Measurement of Redox States in Drosophila melanogaster Circadian mutants

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Bachelor thesis

Rijeka, 2020.

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SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU
Preddiplomski sveučilišni studij
"Biotehnologija i istraživanje lijekova"

Eva Mihelec

Mjerenje redoks statusa cirkadijalnih mutanata *Drosophile melanogaster*

Završni rad

Rijeka, 2020.

Mentor rada: dr. sc. Rozi Andretić-Waldowski
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This thesis was defended on the 24.9.2020.

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This thesis contains _26_ pages, _7_ pictures, _1_ tables and _16_ references.

Abstract

Circadian rhythms are oscillations that occur in a 24 hour period as a result of an endogenous biochemical oscillator known as the circadian clock. It regulates physiological and behavioral processes in all mammals. Hydrogen peroxide (H_2O_2) is a reactive oxygen species (ROS) molecule that also acts as a signaling molecule. We wanted to look at the connection between oxidative stress and the involvement of circadian genes to then be able to further investigate the effects of methamphetamine (METH) on the redox states in *Drosophila.* Disruptions in circadian rhythms are often seen in substance abuse disorders. In order to measure oxidative stress we used fluorescent dihydroethidium (DHE) staining and studied the variations in the levels of H_2O_2 in the *wild type* (*wt*) and circadian mutants Clk^{Jrk}, per^{01}, tim^{01} and cyc^{01} of *Drosophila melanogaster.* Furthermore, we wanted to know if there was a difference in levels of H_2O_2 circulating in the head and body of the fly and whether it was under the influence of the time of day. Our data suggests that H_2O_2 levels vary between *wt* and circadian mutants and that in the circadian mutants the concentration of H_2O_2 varies depending on the time of day, while in *wt* it does not. More H_2O_2 is found in the head.

Keywords: *Drosophila melanogaster*, circadian rhythms, hydrogen peroxide, oxidative stress

Sažetak

Cirkadijalni ritmovi su oscilacije koje se zbivaju u periodu od 24 sata kao rezultat endogenog biokemijskog oscilatora poznatog kao cirkadijalni sat. On regulira fiziološke i bihevioralne procese u svim sisavcima. Vodikov peroksid (H_2O_2) je jedna od molekula reaktivnih kisikovih vrsta koja ujedno djeluje i kao signalna molekula u mnogim procesima. Htjeli smo vidjeti poveznicu između oksidativnog stresa i cirkadijalnih gena kako bi mogli dalje istražiti utjecaj metamfetamina (METH) na redoks status kod *Drosophile.* Poremećaji u cirkadijalnim ritmovima se često viđaju kod zloupotrebe substanci. Kako bismo izmjerili oksidativni stres koristili smo se metodom bojanja fluorescentnom dihidroetidij (DHE) bojom te smo mjerili koncentraciju H_2O_2 koji se nalazi u *wild type* (*wt*) mušicama i cirkadijalnim mutantima Clk^{Jrk}, per^{01}, tim^{01} and cyc^{01} *Drosophile melanogaster.* Nadalje, htjeli smo vidjeti postoji li razlika u količini H_2O_2 koji se nalazi u glavi i u tijelu mušica i je li pod utjecajem doba dana. Naši podatci ukazuju na to da količine H_2O_2 variraju između *wt* i cirkadijalnih mutanata i da kod cirkadijalnih mutanata koncentracija H_2O_2 varira ovisno o dobu dana, dok kod *wt* ne. Veća je koncentracija H_2O_2 u glavi.

Ključne riječi: *Drosophila melanogaster*, cirkadijalni ritmovi, vodikov peroksid, oksidativni stres

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1. Introduction

An organism changes its behavior and physiology such as sleep-wake cycles, body temperature and hormonal and nervous activity over the course of 24 hours according to the changes in its environment. That is due to the presence of cellular circadian clocks, which perceive changes in the light:dark cycle and adjust to it. This endogenous biochemical oscillator has been discovered in all organisms that have been studied so far. A number of genes are involved in circadian timekeeping and function in a cell-autonomous manner. Clock genes in *Drosophila* are expressed in around 150 neurons in the brain (out of ~250 000), but also in in glia cells, sensory neurons and peripheral organs [1]. All cells in *Drosophila*, including the central clock neurons in the head, are equipped with a genetic time-keeping mechanism that involves rhythmic transcription of genes whose protein products then inhibit their own transcription, negative transcription/translation feedback loops (TTFLs). *period (per)* and *timeless (tim)* are the 2 clock genes in *Drosophila* that autoregulate their transcription by inhibiting transcriptional activity of a heterodimer composed of subunits *Clock (Clk)* and *cycle (cyc)* [2]. The *Clk and cyc* heterodimers are the activators which then initiate the transcription of *per* and *tim* genes by binding to their promoters called E-boxes. This happens from mid-day to early night [3]. After translation, PER and TIM act as a supstrate of several kinases and phosphatases and dimerize. PER and TIM form a complex with kinase Dbt and translocate into the nucleus to repress the CLK/CYC complex by binding to it, which ultimately, inhibits *per* and *tim* transcription. PER and TIM are destabilized and degraded. Some CLK proteins are accumulated in their non-phosphorylated form and heterodimerize with CYC to start another cycle of *per* and *tim* transcription [3]. (Fig. 1)

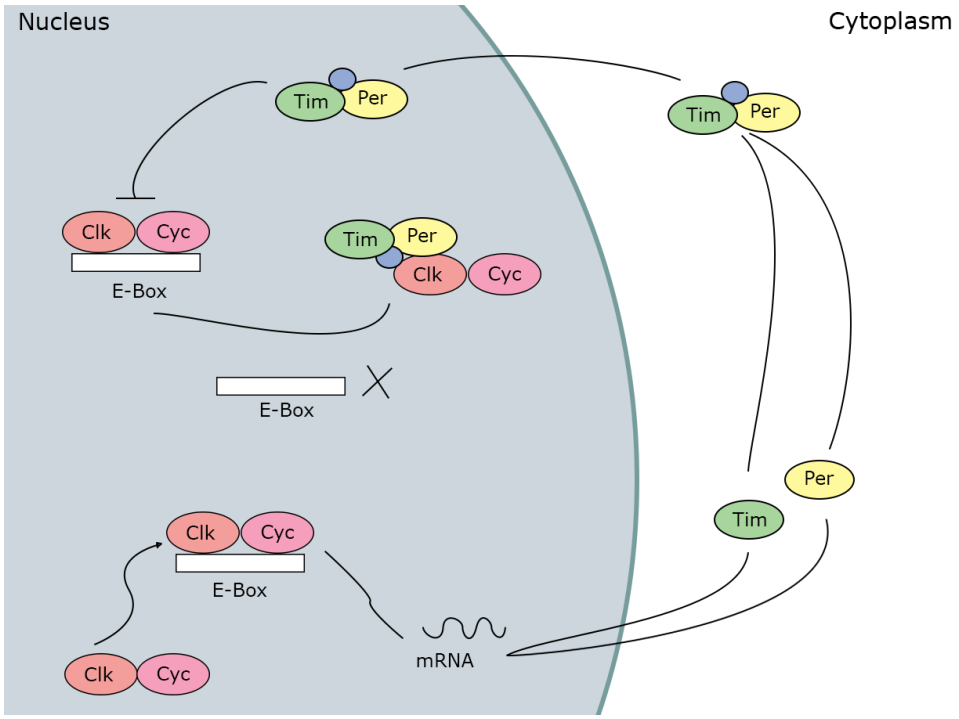

Figure 1: The negative transcription/translation loop

The blue-light sensitive flavoprotein cryptochrome (CRY) plays an essential role in regulating the circadian clock in *Drosophila,* as well as other animals. When light is apsorbed CRY changes its conformation which enables it to bind to TIM, resetting the phase of the circadian rhythm.

In a 12:12 hour light:dark cycle, the locomotor activity of the flies reaches peak during dawn and dusk. The levels of *per* and *tim* mRNA rise during the day and are most elevated in the early to late evening. [3] That is when the PER and TIM proteins start to accumulate, first in the cytoplasm and then around the middle of the night in the nucleus. In the cytoplasm, TIM stabilizes PER and aids in transporting it to the nucleus. TIM levels are the lowest in the early morning and by midafternoon PER levels also decrease notably. With the decrease of PER levels, the negative feedback loop is lifted which allows for a new cycle of transcription [4].

1.1. Reactive oxygen species

Reactive oxygen species (ROS) such as superoxide radicals (O_2^-), peroxides (ROOR), and hydroxyl radicals (OH^-), are byproducts of normal cellular metabolism that have a critical role in physiological processes like signaling, biosynthesis and cellular defence in the body. They are normally produced in the mitochondria as a byproduct of metabolism or by cause of environmental stressors such as UV light or chemical pollution [5]. They can be neutral molecules, ions or radicals. Low ROS levels may result in a lack of signaling, decreased host defence and pathological compensation and high levels can increase the chances of developing neurological disorders, cancer, chronic inflammation and cardiovascular disease. At high concentrations they react with proteins, lipids, carbohydrates and nucleic acids, often resulting in irreversible damage. Cellular respiration and metabolic processes are the main sources of ROS. ROS are highly reactive and quickly transition between species [6].

1.2. Link between ROS and circadian rhythms

The production of antioxidants and protective enzymes has been reported to be regulated or expressed in a rhythmic manner. This links oxidative stress and circadian rhythms. A great deal of data has shown that the circadian regulation of expression of proteins plays a significant role in the cellular response to oxidative stress [5].

In 2008, Krishnan et al. showed that mortality rates varied when flies were exposed to acute oxidative stress induced by H_2O_2 at different times during the day/night cycle. They found that there was a higher mortality rate in flies exposed to H_2O_2 during the day and that those flies had higher levels of protein carbonylation, which is a result of the oxidation of proteins.

Most of the numerous complex behaviors that *Drosophila* exhibits such as walking, flying, grooming, fighting, mating and egg-laying are under control of the circadian rhythm. Both feeding or direct injection of H_2O_2

results in increased activity and changes in behaviour which implies an effect on daily locomotor rhythms [7].

The cell produces antioxidants and protective enzymes in order to counteract reactive oxygen species produced in the body. Those antioxidants and protective enzymes seem to be generated in a rhythmic fashion [5].

1.3. H_2O_2 as a signaling molecule

One of the important cellular processes that ROS are involved in, especially O_2^- and hydrogen peroxide (H_2O_2), is in cellular signaling controlling a variety of biological processes. They have been shown to be involved in many pathways, such as kinase activation and insulin action, and have qualities that make them great signaling molecules. [5] While some of them are extremely unstable, others, such as H_2O_2, are freely diffusible and relatively long-lived [8]. Hydrogen peroxide is a non-radical oxidant that can be found in all aerobic organisms. It used to be viewed as a detrimental byproduct of oxidative metabolism, but today it is known that it plays an important role in cellular signaling. It controls signaling pathways in cells by oxidative modulation of the activity of redox sensitive proteins called redox switches. [9]

2. Aims

The main aim of this thesis is to determine if there is a difference in the amount of hydrogen peroxide (H_2O_2) present in the wild type and circadian mutants of *Drosophila melanogaster*. It was previously shown that there is a connection between circadian genes and ROS production and the cell's attempt to counteract ROS molecules. Circadian mutant strains used in this thesis are: Clk^{Jrk}, per^{01}, tim^{01} and cyc^{01}.The main aim has three sub-aims.

The first one is to determine whether there is a difference in levels of H_2O_2 in the heads versus bodies of the flies. This will be carried out by beheading the flies and measuring the amount of H_2O_2 in the heads and bodies separately. Since METH has an effect on the brain, this information will be useful for comparing the changes of concentration of H_2O_2 after METH feeding in a following research. The second aim is to determine if there is difference in concentration of H_2O_2 depending on the time of the day. This experiment will be conducted at 09:00 and 19:00. The third aim is to compare the amount of H_2O_2 in circadian mutants *per*^{01} and *tim*^{01} versus *Clk*^{Jrk} and *cyc*^{01}.

3. Materials and methods

3.3. Fly Stocks

All assays were performed on male flies from circadian mutant strains of *Drosophila melanogaster Clk*^{Jrk}, *per*^{01}, *tim*^{01} and *cyc*^{01} raised on a standard cornmeal/agar medium at 25°C with 70% humidity in a 12-hour light/12-hour dark cycle. The results were then compared to the values of H_2O_2 concentrations in wild type (*wt) Drosophila* of *CantonS* background.

3.4. Homogenization and extraction of native proteins

The process consisted of weighing empty, 1.5ml Eppendorf tube, beheading the anesthetized flies and then weighing the same tubes with fly heads or bodies in order to measure the weight of those tissues. The extraction buffer solution was prepared with PBSx1 (Phosphate Buffered Saline) and 0.1% TritonX-100. PBSx1 was prepared using 8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24g of KH_2PO_4. With a known weight of fly tissue, we then calculated the needed amount of buffer using the equation:

$$\frac{\mathbf{5}\boldsymbol{mg}}{\mathbf{300}\mu\mathbf{l}} = \frac{\boldsymbol{m(fly\ sample)}}{\boldsymbol{x}}$$

where x is the amount of buffer required and m is the mass of the fly sample.

After weighing, the fly heads and bodies were homogenized using a motorized pestle and the buffer was added to form a homogenous mixture. (Fig.1)

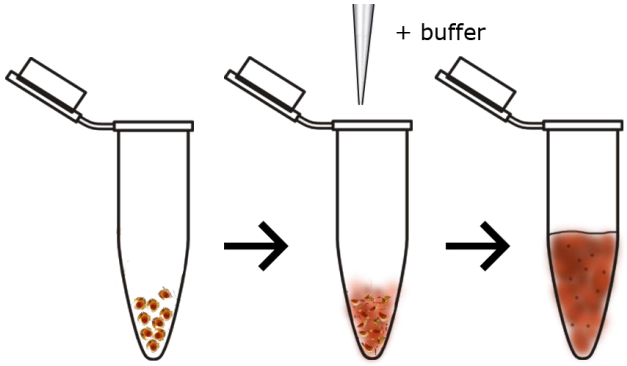

Figure 2: Homogenization of fly heads in buffer (PBSx1+TritonX) using a motorized pestle

3.5. Measuring the concentration of hydrogen peroxide

An array of methods for the detection of ROS have been developed over the years. One of them is dihydroethidium (DHE) staining. DHE is a cell permeable dye which is oxidized by O_2^- to form red fluorescent 2-hydroxyethidium (2-OH-E^+) or by other oxidants such as H_2O_2 to form the non-specific red fluorescent ethidium (E^+). [10]

The tubes were centrifuged for 35 minutes at 4°C and 14000rpm. In the meantime, a 10μM solution of DHE was prepared in PBSx1 with minimal light exposure because of the light sensitive dye by covering the tube with aluminum foil. 200μl of the DHE solution was then pipetted into the wells of a 96-well Black Polyethylene Terephthalate plate in triplicate. After the centrifugation was finished, 5μl of supernatant from each tube was pipetted onto the wells with the DHE solution along with a control sample (DHE solution without the *Drosophila* sample in a triplicate). The reaction was then incubated for 30 minutes at room temperature.

In order to measure the fluorescence intensity we used the Tecan Infinite 200 Pro multimode plate reader (top reading) at an excitation wavelength of 480 nm and emission wavelength of 625 nm.

4. Results

In order to be able to determine the concentration of hydrogen peroxide in the selected tissues we made a calibration curve with relative fluorescence unit (RFU) versus concentration of H_2O_2 in pM. The higher the fluorescence intensity, the higher the concentration of of H_2O_2.

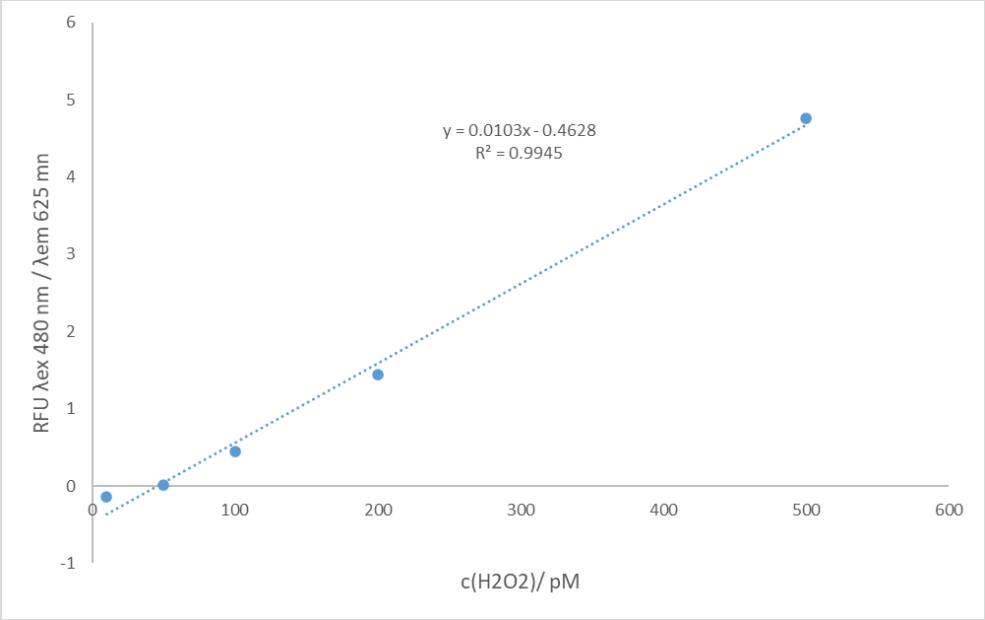

Figure 2: **Calibration curve for determining the concentration of H_2O_2 in an unknown sample**

The relative fluorescence unit (RFU) at an excitation wavelength of 480 nm and emission wavelength of 625 nm was used for determining the concentration of H_2O_2 in tissue samples.

We prepared the tissues; fly heads and bodies at 09:00 and 19:00, for the quantification of the amount of peroxide present. The experiments were conducted over a span of 4 days: starting at 09:00 for *per*^{01} and *tim*^{01}, then

Clk^{Jrk} and *cyc*^{01}, repeating the process starting at 19:00. The obtained values can be seen in Table 1.

Table 1: **Concentrations of H_2O_2 in circadian mutants *per*^{01}, *tim*^{01}, *Clk*^{Jrk} and *cyc*^{01} and *wt.*** Average of 3 samples ± standard error

		c(H_2O_2) / nM	
		09:00	**19:00**
***per*^{01}**	body	4.54±0.14	2.94±0.06
	head	5.64±0.23	2.8±0.12
***tim*^{01}**	body	3.87±0.09	1.87±0.08
	head	4.07±0.1	2.29±0.06
***Clk*^{Jrk}**	body	1.14±0.3	2.96±0.1
	head	0.9±0.25	3.18±0.07
***cyc*^{01}**	body	1.38±0.23	2.01±0.08
	head	0.65±0.14	3.12±0.11
wt	body	3.39±0.19	3.17±0.12
	head	4.78±0.38	5.26±0.38

Levels of H_2O_2 differed among tested genotypes. The biggest difference in the amount of H_2O_2 in the bodies at 09:00 was observed between *Clk*^{Jrk} and *cyc*^{01} mutants relative to *wt*. *Clk*^{Jrk} and *cyc*^{01} mutants had similar amounts of H_2O_2 which was significantly lower than in *wt. per*^{01} mutants had higher levels of H_2O_2 than *wt* and *tim*^{01} mutants. (Fig. 3)

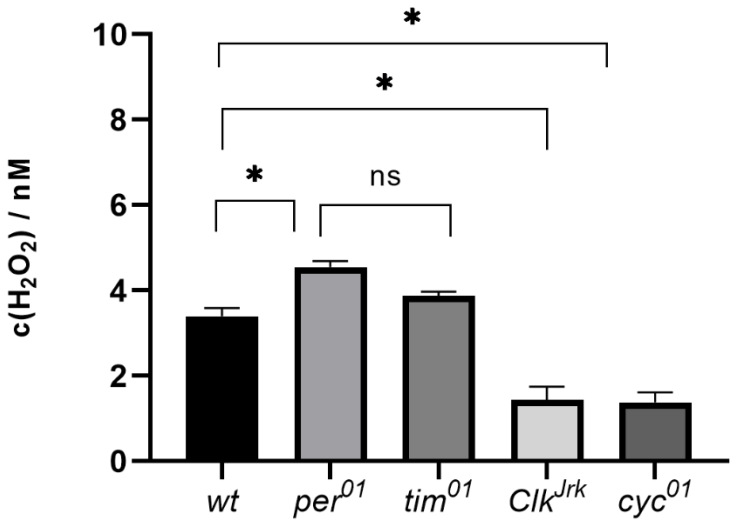

Figure 3: **Circadian mutants differ from *wt* in the amount of H_2O_2 in the bodies at 09:00.** Concentration of H_2O_2 (fluorescence intensity) in the bodies of *Drosophila wt* and circadian mutants per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01} measured at 9:00. The fluorescence reading was done at an excitation wavelength of 480 nm and emission wavelength of 625 nm. One-way ANOVA with post-hoc Bonferroni's multiple comparisons test showed a statistical significance, $P<0.0001$ (*).

The main difference observed at 19:00 in the bodies is that per^{01} and Clk^{Jrk} had similar levels of H_2O_2 relative to *wt*, while it was significantly lower in the bodies of tim^{01} and cyc^{01}. The concentration of H_2O_2 in per^{01} was higher than in tim^{01} and the concentration in Clk^{Jrk} higher than in cyc^{01}. (Fig. 4)

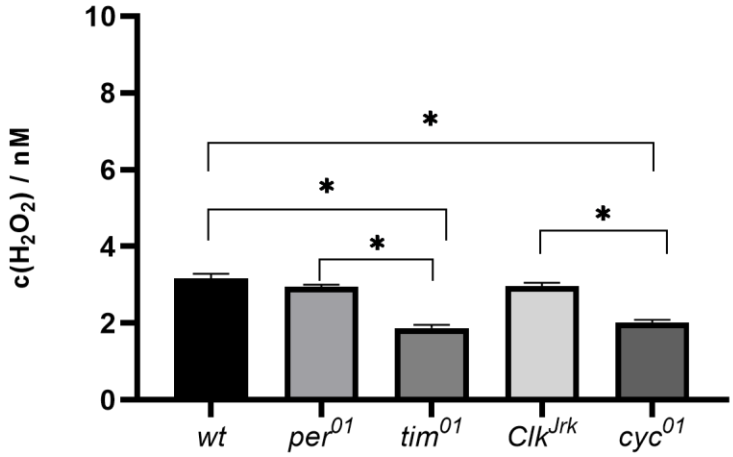

Figure 4: **The amount of H_2O_2 in the bodies at 19:00 is lower in *tim*^{01} and *cyc*^{01} relative to other genotypes.** Concentration of H_2O_2 (fluorescence intensity) in the bodies of *Drosophila wt* and circadian mutants *per*^{01}, *tim*^{01}, *Clk*^{Jrk} and *cyc*^{01} measured at 19:00. The fluorescence reading was done at an excitation wavelength of 480 nm and emission wavelength of 625 nm. One-way ANOVA with post-hoc Bonferroni's multiple comparisons test showed a statistical significance $P<0.0001$ (*)

Following that, we were interested in comparing the concentration of peroxide in the bodies to the concentration found in the heads.

Circadian mutants differed from *wt* in the amount of hydrogen peroxide present. The concentrations of H_2O_2 in the heads at 9:00 are similar to the ones in the bodies at 9:00, but accentuated. The level of H_2O_2 in the heads of *wt* is higher than that in the body. Just like in the body, the concentration of peroxide in *Clk*^{Jrk} and *cyc*^{01} heads was significantly lower compared to *wt* and other mutants. There is no statistical significance between the concentration found in *wt* compared to *tim*^{01} or *per*^{01}. The level of peroxide is significantly higher in *per*^{01} than in *tim*^{01}. (Fig. 5)

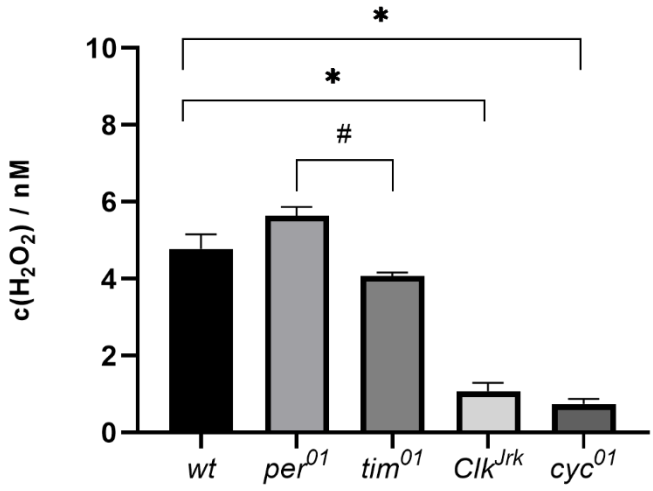

Figure 5: **Circadian mutants differ from *wt* in the amount of H_2O_2 in the heads at 09:00.** Concentration of H_2O_2 (fluorescence intensity) in the heads of *Drosophila wt* and circadian mutants per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01} measured at 09:00. The fluorescence reading was done at an excitation wavelength of 480 nm and emission wavelength of 625 nm. One-way ANOVA with post-hoc Bonferroni's multiple comparisons test showed a statistical significance, $P<0.0001$ (*) and $P\leq0.0001$ (#)

The main difference at 19:00 is that the amount of H_2O_2 in the heads was shown to be significantly higher in *wt* in comparison to all circadian mutants tested. The values seen in the circadian mutants were shown to be similar and non-significant. (Fig. 6)

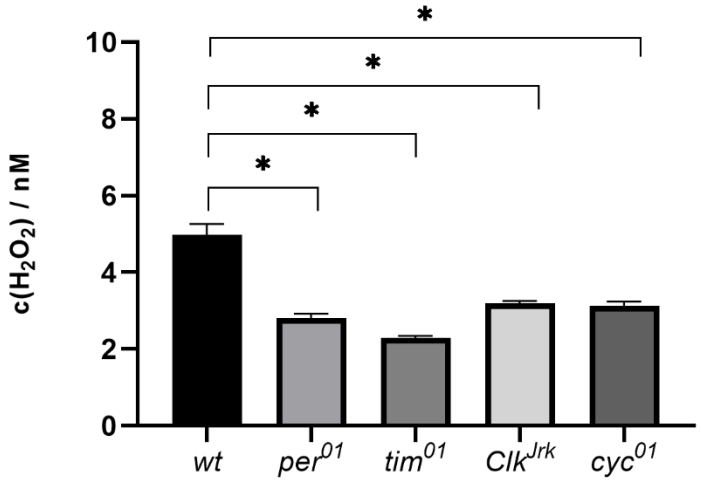

Figure 6: **Circadian mutants differ from *wt* in the amount of H_2O_2 in the heads at 19:00.** Concentration of H_2O_2 (fluorescence intensity) in the heads of *Drosophila wt* and circadian mutants per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01} measured at 19:00. The fluorescence reading was done at an excitation wavelength of 480 nm and emission wavelength of 625 nm. One-way ANOVA with post-hoc Bonferroni's multiple comparisons test showed a statistical significance, $P<0.0001$ (*).

The results depicted in figure 7 are all previous results incorporated in one figure in order to visualize the difference in concentration of H_2O_2 in head versus body and the difference in the time of day. *wt* has a consistent difference in head compared to body, while the circadian mutants show no significant difference between head and body. The amount of H_2O_2 does not change as a function of time of day in *wt*. In circadian mutants it does. The mutants that exhibited a higher concentration of H_2O_2 in the morning, had a lower concentration in the evening and vice versa. per^{01} and tim^{01} show an increase in concentration of H_2O_2 at 09:00 in both heads and bodies compared to 19:00, while Clk^{Jrk} and cyc^{01} have lower concentrations in the morning. (Fig. 7)

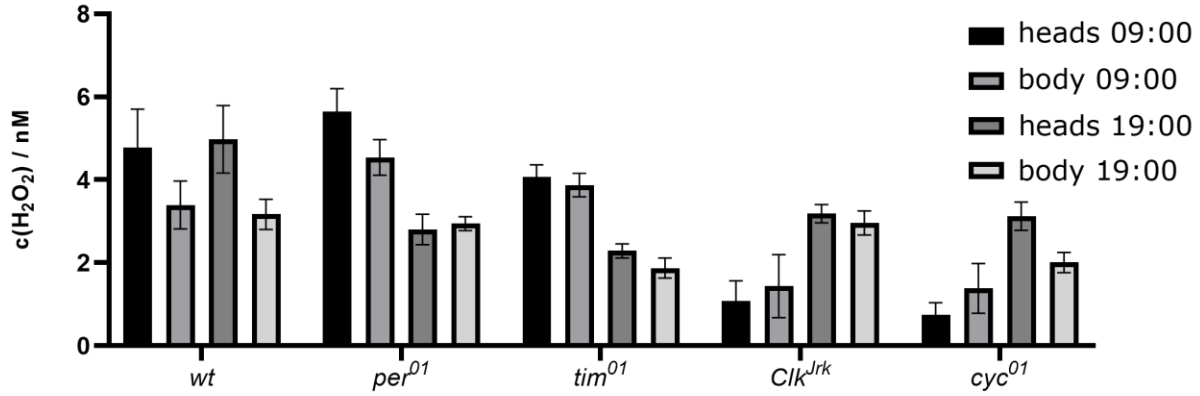

Figure 7: **Concentrations of H_2O_2 in the heads and bodies of *Drosophila wt* and circadian mutants at morning and afternoon time points.** Comparison of all read fluorescence intensities (concentration of H_2O_2). Statistical test used 2-way ANOVA with post-hoc Bonferroni's multiple comparisons test.

5. Discussion

Sleep/wake cycles and locomotion are both under the influence of the circadian clock. The administration of METH causes an increase in activity when awake and shorter sleep times in *Drosophila* [11]. This means that METH has an influence on the circadian clock. The information acquired in this thesis will aid in better understanding what impact METH has on the production of H_2O_2 in *Drosophila.*

The main aim of this work was to determine whether there is a difference in the amount of hydrogen peroxide found in *wt* and circadian mutants Clk^{Jrk}, per^{01}, tim^{01} and cyc^{01} of *Drosophila melanogaster.* Our data indicates that the levels of H_2O_2 in circadian mutants differ from the levels in *wt.* This shows that circadian genes influence the level of H_2O_2 and supports the connection between circadian rhythms and oxidative stress.

We wanted to know if the amount of peroxide in the heads differs from the amount accumulated in the bodies. All drugs of abuse, including methamphetamine, exert their action by having an influence on dopaminergic

circuitry and thus showcasing its rewarding properties [12]. The sources of the neurotransmitters with rewarding properties, such as dopamine, whose production is induced by those drugs are found in the brain. The differences in head and body will also be compared when administering METH. Our data suggests that there is more H_2O_2 in the heads of *wt* in both morning and evening. There is increased metabolic activity in the head compared to the body probably because the small and rapidly diffusible H_2O_2 also acts as a signaling molecule. Total protein carbonylation, a reliable marker for oxidative stress was shown to be significantly higher in the heads of flies, which has been demonstrated to be related to the levels of endogenous production of H_2O_2 by the mitochondria. [1]

The second aim was to find out if there is a difference in concentration depending on the time of day. In *wt* the concentration of peroxide shows no significant difference between the morning and evening. On the other hand, per^{01} and tim^{01} have both shown to have much higher concentrations in the morning in both heads and bodies and Clk^{Jrk} and cyc^{01} in the evening.

It is interesting that while *wt* exhibits a significant difference between the head and body, in the circadian mutants the concentration in the head and body remains similar but varies depending on the time of day. The mutants lack proteins necessary to have working cellular clocks which results in a disrupted circadian rhythm. This could be due to the varied H_2O_2 production by the mitochondria compared to flies with a fully functional circadian clock [1].

Apart from bodies at 19:00, circadian mutants per^{01} and tim^{01} correlate in values and so do Clk^{Jrk} and cyc^{01}. (Fig.7) This finding can be explained by the fact that *per/tim* and *Clk/cyc* form a feedback loop where *per* and *tim* work together *Clk* and *cyc* are subunits of a heterodimer which then activate the

transcription for other clock proteins. per^{01} and tim^{01} have high concentrations in the morning and low in the afternoon, while it is the opposite in Clk^{Jrk} and cyc^{01}. The results for bodies in the afternoon differ from others in this case. Repeating the experiment to confirm whether that is the case would be desirable.

Drosophila exhibits peaks of locomotor activity during dawn and dusk (morning and evening), which is why we chose 09:00 and 19:00 for the experiments. We will be administrating methamphetamine at the same times and thus were interested in the normal variations of H_2O_2 at those times of day. Repeating the experiments at one more time stamp in order to compare the differences would give more insight. There are a few methods and different dyes that can be used for ROS detection. By using more than one method or dye we would have more data to compare and achieve more accurate results.

6. Conclusion

Various previous works have been consistent with a connection between circadian rhythms and oxidative stress. With the help of this data, we will be able to better understand how ROS levels change with the administration of METH in *Drosophila.* Based on the data acquired with the experiments we have concluded that:

- H_2O_2 levels undergo circadian fluctuations in *Drosophila melanogaster,* which further links circadian rhythms to oxidative stress.
- Because of the increased metabolic activity and protein carbonylation, there is more H_2O_2 in the heads.
- Unlike in *wild type,* in circadian mutants, the concentration of H_2O_2 depends on the time of day.
- Circadian mutants per^{01} and tim^{01}/ Clk^{Jrk} and cyc^{01} mostly correlate in values. The loss of one of the two main proteins in each feedback loop results in similar amounts of H_2O_2 being formed.

Nevertheless, for a better understanding of how circadian rhythms are linked to changes in levels of H_2O_2 and other oxidative molecules more experiments are needed.

Having gained this information, we will be administering methamphetamine (METH) to flies to be able to see what kind of effect METH has on the redox states in *Drosophila* and its circadian mutants. Those values will then be compared to normal variations of H_2O_2 presented in this thesis.

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Call centre agent

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Handling incoming calls from Vodafone users from Italy

Shop assistant

Rijeka Airport

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- Rijeka, Croatia

Assisting shoppers in Duty Free Shop, handling goods, help with flight information

Bartender

La Bodega Rijeka

23/06/2018 – 12/07/2018

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Bartending and waitressing

EDUCATION AND TRAINING

Department of Biotechnology, University of Rijeka

Biotechnology and drug research, Bachelor's degree

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High School Diploma
2013 – 2017
Francuska Alijansa
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LANGUAGE SKILLS

Mother tongue(s)

Croatian

Other language(s)

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Listening
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Reading
C2
Spoken interaction
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Spoken production
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B2
Spoken production
B2
Writing
B2

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Listening
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Spoken interaction
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DIGITAL SKILLS

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