University of South Bohemia in České Budějovice

Faculty of Science

The Role of Juvenile Hormone during Immune Response in *Drosophila melanogaster*

Bachelor Thesis

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Annotation: The role of Juvenile hormone during immune response was investigated in *Drosophila melanogaster* larvae. The levels of Juvenile hormone were measured directly by mass spectrometry and indirectly by monitoring Krüppel Homologue expression. Neither of those approaches were successful. We genetically manipulated JH production and signalling, infected the larvae with parasitoid wasps and found a significant difference in immune strength between larvae with and without functional JH production.

Declaration:

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography.

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České Budějovice, 11.12.2019 ……

Sebastian Tschernuth

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List of Abbreviations:

AdoR	Adenosine Receptor
ATP	Adenosine Triphosphate
Aug	August
CA	Corpora allata
cDNA	complementary DNA
CyO	Curly of Oster
dILPs	drosophila Insulin-like peptides
DNase	Deoxyriboniclease
dNTPs	deoxyribonucleic acids
DTT	Dithiothreitol
e-Ado	extracellular Adenosine
E-Cad	Epithelial cadherin
FOXO	Forkhead Box O
Gal-4	Galactose induced gene 4
gce	Germ-cell expressed
GFP	Green fluorescent protein
GLUT4	Glucose transporter type 4
hid	head involution defective
ILPs	Insulin like peptides
JH	Juvenile Hormone
JH III	Juvenile Hormone III
JHAMT	Juvenile-hormone-acid-O-methyl-transferase
JHB3	bis-epoxide Juvenile Hormone III
KrH	Krüppel homologue
Met	Methoprene tolerant
MF	Methyl farnesoate
TOR	Target of Rapamycin
PBS	Phosphate Buffered Saline
RFP	Red fluorescent protein
RNase	Ribonuclease
rpr	reaper
RT-q-PCR	Real-time quantitative polymerase chain reaction
UAS	Upstream activating sequence
WT	Wild type

Abstract:

Prioritizing cells or tissues at the expense of others is a crucial physiological mechanism for animals to ensure survival during periods of stress. In *Drosophila melanogaster*, the immune system is capable of suppressing energy uptake in other tissues via e-Ado signaling, probably inducing insulin resistance. The mechanism for the induction of insulin resistance is unknown, but Juvenile Hormone (JH) may be a candidate for it. Our goal was to find out whether JH levels change upon immune stress and if impaired JH signaling affects the strength of the immune response. We measured JH levels indirectly by monitoring Krüppel Homologue expression, but the results were too variable. We unsuccessfully measured JH directly using mass spectrometry. We used several genetic manipulations to block JH production and signaling, which was done successfully with one of three manipulations. The larvae with impaired JH signaling were infected with parasitoid wasps and immune cells differentiating to combat the intruder were counted. Overall, we could observe only 52% of immune cells in larvae without functioning JH signaling compared with control groups. This implies that JH plays a role in immune response and could possibly be responsible for inducing insulin resistance.

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1. Background:

When thinking about biological organisms, for example animals, we can perceive them as a harmonious entity consisting of tissues working altruistically for one another, always with the best interest of the organism in mind [1]. An organism seems to be inherently free from conflict of interests, its tissues never at war between each other [1]. But this romantic picture of a collective network of cells, tissues and organs all working together may be incorrect [1]. According to Richard Dawkins, an altruistic act of any biological entity is done, because it in turn serves the entity itself, and not because it has the best interest of a greater entity in mind [2]. Such entities could be genes as well as whole organisms, but can this also be true for cells or tissues within organisms?

From an evolutionary standpoint, prioritising certain aspects at the expense of others is very favourable for the survival of all organisms and developed over the course of evolution [2]. For example, when an animal faces extended periods of starvation, it would be potentially lethal for the animal not to adapt its behaviour and metabolism [3].

When looking at an animal as a collection of tissues, this collection of tissues also responds in a way which ensures the organisms survival at the expense of less crucial tasks. In other words, under stressful circumstances, there are tissues which´s wellbeing is sacrificed for the preservation of more important tissues [4].

In this bachelor thesis, we tried to find out more about how immune cells acquire energy while other tissues of the organism are temporarily almost incapable to do so. We used the fruit fly, *Drosophila melanogaster*, as a model organism and studied the interactions between immune cells and the rest of the body. In order to get a more detailed picture of what this research was about, we need a basic understanding of three molecules and the roles they play in our model organism:

- Extracellular Adenosine
- Insulin
- Juvenile hormone

1.1. Extracellular Adenosine:

Extracellular Adenosine (e-Ado) is an important regulator of metabolism in mammals as well as in insects [5] [6]. Usually, e-Ado is present at very low concentrations in the circulatory system [5]. However, large amounts of e-Ado are produced and released under

stressful circumstances by damaged or metabolically stressed cells [5] [6]. This is done so in order to ensure sufficient energy supply in the form of glucose for the cells/tissues [5] [6].

When a Drosophila larva is attacked by a pathogen, i.e. a parasitoid wasp egg, haemocytes are needed to divide and differentiate into lamellocytes quickly to combat the intruder. Lamellocytes are specialized immune cells, which form a capsule around the parasitoid wasp egg and release toxins into the capsule, possibly destroying the egg [5] [6] [7]. The immune system´s energy demand relative to the total consumption of energy in the larva raises from 10% to approximately 30% [5]. This massive increase is due to a change in ATP production. Instead of the very efficient aerobic respiration, haemocytes rely on the much quicker and less efficient anaerobic glycolysis, where glucose is converted into two molecules of pyruvate, which is subsequently converted to lactate [5] [8]. A slow immune response is almost certainly lethal for the animal, haemocytes therefore have got no choice but to use the faster way of energy production [6]. Via Adenosine receptor (AdoR) signalling cascade, this **e-Ado might induce a temporary insulin resistance in some tissues**. We expect e-Ado to do so, because the influence of e-Ado on metabolism is very similar to the effect of insulin resistance [6] [9]. My task was to evaluate if JH could be the link between e-Ado and insulin resistance. Examples for affected, metabolically downregulated tissues are the fat-body, muscle cells and imaginal discs [5].

1.2. Insulin:

Among the most momentous topics related to human health is insulin; according to the WHO, more than 8% of the worldwide population suffer from diabetes mellitus, the disease related to a failing insulin signalling [10]. For this reason, it is of utmost importance to understand the underlying mechanisms of how insulin is created, how it interacts, which influences it has on all kinds of cells and how it is catabolised.

Insulin, a peptide hormone, regulates blood sugar levels in mammals [11] [12]. Similarly, insulin-like-proteins (ILPs) regulate haemolymph sugar levels in insects [12]. Insulin and ILPs are produced as pre-propeptides and modified, which results in two peptide chains linked by disulfide bonds. In *Drosophila melanogaster*, 8 ILPs (drosophila-Insulin-Like-Peptides, dILPs) are known [12].

When animals take up nutrients, carbohydrates are transported into the circulatory system and thereby raising circulating sugar levels. The most abundant form of sugar among all organisms is glucose, therefore I will continue using glucose instead of sugars. When glucose levels rise in animals, insulin/ILPs are produced and excreted into the circulatory

system [13]. Next, insulin/ILPs trigger the activation of an insulin dependent glucose transporter [13] [14]. These glucose transporters are responsible for providing cells with energy in the form of glucose. In mammals, glucose transporter type 4 (GLUT4) is expressed by fat cells and muscle cells and is among the most important mediators for insulin removal from the circulatory system [15]. It is a one of the insulin dependent glucose transporters [13]. Activated GLUT4 transports glucose from the circulatory system into the cell, thereby diminishing excessive glucose in the circulatory system [13]. Once glucose levels in the circulatory system reach ordinary concentrations, insulin/ILP levels also fall, and glucose levels are kept constant, because fewer cells can take up glucose. Nerve cells, as well as immune cells, express a different kind of glucose transporters [14]. The key difference between different glucose transporters is the dependence or independence of insulin [14]. The brain can take up glucose even during absence of insulin [14]. This is one of the ways to ensure a differentiated energy supply depending on the expression of different glucose transporters. While less important tissues can take up glucose only when glucose is sufficiently present in the circulatory system, more important tissues are always able to take up glucose, if there is at least some circulating glucose present.

Insulin resistance is a term used when a cell or tissue is unable to respond according to insulin [11]. This insulin resistance is a state which can be both temporary as well as chronic, whereas the latter is associated with starvation of the cell/tissue and therefore not perceived as very desirable. Referring to the concept of privileging certain tissues, a temporary insulin resistance can be a great pathway for the organism to do so – in other words – to regulate energy consumption in times of acute stress.

Even though Insulin/ILPs are best known as a regulator of circulating sugar levels, they also regulate several other processes. ILPs in our model organism, the fruit fly, are known to interact with most major regulatory pathways, such as TOR, FOXO and the development-hormones ecdysone and JH [16].

1.3. Juvenile Hormone:

The sesquiterpenoid Juvenile Hormone (JH) is known as an important regulator of growth, reproduction and diapause in insects [17]. Its most prominent role is in promoting larval growth while preventing metamorphosis [17]. The structure of JH varies; there are three homologues present in *Drosophila melanogaster*: JH III, bis-epoxide JH III (JHB3) and methyl farnesoate (MF). The structures can be seen in figure 1.

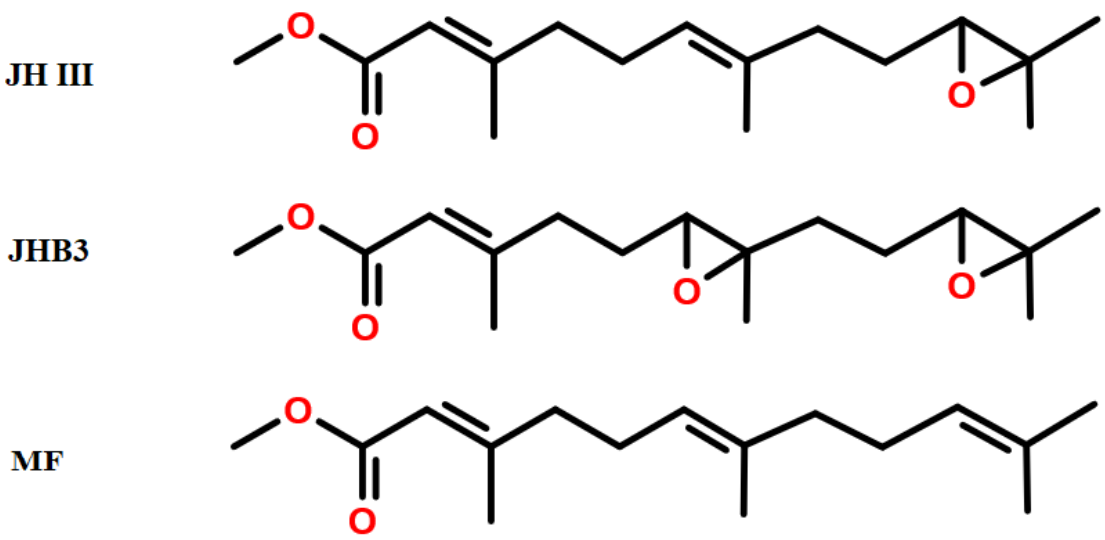

Figure 1, Structural representation of JH III, JHB3 and MF.

JH is produced in the *corpora allata* (CA), which is located in the ring gland (Figure 2) [17]. The ring gland is a collective of endocrine glands, namely the *corpora cardiaca*, CA and the prothoratic gland [17]. The ring gland is located in close proximity to the brain, as seen in Figure 3 [17]. Figure 2 and 3 were obtained from larvae expressing GFP in the CA.

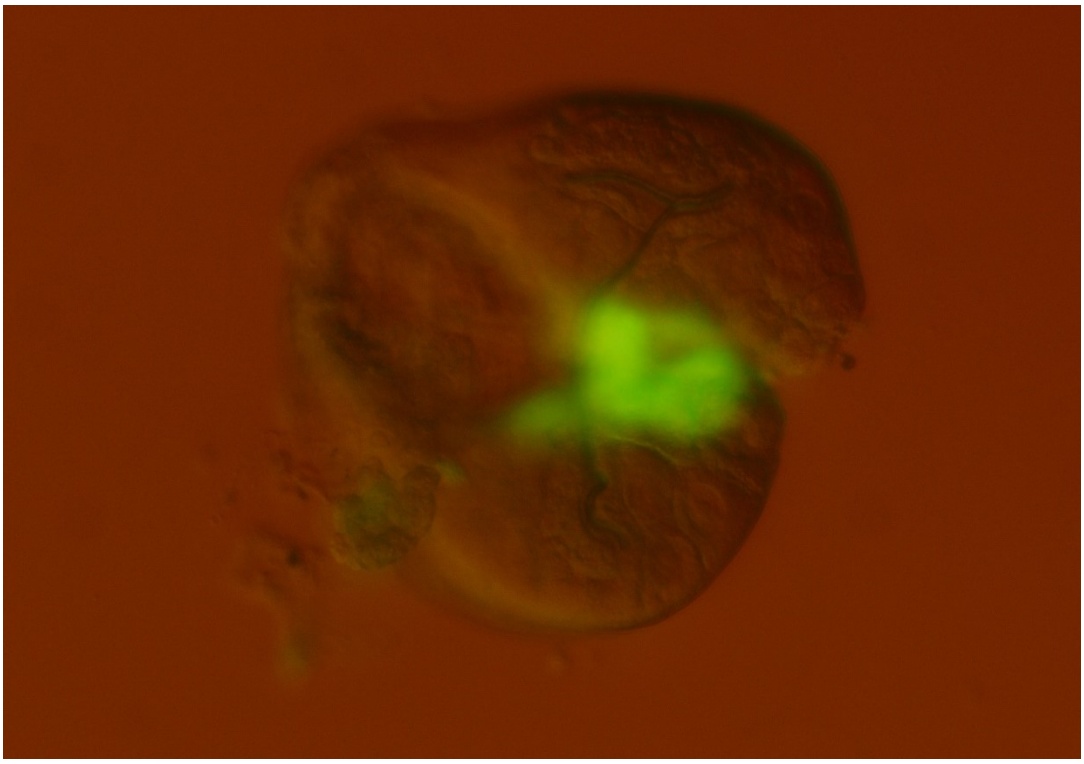

Figure 2, Ring gland with CA expressing GFP, making use of the UAS-Gal4 system.

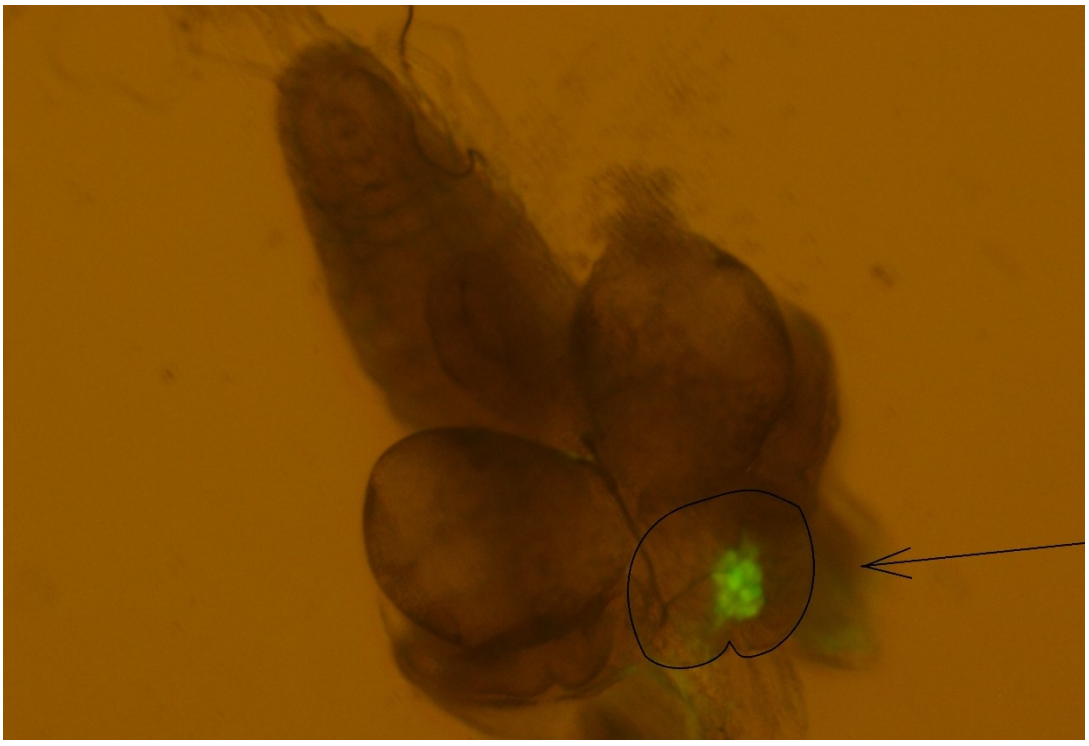

Figure 3, Ring gland (circled in black) with the CA expressing GFP, next to the central nervous system of drosophila larva.

JH is transported to its target regions, specific loci on certain genes, by two possible JH-receptors, Methoprene-tolerant (Met) or Germ cell-expressed (Gce) [17]. The JH-receptors form heterodimers which, combined with JH, act as a transcription factor on genes [17] [18]. In *Drosophila melanogaster*, a protein able to form a transcription factor complex is Taiman [17].

The ring gland highly expresses adenosine receptors [19]. Recently, JH has been found to counteract the adiponectin signalling from Adiponectin Responsive Neurons, i.e. reduce insulin sensitivity [20].

2. Aim of this Thesis:

As described in the introduction, recent findings suggest that JH could act as a link between e-Ado signalling and insulin resistance during immune response. Our aim was to find out whether a strong immune response would elevate JH-levels and to test if interrupted JH-signalling would impair the strength of the immune response.

3. Methodology:

To find out whether interrupting JH-signalling would have an effect on the strength of the immune response, we used several different genetic manipulations to block JH-signalling and we tried to measure the strength of their immune response.

3.1. Infection with parasitoid Wasps:

To trigger a strong immune response, we used the parasitoid wasp infection. Therefore, eggs were laid by flies for approximately four hours on a petri dish with fly-food. After they had done so, the petri dish was incubated for 72 hours. Right before they were 72 hours old, larvae of the right size were selected, e.g. the larvae which have just entered the third instar. These were placed on another petri dish, which is then assembled with a plastic cage containing approximately 100 female wasps, *Leptopilina boulardi*. The cage is then left under a cardboard box for one hour.

3.2. Counting Lamellocytes:

After the infection all wasps were removed from the petri dish, which was again incubated for 18 hours. Lamellocytes are counted 18 hours after the infection for two reasons. Firstly, haemocytes need time to differentiate into lamellocytes, and secondly, once they are differentiated, they attach themselves to the parasitoid wasp egg(s) as seen in figure 4. Therefore, the maximum number of lamellocytes flowing through the haemolymph is approximately 18 hours post infection.

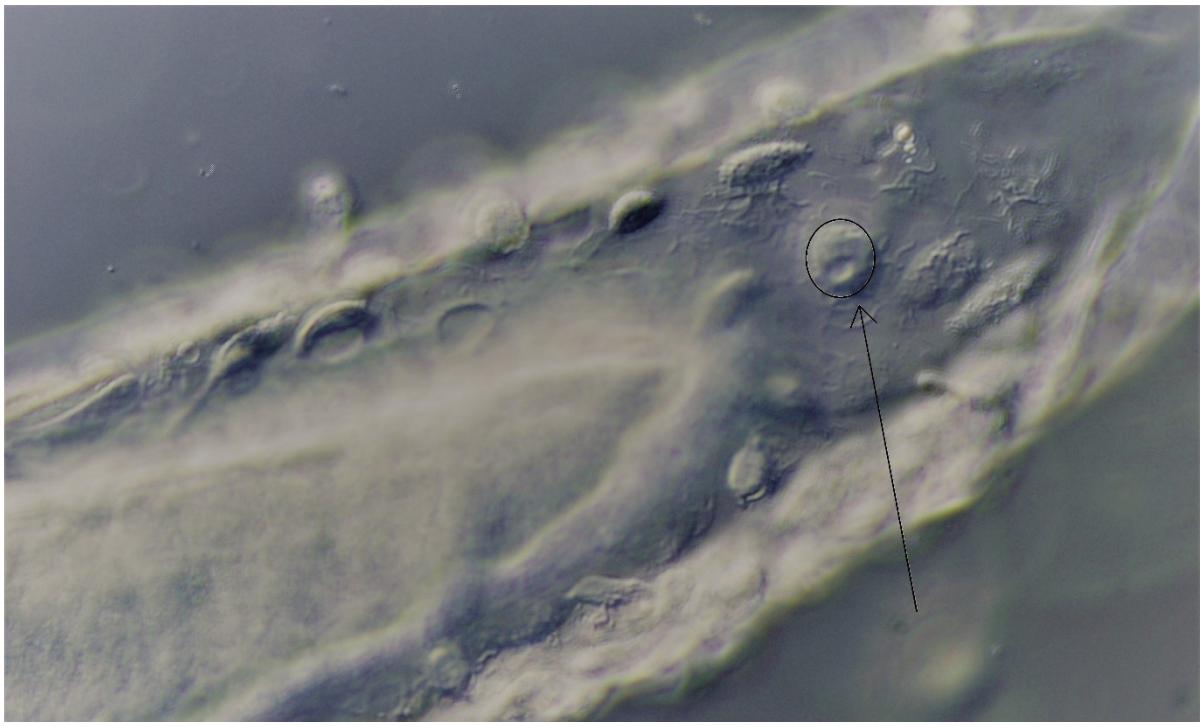

Figure 2, Lamellocytes on parasitoid wasp egg, one lamellocyte circled in black with an arrow indicating its location.

Then, the larvae were washed in Phosphate Buffered Saline (PBS) once, and one larva was placed into a 15 µL drop of PBS. The larvae were dissected using sharp forceps. It was ripped open at the lower dorsal side. As a result, a part of the gut was pushed out of the cuticle. The gut was further pulled out of the cuticle and linearized, and the number of parasitoid wasp eggs was counted. Most often, eggs are found in a specific fold of the gut. If more than 4 eggs were found, the cuticle was inverted. This inversion of the cuticle was done to ensure that the haemolymph is mixed properly with the solution, and to enable us to check the ring gland and recognise the state of the CA. Next, 10 µL of the solution with haemolymph was taken after mixing the solution by pipetting up and down. The 10 µL drop is placed onto an Improved Neubauer hemocytometer and fixed by putting a glass slide on top. The hemocytometer was incubated for five minutes, to let the lamellocytes attach to the surface of the counting chamber. Finally, the chamber was placed on a Differential interference contrast microscope and the lamellocytes were counted.

3.3. Measuring Gene Expression:

The measurement of gene expression was done by RNA isolation, cDNA production and real time quantitative polymerase chain reaction (RT-q-PCR). Therefore, four to five larvae were placed in an 1,5 mL RNA-free Eppendorf tube and frozen in liquid Nitrogen. Once all needed larvae were frozen, they were kept at -80°C until the RNA isolation was performed. The larvae were homogenized in Trizol and nucleic acids were extracted using a phenol,

chloroform, isoamyl-alcohol mixture. After purification, DNase was added and the RNA content was measured using Nanodrop. For cDNA production, RNA was diluted to yield a mixture of 2 µg RNA with oligo-dT-primers, Deoxyribonucleic acids (dNTPs) reverse transcriptase, Dithiothreitol (DTT) and 1x Frist-strand buffer. After incubation for 50 min in a 42°C warm water bath, the reverse transcriptase was deactivated by incubation in a 70°C water bath. RNase was added and the resulting cDNA was used for RT-q-PCR.

3.4. Collection of Haemolymph:

For the collection of haemolymph, a glass slide covered with parafilm was placed on ice under a microscope. Next, larvae were picked from a petri dish, washed twice in distilled water and placed on the parafilm. The cuticle of the larva was ripped open and placed on one spot on the parafilm. After this step was repeated several times and enough larvae were placed on this spot, a few µL of haemolymph could be extracted into an Eppendorf tube with a 1:1 mixture of acetonitrile and 0,9 M sodium chloride solution or with Methanol. The sample was immediately placed on ice or stored at 4°C until it was processed for mass spectrometry.

4. Results:

4.1. Relative KrH Expression:

As shown previously, elevated JH levels induce Krüppel-homologue (KrH) expression, therefore KrH-expression is a frequently used indirect readout for JH levels [17] [21]. If a strong immune response requires high JH levels, KrH-expression should also rise. We used white eyed flies as a wild type control, infected them using parasitoid wasps and 4, 8, 16 and 24 hours after the infection we collected the larval RNA. KrH-expression was measured relative to a housekeeping gene, Epithelial cadherin (E-Cad). Cadherins are known to play a major role in tissue organisation [22].

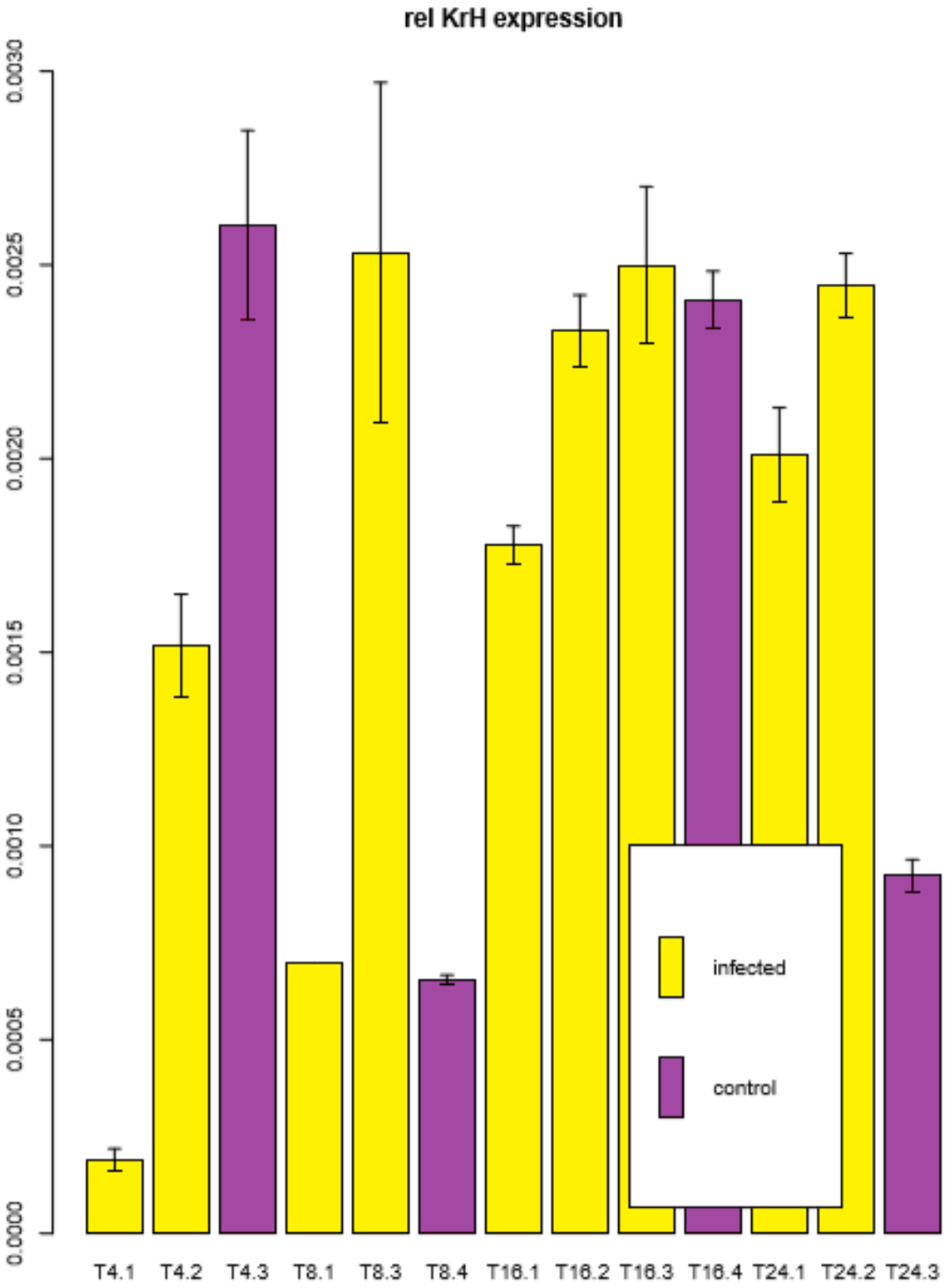

Figure 3, Relative KrH-expression ± standard deviation. The x-axis shows different samples named TA.B, for A hours post infection and B as the sample number within one timepoint TA. The y-axis shows the expression relative to E-Cad, the yellow bars show samples with infected larvae, the purple bars show samples with non-infected larvae.

As for the first timepoint, 4 hours post infection, we can clearly see that the two measurements with infected larvae differ too much to give any attention to the actual numbers. When comparing to other timepoints, one can also see that the sample T4.2 may be

representative for the level of KrH-expression. Comparing to control larvae, the infected ones show a lower expression of KrH. However, when comparing the control measurements with one another, one can also see a great difference, which implies that the KrH-expression varies a lot during early third instar. The second timepoint, 8 hours post infection, shows a similar variety, leaving no other option but to disregard them. The third timepoint, 16 hours post infection, varies less compared to the other timepoints and is worth a closer look. What it shows is that there seems to be no significant difference – or no difference at all – between control and infected larvae. The last timepoint, 24 hours post infection, shows a similar relative expression of KrH compared to the previous timepoint. The control measurement T24.3 is very low in comparison to the infected larvae, which could be interpreted in a way that infected larvae produce more JH compared to non-infected larvae. This is of course wild speculation. It can be said that due to the complexity of immune response, KrH-expression is not an appropriate readout for JH in our model. We can conclude that the relative KrH-expression is not usable and therefore we did not further attempt to measure KrH-expression.

4.2. Parasitoid Wasp Infection and Counting Lamellocytes in genetically modified Larvae:

Using another approach to examine the role of JH during immune response, we let parasitoid wasps infect larvae counted the number of differentiated immune cells (lamellocytes) in the haemolymph. To see an effect of JH during immune response, we had to interfere with JH production or signalling. In order do so, we took several different genetic approaches.

4.2.1. Met-Gce Mutation:

Our first try to interfere with JH signalling was to use JH receptor knockouts, *Met* and *gce* [17]. This line contained a Met^{27}-$Gce^{2.5k}$ knockout balanced by *FM7i, GFP* on the X chromosome. On the second chromosome was an Armadillo-Gal4 driver. Virgins of this line were crossed with male *UAS-*Met^{WT}, *UAS-*Gce^{WT} flies, which represent the wild type (WT) versions, and with *UAS-*Met^{T406Y} and *UAS-*Gce^{T272Y} flies, mutant receptors unable to bind JH. T406Y and T272Y means that the 406th and the 272nd position of the amino acid chain of the respective JH-receptors have been changed from threonine to tyrosine. The goal was to successfully rescue the Met^{27}-$Gce^{2.5k}$ knockout by expression of the wild-type JH receptor (control), and not to rescue the Met^{27}-$Gce^{2.5k}$ knockout by expression of a JH receptor, which is unable to bind JH. The obvious drawback of these crosses was that we expected that only 25% of larvae carried the desired mutation. This is because the Met^{27}-$Gce^{2.5k}$ knockout is

located on the X chromosome, therefore all female larvae also carried the wild-type X chromosome of their father. Because the *Met*^{27}-*Gce*$^{2.5k}$ knockout was balanced by *FM7i, GFP*, only half of the male larvae were expected to possess the knockout, the other half expressed GFP, as seen in figure 4 to 7:

$$\frac{Met^{27}Gce^{2.5k}}{FM7i;GFP};arm\ Gal4\♀\otimes wt;;\ UAS\ Met^{WT}\♂$$

$$\rightarrow \frac{Met^{27}Gce^{2.5k}}{wt};arm\ Gal4;UAS\ Met^{WT}\♀$$

$$\rightarrow \frac{FM7i;GFP}{wt};arm\ Gal4;UAS\ Met^{WT}\♀$$

$$\rightarrow FM7i;GFP;arm\ Gal4;UAS\ Met^{WT}\♂$$

$$\rightarrow Met^{27}Gce^{2.5k};arm\ Gal4;UAS\ Met^{WT}\♂$$

Figure 4, Crossing Met^{27}*-Gce*$^{2.5k}$ *virgins with UAS-Met*^{WT} *receptor males resulted in 4 different genotypes, the last genotype - shown in red - was the desired genotype. The phenotype of the desired larvae were males without GFP*

$$\frac{Met^{27}Gce^{2.5k}}{FM7i;GFP};arm\ Gal4\♀\otimes wt;;\ UAS\ gce^{WT}\♂$$

$$\rightarrow \frac{Met^{27}Gce^{2.5k}}{wt};arm\ Gal4;UAS\ gce^{WT}\♀$$

$$\rightarrow \frac{FM7i;GFP}{wt};arm\ Gal4;UAS\ gce^{WT}\♀$$

$$\rightarrow FM7i;GFP;arm\ Gal4;UAS\ gce^{WT}\♂$$

$$\rightarrow Met^{27}Gce^{2.5k};arm\ Gal4;UAS\ gce^{WT}\♂$$

Figure 5, Crossing Met^{27}*-Gce*$^{2.5k}$ *virgins with UAS-gce*^{WT} *receptor males resulted in 4 different genotypes, the last genotype - shown in red - was the desired genotype. The phenotype of the desired larvae were males without GFP*

$$\frac{Met^{27}Gce^{2.5k}}{FM7i;GFP};\ arm\ Gal4♀ \otimes wt;;\ UAS\ Met^{T406Y}♂$$

$$\rightarrow \frac{Met^{27}Gce^{2.5k}}{wt};\ arm\ Gal4;\ UAS\ Met^{T406Y}♀$$

$$\rightarrow \frac{FM7i;GFP}{wt};\ arm\ Gal4;\ UAS\ Met^{T406Y}♀$$

$$\rightarrow FM7i;\ GFP;\ arm\ Gal4;\ UAS\ Met^{T406Y}♂$$

$$\rightarrow Met^{27}Gce^{2.5k};\ arm\ Gal4;\ UAS\ Met^{T406Y}♂$$

Figure 6, Crossing Met^{27}-$Gce^{2.5k}$ virgins with UAS-Met^{T406Y} receptor males resulted in 4 different genotypes, the last genotype - shown in red - was the desired genotype. The phenotype of the desired larvae were males without GFP

$$\frac{Met^{27}Gce^{2.5k}}{FM7i;GFP};\ arm\ Gal4♀ \otimes wt;;\ UAS\ gce^{T272Y}♂$$

$$\rightarrow \frac{Met^{27}Gce^{2.5k}}{wt};\ arm\ Gal4;\ UAS\ gce^{T272Y}♀$$

$$\rightarrow \frac{FM7i;GFP}{wt};\ arm\ Gal4;\ UAS\ gce^{T272Y}♀$$

$$\rightarrow FM7i;\ GFP;\ arm\ Gal4;\ UAS\ gce^{T272Y}♂$$

$$\rightarrow Met^{27}Gce^{2.5k};\ arm\ Gal4;\ UAS\ gce^{T272Y}♂$$

Figure 7, Crossing Met^{27}-$Gce^{2.5k}$ virgins with UAS-gce^{T272Y} receptor males resulted in 4 different genotypes, the last genotype - shown in red - was the desired genotype. The phenotype of the desired larvae were males without GFP

The problem with these crosses was that after 3 days, there were almost no male, non-GFP larvae to be found. As an example, the following table shows the obtained phenotypes in the $\frac{Met^{27}Gce^{2.5k}}{FM7i;GFP};\ arm\ Gal4♀ \otimes wt;\ UAS\ Met^{T406Y}♂$ cross:

Table 1, resulting phenotypes of larvae, checked 72 hours after egg-laying

	GFP	Non-GFP
Female	16	9
Male	24	1

Clearly, the number of desired larvae was far below the expected 25%.

In order to find out whether the one male, non-GFP larva was carrying the mutation and was not a recombinant, we let the larvae develop. 24 h after the flies hatched, we performed similar counts. Additionally, we checked the eye-shape, hoping to prove that recombination happened. This time the numbers were similar:

Table 2, resulting phenotypes of flies after hatching

	GFP		Non-GFP	
	Bar-eyes	WT-eyes	Bar-eyes	WT-eyes
Female	53	0	0	71
Male	51	0	0	2

The two non-GFP males showed no phenotypic sign of a recombination other than their survival until adulthood. Because the desired genetically modified larvae could not be found, we decided to focus on different crosses.

4.2.2. JHAMT x GrimII:

Juvenile-hormone-acid-O-methyl-transferase (JHAMT) is an enzyme responsible for catalysing the last step of active JH synthesis. It is expressed predominantly in the CA [17] [23].

Grim II is a gene which, upon expression thereof, induces cell death (apoptosis) [24] [25].

The rationale of crossing these two genetically modified flies was to induce apoptosis in the CA. With the UAS-Gal4 system, Grim II was expressed only in the CA and we expected to observe larvae without a functional CA. The problem with this cross was very similar to the Met-Gce approach, with the few non-GFP larvae surviving until 3rd instar, all non-GFP larvae developed until adulthood. This means that for an unknown reason, the genetic manipulation failed. We tried to visually observe apoptosis in the CA, however the attempts to do so failed when trying to dissect the rind gland. It seemed that it was not as stable as the ring gland of wild type larvae, however a clear degeneration of the CA could not be observed.

One of the possible causes for the failure of the experiment could have been a recombination. Another problem could have been the expression of Gal4 in regions other than the CA. Especially the salivary gland was prawn to express Gal4.

4.2.3. Aug-Gal4 x UAS-GFP:

The demand for a clearer sign of presence or absence of a CA was obvious to us. We decided to create flies with a CA-specific Gal4 driver, August-Gal4 (Aug-Gal4), which also express UAS-GFP so that the CA can be located under a fluorescent microscope. Aug almost exclusively expressed in the CA. This allows for a much simpler observation of the CA or the lack thereof. To do so, we first crossed Aug-Gal4 flies balanced on Curly of Oster (CyO) with UAS-GFP flies. These transgenic constructs are all found on the second chromosome. 50% of the resulting larvae carried both transgenic constructs. Next, we crossed these virgins with flies carrying transgenic constructs balanced by Cyo; Red Fluorescent Protein (RFP). We expected recombination to occur in virgins carrying Aug-Gal4 and UAS-GFP, such that the next generation would carry Aug-Gal4 on the same chromosome as UAS-GFP. By selecting flies expressing GFP and picking the CyO adults, we were able to keep these flies as stock. They were expressing both GFP and RFP, making them the perfect flies for further work. For better readability I will continue referring to this stock as Aug-Gal4. The genotype of these animals is depicted in figure 8.

$$\frac{Aug\ Gal4, UAS\ GFP}{Cyo, RFP}$$

Figure 8, Genotype of the second chromosome of the larvae used for further crosses.

4.2.3.1. GrimII virgins x Aug-Gal4 males:

Crossing the Aug-Gal4 flies with UAS-GrimII virgins was a similar approach as described in section 4.2.2, but this time we would be able to clearly see whether the manipulation was successful. By a simple dissection we were able to observe a shining CA in the center of the ring gland (figure 10), or we were able to see traces of GFP in the region around the salivary gland (figure 11).

We were able to observe a lot of larvae without a functional CA, making this one of the successful models to study the influence of JH on the immune strength. Using parasitoid wasps to infect the larvae, we counted lamellocytes in genetically modified larvae and in control larvae expressing RFP (figure 9).

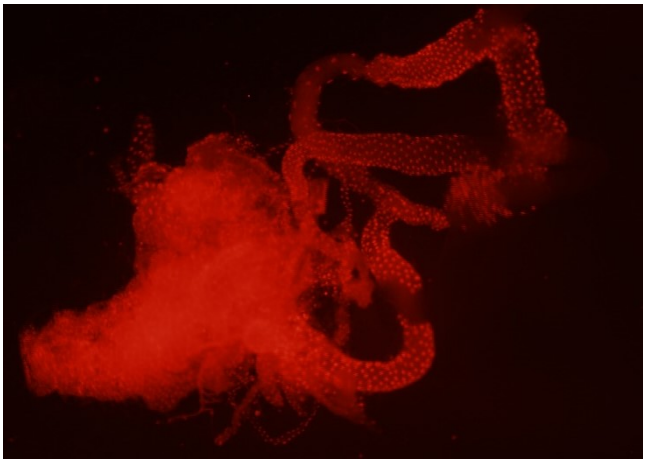

Figure 9, RFP in an inverted larva, with gut-folds outside the cuticle.

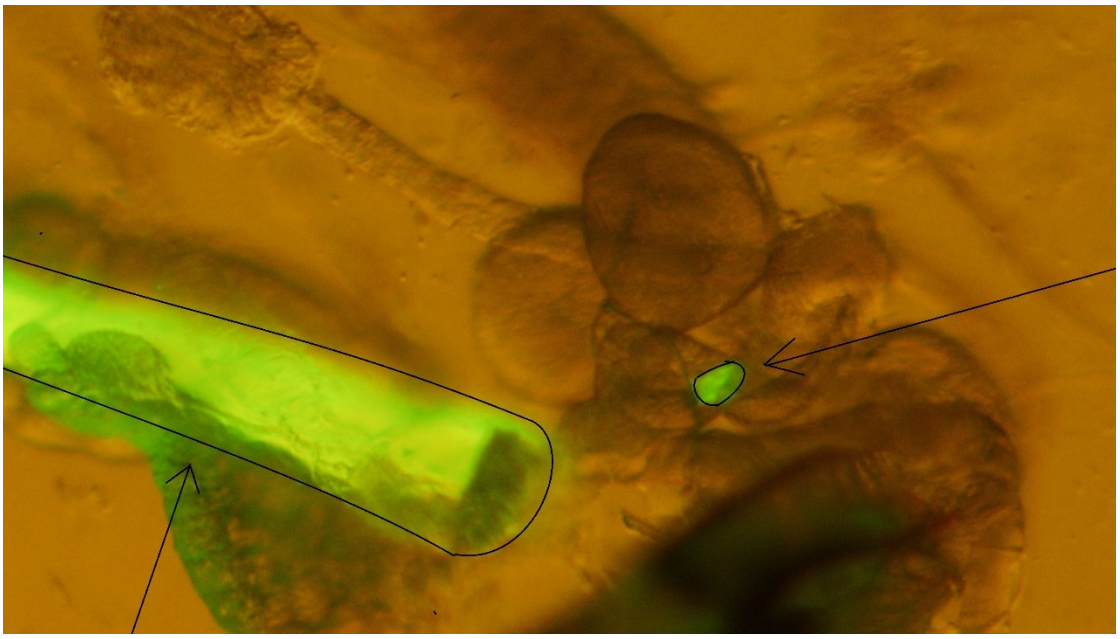

Figure 10, GFP in the ring gland (small ball-shaped tissue located in the center right) and the salivary gland (rod-shaped tissue on the left side) On the bottom right the black tissue is the mouth part.

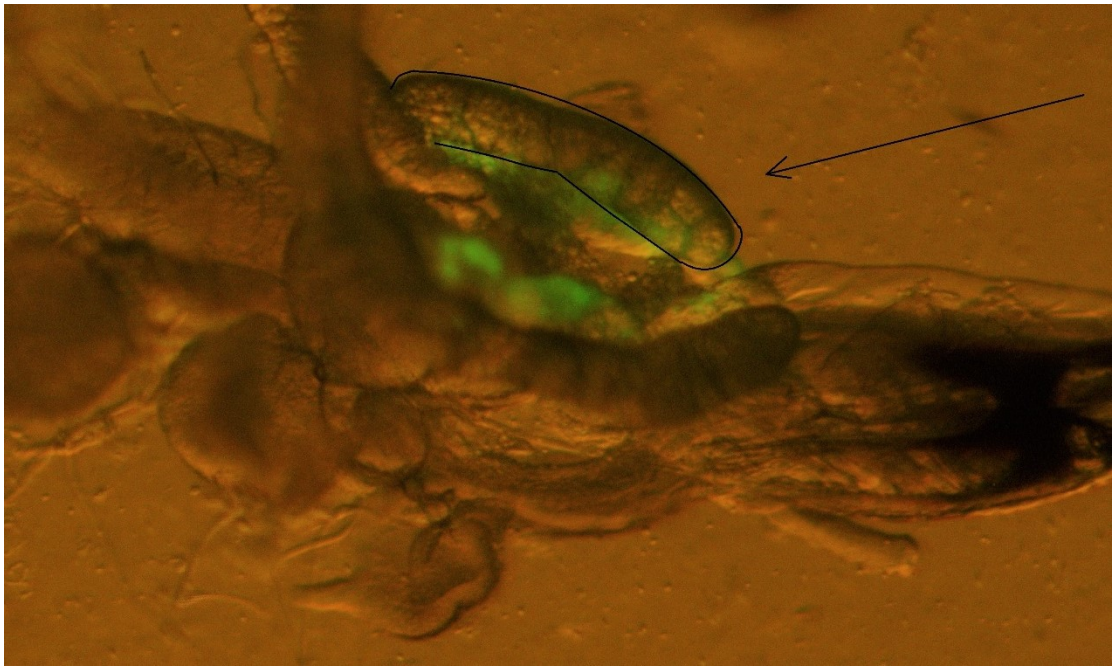

Figure 11, Traces of GFP visible in proximity to the salivary gland indicated as rod-shaped tissue, ring gland not visible. On the bottom right one can see the mouth part (black tissue).

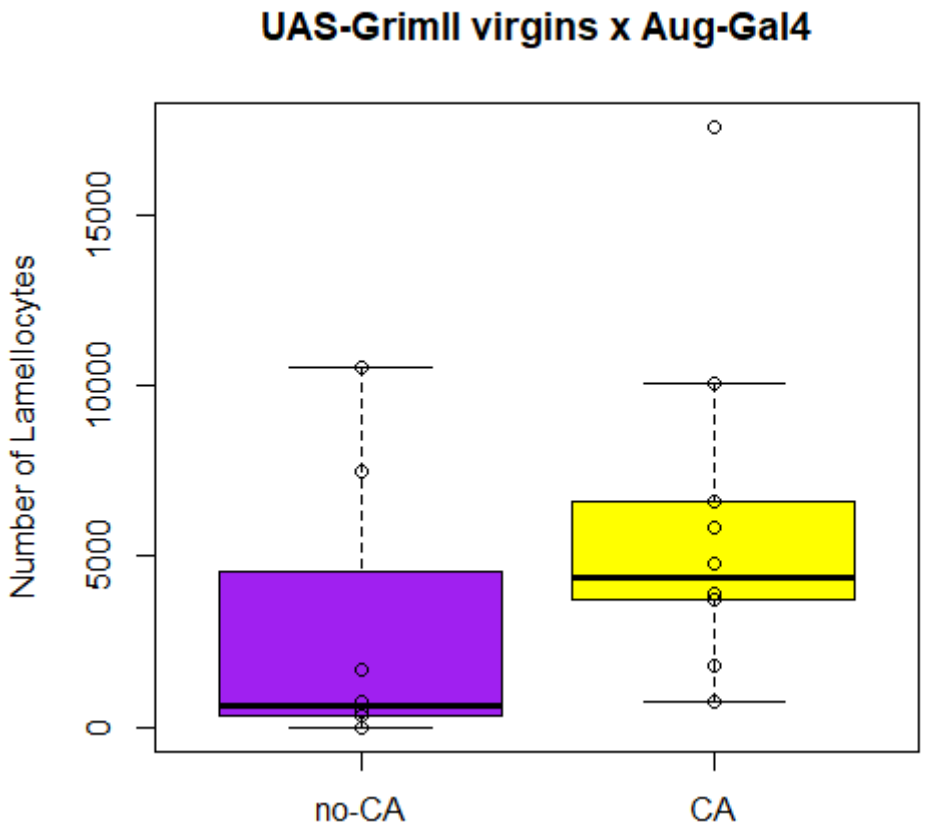

Figure 12, Number of Lamellocytes of CA-deficient larvae (UAS-GrimII females x Aug-Gal4 males) and of larvae with functional CA. Representation in a Boxplot.

Larvae were infected with parasitoid wasps and lamellocytes were counted. The results are shown in figure 12. When performing a Student´s t-Test to compare the difference in means, we obtain a p-value of 0,143. This implies that the difference between means is insignificant but is also a promising indicator that the manipulation of JH signalling did interfere with the differentiation of lamellocytes. Table 3 compares the average number of lamellocytes between genetically modified and control larvae.

Table 3, Average number of lamellocytes in of CA-deficient larvae (UAS-GrimII females x Aug-Gal4 males) and of larvae with functional CA.

	Average number of lamellocytes
CA-ablated (noCA)	2681
Control (CA)	5895

4.2.3.2. Rpr-Hid virgins x Aug-Gal4 males:

We then continued to use the approach of inducing apoptosis in CA with a combination of two other genes inducing apoptosis, reaper (rpr) and head involution defective (hid). [24]

Figure 13 shows the number of lamellocytes in both CA-ablated- and control larvae of crossing virgins of the UAS-Rpr-Hid stock with males of the Aug-Gal4 stock.

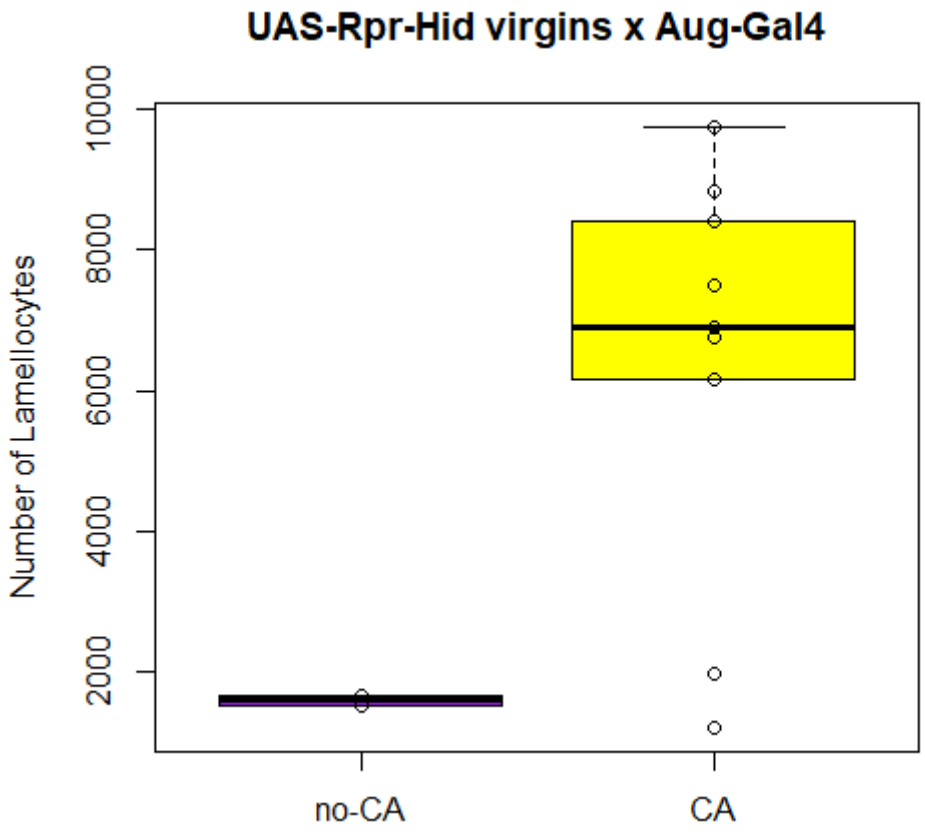

Figure 13, Number of Lamellocytes of CA-deficient larvae (UAS-Rpr-Hid females x Aug-Gal4 males) and of larvae with functional CA. Representation in a Boxplot.

Unfortunately, I was unable to count lamellocytes from more than two larvae without CA from this cross, therefore the statistical testing is trivial. The problem was that larvae without functional CA were rare and therefore a lot more experiments would have to be performed to increase statistical significance. It seems, however, that if I had counted more larvae, there would have been a difference between larvae without and with CA. Table 4 shows the large difference in means between the two groups.

Table 4, Average number of lamellocytes in of CA-deficient larvae (UAS-Rpr-Hid females x Aug-Gal4 males) and of larvae with functional CA.

	Average number of lamellocytes
CA-ablated (no-CA)	1575
Control (CA)	6435

4.2.3.3. Aug-Gal4 virgins x UAS-Rpr-Hid males:

This time we used virgins of the Aug-Gal4 stock and crossed them with UAS-Rpr-Hid males. Figure 14 shows the number of lamellocytes in both CA-ablated larvae and the control larvae expressing RFP.

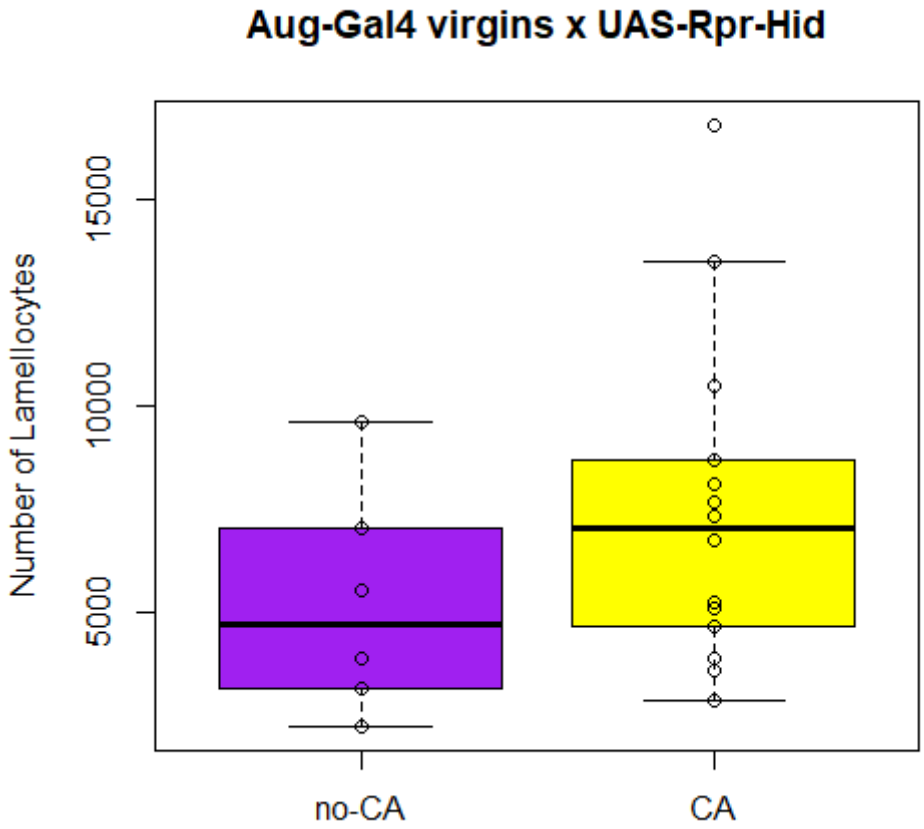

Figure 14, Number of Lamellocytes of CA-deficient larvae (Aug-Gal4 females x UAS-Rpr-Hid males) and of larvae with functional CA. Representation in a Boxplot.

The observed difference in means between the two groups is not significant, with a p-value of 0.1696. There is only a slight difference in means, but further testing would probably yield in a significant difference. Table 5 depicts the difference in means between mutans and control larvae.

Table 5, Average number of lamellocytes in of CA-deficient larvae (Aug-Gal4 females x UAS-Rpr-Hid males) and of larvae with functional CA.

	Average number of lamellocytes
CA-ablated (no-CA)	5250
Control (CA)	7479

4.2.3.4. Both Rpr-Hid crosses:

Since the two crosses described above are subject to the same genetic manipulation, we combined the numbers of both crosses to check if more counts would allow for a significant difference between CA-ablated larvae and control larvae. Figure 15 shows the results in a boxplot.

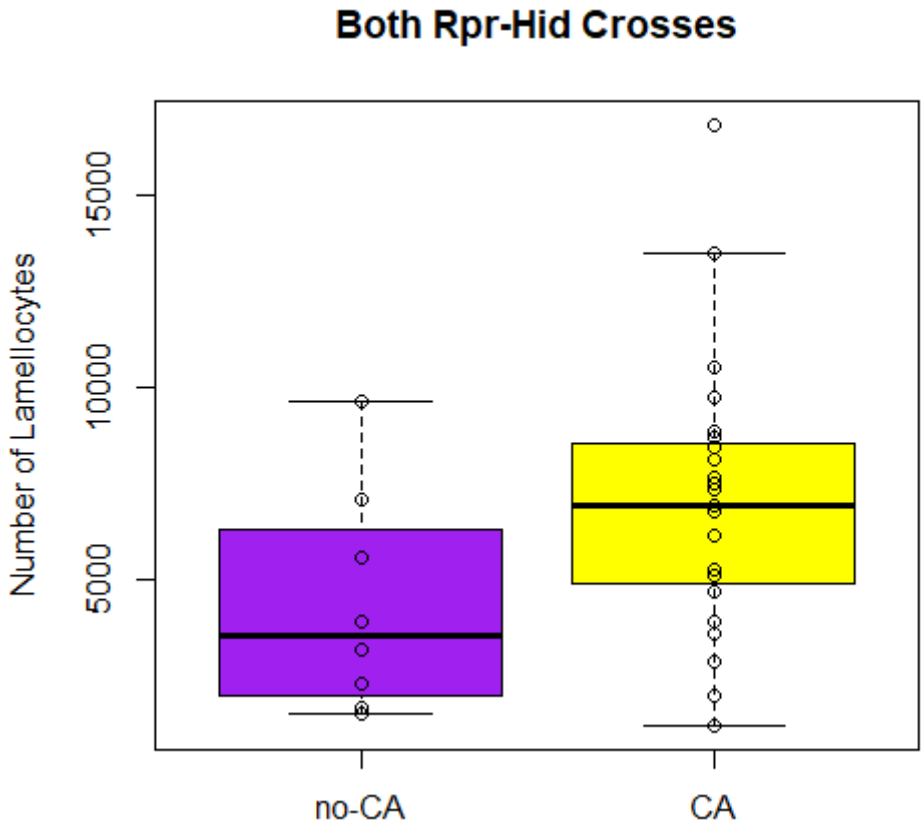

Figure 15, Number of Lamellocytes of CA-deficient larvae (Aug-Gal4 x UAS-Rpr-Hid) and of larvae with functional CA. Representation in a Boxplot.

The p-value of 0,0455 is right below the 0,05-cut-off value, indicating that there is a statistically significant difference between the test and control group. Table 6 compares the difference in means between two groups.

Table 6, Average number of lamellocytes in of CA-deficient larvae (Aug-Gal4 x UAS-Rpr-Hid) and of larvae with functional CA.

	Average number of lamellocytes
CA-ablated (no-CA)	4331
Control (CA)	7043

4.2.3.5. All crosses combined:

Because we induced apoptosis in all the genetically modified larvae described in section 4.2.3, we combined all the numbers from the three crosses and performed the t-test to see if there is a difference between CA-deficient larvae and the control larvae. The resulting numbers are summarized in figure 16.

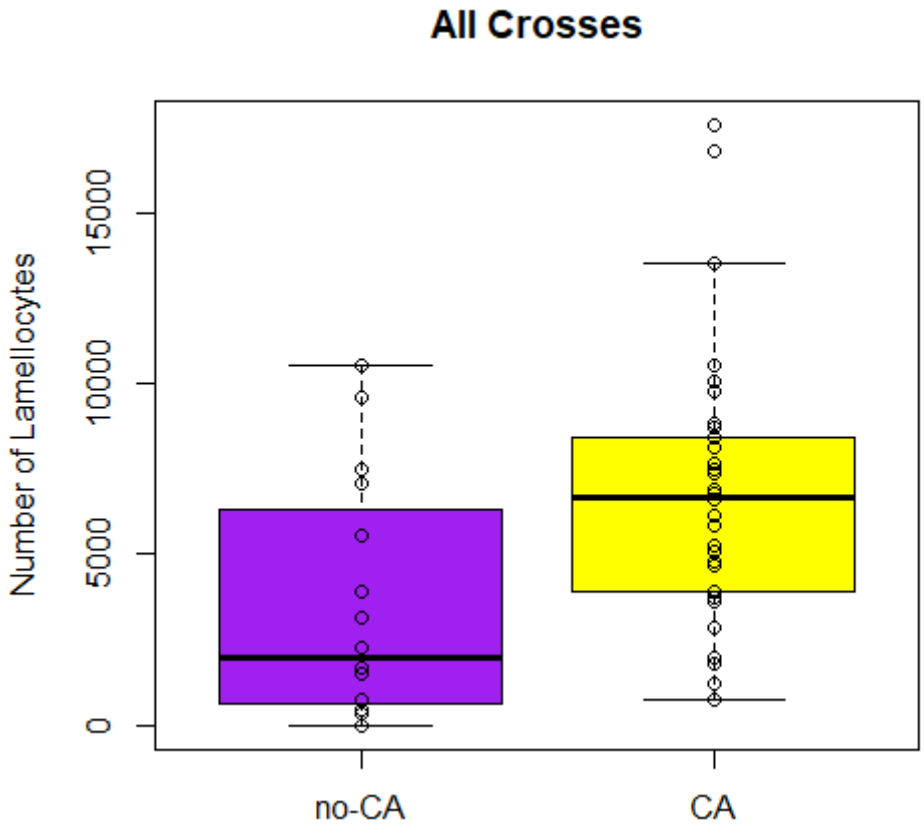

Figure 16, Number of Lamellocytes of CA-deficient larvae and of larvae with functional CA. Representation in a Boxplot.

We obtained a p-value of 0,006281, implying a high degree of significance. On average, larvae without functional CA can produce only half as many lamellocytes as those of the control group. Even if the phenotypic effect of the genetic manipulation seems to be the same, the different genetic backgrounds do not allow a definite conclusion. The combination of counts from all three crosses must not be seen as a steadfast approach to test our hypothesis, but rather as a supplementation in response to the low number of samples. Table 7 gives the average numbers of lamellocytes for CA-deficient larvae and for those with functional CA.

Table 7, Average number of lamellocytes in of CA-deficient larvae and of larvae with functional CA.

	Average number of lamellocytes
CA-ablated (no-CA)	3506
Control (CA)	6705

4.3. Direct measurement of JH using Mass-spectrometry:

Unfortunately, none of our efforts to measure JH levels in the haemolymph directly using mass spectrometry worked. This may have been due to the JH-transporters Met and Gce, which might bind JH and thereby remove JH during purification of the haemolymph done by Dr. Martin Moss.

5. Discussion:

The levels of JH during larval development have been previously measured indirectly by monitoring expression of KrH using southern blots. [26] It shows a relatively stable level of KrH during early third instar, implying a stable level of JH. [17] This can be said because KrH corresponding directly with JH levels. [17] Due to obviously occurring but unidentified problems in our RT-q-PCR experiment, we were unable to use the data and therefore did not continue with this experiment. One possible reason for the problematic RT-q-PCR results may be that the developmental stage of the larvae was too variable. This might have caused that we could not record potential tendencies in JH-levels. Although the eggs were laid within a narrow timespan (2-4h), the developmental speed of each individual larva is too variable from another larva.

Because the first tries to interrupt JH signalling (via Met-Gce knockout and JHAMT-Gal4) proved to be difficult to execute and did not promise clear results, they were discontinued. In the past, Met-Gce crosses were used to prove that Gce is a JH receptor and therefore were a very promising prospect in our experiments. [17] For unknown reasons, none of these crosses gave us the desired outcome.

Counting lamellocytes using GrimII or Rpr-Hid induced apoptosis worked very well. The flies laid enough eggs for an infection, enough larvae without functional CA could be found, and it has been very simple to check if the genetic manipulation really worked. The overall number of larvae, which were dissected and used for counting lamellocytes, was not high enough. This was due to a lack of successful infections by the parasitoid, which usually failed to infect larvae with enough eggs.

The summation of lamellocyte counts shows that functional CA is not a vital requirement for the differentiation of lamellocytes. There were still larvae which contained several thousand lamellocytes. However, larvae without a functional CA - and therefore without JH - struggled to differentiate as many lamellocytes as the control larvae did. Comparing the mean of both groups, larvae with genetically ablated CA only contained 52% of lamellocytes compared to the control group. This is a strong indicator that JH does play a role in immunity.

Comparing these results to former experiments is difficult, since the role of JH in *Drosophila* larvae during immune response has not yet been explored. In adult flies, JH has been found to act as an immuno-surpressor. [27] These findings do not necessarily contradict

our hypothesis considering the different circumstances. Their experiments were done in adult females, in which JH plays also a crucial role in enhancing oogenesis. Also, they used microbes to infect the flies, which suggests that the flies may utilize different molecular pathways to respond to the pathogen. [27]

One can compare the results of this work with other papers describing the role of e.g. extracellular Adenosine in immune response. The average difference of number of lamellocytes in larvae without a functional AdoR and its control group shows a difference of about 35 to 50 %. [5] The AdoR mutation seems to be a similar impairment for the organism and shows that the observed difference between CA-deficient larvae and control larvae as described in section 4.2.3 is similar to other experiments evaluating lamellocyte counts.

Because the expected and observed phenotypic effect of the three different crosses are the same, namely the loss of CA, we can conclude that the results of the lamellocyte counts is a promising indicator that JH does play an important role in immune response. However, there are also major drawbacks from this model. One of them is that we could observe the GFP not only in the CA, but also in the salivary glands (figure 9 and 10), meaning that the genetic manipulation maybe affected the salivary glands too.

The expression of GFP in salivary glands is frequently occurring with several Gal4 drivers [28]. What this means is that we cannot rule out the possibility that the reduced number of lamellocytes in CA deficient larvae is a consequence of inducing apoptosis in the salivary glands. In order to rule out this possibility, we would need to find a Gal4 driver targeted exclusively at the CA, or to test the effect of apoptosis in salivary glands only.

6. Conclusion:

Measuring JH-levels of the haemolymph would be of great significance for our research. We tried to monitor JH during infection directly by mass spectrometry and indirectly, by measuring the expression of KrH, one of the targets of JH. Despite numerous attempts to collect haemolymph, storing it in different solvents and using mass spectrometry to measure JH, so far none of our experiments were successful. We were able to measure KrH expression, however the results were highly variable, probably due to different developmental stages of the larvae. A possible solution for a better RT-q-PCR result would be to re-synchronize the larvae at the L2-L3 transition, which can be done by looking at the morphology of the mouth part. Perhaps this re-synchronization would allow for a more sophisticated evaluation of the JH-levels.

To further test the role of JH during infection, we used genetic manipulation to interrupt JH signalling. Manipulating the receptors of JH was not successful for us but has been shown to work previously. [17] The genetic manipulation through apoptosis of the CA worked well and we were able to observe a weaker immune response in those larvae compared to those with CA.

Several other experiments should be done to evaluate JH during immune response. One of the experiments would be to use RNA interference (RNAi) to target Met and Gce or either one of the JH receptors. If successful, this manipulation would be a great way to evaluate the role of JH during immune response. Another one should be the evaluation of survival of larvae after infection.

To put these findings into perspective: JH is among the most versatile hormones in insects, playing a role in almost every biochemical pathway. Therefore, it does not come to a surprise that interrupting one of the most important signals has effects on basically every tissue. The severity of its effect however points towards the same direction as we expected, which is that JH is produced in response to higher e-Ado levels, possibly by induction of insulin resistance.

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