WAGENINGEN UNIVERSITY
LABORATORY OF ENTOMOLOGY

Artificial selection on learning rate in *Cotesia glomerata*: a behavioral and molecular analysis

No.: 08.12
Naam/Name: Loes Duivenvoorde
Period: October - April 2008
1st Examiner: Ir. Michaël van den Berg
2nd Examiner: Dr. Hans Smid

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Preface

This thesis project forms a part of my Master studies in Biology. By finishing this report, I am close to finishing my first 'real' scientific project. I really enjoyed my time at Entomology. In particular, I liked the combination between the behavioral experiments and the molecular work because this enabled me to really connect the insect's behavior to actual neuronal changes. Working with the wasps was sometimes a bit frustrating, but, in overall, it was amazing to observe their behavior. I think that *C. glomerata* and *C. rubecula* form suitable organisms to study the evolution of learning, as well as the neuronal background of memory formation.

In this thesis I learned a lot about the behavior of insects, I got familiar with several molecular techniques and with scientific work in general. Therefore, I would like to thank my supervisors Michaël van den Berg and Hans Smid for their help and advice during the experimental work, and for their useful comments, while writing this report. Furthermore, I would like to thank Tibor Bukovinszky for his help with the statistical analysis and, finally, I would like to thank everyone of the Entomology group for the nice working atmosphere.

Summary

This thesis project is dedicated to learning behavior of the parasitic wasp species *Cotesia glomerata*. In adult life the females of this species depends on olfactory cues to find suitable hosts. Environmental circumstances during this important task change constantly, so remembering on which plant species hosts can be found, is very useful. The natural tendency of *C. glomerata* is to search for cabbage plants, like Brussels sprouts, because the chance to find host larvae on this plant species is large. However, C*. glomerata* can change its preference for cabbage when suitable hosts are found on other plant species, such as nasturtium. This process occurs according to the principle of olfactory associative learning, which can subsequently lead to memory formation. Memory formation can be divided in three different phases: short-term memory (STM), anesthesia-resistant memory (ARM), and long-term memory (LTM). In most insect species, LTM can only be formed after multiple learning experiences, spaced in time (spaced learning). However, *C. glomerata* learns exceptionally fast; it forms LTM already after one oviposition experience, while the closely related species *Cotesia rubecula*, for example, needs at least three experiences to form LTM. Furthermore, the ARM phase is expected to be reduced in *C. glomerata,* while it is present in *C. rubecula*

Under natural circumstances, it is expected that learning evolves according to the demands that are imposed on an animal by its ecology. Ultimate factors that may have contributed to the evolution of *C. glomerata* towards fast learning are associated to its life style. Larvae of the preferred host of *C. glomerata,* for example, are spread in a more predictable way than the larvae of the host of *C. rubecula.* Due to this difference in host distribution, information during host searching behavior is less variable for *C. glomerata* than it is for *C. rubecula*. High predictive values of information acquired during host searching of *C. glomerata* may have favored the evolution of fast learning in this species, while slow learning was a useful characteristic during evolution of *C. rubecula*.

Under laboratory circumstances, phenotypical traits, such as learning rate, can be altered by applying artificial constrains. Successful artificial selection on learning has already been reported in both fruit flies and Cape bees.

At the molecular level, differences in the efficiency at which LTM is formed, can be correlated to the expression of a protein in the brain called cAMP responsive element binding protein (CREB). CREB regulates protein synthesis by activating other genes, which is a prerequisite for the formation of LTM. In many animals, different isoforms of the CREB gene transcript exist; activating isoforms stimulate gene transcription, and thereby LTM formation, while repressor isoforms inhibit gene transcription. If LTM is formed is thought to depend on the balance between activating CREB isoforms and repressor isoforms. Therefore, the relative concentration of repressor CREB isoforms in the brain of *C. glomerata* is expected to be lower than in other parasitic wasps, such as *C. rubecula*.

Aim of this thesis was to assess the possibility to create a line of *C. glomerata* wasps that do not form long term memory after one oviposition experience. This was executed by applying artificial selection on learning behavior of females over five consecutive generations. Differences in learning performances were monitored using behavioral tests in the wind tunnel, as well in molecular experiments on CREB expression in the brain. Selection was upon LTM formation after a single learning experience, or no LTM formation after this experience and resulted in two different lines of wasps; a slow-learning line and a fast-learning line. It was hypothesized that learning behavior as well as the molecular background of this behavior should eventually shift towards that of slow learner *C. rubecula.* Into more detail, we expected that every generation, a smaller part of the slow learning line would form LTM after one experience, compared to the fast learning line. A second hypothesis was that there should appear a shift in the ratio of CREB isoforms in the brains of the slow learning line, compared to the ratio of CREB isoforms in the fast learning line. It is expected that inhibititory isoforms would occur in higher concentrations in the selected line. Furthermore, we hypothesized that females of slow learning line would need more time to consolidate LTM and that the females of the fast learning line would have a decreased longevity, as a result of evolutionary costs of learning.

Despite some problems during the first selection rounds, the selection on learning rate proved to be successful because, from the third generation on, differences in learning behavior between the two lines became visible. At this point, the learning rate of the fast learning line remained constant at about 60%; the learning rate of the slow learning line, on the contrary, started to decrease until an average learning rate of 40% in the fifth generation.

Furthermore, it was suggested from an experiment, in which LTM formation was specifically blocked with the protein synthesis blocker anisomycin, that the ARM phase is prolonged in the slow learning line.

In addition to the differences in learning rate, the selection experiment revealed that the fast-learning wasps possibly suffered from the fitness cost of learning; since the slow learning line had, in average, a longer lifespan, and wasps from the slow learning line that resulted from the second parasitization needed less time to complete larval development.

The qPCR analysis with specific CREB isoform primers proved to be a successful way to measure CREB isoform expression in the brain. However, the differences in learning behavior between the fast learning line and the slow learning line could not be coupled to differences in CREB isoform expression in the brain. Partly because of the high variation of isoform expression that was observed between replicates of the same group, and partly because not enough time was available to produce enough replicates, no clear conclusions could be drawn on the correlation between CREB expression and the efficiency of LTM formation.

1. Introduction

1.1 Learning in insects

The ability to learn is an important factor in evolution of all animals because it enables an animal to adjust its behavior to changes in the environment. To what extent learning occurs, depends on various factors such as the life span of the animal, the number of foraging decisions the animal has to make during life time and the variability of the environment (Smid, 2006). In general, learning takes place when an animal has to make a large number of foraging decisions and when the variability of the environment is predictable (Geervliet et al., 1998) Insects are no exception to this rule and they are, despite their less complex brains, well equipped for learning. In insects, learning is often associated with chemical cues, such as infochemicals, that enable the insect to associate environmental stimuli with the presence of food or hosts. The association between these two stimuli can be stored in the brain and, therefore, this type of learning is referred to as associative olfactory learning. Just a few examples of olfactory learning in insects are the conditioning of the proboscis-extension response in honeybees (Eisenhardt, 2006) and preference learning in both crickets (Matsumoto and Mizunami, 2006) and fruit flies (Mery and Kawecki, 2002).

Parasitoid insects form a widespread, and highly successful group in the class of insects. Parasitoids are insects that depend on other arthropods for their larval development. Eggs can be laid on or inside the host eggs, larvae or adults (Quicke, 1997). In parasitoids, learning plays an important role during host searching behavior. Learning in these species is based on visual cues and volatiles emitted by various sources associated with the host; responses to these volatiles are species-specific (Geervliet et al., 1998). Two examples of species, of which separate individuals have the ability to adjust innate behavior to a more profitable and environmental-specific behavior, are the closely related parasitic wasps *Cotesia glomerata* and *Cotesia rubecula*. Despite their genetic similarity, there are some interesting differences in learning and memory formation between these two species (Geervliet et al., 1998). These differences, which will be discussed below, form the basis of an extended study to the neural mechanism of memory formation in insects.

1.2 Associative learning

The process of associative learning in insects is intensively studied. The majority of attention has gone to learning in the fruit fly *Drosophila melanogaster* (Kolss et al., 2006; Mery and Kawecki, 2002; Yin et al., 1995a; Yin et al., 1994; Yin et al., 1995b) but also to non-model organisms such as the parasitic wasps *C. glomerata* and *C. rubecula* (Bleeker et al., 2006; Geervliet et al., 1996; Geervliet et al., 1998; Smid, 2006; Smid et al., 2007). For associative learning, two stimuli are needed to form memory. This is in contrast to simpler forms of learning like habituation and sensitization where only one stimulus is required. The first stimulus is, at first, a neutral stimulus. After an association has been made, this stimulus is referred to as the conditioned stimulus (CS) because the CS then triggers a specific behavior which it previously did not, but now it has been conditioned. The second stimulus consists of an already meaningful stimulus for the animal and acts as a reward or punishment. This stimulus is called the unconditioned stimulus (US) because it already triggered behavior before an association was made, so it does not have to be conditioned. In the case of *Cotesia* the US can be an oviposition experience, which acts as a reward. The smell of a plant infested with hosts serves as the CS (Smid, 2006). In this example classical or Pavlovian conditioning occurs.

Apart from classical conditioning, associative learning can also occur in the form of operant conditioning. In operant conditioning the CS is followed by a specific behavior (the operant), which is followed by the reinforcer (Smid, 2006). An example of operant conditioning is when *Cotesia* is released in the wind tunnel and perceives the smell of nasturtium with caterpillars (CS), in response to this, the wasp flies in the direction of the odor source, lands on it (specific behavior), and oviposits in the host (reinforcer). In this situation, an association is formed between the flight response and the reward.

1.3 Biology of Cotesia glomerata

Cotesia glomerata is a common parasitoid of large cabbage white larvae (*Pieris brassicae*) in The Netherlands. *C. glomerata* is a generalist; apart from caterpillars of *Pieris brassicae* it can also develop in larvae of *Pieris rapae, Pieris napi* and *Aporia crataegi* (Geervliet et al., 2000). Females prefer to lay eggs in young larvae in the first (0-4,5 days) or second (4,5-7,5 days) stage of development. Parasitized host larvae develop in the same way as unparasitized larvae. Larvae of the parasitoid emerge when the caterpillar prepares for pupation. Inside the host the *C. glomerata* larvae undergo two larval stages; the first instar

(from day 0 to day 9 or 10) and the second instar (from day 11 until emergence from the host). During development the larvae stay in the body cavity of the caterpillar and feed on hemolymph and the fat body of the host, leaving the vital organs unharmed. After approximately 15 days after oviposition the larvae start to attack the vital organs and cuticle of the caterpillar in order to emerge. Immediately after emergence, the *Cotesia* larvae start to spin cocoons and pupate (Smith and Smilowitz, 1976).

In the search for suitable hosts, *C. glomerata* depends on detection of the smell of a host plant in combination with its host. The female can detect its host on different plants, such as red and white cabbage, Brussels sprouts, and nasturtium; but the parasitoid seems to have a preference for *P. brassicae* larvae on Brussels sprout plants. This preference that is often refered to as naïve preference (Geervliet et al., 1996), can be changed after an oviposition experience on other plant-host combinations. In such a case, the insect associates the host plant odor with the presence of suitable hosts and as a result, associative olfactory learning occurs. The ability of the wasp to change its naïve preference to a specific, more prevalent host-plant combination increases its fitness because it contributes to a larger offspring.

1.4 Differences in host searching behavior and memory formation between *C. rubecula* and *C. glomerata*

To what extent learning evolves in an animal depends on ultimate factors and constraints during evolution. First of all, differences in the quality and quantity of learning of an animal can be explained in terms of energy costs. Learning involves energy consuming processes, such as the consolidation of memory and the maintenance of memory. Spending energy on learning means there is less energy available for other important life history traits. As a results, certain traits, such as reproduction or protection against parasitoids, can trade-off with learning ability (Kolss, 2007). Secondly, learning is associated with ecological costs, such as the risk of making mistakes and the risk of learning the wrong associations. The impact of ecological cost depends on the reliability of the environment; when the information in the environment is reliable, the risk of learning the wrong association is small. In such a situation, an animal needs less learning experiences to form memory (Smid, 2006).

Cotesia rubecula, another parasitoid of *Pieris* in The Netherlands, is closely related to *C. glomerata*. However, host searching behavior and memory formation in both species are different. First of all, *Cotesia glomerata* is a generalist with a preference for *P. brassicae* (Geervliet et al., 2000). The environment where *P. brassicae* caterpillars can be found, can be considered fairly predictable because *P. brassicae* females tend to lay several eggs on

one plant. When the eggs hatch, the emerged caterpillars eat almost the whole host plant and need to migrate to neighboring plants to continue eating and complete their development. Therefore, *P. brassicae* searches for clusters of host plants to lay her eggs. So when a female of *C. glomerata* finds a suitable host, more hosts on the same plant species are likely to be within reach and learning after one oviposition experience will turn out in her advantage. Second, *C. glomerata* lays several eggs in one caterpillar, making the reward of finding a suitable host large.

For *C. rubecula* the situation is different. This species is specialized on *Pieris rapae* and lays only one egg per caterpillar. *P. rapae* females lay only one egg per plant and spread their eggs in a more unpredictable way. The predictive value of finding a suitable host on a plant-host combination is therefore much smaller and fast learning is expected to be disadvantageous (Smid, 2006).

Due to differences in host searching behavior, evolution has driven the learning behavior of these two species in two different directions. An important difference between the two *Cotesia* species is that both species remember one oviposition experience for a short time, but only *C. glomerata* (see fig. 1) is able to remember a single experience for several days (Smid et al., 2007).

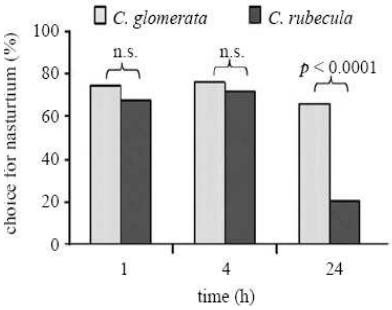


Fig. 1: Memory retention in C. glomerata (represented by dark bars) and C. rubecula (represented by light bars) 1h, 4 h and 24h after a single learning experience. After 1h and 4h both species show a similar preference for nasturtium, but 24h after the experience, memory is almost gone in C. rubecula, *while it is still present in* C. glomerata *(Freely adapted from Smid, et. al., 2007)*

1.5 Artificial selection on learning

Under laboratory conditions artificial constraints can be used to trigger evolution. In such an experiment, the presence or absence of a phenotypical trait is used to decide whether an animal is allowed to breed. Basis of these experiments is the assumption that phenotypical traits are fixed in an animal's genome and selection on this trait should therefore increase its abundance in the next generation. An example of such an experiment is the study of Mery and Kawecki (2002). In this study, fruit flies were selected on the ability to associate between the quality of an oviposition substrate and the smell of the substrate. Only flies that were able to remember this association, could contribute their genes to the next generation. In this example, the choice for an oviposition substrate had a direct impact on the fitness of the flies and, after approximately 15 generations, the experimental populations showed an

increased ability to learn to avoid oviposition substrates that were of a less quality for the development of their offspring. This experiment demonstrates that there exists genetic variation for associative olfactory learning in natural populations. Another interesting result of research to this 'high-learning' selection lines was that the learning performances of the fruit flies seemed to be correlated to their fitness. In fact, flies that were selected on improved learning ability had a decreased larval competitive ability, compared to normal flies; under competitive circumstances less larvae of the selected group survived until adulthood (Mery and Kawecki, 2003).

An additional study, on learning in Cape bees (*Apis mellifera capensis*), also reported a successful artificial selection on associative learning in this species (Brandes et al., 1988). In this experiment, worker bees of the Cape bee were selected on the ability to associate food (sucrose) with an odor. Because the selected bees were bred parthenogenetically, differences between the low and high learning line were already visible within a few generations. The good learning line scored better on the conditioning test and remembered the association for a longer period, compared to the poor learning line.

1.6 Phases in memory formation

Apart from the evolutionary background of learning, research has also focused on its molecular and neuronal basis. An important result from this research is that memory in insects, and also in other organisms, can be divided in three different phases; short term memory (STM), anesthesia-resistant memory (ARM), and long term memory (LTM). STM and ARM last only for a limited period of time and can be consolidated into LTM that is considered to be more stable. In most species, both STM and ARM arise after a single learning experience, while a spaced learning experience leads, next to STM and ARM, to the formation of LTM. Furthermore, LTM requires protein synthesis in nerve cells while STM and ARM do not (Kandel, 2001). Therefore, STM and ARM are considered 'low costs' forms of learning compared to LTM (Smid et al., 2007).

Two contradicting opinions exist about the interactions between the three different memory phases after a learning experience. Two separate studies in *Drosophila* report that STM is replaced by both LTM and ARM, so according to this model, LTM and ARM are produced in parallel and coexist at the same time in nerve cells (Tully et al., 1994); (Margulies et al., 2005). A third study, on the contrary, found evidence for the idea that ARM and LTM function as competitive elements and, therefore, never coexist in nerve cells in *Drosophila* (Isabel et al., 2004). The first pattern of memory formation is hypothesized to be present in

C. rubecula, while the second pattern is suggested to be present in *C. glomerata* from a study by Smid *et al.* (2007). In this study it was discovered that *C. glomerata* consolidates STM into LTM within 4 hours after a single learning trial (see fig. 2a). In *C. rubecula,* on the contrary, a single trial-induced STM is not consolidated into LTM; it needs three spaced trials and several days of consolidation before LTM is established (see fig. 2b). These results led to the assumption that ARM might be reduced or even absent in *C. glomerata,* and that STM might exclusively consolidated into LTM in this species and that, in *C.rubecula,* LTM is formed in parallel with ARM (Smid et al., 2007).

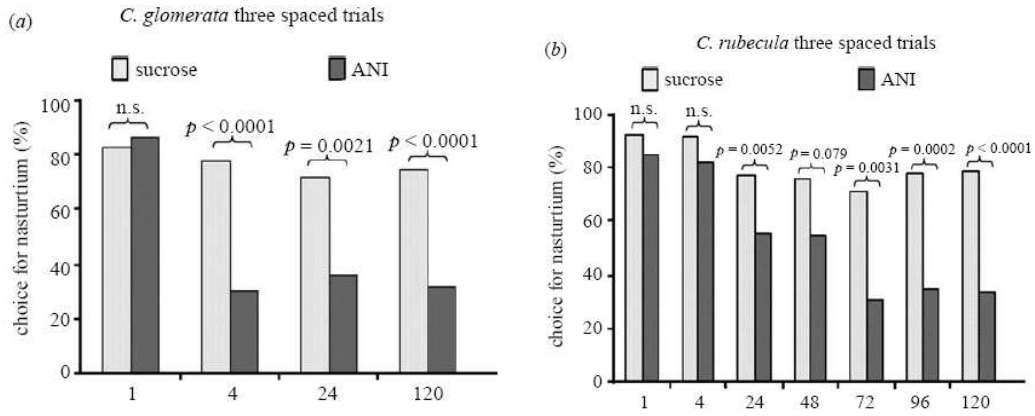


Fig. 2: Memory retention in C. glomerata (a) and C. rubecula (b) after three spaced experiences on nasturtium and fed on either sucrose or sucrose with the protein synthesis blocker anisomycin (ANI). In both species, ANI treatment has no effect 1 h after learning. In C. glomerata*, at 4 h and later memory is inhibited by ANI. In* C. rubecula*, inhibitory effects of ANI become only apparent at 24 h, and maximum inhibition is reached after 72 h. Freely adapted from Smid et al. (2007)*

1.7 The role of VUM neurons and CREB in the formation of long-term memory

Next to the description of the different phases in memory formation, research has also focused more specifically on the molecular neurobiology of memory formation. Two important results from this research are, at first, that VUM neurons in the brain play an essential role in the process of associative olfactory learning and the formation of LTM in insects. And second, that, on the molecular level, the cAMP responsive element binding protein, or CREB, plays a key role in this process (graphic representations of the brain parts involved in learning and the regulation of gene transcription by CREB are given in, respectively, fig. 3 and fig. 4).

The process of associative olfactory learning starts, first of all, with the presence of an odor in combination with a reinforcing stimulus, for example, the smell of an infested plant in combination with the rewarding experience of oviposition (Smid, 2006). The odor is detected by sensory cells in the antennae and sent to the antennal lobe in the brain. Information from

this brain centre is sent via the antennal-glomerular tract to the mushroom bodies (MB) and to the lateral protocerebral lobe (LPL). The mushroom body, a dense network of neural cells situated in the protocerebrum, is important in olfactory memory formation because it integrates olfactory stimuli to behavioral responses (Dubnau et al., 2003). The activated MB processes the olfactory information and projects it to the LPL. The LPL, on its turn, activates the ventral unpaired median interneurons (VUM) of the subesophageal ganglion (SOG) via descending neurons.

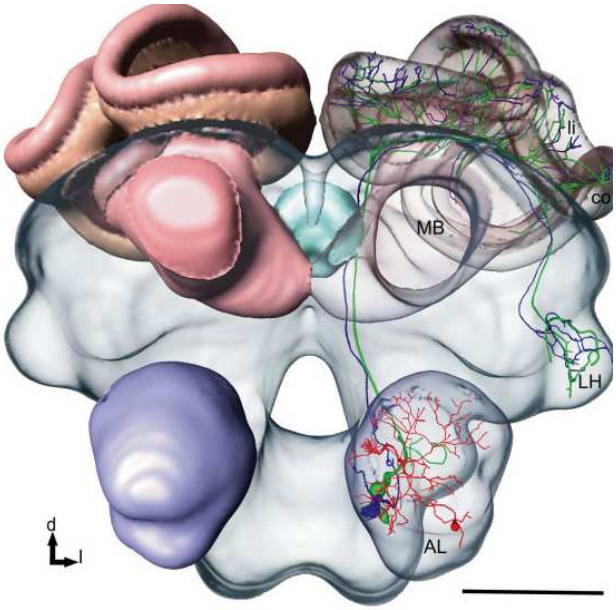


Fig. 3 Schematic view of the honeybee brain. Abbreviations: AL= antennal lobe, MB= mushroom body, LH= lateral horn of protocerebral lobe (LPL). Two projection neurons of the antennal-glomerular tract are depicted in blue and green. Freely adapted from Brandt (2005)

Since VUM neurons project to glomeruli of the AL, to the calyces of the MB and to the LPL, these structures are thought to play an essential part in the process of olfactory memory formation in honeybees (Hammer, 1997). VUM neurons have also been discovered in the antennal lobes of *C. glomerata* (Smid et al, 2003), and it is therefore hypothesized that VUM neurons have the same function in olfactory learning in *Cotesia* as in the honeybee.

The actual process of LTM formation starts with the release of the neuromodulator octopamine (OA) in the above named structures. When OA reaches a receptor on a sensory neuron, an intracellular pathway is initiated that starts with the activation of adenylyl cyclase (AC) by a G protein subunit. AC catalyzes the conversion from ATP into cAMP. Cyclic AMP functions as a second messenger and diffuses through the cytoplasm to activate protein kinase A (PKA). Repeated stimulation of the sensory neuron leads to increasing

concentrations of cAMP that can persist and act for several minutes. Activated PKA units can then translocate to the nucleus and activate the mitogen-activated protein kinase (MAPK) (Kandel, 2001). PKA and MAPK diffuse through the nucleus and phosphorylate, and thereby activate, the CREB. The phosphorylated CREB binds to the CREB binding protein (CBP), which is also present in the nucleus. The CREB-CBP complex can only bind to genes with a CRE site, where it initiates gene transcription by recruiting basal transcription factors and RNA polymerase II (Silva et al., 1998). Activation of genes with CRE sites leads to the formation of proteins that stimulate the growth of new synaptic connections. Changes in the number of synaptic connections between nerve cells are thought to be the key process in long term memory formation (Kandel, 2001).

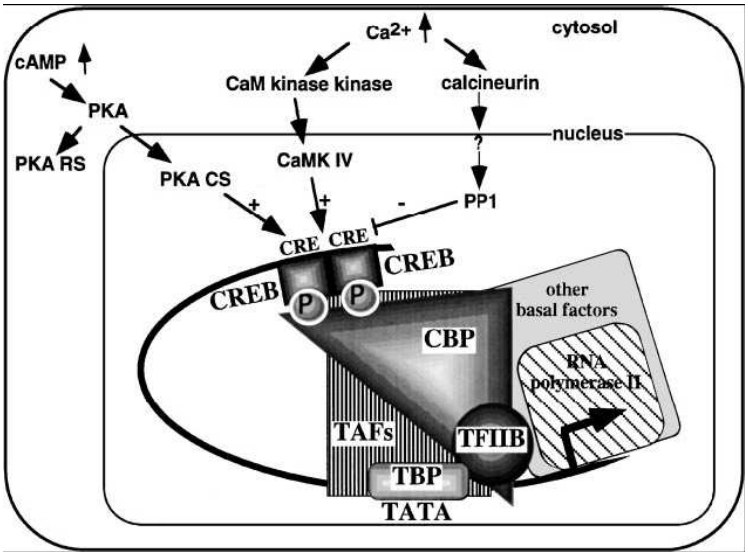


Fig. 4 Schematic view of the regulation of gene expression by cAMP, PKA, CREB and CBP. Freely adapted from Silva (1998))

CREB modulates transcription of certain genes in the nucleus of all body cells and is, apart from memory formation, involved in glucose homeostasis, growth factor dependent cell survival, circadian rhythms and spermatogenesis (Eisenhardt, 2003). The CREB protein consists of two major domains; a basic region-leucine zipper (bZIP) domain and the activation domain. The bZIP domain can bind to specific parts of the DNA; the cAMP response element (CRE) sites while the activation domain can interact with other components involved in transcription. Interaction of CREB with CRE sites is necessary for cAMP responsiveness (Yin et al., 1995b).

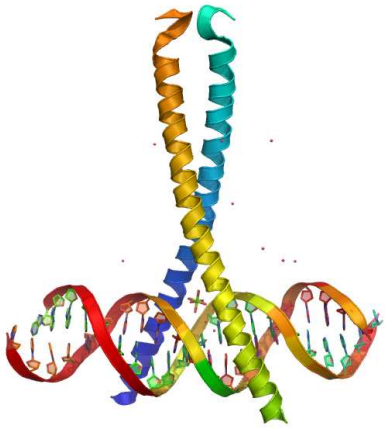


Fig. 5: The two chains of CREB binding to DNA (Freely adapted from (Berg, 2002)

Research in *Drosophila*, *Aplysia* and mice indicated that CREB serves as a switch between LTM and STM in the

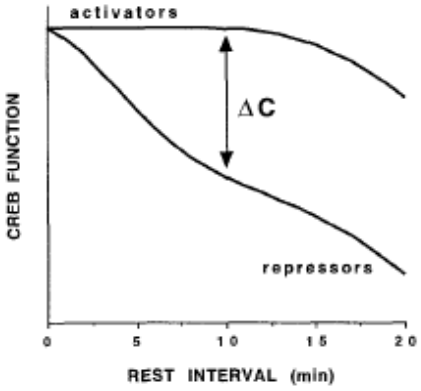
brain (Yin and Tully, 1996). A similar mechanism of LTM formation is expected to be present in *C. glomerata*, since protein synthesis blockers were found to inhibit the formation of LTM in this species, whereas it did not block STM (Smid et al., 2007).

1.8 CREB isoforms

As a result of alternative splicing during the transcription of the CREB gene, different isoforms of the protein exist. Depending on the type of isoform, CREB can have an inhibitory or excitatory effect on the transcription of genes bearing a CRE site. How fast LTM is formed is thought to depend on the ratio of activator and suppressor isoforms present in the brain after a learning experience (Yin et al., 1995a).

The effect of the ratio of CREB isoforms on LTM formation in insects has extensively been studied in the fruit fly (*Drosophila melanogaster*) and the honeybee (*Apis mellifera*). Long-term memory is in both species formed only after multiple trainings, spaced in time and CREB was found to play an important role in this process.

The CREB gene in *Drosophila* shares numerous similarities in sequence and function with the mammalian CREB gene (Yin et al., 1994). Two CREB-like genes have been described in *Drosophila*; dCREB-A and dCREB2. dCREB-A is known to bind to CREs and can activate transcription in a cellular system. The dCREB2 gene gives rise to 7 different isoforms (a, b, c, d, q, r and s), which are all PKA-dependent regulators of gene expression. In order to be activator of CRE sites, the isoform requires the presence of exons 2 and 6. The dCREB2-a isoform contains all exons and is thereby a PKA- responsive transcriptional activator (Poels et al., 2004; Yin et al., 1995b). A study from Yin and del Vecchio *et al*. (1995) confirmed that induced expression of dCREB2-a enhances the formation of LTM in transgenic fruit flies. The dCREB2-b isoform lacks exon 2 and thus blocks PKA- responsive transcription. This was proven by a second study from Yin *et al*. (1994), where induction of the dCREB2-b isoform successfully blocked the formation of LTM. dCREB2-c , -d and –e lack exon 2 and should therefore also act as a suppressor of gene expression but this has not been proven until now. Inhibition can occur by competitive binding and hence occupying available CRE sites or direct contact between the isoforms (Poels et al., 2004). Activation of CRE-dependent gene expression via dCREB2 depends on the ratio of activator to repressor isoforms, as well as on dephosphorylation of the PKA-site of the isoform so it can bind to its CRE-site (Poels et al., 2004).



After an associative learning experience both CREB activator and repressor isoforms are produced. After the training the repressor isoforms degrade faster than the stimulating isoforms (*fig. 5*), making the formation of memory possible (Yin et al., 1995a). Repetition of the experience leads to an accumulation of stimulating isoforms, so the formation of LTM can take place.

Fig. 6 Ratio of CREB activator and repressor isoforms after a learning experience in Drosophila. Freely adapted from Yin et al. (1995a))

The dCREB2 isoforms are not expressed uniformly across tissues in *Drosophila* and the expression of isoforms in a specific tissue can differ between flies of different age. The dCREB2-a isoforms has, for example, been detected in a variety of tissues of *Drosophila* but not in *Drosophila* heads (Poels et al., 2004).

In the honeybee another gene homologous to the mammalian CREB gene was discovered; the AmCREB gene. Of this gene, eight different isoforms were identified (AmCREB 1–8). All eight isoforms were found to be present in different parts of the brain involved in olfactory learning, as the mushroom body, the glomeruli of the antennal lobes and the protocerebral lobes (Eisenhardt et al., 2003). In the honeybee it is not yet known which of the eight isoforms works as a suppressor or activator of genes with CRE sites.

In *C. glomerata and C. rubecula* 9 different isoforms of CREB have been discovered until now; CREB isoforms 1 to 9 (see figure 7). Ongoing research tries to unravel the function of each isoform in memory formation. Since *C. glomerata* forms LTM after one experience, the ratio of repressor CREB isoforms in the brain is thought to be lower than in other species that need repeated stimulation to create LTM (Smid, 2006).

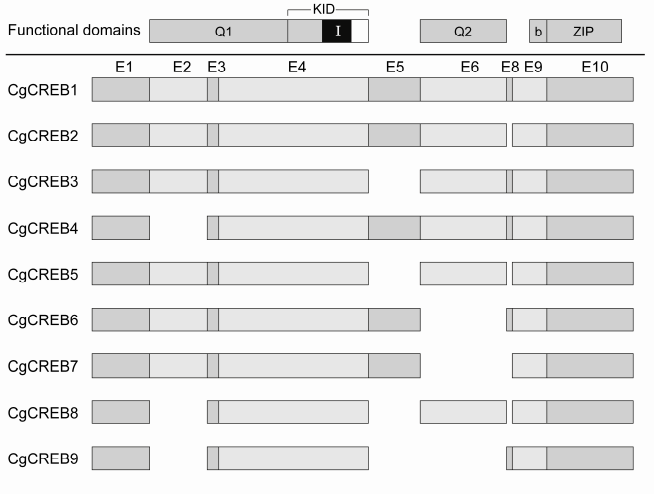


Fig. 7 The nine isoforms, depicted in open reading frames, of the CREB gene in C. glomerata and C. rubecula (van den Berg, et. al., in preparation).

1.9 Aim of the thesis

This thesis project is a part of ongoing research to the behavioral, neural, and molecular basis of memory formation in parasitic wasps. This thesis tries to combine the knowledge of the evolutionary background of learning with the knowledge of the molecular basis of memory formation in *Cotesia glomerata*. It was tried to link fitness consequences to learning rate, using artificial selection constrains, which resulted in two lines of wasps. The first line of wasps was selected on slow learning, or no LTM formation after a single learning experience. The second line was selected on fast learning, or forming LTM after one experience. The selection experiment consisted of a sequence of selections over five consecutive generations in which fast-learning wasps were eliminated after each selection, to create the slow learning line, and slow learning wasps were eliminated after each selection, to create the fast learning line. The two lines of wasps were kept separately during all generations to avoid breeding between individuals of the both groups. Additionally, the two resulting lines of wasps were used to study CREB isoform expression in the brain; the pattern of LTM formation, by comparing the time that is needed to consolidate LTM; and the evolutionary costs of learning, by comparing the longevity of both lines.

We expected that the learning capacity of the slow-learning line would eventually approach or even equal the learning capacity of *C. rubecula.* This was analyzed with behavioral tests,

performed in the wind tunnel, as well as molecular experiments on CREB expression. The hypotheses were formulated as follows:

- Selection on slow learning will:

-Decrease learning rate 24h after one learning experience;
-Increase longevity due to lower costs for learning;
-Increase the time needed to consolidate LTM, compared to selection on fast learning.

- CREB isoform expression in the brain of the slow learning line will differ from the CREB isoform expression of the fast learning line; stimulating isoforms will become less abundant, inhibitory isoforms will become more abundant.

2. Materials and methods

2.1 Rearing and use of plants

Plants (Brussels sprouts, *Brassica oleracea* var. gemmifera L. cv. Cyrus and nasturtium, *Tropaeolum majus* L. cv. Glorious Gleam) were reared as described previously by (Geervliet et al., 1998). For the wind tunnel experiment plants of approximately the same size were used (nasturtium of approx. 5 weeks old and cabbage of approx. 8 weeks old). Because cabbage is larger, two nasturtium plants (in a single pot) were used to test against one cabbage plant. Two leafs of each plant were infested with 20 caterpillars, so each odor source contained 40 caterpillars. Caterpillars were allowed to feed for 24 h on the plant before testing.

For the learning trials, entire nasturtium plants were used. These plants had the same caterpillar treatment as the plants used for the wind tunnel tests. However, shortly before use, caterpillars were removed and new 0-1 day old caterpillars were replaced on the leaf. In this manner, the leafs retain the characteristic smell of infestation and thus provide the CS, while the parasitoids cannot be harmed by aggressive behavior of large caterpillars. After 5 to 10 wasps received an experience, the leaf was exchanged for a fresh leaf of the plant.

2.2 Rearing and treatment of insects (parasitoids and hosts)

Parasitoid and herbivore species originated from individuals collected on Brussels sprouts gemmifera (*Brassica oleracea* cv. Titurel) fields near Wageningen, The Netherlands. *Pieris brassicae* larvae and the start population of *Cotesia glomerata* were reared as described previously (Geervliet et al., 1998).

Upon emergence males and females were caged for one day together to allow mating. After one day the majority of males was removed to ensure the fitness of the females. Cages (40 x 30 x 30 cm) were supplied with wet paper and honey.

Caterpillars used for the oviposition experiences were always 0-1 day old. The learning experiences were offered when the females were between 3 to 10 days old.

After the separate wind tunnel tests, the females were kept in glass cages that were provided with water and honey. After all selection steps, the remaining females were offered pieces of Brussels sprouts leafs with three 1-2 day old host larvae for parasitization. Afterwards, the parasitized caterpillars were transferred to cages with Brussels sprouts

plants and were allowed to feed until the larvae of the parasitoids emerged. After emergence from the caterpillars, cocoons were kept in petri dishes (inner diameter 9 cm) at 20–22 ˚C, 50–70% r.h. and a L16:D8 photoperiod.

2.3 Training

2.3.1 Single trial learning

Every female wasp received the opportunity to oviposit in a 0-1 day old larvae on an infested nasturtium leaf. The wasp was transported to the leaf with a glass vial and directly confronted with a caterpillar on the leaf. After oviposition the wasp was removed and transported to a glass cage, where it was provided with water and honey. Parasitized caterpillars were removed, too.

Fig. 8: Single trial learning; a C. glomerata female encounters a 0-1 day old P. brassicae larva on an infested nasturtium leaf

2.3.2 Spaced learning

The spaced learning trials were performed similar to the single learning trial but after oviposition the wasp was removed with an empty vial, where it was kept for 10 min and brought back to a new caterpillar on the nasturtium leaf for another oviposition. In total, the female received three opportunities to oviposit in a unparasitized caterpillar.

2.4 Experimental design of the selection

For the slow learning line, only females that did not form LTM memory after one learning experience, but that *did* form LTM after three spaced experiences were allowed to breed and contribute their genes to the next generation. The selection was continued over five consecutive generationsand the selection consisted of three different tests in the wind tunnel (for a schematic overview of the selection on slow learning, see figure 9. In each wind tunnel test, females received the choice between the naturally preferred Brussels sprouts plant and a nasturtium plant. The aim of the three tests will now be further explained.

2.4.1 First test

To check if females can change their innate preference for cabbage into preference for nasturtium, it is important to know if a female indeed has an innate preference for cabbage. This to prevent that selection for wasps that fly towards nasturtium results in a line with a naïve preference for nasturtium odor. This is the aim of the first selection step. In this step naïve females from 2-7 days old received the choice between an uninfested, mechanically damaged cabbage plant and an uninfested, mechanically damaged nasturtium plant in the wind tunnel. For the testing on innate preference 2-7 days old females were used. Only females that had a naïve preference for cabbage were used for the further tests.

2.4.2 Second test

Prior to the second test, all females received a single oviposition experience on nasturtium. After 24 h a selection was made between females that formed LTM, and flew to nasturtium, and females that did not. For the slow learning line, the latter group was subjected to the final selection step.

2.4.3 Third test

The last step is used to ensure that selection is on learning speed and not on learning deficiency. In this step the slow learning line received three spaced learning trials on nasturtium. The females that showed a preference for nasturtium after spaced learning were used for breeding of the next generation.

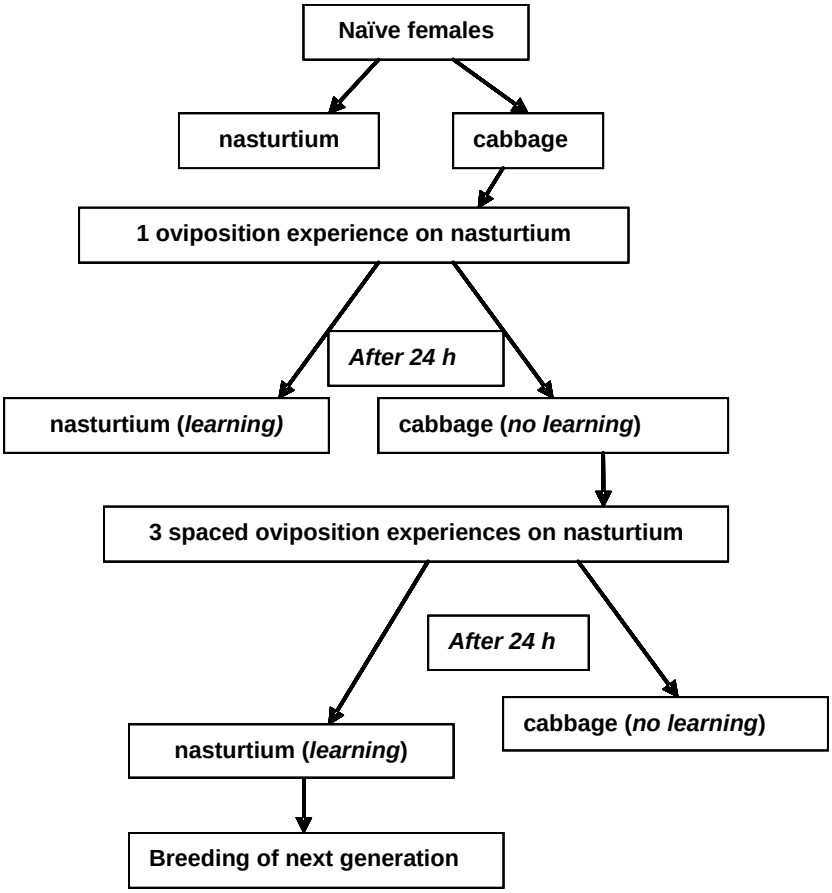


Fig. 9: Schematic overview of the selection on slow learning rate

2.5 Fast learning line

For the fast learning line, a second population of *C. glomerata* was held, separately from the slow learning line. This group consisted of wasps that did show memory retention 24 h after the single trial learning. In order to obtain the biggest contrast between the fast learning line and the slow learning line, the fast learning line was tested each time when the slow learning line was tested. Instead of three tests, only two tests were necessary with the fast learning line; the selection on innate preference and the selection 24h after one learning experience. The females that remained after these two tests were used for breeding (performed in the same way as in the slow learning line). For a schematic overview of the selection on fast learning, see figure 10. Breeding of the fast learning line took place simultaneously with the slow learning line and the amount of females that was used to produce the next generation was equal for both groups (around 15 to 20 wasps per group).

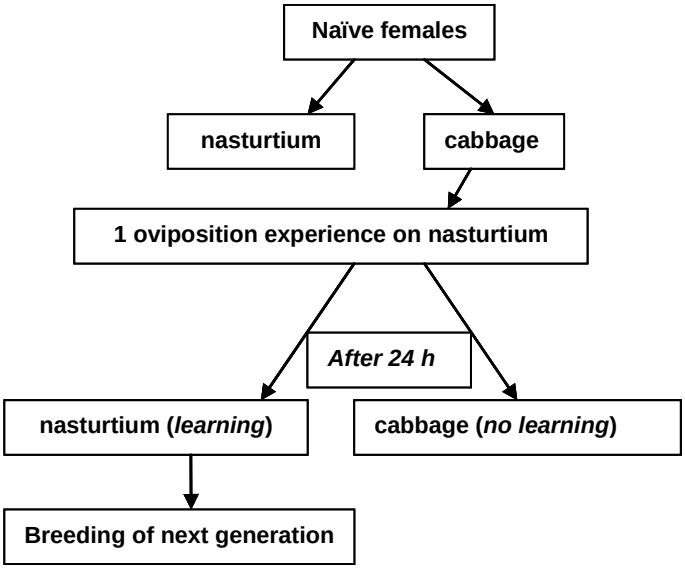


Fig. 10: Schematic overview of the selection on fast learning rate

The methods used in the different steps of the selection cycle will be further explained below.

2.6 Wind tunnel tests

The wind tunnel assay was described previously by (Geervliet et al., 1994). Wind speed was set at 17 cm/s. Wasps were tested at a relative humidity between 40-60% and temperatures between 23 and 25 ºC. In all wind tunnel tests, females received the choice between two different odor sources; a nasturtium and a cabbage plant. Naïve females were tested on artificially damaged plants. The damage was inflicted by giving four leafs of each odor source 10 strikes with a pattern wheel, 24 h before testing. Experienced females were tested on plants infested by caterpillars and 24 hour after training.


Fig. 11: The wind tunnel set up with two odor sources

Each wasp was collected from the cage with a glass vial and transferred to the wind tunnel. A female was released in the middle of a release cylinder at approximately 25 cm from the odor sources. In each test the wasps received

5 min. to show preference for one of the two plants. Landing elsewhere in the wind tunnel or no flight at all counted as no-response.
After testing 5 wasps the plants were switched position, to prevent bias towards one side of the wind tunnel.

2.7 Breeding

To maintain genetic variation in the breeding group, inbreeding was prevented as much as possible. Therefore each selected female received the same opportunity to lay eggs. After step 5 in the selection cycle the remaining females were allowed to parasitize three 1-2 days old caterpillars of *P. brassicae* each. The parasitization was separated over three subsequent days, in order to spread the emergence of adult wasps in the next generation and, thereby, to increase the time window for experiments of that generation. After the parasitized caterpillars were fully grown (after approx. 15 days), cocoons of 15-30 caterpillars (depending on the amount of caterpillars available) were collected and placed in another cage. After approx. 8 days the new wasps emerged from the cocoons and these wasps were allowed to mate freely.

2.8 Additional test on memory formation

To analyze further differences in memory formation after a single learning experience, females of the fourth and fifth generation were used for two additional tests: a test on memory formation after treatment with a protein synthesis blocker (anisomycin) and a test to assess the memory decay over 72 hours. Because of the limited time available, females were not tested on naïve preference for cabbage beforehand in these two experiments.

2.8.1 Anisomycin treatment

To inhibit LTM formation, the protein synthesis blocker anisomycin was used. Anisomycin inhibits translation, and thereby translation-dependent LTM formation, without blocking the other memory phases, STM and ARM. To make sure that all females received the same amount of blocker, females were deprived from water and honey overnight (+/-14h.). The next morning all females were placed in separate vials and received 2 hours to consume a sucrose solution. Half of the females received 0,5 µL of a solution containing only sucrose and served as a control group. The other half of the group received 0,5 µL of sucrose solution containing 5 mM anisomycin.

After the entire solution had been consumed, all females receive a single oviposition experience on nasturtium and were transferred to little glass cages, where they were supplied with water and honey.
Memory retention was tested in a wind tunnel test. Both groups (sucrose and anisomycin) were tested 4h and 24h after the learning experience.

2.8.2 Decay of memory over 72 hours

During the selection experiment, females were always tested 24h after one learning experience. In this experiment, which took place with wasps of the fifth generation, we were curious about the differences in decay of memory after 24 h between the slow learning line and fast learning line. For this experiment, learning experiences were offered as described before. Separated groups of wasps were tested 24h, 48h and 72h after a learning experience, using the same wind tunnel set-up as described before.

2.9 Fitness-related experiments

To compare the fitness of the slow learning line with the fitness of the fast learning line, two additional tests were performed in the fourth and fifth generation. The first experiment was aimed at the average lifespan of the wasps; the second experiment was used to compare the time needed for larval development.

2.9.1 Comparison of average lifespan

The average lifespan of the wasps in days, was measured for both groups, during the fourth generation. For this experiment, wasps that emerged within two successive days were placed together in glass cages and supplied with water and honey. This resulted in four cages of 25 wasps (3 males and 22 females) per group. The cages were placed in an incubator, where they were held on a constant temperature of 20˚C and with a photoperiod of L16:D8. Survival per cage was monitored on a daily base.

2.9.1 Comparison of larval development time

In this experiment, larval development time in the host was monitored for both groups. The experiment took place in the fourth, as well as in the fifth generation. The parasitization of new generations always took place on the same days for both the fast learning line and the slow learning line. Since the parasitized caterpillars of both groups were reared under the same circumstances and were of the same age, larval development time of the wasps could

easily be compared between the two groups. The larval development time of the parasitoids was described as the number of caterpillars that were abandoned by the parasitoids per day.

2.10 Molecular work

Naïve females of both groups were analyzed each generation for CREB mRNA expression in the brain. In addition, females were analyzed on CREB mRNA expression in the brain 24 hour after a single learning experience on an infested nasturtium leaf. The last procedure only occurred in the first, third and fifth generation.

To analyze the expression of CREB in the brains, either dissected brains or total heads of the wasps can be used. Dissection of the brains of large numbers of wasps is time-consuming and therefore, complete heads were used. The head of the wasp is removed and disposed of the antennae and mouth parts using a binocular microscope and forceps. Two replicates of 15 heads were made of each group and each treatment. After dissection, the heads were collected in 500 µL RNAwiz/Trizol, and RNA was purified according to protocol (Tri Reagent, Ambion cat. # AM9738), and cDNA was prepared using the Verso™ kit (ABGene cat. # AB-1453). The RNA samples were checked on both quality and quantity, using gel electrophoresis and NanoDrop-spectrophotometry, respectively.

The cDNA was analyzed for CREB expression using *real-time* quantitative PCR (qPCR), using the Corbett RotorGene 6000 qPCR machine. In real-time qPCR cDNA is amplified and detected in the same machine. Because a fluorescent marker is added to the cDNA before amplification, the amount of fluorescence of the sample during amplification will increase. For the CREB analysis it was decided to use the ABsolute™ qPCR SYBR® Green Mix (ABGene, cat. # AB-1159/a). In a qPCR reaction with SYBR Green, all double-stranded DNA that is synthesized during the reaction is labeled with the fluorescent marker, including non-specific PCR product such as primer-dimers. Therefore, a so-called melting curve is constructed after finishing the PCR reaction. Ideally this curve should show only one peak, meaning that the product is pure. Additional peaks indicate the presence of non-specific products and thus a suboptimal performance of the PCR reaction.

The PCR reaction is characterized by the cycle threshold (Ct). Ct stands for the time (cycle) in the reaction at which the fluorescence intensity becomes larger than the baseline fluorescence and is thus correlated to the amount of cDNA in the sample. Relative

expression is calculated using Ct values and reaction efficiencies. In order to differentiate between the nine isoforms, we use isoform-specific primers, that anneal to the unique exon-exon junctions present in each isoform. Each replicate was tested in duplicate, and the expression of each isoform was expressed as the ratio of the total amount of CREB in the sample. The sequences of the used primers sequences can be found in figure 12.

primer	Sequence	Isoforms	Orientation
E1E3ForD	5'-GCAATCGCTACCTCGGTACAATCAGTTATC-3'	4, 8, and 9	Sense
E2E3ForE	5'-ACGTCGGGGACGACGCAGGTAC-3'	1, 2, 3, 5, 6, and 7	Sense
E4E6RevC	5'-GCCGGTATTACGGCTATTTCACTCGC-3'	3, 5 and 8	Antisense
E4E8E9RevB	5'-CCGTGGCCTGTGTAGTCGGCTATT-3'	9	Antisense
E5E6RevB	5'-CCCGCCGGTATTACTGTTTGGTATTGC-3'	1, 2, and 4	Antisense
E5E8E9RevC	5'-CTCCGTGGCCTGTGTAGTCTGTTTGG-3'	6	Antisense
E5E9RevB	5'-CAACAACGACTCCGTGGCCTGTTTG-3'	7	Antisense

Fig. 12: Sequences of the primers that were used to measure CREB isoform expression.

The isoforms 1 and 2 cannot be distinguished from each other in our qPCR setup and neither can isoforms 3 and 5. Therefore, isoforms 1 and 2 were detected using the same primer combination (E2E3ForE + E5E6RevB), and isoforms 3 and 5 were detected using primers E2E3ForE + E4E6RevC.

3. Results

3.1 Wind tunnel results

Female wasps from both the slow learning line and fast learning line were tested on naïve preference and preference after one learning experience on nasturtium. The wasps of the slow learning line were also tested on preference after three spaced learning experiences on nasturtium. Experienced females were tested 24 hours after the learning experiences. If a female showed a preference for nasturtium in the wind tunnel it was expected to have formed long-term memory. Besides from preference for an odor source, the response rates and the time needed for response (response time) were documented for each wind tunnel test.

Apart from the selection experiments, two additional wind tunnel tests were performed with wasps of the fourth and fifth generation: an experiment to study the effects of anisomycin on memory formation and an experiment to study the decay of memory over 72 hours.

3.1.1 Naïve preference for cabbage

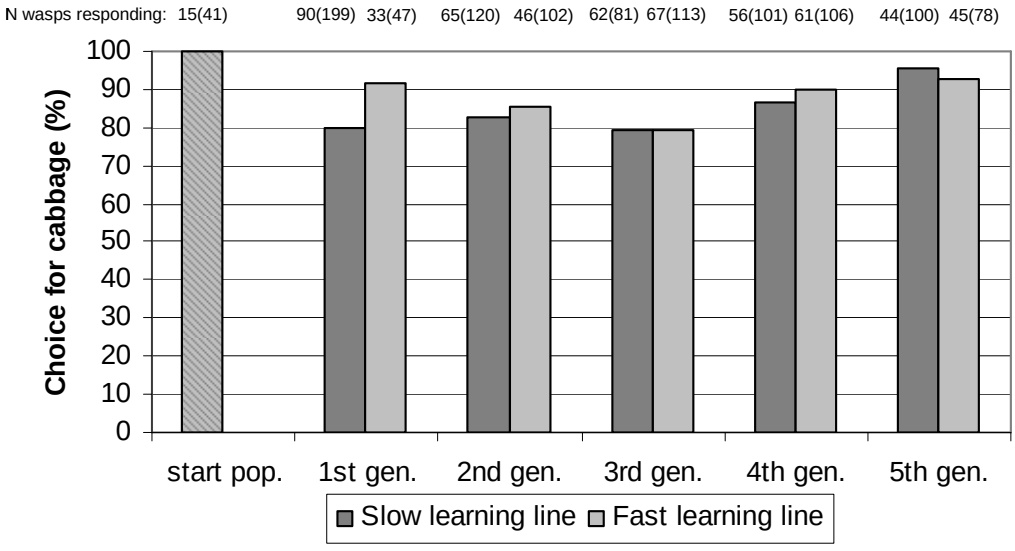


Fig. 13: Average preference for cabbage of naïve females. Individual females were tested in a dual choice test for preference for either an uninfested cabbage plant or an uninfested nasturtium plant. No significant differences in naïve preference were found between the slow learning line and the fast learning line (Independent sample T-test, P-values: 1st gen.: 0.087, 2nd gen.: 0.696, 3rd gen.: 0.879, 4th gen.: 0.712, 5th gen.: 0.414). Both groups were tested on several different days in each generation, except for the start population that was only tested on 1 day. Only the results of tests in which eight or more wasps showed a response, were used to calculate the average preference. Numbers above the bars indicate the numbers of responding females, compared to the total amount of tested females (within brackets).

Naive females showed to have a clear preference for cabbage; choice for cabbage was between 80 to 100% and stayed more or less constant within and between each generation. The slow learning line and the fast learning line did not differ significantly in their naïve preference for cabbage.

3.1.2 Naïve response rates

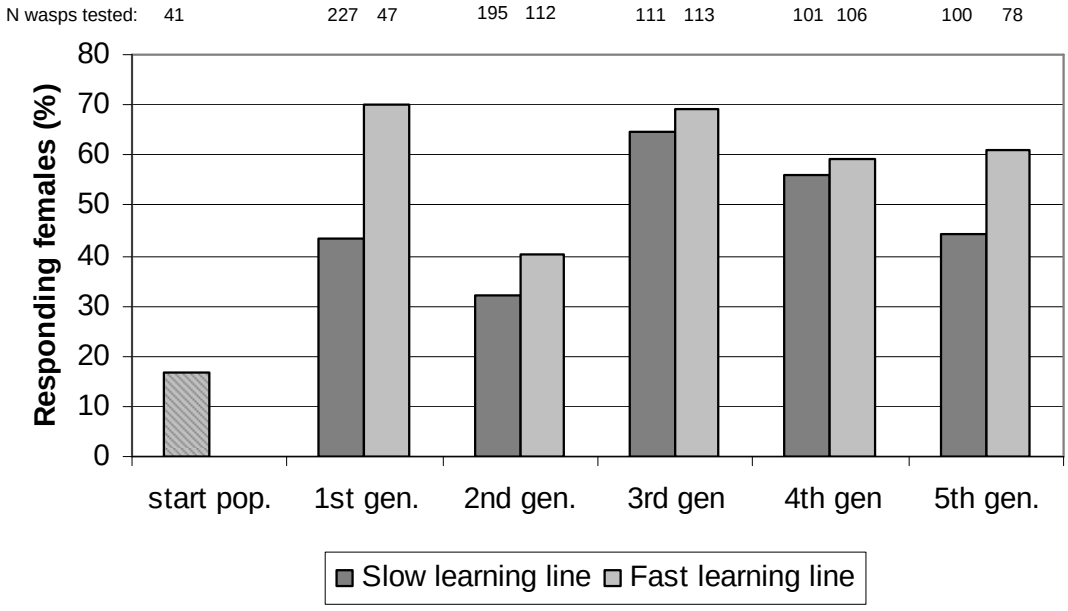


Fig. 14: Average response rates of naïve females. Individual females were tested in a dual choice test for preference for either an uninfested cabbage plant or an uninfested nasturtium plant. No significant differences were found between the slow learning line and the fast learning line (Independent sample T-test, P-values: 1st gen.: 0.097, 2nd gen.: 0.369, 3rd gen.: 0.637, 4th gen.: 0.694, 5th gen.: 0.057). Both groups were tested on several different days in each generation. Only the results of tests in which eight or more wasps were tested, were used to calculate the average response rate. Numbers above the bars indicate the total amount of tested females.

A large variation in the response rates of naive females was observed between individual tests executed on different days (between 0 and 90%; individual data available in chapter 1 of the appendix). No-response wasps stayed in the release cylinder for the total duration of the experiment or landed on the roof or on the back side of the wind tunnel. Overall, the fast learning line performed better than the slow learning line, although these differences were not significant.

3.1.3 Naïve response times

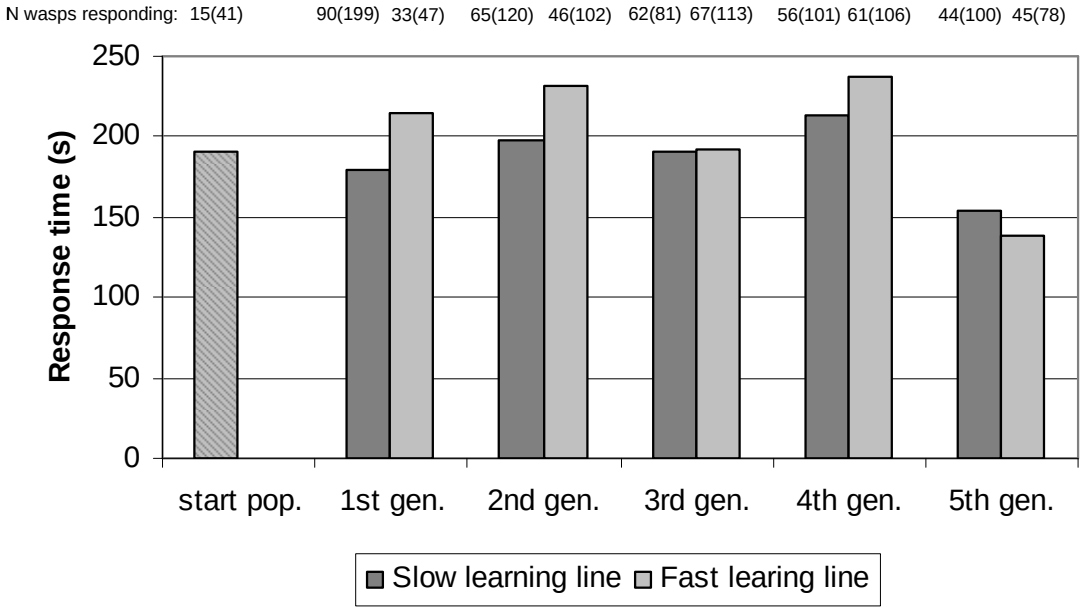


Fig. 15: Average response time of naïve females. Individual females were tested in a dual choice test for preference for either an uninfested cabbage plant or an uninfested nasturtium plant. No significant differences in naïve preference were found between the slow learning line and the fast learning line (Independent sample T-test, P-values: 1st gen.: 0.077, 2nd gen.: 0.111, 3rd gen.: 0.929, 4th gen.: 0.248, 5th gen.: 0.455). Both groups were tested on several different days in each generation, except for the start population that was only tested on one day. Only the results of tests in which eight or more wasps showed a response, were used to calculate the average response time. Numbers above the bars indicate the numbers of responding females, compared to the total amount of tested females (within brackets).

Average response times of naïve females varied from 130 to 230 seconds; there were no consistent differences between the two groups within generations. Response times of naïve females were higher than the response times of experienced females (also see figure 17). Wasps that needed a long time to respond, stayed in the release cylinder for several minutes, before initiating their flight, or took a long flight.

3.1.4 Response rates after one learning experience

Response rates after one learning trial were high (between 85 and 100%). It is interesting to note that after an experience, the wasps did no longer suffer from unfavorable outside circumstances, such as outside temperature or air pressure, as naïve wasps did. A significant difference in response rate was observed in the second and fifth generation. However, this difference was not consistent over the other generations. In fact, the females of the slow learning line performed better in the second generation, while in the fifth generation the fast learning line performed better. In the third and fourth generation, no significant differences were found between the slow learning line and the fast learning line.

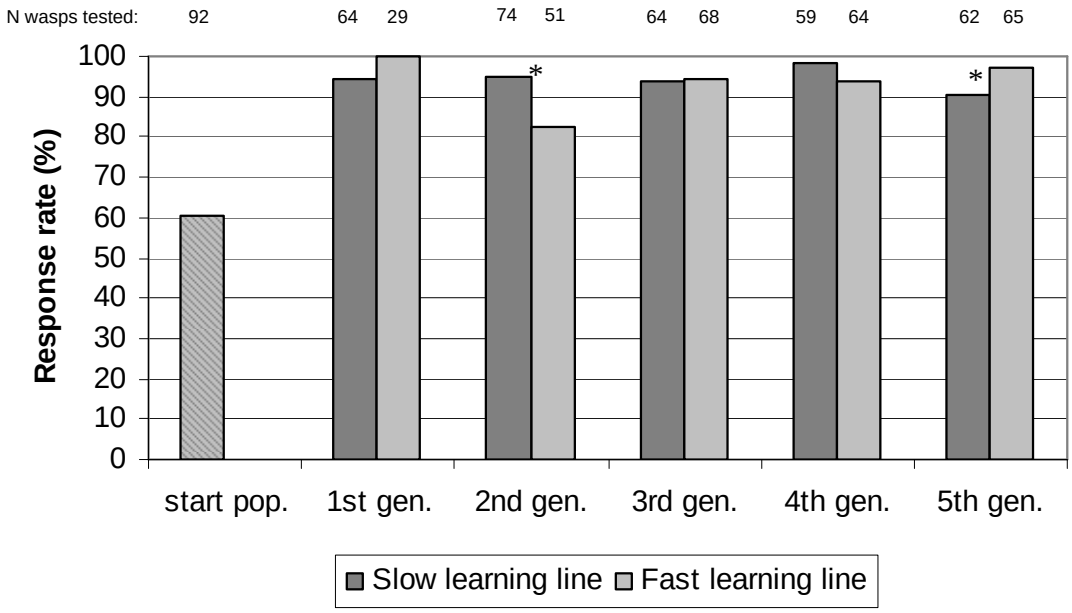


Fig. 16: Average response rates of experienced females. Individual females received one oviposition experience on a P. brassicae larva and were tested 24 hours afterwards in a dual choice test for preference for either an infested cabbage plant or an infested nasturtium plant. Asterisks indicate a significant difference between the two groups within one generation. No significant differences were found in the first, third and fourth generation (Independent sample T-test, P-values: 1st gen.: 0.449, 3rd gen.: 0.,842 4th gen. :0.973). In the 2nd and 4th generation the two groups differed significantly in response rate (Independent sample T-test, P-values: 2nd gen.: 0.002, 5th gen.: 0.,016) but the differences were not consistent. Both groups were tested on several different days within each generation. Only the results of tests in which eight or more wasps were tested, were used to calculate the average response rate. Numbers above the bars indicate the total amount of tested females.

3.1.5 Response times after one learning experience

The fast learning line needed significantly more time to respond in the second and fourth generation. This difference was also present in the third generation, but there it was not significant; in the fifth generation the difference seemed to have disappeared.

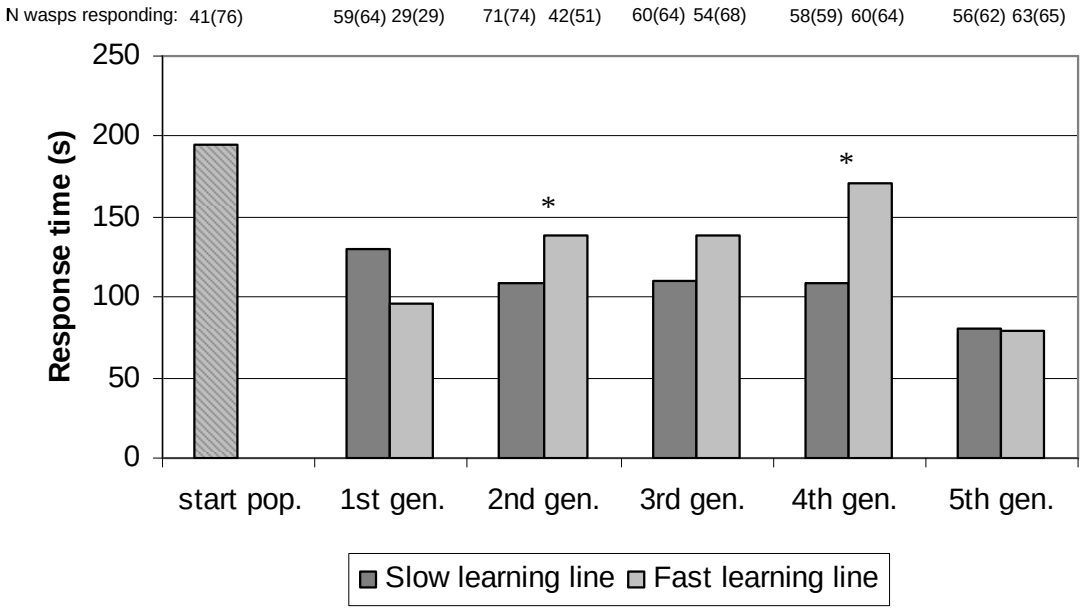


Fig. 17: Average response time of experienced females. Individual females received one oviposition experience on a P. brassicae *larva and were tested 24 hours afterwards in a dual choice test for preference for either an infested cabbage plant or an infested nasturtium plant. Asterisks indicate a significant difference between the two groups within one generation. No significant differences were found in the first, third and fifth generation (Independent sample T-test, P-values: 1st gen.: 0.249, 3rd gen.: 0.079, 5th gen. :0.935). In the 2nd and 5th generation the fast learning line needed significantly more time to respond than the slow learning line (Independent sample T-test, P-values: 2nd gen.: 0.044, 5th gen.: 0.001. Both groups were tested on several different days within each generation. Only the results of tests in which eight or more wasps showed a response, were used to calculate the average response time. Numbers above the bars indicate the numbers of responding females, compared to the total amount of tested females (within brackets).*

3.1.6 Learning rates 24 hour after a single oviposition experience

The slow learning line and fast learning line differ significantly in learning rate after one learning experience (see figure 18) (GLM: generation: χ^2_4= 0.94, p= 0.3336; group: χ^2_1= 6.60, p= 0.0102). In the first generation a large difference in learning rate between the control and slow learning line was observed. The fast learning line had learned better than the slow learning line, as hypothesized. However, the fast learning line was only tested on two different days in this generation, which makes the results less reliable. In the second generation, there was no clear difference between the learning performances of the two groups. The slow learning line, on average, even seems to have learned better than the fast learning line. In the third generation, on the other hand, the fast learning line performed better on three of the five testing days and the average learning rate of the slow learning line was higher than the learning rate of the fast learning line. In generation four the slow learning line displayed lower learning rates on four of the five testing days, and displayed, again, a learning rate that was in average lower than that of the fast learning line. Finally, during the selection of the fifth generation, the fast learning line performed better on all

testing days. In the fifth generation, the average difference in learning rate between the control and the slow learning line was, approximately, 20%. In this generation, learning rates of the fast learning line differed from 53 to 75% on individual testing days, while the learning rates of the slow learning line differed analogously between 27 and 55%.

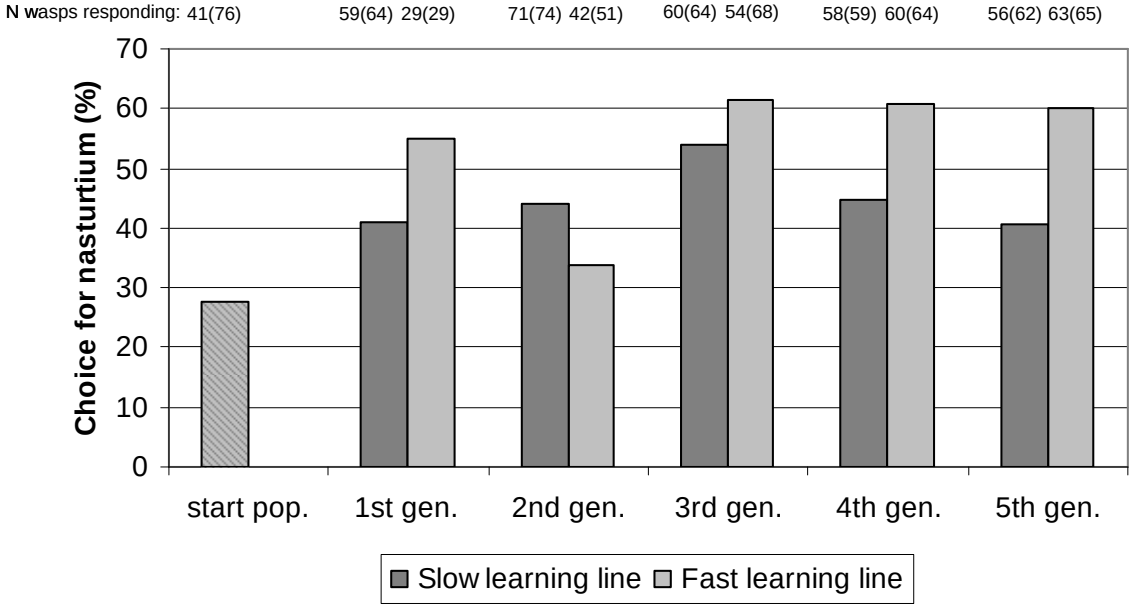


Fig. 18: Average preference for nasturtium of experienced females. Individual females received one oviposition experience on a P. brassicae *larva and were tested 24 hours afterwards in a dual choice test for preference for either an infested cabbage plant or an infested nasturtium plant. From the third generation on, the slow learning line displayed significantly lower learning rates than the fast learning line (GLM: generation: χ^2_4= 0.94, p= 0.3336; group: χ^2_1= 6.60, p= 0.0102). Both groups were tested on several different days within each generation. Only the results of tests in which eight or more wasps showed a response, were used to calculate the average preference. Numbers above the bars indicate the numbers of responding females, compared to the total amount of tested females (within brackets).*

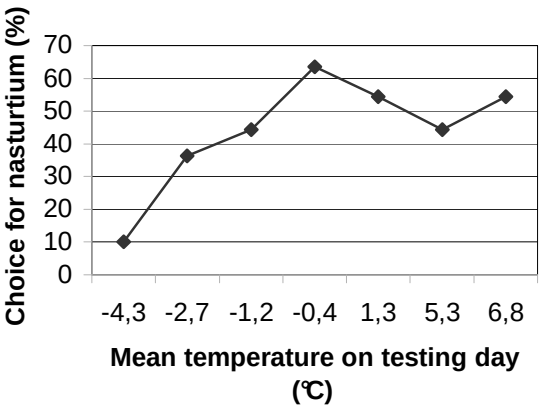


Fig. 19: Preference for nasturtium of the slow learning line in the second generation after one learning experience, plotted against outside temperatures

During the selection of the start population, and the first and second generation, a large variation in learning rate was observed (see fig. 20 for individual test results). The existence of variation within one generation implies that there is, just as with the tests on naïve preference, a day effect. When plotted against outside temperatures (see figure 19), there, indeed, seems to exist a relationship between the temperature outside the building (and thereby the humidity inside the building) and the learning performances of

the wasps. In order to compare the flight results of both groups, the tests on preference after one experience were, as often as possible, performed on the same day and the same plants. In these tests, the circumstances in which both groups received the learning experience were the same and the results should, therefore, be suitable for comparison.

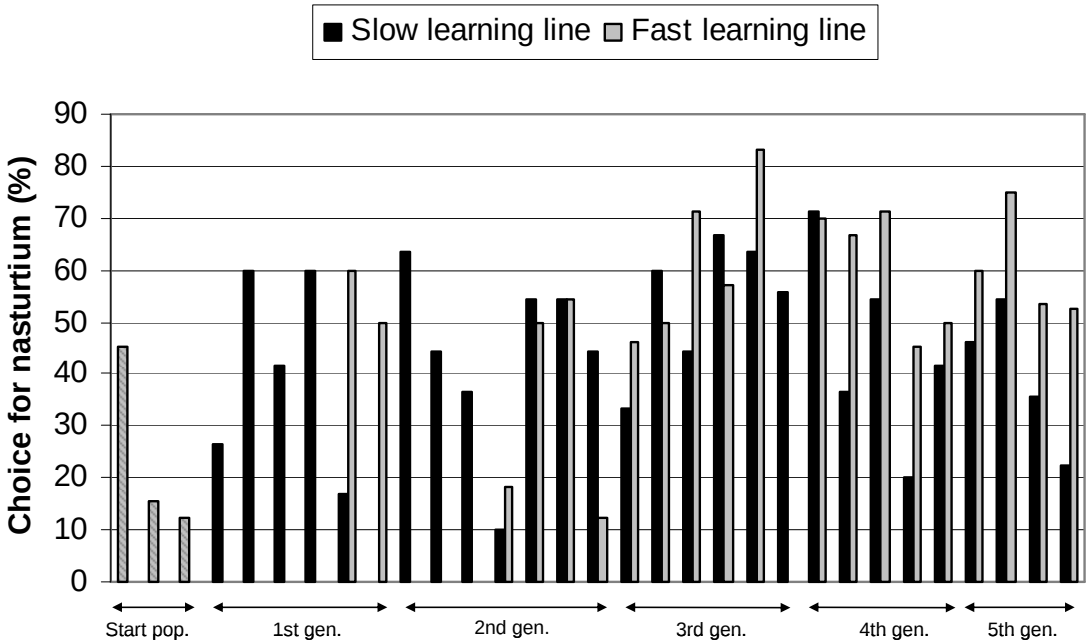


Fig. 20: Preference for nasturtium twenty-four hours after one oviposition experience of individual tests. All data represent tests that were performed on the same day, with the same plants, and with, at least, eight responding wasps. A large variation exists in the learning rates of tests within one generation; however, in overall, when the selected line showed low learning rates, the learning rate of the fast learning line was low, too.

3.1.7 Learning rates 24h after three spaced learning experiences

Only females of the slow learning line that showed a preference for cabbage after a single learning experience were tested after three spaced oviposition experiences. Memory retention after this treatment was, in general high. The learning rate after three experiences increases from approximately 55% in the first generation, to approximately 85% in the fifth generation; although, the differences between the generations were not significant.

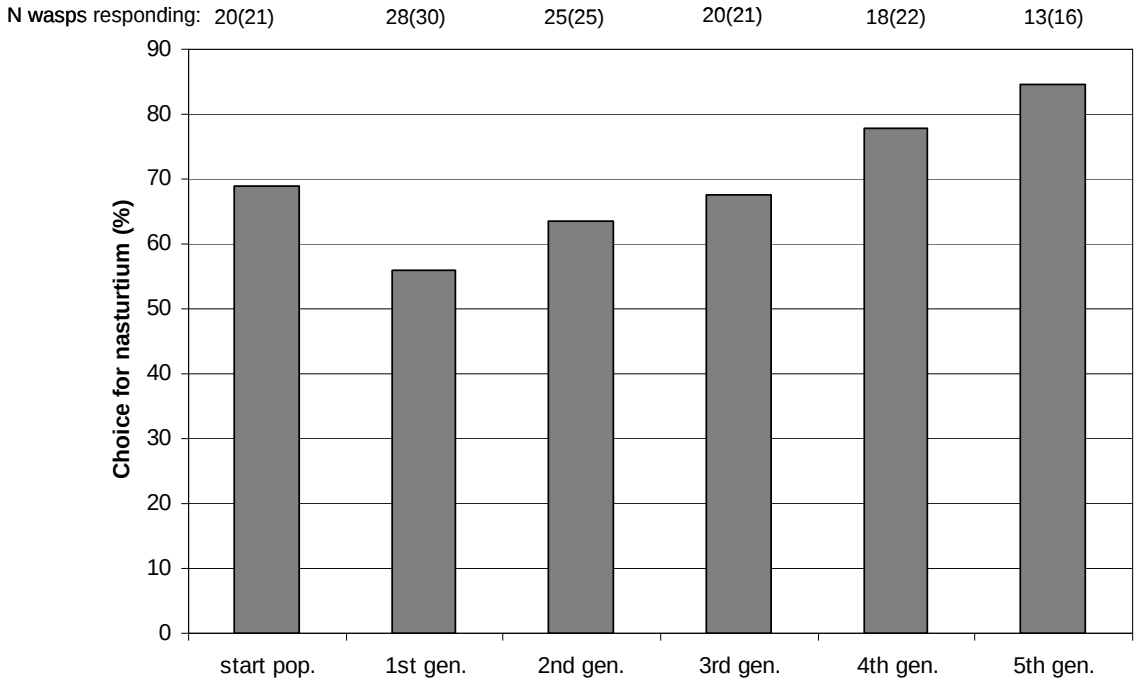


Fig. 21: Average preference for nasturtium of experienced females. Individual females (of the slow learning line) received three spaced oviposition experiences on a P. brassicae *larva and were tested 24 hours afterwards in a dual choice test for preference for either an infested cabbage plant or an infested nasturtium plant. There is no significant difference in preference for nasturtium between the generations (One-way ANOVA, p= 0.785) Females were tested on two or three different days within each generation, except for females of the 5th generation that were only tested on one day. Only the results of tests in which eight or more wasps showed a response, were used to calculate the average preference. Numbers above the bars indicate the numbers of responding females, compared to the total amount of tested females (within brackets).*

3.1.7 Anisomycin treatment

The anisomycin treatment was used to reveal possible differences in memory consolidation between both lines, and was executed with wasps of the fourth and fifth generation. In both generations the same pattern of memory consolidation was observed. Wasps of the slow learning line that were treated with anisomycin showed similar memory retention four hours after the learning experience as wasps of the same group that were fed on sucrose only. This led to the assumption that, at this time point, no LTM is formed in this group. Memory of the fast learning line was, on the other hand, efficiently blocked by anisomycin four hours after the experience. This implies that the fast learning line formed LTM within four hours after the learning experience. The differences in memory formation between the two groups were not significant; probably because the GLM was based on only two measure points in time (the test in the fourth and fifth generation). However, an interaction seems to exist between the factors group and time. (GLM: group: χ^2_1= 0.13, *p*= 0.7152; treatment: χ^2_1=

6.10, *p*= 0.0135; time: χ^2_1= 3.80, *p*= 0.0513; generation: χ^2_1= 9.39, *p*= 0.0022; group X time: χ^2_1= 6.05, *p*= 0.0139).

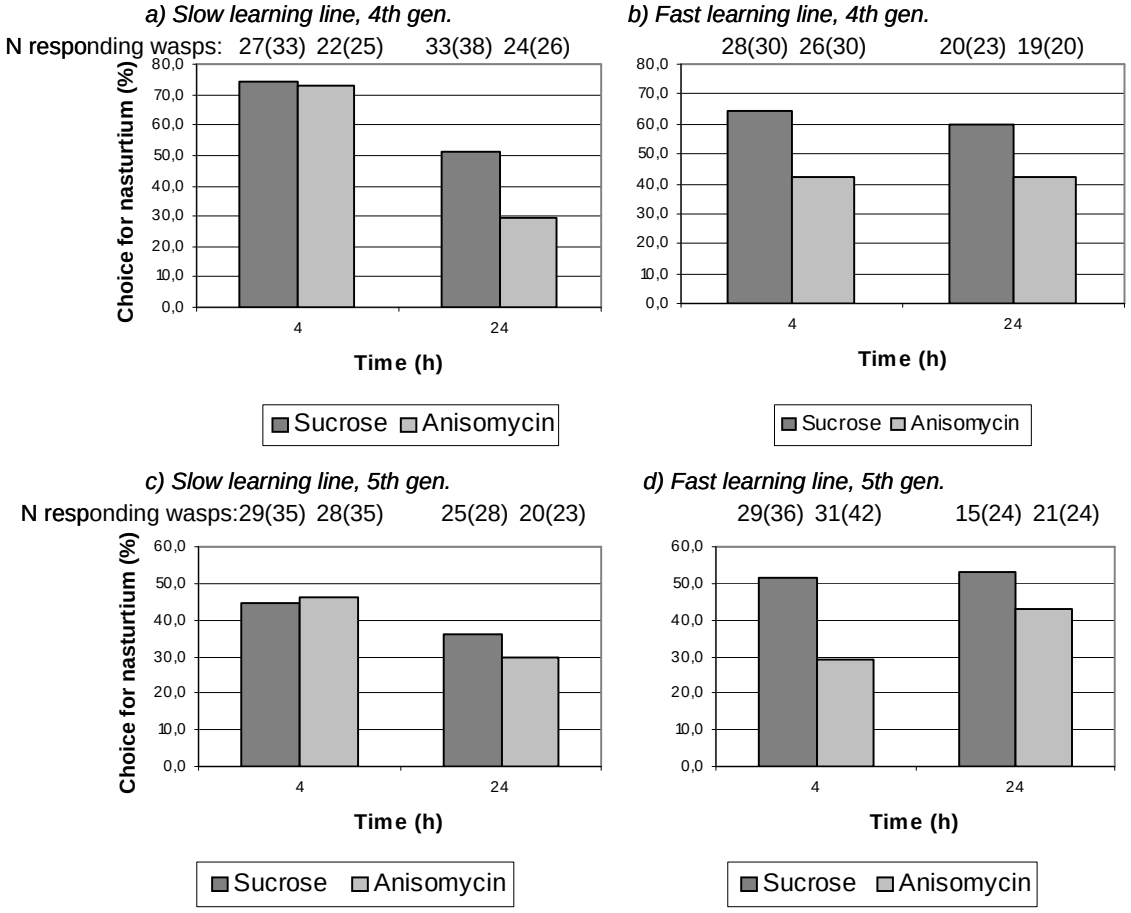


Fig. 22: Preference for nasturtium of females of the (a) slow learning line and (b) fast learning line of the fourth generation, and of the (c) slow learning line and (d) fast learning line of the fifth generation after one learning experience. Individual females received were fed on a sucrose solution or a sucrose solution containing anisomycin (protein-synthesis inhibitor), prior to learning. All females received one oviposition experience on a P. brassicae larva and were tested in the wind tunnel either 4 hours or 24 hours afterwards. In the slow learning line, anisomycin treatment has no effect 4 hours after learning, while in the fast learning line, memory formation was inhibited by anisomycin 4 hours after learning. Statistical analysis did not reveal significant differences between the two groups however if the factors 'generation' and 'treatment' were left out, both groups differed significantly in memory formation (GLM: group: χ^2_1= 0.13, p= 0.7152; treatment: χ^2_1= 6.10, p= 0.0135; time: χ^2_1= 3.80, p= 0.0513; generation: χ^2_1= 9.39, p= 0.0022; group X time: χ^2_1= 6.05, p= 0.0139). Numbers above the bars indicate the numbers of responding females, compared to the total amount of tested females (within brackets)..

3.1.8 Decay of memory over 72 hours

Females from the fifth generation were used to test for possible differences between the two selected lines in the decay of LTM after a single learning experience. For the first measurement (memory retention after 24h), the results of the selection experiment of the whole generation were used. The slow learning line displayed significantly lower learning rates than the fast learning line (GLM: group: χ^2_1= 4.82, p= 0.0281; time: χ^2_2= 2.05, p=

0.3582). The learning rate of the fast learning line decreases from 60% at 24h to 38% at 72h. The learning rate of the slow learning line stays, more or less constant, around 35%.

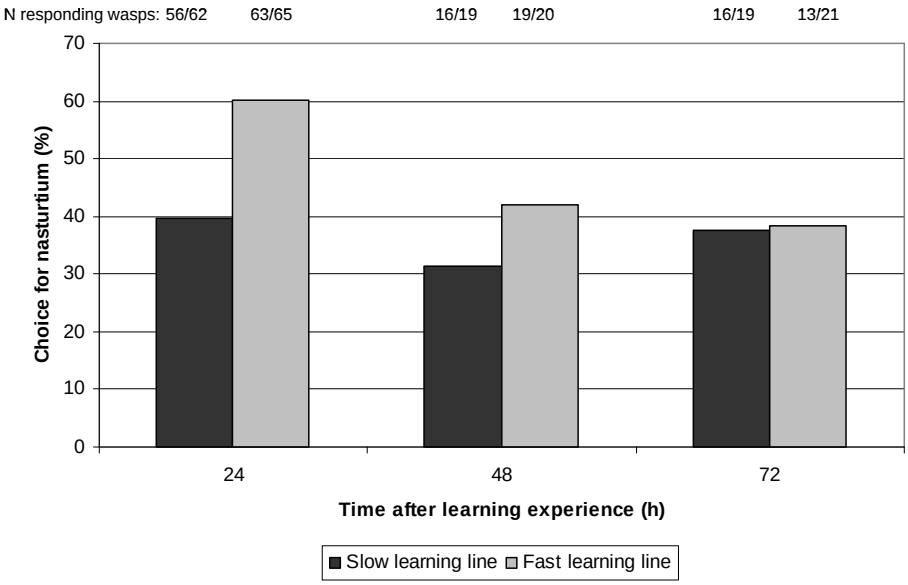


Fig. 23: Average preference for nasturtium of experienced females. Individual females received one oviposition experience on a P. brassicae *larva and were tested 24 hours, 48 hours or 72 hours afterwards in a dual choice test on preference for either an infested cabbage plant or an infested nasturtium plant. The slow learning line displayed significantly lower learning rates than the fast learning line (GLM: group: χ^2_1= 4.82, p= 0.0281; time: χ^2_2= 2.05, p= 0.3582) The result of the after 24 hours includes the test results of the corresponding tests from the selection experiment of the fifth generation. Numbers above the bars indicate the numbers of responding females, compared to the total amount of tested females (within brackets).*

3.2 Fitness costs of learning

3.2.1 Survival rates

The slow learning line proved to have a significantly longer lifespan than the fast learning line ($p < 0.001$). This difference was found in both males and females. Two problems occurred during the execution of this experiment. At first, some wasps managed to escape from the cage, resulting in only 46 and 68 wasps per group. A second problem was that the cages became infected with fungi after several days. The paper and water of the water bottles were exchanged repeatedly, but this did not solve the problem entirely. It is unknown what effect fungi have on the survival of the wasps. Besides these problems, a clear difference became visible between the survival of the slow- and fast-learning wasps. In average, males of the slow learning line lived for 19.2 days, while the males of the fast learning line lived for 13.8 days. The females of the slow learning line had an average

lifespan of 26.8 days, while the females of the fast learning line had an average lifespan of 21.2 days.

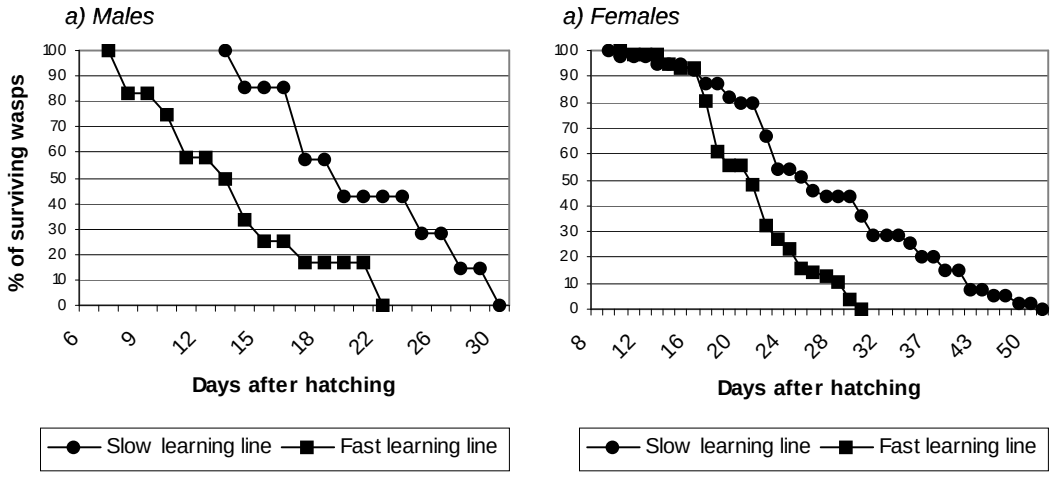


Fig. 24: Survival rates of (a) males and (b) females of the slow learning line and the fast learning line. The slow learning line proved to have a significant longer lifespan than the fast learning line ($p< 0.001$). The average lifespan of the males of the slow learning line was 19.2 days, the females of this group lived for 26.8 days. The average lifespan of the males of the fast learning line was 13.8 days, the females of this group lived for 21.2 days.

3.2.2 Larval development

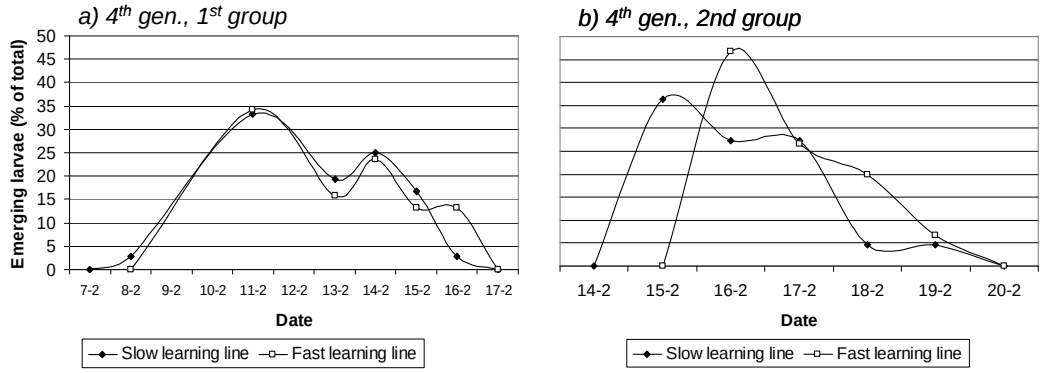


Fig. 25: The emergence from the hosts by wasps of (a) the first and (b) second group of the fourth generation. Emergence is expressed as the number of abandoned caterpillars per day compared to the total number of abandoned caterpillars. The parasitization of group 1 occurred one week before the parasitization of group 2 and is performed by the same females. Parasitization occurred on three consecutive days, on which the same number of females parasitized the same number of caterpillars for both groups. Data from the 9th and the 10th of February are missing.

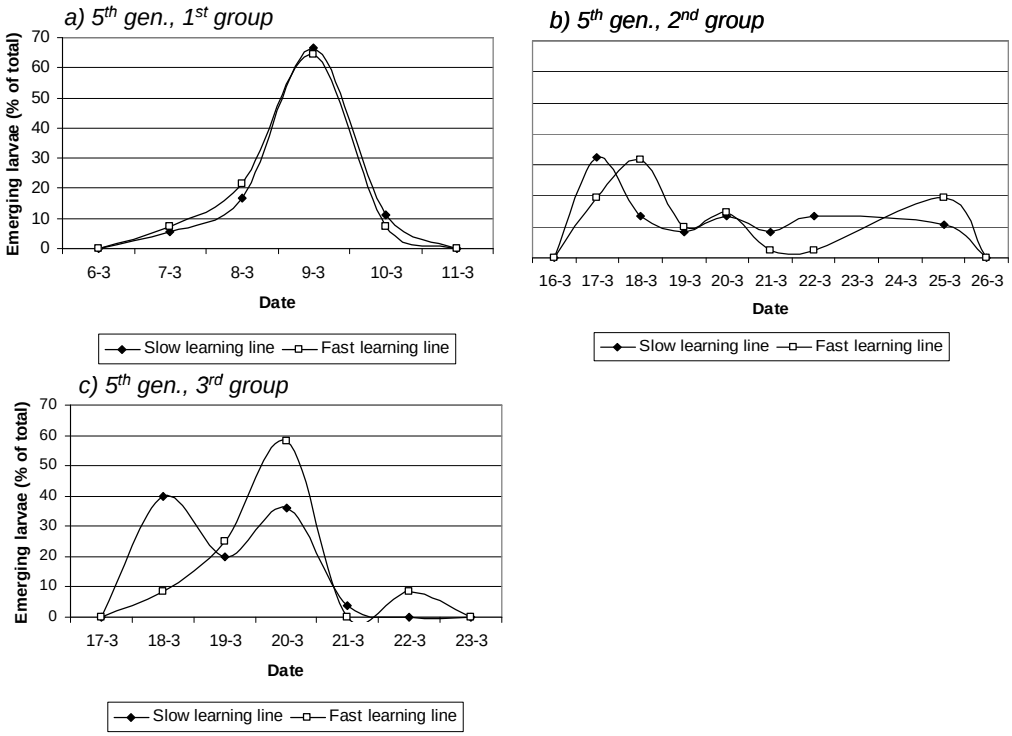


Fig. 26: The emergence from the hosts by wasps of (a) the first, (b) second group, and (c) third group of the fifth generation. Emergence is expressed as the number of abandoned caterpillars per day compared to the total number of abandoned caterpillars. The parasitization of group 1 occurred one week before the parasitization of group 2 and 3 and was performed by the same females. Parasitization occurred on three consecutive days, on which the same number of females parasitized the same number of caterpillars for both groups. Data from the 23rd and the 24th of March are missing.

A difference was observed in the time that both groups needed for larval development. The parasitization, used to create the next generation, took place within two periods of three subsequent days that were separated by one week, in order to increase the time in which wasps are available for experiments. Differences in larval development time were only present in the group that resulted from the second parasitization. In the fourth generation, the second group of the fast learning line started to emerge a day later than the second group of the slow learning line. In the fifth generation, the parasitization took place within three periods of three subsequent days. In this generation, both lines of wasps of the second and third group started to emerge on the same day; however, the first peak in emergence of the fast learning line is situated several days later than the first peak in emergence of the slow learning line. These data suggest that the fast learning lines need more time to complete larval development inside its host; however, because parasitization did not occur on one day but on three consecutive days instead, the actual time needed for

larval development could not be expressed in days and no statistical analysis could be performed on these data.

3.3 CREB isoform expression

CREB isoform expression was measured in every generation in both naïve and experienced females. In the analysis of the samples of generation one and two, a large variation was observed in the expression of specific isoforms between samples and between replicates of the same sample (see chapter 3 of appendix). Based on the results that were earlier obtained by van den Berg, et. al. (in preparation) in the same species, the results of two samples of the first generation and all samples of the second generation (deleted samples are presented in italics in the appendix) were classified as abnormal and were, therefore, not used for further analysis.

3.3.1 CREB expression in naïve females

CREB expression was, in the first place, calculated as the percentage of one isoform to the total amount of isoforms in the same sample. This led to an overview of isoform expression per generation, in which the fast learning line and slow learning line can be compared (see figure 27). The comparison of CREB expression in naïve females between the slow learning line and the fast learning line did not reveal significant differences.

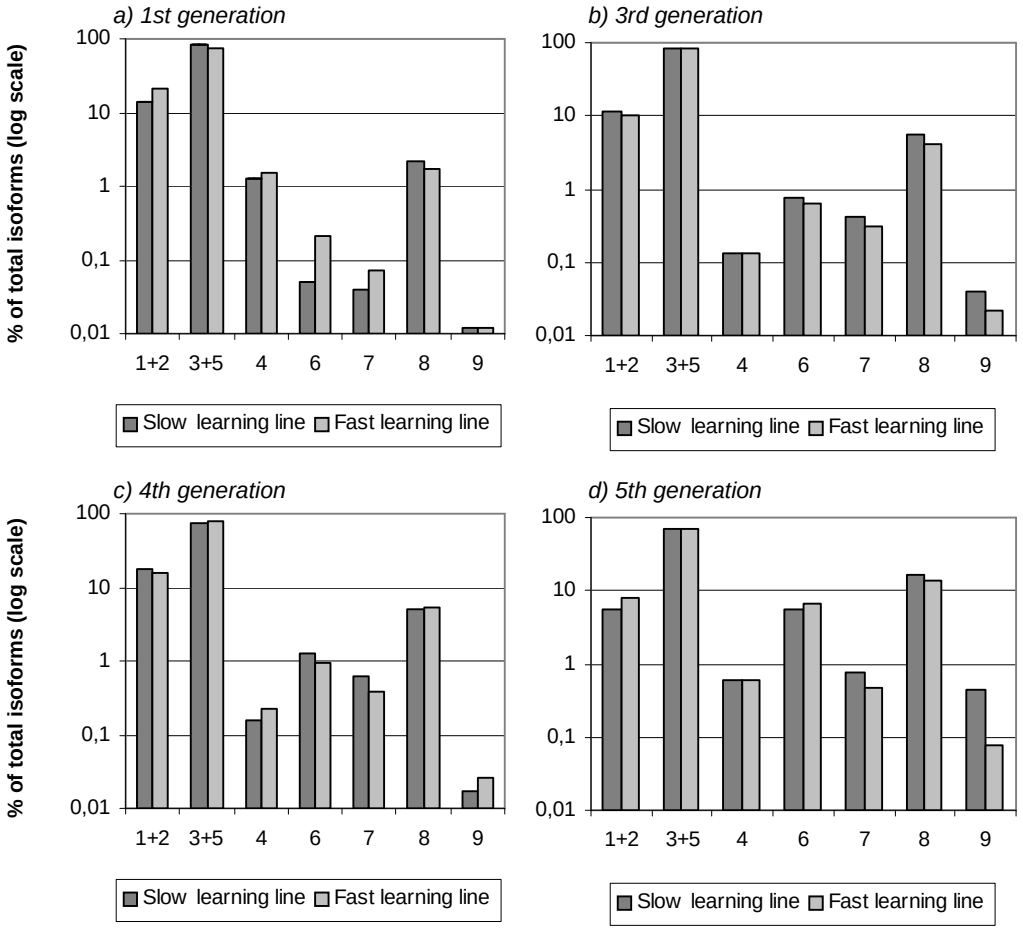


Fig. 27: CREB isoform expression in experienced females of (a) the first generation, (b) the third generation, (c) the fourth generation, (d) and the fifth generation. Asterisks indicate a significant difference between the two groups within one generation. No statistical analysis could be performed with the results of the first generation because the results are based on only one sample per group. For the third generation, results are based on two samples per group. No significant differences were found between the slow learning line and the fast learning line (Independent sample T-test, P-values: 1+2: 0.740, 3+5: 0.608, 4: 0.919, 6:0.724, 7: 0.664, 8: 0.355, and 9: 0.471). For the fourth generation, results are based on two samples per group. No significant differences were found between the slow learning line and the fast learning line (Independent sample T-test, P-values: 1+2: 0.438, 3+5: 0.179, 4: 0.178, 6:0.289, 7:0.444, 8: 0.860, and 9: 0.150). For the fifth generation, results are based on two samples per group. No significant differences were found between the slow learning line and the fast learning line (Independent sample T-test, P-values: 1+2: 0.295, 3+5: 0.888 , 4: 0.683 , 6: 0.454, 7: 0.152, 8: 0.200, and 9: 0.230)

The relative expression of all isoforms is expected to be close to zero in the first generation because the difference between the slow learning line and the fast learning line is expected to be the smallest at this point. Subsequently, the relative expressions are hypothesized either to decrease, or increase, or to stay constant in the successive generations.

This pattern was only observed in isoform seven; for the other isoforms, the patterns are less consistent. In the first generation, isoforms one and two were decreased in the slow learning line; in the third and fourth generation, they were decreased, compared to the fast

learning line. In the fifth generation the relative expression decreased to the value of the first generation. For isoforms three and five the opposite seems true; they were increased in the first generation, but a decreased expression was observed in the third, fourth and fifth generation. Isoform four was, in the first four generations, decreased in the slow learning line. However, in the fifth generation the expression is slightly increased in the slow learning line, compared to the fast learning line. Similar to isoform four, isoform six has a negative relative expression in the first four generations; the expression of this isoform in the slow learning line is, in these generations, 55% to 75% lower than the expression of this isoform in the fast learning line. However, in the fifth generation this difference has disappeared. A more consistent trend is seen in the relative expression of isoform seven; in the first generation, the expression of this isoform in the slow learning line is found to be 45% lower than the expression in the fast learning line. The relative expression decreases rapidly in the successive generations to a relative expression of 65% in generation four and 59% in generation five. Finally, neither isoform eight nor isoform nine showed a consistent pattern in relative CREB expression. The relative expression of isoform eight seems to be constant at 30% during the first three generation, but in the fourth generation the difference between the two groups has disappeared and is close to zero. In the fifth generation it is, again, increased to 26%. The difference in expression of isoform nine is small in the first four generations. In the fifth generation, the expression of isoform nine is almost 500% higher in the selected as in the fast learning line; this is seen as an abnormal increase.

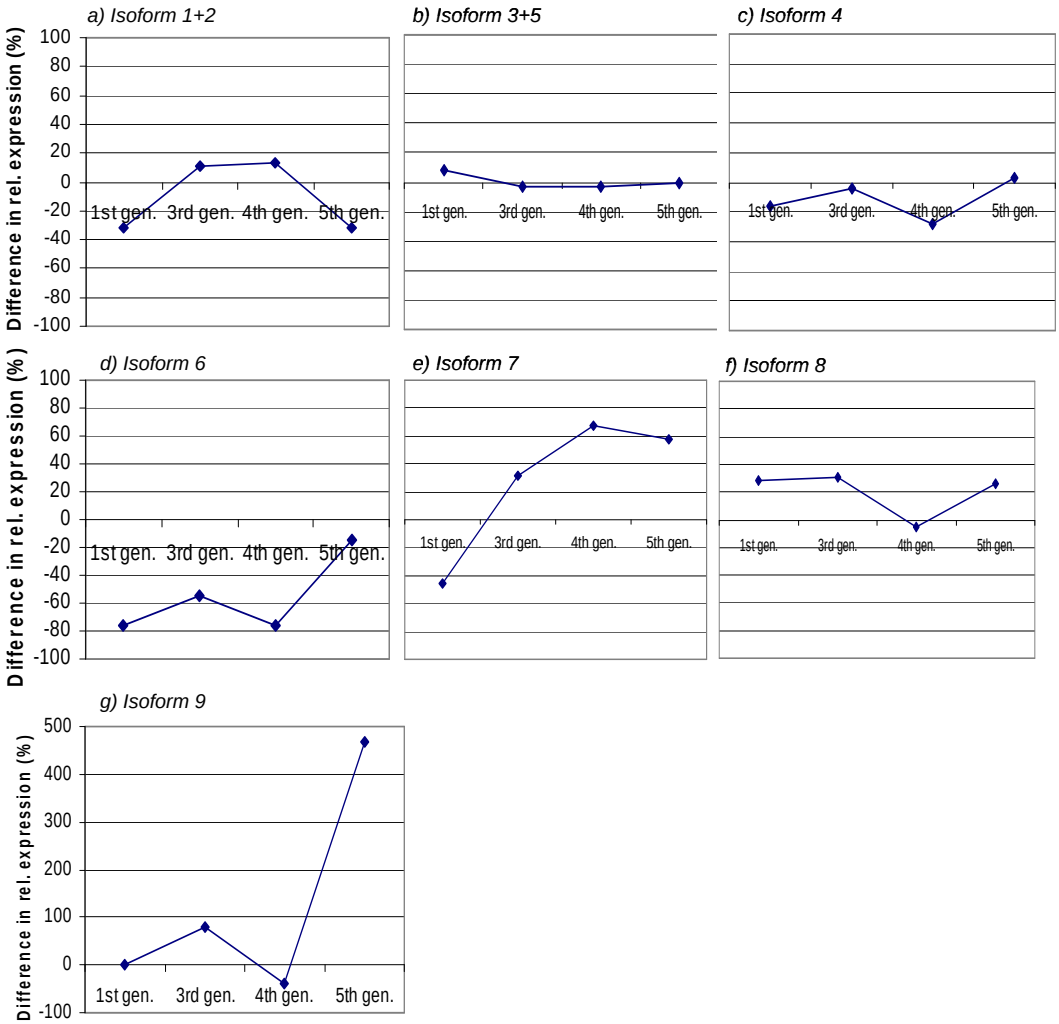


Fig. 28: The difference in relative CREB expression between the fast learning line and the control line of isoform (a) 1+2, (b) 3+5, (c) 4, (d) 6, (e) 7, (f) 8, and (g) 9 in naive females. Data from generation 2 are missing. A negative value in the graphs indicates that the expression of the isoform in that generation of the fast learning line was lower than the expression of that isoform in the slow learning line; a positive value indicates that the expression of an isoform in the slow learning line was higher than the expression of that isoform in the fast learning line, while a negative values indicates that the expression of an isoform was lower in the slow learning line than in the fast learning line.

3.3.2 CREB expression in experienced females

For the CREB expression in experienced females, the same display of results was used (see figure 29). The comparison of CREB expression in experienced females between the slow learning line and the fast learning line did not reveal significant differences, except for the expression of isoform nine in the fifth generation.

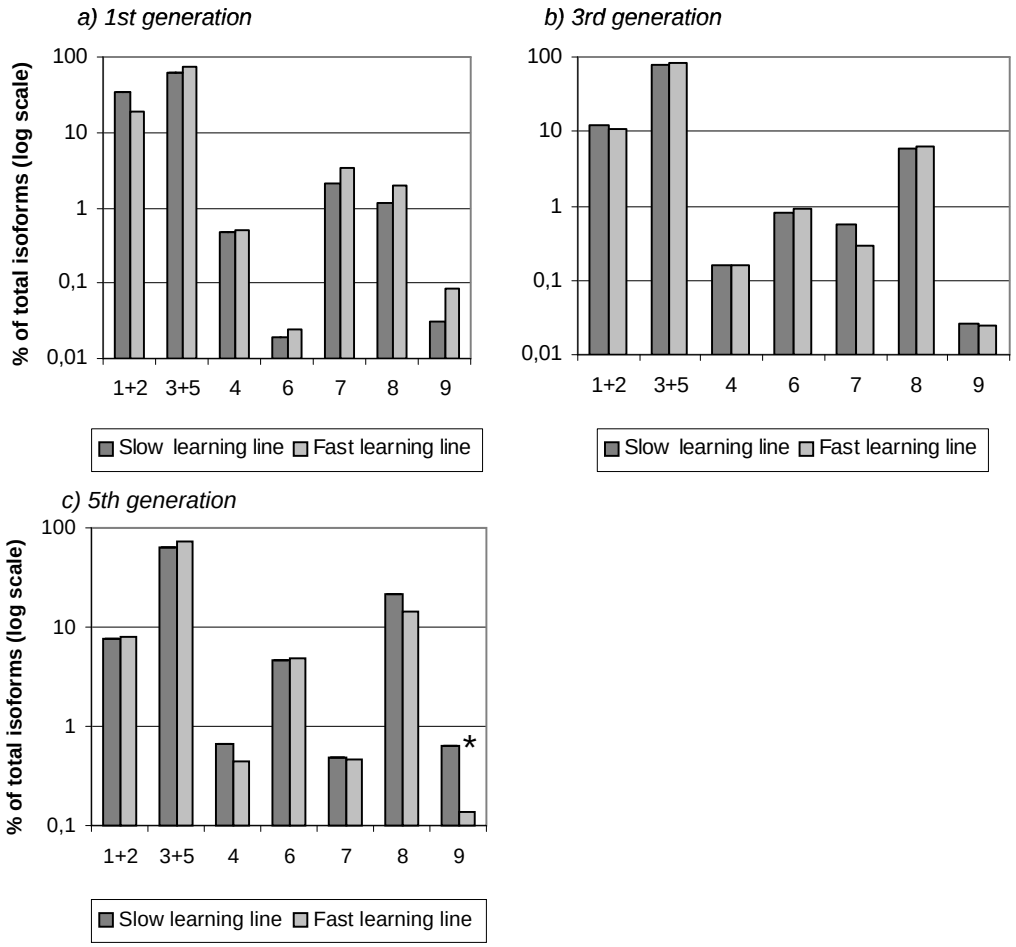


Fig. 29: CREB isoform expression in experienced females of (a) the first generation, (b) the third generation, and (c) the fifth generation. Asterisks indicate a significant difference between the two groups within one generation. For the first generation, test results are based on one sample per group for the slow learning line and two samples per group for the fast learning line. No significant differences were found between the slow learning line and the fast learning line (Independent sample T-test, P-values: 1+2: 0.145, 3+5: 0.217, 4: 0.389, 6:0.584, 7:0.337, 8: 0.249, and 9: 0.443). For the third generation, test results are based on two samples per group. No significant differences were found between the slow learning line and the fast learning line (Independent sample T-test, P-values: 1+2: 0.296, 3+5: 0.271, 4: 0.972, 6:0.254, 7:0.098, 8: 0.849, and 9: 0.992). For the fifth generation, test results are based on two samples for the fast learning line and one sample for the slow learning line group. The expression of isoform 9 in the slow learning line was significantly higher than the expression of this isoform in the fast learning line (Independent sample T-test, P-value: 0.011). No significant differences were found in the expression of the other isoforms (Independent sample T-test, P-values: 1+2: 0.755, 3+5: 0.165, 4: 0.112, 6:0.911, 7:0.855 and 8: 0.380).

The difference in relative CREB expression in experienced females can be found in figure 30. CREB expression in experienced females was only measured in three generations. In isoforms four and six difference in expression over the three generations seems to be consistent. In the first generation, the relative expression of these isoforms is close to zero (-5% for isoform four and –20% for isoform six). The relative expression of isoform four increases in the subsequent generations, until 50% in the fifth generation. The relative

expression of isoform six, on the other hand, decreases in the subsequent generation, until –100% in the fifth generation. The relative expression of isoform eight and nine shows a similar pattern, however, it is expected to be less reliable; since the relative expression of isoform eight increases from -40% in the first generation, to 40% in the fifth generation, and the relative expression of isoform nine increase from –80% in the first generation, to 370% in the fifth generation. The relative expressions of the other isoforms do not show consistent patterns because the difference in expression between the two groups is in the first generation higher than in the third and fifth generation.

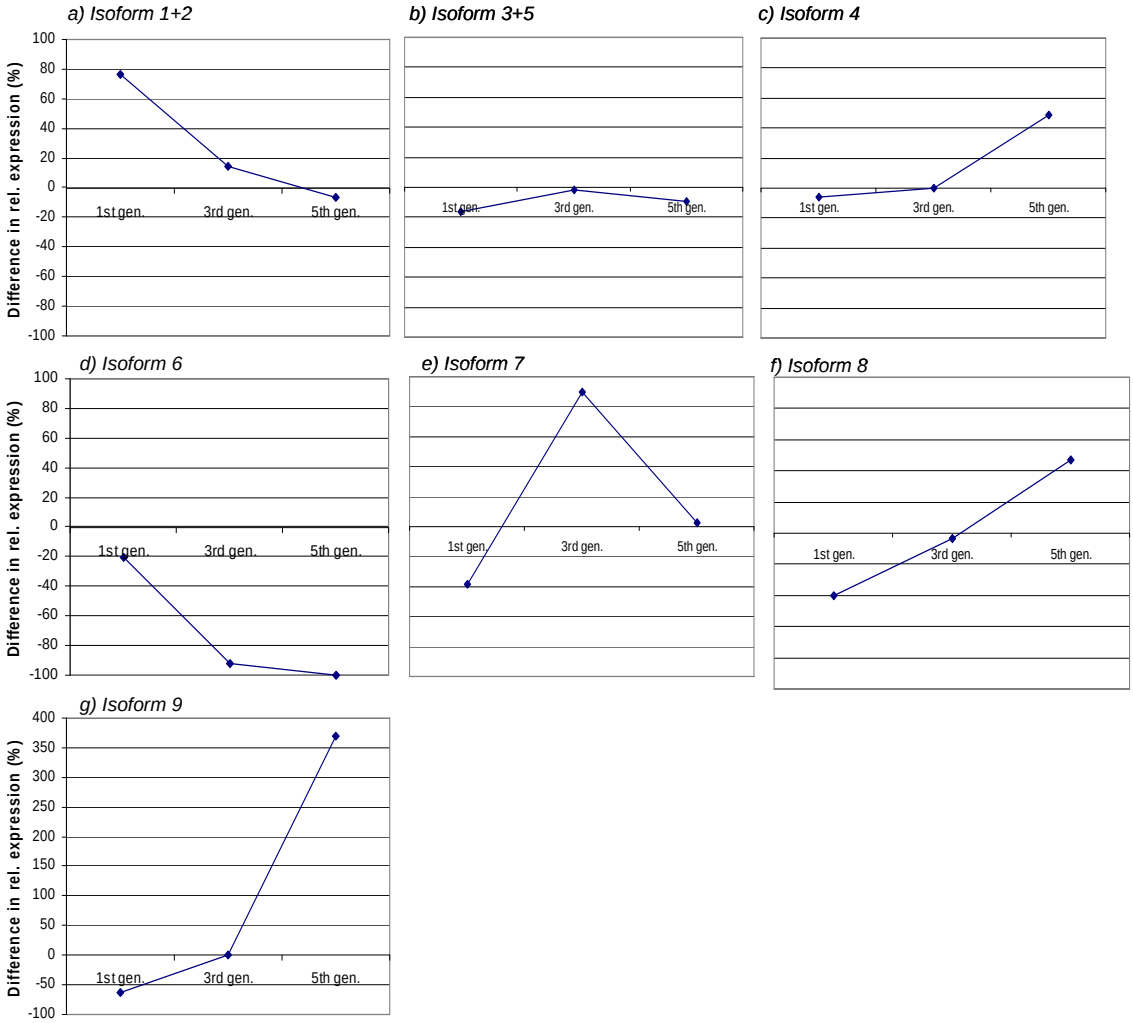


Fig. 30: The difference in relative CREB expression between the fast learning line and the control line of isoform (a) 1+2, (b) 3+5, (c) 4, (d) 6, (e) 7, (f) 8, and (g) 9 in experienced females. CREB expression in experienced females was measured 24 hours after a single oviposition experience on nasturtium and was only measured in females of generation one, three and five. A negative value in the graphs indicates that the expression of the isoform in that generation of the fast learning line was lower than the expression of that isoform in the slow learning line; a positive value indicates that the expression of an isoform in the slow learning line was higher than the expression of that isoform in the fast learning line.

3.3.3 Change in isoform expression after one experience

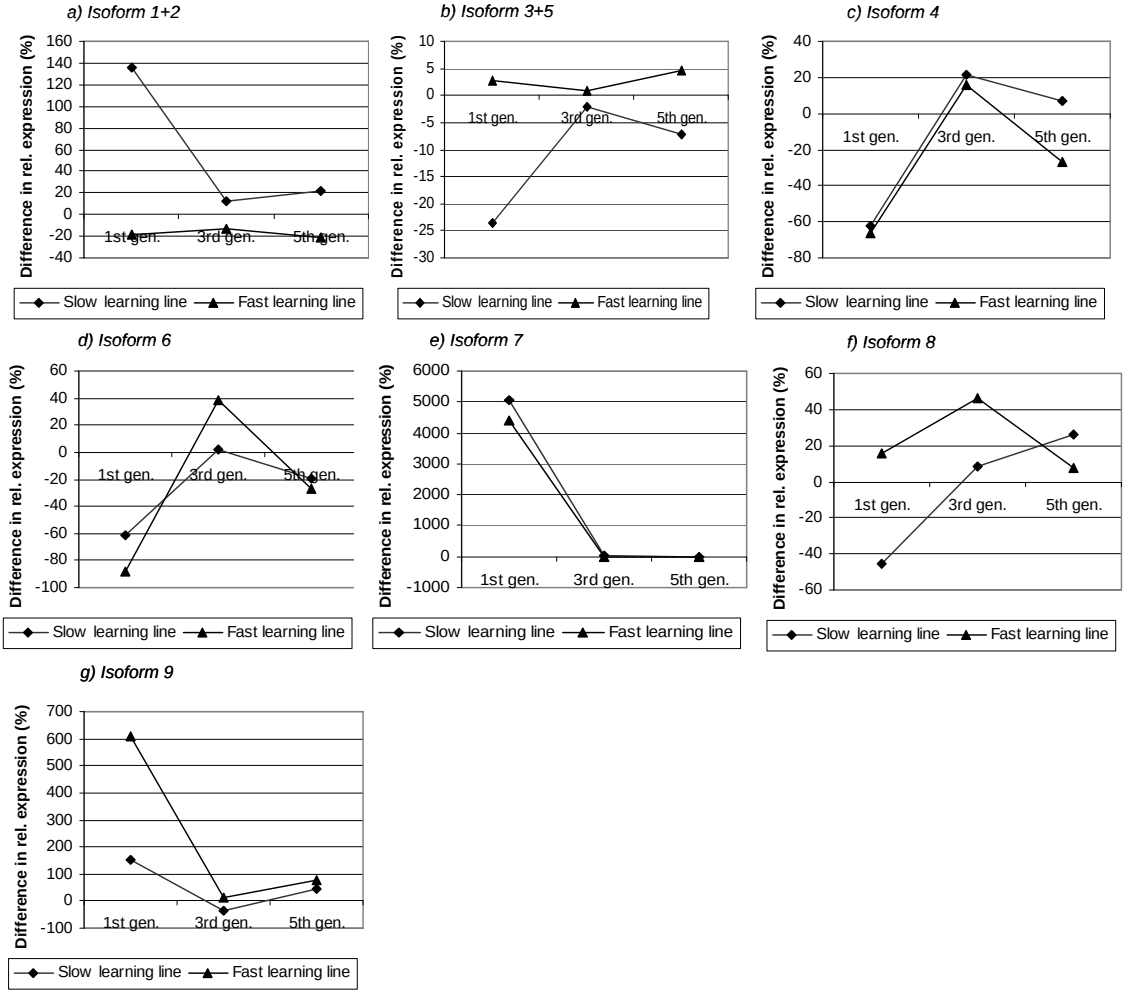


Fig. 31: The difference in relative CREB expression between naïve females and experienced females of isoform (a) 1+2, (b) 3+5, (c) 4, (d) 6, (e) 7, (f) 8, and (g) 9 in both the slow learning line and the fast learning line. CREB expression in experienced females was measured 24 hours after a single oviposition experience on nasturtium and was only measured in females of generation one, three and five. A negative value in the graphs indicates that the expression of the isoform in that generation in experienced females was lower than the expression of that isoform in naïve females; a positive value indicates that the expression of an isoform in experienced females was higher than the expression of that isoform in naïve females.

Finally, CREB expression was expressed as the difference in relative expression between experienced and naïve females for both selected lines. This comparison could reveal the change in isoform expression after an experience. The differences expression of isoforms four, six, seven and nine seem to change in the same pattern in both the slow learning line and the fast learning line. Isoforms one and two were increased in every generation in experienced females of the slow learning line, while they were decreased in experienced females of the fast leaning line. Isoforms three and five are, on the contrary, decreased in every generation in experienced females of the

slow learning line, while they were increased in experienced females of the fast leaning line. Isoform eight is increased in experienced in all generations of the both lines, except for the first generation of the slow learning line, where it is decreased.

4. Discussion and conclusions

The main purpose of this thesis project was to investigate the possibility to select on learning rate in the parasitoid wasp *C. glomerata*, by using artificial selection constrains*. C. glomerata* learns, compared to other insect species, fast because LTM is formed after only one associative learning experience (Bleeker et al., 2006; Geervliet et al., 1996; Geervliet et al., 1998; Smid, 2006; Smid et al., 2007). Therefore, selection was aimed at either the ability or disability to form LTM after a single oviposition. One group of wasps was selected on slow learning or, not forming LTM after a single experience; and a second group was selected on fast learning or, forming LTM after a single experience. The selection continued over five generations. It was hypothesized that every generation a smaller portion of the slow-learning line would form LTM after a single experience. Furthermore, fitness parameters of both groups were monitored by comparing both the time needed for larval development, and the average lifespan of the wasps with wasps of the fourth and fifth generation of the selection. Fast learning wasps were hypothesized to have a reduced fitness, compared to slow learning wasps because of the evolutionary costs of learning.
Finally, it was tried to determine the role of CREB isoform expression on LTM formation, by comparing the CREB isoform expression in the brain between females of the slow learning line and females of the fast learning line in both naïve and experienced females.

During the selection of the start population, as well as during the selection of the first and second generation, a high variation was observed between replicates of the wind tunnel test that monitored memory retention after 24 hours. Because of this variation, it was difficult to distinguish differences between the two selected lines. The variation might be a result of seasonal effects that affected the quality of the wasps, as well as the quality of their hosts. Similar seasonal patterns were observed in the response rates of naïve females. These were, in fact, also decreased in the start population and first two generations, compared to the latter three generations. Response rates are expected to be influenced by outside temperatures. When the outside temperatures are low, the air inside becomes dehydrated because of the heating. During a wind tunnel experiment the humidity of the air is maintained constant between 40 and 60% but before and after the experiment the wasps are held in an incubator, where the humidity cannot be not regulated. On a cold day or night, humidity inside the building can be down to 20 or 30%, which could interfere with the quality of the wasps and thereby with their testing results. Especially during the testing of the

second generation, but also during the selection of the start population and the first generation, outside temperatures were low. Another Master student who worked on comparable learning experiments with *C.glomerata* in the same period last year, reported similar problems. Therefore, seasonal circumstances are likely to be the cause of the variation in test results that were observed during the first three selection rounds. Despite these problems, the selection on learning rate was still ongoing. The average learning rate was low in the start population and increased in both lines during the selection of the first, second and third generation. From the third generation on, differences in learning behavior between the two lines became visible. At this point, the learning rate of the fast learning line stayed constant around 60%. These results are comparable to earlier experiments in *C. glomerata* that used the same wind tunnel set up and training methods. Smid *et al*. (2007), for example, observed average memory retention of 65%, twenty-four hours after a single experience. The learning rate of the slow learning line, on the other hand, started to decrease until an average learning rate of 40% in the fifth generation.

Furthermore, an experiment, in which LTM formation was specifically blocked with the protein synthesis blocker anisomycin, suggested that the consolidation of LTM after one learning experience was delayed in the slow learning line. In the same experiment, the fast learning line showed a similar pattern of LTM formation as was observed in earlier experiments on *C. glomerata,* performed by Smid *et al*. (2007). In addition, an experiment to test the decay of memory after one learning experience further confirmed the difference in learning rate between the two lines of wasps.

In addition to the differences in learning rate and in the pattern of memory formation, the selection experiment revealed that the fast-learning wasps possibly suffered from the fitness cost of maintaining a high learning rate; since the slow learning line had, in average, a longer lifespan, and wasps from the slow learning line that resulted from the second parasitization needed less time to complete larval development.

CREB isoform expression in naïve females was measured in all five generations. The results confirmed that there exists no correlation between CREB isoform expression and learning rate in naïve females. Secondly, CREB expression was measured in experienced females (twenty-four hours after a single experience) of the first, third and fifth generation. No correlation between CREB isoform expression and learning rate was found in experienced females; however, not enough time was available to produce enough replicates per group, which makes the results less reliable.

4.1 Differences in memory formation between the slow learning line and fast learning line

Two remarkable differences were observed in memory formation between the two selected lines. First of all, the learning rate after one experience of the slow learning line appeared to be significantly lower than the learning rate of the fast learning line after one experience. The tests on naïve preference that were performed in each generation, proved that the innate preference for nasturtium did not differ significantly between both groups and, therefore, can not be held responsible for differences in learning rate. Furthermore, memory retention after three experiences was observed to be high in each generation of the slow learning line, proving that these wasps were not selected on the disability to learn.
Secondly, in an additional test on memory formation, in which LTM formation was specifically blocked with the protein synthesis inhibitor anisomycin, it was suggested that the lines differ in the pattern of LTM formation. In this experiment, the fast learning line formed LTM within four hours after the learning experience, as was observed earlier in 'normal' wasps. The slow learning line, on the other hand, did not form LTM in this time period, but differences between the two groups that were treated with either with sucrose+anisomycin or sucrose only, became visible only twenty hours afterwards. Based on these results, we hypothesized that the period, in which ARM is consolidated into LTM, is prolonged in the slow learning line. In other words, females of the slow learning line needed more time to accomplish structural changes in the brain that are the result of learning. The two differences in memory formation between the two selected lines are expected to be interrelated. It is still unclear whether ARM and LTM appear in parallel or sequentially, in which ARM is consolidated in LTM afterwards. Isabel *et al*. (2004) stated that ARM and LTM formation can be seen as competitive states that never coexist at the same moment in the brain, while Tully et al. (1994) and Margulies et. al. (2005) stated that LTM and ARM are produced in parallel and do coexist at the same time in nerve cells. It is expected that LTM formation in *C. glomerata* occurs according to the model of Isabel, while LTM formation in *C.rubecula* occurs according the model of Tully and Margulies (Smid, et al., 2007). The results from the present study suggest that the learning behavior of the slow learning line has shifted towards the learning behavior of *C. rubecula.* The selection regime made the information during host searching highly unpredictable for the slow learning line; although, at first, suitable host were found on nasturtium, the females could only reproduce if they flew to cabbage in the subsequent wind tunnel test. It was, therefore, disadvantageous for females of the slow learning line to consolidate LTM directly after one experience. By increasing the

time in which LTM is consolidated, an animal receives more time to evaluate if the information from the environment is reliable (Smid, 2006). It is difficult to draw conclusions about the present pattern of memory formation in the slow learning line because it is not entirely clear when ARM disappears in this species. Presumably, ARM, is present up to 4 hours in the slow learning line but it is not clear what happens afterwards. In fact, the anisomycin treated group that was tested 24 hours after the experience has a decreased learning rate but it is not known if this is the result of the blocker or of the difference in learning rate that was already present between the two lines. It can, however, be hypothesized that the prolongation of the less stable ARM phase in this group has led to a less efficient LTM formation. This implies that, although ARM and LM may exist in parallel, they do not work as independent traces.

It might be interesting to further investigate the exact duration of the ARM phase in the slow learning line by repeating the anisomycin treatment after three leaning experiences, instead of a single experience, to possibly reveal more evidence for this hypothesis.

4.2 Artificial selection on learning rate

The selection procedure, performed in three different steps, that was used for this project appeared to be a successful way to select on learning rate in *C. glomerata.* Because similar selection procedures were used for both the slow learning line and the fast learning line, most plausible alternative explanations for the differences between the two lines can be excluded. First of all, it is expected that the differences between the two lines are not the result of changes in outside circumstances. During the selection of the start population and the first two generations, outside circumstances were expected to influence both the response rate and the learning rate of the wasps. However, the effect of these circumstances is expected to be equal for both groups and can, therefore, not be held responsible for differences between groups that were tested on the same day. Second, next to the improved outside circumstances, response rates might also be improved by a selection on the ability to fly in the wind tunnel. In the start population, several wasps were observed that did not seem capable of initiating a directed flight. Instead of flying to an odor source directly, these wasps flied in, what seemed, uncoordinated circles. The number of wasps that initiated similar uncoordinated flights, was drastically reduced in the first generation and negligibly small in the successive ones. This suggests that the wasps were, next to learning rate, selected on the ability to fly towards a specific odor source in the wind tunnel. This selection might have increased the quality of the wasps, which could have

influenced learning performances, as well. The selection procedure of the slow learning line contained three separate wind tunnel tests, while that of the fast learning line contained only two. However, the response rates in the additional test on memory retention after three spaced experiences in the slow learning line were as high as the response rates in the preceding test on memory retention after a single experience. Therefore, it is not expected that this additional test has increased selection on the ability to respond in the wind tunnel, and this factor cannot be held responsible for differences in learning rates. Finally, inbreeding is rejected as the cause of differences between the two groups because breeding strategies were the same for both groups.

Some weak points of the selection procedure were observed, too. In the first place, memory retention within one generation was not constant over the different testing days. This variation might be the result of variation in the damage that the caterpillars inflicted to the odor sources. The amount of caterpillars per plant can be specifically regulated; however, it can not be regulated to what extent the caterpillars inflict damage to the leaf; neither can it be regulated at which time period before the actual test, the damage is inflicted. Although, to the naked eye, the plants did not really differ in herbivore damage, the odor production between plants used on different days might have been different. To solve this problem, the two lines were tested on the same day and the same plants, as often as possible. To further decrease the variation in wind tunnel test results and, possibly, to reveal larger differences between the two lines, it might be an idea to test experienced females on artificially damaged plants. Furthermore, the preference for nasturtium of experienced females of the slow learning line was not as low as the preference for nasturtium of naïve females. The average preference for nasturtium of naïve females of both groups differed between 10 to 20%; moreover, in similar experiments with experienced females of *C. rubecula,* memory retention after twenty-four hours was observed to be around 20% (Smid et al., 2007). However, in the present study, the preference for cabbage of experienced females of the slow learning line did not get below 40%, suggesting that the ability to form long-term memory after one experience is not completely absent in this group. This might be explained by the following two reasons. First of all, it is very likely that the selection is not completely fulfilled yet; further selection might result in lower rates of learning in the slow learning line. Secondly, the infestation of the odor sources makes, even the non-attractive nasturtium, more attractive for the wasps. The same effect was observed in the anisomycin experiments; while LTM formation was expected to be totally blocked in the fast-learning

group four and twenty-four hours after the learning experience, the choice for nasturtium was observed to be around 30 to 40%.

The selection on learning rate in *C. glomerata* occurred relatively fast compared to similar experiments in other insect species. Mery *et al.* (2002), for instance, conducted a study on *Drosophila*, in which artificial selection was performed on the ability to avoid specific oviposition substrates. In this study at least 15 generations were needed to create a strain of flies that showed significant better learning performances, compared to the control group. This experiment has several similarities to the experiment executed in the present study. Both studies were, for instance, aimed at olfactory associative learning and used a dual choice test to accomplish and to monitor selection. A difference between the studies is that the study in *Drosophila,* was only aimed at increasing learning ability, while the control group consisted of non-selected flies. In the present study, on the contrary, selection was aimed at both increasing and decreasing learning ability, resulting in a slow learning line and a fast learning line. The differences between the lines became visible in the third generation; from this generation on the learning rate of the slow learning line started to decrease, while the learning rate of the fast learning line stayed, more or less, constant in the last three generations. These results suggest that, for an insect, it takes more time, or a higher selection pressure, to upgrade its learning ability, rather than to down-regulate this ability. More evidence for this hypothesis was found in a similar study on learning in *Drosophila* (Lofdahl, 1992). In this experiment, two strains of *Drosophila* were selected on good learning and poor learning, respectively. Learning rate was measured as the number of flies per generation that had successfully learned to extend their proboscis, as a response to a specific odor. The percentage of the start population that showed the conditioned response was observed to be 19%. After five generations of selection, the learning rate of the poor-learning line was brought back to, approximately, 0 to 4%, while the learning rate of the good-learning line had only increased to, approximately, 23%. During the prolongation of the experiment, the percentage of flies of the good-learning line that showed good learning, increased until 77%, while this percentage in the group of flies that were selected on poor learning stayed constant around 0 to 4%.

From an evolutionary perspective, this would mean that, for an insect, it is easier to adapt to an environment in which the predictive value of a learning experience is low, rather than to adapt to an environment in which the predictive value of a learning experience is high. In other words, when *C. glomerata* would be released in an environment where its hosts are

distributed in an unpredictable way, it will experience less difficulties to evolve slow learning, than *C. rubecula* will experience to evolve fast learning, in the case it would be released in an environment where its hosts are distributed in a predictable way.

A second hypothesis that can explain why learning rate was not increased in the fast learning line is related to the initial situation of memory retention in the start population. The 'natural Wageningen' strain of wasps might already exhibit the maximal level of memory retention that is possible for this species. Therefore, it is not possible to further increase learning rate. Further selection of the wasps will possibly reveal more knowledge on the process of the evolution of learning in both *C. glomerata* and *C.rubecula.*

4.3 Evolutionary costs of learning

In the past few years, several studies have shown that learning can have both direct and constitutive costs on the fitness of insects. *Drosophila* flies, for example, that were selected on high-learning had a reduced larval competitive ability (Mery and Kawecki, 2003), a lower fecundity (Mery and Kawecki, 2004) and lower resistance to extreme stress (Mery and Kawecki, 2005). Additionally, a study from the same group revealed that selection on poor food led to higher survival rates and a reduced learning ability; however, selection on improved learning ability, did not lead to lower survival rates (Kolss et al., 2006).

In the present study, some possible constitutive costs of learning were observed as well. The slow learning line proved to have a significantly higher lifespan than the fast learning line. Furthermore, relatively old wasps of the fast learning line produced offspring that needed more time to complete their larval development; although, this difference between the two lines could not be tested on significance. Before clear conclusions can be drawn from these results, it will be important to investigate if changes in body size and body weight have occurred between both groups, as well. It might, for example, be possible that the selection on slow learning has led to an increase in body size and, consequently, to an increase in lifespan. Comparison of body size and brain size is, furthermore, interesting because it is assumed that enhanced memory ability is associated with a larger volume of the brain (Harvey, 1990; Healy, 1990; Dukas, 1999). It might, therefore, be possible that the wasps of the fast learning line have developed a larger brain than the wasps of the slow learning line, or that the brain size of the slow learning line has decreased. Additionally, the selection on learning rate may also have led to differences in brain anatomy. Previous studies to the differences in brain anatomy between *C. glomerata* and *C.rubecula,* reported that both species have a comparable anatomy of the olfactory processing pathway. The two

wasp species are highly similar in the morphology and distribution of olfactory sensilla in the antennae, as well as in the organization of the glomeruli in the antennal lobe (Smid et al, 2003; Bleeker et al, 2004). Based on these results, it was hypothesized that the differences in learning rate between the two species might be the result of chemical differences in specific brain parts, rather than on structural differences. VUM neurons in the antennal lobe of *C. glomerata*, might, for example, be more sensitive to octopamine than VUM neurons of *C. rubecula.* Differences in learning rate might also be a result of differences in the amount of octopamine that is secreted after a learning experience (Bleeker et al, 2005). Similar comparisons between wasps of the fast learning line and the slow learning line might provide more information about the correlation between learning rate and brain morphology and size. Because the development of a larger brain is probably associated with an increase in energetic and nutritional costs (Dukas, 1999), this may have led an increase in body mass in the slow learning line. To further investigate this hypothesis, it will be interesting to compare brain size, as well as brain composition, between both groups and to couple this to other characteristics of both groups, such as wing length, fecundity and the effectiveness of parasitization.

Furthermore, direct costs of learning were suggested from the difference in response time between experienced females of the two lines. The response time of naïve females did not differ significantly between both groups, but in experienced females, a significant difference in response time was observed in the second and fourth generation. In these generations, females of the fast learning line needed more time to respond; in the sense that they stayed (walking or standing) in the release cylinder for several minutes and took longer flights. In a study by Dukas and Duan (2000), on potential fitness costs of associative learning in the parasitoid wasp *Biosteres arisanus*, a correlation was found between the experience of a wasp and, consequently, the duration of searching behavior and the number of eggs laid on a single odor source of this wasp. In this experiment, experienced and naive wasps received the choice between an infested (and conditioned) odor source and an uninfested odor sources. As expected, the experienced wasps encountered the conditioned odor source more often than the unconditioned one and, therefore, spent less time on searching behavior. Furthermore, experienced wasps spent more time on the odor source after landing and laid significantly more eggs per odor source. Naïve wasps encountered both odor sources but spent less time on searching behavior, once landed. A resulting hypothesis from this study was that learning only improves fitness if it results in significant time savings

during host searching behavior and in an increased parasitization (Dukas and Duan, 2000). In the present study, learning allowed the wasps to restrict host search to nasturtium. However, the results were not consistent to the result of Dukas and Duan, since fast learning females spent more time on searching behavior. In fact, when the degree of experience would be coupled to the degree of memory retention, the slow learning line would show increased searching behavior. This inconsistency might be caused by the used wind tunnel set up that contained two odor sources that were both damaged by suitable hosts. It is, therefore, practically seen, not disadvantageous for a female to land on the unconditioned odor source. A second explanation might be that the females of the fast learning line spent relatively more energy on learning and had, therefore, less energy available for flying. It would be interesting to study the behavior of both groups in a setup with greater analogy to the natural situation to investigate if, indeed, changes have occurred in the host searching behavior between the slow learning line and the fast learning line.

4.4 CREB isoform expression and the formation of LTM

CREB isoform expression in naïve females was measured in each generation. Two of the four sample of the first generation and all samples of the second generation were left out from analysis because they showed abnormal results, compared to similar measurement that were earlier performed in *C. glomerata* (van den Berg, et al., in preparation). The results confirmed that there exist no correlation between CREB expression and learning rate in naïve females since none of the differences between the two selected lines were significant. The most remarkable changes (based on P-values) in naïve females were observed in the fourth generation in the expression of isoforms three and five, four, and nine; and in the fifth generation in isoform seven. However, the nature of the differences in the expression of isoforms three and five, four, and nine is not consistent over all generations. The difference in the expression of isoform seven seems to be consistent but due to the high variation in test results of this isoform, the differences were not significant.

CREB isoform expression was also measured in experienced females. However, not enough time was available to produce more than two replicates per group and CREB expression was only measured in the first, third and fifth generation. Therefore, not enough samples were available to produce reliable results. Experienced females of the fifth generation of the two selected lines differed significantly in the expression of isoform nine. More remarkable, although not significant, differences (based on P-values) were found in the first generation in isoforms one and two, in the third generation in isoform seven, and in

the fifth generation in isoforms three and five and isoform four. When looking at the differences in relative expression between the two lines, the changes in isoform four and isoform six seem to be most consistent. Isoforms four was increased in experienced females of the slow learning line, while isoform six was decreased in the slow learning line, compared to the fast learning line.

Finally, CREB expression was expressed as the difference in relative expression between experienced females and naïve females of both lines. Before conclusions can be drawn from these results and the previous ones, the following two remarks should be taken into consideration. First of all, isoforms that are available in relatively small amounts are difficult to detect. It was observed that even in the no-template controls, which only contain the primers and the SYBR green master mix, fluorescence increases after a certain amount of cycles due to the formation of primer-dimers and contaminations of the master mix. Measurements at high Ct values are, therefore, less reliable, especially when they are within the same range as where no-template control curves come up. This was the case for isoforms four, six and nine because the Ct values of these isoforms were, in overall, within the same range as their no-template controls. To be more concrete; whereas the difference in repetitions cycles, before fluorescence passes the threshold, between the sample and the no-template control was observed to be more than ten cycles in isoforms one and two, isoforms three and five, isoform seven and isoform eight; the same difference appeared to be around five in the measurement of isoform six, around three in the measurement of isoform nine, and around two in the measurement of isoform four. This effect also became visible by calculating the difference in relative expression between experienced and naive females. In fact, it appeared that the difference in isoforms four, six, seven, and nine between experienced females and naïve females of both lines changed in the same pattern. This implies that measurements of these isoforms depend more on the composition of the master mix, than on the composition of the template. To possibly solve this problem it could be suggested to increase the concentration of cDNA in the template by, for example, using more wasps per sample or by increasing the amount of cycles during cDNA amplification.

Secondly, when looking at relative isoform expression, it should be noted that isoforms one and two and three and five form approximately 95% of all CREB. So if one of these two combinations increases, the relative contribution of the other isoforms automatically decreases, even if the absolute concentrations do not change that much. Also this effect became visible by calculating the difference in relative expression between experienced and naive females. Isoforms one and two and isoforms three and five seem to be each other's

mirror; isoform one and two are increased in experienced females of the slow learning line, while isoforms three and five are decreased in this group, and isoform one and two are decreased in experienced females of the fast learning line, while isoforms three and five are increased in this group.

When taking these remarks into account and neglecting the fact that none of the observed differences proved to be significant, except the difference between the two lines in isoform nine in generation five, the data suggest a stimulatory role in single trial LTM formation for isoforms three and five. Since these isoforms are increased in experienced females of the fast learning line and decreased in experienced females of the slow learning line. Furthermore, isoforms three and five are decreased in experienced females of the fast learning line, compared to females of the slow learning line in all three generations. This could imply that LTM formation can only take place when the concentration of stimulatory isoforms, such as isoforms three and five, pass a certain threshold. Based on the difference in learning rate between the two lines, it can be assumed, that this threshold is not passed in the slow learning line after a single learning experience, while it is in the fast learning line.
An inhibitory role in LTM formation could be suggested for isoforms one and two, although the results of these isoforms are less consistent. The isoforms are decreased in experienced females of the fast learning line, compared to naïve females. Alternatively, they are increased in experienced females of the slow learning line, compared to naïve females. Furthermore, the isoforms are decreased in experienced females of the slow learning line compared to the fast learning line in all three generations, except for generation five. Presumably, inhibitory isoforms, such as the isoforms one and two, limit the function of stimulatory isoforms, which makes the ratio of isoforms crucial in the decision if LTM is formed. If the isoforms one and two are, indeed, increased in experienced females of the slow learning line, compared to experienced females of the fast learning line, this might explain why memory retention after a single learning experience is decreased in these females.

That no significant correlation was found between CREB expression and memory formation in this project does not mean that there does not exist such a correlation. Changes in CREB expression, caused by a learning experience, might, for example, only occur in specific parts of the brain. This local enhancement in CREB expression might be too small to detect with the used methods. Another possibility is that changes in CREB expression occur only

shortly after the learning experience and have already disappeared twenty-four hours afterwards. A similar model was, in fact, found in a study on memory formation in *Drosophila* by Yin et al. (1995a). In this study both inhibitory and excitatory isoforms of the CREB gene were found that, respectively, block and stimulate LTM formation. It was observed that after an associative learning experience both CREB activator and repressor isoforms are produced. After the training the repressor isoforms degrade faster than the stimulating isoforms, making the formation of memory possible. Actual changes in isoform expression were, therefore, the largest within twenty minutes after the experience. Ongoing research to the expression of CREB isoforms within different time periods after a learning experience in *C. glomerata and C. rubecula* will provide more information on this subject.

4.5 Conclusions

The used procedure for selection on learning ability proved to be an effective way to decrease learning rate after a single learning experience in *C. glomerata*. Apart from the differences found in learning rate, the slow learning line and the fast learning line differed in survival rate and, possibly, in the pattern of memory formation and the time needed for larval development. No clear conclusions could be drawn about the correlation between CREB isoform expression and LTM formation.

It will be interesting to continue the selection experiment to see if the learning rate of the slow learning line can be further decreases, and if the learning rate of the fast learning line can be increased. Secondly, it will be interesting to further investigate the difference in fitness of the two lines and to see if characteristics, such as longevity and larval development, indeed, trade-off with learning ability in this species. Similar experiments can be invented to investigate the effect of an increased learning rate on other characteristics, such as brain size and composition, body size and resistance to parasitoids. Thirdly, a more precise determination of the time that wasps of the slow learning line need to consolidate LTM, can provide more information about both the neurobiological end evolutionary background of LTM formation in *C. glomerata*. Finally, the two line can be used to further investigate the role of CREB isoforms in LTM formation. When the two lines appear to differ significantly in the expression of a specific isoform after learning, most alternative explanations for a difference in expression can be excluded, since both groups are of the same species. Beforehand it will be important to determine at which time point after the experience the difference in expression is maximal because this information can make the comparison between the two lines more specific.

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Appendix

This appendix contains all 'raw' data from the wind tunnel experiments, RNA isolations and RT-qPCR. Moreover, it contains the results of an additional experiment that was performed with 'normal' wasps on the effect of host species on LTM formation. Finally, an overview of all used molecular techniques is given.

1. Results selection experiments

1.1 Results first selection

Tested females emerged from cocoons collected on 1-10 and 7-10 and hatched respectively around 6-10 and 12-10.
In the first week, small plants were used as odor sources (of approximately four to five weeks old). The response of females was very low in this week. Because of the low response levels, it was decided to skip the first test and to start directly with the learning trials
To increase the flying response during the second week, larger plants were used (cabbage of approximately ten weeks old and four Nasturtium plants of four to five weeks old). Plants were also mechanically damaged 24h before the first test and each wasp was offered a bit of honey before releasing it in the wind tunnel. This resulted in remarkably higher flying responses during the second week. In the second week the first test was thus included again in the selection.

Wind tunnel results

Naïve females

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
9-okt	-	11,1	-	27
10-okt	-	0	-	10
15-okt	-	26,1	-	23
16-okt	-	9,1	-	11
17-okt	100	36,6	191	41
	Average: 100	**Average: 16,6**	**Average: 191**	**Total: 112**

After one experience

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
11-okt	-	37,5	-	16
12-okt	45	40	193	50
16-okt	-	-	-	4
18-okt	15,4	76,5	209	17
19-okt	12,5	88,9	183	9
	Average: 24,3	**Average: 60,7**	**Average: 195**	**Total: 92**

After three experiences

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
16-okt	81,8	100	157	11

17-okt	-	-	-	3
19-okt	55,6	90	154	10
	Average: 68,7	**Average: 95**	**Average: 156**	**Total: 21**

After all selection steps, fourteen wasps remained for the slow learning line and another fourteen wasps could be used as a fast learning line.
All females of the fast learning line parasitized two caterpillars, this was spread over three days (17-,18 and 19-10). The parasitization by the Slow learning line took place on 18 and 19-10.

1.2 Results second selection

Wasps of the second generation developed in caterpillars parasitized between 17-10 and 19-10. 28. The first larvae (of the fast learning line) emerged around 2-11 and three days later the first larvae of the Slow learning line emerged. On 20-10 twelve wasps of the slow learning line parasitized again 2 caterpillars each to ensure that enough female wasps will be available for the second generation. The larvae of this group emerged around 6-11. After the larvae had spun their cocoons, all cocoons were collected and placed in a new cage. For the fast learning line cocoons of 19 different caterpillars could be collected. For the Slow learning line the cocoons of 26 caterpillars were available and from 24 caterpillars from the second group of the Slow learning line.
The first wasps of the fast learning line emerged on 9-11. On 11-11 forty wasps were taken from the cage at random and the sex ratio was determined. 3 X 15 females were used for RNA isolation on the same day. The remaining wasps stayed in the cage but the majority of males was removed to ensure the fitness of the females.
On 10-11 the first wasps of the slow learning line emerged. On 12-11 forty wasps were taken from the cage at random and the sex ratio was determined. Surprisingly the sex ratio of the slow learning line was exactly the same as the sex ratio of the fast learning line (♀:♂= 0,325:0,675). 3 X 15 females were used for RNA isolation the same day. The remaining wasps stayed in the cage and the majority of males was removed again. The wasps of the second group of the slow learning line emerged on 14-11. Also in this group the majority of males was removed after two days.

Wind tunnel results:

Naive females

Slow learning line

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
13-nov	71,4	56	171	25
14-nov	87,5	42,1	152	38
15-nov	81,8	27,5	171	40
16-nov	-	20	-	20
18-nov	-	37,5	-	8
19-nov	26,7	53,8	179	26
20-nov	84,6	43,3	172	30
21-nov	-	45	-	20
22-nov	84,6	65	-	20
	Average: 72,8	**Average: 43,4**	**Average: 179**	**Total: 227**

Fast learning line

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
22-nov	100	68,2	219	22
23-nov	83,3	72	209	25
	Average: 91,65	**Average: 70,1**	**Average: 214**	**Total: 47**

After one experience

Slow learning line

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
15-nov	26,7	78,9	116	19
19-nov	60	90,9	185	11
20-nov	41,7	100	133	12
21-nov	60	100	130	10
23-nov	16,7	100	88	12
	Average: 41,0	**Average: 94,4**	**Average: 130**	**Total: 64**

Fast learning line

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
23-nov	60	100	88	15
24-nov	50	100	103	14
	Average: 55	**Average: 100**	**Average: 96**	**Total: 29**

After three experiences

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
16-nov	55,6	81,8	129	11
21-nov	90	100	141	10
26-nov	33,3	100	100	9
	Average: 59,6	**Average: 93,9**	**Average: 123**	**Total: 30**

After all selection steps, fifteen wasps remained for the slow learning line (the wasps of the slow learning line with preference for Nasturtium after three experiences) and another fifteen wasps could be used as a fast learning line (the females of the fast learning line with preference for Nasturtium after one experience). All females parasitized four caterpillars. The parasitization by the slow learning line took place on 21–11 and was repeated on 27–11. The parasitization by the fast learning line took place on 24–11 and for the second time on 27–11.

1.3 Results third selection

Wasps of the third generation developed in caterpillars parasitized between 21-11 and 24-11. 15 wasps of both the slow learning and fast learning line parasitized four caterpillars each. The first larvae (of the slow learning line) emerged around 06-12 and four days later the first larvae of the fast learning line emerged. On 27-11 eleven wasps of the slow learning line and the fast learning line again parasitized 4 caterpillars to create two back-up groups. The larvae of this group emerged around 12-12. After the larvae had spun their cocoons, all cocoons were collected and placed in a new cage. For the fast learning line cocoons of 50 different caterpillars could be collected. For the slow learning line the cocoons of 50 caterpillars were available. Both back-up groups consisted of wasps from 30 different caterpillars.

The first wasps of the slow learning line emerged on 13-12. 2 X 15 females were used for RNA isolation on 14-12 (samples S2N I and II). The remaining wasps stayed in the cage but the majority of males was removed to ensure the fitness of the females.
On 17-12 the first wasps of the fast learning line emerged. 2 X 15 females were used for RNA isolation on 18-12 (samples C2N I and II). The remaining wasps stayed in the cage and the majority of males was removed again. The wasps of the back-up groups emerged around 19-12. Also in these groups the majority of males was removed after two days.

Wind tunnel results

Naïve females

Slow learning line:

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
15-12	-	15	-	20
16-12	69.2	65	159	20
17-12	76.5	63	232	27
18-12	80	48.4	207	31
19-12	-	6.7	-	15
20-12	-	20	-	20
21-12	-	10	-	10
23-12	100	55	172	20
24-12	-	10	-	10
28-12	88.9	40.9	221	22
	Average: 82.9	**Average: 32.1**	**Average: 198**	**Total: 195**

Fast learning line:

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
19-12	-	20	-	10
20-12	80	40	245	25
21-12	90.9	48	242	25
23-12	-	-	-	5
24-12	90.9	43.3	200	30
28-12	80	50	241	22
	Average: 85.5	**Average: 40.3**	**Average: 232**	**Total: 117**

Preference after 1 learning experience

Slow learning line:

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
17-12	63.6	84.6	97	13
18-12	44.4	100	86	9
19-12	36.4	100	116	11
21-12	10	90.9	115	11
24-12	54.5	100	122	9
28-12	54.5	100	126	11
30-12	44.4	90	103	10

	Average: 44,0	**Average: 95.1**	**Average: 109**	**Total: 74**

Fast learning line:

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
21-12	18.2	78.6%	138	14
24-12	50	85.7%	147	14
28-12	54.5	84.6%	130	13
30-12	12.5	80%	141	10
	Average: 33.8	**Average: 82.2**	**Average: 139**	**Total: 51**

Preference after 3 spaced learning experiences

Date	preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
20-12	66.7	100	107	15
30-12	60	100	173	10
	Average: 63.3	**Average: 100**	**Average: 140**	**Total: 25**

1.4 Results fourth selection

After the third selection, 15 females remained to create a new generation for both groups (slow learning line and fast learning line). Each female parasitized three caterpillars, this took place between 22-12 and 30-12.
The larvae of the fast learning line emerged between 05-01 and 11-01 and the larvae of the slow learning line between 07-01 and 10-01. For the fast learning line, cocoons of 38 different caterpillars could be collected. For the slow learning line the cocoons of 19 caterpillars were available.
To create the backup group, females of the third selection parasitized again three caterpillars each, on 30 and 31-12. The larvae of these groups emerged from 14-01 until 17-01. For the fast learning line, cocoons of 31 different caterpillars could be collected. For the slow learning line the cocoons of 37 caterpillars were available.
The first wasps of the fast learning line emerged on 14-01. On 16-01 2 X 15 females were used for RNA isolation (samples C3N I and II). The first wasps of the slow learning line emerged on 16-01. On 18-01 2 X 15 females were used for RNA isolation (samples S3N I and II). The remaining wasps stayed in the cage and the majority of males was removed to ensure the fitness of the females.
The first wasps of the backup groups emerged on 21-01 (slow learning line) and 22-01 (fast learning line). Also in this group the majority of males was removed after two days.
On 30-01 2X15 wasps were used for RNA isolation, 24h after an oviposition experience (samples S3e I and II and C3e I and II).

Wind tunnel results

Naïve females

Slow learning line:

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
18-Jan	81.3	79.2	208	24
21-Jan	100.0	64.3	157	14

22-Jan	68.8	80	164	20
23-Jan	66.7	78.3	234	23
25-Jan	-	46	-	13
28-Jan	-	41.2	-	17
	Average: 79.2	**Average: 64.8**	**Average: 191**	**Total: 111**

Fast learning line:

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
17-Jan	70.0	66.7	239	30
18-Jan	66.7	56.3	192	16
21-Jan	90.9	91.7	179	12
23-Jan	66.7	52.9	190	17
24-Jan	100.0	76.9	169	13
25-Jan	66.7	60	190	15
28-jan	75	80	188	10
	Average: 76.6	**Average: 69.2**	**Average: 192.3**	**Total: 113**

24h after one experience

Slow learning line:

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
22-Jan	33.3	100	114	12
23-Jan	60.0	90.9	71	11
25-Jan	44.4	100	121	9
28-Jan	66.7	90	92	10
29-Jan	63.6	91.7	154	12
30-Jan	55.6	90	112	10
	Average: 53.9	**Average: 93.8**	**Average: 111**	**Total: 64**

Fast learning line:

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
22-Jan	46.2	100	188	13
23-Jan	50.0	80	176	15
25-Jan	71.4	93	120	14
28-jan	57.1	100	110	14
29-jan	83.3	100	97	12
	Average: 61.6	**Average: 94.6**	**Average: 138**	**Total: 68**

24h after 3 learning experiences

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
25-jan	56.7	100	37	9
30-jan	78.3	91.7	107	12
	Average: 67.5	**Average: 95.9**	**Average: 72**	**Total: 21**

From the slow learning line, 17 females remained to create the next generation. For the fast learning line 31 wasps remained.

1.5 Results fifth selection

Parasitization and emergence from caterpillars

1st group:

Parasitized	25-Jan	29-Jan	30-Jan	31-jan
Slow learning line	5*3	3*3	9*3	3*3
Fast learning line	6*3	6*3	6*3	

Date	Emergence from caterpillar		Emergence of first wasps	
	Slow learning line	Fast learning line	Slow learning line	Fast learning Line
08-02	1	0	15-02	16-02
11-02	12	13		
13-02	7	6		
14-02	9	9		
15-02	6	5		
17-02	1	5		

2nd group:

Parasitized	1-feb	4-feb
Slow learning line	6*3	4*3
Fast learning line	6*3	7*3

Date	Emergence from caterpillar		Emergence of first wasps	
	Slow learning line	Fast learning line	Slow learning line	Fast learning line
15-02	36,4% (8)	0	22-02	23-02
16-02	27,3% (6)	46,7% (14)		
17-02	27,3% (6)	26,7% (8)		
18-02	4,6% (1)	20% (6)		
19-02	4,6% (1)	6,7% (2)		
15-02	36,4% (8)	0		

On 18-02 2 X 15 females of each group were used for RNA isolation (samples C4N I and II and S4N I and II). The remaining wasps stayed in the cage and the majority of males was removed to ensure the fitness of the females.

Wind tunnel results

Naïve females

Slow learning line:

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
19-feb	66.7	42.9	235	21
20-feb	100.0	47.6	188	21
25-feb	66.7	63.2	203	19
26-feb	100.0	57.1	263	21
27-feb	100.0	68.4	175	19
	Average: 86.7	**Average: 55.8**	**Average: 212.7**	**Total: 101**

Fast learning line:

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
19-feb	81.8	47.6	217	21
20-feb	91.7	39.3	243	28
25-feb	93.8	80	219	20
26-feb	100.0	63.2	230	19
27-feb	83.3	66.7	275	18
	Average: 90.1	**Average: 59.4**	**Average: 237.0**	**Total: 106**

24h after one experience

Slow learning line:

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
21-feb	71.4	100	136	14
22-feb	36.4	100	111	11
26-feb	54.5	100	65	11
27-feb	20.0	100	115	10
1-mrt	41.7	92.3	118	13
	Average: 44.8	**Average: 98.5**	**Average: 108.9**	**Total: 59**

Fast learning line:

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
21-feb	70.0	76.9	237	13
22-feb	66.7	100	153	12
26-feb	71.4	100	132	14
27-feb	45.5	100	177	12
1-mrt	50.0	92.3	152	13
	Average: 60.7	**Average: 93.8**	**Average: 170.3**	**Total: 64**

24h after three experiences

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
24-feb	77.8	81.8	137	11
1-mrt	77.8	81.8	62	11
	Average: 77.8	**Average: 81.8**	**Average: 99.4**	**Total: 22**

1.6 Results sixth selection

Parasitization and emergence from caterpillars

1st group:

Parasitized	23-feb	24-feb
Slow learning line	6*3	3*3
Fast learning line	5*3	3*3

Emergence	Slow learning line	Fast learning line
7-mrt	1	1
8-mrt	3	3
9-mrt	12	9
10-mrt	2	1

2nd group:

Parasitized	5-mrt	6-mrt	7-mrt
Slow learning line	6*3	6*3	6*3
Fast learning line	6*3	6*3	6*3

Emergence	Slow learning line	Fast learning line
17-mrt	12	8
18-mrt	5	13
19-mrt	3	4
20-mrt	5	6
21-mrt	3	1
22-mrt	5	1
25-mrt	4	8

3rd group:

Parasitized	4-mrt	5-mrt
Slow learning line	6*3	6*3
Fast learning line	6*3	6*3

Emergence	Slow learning line	Fast learning line
18-mrt	10	1
19-mrt	5	3
20-mrt	9	7
21-mrt	1	0
22-mrt	0	1

Wind tunnel results

Naïve females

Slow learning line:

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
18-mrt	100.0	40	158	25
19-mrt	100.0	40	160	25
20-mrt	90.9	50	142	22
21-mrt	92.3	46.4	156	28
	Average: 95.8	**Average: 44.1**	**Average: 154**	**Total: 100**

Fast learning line:

Date	Preference for cabbage (%)	Response rate (%)	Time for response (s)	Total amount tested
15-mrt	100.0	50	109	30
17-mrt	92.9	66.7	186	15
18-mrt	90.0	52.6	113	19
19-mrt	87.5	71.4	146	14
	Average: 92.6	**Average: 60.1**	**Average: 139**	**Total: 78**

24h after one experience

Slow learning line:

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
20-mrt	46.2	86.7	96	15
21-mrt	54.5	91.7	66	12
22-mrt	35.7	93.3	41	15
27-mrt	22.2	90	117	20
	Average: 39.7	**Average: 90.4**	**Average: 80**	**Total: 62**

Fast learning line:

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
20-mrt	60.0	100	91	15
21-mrt	75.0	100	84	12
22-mrt	53.3	94.4	50	18
27-mrt	52.6	95	88	20
	Average: 60.2	**Average: 97.4**	**Average: 79**	**Total: 65**

24h after three experiences

Date	Preference for nasturtium (%)	Response rate (%)	Time for response (s)	Total amount tested
26-mrt	84.6	81.3	113	16

2. Results RNA isolations

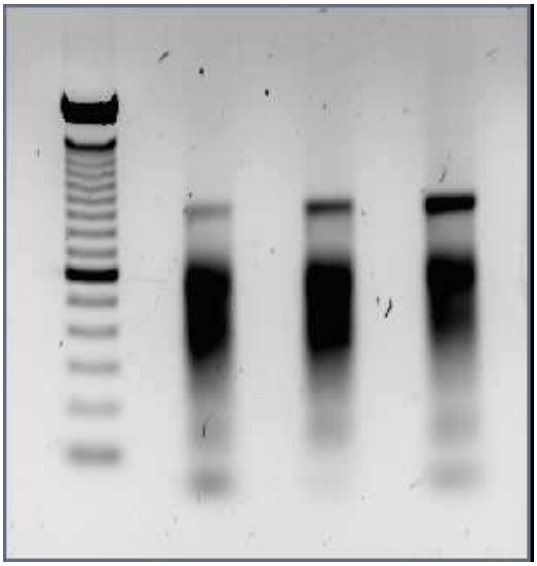
2.1 First generation

RNA isolations were performed after two different treatments. The first with naïve females of 1 day old and the second with females 24h after one learning experience. The females of the second treatment were approximately two weeks old. Dissected heads were kept overnight in RNAwiz before continuing with the following steps.
For the isolation with naïve females 15 wasps were used per sample and of each group (slow and Fast learning line) 3 samples were made. The isolation of the second group did not proceed as planned. Of the fast learning line not enough wasps were available to make 3 samples of 15 wasps so 3 samples of 11 heads were made instead. The three isolations of the slow learning line were made without Glycoblue.

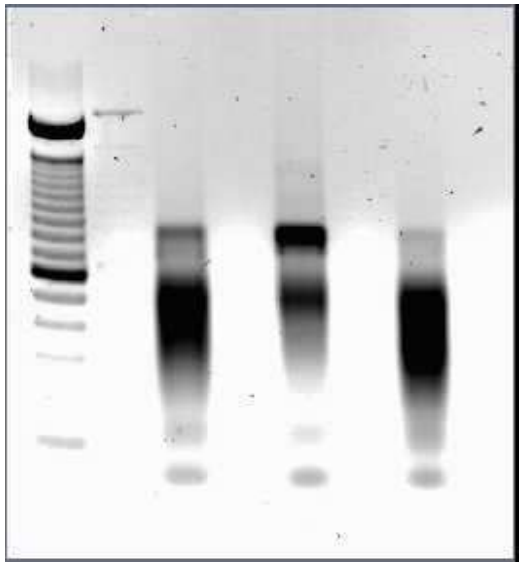
RNA concentrations of naïve females (S1nI, S1nII, S1nIII = slow learning line, C1nI, C1nII, C1nIII = fast learning line): (concentrations in 25 µL)

	RNA concentration (ηg/µL)	260/280
S1nI	277,9	1,89
S1nII	221,5	1,88
S1nIII	294,6	1,90
C1nI	255,0	1,90
C1nII	216,9	1,89
C1nIII	261,1	1,90

Gel electrophoresis:



RNA gel 1:100 bp ladder, C1nI, C1nII and C1nIII

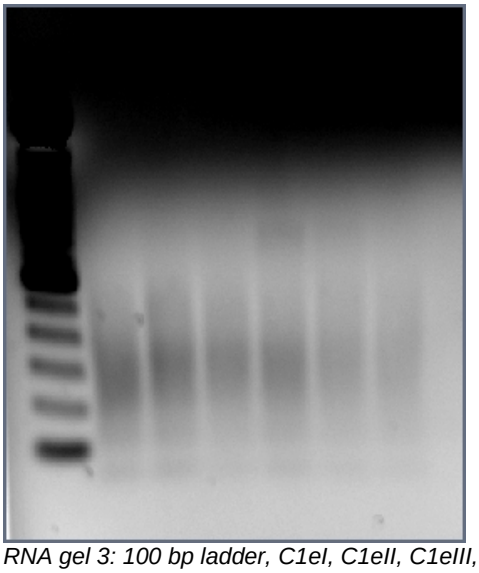


RNA gel 2: 100 bp ladder, S1nI, S1nII and S1nIII

RNA concentrations of females 24h after 1 experience (S1eI, S1eII, S1eIII = slow learning line, C1eI, C1eII, C1eIII = fast learning line): (concentrations in 25 µL)

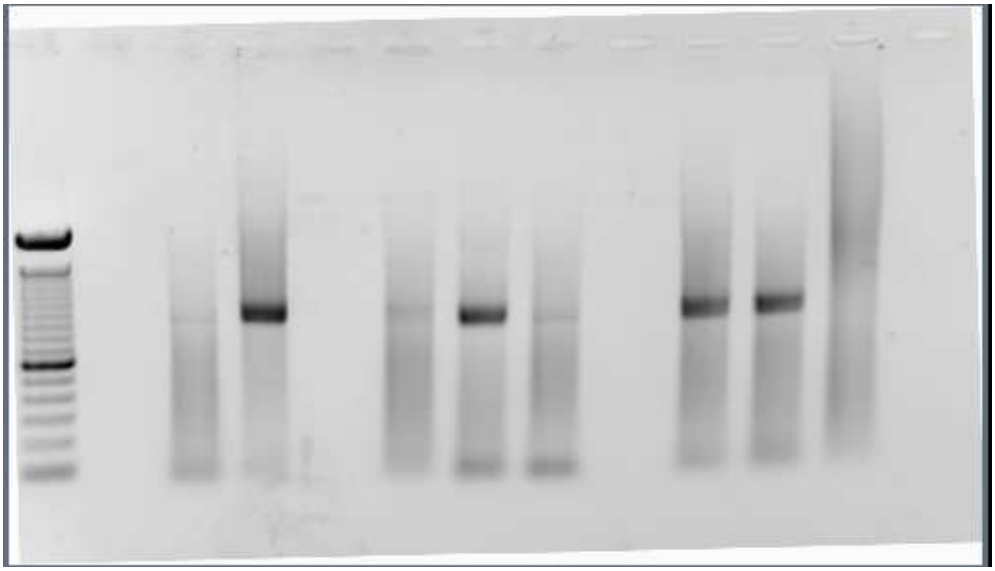
	RNA concentration (ng/µL)	260/280
S1eI	73,2	1,68
S1eII	43,5	1,69
S1eIII	32,8	1,64
C1eI	133,5	1,74
C1eII	102,0	1,74
C1eIII	77,3	1,72

Gel electrophoresis:



RNA gel 3: 100 bp ladder, C1eI, C1eII, C1eIII, S1eI, S1eII and S1eIII

The concentration of RNA in the last six samples is very low. This is probably the reason that the RNA gel does not show clear bands. To be sure about the quality of the RNA, cDNA was produced and a second PCR reaction was performed, using the CREB ForA and RevC primers. The PCR products were tested on gel:



DNA gel 1: 100 bp ladder, C1nI, S1nI, C1eI, C1eII, C1eIII, S1eI, S1eII and S1eIII

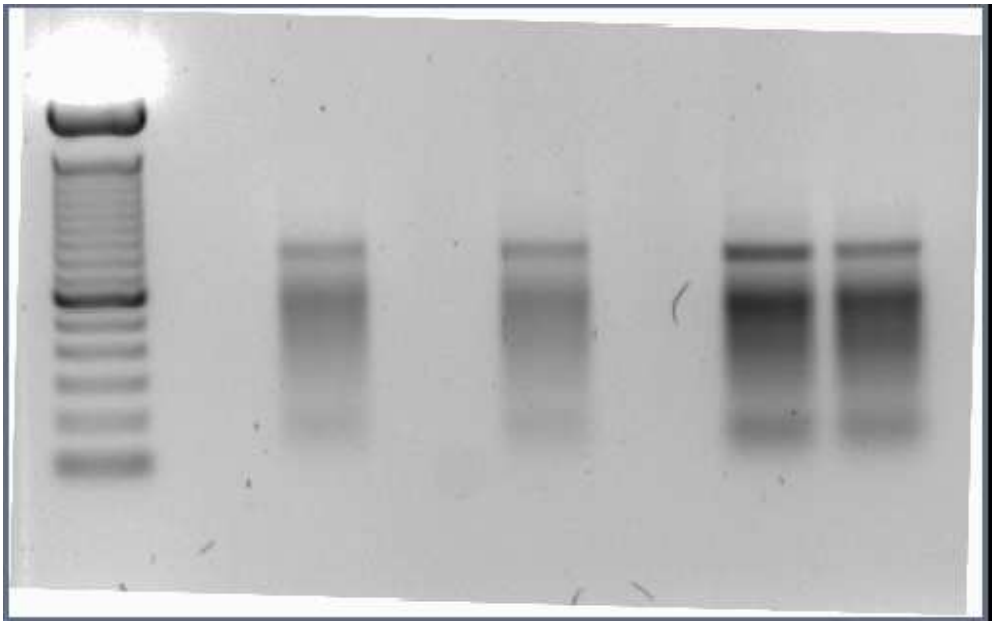
The primers bind to the beginning 5' and 3' ends of the CREB RNA so the whole sequence of the isoforms will be amplified, resulting in strands of DNA from approximately 900 bp. The picture of the DNA gel shows that CREB RNA was present in all samples, except from sample S3e. The bands of sample C1e and C3e are not very clear but seem to be on the right position. The other samples of the naïve females will also be tested on quality. For the q-PCR of each sample only the two samples with the best quality from the three repetitions will be used, resulting in 8 different samples.

2.2 Second generation

RNA concentrations:

Sample	RNA concentration (ng/µL)	260/280
C2n I	172.7	1.81
C2n II	157.6	1.76
S2n I	218.6	1.88
S2n II	237.2	1.85

Gel electrophoresis



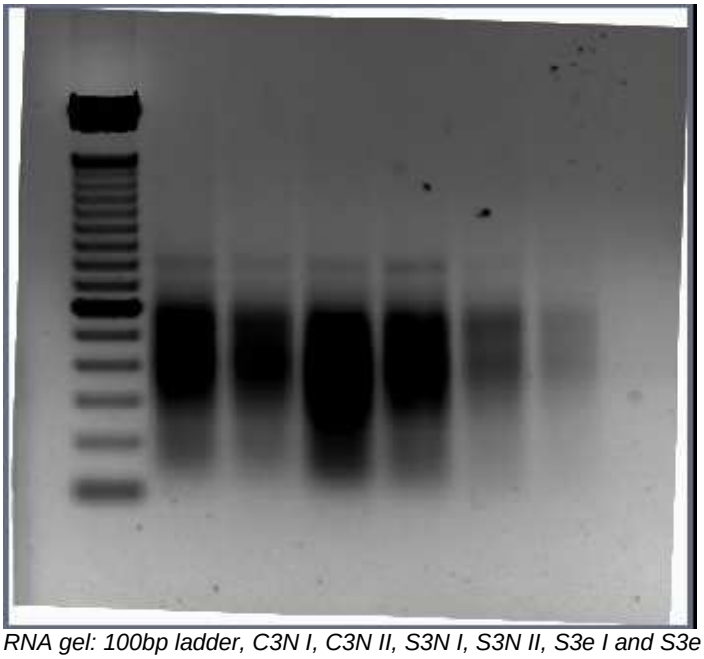
RNA gel: 100bp ladder, C2N I, C2N II, S2N I and S2N II

2.3 Third generation

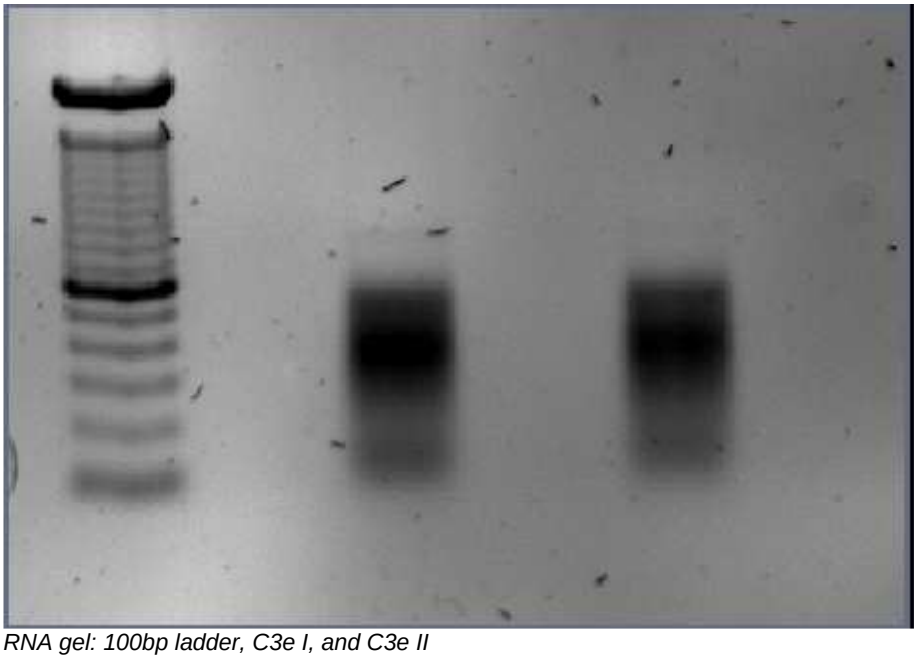
RNA concentrations:

Sample	RNA concentration (ηg/μL)	260/280
C3N I	182.7	1.82
C3N II	127.6	1.77
S3N I	318.6	1.88
S3N II	197.2	1.87
C3e I	106.3	1.63
C3e II	105.8	1.63
S3e I	107.4	1.74
S3e II	68.8	1.73

Gel electrophoresis



RNA gel: 100bp ladder, C3N I, C3N II, S3N I, S3N II, S3e I and S3e II



RNA gel: 100bp ladder, C3e I, and C3e II

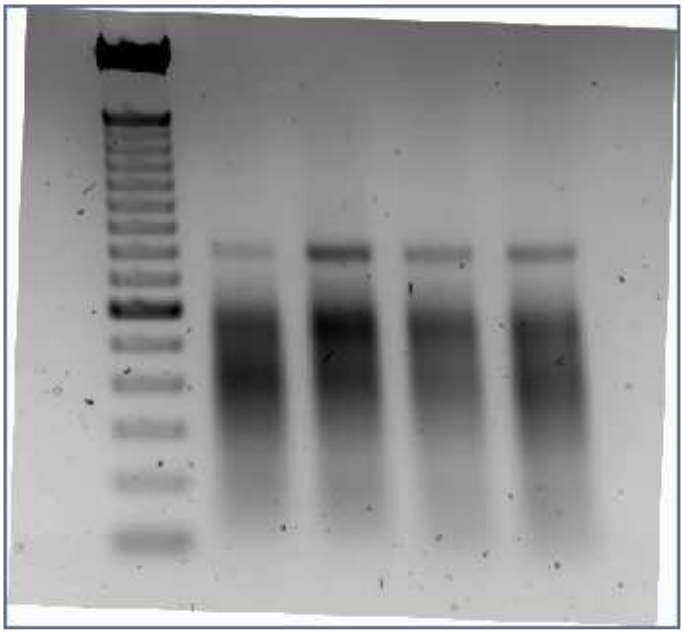
2.4 Fourth generation

RNA concentrations

Sample	RNA concentration (ŋg/µL)	260/280
C4N I	153,7	1,77
C4N II	185,7	1,73
S4N I	134,3	1,78

S4N II	152,0	1,75

Gel electrophoresis



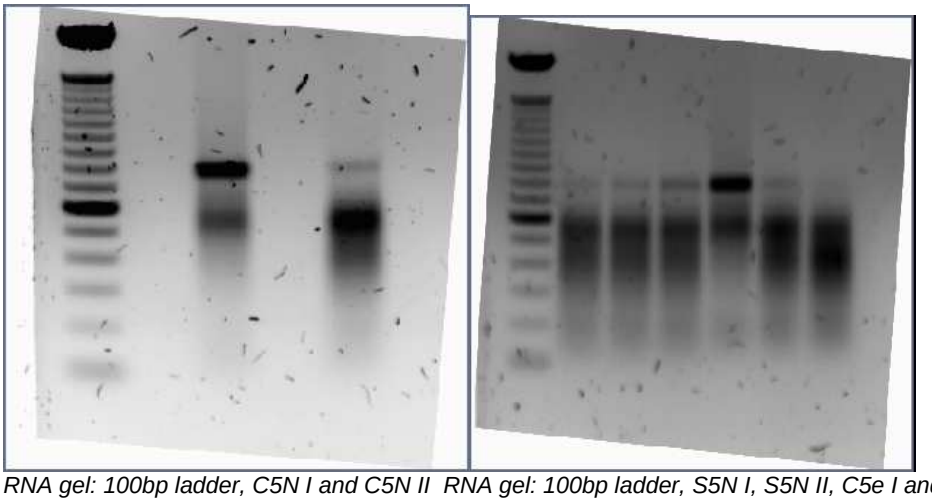
RNA gel: 100bp ladder, C4N I, C4N II, S4N I and S4N II

2.5 Fifth generation

RNA concentrations

Sample	RNA concentration (ηg/µL)	260/280
C5N I	203,6	1,83
C5N II	201.4	1,81
S5N I	192,7	1,84
S5N II	194,6	1,79
C5e I	202,6	1,82
C5e II	204	1,80
S5e I	206,5	1,81
S5e II	213,7	1,84

Gel electrophoresis



RNA gel: 100bp ladder, C5N I and C5N II RNA gel: 100bp ladder, S5N I, S5N II, C5e I and C5e II, S5 I and S5e II

3. RT-qPCR results

Isoforms are expressed as the percentage of one isoform of the total amount of isoforms in one sample

3.1 Naïve expression

First generation

Isoform	**Slow learning line**		**Fast learning line**	
	S1nI	S1nII	C1nI	C1nII
1+2	1.41E+01	*8.05E+01*	2.08E+01	*5.54E+01*
3+5	8.23E+01	*1.36E+01*	7.57E+01	*3.81E+01*
4	1.30E+00	*3.34E+00*	1.54E+00	*2.90E+00*
6	5.06E-02	*1.08E-01*	2.12E-01	*1.39E-01*
7	4.01E-02	*5.34E-02*	7.40E-02	*2.16E-01*
8	2.14E+00	*2.38E+00*	1.67E+00	*3.25E+00*
9	1.21E-02	*5.39E-02*	1.19E-02	*3.74E-02*

Second generation

Isoform	**Slow learning line**		**Fast learning line**	
	S2nI	S2nII	C2nI	C2nII
1+2	*6.24E+01*	*6.27E+01*	*1.63E-02*	*8.55E-02*
3+5	*3.55E+01*	*3.45E+01*	*1.10E-04*	*1.50E+01*
4	*1.28E+00*	*2.09E+00*	*2.33E+01*	*5.06E+00*
6	*2.75E-01*	*2.38E-01*	*8.63E-01*	*6.80E-01*
7	*4.95E-01*	*4.07E-01*	*4.03E+00*	*0.00E+00*
8	*0.00E+00*	*0.00E+00*	*0.00E+00*	*0.00E+00*
9	*0.00E+00*	*4.26E-02*	*2.45E-01*	*1.09E-01*

Third generation

Isoform	**Slow learning line**		**Fast learning line**	
	S3nI	S3nII	C3nI	C3nII
1+2	1.08E+01	1.23E+01	1.31E+01	7.66E+00
3+5	8.17E+01	8.16E+01	8.05E+01	8.81E+01

4	1.23E-01	1.38E-01	1.81E-01	9.11E-02
6	9.84E-01	5.60E-01	8.55E-01	4.52E-01
7	5.87E-01	2.47E-01	3.12E-01	3.23E-01
8	5.80E+00	5.13E+00	5.04E+00	3.31E+00
9	5.63E-02	2.36E-02	2.25E-02	2.16E-02

Fourth generation

Isoform	**Slow learning line**		**Fast learning line**	
	S4nI	S4nII	C4nI	C4nII
1+2	1.86E+01	1.77E+01	1.42E+01	1.78E+01
3+5	7.56E+01	7.41E+01	7.80E+01	7.63E+01
4	1.53E-01	1.67E-01	2.00E-01	2.41E-01
6	1.41E+00	1.09E+00	1.02E+00	8.96E-01
7	4.06E-01	8.44E-01	4.88E-01	2.58E-01
8	3.89E+00	6.15E+00	6.08E+00	4.51E+00
9	1.36E-02	1.98E-02	2.96E-02	2.35E-02

Fifth generation

Isoform	**Slow learning line**		**Fast learning line**	
	S5nI	S5nII	C5nI	C5nII
1+2	6,23E+00	4,76E+00	9,45E+00	6,60E+00
3+5	7,00E+01	7,04E+01	6,74E+01	7,42E+01
4	4,02E-01	8,19E-01	7,06E-01	4,86E-01
6	5,50E+00	5,86E+00	7,45E+00	5,80E+00
7	7,29E-01	7,88E-01	4,01E-01	5,64E-01
8	1,68E+01	1,67E+01	1,45E+01	1,23E+01
9	3,07E-01	5,85E-01	7,73E-02	7,98E-02

3.2 Expression after one experience

First generation

Isoform	**Slow learning line**		**Fast learning line**	
	S1eI	S1eII	C1eI	C1eII
1+2	3.34E+01	*5.02E+01*	1.69E+01	2.08E+01
3+5	6.29E+01	*4.30E+01*	7.77E+01	7.27E+01
4	4.89E-01	*1.17E+00*	5.30E-01	5.07E-01
6	1.98E-02	*8.17E-02*	2.10E-02	2.85E-02
7	2.06E+00	*2.48E+00*	2.90E+00	3.76E+00
8	1.16E+00	*3.12E+00*	1.75E+00	2.12E+00
9	3.06E-02	*7.13E-03*	1.11E-01	5.85E-02

Third generation

Isoform	**Slow learning line**		**Fast learning line**	
	S3eI	S3eII	C3eI	C3eII
1+2	1.18E+01	1.23E+01	1.14E+01	9.79E+00
3+5	8.00E+01	8.10E+01	8.12E+01	8.27E+01
4	1.46E-01	1.71E-01	1.68E-01	1.47E-01
6	7.42E-01	8.37E-01	9.62E-01	8.51E-01
7	5.21E-01	6.06E-01	2.93E-01	3.02E-01
8	6.74E+00	5.07E+00	5.99E+00	6.23E+00
9	5.23E-03	4.62E-02	3.59E-02	1.49E-02

Fifth generation

Isoform	**Slow learning line**		**Fast learning line**	
	S5eI	S5eII	C5eI	C5eII
1+2	7,61E+00	2,27E+00	7,39E+00	8,85E+00
3+5	6,49E+01	5,26E+01	7,06E+01	7,27E+01
4	6,51E-01	4,48E-01	4,59E-01	4,15E-01
6	4,60E+00	2,63E+00	3,94E+00	5,69E+00
7	4,88E-01	6,10E-01	4,41E-01	5,09E-01
8	2,11E+01	4,09E+01	1,70E+01	1,17E+01
9	6,49E-01	4,81E-01	1,43E-01	1,33E-01

4. The effect of host species on memory formation

4.1 Introduction

Cotesia glomerata is a generalist with a preference for *Pieris brassicae* caterpillars. However, P*ieris rapae* can also be used as host. *P. brassicae* females lay several eggs on one plant and spread their eggs on clusters of host plants. *P. rapae* females lay only one egg per plant and spread their eggs in an unpredictable way through the field.
When a female of *C. glomerata* finds a *P. brassicae* larvea , more hosts on the same plant species are likely to be within reach. The predictive value of finding a suitable host on the same plant-host combination is therefore large. Learning after one oviposition experience in *P. brassicae* will turn out in advantage of the female, while learning after one experience in *P. rapae* may not.

Aim of the experiment was to determine if there is a difference in reward between an oviposition experience in the solitary *P. rapae* caterpillar and an oviposition experience in the gregarious *P. brassicae* caterpillar.
It was examined if the type of host species offered as reward in an oviposition experience can affect the subsequent plant odor preference behaviour of the parasitoid.
This was done by comparing two groups of *C. glomerata* wasps that underwent different treatments. The first group received a single learning experience on a 0-1 day old *P. rapae* caterpillar, while the second group received a single learning experience in a 0-1 day old *P. brassicae* caterpillar. All oviposition experiences took place on a nasturtium leaf, damaged by caterpillars. It is assumed that the majority of *C. glomerata* females has a naïve preference for Brussels sprouts plants over nasturtium plants. 24h after the oviposition experience, the plant odor preference of the wasps will be tested in the wind tunnel.

4.2 Experimental design

Treatment of the parasitoids
All wasps used for this experiment were between 1 and 5 days old. On one day three different groups of wasps were tested; naïve wasps tested on plants infested with *P. rapae*, naïve wasps tested on plants infested with *P. brassicae,* wasps with a learning experience on *P. brassicae* tested on plants infested with *P. brassicae,* and wasps with a learning experience on *P. rapae* tested on plants infested with *P. rapae*.

Learning experiences

One day before testing, oviposition experiences were offered on nasturtium leaves, damaged by caterpillars. Before each experience a new 0-1 day old caterpillar was placed on the leaf. Each female was placed on the leaf individually and was directly confronted with the caterpillar. One group received an oviposition experience on *P. brassicae,* while another group received an experience on *P. rapae.*

Odor sources

For the wind tunnel tests two different odor sources were used; Brussels sprouts plants and nasturtium plants. Two sets of plants were infested with 40 0-1 day old *P. brassicae* larvae or 40 0-1 day old *P. rapae* larvae, 24h before testing and distributed over two different leafs.

Wind tunnel tests

The parasitoids were be tested on two different odor sources of approximately the same size. Temperature, humidity and wind speed in the wind tunnel were regulated at respectively 21-24˚C, 40-60% and 17 cm/s.

Each wasp was collected from the cage with a glass vial and released in the wind tunnel in the middle of a release cylinder at approximately 30 cm from the odor sources, where it received 5 minutes to land on one of the odor sources. If a wasp did not land on a plant within this time frame, or did not initiate flight, this was considered as a no-response.

4.3 Results

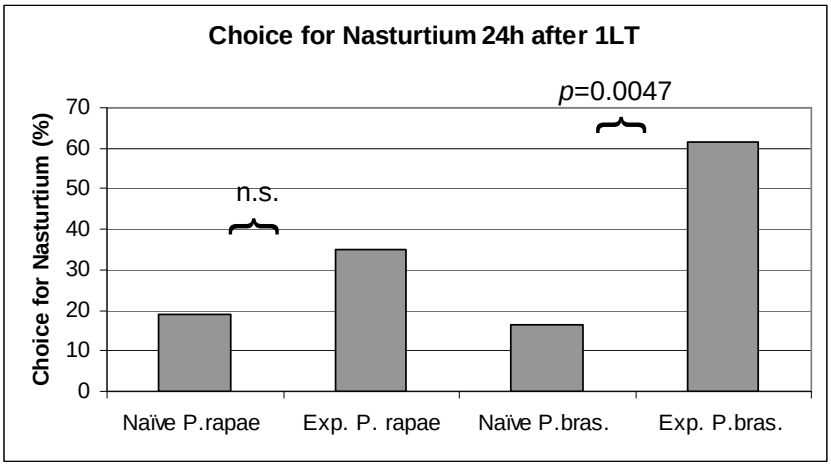


Fig 1. Choice for nasturtium 24h after an oviposition experience on either P. brassicae *or* P. rapae, *compared to the choice for nasturtium of naïve females. There was no significant difference between naïve females, tested on plants infested with* P. rapae*, and females with an experience on* P. rapae *that were tested on the same plants. There was a significant difference between naïve females, tested on plants infested with* P. brassicae*, and females with an experience on* P. brassicae *that were tested on the same plants. Wasps were tested on three different days. A total number of 43, 48, 40 and 40 wasps were used for each group, respectively.*

4.4 Discussion and conclusion

The results show that a single learning experience on a *P. rapae* larva on nasturtium does not lead to an increased preference for this odor source, twenty-four hours afterwards. A single learning experience on a *P. brassicae* larva on nasturtium, on the other hand, does lead to an increased preference for this odor source, after the same time period. These results imply that *C.*

glomerata does not form LTM after a single oviposition experience on *P. rapae* and further support the idea that this type of memory is formed after a single oviposition experience on *P. brassicae.*
These results support the hypothesis that the predictive value of an oviposition experience on *P. rapae* is lower than an oviposition experience on *P. brassicae.*

5. Molecular protocols

5.1 Dissection of the insect brain

1. Place cage with the backside to a light source (e.g. window) to ensure that the wasps will not fly away when the cage is opened, or cover the backside and allow the wasps to walk on the top glass plate. This makes it easier to differentiate between the sexes, and to catch the wasps.
2. Put the wasps in glass tubes, closed with cotton wool.
3. Anesthetize the wasps by putting the glass tubes in ice. Not too long, the wasps only need to be immobilized, not killed.
4. Dissect with two pairs of forceps, which need to be sharpened (stone/water) *intensively* after every insect.
5. Decapitate immobilized wasps with forceps. Remove antennae and mouth parts with forceps under cold Ringer solution.

5.2 RNA isolation

For *C. glomerata*, use 15 heads.

- Use 2.0 mL cups. Never pipette with the tip in the solution!
- Dissect the brains in cold Ringer's and transfer to 1000 µL RNAwiz/Trizol in an Eppendorf cup. Dissect thoracic tissue as a control for the dissection step.
- Homogenize with sonicator for approximately 10 seconds at level 4. Turn the sonicator on when its tip is inserted halfway in the solution. Clean the sonicator with alcohol before and after use.
- Incubate the homogenate for at least 10 minutes at RT to allow dissociation of nucleoproteins from nucleic acids.
- Add 0.2 x starting volume of chloroform to the homogenate. Shake vigorously.
- Incubate at RT for at least 10 minutes.
- Centrifuge at 14000 rpm at 4℃ for 15 minutes. The mixture will separate in 2 or 3 phases: the upper aqueous phase containing RNA, a semi-solid phase containing DNA, and a layer of organic 'debris'.
- Transfer the aqueous phase (150-200 µL) into an RNase-free tube.
- Add 0.5 x starting volume of RNase-free H_2O. Shake for 20 seconds.
- Add 5 µL of glycogen for co-precipitation.
- Add 1.0 x starting volume of isopropanol (2-propanol). Shake for 20 seconds.
- Vortex at medium speed for 10-15 seconds.
- Incubate at RT for 10 minutes. Shake gently every now and then.
- Centrifuge at 14000 rpm at 4℃ for 15 minutes.
- Remove the supernatant with an RNase-free tipped pipet.
- Wash the pellet by shaking gently with at least 1.0 x starting volume of cold 70-75% alcohol. This can be stored at -80℃.

- Centrifuge at 14000 rpm at 4℃ for 5 minutes. The supernatant can be discarded again. Remove all remaining drops from the tube! The pellet must be 'dry'; otherwise, the RNA will dissolve in the remaining ethanol. Cool on ice.
- Air-dry upside down for at least 5 minutes. Any residual ethanol must be gone; otherwise the enzymes in subsequent PCR reactions will not work. Cool on ice.
- Resuspend the RNA in 25 µL (brains) or 100 µL (thoraxes) of RNase-free H_2O. Repeatedly pipet to aid resuspensation.
- Store at -80℃.

5.3 cDNA synthesis - Verso™ kit

4.0 µL 5x cDNA synthesis buffer
2.0 µL dNTP mix
1.0 µL oligo dT primer
1.0 µL Verso enzyme mix
8.0 µL H_2O
1.0 µL SuperAse In
3.0 µL RNA template +
20.0 µL

PCR:

30 minutes @ 42℃ cDNA synthesis
2 minutes @ 95℃ inactivation (of enzyme)

5.4 PCR - Clontech Advantage® 2 Polymerase kit

Standard amounts for PCR mix (equals the amount necessary per PCR-cup):

18.5 µL H_2O (may vary if template volume varies)
2.5 µL 10 x Advantage BD PCR buffer
0.5 µL dNTPs
0.5 µL sense primer
0.5 µL antisense primer
0.5 µL Taq
2.0 µL template DNA +
25.0 µL mix

- First, make a stock PCR-mix in a standard 1.5 mL Eppendorf cup. Multiply the standard amounts described above with the number of samples needed, and make a little extra because during pipetting, some solution will get lost. So for three cDNA samples, use 4x the standard amounts.
- After adding Taq, vortex (since Taq is dissolved in glycerol). Make sure the mixture is homogenous.
- Divide the mix over the PCR-cups (in the example above, three).
- Add the DNA templates to every cup.
- Perform the PCR.
- Store at - 20°.

Standard PCR hot start/touchdown protocol ('sarace3') for CREB amplification with primers ForC/RevB or ForA/RevC:

1. T=94° 5 minutes (hot start)
2. T=94° 30 seconds
3. T=70° 30 seconds
4. T=72° 3 minutes
5. GOTO 2 REP 4
6. T=94° 30 seconds
7. T=70° 30 seconds
   -1° +0 seconds
   R=3℅s +0℅s
   G=0°
8. T=72° 3 minutes
9. GOTO 6 REP 10
10. T=94° 30 seconds
11. T=60° 30 seconds
12. T=72° 3 minutes
13. GOTO 10 REP 29
14. T=72° 5 minutes
15. HOLD 4°ENTER

5.5 Protocol for qPCR

For each qPCR reaction, you need the following:

SYBR Green master mix	12.5 µL	
Forward primer	1.0 µL	
Reverse primer	1.0 µL	
cDNA template	1.0 µL	
Water	9.5 µL	+
	25.0 µL	