Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

# Testing knockdown of nucleotidases and the effect on e-Ado production during immune response in *Drosophila melanogaster* larvae

Bachelor´s thesis

**Thomas Stehrer**

Supervisor: doc. Mgr. Tomáš Doležal, Ph.D.

Co-supervisor: Mgr. Adam Bajgar, Ph.D.

České Budějovice 2018

Stehrer T. 2018: Testing knockdown of nucleotidases and the effect on e-Ado production during immune response in *Drosophila melanongaster* larvae. Bc. Thesis, in English – 28 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

## Annotation

The aim of this thesis was to measure adenosine production from lymph glands in *D. melanogaster* larvae ex vivo and to assess the differences upon infection with parasitic wasps *L. boulardi*. The second aim was to investigate the influence of two different nucleotidases on adenosine levels upon gene knockdown of their respective genes with and without a triggered immune response.

## Declaration

I hereby declare that I have worked on my bachelor 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 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 defence 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.

České Budějovice, 11.05.2018

……...

Thomas Stehrer

## Acknowledgement

I would like to thank doc. Mgr. Tomáš Doležal, Ph.D. and Mgr. Adam Bajgar, Ph.D. for their support during preparation, practical work as well as during writing the thesis. Furthermore, I would like to express my gratitude to all members of the laboratory for being a tremendous help during my work. Besides the scientific support, I would also like to thank my family and friends for their support during writing the thesis and my studies in general.

## Abstract

Extracellular adenosine is involved as a signalling molecule during immune response in *Drosophila melanogaster* mediating a systemic metabolic switch from development towards differentiation of immune cells. Two pathways for generation of adenosine, the SAM cycle and production via nucleotidases, are known. First, a method to assess e-Ado from lymph glands with spectrophotometry was replicated to show differences of infected and uninfected wild-type larvae. Immune response was triggered upon infection by parasitic wasps. To assess the contribution of the individual pathways of adenosine production the same method was repeated additionally with larvae having reduced gene expression for two nucleotidases and were compared to wild-type strains. Gene knockdown for genes *CG11883* and *NT5E-2* was achieved with a specific RNAi and activation using a GAL4 driver line. Preliminary measurements showed a difference of adenosine levels in infected and uninfected wild-type larvae, demonstrating a proper methodology. Further measurements including wild-type and mutant larvae did not show any differences and therefore did not allow any conclusions on the contribution of the individual nucleotidase pathways to e-Ado formation.

## Abbreviations

| | |
|---|---|
| ADA | adenosine deaminase |
| ADGF | adenosine deaminase-related growth factor |
| *adoR* | adenosine receptor; *D. melanogaster* mutant flies without adenosine receptors |
| AHCy | S-adenosyl homocysteine hydrolase |
| AMP | adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| ATP | adenosine triphosphate |
| bp | base pair |
| cDNA | complementary DNA |
| *CG11883* | gene – cytosolic nucleotidase |
| DEPC | diethylpyrocarbonate |
| dNTP | deoxynucleotides |
| dsRNA | double-stranded RNA |
| DTT | 1,4-dithiothreitol |
| EHNA | erythro-9-(2-hydroxy-3-nonyl)adenine |
| ENT2 | equilibrative nucleoside transporter 2 |
| Fwd | forward |
| GAL4 | yeast transcription factor |
| GC-MS | gas chromatography-mass spectrometry |
| GFP | green fluorescent protein |
| hpi | hours post infection |
| Imd | immune deficiency |
| LC-MS | liquid chromatography-mass spectrometry |
| mRNA | messenger RNA |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| *NT5E-2* | gene – Ecto-5'-nucelotidase 2 |
| oligo-dT | sequence of deoxythymidine nucleotides |
| Rev | reverse |

| | |
|---|---|
| RISC | RNA-induced silencing complex |
| RNAi | RNA interference |
| *Rp49* | Gene – Ribosomal Protein L32 |
| RT-qPCR | Reverse transcription real time polymerase chain reaction |
| SAH | S-adenosyl homocysteine |
| SAM | S-adenosyl methionine |
| SSIII RT | SuperScript III reverse transcriptase |
| Toll | Class of receptors; part of immune system |
| UAS | upstream activation sequence |

# Table of contents

# 1 Introduction

The immune system upon activation requires an increased amount of energy as quickly as possible. To cope with that demand, the metabolism of immune cells can switch from oxidative phosphorylation to aerobic glycolysis. The energy yield via oxidative phosphorylation is higher per glucose molecule than compared to aerobic glycolysis. Due to a high metabolic rate, aerobic glycolysis can still be more effective, yielding higher amounts of ATP than oxidative phosphorylation (Heiden et al., 2009). This effect is called the Warburg effect and occurs in immune and cancer cells (Garber, 2004; Herranz & Cohen, 2017). Therefore, the understanding of key principles of the immune system and its response can be important for understanding the behaviour of cancer cells (Cheng et al., 2014; DeBerardinis et al., 2008; Delmastro-Greenwood & Piganelli, 2013; Pandey & Nichols, 2011). To study the immune response, *Drosophila melanogaster* is an excellent choice. It is easy to maintain fly stocks, many genetic tools are available and widely tested and it can serve as a model organism due to similarities to human metabolic pathways (Herranz & Cohen, 2017).

## *1.1 Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* is used in medical and scientific research for more than 100 years. The discovery of the *White* gene by Thomas Hunt Morgen marked an important starting point for using *D. melanogaster* as a model organism in genetics and biology (Stephenson & Metcalfe, 2013). A few characteristics of *D. melanogaster* make this species a universal and successful organism in genetics. Many identified genes from *D. melanogaster* are conserved in other organisms including humans. This is of significant help investigating for example genes suspected to be responsible for a certain disease (Dahmann, 2008). Moreover, having the whole genome sequenced with great accuracy is very beneficial for any genetic experiments as well (Myers et al., 2000). Furthermore, genetic tools like the GAL4/UAS system for targeted gene expression or the use of specific RNAi to destruct mRNA are well developed for *D. melanogaster* and are of immense help for genetic research (Duffy, 2002; Kavi et al., 2008).

### 1.1.1 Immune response

In general, different response mechanisms to several types of infections are described for *D. melanogaster.* A systemic immune response is triggered upon an activation of different pathways, mainly Toll and Imd, encoding for proteolytic enzymes (Schlenke et al., 2007). On a cellular level, three different cell types of hemocytes play distinct roles for immune responses. Plasmatocytes represent 90 to 95 % of all hemocytes in *D. melanogaster* larvae and are responsible for phagocytosis of dead cells and microbial pathogens. Lamellocytes are involved in the encapsulation of larger pathogens and therefore play a key role in the defence against parasitic wasps. Crystal cells play generally only a minor role and are part of melanisation processes (Lemaitre & Hoffmann, 2007). In defence against parasitic wasp eggs, Lamellocytes and crystal cells are the main contributors on a cellular level. These cells are differentiated from lymph gland hemocytes of *D. melanogaster* larvae and lead to encapsulation and melanisation of the parasitic eggs to prevent further development of the parasite (Sorrentino et al., 2002).

### 1.1.2 GAL4/UAS system

The GAL4/UAS system for targeted gene expression is a diverse tool in genetic research (Busson & Pret, 2007). It is based on using the GAL4 transcription factor, which binds to an upstream activation sequence of a specific insertion. GAL4 is a yeast protein but since it is not species specific, it is possible to use it in other species like *D. melanogaster*. One of the major advantages of this system is the separation of the GAL4 gene in a driver line and the gene of interest in a responder line. The GAL4 protein itself has no harmful effects to the fly and the targeted gene stays silent until both driver and responder lines are crossed (Southall et al., 2008). Furthermore, spatial specific targeting can be achieved by using different tissue specific GAL4 driver lines (Duffy, 2002). Combining the GAL4/UAS system with RNAi provides a very useful and versatile tool for gene knockdown (Southall et al., 2008)

### 1.1.3 RNAi

RNA interference is a process where double stranded RNA is used to specifically induce a degradation of a complementary mRNA. It was first discovered 1994 in *Caenorhabditis elegans* and it can be induced either artificially or by endogenous synthesis (Kavi et al., 2008).

In *Drosophila* it is widely used for gene silencing in vivo as well as in vitro. In 2007 a genome wide RNAi library was established, providing a powerful tool for gene inactivation even on a tissue specific level (Dietzl et al., 2007). When using it in combination with the GAL4/UAS system, gene knockdown in different tissues or at different developmental stages can be achieved. The dsRNA is fragmented into roughly 21 bp long interference RNA sequences, which bind to a complementary mRNA and trigger the binding of the RISC protein complex and subsequent degradation (Yamamoto-Hino & Goto, 2013).

### *1.2 Leptopilina boulardi*

A common way to induce an infection in *D. melanogaster* larvae and furthermore trigger an immune response is the parasitization by the parasitic wasp *Leptopilina boulardi*. It is a natural parasite of *D. melanogaster* and an immune response is induced upon injection of parasitic eggs into the larvae, leading to an immune response (Neyen et al., 2014). This consists of the differentiation of blood cells and subsequent encapsulation and melanisation of the parasitic egg. If this process fails, the parasitic egg hatches and develops into an adult (Small et al., 2012; Sorrentino et al., 2002).

### 1.3 Aerobic glycolysis

Aerobic glycolysis is a mechanism in proliferating cells, for instance immune or cancer cells, for production of ATP under the presence of oxygen. Normally oxidative phosphorylation via the Krebs cycle is used in most cell types (Herranz & Cohen, 2017). In contrast to the oxidative phosphorylation processes, the effectiveness of aerobic glycolysis in terms of ATP production per glucose molecule, is significantly lower. The whole process is described as The Warburg effect and the similarities of cancer and immune cells require closer investigation of the metabolism of immune cells for subsequent strategies for therapeutic use (Cheng et al., 2014; Delmastro-Greenwood & Piganelli, 2013). The disadvantage of aerobic glycolysis being less effective than oxidative phosphorylation is compensated by a high metabolic rate. Besides that, important biomolecules are also synthesised during aerobic glycolysis. Ribose, an essential part of nucleotides, glycerol and citrate being components of

lipids or the reducing agent NADPH to name a few (DeBerardinis et al., 2008; Pfeiffer et al., 2001).

## 1.4 Extracellular adenosine

The immune system upon activation consumes a lot of energy which leads to a metabolic switch in immune cells called the Warburg effect (Cheng et al., 2014), as described in section 1.3. Extracellular adenosine is suspected to play a key role as a signalling molecule within the this process (Novakova & Doležal, 2011; Zuberova et al., 2010). Due to the higher energy demand of immune cells, the energy regime of the whole organism changes as well. Energy resources are prevalently used for differentiation of immune cells and energy use for storage and development is almost stopped (Bajgar et al., 2015). This selfish behaviour of immune cells in *D. melanogaster* is shown to be dependent on adenosine signalling (Doležal, 2015). Further investigation of exact mechanism and the pathway of adenosine origin are important to better understand the whole signalling cascade. The following part describes the current research on the role of extracellular adenosine during immune response in *D. melanogaster.*

### 1.4.1 Role of e-Ado during immune response

To investigate the role of extracellular adenosine as a signalling molecule within the immune system, several experiments were performed. It was revealed that upon knockout of *ADGF* genes and subsequent inactivation of adenosine deaminase activity (ADA), a drastic depletion of energy stores in *D. melanogaster* larvae occurred, which subsequently lead to death of the larvae (Zuberova et al., 2010). This was confirmed to be associated with increased adenosine levels in the hemolymph of such mutant larvae deficient in ADA activity. In a further experiment the increased expression of *ADGF* at inflammatory sites was proven by insertion of a GFP reporter. This pointed at the importance of adenosine during the immune response of *D. melanogaster* larvae (Novakova & Doležal, 2011). In ensuing experiments larvae lacking adenosine receptors were used (*adoR*) to investigate e-Ado signalling effects. Metabolite concentrations in hemolymph and different tissues together with pupation rate and time, were compared between wild-type and *adoR* mutant larvae. These experiments showed that *adoR* mutant larvae did not react upon immune stress, suggesting extracellular adenosine

to be an integral component of signalling during immune response. Besides mutations of adenosine receptors, experiments with adenosine transporters were conducted. Specifically, *ENT2* was knocked down, decreasing transport of adenosine from the inside to the outside of a cell. This resulted for instance in lower lamellocyte abundance in mutant larvae post infection, indicating e-Ado to be a signal sent by immune cells. Resistance against the parasite was reduced as well leading to less adult flies developing from larvae with knocked down *ENT2* expression post infection compared to wild-type larvae. Furthermore, increased expression of *ENT2* mRNA was observed in wild-type larvae post infection verifying the effects on a molecular level (Bajgar et al., 2015; Doležal et al., 2005).

### 1.4.2 Metabolic pathways of e-Ado

Two different pathways of extracellular adenosine origin are known. The first is the formation of e-Ado via nucleotidases from AMP, and the second involves methylation processes within the so-called SAM cycle (see Figure 1) Figure 1: Scheme of adenosine formation via SAM-cycle and cytosolic-/ecto-nucleotidases(Chiang et al., 1996; Collis, 1989). The individual contribution of both pathways within the adenosine signalling remains unclear and requires further investigation (Doležal, 2015).

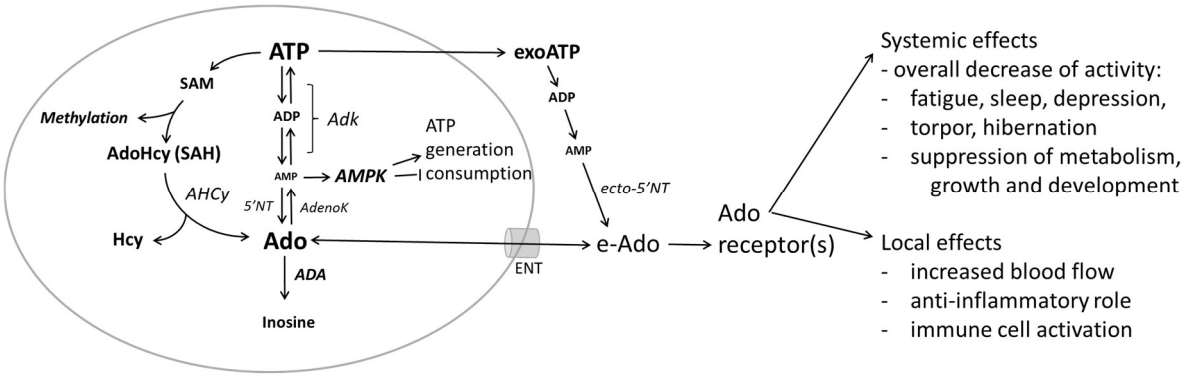


Figure 1: Scheme of adenosine formation via SAM-cycle and cytosolic-/ecto-nucleotidases and overview of e-Ado signalling effects. (Source: Tomáš Doležal)

#### 1.4.2.1 Conversion via nucleotidases

Nucleotidases are proteins converting AMP to adenosine, either extra- or intracellular. They are suspected to play a role during immune response in controlling AMP and adenosine

levels (Antonioli et al., 2008; Novakova & Doležal, 2011). The two enzymes considered in this thesis are an extracellular nucleotidase from gene *NT5E-2*, and a cytosolic nucleotidase from gene *CG11883* (Fenckova et al., 2011; Zimmermann, 1992). An increased consumption of ATP leads to an increase of AMP and subsequent AMPK activity. AMPK in turn stimulates ATP production and lowers ATP consumption. This process usually occurs in non-immune cells. Due to the need of extra energy a conversion of AMP to adenosine appears more likely in immune cells excluding AMPK activity as a possibility. (Bajgar et al., 2015; Collis, 1989; Krawczyk et al., 2010).

#### 1.4.2.2 SAM cycle

S-adenosyl methionine (SAM) is generated from ATP and methionine and during further methylation processes adenosine is produced. Upon donation of a methyl group, SAM is converted to S-adenosyl homocysteine (SAH), which is further converted by hydrolase activity to adenosine and homocysteine. SAM is a key methyl donor and in general, a high methylation rate occurs in activated immune cells. Therefore a high methylation rate reflects the overall cell activity (Chiang et al., 1996; German et al., 1983; Wu et al., 2005). This would support the selfish behaviour of adenosine signalling from immune cells by formation via the SAM Cycle due to the increased activity of immune cells during stress.

### 1.4.3 Method for adenosine measurements ex-vivo

The main purpose of the previous experiments done by Paul Strasser was the establishment of a method to measure lactate, adenosine and inosine levels ex vivo, which are produced in lymph glands of *D. melanogaster* larvae (Strasser, 2016). Furthermore, it was attempted to detect the effects on adenosine levels upon parasitic wasp infection and the subsequent immune reaction. Among important parameters to establish were to find a good strength of infection suitable for the method, a suitable buffer for incubation of the lymph glands, optimal incubation time of the lymph glands in the buffer solution and a suitable concentration of the adenosine deaminase inhibitor EHNA. For all experiments the fly strain $w^{1118}$ and parasitic wasp *L. boulardi* were used. An infection with 2 to 4 parasitic eggs, PBS buffer containing 200 µM glucose and 6 mM trehalose and EHNA concentrations ranging from 0.015 mM to 0.15 mM were used. Adenosine measurements were carried out with a nanodrop spectrophotometer at a wavelength of 260 nm. Lymph glands from infected larvae

were collected 6 hpi. Number of lymph glands, volume of incubation media and incubation time were varied to find optimal settings. Using this method, an increased release of adenosine in lymph glands of infected larvae compared to uninfected larvae was detected. This suggested this method as a useful tool for assessment of e-Ado ex-vivo. Based on this method, e-Ado measurements were carried out for this thesis.

# 2 Aims

- To measure adenosine levels of lymph glands from D. melanogaster larvae ex vivo.
- To detect changes of adenosine levels upon parasitic wasp infection.
- Gene knockdown of *CG11883* and *NT5E-2* encoding for nucleotidases using a RNAi/GAL4/UAS system and verification of the knockdown using RT-qPCR.
- To measure adenosine levels of lymph glands from mutant larvae, detect changes upon parasitic wasp infection and to compare with wild-type larvae.

# 3 Materials and methods

## 3.1 Flies

Four different strains of *Drosophila melanogaster* were used for the conducted experiments. The first strain $w^{1118}$ possessing a mutation on the *White* gene causing white eyes, was used as a control fly. This strain possesses a fully functional immune system. Furthermore, two fly strains containing inserts for RNAi with a UAS regulation sequence for genes *CG11883* and *NT5E-2* respectively from the Vienna Drosophila Resource Center were used. In this thesis the line for *CG11883* will be further abbreviated with 1048 and the line for gene *NT5E-2* with 1378. A line possessing both mutations was obtained by crossing 1048 and 1378 and is furthermore abbreviated with 1315. To trigger the expression of the RNAi´s a GAL4/UAS system was used. Specifically, a Serpent/Gal4 driver line was used, where GAL4 is expressed in hematopoietic cells of *D. melanogaster* larvae including cells of the lymph gland (Bajgar et al., 2015; Crozatier et al., 2004). The used fly strains are summarized in Table 1.

Table 1: Overview of used fly strains

| Abbreviation | Description | Reference |
|---|---|---|
| $w^{1118}$ | mutation in the *White* gene (CG2759), used as control group for all experiments | FlyBase ID: FBst0003605 |
| 1048 | RNAi for *CG11883*, $w^{1118}$; P{GD7418}[v38590] | FlyBase ID: FBst0462588 |
| 1378 | RNAi for *NT5E-2*, $w^{1118}$; P{KK107939}VIE-260B | FlyBase ID: FBst0478464 |
| 1315 | both RNAi´s, $\frac{\text{P\{KK107939\}VIE-260B}}{\text{Cyo}}; \frac{\text{P\{GD7418\}[v38590]}}{\text{TM3 Ser GFP}}$ | Provided by Tomáš Doležal |
| 1420 | Srp/GAL4 driver line, $\frac{\text{lat-}}{\text{FM7 GFP}}; \frac{\text{SrpGal4}}{\text{TM3 Ser GFP}}$ | FlyBase ID: FBtp0020112 |

## 3.2 Infection

For triggering an immune response, an infection with parasitic wasps *L. boulardi* was used. Therefore, early third instar *D. melanogaster* larvae were collected and spread on an agar plate. Time for infection was set to ten minutes to induce a strong infection of the larvae. A proper infection was monitored by dissection of larvae and counting of the parasitic eggs. Only if 3 to 5 eggs on average were detected, the larvae were used for further experiments. Ensuing dissection of infected larvae was performed 2 hpi.

## 3.3 E-Ado production measurement

To measure e-Ado production of the lymph gland in *D. melanogaster* larvae, dissection of lymph glands using a light microscope was performed. For all experiments, three lymph glands were collected and incubated for 20 minutes in 10 µl buffer solution. For further analysis, 7 µl of the buffer solution was pipetted to a new Eppendorf tube while monitoring it under the microscope, to ensure that no transfer of tissue occurred. Afterwards, extracts were kept in the freezer till adenosine measurements were performed at once. For adenosine measurements, the absorbance at a wavelength of 260 nm using a nanodrop spectrophotometer was measured. For quantification, external standard calibration with five standards of known adenosine concentrations, ranging from 0.01 µM to 0.1 µM, were used. For preparation of calibration standards, adenosine was added to the buffer as described in section 3.3.1.

### 3.3.1 Buffer

The dissection and the subsequent incubation of lymph glands from *D. melanogaster* larvae were carried out in a PBS Buffer with 200 µM glucose and 6 mM trehalose. To inhibit adenosine deaminase activity during dissection and incubation, the adenosine deaminase inhibitor EHNA was added to a final concentration of 0.15 mM.

## 3.4 Crosses

To test the influence of nucleotidases on e-Ado release from lymph glands, following genetic crosses were performed. For all crosses around 50 female and 30 male flies were housed together for 5 hours. Virgin female flies were collected after removal of adults from the vial in the morning and subsequent collection 4 and 8 hours afterwards. Male flies from strain 1420 were selected phenotypically upon the absence of notched wings to ensure homozygosity on the third chromosome.

### 3.4.1 *w*$^{1118}$ x 1420

To ensure a comparable genetic background and thus exclude potential errors, virgin females of the control strain *w*$^{1118}$ were crossed in the same way as the strains carrying RNAi´s with males of the GAL4 driver line.

$$\text{♀♀ } w^{1118} \quad \text{x} \quad \text{♂♂ } \frac{\text{lat-}}{\text{FM7 GFP}};\frac{\text{SrpGal4}}{\text{TM3 Ser GFP}}$$

$$\downarrow$$

$$\frac{\text{w}^{1118}}{\text{lat-/ FM7 GFP}};\text{SrpGal4}$$

### 3.4.2 1315 x 1420

In a first step a cross of a fly strain possessing both RNAi´s with the GAL4/UAS driver line was set up to achieve the gene knockdown for both *CG11883* and *NT5E-2*. However, none of several repeats of this cross lead to viable progeny. Further development always stopped before reaching larval stages. Virgin flies from the strain 1315 were selected upon missing markers curly and notch to ensure homozygosity for both RNAi´s.

$$\text{♀♀}\frac{\text{P\{KK107939\}VIE-260B}}{\text{Cyo}};\frac{\text{P\{GD7418\}[v38590]}}{\text{TM3 Ser GFP}} \quad \text{x} \quad \text{♂♂ } \frac{\text{lat-}}{\text{FM7 GFP}};\frac{\text{SrpGal4}}{\text{TM3 Ser GFP}}$$

### 3.4.3 1048 x 1420 and 1378 x 1420

Due to non-viable progeny from the cross 1315 x 1420 (section 3.4.2), experiments were continued with crosses using strains carrying only one RNAi. These strains were again crossed with the Gal4 driver line as depicted below.

*CG11883*

$$\text{♀♀ } w^{1118};\ P\{GD7418\}[v38590] \ \text{x ♂♂ } \frac{\text{lat-}}{\text{FM7 GFP}};\frac{\text{SrpGal4}}{\text{TM3 Ser GFP}}$$

$$\downarrow$$

$$\frac{w^{1118}}{\text{lat-/ FM7 GFP}};\frac{P\{GD7418\}[v38590]}{\text{SrpGal4}}$$

---

*NT5E-2*

$$\text{♀♀ } w^{1118};\ P\{KK107939\}VIE\text{-}260B \ \text{x ♂♂ } \frac{\text{lat-}}{\text{FM7 GFP}};\frac{\text{SrpGal4}}{\text{TM3 Ser GFP}}$$

$$\downarrow$$

$$\frac{w^{1118}}{\text{lat-/ FM7 GFP}};\frac{P\{KK107939\}VIE\text{-}260B}{\text{SrpGal4}}$$

## 3.5 RT-qPCR

### 3.5.1 RNA isolation

The progeny of these crosses was used for the analysis. Therefore, from each group of $w^{1118}$ x 1420, 1048 x 1420 and 1378 x 1420, third instar larvae were collected and 30 lymph glands each were dissected and transferred into Eppendorf tubes. RNA extraction was performed for each group as follows. 200 µl TRIzol buffer was added and the tissue was homogenized using a pestle. 800 µl TRI reagent was added and the solution was left for 5 minutes at room temperature. Afterwards, 500 µl chloroform was added and the tube was vortexed. Subsequently, the samples were centrifuged in a pre-cooled centrifuge at 14000 RPM for 15 minutes. The upper aqueous phase was transferred into a new Eppendorf tube. 500 µl isopropanol was then added to each solution and cooled on ice for 10 minutes. After cooling, tubes were again centrifuged at 14000 RPM and 4° C for 10 minutes. Supernatant was discarded, and RNA pellet was washed with 500 µl ethanol. Finally, the air-dried pellet was dissolved in water treated with DEPC.

### 3.5.2 Reverse transcription

For reverse transcription, extracted RNA from each group was mixed according to Table 2.

Table 2: Reverse transcription protocol

| **Solution** | **Volume [µl]** | **Treatment** |
|---|---|---|
| RNA | 4 | 65° C for 5 minutes, followed by 1 minute on ice |
| oligo-dT Primer | 1 | |
| dNTP | 4 | |
| dH2O | 5 | |
| 5X SSIII buffer | 4 | 50° C for 60 minutes, 75° C for 15 minutes |
| DTT | 1 | |
| SSIII RT | | |

### 3.5.3 PCR

After reverse transcription of the RNA to cDNA, PCR was performed according to the protocol described in Table 3. Each extract was mixed nine times with both primer sets (*CG11883* and *Rp49*, *NT5E-2* and *Rp49*). Furthermore, all used primers are summarized in Table 4.

Table 3: Mixture and time/temperature program for PCR

| **Solution** | **Volume [µl]** |
|---|---|
| dH2O | 2.5 |
| Primers (forward and reverse) | 0.5 |
| SYBR GREEN supermix | 6 |
| DNA template | 3 |

**Temperature program:**

| **T [°C]** | **Time [sec]** | **Description** |
|---|---|---|
| 94 | 240 | Denaturation, 1 cycle |
| 94 | 30 | 40 cycles: denaturation, annealing, elongation |
| 56 | 30 | |
| 72 | 30 | |
| 72 | 600 | Final elongation |

Table 4: Overview of used primers for RT and PCR

| **Primer** | **Sequence** |
|---|---|
| *Rp49* Fwd | 5′-AAGCTGTCGCACAAATGGCG-3′ |
| *Rp49* Rev | 5′-GCACGTTGTGCACCAGGAAC-3′ |
| *CG11883* Fwd | 5′-CCCATTCGCAAATTGTCCGTG-3′ |
| *CG11883* Rev | 5′-CTCCACATCGGAACCTGTCCC-3′ |
| *NT5E-2* Fwd | 5′-CGGTGATTTCTGGACAGATGC-3′ |
| *NT5E-2* Rev | 5′-TCGGGATAGACATAGTCACGC-3′ |
| oligo(dT)20 primer | 5′-d(TTTTTTTTTTTTTTTTTTTT)-3′ |

# 4 Results

## 4.1 First measurements

During the first stage of the thesis, the previous established method by (Strasser, 2016) was repeated. After sufficient dissection skills were acquired, third instar larvae of $w^{1118}$ flies were collected and an immune response was triggered as described in section 0. As a control group, lymph glands of non-infected larvae where dissected as well. In Figure 2 the results of the first successful repetition with elevated adenosine levels from infected larvae is shown.

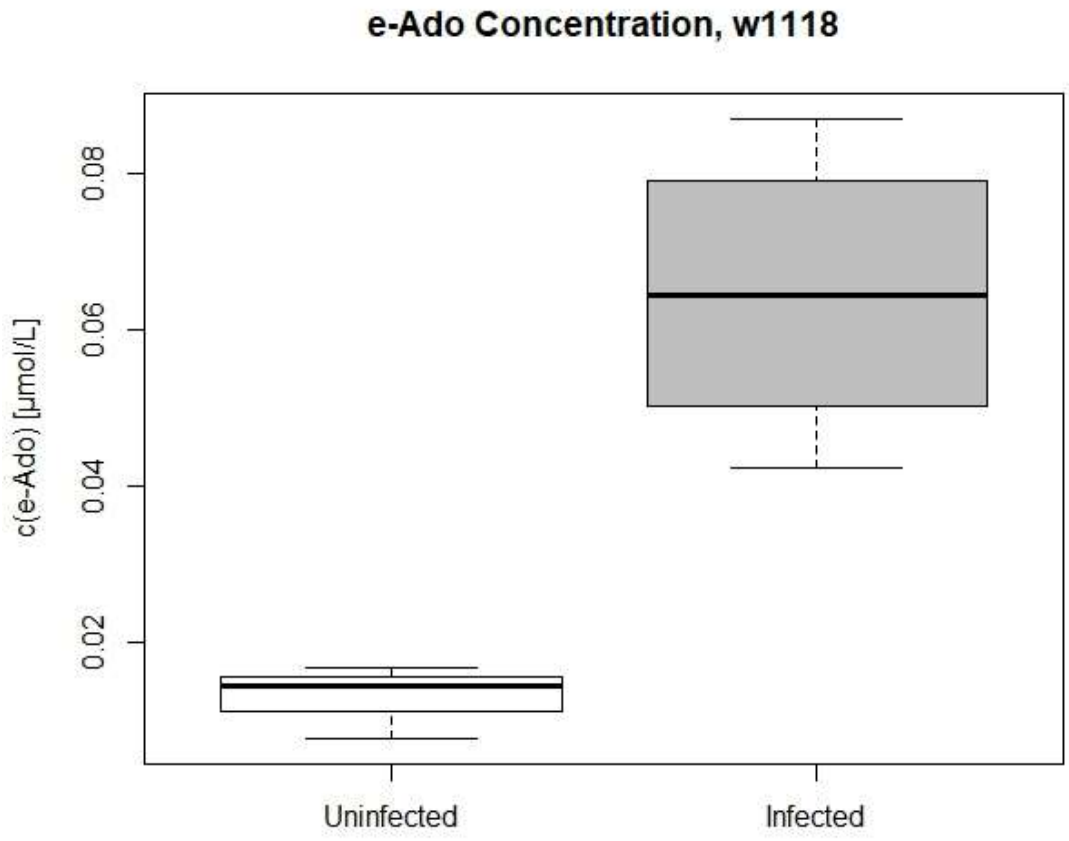


Figure 2: Measured e-Ado in µmol/L ex vivo from lymph glands of infected and uninfected $w^{1118}$.larvae.

For this experiment 3 extracts from uninfected larvae and four extracts from infected larvae respectively were measured. Mean concentrations of 0.013 µM e-Ado for the control group and 0.065 µM e-Ado for the infected larvae were obtained.

A two-sample t-test indicated a significant difference for the infected and uninfected larvae ($t(3.5) = -5.2$; $p = 0.009$, in R 1.1.383). Therefore, a successful repetition of the desired method was assumed. Following this result, the further experiments were conducted using the same procedure.

## 4.2 Double mutation

No adenosine results were obtained from the cross involving the fly strain carrying both RNAi´s due to non-viability of the progeny.

## 4.3 RT-qPCR

The results of the RT-qPCR were analysed using the double delta analysis method. The results indicating the relative abundance of the genes of interest compared to the used housekeeping gene are shown in Figure 3 and Figure 4. For the genotype 1378 two results were obtained (1378a and 1378b), since from a first cross only two lymph glands were obtained. Results from 1378a were not considered for further calculations but are shown in the graph for completeness.

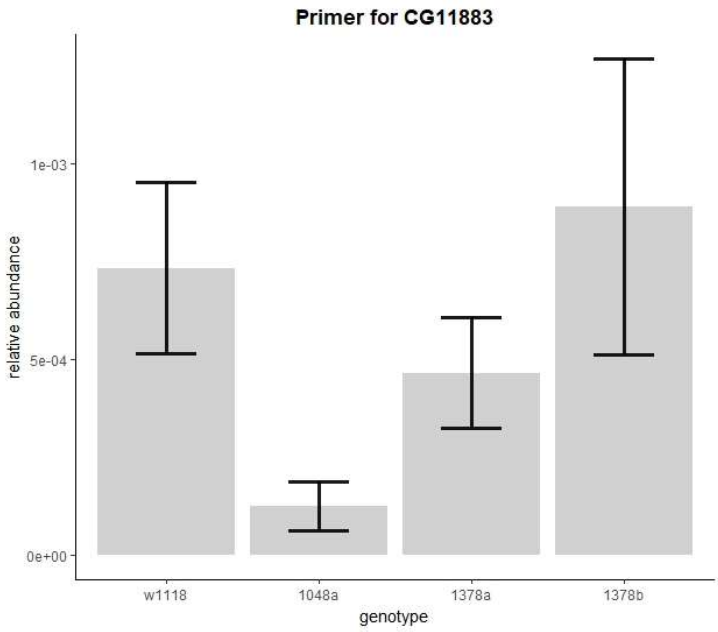


Figure 3: Relative abundance of mRNA for gene *CG11883* in $w^{1118}$ x 1420, 1048 x 1420 and 1378 x 1420 larvae.

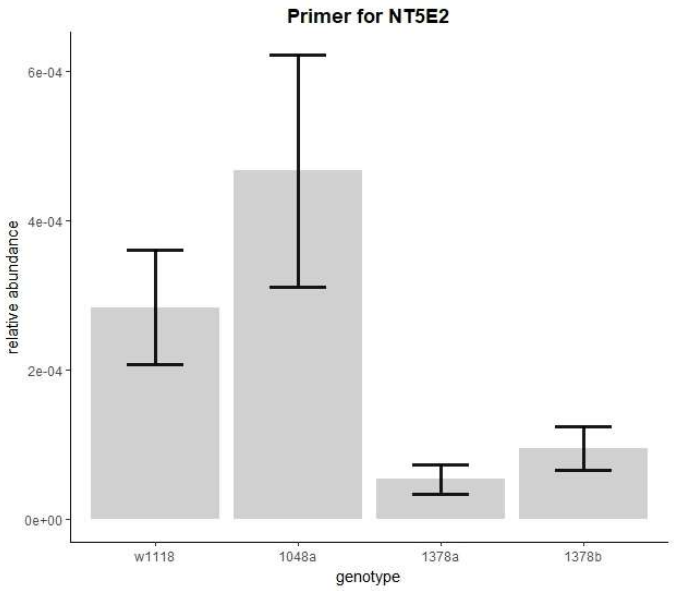


Figure 4: Relative abundance of mRNA for gene *NT5E-2* in $w^{1118}$ x 1420, 1048 x 1420 and 1378 x 1420 larvae.

To assess the rate of gene knockdown the percentages of relative abundance of the genotype compared to the control group were calculated. For the larvae of cross 1378 x 1420, only the value from 1378b was used.

Table 5: Relative abundance (in percent) of mRNA of genes *CG11883* and *NT5E-2*

| **Gene** | **Cross** | **Abundance [%]** |
|---|---|---|
| *CG11883* | $w^{1118}$ x 1420 | 100 |
| | 1048 x 1420 | **17** |
| | 1378 x 1420 | 121 |
| *NT5E-2* | $w^{1118}$ x 1420 | 100 |
| | 1048 x 1420 | 164 |
| | 1378 x 1420 | **33** |

## 4.4 e-Ado upon nucleotidases knock-down

To assess the effect of the two different nucleotidases on the e-Ado production from lymph glands, the same procedure as used for the previous measurements was used with larvae possessing RNAi´s against genes *CG11883* and *NT5E-2*. Larvae of strain $w^{1118}$ x 1420 served as a control group. Results are summarized in Figure 5. For this experiment, several runs were performed with progeny of different crosses. The number of analysed extracts were 17, 16, 18, 15, 16 and 13 four each group following the order of Figure 5. Unlike expected, there was no significant difference in e-Ado production of lymph glands osbserved in any group. This was confirmed by analysis of variance (ANOVA, $F_{(5, 89)} = 0.928$, $p = 0.467$, in R 1.1.383), which didn´t reveal any significant difference.

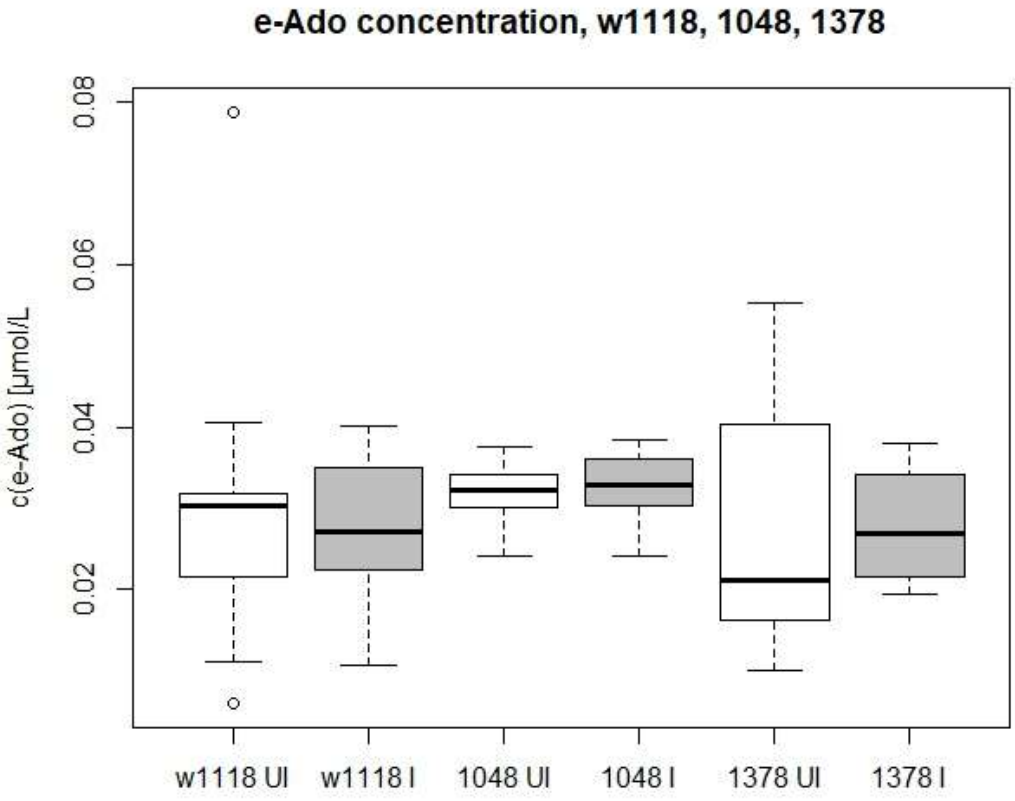


Figure 5: Measured e-Ado concentration in µmol/L from lymph glands ex vivo from $w^{1118}$ x 1420, 1048 x 1420 and 1378 x 1420 larvae.

# 5 Discussion

The first aim of the thesis was to partly replicate the methodology set-up for ex-vivo assessment of adenosine in lymph glands from *D. melanogaster* larvae, which was established in prior work by (Strasser, 2016). After learning general techniques of maintaining fly stocks, infection of larvae with parasitic wasps and training the dissection of lymph glands, first measurements were performed. The first several measurements provided no meaningful results. Problems mainly occurred due to difficulties with proper dissection and careful manipulation with lymph gland tissue to keep it intact. Finally, promising results with a significant difference in adenosine levels were achieved as described in section 4.1, confirming a proper methodology set-up. Furthermore, it supports the theory of adenosine being a signal of immune cells during immune response (Bajgar et al., 2015; Doležal, 2015; Doležal et al., 2005; Novakova & Doležal, 2011; Zuberova et al., 2010). Subsequently, the principle procedure was kept for all ensuing experiments.

Therefore, experiments were continued with using the cross 1315 x 1420 (see section 3.4.2). Because no viable progeny of this cross including flies with both RNAi´s was obtained, no adenosine measurements could have been performed. Since no influence on the development of larvae with a single nucleotidase knockdown was observed, the overall greater decrease of nucleotidase activity in the double mutation seemed to be the reason for the non-viability. Nucleotidases are involved in a complex system catalysing the hydrolysis of ATP to adenosine, whereas their individual role is not fully understood (Fenckova et al., 2011) Therefore, a detailed prediction on the exact mechanism stopping embryonal development in the double mutant larvae is not possible. Consequently, experiments were continued with larvae from crosses 1048 x 1420 and 1378 x 1420 (see section 3.4.3).

To check the functionality of the gene knockdown, lymph glands of larvae from the crosses $w^{1118}$ x 1420, 1048 x 1420 and 1378 x 1420 (see section 3.4.1 and 3.4.3) were dissected and the rate of gene knockdown was investigated using RT-qPCR. Gene knockdown to a rest activity of 17 % for *CG11883* and 33 % for *NT5E-2* were obtained. This indicated a proper gene knockdown serving as a good basis for further experiments. Differences in gene expression for *CG11883* in larvae from $w^{1118}$ x 1420 and 1378 x 1420 and for *NT5E-2* in $w^{1118}$ x 1420 and 1048 x 1420 (see Figure 3 and Figure 4), can be explained by different genetic backgrounds of the different larvae.

Ensuing the confirmation of the functionality of the RNAi/Gal4 construct, experiments with crosses $w^{1118}$ x 1420, 1048 x 1420 and 1378 x 1420 (see section 3.4.1 and 3.4.3) were repeatedly performed. The same procedure for dissection and incubation as for the first measurements was used. Surprisingly, no difference in adenosine release from lymph glands in any group was observed (see Figure 5). The expected difference in infected and uninfected $w^{1118}$ larvae, as observed in first measurements and in the study of (Strasser, 2016), was not detected. This in turn limits any further interpretation of detected adenosine levels in mutant larvae. Most probably degradation of adenosine to inosine due to the presence of ADA did occur which lead to comparably low concentrations in all groups. Since inosine concentrations were not assessed, it is not possible to prove this hypothesis. However, several reasons could have enhanced the degradation. For instance, long incubation time of lymph glands in the buffer solution or problems with the proper functionality of the adenosine deaminase inhibitor. Furthermore, uncertainties due to improper dissection, damage of tissue, as well as different speed of dissection could have influenced the results as well.

The ability to only assess adenosine, inosine and lactate is one of the biggest disadvantages of this method. To simultaneously assess various metabolites, other techniques, namely GC-MS or LC-MS can be used. Many different metabolites can be analysed from one sample allowing extensive screenings. Potential applications are disease modelling, tissue or cell specific metabolite analysis or the assessment of different environmental factors as for example immune response (Cox et al., 2017; Smart et al., 2010; Zhou et al., 2012).

# 6 Conclusion

During the first stage of the work, the prior established method by Paul Strasser was successfully replicated. It represents a rather quick and easy method to measure adenosine ex-vivo with potential in further use to investigate the role of adenosine during immune response in *D. melanogaster*. With the introduction of a gene knockdown for genes *CG11883* and *NT5E-2* a further application of this method was established. Results of this work could unfortunately not help to understand the metabolic pathway of e-Ado during immune response. The method could be improved with shorter incubation times, further repetition and optimization to obtain valuable results. On the other hand, the method is limited to the measurement of adenosine, inosine and lactate. In that respect, other methods as for example GC-MS or LC-MS can be more beneficial as they have the potential to analyse a wider spectrum of metabolites. Moreover, they are more time efficient providing a high rate of sample throughput.

# 7 References


Antonioli, L., Fornai, M., Colucci, R., Ghisu, N., Tuccori, M., Del Tacca, M., & Blandizzi, C. (2008). Pharmacological modulation of adenosine system: Novel options for treatment of inflammatory bowel diseases. *Inflammatory Bowel Diseases*, *14*(4), 566–574.

Bajgar, A., Kucerova, K., Jonatova, L., Tomcala, A., Schneedorferova, I., Okrouhlik, J., & Doležal, T. (2015). Extracellulas adenosine mediates a systemic metabolic switch during immune response. *PLoS Biology*, *13*(4), 1–23.

Busson, D., & Pret, A. M. (2007). GAL4/UAS targeted gene expression for studying *Drosophila* hedgehog signaling. *Methods in Molecular Biology*, *397*, 161–201.

Cheng, S. C., Joosten, L. A. B., & Netea, M. G. (2014). The interplay between central metabolism and innate immune responses. *Cytokine and Growth Factor Reviews*, *25*(6), 707–713.

Chiang, P. K., Gordon, J. T., Zeng, G. C., Doctor, B. P., Pardhasaradhi, K., & McCann, P. P. (1996). S-Adenosylmethionine and methylation. *FASEB Journal*, *10*(4).

Collis, M. G. (1989). The vasodilator role of adenosine. *Pharmacology and Therapeutics*, *41*(1–2), 143–162.

Cox, J. E., Thummel, C. S., & Tennessen, J. M. (2017). Metabolomic studies in *Drosophila*. *Genetics*, *206*(3), 1169–1185.

Crozatier, M., Ubeda, J. M., Vincent, A., & Meister, M. (2004). Cellular immune response to parasitization in *Drosophila* requires the EBF orthologue collier. *PLoS Biology*, *2*(8), 1107–1113.

Dahmann, C. (2008). *Drosophila: Methods and protocols*. Humana Press.

DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G., & Thompson, C. B. (2008). The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. *Cell Metabolism*, *7*(1), 11–20.

Delmastro-Greenwood, M. M., & Piganelli, J. D. (2013). Changing the energy of an immune response. *American Journal of Clinical and Experimental Immunology*, *2*(1), 30–54.

Dietzl, G., Chen, D., Schnorrer, F., Su, K. C., Barinova, Y., Fellner, M., … Dickson, B. J. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature*, *448*(7150), 151–156.

Doležal, T. (2015). Adenosine: a selfish-immunity signal? *Oncotarget*, *6*(32), 32307–32308.

Doležal, T., Dolezelova, E., Zurovec, M., & Bryant, P. J. (2005). A role for adenosine deaminase in *Drosophila* larval development. *PLoS Biology*, *3*(7), 1213–1224.

Duffy, J. B. (2002). GAL4 system in *Drosophila*: A fly geneticist's swiss army knife. *Genesis*, *34*, 1–15.

Fenckova, M., Hobizalova, R., Fric, Z. F., & Doležal, T. (2011). Functional characterization of ecto-5′-nucleotidases and apyrases in *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology*, *41*(12), 956–967.

Garber, K. (2004). Energy boost : The Warburg effect. *Journal of the National Cancer Institute*, *96*(24), 1805–1806.

German, D. C., Blochsj, C. A., & Kredich, N. M. (1983). Measurements of S-adenosylmethionine and L-homocysteine metabolism in cultured human lymphoid cells. *Journal of Biological Chemistry*, *258*(18), 10997–11003.

Heiden, M. G. Vander, Cantley, L. C., & Thompson, C. B. (2009). Understanding the warburg effect: The metabolic requirements of cell proliferation. *Science*, *324*(5930), 1029–1033.

Herranz, H., & Cohen, S. (2017). *Drosophila* as a model to study the link between metabolism and cancer. *Journal of Developmental Biology*, *5*(4), 15.

Kavi, H. H., Fernandez, H., Xie, W., & Birchler, J. A. (2008). Genetics and biochemistry of RNAi in Drosophila. *Current Topics in Microbiology and Immunology*, *320*(February), 37–69.

Krawczyk, C. M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R. J., … Pearce, E. J. (2010). Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood*, *115*(23), 4742–4749.

Lemaitre, B., & Hoffmann, J. (2007). The host defense of *Drosophila melanogaster. Annual Review of Immunology*, *25*(1), 697–743.

Myers, E. W., Sutton, G. G., Delcher, A. L., Dew, I. M., Fasulo, D. P., Flanigan, M. J., … Venter, J. C. (2000). A whole-genome assembly of *Drosophila*. *Science*, *287*(5461), 2196–2204.

Neyen, C., Bretscher, A. J., Binggeli, O., & Lemaitre, B. (2014). Methods to study *Drosophila* immunity. *Methods*, *68*(1), 116–128.

Ni, J. Q., Markstein, M., Binari, R., Pfeiffer, B., Liu, L. P., Villalta, C., … Perrimon, N. (2008). Vector and parameters for targeted transgenic RNA interference in *Drosophila melanogaster*. *Nature Methods*, *5*(1), 49–51.

Novakova, M., & Doležal, T. (2011). Expression of *Drosophila* adenosine deaminase in immune cells during inflammatory response. *PLoS ONE*, *6*(3).

Pandey, U. B., & Nichols, C. D. (2011). Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. *Drug Delivery*, *63*(2), 411–436.

Perrimon, N., Ni, J., Perkins, L., Noller, H. F., Volpe, T., & Martienssen, R. A. (2010). In vivo RNAi : Today and Tomorrow. *Cold Spring Harbor Perspectives in Biology*, *2*(8), 1–11.

Pfeiffer, T., Schuster, S., & Bonhoeffer, S. (2001). Cooperation and competition in the evolution of ATP-Producing pathways. *Science*, *292*(5516), 504–507.

Schlenke, T. A., Morales, J., Govind, S., & Clark, A. G. (2007). Contrasting infection strategies in generalist and specialist wasp parasitoids of *Drosophila melanogaster*. *PLoS Pathogens*, *3*(10), 1486–1501.

Small, C., Paddibhatla, I., Rajwani, R., & Govind, S. (2012). An introduction to parasitic wasps of *Drosophila* and the antiparasite immune response. *Journal of Visualized Experiments*, (63), 1–9.

Smart, K. F., Aggio, R. B. M., Van Houtte, J. R., & Villas-Bôas, S. G. (2010). Analytical platform for metabolome analysis of microbial cells using methyl chloroformate derivatization followed by gas chromatography-mass spectrometry. *Nature Protocols*, *5*(10), 1709–1729.

Sorrentino, R. P., Carton, Y., & Govind, S. (2002). Cellular immune response to parasite infection in the *Drosophila* lymph gland is developmentally regulated. *Developmental Biology*, *243*(1), 65–80.

Southall, T. D., Elliott, D. A., & Brand, A. H. (2008). The GAL4 system: A versatile toolkit for gene expression in *Drosophila*. *Cold Spring Harbor Protocols*, *3*(7), 1–9.

Stephenson, R., & Metcalfe, N. H. (2013). *Drosophila melanogaster*: a fly through its history and current use. *The Journal of the Royal College of Physicians of Edinburgh*, *43*(1), 70–75.

Strasser, P. (2016). Warburg effect in lymph gland of *Drosophila melanogaster* upon parasitoid wasp infection.

Wu, Q., Fu, Y., Zhou, W., Wang, J., Feng, Y., Liu, J., … Zuo, J. (2005). Inhibition of S - Adenosyl- L -homocysteine hydrolase induces immunosuppression. *The Journal of Pharmacology and Experimental Therapeutics*, *313*(2), 705–711.

Yamamoto-Hino, M., & Goto, S. (2013). In vivo RNAi-based screens: Studies in model organisms. *Genes*, *4*(4), 646–665.

Zhou, B., Xiao, J. F., Tuli, L., & Ressom, H. W. (2012). LC-MS-based metabolomics. *Molecular BioSystems*, *8*(2), 470–81.

Zimmermann, H. (1992). 5'-Nucleotidase: molecular structure and functional aspects. *The Biochemical Journal*, *285*, 345–365.

Zuberova, M., Fenckova, M., Simek, P., Janeckova, L., & Doležal, T. (2010). Increased extracellular adenosine in *Drosophila* that are deficient in adenosine deaminase activates a release of energy stores leading to wasting and death. *Disease Models & Mechanisms*, *3*(11–12), 773–784.

# 8 Annex

Table 6: Results from the first successful adenosine measurement with calculated concentrations.

| | absorbance (260 nm) | | | avg. absorbance | c (e-Ado) [µM] |
|---|---|---|---|---|---|
| $w^{1118}$ **(uninfected)** | 0.604 | 0.600 | 0.672 | 0.625 | 0.015 |
| | 0.469 | 0.543 | 0.561 | 0.524 | 0.008 |
| | 0.655 | 0.649 | 0.678 | 0.661 | 0.017 |
| $w^{1118}$ **(infected)** | 1.254 | 1.281 | 1.279 | 1.271 | 0.058 |
| | 1.034 | 1.039 | 1.045 | 1.039 | 0.042 |
| | 1.694 | 1.698 | 1.720 | 1.704 | 0.087 |
| | 1.439 | 1.482 | 1.475 | 1.465 | 0.071 |

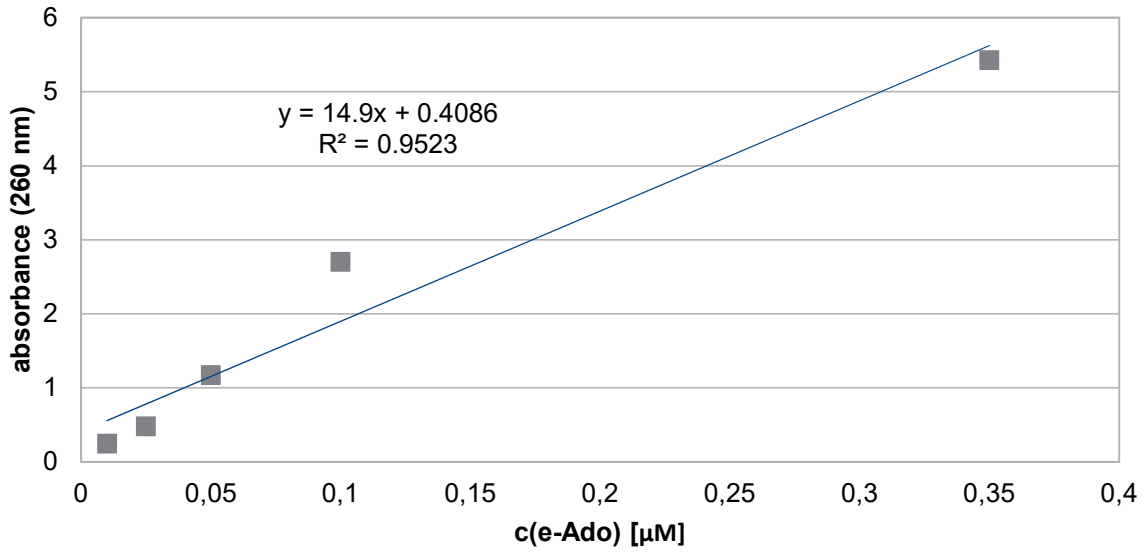


Figure 6: Calibration curve for the first adenosine measurements.

Table 7: Summary of adenosine measurements and calculated concentrations from experiments described in section 4.4.

| Cross | absorbance (260 nm) | | | avg. absorbance | c (e-Ado) [µM] |
|---|---|---|---|---|---|
| $w^{1118}$ **x 1420 (uninfected) 4.7.2016** | 0.579 | 0.997 | 0.830 | 0.802 | 0.031 |
| | 0.434 | 0.643 | 0.589 | 0.555 | 0.018 |
| | 0.429 | 0.428 | 0.397 | 0.418 | 0.011 |
| | 0.282 | 0.321 | 0.348 | 0.317 | 0.006 |
| | 0.518 | 0.875 | 0.919 | 0.771 | 0.029 |
| | 0.674 | 0.496 | 0.496 | 0.555 | 0.018 |
| $w^{1118}$ **x 1420 (infected) 4.7.2016** | 0.355 | 0.424 | 0.486 | 0.422 | 0.011 |
| | 0.344 | 0.447 | 0.431 | 0.407 | 0.011 |
| | 0.603 | 0.726 | 0.754 | 0.694 | 0.025 |
| | 0.701 | 0.849 | 0.949 | 0.833 | 0.421 |
| | 0.314 | 0.472 | 0.476 | 0.421 | 0.011 |
| $w^{1118}$ **x 1420 (uninfected) 7.7.2016** | 0.559 | 0.568 | 0.644 | 0.590 | 0.024 |
| | 0.852 | 0.821 | 0.860 | 0.844 | 0.033 |
| | 0.698 | 0.802 | 0.816 | 0.772 | 0.031 |
| | 0.490 | 0.548 | 0.570 | 0.536 | 0.021 |
| | 0.600 | 0.586 | 0.551 | 0.579 | 0.023 |
| | 0.775 | 0.789 | 0.839 | 0.801 | 0.032 |
| $w^{1118}$ **x 1420 (infected) 7.7.2016** | 0.462 | 0.539 | 0.543 | 0.515 | 0.021 |
| | 0.597 | 0.577 | 0.616 | 0.597 | 0.024 |
| | 0.641 | 0.736 | 0.658 | 0.678 | 0.027 |
| | 0.609 | 0.655 | 0.658 | 0.641 | 0.885 |
| | 0.842 | 0.881 | 0.933 | 0.885 | 0.035 |
| | 0.938 | 1.037 | 1.074 | 1.016 | 0.040 |
| **1048 x 1420 (uninfected) 7.7.2016** | 0.636 | 0.622 | 0.659 | 0.639 | 0.026 |
| | 0.860 | 0.961 | 0.956 | 0.926 | 0.037 |
| | 0.735 | 0.748 | 0.787 | 0.757 | 0.030 |
| | 0.856 | 0.892 | 0.865 | 0.871 | 0.035 |
| | 0.808 | 0.925 | 0.926 | 0.886 | 0.035 |
| | 0.813 | 0.760 | 0.780 | 0.784 | 0.031 |
| **1048 x 1420 (infected) 7.7.2016** | 0.773 | 0.822 | 0.900 | 0.832 | 0.033 |
| | 0.866 | 0.893 | 1.076 | 0.945 | 0.037 |
| | 0.754 | 0.793 | 0.785 | 0.777 | 0.031 |
| | 0.548 | 0.628 | 0.634 | 0.603 | 0.024 |
| **1048 x 1420 (uninfected) 8.7.2016** | 0.772 | 0.808 | 0.648 | 0.743 | 0.030 |
| | 0.838 | 0.851 | 0.740 | 0.810 | 0.032 |
| | 0.888 | 0.676 | 0.697 | 0.754 | 0.030 |
| | 0.962 | 0.697 | 0.929 | 0.863 | 0.034 |
| | 0.859 | 1.123 | 0.489 | 0.824 | 0.033 |
| | 0.989 | 0.986 | 0.872 | 0.949 | 0.038 |
| **1048 x 1420 (infected) 8.7.2016** | 0.971 | 0.935 | 0.847 | 0.918 | 0.036 |
| | 0.917 | 0.710 | 0.662 | 0.763 | 0.030 |
| | 0.783 | 0.823 | 0.885 | 0.830 | 0.033 |
| | 0.904 | 0.961 | 0.856 | 0.907 | 0.036 |

| | | | | | |
|---|---|---|---|---|---|
| | 0.702 | 0.774 | 0.653 | 0.710 | 0.028 |
| | 0.619 | 0.666 | 0.710 | 0.665 | 0.027 |
| **$w^{1118}$ x 1420 (uninfected) 8.7.2016** | 0.750 | 0.774 | 0.876 | 0.800 | 0.032 |
| | 1.055 | 1.058 | 0.978 | 1.030 | 0.030 |
| | 0.726 | 0.775 | 0.780 | 0.760 | 0.030 |
| | 0.958 | 0.974 | 0.934 | 0.955 | 0.038 |
| | 2.710 | 1.559 | 1.791 | 2.020 | 0.079 |
| **$w^{1118}$ x 1420 (infected) 8.7.2016** | 0.961 | 0.977 | 0.978 | 0.972 | 0.038 |
| | 0.973 | 0.883 | 1.032 | 0.963 | 0.038 |
| | 0.800 | 0.873 | 0.901 | 0.858 | 0.034 |
| | 0.813 | 0.924 | 0.900 | 0.879 | 0.035 |
| | 0.662 | 0.699 | 0.690 | 0.684 | 0.027 |
| **1048 x 1420 (uninfected) 11.7.2016** | 0.837 | 0.788 | 0.787 | 0.804 | 0.032 |
| | 0.847 | 0.729 | 0.840 | 0.805 | 0.032 |
| | 0.645 | 0.546 | 0.606 | 0.599 | 0.024 |
| | 0.787 | 0.827 | 0.811 | 0.808 | 0.032 |
| | 0.867 | 0.874 | 0.759 | 0.833 | 0.033 |
| | 0.843 | 0.904 | 0.799 | 0.849 | 0.034 |
| **1048 x 1420 (infected) 11.7.2016** | 0.882 | 0.662 | 0.749 | 0.764 | 0.030 |
| | 0.826 | 0.822 | 0.792 | 0.813 | 0.032 |
| | 0.861 | 0.957 | 0.932 | 0.917 | 0.036 |
| | 0.819 | 0.873 | 0.930 | 0.874 | 0.035 |
| | 1.002 | 0.980 | 0.940 | 0.974 | 0.038 |
| **1378 x 1420 (uninfected) 11.7.2016** | 1.113 | 1.201 | 1.077 | 1.130 | 0.044 |
| | 1.083 | 1.099 | 1.055 | 1.079 | 0.043 |
| | 1.148 | 1.209 | 1.130 | 1.162 | 0.046 |
| | 1.423 | 1.387 | 1.422 | 1.411 | 0.055 |
| **1378 x 1420 (infected) 11.7.2016** | 0.882 | 0.977 | 0.967 | 0.942 | 0.037 |
| | 0.964 | 0.969 | 0.947 | 0.960 | 0.038 |
| **1378 x 1420 (uninfected) 14.7.2016** | 0.627 | 0.626 | 0.555 | 0.603 | 0.020 |
| | 0.343 | 0.425 | 0.356 | 0.375 | 0.010 |
| | 1.027 | 1.032 | 1.029 | 1.029 | 0.038 |
| | 0.668 | 0.666 | 0.635 | 0.656 | 0.022 |
| | 0.533 | 0.607 | 0.583 | 0.574 | 0.019 |
| | 0.780 | 0.840 | 0.771 | 0.797 | 0.028 |
| **1378 x 1420 (infected) 14.7.2016** | 0.579 | 0.569 | 0.633 | 0.594 | 0.019 |
| | 0.841 | 0.896 | 0.879 | 0.872 | 0.032 |
| | 0.688 | 0.745 | 0.683 | 0.705 | 0.024 |
| | 0.774 | 0.793 | 0.706 | 0.758 | 0.027 |
| | 0.803 | 0.779 | 0.785 | 0.789 | 0.028 |
| **1378 x 1420 (uninfected) 17.7.2016** | 0.353 | 0.619 | 0.358 | 0.443 | 0.013 |
| | 0.509 | 0.603 | 0.510 | 0.541 | 0.017 |
| | 0.500 | 0.357 | 0.504 | 0.454 | 0.013 |
| | 0.523 | 0.562 | 0.621 | 0.569 | 0.018 |
| | 0.425 | 0.537 | 0.523 | 0.495 | 0.015 |
| | 0.707 | 0.768 | 0.698 | 0.724 | 0.025 |

| | | | | | |
|---|---|---|---|---|---|
| **1378 x 1420 (infected) 17.7.2016** | 0.706 | 0.720 | 0.867 | 0.767 | 0.027 |
| | 0.519 | 0.658 | 0.628 | 0.602 | 0.020 |
| | 0.566 | 0.725 | 0.626 | 0.639 | 0.021 |
| | 0.921 | 0.956 | 0.920 | 0.932 | 0.034 |
| | 0.582 | 0.650 | 0.688 | 0.640 | 0.021 |
| | 0.895 | 0.964 | 0.947 | 0.935 | 0.034 |

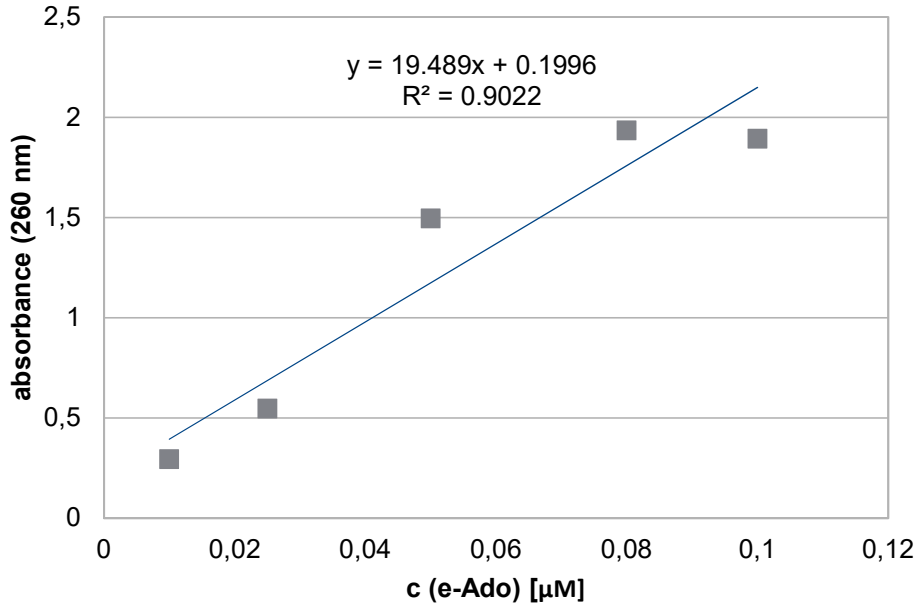


Figure 7: Calibration curve for experiments from 4.7.2016.

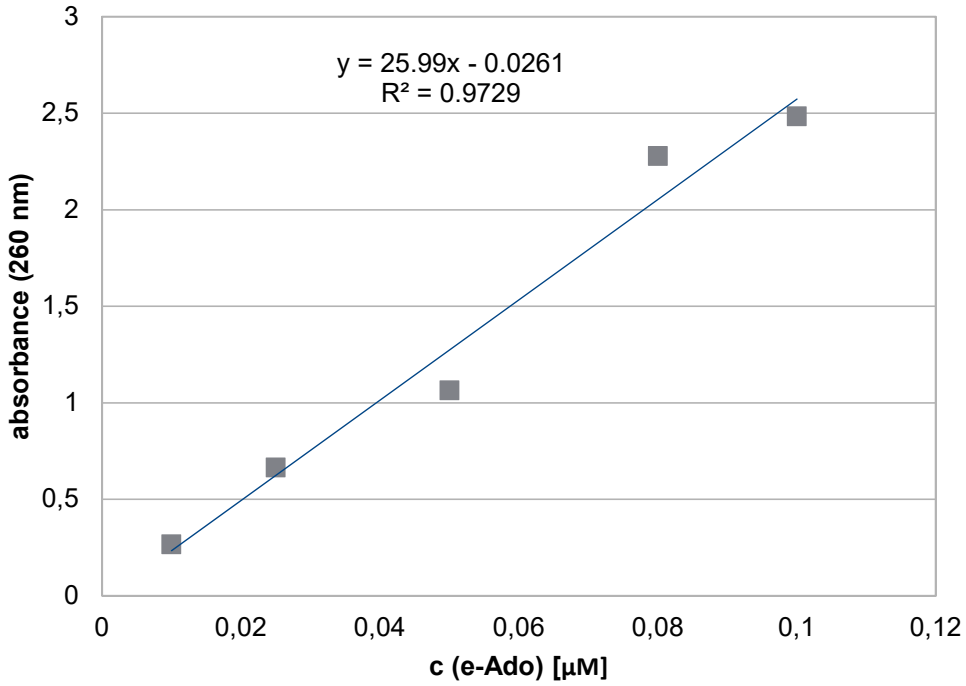


Figure 8: Calibration curve for experiments from 7.7., 7.8. and 11.7. 2016.

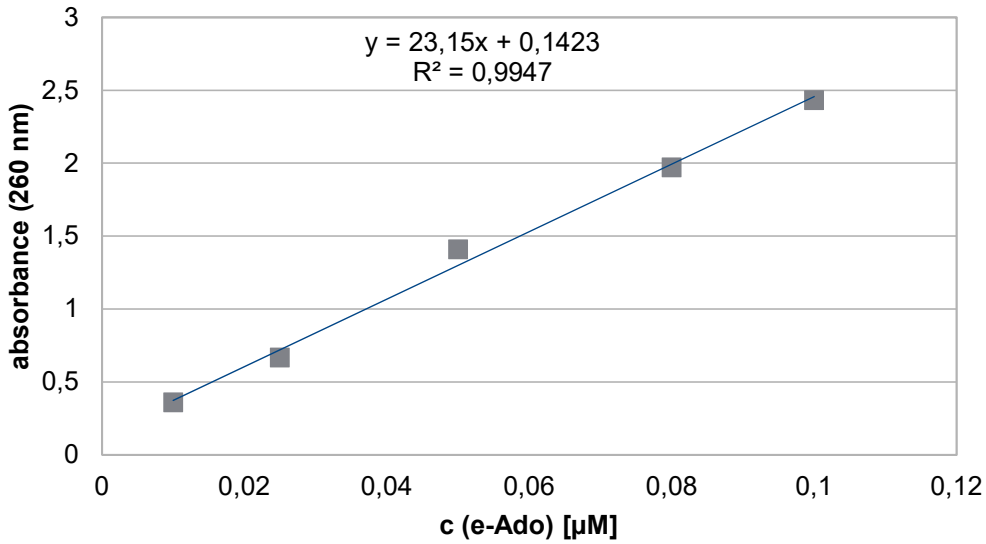


Figure 9: Calibration curve for experiments from 14.7. and 17.7. 2016.