 (***)	0,0002435 (***)
29 h T-cycle		CS vs. *per^{L}*	0,04507 (*)	0,4019 (n.s.)	0,1188 (n.s.)	0,001438 (***)
LL		CS vs. *per^{01}*	0,0002268 (***)	0,001857 (***)	0,1011 8 (n.s.)	0,0001736 (***)
24h vs. 19h T-cycle	maximum medium	CS	0,03051 (*)	0,1025 (n.s.)	0,01696 (**)	0,006441 (***)
		per^{S}	0,762 (n.s.)	0,733 (n.s.)	0,5703 (n.s.)	0,705 (n.s.)
24h vs. 29h T-cycle	maximum medium	CS	0,01363 (**)	0,008594 (***)	0,0007339 (***)	0,001123 (***)
		per^{L}	0,4048 (n.s.)	0,384 (n.s.)	0,1293 (n.s.)	0,001672 (***)
24h vs. LL	maximum medium	CS	0,02065 (*)	0,05869 (n.s.)	0,03039 (*)	0,01537 (**)
		per^{01}	0,7247 (n.s.)	0,2705 (n.s.)	0,009606 (***)	0,0004224 (***)

The following two tables (Tab. 12 and 13) display the data of all humidity eclosion and wing expansion experiments as well as the statistics, respectively.

Table 12: all data of successful eclosion and wing expansion of wild type CS and *period* mutant flies at different relative humidity values (2%, 60%, 80 % rH).

genotype	% rH	percentage of successfully eclosed flies [%]	percentage of properly unfolded wings [%]
CS	2	89,22	95,72
	60	96,58	99,70
	80	97,92	98,87
per^{01}	2	95,55	89,88
	60	97,94	97,02
	80	97,95	94,30
per^{S}	2	87,89	95,70
	60	92,31	97,61
	80	96,13	98,53
per^{L}	2	93,46	98,16
	60	97,91	97,41
	80	97,52	97,05

Table 13: all statistic p-values and level of significance of successful eclosed flies and successfully unfolded wings at 2 %, 60 % and 80 % rH

	comparison: genotype/rH vs. genotype/rH	eclosion success		wing expansion success	
		p-value	level of significance	p-value	level of significance
differences between 2, 60 and 80 % rH within a genotype	CS/2% vs. CS/60%	0,008658	***	0,004772	***
	CS/2% vs. CS/80%	0,001332	***	0,007992	***
	CS/60% vs. CS/80%	0,01265	**	0,01364	**
	per^S/2% vs. per^S/60%	0,229	ns	0,2403	ns
	per^S/2% vs. per^S/80%	0,003853	***	0,0675	ns
	per^S/60% vs. per^S/80%	0,08691	ns	0,05162	ns
	per^L/2% vs. per^L/60%	0,004329	***	0,3776	ns
	per^L/2% vs. per^L/80%	0,01199	**	0,4086	ns
	per^L/60% vs. per^L/80%	0,607	ns	0,3121	ns
	per^{01}/2% vs. per^{01}/60%	0,06494	ns	0,004329	***
	per^{01}/2% vs. per^{01}/80%	0,007592	***	0,1135	ns
	per^{01}/60% vs. per^{01}/80%	0,9546	ns	0,5287	ns
differences between genotypes at 2 % rH	CS/2% vs. per^S/2%	0,5887	ns	1	ns
	CS/2% vs. per^L/2%	0,3095	ns	0,09307	ns
	CS/2% vs. per^{01}/2%	0,04113	*	0,04113	*
	per^S/2% vs. per^L/2%	0,06494	ns	0,09307	ns
	per^S/2% vs. per^{01}/2%	0,004329	***	0,02597	*
	per^L/2% vs. per^{01}/2%	0,5887	ns	0,002165	***
differences between genotypes at 60 % rH	CS/60% vs. per^S/60%	0,09213	ns	0,004772	***
	CS/60% vs. per^L/60%	0,3095	ns	0,213	ns
	CS/60% vs. per^{01}/60%	0,1797	ns	0,007796	***
	per^S/60% vs. per^L/60%	0,02002	*	0,1727	ns
	per^S/60% vs. per^{01}/60%	0,02002	*	0,4848	ns
	per^L/60% vs. per^{01}/60%	0,6991	ns	0,229	ns
differences between genotypes at 80 % rH	CS/80% vs. per^S/80%	0,09199	ns	0,8098	ns
	CS/80% vs. per^L/80%	0,2479	ns	0,07487	ns
	CS/80% vs. per^{01}/80%	0,5003	ns	0,002468	***
	per^S/80% vs. per^L/80%	0,4524	ns	0,1556	ns
	per^S/80% vs. per^{01}/80%	0,2327	ns	0,02162	*
	per^L/80% vs. per^{01}/80%	0,8946	ns	0,2891	ns

6.5 Abbreviations

#	number
%	percent
°C	degree Celsius
A	adenosine
aa	amino acid
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid/aspartate
C	Cytosine
ccg	clock-controlled genes
CCID	CLK:CYC inhibition domain
CEST	Central European Summer Time
CLD	Cytoplasmatic localization domain
clk	*clock* gene
CLK	Clock protein
CNS	Central nervous system
CO_2	carbon dioxide
c*ry*	cryptochrome gene
CRY	Cryptochrome protein
CT	circadian time
cyc	*cycle* gene
CYC	Cycle protein
Cys	Cysteine
DAM	Drosophila activity monitoring
D	darkness
DD	constant darkness
DNs	dorsal neurons
E	evening
e.g.	for example (lat. “exempli gratia”)
et al.	and others (lat. “et alii”)

Fig.	figure
G	Guanine
GFP	Green Fluorescent Protein
Gln	Glutamine
Glu	Glutamic Acid/Glutamate
Gly	Glycine
h	hour
His	Histidine
Ile	Isoleucine
LD	light-dark
LD-cycle	light-dark cycle
LDR	light-dark cycle with simulated twilight (R= ramp)
LED	light emitting diode
Leu	Leucine
LL	constant light
l-LNvs	large ventrolateral neurons
s-LNvs	small ventrolateral neurons
LNds	dorsolateral neurons
LPN	lateral posterior neurons
Lys	Lysine
M	morning
µm	micrometer
max med	maximum medium, nutrient-rich
Met	Methionine
ml	milliliter
min	minute
min med	minimum medium, nutrient-poor
mm	millimeter
n	sample size
NLS	nuclear localization signal
PAS-A/-B	PAS domain
PBS	Phosphate buffered saline

PDF	Pigment dispersing factor
per	*period* gene
PER	Period protein
Phe	Phenylalanine
Pro	Proline
rH	relative humidity
ROI	region of interest
RT	room temperature
SCN	suprachiasmatic nuclei
SD	standard deviation
SEM	standard error of the mean
Ser	Serine
τ	free-running rhythm / endogenous period
T	Thymine
T-cycle	time cycle
TG	Threonine-Glycine repetition
Thr	Threonine
tim	*timeless* gene
TIM	Timeless protein
Tyr	Tyrosine
Val	Valine
vri	*vrille* gene
VRI	Vrille protein
χ^2	Chi square
ZT	Zeitgeber time [T]

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"It is the time you have wasted for your rose that makes your rose so important." – Antoine de Saint-Exupéry, The Little Prince.

Curriculum Vitae

Publication list

1. Horn M, Mitesser O, Hovestadt T, Yoshii T, Rieger D, Helfrich-Förster C (2019); The Circadian Clock Improves Fitness in the Fruit Fly *Drosophila melanogaster*. Frontiers in Physiology 10.

2. Schäbler S, Amatobi KM, Horn M, Rieger D, Helfrich-Förster C, Mueller MJ, Wegener C, Fekete A (2020); Loss-of-function in the *Drosophila* clock gene *period* results in altered intermediary lipid metabolism and increased susceptibility to starvation. Cellular and Molecular Life Sciences.

3. Ruf F, Mitesser O, Mungwa ST, Horn M, Rieger D, Hovestadt T, Wegener C (in submission); Natural Zeitgebers cannot compensate for the loss of a functional circadian clock in timing of a vital behavior in *Drosophila* under temperate conditions. Proceedings of the Royal Society B.

Affidavit

I hereby confirm that my thesis entitled “The impact of *Drosophila melanogaster*`s endogenous clock on fitness: Influence of day length, humidity and food composition” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.
Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

_____ _____

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Auswirkungen von *Drosophila melanogaster*`s Innerer Uhr auf die Fitness: Einfluss von Tageslänge, Luftfeuchtigkeit und Ernährung“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.
Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

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Ort, Datum Unterschrift