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The basis of learning rate differences between *C. glomerata* and *C. rubecula*: Local CREB splice variant expression and global ratio of induced expression of CREB activator and repressor splice variants in the brain

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Abstract

Non-associative and associative learning are forms of learning that exist in the Animal Kingdom. Habituation and Sensitisation are examples of non-associative learning. Examples of associative learning are classical and operant conditioning. Learning leads to memory. The three different forms of memory that differ in consolidation and anasthesia resistance are short-term memory, long-term memory and anasthesia resistant memory. Short term memory requires modifications of pre-existing proteins. LTM requires protein synthesis. The two-closely related parasitic wasp species *C. glomerata* and *C. Rubecula* differ in learning and memory formation. Both parasitic wasps lay their eggs in young larvae of cabbage white butterflies. The larvae have several host plants. *C. glomerata* and *C. Rubecula* locate their host-caterpillars by responding on host-plant volatiles. Both parasitic wasps have a naive response to volatiles of cabbage. However, they can learn to respond to other plant volatiles. By operant conditioning the parasitic wasps learn to associate specific plant odours with host-caterpillars. *C. glomerata* needs just one learning event to form LTM. *C. Rubecula* requires three spaced learning events. After fewer spaced learning events or massed learning events *C. rubecula* only forms STM. In this thesis the influence of CREB expression on LTM formation was studied in these two parasitic wasp species. It is thought that the switch between STM and LTM depends on the ratio of gene-transcription-activating CREB isofroms and gene-transcription repressor CREB isoforms. With an in situ PCR of the brain of both *Cotesia* species the changes in CREB splice variants expression were located in naive wasps. The induced CREB splice variants expression of the whole brain was determined with a real-time quantative PCR.

1. Introduction

Cotesia glomerata and Cotesia rubecula are two closely related parasitoid wasp species. Both species parasitize *Pieris* larvae. White cabage is in the Netherlands the main host plant of *Pieris* larvae. Both parasitoid wasp species have an naive association between the odour of white cabbage and *Pieris* larvae. However, *Pieris* larvae have more host plants, for example *Nasturtium*. To adapt to the other host plants of the *Pieris* larvae, the wasps can learn to associate the odour of other host plants with the possibility of parasitizing. *C. glomerata* and *C. rubecula* differ in the process of associative learning. *C. glomerata* associates a host and a new host plant after one ovopositioning experience. *C. rubecula* requires three ovopositioning experience which are spaced by time for this association. This difference could be based on differences in expression of CREB transcripts. **C**yclic AMP **R**esponsive **E**lement **B**inding (CREB) protein is a transcription factor that has an essential role in memory formation (Yin et al., 1996). Earlier research to CREB looked at the effect of *CREB* transcript expression on memory formation in transgenic animals. *C. glomerata* and *C. rubecula* naturally differ in **L**ong **T**erm **M**emory (LTM) formation. This makes these species interesting for research to CREB. In my thesis I looked at CREB expression in these species in two ways. For one experiment my aim was to localize CREB expression in the brains of both wasp species. The aim of the other experiment was to determine the differences in induced CREB transcript expression between *C. glomerata* and *C. rubecula*. The base of the difference in learning and memory formation has to be understood to understand the base of the possible differences in CREB transcripts expression. In the next paragraphs I will give background information that is necessary to understand the base of the differences.

1.1 Learning

A learning event occurs in advance of the process of memory formation. So this thesis will start with attending to different forms of learning and the costs and benefits of learning. Learning leads to memory. Later in this thesis three different forms of memory will be discussed. Much behaviour is fully functional without previous practice or the behaviour develops during maturation. For example, female fruitflies fly without learning toward aggregation pheromones. These aggregation pheromones are deposited by females that already arrived at a food source (Dukas, 2008). However, a lot of behaviours change by experience. Learning of behaviour is often compared to evolution of behaviour. Both learning and evolution induce more effective behaviour. Learning improves behaviour in a generation and evolution improves behaviour between generations by selection.

1.1.1 Different forms of learning

In the Animal Kingdom different forms of learning exist. According to the definition of Dukas (2008), learning is the acquisition of neuronal representations of new information. New spatial environmental configurations, sensory information, associations between perceived stimuli and environmental states and motor patterns are kinds of information that are formed to neuronal representations.

Non-associative learning is generally considered as a simple form of learning and associative learning is considered as a more complex form of learning. Non-associative forms of learning are habituation and sensitization. Both habituation and sensitization are a result of repeated exposure of the animal to a stimulus. Habituation is defined as a decrease in responding to a specific repeated stimulus (Rankin, 2009). In the environment of an animal a lot of different stimuli are present. Only a small part of those stimuli requires a response. Habituation eliminates responses of stimuli that are generally not important. It is stimulus-specific and only reduces the response to the habituating stimulus or a stimulus that is similar to that (Barnard, 2004). The opposite of habituation is called sensitisation, which is also the result of repeated exposure to a single event. Sensitisation is defined by de Boer (de Boer et al., 2006) as an increased response to a stimulus after exposure to that stimulus without the need for association with another stimulus, whereas for associative learning, an association between at least two stimuli is required. Sensitization more likely occurs after a strong stimulus, like an electric shock, habituation occurs more often after repeated weak stimuli. Another difference between sensitization and habituation is that only the first few exposures to a stimulus can cause sensitization, while habituation keeps occuring every time an animal is exposed to a stimulus (Barnard, 2004).

Besides non-associative learning, several types of associative learning are known. In this thesis classical conditioning and operant conditioning will be discussed. In associative learning, at least two stimuli are associated with each other (Barnard, 2004). Classical conditioning implies an association between a stimulus that initially does not lead to a response and a stimulus that does. In classical conditioning an association between two stimuli is learned. The stimulus that elicits a response without learning is called the unconditional stimulus (US). The response that is the result of exposure to the US is called the unconditional response (UR) The stimulus that is associated with US is called the conditional stimulus (CS). The response that is the result of exposure to the CS after learning is called the conditional response (CR) The associative learning of the dogs of Pavlov is an example in which classical conditioning was used (Barnard, 2004). In lab conditions *Cotesia* learn to associate a new host by classical conditioning. The insect is placed on a leaf with *Pieris* larvae. The wasp learns to associate the taste of caterpillar-derived substances, the US, with the odour of the leaf, the CS (Geertvliet et al., 1998). An ovopositioning experience acts in this example as reinforcer of the association (Takasu and Lewis, 2003)

Another form of associative learning is operant conditioning. In this form of learning an association between a stimulus and a behavioural response is learned instead of between two stimuli. A CS triggers a specific behavioural response (BR). If the right BR is triggered by CS, the BR will be followed by the US that acts as reinforcer. The reinforcer can be either a reward or a punishment. After learning by operant conditioning an association is formed between BR, CS and the reinforcer, US. In natural conditions

Cotesia learn to associate odours of a plant with host caterpillars by operant conditioning. In this example the odour of a host-infested plant is the CS. The right BR is landing on a leaf of the plant. The reward for landing on a leaf is the presence of *Pieris* larvae, the US. (Smid, 2006).

1.1.2 Costs and benefits of learning

Learning enables an animal to adapt to circumstances that vary in or in between generations. Environmental features differ for each time and place. The proper response to those factors can not be genetically determined, so learning in that case is beneficial. Learning increases the type and the amount of information an animal can respond to (Dukas, 2008). The number of learning events required differs between animals and species. The learning rate of an animal defines the number of learning events required for memory formation. The activity level of memory suppressor genes determines the learning rate of an animal. These genes have an inhibitory effect on the formation of memory. Their activity prevents storage of unreliable information. Due to memory suppressor gene activity repeated learning experiences are required for memory formation (Abel et al., 1998).

In rats, blowflies, honeybees and *Drosophila* and parasitic wasps it is shown that learning rate can be improved by selection on learning rate (Burger et al., 2008). This shows that in a natural environment the possibilities of heritable learning rates are not totally utilized. An explanation could be that a high learning rate is accompanied by high costs. In that case a high learning rate would lower the fitness of an animal. The costs of learning can be divided in two kinds of costs; constitutive and operating costs. Constitutive costs are related to having learning ability. These genetically-based costs are likely the result of the development and the maintenance of a sensory system and a nervous system. Operating costs only arise when the sensory system and the nervous system are actually used to learn. These costs are then related with obtaining and memorizing of new information (Burger et al., 2009). Memory formation is high energy taking process. Learning the wrong information spills the energy that memory formation takes. More importantly, however, is that wrong learning leads to maladaptive behaviour. Maladaptive behaviour can lead to decrease of fitness of the animal. A high learning rate prevents learning wrong information and thus prevents forming maladaptive behaviour. (Smid, 2006) (Smid et al., 2007).

1.2 Memory

Memory is divided in three different forms based on consolidation of memory and whether it can be disrupted by anaesthesia. In the first hours after the learning event the only memory present is **S**hort-**T**erm **M**emory (STM). STM is the most labile form of learning. It can be disrupted by anaesthesia given shortly after the learning experienceTo form STM covalent modifications of pre-existing proteins are required. These covalent modifications change the strength of pre-existing synaptic connections between motor and sensory neurons (Bartsch et al., 1995), (Smid, et al., 2007). Hours after the learning event, memory changes into a form of memory that is resistant to anaesthesia. Memory that is insensitive for anaesthesia is called consolidated memory. Two forms of consolidated memory exist; **A**naesthesia **R**esistant **M**emory (ARM) and **L**ong **T**erm

Memory (LTM). ARM is induced by single learning events or a continuous series of learning events. This type of learning is called massed learning. ARM does not require protein synthesis. LTM is usually only induced by learning events spaced by intervals. In most animals single or massed learning events do not induce LTM (Smid et al., 2007). When memory is formed after learning experiences spaced in time it is called spaced learning. LTM formation requires protein synthesis and gene expression. The number of events needed for LTM is different between species and is defined by the expression of inhibitory factors on memory formation. It can also be influenced by changes in expression of keygenes involved in learning and memory (Bartsch, 1998). Examples of those keygenes are ATF-1 and CREB. Formation of LTM is based on long-lasting changes in the synaptic strength, which require translation and transcription of genes. Sometimes these changes are accompanied by with growth of new synaptic connections (Bartsch et al., 1998).

1.3 Learning and memory in *Cotesia*

Current model organisms for the influence of CREB expression on LTM are *Drosophila* (Yin et al., 1994),(Yin et al., 1995), mice (Bourtchuladze et al., 1994), rats and *Aplysia* (Bartsch et al., 1995)(Bartsch et al., 1998). All studies used transgenic animals to determine the effect of *CREB* expression on LTM. In this study the parasitic wasp species *C. rubecula* and *C. glomerata* were used. These closely related parasitic wasps differ in LTM formation. By comparing these two species it is possible to study the *CREB* expression in organisms that naturaly differ in LTM formation. Both parasitic wasp species lay their eggs in young larvae of cabbage white butterflies (Smid, 2006). The eggs of the parasitic wasp larvae hatch inside the body of the caterpillar. When the caterpillar and the parasitic wasp larvae have reached the final larval instar, the parasitic wasp larvae eat their way trough the cuticle of the caterpillar. Afterwards the caterpillar dies and the parasitic wasp larvae spin a cocoon and moult into a pupa (Smid, 2006). The difference in LTM formation is related to the host distribution and the foraging behaviour. *C. glomerata* is a gregarious wasp that lays up to 20 eggs in each host. It can develop successfully in several *Pieridae* species, so it is a generalist. However, in The Netherlands it prefers to parasitize *Pieris brassicae*. *P. brassicae* is also a gregarious species. This species of cabbage white lays a cluster of up to 150 eggs on each plant. In contrary to *C. glomerata, C. rubecula* is a solitary species and a specialist. The only host of *C. rubecula* is *P.* rapae in which it lays a single egg. *P. rapae* is also a solitary species, which lays single eggs on a plant (Smid et al, 2007). Later in this chapter the ecology of both wasp species and their host will be discussed in more detail.

Host caterpillars of *C. glomerata* and *C. rubecula* minimize the production of attractants. The colour of the caterpillars also camouflages them on the leaf, so it is difficult for the parasitic wasp to detect them. *C. glomerata* and *C. rubecula* can only parasitize first and second instar larvae. In the host the larvae of the parasitic wasps need time to develop. So the host can not be too old at the start of the development of the parasitic wasp. Feeding damage, caused by the catterpillars, induces plant volatile excretion. A naive wasp will respond on the volatiles excreted by cabbage, but will not respond to volatiles from other host plants. Parasitic wasps are able to learn to associate volatiles of other plants with their hosts. An association is made when the parasitic wasp

is exposed to volatiles of a plant in combination with an oviposition experience (Smid, 2006).

To make this association between a specific plant volatile and an ovoposition experience *C. glomerata* just needs one learning event. In experiments the preference for host plants is tested after one learning experience. The wasps were given an ovopositioning experience on a *Nasturtium* leaf with feeding damage. One day later the host preference of the wasps was tested in a wind-tunnel. The wasps could choose between cabbage, their innate preference, and *Nasturtium*. One day after the ovopositioning experience *C. glomerata* showed an preference for *Nasturtium*. In a single choice experiment *Nasturtium* evoked a flight respons in *C. glomerata* at least 5 days after the learning experience. These results indicate that *C. glomerata* changes its innate preference after learning. *C. rubecula* on the other hand did not prefer *Nasturtium* in the dual choice experiment, not even after more ovopositioning experiences. In a single choice experiment with *Nasturtium* C. rubecula showed a fly respons to *Nasturtium* till one day after the learning experience. This shows that *C. rubecula* does learn to associate *Nasturtium* with its host by associative learning. However, in the dual choice was shown that the innate preference does not changes after associative learning. *C. rubecula* only forms STM after one ovopositioning experience. For LTM formation three space learning events are required (Smid, 2006).

The differences in learning strategy can be explained by looking at the ecology of the parasitic wasps and the ecology of their hosts. The generalist and gregarious species *C. glomerata* parasitizes the gregarious *P. brassicae* and the specialist and solitary species *C. rubecula* parasitizes the solitary *P. rapae*. In a lifetime *C. glomerata* has the fecundity of 500 – 2200 eggs (Smid et al., 2007). A female parasitizes most of the caterpillars on a host infested plant. An infested plant can contain up to 150 caterpillars and *C. glomerata* lays up to 20 eggs per host. This means that a female wasp can lay half of her offspring on one host infested plant. This makes consecutive ovopositioning experiences reliable enough to make an association between the specific plant and the host. Even a single event will therefor lead to LTM formation. *C. rubecula* will not form LTM after one massed learning event. *C. rubecula* lays a single egg in a host and each host plant will only contain one host. So it has to find a host plant per ovopositioning possibility. One *P. rapae* larvae found on a specific host plant does not give a high change on finding other P. rapae larvae on the same plant species. One caterpillar per plant means for *C. rubecula* that it can only lay one egg per infested host plant, so before LTM is formed the association must be more reliable. The more times a *P. rapae* larva is found on specific host plant species, the more reliable the association between that host plant and *P. rapae* is (Smid et al., 2007).

As mentioned above the number of learning events required for memory formation differs between *C. glomerata* and *C. rubecula*. For *C. glomerata* just one ovopositioning experience is sufficient for LTM formation, altough in *C. rubecula* LTM is only formed after three spaced ovopositioning experiences. So the learning rate of *C. glomerata* is higher than the learning rate of *C. rubecula*. This means that the expression of memory suppressor genes differs between these two species. A gene that is possibly responsible for this difference in learning rate is CREB (Smid et al., 2007).

1.4 CREB

CREB is a protein that belongs to a family of proteins that functions as a transcription factor (Carlezon et al., 2005). Members of the CREB family participate in controlling cAMP dependent gene expression. All proteins in this family contain two important regions, a basic region-leucine zipper (bZIP) domain and an activation domain. The bZIP domain, which is involved in sequence specific DNA binding and dimerization of the protein, is located at the carboxyl- terminal site of the protein. The remaining part of the protein is the activation domain (Yin et al., 1995). This activation domain consists of two activation regions. One region is the kinase inducible (KID) domain or phosphorylation box (P-box), which contains several phosphorylation sites. This KID domain is flanked by two glutamine-rich motifs called Q1 and Q2 (Haus-Seuffert et al., 2000).

In many brains cells CREB is a mediator in gene transcription, however the processes in which gene transcription is mediated by CREB differs between cell types (van den Berg et al., 2010). The key steps in mediating in gene expression are phosphorylation of the CREB protein, dimerization of the protein and binding at response elements in the DNA. On the CREB protein several phosphorylation sites are present. CREB is phosphorylated at serine 133 by protein kinase A (PKA), Ca_{2+}/ calmodulin-dependent kinase (CaMK) IV, and mitogen activated protein kinase (MAPK) – activated ribosomal S6 kinases (RSKs). In the CREB family CREB, CREM and ATF-1 are the only members that encode for PKA-responsive activators, so these are the only genes that can be activated by PKA (Yin et al., 1995). Phosphorylation at serine 133 increases recruitment of CREB-binding protein (CBP), which results in activation of gene transcription. CaMK II phosphorylates CREB at serine 142. This enzyme, in contrast to the earlier mentioned enzymes, decreases CREB mediated gene transcription by dissociation of CREB dimers. CREB dimerization is essential for activation of CREB-mediated gene expression, because, CREB can only bind as dimer to **c**AMP-**r**esponse **e**lement (CRE) (Carlezon et al., 2005). This is a conserved DNA sequence, TGACGTCA (van den Berg et al., 2010). CRE is found in regulatory regions of several genes. If CRE is located in a promoter region, regulation by CREB will be possible (Carlezon et al., 2005).

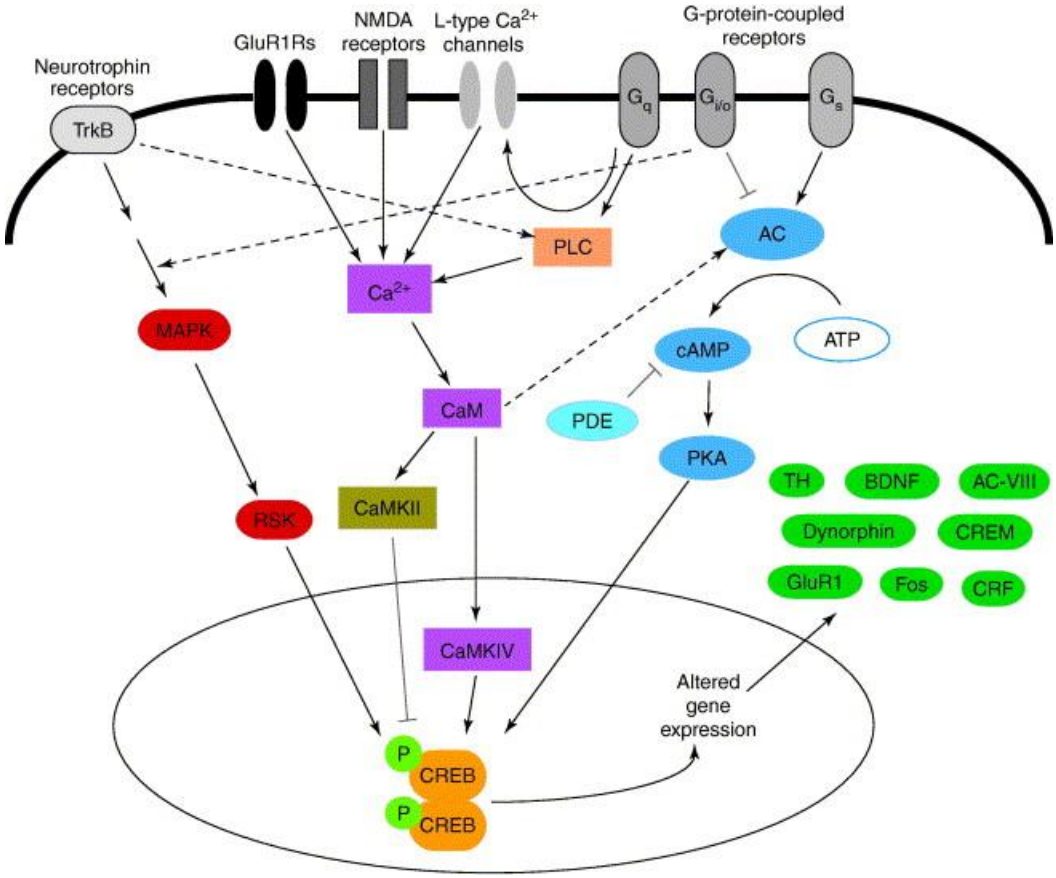

Figure 1. Some of the molecular steps involved in regulation of CREB activation. The intracellar signaling cascades that regulate CREB phosphorylation of CREB are triggered by neurotransmitters and neurotrophins acting at the membrane receptors. PKA, CaMKIV and MAPK-activated ribosomal S6 kinases phosphorylate CREB at serine 13. Phosphorylation of CREB at serine 133 activates CREB-mediated gene expression. CaMKII phosphorylates CREB at serine 142, which results in dissociation of CREB dimers. CREB dimer dissociation reduces CREB-mediated gene expression (Carlezon et al., 2005).

1.4.1 CREB expression in Drosophila

The *Drosophila* CREB family gene is called dCREB2. Several alternatively spliced transcripts can by produced by dCREB2. The resulting isoforms of dCREB2 are homolog in the overall structure. In the bZIP domain the isoforms have almost complete amino identity of mammalian CREB (Yin et al., 1994) An example of an isoform is dCREB2-a, which contains all seven exons of dCREB2. The isoform dCREB2-a has several consensus phosphorylation sites, also one for PKA. dCREB2-a is the only isofrom of dCREB2 that contains a PKA-responsive transcription factor. So it is the only cAMP-responsive *Drosophila* CREB transcription factor. dCREB2-b, in contrast with dCREB2-a, is an antagonist of PKA-responsive transcriptional activation. dCREB2-b can not activate PKA-responsive transcription because it lacks a PKA-responsive transcriptional factor. It does contain exons 1, 3 and 7. Two other examples are dCREB2-c and dCREB2-d, both proteins miss a PKA-responsive transcriptional factor. CREB2-c lacks exon 2 and 6 and CREB2-d consists of al exons except exon 2. The isoform with exons 2

and 6 activate PKA-responsive transcription and the isofroms that lack one or both of those exons do not activate PKA-responsive transcription. This suggests that a both exon 2 and 6 are required for activation of PKA-responsive transcription (Yin et al., 1995).

Yin et al., 1994 tested the role of dCREB2-b in memory formation in *Drosophila*. A dominant-negative line was generated. The transgenic flies expressed dCREB2-b under control of a heat shock promoter (hs-dCREB2-b). Wild-type flies, induced hs-CREB2-b and uninduced hsCREB2-b flies were tested on memory retention after Pavlovian olfactory learning. In wild-type flies, and uninduced flies only ARM is formed after massed training. A combination of ARM and LTM is formed after spaced training in wild-type flies. In transgenic flies a heat shock immediately increases dCREB2-b expression. dCREB2-b protein is also immediately detectable after a heat shock. The dCREB2-b protein concentration reaches a peak 9 hours after the heat shock and is still detectable 24 hours after the heat shock. The effect of hs-CREB2-b induction on one day memory retention was tested in the transgenic flies. The induction of hs-dCREB2-b reduced one day memory retention after spaced training significantly. One day retention after massed training was comparable in heat shock induced transgenic flies and wild-type flies. In wild-type flies the heat shock did not influence one day retention after massed or spaced training. So the heat shock alone does not influence 1 day retention. To show that the reduced one day memory retention was not caused by a particular genomic insertion site of the transgene another transgenic line is tested. This transgenic line also showed reduction of one day retention after spaced learning.

The effect of a protein synthesis inhibitor cycloheximide (CXM) feeding on one day retention was tested in wild type flies. Feeding of CXM before and after spaced training reduced one day retention. Feeding CXM before and after massed training did not reduce one day retention. These results suggest that ARM is not influenced by a protein synthesis inhibitor and LTM is. LTM requires protein synthesis. The reduction in one day retention caused by CXM feeding in wild type flies is similar to the reduction in one day retention seen in hs-dCREB2-b induced flies. So it is likely that induction of hs-dCREB2-b blocks LTM, but leaves ARM intact. ARM and learning behaviour were not affected by induction of hs-dCREB2-b. Both ARM and learning do not require new protein synthesis. That is why they are not affected by hs-dCREB2-b induction (Yin et al.,1994).

The effect of dCREB2-a, dCREB2-b and PKA on CRE reporter activity was tested in an assay of F9 cells. Cotransfection of F9 cells with either PKA or dCREB2-a hardly elevated activation of the CRE reporter above base lines. However cotransfection of F9 cells with dCREB2-a in combination with PKA resulted in a level of CRE reporter activity of 5.4 times higher than in the cotransfection with only PKA. dCREB2-b does not encode for a PKA-responsive transcription factor. The effect of dCREB2-b on the activity of the CRE reporter was not influenced by presence of PKA. dCREB2-b did not affect the CRE reporter activity in the F9 cell assay with and without presence of PKA. However, adding dCREB2-b expression to a F9 cell assay combined with expression of dCREB2-a and PKA leaded to almost total blockage of the PKA-dependent activation of the CRE reporter that was shown in the assay with the combination of dCREB2-a and PKA. dCREB2-b acts in this assay as an antagonist of PKA-dependent activation (Yin et al., 1995).

1.4.2 CREB expression in Aplysia

The switch between **s**hort-**t**erm **f**acilitation (STF) and **l**ong-**t**erm **f**acilitation (LTF) was also studied in the marine snail *Aplysia californa* at the molecular level by using the gill-withdrawal reflex. This reflex is representation of STF and LTF. STF and LTF are studied in cocultures consisting of a single sensory and a single motor neuron. In *Aplysia,* STF is enhanced after a single exposure to 5-hydroxytryptamine (5-HT), also called serotonin. STF enhancement is a consequence of strengthening of the preexisting synaptic connections by covalent modifications of pre-existing proteins. In contrast to STF, LTF requires 5 exposures to 5-HT spaced by 20 minutes in *Aplysia*. 5 Spaced exposures to 5-HT result in an increase in the number synaptic connections between the sensory neuron and the motor neuron. Repeated exposure to 5-HT increases the intracellular cAMP concentration. An enlarged intracellular cAMP concentration activates CREB through activation of PKA. Activated CREB results in activation of cAMP mediated gene expression. (Bartsch et al., 1995).

CREB1 is the only member of the CREB, CREM and ATF family that is expressed in *Aplysia*. Three isoforms of CREB1 are involved in the regulation of the LTF production process, called CREB1a, CREB1b and CREB1c (Bartsch et al., 1998). The function of the isoforms in the regulation of the LTF process will be discussed in the next paragraphs.

Studies in *Aplysia* showed that phosphorylation of CREB1a is necessary and sufficient for inducing LTF. CREB1a contains a T-terminal bZIP domain and a N-terminal P box. At the bZIP domain DNA binding and dimerization take place and at the P box CREB1a is phosphorylated. CREB1a can be phosphorylated by binding of PKA, PKC or CaMKII. Phosphorylated CREB1a is required for LTF. This was shown by injection antisense oligonucleotide (AS IV/V) into the sensory neurons of *Aplysia*. By binding to the boundary between exon 4 and exon 5 AS IV/V interferes with CREB1a expression. Injection of AS IV /V leaded to blockage of LTF. In *Aplysia* neurons CREB1a activation increases after exposure to 5-TH. CREB1a activation is likely to be the first step in the transcriptional switch from the STF to the LTF process. If activated CREB1a binds as CRE as homodimer gene transcription will take place (Bartsch et al., 1998). Data of Bartsch et al., 1998 show that CREB1a also has a role in maintenance of LTF. After exposing *Aplysia* to 5-HT, CREB1a is phosphorylated at least in two phases. Only in the second phase CREB1a phophorylation is accompanied by an increase in CREB1a protein concentration. These results suggest that CREB1a expression may be autoregulated directly by CREB1a binding to CRE in the *CREB1* gene.

A repressor of CREB1a mediated gene activation is CREB1b. This protein lacks a P box, so no phosporylation of CREB1b is possible. The bZIP domain of CREB1b can bind DNA and can form dimers. It forms homodimers and heterodimers with CREB1a. Binding of these dimers to CRE inhibits CREB1a mediated gene expression. By determining the role of CREB1b it was found that injection of the CREB1b protein into the sensory neurons reduces LTF induction of 5 spaced 5-HT pulses. STF is not influenced by CREB1b protein injection. Injection of an antisense oligonucleotide that targets the boundary between exon 3 and 5 in CREB1β lowered the threshold for LTF (Bartsch et al., 1998).

The third isoform that has an important role in STF and LTF is CREB1c. CREB1c is a cytoplasmic regulatory protein, that does not contain a bZIP domain, so DNA binding and dimerization of CREB1c is not possible. It contains a P box, which can be phosphorylated by PKA and PKC. Phosphorylation of this P box inhibits the facilitatory activity of CREB1c. Unphosphorylated CREB1c modulates STF and CREB1a-mediated LTF and transcription directly by inhibiting CaMKII activity. The inhibited CaMKII activity lowers the threshold for STF and LTF. Unphosphorylated CREB1c also modulates CREB1a-mediated LTF indirectly. Since CREB1c is not transcriptional active, it can not interfere on the transcription process itself. CREB1c modifies the core cytoplasmic signal transduction pathways that activate CREB1a. This activated CREB1a still needs to be combined with a 5-HT pulse before LTF is modulated (Bartsch et al., 1998).

1.4.3 CREB expression in Cotesia

Studies, in other animals than Cotesia, have validated the hypothesis that CREB functions as part of a molecular switch between STM and LTM. It is thought that this switch depend on the ratio of gene-transcription-activating CREB isoforms and gene-transcription-repressing CREB isofroms. These assumptions were based on the studies on *Drosophila* (Yin et al., 1995)(Yin et al., 1996) and *Aplysia* (Bartsch et al., 1995)(Bartsch et al., 1998) mentioned above. In these studies CREB concentration were artificially altered by over expression of certain CREB isoforms. It is unknown whether natural variation in CREB isoform concentration is related to differences in learning rate. This relation could be studied by comparing natural CREB isoform concentrations in *C. rubecula* and *C. glomerata*. These parasiticf wasps differ naturally in learning rate, so if the hypothesis phrased in earlier studies is right, the CREB isoform concentrations will have to differ between *C. rubecula* and *C. glomerata*. At the moment it is unclear at what level of expression CREB isoform ratio influences the switch. It could be at the level of transcription, mRNA stabilization and/or translocation of CREB. It is also unknown whether the differences appear locally or globally in the brain (van den Berg, 2009).

In *Cotesia* CREB has been sequenced and it is called *CgCREB* in *C. glomerata* and *CrCREB* in *C. rubecula* (van den Berg. 2009). *CgCREB* and *CrCREB* differ at 10 nucleotide positions; the predicted amino sequences are identical. mRNA transcripts of *CgCREB* are alternatively spliced in at least 9 different transcripts, of which one presents the whole ORF. All transcripts are expressed in both species. The homology of *CgCREB/CrCREB* with CREB in other species is also determined. In figure 2 an alignment of CgCREB/CrCREB to AmCREB5, human and mouse CREBα is shown The overall identity in amino acid sequences with dCREB2-a, 28%, and with mouse CREBα, 31% is low compared with CgCREB/CrCREB. However, in the basic region and the ZIP domain of dCREB2-a the amino acid identity is very high compared with *CgCREB/CrCREB*, respectively 91% and 88%. Overall amino acid identity with AmCREB (*Apis mellifera*) is high, 82%. The majority of the differences in amino acid sequences in AmCREB, dCREB2-a and CgCREB/CrCREB are positioned outside the functional domains. The functional domains define the CREB gene family. So a high conservation in these domains confirms that CgCREB/CrCREB belongs to the CREB gene family (van den Berg et al., 2010).

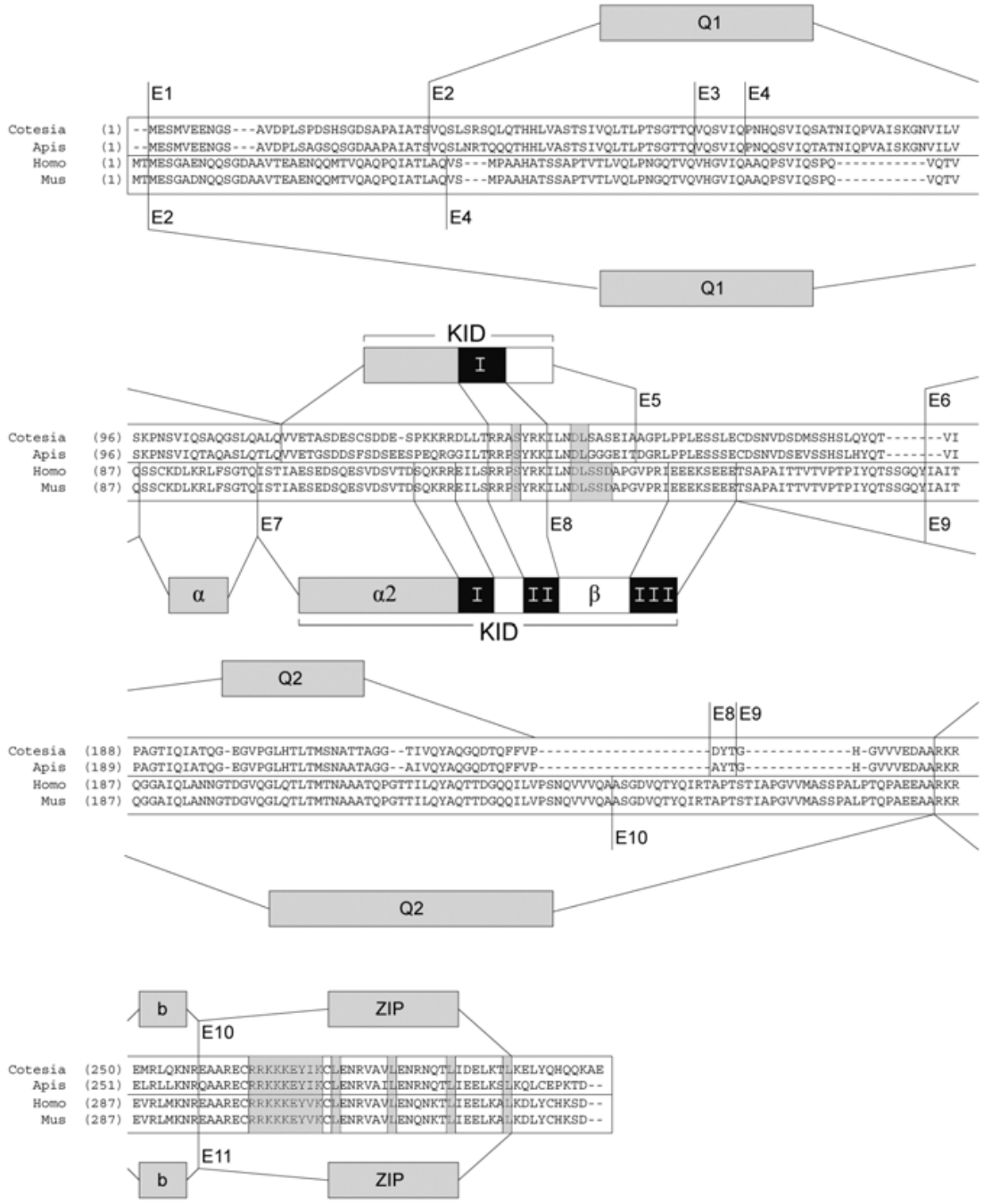

Figure 2 An alignment of CgCREB/CrCREB to AmCREB5, human and mouse CREBα. All these genes contain two glutamine rich regions (Q1 and Q2), a kinase- inducible domain (KID) and a bZIP domain region. Human and mouse CREB have three putative phosphorylation sites in the KID region. The PKC motif is located at the first phosphorylatyion site (site I) of the KID region, site II contains a PKA motif and the casein kinase II motif is located at site III. In mammals the phosphorylation of the serine residue at position 133 is essential for CREB activation. In AmCREB5 this residue is positioned at 142 and in CgCREB/CrCREB at 141. These positions at site II are shaded in the figure. No more phosphorylation sites in CgCREB/CrCREB are experimentally confirmed, but the phosphorylation motif compendium predicts other

phosphorylation sites. At the β part of the KID region, the sequence DLSSD, which is also shaded, was shown to be essential for CREB activation. The first residue of this sequence, that has a critical role in CREB activation, is conserved in AmCREB5 and CgCREB/CrCREB. The bZIP domain, which is responsible for DNA binding, is highly conserved in all genes. Two parts of the bZIP domain are shaded; the leucine residues that occur in the repeating heptads of the leucine zipper and the conserved nuclear translocation signal (van den Berg et al., 2010).

The exons of CgCREB/CrCREB are named based on the exons of AmCREB. Figure 3 represents the nine identified isoforms and the exons the isoforms contain. All CgCREB/CrCREB isoforms contain exons 1, 4, 9 and 10. Exon 2 is part of the glutamine-rich Q1 region. Exon 3 is not spliced out in any of the isofroms of *CgCREB/CrCREB*. In the honeybee just one isoform without exon 3 was found. Exon 5 is located adjacent to the KID domain. However, no role in the regulation of the transcriptional activity is known for exon 5. Part of the glutamine-rich Q2 region is exon 6. The Q2 region enables basal transcriptional activity by interaction with a component of the transcription initiation complex, TAFII130. Three isoforms of *Cotesia* lack this exon, which is interesting because this exon encodes for a functional domain. In honeybees none of the isoforms has this exon spliced out. Exon 7 is only present in one isoform of AmCREB. None of the isoforms of CgCREB/CrCREB encode for exon 7. Exon 8 only codes for 3 amino acids of which the function is unknown. The only difference between CREB 1 and 2 and between CREB 3 and 5 is having or lacking exon 8 (van den Berg, 2009).

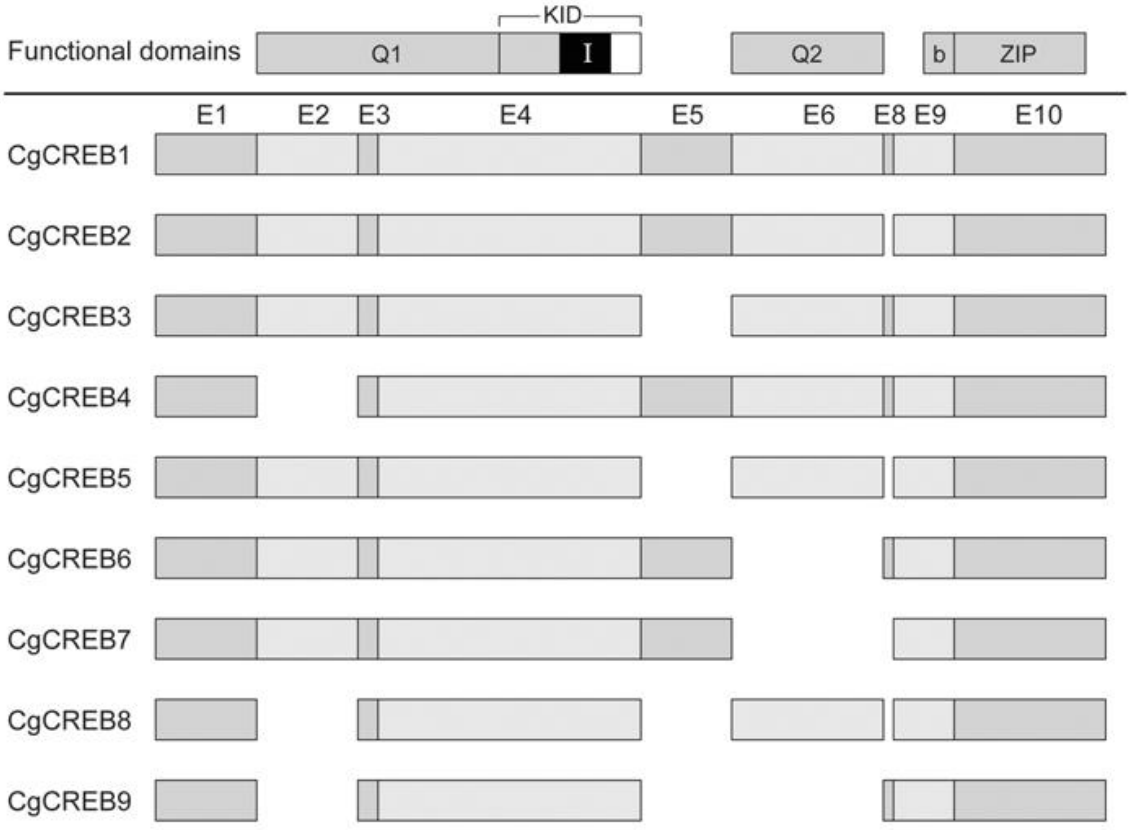

Figure 3 Schematic representation of the nine identified CREB isoforms in C. glomerata and C. rubecula. The complete ORF is translated in CgCREB1. (van den Berg 2009)

Van den Berg, 2009, determined the concentration of mRNA coding for the CgCREB/CrCREB splice variants in brain homogenates of unconditioned and conditioned females of *C. rubecula* and *C. glomerata* by using a **r**eal **t**ime **q**uantative PCR (RT qPCR). A RT qPCR was used because it enables quantification of changes in mRNA-transcripts coding for the different CREB splice variants. Quantified changes can be analyzed statistically. If the switch from STM to LTM is indeed defined by the ratio of activator and repressor CREB isoforms, then a quantative approach will be necessary. In unconditioned wasps the constitutive expression of the CgCREB/CrCREB splice variants was determined. In the conditioned wasps the relative induced CgCREB/CrCREB splice variants concentration was determined.

Earlier research showed that the most intact isoforms generally act as activators in CREB-activated transcription. Alternative splicing can lead to loss of functional domains, so it seems logic that the most intact isoforms are activators. Van den Berg, 2009, found that the major part (90%) of the CREB splice variants in the brains of both wasps, unconditioned and conditioned, consist of CREB splice variants 1, 2, 3 and 5. Splice variants 1 and 2 can not be measured separately, neither can splice variants 3 and 5. Splice variants 1 and 2 are the most intact isoforms found in both wasps. Splice variant 1 encodes for the whole ORF and in splice variant 2 only exon 8 is spliced out. So splice variants 1 and 2 were assumed to be activators of CREB-mediated transcription. Splice variants 3 and 5 were assumed to be repressor of CREB-mediated transcription. Measurements of CREB splice variant concentration in brain homogenates showed lower levels of mRNA transcripts coding for splice variants 1 and 2 than for splice variants 3 and 5 (van den Berg, 2009). If splice variants 1 and 2 are indeed activators and splice variants 3 and 5 repressors than these findings are congruent with founding's in *L. Stagnalis*. In *L. Stagnalis* mRNA coding for the activator LymCREB1 was only found in some neurons, high concentrations mRNA coding for the repressor LymCREB2 were found throughout the whole nervous system (Wagatsuma et al., 2006).

Between the two species the constitutive expression of CREB isoforms only differed for the isoforms 4 and 9. The relative expression levels found for isoform 4 and 9 were significantly higher in *C. glomerata* than in *C. rubecula*. In both species isoform 4 and 9 are expressed in minor quantities. Still it is possible that these two isofrom have an important role in the molecular switch between STM and LTM. In situ PCR of the brain of both wasp species could reveal more information about the role of the different splice variants of CgCREB/CrCREB in the regulation of LTM formation (van den Berg, 2009).

After a single learning event the relative abundance of the CREB splice variants was determined by van den Berg, 2009. A single learning event consisted of one ovopositioning experience on a *Nasturtium* leaf. In *C. glomerata* the relative abundance of isofroms 1 and 2 and isoforms 3 and 5 changed significantly after one learning event. One minute after the learning event the relative concentration of isoforms 1 and 2 decreases significantly compared to the control group. After 2 minutes this concentration increases again to a level that does not differ significantly with the control group. The concentrations of isoforms 3 and 5 increase and decrease in the complementary way. This was expected since the concentration was determined relative to the total concentration of CREB. In *C. rubecula* only significant difference was found between the replicates. In

the replicate the relative abundance of isoforms 1 and 2 were higher and thus the relative abundance of isoforms 3 and 5 were lower after a single learning event. The results of both species correspond to the differences in LTM formation between *C. glomerata* and *C. rubecula*. In *C. glomerata* LTM is formed after a single ovipositioning experience and thus changes in the relative concentrations of the CREB isoforms were expected. In *C. rubecula* LTM is only formed after three spaced ovipositioning experiences, thus no changes in the relative concentrations of the CREB isoforms were expected. However, the significance of the changes in the relative concentrations of the CREB isoforms in *C. rubecula* could be masked by the significant difference in the replicate. After a single learning event, the relative abundance of splice variants 1 and 2 decreased in *C. glomerata.* An increase was expected since splice variants 1 are assumed to be activators of CREB-mediated gene expression. It is possible that immediately after conditioning mRNA coding for isoform 1 and 2 takes place is translated. A part of this mRNA is present before learning and a part is formed after learning. Translation of mRNA leads to a decrease in mRNA concentrations and to an increase in CREB protein of isoform 1 and 2. The mechanisms for mRNA degradation are well understood. It is thought that the transient activity and the mRNA stability are linked. mRNA degradation is an efficient mechanism for transient protein expression which links protein synthesis directly to gene transcription rate.

Also the relative abundance of CREB splice variants after spaced learning is determined (van den Berg, 2009). For spaced learning three ovipositioning experiences were used, spaced by 10 minutes. In *C.* rubecula again the only a significant difference between the replicates was shown. This time the relative abundance of isoform 1 and 2 was lower and the relative abundance of isoform 3 and 5 was higher in the replicate. In *C. glomerata*, against expectations, no significant changes were shown. The first learning event is the same as in the experiment with a single learning event, so the same decrease in relative concentrations of isoform 1 and 2 was expected. This suggests that the crucial translation steps take place between the first and the third learning event. So measuring the relative abundance of the CREB isoforms between the first and third learning event would be ideal. However, mRNA extraction requires killing of the animal. This makes measuring between the first and last trial impossible.

Still the process from learning to LTM formation is not totally understood. LTM formation can depend on either phosphorylation of newly synthesized CREB or phosphorylation of CREB present before learning or even on a combination of both. In *L. Stagnalis* (Wagatsuma et al., 2006) and *Drosophila* (Yin et al., 1996)newly synthesized CREB was required for memory consolidation. If newly synthesized CREB is required for LTM formation CREB mRNA translation must take place between conditioning and the transcription of downstream genes influenced by CREB. Quantifying changes in CREB mRNA may lead to more understanding of the process from learning and LTM formation.

Van den Berg measured relative abundance of the CREB splice variants. The disadvantage of that is when the relative concentration of isofroms 1 and 2 increases, the relative concentration of isoforms 3 and 5 automatically decreases. Therefore I tried to determine the absolute abundance of the CREB isoforms by comparing the abundance of the CREB isoforms with the abundance of 18 S in the each sample. The absolute abundances of the CREB isoforms of the samples can be compared without influence of

the differences between the samples in amount of tissue used for RNA extraction. By measuring the absolute abundances of the CREB isoforms I tried to answer the question; is the difference in learning rate between *C. glomerata* and *C. rubecula* caused by differences in the ratio of induced CREB activator and induced CREB repressor transcript concentrations in the time interval 0 to 30 minutes after a single learning event? My hypothesis is that after induction of CREB isoform expression by a single learning event the ratio of activator CREB /repressor CREB transcript concentrations is higher in *C. glomerata* than in *C. rubecula*.

It is possible that only a small fraction of the total CREB present in the brain is involved in the molecular switch to LTM formation. The changes in CREB isoform concentrations might only be effective for the small subset of neurons that is involved with LTM formation. By using a RT qPCR to determine the CREB isoform concentrations of homogenates of the brain the total CREB in the brain and its surrounding tissues is measured. It is possible to locate the changes in CREB splice variants expression by executing an in situ PCR of the brain (van den Berg et al., 2010; Abdel-Latief et al., 2008). The role in regulation of LTM formation of the differences in the constitutive expression of splice variants 4 and 9 between *C. glomerata* and *C. rubecula* might become more clear by locating the expression of all CREB isoforms. This raises another question; Can the differences in the local CREB transcript concentrations in the brain explain the differences in learning rate between *C. rubecula* and *C.* glomerata? I think that in *C. glomerata* the activator CREB transcript concentration will be higher than the repressor CREB transcript concentration in the regions of the brain that are involved in memory formation. In *C. rubecula* the activator CREB transcript concentrations will be lower than the repressor CREB transcript concentrations in the regions of the brain that are involved in LTM formation.

2. Materials and Methods

2.1 Animals

For my research naïve *C. rubecula* and *C. glomerata* from 1 to 7 days old were used. The insects came from a colony that originated from individuals collected in cabbage fields in the environment of Wageningen, the Netherlands. *C. rubecula* was reared on *Pieris rapae* and *C. glomerata* was reared on *Pieris brassicae* (Smid et al., 2007). The caterpillars used for the learning experience of the second experiment and for rearing of the *Cotesia* were reared on cabbage plants (*Brassica oleracea* var. Gemmifera) as described earlier (Geervliet et al., 1998).

2.2 Plants

Nasturtium was used to feed the caterpillars that were used for the learning experience for the parasitic wasps. These plants were reared as described earlier (Geervliet et al., 1998).

2.3 In situ reverse transcriptase PCR

The procedure was used as described by Abel-Latief et al., 2008. The brains were dissected in an embryo block filled with PBS. After dissection the brains were transferred to an embryo block filled with a fixation buffer (130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4, and 4 %(v/v) formaldehyde) to fixate the brains. After brain fixation the tissue was dehydrated with a series of 70, 90, 96, 90 and 70% ethanol/ H_2O followed by three washing steps with PBS (130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4). The washed brains were incubated for 60 minutes in RNAse free proteinase K/PBS 2µg/ml for protein digestion at room temperature. After three more washing steps with PBS, proteinase K was heat inactivated at 90°C for 2 minutes. Then the brains were transferred to an eppendorf tube filled with 40 µl DNA digestion solution composed of 4µl 10x DNase I buffer (Fermentas), 2µl RNase free DNase I with 10U/ml (Promega), 1µl RNase inhibitor with 40 U/ml (Promega) and 33µl DEPC water. The digestion solution for the negative control the RNAse inhibitor was replaced by RNAseA. For the positive control the DNA digestion step was omitted. The brains were incubated overnight in the digestion solution at 37 °C to digest the DNA. The digestion step was followed by two times washing with RNAse free PBS and incubation at 95°C for 2 minutes in DEPC water to inactivate DNAse. Then the brains were incubated in 40µl verso transcriptase mix (Thermo fisher scientific, Surrey United Kingdom – cat # AB-1453/B) composed of 8µl 5x buffer, 4µl dNTP's, 2µl gene specific primers, 2µl enhancer, 2µl RT enzyme mix and 22 µl DEPC water for 60 minutes at 50°C and 2 minutes at 90°C. The gene specific primers used were primers for CREB 1 and 2 and CREB 3 and 5. The sequences of these primers are given in table 1. The used primer concentration was not known.[1] The verso transcriptase mix is replaced by the PCR mix composed of 5µl 10x buffer, 1µl primers, 1µl dNTP's (50% biotin-labeled UTP was added to the standard amount of TTP), 1µl taq polymerase and 41µl DEPC water. The PCR was performed with the temperature profile of 3 minutes at 94°C, 25 rounds at 30 seconds at 94°C, 1 minute at 60°C and 1 minute at 68°C. After the PCR the brains were washed 4 times with PBS-T (PBS solution with 0.25% tween). The washed brains were transferred to a BSA blocking solution (1% **b**ovine **s**erum **a**lbumin in PBS-T) in an embryo block. After a blocking step of an hour the brains were incubated overnight in PBS-T-BSA with 1:200 strepatividin/Cye and 1:200 propidium iodide. After the incubation the brains were washed again with PBS and dehydrated with a series of 70, 90 96, 100, 100% ethanol/ H_2O. Then the brains were cleared with xylene before mounting with DPX (Abdel-Latief et al., 2007). With a confocal laser scanning microscope the result of whole mount in situ PCR was observed. The idea was to compare the pictures made with the confocal laser scanning microscope with 3D models of *Cotesia* brains. By comparing the results with the 3D models of *Cotesia* brains it is possible to see in what functional domains of the brains the CREB isoforms are most expressed.

The primers used in the whole mount in situ RT PCR were the same that were used in real time PCR (van den Berg et al., 2010). The primers are shown in table 1. It is not possible to distinguish between transcripts containing exon 8 and transcripts without exon 8, because this difference covers just nine base pairs. So no distinction could be

[1] Old primer dilutions with an unknown concentration were used. For the experiments this was not a problem because these were a trial for the reaction.

made between splice variants that only differ in having exon 8 and lacking exon 8. This is the case for cgCREB1 and cgCREB2 and for cgCREB3 and cgCREB5.

Table 1 Splice variant specific primers (van den Berg, 2009)

Primer	**Sequence**	**Splice variants**	**Orientation**
E1E3For	5'-CGCTACCTCGGTACAATCAGTTATCCAACC-3'	4, 8, 9	sense
E2E3For	5'-ACGTCGGGGACGACGCAGGTAC-3'	1-3, 5-7	sense
E4E6Rev	5'-GCCGGTATTACGGCTATTTCACTCGC-3'	3, 5, 8	antisense
E4E8E9Rev	5'-CCGTGGCCTGTGTAGTCGGCTATT-3'	9	antisense
E5E6Rev	5'-CCCGCCGGTATTACTGTTTGGTATTGC-3'	1, 2, 4	antisense
E5E8E9Rev	5'-CTCCGTGGCCTGTGTAGTCTGTTTGG-3'	6	antisense

2.4 Real time quantative PCR

2.4.1 Learning experience

Learning experiences were given for the RT qPCR experiment to evoke gene expression. The learning experience existed of one ovopositioning experience. An ovipositioning experience was given to the parasitic wasps by using a nasturtium leaf on which caterpillars had been feeding for three days to induce feeding damage and volatile production. One-day-old caterpillars were placed on the infested nasturtium. The parasitic wasps then were placed on the same leaf and were allowed to oviposit one time on one caterpillar and than they were removed again. For *C. rubecula, P. rapae* was used and *P. brassicae* in the case of *C. glomerata*.

2.4.2 Sedation

After the learning experience, the wasps were sedated to enable decapitation. Sedation took place after a time interval. The time intervals used were 0, 1, 5, 10, 15, 30 minutes after ovopositioning. For each time interval 10 *C. rubecula* and 15 *C. glomerata* were used. As control group, naive wasps were used. So for both species 7 different treatments were given. For each treatment two or three repeats were made. For sedating the parasitic wasps CO_2 was used. CO2 was used because this sedates the animals in a very short time (10-20 seconds). This is important because the time after the learning experience is a factor that is looked at in the experiment.

2.4.3 Decapitation

After sedation the head of the parasitic wasp was removed by using tweezers. The antenna were also removed until the last segment. Removale of the antennae was done

for two reasons; no gene expression takes place in the antennae, so they have no use for the experiment and by removing the antennae the chemicals could better diffuse to the brains. After decapitation the heads were immediately transferred to an eppendorf tube with 1 ml RNAwiz™ (Ambion, Austin, Texas - cat. #9736). After collecting 15 heads for *C. glomerata* or 10 heads for *C. rubecula* the heads were crushed by using a masher.

2.4.4 mRNA extraction

According to the protocol of RNAwiz™ (Ambion, Austin, Texas - cat. #9736) total RNA was isolated from *C. glomerata* and *C. rubecula.* For the calibration curves total RNA was isolated from 15 thoraxes for *C. glomerata*, male and female, and from 10 thoraxes for *C. rubecula*, male and female. For the treatments total RNA from female heads was isolated, 15 for *C. glomerata* and 10 for *C. rubecula*. Different from the protocol of the manufacturer is that 1µl glycoblue (Ambion, Austin, Texas - cat. #9736) was added to visualize the RNA pellet. Colouration of the pellet was used to make the small pellet better visible. After RNA isolation the RNA was dissolved in 20 µl RNase free water and stored at -80° C till cDNA synthesis.

2.4.5 cDNA synthesis

For the cDNA synthesis the Verso™ cDNA synthesis kit (ABGene, Epsom, United Kingdom - cat. # AB-1453) was used with random hexamers. For synthesis of the reference cDNA for the calibration line of the qPCR a double reaction is used, the total reaction volume was 40 µl. The 40 µl cDNA was diluted till a volume of 1 ml and divided in 50 aliquots of 20 µl. The reference cDNA was synthesised of thorax RNA extracted from *C. rubecula* and C. *glomerata*.

2.4.6 Real-time quantative PCR

Using a real-time quantative PCR (qPCR) the absolute amount of two CREB splice variants was determined by comparing these with the amount of 18S present in the sample. The primer sequences of 18S are given in table 2. The implementation of the qPCR was done with a Corbett RotorGene 6000 system. For all the reactions the ABsolute™ qPCR SYBR® Green Mix (ABGene, cat. # AB-1159/a) was used in combination with the splice variant-specific primers or the primers for 18S according to the protocol of the ABsolute™ qPCR SYBR® Green Mix manufacturer for an end volume of 25 µl. It was tried to determine the optimal concentration for the primers. The determination of the optimal primer concentration will be discussed later in this thesis. All samples were analyzed in duplicate. Melting curve analyses were done after each run to see whether the right RT qPCR product or primer-dimer was formed.

Table 2 18S primer sequences

Orientation	**Sequence**
Sense	5’ CAGCCGCGGTAATTCCAGC 3’
Antisense	5’ CRTHYTYGGCAAATGCTTTCGC 3’

3. Results

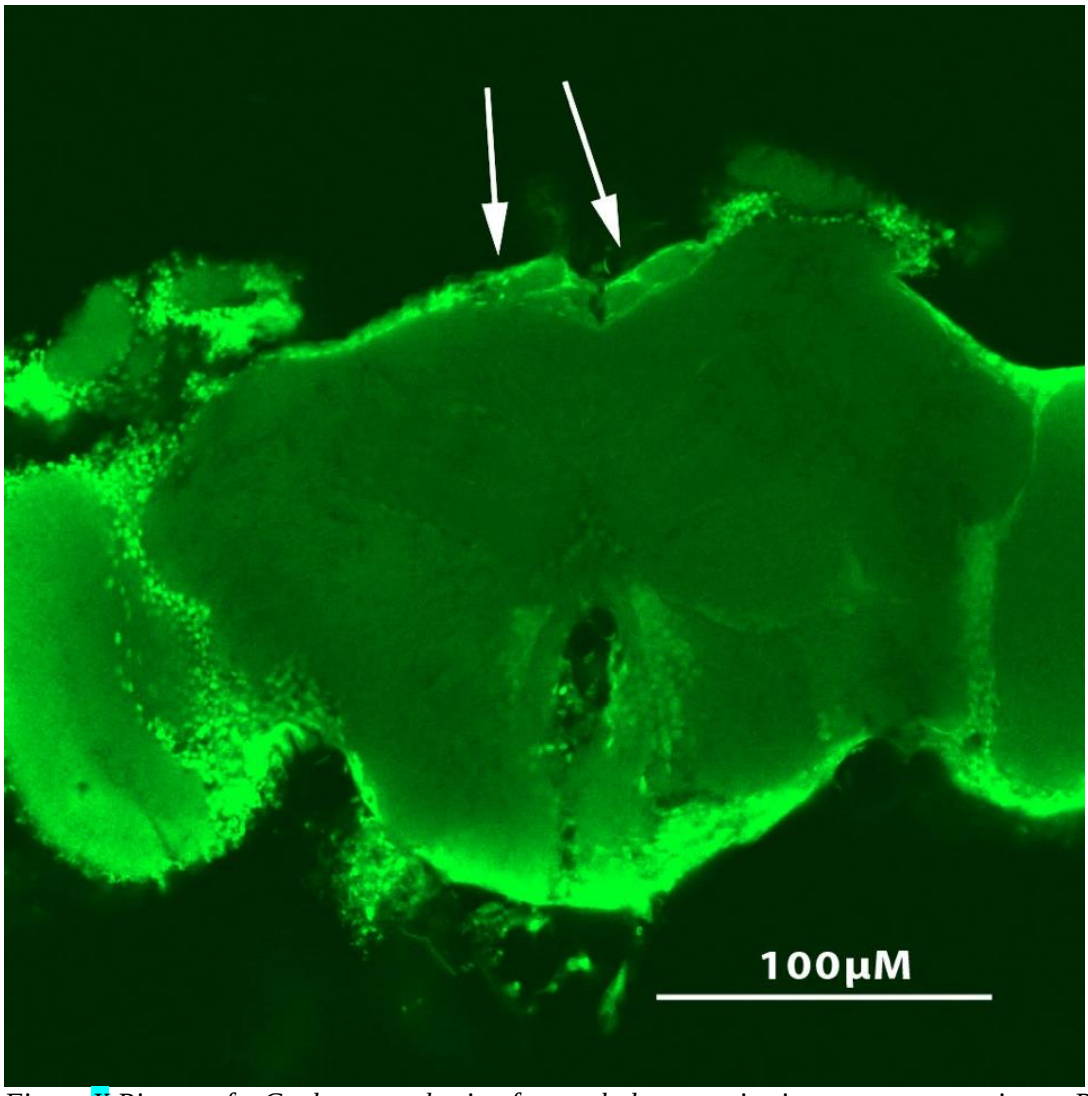

Figure X Picture of a C. glomerata brain after a whole mount in situ reverse transcriptase PCR with primers for CREB 3 and 5.The transcripts of CREB 3 and 5 are expressed at the parts of the brain in which the fluorescence is high (the highlighted light green parts of the brain). The arrowheads point at the kenyon cells. Kenyon cells are compact clustered neurons of which the mushroom body exists. The mushroom body is the part of the brain in which the odour learning takes place. The odour-evoked activity patterns from the lower parts of the brain are transmitted to the kenyon cells in the mushroom body. The differences in patterns of active Kenyon cells evoked by different odours able the insect to discriminate between odours (Galizia et al,. 2008).

3.1 Whole mount in situ reverse transcriptase PCR

The labelling of the RNA transcripts of CREB isoforms 1 and 2 and CREB isoforms 3 and 5 was successful, figure 3. Under the confocal laser scanning microscope high fluorescence was seen in the parts of the brain in which CREB tramscripts 1 and 2 and the CREB transcipts 3 and 5 were expressed. The first time the whole mount RT PCR was performed the brains proved to be too damaged during dissection and the PCR. By

dissection the brains, disruption of the neurilemma can easily occur. During the PCR the brains are heated and cooled 20 times, this causes damage to the brains, especially when the membrane is disrupted by the dissection. These damages and the enzymatic treatments before the PCR cause the brain to disintegrate. In damaged brains it is not possible to determine exactly at which parts of the brain the transcipts are expressed. So damaged brains are not longer useful for localizing CREB in the brain. After dissecting the brains were fixated with formaldehyde 4%. It was tried to fixate the brains in formaldehyde 4% + glutaraldehyde 0.25%. This had little to no effect on the disintegration of the brain and a negative effect on the permeability of the cell brain. The labelling was not as effective as needed. Probably the diffusion of biotin-labelled UTPs, Streptadivin and Cye2 was decreased by the glutaraldehyde.
After dissecting and labelling 50 insect brains in 3 PCR runs, I decided to stop with dissecting the brain because the quality of dissecting was not good enough to use the confocal laser scanning microscope images for localizing the different CREB isoforms.

3.2 Real time quantitive PCR

A qPCR was performed to make a calibration curve. To make this calibration curve the Ct values of at least 3 dillutions of thorax cDNA have to be used. In every qPCR run of the samples a dilution of the calibration curve is also included to be able to determine the RNA concentration. The qPCR for the Ct values for the calibration curve of 18S was performed for *C. glomerata* (figure 4) and *C. rubecula* (figure 5). The figures show at which number of PCR rounds the dilutions were amplificated. The annealing temperature was 60 °C. Normally a no template control is included in the run to check for contamination of the samples. However, in this run there was no no-template control included, so it is not possible to say whether this run was contaminated. Therfor, the calibration curve with the Ct values was not made.

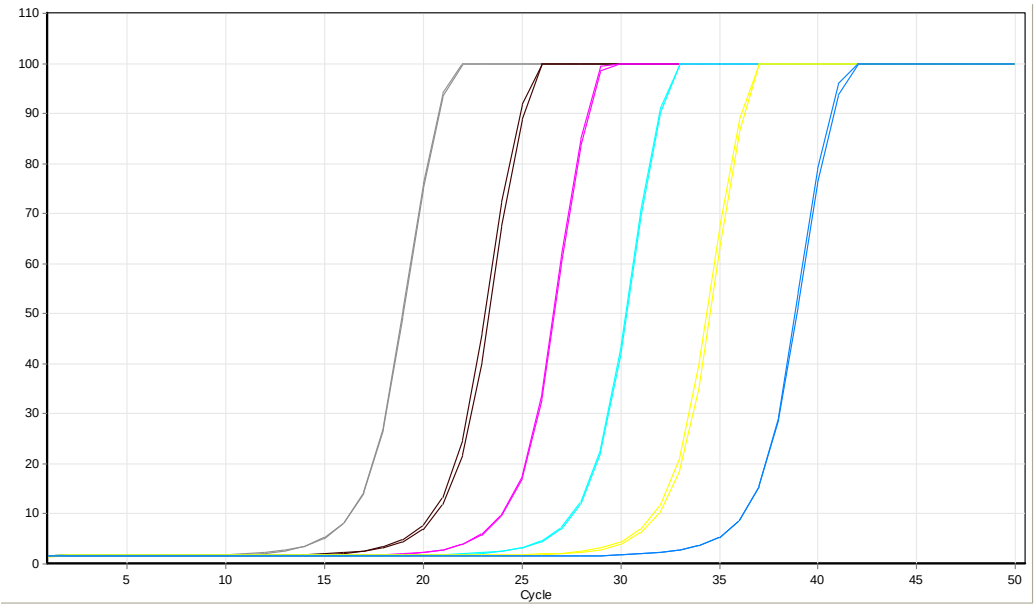

Figure 4 qPCR with 18S primer and thorax cDNA of C. glomerata. (The grey lines;, 10^{-1} aliquot of thorax cDNA of C. glomerata, brown lines; 10^{-2} aliquot, pink lines; 10^{-3} aliquot, green lines; 10^{-4} aliquot, yellow lines; 10^{-5} aliquot, blue lines; 10^{-6} aliquot)

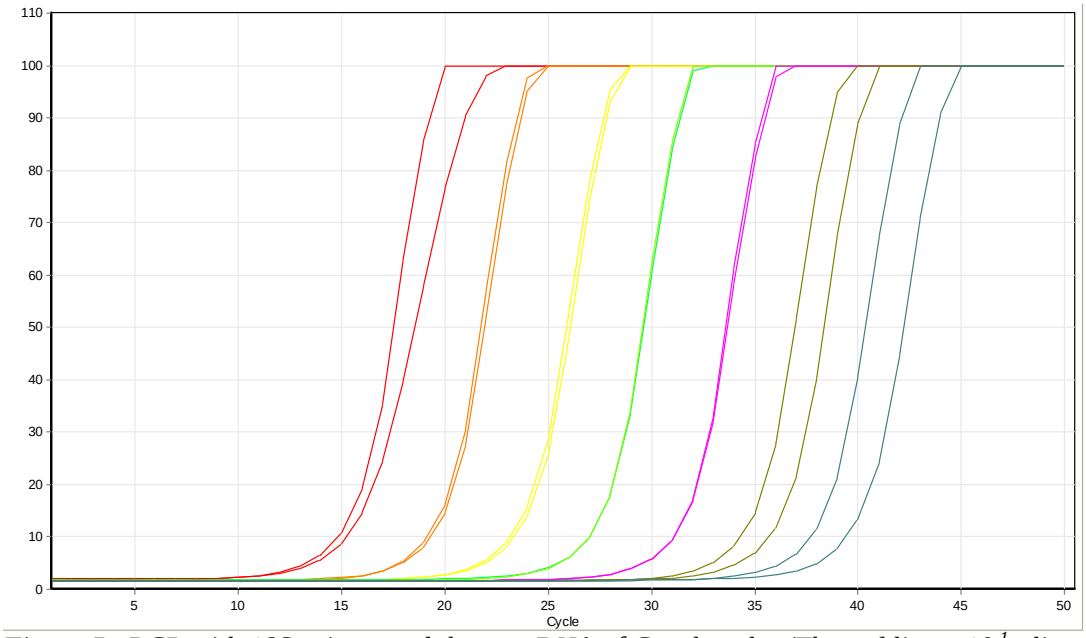

Figure 5 qPCR with 18S primer and thorax cDNA of C. rubecula. (The red lines; 10^{-1} aliquot of thorax cDNA of C. rubecula, orange lines; 10^{-2} aliquot, yellow lines; 10^{-3} aliquot, green lines; 10^{-4} aliquot, pink lines; 10^{-5} aliquot, dark green lines; 10^{-6} aliquot, blue lines; 10^{-6} aliquot)

By making the calibration curve for CREB 1+2 and CREB 3+5 some problems occurred. This RT qPCR has been done before (van den Berg, 2009), but the primer concentration of the stock solution was unclear. Therefore the optimal primer concentration first had to be determined. The old primer dilutions were still available and were used as a positive control. First, a too high primer concentration was used being 20 µM. According to the protocol of ABsolute™ qPCR SYBR® Green Mix (ABGene, cat. # AB-1159/a) optimal primer concentrations for RT-qPCR should be in the range of 50 nM to 300 nM. In this range a conventional PCR machine was used with Gotaq Flexi DNA Polymerase (Promega, cat. # MP305) instead of ABsolute™ qPCR SYBR® Green Mix with a Corbett RotorGene 6000 system to determine optimal primer concentration. Using a conventional PCR machine with Gotaq Flexi DNA Polymerase is a less expensive method to determine optimal primer concentrations than using a Corbett RotorGene 6000 system with ABsolute™ qPCR SYBR® Green Mix for this. A range of primer concentrations, 100 nM, 200nM, 400nM en 800nM, of CREB 1+2 was used to determine which primer concentrations resulted in product formation. The old primer with the unclear concentration was used as a positive control. In case of the concentrations 200nM, 400nM en 800nM and the positive control product was formed in the PCR, (figure 6).

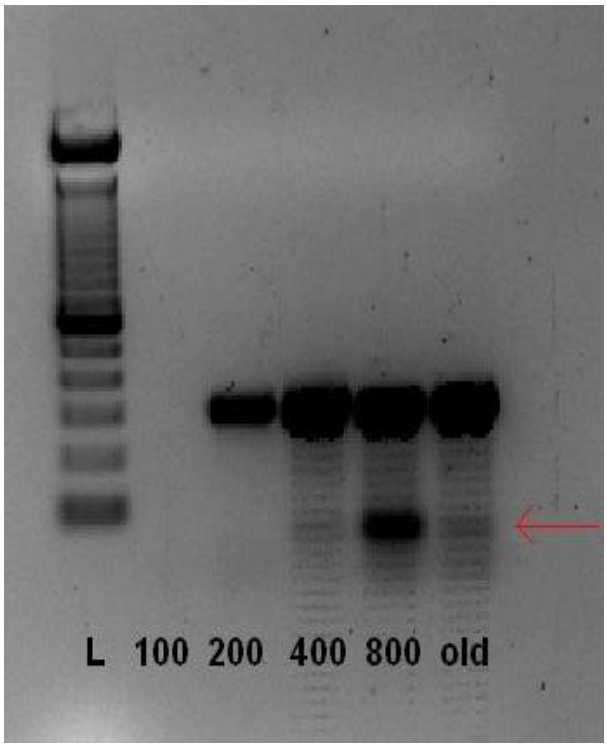

Figure 6 PCR with goTaq Flexi DNA polymerase with a range of primer concentrations of CREB 1+2. cDNA of rubecula was used in the PCR. The numbers in the figure indicate the nM primer used, old stands for the old primer. The concentration of the old primer was unclear. With the primer dillution 100 nM no product or primer-dimer was formed. In the reactions with the primer dillution 100 nM, 200nM and the old primer dillution mainly product was formed. In the reaction with the 800 nM dillution product and primer-dimer was formed. At the height of the red arrow the band of the primer-dimer is positioned.

Too much primer can also inhibit product formation. Thus another PCR was performed with the following range of primer concentrations, 200 nM, 280 nM and 360 nM and the old primer as positive controll, (figure 7). In earlier runs the reaction with 360 nM formed product as well as primer-dimer, this means that after product formation still a lot of primer is left in the reaction, which reacts with itself. In the reaction with 200 nM not always product was formed in earlier PCR runs. Therefore 280 NM was picked for both primers to use in the RT qPCR. Hereafter, this primer concentration was used in the RT qPCR machine with thorax cDNA of *C. glomerata* and *C. rubecula* as template and with primers for CREB 1 and 2.

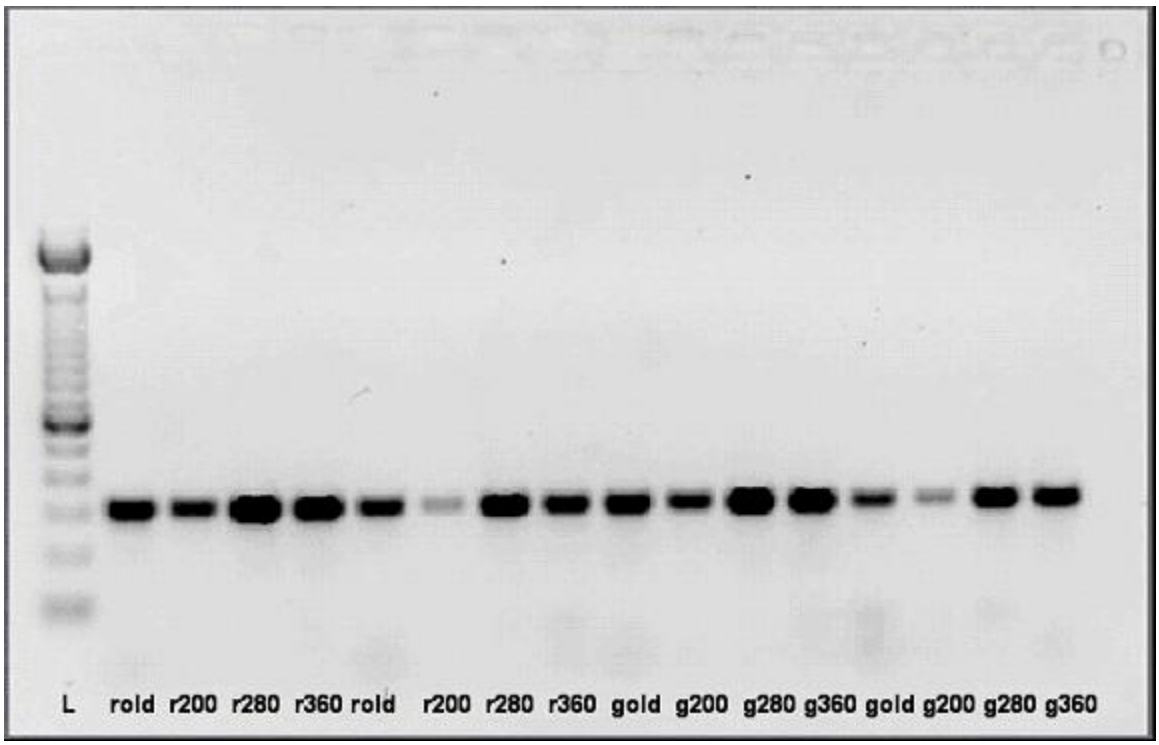

Figure 7 PCR with Gotaq Flexi DNA polymerase with a range of primer concentrations of CREB 1+2. In the picture the r stands for thorax cDNA of C. rubecula and the g stands for thorax cDNA of C. glomerata. Thorax cDNA of C. rubecula and C. glomerata was used in two concentrations, for the first dilution 10 µl RNase free water was added to 20 µl aliquot and the second dilution was a 4 x dilution of the first dilution.The first series of both r and g represent the product formed with the first cDNA dillution, the second series of both r and g re present second cDNA dillution. The DNA ladder in lane L was used to compare the sizes of the products with.The numbers indicate the primer dilution used for the sample. The primer-dimer is smaller than 100 base pairs and can be seen as a smear, because the base pairs number varies. The product is present in a higher concentration so it can be seen as a bright band. Variation in the product concentration can be seen as variation in the intensity of the bands. The density of the bands follows the same pattern for C. rubecula as well as C. glomerata. In ascending density of the bands the primer dilutions were 200nM, old primer, 280nM, 360nM. Between the two dilutions of thorax cDNA only a difference in intensity can be seen for the samples with 200nM primer dilution. The intensity shows that the concentration of the old primer lays between 200nM and 280nM.

With this primer concentration again it was tried to make a calibration curve with Ct values of several cDNA dillutions for CREB1+2. A RT qPCR was done two times with a 280 nM CREB 1+2 primer concentration with thorax cDNA of *C. glomerata* and *C. rubecula*. This primer-dimer is also a factor in the fluorescence signal measured by the Corbett RotorGene 6000 system. The Sybr green fluoresces when bound to double stranded DNA. Since primer-dimer is also double stranded the Sybr green dye bound to the primer-dimer will also fluoresce. So it is not possible to distinguish the part of the signal that originates from the product and the part of the signal that originates from the primer-dimer. Therefore, a RT qPCR was done with a series of primer concentrations 100 nM, 200 nM, 280 nM and 360 nM for CREB 1+2 and CREB 3+5 and thorax cDNA of *C. glomerata* and *C. rubecula* to see whether another primer concentration forms less

primer dimer and still forms product. On the gel it showed that all the primer concentration formed primer-dimer and product (figure 8). Despite none of the primer concentrations did not form primer-dimer, one primer concentration had to be used for following RT qPCR's. The choice was to use 200 nM. Higher concentrations lead to more primer dimer and lower concentration lowers the amount of product. At 200nM the ratio between primer-dimer and product formed is the best of the four concentrations. It is however likely that the primer-dimer was formed at the end of the RT qPCR. Since at the end of the RT qPCR there is no target DNA left for the primer to bind to. The concentration of the product is determined at the beginning of the exponential phase. So may be the primer-dimer formation did not influence the signal measured in the RT qPCR.

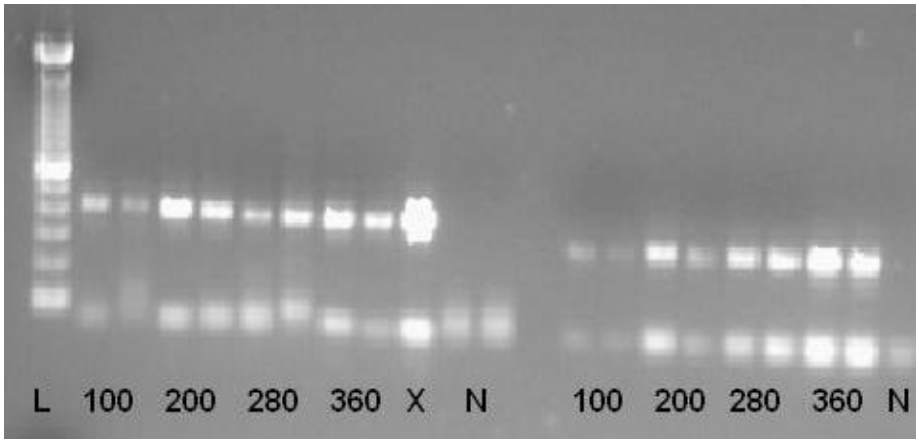

Figure 8 RT qPCR with thorax cDNA of C. rubecula and several primer concentrations of primers for CREB 1+2 and CREB 3+5. The number indicate the nM primer dillution. The first dillution series is the result of primers primer dillutions of CREB 1+2, the second series is the result of primer dillutions of CREB 3+5.The X stands for a pipet mistake and the N stands for NTC. In al the samples both product and primer-dimer was formed. The density of the bands of the product for the 100 nM primer dillution is low. This means that the product concentration was low. The 200 nM primer dillution was choosed to use for the RT qPCR for the calibration curve. In the sample of this primer dilluiton enough product was formed. Unfortunatly primer-dimer formation could not be prevented, since already at the lowest primer dillution primer-dimer is formed.

Again a RT qPCR was done to make calibration curves, this time with 200 nM primer concentration for CREB 1+2 and CREB 3+5 for both parasitic wasp species. Still a lot of primer dimer was formed. So the Ct values of the dilutions could not be used to make a calibration curve. To increase the amount of cDNA without increasing the amount of primer-dimer a nested PCR was done. For this nested PCR nested gene-specific primers were used, the primer sequences are given in table 3. The nested PCR exist of two PCR runs, in the first run primers For A and Rev C were used and in the second run with primers for For C and Rev B were used. With go Taq the first run was performed once, the second run is performed twice. The second run was performed twice because the first time no product was formed in the second run. After doing the second run twice, the product of the PCR with the For A and Rev C and the product of the second run with For

C and Rev B were runned on a gel. In none of the runs product was formed. The second run can not form any product when in the first run no product is formed. There was also a problem with the primers order. In the first run the nested primer Rev C was used and in the second run the primer Rev C was used. If in the first run the nested primer is already used, product formation in the second run will not be possible. To test whether the cause of lack of product formation was caused by using the goTaq kit, the Advantage® 2 Polymerase Mix (Clontech, cat. # 639201) was used. The nested PCR was performed with old cDNA as controll and thorax cDNA of *C. glomerata.* In this nested PCR a sense nested primer was used in the second run and the antisense primer was the same as in the first run. So For A and Rev C were used in the first run and For C and Rev C were used in the second run. In this run no problems occurred. The PCR product of the PCR with the primer For a and Rev C and the PCR product of the PCR with the primers For C and Rev C both gave a band on the gel (figure 9).

Table 3 Nested gene specific primers (van den Berg, 2009)

Primer	**Sequence**	**Orientation**
Forward A	5'-CGCTTGCCCACTCGTATTGC-3'	sense
Forward C	5'-TGGTGACGAGAAATACGTAACTGG-3'	sense
Reverse B	5'-CGCTGCATAAGATTGTT-3'	antisense
Reverse C	5'-CATTGGTCTGCTTAAAGACATCCG-3'	antisense

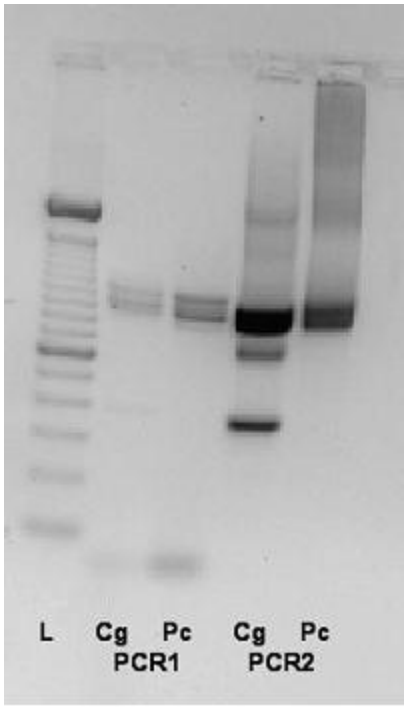

Figure 9 Nested PCR of thorax cDNA of C. glomerata and old cDNA as positive controll with Advantage® 2 Polymerase Mix. *Cg stands for thorax cDNA of C. glomerata, Pc for the positive control. The product of both the first PCR reaction and the second PCR reaction were runned on the gel.*

The product of the nested PCR with thorax cDNA of *C. glomerata* and clontech Advantage® 2 PCR Kit was diluted 100x and divided into aliquots of 20 µl. These dilutions were used to make the calibration curves of the Ct-values for CREB isoforms 1 and 2 and CREB isoforms 3 and 5. A qPCR is done with a primer concentration of 280 nM. The first dilution was a 10^{-5} dilution of the nested PCR product. Five dilutions were used with steps of 10x. The duplicates of the dilutions differed too much and the no-template controll (NTC) was contaminated. The first dilution formed product after 8 rounds of the RT qPCR. This is too early to be able to use this dilution for the calibration curve. The samples will not amplificate at this PCR round because CREB is low abundant and thus present in low concentrations. Another RT qPCR was done starting with 10^{-6} dilution to make the calibration curve. The 10-6 dilution forms product after 13 rounds in the RT qPCR. In this RT qPCR all dilutions were pipetted in triplo, to be able to exclude one of the triplo's when it deviated too much. For making the calibration curves the samples used have to be at least in duplo. To get rid of the contamination the primers were diluted again from the original stock under UV light. On the agarose gel of this qPCR showed no contamination of the NTC and a remarkable result for the dilution series. Every dilution was done in triplo. Of every dilution one sample showed a band related to the expected PCR product, one sample showed a band related to primer-dimer and one sample did not show any band (figure 10).

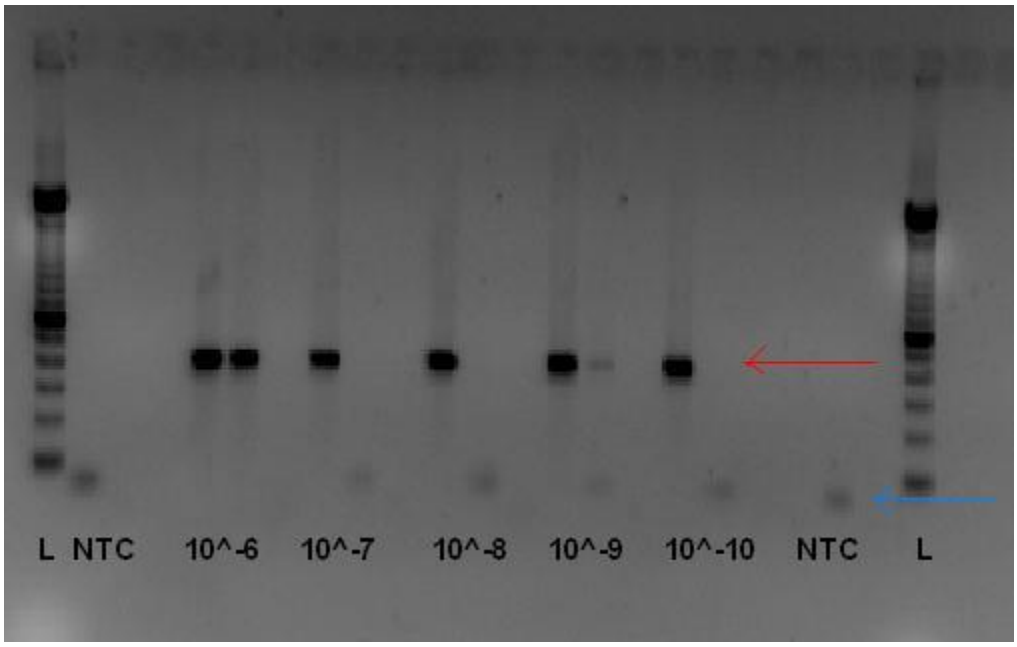

Figure 10 RT qPCR with product of Nested PCR of thorax cDNA of C. glomerata with 100 nM primers of CREB 1+2. Every dillution was runned three times in the RT qPCR. The product of every sample was runned on the gel. The numbers in the picture indicate the dillution of thorax cDNA. The first triplo of NTC was pippetted under UV ligth to prevent contamination. The second triplo of NTC was pipetted after mixing the dillutions of cDNA into the tubes. This was a control to see whether more contamination occured after mixing the cDNA. The red arrow points place of the product in the gel, the blue arrow points the place where the primer- dimer is located on the gel. The gel shows that for every dillution either product, or primer-dimer or nothing was formed in the RT qPCR. This shows that the repeats are not the same. As long as these are not the same the Ct values of the dillution can not be used for making a calibration curve.

This RT qPCR was repeated, but the results were not better than in the previous RT qPCR. Somebody else did this qPCR again to check whether the cause of the unexpected results where to blame on me. Also in this qPCR the triplo's differed a lot from each other and still a lot of primer-dimer was formed. Again the samples differed in what kind of products were formed, primer-dimer, no product or the product. The best results with ABsolute™ qPCR SYBR® Green Mix (ABGene, cat. # AB-1159/a) and primers for CREB 3+5 are given in figure 11 and 12.

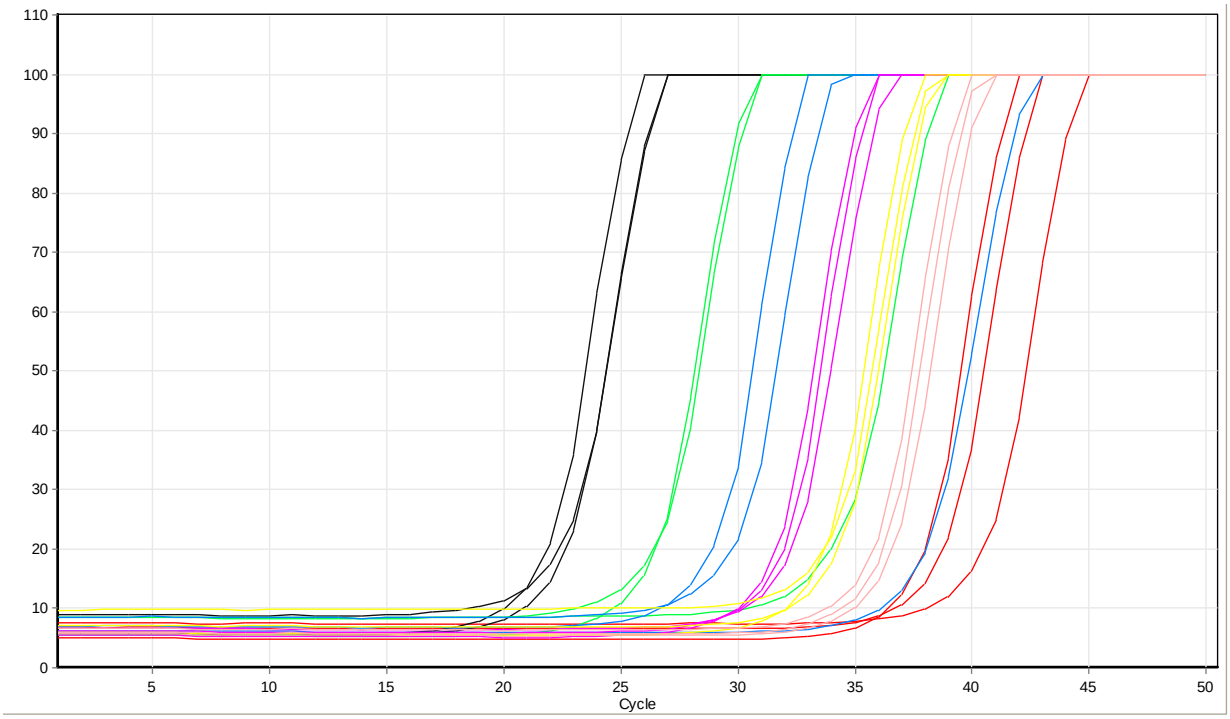

Figure 11 RT qPCR with RT qPCR with product of Nested PCR of thorax cDNA of C. glomerata and 100 nM primer for CREB 3+5. The dillutions were runned in triplo. In a succesful run all triplo's are located aproxiamatly at the same position in the graph. In all the repeats the same amount of thorax cDNA should be present. Therefor the repeats should aproxiamatly have the same Ct-value. In this run this was not the case. The graph shows that on of the repeats of the 10^{-7} (green) and the 10^{-8}dilution (blue) differs a lot from the other two. The NTC's (red and pink) should not give a signal because in the NTC's no cDNA is present. Sometimes the NTC's give a signal because primer-dimer is formed. In this run the NTC's have such a low Ct-value that contamination is most likely. This is also shown in figure 12. (The grey lines; 10^{-6} dilution, green lines; 10^{-7} dilution, blue lines; 10^{-8}, bright pink lines; 10^{-9} dilution, yellow lines; 10^{-10} dilution, light pink lines; NTC mixed under UV lamp, red lines NTC mixed after mixing dilutions)

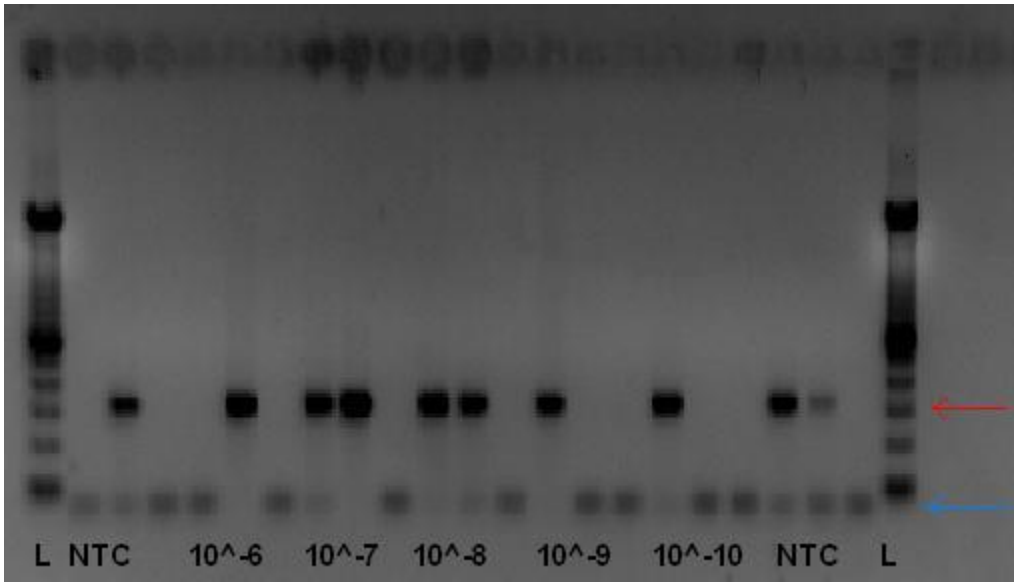

Figure 12 RT qPCR with RT qPCR with the product of Nested PCR of thorax cDNA of C. glomerata and 100 nM primer for CREB 3+5. The first triplo of NTC's was mixed under UV light, the second triplo of NTC's was mixed after the dillutions of cDNA were mixed. The numbers in the picture indicate the dillution of the cDNA.The red arrow points the location of the product band, the blue arrow shows the location of the primer-dimer bands. Again in this RT qPCR either product, either primer-dimer or nothing was formed. The repeats are not the same, so also the Ct values of this RT qPCR could not be used for a calibration curve.

The unstable results could mean that the kit is not suitable for this RT qPCR. So a RT qPCR with two other kits was done; Sensimix Sybr No-Rox (Bioline, cat. # QT650-02) and IQ^{tm} sybr green supermix (Biorad, cat. # 170-8884). The product of the RT qPCR of both kits was put on an agarose gel to compare the results (figure 13). The triplo's of both RT qPCR's differed less than of the RT qPCR's with the kit used earlier. Biorad IQ^{tm} sybr green supermix was used for the subsequent qPCR's because the qPCR Sensimix Sybr No-Rox gave more smear above the bands.

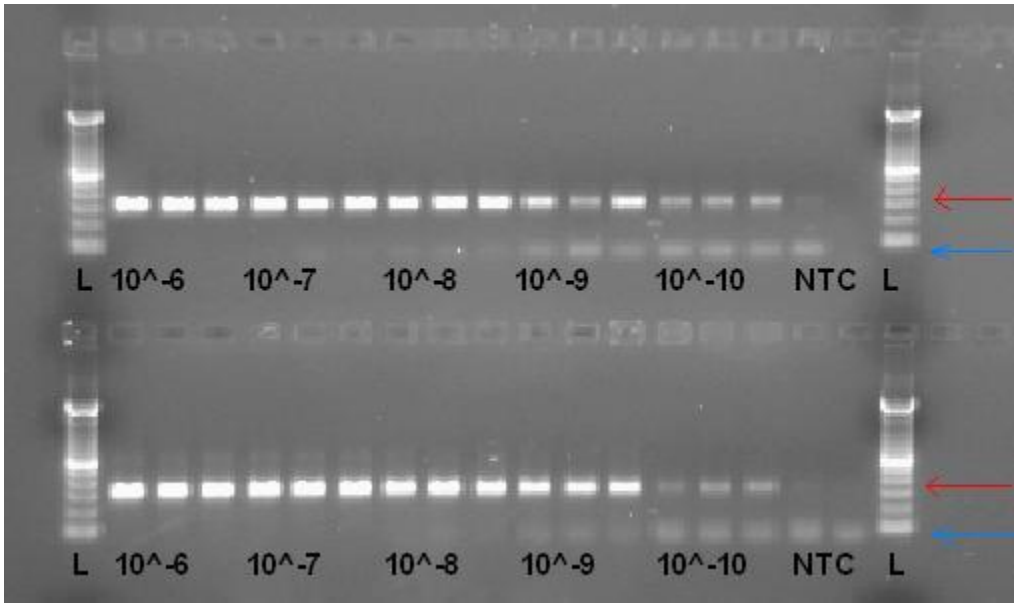

Figure 13 RT qPCR with the product of Nested PCR of thorax cDNA of C. glomerata and different concentrations of primer for CREB 3+5. The upper lanes contain samples from the RT qPCR with Biorad IQ^{tm} sybr green supermix, the lower lanes contain samples from the RT qPCR with Sensimix Sybr No-Rox. The numbers in the picture indicate the dillution of the cDNA. Every dilution was runned in triplo, the NTC's were runned in duplo. The red arrow points at the location of the product in the gel, the blue arrow points at the location of the primer-dimer in the gel. The NTC's of both Sybr green mixes were contaminated with cDNA. A band is visible at the height of the product.

A next qPCR was performed with Biorad IQ^{tm} sybr green supermix. In this qPCR the annealing temperature was raised two degrees compared to the earlier run, because in the first run with the IQ^{tm} sybr green supermix primer-dimer was still formed. On the gel of this qPCR a lot of contamination was present in the NTC's and primer-dimer was formed from the second dilution, 10^{-7}. All the previous RT qPCR's were performed with 50 rounds. It was tested whether primer-dimer formation already appears at the Ct-values of the dillutions. The Ct-value of the lowest dillution lays between 25 and 30 rounds. So to test whether primer dimer is already formed at this number of RT qPCR rounds a RT qPCR with 35 instead of 50 rounds was performed. The graph of the RT qPCR is presented in figure 14 and the gel with products of this RT qPCR is presented in figure 15.

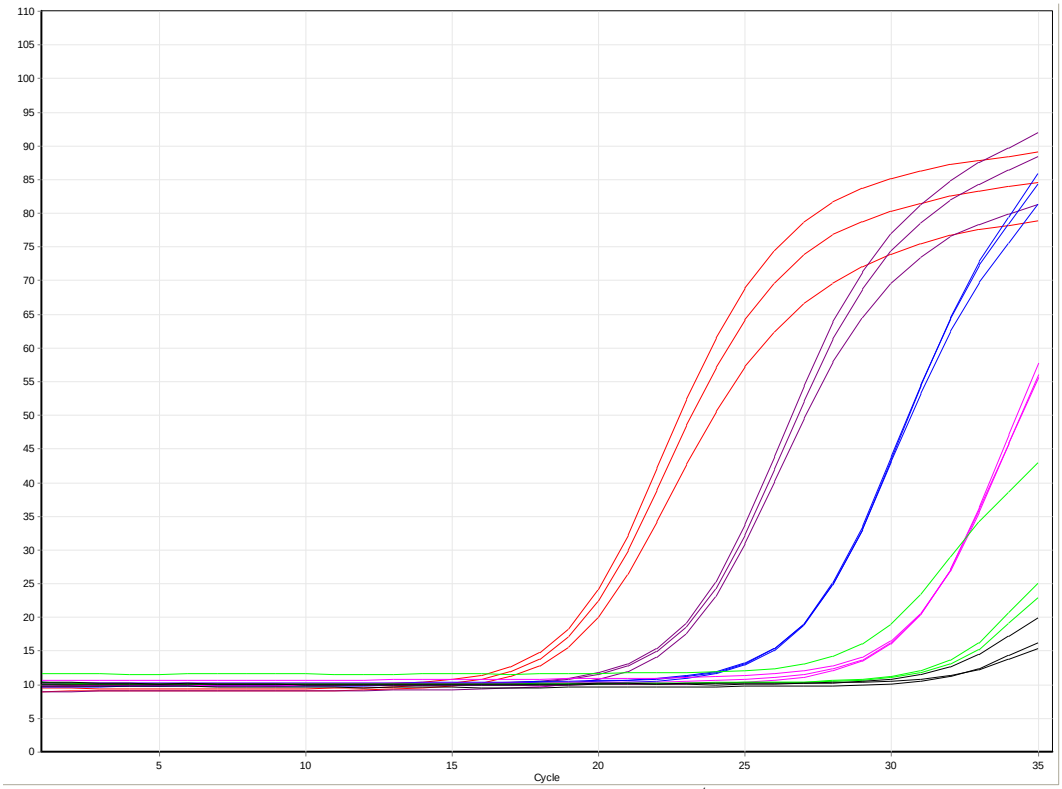

Figure 14 RT qPCR graph of the RT qPCR with Biorad IQ^{tm} sybr green supermix, the product of Nested PCR of thorax cDNA of C. glomerata and 100 nM primer for CREB 3+5 after 35 rounds Therepeats of first four dillutions are positioned at the same point in the graph. If the NTC's were not contaminated this run could be used for making a calibration curve of theCt-values. However, in this run the NTC's (black) are contaminated. The contamination is also visible in figure 15. (Red lines; 10^{-7} dilution, purple lines; 10^{-8} dilution, Blue lines; 10^{-9} dilution, Pink lines; 10^{-10} dilution, Green lines; 10^{-11} dilution, Black lines; NTC)

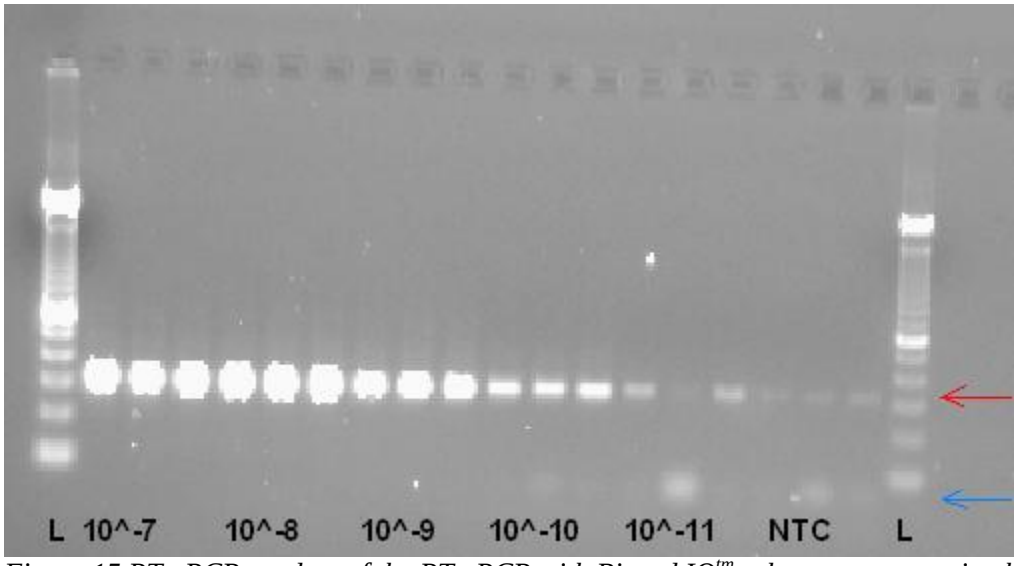

Figure 15 RT qPCR product of the RT qPCR with Biorad IQ^{tm} sybr green supermix, the product of Nested PCR of thorax cDNA of C. glomerata and 100 nM primer for CREB 3+5 after 35

rounds. The numbers indicate the dillution of cDNA used in the mix. The NTC was mixed after mixing the dillution samples. The red arrow shows the location of the product, the blue arrow points at the location of the primer-dimer. Only primer-dimer was formed in two samples of the lowest dilutions of cDNA. The NTC's are again contaminated with cDNA.Only without contamination the Ct values of the dillutions can be used for making a calibration curve.The contamination remained a problem. However, when 35 rounds in the RT qPCR were runned instead of 50 rounds, primer-dimer formation is absent in the first four dillutions. So the primer-dimer formation showed in the previous runs was probably formed after product formation.

Since all the dillutions yielded product at 35 rounds of the RT qPCR this number of rounds was used. In this qPCR the triplo's overlapped each other in exception of one sample of the last dilution, 10^{-11}. However, the melting curve of the first and the second dilution where placed more to the left than the melting curve of the third and fourth dilution. In this RT qPCR's there was looked at isoform 3 and 5 at the same time. No primer can be designed in such a way that these two isoforms are separated. The only difference between CREB 3 and 5 is having or lacking exon 8. Exon 8 encodes for 3 amino acids. Isoform 3 contains exon 8 and so it is 9 base pairs longer. To test whether in the first two dilutions one isofrom was dominant and in the third and fourth dilution the other isoform was dominant the PCR products were runned on a 3% metaphor agarose gel. After running this gel for 2 hours at 60 V and for 1,5 hour at 80 V it was shown that the dilutions did not differ in amount of base pairs (figure 16).

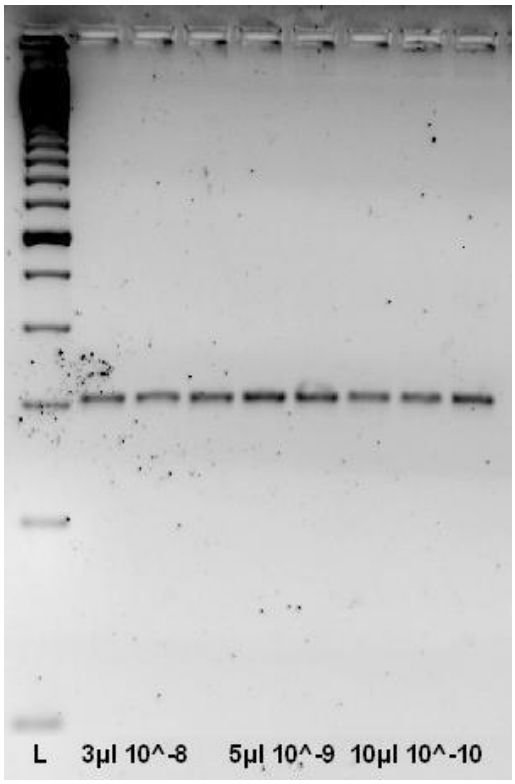

Figure 16 RT qPCR product of last RT qPCR with Biorad IQ^{tm} sybr green supermix runned on a metaphor agarose gel for 2 hours at 60 V and for 1,5 hour at 80 V. The amount of product runned on the gel is mentioned in the figure as wel as the dilutions runned. The products are positioned on the same heigth in the gel. This indicates that all the product consist of the same amount of basepairs.

4. Conclusion and discussion

The principle of the in situ PCR experiment worked. The parts of the brain in which CREB expression is present, were high in fluorescence. However, the quality of my brain dissection was not high enough to result in brain images in which the expression of CREB isoforms could be compared to 3D models of *Cotesia* brains. So the research question formulated for the in situ PCR experiment could not be answered.

In previous research to CREB expression in *C. glomerata* and *C. rubecula* van den Berg, 2009 showed that the major part of the CREB transcripts in the brains of both wasp species, conditioned and unconditioned, consist of splice variants 1,2, 3 and 5. Van den Berg, 2009 also showed lower levels of mRNA transcripts coding for splice variants 1 and 2 than for splice variants 3 and 5 in both wasp species. The only difference in constitutive expression of CREB transcripts between *C. glomerata* and *C. rubecula* are the relative expression levels for the splice variants 4 and 9. These levels were higher in *C. glomerata* and *C. rubecula*. In *C. glomerata* the relative abundance of CREB splice variants 1,2 and 3,5 changed significantly after a single learning event. After three spaced learning events this change was absent. The relative abundance of these splice variants did not significantly change in *C. rubecula* neither after one single learning event nor after three spaced learning events. Van den Berg showed all these differences with the RT qPCR method.

However, the RT qPCR method used in my experiments was not suitable to produce results that gave an answer on my research questions. The calibration curves, needed for determining the abundance of the CREB isoforms, could not be made. The abundance of 18S and the CREB isoforms and the ratio between activator and repressor isoforms could not be determined. Determining the ratio between absolute abundance of activator and repressor isoforms was crucial to answer the research question. In the RT qPCR several problems occurred. One of the problems was contamination of the samples. The NTC's gave a signal in the RT qPCR. Partly this signal originated from primer-dimer formation and partly this signal originated from contamination. By running the product of the RT qPCR on a gel, it was possible to see whether the signal originated from primer-dimer or from contamination. A lot of contamination was shown in the NTC's. As long as the NTC's are contaminated, the results can not be used for a calibration curve. To get rid of the contamination fresh ingredients for the mix can be used and the mix must be prepared under UV-light. For the calibration curves it is also important to work form the lowest dilution to the highest dilution during mixing the template with the mix. When contamination then occurs between first and last dilution the contamination has no effect. The NTC's must be free of contamination before the data can be used for the calibration curve. When the NTC's are not free of contamination it is not sure whether the signal of the dilution only results of the dilution or that the contamination contributes to the signal. The biggest problem in the RT qPCR was shown to be primer-dimer formation. The

signal produced by the primer-dimer could not be separated from the signal produced by the product. The high rate of primer-dimer formation has two causes, the low abundances of the CREB isoforms 1 +2 and 3+5 and the primers were not highly specific. A nested RT qPCR, with thorax cDNA as template, had to be done to form enough product that could be diluted for the calibration curve. The low primer specificity can not be changed, because the primer has to be developed on the borders of the exons. This results in a small amount of base pairs that can be used for primer development. A possibility to decrease the amount of primer-dimer formation is using fewer rounds for the RT qPCR. A lot of the primer-dimer was formed in the last 15 rounds of the RT qPCR. In those last rounds the entire template has reacted and then it is more likely that the primers react with each other. Another solution to get rid of the signal produced by the primer-dimer is using a sequence-specific probe. Sybr green detects all double-stranded DNA, so it detects the product as well as the primer-dimer. A fluorescent-labelled sequence-specific probe only binds to the product. This results in signal only originating from the product and not from the primer-dimer. By using a probe the primer-dimer formation in the RT qPCR can be ignored.

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