University of South Bohemia in České Budějovice, Faculty of Science,
Department of Molecular Biology and Genetics

The effect of CG18446 gene on the specification of circulating immune cells in *Drosophila melanogaster*

Bachelor Thesis

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Annotation:
Investigation of the effect of CG18446 gene on the number of crystal cells and lamellocytes in the larvae of *Drosophila melanogaster* in Hop^{tum} genetic background.

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In České Budějovice 11th of December 2018

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Anna Rázková

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Table of content

Summary

The immune system plays an important role in development and protection of organism. It is crucial for surviving and further evolution of species. Therefore, it is of great importance to understand the mechanisms behind. For this purpose *Drosophila melanogaster* was selected as one of the model organisms. Not only its short life cycle, and so enabling study of mutations and its consequences in relative short time period, but also the easiness to modify the fruit fly's genome are great advantages. The conclusions driven from the detailed studying of this specie can be transferred to higher organisms like humans.

The Laboratory of Developmental Biology at JČU, Faculty of Science in Budweis is characterizing the CG18446 gene in *Drosophila melanogaster* and its influence on the immune system of the fly. For better understanding of the CG18446 gene's function, two different mutations were used ($CG18446^{Mio2952}$ and $CG18446^{ExD}$). To mimic immune challenging conditions the CG18446 alleles were used in combination with the Hopscotch gene that codes for the JAK kinase involved in JAK/STAT pathway. Hopscotch mutations that makes the JAK kinase constitutively active (Hop^{tum}) were used. This temperature sensitive mutation is working weakly at 25°C and strongly at 29°C. As JAK/STAT pathway is important for proper development of immune system especially during the immune challenging conditions, the Hop^{tum} flies are characteristic by black melanotic tumour formations caused by overproliferation of lamellocytes (Harrison, et al., 1995) (Nappi, et al., 1984). Preliminary data from Krejčí group suggested that Hop^{tum} flies lacking the CG18446 gene had fewer black melanotic tumours (dots). As the melanotic tumours may come either from lamellocytes or crystal cells the purpose of my thesis was to count the number of lamellocytes and crystal cells in Hop^{tum} flies and see if CG18446 mutation influences the formation of these immune cell types. As the very first experiment however, I also repeated the preliminary data obtained earlier by a different lab member where I analyzed the number of melanotic dots in Hop^{tum} larvae and Hop^{tum} larvae with CG18446 mutation by the heating assay (Figure 5).

During the hemocyte analysis, I also used the Hop^{tum} and Hop^{tum};$CG18446^{Mio2952}$ larvae but I also introduced red fluorescent reporters for lamellocytes or crystal cells. The L3 larvae were bled and fluorescence signal from haemocytes were analyzed under fluorescence microscope or on the counting chamber. The intensity of red shining spots within the larvae (coming out of lamellocytes) was higher in Hop^{tum} larvae than the intensity of Hop^{tum}; $CG18446^{Mio2952}$ mutant larvae, as it can be seen in

Figure 6. This finding suggests that the mutants for CG18446 gene contain less lamellocytes. In order to assess the number of crystal cells a new stock had to be constructed, stock containing the marker for crystal cells (Lozenge-RFP) and CG18446^{Mio295} mutation. Less crystal cells in CG18446^{Mio2952} mutants were found as well. Taken together, the CG18446 gene seems to affect both the formation of lamellocytes as well as the formation of crystal cells.

1 Introduction

1.1 *Drosophila melanogaster* as model organism

The fruit fly *Drosophila melanogaster* is used in various studies of diverse biological processes like genetics and inheritance, embryonic development, behaviour and learning and last but not least aging. Fundamental biological mechanisms and pathways controlling development and survival were preserved during evolution in almost all species and *Drosophila melanogaster* is suitable to study them.

The first documented research done on *Drosophila melanogaster* was performed by William Castle's group at Harvard University in 1901. Nearly a century later, the sequenced genetic code of *Drosophila melanogaster* was released in March 2000 just eleven months before human's genetic code (Anon., 15.11.2018).

The genome of fruit fly consists of approximately 15 500 genes (Elgin & et, 13.11.2018) concentrated on four chromosomes whereas the human genome consists of approximately 20 000 – 25 000 genes (Anon., 13.11.2018) concentrated on 23 chromosomes. The gene density of fruit fly's genes on its chromosomes is much higher than the one of human. However, because of our common ancestor the genomes of human and fruit fly have approximately 60% homologous genes (Elgin & et, 13.11.2018). Furthermore, while comparing human's and *Drosophila melanogaster's* genome it was determined that approximately 75% of known human disease genes match to unique *Drosophila melanogaster's* sequences (Reiter, et al., 2001). This underlines reasonability of studying *Drosophila melanogaster* with subsequent relationship to human.

Nowadays it is possible to manipulate a certain gene and increase or reduce its expression, a consequence called Gain-of-function or Loss-of-function. By Gain-of-function it is possible to overexpress genes and transgenic lines like it is done with green florescent protein (GFP) or β-galactosidase (LacZ) in specific tissues or in the pattern of specific genes

(Tweedie, et al., 2008). The Loss-of-function method allows us to study the role of individual genes in diverse biological processes.

Just very little genes in *Drosophila melanogaster's* genome code for members of the same protein class, we speak of genome with low redundancy. In contrast higher organisms usually have several paralogous genes coding for closely related proteins (Prokop, version 2).

Furthermore, regardless of the short fruit fly's life cycle the fruit fly is highly fertile. An adult female is able to lay up to 100 eggs per day. Therefore the development of mutations takes much shorter time period in comparison to vertebrates (e.g. mice, zebra-fish, …)

Fruit fly as a small animal with low complexity is very handy for observation and manipulation of cells and tissues in vivo or if not possible, fixed and stained as whole organism or just parts of it. The care of it is quite simple, it does not need much space and thus big amounts of various stocks can be kept in laboratories at low costs. Moreover the diet of *Drosophila melanogaster* is very simple and cheap. All in all, huge data can be produced at low cost and in short period of time.

1.2 Life cycle of *Drosophila melanogaster*

Drosophila melanogaster needs approximately 10 days to grow into an adult at room temperature – around 25°C. The length of the life cycle can be varied by temperature (at 18°C the speed of the development is approximately halved).

Female adult fruit flies are able to keep sperm in the *receptaculum seminis* and lay up to 750 – 1 500 eggs in their lifetime (Elgin & et, 13.11.2018). The eggs are deposited into the food down the container they are kept in.

The development (depicted in Figure 1) from fertilized cell to embryo takes approximately 21 hours (at 25°C). The 1st instar larvae (L3) are hatching after one day, followed by 2nd instar larvae (L2). The final 3rd instar larvae (L3) in the foraging stage continues to feed and crawl in food for another day. Afterwards it migrates up the container (wandering stage) to puparіate – prepupa followed by pupa. At pupa stage major reconstruction of the body is happening. First all present organs degenerate followed by restructuration into the adult shape. Ten days after egg-lay the adult fly emerges. Newly hatched adults are considered as virgins for next 8 hours since males need at least 8 hours to sexually mature. Females are capable of laying eggs 48 hours after hatching (Prokop, version 2).

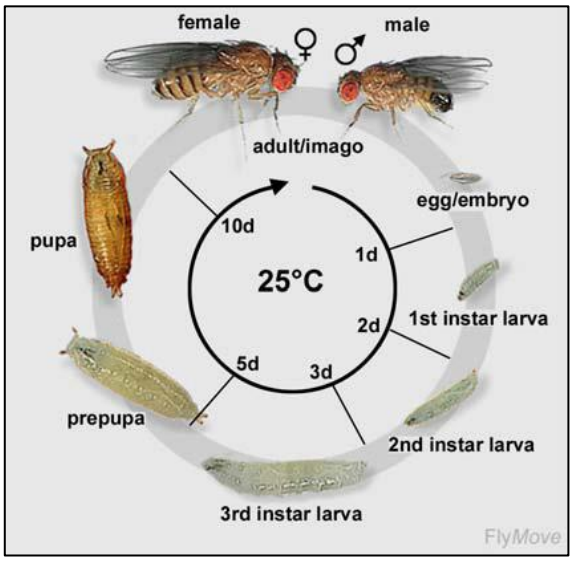

Figure 1: Life cycle of *Drosophila melanogaster* (Prokop, version 2)

1.3 CG18446 gene in *Drosophila melanogaster*

According to Flybase the location of CG18446 gene is on the right arm of the second chromosome. It is a protein coding gene with Zinc fingers C2H2 type as depicted in Figure 2. Zinc finger C2H2 transcription factors are sequence-specific DNA-binding proteins that regulate transcription. They possess DNA-binding domains that are formed from repeated Cys_2His_2 Zinc finger motifs. According to Flybase (Anon., 19.11.2018) and (Fisher & et, 2012) the CG18446 transcript expression is present in 11^{th}-12^{th} embryonic stage in plasmatocytes primordium as reporter for plasmatocytes anlage and in 13^{th}-16^{th} embryonic stage in crystal cells, plasmatocytes and in fat body. These data point towards the fact that CG18446 gene plays important role in *Drosophila melanogaster's* immune system. According to (Tanasić, 2014) the CG18446 expression was found in fruit fly's lymph gland, its organ of immune system. Further enhanced expression of CG18446 gene in the lymph gland of flies infected by a parasitic wasp was shown. The expression of CG18446 gene was also found on the wasp's eggs dissected directly from the infected 3L larvae of *Drosophila melanogaster*. The presence of plasmatocytes, lamellocytes and possibly crystal cells, of the fruit fly on the wasp's eggs was assumed (Tanasić, 2014).

Detailed study of the influence of this gene was studied in mutants. First mutation used was $CG18446^{MI02952}$ which has 3 kbp long sequence inserted in its ORF, which abolishes the translation of CG18446 gene. Second mutation – $CG18446^{ExD}$ was created by deletion of CG18446 gene, lncRNA:CR46138 and one of the promoters for CG46338 (Tanasić, 2014).

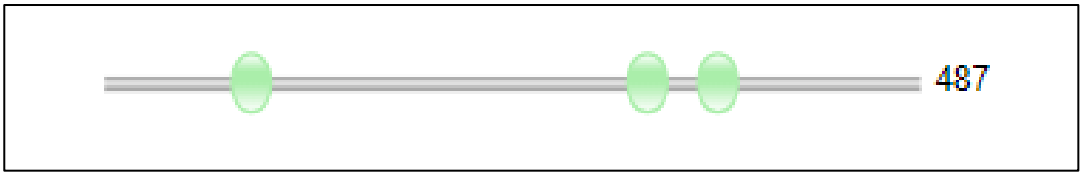

Figure 2: Predicted structure of CG18446 gene with 3 Zinc fingers C2H2-type at positions: 79-101, 315-338 and 356-379, starting with 1 and ending with 487 (Anon., 19.11.2018)

1.4 Janus kinase/Signal transducers and activators of transcription JAK/STAT

The JAK/STAT signalling pathway is one of several pathways which are employed in signals transductions important for development and homeostasis in animals. In mammals it is a signalling mechanism for a wide scale of cytokines and growth factors. JAK activation stimulates cell proliferation, differentiation, cell migration and apoptosis. It is crucial for haematopoiesis, immune development and response, stem cell maintenance and much more. Mutations which reduce JAK/STAT pathway activity affect these processes (Igaz, et al., 2001).

In vertebrates four JAKs (JAK1, JAK2, JAK3 and TYK2) and seven STATs (STAT1, STAT2, STAT3, STAT5a, STAT5b and STAT6) are discovered. In *Drosophila melanogaster* three related ligands called Unpaired (Upd), Upd2 and Upd3 are present but only one STAT protein: STAT92E which is activated by Hopscotch (Hop), the only JAK family kinase (Mankhina, et al., 2011). Upd was first identified as a secreted molecule which activates the JAK/STAT pathway during *Drosophila melanogaster* embryogenesis. Upd3 is a gene coding for a cytokine that is expressed in haemocytes and is produced in response to immune challenge (Agaisse, et al., 2003).

The Upd, Upd2 and Upd3 bind to Domeless (Dome) receptor stimulates signalling and causes the receptor associated JAK tyrosine kinase Hop to phosphorylate both itself and the cytoplasmic tail of the Dome receptor to create docking sites for the latent STAT92E proteins (as depicted in Figure 3). STAT92E is in turn phosphorylated, dimerises and translocate into the nucleus where it binds to a palindromic response element to induce target gene expression as it can be seen on Figure 3 (Bina & Zeidler, 2009).

The participation of the STAT pathway in immune responses in insects was first documented by (Barillas-Mury, et al., 1999). Since then several mutations of the JAK/STAT

pathway were performed to study and better understand the principals behind. One of them is Gain-of-function mutation. Hop$^{Tumorous-Lethal}$ (Hop^{tum}) flies hold Gain-of-function mutation in the JAK kinase – Hopscotch. This overexpression causes haemocytes overproliferation, especially overproduction of lamellocytes, which leads to invasion of normal larval tissues causing subsequent encapsulation and melanisation (Harrison, et al., 1995) (Nappi, et al., 1984). On the other hand Loss-of-function alleles result in lethality and under proliferation of diploid tissues in the larvae (Harrison, et al., 1995).

Hop^{tum} is and X-linked, dominant mutation that results in two phenotypes: i) formation of melanotic tumours, ii) temperature-sensitive lethality. At temperature higher than 25°C is recessive lethal and induces melanotic tumour formations in dominant fashion but under 25°C is recessive (Harrison, et al., 1995).

For our purposes Hop^{tum} flies were used and kept in temperatures above 25°C to ensure immune challenging conditions.

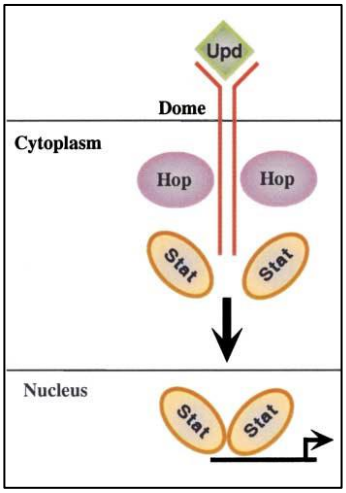

Figure 3: JAK/STAT signalling pathway (Agaisse, et al., 2003)

1.5 Immune system of *Drosophila melanogaster*

Immune system of living organisms is divided into two parts: innative and adaptive. Innative immune system is present in all metazoans and it is essential to health and life of multicellular organisms. The adaptive immune system is more complex since its mechanism contains creation of antibodies and memory cells, insuring faster recognition of pathogen and faster begin of fighting against it after renewed attack.

Detailed research of *Drosophila melanogaster* leads to understanding of its immune system. It consists of several components. During immune challenge the barrier tissue like cuticle gut and trachea are first attacked. Fruit flies also possesses a cellular immune system divided into three different blood cells groups. Circulating cells, cells settled in sessile

compartments under the segmented cuticle and cells within lymph gland. Lymph gland of *Drosophila melanogaster* is an organ consisting out of clusters of haematopoietic cells called lobes. These lobes are arranged segmentally in pairs along the dorsal vessel that runs along the anterior-posterior axis of the fruit fly. As third the humoral immune system responses to immune challenge, namely the fat body. This large multifunctional organ secrets antimicrobial peptides in response to microbial and metazoan infections.

1.5.1 ***Drosophila's* haematopoiesis**

The body of fruit fly is filled with haemolymph which consist of circulating and sessile haemocytes (Parsons & Foley, 2016). The haemolymph contains undifferentiated prohaemocyte progenitors and at least 2 differentiated blood cell types. More than 90% of the haemolymph of the fruit fly (except in early embryonic stage) is composed of differentiated macrophages known as plasmatocytes (Gold & Brückner, 2014). The rest is formed by crystal cells. In case of immune challenge or stress conditions lamellocytes are also present (Crozatier & Vincent, 2011).

The haematopoiesis of *Drosophila melanogaster* consists of three phases, embryonic, larval phase and a phase within lymph gland, see Figure 4 part A and B below (Gold & Brückner, 2014).

The first embryonic phase starts in the head mesoderm establishing the first blood cell pool. The larval phase takes place form the first larval instar till the third. In this phase quick increase in number of cells is observed. The lymph gland originating from the thoracic mesoderm of the embryo (Mandal, et al., 2004) forms a small, bilaterally paired clusters of undifferentiated blood progenitors. The progenitors cease proliferation and differentiation into 600 – 700 macrophages and low number of crystal cells (Gold & Brückner, 2014). The last phase take place within the lymph gland. Blood progenitors undergoes four divisions in embryo subsequently the rate of proliferation drops until second larval instar when the haemocytes in cortical zone (CZ) of the primary lobes have differentiated into macrophages, small number of crystal cells and occasionally lamellocytes. These expands further by proliferation. After immune challenge or under stress conditions the haemocytes are released into the circulation of the *Drosophila melanogaster* (Grigorian, et al., 2011). Under nonimmune conditions haemocytes remain in the lymph gland throughout the larval stage. This points towards the fact that all haemocytes found within the healthy larval haemocoel represent embryonic haemocytes (Holz, et al., 2003) (Wood & Jacinto, 2007). However, in case of immune challenge large amounts of haemocytes are released into the circulation from

lymph gland and the sessile cells (Lanot, et al., 2001). At the onset of pupation, the lymph gland releases large numbers of active haemocytes that have a crucial role in tissue remodelling. The released haemocytes phagocyte cells of doomed larval structures including the remainders of the lymph gland and thus no haematopoietic organs are found in adults (Lanot, et al., 2001).

Haematopoiesis in adults is known as well. In abdomen of adult flies active haematopoietic hubs can be found (Figure 4 part C). The progenitors within these hubs originate from posterior lobes of lymph gland and can produce crystal cells and plasmatocytes which shows immune response and proliferation upon infection. The differentiation within these hubs is controlled by Notch signalling pathway (Ghosh, et al., 2015).

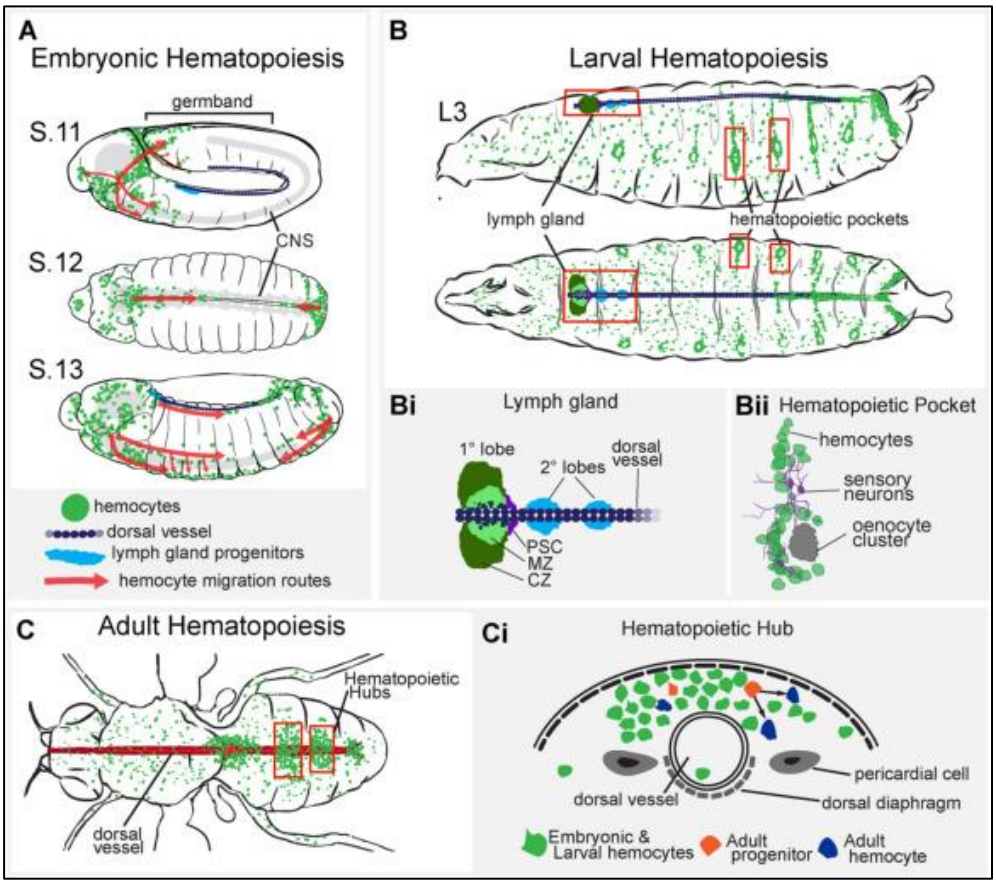

Figure 4: Stages of *Drosophila melanogaster's* haematopoiesis (Parsons & Foley, 2016) A) shows embryonic haematopoiesis, B) shows larval haematopoiesis, Bi) the lymph gland of fruit fly, Bii) the haematopoietic pockets present in larvae, C) shows the haematopoiesis in adult fruit fly, Ci) depict the haematopoietic hubs present in fruit fly adults

1.5.2 Haemocyte linages

During the blood cell proliferation two main linages of blood cells arises: the plasmatocytes and crystal cells. Over 90% of haemolymph of *Drosophila melanogaster* is formed by plasmatocytes (Tepass, et al., 1994). The rest are crystal cells. There are also another blood cells – lamellocytes. These are mostly not present in healthy organism, but differentiate after infection (Crozatier & Vincent, 2011). The lineage specification of *Drosophila melanogaster's* haemocytes is governed by several transcription factors.

1.5.2.1 Plasmatocytes

Plasmatocytes are extremely long living monocyte-like cells involved in phagocytosis of apoptic bodies and pathogens. The diameter is approximately 10 μm but they extend broad lamellipodian protrusions and form dynamic, far reaching filopodia (Parsons & Foley, 2016). For plasmatocytes specification the *Drosophila melanogaster's* GATA protein Serpent (Srp) is required. This protein is necessary for expression of two different factors, factor needed for plasmatocytes differentiation – glial-cells missing (GCM) and GCM2 and factor needed for crystal cell differentiation – Lozenge (Lz). On the other hand Lz and GCM are required only for lineage-specific differentiation (Lebestky, et al., 2000).

Plasmatocytes are very important part of *Drosophila melanogaster's* haemolymph. Beside the release of components essential for embryonic development they secrete immune defence peptides and so are an important part of the humoral immune system (Defaye, et al., 2009).

The initial recognition of the intruder is ensured by the plasmatocytes which are responsible for the immune surveillance in *Drosophila's* larvae. Upon massive release from the 1st lobes of lymph gland, they rapidly attach to the chorion of the wasp egg. After few hours lamellocytes appears in the haemolymph (Lanot, et al., 2001).

1.5.2.2 Lamellocytes

According to (Holz, et al., 2003) lamellocytes are large cells that presumably differentiate from plasmatocytes. For their differentiation the transcription factor Collier (Col) seems to be needed (Crozatier, et al., 2004).

They are not present in healthy organism and could not be found in adult or pupae (Crozatier & Vincent, 2011). They occur as a response to specific immune challenge such as infection by parasitic wasp or stress condition as increase of reactive oxygen species or mutant background in which their massive proliferation leads to formation of black "melanotic tumours" that result from encapsulation of *Drosophila melanogaster's* own tissue by

lamellocytes (Crozatier & Vincent, 2011). Lamellocytes encapsulate around invader, which is too big to be phagocytosed, and isolate it from the rest of the organism. Lamellocytes are capable of melanisation (Lanot, et al., 2001) as well as crystal cells.

1.5.2.3 Crystal cells

Crystal cells are insect-specific immune system but also play a role in wound healing (Crozatier & Vincent, 2011). Crystal cells appear in 17th embryonic stage as a cluster of crystal cells around the proventriculus. For the differentiation of crystal cells expression of the transcription factor Lz is needed. Lz is also needed for the specification of crystal cells in the lymph gland (Wood & Jacinto, 2007) and it seems to be under control of Notch signalling pathway (Lebestky, et al., 2003). On the other hand the misexpression of GCM causes crystal cells transformation into plasmatocytes (Lebestky, et al., 2000). Similarly the friend-of-GATA (FOG) homologue U-shaped has been shown to antagonize crystal cell development in the embryo (Fossett, et al., 2001).

Crystal cells contain large crystalline inclusions. They produce zymogen phenol oxidase (PO) which is activated by cleavage during the immune response cascade – known as melanisation. Melanisation occurs not only during encapsulation of parasites but also during healing process (Parsons & Foley, 2016).

2 Aims of the thesis

1. Comparing the melanisation abilities of larvae with Hop^{tum} allele alone and larvae where Hop^{tum} allele is combined with homozygous mutation in CG18446 gene (boiling assays and fluorescence microscopy using the Msn-RFP reporter for lamellocytes).

2. Quantification of circulating lamellocytes in larvae with Hop^{tum} allele alone and larvae where Hop^{tum} allele is combined with two different homozygous mutations in CG18446 gene ($CG18446^{Mio2952}$ and $CG18446^{ExD}$). This will be achieved by two methods - (1) counting lamellocytes on Neubauer improved counting chamber and (2) quantification of Msn-RFP positive cells under confocal microscopy.

3. Quantification of crystal cells in larvae with Hop^{tum} allele alone and larvae where Hop^{tum} allele is combined with homozygous mutation in CG18446 gene ($CG18446^{Mio2952}$). This will be achieved by counting crystal cells on Neubauer improved counting chamber. For this purpose I will need to generate the Lz-Gal4, UAS-RFP;$CG18446^{Mio2952}$ fly stock.

3 Material and methods

3.1 Fly techniques

All flies stocks were kept in incubators either at 18°C, 25°C or 29°C with natural day/night cycles. All flies were fed with same standard diet.

The food was containing:

- 9 g of agar
- 150 g of glucose
- 160 g of cornmeal
- 30 g of yeast
- 50 mL of methylparaben
- 1900 mL of water
- On top of the food additional grains of dry yeast.

Following *Drosophila* stocks were used:

- CG18446^{Mlo2952} (Minos transposon insertion on II chromosome, mutant for CG18446 gene)
- CG18446^{Mlo2952} /CyOGFP;MsnF9>mCherry/TM3
- CG18446^{ExD} (deletion of CG18446 gene and genes found nearby the gene on II chromosome, mutant for CG18446 gene)
- Hop^{tum}/FM7 (point mutation of Hopscotch gene on the X chromosome, mimics the infection conditions, with FM7 balancer)
- Hop^{tum};MsnF9>mCherry (point mutation of Hopscotch gene on the X chromosome, mimics the infection conditions, with marker for lamellocytes)
- Hop^{tum}/FM7;CG18446^{ExD}
- Hop^{tum};CG18446^{MI02952}/cyo
- Hml-GFP (reporter for all mature haemocytes)
- CG18446^{Mlo2952},Hml-GFP
- MsnF9>mCherry (reporter for lamellocytes)
- Tub-Gal80/FM7;Sco/CyO
- y/FM7;Bil/CyO
- y/FM7;CG18446^{Mlo2952}/Sco
- Lz-RFP,Stinger/FM7;+/CyO

- Lz-Gal4,UAS-red Stinger (reporter for crystal cells)
- Lz-Gal4,UAS-red Stinger;CG18446^{Mlo2952}
- w^{11118} (used as controls)

3.2 Composition of fly stock

Creation of CG18446^{Mlo2952}>Lz-RFP stock was done. Since the expression of the transcription factor Lz is needed for specification of crystal cells (as stated above in 1.5.2.3), construction of stock tagged with red-fluorescence-protein (Red Stinger, form of RFP) on this gene was performed according to scheme below.

♂ CG18446^{Mlo2952} x ♀TubGal80/FM7;Sco/CyO ♀Lz-Gal4,UAS-Red Stinger x ♂y/FM7;Bil/CyO

♂ y/FM7; CG18446^{Mlo2952}/Sco ♀ Lz-Gal4, UAS-Red Stinger/FM7;+/CyO

Lz-Gal4, UAS-Red Stinger/FM7; CG18446^{Mlo2952}/CyO

3.3 Heating larvae to activate phenol oxidases (in crystal cells and lamellocytes)

Crawling L3 larvae were selected and washed. Each larvae was placed onto a bottom of PCR tube and the PCR program was run. The program heated the larvae 15 minutes at 65°C. The larvae was aligned on a white piece of paper and pictures on the binocular microscope was done. The larvae that climbed above the heated collar of the PCR machine were discarded.

3.4 Analysis of circulating haemocytes – preps for confocal microscope

Bleeding larvae for analysis of circulating haemocytes

1. L3 larvae were taken out gently from the vial or bottle to prevent release of haemocytes from the sessile compartments into the circulation.
2. Larvae were transferred to small sieve and washed in 3 big beakers with water (to get rid of all yeast sticking to the larva).
3. Larvae were transferred to an Eppendorf tube with a piece of wet tissue, left 30 minutes on room temperature (to get any sessile cells back to their compartments).

4. 15 µL of Shneider medium + NucBlue was put on in a glass shallow dish (one drop of NucBlue in 0,5 mL media).
5. One larvae was very gently taken out of the tube and by holding its cuticle on the ventral side (belly) by two tweezers a hole was pinched in its abdomen without touching the gut or squeezing the larvae too much. Left bleed for about 20 seconds.
6. The larvae was discarded.
7. The medium was immediately mixed up and down about 6 times and transferred on a cover slip in a humidified chamber.
8. The cells were let to attach for 30 minutes.
9. The cover slip was transferred into a 6-well plate with 4% formaldehyde in PBS to fix the cells for 30 minutes, occasionally gently mixed.
10. The formaldehyde solution was taken out and the slide was washed 5 minutes in PBS (the PBS was not put directly on the coverslip to prevent washing off the attached cells, but slowly in the opposite side of the dish).
11. A small drop of mounting media was put on a clean microscope slide, coverslip was placed on it (cell down) and after 15 minutes sealed with nail polish.
12. The haemolymph was analysed under confocal microscope.

3.5 Analysis of circulating haemocytes – preps for immediate counting

Bleeding larvae for analysis of circulating haemocytes

1. L3 larvae were taken out of the vial or bottle very gently because any mechanical pressure could release haemocytes from the sessile compartments into the circulation and spoil the data.
2. Larvae were transferred to small sieve (for washing embryos), then washed in 3 big beakers with water (to get rid of all yeast sticking to the larva).
3. Larvae were transferred to an Eppendorf tube with a piece of wet tissue, left 30 minutes on room temperature (to get any sessile cells back to their compartments).
4. 15 µL of Shneider medium + NucBlue was put on in a glass shallow dish (one drop of NucBlue in 0,5 mL media).
5. One larvae was very gently taken out of the tube and by holding its cuticle on the ventral side (belly) by two tweezers a hole was pinched in its abdomen without touching the gut or squeezing the larvae too much. Left bleed for about 20 seconds.
6. The larvae was discarded.

7. The medium was immediately mixed up and down about 6 times and transferred on a Neubauer improved counting chamber in a humidified chamber.
8. Cells were counted under fluorescence microscope.

3.6 Methods, microscopy and statistics

For analysis of the larval haemolymph two methods were used. Firstly counting on Neubauer improved counting chamber (for preparation see section 3.5) under fluorescence microscope and secondly preps were prepared (see section 3.4) and subsequently for each drop 3 random placed pictures were made on Olympus confocal microscope. Pictures were then evaluated using FLUOVIEW version 4.2.

Graphs, tables and statistics were done in Microsoft Excel 2016. Two tailed student's t-test was used for comparison of each analysis. P-value > 0,5*; P-value < 0,5**; P-value < 0,1***; P-value < 0,05****; P-value < 0,01*****; P-value < 0,001******.

4 Results

4.1 Heating of Hop^{tum} and Hop^{tum};$CG18446^{Mlo2952}$ L3 larvae

Temperature sensitivity of the Hop^{tum} mutation leads to a mild overproliferation of lamellocytes at 25°C and very high proliferation of lamellocytes at higher temperatures (29°C). It was found out that after heating L3 larvae of these mutants black melanotic tumours appears. These tumours are formed by the larvae's own tissue surrounded and melanised by overexpressed lamellocytes (Harrison, et al., 1995).

To take the advantage from this fact the L3 larvae of Hop^{tum} and Hop^{tum};$CG18446^{Mlo2952}$ mutants kept at 29°C during their life were heated at 65°C for 15 minutes (see section 3.3) and pictures were taken under light microscope (Figure 5).

When comparing the frequency of appeared black spots on the larval body between these two mutants the frequency in Hop^{tum} larvae appeared higher than in Hop^{tum} flies with mutation in CG18446 gene. This was an exciting result that we decided to properly verify. As the melanotic signal can come both from lamellocytes or from crystal cells (both cell types express phenol oxidases) we needed to quantify both the number of lamellocytes and the number of crystal cells in the larvae with the above described genotypes.

Hop^{tum} x MsnF9>mCherry

Hop^{tum};CG18446^{Mio2952} x CG18446^{Mio2952};MsnF9>mCherry

Figure 5: Heating assay of L3 larvae of containing the Hop^{tum} mutation alone or in combination with CG18446^{Mio2952} mutation. Larvae were kept at 29°C before analysis.

4.2 Examining the fluorescent signal derived from lamellocytes in L3 larvae of Hop^{tum} and Hop^{tum};CG18446^{Mio2952} larvae

After crossing Hop^{tum} mutants with MsnF9>mCherry stock we were be able to see the red shining lamellocytes and hence the melanotic tumours under fluorescence microscope (Figure 6).

Firstly the pictures of Hop^{tum} L3 larvae (Figure 6 two upper left pictures) were taken followed by Hop^{tum};CG18446^{Mio2952} L3 larvae under the same exposition time (Figure 6 the two upper right pictures). The captured light from Hop^{tum};CG18446^{Mio2952} larvae was less intense than the light from Hop^{tum}. When comparing pictures taken from Hop^{tum};CG18446^{Mio2952} with enhanced exposition time (Figure 6 the two lower right pictures) it can be seen that the light is emitted from approximately same compartments in larvae of Hop^{tum};CG18446^{Mio2952} mutants as in larvae of Hop^{tum}.

The significant difference in emitting made clear the decreased lamellocyte derived fluorescent signal in CG18446 mutants. Therefore it was expected that the number of these

cells should be lower in CG18446 gene mutants when counting them directly on a cell counting chamber or when analysing the fluorescence signal on confocal microscope after bleeding the larvae.

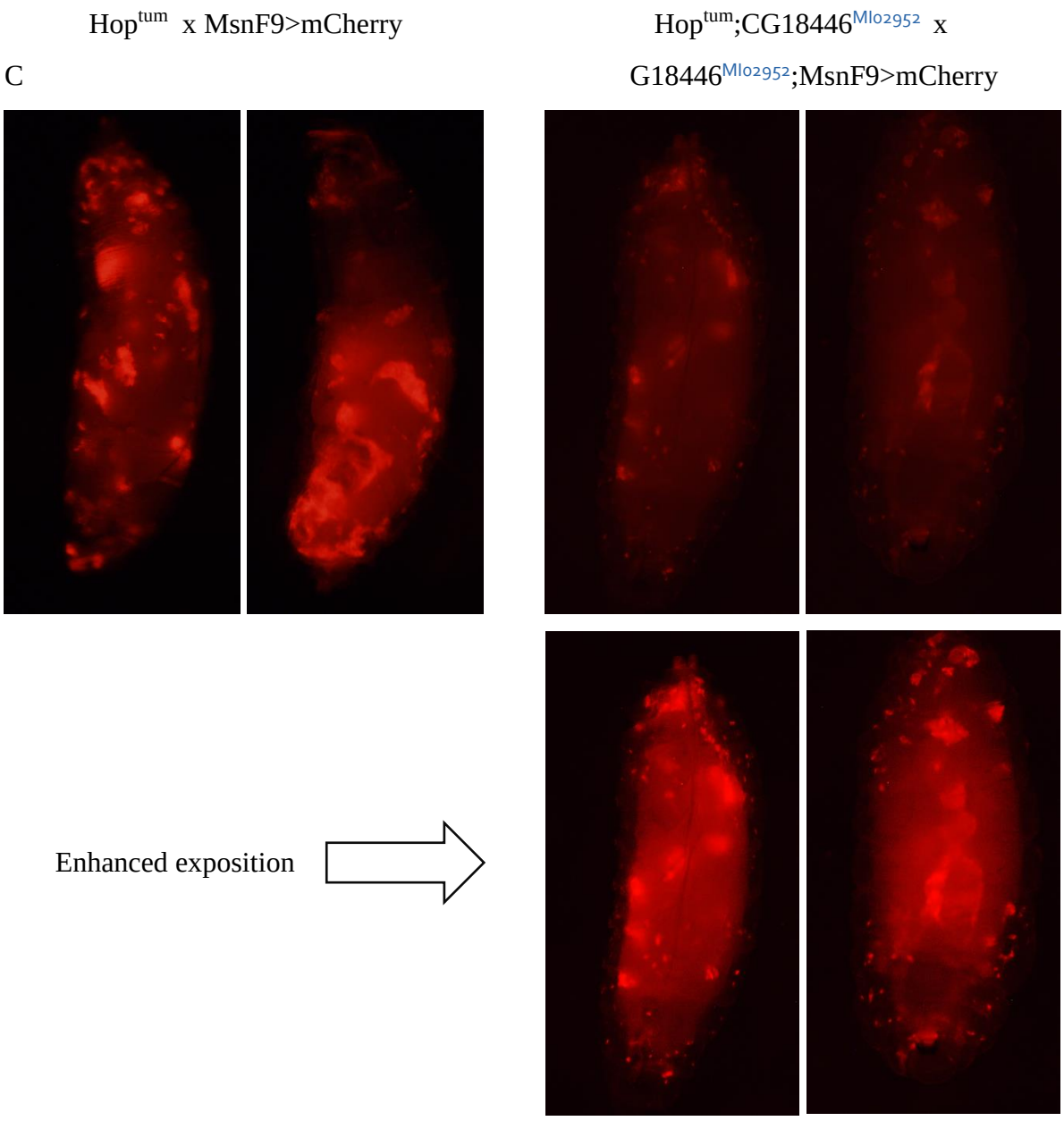

Figure 6: Pictures of Hop^{tum};MsnF9-mCherry and Hop^{tum};CG18446^{Mi02952} x CG18446^{Mi02952};MsnF9-mCherry flies under fluorescence microscope. The two pictures on the bottom right show same larvae as above but under longer exposition.

4.3 Influence of CG18446^{Mio2952} mutation on the total number of circulating immune cells and the number of lamellocytes in Hop^{tum} stock

For the analysis of the number of immune cells in Hop^{tum} and Hop^{tum};CG18446^{Mio2952} L3 larvae two different methods were used. Firstly counting on Neubauer improved counting chamber under fluorescence microscope (once) and secondly taking 3 randomly placed pictures of larval haemolymph under confocal microscope (twice), see section 3.5 and 3.4 for protocols. The difference in these two methods is following: when counting on the Neubauer improved counting chamber the total number of haemocytes present in haemolymph can be estimated, whereas by analysis on confocal microscope only the ratio between lamellocytes and all haemocytes can be calculated (as it is not possible to image the whole drop but just random parts of it).

The Figure 7 shows the number of all haemocytes present in L3 larvae of Hop^{tum} and Hop^{tum};CG18446^{Mio2952} mutants. Difference in the two medians is observed, for Hop^{tum} 13.125 and for Hop^{tum};CG18446^{Mio2952} 16.125 (data obtained by counting on Neubauer improved chamber). Simultaneously difference in number of observed lamellocytes is observed. According to Figure 8 the median for Hop^{tum} is 4.725 and for Hop^{tum};CG18446^{Mio2952} is 3.075. These data correspond with data obtained from previous experiments, CG18446 mutants contained lower number of fluorescent lamellocyte derived signal when comparing with larvae with no mutation on that gene (Figure 8). Correspondingly, also the ratio between lamellocytes and total number of cells for Hop^{tum};CG18446^{Mio2952} is significantly lower than the ratio of Hop^{tum} larvae (35,83% lower). For the analysis on Neubauer improved counting chamber 16 larvae for each genotype were taken.

Taking pictures of Hop^{tum} and Hop^{tum};CG18446^{Mio2952} mutants on confocal was two times performed. The data obtained from both analysis of haemocytes from pictures taken on confocal microscope are summarized in Figure 10 and Figure 11. The results are expressed as the average ratio between the number of lamellocytes and the number of all haemocytes analyzed in each confocal picture. In this analysis 18 larvae of CG18446^{Mio2952} and 20 larvae of Hop^{tum} were bled, a drop containing all haemocytes was fixed on a slide, then 3 random confocal pictures were taken for each drop and the ratio of lamellocytes to the total number of cells was calculated for each picture and each larvae. The results again confirm that there is lower percentage of lamellocytes present in flies with CG18446^{Mio2952} mutation.

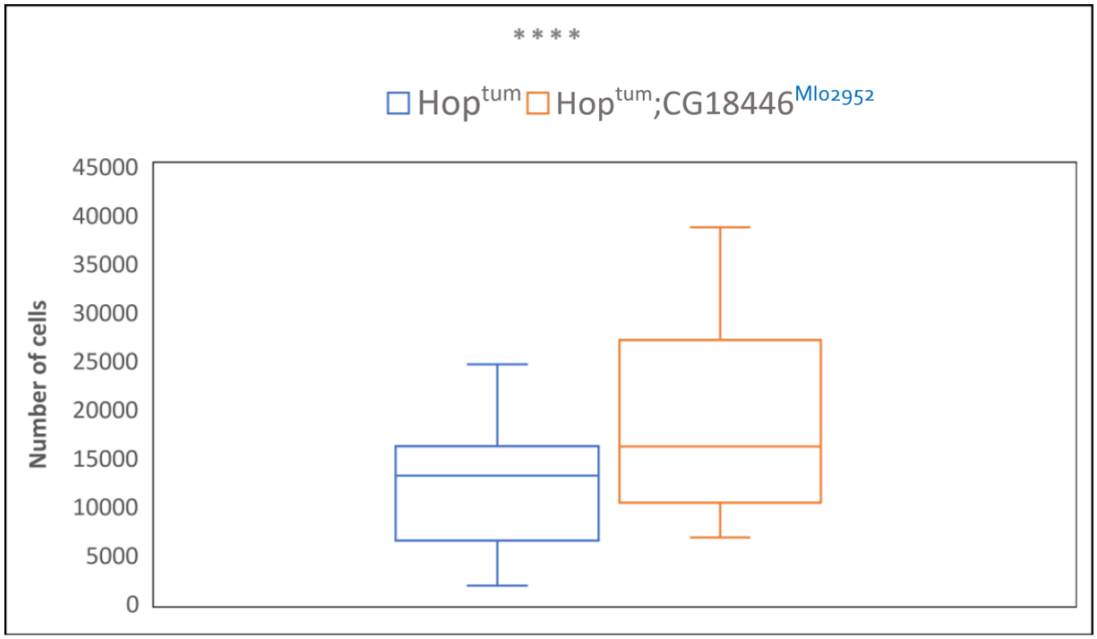

Figure 7: Total number of haemocytes present in L3 larvae of Hop^{tum} and $Hop^{tum};CG18446^{Mio2952}$ mutants counted on Neubauer improved counting chamber. Median for Hop^{tum} is 13.125, median for $Hop^{tum};CG18446^{Mio2952}$ is 16.125. The P-value is 2,76e-2 (P-value < 0,05)

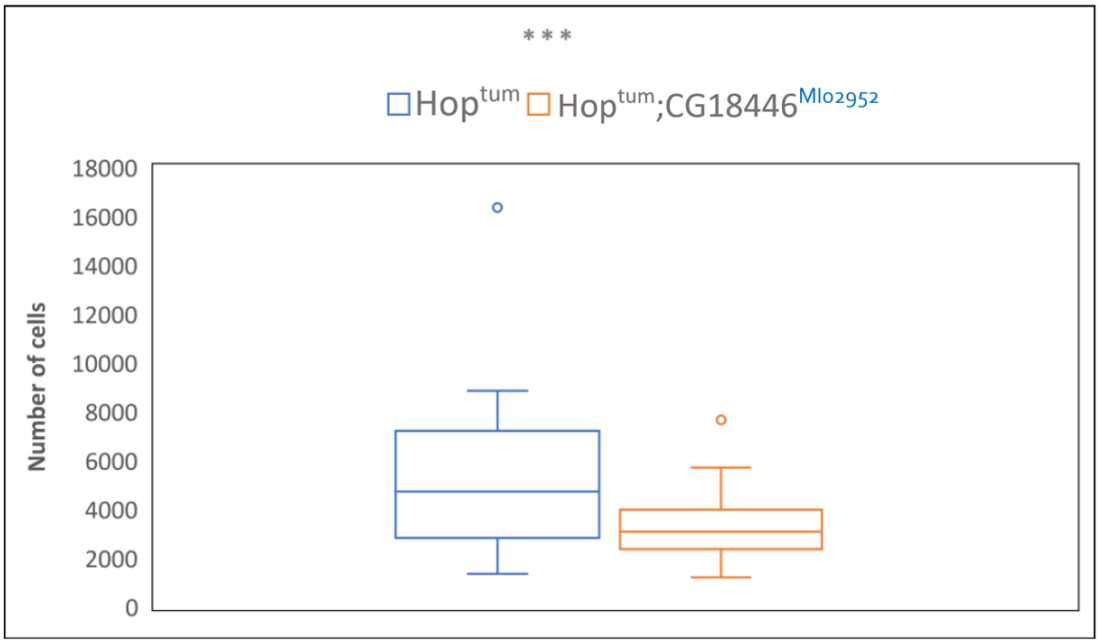

Figure 8: Number of lamellocytes present in L3 larvae of Hop^{tum} and $Hop^{tum};CG18446^{Mio2952}$ mutants counted on Neubauer improved counting chamber. Median for Hop^{tum} is 4.725, median for $Hop^{tum};CG18446^{Mio2952}$ is 3.075. The P-value is 6,05e-2 (P-value < 0,1)

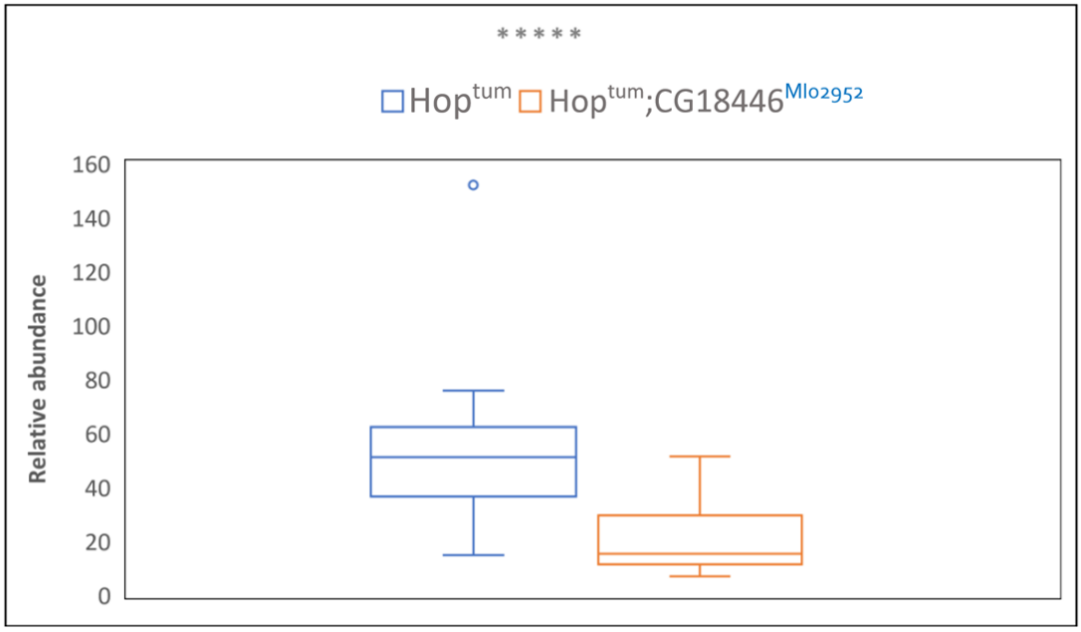

Figure 9: The ratio of the number of lamellocytes to the number of all haemocytes present in larval haemolymph for Hop^{tum} and Hop^{tum};$CG18446^{Mi02952}$ counted on Neubauer improved counting chamber. The median for Hop^{tum} is 51,14% and the median for Hop^{tum};$CG18446^{Mi02952}$ is 15,31%. The P-value is 1,89e-3 (P-value < 0,01)

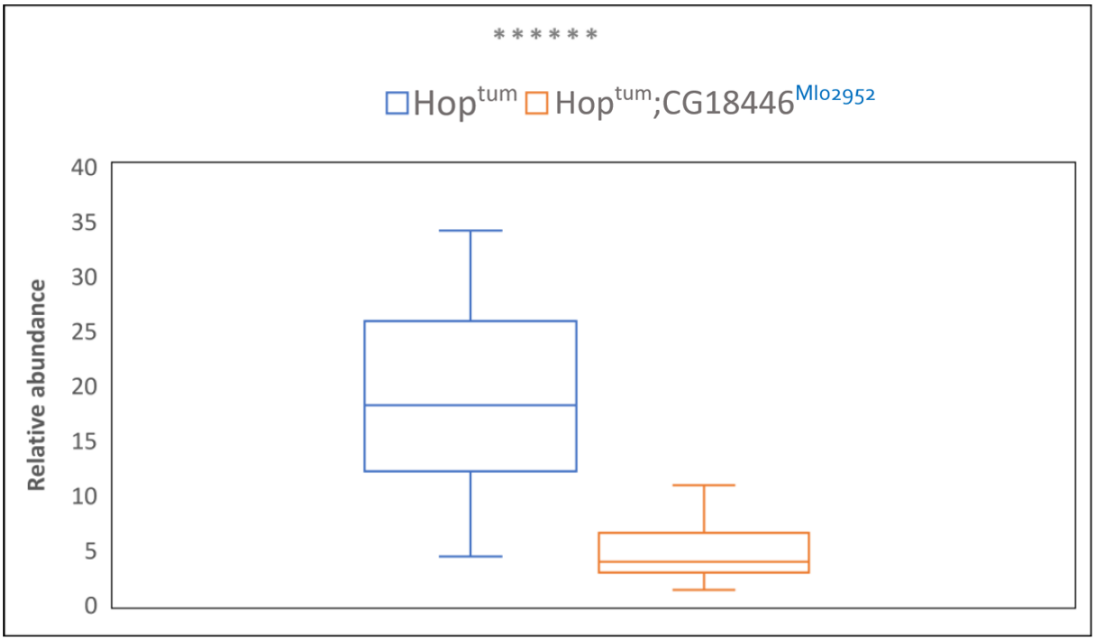

Figure 10: The ratio of the number of lamellocytes to the number of all haemocytes present in larval haemolymph for Hop^{tum} and Hop^{tum};$CG18446^{Mi02952}$ calculated from pictures taken on confocal microscope, first measurement. The median for Hop^{tum} is 18,10% and 3,94% for Hop^{tum};$CG18446^{Mi02952}$. The P-value is 1,00e-5 (P-value < 0,001)

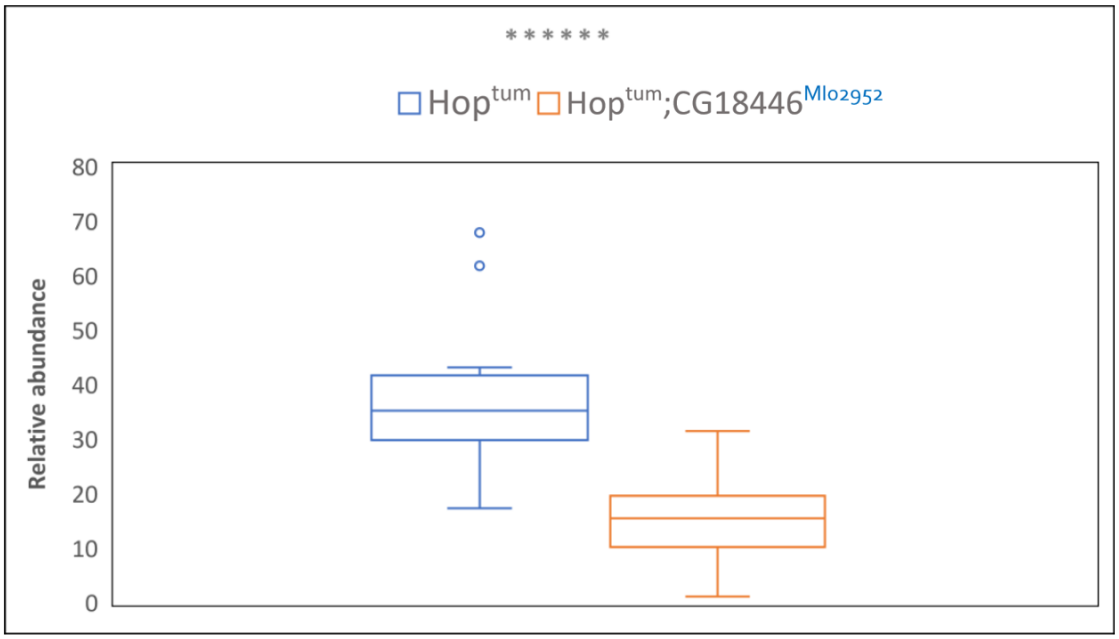

Figure 11: The ratio of the number of lamellocytes to the number of all haemocytes present in larval haemolymph for Hop^{tum} and Hop^{tum};$CG18446^{Mi02952}$ calculated from pictures taken on confocal microscope, second measurement. The median for Hop^{tum} is 37,78% and 13,54% for Hop^{tum};$CG18446^{Mi02952}$. The P-value is 6,66e-10 (P-value < 0,001)

4.4 Influence of $CG18446^{ExD}$ mutation on the total number of circulating immune cells and the number of lamellocytes in Hop^{tum} stock

To confirm the influence of CG18446 gene on the immune system of fruit fly exactly the same experiments and analysis as with $CG18446^{Mi02952}$ larvae were done on larvae with another mutation in this gene, the $CG18446^{ExD}$. The $CG18446^{ExD}$ mutants lack the whole CG18446 gene which should result to the same phenotype as $CG18446^{Mi02952}$ mutants.

Below in Figure 12 the number of all haemocytes present in larval body in Hop^{tum} and Hop^{tum};$CG18446^{ExD}$ mutants is presented from analysing of the haemolymph on the Neubauer improved counting chamber. The two medians for Hop^{tum} and Hop^{tum};$CG18446^{ExD}$, 25.800 and 10.500 respectively, are significantly different (difference of 15.300 cells). This suggest that the CG18446 is needed to establish proper number of haemocytes in larval haemolymph.

Figure 13 present the number of lamellocytes in larval body of Hop^{tum} and Hop^{tum};$CG18446^{ExD}$ mutants. The respective medians for Hop^{tum} 5.625 and Hop^{tum};$CG18446^{ExD}$ 3.975 are in accordance with results presented till now in this thesis, namely the number of lamellocytes is lower in $CG18446^{ExD}$ mutants than in larvae without mutation. The ratios obtained from counting on Neubauer improved counting chamber are

presented in Figure 14. For the analysis on Neubauer improved counting chamber 20 larvae for each genotype were bled and analysed.

The ratio obtained from analysis of the pictures captured under confocal microscope are presented in Figure 15. The median for Hop^{tum} is 29,41% and for Hop^{tum};$CG18446^{ExD}$ 15,29%. These results again confirm that the CG18446 mutant develops lower number of lamellocytes. Nine larvae from each genotype were bled, the haemocytes were fixed on slide and 3 random pictures pro larvae were taken.

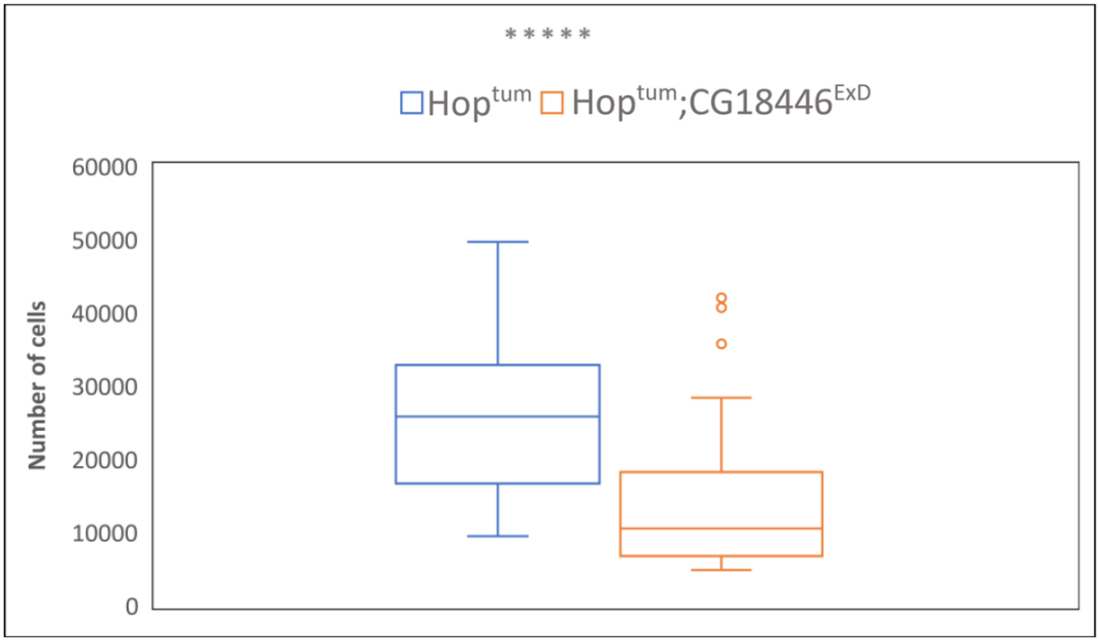

Figure 12: Number of haemocytes present in L3 larvae of Hop^{tum} and Hop^{tum};$CG18446^{ExD}$ mutants counted on Neubauer improved counting chamber. Median for Hop^{tum} is 25.800, median for Hop^{tum};$CG18446^{ExD}$ is 10.500. The P-value is 7,05e-3 (P-value < 0,01)

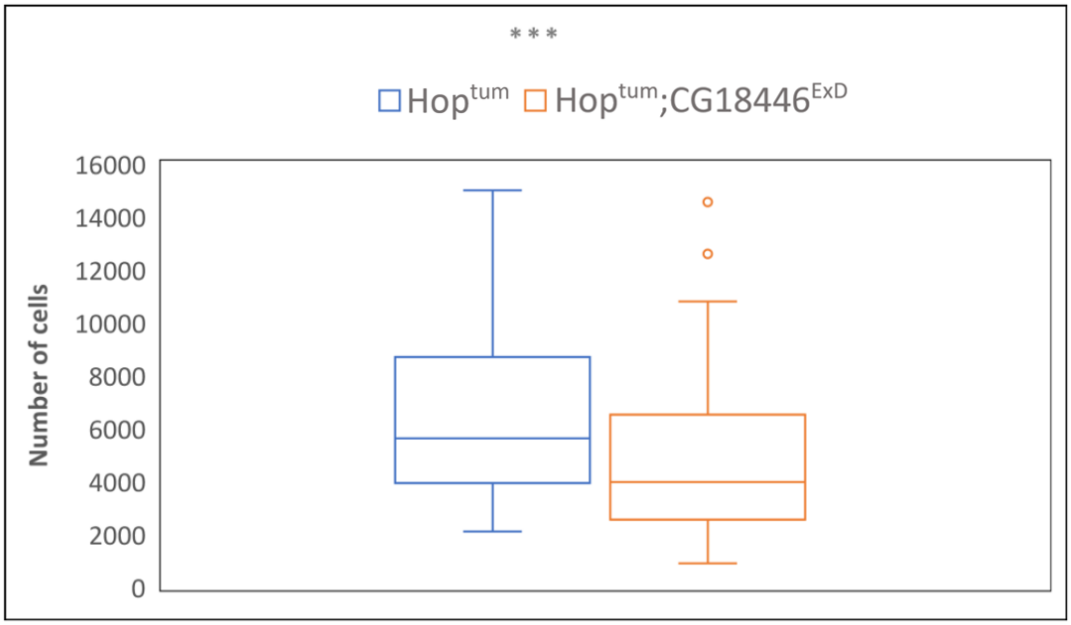

Figure 13: Number of lamellocytes present in L3 larvae of Hop^{tum} and $Hop^{tum};CG18446^{ExD}$ mutants counted on Neubauer improved counting chamber. Median for Hop^{tum} is 5.625, median for $Hop^{tum};CG18446^{ExD}$ is 3.975. The P-value is 2,61e-1 (P-value > 0,1)

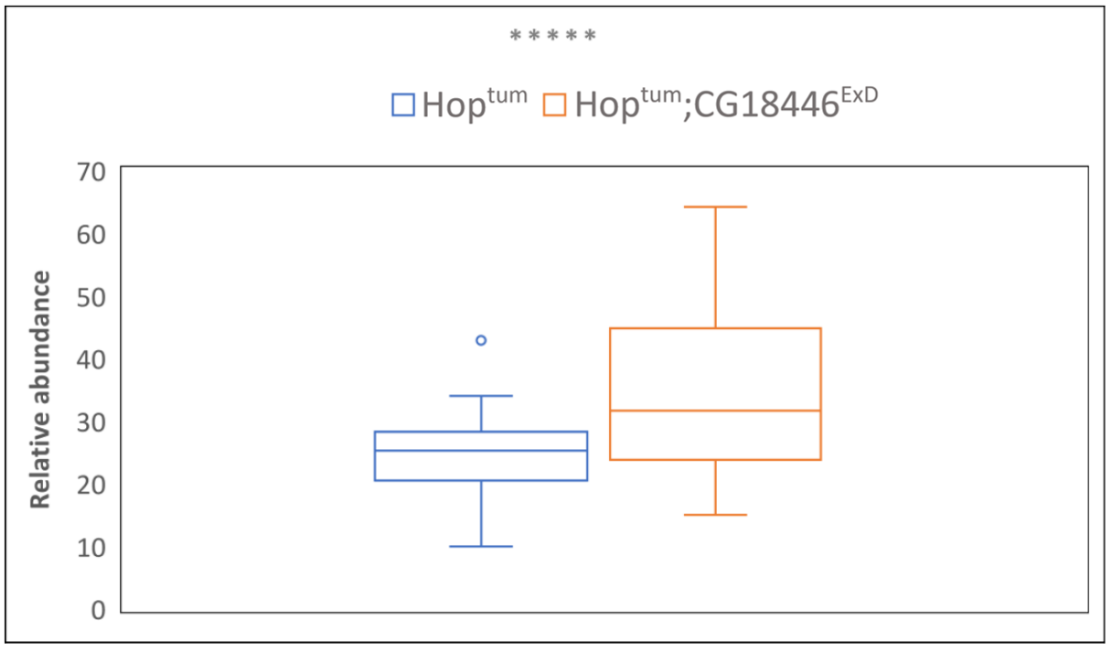

Figure 14: The ratio of the number of lamellocytes to the number of all haemocytes present in larval haemolymph for Hop^{tum} and $Hop^{tum};CG18446^{ExD}$ counted on Neubauer improved counting chamber. The median for Hop^{tum} is 25,23% and the median for $Hop^{tum};CG18446^{ExD}$ is 31,63%. The P-value is 9,17e-3 (P-value < 0,01)

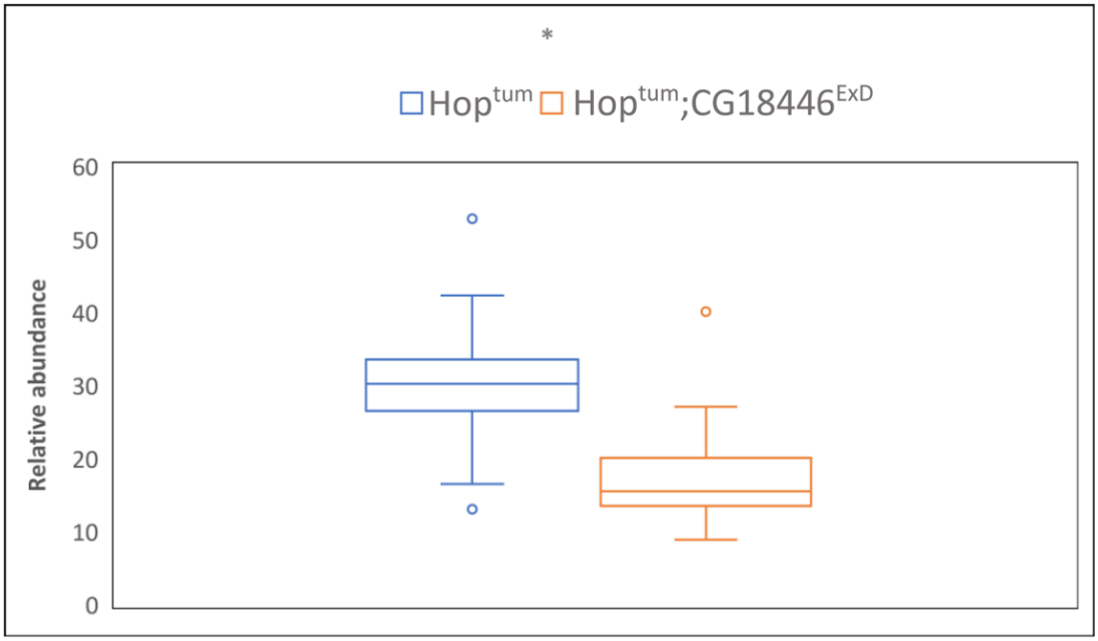

Figure 15: The ratio of the number of lamellocytes to the number of all haemocytes present in larval haemolymph for Hop^{tum} and Hop^{tum};$CG18446^{ExD}$ calculated from pictures taken on confocal microscope. The median for Hop^{tum} is 29,41% and 15,29% for Hop^{tum};$CG18446^{ExD}$. The P-value is 6,41e-1 (P-value > 0,5)

4.5 Influence of $CG18446^{Mlo2952}$ mutation on the number of crystal cells

Creation of fly stock bearing the Lz-mCherry fluorescent reporter for crystal cells was required for further analysis. The mating scheme which was performed can be seen in section 3.2. Counting of red positive cells in larval haemolymph was performed on Neubauer improved counting chamber under fluorescence microscope. The total number of cells present in 16 larvae was counted (depicted in Figure 16) and subsequently the number of crystal cells were counted (depicted in Figure 17).

The number of haemocytes in the two mutants is quite similar – larvae from Lz-Stinger have 2.025 cells and Lz-Stinger;$CG18446^{Mlo2952}$ have 2.100, as depicted in Figure 16. This would on the other hand suggest that the mutation of CG18446 gene does not play role in the number of all haemocytes present in larval haemolymph.

Whereas the number of crystal cells in the two mutants differs significantly, see Figure 17. The number of crystal cells present in Lz-Stinger larvae is 150 where by contrast the number in Lz-Stinger;$CG18446^{Mlo2952}$ larvae is 0. This result support the assumption that the mutation of CG18446 gene does play role in number of crystal cells present in larval haemolymph.

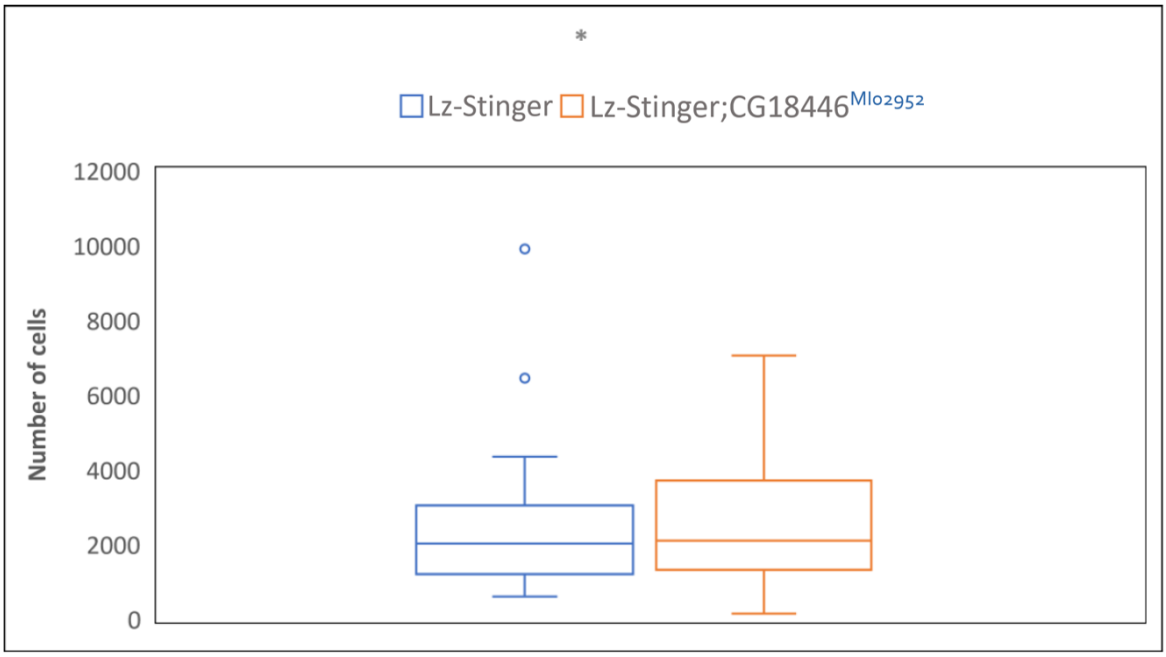

Figure 16: Total number of haemocytes present in L3 larvae of Lz-Stinger and Lz-Stinger;CG18446^{Mio2952} mutants counted on Neubauer improved counting chamber. Median for Lz-Stinger is 2.025, median for Lz-Stinger; CG18446^{Mio2952} is 2.100.

The P-value is 7,86e-1 (P-value > 0,5)

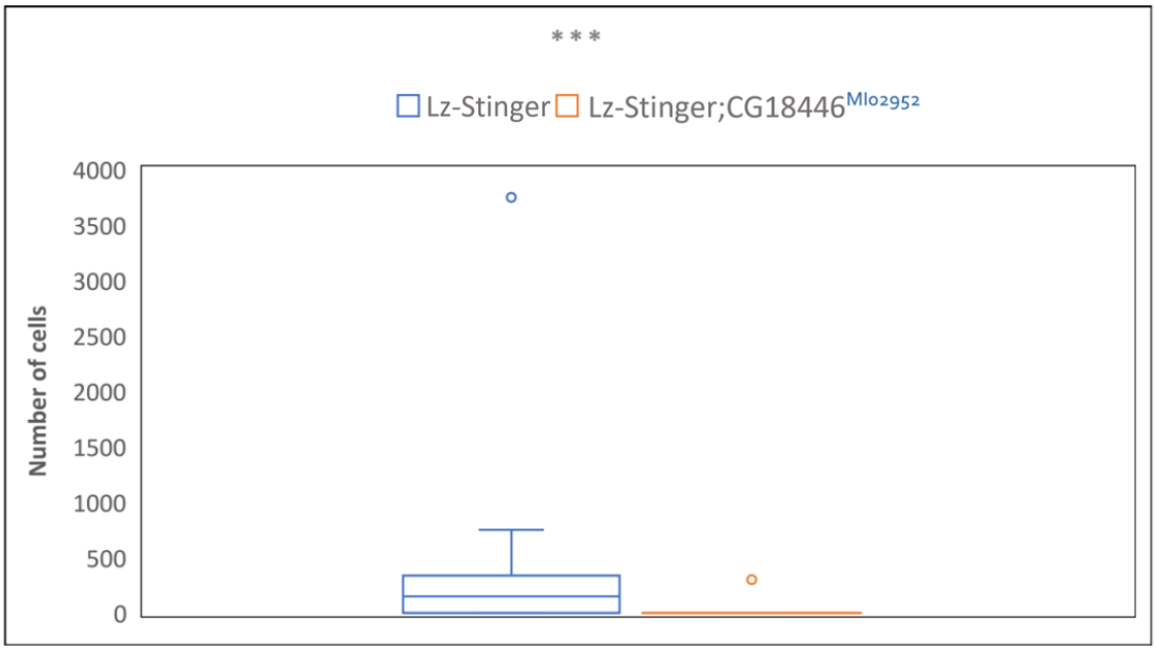

Figure 17: Number of crystal cells present in L3 larvae of Lz-Stinger and Lz-Stinger; CG18446^{Mio2952} stock counted on Neubauer improved counting chamber. The median for Lz-Stinger is 150 and the median for Lz-Stinger; CG18446^{Mio2952} is 0.

The P-value is 9,81e-2 (P-value < 0,1)

5 Discussion

Previous research done in Krejčí lab suggested that Hop^{tum} flies with mutation on CG18446 gene produce lower number of lamellocytes. For confirmation of this assumption repetition of previous experiment was performed. Heating of L3 larvae of Hop^{tum} and Hop^{tum};$CG18446^{Mlo2952}$ was performed (see section 3.3 for protocol) and subsequently photos from these two genotypes were taken (Figure 5) and compared. It is hard to quantify the number of occurred melanotic tumours, but by comparing the two pictures the difference is clear. The mutants for CG18446 gene contain significantly lower number of black melanotic tumours.

The melanotic tumours are caused by melanisation of larval own tissue (Harrison, et al., 1995) (Crozatier & Vincent, 2011) initiated by heat. There are two haemocyte lineages which contain phenol oxidases which are activated during melanisation i) the lamellocytes and ii) the crystal cells (Parsons & Foley, 2016).

As next another quite simple assay was performed. The Hop^{tum} and Hop^{tum};$CG18446^{Mlo2952}$ flies were crossed with MsnF9>mCherry, marker for lamellocytes. The L3 larvae were subsequently examined under fluorescence microscope and pictures were taken (Figure 6). The signal coming out of lamellocytes was captured. Based on the intensity of captured light the number of lamellocytes could be estimated. As it can be seen in the Figure 6 the intensity of emitted light from Hop^{tum} flies is much stronger than the light captured coming from Hop^{tum};$CG18446^{Mlo2952}$ larvae. The lower intensity of emitted light is caused by lower number of lamellocytes present in the larval body.

To be absolutely sure, that the results are right, haemocyte analysis of larval haemolymph had to be performed. The number of lamellocytes and subsequently number of crystal cells present in the larval body fluid was determined. For the analysis of larval haemolymph several fly strains were used. Hop^{tum} strain was used as standard since the mutation holds Gain-of-function in the JAK kinase Hopscotch and so the mutants overproduce lamellocytes (Harrison, et al., 1995), which are not normally present in healthy flies. To be completely sure that the observed effects are caused by CG18446 gene disruption, two different mutations of it were used ($CG18446^{Mlo2952}$ and $CG18446^{ExD}$).

5.1 Effect of CG18446 gene on the number of lamellocytes.

It is known that the CG18446 gene expression is preserved through the development of fruit fly from embryo till the adult (Fisher & et, 2012). Its expression is found in plasmatocytes, lamellocytes, lymph gland and in fat body of infected flies (Tanasić, 2014). All named cells and organs are important part of *Drosophila melanogaster's* immune system. However it wasn't clear which role does the CG18446 gene plays in the fly's immune system. Experiments presented in this thesis and listed results try to clarify part of the CG18446 gene's role in immune system of *Drosophila melanogaster*.

The results from haemocyte analysis performed on Neubauer improved chamber show that the CG18446 mutants are not able to produce such numbers of lamellocytes as flies without mutation on that gene. The number of lamellocytes present in both mutants ($CG18446^{Mlo2952}$ and in $CG18446^{ExD}$) is significantly lower (see Figure 8 and Figure 13).

The ratios obtained by analysis of the pictures of haemolymph taken under confocal microscope presented in sections 4.3 and 4.4 support the results obtained by previous experiments. Namely the ratios of the number of present lamellocytes to the number of all haemocytes present in the pictures are lower in CG18446 mutants than the ratios obtained for Hop^{tum} flies.

Therefore it can be concluded that the lower number of lamellocytes present in the CG18446 mutants is caused by disruption of the function of this gene.

5.2 Effect of CG18446 gene on the number of crystal cells.

After seeing the results obtained by heating assay, it was clear that the number of crystal cells has to be analysed in $CG18446^{Mlo2952}$ as well as in flies without the mutation. For being able to count the crystal cells new stock with marker for crystal cells (Lz-Stinger) had to be constructed (for used mating scheme see section 3.2). The crystal cells were counted on Neubauer improved counting chamber under fluorescence microscope and are present in Figure 17. It is obvious that the $CG18446^{Mlo2952}$ mutants obtain less crystal cells than the flies without mutation on that gene.

By combining all results together the prime assumption is confirmed. Namely that the number of lamellocytes and crystal cells is lower in CG18446 mutants. Therefore it can be said that the CG18446 gene is important for the haemocyte differentiation in *Drosophila melanogaster*. This gene – CG18446, is important for the differentiation of lamellocytes and

crystal cells based on presented results. However based on the obtained results it cannot be concluded if the CG18446 gene plays also role in maintaining the total number of all haemocytes present in the larval body, since the results are not uniform.

6 Conclusion

The overexpression of constitutively active Hopscotch gene, the only JAK kinase in *Drosophila melanogaster*, by using Hop^{tum} mutation, causes increasing of JAK/STAT pathway signalling. This is followed by massive overproliferation of lamellocytes (Harrison, et al., 1995) (Nappi, et al., 1984) (Crozatier & Vincent, 2011). My results showed that when CG18446 gene is not expressed, the number of proliferated lamellocytes is lower and so is the number of crystal cells. The effect of CG18446 mutation on total number of all haemocytes present in larval haemolymph cannot be concluded from data presented as different experiments showed opposing results. For confirmation of the results presented in this thesis and for deeper understanding of the role of CG18446 gene in *Drosophila melanogaster's* immune system, infections of $CG18446^{Mlo2952}$ and $CG18446^{ExD}$ larvae by parasitic wasp could be performed and the number of lamellocytes and all haemocytes in larval haemolymph could be monitored during the infection.

7 Table of content

Agaisse, H. et al., 2003. Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septitc injury.

Anon., 13.11.2018. *ScienceDaily.* [Online]
Available at: https://www.sciencedaily.com/terms/human_genome.htm
[Accessed 13 11 2018].

Anon., 15.11.2018. *National Human Genome Research Institute.* [Online]
Available at: https://www.genome.gov/10002081/2000-release-fruitfly-genome-sequenced/
[Accessed 15 11 2018].

Anon., 19.11.2018. *Flybase.* [Online]
Available at: [http://flybase.org/reports/FBgn0033458]
[Accessed 19 11 2019].

Anon., 19.11.2018. *Flybase.* [Online]
Available at: http://flybase.org/reports/FBpp0087464
[Accessed 19 11 2019].

Barillas-Mury, C., Han, Y. S., D., S. & Kafatos, F. C., 1999. Anopheles gambiae Ag-STAT, a new insect member of the STAT family, is activated in response to bacterial infection.

Bina, S. & Zeidler, M., 2009. JAK/STAT pathway signalling in Drosophila melanogaster.

Crozatier, M., Ubeda, J.-M., Vincent, A. & Meister, M., 2004. Cellular immune response to parasitization in Drosophila requires the EBF otrhologue Collier.

Crozatier, M. & Vincent, A., 2011. Drosophila: a model for studying genetic and molecular aspects of haematopoiesis and associated leukaemias.

Defaye, A. et al., 2009. Genetic ablation of Drosophila phagocytes reveals their contribution to both development and resistance to bacterial infection.

Elgin, S. C. R. & et, a., 13.11.2018. *modENCODE.* [Online]
Available at: http://modencode.sciencemag.org/drosophila/introduction
[Accessed 13 11 2018].

Fisher, B. & et, a., 2012. http://flybase.org/reports/FBrf0219253.html.

Fossett, N. et al., 2001. The friend of GATA proteins U-shaped, FOG-1, and FOG-2 function as negative regulators of blood, heart,and eye development in Drosophila.

Ghosh, S., Singh, A., Mandal, S. & Mandal, L., 2015. Active hematopoietic hubs in drosophila adults generate hemocytes and contribute to immune response.

Gold, K. S. & Brückner, K., 2014. Drosophila as a model for the two myeloid blood cell systems in vertebrates.

Grigorian, M., Mandal, L. & Hartenstein, V., 2011. Hematopoiesis at the onset of metamorphosis: terminal differentiation and dissociation of the Drosophila lymh gland.

Harrison, D. A. et al., 1995. Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects.

Holz, A. et al., 2003. The two origins of hemocytes in Drosophila.

Igaz, P., Tóth, S. & Falus, A., 2001. Biological and clinical significance of the JAK-STAT pathway; lessons from knockout mice.

Lanot, R., Zachary, D., Holder, F. & Meister, M., 2001. Postembryonic hematopoiesis in Drosophila.

Lebestky, T., Chang, T. & Hartenstein, V., 2000. Specification of Drosophila hematopoietic leneage by conserved transcription factors.

Lebestky, T., Jung, S.-H. & Banerjee, U., 2003. A Serrate-expressing signaling center controls Drosophila hematopoiesis.

Mandal, L., Banerjee, U. & Hartenstein, V., 2004. Evidence for a fruit fly hematogioblast and similarities between lymph-gland hematopoiesis in furit fly and mammal aorta-gonadal-mesonephrons mesoderm.

Mankhina, S., Tan, W. & Steward, R., 2011. JAK/STAT and the GATA factor Pannier control hemocyte maturation and differentiation in Drosophila.

Nappi, A., Kmiecik, J. & Silvers, M., 1984. Cellular immune competence of a Drosophila mutant with neoplastic hematopoietic organs.

Parsons, B. & Foley, E., 2016. Cellular immune defenses of drosophila melanogaster.

Prokop, A., version 2. A rough guide to Drosophila mating schemes.

Reiter, L. et al., 2001. A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster.

Tanasić, D., 2014. The expression pattern of CG18446 fene in Drosophila melanogaster.

Tepass, U., Fessler, L. I., Aziz, A. & Hartenstein, V., 1994. Embryonic origin of hemocytes and their relationship to cell death in Drosophila.

Tweedie, S. et al., 2008. FlyBase: enhancing Drosophila gene ontology annotations.

Wood, W. & Jacinto, A., 2007. Drosophila melanogaster embryonic haemocytes: masters of multitasking.