ROSKILDE UNIVERSITY

MOLECULAR BIOLOGY DEPARTMENTS

BACHELOR MODULE PROJECT

NEUROENDOCRINE REGULATION OF DEVELOPMENT TIMING AND BODY SIZE IN *DROSOPHILA MELANOGASTER*

EXAMINATION PERIOD **:** Winter Examination 2010-11

ACADEMIC SUPERVISIOR **:** Kim REWITZ

GROUP **:** Arunagirinathan, N., Henden, G., Jensen, C.H., Jensen, R.J., Mika, K., Mitternöckler, M.F., Sloth, S.H.

Abstract

The process of development and metamorphosis in *Drosophila melanogaster* is a complex interaction of several different hormones, proteins and growth factors. It is known that growth and molting is mainly regulated by three hormones, namely the juvenile hormone (JH), the prothoracicotropic hormone (PTTH) which triggers the release of ecdysone, the third important hormone. The release of PTTH is thought to be influenced by a number of proteins and growth factors, although the precise effects are mostly unknown.

Therefore, the aim of this project is to reach a better understanding of the effects of Ras signalling on PTTH and in turn on ecdysone and the developmental timing of *D.melanogaster*. Such studies on *D.melanogaster* may provide insight about the regulation of growth and development in humans. In order to identify the role and importance of the mentioned hormones and proteins, crosses have been set up to over-express or reduce the activities of Ras signalling components in the PTTH producing neurons. Subsequently, the timing of metamorphosis was observed and the levels of PTTH and ecdysone-inducible genes were measured by proceeding quantitative real-time polymerase-chain-reaction (qPCR).

The results suggest that the Ras signalling has an impact in the synthesis and/or release of PTTH and therefore in the timing of development. The pathway is influenced in a positive way by over-expressing Ras whereas it is affected negatively by reducing Raf signalling. This reveals an important step within the regulation of PTTH signalling during development of *D. melanogaster.*

Resumé

Hos *Drosophila melanogaster* er udviklingen og metamorfosen en kompleks interaktion mellem flere forskellige hormoner, proteiner og vækstfaktorer. Det er allerede kendt at vækst og molting hovedsageligt bliver reguleret af tre hormoner; juvenil-hormon (JH), prothoracicotropic hormon (PTTH), som igangsætter frigivelsen af ecdyson, hvilket er det tredje vigtige hormon. Man mener frigivelsen af PTTH er påvirket af flere forskellige proteiner og vækstfaktorer selvom den nøjagtige mekanisme endnu ikke er kendt.

Målet med dette projekt er derfor at undersøge hvilken effekt Ras signaleringen har på PTTH og dermed på ecdyson og den tidsmæssige udvikling af *D.melanogaster*. Dette kan måske på sigt give en bedre forståelse af reguleringen af humane vækstfaktorer og dermed den menneskelige udvikling. For at kunne identificere de nævnte hormoner og proteiners rolle er et forsøg med krydsninger blevet udført, hvor effekten af henholdsvis øget og reduceret Ras signalering i de PTTH producerende neuroner er blevet undersøgt. Efterfølgende er metamorfosens tidsmæssige forløb blevet observeret og niveauet af PTTH og ecdyson inducerende gener er blevet målt ved at udføre kvantitativ PCR (qPCR).

De opnåede resultater viser at Ras signaleringen har indflydelse på syntesen og/eller frigivelsen af PTTH og dermed på det tidsmæssige forløb af metamorfosen. Forløbet bliver positivt påvirket ved at opregulere Ras og negativt påvirket ved nedregulering af Raf, hvilket tyder på, at dette har en vigtig funktion i PTTH signaleringen og dermed på udviklingen af *D.melanogaster*.

Preface

This project is a contribution to the research in developmental biology. Using *D.melanogaster* as a model organism the mechanisms underlying the neuroendocrine control of steroid hormone production were explored.

We would like to thank our supervisor Kim Rewitz, assistant professor at the Department of Science, Systems, and Models at Roskilde University, for his greatly appreciated assistance and guidance during this project. Furthermore we thank Mette Riisager, laboratory technician at the Department of Science, Systems, and Models at Roskilde University, for her help on qPCR; Johanne Gudmand Høyer and Guilin Ren, master students at the Department of Science, Systems, and Models at Roskilde University.

A complete list of abbreviations can be found in appendix I.

Christina Holm JENSEN

Gizem HENDEN

Magdalena MITTERNÖCKLER

Kaan MIKA

Nathiya ARUNAGIRINATHAN

Rie Juul JENSEN

Stine Hesselholt SLOTH

Table of content

1 Introduction 9

1.1 *D. melanogaster* as a model organism 9

1.1.1 Examples of D. melanogaster as a model organism 10

1.1.2 Huntington´s disease 11

1.1.3 Alzheimer´s disease 11

1.1.4 Parkinson´s disease 12

1.2 Molting and Metamorphosis in Insects 12

1.2.1 Imaginal Discs of *D . melanogaster* 14

1.2.2 The Hormones affecting metamorphosis in *D. melanogaster* 15

1.2.3 Metamorphic stages of *D. melanogaster* 15

1.3 The role of PTTH, Ecdysteroids and Juvenile Hormone 16

1.3.1 Ecdysone production 17

1.3.2 Halloween genes and the synthesis of ecdysone in the PG 18

1.3.3 Conversion of ecdysone to 20-hydroxyecdysone 20

1.4 Ras Signalling and MAP-Kinase Pathway 20

1.4.1 Ras-signalling in neurons and the effect of Raf 22

1.4.2 PTTH synthesis 23

1.4.3 Control of PTTH release 24

1.4.4 PTTH and ecdysone release 24

1.4.5 ISS and ecdysone release 26

2 Method 27

2.1 GAL4-UAS System 27

2.2 Selecting Larvae and Monitoring Pupariation 28

2.2.1 Collecting flies and setting up crosses 28

2.2.2 Egg-lay 29

2.3 The principle of qPCR....30
2.3.1 Conventional PCR....30
2.3.2 Choice of the primers....32
2.3.3 qPCR....32
2.3.4 Melting curve....33
2.4 qPCR Procedure....34
2.4.1 RNA extraction....34
2.4.2 cDNA preparation....35
2.4.3 qPCR measurement....36
3 Results....37
3.1 Pupae Monitoring....37
3.2 Does Ras signalling in the PTTH producing neurons affect PTTH expression and ecdysone levels?....41
3.2.1 PTTH expression....41
3.2.2 Expression of Ecdysone-inducible genes....42
4 Discussion....45
4.1 Discussion of methods....45
4.1.1 Setting up fly-crosses and egg-lay....45
4.2 Discussion of results....46
4.2.1 Temperature effects....46
4.2.2 Choice of primers....46
4.2.3 Amount of primer....48
4.2.4 Measurement of rpl23 and RNA....48
4.3 The effect of Ras-signalling on PTTH and ecdysone in larvae....49
4.4 The effect of Ras signalling on PTTH and ecdysone in adult female flies....51
4.5 Regulation of ecdysone production in the PG....51

4.6 Ras-signalling in the neurons 52
5 Conclusion 55
6 Future Prospects 56
7 References 58
8 Appendix 67
8.1 Appendix I- Abbreviations 67
8.2 Appendix II- Classification of *D.melanogaster melanogaster* 68
8.3 Appendix III- qPCR Melting Curves 69
8.4 Appendix IV- qPCR Amplification Curves 70
8.5 Appendix V- Tables of Mean, SD, SE 71
8.6 Appendix VI- PTTH Expression Results 72

1 Introduction

Within the last years it has been observed that the timing of development and therefore the onset of puberty has undergone a remarkable change. This is suggested to be due to several environmental influences, like nutrition or increased impact of chemical pollution. Indeed, there have been intense studies about the effect of obesity and endocrine disruptors on the timing of puberty (Biro et al., 2005; Landrigan et al., 2003). However, the way these factors influence the hormonal regulation of growth is for the most part still unknown.

In order to get a better understanding of the regulatory pathways in human, the aim of this study is to determine the basic regulation of growth and developmental timing and the effect of Ras signalling in *D. melanogaster*. In respect to that, the main focus lies on PTTH. This hormone represents one significant step within growth-control as it triggers the release of ecdysone, which in turn is known to determine the duration of the growth period. Therefore the effect of Ras signalling on PTTH is investigated.

By studying the effect of these hormones and proteins on *D. melanogaster* the first step to pave the way for understanding the growth regulation in humans is done. *D. melanogaster* has been chosen as a model organism due to the high rate of similarity between the fly and the human genome as well as the easy and comfortable handling.

1.1 *D. melanogaster* as a model organism

D. melanogaster has been used as a model organism for more than 100 years and is the preferred organism for thousands of scientists all over the world. One of the main reasons for scientists today to choose *D. melanogaster* is the long history of working with the fruit fly. Because of the intense studies within the last century, the knowledge on *D. melanogaster* is immense and the methods are well established. The fact, that the complete genome was published in 2001, makes the fruit fly even more popular as a model organism (Twyman, 2002). Additionally, there are several public stock centres, which are hosting a constantly growing variety of mutants and transgenic lines (Toivonen et al., 2009).

The advantages of using *D. melanogaster* as a model organism for this project are manifold. Within the last century the fruit fly has grown in its importance for endocrine research. This is not only because of the short life cycle and the easy and cheap rearing, but also because of the presence of

polytene chromosomes, which result from multiple DNA replication cycles without cell division (Gilbert, 2004; Beckingham et al., 2005). Apart from these facts, the most important aspect of *D. melanogaster* as a model organism is the fact that approximately 70% of the genome is identical to the human genome (Gilbert, 2004).

Although the fruit fly is not an ideal organism in every section within the field of endocrinological research, especially not when using methods like microsurgery or tissue transplantation, where the small size could cause some problems, it has proven of value for other kinds of endocrinological manipulation. Such as genetic ablation, could be described as "the removal of something", which means to make a gene silent. Another way of proceeding ablation is to create a new mutant line by either crossing two mutants, which results in a loss of a specific gene, or expressing a proapoptotic gene, which leads to apoptosis, programmed cell death (Grießhammer et al., 1998). Additionally, tissues of *D. melanogaster* resemble mammal tissues more strongly than those of the nematode *Caenorhabditis elegans*, another important model organism for endocrinological research (Guarente et al., 2008).

1.1.1 Examples of D. melanogaster as a model organism

One of the main fields of studies, using *D. melanogaster* as a model organism, is the research on human diseases. The prominence of the fruit fly for a better understanding of specific human diseases, especially neurodegenerative diseases, has become stronger just recently (Adams et al., 2000). When about 282 genes which are associated with human mental retardation were tabulated several years ago, it was revealed that 76% of those genes have at least one functional ortholog in *D. melanogaster*. Therefore researchers are suggesting that one could use the fruit fly in order to develop functional assays leading to the invention of new drugs for treating mental retardation (Inlow and Restifo, 2004).

Within neurodegenerative diseases there have been successful experiments for three classes, namely Alzheimer´s, Parkinson`s and Huntington`s disease, where the protein product associated with a particular disease state was expressed in the fly and it was determined whether the characteristics of the disease appeared in the organism (Beckingham et al., 2005).

1.1.2 Huntington´s disease

Named after Dr. George Huntington, Huntington´s disease is a hereditary degenerative brain disorder, which affects cognitive ability or mobility and causes depression, mood swings, involuntary twitching, forgetfulness, clumsiness, and lack of coordination (Gusella and McDonald, 1995). Furthermore, concentration and short-time memory is reduced whereas involuntary movements of the head, trunk, and limbs increase (Warrell et al., 2003).

Research has revealed that individuals, suffering from Huntington´s disease, are carrying a CAG tract, which is substantially longer than in wild type controls (Gusella and McDonald, 1995). This leads to the gene product huntingtin (Htt) which has a string of polyglutamines (poly Q) much longer than normal. In fact, it was discoverd that the length of the CAG tract can be associated with the characteristic symptoms of Huntington´s disease. There have been studies, using *D. melanogaster* as a model where Htt-associated proteins were tested as genetic modifiers of neurodegeneration. These studies are the basis of identifying novel modifiers that modulate poly Q toxicity (Gilbert, 2008).

1.1.3 Alzheimer´s disease

Alzheimer´s disease (AD) is the most common form of dementia, which causes loss of memory and other intellectual abilities in a progressive way, meaning that the symptoms are gradually worsen over time (Warrell et al., 2003).

It is observed that a wide range of proteins convert into aggregates, which have characteristics of amyloidal fibrils. This aggregation results in two different forms, namely extracellular fibrils, also called plaques, or amyloid-like intracellular inclusions. In case of AD it is assumed that these aggregations are responsible for the typical symptoms of AD (Chiti et al., 2007).

Using *D. melanogaster* as a model for Alzheimer´s is quite emerging. Crowther et al., (2005) carried out intense studies about the tendency of proteins to aggregate and protein misfolding. They concluded that there is a close relation between protein aggregation, neuronal dysfunction and neurodegeneration in fruit flies. Their experiments concerned the pathogenicity of Aβ42 (parenchymal and cerebrovascular aggregates of β-amyloid 42 peptide). These aggregates compose to senile plaques, which are known to cause Alzheimer´s (GouRas et al., 2000). The Aβ42 aggregation therefore leads to neurodegeneration, loss of memory, reduced life span and other

symptoms typical for Alzheimer`s. As a conclusion of the experiments of the Crowther laboratory, *D. melanogaster* is found to be an adequate model for drug testing concerning human Alzheimer´s disease (Crowther et al., 2005).

1.1.4 Parkinson´s disease

Parkinson`s disease is a motor system disorder, which causes tremor, rigidity, postural instability, bradykinesia, which can be described as the slowness of motion and it could be a side-effect of medication of parkinson's disease's symptom (Warrell et al., 2003). Other symptoms are depression and other emotional changes, decreased ability of speaking, swallowing and chewing, urinary problems, skin problems and sleep disruptions. Furthermore, about one third, up to one half, of all Parkinson´s patients suffer from dementia (Warrell et al., 2003).

In the case of Parkinson´s disease dopamine producing neurone cells are lost, which is accompanied with the formation of cellular inclusions, the so called Lewy-bodies (Botella et al., 2004). The protein α-synuclein is part of those Lewy-bodies and several mutations of synuclein already have been detected for rare familiar cases of Parkinson´s disease, but are thought to be present in most cases of Parkinson´s. The use of *D. melanogaste*r as a model was established several years ago, where the human synuclein was over expressed in a fly´s central nervous system (CNS). It was observed, that the neurodegenerative effect solely took place in the CNS, which interconnect with Parkinson´s disease, although synuclein was expressed in all neurons. Additionally, the affected neurons showed inclusions, which are comparable to Lewy-bodies (Botella et al., 2004).

1.2 Molting and Metamorphosis in Insects

As all complex organisms, insects need to feed, grow, and differentiate in varies stages and at last end up in adult forms. Metamorphosis is the biological term for the process where the structures of the body undergo several changes. Insects with a complete metamorphosis (holometabolous development) have four main life stages; the embryonic, larval, pupal, and adult stage (Gilbert, 2000). Embryonic stage starts after the egg lay and ends with the hatching from the egg. After this stage, the larvae begin to feed and grow until they reach a specific threshold. Insects have a rigid exoskeleton, which gives the mechanical resistance and shape of the insect, attached to underlying epidermal cells. In every ecdysis process, the old cuticle undergoes apolysis by molting fluid and

separates from the epidermis. Then the new cuticle expands instead of the old one. All these processes are controlled by ecdysone levels in insects.

After egg lay (AEL), hatching occurs approximately 24 hours later, then the 1st instar larvae stage begins and it finishes with the first larval-larval molting. In the 1st instar, larvae are feeding with one tooth and the larvae have small salivary glands. Then, the 2nd instar larvae stage occurs between the first and second larval molt. In the 2nd instar, mouth hooks are getting bigger and the larvae have 2-3 teeth, additionally the salivary glands increase in size and imaginal discs start to grow (Andres, A.J, Thummel, C.S., 1994). Finally, the 3rd instar is ended with the puparium formation which is the last molting process. The most obvious change between 2nd instar larvae and the 3rd instar larvae is the modification of spiracles. Anterior spiracles become brunched and the tips of posterior spiracles become dark orange. 3rd instar larvae continue feeding for approximately 24 h before ceasing feeding (see figure 1 for the life cycle of *D. melanogaster*). At this point the larvae crawl out of the nutrient in search of a dry place to undergo metamorphosis. Then the larval- pupal molting occurs. The pupal stage causes a drastic transformation. In every larval molt, cuticular structures as mouth armature and spiracles are shedded and reformed again, but the imaginal organs continue to increase their sizes by cell multiplication independently from cuticular structures (Demerec, 1994).

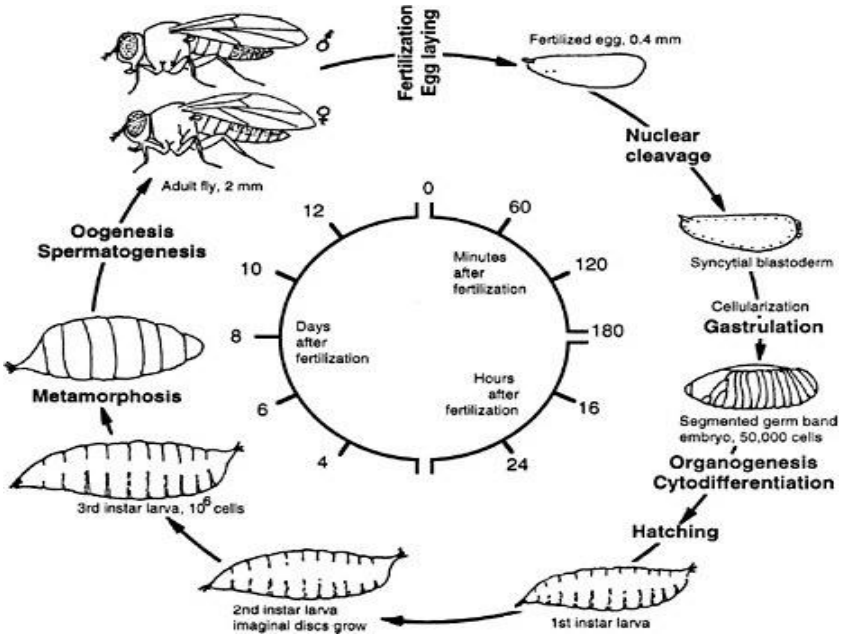


Figure 1 *The life Cycle of D. melanogaster. After egg-lay, D. melanogaster completes embryonic development and hatches. Then the 1st and the 2nd instars both last a day. Larvae spend 2 days at the 3rd instar until they enter the metamorphosis. The last process, which is metamorphosis, takes 4 days and ends with adult flies enclosing -from (Scientific Frontiers in Developmental Toxicology and Risk Assessment)*

1.2.1 Imaginal Discs of *D . melanogaster*

An adult *D. melanogaster* is constituted from three main body sections; head, thorax and abdomen. The *D. melanogaster* head contains the antenna, frons, vertex, clypeus, labrum, eyes, and labellum. The thorax consists of wings, legs haltere, and thoracic segments as prothorax, metathrorax, and mesothorax. Lastly, the abdominal part of the fly includes the tail, anal plate, vaginal plate (if the fly is female), the dorsal tegnum and lateral spegnum (Bainbridge, 1981). Figure 2 shows the changes from larval to adult stage.

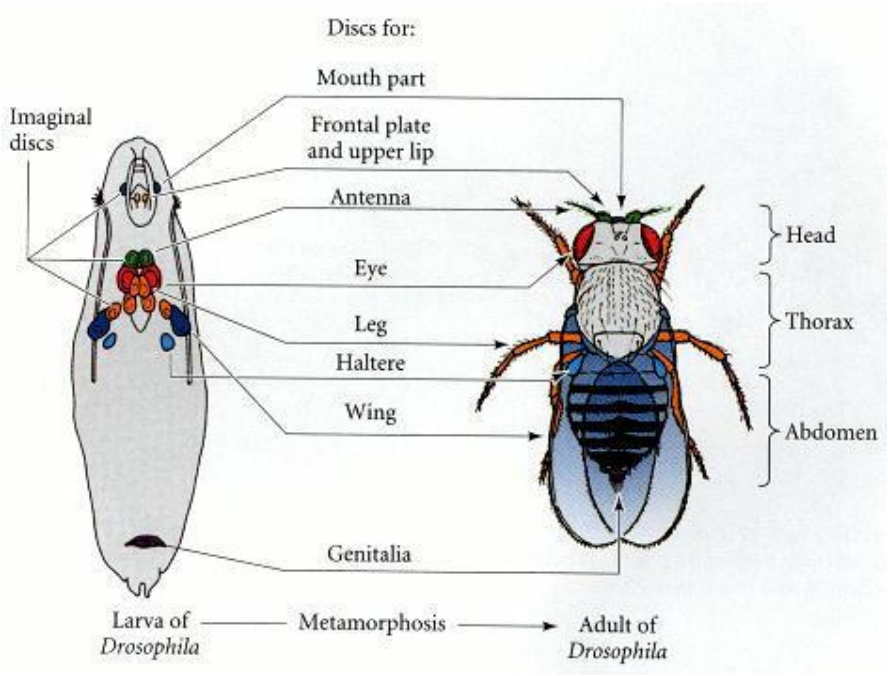


Figure 2 *Transformation of Imaginal Discs in D.melanogaster . This figure shows how D.melanogaster develops from larva's imaginal discs and emphasizes the importance of imaginal discs in metamorphosis from Gilbert et al., 1994*

The development of adult fruit flies is formed from imaginal cells, which are formed as clusters of cells during embryonic development. Imaginal discs, which are formed by histoblasts and imaginal rings, play a significant role in forming external structures of the fly such as legs, wings, eyes and antennas. During larval stages, the imaginal discs await signals to develop into adult structures. During metamorphosis larval structures undergo apoptosis, and the imaginal discs divide rapidly to construct the new adult structures (Gilbert, 1994). The Malpighian tubules and the brain do not change dramatically during metamorphosis, but most of the other larval organs and tissues are completely histolyzed (Demerec, 1994). On the contrary to imaginal discs, integral organs such as foregut, hindgut, midgut, salivary gland and trachea are structured by imaginal rings during embryonic development.

1.2.2 The Hormones affecting metamorphosis in *D. melanogaster*

D. melanogaster has to reach a necessary check-point in order to enter metamorphosis at the end of the 3rd larval instar which is called "critical weight" (Davidowitz, 2003). Some hormones, the pathways stimulated by these hormones, and diet quality have a significant effect on final body size (Davidowitz, 2003). Juvenile hormone (JH), which is secreted by corpus allatum, prevents the flies from entering metamorphosis (McBrayer et al., 2007). Secreting cells in corpus allatum are active during the larval molts, but these secreting cells become inactive during the metamorphic molt. When the larvae reach the critical weight, the level of JH decreases because the glands, which produce JH, are inhibited by the brain (Safranek and Williams, 1989). After this inhibition, the level of JH decreases and PTTH is released from bilateral neurosecretory cells into the brain at the first photoperiodic gate, which occurs eight hours a day (Mizoguchi A, Kataoka H., 2005). As a result of high level of PTTH, ecdysteroid synthesis is triggered leading to the cessation of feeding. After the larvae stop feeding, the growth stops. Therefore, final body size of the adult flies is determined by the time larvae reach critical weight and stop feeding before entering metamorphosis. Larvae starving before attaining the critical weight will eventually become smaller adults. At the end of the third instar, there is a high titer of ecdysone which induces puparium formation and determines the start of the prepupal stage and metamorphosis. The level of ecdysone is low at prepupae stage of *D. melanogaster*, however, the level rises 12 hours after the white puparium stage. These peaks continue also after the puparium.

1.2.3 Metamorphic stages of *D. melanogaster*

Metamorphosis in *D. melanogaster* can be explained as a specific process which transforms the larvae after 3rd instar to the reproductively mature adult form. During the 1st and 2nd instar larvae feed and increase in body size. At the end of the 3rd instar they stop the consumption of food and start preparing for metamorphosis. Hereafter the larvae stop moving, evert spiracles and start to form puparium which is a larval cuticle that protects insects while they are transforming (Bodenstein et al., 1943). The white puparium stage marks the beginning of metamorphosis. This stage lasts approximately 15 minutes, during which larvae evert anterior spiracles to provide gas exchange between the fly and the environment. Then the body shortens and it attaches to the wall of the substratum. After the white puparium stage, 3rd larval instar has reached its end and the prepupal

stage is initiated. The movement of the larvae stops completely and the cuticle turns dark brown. When larval-pupal stage begins, imaginal discs are developing into the wings, legs and start forming the main structures of the adult body, such as head, thorax and abdomen (Bainbridge et al., 1981). However, it is not possible to see any external limbs and head of the flies within the first 12 hours during the prepupal stage. First the gas bubble which evolves in the abdomen becomes visible. After 12 hours, pupal formation is triggered by ecdysone titers and the bubble translocates from the abdomen to the anterior end. The muscles in the abdomen push the head of *D. melanogaster* to the anterior end and this crucial point is referred to as the prepupal-pupae transition of the *D. melanogaster*. After the formation of the head, the pupal stage approximately lasts additional 84 hours. Then Malpighian tubules migrate from thorax to the abdominal part of the fly. Malpighian tubules become visible dorsally. Next, the colour of the tubules changes from white into green. The eye colour changes from bright colour to amber, then it darkens and first becomes pink, and hereafter turns dark red. After the development of eye pigmentation, first head bristles and then tergite bristles start to become visible (Bainbridge, 1981). Thereafter the colour of the wing tips changes from grey to black. Next meconium, which can be described as first excrement, becomes visible at the tip of the abdomen. Later Tarsal structure is formed and darkens. Firstly, wings and legs are developed, then all cells and tissues start changing and forming bristles, sockets, eye pigments, organs and neural maturation occurs (Bainbridge, 1981). At the end of the metamorphosis, adult flies eclose.

1.3 The role of PTTH, Ecdysteroids and Juvenile Hormone

Molting and metamorphosis are mainly controlled by three hormones in insects: PTTH which is produced in the neurosecretory cells, ecdysteroids produced in the prothoracic glands (PG) and finally JH released from the corpora allata (Nation, 2008).

When PTTH is secreted the PG in response synthesizes and secretes ecdysteroids. These ecdysteroids combine with a receptor protein in the nucleus. The complex binds to DNA and as a result, master genes undergo transcription. This initiates a cascade process resulting in increased gene activity. Consequently, the epidermal cells start cell division, secretion of molting fluid occurs as well as secretion of a new cuticle. Depending on the age of the insect numerous changes in morphology and physiology occur in the nervous system, gut and reproductive organs (Nation, 2008).

JH modulates the effects of ecdysteroids depending on which type of transformation the insect is subjected to. The extend of the changes in the internal organs depends on the kind of transformation occurring. A change from larval to larval stage is not as drastic as from pupal to adult. The specific way JH effects the molting and metamorphosis is still not known.

In *D. melanogaster* there are six pulses of ecdysteroid production during the development from embryo to adult. The ecdysteroid secretion is induced by a preceding pulse of PTTH release. JH is also secreted in conjunction to the ecdysteroid pulses but early in the last instar the release of JH is reduced compared to the other instars and finally becomes nondetectable at the end of the last instar. This is the time when the pupa molts into adult. The interaction of the ecdysteroid pulses, PTTH and JH determine the development of *D. melanogaster* between the different instars and without perfect timing of the three hormones an accurate molting process cannot occur. Figure 3 shows the hormone titers during the development of *D. melanogaster*.

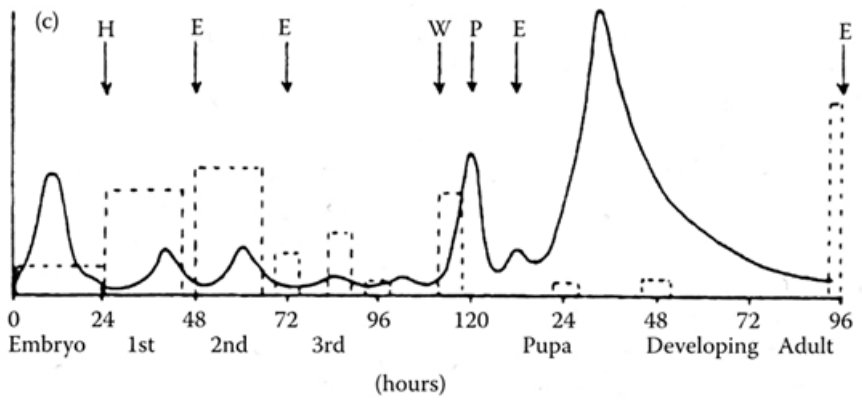


Figure 3 *Hormone titers during development. Solid line shows ecdysone titer, dotted line shows JH titer. The figure shows an increase in ecdysone during each instar. The decrease in JH during development from pupa to adult is clearly seen in the figure. H refers to hatching. E refers to ecdysis. W refers to wandering stage. P refers to puparation from Nation 2008*

1.3.1 Ecdysone production

Ecdysone is also known as the molting hormone in insects. Ecdysone is a steroid hormone of which insects need a sufficient production to be able to execute a normal development. Insects need to take up cholesterol from their diet in order to produce steroids since they lack the ability to

synthesize it from smaller precursor molecules. Meat eating insects can directly take up cholesterol, but plant-eating insects need to rely on the uptake of primarily phytosterols such as sitosterol or campesterol which they can then dealkylate to cholesterol (Gilbert, 2004). *D. melanogaster* is an exception as it seems as it cannot dealkylate plant sterols as other plant-eating insects, instead it uses that small amount of cholesterol plants do contain.

1.3.2 Halloween genes and the synthesis of ecdysone in the PG

A family of genes in *D. melanogaster* has been identified to code for cytochrome P_{450} enzymes in the ecdysteroidogenic pathway (biosynthesis of ecdysone from cholesterol). The genes include the following: spook (*spk*), spookier, phantom (*phm*), disembodied (*dib*), shadow (*sad*), and shade (*shd*). Mutations in these genes have been identified to cause embryonic lethality (Gilbert, 2004).

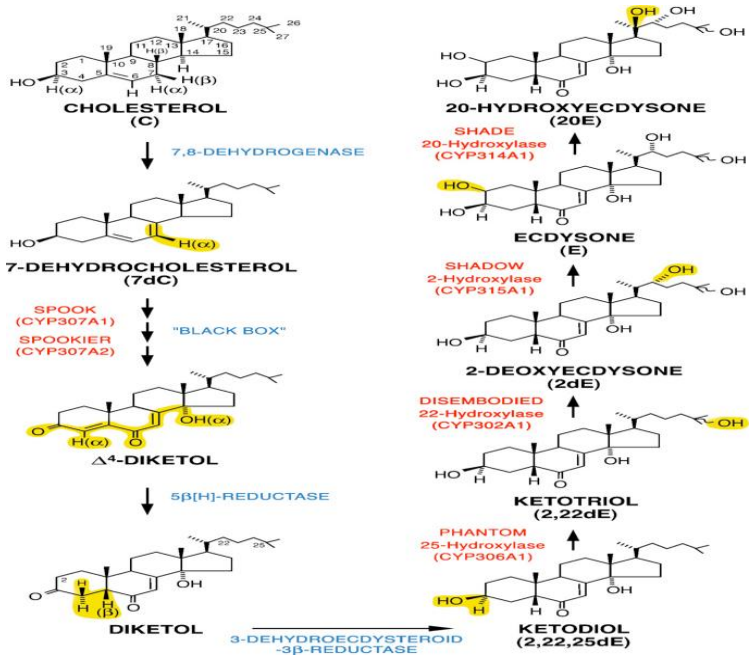


Figure 4 *shows the ecdysteroidogenic pathway and the role of the Halloween genes in the conversion from cholesterol to 20E from Gilbert et al., 2008*

First, cholesterol is converted to 7-dehydrocholesterol (7dC) by 7,8 dehydrogenase. The irreversible reaction involves removal of both the 7β and 8β hydrogens and as a result a double bond is created. Neverland (*nvd*) has been proposed as being the responsible gene for the conversion (Yoshiyama, 2006). Another important aspect is the transcription factor called *woc* (without children) and if this

is missing or malfunctioning, it is lethal for the *D. melanogaster* larvae and they cannot undergo proper development. However, the mutant *woc* larvae can develop normally if they are fed 7-dehydrocholesterol (Warren, 2001). In the next step 7-dehydrocholesterol is shuttled from the cytoplasm into the mitochondria where oxidative and hydroxylation steps occur (Nation, 2008). These reactions are also known as the "Black box" since the pathway is not yet fully understood. This means that 7dC is oxidized to Δ^4-diketol by a number of uncharacterized enzymes. The genes *spook* and *spookier* have been proposed as being part of the black box, but the actual substrates have not yet been established (Gilbert, 2008). A 5β[H]-reductase will convert Δ^4-diketol to a diketol having a cis-A, B-ring fusion. Subsequently a 3-dehydroecdysteroid – 3β-reductase synthesizes a ketodiol (2,22,25dE).

Phantom (*phm*) is the gene coding for the 25-hydroxylase that converts ketodiol (2,22,25dE) to a ketotriol (2,22dE). *Phm* is expressed as early as in the blastoderm stage (as with *dib* and *sad*) in the epidermal stripes but later when the ring gland is developed the expression is restricted only to the ring gland (Gilbert and Warren, 2005). The conversion of cholesterol to 20E is shown in figure 4.

In situ analysis has shown that *dib* is only expressed in the PG cells of the adult *D .melanogaster* ring gland. The phenotype of mutant homozygous embryos appears to be normal up to mid-embryonic development. Then the embryos show signs of an influenced development in form of undifferentiated cuticle, failure of head involution and dorsal closure, a compact appearance and abnormal looping of the hindgut. As a consequence the animals die before the embryogenesis is finished. *Dib* has been established to be the C22-hydroxylase that converts ketotriol to 2-deoxyecdysone (Gilbert and Warren, 2005).

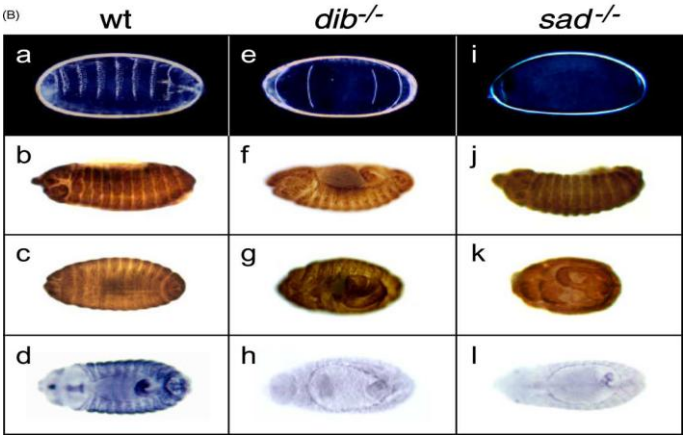


Figure 5 *Phenotypes of sad and dib mutant embryos compared to wildtype. Mutations in sad and dib genes result in embryonic lethality and disrupted developments when compared to wildtype. This is due to their important role in the synthesis of 20- hydroxyecdysone from Gilbert et al., 2004*

Shadow (*sad*) mutants also cause embryonic lethality and it is restricted to expression in the PG cells. It has been shown that shadow is responsible for the conversion of 2-deoxyecdysone to ecdysone by 2-hydroxylase (Gilbert and Warren, 2005). The disturbed development of the mutants compared to the wildtype can be seen in figure 5.

1.3.3 Conversion of ecdysone to 20-hydroxyecdysone

The last reaction step to be covered is the conversion of ecdysone to 20-hydroxyecdysone. The reaction is an oxygenation of C20 (addition of hydroxyl-group). The activity of 20-hydroxylase has been found in various insect tissues in mitochondria and microsomes of the fat body (the insect liver), Malpighian tubules (excretory organ), midgut and several others. It has not been shown to be active in the prothoracic glands, nerve cords or muscles. The mutant Shade (*shd)* gene has been shown to be an embryonic lethal as well with the same developmental consequences as for *dib* and *sad*. Studies have shown that *shd* is not expressed very early in embryogenesis as it is the case for *dib* and *sad*. Five hours later, expression has been seen in the epidermis (like *sad*). Late in the 3rd instar *shd* is expressed in the above mentioned tissues (Nation, 2008).

1.4 Ras Signalling and MAP-Kinase Pathway

The PTTH release that triggers the production of ecdysone is mediated through Ras-signalling and the subsequent activation of the MAP-kinase pathway.

Ras is a small GTPase or monomeric GTP-binding regulatory protein which is a regulator in cell proliferation (Devlin, 2006). Ras is a protein that mediates signal transduction (Nelson et al, 2008). Ras has an "on and off" switch regulated by GTPase proteins which have either a GDP or a GTP molecule bound to them. After the signal has been switched on it needs to be switched off again at some point, and this happens when the Ras protein converts GTP to GDP (Elliott et al., 2002). In order to activate and stimulate Ras an adaptor molecule that recruits and stimulates Ras activating proteins binds to the phosphotyrosine sites of the activated Receptor Tyrosine Kinases (RTK). Ras-activating proteins act to enhance the exchange of GTP and GDP at the guanine binding site of Ras, which happens in the stimulated state (Devlin, 2006). When GTP binds, Ras can activate a protein kinase called Raf-1 which is the first of 3 protein kinases; Raf-1, MEK and ERK (extracellular signal-regulated kinase). These proteins conduct a cascade, where each kinase activates the next by phosphorylation (Nelson et al, 2008). Following this, there is an accumulation of Ras molecules with active GTP-bound conformations. This results in the active Ras proteins temporarily binding to and therefore stimulating a family of serine/threonine protein kinases that trigger the Mitogen Activated Protein kinase cascade (MAPK cascade) (Devlin, 2006).

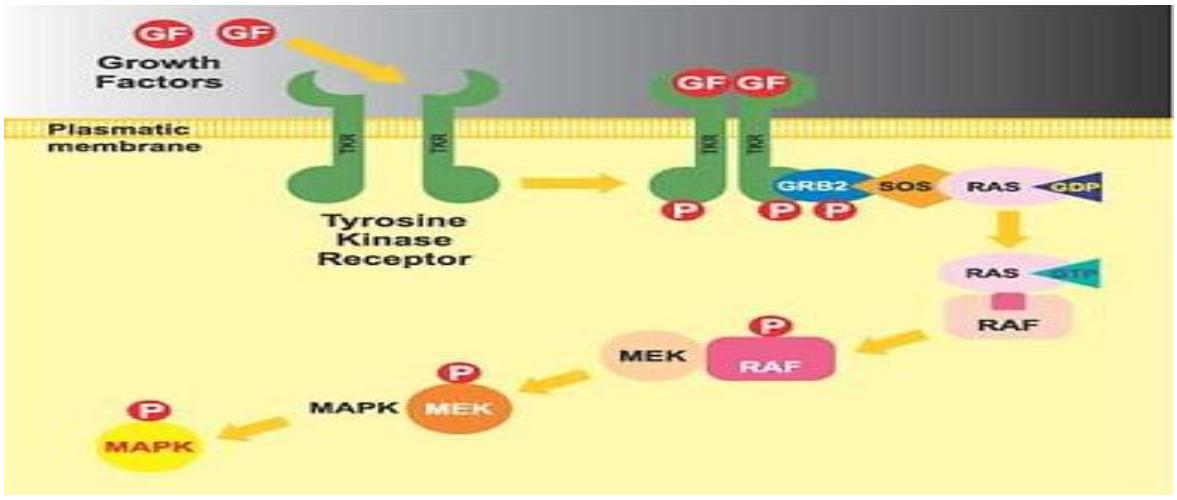


Figure 6 *schematic illustration of how Ras signalling activates the MAPK cascade from Sobera et al., 2002*

MAPK pathways are found in eukaryotes and represent a signalling pathway used by many different growth factors. In short terms the pathway is used by specific tyrosine kinase-associated receptors to activate gene transcription. The first protein in the kinase cascade is Raf, which is

activated by the Ras-GTP and in turn the activated Raf phosphorylates MEK, which is the second protein in the kinase cascade. MEK then phosphorylates the last of the three protein kinases in the cascade called ERK. When ERK is phosphorylated it then enters the nucleus of the cell and phosphorylates target transcription factors so that a transcription of specific genes as well as the synthesis of their cognate proteins takes place (Elliott et al., 2002).

As seen in figure 6 growth factors open up the RTK, which stimulates Ras activating proteins. Ras proteins exchange GDP and GTP. When GTP is bound to Ras, it activates Raf and by phosphorylation Raf activates MEK. Finally Ras-signalling activates the MAPK cascade (Berger et al., 2003).

ERK belongs to MAPK families, which are extracellular signals that induce mitosis and cell division (Nelson et al, 2008). This cascade involves the serial actions of 3 protein kinases; Ras-activated kinases activate an intermediary set of MAPK kinases, which activate the terminal MAP kinase effectors. The terminal MAPK phosphorylate multiple target proteins in the cytosol and the nucleus, plus the transcription factors that regulate the expression of genes required for cell division, cell survival or phenotypic differentiation. In short the GTP-Ras activates a kinase cascade resulting in the phosphorylation and activation of the terminal kinase, MAPK. Activated MAPK enters the nucleus and phosphorylates transcription factors that regulate the expression of proteins involved in S-phase (Devlin, 2006).

The signalling during MAPK pathways occurs via membrane receptors, where the signal binds to a transmembrane domain and a cytoplasmic domain (Elliott et al., 2002). When growth factors bind to the receptors they dimerize in the membrane and thereby bringing their domains together so they can phosphorylate each other (Elliott et al., 2002). Now it is possible for a growth receptor-binding protein (GRB) associated with a protein called SOS (the name is derived from a *Drosophila* genetic mutant known as *son of sevenless*) to bind to the phosphorylated receptor and this GRB/SOS complex in turn activates Ras and the protein kinase cascades can now begin (Elliott et al., 2002).

1.4.1 Ras-signalling in neurons and the effect of Raf

It is known that PTTH activates the MAPK-pathway in PG which affects the synthesis of the molting hormone 20E. There is, however, limited literature on the role of Ras and Raf in the PTTH-producing neurons. Caldwell (2007) investigated the role of Ras-signalling in cholinergic neurons

in *D. melanogaster*. For this experiment they used flies bearing ChaGal4 and a transgene expressing the constitutively active Ras^{v12} under the control of UAS. Chagal4 is proven to be expressed in all cholinergic neurons in adult flies (Salvaterra et al., 2001) and in the study by Caldwell (2007) it is determined that ChaGal4 is not expressed in the PG in larvae. The flies bearing both of the genes where Ras is overactivated in the PTTH-producing neurons were found to be morphologically normal but reduced in size. The size reduction was due to both reduced cell-size and reduced number of cells when wing hair cell size and wing hair cell density were measured (Caldwell, 2007).

To investigate through which pathways Ras^{v12} exerts its effect, Raf^{f20} among others were introduced under UAS-control into flies bearing ChaGal4. The experiment shows that Raf^{f20} has the same reducing effect on body size and to the same extend as Ras^{v12}. In the same experiment by Caldwell (2007) the PI3K and Ral were also investigated as possible pathways for the effect of Ras^{v12}, however none of these showed an effect and Caldwell (2007) therefore concludes that Ras^{v12} only exerts it effect through Raf.

The fact that overexpression of Ras and Raf creates flies with reduced cell size and number only shows that Ras and Raf are sufficient in reducing body size, but it does not show that Ras and Raf are necessary. In order to demonstrate this, the dominant-negative Ras^{N17} and Raf ^{K497M} were introduced under UAS-control into flies bearing ChaGal4. The result of this was an increase in body size in flies expressing the dominant-negative Ras^{N17} and in flies expressing the dominant-negative Raf ^{K497M} (Caldwell, 2007).

The paper does not mention anything about the role of Ras and Raf in the timing of development, however the differences in fly size indicates that the metamorphosis occurred on different times, and therefore Ras and Raf can be assumed to regulate the timing of metamorphosis.

1.4.2 PTTH synthesis

The adult size of *D. melanogaster* is determined by larval size at the time of the pupal molt because all growth occurs during the larval stages. The timing of this molt is regulated by the release of the steroid molting hormone ecdysone from cells of the PG (Caldwell et al., 2005). The release of ecdysone from cells in the PG is a response to the effect the neuropeptide PTTH has on PG. PTTH activates the PG to synthesize and secrete ecdysone (Marchal et al., 2009).

PTTH is synthesized in the two large lateral neurosecretory cells (NSCs) in the lateral region of each hemisphere of the *D. melanogaster* brain. PTTH is synthesized as a prehormone and released as a shorter, glycosylated, homodimeric molecule of between 25 and 30 kDa containing a single intermonomeric cysteine–cysteine bond and three intramonomeric cysteine–cysteine bonds (Gilbert et al., 2002). PTTH is stored in the corpora cardiaca–corpora allata complex (CC–CA), the neurohemal organ for PTTH until release.

1.4.3 Control of PTTH release

The release of PTTH occurs at specific developmental stages during the development of *D. melanogaster*. The release is controlled by a series of physiological factors, such as the nutritional state of the animal. However it is also influenced by certain environmental cues, such as photoperiod, circadian factors, and time of day (Marschal et al., 2009). In some insects artificial inflation of the gut with air can trigger PTTH release and molting. Therefore abdominal stretch receptors are suggested to activate PTTH. In *D. melanogaster* artificial inflation does not induce a molt meaning that stretching of the larval abdomen is not on its own sufficient enough to stimulate PTTH release in *D. melanogaster*. Instead, as mentioned above, the release is controlled by a more complex nutritional assessment where the fly has to achieve a certain size and nutritional state in order to survive the period of starvation during metamorphosis (Edgar, 2006 & Nijhout, 2003).

1.4.4 PTTH and ecdysone release

Metamorphosis of *D. melanogaster* is primarily controlled by the steroid molting hormone ecdysone. Ecdysone pulses trigger two larval molts to accommodate the approximately 200-fold increase in mass that occurs as the larva feeds. Increases in ecdysone seen at the end of the last larval stage terminate feeding and initiate metamorphosis (King-Jones et al., 2005) The release and synthesis of ecdysone is known to be regulated mainly by the neuropeptide PTTH. However the experiment from Mcbrayer et al. (2007) showed that even though PTTH is removed, metamorphosis of the flies will still occur. The flies are larger in size which indicates that the timing of metamorphosis is changed when PTTH is not available. This suggests that PTTH controls that the right amount of 20 E is released at the right time during larval stage because the final fly size depends on the larval size. In addition PTTH is only released when the flies reach a correct

size/nutritional state that enables them to survive starving during metamorphosis (Mcbrayer et al., 2007).

The signalling pathway, responsible for the production of ecdysone, is incompletely defined. In a report by Rewitz et al. (2009), it was demonstrated that Torso, a receptor tyrosine kinase that regulates embryonic terminal cell fate in *D. melanogaster*, is the PTTH receptor. The activation of Torso by PTTH stimulates ERK and it was concluded that this Torso/ERK pathway activated by PTTH is what initiates metamorphosis in insects (Rewitz et al., 2009).

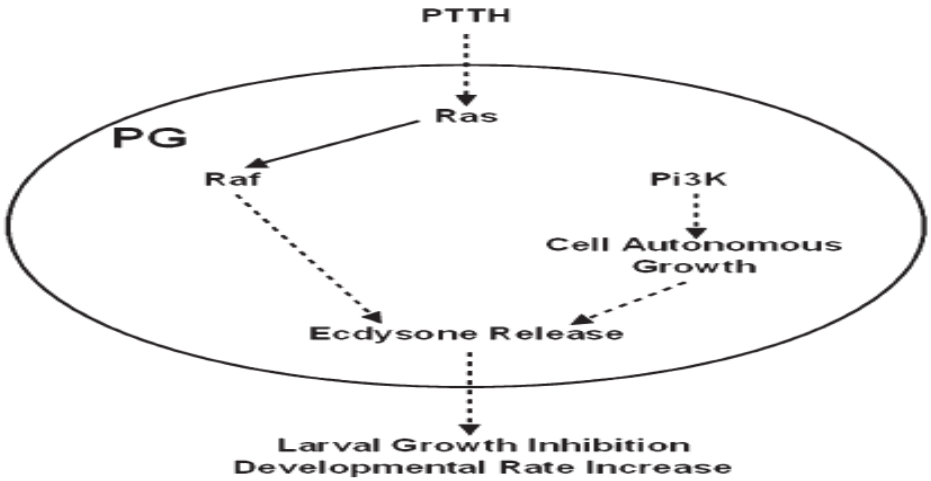


Figure 7 *The possible ways of ecdysone release in PG cells. Figure from Caldwell et al., 2008*

A study by Mcbrayer et al. (2007) shows that metamorphosis occurs and ecdysteroid peaks are observed even though PTTH is not present. This suggests that another factor affects the ecdysone release from PG-cells (Mcbrayer et al., 2007). This could possibly be insulin-signalling.

Figure 7 is describing the effect of Ras signalling on ecdysone release, larval growth rate, and developmental rate. Ecdysone release is possibly regulated by PI3K in PG cells, but also by a PTTH induced Ras/Ras dependent step that involves the transcriptional regulation of ecdysone genes. The release of ecdysone then affects both the growth and developmental rates of larvae, resulting in final body size (Caldwell et al., 2005).

1.4.5 ISS and ecdysone release

In *D. melanogaster*, the insulin/IGF signalling (IIS) pathway is a neuroendocrine signalling pathway that plays an important role in the regulation of growth and metabolism, both during development and in the adult life. In model animals ranging from the fruit fly to the mouse, IIS affects growth and development, metabolic/energy homeostasis, stress resistance, reproduction and lifespan (Colombani et al., 2005).

As it is shown in figure 8, the ISS system is activated by an insulin/IGF like ligand that binds to insulin type receptor on the extracellular side of the cell. The binding results in the activation of the tyrosine-kinase domain placed on the intracellular side of the cell. Next, this initiates phosphoinositide 3 kinase (PI3K) and thereafter the serine-threonine protein kinase AKT which is also called protein kinase B (PKB). Furthermore it leads to the inactivation of the fork head box O transcription factors. The active receptor also activates ERK and in addition AKT seems to interact with the target of rapamycin (TOR pathway) (Colambani et al., 2005).

In *D. melanogaster* insulin-like molecules called Dilp activate the insulinreceptor InR (Colombani et al., 2005). ISS can have an effect on activation of ecdysone production because ISS activates ERK, which is already known to be involved in the production of ecdysone via MAPK activated by PTTH.

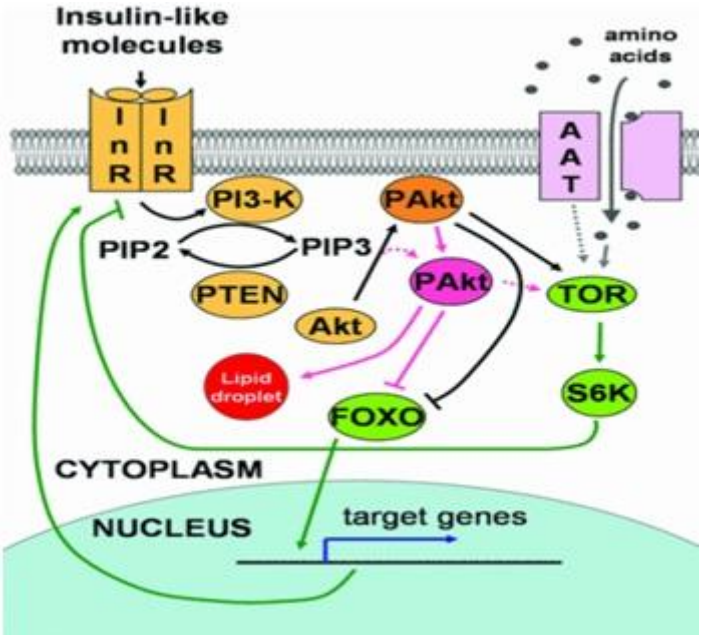


Figure 8 *Insulin-like molecules called Dilp activate the insulin receptor InR which leads to the activation of PI3K and Akt which also interact with the TOR pathway. Figure from Wilson et al., 2007*

2 Method

2.1 GAL4-UAS System

The "Gal4-UAS" technique is an extremely useful tool in the control of where and when specific genes are expressed. Two types of transgenes, which are a Gal4 "driver" and a Gal4-responsive UAS expression vector, are used in this technique. Gal4 encodes a protein of 881 amino acids, which is identified in the yeast *Saccharomyces Cerevisiae* as a regulator of the trancription of GAL10 and GAL1 genes (Johnston et al., 1987). The protein, encoded by Gal4, is binding directly to four related 17 basepair sites located between GAL10 and GAL1 and, in addition these basepairs define the Upstream Activating Sequences (UAS) element (Andrioli et al., 2002).

The Gal4-UAS system can be transferred into other organisms where UAS is essential for the transcriptional activation of these Gal4-regulated genes. Therefore, the Gal4-UAS system can be used to drive expression of inserted cDNA (complementary DNA) sequences when Gal4 is present.

The Gal4-UAS system separates the target gene from its transcriptional activator in two distinct transgenic lines (Chae et al., 2002). The first line contains the target gene which remains silent in the absence of its activator, whereas the second line contains the UAS- responder gene. The Gal4-UAS system is designed to generate lines that express a transcriptional activator.

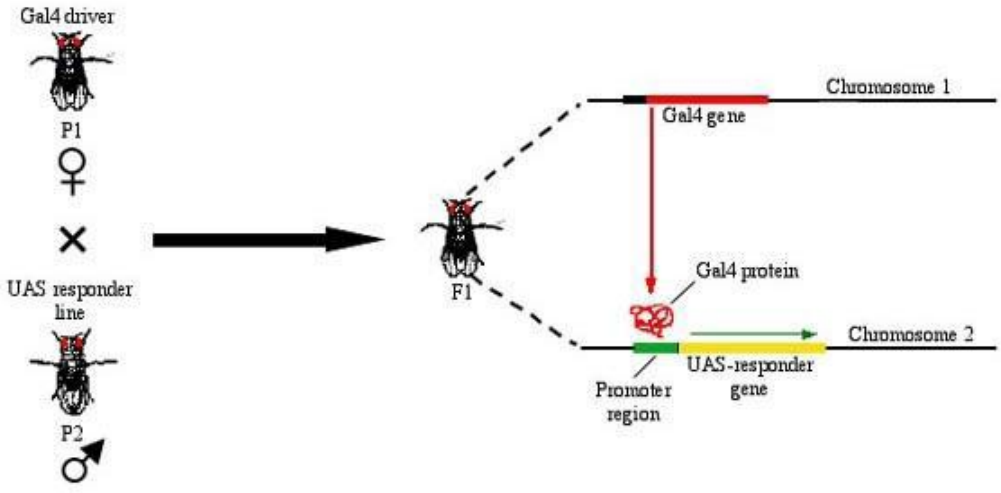


Figure 9 *The Gal4-UAS Technique. Figure from Lanata 2003*

As seen in figure 9, Gal4 protein products directly bind to the UAS element and then drive the expression of genes located downstream to the UAS element. This binding is essential for the trancriptional activation of the Gal-4 regulated genes.

Therefore, Gal4 expression systems have been applied in mice, zebrafish, and plants. The Gal4 system is also used to interfere with gene expression by transcription of RNAi, which provides a powerful approach to the targeted disruption of a given genetic function in *D. melanogaster*, or expression of dominant-negative forms of a protein. Moreover, its other applications include targeted cell ablation, performed by expression of cellular toxins or apoptotic genes and the expression of reporter proteins.

One of the advantages of over-expression techinique is that it allows the rapid generation of individual strains in which ectopic expression can be directed to different tissues or cell types.

2.2 Selecting Larvae and Monitoring Pupariation

2.2.1 Collecting flies and setting up crosses

To create the lines which were studied in this project crosses were set up. From one of the two lines used in one cross, virgin females had to be collected. Virgin females are used to make sure they have not been fertilized from males of their own genotype. The vials from which virgin females were collected were emptied and thereafter females were collected within 18 hours when incubated at 18° C and within 6 hours when incubated at 25° C. At 25° C females do not mate until 8 hours after eclosion (eclosion is the development from pupae to fly). At 18° C the development of the female flies is prolonged, ensuring that no fertilization can occur in up to 16 to 18 hours after eclosion (Raplh J. 1997). The flies were sedated with CO_2 during the process of collecting. The process was repeated until enough flies were collected.

The following crosses (seen in table 1) were set up in order to investigate the role of Ras-signalling in the production and release of the neuropeptide PTTH. Lines expressing a dominant negative form of Raf (Raf D.N.) and lines expressing a constitutively active form of Ras (Ras^{v12}) were used in this study. The knocked-down form of Raf was chosen since there is no dominant negative form of Ras which shows a remarkable effect. By choosing the knocked-down form of Raf it was ensured that

the pathway downstream was inactivated. In addition, Feb211 lines were tested since they bear an analog of the *ptth-Gal4* line. The *UAS-Raf.D.N.2.1/Cyo-GFP. UAS-Raf.D.N. /UAS-Raf.D.N.* was included in order to test a line with two alleles of dominant negative Raf. Controls were set up by crossing W^{118} (wildtype) with the lines of interest (*ptth-Gal4, Feb211-Gal4* or *UAS-Raf.DN.21*). These lines were used as controls because of their similarity with the manipulated lines. The crosses were set up using approximately 15 males and 15 females in each vial. Virgin females were collected from following lines: *ptth-Gal4*, W^{118}, *feb211-Gal4* and males were collected from the *UAS-Ras*^{v12}, *feb211-Gal4*, *Uas-Raf.DN.2.1,* W^{118} *and UAS-Raf.DN.2.1/Cyo-GFP. UAS-Raf.D.N. /UAS-Raf.D.N.-lines.*

Table 1 Crosses between females and males that were made in this study.

Females	Males	Type
Ptth-Gal4	UAS-Ras^{v12}	Over active
W^{118}	Feb211-Gal4	Control
W^{118}	UAS-Raf.DN.2.1	Control
Ptth-Gal4	UAS-Raf.DN.2.1	Dominant negative
Feb211-Gal4	UAS-Raf.DN.2.1	Dominant negative
Ptth-Gal4	W^{118}	Control
Ptth-Gal4	UAS-Raf.DN.2.1/Cyo-GFP.UAS-Raf D.N. /UAS-Raf.D.N.	Dominant negative

2.2.2 Egg-lay

The egg-lay was carried out for 4 hours on applejuice agarplates with yeast added as nutrient. The day before the egg-lay, the flies were moved to applejuice agarplates with yeast to make them familiar with the environment before the actual egg-lay was carried out and therefore optimizing the result of the egg-lay.

Immediately after the first egg-lay the applejuice agar plates were removed and incubated at 25 °C and another egg-lay was made with the same flies. Approximately 26 hours AEL larvae were collected and transferred to fresh vials with standard cornmeal media. This was done for both the first and the second egg-lay. Approximately 32 larvae were transferred to each vial to guarantee optimal growth conditions. Only visibly moving larvae were transferred in order to make sure that they were alive. The larvae from the first egg-lay were incubated at 25 °C and the ones from the second egg-lay were incubated at 29°C.

In this study, the aspect used to monitor the developmental transition was the timing of pupariation, to determine whether the larvae have entered that stage shortening of the animal, everion of anterior spiracles and a darkening of the pupae has been observed.

The monitoring of pupariation started 5 days AEL. The pupaes were counted several times during the day in approximately two hour interval.

2.3 The principle of qPCR

Quantitative real time polymerase chain reaction (qPCR) is a technique based on the regular PCR.

2.3.1 Conventional PCR

PCR is a technique that is based on the DNA-replication. The actual DNA replication in living cells takes up to two months, but the PCR technique is able to make millions of copies of a DNA-fragment within 2-3 hours. Following components are used in a PCR technique (Thougaard et al., 2007).

Template: Template of the target DNA

Primer: 2 short single stranded DNA sequences about 15-20 nucleotides, which are complementary to the 3'ends of the target-DNA.

Heat stable DNA Polymerase: Enzyme that is able to build the single-stranded DNA to double-stranded DNA; it puts the free nucleotides to the strand.

dNTP: Consist of 4 nucleotide; dATP, dTTP, dCTP and dGTP, used as a building stone to build the new DNA strand.

Buffer: Gives a suitable environment for optimal activity and for stabilizing DNA-polymerase (Coyne et al., 2001)

The procedure of PCR is shown in figure 9 step by step and explained in detail below.

Denaturation: Separation of the target DNA strand. The hydrogen binding will denaturize by heating to 94°C, and there will be two single-stranded DNA-molecules.

Annealing: Also called hybridization; the single-stranded complementary DNA sequences assemble to double-strand. This happened by 50-60°C.

DNA- synthesis: By decreasing the temperature, DNA-polymerase will build up a double-stranded DNA from primers 3'end.

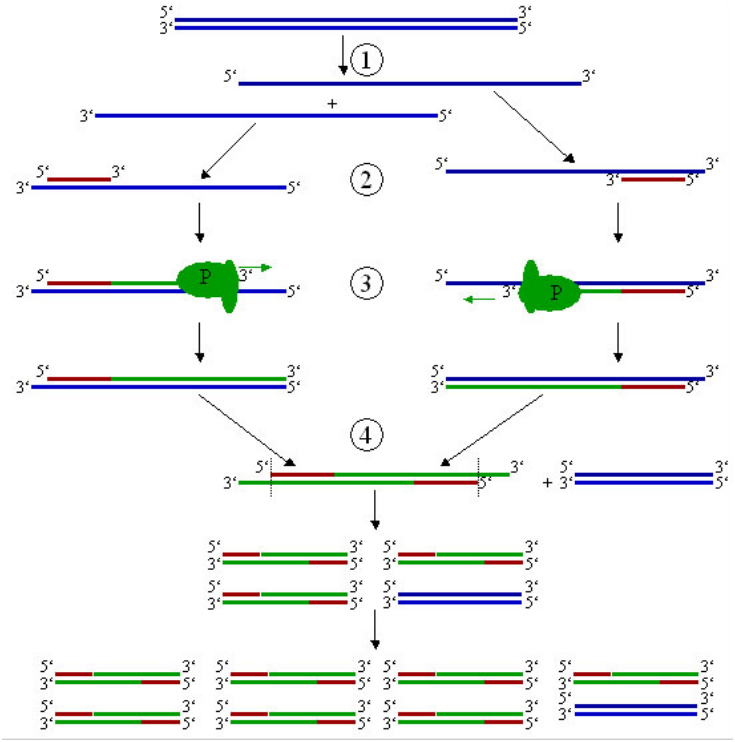


Figure 10 *The PCR method step by step. Step 1: Denaturation, where the double-stranded DNA is divided into two single-strands by heating up the DNA. Step 2: Annealing: Primer is attached to the target-site on the single-stranded DNA. Step 3: DNA-synthesis, where the double-stranded DNA is build up with polymerase. Step 4: Another cycle begins. (Microbial biologi)*

2.3.2 Choice of the primers

The Annealing temperature (T*m*) is fixed by the length and composition of the primer. The length of the primer is very important for the PCR method. If they are too small, the primers can ”anneal” in different position of the DNA template, depending on the nucleotide sequence. And this will result in non-specific copies of the template DNA. At the same time, if the primers are too long, it can cause problems as well. As mentioned before, the T*m* is fixed by the length of the primer, and if the primer is too long, T*m* increases. The polymerase that is used will be less active at temperature higher than 80°C. It is therefore recommended that the length of the primer does not exceed 20-30 nucleotides, which gives a T*m* of 55-65°C. As the annealing for the primer-pair happens at the same time, it is important that both primers have the same T*m*. If one of the primers has a higher T*m* than the other one, it does not hybridize properly which can result in incorrect binding to the DNA sequence. On the other hand, if T*m* is lower in one primer compared to the other, it does not hybridize and thereby no binding to the DNA occurs. It is important to choose specific primers with a nucleotide combination that is not complementary to other primers. If they are complementary to each other, they might anneal in the PCR mix, and form a “primer-dimer” (Coyne et al., 2001).

2.3.3 qPCR

Conventional PCR amplifies a target DNA sequence, and in qPCR it is possible to quantify the target DNA molecule in real time after each PCR cycle. It enables both detection and quantification of one or more specific sequences in a DNA sample.

qPCR takes basis in the traditional PCR, the only difference is that, there is a probe attached. A probe is an oligonucleotide like a primer that is attached to a fluorescent dye and a quencher (a dye that neutralizes the fluorescent dye). The dye will only light up when the polymerase cuts the quencher and the dye from each other. The quantity of the target DNA is directly proportional with the fluorescent dye. In this experiment SYBR Green was used. SYBR Green, is a popular fluorescent DNA binding dye, which is used for detection by measuring the increase in fluorescence throughout the cycle.

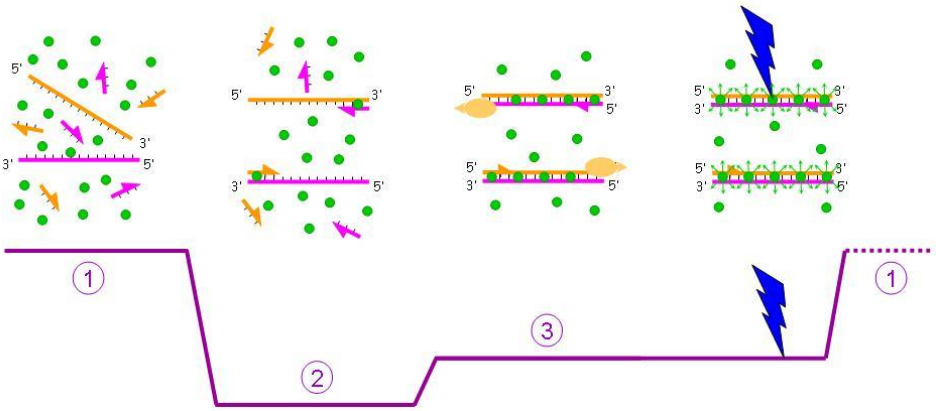


Figure 11 *The procedure of qPCR Step 1: Denaturation. Step 2: Annealing. Step 3: DNA-synthesis. The blue lightning indicated the fluorescence of SYBR Green. (Applied system)*

Figure 11 shows the procedure of qPCR (Applied Biosystems, 2002 & Kielberg et al., 2003). SYBR green is attached to the template. During annealing, the probe will straighten. In the extension part, the polymerase cuts the probe off, so the fluorescent and the quencher divide and thereby a fluorescent light appears which can be detected in the qPCR.

2.3.4 Melting curve

Quantitative real-time PCR was performed. determine the melting point of the product at the end of the amplification reactions. The products should have the same melting temperature, if this is not the case it is possible that there are problems with contamination, the primer-dimer etc. The melting point is also used for quality control. If the melting peaks are similar to each other then the melting temperature is the same for all the products. If the peaks are different from each other, it might suggest contamination, mispairing ect.

The melting temperature of a DNA double helix depends on its base composition. The PCR products with a specific primer pair should have the same melting temperature. At the melting point, the two strands of the DNA separate and the florescence will decrease (Kielberg et al., 2003).

See appendix-III for the melting curves from the qPCR measurement. The melting points was similar for each primer-pair indicating that the PCR reactions succesfully generated a single product of the same size and base pair composition in each sample. This is prerequisite to use the Ct values for calculating relative mRNA levels. Ct can be decribed as the

number of cycles nesseccary for the flourescent signal to cross the threshold. For every sample measured an amplification curve was generated (see appendix IV). The threshold was adjusted to the exponential phase of the amplification reaction.

2.4 qPCR Procedure

2.4.1 RNA extraction

Following crosses were chosen from which RNA was extracted.

- ptth-Gal4 x UAS-Raf.D.N.2.1 (dominant negative)
- ptth-Gal4 x UAS-Ras^{v12} (over-expressing)
- ptth-Gal4 x w^{118} (control)
- w^{118} x UAS-Raf.D.N.2.1 (control)

Out of these lines, one sample of three adult female flies and three samples of three larvae each was collected, which adds up to a total number of 16 samples. Each sample was homogenised with a mixture of β-mercaptoethanol and lysisbuffer (350µl from a mixture of 9,9ml lysisbuffer and 100µl β-mercaptoethanol) and frozen at -80℃. The lysisbuffer will destroy the lipid plasma membrane of cells and β-mercaptoethanol will inhibit the activity of RNases by breakage of disulfide bonds in proteins (Kielberg, 2003).

The RNA extraction was performed by using the RNeasy Mini Kit from Qiagen. This is based on the two-step RT-PCR method. With two-step RT-PCR, the RNA is first reverse transcribed into cDNA using oligo-dT. An aliquot of the reverse-transcription reaction is then added to the real-time PCR. (Qiagen, 2010)

The samples were centrifuged and the supernatant was transferred to a spin column filled with 350 µl 70% RNAse free ethanol. The ethanol will precipitate the RNA and DNA by removal of the polar water molecules. After a second centrifugation (15 sec. at 10000 rpm) and removal of the supernatant, the samples were mixed with 350 µl of Buffer RW1 and again centrifuged - this step was repeated twice. Subsequently, 180 µl DNase 1 stock solution was added to 1260 µl buffer RDD. Out of this mix, 80 µl were transferred into the spin-column, which were then incubated for 15 min at room temperature. DNase will degrade the unwanted genomic DNA and RNA will

remain intact. Next, 350 µl buffer RW1 were added and the columns were centrifuged for 15 sec at 10000 rpm. This was followed by several washing steps, were 500 µl of buffer RPE were added each time and centrifuged for 15 sec at 10000 rpm, first, and then for 2 min at 10000 rpm. In order to dry the spin-column membrane, the columns was centrifuged for 1 min at full speed. Finally the tubes were placed in normal eppendorf-cups and centrifuged again for 1 min at 10000 rpm, with 30 µl RNase free water, to elude the RNA.

2.4.2 cDNA preparation

mRNA is converted to cDNA since the latter is more stable. The oligo $(dT)_{20}$ primer will bind to the mRNA poly(A) tail when heated (Kielberg, 2003).

To set up the cDNA a mastermix was prepared:

- 18 µl Oligo $(dT)_{20}$ (50µM)
- 18 µl DNTP (10mM)
- 108µl DEPC-treated water

8 µl of this mastermix were transferred to PCR tube. After addition of 2 µl RNA the tubes were incubated at 65°C for 5 min and then placed on ice.

A second mastermix, the cDNA synthesis mix, was prepared:

- 36 µl 10xRT buffer
- 72 µl 25mM $MgCl_2$
- 36 µl 0,1 M DTT
- 18 µl RNaseOUT (40U/µl)
- 18 µl superscript (200U/µl)

The cDNA synthesis mix contains superscript which is a reverse transcriptase. Superscript binds to the RNA and use this as a template for the synthesis of cDNA (Kielberg, 2003). 10 µl of the cDNA synthesis mix was added to the tubes and incubated at 50°C for 50 min and then at 85°C for 5min. After leaving the samples on ice for a few minutes 1 µl of RNase H was added and the samples were incubated at 37°C for 20 min, and frozen at -20℃. RNase H will remove the RNA from the

formed RNA/DNA-hybrid (Kielberg, 2003). The single-stranded cDNA was then amplified by qPCR.

2.4.3 qPCR measurement

Following 6 primers were chosen for the qPCR, and diluted 1:10.

- F_1/R_1: Qptth measurement of PTTH expression
- F_2/R_2: Qptth measurement of PTTH expression
- f/r: rpl23 (f/r) reference
- F_3/R_3: QE75A measurement of ecdysone-inducible gene expression
- F_4/R_4: QE75B measurement of ecdysone-inducible gene expression
- F_5/R_5: QE74 measurement of ecdysone-inducible gene expression

A primer-mastermix was prepared for each primer (Qptth, Qptth and rpl23). The calculation is the same for every primer-pair:

- 225 µl SYBR Green (containing DNA polymeRase, buffer, dNTP and SYBR green)
- 18 µl Primer F
- 18 µl Primer R
- 153 µl RNase free water

  2 µl of sample-cDNA and 23 µl of primer-mastermix were transferred to the qPCR sample-plate.

Another primer mastermix was prepared for the primers; QE75A, QE75B and QE74:

- 125 µl SYBR Green
- 10 µl Primer F
- 10 µl Primer R
- 85 µl RNase free water

1 µl of sample-cDNA and 11.5 µl of primer-mastermix were transferred to the qPCR sample-plate.

The qPCR reaction was set up in a qPCR machine, set up to drive 40 cycles, and to measure a melting curve. Negative controls were included to check for contamination.

3 Results

3.1 Pupae Monitoring

To see if Ras signalling plays a role in the control of PTTH production and release, the timing of pupariation was monitored in order to see if the manipulated flies showed differences in the timing of development. The assay was performed with 32 larvae in each vial.

When calculating the results for timing of pupariation the samples where less than 15 larvae pupariated were excluded. This was done because the vials with a small amount of larvae have different nutritional conditions which can affect the timing of pupariation.

The experiment was conducted at both 29 °C and 25 °C. It turns out that groups containing less than 15 larvae were the same at both temperatures and these were excluded from the results. The excluded groups are *ptth-Gal4* crossed to *UAS-Raf.DN.2.1/Cyo-GFP;UAS-Raf.DN/UAS-Raf.DN* (a line expressing two copies of UAS-Raf-DN*)*, *ptth-Gal4* crossed to w^{1118}, and w^{1118} crossed to *feb211-Gal4*.

Not all of the larvae seeded in the vials pupariated during the monitoring. Death or maybe the loss of some larvae during the transfer to vials can be a possible explanation to this. Therefore it was chosen that the number of pupariated larvae equals 100% of the larvae having pupariated.

The timing of pupariation is shown in figure 12 (29°C) and figure 14 (25°C), respectively.

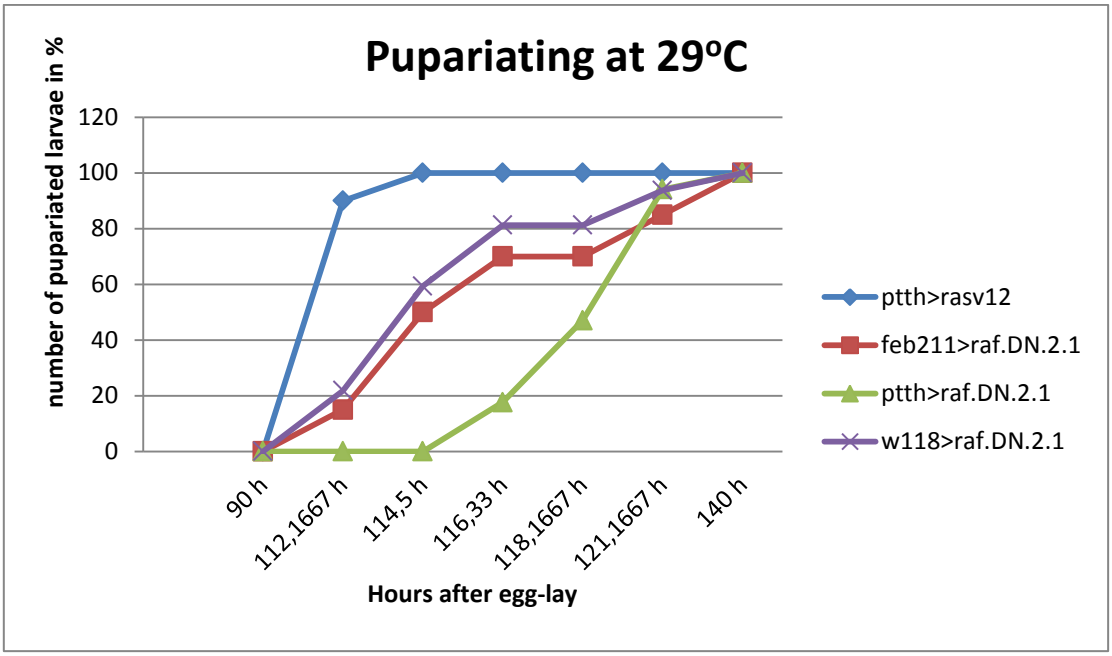


Figure 12 *The timing of development in different lines.* ptth>Ras^{V12} *is a line which expresses a constitutively active Ras in the PTTH producing neurons which results in precocious pupariation. The control line is* w^{1118} *crossed to* UAS-Raf DN.2.1. *The lines expressing an inactivated form of Raf (UAS-Raf.DN.2.1) in the PTTH producing neurons are* feb211>Raf.DN.2.1 *and* ptth>Raf.DN.2.1

To get a more precise view on the changes in timing average pupariation time were calculated for all.

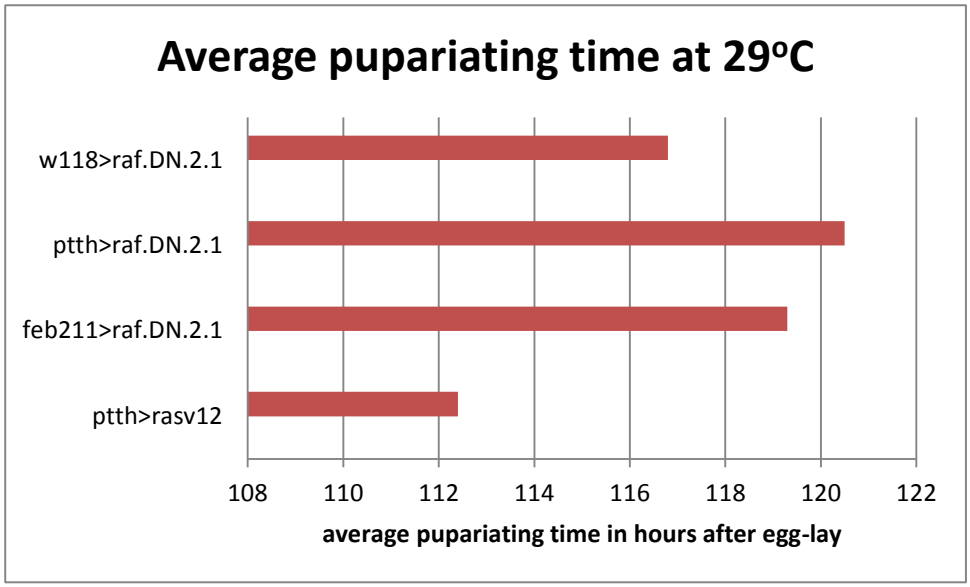


Figure 13 *The average pupartiating time at 29°C for each cross.* ptth>Ras^{v12}*pupariate after 112.4 hours which is 4.4 hours earlier than* w^{118}>Raf.DN.2.1 *which pupariate at 116.8 hours. Both the lines with a dominant negative form of Raf are delayed. They pupariate at 120.5hours (*ptth>Raf.DN.2.1*) and 119.3 hours (*feb211>Raf.DN.2.1*)*

The ptth>Ras^{V12} animals pupariated in average after 112.4 hours whereas the control animals pupariate after 116.8 hours (figure 13). This suggests that expression of a constitutively active Ras results in precocious pupariation compared to the control. If activation of Ras signalling in the PTTH producing neurons induces pupariation it could be expected that inactivation of this signalling pathway would delay pupariation. To test this, a dominant negative form of Raf (*UAS-Raf.DN.2.1*) was expressed, a downstream component of Ras signalling pathway in the PTTH producing neurons (*ptth>Raf.DN.2.1*). Larvae with reduced Raf activity (*ptth>Raf.DN. 2.1*) pupariated on average after 120.5 hours. This indicates that inactivation of Raf in the PTTH producing neurons causes a delay of pupariation.

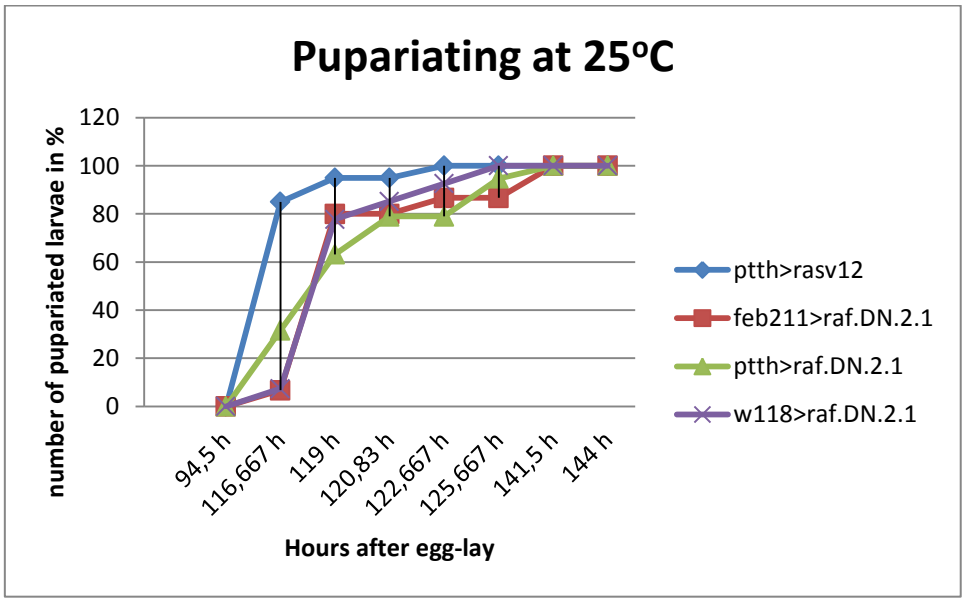


Figure 14 *The timing of pupariation at 25°C*

The exact same lines were examined at 25 °C. In figure 14, when looking at the timing of pupariation at 25°C, pupariation occurs earlier in *ptth>ras^{v12}* compared to the control animals. However there is not a clear delay of timing in *ptth>raf.DN.2.1* and *feb211>raf.DN.2.1*

The results of this assay also show a difference in timing of development in the different lines when looking at figure 15. When looking at the calculations of average pupariating time the line with the constitutively active ras (*ptth>UAS-ras^{v12}*) puparitate after 117.2 hours. The controls pupariated after 119,7 hours. And again the larvae expressing the dominant negative form of raf were slightly

delayed as they pupariated after 120.8 hours (ptth-gal4xuas-raf.DN.2.1) and 122.1 hours (feb211-gal4xuas-raf.DN.2.1)

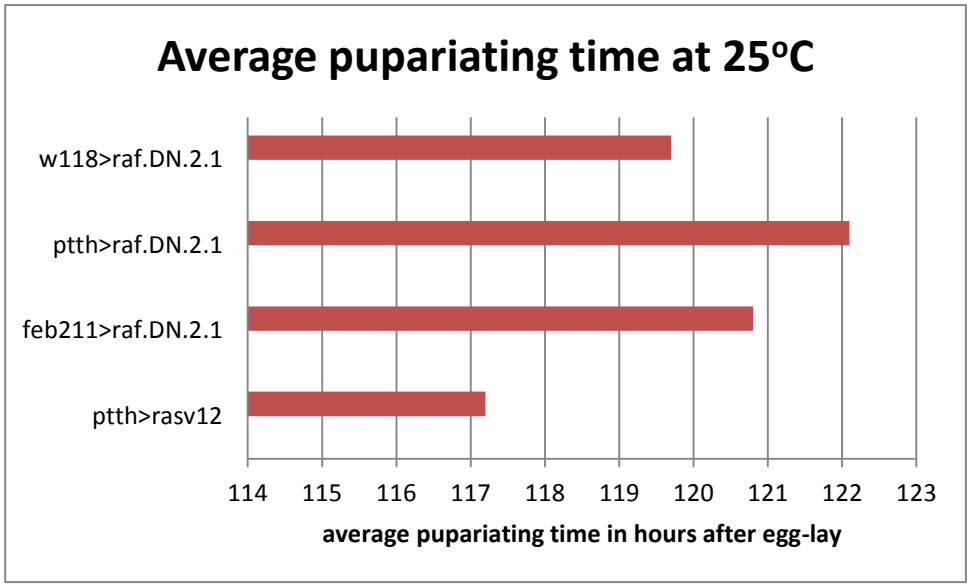


Figure 15 *The average pupartiating time at 25°C for each cross. ptth*>ras^{v12}pupariate *after 117.2 hours which is 2.5 hours earlier than* w^{118}>raf.DN.2.1 *which pupariate at 119.7 hours. Both the lines with a dominant negative form of Raf are delayed. They pupariate at 120.8hours (*ptth>raf.DN.2.1*) and 122.1 hours (*feb211>raf.DN.2.1*)*

The results for average time of pupariation show a clear difference between the lines with the constitutively active form of Ras and the control at both temperatures. At 29°C a difference of 4.4 hours is seen and at 25°C the difference is 2.5 hours indicating that an active form of Ras results in precocious metamorphosis. Similar differences are, as mentioned above, seen between the control and the lines with dominant negative form of Raf at both temperatures. At 29°C *feb211-Gal4xUAS-Raf.DN.2.1* is 2.5 hours delayed and *ptth-Gal4xUAS-Raf.DN.2.1* is 3.7 hours delayed. These lines are also delayed at 25°C with 2.3 hours and 1.06 hours respectively. So overall the differences observed in animals kept at 29 °C are greater than those maintained at 25 °C. The differences are not statistically verified, because of an sufficient number of independent samples, due to time limitations.

3.2 Does Ras signalling in the PTTH producing neurons affect PTTH expression and ecdysone levels?

The results from the samples were normalized to rpl23 as a reference. The efficiency of the primers was set by using the delta-delta Ct method, which is an approximation method. (Microbiology and immunology online). The expression of PTTH was then determined by dividing the relative PTTH mRNA level with the relative reference gene (rpl23) mRNA level, in this way the Ct value is normalized to an appropriate endogenous housekeeping gene in order to adjust for unequal quantities of starting RNA

3.2.1 PTTH expression

The expression of PTTH levels in the selected crosses was measured by using qPCR with the qPTTH primers. The crosses are *ptth-Gal4 x UAS Raf D.N 2.1* and *ptth-Gal4 x UAS Ras^{v12}*, with *ptth-Gal4 x w^{118}* and *w^{118} x UAS Raf DN 2.1* as the control crosses.

The expression of the PTTH is shown in figure 16 including the standard error (SE). The SE gives an indication of the sample mean, and give an idea about the variation, which is useful because it give an idea about the interval in which the mean is. The SE was only calculated for the larvae and not for the adults as there is only one adult sample.

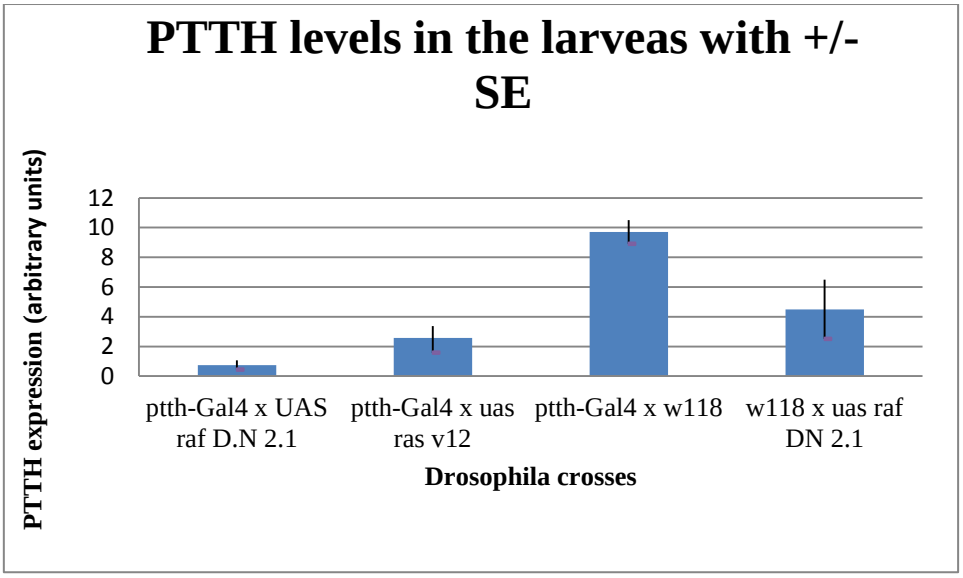


Figure 16 *Expression PTTH in the larvae from the selected crosses. Values are expressed as means,and error bars indicate SE of the mean. SE (n=3). See appendix-V for information about the mean, SD and SE values.*

In order to measure if there is a PTTH activity in adult flies, one adult sample was measured. The expression of PTTH is shown in figure 17 in each different cross.

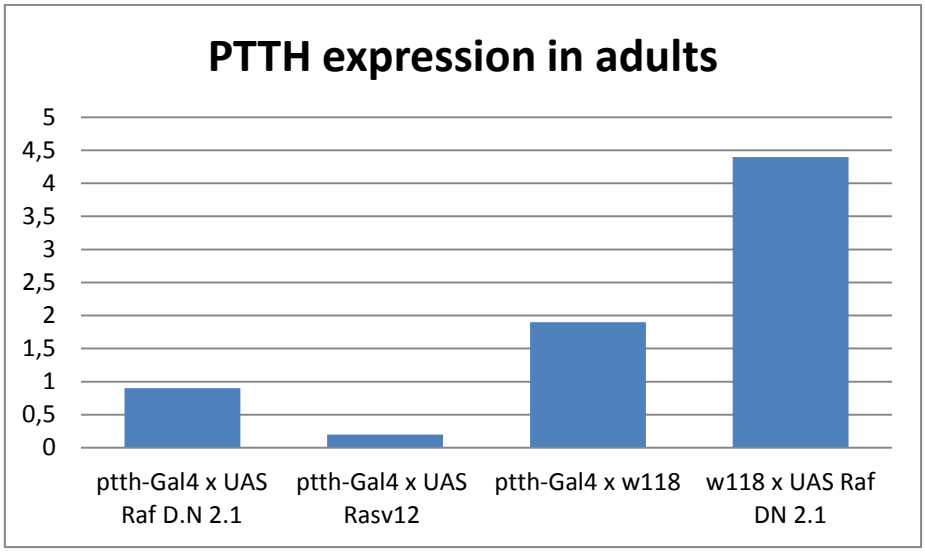


Figure 17 *Expression of PTTH in adults, these levels are measured from three adult flies.*

3.2.2 Expression of Ecdysone-inducible genes

If PTTH signaling is controlled by Ras in the PTTH producing neurons, it would be expected that the observed differences in the timing of pupariation is associated with changes in the levels of 20E. To test this possibility, the expression of ecdysone-inducible genes, E75 and E74, was measured using three different primer-pairs, namely QE75A, QE75B and QE74. E75 was detected with primers that recognize the two different isoforms, the E75A and the E75B (Rewitz et al. 2010). On the other hand both isoforms of E74, E74A and E74B, were detected with one primer pair targeting a common region.

The concentration of ecdysone-inducible genes in the larvae was determined as described for PTTH above.(Figure 18). It shows the expression of the ecdysone – inducible genes in the *ptth>Ras*^{v12} cross, in which an activated Ras was overexpressed. The control for the cross *ptth-Gal4 x w*^{118} is given as well. The figure shows that increased expression of E75A, E75B and E74 in the *ptth>ras*^{v12} larvae suggest that these animals have elevated ecdysone level.

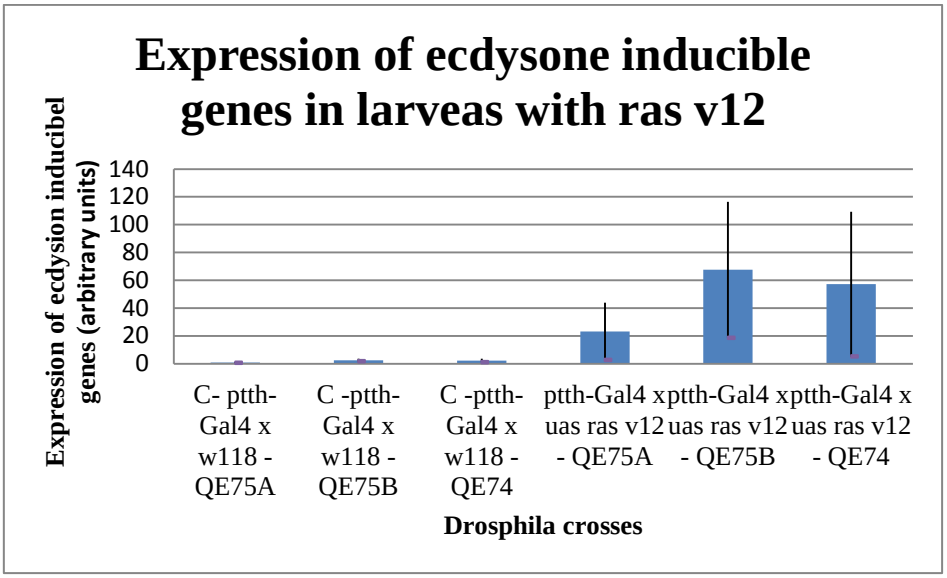


Figure 18 *The expression of the ecdysone-inducible genes E75 (isoform E75A and E75B) and E74) in larvae overexpressing activated Ras. Sample 1-3 are the controls (C). Sample 4-6 are (ptthxras^{v12}) measured with primer Qe75A, QE75B and QE74. Values are expressed as means,and error bars indicate SE of the mean. SE (n=3). See appendix-V for mean, SD and SE values*

The SE was also calculated and illustrated for the crosses where the Ras signalling was knocked down, respectively *ptth-Gal4 x UAS Raf. D.N. 2.1* and *w^{118} x UAS Raf. D.N. 2.1*, which is the control. Figure 19 shows the expression of the ecdysone inducible genes in *D.melanogaster*, where the Ras signalling was reduced. The figure shows that the ecdysone level is reduced in dominant negative larvaes. .

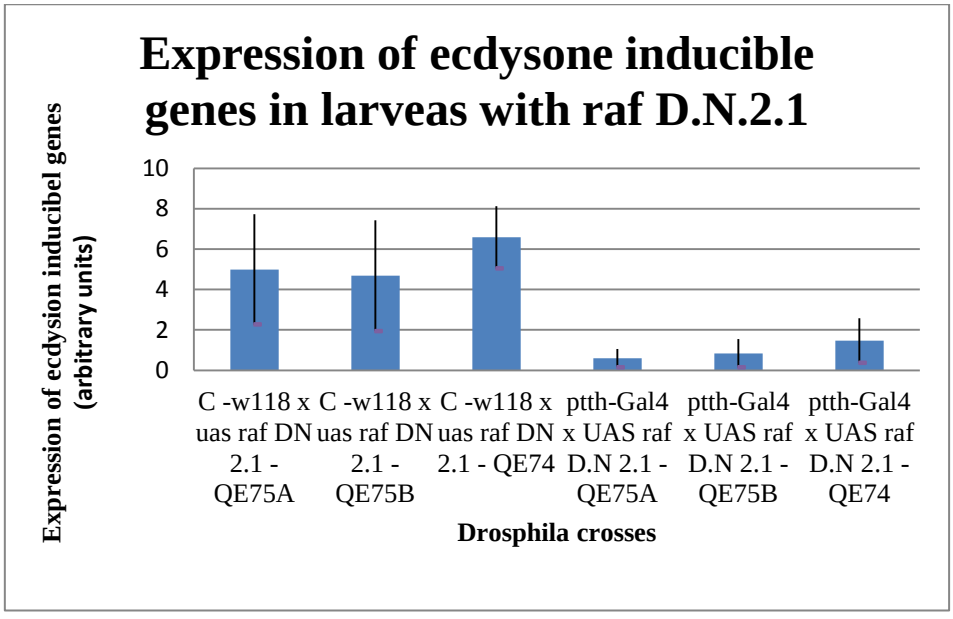


Figure 19 *The expression of the ecdysone-inducible genes E75 (isoform E75A and E75B) and E74) in larvae expressing a dominant negative Raf Sample 1-3 are the controls (C). Sample 4-6 are the fly lines Values are expressed as means,and error bars indicate SE of the mean. SE (n=3). See appendix-V for the extact mean, SD and SE values.*

The mean from the *ptth-Gal4 x UAS Raf. D.N. 2.1* measured with the primer QE75B was calculated from only 2 values, because there was no Ct value in one of the samples. Figure 18 and 19 show the expression of ecdysone inducible genes in the different crosses that were set up for the project. The results show how the release of steroid is affected by Ras. Both the figures shows that the level of ecdysone is related to Ras. Level of ecdysone is elevated in ptth>ras^{v12} and decreased in ptth>Raf.D.N.2.1.

The measurement of expression of ecdysone inducible genes in female adults was also made. Following figure (figure 20) will show the expression of ecdysone inducible genes in each cross, measured with three different primer-pair. These result shows that ecdysone level in ptth>Raf.D.N.2.1 female adults is elevated compared to the ecdysone level in ptth>ras^{v12} female adults from the

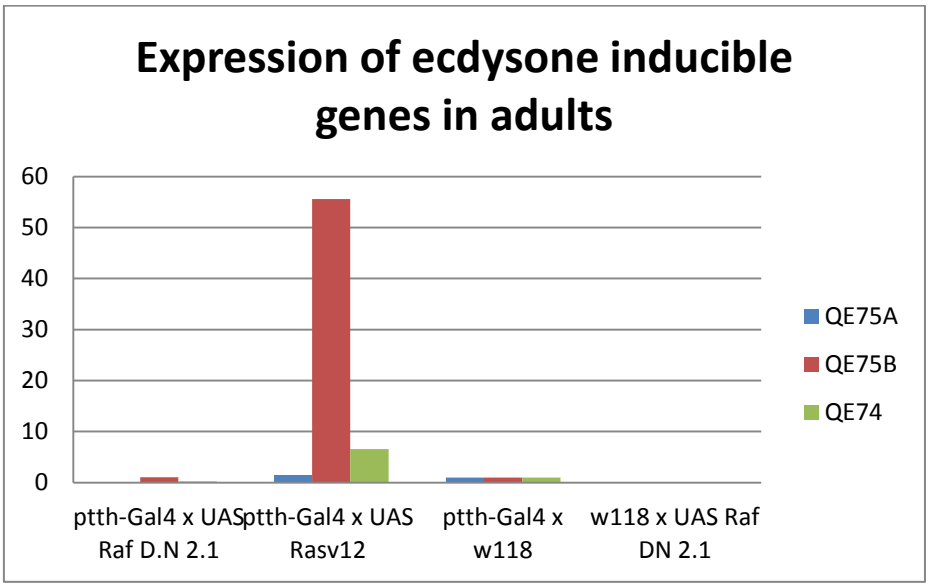


Figure 20 *The expression of the ecdysone-inducible genes E75 (isoform E75A and E75B) and E74) in adult flies. These results are measured from a pooled sample of three female adult flies.*

4 Discussion

4.1 Discussion of methods

4.1.1 Setting up fly-crosses and egg-lay

When conducting the experiment for timing of development, egg-lays were made to obtain approximately 30 eggs from each line to have enough larvae to monitor. In order to ensure that enough eggs were laid, eggs were collected from a 4 hour period. This is a large time span which means that eggs categorized as laid at the same time can actually have been laid with a 4 hour difference. These differences cause an uncertainty when looking at the timing of development. Preferably the larvae would have been precisely timed either by shortening the egg-lay period or synchronizing the larvae when entering the third larval instar. This was, however, not possible in this study due to the limited number of eggs laid.

The fact that the egg-lay lasts for 4 hours can also have an impact on the qPCR results because *ptth* is expressed throughout the third instar stage. The expression varies throughout the instar showing a cyclic pattern with an approximately 8 hour time span per interval (Mcbrayer et al, 2007). Furthermore, *ptth* expression is upregulated approximately 12 hours before pupariation. In addition to shortening the egg lay, the larvae could have been monitored with shorter and more precise intervals both night and day to more precisely determine the differences. In this study there is a very large time span from the point were no larvae have pupariated to the next point where the majority of the *ptth>Ras*^{v12} animals have pupariated. If the lines had been checked in between these two points it is possible that one would have observed that animals expressing an activated Ras pupariate more than 4.4 hours earlier than the control and therefore show bigger difference. This was a problem in both experiments (29°C and 25°C).

In order to strengthen the results it would have been preferred to conduct a larger number of timing assays with more larvae and take out more larvae for qPCR to decrease the variance between the samples. However this was not possible due to time limits. This would likely also have made it possible to demonstrate statistically significant differences.

A disadvantage of this study is the fact that not all larvae pupariated during the monitoring. Some of the larvae could have been lost during transfer from vial to vial or they could have died or maybe never have entered metamorphosis. In a study of Mcbrayer et al. (2007) a great amount of the manipulated larvae also die before eclosing. Approximately 50 % of the larvae with ablated PTTH-

production died before pupariating. It is suggested that this could be due to disruption in the expression of genes regulated by 20-E. This could also be thought to be the case in this present study. The manipulation of the flies could cause disruptions in ecdysone production (maybe via PTTH). Therefore it is possible that the lack of PTTH in the 1st and 2nd instar does play a role in regulating ecdysone production and that is why 50% of these animals die as larvae. This is suggested since the result in Mcbrayer et al. (2007) indicates that lack of PTTH is not lethal but plays a great role in timing of metamorphosis; instead ecdysone production is essential in the development. It is difficult to say what effect this has on the results; however it needs to be kept in mind that the present results are only calculated for the surviving animals. Vials with less than 15 larvae pupariating were excluded because the surviving larvae's nutritional and growing conditions would vary too much compared to the other lines and therefore possibly have an impact on developmental timing. Nutrition is shown to have an impact on developmental timing in a study by Layalle et al. (2008). In this way similar growth conditions, which are required in order to compare timing, are ensured.

4.2 Discussion of results

4.2.1 Temperature effects

The average pupariating time in hours AEL at the two different temperatures is shown in figure 13 and 15. A closer look at these figures indicates that differences in pupariation time between the 4 lines are greater when rearing the flies at 29°C rather than at 25°C. So in total the effect of the manipulations show a tendency to be stronger at 29°C than at 25°C. In a study by Long and Griffith (2000) it is proved that the Gal4 driver is temperature dependent and it seems to be more stable at 29°C compared to 25°C (Long and Griffith, 2000). This is a possible explanation to why a greater effect of both Ras^{v12} and Raf.DN.2.1 is seen at 29°C. This is also the reason why qPCR was only conducted on flies reared at 29°C.

4.2.2 Choice of primers

The choice of adequate primer-pairs is one of the most important steps, while performing qPCR. Therefore one has to consider some significant properties of the primers such as the sequence, which was given by the purpose of the experiment, in this case, the annealing temperature and the

length of the amplicon – the piece of cDNA to be amplified. Moreover it is necessary to create a balance between the two most important factors when it comes to choose a primer-pair, namely the efficiency and the specificity (Hyndman and Mitsuhashi, 2003). Efficiency can be shortly described as the amount of template, which is acquired to synthesize a new strand. However, one could also think of it as the efficiency at which the primers copy the DNA in each cycle. Specificity is known as the affinity of the primer to bind to its definite target (Hyndman and Mitsuhashi, 2003).

To take in consideration which impact these attributes took in the decision-making-process in this experiment, one has to have a closer look at the used primers again. In order to measure the level of PTTH and the expression of ecdysone-inducible genes in adult flies as well as in larvae, the primer *Qptth*, *Q74E*, *QE75A* and *QE75B* were chosen whereas *rpl23* was chosen as a reference for normalization. The primers have been described previously by Rewitz et al. (2010)

In other studies E74A and E74B, which are known as early puff genes encoding site specific DNA-binding proteins which are functioning as transcription factors are used to study the regulation of body size and developmental timing of *D. melanogaster* and the role of ecdysone. Both E74A and E74B are ecdysone-inducible genes. When looking at the results in these studies high levels of E74A and E74B were measured just before pupariation in lines expressing an active form of Ras in PTTH producing neurons. These lines also pupariated earlier than the controls. The primers used in these studies and the present experiment are designed to bind to ecdysone signalling targets. The results from studies by Caldwell et al. (2005) and McBrayer et al. (2007) have shown that measuring the expression of these genes can be used for detection of changes in ecdysone levels. In addition the method likely gives a reflection of PTTH levels due to the assumption that the release of PTTH triggers the release of ecdysone.

Mcbrayer et al.(2007) and Caldwell et al. (2005) used the similiar primer-pair as the present experiment, it is therefore expected that the primers will work in the same way as in their study. In the present experiment the line expressing a constitutively active form of Ras shows a precocious pupariation, as well as high levels of ecdysone-inducible genes are measured in the line with early pupariation.

Furthermore, the ribosomal protein rpl23 has previously been used as a reference gene for normalization (McBrayer et al. 2007; Rewitz et al. 2010). This housekeeping protein is well studied and therefore it seemed to be a good choice to use in this experiment (Thellin et al, 1999).

4.2.3 Amount of primer

The qPCR experiments were carried out on two different days. The first three pairs of primer – F_1/R_1: Qptth, F_2/R_2: Qptth, f/r: rpl23 – were used on the first day, whereas the last three pairs – F_3/R_3: QE75A, F_4/R_4: QE75B, F_5/R_5: QE74 – were used on the second day of the experiment. Two different qPTTH primer-pairs were tested and it was observed that the F_2/R_2 was the more efficient. That is why this primer-pair was used in the further analysis.

In order to save on the amount of reagents (especially SYBR Green PCR Mix), it was decided to reduce the reaction volume to 50% for the second set of qPCR reactions. Therefore the amount of primers and the different constituent parts of the mastermix was cut down to half of the amount used for the first three primers. Following this procedure the concentrations are the same in both runs of the qPCR. Therefore reducing the volume should not affect the conditions of the PCR reactions. Though it needs to be kept in mind that the risk of pipetting errors increase when the amounts decreases. Therefore it would have been better to create one mastermix for both qPCR runs.

Of cause the SE cannot be compared for the two runs because it is two different things that were measured. But it is just needed to be emphasized that a larger part of SE of QE75A, QE75B, and QE74 than SE of QPTTH could be due to pipetting errors because of the small amounts.

4.2.4 Measurement of rpl23 and RNA

Usually, while proceeding qPCR, the quality and quantity of RNA is measured beforehand. (Qiagen, 2010).When measuring the amount of RNA one is able to reverse transcribe a known amount of RNA into cDNA. In addition, the quantity of housekeeping gene, *rpl23* is also often measured. This is done to guarantee a correct correlation between RNA and the housekeeping gene (Thellin et al., 1999). In experiments, where samples containing the same amount of RNA are examined, the Ct values of rpl23 should be very similar in all samples. In case of this experiment the measurement of RNA quantity was not done and solely the amount of rpl23 was determined. However, this should not have an effect on the experiment because all the results were normalized to the values of rpl23.

4.3 The effect of Ras-signalling on PTTH and ecdysone in larvae

The timing experiment demonstrates that *ptth>ras^{v12}* larvae over-expressing an activated form of Ras in the PTTH producing neurons causes the larvae to enter metamorphosis earlier than the control larvae (*w^{118}xUAS-Raf.D.N.2.1*). It can also be seen that expression of a dominant negative form of Raf caused a delayed metamorphosis when driven in the PTTH producing neurons (*feb211-Gal4xUAS.Raf.D.N.2.1* and *ptth-Gal4xUAS-Raf.D.N.2.1)*. This suggests that Ras regulates PTTH expression, because the larvae expressing Ras^{v12} pupariate precociously which must be expected to be a result of elevated ecdysone levels. Mcbrayer et al. (2007) suggest that the primary function of PTTH in *D. melanogaster* is to be a regulator of the ecdysone levels which is important in the 3rd instar where onset of metamorphosis needs to be properly timed. This indicates that there must be some differences in the levels of PTTH and ecdysone expression in the manipulated lines. Otherwise they would not show differences in timing of development. It needs to be kept in mind that the differences in time of pupariation between the lines have not been statistically proven; due to the fact that only one timing assay was conducted. In the study by Mcbrayer et al. (2007) it was observed that loss of PTTH results in a prolonged developmental period if the larvae had not died prior to pupariation. This supports the present observation of a delayed metamorphosis as seen in the larvae expressing a dominant negative form of Raf.

Even though the timing results indicate that Ras-signalling regulates PTTH, the qPCR results do not match this perfectly; the expression of PTTH levels (seen in figure 16) does not seem to fit with the timing results. The *ptth>ras^{v12}* larvae do not express the highest level of PTTH. Instead the controls (w^{118}>raf.DN.2.1 and ptth>w^{118}) show the highest level of PTTH expression. However the low level of PTTH expression in the ptth>raf.DN.2.1 larvae that are delayed does fit into this model since they have the lowest level of PTTH expression. These results from qPCR are unexpected due to the fact that a difference in timing was seen and timing is assumed to be controlled by ecdysone (which is controlled by PTTH). A possible explanation for the low level of PTTH expression in ptth>ras^{v12} larvae could be caused from the pulsatile release of PTTH from the neurons. According to an experiment by McBrayer et al (2007), PTTH is expressed in a cyclic pattern in the 3rd instar *D. melanogaster* larvae. Furthermore, increased expressions occur at a specific 8 h window during the day (photogate), but reach a zenith at the final instar just before pupariation. The reason for the low level of PTTH expression in *ptth>ras^{v12}* larvae might therefore be a consequence of the time that they were collected from the vials. After the photoperiodic gate, the expression of PTTH starts decreasing until the next gate. Experiments performed by Mcbrayer et al. (2007) indicate that

different periodic time of the flies could cause some unexpected results. In the present experiment, it might be possible that the *ptth>ras^{v12}* larvae were collected in the decreasing phase of PTTH release. If the *ptth>ras^{v12}* larvae had been collected earlier it is therefore possible that the PTTH expression had been the highest observed of all larvae lines. The pulsatile regulation of PTTH is thought to be caused by PDF-producing neurons that synapse with the PTTH-producing neurons. Mcbrayer et al. (2007) experienced an altered PTTH periodicity when *ptth* expression was compared in *pdf^{01}* mutants and wildtype larvae. This led them to suggest that PDF-signalling could act as a regulator of *ptth* transcription and by that to the periodic release of PTTH.

There is reason to believe that PTTH expression is regulated by Ras signalling even though some of the qPCR results were unexpected. PTTH is assumed to regulate ecdysone and apparently there is a difference in expression of ecdysone inducible genes in *ptth>ras^{v12}* compared to the control (figure 18). Therefore the level of ecdysone must be elevated in ptth>ras^{v12} compared to the control. Since the larvae were collected at the same time for qPCR it is demonstrated that ptth>ras^{v12} larvae seem to have gone further in their development than the controls because of the elevated ecdysone level. This fits with the observation of decreased pupariating time for *ptth>ras^{v12}* larvae. Expression of ecdysone inducible genes in the larvae with inactivated Ras pathway also shows an apparent difference from the control (figure 19). The level of ecdysone must be lower in the *ptth>raf.DN.2.1* lines than in the controls since the ecdysone inducible genes show a lower expression than in the controls. Therefore it may be seen as if they are delayed in the development compared to the controls. This fits with the timing experiment where a prolonged pupariating time was observed for ptth>raf.DN.2.1 larvae. This indicates that low levels of PTTH and ecdysone results in a prolonged pupariating time in larvae with inactivated Ras pathway.

Since PTTH is the trigger of ecdysone release it would be expected that there would also be correlation of the ecdysone and PTTH expression levels in the manipulated lines. For instance a high ecdysone expression level would correlate to a high PTTH expression in early pupariating larvae and a low ecdysone expression would correlate to a lower PTTH expression in delayed pupariating larvae. One reason to why PTTH levels do not correlate with the ecdysone levels in the line expressing Ras^{v12} could be due to negative feedback. PTTH activates the PG and when it is activated, it starts ecdysone synthesis independently from PTTH levels for several hours (Mizhoguchi and Dedos, 2002). In the present experiment, the *ptth>ras^{v12}* larvae did not express the highest PTTH levels, but ecydsone levels are dramatically high judged from the

high expression of ecdysone-inducible genes. This might be explained as a negative feedback effect which causes a decline in PTTH expression as a result of high amount of ecdysone. The negative feedback mechanism of ecdysone on PTTH release has not been verified in *D.melanogaster* at the moment. Takaki and Sakurai have shown the negative feedback of 20E to PG in lepidopterans by injecting 20E at different larval instars. According to their results, high level of 20E affects the responsiveness of PG in a negative way rather than affecting stimulation of PTTH (Takaki and Sakurai, 2003). Since the mechanism of negative feedback on PTTH by high amounts of ecdysone is still unidentified, this experiment's results give a significant opinion for the future studies. To fully elucidate the interactions between PTTH and ecdysone levels will be important understanding developmental timing of insects.

4.4 The effect of Ras signalling on PTTH and ecdysone in adult female flies

In this experiment only female adults were used for qPCR. In this way the result would not be affected by the differences in PTTH production between male and females. Through a not well characterized mechanism adult females can produce ecdysteroids in the ovaries and adult males are believed to synthesize ecdysteroids in the testes (Marchal et al., 2010). It is seen in figure 17 that PTTH is expressed in female adult flies. The level of PTTH expression in the control lines and in *ptth>raf.DN.2.1* are increased compared to the level of *ptth>ras*^{v12}. The decreased level of PTTH expression in *ptth>ras*^{v12} may be an indication of negative feedback of ecdysone on the PTTH level as suggested above. This can further be supported by looking at the expression of ecdysone inducible genes in female flies. In figure 20 it is clear that the ecdysone level of *ptth>ras*^{v12} is increased compared to both *ptth>raf.DN.2.1* and control lines. This indicates that PTTH has a role in the regulation of ecdysone in the adult and that ecdysone possibly can influence PTTH through negative feedback.

4.5 Regulation of ecdysone production in the PG

This project deals with ras-signalling in neurons and how this affects PTTH-production. PTTH activates ecdysone-synthesis which is essential for the flies in order to enter metamorphosis. In this project developmental timing is studied and measurements of ecdysone inducible genes are measured in order to determine whether ras-signalling controls PTTH-production.

Even though this study does not deal with PI3K in PG in relation to ecdysone production, it is interesting to mention this aspect because ecdysone levels were measured in the qPCR despite the fact that Raf was expressed in a dominant negative form in neurons. Ecdysone production is to be inhibited because it is assumed that Ras signalling controls PTTH release which is involved in the transcriptional regulation of ecdysone genes (Caldwell et al., 2005). It is indicated in figure 19 that ecdysone is present in the larvae with D.N Raf. This is not expected, however the efficiency of the D.N Raf is not always 100%, which could explain why both PTTH and ecdysone levels are detectable. They also pupariate, although later, which they only can do if they produce ecdysone.

Ecdysone production is possibly also regulated via PI3K/ISS system. In a study by Colombani et al., experiments indicate that ectopic expression of PI3K accelerates cell growth, whereas the expression of the dominant negative form $PI3K^{DN}$ hinders cell growth.

Growth of the ring gland was observed in the line expressing PI3K but the flies showed reduced growth at all stages of development. In addition the adults were reduced in both size and weight and produced a larger amount of ecdysone. The flies ($PI3K^{DN}$) with reduced size of ring gland showed an increase in growth and the adults were about 17% larger in weight in average. This line showed a decrease in ecdysone production. This suggest that the effect of PG insulin activity on the body size are mediated by changes in ecdysone levels, and it is likely that the InR-PI3K signalling pathway can specifically activate ecdysone production from the PG (Colombani et al., 2005).

It is important to consider that the PI3K possibly takes part in the production of ecdysone. Therefore Ras signalling in neurons via PTTH might not be the only source to release of ecdysone.

4.6 Ras-signalling in the neurons

In this project it is investigated whether Ras signalling in neurons plays a role in PTTH-production and/or release. Only a few studies related to this topic has been found, so therefore there is limited literature regarding this area. Other literature about signalling in neurons in general and not necessarily concerning the link between Ras signalling and PTTH production has therefore been used for this part of the discussion.

The results from the present study indicates that over-expressing Ras in *D. melanogaster* causes the flies to pupariate earlier than control lines whereas lines with Raf.D.N expression shows delayed pupariation. This result together with the results obtained by qPCR indicates that developmental timing is controlled by Ras signalling in neurons via PTTH.

In a study by Lee et al. it was found that the activation of ERK in insulin-producing cells (IPC) of the brain increases the expression of Dilp1 and Dilp2 whereas the transcripts were repressed by the expression of an ERK inhibitory phosphatase in IPCs (Lee et al., 2008). They conclude that short neuropeptide F (sNPF) and short neoropeptide F receptor 1 (sNPFR1) regulate activation of ERK in IPC which in turn modulates expression of Dilp1 and Dilp2 (Lee et al., 2008). The results obtained from the study by Lee et al. support that Ras signalling plays a role in regulation of neuropeptide release from neurosecretory cells. If activation of ERK in IPC can modulate the expression of Dilp1 and Dilp2 which are neuropeptides it is possible to imagine that a similar mechanism can be present in PTTH-producing neurons. In both cases the activation of ERK in specialised neurons are involved and results in the expression and/or release of a neuropeptide.

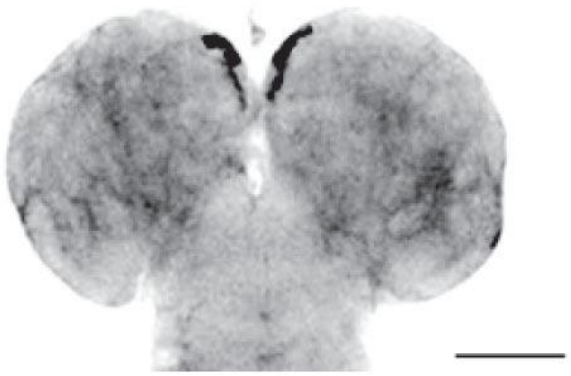


Figure 21 *The larval brain of a D. melanogaster; The black staining indicates Dilp2 expression in the IPCs. From Lee et al., 2008*

Figure 21 shows where the expression of Dilp2 is located in the brain of *D.melanogaster*. This is located near the PTTH neurons (Kim Rewitz, personally communicated). Because the two types of neurons are neurosecrotory neurons that produces peptide hormones, it is reasonable to assume that they are very similar in their function meaning that it is possible that MAPK pathway is involved in the expression of the neuropeptide in PTTH-producing neurons as well as it is in IPC.

Another study by Walker et al. 2006) demonstrates that the function in specific neurons of the larval CNS is significant for its ability to regulate the growth. The neurons are regulated by NF1 which is a GTPase activating protein. NF1 inactivates Ras signalling by exchanging GTP to GDP. Ras bound to GDP is inactive. Loss of NF1 in flies can give a high activity of Ras and results in small fly body size (Walker at al. 2006). Walker et al. (2006) investigates NF1's ability to regulate growth

by examine UAS-NF1 (expressing a transgene in defined larval tissues) crossed to *en*-GAL4 (driving the wing expression in imaginal discs) and UAS-NF1 crossed to *elav*-GAL4 (driving neuronal expression). When they measure the size of the pupa in these crosses, they find that the pupal size was reduced in the cross UAS-NF1 x *en*-GAL4 compared to the ^{w1118} used as a control. In contrast animals of the cross with *elav*-GAL4 had a normal size (the same size as the control). This indicates that NF1 is required in the neurons to control size and not elsewhere (Walker et al., 2006).

NF1 regulates the activity of Ras which means that Ras is of importance in neurons in relation to the pupal size. Over-expression of Ras in the PTTH-producing neurons observed to results in a precious pupariation in the present study, also results in a small adult body size (Kim Rewitz-personally communication). The size of the body is affected by Ras activity in the neurons. It is known that Ras activates Dilp1 and Dilp2 via MAPK and therefore it should activate PTTH in the same way. This means that changing Ras signalling in the PTTH producing neurons alone can affect body size and perhaps be part of the explaination that the NF1 mutants are small if they have increased Ras signalling in the PTTH producing neurons.

5 Conclusion

As it is known that PTTH has an effect on the developmental timing in *D. melanogaster*, it is a reasonable ambition to find out what influences the synthesis and release of PTTH as well as studying the pathways involved. Therefore the purpose of the present experiment was to determine the impact of Ras signalling in the neuro-secretory neurons producing PTTH.

The results suggest that Ras signalling controls PTTH expression and/or release from the PTTH producing neurons. This was demonstrated in the timing experiment where larvae with over-expressed Ras in fact showed a precocious metamorphosis as well as the ones bearing the dominant negative form of Raf showed a delayed development.

In order to strengthen the hypothesis gained from the timing assays, qPCR was conducted. The qPCR data for PTTH does not fit for the over-expressed Ras, although it does fit for the inactivated Ras where a low level of PTTH-expression was seen to correspond to a low level of ecdysone indirectly reflected by expression of ecdysone inducible genes. It suggests that increasing Ras^{v12} signalling in the neurons increases PTTH signalling and vice versa. The low level of PTTH expression was also seen in the adult females with over-expressed Ras-signalling and might be a consequence of either the pulsatile release of PTTH or due to negative feedback.

6 Future Prospects

The timing of puberty in humans as for the timing of metamorphosis in insects like *D.melanogaster* is not yet fully understood. In humans gonadotropin-releasing hormone (GnRH) activates the synthesis and release of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Gharib et al., 1990). GnRH is released from the hypothalamus in a pulsatile manner to regulate pituitary gonadotrope function (Clarke et al., 1987). This may be the equivalent of the PTTH release from the neurosecretory cells in the insect brain which then triggers the ecdysone release from the prothoracic glands. When the pathway is completely elucidated in *D. melanogaster* it may prove useful in the research of human development since it is easier to manipulate genes in a model organism like *D.melanogaster*. In future studies of metamorphosis of *D. melanogaster* the results obtained in this project are very relevant. If the pathways mentioned above are comparable it is relevant to examine what activates the release of hormones in *D. melanogaster*, which is what was done in present study. The results suggest that Ras signalling has an influence on the release of PTTH in *D. melanogaster*. This is a step in the direction of establishing which pathways activate hormone release.

The results in this project indicate that Ras signalling affects the release of PTTH but in order to ensure that this is the case further studies are needed. It is necessary to do timing assays with increased number of animals and samples in order to get statistical evidence. QPCR results showed a low PTTH level in larvae with constitutively active form of Ras which was unexpected. When comparing to the other lines a possibility in future studies would be to take out larvae a little earlier than in this study to make sure that PTTH is measured at its zenith just before pupariation. When conducting more timing assays at both temperatures the impact of temperature could also be solved. This would make it easier to suggest at which temperatures the larvae should be reared in order to get the best qPCR results.

The fact that the results show that Ras-signalling in neurons are likely to control PTTH, paves the way to further examination of what regulates PTTH. The information could be used to investigate what activates Ras-signalling in PTTH-producing neurons. Ras must possibly be activated by a receptor on the surface of PTTH-producing neurons. In other cells Ras is often activated by receptor Tyrosine Kinases, and as mentioned earlier it can be activated by G-protein coupled receptor in IPC's. At this moment it is known that PTTH release is under the control of PDF, and it could

therefore be possible that the PDF-receptor is activating the Ras-signalling in PTTH-producing neurons.

When comparing to how much knowledge there is about the mechanisms in PG, very little is known about PTTH-producing neurons. In order to understand developmental timing a key issue is to understand what activates PTTH because PG responds to PTTH. The results obtained from this project are hopefully the first step on the way in understanding what regulates PTTH.

7 References

Adams et al, 2000. The Genome Sequence of *Drosophila melanogaster*. Science Vol. 287

Andres, A.J, Thummel, C.S., 1994, *Drosophila melanogaster*: practical uses in cell and molecular biology, p.p. 566-567

Andrioli et al. 2002. Anterior repression of a *Drosophila* stripe enhancer requires three positionspecific mechanism. Development 129, 4931-494

Applied Biosystems 2002, '*Real-Time PCR Vs. Traditional PCR*' Applied Biosystemsretrived December 20 2010-12-20 retrived from http://www.appliedbiosystems.com/support/tutorials/pdf/rtpcr_vs_tradpcr.pdf

Bainbridge, S.P., Bownes, M., 1981. Staging the metamorphosis of *Drosophila melanogaster*. Embryol. exp. Morph. Vol. 66, pp. 61-67.

Beckingham et al, 2005. *Drosophila melanogaster*- the model organism of choice for the complex biology of multi-cellular organisms. Gravitational and Space Biology 18(2)

Berger, D.M., Mallon, R., 2003. Kinase components of the Ras-MAPK signaling cascade as potential targets for therapeutic intervention. Drugs of the Future. 28: 1211

Biro et al, 2005. Influence of obesity on timing of puberty; international journal of andrology. ISSN 0105-6263

Bodenstein, D., 1943. The Induction of Larval Molts In *Drosophila,* Columbia University, NY. Pp.120-123.

Botella et al, 2004. *Drosophila melanogaster*: Ein Modellsystem zur Analyse neurodegenerativer Erkrankungen. BIOspektrum · 5/04

Caldwell, P.E., Walkiewicz, M., Stern, M, 2005. Ras Activity in the *Drosophila* Prothoracic Gland Regulates Body Size and Developmental Rate via Ecdysone Release; Current Biology, Vol. 15, 1785–1795

Caldwell, P.E., 2007. Ras signaling in Either Prothoracic Gland Cells or Cholinergic Neurons of *Drosophila melanogaster* Regulates Fly Size. A thesis submitted in partial fulfillment of the requirements for the degree doctor of philosophy.

Chae et al, 2002. Inducible control of tissue-specific transgene expression in Xenopus tropicalis transgenic lines. Mechanisms of Development. Volume 117, Issues 1-2, p.p. 235-241

Chiti et al. 2007: Protein Misfolding, Functional Amyloid, and Human Disease; Annu. Rev. Biochem. 2006. 75:333–66.

Clarke, I.J., Cummins, J.T., 1987. Pulsatility of reproductive hormones: physiological basis and clinical implications. Baillieres Clinical Endocrinology and Metabolism 1, 1–21.

Colombani J, Bianchini L, Layalle S, Pondeville E, Dauphin-Villemant C, Antoniewski C, Carre C, Noselli S, Leopold P (2005); Antagonistic Actions of Ecdysone and Insulins Determine Final Size in *Drosophila*; Sciencemag.org, Vol 310; 667-670

Coyne VE, James MD, Reid SJ og Rybicki EP 2001, ' *PCR primer design and reaction optimisation* University of Cape Town Department of Molecular and Cell Biology. Retrived on December 20, 2010 at http://www.mcb.uct.ac.za/pcroptim.htm

Crowther et al. 2005. Intraneuronal a non-amyloid aggregates and neurodegeneration in a *Drosophila* model of Alzheimer's disease. Neuroscience 132 (2005) 123–135.

Davidowitz, G., D'Amico, L.J., Nijhout, H.F., 2003. Critical weight in the development of insect body size. Evolution & Development 5:188-197.

Demerec, M., 1994. Biology of *Drosophila*, Facsmile edition, Cold Spring Harbor Laboratory Press, NY pp. 283-288

Devlin, T.M., 2006. Textbook of Biochemistry – with clinical correlations. Wiley-Liss, USA, 6th edition.

Edgar, B.A.,2006. How flies get their size: genetics meets physiology. Nat. Rev. Genet. 7, 907–916.

Elliott, W.H, Elliott, D. C., 2002. Biochemistry and Molecular Biology: Oxford University Press; second edition.

Fletcher et al. 1997: A steroid-triggered switch in E74 transcription factor isoforms regulates the timing of secondary-response gene expression; Genetics, Vol. 94, pp. 4582–4586.

Fly pushing : the theory and practice of Drosophila genetics / Ralph J. Greenspan, New York : Cold Spring Harbor Laboratory Press, 1997

Gharib, S.D., Wierman, M.E., Shupnik, M.A., Chin, W.W., 1990. Molecular biology of the pituitary gonadotropins. Endocrine Reviews 11 177–199.

Gilbert, Lawrence I., 2004, Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*, Molecular and Cellular Endocrinology, 215, 1-10.

Gilbert, Lawrence I., 2008. *Drosophila* is an inclusive model for human diseases, growth and development, Molecular and Cellular Endocrinology, 293, 25-31.

Gilbert, L.I.,Warren, J.T., 2005. A molecular genetic approach to the biosynthesis of the insect steroid molting hormone, Vitamins and Hormones, 73, 31-57.

Gilbert, L.I., Rybczynski, R., Warren, J.T., 2002. Control and biochemical nature of the ecdysteroidogenic pathway. Annu Rev Entomol., 47:883-916.

Gilbert, S. F. (1994). Developmental biology (4th ed.). Massachusetts: Sinauer Associates.

Gilbert S.F, 2000, Developmental Biology, 8th edition, Chapter 18: Insect development, *Drosophila* metamorphosis.

Gouras et al. 2000: Intraneuronal Ab42 Accumulation in Human Brain; American Journal of Pathology, Vol. 156, No. 1.

Grießhammer et al. 1998: Muscle-Specific Cell Ablation Conditional upon Cre-Mediated DNA Recombination in Transgenic Mice Leads to Massive Spinal and Cranial Motoneuron Loss; Dev. Bio. 197, 234–247 (1998)

Guarente et al. 2008: Molecular biology of aging; Cold Spring Harbor Laboratory Press

Gusella, J.F., McDonald, M.E., 1995, Huntington's disease. CELL BIOLOGY, Vol 6.

Hyndman and Mitsuhashi, 2003: PCR Protocols, second edition; Methods in molecular biology, v. 226, Chapter 19.

Inlow, J.K, Restifo, L.L., 2004, Molecular and Comparative Genetics of Mental Retardation; Genetics 166: 835–881

Johnston et al. 1987. Interaction of positive and negative regulatory proteins in the galactose regulon of yeast. Cell, Volume 50, Issue 1, 143-146.

Kielberg, S.N, Rasmussen, L; DNA og RNA – en håndbog; Gads forlag; 2003

L.A. Sorbera, J. Castañer, J. Bozzo, P.A. Leeson.2002. *Oncolytic, Raf kinase inhibitor.* Drugs of the future. 27(12)

Lanata, S. 2003. Head Involution Defective (hid) Iap Antagonist - A Possible Transcriptional Gene Target for Tumor Suppressor Gene p53. Journal of Undergraduate ResearchVolume 5, Issue 3

Landrigan et al. 2003: Assessing the Effects of Endocrine Disruptors in the National Children's Study; Environmental Health Perspectives, Vol. 111 | Number 13.

Layalle 2008: Layalle S, Arquier N and Leopold P.2008.The TOR Pathway Couples Nutrition and Developmental Timing in Drosophila. Developmental Cell 15: 568–578

Lee K, Kwon O, Lee J H, Kwon K, Min K, Jung S, Kim A, You K, Tatar M and Yu K (2008); Drosophila short neuropeptide F signaling regulates growth by ERK-mediated insulin signaling: Nature Cell Biology; Vol 10: 468-475

Long, X., Griffith, L.C. (2000). Identification and characterization of a SUMO-1 conjugation system that modifies neuronal calcium/calmodulin-dependent protein kinase II in Drosophila melanogaster. J. Biol. Chem. 275(52): 40765--40776.

Marchal, et al., 2010, Control of ecdysteroidogenesis in prothoracic glands of insects: A review, peptides 31, p 507.

McBrayer et al. 2007: Prothoracicotropic Hormone Regulates Developmental Timing and Body Size in *Drosophila*; Developmental Cell 13, 857–871

Microbiology and immunology online, Realtime PCR. Retrieved on December 13, 2010 from http://pathmicro.med.sc.edu/pcr/realtime-home.htm

Mizoguchi A, Kataoka H., 2005. An in vitro study on regulation of prothoracic gland activity in the early last-larval instar of the silkworm *Bombyx mori*. J Insect Physiology; 51:871–9.

Mizoguchi, A. et al.,2002, Basic pattern of fluctuation in hemolymph PTTH titers during larval–pupal and pupal–adult development of the silkworm, *Bombyx mori*, General and Comparative Endocrinology 17 p. 185

Nation, James L., 2008. Insect Physiology and Biochemistry, 127-128, CRC Press, 2nd edition.

Nelson, D.L., Cox, M.M., 2008. Lehninger, Principles of Biochemistry. W. H. Freeman and company, USA, 5th edition.

Nijhout, H.F. (2003). The control of body size in insects. Dev. Biol. 261,1–9.

Polymerase Chain Reaction (PCR), Microbial biologi, edicational resources, retrived on December 20 from http://serc.carleton.edu/microbelife/research_methods/genomics/pcr.html

Qiagen, July,2010. PDF-file: Critical factors for successful Real-Time PCR. Retrieved on December 13, 2010 from http://www.qiagen.com/literature/render.aspx?id=23490.

Rewitz K. F, Yamanaka N, Gilbert L. I, O'Connor M. B. (2009); The Insect Neuropeptide PTTH Activates Receptor Tyrosine Kinase Torso to Initiate Metamorphosis; Sciencemag.org, Vol 326; 1403-1405

Rewitz, K.F, Yamanaka N, O'Connor M.B, 2010 Steroid Hormone Inactivation Is Required during the Juvenile-Adult Transition in Drosophila, Developmental Cell 19, 1–8

Safranek, L. and Williams, C. M. 1984. Critical weights for metamorphosis in the tobacco hornworm, Manduca sexta. Biol. Bull. 167: 555-567.

Salvaterra PM, Kitamoto T.,2001. Drosophila cholinergic neurons and processes visualized with Gal4/UAS-GFP. Gene Exp. Patt. 1, 1-9.

Takaki, K., Sakurai, S., 2003, Regulation of prothoracic gland ecdysteroidogenic activity leading to pupal metamorphosis, Insect Biochemistry and Molecular Biology 33 (2003) 1189–1199

The Life Cycle of *Drosophila*, 2000. Scientific Frontiers in Developmental Toxicology and Risk Assessment. Retrieved on December 20, 2010 from http://www.nap.edu/openbook.php?record_id=9871&page=162

Thellin et al. 1999: Housekeeping genes as internal standards: use and limits; Journal of Biotechnology 75.

Thougaard H, Varlund V,l Madsen RM. Mikrobiologi. 2. udgave. København. Nyt teknisk forlag; 2007.

Toivonen et al. 2009: Endocrine regulation of aging and reproduction in *Drosophila*, Molecular and Cellular Endocrinology, Volume 299, Issue 1.

Toma, D.P., White, K.P., Hirsch, J., Greenspan, R.J., 2002. Identification of genes involved in *Drosophila melanogaster* geotaxis, a complex behavioral trait. Nature Publishing Group, doi:10.1038/ng893

Twyman, R., 2002. Model Organisms: The fruit fly. Retrieved December 1, 2010 from http://genome.wellcome.ac.uk/doc_WTD020807.html

Walker J A, Tchoudakova A V, McKenny P T, Brill S, Wu D, Cowley G S, Hariharan I K and Bernards A (2006); Reduced growth of Drosophila neurofibromatosis 1 mutants reflects a non-cell-autononous requirement for GTPase-Activating Protein activity in larval neurons: GENES & DEVELOPMENT; Vol 20: 3311-3323

Warrell, D.A., Cox, T.M. Firth, J.D., 2003. Oxfort Textbook of Medicine. Oxford University Press. Vol. 3, 4th Edition.

Warren, J.T. et al, 2001. WOC (without children) gene control of ecdysone biosynthesis in *Drosophila melanogaster*. Molecular and Cellular Endocrinology, 181, 1-14.

Wilson C, Vereshchagina N, Reynolds B,. Meredith D, Boyd CAR, and Goberdhan DCI, 2007. Extracellular and subcellular regulation of the PI3K/Akt cassette: new mechanisms for controlling insulin and growth factor signalling. Biochemical society transactions. 35; 219-221.

Yoshiyama, Takuji et al, 2006. Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. Development, 133, 2565-2574

8 Appendix

8.1 Appendix I- Abbreviations

7dC: 7-dehydrocholesterol

20E: 20-hydroxyecdysone

AD: Alzheimer's disease

AEL: After egg lay

Aβ42: β-amyloid 42 peptide

CA: Corpus allata

CC: Corpus cardiac

cDNA: Complementary DNA

CNS: Central nervous system

Dib: Disembodied

DILPs: D.melanogaster insulin like peptides

DN: Dominant negative

ERK: Extracellular-signal-regulated kinase

FSH: Follicle-stimulating hormone

GFP: Green fluorescent protein

GnRH: Gonadotropin releasing hormone

GRB: Growth receptor-binding protein

Htt: Huntingtin

IGF: Insulin growth factor

IIS: Insulin/Insulin growth factor signalling

JH: Juvenile hormone

MAPK: Mitogen activated protein kinase

NSC: Neurosecretory cell

Nvd: Neverland

PG: Prothoractic gland

Phm: Phantom

PI3K: Phosphatidylinositide 3-kinase

PKB: Protein kinase B

Poly Q: Polyglutamines

PTTH: Prothoracicotropic hormone

RNAi: RNA interference

RTK: Receptor tyrosine kinase

RPL23: Ribosomal protein L23

Sad: Shadow

SE: Standard errors

Shd: Shade

Spk: Spook

TOR: Target of rapamycin

UAS: Upstream activation sequence

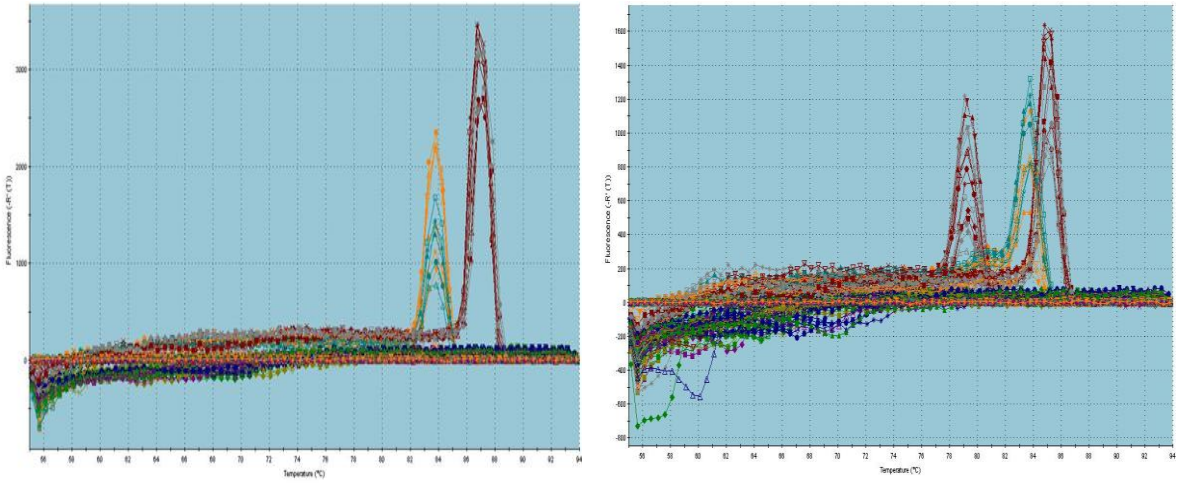
Woc: without children

8.2 Appendix II- Classification of *D.melanogaster melanogaster*

Table.1 Classification of *D.melanogaster melanogaster*

Domain	Eukarya
Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Diptera
Suborder	Brachycera
Family	Drosophilidae
Subfamily	Drosophilinae
Genus	D.melanogaster
Species	Melanogaster

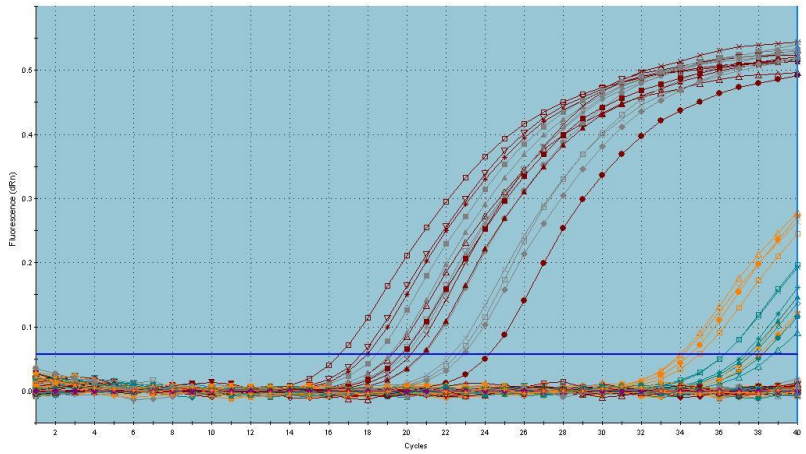
8.3 Appendix III- qPCR Melting Curves



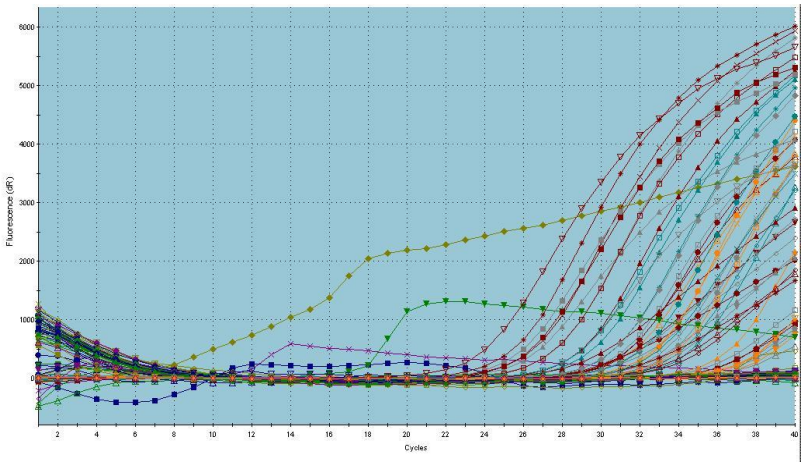
Graph.1 -A *The melting curve for the first PCR measurement, there are 2 peaks, one for the rpl23 primer-pair and the second one for the qPTTH primer-pair.*

Graph.1-B *The melting curve the second PCR measurement, there are 3 peaks, one for each primer-pair (QE75A, QE75B and QE74).*

8.4 Appendix IV- qPCR Amplification Curves



Graph.22 *The curve with the PCR reaction run with the primer qPTTH and rpl23, after approximately 20 cycles the amplification is starting to increase, which it is suppose to. The points below the blue line are noise.*



Graph.3 *The graph for the PCR reaction run with the primer-pair QE75A, QE75B and QE74. Some of the line does not fit in with the others, but otherwise it look like the PCR reactions did run well.*

8.5 Appendix V- Tables of Mean, SD, SE

Primer qPPTH F2/R2	Mean	SD	SE
ptth-Gal4 x UAS Ras D.N 2.1	0,73	0,59	0,34
ptth-Gal4 x uas Ras v12	2,57	1,40	0,81
ptth-Gal4 x w118	9,7	1,4	0,81
w118 x uas Ras DN 2.1	4,5	3,49	2,01
QE75A			
ptth-Gal4 x UAS Ras D.N 2.1	0,6	0,78	0,45
ptth-Gal4 x uas Ras v12	23,21	35,76	20,64
ptth-Gal4 x w118	0,86	0,731186	0,42
w118 x uas Ras DN 2.1	4,99	4,74	2,73
QE75B			
ptth-Gal4 x UAS Ras D.N 2.1	0,835	0,98	0,70
ptth-Gal4 x uas Ras v12	67,52	84,86	48,99
ptth-Gal4 x w118	2,49	1,97	1,14
w118 x uas Ras DN 2.1	4,69	4,75	2,74
QE74			
ptth-Gal4 x UAS Ras D.N 2.1	1,47	1,9	1,09
ptth-Gal4 x uas Ras v12	57,15	90,24	52,10
ptth-Gal4 x w118	2,29	2,17	1,25
w118 x uas Ras DN 2.1	6,58	8	1,55

8.6 Appendix VI- PTTH Expression Results

Table.2 *The PTTH level for each cross. The first line is the PTTH level in the adults, and the 3 other racks is the triplets. These were determined with the primer qPTTH.*

qPTTH	*ptth-Gal4* x *UAS Raf D.N 2.1*	*ptth-Gal4* x *UAS Ras^{v12}*	*ptth-Gal4* x *w^{118}*	*w^{118}* x *UAS Raf DN 2.1*
Adults	0,9	0,2	1,9	4,4
Larvae 1	0,5	2,7	8,7	6,9
Larvae 2	1,4	1,1	11,3	6,1
Larvae 3	0,3	3,9	9,1	0,5

Table 3 shows the expression of ecdysone-inducible genes, measured by using the primer QE75A. The expression of ecdysone-inducible genes was determined by dividing the level of ecdysone-inducible genes with the reference (rpl23).

Table.3 *The steroid level for each crosses, these were determined using the primer QE75A.*

QE75A/QE75A (F_3/R_3)	*ptth-Gal4* x *UAS Raf D.N 2.1*	*ptth-Gal4* x *UAS Ras^{v12}*	*ptth-Gal4* x *w^{118}*	*w^{118}* x *UAS Raf DN 2.1*
Adults	0.07	1.47	1.00	0.05
Larvae 1	0.22	4.67	1.04	10.28
Larvae 2	0.08	64.43	1.49	3.56
Larvae 3	1.50	0.53	0.06	1.14

Table 4 below shows the expression of ecdyson-inducible genes, measured by using the primer QE75B.

Table.4 *The steroid level for each crosses, these were determined using the primer QE75B, "low" in ptth-gal4 x UAS Ras D.N 2.1 indicate that there is any CT present it is under detection limit.*

QE75B/QE75B (F_4/R_4)	*ptth-Gal4 x UAS Raf D.N 2.1*	*ptth-Gal4 x UAS Ras^{v12}*	*ptth-Gal4 x w^{118}*	*w^{118} x UAS Raf DN 2.1*
Adults	1.04	55.59	1.00	0.06
Larvae 1	low	14.26	2.36	10.41
Larvae 2	0.14	187.00	4.79	2.92
Larvae 3	1.53	1.31	0.34	0.73

Table 5 below shows the expression of ecdysone-inducible genes, measured by the third primer-pair QE75B.

Table.5 *The steroid level for each crosses, these were determined using the primer QE74.*

QE74/QE74 (F_4/R_4)	ptth-Gal4 x UAS Raf D.N 2.1	ptth-Gal4 x UAS Ras^{v12}	ptth-Gal4 x w^{118}	w^{118} x UAS Raf DN 2.1
Adults	0.20	6.56	1.00	0.05
Larvae 1	0.58	6.99	1.86	8.48
Larvae 2	0.18	161.33	4.64	7.75
Larvae 3	3.66	3.13	0.36	3.52