THE ANALYSIS OF SERINE PROTEASES AFFECTING THE IMMUNE RESPONSE IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

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KAULIO, EVELIINA:
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Drosophila melanogaster, the fruit fly, is a widely used model organism for studying mammalian immunity. The objective of this study was to investigate the function of serine proteases in fly immunity. Five serine proteases were knocked down in the blood cells and fatbody of fly larvae, and they were infected with parasitoid wasps, *Leptopilina boulardi*. The purpose of this study was to cross, infect and dissect *Drosophila melanogaster* larvae to study the effects of wasp infection.

The data of this study were collected from the larvae of crosses of five serine protease-RNAi constructs with haemocyte driver, fatbody driver, and control fly lines. The empirical part of the study consisted of crossing flies and encapsulation experiments with parasitoid wasps, where the investigation was performed by following the ability of *Drosophila* larvae to encapsulate and kill the parasitoid wasp eggs. The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was performed to investigate gene expression.

The results of this study suggested that if the serine protease *spirit* is knocked down in both haemocytes and fatbody, the ability of the immune system to kill the parasitoids is reduced. As regards the knockdown of the other serine protease-RNAi constructs, significant changes in these immune responses were not observed.

The findings indicated that *spirit* is part of the serine protease cascade that cleaves and thereby activates *Spätzle*, which leads to the activation of *Toll*-signaling. When *spirit* was knocked down, the encapsulation process did not function correctly. Further research is required to find out why knocking down *spirit* has an influence on both haemocytes and fatbody.

Key words: *Drosophila melanogaster*, immune response, *Leptopilina boulardi*, RNAi, serine protease, *spirit*.

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Drosophila melanogaster – banaanikärpänen, on maailmanlaajuisesti käytetty malliorganismi. Se on genomiltaan hyvin samankaltainen nisäkkäiden, mukaan lukien ihmisen, kanssa. Tämän opinnäytetyön tavoitteena oli kerätä tietoa *Drosophila melanogasterin* immuunipuolustuksen toiminnasta. Työn tarkoituksena oli hiljentää useiden seriiniproteaasi geenien ilmentyminen käyttäen RNAi-tekniikkaa joko verisoluissa tai rasvakudoksessa, infektoida banaanikärpäsen toukat *Leptopilina boulardi* -loispistiäisillä sekä avata toukat infektion vaikutuksen selvittämiseksi.

Tämän opinnäytetyön aineisto kerättiin risteyttämällä RNAi-linjan banaanikärpäset verisolu- ja rasvakudoksen ajurilinjoihin sekä kontrollilinjoihin. Pistiäisinfektion vaikutusten tutkimiseksi banaanikärpästen toukissa seurattiin pistiäisen munien ja toukkien kapseloitumista, joka kertoo Drosophila-toukan kyvystä päästä eroon loisesta. Kvantitatiivisella käänteistranskriptiopolymeraasiketjureaktiolla tutkittiin, toimivatko RNAi-linjat eli oliko halutun geenin hiljentäminen onnistunut sekä ilmentyykö kyseinen geeni verisoluissa vai rasvakudoksessa.

Kun *spirit* hiljennettiin joko verisoluissa tai rasvakudoksessa, *Drosophila*-toukan immuunipuolustus ei pystynyt taistelemaan pistiäisinfektiota vastaan normaalisti. Muiden työssä käytettyjen geenien kohdalla vastaavaa, huomattavaa muutosta ei havaittu.

Tuloksista voidaan päätellä, että *spirit* on osa signalointireittiä, joka valmistelee Spätzle-ligandin kiinnittymään Toll-reseptoriin. Kun *spirit* hiljennetään, tämä reitti ei toimi ja sen seurauksena *Drosophilan* immuunipuolustus ei pysty kapseloimaan pistiäisen toukkia. Jatkotutkimuksissa voitaisiin selvittää, miksi *spirit* vaikuttaa immuunipuolustukseen sekä verisoluissa että rasvakudoksessa. Selvitettävänä voisi olla myös Toll -signalointireitin osallisuus *Drosomycin*-GFP:n avulla.

Asiasanat: *Drosophila melanogaster*, immuunipuolustus, *Leptopilina boulardi*, RNAi, seriiniproteaasi, *spirit*.

CONTENT

1 ABBREVIATIONS

AMP	antimicrobial peptide
anterior - posterior	head - tail axis
C_T	cycle threshold
dorsal - ventral	back - belly axis
E	amplification efficiency
Fb	Fatbody driver
GAL4	transcriptional activator from yeast
GFP	green fluorescent protein
HH	Hemolectin Δ Hemese double driver
L3	third instar larva
RpL32	housekeeping gene, ribosomal protein L32
SP	serine protease
spirit	serine protease immune response integrator
UAS	upstream activating sequence
W_{RHP}	wild type/ control line

2 INTRODUCTION

Drosophila melanogaster, also known as common fruit fly, is a useful animal model. It is small and easy to take care of in laboratory environment. Other important features are the short life cycle, the female fly lays many eggs and the fly can be examined in all phases of its life cycle.

Its generation time is short, enabling evolutionary research. There are genetic mutants and specially created lines of *Drosophila*. The entire genome of *Drosophila* has been sequenced and that gives a basis for specific studies of genetics and evolution. It is widely used as an experimental tool because it is a simplified and well-studied genetic model system.

The bachelor's thesis was done in the Genetic immunology research group under supervision of Dip. Biol. Ines Anderl and Professor Dan Hultmark at the Institute of Biomedical Technology, University of Tampere.

The objective of the study was to investigate the effects of parasitoid wasp infection on the *Drosophila melanogaster* immune system. This study resulted in important knowledge on the functioning of the *Drosophila melanogaster* immune system and the results of this study will be included in a scientific article.

The purpose of the thesis was to cross, infect, and dissect *Drosophila melanogaster* larvae to analyse the immune response in response to parasitoid wasp infections. Male flies that contained UAS-RNAi constructs were crossed with females carrying a GAL4-driver. In the offspring the expression of a specific gene was blocked by means of the GAL4-UAS system.

3 BACKGROUND

3.1 *Drosophila melanogaster*

An optimal condition for normal development of *Drosophila* is at 25 °C. Females lay eggs usually right after mating. The egg grows into a larva within 22-24 hours and the larval stages L1, L2 and L3 last about 24, 24 and 48 hours, respectively. Pupa stages last four to four and half days before the fly ecloses. The flies are long, thin and pale after eclosure and the wings are unexpanded. The flies hatch from pupa as virgins and females become sexually mature within 12-14 hours. (Ashburner 2005, 122.) The collection of virgin females is required for crosses for genetic experiments. The female flies are able to store sperm from a single insemination, which is sufficient to fertilize the eggs that will be laid during the following six to eight days. During this time, females are not showing attention to males but are unprotected against rapes. (Ashburner 2005, 123.)

Drosophila has three developmental stages in its advancement from egg to the adult fly. The egg cell grows and develops in the female fly's ovary. The female fly lays eggs one at a time into the food. Mitosis begins directly after the fertilization and egg laying. During the egg stage, the embryo goes through early and late blastula phases protected by the eggshell. After embryogenesis, a larva hatches out of the shell. (Campbell, Reece & Mitchell 1999, 398.)

The larval stage consists of L1, L2 and L3. During these stages the larva eats, grows and forms a tough outer layer in which it continues its transformation to the pupa stage. During the pupa stage, the metamorphosis occurs; the larva is changing into the adult fly. The adult *Drosophila* has three pairs of legs, a pair of wings and a pair of halteres, which measure speed and balance the fly during flying. (Campbell, Reece & Mitchell 1999, 398.)

As well as other arthropods, the *Drosophila melanogaster* body consists of three segments: the head, the thorax and the abdomen. The thorax is the middle part of the body where the wings and legs extend. The abdomen is the final part of the body. All bilaterally symmetrical animals, also *Drosophila*, have an anterior-posterior axis that consists of head-tail axis and dorsal-ventral axis, which means back-belly axis. (Campbell, Reece & Mitchell 1999, 397-398.) Figures 1 to 3 show L1, L2 and L3 larvae, pupae of different ages and female and male flies.

FIGURE 1. L3, L2, L3 larval stages

FIGURE 2. White prepupa, empty pupal case and pupa prior to eclosure

FIGURE 3. *Drosophila melanogaster* male (left) and female (right)

Drosophila have four pairs of chromosomes the X/Y, 2L, 2R, 3L, 3R and 4; L stands for left arm and R for right arm. Chromosomes 4 and X have a large left arm and a small right arm. The sizes of chromosomes X, 2L, 2R, 3L and 3R are comparable but chromosome 4 is only about one-fifth of the size of the other chromosomes. (Greenspan 2004, 3-4.) Female flies have two X chromosomes and males an X and a Y chromosome. *Drosophila* sex is determined based on the ratio of X chromosomes to autosomal sets. Chromosome Y does not function in male development but is needed for proper sperm motility. (Greenspan 2004, 4.)

The notable advantages of using *Drosophila* as a model organism are short life cycle and inexpensive costs of fly keeping as well as the existence of balancer chromosomes and a sequenced genome. The *Drosophila* genome is homologous to the mammalian genome, and there are many genetic mutants available. This makes it a useful model organism to study the genetic basis of diseases. (Wolf & Rockman 2008.)

The *Drosophila* genome is smaller but it shares several common genes and signaling pathways to vertebrate genome. Researchers have proven that *Drosophila* has about 80 % of identified human disease genes. Human diseases where the *Drosophila* model has been used successfully, include neurodegenerative diseases; for example, Parkinson's disease, Fragile-X syndrome, Alzheimer's disease, moreover cancers, infectious diseases, diabetes and cardiovascular disease. (Wolf & Rockman 2008.)

Specific phenotypes and genetic mutations can be investigated through broad collections of *Drosophila* stocks. Some of these stocks use P-elements to disrupt gene function. The P-element is a foreign piece of DNA. The stock collections contain molecularly-defined genomic deficiencies and chemically and radiation-induced mutants. Moreover, transgenic fly mutants contain tissue specific small interfering RNAs for gene knockdown and GAL4-UAS transgenic flies for specific transgene expression. These fly lines can be obtained from public stock centers. In addition, very good databases, for example Flybase, support the fly community. (Wolf & Rockman 2008.)

3.2 *Leptopilina boulardi*

Leptopilina boulardi is a parasitoid wasp species that belongs to the order Hymenoptera. They have two pairs of membranous wings, three pairs of legs, a mobile head with chewing mouthparts and a posterior stinging organ on females (Campbell, Reece & Mitchell 1999, 620). A *Leptopilina boulardi* female and male is shown in figure 4.

FIGURE 4. *Leptopilina boulardi* female on the left and male on the right

The female parasitoid wasp deposits its egg, one or more, inside the haemocoel of second to early third-instar *Drosophila* larvae (Meister 2004, 13; Dubuffet et al 2009, 148). Host development is slightly delayed because of infection. The eggs of *L. boulardi* are generally found attached to host tissues whereas *L. heteroma* or *L. victoriae* eggs are floating freely in host haemolymph. (Dubuffet et al 2009, 126.) If the host immune system recognizes the invader, it is killed by the immune response but if not, the parasitoid kills its host by eating it from inside. These parasitoids are called endoparasitoids because they develop inside the body cavity of their hosts. The defence mechanism of the host is called encapsulation, which means that the invader is encapsulated inside a multicellular and melanized capsule. (Dubuffet et al 2009, 148.) Figures 5 to 7 show a living wasp larva, a half-melanized wasp larva and completely melanized wasp eggs.

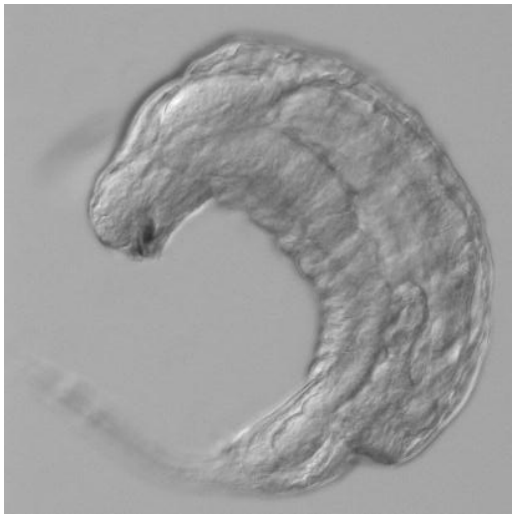

FIGURE 5. Living wasp larva

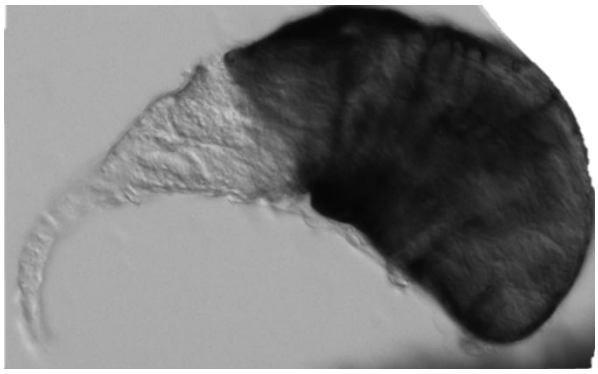

FIGURE 6. Melanized but living wasp larva

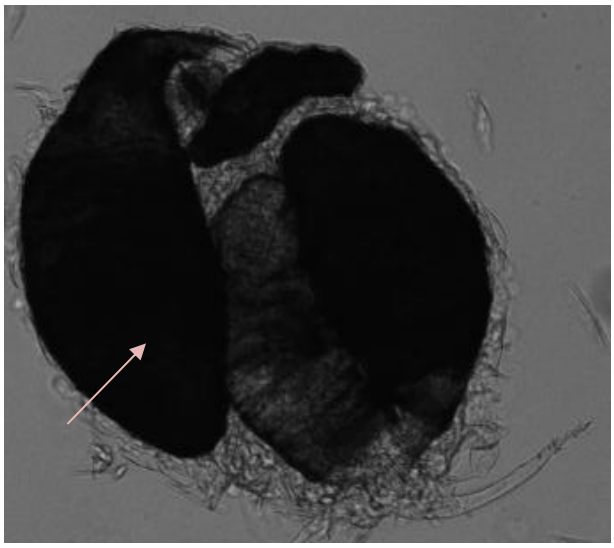

FIGURE 7. Dead, melanized wasp egg marked with an arrow

The parasitoid wasp larval development passes through three larval instars excluding some species that have four to eight instars. After the last larval phase, the pupa is directly formed. (Quicke 1997, 111, 117, 146.) In the case of an avirulent parasitoid, the egg is encapsulated and the melanotic capsule remains in the body of the host through its development. Whereas, in the case of a virulent parasitoid, the parasitoid's offspring escapes from the immune system of the host and ecloses as an adult from host puparium. (Vass & Nappi 2000, 1260.)

Parasitoids have virulence strategies to escape encapsulation and some of them have been characterized. However, the mechanism for the success/failure of parasitoids is not known. (Dubuffet et al 2009, 148-149.) The most commonly used host-parasitoid combination is *Drosophila melanogaster* and *Leptopilina boulardi* or *Asobara tabida* (Dubuffet et al 2009, 149). The encapsulation process is rapid, six hours post-infection, visualized by a thin melanin layer on the surface of the parasitoid egg. 24 hours post-infection, plasmatocytes have completely surrounded the wasp egg. After 40 hours of infection, lamellocytes have appeared around the wasp egg and 48 hours post-infection a melanotic capsule can be seen in the host haemocoel. (Dubuffet et al 2009, 158-159.)

There are three sources for variation of any host-parasite interaction; the variation in host resistance, the variation in the parasite ability to escape host resistance that is called parasite virulence. Additionally, there is increasing evidence for complex interactions between host and parasite genotypes. (Dubuffet et al 2009, 149.) There are also some abiotic and biotic factors that can affect encapsulation. Examples of abiotic factors would be temperature and host diet. An example for a biotic factor would be the existence of another parasitoid inside the host. The existence of other parasitoids is thought to strengthen the success to survive. (Dubuffet et al 2009, 150.)

There are parasitoid wasps that inject virus-like particles or venom together with the egg into the larva to block the larval immune. Virus-like particles can enter into host plasmatocytes and lamellocytes and are able to kill plasmatocytes or cause morphology formation in lamellocytes. (Dubuffet et al 2009, 167, 169, 171.)

3.3 Fly work tools

3.3.1 RNA interference

The first observation of RNA interference (RNAi) was made accidentally in petunias. The researchers tried to deepen the purple colour of the plant by introducing a pigment-producing gene. Instead of the perfect purple colour, the inserted gene suppressed it, and the result was a totally white petunia flower. The phenomenon is a natural process where specific genes are turned off or silenced. RNA interference was identified a few years later in the nematode, *C. elegans*. It became a powerful research tool at short notice and nowadays it is used worldwide. In 2006 two scientists Andrew Fire and Craig Mello were awarded the Nobel Prize in physiology or medicine for fundamental work in RNA interference and its therapeutic potential. The RNA interference is found in single-celled organisms to plants and humans. It is thought to work as a cellular defense mechanism towards invaders, for example RNA viruses or transposons. (Davis 2012; NIGMS 2012.)

Double stranded RNA causes gene silencing in a sequence-specific way (Campbell, Reece & Mitchell 1999, 394). RNA interference functions by destroying messenger RNAs (mRNA) (Davis 2012; NIGMS 2012). An enzyme, called Dicer, is a member of the RNase III class. It binds to double-stranded RNA and cleaves it into small fragments called small interfering RNAs (siRNA). These fragments guide the RNA interference machinery called RISC (RNA-induced silencing complex) to complementarily matching mRNA and cleaving the messengers and thereby terminating translation. (Campbell & Farrell 2008, 394; Davis 2012; NIGMS 2012.)

After siRNA mechanism was found, scientists discovered another, related pathway that is called microRNA (miRNA) (NIGMS 2012). The miRNA pathway is not exactly similar to siRNA pathway but it uses the same components. miRNAs originate from endogenous genome DNA sequences and are non-protein coding. They are processed from pri-miRNAs or small hairpin microRNAs (shmiRNA) to pre-miRNAs by the ribonuclease Drosha and transported out of the nucleus. In the cytoplasm, Dicer produces miRNAs. Both, the siRNA and miRNA pathways are combined to the RISC (RNA-induced silencing complex); consequently, the mRNA is cleaved and degraded. However, the miRNA matches only partially to mRNA's sequence whereas siRNA matches complementary. The mechanism of miRNA is not fully understood. In research, several tools for gene silencing that use RNA interference such as synthetic siRNAs, small hairpin RNAs (shRNA) or long dsRNAs are available. (Guo et al 2010, 4-5; NIGMS 2012; Perrimon 2010, 2.) The mechanism of RNA interference is presented in figure 8.

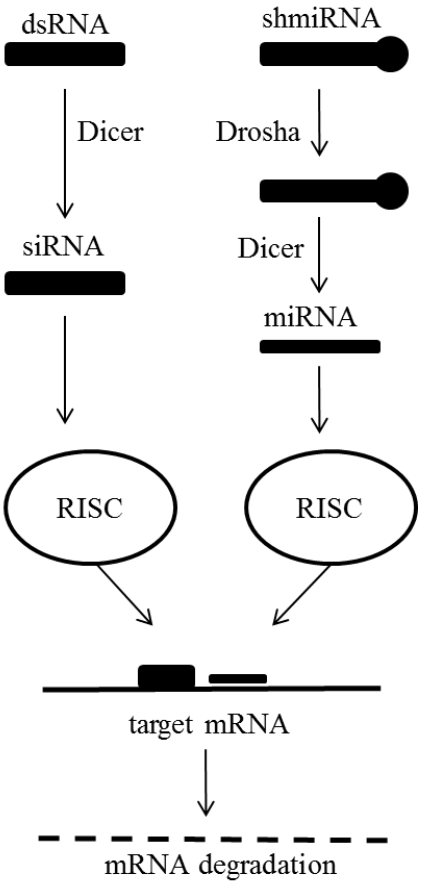

FIGURE 8. RNA interference (Perrimon et al 2010, 3, modified)

3.3.2 GAL4-UAS system

The GAL4-UAS system is a biochemical method and is used to study targeted gene expression in organisms such as *Drosophila*. The GAL4 system comprises two components; GAL4, a transcriptional activator from the yeast (*Saccharomyces cerevisiae*) and UAS, (Upstream Activation Sequence), a promoter to which the GAL4 binds to activate gene transcription. (Elliot & Brand 2008, 79).

The function of GAL4-UAS system is depicted (figure 9). The "driver" line is the fly line, where the GAL4 is expressed in cell- or tissue specific pattern. The "responder" fly is the line that contains the UAS upstream of the target gene sequence. To activate the GAL4-UAS system, these fly lines are crossed. When the GAL4 binds to the UAS binding site, the transcription of the defined gene starts in the progeny. (Brand & Perrimon 1993, 402.)

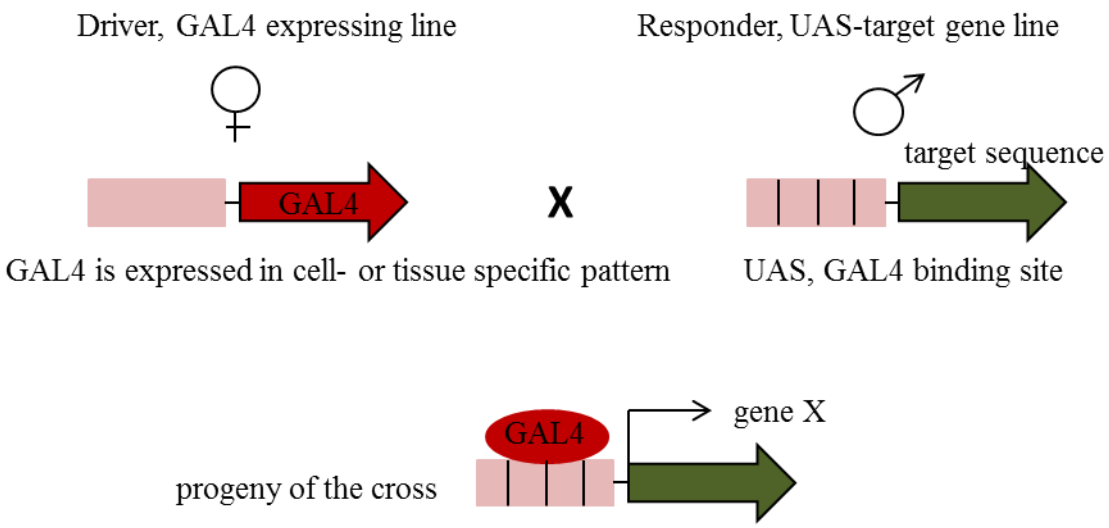

FIGURE 9. GAL4-UAS system (Elliot & Brand 2008, 81, modified)

The GAL4-UAS system is widely used for gene expression in tissue and cell-specific patterns because it enables selective activation of any cloned gene. A few problems can occur when using the GAL4-UAS system. Tissue-specific promoters enable transcription to be limited to a determined subset of cells. However, availability of cloned and characterized promoters can be restricted and that can influence the desired pattern. In addition, the gene product that is expressed can be toxic to the organism. Then it is not possible to use or create a stable transgenic line that carries the gene of interest. (Brand & Perrimon 1993, 401, 404.)

To regulate the GAL4-UAS system, the protein GAL80 is used. In yeast, GAL80 binds to transactivation domain of GAL4 and prevents activation. In *Drosophila*, researchers have developed GAL80 that functions under the control of *tubulin 1α* promoter that represses function of GAL4 in all tissues. (Elliot & Brand 2008, 83.) GAL80 under the control of *tubulin 1α* promoter can also be used as a temperature sensitive version. For example at 18 °C, expression of GAL4 is blocked and at 29 °C, expression of GAL4 is on. (Elliot & Brand 2008, 81, 84.) The use of the GAL4-UAS system allows for connections with several other tools; for example, RNA interference and green fluorescent protein (GFP) (Duffy 2002, 3-9).

3.3.3 Green fluorescent protein

The green fluorescent protein (GFP) is produced by many coelenterates. The GFP of jellyfish *Aequorea victoria,* is the most studied GFP. When the first light-producing protein was purified and characterized from *A. victoria,* researchers noticed that activated photoprotein; aequorin, produced blue instead of green light. (Chalfie 1995, 651.)

Green fluorescent protein is used for visualizing gene expression and protein localization in living cells as well as fixed tissues. A single gene encodes GFP and when it is translated, it undergoes self-catalyzed modification that results in strong, green fluorescence. The gene can be cloned and expressed in any organism, for example in bacteria, plants and animals, and it is shown not to be harmful to the cells. (Willey, Sherwood & Woolwerton 2009, 367; Chalfie 1995, 651; Chalfie et al 1994, 803.) The experiments with *C. elegans* showed that GFP can be used to study gene expression *in vivo* without cell permeabilization or toxic detection methods. The experiments also indicated that specific cells can be marked by GFP. This enables the use of GFP in living animals. (Chalfie 1995, 651.)

3.3.4 Drosophila immune system

The *Drosophila melanogaster* immune system consists of a cellular and humoral branch (Fauvarque & Williams 2011, 1374; Morales-Hojas et al 2009, 573). The blood cells or haemocytes encompass three types: lamellocytes, plasmatocytes and crystal cells. The haemocytes are involved in phagocytosis, encapsulation, coagulation and antimicrobial peptide secretion whereas the fatbody produces antimicrobial peptides. (Fauvarque & Williams 2011, 1374; Morales-Hojas et al 2009, 573.)

In addition, the epithelia of the alimentary tract and tracheae fight against invading microorganisms by functioning as a physical barrier and locally by producing antimicrobial peptides (AMP) and reactive oxygen species (ROS). (Lemaitre & Hoffmann 2007, 699.) The fatbody is functionally similar to the mammalian liver. It produces humoral response molecules like antimicrobial peptides (AMPs). The fatbody tissue is responsible for immune response. (Lemaitre & Hoffmann 2007, 699.)

The *Drosophila* body cavity is filled with circulating haemolymph that comprises free-floating and sessile blood cells (Lemaitre & Hoffmann 2007, 712). The haemocytes are located in three main sites in the larvae, in circulation, in the lymph gland and in a sessile population. The lymph gland, also called hematopoietic organ, is comprised of multiple pairs of lobes and is located behind the brain. The sessile population or sessile cell pattern is found just underneath the larval cuticle. (Fauvarque & Williams 2011, 1373.)

Plasmatocytes function in phagocytosis and are also involved in wound healing. Plasmatocytes produce antimicrobial peptides (AMPs) and secrete extracellular matrix components. In addition, they are the most common haemocytes in the circulation of healthy larvae. (Fauvarque & Williams 2011, 1373.) They comprise 90 – 95 % of all mature larval haemocytes (Lemaitre & Hoffmann 2007, 712). The rest, 5 %, of larval circulating haemocytes are crystal cells. These are nonphagocytic but are involved in the melanization process as well as wound healing and coagulation. Mature crystal cells express prophenoloxidase (proPO) that is released into the haemolymph upon activation. Crystal cell are fragile and rupture easily. Crystal cells seem to be a storage cell for proPO that is expressed in their cytoplasm in crystallized form. (Lemaitre & Hoffmann 2007, 713.) The *Drosophila* larval immune response is presented in figure 10.

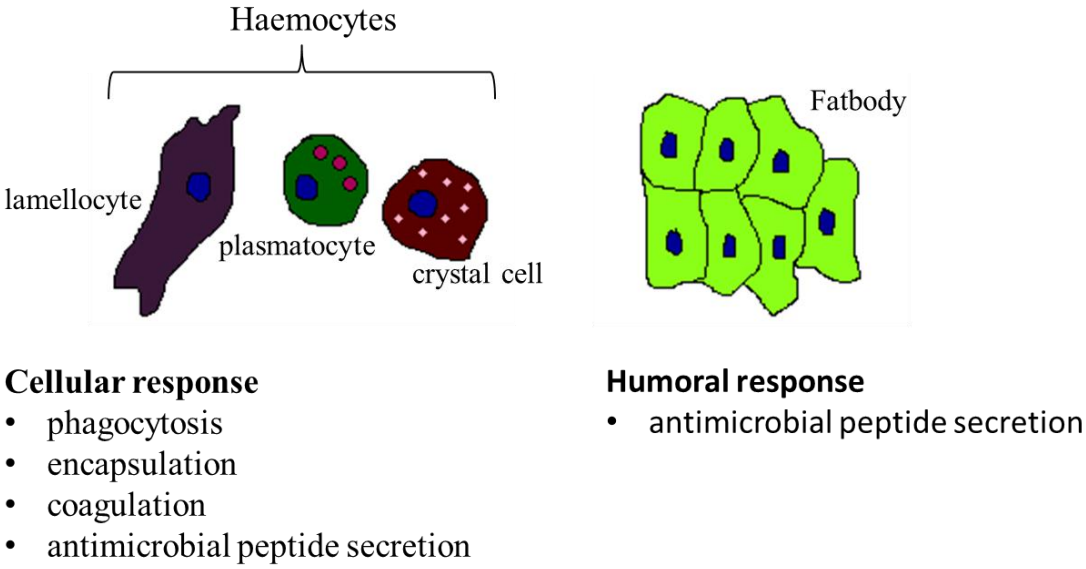

FIGURE 10. *Drosophila* larva immune system (Fauvarque & Williams 2011, 1374, modified)

The specialized haemocytes that take care of encapsulation are called lamellocytes. Lamellocytes are fairly large, flat cells that form a melanized capsule around the parasite or objects too large to be phagocytosed. (Lemaitre & Hoffmann 2007, 699, 712.) Melanized capsule is shown in figure 11. Lamellocytes are absent in embryos, adult flies and healthy larvae. They are inducible after larvae have been parasitized by wasps. It is not clear where lamellocytes originate but it is thought that plasmatocytes turn directly into lamellocytes. (Fauvarque& Williams 2011, 1373.)

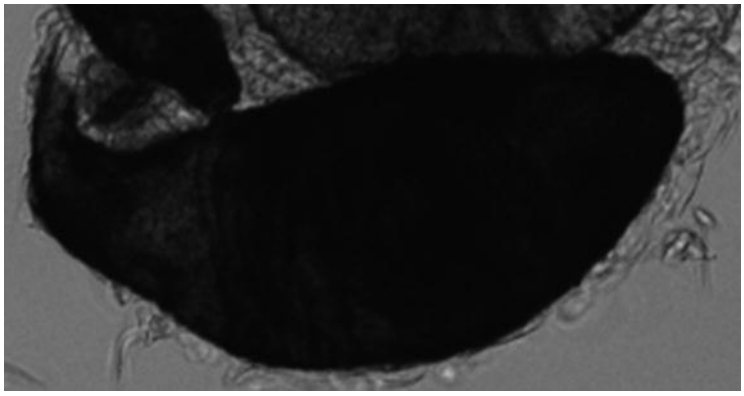

FIGURE 11. Melanized capsule around wasp egg

Plasmatocytes detect the wasp egg inside the larval haemocoel and attach to the egg chorion. Within a few hours a strong cellular reaction can be observed in the lymph gland. (Meister 2004, 13.) During the encapsulation process, lamellocytes form a multilayer capsule around the invader (Marmaras & Lampropoulou 2008, 187). The encapsulation process eventually causes parasite death. The parasite dies either of asphyxiation or formation of cytotoxic free radicals, quinones or semiquinones. (Meister 2004, 13.)

A serine protease cascade controls the melanization process by cleaving the zymogen prophenoloxidase and thereby converting it into its active form. The reaction continues when phenoloxidase catalyzes the oxidation of tyrosine-derived phenols to quinones. Melanin forms by non-enzymatic polymeration. The melanization process is thought to be involved in the pathogen killing as some of the compounds that are formed during the process, are cytotoxic. (Meister 2004, 13.)

The melanization process requires both, the humoral and cellular response. There are three genes that encode prophenoloxidases in the *Drosophila* genome. In larvae two of these genes are expressed in crystal cells. As mentioned earlier, crystal cells are fragile and easily rupture. They are ready to deliver their content into the haemolymph where zymogens are activated. (Meister 2004, 13.) The sessile cells are released into the circulation during the infection and sessile cells are not found in wasp-infected larvae (Márkus et al 2009, 1).

3.3.5 Serine proteases

Proteases are enzymes that cleave peptide bonds between amino acids in proteins. Serine proteases can be found in every type of organisms, and are involved in many biological processes like digesting food, clotting blood, fighting infections as well as helping sperm to enter the eggs. They also regulate the development of organisms. (Neitzel 2010, 21.) Some serine proteases are involved in the defense responses against bacteria or positive regulation of Toll signaling pathway (Kambris et al 2006).

They form the second largest gene family in the *Drosophila melanogaster* genome. There are 147 studied serine proteases. 84 of them have less than 300 amino acid residues and most of these are probably digestive enzymes. Larger serine proteases may contain important regions for protein protein interactions like clip domains. Most of the serine proteases are protein coding genes, and their molecular function is reported to be serine-type endopeptidase activity (Flybase 2012). The majority of them are thought to be trypsin-like and are activated when specific arginine or lysine residues are cleaved. (Ross et al. 2002, 117.)

The *Drosophila* genome also contains serpins (serine proteinase inhibitors) that have a unique cleavage mechanism. Serpins are found in plants, animals and viruses and it is a family containing over 1000 proteins. In *Drosophila*, there are 29 serpin genes. The function of these genes is to inhibit their target serine proteases but only little is known about them. Serpins in *Drosophila* are classified according to their chromosomal location. More than half of *Drosophila* serpins are located in clusters of adjacent transcripts. (Garret et al 2009, 2.)

3.3.6 Toll-signaling

All living organisms, as well as *Drosophila*, have several intra and intercellular signaling pathways to respond to signal from outside and within. The expression and the secretion of antimicrobial peptides by the fatbody are controlled by Toll and Imd (Immune deficiency) pathways. (Marmaras & Lampropoulou 2008, 193.) Bacteria and fungi activate the Toll and Imd pathways. The Toll and Imd are the only reported intracellular pathways that are activated by microbial ligands. Studies have shown that these pathways control about 80 % of genes induced during septic injury. There are some immune-induced genes dependent on only one pathway and others can be induced by both pathways. (Lemaitre & Hoffmann 2007, 703-704.) In both pathways, the intracellular signaling cascades end with nuclear translocation of a transcription factor. (Shia et al 2009, 4505.) The Toll-signaling pathway is required for encapsulation. (Marmaras & Lampropoulou 2008, 193.) Toll is activated by a cytokine whereas Imd is activated by straight interaction with microbes (Hoffman 2003, 33).

Immune deficiency also called *imd* got its name according to similarly named mutation. This mutation weakens the expression of some antibacterial peptides but is not affecting much induction of *Drosomycin*. (Lemaitre & Hoffmann 2007, 703-704.) The Imd pathway can activate JNK pathway via TAK1 pathway (Marmaras & Lampropoulou 2008, 193). The Toll and Imd are the only reported intracellular pathways that are activated by microbial ligands. Studies have shown that these pathways control about 80 % of genes induced during septic injury. There are some immune-induced genes dependent on only one pathway and others can be induced by both pathways. (Lemaitre & Hoffmann 2007, 703-704.)

The Toll pathway is activated by a ligand called Spätzle, which is an extracellular cytokine. A serine protease cascade leads to the cleavage of pro-Spätzle, hence activating Spätzle. Toll is a transmembrane receptor on the fatbody cells, and binding of Spätzle causes a formational change and Myd88 and Tube adaptors are recruited. Toll/Interleukin-1 receptor homology (TIR) domain is found in Toll receptor and MyD88. The kinase Pelle is activated. Toll signaling leads to the degradation of the inhibitor molecule Cactus and the transcription factors Dorsal and Dif are translocated into the nucleus. (Marmaras & Lampropoulou 2008, 193; Lemaitre & Hoffmann 2007, 701, 703.) In the nucleus, antimicrobial peptide expression starts. One of the antimicrobial peptides is Drosomycin that can be used as a marker for Toll activity. (Shia et al 2009, 4505.) Activation of the Toll-signaling pathway is presented in figure 12.

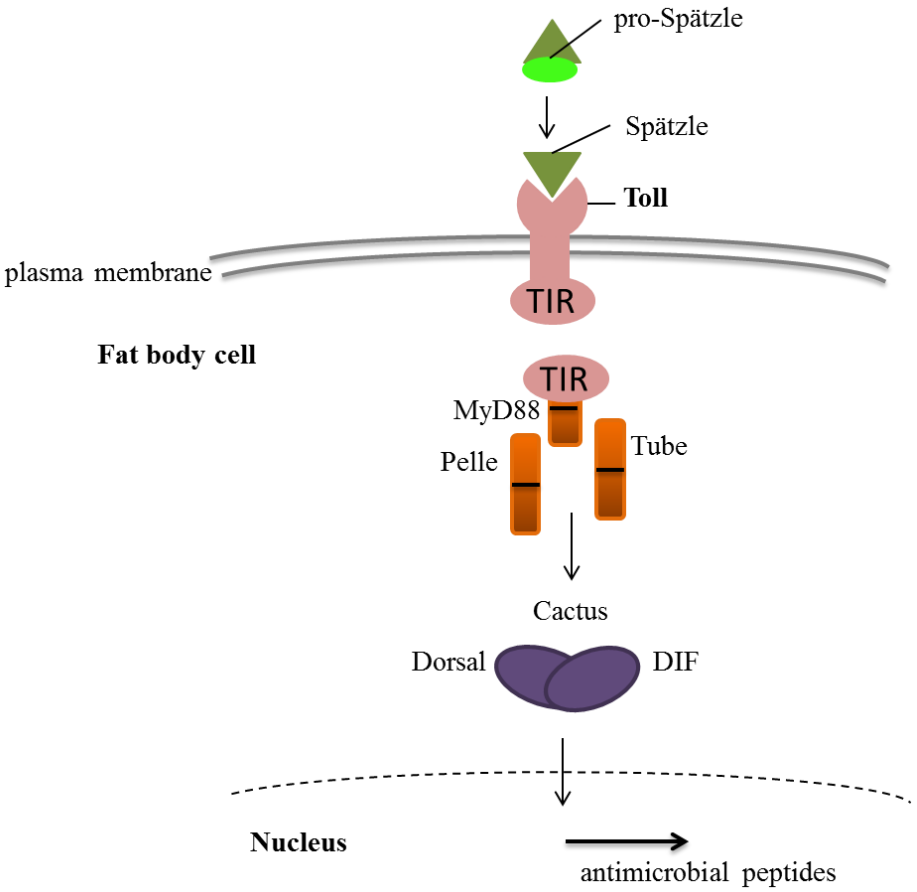

FIGURE 12. Toll-signaling pathway (Lemaitre & Hoffmann 2007, 702, modified)

3.4 Quantitative reverse transcriptase polymerase chain reaction

Polymerase chain reaction (PCR) is a way to increase the amount of a DNA sample over a million times. The polymerase chain reaction process has three parts, denaturation, annealing and extension (Campbell & Farrel 2008, 386.), shown in figure 13.

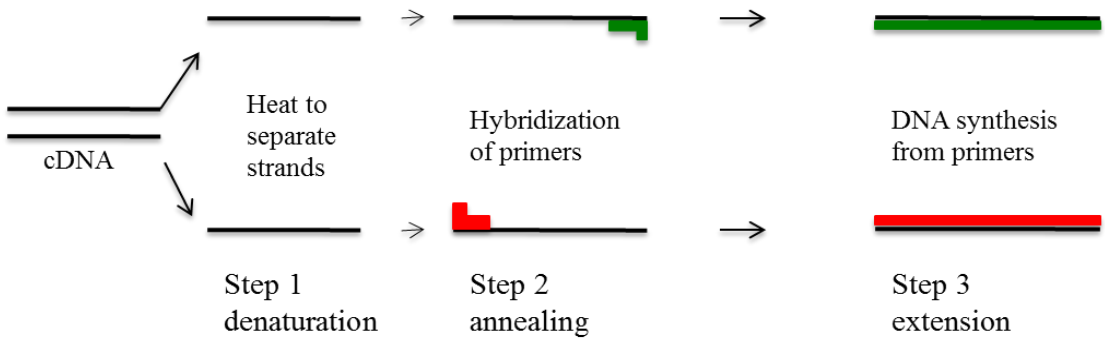

FIGURE 13. Polymerase chain reaction (Alberts et al 1998, 333, modified)

In the first step, denaturation, double stranded DNA is separated by heating. The second step, annealing, decreases the temperature and allows short oligonucleotide primers to bind to the single stranded DNA. The primers are complementary to the ends of the chosen sequence of the DNA. The third step, extension, raises the temperature again and *Taq* polymerase starts to synthetize the new DNA. Every round doubles the amount of the DNA. (Campbell & Farrel 2008, 386.) PCR cycles are shown in figure 14.

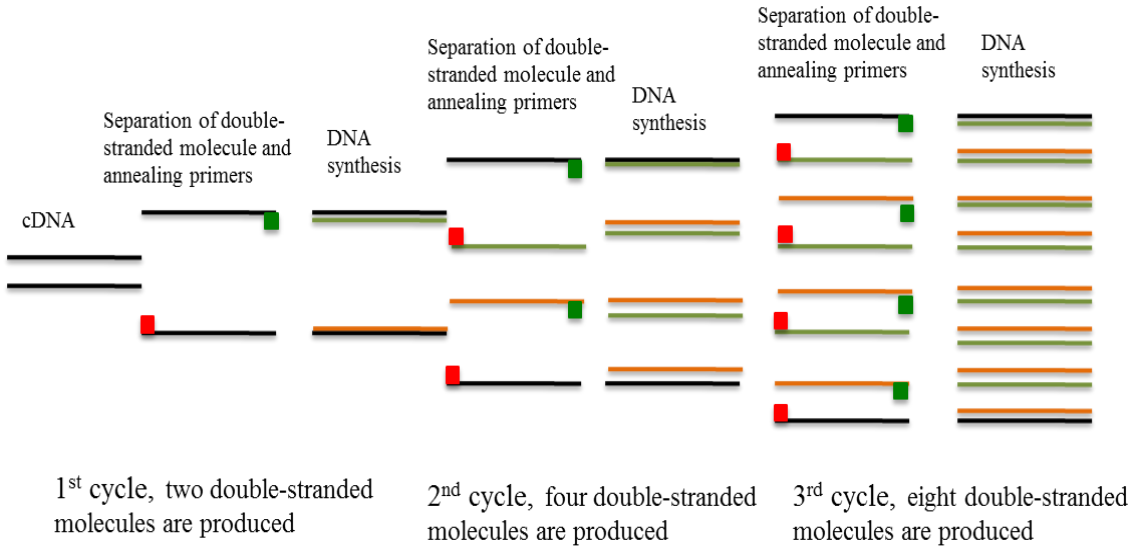

FIGURE 14. PCR cycles (Alberts et al 1998, 333, modified)

The idea of multiplying the DNA with the quantitative polymerase chain reaction is the same. To find out the result of the conventional PCR, the samples need to be run on an agarose gel but the qPCR, also called real-time PCR, allows detection and measurements during the run. (Bio-rad 2006, 2.) The detection of the real-time PCR products during the run is made possible by fluorescent labels. The fluorescent labels give a fluorescent signal that shows the increase in the amount of the DNA. The fluorescence is proportional to the detected product. The fluorescent dye that is the most commonly used in qPCR is SYBR Green I. This dye binds non-specifically to double-stranded DNA. The equipment has to be specialized to detect the fluorescent signal. (Bio-rad 2006, 2.)

There are several advantages when using qPCR. One of them is a possibility to determine the starting template copy number with precision and high sensitivity. The qPCR run can be either qualitative or quantitative. Real-time PCR data can be used and evaluated without gel electrophoresis. Closed-tube system reduces contaminations and eliminates postamplification manipulation. (Bio-rad 2006, 2.)

The result plot is shown in figure 15. On the x-axis there is the PCR cycle number and on the y-axis is the fluorescence from the amplification reaction called RFU, relative fluorescent units. In the result plot two phases are seen, the exponential phase and the non-exponential plateau phase. In the exponential phase, the amount of the products roughly doubles in each cycle. In the non-exponential plateau phase one or more of the starting-substances becomes limiting and the reaction slows and turns to non-exponential. (Bio-rad 2006, 3.) In the beginning of the run, the fluorescence remains at background levels, where the detection of the fluorescence is not possible. The cycle number, where the product becomes detectable, is called the threshold cycle, C_T. (Bio-rad 2006, 3.)

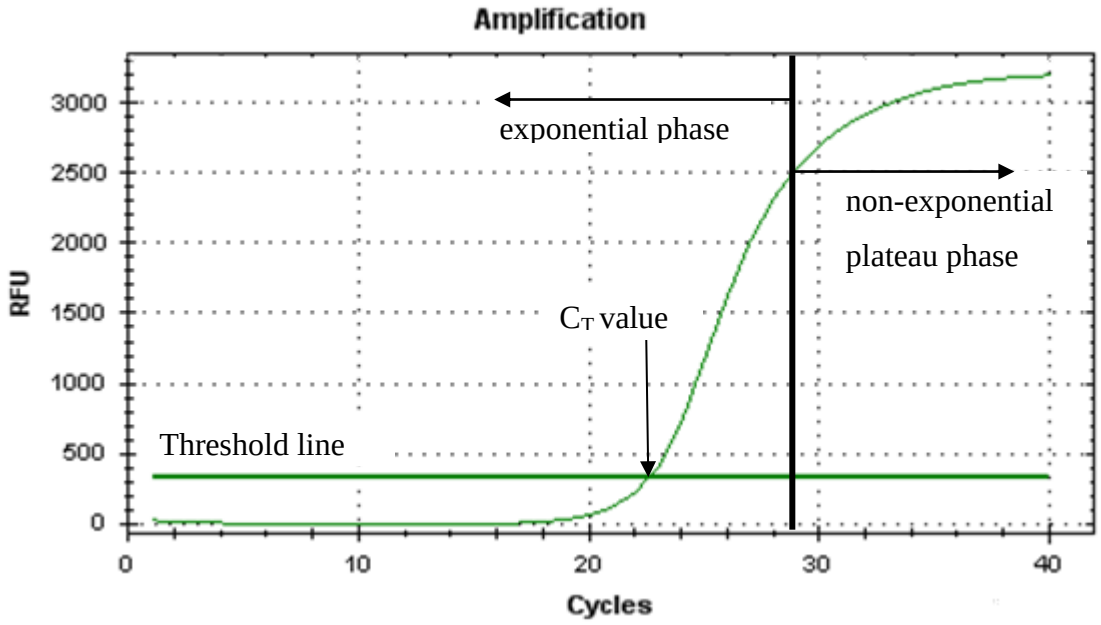

FIGURE 15. The PCR result plot

The C_T value of the reaction tells approximately the amount of the template present at the beginning of the run. If there is a large amount of the template at the start of the reaction, it needs only a few amplification cycles to give a fluorescent signal above the background, the reaction has a low or early C_T. Thus, low amount of the template in the beginning of the run needs more cycles before it gives a detectable signal. This reaction has high or late C_T. (Bio-rad 2006, 4.)

The primer design is the most important part of the qPCR. Primers have to be long enough to be specific for the target sequence but not too long. Normally primers are 18 to 30 bases long and they should contain more Gs and Cs than As and Ts because of the binding properties and melting temperature. The optimal melting temperature is between 55-60 °C and difference between forward and reverse primer should not be more than 4 °C. (Campbell & Farrel 2008, 388; Eurogentec, 28.) The ratio between CG and AT content should be 50-60 % for CG. There should not be repeats of Gs or Cs longer than three bases. Gs and Cs should be located at the end of the primer sequences instead of As or Ts. (Bio-rad 2006, 10.)

The primer sequences must not form secondary structures with it or with other primers. Unwanted primer sequences are hairpin sequences with itself or primer dimers with other primer, in consequence the DNA sample does not amplify. Primer testing is performed to find the optimal primer pairs. Even the primer pairs that have checked against secondary structures or primer dimers might not suit the purpose. (Campbell & Farrel 2008 388.)

To continue the qPCR optimization process dilution series of the template are run. In an ideal situation of the dilution series, amplification curves should occur evenly spaced. Equation that can be used is

$$2^n = \text{dilution factor}, \quad (1)$$

where n is the difference between the C_T values of the curves. For example, dilution series with 10-fold dilutions of DNA, $2^n = 10$, when n=3.32. This means that C_T values should be separated by 3.32 cycles. (Bio-rad 2006, 4-5.)

In an ideal situation, where C_T values are evenly spaced and the product has doubled perfectly, the standard curve will be linear. The standard curve is gathered by plotting the log of the dilution factor against the C_T value. The r or R^2 value shows the linearity of the data and this represents onwards the variability of the replicates. R^2 value should be >0.980. (Bio-rad 2006, 5.)

The standard curve gives an equation and there the slope is required to calculate the amplification efficiency (E) using the formula:

$$E = 10^{-1/slope}. \quad (2)$$

In the ideal case, the efficiency of the reaction is 2 and then the slope of the equation will be -3.32. The amplification efficiency is presented also as a percentage using formula:

$$\% \text{ Efficiency} = (E-1)*100\%. \quad (3)$$

In the ideal case the % efficiency will be 100% when (2-1)*100% = 100 %. In practice, the efficiency percent should be 90 - 105 %. If low efficiencies occur, primer design may have been poor or the conditions of the reaction have not been optimal. When the efficiency percent is too high, there may be a pipetting error or nonspecific products like primer-dimers have appeared. (Bio-rad 2006, 6.) The other reference is presenting the efficiency being acceptable between 90 - 110 %, when the slope should be between 3.1 - 3.58 (Eurogentec, 46). After the primers are designed, the assay has to be validated and optimized. This can be performed by identifying the optimal annealing temperature and/or constructing a standard curve to evaluate assay performance. (Bio-rad 2006, 11.)

Annealing temperature optimization is performed because SYBR Green I binds to all double-stranded DNA and the specificity of performed assay has to be checked by analyzing the reaction products. The products are run on an agarose gel as well as the melting curves are examined with the function of the qPCR instrument. The melting curves should show a single peak such as the single band on the agarose gel. The standard curve evaluation with dilution series were performed as showed earlier. (Bio-rad 2006, 12.)

There are different methods to calculate relative gene expression, for example the $2^{-\Delta\Delta CT}$ (Livak) method, the ΔC_T method using a reference gene and the Pfaffl method. The first two methods can be used and are valid when the amplification efficiencies of the target and reference genes are similar. The last method can be used when the target and reference gene efficiencies differ from each other. (Bio-rad 2006, 25; Eurogentec, 42.) The formula that is used in the last method is

$$R = \frac{(E_{target})^{\Delta C_T,\ target(calibrator-test)}}{(E_{ref})^{\Delta C_T,\ ref(calibrator-test)}}, \qquad (4)$$

where E_{target} = efficiency of the target gene, E_{ref} = efficiency of the reference gene, calibrator = control cross, test = knock-down cross, target = gene assayed, ref = reference gene

3.4.1 Reference gene

A reference gene is needed for successful quantitative polymerase chain reaction. The reference gene is used to normalize changes in expression levels of target genes during RNA extraction and cDNA synthesis when using qRT-PCR. In the ideal case, reference gene is expressed in all cell types and it should not affect the process. However, there is no universal reference gene. (Kriegova et al 2008, 2.)

As reference genes, the so-called housekeeping genes (HKGs) are used for normalization and are most commonly used as reference genes. When using housekeeping gene as a reference gene it needs to be validated as it is expressed in all cells but variation between different cell types and organs may be high. (Kriegova et al 2008, 2.)

4 MATERIALS AND METHODS

4.1 Flies and wasps

Most UAS fly stocks included in this study were obtained from the VDRC (Vienna Drosophila RNAi center). Hemolectin Δ-GAL4 was described in Sinenko & Mathey-Prevot (2004). Hemese-GAL4 was described in Zettervall et al (2004).The drivers were used to construct HmlΔ-GAL4;He-GAL4. Fatbody fly line wRHP;Fb-GAL4 line was obtained from the Bloomington stock center as P{w[+mW.hs]=GawB}FB. The control lines were $w1118_{ISO}$, $w1118_{empty}$ and $w1118_{RHP}$. The driver fly lines were backcrossed to the control line $w1118_{RHP}$ excluding Hemolectin Δ Hemese. *Leptopilina boulardi* line G486 was described by Russo et al (1996).

The flies were kept on a standard mashed potato diet at room temperature. The fly food recipe is seen in the appendices (Appendix 1, p.60). The GAL4-UAS system combined to RNA interference was used for tissue specific expression of transgenes. The wasps were kept also at room temperature and were harvested twice a week to apple juice food vials.

4.2 Encapsulation

Twenty virgin females and ten males were collected for one cross. Males did not need to be virgins. W_{RHP}, Hemolectin Δ Hemese (HH) and Fatbody (Fb) females were crossed to the RNAi males and the control males W_{EMPTY} and W_{ISO}. The crosses were repeated three times. The crosses are shown in table 1.

TABLE 1. Crosses for encapsulation. (wild type line W_{RHP}, Hemolectin Δ Hemese (HH), Fatbody (Fb). W_{ISO} and W_{empty} = genetic backgrounds)

Females	Males
wRHP;HmlΔ-GAL4;He-GAL4	w_{ISO};RNAi
wRHP;Fb-GAL4	w_{empty};RNAi
wRHP;HmlΔ-GAL4;He-GAL4	w_{ISO}
wRHP;Fb-GAL4	w_{empty}
wRHP	w_{ISO};RNAi
	w_{empty};RNAi

The flies were anaesthetized with CO_2 gas on the CO_2 pad and the virgins were separated from older flies using a dissecting microscope Olympus SZ61. Twenty virgin flies were put into each food vial. Male flies were separated similarly and added to the food vials to the females.

Crossed flies were allowed to lay eggs for one day before the "flipping" was started. The parental flies were transferred into the new food vials every day and the eggs were shifted to the 29 °C incubator. Three day old fly larvae were infected with parasitoid wasps for two hours. Twenty female and twenty male wasps were used for the infection and the wasps spent two hours in the same vial with the fly larvae. Female wasps deposited their eggs inside the fly larvae.

The infected fly larvae were incubated at 29 °C for 48 hours, and dissected after 48 hours during two hours. The dissection was performed using a dissecting microscope Olympus SZ61. Water was added to the food vial where the five days old fly larvae were. Water brought the larvae out from the food and the larvae were poured from the vial into a petridish. The fly larvae were picked up one by one and put in to a water drop on the object glass. The dissection was performed with two forceps by opening the larvae from the back and releasing the content to the water drop. The data sheet on which the results were marked can be found in the appendices (Appendix 2, p.61).

The wasp encapsulation assay was performed by dissecting 100 infected larvae of each cross. The results were gathered from three independent crosses of the RNAi constructs to the haemocytes and fatbody drivers, respectively. The dissected larvae were categorised as living, non melanized (living wasp larvae with no melanization); living, melanized (living wasp larvae, but melanisation present) and dead, melanized (dead encapsulated wasp egg or larvae). For the results, average values in percentages were calculated from dead melanized. Standard errors of the mean of dead melanized were also calculated.

A generalized linear mixed model was used to detect significant changes of the different treatments. The data were Arctangents-transformed and T-tests were used to compare the fraction of "dead-melanized" of individual crosses with each other.

4.3 Quantitative reverse transcription (real time) polymerase chain reaction

4.3.1 Primers

Primers were designed according to the PCR manual (Bio-Rad 2006). In this study five different serine proteases were used and for each protease three to four primer pairs were designed. The sequences of the genes were found in Flybase (Flybase 2012.) These genes are identified by their cognate gene (GC) numbers or their names. Serine proteases and the genetic backgrounds of the RNAi construct used in the study are presented in table 2.

TABLE 2. Serine proteases and the genetic backgrounds of their RNAi constructs used in the study. Others are identified by their CG -numbers except spirit.

Serine protease	Background
CG2056 also known as spirit	W_{empty} (KK)
CG1299	W_{empty} (KK)
CG18477	W_{ISO} (GD)
CG18478	W_{ISO} (GD)
CG31780	W_{ISO} (GD)

The primers were designed according to the primer design program Primer3 (Rozen & Skaletsky 2000, 365-386). The reverse primers were made with a reverse complement program (Stothard 2000). All the designed primers were checked with the Blast (Basic Local Alignment Search Tool) tool in Flybase (Flybase 2012). Sequences are shown in the Tables 3-7. Forward primers are presented as F and reverse primers as R. Melting temperatures (Tm) are added to tables.

TABLE 3. Sequences of Spirit (F = forward primer, R = reverse primer, Tm = melting temperature)

	Primers	**Tm (°C)**
F1	ACACACGAGTGGGTATGCAATGGG	64,4
R1	GGCAACTTCCAACATCCAAGTGCC	64,4
F2	TAACGGTGAAAATGCGAACAGTGTGC	63,2
R2	GCCAGTGCTTCTTGTTTGCCTGTTG	64,6
F3	TGGCTCATTTCAAGTGCAACCC	60,3
R3	GCATTCAGAAGATCGCACAA	55,3
F4	CCTCGTCATTCCATCTTCGT	57,3
R4	ACCAAAGTGGCCCTGTATTG	57,3

TABLE 4. Sequences of CG1299 (F = forward primer, R = reverse primer, Tm = melting temperature)

	Primers	**Tm (°C)**
F1	TTTCGGCGAGAGAGTGAAATCGG	62,4
R1	CTTCTCACCCACAACCCACT	59,4
F2	CACAATCTTGTTGCCCCTTT	55,3
R2	AGTTCACCAGCAAGATCGCT	57,3
F3	AAGTGCGGAGGAACCCTAAT	57,3
R3	ACTCACAATCTTGTTGCCCC	57,3
F4	GGAATGCTTTTCCACCTTGA	55,3
R4	AAAGTTCGCTGGAGTGCACT	57,3

TABLE 5. Sequences of CG18477 (F = forward primer, R = reverse primer, Tm = melting temperature)

	Primers	**Tm (°C)**
F1	CACAGTCGAGATGGCTTTGA	57,3
R1	TGTGTTCCGTTCGTTGTGAT	55,3
F2	GAGAGACAGACCGCAAGGAC	61,4
R2	CACCATCAAAAACTTGGGCT	55,3
F3	TGGTGACTTGCGCTCTAATG	57,3
R3	ATTAGGGGCTCAGGAGGAAA	57,3
F4	TTTGCCCTGATTTTCCTCAC	55,3
R4	GAAAAAGAAGCAGGCGAATG	55,3

TABLE 6. Sequences of CG18478 (F = forward primer, R = reverse primer, Tm = melting temperature)

	Primers	**Tm (°C)**
F1	AATTGCATTAGGGCCAACAG	55,3
R1	CTAAGCCGAGCCATTCAAAG	57,3
F2	CACAGGCATTCCACACAAAC	57,3
R2	CATAACTGAACGTGTGCGCT	57,3
F3	TACAGCAAGGCAACAGCAAC	57,3
R3	GCATGTCCTTTGTCCCTTGT	57,3

TABLE 7. Sequences of CG31780 (F = forward primer, R = reverse primer, Tm = melting temperature)

	Primers	**Tm (°C)**
F1	CACAGTCGAGATGGCTTTGA	57,3
R1	TGTGTTCCGTTCGTTGTGAT	55,3
F2	GAGAGACAGACCGCAAGGAC	61,4
R2	CACCATCAAAAACTTGGGCT	55,3
F3	TGGTGACTTGCGCTCTAATG	57,3
R3	ATTAGGGGCTCAGGAGGAAA	57,3
F4	TTTGCCCTGATTTTCCTCAC	55,3
R4	GAAAAAGAAGCAGGCGAATG	55,3

4.3.2 Primer testing

Primer testing was done to find the primer pairs, which functioned the best. There were four designed primer pairs for each gene, except for CG18478 where there were three. The samples were pipetted into 96-well plate as three technical replicates and master mix containing no reverse transcriptase solution was used as a control. The pipetting scheme is shown in table 8.

TABLE 8. Pipetting scheme for primer testing. (p = primer, 1.1, 1.2, 1.3 = technical replicates, no RT = no reverse transcriptase added (negative control)).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	p1.1	p2.1	p3.1	p4.1	p1.1	p2.1	p3.1	p4.1	p1.1	p2.1	p3.1	p4.1
B	p1.2	p2.2	p3.2	p4.2	p1.2	p2.2	p3.2	p4.2	p1.2	p2.2	p3.2	p4.2
C	p1.3	p2.3	p3.3	p4.3	p1.3	p2.3	p3.3	p4.3	p1.3	p2.3	p3.3	p4.3
D	p1.1 no RT	p2.1 no RT	p3.1 no RT	p4.1 no RT	p1.1 no RT	p2.1 no RT	p3.1 no RT	p4.1 no RT	p1.1 no RT	p2.1 no RT	p3.1 no RT	p4.1 no RT
E	p1.2 no RT	p2.2 no RT	p3.2 no RT	p4.2 no RT	p1.2 no RT	p2.2 no RT	p3.2 no RT	p4.2 no RT	p1.2 no RT	p2.2 no RT	p3.2 no RT	p4.2 no RT
F	p1.3 no RT	p2.3 no RT	p3.3 no RT	p4.3 no RT	p1.3 no RT	p2.3 no RT	p3.3 no RT	p4.3 no RT	p1.3 no RT	p2.3 no RT	p3.3 no RT	p4.3 no RT
G												
H												

For the primer testing, the primers were diluted in a ratio of 1:10. All PCR runs were performed in the 96-well plate. A commercial Bio-Rad iScript™ One-Step RT-PCR Kit With SYBR® Green was used for master mix preparation. The kit contained PCR grade water, 2XSYBR® green containing 0.4 mM of each dNTPs, magnesium ions, iTaq DNA polymerase, 20 nM fluorescein, SYBR® Green I dye and stabilizers, and reverse transcriptase solution containing optimized 50X formulation of iScript MMLV for one-step RT-PCR procedures. The reagents and quantities are shown in table 9.

TABLE 9. Master mix for one reaction

Reaction components	Volume (µl)
PCR grade water	10
2X SYBR Green RT-PCR-reaction mix	12.5
iScript reverse transcriptase for one-step RT-PCR	1
Forward primer	0.5
Reverse primer	0.5
RNA template	1

Two master mix solutions were made, one containing reverse transcriptase solution and one without. Master mix solutions were divided into 12 eppendorf tubes because there were four different primers and each of them contained four different primer pairs. The forward and reverse -primers were added into tubes. Mixtures were divided into single eppendorf tubes where the template was added and the mixtures were pipetted into 96-well plate according to the pipetting scheme. In the primer testing, W_{RHP} fly larvae was used as template RNA.

The primer testing showed which primer pairs were functional and those which were not. The PCR run was always performed with the same run program. The program is shown in table 10.

TABLE 10. PCR run program

Temperature (°C)	Time	
50	10 minutes	
95	3 minutes	
95	10 seconds	Repeated 40 times
60	30 seconds	
65	0.05 seconds	
95		

4.3.3 Dilution series

A dilution series was pipetted to determine the efficiency for each primer pair. Depending on the results of the dilution series the best primer pairs were chosen for the study.

The master mix solution was made as presented earlier, see table 3, p. 37. Three technical replicates of each primer pair were pipetted into the 96-well plate. The master mix was made and divided into four eppendorf tubes because of four different primer pairs. Forward and reverse -primers were added into the tubes. Solutions were divided into 21 eppendorf tubes and template was added.

The template was RNA from whole W_{RHP} fly larvae and diluted for the dilution series six times in ratio 1:10. Sample solutions were pipetted into the 96-well plate according to the pipetting scheme, shown in table 10.

TABLE 10. Pipetting scheme for dilution series. The template was diluted six times in ratio 1:10

	primer_1			**primer_2**			**primer_3**			**primer_4**		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1	1	1	1	1	1	1	1	1	1	1	1
B	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}
C	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}
D	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}
E	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
F	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}
G	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}
H												

According to the results of the dilution series, the efficiencies of the primers and efficiency percentages were calculated. The equations 2 and 3 were used and are seen on page 28. All the data from dilution series results are presented in the appendices (Appendix 3, p.62-73). Results from calculations are presented in table 11. The slope is added to the table because it is needed in the calculations.

TABLE 11. Efficiencies of primer pairs

Primer	**slope**	**E**	**E %**
spirit_1	-3,175	2,0652	106,5193
spirit_2	-2,95	2,1826	118,2645
CG1299_3	-3,91	1,8020	80,1999
CG1299_4	-3,63	1,8857	88,5741
CG18477_1	-3,315	2,0029	100,2899
CG18477_3	-3,99	1,7808	78,0847
CG18477_4	-3,61	1,8924	89,2380
CG18478_2	-3,125	2,0893	108,9296
CG18478_3	-2,706	2,3418	134,1796
CG31780_1	-3,539	1,9168	91,6751
CG31780_2	-2,369	2,6431	164,3133
CG31780_3	-3,0865	2,1086	110,8587

Agarose gel electrophoresis was done to check that there was only one product for each primer pair. Gel and buffer components and quantities are shown in tables 12 and 13.

TABLE 12. TAE buffer

10X TAE buffer components	**Quantity**
TRIS base	48.8 g
Acetic acid (17,4 M)	11.4 ml
EDTA	3.7 g

TABLE 13. Agarose gel

Agarose gel components	**Quantity**
1X TAE buffer	100 ml
Agarose powder	1.5 g
Ethidium bromide solution	3 µl

Two gels and 10X TAE buffer were prepared. 10X TAE buffer was diluted to 1X TAE and it was used for gel preparation. The gels were run for 1 hour and 45 minutes at 90 V.

Samples that were chosen for gel runs, had the lowest CT-values determined based by the dilution series. 25 µl of sample and 4 µl of loading dye were mixed and 20 µl of the mixture was loaded to the gel. Gene ruler^{TM} 50 bp DNA ladder was used as a standard and it was pipetted in the both sides of the gels. Loading schemes are shown in tables 14 and 15.

TABLE 14. Gel I loading scheme

standard		spirit_1	spirit_2	cg1299_3	cg1299_4	cg18477_1	cg18477_2	cg18477_4	cg18478_2		standard

TABLE 15. Gel II loading scheme

	standard	cg18478_3	cg31780_1	cg31780_2	cg31780_3	standard					

According to the agarose gel electrophoresis, the best primer pairs were chosen for the study. Results from the agarose gel electrophoresis are shown in figures 16 and 17 p. 40. Chosen primers are marked with the arrows.

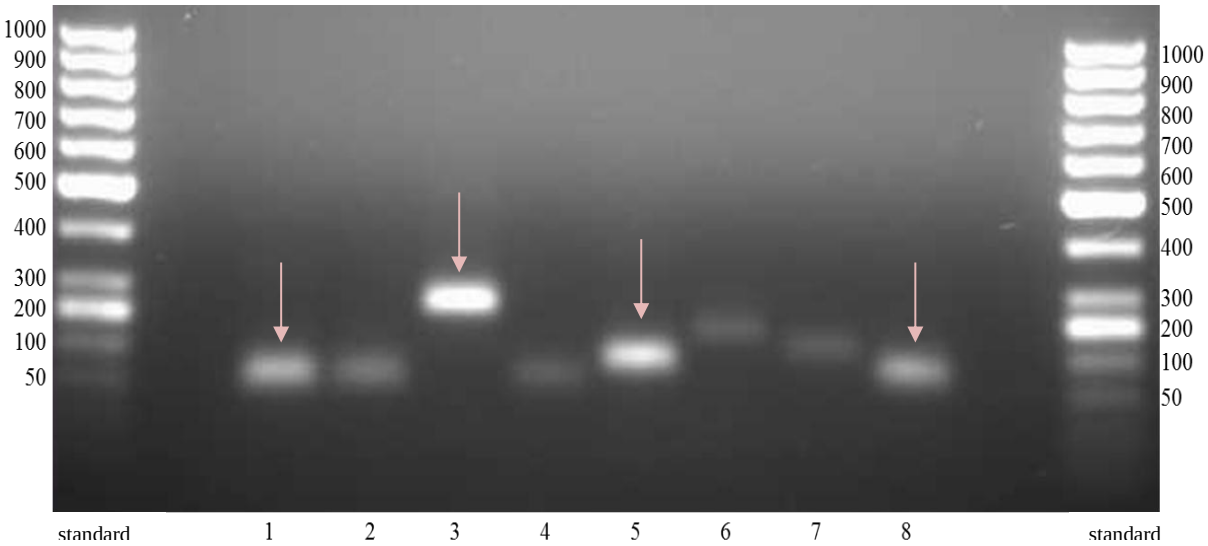

FIGURE 16. Gel I. Numbers indicate different serine protease primers (1 = spirit_1, 2 = spirit_2, 3 = CG1299_3, 4 = CG1299_4, 5 = CG18477_1, 6 = CG18477_2, 7 = CG18477_4, 8 = CG18478_2)

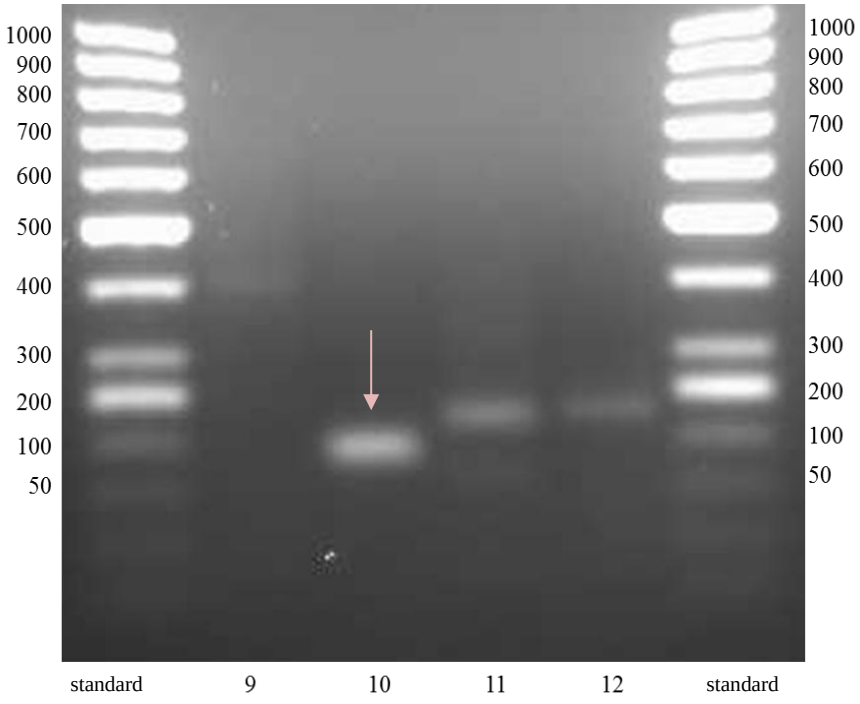

FIGURE 17. Gel II. Numbers indicate different serine protease primers (9 = CG18478_3, 10 = CG31780_1, 11 = CG31780_2, 12 = CG31780_3)

4.3.4 RNA extraction

RNA samples were taken from infected and uninfected *Drosophila* late L3 larvae. The samples were collected from the same crosses as used for encapsulation, excluding fatbody crosses (table 1, chapter 4.3, page. 31). These crosses were made three times and the samples were collected from each cross. Haemocyte samples were collected from 20 larvae, fatbody samples were collected from five larvae, and also five whole larvae were collected.

The larvae were washed three times in water with the help of a brush. The whole larvae were put into a 1.5 ml eppendorf tube. The blood cell samples were taken by bleeding each larva in 20 µl ice-cold PBS, transferring the blood cells into a 1.5 ml eppendorf tube and centrifuging at 700 rcf for five minutes. Supernatant was discarded and pellet was diluted in a small volume of PBS. Fatbody samples were taken by bleeding the larvae and separating the fatbody to the ice-cold PBS. The samples were stored at -80 °C before use. (Bio-Rad.)

RNA extraction was performed according to manufacturer's protocol, and using a commercial kit (Aurum^{TM} Total RNA Mini Kit, Bio-Rad). To extract the RNA from the sample, the samples were centrifuged through filter membrane. RNA binds to the RNA binding column and after three washes and removing the DNA with the DNase, the clean RNA sample was eluted into an eppendorf tube by elution solution. (Bio-Rad.)

4.3.5 Quantitative polymerase chain reaction of serine proteases

The best primers for the study were chosen based on the results of dilution series. The chosen primer pairs can be seen in figures in page 40. In the study the reference gene *RpL32* was used to set the baseline. *RpL32* stands for ribosomal protein L32 and it is a member of the 80 different ribosome proteins (Zhang, Ding, Sandford 2005, 2). Sequences of *RpL32* are shown in table 16.

TABLE 16. *RpL32* sequences (F = forward primer, R = reverse primer, Tm = melting temperature)

	Primer	**Tm (°C)**
F	TTC TGC ATG AGC AGG ACC TC	65,6
R	GGT TAC GGA TCG AAC AAG CG	66,5

The pipetting scheme is shown in table 17. One of the RNAi constructs was examined at a time. In the table 'w' stands for the W_{RHP} crossed to the RNAi constructs and 'HH' for HH crossed to the RNAi constructs.

There were three different type of samples, blood cells (BC), fatbody (FB) and whole larvae (WL) and all the samples were either uninfected or infected. Infected sample was marked as i. The samples were taken from three different crosses, marked as I, II and III. All the samples were pipetted as three replicates, marked as 1.1, 1.2 and 1.3. The negative controls in the study were samples without the template and samples containing no reverse transcriptase solution, marked as no RT and no template. Reactions without template were performed to control the quality of the master mix and the primer pairs. This showed that the master mix or the primers were not contaminated with RNA. Reactions without the enzyme were performed to control the quality of the templates. This showed that the prepared RNA samples were not contaminated with genomic DNA.

TABLE 17. Pipetting scheme of serine proteases (w = W_{RHP}, HH = Hemolectin Δ Hemese, no RT = sample without reverse transcriptase solution, no template = sample without template, I, II and III = different crosses, 1,1, 1,2, 1,3 = technical replicates)

	non-infected						**infected**					
	serine proteases			RpL32			serine proteases			RpL32		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	w I 1.1	w II 1.1	w III 1.1	w I 1.1	w II 1.1	w III 1.1	w I 1.1i	w II 1.1i	w III 1.1i	w I 1.1i	w II 1.1i	w III 1.1i
B	w I 1.2	w II 1.2	w III 1.2	w I 1.2	w II 1.2	w III 1.2	w I 1.2i	w II 1.2i	w III 1.2i	w I 1.2i	w II 1.2i	w III 1.2i
C	w I 1.3	w II 1.3	w III 1.3	w I 1.3	w II 1.3	w III 1.3	w I 1.3i	w II 1.3i	w III 1.3i	w I 1.3i	w II 1.3i	w III 1.3i
D	no RT	no RT	no RT	no RT	no RT	no RT	no RT	no RT	no temp	no RT	no RT	no temp
E	HH I 1.1	HH II 1.1	HH III 1.1	HH I 1.1	HH II 1.1	HH III 1.1	HH I 1.1i	HH II 1.1i	HH III 1.1i	HH I 1.1i	HH II 1.1i	HH III 1.1i
F	HH I 1.2	HH II 1.2	HH III 1.2	HH I 1.2	HH II 1.2	HH III 1.2	HH I 1.2i	HH II 1.2i	HH III 1.2i	HH I 1.2i	HH II 1.2i	HH III 1.2i
G	HH I 1.3	HH II 1.3	HH III 1.3	HH I 1.3	HH II 1.3	HH III 1.3	HH I 1.3i	HH II 1.3i	HH III 1.3i	HH I 1.3i	HH II 1.3i	HH III 1.3i
H	no RT	no RT	no RT	no RT	no RT	no RT	no RT	no RT	no RT	no RT	no RT	no RT

The master mix was prepared as presented in the primer testing section (table 9, chapter 4.4.2, page 37). Master mix solutions were prepared into the four tubes because of two different primer pairs with and without reverse transcriptase solution. These four tubes were divided into single tubes and template was added. The solutions were pipetted into the 96-well plate according to the pipetting scheme. The run performed as presented earlier (table 10, chapter 4.4.2, page 37).

5 RESULTS

5.1 Wasp assay

The objective of the wasp assay was to determine whether the larval immune system was able to kill the wasp larvae after serine proteases had been knocked down in haemocytes or fatbody. The results of the dissected *Drosophila* larvae were classified as "living, non-melanized", "living, melanized" and "dead, melanized" wasp larvae. "Living, non-melanized" wasp larvae indicated that the *Drosophila* immune system was not able to kill the parasitoid, whereas "dead, melanized" wasp larvae indicated a functional *Drosophila* immune system. The results are presented in tables 18 and 19 and figures 18 and 19. All data from wasp assay can be seen in the appendices (Appendix 4, p. 74-76).

When *spirit* was knocked down in the fatbody, the larval immune response responded weaker towards the infection than in wild type situation (figure 18). The same was seen when spirit was knocked down in haemocytes (figure 19). There was significant difference compared to its wild type and background.

The knock-down of CG1299 in haemocytes and fatbody did not give very notable reaction in either case. There was no significant difference when comparing CG18477, CG31780 and CG18478 to their backgrounds in both crosses; fatbody and haemocytes. In the fatbody crosses, serine proteases CG18477 and CG31780 showed differences when comparing them to wild type situation.

TABLE 18. The results from fatbody crosses (w_KK and w_GD = control lines, Fb = fatbody driver and w = W_{RHP})

	mean of dead melanized (%)	mean of dead melanized (%)	standard error of the mean of dead melanized (%)	standard error of the mean of dead melanized (%)
males/females	**Fb**	**w**	**Fb**	**w**
w_KK	30,63	44,19	12,14	8,73
spirit_KK	13,19	39,05	4,33	6,34
CG1299_KK	31,20	38,14	8,56	7,40
w_GD	34,05	55,41	2,94	3,86
CG18477_GD	39,12	64,11	8,06	6,32
CG31780_GD	50,79	71,10	4,78	0,54
CG18478_GD	51,27	57,84	9,27	4,48

TABLE 19. The results from haemocyte crosses (w_KK and w_GD = control lines, HH = Hemolectin Δ Hemese driver, w = W_{RHP})

	mean of dead melanized (%)	mean of dead melanized (%)	standard error of the mean of dead melanized (%)	standard error of the mean of dead melanized (%)
males/females	**HH**	**w**	**HH**	**w**
w_KK	23,09	44,19	11,13	8,73
spirit_KK	8,04	39,05	1,98	6,34
CG1299_KK	37,48	38,14	8,94	7,40
w_GD	49,57	55,41	13,82	3,86
CG18477_GD	56,23	64,11	6,93	6,32
CG31780_GD	72,34	71,10	6,36	0,54
CG18478_GD	58,81	57,84	2,53	4,48

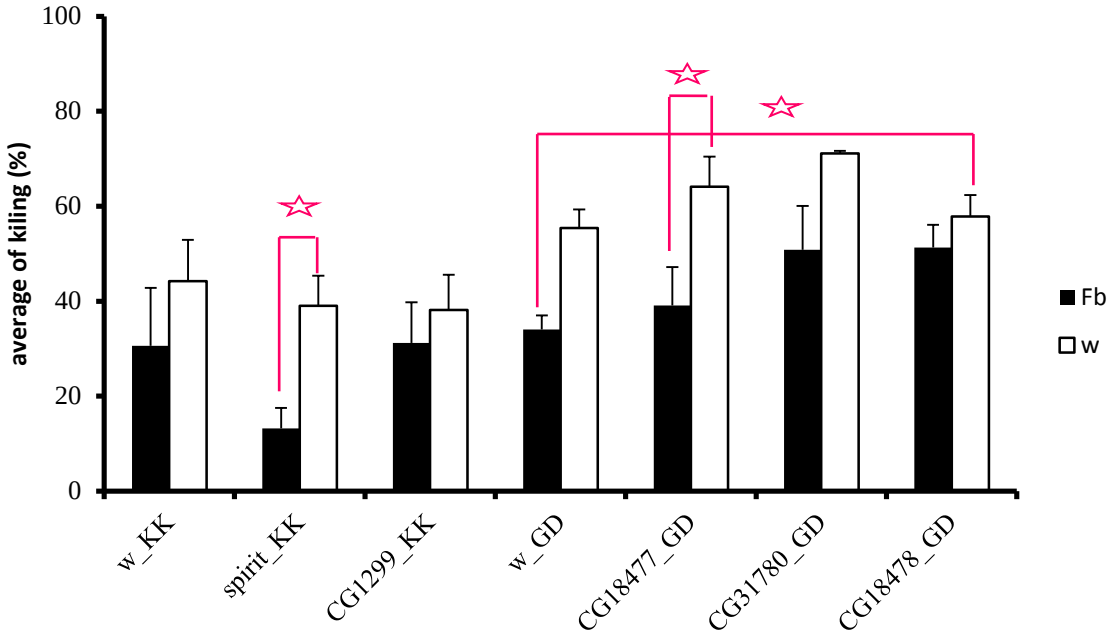

FIGURE 18. Fatbody-GAL4 crossed to serine protease RNAi costructs. Error bars are standard error of the mean of three different experiments. (w_KK = the genetic background of the VDRC-KK lines, w_GD = the genetic background of GD lines, ☆ = significant difference)

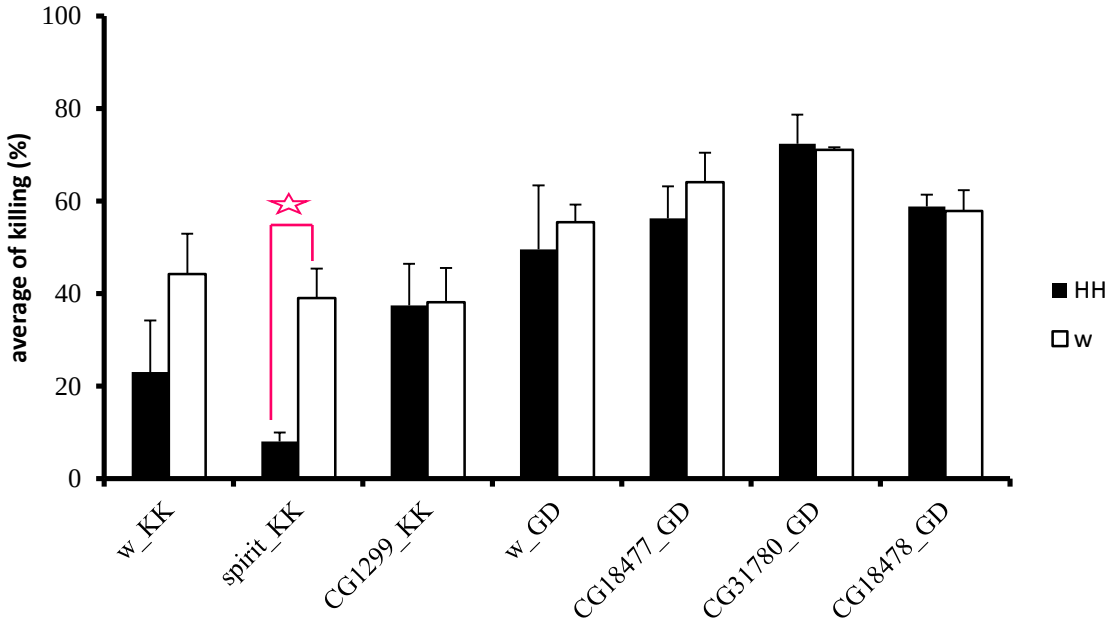

FIGURE 19. Haemocyte-GAL4 crossed to serine protease RNAi constructs. Error bars are standard error of the mean of three different experiments. (w_KK = the genetic background of the VDRC-KK lines, w_GD = the genetic background of GD lines, ☆ = significant difference)

5.2 Quantitative polymerase chain reaction assay

The qPCR experiments were performed to find out whether the RNAi constructs worked and if the genes were induced after wasp infection. All the qRT-PCR data is in the appendices (Appendix 5, p. 77-84). In table 21 the reduction of template is presented. Means are the percentages of template present after knock down of respective genes. Negative mean values indicate an increase of the RNA of the respective genes. The result is also presented in figure 20.

Only the knock down of *spirit* and CG18478 RNAi in the haemocytes reduced their respective messengers. When the reduction in the amount of template was calculated for the RNAi constructs of CG1299, CG18477 and CG31780, the knock down seemed to have increased the amount of RNA present for each of the genes. They are not plotted in figure 20.

TABLE 21. Reduction of template in haemocytes.

Tissue	**RNAi**	**mean**	**SEM**
haemocytes	spirit	58,15	0,03
haemocytes	CG1299	-46,60	
haemocytes	CG18478	56,54	10,01
haemocytes	CG18477	-191,31	
haemocytes	CG31780	-63,37	

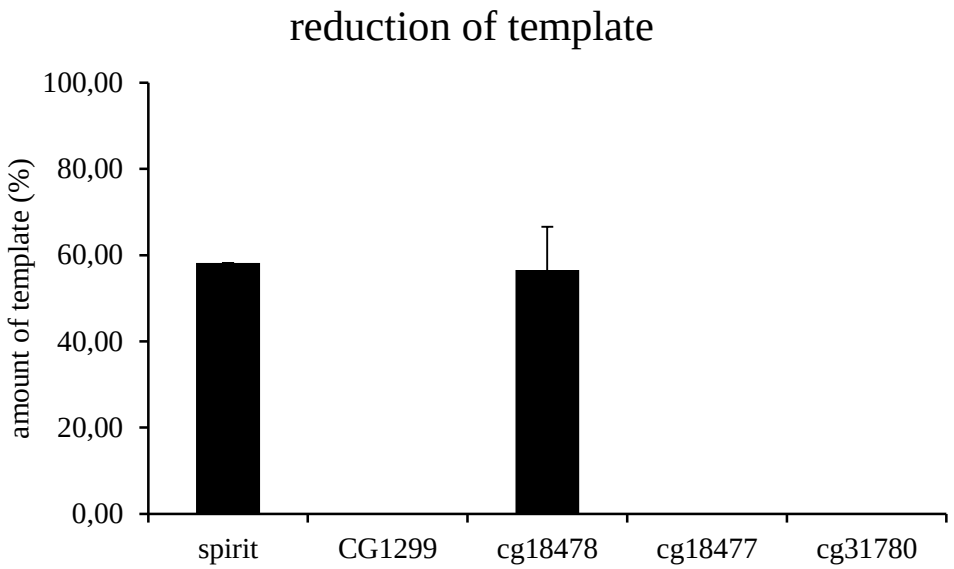

FIGURE 20. Reduction of template in haemocytes. The error bars indicate the standard error of the mean (SEM).

Table 22 and 23, p. 51, figure 21, p. 51 and figure 22, p. 52 show how much the genes were induced 48 hours after wasp infection in haemocytes and fatbody. The error bars are included, indicating the standard error of the mean (SEM). *Spirit* and CG1299 were not induced in the haemocytes. CG18478 was induced but the variation among the three different replicates was big. Also CG18477 and CG31780 were induced in haemocytes. The results indicated that *spirit* was not induced in fatbody either. CG1299 and CG18478 were induced very little in the fatbody. CG18477 was induced but CG31780 was not.

TABLE 22. Fold induction of *spirit*, *CG1299*, *CG18478*, *CG19477* and *CG31780* transcripts in haemocytes 48 hours after wasp infection. SEM represents the variation between the replicates

Tissue	**RNAi**	**mean**	**SEM**
haemocytes	spirit	0,83	0,48
haemocytes	CG1299	0,40	0,06
haemocytes	CG18478	30,74	27,19
haemocytes	CG18477	19,71	18,74
haemocytes	CG31780	9,81	8,38

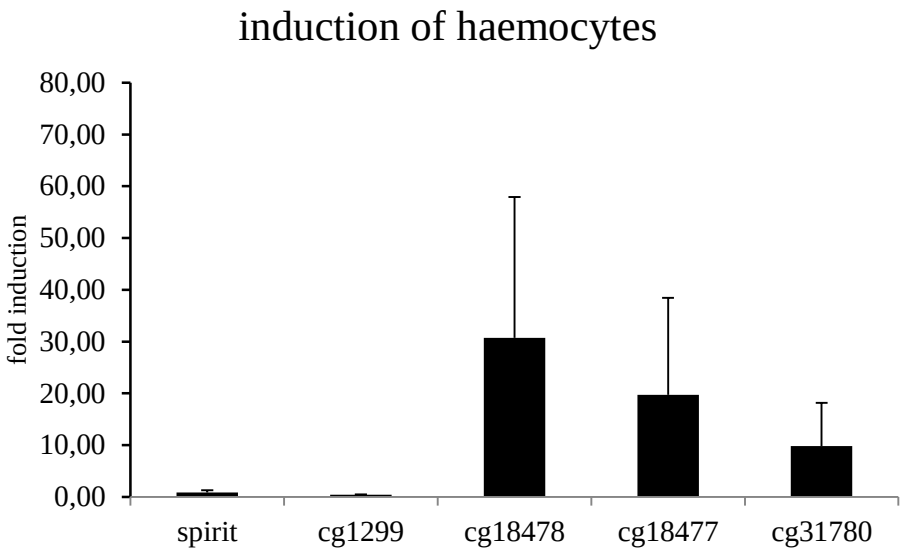

FIGURE 21. Fold induction of the indicated genes in haemocytes 48 hours after wasp infection. Error bars are SEM of three independent experiments

TABLE 23. Fold induction of *spirit*, *CG1299*, *CG18478*, *CG19477* and *CG31780* transcripts in fatbody 48 hours after wasp infection. SEM represents the variation between the replicates

Tissue	**RNAi**	**mean**	**SEM**
fatbody	spirit	0,81	0,30
fatbody	CG1299	2,79	1,53
fatbody	CG18478	1,15	0,56
fatbody	CG18477	19,90	8,72
fatbody	CG31780	2,51	0,40

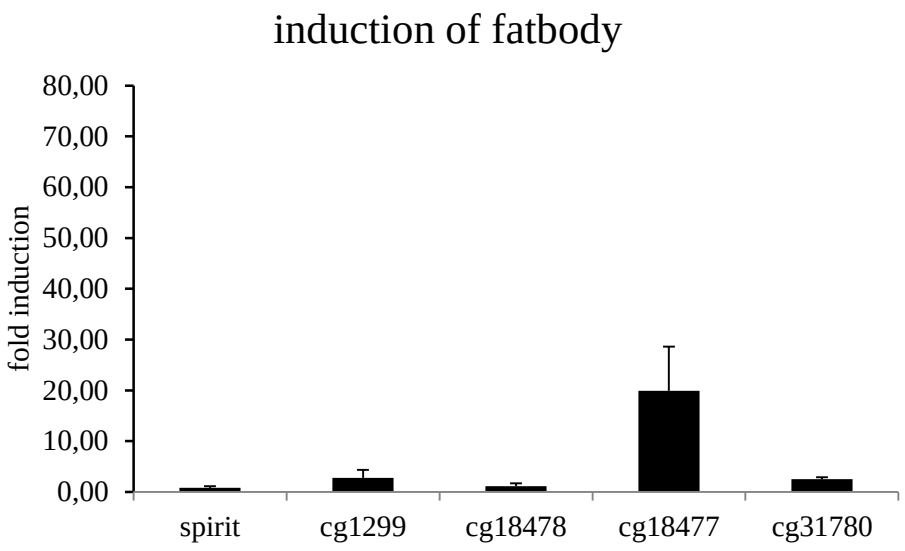

FIGURE 22. Fold induction of the indicated genes in fatbody 48 hours after wasp infection. Error bars are SEM of three independent experiments

6 DISCUSSION

The objective of this study was to investigate whether the serine proteases *spirit*, *CG1299*, *CG18478*, *CG18477* and *CG31780* played a role in the *Drosophila melanogaster* immune system during parasitoid wasp infection. The serine proteases were chosen for the study based on earlier experiments. *CG18478*, *CG18477* and *CG31780* were highly induced after wasp infection in a so far unpublished microarray experiment carried out in Dan Hultmark's group. *CG1299* was found as a suppressor in an unpublished screen for modifiers of an activated immune system. *Spirit* was also a suppressor of that activated immune phenotype, but not found in the screen. The hypothesis was that haemocytes would signal to fatbody to turn on Toll signalling in response to the attack by the parasitoid wasps. Spätzle, the only known Toll ligand, is activated by a serine protease cascade. Thus, serine proteases would be needed in the signalling process from haemocytes to fatbody. (Lemaitre & Hoffmann 2007; Shia et al 2009.)

The wasp assay part of the study gave important knowledge on the *Drosophila* immune system. The serine protease *spirit* was the most interesting. It showed a significant difference when knocked down in fatbody and haemocytes. In both cases the immune response could not fight properly against the wasp infection. Thus, *spirit* can be assumed as a part of the serine protease cascade that activates and cleaves Spätzle, which induces the Toll signaling pathway and leads immune response to fight against wasp infection. As Marmaras and Lampropoulou (2008, 193) mentioned, the Toll-signaling pathway is required for accurate encapsulation.

It has been shown earlier that the serine protease *spirit* is a part of Toll-signaling pathway in response to bacterial and fungi infections by RNA interference screen and microarrays. It is proven that *spirit* is involved in the *Drosophila* immune system. (Morales-Hojas, Vieira & Vieira 2009.) However, *spirit* was not induced in haemocytes or fatbody after wasp infection and thus might not be the serine protease that starts the cascade. Probably there is another serine protease that would be produced after wasp infection could activate *spirit*. It appeared in the end of the study that *spirit* has four annotated transcripts. The four transcripts are similar except the signaling peptides. This can be an explanation why *spirit* influences haemocytes and fatbody.

The female flies caused problems when they did not lay eggs properly. For the most part, this problem occurred with *spirit* and it can be seen in the data of wasp assay (Appendix 4, p. 74-76). In these cases there were only few larvae to infect and dissect. The explanation could be too old males, incorrect conditions such as too high temperature or somehow sick flies. Also, *spirit* crosses finished egg laying days earlier than other crosses.

The other serine proteases did not seem to have a big effect in either case but some weak changes in the immune response could be seen when *CG18477*, *CG31780* and *CG18478* were knocked down in the fatbody. The RNA interference constructs did not seem to function in the cases of *CG18477* and *CG31780*, explanation is given later. In the end of the study it turned out that *CG18477* and *CG31780* are gene duplications; this lead to the decision that they could be counted as the same gene. *CG18478* also has a duplicate gene but it was not included in this study. This, however, did not have an effect on the wasp assay results.

The idea of qRT-PCR was to find out how efficiently the RNAi constructs functioned. This could be seen by, how much the transcripts were reduced after RNAi, and how much and where the serine proteases were induced after wasp infection. The efficiencies of the primers were calculated based on dilution series. The ten-fold dilutions required very careful mixing and pipetting. The acceptable efficiencies are between 90-105 % according to Bio-Rad (2006) and 90-110 % according to Eurogentec. Two of the primers were outside of Bio-Rad range and CG1299 was outside of both. *Spirit* E % was 106.52, CG18478 E % was 108.93 and CG1299 E % was 80.20. The values of the range may indicate technical issues or primer dimers and the value below the range as for poor primer design or unfavorable reaction conditions (Bio-Rad 2006, 6). In the primer efficiency data, can be seen that CG1299_4 had higher efficiency (88.57 %) than chosen CG1299_3 (80.20 %). CG1299_3 was chosen because it had better result in the agarose gel electrophoresis, it gave higher C_T values from qRT-PCR and it was more reliable than CG1299_4. The crosses to test the efficiency of the serine protease RNAi construct analysis by qRT-PCR were only done with Hemolectin Δ Hemese and wRHP crossed to RNAi and control lines. For the further research the qRT-PCR analysis needs to be performed with fatbody driver as well to see if the RNAi constructs reduce the transcript in the fatbody.

The results are clear with *spirit* and *CG18478*, even though there was variation in reduction of template and induction of haemocytes. *CG1299*, *CG18477* and *CG31780* gave negative values in reduction of template. Presumably, the RNAi constructs of *CG18477* and *CG31780* did not work. The values of *CG18477* and *CG31780* were inconsistent. They should give similar results because they are gene duplications. The average value in reduction of template of *CG18477* was three times bigger than the average value of *CG31780*. Also differences in values between different crosses in reduction of template were notable. It can be seen in appendices (Appendix 5, p.82). Also *CG1299's* values vary between different crosses.

In haemocytes, the induction of *CG18477* was twice that of *CG31780* and the variation was also big. In the fatbody, the induction of *CG18477* was almost ten times bigger than the induction of *CG31780*. The reason for this is unclear but there are theories that it might be caused by biological variation. To clarify the results, the induction parts need to be repeated with *CG18477* and *CG31780*, and "living, non-melanized" and "dead, melanized" samples should be analysed separately. Before repeating the foregoing, qRT-PCR analysis needs to be performed using samples from fatbody driver crosses. Even though *CG18477* and *CG31780* varied in their fold inductions, they might be important for the immune response because they were induced in the previous microarray experiment. These RNAi constructs did not affect to the killing of wasp larvae and this may be caused that RNAi construct did not work.

CG18478 was strongly induced in the wasp microarray and that was the reason it was included in this study. The qPCR analysis showed that it was reduced 57 % but the encapsulation assay showed that it did not have an effect on the immune response. *CG18478* was induced in haemocytes but not in fatbody after wasp infection. This may indicate that it functions in haemocytes. However, further experiments are needed to clarify its role in the cellular immune response. *CG1299* was a suppressor in the genetic screen. It may not be involved in the immune response because it was not induced in haemocytes or fatbody after wasp infection. Because of variation the experiment should be repeated.

There are many factors in qRT-PCR that can affect the results, for example primer design, selection of reference gene and the run program. Temperatures in the run program if melting temperatures are variable between different primer pairs can effect. (Bio-rad 2006; Eurogentec.) Primers were designed with primer design program and were checked with different programs against, for example, primer dimers. The primer pairs were high-quality. The reference gene RpL32 was only reference gene for this study. The run program was made according to Bio-Rad instructions. To change conditions of run program may have an effect on results and could be worth to trying in further research.

The sampling was performed first and all the samples were frozen rapidly and put in the 80 °C freezer to prevent the RNA degradation. After all the samples were collected, the RNA preparation was started; it required melting and freezing samples again. The RNA extraction was performed on ice but centrifuging was performed at room temperature. This may have caused RNA degradation and have an effect on results. In the qRT-PCR part, the C_T values of the three technical replicates of one cross did not differ much but between different crosses they varied. Even though the samples were collected at the same time point, 48 hours post infection, the crossed flies were different in the three crosses. And even though the background of the crossed flies was always same, they were individuals and some of the flies could be genetically stronger or different than the others. The wasp also tries to block the larval immune system (Dubuffet et al 2009). These factors could explain the variation between the replicates. Sometimes the C_T values did not appear; in the results these were noted as N/A, which may indicate technical issues.

Melting temperatures of the primer pairs were inside the range of 55-60 °C excluding spirit_1, which melting temperature was 64.4 °C in both forward and reverse primers. Spirit_1, however, was chosen for the study because it was the best primer for its features. Also the reference gene had high melting temperatures, forward primer T_m was 65.6 °C and reverse primer T_m was 66.5 °C. These temperatures were outside of the given, ideal range and may have had an effect on the results. The melting temperatures between different primer pairs were similar. There was only 9.1 °C difference between the highest and lowest value.

The next step of this study could be to find out the reasons for *spirit* influencing both, haemocytes and fatbody. Also, cell counting could be performed to compare haemocyte levels in the non-infected and infected *Drosophila* larvae. Cell counting could be performed by FACS and fluorescent microscopy. To investigate whether the serine proteases used in the study, activate and cleave *Spätzle* and consequentially affect the Toll-signaling pathway functions, needs to be further examined with the reporter gene *Drosomycin* connected to green fluorescent protein (GFP). For example, knocking down *spirit* in haemocytes or in fatbody, the activation of Toll signalling should be blocked and the reporter gene would not be activated.

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APPENDICES

Appendix 1. Fly food recipe

Potato-mash food

5,5 l water
200 g mash potato powder
50 g agar
250 ml sugar syrup
80 g yeast
42 ml nipagin (10 % in 96 % ethanol) (100 g powder/ 1 liter 95 % Ethanol)
1,25 tsp ascorbic acid

Bring water to a boil, add yeast, syrup, mash potato powder, and agar. Boil up shortly and then reduce the heat and cook for additional 15 min. Cool the potato mash to 60 °C, add nipagin and ascorbic acid.

Ca. 500 vials or 100 bottles.

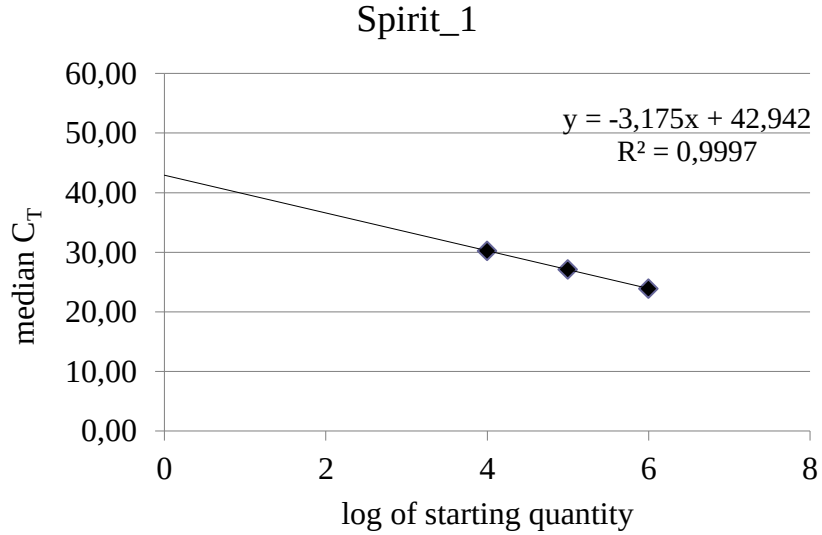
Appendix 2. Wasp data collecting form

crossing date infection date infection time				
genotypes	living	living mel.	dead mel.	sum
crossing date infection date infection time				
genotypes	living	living mel.	dead mel.	sum
crossing date infection date infection time				
genotypes	living	living mel.	dead mel.	sum
crossing date infection date infection time				
genotypes	living	living mel.	dead mel.	sum

Appendix 3. Dilution series data, spirit_1 1(12)

well	fluor	primer	dilution	C_T	Median C_T
A01	SYBR	spirit_1	10^0	23,67	23,86
A02	SYBR	spirit_1	10^0	23,90	
A03	SYBR	spirit_1	10^0	23,86	
B01	SYBR	spirit_1	10^1	27,13	27,13
B02	SYBR	spirit_1	10^1	27,18	
B03	SYBR	spirit_1	10^1	26,92	
C01	SYBR	spirit_1	10^2	30,35	30,21
C02	SYBR	spirit_1	10^2	30,21	
C03	SYBR	spirit_1	10^2	30,11	
D01	SYBR	spirit_1	10^3	32,40	32,29
D02	SYBR	spirit_1	10^3	32,23	
D03	SYBR	spirit_1	10^3	32,29	
E01	SYBR	spirit_1	10^4	32,69	32,72
E02	SYBR	spirit_1	10^4	32,72	
E03	SYBR	spirit_1	10^4	33,07	
F01	SYBR	spirit_1	10^5	32,76	32,71
F02	SYBR	spirit_1	10^5	32,71	
F03	SYBR	spirit_1	10^5	32,64	
G01	SYBR	spirit_1	10^6	32,83	32,83
G02	SYBR	spirit_1	10^6	32,99	
G03	SYBR	spirit_1	10^6	32,51	

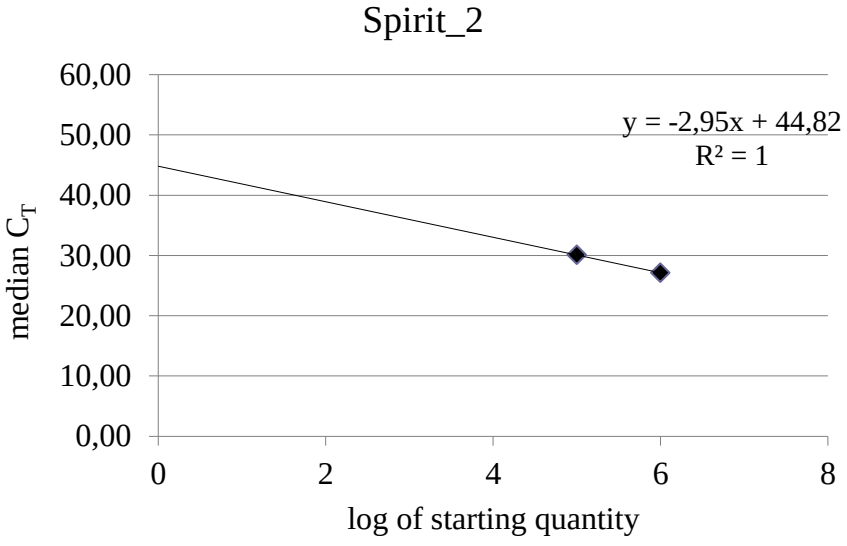
spirit_1			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	23,86	
10^1	5	27,13	
10^2	4	30,21	
10^3	3		32,29
10^4	2		32,72
10^5	1		32,71
10^6	0		32,83
slope	-3,175		
E(10^-1/slope)	2,065192931		
% Efficiency (E-1)*1(	106,5192931		



continued

well	fluor	primer	dilution	C_T	Median C_T
A04	SYBR	spirit_2	10^0	29,43	27,12
A05	SYBR	spirit_2	10^0	27,12	
A06	SYBR	spirit_2	10^0	26,27	
B04	SYBR	spirit_2	10^1	33,56	30,07
B05	SYBR	spirit_2	10^1	30,07	
B06	SYBR	spirit_2	10^1	29,58	
C04	SYBR	spirit_2	10^2	34,50	31,02
C05	SYBR	spirit_2	10^2	31,02	
C06	SYBR	spirit_2	10^2	30,28	
D04	SYBR	spirit_2	10^3	34,27	31,06
D05	SYBR	spirit_2	10^3	31,06	
D06	SYBR	spirit_2	10^3	30,71	
E04	SYBR	spirit_2	10^4	32,19	30,86
E05	SYBR	spirit_2	10^4	30,86	
E06	SYBR	spirit_2	10^4	30,55	
F04	SYBR	spirit_2	10^5	32,69	30,83
F05	SYBR	spirit_2	10^5	30,83	
F06	SYBR	spirit_2	10^5	30,57	
G04	SYBR	spirit_2	10^6	33,05	30,45
G05	SYBR	spirit_2	10^6	30,45	
G06	SYBR	spirit_2	10^6	30,41	

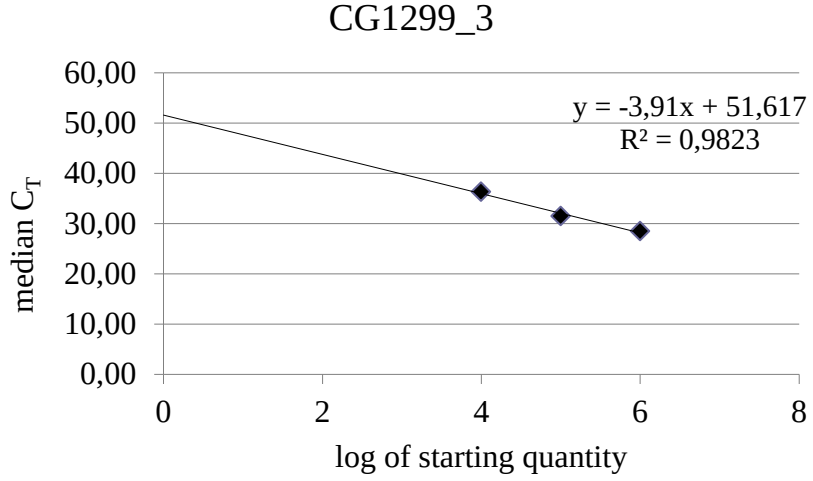
spirit_2			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	27,12	
10^1	5	30,07	
10^2	4		31,02
10^3	3		31,06
10^4	2		30,86
10^5	1		30,83
10^6	0		30,45
slope	-2,95		
E(10^-1/slope)	2,182644728		
% Efficiency (E-1)*1(	118,2644728		



CG1299_3 3(12)

well	fluor	primer	dilution	C_T	Median C_T
A07	SYBR	cg1299_3	10^0	30,57	28,46
A08	SYBR	cg1299_3	10^0	28,46	
A09	SYBR	cg1299_3	10^0	27,75	
B07	SYBR	cg1299_3	10^1	32,86	31,46
B08	SYBR	cg1299_3	10^1	31,46	
B09	SYBR	cg1299_3	10^1	31,18	
C07	SYBR	cg1299_3	10^2	37,31	36,28
C08	SYBR	cg1299_3	10^2	34,25	
C09	SYBR	cg1299_3	10^2	36,28	
D07	SYBR	cg1299_3	10^3	N/A	36,34
D08	SYBR	cg1299_3	10^3	37,07	
D09	SYBR	cg1299_3	10^3	35,60	
E07	SYBR	cg1299_3	10^4	N/A	
E08	SYBR	cg1299_3	10^4	N/A	
E09	SYBR	cg1299_3	10^4	N/A	
F07	SYBR	cg1299_3	10^5	N/A	
F08	SYBR	cg1299_3	10^5	N/A	
F09	SYBR	cg1299_3	10^5	N/A	
G07	SYBR	cg1299_3	10^6	N/A	
G08	SYBR	cg1299_3	10^6	N/A	
G09	SYBR	cg1299_3	10^6	N/A	

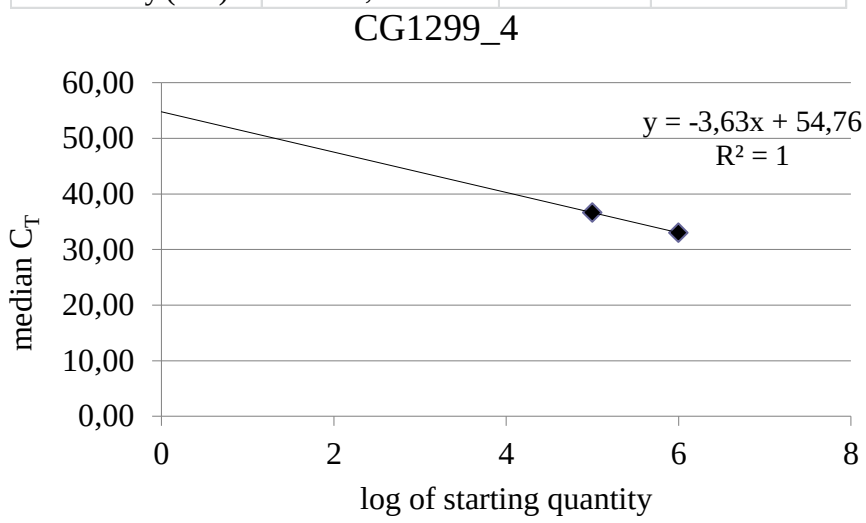
CG1299_3			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	28,46	
10^1	5	31,46	
10^2	4	36,28	
10^3	3		36,34
10^4	2		
10^5	1		
10^6	0		
slope	-3,91		
E(10^-1/slope)	1,80199871		
% Efficiency (E-1)*1	80,19987098		



CG1299_4 4(12)

well	fluor	primer	dilution	C_T	Median C_T
A10	SYBR	cg1299_4	10^0	35,20	32,98
A11	SYBR	cg1299_4	10^0	32,98	
A12	SYBR	cg1299_4	10^0	31,37	
B10	SYBR	cg1299_4	10^1	38,53	36,61
B11	SYBR	cg1299_4	10^1	35,45	
B12	SYBR	cg1299_4	10^1	36,61	
C10	SYBR	cg1299_4	10^2	N/A	
C11	SYBR	cg1299_4	10^2	N/A	
C12	SYBR	cg1299_4	10^2	N/A	
D10	SYBR	cg1299_4	10^3	N/A	
D11	SYBR	cg1299_4	10^3	N/A	
D12	SYBR	cg1299_4	10^3	N/A	
E10	SYBR	cg1299_4	10^4	N/A	
E11	SYBR	cg1299_4	10^4	N/A	
E12	SYBR	cg1299_4	10^4	N/A	
F10	SYBR	cg1299_4	10^5	N/A	
F11	SYBR	cg1299_4	10^5	N/A	
F12	SYBR	cg1299_4	10^5	N/A	
G10	SYBR	cg1299_4	10^6	N/A	
G11	SYBR	cg1299_4	10^6	N/A	
G12	SYBR	cg1299_4	10^6	N/A	

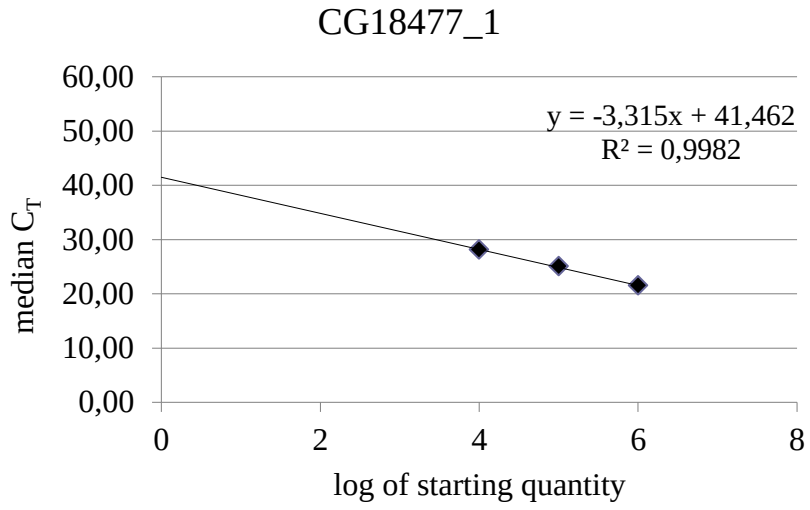
CG1299_4			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	32,98	
10^1	5	36,61	
10^2	4		
10^3	3		
10^4	2		
10^5	1		
10^6	0		
slope	-3,63		
E(10^-1/slope)	1,885741217		
% Efficiency (E-1)*1(	88,57412172		



CG18477_1 5(12)

well	fluor	primer	dilution	C_T	Median C_T
A01	SYBR	cg18477_1	10^0	23,49	21,49
A02	SYBR	cg18477_1	10^0	21,49	
A03	SYBR	cg18477_1	10^0	21,26	
B01	SYBR	cg18477_1	10^1	27,32	25,05
B02	SYBR	cg18477_1	10^1	24,98	
B03	SYBR	cg18477_1	10^1	25,05	
C01	SYBR	cg18477_1	10^2	30,24	28,12
C02	SYBR	cg18477_1	10^2	28,12	
C03	SYBR	cg18477_1	10^2	28,07	
D01	SYBR	cg18477_1	10^3	31,10	29,50
D02	SYBR	cg18477_1	10^3	29,50	
D03	SYBR	cg18477_1	10^3	29,14	
E01	SYBR	cg18477_1	10^4	30,00	29,54
E02	SYBR	cg18477_1	10^4	29,54	
E03	SYBR	cg18477_1	10^4	29,18	
F01	SYBR	cg18477_1	10^5	30,39	29,40
F02	SYBR	cg18477_1	10^5	29,40	
F03	SYBR	cg18477_1	10^5	29,13	
G01	SYBR	cg18477_1	10^6	30,71	29,46
G02	SYBR	cg18477_1	10^6	29,18	
G03	SYBR	cg18477_1	10^6	29,46	

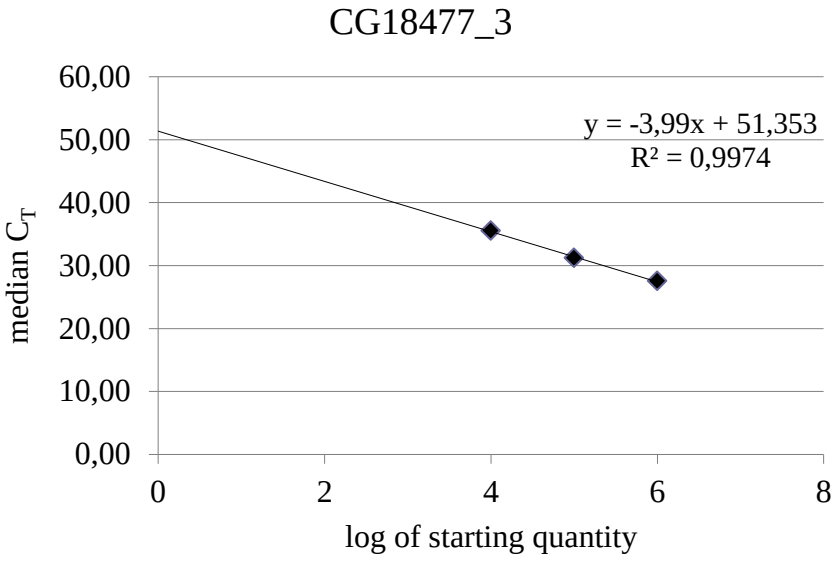
CG18477_1			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	21,49	
10^1	5	25,05	
10^2	4	28,12	
10^3	3		29,5
10^4	2		29,54
10^5	1		29,40
10^6	0		29,46
slope	-3,315		
E(10^-1/slope)	2,002899348		
% Efficiency (E-1)*1(	100,2899348		



CG18477_3 6(12)

well	fluor	primer	dilution	C_T	Median C_T
A04	SYBR	cg18477_3	10^0	31,71	27,53
A05	SYBR	cg18477_3	10^0	27,53	
A06	SYBR	cg18477_3	10^0	27,23	
B04	SYBR	cg18477_3	10^1	35,37	31,17
B05	SYBR	cg18477_3	10^1	31,17	
B06	SYBR	cg18477_3	10^1	30,39	
C04	SYBR	cg18477_3	10^2	37,61	35,51
C05	SYBR	cg18477_3	10^2	34,57	
C06	SYBR	cg18477_3	10^2	35,51	
D04	SYBR	cg18477_3	10^3	N/A	
D05	SYBR	cg18477_3	10^3	N/A	
D06	SYBR	cg18477_3	10^3	N/A	
E04	SYBR	cg18477_3	10^4	N/A	
E05	SYBR	cg18477_3	10^4	N/A	
E06	SYBR	cg18477_3	10^4	N/A	
F04	SYBR	cg18477_3	10^5	N/A	
F05	SYBR	cg18477_3	10^5	N/A	
F06	SYBR	cg18477_3	10^5	N/A	
G04	SYBR	cg18477_3	10^6	N/A	
G05	SYBR	cg18477_3	10^6	N/A	
G06	SYBR	cg18477_3	10^6	N/A	

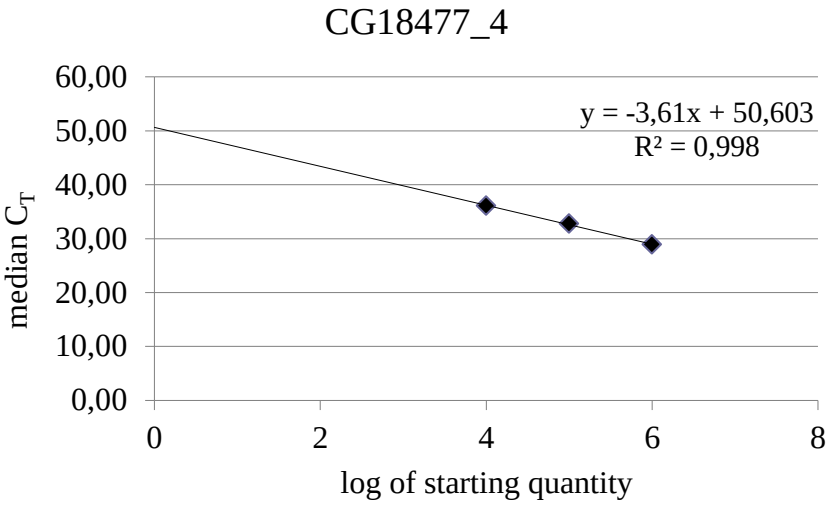
CG18477_3			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	27,53	
10^1	5	31,17	
10^2	4	35,51	
10^3	3		
10^4	2		
10^5	1		
10^6	0		
slope	-3,99		
E(10^-1/slope)	1,780846825		
% Efficiency (E-1)*1	78,08468253		



CG18477_4 7(12)

well	fluor	primer	dilution	C_T	Median C_T
A07	SYBR	cg18477_4	10^0	31,22	28,85
A08	SYBR	cg18477_4	10^0	28,85	
A09	SYBR	cg18477_4	10^0	28,17	
B07	SYBR	cg18477_4	10^1	33,20	32,74
B08	SYBR	cg18477_4	10^1	32,74	
B09	SYBR	cg18477_4	10^1	32,19	
C07	SYBR	cg18477_4	10^2	37,14	36,07
C08	SYBR	cg18477_4	10^2	36,07	
C09	SYBR	cg18477_4	10^2	35,27	
D07	SYBR	cg18477_4	10^3	N/A	38,37
D08	SYBR	cg18477_4	10^3	37,68	
D09	SYBR	cg18477_4	10^3	39,06	
E07	SYBR	cg18477_4	10^4	N/A	
E08	SYBR	cg18477_4	10^4	N/A	
E09	SYBR	cg18477_4	10^4	N/A	
F07	SYBR	cg18477_4	10^5	N/A	
F08	SYBR	cg18477_4	10^5	N/A	
F09	SYBR	cg18477_4	10^5	N/A	
G07	SYBR	cg18477_4	10^6	N/A	
G08	SYBR	cg18477_4	10^6	N/A	
G09	SYBR	cg18477_4	10^6	N/A	

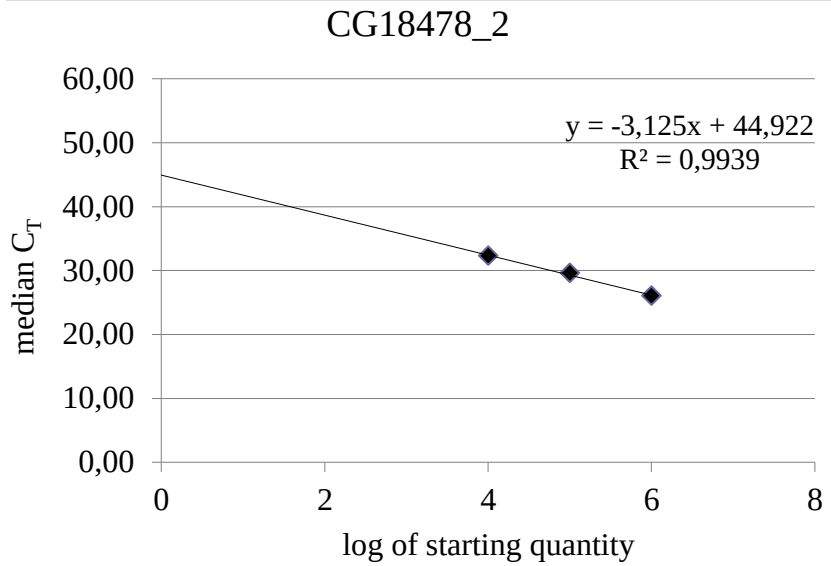
CG18477_4			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	28,85	
10^1	5	32,74	
10^2	4	36,07	
10^3	3		38,37
10^4	2		
10^5	1		
10^6	0		
slope	-3,61		
E(10^-1/slope)	1,892379829		
% Efficiency (E-1)*1	89,23798291		



CG18478_2 8(12)

well	fluor	primer	dilution	C_T	Median C_T
A10	SYBR	cg18478_2	10^0	27,08	26,03
A11	SYBR	cg18478_2	10^0	26,03	
A12	SYBR	cg18478_2	10^0	25,79	
B10	SYBR	cg18478_2	10^1	30,39	29,58
B11	SYBR	cg18478_2	10^1	29,57	
B12	SYBR	cg18478_2	10^1	29,58	
C10	SYBR	cg18478_2	10^2	34,10	32,28
C11	SYBR	cg18478_2	10^2	32,28	
C12	SYBR	cg18478_2	10^2	32,15	
D10	SYBR	cg18478_2	10^3	35,54	35,21
D11	SYBR	cg18478_2	10^3	35,21	
D12	SYBR	cg18478_2	10^3	34,15	
E10	SYBR	cg18478_2	10^4	35,62	34,70
E11	SYBR	cg18478_2	10^4	34,70	
E12	SYBR	cg18478_2	10^4	33,69	
F10	SYBR	cg18478_2	10^5	37,74	35,78
F11	SYBR	cg18478_2	10^5	35,78	
F12	SYBR	cg18478_2	10^5	34,18	
G10	SYBR	cg18478_2	10^6	35,90	35,90
G11	SYBR	cg18478_2	10^6	34,87	
G12	SYBR	cg18478_2	10^6	37,05	

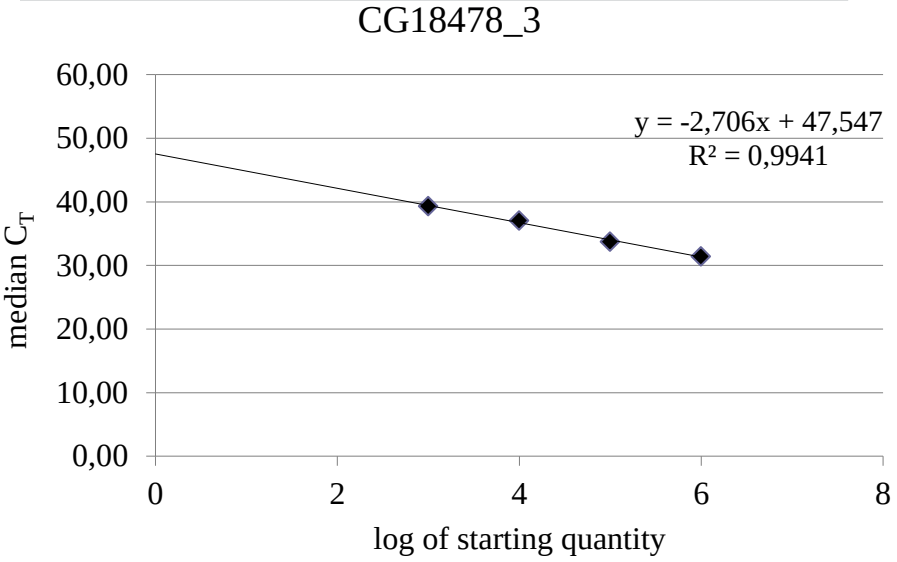
CG18478_2			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	26,03	
10^1	5	29,58	
10^2	4	32,28	
10^4	3		32,21
10^3	2		34,70
10^5	1		35,78
10^6	0		35,9
slope	-3,125		
E(10^-1/slope)	2,089296131		
% Efficiency (E-1)*1(	108,9296131		



CG18478_3 9(12)

well	fluor	primer	dilution	C_T	Median C_T
A01	SYBR	cg18478_3	10^0	35,76	31,4
A02	SYBR	cg18478_3	10^0	31,4	
A03	SYBR	cg18478_3	10^0	31,08	
B01	SYBR	cg18478_3	10^1	36,24	33,72
B02	SYBR	cg18478_3	10^1	33,72	
B03	SYBR	cg18478_3	10^1	33,12	
C01	SYBR	cg18478_3	10^2	37,05	37,05
C02	SYBR	cg18478_3	10^2	37,17	
C03	SYBR	cg18478_3	10^2	36,55	
D01	SYBR	cg18478_3	10^3	39,59	39,31
D02	SYBR	cg18478_3	10^3	N/A	
D03	SYBR	cg18478_3	10^3	39,03	
E01	SYBR	cg18478_3	10^4	N/A	
E02	SYBR	cg18478_3	10^4	N/A	
E03	SYBR	cg18478_3	10^4	N/A	
F01	SYBR	cg18478_3	10^5	N/A	
F02	SYBR	cg18478_3	10^5	N/A	
F03	SYBR	cg18478_3	10^5	N/A	
G01	SYBR	cg18478_3	10^6	N/A	
G02	SYBR	cg18478_3	10^6	N/A	
G03	SYBR	cg18478_3	10^6	N/A	

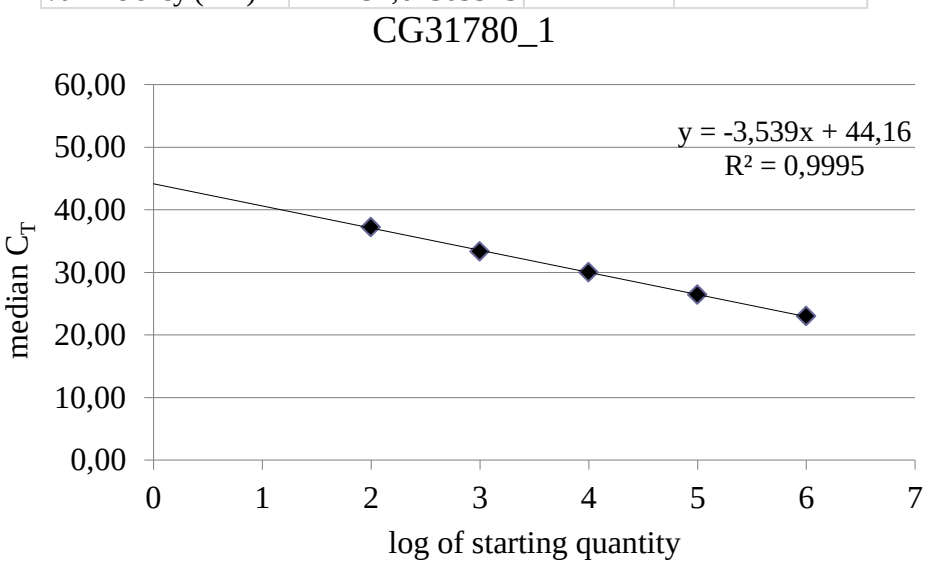
CG18478_3			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	31,40	
10^1	5	33,72	
10^2	4	37,05	
10^3	3	39,31	
10^4	2		
10^5	1		
10^6	0		
slope	-2,706		
E(10^-1/slope)	2,341796486		
% Efficiency (E-1)*1(	134,1796486		



CG31780_1 10(12)

well	fluor	primer	dilution	C_T	Median C_T
A04	SYBR	cg31780_1	10^0	23,72	23
A05	SYBR	cg31780_1	10^0	23	
A06	SYBR	cg31780_1	10^0	22,51	
B04	SYBR	cg31780_1	10^1	28,3	26,42
B05	SYBR	cg31780_1	10^1	26,42	
B06	SYBR	cg31780_1	10^1	25,99	
C04	SYBR	cg31780_1	10^2	31,16	30,02
C05	SYBR	cg31780_1	10^2	30,02	
C06	SYBR	cg31780_1	10^2	29,6	
D04	SYBR	cg31780_1	10^3	35,24	33,35
D05	SYBR	cg31780_1	10^3	33,35	
D06	SYBR	cg31780_1	10^3	32,85	
E04	SYBR	cg31780_1	10^4	37,18	37,23
E05	SYBR	cg31780_1	10^4	37,28	
E06	SYBR	cg31780_1	10^4	N/A	
F04	SYBR	cg31780_1	10^5	N/A	
F05	SYBR	cg31780_1	10^5	N/A	
F06	SYBR	cg31780_1	10^5	N/A	
G04	SYBR	cg31780_1	10^6	N/A	
G05	SYBR	cg31780_1	10^6	N/A	
G06	SYBR	cg31780_1	10^6	N/A	

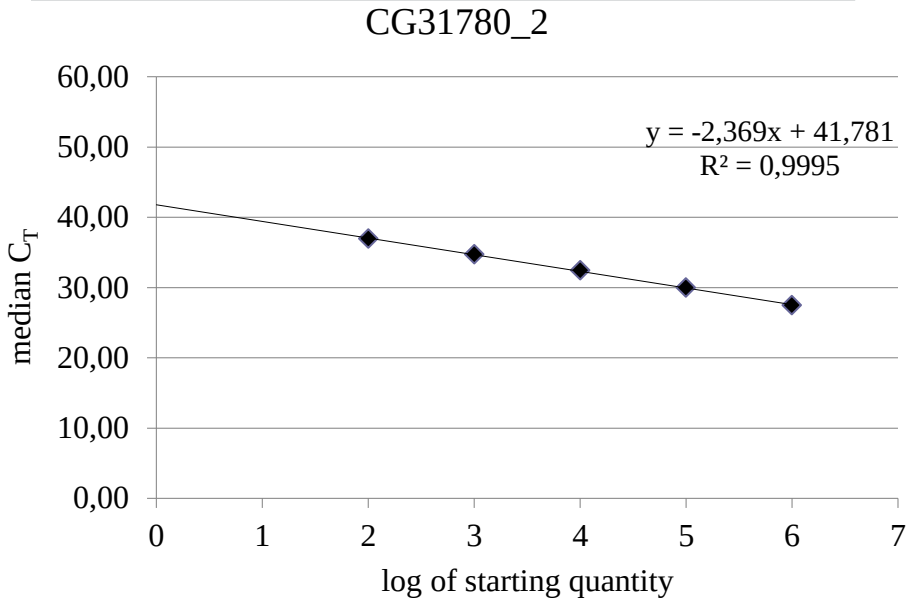
CG31780_1			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	23,00	
10^1	5	26,42	
10^2	4	30,02	
10^3	3	33,35	
10^4	2	37,23	
10^5	1		
10^6	0		
slope	-3,539		
E(10^-1/slope)	1,916750991		
% Efficiency (E-1)*1(	91,67509913		



CG31780_2 11(12)

well	fluor	primer	dilution	C_T	Median C_T
A07	SYBR	cg31780_2	10^0	N/A	27,48
A08	SYBR	cg31780_2	10^0	27,95	
A09	SYBR	cg31780_2	10^0	27,01	
B07	SYBR	cg31780_2	10^1	N/A	29,97
B08	SYBR	cg31780_2	10^1	29,97	
B09	SYBR	cg31780_2	10^1	N/A	
C07	SYBR	cg31780_2	10^2	N/A	32,405
C08	SYBR	cg31780_2	10^2	32,72	
C09	SYBR	cg31780_2	10^2	32,09	
D07	SYBR	cg31780_2	10^3	N/A	34,72
D08	SYBR	cg31780_2	10^3	35,14	
D09	SYBR	cg31780_2	10^3	34,3	
E07	SYBR	cg31780_2	10^4	38,06	36,95
E08	SYBR	cg31780_2	10^4	36,95	
E09	SYBR	cg31780_2	10^4	36,48	
F07	SYBR	cg31780_2	10^5	38,23	37,05
F08	SYBR	cg31780_2	10^5	36,12	
F09	SYBR	cg31780_2	10^5	37,05	
G07	SYBR	cg31780_2	10^6	39,51	36,53
G08	SYBR	cg31780_2	10^6	36,28	
G09	SYBR	cg31780_2	10^6	36,53	

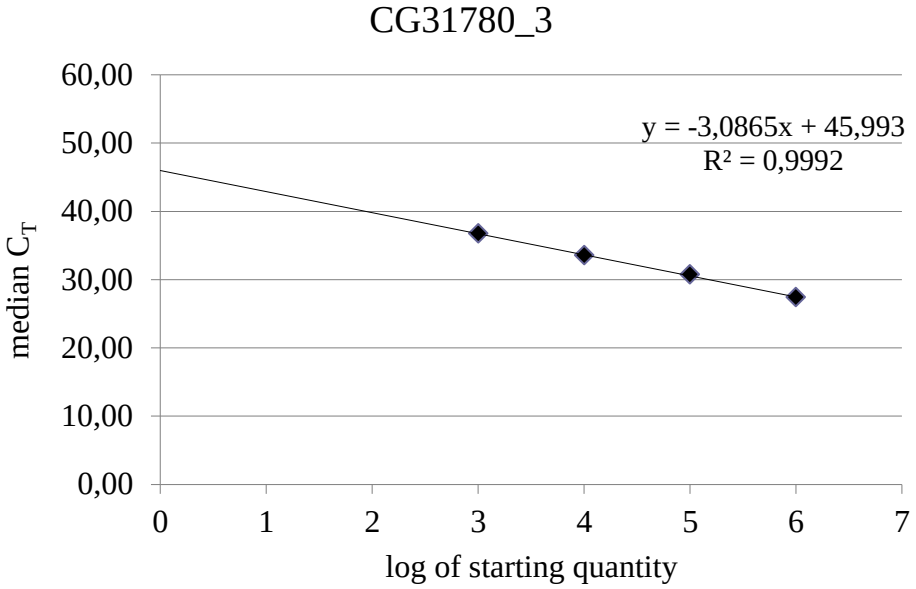
CG31780_2			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	27,48	
10^1	5	29,97	
10^2	4	32,41	
10^3	3	34,72	
10^4	2	36,95	
10^5	1		37,05
10^6	0		36,53
slope	-2,369		
E(10^-1/slope)	2,643133125		
% Efficiency (E-1)*1	164,3133125		



CG31780_3 12(12)

well	fluor	primer	dilution	C_T	Median C_T
A10	SYBR	cg31780_3	10^0	37,98	27,4
A11	SYBR	cg31780_3	10^0	27,4	
A12	SYBR	cg31780_3	10^0	26,91	
B10	SYBR	cg31780_3	10^1	35,09	30,72
B11	SYBR	cg31780_3	10^1	30,72	
B12	SYBR	cg31780_3	10^1	30,55	
C10	SYBR	cg31780_3	10^2	N/A	33,55
C11	SYBR	cg31780_3	10^2	33,73	
C12	SYBR	cg31780_3	10^2	33,37	
D10	SYBR	cg31780_3	10^3	N/A	36,745
D11	SYBR	cg31780_3	10^3	36,93	
D12	SYBR	cg31780_3	10^3	36,56	
E10	SYBR	cg31780_3	10^4	N/A	
E11	SYBR	cg31780_3	10^4	N/A	
E12	SYBR	cg31780_3	10^4	N/A	
F10	SYBR	cg31780_3	10^5	N/A	38,12
F11	SYBR	cg31780_3	10^5	N/A	
F12	SYBR	cg31780_3	10^5	38,12	
G10	SYBR	cg31780_3	10^6	N/A	38,33
G11	SYBR	cg31780_3	10^6	N/A	
G12	SYBR	cg31780_3	10^6	38,33	

CG31780_3			
dilution	log starting quantity	median_Ct	excluded values
10^0	6	27,40	
10^1	5	30,72	
10^2	4	33,55	
10^3	3	36,75	
10^4	2		
10^5	1		38,12
10^6	0		38,33
slope	-3,0865		
E(10^-1/slope)	2,10858725		
% Efficiency (E-1)*1(	110,858725		



Appendix 4. Wasp data, fatbody 1(3)

females X males	[1]	[2]	[3]	sum	dead melanized [%]	mean score	mean_dead melanized [%]	mean of mean score	SD_mean of dead melanized [%]	SD_mean of mean score	SEM_mean of dead melanized [%]	SEM_mean of mean score
Fb x cg1299	60	31	15	106	14,15	1,58	31,20	1,89	14,82	0,28	8,56	0,16
Fb x cg1299	43	16	41	100	41,00	1,98						
Fb x cg1299	27	37	40	104	38,46	2,13						
Fb x cg18477	29	28	50	107	46,73	2,20	39,12	2,09	13,96	0,31	8,06	0,18
Fb x cg18477	48	29	23	100	23,00	1,75						
Fb x cg18477	15	40	50	105	47,62	2,33						
Fb x cg18478	16	29	59	104	56,73	2,41	51,27	2,32	8,28	0,17	4,78	0,10
Fb x cg18478	30	30	43	103	41,75	2,13						
Fb x cg18478	14	32	57	103	55,34	2,42						
Fb x cg31780	12	22	66	100	66,00	2,54	50,79	2,36	16,06	0,18	9,27	1,36
Fb x cg31780	16	50	34	100	34,00	2,18						
Fb x cg31780	16	34	55	105	52,38	2,37						
Fb x spirit	72	11	22	105	20,95	1,52	13,19	1,40	7,49	0,20	4,33	0,81
Fb x spirit	89	5	6	100	6,00	1,17						
Fb x spirit	65	25	13	103	12,62	1,50						
Fb x Wiso	45	18	30	93	32,26	1,84	34,05	1,98	5,10	0,12	2,94	1,14
Fb x Wiso	24	48	31	103	30,10	2,07						
Fb x Wiso	38	24	41	103	39,81	2,03						
Fb X w_empty	5	41	56	102	54,90	2,50	30,63	2,10	21,03	0,35	12,14	1,21
Fb X w_empty	35	53	19	107	17,76	1,85						
Fb X w_empty	24	60	20	104	19,23	1,96						

(continued)

Haemocytes 2(3)

females X males	[1]	[2]	[3]	sum	dead melanized [%]	mean score	mean_dead melanized [%]	mean of mean score	SD_mean of dead melanized [%]	SD_mean of mean score	SEM_mean of dead melanized [%]	SEM_mean of mean score
HH x CG1299	34	46	37	117	31,62	2,03	37,48	2,09	15,49	0,21	8,94	1,21
HH x CG1299	33	39	25	97	25,77	1,92						
HH x CG1299	24	25	60	109	55,05	2,33						
HH x CG18477	14	56	52	122	42,62	2,31	56,23	2,47	12,01	0,14	6,93	0,08
HH x CG18477	1	10	17	28	60,71	2,57						
HH x CG18477	13	22	66	101	65,35	2,52						
HH x CG18478	17	16	48	81	59,26	2,38	58,81	2,42	4,39	0,06	2,53	0,03
HH x CG18478	12	26	45	83	54,22	2,40						
HH x CG18478	15	25	68	108	62,96	2,49						
HH x CG31780	9	18	79	106	74,53	2,66	72,34	2,69	11,02	0,12	6,36	0,07
HH x CG31780	2	38	61	101	60,40	2,58						
HH x CG31780	0	17	78	95	82,11	2,82						
HH x spirit	25	39	3	67	4,48	1,67	8,04	1,60	3,42	0,07	1,98	0,04
HH x spirit	40	37	7	84	8,33	1,61						
HH x spirit	67	35	13	115	11,30	1,53						
HH x empty	35	52	16	103	15,53	1,82	23,09	1,76	19,28	0,51	11,13	0,29
HH x empty	89	5	9	103	8,74	1,22						
HH x empty	22	33	45	100	45,00	2,23						
HH X w_iso	2	28	78	108	72,22	2,70	49,57	2,35	23,94	0,45	13,82	0,26
HH X w_iso	2	47	53	102	51,96	2,50						
HH X w_iso	43	37	26	106	24,53	1,84						

Whole larvae 3(3)

females X males	[1]	[2]	[3]	sum	dead melanized [%]	mean score	mean_dead melanized [%]	mean of mean score	SD_mean of dead melanized [%]	SD_mean of mean score	SEM_mean of dead melanized [%]	SEM_mean of mean score
w x CG1299	48	28	29	105	27,62	1,82	38,14	2,05	12,83	0,24	7,40	0,14
w x CG1299	20	22	22	64	34,38	2,03						
w x CG1299	24	25	54	103	52,43	2,29						
w x CG18477	16	35	59	110	53,64	2,39	64,11	2,58	10,94	0,17	6,32	0,10
w x CG18477	1	31	55	87	63,22	2,62						
w x CG18477	4	22	80	106	75,47	2,72						
w x CG18478	20	50	68	138	49,28	2,35	57,84	2,46	7,76	0,13	4,48	0,08
w x CG18478	4	33	67	104	64,42	2,61						
w x CG18478	18	25	64	107	59,81	2,43						
w x CG31780	8	22	71	101	70,30	2,62	71,10	2,67	0,93	0,04	0,54	0,03
w x CG31780	3	27	73	103	70,87	2,68						
w x CG31780	1	28	75	104	72,12	2,71						
w x spirit	1	38	39	78	50,00	2,49	39,05	2,22	10,99	0,34	6,34	0,20
w x spirit	1	13	9	23	39,13	2,35						
w x spirit	59	36	37	132	28,03	1,83						
w x empty	32	45	30	107	28,04	1,98	44,19	2,17	15,12	0,19	8,73	0,11
w x empty	30	24	47	101	46,53	2,17						
w x empty	22	20	58	100	58,00	2,36						
w x w_iso	14	38	49	101	48,51	2,35	55,50	2,46	6,76	0,10	3,90	0,06
w x w_iso	12	26	62	100	62,00	2,50						
w x w_iso	3	41	56	100	56,00	2,53						

Appendix 5. Serine protease data spirit_1 1(8)

cross	tissue	RNAi	primer	genotype	repl1	repl2	repl3	median C_T
1	hemocytes	spirit	spirit_1	RNAi x W	32,99	32,60	32,48	32,60
2	hemocytes	spirit	spirit_1	RNAi x W	30,34	30,31	30,02	30,31
3	hemocytes	spirit	spirit_1	RNAi x W	32,91	32,14	31,76	32,14
1	hemocytes	spirit	RpL32	RNAi x W	29,08	28,93	28,74	28,93
2	hemocytes	spirit	RpL32	RNAi x W	26,38	26,12	25,70	26,12
3	hemocytes	spirit	RpL32	RNAi x W	30,04	29,92	29,59	29,92
1	hemocytes	spirit	spirit_1	RNAi x HH	31,88	31,58	31,85	31,85
2	hemocytes	spirit	spirit_1	RNAi x HH	31,53	31,24	31,09	31,24
3	hemocytes	spirit	spirit_1	RNAi x HH	32,08	32,03	32,01	32,03
1	hemocytes	spirit	RpL32	RNAi x HH	27,75	28,01	27,94	27,94
2	hemocytes	spirit	RpL32	RNAi x HH	25,79	25,71	25,73	25,73
3	hemocytes	spirit	RpL32	RNAi x HH	27,80	27,91	27,77	27,80
1	hemocytes	spirit	spirit_1	RNAi x W i	30,29	30,18	30,06	30,18
2	hemocytes	spirit	spirit_1	RNAi x W i	32,44	32,01	31,81	32,01
3	hemocytes	spirit	spirit_1	RNAi x W i	32,08	31,86	31,53	31,86
1	hemocytes	spirit	RpL32	RNAi x W i	27,01	27,12	26,71	27,01
2	hemocytes	spirit	RpL32	RNAi x W i	25,72	25,72	26,00	25,72
3	hemocytes	spirit	RpL32	RNAi x W i	26,09	25,88	25,85	25,88
1	hemocytes	spirit	spirit_1	RNAi x HH i	29,49	30,03	29,69	29,69
2	hemocytes	spirit	spirit_1	RNAi x HH i	28,23	28,08	28,16	28,16
3	hemocytes	spirit	spirit_1	RNAi x HH i	30,47	30,33	30,34	30,34
1	hemocytes	spirit	RpL32	RNAi x HH i	26,31	26,20	26,12	26,20
2	hemocytes	spirit	RpL32	RNAi x HH i	24,37	24,36	24,17	24,36
3	hemocytes	spirit	RpL32	RNAi x HH i	25,15	25,03	24,92	25,03
1	whole larvae	spirit	spirit_1	RNAi x W	23,43	23,45	23,33	23,43
2	whole larvae	spirit	spirit_1	RNAi x W	24,82	24,91	24,66	24,82
3	whole larvae	spirit	spirit_1	RNAi x W	24,20	24,09	24,02	24,09
1	whole larvae	spirit	RpL32	RNAi x W	24,08	24,07	23,71	24,07
2	whole larvae	spirit	RpL32	RNAi x W	24,61	24,43	24,37	24,43
3	whole larvae	spirit	RpL32	RNAi x W	24,51	24,40	24,01	24,40
1	whole larvae	spirit	spirit_1	RNAi x HH	24,13	24,05	24,11	24,11
2	whole larvae	spirit	spirit_1	RNAi x HH	23,62	23,79	23,63	23,63
3	whole larvae	spirit	spirit_1	RNAi x HH	23,70	23,52	23,67	23,67
1	whole larvae	spirit	RpL32	RNAi x HH	24,00	24,05	24,76	24,05
2	whole larvae	spirit	RpL32	RNAi x HH	23,91	24,06	24,57	24,06
3	whole larvae	spirit	RpL32	RNAi x HH	24,21	24,27	24,32	24,27
1	whole larvae	spirit	spirit_1	RNAi x W i	24,37	24,40	24,03	24,37
2	whole larvae	spirit	spirit_1	RNAi x W i	27,39	26,93	26,55	26,93
3	whole larvae	spirit	spirit_1	RNAi x W i	24,92	25,53	24,71	24,92
1	whole larvae	spirit	RpL32	RNAi x W i	25,18	24,94	24,79	24,94
2	whole larvae	spirit	RpL32	RNAi x W i	27,54	27,47	27,25	27,47
3	whole larvae	spirit	RpL32	RNAi x W i	24,84	24,92	24,64	24,84
1	whole larvae	spirit	spirit_1	RNAi x HH i	23,23	23,32	23,49	23,32
2	whole larvae	spirit	spirit_1	RNAi x HH i	24,99	25,00	25,02	25,00
3	whole larvae	spirit	spirit_1	RNAi x HH i	22,72	23,06	22,91	22,91
1	whole larvae	spirit	RpL32	RNAi x HH i	24,43	24,51	24,63	24,51
2	whole larvae	spirit	RpL32	RNAi x HH i	25,68	25,76	25,81	25,76
3	whole larvae	spirit	RpL32	RNAi x HH i	24,95	24,76	24,99	24,95

(continued)

CG1299_3 2(8)

cross	tissue	RNAi	primer	genotype	repl1	repl2	repl3	median C_T
1	hemocytes	cg1299	cg1299_3	RNAi x W	N/A	38,28	N/A	38,28
2	hemocytes	cg1299	cg1299_3	RNAi x W	37,35	36,95	36,43	36,95
3	hemocytes	cg1299	cg1299_3	RNAi x W	37,80	38,38	N/A	38,09
1	hemocytes	cg1299	RpL32	RNAi x W	27,79	27,31	27,22	27,31
2	hemocytes	cg1299	RpL32	RNAi x W	28,09	27,35	27,37	27,37
3	hemocytes	cg1299	RpL32	RNAi x W	29,18	28,83	28,53	28,83
1	hemocytes	cg1299	cg1299_3	RNAi x HH	35,88	37,97	36,08	36,08
2	hemocytes	cg1299	cg1299_3	RNAi x HH	N/A	37,79	38,38	38,09
3	hemocytes	cg1299	cg1299_3	RNAi x HH	35,08	36,63	35,48	35,48
1	hemocytes	cg1299	RpL32	RNAi x HH	26,70	26,52	26,59	26,59
2	hemocytes	cg1299	RpL32	RNAi x HH	25,28	25,26	25,53	25,28
3	hemocytes	cg1299	RpL32	RNAi x HH	26,08	26,30	26,65	26,30
1	hemocytes	cg1299	cg1299_3	RNAi x W i	28,14	28,06	27,75	28,06
2	hemocytes	cg1299	cg1299_3	RNAi x W i	35,94	38,00	N/A	36,97
3	hemocytes	cg1299	cg1299_3	RNAi x W i	36,89	37,56	37,24	37,24
1	hemocytes	cg1299	RpL32	RNAi x W i	25,59	25,21	25,28	25,28
2	hemocytes	cg1299	RpL32	RNAi x W i	25,91	26,03	25,86	25,91
3	hemocytes	cg1299	RpL32	RNAi x W i	25,81	25,82	25,49	25,81
1	hemocytes	cg1299	cg1299_3	RNAi x HH i	27,39	27,54	27,54	27,54
2	hemocytes	cg1299	cg1299_3	RNAi x HH i	33,98	37,79	34,37	34,37
3	hemocytes	cg1299	cg1299_3	RNAi x HH i	36,02	33,78	36,20	36,02
1	hemocytes	cg1299	RpL32	RNAi x HH i	24,22	24,16	24,10	24,16
2	hemocytes	cg1299	RpL32	RNAi x HH i	24,77	24,56	24,42	24,56
3	hemocytes	cg1299	RpL32	RNAi x HH i	24,38	24,03	23,89	24,03
1	whole larvae	cg1299	cg1299_3	RNAi x W	28,07	27,63	28,24	28,07
2	whole larvae	cg1299	cg1299_3	RNAi x W	27,87	27,80	27,52	27,80
3	whole larvae	cg1299	cg1299_3	RNAi x W	26,15	26,03	25,76	26,03
1	whole larvae	cg1299	RpL32	RNAi x W	22,38	22,15	22,06	22,15
2	whole larvae	cg1299	RpL32	RNAi x W	21,65	21,53	21,27	21,53
3	whole larvae	cg1299	RpL32	RNAi x W	21,77	21,83	21,46	21,77
1	whole larvae	cg1299	cg1299_3	RNAi x HH	26,02	26,36	26,15	26,15
2	whole larvae	cg1299	cg1299_3	RNAi x HH	25,09	25,15	25,04	25,09
3	whole larvae	cg1299	cg1299_3	RNAi x HH	27,51	27,60	27,63	27,60
1	whole larvae	cg1299	RpL32	RNAi x HH	21,92	21,67	22,09	21,92
2	whole larvae	cg1299	RpL32	RNAi x HH	21,04	21,19	21,20	21,19
3	whole larvae	cg1299	RpL32	RNAi x HH	21,68	21,77	21,90	21,77
1	whole larvae	cg1299	cg1299_3	RNAi x W i	27,50	27,54	27,16	27,50
2	whole larvae	cg1299	cg1299_3	RNAi x W i	25,64	25,30	25,20	25,30
3	whole larvae	cg1299	cg1299_3	RNAi x W i	26,91	26,84	26,68	26,84
1	whole larvae	cg1299	RpL32	RNAi x W i	23,07	22,78	22,50	22,78
2	whole larvae	cg1299	RpL32	RNAi x W i	22,04	22,22	21,85	22,04
3	whole larvae	cg1299	RpL32	RNAi x W i	22,55	22,59	22,21	22,55
1	whole larvae	cg1299	cg1299_3	RNAi x HH i	26,22	26,42	26,61	26,42
2	whole larvae	cg1299	cg1299_3	RNAi x HH i	26,12	26,05	26,14	26,12
3	whole larvae	cg1299	cg1299_3	RNAi x HH i	26,95	27,22	27,00	27,00
1	whole larvae	cg1299	RpL32	RNAi x HH i	21,58	21,55	21,50	21,55
2	whole larvae	cg1299	RpL32	RNAi x HH i	21,69	21,50	21,46	21,50
3	whole larvae	cg1299	RpL32	RNAi x HH i	22,24	21,85	21,86	21,86

CG18478_2 3(8)

cross	tissue	RNAi	primer	genotype	repl1	repl2	repl3	median C_T
1	hemocytes	cg18478	cg18478_2	RNAi x W	37,01	N/A	36,95	36,98
2	hemocytes	cg18478	cg18478_2	RNAi x W	36,81	N/A	38,24	37,53
3	hemocytes	cg18478	cg18478_2	RNAi x W	36,44	38,47	37,38	37,38
1	hemocytes	cg18478	RpL32	RNAi x W	29,91	29,67	29,60	29,67
2	hemocytes	cg18478	RpL32	RNAi x W	27,83	27,35	27,19	27,35
3	hemocytes	cg18478	RpL32	RNAi x W	33,95	33,59	33,56	33,59
1	hemocytes	cg18478	cg18478_2	RNAi x HH	N/A	36,23	37,64	36,94
2	hemocytes	cg18478	cg18478_2	RNAi x HH	N/A	37,02	35,14	36,08
3	hemocytes	cg18478	cg18478_2	RNAi x HH	34,30	36,21	34,29	34,30
1	hemocytes	cg18478	RpL32	RNAi x HH	27,50	27,52	27,62	27,52
2	hemocytes	cg18478	RpL32	RNAi x HH	26,14	26,05	26,34	26,14
3	hemocytes	cg18478	RpL32	RNAi x HH	27,71	27,84	28,04	27,84
1	hemocytes	cg18478	cg18478_2	RNAi x W i	28,37	29,09	28,69	28,69
2	hemocytes	cg18478	cg18478_2	RNAi x W i	34,12	34,11	34,10	34,11
3	hemocytes	cg18478	cg18478_2	RNAi x W i	34,32	35,85	35,12	35,12
1	hemocytes	cg18478	RpL32	RNAi x W i	26,19	26,49	26,76	26,49
2	hemocytes	cg18478	RpL32	RNAi x W i	26,29	26,35	26,24	26,29
3	hemocytes	cg18478	RpL32	RNAi x W i	26,67	26,70	26,61	26,67
1	hemocytes	cg18478	cg18478_2	RNAi x HH i	28,28	28,27	28,21	28,27
2	hemocytes	cg18478	cg18478_2	RNAi x HH i	34,96	33,72	34,35	34,35
3	hemocytes	cg18478	cg18478_2	RNAi x HH i	36,62	37,55	N/A	37,09
1	hemocytes	cg18478	RpL32	RNAi x HH i	26,09	26,04	26,29	26,09
2	hemocytes	cg18478	RpL32	RNAi x HH i	25,70	25,78	25,44	25,70
3	hemocytes	cg18478	RpL32	RNAi x HH i	27,49	27,51	27,42	27,49
1	whole larvae	cg18478	cg18478_2	RNAi x W	27,63	26,91	27,40	27,40
2	whole larvae	cg18478	cg18478_2	RNAi x W	27,99	28,31	28,19	28,19
3	whole larvae	cg18478	cg18478_2	RNAi x W	27,76	28,42	28,06	28,06
1	whole larvae	cg18478	RpL32	RNAi x W	22,10	21,80	21,50	21,80
2	whole larvae	cg18478	RpL32	RNAi x W	21,98	21,63	21,36	21,63
3	whole larvae	cg18478	RpL32	RNAi x W	22,14	21,54	21,43	21,54
1	whole larvae	cg18478	cg18478_2	RNAi x HH	27,14	27,85	27,70	27,70
2	whole larvae	cg18478	cg18478_2	RNAi x HH	28,05	28,00	28,03	28,03
3	whole larvae	cg18478	cg18478_2	RNAi x HH	28,58	28,16	28,96	28,58
1	whole larvae	cg18478	RpL32	RNAi x HH	21,33	21,37	21,30	21,33
2	whole larvae	cg18478	RpL32	RNAi x HH	21,79	21,92	22,00	21,92
3	whole larvae	cg18478	RpL32	RNAi x HH	21,85	22,02	22,05	22,02
1	whole larvae	cg18478	cg18478_2	RNAi x W i	27,72	27,82	27,41	27,72
2	whole larvae	cg18478	cg18478_2	RNAi x W i	27,71	27,37	27,13	27,37
3	whole larvae	cg18478	cg18478_2	RNAi x W i	27,87	28,04	28,10	28,04
1	whole larvae	cg18478	RpL32	RNAi x W i	22,04	21,76	21,72	21,76
2	whole larvae	cg18478	RpL32	RNAi x W i	22,08	22,09	22,06	22,08
3	whole larvae	cg18478	RpL32	RNAi x W i	22,43	22,53	22,07	22,43
1	whole larvae	cg18478	cg18478_2	RNAi x HH i	27,02	27,17	27,19	27,17
2	whole larvae	cg18478	cg18478_2	RNAi x HH i	27,95	27,98	27,93	27,95
3	whole larvae	cg18478	cg18478_2	RNAi x HH i	28,45	28,41	28,49	28,45
1	whole larvae	cg18478	RpL32	RNAi x HH i	22,17	22,24	22,10	22,17
2	whole larvae	cg18478	RpL32	RNAi x HH i	21,90	21,78	21,62	21,78
3	whole larvae	cg18478	RpL32	RNAi x HH i	22,78	22,72	22,51	22,72

CG18477_1 4(8)

cross	tissue	RNAi	primer	genotype	repl1	repl2	repl3	median C_T
1	hemocytes	cg18477	cg18477_1	RNAi x W	38,03	35,29	36,53	36,53
2	hemocytes	cg18477	cg18477_1	RNAi x W	34,85	34,09	36,15	34,85
3	hemocytes	cg18477	cg18477_1	RNAi x W	35,82	34,13	34,32	34,32
1	hemocytes	cg18477	RpL32	RNAi x W	29,32	28,94	28,78	28,94
2	hemocytes	cg18477	RpL32	RNAi x W	27,26	26,81	27,04	27,04
3	hemocytes	cg18477	RpL32	RNAi x W	30,24	N/A	30,01	30,13
1	hemocytes	cg18477	cg18477_1	RNAi x HH	35,98	34,51	34,35	34,51
2	hemocytes	cg18477	cg18477_1	RNAi x HH	31,27	33,21	31,61	31,61
3	hemocytes	cg18477	cg18477_1	RNAi x HH	33,71	32,59	33,28	33,28
1	hemocytes	cg18477	RpL32	RNAi x HH	27,55	27,63	27,69	27,63
2	hemocytes	cg18477	RpL32	RNAi x HH	26,14	26,11	26,27	26,14
3	hemocytes	cg18477	RpL32	RNAi x HH	28,20	28,21	28,23	28,21
1	hemocytes	cg18477	cg18477_1	RNAi x W i	29,90	28,70	28,83	28,83
2	hemocytes	cg18477	cg18477_1	RNAi x W i	33,65	33,21	33,30	33,30
3	hemocytes	cg18477	cg18477_1	RNAi x W i	32,93	32,76	32,01	32,76
1	hemocytes	cg18477	RpL32	RNAi x W i	26,19	26,48	26,57	26,48
2	hemocytes	cg18477	RpL32	RNAi x W i	25,60	25,96	25,85	25,85
3	hemocytes	cg18477	RpL32	RNAi x W i	26,16	26,28	26,09	26,16
1	hemocytes	cg18477	cg18477_1	RNAi x HH i	28,48	28,46	27,88	28,46
2	hemocytes	cg18477	cg18477_1	RNAi x HH i	31,85	31,58	31,03	31,58
3	hemocytes	cg18477	cg18477_1	RNAi x HH i	31,89	31,88	31,17	31,88
1	hemocytes	cg18477	RpL32	RNAi x HH i	25,63	25,53	25,57	25,57
2	hemocytes	cg18477	RpL32	RNAi x HH i	24,94	24,79	24,69	24,79
3	hemocytes	cg18477	RpL32	RNAi x HH i	25,48	25,74	25,64	25,64
1	whole larvae	cg18477	cg18477_1	RNAi x W	26,23	25,74	25,52	25,74
2	whole larvae	cg18477	cg18477_1	RNAi x W	24,88	25,07	24,65	24,88
3	whole larvae	cg18477	cg18477_1	RNAi x W	26,08	25,14	25,99	25,99
1	whole larvae	cg18477	RpL32	RNAi x W	21,44	21,13	20,99	21,13
2	whole larvae	cg18477	RpL32	RNAi x W	21,47	21,22	20,81	21,22
3	whole larvae	cg18477	RpL32	RNAi x W	21,38	21,16	20,84	21,16
1	whole larvae	cg18477	cg18477_1	RNAi x HH	24,50	23,80	23,52	23,80
2	whole larvae	cg18477	cg18477_1	RNAi x HH	25,52	25,23	25,40	25,40
3	whole larvae	cg18477	cg18477_1	RNAi x HH	25,03	24,65	25,04	25,03
1	whole larvae	cg18477	RpL32	RNAi x HH	21,10	21,16	21,07	21,10
2	whole larvae	cg18477	RpL32	RNAi x HH	21,20	21,18	21,23	21,20
3	whole larvae	cg18477	RpL32	RNAi x HH	21,13	21,11	21,04	21,11
1	whole larvae	cg18477	cg18477_1	RNAi x W i	27,32	27,56	26,86	27,32
2	whole larvae	cg18477	cg18477_1	RNAi x W i	25,41	25,32	24,53	25,32
3	whole larvae	cg18477	cg18477_1	RNAi x W i	23,87	23,88	23,50	23,87
1	whole larvae	cg18477	RpL32	RNAi x W i	23,65	23,56	23,47	23,56
2	whole larvae	cg18477	RpL32	RNAi x W i	22,19	22,22	21,93	22,19
3	whole larvae	cg18477	RpL32	RNAi x W i	21,83	21,83	21,47	21,83
1	whole larvae	cg18477	cg18477_1	RNAi x HH i	24,98	24,93	24,69	24,93
2	whole larvae	cg18477	cg18477_1	RNAi x HH i	21,80	21,72	21,75	21,75
3	whole larvae	cg18477	cg18477_1	RNAi x HH i	23,10	23,12	23,02	23,10
1	whole larvae	cg18477	RpL32	RNAi x HH i	23,11	23,08	23,15	23,11
2	whole larvae	cg18477	RpL32	RNAi x HH i	21,21	21,08	21,10	21,10
3	whole larvae	cg18477	RpL32	RNAi x HH i	21,49	21,21	21,16	21,21

CG31780_1 5(8)

cross	tissue	RNAi	primer	genotype	repl1	repl2	repl3	median C_T
1	hemocytes	cg31780	cg31780_1	RNAi x W	36,62	37,09	35,69	36,62
2	hemocytes	cg31780	cg31780_1	RNAi x W	36,15	35,98	36,65	36,15
3	hemocytes	cg31780	cg31780_1	RNAi x W	37,96	37,27	36,77	37,27
1	hemocytes	cg31780	RpL32	RNAi x W	29,25	28,57	28,62	28,62
2	hemocytes	cg31780	RpL32	RNAi x W	27,71	27,85	27,48	27,71
3	hemocytes	cg31780	RpL32	RNAi x W	31,27	31,19	30,68	31,19
1	hemocytes	cg31780	cg31780_1	RNAi x HH	35,85	37,80	37,00	37,00
2	hemocytes	cg31780	cg31780_1	RNAi x HH	33,88	33,82	33,54	33,82
3	hemocytes	cg31780	cg31780_1	RNAi x HH	34,43	34,22	34,07	34,22
1	hemocytes	cg31780	RpL32	RNAi x HH	28,12	28,34	28,28	28,28
2	hemocytes	cg31780	RpL32	RNAi x HH	26,45	26,22	26,40	26,40
3	hemocytes	cg31780	RpL32	RNAi x HH	28,09	27,99	28,16	28,09
1	hemocytes	cg31780	cg31780_1	RNAi x W i	29,45	29,22	28,71	29,22
2	hemocytes	cg31780	cg31780_1	RNAi x W i	33,27	33,19	32,76	33,19
3	hemocytes	cg31780	cg31780_1	RNAi x W i	33,36	33,37	33,05	33,36
1	hemocytes	cg31780	RpL32	RNAi x W i	25,67	25,86	25,31	25,67
2	hemocytes	cg31780	RpL32	RNAi x W i	25,66	25,29	25,11	25,29
3	hemocytes	cg31780	RpL32	RNAi x W i	26,25	26,19	25,59	26,19
1	hemocytes	cg31780	cg31780_1	RNAi x HH i	29,51	29,50	29,82	29,51
2	hemocytes	cg31780	cg31780_1	RNAi x HH i	31,40	32,07	31,64	31,64
3	hemocytes	cg31780	cg31780_1	RNAi x HH i	33,70	33,72	33,43	33,70
1	hemocytes	cg31780	RpL32	RNAi x HH i	25,60	25,42	25,44	25,44
2	hemocytes	cg31780	RpL32	RNAi x HH i	25,08	25,03	24,80	25,03
3	hemocytes	cg31780	RpL32	RNAi x HH i	27,41	27,07	27,12	27,12
1	whole larvae	cg31780	cg31780_1	RNAi x W	24,43	24,36	24,01	24,36
2	whole larvae	cg31780	cg31780_1	RNAi x W	25,07	25,01	24,88	25,01
3	whole larvae	cg31780	cg31780_1	RNAi x W	24,71	24,51	24,24	24,51
1	whole larvae	cg31780	RpL32	RNAi x W	23,41	23,11	22,29	23,11
2	whole larvae	cg31780	RpL32	RNAi x W	23,26	23,05	22,86	23,05
3	whole larvae	cg31780	RpL32	RNAi x W	23,25	23,14	22,55	23,14
1	whole larvae	cg31780	cg31780_1	RNAi x HH	24,24	24,29	24,34	24,29
2	whole larvae	cg31780	cg31780_1	RNAi x HH	24,24	24,31	24,25	24,25
3	whole larvae	cg31780	cg31780_1	RNAi x HH	25,79	25,82	25,78	25,79
1	whole larvae	cg31780	RpL32	RNAi x HH	22,19	21,69	21,98	21,98
2	whole larvae	cg31780	RpL32	RNAi x HH	22,09	21,77	21,49	21,77
3	whole larvae	cg31780	RpL32	RNAi x HH	23,45	23,46	23,20	23,45
1	whole larvae	cg31780	cg31780_1	RNAi x W i	23,12	23,21	23,10	23,12
2	whole larvae	cg31780	cg31780_1	RNAi x W i	23,86	23,71	23,37	23,71
3	whole larvae	cg31780	cg31780_1	RNAi x W i	22,72	22,95	22,47	22,72
1	whole larvae	cg31780	RpL32	RNAi x W i	22,05	21,66	21,70	21,70
2	whole larvae	cg31780	RpL32	RNAi x W i	22,28	22,19	22,09	22,19
3	whole larvae	cg31780	RpL32	RNAi x W i	22,50	22,29	22,14	22,29
1	whole larvae	cg31780	cg31780_1	RNAi x HH i	22,51	22,66	22,90	22,66
2	whole larvae	cg31780	cg31780_1	RNAi x HH i	22,73	22,62	22,53	22,62
3	whole larvae	cg31780	cg31780_1	RNAi x HH i	22,47	22,68	26,90	22,68
1	whole larvae	cg31780	RpL32	RNAi x HH i	21,41	21,43	21,47	21,43
2	whole larvae	cg31780	RpL32	RNAi x HH i	21,75	21,65	21,61	21,65
3	whole larvae	cg31780	RpL32	RNAi x HH i	22,13	22,11	21,80	22,11

Reduction										
	dCT_target (cal - test)	**dCT_ref (cal - test)**	**E_target^dCT_target**	**E_ref^dCT_ref**	**E_target/E_ref**	**reduction [%]**	**mean**	**SD**	**SEM**
spirit	0,75	0,99	1,72	1,68	1,02	-2,28	38,15	35,01	20,21
	-0,93	0,39	0,51	1,23	0,42	58,39			
	0,11	1,82	1,08	2,60	0,42	58,33	58,36	0,04	0,03
CG1299	2,20	0,72	3,64	1,46	2,50	-149,76	-46,60	118,52	68,43
	-1,14	2,09	0,51	2,99	0,17	82,86			
	2,61	1,88	4,64	2,68	1,73	-72,92			
CG18478	0,05	2,15	1,03	3,09	0,33	66,55	19,77	64,47	37,22
	1,45	1,21	2,90	1,89	1,54	-53,77			
	3,08	5,52	9,68	18,11	0,53	46,53	56,54	14,15	10,01
CG18477	2,02	1,31	4,06	1,99	2,04	-103,96	-191,31	265,20	153,11
	3,24	0,90	9,45	1,60	5,89	-489,17			
	1,04	1,78	2,06	2,54	0,81	19,20			
CG31780	-0,38	0,34	0,78	1,20	0,65	34,71	-63,37	86,72	50,07
	2,33	1,31	4,57	1,99	2,30	-129,91			
	3,05	2,52	7,31	3,75	1,95	-94,89			

Induction of serine proteases in haemocytes 7(8)

Induction	hemocytes								
	dCT_target (cal - test)	**dCT_ref** (cal - test)	**E_target^dCT_target**	**E_ref^dCT_ref**	**E_target/E_ref**	**mean_ddCT**	**SD_ddCT**	**SEM_ddCT**	
spirit	2,42	1,92	5,75	2,74	2,10	0,83	1,10	0,64	
	-1,70	0,40	0,29	1,23	0,24	0,19	0,06	0,04	
	0,28	4,04	1,22	8,33	0,15				
CG1299	10,22	2,03	406,34	2,90	140,05	46,95	80,63	46,55	
	-0,02	1,46	0,99	2,15	0,46	0,40	0,09	0,06	
	0,85	3,02	1,65	4,88	0,34				
CG18478	8,29	3,18	450,83	5,30	84,98	30,74	47,10	27,19	
	3,42	1,06	12,40	1,74	7,11				
	2,26	6,92	5,29	37,76	0,14				
CG18477	7,70	2,46	207,94	3,64	57,19	19,71	32,47	18,74	
	1,55	1,19	2,93	1,87	1,57				
	1,56	3,97	2,95	8,01	0,37				
CG31780	7,40	2,95	124,86	4,70	26,56	9,81	14,51	8,38	
	2,96	2,42	6,90	3,56	1,94				
	3,91	5,00	12,81	13,79	0,93				

Induction of serine proteases in fatbody 8(8)

Induction	fatbody								
	dCT_target (cal - test)	**dCT_ref** (cal - test)	**E_target^dCT_target**	**E_ref^dCT_ref**	**E_target/E_ref**	**mean_ddCT**	**SD_ddCT**	**SEM_ddCT**
spirit	0,47	0,89	1,40	1,60	0,88	0,81	0,52	0,30
	-2,14	-0,34	0,21	0,84	0,25			
	-0,06	-0,56	0,96	0,75	1,28			
CG1299	2,99	0,03	5,80	1,02	5,71	2,79	2,65	1,53
	0,74	-0,63	1,54	0,72	2,15			
	-0,94	0,18	0,58	1,10	0,52			
CG18478	-1,37	0,48	0,36	1,29	0,28	1,15	0,97	0,56
	-0,90	-1,21	0,52	0,53	0,97			
	1,02	-0,07	2,12	0,96	2,20			
CG18477	4,02	-1,09	16,22	0,56	28,74	19,90	15,10	8,72
	0,28	-1,35	1,21	0,49	2,47			
	4,37	-0,61	20,68	0,73	28,48			
CG31780	1,19	0,44	2,17	1,26	1,73	2,51	0,69	0,40
	1,47	-0,12	2,61	0,94	2,78			
	2,18	0,60	4,15	1,37	3,03			