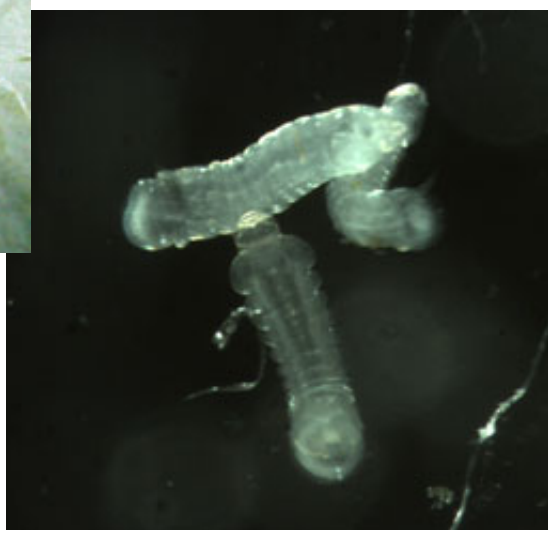
WAGENINGEN UNIVERSITY

LABORATORY OF ENTOMOLOGY

The role of *PKG* expression on larval mobility of parasitoid wasps:

A comparison of *Cotesia glomerata* and *Cotesia rubecula*



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Preface

This thesis report describes all the research that I have done in the past 7 months on larval mobility of *C. glomerata* and *C. rubecula* and on the expression of the *PKG* gene. It is amazing that differences in expression or activity of only a single gene can have such large effects on behaviour of animals, while at the same time many genes of different pathways are likely to be involved in this behaviour. I have found working on this subject very interesting and I'am happy to have learned so much on this type of research and on molecular work. Off course, 7 months is far too less time to be working on this subject, as there are still so many aspects of larval mobility and *PKG* that would be nice to further investigate.

In my opinion the parasitoid wasps *C. glomerata* and *C. rubecula* are very good organisms for the research on the *PKG* gene and larval mobility. They are quite easy to handle and to rear in large numbers because of their small size, but they are still large enough to do research on the brains and on the larvae. Beside this *C. glomerata* and *C. rubecula* are interesting to observe. The front page of this report shows a female *C. glomerata* wasp that is parasitizing *Pieris brassicae* larvae and next to it is a picture of larvae of *C. rubecula* which are attacking each other.

I would like to thank my supervisors Hans Smid and Louise Vet, from whom I've learned so much on this subject and on the scientific work in general. Furthermore I would also like to thank my 'unofficial' supervisors Patrick Verbaarschot and Michaël van den Berg, from whom I have learned many things on the experimental procedures that I have used and who have helped me to become a real 'professional' on molecular work.

Summary

Solitary larvae of endoparasitoids live alone in their host and they display aggressive behaviour against other larvae, whereas gregarious larvae live together in a host without killing each other. Gregarious larvae have, however, the ability to fight against other larvae when being attacked by solitary larvae. Research on solitary and gregarious parasitoids showed that solitary behaviour is the ancestral state and that gregariousness most likely evolved due to a reduced mobility of the larvae. The theory implies that gregarious larvae do not kill each other, because they do not move and thereby will not encounter each other. Research on the gregarious wasp *Cotesia glomerata* and the solitary wasp *C. rubecula* has shown that there is indeed a difference in larval mobility between these two species: larvae of *C. rubecula* are significantly more mobile at the first day after hatching, but this mobility decreases over the following three days. The mobility of *C. glomerata* larvae remains more or less the same in these four days.

The question is how this difference in mobility has evolved and what the genetic basis is of the differences in mobility. The aim of this thesis was therefore to identify a gene that may be related to these differences in mobility. Several candidate genes might explain the differences in larval mobility. One of these genes is the *PKG* gene encoding a cGMP-dependent protein kinase (PKG). This gene was found to have a causal effect on mobility of *D. melanogaster* and the honey bee, *A. mellifera*. In these species a positive correlation of *PKG* expression and activity with mobility was found: a higher expression of *PKG* is correlated to a higher mobility of these species. Research on *C. elegans* and the harvester ant, *P. barbatus*, also showed an effect of *PKG* expression and mobility, but this correlation was found to be negative. The *PKG* gene plays an important role in mobility of several species although the regulation and the correlation of the gene with mobility is different between these different species.

The mobility of *C. rubecula* larvae decreases during the first four days after hatching, while the mobility of *C. glomerata* larvae remains constant during this time. It is therefore expected that the expression of the *PKG* gene will change in *C. rubecula* larvae, while the expression in *C. glomerata* larvae will remain the same. The expression of *PKG* in *C. rubecula* larvae might decrease with the decreasing mobility. It is, however, also possible that the expression of *PKG* is negatively correlated to mobility. This thesis will therefore focus on the following hypothesis: The *PKG* gene is involved in mobility of larvae of the solitary parasitoid wasp *C. rubecula* and the gregarious parasitoid wasp *C. glomerata*. The correlation between the expression of the *PKG* gene and the mobility of the larvae can either be positive or negative.

The *PKG* gene of both *C. glomerata* and *C. rubecula* was sequenced and several fragments of different length were found in both the 3' and the 5' RACE procedure. These fragments all have an identical sequence, they only differ in length. These fragments may therefore be 5' or 3' truncated isoforms of the *PKG* gene. It is, however, also possible that a number of these different fragments resulted from artifacts of the experimental procedure. Further investigation of isoforms of the gene was done using primers that were located on the flanks of the gene in PCR. This procedure also revealed several possible isoforms, but due to sequencing problems only two of these fragments could be sequenced. The largest fragment had a size of 2 kb, while the smaller fragment had a size of only 1 kb. The smaller fragment had a identical sequence of the 2 kb fragment, except for 1 kb located almost at the 5' end of the fragment that was not present in this smaller isoform.

More information on the sequence and the possible isoforms of the *PKG* gene was obtained from performing blots. The Southern blot failed, but the Northern blot showed that both species have at least three transcripts of the *PKG* gene. The largest transcript has a size of 2,5 kb, whereas the smaller transcripts have a size of 1,8 kb and 1,2 kb. It can, however, not be determined with certainty what the sequence of the transcripts is, because many different fragments were found in the 5' and 3' RACE and in the flank PCR, which could be combined into to the length of the different transcripts. No apparent differences in expression appeared to be present between *C. glomerata* and *C. rubecula* on the Northern blot, however.

In order to quantify differences in expression of the *PKG* gene in larvae of the two *Cotesia* species, Real-time quantitative PCR (RT-qPCR) experiments were done. The *18S* RNA was used as a reference in these experiments. When experiments were done using RNA from complete larvae, it was found that there is no significant difference in *PKG* expression between *C. glomerata* and *C. rubecula* larvae in the first four days after hatching. The larvae that were used in the experiment were not all collected on the same time, but from a number of different oviposition events in which a large number of caterpillars were parasitized by adult *Cotesia* wasps. Both species show a similar pattern for each oviposition event: the expression of the *PKG* gene increases a lot on day 3 and 4 after hatching in comparison to day 1 and 2. The differences between the different oviposition events are very large, however. It is unknown what caused the variation between the different events.

The large variation between the different samples in the RT-qPCR experiments makes it impossible to draw a conclusion about the correlation of *PKG* expression and larval mobility of *Cotesia*. It should be investigated what causes the large variation between the samples. I suggest, however, that future research on *PKG* and larval mobility of *Cotesia* focuses on the

enzyme activity of PKG, because it appears from research on ants and fruit flies that PKG enzyme activity correlates better to different behavioural patterns than the expression of the *PKG* gene. Depending on the results of these experiments either the relation between the *PKG* gene and larval mobility can be further investigated or another candidate genes can be tested.

1. Introduction

The *PKG* gene is thought to play an important role in larval mobility. PKG is a protein kinase that is activated by cGMP. In this thesis, I have investigated whether there is a correlation between the expression of the *PKG* gene and the mobility of *Cotesia* larvae.

1.1 The evolution of gregarious parasitoid species

A parasitoid is a parasite that spends a large amount of its life history within or attached to a single host organism. The host is eventually killed by the parasitoid, which often also consumes its host. Many different parasitoid insects are known including parasitoid wasps. Different types of behaviour can be recognized in the development of parasitoid larvae. The difference between solitary and gregarious parasitoids has been extensively investigated. In most cases the adult females of solitary parasitoids only lay one egg per host and the larvae of solitary parasitoids each develop alone in a host. If more eggs are laid in a single host, the behavior of these solitary larvae involves the killing of broodmates by physical attack, physiological suppression or resource competition, in order to become the only larva in the host (Godfray, 1994). In case of superparasitism, when multiple parasitoid females lay their eggs in a single host, a solitary larva also kills other larvae. In both cases, the young, first instar solitary larva moves towards another larva and upon encounter they will fight using their strong mandibles until one of the larvae is killed. If the solitary larva is older, then usually indirect methods are used to eliminate the other larvae (Godfray, 1994). An advantage of this aggressive behaviour is an increase of fitness when the host is small, because there is in this case not enough food for multiple larvae to reach maturity. For the adult female, however, the solitary behaviour of the larvae has a negative effect, as the female is forced to have a low clutch size per host and therefore she has to search for many hosts (Mayhew & Alphen, 1999)

The larvae of gregarious parasitoids have a quite different behaviour. Larvae of gregarious parasitoids do not kill broodmates or other parasitoids, but live together in the same host (Mayhew & Alphen, 1999). Research has revealed that gregariousness occurs quite often (Boivin & Baaren, 2000). Furthermore, it was found that the solitary state was most likely to be the ancestral state, from which gregariousness has evolved (Boivin & Baaren, 2000). This evolution of gregariousness is thought to be facilitated by laying multiple eggs within one

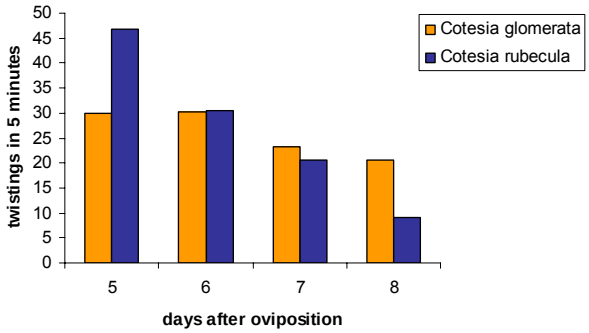
host. These multiple egg-clutches can occur by superparasitism of an already parasitized host by another female. It is also possible that the parasitoid females lay several eggs in one host. Both events were found to occur occasionally in solitary species (Mayhew & van Alphen, 1999; Boivin & Baaren, 2000). An advantage of laying multiple eggs within one host organism is that it is a sort of insurance in case an egg or larva fails to develop (Boivin & Baaren, 2000). The occurrence of multiple egg-clutches itself does not explain the evolution of gregariousness, however, since solitary larvae will kill each other upon encounter. Two theories were therefore proposed to explain the evolution of gregariousness.

One theory on the evolution of gregariousness proposes that gregarious behaviour evolved through a reduction in aggression of the larvae. Gregarious larvae do not fight with each other upon encounter and therefore they are able to develop together in one host. A consequence of this theory is that it is very difficult for gregarious parasitoids to invade a population of solitary parasitoids. Solitary offspring will kill the gregarious offspring, which will not defend themselves because of the reduced aggressiveness (Mayhew & Alphen, 1999).

Another theory proposes a different mechanism for the evolution of gregariousness. This theory proposes that gregarious species have evolved due to a reduced mobility instead of due to a reduced aggression. According to this theory, gregarious larvae survive because they do not move and thereby do not encounter each other. This theory further implies that if both solitary and gregarious offspring have the same fighting capacity, then each of them has a 50% chance of survival in case of a fight. This would make it much more easy for gregarious larvae to invade a population of solitary larvae, because several gregarious larvae are present in a host which each have a chance to kill the single solitary larva. The chances of survival of solitary larvae are then even lower since in each fight it has a 50% chance to die. The lower mobility of gregarious larvae will decrease the chance that they will encounter and kill their siblings and therefore they can exist in the same host (Boivin & Baaren, 2000; Pexton & Mayhew, 2001). Such reduced mobility was indeed observed when studying the gregarious parasitoid *Anaphes listronoti* in comparison to the closely related, solitary parasitoid *Anaphes victus* (Boivin & Baaren, 2000) and also modeling studies revealed that a reduction of mobility can provide a good explanation for the evolution of gregarious species (Pexton et al, 2003)

Much research is done on two closely related parasitoid wasps at the laboratory of Entomology of the Wageningen University and Research Centre (WUR). These are *Cotesia glomerata* and *C. rubecula*. *C. glomerata* is a gregarious parasitoid wasp and it can parasitize several species of *Pieris* butterflies. Its preferred host in Europe is, however, *P. brassicae*. *C.*

rubecula is a solitary parasitoid and it is a specialist of *P. rapae* (Wiskerke & Vet, 1994). Larval mobility was observed in these two species by counting the number of twistings that the larvae made after taking them out of their host (Tribuhl, 2007). These observations indeed show that the gregarious *C. glomerata* and the solitary *C. rubecula* have a different larval mobility (Graph 1). The mobility of C. rubecula larvae is significantly higher than the mobility of *C. glomerata* larvae at day 5 after oviposition, which is the first day after emergence of the larvae. This mobility decreases, however, during the following days. At day 8 the mobility of *C. rubecula* is even lower than that of *C. glomerata*. These observations indeed support the theory that differences in larval mobility have played a role in the development of gregariousness. The aggressiveness of *Cotesia* larvae has not yet been investigated as thoroughly as the mobility of the larvae, but some observations have shown that larvae of *C. glomerata* do not display aggressive behaviour towards other larvae of both *C. glomerata* and *C. rubecula*. The larvae of *C. rubecula* have been observed to display aggressive behaviour to other larvae a number of times. Furthermore, the larvae of *C. glomerata* were found to have only maxillary palpes at day 7 and 8 after oviposition, whereas *C. rubecula* larvae were found to have also large mandibles and labial palpes, beside maxillary palpes at 5 to 8 days after oviposition (Tribuhl, 2007). It therefore appears that the larvae of *C. glomerata* and *C. rubecula* also differ in their aggressiveness and ability to fight, suggesting that both differences in mobility and aggressiveness have been involved in the evolution of gregarious behaviour. More research should still be done on aggressive behaviour of *Cotesia* larvae. This research will, however, only focus on the difference in mobility and what the genetic basis is of these differences in mobility.



Graph 1: Differences in mobility of *Cotesia glomerata* and *C. rubecula* measured as the number of twistings of the larva in 5 minutes. *C. rubecula* has a significant higher mobility at day 5, but this larval mobility decreases fast over the following days. The mobility of *C. rubecula* is significantly lower than the mobility of *C. glomerata* at day 8 (Tribuhl, 2007).

1.2 Finding candidate genes for larval mobility

When related solitary and gregarious species differ in larval mobility, the candidate gene approach can be used to identify genes that are possibly involved in this mobility. Candidate

genes are genes that influence similar phenotypes in multiple species as they are conserved genes in evolution (Fitzpatrick et al, 2005). Genes that were investigated in model organisms, such as the fruit fly, the mouse and nematodes can serve as candidate genes and the effect of the genes can then also be studied in non-model organisms. When working with the candidate gene approach it should, however, be considered that interactions such as the gene-by-environment interaction, pleiotropic effects of genes and epistatic interactions can have a large affect on the function of a gene. Especially when working on complex traits such as behaviour, these interactions are likely to have a large impact. Beside these interactions, many behavioural traits are controlled by quantitative trait loci (QTL), which often consist of a major gene and several minor genes (Fitzpatrick et al, 2005).

Four species are described in the paragraphs below, showing differences in mobility that are, to some extent, comparable to the differences in mobility of the *Cotesia* larvae. These are the fruit fly, nematodes, honey bees and harvester ants. Several genes have been found to be involved in the behaviour and mobility of these species and these genes are therefore possible candidate genes for explaining the differences in mobility between *C. glomerata* and *C. rubecula*. It is described how research was done on these genes, in order to show which types of experiments should be done to prove that a certain gene is involved in a certain behaviour.

1.2.1. The foraging behaviour of the fruit fly

Research on so called foraging behaviour first started when differences in foraging strategy were observed in larvae of the fruit fly, *Drosophila melanogaster*. The larvae could be divided in two types of feeding behaviour: ‘rovers’ and ‘sitters’. Rover larvae move over a large area while feeding, whereas sitter larvae cover only small areas and remain on a food source once they have encountered it. This difference in foraging strategy provides an advantage for the two types of larvae in different environments. If the food distribution is discontinuous, the ‘rovers’ have an advantage since they move large areas and have a better chance to find the food. If the food is evenly distributed, however, the sitters have an advantage because they waste less energy on moving around. Both in nature and in laboratory stocks, approximately 70% of the larvae were found to be ‘rovers’ and 30% were found to be ‘sitters’(Sokolowski, 1980). Later is was found that the different foraging strategies were also present in adult flies: ‘Rovers’ walk further distance after eating then ‘sitters’. Furthermore the differences between ‘rovers’ and ‘sitters’ could only be observed in the presence of food. If no food is available, both ‘sitters’ and ‘rovers’ move similar distances, indicating that the

differences in behaviour are involved in foraging and not in general locomotion (Pereira and Sokolowski, 1993). Although it is not known if food has a role in the mobility of *Cotesia* larvae, it is interesting that differences in mobility have a role in behaviour of both *Drosophila* and *Cotesia*.

Figure 1: *Drosophila melanogaster*, the fruit fly.

The differences in foraging strategy of *D. melanogaster* appeared to be caused by one gene on the second chromosome, which was called *foraging* (*for*). This gene was thought to have two alleles: the dominant 'rover' allele, for^R, and the recessive 'sitter' allele, for^s. (Sokolowski, 1980). It took, however, many years before the *foraging* gene could be characterized with the help of molecular tools. Osborne et al mapped the *foraging* gene by mutational analysis, meaning that they determined the location of mutations that affected foraging behaviour. These mutations were found to be located in the *dg2* gene. This gene is one of the two known *PKG* genes in *Drosophila*. PKG is a kinase that is dependent on cGMP for its function. PKG is involved in signaling pathways and it activates receptors or other enzymes. Three major transcripts of *PKG* were identified (see figure 2). These transcripts were named T1, T2 and T3 and they all have the same 3' region, which contains the kinase domain of PKG. This kinase domain is essential for the activity of PKG as it is the enzymatic part that makes PKG a kinase (Osborne et al, 1997).

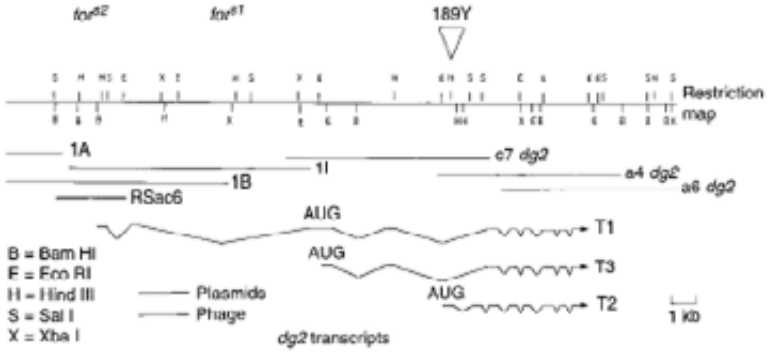


Figure 2: The three major transcripts of the *dg2* gene, T1, T2 and T3, are shown on the molecular map of *dg2*. The three transcripts have similar 3' regions, which contains the kinase domain. They differ in their 5' regions. Figure taken from Osborne et al, 1997.

In order to determine if the *PKG* is directly responsible for the differences in foraging behaviour, a mutant strain of *D. melanogaster* was made which had a transposable P-element inserted in the 5' end of the T2 transcript. This element was then excised from the transcript using transposase, which increased the expression of the transcript. It was found that this increase in expression reverted the larval foraging behaviour from sitter to the rover phenotype. In another experiment, *dg2* in sitter larvae was overexpressed, which again resulted in a reverted behaviour to the rover phenotype. Only the foraging behaviour was

changed during these experiments, indicating that PKG is indeed directly responsible for the difference in foraging behaviour (Osborne et al, 1997).

PKG enzyme assays were performed using heads extracts of adult fruit flies. The activity of PKG was compared between rover and sitter strains. The activity in rovers was found to be significantly higher than the activity in sitters strains. Beside the activity of PKG, the expression of the *dg2* gene was also estimated from Northern blots. Sitters were found to have a small reduction in the abundance of the T1 transcript relative to rovers. The other two transcripts, T2 and T3, were also slightly reduced in sitters, but the differences were very small (Osborne et al, 1997).

The difference in foraging behaviour in rovers and sitters appears to be caused mainly by a difference in enzyme activity of PKG and it is therefore expected that the for^R and the for^s allele differ in a regulatory region. So far, only the T2 transcript of *dg2* was sequenced, however, and no differences were found in the sequences of rovers and sitters. It therefore still remains a question what causes the difference in PKG activity between the rover and sitter phenotype (Osborne et al, 2001).

Several other investigations have recently been carried out on foraging behaviour and also other species have been investigated for this purpose.

1.2.2. Different mobility states in nematodes

In the nematode, *Caenorhabditis elegans*, two alternating states of behaviour can be distinguished that have a similarity with the rover and sitter phenotype in fruit flies. These two types of behaviour alternate within an individual. The first type of behaviour is 'roaming': the nematode moves over a large distance with high speed and low turning. The second type is 'dwelling': the nematode moves only small distances at a low speed and with lots of turning. Both types of movement can be distinguished when observing a nematode. Roaming and dwelling were only observed in the presence of food, it is not known whether these states also exist when food is absent (Fujiwara et al, 2002).

Figure 2: *Caenorhabditis elegans*, a nematode.

A study on this behaviour started with the generation of mutant nematodes that had a defect in their ciliated sensory neurons. Nematodes have in total 302 neurons of which 60 have cilia. These neurons are sensory neurons and their cilia are involved in the transduction of signals. The mutants, which are called *che-2* mutants, lack a normal sensory cilium

structure and they have several defects due to this mutation. The mutants have diminished responses to chemicals and pheromones, they have a smaller body size and they have a different locomotive behaviour. The relative time spent 'roaming' is decreased in these mutants. These differences in the *che-2* mutant appear to be due to a decreased sensory perception, the ability to localize food did not appear to be affected. (Fujiwara et al, 2002).

In order to identify genes that act downstream in the processing of sensory input, mutants were generated in the already mutated *che-2* strain. These new mutants were screened for suppressors of the *che-2* mutation, meaning that the second mutation can rescue the defects of the initial *che-2* mutation. These nematodes will then have similar behaviour as the initial strain without mutations. One of the mutations appeared to be in the *egl-4* gene of *C. elegans*, encoding PKG, the same protein that was found to be involved in foraging behaviour of *D. melanogaster*. All defects that had resulted from the initial *che-2* mutation were rescued due to the mutation in the *egl-4* gene, indicating that PKG acts downstream in the processing of sensory input. Two major transcripts were found of *egl-4* in *C. elegans*. The first transcript was named *egl-4.a* and it consists of ten exons with a size of 2343 bp. A second transcript differed in the first exon, but shared the second to the tenth exon with *egl-4.a*. This second transcript was named *egl-4.b*. Interestingly, only a mutation in *egl-4.a* was found to restore the defects of the *che-2* mutation: lower concentrations of *egl-4.a* result in an increase of body size and the relative time spent 'roaming' (Fujiwara et al, 2002).

Beside this interesting correlation between a *PKG* transcript and mobility in nematodes, a correlation was also found between another gene and differences in mobility. Nematode strains can be classified as solitary strains or as social strains. Individuals of solitary strains remain dispersed evenly over the area, while social strains will aggregate into clumps at locations were most food is available. These social nematodes move twice as slow as solitary nematodes when clumped together. If nematodes of social strains are placed on a food source alone, however, they move twice as fast as solitary nematodes. These differences between solitary and social strains only become visible in the presence of plentiful food. If no food is present, social and solitary strains have similar behaviour: the individuals disperse evenly over the area in search of food. The differences between solitary and social strains did not appear to be caused by a decrease in olfactory sensitivity of social nematodes, as these nematodes have similar responses to a number of odors as solitary nematodes (de Bono & Bargmann, 1998).

Mutations that reverted solitary behaviour to social behaviour were found to be located in the *npr-1* gene. This gene is predicted to be a G protein-coupled receptor of the neuropeptide

Y (NPY) receptor family. NPY is a neurotransmitter that has been associated with several processes in the brain including the regulation of food consumption, memory and blood pressure (de Bono & Bargmann, 1998). The *npr-1* gene encodes a receptor with seven transmembrane domains and has a size of 1,4 kb. Only one amino acid, at position 215, was found to be different between solitary and social strains. This amino acid is located close to a transmembrane domain in a region that is important for G-protein coupling. Social nematodes have a phenylalanine at this position, while solitary nematodes have a valine. The differences between social and solitary strains were indeed found to be caused by this difference in the amino acid at location 215. When *npr-1* mutants were injected with *npr-1* with phenylalanine at location 215, the solitary behaviour was not restored in contrast to injections with *npr-1* with valine. It is not known exactly what the effect of this amino acid is on the receptor, but is expected to be involved in the strength or specificity of the G-protein coupling (de Bono & Bargmann).

It can be concluded that two genes of the nematode *C. elegans* have an effect on mobility of the nematodes. The *egl-4* gene, encoding PKG, has an effect on 'roaming' and 'dwelling', two alternating states of locomotive behaviour. A decrease in PKG will cause the nematode to display relatively more 'roaming', which is comparable to the behaviour of 'rovers' of *D. melanogaster*. The *npr-1*gene, encoding a G-protein coupled receptor, is involved social and solitary behaviour of nematodes. Social and solitary nematodes differ in their mobility.

1.2.3. Division of labor in honey bees

Honey bees (*Apis mellifera*) are social insects that live in colonies with a division of labor. The division of labor is age-dependent, but it also depends on the needs of the colony. Young adult bees are nurse bees which remain in the hive in order to take care of the larvae. After two or three weeks, however, they become foraging bees that search for food outside the hive. This transition involves many changes in brain chemistry, brain structure, endocrine activity and gene expression (Ben-Shahar et al, 2002; Robinson & Ben-Shahar, 2002).

Figure 3: The honey bee, *Apis mellifera*

The behaviour of nurse bees and forager bees can be compared to some extend to that of rovers and sitters of *Drosophila*. Nurse bees remain in the hive and they move therefore only in a small area, which is comparable to the behaviour of 'sitter' flies. Foragers, on the other

hand, move over large distances in order to collect food, similar as the behaviour of 'rover' flies. It was therefore decided to investigate the role of the *PKG* gene in the behaviour of bees. The *Amfor* gene, the *for* ortholog of honey bees, was sequenced and it was found that only one transcript of *Amfor* is present in the heads of the bees (Ben-Shahar et al, 2002).

Experiments showed that forager bees had on average a four times higher expression of *Amfor* in their brains than nurse bees. Also the activity of the PKG was four times higher in brains of forager bees. This correlation between higher expression of the *Amfor* gene and a higher activity of the bees does, however, not necessarily indicate a causal relation. It is possible that both the expression and activity increase with age. In order to rule out such an age-dependent effect, single cohort colonies were established. These colonies are composed of only young, adult bees of the same age. These bees would normally all be nurse bees for the first two or three weeks of their life, but due to the lack of forager bees in the single cohort colonies, some of the young bees become foragers as much as two weeks earlier than normal. These 'young' forager bees had a higher expression of *Amfor* than nurse bees of the same age (Ben-Shahar et al, 2002).

It can be concluded that the *Amfor* gene is correlated to social behaviour in honey bees, but in order to test whether an increased PKG activity causes the transition from nurse to foraging, another experiment had to be done. Nurse bees were treated with 8-Br-cGMP, a membrane-permeable analog of cGMP, which activates PKG. The downside of treatment with cGMP is, however, that it is a very common second messenger molecule, which could also activate other molecules. Nursing bees treated with the cGMP analog were indeed found to have a higher activity of PKG and these nursing bees were also more likely to display foraging behaviour at a younger age, suggesting a direct effect of PKG activity on behaviour (Ben-Shahar et al, 2002).

The transition from nurse to forager is correlated with the *Amfor* gene, but also thousands of other genes change in expression during the transition. Besides the pathway associated with *foraging*, also the pathway associated with the *malvolio* gene is thought to play an important role in behavioural maturation of honey bees (Whitfield et al, 2006). The *malvolio* gene encodes a manganese transporter protein and it also was found to be correlated with foraging behaviour of bees. The expression of *malvolio* is higher in forager bees than in nurse bees, and treatment with manganese also induces early foraging behaviour. This suggests that multiple pathways affect behavioural maturation simultaneously (Ben-Shahar et al, 2004; Whitfield et al, 2006). Furthermore, hormones and pheromones such as juvenile hormone, brood pheromone and queen mandible pheromone also have an influence on behaviour (Le

Conte et al, 2001; Whitfield et al, 2006). As shown, it appears that many factors can influence the transition from nurse to foraging.

1.2.4. Division of labor in harvester ants

The research on honey bees has indicated that PKG has a role in the colony organization. Therefore, the role of PKG in colony organization of the red harvester ant, *Pogonomyrmex barbatus*, was also investigated recently. Colony organization evolved independently in bees and ants (Ingram et al, 2005), but there are similarities in the division of labor between honey bees and harvester ants. Young worker start as callow workers and after that they will perform brood care inside the nest. Later they will perform maintenance tasks and patrolling tasks. Finally, the ants will perform foraging tasks outside the nest. This division of labor is, however, more variable in ants than it is in honey bees and somewhat less related to age (Ingram et al, 2005).

Figure 4: The red harvester ant, *Pogonomyrmex barbatus*

The gene encoding PKG was sequenced and it was named *Pbfor*. As in honey bees, only one transcript of *Pbfor* was found, which had a size of approximately 3 kb. The expression of *Pbfor* was significantly higher in callow worker than in foragers in all colonies tested. The expression patterns of the other groups, however, did not show any clear differences in expression. The differences in expression were furthermore quite large between different colonies (Ingram et al, 2005).

1.3. A role for the *PKG* gene in mobility of *Cotesia* larvae?

As shown in the paragraph above, several genes have been shown to be involved in food-related behaviour of several species and differences in mobility are characteristic for distinct behavioural states. Although the differences in behaviour in *Cotesia* larvae might not be related to food, the mobility is clearly different between *C. glomerata* and *C. rubecula*. The genes that were mentioned are therefore considered good candidate genes. The candidate gene that was investigated most in detail and in most species is the highly conserved gene encoding PKG. The *PKG* gene is therefore considered the best gene to start investigating in correlation with mobility of *Cotesia* larvae, although several other genes and pathways are also likely to be involved.

PKG is the guanosine 3', 5'-monophosphate (cGMP)-dependent protein kinase and phosphorylates specific serine/threonine sites. Phosphorylation can activate a receptor or another enzyme. PKG itself is activated by cGMP, which is a very common second messenger molecule and has a role in many different signaling pathways (Hofmann et al, 2006).

The *PKG* gene is widely expressed in animals and it is involved in many processes. It is involved in the relaxation of smooth muscle in many tissues. PKG therefore has an important role in the cardiovascular system, but also in other organs such as the urinary bladder, the kidneys and the penis. A well known medical application which involves PKG is Viagra, which raises the level of cGMP in order to stimulate PKG. This causes the relaxation of smooth muscle which allows an increased flow of blood. Furthermore PKG has several roles in signaling in the nervous system and it is involved in regulation of different aspects of behaviour, as was shown. Beside its role in foraging behaviour, PKG also plays a role in learning and in circadian rhythms of animals (Hofmann et al, 2006; Shafer, 2002).

Research on nematodes, fruit flies, honey bees and harvester ants has shown that, although the *PKG* gene affects similar behaviour in all four species, the regulation of the gene and the correlation with mobility differs between the species. In *D. melanogaster* the more active 'rovers' have a higher expression and activity of the *PKG* gene than the less mobile 'sitters'. A similar situation can be observed in adults of *A. mellifera* in which the more mobile forager bees also have a higher expression of the *PKG* gene than the nurse bees which perform their tasks only in the hive. In these two species there is therefore a positive correlation between the expression of *PKG* and mobility.

The opposite situation is observed in *C. elegans* and *P. barbatus*, in which there is a negative correlation between the activity of *PKG* and mobility. In *C. elegans* an increase in *PKG* expression causes the nematodes to display relatively more dwelling, which is the behavioural state with a lower mobility in comparison to roaming. In *P. barbatus* the callow workers that perform tasks inside the nest have a lower mobility than the foragers that forage for food outside the nest, which is a similar division of labor as there is in hives of *A. mellifera*. The callow workers have, however, a higher expression of *PKG* than the foragers.

It is expected that the *PKG* gene is also involved in the differences in mobility in *C. rubecula* and *C. glomerata*. The correlation between *PKG* expression and mobility could, however, be either positive or negative.

1.4 Hypothesis

It is clear that the mobility patterns are different between the larvae of the closely related *Cotesia* parasitoids. The mobility of the solitary larvae of *C. rubecula* is significantly higher than that of *C. glomerata* larvae on the first day after hatching of the larvae, but the mobility of the larvae of *C. rubecula* decreases significantly during the following three days, while the mobility of the gregarious larvae of *C. glomerata* remains constant. These differences in mobility were likely to be involved in the evolution of gregariousness. Several candidate genes have been proposed to be involved in these mobility patterns, such as the *PKG* gene (Osborne et al, 1997; Ben-Shahar et al; 2002, Fujiwara et al, 2002; Ingram et al, 2005), the *npr-1* gene (de Bono & Bargmann, 1998) and the *malvolio* gene (Ben-Shahar et al, 2004). The gene that encodes the enzyme PKG has, so far, been investigated most thoroughly in relation to mobility and food-related behaviour and it has been found to be involved in behaviour of fruit flies, nematodes, honey bees and ants. This makes the *PKG* gene a likely candidate to be involved in mobility of *Cotesia* larvae as well. I will therefore focus on this gene by comparing the expression of the *PKG* gene in larvae of *C. glomerata* and *C. rubecula*. I will focus on the following hypothesis:

The *PKG* gene is involved in mobility of larvae of the solitary parasitoid wasp *C. rubecula* and the gregarious parasitoid wasp *C. glomerata*. The correlation between the expression of the *PKG* gene and the mobility of the larvae can either be positive or negative.

2. Materials and methods

In order to measure the expression of the *PKG* gene of the parasitoid wasps, the sequence of the complete *PKG* gene has to be determined first. I started my research therefore with sequencing the *PKG* gene. After approximately 3 months of sequencing I started with experiments to search for possible isoforms of the *PKG* gene and experiments to compare the expression of the *PKG* gene between *C. glomerata* and *C. rubecula*.

2.1 General precautions against cross contamination

When doing molecular experiments in a laboratory, it is very important to avoid contaminations. Only small samples of DNA or RNA are used and even minor contaminations with other genetic material could alter the results significantly. This risk is especially high, when genetic material is amplified using the Polymerase Chain Reaction (PCR). Therefore, several precautionary measures have to be taken to work as sterile as possible. These include of course the use of gloves and sterile eppendorf tubes, but also barriers pipet tips have been used a lot. Barrier pipet tips are sterile, RNAse free tips, which have a barrier in the tip to avoid contamination with aerosols from within the pipet. I have used these barrier tips extensively during my experiments: during the sequencing procedure and the search for isoforms and during the RT-qPCR experiments. For sequencing it is important to avoid any contamination, otherwise you might obtain sequences from another organism.

Many different protocols are used in the several experimental procedures. Most protocols were performed according to the instructions of the manufacturer, but a number of protocols were altered in order to have better results. Procedures that are not performed according to instruction of the manufacturer, but according to standard protocols of the laboratory of Entomology, are described in the appendices.

2.2 Sequencing procedure

Several steps can be distinguished when sequencing a gene. The following paragraphs describe these steps of sequencing of the *PKG* gene of *C. glomerata* and *C. rubecula*. Also the sequencing of part of the *18S* gene is described. The *18S* gene encodes a ribosomal RNA and

it is used in the RT-qPCR experiments in order to normalize the results from different RNA samples.

RNA was isolated from adult *Cotesia* wasps and reverse transcribed to cDNA, which can then be used as template in a PCR. A small piece of the *PKG* gene is amplified with PCR using specific primers designed using known sequences form other species. After sequencing of this first piece of *PKG*, gene specific primers can be designed that can be used to amplify the 3' and the 5' ends of the *PKG* gene. The sequence of the entire *PKG* gene can be obtained in this way. How this sequencing process is done exactly is described in the following paragraphs.

2.2.1. Preparing template for the sequencing process

RNA was isolated from the thoraxes of adult *Cotesia* wasps. Both male and female wasps were used up to an age of 1 week. Thoraxes were collected in RNAwiz/TRI reagent (Ambion, Austin, USA), which is a phenol-based solution for the isolation of RNA. The standard protocol of the laboratory of Entomology for RNA isolation was used. This protocol is an adjusted version of the protocol that is suggested by the manufacturer of RNAwiz (see appendix 1). Purified RNA was stored in RNAse free water at –80^{o}C.

RNA was then reverse transcribed into cDNA. This was done using the SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech, Palo Alto, USA). This kit uses Powerscript reverse transcriptase for the reverse transcription of mRNA. Only mRNA is then reverse transcribed into cDNA (see appendix 2).

2.2.2. The first step of sequencing the PKG *gene: the CODEHOP method.*

Before the *PKG* gene can actually be sequenced, it has to be specifically amplified by PCR from cDNA. For this purpose, specific primers are needed that anneal to the *PKG* cDNA. These primers were designed using the sequences of PKG proteins of related species. The sequences of *Apis mellifera* and *Drosophila melanogaster* were used for this purpose. The reason that amino acid sequences are used and not nucleotide sequences, is that several amino acids are encoded by multiple codons. A mutation in such a codon may thus still result in the same amino acid. There is therefore no selection against these mutations, because the composition and the activity of the protein are not changed. This results in high levels of variation in the nucleotide sequences, which are not present at the protein level.

Once PKG sequences have been aligned, conserved regions are located within the protein. The next step is designing PCR primers at these conserved regions that were found after aligning the PKG protein of *A. mellifera* and *D. melanogaster*. The selected conserved amino acid sequence first has to be retrotranslated to the nucleotide sequence for this purpose. Since many amino acids are encoded by more than one codon, the result is a set of several different possible nucleotide sequences that encode this amino acid sequence. This set of sequences is called a degenerate sequence combination, and primers derived from such sequences are called degenerate primers. A too large number of different sequences results in poor PCR amplification, therefore a special procedure was used to minimize the number of degenerations. It is very important that the 3' end of the primer matches the sequence of *Cotesia* exactly, because otherwise the primer cannot be extended by the DNA polymerase in the PCR. Mismatches at the 5'end of the primer are less important for the proper functioning of the primer, because this end in not to be extended by DNA polymerase; it merely adds to the specificity of primer binding. This knowledge is used to create primers that have a low level of degeneration. Special primers are therefore designed: consensus-degenerate hybrid oligonucleotide primers (CODEHOP). A CODEHOP primer has a 3' degenerate core with every possible codon combination in a conserved amino acid region of 3 to 4 amino acids, therefore this part will fit 100% to the target template cDNA.. Furthermore it has a 5' consensus clamp derived from the most probable sequence for stabilizing the annealing process of the primer. This results in a mixture of primers with 3' degenerate cores and 5', nondegenerate, consensus clamps. CODEHOP primers have been used successfully for PCR amplification of several distantly related gene sequence (Rose et al, 2003).

Some effort had already been put in sequencing the *PKG* gene of *C. glomerata*. CODEHOP primers were designed by Arjan Bergsma (2005) for obtaining the *PKG* sequence of *C. glomerata*. He succeeded in amplifying a gene product using CODEHOP primers in a PCR and the product was sequenced. The sequence indeed was part of the *PKG* gene, considering the high resemblance of the sequence with the *PKG* gene sequence of *Apis mellifera*. My research started after this step, by using this *PKG* sequence to design gene specific primers for amplification of 5' and 3' ends of the *PKG* gene.

2.2.3. Amplifying the 5' and 3' ends of the PKG *gene*

After obtaining a small piece of sequence of the *PKG* gene, it is necessary to amplify the ends of the gene at the 3' and 5' side in order to sequence these parts of the gene as well. For this

procedure it is important to use cDNA with a known sequence at the 3' or 5' ends, and this was produced using the SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech, Palo Alto, USA). It is then possible to use a universal primer that will anneal to this know sequence in a PCR reaction.

The 3' end of mRNA has a poly-A tail, which can be used to add a known sequence to the 3' end of the mRNA. The 3' end of the *PKG* gene can then be amplified in a 3' RACE PCR, by using a primer that will anneal to the known sequence at the 3'-end and a primer that specifically binds to the *PKG* gene. The 5' end of mRNA does not have such a poly-A tail or another common sequence and it is therefore needed to first add such a tail to the 5'-end of the mRNA before it is possible to add the known sequence. Powerscript reverse transcriptase, which is used in the SMART PCR cDNA Synthesis Kit, causes such a 5' overhang of poly-C. An oligonucleotide with a poly G at its 3' end, followed by of a known sequence anneals to this overhang and a special template switching mechanism finally leads to the reverse transcription of the added oligonucleotide (Nucleotide Switching Mechanism At 5' end of RNA Transcript, SMART) (see figure 5). The 5' end of the *PKG* gene can then be amplified in a 5' RACE PCR, by using a primer that will anneal to the known sequence at the 5'-end and a primer that specifically binds to the *PKG* gene.

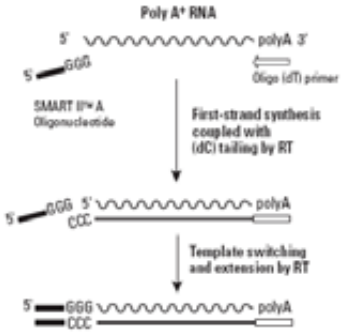


Figure 5: Mechanism of BD SMART cDNA synthesis. From: User Manual of BD SMART RACE cDNA Amplification Kit (www.clontech.com)

Using gene specific primers that are designed to anneal to the sequenced part of the PKG, it is then possible to amplify the 5' or the 3' ends of the mRNA in a PCR (rapid amplicifation of cDNA ends, RACE) (see figure 6).

Three gene specific primers were designed for the 5'RACE and also three gene specific primers were designed for the 3'RACE of *Cotesia*. The sequences of these primers are shown in table 1. I started my thesis with this procedure. I first focused on sequencing the *PKG* gene of *C. glomerata* and later I also focused on sequencing the *PKG* gene of *C. rubecula*.

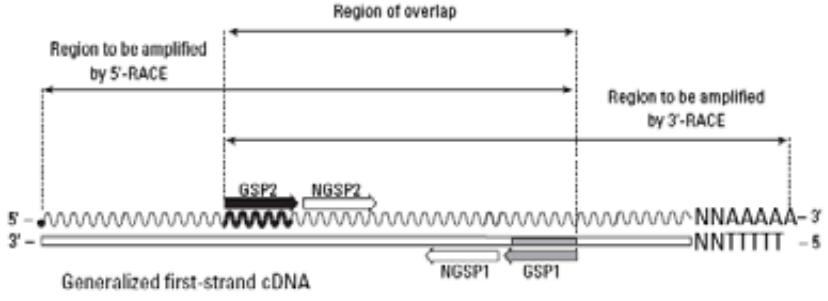


Figure 6: Location of the gene specific primers in relation to the cDNA template. From: User Manual of BD SMART RACE cDNA Amplification Kit (www.clontech.com)

After some testing of different PCR-protocols, it was found that two successive PCR-reactions

name	sequence of primer
3'RACE primers (forward primers)	
3'1	5' GTAGAGACTCGTCAGCAGCAGCACATAAT 3'
3'2	5' ACCGGATGTGTCGTTGAAGCTTTTGATTA 3'
3'3	5' TTACTTGCACTCGCGGAATATCATCTACC 3'
5'RACE primers (reverse primers)	
5'1	5' TCTACTCGACCGAATCCACCAACTCCTAAG 3'
5'2	5' TGGAGGGCAAACGAGCGGCTACTGTCACC 3'
5'3	5' TGGAGGTGCACCGGTCAATAATTCAAACAT 3'

Table 1: Sequences of the 3'RACE and 5'RACE primers. The names of the primers are the names that are written on the tubes containing the primers.

PCR-1		PCR-2	
1. 94°C	10 sec.	1. 94°C	10 sec.
2. 68°C	30 sec.	2. 70°C	30 sec.
3. 68°C	3,5 min.	3. 68°C	3,5 min.
step 1-3: 30 cycles		step 2: -0,5°C/cycle step 1-3: 20-40 cycles	
4. 68°C	6 min.	4. 68°C	6 min.

Table 2: PCR-protocol that was used for the 3' and 5' RACE of *Cotesia*. PCR-product from the first PCR was diluted 100 times and then used as template for the second PCR. Furthermore, nested primers were used in the second PCR.

(nested PCR) yielded the best results. The exact PCR-protocol is shown in table 2. The PCR-product from the first PCR was diluted 100 times in RNAse-free water and then used as template for the second PCR. Nested primers were used in this second PCR in order to diminish the number of products that result from non-specific binding of the primers. In the 3'RACE, primers 3'1 or 3'2 were used in the first PCR and in the second PCR primers 3'2 or 3'3 were used as nested primers. In the 5'RACE, primers 5'3 or 5'2 were used in the first PCR, whereas primers 5'2 or 5'1 were used as nested primers. Furthermore, a touchdown protocol was used in this second PCR. In a touchdown PCR, the annealing temperature is high in the first cycles of the PCR, but it is gradually lowered in successive PCR cycles. At a highest temperature the primer will be able to anneal only to sequences that are exactly similar to the sequence of the primer. Sequences that are highly similar to the primer can therefore be amplified earlier in the PCR than sequences that have some mismatches, which will result in a higher amount of product at the end of the PCR.

A special taq polymerase was used in these PCR-reactions: Advantage 2 polymerase (BD Biosciences Clontech, Palo Alto, USA). This polymerase is well suited for amplifying large fragments of cDNA and it has a low error rate, which makes it very suitable for sequencing purposes.

2.2.4. Preparing the amplified PCR-products for sequencing

The amplified PCR-products that resulted for the 3' and 5'RACE were separated using agarose gel electrophoresis. The different PCR-products are visible on the gel as distinct bands of different sizes using a camera with UV-light. You can than see bands of products, which have different sizes. The bands of interest were cut out of the gel and the PCR-products were extracted from the gel using the Qiaex Gel Extraction Kit (Qiagen Benelux B.V., Venlo,

The Netherlands). This kit uses beads that will bind the PCR-products in order to isolate the products from the gel. This extraction was done according to instructions of the manufacturer.

The amount of cDNA that results from the extraction is too low for sequencing. The PCR-fragments therefore have to be multiplied first. The general procedure for this is shown in figure 7. In order to increase the number of product, the extracted PCR-products were first ligated into a pGEM-T easy vector (Promega Corporation, Madison, USA). This results in a vector that includes the sequence of the PCR-product. This vector, which hopefully has the desired PCR-fragment ligated into it, is then used in the transformation of competent *E. coli* cells. This means that the *E. coli* cells are genetically altered by the uptake and expression of foreign DNA, in this case the vector and the *PKG* fragment. The *E. coli* cells are transformed by heat shocking them and then grown on agar plates. Only the transformed E. coli cells will be able to grow on agar plates that contain the antibiotic ampicillin, since only the vector has a ampicillin-resistance gene. It is very easy to distinguish colonies that contain a plasmid with a PCR-fragment inserted on the agar plates, because the fragment inactivated a coding region for β-galactosidase when it was ligated into the vector. Colonies that contain a PCR-fragment will thus be white, while colonies that contain an 'empty' plasmid are blue.

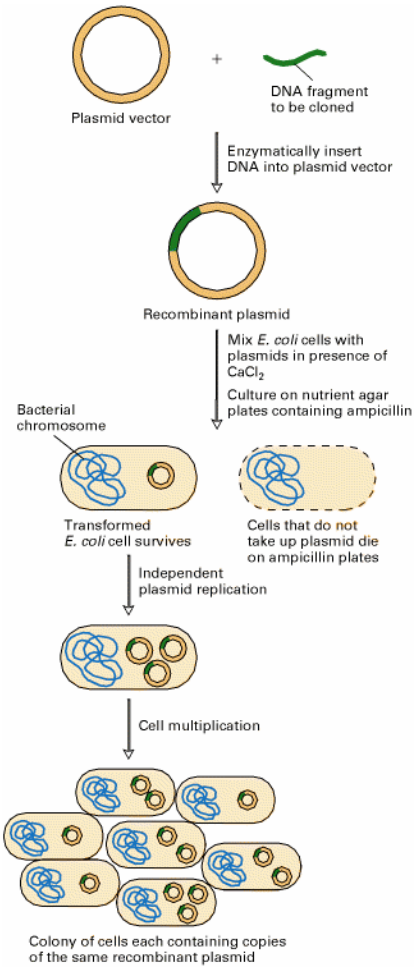


Figure 7: General overview of the ligation of a DNA fragment into a vector and of the transformation of E. coli cells. From: tainano.com.

White colonies that contain the PCR-fragment are transferred to liquid medium in which they can grow overnight. This also means that the plasmid containing the PCR-fragment is multiplied. The colonies were grown in 3,5 ml of liquid LB-medium with ampicillin added in plastic tubes. In order to sent the fragments to the sequencing company, it is then needed to purify the plasmid DNA from the *E. coli* cells, using the Qiaprep Spin Miniprep kit (Qiagen Benelux B.V., Venlo, The Netherlands). Two adjustments were made to the protocol: step 7 (washing with buffer PB) was not done, step 10 was adjusted (40 µl of 5 times diluted EB buffer was used for elution for 30 minutes).

After this plasmid isolation it is only needed to check whether the plasmid contained the right insert. The pGEM-T easy vector has two EcoRI restriction sites, flanking the inserted

fragment. EcoRI restriction enzymes were therefore used to cut the fragment from the plasmid and the size of the fragment was visualized using gel electrophoresis. If the size of the fragment is as expected, it can finally be sent out for sequencing.

2.2.5. Sequencing the housekeeping gene 18S

In parallel with sequencing of the *PKG* gene, a housekeeping gene had to be sequenced as well. A housekeeping gene is a gene that is constitutively expressed in tissues that will be analysed later on for *PKG* expression. Such a gene can encode for example a ribosomal RNA. The gene encoding 18S rRNA was chosen for this purpose. The expression of 18S has to be measured later in the experiments on PKG expression in order to normalize the results. The sequence of 18S of many species was already known and it was easy to design primers targeting a conserved region of the gene. It was not possible to use the CODEHOP method for designing primers, because a ribosomal RNA is not transcribed into a protein. Two sets of degenerate primers were therefore designed targeting the 18S region.

The first set of primers was designed to amplify a fragment of 1500 bp, while the second set of primers only amplified a fragment of 400 bp. The sequences of these two primer sets are shown in table 3. cDNA was diluted 100 times in RNAse free water, before using it for PCR, because 18S rRNA is very abundant and too large amounts of template will inhibit the PCR. The PCR-protocol that was used was actually a protocol that was designed for the CODEHOP-primers (see table 4), but it also worked properly for the 18S primers. Primer set 1 (1500 bp) did not result in any bands after visualizing the PCR products using gel electrophoresis, but primer set 2 (400 bp) did show a band of the expected size. These bands were therefore cut out of the gel and prepared for sequencing, following all the steps described in paragraph 2.2.4. This small part of the *18S* gene was sequenced of both *C. glomerata* and *C. rubecula* simultaneously and also *Pieris brassicae* and *P. rapae* were sequenced.

PCR-protocol	
1. 94°C	2 min.
2. 94°C	30 sec.
3. 50°C	30 sec.
4. 72°C	2 min.
step 2-4: 40 cycles	
5. 72°C	7 min.

Table 4: PCR-protocol that was used to amplify 18S. The protocol was initially designed for use with CODEHOP-primers.

name	size of fragment	Sequence of primer
18S 1	1500 bp	Forward: 5' TGGTTGATCCTGCCAGTAG 3'
		Reverse: 5' CTTCYGCAGGTTCACCTAG 3'
18S 2	400 bp	Forward: 5' CAGCCGCGGTAATTCCAGC 3'
		Reverse: 5' CRTHYTYGGCAAATGCTTTCGC 3'

Table 3: Sequences of the 18S primers.

2.3 Looking for different isoforms of *PKG*

Isoforms are different forms of mRNA and protein that result from the same gene. A gene usually consist of several introns and exons. The exons encode the sequence of the mRNA and the protein, but they are interrupted by introns, which are non-coding sequences. When the gene is translated to mRNA only the exons are translated. Alternative splicing can, however, occur, which will then cause the exons to be spliced in different arrangements. This can result in several structurally and functionally distinct variants of mRNA and protein (Lee & Irizarry, 2003; Blencowe, 2006). Alternative splicing is a common process, it occurs for example in 40 to 60% of all human genes. The majority of these genes were found to be involved in transmission or regulation of signals. Especially genes involved in the nervous system and the immune system appear to have a lot of alternative splicing (Lee & Irizarry, 2003). Because differentially spliced isoforms can have a different functions, it is very important to investigate whether the *PKG* gene also has different isoforms. It might be that the expression of only one isoform differs between *C. glomerata* and *C. rubecula* and then it would be best to focus on this isoform alone instead of focusing on all PKG isoforms.

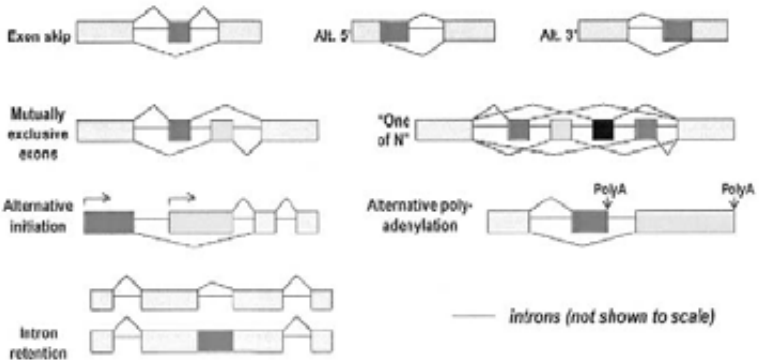


Figure 8: Overview of several types of alternative splicing. The regions that are added or removed by alternative splicing are shaded (more or less, resp.). From: Lee & Irizarry, 2003

Several types of alternative splicing can occur (figure 8). The first type involves cassette-type alternative exons, which accounts for at least one third of all alternative splicing events. This means that an exon can either be skipped or included in an isoform. This type of alternative splicing will result in a group of isoforms that result from a different set of exons. The second type involves alternative 5' or 3' splicing, accounting for at least a quarter of all alternative splicing events. An exon can then be either lengthened or shortened, again resulting in different isoforms. Another important type of alternative splicing is alternative initiation or alternative poly-adenylation in which isoforms differ at their 5' or 3' end. Beside these three important types of alternative splicing, there are also some other types, but these are quite rare and I will therefore not describe them here (Lee & Irizarry, 2003; Blencowe, 2006).

PCR-1		PCR-2	
1. 94°C	10 sec.	1. 94°C	10 sec.
2. 63°C	30 sec.	2. 65°C	30 sec.
3. 68°C	4 min.	3. 68°C	4 min.
step 1-3: 30 cycles		step 2: -0,5°C per step 1-3: 10 cycles	
3. 68°C	6 min.	4. 94°C	10 sec.
		5. 60°C	30 sec.
		6. 68°C	4 min.
		step 4-6: 25 cycles	
		68°C	6 min.

Table 6: PCR-protocol that was used for PCR with flank primers. PCR-product from the first PCR was diluted 100 times and then used as template for the second PCR. Furthermore, nested primers were used in the second PCR.

The 5'RACE and 3'RACE experiments might already reveal some isoforms of PKG. Using the CODEHOP-primers, the sequence of a conserved part of the *PKG* gene was discovered and the primers for the RACE experiments were designed using this sequence. By amplifying the 5' ends of *PKG*, isoforms might be found with an alternative initiation site. Amplification of the 3' ends of *PKG*, might reveal isoforms with alternative poly-adenylation. A problem of this method is that you only find either the 3' or the 5' sides of these isoforms, because not the entire *PKG* gene is amplified in a RACE-PCR. In order to obtain the sequence of the entire gene and in order to further investigate possible isoforms, primers were designed that are located on the flanks of the PKG gene (table 5). These primers were located outside the open reading frame (ORF), which has a size of approximately 2 kb. By locating the primers just outside the ORF, all isoforms with the same sequence at their 5'side and at their 3'side can be found. Any isoforms that are found by using these primers are likely to differ in their length and exon composition.

name	sequence of primer
Forward flank primers (5' end)	
CPKG_forw1	5' GGCTGTTCCCGTTCCTGGTC 3'
CPKG_forw2	5' CGGCGACCACTTGCAGCG 3'
CPKG_forw3	5' CAACAACTCTACCTTGACTCCTGC 3'
Reverse flank primers (3' end)	
CPKG_rev1	5' GCACAATAATAAATCCCTAGTGGCC 3'
CPKG_rev2	5' CAATAGAGCGATTGGAGACCAAC 3'
CPKG_rev3	5' CATTGTCCCAGCCAGAGACGTC 3'

Table 5: Sequences of the flank primers. These primers are located just outside the large ORF of the PKG gene.

It was again chosen to use two successive PCR-reactions (nested), because very large fragments had to be amplified, even up to 2 kb or more. The used PCR-protocol is shown in table 6. The PCR-product from the first PCR was diluted again 100 times in RNAse-free water and then used as template for the second PCR, which had a touchdown protocol. Nested primers were used in this second PCR in order to diminish the number of products that result from a-specific binding of the primers. Primer set 3 was nested to both primer sets 1 and 2. Primer set 2 was only nested to primer set 1. Again the Advantage 2 polymerase was used in these PCR-reactions.

PCR-program: M13	
1. 95°C	3 min.
2. 95°C	30 sec.
3. 55°C	30 sec.
4. 72°C	4 min.
step 2-4: 35 cycles	
5. 72°C	5 min.

Table 7: PCR-protocol for the colony-PCR using M13 primers.

After amplification of fragments with the flank primers, the bands that were visible were cut out of the gel and prepared for sequencing, following all the steps described in paragraph

2.2.4. Unfortunately, it appeared to be very difficult to ligate large fragments of 2 kb into the pGEM-T easy vector and to transform the competent E. coli cells with these large plasmids. It was therefore needed to screen hundreds of white colonies, in order to find a colony in which a large fragment had been inserted. This took a lot of time, because all the colonies had to be mini-prepped first, before the size of the insert could be determined. Therefore it was only possible to screen around 30 colonies a day.

It was therefore chosen to screen many colonies first, before preparing the samples for mini-prep. This was done by transferring cells of 96 white colonies on a 96 wells plate. Each well contained 200 µl of LB-freeze medium to which ampicillin had been added. The cells were then grown at 37^{o}C for eight hours and 1 µl of medium containing the cells was then used directly as template in a PCR. For this colony-PCR, M13 primers were used, that are located next to the insert of the pGEM-T easy vector. Furthermore, a cheaper polymerase was used, because of the large amount of samples: GoTaq (Promega, Madison, USA). GoTaq is less accurate than Advantage 2 polymerase and it has more trouble amplifying large fragment, but it was used anyway since the purpose of the colony-PCR was only to screen the colonies. If colonies are found that have the right insert, these can be prepared for mini-prep and the fragment can subsequently be sent away for sequencing.

2.4 Performing blots in order to gain more information on the *PKG* gene

The principle of blotting is that a sample of total DNA, RNA or protein is separated using gel electrophoresis. After separating the fragments according to size, an image of the gel is made by transferring the fragments to a membrane. A gene-specific, labelled probe can be bound to the gene or protein of interest and this probe can then be visualized.

A Southern blot is done using DNA. With such a Southern blot it van be investigated whether there is only one copy of the *PKG* gene or perhaps more copies. A Northern blot is done using RNA and it was performed in order to gain additional information on isoforms of *PKG* and on expression of *PKG*. The procedures of these Northern and Southern blot are described in the paragraphs below.

2.4.1 Making probes for blotting

Before the blotting can begin, first a probe has to be made. For both the Southern and the Northern blot the same probe was used. This probe was made by amplifying a small fragment of cDNA using PCR. Two sets of primers were designed, which could be used to make probes for blotting and which also could be used in the RT-qPCR experiments (see paragraph 2.5). Both for blotting as for RT-qPCR, is it optimal to use primers that amplify approximately 200 to 300 bp. Both sets of primers amplify a fragment of the *PKG* gene that is located at a domain that was identified as the enzymatic kinase domain of the *PKG* gene. This is an important domain of the gene, as PKG is an enzyme and it is therefore expected to be present in all isoforms of the gene. The probes that are made with these primers are thus expected to detect all isoforms.

Name	Size of fragment	Sequence of primer
ExpCot1	243 bp	forward: 5' CGGCACGCCGGAGTACGTTG 3'
		reverse: 5' GCAGGGTTATCGCGGCAGAG 3'
ExpCot2	334 bp	forward: 5' GCCGATTATTGGTCACTGGGTGTTC 3'
		reverse: 5' CGAAGTTAGCGGTGTCAGTGGC 3'

Table 8: Sequences of the primers that are used to make probes and for use in qRT-PCR experiments.

probe-PCR	
1. 94°C	15 sec.
2. 65°C	30 sec.
3. 68°C	1,5 min.
step 2: -1°C per cycle step 1-3: 10 cycles	
4. 94°C	15 sec.
5. 55°C	30 sec.
6. 68°C	1,5 min.
step 4-6: 25 cycles	
7. 68°C	6 min.

Table 9: PCR-protocol for making probes

The primers are, as said, used in a PCR in order to amplify a small fragment of the *PKG* gene. During this PCR the fragments are labeled with DIG-11-dUTP (Roche Diagnostics, Mannheim, Germany), which can partly replace dTTP. For this purpose a dNTP mix is made with normal concentrations of dATP, dGTP and dCTP, but with only 75% or 50% of the normal concentration of dTTP. This lower amount of dTTP is compensated by adding DIG-11-dUTP, which will then be incorporated into the amplified fragments instead of dTTP. The amplified fragments can than be used as probes in a blotting procedure. The probes hybridize to DNA of RNA with the same sequence as the probe. After the hybridization anti-DIG-AP is added to the blot, which will bind to the DIG-11-dUTP. The probes can then be detected with CSPD, which will generate a chemiluminescent signal that can be detected with a camera.

After some testing of different PCR-protocols, templates and PCR mixes (see appendix 4), it was found that it is best to use plasmid DNA as a template rather than cDNA. Therefore material was used that was obtained after mini-prep. Furthermore GoTaq was used as polymerase and a 17% higher amount of $MgCl_2$ (3,5 µl instead of 3 µl as was used in the original protocol) was added to the PCR mix. A touchdown-protocol (see table 9) was then used in the PCR to make the probe.

2.4.2 Performing the Southern blot

The purpose of the Southern blot is to investigate whether there is only one or several copies of the *PKG* gene present in *C. glomerata* and *C. rubecula*. A Southern blot is done using DNA of *C. glomerata* and *C. rubecula*. Genomic DNA was isolated from complete adult wasps using the Genomic DNA Purification Kit (Gentra Systems, Minneapolis, USA). The DNA isolation was done according to the mouse tail protocol, provided by the manufacturer. Approximately 30 µg of DNA was isolated from around 30 *C. glomerata* or 25 *C. rubecula* wasps using this kit. If the DNA was not concentrated enough, it was concentrated using the Speed Vac Plus SC110A.

The first step of a Southern blot is cutting the genomic DNA using restriction enzymes to generate fragments of DNA. If there are more than one copies of the *PKG* gene present in *Cotesia*, they will be present on different locations in the genome, on the same chromosome or on different chromosomes. These copies will be present on different DNA fragments after restriction enzyme digestion and these fragments are likely to have a different size. This will then be visible on the blot as two bands. Therefore several samples of the *Cotesia* DNA are cut with different restriction enzymes and if there are indeed more than one copies of the *PKG* gene, this will then be visible on the blot. Three different restriction enzymes were used: EcoRI, EcoRV and XbaI. None of these three restriction enzymes has a restriction site that is located within the sequence of the probe. Beside samples for each of the restriction enzymes, also a sample to which no restriction enzymes were added and a positive control were made. The positive control was plasmid DNA containing a *PKG* fragment and it was cut with EcoRI, in order to cut the PKG fragment from the vector.

After an hour, the restriction reaction was stopped and the DNA fragments then are separated on a 0,6% agarose gel by gel electrophoresis. The gel is then denatured and neutralized in order to make the DNA single stranded and after that a nylon blot is placed on the gel. By absorption of buffer, the DNA fragments are transferred to the blot overnight. The next morning, the DNA can than be bound to the blot by UV-crosslinking (UV Stratalinker 2400, 120000 µ joules).

The probe can now be hybridized to the blot. The probe has to be boiled in order to make it single stranded and then it is hybridized to the blot in Church buffer (also see appendix 5) overnight. After this hybridization, the blot first has to be washed in order to remove any probe that has hybridized a-specifically to the blot. Next is the blocking of unhybridized

area's of the blot. This is important, because otherwise the anti-DIG-AP will also bind to these area's and this will then result in a lot of background noise. The probes, to which anti-DIG-AP will be bound can now be detected with CSPD, which will generate a chemiluminescent signal that can be detected with a camera.

When doing a Southern blot, there are several variables that can be varied in order to have a good result. These are the amount of DNA, the amount of probe and the hybridization temperature and buffer. The protocol that was used for the Southern blots is the standard protocol of the laboratory of Entomology (appendix 5).

2.4.3 Performing the Northern blot

The procedure of the Northern blot is very much the same as the procedure for the Southern blot. The main difference is that RNA is used as template instead of DNA. The purpose of the Northern blot is to look at differences in expression between the two *Cotesia* species or between different isoforms of PKG. Furthermore it is possible to see whether there are isoforms on a Northern blot. This is because the probe will bind all RNA that has a similar sequence as the probe. When doing a Northern blot it is important to work clean and carefully, because the RNA can easily be degraded by RNAse.

The RNA that was used in the Northern blot, had to be quite concentrated and therefore a lot of RNA had to be collected. Larvae were collected for this purpose, which were 4 days old. RNA was isolated from these larvae following the standard protocol of the laboratory of Entomology. The concentration of the RNA was measured and exactly 10 μg of RNA of both *C. glomerata* and *C. rubecula* was used in the blotting procedure. PCR-product that resulted from the control sample of the probe-PCR was used as a positive control. This PCR-product had to be boiled first in order to make it single stranded.

The RNA and the positive controls were loaded on an agarose/formaldehyde gel and after running it, a nylon blot was placed on the gel. By absorption of buffer, the RNA fragments are transferred to the blot overnight. The next morning, the RNA has to be bound to the blot by UV-crosslinking. The hybridization, the washing and the detection of a Northern blot are very similar as in a Southern blot. An important difference is, however, that the probe is hybridized in ULTRAhyb (Ambion, Austin, USA), which is a buffer that results in a much more efficient hybridization. The hybridization temperature for this buffer is 42^{o}C. At the end of the blotting procedure, CSPD will generate a chemiluminescent signal that can be detected with a camera, similar to the signal of the Southern blot.

When doing a Northern blot, there are again several variables that can be varied in order to have a good result. The protocol that was used for the Northern blots is the standard protocol of the laboratory of Entomology (appendix 6).

2.5 Measuring *PKG* expression in *C. glomerata* and *C. rubecula* larvae

One of the most interesting parts of this thesis is comparing the PKG expression between larvae of *C. glomerata* and *C. rubecula*. That is after all main question of this research.

2.5.1 Collecting larvae

In order to investigate the correlation between larval mobility and *PKG* expression, first of all larvae of *C. glomerata* and *C. rubecula* have to be collected. For this purpose, caterpillars were parasitized by adult *Cotesia* females. *C. glomerata* wasps were offered caterpillars of *P. brassicae* for parasitizing and *C. rubecula* wasps were offered caterpillars of *P. rapae* for parasitizing. These caterpillars were offered on leaves of Brussels sprout plants (Brassica oleracea L. var. gemmifera cv. Cyrus), on which they had hatched approximately half a day or a day earlier. After a few hours of parasitizing, the wasps were removed from the leaves and the caterpillars were transferred to a new Brussels sprout plant. The plants with the parasitized caterpillars were then placed in a climate cabinet, with a constant temperature of 21^{o}C and a 16 hours light, 8 hours darkness regime. Five days after the oviposition, the larvae hatch from their eggs and then they can be collected.

Initially, only larvae of one day old (day 5 after oviposition) were collected, in order to investigate whether there was a difference in *PKG* expression on that day. The mobility differs significantly on this day between *C. glomerata* larvae and *C. rubecula* larvae. Entire larvae extracts were used for this purpose. It would be more relevant to only analyze the brains of the larvae, but these are very small and rather difficult to collect. Therefore it was decided to first test the entire larvae in order to see if it was possible to find significant differences in this way. The larvae were collected by opening caterpillars in Ringer solution and releasing the larvae in the solution. RNA was isolated from the larvae according to the standard protocol. In total 5 samples of larvae of both species were collected; a sample of *C. rubecula* consisted of 5 larvae and a sample of *C. glomerata* consisted of 15 larvae collected from 5 different caterpillars. The larvae of *C. glomerata* were approximately three times smaller than the larvae of *C. rubecula* and it was therefore decided to collect three times as

many larvae of *C. glomerata* for each sample. The 5 samples resulted from 4 oviposition events.

As an extra experiment, also some brain samples of male adult wasps were collected. Male wasps of *C. glomerata* usually hatch some time before the female wasps and these males then wait with the cocoons, until the females hatch. Male wasps of *C. rubecula* hatch alone and therefore will begin searching females immediately. Brains of male wasps up to an age of one week were therefore also collected in order to measure *PKG* expression. The dissection of the brains was done in Ringer solution. Three samples of brains were collected of each species: 15 brains of *C. glomerata*, 10 brains of *C. rubecula*. RNA was isolated from these brains according to the standard protocol.

After the first experiments on *PKG* expression, it was decided to look also at *PKG* expression in the larvae in each of the first four days after hatching. This makes it possible to see the differences in *PKG* expression over time. Larvae were reared the same way as the earlier larvae. The mobility of the larvae was also observed to repeat and confirm earlier experiments from Tribuhl, 2007 with the current *Cotesia* strain, because there had been some problems with the *Cotesia* populations that might affect mobility. The number of twisting of 15 larvae per day was therefore counted during a period of 5 minutes and also the length of the larvae was measured. The larvae were released from the caterpillars in Ringer solution, the same conditions at which the mobility of the larvae was measured before. Larvae were also collected for RNA isolation following again the same procedure as the earlier larvae. In total eight samples of *C. rubecula* larvae per day were collected. These samples resulted from three oviposition events. Six samples per day of *C. glomerata* larvae per day were collected, which resulted from two oviposition events.

Because *PKG* expression of the entire larvae may differ from the *PKG* expression in the brains of the larvae, also one sample per day of larval brains was collected. The brains of the larvae were dissected for this purpose in Ringer solution. Five brains of *C. rubecula* were collected per sample and fifteen brains of *C. glomerata* were collected per sample. RNA was also isolated from these samples according to standard protocol.

After RNA had been isolated from a sample of larvae or brains, cDNA was synthesized from the RNA using the SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech, Palo Alto, USA). The cDNA was synthesized according to the protocol for synthesis of 5'RACE ready cDNA, but without the SMART Oligo added. The integrity of the cDNA was tested by performing two PCR's with flank primers (paragraph 2.3, using primersets 2 and 3). After that the cDNA was ready for use in the RT-qPCR experiments.

2.5.2 Performing RT-qPCR experiments

Real-time quantitative PCR (RT-qPCR) is a quite novel and accurate method for measuring the expression of a gene. The main principle of RT-qPCR is that fluorescent probes or dyes anneal to the template and then emit a fluorescent signal. This signal is measured by the RT-qPCR machine after each cycle and this makes it possible to see the amplification of your template over time. In the PCR mixture the template and product are initially at such low levels that renaturation does not compete with primer binding. Amplification then proceeds at an exponential rate. After a number of PCR rounds, renaturation of strands starts competing with primer binding due to the increase in copies. The reaction than enters a linear phase (see figure 5).By comparing the curves of different samples, it is possible to compare differences in expression between samples. The higher the amount of target template that is initially present, the faster the curve will reach the detection threshold and will become visible on the graph.

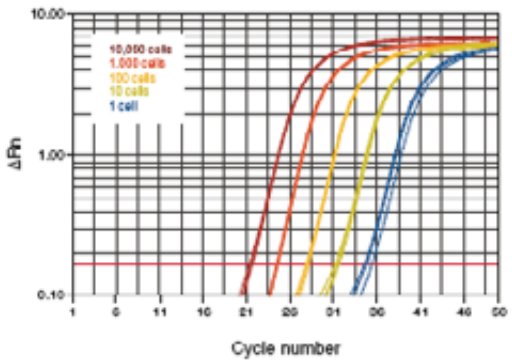


Figure 9: Graph produced during a RT-qPCR reaction. The higher the amount of cells that is initially present, the faster the curve will reach the detection threshold and will become visible on the graph. Figure taken form qPCR and RT-qPCR Brochure (www.invitrogen.com)

There are several types of fluorescent probes and dyes available for RT-qPCR. Using fluorescent dyes that generate a signal upon binding to double stranded DNA is the most simple and cheapest way for measuring expression of a gene. There are also fluorescent probes available that anneal only to specific sequences. Upon binding to this specific sequence, the probes will emit a fluorescent signal. As the target sequence increases during the PCR, more probes will be able to anneal and a larger fluorescent signal will be emitted. A probe can be designed that will only anneal to your gene. Using probes, it is possible to measure different signals from different probes in one sample and there is less chance of measuring signal from non-specifically amplified template. Probes are, however, much more expensive. It was decided to use SYBR-green, which is a fluorescent dye that will emit a fluorescent signal when binding double stranded DNA, for measuring the *PKG* expression. This was decided because SYBR-green is relatively cheap in comparison to gene-specific

probes. The SYBR-green mix contains polymerase, dye, buffer and dNTP and it is therefore only needed to add primers, cDNA and water to the SYBR-green mix (see appendix 3.)

In RT-qPCR it is needed to measure, besides measuring the expression of *PKG*, the expression of an appropriate reference gene as well. A housekeeping gene is a gene that is always expressed in a cell at a more or less constant amount. Such a gene can encode for example a ribosomal RNA. The gene encoding *18S* rRNA was chosen for this purpose. A part of this gene was sequenced in earlier experiments and a set of primers was designed to amplify a fragment of *18S* of approximately 200 bp (see table 10).

Name	Size of fragment	Sequence of primer
ExpCot1	243 bp	forward: 5' CGGCACGCCGGAGTACGTTG 3'
		reverse: 5' GCAGGGTTATCGCGGCAGAG 3'
Cot18S	234 bp	forward: 5' CGCGGTGCTCTTAATTGAGTGTCG 3'
		reverse: 5' CGCTTCTGTTCGTCTTGCGACG 3'

Table 10: Sequences of the primers that are used in RT-qPCR experiments.

The first step of the RT-qPCR experiments was testing the efficiency of the primers at different concentrations. For this purpose standards of known concentration were made using plasmid DNA which contained a fragment of *18S* or *PKG*. All three sets of primers had good efficiencies and it was decided to use primer set 1 for measuring the *PKG* expression, because the efficiency of the set of primers was slightly higher than that of set 2. After testing the primers, the RT-qPCR experiments could begin. Triplicates were tested for each sample, for both *PKG* and *18S*. The samples were tested using the Rotor Gene RT-qPCR machine (Corbett Life Science, Sydney, Australia). The first samples were tested in a Rotor Gene 3000, the last samples were tested in a Rotor Gene 6000. The PCR-protocol that was used was the same for all samples (see table 11).

qRT-PCR protocol	
1. 95°C	15 min.
2. 94°C	30 sec.
3. 63°C	30 sec.
4. 72°C	60 sec.
step 2-4: 45 cycles	
5. Melt analysis: 58°C-95°C	

Table 11: PCR-protocol for the RT-qPCR experiments

2.5.3 Analyzing RT-qPCR data

Different methods can be used to analyze and quantify the results of the reaction. The standard curve method compares the unknown concentration of the sample to a standard curve that was constructed from RNA of known concentration. It is, however, also possible to compare the C_t values of different samples, which is the number of cycles at which the fluorescent signal starts to become larger than the background signal (C_t). A relative comparison is then made between the two *Cotesia* species and between the *PKG* expression on different days instead of determining the actual amount of *PKG*. The C_t values of *PKG* have to be normalized to an appropriate endogenous housekeeping gene.

The comparative C_t method was the preferred method to use for analysing the data, because then there is no need to use standard curves in the analysis. The C_t values can, however, only be used under certain circumstances. It is very important that the primers of both the gene of interest and of the reference gene have the same efficiency. The efficiency of a primer is calculated using the results of the standard curves. It was found that the difference in efficiency between the *18S* primers and the *PKG* primers is large and there are also differences in efficiency of the *PKG* primer between *C. glomerata* and *C. rubecula*. The efficiency of the *18S* primer was found to be 93%, whereas the efficiency of the *PKG* primers was found to be 85% for *C. rubecula* and 79% for *C. glomerata*. Especially the difference in efficiency of the PKG primer between *C. glomerata* and *C. rubecula* was unexpected, since the primer targets the same sequence in both species. Because of the differences in the efficiency of the primers the C_t values could not be used.

The standard curve method therefore had to be used. In this method, the concentration of *18S* and *PKG* is determined of each sample by comparing the curve with the standard curves with a range of concentrations. For the construction of these standard curves, plasmid DNA with inserts of *PKG* and *18S* cDNA is used, of which the concentration was determined and then diluted to obtain the desired range of concentrations. The concentration of *PKG* of the sample is then normalized to the concentration of *18S* of the sample, by dividing the *PKG* concentration by the *18S* concentration. This way, the differences that are caused by a difference in initial concentration of the cDNA are erased. Furthermore, the relative expression of all samples is calculated as a percentage in comparison to the sample 1 of *C. rubecula*. All these analyses and calculations of concentration are done using the Rotor-Gene 6000 series software, version 1.7 (Corbett, Sydney, Australia).

The SPSS statistics program (version 12.0.1, SPSS Incorporation, Chicago, USA) is used to test the significance of the relative expression of *PKG* of the different samples. For comparing samples that differ in only one variable (such as comparing two different days or two species) the Mann-Whitney test was. When more variables were analysed at the same time a General Linear Model (GLM) was used.

3. Results

3.1 Sequence of the *PKG* gene of *C. glomerata* and *C. rubecula*

The first part of the sequencing process of the *PKG* gene, by using the CODEHOP primers, was already done at the beginning of my thesis. I will therefore not focus anymore on the sequence that resulted from that first part. The sequence can, however, be found in the appendix 8. Both the 3'RACE and the 5'RACE resulted in a number of sequences that closely resembled the *Amfor* gene of *A. mellifera*, but these sequences did not overlap, making it difficult to construct the sequence of the entire *PKG* gene. After performing the PCR with flank primers all sequences could be aligned into one piece and the sequence of the entire *PKG* gene was then known.

First the results of the 3'RACE and 5'RACE will be described, followed by the results from the flank-PCR. Finally the characteristics of the entire *PKG* gene will be given.

3.1.1 Sequence of the 3'RACE and 5'RACE products

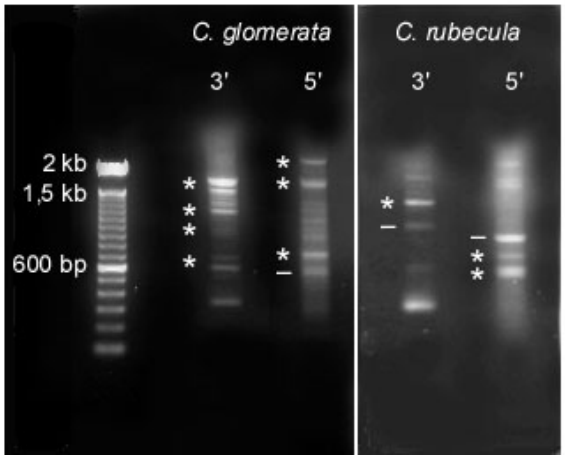


Figure 10: Picture of the 3' and 5' RACE fragments on an agarose gel. The first lane is a DNA ladder. Lane two and three are the products from the 3' and 5' RACE resp. of *C. glomerata* and lane four and five are the products from the 3' and 5' RACE resp. of *C. rubecula*. The bands that are marked with an asterisk are sequenced and were found to be *PKG*. The bands that are marked with a minus sign were also sequenced, but these bands were found to be another sequence than *PKG*.

The sequence that was obtained by the CODEHOP procedure is located in the kinase domain of the *PKG* gene. Both in *D. melanogaster* and in *A. mellifera* the kinase domain is located at the 3' end of the gene encoding PKG. The 3'RACE, as well as the 5'RACE, of both *C. glomerata* and *C. rubecula* resulted in the amplification of several products (see figure 10). As many products as possible were sequenced, but only a small number of sequences of *C. rubecula* were obtained, because the transformation of *E. coli* cells proved to be very inefficient. After sequencing the products of the 3'RACE, it was found that the fragments do not differ in any nucleotides, the only difference is in the length of the sequence. All fragments continue after the stop-codon of the ORF that was found, but the shortest fragments, however, have only a

length of around 300 bp after the stop-codon, while the longest fragments have a length of approximately 1 kb after the stop-codon (see figure 11). These different fragments might be different isoforms of *PKG* that resulted through alternative poly-adenylation. Another option is that the differences in length resulted because of some artifact of the cDNA synthesis, although this is not very likely since all the different fragments were found twice, from two different 3'RACE reactions.

The sequences of the 5'RACE fragments are, like the sequences of the 3'RACE, all identical except that they differ in the length of the sequence (see figure 12). The longest fragments have a length of approximately 1,5 kb, while the smallest fragments are only around 400 bp long. For *C. rubecula*, we only succeeded in sequencing fragments of 600 bp, due to problems with the transformation, but longer fragments were visible on gel, indicating that both *C. rubecula* and *C. glomerata* had fragments up to 1,5 kb. After analyzing the sequences for possible ORF's, it was found that there are several large ORF's that do not have a stop-codon in the 5'RACE sequences and it is expected that these ORF's continue until the stop codon that was found in the 3'RACE sequences. These ORF's are all located in the same reading frame, which could indicate that the smaller fragments of *PKG* are truncated 5' isoforms (see figure 12). It is, however, also possible that the smaller fragments resulted from degradation of RNA. This is not very likely for most of the fragments, since these different fragments were found twice. Only fragment 5PKG9 (see figure 12) might be degraded on its 5' end, because no SMART Oligo was found in the sequence of this fragment.

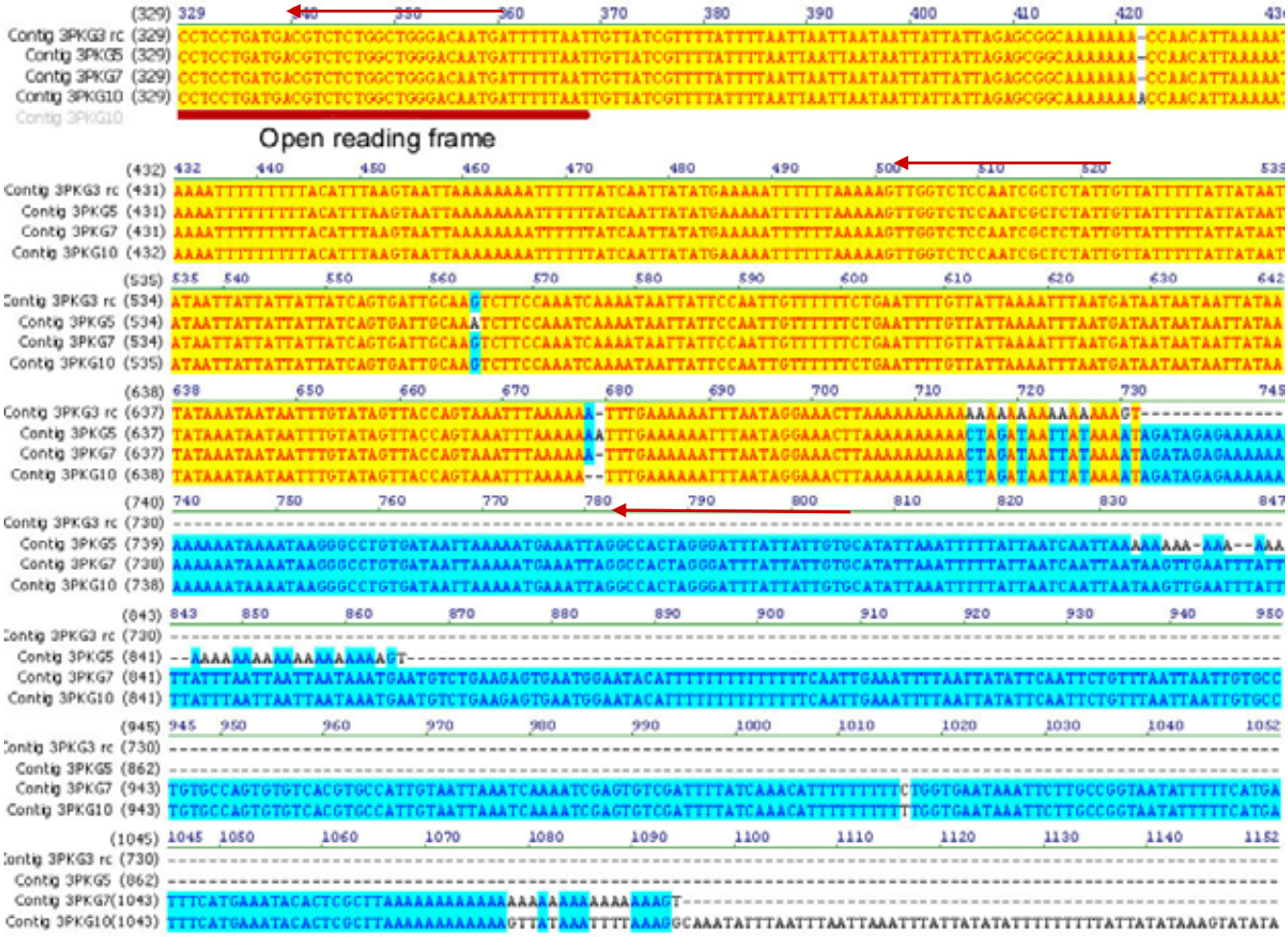
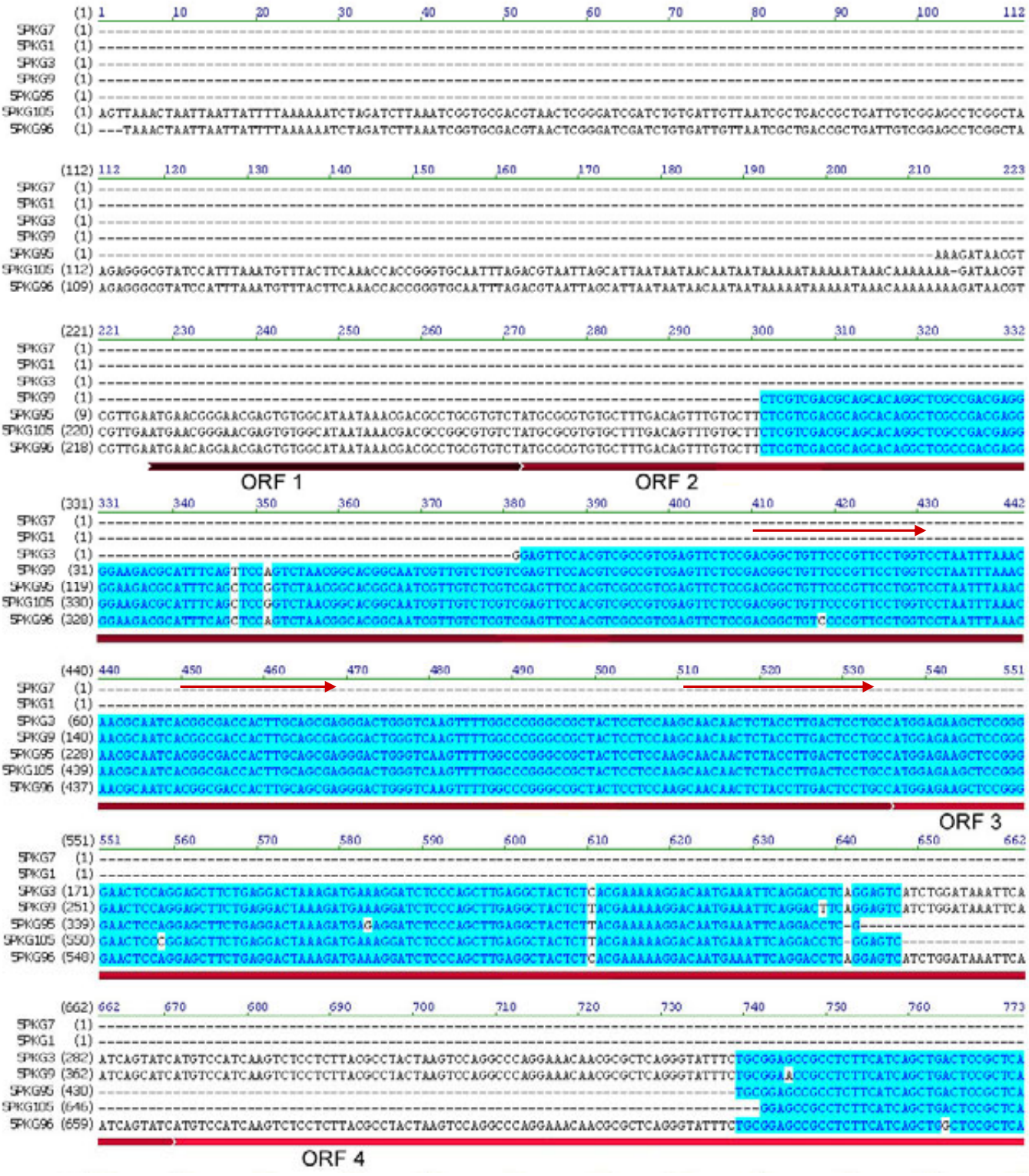


Figure 11: 3'RACE fragments of *C. glomerata* of different lengths. All fragments have the same ORF, but they differ in their length after the stop-codon. The smallest fragments have around 300 bp after the stop-codon, the longest fragments around 1 kb. The red arrows indicate the locations were the reverse flank primers anneal. The 5' end of primer 1 anneals at 805 bp, primer 2 at 523 bp and primer 3 at 358 bp.


SPKG7
SPKG1
SPKG3
SPKG9
SPKG95
SPKG105
SPKG96
ORF 1
ORF 2
ORF 3
ORF 4

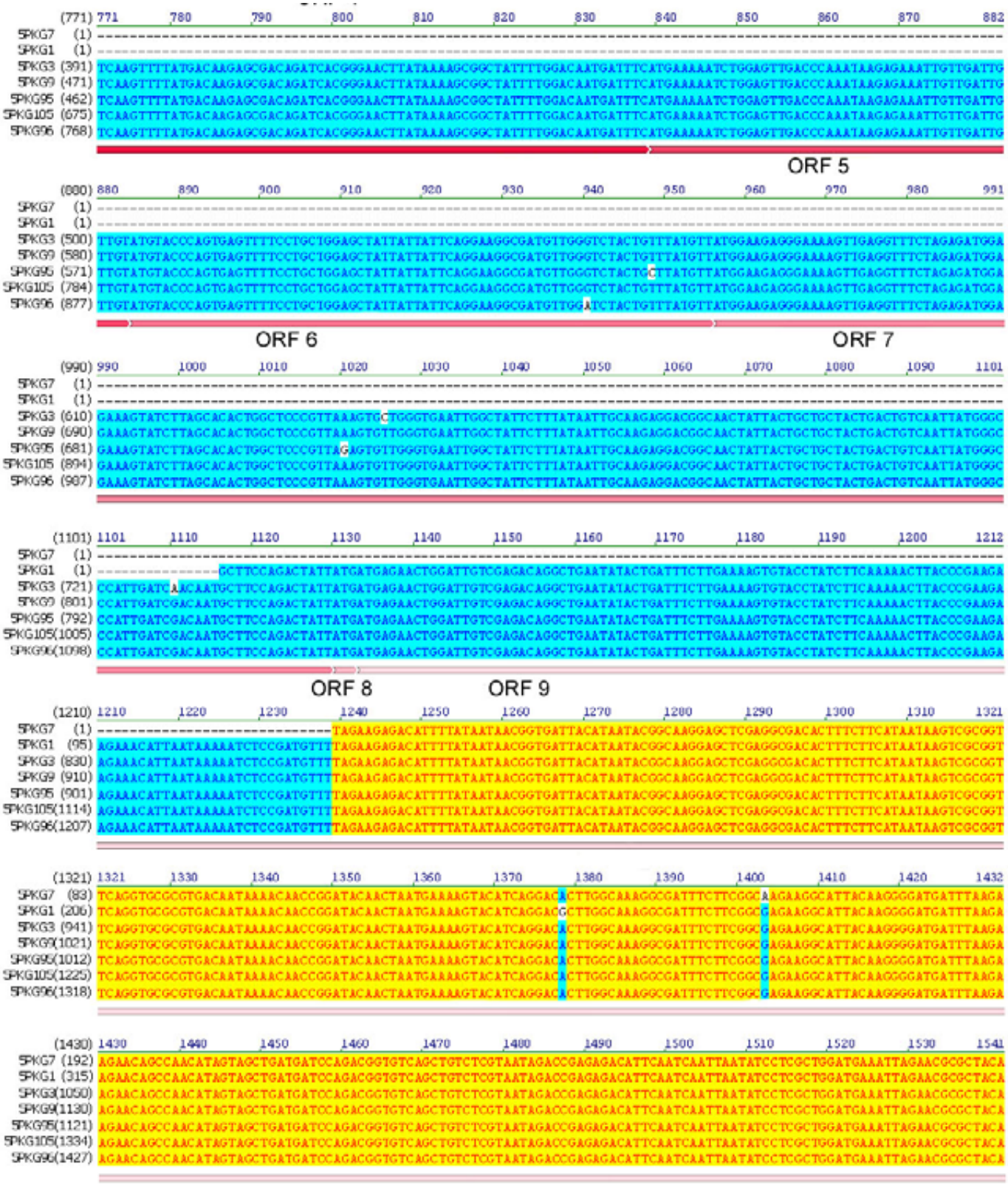


Figure 12: The sequence of the fragments from the 5'RACE of *C. glomerata*. The ORF's are indicated with the line that is beneath the sequences. Nine possible ORF's were found in total and all these ORF's are located in the same reading frame and continue until after the end of the sequence. The red arrows indicate the location were the forward flank primers anneal. The 5' end of primer 1 anneals at 510 bp, primer 2 at 450 bp and primer 3 at 411 bp.

3.1.2 Sequence of the flank-PCR products

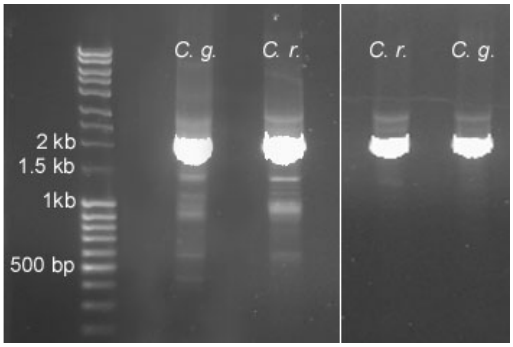


Figure 13: Picture of the flank-PCR fragments on an agarose gel. The first lane is a DNA ladders. Lane two and three are the products from flank-PCR of resp. *C. glomerata* and *C. rubecula*. Lane four and five are the products from of a later flank-PCR on cDNA from larvae of resp. *C. rubecula* and *C. glomerata*. Interestingly, in the PCR of larvae cDNA, no smaller fragment have been amplified.

The so called flank-PCR was done with the flank primers. The sense primers were designed to anneal upstream of ORF 3 of the 5' sequence (see figure 12 for the precise locations of the primers). This was done because at the time that the primers were designed this was the first and largest ORF that was found in the 5' sequence. The longer fragments were sequenced at a later moment and two more possible ORF frames (ORF 1 and ORF 2) were then found. The antisense primers are located shortly after the stop-codon (see figure 11). After the PCR, several fragments were observed on gel. Both *C. glomerata* and *C. rubecula* samples showed a large amount of product with a size of 2 kb, but also several smaller fragments could be observed (see figure 13). Major problems, however, occurred during the transformation of *E. coli* cells. The transformation was very inefficient and many colonies had to be screened in order to find a colony with the right insert. Therefore only the 2kb fragment of *C. glomerata* was sequenced, but of *C. rubecula* also a smaller fragment of approximately 1 kb was sequenced. Interestingly, this smaller fragment is almost completely identical to the large fragment, except for 5' end of the fragment. The 3' end of the fragment aligns with the 3' end of the 2 kb fragment and the first 42 basepairs align with the 5' end of the large fragment. Between the 42 basepairs at the 5' end and the remaining sequence, 1 kb of sequence is not present in comparison to the 2 kb fragment (see figure 14 and 15). The 1 kb fragment is a small isoform of *PKG*, which is missing one or more exons. Both fragments have the same start- and stop-codon and one large ORF.

Figure 14: Schematic overview of the sequence of the 2kb fragment and the 1 kb fragment that was obtained with the flank-PCR. The first 40 bp of 1kb fragment are similar to the 5' end of the 2 kb fragment. The start-codon of both fragments is marked with the 'm'.

The flank primers were later also used in order to test the quality of cDNA of larvae and, remarkably, only one large fragment of 2kb was amplified in this PCR. The smaller fragments can not be seen on gel. This could mean that only the large *PKG* product is present in larvae, but it could also mean that the smaller fragments that were observed earlier, were caused by degeneration of the RNA or cDNA. Degeneration does, however, not explain the 'missing'

part of almost the 1 kb in the small fragment that was sequenced. It is therefore still not entirely clear which isoforms of *PKG* there are. The Northern blot can give more insight in the number and size of different transcripts of *PKG*.

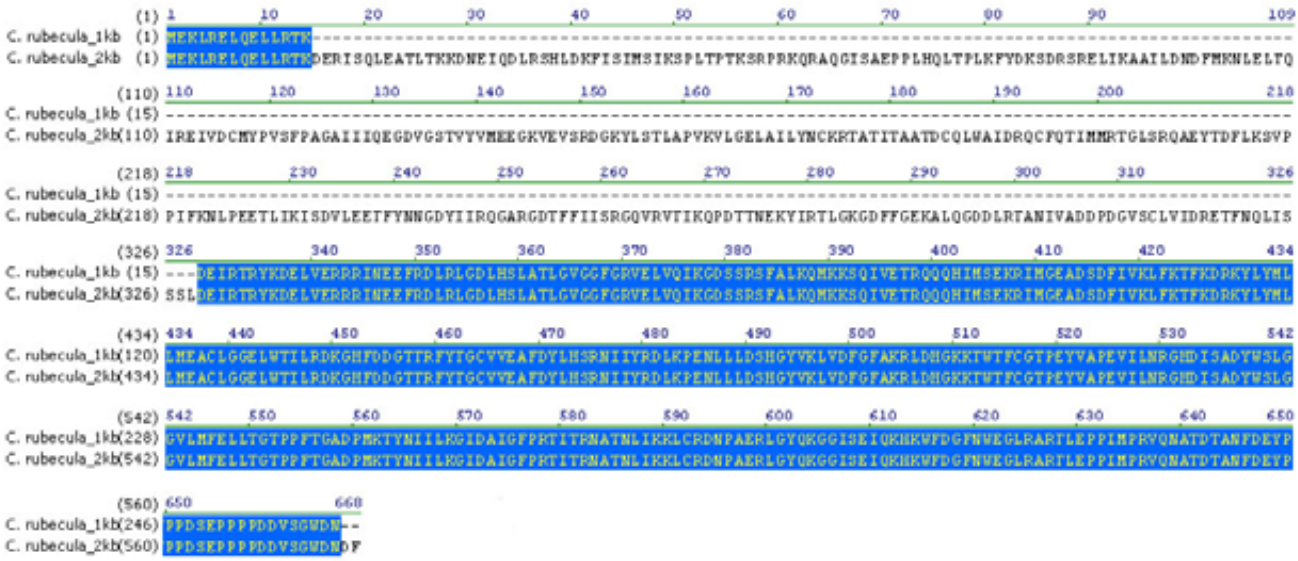


Figure 15: Overview of the protein sequence of the 2kb fragment and the 1 kb fragment of *C. rubecula* that was obtained with the flank-PCR. The first 14 amino acids of 1kb fragment are similar to the 5' end of the 2 kb fragment.

3.1.3 Characteristics of the entire PKG *gene*

After analyzing and aligning the different sequences of *PKG* of *C. rubecula* and *C. glomerata* using the software program Vector NTI 10.3.0 (Invitrogen Corporation, Carlsbad, California), the sequences that were obtained in the RACE-PCR's and the flank-PCR's could be combined into large sequences. All sequences that were found have the same, single stop-codon, but the length of the ORF differs as the size of the 5' sequences differs (see appendix 8 for the sequences). The largest ORF that was found in *C. glomerata* has a size of 2,5 kb. This large ORF is constructed by combining the largest sequences of the 3' and 5' RACE and the flank-PCR. This ORF is therefore expected to be the largest ORF that there is in the *PKG* gene. The largest ORF that was found in *C. rubecula* is only 2 kb, but this is most likely due to the fact that only few large sequences of the RACE-PCR's were obtained from *C. rubecula*.

The sequences of the 2,5 kb ORF of *C. glomerata* and the 2 kb and 1 kb ORF's of *C. rubecula* were translated and then aligned to the *PKG* sequences of a number of other organisms in order to investigate the similarities between the different sequences. The functional domains of the *PKG* genes were also determined for this purpose (see figure 16). The kinase protein domain is the most conserved region in the *PKG* gene and the sequences

are therefore most similar in this domain. The 2,3 kb ORF of *C. glomerata* and the 2 kb ORF of *C. rubecula* both have the complete protein kinase domain, the AGC-kinase C-terminal domain and two cGMP-binding domains. The 1kb ORF of *C. rubecula* also has the protein kinase domain and the AGC-kinase C-terminal domain, but only a small part of a single cGMP-binding domain

The similarity of the different sequences was determined by comparing the kinase protein domains of the different species (see table 12). This was calculated as the percentage of similar amino acids between the sequence of *C. glomerata* and the sequences of the other species. The *PKG* gene of *Cotesia* is most similar to that of the *foraging* gene of *A. mellifera*, with 93% similarity. This is not unexpected, since both species belong to the order of *Hymenoptera*.

Despite the several differences in the nucleotide sequence between both *Cotesia* species, there is only one difference in amino acid sequence at position 703 (see figure 16, the red arrow). *C. glomerata* has a alanine (A) whereas *C. rubecula* has a threonine (T) at this location. This difference in sequence is present in the kinase protein domain of the gene. Alanine and threonine both have neutral side-chains, but the side-chain of alanine is nonpolar, whereas threonine has a polar side-chain. It is unknown if this difference has any effect on the function of PKG, but it might have an effect on the activity of the protein. When observing the amino acids of other species at position 703, it is seen that only *C. glomerata* has an alanine at this position. *C. rubecula*, *A. mellifera* and *D. melanogaster* all have a threonine.

Species	Gene name and information	NCBI accession number	Similarity to *C. glomerata*
Cotesia rubecula	n.a.	n.a.	99,6%
Apis mellifera	*foraging* (*For*)	NM_001011581	93,8%
Drosophila melanogaster	*foraging*, isoform B	NP_477488	89,2%
Caenorhabditis elegans	*egl-4*, isoform A	AAD36954	65,0%
Mus musculus	*PKG*, type I beta isoform	NP-035290	75,0%

Table 12: Similarities between the amino acid sequence of the kinase protein domain of the *PKG* gene. All species were compared to *C. glomerata* and the similarity was determined as the percentage of similar amino acids. The sequence of *Cotesia* is most similar to that of *A. mellifera* and least similar to that of *C. elegans*.

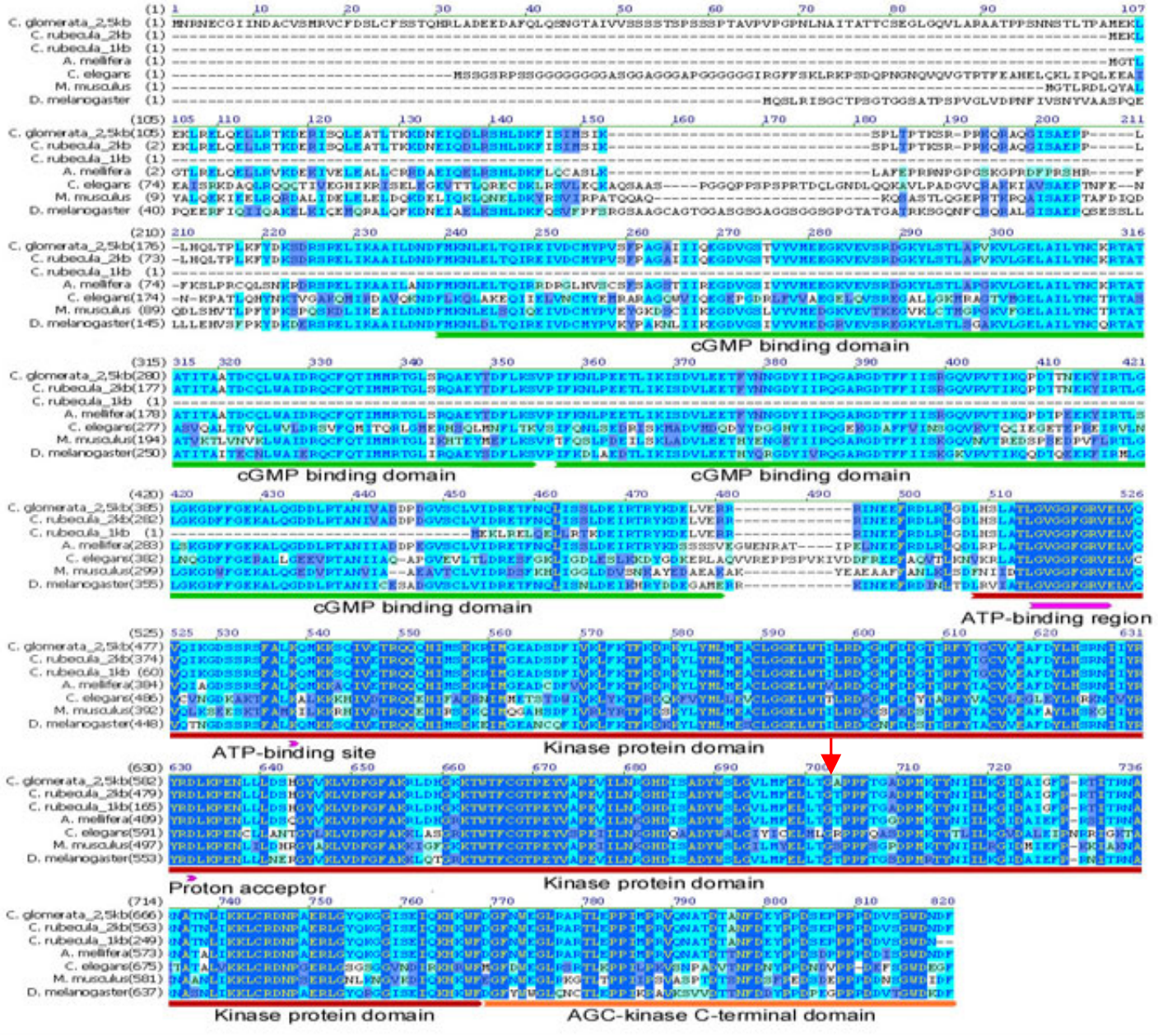


Figure 16: Alignment of the sequences of *C. glomerata* (2,3 kb), *C. rubecula* (2 kb and 1 kb), *A. mellifera*, *C. elegans*, *M. musculus* and *D. melanogaster*. The dark blue regions are regions which are similar in all species, the lighter regions are regions that are similar in a number of species, but not in all species, and the white regions are sequences that are not similar among a majority of the species. The red arrow in the kinase protein domain marks the location of the different amino acid between *C. glomerata* and *C. rubecula*.

3.2 Sequence of the *18S* gene of *C. glomerata* and *C. rubecula*

Part of the 18S rRNA was sequenced of both *C. glomerata* and *C. rubecula*. This sequence was approximately 400 bp long and it was found that the sequences of *C. glomerata* and *C. rubecula* were 98% similar. The sequences can be found in appendix 8.

3.3 Southern blot results

Genomic DNA was isolated from complete adult wasps using the Genomic DNA Purification Kit (Gentra Systems, Minneapolis, USA). For each blot, DNA had to be isolated from approximately 50 *C. glomerata* and *C. rubecula* adults. Purified DNA appeared dark reddish, presumably resulting from contamination of the eye pigments, which may have a negative effect on the quality of the DNA. I therefore suggest that DNA is isolated from larvae of *Cotesia* in future experiments. The larvae do not yet have an cuticle or eye-pigments and I expect that the efficiency of the isolation will be much higher. The age of the larvae does not matter, since DNA does not change over the lifetime of the larvae and it would, therefore, be easiest to use larvae that already are quite large.

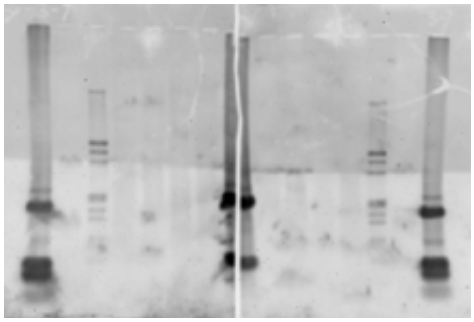


Figure 17: Picture of the final Southern blot, which was hybridized a second time using the Ultrahyb buffer. The lanes at the left and right side and the lane in the middle are non-labeled markers. The left part of the blot contain DNA from *C. rubecula*: the positive control, the dig-marker, the three cutted DNA samples and the uncut samples. The right side of the blot contains the samples of *C. glomerata* and it is mirrored in comparison the samples of *C. rubecula*.

In the first Southern blot, 5 µg of genomic DNA and 3 µl probe (25% labelled) were used and the hybridization temperature was 65^{o}C. After the cutting of the DNA and the running on gel, a picture was made of the gel in order to make sure that the DNA had moved properly through the gel and everything looked alright. When the blotting process was finished, however, no fragments were visible on the blot, not even for the positive control, which was plasmid DNA containing a *PKG* fragment. The dig-marker (DNA molecular weight marker III, Digoxigenin-labeled, Roche Diagnostics GmbH, Mannheim, Germany), however, was clearly visible, showing that the electrophoresis, blotting and detection procedures were successful. Therefore, either the amount or quality of DNA was insufficient, or hybridization had failed. In order to increase the signal from the *Cotesia* DNA, the amount of DNA and probe was increased in the next Southern blots. Furthermore, the hybridization temperature was lowered and the washing was

done less stringent. All these factors are known to enhance the signal, but it also increases the background.

The second Southern blot was done using 10 µg of DNA and 10 µl of probe (25% labelled). The hybridization temperature was lowered until 60°C and the washing was done less stringent. When visualising the signal of the blot, again no fragments were visible on the blot, except for the dig-marker. The upper side of the blot did have a weird dark staining of which the cause was unknown (not shown). It was thought that this staining might have been caused by a contamination in the gel tray and therefore the second Southern blot was repeated. This third time, the gel tray was first cleaned, though. This, however, did not prevent a dark staining in the third blot and also no fragments were visible. Another idea was that the dark staining was caused by the ethidium bromide and therefore in the fourth Southern blot procedure, no ethidium bromide was used in the gel. Also the hybridization temperature was lowered to 55°C and 10 µl of 50% labelled probe was used. The dark staining was very small this time, but still no fragments could be seen on the blot.

It can be concluded that there is a problem in the hybridization between the DNA and the probe, because the dig-marker has a very good signal under all circumstances. In a final attempt to obtain results from the Southern blot, the two latest blots were hybridized again. This time, however, the Ultrahyb buffer (see Northern blot procedure) was used. The manufacturer of this buffer states that it increases the efficiency of the hybridization 20 to 40 times and although it has been used only in the Northern blots, it can also be used in Southern blots just as well. This buffer was used according to instructions of the manufacturer, but still no fragments were visible (see figure 16). It is possible that the hybridization failed, because the blots were already used once and because the were not stripped before the hybridization in Ultrahyb. I therefore suggest that next Southern blots are hybridized using Ultrahyb and that the DNA is isolated from larvae in order to obtain higher amount of DNA which is probably less contaminated. It also may be useful to use a different type of probe or a probe at a different location of the *PKG* gene. If the probe is located on two different exons, it may not bind properly to the DNA if an intron is present between the two exons.

3.4 Northern blot results

The Northern blot was performed using 10 µg of RNA and 10 µl of probe (50% labeled). Both *C. rubecula* and *C. glomerata* showed at least three fragments on this blot (see figure 18). Two markers had been used (RNA molecular weight marker I and III, digoxigenin-labeled, Roche Diagnostics GmbH, Mannheim, Germany) in order to determine the size of the transcripts of *PKG*. The largest transcript has a size of approximately 2,5 kb, the smaller transcript has a size of around 1,8 kb and the smallest fragment has a size of approximately 1,2 kb. These lengths are approximately and the fragments may be around 200 bp larger or smaller. The largest transcript appears to be expressed in a slightly higher amount than the fragment of 1,8 kb, whereas the smallest fragment appears to be expressed much less than the other two fragments. The large transcript may be the large 2,5 kb ORF with a small poly-A tail, but it may also have a smaller ORF and a longer poly-A tail. The smaller transcripts could be the small 1kb transcript that was found in *C. rubecula*. They could, however, also be a 5' truncated isoform. It can not be determined with certainty what the sequence of the two transcripts is, because there was a lot of variation in the length of the different fragments that were found in the 3' and 5' RACE and several combinations of fragments would result in transcripts of the expected length. Furthermore, the bands that are seen on the Northern blot are not very distinct and their size can not be determined accurately.

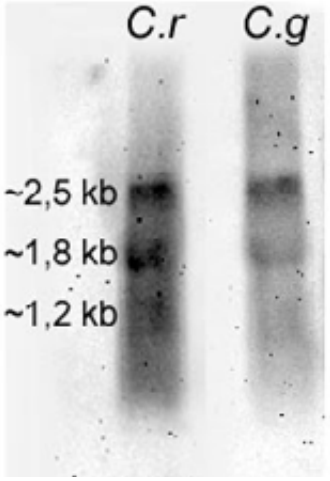


Figure 18: Picture of the Northern blot. The left lane is *C. rubecula* and the right lane is *C. glomerata*. Three transcripts can be seen: the largest transcript is approximately 2,5 kb, the second transcript is approximately 1,8 kb and the smallest transcript is around 1,2 kb. The transcripts can, however, be around 200 bp larger or smaller.

3.5 *PKG* expression of *C. glomerata* and *C. rubecula*

After the determination of the sequence and the isoforms of the *PKG* gene in the previous paragraphs, the results of *PKG* expression are given in the paragraphs below.

3.5.1 Comparing PKG *expression of larvae of 1 day old*

The first RT-qPCR experiments revealed that the expression in 1 day old larvae differs significantly between *C. rubecula* and *C. glomerata*. The relative expression of *PKG* was found to be

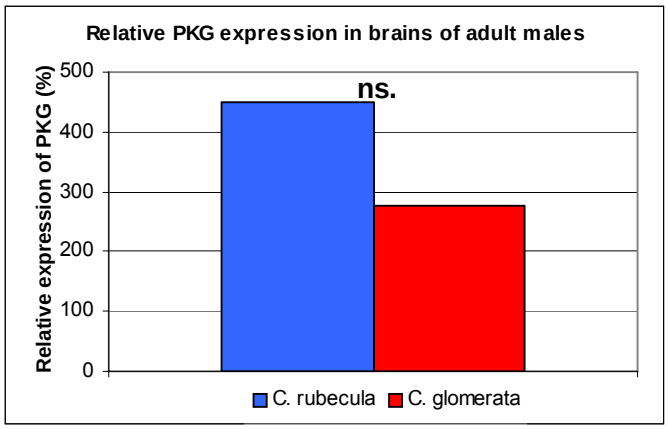
	Relative expression of the *PKG* gene (%)		
	C. rubecula (using *C. rubecula* standard curves)	*C. glomerata* (using *C. glomerata* standard curves)	*C. glomerata* (using *C. rubecula* standard curves)
sample 1	100	11112	248
sample 2	111	3112	57
sample 3	87	6701	126
sample 4	138	13194	386
sample 5	65	13332	418
Average	100	9490	247

Table 13: The differences in efficiency between the standard curves of C. glomerata and C. rubecula is found that have a major effect on the relative expression of the PKG gene that is calculated. The relative expression of a sample is calculated by dividing the concentration of *PKG* by the concentration of *18S*. The relative expression of all samples was furthermore calculated as a percentage in comparison to sample 1 of *C. rubecula*.

100 times higher on average in *C. glomerata* larvae than in *C. rubecula* larvae. These results were obtained by analyzing the *C. glomerata* samples using the standard curves of *PKG* of *C. glomerata* and by analyzing samples of *C. rubecula* with standard curves of *C. rubecula*. This difference in expression between both species seems quite high and therefore the relative expression of the *C. glomerata* samples was also determined using the *C. rubecula* standard curve. The effect of the differences between the standard curves of PKG then becomes clear: there was no significant difference anymore between relative *PKG* expression of both larvae species (Mann-Whitney, P=0.175)(see table 13). This clearly shows the weakness of using standard curves: the quantification is highly dependent on the efficiency of the primers on the plasmid DNA, which might not be similar to the efficiency of the primers on cDNA. Since it was already found rather strange that there were differences in efficiency of the *PKG* primers between the two species, it was chosen to analyze all the following samples using the standard curves for *PKG* of only one species. The standard curve of *PKG* of *C. rubecula* was chosen for this purpose, because the efficiency of this standard curve is more similar to the efficiency of the *18S* primers. The concentrations that are calculated for *18S* and *PKG* using these standard curves may differ from the actual concentrations, but that does not matter for this analysis, since we are interested in the relative expression of *PKG* between both *Cotesia* species.

3.5.2 Comparing PKG *expression of brains of adult male wasps*

The expression of brains of adult male wasps of *C. glomerata* and *C. rubecula* was compared. There appeared to be no significant difference between both species (Mann-Whitney, P=0.275), but the average *PKG* expression of *C. rubecula* was found to be higher than that of *C. glomerata* (see graph 2). It was hypothesized that *C. rubecula* males would have a higher expression of *PKG* in their brains, because these males do not remain at the location were they hatch in order to wait for females to hatch. Since only three samples of male brains were compared, some more samples should be tested in order to investigate whether there is indeed

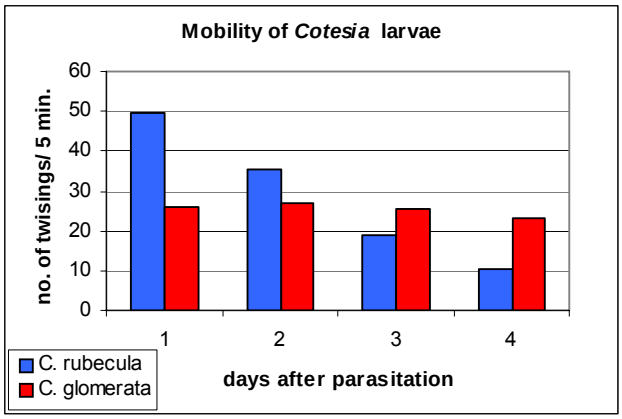


Graph 2: The relative expression of *PKG* in brains of adult males. The average of three samples is shown in the graph. The *PKG* expression appears to be higher in *C. rubecula* males, but the difference is not significant. The relative expression is given as a percentage of the expression of sample 1 of *C. rubecula* larvae.

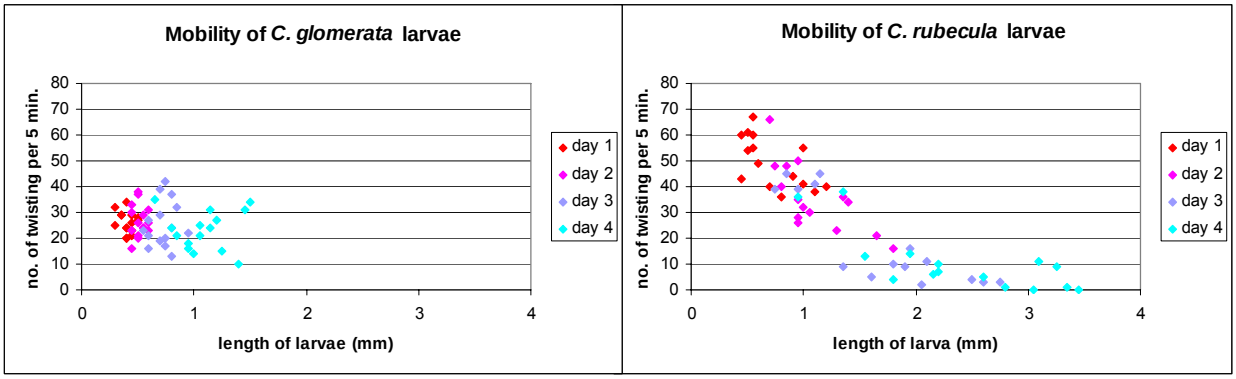
a difference between the two species or not. I suggest that very young adult males are used for this purpose, because only very young *C. glomerata* males remain with their cocoon until the females start hatching.

3.5.3 Measuring mobility of larvae

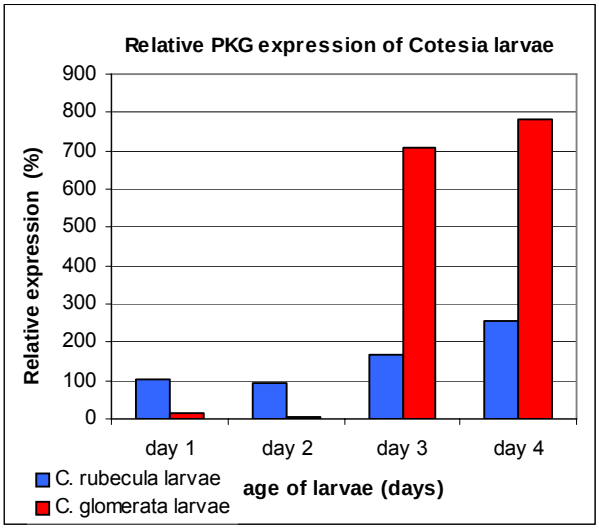
Problems had occurred with the rearing of *C. glomerata* and *C. rubecula* wasps after the mobility of the larvae was measured by Tribuhl, 2007. Therefore it was decided to measure the mobility of the larvae again, in order to see if the differences in mobility were still present. Beside the number of twistings, also the length of the larvae was determined to get an overview of the differences in growth rate between the larvae. As can be seen in graph 3 the mobility of both *C. glomerata* and *C. rubecula* are similar as observed before. The mobility of *C. rubecula* larvae is higher than the mobility of *C. glomerata* larvae at day 1, the first day after hatching of the eggs. The mobility of the larvae of *C. rubecula* decreases significantly over the next three days



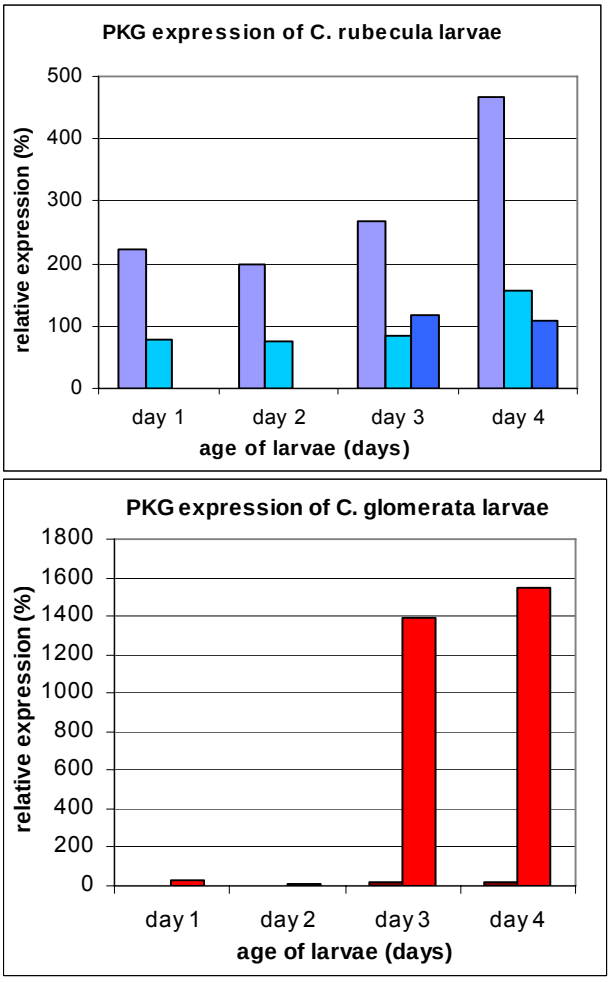
Graph 3: The mobility of the larvae is shown as the number of twistings per 5 minutes. As shown, the larvae of *C. rubecula* have a higher mobility at day 1, but this mobility decreases significantly over the next three days.



Graph 3: The left graph shows the mobility and length of *C. glomerata* larvae. The right graph shows the mobility and length of *C. rubecula* larvae. As can be seen, larvae of *C. rubecula* grow much more in the first four days than the larvae of *C. glomerata*.



Graph 5: The relative expression of *PKG* of all larvae of *C. glomerata* and *C. rubecula*. Both species show an increase in *PKG* expression in day 3 and 4, but only the increase in *C. glomerata* is significant.



Graph 6: The graph shows the differences in relative *PKG* expression between samples of larvae derived from different oviposition events. The upper graph shows the average *PKG* expression of the three oviposition events of *C. rubecula*. The lower graph shows the average *PKG* expression of the two oviposition events of *C. glomerata*.

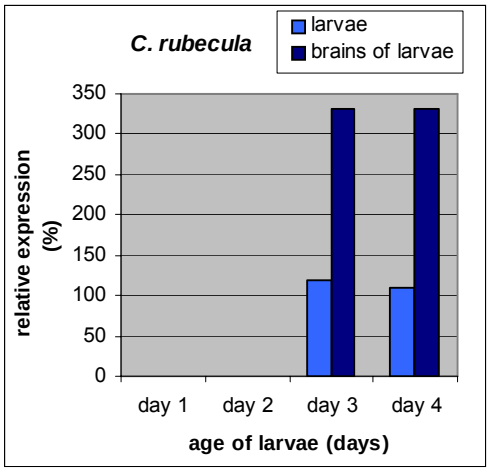
(GLM, $F_{3,56}$= 25,85, P<0,001) and at day 4, the mobility is even lower than that of *C. glomerata* larvae. The mobility of *C. glomerata* larvae does not change in the first four days after hatching (GLM, $F_{3,56}$= 0,799, P=0,500).

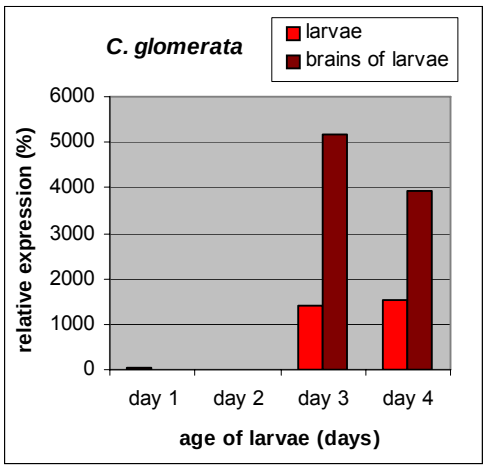
3.5.4 Comparing PKG *expression of larvae from 1 to 4 days old*

After analyzing 8 samples of *C. rubecula* (3 oviposition events) and 6 samples of *C. glomerata* (2 oviposition events), it was found that larvae of both species show an increased *PKG* expression in days 3 and 4 in comparison to day 1 and 2 (see graph 5). This increase was significant for *C. glomerata* (GLM, $F_{3,20}$=6,731, P=0,004), but not for *C. rubecula* (GLM, $F_{3,28}$=2,359, P=0,102). Although, the expression of *PKG* appears to increase much more in *C. glomerata* than in *C. rubecula*, the difference between both species is not significant (GLM, $F_{1,54}$= 3,132, P=0,083).

Statistical analysis furthermore revealed that the different oviposition events had a significant effect on the *PKG* expression of both species (GLM, $F_{4,53}$=11,031, P=0,001). When the different oviposition events were analyzed separately, it was found that all events show a similar trend: the *PKG* expression increases at day 3 and 4 in comparison to day 1 and 2. The differences in relative expression are, however, very large between the different events (see graph 6). In *C. glomerata* the expression differs

even 170 times between larvae of the two oviposition events (day 3). The differences in *PKG* expression are hard to explain, because they seem to be too large to be just normal variation in expression. Perhaps, somehow some variation has occurred during one of the several experimental procedures, although no deliberate changes were made. Another point of concern is reference gene. The *18S* gene was used as a reference, but it turned out that the concentrations of this gene were very high in the samples. The signal of *18S* was almost immediately detectable in the RT-qPCR and this may have caused an inaccurate determination of the concentration of the 18S. The highest concentrations were furthermore up to a 10000 times more concentrated than the highest concentrations of the standard curve that was made for 18S and it is not certain that the efficiency of the primer is the same at these high concentrations. As already seen, differences in efficiency of the primer can have a major impact on the calculated concentrations of the samples. It would therefore be wise to lower this concentration of the cDNA before it is used in the RT-qPCR in order to lower the concentration of 18S. This should, however, be done with caution, because otherwise the concentration of *PKG* will become too low to be determined accurately. Another possibility is to use another endogenous housekeeping gene as a reference with a lower expression, that is more similar to the expression of *PKG*.





Graph 7: The differences between *PKG* expression of entire larvae and brains of larvae are shown in the two graphs. The upper graph shows *C. rubecula* and the lower graph shows *C. glomerata*. The expression of *PKG* appears to be higher in the brains of the larvae compared to the expression in entire larvae.

3.5.5 Comparing PKG *expression of brains of larvae*

As a pilot experiment, one sample of brains of larvae was analyzed for each of the four days after hatching. No statistical analysis can be used on these samples, since there is only one replicate, but it can show if there are large differences between the expression in entire larvae and brain of larvae. This way it can be decided whether it will be useful to test more brains of larvae or not. The brains of the larvae were isolated from larvae of the last oviposition event of *C. rubecula* and *C. glomerata* and their expression was therefore compared only to the expression of samples

of entire larvae of this oviposition event. It appears that the expression of *PKG* is higher in the brains of the larvae compared to the expression in the entire larvae. The pattern of expression, appears, however, to be more or less the same: the expression is much higher in day 3 and 4 in comparison to day 1 and 2. I therefore suspect that, although the expression may be higher, the pattern of expression will be the same in brains of larvae and it would then not be necessary to analyze any more brain samples.

4. Discussion and conclusion

The main purpose of this thesis was to investigate the role of the *PKG* gene on larval mobility of the two closely related parasitoid wasps *C. glomerata* and *C. rubecula*. The expression of *PKG* was hypothesized to be, either positively or negatively, correlated to the mobility of the larvae. The mobility of *C. glomerata* larvae was found to be constant over the first four days after hatching and the *PKG* expression was therefore also expected to be constant. On the other hand, the mobility of *C. rubecula* was high at the first day after hatching, but the mobility decreased significantly in the following three days. It was expected that either a positive or a negative correlation of *PKG* expression would be found with this decreasing mobility.

The *PKG* gene of both *C. glomerata* and *C. rubecula* was sequenced. The 3' and 5'RACE PCR reactions resulted in the amplification of a number of different fragments. Four fragments of the 3'RACE and also four fragments of the 5'RACE of *C. glomerata* were sequenced. The 3'RACE fragments were all identical in sequence, but they differed in length. These different fragments might be different isoforms of *PKG* that resulted through alternative poly-adenylation and all fragments continue after the stop-codon of the ORF that was found. The differences in length after the stop-codon might have an effect on the localization or the stability of the mRNA, without having an effect on the actual sequence of the translated protein (Lee & Irizarry, 2003).The 3' UTR may also be involved in mRNA silencing by RISC. This silencing was found to control the long term memory formation in *D. melanogaster* (Ashraf et al, 2006). The 5'RACE fragments are also all identical in sequence and they differ in length. Several possible ORF's were found and these ORF's are all located in the same reading frame, which could indicate that the smaller fragments of *PKG* are truncated 5' isoforms. Not all fragments that were observed on gel after the 3' and 5' RACE were sequenced, however, and it may therefore be possible that there are more isoforms of *PKG*. Many problems occurred during sequencing of 3' and 5' fragments of *C. rubecula* and only few fragments were sequenced for this reason. It is therefore needed to repeat the 3' and the 5' RACE PCR for both *C. glomerata* and *C. rubecula* in order obtain the sequences of all possible isoforms of both species.

The sequence of *PKG* was furthermore investigated using flank primers and several fragments were amplified during the PCR. Two of the fragments were sequenced and the shortest of the two fragments was found to be identical in sequence and both fragments have the same start- and stop-codon and one large ORF. The short fragment is missing one or more

exons causing the fragment to be 1 kb shorter. There were, however, also many problems during the sequencing of the fragments of the flank PCR and several of the fragments were therefore not sequenced. The flank PCR should be repeated as well in order to obtain the sequence of all the fragments. Another point of concern is the fact that the flank primers were designed based on the sequence of a single 5'RACE fragment and it might be possible that there are also still other isoforms that have a different sequence on their 5' end.

The Northern blot has shown that there are at least three different transcripts of *PKG* for as well *C. glomerata* and *C. rubecula*. The size of the transcripts on the blot could not be determined very accurately, due to the fact that there was quite a lot of background signal on the blot which makes it difficult to determine the boundaries of the transcripts precisely. The size of the transcripts was, however, estimated to be 2,5 kb, 1,8 kb and 1,2 kb. The sequence of these three transcripts is not known, because several of the isoforms that were sequenced have a size that correlates to the size of the transcripts on the Northern blot. The Northern blot should therefore also be repeated in order to get a better view on the different transcripts.

When comparing the number of transcripts of *C. glomerata* and *C. rubecula* to that of other species it is found that *D. melanogaster* also has three transcripts of *PKG*. The honey bee and the harvester ant had only one transcript and the nematode, *C. elegans*, had two transcripts of *PKG* (Ben-Shahar et al, 2002; Fujiwara et al, 2002; Ingram et al, 2005) . Perhaps the situation concerning different isoforms is therefore similar in *Cotesia* and *D. melanogaster*. The three different isoforms of *D. melanogaster* are 5' truncated isoforms of *PKG* (Osborne et al, 1997) and it was recently found that the smallest transcript, the T2 transcript, is most important for the differences in behaviour between 'rovers' and 'sitters' of *D. melanogaster* (Sokolowski, pers. comm.). It is therefore very important to determine the sequence of the different transcripts of *PKG* of *Cotesia* and to investigate the effect of each of the transcripts on mobility of the larvae, because only one of the transcripts may be responsible for the differences in mobility.

The expression of *PKG* was investigated by both the Northern blot and by RT-qPCR experiments. The Northern blot, which was done using RNA from entire 4 days old larvae of *C. glomerata* and *C. rubecula*, did not reveal any large difference in expression in one of the three transcripts between the two species. The transcripts were, however, rather vague on the blot and it is also possible that the use of entire larvae masks differences between the two species. The Northern blot should therefore also be repeated using RNA from brains of the larvae only.

Before the RT-qPCR experiments were performed, first the mobility of the larvae was determined again in order to repeat and confirm the results form Tribuhl, 2007. Beside mobility, also the length of the larvae was measured in order to determine the variation in growth rate between the larvae. The mobility of the larvae was found to be comparable to the mobility that was measured by Tribuhl, 2007. It was furthermore found that the larvae of *C. rubecula* grow much faster than the larvae of *C. glomerata*. This can be explained by the fact that the gregarious larvae of *C. glomerata* can not grow too fast, otherwise they will outgrow their host. The larvae of *C. rubecula* are alone in their host and they therefore they can grow much faster.

The RT-qPCR experiments did not reveal any differences in *PKG* expression between *C. glomerata* and *C. rubecula*. The RT-qPCR on both species shows an increase of *PKG* expression at day 3 and 4 after hatching in comparison to day 1 and 2, although this increase was found not to be significant in *C. rubecula*. This increase may rather be an age-dependent effect instead of a mobility-dependent effect. Although all the different oviposition events show a similar pattern, the variation between the samples of the different events is large. It is not known what caused this variation, but possibly inaccuracies of the standard curves had an effect on the results from the experiments. The concentrations of *18S* were found to be higher than the range of the standard curve and it is unknown whether the efficiency of the primer is similar in such higher concentrations. Differences in efficiency can have a large effect on the results of the analysis and the efficiency of the *18S* primers should therefore also be determined in higher concentrations of template. It is furthermore possible that differences in *PKG* expression will be found if the three different transcripts of the *PKG* gene are measured separately, although the Northern blot did not reveal any large difference in expression between the transcripts of *C. glomerata* and *C. rubecula*. The Northern blot should, however, be improved first before it is possible to draw any conclusions on differences in expression of the different transcripts. At the moment, it is therefore not possible to draw a conclusion on the correlation of *PKG* expression with the mobility of larvae of *C. glomerata* and *C. rubecula*.

4.1 The role of PKG activity in larval mobility

A lack of correlation of *PKG* expression and larval mobility does not totally exclude that the gene encoding PKG has a role in larval mobility. As mentioned in the introduction, significant differences were found between ‘rovers’ and ‘sitters’ in a PKG enzyme assay performed on

extracts of adult heads of *D. melanogaster*. These differences were, however, not that obvious when RNA of entire adult flies was analyzed on a Northern blot. The expression of the three transcripts of the *dg2* gene was estimated from the Northern blot and 'sitters' were found to have a small reduction in the abundance of the T1 transcript relative to 'rovers'. The other two transcripts, T2 and T3, were also slightly reduced in sitters, but the differences were very small (Osborne et al, 1997). It would be better to quantify the expression of *PKG* using RT-qPCR experiments on brains of adult flies, but this has not been done so far. From the results of the Northern blot, it is not possible to conclude that there are significant differences in expression of the *dg2* gene between rovers and sitter, whereas there are significant differences in PKG activity. (Osborne et al, 1997). Recent experiments have shown that the T2 transcript contributes most to these differences in activity, while the other two transcripts contributed less (Sokolowski, pers. comm.).

Research on ants also showed that that *PKG* expression did not correlate very well with differences in behaviour (Ingram et al, 2005). The expression of *Pbfor* in the brains of ants from different task behaviour groups was quantified using RT-qPCR experiments. The different groups are the callow workers and interior worker, which perform tasks inside the nest, and nest maintenance workers, patrolling ants and forager ants, which perform their tasks outside the nest. It was found that the expression of *Pbfor* was to significantly higher in callow workers, which perform tasks inside the nest, than in foragers in all of the colonies that were tested. The expression patterns of the other groups, however, did not show any clear differences in expression as there was a large variation in expression between different colonies (Ingram et al, 2005). In another recent study on colony organization and mobility of ants, the PKG activity of brain extracts was compared between ants that perform tasks inside the nest and ants that perform tasks outside the nest. These enzyme assays do show a consistent difference in PKG activity between the two groups of ants (Lucas et al, in prep.; Sokolowski, pers. comm.).

These results from research on fruit flies and ants suggest that PKG enzyme activity correlates better to different behaviour patterns than the expression of the *PKG* gene. I therefore propose that the next step in the investigation of larval mobility of *Cotesia* focuses on PKG activity. The PKG enzyme assay protocol that is described by Belay et al (in prep.) can be used for this purpose. This protocol was also successfully used in the research on ants.

If the PKG activity of *Cotesia* larvae is indeed found to correlate with the mobility of the larvae, then it would be interesting to investigate whether PKG is also directly responsible for the differences in mobility. One possibility that is likely to increase the activity of PKG is

feeding of an analog of cGMP to the larvae, in order to see whether the activity of PKG will change. This is because PKG is dependent of cGMP for its function. Ben-Shahar et al, 2002 used 8-Br-cGMP, a membrane-permeable analog of cGMP, in order to increase the expression of PKG in bees and it was indeed found that this activity was increased (Ben-Shahar, 2002). This analog of cGMP could also be used on *Cotesia* larvae. The analog has to be fed to the larvae very carefully, because the host of the larvae should not be damaged. The analog could be injected into the caterpillars of *P. brassicae* and *P. rapae* and it is then expected to diffuse throughout the host and reach the larvae. It is important to disturb the natural behaviour of the larvae and the caterpillars as less as possible in this experiment. The larvae can then be collected after hatching from their eggs as is described in the materials and methods and the PKG activity can then be measured. It is also recommended to observe the mobility of the larvae after the cGMP-treatment in order to see whether the mobility has indeed changed. A major disadvantage of using a cGMP-analog is, however, that it is a common second messenger molecule and that it is difficult find out what the possible effects on other genes or pathways are.

A better way to test whether PKG has a direct effect on larval mobility would be to specifically activate or inhibit PKG, without the use of a common second messenger. An exciting technique that could be used for this purpose is RNAi. RNAi is a technique that can be used to silence a gene in animal cells. Animal cells have a natural defense system against foreign RNA. Small pieces of double stranded RNA (siRNA) are incorporated in the RNA induced silencing complex (RISC). This complex is then activated and will it will recognize and destroy all mRNA that is similar to the foreign RNA. This mechanism can be used to silence genes within a cell. Adding RNA, that is similar to the mRNA of the gene, to the cell will induce the RNAi mechanism and the gene will be silenced (Aigner, 2007). The *PKG* gene of *C. glomerata* and *C. rubecula* can be silenced using this method, resulting in a lower expression and activity of PKG.

4.2 The role of PKG in the brains of larvae

Research on the spatial distribution of PKG can give additional information on the possible place of action of the enzyme. Furthermore it will show whether differences in distribution occur in the first 4 days of the larvae after hatching. In situ hybridizations can be used to make the PKG in the brains visible. In situ hybridization is a technique in which the brain is incubated in a solution containing a *PKG*-specific probe. The probe will disperse throughout

the brain and it will hybridize to the *PKG* that is expressed in the brain. The probe emits a fluorescent signal and this is used to determine were the *PKG* is expressed in the brain.

The distribution of PKG in brains of *A. mellifera* was determined and it was found that PKG is widely expressed in the brains and most prominently in certain cells of the mushroom bodies and optical lamina. The mushroom bodies are a region in the brains that is important for the processing of various stimuli and learning and memory. The optical lobes are involved in the processing of visual information. No obvious differences in spatial expression of PKG were found when nursing bees and forager bees were compared, which indicates that the expression of *PKG* increased overall (Ben-Shahar, 2002). Because of the expression of *PKG* in the optical lobes and in the Kenyon cells, which are cells in the mushroom bodies that receive mainly visual input, it was thought that the *Amfor* gene was involved in the integration of visual information. Beside this, it was also found that nurse bees are negative phototactic, while foragers are positive phototactic, which strengthened the believe that visual stimuli were involved in the division of labor (Ben-Shahar et al, 2003; Ben-Shahar, 2005). Further research has showed that exposure to light itself does not increase expression of *Amfor* and that PKG is not expressed in the photoreceptor cells. It is at the moment not yet know how visual stimuli are involved exactly in the behavioural maturation in bees.

In *Drosophila*, the role of the mushroom bodies in foraging behaviour was also investigated. This was done by removal of the mushroom bodies. It was found that the larval mobility was not significantly changed by the treatment, suggesting that the mushroom bodies are not involved in larval mobility (Osborne et al, 2001). Therefore it might also be possible that the stimuli that are involved in *PKG*-mediated differences in behaviour differ for each species. Beside visual stimuli, also other stimuli are likely to be involved in the differences in behaviour. In case of larvae of *Cotesia* visual stimuli are not very likely to have an effect on the differences on mobility, since the larvae do not yet have eyes or optical lobes. The larvae can perceive light through light sensitive cells, but since the larvae are located in their host, it seems more likely that other stimuli will affect mobility. Since the behaviour is, to some extend, related to food, it is expected that either odors or taste also have an effect (Ben-Shahar et al, 2003).

Both in *A. mellifera* and in *Drosophila*, sucrose responsiveness was investigated in relation to differences in foraging behaviour. This sucrose responsiveness was determined by stimulating the antennae of adult bees or the tarsus of the leg of an adult fly with a sucrose solution. The insect will extend its proboscis reflexively upon stimulation and the number of extensions after a stimulation was recorded. A greater responsiveness to sucrose is reflected

by a higher number of extensions (Ben-Shahar et et, 2004; Scheiner et al, 2004). It was found that forager bees were more responsive to sucrose than nursing bees (Ben-Shahar et al, 2004) and also adult 'rover' flies were found to be more responsive to sucrose than 'sitters' (Scheiner et al, 2004). The study on *Drosophila* further showed that the 'rovers' showed on average less habituation than 'sitters' to sucrose. These differences in habituation were not related to differences in responsiveness to sucrose, as the habituation tests were done on 'rovers' and 'sitters' with similar responsiveness. This suggests that the *PKG* gene is involved in sucrose responsiveness and in nonassociative learning, which supports that theory that the differences in behaviour are the result of a variation in the sensory perception of stimuli or differences in higher brain-determined 'evaluation' responses (Scheiner et al, 2004; Ben-Shahar, 2005). An adult 'rover' might evaluate a sucrose droplet differently than a 'sitter' which causes the difference in foraging strategy. This idea is further supported by research on neurons of 'rovers' and 'sitters' of *Drosophila*. This research was done on two types of neurons: cultured embryonic central neurons and motor axons of larvae. The neurons of 'sitters' were found to be hyperexcitable compared neurons of 'rovers'. Beside this, also synaptic transmission was found to be hyperexcitable in 'sitters' and also the neuronal connectivity patterns were found to be different in 'rovers' and 'sitters' (Renger et al, 1999). Finally, research on olfactory adaptation of the nematode, *C. elegans*, also revealed that PKG is involved in behavioural adaptation. Nematodes with mutations in the *egl-4* gene did not show a slow, long-lasting behavioural adaptation to odors, but a rapid, short-term adaptation was still possible (L'Etoile et al, 2002).

PKG is a protein kinase and it is therefore likely to be involved in responsiveness or adaptation to certain stimuli. The question remains, however, which stimuli the PKG of *Cotesia* larvae processes. In situ hybridizations on the brains of *Cotesia* larvae can provide a clue on what stimuli are involved in the larval mobility and why the mobility differs between *C. glomerata* and *C. rubecula*.

4.3 Factors that influence the mobility of *Cotesia* larvae

Several experiments have been done on the correlation of *PKG* expression and larval mobility of *Cotesia*, but the mobility of the larvae itself has not yet been investigated much. The mobility of *C. rubecula* decreases in the first four days after hatching, while the mobility of *C. glomerata* remains more or less constant. The larvae of *C. rubecula* also grow a lot during these first four days, while the larvae of *C. glomerata* grow much less. Perhaps the mobility of *C. glomerata* larvae also decreases as it grows older, but at a slower rate due to its lower

growth rate. Mobility is especially important for young, first instar larvae, because these young larvae will move towards other larvae and they will fight until one of the larvae is killed. If larvae are older, then usually indirect methods are used to eliminate the other larvae (Godfray, 1994). This offers a good explanation for the rapid decline in mobility of *C. rubecula*: larvae of *C. rubecula* have a high mobility immediately after hatching in order to find and attack other larvae. Due to the high growth rate of the larvae, this mobility decreases rapidly and indirect methods for attack and defense can also be used rapidly. The lower mobility of *C. glomerata* larvae immediately after hatching suggest that these larvae are indeed able to live gregariously in their host, because they will not encounter each other.

More observations on the 'normal' mobility of the *Cotesia* larvae could be done, but beside this it would also be interesting to investigate if certain stimuli can alter the mobility of the larvae. Perhaps the larvae increase their mobility if they perceive other larvae. A possible hypothesis would then be that larvae of *C. rubecula* increase their mobility when they perceive other larvae, while larvae of *C. glomerata* do not, as they live gregarious with many other sibling larvae. It is important that the mobility of the larvae is measured under conditions that are a natural as possible, since otherwise the effects of the experimental procedure on the mobility may be large. Ideally, the mobility of the larvae would be measured in the host, but this is off course practically impossible. The medium in which the larvae is measured should be suited for the larvae to live in for a while, but it should not be extremely poor or rich in nutritives compared to the host, as this might also affect mobility. A possible medium is Grace's insect medium, which was successfully used to observe aggressiveness and mobility of *Cotesia* larvae in earlier experiments (Kalkman & van Groningen, 2007). If the larvae indeed increase their mobility in the presence of other larvae, then it is of course possible to investigate if the activity of PKG has also changed. Another option is screening a large number of genes for a difference in expression using microarrays. Of course, also other stimuli could be tested for a possible effect on mobility.

4.4 Other candidate genes that might affect larval mobility of *Cotesia*

It is possible that the *PKG* gene is not found to be correlated to mobility of *Cotesia* larvae in any way and then the next step would be to find another gene that might explain the differences in mobility. It is an option to investigate other genes that are involved in the *PKG*-mediated pathway. Differences in expression or activity of gene that has a function upstream or downstream of the *PKG* gene can also cause the differences in mobility of *Cotesia* larvae. A difficulty is, however, that this pathway is not unraveled yet. One study on *Drosophila*,

investigated the role of guanylyl cyclase on foraging behaviour of larvae. Guanylyl cyclase is an enzyme that catalyses the formation of cGMP, which is needed to activate PKG. When the encoding gene guanylyl cyclase was mutated, it was, however, found that the activity of PKG increased, both in 'rover' and in 'sitter' larvae. This increase in PKG activity might have been caused by an overcompensation of cGMP formation of another gene, but no increased transcription of any known or predicted gene encoding guanylyl cyclase or PKG was found (Riedl et al, 2005). This illustrates that the exact pathway in which PKG is active is still largely unknown and it will therefore be difficult to point out genes that are likely to be involved in this pathway.

A number of candidate genes have already been described briefly in the introduction chapter and expression or activity of these genes could be investigated in order to find out if there is a correlation with larval mobility. The role of the *malvolio* gene was investigated in honey bees and also in *Drosophila*. The *malvolio* gene encodes a manganese transmembrane transporter and mutations in this gene were found to affect sucrose responsiveness in *Drosophila*. The exact role of manganese in neural and behavioural plasticity is, however, poorly understood (Ben-Shahar et al, 2004). In honey bees, the levels of *malvolio* were found to be higher in foragers than in nursing bees, suggesting a correlation between the gene and behavioural maturation. The sucrose responsiveness also increased in foragers. In order to determine whether *malvolio* had a direct effect on sucrose responsiveness and behavioural maturation, bees were treated with manganese. This treatment indeed increased sucrose responsiveness and caused precocious foraging of young bees (Ben-Shahar et al, 2004). The *malvolio* gene may therefore also have an effect on larval mobility of *Cotesia* larvae. I, however, suggest that the sucrose responsiveness is first tested in *C. glomerata* and *C. rubecula*, before investigating this gene extensively. If there are no differences in sucrose responsiveness in the larvae, then it would be better to investigate another candidate gene first.

Research on *C. elegans*, a nematode, has also resulted in a good candidate gene for the differences in larval mobility between *C. glomerata* and *C. rubecula*. The *npr-1* gene was found to be responsible for differences between social and solitary strains of *C. elegans*. In the presence of plentiful food solitary strains remain dispersed evenly over the area, while social strains will aggregate into clumps at locations were most food is available. These social nematodes move twice as slow as solitary nematodes when clumped together, but when the nematodes were placed on a food source alone they move twice as fast as solitary nematodes.

This difference in behaviour was found to be caused by differences in response to other nematodes (de Bono & Bargmann, 1998).

The *npr-1* gene is predicted to be a G protein-coupled receptor of the neuropeptide Y (NPY) receptor family. NPY is a neurotransmitter that has been associated with several processes in the brain including the regulation of food consumption, memory and blood pressure. Neuropeptide Y receptors activate G proteins, which then modulate the activity of enzymes, ion channels or second messenger pathways. Social and solitary strains were found to differ one amino acid in the protein sequence of the receptor. This difference is suggested to decrease the strength or the specificity of the receptor of the protein of social nematodes, which will result in a less active version of the protein (de Bono & Bargmann, 1998).

Research on the location of the NPR-1 receptor showed that it is widely expressed in neurons of *C. elegans*. It is present in several sensory neurons, interneurons and motorneurons throughout the nematode. Specific expression of the *npr-1* gene in small subsets of these neurons was used to determine which of the neurons were involved in the regulation of the social behaviour of the nematodes and three body cavity neurons were found to be involved in this behaviour (Coates & de Bono, 2002).

One study on stimuli that may be involved in the regulation of social and solitary behaviour focused on oxygen. The oxygen concentration is relevant to nematodes, as bacteria can locally alter oxygen levels. The oxygen concentration can therefore be a measure for the amount of food that is present at a certain location (Gray et al, 2004). Whether oxygen has any role in the larval mobility of *Cotesia* is unknown, but it might be interesting to test this.

Further research on other genes in *C. elegans* that might be involved in the regulation of social behaviour focused on cyclic nucleotide-gated ion channels, because these channels are known to be important in the transduction of sensory signals. The *tax-2* and *tax-4* genes encode the β- and α-subunits respectively of the cGMP-gated cation channel and were found to be expressed also in the body cavity neurons in which *npr-1* is expressed. These two genes were found to promote aggregation, while the *npr-1* gene suppresses aggregation. It is therefore proposed that the two antagonistic signaling pathways in the body cavity neurons together regulate the social and solitary feeding behaviour of the nematodes (Coates & de Bono, 2002). Interestingly, the *tax-2* gene was also investigated in relation to olfactory adaptation in *C. elegans*. It was found that the gene is involved in a rapid, short-term adaptation, while the *egl-4* gene, encoding PKG, was found to be involved in slow, long-term adaptation to odors (L'Etoile et al, 2002). The pathways of *npr-1*, *tax2/tax4* and *egl-4* therefore appear to act somewhat parallel to each other to regulate the mobility and behaviour

of nematode, by regulation of the responses to relevant stimuli. If these pathways are similar in *Cotesia*, then the *npr-1* and *tax2/tax4* genes are good candidate genes, in case that no correlation is found of the *PKG* gene and larval mobility.

4.5 Investigating the correlation of PKG activity and mobility of Lepidopterans

Differences between solitary and gregarious species do not only occur within parasitoid insects, also in the order of Lepidoptera solitary and gregarious species occur. Gregariousness in Lepidopterans is also assumed to be an adaptive trait, as it is in parasitoid insects and research has been done on the evolution of gregariousness as well. Many moths and butterflies are gregarious in early larval stages, but solitary in later larval stages. Gregarious caterpillars live together at close distance to each other and also remain close to each other when moving to a new leaf. Solitary caterpillars do not live in close distance to siblings and also do not remain in groups if they encounter other caterpillars (Reader & Hochuli, 2003). Mobility thus appears to play a role in Lepidopteran larval mobility as well.

A possible advantage of gregariousness for Lepidoptera caterpillars is that large groups of caterpillars are more successful in feeding on tough leaves, which will raise the food consumption of all caterpillars. It is also possible that large groups of caterpillars are better defended against predators due to aposematism. Aposematism is a means of defense in which for example bright colors on the insect body are being used as a warning signal for enemies. Such a warning signal is after all more prominent when the caterpillars are in a large group. Beside aposematism, the use of poisonous or irritating substances can be more effective when accomplished by a large group of caterpillars. Fewer caterpillars will then be killed or parasitized in comparison to the situation in which the caterpillars have a solitary lifestyle. Another possible advantage is that the presence of many caterpillars can change the local microclimate, which will result in a better habitat for the caterpillars. The transition of gregariousness to a solitary state in late instars is possibly the result of an increased ability to feed on though leaves and an increased need for food (Reader & Hochuli, 2003).

Much of the research on Lepidopterans has been done on *Pieris* butterflies and these butterflies are also used extensively in many different research topics at the Laboratory of Entomology of Wageningen University and Research Centre. Two species are especially interesting for research on larval mobility, namely *P. brassicae* and *P. rapae*. *P. brassicae* is a gregarious butterfly. The eggs are being laid in large clutches and the caterpillars tend to remain together in large groups until their later instars. At their larval stage L3 or L4, these caterpillars will make a transition to a solitary state at which they will move large distances in

search for food. *P. rapae*, on the other hand, is a solitary butterfly. The eggs are not being laid in large clutches, but solitary and the caterpillars also do not tend to assemble in groups (Wiskerke & Vet, 1994).

It would be interesting to investigate whether these differences in mobility between *P. brassicae* and *P. rapae* are also correlated to PKG activity or *PKG* expression.

4.6 Conclusion

The main conclusion of this thesis is that no conclusion can be drawn on the correlation between expression of the *PKG* gene and larval mobility of *Cotesia*. The first experiment that should be done now is testing whether there is a correlation between the activity of PKG and larval mobility, because it appears that PKG enzyme activity correlates better to different behaviour patterns than expression of the *PKG* gene. Depending on the results of these experiments either the relation between the *PKG* gene and larval mobility can be further investigated or another candidate gene can be tested.

References

Aigner, A. (2007) Applications of RNA interference: current state and prospects for siRNA-based strategies in vivo. *Applied Microbiology and Biotechnology*, vol. 76, 9-21.

Ashraf, S. I., McLoon, A. L., Sclarsic, S. M. and Kunes, S. (2006) Synaptic protein synthesis associated with memory is regulated by the RISC pathway in *Drosophila*. *Cell*, vol. 124, 191-205.

Ben-Shahar, Y (2005) The *foraging* gene, behavioral plasticity, and honeybee division of labor. *Journal of Comparative Physiology A*, vol. 191, 987-994.

Ben-Shahar, Y, Dudek, N. L. and Robinson, G. E. (2004) Phenotypic deconstruction reveals involvement of manganese transporter *malvolio* in honey bee division of labor. *The Journal of Experimental Biology*, vol. 207, 3281-3288.

Ben-Shahar, Y., Leung, H. –T., Pak, W. L. Sokolowski, M. B. and Robinson, G. E. (2003) cGMP-dependent changes in phototaxis: a possible role for the *foraging* gene in honey bee division of labor. *The Journal of Experimental Biology*, vol. 206, 2507-2515.

Ben-Shahar, Y., Robichon, A., Sokolowski, M. B. and Robinson, G. E. (2002) Influence of gene action across different time scales of behavior. *Science*, vol. 296, 741-744.

Blencowe, B. J. (2006) Alternative splicing: new insights from global analyses. *Cell*, vol. 126, 37-47.

Boivin, G. and Baaren, J. van (2000) The role of larval aggression and mobility in the transition between solitary and gregarious development in parasitoid wasps. *Ecology Letters*, vol. 3, 469-474.

Bono, M. de and Bargmann, C. I. (1998) Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in *C. elegans*. *Cell*, vol. 94, 679-689.

Coates, J. C. and Bono, M. de (2002) Antagonistic pathways in neurons exposed to body fluid regulate social feeding in *Caenorhabditis elegans*. *Nature*, vol. 419, 925-929

Fitzpatrick, M. J., Ben-Shahar, Y., Smid, H. M., Vet, L. E. M., Robinson, G. E. and Sokolowski, M. B. (2005) Candidate genes for behavioural ecology. *Trends in Ecology and Evolution*, vol. 20, 96-104.

Fitzpatrick, M. J. and Sokolowski, M. B. (2004) In search of food: exploring the evolutionary link between cGMP-dependent protein kinase (PKG) and behaviour. *Integrative and Comparative Biology*, vol. 44, 28-36.

Fleige, S., Walf, V., Huch, S., Prgomet, C., Sehm, J. and Pfaffl, M. W. (2006) Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. *Biotechnological Letters*, vol. 28, 1601-1613.

Fujiwara, M., Sengupta, P. and McIntire, S. L. (2002) Regulation of body size and behavioral state of *C. elegans* by sensory perception and the EGL-3 cGMP-dependent protein kinase. *Neuron*, vol. 36, 1091-1102.

Godfray, H. C. J. (1994) Parasitoids: behaviour and evolutionary ecology. *Princeton University Press*.

Gray, J. M., Karow, D. S., Lu, H., Chang, A. J., Chang, J. S., Ellis, R. E., Marletta, M. A. and Bargmann, C. I. (2004) Oxygen sensation and social feeding mediated by a *C. elegans* guanylate cyclase homologue. *Nature*, vol. 430, 317-322.

Hofmann, F., Feil, R., Kleppisch, T. and Schlossmann, J. (Function of cGMP-dependent protein kinases as revealed by gene deletion. *Physiological Reviews*, vol. 86, 1-23.

Ingram, K. K., Oefner, P. and Gordon, D. M. (2005) Task-specific expression of the *foraging* gene in harvester ants. *Molecular Ecology*, vol. 14, 813-818.

Kalkman, B. and van Groningen, K. (2007) Mobiliteit en agressiviteit van de larven van *Cotesia glomerata* en *Cotesia rubecula*. Pilot experiment during student project, Laboratory of Entomology, Wageningen University and Research Centre.

Le Conte, Y., Mohammedi, A. and Robinson, G. E. (2001) Primer effects of a brood pheromone on honeybee behavioural development. *Proceedings of the Royal Society of the Royal Society of London Series B-Biological Sciences*, vol. 268, 163-168.

Lee, C. J. and Irizarry, K. (2003) Alternative splicing in the nervous system: an emerging source of diversity and regulation. *Biological Psychiatry*, vol. 54, 771-776.

L'Etoile, N. D., Coburn, C. M., Eastham, J., Kistler, A., Gallegos, G. and Bargmann, C. I. (2002) The Cyclic GMP-dependent protein kinase EGL-4 regulated olfactory adaptation in *C. elegans*. *Neuron*, vol. 36, 1079-1089.

Mayhew, P. J. and Alphen, J. M. van (1999) Gregarious development in alysiine parasitoids evolved through a reduction in larval aggression. *Animal Behaviour*, vol. 58, 131-141.

Osborne, K. A., Robichon, A., Burgess, E., Butland, S., Shaw, R. A., Coulthard, A., Pereira, H. S., Greenspan, R. J. and Sokolowski, M. B. (1997) Natural behavior polymorphisms due to a cGMP-dependent protein kinase of *Drosophila*. *Science*, vol. 277, 834-836

Osborne, K. A., Steven de Belle, J. and Sokolowski, M. B. (2001) Foraging behaviour in *Drosophila* larvae: mushroom body ablation. *Chemical Senses*, vol. 26, 223-230.

Pereira, H. S. and Sokolowski, M. B. (1993) Mutations in the larval *foraging* gene affect adult locomotory behaviour after feeding in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, 5044-5046.

Pexton, J. J. and Mayhew, P. J. (2001) Immobility: the key to family harmony? *Trends in Ecology & Evolution*, vol. 16, 7-9.

Pexton, J. J., Rankin, D. J., Dytham, C. and Mayhew, P. J. (2003) Asymmetric larval mobility and the evolutionary transition from siblicide to nonsiblicidal behavior in parasitoid wasps. *Behavioral Ecology*, vol. 14, 182-193.

Reader, T. and Hochuli, D. F. (2003) Understanding gregariousness in a larval Lepidopteran: the roles of host plant, predation, and microclimate. *Ecological Entomology*, vol. 28, 729-737.

Renger, J. J., Yao, W.-D., Sokolowski, M. B. and Wu, C.-F. (1999) Neuronal polymorphism among natural alleles of a cGMP-dependent kinase gene, *foraging*, in *Drosophila*. *The Journal of Neuroscience*, vol. 19, RC 28.

Riedl, C. A. L., Neal, S. J., Robichon, A., Westwood, J. T. and Sokolowski, M. B. (2005) *Drosophila* soluble guanylyl cyclase mutants exhibit increased foraging locomotion: behavioral and genomic investigations. *Behavior Genetics*, vol. 35, 231-244.

Robinson, G. E. and Ben-Shahar, Y. (2002) Social behavior and comparative genomics: new genes or new gene regulation. *Genes, Brains and Behavior*, vol.1, 197-203.

Rose, T. M., Henikoff, J. G. and Henikoff, S. (2003) CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. *Nucleic Acids Research*, vol. 31, 3763-3766.

Schafer, W. R. (2002) PKG and the neural basis for behavioural phenotypes. *Neuron*, vol. 21, 463-466.

Scheiner, R., Sokolowski, M. B. and Erber, J. (2004) Activity of cGMP-dependent protein kinase (PKG) affects sucrose responsiveness and habituation in *Drosophila melanogaster*. *Learning & Memory*, vol. 11, 303-311.

Sokolowski, M. B. (1980) Foraging strategies of *Drosophila melanogaster*: a chromosomal analysis. *Behavior Genetics*, vol. 10, 291-302

Tribuhl, S. (2007) Comparative study on memory phases in the parasitic wasps *Cotesia glomerata* and *Cotesia rubecula*. Master thesis project, Laboratory of Entomology, Wageningen University and Research Centre

Utsunomiya, A. and Iwabuchi, K. (2002) Interspecific competition between the polyembryonic wasp *Copidosoma floridanum* and the gregarious endoparasitoid *Glyptapanteles pallipes*. *Entomologia Experimentalis et Applicata*, vol. 104, 353-362.

Whitfield, C. W., Ben-Shahar, Y., Brillet, C., Leoncini, I., Crauser, D., LeConte, Y., Rodriguez-Zas, S. and Robinson, G. E. (2006) Genomic dissection of behavioral maturation in the honey bee. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, 16068-16075.

Wiskerke, J. S. C. and Vet, L. E. M. (1994) Foraging for solitarily and gregariously feeding caterpillars: a comparison of two related parasitoid species (Hymentoptera: *Braconidae*). *Journal of Insect Behavior*, vol. 7, 585-603.

Appendices

Appendix 1: Protocol for RNA isolation

1. The tissue of interest is collected in 1 ml of RNAwiz/TRI reagent (Ambion, Austin, USA) and is homogenized using the Sonifier B-12.
2. The solution is incubated at room temperature (RT) for at least 30 minutes to dissociate the nucleoproteins form the nucleic acids.
3. 0.2 times starting volume of RNAse free chloroform (without isoamyl alcohol or other additives) is added to the homogenate.
4. Shake the sample vigorously for 20 seconds and incubate at RT for 10 minutes.
5. Centrifuge the mixture at 14.000 rpm for 15 minutes at 4°C. The mixture separates into 3 phases: the colorless upper aqueous phase (containing the RNA), the semi-solid interphase (containing most of the DNA) and the lower organic phase.
6. Without disturbing the interphase, carefully transfer the aqueous phase into a clean, RNAse free tube.
7. Add 0.5 times starting volume of RNAse free water and mix well.
8. Add 5 µl glycoblue (Ambion, Austin, USA)
9. Add 1 times starting volume of isopropanol and mix well. Incubate for 10 minutes at RT.
10. Centrifuge 15 minutes at 4°C to pellet the RNA.
11. Decant the supernatant and wash the pellet with at least 1 times starting volume of cold 70% ethanol. Gently flick the tube to wash the pellet.
12. Centrifuge 5 minutes at 4°C to pellet the RNA and discard the supernatant.
13. Air dry the pellet for about 10 min.
14. Resuspend the RNA in an appropriate amount of RNAse free water and store at –80°C. The samples of thoraxes, larvae and brains were all resuspended in 20 µl of RNAse free water.

Appendix 2: Protocol for cDNA synthesis

Prepare master mix, per reaction, and keep on ice until step 3:

- 2 µl 5x First-Strand Buffer
- 1 µl DTT (20mM)
- 1 µl dNTP Mix (10mM)
- 1 µl BD PowerScript Reverse Transcriptase

Combine the following in separate 0.5 ml microcentrifuge tubes:

5'RACE-ready cDNA:	3'RACE-ready cDNA:
- 2 µl RNA sample	- 3 µl RNA sample
- 1 µl 5'-CDS primer	- 1 µl 3'-CDS primer A
- 1 µl RNAse inhibitor	- 1 µl RNAse inhibitor
- 1 µl BD SMART II A Oligo	

1. Mix contents and place in thermocycler.
2. Incubate the tubes at 70°C for 3 minutes.
3. When the temperature has reached 42°C, place mastermix in thermocycler, wait 30 seconds and then add 5 µl to each reaction. Mix by pipetting and incubate with hot lid closed for 1 hour.

Appendix 3: PCR-mix

PCR-mix for Advantage 2 polymerase (1 reaction)		PCR-mix for GoTaq (1 reaction)	
Advantage Taq	0,5 µl	GoTaq	0,125 µl
dNTP	0,5 µl	dNTP	0,5 µl
forward primer (10 nmol ml^{-1})	0,5 µl	forward primer (10 nmol ml^{-1})	0,5 µl
reverse primer (10 nmol ml^{-1})	0,5 µl	reverse primer (10 nmol ml^{-1})	0,5 µl
Advantage buffer	2,5 µl	GoTaq buffer	5 µl
template	1 µl cDNA or 0,5 µl plasmid DNA	template	1 µl cDNA or 0,5 µl plasmid DNA
		$MgCl_2$	1,5 µl
H_2O	to a total volume of 25 µl	H_2O	to a total volume of 25 µl

Appendix 4: PCR-mix for making probes

PCR-mix for probes with 25% DIG		PCR-mix for probes with 50% DIG	
GoTaq	0,25 µl	GoTaq	0,25 µl
AGCT-mix (75% T)	2 µl	AGCT-mix (50% T)	2 µl
DIG-11-dUTP	1 µl	DIG-11-dUTP	2 µl
forward primer (10 nmol ml^{-1})	1 µl	forward primer (10 nmol ml^{-1})	1 µl
reverse primer (10 nmol ml^{-1})	1 µl	reverse primer (10 nmol ml^{-1})	1 µl
GoTaq buffer	10 µl	GoTaq buffer	10 µl
plasmid DNA	0,5 µl	plasmid DNA	0,5 µl
$MgCl_2$	3,5 µl	$MgCl_2$	3,5 µl
H_2O	30,75 µl	H_2O	29,75 µl

When making a probe, you always make a control sample. For this purpose twice the amount of a normal PCR-mix is used, with the same primers and template as in the mix for the probe. The AGCT-mix can be made by mixing 65 µl H_2O, 4 µl of dATP, dGTP and dCTP and 2 µl (50%) or 3 µl (75%) of dTTP

Appendix 5: Protocol for Southern blotting (see appendix 7 for blot buffers)

* ***Cutting the genomic DNA***

1. Cut 5 to 10 µg of genomic DNA with restriction enzymes for 1 hour at 37°C. The mixture should have a total volume of 20 µl. Add buffer, restriction enzyme and BSA as instructed by the manufacturer. Also make a control sample, containing at least 10 pg of plasmid DNA.
2. Stop the reaction by adding 1 µl 0,5 M EDTA and incubate at 65°C for 10 minutes.

* ***Running the DNA on agarose gel***

1. Run the samples on a 0.6% agarose gel in 1x TAE, with 3,5 µl ethidium bromide. Also run markers to determine the length of the fragments.
2. Run the gel at 60 V for approximately two hours.
3. Make a picture of the gel and use it for blotting.

* ***Pretreatment of the gel***

1. Denature the gel 2x15 minutes in 0,5 M NaOH; 1,5 M NaCl (while shaking). Rinse the gel in mQ.
2. Neutralize the gel 2x15 minutes in 0,5 M Tris-HCl (pH 7,5); 1,5 M NaCl (while shaking). Rinse the gel in mQ.
3. Incubate the gel in 20xSSC (Invitrogen, Paisly, United Kingdom) for at least 5 minutes.

* ***Pretreatment of the Hybond-N***

1. Wet the Hybond-N (Amersham pharmacia biotech, Buckinghamshire, United Kingdom) in mQ.
2. Incubate the Hybon-N in 20xSSC for at least 5 minutes.

* ***Blotting***

1. Put a gel tray upside-down in a plastic holder and fill the holder with 20xSSC.
2. Wet three Whatmann papers and put them on the tray. Remove air bubbles (1 paper should be longer than the tray in order to have contact with the 20xSSC, all other papers should have a similar size as the gel).
3. Put the gel (slots down) on the tray and put the wetted Hybond-N on the gel.
4. Wet three Whatmann papers and put them on the Hybond-N, remove air bubbles.
5. Put a pile of papers on the on top of the gel and Hybond-N and put a plastic lid on the pile. Put a bottle (500 to 750 gram) on top of the lid and blot overnight.

* ***(Pre-)Hybridization***

1. Turn the gel and the blot upside-down and put them on a dry piece of Whatmann paper. Mark the lanes of the gel on the blot with a pencil.
2. Put the blot on a piece of Whatmann paper (pre-soaked in 2xSSC) and UV-crosslink the blot (UV Stratalinker 2400, 120000 µ joules). Rinse the blot in mQ and put the blot in a hybridization-tube, with the DNA on the inside.
3. Pre-hybridize the blot in 75 ml of Church buffer for 1 hour at 70^{o}C.
4. Dilute the labeled probe (10 µl) in RNAse free water to a volume of 20 µl. Boil the labeled probe for 5 minutes and chill shortly on ice.
5. Hybridize the blot overnight at 60-70^{o}C in 15 ml Churchbuffer with the 20 µl labeled probe.

* ***Stringent wash***

1. Pour off the probe/buffer. This can be stored at –20^{o}C and be used in a next blot.
2. 2x5 minutes washing with 2xSSC, 0,1% SDS (100 ml per wash).
3. 2x5 minutes washing at 65^{o}C with 0,5xSSC, 0,1% SDS (or more stringent with 0,1xSSC, 0,1% SDS).

* ***Detection (at RT)***

1. 2 minutes washing with Washing buffer.
2. Incubate 30 minutes in 100 ml Buffer 2 (freshly made).
3. Dilute anti-DIG-AP conjugate (vial 3, Roche Diagnostics, Mannheim, Germany) 1:10000 in Buffer 2: 2 µl anti-DIG-AP in 20 ml Buffer 2.
4. Incubate 30 minutes in 20 ml antibody solution.
5. 2x15 minutes washing in 100 ml Washing buffer.
6. 3 minutes washing in 20 ml Buffer 3.
7. Dilutes CSPD (vial 5, Roche Diagnostics, Mannheim, Germany) 1:100 in Buffer 3: 20µl CSPD + 1980 µl Buffer 3.
8. Seal the membrane in plastic with 2 ml CSPD solution for 5 minutes.
9. Pour off the solution and blot the membrane shortly on Whatmann paper (DNA side up!!)
10. Seal the damp membrane in plastic and incubate 5 to 15 minutes at 37^{o}C to increase the luminescent reaction.
11. Record the chemiluminescent reaction using the photograph machine.

Appendix 6: Protocol for Northern blotting (see appendix 7 for blot buffers)

1. Agarose/Formaldehyde Gel Electrophoresis

- Prepare gel (100 ml): Dissolve 0.8 g agarose in 72 ml water and cool to 60 °C in a water bath. When the flask has cooled to 60 °C, place in a fume hood and add 10 ml of 10xMOPS-buffer (Eppendorf, Hamburg, Germany), 18 ml formaldehyde. Pour the gel and allow it to set. Remove the comb, place the gel in the gel tank, and add sufficient 1xMOPS-buffer to cover to a depth of ~ 1mm.
- Prepare sample: Adjust the volume of each RNA sample to 6 µl with water. Boil the positive control (control PCR product from the probe PCR) for 10 minutes and chill shortly on ice. Then add 2.5 x 6 µl RNA gel loading buffer (eppendorf, stored at -20°C). Mix by vortexing, microcentrifuge briefly to collect liquid, and incubate 10 min at 65 °C. Load on the gel.
- Run gel: Run the gel in 1xMOPS-buffer at 100 volt for 10 min, then at 65 volt for 90 min. Take picture.

2. Transfer of RNA from Gel to Membrane

- Incubate gel 5 min. in 20x SSPE (Invitrogen, Paisly, United Kingdom). Fill a clean RNAse-free tray (large square Petri-dish) half with 20x SSPE. Place an upside down gel tray. Wet 3 pieces of Whatman paper (gel size) in 20x SSPE buffer and place on the tray. The first piece should hang over the 2 edges of the tray so it comes into contact with the buffer. Remove air-bubbles with Pasteur pipette. Place gel upside down on the paper (cut one corner of the gel). Remove air-bubbles. Cut 4 pieces of foil and place it around the gel. Cut a piece of nylon membrane (Hybond-N+) just large enough to cover the gel and wetted in water and then in 20x SSPE. Place the wetted membrane on the surface of the gel. Try to avoid getting air bubbles under the membrane. Wet 3 pieces of Whatman paper (gel size) in buffer and place on the gel. Remove air-bubbles with Pasteur pipette. Place stack of paper (at least 6 cm high) and

the plastic lid on top of the Whatman paper. Add weight to keep everything in place and incubate overnight.

3. Hybridization Analysis

- Prepare membrane for hybridization: Remove paper towels and filter papers and recover the membrane and flattened gel. Mark the position of the wells on the membrane with pencil and ensure that the up-down and back-front orientation are recognizable. Rinse the membrane in 5xSSPE, then place it on a sheet of Whatman paper (RNA-side up) and allow drying (not completely). Place on a wetted (in 2x SSPE) Whatman paper and place in UV-crosslinker (UV Stratalinker 2400, 120000 μ joules), irradiate using the auto settings. Wash blot in water and place in a hybridization tube RNA-side up.
- Pre-hybridization: preheat ULTRAhyb (Ambion, Austin, USA) to 68°C. Swirl the buffer until all precipitated material has dissolved. Add 15 ml ULTRAhyb-hybridization solution, then place the tube in the hybridization oven and incubate with rotation for 30 min at 42 °C.
- Probe preparation: Denature Double-stranded probe (10 μl probe + 10 μl RNAse-free water) by heating in a water bath for 10 min at 100 °C, then transfer to ice. Centrifuge shortly.
- Hybridization: add the probe to the ULTRAhyb solution. Continue to incubate with rotation overnight at 42 °C
- Stringent wash: 2 x 5 min. wash with 2 x SSC, 0.1% SDS at 42°C(100 ml each). 2 x 15 min. wash with 0.1 x SSC, 0.1% SDS at 42 °C(100 ml each, pre-warm the wash solution).
- Detection (at RT): similar to detection in Southern blotting

Appendix 7: Buffers for blotting

*** 0,5 M NaOH; 1,5 M NaCl** (1st step pretreatment gel, Southern blot)
- 20 gram NaOH (solid pellets)
- 87,66 gram NaCl
- Add mQ to a volume of 1 liter and autoclave

*** 0,5 M Tris-HCl (pH 7,5); 1,5 M NaCl** (2nd step pretreatment gel, Southern blot)
- 60,57 gram Tris (Trizma base)
- 87,66 gram NaCl
- Add 800 ml of mQ and dissolve the ingredients
- Lower the pH to 7,5 using concentrated HCl
- Add mQ to a volume of 1 liter and autoclave

*** 10% SDS** (for use in stringent wash)
- 100 gram SDS
- Add 900 ml mQ and heat to 68°C to dissolve the SDS
- Lower the pH to 7,2 with concentrated HCl
- Add mQ to a volume of 1 liter and autoclave

*** 1 M NaHPO4 (pH 7,2)** (needed for preparation of Churchbuffer)
- 89 gram $Na_2HPO_4 \cdot 2H_2O$
- 4 ml 85% H_3PO_4
- Adjust pH to 7,2
- Add mQ to a volume of 1 liter and autoclave

*** Church buffer** ((pre)hybridization of Southern blot)
- 2 ml 0,5 M EDTA
- 70 gram SDS
- 500 ml 1 M $NaHPO_4$ (pH 7,2)
- Add mQ to a volume of 1 liter. Does not need to be autoclaved, but can be heated to dissolve ingredients.

*** 2 x SSC; 0,1% SDS** (first step stringent wash, make freshly)
- 10 ml 20 x SSC
- Add mQ to 99 ml
- 1 ml 10% SDS

*** 0,1 x SSC; 0,1% SDS** (second step stringent wash, make freshly)
- 0.5 ml 20 x SSC
- Add mQ to 99 ml
- 1 ml 10% SDS

*** Buffer 1 (Maleic acid buffer, 0,1 M maleic acid; 0,15 M NaCl (pH 7,5) with solid NaOH))**
- 11,607 gram Maleic acid
- 8,766 gram NaCl
- Add 800 ml mQ and dissolve the ingredients

- Adjust pH to 7,5 (20°C) using solit NaOH
- Add mQ to a volume of 1 liter and autoclave

* **Washing buffer (Maleic acid buffer + 0,3% Tween 20 (v/v))**
  - 3 ml Tween 20
  - 997 ml Maleic acid buffer (buffer 1)
* **Blocking Stock solution (10x)(10% (w/v) blocking reagent (vial 4), in Maleic acid buffer**)
  - 10 gram Blocking reagent (vial 4, Roche Diagnostics, Mannheim, Germany)
  - Add Maleic acid buffer (buffer 1) to a volume of 100 ml
  - Dissolve by stirring at 65°C or by heating in the microwave
  - Autoclave and store at 4°C
* **Buffer 2 (1x blocking solution: 10x diluted blocking stock solution in Maleic acid buffer)**
  - 10 ml 10x Blocking Stock solution
  - 90 ml Maleic acid buffer (buffer 1)
* **Buffer 3 (Detection buffer = 0,1 M Tris-HCl; 0,1 M NaCl; 50 mM $MgCl_2$ (pH 9,5, 20°C))**
  - 6,057 gram Tris (Trizema base)
  - 2,922 gram NaCl
  - Add 400 ml of mQ and dissolve ingredients
  - Adjust pH to 9,5 (20°C)
  - 5 ml 1 M $MgCl_2$ (4°C)
  - Add mQ to a volume of 500 ml en autoclave

Appendix 8: Sequences of *C. glomerata* and *C. rubecula*

1. *PKG* sequence obtained from the CODEHOP procedure

cagcccaacatagtagcctgatggatccagaccggtgtcagctgtctcgttaatagaccgagagacattcaatcaattaatatcctcggctggatgaaattagaacgc
gctacaaagatgattagttgagcgcagaagaataaacgaagaattccgtgatttacgtctgggagatctccattccttagcaaccttaggagttggtggattcggtcga
gtagaattagtccaaataaagggtgacagtagccgctcgtttgccctccaagcaaatgaagaaatcacaaattgtagagactcgtcagcagcagcacataatgtcag
aaaaaagaatcatgggtgaagccgattcggactttattgtaaaactgttcaagacatttaaagaccgcaagtatctgtacatgcttatggaagcctgccttggtggaga
attgtggaccatcttacgtgacaaaggtcactttgatgacggtaccacccaggttctacaccggatgtgtcgttgaagcttttgattacttgcactcgcggaatatcatct
accgcgatttaaagccggagaacttactgctagatagtcatggttatgttaaattagtcgactttggtttcgcaaaacgtctggatcacggtaaaaaaacttgggacattc
tgcgcgcgccggagtacgttgcgccagmagttatttaaatagaggtcacgatattagtgccgattattggtcactgggtgttctaatgtttgaattattgaccggtgca
cctccatttactggcgcagaccccatgaaaacttacaatattattcstaaagggaattgacgctataggatttcctagaactattaccagaaatgctactaatttaattaaa
aaactctgccgcgataaccc

2. The longest fragment of *PKG* from the 3'RACE (*C. glomerata)*

ccggatgtgtcgttgaagcttttgattacttgcactcgcggaatatcatctaccgcgatttaaagccggagaacttactgctagatagtcatggttatgttaaattagtcga
ctttggtttcgcaaaacgtctggatcacggtaaaaaaacttggacattctgcggcacgccggagtacgttgcgccagaagttattttaaatagaggtcacgatattagt
gccgattattggtcactgggtgttctaatgtttgaattattgaccggtgcacctccatttactggcgcagaccccatgaaaacttacaatattattctaaagggaattgacg
ctataggatttcctagaactattaccagaaatgctactaatttaattaaaaaactctgccgcgataaccctgctgagcgattgggatatcaaaaaggcggaattagtgaa
attcaaaaacacaaatggttcgatggtttcaactgggaaggtctgagagcaaggacactggagcctccaataatgcccagggtacaaaacgccactgacaccgcta
acttcgacgaataccctccagactcagaacctccacctcctgatgacgtctctggctgggacaatgatttttaattgttatcgttttattttaattaattaataattattattaga
gcggcaaaaaaaccaacattaaaaatttttttttacatttaagtaattaaaaaaaatttttatcaattatatgaaaaatttttaaaaagttggtctccaatcgctctattgttatt
tttattataattattattattatcagtgattgcaagtcttccaaatcaaaataattattccaattgtttttctgaattttgttattaaaatttaatgataataataattataaataataat
ttgtatagttaccagtaaatttaaaaaatttgaaaaaatttaataggaaacttaaaaaaaaaactagataattataaaatagatagagaaaaaataaaataagggcctgtg
ataattaaaaatgaaattaggccactagggatttattattgtgcatattaaatttttattaatcaattaataagttgaatttatttaattaattaataaatgaatgtctgaagagtg
aatggaatacatttttttttttttcaattgaaatttaattatattcaattctgtttaattaattgtgccagtgtgtcacgtgccattgtaattaaatcaaaatcgagtgtcgattttat
caaacatttttttttctggtgaataaattcttgccggtaatatttttcatgaaatacactcgcttaaaaaaaaaaaaaagttataaatttttaaaggcaaatatttaatttaattaaatt
tattatatatttttttttattatatataaagtatatgtatttttcattgatttattgtaaagaatatattaacttttaaatattgtaaataaaaatatacaattgtatttaatgagtgtaatgct
ctaattctttaataaaaatcaacaaaatataaaaaaaaaaaaaaaaaaaaaaaaaaagt

3. The longest fragment of *PKG* from the 5'RACE (*C. glomerata)*

agttaaactaattaattattttaaaaaatctagatcttaaatcggtgcgacgtaactcgggatcgatctgtgattgttaatcgctgaccgctgattgtcggagcctcggcta
gagggcgtatccatttaaatgtttacttcaaaccaccgggtgcaatttagacgtaattagcattaataataacaataataaaaataaaaataaacaaaaaaagataacgtt
gaatgaacgggaacgagtgtggcataataaacgacgccggcgtgtctatgcgcgtgtgctttgacagtttgtgcttctcgtcgacgcagcacaggctcgccgacga
ggaagacgcatttcagctccggtctaacggcacggcaatcgttgtctcgtcgagttccacgtcgccgtcgacttctccgacggctgttcccgttcctggtcctaatttaa
acgcaatcacggcgaccacttgcagcgagggactgggtcaagttttggcccgggccgctactcctccaagcaacaactctaccttgactcctgccatggagaagct
ccgggaactccaggagcttctgaggactaaagatgaaaggatctcccagcttgaggctactcttacgaaaaaggacaatgaaattcaggacctcaggagtcatctg
gataaattcatcagtatcatgtccatcaagtctcctcttacgcctactaagtccaggcccaggaaacaacgcgctcagggtatttctgcggagccgcctcttcatcagct
ggctccgctcaacttttatgacaagagcgacagatcacgggaacttataaaagcggctattttggacaatgatttcatgaaaaatctggagttgacccaaataagaga

aattgttgattgtatgtacccagtgagttttcctgctggagctattattattcaggaaggcgatgttggatctactgtttatgttatggaagagggaaaagttgaggtttcta
gagatggaaagtatcttagcacactggctcccgttaaagtgttgggtgaattggctattctttataattgcaagaggacggcaactattactgctgctactgactgtcaat
tatgggccattgatcgacaatgcttccagactattatgatgagaactggattgtcgagacaggctgaatatactgatttcttgaaaagtgtacctatcttcaaaaacttacc
cgaagaaacattaataaaaatctccgatgttttagaagagacattttataataacggtgattacataatacggcaaggagctcgaggcgacactttcttcataataagtc
gcggtcaggtgcgcgtgacaataaaacaaccggatacaactaatgaaaagtacatcaggacacttggcaaaggcgatttcttcggcgagaaggcattacaagggg
atgatttaagaacagccaacatagtagctgatgatccagacggtgtcagctgtctcgtaatagaccgagagacattcaatcaattaatatcctcgctggatgaaattag
aacgcgctacaaagatgaattagttgagcgcagaagaataaacgaagaattccgtgatttacgtctgggagatctccattccttagcaaccttaggagttggtggattc
ggtcgagtaga

4. The ORF of the *PKG* gene of *C. glomerata* (2,3 kb)

atgaacaggaacgagtgtggcataataaacgacgcctgcgtgtctatgcgcgtgtgctttgacagtttgtgcttctcgtcgacgcagcacaggctcgccgacgagg
aagacgcatttcagctccagtctaacggcacggcaatcgttgtctcgtcgagttccacgtcgccgtcgagttctccgacggctgttcccgttcctggtcctaatttaaac
gcaatcacggcgaccacttgcagcgagggactgggtcaagttttggcccgggccgctactcctccaagcaacaactctaccttgactcctgccatggagaagctcc
gggaactccaggagcttctgaggactaaagatgaaaggatctcccagcttgaggctactcttacgaaaaaggacaatgaaattcaggacctcaggagtcatctgga
taaattcatcagtatcatgtccatcaagtctcctcttacgcctactaagtccaggcccaggaaacaacgcgctcagggtatttctgcggagccgcctcttcatcagctga
ctccgctcaagttttatgacaagagcgacagatcacgggaacttataaaagcggctattttggacaatgatttcatgaaaaatctggagttgacccaaataagagaaat
tgttgattgtatgtacccagtgagttttcctgctggagctattattattcaggaaggcgatgttgggtctactgtttatgttatggaagagggaaaagttgaggtttctagag
atggaaagtatcttagcacactggctcccgttaaagtgttgggtgaattggctattctttataattgcaagaggacggcaactattactgctgctactgactgtcaattatg
ggccattgatcgacaatgcttccagactattatgatgagaactggattgtcgagacaggctgaatatactgatttcttgaaaagtgtacctatcttcaaaaacttacccga
agaaacattaataaaaatctccgatgttttagaagagacattttataataacggtgattacataatacggcaaggagctcgaggcgacactttcttcataataagtcgcg
gtcaggtgcgcgtgacaataaaacaaccggatacaactaatgaaaagtacatcaggacacttggcaaaggcgatttcttcggcgagaaggcattacaaggggatg
atttaagaacagccaacatagtagctgatgatccagacggtgtcagctgtctcgtaatagaccgagagacattcaatcaattaatatcctcgctggatgaaattagaac
gcgctacaaagatgaattagttgagcgcagaagaataaacgaagaattccgtgatttacgtctgggagatctccattccttagcaaccttaggagttggtggattcggt
cgagtagaattagtccaaataaagggtgacagtagccgctcgtttgccctcaagcaaatgaagaaatcacaaattgtagagactcgtcagcagcagcacataatgtc
agaaaaaagaatcatgggtgaagccgattcggactttattgtaaaactgttcaagacatttaaagaccgcaagtatctgtacatgcttatggaagcctgccttggtgga
gaattgtggaccatcttacgtgacaaaggtcactttgatgacggtaccaccaggttctacaccggatgtgtcgttgaagcttttgattacttgcactcgcggaatatcatc
taccgcgatttaaagccggagaacttactgctagatagtcatggttatgttaaattagtcgactttggtttcgcaaaacgtctggatcacggtaaaaaaacttggacattc
tgcggcacgccggagtacgttgcgccagaagttatttaaatagaggtcacgatattagtgccgattattggtcactgggtgttctaatgtttgaattattgaccggtgca
cctccatttactggcgcagaccccatgaaaacttacaatattattctaaagggaattgacgctataggatttcctagaactattaccagaaatgctactaatttaattaaaa
aactctgccgcgataaccctgctgagcgattgggatatcaaaaaggcggaattagtgaaattcaaaaacacaaatggttcgatggtttcaactgggaaggtctgaga
gcaaggacactggagcctccaataatgcccagggtacaaaacgccactgacaccgctaacttcgacgaataccctccagactcagaacctccacctcctgatgac
gtctctggctgggacaatgatttttaa

5. The protein sequence from the ORF of the *PKG* gene of *C. glomerata* (2,3 kb)

mnrnecgiindacvsmrvcfdslcfsstqhrladeedafqlqsngtaivvsssstspsssptavpvpgpnlnaitattcseglgqvlaraatppsnnstltpamekl
relqellrtkderisqleatltkkdneiqdlrshldkfisimsiksspltptksrprkqraqgisaepplhqltplkfydksdrsrelikaaildndfmknleltqireivd
cmypvsfpagaiiiqegdvgstvyvmeegkvevsrdgkylstlapvkvlgelailynckrtatitaatdcqlwaidrqcfqtimmrtglsrqaeytdflksvpif
knlpeetlikisdvleetfynngdyiirqgargdtffiisrgqvrvtikqpdttnekyirtlgkgdffgekalqgddlrtanivaddpdgvsclvidretfnqlisslde
irtrykdelverrrineefrdlrlgdlhslatlgvggfgrvelvqikgdssrsfalkqmkksqivetrqqqhimsekrimgeadsdfivklfktfkdrkylymlme
aclggelwtilrdkghfddgttrfytgcvveafdylhsrniiyrdlkpenllldshgyvklvdfgfakrldhgkktwtfcgtpeyvapevilnrghdisadywslg
vlmfelltgappftgadpmktyniilkgidaigfprtitrnatnlikklcrdnpaerlgyqkggiseiqkhkwfdgfnweglrartleppimprvqnatdtanfde
yppdsepppddvsgwdndf

6. The ORF of the *PKG* gene of *C. rubecula* (2 kb)

atggagaaactccgggaactccaggagcttctgaggactaaagatgaaaggatttcccagcttgaggctactctcacgaaaaaggacaatgaaattcaggacctca
ggagtcatctggataaatttattagtatcatgtccattaaatcacctcttacgcctactaagtccaggcccaggaaacaacgggctcagggtatttctgcggagccgcct
cttcatcagctgactccgctcaagttttatgacaagagcgacagatcacgggaacttataaaagcggctattttggacaatgattttatgaaaaatctggagttgaccca
aataagagaaattgttgattgtatgtacccagtgagttttcctgctggagctattattattcaggaaggagatgtcgggtctactgtttatgttatggaagagggaaaagtt
gaggtttctagagatggaaagtatcttagcacactggctcccgttaaagtgttgggtgaattggctattctttataattgcaagaggacggcaactattactgctgctact
gattgtcaattatgggccattgatcgacaatgcttccagactattatgatgagaactggattgtcgagacaggctgaatatactgatttcttgaaaagtgtacctatcttca
aaaacttacccgaagaaacattaatcaaaatctccgacgttttagaagagacattttataataacggtgattacataatacggcaaggagctcgaggcgacactttcttc
ataataagtcgcggtcaggtgcgcgtgacaataaaacaaccggatacaactaatgaaaagtacatcaggacacttggcaaaggcgatttctttggcgagaaggcatt
acaaggggatgatttaagaacagccaacatagtagctgatgatccagacggtgtcagctgtctcgtaatagaccgagaaacattcaatcaattaatatcctcgctgga
tgaaattagaacgcgttacaaagatgaattagttgagcgtagaagaataaacgaagaattccgtgatttacgtctgggagatctccattccctagcaaccttaggagtt
ggtggatttggtcgagtagaattagtccaaataaaaggtgacagtagccgttcgtttgccctcaagcaaatgaagaaatcacaaattgtagagactcgtcagcagcag
cacataatgtcagaaaaacgaatcatgggtgaagctgattcggactttatcgtaaaactgttcaagacattcaaagaccgcaagtatctgtacatgctgatggaagcct
gtcttggtggagaattgtggaccatcttacgtgacaaaggtcactttgatgacggtaccaccaggttctacaccggatgtgttgttgaagcttttgattacttgcactccc
ggaatatcatctaccgcgatttaaagccggaaaacttattgctagatagtcatggttatgttaaattagtggactttggtttcgcaaaacgtctggaccacggaaaaaaa
acttggacattctgcggcacgccggagtacgttgcgccagaagttatttaaacagaggtcacgatattagtgccgattattggtcactgggtgttctaatgtttgaatta

ttgaccggtacacctcctttcactggcgcagaccccatgaaaacttacaatattattttgaagggaattgacgctataggatttccaagaactatcaccagaaatgctact
aatttaattaaaaaactctgccgcgataaccctgccgaacgattgggatatcaaaaaggcggaattagtgaaattcaaaaacacaaatggttcgatggtttcaactggg
aaggtctgagagcaaggacactggagcctccaataatgccccgggtacaaaacgccactgacaccgctaacttcgacgaataccctccagactcagaacctccac
ctcctgatgacgtctctggctgggacaatgatttt

7. The protein sequence from the ORF of the *PKG* gene of *C. rubecula* (2 kb)

meklrelqellrtkderisqleatltkkdneiqdlrshldkfisimsikspltptksrprkqraqgisaepplhqltplkfydksdrsrelikaaildndfmknleltqir
eivdcmypvsfpagaiiiqegdvgstvyvmeegkvevsrdgkylstlapvkvlgelailynckrtatitaatdcqlwaidrqcfqtimmrtglsrqaeytdflks
vpifknlpeetlikisdvleetfynngdyiirqgargdtffiisrgqvrvtikqpdttnekyirtlgkgdffgekalqgddlrtanivaddpdgvsclvidretfnqlis
sldeirtrykdelverrrineefrdlrlgdlhslatlgvggfgrvelvqikgdssrsfalkqmkksqivetrqqqhimsekrimgeadsdfivklfktfkdrkylym
lmeaclggelwtilrdkghfddgttrfytgcvveafdylhsrniiyrdllkpenllldshgyvklvdfgfakrldhgkktwtfcgtpeyvapevilnrghdisady
wslgvlmfelltgtppftgadpmktyniilkgidaigfprtitrnatnlikklcrdnpaerlgyqkggiseiqkhkwfdgfnweglrartleppimprvqnatdta
nfdeyppdsepppddvsgwdndf

8. The ORF of the *PKG* gene of *C. rubecula* (1 kb)

atggagaaactccgggaactccaggagcttctgaggactaaagatgaaattagaacgcgttacaaagatgaattagttgagcgtagaagaataaacgaagaattcc
gtgatttacgtctgggagatctccattccctagcaaccttaggagttggtggatttggtcgagtagaattagtccaaataaaaggtgacagcagccgttcgtttgccctc
aagcaaatgaagaaatcacaaattgtagagactcgtcagcagcagcacataatgtcagaaaaacgaatcatgggtgaagctgattcggactttatcgtaaaactgttc
aagacattcaaagaccgcaagtatctgtacatgctaatggaagcctgtcttggtggagaattgtggaccatcttacgtgacaaaggtcactttgatgacggtaccacca
ggttctacaccggatgtgttgttgaagcttttgattacttgcactcccggaatatcatctaccgcgatttaaagccggaaaacttattgctagatagtcatggttatgttaaa
ttagtggactttggtttcgcaaaacgtctggaccacggaaaaaaaacttggacattctgcggcacgccggagtacgttgcgccagaagttatttttaaacagaggtcac
gatattagtgccgattattggtcactgggtgttctaatgtttgaattattgaccggtacacctcctttcactggcgcagaccccatgaaaacttacaatattattttgaaggg
aattgacgctataggatttccaagaactatcaccagaaatgctactaatttaattaaaaaactctgccgcgataaccctgccgaacgattgggatatcaaaaaggcgga
attagtgaaattcaaaaacacaaatggttcgatggtttcaactgggaaggtctgagagcaaggacactggagcctccaataatgccccgggtacaaaacgccactg
acaccgctaacttcgacgaataccctccagactcagaacctccacctcctgatgacgtctctggctgggacaatg

9. The protein sequence from the ORF of the *PKG* gene of *C. rubecula* (1 kb)

meklrelqellrtkdeirtrykdelverrrineefrdlrlgdlhslatlgvggfgrvelvqikgdssrsfalkqmkksqivetrqqqhimsekrimgeadsdfivklf
ktfkdrkylymlmeaclggelwtilrdkghfddgttrfytgcvveafdylhsrniiyrdllkpenllldshgyvklvdfgfakrldhgkktwtfcgtpeyvapevil
nrghdisadywslgvlmfelltgtppftgadpmktyniilkgidaigfprtitrnatnlikklcrdnpaerlgyqkggiseiqkhkwfdgfnweglrartleppim
prvqnatdtanfdeyppdsepppddvsgwdn

10. The sequence of the *18S* gene (400 bp) of *C. glomerata*

cagccgcggtaattccagctccaatagcgtatattaaagttgttgcggttaaaaagctcgtagttgaatttgtgtttcatactattggttcatcgctttgcggtgttaactggt
atgttatgaaacgtcctaccggtggatttagctgtaaaaggcgacatccaatttaatcctatcgcggtgctcttaattgagtgtcgaggtaggccggtacgcttactttga
acaaattagagtgcttaaagcaggcttatttcgcctgaatactgtgtgcatggaataatagaataggacctcagttctattttgttggttttcggaatactgaggtaatgatt
aatagggacagatgggggcattcgtattgcgacgttagaggtgaaattcttggatcgtcgcaagacgaacagaagcgaaagcatttgccaaaaatg

11. The sequence of the *18S* gene (400 bp) of *C. rubecula*

cagccgcggtaattccagctccaatagcgtatattaaagttgttgcggttaaaaagctcgtagttgaatttgtgtttcatactattggttcatcgctttgcggtgttaactggt
atgttatgaaacgtcctaccggtggatttagctgtaaaaggcgacatccaatttaatcctatcgcggtgctcttaattgagtgtcgaggtaggccggtacgtttactttga
acaaattagagtgcttaaagcaggcttatttcgcctgaatactgtgtgcatggaataatagaataggacctcagttctattttgttggttttcggaatactgaggtaatgatt
aatagggacagatgggggcattcgtattgcgacgttagaggtgaaattcttggatcgtcgcaagacgaacagaagcgaaagcatttgccgaggacg

Appendix 9: RT-PCR mix

RT-PCR-mix (1 reaction)	
Absolute QPCR SYBR-green mix (Thermo Fisher Scientific, ABgene, Epsom, United Kingdom)	12,5 µl
forward primer (10 nmol ml^{-1})	0,5 µl
reverse primer (10 nmol ml^{-1})	0,5 µl
cDNA	1 µl
H_2O	10,5 µl