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Trehalose as an important energy source for immune cells in *Drosophila melanogaster*

Bachelor´s Thesis

Laboratory of Molecular Integrative Physiology in *Drosophila*

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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BACHELOR THESIS

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ANNOTATION

The aim of this thesis was the identification of the change of trehalase gene expression in hemocytes of *Drosophila melanogaster* in uninfected and infected larvae using parasitoid wasp infection by *Leptopilina boulardi.* Moreover, the energy supply was shut down by deactivating the trehalose transporter and measuring the lamellocyte production, again in uninfected and infected larva to test the role of trehalose for the selfish immune system.

DECLARATION

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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LIST OF ABBREVIATIONS

HPI	**hours post-infection**
RT	**room temperature**
PCR	**polymerase chain reaction**
Treh	**Trehalase**
sTreh	**secreted trehalase**
cTreh	**cytoplasmic trehalase**
cDNA	**complementary DNA**
GAL4	**yeast transcription factor**
Rp49	**Gene – Ribosomal Protein L32**
qPCR	**quantitative PCR**
MM	**Master mix**
Rp49	**Ribosomal protein 49**
RNAi	**RNA interference**
UAS	**Upstream activation sequence**
TreT	**Trehalose transporter**
mRNA	**messenger RNA**
siRNA	**small interfering RNA**
RISC	**RNA-induced silencing complex**
Tps1	**Trehalose-6-phosphate synthase**
GluT	**Glucose transporter**
ATP	**Adenosine-triphosphate**
e-Ado	**extracellular adenosine**
AdoR	**Adenosine receptor**
DIC	**Differential interference contrast**
PBS	**Phosphate-buffered saline**
bp	**base pair**
DEPC	**Diethylpyrocarbonate**
dNTP	**deoxynucleotides**
FW	**forward**
RV	**reverse**
GFP	**Green fluorescent protein**
oligo-dT	**sequence of deoxythymidine nucleotides**
Inf	**infection**

TALE OF CONTENT

1. INTRODUCTION

1.1. Overview

An organism can only survive an infection if it has a well- functioning immune system. The immune response requires a great deal of energy. This study tried to show the energy demand of immune cells upon infection. During infection, provoked by the parasitoid wasp *Leptopilina boulardi*, immune cells receive a command to switch to a faster but less effective kind of metabolism [1]. To ensure that its high demand for energy is fulfilled, the immune cells must be privileged over the rest of the organism. First, all other processes like growth and energy storage are shut down. Besides, immune cells must have a privileged access to energy and, or nutrients [2]. This is known to occur in mammals but had not yet been found in insects [3].

Drosophila melanogaster was chosen as a model because it is one of the most known and best-studied organisms in the world [4]. It is a great model for genetic research since many identified genes are also found in other organisms, like the human. The fly has been used in a large range of study's for better understanding e.g. of cellular metabolism for cancer or immunity and also for circadian clocks [5] [6]. Other advantages are the easy handling of the fly and a huge number of genetic tools which are available [7]. The genetic tools like GAL4/UAS system and the RNA interference (RNAi) are well established in *Drosophila melanogaster* and are tremendously important for genetic studies, they also have been used in this study [8].

This thesis demonstrated that immune cells are selfish, they usurp the energy. The main energy, in this case, is the sugar trehalose. It's the most important one in insects. So how is it possible to make the immune system selfish? It is believed that only important or privileged tissues like the immune cell can take up energy, during immune response. Trehalose is used by the immune cell to produce energy; the other cells are only able to utilize glucose. Trehalose is taken into the immune cell where it is converted to glucose. Therefore, the converted glucose is only available for the immune cell, and the glucose is not accessible for other tissues.

1.2. Genetic tools in *Drosophila melanogaster*

RNA interference (RNAi) together with Gal4-UAS (Upstream activation sequence) was used in this study to shut down the trehalose transporter (TreT). This was done, to show the dependency of the immune cell on trehalose. Due to the knocked-down TreT, which specifically occurs in the fruit flies' immune cells, the uptake of trehalose was decreased leading to a lower production of specialized immune cells to counteract the infection. This could be achieved by a simple cross of one fly line inserted with the Gal4- system and one with the RNAi TreT silencing. The progeny of this cross then has the silenced TreT.

1.2.1. RNAi

Generally, RNAi is a natural mechanism in the cell of eukaryotes for targeted silencing of genes. RNAi is based on interactions of short pieces of RNA to specifically induce degradation of an mRNA (messenger RNA) involving several enzyme complexes [9]. The procedure for RNAi works as follows: First, double-stranded RNA molecules are cut into small interfering RNA (siRNA) with ribonuclease enzymes Dicer or Drosha. The double-stranded siRNA then binds an Argonaut protein. One strand of the siRNA is selected and remained bound to Argonaut, this is the guide strand. Combining the siRNA and Argonaut with other proteins is named RNA induced silencing complex (RISC). The siRNA guide RISC; to bind to specific mRNA. Targeting is accurate because it by base pairing between siRNA and the target mRNA. Most siRNAs and micro RNAs are approximately 21 nucleotides long. siRNA often have identical base pairs to the target sequence. Once the siRNA is bound, Argonaut catalyses the cleavage of mRNA, which leads to degradation of mRNA [10] [11].

1.2.2. Gal4-UAS system

The Gal4-UAS system is used for targeting specific gene expressions and is one of the most powerful mechanisms. It origins from the yeast GAL4 transcription factor, which binds to an upstream initiation sequence to a particular insert. Since the GAL4 is not species-specific it can be used in many other models i.e. *Drosophila melanogaster*. This system has two parts, as the name says the Gal4 gene, which encodes the protein Gal4 and the enhancer UAS, to which Gal4 particularly binds, this then activates the expression of the target gene [12].

The GAL4 system is used in a broad range like expresses altered forms of proteins, structure/functions analysis toxins for cell ablation, inhibiting of cellular functions, RNAi, etc.

When separating the GAL4 from the gene of interest in different fly lines, it guarantees that the gene of interest is silent until GAL4 is inserted by a genetic cross [13].

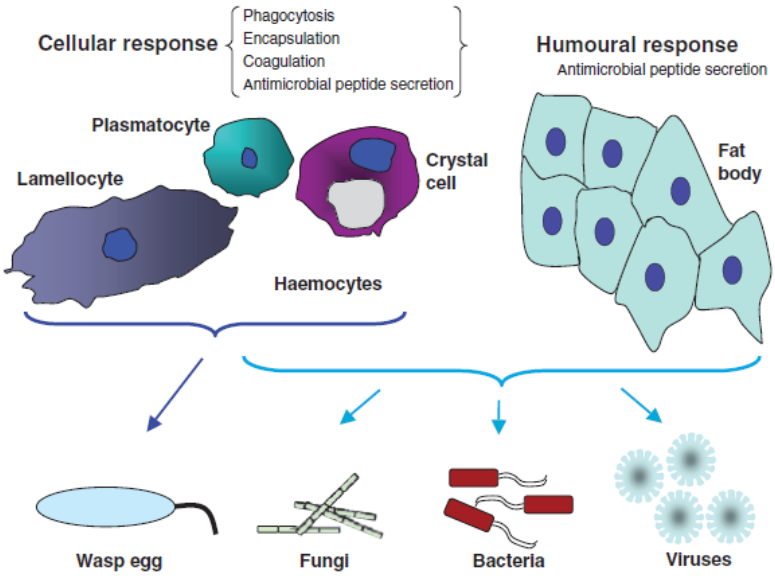
1.3. Leptopilina boulardi

To study the immunity, the larva must be made "sick". This was achieved by using the parasitoid wasp *Leptopilina boulardi*. Parasitic wasps have different strategies, either attack the larval or pupal stage of *Drosophila*. Whilst *Trichopria drosophilae* invade the pupal stage of the fruit fly, females of the wasp *Leptopilina* and *Ganaspis* infect the larval stage (Figure 1). The parasitoid wasp needs a host for their reproductivity. For laying eggs, a sharp needle-like structure is needed, the female wasp has an ovipositor which makes this possible, it lays eggs into the hemocoel. The first line of defence from the larva are movements, further the larva has a cuticle. After injection of the eggs, the cuticle of the larva starts wound healing. Depending on the wasp line, most lay 2 or more eggs into one larva, however, only one egg can emerge into a wasp. The recently injected wasp egg has now two endings, relying on the fly strain and wasp type. Either the egg is encapsulated, the host survives. Or the wasp egg emerges into an adult, resulting in killing the fly [14]. *L. boulardi* has difficulties with surviving in the host since mostly the larva succeeds in encapsulating their eggs. In *L. heterotoma* for example, the eggs of this species are able to form a virus-like particle, which changes the properties of specialized immune cells, which lose their ability to form capsules, this then increases the survival of the wasp egg [15].

Figure 1: *Drosophila* larvae infected by parasitoid wasp [16].

1.4. *Drosophila* immunity

Understanding the immunity of *Drosophila* is necessary for this study. When pathogenic organisms, like fungi or bacteria, invade the host *Drosophila*, initiation of humoral response induces production of antimicrobial peptides (Figure 3) into the hemolymph. On the other hand, there are parasitoid wasp eggs (Figure 4), which are another parasite. Those are then surrounded by hemocytes, which belong to the cellular response [17] [18] [19] [20]. The circulating hemocytes, especially lamellocytes and plasmatocytes, then try to phagocytise or encapsulate the intruder.



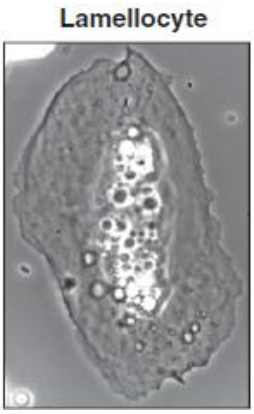


Figure 2: Picture of a lamellocyte,they are larger than other hemocytes, adapted from M.-O. Fauvarque and M. J. Williams [19].

Figure 3: Schematic representation of *Drosophila* larval immune response, there are two types, the cellular response, including lamellocytes, plasmatocytes, and crystal cells and the humoral response which secrete antimicrobial peptides from the fat body, adapted from M.-O. Fauvarque and M. J. Williams [19].

Even though antimicrobial peptide secretion is done by the fat body, cellular response is done by hemocytes, consisting of three types, lamellocytes, plasmatocytes and crystal cells, where each of them provides specific features, i.e. encapsulation, coagulation and phagocytosis [19] [17]. In the larvae of *Drosophila*, hemocytes can be found in three main segments, initially in circulation, secondly in the lymph gland (based behind the brain) and lastly bellow the larval cuticle [19] [20].

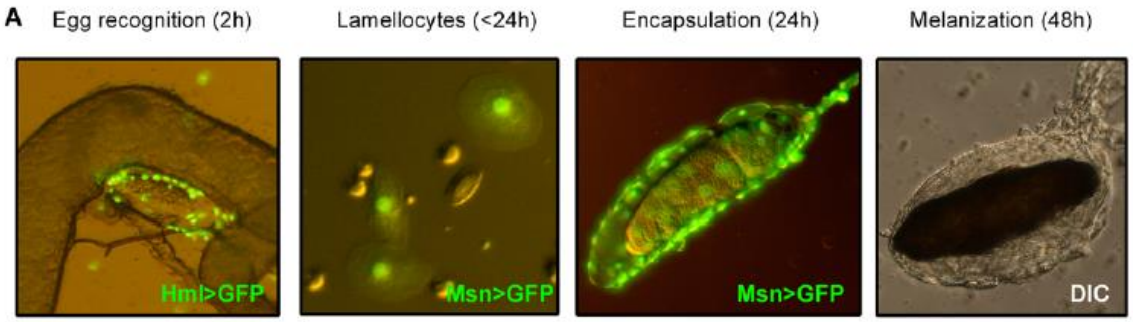


Figure 4: Immune response of *Drosophila* to parasitoid egg of wasp The pictures show that eggs like to hide particularly in the gut folds, the produced lamellocytes which encapsulate the wasp egg which is then followed by melanisation. Adapted from Bajgar et. al [2].

Lamellocytes (Figure 2) are only found in the larval stage and tremendously differentiate in response to parasitism (Figure 5). They are unusual in healthy larva. They form a multi-layered wall (Figure 4) around the parasitoid egg, which has been previously detected by plasmatocytes [17]. Often lamellocytes derive from plasmatocytes [19]. Giving those aspects, lamellocytes were chosen to be the significant criteria for immune response in third instar larvae which are infected with parasitoid eggs in this study. The maximum number of lamellocytes flowing through the hemolymph is approximately 18 hours post-infection (hpi). Therefore, lamellocytes were counted at this time point.

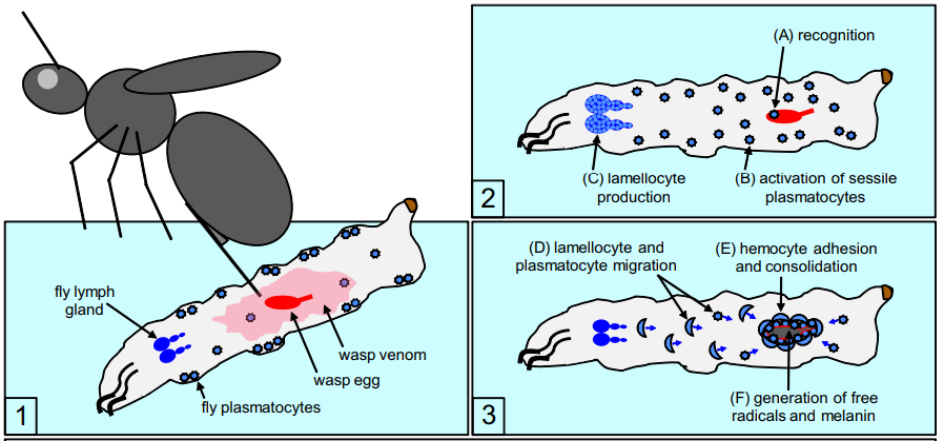


Figure 5: Interaction between larvae of *Drosophila* and parasitoid wasp egg, it visualizes (1) the infection from the wasp, (2) the recognition of the foreign body and (3) the immune response, adapted from Keebaugh et al [21].

1.4.1. Trehalose as an energy source

Trehalose is the primary sugar in insect hemolymph and is present in much higher concentrations than glucose [22] [23] [24]. Trehalose was the main focus of this study. We wanted to show that trehalose is the dominant sugar used during immune response.

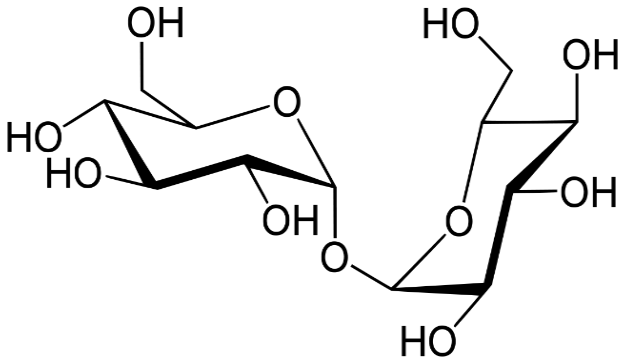


Figure 7: Structure of trehalose.

Trehalose is a disaccharide consisting of two glucose molecules, the structure is shown in Figure 7.

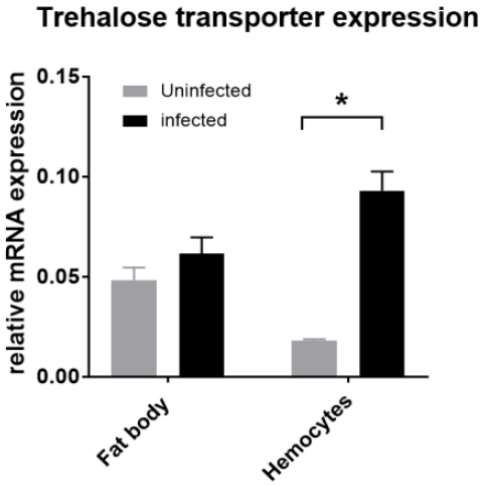


Figure 6: Relative trehalose transporter expression in fat body and hemocytes, during infected and uninfected stage, adapted from Bajgar et. al [2].

It is found in numerous life forms such as bacteria, fungi, insects, plants and invertebrates. Trehalose plays a vital role in stress situations like starvation, freezing, desiccation, and osmopressure. Since trehalose has a high thermostability and a broad pH stability range, this factor makes trehalose a very stable sugar [24]. In insects, where it is the main sugar, it is produced in the fat body. The advantage of trehalose is that it can be stored in high amounts in the insect hemolymph and is used to keep optimal glucose levels low, otherwise it can cause hyperglycemia. [22] [25]. It can be rapidly converted to glucose which is crucial for insect flight for example. In *Drosophila* fat body, the enzyme trehalose-6-phosphate-synthase (Tps1) synthesizes trehalose from glucose and is transported via the trehalose transporter (TreT) [26] [27]. Since trehalose is synthesized in the fat body, a strong expression of TreT can be found, another high expression was found in hemocytes during an immune response (Figure 6). However, the expression levels of glucose transporter (GluT) were lower than the ones from TreT. This obtained data from Bajgar et al [2] pops up the question if immune cells, during infection choose the direct uptake of trehalose and convert it into glucose inside their cell as their own source for increased energy demand.

1.4.2. Trehalase

Trehalase (Treh), is the hydrolyzing enzyme of trehalose and converts trehalose into glucose. But still, the physiological role of the enzyme remains unknown [26]. There are two enzymes that convert trehalose into glucose, via alternative splicing (Flybase) which can then be used directly for metabolic processes: cytoplasmic trehalase (cTreh), which can e.g. be found inside the immune cell, and secreted trehalase (sTreh), which is available outside the cell. The sTreh is used to keep circulating glucose level constant and is part of the systemic regulations. Figure 8 shows the cTreh and sTreh and their position. To fulfill high energy demands during immune response, more trehalose must be converted into glucose, therefore an increase of trehalose gene expression could be expected.

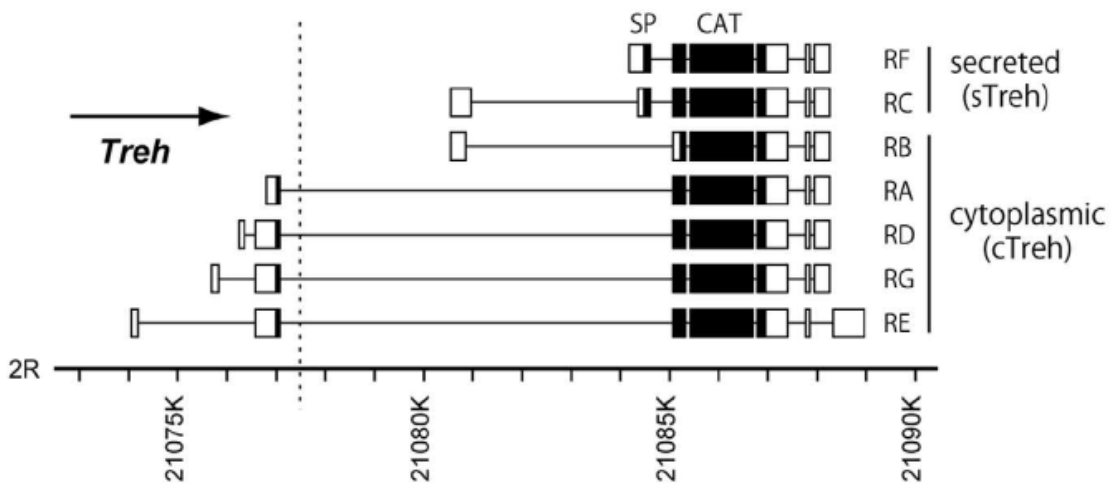


Figure 8: Schematic sketch of Treh locus and two different enzymes which synthesize trehalose, secreted (sTreh) and cytoplasmic (cTreh) [23]. Further, it presents the protein-coding regions and untranslated region, which are represented by filled and open boxes (Flybase).

1.5. Energy requirements of immune response

Fighting a parasite and starting immune response is an energy-consuming process. Energy demands can rise from 10% to 30% of the total body consumption [2]. Activating immune response during infection means that many new cells and molecules must be produced, this requires a great deal of energy [18]. Immune cells experience a metabolic switch that allows the fast production of ATP (Adenosine triphosphate) [1]. This switch is known as the Warburg effect [1]. At normal circumstances, ATP is produced under aerobic respiration. However, hemocytes need a faster but less effective way to produce ATP, therefore a change to increased glycolysis occurs. This metabolic switch is very nutrient demanding, it mostly

needs glucose and glutamine [18]. This life-preserving strategy must be privileged during immunity over the rest of the non-immune tissues [18] [1].

All organism, insects or humans can trigger an immune response, it is one of the most fundamental abilities. Keeping hematopoietic cells in the fruit fly at a steady level requires a great deal of the whole available glucose from the body [28]. The first energy demand during immune response, is the activation of this system. Then, detecting a pathogen triggers further immune reactions, like newly synthesized molecules for signalling or antimicrobial functions and also the production of new immune cells like lamellocytes or plasmatocytes [18] [2].

To fulfil the increased energy demand, it must be primary accessible for the immune system. This means that the immune cells become selfish in terms of energy supply. Extracellular adenosine (e-Ado) is a good example of how immune cells could make themselves selfish. It is used as a signal for stress or injury [29]. During inflammation or cell damage, e-Ado is released from ATP and concentrations increase. The released e-Ado works via specific adenosine receptors (AdoR). With the help of the AdoR, temporary metabolic suppression in some tissues is provoked. How exactly e-Ado induces this suppression is unknown. Examples for affected, metabolically downregulated tissues are imaginal discs, the fat-body, and the muscle cells. The activation of AdoR also ensures the release of glucose from glycogen stores [2] [29].

The first confirmation for the privileged immune cells was shown by Bajgar et al. [2]. Extracellular adenosine is used as a signal in *Drosophila melanogaster*. When obstructing the signal for the energy-demanding metabolic switch, the development, and growth of the fly continued at normal rates. Leading to less energy left for producing enough lamellocytes, which led to a reduced survival rate. This experiment showed the essential meaning of a well-balanced energy supply between immune response and development. [1]

2. AIMS

- To shut down the transporter of trehalose to test the importance of trehalose as a source of energy in immune cells during infection
- To count the produced specialized immune cells (lamellocytes) upon infection
- To optimize the conditions for q-PCR to check the expression levels of transcripts encoding two enzymes that convert trehalose into glucose, cytoplasmic- and secreted-trehalase
- To check the expression levels of those two enzymes during infection

3. MATERIALS AND METHODS

3.1. Fly strains

For investigation, four different fly strains 1252, 1498, 1527 and 1586 were used. 1252 and 1586 have the Gal4-UAS system and 1527 the inserted TreT-RNAi construct. Moreover, for comparison and excluding possible errors the control 1498, with a TRIP genetic background used for the generation of 1527 RNAi line, was crossed with 1252 and 1586 the same way as the strain caring the RNAi. All fly strains and their specific mutations are listed in Table 1. The control 1498 had a well-functioning immune system. Two fly lines 1252vand 1527 had the Gal4/ UAS -system. 1527 carried the TreT- RNAi. The progeny of the crosses $\frac{1252}{1527}$ and $\frac{1586}{1527}$ had the shutdown TreT transporter.

Table 1: Overview of used *Drosophila melanogaster* fly strains.

Abbreviation	**Description**	**Genotype**
1252	HmlΔ-Gal4 UAS-GFP	w; HmlΔ-Gal4 UAS-GFP
1498	TRIP background	y[1] v[1]; P{y[+t7.7]=CarryP}attP2
1527	TreT1-1 RNAi	y[1] sc v[1]; P {TRiP.HMS02573}attP2
1586	Srp-Gal4	w; Srp-Gal4

All fly stocks were held in glass vials and fed with cornmeal medium, the vials were closed with a cotton plug. To test the influence of TreT knock-down during the immune response, the following genetic crosses were performed. From each fly strains 1252 and 1586 approximately 50 female virgins were collected. Those 50 virgins of 1252 and 1586 were put into embryo collecting cages with 30 males of 1527, on agar plates for 2 days. To collect eggs in a short time range, the agar plates were changed after every two hours. Then the eggs were incubated at 25°C. The larvae, which now had the blocked TreT, of the crosses $\frac{1252}{1527}$ and $\frac{1586}{1527}$ were then further used.

3.2. Parasitoid wasp infection

In order to provoke the immune response, the parasitoid wasp *Leptopilina boulardi* was used. Early third-instar *Drosophila* larvae (~ 72h after egg laying) were collected and put onto an agar plate. Around 100 female fertilized wasps were put into that cage and the larvae were infected for 40 min to 1 hr. The cage was immediately put into a cardboard box to ensure darkness, which is crucial for a good infection.

3.3. Lamellocytes counting

After infection, the parasitoid was removed from the cage. The agar plate was then again incubated for 18 hrs. Later, PBS (Phosphate-buffered saline) was used to wash the larvae once, to ensure that all agar was removed. Then one larva at a time was put into a 15 µL drop of PBS and dissected using two forceps. The lower abdominal segment was ripped open and the gut was carefully pulled out of the abdomen. The parasitoid wasp likes to put their eggs in folds of the gut, therefore the gut was straightened, and the number of eggs was counted. 4 to 5 eggs give a strong enough infection. The cuticle was inverted in order to mix the hemolymph with the buffer. Following, 10 µL of the mixed solution was pipetted onto a counting chamber (Improved Neubauer hemocytometer). The slide was left for 5 min to ensure the lamellocytes attach on the surface of the counting chamber. Lastly, the lamellocytes were counted by putting the chamber on a Differential interference contrast (DIC) microscope.

3.4. Gene expression analysis

3.4.1. Hemolymph collection

Three different samples were collected, 0 hpi, 18 hpi, and 18 hpi infected. Around 50 larvae were collected and washed twice with PBS buffer (1x) and once with distilled water. Then the larvae were assembled to make a pile on a glass slide which is covered with a parafilm which is laid on ice. As already described in 3.3 the larvae were ripped, and the hemolymph was squeezed out in a few droplets of PBS. Shortly after this is done the hemolymph of the new pile is collected with a pipette and put into an Eppendorf tube filled with a 1:1 mixture of acetonitrile and 0,9 M NaCl solution. Promptly after, it was stored in -80°C until it was used for further procedures.

3.4.2. Quantitative reverse transcription PCR (RT-qPCR)

The level of gene expression of the cytoplasmic (cTreh) and secreted (sTreh) trehalase were measured using RT-qPCR (quantitative reverse transcription PCR). Prior to qPCR, normal

PCR was done, the RNA was isolated first from the whole larva for PCR condition optimization and later for q-PCR from collected hemolymph (3.4.1). cDNA (complementary DNA) was produced by reverse transcription for both. The quality and quantity of the RNA were evaluated by gel electrophoreses together with measuring the concentration on a spectrophotometer (UVS-99 ACTGene) for the primer products.

3.4.3. RNA isolation

For RNA isolation, the collected samples of hemolymph or the whole larvae were mixed with 0.2 µL TRIzol and homogenized using RNAse free pestles. Then again 0.8 µL TRIzol and 0.2 µL chloroform were added. Immediately after, it was vortexed for 1 min and left for 10 min at room temperature. It was put into the centrifuge at 13,000 rpm for 15 min at 4°C. The upper aqueous phase was carefully transferred into a new Eppendorf tube, the interphase as seen in Figure 9 should not be disturbed. Then 0.5 µL of isopropanol was added and vortexed, it was then directly put on ice for 10 min. Afterward, it was centrifuged at 13,000 rpm for 10 min at 4°C, a grey precipitate should form at the bottom. The supernatant was removed and 0.5 µL of 75% ethanol (96% ethanol in DEPC H_2O) was added for washing and the sample was centrifuged at 13,000 rpm for 5 min at 4°C. This step was repeated, and at the end, the ethanol was removed and air-dried for 3 min at RT. The isolated RNA was dissolved in DEPC H_2O and stored in -80°C.

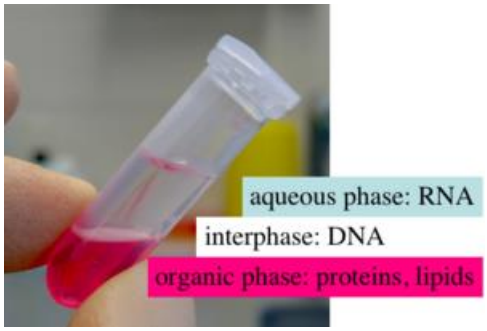


Figure 9: Three phases can be seen for RNA isolation, adapted from Openwetware website [32]

For checking the quality of the isolated RNA, the spectrophotometer was used. This was done by checking the 260/280 and 260/230 wavelength ratio. Moreover, the concentration of the RNA was noted.

The absorbance ratio of 260/280 nm is used to check the purity of RNA. RNA can be seen as pure at a ratio of ~ 2.0. If the value is lower it may identify proteins, phenol or other contaminants that absorb at 280 nm.

The second ratio can be used as another number for nucleic acid purity. Estimated values are around 2.0-2.2. If the value is lower, it again indicates the presence of contaminations that absorb at 230 nm.

3.4.4. Reverse transcription

To reverse transcribe RNA to cDNA, a master mix 1 (MM1) was prepared. The composition of the MM1 is shown in Table 2. The MM1 was prepared on ice, and after adding all the components, gently mixed. The Eppendorfs were then placed in a thermoblock and heated to 65°C for 5 min, they then were cooled on ice immediately and gently centrifuged again.

Table 2: cDNA master mix 1 (MM1) composition.

Solution	**Volume / µL**
DEPC H_2O	2
Oligo dT primer (50 µM)	1
dNTPs (20 mM)	4
Template RNA	7

After this, the master mix 2 (MM2) was prepared according to Table 3 and added to each sample of MM1. The samples were then incubated using a thermoblock (50°C for 50 min, then 75°C for 15 min), and if not used for a longer period they are stored in -80° C.

Table 3: cDNA master mix 2 (MM2) composition.

Solution	**Volume / µL**
DTT (0,1 µM)	1
5x FS III Buffer	4
SS III Reverse Transcriptase	1

3.4.5. PCR for primer adjustment

Before doing q-PCR, normal PCR, followed by gel electrophoresis, was done in order to test all possible primers which are available for the enzyme trehalase, Table 6. For this PCR, the whole larva RNA was collected and treated as described in 3.4.3 and 3.4.4. First, a master mix (MM3) was prepared and mixed with 1 µL sample. The thermocycler protocol is listed in Table 5. For each enzyme, cTreh, and sTreh, the best primer is used for further analysis with q-PCR.

Table 4: q-PCR master mix 3 (MM3) composition.

Solution	**Volume / µL**
DEPC H_2O	13.7
5X One*Taq* Standard Reaction Buffer	4
Primers (10 µM)	0.4
One*Taq* DNA Polymerase	0.1

Table 5: Thermocycler program for PCR.

Step	**Temperature**	**Time**		**Cycling step**
1.	94°C	5 min 0 sec		*Denaturation*
2.	94°C	0 min 30 sec		*Denaturation*
3.	56°C / 58°C / 60°C	0 min 30 sec		*Annealing (primers)*
4.	72°C	1 min 30 sec	return to step 2, 35x	*Elongation (OneTaq Poly)*
5.	72°C	5 min 0 sec		*Elongation („final “)*
6.	12°C	Pause		Stop

Table 6: Overview of used primers for secreted and cytoplasmic Trehalase to optimize primer conditions.

Primer	**Sequence**	**Description**
Treh-Fw1	5´-CGAGCAATCACAAAATGAACGG-3´	
Treh-RA-Fw	5´-CGGAAATGGTAAAATCTACTGCG-3´	exon spanning on cTreh
Treh-RB-Fw1	5´-AATAAAATAAAATCTACTGCGAGGGC-3´	exon spanning on cTreh
Treh-RB-Fw2	5´-CTGGTGCACAAAACAATACAGAT-3´	
Treh-RF-Fw	5´-TCGCCAGCTAGTAAAATCTACTGCG-3´	exon spanning on sTreh
Treh-RF-Fw3	5´-CGACTATAACAATGCCATTCCCG-3´	

Treh-Rv1	5´-CTGATTCTTGGCCTCCATCATG-3´	
Treh-Rv2	5´-TTTGGATGGTGTGCAGCAGATT-3´	

3.4.6. Quantitative PCR (qPCR)

The previously produced cDNA from hemocytes of the uninfected and infected larvae was used for quantitative PCR (qPCR) and diluted with 230 µL of dH_2O. 3 µL of each sample was mixed with a qPCR master mix (MM 4, seen in Table 7), again it was worked on ice.

After analysing all primers and evaluation, RA-FW was used as the forward primer and RV1 as the reverse primer for the gene cTreh, as mentioned in 4.2.1. For the sTreh, RF-FW was used as the forward one and RV1 as the reverse primer. The tube was sealed and centrifuged for 2 minutes at 500 rpm at 4°C before performing qPCR. The program of the qPCR was set to the following, see Table 8.

The same marker (Figure 10) was used in each gel electrophoresis (N32313, from England Biolabs). The size ranges from 100 to 1,517 bp.

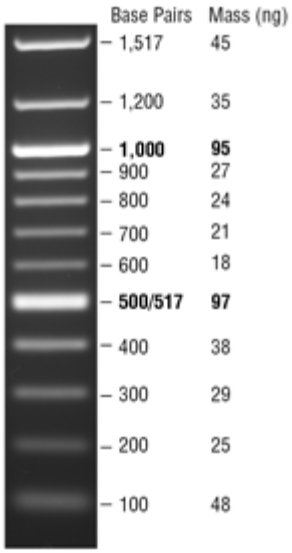


Figure 10: Used marker in PCR for optimizing primer conditions, on the left side is the gel with the length of the base pairs, on the right side are the corresponding masses / g, adapted from England Biolabs.

Table 7: q-PCR master mix 4 (MM4) composition.

Solution	**Volume / µL**
PCR ultra H_2O	2.5
2x SYBR Master Mix	6
Reverse Primer (20 µM)	0.25
Forward Primer (20 µM)	0.25
One*Taq* DNA Polymerase	0.1
cDNA	1

Table 8: Thermocycler program for q-PCR.

Step	**Temperature**	**Time**		**Cycling step**
1.	94°C	3 min 0 sec		*Denaturation*
2.	94°C	0 min 15 sec		*Denaturation*
3.	56°C	0 min 30 sec		*Annealing (primers)*
4.	72°C	1 min 30 sec	return to step 2, 40x	*Elongation*
5.	72°C	5 min 0 sec		*Elongation („final “)*
6.	12°C	Pause		Stop

4. RESULTS

4.1. TreT -RNAi

Normally, after infection with parasitoid wasp, the number of lamellocytes increase to counteract the intruder. Since we speculate that the immune cells of the larvae use trehalose as an energy source for the production of lamellocytes, we tested if there would be a significant decrease in the number of lamellocytes due to the lack of this particular sugar, because of knocking down the TreT. First, proper dissection skills and good infection with parasitoid wasp were gained. In the beginning, it was thought that three eggs were sufficient enough to induce a strong immune response. However, due to high variability of lamellocyte numbers, infection was increased up to four-five eggs per larva, to induce a more robust and uniform infection. Although good infection was problematic in the beginning, reliable results were observed in the end. Third instar larvae of the crosses $\frac{1252}{1527}$ and $\frac{1586}{1527}$ were collected and an immune response was triggered, and dissected after 18hpi, as previously discussed in 3.2 and 3.3. As a control, the fly strain 1498 was also infected and lamellocytes were counted.

Infections with lower numbers of eggs were excluded and only attempts with at least 4 eggs per larva are shown in Figure 11. Numbers of lamellocytes in Figure 11 are extrapolated per one larva. There are three repetitions (combined in Figure 10) for the Srp-Gal4-control and one with Srp-Gal4-TreT-RNAi. The average number of lamellocytes plus standard error of the mean (SEM) for the Srp-Gal4-control is 7659 ± 870, with a sample size of n=34. And the average + SEM for the Srp-Gal4-TreT-RNAi is 3525 ± 589, n=12. The p-value for Srp-Gal4 is 0,0091. This means that Srp-induced TreT-RNAi leads to a significantly lower number of lamellocytes.

Hml-Gal4 has a different genetic background than Srp-Gal4 and therefore the number of lamellocytes is different. However, Hml-induced TreT-RNAi again leads to a significantly lower number of lamellocytes when compared to the appropriate control. The mean + SEM for the control is 4626± 479, the sample size is n=25. For the TreT-RNAi the mean is 2784, the SEM is 256 and n=25. The p-value for Hml-Gal4 is 0,0014. This means the difference is meaningful.

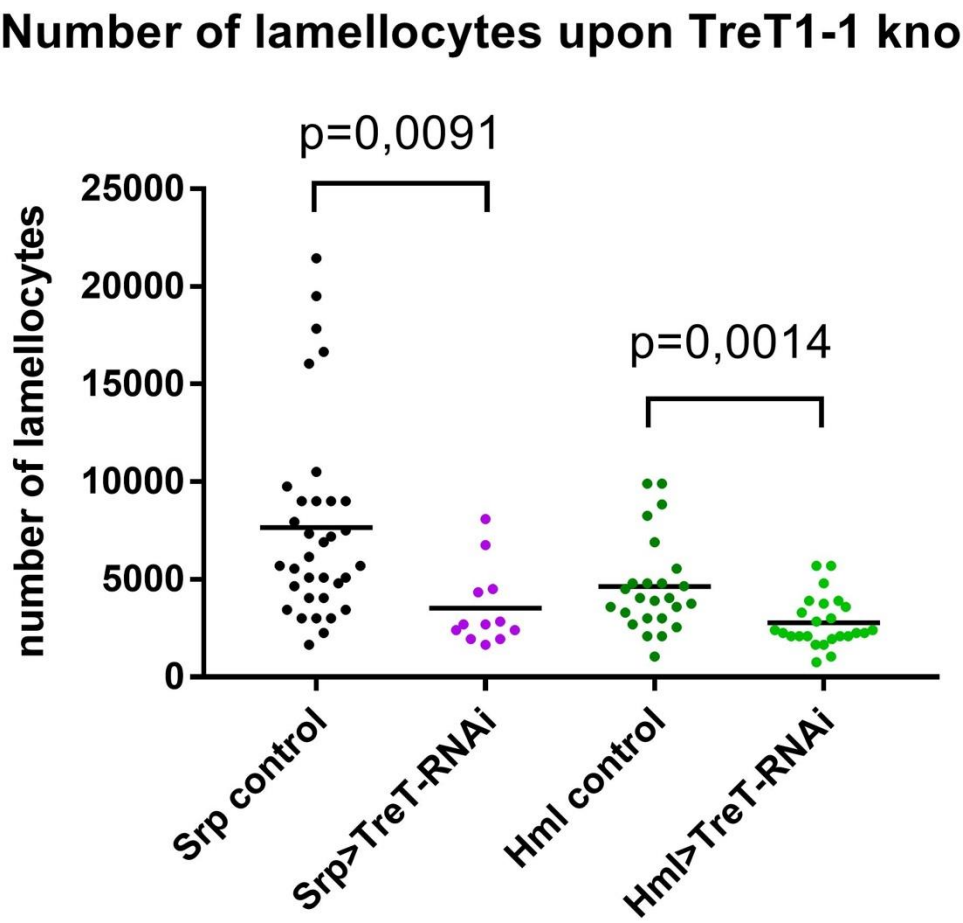


Figure 11: Number of lamellocytes in control larvae and larvae with TreT being knocked-down, where each dot represents one dissected and used larva. 1252 possess the Hml- Gal4, whereby 1586 possess the Srp – Gal 4. The y-axis shows the number of lamellocytes per one larva.

In general, it must be pointed out, that the small number of biological replicates does not allow valid statement about the lamellocytes deficiency during immune response. So, this work serves as a preliminary study to elucidate the importance of trehalose in immune response. Nevertheless, a clear trend can be seen. There are no comparable data from other studies yet. But Previous studies show similar trends.

In Bajgars paper [2], where the energy regulation was also studied with the parasitoid wasp. A lower level of lamellocytes was seen, but for a different reason. In his paper, the regulator e - Ado, was switched off. The flies used their energy for growth and other tissues, rather than for immune response. Bajgar showed, that the immune cells need the energy, and if it is not received, the flies die.

Bajgars paper showed that if you suppress this systemic metabolic switch, then the lamellocyte number during immune response is also affected. In our study, we were not

manipulating the systemic energy, but the energy supply specifically to hemocytes was interrupted, by shutting down the trehalose transporter.

Concluding, in Bajgars study, the systemic energy being available was shut down, in this study, the intake of the energy, only in the immune cell, was shut down. Both studies showed a decrease in lamellocytes upon infection. Due to this data, one can say that immune cells, during infection, receives their main energy source from trehalose, which could hint to the selfish immune system. Another test for independence was to check the expression levels of the specific two enzymes that convert trehalose into glucose.

4.2. Trehalase gene expression analysis

In *Drosophila*, there are two versions of the enzyme trehalase, cytoplasmic and secreted, both originate from one gene as alternative transcripts. In this part of work, we tried to find which version is expressed in hemocytes.

4.2.1. PCR condition optimization

First, 8 different primers (Table 6) had to be tested. Three of them are exon spanning, those would give a more secure result because they cannot bind to genomic DNA (gDNA) which contains introns between exon-exon junctions. The respective forward and reverse primer, their length, and the product are shown in Table 9 and Figure 12.

Table 9: Overview of the set of primers, the respective product, and length.

Forward and Reverse Primer	**Description**	**Length / bp**
Fw1 + Rv1	cDNA	182
RA-Fw + Rv1	cDNA, no gDNA	163
RB-Fw1 + Rv1	cDNA, no gDNA	161
RB-Fw2 + Rv2	cDNA	118/384
RF-Fw + Rv 1	cDNA, no gDNA	164
RF-Fw3 + Rv1	cDNA	715
RF-Fw3 + Rv2	cDNA	600

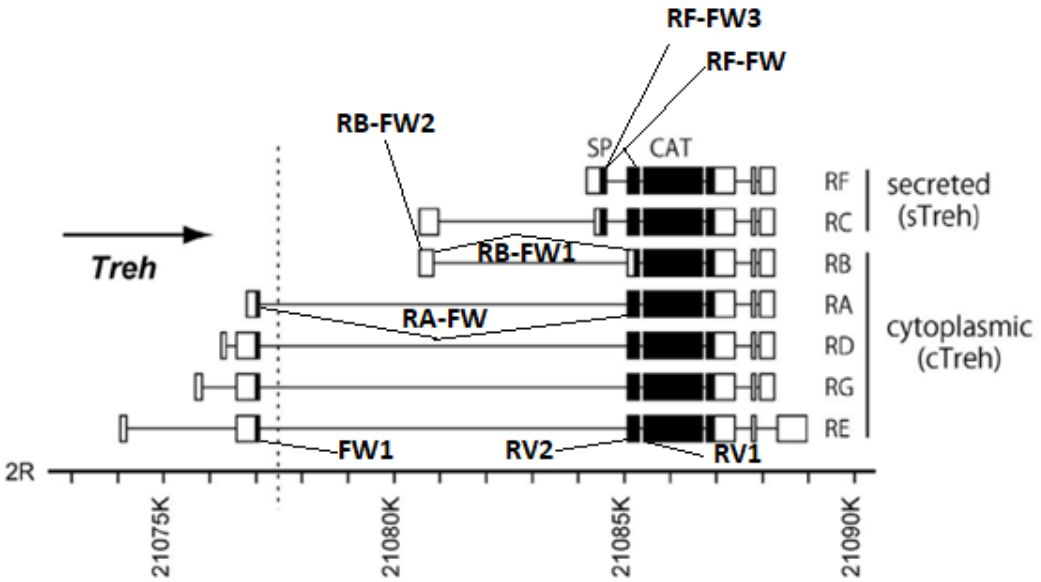


Figure 12: All analysed sets of forward and reverse primers from Table 9, lines with an angle are the exon spanning primers with only cDNA. adapted from Flybase.

Each primer was tested at three different annealing temperatures, 56°C, 58°C and 60°C. All samples were cDNA, made from whole larvae RNA, which was treated as described in 3.4.2, 3.4.3, 3.4.4 and 3.4.6. The same marker was used in each measurement, Figure 10. Most of the bands lay between 100 bp to 200 bp. In all gels, the products which are measured are abbreviated as either S for sample, C for control or N for negative control.

Figure 13 shows two cDNA samples and one control, one of the cDNA samples and the cDNA control were provided by a student from the lab. The control cDNA was a previously analysed sample which showed no contaminations and worked well for gel electrophoresis. All samples were collected from whole larvae. Further, the first measurement (Figure 13) did not include a negative control, following primers where used:

FW1 + RV1 … cTreh

RA-FW + RV1 … cTreh-exon spanning

At 58°C, the primers were most visible, therefore this temperature setting is the preferred one. RA-FW + RV1 also worked fine at 56°C.

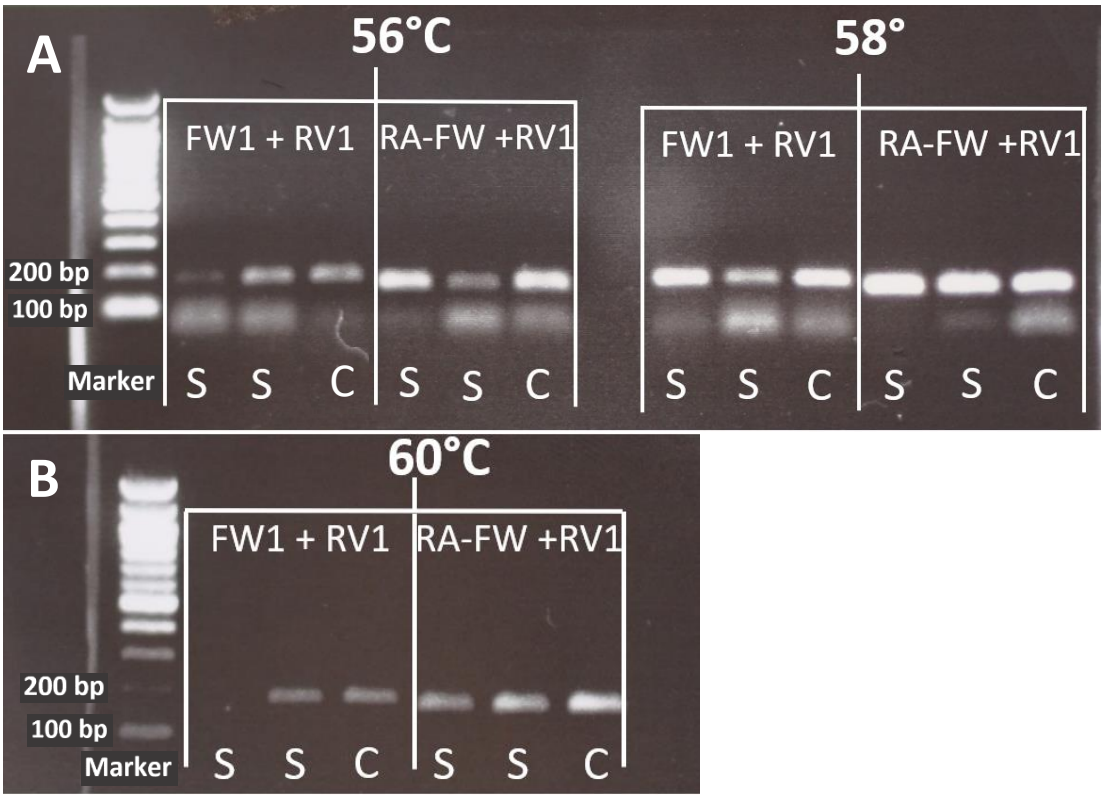


Figure 13: On the bottom are the PCR products of two sample cDNAs, abbreviated as S, next to the samples is the control cDNA, abbreviated as C, this measurement did not use a negative control. Here the FW1 + RV1 for the cTreh, and RA-FW + RV1 for the sTreh, were analysed. Each set of primer was measured at 56°C, 58°C (Picture A) and 60°C (Picture B). A and B have a marker with a length of 100 bp to 1,517 bp on the left side. Samples and the control are between 100 bp to 200 bp long.

The second measurement (Figure 14) shows three different primers:

RF-FW3 + RV2 … sTreh

RF-FW3 + RV1 … sTreh

RF-FW + RV1 … sTreh-exon spanning

All are from sTreh. Here, only one sample together with a control and a gDNA as negative control were analysed. The negative control worked in all primers except in RF-FW + RV1, here gDNA was provided by a student, the contamination, probably cDNA, happened during preparing and pipetting the mixes, but was due to time shortage not further analysed (later, the contamination was confirmed by other lab members, data not shown). 60°C was too high, but 56°C and 58°C worked equally well.

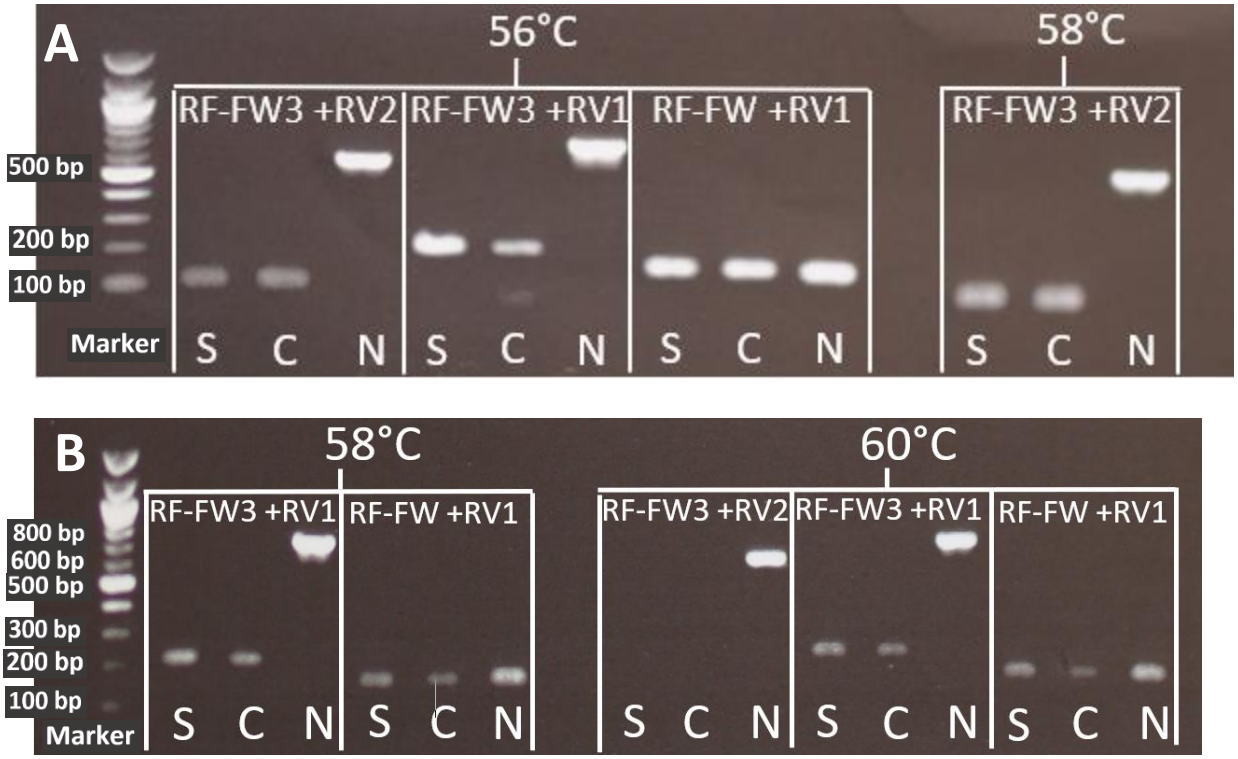


Figure 14: On the bottom are the PCR products of sample cDNA, abbreviated as S, control cDNA, abbreviated as C, followed by the negative control gDNA, abbreviated as N, of RF-FW3 + RV2 (sTreh), RF-FW3 + RV1 (sTreh) and RF-FW + RV1 (sTreh-exon spanning). Each primer was analysed at 56°C, 58°C (Picture A) and 60°C (Picture B). A and B have a marker with a length of 100 bp to 1,517 bp on the left side. The negative control had contamination in the RF-FW + RV1. The samples and the positive controls are between 100 bp and 250 bp long. The negative controls in RF-FW3 + RV2 and RF-FW3 + RV1 are around 600 bp to 800 bp long.

The last primers in Figure 15 are:

RB-FW1 + RV1 … cTreh-exon spanning

RB-FW2 + RV2 … cTreh

One can see a contamination for the negative control in both primers, due to the same reasons as in the second measurement (Figure 14). The temperature 56°C worked best. RB-FW1 + RV1 is brighter and more visible than RB-FW2 + RV2.

RA-FW + RV1 and RF – FW + RV1 were picked for the two enzymes to be used for analysing the gene expression. Both are exon spanning which eliminates a possible error source. Exon spanning primers are specific for cDNA, because of the cut-out introns.

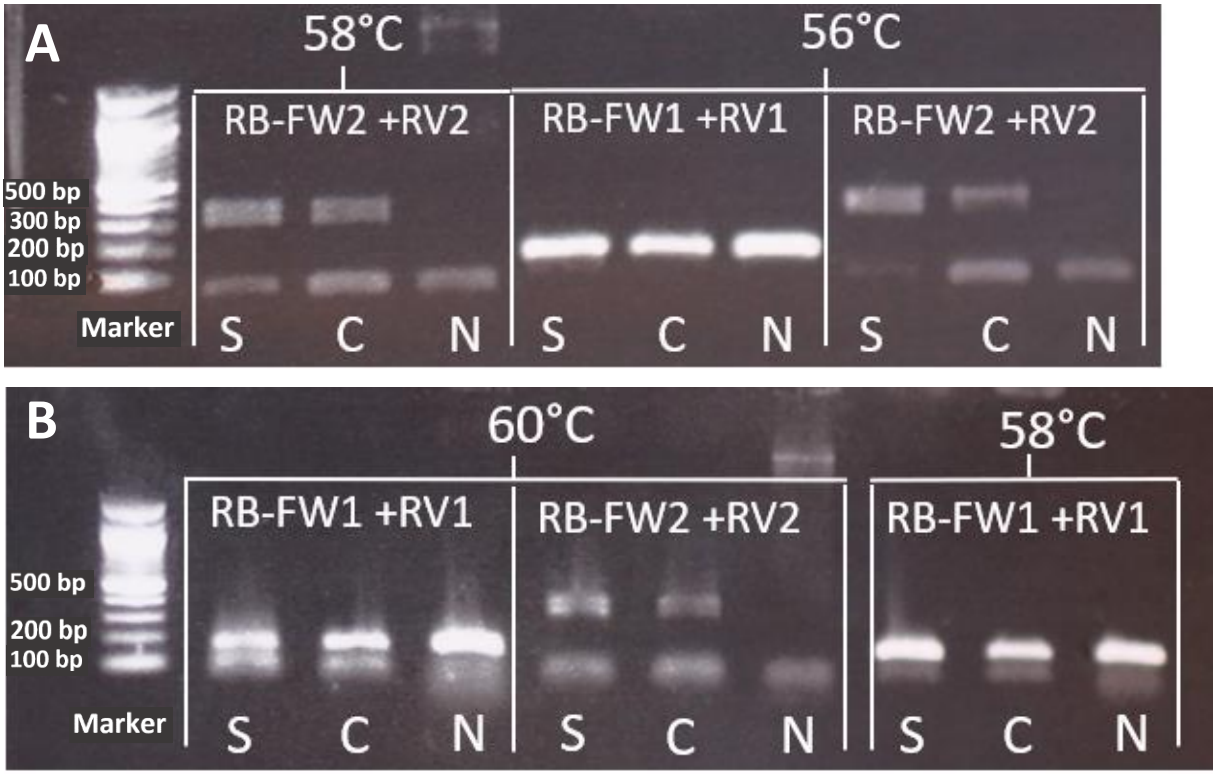


Figure 15: On the bottom are the PCR products of sample cDNA, abbreviated as S, control cDNA, abbreviated as C, followed by the negative control gDNA, abbreviated as N, of RB-FW1 +RV1 (cTreh-exon spanning) and RB-FW2 +RV2 (cTreh).Each primer was analysed at 56°C, 58°C, and 60°C (Picture A and B). A and B have a marker with a length of 100 bp to 1,517 bp on the left side. The sample and the positive control of the two primers are between 100 bp and 250 bp long. The negative control had a contamination in both primers.

4.2.2. cTreh and sTreh gene expression

From 4.2.1 two primers were used. For cTreh, RA-FW + RV1 and RF – FW + RV1 for sTreh, those were the most reliable ones. They were chosen because they work reasonably well at 56°C and are both exon spanning.

The average c_t values of the samples were calculated and Δc_t was determined using actin as a reference gene. Figure 16 nicely shows, that sTreh is much lower in hemocytes than cTreh, in 0 hpi and 18 hpi, even without infections. Looking at the infected 18hpi, there is again a great difference in the expression levels of the two enzymes, the expression of cTreh is 4 times higher than sTreh.

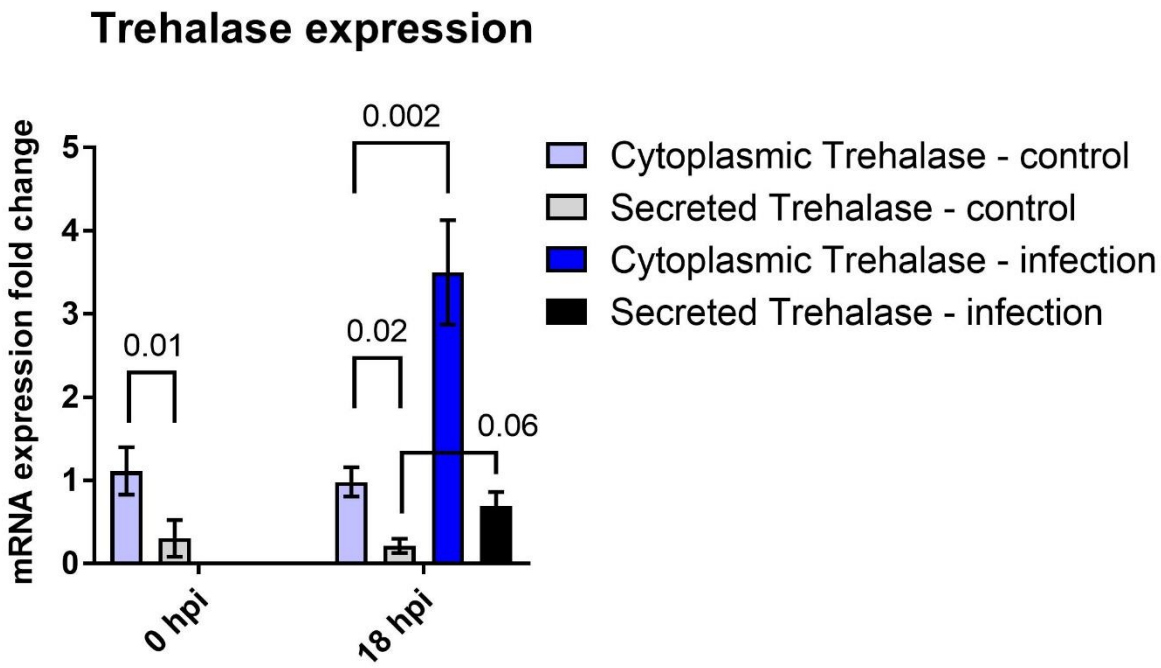


Figure 16: Relative cTreh and sTreh expression ± standard deviation and p-value above the graphs. The x-axis shows the different time points (0 hpi and 18 hpi), at 0 hpi cTreh and sTreh uninfected. At 18hpi, one can see the controls and the infections. The y-axis shows the double delta, when first normalized by Act, relative to cTreh at 0 hpi the relative mRNA amount.

Given the fact that the cTreh expression is much higher, this must mean that during infection the immune cell prefers an uptake of trehalose and conversion to glucose inside the cell by the cTreh and thus be independent and does not need glucose from outside the cell.

5. CONCLUSION

The analysis of the immune cells using *Drosophila melanogaster* gives interesting insights on how the energy demand is fulfilled during immune response. Due to time limitation and limited experiment repetitions, this study cannot give reliable proof for the energy household of immune cells. However, they support the hypothesis of the selfish immune system.

To study the immunity, the fly must be made sick, this was done by using the parasitoid wasp *Leptopilina boulardi*. This wasp lays eggs inside the larva of the fruit fly, for reproduction purposes. Lamellocytes are specialized immune cells that fight this intruder. For high survivability, high numbers of lamellocytes are profitable. During infection, one should actually see an increase in numbers of those specialized immune cells, in order to counteract the intruder. The immune cell has a high energy demand during immune response to produce lamellocytes, this energy can be gained either from trehalose or glucose. However, during knockdown of the Trehalose transporter, a lower number of lamellocytes showed. This gives knowledge that in fact, the immune cells obtain their energy preferentially from trehalose and not from glucose. To further test this theory of the selfish immune system, the two versions of trehalase in *Drosophila* were analysed by gene expression upon infection, using q-PCR. Both trehalase enzymes convert trehalose into glucose. Cytoplasmic (cTreh), which can be found inside the immune cell and secreted (sTreh), which is outside the cell. First forward and reverse primers have been tested for each trehalase enzyme. If a cell would express both, trehalose transporter and its own cytoplasmic trehalase, then it could be independent of the systemic regulation and could have access to a very rich source since there is much more trehalose in circulating hemolymph than glucose. For further investigation, more biological repetitions should be performed for a valid statement. Also knocking down the cTreh or sTreh would give interesting insights into how the immune cell receives its energy. All in all, the theory of the selfish immune system was tested, the expression levels of those two enzymes upon infection were monitored. cTreh was way more expressed during infection than sTreh. Immune cells prefer and need trehalose for efficient production of lamellocytes. This thesis gives hints that immune cells are selfish, the usurp the energy, to improve the chances of surviving.

6. LITERATURE

[1] P.-M. EM und O. LAJ, „The Warburg effect then and now: From cancer to inflammatory diseases," *Review essays. BioEssays,* Nr. 35, pp. 965-973, 2013.

[2] A. Bajgar, K. Kucerova, L. Jonatova, A. Tomcala, I. Schneedorferova, J. Okrouhlik und T. Dolezal, „Extracellular Adenosine Mediates a Systemic Metabolic Switch during Immune Response," *PLoS Biol.,* Bd. 13, 2015.

[3] M. PE und B. R, „Stress hyperglycemia: an essential survival response!," *Crit Care,* Nr. 17, 2013.

[4] R. Stephenson und N. Metcalfe, „Drosophila melanogaster: a fly through its history," *J R Coll Physicians Edinb,* p. 43:70–5, 2013.

[5] S. M. C. Héctor Herranz, „Drosophila as a Model to Study the Link between Metabolism and Cancer," *J. Dev. Biol.,* 2017.

[6] R.-C. Huang, „The discoveries of molecular mechanisms for the circadian rhythm: The 2017 Nobel Prize in Physiology or Medicine," *Biomedical Journal,* 2018.

[7] S. H und G. P., „Getting started : an overview on raising and handling Drosophila.," *Methods Mol Biol.,* pp. 27-44, 2008.

[8] U. B. Pandey und C. D. Nichols, „Human Disease Models in Drosophila melanogaster and the Role of the Fly in Therapeutic Drug Discovery," *Drug Deliv,* Nr. 63, pp. 411-436, 2011.

[9] H. Siomi und M. C. Siomi, „On the road to reading the RNA-interference code," *Nature,* Nr. 457, pp. 396-404, 2009.

[10] E. Bernstein, A. A. Caudy, S. M. Hammond und G. J. Hannon, „Role for a bidentate ribonuclease in the initiation step of RNA interference," *Nature,* Nr. 409, pp. 363-366, 2001.

[11] C. C. Mello, „RNA interference (RNAi) : by Nature Video," Nature viedeo, 2011.

[12] D. Busson und A.-M. Pret, „GAL4/UAS Targeted Gene Expression for Studying Drosophila Hedgehog Signaling," *Methods in Molecular Biology, Hedgehog Signaling Protocols,* pp. 161-201, 2007.

[13] T. D. Southall, D. A. Elliott und A. H. Brand, „The GAL4 System: A Versatile Toolkit for Gene Expression in Drosophila," *Cold Spring Harb Protoc,* 2008.

[14] C. Small, I. Paddibhatla, R. Rajwani und S. Govind, „An Introduction to Parasitic Wasps of Drosophila and the Antiparasite Immune Response," *J. Vis. Exp.,* Nr. 63, 2012.

[15] T. M. Rizki und R. M. Rizki, „Lamellocyte differentiation in Drosophila larvae parasitized by Leptopilina.," *Developmental & Comparative Immunology,* pp. 103-110, 1992.

[16] T. Doležal, „From Drosophila moel to human diesease and back again," Habilitation Thesis, University of South Bohemia in České Budějovice, 2015.

[17] M. Crozatier und M. Meister, „Drosophila haematopoiesis," *Cellular Microbiology,* pp. 1117-1126, 2007.

[18] T. Dolezal, G. Krejcova, A. Bajgar, P. Nedbalova und P. Strasser, „Molecular regulations of metabolism during immune response in insects," *Insect Biochemistry and Molecular Biology. ,* 2019.

[19] M.-O. Fauvarque und M. J. Williams, „Drosophila cellular immunity: a story of migration and adhesion," *Journal of Cell Science,* Bd. 124, pp. 1373-1382, 2011.

[20] S.-H. Jung, C. J. Evans, C. Uemura und U. Banerjee, „The Drosophila lymph gland as a developmental model of hematopoiesis," *Development,* pp. 2521-2533, 2005.

[21] E. S. Keebaugh und T. A. Schlenke, „Insights from natural host–parasite interactions: The Drosophila model," *Developmental and Comparative Immunology,* pp. 111-123, 2014.

[22] E. Shukla, L. J. Thorat, B. B. Nath und S. M. Gaikwad, „Insect trehalase: Physiological significance and potential applications," *Glycobiology,* Nr. 25, pp. 357-367, 2015.

[23] M. Yoshida, H. Matsuda, H. Kubo und T. Nishimura, „Molecular characterization of Tps1 and Treh genes in Drosophila and their role in body water homeostasis," Scientific Reports, 2016.

[24] T. Higashiyama, „Novel functions and applications of trehalose," Pure Appl. Chem, 2002.

[25] S. N. Thompson, „Trehalose – The Insect 'Blood' Sugar," *ADVANCES IN INSECT PHYSIOLOGY,* Nr. 31, 2003.

[26] T. Yasugi, T. Yamada und T. Nishimura, „Adaptation to dietary conditions by trehalose metabolism in Drosophila," Bd. 7, 2017.

[27] T. Kikawada, A. Saito, Y. Kanamori, Y. Nakahara, K.-i. Iwata, D. Tanaka, M. Watanabe und T. Okuda, „Trehalose transporter 1, a facilitated and high-capacity trehalose transporter, allows exogenous trehalose uptake into cells," *Proc Natl Acad Sci U S A,* Nr. 104, pp. 11585-11590, 2007.

[28] K. A. McKean, C. P. Yourth, B. P. Lazzaro und A. G. Clark, „The evolutionary costs of immunological maintenance and deployment," *BMC Evol. Biol.,* Nr. 8, pp. 76-76, 2008.

[29] H. G., „Adenosine: an endogenous regulator of innate immunity," *Trends in Immunology,* Nr. 25, pp. 33-39, 2004.

[30] L. T. Reiter, L. Potocki, S. Chien, M. Gribskov und E. Bier, „A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster," *Genome Research,,* Bd. 11, pp. 1114-1125, 2001.

[31] S. RH, „Insulin resistance, selfish brain, and selfish immune system: an evolutionarily positively selected program used in chronic inflammatory diseases," *Arthritis Research Therapy,* Nr. 16, 2014.

[32] J. Suckale, „Openwetware," 2008. [Online]. Available: https://openwetware.org/wiki/RNA_extraction_using_trizol/tri. [Zugriff am 03 August 2019].

7. ATTACHMENTS

$\frac{1252}{1527}$ Hml-Gal4 / TreT- RNAi

Table 10: Number of counted lamellocytes for Hml - Gal4/ TreT-RNAi.

Number	Lamellocytes	Number	Lamellocytes
1	14	1	11
2	14	2	25
3	38	3	26
4	14	4	32
5	16	5	15
6	15	6	14
7	24	7	22
8	15	8	11
9	7	9	20
10	26	10	19
11	38	11	16
12	13	12	14
13	5		

$\frac{1252}{1498}$ Hml-Gal4 / control

Table 11:Number of counted lamellocytes for Hml - Gal4 / control.

Number	Lamellocytes	Number	Lamellocytes
1	37	1	66
2	66	2	59
3	20	3	24
4	46	4	7
5	25	5	30
6	14	6	31
7	32	7	27
8	20	8	22
9	17	9	55
10	24	10	27
11	32	11	26
12	32	12	18
13	14		

$\frac{1586}{1527}$ Srp-Gal4 / TreT-RNAi

Table 12: Number of counted lamellocytes for Srp-Gal4 / TreT-RNAi.

Number	Lamellocytes
1	54
2	13
3	18
4	13
5	29
6	45
7	16
8	16
9	11
10	30
11	19
12	18

$\frac{1586}{1498}$ Srp-Gal4 / control

Table 13: Number of counted lamellocytes for Srp-Gal4 / control.

Number	Lamellocytes	Number	Lamellocytes	Number	Lamellocytes
1	143	1	27	1	34
2	20	2	23	2	34
3	107	3	41	3	15
4	32	4	38	4	60
5	48	5	130	5	20
6	53	6	46	6	111
7	31	7	34	7	65
8	11	8	60	8	70
9	38	9	60	9	119
10	20	10	37	10	60
11	50	11	27		
12	23	12	49		
13	14				

Table 14: c_t values for gene expression of cTreh, sTreh, Rp49 and Act. There are 3 c_t values for each sample from reaction. 5 biological replicates were measured (I-V), for each time and treatment (control/ infected). Each gene was measured 45 times in total. 5x3 times at 3 different points 0 hpi, 18 hpi uninfected and 18 hpi infected.

		Ct values							
		Treh C		**Treh S**		**Act**		**Rp49**	
0 hpi	I	25,92 25,77 25,42	25,703	28,75 28,73 28,67	28,717	19,04 19,18 18,82	19,013	18,84 18,78 18,8	18,807
	II	25,23 25,09 25,07	25,130	29,06 28,27 28,22	28,517	19,22 18,9 18,98	19,033	19,09 19,08 19,11	19,093
	III	30,66 30,47	30,565	32,48 32,54	32,510	25,44 25,3 25,25	25,330	23,53 23,55 23,56	23,547
	IV	29,87 30,41 30,12	30,133	33,71 33,01 33,41	33,377	23,37 23,26 23,42	23,350	22,52 22,67 22,53	22,573
	V	28,32 28,19 28,27	28,260	32,01 32,05 32,48	32,180	21,12 21,21 21,41	21,247	20,34 20,22 21,07	20,543
18 hpi	I	33,39 33,53	33,460	35,25 35,76 34,95	35,320	27,51 27,32 27,44	27,423	20,13 20,12 20,09	20,113
	II	23,95 24,11 24,05	24,037	27,1 27,24 27,27	27,203	17,82 18,07 18,31	18,067	18,27 18,43 18,22	18,307
	III	27,09 27,13 27,29	27,170	 30,81 31,14	30,975	23,47 23,56 23,59	23,540	20,19 20,26 20,27	20,240
	IV	25,45 26,14 25,56	25,717	29,72 30,05	29,885	18,87 18,87 18,94	18,893	17,45 18,35 18,17	17,990
	V	28,34 28,19 28,36	28,297	31,66 31,71 31,77	31,713	21,14 21,22 21,44	21,267	20,62 20,67 20,64	20,643
18 hpi inf	I	 21,8 21,82	21,810	25,01 25,1 25,13	25,080	15,8 15,94 15,88	15,873	16,5 16,66 16,77	16,643
	II	17,49 17,72 17,82	17,677	20,43 20,65 20,66	20,580	13,69 13,5 13,64	13,610	15,98 16,01 16,14	16,043
	III	19,29	19,353	21,11	21,307	15,04	15,183	17,65	17,680

		19,37		21,29		15,11		17,75	
		19,4		21,52		15,4		17,64	
		25,03		27,01		20,36		22,03	
	IV	25,04	25,100	27,17	27,133	20,4	20,433	22,04	22,070
		25,23		27,22		20,54		22,14	
		19,01		21,27		14,45		16,95	
	V	19,05	19,097	21,46	21,400	14,63	14,510	17,26	17,160
		19,23		21,47		14,45		17,27	